Exploring the developmental roles of plk4

Candace Rapchak

*University of Windsor*

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation


https://scholar.uwindsor.ca/etd/5138

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
Exploring the Developmental Roles of \textit{plk4}

By

Candace Rapchak

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2014

© 2014 Candace Rapchak
Exploring the Developmental Roles of \textit{plk4}

by

Candace Rapchak

APPROVED BY:

________________________

Dr. M. Boffa
Department of Chemistry and Biochemistry

________________________

Dr. A. Swan
Department of Biological Sciences

________________________

Dr. M. Crawford, Advisor
Department of Biological Sciences

________________________

Dr. J. Hudson, Advisor
Department of Biological Sciences

May 9, 2014
DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
ABSTRACT

Plk4 is a member of the Polo-like kinase family of cell cycle regulators that has well established roles in the regulation of centrosome duplication, cytokinesis and the DNA damage response in adult cells. Mice with homozygous Plk4 null mutations are embryonic lethal at E7.5 and do not develop somites, notochord nor a proper neural tube. In this study, plk4 was cloned and characterized for expression and function during the embryonic development of Xenopus laevis. Xenopus plk4 is expressed in a diverse number of tissues including the somites, lens and retina. Translational knockdown of plk4 via morpholinos causes perturbations to somitogenesis and precludes lens placode formation by inhibiting cellular elongation in presumptive somite and lens cells. Additionally, centrosome polarity is not established in somitic cells. These results point to a role for plk4 in the establishment of cellular polarity during development and highlight its multi-functionality.
DEDICATION

To my family for their unconditional support and encouragement.
ACKNOWLEDGEMENTS

I would first like to thank my graduate supervisors, Dr. John Hudson and Dr. M. Crawford, for their guidance and support throughout this project. Your insights and advice have helped me to become a better scientist and given me a greater appreciation for the value of basic research. I would like to extend additional gratitude to Dr. M. Crawford for providing me with not only guidance in science but in the other endeavours of my life, and for always providing good conversation and laughter. Thank you to my committee members for providing me with helpful insights during this project, your guidance is much appreciated.

I extend my gratitude to past and present members Crawford labs, Lara, Jerant, Cristine Smoczer for teaching me the ins and outs of molecular biology, with a special thank you to Saqib Sachani for always providing a helping hand and for his friendship. To present members of the Hudson lab and Crawford labs, Alex, Gayathri, Braden, Natalie, Mohamad, Mike, Andrei and Vaishali, you have provided me with endless laughter, support and friendship that I cannot put into words. Some of my fondest memories and friendships involve my time here. I am grateful for the opportunity to be a part of these labs as the experiences I have had during my time here have made me a better scientist and shaped me into the person I am today.

I must give special credit to my family. You have always believed in my abilities and supported my desire to pursue the sciences with enthusiasm. I am grateful to have such wonderful people in my life.

Last I would like to acknowledge my better half, Chris Swartz, for his unwavering support throughout this journey. You have kept me sane during this project with your humour and encouragement. You have always bought out the best in me and I would not be where I am today without you.
# TABLE OF CONTENTS

DECLARATION OF ORIGINALITY ........................................................................ iii
ABSTRACT ............................................................................................................... iv
DEDICATION ........................................................................................................... v
ACKNOWLEDGEMENTS ........................................................................................ vi
LIST OF TABLES ..................................................................................................... x
LIST OF FIGURES .................................................................................................. xi
LIST OF APPENDICES .......................................................................................... xii
LIST OF ABBREVIATIONS/SYMBOLS ................................................................. xiii

CHAPTER 1: Introduction ....................................................................................... 1
  Plk4 ..................................................................................................................... 1
    Protein Structure .............................................................................................. 2
    Genomic Structure ............................................................................................ 5
    Mouse Models .................................................................................................... 6
  Functions of Plk4 ................................................................................................. 8
    Plk4 and Centrosome Duplication ..................................................................... 8
    Cell Cycle Progression and Plk4 ....................................................................... 13
    Plk4 and Tumourgenesis ................................................................................. 15
    Plk4 and Development .................................................................................... 16
  Project Outline ..................................................................................................... 17
  The Centrosome and Polarity .............................................................................. 18
  Somitogenesis ..................................................................................................... 21
    Clock and Wavefront Model of Segmentation ................................................ 21
      The Wavefront ............................................................................................... 22
      The Segmentation Clock .............................................................................. 23
      When Clock and Wavefront Meet .................................................................. 26
    Morphological Changes ................................................................................... 27
  Eye Development ................................................................................................. 30
Eye Morphogenesis ................................................................. 30
Lens Placode Formation .......................................................... 32
Centrosome Polarity in Eye Development ................................. 35

CHAPTER 2: Materials and Methods ........................................... 37
Embryo Collection .................................................................. 37
Cloning ................................................................................... 37
In Situ Hybridization ............................................................... 38
Antibody Development ............................................................. 38
Protein Isolation and Western Blotting ....................................... 39
Antibody Pre-absorption .......................................................... 40
Morpholino Design and Microinjection ..................................... 40
Immunohistochemistry ............................................................. 41
Sectioning and Hoechst Stain .................................................... 42

CHAPTER 3: Results ................................................................ 45
Plk4 has a Diverse Spatio-temporal Pattern of Expression During Development ......................................................... 45
Production of a Xenopus laevis specific plk4 antibody .................. 49
Knockdown of plk4 Affects Multiple Developmental Systems ..... 51
Depletion of plk4 Disrupts Somite Morphology and Patterning .... 56
Plk4 Knockdown Precludes Lens Development ......................... 61
Centrosome Localization is Lost Upon plk4 Depletion ................ 67

CHAPTER 4: Discussion ............................................................. 71
Plk4 expression during embryogenesis indicates cell cycle as well as polarity functions during organ morphogenesis ................................................. 71
Morpholino mediated knockdown of plk4 results in disruptions to somite and lens formation ................................................................. 74
Loss of cell polarity may underlie plk4 knockdown phenotypes .......... 79
A prmt5-plk4 signalling axis may exist in Xenopus laevis ............ 82
Xenopus laevis may express an unusual plk4 isoform .................. 83
Future Directions .................................................................... 86
Conclusions ............................................................................. 90

REFERENCES/BIBLIOGRAPHY ................................................. 92

APPENDICES .......................................................................... 102
Appendix A: Plasmid Maps.............................................................................................................102
Appendix B: Preliminary phenotype scoring.............................................................................104

VITA AUCTORIS .................................................................................................................................106
LIST OF TABLES

Chapter 2

- **Table 2.1**: List of plasmids used for *in situ* hybridization

Appendix B

- **Table B.1**: Effect of *plk4* morpholino mediated knockdown on eye development
- **Table B.2**: Effect of *plk4* morpholino mediated knockdown on somitogenesis
LIST OF FIGURES

Chapter 1

- **Figure 1.1**: Comparison of the four human Polo-like kinase proteins.
- **Figure 1.2**: Centrosome Duplication.
- **Figure 1.3**: Somitogenesis in *Xenopus laevis*.
- **Figure 1.4**: Eye morphogenesis.

Chapter 3

- **Figure 3.1**: *plk4* has a diverse spatial-temporal expression pattern during *Xenopus* embryogenesis.
- **Figure 3.2**: Production of a *Xenopus laevis* specific plk4 antibody
- **Figure 3.3**: Depletion of plk4 affects multiple organ systems.
- **Figure 3.4**: Depletion of plk4 perturbs both morphological and molecular aspects of somitogenesis.
- **Figure 3.5**: Scoring of *plk4MO* embryos with perturbations to somitic patterning
- **Figure 3.6**: plk4 depletion causes eye development defects.
- **Figure 3.7**: Scoring of *plk4MO* injected embryos with perturbed lens development
- **Figure 3.8**: Depletion of plk4 disrupts only somitic \(\gamma\)-tubulin localization.

Appendix

- **Figure A.1**: pCMV-Sport6-\(plk4\)
- **Figure A.2**: pCMV-Sport6-\(plk4\Delta2354bp\)
LIST OF APPENDICES

Appendix A: Plasmid maps

Appendix B: Preliminary phenotype analysis
LIST OF ABBREVIATIONS/SYMBOLS

+/ - heterozygous mutation
µm- micrometers
12/101- mono-clonal anti-body that stains myogenic cells
aa- amino acid
ABP- apical-basal polarity
ActR-1- ARP1- actin related protein 1 homolog
ANP- anterior neural plate
ATG- triplet codon for amino acid methionine; nucleotide translational start codon
B- brain
BA- branchial arches
Bcan- brevican
BLAST- basic local alignment search tool
Bp- base pairs
BSA- bovine serum albumin
cc- cubic centimeters
C. elegans- Caenorhabditis elegans
Cdc genes- cell division cycle
CDK proteins- cyclin dependant kinases
cDNA- complimentary deoxyribonucleic acid
CEP152- centrosomal protein of 152Kd
CEP192- centrosomal protein of 192Kd
Chk2- checkpoint kinase 2
CpG- cystein-phosphate-guanine
C-terminal- carboxy terminal of protein
Cyp26AI- cytochrome P450. Family 26, subfamily A, polypeptide 1
DAB- 3,3'-Diaminobenzidine
DNA – deoxyribonucleic acid
DRE- downstream regulatory element
E7.5- embryonic day 7.5 of mouse development
Ect2- epithelial cell transforming sequence 2 oncogene
efna4- ephrin-A4
epha4- EPH receptor A4
ephB2-EPH receptor 2
esr genes- BHLB-WRPW transcription factor ESR
EST- expressed sequence tag
FB- forebrain
FBS- fetal bovine serum
FBXW5- F-box and WD repeat domain containing 5
Fgf- fibroblast growth factor
G1 phase- growth phase 1 of cell cycle
G2 phase- growth phase 2 of cell cycle
GCP6- gamma complex associated protein 6
h2afy- 2A histone family, member Y2
H3- histone 3
H3R8- histone 3 arginine 8
H4R3- histone 4 arginine 8
Hamd1- hand and neural crest derivative 1
hCGH- human chorionic gonadotropin hormone
HCT116- human colon cancer cell line
HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hes genes- hairy and enhancer of split
hnf1a- HNF homeobox A
HRP- horse radish peroxidase
IMZ0- involuting marginal zone
kDa- kilo Daltons
L- lens
LE- lens
LF- lens fibre
LP- lens placode
LPM- lateral plate mesoderm
LV- lens vesicle
M phase- mitosis phase of cell cycle
MBS- modified barth’s saline
MEF- mouse embryonic fiberblast
MEMPFA- mops, Ethylenediaminetetraacetic acid, paraformaldehyde solution
mesp2- mesoderm posterior 2 homolog
mespo- mesogenin 1
mL- milliliters
mM- micromolar
MO- morpholino
mRNA- messenger ribonucleic acid
N- notochord
NCAM- neural cell adhesion molecule
Neddl- neural precursor cell expressed, developmentally down-regulated 1
NF- neural fold
ng- nanogram
NIBB- national institute for basic biology
nL- nanoliter
NT- neural tube
N-terminal- amino terminal of protein
OP- otic placode
OtV- otic vesicle
OV- optic vesicle
p53- tumour protein p53
PAPC- protocadherin 8
pax6- paired box 6 gene
PB- polo-box
PBD- polo-box domain
PCM- peri-centriolar material
PCP- planar cell polarity
PEST- amino acid sequence concentrated in proline, glutamic acid, serine and threonine
pitx3- pituitary homeobox 3
PLE- presumptive lens ectoderm
plk genes- polo-like kinase genes
PP2a/Twins- protein phosphatase 2-twins complex
Prmt5- protein arginine methyltransferase 5
PSM- presomitic mesoderm
R- retina
RaldH2- retinal dehydrogenase 2
rax1- retina anterior neural fold homeobox 1
RhoA- ras homolog family member A
RPE- retinal pigmented epithelium
S- somite
SAS proteins- spindle assembly homolog
SCF- ubiquitin ligase complex
SCMO- standard control morpholino
SEM- scanning electron microscopy
six3 - sine oculis homeobox gene
sox2 - SRY (sex determining region) - box
stg. - stage
TUNEL - Terminal deoxynucleotidyl transferase dU TP nick end labeling
UTP - uridine triphosphate
UTR - untranslated region
Wnt - wingless type
γ - gamma
β - beta
CHAPTER 1:

Introduction

Plk4

*Plk4* is a member of the *polo*-like family of serine-threonine kinases, which contain four other members, *Plk1/2/3/5*, with *Plk5* being the newest and most divergent member of the family. The *Polo*-like kinases are best known for their roles in cell cycle regulation and more specifically in mitotic progression and centrosome duplication (Archambault and Glover, 2009). They were first discovered in *Drosophila*, where *polo*, a homologue of mammalian *Plk1* was found to have profound effects on cell cycle progression (Sunkel and Glover, 1988). They have since been characterized in a diverse number of organisms, indicating the likely importance of these proteins to basic cellular function. The *Polo*-like kinase family seems to have arisen from an ancestral *Polo*-like gene that has since duplicated to give rise to the five modern day family members (Carvalho-Santos et al., 2010). The emergence of new *Polo*-like kinase family members in more complex organisms has allowed each protein to develop a specific niche for them to toil within the cell cycle and to subsequently sub-functionalize. The number of *Polo*-like kinase genes an organism possesses seems to be dependent upon the complexity of their cell cycle. Less complex organisms such as the yeast, *Saccharomyces cervisiae*, contain only one *polo*-like gene, *Cdc5*. In contrast, more complex organisms like *Drosophila* have at least two (*polo*, *Sak*), while vertebrates such as *Xenopus* (*plk1-5*), mouse (*Plk1-5*) and human (*PLK1-5*) have five.
*Plk1* is the most studied member of the family with multiple functions within the cell cycle. Most importantly it is involved in entry into mitosis, centrosome maturation, chromosome separation and cytokinesis among many other functions (Abrieu et al., 1998; Alexandru et al., 2001; Burkard et al., 2007; de Carcer et al., 2011; Kang et al., 2006; Lane and Nigg, 1996; Neef et al., 2003; Petronczki et al., 2007; Seong et al., 2002; Sumara et al., 2002; van Vugt et al., 2004). The function of *Plk2* and *Plk3* are less well known however it appears that *Plk2* is involved in centrosome duplication (Chang et al., 2010; Warnke et al., 2004) and *Plk3* in promoting entry into S phase, DNA replication, and in mediating the stress response via p53 (Bahassi el et al., 2004; Wang et al., 2008; Xie et al., 2001; Zimmerman and Erikson, 2007). *Plk4* performs a specific role in the cell cycle as the master regulator of centrosome duplication, as well as functioning to regulate mitotic progression and cytokinesis (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Hudson et al., 2001; Ko et al., 2005; Rosario et al., 2010).

**Protein Structure**

All of the Polo-like kinases share a similar protein structure; however Plk4 has diverged slightly from other family members. In its N-terminal, like all other Polo-like kinases, Plk4 contains a kinase domain. This domain is responsible for phosphorylating protein substrates, however Plk4 has a unique phosphorylation motif that is distinct from other members of the family (Leung et al., 2007). Unlike other family members, which possess serine/threonine kinase domains, the Plk4 kinase domain resembles both a serine/threonine and tyrosine kinase domain (Leung et al., 2007) and Bing, unpublished). It is in the C-terminal domain that Plk4’s structure has diverged the most. The defining characteristic of the Polo-like kinases is their polo-box domain which is a conserved 64
amino acid motif that determines protein-protein interactions that regulate the protein’s function. While other Polo-like kinases contain two polo-boxes at their C-terminal ends, Plk4 only contains one bona fide polo box and two cryptic polo boxes that act as one unit (Leung et al., 2002; Slevin et al., 2012) (Fig.1.1). Both the polo-box and cryptic polo-box can independently localize Plk4 to the centrosome, and only when both are removed from Plk4 does it lose its localization capabilities (Habedanck et al., 2005; Leung et al., 2002). The cryptic polo-box may also contribute to Plk4’s unique regulation. Polo-boxes are able to dimerize with each other leading to intermolecular homodimers of Plk4 and intra-molecular polo-box folding within Plk1-3 (Elia et al., 2003; Leung et al., 2002). This intra-molecular dimerization of Plk1-3 serves to inactivate kinase activity until the polo-box domain (PBD) binds to its substrate recognition site thereby activating the kinase domain (Elia et al., 2003). However, the polo-box and cryptic polo-box of Plk4 cannot dimerize, thereby preventing the intra-molecular dimerization seen with the other Polo-like kinases (Leung et al., 2002). Thus, it would seem the divergence in Plk4 sequence has put it under a different set of regulatory mechanisms that could permit it to take on new functions and substrates in the cell.

Plk4 also possess three PEST sequences, one of which is conserved in multiple organisms including mouse, *Xenopus*, and human pointing to a conserved mechanism of regulation. These sequences are important for regulating Plk4 stability, as the ubiquitin ligase SCF recognizes the phosphorylated form of this sequence and targets Plk4 for
All Polo-like kinase family members share the same basic protein structure; an N-terminal kinase domain and C-terminal PBD. Plk1-3 contain two polo boxes while Plk4 contains three distinct polo boxes. In Plk4, PB1 and PB2 comprise the cryptic polo box domain which share little structural homology to the polo-box domain found in other polo-like kinases. Together, both PB1 and PB2 are required for localization to the centriole. The amino acid residues that comprise the entire protein are listed at the C-terminal end of the structure. The DRE (downstream regulatory element) regulates Plk4 stability. This is the region that contains the PEST sequences that Plk4 trans-autophosphorylates, leading to its recognition by the SCF ubiquitin ligase and subsequent degradation.

**Figure 1.1: Comparison of the four human Polo-like kinase proteins**

[Diagram showing the comparison of four human Polo-like kinase proteins, with each protein labeled as Human Plk1, Human Plk2, Human Plk3, and Human Plk4, indicating their respective number of polo boxes (PB1, PB2, PB3) and total amino acid count (603aa, 685aa, 646aa, 970aa).]
degradation by the proteosome (Cunha-Ferreira et al., 2009; Rogers et al., 2009). This becomes important for regulating Plk4 activity and ensuring centrosomes are duplicated only once per cell cycle. Plk4 also has the ability to auto-phosphorylate upon kinase activation (Sillibourne et al., 2010). This auto-phosphorylation is believed to contribute to Plk4 degradation and regulation by providing the phosphorylated recognition sequence required by the SCF ubiquitin ligase. Phosphorylation by other kinases has not yet been ruled out however there is mounting evidence to indicate that it is indeed Plk4 auto-phosphorylation that leads to its own degradation: deletion of the N-terminal PEST sequence leads to increased Plk4 stability (Holland et al., 2010). In addition, expression of a kinase dead Plk4 in Drosophila and mammalian cells leads to increased Plk4 protein stability (Brownlee et al., 2011; Guderian et al., 2010).

**Genomic Structure**

Plk4 was first cloned in mouse where it was discovered to contain two isoforms, formerly, Sak-a and Sak-b (Fode et al., 1994). Sak-a consists of 15 coding exons and is the predominant isoform at 3567 base pairs and 925 amino acids. Sak-b encodes a 465 amino acid truncated protein due to early termination of transcription and alternative splicing of exons 5 and 6. It contains the N-terminal catalytic domain coded by exons 1-5, just as in Sak-a, however it lacks the C-terminal polo-box and cryptic polo-box domains and instead contains a portion of exon 5’s flanking intron incorporated into the mRNA by alternative splicing. This intron contains the stop codon leading to its early termination. Human Plk4 mRNA, by comparison, encodes a protein that is highly homologous to the sequence encoding the mouse kinase domain (94% amino acid identity) however; it differs in the sequence encoding the C-terminal domain, sharing
only 77% amino acid identity. Interestingly, human Plk4 contains an insert after exon 5 that is highly similar to the intron sequence that is retained in mouse Sak-b (Hudson et al., 2000). Mouse and Xenopus Plk4 appear to share a similar genomic organization of exons. While the Xenopus laevis genome has only recently been sequenced, predictive analysis of Xenopus laevis plk4 indicates a transcript with 16 exons, similar to what is predicted in mouse and human (mouse: Accession no. NM_011495.2, human: Accession no. NM_001190799.1, Xenopus: Accession no. NM_001089677, Xenopus genomic sequence available at xenbase.org)

Expression studies have revealed Plk4 mRNA to be highly expressed in the testes of adult mice as well as in the ovary, spleen and thymus. Analysis of its spatio-temporal expression during embryogenesis reveals Plk4 to be expressed during the proliferative stages of murine organ development, particularly in the liver, skin, thymus, small intestine and kidney. In addition, Plk4 expression is also seen in non-proliferative tissues such as the nasal and olfactory mucosa and respiratory epithelium (Fode et al., 1994).

**Mouse Models**

Studies into Plk4 gene function began with the generation of a Plk4 knockout mouse. These embryos were embryonic lethal at day E7.5 just after gastrulation. They lacked somites, notochord and neural tube even though neural crest had developed (Hudson et al., 2001). Plk4-null mice survive longer into development than Plk1-null mice which are embryonic lethal at the morula stage (Lu et al., 2008). In contrast, Plk2-null and Plk3-null mice are viable, however Plk3-nulls are prone to tumour development (Ma et al., 2003; Yang et al., 2008). Plk4-null embryos most likely survived through to
gastrulation because of redundancy with other Polo-like kinase family members (Swallow, 2005). On the other hand, the inability of embryos to survive post gastrulation indicates a specific role for Plk4 in the later stages of development when organs begin to pattern and organize themselves. Plk4-null embryos displayed several mitotic defects including a high number of cells arrested in anaphase of mitosis as well as a higher number of apoptotic cells, hinting at a role in cell cycle progression. Additionally, cells exhibited chromosomes that were only partially separated (Hudson et al., 2001).

In contrast to Plk4-null mice, heterozygote embryos develop normally without any externally obvious morphological phenotypes. However, elderly mice have an increased incidence of spontaneous liver and lung tumours indicating Plk4 is haploinsufficient for tumour suppression. After partial hepatectomy experiments, regenerating liver tissue showed mitotic delay along with irregular spindle poles, and the tissue itself was disorganized. Embryonic fibroblasts of Plk4+/- mice showed centrosome amplification, multi-polar spindles and aneuploidy (Ko et al., 2005). The presence of supernumerary centrosomes is not in agreement with other reports describing a loss of centriole number in cells with reduced Plk4 levels (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). This could be explained by the fact that Plk4 is also important for cytokinesis. The Plk4+/− MEFs may not be undergoing cytokinesis leading to accumulation of centrosomes and DNA within the cells (Rosario et al., 2010; Swallow et al., 2005).

It was clear from these mouse models that Plk4 plays a decisive role in the cell cycle and that a reduced Plk4 gene dosage leads to not only mitotic defects that affect
cellular proliferation, but also to chromosomal defects that greatly increase the risk of developing cancer.

**Functions of Plk4**

1. **Plk4 and Centrosome Duplication**

   Plk4’s role in centrosome duplication first became evident from gain-and loss-of function studies: PLK4 over-expression leads to the formation of supernumerary centrioles while loss of PLK4 leads to loss of centrosomes and mitotic defects (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Ko et al., 2005; Rodrigues-Martins et al., 2007). Additionally, Plk4 can induce de novo centrosome formation in both *Drosophila* and *Xenopus* (Eckerdt et al., 2011; Rodrigues-Martins et al., 2007). PLK4 protein has been shown to localize to the centrosome during the cell cycle and recently it has been shown that PLK4 physically interacts with several components of the centrosome biogenesis machinery such as CEP152, CEP192, CDK11 and GCP6 and FBXW5, providing further evidence for its role in centrosome duplication (Bahtz et al., 2012; Franck et al., 2011; Hatch et al., 2010; Hudson et al., 2001; Leung et al., 2002; Puklowski et al., 2011; Sonnen et al., 2013).

   Centrosomes consist of two centrioles, which are microtubule based barrel structures that are surrounded by a cloud of pericentriolar material. Centrosomes are duplicated once per cell cycle so that each can migrate to opposite poles of the cell to initiate spindle assembly and subsequent chromosome segregation in mitosis. Therefore, centrosome duplication must be carefully regulated to ensure faithful chromosome separation. To date, the process of centrosome duplication is not fully understood,
however recent work has shed light on the potential functions of PLK4 during this process. In mammals, PLK4 appears to be important during the initiation of procentriole formation from an existing centriole template (reviewed in (Nigg and Stearns, 2011) (Fig. 1.2). Centrosome duplication begins at the G1-S phase transition using the existing centrioles as templates. These centrioles are referred to as the mother centrioles and both will act as templates to form two daughter centrioles. Two newly forming procentrioles will form perpendicularly off of each mother centriole at the proximal end, a process which is regulated by PLK4. Important for PLK4 recruitment to the mother centriole are the procentriolar proteins CEP192 and CEP152. Both CEP192 and CEP152 interact with PLK4 in a hierarchal manner creating a scaffold for procentriole nucleation (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010; Kim et al., 2013; Sonnen et al., 2013). Once at the centrosome, PLK4 is proposed to phosphorylate FBXW5 leading to the subsequent stabilization of SAS-6, which is a target of FBXW5 mediated degradation (Nigg and Stearns, 2011; Puklowski et al., 2011). The stabilization of SAS-6 allows for the formation of the procentriole with its distinct cartwheel assembly (Gopalakrishnan et al., 2010; Kitagawa et al., 2011; Leidel et al., 2005; Nakazawa et al., 2007). SAS-6 has self assembly properties similar to the observed procentriole cartwheel structure, leading many to believe it is critical for procentriole assembly (van Breugel et al., 2011). SAS-5, SAS-4 and CEP135 are also believed to be important for procentriole stabilization along with SAS-6 (Gopalakrishnan et al., 2011). At this point the foundation has been laid as a cartwheel structure with nine fold symmetry. The centrosome then elongates during G2 phase with the aid of SAS-4 and is capped with proteins such as CP110 and CEP97, to prevent further procentriole
elongation (Kirkham et al., 2003; Kohlmaier et al., 2009; Leidel and Gonczy, 2003; Schmidt et al., 2009). This structure is only an immature centriole as it does not yet have the ability to form pericentriolar material (PCM). Towards the end of G2 phase the immature centriole goes through a maturation process under the guidance of Aurora A kinase and PLK1, which will allow it to form PCM and subsequently nucleate microtubules (Hannak et al., 2001; Lane and Nigg, 1996).

Centrosome duplication must be carefully regulated so that only one centrosome duplicates during the cell cycle. This is likely accomplished by a licensing event that occurs in mitosis prior to the initiation of duplication in the subsequent S phase. Mother and newly replicated daughter centrioles are tethered together throughout the cell cycle; a structural identity referred to as engaged. After mitosis the centrioles are released from each other or disengaged, a process likely regulated by PLK1 and separase (Loncarek et al., 2010; Tsou et al., 2009; Wang et al., 2011) (Fig. 1.2). Centriole disengagement is thought to be essential for centrosome duplication and has been proposed, with support from multiple experiments, as part of the licensing event (Loncarek et al., 2008; Tsou et al., 2009; Wong and Stearns, 2003). Additionally, PCM is required for duplication and therefore, the accumulation of PCM along with centrosome disengagement is required for the licensing of centrosome duplication (Wang et al., 2011). However, another mechanism involving PLK4 as the licensing factor has also been proposed (Brownlee and Rogers, 2013). This model takes into consideration the fact that PLK4 protein levels are tightly regulated during the cell cycle resulting in its expression only during late G1 to its highest levels at mitosis. Once mitosis is over its expression sharply decreases by interphase where upon it rises again at late G1 (Fode et al., 1996). Also considered in
**Figure 1.2: Centrosome Duplication**

This diagram depicts the process of centrosome duplication which is described in detail within the text. Centrioles are represented by green cylinders and pericentriolar material by grey clouds surrounding centrioles. Newly replicated mother and daughter centrioles are tethered together, represented by the red lines. Disengagement is the severing of this connection in mitosis, which is believed to be part of a licensing event required for centrosome duplication in the next cell cycle. The proteins believed to be a part of disengagement are shown in the blue box. Question marks are present to indicate that these proteins are only speculated to be involved. Procentriole formation occurs during the G1-S phase transition. It is initiated off of the centrioles that were segregated to the cell during the previous cell cycle. Elongation of procentrioles into fully formed centrioles begins in G2 phase. The proteins important for centrosome duplication are listed in the green boxes at the time in the cell cycle that they are active. In cells that have exited the cell cycle the centrosome can also go onto form the basal body for ciliogenesis. Only the more mature centriole which has obtained distal appendages (black triangles) in the previous cell cycles can form the basal body.

Figure adapted and modified from: (Nigg and Stearns, 2011)
this model is the fact that PLK4 is de-phosphorylated by PP2a/Twins during mitosis resulting in a brief stabilization of PLK4 during this time (Brownlee et al., 2011) (Fig. 1.2). This stabilization may allow PLK4 to somehow “license” the daughter centriole through a phosphorylation event, allowing for disengagement later in mitosis by PLK1 (Brownlee, 2013). In this regard, it could be possible that licensing is achieved by the activity of both PLK1 and PLK4 together. This model is appealing since PLK4 over-expression leads to supernumerary centrosomes, indicating high levels of PLK4 can override the licensing mechanism, however no work exists pointing to a functional role for PLK4 in licensing (Basto et al., 2008; Kleylein-Sohn et al., 2007). More work needs to be done to determine if this is the case and to show that PLK4 plays a functional role in centrosome licensing and this is not just reflective of an epiphenomenon.

2. **Cell Cycle Progression and Plk4**

Plk4’s role in cell cycle progression is evident from the phenotypes of its mouse models. Both the null and heterozygote cells exhibit a delay in mitotic progression (Hudson et al., 2001; Ko et al., 2005). The interaction of Plk4 with mitotic and DNA damage proteins add further support for its role in regulation of the cell cycle (Bonni et al., 2008; Ko et al., 2005; Petrinac et al., 2009; Rosario et al., 2010). Through *in vitro* kinase assays human PLK4 has been shown to interact with and phosphorylate CDC25C, a key protein in the regulation of mitotic entry (Bonni et al., 2008). Although not much is known about this interaction it is hypothesized that PLK4 phosphorylation along with phosphorylation by PLK1 and PLK3 exert additional controls that would regulate CDC25C stability and/or activity and, entry into mitosis (Bonni et al., 2008). Plk4 also interacts with Ect2 indicating that it may function in cytokinesis. *Plk4*+/- MEFs
frequently fail to undergo cytokinesis due to lack of Ect2 at the central spindle and a consequent lack of RhoA activity (Rosario et al., 2010).

In addition to functioning within the signaling pathways that regulate mitotic entry and cytokinesis, Plk4 also works within the DNA damage signaling pathway. While not completely elucidated, it appears that Plk4 functions to sense certain genotoxic stresses and then through a phosphorylation event, activates p53 to initiate a DNA damage response (Nakamura et al., 2013; Ward and Hudson, 2014). Support for this model comes from recent studies showing that Plk4 protein activity is up-regulated in response to certain genotoxic stresses (Nakamura et al., 2013; Ward and Hudson, 2014). Additionally, Plk4 has been shown to phosphorylate p53 and Chk2, both of which are important signaling proteins in the initiation of the DNA damage response (Ko et al., 2005; Petrinac et al., 2009; Swallow et al., 2005). Plk4 phosphorylation of p53 appears to be activating since levels of active p53 are reduced in the livers of Plk4+/- mice that have undergone a partial hepatectomy and, cells lacking Plk4 are unable to respond with a p53 response when provoked by certain DNA damage cues, such as UV radiation (Ko et al., 2005; Ward and Hudson, 2014). The relationship between p53 and Plk4 appears to be that of a negative feedback loop as p53 can down-regulate Plk4 upon DNA damage in multiple cell lines (Ward and Hudson, 2014). The Plk4 locus contains CpG islands that are hypermethylated following DNA damage indicating that its regulation via p53 may be done indirectly through epigenetic regulation to decrease Plk4 transcription (Afonin et al., 2006; Ward and Hudson, 2014; Ward et al., 2011). Histone deacetylases have also been shown to prevent transcription of Plk4 (Swallow et al., 2005). These data point to a model where Plk4 is briefly activated in response to certain genomic stresses: activated
Plk4 will phosphorylate p53 thereby activating it and the stress response. This will in turn permit p53 to up-regulate epigenetic modulators that will shut down Plk4 and prevent further progression in the cell cycle.

3. Plk4 and Tumourgenesis

Plk4’s function as the master regulator of centrosome duplication indirectly impacts genomic stability as well. As has been stated, loss of Plk4 regulation leads to supernumerary centrosomes and multi-polar spindles (Hudson et al., 2001). In Plk4 +/- mice and in human colorectal cancer cell lines, these phenotypes lead to aneuploidy and chromosomal instability which drives cancer progression (Swallow et al., 2005).

Moreover, chromosomal instability and centrosome amplification have been identified as hallmarks of cancer cells (Anderhub et al., 2012; Ganem et al., 2009). There are several in vivo animal models published describing the effects of aberrant Plk4 expression on tumour initiation or progression. Plk4 heterozygous mice are susceptible to spontaneous liver and lung tumours and, over-expression of Plk4 in flies leads to centrosome amplification and causes tumours (Basto et al., 2008; Ko et al., 2005). It is, therefore, not surprising that Plk4 is mis-regulated in several different human cancers. Plk4 protein levels were found to be decreased in hepatocellular carcinomas and this is associated with poor prognosis (Liu et al., 2012). Additionally, the Plk4 locus is frequently associated with loss of heterozygosity in hepatocellular carcinomas (Rosario et al., 2010; Swallow et al., 2005). In contrast, Plk4 levels are increased in human colorectal cancer tissue (Macmillan et al., 2001). Microarray analysis of breast tumours reveals that Plk4 is up-regulated in the aggressive triple negative subgroup and that its increased levels are associated with poor survival (Hu et al., 2006; Laufer et al., 2013). Given that Plk4
levels are up-regulated or down-regulated in different cancers lends to the idea that it is both a tumour suppressor and oncogene depending on the cellular context.

4. Plk4 in Development

While much work has been focused exclusively on Plk4’s role within the cell cycle it also has a crucial role during embryonic development. Plk4 null mice are embryonic lethal postgrastrulation, signifying a role for Plk4 in development (Hudson et al., 2001). This hypothesis can be further supported by the fact that Plk4 directly phosphorylates Hand1 to influence trophoblast stem cell differentiation during mouse placental development (Martindill et al., 2007). Hand1 is a transcription factor that localizes to the nucleolus, which serves to prevent its activity during trophoblast cell proliferation. Plk4 is predominately localized to the centrosome, however in G2 of the cell cycle it briefly moves to the nucleolus, an action that coincides with the differentiation of trophoblast stem cells (Hudson et al., 2001; Martindill and Riley, 2008). Plk4 initiates trophoblast stem cell differentiation by phosphorylating Hand1, allowing it to localize to the nucleus and drive expression of genes required for differentiation. Interestingly, Plk4 null embryos have a normal population of trophoblast cells, however, the number of differentiated giant cells is reduced, commensurate with a lack of Hand1 localization to the nucleolus, leading to loss of differentiation (Martindill et al., 2007). Clearly, while Plk4 has important functions within the cell cycle and as a tumour suppressor it has equally important roles during embryonic development that need to be unraveled.
Project Outline

To date much work has gone into understanding the role of Plk4 during the cell cycle and in cancer progression. However, Plk4 also has an important role during development: Plk4 null mice are embryonic lethal at E7.5. This study aimed to elucidate the functional roles of Plk4 during embryonic development. However, the lethality of Plk4 null mice makes further study of its role in development impossible. To circumvent this issue, the Plk4 loss of function phenotype was characterized in the model system Xenopus laevis. Using morpholino anti-sense technology, the plk4 loss of function phenotype was characterized with a focus on understanding its function in localizing the centrosome to establish cell polarity.

The study of gene function during Xenopus development has several advantages over studies on mouse. First, embryo collection is much easier as embryos develop externally and their developmental time frame is much shorter compared to mouse. Second, injection of agents that can alter gene expression can be performed by simple microinjection into one cell or multi-cell embryos. Injections into two cell embryos offer the opportunity to produce chimeric embryos in which the mutant phenotype is expressed on only one side while the other can be used as a contra-lateral control. Finally, the amount of depletion can be carefully manipulated in order to prevent lethality while still depleting plk4 protein levels, allowing for characterization of its mutant phenotypes and determination of its roles during embryonic development.
The Centrosome and Polarity

A centrosome is a microtubule-based structure comprising two centrioles and their surrounding pericentriolar material (PCM). Centrioles are composed of proteins that organize microtubules into a cartwheel structure with nine fold radial symmetry. PCM is then formed in a cloud around the centrioles and contains the proteins necessary for microtubule nucleation. The centrioles of a centrosome are not functionally equal; the older centriole is termed the mother centriole and has the ability to form primary cilia due to the presence of distal appendages, while the newly formed centriole is referred to as the daughter centriole (Nigg and Stearns, 2011) (Fig. 1.2).

The centrosome is responsible for nucleating microtubules and forming the spindle apparatus during mitosis which will segregate chromosomes to each new daughter cell (Avidor-Reiss and Gopalakrishnan, 2013). However, the centrosome has other important functions outside of the cell cycle, such as forming the basal body for ciliogenesis, defining cell polarity, participating in cell migration, and determining cell fate (Feldman and Priess, 2012; Januschke and Gonzalez, 2010; Kim and Dynlacht, 2013; Musch, 2004; Rusan and Peifer, 2007; Sepich et al., 2011; Sorokin, 1962; Tanos et al., 2013; Wakida et al., 2010; Wang et al., 2009; Yamashita et al., 2003; Yamashita et al., 2007; Zonies et al., 2010). These non-cell cycle functions can be attributed to specific positioning of the centrosome within the cell resulting in changes to cellular geometry through microtubule re-orientation (Tang and Marshall, 2012). With so many functions it has become apparent that the centrosome is important in embryonic cells as well as in differentiated cells. As such, the loss of key centrosomal proteins has been linked to several human disorders such as ciliopathies, microcephaly, respiratory disease and
cancer (Basto et al., 2008; Guernsey et al., 2010; Kim and Dynlacht, 2013; Marthiens et al., 2013; Pihan et al., 2003; Rutland and de Iongh, 1990; Yu et al., 2010).

One important yet understudied function of the centrosome is its role in establishing cellular polarity. Cell polarity can be defined as the asymmetric distribution of cellular factors that will allow the cell to properly orient itself within a tissue in order to carry out specialized functions. In the case of a zygote, polarity is sometimes established to define the domains of the embryo: that is its anterior-posterior, dorsal-ventral and left-right domains. In *C. elegans* the centrosome has been directly implicated in establishing anterior-posterior polarity. During fertilization, the point at which the sperm penetrates the zygote and donates its centrosome defines the posterior pole of the embryo. The mechanism by which this is achieved is unclear, however it appears that the centrosome provides a microtubule-independent cue at the cell cortex that initiates a cortical flow towards the anterior of the embryo that will asymmetrically distribute polarity proteins (Zonies et al., 2010).

Establishment of cellular polarity within a developing tissue is also an important developmental tool. One such example is in the case of epithelial cells which exhibit an apico-basal polarity. The establishment of apico-basal polarity (ABP) is an important aspect of embryonic development, particularly in the formation of epithelial and glandular structures. Several changes occur during establishment of this polarity, one of which involves the re-positioning of the centrosome to the apical surface of the cell. In most epithelial cell types this allows for the formation of a non-centrosomal, apical-basal microtubule array and primary cilia (Musch, 2004; Satir and Christensen, 2007; Tang and Marshall, 2012). Polarization of the microtubule array within the apico-basal axis allows
for vesicular transport between apical and basal ends of the cell (Musch, 2004). This process of apical centrosome relocation is conserved throughout the development of most vertebrate epithelial structures. The centrosome is polarized to the apical surface during neural, lens, retinal, mammary, intestinal and cochlea development (Manning et al., 2008; Musch, 2004; Tang and Marshall, 2012). The centrosome appears to be required for the establishment of apico-basal polarity by providing nucleated microtubules to the cell (Feldman, 2012; Tang, 2012). Recent work in C. elegans intestinal cells points to a functional role for the centrosome in establishing the apical end of the cell by depositing microtubule nucleating proteins to the apical surface. When the centrosome is laser ablated, apical polarity is disrupted due to a lack of apical microtubule distribution (Feldman and Priess, 2012).

During vertebrate gastrulation, cells undergo dramatic movements through convergent extension to organize into the three germ layers (endoderm, mesoderm, and ectoderm). Recently it has been shown that cell polarity defined by the centrosome is important to this process (Sepich et al., 2011). In zebra fish the planar cell polarity (PCP) pathway creates cell polarity by polarizing the centrosome posteriorly and medially at the transition from mid to late gastrulation. When centrosome position is randomized by down regulating components of the PCP pathway, disturbed cell division, misaligned mesoderm cells and reduced elongation are observed (Sepich et al., 2011).

Presumably, centrosome polarity is important to other developmental processes that require the coordinated movement of whole cohorts of cells, however little work has gone into understanding the exact function of the centrosome in establishing polarity. In
the following study I investigate the effect loss of centrosomes has on polarity during embryonic development.

**Somitogenesis**

One of the structures missing in *Plk4* null mice are the somites. During vertebrate development the dorsal axis is segmented into balls of mesoderm cells called somites that will give rise to the skeletal muscle, vertebrae, and dermis of the body. Under the control of several gene networks, somites bud off from pre-somitotic mesoderm (PSM) located on either side of the neural tube (Pourquie, 2011). The pace of somite formation is exquisitely timed with a new somite pair forming at a constant rate, however, this pace is differs according to species; in mouse a pair forms every 2 hours while in *Xenopus*, somites form every 45 minutes. The combination of several signaling networks along with morphological changes in cell shape and position will give rise to a mature somite from undefined pre-somitotic mesoderm. This process of somite formation occurs in almost all vertebrate species (Dequeant and Pourquie, 2008; Holley, 2007; Keller, 2000). The general machinery that controls somite formation appears to be conserved across multiple organisms; however there are some species-specific differences in the process.

**Clock and Wave front Model of Segmentation**

The molecular events that govern segmentation are only now beginning to be elucidated. Consensus is building for a Clock and Wave front mechanism, first proposed conceptually by Cooke and Zeeman in 1976 (Cooke and Zeeman, 1976). The proposed mechanism contains two components: (1) a wave front of maturation that moves from
anterior to posterior along the PSM of the embryo; (2) gene oscillatory (clock-like) activity in the PSM that propagates in anti-parallel fashion from the posterior to anterior of the embryo (Cooke and Zeeman, 1976). When cells in the correct phase of the oscillator intersect with the wave front of maturation, a cascade of signaling events begins the segmentation of modeling a new somite pair. The existences of both components of this model have been supported by molecular experiments in a variety of organisms (Pourquie, 2011).

The Wave front

The wave front is formed by the opposing activities of three signaling pathways (reviewed in (Pourquie, 2011)) (Fig. 1.3). Fgf and Wnt signaling gradients emanate from the posterior PSM and their expression becomes progressively less intense towards the anterior. The FGF gradient is produced by mRNA decay in conjunction with embryo elongation (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004). Fgf8 is only synthesized in the most posterior cells of the PSM. As the embryo elongates cells are displaced towards the anterior PSM and cease to produce Fgf8 mRNA, creating a posterior to anterior gradient of cells that express Fgf8. It is believed the Wnt gradient is established in the same manner; however, there is no experimental evidence to confirm this (Aulehla et al., 2003).

In contrast, retinoic acid is maintained high concentrations in the formed somites as well as the anterior PSM. The retinoic acid signaling gradient is established in the anterior PSM by anti-parallel expression of its biosynthesis components within the entire PSM. Raldh2, the enzyme responsible for retinoic acid synthesis, is expressed at high
levels in the anterior PSM and somites (Niederreither et al., 1997). The synthesized retinoic acid will then diffuse toward the posterior PSM where it is met by high levels of Cyp26AI expression, which will result in the catabolism of retinoic acid (Sakai et al., 2001). The opposing action of its biosynthesis components leads to the establishment of a retinoic acid gradient that will oppose the activity of the Fgf and Wnt gradients encroaching from the posterior.

Together these opposing gradients will form a threshold or determination point permissive for the changes in gene activity required for somite formation (Pourquie, 2011). In *Xenopus*, the determination point has been mapped to an un-segmented domain that sits 9 prospective somites away from the newest formed boundary (Sparrow, 2008).

**The Segmentation Clock**

The second component of the Clock and Wave front model is the segmentation clock or oscillator. Cook and Zeeman proposed that an oscillator of gene expression traverses the PSM setting the pace of somite formation such that one oscillation corresponds to the time it takes to form one somite (Cooke and Zeeman, 1976). While the pace of somite formation is different for all vertebrates the molecular machinery involved is astonishingly conserved in most organisms.

Oscillatory gene syntax expression is seen in the PSM of almost all vertebrates indicating this mechanism is conserved. These oscillations are first activated in cells of the posterior PSM that then propagate anteriorward like a wave into the anterior PSM. The wave of oscillatory gene expression meets the determination point, where, if the cells are properly synchronized, they establish the persistent gene expression necessary to
Figure 1.3: Somitogenesis in *Xenopus laevis*

Somite maturation begins after a cohort of cells has been specified to become the next somite at the determination point. The determination point is determined by opposing gradients of FGF and WNY signaling from the posterior (dark and light blue) and retinoic signaling from the anterior (red) PSM. After a cohort of cells has been determined to form a somite they will go through a series of morphological changes allowing them to rotate 90°. Rotated somites possess nuclei in a pattern that will appear as a stripe perpendicular to the neural tube.

Figure adapted and modified from: (Sparrow, 2008)
Anterior

Retinoic Acid Gradient

Determination Point

FGF and WNT Gradients

Posterior

Mature Somites

Determined Somites

Unsegmented Mesoderm
differentiate somites. During this oscillation, cells along the PSM will experience a transient receptive state of cyclic gene expression. The majority of cycling genes belong to the Wnt, FGF and Notch signaling pathways. Of note, the Wnt and FGF signaling pathways are also components of the wave front pointing to a relationship between the two components of the model. Negative feedback seems to be a common mechanism for maintaining cyclic gene expression, although signaling between networks also seems to be important for registration of all components. In all the pathways involved in somitogenesis, negative regulators of signaling are found to have cyclic gene expression (Kageyama et al., 2012).

When Clock and Wave front Meet

Together the clock and wave front are able to initiate segmentation resulting in the formation of somites that bud bilaterally from the anterior PSM at a specific pace thereby producing somites that are the same size every time. In this model the molecular clock sets the pace of somite formation and the wave front determines both where somites will form as well as their size, although there has been some recent debate about whether this is true (Kageyama et al., 2012; Niwa et al., 2011). As the clock oscillates towards the anterior PSM it will meet the determination point that is moving towards the posterior PSM. In this location a specified cohort of cells will be released from the constraints of FGF and Wnt signaling that have kept them in an undifferentiated state and thus will be able to respond to the signals of the molecular clock and retinoic acid resulting in changed gene expression in these cells (Sparrow, 2008).
While only a certain number of somites may have actually budded off from the PSM, the un-segmented PSM will already be pre-patterned into several prospective somites. This pre-pattern can be seen as stripes of gene expression in the un-segmented PSM. In *Xenopus*, examples of common segmentally expressed genes include *Thylacine*, *Mesogenin1/Mespo*, *Paraxis*, *Hes6*, *Hairy2b*, *ESR4*, *ESR5* and *Bowline* (Jen et al., 1999; Jen et al., 1997; Kondow et al., 2006; Koyano-Nakagawa et al., 2000; Sparrow et al., 1998; Yoon et al., 2000). This pre-pattern of genes will then initiate the changes required for somite maturation, differentiation and border formation. Genes such as *PAPC*, *C-cadherin*, *EphA4*, *EphB2*, *EphLI*, *integrin-alpha5* and *NCAM*, are activated downstream of the clock and are important for border formation and somite organization (Barrios et al., 2003; Durbin et al., 1998; Giacomello et al., 2002; Kim et al., 2000; Kintner, 1988; Kragtorp and Miller, 2007).

**Morphological Changes during *Xenopus* Somitogenesis**

Before somites can emerge, the PSM must be formed within the dorsal axis of the embryo. In *Xenopus*, the PSM derives from the deep layer of marginal zone (IMZ) which produces the somites and notochord (reviewed in (Keller, 2000)). These cells involute and then undergo convergence and extension along the anterior-posterior axis. The PSM and consequently the embryo will then elongate throughout somitogenesis. PSM elongation is not due to cell division but instead to intercalation of the involuting cells. This continuous extrusion of posterior PSM causes the embryo to elongate with little change to the embryo’s width.
The morphological changes that accompany somitogenesis have been extensively studied in *Xenopus laevis* through histology, SEM, live imaging and confocal imaging (Afonin et al., 2006; Hamilton, 1969; Wilson et al., 1989; Youn and Malacinski, 1981). Two important morphological changes occur in the PSM in order to form a somite. (1) A fissure must be produced to physically separate the newly formed somite from the rest of the PSM and (2) the cells of the new somite must undergo changes to make them morphologically distinct from the rest of the un-segmented PSM. How these changes come about is different among vertebrates.

In *Xenopus*, fissure formation begins in the lateral portion of the future somite and moves medially until it reaches the notochord, with very little in the way of cell rearrangements (Wilson et al., 1989). This also seems to be the case during zebra fish fissure formation as well (Henry et al., 2000; Wood and Thorogood, 1994). These movements are in contrast to chick where the fissure forms both medially and laterally whereupon they meet in the middle of the somite. Also, this fissure is not straight and forms a ball and socket shape with the PSM (Kulesa and Fraser, 2002). Eventually the cells of the somite will pull away from the PSM and the gap is filled with cells from the lateral anterior edge of the PSM (Kulesa and Fraser, 2002).

As fissure formation proceeds in *Xenopus*, the cells of the newly forming somites are also rotating 90°. This was first described by Hamilton (Hamilton, 1969). Cells closest to the fissure move towards the notochord while cells in the posterior compartment move laterally (Hamilton, 1969). If one were looking from above at a cohort of cells that were rotating on the right side of the notochord, they would appear to be rotating in a clockwise direction (Fig. 1.3). As a result, cells once aligned with their
anterior-posterior axis perpendicular to the notochord are rotated $90^\circ$ such that this axis is now parallel to the notochord. From Hamilton’s analysis it would seem that cells of a somite rotate as a coherent cohort, however this is not the case. Confocal analysis revealed that when dorsal-ventral aspects of the somite are taken into account, rotation is initiated in the dorsal cells at the level of the neural tube and then progresses ventrally throughout the somite (Afonin et al., 2006).

A recent report in zebra fish describes cell rotation required for the formation of a muscle progenitor cell compartment that is reminiscent of the rotation seen in *Xenopus laevis* (Hollway et al., 2007): rotation may not be a species specific aspect of *Xenopus* somitogenesis as once previously thought. The author’s noted that while rotation occurred at different times in somitogenesis, in both zebra fish and *Xenopus* rotation occurred around the time muscle differentiation occurs leading them to suggest rotation is quite similar between the two species (Hollway et al., 2007).

Distinct from *Xenopus*, chick and mouse somites undergo an epithelialization event during somite formation as opposed to cell rotation (Duband et al., 1987; Gossler and Hrabe de Angelis, 1998; Kimmel et al., 1995). In these vertebrates, a budding somite starts with a population of identical mesodermal cells. Under the control of *Paraxis* and *Mesp2*, a small group of cells will undergo a mesenchyme to epithelial transition to form a layer of epithelial cells around the newly formed somite (Barnes et al., 1997; Takahashi and Sato, 2008). This transition from a mesenchymal to epithelial state is governed by changes in the actin cytoskeleton and the establishment of cell polarity (Nakaya et al., 2004). It is unknown whether this morphological difference between frog and other vertebrates is functionally significant to somitogenesis.
The establishment of cellular polarity is important for most cellular movements. The cell shape changes that occur during vertebrate somitogenesis presumably require the establishment of a cellular polarity. In *Xenopus* just prior to rotation cells elongate along their medio-lateral axis and form filopodial projections at their posterior ends, which will physically aide in rotation (Afonin et al., 2006). In mouse, the centrosome is apically polarized during the epithelialization of somites (Barrios et al., 2003). To date little work has gone into understanding the molecular events driving cell polarity, or how the positioning of organelles contributes to it. In the following study, I investigate the role of *plk4* in regulating cell polarity during *Xenopus laevis* somitogenesis.

**Eye Development**

Vertebrate eye development is a complex process involving many gene networks and morphological changes. Eye formation is remarkably conserved in vertebrates: eye develops from both neural and non-neural ectoderm as multiple signaling pathways specify and organize a functional eye. Lens formation represents a classic case of embryonic induction in which signaling from one tissue directs the development of adjacent tissue. In addition, although not extensively studied, establishment of polarity in the individual cells of the lens and retina is also important for proper eye formation.

**Eye Morphogenesis**

The first morphological sign of eye development occurs early in neurulation as the developing diencephalon begins to bilaterally evaginate to form two optic
protuberances. These optic vesicles extend laterally towards the non-neural surface ectoderm. This overlying ectoderm will eventually give rise to the lens and cornea and thus is referred to as the presumptive lens ectoderm (PLE) (Fig. 1.4A). The optic vesicles displace the intervening mesenchyme as they move towards the surface ectoderm: this area represents a very rare instance of ectoderm coming into direct contact with neural primordia. In *Xenopus*, this occurs at stage 20 as the neural tube finishes closing (Chow and Lang, 2001). This direct contact permits inductive signals from the optic vesicle to stimulate ectoderm to thicken via cellular columnarization to form a placode (Fig. 1.4A). The lens placode is a thickening of the ectoderm due to cell proliferation and elongation in a confined area (Chow and Lang, 2001; Huang et al., 2011; Zwaan and Pearce, 1971). Lens placode forms at around stage 28 in *Xenopus*. While the PLE is already competent and biased to form lens from previous inductive events, induction by the underlying optic vesicle at this juncture is critical to specification and eventual differentiation. Once the lens placode is formed reciprocal inductive signals cause the optic vesicle and placode to invaginate (Grainger, 1992) (Fig. 1.4B, C). Invagination of the optic vesicle leads to the formation of the optic cup which becomes a double layered structure. The outer layer will differentiate into the retinal pigmented epithelium (RPE) while the inner layer will differentiate into the neural retina (Chow and Lang, 2001; Lang, 1999). Invagination of the lens placode forms the lens vesicle which eventually detaches from the head ectoderm to complete its development as a lens within the optic cup (Chow and Lang, 2001; Grainger, 1992; McAvoy, 1980) (Fig. 1.4D). Overt eye formation can be seen in *Xenopus* at around stage 34 where the pigmented epithelium and lens are visible.
Once the lens vesicle has detached from the surface ectoderm it will begin its differentiation into primary lens fibre cells (Fig. 1.4E). Lens fibre cell differentiation is characterized by the synthesis of crystallin proteins as well as by cell polarization and elongation (Kuszak, 1995; Piatigorsky, 1992). Differentiation occurs in the posterior half of the lens vesicle leading to a polarization of the lens as a whole. In the anterior a monolayer of proliferating epithelial cells lines the differentiated lens fibre mass of the posterior. These anterior epithelial cells will act as the progenitors of the lens fibre cells throughout the adult life of the organism (Chow and Lang, 2001; Lang, 1999).

**Lens Placode Formation**

Placode formation is the first step in the morphogenesis of cranial sensory structures from non-neural ectoderm. For example, the lens, nose and inner ear all arise from placodes. The lens placode is characterized by the thickening of a specified group of cells in the ectoderm overlying the optic vesicle. These cells transform from a cuboidal to a columnar shape and then elongate in this confined area (McKeehan, 1951). In the case of lens, placode formation appears to be critical for further eye development. In Pax6-null mice or pitx3 depleted Xenopus laevis embryos, a lens placode fails to form and further eye development is halted (Hill et al., 1991; Hogan et al., 1986; Khosrowshahian et al., 2005).

Formation of the lens placode begins early in embryogenesis when cell-autonomous and inductive events specify which cells of the ectoderm will give rise to the lens placode and ultimately to lens (reviewed in (Chow and Lang, 2001; Grainger et al., 1992; Zuber et al., 2003)). This specified group of cells defines the presumptive lens
Figure 1.4: Eye morphogenesis

(A) The first morphological signs of eye development are indicated by the formation of the optic vesicle (OV). The OV will come into contact with the overlying non-neural ectoderm known as the presumptive lens ectoderm (PLE) that has previously been biased to become lens cells. The OV sends inductive signals to the PLE indicated by the red arrow. (B) The lens placode (LP) begins to form due to cell proliferation and elongation in a confined area. The lens placode also sends inductive signals (red arrow) back to the OV resulting in invagination of both structures (C and D). The invagination of the OV and lens placode results in the formation of the optic cup (OC) and lens vesicle (LV). (E) The lens becomes polarized as it begins to organize itself. The lens epithelial cells (LE) line the anterior of the lens while the posterior of the LV has differentiated into primary lens fibres (LF) which express crystallins giving the lens its characteristic transparency.

Figure adapted and modified from: (Ogino et al., 2012)
ectoderm by expressing genes that will govern further lens morphogenesis. The transcription factors *Pax6* and *Six3* are especially important during lens ectoderm specification and differentiation. *Pax6* has been shown to activate genes that are required for lens placode formation and subsequent differentiation, such as *Sox2* and the *crystallins* (Ashery-Padan et al., 2000; Cvekl et al., 1995; Ogino et al., 2012). *Six3* is important for maintaining *Pax6* expression in the presumptive lens ectoderm and also for sustaining cellular proliferation through inhibition of negative cell cycle regulators (Gestri et al., 2005; Goudreau et al., 2002; Ogino et al., 2012).

While the genetic pathways that govern lens placode specification have been extensively studied, little work has gone into understanding the cellular changes that regulate the process. To date this understanding comes from studies in chick embryos. Just prior to placode formation the cells that comprise the PLE undergo changes to cell shape and develop a specific polarity (Byers and Porter, 1964; McKeehan, 1951). PLE cells transition from a cuboidal to columnar shape and undergo elongation in a direction parallel to the direction of future invagination (McKeehan, 1951). This is the first morphological evidence of lens placode formation. These cells are also characterized by a shift in cell polarity, creating an apico-basal polarity presumably important for placode formation and invagination. This apico-basal polarity is created by shifting most of the organelles, including the nucleus, towards the basal end of the cell (Byers and Porter, 1964; McKeehan, 1951). Proteins such as PAR3, Shroom3, RhoA and ActR-1, as well as actin foci are polarized apically, indicating that indeed an apico-basal polarity is established within the cells of the PLE (Borges et al., 2011; Plageman et al., 2011; Yoshikawa, 2000). Additionally, microtubules appear to anchor themselves at the apical
end of the cells and then to orient themselves parallel to the cell’s longitudinal axis (Byers and Porter, 1964). It appears that microtubules are important for elongation. Cultured lens epithelial cells treated with colchicine, which inhibits microtubule assembly, fail to elongate (Piatigorsky et al., 1972). The case is presumably the same in PLE cells (Pearce and Zwaan, 1970; Piatigorsky et al., 1972). Once the microtubule cytoskeleton is properly established it is believed to aide in process termed inter-kinetic nuclear migration. During this process the nucleus migrates to the apical end for mitosis then returns to the basal end for DNA synthesis (Pearce and Zwaan, 1970). After the establishment of apico-basal polarity and completion of lens placode formation, the placode invaginates through apical-constriction, internalizing the lens rudiment within the optic cup where it will develop further.

**Centrosome Polarity in Eye Development**

Once the lens placode has internalized it organizes into the lens vesicle and begins to establish a polarity between the types of cells in the anterior and posterior of the vesicle. In the anterior of the vesicle, a proliferative monolayer of lens epithelial cells forms that will give rise to the differentiated fibre cells of the lens throughout life. The cells in the posterior of the lens vesicle begin to differentiate into the primary lens fibres (Lang, 1999). During the differentiation process these cells will lose organelles, such as the nucleus and centrosome (Wride, 2000). In addition, they begin to elongate through a process involving major cytoskeleton reorganization (Kuszak, 1995). The centrosome has been shown to be apically polarized in the anterior epithelial and posterior fibre cells of adult lenses (Dahm et al., 2007; Sugiyama et al., 2011). Reports in mouse, chick and zebra fish indicate that the centrosome is also apically polarized during embryonic eye
development, in both the retina and lens (Malicki and Driever, 1999; Manning et al., 2008; Rizzolo and Joshi, 1993; Zolessi et al., 2006). The importance of centrosome polarization in the lens is unknown, however; the centrosome is important for regulating cell shape changes and cell polarity, suggesting the centrosome is likely important for the differentiation of lens epithelial cells (reviewed in (Tang and Marshall, 2012). When *Nedd1*, a gene that encodes a centrosomal protein involved in the recruitment of $\gamma$-tubulin, is translationally knocked down in zebra fish severe morphants fail to form eyes (Manning et al., 2010). This result seems to point to an important role for the centrosome during eye development though, whether it is the loss of centrosome polarization or some other function of the centrosome that contributes to this eye phenotype is unknown.
CHAPTER 2:
Materials and Methods

Embryo Collection

*Xenopus laevis* females were induced to ovulate with injection of 0.6-0.8cc of human Chorionic Ganadotrophin hormone (hCGH) (Intervet Canada Corp.). Embryos were obtained via *in vitro* fertilization and de-jellied in 2% cysteine (pH 8) as previously described (Drysdale and Elinson, 1991). For wild type analysis embryos were left to develop in 0.1x MBS (88mM NaCl, 1mM KCl, 0.7mM CaCl, 1mM MgSO$_4$, 5mM HEPES pH7.8, 2.5mM NaHCO$_3$) at either 12°C or 17°C. Embryos to be used for morpholino injection were always incubated at 12°C post-injection. Embryos were staged according to Nieuwkoop and Faber, fixed in MEMPFA (0.1M MOPS pH 7.4, 1mM MgSO$_4$, 2mM EDTA, 4% paraformaldehyde) at 4°C overnight, and stored long term in 70% methanol (Nieuwkoop, 1967).

Cloning

A *plk4* truncation construct containing the first 1200bp of the *plk4* cDNA was cloned for riboprobe production. *Plk4*-Sport6 was digested with *Hind*III to remove 2354bp from the 3’ end of the *plk4* cDNA insert. The larger fragment, containing the Sport-6 vector backbone and the first 1200bp of *plk4* cDNA, was gel extracted using Sigma’s gel extraction kit. This fragment was then re-circularized using Thermo-Scientific’s rapid ligation kit.
**In Situ Hybridization**

Staining to detect mRNA localization via *in situ* hybridization was performed according to standard protocols (Nieuwkoop, 1967). Briefly, linearized plasmids were transcribed into RNA probes labeled with digoxigenin-UTP (Roche). Embryos were incubated with probe overnight at high stringency (68°C) to allow binding. Embryos were washed and incubated overnight at 4°C with an anti-digoxigenin secondary antibody linked to alkaline phosphatase (Roche). Excess antibody was washed away with maleic acid buffer (100mM Maleic acid, 150mM NaCl pH 7.5) and incubated with BM purple as a substrate for alkaline phosphatase (Roche). The colour reaction was stopped by addition of maleic acid and embryos were fixed in MEMPFA and bleached (1% H2O2, 5% formamide, 0.5x SSC). Embryos were cleared with BABB (2 parts benzoic acid: 1 part benzyl benzoate) prior to image collection. Images were collected on a Leica MZFLIII stereoscope using northern eclipse software (Empix, Canada). Refer to table 2.1 for information regarding procurement of plasmids, plasmid linearization and, RNA transcription.

**Antibody Development**

A polyclonal anti-plk4 antibody was produced by the company GenScript (Piscataway, NJ, USA) using rabbits as the host. GenScript performed peptide synthesis, animal injections and antibody purification. The peptide sequence synthesized for use as the antigen corresponds to the following 14 amino acid sequence within the N-terminal kinase domain of *Xenopus laevis* plk4: NRYLKNRKKPFAED.
Protein Isolation and Western Blotting

Protein lysates were obtained from batches of 20 embryos. Embryos were lysed (20mM Tris pH8, 100mM NaCl, 1mM EDTA, 0.5% TritonX, 10% glycerol and supplemented with Roche protein inhibitor tablets at 1:20 dilution- protocol kindly provided by Dr. Kristen Kroll), sonicated with 10 pulses (repeated three times) on ice then centrifuged for 15 minutes. Sonification followed by centrifugation was repeated twice. Protein concentration was determined by Bradford assay. Samples were run with 30µg of protein on an 8% poly-acrylamide gel at 160V for approximately one hour. The gel was then washed in 1x transfer buffer (48mM Tris, 39mM glycine, 20% methanol, 0.004% SDS) for 15 minutes and proteins were then transferred to a PVDF membrane (Roche) using a semi-dry apparatus at 10V for 45 minutes. For all blots blocking was done for one hour at room temperature with 5% milk in TBST (50mM Tris base, 150mM NaCl, 0.1% Tween-20, pH 7.6) prior to antibody incubation. Anti-plk4 (rabbit) and anti-Prmt5 (rabbit- Millipore) were both diluted 1:1000 in 5% milk and anti-actin (Sigma, A2066) diluted 1:10 000 in TBST. All primary antibody incubations were done overnight at 4°C. Blots were washed three times for a minimum of 10 minutes and incubated with anti-rabbit HRP (Cell Signaling) at a dilution of 1:10 000 at room temperature for one hour. Blots were washed three times for a minimum of 10 minutes and exposed using super signal chemiluminescence reagents (Thermo-Fisher West Pico) for 8-20 minutes. Anti-plk4 blots were stripped prior to actin staining. Briefly blots were incubated at 50°C in stripping solution (B-mercaptoethanol, SDS and Tris pH6.8) for 30 minutes then washed with TBST another 30 minutes. Blots were then washed for one hour at room temperature in TBST and then blocked in 5% milk-TBST for one hour.
After blocking blots were incubated with the above mentioned dilution of actin overnight at 4°C and incubated with anti-rabbit secondary as described above.

**Antibody Pre-absorption**

Western blotting to obtain a PVDF protein blot was done as described above. Prior to primary antibody incubation the plk4 peptide was incubated with the plk4 antibody in 1mL of TBST for 2-3 hours at room temperature with gentle rocking. The peptide was in 100 molar excess of the plk4 antibody. This mixture was then added to 9mL of 5% milk in TBST and incubated with the blot overnight at 4°C. Procedures for secondary antibody and chemiluminescence were done as described above. Secondary antibody used was HRP anti-rabbit at 1:10000 dilution in 5% milk and TBST.

**Morpholino Design and Microinjection**

For translational knockdown assays anti-sense morpholino oligonucleotides were designed and ordered through GeneTools, LLC (Orlando, USA). To confirm specificity two plk4 specific morpholinos were designed to target different regions of the plk4 transcript. Plk4.upMO sequence targeting the 5' UTR was 5’-TCTTTTTCTCTCGCTGCTCGCGCCC-3' and Plk4.1MO sequence targeting the ATG start site was 5’-CTCTCTCCTATGCTGCCCGCATGC-3’. The mis-match control sequence corresponding to Plk4.1 was 5’-CTCTCTTCgTATcCTcCCCcCATc-3’. A standard control morpholino targeted against the human β-globin gene was also ordered from GeneTools with sequence 5’-CCTCTTACCTCAGTTACAATTATA-3’. All morpholinos were tagged with a 3’-Carboxyfluoroscein moiety for lineage tracing.
Embryos to be used for morpholino microinjection were incubated briefly in 0.3x MBS and 2% Ficoll-400 (Sigma) prior to injection and then microinjected into their animal pole at either the 1 or 2 cell stage. Microinjections were performed using a Drammond nanoinjector maintained at a volume of 4.6nL. Post-injected embryos were incubated in 0.3xMBS and 2% Ficoll-400 at 12°C for at least 1.5 hours to allow healing. Embryos were subsequently moved into 0.1x MBS for incubation at 12°C degrees until they developed to appropriate stages for analysis. Experimental embryos were staged according Nieuwkoop and Faber and fixed in MEMPFA for either 2 hours at room temperature or overnight at 4°C (Nieuwkoop, 1967).

**Immunohistochemistry**

Immunostaining was performed using standard protocols (Sive, 2000). Briefly, whole embryos were rehydrated to 25% methanol and washed three times in PBT (1xPBS, 2mg/mL BSA, 0.1% TritonX-100). Embryos were blocked with 10% lamb or fetal bovine serum in PBT for one hour at room temperature. Embryos were then incubated with overnight at 4°C with primary antibodies to either anti-β1 integrin (Drs. P. Hausen and V. Gawanta- 8C8, Developmental Studies Hybridoma Bank, Iowa City, USA) at 1:400 dilution, anti-12/101 (Dr. JP. Brockes-12/101, Developmental Studies Hybridoma Bank, Iowa City, USA) at 1:1000 dilution or, anti-Ɣ- tubulin (Sigma GTU-88) at 1:100 dilution. All primary antibody dilutions were made in PBT and 10% lamb or fetal bovine serum. The next day embryos were washed 5 times with PBT for a minimum of 20 minutes and incubated with secondary antibodies either overnight at 4°C or for 4 hours at room temperature. Alexa-568 (Life Technologies) secondary antibody
diluted 1:500 in PBT was used for β1-integrin and γ-tubulin stained embryos. Anti-mouse-HRP secondary (Sigma) diluted to 1:200 in PBT was used for anti-12/101 embryos. After secondary antibody incubations embryos were washed at least 5 times for a minimum of 20 minutes. Embryos stained with Alexa-568 secondary were dehydrated to 70% methanol to be sectioned later. Embryos stained with anti-12/101 were washed twice for 5 minutes with 1x PBS. Antibody detection was achieved by addition of DAB (Sigma) diluted to 1mg/ml in PBT supplanted with 0.003% H2O2. The colour reaction was stopped by two five minute washes with PBT. Embryos were then dehydrated to 70% methanol and imaged as whole mounts using a Leica MZFLIII stereoscope and Northern Eclipse software.

**Sectioning and Hoechst Stain**

Whole embryos were dehydrated in methanol, cleared in xylene and then embedded into paraffin wax (McKormick Scientific). Embryos were then oriented into paraffin blocks to allow for frontal or transverse sections and sectioned using a manual rotary microtome (American Optical Company, 820 Spenser). Sectioning was done at 7µm for embryos previously stained with anti-γ tubulin, at 13µm for unstained embryos to be visualized via Hoechst, and 15µm to 20µm for embryos stained with riboprobes. Sections were then left on a warmer set to 42°C overnight. Sectioned embryos to be Hoechst stained were then de-waxed in xylene and rehydrated into 25% methanol. Sections were then washed once in PBS, permeablized in PBST (0.1% tween), stained with Hoechst 33342 (Invitrogen) diluted 1:1000 in PBST and washed with PBST. Fluorescently stained sections were cover slipped with fluoromount G and imaged on a
Zeiss Axioscope fluorescent microscope using northern eclipse software. Sectioned embryos stained with riboprobe were washed three times in xylene to remove the wax and then cover slipped with Paramount (Fisher Scientific). Bright field images were taken with a Zeiss Axioscope using Northern Eclipse software.
Table 2.1: List of plasmids used for *in situ* hybridization

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzyme used for linearization</th>
<th>RNA polymerase used for transcription</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>plk4</em></td>
<td>SalI</td>
<td>T7</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td><em>plk4-5’</em></td>
<td>SalI</td>
<td>T7</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td><em>notch</em></td>
<td>Cla</td>
<td>Sp6</td>
<td>Dr. Kintner</td>
</tr>
<tr>
<td><em>hairy/2b</em></td>
<td>EcoRI</td>
<td>T7</td>
<td>NIBB</td>
</tr>
<tr>
<td><em>pax3</em></td>
<td>EcoRI</td>
<td>T7</td>
<td>Dr. M. Crawford</td>
</tr>
<tr>
<td><em>pax6</em></td>
<td>EcoRI</td>
<td>SP6</td>
<td>Dr. Harris</td>
</tr>
<tr>
<td><em>rax1</em></td>
<td>HindIII</td>
<td>T3</td>
<td>Dr. Zuber</td>
</tr>
</tbody>
</table>
CHAPTER 3:

Results

*Plk4* has a diverse spatio-temporal pattern of expression during *Xenopus* development

The spatial temporal expression of *plk4* mRNA was determined via riboprobe *in situ* hybridization. Two anti-sense probes were developed to confirm that its expression pattern was specific along with the use of sense probes. One probe was developed using the entire full length sequence of *plk4* cDNA. The *plk4* spatial-temporal pattern seen with this probe was consistent with another probe made by a previous colleague in the lab. The second probe was developed using only the first 1200bp of 5' sequence. Sense probes were developed as controls for both probes. Unfortunately, both sense probes did not provide an optimal control as they produced the same expression pattern as was seen for the anti-sense probes. For example both sense probes were expressed in the somites, lens and retina, among other structures.

*Plk4* was expressed rather ubiquitously in the early stages of development. At approximately stage 13 *plk4* expression restricts to the anterior and dorsal domains of the embryo (Fig. 3.1 A). At around stage 17 the *plk4* expression domain becomes more intense. During this stage *plk4* expresses the anterior neural plate, neural tube, pre-somitic mesoderm and lateral plate mesoderm (Fig. 3.1 B, C). Around stage 20 *plk4* expression becomes more restricted in the anterior neural plate with expression in the optic vesicles and brain (Fig. 3.1 D). Faint expression in the pre-somitic mesoderm (PSM) remains throughout the processes of segmentation as well as in newly formed
somites (Fig. 3.1 F, G, H, I, L). In mature somites $plk4$ expresses along the entire anterior-posterior length of the somite. Interestingly, $plk4$ expression concentrates at the centre of the somite, in a location where the nuclei are seen to align after rotation (Fig. 3.1 H). The expression of $plk4$ decreases significantly around stage 24 of development. At this stage expression is restricted to the developing optic vesicle, brain, somitic mesoderm and can also be seen in the newly forming otic placode (Fig. 3.1 E, F). As development progresses $plk4$ is expressed in the branchial arches, lens placode and future retina, as well as the in the brain and otic vesicle (OtV) (Fig. 3.1 G). $Plk4$ continues to express in the late stages of eye development. At stage 31 it is seen in the lens vesicle as well as the invaginating retina (Fig. 3.1 I). When viewed laterally, its expression in the lens vesicle appears to be concentrated within a circle surrounding the lens. A similar pattern is seen in the otic vesicle as well. A closer look at the eyes through sectioning of a stage 34 embryo reveals expression of $plk4$ in the neural retina as well as the pigment retinal epithelium. Through sectioning it can also be seen that $plk4$ expression in the lens is concentrated within the mitotic centre of the lens (Fig. 3.1 K). $Plk4$ also expresses in the notochord and neural tube, where it persists into the later stages of development (Fig 3.1 J). $Plk4$ expression remains within the above mentioned structures even into the late tail bud stages (Fig. 3.1 L).
Figure 3.1: *plk4* has a diverse spatial-temporal expression pattern during *Xenopus* embryogenesis

The spatial-temporal pattern of *plk4* mRNA expression was analyzed via riboprobe *in situ* hybridization. In all panels the anterior of the embryo is oriented to the left and the dorsal side upwards. Arrows point to areas of significant expression.  
(A) Dorsal view of stage 14 embryo expressing *plk4* in the anterior neural plate (ANP) and neural folds (NF).  
(B) At stage 17 *plk4* expression is restricted dorsally with expression in the pre-somitic mesoderm (PSM) and neural tube (NT). Expression remains within the anterior neural plate.  
(C) Lateral view of stage 17 embryo with *plk4* expression in the lateral plate mesoderm (LPM).  
(D) *Plk4* expression becomes more defined at stage 20 with expression in the optic vesicle (OV) and forebrain (FB).  
(E and F) At stage 24 *plk4* can be seen in the otic placode (OP) and somites (S). Its expression continues in the neural tube, optic vesicle, and brain (B).  
(G) As embryogenesis progresses to stage 28 *plk4* expression remains within the somites and can be seen in the newly formed lens placode (LP), otic vesicle (OtV), retina (R) and branchial arches (BA).  
(H) *Plk4* is expressed throughout the entire somite, with strongest expression at the centre of the somite (outlined by black box).  
(I) By stage 32 *plk4* expression persists within the somites, branchial arches, otic vesicle and retina. It is expressed in the newly forming lens with strongest expression along the outer surface. A similar pattern is observed in the otic vesicle. These patterns of expression remain at stage 37 (L).  
(J) Frontal section of stage 31 embryo confirming *plk4* expression in the neural tube and somites. It is also expressed in the notochord (N).  
(K) Frontal section of stage 34 embryo confirming expression of *plk4* within the forebrain (FB), neural retina (NR) and lens (L). Its expression in the lens appears to be concentrated at the centre of the structure. *Plk4* is also expressed within the retinal pigmented epithelium (RPE).
Production of a *Xenopus laevis* specific plk4 antibody

There is no commercially available antibody for *Xenopus* plk4 so we sought to produce an antibody through the company GenScript. A small peptide fragment corresponding to a region within the N-terminal of plk4 was used to produce the antibody in rabbits. Western blot analysis failed to produce a signal at the expected size of 109kDa for plk4. Instead, we obtained a strong signal in the 42kDa region (Fig. 3.2, lane 1). To confirm that the antibody was specific to plk4 we performed western blots in which the antibody had been pre-absorbed with the plk4 antigen used to produce the antibody. If the antibody is indeed specific to the plk4 peptide used for its production pre-absorption would serve to soak up the antibody and prevent a signal. This indeed was the case as pre-absorption with 100 molar excess of peptide prevented antibody binding as can be seen by the loss of the 45kDa signal (Fig. 3.2, lane 2). The protein identified by this antibody is also present in control and *plk4MO* injected embryos (Fig. 3.2, lane 3 and 4, respectively).
Figure 3.2: Western blot analysis of GenScript plk4 antibody

The GenScript anti-plk4 antibody was tested via western blot analysis of wildtype (lane 1) stage 20 protein. The antibody identified a protein ~42kDa in size. Staining of the unknown protein was lost when anti-plk4 was preabsorbed with the plk4 peptide used to produce it (lane 2). The unknown protein identified by the GenScript anti-plk4 antibody is present in SCMO and plk4MO injected embryos (lanes 3 and 4). Actin was used as a loading control.
Knockdown of plk4 affects multiple developmental systems

To assess the role of plk4 during development we sought to manipulate its expression in the model system Xenopus laevis. Antisense morpholino oligonucleotides were used to inhibit protein expression of plk4, the amphibian homologue of Plk4. Morpholino oligonucleotides work by binding with base pair complimentarity to a specific region of a target mRNA transcript, thereby preventing their translation into protein (Summerton, 1999). Morpholino injections were made into one blastomere of a 2 cell embryo. At the 2 cell stage one blastomere will give rise to all the cells of either the left or right side of the body. By injecting morpholino into one blastomere of a 2 cell embryo we were able to obtain embryos with unilateral knockdown of plk4, allowing the un-injected side to serve as a contra-lateral control. To ensure the phenotypes observed were due to specific plk4 knockdown via morpholinos, two antisense morpholinos were developed against the immediate upstream region of the plk4 transcription start site (Fig. 3.3 A). For all phenotypes discussed both morpholinos produced the same phenotypes at similar dosages (preliminary results in tables B.1 and B.2 in appendix B). In a second control experiment for morpholino specificity, both morpholinos were co-injected at a concentration one-tenth of the normal dose used to produce a phenotype. Morpholinos targeted to the same transcript can act synergistically to produce a phenotype and since they are injected at reduced doses they should not produce non-specific effects. This was the case when both plk4 targeted morpholinos were co-injected; the phenotypes seen in these experiments were the same as what was seen when both morpholinos were injected independently (preliminary results in tables B.1 and B.2 in appendix B). To ensure non-specific phenotypes did not arise due to the stress of injection or nucleic acid toxicity,
embryos were injected at the 2 cell stage with a standardized control morpholino directed against the human $\beta$-globin gene. Injections with standard control morpholinos, at the same or higher doses compared to $plk4$ morpholinos rarely produced non-specific effects (tables B.1 and B.2 in appendix).

Embryos injected with 5ng to 10ng of either $plk4$ morpholino developed normally through gastrulation and neurulation; however some embryos displayed a curved neural tube and occasional neural tube closure defects (data not shown). As development progressed depletion of $plk4$ perturbed the development of multiple organ systems. Most notably, loss of $plk4$ resulted in perturbed somitogenesis and eye development. While control morpholino injected embryos developed almost exactly like their wildtype counterparts (Fig. 3.3 B), $plk4MO$ injected embryos displayed a bent dorsal axis indicating a perturbation to somitogenesis (Fig. 3.3 C). Occasionally, control morpholino injected embryos did bend toward the side of injection but this bend was slight in severity compared to $plk4$ morpholino injected embryos, and somitogenesis appeared normal. At the later stages of somitogenesis, injected embryos lacked the morphological signs of somite development. When stimulated to move, embryos were unable to swim and could only twitch their bodies towards the un-injected sides. This behaviour was not observed in standard control injected embryos (data not shown).

In terms of eye development, examination of gross morphology revealed embryos that displayed varying eye phenotypes that could be categorized as moderate or severe. Compared to the un-injected side (Fig. 3.3 D), $plk4MO$ injected sides with moderate eye phenotypes possessed eyes that were smaller and also displayed dorsal-ventral defects in the development of the retinal pigmented epithelium (Fig. 3.3 E). In contrast, embryos
with the severe phenotype had no observable eyes (Fig. 3.3 F). The dosage of morpholino did not seem to correlate with the severity of the eye phenotype as both categories of eye phenotype were observed for low dosages (5ng) as well as high dosages (10ng).

In addition to the phenotypes described above, embryos also displayed disrupted development of the branchial arches as well as the otic vesicle, both of which derive from placodes. At high doses (10ng) embryos did not develop branchial arches and had either smaller or absent otic vesicles on the injected side (data not shown).

Depletion of plk4 protein levels affected the abundance of prmt5 protein during development. Western blot analysis of wildtype and morpholino injected embryos was used to evaluate the levels of prmt5 at stage 20 of development. Protein levels of prmt5 in wildtype and control morpholino injected embryos are extremely similar (Fig. 3.3G lanes 1 and 2), while prmt5 levels are severely decreased in plk4MO injected embryos (Fig. 3.3G lane 3).

Lastly, injection of both morpholinos, either independently or together, produced embryos with impaired axis elongation, spinal curvature, heart edema and reduced head size (data not shown). These phenotypes resemble those of increased p53 activation (personal communication with gene tools and Robu, 2007). Both knockdown morpholinos used to control for morpholino specificity produced identical phenotypes while injections with the standard control did not. These results suggest depletion of plk4 specifically leads to increased activation of p53.
Figure 3.3: Depletion of plk4 affects multiple organ systems

(A) Diagram showing binding sites of plk4-specific morpholinos used to inhibit plk4 protein translation. Red and grey arrow indicates 5’UTR of plk4 and blue box indicates start of plk4 coding sequence. ATG start site is indicated by a green box. Plk4 specific morpholinos are represented by aqua coloured boxes. (B) Right injected embryo with 20ng standard control morpholino (SCMO). Embryo shows no signs of a somite or eye phenotype indicating that morpholino concentration is not toxic nor does it cause non-specific effects. (C) Left injected embryo with 10ng of plk4 morpholino. Embryo is bent along its dorsal axis unlike control embryos (Compare B to C). (D) Un-injected contra-lateral control eye of a plk4 morpholino injected embryo. Eye has developed normally with defined retina and lens structures. (E) Plk4 morpholino injected eye with moderate phenotype. Eye displays dorsal-ventral defects in retinal pigmented epithelium formation indicated by arrow (compare D to E). (F) Plk4 morpholino injected eye displaying severe phenotype. There is no obvious formation of lens or retinal structures. (Compare D to F). (G) Western blot of stage 20 wild type (lane 1), control morpholino (lane 2) and plk4MO (lane 3) injected embryos. Wildtype and control embryos contain similar levels of prmt5 protein. Depletion of plk4 leads to a decrease in prmt5 protein levels. Actin was used as a loading control.
Depletion of plk4 disrupts somite morphogenesis and patterning

Examination of the gross morphology of plk4MO injected embryos indicated that loss of plk4 protein inhibited somitogenesis. To further examine this phenotype stage 22 plk4MO injected embryos were stained with Hoechst nuclear stain to visualize somite rotation. Injections with high doses of plk4MO (10ng) into 2 cell embryos completely inhibited somite rotation on the injected side (Fig. 3.4 A). The nuclei within the somitic mesoderm of the plk4MO injected side appeared to be randomly distributed, in comparison to the un-injected control somitic mesoderm, where nuclei have specific localizations. In milder cases (7ng dose), embryos appeared to have small groups of cells in the ventral portion of the somite undergo rotation while cells in the dorsal portion did not (data not shown). This may have been due to uneven plk4MO distribution to these embryos. Next, plk4MO injected embryos were stained with the myofibre marker, antibody 12/101, to visualize somite morphology and muscle differentiation. Un-injected control somites have a normal somite morphology characterized by defined somite borders and striated myofibre cells (Fig. 3.4 B). Plk4MO injected embryos stained with the antibody indicating muscle differentiation had been initiated however; the stain was faint indicating fewer differentiated muscle cells are present. Additionally, these somites did not have their usual striated appearance and did not appear to have well defined somite borders (Fig. 3.4 C, n=20/23). Staining with a β1-integrin antibody revealed that somite borders were present in plk4MO injected somites, however its expression was very faint compared to un-injected somites (Fig. 3.4 D).

After determining that knockdown of plk4 results in irregular somite morphology and organization, we next wanted to determine whether plk4 knockdown also perturbed
the molecular patterning of somites. *Plk4MO* injected embryos were probed with *notch* or *hairy2b* by *in situ* hybridization to assess the affect of plk4 depletion on the early and late stages of the segmentation clock, respectively. Surprisingly, depletion of plk4 perturbed both early and late molecular patterning. Both *notch* and *hairy2b* have characteristic banding patterns within the developing PSM which were completely abolished in the *plk4* morpholino injected side of embryos, while normal patterning was still seen on the un-injected side (Fig. 3.4 E, F and Fig. 3.5). Both genes were expressed within the PSM however they were not confined to their characteristic pre-patterns. Aberrant somitogenesis, as measured by defective *notch* expression, was seen at a statistically significant higher incidence in three biological replicates of *plk4MO* compared to control morpholino 2 cell injected embryos (Figure 3.5).
Figure 3.4: Depletion of plk4 perturbs both morphological and molecular aspects of somitogenesis

Stage 23-24 embryos injected at the 2 cell stage with 10ng of plk4MO were evaluated to determine the extent of the somitogenesis phenotype. For panels A, D, E and, F the un-injected side of the embryo is oriented to top and the plk4MO injected side towards the bottom. In all panels the anterior of the embryo is oriented to the left and posterior to the right. (A) Dorsal view of embryos were stained with Hoechst nuclear stain to visualize pre-somitic rotation. The nuclei of plk4MO somitic cells fail to align perpendicular to the neural tube indicating impaired rotation. Nuclei of plk4MO cells appear disorganized compared to the un-injected control side in which somite rotation occurs normally (white arrows). (B) Dorsal view of somites of un-injected control side stained with antibody 12/101. Black arrow points to normal somite with defined borders and organized morphology. Brown staining indicates striated muscle differentiation. (C) Lateral view of same embryo in B showing the plk4MO side stained with antibody 12/101. The somitic mesoderm did not develop clearly defined somites with elongated striated cells and defined borders (black arrow heads). (D) Embryos stained with β1-integrin (red) to visualize somite borders and counter-stained with Hoechst (blue) to visualize nuclei. Plk4MO pre-somitic mesoderm appeared to express β1-integrin very faintly at presumed somite borders (white arrow heads). In un-injected control somitic mesoderm borders are clearly defined by strong β1-integrin expression (white arrows). (E and F) Dorsal view of in situ hybridization for early somite clock gene notch, and late clock gene hairy2b. Both genes were expressed in discrete regions of the pre-somitic mesoderm in control sides (indicated by black arrows in E and F, respectively). In contrast to control sides, plk4MO somitic mesoderm did not contain discrete bands of notch or hairy2b expression, although both genes are still expressed (E and F respectively). Arrows indicate normal control somites while arrow heads indicate plk4MO effected somitic mesoderm.
Scoring of defects to somitic patterning was done on embryos obtained by 2 cell injection of either 10ng of \textit{plk4MO} or 20ng of \textit{SCMO} and, then collected at approximately stage 22. Injections of \textit{plk4MO} and \textit{SCMO} were done on the same day in three separate experiments to obtain biological replicates. Embryos were scored based on the presence of striped \textit{notch} expression in the PSM after \textit{in situ} hybridization. The raw numbers were then calculated as a percentage of the population scored for each replicate. The average percentage of all three replicates is presented in the graph. Injection of 10ng of \textit{plk4MO} perturbs somitic patterning in 92% of the \textit{plk4MO} injected embryos scored. This phenotype is statistically significant when compared to controls with only 17% showing the phenotype on average (p <0.05, using one-way ANOVA). An asterisk marks statistical significance.

Figure 3.5: Scoring of \textit{plk4MO} embryos with perturbations to somitic patterning
Plk4 knockdown precludes lens development

After the surprising discovery that depletion of plk4 perturbs eye development we next wanted to determine what aspects of eye development were disrupted. Eye development begins early during embryogenesis and involves precise regulation of transcription factor gene networks that will establish the signaling required for lens and subsequent retina morphogenesis and differentiation. Judging by the gross morphology of plk4 depleted embryos it appeared that one of the initial and critical steps of eye development, formation of the optic vesicle, was not impaired as an optic bulge could be seen on the plk4MO injected side. Also, in stage 34 plk4MO injected embryos, the expression of pax6 and rax1, two important genes required in the early stages of eye development, were still expressed (Fig 3.6 A, B). Hoechst staining confirmed the existence of retina-like structures indicating that the expression of eye transcription factors that regulate early eye formation are indeed expressed in the correct spatial-temporal pattern (data not shown).

We next wanted to determine if a lens formed in plk4MO injected embryos; since this is a crucial step for further differentiation of the retina and morphogenesis of the future eye. The lens marker pitx3 was used to evaluate the presence of a lens in two cell stage 34 plk4MO injected embryos. Compared to the un-injected side, plk4 depletion almost always results in a complete loss of pitx3 expression, indicating lens had not formed (Fig 3.6 C and Fig. 3.7). Occasionally, embryos still produced a lens but it was much smaller in size (Fig. 3.7). These lens phenotypes occurred at a statistically higher frequency in plk4MO injected embryos compared to controls (Fig. 3.7).
Since *plk4* morpholino injected embryos did not form a lens but still seemed to exhibit normal expression patterns of eye field transcription factors, we next wanted to determine if a lens placode formed. In two cell stage 27 *plk4MO* injected embryos, optic bulges did not flatten like their un-injected counterparts indicating a lens placode may not have formed. Staining of two cell *plk4MO* injected embryos at placode stages with *pitx3*, a marker of lens placode formation, was completely abolished in the *plk4MO* injected sides, suggesting lens placode had not formed (Fig. 3.6 D, n=7/8). This was confirmed by Hoechst stain (Fig. 3.6 E). Unlike their un-injected counterparts, *plk4MO* injected optic vesicles did not invaginate, nor did they form a placodal thickening. Taken together, these results indicate that a loss of *plk4* inhibits differentiation of normal eye structures by inhibiting normal development of the pre-lens placode. The effects of *plk4* on eye markers are indirect— they can still express, but the failure of normal induction leaves the field disorganized.
Figure 3.6: plk4 depletion causes eye development defects.

plk4MO embryos injected at the two cell stage were evaluated for the expression of lens and retinal eye genes via in situ hybridization. In all panels anterior views are shown with a dashed white line to designate left from right. Un-injected control sides are found to the left of the line and plk4MO injected sides on the right.  

(A) Retinal and lens marker pax6 is expressed only in the retina with no defined lens expression. Retinal expression in plk4MO cells is indicated by the black arrow.  

(B) Rax1 expression remains in plk4MO retinas (black arrow).  

(C) The lens marker pitx3 it not expressed in late stages of eye development (indicated by black arrow). Compare to normal pitx3 expression seen on the un-injected control side left of the dashed line.  

(D) Placode stage embryos do not express pitx3 in presumptive placode cells. Black arrow indicates lack of pitx3 expression in placodal region.  

(E) Hoechst nuclear stain reveals that plk4MO embryos fail to form a placode and optic vesicles do not invaginate. The white oval indicates the absence of the lens placode. The white line indicates the optic vesicle has not invaginated. Line on the plk4MO injected side is still convex compared to concave line in control optic cup (compare left to right.)
Figure 3.7: Scoring of *plk4MO* injected embryos with perturbed lens development

Lens defects were scored via *in situ* hybridization with the lens marker *pitx3*. All embryos scored were at stage 34 and were 2 cell injected with 10ng of *plk4MO* or 20ng of *SCMO*. Injections of *plk4MO* and *SCMO* were done on the same day in three separate experiments to obtain biological replicates. Lens phenotypes were categorized into three groups: (1) no lens, (2) reduced lens size and, (3) total lens aberrations. The total lens aberrations category is the total of the previous two groups. After the initial scoring the raw data were converted into percentages of the population. The average percentage for all three replicates is presented in the graph. Standard deviation is represented by error bars. The no lens phenotype was seen in 72% of *plk4MO* injected embryos while the reduced lens phenotype was seen in 14%. This is compared to 2% and 5% respectively for control embryos. When total lens phenotypes are calculated together, 86% of *plk4MO* embryos display the phenotype while only 4% of controls do. Compared to controls both phenotypes were statistically significant for *plk4MO* injected embryos (p<0.05, one-way ANOVA).
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>plk4MO 10ng, n=74</th>
<th>SCMO 20ng, n=63</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lens</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Reduced Lens</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Total Abberent lens</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Percentage of embryos with phenotype.
Centrosome localization is lost upon plk4 depletion

Due to Plk4’s role in mammalian centrosome biogenesis we sought to determine if there was a loss of centrosomes following plk4 depletion during *Xenopus* development. To detect centrosomes, 2 cell injected embryos were stained with an antibody against the centrosome marker γ-tubulin. γ-tubulin staining revealed the presence of centrosomes in the *plk4MO* injected side, even in the late stages of embryogenesis, long after maternal transcripts have been depleted (Fig 3.8 C, D, H, I).

Staining with γ-tubulin also revealed that the centrosome has a dynamic localization pattern among different tissues during development. Localization of the centrosome in somites has not been carefully studied. Here we report that after somite rotation the centrosome normally localizes centrally within a somitic cell, in close proximity to the nucleus (Fig. 3.8 A and B). When looking at the somite from the dorsal aspect, this central pattern of localization gives the centrosomes the appearance of forming a line perpendicular to the neural tube; similar to how the nuclei align at this time (Fig. 3.8 A, B). When *plk4* is depleted this centrosome localization is lost and, instead centrosomes are distributed randomly within the cells of the somitic mesoderm (Fig. 3.8 C). The disorganized pattern of centrosome localization upon plk4 depletion is similar to what is seen for the nuclei (Fig. 3.8 D). This loss of centrosome localization could indicate a loss of polarity within the somite, which may be required for rotation.

Upon examination of late stage eyes, we noted a distinct localization pattern for the centrosome on the un-injected control side that is in agreement with what has been reported in the literature for lens and retina (Manning et al., 2008). In the retina, the

67
centrosome is localized to the outer surface of the RPE (data not shown). In the lens, centrosomes localize within the anterior of the lens, most likely in the lens epithelial cells. Centrosomes were also seen in the cells that had migrated into the transition and germinal zones to begin differentiation into primary lens fibres. Centrosomes were not seen in the fully differentiated lens fibres (Fig. 3.8 F, G). Plk4MO injected embryos that contained only the moderate dorsal-ventral abnormalities still developed lenses. The eyes of these embryos also contained normal centrosome localization in both the lens and retina (Fig. 3.8 H, I). Interestingly, centrosomes in the retina-like structures of the plk4MO injected embryos with the severe phenotype still localized normally to the outer surface of the RPE (data not shown).
**Figure 3.8: Depletion of plk4 disrupts only somitic γ-tubulin localization.**

Stage 22 and 34 two cell plk4MO injected embryos were stained with γ-tubulin to detect the presence of centrosomes (green). Hoechst nuclear stain was used as a counter stain (blue). (A) γ-tubulin stained un-injected somite. Normal centrosome localization can be seen with centrosomes forming a straight line across the medio-lateral axis of the somite. (B) Merge of γ-tubulin stain from A with Hoechst counter-stain of same somite. Nuclei are aligned perpendicular to the neural tube indicating rotation has occurred. Centrosomes can be seen in close proximity to nuclei and aligned in the centre of the somite. Red arrows point to γ-tubulin foci. (C) γ-tubulin stain of plk4MO injected somite. Centrosomes are scattered throughout the somite indicating polarity is not established. (D) Merge of γ-tubulin stained somite in C with Hoechst counter-stain of same somite. Nuclei are not aligned along the medio-lateral axis indicating rotation has not occurred. Some centrosomes do not associate with a nucleus. White arrows indicate γ-tubulin foci. (E) Bright field image of normal eye. Box represents region examined in panels F-I. (F) γ-tubulin stained un-injected lens. Centrosomes localize to anterior and boarder cells within the lens. (G) Merge of γ-tubulin stained lens in F with Hoechst stain of same lens. Centrosomes are not always associated with the nucleus. (H) γ-tubulin stain of plk4MO injected lens. Centrosomes show a similar localization in the lens that seen in un-injected controls (compare to G). (I) Merge of γ-tubulin stained lens in H with Hoechst counter-stain of same lens. Centrosomes are not always associated with a nucleus, similar to controls. White arrows indicate γ-tubulin foci.
CHAPTER 4:

Discussion

Plk4 expression during embryogenesis indicates cell cycle as well as polarity functions during organ morphogenesis

To date the spatio-temporal expression pattern of plk4 during development has only been evaluated during mouse embryogenesis (Fode et al., 1994). Here we report the expression pattern of plk, the amphibian homologue, during the development of *Xenopus laevis*. Early in mouse embryogenesis Plk4 is expressed in the embryonic and extra-embryonic tissues. During mouse organogenesis Plk4 is restricted mostly to proliferative zones. Specifically, Plk4 is expressed in the ventricular zones of the brain and spinal cord, as well as in the skin, liver thymus, small intestine, cortical layer of the kidney, olfactory and nasal mucosa, and within the spermatocytes of the testis. However, it is also expressed in the somites, a structure not considered to be highly proliferative (Fode et al., 1994).

While the mouse study focused strictly on the expression of Plk4 in the later stages of development, our study in *Xenopus* documents plk4 expression throughout the early stages of development and into organogenesis. During neurulation, plk4 is highly expressed in the dorsal and anterior regions of the embryo. At the onset of organogenesis, its expression is restricted to somites, notochord, neural tube, brain, optic cup, lens and otic placodes, and branchial arches. In the later stages of organogenesis
plk4 is expressed in the lens, retina and otic vesicle. It also continues to express in the brain, neural tube, somites and branchial arches.

The expression of Plk4 during frog and mouse development has some similarities. For example, in both animals Plk4 is expressed in proliferative tissues such as the brain. Our analysis shows that plk4 expression in proliferative tissues begins earlier in the formation of this organ and from anterior neural plate stage through to the development of the optic cups. This is not surprising as plk4 is a critical player in the cell cycle, regulating mitotic progression and centrosome duplication (Sillibourne and Bornens, 2010). Additionally, in both mouse and frog, Plk4 is expressed in the somites, a structure that is not considered to be highly proliferative. In Xenopus we find that plk4 is also expressed in notochord, branchial arches, otic placode and in the lens and retina throughout their development, something that has not been documented in mouse. However, the late development time points at which Plk4 expression was assessed in mouse make comparison to its expression in frog difficult. A complete analysis of embryonic Plk4 expression in mouse over several stages of early organ morphogenesis, such as E8 and E10, would aid in determining if Plk4 expression is conserved in the above mentioned structures.

During the later stages of organogenesis, plk4 transcripts express in specific patterns within cells that are strikingly similar to the pattern of centrosome localization in that structure. For example, in somites plk4 transcripts localize with higher intensity in peri-nuclear locations at the centre of the somite, forming a straight line perpendicular to the neural tube, similar to how the nuclei themselves align after rotation. Similar patterns of shared plk4 transcript and centrosome localization are also seen in the lens and otic
vesicle. Mouse and human Plk4 protein has been shown to localize to the centrosome (Hudson et al., 2001; Leung et al., 2002). Taken together, these results point to a scenario where \textit{plk4} transcripts may localize in close proximity to centrosomes. As long as free ribosomes are in the immediate area, \textit{plk4} transcript localization close to the centrosome could allow for quick plk4 protein localization to the centrosome and efficient centrosome duplication. Alternatively, it could serve to protect the transcript from degradation or to act as a regulatory mechanism to control the spatial and temporal activation of \textit{plk4} translation.

Our analysis of \textit{plk4} expression during \textit{Xenopus} embryogenesis complements the mouse study while also providing new insights into the expression and possible function of \textit{plk4} during vertebrate development. \textit{Plk4’s} conserved expression in vertebrate somites may indicate it plays an important role in the morphogenesis of this structure outside of its role in the cell cycle. The phenotypes seen in both mouse and frog also point to a role for \textit{plk4} in the formation of somites as \textit{plk4} mutants in both species do not form proper somites (Hudson et al., 2001). \textit{Plk4} expression was noted in the lens and otic cranial placodes. Placodes are epithelial thickenings that will develop into the neurons and ancillary structures of organs such as eye, ear, olfactory and lateral line organs. Placodes enjoy a feature in common with somites: namely extreme cellular elongation and alignment. This elongation is followed by the establishment of an apical-basal polarity which allows for apical constriction leading to invagination or in the case of somites, rotation and rounding. The increased expression of \textit{plk4} in these placodes suggests a functional role for its protein product in placode formation (discussed later).
One of the pitfalls of this expression analysis was that our control sense probes produced an expression pattern that was identical to the anti-sense plk4 specific probes. This could be explained by the presence of a microRNA or long non-coding RNA (lncRNA). In humans there are several predicted microRNAs targeted to PLK4 (http://bioinformatics.ekmd.huji.ac.il/reptar/gene_report.php?species=human&id=13340). A lncRNA species has been reported for Plk1 (Saito et al., 2011). Possibly, the presence of a long span of repetitive sequence within the plk4 sequence may allow the sense probe to bind to the endogenous plk4 mRNA. Analysis using ClustalW2 revealed this scenario to be highly unlikely as there were no long stretches of contiguous base pairing among plk4 and its reverse strand. In humans, PLK4 is a rare transcript. It is possible the expression patterns revealed in frog were the product of an over-developed colour reaction during the in situ hybridization process. However, we reject this hypothesis for the following reasons: the same spatial-temporal expression of plk4 was derived using multiple probes produced by different lab members; and the hybridization of probe was conducted at high stringency. Production of a control probe consisting of an inverted or scrambled plk4 sequence may serve as a better control.

**Morpholino mediated depletion of plk4 results in disruptions to somite and lens formation**

To knock down plk4 during *Xenopus laevis* development we sought to prevent its translation into protein via anti-sense morpholino mediated translational knockdown. Morpholinos work by binding to a complementary sequence located just 5’ to the ATG start site within the target mRNA, and they act to physically prevent mRNA translation. Morpholino binding does not lead to degradation of the mRNA (Summerton, 1999). This
property makes morpholinos an especially advantageous knockdown strategy: since the mRNA is not removed from the cell it can still bind with other proteins and miRNAs thereby causing little disruption to other cellular processes while creating an environment in which protein-inhibited knockdown phenotype can be studied.

As with any technology relying on complementary base pairing, there are concerns regarding the specificity of morpholinos. Even with careful design, morpholinos have the potential to bind to off-target genes and cellular components that could produce non-specific phenotypes. There are many controls in place in order to confirm the specificity of morpholinos and to ensure the phenotypes seen are indeed due to loss of the desired protein (reviewed in (Eisen and Smith, 2008).

Translational knockdown of plk4 in *Xenopus laevis* caused disruptions to the morphogenesis of somites. Somites of plk4 depleted embryos did not rotate and did not form proper somitic borders. These somitic cells also did not elongate nor did they position their centrosomes correctly, suggesting that cellular polarity was not properly established. This was evident in horizontal sections through pre-somitic mesoderm where nuclei appeared disorganized instead of aligned parallel to the neural tube. This defect in polarity induced by plk4 depletion most likely underlies the failure of somites to rotate. Additionally, molecular patterning of the somite was lost even though genes deployed by the clock and wave-front signaling cascade that is required for somite formation were still expressed. Depletion of plk4 also precludes lens development: presumptive lens cells fail to elongate and form a placode. Failed lens placode formation inhibits further lens and optic cup induction resulting in embryos that lack eyes. Additionally, *pitx3*, a transcription factor required for lens placode formation in *Xenopus
*laevis*, is not expressed in the presumptive lens placode cells. Plk4 depleted embryos also display spinal curvature, shortened anterior-posterior axis and heart edema, phenotypes similar to those observed in zebrafish morphants with non-specific activation of p53, indicating that p53 signaling may be activated upon plk4 depletion in *Xenopus* (Robu et al., 2007) and personal communication with Gene Tools). In this instance p53 activation upon plk4 depletion is not surprising: *Plk4*+/− MEFs display heightened levels of p53, likely due to the inherent genomic instability caused by aberrant centrosome duplication (Morettin et al., 2009). We believe these phenotypes to be specific for several reasons. Two unique morpholinos targeted to different regions of the 5’UTR produced the same phenotype when injected individually. The somite phenotype seen in *Xenopus* is similar to what is seen in *Plk4* null mice which also do not form somites. We also injected both morpholinos together at smaller doses, which is the newest control for specificity. The two morpholinos acted synergistically to produce the same phenotypes. In addition, the phenotypes we are seeing are occurring in cells where plk4 is expressed. Taken together, these controls indicate that is highly likely our phenotypes are legitimate.

Activation of p53 is a common off-target effect of some morpholinos. In zebrafish, approximately 18% of morpholinos produce off-target p53 activation phenotypes (Ekker and Larson, 2001; Robu et al., 2007). This is most likely due to the fact that p53 is the major signaling centre for the DNA damage response so there is the potential for off-target effects to funnel through p53 signaling. In zebrafish potential non-specific p53 activation phenotypes are teased apart from target specific phenotypes through co-injections of the experimental morpholino and a *p53* morpholino (Robu et al., 2007). This has never been documented in *Xenopus*, most likely due to the fact that p53 is
required for early embryo development (Takebayashi-Suzuki et al., 2003). Additionally, mammalian Plk4 has been shown to be involved in a p53 signaling pathway (Ward and Hudson, 2014). If this interaction is occurring in *Xenopus* it would be impossible to separate potential p53 off-target activation from specific p53 activation caused by plk4 knockdown.

Another popular control for morpholinos experiments is the use of a five base pair mis-match morpholino (Eisen and Smith, 2008). This morpholino is almost identical to the original experimental morpholino except for five base pairs which have been strategically changed to the pyrimidine/purine counterpart. These changes theoretically prevent the control morpholino from binding to the desired target while controlling for potential off target effects. We attempted this strategy however our mis-match morpholino was lethal to the embryos early in development. The mis-match sequence shared significant homology with efna4, h2afy2, bcan, hnf1a-a, and ribokinase, which could explain its lethality. Instead we resorted to the standard control morpholino provided by Gene Tools. We witnessed no off-target or non-specific effects with this morpholino.

Plk4 is best known for its role as the master regulator of centrosome duplication. The dose of *plk4* morpholino used in our experiments did not inhibit centrosome formation as γ-tubulin containing foci were still present in *plk4* morpholino injected cells. We had predicted that depletion of plk4 would lead to the loss of centrosomes in later stages of development when maternal transcripts have long been depleted, and cells had begun to lose their centrosomes due to lack of duplication and repeated cell cycles.
We speculate their presence may be due to incomplete depletion of plk4 resulting in the deregulation of centrosome duplication. This is observed in mice and cell culture, where haploid levels of Plk4 lead to centrosome amplification (Holland et al., 2012; Ko et al., 2005). Alternatively, the centrosome-like localization of $\gamma$-tubulin may reflect assembly of centrosomal proteins and $\gamma$-tubulin, resulting centrosome-like structures that cannot nucleate microtubules. This has been reported in a human colon cancer cell line and correlated with low levels of Plk4 in those cells (Kuriyama et al., 2009). It is also possible that plk4 is not exclusively required for centrosome duplication in *Xenopus laevis*. This scenario seems unlikely as plk4 is required for the *de novo* duplication of centrosomes in *Xenopus laevis* cell extracts; however, cell extracts are, by their very nature, only approximations of normal cell behaviour (Eckerdt et al., 2011). There is the potential that functional redundancy and replacement by other polo-like kinase family members can account for the presence of the $\gamma$-tubulin foci.

Overall, the controls utilized in this study indicate the phenotypes produced by *plk4* targeted morpholinos are specific. In the future, development of an antibody specific to *Xenopus laevis* plk4 would aide in determining the efficacy of the morpholinos in preventing *plk4* translation. In addition, rescue experiments in which embryos are co-injected with the morpholino and a *plk4* mRNA lacking the morpholino binding site need to be completed.
Loss of cell polarity may underlie plk4 knockdown phenotypes

The disruptions to somite and lens placode formation caused by plk4 depletion are strikingly similar. In both structures, cells fail to elongate and, in the case of somites, they do not establish proper polarity. However, lens and somites seem to develop normally if they are able to get past the initial effects caused by plk4 depletion, suggesting if polarity is established normal patterning continues. To establish polarity in lens placode cells, organelles localize to specific positions and microtubules are anchored apically and orient themselves parallel to the cells’ longitudinal axis (Byers and Porter, 1964; McKeehan, 1951). In somites, the internal polarization of organelles, other than the nuclei, is not well documented, however cells do develop posterior filopodial extrusions and an obvious morphological polarization of their prospective anterior-posterior axis (Afonin et al., 2006; Hamilton, 1969; Youn and Malacinski, 1981).

We speculate that plk4 is exerting its effect on polarity in somitic and presumptive lens placode cells is likely by mediating polarization of the centrosome and subsequent positioning of microtubules. Somitic cells have been described to take on morphologies similar to those of migrating cells, which usually position their centrosomes centrally and ahead of the nucleus (Tang and Marshall, 2012; Youn and Malacinski, 1981). We find that the centrosome in normal somitic cells localizes centrally after rotation, which may reflect the migratory nature of the cells. Mammalian Plk4 has recently been implicated in establishment of polarity and migration. Plk4+-/- MEFs show delays in migration and do not orient their mitotic organizing centres properly (Rosario, dissertation, 2011). This phenotype is similar to what is seen in our Xenopus plk4 depleted somites, suggesting Plk4’s role in establishing polarity is
conserved in vertebrates and further supports the notion that Plk4 is required for centrosome positioning. In this latter study Plk4+/− MEFs also experienced delays in actin cytoskeleton rearrangement during migration (Rosario, dissertation, 2011). The authors speculate that this is due to defective RhoA activation since Plk4+/− MEFs had decreased levels of active RhoA during migration. This relationship should be investigated further in *Xenopus* as it may contribute to the polarity and elongation deficiencies seen in plk4 depleted somites and presumptive lens placode cells. In lens placode cells, plk4 may aide in anchoring microtubules to the apical side of the cell. Once apically localized, the microtubules can orient themselves within the longitudinal axis. This may be important for establishing further polarity, possibly by allowing for polarized vesicular transport (Musch, 2004).

While we found that polarity is lost in somites, it is possible that the mechanism may be indirect: centrosomes are critical to microtubule nucleation and many of the observed phenotypes could be explained by microtubule defects. Microtubules are essential for somitogenesis, as their inhibition in both *Xenopus* and *chick* prevent further segmentation (Chernoff and Hilfer, 1982; Sparrow, 2008). The importance of microtubules to lens placode formation is not well established; however, when inhibited in lens epithelial cells they fail to elongate (Piatigorsky et al., 1972). Since the cellular mechanisms involved in elongation are similar between structures it is likely microtubules are essential for presumptive lens placode elongation as well. Given plk4’s role in centrosome duplication we speculate that loss of plk4 precludes proper centrosome formation resulting in aberrant microtubule nucleation (Habedanck et al., 2005). This loss of proper microtubule formation could affect presumptive lens placode
cell elongation, similar to how loss of microtubule function in cultured lens epithelial cells prevents their elongation. Interestingly, centrosome-like structures, however imperfect, still exist in plk4 depleted embryos. Even though γ-tubulin foci are still present in plk4 depleted embryos they may not be able to nucleate microtubules. As mentioned above, this has been documented in a human cancer cell line, HCT116 (Kuriyama et al., 2009). Without knowing the microtubule nucleating ability of these centrosome-like structures it is hard to definitively conclude that the phenotypes seen are due to a lack of functional microtubules.

Depletion of plk4 seems to affect the subsequent molecular patterning cues required for further development of the somites and lens placode. This is evident in plk4 depleted presumptive lens placode cells which do not activate pitx3 signaling, a requirement for further lens placode formation in Xenopus (Khosrowshahian et al., 2005). In fact, the phenotypes seen in plk4 depleted embryos are similar to those seen when pitx3 is depleted. Loss of either pitx3 or plk4 completely inhibits eye development due to failure of lens placode formation and subsequent failure to induce retina (Khosrowshahian et al., 2005). Just as with pre-lens placode, it appears that it is the early patterning of somites that is most perturbed. β1-integrin is still faintly expressed and muscle differentiation is initiated, suggesting that the latter differentiation pathways still seem to perform, albeit in a disorganized manner. These phenotypes indicate that plk4 exerts its functions prior to the initiation of the signaling cascades that partition the somitic mesoderm and that loss of plk4 prevents normal segmentation but not necessarily differentiation. The question then remains: how does loss of plk4 prevent the later patterning of these structures? It may be that in somites and presumptive lens cells the
establishment of polarity is required for elongation, and that if these two cellular changes do not occur than further development of their structure is aborted. However, another possibility is that plk4 somehow indirectly regulates pitx3 expression specifically: pitx3 depleted embryos also show defects in somite rotation and spatio-temporal patterning of late somitogenesis genes.

A prmt5-plk4 Signaling axis may exist in Xenopus laevis

Recent work in our lab has identified a potential signaling axis between Plk4 and Prmt5. Prmt5 is a type II arginine methyltransferase, meaning it symmetrically dimethylates its substrates at arginine residues (Karkhanis et al., 2011). Prmt5 is well known for its role in epigenetic regulation via methylation of histones H3R8 and H4R3, leading to transcriptional repression and to the recruitment of other epigenetic regulators that further repress transcription at the DNA level (Pal et al., 2004). Work by colleagues in our lab has demonstrated that murine Prmt5 and Plk4 co-localize at the centrosome and, that Plk4 phosphorylates Prmt5 in vitro. Additionally, in Plk4+/- MEFs the levels of Prmt5 are depleted to haploid levels, which in turn affect the global methylation of proteins within the cell (Ward, dissertation, 2014). We see a similar pattern of Prmt5 protein depletion in Xenopus laevis embryos, suggesting this interaction is conserved in vertebrates. This finding could have important implications for embryonic development. Epigenetic regulation is an important aspect of embryonic development and being an epigenetic regulator, prmt5 may play a role in regulating the activation or repression of developmental genes. Moreover, Prmt5 has been shown to be involved in initiating muscle differentiation in zebrafish by epigenetically regulating the activation of myogenin transcription (Batut et al., 2011). Prmt5 regulates expression of myogenin by
di-methylating H3A8 which allows for recruitment of the SWI/SNF ATPase Brg1 to the
*
myogenin
*
promoter. This event then allows for further chromatin modeling and eventual
activation of *myogenin* expression (Dacwag et al., 2007). This could explain why,
although muscle differentiation still seems to occur upon plk4 depletion, it is occurring at
a reduced level compared to controls. There is potential that prmt5 could also regulate
the spatial-temporal expression of other genes during embryonic development.
Considering that plk4 regulates the stability and activity of prmt5 in mouse, and that its
depletion leads to reduced Prmt5 levels in both mouse and frog, it is a conceivable that
plk4 could mediate the epigenetic regulation of gene activation during embryonic
development.

Interestingly, Prmt5 can also arginine-methylate p53 leading to its stabilization
and activation during genotoxic stress and, recent work in our lab has indicated that a
Plk4-Prmt5-p53 interaction is important for the DNA damage response (Jansson et al.,
2008). My colleagues propose a signaling axis where Plk4 and Prmt5 work together to
activate p53, leading to faster activation of DNA damage repair pathways. However, it is
doubtful that this interaction would be important during embryonic development.

**Xenopus laevis may express an unusual plk4 isoform**

In an attempt to produce a *Xenopus laevis* plk4 specific antibody we identified a
possible polypeptide sequence of plk4 that is smaller than the expected plk4 protein
product. We produced a plk4 antibody through the commercial company GenScript that
ultimately bound to an unknown 42kDa protein in western blot instead of the expected
109Kd plk4 protein. The presence of a 109kDa plk4 protein in *Xenopus laevis* has been
confirmed by another group of investigators (Hatch et al., 2010). Their plk4 antibody was produced using the C-terminal of plk4, which was designed using the published plk4 protein sequence (Accession no. NM_001083146). In contrast, our antibody was produced using a short polypeptide consisting of 14 amino acids located within the N-terminal kinase domain. Our antibody’s specificity to the 42kDa unknown protein was confirmed by pre-absorption of the antibody with the peptide used to produce it. However, it is odd that our antibody does not reveal the expected isoform of plk4. The design of the peptide used to produce the antibody was done using the published protein sequence of plk4, so the 109kDa expected plk4 protein should also contain this recognition site. It is possible that the folding patterns of the two proteins are different, creating a situation where only the 42kDa isoform presents this antigen to the antibody. To confirm the identity of the 42kDa unknown protein a co-immunoprecipitation using our GenScript antibody could be done to isolate the protein, followed by mass spectrometry to determine its sequence. Another company was also asked to produce a plk4 antibody for us using the same peptide as an antigen; however they were unable to produce one.

It is unlikely that our antibody is specific to other members of the polo-like kinase family because the size of the unknown protein is much smaller than that of *Xenopus laevis* plk1 or plk2, which are 66kDa and 73kDa respectively. Plk3 has not been isolated in *Xenopus laevis*; however, in *Xenopus tropicalis* it is present as a 39kDa or 73kDa protein. In addition, the antibody was produced using a region of the plk4 N-terminal sequence that showed very low homology to other *Xenopus laevis* polo-like kinase family members.
Hypothetically, an isoform of plk4 could be produced from alternative splicing of the plk4 transcript. In mouse, Plk4 can be alternatively spliced to produce two different protein coding transcripts, Sak-a and Sak-b (Hudson et al., 2000). Sak-a is the predominant form and contains all 15 exons which encodes the full length protein of 930 amino acids and 102kDa in size. Sak-b, which is usually found in much lower abundance, is produced from alternative splicing of exon 5 and 6, which incorporates an early stop codon found in the intron flanking exon 5. The resulting Sak-b transcript encodes a protein that contains only the N-terminal kinase domain leading to a protein product that is only 462 amino acids long and approximately 62kDa in size (Fode et al., 1994; Hudson et al., 2000). We do not believe the unknown protein identified in Xenopus laevis was produced from alternative splicing of plk4. First, the unknown protein identified here is approximately 90 amino acids smaller than the mouse Sak-b protein product. Second, while the genomic structure of plk4 is very similar between Xenopus and mouse, there is no indication that Xenopus plk4 is spliced in a manner similar to mouse Plk4. Alternative splicing of Xenopus plk4 to incorporate only the first 5 exons would produce a product of approximately 55kDa, which is much larger than the unknown protein we identified.

The Xenopus laevis genome is pseudo-tetraploid due to a whole genome duplication event (Bisbee et al., 1977). This has created the opportunity subsequent functional divergence of the duplicated gene (Koonin, 2005). It possible that the unknown protein we identified here is actually an isoform of plk4 generated by the duplication event. The duplicated plk4 gene could significantly diverge over time from the ancestral form located on a different chromosome, resulting in the protein product we
have identified here. The *Xenopus laevis* genome has been partially sequenced allowing for analysis of *plk4*’s genomic structure. Searching the *Xenopus laevis* EST database against the genomic *plk4* sequence does not reveal any ESTs that indicate an alternative splicing event is occurring, especially not one that could produce the unknown protein we have identified. However, the genome is still in the early stages of its annotation so any information garnered from EST searches should be evaluated with caution.

Of course it is possible the protein we identified is not related to *plk4* at all. The peptide used to create the antibody did show some homology to other proteins in a BLAST search. Some of the proteins are in the approximate size range required to produce a 42Kd protein. Additionally, our unknown protein was not depleted by *plk4* morpholinos suggesting it may not be a *plk4* isoform. I conclude that the presence of the cryptic protein identified by our antibody in *plk4* depleted embryos likely indicates that the antigen is not *plk4* specific and our antibody is recognizing some unknown protein.

**Future Directions**

The specificity of our morpholinos has not yet been concretely validated. At this point the results indicate but do not exclusively prove that the phenotypes are legitimate. Specificity of our phenotypes can be confirmed by rescue experiments in which *plk4* mRNA lacking the morpholino binding site is co-injected with the *plk4* targeting morpholino. A $^{35}$S methionine *in vitro* translation knockdown assay can also be done to confirm specificity. Although not as robust as rescue experiments it will provide further support in confirming the specificity of the morpholinos.
A proper antibody will also need to be developed so that the specificity and efficacy our morpholinos can be quantified. At this time we do not know if *plk4* translation is completely inhibited at the 10ng dose we used throughout our experiments. The ability to quantify protein levels of *plk4* would greatly aide in understanding and interpretation of the phenotypes we see here. Procurement of an antibody suitable for immunohistochemistry would be particularly advantageous. This would allow for *plk4* localization studies during embryogenesis. Having an understanding of *plk4* localization might shed light on its role in establishing polarity. In addition, a *plk4* antibody would allow for co-localization and co-immunoprecipitation studies to search for any *plk4* binding partners. This would be useful in validating the *plk4*-prmt5 signaling axis that is seen in mouse.

Experiments to assess the effect of *plk4* over-expression during embryogenesis should also be done. This can be achieved by cloning *plk4* cDNA into an RNA expression vector and subsequent *in vitro* translation into mRNA and injection into one or two cell embryos. Characterizing the effect of *plk4* over-expression may aide in understanding exactly what *plk4*’s role is during polarity establishment.

The results examining the consequences of *plk4* depletion upon cell polarity need to be more refined. It is clear that cellular elongation is not occurring during neither lens nor somite development. Somite cells adopt a clear morphological polarity in that their anterior ends are flat and broad while their posterior ends become narrow, cytoskeletal staining would reveal if this is impaired by *plk4* depletion (Youn and Malacinski, 1981). Staining with phalloidin would also determine if the actin cytoskeleton is affected by loss of *plk4* as this has been documented in *Plk4*+/- MEFs (Rosario, dissertation, 2011).
There is a possibility that the phenotypes we see in plk4 depleted embryos are due to the loss of functional microtubules since plk4’s main function is to duplicate the centrosome, which will go on to nucleate microtubules (Habedanck et al., 2005). However, we did not address the impact plk4 depletion had on microtubules. Immunofluorescent staining of plk4 depleted embryos for alpha-tubulin could indicate if microtubule dynamics are impaired.

At present, our results only suggest that plk4 is involved in establishing polarity. The next step in understanding plk4 mediated polarity would be to determine how it re-localizes the centrosome. The Par protein family is well known for their role in polarizing cells in a diverse number of tissues and organisms (Goldstein and Macara, 2007). Par-3 has been shown to polarize the centrosome during neurulation in zebra fish and, with the aid of Par-6, in the intestinal cells of C. elegans (Feldman and Priess, 2012; Hong et al., 2010). Given that members of the Par family have been shown to polarize the centrosome, they are ideal candidates for positioning the centrosome during Xenopus laevis somite and lens placode morphogenesis. There is no evidence to suggest par-3 is expressed during somitogenesis or lens development in Xenopus laevis, however, par-6 expresses in somites and during eye development (Choi et al., 2000). It is possible that plk4 may mediate par-6 driven centrosome polarization, possibly by influencing its localization with the centrosome or by regulating its activity through phosphorylation. The first step to confirm this hypothesis would be to determine if plk4 and par-6 proteins co-localize at the centrosome prior to somite rotation or lens placode formation using immunofluorescence histochemistry.
Depletion of plk4 in *Xenopus laevis* causes decreased expression of Prmt5 protein and may activate p53 signaling. This should be further studied as mammalian Plk4 has been shown to function in a signaling cascade with Prmt5 and p53 (Ward, dissertation, 2014). First, the levels and activation of p53 should be characterized to confirm p53 activation. This can be done using western blot analysis to characterize the protein levels of p53 and p21, which is a downstream effector of p53 signaling. TUNEL assay can also be done to determine if p53-mediated activation of apoptosis is occurring. Contingent upon the availability of a plk4 antibody, in the context of prmt5, co-immunoprecipitations can be done with plk4 to determine if they interact, something that is confirmed in mouse (Sivakumar, dissertation, 2014). Also, an analysis of the global arginine methylation of proteins from plk4 depleted embryos would also indicate if prmt5 activity is affected by loss of plk4. In mouse, Plk4 appears to act upstream of Prmt5 which may lead to global epigenetic regulation by Plk4 (Ward, dissertation, 2014). It would be interesting to determine if there are any changes to epigenetic regulation upon plk4 depletion during embryogenesis, as this might have detrimental affects to development. By studying the plk4-prmt5-p53 signaling axis during development we may be able to better understand how de-regulation of these proteins affects cancer progression.

Plk4 is actively involved in the cell cycle, regulating centrosome duplication, DNA damage signaling and mitotic progression. *Plk4* null mice suffer from increased apoptosis, chromosomal abnormalities and are arrested in anaphase (Hudson et al., 2001). It is quite possible that the phenotypes seen here are due to the loss of cell cycle regulation leading to DNA damage and subsequent p53-mediated apoptosis. Staining via TUNEL and phosphorylated histone H3, as was done in the *Plk4* null mouse study, would
indicate if plk4 depletion in *Xenopus laevis* causes the same cell cycle problems. It is likely that the cause of embryonic lethality in *Plk4* null mice is due to the accumulation of cell cycle defects. However, in *Xenopus laevis* any cell cycle defects that may be present due not cause lethality. This has functionally allowed us to study the function of plk4 outside of its role in the cell cycle.

**Conclusions**

In this study we examine the effects of plk4 depletion on *Xenopus laevis* embryogenesis. We show that depletion of plk4 precludes proper somite and lens placode formation and that the underlying cause of these phenotypes is potentially due to a loss of polarity and subsequent elongation. This is the first time plk4 has been linked to cellular polarity *in vivo*. We also find that plk4 depletion leads to a decrease in Prmt5 protein levels and that plk4 depleted embryos also displayed p53 phenotypes indicating its activity may be increased. These results suggest that a plk4-prmt5-p53 signaling cascade, which has been identified in mouse, may also be conserved in frog. Our results highlight the multi-functionality of plk4 and indicate that it is critical to proper tissue morphogenesis. With its ability to localize to the centrosome, which has been proposed to be a hub for cellular signaling, plk4 could integrate multiple signaling pathways to ensure that cellular reorganization is occurring properly and at the correct time (Sumiyoshi and Sugimoto, 2012). This could be become very important during development when cellular proliferation and tissue morphogenesis must be tightly regulated to ensure the proper formation of highly complex structures. With an
understanding of the multiple functions plk4 has within a cell we may be able to better understand its role in cancer progression, leading to the development of appropriate therapeutic strategies.
REFERENCES/BIBLIOGRAPHY


Figure A.1: pCMV-Sport6-plk4

Depiction of Sport6 plasmid containing full length *Xenopus laevis plk4* cDNA (Accession no. NM_001083146). This plasmid was used for production of riboprobes for in situ hybridization. *Plk4* coding sequence is represented by a solid blue arrow. The 5’ and 3’ UTRs are represented by grey arrows outlined in red (5’) or green (3’). The SP6 and T7 promoters used for RNA transcription are indicated by yellow and green rectangles, respectively. The plasmid CMV promoter is indicated by a purple box. The plasmid’s selectable marker is ampicillin (AmpR) and is represented by a solid red arrow.
Figure A.2: pCMV-Sport6-plk4Δ2354bp

Depiction of Sport6 plasmid containing $plk4\Delta2354$ bp cDNA. Using HindIII 2354 bp of the $plk4$ cDNA was digested out of pCMV-Sport6-plk4. The plasmid was re-ligated creating a new construct that contains only the 5’UTR and first 1014 bp of $plk4$ coding sequence. This plasmid was used for production of a second riboprobe to act as a control for potential non-specific staining during in situ hybridization. $Plk4$ coding sequence is represented by a solid blue arrow. The 5’ UTR is represented by a grey arrow outlined in red. The SP6 and T7 promoters used for RNA transcription are indicated by yellow and green rectangles, respectively. The plasmid CMV promoter is indicated by a purple box. The selectable marker is ampicillin (AmpR) and is represented by a solid red arrow.
APPENDIX B: Preliminary phenotype analysis

<table>
<thead>
<tr>
<th></th>
<th>10ng plk4.upMO</th>
<th>10ng plk4.IMO</th>
<th>1ng Additive</th>
<th>20ng SCMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>124</td>
<td>44</td>
<td>17</td>
<td>143</td>
</tr>
<tr>
<td>Small eyes (moderate)</td>
<td>25%</td>
<td>27%</td>
<td>0%</td>
<td>13%</td>
</tr>
<tr>
<td>Dorsal-Ventral Defects (moderate)</td>
<td>25%</td>
<td>36%</td>
<td>35%</td>
<td>17%</td>
</tr>
<tr>
<td>No eyes (severe)</td>
<td>67%</td>
<td>36%</td>
<td>65%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table B.1: Effect of plk4 morpholino mediated knockdown on eye development

Embryos injected with control (SCMO), or one of two plk4 targeted morpholinos, were scored at stage 37 for eye defects. Eye phenotypes caused by plk4MO injection were categorized as moderate (small eyes and/or dorsal ventral defects) or severe (no eyes). The results for when two morpholinos targeted to different regions of the plk4 transcript are injected individually or together at low doses are shown. Both plk4 morpholinos cause moderate eye defects at a similar frequency; however the plk4 morpholino targeted to the upstream region causes severe phenotypes at a higher frequency. A standard control morpholino was used to control for non-specific and toxic effects and rarely caused eye defects. The results shown are for one replicate except for the plk4.upMO which is the average of two replicates. All embryos scored were injected at the 2 cell stage.
Table B.2: Effect of *plk4* morpholino mediated knockdown on somitogenesis

Hoechst nuclear stain of stage 37 whole embryos was used to evaluate the somite phenotype. Somitogenesis was determined to be aberrant if somites had not rotated. Both *plk4* targeted morpholinos cause somitogenesis defects at a higher frequency than control morpholinos. When injected together at small doses both morpholinos act synergistically to cause somitogenesis defects at a slightly higher frequency than when injected individually. Control morpholino injections rarely cause defects to somitogenesis. These results were obtained from one replicate except for the standard control which is the average of two replicates. All embryos scored were injected at the 2 cell stage.

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>66</th>
<th>54</th>
<th>22</th>
<th>174</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curved axis</td>
<td>88%</td>
<td>83%</td>
<td>100%</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Aberrant Somitogenesis</td>
<td>79%</td>
<td>71%</td>
<td>91%</td>
<td>11%</td>
<td></td>
</tr>
</tbody>
</table>

Table B.2: Effect of *plk4* morpholino mediated knockdown on somitogenesis
VITA AUCTORIS

NAME: Candace Raphak
PLACE OF BIRTH: Chatham, ON
YEAR OF BIRTH: 1989
EDUCATION: Belle River High School, Windsor, ON, 2007
University of Windsor, B.Sc., Windsor, ON, 2011
University of Windsor, M.Sc., Windsor, ON, 2014