EphA4 Receptor Tyrosine Kinase and PAK1 Signaling: Novel Regulators of *Xenopus laevis* Brachyury Expression and Involution Movements During Gastrulation

by

Sevan Evren

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Cell and Systems Biology
University of Toronto

© Copyright by Sevan Evren 2010
EphA4 Receptor Tyrosine Kinase and PAK1 Signaling: Novel Regulators of *Xenopus laevis* Brachyury Expression and Involution Movements during Gastrulation

Sevan Evren

Masters of Science
Cell and Systems Biology
University of Toronto
2010

Abstract

Gastrulation is a highly complex series of cellular rearrangements that leads to the internalization of the mesoderm and endoderm. The cellular behaviors that underlie morphogenesis are dependent upon changes in cell motility and polarity. Eph receptors belong to a family of receptor tyrosine kinases that are involved in a variety of developmental processes. This study is the first to examine the role EphA4 during *Xenopus* gastrulation.

Morpholino oligonucleotide (MO) mediated knockdown of EphA4 resulted in attenuated mesoderm involution and reduced the expression of the posterior mesoderm marker brachyury (Xbra). Expression of EphA4 in the blastocoel roof was sufficient to promote ectopic Xbra expression. I show that EphA4 can regulate Xbra expression and involution movements by signaling through PAK1. Temporal regulation of Xbra was sufficient to rescue EphA4 induced gastrulation defects. This study has uncovered a novel EphA4/PAK1 pathway which is required for mesoderm involution and Xbra expression during *Xenopus* gastrulation.
ACKNOWLEDGEMENTS

I would like to first and foremost thank Dr. R. Winklbauer for a giving me an opportunity to work in his lab.

I would also like to thank Olivia Luu, Erich Damm, Tina Negal, Katherine Sodak, and Hiro; my committee members, Dr. U. Tepass and Dr. A Bruce for all there constant help, guidance, and providing answers to all my questions throughout the years.

But more importantly, I’d like to thank my parents, Jirayr and Bahar, and my sister, Serda for helping me out and supporting me during this project. I could not have done it without you guys.
# TABLE OF CONTENTS

## LIST OF FIGURES

VII

## INTRODUCTION

1. Gastrulation Movements in *Xenopus laevis* ........................................... 1
2. FGF Signaling during Mesoderm Induction ........................................... 5
3. Eph Receptor Tyrosine Kinases .............................................................. 10
4. EphA4 in the *Xenopus* Embryo ............................................................... 15
5. PAK1 is a Downstream Target of EphA4 ................................................ 17

## MATERIALS AND METHODS

20

1. *Xenopus laevis* Embryos ................................................................. 20
2. *In Vitro* Fertilization ........................................................................... 20
3. Microinjection Technique ..................................................................... 21
4. Fixation and Fracturing of Embryos ..................................................... 21
5. Dorsal Blastopore Lip (DBL) and Convergent Extension Assays .......... 24
6. Roof Induction Assays ......................................................................... 24
7. Whole Mount *In situ* Hybridization .................................................. 27
8. Morpholino Antisense Oligonucleotides ............................................. 28
9. RNA constructs .................................................................................... 29
10. Transformation and Miniprep ............................................................. 30
11. Digestion .............................................................................................. 31
12. DNA Purification and Extraction ......................................................... 31
13. *In Vitro* Transcription ....................................................................... 31
14. Hormone Inducible Xbra .................................................................... 32
15. SU5402 Treatment ................................................................................ 32
16. Mesoderm Induction Assays ............................................................... 33
17. Imaging and Measuring ....................................................................... 33
18. Statistics ............................................................................................... 33
RESULTS......................................................................................................................................34
1. Knockdown of EphA4 receptor Tyrosine Kinases Leads to Severe Gastrulation Defects that are a Result of Improper Involution.................................................................34
2. MO Knockdowns of EphA4 can have Profound Effects on the Maintenance of Xbra expression during Involution without Affecting Xbra Induction.................................50
3. EphA4 can Promote Localized Xbra Expression.................................................................60
4. Wild type (wt) PAK1 is a Downstream Target of EphA4..................................................68
5. Precise Tempo-Spatial Regulation of Xbra is Essential for Involution.........................80
6. Constitutively active Mek cannot Rescue EphA4 MO-induced Gastrulation defects. Proper tempo-spatial regulation of Mek/Xbra is required for involution...............91
7. Xbra Expression Independent of FGFR Signaling...............................................................96

DISCUSSION.............................................................................................................................102
1. EphA4 acts through Pak1 to Promote Mesoderm Involution.............................................102
2. EphA4 acts through Pak1 to Control Xbra Expression.....................................................103
3. EphA4 and Pak1-dependent Xbra Expression is Necessary for Mesoderm Involution.................................................................107
4. Conclusion.........................................................................................................................111
5. Future Studies.....................................................................................................................114

REFERENCES..........................................................................................................................117
LIST OF FIGURES

Figure 1: Germ Layer Organization during Early Xenopus Development……………………………..3
Figure 2: Gastrulation Movements in Xenopus……………………………………………………………..7
Figure 3: Eph-ephrine Receptor and Ligands……………………………………………………………..13
Figure 4: Isolation Method of novel Dorsal Blastopore Lip (DBL) Explants used for Involution assays………………………………………………………………………………………………………..23
Figure 5: A. Xbra Induction in EphA4 RNA Expressing Animal Caps……………………………...27
B. Methodology of Roof-Leading Edge Mesoderm Combination Experiments……………………………...27
Figure 6: A. Effects of EphA4 MO Injection when viewed at stage 11……………………………………36
B. Effects of EphA4 MO Injection when viewed at stage 11.5…………………………………………..36
Figure 7: A. EphA4 MO, mouse EphA4 RNA and rescue experiments when viewed at stage 11…………………………………………………………………………………………………39
B. EphA4 MO, mouse EphA4 RNA and rescue experiments when viewed at stage 11.5…………………………………………………………………………………………………40
Figure 8: A. EphA4 MO, Xenopus EphA4 RNA and Rescue Experiments when viewed at stage 11……………………………………………………………………………………………42
B. EphA4 MO, Xenopus EphA4 RNA and Rescue Experiments when viewed at stage 11.5 ………………………………………………………………………………………………43
Figure 9: EphA4 RNA can rescue Higher Concentrations of EphA4 MO……………………………………………………………………………………………………………………………45
Figure 10: Epiboly Movements in EphA4 MO Injected Embryos………………………………………………47
Figure 11: Convergent Extension Movements in EphA4 MO Injected Embryos…………………………49
Figure 12: Involution Behavior in EphA4 MO Injected Embryos……………………………………………52
Figure 13: Xbra Expression in EphA4 MO Injected Embryos at stage 9…………………………………55
Figure 14: Xbra Expression in EphA4 MO Injected Embryos at stage 10.5………………………………57
Figure 15: Xbra Expression in EphA4 MO and PAK1 Injected Embryos at stage 11.5………………..59
Figure 16: Involution Behavior and Xbra Expression in DBL Explants of EphA4 MO Injected Embryos

Figure 17: In situ hybridization of Various Mesoderm and Neural markers

Figure 18: Localized Xbra expression in EphA4 injected roof-mesoderm combinations

Figure 19: EphA4 MO Gastrulation Defects were Rescued with wtPAK1 co-injection at stage 11

Figure 20: EphA4 MO Involution Movements Rescued with co-injection of wtPAK1

Figure 21: Xbra Expression in EphA4 MO and wtPAK1 Injected Embryos at stage 10.5

Figure 22: PAK1 cannot Rescue Xbra Expression in Higher EphA4 MO injections

Figure 23: Strong Marginal Zone Xbra Expression in caPAK1 injected embryos

Figure 24: Delayed Gastrulation Movements after Xbra RNA Injections

Figure 25: Involution Movements in DBL Explants injected with EphA4 MO, Xbra RNA, and EphA4 MO + Xbra

Figure 26: Rescue of EphA4 MO Mutant Phenotypes using Xbra-GR RNA Constructs

Figure 27: Mesoderm Induction in Animal Caps

Figure 28: caMEK cannot Rescue of EphA4 MO or kdPAK1 Gastrulation Defects

Figure 29: Widespread Xbra Expression in EphA4 MO and caMEK Injected Embryos

Figure 30: Gastrulation Defects after SU5402 Treatment

Figure 31: Reduced Xbra Expression after Early SU5402 Treatment and EphA4 MO Injection after late treatment

Figure 32: Proposed Model of Xbra Induction and Involution during Xenopus Gastrulation
Chapter 1
Introduction

1. Gastrulation Movements in *Xenopus laevis*

Gastrulation is a highly conserved, fundamental developmental process that establishes the characteristic internal spatial arrangement of the germ layers: with the ectoderm on the outside, endoderm inside, and positioned in between these two layers is the mesoderm (Montero and Heisenberg, 2004, Leptin, 2005, Solnica-Krezel, 2005).

Embryos of the African clawed frog, *Xenopus laevis*, have been widely used by developmental biologists to study the cellular and molecular processes of gastrulation. *Xenopus laevis*’s rapid external development, and large egg size easily allows for molecular and micro-surgical studies. After fertilization, *Xenopus* embryos enter a period of rapid cell division or cleavage. This stage is followed by the formation of a fluid-filled space, the blastocoel, marking the blastula-stage embryo. Developmental stages of *Xenopus* have been mapped according to Nieuwkoop and Faber (1967) and the blastula-stage embryo corresponds to stage 9 (Nieuwkoop and Faber, 1967). At this stage the embryo is divided into 3 distinct regions. These regions include the animally located ectoderm, the vegetally located endoderm, and positioned in between these two layers is the mesoderm. The prospective mesoderm is located in the marginal zone in an equatorial band separating both hemispheres (Figure 1).

Initial gastrulation movements start on the dorsal side of the embryo before spreading laterally and ventrally. Formation of pigmented bottle cells (BC), at stage 10 or 9 hours post fertilization (hpf) at room temperature, are the first visible signs of gastrulation. The prospective BCs are 6 to 8 tiers of superficial epithelial cells that exhibit apical contractions along the animal-vegetal direction (Hardin and Keller, 1988). During this stage, peripheral upwelling of vegetal cells allows the blastocoel floor (BCF) and outer marginal zone, containing cerberus (cer)-positive mesoderm to be positioned against the blastocoeel roof (BCR) by vegetal rotation (Winklbauer and Schürfeld, 1999). The leading edge of the migrating mesoderm therefore contains a mixture
Figure 1: Germ Layer Organization during Early *Xenopus* Development. At stage 9, the *Xenopus* gastrula has established the characteristic spatial arrangement of germ layers with the ectoderm (black) animally located, the endoderm (white) vegetally, and mesoderm (grey) in between these two layers.

*Adapted from Winklbauer, 2009.*
Figure 1- Germ Layer Organization during Early Xenopus Development
of endoderm and Cer-positive mesoderm and is often referred to as the mes-endoderm. Cer is a secreted polypeptide that belongs to a family of BMP antagonists with unique head-inducing properties (De Souza and Niehrs, 2000) and has traditionally been used as a marker for the leading edge mes-endoderm (Bouwmeester et al., 1996). The dual action of vegetal rotation and BC formation helps to create an indentation which forms the dorsal blastopore lip (DBL) at stage 10+ or 10hpf. Pre-involuting mesoderm populations at this stage must also internalize. Upon reaching the DBL, the prospective mesodermal mass is internalized by involution (Figure 2, red). Involution refers to a folding inward and rolling over movement which leads to the internalization of the mesoderm and endoderm (Shih and Keller, 1994, Ibrahim and Winklbauer, 2001).

Goosecoid (gsc)-expressing prechordal head mesoderm cells are the first to involute (stage 10+ or 10hpf) (De Robartis et al., 1992). Active and passive (vegetal rotation) cellular movements help to position this mesoderm population to the inside of the embryo (Winklbauer, personal communication). These movements are then followed by active involution of the brachyury (Xbra)-expressing chordamesoderm and lateral/ventral posterior mesoderm (stage 10.5 or 11 hpf) (Smith et al., 1991).

Involution is a highly dynamic and active morphogenetic movement that is essential for gastrulation. It has previously been demonstrated that involution of the Xbra-positive mesoderm is not a consequence of BC formation, as the removal of these cells does not effect gastrulation (Keller, 1981, Keller and Jansa, 1992). Furthermore, involution is not passively driven by an active external pushing or pulling force, as removal of the non-involuting marginal zone (NIMZ) or already involuted mesoderm, respectively, does not delay gastrulation (Cooke, 1975, Keller and Jansa 1991, Shih and Keller, 1994). Shih and Keller (1992) have demonstrated that prior to involution, cells at the marginal zone acquire distinct bipolar morphologies and form the vegetal alignment zone (VAZ) (Shih and Keller, 1992). The VAZ cells are believed to intercalate mediolaterally allowing the sheet-like mass to roll over the blastopore lip and involute. Perturbation of the VAZ using nocodazole, a microtubule depolymerizing agent, can disrupt bipolar cell morphologies and inhibit involution (Lane and Keller, 1997). These results suggest that involution is a highly active cellular process that is required for gastrulation. To date, few studies have identified the molecular players involved in regulating this cellular behavior.
Once internalized, gsc positive cells are exposed to a rich fibronectin-fibril matrix that functions as a substratum for cell spreading and lamellipodia and filopodia extensions (Winklbauer and Nagel, 1991). In addition, guidance cues, such as platelet derived growth factor alpha (PDGF-α) allow cells to become polarized and migrate animaly with a characteristic shingle arrangement (Nagel et al., 2004). In contrast, the chordamesoderm undergoes convergent extension (CE) movements (Figure 2, green). During CE, cells intercalate along the mediolateral axis, thereby thinning and elongating the chordamesoderm (Keller and Tibbetts, 1989). CE movements can be visualized in *Xenopus* embryos by removing the dorsal marginal zone and culturing them in isolation (Keller and Danilchik, 1988). Subsequently at this stage, the animaly located ectoderm continually moves vegetally to replace previously internalized mesoderm by epiboly (Figure 2, blue). The ectoderm, however, does not involute and marks the edge of the NIMZ boundary.

2. FGF Signaling during Mesoderm Induction

As mentioned previously, the prospective mesoderm is located in the marginal zone at an equatorial band separating animal and vegetal hemispheres (Figure 1). The mesoderm precursors are believed to originate as a result of inductive signals originating from the vegetal and animal halves. Cooperative inductive signals between several families of animaly located Fibroblast Growth Factor (FGF) and vegetally located Transforming Growth Factor-beta (TGF-β) ligands, such as activin, help to establish the mesoderm in its correct position (Cornell et al., 1995, Isaacs, 1997, Agius et al., 2000). Ectopic applications of FGF and activin can induce mesoderm in explanted animal caps (Chang et al., 1995). The concentration of activin can have profound effects on the type of mesoderm that forms. High concentrations of activin can suppress the expression of the posterior mesoderm marker, Xbra, by stimulating anterior mesoderm formation (goosecoid, gsc) (Latinkic and Smith, 1999, Artinger et al., 1997), while intermediate concentrations can promote Xbra expression, and low concentrations form the native ectoderm tissue (Latinki et al., 1997, Chang et al., 1997, Kurth and Hausen, 2000, Piepenburg, 2004). Application of FGF can induce dorsal and ventral mesoderm in animal caps (Kimelman and Maas, 1992). Furthermore, activin induced mesoderm induction has been shown to require FGF
Figure 2: Gastrulation Movements in *Xenopus*. The *Xenopus* embryo utilizes a variety of morphogenetic movements to internalize the presumptive mesoderm (red) and endoderm (light yellow), including epiboly (blue), vegetal rotation (yellow), involution (red), and convergent extension (green). The ectoderm is highlighted in light blue.

*Adapted from Winklbauer and Schufeld, 1999.*
Figure 2 - Gastrulation Movements in Xenopus

Epiboly
Vegetal Rotation
Involution
Convergent Extension
signaling, as dominant negative FGF receptor (XFD) constructs can repress mesoderm formation (Cornell and Kimelman, 1994). FGF receptors (FGFR) constitute a large family of growth factor receptors that contain an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular ligand binding domain of FGFRs contain two or three immunoglobulin-like domains (Böttcher and Niehrs, 2005). Alternative splicing of helps to create several isoforms of FGFRs with unique ligand binding properties and specificities (Eswarakumar et al., 2005). Upon ligand binding, FGF receptors dimerize and exhibit conformational changes. Transphosphorylation of intracellular tyrosine residues help to create docking sites for adapter proteins which leads to the activation of various downstream pathways, including MAPK signalling (Turner and Grose, 2010).

Phospho-tyrosine residues on the receptor are bound by the adapter protein Grb2. The guanine nucleotide exchange factor (GEF), Son of Sevenless (SOS) is recruited to the plasma membrane by binding to Grb2 and is able to activate Ras by GTP exchange due to its close proximity to membrane-bound Ras (Böttcher and Niehrs, 2005). The small GTP-bound Ras can bind to Raf using the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) and recruits it to the cell membrane for further phosphorylation and activation of the mitogen-activated protein kinase (MAPK) cascade (Chong et al., 2003). Raf (MAPKKK) activates Mek (MAPKK) by phosphorylation of its activation loop (ser218 and ser222 (Cowley et al., 1994, Mansour et al., 1994)). Furthermore, Mek activates the extracellular signal-regulated kinase/mitogen-activated protein kinase Erk (MAPK) by phosphorylation. Upon activation, Erks dimerize and migrate into the nucleus to activate various downstream targets including the posterior mesoderm marker, Xbra (Yang et al., 2003). In addition, Ras can promote PI3K signaling in Xenopus (Carballada et al., 2001). PI3K can synergize with Raf to activate Erk and promote Xbra expression (Nie and Chang, 2007, Caraballada, 2001). Microarray analysis has shown that the FGF/MAPK pathway can positively regulate at least 67 genes, including Xbra (19-fold increase) and EphA4 (4.8-fold increase) during Xenopus gastrulation (Branney et al., 2009). Furthermore, applications of the FGFR inhibitor, SU5402, can suppress EphA4 expression, as demonstrated by in situ hybridization analysis (Branney et al., 2009).
Xbra belongs to a member of the DNA-binding T-box transcription factor family of genes that can positively regulate a variety of targets in *Xenopus*, including embryonic FGF (eFGF) (Isaacs *et al.*, 1994). In turn, eFGF can bind to the FGFR and re-stimulate Xbra expression, forming a positive feedback loop (stage 11.5 or 12.5hpf) (Schulte-Merker and Smith, 1995, Isaacs *et al.*, 1994). Xbra expression is initially observed in the marginal zone of embryos shortly before the beginning of gastrulation (stage 8 or 8 hpf) (Smith *et al.*, 1991). Once the posterior mesoderm is internalized, Xbra is down regulated in the presomitic and lateral plate mesoderm, but maintained in the notochord via the Xbra-eFGF positive feedback loop (Smith *et al.*, 1991). At the end of gastrulation, Xbra is detected only in the notochord and tail bud (Gont *et al.*, 1993). Overexpression of Xbra animals can induce mesoderm formation (muscle, mesothelium, mesenchyma) and disrupt normal mesoderm involution (Cunliffe and Smith 1992, Isaacs, 1994). Although ectopically expressed and at levels similar to endogenous Xbra, injected Xbra mRNA is believed to be translated immediately without proper temporal regulation, thereby giving rise to nonspecific secondary defects (Cunliffe and Smith 1992, Tada *et al.*, 1997). Precise temporal and spatial regulation of Xbra is observed during *Xenopus* gastrulation (Artinger *et al.*, 1997, Latinkic *et al.*, 1997, Lerchner *et al.*, 2000). Currently, temporal regulation of Xbra has been achieved using hormone-inducible Xbra constructs (Xbra-GR) (Tada *et al.*, 1997). The coding region of the Xbra gene can be attached to the hormone-binding domain of the glucocorticoid receptor (GR). In the absence of hormone, the protein construct is sequestered by heat-shock proteins, while the addition of dexamethasone (DEX) can release inhibitory constraints and allow for proper signalling (Hollenberg *et al.*, 1996). Applications of Xbra-GR with DEX can promote mesoderm formation and ectopic tail-like protrusions, consistent with Xbra’s role in early tail bud formation (Gont *et al.*, 1993, Tada *et al.*, 1997).

FGF activity is essential for mesoderm formation in *Xenopus* (Kimelman and Maas, 1992). Dorsal injections of dominant negative FGF receptor (XFD) can suppress involution and Xbra expression without affecting the anterior mesoderm, as demonstrated by gsc expression (Amaya *et al.*, 1993). As mentioned previously, tyrosine kinase activation and phosphorylation are dependent on FGF receptor dimerization in response to ligand binding. XFD is a truncated FGFR that contains only the transmembrane and extracellular ligand-binding domain, thereby suppressing downstream signaling events (Amaya *et al.*, 1991). It is believed that FGF activity is required for initial mesoderm induction and maintenance of Xbra (Fletcher and Harland, 2008).
Extensive active MAPK (Curran and Grainger, 2000, Schohl and Fagotto 2002) and FGFR activity has been observed animally during gastrulation (Lea et al., 2009). Furthermore, injections of constitutively active (ca) Mek can stimulate Xbra expression in animal caps (LaBonne et al., 1995). In contrast, low FGFR activity and expression (Cornell et al., 1995), low MAPK signaling (Kurth and Hausen, 2000) and reduced expression of the FGF downstream target, ER81, has been documented in the vegetal half of embryos (Münchberg and Steinbeisser, 1999). Furthermore, FGF can inhibit endoderm formation and promote Xbra expression in the vegetal mass (Cornell et al., 1995, Cha et al., 2008), suggesting that FGF induced mesoderm induction is primarily restricted to the animal pole.

Vegetally localized TGF-β ligands are critical for mesoderm formation in Xenopus embryos. TGF-β functions by binding to its respective type II serine/threonine kinase receptor. Upon ligand binding, type II receptors recruit and phosphorylate type I receptors, which then leads to the activation of various downstream targets, including Smad signaling (Heldin, et al., 1997). The maternal factor, VegT, is vegetally localized and can stimulate the expression of various mesoderm inducing TGF-β ligands such as, Xnr1, 2, 4 and derriere, and mesoderm inducing FGF ligands, FGF3, FGF8, and can partially activate eFGF, activin, and Xnr 3 (Zhang and King, 1996, Zhang, 1998, Kofron et al., 1999). The TGF-β1 factor, Vg1, is also vegetally localized and is essential for proper mesoderm formation (Joseph and Melton, 1998). Mesoderm induction by TGFβ1 has been shown to require FGF signaling (Cornell and Kimelman, 1994).

It is believed that the cooperative interaction between vegetally localized TGFβ1 signals, regulated by VegT and Vg1, and animally localized FGF signaling is responsible for the formation of the mesoderm along an equatorial band in the embryo.

3. Eph Receptor Tyrosine Kinases

Gastrulation is a highly complex and coordinated series of cellular rearrangements that leads to the internalization of the presumptive mesoderm and endoderm. The cellular behaviors that underlie morphogenesis occur as a result of alternation in the cell’s cytoskeletal organization, cell adhesion and polarity. Each of the aforementioned gastrulation movements, including
vegetal rotation, involution, directional migration, convergent extension (CE), and epiboly require dynamically regulated cellular molecules that can properly control tissue adhesion and motility, thereby allowing sheets of cells to fold and for cells to migrate past one another. The erythropoietin-producing hepatocellular (Eph) receptor tyrosine kinases (RTKs) and their membrane bound Eph receptor interacting protein (ephrin) ligands are involved in a variety of developmental processes that regulate tissue migration, adhesion and polarity, making them ideal candidates to study the morphogenetic movements required for gastrulation (Holder and Klien, 1999, Mellitzer et al., 2000, Kullander and Klein, 2002).

Eph RTKs are divided into two subgroups, type A and B, based on their homologies and binding affinities. To date, 16 Eph receptors (10 EphA and 6 EphB) and 9 ephrins (5 ephrin A and 4 ephrin B) have been characterized in vertebrates. The functional divergence between ephrin A and B ligands occur as a result of structural differences between the two ligands. Type B ligands are bound to the membrane by transmembrane domains and contain a short intracellular cytoplasmic domain (Figure 3). In contrast, ephrin A ligands lack a transmembrane domain and are tethered to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Figure 3). In addition, ephrin ligands contain extracellular receptor binding domains, which is partitioned into areas of high and low affinity Eph binding (for a review see Kullander and Klein, 2002, Vearing and Lackmann 2005).

Eph RTKs are transmembrane-spanning receptors that are composed of an extracellular ligand binding domain and an intracellular cytoplasmic domain. The extracellular domain is composed of an N-terminal ligand binding globular domain (LBG), a cystine-rich region, and two fibronectin type III repeats. The LBG is further subdivided into areas of high and low affinity ephrin ligand binding. The intracellular catalytic domain includes the juxtamembrane segments, a tyrosine kinase domain, and a sterile alpha-motif (SAM) domain (Figure 3). In its native unbound state, EphA4 receptors exist as type A or B conformers (Qin et al., 2008). Both free state conformers are superimposable over the entire sequence of the receptor, aside from slight differences within the J-K loop of the molecule (Qin et al., 2008, Qin et al., 2010). Cell to cell contact allows high affinity regions of the LBG domain of Eph receptors to interact with the high affinity receptor binding areas of the ephrin ligand, creating areas of high affinity interface and the formation of Eph-ephrin dimers.
**Figure 3: Eph-ephrin Receptor and Ligands.** Eph RTKs are composed of an extracellular, transmembrane, and intracellular domain. The intracellular domain is further subdivided into the juxtamembrane, kinase domain, and SAM domain, which helps to mediate receptor-receptor interactions and activation of various downstream signaling components. Various Phosphotyrosine sites have been identified within the juxtamembrane, kinase, SAM domain of Eph receptors. Eph RTKs bind to their respective ephrin ligands. Type A ligands are linked to the membrane using a GPI anchor while type B ligands have a transmembrane and intracellular domain. EphA4 can bind to both type A and B ligands.
Figure 3- Eph-ephrin Receptor and Ligands

Globular domain
Cystine rich region
Fibronectin type II repeats

Juxtamembrane region
Kinase domain
SAM domain

Eph RTK

Ephrin A  Ephrin B

GPI anchor
Ligand binding domain
Intracellular domain
Transmembrane domain
PDZ-like binding motif

P
P
P
Eph-ephrin binding occurs as a result of polar interactions between the G-H loop of the ephrin ligand, which is inserted into the hydrophobic Eph receptor channel (Himanen et al., 2009, Qin et al., 2010). A second class of surface binding sites have also been identified, but these interactions are primarily involved in Eph-ephrin B receptor and ligand binding (Qin et al., 2010). Furthermore, low affinity LGB domains of receptor and ligands, allow the interaction of Eph-ephrin dimers with adjacent complexes, creating tetramers. In addition, interaction between neighboring Eph RTKs intracellular domains or through lipid raft microdomains can lead to the formation of massive Eph-ephrin clusters or higher-order complexes that can enhance signaling and diversify downstream responses (Torres et al., 1998, Thanos et al., 1999). Interestingly, exposure of HEK293 cells to ephin A5-coated beads can trigger the formation of EphA3 cluster that often exceed the size of the interacting ephrin surface by several folds (Wimmer-Kleikamp et al., 2004).

The unique structure (intracellular domain) of ephrin B ligands can allow signals to be transduced to not only the receptor, but ligand bearing cell as well, a process refered to as bi-directional signaling (Tanaka et al., 2003, Dravis et al., 2004). For conventional purposes, the receptor bearing cell exhibits forward signaling while the ligand bearing cell displays reverse signaling. Depending on the state of Eph-ephrin clusters and their composition, numerous distinct biological responses can be activated in both the receptor and ligand bearing cell, owing to the functional diversity of this molecule.

Eph RTKs exhibit limited promiscuity with respect to binding capabilities outside of their subgroup. Generally, type A receptors bind to type A ligands, and type B receptors bind to type B ligands. The only exception is EphA4, which can bind to both type A and type B ligands, although more weakly to ephrin B than to ephrin A ligands (Bowden et al., 2009, Qin et al., 2010). The stronger EphB-ephrin B interaction is believed to occur as a result of an additional aromatic-hydrophobic interactions with the G-H lopp of the ligand and the channel of the receptor (Qin et al., 2010). The extensive promiscuity of EphA4 receptors has been attributed to additional surface binding sites between EphA4 and ephrin B ligands (Bowden et al., 2009, Qin et al., 2010). Upon ligand binding and multimerization, Eph receptors facilitate conformational changes that trigger auto-phosphorylation of tyrosine residues in the juxtamembrane region, the kinase domain and the SAM domain (Wiesner, 2006). Type B receptors exhibit vast structural
conformational changes while minor changes have been observed after EphA ligand binding 
(Qin et al., 2008, Himanen et al., 2009). Deletion of the SAM domain of EphA4 has not revealed 
any structural function in Xenopus (Park et al., 2004), however, this domain may be involved in 
high order receptor clustering (Thanos et al., 1999). Phospho-tyrosine residues are then used as 
docking sites for various downstream signaling molecules including the Rho family of GTPases, 
RAS/MAPK pathway, and PI3K (for a review see, Vearing and Lackmann 2005, Noran and 
Pasquale, 2004). EphA2 and EphB1 have both been shown to recruit Grb and reduce cell to 
matrix adhesion and increase cell migration by activating the MAPK signaling pathway (Zisch et 

4. EphA4 in the Xenopus Embryo

In the Xenopus embryo, Eph and ephrin expression has been described during gastrulation 
however, limited studies have examined the role of EphA4 during Xenopus gastrulation.

EphA4 expression is first observed at the onset of zygotic transcription before gastrulation, and 
no expression was observed maternally (Winning and Sargent, 1994). During gastrulation, 
EphA4 expression is spatially localized as a concentric ring around the marginal zone, similar to 
Xbra (Smith et al., 1991, Winning and Sargent, 1994). Overexpression of EphA4 can lead to 
animal cell dissociations and blastocoel occlusions (Park et al., 2004).

Inducible forms of EphA4 (EPP) have been generated by replacing the extracellular domain of 
EphA4 with the ligand binding domain of the human epidermal growth factor receptor (EGFR) 
(Winning et al., 1996). Application of growth factors to animaly injected EPP embryos can lead 
to loss of adhesion phenotypes at much higher frequencies than with EphA4 overexpression 
(Winning et al., 1996, Park et al., 2004). Furthermore, these defects were shown to be highly 
specific as tissue integrity was maintained after the introduction of a mutant form of the chimeric 
receptor (Winning et al., 1996). Scanning and transmission electron microscopy have shown that 
in the blastula, EPP induced loss of adhesion phenotypes exhibit an absence of apical basolateral 
boundaries, a decrease in overall flatness, reduction of apical microvilli density, and loss of 
aderance and disruption of tight junctions (Winning et al., 2001).
It has been proposed that EphA4 can modulate cell adhesion in *Xenopus* by negatively regulating the Rho family of GTPases. Loss of adhesion phenotypes have been observed using GTPase inhibitors (C3 transferase- Rho A, B, C inhibitor and toxin A- Rac, Cdc42, and RhoA inhibitor) (Winning et al., 2002). Furthermore, embryos exhibiting loss of adhesion phenotypes induced by EPP can be rescued with the addition of C-cadherin and constitutively active forms of Rac, RhoA, or Cdc42 (Winning et al., 2001, Winning et al., 2002, Bisson et al., 2007). These results suggest that active ectopic EphA4 expression can regulate cell adhesion pathways by repressing the Rho family of GTPases and cadherin mediated adhesion in *Xenopus*.

Furthermore, animal injections of EPP and EphA4 were sufficient to induce ectopic tail-like protrusions in neural stage embryos (Park et al., 2004). These protrusions have also been observed with overexpression of Xbra, Xbra-GR, active FGF receptor, and by the FGF downstream target Laloo, highlighting EphA4’s potential role within the FGF/MAPK/Xbra cascade (Tada et al., 1997, Wienstien, 1998, Park et al., 2004). These results are consistent with Xbra’s localized expression within the tail bud of developing *Xenopus* embryos (Gont et al., 1993).

EPP induced ectopic tail protrusions were positive for FGFR1, FGFR4a, and FGF8 expression, as demonstrated by *in situ* hybridization analysis (Park et al., 2004). In addition, expression of EPP in animal caps can induce eFGF and FGF8b in *Xenopus* (Park et al., 2004), both of which are prominent mesoderm inducing factors (Isaacs et al., 1994, Fletcher et al., 2006). The FGF family of receptors and ligands are essential for initiating and maintaining mesoderm (Fletcher and Harland, 2008), further highlighting EphA4’s potential role within the FGF/MAPK signaling cascade.

Furthermore, the kinase domain of EphA4 receptors can physically interact with the juxtamembrane region of the FGFR and promote MAPK signaling in the absence of FGFR kinase activity (Yokote et al., 2005). Although the EphA4-FGFR1 interaction was observed both *in vitro* (HEK293 cells) and *in vivo* (P19 neuronal cells), full MAPK activity (EphA4 was shown to synergistically activate MAPK *in vivo*) was limited to *in vitro* settings (Yokote et al., 2005). In human glioblastoma cell lines, tumors were shown to positively regulate cell survival and
migration using a novel EphA4-FGFR1 heteroreceptor complex (Fukai et al., 2008). EphA4 overexpression has also correlated with FGFR overexpression in human gastric cancers (Oki et al., 2008). These findings emphasize a potential interaction between EphA4 and FGFR.

4. PAK1 is a Downstream Target of EphA4

Loss of adhesion phenotypes induced by ectopic active EphA4 was further used to elucidate downstream targets of the EphA4 cascade. Bisson and colleagues (2007) were able to generate loss of adhesion phenotypes using wild-type (wt) and kinase dead (kd, M4) forms of p21 activated kinase (PAK1) (Bisson et al., 2007). Furthermore, kdPAK1 and dn versions of the adapter protein nckβ were sufficient to rescue EPP induced loss of adhesion, suggesting that EphA4 can regulate cell adhesion by recruiting nckβ and PAK1.

PAK1 is a serine/threonine protein kinase that is involved in a variety of morphogenetic processes, including regulating mes-endoderm cell morphology, polarity, directional migration, and tissue separation in Xenopus (Nagel et al., 2009, Macanovic et al., unpublished). Structurally, PAK1 is composed of an N-terminal GTPase binding domain (PBD) overlapping an autoinhibitory domain (PID) and a highly conserved C-terminal kinase domain. In its nonactive state, PAK1 is maintained within an autoinhibitory dimer, whereby the PID domain of one PAK molecule interacts with the PBD domain of another (Bagrodia and Cerione, 1999, Bokoch, 2003). Binding of active Cdc42 or Rac to the PBD domain can disrupt dimerization and allow autophosphorylation of the activation loop (thr423) of PAK1 thereby leading to full kinase activity (Manser et al., 1994). Repression of the inhibitory lock of PAK1 can lead to the activation of various downstream targets including cytoskeletal regulators (myosin light chain kinase) (for a review see, Daniels and Bokoch, 1999), and PAK1 has therefore been implicated in controlling cell migration and polarity (Sells et al., 1991, Nagel et al., 2009).

PAK1 can also regulate gene transcription by targeting the Ras/MAPK signalling pathway. PAK1 can phosphorylate Mek on S298 and Raf on S338. These phosphorylation events are thought to increase the affinity of Mek for Erk and/or Ras and are believed to relieve autoinhibition of Raf (Chaudhary et al., 2000, Slack-Davis et al., 2003, Beeser et al., 2005).
Inactive forms of PAK can attenuate Ras, Mek, and Erk signaling and both Rac and Cdc42 synergize with Ras to promote Erk activation using PAK1 (Besser et al., 2005, Frost et al., 2006). During Xenopus development, maternal and zygotic PAK1 RNA and protein expression has been observed ubiquitously in the embryo (Islam et al., 2000).

Cdc42, Rac and RhoA belong to a highly diverse family of Rho GTPases. The Rho family of GTPases shuttle between active (GTP-bound) and inactive (GDP-bound) states. Guanine nucleotide exchange factors (GEFs) help to catalyze the exchange of Rho-GDP to Rho-GTP, conversely, GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho proteins and maintain them in an inhibitory GTP-bound state. The Rho family of GTPases are involved in a variety of biological and developmental processes including the regulation of gene transcription and actin cytoskeleton organization (for a review see Van Aelst and Symons, 2002).

In addition, PAKs can be activated through GTPase independent mechanisms. Membrane recruitment of PAKs can occur using their N-terminal PXXP motif which allows binding to various SH2/SH3 containing adaptor proteins, such as nckβ (Bokoch, 2003). Membrane localized PDK1 has a propensity to activate PAK1 and interactions with sphingolipids and sphingo-derived lipids can relieve the inhibitory lock of PAK1 and mediate its signaling (Bokoch et al., 1998, King et al., 2000). Membrane recruitment of PAK1 can also increase its proximity to Raf and thereby enhance the MAPK signaling pathway (Frost et al., 2006). Elevated PAK1 kinase activity has been observed after EphA4-nckβ mediated membrane recruitment in Xenopus (Bisson et al., 2007).

PAK1 can also function as a scaffold protein, acting upstream or downstream of Cdc42/Rac (Obermeier et al., 1998). Both wtPAK and kdPAK1 are sufficient to induce loss of adhesion phenotypes (Bisson et al., 2007). Kinase dead mutants of PAK1 have been shown to regulate EPP induced loss of adhesion by sequestering Cdc42-GTP and thereby leading to reduced Rho-GTP activity and cell adhesion (Bisson et al., 2007).

In contrast, the kinase domain of PAK1 was shown to positively regulate cell polarity and lamellipodia formation through Cdc42 and Rac within the leading edge mesoderm of gastrulating Xenopus embryos (Nagel et al., 2009). These results further highlight the complexity of PAK1
signaling during *Xenopus* development. However, differences between both findings can be attributed to the specific cell type, ectoderm versus leading edge mesoderm, as well as ectopic application of active EPP versus the endogenous role of PAK1, used by researchers (Bisson *et al.*, 2007, Nagel *et al.*, 2009). Both EphA4 and PAKs have also been implicated in cancer tumorigenesis and metastasis (Carter, 2004, Fukai, 2008,)

Although these previous studies have helped to uncover the role and downstream signaling targets of EphA4 in regulating cell adhesion in *Xenopus* embryos, all experiments were done using ectopic, constitutively active forms of EphA4, providing limited information on the role of endogenous EphA4 during gastrulation.

This study presents the first morpholino oligonucleotide (MO) loss of function experiments of endogenous EphA4 within the dorsal marginal zone (DMZ) tissue of gastrulating *Xenopus* embryos. MOs are frequently used by developmental biologists to knockdown gene expression by inhibiting protein synthesis. MO can bind to nucleic acid sequences by complementary base pairing and are resistant to nucleases (Genetools). In addition, their lack of negative charge prevents nonspecific interaction with cellular proteins. MO can block translation (protein coding) by targeting the 5' untranslated region (UTR) of mRNA sequences or generating altered splice variants that are eventually degraded (Genetools). Our results demonstrate that EphA4 is actively involved in regulating morphogenetic movements required for proper gastrulation. Loss of EphA4 function was shown to delay gastrulation movements that were rescued by expression of wtPAK1 and temporal regulation of Xbra (Xbra-GR). I was able to show that defective gastrulation movements were a result of improper tissue involution without affecting mesoderm induction or convergent extension movements. Overexpression of EphA4 was sufficient to induce Xbra expression during involution stages. Our results demonstrate that EphA4 temporally and spatially regulates Xbra expression using PAK1, thereby leading to normal involution behavior and proper gastrulation movements.
Chapter 2
Materials & Methods

1. *Xenopus laevis* Embryos

Female *Xenopus laevis* (*Xenopus Express*) were injected subcutaneously with 0.2ml of Human Chorionic Gonadotropin (HCG) (Sigma-Aldrich, St. Louis, MO, USA) near the dorsal lymph sac approximately 16 hours prior to ovulation. Frogs were kept in 21°C water chambers and hormone-induced ovulations were scheduled every 3 months. Testes were obtained by euthanization. Testes were isolated from the abdominal cavity and stored in Steinberg’s solution at 4°C. All animals were cared for under the guidelines set out by the Canadian Council of Animal Care.

2. *In Vitro* fertilization

Oocytes were isolated from female frogs by applying mechanical pressure above the cloaca. Eggs were deposited directly into a 60mm Petri dish. *In vitro* fertilization allowed us to fertilize a large quantity of eggs at roughly the same developmental stage. Eggs were mixed with shredded testes in 1X modified Barth’s solution (MBS) and left standing for 5 minutes, after which, the solution was diluted using 1/10X MBS and incubated for 30 minutes at 15°C. Fertilized eggs were de-jellied using 2% L-cysteine in 1/10X MBS, ph 8, for 7 minutes. De-jellied embryos were rinsed 3 times with 1/10X MBS and stored in 1/10X MBS at 15°C until the desired developmental stage. Embryos were incubated at lower temperatures to reduce the rate of development. All embryos were staged according to Nieuwkoop and Faber (1967) and all experiments were done using 2 or more batches of embryos (Nieuwkoop and Faber, 1967).
3. Microinjection

Unless otherwise stated all injections were carried out on the late 4-cell stage embryos, at the animal – vegetal boundary, marking the future site of the dorsal marginal zone. Dorsal blastomeres were identified based on their relative small size and weaker pigmentation. Injections were carried out using a Nangel 2 microinjector (Fisher Scientific, Ottawa, ON, CAN). The same injector was used in all experiments to reduce variability.

Needles were fashioned from 3.5’’-glass capillaries (Drummond, Broomall, PA, USA), using a needle puller (Narishige, Los Angeles, CA, USA). Needle tips were generated at a 35º angle, roughly 3-4 chips, and sharpened at 25º using a needle grinder (Narishige).

Glass tips were filled with mineral oil (Fisher, Toronto, ON, CAN) and placed into the injector needle. Unless otherwise stated, the total injection volume was 6.9 nL. At the time of injection, fertilized embryos were placed in a 5% ficoll solution (Sigma, Oakville, ON, CAN). After injection, embryos were transferred into a new solution of 5% ficoll and left for 1 hour at 15°C, to promote healing. Embryos were washed, 3 times with 1/10X MBS and stored overnight in 1/10X MBS at 15°C. Unless otherwise stated, the following concentrations were routinely used for each experiment: EphA4 morpholino antisense oligonucleotides MO (15ng), EphA4 RNA (500 picograms (pg)), wild type (wt) PAK1 (450 pg), kinase dead (kd) PAK (400 pg), Xbra (10 pg), and Xbra-GR (10 pg) into two dorsal blastomeres.

4. Fixation

Embryos and explants were fixed in 4% formaldehyde (in MBS) at the desired developmental stage.
Figure 4. Isolation Method of novel Dorsal Blastopore Lip (DBL) Explants used for Involution assays. Normal gastrulation movements can be visualized in whole embryos. At stage 10 (A), bottle cells (black arrow) are situated at the outside of the dorsal marginal zone. As gastrulation proceeds to stage 11 (B), the mesoderm involutes and superimposes over itself. At this stage, bottle cells are located inside of the embryo and the archenteron makes a distinct elbow curve (inset zoom). The formation of Brachet’s cleft is also visible (red arrow). Similarly, involution movements can be analyzed in *Xenopus* embryos by excising the DBL at stage 10 and culturing them in isolation until stage 11 (C). I used these novel DBLs explants to assess involution movements in *Xenopus* embryos.
Figure 4- Isolation Method of novel Dorsal Blastopore Lip (DBL) Explants used for Involution assays.
5. Dorsal Blastopore Lip (DBL) and Convergent Extension (CE) Assays

Stage 10 embryos were isolated and placed in plasticine covered petri dishes containing 1X MBS. Left and right incisions were made using an eyelash tool at bottle cells (BC) edges. In cases where BC had difficulty forming, this area was extended. Embryos were then cut in half, such that the tip of the BC marked the edge of the cut site (Figure 4A). Internalized leading edge mes-endoderm was shaved off. DBL were then fixed, for photography or in situ hybridization, at approximately stage 10.5-11 (Figure 4B). Control non dissected embryos were used to reference staging. Convergent extensions assays followed similar experimental procedure. However, explants were placed overnight at 15°C in 1X MBS until stage 19 and fixed.

Involution behavior was scored based on three prominent phenotypes observed: an involution phenotype, when BC/archenteron made a distinct elbow curve with an observable c-shape bend, an intermediate involution phenotype, when BC/archenteron did not curve inward but translocated laterally, and a no involution phenotype, when BC/archenteron was situated outside of the mesoderm. The total number of explants was counted and the percentage was graphed. For CE assays, explants were photographed using the Axiocam MRc (Carl Ziess International, Toronto, ON, CAN) camera and the length to width ratios were determined using the AxioVision LE Rel 4.2 (Carl Ziess International) length measure tool.

6. Roof Induction Assays

**Roof:** Embryos at stage 10 were placed in 1X MBS plasticine covered mini petri dishes. Using an eyelash tool, horizontal cuts were made along the animal boundary. In cases where non-pigmented areas were excised, these roofs were either discarded or only white areas (mesoderm) were removed (Figure 5Ai).

**Leading edge isolates:** Embryos at stage 10 were placed in 1X MBS plasticine covered dishes. DBL explants were isolated as previously mentioned, however for this study the leading edge
Figure 5. Methodology of Roof- Leading Edge Mesoderm Combination Experiments (A) and Xbra Induction in EphA4 RNA Expressing Animal Caps (B). A. Stage 10 mesoderm (green) and roofs (yellow) were excised and combined to assess Xbra expression using *in situ* hybridization. B. Proposed model of Xbra induction along the BCR. The leading edge mesoderm (green) is positioned against the roof (yellow) by vegetal rotation. Normal Xbra (blue) expression is localized as a concentric ring around the blastopore. Expression of EphA4 mRNA in the BCR (red) can induce ectopic Xbra expression (blue) between the mesoderm (grey) and roof. I hypothesize the presence of an endogenous EphA4 ligand in the mesoderm (grey) which can stimulate Xbra (blue) expression at the interface of EphA4 expressing roofs (red) and mesoderm (grey).
Figure 5A- Methodology of Roof- Leading Edge Mesoderm Combination Experiments

Figure 5B- Proposed Model of Xbra Induction Along the BCR
mesoderm was excised and placed into the cup-like roof. Explants were allowed to develop till stage 10.5 in 1X MBS and in situ hybridization was performed (Figure 5Aii).

7. Whole Mount In situ Hybridization

Briefly, Xenopus laevis embryos or explants at a desired developmental stage were fixed for 30 minutes in 1X MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) in DEPC water: 1/10 10x MEMFA, 1/10 formaldehyde, and 8/10 DEPC water. Embryos were transferred to 1/10X MBS solution and vitelline membranes were removed. Embryos were refixed for another 1-1.5 hours in MEMFA. Samples were then transferred to a 50% ethanol and 50% MEMFA solution for 1 hour and stored overnight at -40°C in 100% ethanol. At the time of experimentation, embryos were gradually rehydrated with decreasing concentrations of ethanol and increasing concentrations of Ptw (1X PBS + 0.1% Tween-20). Proteinase K (7.5µg/ml) (Sigma) was added to 2ml of Ptw solution and incubated on a nutator for 10 minutes. Proteinase K was used (on embryos) for no longer than 10 minutes to maintain structural integrity. Samples were rinsed 2 times with triethanolamine (TEA) for 10 minutes at room temperature. A third and final wash of TEA was incubated with acetic anhydride for 5 minutes. Acetic anhydride was added to neutralize positive charges, preventing nonspecific probe binding due to electrostatic interactions with RNA or proteins.

Embryos were washed 2 times with Ptw for 5 minutes each. Afterward, embryos and explants were refixed using 4% paraformaldehyde solution in Ptw for 30 minutes at room temperature. This was then followed by 5 washes in Ptw for 5 minutes. Ptw was removed and samples were incubated with hybridization buffer for 4 hours at 60°C (prehybridization). Prehybridization helps to linearize RNA and allows for better probe binding.

Hybridization buffer was replaced and a new solution of hybridization buffer and probe was added and left overnight at 60°C. Probes used in the experiments were Xbra (concentration used: 0.5µg/ml), goosecoid (1µg/ml), cerberus (1µg/ml), chordin (1µg/ml), Sox2 (1µg/ml) or Xnot (1µg/ml).
Samples were washed 3 times using a low stringency solution, 2X SSC (sodium chloride/sodium citrate) for 20 minutes each at 60°C, to remove excess probe and hybridization buffer. Embryos where then washed twice using a high stringency solution, (0.2X SSC) at 60°C for 30 minutes and twice in MAB (maleic acid buffer, ph 7.5) for 15 minutes on a nutator.

Non-specific antibody binding was blocked using a MAB with 2% blocking reagent (Hoffman-LaRoche, Mississauga, ON, CAN) and 20% goat serum (Gibco/Invitrogen, Carlsbad, CA, USA) solution for 1 hour at room temperature. Goat serum was pretreated for 30 minutes at 55°C to inactivate endogenous proteasese and nucleases. Embryos were removed from the blocking buffer and incubated in a new blocking buffer with 1/2000 anti-digoxygenin alkaline phosphastase antibody (Sigma) for 4 additional hours. Samples were then washed 3 times with MAB and incubated overnight.

Endogenous phosphatase activity was inhibited using an Alkaline Phosphatase buffer (ALP). The ALP solution was removed and BM purple (Roche) was added. BM purple is chromogenic substance that can precipitate in the presence of anti-DIG antibodies that are conjugated to an alkaline phosphatase.

Embryos were left at room temperature and monitored every 15-20 minutes for color development. The reaction was stopped with the addition of MEMFA. Typical incubation time for Gsc, Cer, Chd, Sox2, Xnot was 1 to 1.5 hours and for Xbra 30 minutes to 45 minutes.

8. Morpholino Antisense Oligonucleotides

Morpholino antisense oligonucleotides (MO) are nonionic DNA analogs containing a nucleic acid base, a morpholino ring and a non-ionic phosphorodiamidate intersubunit (Genetools, Philomath, OR, USA). The sequence for the EphA4 MO (Genetools) used for this study was 5’-CGGAGGTGGATTAAGAGATGCCTAT- 3’. Both the *Xenopus* and mouse EphA RNA was sequenced (The Centre for Applied Genomics (TCAG), Sickkids, Toronto, Canada), and no MO binding site was identified in the coding region of either RNA. These results suggest that EphA4 MO binds to the 5’ UTR, without binding to the start or coding sequence. This allowed us to
inject both MO and RNA simultaneously into embryos, without having the MO binding to our RNA.

9. RNA Constructs

9.1 Injectable RNA

Mouse and *Xenopus* EphA RNA (provided by Dr. Moss), pCS2+ vector, were cut with Xba I and transcribed using an SP6 polymerase. The cDNA coding the entire wildtype *Xenopus* PAK1 or a kinase-dead mutant (kd, M4) of PAK1 (K281: lysine 281 was mutated to alanine, to inactivate the catalytic domain) (provided by Dr. Moss), pT7TS vector, was linearized with XbaI and transcribed with T7 polymerase (Bisson et al., 2007, Bisson et al., 2003, Macanovic et al., unpublised). Constitutively active (ca) PAK1 (M3) (provided by Dr. Moss), pTSHAxPAK1 vector, was linearized using Xba1 and transcribed with a T7 polymerase. Active PAK1 (M3) constructs can be achieved by mutating leucine 98 to phenylalanine (L98F) within the GTPase Binding Domain, which allows it to by-pass GTPase binding (and most likely autoinhibitory PAK1 locks) and renders it fully active (Brown et al., 1996, Bisson et al., 2003, Bisson et al., 2007). *Xenopus* brachyury (Xbra) RNA (provided by Dr. Smith), vector pSP64T, was linearized with HindIII and transcribed with SP6 polymerase. caMek RNA (provided by Dr. Whitman), pSP64 poly (A) vector, was linearized with EcoR1 and transcription with SP6 polymerase. Active Mek constructs can be generated by mutating two regulatory serines, ser218 and ser222 (which are phosphorylated by MAPKKK), to aspartic or glutamic acid within the activation loop and deleting the N-terminal catalytic domain, which helps to stabilize the active state of the molecule (Cowley et al., 1994, Mansour et al., 1994, LaBonne et al., 1995). Xbra-GR RNA (purchased from the Developmental Biology Plasmid Stalk), pSP64T vector, was linearized using SAL1 and transcribed with SP6 polymerase. The Xbra-GR RNA construct was generated by fusing the coding region of Xbra to the hormone binding domain of the human glucocorticoid receptor (Tada et al., 1997).
9.2 Probe RNA

Xbra digoxigenin probe, vector P2P73 (provided by Dr. T. Sargent), was linearized with BgIII and transcribed with T7 RNA polymerase. The gsc RNA probe (provided by Dr. Heidelberg), clone H7 in pBluescript SK(-) vector, was linearized with EcoRI and transcribed with T7 polymerase. The Cer RNA probe (provided by Dr. De Robertis), pBSSK vector, was linearized using EcoRI and transcribed using a T7 polymerase. Chd RNA probe (provided by Dr. De Robertis), clone H7 in pBluescript SK(-) vector, was linearized with EcoRI and transcribed with T7 polymerase. The Xnot probe (provided by Dr. Kimelman), Bluescript KS + vector, was linearized with Hind III and transcribed with a T7 polymerase. The Sox2 RNA (provided by Dr. Sasai), pCS2-Sox2 vector, linearized with EcoRI using T7 RNA polymerase.

10. Transformation and Miniprep

DH5 alpha E. coli bacteria were thawed on ice and 1-20 ng of bacteria was added to 20 ng/µl of plasmid. Samples were lightly mixed and placed on a 42°C water bath for 42 seconds. Samples were placed on ice for 2 minutes, incubated with Luria broth (LB) and placed on a shaker for 1 hour. Bacterial plates were made by adding 40 grams of LB agar to 1l of water and 50 µl of ampicillin, to give a final concentration of 50 µg/ml. The broth mixture was placed on petri dishes, cooled and stored in the fridge. At the time of experiment, an additional 5 µl of ampicillin was spread on plates. Plates were divided in half and 50 µl (low colony) or 100 µl (high colony) of the bacterial mixture was added on either side of the plate. Samples were incubated at 37°C overnight. Maximum storage time for bacterial cultures was less than 16 hours. A positive colony was inoculated in LB with 50 µg/µl amp for overnight at 37°C. Samples were then centrifuged for 15 minutes at 5000 rpm. The supernatant was removed and bacterial digestions were done according to instructions provided by Qiaprep Spin Miniprep Kit (Qiagen, Mississauga, ON, CAN). The concentration of DNA was determined using a spectrophotometer (1 µl of sample in 3 µl of DEPC water). Samples were then stored at -80°C.
11. Digestion

Briefly, 5 units of enzyme (Amersham-Biosciences) and 10X buffer was added to 1-2µg of plasmid. The sample was mixed and centrifuged. In addition, a control no enzyme sample was also added for all digestion experiments. Samples where incubated in 37°C for 1-2 hours in a water bath. Samples were then run on a 0.8% agarose gel to verify linearization.

12. DNA purification and Extraction

Fifty microliters of chloroform and 50µl of phenol were added to the linearized plasmid. Samples were mixed and centrifuged for 1 min at 13000rpm at room temperature. Top phase of the sample was transferred and an addition chloroform-phenol wash was performed. Top phase of samples were removed again and this time only 50µl of chloroform was added. Samples were centrifuged again for 1 min. The top phase was removed, and to 1/10th of the volume, 3 M of sodium acetate, and to 2 times the volume, 100% ethanol was added. Samples were mixed and incubated at -20°C for 30 minutes and centrifuged for 15 minutes at 4°C. Ethanol was removed and 70% cold ethanol was added. Samples were centrifuged at 4°C for 15 minutes. Finally, all the ethanol was removed, air dried and 5µl of DEPC water was added to extracted DNA samples.

13. In Vitro Transcription

Transcription reactions were carried out in order to generate RNA for injection or probes for in situ hybridization. Briefly, 2µl of reaction buffer (Ambion) was thawed and added to 10µl of 2X NTP/CAP (for RNA injection) or 2µl of 10X DIG (Roche) (for probe generation). Furthermore, 1ug of lineaized DNA and 2µl of enzyme mix (Ambion) was added to give a final volume of 20µl. A control reaction with no enzyme mix was also done for all transcription reactions. Samples were mixed by hand, centrifuged and incubated in 37°C for 1 hour (T3) or 2 hours (SP6). Samples were then run on a 1 % agarose gel to see if transcription was complete. The transcription reaction was stopped with the addition of 30µl of LiCl and stored in -20°C for 1
hour. In addition, 33µl of Glycoblué (final concentration, 50 ug/ml) (Ambion) was added for easy visualization of the RNA pellet.

Samples were centrifuged for 20 minutes at 4°C, to which, 70% cold ethanol was added. Samples were then centrifuged for 10 minutes at 4°C and washed twice with 70% ethanol and centrifuged. Finally, all the ethanol was removed and air dried. Twenty microliters of RNase free water was added to samples and the concentration of RNA was determined using a spectrophotometer (1µl of sample in 3µl of DEPC water). RNA was stored in ethanol and sequenced by The Centre for Applied Genomics (TCAG) (Sickkids, Toronto, ON, Canada).

14. Hormone inducible Xbra Expression

Dexamethasone (DEX) (Sigma) was initially dissolved in ethanol to give a stock concentration of 100 mM. This stock solution was then further diluted with ethanol to give a working solution of 2 mM. Twenty microliters of the working solution was added to 1/10X MBS for whole embryos and 1X MBS for explants, to give a final concentration of 8µM. Stock, working and embryos exposed to DEX were all kept under dark conditions to reduce hormone break-down. All experiments using DEX was done within 3-4 weeks of the opened date, as per requested by the manufacturer.

15. FGFR inhibitor (SU5402) Treatment

SU5402 (provided by Dr. Tropepe) inhibitor treatment followed the same microinjection protocol, as previously mentioned. Embryos were injected animally into the blastocoel at stage 8, 9, or 11 with 13.9 nl (half dose) or 27.8nl of Dimethyl sulfoxide (DMSO) stock or 2 mM SU5402 (Calbiochem). Embryos were then transferred into a 20µm (half dose) or 40µm SU5402 media and incubated till stage 10.5 or stage 11.
16. Mesoderm Induction Assays

Control and Xbra, constitutively active (ca) Mek, or EphA4 MO + caMek injected embryos were transferred to 1X MBS. Animal caps were excised by a straight horizontal cut along the pigmented-animal boundary using an eyelash tool. Explants there then transferred to a new solution of 1X MBS and left at room temperature for 4 hours. Explants were then transferred to 1/10X MBS and left overnight.

17. Imaging and measuring

Images were viewed under a 200m Axio-Vert (Carl Zieß International) inverted microscope and images were photographed using the Axiocam MRc (Carl Zieß International) camera. The AxioVision LE Rel4.8 (Carl Zieß International) software was used to measure archenteron length in sagittally sectioned whole embryos.

18. Statistics

All numerical data was analyzed using a two-tailed Student’s unpaired t-test. All values below p < 0.05 were considered to be statistically significant.
Chapter 3
Results

1. Knockdown of EphA4 Receptor Tyrosine Kinases leads to Severe Gastrulation Defects that are a Result of Improper Involution

Previous studies have analyzed the role of EphA4 by expressing constitutively active forms in ectopic locations. This study is the first to examine the endogenous role of EphA4 within the native dorsal marginal zone (DMZ) using morpholino oligonucleotide (MO) mediated loss of function experiments during *Xenopus laevis* gastrulation. EphA4 MO was injected dorsally at the late 4 cell stage in 2 blastomeres.

I assessed gastrulation defects, in particular mesoderm internalization, by examining the location of bottle cells (BC) and the length of the archenteron as a measure of mesoderm involution. Whole embryo and dorsal blastopore lip explants *in situ* hybridization results (presented in section 2), validate that EphA4 induced gastrulation defects were a result of improper involution. Our dorsal blastopore lip (DBL) explants also confirmed that EphA4 induced gastrulation defects were tissue autonomous, consistent with attenuation of involution.

When compared to controls, embryos injected with 15ng or 30ng of EphA4 MO exhibited a statistically significant concentration dependent reduction in archenteron length when viewed at stage 11 and stage 11.5 (*Figure 6A and B*). No significant differences in archenteron length were observed between control and embryos injected with 5ng of EphA4 MO. These results suggest that EphA4 may play a significant role during *Xenopus laevis* gastrulation.
Figure 6. Effects of EphA4 MO Injection when viewed stage 11 (A) and 11.5 (B). Injection of 15ng and 30ng of EphA4 MO significantly perturbed gastrulation movements (p < 0.0001 for 15ng and p < 0.0001 for 30ng) (black arrow, bottle cells), in a concentration dependent manner. No defects were observed with the injection of 5ng of EphA4 MO.
Figure 6A- Effects of EphA4 MO Injection when viewed at stage 11

Figure 6B- Effects of EphA4 MO Injection when viewed at stage 11.5
In order to show that EphA4 MO induced gastrulation defects were specific to EphA4 knockdown, I performed rescue experiments by injecting mouse or *Xenopus* EphA4 mRNA. We assessed rescue concentrations by initially injecting EphA4 RNA alone into embryos. Expression of either mouse or *Xenopus* wild type (wt) EphA4 RNA led to severe gastrulation defects when viewed at stage 11 and 11.5, in a concentration dependent manner (Figure 7A and B, Figure 8A and B). The most marked reductions in archenteron length occurred with 500 pg or 800 pg of EphA4 RNA.

Mutant phenotypes induced by EphA4 MO injected embryos were shown to be highly specific, as gastrulation defects induced by 15ng of EphA4 MO could be rescued with the addition of 500 pg of either *Xenopus* or mouse EphA4 RNA (Figure 7A and B, Figure 8A and B). Embryos injected with 15ng of EphA4 MO + 500 pg of mouse or *Xenopus* EphA4 RNA were not significantly different from controls. Furthermore, gastrulation defects induced with injections of 30ng of EphA4 MO could be rescued with the addition of 500 pg of wt EphA4 RNA, suggesting that mutant phenotypes were highly specific for EphA4 (Figure 9).

EphA4 MO and RNA injected embryos displayed normal leading edge mes-endoderm populations, suggesting that defects were not a result of improper invagination or vegetal rotation (Winklbauer and Schürfeld, 1999). I observed normal BC formation when 15ng of EphA4 MO was injected into embryos (data not shown). However, at a low frequency I did observe reduced BC with 30ng of EphA4 MO injections (data not shown). Furthermore, I assessed epiboly movements by measuring the animal and dorsal blastocoel roof (BCR) thickness. I did not observe any statistically significant differences between control and 15ng or 30ng of EphA4 MO injected embryos (Figure 10). These results suggest that gastrulation defects induced by EphA4 knockdown experiments are not a result of defective mes-endoderm, BC formation or abnormal epiboly movements.

In addition, I was able to show that EphA4 induced perturbation in gastrulation movements were not a result of defect convergent extension (CE). I did not observe any statistical differences in the length-to-width ratio between controls or in explants injected with 15ng of EphA4 MO (Figure 11A and B). However, I did observe significant reductions in length-to-
Figure 7. EphA4 MO, mouse EphA4 RNA and Rescue Experiments when viewed at stage 11 (A) and 11.5 (B). Severe gastrulation defects were observed with EphA4 MO and mouse EphA4 RNA injections. Delay in gastrulation observed with EphA4 MO was highly specific, as co-injection of EphA4 RNA could rescue movements. No significant differences were observed with control and embryos injected with 15ng of EphA4 MO and 500 pg of EphA4 RNA when viewed at stage 11 (p= 0.1312) or 11.5 (p= 0.0642). Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05).
Figure 7A- EphA4 MO, mouse EphA4 RNA and Rescue Experiments when viewed at stage 11

Lower graph showing average archenteron length with error bars and sample sizes (n) for each condition:
- Control (n=15)
- EphA4 MO- 5ng (n=10)
- EphA4 MO- 15ng (n=48)
- EphA4 MO- 30ng (n=15)
- m44 RNA- 90pg (n=8)
- m44 RNA- 200pg (n=15)
- m44 RNA- 500pg (n=57)
- m44 RNA- 800pg (n=15)
- EphA4 MO + m44 RNA- 90pg (n=10)
- EphA4 MO + m44 RNA- 200pg (n=10)
- EphA4 MO + m44 RNA- 500pg (n=24)
- EphA4 MO + m44 RNA- 800pg (n=40)

Note: All conditions are compared to the control group with statistical significance indicated by asterisks (*) for differences.
Figure 7B- EphA4 MO, mouse EphA4 RNA and Rescue Experiments when viewed at stage 11.5
Figure 8. EphA4 MO, *Xenopus* EphA4 RNA and Rescue Experiments when viewed at stage 11 (A) and 11.5 (B). Gastrulation defects were observed with EphA4 MO and *Xenopus* EphA4 RNA injections. Delay in gastrulation movements was specific, as co-injection of EphA4 MO with *Xenopus* EphA4 RNA led to a rescue. No significant differences were observed with control and embryos injected with 15ng of EphA4 MO and 500 pg of EphA4 RNA when viewed at stage 11 (p= 0.0682) or 11.5 (p= 0.0649). Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05).
Figure 8A- EphA4 MO, Xenopus EphA4 RNA and Rescue Experiments when viewed at stage 11

Control  | EphA4 MO- 5ng  | EphA4 MO- 15ng  | EphA4 MO- 30ng  
---|---|---|---
  |  |  |  

EphA4 RNA- 90pg  | EphA4 RNA- 200pg  | EphA4 RNA- 500pg  | EphA4 RNA- 800pg  
---|---|---|---
  |  |  |  

EphA4 MO- 15ng + EphA4 RNA- 500pg  | EphA4 MO- 15ng + EphA4 RNA- 800pg  
---|---
  |  

![Graph showing average archenteron length with error bars and statistical significance symbols (*) for different conditions.](image-url)
Figure 8B- EphA4 MO, Xenopus EphA4 RNA and Rescue Experiments when viewed at stage 11.5
Figure 9. EphA4 RNA can rescue Higher Concentrations of EphA4 MO. Gastrulation defects observed with 30ng of EphA4 MO were rescued with the addition of EphA4 RNA. No statistically significant differences were observed between control and 30ng of EphA4 MO + 500 pg of EphA4 RNA co-injected embryos (p= 0.2005). Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05).
Figure 9- EphA4 RNA can Rescue of Higher Concentration of EphA4 MO

Control   EphA4 MO- 30ng   EphA4 MO- 30ng + EphA4 RNA- 500pg   EphA4 MO- 30ng + EphA4 RNA- 800pg

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Archenteron Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>350</td>
</tr>
<tr>
<td>EphA4 MO- 30ng</td>
<td>150</td>
</tr>
<tr>
<td>EphA4 MO- 30ng + EphA4 RNA- 500pg</td>
<td>300 (n=16)</td>
</tr>
<tr>
<td>EphA4 MO- 30ng + EphA4 RNA- 800pg</td>
<td>320 (n=26)</td>
</tr>
</tbody>
</table>

* Indicates a significant difference compared to the control.
**Figure 10. Epiboly Movements in EphA4 MO Injected Embryos.** Epiboly movements were quantitatively assessed by measuring the dorsal (blue) and animal (red) roof thickness. No statistically significant differences were observed between control (A) and 15ng (B) and 30ng (C) of EphA4 MO injected embryos. Quantitative analysis of gastrulation defects are presented at the bottom panel (D). Results represent the mean +/- SD.
Figure 10- Epiboly Movements in EphA4 MO Injected Embryos

A. Control

B. EphA4 MO-15ng

C. EphA4 MO-30ng

D. Graph showing average roof thickness with error bars for control, EphA4 MO-15ng, and EphA4 MO-30ng groups. The graph compares dorsal and animal roof thicknesses.
Figure 11. Convergent Extension Movements in EphA4 MO Injected Embryos. Convergent extension movements were assessed by measuring the length-to-width ratio of controls and 15ng and 30ng of EphA4 MO injected explants. No significant differences were observed between control and 15ng of EphA4 MO injections (p = 0.2170). However, significant differences were observed between control and 30ng of EphA4 MO injected explants (p = 0.0226). Quantitative analysis of CE movements is presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05).
Figure 11- Convergent Extension Movements in EphA4 MO Injected Embryos

A. Control

B. EphA4 MO- 15ng

C. EphA4 MO- 30ng

![Bar Chart]

- **Average Length to Width Ratio**
  - **Control**: n=15
  - **EphA4 MO- 15ng**: n=14
  - **EphA4 MO- 30ng**: n=10

*Statistical significance*
width ratio between control and 30ng of EphA4 MO injected embryos (Figure 11A and C). Similar data has been presented in a recent abstract by Park and colleagues (2009), although the construct (MO or RNA), concentration, and area of injections (dorsally or animaly) were never mentioned (Park and Han, 2009). These results suggest that gastrulation defects induced by knockdown of EphA4 are not a result of improper CE movements, with effective concentrations of EphA4 MO.

We therefore wondered whether gastrulation defects may be a result of defective involution. I assessed involution behavior using novel dorsal blastopore lip (DBL) assays. Briefly, DBL explants were isolated from whole embryos at stage 10 and fixed at stage 11 (Figure 4). I observed 3 prominent phenotypes, a complete involution (a distinct “elbow” curvature is present), intermediate involution (migration of BC along the mesoderm), and no involution (BC were positioned away from the mesoderm). I observed a significant reduction in explants displaying complete and intermediate involution movements when compared to 15ng and 30ng EphA4 MO injected embryos. The total number of DBL explants showing normal or intermediate involution behavior in control, 15ng and 30ng of EphA4 MO injected DBL was 37/37 (100%), 8/24 (33%), and 8/30 (27%), respectively (Figure 12). These mutant phenotypes were specific as injection of 500 pg of wtEphA4 RNA rescued EphA4 MO induced mutant phenotypes. The average number of DBL explants showing normal or intermediate involution behavior when 15ng of EphA4 MO and 500 pg of *Xenopus* EphA4 RNA was co-injected was 13/16 (81%). These results suggest that gastrulation defects induced by EphA4 MO injections are a result of defective involution movements. Our DBL explants confirm that EphA4-induced defects were tissue autonomous, consistent with a failure to involute.

2. MO Knockdowns of EphA4 can have Profound Effects on Maintenance of Xbra expression during Involution without Affecting Xbra Induction

In order to assess if gastrulation defects were the result of improper mesoderm specification, I performed whole mount *in situ* hybridization for the posterior mesoderm marker, *Xenopus*
Figure 12. Involution Movements in EphA4 MO Injected Embryos. The DBL of control and 15ng and 30ng of EphA4 injected embryos were removed and categorized into 3 main involution phenotypes, normal involution (blue), intermediate involution (red), and no involution (yellow). The total number of explants were counted and the percentage was graphed. EphA4 induced involution defects were highly specific as injection of EphA4 RNA was sufficient to rescue mutant phenotypes.
Figure 12- Involution Movements in EphA4 MO Injected Embryos

Normal Involution  Intermediate Involution  No Involution

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage of Explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100%</td>
</tr>
<tr>
<td>EphA4 MO-15ng n=24</td>
<td>90%</td>
</tr>
<tr>
<td>EphA4 MO-30ng n=30</td>
<td>80%</td>
</tr>
<tr>
<td>EphA4 MO-15ng + EphA4 RNA-500pg n=16</td>
<td>70%</td>
</tr>
</tbody>
</table>

Legend:
- No Involution
- Intermediate Involution
- Normal Involution
brachyury (Xbra), the anterior mesoderm marker, goosecoid (gsc), the mes-endoderm marker, Cerberus (cer), the dorsal mesoderm factor, chordin (chd), neural marker, Sox2, and the notochord marker, Xnot. For Xbra, I assessed expression at stage 9 corresponding to initial mesoderm formation, at stage 10.5 prior to involution, and at stage 11, when dorsal mesoderm was internalized. Our whole mount *in situ* hybridization data was scored based on 3 prominent phenotypes observed, strong/normal Xbra expression, reduced Xbra expression, or a total absence of Xbra expression.

At stage 9, 15/15 (100%) of un-injected embryos, 10/11 (91%) of 15ng of EphA4 MO injected embryos, and 6/9 (67%) of 30ng of EphA4 MO injected embryos displayed strong Xbra expression (Figure 13). Reduced Xbra expression was observed in 1/11 (9%) and 2/9 (22%) of embryos injected with 15ng and 30ng of EphA4 MO, respectively (Figure 13B and C). Absence of Xbra was only observed in 1/8 (11%) of embryos injected with 30ng of EphA4 MO (Figure 13C). I did not observe significant differences in Xbra expression between control and EphA4 MO injected embryos. These results suggest that EphA4 does not play a significant role during initial Xbra induction.

At stage 10.5, I observed a significant reduction in Xbra expression in embryos injected with EphA4 MO. Xbra expression was reduced or absent in 41/51 (83%) or 10/10 (100%) of embryos injected with 15ng or 30ng of EphA4 MO, respectively (Figure 14). These results suggest that EphA4 may be responsible for regulating Xbra specifically during involution. Loss of Xbra expression induced by EphA4 MO injections were highly specific, as Xbra expression could be restored with the addition of wtEphA4 RNA (Figure 14D). Embryos injected with 15ng of EphA4 MO and 500 pg of EphA4 RNA, 18/25 (72%) showed normal Xbra expression.

We assessed Xbra expression at stage 11.5 in EphA4 MO injected embryos. Although the area of Xbra expression was markedly reduced with the injection of 15ng or 30ng of EphA4 MO, the staining intensity of this domain was similar to controls (Figure 15). I believe that defective involution movements and the failure of the Xbra domain to converge medio-laterally may account for differences in Xbra expression between control and MO injected embryos (Figure 15B and C). This domain was restored when involution was rescued, as seen by the addition of 500 pg of EphA4 RNA (Figure 15D). These results suggest that Xbra
Figure 13. Xbra Expression in EphA4 MO Injected Embryos at stage 9. Xbra expression was assessed in control (A) and 15ng (B) and 30ng (C) of EphA4 injected embryos. Staining intensities were categorized into 3 groups: strong (yellow), reduced (red), and not detected (blue), and averaged (D). Results represent the mean.
Figure 13- Xbra Expression in EphA4 MO Injected Embryos at stage 9

A. Control
B. EphA4 MO-15ng
C. EphA4 MO-30ng

D. Percentage of Embryos

- Lost
- Reduced
- Normal

Control: n=15
EphA4 MO-15ng: n=11
EphA4 MO-30ng: n=9
Figure 14. Xbra Expression in EphA4 MO and RNA Injected Embryos at stage 10.5. Xbra expression was assessed in sagittally sectioned control (A) and 15ng (B) and 30ng (C) of EphA4 MO injected embryos and staining intensities were categorized into 3 groups: strong (yellow), reduced (red), and absent (blue). Xbra expression in unfractured embryos is presented in the upper inset. Reduced Xbra expression was observed with 15ng and 30ng of EphA4 MO injections but expression was restored with co-injection of EphA4 RNA (D). Ectopic Xbra expression was also observed with rescue experiments (D, red arrows, side inset zoom). EphA4 mRNA injections alone stimulated ectopic Xbra expression along the BCR (E, red arrows) or marginally (F, red arrows). Quantitative analysis of Xbra expression at stage 10.5 is presented at the bottom panel (H). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 14- Xbra Expression in EphA4 MO Injected Embryos at stage 10.5

A. Control
B. EphA4 MO- 15ng
C. EphA4 MO- 30ng
D. EphA4 MO- 15ng + EphA4 RNA 500pg
E. EphA4 RNA-500pg
Marginal
F. EphA4 RNA- 500pg
BCR

H.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control</td>
<td>100%</td>
</tr>
<tr>
<td>EphA4 MO- 15ng</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO- 30ng</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO- 15ng + EphA4 RNA 500pg</td>
<td></td>
</tr>
</tbody>
</table>

n=20  n=51  n=10  n=25
Figure 15. Xbra Expression in EphA4 MO and PAK1 Injected Embryos at stage 11.5. Xbra expression was assessed in sagittally sectioned and whole embryos (upper inset) in control (A), 15ng (B) and 30ng (C) of EphA4 MO, 15ng of EphA4 MO and EphA4 RNA (D), and kinase dead (kd, M4) PAK1 injected embryos (E). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 15- Xbra Expression in EphA4 MO and PAK1 Injected Embryos at stage 11.5

A. Control  
B. EphA4 MO- 15ng  
C. EphA4 MO- 30ng  
D. EphA4 MO- 15ng + EphA4 RNA- 500pg  
E. M4- 400pg
expression at stage 11.5 is attenuated because of defective involution movements and a failure of this domain to converge. We have shown that EphA4 MO injections severely perturb involution. I therefore performed whole mount in situ hybridization on DBL explants in control and EphA4 MO injected embryos. When compared to controls, the localization and concentration of Xbra was severely perturbed with injections of 15ng of EphA4 MO. I observed reduced Xbra expression at stage 10.5 and abnormal/posterior localization of Xbra at both stage 10.5 and 11 (Figure 16C and D). Uninjected DBL explants showed strong Xbra expression at the edge of the elbow-curve/site of involution, consistent with our findings (Figure 16A and B). These results suggest that EphA4 may play a significant role in regulating Xbra expression and involution movements during Xenopus gastrulation. Furthermore, I assessed the expression of various mesoderm or neural factors using in situ hybridization. I did not observe any differences in Gsc, Cer, Chd, Sox2, or XNOT expression between control and MO injected embryos (Figure 17). Thus, I did not observe cell fate changes in the mesoderm, even within the chordamesoderm, where XNOT is normally expressed and where CE movements normally occur

3. EphA4 can Promote Localized Xbra Expression

Interestingly, I observed ectopic Xbra expansion along the inner surface of the BCR in the rescue experiments at stage 10.5, suggesting that EphA4 can regulate Xbra expression (Figure 14D, red arrows). To further assess EphA4’s Xbra inductive capabilities, I injected EphA4 mRNA in the animal blastomere (future site of the BCR) or dorsal marginally at the late 4-cell stage embryo. In both conditions I observed ectopic Xbra expression (Figure 14E and F, red arrows). With respect to BCR injections Xbra expression was observed, similar to rescue experiments, although at a stronger intensity, along the inner surface of the BCR (Figure 14F, red arrows). Xbra expression was seen at the interface between the roof and the leading edge mesoderm. In marginally injected embryos, Xbra was ectopically expressed in the deep mesoderm tissue. These results suggest that EphA4 can regulate Xbra expression (Figure 14D, E, and F).
Figure 16. Involution Behavior and Xbra Expression in DBL Explants of EphA4 MO injected embryos. In control DBL explants, strong Xbra expression was localized posteriorly at stage 10.5 (A) and at stage 11(B), Xbra expression was observed along the c-shaped curvature, consistent with proper involution and superposition of this domain. Injection of EphA4 MO resulted in reduced (C, stage 10.5) and abnormal localization (D, stage 11) of Xbra. Arrows mark the position of the bottle cells.
Figure 16- Involution Behavior and Xbra Expression in DBL Explants of EphA4 MO Injected Embryos

<table>
<thead>
<tr>
<th>Stage 10</th>
<th>Stage 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>B.</td>
</tr>
</tbody>
</table>

**Control**

<table>
<thead>
<tr>
<th>Stage 10</th>
<th>Stage 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.</td>
<td>D.</td>
</tr>
</tbody>
</table>

**EphA4 MO - 15ng**
Figure 17. *In situ* Hybridization of various mesoderm and neural markers. Goosecoid, Cerberus, Chordin, Xnot, or Sox2 was analyzed in control (A-E) and 15ng of EphA4 MO injected embryos (F-J). Normal Gsc, Cer, Chd, XNOT, or Sox2 expression was observed between EphA4 MO injected and un-injected control embryos. Arrows mark the position of the bottle cells.
Figure 17- in situ hybridization of Various Mesoderm and Neural Markers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EphA4 MO- 15ng</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Goosecoid</strong></td>
<td><img src="imageA" alt="Image" /></td>
<td><img src="imageF" alt="Image" /></td>
</tr>
<tr>
<td><strong>Cerberus</strong></td>
<td><img src="imageB" alt="Image" /></td>
<td><img src="imageG" alt="Image" /></td>
</tr>
<tr>
<td><strong>Chordin</strong></td>
<td><img src="imageC" alt="Image" /></td>
<td><img src="imageH" alt="Image" /></td>
</tr>
<tr>
<td><strong>XNOT</strong></td>
<td><img src="imageD" alt="Image" /></td>
<td><img src="imageI" alt="Image" /></td>
</tr>
<tr>
<td><strong>Sox2</strong></td>
<td><img src="imageE" alt="Image" /></td>
<td><img src="imageJ" alt="Image" /></td>
</tr>
</tbody>
</table>
We hypothesize that an endogenous EphA4 ligand may be present within the involuted mesoderm. Interactions between ectopic EphA4 receptors along the BCR (Figure 5B, red) and endogenous EphA4 ligands within the leading edge mesoderm (Figure 5B, grey) may promote Xbra expression at stage 10.5 (Figure 5B, blue). Vegetal rotation is known to position the leading edge mesoderm against the BCR during Xenopus gastrulation (Ibrahim and Winklbauer, 2001). To confirm EphA4’s ability to promote Xbra expression and to identify potential EphA4 ligands, I performed novel roof-mesoderm combination experiments. In this assay, stage 10 control or EphA4 RNA injected roofs were cut and combined with isolated leading edge mes-endoderm for in situ hybridization of Xbra expression, at stage 10.5 (Figure 5A).

No Xbra was detected when un-injected roofs were combined with un-injected roofs (Figure 18A), when EphA4 RNA injected roofs were combined with EphA4 RNA injected roofs (Figure 18B), and when un-injected roofs were combined with mesoderm (Figure 18C). In contrast, Xbra expression was observed when EphA4 RNA injected roofs were combined with mesoderm (Figure 18D). In all cases when EphA4 RNA was expressed in the roof, I observed localized Xbra expression.

Localized expression of Xbra may be explained by several factors, such as the dorsal-ventral specificity of the animal cap tissue or the presence of endoderm in cer-positive leading edge mesoderm used in this assay. To address the first possibility, I simply bisected animal caps into dorsal and ventral halves and combined them with leading edge mesoderm as previously described and assessed Xbra expression. Dorsal side of embryos was evident by the beginning of BC formation. No Xbra was observed in EphA4 ventral injected roof and mesoderm combined experiments (Figure 18E). Interestingly, Xbra was only present when EphA4 dorsal injected roof were combined with mesoderm (Figure 18F). No Xbra was observed in dorsal or ventral un-injected roof-roof or in dorsal or ventral EphA4 injected roof-roof combined explants (data was similar to Figure 18A). Furthermore, no Xbra expression was observed when EphA4 injected roofs were combined with vegetal endoderm (Figure 18G), confirming the presence of an endogenous EphA4 ligand located within the leading edge mesoderm. When EphA4 RNA was injected ventrally, ectopic posterior elongations of Xbra expression was observed along the inner surface of the BCR (Figure 18H, red arrows), however, Xbra intensity was much lower
**Figure 18. Localized Xbra Expression in EphA4 Injected Roof-Mesoderm Combinations.**

Xbra expression was assessed in un-injected roof (R)-R (A), EphA4 injected roof (R+)-R+ (B), R-mesoderm (M) (C), R+-M (D), ventral EphA4 injected roof (VR+)-M (E), dorsal EphA4 injected roof (DR+)-M (F) and R+-endoderm (VEG) combined explants (G). Localized Xbra expression was observed on the dorsal side of EphA4 injected embryos. Weak ectopic Xbra expression along the BCR was observed (red arrows) in ventral EphA4 mRNA injected embryos (H). Dorsal is to the right and arrows mark the position of the bottle cells.
Figure 18-Localized Xbra Expression in EphA4 Injected Roof-Mesoderm Combinations

A. R/R  
B. R+/R+  
C. R/M  
D. R+/M  
E. VR+/M  
F. DR+/M  
G. R+/VEG  
H. EphA4 RNA-500pg Ventral injection
when compared to dorsal injections (Figure 14, red arrows). These results are consistent with our findings which suggest that EphA4-mediated Xbra induction may be a specific property of the dorsal roof and an endogenous EphA4 ligand may be located in the mesoderm.

4. Wild type (wt) PAK1 is a Downstream Target of EphA4

Bisson and colleagues (2007) have identified an EphA4-PAK1 pathway regulating cell adhesion in the *Xenopus* ectoderm in an artificial situation, by ectopically overexpressing active EphA4 (Bisson *et al.*, 2007). In order to determine if PAK1 is a downstream target of EphA4 in our context, EphA4 MO was co-injected with 450 pg of PAK1 mRNA. Compared to EphA4 MO injected embryos, I observed a statistically significant rescue of gastrulation at stage 11 with co-injection of PAK1 mRNA (Figure 19). I did not observe any statistically significant differences in archenteron length between control and EphA4 MO + wtPAK RNA injected embryos. However, co-injection of 30ng of EphA4 MO with 450 pg or 800 pg of wtPAK1 RNA did not rescue gastrulation in embryos (Figure 19). Injections of 450 pg of wtPAK1 alone resulted in significant reduction in gastrulation movements when compared to controls. Similarly, I observed significant reductions in archenteron length when 400 pg of kinase dead (kd, M4) PAK1 was injected, consistent with Macanovic *et al.*, (unpublished). These results suggest that PAK1 may be a potential downstream target of EphA4 and that PAK1 mediated rescues requires EphA4.

We have demonstrated that EphA4 MO phenotypes were a result of defective involution movements. I therefore tried to rescue EphA4 MO involution phenotypes with co-injection of wtPAK1 mRNA. I observed significant number of explants, 10/11 (91%), showing normal or intermediate involution behavior when 15ng of EphA4 MO was co-injected with wt PAK1 (Figure 20). Furthermore, injections of PAK1 alone lead to involution defects, suggesting that the precise concentration of PAK1 is essential for involution. I observed normal or intermediate involution behavior in 1/19 (5%) of explants injected with PAK1 RNA. Our results present similar findings as Bisson and colleagues (2007) (Bisson *et al.*, 2007), who suggested that PAK1 acts downstream of EphA4 to regulate tissue adhesion and in our case mesoderm involution.
Figure 19. EphA4 MO Gastrulation Defects were Rescued with wtPAK1 co-injection at stage 11. Gastrulation defects induced by 15ng of EphA4 MO were rescued with the addition of wtPAK1. No statistically significant differences in archenteron length were observed between control and EphA4 MO + wtPAK RNA injected embryos (p =0.2495). In contrast, 30ng of EphA4 were not rescued with 450 or 800 pg of PAK1 injections (p < 0.0001 and p < 0.0001, respectively, when compared to controls). Injections of 450 pg of wtPAK1 mRNA alone resulted in significant reduction in gastrulation movements when compared to controls (p <0.001).
Quantitative analysis of Xbra expression at stage 10.5 is presented at the bottom panel. Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 19- EphA4 MO Gastrulation Defects were Rescued with wtPAK1 co-injection at stage 11

![Image of embryos with different treatments showing rescued gastrulation defects]

![Bar graph showing average archenteron length with error bars]

Legend:
- Control
- EphA4 MO- 5ng
- EphA4 MO- 15ng
- EphA4 MO- 30ng
- wtPAK1 RNA- 450pg
- EphA4 MO- 15ng + wtPAK1 RNA- 450pg
- EphA4 MO- 30ng + wtPAK1 RNA- 450pg
- EphA4 MO- 30ng + wtPAK1 RNA- 800pg

*Denotes statistical significance compared to control
Figure 20. EphA4 MO Involution Defects were Rescued with co-injection of wtPAK1.

Defective involution movements induced by EphA4 MO injections were rescued with the addition of wtPAK1. Injection of wtPAK1 alone lead to involution defects. Number of explants were averaged into the 3 categories, normal involution (blue), intermediate involution (red), and no involution (yellow). Bars 1-4 were previously presented in figure 12.
Figure 20- EphA4 MO Involution Defects were Rescued with co-injecton of wtPAK1.
It has previously been demonstrated by Macanovic et al., that kdPAK1 can reduce Xbra expression at stage 11 (Figure 15) (Macanovic et al., unpublished). However, it is also possible that apparent reductions in Xbra expression may be due to defective internalization and improper movement of the Xbra domain. I therefore assessed Xbra expression in kdPAK1 injected embryos at stage 10.5, prior to involution. I observed significant reductions in Xbra expression with kdPAK1 injections (Figure 21E).

To further validate PAK1 as a potential downstream target of EphA4, I injected 15ng of EphA4 MO + 450 pg of wtPAK1 RNA and performed whole mount in situ hybridization for Xbra expression. EphA4 MO has been shown to repress Xbra expression at this stage, however, when 15ng of EphA4 MO and 450 pg of wt PAK RNA were co-injected, I observed Xbra expression, thus confirming our previous observation (Figure 21C). In contrast, I did not observe Xbra expression in embryos injected with 30ng of EphA4 MO and 450 pg of wtPAK1 RNA. In this study I have shown that injections of 450 pg or 800 pg of wtPAK1 was not sufficient to rescue gastrulation defects caused by higher concentrations of EphA4 MO, suggesting that EphA4 is required for PAK1 mediated rescues (Figure 19). These results indicate that an EphA4-PAK1 interaction is critical for Xbra expression (Figure 22C) and gastrulation (Figure 19). Furthermore, ectopic Xbra expression caused by EphA4 RNA 500 pg injections were severely reduced with kd PAK1 co-injection (Figure 21F).

We have shown that EphA4 has a capacity to promote Xbra expression at stage 10.5. Furthermore, both our study and that of Bisson et al., (2007), showed PAK1 as a downstream effector of EphA4 signaling during Xenopus development (Bisson et al., 2007). I therefore wondered if constitutively active (ca) Pak1 constructs can induce ectopic Xbra expression. Injection of 30 pg, 60 pg, and 90 pg of caPAK1 RNA in the animal blastomere at the 4-cell stage (future site of the BCR) resulted in stronger Xbra expression, in a concentration dependent manner (Figure 23 B-D) in the marginal zone, however, I did not observe ectopic Xbra expression along the BCR (Figure 23 B-D). These finding are consistent
Figure 21. Xbra Expression in EphA4 MO and wtPAK1 Injected Embryos at stage 10.5.

When compared to controls (A), reduced Xbra expression was observed with 15ng (B) and 30ng (C) of EphA4 MO injected embryos. Reduced Xbra expression was also observed with kdPAK1 (M4) injections (D). Expression induced by EphA4 MO was restored with the addition of wtPAK1 mRNA (E). Xbra expression along the BCR induced by EphA4 RNA was reduced with the co-injection of kdPAK1 (F). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 21- Xbra Expression in EphA4 MO and wtPAK1 Injected Embryos at stage 10.5

A. Control
B. EphA4 MO- 15ng
C. EphA4 MO- 30ng
D. M4- 400pg
E. EphA4 MO-15ng + wtPAK1 RNA 450pg
F. EphA4 RNA 500pg + M4 RNA 800 pg
Figure 22. PAK1 cannot Rescue Xbra Expression in Higher EphA4 MO Injections. An absence of Xbra expression was observed when compared to controls (A) in embryos injected with 30ng of EphA4 MO (B) and with 30ng of EphA4 MO + 450 pg of wtPAK1 RNA (C).
Figure 22- PAK1 cannot Rescue Xbra expression with Higher EphA4 MO Injections

A. Control
B. EphA4 MO-30ng
C. EphA4 MO-30ng + PAK1 RNA-450pg
Figure 23. Strong Marginal Zone Xbra Expression in caPAK1 Injected Embryos. Xbra expression was assessed in control (A) and embryos injected with 30 (B), 60 (C), or 90 pg (D) of caPAK1 mRNA dorso-animally in the late 4 cell-stage embryo (future site of BCR). No Xbra was observed along the BCR-mesoderm interface. However, Xbra expression was stronger along the native marginal zone, in a concentration dependent manner.
Figure 23- Strong Marginal Zone Xbra Expression in caPAK1 Injected Embryos

A. Control
B. CA PAK- 30pg
C. CA PAK- 60pg
D. CA PAK- 90pg
with those of Dr. Moss (Dr. R. Winklbauer, personal communication). These results suggest that an EphA4-PAK1 interaction is required for Xbra expression and that PAK1 alone is not sufficient. This is consistent with our sagittally sectioned embryos and in situ hybridization findings.

5. Precise Tempo-Spatial Regulation of Xbra is Essential for Involution

In this study I have shown that EphA4 RTKs can regulate Xbra expression and mesoderm involution using PAK1. I therefore wondered whether Xbra expression in EphA4 MO and kdPAK1 injected embryos can rescue gastrulation and involution behavior. To assess rescue concentrations, I overexpressed Xbra dorsal marginally and screened sagittally sectioned embryos and DBL explants. I saw a statistically significant delay in gastrulation with the injection of 100 pg, 50 pg, and 25 pg of Xbra RNA. Expression of 10 pg of Xbra resulted in minimal gastrulation defects, however these were significantly different when compared to controls (Figure 24). Expression of Xbra RNA was sufficient to delay gastrulation movements in whole embryos, suggesting that Xbra concentrations are critical for gastrulation. Injection of Xbra can lead to widespread expression, beyond the normal expression domain, and to nonspecific secondary defects in *Xenopus* embryos (Tada *et al.*, 1997, Cunliffe and Smith, 1992).

Injection of 100 pg, 50 pg, 25 pg, or 10 pg of Xbra RNA significantly perturbed involution movements, as assessed by our DBL explants. On average, I observed normal or intermediate involution behavior in 3/11 (27%), 3/9 (33%), 3/9 (33%), 10/11 (91%) of explants injected with 100 pg, 50 pg, 25 pg, or 10 pg of Xbra RNA, respectively (Figure 25). These results suggest that proper concentration of Xbra is critical for involution and our DBL explants suggest that defects were tissue autonomous: characteristic of attenuation of involution.

As demonstrated, EphA4 MO injections of 15ng and 30ng can result in a significant delay in gastrulation and Xbra expression. When 15ng of EphA4 MO was co-injected with 25 pg or 10 pg of Xbra RNA, I did not observe a rescue in gastrulation movements (Figure 24). As determined by our Xbra overexpression experiments, I used 25 pg or 10 pg of Xbra RNA for
Figure 24. Delayed Gastrulation Movements after Xbra RNA Injections. Addition of Xbra RNA lead to gastrulation defects. Statistically significant differences in gastrulation movements were observed with 100 pg, 50 pg, and 25 pg of Xbra mRNA injection when compared to controls (p < 0.0001 for all conditions). Expression of 10 pg of Xbra resulted in minimal gastrulation defects, however these were significantly different when compared to controls (p < 0.0001). EphA4 induced mutant phenotypes were not rescued with the addition of Xbra RNA. Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 24- Delayed Gastrulation Movements after Xbra RNA Injections
Figure 25. Involution Movements in DBL Explants Injected with EphA4 MO, Xbra RNA, and EphA4 MO + Xbra. Defective involution movements were observed with the addition of EphA4 MO and Xbra RNA or with co-injection of EphA4 MO + Xbra. Phenotypes were grouped into 3 categories: normal involution (blue), intermediate involution (red), and no involution (yellow).
Figure 25- Involution Movements in DBL Explants Injected with EphA4 MO, Xbra RNA, and EphA4 MO + Xbra

<table>
<thead>
<tr>
<th></th>
<th>Normal Involution</th>
<th>Intermediate Involution</th>
<th>No Involution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>EphA4 MO- 15ng</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>EphA4 MO- 30ng</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Xbra RNA- 10pg</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Xbra RNA- 25pg</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>Xbra RNA- 50pg</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>Xbra RNA- 100pg</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
</tr>
<tr>
<td>EphA4 MO- 15ng</td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
<tr>
<td>EphA4 MO- 15ng + Xbra RNA- 25pg</td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
</tr>
</tbody>
</table>

Percentage of Explants

- Control: n=37
- EphA4 MO- 15ng: n=24
- EphA4 MO- 30ng: n=30
- Xbra RNA- 10pg: n=11
- Xbra RNA- 25pg: n=9
- Xbra RNA- 50pg: n=9
- Xbra RNA- 100pg: n=11
- EphA4 MO- 15ng + Xbra RNA- 25pg: n=14
- EphA4 MO- 15ng + Xbra RNA- 10pg: n=9

Legend:
- □ No Involution
- ■ Intermedia Involution
- ● Normal Involution
Figure 26. Rescue of EphA4 MO Mutant Phenotypes using Xbra-GR RNA Constructs.

Gastrulation movements were assessed in EphA4 MO and Xbra-GR injected and EphA4 MO + Xbra-GR co-injected embryos. Xbra-GR + DEX treatments lead to severe gastrulation defects. When compared to controls, injection of 10 pg of Xbra-GR did not significantly delay gastrulation movements in the absence of DEX (p = 0.1036), however, when exposed to DEX, significant reductions in gastrulation movements were observed (p < 0.0001). Co-injection of 15ng of EphA4 MO with 25 or 10 pg of Xbra-GR with DEX treatment at stage 9 did not rescue whole embryos (p < 0.0001 and p < 0.0001, respectively). However, when 15ng of EphA4 MO and 10 pg of Xbra-GR were co-injected and treated with DEX at stage 10+, I observed a statistically significant rescue in gastrulation movements when compared to controls (p = 0.4245). Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 26- Rescue of EphA4 MO Mutant Phenotypes using Xbra-GR

![Image showing rescued phenotypes with EphA4 MO and Xbra-GR treatments with or without DEX addition.](image)

Figure legend: Rescue of EphA4 MO Mutant Phenotypes using Xbra-GR. The images show rescued phenotypes with EphA4 MO and Xbra-GR treatments with or without DEX addition. The graphs below the images represent the average archetypal length with error bars, indicating statistical significance with asterisks.

- **Control**
- **Control + DEX**
- **EphA4 MO- 15ng**
- **Xbra-GR- 10pg**
- **Xbra-GR- 10pg + DEX**
- **Xbra-GR- 25pg**
- **Xbra-GR- 25pg + DEX**
- **EphA4 MO- 15ng + Xbra-GR- 10pg + DEX st. 9**
- **EphA4 MO- 15ng + Xbra-GR- 10pg + DEX st. 10**
- **EphA4 MO- 15ng + Xbra-GR- 25pg + DEX st. 9**
- **EphA4 MO- 15ng + Xbra-GR- 25pg + DEX st. 10**

Graphs show:

- **Average Archetypal Length**
- **Control n=25**
- **Control+DEX n=19**
- **A4 MO-15ng n=48**
- **Xbra-GR-10pg n=7**
- **Xbra-GR-10pg+DEX n=18**
- **Xbra-GR-25pg n=8**
- **Xbra-GR-25pg+DEX n=12**
- **A4MO-15ng+Xbra-GR-10pg+DEX st.9 n=17**
- **A4MO-15ng+Xbra-GR-10pg+DEX st.10 n=24**
- **A4MO-15ng+Xbra-GR-25pg+DEX st.9 n=12**
- **A4MO-15ng+Xbra-GR-25pg+DEX st.10 n=10**

**Statistical Significance:** Indicates significant difference with asterisks.
rescue experiments, because it was between these two concentrations were I had observable effects on gastrulation movements.

Inability of Xbra to rescue EphA4 mutant phenotypes may be a result of improper spatial regulation of Xbra, as injections lead to widespread expression of RNA in the whole embryo. To restrict this effect of ubiquitous Xbra expression, I isolated DBL explants and examined involution behavior. As previously described, injections of 15ng and 30ng of EphA4 MO increased the number of explants displaying incomplete involution behavior, in a concentration dependent manner. Co-injection of 15ng of EphA4 MO with 25 pg or 10 pg of Xbra RNA did not improve involution behavior. The average number of explants co-injected with 15ng of EphA4 MO and 25 or 10 pg of Xbra RNA that displaying normal or intermediate involution was 4/14 (29%) and 2/9 (22%), respectively (Figure 25).

Interestingly, majority of 5ng of EphA4 MO or 10 pg of Xbra RNA injected explants did show either a normal or intermediate involution phenotype. When 5ng of EphA4 MO and 10 pg of Xbra RNA were co-injected, majority of explants showed normal involution behavior, suggesting that Xbra is in fact downstream of EphA4 (data not shown).

Furthermore, injections of RNA are believed to be translated immediately without proper temporal regulation and thereby can have profound effects on surrounding tissues. It is possible that improper temporal regulation of Xbra may allow it to disrupt surrounding tissues and thereby effect involution. I therefore used a hormone-inducible Xbra construct to temporally regulate Xbra expression.

To assess the amount of RNA required to rescue, I over expressed Xbra-GR RNA constructs in whole embryos. Injections of 10 pg of Xbra-GR did not significantly delay gastrulation movements in the absence of DEX, however, when embryos were exposed to DEX, statistically significant reductions in gastrulation movements were observed. Injections of 25 pg of Xbra-GR with or without DEX delayed gastrulation movements (Figure 26).

Embryos were co-injected with 15ng of EphA4 MO and 25 pg or 10 pg of Xbra-GR and DEX was applied at stage 9 (mesoderm formation) or stage 10 (onset of involution). Injection of 25 pg
or 10 pg of Xbra-GR RNA was used for rescue experiments, because it was between these two concentrations were I had observable effects on gastrulation.

Co-injection of 15ng of EphA4 MO with 25 pg or 10 pg of Xbra-GR with DEX treatment at stage 9 did not rescue whole embryos. However, when 15ng of EphA4 MO and 10 pg of Xbra-GR were co-injected and treated with DEX at stage 10+, I observed a statistically significant rescue in gastrulation movements when compared to controls (Figure 26). Our results confirm that EphA4 can regulate Xbra expression and precise spatio-temporal regulation of Xbra is required for proper involution of the mesoderm.

It has been demonstrated that injections of Xbra or constitutively active (ca) Mek constructs were sufficient to induce mesoderm formation in animal caps (LaBonne et al., 1995, Tada et al., 1997). Similarly, I injected 400 pg of Xbra or 1ng of caMek animaly (LaBonne et al., 1995, Tada et al., 1997). I observed mesoderm induction, made evident by a smooth spherical surface, tissue elongation, and fluid-filled sacs, with Xbra or caMek injections (Figure 27B and C, red arrows). I did not observe mesoderm induction in control embryos (Figure 27A, yellow arrows). All of these explants had a wrinkled surface epithelium, consistent with ectoderm formation. These results confirm that our Xbra and caMek RNAs are functional in Xenopus. Furthermore, I observed normal mesoderm induction in caMek RNA constructs that were co-injected with 15ng of EphA4 MO (Figure 27D, red arrows). Consistent with EphA4’s inability to alter early mesoderm induction. I was not able to induce mesoderm in explants injected with caMek RNA and 30ng of EphA4 MO, as all embryos died after treatment.
Figure 27. Mesoderm Induction in Animal Caps. Control (A) and Xbra (B), caMEK (C), or EphA4 MO + caMEK (D) were co-injected into animal caps and mesoderm induction was assessed. Injection of Xbra, caMEK, or EphA4 MO + caMEK promoted mesoderm formation (red arrows), while un-injected caps formed ectoderm (yellow arrows).
Figure 27 - Mesoderm Induction in Animal Caps

Control

Xbra RNA- 500pg

caMEK RNA- 1ng

EphA4 MO- 15ng + caMEK RNA- 1ng

D.
6. Constitutively active Mek cannot Rescue EphA4 MO-Induced Gastrulation Defects. Proper Tempo-Spatial Regulation of Mek/Xbra is Required for Involution.

The FGF/ras/Mek signaling pathway is required for initiating and maintaining Xbra expression (Isaacs et al., 1994, Fletcher and Harland, 2008). The FGF signaling pathway follows a consecutive activation of Ras, Raf, Mek, and Erk. Upon activation, Erk can translocate into the nucleus and activate various downstream targets, including Xbra (Yang et al., 2003). In this study, I have shown that EphA4 can positively regulate Xbra expression using PAK1. I therefore tried to identify the location of the novel EphA4/PAK1/Xbra cascade within the FGF/ras/Mek/Xbra signaling pathway. Co-injection of 15ng of EphA4 MO or 450 pg of kdPAK1 with 100 pg, 150 pg, 200 pg, or 250 pg of caMek was not sufficient to rescue gastrulation movements (Figure 28).

Injections of caMek can lead to widespread RNA expression and delayed gastrulation movements. It is also possible that caMek was not able to rescue EphA4 mutant phenotypes because of improper tempo-spatial-regulation of Mek/Xbra. I therefore assessed Xbra expression in embryos co-injected with 15ng of EphA4 MO and 250 pg of caMek RNA. I performed in situ hybridization on embryos injected with 15ng of EphA4 MO and 150 or 250 pg of caMek dorsal marginally at the late 4-cell stage. As expected, co-injection of caMek lead to widespread Xbra expression (Figure 29 C and D, red arrows). Xbra expression was often noted in the roof, endoderm, and inner mesoderm of embryos. These results also confirm that the MAPK pathway is a downstream target of EphA4 signalling, as Xbra expression was restored after EphA4 MO injection. These results also suggest that proper tempo-spatial regulation of Xbra is essential for involution and gastrulation in Xenopus.
Figure 28. caMek cannot Rescue EphA4 MO or kdPAK1 Gastrulation Defects. EphA4 induced gastrulation defects were not rescued with the addition of caMek (for all conditions, p < 0.0001). Quantitative analysis of gastrulation defects are presented at the bottom panel. Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 28 - caMEK Cannot Rescue of EphA4 MO or kdPAK1 Gastrulation Defects

![Images of gastrulation defects with different treatments]

![Bar graph showing average archenteron length with error bars and asterisks for statistical significance]

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Average Archenteron Length</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-15ng n=48</td>
<td>15</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-30ng n=15</td>
<td>20</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-15ng + caMEK RNA-100pg n=26</td>
<td>15</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-15ng + caMEK RNA-150pg n=20</td>
<td>15</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-15ng + caMEK RNA-200pg n=10</td>
<td>15</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>EphA4 MO-15ng + caMEK RNA-250pg n=9</td>
<td>15</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>M4 RNA-400pg + caMEK RNA-100pg n=18</td>
<td>18</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>M4 RNA-400pg + caMEK RNA-150pg n=14</td>
<td>14</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>M4 RNA-400pg + caMEK RNA-200pg n=18</td>
<td>18</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

Note: * Indicates statistical significance.
Figure 29. Widespread Xbra Expression in EphA4 MO and caMek Injected Embryos. *In situ* hybridization was performed on control (A) and embryos injected with 15ng of EphA4 MO (B) and 15ng of EphA4 MO and 150 pg (C) and 200 pg (E) of caMek RNA. Widespread Xbra expression was observed in embryos injected with caMek + EphA4 MO (C and D, red arrows).
Figure 29- Widespread Xbra Expression in EphA4 MO and caMEK Injected Embryos

A. Control
B. EphA4 MO- 15ng
C. EphA4 MO-15ng + caMEK RNA-150pg
D. EphA4 MO-15ng + caMEK RNA-250pg
7. Xbra Expression Independent of FGFR Signaling

FGF receptor (FGFR) activity is required for initiating and maintaining Xbra expression (Fletcher and Harland, 2008). Upon activation, Xbra can activate various downstream targets including embryonic FGF (eFGF). In turn, eFGF can bind to the FGF receptor and promote Xbra expression, constituting a positive feedback loop (Shulte-Maker and Smith, 1995, Isaacs, 1994).

In this study, I applied the FGF receptor inhibitor 3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl)methylene]-2-indolinone (SU5402) to assess EphA4’s role in regulating Xbra’s expression. SU5402 can bind to the tyrosine kinase domain of FGFR1 and prevent downstream signaling. Hydrogen bonding between SU5402’s oxyindole ring N and various hydrophobic residues on the receptors kinase domain mediate this interaction (Mohammadi et al., 1997).

Injection and exposure of SU5402 to whole embryos from stage 8 to 11 (11hpf) resulted in significant reductions in archenteron length (Figure 30A and B). Injection and exposure at half the SU5402 dose resulted in normal gastrulation movements (Figure 30C).

Normal Xbra expression was observed after DMSO treatment from stages 8-11 (Figure 31A). We applied SU5402 from stages 8 and 9 and assessed Xbra expression at stage 11. SU5402 treatment from stage 8 to 11 completely perturbed Xbra expression in whole embryos (Figure 31B). These results confirm FGFRs role as an initial mesoderm inducer.

SU5402 treatment from 9-11 resulted in normal Xbra expression (Figure 31C). I believe that normal Xbra induction has occurred at earlier stages (stage 8), however, Xbra is maintained in the absence of FGF signaling. SU5402 treatment from 9-11 can inhibit the Xbra-eFGF-Xbra positive feedback loop and subsequent FGFR signaling, suggesting an alternative pathway is involved in Xbra maintenance. I believe that the EphA4-PAK1 pathway, identified in this study, is involved in Xbra induction during this phase. Interestingly, however, in the absence of FGF signaling embryos did not involute but maintained normal Xbra expression.
Figure 30. Gastrulation Defects after SU5402 Treatment. When compared to controls (A) SU5402 treatment of embryos leads to severe gastrulation defects (B) (p < 0.0001). No statistically significant defects were observed with low SU5402 dose treatment when compared to controls (C) (p = 0.203). Quantitative analysis of gastrulation defects are presented at the bottom panel (D). Results represent the mean +/- SD. * marks statistically significant values from the control condition (p < 0.05). Dorsal is to the right, bottle cells are highlighted with black arrows.
Figure 30- Gastrulation Defects After SU5402 Treatment

A. DMSO  
B. SU5402  
C. SU5402 (low dose)

D. 

<table>
<thead>
<tr>
<th></th>
<th>Average Archenteron Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>350 (n=25)</td>
</tr>
<tr>
<td>SU5402</td>
<td>300 (n=18)</td>
</tr>
<tr>
<td>SU5402 (low dose)</td>
<td>250 (n=12)</td>
</tr>
</tbody>
</table>
We believe that FGF is downstream of the EphA4/PAK1/Xbra pathway, identified in this study, and is required for involution.

We have shown that embryos injected with 5ng of EphA4 MO exhibit normal gastrulation movements (Figure 6A and B) and Xbra expression (Figure 31D). Injection of 5ng of EphA4 MO with SU5402 treatment from stage 9-11, however, can suppress Xbra expression, suggesting that the FGF/MAPK pathway and EphA4/PAK1 function synergistically to promote Xbra expression and involution (Figure 31E). SU5402 treatment at half the dose resulted in normal Xbra expression (Figure 31F).

In order to confirm that reduced time of exposure of embryos to SU5402 from stage 8 versus stage 9 did not account for the differences in Xbra expression, I reduced our incubation period and treated embryos with SU5402 from stage 8 and 9 to stage 10 (10 hpf). I observed similar results with reduced exposure time, suggesting that reduced SU5402 exposure could not account for the differences in Xbra staining between the 2 stages (Figure 31G-I). These results also confirm our findings, which suggest that SU5402 treatment from stage 9-11-induced defects are not a result of reduced Xbra expression but convergence of this domain, as normal expression was observed from stage 9-10.
Figure 31. Reduced Xbra expression after Early Treatments, but not with Late Treatments of SU5402. Normal Xbra expression was observed in embryos treated with DMSO from stage 8-11 (A). SU5402 treatment of embryos at stage 8-11 resulted in a loss of Xbra expression (B). In contrast, SU5402 treatment from stage 9-11 lead to normal Xbra expression (C). Injection of 5ng of EphA4 alone did not effect Xbra expression (D). However, injection of 5ng of EphA4 and SU5402 treatment from stages 9-11 attenuated Xbra expression (E). Normal Xbra expression and gastrulation movements were observed after low dose of SU5402 treatment (F). Differences in SU5402 treatment timing did not account for the variation of Xbra expression. Reduced DMSO (G) or SU5402 treatment from stages 8-10 (H) or 9-10 (I) resulted in similar findings.
Figure 31- Reduced Xbra Expression after Early but not late Treatments of SU5402

A. DMSO- st 9-11
B. SU5402- st 8-11
C. SU5402- st 9-11

D. EphA4 MO- 5ng
E. EphA4 MO- 5ng+ SU5402- st 9-11
F. SU5402 (low dose)- st 9-11

G. DMSO- st 9-10
H. SU5402- st 8-10
I. SU5402- st 9-10
Chapter 4
Discussion

1. EphA4 acts Through Pak1 to Promote Mesoderm Involution

Eph receptor tyrosine kinases (RTKs) are involved in a variety of developmental processes, including neural crest cell migration, axon path finding, angiogenesis, hindbrain segmentation, and otic placode invagination (Helbling et al., 1998, Holland et al., 1998, Cooke et al., 2001, Cheng et al., 2002, Barrionuevo et al., 2008). EphA4 expression has been observed in the *Xenopus* gastrula (Winning and Sargent, 1994), making it an ideal candidate to study the morphogenesis of gastrulation.

In this study, I examined the role of EphA4 RTK signaling during *Xenopus laevis* gastrulation using a loss of function study. I observed a concentration dependent reduction in gastrulation movements with the injection of EphA4 morpholino oligonucleotide (MO). EphA4 MO defects were characterized with reduced archenteron formation and elongation in the early gastrula, consistent with diminished mesoderm involution. In this study, I used novel dorsal blastopore lip (DBL) explant to confirm that the defects were tissue autonomous, indicating an attenuation of mesoderm involution movements. This interpretation was also supported by the fact that I did not observe any abnormalities in bottle cell formation, epiboly, or convergent extension (CE). Furthermore, defects were shown to be highly specific, as EphA4-induced mutant phenotypes could be rescued with co-injection of EphA4 mRNA.

Defective gastrulation and involution movements induced by EphA4 MO could also be rescued with co-injection of wtPAK1 mRNA. These results suggest that EphA4 can regulate mesoderm involution by targeting PAK1. PAK1 has been shown to regulate tissue adhesion and cytoskeletal dynamics (Sells et al., 1991, Daniels and Bokoch, 1999) and has therefore been implicated in cell migration and morphogenetic movements in the *Xenopus* gastrula (Sells et al., 1991, Nagel et al., 2009). In addition, EphA4 can regulate cell adhesion dynamics by targeting upstream activators of PAK1 in *Xenopus* (Winning et al., 2001, Winning et al., 1996).
Although in our experiments, PAK1 was able to rescue embryos injected with low, but effective concentrations of EphA4 MO, it was not able to rescue gastrulation defects when higher concentrations of EphA4 MO were used, suggesting that some remaining EphA4 activity is required for PAK1 mediated rescues.

We believe that low level of EphA4 protein are still present with low dose of EphA4 MO injections, but are absent with higher concentration of MO. These results suggest that EphA4 is required for PAK1 signaling. Elevated PAK1 signaling and kinase activity has been observed after EphA4-mediated membrane recruitment in *Xenopus* (Bisson *et al.*, 2007). Furthermore, EphA4, PAK1 and nckβ have been shown to co-immunoprecipitate (Bisson *et al.*, 2007), suggesting a direct functional interaction between the 3 molecules.

Previously, Bisson and colleagues (2007) identified an artificial EphA4-PAK1 pathway regulating cell adhesion in the *Xenopus* ectoderm, by ectopically overexpressing active EphA4 (Bisson *et al.*, 2007). Through a loss-of-function experiment I have identified a novel endogenous EphA4-PAK1 signaling cascade in *Xenopus* embryos that is involved in regulating mesoderm involution during gastrulation.

### 2. EphA4 acts Through Pak1 to Control Xbra Expression

Xbra acts as a transcriptional activator that promotes posterior mesoderm specification along the marginal zone during blastula stages (Smith *et al.*, 1991). In this study I have shown that EphA4 is necessary for mesoderm involution during gastrulation. In order to assess whether EphA4 MO induced gastrulation defects were a result of improper mesoderm specification, I performed whole mount *in situ* hybridization experiments to assess mesoderm specification. I observed normal goosecoid (anterior mesoderm), cerberus (leading edge mesoderm), chordin (dorsal factor), SOX2 (neural), and Xnot (notochord) expression in embryos injected with effective concentrations of EphA4 MO. Furthermore, I also observed normal CE movements with EphA4 MO injections, suggesting that EphA4 mutants exhibited approximately normal mesoderm specification.
We analyzed Xbra (chordamesoderm and lateral/ventral mesoderm marker) expression at 3 stages: stage 9, during initial mesoderm induction, stage 10.5, during involution, and at stage 11.5, after mesoderm internalization. I observed normal Xbra expression at stage 9 in embryos injected with EphA4 MO, suggesting that EphA4 is not involved during initial mesoderm formation. However, I observed markedly reduced Xbra expression at stage 10.5 in embryos injected with EphA4 MO, suggesting that EphA4 plays a critical role in regulating Xbra expression during this stage. I observed normal Xbra expression at stage 11.5. I hypothesize that EphA4 is responsible for promoting and maintaining Xbra expression during involution. In support of this I observed ectopic Xbra expression along the BCR and marginally in rescue experiments and EphA4 RNA injected embryos. Ectopic expression of Xbra along the BCR occurred at the interface of the leading edge mesoderm and roof. Vegetal rotation is believed to position the mesoderm against the roof during gastrulation and may account for this induction (Ibrahim and Winklbauer, 2001). I hypothesize that endogenous EphA4 ligands in the mesoderm can stimulate Xbra expression by activating EphA4 in the marginal zone, or as in our rescue or overexpression experiments, along the BCR.

To further validate EphA4’s Xbra inductive capabilities, I performed novel leading edge mesoderm-roof combined assays and assessed Xbra expression using *in situ* hybridization. Confirming our previous results, I observed Xbra expression when roofs injected with EphA4 mRNA were combined with leading edge mesoderm. In all cases I observed localized Xbra expression in parts of our mesoderm-roof combined explants and I later confirmed that this localization was due to dorsal-ventral differences in the roof and the location of a potential EphA4-ligand in the mesoderm. Various EphA4 ligands have been observed in the mesoderm of gastrulating *Xenopus* embryos (N. Rohani unpublished, Winklbauer, personal communication).

We propose that Xbra induction and maintenance occurs in 3 phases during *Xenopus* development: initially, the FGF pathway stimulates Xbra expression and promotes mesoderm induction. FGF has been implicated as an early mesoderm inducer in *Xenopus* (Amaya *et al.*, 1993, Isaacs *et al.*, 1994, Fletcher and Harland, 2008).
Furthermore, FGF then stimulates EphA4 expression and EphA4 synergistically activates the MAPK pathway to stimulate Xbra expression during the second phase of mesoderm maintenance. FGF has been shown to promote EphA4 expression during *Xenopus* gastrulation, as demonstrated by microarray and *in situ* hybridization experiments (Branney *et al.*, 2009). In addition, EphA4 can compensate for FGF-MAPK signaling *in vitro* in HEK293 cells and synergistically activate MAPK signaling *in vivo* in P19 neuronal cells (Yokote *et al.*, 2005). It is also possible that Xbra may be responsible for initial EphA4 expression. Branney and colleagues (2009) assessed downstream targets of FGF using SU5402 (Branney *et al.*, 2009). Early suppression of FGF can prevent Xbra signaling (Fletcher and Harland, 2008, Branney *et al.*, 2009). EphA4 may therefore be initially activated with Xbra in *Xenopus*, however this hypothesis requires further analysis.

Furthermore, I have shown that EphA4, at this stage, can regulate involution movements during gastrulation. I believe that EphA4 regulates Xbra expression during the second phase of mesoderm maintenance and in this way properly modulates cellular properties which are required for involution. EphA4 could also, in addition, directly affect cellular behaviors required for involution. It has been shown to regulate cell cytoskeletal dynamics and adhesion by targeting the Rho family of GTPases and cadherin mediated adhesion (Winning *et al.*, 1996, Winning *et al.*, 2001). Loss of adhesion has also been observed after ectopic active EphA4 expression (Winning *et al.*, 1996), suggesting that EphA4 could have dual transcriptional and morphogenetic functions in *Xenopus*.

Once the mesoderm is internalized, the FGF/PLC pathway has been implicated in regulating CE movements by activating Xbra (Sivak *et al.*, 2005). In addition, Xbra can activate embryonic FGF (eFGF) (Isaacs *et al.*, 1994), and eFGF can bind to the FGFR and re-stimulate Xbra expression, forming a positive feedback loop (Isaacs *et al.*, 1994, Schulte-Merker and Smith, 1995). The third phase of Xbra induction may therefore involve the FGF/PLC-Xbra pathway required for CE movements and the Xbra-eFGF-Xbra positive feedback loop for mesoderm maintenance.

We have shown that EphA4 can regulate involution movements by targeting PAK1. I therefore assessed Xbra induction in PAK1 rescue experiments. I also observed normal Xbra expression in
embryos co-injected with EphA4 MO and PAK1 RNA. These results suggest that PAK1 is a downstream target of EphA4 and is involved in maintaining Xbra expression during involution.

Nonetheless, I did not observe Xbra expression in embryos injected with higher concentrations of EphA4 MO and wtPAK1. In this study, I have shown that wtPAK1 was insufficient to rescue gastrulation movements when high concentrations of EphA4 MO were used. These results suggest that residual EphA4 is essential for PAK1 mediated involution movements and Xbra expression. I suggest that an EphA4-PAK1 interaction is critical for mesoderm/Xbra induction and involution.

Furthermore, I did not observe ectopic Xbra expression along the BCR using constitutively active (ca) PAK constructs, however, endogenous Xbra expression was enhanced within the marginal zone. I did observe ectopic Xbra expression along the BCR when EphA4 RNA was injected in the BCR. These finding are consistent with those of Dr. Moss (Dr. R. Winklbauer, personal communication). I propose that PAK1 is recruited to the membrane by EphA4 and this step is critical for mesoderm/Xbra induction and involution. EphA4 has been shown to recruit PAK1 using nckβ and this has been implicated in elevated PAK1 kinase activity and signaling (Bisson et al., 2007). PAK1 can also target the MAPK cascade and its close proximity to Ras can enhance signaling of this pathway (Chaudhary et al., 2000, Slack-Davis et al., 2003, Beeser et al., 2005, Frost et al., 2006). In this study, I have also identified the presence of an endogenous EphA4 ligand within the mesoderm, as demonstrated by our novel roof-mesoderm combined experiments. I believe that interactions between EphA4, which is located endogenously within the marginal zone (Winning and Sargent, 1994) and potential EphA4 ligands found in the involuted mesoderm (this study, Nadanine, unpublished, Winklbauer, personal communication) can stimulate/maintain Xbra expression as a concentric ring along the marginal zone (Smith et al., 1991) during Xenopus gastrulation. I have shown that PAK1 is a downstream target of EphA4 and that an EphA4-PAK1 interaction is critical for Xbra expression.
3. EphA4 and Pak1-dependent Xbra Expression is Necessary for Mesoderm Involution

My results have shown that EphA4 can upregulate Xbra expression using PAK1. I therefore wondered whether injections of Xbra can rescue EphA4 mutant gastrulation phenotypes, in particular involution defects. Co-injection of EphA4 MO and Xbra mRNA, however, was not sufficient to rescue gastrulation movements. Our inability to rescue EphA4 mutant phenotypes using Xbra RNA may partly be due to improper spatial localization of Xbra. Injection of low concentrations of Xbra RNA animally can inhibit involution behavior dorsally and lead to widespread ectopic Xbra expression (Cunliffe and Smith, 1992). I therefore tried to localize the effects of Xbra expression in our experiments by isolating DBL explants and assessing involution behavior. I have already shown that EphA4 induced gastrulation defects were a result of improper involution movements.

We were not able to rescue involution in DBL explants and the majority of EphA4 MO and Xbra RNA injected explants displayed aberrant involution behavior. However, injections of 15ng of MO and/or 10 pgs of Xbra RNA did not impede involution in DBL explants when injected together or alone (data not shown), suggesting that Xbra is a downstream target of EphA4.

Alternatively, since injected Xbra mRNA is translated immediately without proper temporal regulation, it is possible that this can lead to nonspecific secondary defects (Cunliffe and Smith, 1992, Tada et al., 1997). I therefore tried to rescue EphA4 mutant phenotypes by temporally regulating Xbra expression using a hormone-inducible Xbra construct (Xbra-GR). Application of dexamethasone (DEX) was sufficient to delay gastrulation movements in embryos injected with Xbra-GR. Importantly, I observed a significant rescue of EphA4 mutant phenotypes with the addition of Xbra-GR and DEX treatment at stage 10 (start of involution), when compared to embryos injected with Xbra-GR and treated with DEX at stage 9 (mesoderm induction). These results suggest that proper temporal regulation of exogenous Xbra is essential for the rescue of involution behavior. Temporal and spatial regulation of Xbra is observed during gastrulation (Latinkic et al., 1997, Lerchner et al., 2000).
A possible explanation of our results is that during the first phase of Xbra expression and mesoderm induction, unrestricted overexpression leads to aberrant mesoderm expression beyond its normal boundaries, which could therefore interfere with orderly involution movements. During the second phase, which depends on EphA4, the embryo is no longer sensitive to ectopic Xbra expression, and the presence of Xbra in the blastopore lip is sufficient for involution. I therefore believe that proper spatial regulation of Xbra during mesoderm induction may be necessary for the proper spatial arrangement of the mesoderm with respect to other tissues. During development, Xbra is expressed widely in the embryo and is actively restricted to the marginal zone by negative regulators (Lerchner et al., 2000). Various repressors of Xbra have been identified in the DMZ, including head mesoderm factor, gsc and otx2 (Latinkic and Smith, 1999, Artinger et al., 1997, Pannese et al., 1995), endoderm maker, mix1 (Latinkic and Smith, 1999, Lemaire et al., 1998), and ectoderm factor, SIP1 (Lerchner et al., 2000). The position of Snail is also believed to play a significant role in mesoderm regulation and involution (Winklbauer, personal communication). The presence of negative regulators may help to position Xbra to its proper location. Once this has occurred, Xbra is necessary in the blastopore lip for involution to take place. In summary, our results suggest that EphA4 can regulate Xbra expression during involution using wtPAK1, and it may be involved in spatial restriction of Xbra as EphA4, similar to Xbra, is confined to the marginal zone (Smith et al., 1991, Winning and Sargent, 1994). I have shown that ectopic EphA4 can lead to ectopic Xbra expression.

The FGF/MAPK signaling pathway is recognized as a critical regulator of Xbra expression (Cornell and Kimelman, 1994, Cornell et al., 1995, Gotoh et al., 1995, LaBonne et al., 1995). I therefore tried to localize the novel EphA4/PAK1 pathway identified in this study with respect to the FGF signaling pathway. I attempted to rescue EphA4 and kinase dead (kd, M4) PAK1 mutant phenotypes with the injection of caMek, a component of the MAP kinase pathway downstream of the FGFR. However, this proved unsuccessful. It is possible that caMek mRNA injections may be translated immediately and thereby leads to widespread Xbra expression and promote disorganized involution movements. Similar problems were also observed with our Xbra RNA injection rescue experiments.

We therefore assessed Xbra localization in caMek and EphA4 MO injected embryos. CaMek was able to rescue Xbra expression in EphA4 MO injected embryos, suggesting that MAPK/Mek
is a downstream target of the EphA4/PAK1 pathway identified in this study. As expected, I observed widespread Xbra expression in EphA4 MO and caMek injected embryos, suggesting again that proper temporal and spatial regulation of Xbra is essential for gastrulation and involution. I have previously shown that injections of EphA4 MO can suppress Xbra expression, it is therefore logical to assume that widespread Xbra expression caused by caMek and EphA4 MO injections would have been more pronounced Xbra expression in the absence of a negative regulator like EphA4 MOs. Temporal/spatial regulation of caMEK may be required to circumvent this problem.

SU5402 is an inhibitor of FGFR signaling (Mohammadi et al., 1997). In this study, I assessed FGFRs role during Xbra induction and involution. Treatment of embryos with SU5402 from stage 8 to 11 completely perturbed Xbra expression. SU5402 treatment at this stage inhibits initial mesoderm/Xbra induction and thereby suppress any downstream events.

In contrast, SU5402 treatment from stage 9-11, lead to normal Xbra expression. In the absence of FGFR signaling at this stage, Xbra was continually maintained, suggesting an alternative pathway can stimulate Xbra expression after its initial induction and I believe that the EphA4-PAK1 pathway, identified in this study, is involved in this event. SU5402 treatment at this stage can also suppress the 3rd phase of mesoderm maintenance: Xbra-eFGF-Xbra positive feedback loop (as the FGFR is no longer functional) and the FGF/PLC/Xbra pathway required for CE, indicating the presence of an additional pathway required for Xbra expression. I show that EphA4/PAK1 can feed into the MAPK pathway and stimulate Xbra expression.

Interestingly, embryos did not involute but maintained normal Xbra expression in the absence of FGF signaling. I believe that FGF is downstream of the EphA4/PAK1/Mek/Xbra pathway, identified in this study, and is directly required for involution. Xbra and EphA4 can both stimulate FGF ligand and receptor expression in Xenopus (Isaacs et al., 1994, Park et al., 2004) and I believe that this step is necessary for involution.

It is possible that FGF may function as a chemoattractant to stimulate proper tissue polarity and involution movements in Xenopus. Platelet derived growth factor alpha (PDGF-α) is required for proper polarization of the leading edge mesoderm during Xenopus gastrulation (Nagel et al.,
and FGF may therefore be acting in a similar manner for the chordamesoderm. Chemoattractant functions of FGF have been described in *Drosophila* tracheal development (Sato and Kornberg, 2002), endothelial tissue regeneration (Grotendorst *et al.*, 2005), and motor axon outgrowth (Shiraski *et al.*, 2002).

Interestingly, CE movements have also been shown to require FGF signaling (Sivak *et al.*, 2005). Xbra mediated activation of FGF may also be critical for involution. Keller and colleagues (1997) have documented similar CE-like movements during mesoderm involution. They showed that cells at the marginal zone acquire distinct bipolar morphologies and intercalate mediolaterally to involute (Lane and Keller, 1997). The use of similar morphogenetic movements between two very distinct biological processes suggests that redundant pathways may be involved in their regulation.

Both EphA4 and PAK1 have been shown to regulate cell adhesion by negatively targeting the Rho family of GTPases and cadherin mediated adhesion. Loss of tissue integrity has been observed with active EphA4 and PAK1 additions (Winning *et al.*, 2001, Bisson *et al.*, 2007). EphA4 and PAK1 signaling have also been involved in regulating growth cone collapse and restricting cell adhesion (Winning *et al.*, 2001, Bisson *et al.*, 2007, Shi *et al.*, 2007). Histologically, the DMZ has been characterized as a highly compact structure (Damm, unpublished). It is possible that the EphA4-PAK1 signaling can reduce cell adhesion and increase the intracellular space within the marginal zone tissue and thereby promote orderly involution movements.

We did run into some unexplained findings in our study, as injections of 5ng of EphA4 MO before SU5402 treatment from stages 9-11 lead to normal Xbra expression. In contrast, injection of 5ng of EphA4 MO alone had no effect on Xbra expression, suggesting that EphA4 functions by synergizing with FGF for Xbra induction at this stage. However, I have shown that EphA4 alone can still maintain Xbra expression in the absence of FGF signaling, based on our stage 9-11 SU5402 treated embryos (above). Differences in our findings can be attributed to the variability in the small sample size used in our 5ng of EphA4 MO + SU5402 treated embryos.
Our results confirm that EphA4 plays a significant role in regulating Xbra expression during involution. Furthermore, I was able to show that SU5402 treatments were not a result of differences in inhibitor exposure times. To address this problem, I reduced our SU5402 incubation period from stage 8-11 to stage 8-10 and from stage 9-11 to stage 9-10. I observed similar results with reduced SU5402 exposure suggesting that differences in Xbra expression were not a result of timing. It is also possible that residual FGF activity is still present in our previous experiment (above) and that EphA4 and FGF synergize to promote Xbra expression and involution movements.

4. Conclusion

We believe that FGF/MAPK signaling is responsible for initial mesoderm induction during the first phase of mesoderm formation. This is supported by the fact that injections of EphA4 MO was not sufficient to inhibit Xbra expression during early stages and in caMek expressing animal caps. Initial Xbra may be sufficient to activate various “mesoderm characteristics” and thereby form the mesoderm. The expression of Xnot and CE movements, a characteristic of the chordamesoderm, after effective concentrations of EphA4 MO injection, also supports the fact that “mesoderm” in these mutants still maintained their “true” mesoderm characteristics.

During the second phase of mesoderm development, FGF may activate EphA4 (Branney et al., 2009) and promote MAPK signaling (Yokite et al., 2005). Alternatively, MAPK signaling may also be regulated by PAK1 (Chaudhary et al., 2000, Slack-Davis et al., 2003, Beeser et al., 2005, Frost et al., 2006) and in this study, I show that EphA4 can regulate PAK1. PAK1 rescues were shown to require residual EphA4 expression, suggesting that a possible EphA4-PAK1 interaction is essential for this response. Our results indicate that EphA4 can signal through PAK1 to promote Xbra expression and involution during *Xenopus* gastrulation (Figure 32). Constitutively active (ca) MEK, a target of FGF/MAPK signaling, was also shown to be a downstream target of EphA4.
Figure 32. Proposed Model of Xbra Expression and Involution during *Xenopus* Gastrulation. During early stages of development the FGF/MAPK pathway is involved in mesoderm initiation. As gastrulation proceeds, FGF or Xbra may activate EphA4 and EphA4 can stimulate Xbra and FGF expression for involution. In this study, I show that EphA4 can regulate Xbra by targeting PAK1 and MAPK signaling. I also highlight an essential EphA4-PAK1 interaction for Xbra expression (red circle). Proper temporal regulation of Xbra is critical for involution movements. RNA injections during early stages can lead to widespread Xbra expression and suppress involution. As development proceeds, activation of various Xbra inhibitors including, goosecoid (red), endoderm marker, mix1 (green), and ectoderm factor, SIP1 (dark brown), can actively restrict Xbra to the marginal zone and thereby help to promote orderly involution movements. The position of Snail (yellow) is also believed to play a significant role in regulating Xbra (blue). Proper tempo-spatial regulation of Xbra is critical for involution.
Figure 32 - Proposed Model of Xbra Expression and Involution during Xenopus Gastrulation

FGFR
  ↓
Ras
  ↓
Raf
  ↓
Mek
  ↓
Erk
  ↓
Xbra
  ↓
Mesoderm Specification

Ras
  ↓
Raf
  ↓
Mek
  ↓
Erk
  ↓
Xbra
  ↓
FGF
  ↓
Involution
  ←
EphA4
  ←
PAK1
We have shown that loss of FGF signaling from stage 9-11 does not suppress Xbra expression, suggesting an alternative pathway may be involved in Xbra regulation. Based on our studies, I believe that the EphA4/PAK1/Mek pathway, identified in this study, controls Xbra expression at this stage. Interestingly, FGF suppression from stage 9-11 inhibited involution movements, suggesting that FGF signaling is required for involution and is downstream of Xbra.

In this study, I also show that precise temporal and spatial expression of Xbra is essential for involution and this can be achieved using hormone inducible Xbra constructs. I believe that Xbra RNA injections in the early gastrula may lead to aberrant mesoderm induction and loss or normal mesoderm-ectoderm and mesoderm-endoderm boundaries and interfere with orderly involution movements. I have shown this using caMEK and Xbra RNA constructs. During development, Xbra is actively restricted to the invoving mesoderm by various negative regulators in the DMZ (Latinkic and Smith, 1999, Artinger et al., 1997, Pannese et al., 1995) and by possibly by EphA4. Expression of FGF at this stage is also critical for involution. Our study has identified a novel EphA4/PAK1/Mek signaling cascade that is involved in Xbra regulation and involution movements during Xenopus laevis gastrulation and has uncovered a novel role of FGF signaling during mesoderm involution.

5. Future Studies

One of the major questions I had during this study had to do with the characterization of Xbra’s role during involution. To date few studies have examined the role Xbra during various morphogenetic movements, including the much studied CE movements. It would be interesting to identify cellular processes that Xbra has been able to orchestrate to mediate involution. One way this could be accomplished would be to analyze scanning electron microscope (SEM) images of the dorsal blastopore in control and embryos that exhibit loss to Xbra function exactly at involution stages. To date, only dominate negative (dn) Xbra RNA constructs (Xbra-EN) are available to repress Xbra expression. Expression of dn Xbra can prevent early mesoderm induction and provide minimal information on Xbra role during involution. A hormone repressive Xbra construct would be required. Hormone repressive Xbra would allow us to identify basic, if any, cellular processes Xbra is able to regulate to mediate involution. I would expect loss of Xbra function to suppress involution by possibly increasing cell adhesion and this
could be visualized in SEM images by analyzing the dense packing of cells in the DMZ. It has been shown, in this study and by others, that EphA4 can regulate involution and reduce cell adhesion and Xbra may be a downstream mediator of this loss of adhesion pathway. In addition, cell to cell and cell to matric adhesion assays, as presented by Nie and Chang (2007), can be used to further validate Xbra role in regulating the packing of cells to their surroundings (Nie and Chang, 2007).

Furthermore, temporally repressive Xbra constructs (provided by Dr. Moss) would also help to identify novel functions in Xbra. Currently, addition of dn Xbra constructs can suppress early mesoderm formation and provide limited information on the role of Xbra during involution. Temporal repression of Xbra may therefore allow us to by-pass early mesoderm induction and mediate suppression at exactly the time of involution. This study would provide a novel role of Xbra during involution. I would expect loss of Xbra function of suppress gastrulation (while normal mesoderm characteristics have been induced), consistent with Xbra role in regulating involution.

It would also be critical in this study to analyze the various assumptions presented, such as the EphA4-PAK1 interaction. The EphA4-PAK1 interaction could simply be identified by using a co-immunoprecipitation assay. Bisson et al., 2007 has already confirmed an EphA4-PAK1 interaction in *Xenopus* (Bisson et al., 2007). In addition, I have suggested that EphA4 is positively regulated by FGF or Xbra. This assumption could be answered by injecting various loss and gain of function Xbra or FGF RNA constructs and examining EphA4 expression using *in situ* hybridization. However, in this study I lacked any sufficient EphA4 RNA probes for *in situ* hybridization assays. I neglected the use of EphA4 antibodies (AB), as no *Xenopus* specific Abs were commercially available and considering the close relationship between the family Eph RTKs, cross-reactivity with other species would be likely. This study would help to complete the EphA/PAK1/Xbra pathway in *Xenopus*. It would also be important to further verify EphA4/PAK1’s Xbra inductive capabilities by using a Western Blot analysis.

Finally, it would also be interesting to identify the role FGF plays in involution. In this study, I have shown that FGF is critical for involution and that Xbra alone is not sufficient. It would be interesting to identify how FGF mediate these cellular movements as well as to identify which
FGF ligands and receptors are involved in involution. One of the major problems with loss of FGF function early would lead to the loss of mesoderm formation. Temporal regulation of FGF suppression using SU5402 can be used to circumvent this problem. Firstly, examination and in situ hybridization for Xbra in sagittally sectioned whole embryos and DBL explants after FGF suppression (SU5402) at 9-10, would help to further validate FGF role in involution. In addition, use of SEM to identify cellular changes with FGF suppression (in addition with Xbra SEM images) may also provide vital information on the role of FGF signaling during *Xenopus* gastrulation. My paper provides a basic analysis of mesoderm involution and helps to uncover the morphogenetic movements required for proper *Xenopus* gastrulation.
Chapter 5
References


Latinkic BV, Umbhauer M, Neal KA, Lerchner W, Smith JC, Cunliffe V. (1997). The *Xenopus* brachyury promoter is activated by FGF and low concentration of activin and suppressed by high concentrations of activin and pair-type homeodomain proteins. Genes & Development. 11, 3265-3276.


