2012

Exploring New Capacities for pitx3 During
Xenopus Embryogenesis

Lara Nicole Hooker

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Exploring New Capacities for \textit{pitx3} During \textit{Xenopus} Embryogenesis

by

Lara Hooker

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

2012

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Exploring New Capacities for \textit{pita}\textsubscript{3} During \textit{Xenopus} Embryogenesis

by

Lara Hooker

APPROVED BY:

\begin{center}
\underline{Dr. T. Drysdale, External Examiner}  
University of Western Ontario
\end{center}

\begin{center}
\underline{Dr. S. Ananvoranich}  
Department of Chemistry and Biochemistry
\end{center}

\begin{center}
Dr. J. Hudson  
Department of Biological Sciences
\end{center}

\begin{center}
Dr. A. Hubberstey  
Department of Biological Sciences
\end{center}

\begin{center}
Dr. M. Crawford, Advisor  
Department of Biological Sciences
\end{center}

\begin{center}
Dr. J. Gauld, Chair of Defense  
Faculty of Chemistry and Biochemistry
\end{center}

2012 08 27
DECLARATION OF CO-AUTHORSHIP/ PREVIOUS PUBLICATION

Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is the result of joint research, as follows:

The following manuscripts in Chapters 2, 4, and 5 incorporate the outcome of joint research undertaken in collaboration with my colleague Cristine Smoczer and previous students Sarah Brode, Marian Wolanski, and Farhad KhosrowShahian, under the supervision of Dr. Michael Crawford, with intellectual contributions from Dr. John Hudson. The key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the authors, Hooker/Smoczer/Crawford jointly. The contribution of co-authors was primarily through the provision of images for figures and gathering of data for tables produced.

In Chapter 2, I contributed all images used to compose Figure 1 (1a, b, c, d, e) showing the effect of morpholinos on dorsal axis differentiation, the image used in Figure 5c of *Pitx2* expression and inlay of sectioned embryo, and Figure 6c depicting immunohistochemistry for β-tubulin and hoescht in wildtype and morphant lenses.

Chapter 4 represents a manuscript that I wrote based on a microarray experiment, of which I contributed one replicate. An initial screen of 80 potential target genes by *in situ* hybridization was jointly conducted by Hooker/Smoczer. Personally, I contributed Figure 1 categorizing the 100 most up- and down-regulated transcripts at two stages of embryogenesis, Figure 2E (*Pitx3*), F, F’, G, G’ (*Vent2*), Figure 3 characterization of *Rbp4l*, Figure 4 characterization of *GalectinIX*, Figure 5 characterization of *Rdh16*, Figure 6B, B’ (*Baz2b*), Figure 7 characterization of *Baz2b*, Figure 8C, C’, D, D’ (*Spr1*),...
Figure 9C, C’, D, D’ (Hes4), and Figure 10C, C’, D, D’ (Spr2), E, E’, F, F’ (Lim1). Two of the novel transcripts have been submitted to GenBank: *X. laevis GalectinIX* JN975639 and *X. laevis Baz2b* JN975638.

The working manuscript comprising Chapter 5 was written by Cristine Smoczer, based on experimental design by all authors: Cristine Smoczer, myself, Dr. Michael Crawford, and Dr. John Hudson. Cristine Smoczer and I have equal contributions to the paper with respect to developing and optimizing the technique with the pitx3: *Tyrosine hydroxylase* positive control system, and cloning, including mutant analysis (Figures 1, 4, and 5. For Figures 2 and 3, comparing a dilution series and time-point experiment, I performed all protein data comprising Western blot analysis. I utilized the novel reporter assay technique for analyzing promoters of *gsc, lhx1*, and *nodal5*, as well as mutants for *lhx1* and *nodal5* (Figures 6, 8A and B). A personal cloning library has been included in Appendix B.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my dissertation, and have obtained written permission from each of the co-authors to contain the included materials in my thesis (Appendix A).

I certify that, with the above qualification, this dissertation, and the research to which it refers, is the product of my own work.

**Declaration of Previous Publication**

This dissertation includes two original papers that have been previously submitted for publication in peer-reviewed journals, as follows:
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<thead>
<tr>
<th>Thesis Chapter</th>
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<tr>
<td>Chapter 2</td>
<td>The <em>Xenopus</em> Homeobox Gene <em>Pitx3</em> Impinges Upon Somitogenesis and Laterity</td>
<td>Accepted by Biochemistry and Cell Biology (Manuscript Number 2012-0057)</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Microarray Based Identification of <em>Pitx3</em> Targets During <em>Xenopus</em> Embryogenesis</td>
<td>Accepted by Developmental Dynamics (Manuscript Number DVDY-12-0073)</td>
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dissertation has not been submitted for a higher degree to any other University or Institution.
ABSTRACT

*Pitx3* encodes a homeodomain transcription factor that represents the causative locus for the *aphakia* phenotype in mouse as well as congenital cataracts and anterior segment mesenchymal dysgenesis in humans. Mutations in *Pitx3* can also lead to the development of Parkinson’s disease. A conserved role for *Pitx3* has been established in the terminal differentiation and maintenance of lens fibres and dopaminergic neurons within the midbrain. *Pitx3* has also been reported to contribute to skeletal muscle differentiation in mice. Through the use of morpholino-mediated knockdown, the *Xenopus* model system has allowed further refining of putative roles for *pitx3* during embryogenesis. We report a novel role for *pitx3* in regulating somitogenesis and laterality pathways and also influences upon retinoic acid signalling. Microarray analysis identified gene networks affected by *pitx3* knockdown within the eye, brain, segmentation patterning, and tailbud region. Early expression of *pitx3* reveals unique involvement in early signaling pathways and subsequent effects on gastrulation. Novel transcripts were also identified and characterized for *Rbp4l, GalectinIX, Rdh16*, and *Baz2b*. Through the development of a novel reporter assay that utilizes flow cytometry, bicistronic vectors, and a three-fluor system, we determined *nodal5, lhx1*, and *crybb1* to be direct targets of *pitx3* regulation. This unique assay allows us to report that *pitx3* operates in an all-or-none mechanism as both an activator and repressor protein.
DEDICATION

I choose to study biology because I believe in science and I have faith in the process of the scientific method. I dedicate my research to my Dad, who has humbly helped science with his own participation in a clinical trial. His openness and willingness, putting risks and unknowns aside, is truly admirable. He is vocal and determined, and I will forever applaud his leadership and follow his example, by marching into the future of the unknown with confidence, and with science.
ACKNOWLEDGEMENTS

My graduate supervisor, Dr. Michael Crawford, is greatly appreciated for his liberal guidance and the freedom he allows all his students for exploring their research interests. Not only has Michael been a mentor to me for studying and questioning biology, but also he has been a wonderful advisor in the greater avenue of life. My colleague Cristine Smoczer has been an extraordinary teammate and is a friend I cherish. I am deeply obliged for the experiences that I have had in the Crawford lab, as they have given me strength in character and as a scientist.

My committee members have provided guidance and support throughout the years, pertaining to my research as well as advising through my teaching endeavours and graduate courses. Thank-you Dr. Hubberstey, Dr. Ananvoranich, and Dr. Hudson. I extend additional gratitude to Dr. Hudson for hands-on instruction with tissue culture and flow cytometry, as well as teaching and comradery.

I must give special credit to my Mom for all of her encouragement and endless belief in my abilities. The considerable support from family and friends has assisted me to completion and I am thankful for all of those believing in me.
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Appendix B: Cloning and Plasmid Construction
LIST OF ABBREVIATIONS AND SYMBOLS

+/− - heterozygous mutation
− - null allele
−/− - homozygous mutation
14-3-3 – intracellular phosphoserine/threonine-binding proteins
3G8 - mouse monoclonal antibody against dissected Xenopus pronephroi (tubules)
4A6 - mouse monoclonal antibody against dissected Xenopus pronephroi (ducts)
ACF – Drosophila ATP-utilizing chromatin assembly and remodelling factor
Ahd – aldehyde dehydrogenase family (Aldh)
ak – aphakia mutant mouse
aldh1a2 – aldehyde dehydrogenase (raldh2)
ANOVA – analysis of variance
AP - Jun oncogene
ASMD – anterior segment mesenchymal dysgenesis
ATCC – global bioresource centre
ATG – triplet codon for amino acid methionine; nucleotide translational start codon
ATP – adenosine triphosphate
atv – antivin (lefty)
βB1-crys – beta-crystallin B1 (crybb1)
BarH1 – Drosophila homeobox gene in Bar eye mutation region
BAZ – bromodomain adjacent to zinc finger
BBE – bicoid binding element
Bcd – bicoid gene
BDNF – brain-derived neurotrophic factor
bHLH – basic helix-loop-helix
bix4 – brachyury-inducible homeobox gene 4
BM – binding mutant
bmp4 – bone morphogenetic protein 4
bp – base pairs
BP – band pass filter
BR - bromodomain
C15 – *Drosophila* clawless homeobox gene, member of the 93DE cluster

cAMP – cyclic adenosine monophosphate
cart - cocaine- and amphetamine-regulated transcript
cDNA – complementary DNA
CF – cardiac field
Cig30 – cold-inducible glycoprotein
Cmo – control-morpholino
CNS – central nervous system
CO₂ – carbon dioxide
Co-IP – co-immunoprecipitation
Cont MO – control morpholino
CpG – cytosine-phosphate-guanine site
CRABP – cellular retinoic acid binding protein
CRBP – cellular retinol binding protein
CRE – cAMP response element
cRNA – complementary RNA
cry - crystallin
crybb1 – βB1-crystallin
CTP – cytidine triphosphate
Cy3 – cyanine dye
cyp26a1 – cytochrome P450, family 26, subfamily A, polypeptide 1
D-ptx1 – *Drosophila* pituitary homeobox gene 1
D3 – human brain endothelial cell line (hCMEC/D3)
DA - dopamine
DAT – dopamine transporter
DDT – DNA binding domain
dhc – dynein axonemal heavy chain
DJ1 – Parkinson disease (autosomal recessive, early onset) 7 (Park7)
Dlx-2 – distal-less homeobox 2
DMEM – Dulbecco’s Modified Eagle Medium
DMZ – dorsal marginal marginal zone
DNA – deoxyribonucleic acid
DNase - deoxyribonuclease
Dpp – Drosophila Decapentaplegic gene
DsRed – Discosoma sp. red fluorescent protein
Dyl – dysgenetic lens mouse mutant
ECM – extracellular matrix
eFGF – embryonic fibroblast growth factor (fgf4)
eGFP – enhanced GFP (F64L/S65T)
Eng – engrailed repressor domain
ESC – embryonic stem cell
EST – expressed sequence tag
eyl – eyeless mouse mutant
F64L/S65T – amino acid substitutions (phenylalanine to leucine; serine to threonine)
fgf – fibroblast growth factor
FL – fluorescence detector
Fox – forkhead box
Gata4 – zinc finger transcription factor that binds DNA sequence 5’-AGATAG-3’
Gbf1 – golgi brefeldin A resistant guanine nucleotide exchange factor 1
GDNF – glial cell line derived neurotrophic factor
GFP – green fluorescent protein
GFRα1 – GDNF family receptor alpha 1
GRP – gastrocoel roof plate
gsc – goosecoid
H+ - hydrogen proton
H+K+ATPase – hydrogen potassium proton enzyme pump that utilizes ATP
H3K27me3 – histone 3 tri-methylation of lysine 27
H3K4me2 – histone 3 di-methylation of lysine 4
hand – heart and neural crest derivatives
HAT – histone acetyltransferase
HeRed – Heteractis crispa red fluorescent protein
HD - homeodomain
HEK293 – human embryonic kidney cell line
Hes – Hairy and enhancer of split
hLamp – human lysosome-associated membrane protein
Hox – homeobox
i.e. – id est
inv – inversion of embryonic turning mouse mutant
IRES – internal ribosome entry site
ISH – in situ hybridization
ISWI – Drosophila chromatin-remodeling protein
iv – situs inversus mouse mutant
JB3 – antibody that recognizes atrioventricular and outflow tract epithelia of the heart
K50 – lysine at position amino acid 50 of the homeodomain
Kb - kilobases
kDA – kilodaltons
L-Maf – lens-specific Maf gene (nrl)
l,b – lens, brain promoter
lens1 – foxe3 homolog
lefty – left-right determination factor
lhx – LIM homeobox (lim1)
lim1 – LIM-class homeodomain transcription factor 1 (lhx1)
LP – longpass filter
LPM – lateral plate mesoderm
lrd – left-right dynein
m – muscle promoter
Maf – musculoaponeurotic fibrosarcoma oncogene family
MBD – methyl-CpG binding domain
MBS – modified Barth’s saline
mDA – mesencephalic dopaminergic
MF20 – mouse monoclonal antibody against sarcomeric myosin heavy chains
MHB – mid-hindbrain boundary
MIP - major intrinsic protein of lens fibre
miR-133b – microRNA gene
MO - morpholino
MRF – myogenic bHLH regulatory factor
mRNA – messenger RNA
MTA1 – metastasis associated 1
mTH – murine Tyrosine hydroxylase
MyoD – myoblast determination protein
NCBI – National Center for Biotechnology Information
NeuroD1 – neurogenic differentiation 1
ng - nanograms
NIBB – National Institute for Basic Biology
Nkx2.5 – homeobox gene homolog to Tinman in Drosophila
NLS – nuclear localization signal
Nono – non-POU-domain-containing, octamer binding protein
nrl – neural retina leucine zipper
Nurr1 – nuclear receptor subfamily 4, group A, member 2 (Nr4a2)
OAR – Otx, Aristaless, Rax (Aristaless protein domain)
Obscn1 – Obscurin gene 1
ODC – ornithine decarboxylase
Oligo(dT) – oligonucleotide of deoxy-thymine nucleotides
Om1D – Drosophila annasae gene of Om mutants (optic morphology)
Otx – orthodenticle homeobox gene
P19 – mouse teratocarcinoma cell line
P27Kip1 – cyclin-dependent kinase inhibitor 1B
P57Kip2 - cyclin-dependent kinase inhibitor 1C
Pax6 – paired box gene 6
pBS – plasmid Bluescript
PD – Parkinson’s disease
PEI - polyethylenimine
PEST – amino acid sequence concentrated in proline, glutamic acid, serine, and threonine
PFA – paraformaldehyde
PHD – zinc finger motif
Pit-1 – POU class 1 homeobox 1 (pituitary-specific positive transcription factor)
pitx – pituitary homeobox gene (Paired-like homeodomain transcription factor)
pitx3MO – pitx3-morpholino
pkd2 – polycystin gene 2
PLOD1 – procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1
Pmo – pitx3-morpholino
Poly(A) - polyadenylation
POMC – pro-opiomelanocortin-alpha
POU – DNA-binding domain, common to Pit, Oct, Unc genes
Prox – prospero homeobox protein 1
PSF – PTB-associated splicing factor
PTB – phosphotyrosine binding group
Q50 - glutamine at position amino acid 50 of the homeodomain
RA – retinoic acid
RACE – rapid amplification of cDNA ends
raldh2 – retinal dehydrogenase (aldh1a2)
RAR – retinoic acid receptor
RARE – retinoic acid response element
ras – rat sarcoma viral oncogene
rax1 – retinal and anterior neural fold homeobox gene 1 (Rx1)
RBP – retinol binding protein
rbp4l – retinol binding protein 4 like
rdh – retinol dehydrogenase
RhoGEF – guanine nucleotide exchange factor with Rho domain (Ras homology)
RISC – RNA-induced silencing complex
RNA – ribonucleic acid
RRF – retrorubral field
RT-PCR – reverse transcription polymerase chain reaction
rx1 – retina and anterior neural fold homeobox (Rax)
RXR – retinoid X receptor
SF-1 – nuclear receptor subfamily 5, group A, member 1
SFPQ – splicing factor prolin/glutamine-rich gene
shh – sonic hedgehog
shox – short stature homeobox gene
Six3 – Sine oculis homeobox homolog 3
SMRT – silencing mediator for retinoid or thyroid-hormone receptors
SNC – substantia nigra pars compacta
Sox – SRY (sex determining region Y)-box
Sp – trans-acting transcription factor
Spr – Sp5-related
t – *Xenopus* brachyury homolog (Xbra)
tbx – T-box transcription factor
TCOF – Treacher Collins-Franceschetti Syndrome
TF – transcription factor
TGF-β – transforming growth factor beta
TH – Tyrosine hydroxylase
TnnC – troponin C
Trkβ – neurotrophic tyrosine kinase receptor type 2
Ubx – Ultrabithorax
UI – International Units
µm – micrometers
UTP – uridine triphosphate
UTR – untranslated region
UV – ultraviolet
V8 – vegetal blastomere 8
VegT – T-box transcription factor, meso-endodermal determinant
Ventx2 – VENT homeobox gene (ventralizing protein) (Xom)
Vg1 – growth differentiation factor 1 (gdf1)
Vmat2 – vesicular monoamine transporter 2
vnd – *Drosophila* ventral nervous system defective
VTA – ventral tegmental area
Wnt – wingless-type
WT – wildtype
Xbra – *Xenopus* brachyury (t)
Xlns1 – foxe3
Xom – Vent homeobox gene 2 (Ventx2)
Xnr - *Xenopus* Nodal-related
XtnIc – *Xenopus* cardiac troponin I type c
Z – zinc finger domain
CHAPTER I
INTRODUCTION

Research Outline and Goals

During embryogenesis, the complex interaction of tissues, cells, signalling pathways, and genes, makes the analysis of gene function difficult. Tissue induction, feedback regulatory loops, and the cooperative influence of transcriptional co-activators, all add to the complexity of gene regulation. Our model system revolves around the role of a homeobox transcription factor, pitx3, in the process of lens induction. Intriguingly, when this gene is perturbed, we observe unexpected phenotypes (Chapters 2 and 3). In order to characterize novel roles for pitx3 in the development of the embryo, we undertook a microarray experiment (Chapter 4). This allowed us to sift through a large data set in search of putative target genes and signalling pathways of pitx3.

Initially, we assessed the list of potential target genes and performed a broad analysis of gene expression patterns in order to determine likeliness of interaction with pitx3. If expression patterns overlapped temporally and spatially, the putative target gene was selected for more exhaustive analysis (Chapters 4 and 5). We also searched for patterns in the microarray data indicative of genes involved in the same developmental pathways, and especially those involved in pathways that could account for the novel phenotypes observed. Concomitantly, where sequences and plasmids were available, we looked at target genes for putative pitx3 binding sites in their promoter/regulatory regions. Finally, we were able to identify and characterize novel genes in the Xenopus laevis genome, and to provide new evidence for pitx3 performing novel roles during embryological development, by directly or indirectly regulating exciting new partners.
Transcription Factors

Transcription factors are proteins that bind DNA to regulate a gene, either by activating or repressing transcription. These proteins are modular, usually containing both a DNA binding domain as well as a transactivation or repressor domain (Latchman, 1990; Frankel and Kim, 1991). They also possess nuclear localization signals, since transcription factors act on DNA located in the nucleus, and can contain PEST sequences (enriched in proline (P), glutamic acid (E), serine (S), and threonine (T)) which signal protein degradation and maintain a tight control on transcription factor concentration (Rogers et al., 1986; LaCasse and Lefebvre, 1995). During embryogenesis, these morphogenetic signals elaborate a network of gene regulation that ultimately sets the developmental agenda for an organism. The transcription factor will eventually regulate, either directly or indirectly, the binding of RNA polymerase and the initiation of transcription of the target locus (Latchman, 1990). Spatial and temporal control of the transcription factor allow for specific timing of target gene transcription. Transcription factor regulation can occur at the level of transcription and/or post-transcriptional mRNA splicing, sequestration or silencing, or at the level of protein binding and post-translational modifications (Latchman, 1990; Lee et al., 2006).

Regulatory transcription factors are categorized into different classes with respect to functional domains or motifs that pertain to how they bind DNA and how they interact with other factors. pitx3 encodes a homeodomain (HD): a relatively small DNA-binding motif (60 amino acids) characteristic of a large gene family. Homeodomain-containing transcription factors, or homeoproteins, are grouped into different families, based on their homeodomain residue sequence and other domain similarities elsewhere in the protein.
Members of the same family may show overlapping expression patterns and/or functional redundancy. Homeoproteins often participate in protein-protein interactions that modulate their transactivation activity (Poulin et al., 2000).

Homeodomains comprise three $\alpha$-helical regions, characterizing a helix-turn-helix motif. This motif mediates DNA binding to a core “TAAT” sequence within the major groove (Qian et al., 1989; Laughon, 1991; Poulin et al., 2000; Baird-Titus et al., 2006). The third HD helix is termed the recognition helix since it is the helix that makes contact with the major groove (Ptashne, 1986). Residue 50 of the homeodomain (position 9 of the recognition helix) is a particularly important determinant of DNA binding specificity (Gehring et al., 1994), and provides an additional criteria for subdivision of this family, as it differentially specifies 3’-dinucleotides adjacent to the recognition site to which the homeoproteins bind (i.e. TAAT-CC) (Baird-Titus et al., 2006).

The Paired-like class of homeodomain proteins have either a glutamine (Q50) or a lysine (K50) residue in position 50 of the HD (Galliot et al., 1999). Rather than the paired-box motif that is found in members of the Paired family, the Paired-like homeoproteins share an OAR or Aristaless domain (Furukawa et al., 1997). This is variously described as a 6-14aa stretch at the C-terminal of the protein sequence, and it is thought to be a transactivation domain or to participate in protein-protein interactions (Semina et al., 1996; Furukawa et al., 1997; Medina-Martinez, 2010). OAR stands for $otp$, $aristaless$, and $rax$, which are homeodomain-containing proteins that share this domain in common with $shox$, $cart$, and $pith$ family members (Furukawa et al., 1997; Meijlink et al., 1999). These homeodomain proteins have been shown to autoregulate themselves. Internal folding via intramolecular binding of the OAR domain to amino
acids in the N-terminal of the protein, encloses the homeodomain (Amendt et al., 1999). The transcription factor is kept in this folded and inhibited state until the binding of the OAR domain to cofactors which then relieves the OAR domain and allows the homeodomain to bind cognate sequence of downstream gene promoters (Amendt et al., 1999).

**Bicoid**

While it might not be useful to review all members of this class of homeobox genes, as the founding member of the K50 subclass of *Paired*-like genes in *Drosophila*, *bicoid* (*bcd*) is a useful exemplar of some general traits. As a maternal transcript, deposited into *Drosophila* eggs prior to fertilization, *bcd* functions as a molecular morphogen to activate zygotic gene transcription, which in turn is responsible for the development of anterior structures in the embryo (Driever et al., 1990). Eggs from females that are void of *bcd*, fail to form head or trunk portions of the fly (Frohnhofer et al., 1986). The presence of K50 within its homeodomain contributes to the preference of this protein to bind DNA as a monomer (Wilson et al., 1993). The *bcd* K50 HD recognizes the hexamer consensus DNA binding site of 5’-TAATCC-3’, known as a *bicoid* Binding Element (BBE) (Wilson et al., 1993). The lysine at position 50 restricts interaction by requiring a 3’-CC dinucleotide next to the core TAAT DNA target motif (Treisman et al., 1989; Treisman et al., 1992). There are also non-consensus binding sites that are essential for the activity of *bcd*. This observation leads to an adaptive model of DNA recognition that allows this protein to function using interchangeable recognition codes in various DNA environments and for the specific regulation of different genes.
(Dave et al., 2000). A PEST domain has also been identified in the bcd protein (Stauber et al., 1999), indicative of signals for proteolytic degradation and a high turnover for this transcription factor (Rechsteiner and Rogers, 1996).

Expressed in an anterior-to-posterior protein concentration gradient, bcd binds the DNA of downstream target genes cooperatively (Ma et al., 1996; Yuan et al., 1996). Bcd preferentially binds to nucleotide consensus motifs that are oriented head-to-head or tail-to-tail, separated by a short 7-15bp sequence, or to sites in tandem if separated by a longer stretch of DNA sequence (Yuan et al., 1999). Once bound, the transcription factor-DNA complex that is formed is quite unstable: bcd actually has very weak transactivation activity (Ma et al., 1999). One theory to explain how the concentration gradient of bcd elicits a threshold-dependent activation of downstream target genes is that intermolecular interactions stabilize the protein on DNA: cooperativity facilitates more protein binding and thus progressively more transactivation (Ma et al., 1999). The bcd gene is a target of autoregulation and has multiple sites for binding bcd protein, which would support this theory (Yuan et al., 1996; Yuan et al., 1999). Bcd is unique to Drosophila, evolving from the duplication of Hox3 (Stauber et al., 1999), another homeodomain transcription factor belonging to the Hox (Homeobox) gene family known for axial patterning (Krumlauf, 1994). However, there are many Bicoid-like homeodomain proteins identified in other organisms such as goosecoid, Otx1 and Otx2, and the Pitx genes (Drouin et al., 1998).

**Pitx/Rieg Family**

The Pitx/Rieg family of Paired-like/K50 homeodomain transcription factors is comprised of three paralogs: Pitx1, Pitx2, and Pitx3. This family is known to be involved
in patterning of the embryo as well as cell differentiation (Poulin et al., 2000). All family members possess nearly identical homeodomains (Figure 1) (Hanes and Brent, 1989). For that reason, these bicoi d-like transcription factors are thought to bind consensus sites similar to bicoi d binding elements BBEs (5′-TAATCC-3′) and to functionally behave similarly to bcd with respect to their transcriptional activation (Lamonerie et al., 1996; Amendt et al., 1998).

Pitx proteins also share the C-terminal OAR domain characteristic of the Paired-like class, and although there are hints that this domain acts as the transactivation domain, it has yet to be solidly proven (Medina-Martinez, 2010). The Pitx family also shares highly conserved regions that are serine and proline rich that have previously been shown to have transactivation abilities (Gerber et al., 1994; Lamonerie et al., 1996; Drouin et al., 1998). The identity between the three Pitx paralogs is quite high, especially in the homeodomains which are 98% identical. Over the entirety of the proteins, pitx1 and pitx2 share 66.1%, pitx2 and pitx3 share 72.9%, and pitx1 and pitx3 share 62.9% amino acid identities. They vary most in the N-terminal region and the sequence divergence, in conjunction with their unique expression patterns, may account for the differing functions of each Pitx protein during development.

Role of Pitx Genes

The pituitary gland develops primarily from the portion of anterior neural ridge that forms the stomodeal placode, stomodeum, and then subsequently Rathke’s pouch (which is the anterior pituitary anlage) (Schwind, 1928). Because of the expression of Pitx1 in Rathke’s Pouch (Lamonerie et al., 1996) and its ability to regulate the activation
Figure 1: Alignment of homeodomains from *Xenopus laevis* pitx family members with bicoid. Alignment shows one differing residue between pitx members (Box I) and the conserved lysine (K) among all proteins at the amino acid in position 50 (Box II). Percent identities are listed in reference to pitx3 homeodomain (HD) (right). Notably, the pitx sequences differ among themselves by a single residue. Red shows conserved residues and green/blue designate differences between protein sequences. GenBank numbers for the protein sequences from which the HD sequences were extracted are as follows: bicoid (AAL77032), pitx1 (AAI69747), pitx2 (AAC29426), pitx3 (AAI70394). MegAlign from DNASTAR Lasergene 8, Clustal W Method used for alignment.

Figure 2: Family tree of the *Pitx* members identified across different species. Right brackets show the segregation of each paralog and the conservation between protein amino acid sequences is along the x-axis. Identification of *Drosophila* Pitx protein relates to an emergence of this protein family prior to the divergence of protostome and deuterostome organisms. GenBank numbers: AAF57099, NP_001035436, CAC12834, AAH03685, NP_035227, NP_001161156, AAF00486, AAC27322, AAC29426, AAK15048, AAH75660, AAT68296, AAB87380, AAI70394, NP_005020, NP_001027689, ACZ55229, BAE07208, BAE66654, CAP22817. MegAlign from DNASTAR Lasergene 8, Clustal W Method used for alignment.
of several pituitary-specific genes (Tremblay et al., 1998), the name conferred to this family was Pituitary Homeobox (Pitx). Although this family is termed bicoid-like, a Pitx homolog has subsequently been identified in Drosophila (D-px1) (Figure 2) (Vorbruggen et al., 1997). Although the homeodomain of D-px1 places it in the bcd-like subgroup, its expression differs from bcd in that it expresses in the posterior versus the anterior of the embryo (Vorbruggen et al., 1997). Also, no morphology has been described for the loss of this gene in the fly, indicating that the function of D-px1 is either redundant in the embryo, or it may play a more physiological role (Vorbruggen et al., 1997). A Pitx ortholog has also been identified in echinoderms (Hibino et al., 2006) and tunicates (Ascidians) (Christiaen et al., 2002) showing high conservation of Pitx during the evolution of the various deuterostome phyla (Figure 2). The discovery of similar Pitx orthologs in worm (Westmoreland et al., 2001) and snail (Grande and Patel, 2009b) further categorizes the Pitx gene family as having evolved prior to the divergence of protostomes and deuterostomes (Figure 2).

In vertebrates, the three Pitx members have distinct but overlapping expression patterns. Pitx1 and Pitx2 overlap in the Rathke’s pouch, the branchial arches, tooth germ, and hindlimb mesenchyme (Gage et al., 1999a). Pitx2 and Pitx3 expression overlaps in the eye and brain (Gage et al., 1999a). Given the highly conserved nature of their homeodomains (Figure 1), it might be reasonable to expect functional redundancy where expression domains overlap. All members appear to bind bicoid binding elements and appear to enjoy similar protein-protein interactions that modify their transactivation properties (Lamonerie et al., 1996; Poulin et al., 1997; Amendt et al., 1998; Tremblay et al., 1998; Amendt et al., 1999; Cox et al., 2002; Grande and Patel, 2009a; Medina-
Martinez, 2010). Their non-overlapping expression domains in unique tissues, suggests novel functions having evolved more recently for specific members of this transcription factor family.

**Pitx1**

*Pitx1* was first detected in the mouse, expressed in the pituitary anlage Rathke’s pouch, throughout pituitary organogenesis, and in adult corticotrophic cells (Lamonerie et al., 1996; Lanctot et al., 1997). Being expressed in the most anterior domain of the developing murine embryo, the stomodeal epithelium, supports a functional relationship similar to *bicoid* in *Drosophila* for determining the development of anterior structures. Murine *Pitx1* is also expressed in the first branchial arch, tongue, palate, teeth, and craniofacial structures, as well as the olfactory system and the primitive streak (Lanctot et al., 1997). Later in development, *Pitx1* is expressed in the posterior lateral plate mesoderm (LPM), which gives rise to the bladder and hindgut, as well as muscles of the lower body (Crawford et al., 1997), and in proliferating mesenchyme of the hindlimb (Lanctot et al., 1997; Chang et al., 2006). In the chick, similar expression patterns are observed (Lanctot et al., 1997). In *Xenopus*, *pitx1* is expressed additionally within the cement gland anlage and lens placode, and similarly in the anterior neural ridge, the stomodeal-hypophyseal anlage, and oral epithelia (Hollemann and Pieler, 1999). *Pitx1* was found to co-express with *Pitx3* in the developing lens in mouse as well, however starting at the late lens vesicle stage (Semina et al., 2000).

*Pitx1* transcriptionally activates the *pro-opiomelanocortin (POMC)* gene in differentiating cells of the anterior pituitary and this *Pitx1* activation of the *POMC* gene serves as a model for *Pitx* family transcription factor behaviour (Lamonerie et al., 1996).
Like *bicoid*, *Pitx1* binds target DNA sequences as a monomer (Lamonerie et al., 1996), and almost all promoters for hormone-producing genes in the pituitary are responsive to *Pitx1* (Tremblay et al., 1998). Different co-factors are necessary for synergistic gene activation by *Pitx1*, including *NeuroD1*, *Pit-1*, and *SF-1* (Poulin et al., 1997; Tremblay et al., 1998). *NeuroD1* is a basic helix-loop-helix (bHLH) factor that is known to have a role in neurogenesis, specifically for cell differentiation (Lee et al., 1995). *Pit-1* is a pituitary POU-domain transcription factor that is important for the expression of various hormones from the pituitary, including growth hormone, thyroid stimulating hormone and prolactin (Simmons et al., 1990). The orphan nuclear receptor *SF-1* is specifically expressed in gonadotrope cells of the pituitary and acts as a transcription factor for the differentiation of these cells (Asa et al., 1996). A combinatorial mechanism allows the six different cell lineages of the pituitary to differentiate, and secrete distinct hormones, with modulatory input by *Pitx1* (Tremblay et al., 1998).

*Pitx1* plays a role in defining the formation of the pituitary and the differentiation and maintenance of pituitary cells into different lineages based on the hormone produced, and also the maintenance of *POMC* expression in adulthood (Lamonerie et al., 1996; Tremblay et al., 1998). *Pitx1* may also play a role during early gastrulation, as seen from its expression in the primitive streak (Lanctot et al., 1997). This transcription factor is also important for determining hindlimb identity in the developing embryo, through regulation of *Tbx4* (Logan and Tabin, 1999; Chang et al., 2006). This latter T-box transcription factor has restricted expression in the hindlimb buds of developing embryos and is required for hindlimb identity, versus the forelimb identity that is specified by *Tbx5* (Gibson-Brown et al., 1996).
In humans, \textit{PITX1} localizes to chromosome 5q31, which is adjacent to the locus for Treacher Collins Fraceschetti (TCOF) Syndrome (5q31-35) (Crawford et al., 1997). TCOF syndrome presents with craniofacial abnormalities, including those affecting the mandible, the palate, the eyes (slanting) and the ears, and can result in hearing loss (Dixon, 1995). These malformations occur in regions where \textit{Pitx1} expresses during embryonic development, and thus begs the question whether the adjacent and ubiquitously expressed TCOF1 gene identified as the causative locus for TCOF syndrome (Valdez et al., 2004) is the sole effector in these patients. Mutations that inactivate \textit{Pitx1} in mice give rise to Treacher Collins-like mandible malformations, in addition to hindlimb abnormalities (Lanctot et al., 1999).

\textit{Pitx2}

Unlike the other \textit{Pitx} family members, there are three different isoforms of \textit{Pitx2}: \textit{Pitx2a, b,} and \textit{c}, expressed in all organisms studied to date (Cox et al., 2002). A fourth isoform, \textit{PITX2D}, is only found in humans and acts to inhibit the activity of the other isoforms (Cox et al., 2002). Differential promoter usage regulates \textit{Pitx2} expression and results in different isoforms via alternative splicing events (Cox et al., 2002). In all isoforms the homeodomain and C-terminals are the same, however differing N-terminals affect DNA-binding capabilities, resulting in transactivational differences (Cox et al., 2002). Like \textit{Pitx1}, \textit{Pitx2} binds the \textit{bicoid} binding element and causes activation of promoters/enhancers containing these sequences (Amendt et al., 1998). These isoforms can create homodimers with themselves or heterodimerize with the \textit{Pitx2b} isoform (Cox et al., 2002).
The role and specificity of each Pitx2 isoform varies depending on the organism being studied. Generally, Pitx2 has been shown to express in the eye, brain, Rathke’s pouch and pituitary, mandible and maxillary areas, dental epithelium, the umbilicus, cement gland, and in particular the left lateral plate mesoderm (LPM) (Arakawa et al., 1998; Hjalt et al., 2000). Pitx2c specifically expresses asymmetrically on the left side of the developing heart, gut, and lungs (Hjalt et al., 2000; Schweickert et al., 2000). Later during myogenesis, it is expressed in muscle progenitor and precursor cells in myotome and limb buds, just prior to Pitx3 expression, where the two Pitx transcription factors play a coordinated role (L'Honore et al., 2007). Pitx2 is able to compensate in the event of a loss of Pitx3, and runs the myogenic program alone by expressing longer than normal to maintain muscle differentiation in adult muscles (L'Honore et al., 2007).

Pitx2 has been shown to bind and activate the prolactin promoter in concert with the Pit-1 cofactor, which specifically heterodimerizes with Pitx2b (Amendt et al., 1998; Amendt et al., 1999; Cox et al., 2002). The ability of Pitx2 to activate pituitary-specific hormone genes is similar to the ability of Pitx1 (Drouin et al., 1998), however the two transcription factors likely interact with different cell-specific cofactors. Their functions may be redundant in the pituitary, since there is no deficit of pituitary function in either Rieger (PITX2) or PITX1 mutants, where only one Pitx factor is affected (Crawford et al., 1997). Pitx2 isoforms a and c can activate genes PLOD1 and Dlx2, and are each able to create synergistic activation via heterodimerization with Pitx2b (Cox et al., 2002).

PLOD1 is an enzyme necessary for modifying collagens (Knippenberg et al., 2009). Pitx2 therefore plays an indirect role in constituting extracellular matrix and providing the appropriate substrate for tissue and organ development (Cox et al., 2002). Dlx2 is a
transcription factor known to regulate the development of branchial arches and shares
similar expression domains with Pitx2 (Cox et al., 2002). Synergistic activation
capabilities appear to be a theme with Pitx factors.

Similar to its paralog Pitx1, Pitx2 is involved in the proper functioning of the
pituitary and together they are the earliest markers of pituitary development (Tremblay et
al., 1998). The difference lies in Pitx1 expressing in the mandible and its derivatives,
while Pitx2 expresses in the maxillary and its respective derivatives (Drouin et al., 1998).
Pitx2 plays a role in the determination of left-right body asymmetry, possibly not by
directing the looping of asymmetric organs such as the heart, but by positioning the heart
after it has formed as well as giving organs such as the lung a left-side identity (for
example, different number of lobes of the left and right lung) (Lin et al., 1999). Pitx2
expression in bone marrow implicates this transcription factor in the development of
haematopoietic lineages (Arakawa et al., 1998). The dosage and combinatorial isoform
and cofactor expression for Pitx2 regulates the development of the pituitary, heart and
gut, lung, brain, and teeth: a threshold concentration of Pitx2 may be required for normal
organ formation (Gage et al., 1999b; Cox et al., 2002).

In humans, PITX2 maps to 4q25 near the Rieger locus at 4q21-22 (Semina et al.,
1996). Point mutations in the homeodomain of the PITX2 gene, resulting in proteins that
either lose binding specificity or become unstable, give rise to the developmental disorder
Axenfeld-Rieger syndrome (Amendt et al., 1998). This autosomal dominant disorder,
presents with dental, ocular, craniofacial, and umbilical abnormalities, as well as defects
in heart, limb, and pituitary development (Rieger, 1935). Ocular defects can include
glaucoma resulting from developmental problems of the anterior chamber of the eye
(Amendt et al., 1998). Similarly, in mouse Pitx2\textsuperscript{+/\textminus} mutants, there are defects in the ventral body wall, heterotaxia phenotypes including different positioning of the heart, as well as a decrease in cellular proliferation in the pituitary gland and teeth (Lin et al., 1999).

**Pitx3**

*Pitx3* has been extensively studied for its roles in the development of the lens and a specific subset of neurons arising from the midbrain. During lens development, from placode into adulthood, *Pitx3* functions in the maintenance of lens epithelial cells and the differentiation of lens fibre cells (Medina-Martinez, 2010). *Pitx3* is necessary for the establishment of midbrain neurons that produce dopamine (Jacobs et al., 2007). The mesencephalic dopaminergic (mDA) neurons regulate behaviour and movement control, and interestingly, *Pitx3*-expressing mDA neurons are specifically reduced in patients diagnosed with Parkinson Disease (Smidt et al., 1997). *Pitx3* regulates the identity of these neurons by regulating their terminal differentiation and survival (Hwang et al., 2003). *Pitx3* is able to operate with assorted cofactors for the development of various structures in an organism, and therefore it is suggested to function by different modes of action, depending on its cellular and co-factor context (Medina-Martinez, 2010).

**Genomic Structure of Pitx3**

Semina et al. (1997) mapped the mouse *Pitx3* gene to chromosome 19 within close proximity to a region responsible for the *aphakia (ak)* phenotype. Four exons were identified (Figure 3A): the first is a non-coding exon; the second contains the ATG translational start; the third contains the homeobox sequence, and the fourth encodes the OAR domain (Coulon et al., 2007). Another group discovered that there are in fact two different starting exons that are noncoding: one specific for lens and brain expression
(l,b) and one for muscle expression (m) (Coulon et al., 2007). Since the differing first exons are noncoding, the resulting Pitx3 translated protein is identical in both (lens, brain) and muscle. Two separate promoters have been proven to regulate the expression of Pitx3: a lens, brain (l,b) and a muscle (m) promoter (Figure 3A) (Coulon et al., 2007). There is a muscle-specific promoter/enhancer located in between exon 1(l,b) and exon 1m, that can direct Pitx3 expression in muscle (Coulon et al., 2007). However, even when transcription is activated by muscle-specific transcription factors, exon 1 (l,b) instead of exon 1m is preferentially transcribed for the mRNA transcript (Coulon et al., 2007).

Mouse genomic DNA sequence reveals that there are two genes in close proximity to Pitx3, both running in the opposite orientation: Gbf1 in the 5’- untranslated region (UTR) of Pitx3 and Cig30 in the 3’-UTR of Pitx3 (Figure 3) (Tvrdik et al., 1999; Semina et al., 2000). This bidirectional transcription and the fact that the 3’-ends of Pitx3 and Cig30 transcripts are overlapping and complementary for at least 10 nucleotides could mean that these molecules hybridize within the cell if transcribed at the same time; although no such interaction has been described (Tvrdik et al., 1999). It is odd to have three genes that are not functionally related so close to each other in vertebrate genomic DNA. These genes do however appear to be embedded within a genomic regulatory block, nested in a region that is rich in highly conserved non-coding elements, indicating that they do express at the same time and in the same place at some point in development (http://anca.romaneg.net/). However, what is known about the functions of the protein products of these three genes arranged in tandem appears unrelated. Cig30 encodes a membrane glycoprotein that is expressed in the skin, the liver and brown fat, and may be
involved in recruiting brown adipose tissue (Tvrdik et al., 1997). *Pitx3* is not expressed in these tissues and is therefore unlikely to be functionally linked or involved with *Cig30*. *Gbfl* is a ubiquitously expressed gene that has possible housekeeping functions (Mansour et al., 1998), but again, no relation to *Pitx3* is obvious.

*Pitx3* has since been cloned and characterized in zebrafish (*Danio rerio*) (Zilinski et al., 2005), frog (*Xenopus laevis*) (Pommereit et al., 2001; Khosrowshahian et al., 2005), and human (Semina et al., 1998). The genomic structure of the human *PITX3* gene is the same as in mouse (Figure 3), however is unknown as of yet for *Xenopus* due to lack of sequencing.

*Pitx3* has been described as a bivalent gene (Konstantoulas et al., 2010), in that its chromatin is primed for expression upon receipt of a differentiation signal, but is kept silent up until that point (Bernstein et al., 2006). This occurs thorough chromatin modification involving a combination of *H3K4me2* (active chromatin) and *H3K27me3* (inactive chromatin) histone methylation factors (Konstantoulas et al., 2010).

**Protein Structure of Pitx3**

In addition to the *bicoid*-like/K50 homeodomain and a 14-amino acid OAR domain (Figure 3B), *Pitx3* possesses a consensus nuclear localization signal (NLS) in its C-terminal (Smidt et al., 1997; Zilinski et al., 2005). Another resides within the recognition helix of the homeodomain, right beside the K50 residue (Figure 3B) (Sakazume et al., 2007). *Pitx3* has been shown to exclusively localize to the nucleus, albeit with the caveat that this was shown utilizing a GFP-tagged *Pitx3* protein construct (Messmer et al., 2007).
Figure 3: Schematic representation of the genomic structure of Pitx3 (A) and of the Pitx3 protein (B) for Mus musculus. (A) Exons are boxes (numbered for Pitx3) and coding regions are shaded. Lens, brain (l,b) and muscle (m) specific exon1 are noted. Initiation codon (ATG), termination codon (TGA), poly-adenylation signal (AATAA), ak deletions (Del1 + Del2), and the direction of transcription (arrows) are designated. (B) Protein domains are boxed and identified. Lysine residue at position 50 in the homeodomain (K50) and nuclear localization signals (NLS) are specified. Numbers of amino acid residues are represented in each segment of the protein. (Semina et al., 1997; Smidt et al., 1997; Semina et al., 2000; Rieger et al., 2001; Coulon et al., 2007; Sakazume et al., 2007). Homeobox domain: PF00046, OAR domain: PF03826 (NCBI CCD conserved domains).

Figure 4: In situ hybridization utilizing an antisense riboprobe allows the visualization of pitx3 transcripts in X. laevis embryos. (A) Stage 23 neurula embryo. (B) Stage 35 tailbud embryo. (C) Stage 43 tadpole. B - branchial arches, H - heart, I - intestine, L - lens, O - otic vesicle, S - somites, dotted line represents midline and shows the connected heart primordia.
There is significant homology for the *Pitx3* protein amino acid sequence across species, especially within the homeodomain, suggesting an evolutionarily conserved function for this gene. This is supported by its consistent expression pattern and mutant phenotypes across species. The percent amino acid identity between frog *pitx3* and human (66.8%), rat (66.5%), mouse (67.2%) *Pitx3* proteins is quite strong, but identical (100%) in the homeodomain (Pommereit et al., 2001). The size of the *PITX3* protein is approximately 37kDA in humans (Sakazume et al., 2007), and 32kDa in both zebrafish (Shi et al., 2005) and frog (Chapter 5). This protein binds *bicoid* binding element sequences as a monomer or creates homo- or hetero-dimer complexes on DNA, via protein-protein interactions with cofactors and/or other transcription factors (Sakazume et al., 2007).

**Pitx3 Gene Expression**

In zebrafish, *pitx3* first expresses at the anterior end of the embryo in the shape of a crescent, representing a common placodal structure, that later bifurcates to form the primordia of the pituitary, lens, olfactory epithelium, and cranial ganglia (Shi et al., 2005; Zilinski et al., 2005). Expression is also reported in the diencephalon, pectoral fins, cartilage surrounding the mouth (later the lower jaw and branchial arches), and in the muscles along the body trunk (Shi et al., 2005). *Pitx3* expression in the lens becomes restricted to the equatorial region where the secondary fibre cells are produced, as the lens develops and grows (Shi et al., 2005).

In *X. laevis* embryos, this transcription factor is seen first in the pituitary anlage, as well as the stomodeal-hypophyseal anlage, and is most concentrated in the lens
placode (Pommereit et al., 2001). *pitx3* is also expressed in the head mesenchyme and lateral plate mesoderm (Pommereit et al., 2001), as well as in the otic vesicles, somites, lower jaw region and branchial arches (Figure 4) (Khosrowshahian et al., 2005). Later during lens development in frog, and in contrast to zebrafish, *pitx3* expresses in the epithelial cells only, and not in the primary lens fibres (Pommereit et al., 2001). At tadpole stages, *pitx3* is expressed in the heart and gut, including the stomach and the second coil of the intestine (Khosrowshahian et al., 2005). Temporally, although not yet visualized via *in situ* hybridization preceding stage 17 in *Xenopus* embryos (Pommereit et al., 2001), *pitx3* transcripts can be detected by RT-PCR at stage 8, well prior to gastrulation (Khosrowshahian et al., 2005). Midblastula transition occurs at stage 7.5 (Newport and Kirschner, 1982), indicating that *pitx3* is among the first genes activated following the mid-blastula transition and thus might be necessary for early development of the embryo.

In the mouse, *Pitx3* expression is most clearly visualized at all stages of the developing lens, persisting in the fibre cells, anterior epithelium, and equator region, but also in the eye muscles and eyelid (Semina et al., 1997; Semina et al., 1998; Ho et al., 2009). Expression in the mouse lens persists into the adult organism (Semina et al., 2000). *Pitx3* in mouse can also be detected in head muscles, tongue, mesenchyme, as well as within the midbrain region, incisors and surrounding the vertebrae and sternum (Semina et al., 1998; Semina et al., 2000). Within the mesencephalon, or midbrain, there are three different areas containing neurons that express dopamine (DA): the retrorubral field (RRF), the ventral tegmental area (VTA), and the *substantia nigra compacta* (SNC) (Alavian et al., 2008). In the midbrain of the rat, *Ptx3* is expressed in the SNC and VTA,
specifically in the mesencephalic dopaminergic (mDA) neurons (Smidt et al., 1997). In these neurons Ptx3 overlaps with and regulates the expression of Tyrosine hydroxylase (TH), a rate-limiting enzyme necessary for the production of dopamine (Smidt et al., 1997; Lebel et al., 2001; Messmer et al., 2007). Also, Pitx3 is expressed within developing myotomes and limb buds specifically in the differentiating myoblasts and into adulthood, where it is expressed in muscle fibre cells and satellite cells, which are muscle progenitors that lie quiescent in adult muscle until needed for repair (Buckingham et al., 2003; Coulon et al., 2007; L'Honore et al., 2007). The expression pattern of Pitx3 in the developing mouse embryo was confirmed using a GFP knock-in mouse, where GFP is being transcribed in place of Pitx3, and seen in lens, somites, midbrain, muscles, tongue, and craniofacial regions (Zhao et al., 2004).

A main difference in the expression of Pitx3 between species appears to be in the degree of expression in the developing lens and brain, indicating a changing role for this transcription factor through evolution.

**Pitx3 Loss of Function Analysis**

In order to deduce the function of a gene, developmental geneticists make use of mutant models to characterize phenotypes due to the change in gene concentration and/or activity. Mutants consist of: naturally occurring mutations to the gene, the introduction of ectopically expressing alleles, or genetic disruption by homologous recombination or transgenesis. Alternative approaches include, for example, translational knockdown by morpholinos.
Zebrasfish morphants exhibit morphological phenotypes reflective of pitx3 expression domains: small eyes, abnormal jaws, body axis deviation, and malformed pectoral fins (Shi et al., 2005), as well as misshapen small heads, lens degeneration, and retinal defects (Sorokina et al., 2011). In morphant fish the lens fibre cells fail to lose nuclei, a necessary step that normally occurs with elongation during differentiation from epithelial cells (Shi et al., 2005). Moreover, the retinal layers are unorganized and contain pyknotic nuclei, a sign of apoptosis (Shi et al., 2005). Both retina and lens show degeneration, which could be due to a lack of normal retinal induction by lens placode or a failure to support proliferation by both direct and indirect means.

In *Xenopus*, overexpression and morphant studies reveal phenotypes including abnormal eye development and microcephaly (Khosrowshahian et al., 2005). In addition, phenotypes arising from a compromised midline or laterality defects are evident (Khosrowshahian et al., 2005). Ectopic expression of pitx3 in vivo, results in a bent body axis, craniofacial malformations, a diminished diencephalon leading to a reduction of the midline between the developing eye fields, and an extension of the retina leading occasionally to cyclopia (Khosrowshahian et al., 2005). Since homeodomains between *Xenopus* pitx1, pitx2 and pitx3 are identical and these proteins are shown to be able to activate the same consensus binding sites (Smidt et al., 1997), overexpressing pitx3 may elicit defects by mimicking the other paralogs. Ectopic cement glands seen in pitx3 overexpressing embryos likely reflect this, as both pitx1 and pitx2 express in cement gland, whereas pitx3 does not (Arakawa et al., 1998; Hollemann and Pieler, 1999; Hjalt et al., 2000; Khosrowshahian et al., 2005). By inhibiting pitx3 using an engrailed repressor chimera or morpholino, Khosrowshahian et al. (2005) observed inhibited lens and retina
Figure 5: Comparison of wildtype and pitx3-morphant X. laevis embryos. (A-D) Curved or bent dorsal axis is seen when pitx3-morpholino (Pmo) is injected into one side of the embryo (L-left or R-right) or completely knocking down pitx3 in the embryo (ALL). Arrows show loss of eye structures as well as bent axis in pitx3 morphants. (E, F) Comparing a control-morpholino (Cmo) injected on the left (L) side with pitx3-morpholino (Pmo) shows that the curved axis is due to the loss of pitx3 protein versus the injection site. (G, H) Using Hoechst stain to identify cell nuclei in the lenses of an embryo injected with Pmo on one side shows the loss of organization of lens cells and the absence of the vesicle lumen. (I, J) A pitx3-morphant embryo shows reversed heart looping compared to wildtype (WT).
development. In our lab, we have documented morphant phenotypes (Figure 5), including the bent dorsal axis on the side of pitx3 knockdown, and lens irregularities, as well as heterotaxia phenotypes, resulting in abnormal heart and gut (not shown) looping.

*Aphakia* (*ak*) is a natural homozygous recessive mutant (*ak/ak*) in mouse that consists of a promoter mutation for the *Pitx3* gene locus (Varnum and Stevens, 1968). The mutation consists of a 652bp deletion in the 5'-promoter sequence of *Pitx3* approximately 2.5kb upstream of the transcriptional start site (Figure 3A) (Semina et al., 2000). Another group identified a second 1423bp deletion within the *Pitx3 ak* allele; one that ablates all of exon1, part of the promoter on the 5'-end and part of intron 1 on the 3'-end of the deletion (Figure 3A) (Rieger et al., 2001). This large deletion removes the lens/brain promoter region such that lens and brain *Pitx3* expression is abolished, but expression in the muscles remains and Pitx3 protein is detected in these tissues as normal (Coulon et al., 2007). This mutation is responsible for a lack of *Pitx3* expression in the developing lens (Semina et al., 2000) and severely reduced *Pitx3* transcripts (down to 5% of wild-type) in the entire organism (Rieger et al., 2001). Neither deletion of the *ak* mutation affects the translational start site of *Pitx3* located in exon 2 (Figure 3A). Since some residual *Pitx3* transcripts can be detected in the mouse (Rieger et al., 2001), outside of the lens and midbrain regions, some amount of *Pitx3* protein could be made under direction of the muscle promoter (Coulon et al., 2007), which is unaffected by the mutated regions of the *aphakia* allele. Interestingly, there is no affect on *Gbf1* transcript levels, however *Cig30* expression in reduced to 50%, in *ak* mice, indicating some sort of relationship between the regulation of *Cig30* and *Pitx3*, perhaps lending support to these
genes being part of a genomic regulatory block (Rieger et al., 2001) (http://ancora.genereg.net/).

In ak mutants, lens development, arresting at the stage of lens vesicle, produces the characteristic ak phenotype of small eyes that lack lenses (Varnum and Stevens, 1968). Prior to the complete development of the lens vesicle, there is a problem with detachment between lens epithelial cells and the overlying ectoderm (corneal epithelium), as the lens stalk persists between the two cell layers (Varnum and Stevens, 1968). There is a lack of organization of the lens cells, including cells filling the lens pit without the development of primary fibre cells. The retinal cup appears normal, yet at advanced stages of this phenotype, there is abnormal folding of the retinal layers, which may be a secondary morphology subject to the failure of lens development (Varnum and Stevens, 1968). Also secondary to the lens phenotype, there is no iris or pupil formed, as the anterior chamber never develops (Rieger et al., 2001).

The aphakia phenotype is also reported to have a deficit of dopaminergic neurons (DA) in SNc region of the midbrain, as well as a 50% reduction of DA neurons in the neighbouring VTA, where Pitx3 is expressed (Smidt et al., 1997; Hwang et al., 2003). There is virtually no Pitx3 protein observed in the midbrain of these mutant mice, and they consequently display a kinetic phenotype, including a decrease in locomotor movement, as a result of the decreased amounts of dopamine production (van den Munckhof et al., 2003). These mutants are responsive to Levodopa, which can replace dopamine function temporarily (van den Munckhof et al., 2006). This attribute of ak mice mimics the loss of motor movement in patients with Parkinson’s disease (PD). Phenotypes affecting other organs in the ak mouse have not been reported to date.
Another natural mouse mutant has recently been reported to exhibit closed eyelids, reduced eye development, and behavioural disorders (Rosemann et al., 2010). This mutant model termed eyeless (eyl) is thought to provide a better model for Parkinson’s disease, due to some common features in lowered pain tolerance and problems with grip (Rosemann et al., 2010). The recessive mutation is mapped to exon 4 of Pitx3 and consists of a single nucleotide insertion (416insG) that results in a frame-shift that does not affect the homeodomain, but does change the C-terminal amino acids (including the loss of the OAR domain) (Rosemann et al., 2010). Microphthalmia and a lack of DA neurons specifically in the SNc results in the eyl mutant (Rosemann et al., 2010). Since ak mutant mice retain Pitx3 expression in the muscles, their akinesia phenotype can be considered due to a loss of these specific neurons, however the eyl mutant would result in lack of wild-type Pitx3 in the muscles and the difference in behaviour could lead to clues about the roles of Pitx3 in muscle development and function (Rosemann et al., 2010). Other phenotypes include problems with the bones, liver, and lungs, which again expands the likely repertoire of roles for this transcription factor, or could be attributed to secondary features arising from muscle and nerve damage (Rosemann et al., 2010).

In humans, two PITX3 mutations have been discovered that give rise to eye development phenotypes leading to impaired vision or blindness. The first, a frame-shift mutation due to a 17bp insertion that truncates the OAR domain at the C-terminus of the protein product, causes anterior segment mesenchymal dysgenesis (ASMD) (Sakazume et al., 2007). This is an inclusive term used to describe any defects in the eye structures formed from mesenchyme arising from the neural crest, including the cornea and iris.
(Hittner et al., 1982). The second mutation in humans is a substitution that occurs in the N-terminal of \( \textit{PITX3} \) and gives rise to autosomal-dominant congenital cataracts (Sakazume et al., 2007). Both mutations exist outside of the homeodomain region of the transcription factor, and therefore give rise to different developmental defects, but do not abolish lens development altogether, probably due to some partially retained functionality (Semina et al., 1998). Both human conditions represent hypomorphic mutations. Humans homozygous for \( \textit{PITX3} \) mutations have more severe phenotypes compared to those with one wild-type allele (Sakazume et al., 2007). Heterozygous mutations for \( \textit{PITX3} \) lead to cataracts, but homozygous mutations lead to more severe developmental phenotypes in the brain and microphthalmia, justifying a need for at least one functional allele of this gene for partial retention of tissue development (Huang and He, 2010). No \( \textit{PITX3} \) mutations that affect the homeodomain have been reported and this implies that non-functional \( \textit{PITX3} \), via the inability to bind DNA properly, may be embryonic lethal.

Some common themes arise when looking at the phenotypes produced by impaired action of \( \textit{Pitx3} \). Gene dosage appears to be an important factor for the proper function of \( \textit{Pitx3} \) in development. Morphant phenotypes, when related to the concentration of morpholino that is utilized to knockdown \( \textit{Pitx3} \) protein expression, appear to be dose-dependent, with more severe phenotypes resulting from higher concentrations of morpholino used (Khosrowshahian et al., 2005; Shi et al., 2005). This shows that residual \( \textit{Pitx3} \) protein that is being translated from uninhibited mRNA transcripts, can still contribute towards the development of lens, mDA neurons, and other structures.
Gene dosage effects can explain phenotypes in some Pitx3+/− heterozygous mutations: one functional allele might not compensate by producing more protein, or the mutated allele produces a negative effect. The human PITX3 mutations that lead to eye phenotypes are heterozygotes, implying that the phenotypes are due to diminished, but not abolished, PITX3 protein concentrations (Rosemann et al., 2010). However, this may be a species-specific effect.

By contrast, heterozygous ak mice appear unaffected (Varnum and Stevens, 1968; Semina et al., 1997), and therefore, despite a reduction in Pitx3 protein, proper development is restored, and so gene dosage is not at play. L’Honore et al. (2007) produced a Pitx3 mutant mouse in which the mutation extracted exon 3 containing the homeobox sequence, thus resulting in a putative total loss-of-function mutant. Heterozygotes (+/−) for this allele showed no obvious phenotype, yet homozygotes (−/−) resulted in eye dysmorphogenesis and again selective loss of mDA neurons. One functional allele in these studies was able to compensate for proper embryonic development. Similarly, the eyl mutant allele of Pitx3 produces a loss-of-function protein, yet heterozygotes for this mutation have no phenotype (Rosemann et al., 2010).

**Activation of Pitx3**

In order to determine what factors act upstream of Pitx3, we look to genetic mutants that abolish Pitx3 expression. Pitx3 activation in zebrafish appears to be dependent on both nodal and hedgehog signalling pathways, since pitx3 expression is severely reduced or absent in embryos mutant for these genes (Zilinski et al., 2005). Both sonic hedgehog (shh) and nodal are important for regulating midline integrity and
anteror development, implying that pitx3 is activated to uphold roles in these pathways (Muller et al., 2000). In support of this, Khosrowshahian et al. (2005) determined that pitx3-morphant phenotypes exhibit compromised midline characteristics. Shh expression is also required for the proper formation of mDA neurons, which later express Pitx3 in specific regions, again situating Pitx3 downstream of Shh signalling (Ericson et al., 1997; Lebel et al., 2001). Recently, neural stem cells have been induced to form dopaminergic neurons utilizing Shh to trigger genetic cascades that lead to the activation of Pitx3 (Rossler et al., 2010).

Analysis of the deleted region in the mouse Pitx3 promoter responsible for the ak phenotype shows putative binding sites for both AP-2 and Maf (i.e. L-Maf) transcription factors (Semina et al., 2000). AP-2 acts early in lens development, since lens detachment from the ectoderm is not complete in AP-2 null mice (West-Mays et al., 1999). Maf appears to be important for the terminal differentiation of lens fibre cells via the activation of crystallin genes (Ring et al., 2000). This suggests that Pitx3 is possibly directly regulated by these factors, which themselves have been shown to be implicated in lens development (Figure 6). Pax6, another homeodomain transcription factor necessary to lens development and that shows overlapping expression with Pitx3, affects the expression of Pitx3 in mouse lenses as shown by microarray analysis; in lenses of Pax6 heterozygous mice, Pitx3 transcript levels decrease 3.9 fold, which is confirmed by RT-PCR (Chauhan et al., 2002a; Chauhan et al., 2002b). Pax6 mutations give rise to small eye phenotype in mouse (Graw et al., 2005) and aniridia in humans (Jordan et al., 1992; Semina et al., 1998). Pax6 is also suggested to be upstream of Pitx3 in the eye
Figure 6: Simplified diagram of the Pitx3 gene regulatory network during lens development. (?) Denotes unknown direct regulation. (β,γ) designate crystallins.

Figure 7: Simplified diagram of the Pitx3 gene regulatory network during development of dopaminergic neurons in the midbrain (mDA) of the Substantia Nigra compacta (SNc) region. DA, dopamine; RA, retinoic acid; (?) denotes unknown direct regulation.
development pathway due to conserved putative Pax6 binding sites located in the promoter region of Pitx3 (Chauhan et al., 2002b; Ho et al., 2009). However, these binding sites are not within the deleted regions associated with aphakia, and which abolish Pitx3 expression in the lens (Semina et al., 2000). This implies a parallel signalling pathway for regulating lens development between Pax6 and Pitx3.

Myogenic bHLH regulatory factors (MRF’s) are capable of activating Pitx3 expression in muscle tissues and engage in a positive feedback loop leading to upregulation of Pitx3 (Coulon et al., 2007). MyoD was shown to locate to the Pitx3 upstream region and to bind the muscle-specific promoter via MRF binding sites to transactivate Pitx3 directly (Blais et al., 2005; Coulon et al., 2007). MyoD is known as the master switch of muscle development, as it can direct myogenesis (Tapscott and Weintraub, 1991). Myogenin, another MRF that is necessary for the differentiation of myoblast to muscle fibre cells, is co-expressed in Pitx3-positive myoblasts (Buckingham et al., 2003; L'Honore et al., 2007). Myogenin binds the Pitx3 promoter and thus can be a possible upstream regulator of Pitx3, directing expression in differentiating skeletal muscle cells (Coulon et al., 2007; L'Honore et al., 2007).

Pitx3 and the microRNA miR-133b act in a negative feedback circuit, as miR-133b binds the 3’-UTR of Pitx3 mRNA transcripts to regulate Pitx3 protein production and Pitx3 in turn binds the promoter of miR-133b to activate its expression (de Mena et al., 2010). MicroRNAs operate by enlisting the RNA-induced silencing (RISC) complex, which eliminates RNA duplexes created by the binding of a microRNA and its target mRNA sequence (Tijsterman and Plasterk, 2004). Since miR-133b expression is seen
specifically in DA neurons of the midbrain (Kim et al., 2007), it is suggested to regulate Pitx3 transcription factor levels in this region (de Mena et al., 2010), ultimately regulating the development of mDA neurons in the SNc and affecting dopamine production in mammals (Figure 7).

FoxP1, a winged-helix transcription factor from the forkhead domain family, is a marker of mDA neurons and is present in the same mDA neurons that express Pitx3 (Konstantoulas et al., 2010). The chromatin of the Pitx3 gene is modified in the presence of FoxP1 to a more active state via H3K4me2 (Konstantoulas et al., 2010). FoxP1 directly binds the Pitx3 promoter via two sites and activates its transcription up to 76% in reporter assays in vitro (Figure 7) (Konstantoulas et al., 2010). Thus FoxP1 plays a major role initiating the specification events that lead to the dopamine-type differentiation of mDA neurons, via activation of Pitx3.

In summary, Pitx3 is regulated directly by particular factors that vary depending upon tissue type. This signifies that there is not one mode of Pitx3 activation, but rather that multiple pathways may lead to its activation and regulation for particular functions in a context specific manner.

Pitx3 Targets

Transcription factors can sometimes act as both activators and repressors of gene transcription, and this regulation often depends upon the environment and what other factors are available for interaction. Smidt et al (1997) determined that Pitx3 in rat is able to act as a transcriptional activator, with its ability to bind and transactivate the POMC promoter in a luciferase reporter assay, a known target gene of Pitx1 (Lamonerie
et al., 1996). We have been able to show that xPitx3 functions as a repressor of Tyrosine hydroxylase in a reporter assay of our own, reflecting that Pitx3 is able to act in either a positive or inhibitory role, depending on the target and cellular context (Chapter 5).

In mDA neurons in the SNC of the midbrain, Pitx3 is necessary to directly regulate multiple factors necessary for neuron development, differentiation, and maintenance or survival. One of the most extensively studied downstream targets of Pitx3 is Tyrosine hydroxylase (TH) (Figure 7). Pitx3 expression in mDA neurons appears a half-day before TH in mouse central nervous system (CNS) development (Lebel et al., 2001). TH is the first enzyme necessary for the production of dopamine, and is also the rate-limiting step for its synthesis (Landis et al., 1988). Therefore, in order for dopaminergic cells to differentiate, they must express TH. Lebel et al. (2001) show that Pitx3 directly binds and transactivates the TH gene via a high-affinity binding site (TAATCC). This study utilized murine protein and DNA in transient transfection assays using undifferentiated P19 cells (mouse embryonic teratocarcinoma cells that differentiate into neuronal lineages upon application of RA). Messmer et al. (2007) confirmed this interaction by showing that Pitx3 overexpression endogenously activates TH in HEK293 (human embryonic kidney cells) and D3 cells (mouse embryonic stem cells).

In the same neuron population, human PITX3 directly regulates the expression of VMAT2 (vesicular monoamine transporter 2) and DAT (dopamine transporter) (Hwang et al., 2009), which are responsible for the storage and reuptake of dopamine, respectively (Figure 7) (Harrington et al., 1995). VMAT is responsible for packaging dopamine into vesicles that can then be recycled, leading to a function in mDA neuron homeostasis (Vergo et al., 2007). As a regulator of these two genes, Pitx3 is responsible for the
function and homeostasis of mDA neurons as they communicate with their neighbours (Hwang et al., 2009). In eyl mutant mice that lack functional Pitx3 in the mDA neurons of the SNc, there is a reduction of Th and Dat, providing evidence for these genes being downstream of Pitx3 (Rosemann et al., 2010).

Overexpression studies of Pitx3 in cell-cultured neurons show that transcript and protein levels for two neurotrophic factors increase in response to elevated Pitx3 (Peng et al., 2007). This places GDNF and BDNF, glial cell line- and brain-derived neurotrophic factors, downstream of Pitx3 in the mDA neuron developmental pathway. As neurotrophic factors, these ligands help maintain the differentiation and survival of these neurons, as they protect against neurotoxins (Knusel et al., 1991; Gash et al., 1996). Their respective receptors, Trkβ and GFRα1, reduce in concentration in response to Pitx3, most likely due to a negative feedback loop with their signalling proteins (Peng et al., 2007). More recent data supports this pathway, however placing Pitx3 as a mediator between GDNF and BDNF (Peng et al., 2011). BDNF expression inhibits apoptotic signals, preventing neuron death and promoting survival (Peng et al., 2011). In regulating neurotrophic factors, Pitx3 appears to function in the survival of mDA neurons.

Pitx3 has also been shown to directly regulate Aldehyde dehydrogenase 2 (Ahd2/Aldh1a1) by binding a highly conserved non-consensus site within the promoter region of this gene (Figure 7) (Jacobs et al., 2007). Ahd2 is an enzyme that regulates the production of retinoic acid (RA) from retinol, and it is necessary for the proper development and maintenance of the mDA neurons (McCaffery and Drager, 1994). Low amounts of Ahd2 are characteristic of patients diagnosed with Parkinson’s disease, showing a persistent requirement of RA in mDA neurons in order to maintain proper
function (Chung et al., 2005). In mice, loss of these neurons can be rescued in the developing embryo by maternal ingestion of RA (Jacobs et al., 2007). An increase of TH-positive mDA neurons can also be produced when aphakia mDA embryonic stem cells that are deficient in Pitx3, are treated by addition of RA to the cell culture medium (Jacobs et al., 2007). RA, being downstream of Pitx3, can rescue mDA neuron development when Pitx3 is absent. Pitx3 is maintaining mDA neurons by regulating RA metabolizing enzymes and thus the amount of RA produced in these cells.

Through morpholino studies performed in zebrafish, it has been shown that foxe3, a winged helix/forkhead domain transcription factor, lies genetically downstream of pitx3 in the lens development pathway (Figure 6) (Shi et al., 2006). This is supported by studies in mouse Pitx3-null lenses, where no Foxe3 is detectable (Ho et al., 2009). Foxe3, functional homolog to foxe3 (lens1) in Xenopus, functions in the transition from lens epithelial cells into enucleated secondary lens fibres (Shi et al., 2006). Mutations of this transcription factor which is responsible for the mouse dysgenetic lens (dyl) phenotype, are phenotypically characterized by small lenses, a reduction in the lens epithelial cell population, as well as by premature differentiation of fibre cells (Sanyal and Hawkins, 1979). Zebrafish foxe3-morphants show normal retinal development, however they possess small lenses that lack proper lens fibre morphology and organization, and they display multilayered epithelial cells with increased numbers retaining their nuclei (Shi et al., 2006). This suggests a lack of transition from lens epithelial to lens fibre cells. Being upstream of foxe3, pitx3 likely regulates the terminal differentiation of lens cells into the organized, clear fibres that will ultimately be necessary for lens function. It has also been suggested that Pitx3 is responsible for
differentiation by regulating Prox1, which is mis-expressed in Pitx3 null lenses, and in turn would regulate crystallin expression (Figure 6) (Ho et al., 2009). Crystallins are structural proteins that are expressed by terminally differentiated lens fibres, and that function to prevent apoptosis and maintain the integrity of the cells necessary for optics (Andley, 2007). In Xenopus, pitx3 perturbation affects the expression domains of lens genes pax6, six3, rax1, and foxe3 (lens1), and crystallins, possibly stimulating the expression of six3, rax1, and foxe3 (Khosrowshahian et al., 2005). This shows the ability of Pitx3 to regulate multiple genes in the eye developmental pathway that ultimately leads to a functional lens.

Also in the lens developmental pathway, Pitx3 directly activates MIP/Aquaporin O (major intrinsic protein of lens fibres) (Figure 6) (Sorokina et al., 2011). In zebrafish pitx3-morphants, mip1 expression is reduced and PITX3 in human cell lines activates MIP expression 5.2-fold via two conserved bicoid binding sites (Sorokina et al., 2011). MIP is one of the most abundant integral membrane proteins expressed in the lens in both primary and secondary lens fibres on into adulthood (Huang and He, 2010). MIP functions as both an inter-fibre adhesion molecule as well as a water channel, regulating osmotic permeability and cell hydration (Chepelinsky, 2009). Mutations in human MIP can give rise to dominant lens defects, leading to cataracts (Huang and He, 2010). This shows that Pitx3 is regulating important proteins that are necessary for lens composition and function.

In the absence of Pitx3 in lenses of the ak mouse mutant, cell cycle inhibitors are prematurely activated, and lens epithelial cells lose mitotic activity and terminally differentiate by expressing crystallin proteins (Ho et al., 2009). In a wild-type lens, β-
crystallin is restricted to fibre cells solely at the centre of the lens vesicle, and in Pitx3 mutant embryos, \( \beta \)-crystallin is expressed throughout the lens (Ho et al., 2009). Pitx3 could be suppressing the expression of \( \beta \)- and \( \gamma \)-crystallins in lens epithelial cells, since without Pitx3 there is premature expression of both molecules (Ho et al., 2009). Pitx3 has roles in maintaining the mitotic ability of lens epithelial cells, which is necessary for the replenishment of lens fibre cells throughout life, as well as a role in the terminal differentiation of lens fibre cells with respect to crystallin production.

**Pitx3 Cofactors**

Members of the Pitx transcription factor family have shown the ability to work in tandem with other molecules or transcription factors to synergistically activate gene transcription (Amendt et al., 1999; Poulin et al., 2000). The homeodomain of Pitx factors can interact directly with Class A bHLH transcription factors, which are ubiquitously expressed and are involved in cell differentiation (Poulin et al., 2000). Poulin et al. (2000) suggest a mechanism of interaction between these two families of transcription factors that may regulate differentiation of tissues where they are commonly co-expressed, such as in muscles and neurons. This group has specifically hypothesized a Pitx HD: bHLH direct interaction during the myogenic program in muscle development (Poulin et al., 2000). This means that co-factors possibly have the ability to modulate the DNA-binding and transactivation activities of Pitx3 (Amendt et al., 1998). Also in support of this theory is that the ability of Pitx3 to activate TH expression is cell-type dependant, implying that co-factors are needed for this interaction (Lebel et al., 2001). Transcriptional synergism may be a common theme among the Pitx family.
There appears to be controversy over whether *Pitx3* and *Nurr1*, an orphan nuclear receptor, are working synergistically or independently for the regulation of mDA neuron differentiation. *Pitx3* has been shown to interact with the protein *PTB-associated splicing factor* (*PSF*) and the non-POU-domain–containing, octamer binding protein (*Nono*) (Jacobs et al., 2009), which can repress transcription of nuclear receptor-mediated gene activation (Mathur et al., 2001). *PSF* can also bind to *Nurr1*, and may bridge the two proteins in a transcriptional complex (Jacobs et al., 2009). Both *Pitx3* and *Nurr1* can co-localize on the same promoters, including *Vmat2*, but not on those of *Th*, *Dat*, or *Ahd2* (Jacobs et al., 2009). *Nurr1*, by activating dopamine-specific genes such as *TH*, *VMAT2* and *DAT*, is important, but not essential, for establishing the identity of mDA neurons (Hwang et al., 2009). Although *Nurr1* is expressed in midbrain neurons prior to *Pitx3*, it is unable to activate its target genes, including *DAT* and *VMAT2*, until *Pitx3* is expressed. Once expressed, *Pitx3* can co-localize to the *Nurr1* downstream target gene promoter where *Nurr1* is already bound, and release *Nurr1* repression by *SMRT* (silencing mediator of RA and thyroid hormone receptor) (Jacobs et al., 2009). Since *Vmat2* expression is greatly reduced in *Pitx3* null mDA cells and both *Pitx3* and *Nurr1* transcription factors bind the *Vmat2* promoter (Jacobs et al., 2009), we can identify *Nurr1* as a necessary cofactor for *Pitx3* activation of this downstream gene (Figure 7). Although they do not co-localize on the promoters of *Ahd2*, *Th*, or *Dat*, neither of these are expressed in *Nurr1* null mice, where *Pitx3* is still expressed (Smits et al., 2003), indicating that *Pitx3* is unable to activate them alone (Jacobs et al., 2009). However, *Pitx3*’s ability to activate *Th* was unaffected by the overexpression of *Nurr1* (Messmer et al., 2007). Possibly, *Pitx3* and *Nurr1* act as cofactors in a transcriptional complex solely
for the activation of genes responsible for the dopamine phenotype in the SNc region of the midbrain. Both transcription factors are expressed in mDA neurons on into adulthood (Hwang et al., 2009), so they are necessary past initial development and are thought necessary for maintenance and/or survival of mDA neurons.

Also in this subset of mDA neurons, Pitx3 has been shown to interact via its homeodomain with metastasis-associated protein 1 (MTA1), a chromatin modifier that is ubiquitously expressed, but cannot itself bind to DNA (Reddy et al., 2011). MTA1 acts in conjunction with DJ1 (Parkinson’s Disease 7 gene) in an MTA1/DJ1 complex, which is recruited to the Th promoter region by Pitx3, to coactivate Th expression (Figure 7) (Reddy et al., 2011). Upon recruitment, epigenetic changes cause histone acetylation and consequently increased availability of the gene for subsequent transcription (Reddy et al., 2011).

Pitx3 Function at the Tissue Level: Possible Signalling Pathways

Some transcription factors are expressed in precursor cells to regulate cell-specific transcription. As a cell differentiates, it becomes post-mitotic, exiting the cell cycle, and expresses proteins necessary for its differentiated function. A common theme in Pitx3 activity is its expression in precursor cells in different tissues, including those of lens, midbrain, and muscle, and its role in the terminal differentiation of these cells via activation of cell-specific proteins that define cell type.

In zebrafish, it is suggested that pitx3 expression may be a common feature of all placodes that develop in the embryo, leading to a unanimous role in turning on a transcriptional profile for the development of placodal-derived structures, such as the
pituitary, lens, and olfactory systems (Zilinski et al., 2005). Since pitx3 expression is seen in all of these placodes at a similar time, specificity of gene transcription activated by pitx3 may be defined by other factors in the different placodes; environmental or positional integration may be at play.

**Lens**

The lens is a placode-derived structure that forms a lens vesicle upon induction from the protruding optic vesicle (McAvoy et al., 1999). Further development allows the lens to detach from the overlying ectoderm and settle into the optic cup (McAvoy et al., 1999). Outer mitotic epithelial cells continually replenish inner lens fibre cells throughout life. Functional lenses must be transparent, composed of fibres where nuclei or organelles are aligned with one another so that light passes through this cellular architecture with minimal diffraction and refraction (Huang and He, 2010). Any disturbances in the homeostasis of the arrangement, composition, or function of these lens cells can lead to cataract opacities and difficulties leading to blindness. (Huang and He, 2010)

*Pitx3* has a general function in lens development that appears to be conserved from teleost fish, to amphibians, to mammals. Since the lack of functional *pitx3* in the developing embryo results in the loss of proper development of both lens and retina, *pitx3* likely acts as a transcriptional activator of genes involved in the inductive pathways leading to the development of both these structures (Khosrowshahian et al., 2005). There is a loss of differentiated retinal structures, and it can be suggested through grafting experiments that *pitx3* is required in lens ectoderm prior to placode development in order
for the presumptive lens cells to be competent to responding to optic cup induction (Khosrowshahian et al., 2005).

*Pitx3* functions in three separate pathways in the developing eye; an indirect path that influences retinal induction, and two direct routes, one to regulate *foxe3* for secondary lens fibre cell differentiation and organization, and another for lens cell survival (Shi et al., 2006). Since *Pitx3* expression is concentrated in the anterior epithelium and equator regions of the developing lens, it is thought to be involved in cytodifferentiation, since this is where epithelial cells differentiate into secondary lens fibre cells (Semina et al., 1997). Also, the lack of lens fibre differentiation in both zebrafish *pitx3* morphants and *ak* mice indicates a role for *pitx3* in primary lens cell differentiation through elongation and loss of nuclei (Varnum and Stevens, 1968; Shi et al., 2005). Since in the *ak* mouse lens no lens fibres are developed (Varnum and Stevens, 1968), this implicates *Pitx3* in the regulation of lens fibre differentiation.

There is an increase in cell-cycle inhibitors *p27Kip1* and *p57Kip2* in *Pitx3*-deficient versus wild-type lenses (Figure 6) (Ho et al., 2009), implying an exit from cell cycle leading to precocious differentiation. This in turn would lead to fewer cells and thus smaller lenses. Moreover, fibre cells die without *Pitx3* expression, suggesting *Pitx3* also has a role in cell survival (Ho et al., 2009). It can be concluded from the expression pattern, upstream regulators, and downstream target genes, that *Pitx3* plays a role in the differentiation of primary and secondary lens fibres, as well as the maintenance of lens function. Lens fibre cells are constantly regenerated in the adult lens (McAvoy et al., 1999), and if *Pitx3* is responsible for lens fibre differentiation, which would be necessary
for new fibre cells to be produced from the mitotic epithelium, this could explain a role of Pitx3 mutation in the development of cataracts (Hwang et al., 2009).

Shi et al (2005) suggest that when zebrafish pitx3 morphants experience a reduction in the number of cells in the developing retina, pitx3 may play a role in cell differentiation and/or survival within the retina as well as the lens. This is also seen in *Xenopus*, where retina formation is inhibited by a loss of pitx3 expression in the lens placode and subsequent loss of lens formation, however this loss of retinal induction by improper lens development could be considered an indirect phenotype (Khosrowshahian et al., 2005). It remains undetermined whether Pitx3 plays a role solely in lens development, or also directly influences development of the retina as well.

**Midbrain**

In the midbrain, specifically in the SNc, Pitx3 plays a role in the terminal differentiation, maturation and survival of mesencephalic dopaminergic (mDA) neurons. In embryonic stem cells (ESC) produced from aphakia SNc, Papanikolaou et al. (2009) showed a reduction of Th, VMAT2, and DAT, which are necessary to synthesize, transport, and re-uptake dopamine; necessary features of mature mDA neurons. This identifies Pitx3 as a gene regulator of dopamine neurotransmission and the survival of mDA neurons (Hwang et al., 2009). Although there were still Th+ neurons in this region of the ak midbrain, there were 68% less, indicating a role for Pitx3 in the generation of at least part of this DA neuronal population (Papanikolaou et al., 2009). However, in the Th+ cells that are developed in ak mice midbrain, there is a decrease in DAT and VMAT2, indicating that they will not be functional in the proper administration of dopamine (Papanikolaou et al., 2009). While Th gives mDA neurons their identity through
expressing dopamine, VMAT2 and DAT give these neurons their function of neurotransmission via dopamine signalling. Pitx3 is shown to be required for all of these factors.

**Muscle**

L’Honore et al. (2007) created a Pitx3 mouse mutant, where exon 3 containing the HD is conditionally removed, resulting in a non-functional protein product. Pitx3 is apparently not required for muscle development, since muscles develop normally in these mice, even when eye and mDA neuron morphogenesis mimics aphakia phenotype (L'Honore et al., 2007). This is due to the redundant function of Pitx2, or at least its ability to compensate for loss of Pitx3 function in the myogenic program by upregulation (L'Honore et al., 2007). However, under normal conditions, Pitx3 expression does appear in differentiated muscle cells during development and on into adulthood, contributing to the final differentiation and maintenance of the muscle cells (L'Honore et al., 2007). It is suggested that Pitx3 expresses in post-mitotic muscle cells, induced after muscle precursors, or myoblasts, exit the cell cycle for final differentiation and maintenance of the muscle phenotype (L'Honore et al., 2007). Due to genetic redundancy, absolute determination of Pitx3 function in this tissue is difficult and requires more attention.

**Unexplained Pitx3 Phenotypes/Observations**

The research in this thesis is focussed around finding novel roles for pitx3 in the development of Xenopus laevis. Some of the expression patterns of pitx3 have not been explored and certain morphant phenotypes have not been explained, suggesting there are functions of this transcription factor that remain unknown.
It was observed in our lab that embryos injected with \textit{pith3}-morpholino developed a curved spine or a bent dorsal axis (Figures 5B, C, D, F). This was a common theme in zebrafish morphants as well (Shi et al., 2005). Embryos injected with the control-morpholino developed a normal, straight anterior-posterior body axis (Figure 5E). This suggests that \textit{pith3} knockdown is causing a perturbation in the somites, causing them to develop irregularly, such that uniform morphogenesis between left and right sides of the embryo is not achieved (explored in Chapter 2). In addition to the curved body axis, morphant embryos exhibited a twitching movement by tailbud stages, versus the movements that their wild-type siblings were displaying (data not shown). This indicates that the muscles of the trunk of the embryo are not able to work together to create sigmoidal movement required for proper swimming.

One of the tissues that has not been looked at for \textit{pith3} function is the lateral plate mesoderm (LPM), which has been reported to have a modest expression of \textit{pith3} symmetrically on both left and right sides of the embryo (Pommereit et al., 2001; Khosrowshahian et al., 2005). This differs from the expression of paralog \textit{pith1} in the posterior LPM (Lanctot et al., 1997) and the left-specific expression of paralog \textit{pith2c} (Schweickert et al., 2000). The lateral plate mesoderm has shown to be important for patterning the left-right body axis that is necessary for proper internal organ \textit{situs} (Levin, 2005). Asymmetric \textit{pith3} expression was reported in the heart, stomach, and the second coil of the intestine (Khosrowshahian et al., 2005). To date, no role for \textit{pith3} in these tissues has been established. Our lab has also observed laterality phenotypes in \textit{Xenopus} morphant embryos, pertaining to cardiac (Figure 5I and J) and gut malrotations (explored in Chapters 2 and 3).
In addition, _pith3_ expression is detectable at stage 8 of development by RT-PCR however the earliest that transcripts can be visualized via _in situ_ hybridization is at stage 17 (Khosrowshahian et al., 2005). The function of this transcription factor arising concomitantly with the mid-blastula transition suggests an early role for this gene in patterning the pre-gastrulation embryo (explored in Chapters 3 and 4).

It is the intention of this research to explore a possible early patterning role, find novel downstream target genes of _pith3_ and assess new developmental pathways that _pith3_ may be regulating during embryogenesis. The possibilities for roles of this transcription factor has been made apparent by a microarray experiment conducted in our lab, that has not only identified novel genes that have yet to be characterized in _Xenopus_ [GenBank JN975639 (xGalectin IX) and JN975638 (xBaz2b)], but also unique genetic cascades that are perturbed in the absence of _pith3_ (Chapter 4). We have established a unique reporter assay that allows us to view how _pith3_ operates on different promoters and in different cell lines, with respect to cooperativity (Chapter 5). Through the use of this assay, we have detected new direct target genes for _pith3_, which strengthen its role in the development of organs unexplored thus far.
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CHAPTER II
THE XENOPUS HOMEBOX GENE PITX3 IMPINGES UPON SOMITOGENESIS AND LATERALITY

Cristine Smoczer*, Lara Hooker*, Sarah Brode, Marian Wolanski, Farhad Khosrowshahian, Michael Crawford

*Equal first authors
1 Author for Correspondence

Introduction

During a study on the role of Pitx3 in Xenopus lens development we noticed several other defects that indicated diverse roles for the gene, particularly during the segmentation of paraxial mesoderm and the development of organ asymmetries (Khosrowshahian et al. 2005). The Pitx genes encode paired-like/K50 homeodomain proteins, and three members of the Pitx family (Pitx1, Pitx2, and Pitx3) have been cloned in vertebrates. Pitx1 plays an important role in the development of the pituitary gland, lower mandible, and hindlimb (Lamonerie et al. 1996; Szeto et al. 1996; Lanctot et al. 1997; Tremblay et al. 1998; Hollemann and Pieler 1999; Lanctot et al. 1999; Logan and Tabin 1999; Szeto et al. 1999; Chang et al. 2001). Similarly, Pitx2 plays a role in the development of pituitary, eye, dentition and the maxilla, however, it also regulates the establishment of left-right asymmetry during development (Semina et al. 1996; Gage and Camper 1997; Logan et al. 1998; Yoshioka et al. 1998; Campione et al. 1999; Lin et al. 1999; Essner et al. 2000; Schweickert et al. 2000; Campione et al. 2001).

In mice, Pitx3 is unique in the family for not expressing in the mammalian Rathke’s pouch or in pituitary adenomas. It is expressed primarily in mesencephalic dopaminergic neurons of midbrain, in somites, lens placode, and forming lens pit
In mice, \textit{Pitx3} has been identified as the causative locus for \textit{aphakia}, a recessive deletion mutation resulting in small eyes that lack lenses, however no vertebral anomalies arise despite its expression during normal somitogenesis (Semina et al. 1998). In humans mutation of \textit{Pitx3} has been tied solely to \textit{substantia nigra} deficits, autosomal dominant mesenchymal dygenesis, and congenital cataracts (Semina et al. 1998; van den Munckhof et al. 2003). During myogenesis, both \textit{Pitx2} and \textit{Pitx3} participate in the differentiation of skeletal muscles (Coulon et al. 2007; L'Honore et al. 2007). In frog, \textit{Pitx3} expresses in lens, lateral plate mesoderm, differentiating somites, craniofacial regions, and in looping heart and gut (Pommereit et al. 2001; Khosrowshahian et al. 2005).

When we manipulated \textit{Pitx3} expression in frog embryos (Khosrowshahian et al. 2005) we frequently observed craniofacial, and midline phenotypes reminiscent of \textit{Shh} mutants (Ahlgren and Bronner-Fraser 1999), as well as impaired midline integrity and/or laterality (Chiang et al. 1996; Essner et al. 2000; Dubourg et al. 2004). In addition, \textit{Pitx3} and morpholino (\textit{PitxMO}) injected embryos frequently exhibited a bent dorsal axis – embryos reflect inwards on the side of injection and often develop spinal kinks by the time somites had differentiated. Severely kinked embryos die by the time cardiac looping should have completed.

The mechanisms underlying these additional \textit{Pitx3} defects are unknown, and are not seen in the human and mouse mutants. Indeed irrespective of whether the whole coding region or just the homeodomain is disrupted, \textit{Pitx3} null mutant mice are both fertile and superficially appear morphologically normal except for the eye defects (Zhao et al. 2004; L'Honore et al. 2007). Why would \textit{Xenopus} present a different phenotype?
We used a panel of probes, some of which we had archived from a subtractive cloning project, to monitor the changes that result as a consequence of Pitx3 mis-expression. Our hope was to distinguish whether in Xenopus, Pitx3 uniquely impedes the evolutionarily conserved molecular clock mechanism that underlies segmentation, or if the later phase of pre-somitic rotational behaviour is affected. We identified markers of somitogenesis by subtractive cloning, namely desmin, creatine kinase, and a troponin C variant, each of which undergo modified expression during somitogenesis as a consequence of Pitx3 mis-regulation. This modification of gene activity is preceded by anomalies in pre-somitic rotation and organization in Pitx3-expressing pre-somitic mesoderm, however the early molecular signaling steps necessary to initiate the segmentation clock appear to function relatively normally.

Results

Unilateral injection at the two cell stage using in vitro transcribed Pitx3 RNA, repressor chimeras lineage tagged with GFP, or Pitx3 antisense morpholinos, causes embryos to undergo abnormal dorsal axis formation: embryos curve inwards on the side of injection. Phenotypes vary depending upon morpholino and mRNA concentration, and upon the degree of dispersion and longevity of the reagent in the injected embryos. Optimal concentrations for generating phenotypes using morpholinos or RNA were obtained in a previous study (Khosrowshahian et al. 2005). For example, cardiac and gut laterality deficits required substantially more injected mRNA to generate an effect than needed to reliably produce the bent axis phenotype (300 versus 100 pg). This likely reflects the longer developmental time and the attrition of RNA by degradation between
the early somite versus later cardiac stages. In those studies where long cultivation was required solely in order to study somite differentiation, we elected to minimize cardiac deficits (and later stage lethality) and to inject RNA at a lower dose.

Hypothetically, injection could cause a small degree of cytoplasmic leakage resulting in a slightly smaller volume of blastomeres being available to contribute to the embryo on the injected side. This population anomaly might persist and later engender impediments to normal morphological modeling either through a reduction in blastomere number, or by alterations in the bilateral timing of the mid-blastular transition due to altered nuclear:cytoplasmic ratios. Moreover, the action of RNA species injected might not be specifically attributable to Pitx3, but rather the result of ectopic expression and mimicry of other Pitx gene family members, or even of other paired-like relatives.

Having previously established parameters for the use and specificity of a Pitx3 antisense morpholino (Pitx3MO) (Khosrowshahian et al. 2005), we injected embryos at the two cell stage so that one side was Pitx3-impaired and the other normal. Experiments were further controlled through deployment of a second Pitx3 morpholino (no difference in effect discernable compared to the first), and a mis-match control. Injection of antisense morpholino results in curvature of the dorsal axis so that the injected side is convex relative to control side. This occurred more frequently in Pitx3MO than in control injected embryos (Table 1; Figs 1, 2, 3). Moreover, when the progression of somitogenesis was monitored using morphological or molecular markers, only Pitx3, Pitx3-engrailed, or either of the two Pitx3MO injected embryos underwent anomalous segmentation and patterning: control injected embryos underwent normal and bilaterally symmetrical somitogenesis (compare Fig 1a - control injected with 1b - Pitx3MO
injected, and Fig 3). The effects of *Pitx3* perturbation by means of morpholino mediated knockdown were rescue-able by co-injection with *Pitx3* mRNA. The consequence of this early perturbation was irregular axis formation both in the dorso-ventral, but particularly in the lateral planes (Fig 1b, c, e). Somitogenesis was perturbed irrespective of whether *Pitx3* mRNA or *Pitx3MO* was injected unilaterally into the left or right blastomere at the two cell stage (Table 1). Experiments were repeated a minimum of three times, although for the controls, several more repetitions were employed to garner a larger sample size. Given the similar effects elicited by either morpholino or mRNA, it is perhaps surprising that the two together nullify to some extent.

*Pitx3MO* injected embryos displayed movement disorders. While the severely curved embryos would no doubt be mechanically inhibited from swimming normally, even mild phenotype embryos responded to startle by twitching spasmodically – swimming movements were not sigmoidal.

In addition to dorsal axis patterning anomalies, injection of *Pitx3* transcript or *Pitx3* morpholino had effects upon the patterning of left/right asymmetrical organs. These anomalies were induced if injections were made at either the 1 or 2 blastomere stages of development. If injections were performed unilaterally at the 2 cell stage, both treatments had the potential to randomize *situs* irrespective of the side of injection. Incomplete inversion often occurred, and this was manifest in the abnormal morphologies that were the consequence of abnormal cardiac and gut looping (Table 2).
**Figure 1: Effect of morpholinos on dorsal axis differentiation.** Embryos injected unilaterally on the left at the two-cell stage with control morpholino (Cont MO) develop normally (a) while those injected with Pitx3 MO reflex inward on the side of injection (b). Late into somitogenesis, Pitx3MO injected embryos exhibit abnormal lateral curvature (compare c to d) as well as dorso-ventral kinks (compare e with d). White line demarcates left from right sides of the embryos.
Table 1: Effect of unilateral injection of morpholino upon dorsal axis patterning.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cont MO (20ng)</th>
<th>Pitx3 MO (20ng)</th>
<th>Pitx3 MO (15ng)</th>
<th>Pitx3 MO (11.5 ng) + GFP RNA (100 pg)</th>
<th>Pitx3 MO (11.5 ng) + Pitx3 RNA (100 pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bent Axis</td>
<td>19% (n=143)</td>
<td>76% (n=186)</td>
<td>38% (n=51)</td>
<td>57% (n=33)</td>
<td>26% (n=32)</td>
</tr>
<tr>
<td>Suppressed Rotation/Depressed Somitogenesis</td>
<td>0%</td>
<td>100%</td>
<td>100%</td>
<td>85%</td>
<td>59%</td>
</tr>
</tbody>
</table>

Table 2: Effects of ectopic Pitx3 expression/knockdown on the patterning of asymmetrical organs. Percentages in brackets represent the subset of organs that, although inverted, are otherwise normally patterned. The compound nature of the phenotypes means that the different categories of anomalies can sum to more than 100% if a single embryo is affected in more than one organ system.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Injected Transcript (Injected at 2 cell stage)</th>
<th>Injected Morpholino (Injected at 1 cell stage)</th>
<th>Control (18ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pitx3 left side (300pg)</td>
<td>Pitx3 right side (300pg)</td>
<td></td>
</tr>
<tr>
<td>Aberrant Heart Looping</td>
<td>31% (15%)</td>
<td>22% (7%)</td>
<td>47% (22%)</td>
</tr>
<tr>
<td>Aberrant Gut Looping</td>
<td>38% (18%)</td>
<td>31% (14%)</td>
<td>71% (3%)</td>
</tr>
<tr>
<td>Complete Situs inversus (all visceral organs inverted)</td>
<td>12%</td>
<td>6%</td>
<td>12%</td>
</tr>
</tbody>
</table>

\( n \) | 206 | 217 | 80 | 186 | 51 | 120 |
Three of the *Xenopus* subtraction clones isolated had been similarly identified in a zebrafish study as early markers of myogenic lineages in somites, namely *desmin, fast skeletal troponin C, and creatine kinase*, (Xu et al. 2000). Desmin is a very early marker of the myotome and serves to couple one somite to the next (Cary and Klymkowsky 1994). Although inhibition of desmin impedes myoblast fusion (Li et al. 1994), it does not appear to impede the early stage of somitogenesis (Cary and Klymkowsky 1995). Both *creatine kinase* and *troponin C* express slightly later during somitogenesis. In embryos that were unilaterally injected with control morpholino at the 2-cell stage, both left and right sides of the embryos demonstrate equivalent expression of *desmin* (Fig 2a), and identical results were seen for *troponin C* and *creatine kinase* control injected embryos. Ectopic over-expression or inhibition of *Pitx3* activity appears to have roughly similar effects: when either *Pitx3* transcript or *PitxMO* are injected, expression of all three myogenic marker genes are inhibited, although generally speaking, antisense morpholino has more severe inhibitory effects (Figs 2b-g). This pattern of unilateral inhibition persists through to stages in the mid 30s. Whether or not the resultant somite perturbation evident at stages 22-32 would eventually lead to vertebral column dysgenesis could not be reliably determined: attendant laterality defects precluded survival to stages past cardiogenesis. Where the laterality phenotypes were mild, and embryos survived to feeding stage, the spinal column posterior to the abdomen was kinked.
Figure 2: Effect of Pitx3 perturbation upon myogenic/somite markers. Pitx3 ectopic mRNA expression as well as Pitx3 MO inhibit expression of the early myogenic marker desmin (compare a to b, c), as well as the fast and slow skeletal markers troponin C (d, e), and creatine kinase (f, g). Dorsal view of left injected whole embryos with head oriented to the top. All embryos have been unilaterally injected on the left side.
**Figure 3: Effect of Pitx3 perturbation upon somite formation.** *Pitx3* ectopic mRNA expression as well as *Pitx3 MO* inhibit the normal assembly of cells into somites indicated in Hoechst-stained coronal sections. Compare controls to left injected *Pitx3* mRNA and *Pitx3 MO* embryos (compare a to b). In coronal sections (top is rostral), both treatments appear to impair the organized rotation of pre-somitic mesodermal cohorts on the left injected side (c, d). The poor organization of somites into aligned and rotating cellular cohorts is evident at higher magnification (e) where nuclei are stained with DAPI (blue) and inter somitic borders are indicated by b1-integrin staining (red).
Not only is expression of somitic and myogenic markers diminished, but somite organization is impaired: in Hoechst labeled longitudinal coronal sections, somitogenesis is both retarded and out of registry on the injected relative to the contralateral control side (compare control Fig 3a to b, c, d). By the time somites have formed discrete bodies on the control side, disorganization of intersomitic adhesion and somites is severe on the morphant side (Fig 3e). The same effect is elicited by injection of Pitx3-engrailed repressor mRNA (not shown). Instead of organizing into a smoothly rotating cohort with elongating nuclei, pre-somitic cells instead seem to aggregate slowly and clumsily, and their nuclei remain small and fail to elongate. Counts of nuclei in laterally matched somite-forming regions indicate that there is no statistically significant difference between experimental and control sides at the axial level at which presomitic mesoderm begins to rotate (Fig 4). Apoptosis is not the cause of retarded somitogenesis.

Pitx3 is expressed just prior to gastrulation – the image in Fig 5a illustrates light staining in stage 10 and 12 embryos, and little background staining in a stage 35 embryo processed in the same vial. Although mesodermal and somitic expression of Pitx3 is not superficially evident during the early stages of somitogenesis, nevertheless Pitx3 is visible in cleared whole mounts (Fig 5b). This agrees with RT-PCR data which reveals expression of Pitx3 as early as stage 8, and that substantially increases by stage 18 and into somitogenesis (Khosrowshahian et al. 2005). In cleared specimens, somite expression gradually wanes until stage 31 whereupon it almost immediately re-expresses at higher levels coincident with the myogenic program, and commencing at the anterior end. Both Pitx2 as well as Sonic hedgehog (Shh) expression are perturbed by Pitx3 misregulation, and in the case of Shh, both Pitx3-engrailed repressor as well as antisense
morpholino injection have a similar inhibitory effect (Figs 5 c, d, e). Genes in the upstream portion of the segmentation clock appear to express in a normally arrayed and periodic fashion, although in extreme cases, the downstream effectors can be diminished in intensity or even abolished (Figs 6a, b).

**Figure 4:** Graph comparing pre- and post somitic nuclear counts on either side of unilaterally injected *Pitx3 MO* embryos. Axially paired counts were made using coronally sectioned embryos stained with the nuclear stain Hoechst. There is no significant difference in cell number between control and *Pitx3 MO* injected sides for pre-somitic (rotating) nor for post-rotation perpendicular arrays of somite cells. The sample size for each treatment was 10 specimens.

Perturbation of *Pitx3* activity in both embryos and tissue culture suggests that *Pitx3* plays a role in mediating cytoskeletal architecture (Fig 3 e and 6c, d, e). *Pitx3MO* appears to inhibit the normal morphological progression of lens fiber differentiation, and this appears to have its roots early since cells fail to enter into the lumen post-vesiculation, a step coincident with primary lens fiber elongation (Zelenka, 2007). Over-expression in tissue culture causes changes in cell shape – cells are retracted and more compact relative
to un-transfected peers, and they appear poorly equipped to form intercellular contacts (Fig 6 d, e).

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Figure 5: Pitx3 is detectable by *in situ* hybridization in early stages commencing just before gastrulation and through stages 10 and 12 (a). These four embryos were processed in the same vial, and the specificity of staining in the stage 35 embryo (lens and somites) as well as low background serves to indicate the legitimacy of staining at the earlier stages. Pitx3 expression is expressed in pre-somitic mesoderm and transiently in somites (b). Its activity affects both Pitx2 as well as Sonic hedgehog. Pitx2 normally expresses in paired arrays along the dorsal axis, however this is abolished by Pitx3 knockdown on the injected side (c). A section through Pitx2 expressing somites is provided in the insert. Pitx3-ENGRAILED repressor mRNA as well as Pitx3MO inhibit Sonic hedgehog on the injected side (d, e).
Figure 6: *Pitx3MO* has mixed effects upon the segmentation clock and alters patterns of tissue differentiation. The characteristic periodic expression patterns of the upstream element of the clock (*Delta2*) are unaffected, while the downstream effector (*Hairy2b/Hes4*) was reduced or abolished by *Pitx3MO*. (a, b). Lens vesicle formation is similarly impaired on the left side compared to the control right (c) in lens, β-tubulin is red, blue is Hoechst. Transfected HEK293 cells (arrows) acquire a normal morphology when they express GFP alone, but cells co-expressing GFP and Pitx3 are less stellate and tend to form fewer and smaller intercellular junctions (compare d with e). Actin filaments are red, and nuclei stained with Hoechst are blue.
Discussion

In vertebrates, segmentation of the presomitic paraxial mesoderm is the first overt step in the generation of vertebrae. Generally, it occurs in an anterior to posterior direction as two long bars of mesoderm on either side of the notochord and neural tube synchronously pinch off to form pairs of epithelialized balls called somites. As the somites mature, they lose their epithelial morphology and differentiate into three distinct populations: sclerotome, myotome, and dermatome which migrate and re-segment to contribute respectively to the vertebra and proximal ribs, the skeletal muscle precursors, and dorsal dermis and skeletal muscle (Brent and Tabin 2002). There are qualitative differences in the somitogenic process in the trunk versus the tail (Cunningham et al. 2011).

In *Xenopus laevis*, somitogenesis proceeds over a long time in developmental terms – somites segment as matched pairs from paraxial mesoderm from stage 19 to 42 – one pair emerges approximately every 45 minutes. Interestingly, rotation of pre-somitic cellular cohorts by means of cell elongation and bending to form somites is slower during early compared to late somitogenesis (Afonin et al. 2006). Somitogenesis in *Xenopus* is different from amniotes in substantial ways. There is little in the way of an obvious dermatome – this is present as a separate sheet of cells lying between the myotome and dermis (Hamilton 1969). The somites don’t ball up and pinch off as with chicks and mammals, but rather, from a long file of cells along the dorsal axis, cohorts of approximately ten cells undergo coordinated rotation on either side of the neural tube - nuclei that were formerly aligned along the dorsal axis are broken into smaller groups that become perpendicularly arrayed (Hamilton 1969). Each rotating cellular cohort
defines a somite pair with a somite forming on either side of the dorsal axis. Another difference resides in the myotome: it comprises the dominant component of *Xenopus* somites until the tailbud stage (Newman et al. 1997).

The conserved cues that drive segmentation are thought to involve the rostral to caudal progression of a wave front of intersecting and anti-parallel gradients that render presomitic mesoderm competent to respond to a molecular oscillator. Anterior expression of a *retinaldehyde dehydrogenase* (like *Raldh2*) builds a gradient of retinoic acid synthesis that is anti-parallel to the regressing and posterior dominance of *FGF* and *Wnt* – the gradients intersect at a threshold called determination wave front (reviewed by Pourquie, 2011; Dubrulle and Pourquie 2004). Cyclically expressed members of the *Notch/Delta*, *Wnt*, and *FGF* pathways induce segmentation behaviour in cells at the determination wave front as it moves caudally. Retinoic acid plays a role in generating the wave front, but it also buffers the symmetrically emerging somite pairs from the asymmetrical cues necessary to organ asymmetry (Pourquie, 2011). When we manipulate *Pitx3* expression in frog embryos we frequently notice somite and laterality defects.

Embryos with a mild phenotype (straight axis) also manifest spastic behaviour when stimulated to a startle reflex. Interestingly, disruption of *Pitx3* activity in humans sometimes leads to movement disorders and spasticity, presumably reflecting abnormal patterns of neuronal differentiation that extend beyond the *substantia nigra* (Bidinost et al. 2006).

**Is pre-somitic recruitment and rotation affected by *Pitx3* mis-regulation?**

In cleared Hoechst stained whole mounts as well as in sectioned material, it is apparent that although pre-somitic mesoderm aligns parallel to the dorsal axis, cells
experiencing Pitx3 mis-regulation do not rotate normally in cohorts. Non-specific morpholino effects (Robu et al. 2007) are unlikely to be the cause: first, the same effects are elicited by two different Pitx3 morpholinos but not by mis-match nor general controls; second, the same phenotype is elicited in specific manner by Pitx3-engrailed repressor as well as by Pitx3 mRNA. Moreover, cohorts of the correct cell number are both recruited and attempt to rotate perpendicular to the dorsal axis, even though subsequent differentiation is adversely affected. In the milder phenotypes that survive past cardiac development, deficits are posterior to the trunk. In those embryos where the phenotype is anterior and profound, somites do not form normally, and the resulting structures show poor definition and integration. Given the consistency of population numbers, neither apoptosis nor altered rates of cell division seem likely to be acting to retard somite differentiation. These effects are manifested during a phase of development when Pitx3 is transiently expressing in both pre-somitic mesoderm and in new somites where it gradually fades as they differentiate. By the end of stage 25, Pitx3 is hard to detect, and by stage 31, expression in somites has disappeared until it resurges again in the mid-30s (Khosrowshahian et al. 2005), presumably as part of the myogenic program (L'Honore et al. 2007).

Ectopic mRNA or dominant negative constructs might exert non-specific and ectopic effects (competing for Pitx2 response elements for example), however it is hard to imagine how the highly specific Pitx3 morphants could elicit similar results. We propose two explanations. First, analogous to the clock/wavefront model for somitogenesis, exquisitely regulated and transient expression of Pitx3 might be required for pre-somitic mesoderm to remodel to form somites. If the timing and pattern of
somitogenesis is dose-sensitive, then either protracted elevation or depletion of transcript by ectopic agents would obscure necessary differentiation cues. Certainly, Pitx3 has markedly different effects upon the tyrosine hydroxylase promoter in different cell lines – it can either activate or repress the reporter (Messmer et al. 2007). Perhaps some of these regulatory differences are attributable not merely to the presence or absence of transcriptional co-factors, but to levels of Pitx3 relative to partners in these different contexts. A sensitivity to multiple thresholds is not without precedent: dpp mediates three different threshold-dependent responses upon the target gene C15 that are mediated by cumulative and combinatorial effects of its activating and repressing partners (Lin et al. 2006).

The second possibility relates to the ability of the Pitx2 isoforms to heterodimerize (Cox et al. 2002; Saadi et al. 2003; Lamba et al. 2008). Perhaps Pitx3 and Pitx2 isoforms form heterodimers that are necessary for somite differentiation - when Pitx3 is either too scarce or superabundant, the regulation of targets that require heterodimers are impaired.

**Does Pitx3 mis-regulation perturb the segmentation clock?**

We assessed a broad panel of segmentation genes, and found that while there are Pitx3MO-induced changes to the size of some expression domains, nevertheless the placement and periodicity of segmentation signals remains intact for the primary patterning genes such as Delta2. For at least one of the induced downstream players, Hairy2b/Hes4, expression was blurred and often obliterated. Recently, in a microarray study, we have also identified a second gene, Hes7 as well as confirmed that Hairy2b/Hes4 are perturbed by Pitx3 mis-expression (Hooker et al. 2012): the two Hes-related genes perform in the Notch/Delta pathway. Therefore it seems unlikely that Pitx3
affects the initiators of the conserved segmentation clock in frog, but that they may disrupt the effectors necessary to segmentation. In our experiments, Pitx3 mis-regulation in frog results in changes to the expression of Sonic hedgehog – a gene that has been implicated in modulation of both laterality and the segmentation clock (van den Eeden et al. 1998; Tsukui et al. 1999; Christ et al. 2000; Dubrulle et al. 2001; Roessler and Muenke 2001).

**Post-segmentation differentiation of somites**

Once somites have formed, Pitx3 is activated by myogenic bHLH proteins, and in turn it likely activates some of their number too (Coulon et al. 2007; L'Honore et al. 2007). Myogenesis and muscle patterning, as well as laterality, appear to proceed normally, and this has been ascribed to a compensatory increase of Pitx2 expression (L'Honore et al. 2007). In this respect, *Xenopus* somitogenesis is distinct: not only does Pitx3 mis-regulation result in anomalous development, morpholino mediated translational knockdown is not compensated by increased Pitx2 activity. Indeed in frogs, Pitx3 appears to be necessary for Pitx2 expression in early stage somites since Pitx3 morphants demonstrate abolition of Pitx2 in somites on the injected side: this particular regulatory link must behave differently than in mice.

In embryos where desmin, TnnC, or creatine kinase have been knocked down, levels remain persistently low up beyond stages in the late 20s. All three of these genes serve as markers of later somite differentiation (well past segmentation). Desmin, an early myogenic marker, is suppressed by both Pitx3MO and Pitx3 mRNA. This suppression alone, however, is unlikely to be the cause of early somite perturbation: early stage somites are normal looking in desmin null mutant mice (Li et al. 1994), and
although interference with the transcript in frogs impedes later stage myogenesis and inter-somitic adhesion, anomalous rotation of somites has not been reported (Cary and Klymkowsky 1995). Whatever the impact of these marker genes upon myogenesis, the effects on somitogenesis preceded their expression. In this context, it is interesting to note that the effects of Pitx3 knockdown are more severe than Pitx3 ectopic expression when assessed by creatine kinase expression. Presumably, Pitx3 is playing a role not merely in the inhibition of normal segmentation, but also in regulation of the myogenic differentiation that subsequently occurs (compare Fig 2f and g).

Some “recovery” of somite segmentation appears possible: in our experiments, stage 27 somitic nuclei are grouped, but inter-cellular adhesion is impaired such that individual somites do not form monolithic aggregates, but display aberrant clefts, cellular mis-alignments, and inter-compartmental bridges of tissue. Often, there is no clear delineation of somites whatsoever. The result of this disorganization is curvature of the dorsal axis – a phenotype previously reported for Pitx3 morphant zebrafish (Shi et al. 2005). β1-integrin stained specimens demonstrate many attributes of normal somites, but on the whole, lack normal organization: the adherent complexes that normally form between somites are either absent or lack focus. Similarly, lens vesicles are disorganized on the morphant side, but in contrast to affected somites, demonstrate cavities that appear to be the remnants of cells, as well an unusual distribution of the remaining cells that is suggestive of aberrant cell sorting and cell shape changes. For example, the coincident elongation and migration of primary lens fibers into the lumen does not take place. Previous studies on Pitx3 morphants in zebrafish demonstrated an identical lens phenotype (Shi et al. 2005).
We wondered if these effects were induced or cell autonomous. We turned to tissue culture (HEK293 cells) to see if ectopically expressed Pitx3 affected morphology. HEK293 cells were used because they are serve as a mesoderm model, and because preliminary experiments suggested that partners necessary to Pitx function were present (data not shown). Transfected cells are less stellate and appear to form fewer and smaller junctions with their counterparts. This phenomenon resonates with the frayed appearance of affected myotome in embryos. Apparently Pitx3 normally modulates either cytoskeletal architecture, cell-cell, or cell-substrate adhesion: mis-regulation appears to change behaviour sufficiently that presomitic mesoderm cannot rotate in an organized fashion. Interestingly, Shroom3, a mediator of cytoskeletal remodeling, is activated by Pitx1, 2, and 3 (Chung et al. 2010), so we might speculate that Pitx3 perturbation effects are mediated by one of the growing family of Shroom genes.

What causes the Xenopus laterality defects?

Pitx3 can have either activating or repressive effects upon target genes in a context-specific manner (Messmer et al. 2007). In the context of these experiments, injection of Pitx3MO or of Pitx3-engrailed mRNA have similar effects upon Sonic hedgehog expression, suggesting that one role for Pitx3 is to activate the pathway for this gene. Shh plays a relatively upstream role in the cascade of signals that direct laterality and midline integrity in several organisms (Casey and Hackett 2000), so the abrogation of early Shh expression by Pitx3 mis-expression could have elicited the observed laterality defects. Cyclopia and laterality defects can be elicited by mutation of several different genes, but a unifying theme for several appears to be that they impair midline integrity (Chiang et al. 1996; Ahlgren and Bronner-Fraser 1999; Essner et al. 2000; Dubourg et al. 2004). In our
embryos, elicited phenotypes include heterotaxia in addition to eye defects – the effect upon Shh suggests a role in midline integrity. Given the catastrophic effects of Pitx3MO upon somite formation, it is easy to imagine that it might also undermine integrity of the midline and render it leaky to asymmetric cues. Sonic hedgehog signaling is multifaceted though – perturbation of its pathway also retards the segmentation clock in chicks (Resende et al. 2010), albeit only to a recoverable degree. Finally, laterality deficits in morphants could indicate that symmetrically expressed Pitx3 protein modulates activity of asymmetrically expressed Pitx2 in lateral plate mesoderm, perhaps by means of heterodimerization.

We have recently completed a microarray-based screen to identify possible downstream targets of Pitx3 in both the segmentation and laterality pathways (Hooker et al. 2012). The results confirm a role for the gene in mediating both the retinoid as well as segmentation clock/wave front pathways. We are presently working to characterize the promoters of candidate target genes.

Materials and Methods

Subtractive Cloning

The subtractive cloning was undertaken in an earlier project to identify eye-specific genes. Uncharacterized clones were archived and resurrected to serve as probes in this project. Briefly, RNA samples were derived from RNA pooled from stage 14, 20, 27, and 32 embryos. Embryos had been injected at the 1 cell stage with either Pitx1 or Pitx3, and all were co-injected with GFP which served as a marker for successful injection and distribution of transcript. RNA was purified using RNAwiz (Ambion/ Life Technologies
Inc. Burlington, Canada), and poly-adenylated RNA was isolated from the aqueous fraction using columns (Ambion, Poly(A)Purist). RNA was reverse transcribed and the cDNA library was constructed according to manufacturer’s instructions (Clontech, Mountain View, USA: PCR-Select). To confirm the legitimacy of the candidate clones, dot blots were performed and successively probed with radio-labeled cDNA derived from one or other of the original stocks of pooled RNAs (*Pitx1* vs *Pitx3* injected embryos). Clones that demonstrated different hybridization profiles were submitted for sequencing. Selected clones of interest were then tested by in situ hybridization to confirm that they did indeed undergo differential expression following mis-regulation of *Pitx3*.

**Embryos**

Embryos were staged, fertilized, dejellied in 2% cysteine and cultured as previously described (Nieuwkoop and Faber, 1967; Drysdale and Elinson, 1991). Animals were reared and used in accordance with University, Provincial, and Federal regulations.

**Microinjection**

Synthetic capped mRNA of *Pitx3*, *Pitx3*-engrailed repressor (Khosrowshahian et al. 2005), and/or *Green Fluorescent Protein* (GFP) transcript was made from linearized template using mMessage Machine (Ambion, Life Technologies Inc. Burlington, Canada) driven by a SP6 promoter. Capped mRNA or morpholino was resuspended in water and injected into embryos with a Drummond nanoinjector (Drummond Scientific Co., Broomall, USA). Injections were made into the animal pole of embryos at either the 1-cell or 2-cell stages. Concentrations of the capped mRNA injected ranged from 60 pg to 1.2 ng. Injection volumes never exceeded 9.2 nl. Injected embryos were cultured in 0.3 X MBS (1X Modified Barth’s Saline: 88 mM NaCl; 1 mM KCl; 1 mM MgSO4; 5mM
HEPES pH 7.8; 2.5mM NaHCO$_3$; 0.7mM CaCl$_2$) and 2% Ficoll-400 (Sigma-Aldrich Canada Ltd., Oakville, Canada) at 12 °C for at least 1 hr to allow healing before being removed and allowed to develop at room temperature. At this point the solution was changed to 0.1 X MBS. When injected embryos were intended for comparisons of one treatment to a control, the embryos were injected in one blastomere at the 2-cell stage with the transcript of interest and GFP marker for identification and separation later. The contra-lateral side served as a control. For translation knockdown assays, a previously characterized and specific Pitx3 antisense morpholino oligonucleotide sequence was employed and in addition, a second morpholino was designed to confirm specificity as well as a mis-match control (Khosrowshahian et al. 2005). Morpholinos employed were: Pitx3 specific- TGGGCTAATCCTGGTTGAAGGGAAT and CCTCTATTTGTAAATCCTTCCTGC; mis-match control CCaCaATTTcTTAAATCCTTCgTC; and general morpholino control CCTCTTACCTCAGTTACAATTTATA (Gene Tools LLC, Philomath, USA).

**Whole-mount in situ hybridization and sectioning**

*In situ* hybridizations were performed according to established protocols (Harland 1991) using digoxygenin labeled riboprobes. Delta2 and Hairy2b/Hes4 were kind gifts of Dr. T. Kinoshita and the NIBB respectively. Hybridizations were conducted at high stringency (65° C). After photography, whole mount specimens were embedded either in 5% agarose or paraffin, and then sectioned either at 30 um using a vibratome (Leica VT 1000s, Leica Microsystems, Oakville, Canada), or at 10 um using a manual rotary microtome (American Optical Co. 820 Spenser).

**Hoechst Stain**
Hoechst 33258 (bis benzamide) dissolved in methanol (5μg/ml) was employed to
stain specimens of embryos either after fixation or following riboprobe in situ
hybridization. After sectioning, nuclei were visualized under filtered UV light and
photographed.

**Embryo and Immunocytochemistry**

Whole embryos were fixed in 4% MEMPFA overnight at 4°C and incubated with
mouse β1-integrin antibody (Drs. P Hausen and V. Gawantka - 8C8 diluted 1:400,
Developmental Studies Hybridoma Bank, Iowa City, USA). After extensive washing, the
whole embryo preparations were stained with a secondary antibody, namely anti-mouse
Cy3 conjugate (Sigma C-2181, 1:200). Nuclei were stained with Hoechst 33285 (1:1000).
Embryos were imaged as whole-mounts or subsequent to paraffin-embedding and
sectioning at 14μm thickness. The images were captured on a Zeiss Axioskope
fluorescent microscope using Northern Eclipse software (Empix, Mississauga Canada).
Sections through lens were developed from the same specimens, but were stained with
anti mouse β-tubulin as primary (Dr. M. Klymkowsky - antibody E7 diluted 1:200;
Developmental Studies Hybridoma Bank) followed by anti-mouse Cy3 conjugate (diluted
1:200, Sigma C-2181). Nuclei were stained with Hoechst (diluted 1:1000, Sigma H-
33258).

**Tissue Culture Immunocytochemistry**

HEK 293 cells were grown on glass coverslips in 60mm dishes, in Dulbecco’s
modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 100
units/ml Penicillin, 100 μg/ml Streptomycin and 2.5μg/ml Amphotericin B at 37°C in a
humidified 5% CO2 incubator. HEK293 cells were transfected either with pCINeo/RES-
GFP vector (kind gift of Dr. Jan Eggermont, from University of Leiwen, Belgium) or pCINeo/ xPitx3-IRES-GFP vector using the polyethylenimine method. Shortly before transfection, cells were transferred to serum-free and antibiotic-free medium. The PEI-DNA complexes were prepared by diluting 6.5ug of plasmid DNA in 250ul serum-free DMEM and adding 12.5ul PEI. The mixture was incubated for 20 min at room temperature prior to adding to the cells. Four hours later the serum-free medium was replaced with complete medium with antibiotics and cells were incubated for an additional 48 hours. Post-transfection, HEK293 cells were fixed with 3.7% PFA: cells were stained for actin filaments (30min) with Phalloidin Alexa 647 (A22287 diluted 5:200; Molecular Probes, Life Technologies Inc. Burlington, Canada). Nuclei were stained with Hoechst 33285 (1:1000) followed by mounting of the cover slips for fluorescence microscopy analysis.

**Cell Counts in Somites**

In the segmentation zone, boxes were superimposed over images of Hoechst stained longitudinal coronal sections of newly emerging somites. The box borders were centred between somites and they were registered to enclose two of them. Once control nuclei were counted, the boxes were then moved to cover the contralateral region on the injected side of the embryo to delimit an equivalent area of counting for the injected side. The number of nuclei per section was averaged for areas spanning two somite-equitants on either side of section in both pre- and post-somatic regions. Ten specimens were assessed.

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CHAPTER III
THE EFFECT OF PITX3 ON THE GENETIC NETWORK OF THE ORGANIZER
AND LATERALITY

Introduction

The prior chapter reports a relationship between laterality and somitogenesis phenotypes observed in pitx3-morphants. To explore perturbations to the left-right axis in more detail, I conducted further experiments on this subject. It is generally assumed that after the dorsal-ventral and antero-posterior axes are established, bilateral symmetry in the embryo is disrupted as the left-right axis forms and signals asymmetric organ development (Sutherland and Ware, 2009). The initial symmetry-breaking event across species remains controversial (Burdine and Schier, 2000; Tabin, 2005). However, as early as the 4-cell stage in chick and frog there is asymmetric accumulation of serotonin in right blastomeres and this concentration is reliant on gap-junction communication (Fukumoto et al., 2005). As well, in Xenopus there is asymmetrical localization of H\(^+\)/K\(^+\)-ATPase within the first two cell divisions and 14-3-3, a ubiquitously expressed regulatory protein that mediates signalling cascades and is necessary for producing an H\(^+\) flux between blastomeres (Baldin, 2000; Fu et al., 2000; Levin et al., 2002; Bunney et al., 2003). There are unique as well as conserved components of the laterality pathway across the commonly used developmental model organisms (zebrafish, Xenopus, chick, mouse). There appear to be three separate stages of left-right axis establishment: 1) development of a node/organizer structure that initiates the genetic symmetry breaking event; 2) initial break of left versus right gene expression involving a triad of genes expressing in the left lateral plate mesoderm; and 3) interpretation of left signals during organ morphogenesis, including heart and gut directional looping (Burdine and Schier, 2000; Sutherland and
Ware, 2009). A midline barrier is also of utmost importance during this axis development: it maintains separation of left and right signalling domains (Lenhart et al., 2011). In addition, the retinoic acid pathway sets up an antero-posterior gradient that also influences asymmetry (Durston et al., 1989; Wasiak and Lohnes, 1999). It is at each of these steps that I explore the effect of pitx3 on the laterality pathway in order to generate an overall view of the role for this transcription factor in perturbing organ situs.

In *Xenopus*, just past the stage of midblastula transition, when zygotic gene transcription begins, the Spemann organizer develops at the presumptive dorsal lip (Spemann and Mangold, 1924; Gerhart et al., 1989). This organizer is necessary for initiating gastrulation and establishing the dorsal axis (Spemann and Mangold, 1924). Recently, a second transient laterality structure called the gastrocoel roof plate (GRP) has been described, which develops during neurulation in *Xenopus* (Shook et al., 2002; Schweickert et al., 2007). In chick and mammals these two organizers appear to be functionally combined, both initiating gastrulation and breaking symmetrical gene expression (Beddington, 1994; Tsang et al., 1999; Burdine and Schier, 2000; Gros et al., 2009). Signals pass from the laterality organizer through the lateral plate mesoderm and distribute left-right axis patterning information throughout the embryo. Internal organs, such as the heart and the intestines, develop using these genetic signals to facilitate organ positioning and looping (*situs solitus*) (Burn and Hill, 2009). Any divergence from this patterning can result in intermediate heterotaxia phenotypes (*situs ambiguous*), presenting as the backward looping of organs, isomerism, or the complete inversion of the visceral organs (*situs inversus totalis*) (Burn and Hill, 2009).
Pitx3 appears to play a role in patterning the left-right axis and/or interpreting signals from this axis for proper development of the viscera. Pitx3 expresses prior to Spemann organizer development and then symmetrically along the lateral plate mesoderm, and finally asymmetrically in the looping heart and intestine (Chapter 1, Figure 4C) (Pommereit et al., 2001; Khosrowshahian et al., 2005). Perturbations to pitx3 result in heterotaxia phenotypes such as backwards (sinistral) looping of the heart and aberrant gut development (Chapter 2 Table 2). The earliest expression patterns of pitx3 have yet to be explored for a functional role for this gene during early embryogenesis.

Spemann’s Organizer

The Nieuwkoop Centre marks the future dorsal side of a fertilized Xenopus embryo (Nieuwkoop, 1973). This region is located across from the sperm entry site, where vegetal cells are exposed to localized β-catenin, express Nodal signals, and induce the formation of the Spemann organizer in cells overlying the dorsal marginal zone (Nieuwkoop, 1973; Heasman et al., 1994; Takahashi et al., 2000; De Robertis and Kuroda, 2004). The organizer is a signalling centre, necessary for expressing multiple transcription factors and secreted signalling proteins needed for initiating gastrulation (De Robertis and Kuroda, 2004). This organizer in Xenopus is homologous to the node in mouse, embryonic shield in zebrafish, and Hensen’s node in chick (Blum et al., 2009).

Organizer appearance is signified by the presence of the dorsal lip where bottle cells begin invagination to commence gastrulation (Heasman, 2006). This dorsal lip is marked by the expression of the transcription factor goosecoid (gsc) (Cho et al., 1991). The dorsal lip systematically expands via involution of the marginal zone cells, to
encompass a yolk plug of endodermal cells (Keller and Shook, 2008). It is amongst the cilia surrounding this node structure in amniotes that symmetrical gene expression is broken (Okada et al., 2005). The two organizers in *Xenopus*, Spemann’s organizer and GRP laterality organ, appear to divide the roles of the node in mice. Spemann’s organizer directs gastrulation and antero-posterior patterning, while GRF sets up nodal flow common to other organisms in establishing leftward movement necessary for downstream asymmetrical gene expression (Spemann and Mangold, 1924; Schweickert et al., 2007). It is only the Spemann’s organizer that is analyzed further in this chapter, however future studies will be suggested for the GRP.

At gastrulation, comparisons of wildtype, control morpholino-injected, and *pax3* morpholino-injected embryos reveal significant morphological differences (Figure 1). Following development from the initial appearance of the dorsal blastopore lip at stage 10, to a crescent shape at stage 10.25, and then a fully formed blastopore and yolk plug at stage 10.5, wildtype and control embryos show deep, smooth furrows that evenly expand around the yolk plug (Figures 1A-F). In the *pax3* morphants, the slit-like dorsal lip is less defined, off-centre, and appears discontinuous in its delineation of the yolk plug (Figures 1G-I). The endodermal cells of the yolk plug appear unorganized, large and yolky. Gastrulation defects have previously been reported following morpholino-mediated gene knockdown as a consequence of off-target effects (Coffman et al., 2004; Eisen and Smith, 2008). However, the use of a control-morpholino, as well as a second morpholino designed to knock down *pax3* in this experiment (data not shown), ruled out general morpholino perturbations on the gastrulating embryos: they show a phenotype consistent with loss specifically of *pax3* (Bedell et al., 2011).
Although pitx3 expresses as early as stage 8 in *Xenopus* (Khosrowshahian et al., 2005), several attempts to employ *in situ* hybridization to visualize early expression of pitx3 in the blastula and during early gastrulation stages revealed only general and ubiquitous ectodermal expression (Figure 2A and B). Similar diffuse and wide-spread expression was reported for goosecoid transcript levels at stage 8.5, another homeodomain transcription factor: it could be confirmed by Northern blot analysis only (Cho et al., 1991). Since cell-specific expression of pitx3 is difficult to visualize during late blastula and early gastrula stages, it is difficult to predict a specific function for this gene during these developmental stages. Since knockdown of pitx3 expression affects this early dorsal lip stage of development it is reasonable to expect an early function for pitx3 upon organizer gene expression.
Figure 1: Dorsal lip, blastopore, and yolk plug phenotype comparison between wildtype *Xenopus* embryos, and control- and *pitz3*-morphants, imaged from the vegetal side (bottom). (A-C) Wildtype embryos (wt) at stage 10, 10.25, and 10.5, showing invagination of the dorsal lip, expansion of the blastopore, and distinct endodermal cells in the yolk plug. (D-F) Control morpholino-injected embryos (Cmo) displaying similar phenotypes to the wildtype. (G-I) *pitz3* morpholino-injected embryos (Pmo) present irregular dorsal lip and blastopore formation and disrupted cellular size and arrangement in the yolk plug. (*) Designates dorsal lip.
Figure 2: Early *pitx3* expression shown by *in situ* hybridization in cleared embryos. At gastrulation stage 10.5 from animal view (A) and lateral view (B), *pitx3* expresses slightly in ectodermal cells (arrow). (C) Control embryo at stage 35 indicates specific staining for *pitx3* in lens (arrow) above background precipitation. (*) designates blastocoel.
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* denotes specific markers or features in the images.
Figure 3: Differential gene expression at early gastrula stages (10-10.5). (A-E) Embryos injected with control-morpholino (Cmo) display wildtype gene expression patterns. (F-J) Pitx3-morpholino (Pmo) affects gene expression during the beginning of gastrulation. Dotted lines in A and F demarcate the Organizer region. (*) Show gaps in gene expression around the blastopore. Arrow shows encroaching gene expression within yolk plug.

Goosecoid (gsc) is an organizer-specific homeodomain transcription factor that marks the appearance of the Spemann organizer as well as the spatial extent of organizer tissue (Cho et al., 1991). Cells expressing gsc are mesodermal, and since the organizer cells are the first to involute through the blastopore during gastrulation, gsc-expressing dorsal mesoderm cells define the anterior-most head mesoderm (Gerhart and Keller, 1986). Gsc is not only a marker for organizer tissue, but it also elicits dorsal lip invagination and subsequent gastrulation cellular movements (Cho et al., 1991). In comparison to controls, pitx3-morphant embryos display an enlarged organizer region (Figure 3A and F). Cells expressing gsc comprise a larger width of the emerging blastopore, and the transcription factor signal appears more diffuse (Figure 3F). The size and shape of the organizer remains static as the embryo progresses through early gastrula stages, until these cells themselves involute and internalize (Niehrs et al., 1994; Gorodilov, 2000). Therefore, even slight staging errors of the embryos for gsc staining should not influence organizer domain size. Thus, pitx3 may play an early gastrulation role in helping to establish the boundaries of the Spemann organizer.

Lhx1 is another homeobox gene expressed in the Spemann organizer, as well as within the dorsal mesoderm (Taira et al., 1992). It expresses in the anteriorly involuting mesodermal cells and is necessary during gastrulation for proper cell movement (Taira et al., 1992; Hukriede et al., 2003). Lhx1 is extremely important for anterior development of the embryo: Lhx1 mutant mice present a headless phenotype (Shawlot and Behringer,
1995). *Lhx1* directly targets and activates *gsc* expression and may be responsible for maintained *gsc* expression during late gastrulation within the prechordal plate (Figure 4) (Mochizuki et al., 2000). *Lhx1* expression in *pitx3*-depleted embryos appears relatively unaffected (Figure 3B and G). This suggests that *gsc* expression is being influenced by *pitx3* knockdown via a different route of activation or restriction.

*Lefty* (*antivin* or *atv*) is expressed at the onset of gastrulation at the dorsal lip (Branford et al., 2000). This gene also expresses in the marginal zone later in gastrulation as it confines to the circumference of the blastopore, where it inhibits mesoderm patterning (Branford et al., 2000; Cheng et al., 2000; Tanegashima et al., 2000). *Lefty* is a diffusible TGF-β (transforming growth factor) member that opposes *Nodal* signalling pathways, including that of *activin*. *Activin* induces both *gsc* and *lhx1* (Figure 4) (Cho et al., 1991; Taira et al., 1992; Cheng et al., 2000; Branford and Yost, 2002). It is thought that inhibition by *lefty* is required to restrict the spatial limits of gene expression within the organizer, since without *lefty*, organizer gene expression expands (Branford and Yost, 2002). Also, by confining the organizer region, *lefty* facilitates involution prior to convergent extension movements (Branford and Yost, 2002). In the absence of *lefty*, mesodermal cells are unable to involute before they undergo convergent extension, resulting in exogastrulation (Branford and Yost, 2002).
Figure 4: Diagram of the gene regulatory network at play in and around the dorsal lip of an embryo at the onset of gastrulation. Solid line indicates direct interactions, broken lines are indirect effects. Red area denotes the dorsal marginal zone and the grey circle represents the Spemann Organizer. Lefty inhibits mesoderm formation, t marks mesoderm and represents the trunk organizer, gsc marks the organizer and directs head organization, bix4 induces mesoderm induction via t and endoderm formation via vegT, and nodal elicits mesoderm induction. Red dotted lines show perturbations when pitx3 is knocked down, suggesting possible interactions with these genes.

Within the marginal zone, in addition to inhibiting activin and nodal (Tanegashima et al., 2000), lefty inhibits brachyury (Xbra/t) expression, which is induced by nodall (Figure 4) (Branford and Yost, 2002). Lefty expression in pitx3-morphant embryos shows discontinuity around the blastopore, when compared to the control-morphants (Figure 3E and J). Since this gene suppresses nodal signalling and regulates the mesodermal domain, uneven mesoderm induction and involution could be occurring in pitx3 morphants. Embryos that have depleted lefty expression show not only expanded
mesodermal gene expression domains, but also enlarged endodermal marker domains, and thus exhibit problematic cellular movements during gastrulation (Cha et al., 2006). Since *lefty* is irregularly expressed in *pitx3*-morphants, perhaps this discontinuity contributes to the irregular phenotypes seen at this early gastrula stage, including uneven involution of the blastopore (Figures 1G-I). Also, by inhibiting *activin* and thus indirectly affecting *gsc*, aberrations in *lefty* expression in *pitx3*-morphants could account for changes in *gsc* expression (Figure 4).

Mesodermal patterning in the marginal region of the embryo at the onset of gastrulation also requires *nodal* gene expression (Takahashi et al., 2000; Luxardi et al., 2010). *Activin* induces *nodal* expression in this region, and *nodal* positively regulate this induction via a feedback loop (Tanegashima et al., 2000). To confine the level of *nodal* expression, both *lefty* and *t* must negatively regulate *nodal* levels and inhibit mesoderm induction more peripherally (Cha et al., 2006). *T* is a pan-mesodermal marker gene that acts as a transcriptional activator (Conlon et al., 1996), but *t* indirectly and negatively regulates *nodal* gene expression in the marginal zone (Cha et al., 2006). *Nodal* can act at a distance and is autoregulatory: it must be tightly restricted by *lefty* and *t* (Sakuma et al., 2002; Ohi and Wright, 2007). In *pitx3*-depleted embryos, we see an interrupted pattern of *t* expression around the forming blastopore of gastrulating embryos, compared to the continuous, mesoderm-encompassing expression domain in controls (Figure 3C and H). This likely indicates a break in the patterning of mesoderm during early gastrulation stages.

*T* is expressed in mesodermal cells restricted to become notochord after gastrulation (Smith et al., 1991). *T* and *gsc* are capable of inhibiting each other in late
gastrulae, where they define head and trunk organizer regions for the prechordal plate (gsc) and notochord (t) (Mangold, 1933; Artinger et al., 1997; Mochizuki et al., 2000; De Robertis and Kuroda, 2004). With the gsc domain expressing larger than normal in morphants, and t expression being intermittent, pitx3 morphant embryos may exhibit errors in distinguishing these separate organizer regions for patterning the head and trunk. It would be worth assessing this possibility in the future using antero-posterior probes.

**Bix4** (*brachyury*-inducible homeobox gene) is a homeodomain transcription factor induced by *vegT* prior to the midblastula transition (Figure 4) (Casey et al., 1999). It is preferentially expressed in the dorsal region of the embryo, and lies early in the specification pathway to induce endoderm fate (Casey et al., 1999; Chiao et al., 2005; Skirkanich et al., 2011). Since *bix4* is expressed in the marginal zone downstream of *t* for mesoderm induction (Tada et al., 1998), and since *t* expression expands in pitx3-morpholino treated embryos (Figure 3D and I), it was not surprising that *bix4* expression domains appeared to expand slightly.

Organizer cells are fated to become 1) pharyngeal endoderm and prechordal plate, which later help to pattern forebrain and midbrain development; 2) dorsal mesoderm including notochord that induces hindbrain and trunk regions; and 3) chordoneural hinge, which acts as a secondary organizer to induce the tip of the tailbud formation in *Xenopus* (Gilbert, 2000). *Pitx3* expresses early, appears to affect the organizer region, and thus likely has effects beyond its already established roles in lens, *Substantia nigra*, and muscle development (reviewed in Chapter 1). In Chapter 4 we explore multiple developmental pathways that are affected by pitx3 perturbation, and isolate putative target genes from each of these pathways (early signalling, brain, trunk segmentation, and
tailbud). It may be significant that *pitx3* appears to affect midbrain development (mDA neurons) and potentially the midbrain-hindbrain boundary (MHB or isthmus), somite development along the trunk, as well as skeletal muscle differentiation, and finally, an array of genes marking the tip of the tailbud (Chapter 4). Since the majority of tissues affected by *pitx3* perturbation later in development originate from the dorsal lip organizer region or are induced and patterned by these tissues, and that I am showing here to be developmentally disturbed, we must reserve the possibility that early *pitx3* effects on development carry over to later developmental stages once tissues from this early region differentiate and/or undergo morphogenesis.

**Nodal Flow**

In mammalian and fish embryos a leftward movement of extraembryonic fluid has been detected at the node and Kupffer’s vesicle, respectively, and this has been termed nodal flow (Nonaka et al., 1998; Essner et al., 2005). This flow is hypothesized to cause the initial asymmetric gene expression in this region that then propagates to lateral plate mesoderm (Okada et al., 1999). This flow has also been detected at the gastrocoel roof plate in *Xenopus*, separate from the Spemann’s organizer, and it is necessary for patterning laterality in the embryo (Schweickert et al., 2007). Monocilia present on the epithelial cells of these transient structures create laminar flow when rotating in a coordinated fashion (Okada et al., 2005). Multiple components of this nodal flow system have been explored with mutant models, however the mechanism by which this flow conveys asymmetric gene expression remains unknown.

In *Xenopus*, slowing the flow at the GRP results in phenotypes that are similar to those seen in *pitx3* morphants. Flow-perturbed embryos display *situs inversus* (8% versus
12% for \textit{xPitx3} and heterotaxia (33% versus 25%) (Compare to Chapter 2 Table 2) (Schweickert et al., 2007). Future studies might profitably examine these monocilia and look for altered protein expression such as dhc9 (lrd homolog), inversin, and polycystin-2 (pkd2), necessary for the function of these cilia.

**Laterality Pathway**

Although some of the specific mechanisms that initiate left-right asymmetry can differ between organisms, as does gastrulation, this genetic cascade of downstream signalling is evolutionarily conserved in deuterostomes (Lowe et al., 1996; Ryan et al., 1998; Ohi and Wright, 2007; Grande and Patel, 2009a). Inhibiting “left” signals causes laterality problems (Ryan et al., 1998; Cheng et al., 2000). Even with this pathway elicited, the lateral plate mesoderm nevertheless retains plasticity until organs commence differentiation (Lohr et al., 1998; Ohi and Wright, 2007). This genetic cascade involves a triad of gene expression: \textit{nodal1}, \textit{lefty}, and \textit{pitx2}.

**Nodal1**

In \textit{Xenopus}, \textit{nodal1}, a TGF-\beta family member, is first expressed symmetrically in the posterior tailbud of the embryo, in the area synonymous with the posterior node in mouse (Figure 5) (Lowe et al., 1996). It then expresses asymmetrically in the left lateral plate mesoderm (Lowe et al., 1996). \textit{Nodal1} autoactivates itself and produces a wave of expression that progresses rostrally from the posterior end via planar tissue communication within the left lateral plate mesoderm (Ohi and Wright, 2007). The pulse is further refined by \textit{lefty}, a \textit{nodal} antagonist, which is itself activated by \textit{nodal1} and provides \textit{nodal1} with a negative feedback loop (Figure 5) (Cheng et al., 2000). \textit{Lefty} is
activated soon after nodal1 appears, and expresses behind nodal1 in the left lateral plate mesoderm (Cheng et al., 2000; Ohi and Wright, 2007). Nodal is a dominant effector of left-right information that the body plan relies on for patterning the viscera (Ryan et al., 1998). In mouse, when Nodal is expressed in the right lateral plate mesoderm instead of the left, as is the case of the mouse inv mutant (inversion of embryonic turning), this induces right-side Lefty expression and complete situs inversus (mirror image of the normal internal body plan) (Yokoyama et al., 1993). The inv mouse has an insertional mutation in an uncharacterized intracellular protein, which results in turbulent nodal flow (Yokoyama et al., 1993; Okada et al., 1999). By contrast, if Nodal expression is absent or bilateral, as seen in the mouse iv mutant (inversus viscerum), situs is randomized (Lowe et al., 1996). The iv mutant consists of a spontaneous mutation of a left-right dynein gene (lr'd) that causes immotile cilia in the node, a lack of nodal flow, and a delay in left-sided genetic gene expression (Hummel, 1959; Okada et al., 1999). When comparing nodal1 expression in control- versus pitx3-morphant embryos, expression in the left lateral plate mesoderm is either normal, absent altogether, or shows delayed expression, not appearing until late neurula stages when expression has already expired in control embryos (Figures 6A-D). A delay in Lefty expression is shown in both the iv and inv mutants (Okada 1999), suggesting that there may be a problem with nodal flow in pitx3 morphants.

**Lefty**

We have already come across lefty expressing early in the gastrulating embryo (Figure 4) and we will see it again in the midline and heart discussions to follow. This gene expresses at multiple steps along the laterality pathway and thus has many opportunities to exert an effect on the patterning of asymmetric organs (Branford et al.,
It will be a challenge to isolate the precise stages pitx3 affects this gene’s expression and the possible ramifications of these perturbations, relating to the laterality phenotypes we observe in pitx3 morphant embryos.

**Figure 5**: Diagram of the gene regulatory network that occurs in the left lateral plate mesoderm to propagate the laterality pathway throughout the embryo. Blue designates nodal1 signalling. Although lefty appears downstream in the signalling cascade, expression appears behind nodal1 as it acts to negatively regulate nodal1 and keep its expression transient. Pitx2 is activated once nodal1 signalling reaches the anterior end of the embryo. Red dotted arrow shows where the pathway appears to be affected when xPitx3 is knocked down.
Figure 6: Gene patterning for the laterality gene cascade expressed in left lateral plate mesoderm, comparing control (Cmo) and pitx3 (Pmo) morpholino-injected embryos at developmental stages 24 and 27. (A-D) Nodal1 shows delayed expression in pitx3-depleted embryos. (E-H) Lefty expression in the absence of pitx3 becomes reduced and hazy in the pattern. (I-L) Expression of pitx2 is decreased and absent in the left lateral plate mesoderm. Arrows point to expression in the lateral plate mesoderm, (*) denotes expression in heart primordia.
Lefty, the *Xenopus* homolog for both *Lefty-1* and *Lefty-2* in mouse, is expressed in the left lateral plate mesoderm, as well as dorsally in the left endoderm and later in the left cardiac field and looping regions of the developing heart (Branford et al., 2000; Cheng et al., 2000; Tanegashima et al., 2000). *Lefty* may be involved in the looping development of the heart and gut (Cheng et al., 2000). Left-sided expression of *lefty* may be downstream of left-specific *Vg1* expression, which is unique to *Xenopus* (Branford et al., 2000). Overexpression of *lefty* results in randomization of gut coiling and cardiac looping (Branford et al., 2000). In pitx3-morphants, *lefty* expression is significantly reduced in the left lateral plate mesoderm, likely indicating that a lack or delay in *nodal1* expression adversely affects activation of *lefty* (Figure 6E-H).

**Pitx2**

*Pitx2* expression begins in the left LPM and is also induced by *nodal1* (Figure 5) (Ryan et al., 1998; Cheng et al., 2000). Instead of the transient expression seen for *nodal1*, *pitx2* expression sustains throughout organogenesis (Ryan et al., 1998). *Pitx2* expression corresponds directly with the left region of the developing heart, and later in corresponding left-side anatomy of the looping heart, specifically the inner curvature (Gormley and Nascone-Yoder, 2003). Interestingly, *pitx2* also expresses in areas fated to the inner curvature areas of the early gut (Muller et al., 2003). As a transcription factor, *pitx2* translates this labile left signal into the activation of organ morphogenetic programs, which ultimately fix the left-right axis in the viscera. In severe phenotypes, *pitx3*-depleted embryos do not express *pitx2* in the left lateral plate mesoderm, indicating that the developing heart and gut are not receiving the laterality signals to avoid random looping (Figures 6I-L).
In the absence of these *nodal1* and *pitx2* left signals, organs may be responding to bilaterally symmetric instructions (Cheng et al., 2000). We report a 25% inverted heart looping and 10% inverted gut rotation, with complete *situs inversus* occurring 12% of the time (Chapter 2 Table 2, 18ng *pitx3*-morpholino). Delaying signals would likely cause a low incidence of heterotaxia and *situs inversus*, if complete absence of left signal results in 50:50 randomization and *nodal1* expression shows varying phenotypes in the *pitx3*-morphants. Aberrant heart and gut development with incomplete isomerism occurs at a higher frequency (49% and 53% respectively), in morphants, indicating the importance of *pitx3* for laterality.

*Pitx3* expresses bilaterally in the lateral plate mesoderm, however a function for this transcription factor in this tissue has yet to be explained (Pommeret et al., 2001b; Khosrowshahian et al., 2005). It is difficult to discern at this time whether early expression of *pitx3* is affecting *nodal1* expression directly, or if *pitx3* in the lateral plate mesoderm somehow regulates *nodal1* expression on the left side differently from the right, perhaps due to different cofactors available on either side of the embryo. Similar function has been reported for *Bmp4* during heart development (Branford et al., 2000).

The varying morphant *nodal1* expression phenotypes in lateral plate mesoderm may be due to incomplete penetrance of the morpholino at these stages of development (Heasman, 2002). However, for the *iv* mouse mutant, an array of four different *Nodal* expression patterns can be observed (bilateral, absence, right-side, and normal left-side) (Lowe et al., 1996; Okada et al., 1999). If *pitx3* affects early steps of the laterality cascade, we would similarly expect varying degrees of *nodal1* perturbation. The fact that we never observe right-sided *nodal1* expression suggests that the laterality bias is still in
effect, but that it may not be as powerful as it is in wildtype embryos. Future studies may look at the gastrocoel roof plate organizer, the cilia implicated in generating nodal flow, as well as the quality of leftward flow produced.

**Midline Integrity**

Another player in left-right axis patterning is the dorsal midline. Midline defects cause not only laterality problems, such as reversal of cardiac looping, but also cyclopia. It has been reported for *pitx3* morphants that cyclopia occurs as an extreme phenotype, where the two eye fields are not separated (Khosrowshahian et al., 2005). These two eye primordia are specified by stage 16 as the prechordal plate reaches its anterior limit and induces eye formation in the anterior neural plate (Li et al., 1997; Eagleson et al., 2001). Another phenotype reported in *pitx3* mutants is a bent dorsal axis, indicating that when *pitx3* is knocked down, there are perturbations to the symmetry of left and right aspects of the developing trunk (Chapter 2).

The dorsal midline comprises several structures arising from the embryonic node, including the notochord (mesoderm), the hypochord (endoderm), and the neural tube (formerly ectoderm), which consists of the floor plate and roof plate (Yost, 1998). There are different domains along the embryonic midline, designated by specific genetic pathways unique to the various sections (Bisgrove et al., 2000; Lenhart et al., 2011). Midline integrity is essential to prevent the mixing of left- and right-specific genetic signals (Lohr et al., 1998; Yost, 1998; Kelly et al., 2002). Signals from the midline structures are required for the maintenance of left-side expression of *nodall* in the lateral plate mesoderm, specifically to inhibit *nodall* from being expressed on the right side.
Lohr et al. (1997; Lohr et al., 1998) discovered that if these midline structures were eradicated in *Xenopus*, there was bilateral expression of *nodal1* and subsequent randomization of cardiac looping. These medial structures (notochord, hypochord, and floorplate) are only necessary for these inhibitory effects between stages 15 and 20 of neurulation (Lohr et al., 1997). Regression of the anterior notochord, through loss of dorsal-anterior development, is also correlated with heart reversals, implicating midline structures in maintaining left-right asymmetry (Danos and Yost, 1995). Similarly, within the prechordal plate, the most anterior midline structure that is continuous with the notochord, signals are secreted for the separation of the eye field to prevent cyclopia (Roessler and Muenke, 2001). *Sonic hedgehog (shh)* and *lefty* are two genes expressed within these midline structures and they give clues as to the possible disruption of the midline in *pitx3*-morphant embryos.

*Shh* expression begins in the gastrulating embryo at the dorsal marginal zone (DMZ) and several other genes that are expressed throughout the dorsal midline begin expression in this area (Ekker et al., 1995; Peyrot et al., 2011). *Shh* is expressed in the organizer region and then along the antero-posterior axis within the notochord and prechordal plate of the mesodermal midline, as well as in the floorplate of the neural tube (Roessler and Muenke, 2001; Peyrot et al., 2011). *Xenopus shh*-morphants exhibit cyclopia as well as gut looping defects (Peyrot et al., 2011), similar to those seen in our *pitx3*-morphants (Khosrowshahian et al., 2005). *Pitx3* expression is dependent on *shh* signalling in zebrafish (Zilinski et al., 2005), and *shh* is also required for development of mDA neurons in the midbrain (Ericson et al., 1997; Lebel et al., 2001). This would suggest that *shh* is upstream of *pitx3*, however since *shh* expression also shows
perturbation in *pitx3* morphants (Chapter 2 Figure 5D and E), there may be a feedback loop between these two genetic signals. In *Shh* mouse mutants, there is a complete lack of *Lefty-1* expression along the dorsal midline and as a result, ectopic expression of the laterality cascade (*Nodal*, *Lefty-2*, and *Pitx2*) on the right side of the embryo (Tsukui et al., 1999). Even with this genotype, there was not a problem with the direction of heart looping, but rather delayed or partial looping (Tsukui et al., 1999). Similar laterality effects are seen in *Lefty-1* mouse mutants, signifying the importance of *Lefty* expression in the midline to inhibit “left” signals from spreading (Meno et al., 1998; Tsukui et al., 1999). Therefore, as far as laterality is concerned, *Shh* is required for midline integrity through its regulation of *Lefty-1* to promote a molecular barrier (Tsukui et al., 1999).

The midline is another area of the laterality pathway that we observe perturbations in *lefty* expression. *Lefty* expresses in the neural tube floorplate, notochord, and endodermal hypochord along the axial midline (Branford et al., 2000; Cheng et al., 2000). In the mouse there are two *Lefty* genes: *Lefty-1* is expressed in the left part of the midline floorplate and *Lefty-2* is expressed in the left lateral plate mesoderm (Tsukui et al., 1999). What two *Lefty* genes do in other species, *lefty* does alone in *Xenopus* (Branford et al., 2000). Overexpression of *lefty* causes cyclopia as the formation of the prechordal plate and floorplate compartments of the dorsal midline that require *nodal* signaling, fail to form (Thisse et al., 2000). Opposed to its asymmetric expression in the left lateral plate mesoderm, in *Xenopus lefty* is expressed symmetrically in the midline, possibly acting as a molecular barrier inhibiting the left-specific *nodal1* signals from crossing to the right side of the embryo (Branford et al., 2000). Interfering with TGF-β signalling, by means
of a dominant-negative form of activin type II receptor, blocks *lefty* signalling and allows the spread of *nodal* expression (Ryan et al., 1998).

*Nodal1* expression in the lateral plate mesoderm of *pitx3* morphants is variable (normal, absent, or delayed), yet implies that the midline barrier is functionally intact during these stages since we never see bilateral expression of *nodal1*. Comparing control- and *pitx3*-morphants at early stage 19 when midline integrity is necessary for establishing proper laterality (Lohr et al., 1997), we do not detect changes in *lefty* expression along the midline (Figure 7A and C). We do, however, see a reduction of anterior midline expression for *lefty* at later stages (Figure 7B and D), and this may influence left-right signalling to the developing cardiac *situs*, since in zebrafish anterior expression of *lefty* is required to prevent the spill-over of laterality signals into the rostral and right-side domains (Lenhart et al., 2011). Since cyclopia is already determined at early stages when *lefty* expression appears unaffected, this phenotype may be attributed to perturbed *shh* signalling in *pitx3* morphants.

*Shh* induces *Lefty-1* expression in the midline of chick embryos and *Lefty-1* lies downstream of *Shh* in mice (Tsukui et al., 1999). In *Xenopus pitx3* morphants, *lefty* perturbation in anterior midline structures could be an indirect effect of *pitx3* on *shh* signalling. However, results show that early *lefty* expression in the dorsal midline is not affected in these embryos, suggesting that there is another pathway regulating *lefty* expression in midline other than *shh* in *Xenopus*. These perturbations of *lefty* do not allow for the leak of *nodall* expression to the right side of the embryo and therefore, although reduced, the *lefty* molecular barrier does appear to be intact and not the source of *pitx3* resulting in laterality defects.
**Figure 7:** Midline expression of *lefty* at stages 19 and 27. (A, C) *Lefty* expression does not appear to be affected at stage 19 when injected with *pitx3*-morpholino (P-inj) versus control-morpholino (C-inj) on the left side. Note the bent dorsal axis towards the side of injection with *pitx3* morpholino (C). (B, D) At Stage 27, expression of *lefty* appears less specific to midline features (black arrows) in *pitx3* morphants (Pmo) and reduced in the anterior midline (red arrow) compared to controls (Cmo). (fp) floorplate, (h) hypochord.
Heart and Gut Looping Asymmetry

The processes that lead to development of a functional heart are highly conserved across vertebrates, especially the formation of a linear heart tube and asymmetrical looping (Warkman and Krieg, 2007). Heart precursors are specified at gastrulation in two patches of mesoderm that gradually migrate ventrally, meeting at the midline by stage 16 and fuse to form a linear heart tube by stage 32 (Warkman and Krieg, 2007). This single tube then undergoes rightward or dextral looping, which is a point of asymmetry, and then morphogenesis to form chambers (Harvey, 1998; Gormley and Nascone-Yoder, 2003; Warkman and Krieg, 2007). Such biomechanical mechanisms as cell polarity, adhesion, size and shape, will create structural dissimilarities between sides of the resulting organ (Gormley and Nascone-Yoder, 2003).

The heart is often used as an indicator of laterality, since it is sensitive to problematic laterality signals, but also because changes in development due to left-right cues are easily detected and dextral heart looping is the first morphological marker of asymmetry (Harvey, 1998; Ryan et al., 1998; Breckenridge et al., 2001). Not surprisingly, given that heterotaxia can be threatening to survival, the mechanisms are usually highly controlled and conserved through evolution (Ryan et al., 1998).

Development of the gastrointestinal tract also begins as a single tube, but it runs along the ventral midline. Visceral organs bud off the tube according to signals emanating from the dorsoventral axis, and then asymmetric cues signal the enteric tube to rotate counter-clockwise (Burn and Hill, 2009). There are two types of laterality imposed on the developing gut: position of gut origin and coiling direction (Branford et al., 2000). Counter-clockwise rotation of the gut results in the left concave and right convex portions
of the loop, via asymmetric elongation rates of the endoderm tube (Muller et al., 2003; Burn and Hill, 2009). Differences in left and right sides of the differentiating gut may include convergent extension movements, cellular adhesion, as well as intercalation of cells, and likely involves asymmetric gene expression from lateral plate mesoderm as associated downstream effector genes (Muller et al., 2003; Burn and Hill, 2009). Curvature of the intestine does not appear to be due to left and right differences in cell proliferation or apoptosis (Muller et al., 2003). Therefore, the left-right axis is not necessarily imposing fate cues for gut differentiation properties, rather triggering topological cell behavioural blueprints for gut formation (Muller et al., 2003).

In mice, defects in the laterality gene cascade in the lateral plate mesoderm have been shown to cause gut malrotations (Nodal, Lefty-2, and Ptx2), as well as situs inversus (Lefty-1 / midline expression) (Burn and Hill, 2009). Whereas asymmetric looping of the heart is necessary for chamber formation during cardiac morphogenesis, gut rotation allows for normal packaging of a large organ into a small space, rather than differentiation of the viscera per se (Burn and Hill, 2009). As the gut elongates, it loops and coils, and therefore morphogenesis involves regulating cellular differences on either side of the tube to create bends where necessary (Chalmers and Slack, 2000).

Each visceral organ is regulated independently for left-right orientation (Bisgrove 2000). Since pitx3 affects heart and gut laterality and morphogenesis (Chapter 2), it is necessary to determine if the effect of pitx3 on the laterality cascade is the cause, or if pitx3 plays individual roles in these organs. Expression of pitx3 has been reported throughout the heart and within the concave portion of the counter-clockwise coil of the gut, as well as specifically within the coil of the S-shaped intestine (Pommereit et al.,
2001a; Khosrowshahian et al., 2005). In an attempt to envisage the laterality defects reported for *pitx3* morphants (Chapter 2, Table 2), with the use of an antibody specific for sarcomeric myosin in both skeletal and cardiac muscle, an example of *situs* is visualized in Figures 8 and 9. A multitude of cardiac phenotypes were observed, including reversal of looping (Figure 8), ambiguous looping, irregular thoracic placement, and abnormal chamber development. A common attribute was a lack of fluorescence only in the heart area of the embryo, indicating a deficiency in cardiac muscle fibre differentiation.

Since the heart develops at the ventral midline, embryos are imaged ventrally, with the left-hand side appearing on the right and vice versa. The control-morphant embryo depicts wildtype *situs*, confirmed by dextral cardiac looping of the outflow tract (conotruncus) and counter-clockwise rotation of the intestine with inner S-shaped looping (Figure 8B and C, respectively). The selected *pitx3* morphant displays reversed heart looping of the two-chambered heart and aberrant gut looping (Figure 8F and G, respectively). In the lateral view of the *pitx3* morphant there is also a break in the musculature of the abdominal wall compared to the control embryo (Figure 8H).

Although there were various outcomes for cardiac development among *pitx3*-mopholino injected embryos, one type of aberrant gut looping was consistently represented (Figure 9). The gut looping as well as the initial counter-clockwise coil shows wild-type patterning, however the inner S-shaped looping is horizontal instead of vertical (Figure 9C). This aberration in gut looping may not impinge upon intestinal function, however it does suggest a problem in receiving and/or interpreting asymmetric signals to develop normal gut morphology, specifically in this S-shaped coil that represents the small intestine, and where *pitx3* is specifically
Figure 8: Visualization of sarcomeric myosin with MF20 antibody of control morpholino-(A-D) and pitx3 morpholino-injected (E-H) embryos at stage 46. Arrows point at the looping heart in both ventral (A and E) and lateral (D and H) images. (ct) conotruncus (v) ventricle (*) gap in abdominal musculature.
Figure 9: Differences in gut looping at stage 45 between control-morphants (Cmo, B) and pitx3-morphants (Pmo, C). (A) Nieuwkoop and Faber staging (NF).
expressed (Chalmers and Slack, 2000; Khosrowshahian et al., 2005). Abnormal concavitites can lead to gut malrotation, which is what we see in *pitx3* morphants (Muller et al., 2003).

The caudal part of the heart tube is the first to respond to laterality cues, possibly since the lateral plate mesoderm signals of *nodal1, lefty*, and *pitx2* cease just posterior to the developing cardiac mesoderm (Lowe et al., 1996; Harvey, 1998). *Nodal1* is noted to be a determinant for the direction of cardiac looping, signalling left-specific signals through the lateral plate mesoderm to downstream effector genes (Lohr et al., 1997). *Pitx2* is responsible for Rieger Syndrome in humans, where abnormal umbilical and cardiac development suggests a role for this transcription factor in both heart and gut morphogenesis (Rieger, 1935). *Pitx2* expression in the left lateral plate mesoderm corresponds to regions of the heart that result in concave curvatures (Gormley and Nascone-Yoder, 2003). The left heart field overlaps with left *pitx2* expression and, therefore is subjected to left-specific asymmetrical information. This region forms the inner curvature of the dextral loop and becomes part of the left atrium (Gormley and Nascone-Yoder, 2003). Cells from the right heart field contribute to the outer curvature and the right atrium (Gormley and Nascone-Yoder, 2003). The *pitx2*-expressing cells in the left cardiac field that contribute to the heart have a morphogenetic program that is distinct from those on the right. When ectopically expressed on the right side, *Pitx2* produces heart isomerism, indicating left-sidedness on both sides of the heart, or reversal of heart and gut looping direction (Ryan et al., 1998). During gut development, *pitx2*-expressing cells are found also in concave portions of the looping intestine and overexpression can lead to ectopic concavities, implying that *pitx2* is programming the
cells to elongate slower than those on the right that are not subjected to pitx2 expression (Muller et al., 2003). Pitx2 may act to inhibit elongation of the gut in specific areas, leading the tube to curve creating concavities (Muller et al., 2003). One mechanism that could underlie the morphogenetic anomalies may reside in the effects that pitx family members have upon shroom3 activation, and that subsequently mediates the cytoskeletal remodelling necessary to cellular elongation (Chung et al., 2010).

Pitx2 is not required to initiate the direction of cardiac looping, but instead it may be necessary for fine-tuning looping dynamics, including cell movement, proliferation/apoptosis, polarity, or adhesion/shape (Breckenridge et al., 2001). Pitx2 is known to regulate PLOD1, which modifies collagen in the extracellular matrix (Hjalt et al., 2001). This indicates that pitx2 is involved in both the left-right information as well as the morphological development of the heart. This is reflected in pitx3-morphant embryos where xpitx2 expression is perturbed (Figure 6K and L), resulting in aberrant morphological heart phenotype (49%) as well as directional looping phenotype (25%) (Chapter 2, Table 2). This role in morphogenesis of looping may be congruent with gut looping as well, with pitx2 being expressed in the concave portion of both heart and gut (Gormley and Nascone-Yoder, 2003). Gut morphology in pitx3-depleted embryos also showed morphological abnormalities (53%) more so than a backward direction of looping (10%), implicating a larger role for pitx3 upstream of morphology than looping direction (Chapter 2, Table 2). When we look at the expression of pitx2 in the left cardiac heart field of pitx3 morphants, pitx2 expression is absent in this region (Figure 10C and D). This would leave the cells of the left cardiac field without instructions to become concave, and could result in aberrant hearts.
*Lefty*, with its multiple expression domains, is considered to play multiple roles in the establishment of the left-right body axis (Branford et al., 2000). *Lefty* expresses in the left cardiac field and is seen in the anterior of the dorsal endoderm, an expression domain unique to *Xenopus*, implicating this gene in relaying left messages to the developing gut as well as to the heart (Branford et al., 2000). Overexpression studies show that increased *lefty* expression randomizes the direction of heart and gut looping, presumably due to inhibition of *nodal* signalling and thus a lack of laterality information in the lateral plate mesoderm (Branford et al., 2000). Again when we compare control and *pitz3* morphants, there is a lack of *lefty* expression in the left heart primordial region (Figure 10A and B). Influencing the expression of this laterality factor in the heart field could also account for the heterotaxia observed in *pitz3* morphants.

The heart and gut defects that we see in *pitz3*-morphants cannot be due to *lefty* aberrations alone, although this gene does appear affected at the different stages of the left-right pathway. In embryos with augmented *lefty* expression, regardless of the side, *pitz2* expression in the head and along the dorsal midline is not affected (Branford et al., 2000). In Figure 6I-L, we do see a change in *pitz2* expression pattern both along the dorsal ridge and within the dorsal retina. This implies that another pathway is affecting *pitz2* expression in addition to or in parallel to the *nodal1/lefty* pathway, or perhaps alternate *pitz2* promoters are in use within the different tissues.
Figure 10: Expression of genes in the developing cardiac expression domains at stage 27 of *Xenopus* embryogenesis. Paired heart patches are seen for *bmp4*, *tbx5*, and *ventx2* as the linear heart tube begins to form. Dotted lines represent the ventral midline, arrow shows single, anterior expression domain of *ventx2* in *pitx3*-morphants (Pmo). (Cmo) control-morphants.
Another TGF-β morphogen, *bmp4* (bone morphogenetic protein 4), initially expresses symmetrically in *Xenopus* in the paired cardiac primordia and may be involved in the primary induction of the heart anlage (Breckenridge et al., 2001). It has been proposed that *bmp4* has a bilateral role interacting with *lefty* to deliver specific left and right instructions to the developing heart and gut: synergistic on the left and antagonistic on the right (Branford et al., 2000). *Bmp4* later expresses asymmetrically in the left side of the heart tube, overlapping with *pitx2* in certain regions (Breckenridge et al., 2001). The expression of *bmp4* is affected by *nodal1*, implicating it as an effector molecule for interpreting the left-right information from the lateral plate mesoderm into heart morphogenesis (Breckenridge et al., 2001). *Bmp4* overexpression results in randomization of heart looping, and lack of *bmp4* results in an unlooped heart (Breckenridge et al., 2001). Therefore, both *bmp4* and *pitx2* may play roles in the regulation of looping. At the developmental stage where the two heart fields begin to merge to form the linear heart tube at the ventral midline, there do not appear to be any effects on the spatial distribution *bmp4* when *pitx3* is knocked down, however expression is low compared to control embryos (Figure 10E and F). *Bmp4* appears to be a genetic switch for whether cardiac looping occurs or not, and this decisive genetic pathway does not appear to be perturbed in *pitx3* morphants, since we never observe an unlooped heart. The decision to loop has been made in *pitx3* morphants and so this decrease in *bmp4* expression is not sufficient to override this phenotype. However, *pitx3*’s effect upon *lefty* and *pitx2*, both of which are greatly perturbed, will require further research for its effect on heart development under these conditions.
Tbx5 is a T-box transcription factor that is a marker gene for cardiogenesis, expressing early in the cardiac lineage (Brown et al., 2005; Herrmann et al., 2011). Tbx5 marks the posterior heart tube that will become the left ventricular and atrial region (Horb and Thomsen, 1999; Herrmann et al., 2011). By contrast, bmp4 marks the outflow tract that develops from the anterior heart tube (Gessert and Kuhl, 2009). Tbx5 appears to direct the proliferation and differentiation of cardiomyocytes, and may contribute to the development of the conductive system necessary for coordinated beating (Moskowitz et al., 2004; Herrmann et al., 2011). Enhancing Tbx5 expression decreases the expression of genes in the anterior heart field, including Bmp4 (Herrmann et al., 2011). With reduced levels of tbx5, the heart tube fails to form completely and heart looping does not occur, thus inhibiting chamber formation (Brown et al., 2005). It has been proposed that tbx5 regulates adhesive properties and cell polarity of cardiac cells once the two cardiac fields meet at the ventral midline (Brown et al., 2005). As seen with bmp4, the spatial expression of tbx5 is relatively unaffected in pitx3 morphants (Figure 10G and H). Pitx1 is able to regulate tbx4 in hindlimb (Duboc and Logan, 2011), which could mean that these two families interact. However, we do not observe a change in tbx5 expression in the presumptive heart and the pitx3 morphant phenotypes do not coincide with perturbations of tbx5. Pitx3 appears to affect cardiac development in parallel pathways to those of bmp4 and tbx5, or pitx3 lies downstream of these factors.

Ventx2/Xom is a direct target of bmp4 in a synexpression group, and expresses in a similar pattern as bmp4 throughout the embryo, including within the developing heart (Ladher et al., 1996; Karaulanov et al., 2004). Bmp4 and ventx2 act in a positive feedback loop with each other and ventx2 also has the ability to autoregulate itself, thus
becoming self-reliant from *bmp4* activation (Onichtchouk et al., 1996; Henningfeld et al., 2002). *Ventx2* is reported to show expression in the dorsal region of the gut tube, as well as the pancreas and stomach, indicating a role for this homeodomain transcription factor in visceral development (Costa et al., 2003). We can visualize expression of *ventx2* in both cardiac patches on the ventral side of the *Xenopus* embryo (Figure 10I). In some of the *pitx3* morphants examined, only a single, more anterior region of expression was seen for *ventx2* (Figure 10J). It is unclear what this indicates for cardiac development since little is known about the role of this gene in heart development. However, since *ventx2* is a direct target of *bmp4* and often mimicks *bmp4* expression, this does imply that another effector is influencing *ventx2* expression since *bmp4* expression in the *pitx3*-affected area is unchanged.

**Retinoic Acid**

Retinoic acid (RA) is an upstream effector of *pitx2*, and can also induce both *Lefty-1* and *Lefty-2* expression in mouse embryos (Tsukui et al., 1999; Wasiak and Lohnes, 1999). In line with this, RA antagonists block *Lefty*, *Nodal*, and *Pitx2* expression in mice (Chazaud et al., 1999). It has been suggested that *Shh* and RA act in parallel pathways to regulate *Lefty* expression in the midline (Tsukui et al., 1999). Components of the retinoic acid pathway are expressed symmetrically on left and right sides of the embryo, supporting the suggestion that retinoic acid provides a more general role in the embryo, establishing the correct environment that allows proper expression of genes that constitute the laterality pathway, thus acting early on the left-right axis (Wasiak and Lohnes, 1999; Zile, 2001).
Although RA appears present in early embryogenesis during gastrulation, this morphogen is not required for proper organ development until the primitive heart begins to form (Zile, 2001). Vitamin A deficiency in the developing embryo leads to heart anomalies such as defective outflow tract formation, thin-walls, and a dilated organ without defined chambers (reviewed by (Zile, 2001)). Exogenous application of retinoic acid is shown to affect normal heart asymmetry (Wasiak and Lohnes, 1999). Metabolic enzymes tightly regulate retinoic acid concentrations: families responsible for the production (dehydrogenases, such as \textit{aldh1a2/raldh2}) and breakdown (hydroxylases, such as \textit{cyp26a1}) of active RA. These two enzymes establish a sensitive anteroposterior concentration gradient of active RA, which acts as a transcriptional regulator (Chen et al., 2001).

\textit{Aldh1a2} null mouse embryos are deficient for RA and present with a linear heart that is unlopped (Niederreither et al., 1999). Overexpression of \textit{aldh1a2} and ectopic supply of its substrate produces embryos with microcephaly, indicating the importance of RA signalling on the anteroposterior axis and patterning the central nervous system (Durston et al., 1989a; Chen et al., 2001). When we knock down \textit{pitx3} on one side, developing embryos display asymmetric \textit{aldh1a2} expression (Figure 11A-D). At early neural tube stage, a reduction of \textit{pitx3} causes \textit{aldh1a2} expression to approach the midline (Figure 11A and B), where normally it is excluded (Chen et al., 2001). At later tailbud stages, \textit{aldh1a2} expression markedly increases in the retinal area (Figure 11C and D): this anterior expression domain of \textit{aldh1a2}, surrounding the eye anlagen, is the most sensitive to alteration in RA concentration, compared to expression domains in the lateral plate mesoderm (Chen et al., 2001). The concentration gradient of RA is high in
posterior areas and kept low in anterior regions (Chen et al., 1994), perhaps indicating that anterior structures are very sensitive to RA signalling and levels are tightly controlled. The lateral plate mesoderm expression of *aldh1a2* appears relatively unaffected in *pitx3* morphants, coinciding with the notion that *pitx3* does not affect RA production in the laterality pathway. These results suggest that the genetic components required to generate RA in a specific temporal and spatial pattern, are affected by *pitx3* perturbation, more so in some areas than others.

It has been shown that Pitx3 targets members of the retinoic acid pathway in the midbrain for proper neuron differentiation, where *pitx3* acts as an upstream effector on this pathway (Jacobs et al., 2007). In order to confirm that RA does not effect *pitx3* expression, embryos were treated with exogenous all-trans-retinol and stained for *pitx3* expression. The expression pattern for *pitx3* in retinoic acid-treated embryos appears similar to the control embryo (Figure 11E and F), confirming that RA is downstream of this transcription factor. Transcript levels of *aldh1a2* and *cyp26a1* were tested via RT-PCR after treatment to ensure that the retinoic acid pathway was perturbed (Figure 11G). Although an obvious reduction in *aldh1a2* expression is seen, an anomaly can be noted for *cyp26a1*, which should show a drastic increase in expression due to RA treatment.
**Figure 11:** Expression of *aldh1a2* in *pitx3* perturbed embryos and expression of *pitx3* in RA-treated embryos. (A, B) At Stage 19, *aldh1a1* expression encroaches the dorsal midline (dotted line) on the side injected with *pitx3* morpholino (P-inj) compared to control morpholino (C-inj). (C, D) *pitx3* morpholino-injected side of Stage 27 embryos display an increase in *aldh1a2* expression in the retinal region (arrow), compared to the contralateral control. (E, F) when embryos are treated with retinoic acid (RA), no effect is seen on *pitx3* expression. Control embryos are treated with ethanol (EtOH). (G) RT-PCR for *aldh1a2*, *cyp26a1* and loading control *ODC*, for embryos treated with EtOH and RA, compared to wildtype (WT).
Conclusion

There are many steps within the laterality pathway that together establish correct left-right patterning of the body plan, and so defects at the different stages result in specific phenotypic anomalies (Schneider and Brueckner, 2000). It is through the analysis of these phenotypes and the various steps of the pathway that I attempt to discern the role of *pitx3* for the asymmetric body plan.

*Pitx3* acts on asymmetry at multiple stages since both *situs inversus* and heterotaxy phenotypes are variable in *pitx3* morphants. An early role for *pitx3* in the gastrulating embryo appears to affect the size of Spemann’s organizer and patterning of mesodermal genes. Left-specific gene expression is perturbed, translating to aberrant laterality signals along the length of the embryo to the pre-cardiac field. Early heart genes involved in cardiac looping appear unaffected, yet *pitx3* morphant hearts and guts elicit heterotaxia as well as abnormal morphogenesis.

The expression of asymmetric gene markers *nodal1*, *lefty*, and *pitx2* in *pitx3* morphant embryos, suggest that there is a problem generating the left-specific gene cascade. *Nodal1* is either absent or delayed, thus leading to an absence of *lefty* and *pitx2* in most morphants. *Pitx3* may be regulating genetic pathways that initiate the laterality signal upstream of left-sided *nodal* signalling and perhaps the earlier problems seen in the gastrulating embryos and subsequent aberrant gene expressions may contribute to this phenotype. There is a need to examine gastrocoel roof plate cilia via transmission electron microscopy for analysis of ciliary structure as well as gene expression for cilia function (Sutherland and Ware, 2009).
Figure 12: Summary diagram of the stages of laterality. Pitx3 affects early patterning of Spemann’s organizer, marked by gsc expression. Later in development, monocilia at the gastrocel roof plate (blue) create leftward Nodal flow, initiating the left-specific gene cascade in the lateral plate mesoderm (LPM). Left signals progress to the cardiac field (CF) at the anterior end of the embryo. Midline signalling provides a molecular barrier against the leak of left information to the right side. A gradient of retinoic acid provides competence to the embryonic tissue to respond to laterality signals. Left information is then interpreted individually in heart and gut for laterality as these organs undergo morphogenesis.

The results reported here suggest that pitx3 is impinging upon the laterality pathway at steps individually in the heart and gut for both looping directionality and morphogenesis: situs inversus where both are affected together is rare. For cardiac development, laterality signals in the left pre-cardiac field are perturbed, most likely due to the aberrant nodal signalling cascade, and may be accountable for the problems in
looping direction. Since pitx3 expresses in the heart primordia directly and there are morphological phenotypes for heart development, pitx3 plays a direct role in heart development. Consistent gut looping phenotypes, specifically in a region that expresses pitx3 (S-curve), supports the theory that pitx3 also functions separately in gut for proper morphogenesis.

Midline integrity appears to be sufficient in retaining left signals when they are expressed asymmetrically and retinoic acid appears unaffected in the lateral plate mesoderm where it does not appear to be a cause for laterality phenotypes in pitx3 morphants. The role of bilateral pitx3 expression in the lateral plate mesoderm still remains unclear.

Materials and Methods

Embryos

Embryos were fertilized, dejellied in 2% cysteine, cultured, and staged as previously described (Nieuwkoop and Faber, 1967; Drysdale and Elinson, 1991). Animals were reared and used in accordance with University, Provincial, and Federal regulations.

Whole-mount In situ Hybridization

In situ hybridizations were performed according to established protocols (Harland, 1991) using digoxigenin labeled riboprobe for xPitx3, previously cloned in our lab. Hybridizations were conducted at high stringency (65° C). For clearing, embryos were taken to 100% mthanol and then through a series of washes in BABB (1:2 benzyl alcohol to benzyl benzoate).
Embryo Manipulations

Morpholino was resuspended in water and injected into embryos with a Drummond nanoinjector. Injections were made into the animal pole of embryos at either the 1-cell or 2-cell stages. The contra-lateral side served as a control. Injected embryos were cultured in 0.3 X MBS with 2% Ficoll-400 (Sigma) at 12°C for 1 hr to allow healing before being removed and allowed to develop at 17°C. At this point the solution was changed to 0.1 X MBS.

For translation knockdown assays, a previously characterized and specific xPitx3 antisense morpholino oligonucleotide sequence was employed and in addition, a second morpholino was designed to confirm specificity as well as a mis-match control (Khosrowshahian et al., 2005). Morpholinos employed were: xPitx3 specific-TGGGCTAATCCTGGTTGAAGGGAAT and CCTCTATTTGTTAAATCCTTCCTGC; mis-match control CCaCaATTTcTTAAATCCTTCgTcC and general morpholino control CCTCTTACCTCAGTTACAATTTATA.

For retinoic acid treatment, embryos were administered a 30-minute pulse treatment of 10⁻⁷M all-trans retinoic acid (Sigma) diluted in 0.1xMBS at stage 10, as previously described (Durston et al., 1989). RA was diluted from a 0.1M stock concentration diluted in ethanol. Sibling embryos were cultured in 0.01% ethanol diluted in 0.1xMBS for control.

RT-PCR

After RA treatment, embryos were isolated at stage 13, lysed, and processed in Trizol as per manufacturer’s instructions (Invitrogen). We then used DNAseI to remove genomic DNA, and ran the product over Qiagen RNeasy columns for purification.
cDNA was made using Omniscript reverse transcriptase (Qiagen) and Oligo(dT)\textsubscript{18} primers (Sigma) from 1ug total RNA. RT-PCR was performed at various annealing temperatures and cycle numbers, resulting in 5 time-points that were ultimately graphed. A cycle at the linear phase of amplification was selected for each gene. Primers used are as follows: (ODC forward 5’ – GTC AAT GAT GGA GTG TAT G – 3’) (ODC reverse 5’ – TCC ATT CCG CTC TCC TGA – 3’) (Cyp26 forward 5’ - GCT GCC ACG TCC CTC ACC TCT T - 3’) (Cyp26 reverse 5’ - GCC GAT GCA GCA CCT CAC TCC A – 3’) (Raldh2 forward 5’ - TAT GGG AGC CCT CAT CAA AG – 3’) (Raldh2 reverse 5’ - TCT TCC CAA TGC TTT TCC AC – 3’).

**Immunohistochemistry**

Mouse monoclonal MF20 antibody (kind gift from Dr. Christopher Wright) was diluted 50% in PBT (1xPBS; 2mg/mL BSA; 0.1% Triton X-100). Embryos were staged to 46, fixed in MEMPFA (4% paraformaldehyde) and taken to 70% methanol. Fixed embryos were taken through methanol dilutions in water to PBS, blocked in PBT supplemented with 10 % lamb serum, and incubated with antibody for 2 hours at room temperature. Secondary antibody Anti-mouse IgG Cy3 conjugate (Sigma) was diluted 1:250 in PBT and embryos were imaged using filter 11002v2 (Chroma Technology Corp.) and Leica MZFLIII microscope.
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CHAPTER IV
MICROARRAY BASED IDENTIFICATION OF PITX3 TARGETS DURING XENOPUS EMBRYOGENESIS

Hooker, L., Smoczer, C., Khosrowshahian, F., Wolanski, M., and Crawford, M. J.*

*Author for correspondence

Introduction

*Pitx3 encodes a *bicoid*-like transcription factor that is characterized by a lysine residue at position 50 of the homeodomain. The *aphakia (ak)* mouse represents a natural *Pitx3* mutant model that is the result of two deletions in its regulatory region that abolish eye and brain expression, but leave muscle expression intact (Semina et al., 2000; Rieger et al., 2001; Coulon et al., 2007). This genotype displays microphthalmic eyes that lack developed lenses. They also display impaired differentiation of dopaminergic neurons in the *substantia nigra*: mutants mimic the symptoms of Parkinson’s disease (PD) (Varnum and Stevens, 1968; van den Munckhof et al., 2003). In humans, *PITX3* disruption can lead to congenital cataracts, anterior segment mesenchymal dysgenesis (ASMD), Peter’s anomaly, and/or microphthalmia (Sakazume et al., 2007). This implicates *PITX3* as a major player in the control of gene transcription in lens fibers. In the ventral tegmental area (VTA) and *substantia nigra compacta* (SNc) regions of the midbrain, *PITX3* is necessary for the terminal differentiation and survival of mesencephalic dopaminergic neurons (mDA) (van den Munckhof et al., 2003; Hwang et al., 2009). Zebrafish *pitx3* morphants also exhibit small eyes with lens degeneration, along with misshapen heads, a bent dorsal axis, and reduced jaws and fins (Shi et al., 2005). Disruption of *Pitx3* in *Xenopus laevis* impedes development of lens and retina, and recent evidence suggests an
additional role in dorsal axis segmentation and in laterality (Khosrowshahian et al., 2005; Shi et al., 2005; Smoczer et al., 2011; Smoczer et al., 2012). In zebrafish, Pitx3 expresses in the hypoblast of gastrulating embryos (Dutta et al., 2005), and the transcript is detectable by RT-PCR in pre-gastrula Xenopus (Khosrowshahian et al., 2005). These two studies suggest an earlier involvement for the gene in dorso-anterior patterning than is generally understood.

Pitx3 binds target DNA to regulate transcription of downstream genes via bicoid binding elements (BBE; TAATCC)(Lamonerie et al., 1996; Amendt et al., 1998). Pitx3 directly regulates MIP/Aquaporin O, which encodes an abundant protein in the lens that functions as an osmotic regulator and cell adhesion molecule (Chepelinsky, 2009; Huang and He, 2010; Sorokina et al., 2011). In zebrafish, pitx3 acts upstream of the transcription factor foxe3, which is necessary for the transition of lens epithelial cells into differentiated secondary lens fibres via nuclear degradation (Shi et al., 2005). Pitx3 is also thought to regulate the balance between mitosis and terminal differentiation in the equatorial region of the lens: here it operates upstream of cell cycle inhibitors p27Kip1 and p57Kip2 (Ho et al., 2009). Within midbrain regions, it directly regulates tyrosine hydroxylase (TH) expression, the rate-limiting enzyme in dopamine production (Landis et al., 1988; Lebel et al., 2001; Messmer et al., 2007). It also controls the neurotransmission of dopamine in mDA neurons via regulation of vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) (Hwang et al., 2009). Direct regulation of Adh2 in mDA neurons affects the production of retinoic acid that is necessary for proper neuron development (Jacobs et al., 2007). To complicate matters, Pitx3 is a versatile
transcription factor: depending upon signalling context, it can act as either a transcriptional activator or as a repressor (Cazorla et al., 2000; Messmer et al., 2007).

We performed a microarray analysis to compare the transcriptomes of Pitx3- and control-morphants at stages 19 (when eye development is commencing) and 27 (when lens differentiation begins) (Nieuwkoop and Faber, 1967). We elected to employ morpholinos since ectopic expression and dominant negative approaches could affect the response elements of other Pitx family members: the ectopic expression approach is impossible to restrict solely to Pitx3 expression domains, and the homeodomain sequences of Pitx2 and 3, for example, are identical. Pitx2 and 3 differ from Pitx1 by a single amino acid in the turn between helices I and II.

Although the preponderance of literature regarding the gene relates to lens and mDA neurons, Pitx3 also expresses broadly throughout gastrulation, and later in somites, and lateral plate mesoderm (Pommereit et al., 2001; Khosrowshahian et al., 2005; Smoczer et al., 2012). In zebrafish, Pitx3 expresses in the demarcation of the mesendoderm-derived polster (Dutta et al., 2005). Ectodermal explants have been useful as source material for Xenopus microarray experiments in the past, but this restriction to a single germinal layer would miss some likely Pitx3 targets, and in addition would require the complicating necessity of neural inducing agents. That said, the interpretation of results can also be confounded by the feature that morpholino mediated translational knockdown, unlike RNAi approaches, solely affects translation and does not appear to affect mRNA degradation rates. Indeed, some embryos are suspected to compensate for morpholino mediated knockdown by releasing more transcript into circulation (Eisen and Smith, 2008).
We designed our search for *Pitx3* targets to be as broad as possible, and consequently we sampled from whole embryos. The results generated a long list of genes that are affected by *Pitx3* mis-regulation. We characterized novel transcripts that represent putative targets of Pitx3 and report plausible genetic pathways that are regulated by this multifaceted transcription factor.

**Results and Discussion**

**Microarray Analysis**

Morpholino specificity has been previously published and reported to selectively reduce *Pitx3* transcript and protein levels, with the control-morpholino having none of these effects (Khosrowshahian et al., 2005). This specificity has subsequently been confirmed using a second *Pitx3* morpholino and mis-sense control (Smoczer et al., 2012). *Xenopus* microarray GeneChips (Affymetrix) were employed, and the data were analyzed comparing control-morpholino treatments to *Pitx3*-morpholino treatments. The threshold for consideration was set at a 2-fold cut-off with a p-value of < 0.05. We categorized the top 100 up- and down-regulated transcripts at each stage, with regards to function, and generated pie charts to show their distribution (Fig 1).

Among gene categories, the largest group affected consists of transcripts with unknown function (expressed sequence tags; ESTs). Other transcripts encoded secreted factors and ligands, transport and binding proteins, and modifying enzymes. In sum, changes in expression profiles for these genes implicate *Pitx3* in some of the indirect controls upon morphogenesis such as those exerting an effect via regulation of secreted morphogens.
Figure 1: Microarray data represented according to putative gene function. The 100 most up- and down-regulated transcripts affected by Pitx3-morpholino-mediated knockdown were categorized by sequence analysis for stages 19 and 27 of X. laevis embryonic development. Colors correspond to functional groups in the legend (right).

When assessed in broad strokes, the secreted factors and ligands are notably less up-regulated in morphants at stage 27 than at stage 19, however by contrast, transcription factors are more up-regulated at stage 27. At stage 19, structural proteins were more profoundly affected (both up- or down-regulated) as a consequence by Pitx3 knockdown than at stage 27. A similar picture developed for signal transduction. The disruptions are consistent with embryos experiencing impaired movement, signaling and morphological changes during neurulation at stage 19, when the body plan is arguably at its most ductile
phase. Overall, chromatin modifying genes were up-regulated more than down-regulated at both stages.

Our aim was to use the microarray experiments to deduce novel Pitx3 pathways, so we first focused upon the transcripts that were most up- and down-regulated in response to morpholino-mediated knockdown of Pitx3. In published studies involving samples from rapidly developing systems, microarray and RT-PCR results have occasionally been at odds. Moreover, microarrays are likely to be sensitive to subtle differences in the staging of developmental samples: quantitative data might not be fairly interpreted in absolute terms. We elected to categorize on the basis of trend: if gene expression levels were altered 2 fold or more relative to controls, and this was repeated in a second experiment, we pursued the gene for further analysis using semi-quantitative RT-PCR analysis and riboprobe in situ hybridization. Genes that expressed in expression patterns that overlapped with Pitx3 were deemed possible direct target genes of Pitx3. Of this subset, we focused upon those that also possessed putative Pitx3 binding motifs in their 5’-UTR. *X. tropicalis* sequences. These were employed for the reason that they were uniformly available, and all of the *X. laevis* ESTs and genes that we have examined to date enjoy near perfect homology (Table 1). We then looked deeper into the data set to see if genes in the same signaling pathway or developmental process were similarly affected (Table 2). If the behaviors of the expanded set grouped in a logical manner, and if the behaviors were consistent with the Pitx3 knockdown phenotypes, these genes were further analyzed by RT-PCR or in situ hybridization.
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>UniGene ID</th>
<th>Gene Highest BLASTn Hit (Xenopus laevis)</th>
<th>Microarray Ratio</th>
<th>Concanavalin A Pathway</th>
<th>Inductive Secondary</th>
<th>RT-PCR Confirms</th>
<th>Change in ISH expression pattern</th>
<th>Putative BBE sites in 5'UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bas2b</td>
<td>XI.19899</td>
<td>EST - Moderately similar to bromodomain adjacent to zinc finger domain, 2B</td>
<td>0.400 (19)</td>
<td>✓</td>
<td>✓</td>
<td>No</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>Bix4</td>
<td>XI.399</td>
<td>homeobox protein BIX4 (bix4)</td>
<td>0.363 (27)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Crybb1</td>
<td>XI.21502</td>
<td>Beta B1-crystallin (Crybb1)</td>
<td>0.325 (27)</td>
<td>✓</td>
<td></td>
<td>Yes</td>
<td>No (27)</td>
<td>9</td>
</tr>
<tr>
<td>eFGF</td>
<td>XI.1181</td>
<td>fibroblast growth factor 4B (fgf4-b) XeFGF(iss) embryonic fibroblast growth factor</td>
<td>0.406 (19)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>N/A</td>
<td>4</td>
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<tr>
<td>Galectin IX</td>
<td>XI.15089</td>
<td>EST - Fish-egg lectin-like isoform 1</td>
<td>0.154 (19)</td>
<td>✓</td>
<td>✓</td>
<td>No (19)</td>
<td>Yes</td>
<td>N/A</td>
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<td>Gsc</td>
<td>XI.801</td>
<td>Goosecoid (gsc)</td>
<td>2.329 (19)</td>
<td>✓</td>
<td>✓</td>
<td>No</td>
<td>N/A</td>
<td>14</td>
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<tr>
<td>Hes4 (Hairy 2b)</td>
<td>XI.25977</td>
<td>basic-helix-loop-helix transcription factor hairy2b (hairy2)</td>
<td>0.299 (27)</td>
<td>✓</td>
<td>✓</td>
<td>Yes (27)</td>
<td>No (27)</td>
<td>14</td>
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<tr>
<td>Hes7+ (Est-4)</td>
<td>XI.15142</td>
<td>EST - Highly similar to Xenopus laevis Est-4</td>
<td>0.381 (27)</td>
<td>✓</td>
<td>✓</td>
<td>No (27)</td>
<td>Yes</td>
<td>10</td>
</tr>
<tr>
<td>Hes7.1+ (Nhr1)</td>
<td>XI.12126</td>
<td>EST - Moderately similar to transcription factor HES-7.1-B (XHR1)</td>
<td>0.211 (27)</td>
<td>✓</td>
<td>✓</td>
<td>Yes</td>
<td>No (19)</td>
<td>11</td>
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<tr>
<td>HoxA11</td>
<td>XI.266</td>
<td>Homeobox A11 (HoxA11)</td>
<td>0.423 (27)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>9</td>
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<tr>
<td>Lim1</td>
<td>XI.21652</td>
<td>EST - LIM class homeodomain protein (Lim5:Lhs5) (Bix; xLIM:2B)</td>
<td>2.046 (27)</td>
<td>✓</td>
<td>✓</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>L-Maf</td>
<td>XI.767</td>
<td>neural retina leucine zipper (nrl) bZIP transcription factor L-Maf (nrl)</td>
<td>0.357 (19)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>N/A</td>
<td>12</td>
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<tr>
<td>Obscn</td>
<td>XI.13958</td>
<td>EST - Weakly similar to obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF</td>
<td>6.211 (19)</td>
<td>✓</td>
<td></td>
<td>No (19)</td>
<td>Yes (27)</td>
<td>N/A</td>
</tr>
<tr>
<td>Pax6</td>
<td>XI.647</td>
<td>Paired box 6 (pax6-b)</td>
<td>0.226 (19)</td>
<td>✓</td>
<td>✓</td>
<td>No (19)</td>
<td>Yes</td>
<td>13</td>
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<tr>
<td>Rbp4l</td>
<td>XI.17576</td>
<td>EST - Weakly similar to RET_B Human plasma retinoid-binding protein precursor (PRBP)</td>
<td>6.164 (19)</td>
<td>✓</td>
<td></td>
<td>Yes</td>
<td>N/A</td>
<td>7</td>
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<tr>
<td>Rdh16</td>
<td>XI.5553</td>
<td>EST - retinol dehydrogenase 16 (all-trans; retinal)</td>
<td>6.285 (19)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>N/A</td>
<td>15</td>
</tr>
<tr>
<td>Rippy2</td>
<td>XI.9206</td>
<td>Lederlinicle (Stippy)</td>
<td>2.014 (19)</td>
<td>✓</td>
<td>✓</td>
<td>✓ (19)</td>
<td>Yes</td>
<td>Ambiguous: 20</td>
</tr>
<tr>
<td>RXRa</td>
<td>XI.877</td>
<td>retinoid X receptor, alpha (rrx)</td>
<td>0.319 (19)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Spr1</td>
<td>XI.17379</td>
<td>Sp5 transcription factor (sp5) Sp1-like zinc-finger protein XSPR1</td>
<td>0.441 (19)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>Ambiguous</td>
<td>N/A</td>
</tr>
<tr>
<td>Spr2</td>
<td>XI.2755.1</td>
<td>Sp1-like zinc-finger protein XSPR-2 GLI family zinc finger 1, gene 2 (gl1:2)</td>
<td>0.396 (27)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>N/A</td>
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<tr>
<td>Vent2</td>
<td>XI.37</td>
<td>VENT homeobox 2, gene 2 (ventx2; Xem)</td>
<td>0.406 (27)</td>
<td>✓</td>
<td>✓</td>
<td>Yes</td>
<td>Ambiguous</td>
<td>17</td>
</tr>
<tr>
<td>Wnt1</td>
<td>XI.21471</td>
<td>Wnt1 related (Wnt7c)</td>
<td>0.274 (19)</td>
<td>✓</td>
<td>✓</td>
<td>N/A</td>
<td>N/A</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 1: Data summary for genes analyzed for microarray confirmation.** Combined in situ hybridization results with RT-PCR outcome, compared to the microarray prediction of gene transcript behavior in response to xPitx3 knockdown. Highlighted genes represent the best-fit candidates for putative direct targets of Pitx3 since in situ hybridization and RT-PCR confirm the microarray data. Only the genes that had statistically significant RT-PCR results across 3 replicates were indicated on table as “Yes” confirmed by RT-PCR. For promoter analysis, putative Pitx3 and bicoid-binding elements (BBE) were searched in the 5000bp upstream region from ATG of *X. tropicalis* homologs where available at Ensembl.org (TAATCC, TAATCT, ...
TAATGG, TAATCA, and putative binding sites for Pitx3; (Lebel et al., 2001)). (*) Designate multiple Affymetrix probe sets that identify to the same gene transcript. (†) Hes7 and Hes7.1 are discrete products arising from separate genes and that share only 40% amino acid identity. Hes7 shares 90% identity with murine Hes7.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>UniGene ID</th>
<th>Gene</th>
<th>Highest BLASTn Hit (Xenopus laevis)</th>
<th>Microarray Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rax1</td>
<td>XI.186</td>
<td>Retina and anterior neural fold homeobox (Rax-a)</td>
<td>2.148 (19)</td>
<td></td>
</tr>
<tr>
<td>βB3-crystallin-like</td>
<td>XI.26355</td>
<td>Crystallin, beta B3 (crybb3)</td>
<td>0.366 (27)</td>
<td></td>
</tr>
<tr>
<td>γ-crystallin</td>
<td>XI.23710</td>
<td>Transcribed locus, strongly similar to NP_001088720.1 crystallin, gamma A (X. laevis)</td>
<td>0.140 (19)</td>
<td></td>
</tr>
<tr>
<td>γB-crystallin</td>
<td>XI.21441</td>
<td>Crystallin, gamma B (crygb)</td>
<td>0.298 (19)</td>
<td></td>
</tr>
<tr>
<td>βB3-crystallin-like</td>
<td>XI.26349</td>
<td>60% similar to beta-crystallin B3 (H. sapiens)</td>
<td>3.800 (27)</td>
<td></td>
</tr>
<tr>
<td>βA4-crystallin</td>
<td>XI.19126 (retired) replaced XI.67089</td>
<td>Crystallin, beta 4 (cryba4)</td>
<td>0.223 (19)</td>
<td></td>
</tr>
<tr>
<td>βB1-crystallin-like</td>
<td>XI.1337</td>
<td>Transcribed locus, strongly similar to XP_002938264.1 predicted: beta-crystallin B1-like (X. tropicalis)</td>
<td>2.741 (27)</td>
<td></td>
</tr>
<tr>
<td>Tbx4</td>
<td>XI.21545</td>
<td>T-box 4 (tbx4)</td>
<td>0.436 (27)*</td>
<td></td>
</tr>
<tr>
<td>Tbx5</td>
<td>XI.529</td>
<td>T-box 5 (tbx5-b)</td>
<td>0.237 (27)*</td>
<td></td>
</tr>
<tr>
<td>HoxA10</td>
<td>XI.21639</td>
<td>Homeobox A10 (hoxa10)</td>
<td>0.373 (27)</td>
<td></td>
</tr>
<tr>
<td>HoxA13</td>
<td>XI.21581</td>
<td>Homeobox A13 (hoxa13)</td>
<td>2.537 (27)</td>
<td></td>
</tr>
<tr>
<td>Galactin 1</td>
<td>XI.747</td>
<td>Lectin, galactoside-binding, soluble, 1 (lgals1)</td>
<td>2.264 (19)</td>
<td></td>
</tr>
<tr>
<td>Galactin 1a</td>
<td>XI.17371</td>
<td>Galactin family galactin-Ila (xgalactin-Ila)</td>
<td>0.291 (19)</td>
<td></td>
</tr>
<tr>
<td>Galactin 1b</td>
<td>XI.21879</td>
<td>Galactin 4 (lgals4-a)</td>
<td>2.367 (19)</td>
<td></td>
</tr>
<tr>
<td>Galactin 1Ha</td>
<td>XI.15364</td>
<td>Lectin, galactoside-binding, soluble, 9c (lgals8c-a)</td>
<td>0.414 (27)</td>
<td></td>
</tr>
<tr>
<td>Galactin 1Hb</td>
<td>XI.21878</td>
<td>Lectin, galactoside-binding, soluble, 9c (lgals8c-b)</td>
<td>0.304 (19)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Additional genes identified in the microarray data that pertain to genetic pathways implicated in this study.

The affected genes can be classified as: potential direct targets of Pitx3; genes that operate within a Pitx3 regulated pathway; or genes that are affected indirectly and outside of the domain of Pitx3 expression as a result of grossly perturbed patterns of organ differentiation. Only four genes with putative Pitx3 binding motifs displayed both RT-PCR and riboprobe in situ hybridization patterns that were unequivocally consistent with the microarray trend: Pax6, β b1 Crystallin (Crybb1), Hes7.1, and Hes4. Two others, Vent2, and Ripply2 (aka Ledgerline or Striply) displayed altered in situ hybridization patterns that were difficult to interpret with respect to expression level since their respective patterns were affected differently in disparate domains (Table 1). For example, although Vent2 expression is obliterated in the optic region consistent with the microarray trend, the gene is up-regulated in the posterior endoderm. Similarly, the
banded pattern of Ripply2 expression is anteriorized and delayed by morpholino at early stages, but appears to recover to some extent by stage 27.

In *X. laevis*, Pitx3 expresses in the developing lens, the otic vesicle and head mesenchyme, as well as in the branchial arches and along the anteroposterior axis in the developing somites (Pommereit et al., 2001; Khosrowshahian et al., 2005). Insofar as Pitx3 is critical to lens placode function, it plays a critical role in frog retina induction (Khosrowshahian et al., 2005), so one might expect gene expression in retina to be indirectly affected as well. Eye pathway genes Pax6, L-Maf, and Crybb1, express in the developing lens, and thus are good candidates for Pitx3 targets. Vent2, Rbp4l (purpurin), Galectin IX, and Rax1 express in early retina, and are all affected in morphants. They likely represent examples of the indirect consequences of Pitx3 perturbation. Moreover, a microarray survey of Aphakia mice revealed a link between Pitx3 perturbation and regulation of Pax6 and Rbp4 (Münster, 2005). All of the aforementioned provide validation for the efficacy of the microarray. Unfortunately, none of the previously published and characterized targets of Pitx3 are represented on the microarray, however one of the probe sets is to an EST that has homology to MIP/Aquaporin O, and it is down-regulated consistent with expectation.

**Riboprobe In situ Hybridization**

We assessed the effect of Pitx3 perturbation by injecting embryos at the 2-cell stage such that the left and right sides of the developing embryo could be compared as embryogenesis ensued: morphant phenotypes were monitored on the “mutant” side relative to the contra-lateral control. Candidate gene expression patterns were assessed
for perturbation in morphants and for a role in developing eye (Figs. 2-7), brain (Fig 8), somite (Fig 9), and tailbud (Fig 10).

Eye development

Among other domains, Vent2 (a.k.a. Ventx2) is expressed in the dorsal retina (Fig 2F, G) and it shows structural and functional homology to two Drosophila proteins, Om1D and BarH1, which are necessary for the differentiation of photoreceptor cells in the eye (Ladher et al., 1996). Along with Vent2, Pax6 and Crybb1 are perturbed in Pitx3 morphants (Fig 2H, I, J).

Pax6 is required and sufficient for the initiation of eye development where it specifies the lens and retinal primordia (Halder et al., 1995), and it too is perturbed in our assays. The microarray and RT-PCR data regarding L-Maf’s response to Pitx3 perturbation was ambiguous but is nevertheless worth following up: its relationship to Pitx3 has not been directly assessed, however Maf binding sites are deleted in the promoter of a naturally occurring mouse Pitx3 mutant (Semina et al., 2000) and L-Maf itself appears to reciprocally possesses 12 putative Pitx3 binding motifs in its 5’UTR. L-Maf is expressed in the developing lens in response to inductive events from the optic vesicle, and is directly targeted by Pax6 in chicks (Reza et al., 2002). Maf acts specifically in the lens fiber cells, where it can induce the expression of structural proteins such as the y- and βb1-crystallins (Crybb1) (Ishibashi and Yasuda, 2001; Cui et al., 2004). Given the presence of numerous potential Pitx3 binding sites in the Crybb1 promoter, and the response of this gene in our Pitx3 morphants, we speculate that Maf and Pitx3 act in tandem to activate the Cry genes. It is worth noting that other Cry genes represented on
**Figure 2:** *In situ* hybridization analysis for putative targets of Pitx3 involved in eye development. Visual comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or Pitx3-morpholino (Pmo) embryos and their untreated contralateral control.

**A-E:** *Pitx3* expression patterns are presented for comparison (adapted from Khosrowshahian et al., 2005, and Smoczer et al., 2012). (A) demonstrates faint but detectable signal throughout the ectoderm and in agreement with RT-PCR results. (B) Expression is detectable throughout neural ridge, while at stage 22, the gene is expressed in a cleared specimen where an arrow indicates pre-somatic mesoderm. By stage 27 (D), Pitx3 is detectable throughout much of the head ectoderm, as well as in branchial arches and somites. This pattern restricts later to somites, otic vesicle, lens, and brain (D).

**F-G':** *Vent2* expression is reduced in the developing eye field at stage 19 for the Pitx3-morpholino (Pmo) injected side (A’ white arrow) and at stage 27 (B’ black arrow), when compared to control-morpholino (Cmo) injected embryos (A, B).

**H-I':** *Pax6* shows reduced expression in eye field on Pmo side of embryos at stage 19 (C’ black arrow) and 27 (D’ white arrow).

**J-J':** *Crybb1* shows drastic loss of expression in the eye vesicle on the Pmo treated side of stage 27 embryo (E’) and no difference caused by Cmo treatment (E). Dotted line represents the midline of the embryo, separating injected right side from contra-lateral left side control.
the microarray also underwent significant fractional change, albeit at less spectacular levels, namely: $\gamma$ crystallin (0.14), $\gamma$ B crystallin (0.3), $\beta$ B3 crystallin (3.8), $\beta$ A3 crystallin (2.74), and species weakly similar to human $\beta$ B1 crystallin (2.12), and $\beta$ B3 crystallin (0.37).

**Novel Xenopus retinol-binding protein Rbp4l is expressed in lens**

The microarray indicated that an EST sequence encoding a 197 amino acid protein (GenBank CD362061) was up-regulated at stages 19 and 27 by 6.2 and 4.4 fold, respectively. We obtained a clone from NIBB (XL060f11) and after sequencing it, we identified it as a member of the lipocalin protein family, namely RBP4-like (Retinoid binding protein 4 like -Rbp4l) or purpurin. These small extracellular proteins characteristically bind hydrophobic molecules and are typically known as transport proteins (Flower, 1996). Fig. 3 shows that Rbp4l shares 73% residue identity with goldfish and salmon, 75% identity with zebrafish, and 78% similarity to chick Rbp4l. The similarity to human and murine retinoid-binding protein precursor is on 55 and 54% respectively. Rbp4l consists of three conserved motifs that create a cup-shaped cavity, enabling the protein to bind retinol, and the protein possesses a signal peptide for secretion (Berman et al., 1987). In zebrafish, rbp4l is transcribed in photoreceptor cells, and the protein is diffusely detectable in all retinal layers (Tanaka et al., 2007). As a supplier of retinol, a precursor of retinoic acid, this protein activates the retinoic acid and retinoid receptor pathway (RAR and RXR, respectively) (Nagy et al., 1996). Rbp4l functions as an extracellular matrix protein in the inter-photoreceptor matrix, and it appears to be necessary for cell adhesion and for the survival of photoreceptor cells in the
neural retina (Berman et al., 1987; Nagy et al., 1996). Photoreceptor cells require retinol for phototransduction and retinol is carried to them from the pigmented retinal layer, through the matrix, bound to Rbp4l. In contrast, the other RBP’s, including Rbp4l’s closest human homologue RBP4, are synthesized in the liver, bind to retinol in the blood (serum RBPs), and they transport retinol throughout the body to target cells (Goodman, 1981). Human PITX3 maps to10q25, and this is close to human RBP4 and several retinoid synthetic CYP loci at 10q24 (Gray et al., 1997). According to the Ancora resource, the region near Pitx3 is replete with highly conserved non-coding elements, so it is tempting to speculate that the genes are embedded within a conserved genome regulatory block (Kikuta et al., 2007; Engstrom et al., 2008).

Expression of Rbp4l is first detected by RT-PCR around stage 17 and increases past stage 35 (Fig 3B). In situ hybridization shows that expression of this transcript concentrates in the lens area and as a pronounced spot along the midline on the top of the brain. It expresses at lower levels in the craniofacial region and somites (Fig 3C-E). These expression patterns are distinct from those reported for RBP4 and purpurin. RT-PCR analysis was performed and confirmed microarray trends: morphants demonstrated an increase in expression at stage 19 (1.84 fold) and 27 (2.88 fold) (Fig 3B). Consistent with the microarray and RT-PCR data, the gene undergoes up-regulation as a consequence of Pitx3 knockdown (Fig 3F). Since Rbp4l expression in Pitx3 morphants is broadly up-regulated in the craniofacial region, our supposition is that Pitx3 exerts its effects upon this gene earlier than the lens stage, and when Pitx3 expression is more expansive. The murine homolog, Rbp4, is also affected by Pitx3 depletion in Aphakia mutants (Münster, 2005). Taken together, the results for this novel retinol binding protein
Figure 3: Characterization of a novel transcript, Rbp4l, in X. laevis.

A: Protein alignment showing distinct groups between retinol binding proteins and purpurin family members.

B: Temporal expression of Rbp4l throughout embryonic stages of development, showing slight detection at stages 17 and 24, and an increase in expression at stages 31 and 35. Confirmation of microarray predictions via RT-PCR, showing an increase in Rbp4l expression in response to Pitx3-morpholino (Pmo) at stages 19 and 27, when compared to wild-type (WT) and control-morpholino (Cmo) treatments.

C-E: In situ hybridization with antisense riboprobe against Rbp4l transcript, shows expression at stages 27 (C), 31 (D), and 35 (E) concentrated in the developing lens (white arrows, D and E) and at the dorsal midline of the developing midbrain region.

F: An embryo injected unilaterally with Pitx3 morpholino on its right side (left of the dotted line) displayed enhanced and general expression in the craniofacial region.

G: A schematic diagram of Rbp4l protein depicting a secretory signal at the N-terminus (red) and three characteristic lipocalin motifs (blue) that classify this protein as a member of the kernel subfamily of lipocalins.
GenBank accession numbers used to generate phylogenetic tree (A) are as follows: xRbp4l CD362061 (X. laevis), rRbp4 plasma BC167099 (rat), mRbp4 BC031809 (mouse), hRBP4 plasma AL356214 (human), cRbp4 precursor NM_205238 (chick), xlRbp4 precursor NM_001087726 (X. laevis), xIRb4 plasma NM_001086998 (X. laevis), xtRbp4 plasma NM_001015748 (X. tropicalis), zRbp4 NM_130920 (zebrafish), zpurpurin AB242211 (zebrafish), spurpurin NP_001135080 (salmon), ccpurpurin NP_001187969 (channel catfish), gpurpurin BAD42450 (goldfish), bcpurpurin AD028302 (blue catfish), cpurpurin P08938 (chick).

show the possibility of acting downstream of Pitx3 in lens developmental pathways, where both genes are expressed.

**Galectin IX is expressed in eye field and retina**

One of the EST sequences from the microarray data identified mostly with the *Galectin* family, and represents a new family member (Fig 4). We identify this sequence as a *Galectin IX* (Genbank Accession JN975639). It is related to the tectonin family that encodes beta-propeller repeats: the microarray reports a change in transcript levels at stage 19 (diminished to a fractional level of 0.15) and stage 27 (diminished to 0.25 of its former level). The function of a galectin can be extremely varied: it has intracellular and extracellular functions in cell adhesion, migration, proliferation, and apoptosis and that are stage- and tissue-specific (Cooper and Barondes, 1999).

*Galectin IX*, a gene uncharacterized with regard to expression patterns until this study, expresses in eye field and later in both lens and retina (Fig 4). Little is known of its promoter structure, so it is early to speculate whether or not the gene is a direct target of Pitx3. In *Xenopus* alone, twelve (12) different galectin proteins have been identified, numbered in order of discovery, and can be identified via galactose-binding ability and protein motifs, specifically carbohydrate recognition domains (Shoji et al., 2003). Other *Galectin* family members are expressed throughout the embryo in specific spatiotemporal
Figure 4: Characterization of a novel transcript *Galectin IX* in *X. laevis.*

A: Protein alignment showing amino acid similarities between *Xenopus* Galectin family members.

B: Temporal expression of *Galectin IX* throughout embryonic stages of development, shows expression beginning at gastrulation (stage 10), decreasing at stage 12, and expressing consistently at stages 17 through 35. Confirmation of microarray predictions via RT-PCR, detect an increase in expression at stage 19 and a decrease at stage 27 for *Pitx3* morpholino (Pmo) treated samples, compared to wild-type (WT) and control morpholino (Cmo).

C-E: *Galectin IX* transcript expresses at stages 24 (C), 27 (D), and 31 (E) concentrated in the developing eye (white arrows) and presumptive pronephros, persisting in the nephric tubules and ducts.

GenBank accession numbers used to generate phylogenetic tree (A) are as follows: xGalectinIa AB056478, xGalectinIb AB060969, xGalectinIIa AB060970, xGalectinIIb AB080016, xGalectinIIa AB060971, xGalectinIIb AB080017, xGalectinIVa AB060972, xGalectinVb AB080018, xGalectinVia AB080019, xGalectinVIIa AB080020, xGalectinVIIa AB080021, xGalectinIX BJ056659.
patterns, suggesting varied developmental roles for each protein (Shoji et al., 2003).

Additional galectins were identified in the microarray data: Galectin IIb (St.19 2.37 Fold), Galectin I (St.19 2.26 Fold, St.27 0.44 Fold), Galectin IIIb (St.19 0.30 Fold), Galectin IIa (St.19 - 0.29), Galectin IIIa (St.27 - 0.41). As a candidate Galectin, further functional assessment for galactose-binding affinity will be necessary to firmly classify this novel protein within the galectin family (Cooper and Barondes, 1999). Using an NIBB clone (XL103j23) we performed in situ hybridization to visualize the expression pattern of this novel transcript, which appears to be concentrated in the presumptive pronephros and eye regions (Fig 4C-E). Expression begins at gastrulation, fades and then increases gradually beginning at neurulation (Fig 4B). Curiously, RT-PCR for microarray confirmation (Fig 3B) shows a fractional increase in expression at stage 19 (5.28), but the expected slight decrease at stage 27 (0.83) in morphants. This interaction is likely indirect since even though expression patterns of Pitx3 and Galectin IX overlap, in situ hybridizations do not demonstrate obvious changes of Galectin IX expression in morphants.

**Novel Xenopus Retinol Dehydrogenase (Rdh16)**

An EST sequence found in the microarray data can be identified as retinol dehydrogenase 16 (Rdh16) (Fig 5). Since retinoic acid is pertinent to many developmental processes, and Pitx3 has already been shown to regulate an aldehyde dehydrogenase, AHD2 (Jacobs et al., 2007), this sequence is interesting as a putative downstream target of Pitx3. Retinol dehydrogenases are enzymes that catalyze the
Figure 5: Characterization of a novel transcript, Rdh16, in *X. laevis*.

A: Protein alignment showing amino acid similarities between *Xenopus* retinol dehydrogenase (rdh) family members.

B: Temporal expression of *Rdh16* throughout embryonic stages of development shows faint expression beginning at stage 24 and 27, then increasing at stages 31 and 35. We were unable to confirm the microarray predictions via RT-PCR, as no change in expression was detected between wildtype (WT) control-morpholino (Cmo), or *Pitx3*-morpholino (Pmo) embryos.

C-E: *In situ* hybridization with antisense riboprobe against *Rdh16* transcript, shows expression at stages 27 (C), 31 (D), and 35 (E) concentrated in the eyecup, branchial arches, and otic vesicle, as well as along the lateral plate mesoderm, with a focus on the posterior half (D), and on in the developing myotomes.

GenBank accession numbers used to generate phylogenetic tree (A) are as follows: xRdh16 NP_001083356, xRdh7 NP_001079189, xRdh13 NP_001085680, xRdh5 NP_001086194, xRdh9 NP_001090337, xRdh10 ACN32204.
conversion of retinol (vitamin A) to retinal, an intermediate in the biosynthesis pathway of retinoic acid (Pares et al., 2008). These enzymes belong to the short-chain dehydrogenase/reductase (SDR) family. Their substrate is retinol bound to CRBP (cellular retinol binding protein) (Napoli et al., 1991) and they appear to be differentially expressed in different tissues (Chai et al., 1996). Their differential expression suggests tissue-specific roles for different family members. *Xenopus Rdh16* shows 51% similarity to human 11-cis *RDH*. 11-cis RDH is: expressed in the retinal pigmented epithelium; is necessary for the generation of 11-cis retinaldehyde from retinol; and binds visual pigments in the eye (Wald, 1968; Simon et al., 1995; Simon et al., 1996). Microarray predicts a fold change of 6.288 at stage 19 and 2.758 at stage 27 for this transcript. We were unable to confirm this by RT-PCR (Fig 5B) or in situ hybridization. We rule this gene out as a *Pitx3* target.

The expression of this retinol dehydrogenase appears only in tailbud stages and is concentrated in the retinal layer of the developing retina, peripheral lens, otic vesicle, branchial arches and along the antero-posterior axis in a gradient intensified at the posterior half (Fig 5C-E). If this gene is a homolog of human 11-cis *RDH*, the expression in the eye would support a conserved functional role.

**Novel Xenopus Genes Oscurin-like and Chromatin-Remodeling Protein Baz2b**

Other genes may be indirect targets of *Pitx3* such as *obscurin-like* (*Obscnl*) in the eye field and branchial arches (Fig 6A), and a chromatin remodeling gene *Baz2b* (Figs 6B, 7). *Obscnl* is an EST weakly similar to obscurin, cytoskeletal calmodulin and titin-interacting *RhoGEF*. Since neither gene’s expression pattern is altered in all *Pitx3*-
expressing domains, it seems likely that they are affected by the morphological changes induced by Pitx3 knockdown, and thus should be considered indirectly affected.

The EST with homology to the BAZ family of bromodomain-containing proteins (bromodomain adjacent to zinc finger) is tentatively assigned the designation Xenopus Baz2b (GenBank Accession JN975638). The clone represents the 5’ half of a sequence encoding the N-terminus (921aa). This protein family contains a conserved bromodomain at the C-terminus, adjacent to a PHD zinc finger motif (Fig 7F). Bromodomains, capable of binding acetyl-lysine residues, are often found in proteins with histone acetyltransferase (HAT) activity and they are thought to play a role in chromatin-dependent gene regulation by unwinding histone-DNA complexes (Zeng and Zhou, 2002). Baz2b may have the ability to bind methylated CpG regions through a methyl-CpG binding domain (MBD) (NCBI) (Fig 4F). There is some evidence of BAZ proteins having the ability to interact with human homologs of ISWI which in Drosophila, binds the BAZ1 protein homolog Acf1 to form the ACF chromatin remodeling complex (Ito et al., 1999; Jones et al., 2000a; Jones et al., 2000b).

The microarray predicts that at stage 19 this transcript decreases in morphants to a fraction of 0.4 and at stage 27 to a fraction of 0.27. Unfortunately, by RTPCR stage 19 transcript is just at the limit of detectability. RT-PCR shows expression throughout embryogenesis, beginning as a maternal transcript in the oocyte and persisting through tailbud stages, and confirms the microarray data by showing a drastic decrease in expression at stage 27 (to a fraction of 0.086), with undetected expression at stage 19 (Fig 7B). Its spatial expression pattern, initially quite diffuse (not shown), condenses around the developing eye and pronephric structures during tailbud stages (Fig 7C-E).
**Figure 6**: *In situ* hybridization analysis for putative Pitx3 target genes Obscn1 and Baz2b. Visual comparisons of gene expression patterns between control-morpholino (Cmo) and Pitx3-morpholino (Pmo) right side-injected embryos. *Obscn1* shows a loss of expression in the branchial arches (black arrow), otic vesicle, and retina when treated at stage 27 with Pmo (A’) versus Cmo (A). *Baz2b* is substantially reduced in response to Pmo (B’) in the retinal layer of the optic protuberance (white arrow), as well as in the pronephros and in the anterior region of the dorsal axis, when compared to Cmo (B).

**Figure 7**: Characterization of a novel transcript, *Baz2b*, in *X. laevis*. 

A: Protein alignment showing amino acid similarities between Baz2B homologs across organisms.

B: Temporal expression of *Baz2b* throughout embryonic stages of development show expression as a maternal transcript in the egg “E” and throughout development to tailbud stage, with slight reductions in transcript level at stages 10 and 19. B Confirmation of microarray predictions via RT-PCR show abolished expression at stage 27 in response to Pitx3-morpholino (Pmo) when compared to control-morpholino (Cmo) and wild-type (WT) embryos.
C-E: Baz2b expression at stages 21 (C), 31 (D), and 35 (E) is concentrated in the developing eye, as well as the branchial arches and otic vesicle. Dark expression is seen in the pronephros, persisting in the tubules (E).

F: A schematic diagram of Baz2b protein depicting various domains characteristic of Baz2B: methyl-CpG binding domain (MBD), DNA binding domain (DDT), zinc finger domain (Z), adjacent to the bromodomain (BR).

GenBank accession numbers used to generate phylogenetic tree (A) are as follows: xBaz2b BQ400337 (X. laevis), mBaz2b BC150814 (mouse), rBaz2b NM_001108260 (rat), hBAZ2B NM_013450 (human), cBaz2b NM_204677 (chick), xtBaz2b BC166361 (X. tropicalis).

Since Pitx3 has been shown to play major roles in both the lens and retina development, these genes correlate with a role for this transcription factor in specifying lens placode, initiating lens differentiation, and in inducing retina (Khosrowshahian et al., 2005).

**Brain expression**

One candidate sequence was highly similar to Hes-related 1, and is tentatively re-assigned the name Hes7.1 based upon homology to the X. tropicalis and human genes. This gene likely specifies the frog midbrain/hindbrain boundary, or isthmus (Shinga et al., 2001; Takada et al., 2005). The isthmus is an important organizer of brain regionalization and consequent patterning (Nakamura and Watanabe, 2005). When murine Hes1 is disrupted, brain patterning mediated through the isthmus is damaged, and the mesencephalic dopaminergic (mesDA) neurons fail to thrive. The same authors report that expression of both Pitx3 and tyrosine hydroxylase is abnormal (Kameda et al., 2011). Since the related Xenopus homolog possesses 11 putative Pitx binding motifs, future studies should be sensitive to the possibility that Hes1/Hes7.1 and Pitx3 are engaged in a reciprocally regulatory relationship. Spr1, a Xenopus laevis transcription factor that is related to the human Sp1 and mouse Sp5 zinc finger proteins, is expressed in the forebrain as well as the isthmus, where eFGF also plays a role (Isaacs et al., 1992;
Ossipova et al., 2002). Both Spr1 and Hes7.1 show decreased expression in the isthmus in response to Pitx3-morpholino as assessed by in situ hybridization (Fig 8).

Unfortunately, tyrosine hydroxylase, a gene critical to differentiation of dopaminergic neurons (mDA) of the substantia nigra, is not represented on the microarray. However, Wnt1, an early stage marker for murine isthmus (Würst et al., 1994), is both represented on the microarray and down-regulated (Table 2). Only an unworkably small fragment of the gene has been cloned in frog (Wolda and Moon, 1992). Since Pitx3 is especially pertinent for the differentiation and maintenance of mDA neurons and since the isthmus is critical to development of the substantia nigra (Marchand and Poirier, 1983), it is tempting to speculate that this Pitx3 effect is mediated through control of isthmus patterning at early developmental stages.

The expression patterns of Lim1 will be discussed a greater length later, however it is worth noting in the context of isthmus and substantia nigra (structures that are induced and patterned early by Lim1 (Shawlot and Behringer, 1995)), that although the RT-PCR assays did not confirm the microarray data, nevertheless, in situ hybridization did. Moreover, Lim1 possesses 5 evolutionarily conserved Pitx3 binding motifs. Based upon our preliminary slate of putative signaling partners, our suspicion is that Pitx3 plays a heretofore uncharacterized role during gastrulation to pattern anterior-most structures – previous work has indicated that it expresses in fish hypoblast (Dutta et al., 2005), and somewhere in Xenopus pre-gastrula (RT-PCR, uncharacterized and low-expression location) (Khosrowshahian et al., 2005).
**Figure 8: In situ hybridization analysis for putative brain targets of Pitx3.** Comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or Pitx3-morpholino (Pmo) embryos and their untreated contra-lateral control.

**A-B’: Hes7.1** at stage 19 shows decreased expression in the midbrain hindbrain boundary or isthmus (black arrow) in response to Pmo (A’) versus Cmo (A) and again at stage 27 Pmo (B’) (black arrow) versus Cmo (B).

**C-D’: Spr1** stained embryos show increased expression (black arrow) at stage 19 when treated with Pmo (C’), where no change in expression is observed with Cmo (C). At stage 27, Spr1 expression in the isthmus is abolished on the Pmo side (D’) (white arrow). Dotted line represents the midline of the embryo, separating injected right-side from contra-lateral left-side control.
Segmentation and tailbud signaling

The Ripply family, Ripply 1 (bowline), Ripply2 (ledgerline, stripy) and Ripply3 serve as transcriptional repressors that are necessary for proper boundary formation during somitogenesis. The Ripply genes appear to act by balancing the FGF/RA signaling wave front and thereby regulate the emergence of new somites: this regulation is likely mediated by interaction with T-box genes (Chan et al., 2006; Kawamura et al., 2008; Hitachi et al., 2009). It is interesting that both Tbx4 and Tbx5 go down in our data set (0.237 and 0.436 for each of the two Tbx4 probands, and 0.432 for Tbx5). Ripply2, Hes4, and Hes7 are perturbed in Pitx3 morphants (Fig 9), and Ripply2 possesses 20 Pitx3 binding sites in its 5’UTR. Hes7 expression patterns confirmed the microarray data, however triplicate RT-PCR reactions did not substantiate this statistically. We note that RT-PCR consistency has historically been a problem in microarray studies (Altmann et al., 2001; Buchtova et al., 2010), and given the presence of 10 Pitx3 binding motifs within the 5’UTR of Hes7, we are inclined to pursue this gene’s candidacy further. Perturbation of Hes4 is complex: it appears to up-regulate at early stages, to remain unchanged through neurulation, but to be inhibited at tailbud stages (Smoczer et al., 2012). Hes4 and Hes7 are factors that function downstream of the Notch pathway during somitogenesis and that mediate segmental patterning of the presomitic mesoderm where they serve as components of the segmentation clock (Jen et al., 1999; Tsuji et al., 2003; Murato et al., 2007). Recently, pre-somitic expression has been reported for Pitx3 and its perturbation results in anomalous segmentation presenting as a bent dorsal axis and aberrant somite morphogenesis (Smoczer et al., 2011). Ripply2 morphants also produce bent dorsal axes and shift Hes4 and Hes7 expression patterns anteriorly (Chan et al.,
Further research is necessary to deduce which of these are direct downstream targets of Pitx3, but a good starting point would be to test if Pitx3 modulates Ripply2 and thereby indirectly alters expression of the Hes genes.

Both eFGF and RXRa are transcribed in the tailbud and thus may be factors that are affected by Ripply2 (Chan et al., 2006). eFGF extends to the posterior of the body axis and into the proliferating tailbud where notochord and somites continue to emerge. eFGF is also expressed later in the myotome of the trunk (Isaacs et al., 1992). Both eFGF and RXRa appear regulated by Pitx3 in the microarray dataset, but neither confirm by RT-PCR. The expression levels are too low to be reliably detected by in situ hybridization at stage 19 and 27, however both possess consensus Pitx3 binding motifs in their respective 5’UTR. Given the effects of Pitx3 perturbation upon the somitogenesis- and tailbud-expressing genes HoxA11, Spr2, and Lim1 (Fig 10), it might be worth re-examining their failed candidacy at targets.

Spr2 and HoxA11 are affected by Pitx3 mis-regulation (Fig 10). HoxA11 specifies positional identity along the antero-posterior axis and is largely expressed in the posterior notochord and tailbud mesoderm (Lombardo and Slack, 2001). Other Hox genes are affected to a lesser, though still significant fractional degree: HoxA13 (2.4), and HoxA10 (0.37). The differential effect upon these genes renders an indirect mediation by retinoid metabolism unlikely. Lim1 expression undergoes a complex modulation of expression: lateral mesoderm expression increases, while in paraxial mesoderm, expression is abolished. Spr2 and Vent2 are expressed in the developing tailbud (Ladher et al., 1996; Ossipova et al., 2002), so effects in this domain would also be reflected in the microarray.
**Figure 9:** *In situ* hybridization analysis for putative segmentation targets of Pitx3. Visual comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or Pitx3-morpholino (Pmo) embryos and contralateral control.

**A-B':** *Ripply2* expression, showing as two stripes in the pre-somitic mesoderm, shows an anterior shift (black arrow) in expression at stage 19 when treated with Pmo (A') instead of Cmo (A). At stage 27, *Ripply2* expression pattern loses its distinct shape and becomes unrestricted in response to Pmo (B'), whereas with Cmo treatment, precise patterning of this gene expression remains intact (B).

**C-D':** *Hes4* expression becomes blurred in Pmo treated embryos at stage 19 (C') and at stage 27 (D') *Hes4* expression is absent in the presomitic mesoderm (black arrow) and pronephros areas, compared to Cmo treated embryos (D).

**E-F':** *Hes7* no longer expresses in the most anterior stripe (black arrow), and the remaining two stripes are shifted anteriorly in comparison to the contralateral control (E'). At stage 27, on the Pmo side of the embryo (F'), *Hes7* shows increased expression in the presomitic mesoderm (white arrow) and again an anterior shift of the striped pattern (black arrow). Dotted line represents the midline of the embryo, separating injected right-side from contralateral left-side control.
Figure 10: In situ hybridization analysis for putative tailbud targets of Pitx3.

A-B’: *HoxA11* shows decreased posterior expression in the tailbud region (white arrows) of Pmo embryos at stages 19 (A’) and 27 (B’);

C-D’: *Spr2* displays a broader and larger domain of expression (black arrows) when treated with Pmo, both at stage 19 (C’) and 27 (D’), compared to Cmo treated embryos (C, D).

E-F’: *Lim1* expression disappears from paraxial mesoderm (red arrow) and is up-regulated in lateral mesoderm (black arrow) at stage 19 when treated with Pmo (E’). At stage 27 (F’), Pmo reduces *Lim1* expression in the developing pronephros (black arrow) and in the head mesenchyme and along the dorsal axis.
Indirectly characterized early perturbation effects

Although the microarray data was analyzed for embryos at stages 19 and 27, a significant number of candidates are pertinent for early patterning of the embryo, and moreover, are known to interact with each other in a manner consistent with Pitx3 impinging upon their respective regulatory networks. Pitx3 has been detected at early stages in the embryo (stage 8) (Khosrowshahian et al., 2005) implying an unknown function for this transcription factor at earlier stages. One of our candidate targets, Vent2, provides ventralizing information and perhaps signals for the differentiation of the epidermis (Ladher et al., 1996). This factor directly down-regulates the homeobox gene Goosecoid (Gsc), which is expressed in Spemann’s organizer and then becomes undetectable as the embryo undergoes neurulation (Cho et al., 1991; Trindade et al., 1999). Gsc is responsible for the development of dorsal structures (Cho et al., 1991). These two genes, Vent-2 and Gsc, play antagonistic roles in the establishment of the dorsoventral axis. Lim1 expression peaks at gastrulation in Spemann’s organizer, and has the ability to directly activate Gsc and maintain its expression in the prechordal plate (Mochizuki et al., 2000). All three are represented as Pitx3-sensitive in the microarray, however Gsc expresses too early to have been monitored in our riboprobe in situ hybridization although it should be noted that Gsc possesses 14 Pitx3 motifs in its 5’UTR.

Bix4 is a Brachyury-inducible homeobox-containing gene and is thought to induce both mesoderm and endoderm formation depending on the concentration of its encoded protein (Tada et al., 1998). It expresses earlier than we monitored by in situ hybridization at stages 19 or 27. Similarly, eFGF and RXRα are also expressed early in development,
well before the stages that we assessed. eFGF is most similar to FGF-6 and FGF-4 in mammals, yet may represent a novel FGF secreted factor that has both mesoderm-inducing properties and roles in anterior-posterior patterning (Isaacs et al., 1994). RXRα encodes a retinoid X receptor that is part of the nuclear receptor family that mediates the effects of retinoic acid upon embryos. Expression of RXRα begins as a maternal transcript in the oocyte, and then is temporarily abolished before gastrulation, leading to a role for this receptor in early patterning of the embryo (Blumberg et al., 1992). RA provides positional information and helps to pattern the anteroposterior body axis, mostly by mediating posterior transformation of the embryo (Durston et al., 1989).

**Conclusion**

Microarray analysis is a useful tool to monitor the influence of a gene upon the entire transcriptome of an organism. However, the generated data set is quite elaborate and deducing pertinent trends can be a challenging process. The information represented in this study provides a global view of general developmental processes in which Pitx3 may be involved. New genetic players have been identified as putative Pitx3 targets in the already established eye and brain developmental processes. In addition, based on genes identified by the microarray, novel roles for Pitx3 can be inferred for regulation of early patterning events and the development of the anterior-posterior body axis.

**Experimental procedures**

**Embryo collection and manipulation**
Staging, de-jellying, and culturing of *Xenopus laevis* embryos were conducted as previously described (Nieuwkoop and Faber, 1967; Drysdale and Elinson, 1991). Animals were reared and used in accordance with University, Provincial, and Federal regulations. Fluorescently labeled morpholinos for either control or experimental Pitx3 treatments were injected as previously described (Khosrowshahian et al., 2005; Smoczer et al., 2012). Essentially, 4.6nL injections were made into the animal pole of embryos at the 1-cell stages for RNA collection and 1- or 2-cell stages for in situ hybridization. Injected embryos were cultured in 0.3 X MBS and 2% Ficoll-400 (Sigma) at 17°C for at least 1 hr to allow healing before being removed and allowed to develop at 12°C in 0.1 X MBS.

**RNA Preparation and Microarray Analysis**

At staged intervals, embryos were removed for RNA isolation, lysed, and processed in Trizol as per manufacturer’s instructions (Invitrogen). We then used DNaseI to remove genomic DNA, and ran the product over Qiagen RNeasy columns for purification. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA).

All GeneChips were processed from 2 biological replicates at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). Biotinylated complimentary RNA (cRNA) was prepared from 10 µg of total RNA as per the Affymetrix GeneChip Technical Analysis Manual (Affymetrix, Santa Clara, CA). Double-stranded cDNA was synthesized using SuperScriptII (Invitrogen, Carlsbad, CA) and oligo(dT)24 primers. Biotin-labeled cRNA was prepared
by cDNA in vitro transcription using the BioArray High-Yield RNA Transcript Labeling kit (Enzo Biochem, New York) incorporating biotinylated UTP and CTP. 15 µg of labeled cRNA was hybridized to *Xenopus laevis* GeneChips for 16 hours at 45°C as described in the Affymetrix Technical Analysis Manual (Affymetrix, Santa Clara, CA). GeneChips were stained with Streptavidin-Phycoerythrin, followed by an antibody solution and a second Streptavidin-Phycoerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 400. GeneChips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Signal intensities for genes were generated using GCOS1.2 (Affymetrix Inc., Santa Clara, CA) using default values for the Statistical Expression algorithm parameters and a Target Signal of 150 for all probe sets and a Normalization Value of 1. Normalization was performed in GeneSpring 7.2 (Agilent Technologies Inc., Palo Alto, CA). Data were first transformed, (measurements less than 0.01 set to 0.01) and then normalized per chip to the 50th percentile, and per gene to control samples for each stage. We performed two biological replicates and filtered the data based upon fold change with a cut off P-value set at 0.05.

**RT-PCR**

cDNA was made using Omniscript reverse transcriptase (Qiagen) and Oligo(dT)18 primers (Sigma) from 1µg total RNA for microarray confirmation and from 10uL mRNA further isolated (GenElute Direct mRNA Miniprep Kit – Sigma) for stage analysis of novel EST sequences. RT-PCR was performed at various annealing temperatures and cycle numbers, resulting in 5 time-points that were ultimately graphed. A cycle at the linear phase of amplification was selected for each gene and standardized against ODC.
Fold change for microarray confirmation was determined by comparing gene amplification of control-morpholino treated samples with *Pitx3*-morpholino treated samples. Primers and parameters are outlined in Table 3.

**Whole-mount In situ Hybridization**

*In situ* hybridizations were performed according to established protocols (Harland, 1991) using digoxygenin labeled riboprobes. We probed genes that were either two times up- or down-regulated as a consequence of *Pitx3*-morpholino perturbation, deemed by the microarray analysis. The probes used were generated from plasmids that were either the generous gifts of colleagues, the NIBB/NIG/NBRP *Xenopus laevis* EST project, or were purchased from ATCC (see Table). When a probe revealed a temporal and spatial expression pattern that overlapped with the known activity of *Pitx3*, further in situ hybridizations were conducted on specimens that had been unilaterally injected with morpholino (control- or *Pitx3*-morpholino) at the 2 cell stage: expression on the perturbed side could be compared to the contra-lateral control, and the trend predicted by the microarray thereby confirmed. Probes were prepared from vectors as outlined in Table 4.
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**Table 3:** Parameters and primer sequences used in RT-PCR experiments.
Table 4: Gene-specific information regarding restriction enzymes and RNA polymerases used to generate riboprobes for *in situ* hybridization experiments.

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**Identification of Novel Genes**

Some of the most differentially expressed but previously uncharacterized EST sequences were explored. Their spatial expression pattern was visualized via *in situ* hybridization and the temporal expression pattern was then investigated using RT-PCR throughout embryonic stages of development. Varied stages were utilized to determine specific developmental events: unfertilized egg (E) and stage 5 for maternal transcripts, stage 10 (early gastrula), stage 12 (neural anlage), stage 17 (onset of somitogenesis), stage 19 (neural tube), stage 24 (tail bud), stage 27 (lens differentiation), stage 31 (cardiac looping), stage 35 (blood supply) (Nieuwkoop and Faber, 1967). Phylogenic profiles and functional attributes were deduced using Blastp searches within GenBank and homolog alignments using the Megalign program of DNASTAR Lasergene 7.2.
Acknowledgements

Thanks are due to the Natural Sciences and Engineering Research Council (NSERC) of Canada for grant 203459 to MJC. FKS was supported by an NSERC PGS, and MW was partially supported by an Ontario Graduate Scholarship. The authors express gratitude to the numerous colleagues who provided us with probes, and particularly to Dr. Kitayama and Dr. Ueno, at the NIBB, without whose patient, generous, and numerous gifts this work could not have proceeded. Thanks are also extended to David Carter of the London Regional Genomics Centre, Robarts Research Institute, London, Ontario.
References


CHAPTER V
DIRECT TARGETS OF PITX3 IDENTIFIED USING A NOVEL CELL-SPECIFIC REPORTER ASSAY

Introduction

The Pitx gene family belongs to the OAR (Otx, Arx, Rax) subgroup of paired-like transcription factors (TF). In addition to a paired-like homeodomain, the genes encode a transactivation domain that may also participate in protein-protein interactions, as well as a nuclear localization signal (Medina-Martinez et al., 2009). One member of this family, Pitx3, is expressed in the Substantia nigra compacta area of the midbrain where it is responsible for the maturation and final differentiation of mesencephalic dopaminergic neurons and also for the subsequent regulation of the dopamine rate-limiting enzyme, tyrosine hydroxylase (van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005). Pitx3 also expresses in developing somites, lens placode, and in forming lens pit (Smidt et al., 1997; Semina et al., 1998; Smidt et al., 2004). In mice, Pitx3 has been identified as the causative locus for aphakia, a recessive deletion mutant resulting in small eyes that lack lenses (Semina et al., 1998). In humans, mutations are tied to defective differentiation of dopaminergic cells of the Substantia nigra, and to autosomal dominant anterior eye compartment dysgenesis and congenital cataracts (Semina et al., 1998; van den Munckhof et al., 2003). During myogenesis, both Pitx2 and Pitx3 participate in the differentiation of skeletal muscles (Coulon et al., 2007; L'Honore et al., 2007). In frog, pitx3 expresses additionally in pre-somitic mesoderm, lateral plate mesoderm, differentiating somites, craniofacial regions, and in looping heart and gut (Pommereit et al., 2001; Khosrowshahian et al., 2005; Smoczer et al., 2012).

Pitx3 is directly regulated by FoxP1 (Konstantoulas et al., 2010), myogenic helix...
loop helix proteins (Coulon et al., 2007), and is reciprocally interactive with miR-133b: Pitx3 activates the transcription of miR-133b and miR-133b inhibits translation of Pitx3 (Kim et al., 2007). Many other relationships have been inferred from mutant phenotypes but not proven by direct molecular analysis. Since Pitx1 and Pitx2 generate several different isoforms via differential promoter usage and alternative splicing (7 and 12 respectively) (Cox et al., 2002; Thierry-Mieg and Thierry-Mieg, 2006), we must entertain the possibility of multiple Pitx3 isoforms and heterodimerization.

Pitx3 is known to directly regulate tyrosine hydroxylase (Lebel et al., 2001), however transgenic studies document that Pitx3 is necessary but not sufficient to activate this gene (Zhao et al., 2004). Reporter assays of the tyrosine hydroxylase promoter give results that differ in a context-specific manner: Pitx3 can either activate or repress, presumably depending upon the availability of co-factors in the various cell lines utilized (Messmer et al., 2007). Other characterized targets of Pitx3 include: VMAT2 (vesicular monoamine transporter 2) and DAT (dopamine transporter) (Hwang et al., 2009); Aldehyde dehydrogenase 2 (Ahd2) (Jacobs et al., 2007); and MIP/Aquaporin O (an intrinsic protein of lens fibres) (Sorokina et al., 2011). Pitx3 has the ability to either activate or to repress target genes in a context-specific manner (Messmer et al., 2007). Clearly, the presence or absence of interacting partners must play a role in this regulatory specificity, however to date, Pitx3 interacting partners include only Sox15 (by yeast two hybrid) (Ravasi et al., 2010), SFPQ and NONO (by affinity capture) (Jacobs et al., 2009), and MTA1 and PARK7/DJ1 (by co-IP) (Reddy et al., 2011). So far there does not appear to be overlap among the partners identified for Pitx1, Pitx2, or Pitx3: this may, in part, explain their developmentally specific functions.
We have been studying the role of *pitx3* during *Xenopus laevis* embryogenesis where perturbation has an effect upon eye development as well as upon laterality (left-right organ asymmetry) and somitogenesis. Somite and laterality phenotypes are specific, and remarkably, they are elicited by both gain of function as well as by morpholino-mediated translational knockdown (Smoczer et al., 2012). We performed a microarray-based search for potential downstream target genes and defined a preliminary list of potential target genes based upon near-coincident timing and domain of expression. This list initially comprised roughly 80 candidates, however it was refined using RT-PCR followed by riboprobe *in situ* hybridization to those most likely to perform as legitimate *Pitx3* targets (Hooker et al., 2012). We then further selected a subset of 4 genes that possess *pitx3*-binding motifs in their respective promoter/enhancer regions (based upon elements identified in *X. laevis* or *tropicalis* sequences and conserved in mammalian species). All four are likely to play a role in one or more of patterning the eyes, somites, or early patterning of asymmetry. The 4 newly identified putative target genes possess between 4 to 13 *pitx3* binding motifs (*lhx1, gsc, nodal5, crybb1*), suggesting the possibility for an interaction between this transcription factor and the respective promoter sequences.

A drawback of most reporter assays is that reporter gene expression is assayed in a heterologous population of transfected and untransfected cells, where estimation of the ratio between populations is difficult. To circumvent this shortcoming, a dual luciferase reporter assay was developed where in addition to the reporter vector another bioluminescent gene driven by a constitutive promoter was introduced to serve as control for transfection efficiency (Stables et al., 1999). Although widely employed, this assay
relies upon the presumption that both vectors have identical or at least similar transfection properties. Finally, since lysates function to homogenize and average cellular results of transcription factor activity, it is hard to assess quantitative effects on a per-cell-basis. For example, some of our putative targets have multiple candidate response elements and transcription factor cooperativity would not be easily discerned using standard assays (Beachy et al., 1993).

In order to address these shortcomings, we devised a novel flow cytometry-based protocol that works exquisitely well to link transcription factor input to promoter reporter output on a cell-by-cell basis. By counting only those cells that are co-transfected, we can estimate how promoters work even if responses are non-linear. The system relies upon co-transfection of two plasmids: one comprises a CMV-\textit{eGFP} IRES unit that is bicistronically linked to the cDNA for \textit{Pitx3} (input); the other houses CMV-\textit{HcRed1} in opposite orientation to a test-promoter driven reporter, \textit{DsRed} (output). Since only those cells that are co-transfected are analyzed, differences in transfection efficiency between treatments are rendered irrelevant. In addition, a ratio between the two transfected plasmids can be generated for each cell. As proof of principle, we carefully calibrated our system against a well-characterized promoter, murine \textit{tyrosine hydroxylase (TH)}. We have defined the range of transfection parameters within which the system reports with fidelity and in linear fashion – in other words, the range at which GFP does not accumulate and fluoresce more than \textit{pitx3} is detectable on Western blots.

We can confirm three new direct targets for \textit{pitx3} and show that the factor acts either as an activator or repressor, contingent upon the context of its environment, including the promoter at hand. \textit{Pitx3} can repress both \textit{crybb1}, a lens-specific
differentiation marker, and \textit{nodal5}, an early inducer of mesendoderm formation, while it activates \textit{lhx1}, a factor present early in the Spemann organizer and later in the pronephric kidney (Taira 1992). Previous work has indicated that \textit{pitx3} expresses in fish hypoblast (Dutta et al., 2005), and somewhere in \textit{Xenopus} pre-gastrula (RT-PCR, uncharacterized and low-expression location) (Khosrowshahian et al., 2005), and based upon our preliminary slate of putative signaling targets, our suspicion is that \textit{pitx3} plays a heretofore uncharacterized role during gastrulation.

\textbf{Results}

\textbf{Construction of the expression and reporter vectors.} Our system relies on two participating plasmids. The first is a bicistronic expression vector, which harbors the transcription factor \textit{pitx3} and \textit{GFP} (Figure 1A) and simultaneously produces two proteins from a single mRNA transcript (Trouet et al., 1997). A corresponding \textit{pitx3} binding mutant was constructed by inserting a mutated form of \textit{pitx3} as the first coding sequence of the bicistronic unit. The L99P amino acid substitution within the DNA-binding homeodomain was modeled after one described for \textit{mix1}, shown to hinder binding of the transcription factor to its target DNA sequences and thereby serves as a dominant inhibitor of normal activity (Mead et al., 1996). The second vector harbors the promoter reporter and a transfection calibration fluor (Figure 1B). Mutants were also generated for promoters to serve as specificity controls by prohibiting \textit{pitx3} binding: \textit{Th} mutant (-350bp from ATG: TAATCC to TAccCC), \textit{lhx1} mutant (-709bp from ATG: TAATGG to TccaTGG), \textit{nodal5} mutant (-94bp from ATG: TAAGCT to TcgaCT), \textit{crybb1} mutant (-1156bp from ATG: ACATTA to AgcTTA).
Figure 1: Expression and reporter plasmids. (A) Expression plasmid with pitx3 bicistronically linked to eGFP. (B) Reporter plasmid with the reporter gene DsRed-express driven by the tested promoter, cloned in opposite orientation from the transfection control gene HcRed1 driven constitutively by CMV.
Calibration of pitx3 relative to GFP in cells transfected with the bicistronic expression plasmid. In order to ensure the reliability of the system, we established the correlation between the levels of the two proteins produced by the bicistronic vector. We assessed the ratio of GFP and pitx3 in two separate experiments: one to determine plasmid concentration dependence, and a second to ensure that the ratio remains constant over time.

HEK293 cells were transiently transfected with four different 1.3 fold dilutions of pPitx3-IRES-GFP and assessed by Western blotting. This series allowed the maximum number of dilutions resulting in observable protein by pitx3 antibody. The protein band intensities for GFP and pitx3 proteins were compared and linear regression analysis reveals a strong and consistent correlation between the two proteins across all concentrations (Figure 2A).

Moreover, at these transfection concentrations both proteins have parallel accumulation rates across time. A set amount of pPitx3-IRES-GFP was transfected into HEK293 cells and cell lysates were collected at 24 hours, 36 hours and 48 hours. The ratio between the pitx3 and GFP proteins levels is constant, with no statistically significant differences between time-points (Figure 2B). However it can be noted that a reduction in the ratio between pitx3 and GFP protein levels at the 48 hour time-point could suggest unequal degradation rates for the two proteins.
Figure 2: Correlation between the pitx3 and GFP proteins. The proteins were assayed by Western blotting and the amount of each protein was assessed as the optical density of the respective band. (A) Regression analysis to correlate the levels of pitx3 and GFP proteins in cells transfected with different concentrations of expression vector. (B) Ratios between the levels of pitx3 and GFP protein in cells transfected with a set concentration of expression vector and analyzed at 24, 36 and 48 hours post-transfection.
Figure 3: Correlation between GFP protein and GFP fluorescence. The GFP protein levels were determined by Western blotting and evaluated as the optical density of the band on the blot. A percentage of the total cells were used to detect the fluorescence using flow cytometry. (A) Regression analysis to correlate GFP protein levels and GFP fluorescence in cells transfected with 4 decreasing concentrations of expression vector by 1.3 fold. (B) Linear regression between the GFP protein and fluorescence in cells transfected with equal concentrations of expression vector and evaluated at 3 different times post-transfection.
**GFP protein concentrations correlate with GFP fluorescence in transfected cells.**

The total fluorescence for each population of transfected cells in the dilution and time-point experiments was plotted relative to the GFP protein band intensity analyzed by immunoblotting. This determines if changes in GFP fluorescence are accurately reflecting changes observed at the protein level. In triplicate experiments, regression analysis revealed a very strong correlation between GFP protein and fluorescence irrespective of the amount of vector that was transfected or post-transfection time of analysis (Figure 3A and B).

**Flow cytometry protocol for the three-fluor reporter assay.**

For acquisition of accurate signals from each fluorescent protein, we developed an optimal flow cytometry protocol to separate the three fluors into discrete channels with minimal spectral overlap. The forward versus side scatter data is used to restrict the selection solely to viable cells. Each fluor is analyzed in a separate control and the appropriate voltage necessary for optimal fluor excitation is established (Table 1). As controls to set-up experimental parameters, we used cells transfected separately with each of the vectors pIRES-GFP, pHcRED1, pDsREDN1, as well as with a combination of the pIRES-GFP and pHcRED1 empty vectors. The GFP signal is collected in FL1, the HcRED1 in FL5 and the DsRed in FL2. (Figure 4A and B) This allows us to gate on each fluor in order to minimize background fluorescence and to establish proper compensation for each signal to reduce spillover into other channels. These controls were run prior to each individual experiment. From the cells that were co-transfected with both GFP and HcRED1 control vectors we collected $10^4$ cells in the gate with active signal for both fluors (Figure 4C) and this co-expressing population was plotted on a FL2 histogram to
Figure 4: Flow cytometer set-up to detect the three fluors in the new reporter assay. (A) Gate set-up for eGFP in FL1 for cells transfected with the pCI-Neo/IRES-eGFP control vector (quadrant G4). (B) Cells transfected with pCS2-HcRed1 control plasmid, recorded in FL5 and gated for HcRed1 expression (quadrant G1). (C) Gated population of 10,000 cells expressing both eGFP and HcRed1 (quadrant G2). (D) Histogram of DsRed output in FL2 for the population of eGFP and HcRed co-expressing cells.
collect the total background DsRed fluorescence that was subsequently subtracted from each experimental data set (Figure 4D).

The final step for each reporter experiment was to assess the level of cooperativity of the transcription factor on the tested promoter. The cells expressing all three fluors were represented on a dot-plot with the DsRed as ratio of HcRed fluorescence: this accounted for the amount of promoter plasmid transfected (reporter output) correlated to GFP fluorescence (transcription factor input). A linear regression of the analysis permits us to discriminate between the possibilities of cooperative or linear modes of activation or repression.

**Calibration utilizing the previously characterized Pitx3 and Tyrosine hydroxylase interaction.** To test our new technique, we used the well-studied activity of pitx3 upon the *tyrosine hydroxylase (TH)* promoter. The two players in our system include the 1.5kb mouse *TH* promoter, which is sensitive via an active Pitx3 binding site (Lebel et al., 2001), and the *Xenopus* pitx3 coding sequence. The homeodomains of murine and frog Pitx3 are identical. The HEK293 cell line was used, where Pitx3 is known to act as a repressor for *TH* (Cazorla et al., 2000). The endogenous levels of the *TH* reporter were found to be very low in this cell line and therefore the repression induced by pitx3 was very small, although significant. Given the strong *TH* activation by cyclic AMP independent of pitx3 (Cazorla et al., 2000), we chemically activated the *TH* promoter with forskolin and thus allowed for a potentiation of Pitx3 repressive activity. Using the novel reporter assay, we show that pitx3 represses *TH* output by approximately 80%, while the pitx3 homeodomain mutant (BM) leaves expression unchanged (Figure 5A).
Figure 5: Calibration of the new technique using the known pitx3 – tyrosine hydroxylase (TH) interaction. (A) Pitx3 represses TH in HEK293 cells, under both basal and forskolin-treated conditions. The pitx3 binding mutant (BM) restores the constitutive expression of TH. (B) The mutant for the known Pitx3 binding site on the TH promoter prevents the repressive activity of pitx3 on TH in forskolin-treated cells. (C) Transcription factor cooperativity assessed in cells expressing all three fluors, by plotting the normalized DsRed output to the GFP input and determining the generated trendline. (*) p<0.05 (**) p<0.01 (*** p<0.001 (****) p<0.0001.
Conversely, by site-directed mutagenesis we mutated the known Pitx3 binding site within the TH promoter (Lebel et al., 2001) and, as expected, pitx3 has no significant effect on TH promoter activity in the absence of this particular binding site (Figure 5B). Plotted cells expressing both the expression and the reporter vectors and subjected to linear regression analysis reveal no cooperativity but rather an all-or-nothing repression (Figure 5C).

**Promoters tested as novel direct targets of pitx3.** Selected genes were part of a data set generated in a pitx3 morpholino knockdown microarray experiment. *Xenopus laevis* promoters for *lhx1, gsc, nodal5* and *crybb1* were cloned into the reporter plasmid and when assessed for reporter activity we determined three direct targets of pitx3. *Lhx1* is significantly activated by pitx3 (Figure 6C), while *crybb1* is inhibited in the context of HEK293 cells (Figure 7B). The basal activity of the *nodal5* promoter did not allow for a conclusive assessment, and therefore required an initial activation by *vegT*. Following this activation, we could observe a small, though significant and consistent inhibition of the *nodal5* reporter activity when co-transfected with pitx3 (Figure 6B). *Gsc* showed no significant transcriptional regulation by pitx3 in this environment (Figure 6D). To determine the site responsible for pitx3 binding in each targeted promoter, we started by searching the ENSEMBL.org database for the respective promoter sequences in *Xenopus tropicalis* and zebrafish. They were subsequently aligned using the MULAN software (Ovcharenko et al., 2005) and searched for conserved known Pitx3 binding sequences (TAAT(C/G)N) (Lebel et al., 2001). The sites that were found to be conserved were mutated by site-directed mutagenesis and assessed for where pitx3 binding effects
**Figure 6**: New pitx3 targets in early embryonic development. (A) Graphic representation of the tested promoters with the location of the putative Pitx3 binding sites. Promoter sequences for *X.laevis*, *X.tropicalis* and *D.rerio* were aligned using the MULAN software and conserved binding sites in 2 (*) or 3 (**) organisms are marked on the diagram. Graphs are not to scale. (B) *Nodal5* is repressed by pitx3 by approximately 20% and the pitx3 binding mutant abolishes the repression. By site-directed mutagenesis we have found the site responsible for binding pitx3 at –94bp upstream of ATG. The mutated promoter becomes unresponsive to the repressive activity of pitx3. (C) Pitx3 activates *lhx1* promoter and induces a 50% increase in DsRed output, while the pitx3 binding mutant reverses this effect. The site located at -709bp upstream of the translational start site is found responsible for binding pitx3, since an induced mutation here restores the basal promoter levels. (D) *Gsc* promoter does not show significant response due to pitx3 in HEK293 cells, nor does it display conserved putative Pitx3 binding sites (A). (*) p<0.05  (**) p<0.01 (***) p<0.001 (****) p<0.0001.
**Figure 7:** New pitx3 target during lens development. (A) *Crybb1* promoter represented as a diagram with the location of both conserved and non-conserved possible Pitx3 binding sites. The position of the deletion (SpeI) and site-directed mutagenesis mutants is also shown (arrows). (B) Pitx3 inhibits the *crybb1* activity by 50%, while its binding mutant recovers this effect. (C) Mutant A eliminates the last 750bp of the promoter, harboring 6 possible Pitx3 binding sites, and is shown to not contribute to the binding of pitx3. Mutants B and C were created by site-directed mutagenesis of conserved binding sites and we show that the site responsible for the binding of pitx3 is the sequence obliterated in mutant C. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$ (****) $p<0.0001$.

**Figure 8:** Cooperative mode of action for pitx3 on the new targets. A major benefit of the new reporter assay is the possibility of assessing cooperativity of transcription factors. The major conclusion is that pitx3 does not act in a cooperative way on the tested promoters, producing a linear all-or-nothing effect.
(Figures 6A and 7A). In the case of all three mutated promoters, pitx3 influence on the reporter activity can be abolished and the DsRed output returns to basal levels. Pitx3 input and the reporter output was linear for each of the influenced promoters, pointing towards the absence of pitx3 cooperativity in the regulation of these genes (Figure 8).

Discussion

We have developed a novel and innovative reporter technique and tested its efficacy using a known Pitx3 interaction before then utilizing the assay to assess new potential targets for this transcription factor. An IRES plasmid could introduce a few variables since the two separately translated proteins might be post-translationally modified at different rates. Moreover, the translated products could saturate and degrade at different rates. Before making this plasmid a component of our system, we ensured that the detected GFP fluorescence accurately reflects the titers of pitx3 protein present in cells, by demonstrating that the ratio between pitx3 and GFP is a reliable parameter within the concentration ranges deployed, and was independent of concentration and time of analysis (Figures 2 and 3).

The novelty of the technique is enhanced by the introduction of a reporter plasmid which itself contains a constitutively driven fluorescent protein, HcRed1, to serve as an indicator for transfection efficiency. Flow cytometry permits us to gate such that we analyze only the populations that are co-transfected, and the analysis delivers quantitative data regarding transcription factor concentrations (input) and candidate promoter reporter activity (output) (Figure 4).
To calibrate the specificity and sensitivity of the newly developed method, we tested the interaction between Pitx3 and the tyrosine hydroxylase promoter. Pitx3 operates by association with other co-factors such as MTA1 and Nurr1 to ensure efficient activation of TH (Cazorla et al., 2000; Reddy et al., 2011), and therefore the outcome of this interaction is highly dependent upon the cellular context (Messmer et al., 2007; Medina-Martinez, 2010). We chose the HEK293 cells line where the interaction has been previously analyzed by luciferase assay and where Pitx3 is known to inhibit the TH transcription (Cazorla et al., 2000). In order to increase the basal activity of the TH promoter we used forskolin to boost the levels of cAMP, which is known to bind to the cAMP-response element (CRE) on the TH promoter and induce its activation (Cazorla et al., 2000). Our data confirms a 70-80% repression by Pitx3 in both basal and forskolin-activated states; levels identical to those observed by luciferase assay (Cazorla et al., 2000) (Figure 5A). Mutating a site known to be responsible for Pitx3 binding (Lebel et al., 2001), we were able to also confirm the specificity of our technique by prohibiting pitx3 interaction with the TH promoter (Figure 5B). Finally, we further confirmed specificity by testing a pitx3 binding mutant to show that the mutated homeodomain cannot induce transcriptional repression. These results also suggest that the regulation of dopamine production is conserved across species.

We investigated four genes as possible direct pitx3 targets: lhx1, nodal5, gsc and crybb1, based on the three requirements for a transcription factor (TF)-target relationship to be considered direct (Loose and Patient, 2004). They are all affected by the pitx3 knockdown (changes assessed by in situ hybridization, RT-PCR or both (Hooker et al., 2012), their expression patterns overlap with pitx3 (either during early gastrulation or
during lens development), and all contain multiple putative binding sites in the analyzed promoters.

*Nodal5* is a *Nodal* related ligand/signaling molecule that controls the early mesendoderm induction program (Luxardi et al., 2010). Its expression begins at stage 8.5 and ends around stage 10 (Takahashi et al., 2000), leaving a very short timeframe during early development for a possible interaction with *pitx3*. *Nodal5* represents the first zygotically expressed gene activated by maternal factor *vegT* and it in turn activates *nodal1* and *nodal2* in a feed-forward system that influences the expression of *gsc* and *lhx1* (Luxardi et al., 2010; Skirkanich et al., 2011). We show here that *nodal5* is a direct target of *pitx3*, repressed by 20% in HEK293 cells and we identify the critical one of three putative *Pitx3* binding sites in the 775bp tested promoter located at -94bp from ATG (Figure 6A and B).

*Lhx1* is a LIM-class homeodomain TF that is expressed in two waves, the first at early gastrula in Spemann’s organizer, and the second during tailbud stages in the pronephric kidney and brain (for-, mid-, and hind-brain) where it is responsible for the maintenance of the differentiated state of the neural tissue (Taira et al., 1992; Cirio et al., 2011). Our experiments do not distinguish between these two developmental phases, however *lhx1* shows a strong 50% activation by *pitx3* in the reporter assay and a highly conserved binding site located at -709bp from the translational start site seems to be responsible for this interaction (Figure 6A and C). Pitx3 may therefore exert both a direct and indirect regulation of *lhx1* (by also controlling via *nodal5* activity), however we cannot conclude if this occurs concomitantly or differentially in a tissue-specific manner.
Gsc (goosecoid) is a homeodomain TF, known as an organizer gene since it is capable of producing axis duplications and of executing organizer functions when mis-expressed in ventral cells (Cho et al., 1991). It is expressed as early as stage 8.5 and is not detectable once neurulation begins (Cho et al., 1991). Gsc expression is initially induced in the organizer by dorsalizing Wnt signals and it is then maintained through direct regulation by lhx1 (Taira et al., 1992; Mochizuki et al., 2000). Using our reporter assay to test 1.4kb of the gsc promoter upstream of the translational ATG start codon, we observed no changes in HEK293 cells that can be ascribed to the presence of pitx3, despite 9 putative homeodomain binding sites (Figure 6A and D). Looking at the genetic pathway of the early patterning, we can explain the changes observed in the gsc embryonic expression (Hooker et al., 2012 submitted) in the context of gsc being a player in the pathway governed by nodal5 and lhx1, and therefore an indirect target of pitx3. That said, we are planning to assess the promoter’s activity in other cell lines.

Crybb1 is a lens specific marker, and it represents a major structural protein of the lens. Expression of crybb1 begins in the lens around stage 26 and increases over time until stage 38, when its expression gradually starts to decrease to stable but lower levels in the differentiated primary and secondary fiber cells of the adult lens (Altmann et al., 1997; Zhao et al., 2011). The overlapping expression of pitx3 in the lens and the loss of crybb1 expression in the lenses of pitx3 morphants make it a good candidate for direct interaction (Hooker et al., 2012). Using a 3.5kb promoter previously tested in vivo by transgenesis to reproduce crybb1 expression patterns (Mizuno et al., 2005), we were able to demonstrate direct regulation by pitx3. A 50% decrease in reporter output by pitx3 is maintained in a variety of tested promoter mutants, however the mutation of one conserved site located at
–1165bp from the ATG, in close proximity to the pax6 and prox1 binding sites (Mizuno et al., 2005), abolishes repression by pitx3 (Figure 7).

The described reporter assay is unique in that it has the potential to reveal information regarding cooperativity of transcription factors upon tested promoters, by quantifying the amount of protein and promoter availability present in each cell in relation to promoter output. Cooperativity is a well known process used by transcription factors to enhance binding specificity and subsequently increase their effect on the transcription of the target gene in a combinatorial manner (Courey, 2001). Once the binding of one TF monomer occurs it induces conformational changes in the DNA to facilitate the binding of a second TF on a nearby binding site, through dimerization (Courey, 2001). This results in a sharp increase in transcriptional response even in the smallest changes of the monomeric TF concentration (Georges et al., 2009). Since we examine a homogenous population of cells expressing both the TF and the target promoter, we can easily correlate any increase in TF concentration with the reporter output and draw conclusions regarding cooperativity. Despite the wide array of information regarding homodimerization in Pitx2 (Saadi et al., 2003) and cooperative regulation of transcription by bicoid genes (Beachy et al., 1993), Pitx3 appears so far to operate as a monomer on target genes (Sakazume et al., 2007). Also, it is known that bicoid proteins bind cooperatively to head-to-tail and tail-to-tail DNA target sites separated by 7 to 36bp and to head-to-head sites separated by only 3bp (Yuan et al., 1999). Since none of our tested promoters have neighboring binding sites that meet these criteria, the lack of observable cooperativity in our tested promoters is perhaps not surprising (Figure 8).
Making use of different fluorescent proteins, spread over a wide range of excitation and emission ranges, and the powerful tool of flow cytometry, we created a new tool to evaluate the output of a reporter gene on a cell-by-cell basis. In essence, each cell harbors an individual reporter assay, producing a cumulative, extremely accurate result that is derived from a selective and homogenous population. The assay also confers the benefit of permitting analysis in cases where high transfection efficiency is not possible while also permitting the detection of very slight variations of reporter output that would not be distinguishable by conventional methods.

Materials and Methods

Plasmid constructs

Expression plasmid (pPitx3-IRES-GFP). The pitx3 coding sequence was PCR-amplified from pBSK-Pitx3 homegrown plasmid (NM_001088554) with primers harboring adaptors for XhoI and EcoRI, and cloned into the pCI-Neo/IRES-GFP [F64L/S65T] bicistronic vector (kindly provided by Dr. J. Eggermont). The rationale for using a bicistronic vector as opposed to a fusion protein lies in the known intramolecular folding that occurs in the Pitx2 protein. In the absence of cofactors binding to it, the C-terminal region of the protein comes in direct contact with the N-terminus and masks the homeodomain preventing the transcriptional activation of the target genes (Amendt et al., 1999). A DNA binding mutant (BM) was produced through site-directed mutagenesis, by mutating the leucine into a proline at position 39 of the pitx3 homeodomain sequence – in the hinge region between helix II and III (L99P).
Reporter plasmid. The pCS2-HcRED1 vector was generated through PCR-amplification of the HcRED1 sequence from pCAG-HcRED1 (Add Gene collection) and subsequent ligation into the XhoI/ClaI sites of pCS2-. The reporter cassette was built by PCR-amplifying 1.5kb upstream from ATG of the murine tyrosine hydroxylase promoter off the 3805-4 mTH vector (kind gift from Dr. R. Palmiter). The amplicon was sub-cloned into the EcoRI/SmaI restriction sites of pDsRED-express-N1 (Clontech). Subsequently the mTH-DsRed-express reporter cassette was PCR-amplified out of the previous vector and cloned in opposite orientation to HcRED1 using the SacII/KpnI restriction sites of a second multiple cloning site of pCS2-HcRED1. This produced the dual-fluor vector pHcRED1/mTH-DsRed. For a control, a critical Pitx3 binding motif (underlined) in the TH promoter (Lebel et al., 2001) was mutated (small case) to form a KpnI site (bold) (CTTGGGTAATCCAGC → CTTGGGTAccCCAGC).

Lhx1 promoter and mutant (pHcRed/lhx1-DsRed) The lhx1 reporter plasmid was created by PCR-amplification of the lhx1 promoter from plasmid xLim1:luciferase Ex-1:A (kind gift from Dr. Igor Dawid) and cloned into EcoRI and BamHI sites of pDsRED-express-N1. The lhx1:DsRED transcription cassette was again PCR-amplified and blunt cloned in reverse direction into the PvuII site of pCS2-HcRED1. An lhx1 mutant promoter was generated via site-directed mutagenesis utilizing mutated oligonucleotides to introduce an NcoI restriction site (GTGCTTAATGGTTTA → GTGCTccATGGTTTA).

Nodal5 promoter and mutant (pHcRed/nodal5-DsRed) The nodal5 promoter was PCR-amplified using adaptors for KpnI and BamHI off Xenopus laevis gDNA template isolated from adult Xenopus laevis liver. The resulting 773bp amplicon (-12 to -785 from ATG) was cloned into pDsRed-express-N1. The nodal5:DsRed transcription cassette
was PCR-amplified off nodal5-pDsRed-express-N1 template using adaptors for KpnI and SacII and cloned into pCS2:HcRed1 in opposite orientation. Site-directed mutagenesis was used to create the nodal5 mutant promoter, introducing a novel SalI site (TGAAGTAAGCTTCTG\textarrow{TGAAGTcgaCTTCTG}).

\textit{Gsc} promoter (pHcRed/gsc-DsRed) The \textit{gsc} promoter was PCR-amplified from -1553gsc pOLuc (kind gift from Dr. K.Chow) using adapters for KpnI and BamHI and ligated into corresponding restriction sites of pDsRed-express-N1. The \textit{gsc}:DsRed transcription cassette was again PCR-amplified using gsc-pDsRed-express-N1 as template and inserting adaptors for KpnI and SacII whereupon it was cloned into the pCS2:HcRed1 vector in opposite orientation.

\textit{Crybb1} promoter and mutants (pHcRed/crybb1-DsRed) The \textit{crybb1} reporter cassette was generated by cloning the 3.5kb Sacl/ApaI digested promoter out of the \textit{X. laevis} \textit{βB1-Crys} promoter (kind gift from Dr. H. Kondoh) into the multiple cloning site of pDsRED-express-N1. The transcription cassette was PCR amplified, cloned into the \textit{PvuII} site of the pCS2-HcRed1 and selected for a reporter cassette inserted in reverse orientation to the CMV-HcRed1. \textit{Crybb1} mutant A was generated by deleting the last 750bp containing six binding sites with restriction enzyme \textit{SpeI}. \textit{Crybb1} mutants B and C were produced by site-directed mutagenesis using mutated primers to introduce new \textit{EcoRV} and \textit{HindIII} restriction sites respectively (GTACTGCATTATCAA \textarrow{GTACTGCgaTATCAA} and TTAAAAACATTATTTTC \textarrow{TTAAAAcTTATTTTC}).

All vectors were sequenced for verification of cloning and mutagenesis accuracy.

Plasmid DNA was purified using Qiagen Maxi/Midi preparation columns.
**Cell cultures** HEK293 cells (kindly gifted by Dr. O. Vacratsis) were cultured in high glucose DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum (Invitrogen) and Penicillin-Streptomycin (Sigma-Aldrich), 500UI Penicillin and 500ug Streptomycin, under standard conditions.

**Transient transfections** HEK293 cells were split 24 hours prior to transfection and were 40% confluent at the day of transfection. For the reporter assays, cells grown in 100mm dishes were transfected with 13ug DNA in 750uL DMEM with 25uL 1mg/ml polyethylenimine (Sigma). A combination of 9ug: 4ug reporter vector to expression vector was found to be optimal for the flow cytometric detection of both GFP and HcRed transfection control fluors. The DNA-PEI complexes were introduced to cells in plain media and 4-6 hours post-transfection the serum-free media was replaced with complete media. To the *tyrosine hydroxylase* experiments 10uM forskolin (LLC Lab) was added after 24 hours and cells were analyzed 48 hours post-transfection. To increase the basal activity of the *nodal5* promoter, 2ug of *vegT* plasmid was transfected together with 9ug of reporter vector and 2ug of expression plasmid. For dilution experiments, various concentrations of expression vector were transfected in combination with corresponding titres of pCS2- to total 13ug of DNA. 48 hours post-transfection cells were trypsinized and separated: 2ml were reserved for flow cytometry and 8ml for protein isolation. Time-point experiments were conducted similarly, with cells transfected with 13ug of DNA and analyzed 24, 36 and 48 hours post-transfection by flow cytometry and Western blotting.

**Immunoblotting** Total protein was isolated from cell lysates and 50ug was loaded for SDS-PAGE. Proteins were detected as follows: 32kDa pitx3 1:2000 (ProSci Inc. 1°)
Rabbit Antibody: PAS 3131/3132), 47kDa a-actin 1:10,000 (Sigma 1° Rabbit Antibody: A2066), 27kDa eGFP 1:5000 (Torrey Pines Biolabs Inc. 1° Rabbit Antibody: TP401), Chemicon International 2° Goat Antibody: AQ132P (1:10,000). Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) using an Alpha Innotech imager equipped with AlphaEase Fluor Chem HD2 software.

Flow Cytometry Transfected cells grown for 48 hours in a dark environment were washed with PBS, trypsinized, and re-suspended in the appropriate volume of PBS to conduct flow cytometry utilizing a Beckman Coulter Cytomics FC500 system and the filter/detector system in Table 1 for maximum detection and separation of the three fluors used. Both the uniphase Argon ion and coherent red solid-state diode lasers were enabled. Using CXP software (Beckman Coulter), forward and side scatter enabled the gating of viable single cells. Samples containing each plasmid transfected individually were employed to set gates for the respective fluor, to subtract background fluorescence, and to allow for compensation of their overlapping emission spectra. For each treatment, 10,000 co-transfected cells expressing both GFP and HcRed1 were collected and the total fluorescence intensity for the reporter gene DsRed was calculated. The ratio between fluorescence intensities for the promoter reporter DsRed and its in-vector transfection control gene, HcRed, were related to the fluorescence intensity for GFP (indicative of transcription factor pitx3) using Weasel software (Walter and Eliza Hall Institute of Medical Research). All experiments were conducted in triplicate.
<table>
<thead>
<tr>
<th>Channel Detector</th>
<th>Fluorescent Protein</th>
<th>Colour</th>
<th>Excitation Peak ($\lambda_{\text{max}}$)</th>
<th>Emission Peak ($\lambda_{\text{max}}$)</th>
<th>Filter</th>
<th>Voltage</th>
<th>Gain</th>
</tr>
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<tbody>
<tr>
<td>FL1</td>
<td>eGFP [F64L/S65T]</td>
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<td>510nm</td>
<td>525BP</td>
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<td>579nm</td>
<td>575BP</td>
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<tr>
<td>FL5</td>
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<td>Far-red</td>
<td>588nm</td>
<td>618nm</td>
<td>640LP</td>
<td>500</td>
<td>2.0</td>
</tr>
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**Table 1: Flow cytometry system standardization.** Different types of optical filters (Band-pass (BP) and long-pass (LP)) are employed to achieve optimal fluor separation.

**Statistical calculations** SPSS software was used to assess statistical differences in the total DsRed fluorescence generated in the different conditions of the reporter assay. To determine the effect of pitx3 on a promoter, we used a one-way ANOVA test corroborated with a contrast test to compare the basal levels of the promoter reporter. This was assessed after pitx3 exposure following co-transfection with the wild type or homeodomain binding mutant (BM). For the binding site mutants we employed a T-test to compare the DsRed output of the mutant under basal conditions with the one exposed to pitx3. Tests were considered significant when p<0.05.
References


CHAPTER VI
CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Introduction

6.1.1 *Pitx3*: Novel Expression Patterns/Characterization of Disruption

The data represented in this thesis was elicited by unexplained phenotypes that were observed in *Xenopus pitx3* morphants. Moreover, I identified *pitx3* expression in tissues that have yet to be studied for a functional role for the gene. *Xenopus pitx3* expression is unique compared to mouse in these tissues (Semina et al., 1997; Pommereit et al., 2001; Khosrowshahian et al., 2005) suggesting that some functions of *Pitx3* may be species-specific.

The *pitx3* morphant presents the phenotype of a bent dorsal axis presumably resulting from de-regulated somite development (Chapter 1 Figures 5A-F; Chapter 2 Figures 1A-E), which has also been reported in zebrafish *pitx3* morphant embryos (Shi et al., 2005). The somitogenesis program in both zebrafish and *Xenopus* are dissimilar from that of chick and mammals (Eckalbar et al., 2012). This might explain why disruption elicits similar somitogenesis complications in these two species, and account for the lack of similar reported phenotypes for mouse or human *Pitx3* mutations. This also shows that the function of *Pitx3* has become refined through evolution, possibly losing a role in somitogenesis as the segmentation program evolved before the branch of mammals and birds. A behavioural phenotype that coincides with the disruption in somite patterning is erratic twitching rather than sigmoidal swimming patterns.

We also report the discovery of a very early phenotype of *pitx3* morphants presenting at the early gastrula stage (Chapter 3 Figure 1), where involution appears to be
perturbed and results in disrupted expression of mesodermal genes (Chapter 3 Figure 3). Disruptions to the laterality patterning of the internal organs, specifically heart and gut, are also explored (Chapter 2 Table 2). Such morphogenetic problems have not been reported in mouse mutant models or humans with PITX3 mutations. However, these phenotypes may not have been detected if the phenotypes led to embryonic lethality in mammalian embryos that require a functional embryonic heart at early stages of development for survival. Moreover, the morpholino-mediated knockdown in amphibian embryos can allow for partial knockdown phenotypes to be observed, thereby highlighting functional roles in areas that would have otherwise been missed in complete or embryonic lethal knockdown models.

In frogs, gastrulation stage expression of pitx3 may explain some of the phenotypes reported, yet never explored for function. In Xenopus, pitx3 is detected as early as stage 8 by Northern blot analysis, when the mid-blastula transition is underway (Khosrowshahian et al., 2005). RNA blots are more sensitive to the broad expression of transcripts that are present in low concentration, due to cumulative amplification, where early pitx3 expression is only just detectable by in situ hybridization (Chapter 3 Figure 2). Early pitx3 expression has also been reported in zebrafish (Dutta et al., 2005).

6.2 Mechanisms That Underlie Homeobox Genes and Patterning

Comparisons of Pitx3 with better-characterized homeobox genes provide ample fodder for speculation and future directions. There are many examples of homeobox genes that play a role in patterning the early embryo and that endow different regions with positional information. For example, a family of homeobox genes that confer
positional information in the developing embryo are the Hox genes. This evolutionarily conserved family of homeotic transcription factors operate to pattern the anterior-posterior axis and give identity to different segments of the embryo pertaining to which combinations of Hox genes are expressed: the Hox Code (Kessel and Gruss, 1991). Homeodomain genes can operate by either regulating the development of non-repeating structures or to give positional information to structures that are repeated (Richardson et al., 1998). Since Pitx3 is a member of the family of homeodomain transcription factors, we might draw on what is known for other members to help explain how Pitx3 operates with respect to specific gene regulation as well as coordinating gene networks.

6.2.1 Pitx3: Implications of Binding Motif Distribution and Structure

In Drosophila Ultrabithorax (Ubx) is known to provide positional information to specific segments of the developing larvae by directly binding cognate DNA sequences in the promoters of downstream genes (Beachy et al., 1988). Ubx has been shown to present as a homodimer in solution and it binds tandemly arrayed response elements (Beachy et al., 1988). The binding sites of Ubx in the promoter of Antennapedia contain multiple tandem repeats of tri- and hexanucleotide sequences, and multiple Ubx proteins bind cooperatively to these clusters of DNA sites (Beachy et al., 1988; Beachy et al., 1993).

When looking at putative downstream targets of pitx3, we searched in silico for potential binding sites within the promoter regions (Chapter 4 Table 1). Many of those promoters showed clusters of binding sites that might allow for pitx3 to operate in cooperative fashion like Ubx. However in all promoters tested by our lab thus far (Th, nodal5, lhx1, crybb1), it appears that only one hexameric binding site is required for
transcriptional regulation by pitx3 (Chapter 5). Perhaps different cellular environments
would allow for more complex gene regulation. Future gel mobility shift assays and
immunoblotting will be required to determine if pitx3 acts as a monomer, dimer or
multimer.

6.2.2 Pitx3 Binding Dynamics Need to be Clarified

As the founding member of the K50 class of homeodomain proteins, the
_Drosophila_ morphogen _Bicoid (Bcd)_ acts as a model transcription factor for _pitx3_
behaviour. _Bcd_ originated from a duplication event of _Hox3_ and acts to pattern the
anteroposterior axis (Stauber et al., 1999). In fly, _Bcd_ is a maternally deposited transcript
that localizes to the anterior end of the egg, thus producing a gradient of both mRNA and
protein (Driever et al., 1990). Not only can Bcd act as a transcriptional activator by
binding specific recognition sequences in DNA, it can also transcriptionally repress by
binding mRNA transcripts (i.e. Caudal) (Rivera-Pomar et al., 1996). According to an
adaptive recognition model, Bcd is capable of binding a multitude of DNA sites, both
consensus and non-consensus, which allows for cooperativity to ensue under certain
circumstances of binding site patterns (Treisman et al., 1992; Ma et al., 1996; Yuan et al.,
1996; Dave et al., 2000; Baird-Titus et al., 2006). Thus, the Bcd gradient becomes
important for eliciting different effects: under low concentrations Bcd acts as a weak
activator as a monomer and under high concentrations Bcd can bind several sites more
readily as dimers and multimers to create strong regulation of zygotic gene transcription
(Ma et al., 1996; Ma et al., 1999). Binding site patterns are important for threshold-
dependent regulation (Yuan et al., 1999), but also synergistic effects between other
transcription factors can come in to play (i.e. Hunchback) (Simpson-Brose et al., 1994).
This flexibility of DNA-protein interactions is dependent upon the local environment, including the participation and presence of additional co-activators and repressors (Ma et al., 1999; Baird-Titus et al., 2006).

In Chapter 5 when I assessed the regulation of specific promoters by pitx3, our novel reporter assay allowed us to determine the behaviour of pitx3 under different concentrations. In all circumstances tested, pitx3 acted in an all-or-none manner to regulate transcription: regardless of transcription factor concentration, promoter activity responded in a linear and constant manner (Chapter 5 Figure 5C and 8). If cooperativity was at play for pitx3, exponential changes in transcriptional regulation might have been expected (Beachy et al., 1993).

Varied consensus DNA sites were regulated by pitx3: the consensus K50 binding site (TAATCC) was utilized in the activation of Th whereas the non-consensus site (TAAGCT) was utilized to repress nodal5 transcription. If we compare this behaviour to that of Bcd, under endogenous conditions only high concentrations of pitx3 repress nodal5 via this non-consensus binding site in particular regions of the blastula. It will be interesting to see, as more Pitx3 targets are analyzed in different cell lines, if themes emerge regarding binding motifs more likely to mediate activation, versus motifs more likely to mediate repression.

It will be interesting to screen for possible synergistic activation and/or repression of these promoters by other transcription factors present in specific tissues. For example, it has already been shown that Pitx3 acts synergistically with Nurr1 to transcriptionally activate Vmat2 in mDA neurons (Jacobs et al., 2009). Selecting different cell lines in which to perform this reporter assay will also allow for a diversity of cofactor interaction
to be characterized based on the environment of the interactions and the cofactors present.

6.3 Role of Pitx3 Understood in Characterized Tissues Suggests Opportunities for Study in Other Tissues

6.3.1 Terminal Differentiation

The roles for Pitx3 in lens and midbrain development have been well studied and a common theme appears to be that the gene regulates terminal differentiation in both processes (Figure 1). Maintenance of the differentiated cells also appears to be a recurrent role for Pitx3, where it is expressed even in adult lens and midbrain tissues (Semina et al., 2000; Hwang et al., 2009; Medina-Martinez, 2010). Similarly, although studies of Pitx3 in myogenesis are still in their infancy, and despite its functional redundancy with Pitx2, Pitx3 appears to be involved in both the differentiation and maintenance of muscle cells (Figure 1) (Coulon et al., 2007; L'Honore et al., 2007). We have shown that genes expressed at early stages of myogenesis (Desmin, Creatine kinase, TnnC) and that signal the differentiation of muscle cells within somites show decreased expression in response to mis-expressed pitx3, both during early myogenic patterning, as well as in differentiated tissue (Chapter 2 Figure 2). In keeping with this trend, we might continue searching for roles of Pitx3 in the regulation of terminal differentiation in other tissues.

6.3.2 Known Roles in Lens and Midbrain Differentiation

During lens development pitx3 is expressed in anterior epithelial cells, which are mitotic and give rise to primary lens fibre cells (Pommereit et al., 2001). Pitx3 also expresses at the equator region where fibres proliferate and migrate to the lumen, and
terminal differentiation is initiated whereby fibres elongate and lose their nuclei (Shi et al., 2005). Pitx3 targets Foxe3, which regulates the transition from epithelial cells to secondary fibre cells where amongst other things, it controls nuclear degradation (Shi et al., 2006; Ho et al., 2009). Pitx3 also controls Prox1, which triggers crystallin production marking differentiated lens fibres (Ho et al., 2009). Pitx3 is also upstream of p27kip1/p57kip2 cell cycle inhibitors, so it likely modulates the mitotic ability of proliferating lens cells (Ho et al., 2009). In aphakia mice that lack Pitx3, the lenses lack proliferation, they display premature ubiquitous crystallin production and retention of nuclei, and they consequently fail to differentiate (Varnum and Stevens, 1968; Medina-Martinez, 2010).

Neurons in the midbrain undergo late differentiation to become dopaminergic, by expressing Tyrosine hydroxylase (Th) (Smidt and Burbach, 2009). This requires Pitx3 in the Substantia nigra compacta (SNc) where Pitx3 is required for the terminal differentiation and survival of these specific mDA neurons (Hwang et al., 2003; Smidt et al., 2004; Maxwell and Li, 2005; Jacobs et al., 2007). Pitx3 transcriptionally activates the dopamine phenotype by directly regulating Th expression (Lebel et al., 2001; Messmer et al., 2007), and also regulates the function of these neurons by targeting Vmat2 and Dat expression for dopamine uptake and storage (Hwang et al., 2009). The maintenance of mDA neurons requires Ahd2 expression, also regulated by Pitx3 (Jacobs et al., 2007) and BDNF and GNDF neurotrophic factors are also downstream of Pitx3 (Peng et al., 2007). New evidence has shown that GDNF operates in a positive feedback loop with Pitx3 to mediate BDNF expression, which functions in survival and protection by inhibiting
Figure 1: Pathways depicting Pitx3 regulation of terminal differentiation in different tissue types. Known pathways are boxed and unknown pathways are suggested with red arrows. Solid lines indicate direct pathways, dotted lines propose unknown interactions.
apoptotic cell death (Lei et al., 2011; Peng et al., 2011). All of this supports a role for *Pitx3* in the terminal differentiation of mDA neurons in the SNc.

### 6.3.3 Cardiac Muscle

Some of the cardiac phenotypes seen in *pitx3* morphants display a lack of MF20 antibody staining specifically within the cardiac field, while staining normally in other muscles of the embryos. This implies that the myosin heavy chains that bind MF20 antibody also designate differentiated muscle cells (Schiaffino et al., 1986) are not present in the cardiac fibres of these morphants. Perhaps *Pitx3* is regulating terminal differentiation programs within the heart as well (Figure 1). Future studies might look for other cardiac differentiation markers, such as early heart determination factors *nkx2-5* (Tonissen et al., 1994), *gata4* (Latinkic et al., 2003), *hand1* (Togi et al., 2004) and *hand2* (Thattaliyath et al., 2002), as well as myocardium differentiation markers, such as *cardiac α-actin* (Mohun et al., 1984) and *cardiac troponin I* (*tnni3*) (Drysdale et al., 1994).

### 6.3.4 Pronephros

*Lhx1* (*Lim1*) shows altered expression in the pronephric field of tailbud *pitx3* morphant embryos (Chapter 4 Figure 10F). The kidneys arise from intermediate mesoderm which lies between the paraxial and lateral plate mesoderm in both of which *pitx3* expresses bilaterally (Khosrowshahian et al., 2005). Signals from adjacent tissues could be inducing organ morphogenesis and we have shown the ability of pitx3 to directly bind and transactivate *lhx1* (Chapter 5 Figure 6). *Lhx1* is necessary for specifying the pronephric field during early embryogenesis, however ectopic expression of *lhx1* is detrimental to the terminal differentiation of functional kidneys (Figure 1) (Cirio et al.,
2011). We might also look for kidney differentiation markers in these embryos. Antibodies specific for the proximal (3G8) and distal (4A6) tubules of the differentiating pronephric kidneys will reveal whether kidney development is impaired due to loss of pitx3 expression (Brennan et al., 1998).

6.3.5 Lens

\textit{XL-maf (nrl)} is a transcription factor expressing in the developing lens and shown to regulate crystallin production and rhodopsin expression - clear signs of a differentiated lens (Ishibashi and Yasuda, 2001; Whitaker and Knox, 2004). \textit{Nrl} appears in the microarray data (Chapter 4 Table 1), however perturbation was not confirmed via \textit{in situ} hybridization or RT-PCR experiments due to expression levels below the threshold of detectability at the stages tested (stages 19 and 27) (Ishibashi and Yasuda, 2001). \textit{Nrl} binding sites have been reported in the \textit{Pitx3} promoter region (Semina et al., 2000) and likewise, we have shown an abundance (12) of putative \textit{Pitx3} binding sites in the promoter of \textit{nrl} (Chapter 4 Table 1). Perhaps a reporter assay could help to clarify which transcription factor regulates which, whether they operate in a transcriptional feedback loop, or if these genes act in parallel pathways during lens differentiation (Figure 1). Alternative methods such as ChIP or gel retardation assays could also be used to determine direct binding of transcription factor to promoter DNA.

6.4 Cellular Organization

There is some evidence from the studies of lens development that suggest a role for \textit{Pitx3} in maintaining the organization of cells within tissues (Figure 2). When there is a lack of pitx3 in the embryo, there is an unusual distribution of lens fibre cells (Chapter 1
Pitx3 regulates the expression of *MIP/Aquaporin O*, which is an intercellular adhesion molecule necessary in zebrafish for lens transparency (Froger et al., 2010). Cataracts are a consequence of *Pitx3* mutations across species, and this is phenocopied by *mip* morpholino in zebrafish (Varnum and Stevens, 1968; Shi et al., 2005; Sakazume et al., 2007; Froger et al., 2010). In the *aphakia* mouse mutant, if lenses are present, there is no obvious cellular organization (Varnum and Stevens, 1968).

### 6.4.1 Somitogenesis

The perturbed cellular arrangement that is observed in somites of *pitx3* morphants suggests a similar organizational role for this gene in this tissue (Figure 2). In Chapter 2 we see that cohorts of unsegmented mesodermal cells do not rotate coherently as the somite differentiates, and anomalous patterning of β1-integrin expression demonstrates disorganized adherent complexes between somites (Chapter 2 Figure 3E). The resulting somites show a lack of intrasomatic adhesion and a frayed appearance as the cells within the somite are not packed close to each other (Chapter 2 Figures 3A-E). We show that *Desmin*, which attaches one somite to the next (Cary and Klymkowsky, 1994), is decreased in *pitx3* morphants, yet *Desmin* -/- mice have normal somites (Cary and Klymkowsky, 1995). This suggests that if *pitx3* acts through *Desmin* for somite cellular adhesion, this may be a species-specific function. However, the functions of *Pitx2* and *Pitx3* are redundant in muscles of mice (L'Honore et al., 2007). Perhaps in the absence of *Pitx3* in mammals, *Pitx2* can compensate to regulate organization within somites: a trait that is absent in *Xenopus*. In organisms where Pitx2 compensation is a concern, double
knock-out mutants may be necessary to study gene function. In frog, morpholinos directed against both pitx family members could be deployed.

In tissue culture, intercellular interactions appear reduced in pitx3 overexpressing cells (Chapter 2 Figure 6D and E). Clearly the junctional anomalies in morphant embryos are the product of cell-autonomous processes during mitotic remodelling. The inhibition of normal junctions must be particularly problematic and consequently, muscle disorganization must impair muscle contraction and may account for the spasmic twitching phenotype observed in pitx3 morphant tadpoles. This twitching continues well after the bent axis phenotype straightens out eventually around stage 27, indicating that muscle fibres are not acting cohesively. Twitching or spastic movements reported in mice or humans with Pitx3 mutations are different. Since vertebrae and muscles pattern normally in these organisms, the behaviours most likely result from mDA neuron deficiency and not skeletal muscle formation (Hwang et al., 2003; Bidinost et al., 2006; van den Munckhof et al., 2006). Identifying putative downstream targets of pitx3 in the microarray dataset that may play a role in intercellular adhesion will provide a starting point for suggesting pathways downstream of pitx3 for this function. Further ectopic expression of those genes in pitx3 morphant embryos may show rescuability of the cohesive deficiency between cells.
Figure 2: Pathways depicting Pitx3 regulation of cellular organization in different tissue types. Known pathways are boxed and unknown pathways are suggested with red arrows. Solid lines indicate direct pathways, dotted lines propose unknown interactions. Boxes indicate resulting phenotypes.
6.4.2 Looping Heart and Gut

When we observe heart and gut morphology phenotypes that pertain to aberrant looping, one can also imagine a role for \textit{Pitx3} regulating cell adhesion during this process. The topography of organ looping requires cells to migrate and is heavily reliant on cell adhesion properties, differential cell growth and shape change, as well as interactions between cells and the extracellular matrix (Figure 2) (Manasek, 1983). Vitronectin is an extracellular matrix (ECM) protein that expresses at similar stages of cardiac and gastrointestinal tract development as \textit{pitx3}, corresponding to the looping process, and may serve as a marker for proper ECM formation (Khosrowshahian et al., 2005; Luque et al., 2010). Moreover, the F-22 antibody may be employed to stain Flectin, an ECM protein that expresses asymmetrically in the developing heart (Tsuda et al., 1996; Linask et al., 2002). Other asymmetrically expressing ECM proteins available to monitor heart morphogenesis are hLAMP and JB3 (Smith et al., 1997).

6.4.3 Early Patterning Processes

Cell adhesion also plays a major role during gastrulation movements as cells migrate and undergo convergent extension (Wang and Steinbeisser, 2009) and gastrulation also appears to be perturbed in \textit{pitx3} morphants. It is possible that there is a lack of cohesiveness or coordination between involuting cells of the circumblastoporal ring (Chapter 3 Figure 1). Therefore, future directions might look for cell adhesion proteins such as cadherins and protocadherins which are necessary for cell interactions during gastrulation movements (Figure 2) (Wang and Steinbeisser, 2009). For example, C-cadherin is necessary for complete involution during gastrulation and knockdown can cause cell dissociation and an open blastopore (Lee and Gumbiner, 1995).
6.5 Regulating Early Pathways

In both zebrafish and *Xenopus*, *pitx3* expression has been reported at early developmental stages prior to gastrulation: at stage 95% epiboly and stage 8, respectively (Dutta et al., 2005; Khosrowshahian et al., 2005). This is quite earlier than the processes of lens, midbrain neuron, and muscle differentiation that are the most studied for Pitx3 function. When looking for early phenotypes represented in *pitx3* morphants, it was evident that there was a problem with involution (Chapter 3 Figure 1), and that this aberrant gastrulation affects gene expression around the blastopore (Chapter 3 Figure 3). Also, a number of genes identified in the microarray experiment showing perturbation in response to *pitx3* knockdown (i.e. *bix4*, *nodal5*, and *gsc*) (Chapter 4), only express during this early window of development, strengthening an argument for *pitx3* in an early patterning/morphogenesis role.

6.5.1 Signalling Pathways

Early patterning of the pre-gastrula embryo consists of three major genetic networks stemming from *vegT*, *wnt*, and *bmp* signalling (Xanthos et al., 2002). We have shown that *nodal5*, one of the earliest known transcripts zygotically induced by *vegT* (Takahashi et al., 2000), is not only perturbed by *pitx3* knockdown (microarray predicts *pitx3* morpholino causes a 0.315 fold change in transcript level for *nodal5*), but also shows direct repression by *pitx3* in cell culture (Chapter 5 Figure 6). Downstream of this, Spemann’s organizer is specified by combined *vegT/nodal* (ventral) and *wnt/β-catenin* (dorsal) signalling, which subsequently induce expression of organizer-specific genes, such as *gsc* and *lhx1* (Xanthos et al., 2002). Bmp signals inhibit organizer fate elsewhere.
in the embryo (Xanthos et al., 2002). Gsc was affected in the microarray and is a target of combined vegT and wnt signalling (Chapter 4 Table 1) (Cho et al., 1991; Watabe et al., 1995; Xanthos et al., 2002). Although gsc does not appear to be a direct transcriptional target of pitx3 (Chapter 5 Figure 6), its expression changes to expand upon pitx3 knockdown (Chapter 3 Figure 3F). This suggests that gsc is an indirect target of pitx3, likely downstream of early pitx3 signalling. The expanded gsc domain seen in pitx3 morphants (Chapter 3 Figure 3F) suggests relaxed constraints upon the organizer region. Similar results can be induced via the application of dorsalizing agents, such as lithium chloride (Kao and Elinson, 1988; Deardorff et al., 1998). In the absence of pitx3, embryos are receiving an abundance of dorsal cues, which can later affect dorsal patterning of the anterior head and trunk regions. Endogenously, pitx3 may act to repress dorsal signals that induce organizer formation, such as nodal5 (Figure 3).

Another gene from the microarray, lhx1, is a direct target of pitx3 in tissue culture (Chapter 5, Figure 6), however, its expression pattern only appears to be perturbed later in the pronephros versus early signalling pathways (Chapter 4 Figure 10 versus Chapter 3 Figure 3). Pitx3 most likely influences gsc through nodal signalling pathways (i.e. nodal5 and nodal1). Perhaps double knockdown of pitx3 and nodal5 could rescue the pitx3 gastrulation phenotype and show this to be the pathway through which pitx3 operates at early stages. Knocking down pitx3 will alleviate repression of nodal5 and thus simultaneous knockdown of nodal5 will rescue these early effects, if this is the pathway through which pitx3 acts.
Figure 3: Schematic representation of pitx3 regulating signalling pathways during early embryogenesis. β-catenin and nodal signalling induces gsc expression in the organizer, while lhx1 maintains gsc expression. Early nodal signalling (nodal5) induces mesendoderm formation, while later nodal signalling (nodal1) induces gastrulation movements (large arrows). Red circle represents Spemann’s organizer, green circle represents Nieuwkoop Centre, small arrows symbolize direct gene activation.
The earliest detection of pitx3 in *Xenopus* embryos by RT-PCR is at stage 8, which is later than nodal5 initiates (Yang et al., 2002; Luxardi et al., 2010), yet prior to gsc expression at stage 8.5 (Cho et al., 1991). In order to determine where pitx3 sits hierarchically in the early signalling pathways that initiate gastrulation, it will be necessary to determine the activating signal for pitx3 expression at this early stage. Potential future experiments could include antagonizing vegT and wnt signalling with vegT and β-catenin antisense morpholino oligonucleotides respectively (Xanthos et al., 2002), and then monitoring pitx3 expression. Looking upstream in the pitx3 promoter for putative binding sites of maternal transcription factors may show what factors initially activate pitx3 in the embryo.

6.5.2 Mechanical Processes

In pitx3 morphants, inconsistent involution of the blastopore occurs around the yolk plug, and matching this, there is intermittent expression of both lefty (an inhibitor of mesoderm) and t (a marker of mesoderm formation) (Chapter 3, Figures 3). I suggest that pitx3 is required for cell-to-cell interactions necessary for the involution of mesoderm, which then reflects the perturbed patterns of organizer gene expression in morphants. Evidence for this argument can be found in mutant models for the other genes. Aberrant lefty expression results in phenotypes such as exogastrulation, showing a role for lefty for ensuring that involution begins close enough to the dorsal lip (Branford and Yost, 2002). Exogastrulation is not a phenotype we see in pitx3 morphants, implying that lefty acts parallel to the pitx3 gastrulation phenotype. Similarly, t is expressed in presumptive mesodermal cells surrounding the blastopore (Smith et al., 1991), yet mis-expression of t causes a delay or failure of gastrulation (Conlon et al., 1996).
absence of a delay in pitx3 morphant gastrulation, measured by sibling development, again implies that the pitx3 phenotype of intermittent t expression is based on an involution malfunction. It will be useful to determine if any genes involved in the involution process are affected by pitx3 mis-expression.

Another avenue to explore is the development of bottle cells, which are responsible for the initial involutions that create the blastopore (Lee, 2012). Since uneven involution is occurring, perhaps uncoordinated bottle cell development is to blame. Bottle cells initially form at the dorsal lip and then continually form to create the circular blastopore (Lee, 2012). One might speculate that the contiguous spreading of bottle cell induction from the dorsal lip laterally and then ventrally around the periphery of the embryo is disturbed in the absence of pitx3, since bottle cell shape changes actually form the intermittent indentation of the lip (Sawyer et al., 2009; Lee, 2012). It is thought that nodal signalling controls bottle cell formation (Kurth and Hausen, 2000), and this suggests that nodal growth factors may be disrupted for proper patterning of this process (Figure 3). Continuous expression of nodall is thought to be necessary for the proper timing of bottle cell induction (Agius et al., 2000; Kurth and Hausen, 2000). Nodal1 is downstream of nodal5 in the vegT signalling cascade and thus pitx3 may be influencing this pathway of transcriptional activation (Figure 3) (Takahashi et al., 2000). Early pitx3 expression in the gastrulating embryo does appear to be present in the ectodermal layer (Chapter 3 Figure 2), which is the sole source of bottle cells, yet they arise only in areas of high TGF-β signaling (Kurth and Hausen, 2000). Through direct regulation of nodal5, perhaps pitx3 regulates bottle cell formation at the blastopore where high nodal signals prevail, by generally repressing the signals elsewhere in the ectoderm (Figure 3). The
pitx3 morphant gastrulation phenotype appears most similar to activin-induced ectopic bottle cell formation (Kurth 2000). This supports the hypothesis that pitx3 affects nodal signalling in the early patterning events required for smooth gastrulation. Future experiments would involve assessing pitx3 morphants for the number and distribution of bottle cells, determine if convergent extension appears mechanically confluent, and assess other players in the nodal pathway.

6.6 Regulation of Retinoic Acid Metabolism

As a metabolite of vitamin A (retinol), retinoic acid (RA) is an endogenous morphogen with levels that are tightly regulated in specific areas of the embryo via metabolic synthesis and catabolic degradation: these processes are controlled by families of retinal dehydrogenases (raldh/aldh) and cyp26 family members, respectively. The expression of aldhlα2 and cyp26α1 indicate an anteroposterior gradient of RA (Moreno and Kintner, 2004). Since RA can diffuse into cells, its function is further regulated, protected, and chaperoned by serum and cellular binding proteins (CRABPs) and nuclear receptors (RAR and RXR heterodimers), which together determine when and where RA may affect gene transcription via binding of retinoic acid response elements (RAREs) (Blomhoff, 2005). In the absence of RA these heterodimeric receptors repress gene expression (Koide et al., 2001). Expression of RA metabolism enzymes in different tissues connotes where high and low levels of RA are required for proper patterning of organ development, as the parent retinol molecule is metabolized into active retinoids in target cells (Blomhoff, 2005; Lynch et al., 2011). RA can promote the differentiation of cells and confer positional information to pattern the developing embryo (Niles, 2003;
Spinella et al., 2003; Diez del Corral and Storey, 2004; Blomhoff, 2005). In particular, high concentrations of RA can induce truncation of anterior structures via inhibition of anterior differentiation, implying this teratogen is a posterior determinant (Durston et al., 1989; Drysdale and Crawford, 1994). Likewise, inhibiting RA at early stages can cause expansion of anterior structures (Koide et al., 2001).

Many of the pathways analyzed throughout this thesis are affected by different aspects of the retinoic acid signalling modalities. For example in Chapter 1, Pitx3 was reported to directly activate Ahd2, a retinaldehyde dehydrogenase necessary for converting retinaldehyde to retinoic acid in mDA neurons for downstream signalling (McCaffery and Drager, 1994; Chung et al., 2005). We also see perturbations of aldhl2 in response to pitx3 morpholino (Chapter 3 Figure 11), specifically within the eye fields. In Chapter 4, we introduce two novel genes deemed to be involved in the RA pathway: retinol binding protein 4-like (rbp4l) and retinol dehydrogenase 16 (rdh16). In addition to this, rxra was detected as altered in the microarray dataset (Chapter 4 Table 1). It is likely that Pitx3 helps pattern specific tissues, including lens and retina, through the regulation of RA production and availability. Further research with the use of RA receptor agonists and antagonists will determine how this transcription factor is capable of affecting multiple aspects of these pathways.

6.6.1 Ahd2 and Midbrain Neurons

Pitx3 indirectly affects the metabolism of RA by directly regulating Ahd2/Raldh1 (aldehyde dehydrogenase) expression in midbrain neurons (Figure 4) (Jacobs et al., 2007). Ahd2 generates RA by converting retinaldehyde to retinoic acid (McCaffery and Drager, 1994). In Pitx3+/- mouse embryos as well as within aphakia mDA cultured stem
cells, treatment with RA restores Tyrosine hydroxylase expression in mDA neurons of the SNc, which indicates that RA is downstream of Pitx3 (Papanikolaou et al., 2009; Jacobs et al., 2011). This pathway operates through RAR and/or RAR-RXR nuclear receptor complexes (Jacobs et al., 2011), specifically perhaps by RARβ binding and activating TH (Jeong et al., 2006). Therefore, RA is necessary for the terminal differentiation of these neurons, however in Pitx3 nulls it cannot restore the expression of Pitx3 RA-independent targets such as Ahd2, Dat, and Vmat2 (Figure 1) (Jacobs et al., 2011). Thus, there are RA-dependent and RA-independent Pitx3 signalling pathways present in the midbrain (Jacobs et al., 2011).

In Xenopus, ahd2 (raldh1/aldh1a1) is also shown to express strongly in the pronephric kidney and duct (Lynch et al., 2011), potentially linking pitx3 and pronephros development once again. This avenue could be explored from the perspective of pitx3 regulation of aldh1a1 in tissues in addition to the midbrain.

6.6.2 Raldh2 and Eye Development

In the retinal pigmented epithelium, a retinoid cycle is necessary for nerve impulses to generate the vision process (Saari et al., 1994; Baehr et al., 2003). Retinaldehyde dehydrogenases are present at high levels in the eye, specifically in the ventral portion of the retina, indicating RA production (Marsh-Armstrong et al., 1994; McCaffery et al., 1996). Moreover, there is evidence suggesting a gradient of retinoic acid in the eye (McCaffery et al., 1992; Tsonis et al., 2000). When RA signalling is disrupted in the lens, development is inhibited, while conversely, exogenous RA can lead to ectopic lens formation (Manns and Fritzsch, 1991; Tsonis et al., 2000). Pitx3 may relate to RA metabolism in this developing tissue (Figure 4). Eyes of embryos treated
with RA also show a lack of organization between retinal layers (Drysdale and Crawford, 1994; Eagleson et al., 2001). There are also dopaminergic interneuron cells within the retinal layer of the eye and treatment of RA inhibits differentiation, measured by TH expression (Eagleson et al., 2001). Since pitx3 is necessary for lens placode formation and lens development, it seems likely that RA and Pitx3 signalling are linked in this tissue too.

In pitx3 morphants we see a vast increase in aldha expression in the dorsal retina (Chapter 3 Figure 11D). Cyp26 is expressed specifically in the lens of Xenopus embryos (Lynch et al., 2011), indicating that extremely low levels of RA are necessary for normal formation in this region of the developing eye. Therefore, this increase in aldha-induced RA in close vicinity to the lens could indicate a diffusible challenge to proper lens development and provide new insights into the role of Pitx3 in lens genetic pathways, especially transitioning from relatively undifferentiated and proliferating cells to terminally differentiated lens fibres. Perhaps Pitx3 is operating in the lens similarly as in the midbrain, with RA-dependent and -independent signalling pathways, and this could account for some of the cataract lens phenotype displayed by embryos deficient for Pitx3. This phenotype could also be explained by poor regulation of crystallins, the most predominant protein in the lens.
Figure 4: Pathways depicting \textit{Pitx3} regulation of retinoic acid metabolism in different tissue types. Known pathways are boxed and unknown pathways are suggested with red arrows. Solid lines indicate direct pathways, dotted lines propose unknown interactions. Red circle suggests that \textit{Pitx3} does not regulate retinoic acid signalling pertaining to the lateral plate mesoderm (LPM) and heart morphogenesis.
6.6.3 Lateral Plate Mesoderm and Heart

*Aldh1a2* is also expressed in the lateral plate mesoderm, specifically in the dorsal anterior region, and is necessary for normal heart morphogenesis (Koster et al., 1999; Niederreither et al., 2001; Deimling and Drysdale, 2009; Lynch et al., 2011). Since the lateral plate mesoderm allows RA to reach the heart field, and *Aldh1a2* deficient mice have heart-looping defects (Niederreither et al., 2001), *Aldh1a2* may prove to be yet another factor to confer left-right axis identity to this organ (Lynch et al., 2011). However, in *Aldh1a2*−/− mouse embryos, the laterality triad of gene expression is not perturbed and so RA may confer competence for the heart to loop, rather than looping directionality itself (Niederreither et al., 2001). The lateral plate mesoderm expression domain of *aldh1a2* appears unaffected by *pitx3* morpholino (Chapter 3 Figure 11), so this does not appear to be the genetic pathway by which *pitx3* acts upon heart development (Figure 4). *Aldh1a2*−/− mouse mutants show abnormal *Tbx5* expression in the posterior heart, and in *Xenopus* embryos treated with exogenous RA or RA antagonists *tbx5* expression is reduced substantially (Niederreither et al., 2001; Collop et al., 2006). Since *tbx5* was not affected by *pitx3* morpholino (Chapter 3 Figure 10), this again confirms that *pitx3* is acting independent of RA pathways during heart patterning. In addition, *Pitx2* still expresses in the left heart field of *Aldh1a2*−/− mutant mice, yet *pitx2* is not expressed here in *pitx3* morphants (Chapter 3 Figure 6L).

Lateral plate mesoderm is patterned by specific anterior-posterior expression domains of transcription factors, and these domains are RA-sensitive (Deimling and Drysdale, 2009). Change in *pitx3* expression within the lateral plate mesoderm was not noted in RA-treated embryos (Chapter 3 Figure 11F). It has been suggested that there is a
gradient of RA expression along the anteroposterior axis of the heart tube created by Aldh1a2, and RA-treated heart tubes do not loop and never beat (Drysdale et al., 1997; Collop et al., 2006). RA only appears necessary for heart-looping ability, and therefore it is unlikely that Pitx3 is up- or down-stream of retinoic acid pathways for heart morphogenesis, since the hearts of pitx3 morphants do progress past the decision to loop.

Specification of cardiac cells begins during gastrulation, however differentiation of a heart occurs at tailbud stages, when morphological changes occur and cardiac-specific gene expression commences (Warkman and Krieg, 2007). RA binds RXR-α to inhibit myocardial differentiation (Kastner et al., 1995; Drysdale et al., 1997). With rrxα appearing in the microarray data, we might speculate an RA-dependent pathway for pitx3 for heart differentiation at the cellular level (Figure 1), rather than at the level of organ morphogenesis (Figure 4).

6.6.4 Novel RA Pathway Genes

In Chapter 4 two novel genes were identified in the microarray dataset that may be involved in retinoic acid patterning of developmental tissues. Rdh16 encodes a retinol dehydrogenase and is predicted to be up-regulated by treatment with pitx3 morpholino (Chapter 4 Table 1). The first step towards RA synthesis is the conversion of retinol (vitamin A) to retinaldehyde, and this is done by alcohol dehydrogenases. Since rdh16 expresses in the retina (Chapter 4 Figure 5), this unexplored protein may contribute to the role of retinoic acid in the vision process (Figure 4). Rdh16 is also detected in the pronephros and could therefore be implicated in the retinoic acid patterning of the early kidney (Figure 4); retinoic acid affects the initial specification of the kidney and can directly regulate lhx1 there in Xenopus (Cartry et al., 2006). A decrease in Lhx1
expression is necessary for the terminal differentiation of kidney cells and prolonged expression results in an undifferentiated state (Agrawal et al., 2009). In pitx3 morphants, lhxl expression is reduced in the pronephros and may indicate perturbation of kidney differentiation. Future studies might illuminate the extent to which pitx3-mediated lhxl activity is necessary to kidney differentiation, and whether this is an RA-dependent pathway (Figure 4).

The second transcript affected by pitx3 knockdown is rbp4l, which encodes a retinol binding protein (RBP). Retinol binding proteins transport retinoids throughout the embryo in the plasma and are essential for the mobilization and uptake of retinol by target cells (Kanai, 1986; Quadro et al., 1999; Blomhoff, 2005). These proteins contain a hydrophobic pocket that protects bound retinol as they shuttle the molecule to appropriate tissues (Zanotti and Berni, 2004). The RBP-retinol concentration in plasma is strictly controlled implying the regulation of RBP transcription must be tightly regulated by transcription factors (Blomhoff, 2005). This transcript is highly expressed at late tailbud stages and appears to be up-regulated in the absence of pitx3 (Chapter 4 Table 1). Like pitx3, rbp4l expresses in the lens (Chapter 4 Figure 3). Another correlation between Rbp4 and Pitx3 has already been reported, whereby Rbp4 transcript levels were affected in aphakia lenses as assessed by microarray and RT-PCR analysis (Münster, 2005). In Xenopus, rbp4 and rbp4l have only 43% identity at the amino acid level although these proteins express in similar tissues: both are possibly downstream of pitx3. Rbp4l also expresses in the isthmus (Chapter 4 Figure 3), close to where Pitx3 functions in the midbrain in mammals. It will be interesting to determine whether pitx3 directly regulates this novel protein in order to control the cellular availability of retinol in both lens and
midbrain (Figure 4). This can be determined in the future through employment of our novel reporter assay.

6.7 Conclusion

A common theme in nature appears to be the redeployment of a given gene network to serve at multiple times and places (Stearns, 2010). The pleiotropic consequences of this redeployment might explain how Pitx3 affects multiple different tissues during development. Teasing out which deployment of the gene is exerting each phenotypic effect will present a challenge. It will be necessary to determine if Pitx3 produces one transcript that is useful for multiple functions or whether this transcription factor, like its paralogs Pitx1 and Pitx2, is capable of producing many isoforms, with each polypeptide contributing to its own role in development (Grüneberg, 1938; Tremblay et al., 2000; Cox et al., 2002; He and Zhang, 2006). An in silico search of EST databases might identify alternative transcripts, however 5’-RACE or a nuclease protection assay could help identify if multiple isoforms of pitx3 exist in vivo. Usually pleiotropic genes produce only single products (He and Zhang, 2006), and in this light, it may prove significant that only one Pitx3 isoform has been characterized to date. If Pitx3 is a multifunctional protein with different purposes within various tissue types, then cellular and signalling contexts will be critically important in determining what factors are available that allow this transcription factor to operate in a spatio-temporally specific manner (Stearns, 2010). Discovering co-factors with which Pitx3 synergizes will help to clarify how Pitx3 has adopted so many different roles. For example, future research may be directed towards discovering whether Pitx3 operates as part of protein complexes by
means of tandem-affinity purification and mass-spectrometry to identify interacting proteins.

Looking at the various gene networks in which *Pitx3* has been implicated, it appears that Pitx3 fits the definition of a dating hub protein, binding with different interacting factors in different tissues and at different times (Han et al., 2004; Ekman et al., 2006). Determining the connectivity of Pitx3 pertaining to interacting proteins will be necessary to assign Pitx3 a behavioural category (He and Zhang, 2006). Some phenotypes are more pronounced in *Xenopus* and zebrafish when compared to mice and humans (i.e. muscle and laterality perturbations), lending to the idea that evolution has occurred to refine this protein’s roles in embryogenesis by modifying the spatio-temporal presence of interacting partners.

Microarray experiments only detect changes in the concentrations of transcripts affected by gene perturbation, not changes in expression patterns, or post-transcriptional processing. Since *Pitx3* expresses at multiple stages and in various tissues, in order to study its role in different vicinities, transgenic mis-expression of *pitx3* and/or a dominant-negative form of pitx3 under the direction of time- and tissue-specific promoters may provide a good approach to deciphering specific gene interactions. Future experiments might look to cardiac-specific gene regulation, under regulation of the *nkx2-5* promoter to initiate expression in cardiac precursors (Chen and Schwartz, 1996; Danos and Yost, 1996), or the *cardiac troponin I* promoter for expression in the differentiated myocardium (Drysdale et al., 1994). Perhaps individual microarray experiments for specific embryonic tissues (i.e. lens, heart, gut) could lend clarity to the specific regulatory networks involved.
Morpholino-mediated knockdown of *pitx3* has provided to give clues to its functional roles in tissues that have been missed in other organisms, perhaps due to lethality (i.e. heart morphology). Morpholinos can have a diluted effect at later stages of development and may be losing functional sustainability by tailbud stages due to decreased per nucleus concentration as cells multiply (Heasman, 2002; Eisen and Smith, 2008). Therefore, the later kidney and/or heart phenotypes that we are seeing in *pitx3* morphants may be subdued due to incomplete *pitx3* knockdown by the time these organs undergo morphogenesis. Like other global knockdown methods, affecting gene expression at early stages, especially at gastrulation where many tissues are pre-patterned, may inadvertently affect downstream pathways and organ morphogenesis. For example, perturbation of the Spemann organizer can affect all tissues where *pitx3* expresses at later stages.

It is difficult to deduce the specific role of a gene that expresses at multiple times and in various tissues, without always ascribing phenotypes to the indirect affects of earlier perturbations. Since *lhx1* expresses at early developmental stages that are prior to its role in kidney specification, it might be interesting to target *pitx3* knockdown specifically to cells of the kidney lineage. Fate mapping of the *Xenopus* embryo would allow injecting morpholino in certain blastomeres to affect only certain tissues. For example, the V8 blastomere of the 8-cell embryo is fated for kidney (Moody and Kline, 1990). This will help to distinguish early *lhx1*:pitx3 interactions from later effects on kidney patterning specifically. Another option for *pitx3* knockdown may be to employ an inducible dominant-negative form of *pitx3* that can be activated at specific periods of development. This could be useful for defining how genes such as *lefty* are being affected.
by $pitx3$ perturbations at multiple stages and in different tissues. For example, the glucocorticoid-inducible system allows temporal regulation with the addition of dexamethasone (de Graaf et al., 1998). This would also allow embryos to gastrulate normally and execute proper organizer formation and signalling, so that we can look at later stage roles of $pitx3$ without the confusion of early stage effects.

My research has uncovered novel functions of $pitx3$ during Xenopus embryogenesis. Distinguishing species-specific phenotypes due to $Pitx3$ perturbation gives insight to evolutionarily conserved roles for this transcription factor as well as the evolutionary progression of this gene’s interaction network. Further definition of the proposed regulatory networks influenced by $pitx3$ regulation will give weight to the importance of this gene during multiple facets of embryology.
References:


Kanai M. 1986. [Retinoids and their binding proteins from basic science to clinical medicine]. Rinsho Byori 34:483-500.


APPENDICES

APPENDIX A

PUBLICATION/CO-AUTHORSHIP AGREEMENTS

Chapter II

**Manuscript Title:** “The *Xenopus* Homeobox Gene Pitx3 Impinges Upon Somitogenesis and Laterality”

**Authors:** Smoczer*, C., Hooker*, L., Brode, S., Wolanski, M., KhosrowShahian, F., and Crawford, M.J.

**Submitted to:** Development, Growth & Differentiation

**Manuscript ID:** DGD-00038-2012

Chapter IV

**Manuscript Title:** “Microarray Based Identification of Pitx3 Targets During *Xenopus* Embryogenesis ”

**Authors:** Hooker*, L., Smoczer, C., KhosrowShahian, F., Wolanski, M., and Crawford, M.J.

**Submitted to:** Developmental Dynamics

**Manuscript Number:** DVDY-12-0073

Chapter V

**Working Manuscript Title:** “Direct Targets of *xPitx3* Identified Using a Novel Cell-Specific Reporter Assay ”

**Authors:** Smoczer*, C., Hooker*, L., Hudson, J., and Crawford, M.J.
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April 18th, 2012

#421-3000 Sandwich Street West
Windsor, ON
N9C 4G3

Dear Dr. Cristine Smoczer

I am completing a doctoral dissertation at the University of Windsor entitled "Exploring New Capacities for Pitx3 during Xenopus Embryogenesis." I would like your permission to include in my thesis/dissertation the following material:

**Manuscript Title:** “The Xenopus Homeobox Gene Pitx3 Impinges Upon Somitogenesis and Laterality”

**Authors:** Smoczer*, C., Hooker*, L., Brode, S., Wolanski, M., Khosrowshahian, F., and Crawford, M.J.

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**Manuscript ID:** DGD-00038-2012

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Lara Hooker

Signature: [Signature]

Date: 18.04.2012
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April 18th, 2012

University of Toronto
Division of Respirology
1 King’s College Circle Suite 6263
Toronto, ON
M5S 1A8

Dear Dr. Sarah Brode:

I am completing a doctoral dissertation at the University of Windsor entitled "Exploring New Capacities for Pitx3 during Xenopus Embryogenesis." I would like your permission to include in my thesis/dissertation the following material:

Manuscript Title: "The Xenopus Homeobox Gene Pitx3 Impinges Upon Somitogenesis and Laterality."

Authors: Smocerez*, C., Hooker*, L., Brode, S., Wolanski, M., Khosrowshahi, F., and Crawford, M.J.

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Lara Hooker

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April 13th, 2012

Belmore Neidrauer LLP
TD Waterhouse Tower
79 Wellington Street West
Suite 2401
PO Box 16, TD Centre
Toronto, Ontario
M5K 1A1

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I am completing a doctoral dissertation at the University of Windsor entitled “Exploring New Capacities for Pitx3 during Xenopus Embryogenesis.” I would like your permission to include in my thesis/dissertation the following material:

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**Authors:** Smoover, C., Hooker, L., Brode, S., Wolanski, M., Khoroshowshian, F., and Crawford, M.J.

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South Star Family Dental
9833 Tecumseh Road East
Windsor, ON
N8R 1A5

Dear Dr. Farhad Khosrowshahian

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Lara Hooker

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Department of Biological Sciences
University of Windsor
401 Sunset Avenue
Windsor, ON
N9B 3P4

Dear Dr. Michael Crawford,

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N9C 4G3

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**Submitted to:** Developmental Dynamics

**Manuscript Number:** DVDY-12-0073

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9833 Tecumseh Road East
Windsor, ON
N8R 1A5

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TD Waterhouse Tower
79 Wellington Street West
Suite 2401
PO Box 16, TD Centre
Toronto, Ontario
M5K 1A1

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Submitted to: Developmental Dynamics

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April 18th, 2012

Department of Biological Sciences
University of Windsor
401 Sunset Avenue
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N9B 3P4

Dear Dr. Michael Crawford,

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**Thesis Chapter:** “A Novel Reporter Assay Confirms Direct Targets of xPitx3”

**Authors:** Hooker, L., Smoczer, C., Hudson, J., and Crawford, M.J.

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Department of Biological Sciences
University of Windsor
401 Sunset Avenue
Windsor, Ontario
N9B 3P4

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**Working Manuscript Title:** “Direct Targets of xPitx3 Identified Using a Novel Cell-Specific Reporter Assay”

**Authors:** Smoczer*, C., Hooker*, L., Hudson, J., and Crawford, M.J.

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Department of Biological Sciences  
University of Windsor  
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Windsor, Ontario  
N9B 3P4

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**Authors:** Smočzer*, C., Hooker*, L., Hudson, J., and Crawford, M.J.

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Sincerely,

Lara Hooker

[Signature]

Date: 30 May 2012
APPENDIX B

CLONING AND PLASMID CONSTRUCTION

Flow Cytometry Clones

Figure A: pCINeo/xPitx3-IRES-GFP

xPitx3 coding sequence (889bp) was PCR-amplified from pM53 homegrown plasmid (GenBank sequence NM_001088554) with primers 33.3 (XhoI adapter) (CCG CTC GAG CTG TTG CCA CAT GGA TTT CAA TCT) and 31.6 (EcoRI adapter) (CGG AAT TCC GTC CTT CAT ACT GGC CGA TCC A) and ligated into pCINeo/IRES-GFP vector (6777bp). Total size: 8149bp
Figure B: mTH/pDsRed-express-N1
Murine Tyrosine hydroxylase (mTH) promoter (1516bp upstream from ATG) was PCR-amplified using mTH gDNA clone (18kb) from Dr. Palminter using primers 26.11 (EcoRI adapter) (GGA ATT CCA GTG TTC CCT TTG TAC TG) and 30.21 (SmaI adapter) (TCC CCC GGG GGA AGT GCA AGC TGG TGG TCC) and ligated into pDsRed-express-N1 vector (4700bp) from Dr. Ananvoranich.
Total size: 6201bp
mTH:DsRed transcription cassette was PCR-amplified from mTH/pDsRed-express-N1 plasmid using primers 28.18 (KpnI adaptor) (GGG GTA CCC CAG TGT TCC CTT TGT ACT G) and 30.22 (SacII adaptor) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to give a 2619bp amplicon and ligated into pCS2:HcRed vector (4757bp)
Total size: 7363bp
[Note: sequencing showed a missing 30bp stretch in the MCS, but digestion reveals the sequence is present.]

mTH MUT-DsRed/pCS2-HcRed
Site-directed mutagenesis was used to mutate Pitx3 binding site #3 (ATG -356bp) (Lebel et al., 2001) on the murine Tyrosine hydroxylase promoter using primers 36.1 (ACA TGA ACC CTT GGG TAc c CC AGC ATG GGC GCT CCC) and 36.2 (GGG AGC GCC CAT GCT GG g TA CCC AAG GGT TCA TGT) to introduce a novel KpnI site using mTH:DsRed/pCS2:HcRed plasmid as template. [GGTAATCC mutated to GGTAeeCC]
Total size: 7376bp
Diagnostic digest: Template plasmid (KpnI) = 7376bp
Mutated plasmid (KpnI) = 1168 + 6208bp
**Figure D: xGsc/pDsRed-express-N1**

xGsc promoter was PCR-amplified from -1553gsc pGsc:LUC vector from Dr. K. Chow using primers 28.19 (*KpnI* adapter) (GGG GTA CCC CCA CTG AAA CTG TAC TGA C) and 28.20 (*BamHI* adapter) (CGG GAT CCC GCT CTC CCA TCT GTG CTC C) to produce a 1351bp amplicon and was ligated into pDsRed-express-N1 vector (4700bp). Total size: 6035bp
Figure E: xGsc-DsRed/pCS2-HcRed

xGsc:DsRed transcription cassette was PCR-amplified using xGsc/pDsRed-express-N1 template using primers 28.19 (KpnI adaptor) (GGG GTA CCC CCA CTG AAA CTG TAC TGA C) and 30.22 (SacII adaptor) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to give a 2431bp amplicon that was ligated into the pCS2:HcRed vector (4758bp). Total size: 7194bp
Figure F: xLim1/pDsRed-express-N1

xLim1 promoter was PCR-amplified using primers 31.10 (EcoRI adaptor) (GGA ATT CCT GTA TCT TAT GGT ACT GTA ACT G) and 29.18 (BamHI adaptor) (CGG GAT CCC GCC AAC AGT ACC GGA ATG CC) off xLim1:LUC (Ex-1:A) in pGL2-basic plasmid from Dr. Igor Dawid to obtain a 4093bp amplicon and ligated into the pDsRed-express-N1 plasmid (4700bp). Total size: 8758bp
Figure G: xLim1-DsRed/pCS2-HcRed

xLim1:DsRed transcription cassette was PCR-amplified using primers 36.3 (ApaI adaptor) (AGC TTT GGG CCC CTG TAT CTT ATG GTA CTG TAA CTG) and 30.22 (SacII adaptor) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to result in a 5165bp amplicon derived from xLim1/pDsRed-express-N1 template, that was subsequently blunted, and ligated into the PvuII site of the pCS2:HcRed vector (4.7kb).
Total size: 9969bp
Diagnostic test: Forward clone (ApaI) = 235 + 9737bp (same direction as pCS2:HcRed)
Reverse clone (ApaI) = 5406 + 4566bp (desired)

xLim1 MUT-DsRed/pCS2-HcRed
Site-directed mutagenesis was employed using primers 30.30 (CCC TGG TAA ACC ATg gAG CAC CCC GGC AGG) and 30.31 (CCT GCC GGG GTG CTe caT GGT TTA CCA GGG) off xLim1:DsRed/pCS2:HcRed template to introduce a novel NcoI site. [TAATGG mutated to TccaTGG]
Total size: 9969bp
Diagnostic test: Template plasmid (NcoI) = 3708 + 6261bp
Mutant plasmid (NcoI) = 688bp, 3020bp, 6261bp
**Figure H: Xnr5/pDsRed-express-N1**

Xnr5 promoter was PCR-amplified using primers 30.26 (KpnI adaptor) (ACT AGG TAC CCC TCG GTA ACT TAT CAT ATC) and 28.21 (BamHI adaptor) (CGG GAT CCC GAA GCT TCC AGT GAA TCT T) off Xenopus laevis gDNA template (gDNA isolated from adult Xenopus laevis liver) to give a 773bp amplicon (-12 to -785 from ATG) that was ligated into the pDsRed-express-N1 vector (4700bp).

Total size: 5455bp
Figure I: Xnr5-DsRed/pCS2-HcRed

Xnr5:DsRed transcription cassette was PCR-amplified off Xnr5/pDsRed-express-N1 template using primers 30.26 (KpnI adaptor) (ACT AGG TAC CCC TCG GTA ACT TAT CAT ATC) and 30.22 (SacII adaptor) (TTC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to yield a 1856bp amplicon that was ligated into the pCS2:HcRed vector (4757bp). Total size: 6613bp

Xnr5-DsRed/pCS2-HcRed Mutant A

Unable to mutate the first putative xPitx3 binding site in the Xnr5 promoter using site-directed mutagenesis, a truncation mutant was made instead by excising the 5' end of the promoter that contains this site.

Xnr5:DsRed/pCS2:HcRed (6613bp) was digested with KpnI and AjuI to remove a 276bp fragment, leaving a 499bp promoter fragment. The plasmid was then blunted and re-circularized. Total size: 6299bp

Diagnostic digest: Xnr5:DsRed-pCS2:HcRed (PvuII) = 1581bp + 5032bp

Mutant A (PvuII) = 1267bp + 5032bp

Xnr5-DsRed/pCS2:HCRed Mutant B

Site-directed mutagenesis was used to mutate the second putative xPitx3 binding site in the Xnr5 promoter using primers 34.2 (CAG GTG ACA GGT TCC Cgg ATC CTA TGC TAA TAA G) and 34.3 (CTT ATT AGC ATA GGA Tcc GGG AAC CTG TCA CCT G) to introduce a novel BamHI site. [TAATCC mutated to TA ggg CC]. Total size: 6613bp

Diagnostic test: Template plasmid (BamHI) = 2031+ 4582bp

Mutant plasmid (BamHI) = 2031, 218, 4364bp

Xnr5-DsRed/pCS2:HCRed Mutant C

Site-directed mutagenesis was used to mutate the third putative xPitx3 binding site in the Xnr5 promoter using primers 33.5 (CCT TAG GAA TGA AG Cga CT T CTG AGC ATG ACT) and 33.6 (AGT CAT GCT CAG AAG teg ACT TCA TTT CTA AGG) to introduce a novel SalI site. [TAAGCT mutated to TC gac T].
Total size: 6613bp
Diagnostic test: Template plasmid (SalI) = 6613bp
Mutant plasmid (SalI) = 3432 + 3181bp

Clones with YFP for Future Use with a Yellow Laser

Figure J: mTH-DsRed/pCS2-YFP
mTH-DsRED transcription cassette was PCR-amplified from the mTH/pDsRed-express-N1 plasmid using primers 28.18 (KpnI adapter) (GGG GTA CCC CAG TGT TCC CTT TGT ACT G) and 30.22 (SacII adapter) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC), producing an amplicon of 2612bp, which was blunted and ligated into the pCS2:YFP plasmid (YFP cloned into Clal and Stul sites).
Total size: 7300bp
Diagnostic test: Forward direction (SmaI) = 2074 + 5226bp
Reverse direction (SmaI) = 1636 + 5664bp (*desired)
**Figure K: xGsc-DsRed/pCS2-YFP**

xGSC:DsRed transcription cassette was PCR-amplified from xGsc/pDsRed-express-N1 plasmid using primers 28.19 (*KpnI* adaptor) (GGG GTA CCC CCA CTG AAA CTG TAC TGA C) and 30.22 (*SacII* adaptor) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to produce a 2431bp amplicon, which was blunted and cloned into the pCS2:YFP plasmid. Total size: 7127bp

Diagnostic test: Forward direction (*StuI*) = 2308 + 4131bp

Reverse direction (*StuI*) = 1123 + 5316bp (*desired)*
Clones for Meganuclease-mediated Transgenics

**Figure L: mTH-DsRed/pBSSK+SceI**

mTH:DsRed transcription cassette was PCR-amplified from mTH/pDsRed-express-N1 plasmid using primers 28.18 (*KpnI* adaptor) (GGG GTA CCC CAG TGT TCC CTT TGT ACT G) and 30.22 (*SacII* adaptor) (TCC CCG CGG GGA CCC TAT CTC GGT CTA TTC) to give an amplicon of 2612bp and ligated into the pBSSK+SceI vector (2997bp) from Dr. Thomas Pieler. Total size: 5520bp
Figure M: Xnr5-DsRed/pBSSK+SceI

The Xnr5:DsRed transcription cassette was digested from the Xnr5:DsRed/pCS2:HcRed plasmid with KpnI and SacII resulting in a 1855bp insert, which was ligated into the pBSSK+SceI vector (2997bp).
Total size: 4744bp
The xGsc:DsRed transcription cassette was digested from the xGsc:DsRed/pCS2:HeRed plasmid with *KpnI* and *SacII* resulting in a 2437bp insert, which was ligated into the pBSSK+SceI vector (2997bp).
Total size: 5339bp
The xLim1:DsRed transcription cassette was digested from xLim1:DsRed/pCS2:HcRed plasmid using ApaI and SacII (5172bp) and ligated into the pBSSK+SceI vector (2997bp). Total size: 8085bp

**xLim1-GFP/pBSSK+SceI**

The pBSSK+SceI/CMV-GFP plasmid (kind gift from Dr. Thomas Pieler) was digested with KpnI and BamHI to excise the CMV promoter sequence. Although this plasmid was made with CMV-GFP from pCSGFP3 plasmid, the sequence is unavailable. The xLim1 promoter was digested out of the xLim1-DsRed/pCS2-HcRed plasmid with KpnI and BamHI and then ligated together. Sequencing is pending for this plasmid.
Table 1: Cloning primers utilized to construct this plasmid library. Adapters are underlined, binding sites are bolded, mutations are lower case.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplicon</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.3 F 5' - CCG GTC GAG CTG TTG CCA CAT GGA TTG CAA ACG TCT - 3'</td>
<td>xPt3x CDS</td>
<td>pM53</td>
</tr>
<tr>
<td>31.6 R 5' - CCG AAT TCC GTC CTT CAT ACT GGC CGA TTC A - 3'</td>
<td>mTH Promoter</td>
<td>mTH gDNA clone</td>
</tr>
<tr>
<td>26.11 F 5' - GGA ATT CCA GTG TTC CCT TTT AGG AGC TGG TGG TCC A - 3'</td>
<td>mTH-DsRed transcription cassette</td>
<td>mTH/pDsRed-express-N1</td>
</tr>
<tr>
<td>30.21 R 5' - TTC CCC GGG GGA AGT GCA AGC TGG TGG TCC A - 3'</td>
<td>mTH-DsRed transcription cassette</td>
<td>mTH/pDsRed-express-N1</td>
</tr>
<tr>
<td>28.18 F 5' - GCC GTA CCC CAG CTT TCC CTG TGT ACT G - 3'</td>
<td>mTH mutagenesis primers</td>
<td>mTH/pDsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>30.22 R 5' - CCC TCC GGG GGA CCC TAT CTC GGT CTA TCC T - 3'</td>
<td>mTH mutagenesis primers</td>
<td>mTH/pDsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>36.1 F 5' - ACA TGA ACC CTT GGG TAC cCC AGC ATG GGC GCT CCC - 3'</td>
<td>Xu5 promoter</td>
<td>Xu5/pDSRed-express-N1</td>
</tr>
<tr>
<td>36.2 R 5' - GGG AGC CCC CAT GCT GGG gTA CCC AAG GGT TCA TGT - 3'</td>
<td>Xu5 promoter</td>
<td>Xu5/pDSRed-express-N1</td>
</tr>
<tr>
<td>28.19 F 5' - GCC GTA CCC CCA CTA TCC TTA ACG TTA C - 3'</td>
<td>xGc Promoter</td>
<td>-1553gsc pGsc:LUC</td>
</tr>
<tr>
<td>28.20 R 5' - GGG CAT CCC CCA TCA TCC TTA TTA C - 3'</td>
<td>xGc-DsRed transcription cassette</td>
<td>xGc/pDSRed-express-N1</td>
</tr>
<tr>
<td>31.10 F 5' - GGA ATT CCA GTG TTC CCT TTT AGG AGC TGG TGG TCC A - 3'</td>
<td>xLim1 promoter</td>
<td>xLim1:LUC (Ex-1:A)</td>
</tr>
<tr>
<td>29.18 R 5' - GCC GAT CCC GCC CAA AGC ACC GGA ATG CC - 3'</td>
<td>xLim1 promoter</td>
<td>xLim1:pDSRed-express-N1</td>
</tr>
<tr>
<td>36.3 F 5' - AGC TTT GGG CCC CTC TCT TTT ATG GTA CTG TAA CTG - 3'</td>
<td>xLim1-DsRed transcription cassette</td>
<td>xLim1-DsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>30.22 R 5' - CCC TCC GGG GGA CCC TAT CTC GGT CTA TCC T - 3'</td>
<td>xLim1-DsRed transcription cassette</td>
<td>xLim1-DsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>30.30 F 5' - CCC TGG TAA ACC ATg gAG CAC CCC GGC AGG - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>30.31 R 5' - CTT GCC GGG GTG CTA caT GGT TTA CCA GGG - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>30.26 F 5' - ACT AGG TAC CCC TCC GCA ACT CAT CAT TCC G - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>28.21 R 5' - CCG GAT CCC GGA GCT TCC AGT GAA CTT T - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>30.26 F 5' - ACT AGG TAC CCC TCC GCA ACT CAT CAT TCC G - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>30.22 R 5' - TTC CCG GGG GGA CCC TAT CTC GGT CTA TCC T - 3'</td>
<td>Xu5 promoter</td>
<td>Xenopus laevis gDNA</td>
</tr>
<tr>
<td>34.2 F 5' - CAG GTG ACA GGT TCC Ggg ATC CTA TTC AAT TAA G - 3'</td>
<td>Xu5-B mutagenesis primers</td>
<td>Xu5-DsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>34.3 R 5' - CTT ATT AGC ATA GGA Tcc GGG AAC CTT TCC TCA TCC G - 3'</td>
<td>Xu5-B mutagenesis primers</td>
<td>Xu5-DsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>33.5 F 5' - CTT TAG AAT TAA TTG CAG CAG TGG gTA CCC AAG TCA CTC TCA G - 3'</td>
<td>Xu5-C mutagenesis primers</td>
<td>Xu5-DsRed/pCS2-HeRed</td>
</tr>
<tr>
<td>33.6 R 5' - AGT CAT GCT CAG AAG teg ACT TCA TCA TCA AGG - 3'</td>
<td>Xu5-C mutagenesis primers</td>
<td>Xu5-DsRed/pCS2-HeRed</td>
</tr>
</tbody>
</table>
VITA AUCTORIS

Name: Lara Hooker

Place of Birth: Victoria, British Columbia

Year of Birth: 1981

Lara Hooker attended Reynolds Secondary School in Victoria, B.C., graduating in 1999. From there she moved to Vancouver, B.C. and obtained a Bachelor of Science from the University of British Columbia with a Major in Animal Biology in May 2003. In the Fall of 2004, Lara enrolled in a Master’s Program in the Department of Biological Sciences at the University of Windsor. With two years towards this degree she transferred to the Doctoral program and is looking to graduate in Fall 2012.

Publications/Manuscripts:


Conference Posters/Presentations:

5TH Canadian Developmental Biology Conference: Mont-Tremblant, Quebec

Poster: Direct target genes of Pitx3: going with the flow 04/09/10
Authors: Jerant¹, L., Smoczer, C., Hudson, J.W., Crawford, M.J.

12TH International Xenopus Conference: Leiwen, Germany
Poster: Left-right patterning in *X. laevis*: *xPitx3* and the retinoic acid shield  
**Authors:** Jerant\(^1\), L., Smoczer, C., Wolanski, M., KhosrowShahian, F., Crawford, M.J.

Poster: *xPitx3*, a possible player in the *Xenopus laevis* somitogenesis process  
**Authors:** Smoczer, C., Jerant\(^1\), L., Brode, S., KhosrowShahian, F., Wolanski, M., Crawford, M.J.

**European Society for Evolutionary Developmental Biology: Prague, Czech Republic**

Poster: *Pitx3*: casting a lens on segmentation  
**Authors:** Crawford, M.J., Hooker, L., Smoczer, C., KhosrowShahian, F., Wolanski, M.

**3\(^{RD}\) Canadian Developmental Biology Conference: Mont-Tremblant, Quebec**

Poster: Four novel genes express asymmetrically in *Xenopus laevis* embryos  
**Authors:** Hooker, L., Smoczer, C., Wolanski, M., KhosrowShahian, F., Crawford, M.J.

Poster: *PITX3* impinges upon segmentation clock in *Xenopus laevis* embryos  
**Authors:** Smoczer, C., Hooker, L., Brode, S., KhosrowShahian, F., Wolanski, M., Crawford, M.J.

**Department of Zoology, University of Toronto: Toronto, Ontario**

Presentation: When breaking up is hard to do: does Pitx3 link segmentation and laterality?  
**Authors:** KhosrowShahian, F., Wolanski, M., Brode, S., Hooker, L., Smoczer, C., Crawford, M.J.

*Equal contributions.
\(^1\)Previous name used is Jerant.