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Novel Targets and Functions for Xenopus pitx3 during Embryonic Development

Cristine Smoczer
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Novel Targets and Functions for *Xenopus pitx3* during Embryonic Development

By

Cristine Smoczer

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DECLARATION OF CO-AUTHORSHIP/ PREVIOUS PUBLICATIONS

I. Co-Authorship Declaration

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

The following manuscripts in Chapters II, III, and IV incorporate the outcome of joint research undertaken in collaboration with my colleague Lara Hooker 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, Smoczer/Hooker/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 II, I collaborated to the generation of Table 1 showing the effect of unilateral injection on the dorsal axis and also repeated the experiment with a second *pitx3* morpholino (not shown). Personally, I have contributed the image used in Figure 3e showing immunohistochemistry for β1-integrin and Hoechst in the somites of morphant embryos at stage 27. I also contributed the images used in Figures 6a and b representing *in situ* hybridization for *delta2* and *hes4 (hairy2b)* and the images in Figures 6d and e illustrating immunohistochemistry for phalloidin and Hoechst on HEK293 cells transfected with control and pitx3.

Chapter III represents a manuscript written by Lara Hooker, based on a microarray experiment. An initial screen of 80 potential target genes by *in situ*

hybridization was jointly conducted by Smoczer/Hooker. I collaborated with Lara Hooker to produce Figure 1 categorizing the 100 most up- and down-regulated transcripts at two stages of embryogenesis. Personally, I have contributed Figure 2B and D (Pitx3 expression at stages 19 and 27), H, H', G, G' (*Pax6*), J, J' (*Crybb1*), Figure 6A, A' (*Obscnl*), Figure 8A, A', B, B' (*Hes7.1*), Figure 9A, A', B, B'(*Ripply2*), C, C', D, D' (*Hes4*), E, E', F, F'(*Hes7*) and Figure 10A, A', B, B'(*HoxA11).* Together with Lara Hooker we compiled the *in situ* hibridization and RT-PCR data to generate Table 1.

The working manuscript comprising Chapter IV was written by me, based on experimental design by all authors: Lara Hooker, myself, Dr. Michael Crawford, and Dr. John Hudson. Lara Hooker 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 in HEK293 cells (Figures 1, 4, and 5A, B, C). I have repeated the *Tyrosine hydroxylase* control in the SK-N-BE2c cell line (Figure 5D). For Figures 2 and 3 comparing a dilution series and time-point experiment, I have performed the flow cytometry experiments to generate data for GFP fluorescence. I utilized the novel reporter assay technique for analyzing the promoter and mutants for *crybb1* (Figures 7, 8C). A personal cloning library has been included in Appendix C.

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 thesis, and have obtained written permission from each of the co-authors to include the above materials in my thesis.

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II. Declaration of Previous Publication

This thesis includes two original papers that have been previously submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter II	The *Xenopus* Homeobox Gene *Pitx3* Impinges Upon Somitogenesis and Laterality	Submitted to Biochemistry and Cell Biology (Manuscript Number 2012-0057)
Chapter III	Microarray Based Identification of *Pitx3* Targets During *Xenopus* Embryogenesis	Accepted to Developmental Dynamics (Manuscript Number DVDY-12-0073)

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ABSTRACT

Pitx3 is a homeodomain transcription factor with an expression pattern conserved across phyla: in the dopaminergic neurons of the midbrain, in the lens during all stages of lens development and in the forming somites. In *Xenopus laevis*, this gene shows novel expression areas, such as the pituitary gland, the heart and the gut. Morpholino-based knockdown of *pitx3* results in phenotypes characterized by small or absent lens, bent dorsal axis and randomized coiling of the heart and gut. Comparing gene expression changes in wild-type versus knockdown embryos by microarray, we generated a vast list of genes possible downstream targets for pitx3. Confirming a number of those genes as affected by the absence of *pitx3* allowed for positioning *pitx3* in a variety of pathways. Given that a significant number of these genes were known as major players during somitogenesis, corroborated with the bent dorsal axis phenotype initiated the further discovery for the role of *pitx3* in this developmental process. To determine direct targets for pitx3 we needed a reporter assay to test the protein-promoter interaction. Since the existing assays were deemed unsatisfactory in terms of accuracy and sensitivity we developed a new technique which permits precise detection of the reporter gene in a homogenous population of cells containing both the transcription factor and the reporter. This also enables the assessment of cooperativity for the tested transcription factors. Lastly, this new technique was employed to examine the promoters of some of the microarray candidate genes and to determine new direct targets for pitx3, thus redesigning existing pathways to incorporate the new interactions.

DEDICATION

I dedicate this research to the memory of my father whose legacy of numerous Genetics books has been the stepping stone for my interest in science. I wish he would know that indirectly he got his chance to make a minuscule contribution to the scientific process.

ACKNOWLEDGEMENTS

My profound appreciation and gratitude go to my scientific advisor, Dr. Michael Crawford, for welcoming me in his lab and believing in me despite the shortcomings in my experience. He endorsed all my ideas, firmly believing that a sincere interest in a topic amounts to better results. Michael was always available for guidance without attempting to micromanage my projects and his "hands-off" approach prepared me to be better equipped to function as an independent scientist. However, my respect to him extends beyond the scientific field and I will forever be thankful to have been mentored in all walks of life by such a distinguished intellectual.

I would also like to thank my labmate, Lara Hooker, for being such a reliable and valuable colleague and friend. We have done the majority of this research working side-by-side, encouraging each other when hope was lost and cheering one another for every break-through. I am thankful to have gained a friend for the years to come and a colleague scientist to turn to when one mind is not enough.

I am also thankful to my committee members, Dr. Sirinart Anavoranich and Dr. Andrew Hubberstey for their valuable insight. Special thanks go to Dr. John Hudson for his help with suggestions and moral support during these years.

Lastly, my deepest gratitude and love go to my mother and my husband. I want to thank my mother for her unwavering support in everything I did and for instilling in me the appreciation for superior education. And to my husband, Emilian, words are not enough to express my appreciation for being so understanding, patient and positive throughout this process.

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LIST OF ABBREVIATIONS

Acf1 – ATP-utilizing chromatine assembly 1

ADH2 – aldehyde dehydrogenase 2

AH – adenohypophysis

ak - aphakia mutant

AP-2 alpha – activating enhancer of binding protein 2alpha

ASMD – anterior segmental mesenchymal dysgenesis

Baz2B – bromodomain adjacent to zinc finger domain 2B

BBE – bicoid binding element

Bcd - bicoid

BDNF - brain derived neurotrophic factor

bHLH – basic helix-loop-helix

cdh2 – cadherin2 (N-cadherin)

Cig30 – cold-induced glycoprotein 30

Ck – creatine kinase

Cmo – control morpholino

CPP4 - congenital posterior polar cataracts

crybb1– betaB1 crystallin

DAPT – N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DAT – dopamine transporter

Dlx1 – distal-less homeobox 1

DMEM – Dulbecco's essential medium

ESC – embryonic stem cells

EST – expressed sequence tag

eyl – eyeless mutant

FGF – fibroblast growth factor

FoxE3 – forkhead homeobox E3

FoxP1 – forkhead homeobox P1

Gbf1 - Golgi-specific brefeldin-A guanine exchange factor 1

GDBF - glial derived neurotrophic factor

GFP – green fluorescent protein

Gsc – goosecoid

H3K4me3 - trimethyl-lysine4 histoneH3

HAT – histone acetyltransferase

HD – homeodomain

HDAC – histone deacetylase

HEK293 – human embryonic cells 293

HMG – high mobility group

ISH – *in situ* hybridization

K50 – lysine in the 50^{th} position of the homeodomain

Lef-1 – lymphoid enhancer factor 1

Lmx 1a/1b – LIM-homeobox gene

Maf – musculoaponeurotic fibrosarcoma gene

MBD – methyl CpG binding domain

MesDA – mesenchephalic dopaminergic neurons

MIP – major intrinsic protein of the lens fibers

Mix.1 – mitotic chromosome homeobox 1

MRFs – myogenic regulatory factors

MTA1 –metastasis tumor antigen1

Myf5 – myogenic factor 5

MyoD – myoblast determination gene

NLS – nuclear localization signal

Nurr1 – nuclear receptor related 1

OAR – Otp, Aristaless, Rax motif

Obscnl – obscurin-like

Om1D – optic morphology 1D

Otd – orthodenticle

Pax genes – paired-box homeobox genes

PCP – planar cell polarity

PD – Parkinson's disease

PEI – polyethylenimine

PFA – paraformaldehyde

Pitx genes – pituitary homeobox genes

PKC – protein kinase C

PLOD1- procollagen-lysine 2-oxoglutarate 5-dioxygenase 1

Pmo – Pitx3 morpholino

POMC – pro-opiomelanocortin

Prd – paired

Prox1 - Prospero-related homeobox 1

PSF – pyrimidine tract-binding protein-associated splicing factor

RA – retinoic acid

Rax1 – retina and anterior neural fold homeobox 1

Rbp4l –retinol binding protein 4-like

Rdh16 – retinol dehydrogenase 16

RhoGEF – Rho guanine exchange factor

RT-PCR – reverese transcriptase polymerase chain reaction

RXRa - retinoid X receptor alpha

Shh – sonic hedgehog

SLRP – small leucine-rich proteoglycan

SMRT - silencing mediator of retinoic acid and thyroid hormone receptor

SNc - substantia nigra compacta

SNPs – single nucleotide polymorphisms

TF – transcription factor

TH - tyrosine hydroxylase

TnnC – fast skeletal troponin C

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

UTR – untranslated region

UTR – untranslated region

VMAT2 – vesicular monoamino transporter 2

VTA - ventral tegmental area

CHAPTER I

GENERAL INTRODUCTION

The blueprint of a mature organism is laid during embryonic development in a beautifully orchestrated cascade of genetic interactions that define complex developmental processes. One of the participants to this concert of genes is *pitx3*, a key player in the development of the lens and the dopaminergic neurons of the midbrain, which acts and is expressed in a conserved fashion across phyla. In this body of research I am trying to extend the knowledge about other roles pitx3 might play and factors it might interact with, using the African clawed frog, *Xenopus laevis*, as a developmental model for this endeavor.

1. Homeobox transcription factors

Gene expression and regulation relies on transcription in a well defined temporal and spatial manner and is the result of high specificity recognition and subsequent recruitment of particular transcription factors, which will cooperate in achieving tight and precise transcriptional control. The eukaryotic genome encodes a large number of transcription factors capable of binding to consensus DNA motifs and initiating transcriptional activity.

The homeobox family of genes is highly conserved throughout evolution and encodes homeodomain proteins, named after a 60- amino acid motif, which represents the DNA-binding domain. The 60 amino-acid homeodomain comprises a helix-turn-helix structure, where the third helix binds to the major groove of the target DNA. All

homeodomain proteins recognize the conserved bicoid 4bp motif TAAT, while the binding specificity for individual proteins is conferred by the following 2bp in the motif (Bürglin, 1994; Gehring et al., 1994a).

Based on the phylogenetic commonality between genes and the presence of additional protein domains outside the homeodomain, the homeodomain family has futher been classified in smaller subfamilies. The most recognized subfamilies are ANTP (antennapedia), PRD (paired), LIM and POU, along with a seven other smaller and less homogenous subfamilies (Holland et al., 2007).

The *paired* subfamily is characterized by a second DNA binding domain, the prd domain, and a residue in the 50th position of the homeodomain which further classifies the family further into three subclasses (Treisman et al., 1989). The *Pax* or *prd* genes are specified by a serine residue in position 50 (S50) of the HD and a second DNA-binding prd domain. The second subclass of genes related to *Orthodonticle (Otd)* is characterized by a lysine at position 50 (K50) (Baird-Titus et al., 2006; Hanes and Brent, 1989) and a third subclass class is identified by the glutamine residue in position 50 (Q50) (Gehring et al., 1994b). The last two subclasses are named paired-like genes, due to the absence of sequences to encode the paired domain in their structure, however they encode a second shared C-terminal motif – the aristaless motif (OAR), and a 42 bp structure possibly responsible for protein-protein interactions (Galliot et al., 1999; Medina-Martinez et al., 2009).

2. *Pitx* family

The *Pitx* family of genes belongs to the K50 paired-like subclass and encompasses the three highly conserved paralogs *Pitx1*, *Pitx2*, *Pitx3*. The three members share a high degree of similarity in both the encoded homeodomain and the OAR domain, with *Pitx2* and *Pitx3* having 100% identity in the HD and *Pitx1* sharing 98% similarity to the other two paralogs. (Fig.1.1) The C-terminal regions are also relatively conserved in the three genes (55-70%), while the N-terminal is unique to each one. Despite partially overlapping in expression, each paralog has a unique spatial and temporal expression pattern. (Gage et al., 1999a)

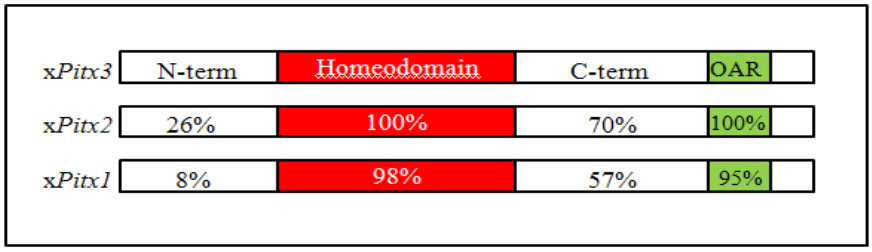

Figure 1.1 Graphic representation of the protein structures of the three *Xenopus laevis* Pitx family members: pitx1 (AAI69747), pitx2 (AAC29426), pitx3 (AAI70172). The homeodomain (in red) and the OAR motif (in green) are approximately identical, while the amino acid sequences are divergent in the C-terminal.

2.1. *Pitx1*

Pitx1 is the first identified gene of the *RIEG/ Pitx* family and has a high degree of conservation of sequence and expression in vertebrates. Murine *Pitx1* is expressed in the stomodeum and the stomodeal derivatives, such as the nasal pit and Rathke's pouch and it also functions to specify the anterior facial structures (lower mandible, tongue, teeth) and to define the identity of the hindlimb mesenchyme (Crawford et al., 1997; Lanctot et al., 1997; Lanctot et al., 1999).

Murine *Pitx1* is also expressed in the anterior pituitary cells and directly activates *the pro-opio-melanocortin (POMC)* and *Pit-1* genes in that region.(Lamonerie et al., 1996) Partial expression and functional overlap of murine *Pitx1* and *Pitx2* in the pituitary region has been shown to permit compensation that allows for a *Pitx*-dosage dependant pituitary development (Gage et al., 1999a). These two *Pitx* genes also work cooperatively to promote the growth of the hindlimb and show redundancy in the process (Marcil et al., 2003). *Pitx1* exerts an auto-regulatory action on its own DNA-binding and transactivating domains (Goodyer et al., 2003) and it inhibits the *Ras* pathway by direct activation of the *RASAL1* gene, thus being categorized as a tumor suppressor gene (Kolfschoten et al., 2005).

Murine null mutants for *Pitx1* exhibit malformed hindlimbs with structures that resemble closely the corresponding forelimb, abnormal anterior pituitary and defects in the facial structures derived from the first branchial arch (Lanctot et al., 1999; Szeto et al., 1999). Defects in human *PITX1* are causative for congenital clubfoot, a limb deformity with adjacent soft tissue aberrations (Gurnett et al., 2008). Murine and human *PITX1* map to a region of high synteny associated with Treacher-Collins syndrome, characterized by craniofacial and hindlimb malformations (Crawford et al., 1997).

In *Xenopus laevis*, *Pitx1* is detected by RT-PCR as a transcript deposited maternally and is first visualized by *in situ* hybridization during the early phases of gastrulation as a weak dorsal streak (Chang et al., 2001). The signal becomes stronger in the neuroectoderm during late gastrulation stages and the expression is consolidated in the cement gland and the stomodeum/pituitary region, lens placode and midbrain tissue during the neurulation and tailbud stages. (Hollemann and Pieler, 1999; Schweickert et

al., 2001). During later stages of frog development, the *pitx1* transcript is detected in the proliferating mesenchyme regions of the hindlimb buds, being required for the hindlimb patterning and regeneration (Chang et al., 2006).

2.2. *Pitx2*

The *Pitx2* gene has three isoforms in almost all studied organisms, that are the result of alternative splicing and differential promoter usage: *Pitx2a*, *Pitx2b* and *Pitx2c* (Cox et al., 2002; Gage and Camper, 1997). A fourth isoform PITX2D has been reported only in humans and exerts a repressive effect on the other isoforms contributing to the tight regulation of the *Pitx2* genes (Cox et al., 2002). The isoforms encode identical homeodomain and C-terminae, while the differences in the N-terminus confer specificity in expression and transcriptional activity (Arakawa et al., 1998; Gage and Camper, 1997). All isoforms can homodimerize or heterodimerize with PITX2B to cooperatively regulate target genes (Amendt et al., 1999; Cox et al., 2002; Saadi et al., 2003). The variety of possible isoform combinations contributes to the dosage-response model suggested for the pituitary and internal organs development (Gage et al., 1999b; Liu et al., 2001)

Structurally, the native C-terminal tail of Pitx2 is intrinsically folded over the N-terminal region, whereby it inhibits the access of the homeodomain to the cognate region. Protein-protein interactions occurring at the C-terminal OAR domain are required to reverse the inhibition and allow for DNA-binding activity during development (Amendt et al., 1999). Phoshorylation of the protein kinase C (PKC) sites in the C-terminus can also release this intramolecular folding and induce increased transactivation, while

phosphorylation of the N-terminus sites results in repressed Pitx2 transcriptional activity (Espinoza et al., 2005).

The murine *Pitx2* isoforms share conserved expression patterns in the anterior pituitary, brain, eye, cement gland, stomodeal and maxillary derivatives, teeth, umbilicus, and in the myoblasts of the trunk and limbs (Hjalt et al., 2000; L'Honore et al., 2007). Only the *Pitx2c* isoform restricts expression to the left side of the lateral plate mesoderm where it contributes to the asymmetrical patterning of the internal organs (heart and gut), downstream of the *Shh*/*nodal*/*lefty1* pathway (Schweickert et al., 2000).

The different isoforms have cell-specific transcriptional activities, depending on the availability of their interacting partners. PITX2A and PITX2C can synergistically activate the promoter of *DLX2*, a transcription factor present in the epithelial cells of the mandibulary and dienchephalic regions and *PLOD1*, a gene responsible for specifying the extracellular matrix (Cox et al., 2002). Pitx2b is the biggest contributor to the regulation of the *prolactin* promoter by interacting with Pit-1 in the OAR domain in mouse (Cox et al., 2002). Human PITX2 isoforms synergistically interact with β-catenin and LEF-1 to activate *LEF-1* transcription during tooth morphogenesis (Vadlamudi et al., 2005). Since LEF-1 is a transcription factor member of the high mobility group (HMG) of proteins, that can only activate targets in collaboration with other DNA-binding proteins to form nucleoprotein complexes (Giese and Grosschedl, 1993), the question of a necessary *PITX* partner in other tissues can be raised.

Complete loss-of-function of murine Pitx2 is generally fatal, resulting in embryonic death around day 13 of development. The embryos fail to close the ventral body wall and present heart valve defects and lung isomerism, enophtalmos and

hypoplastic pituitary (Kitamura et al., 1999). In humans, a *Pitx2* haploinsufficiency is responsible for the Axenfeld-Rieger syndrome, an autosomal dominant disease, characterized by abnormalities in the anterior segment of the eye leading to glaucoma, teeth agenesis and facial and umbilical anomalies (Semina et al., 1996), consistent with the anomalies observed in the heterozygous mouse mutants (Gage et al., 1999a).

In *Xenopus laevis*, the *pitx2* gene is first detected by RT-PCR at stage 10.5 and by ISH around stage 12, anterior of the neuroectoderm (Schweickert et al., 2001). During mid and late neurula, it becomes consolidated in the cement gland and pituitary primordia, while neural expression is detected first around stage 22 (Schweickert et al., 2001). While all isoforms express similarly in the stomodeum, cement gland, pituitary, eye musculature and midbrain, the *pitx2b* expression in the cement gland disappears at mid tadpole stages and *pitx2c* is detected in the lateral plate mesoderm, left side of the heart and head mesenchyme (Schweickert et al., 2000; Schweickert et al., 2001).

2.3. *Pitx3*

The third member of the Pitx/RIEG family is *Pitx3*, a gene sharing a high degree of structural, expressional and functional conservation in most species. The almost 100% identity in the homeodomain between homologs suggests a similar array of targets in various species (Fig.1.2).

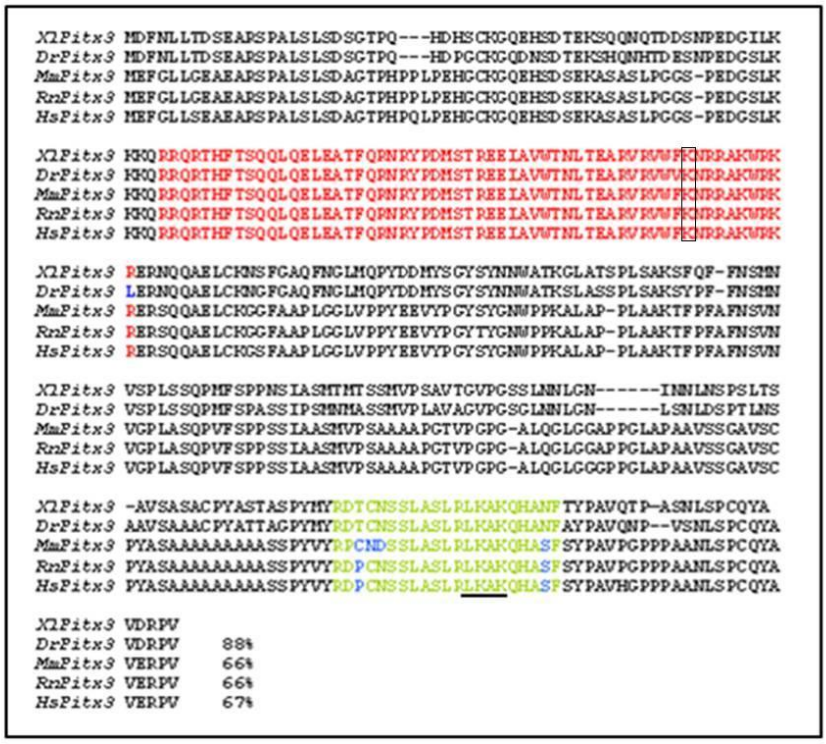

Figure 1.2 Alignment of Pitx3 protein sequences. Pitx3 amino acid sequence from *Xenopus* pitx3 (AAI70172)/ Xlpitx3 is represented aligned to zebrafish (AAT68296)/ DrPitx3, mouse (AAB87380)/ MmPitx3, rat (NP_062120)/ RnPitx3 and human (NP_005020)/ HsPitx3. The consensus sequences include the homeodomain (in red) with the lysine residue in position 50 (K50 –boxed), the OAR motif (in green) with the non-conserved aminoacids (in blue) and a potential nuclear localization signal (NLS) (underlined).

2.3.1. Genomic and protein structure

PITX3 has been mapped to the chromosome 10 in humans, chromosome 19 in mouse and chromosome 13 in zebrafish, in regions of high synteny (Barbazuk et al., 2000; Puttagunta et al., 2000). A similar gene structure has been reported in all the organisms characterized to date, where it comprises four exons, with the first one being untranslated and the translational start site being localized within the second exon. The well conserved homeobox is interrupted by the third intron at the codon encoding for the

amino-acid located at position 46 of the homeodomain (Semina et al., 2000; Semina et al., 1997; Shi et al., 2005).

Close examination of the murine genomic region adjacent to *Pitx3* has revealed two other genes flanking and partially overlapping our transcription factor in its untranslated regions (Semina et al., 2000; Tvrdik et al., 1999). *Cig30* encodes for a brown adipose tissue glycoprotein and is located 4.5kb downstream of *Pitx3* on the opposite strand, and the genes encode 3'-UTRs that overlapp over the last 10bp (Tvrdik et al., 1999). *Gbf1*, a guanine nucleotide exchange factor, member of the ubiquitously expressed *Sec7* domain family (Mansour et al., 1998) is located approximately 4.2kb upstream from the *Pitx3* start site, in the opposite direction in a head-to-head conformation (Semina et al., 2000) (Fig.3A). The expression pattern of both these genes is widely different from that of *Pitx3* and no shared regulatory regions have been reported to date. Also, no additional transcriptional units could be found in the 22kb flanking *Pitx3* on both ends. (Semina et al., 2000).

Functional analysis of the presumed promoter, located upstream of the transcriptional start site revealed two alternative promoters employed by the murine *Pitx3* depending upon the context of expression. Activity on the promoter region upstream and including exon1 is necessary for lens and midbrain expression, while a second promoter, located in the intronic region located between exon1-lens and brain specific (EI-l, b) and the presumptive translational start site ATG on exon2, has been shown to be capable to induce expression in all skeletal muscles (Coulon et al., 2007). That intronic region has also been reported to encompass a new exon1-muscle (EI-m), which together with the alternative promoter allows for muscle-specific gene expression (Fig.1.3A). The

regulatory region within the common promoter and EI-l,b is preferentially utilized even for muscle activity, and it is only when a deletion occurs in this region that the second promoter/exon takes over to maintain expression during muscle development (Coulon et al., 2007). The abundance of active chromatin markers (RNA PolII recruitment, H3K4me3 enrichment) on the lens-/brain promoter explains why this promoter is preferentially utilized to induce gene expression in all tissues, including muscle (Coulon et al., 2007). Following the promoter deletions associated with *aphakia*, the active chromatin signature is transferred to the second promoter, which ensures the maintenance of *Pitx3* muscle expression in the mutant mouse (Coulon et al., 2007).

The 302 animo acid Pitx3 protein follows the same structural pattern as all the K50 paired-like family members, characterized by two conserved regions: the homeodomain, responsible for target DNA recognition and binding and the OAR motif, a 14 amino-acid sequence of a yet unknown function (Fig.1.3B). In the Pitx2 paralog, the OAR motif plays an inhibiting role on the transactivational activity of the homeodomain (Amendt et al., 1999; Brouwer et al., 2003), and was found responsible for the interaction with partnering proteins, such as Pit-1 and Lef-1 (Amendt et al., 1999; Vadlamudi et al., 2005). In *Xenopus*, the absence of the OAR region in the *rax1* gene, resulted however in the repression of the target genes, therefore inferring the role for this motif is highly speculative (Andreazzoli et al., 1999). Pitx3 is expressed and translocated to the nuclei to direct the transcription of downstream targets via a nuclear localization signal (NLS) (Messmer et al., 2007; Sakazume et al., 2007). Two sequences have been previously reported as possible nuclear localization signals in homeodomain genes: a conserved sequence RRMKWKK, located in the 3rd helix of the homeodomain (Kozlowski and

Walter, 2000; Moede et al., 1999) or the conserved motif RLKAK, located inside the OAR region (Shi et al., 2005). There is no precise information on the one used by Pitx3, although Pitx3 was shown to localize to the nuclei (Messmer et al., 2007).

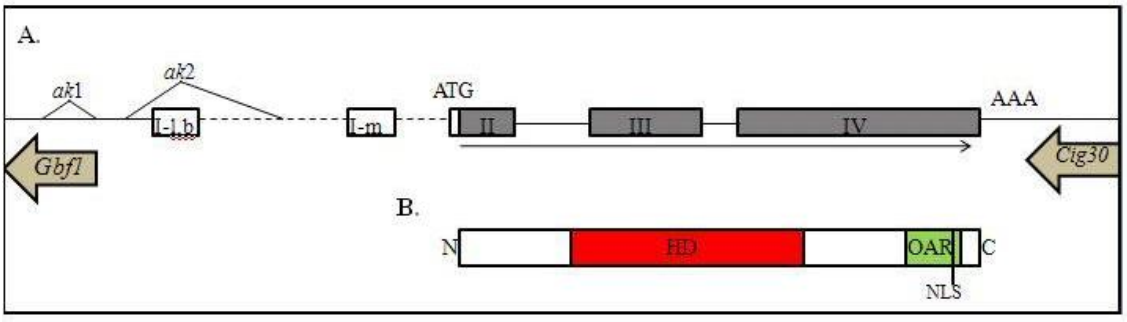

Figure 1.2. Genomic and protein structure of mouse Pitx3. A. The genomic representation of *Pitx3*. *Gbf1* is overlapping the 5'-UTR of *Pitx3* in the opposite direction and *Cig30* overlaps *Pitx3* over a region opposite to the 3'-UTR of *Pitx3*. The translational start site (ATG) is shown in the exon II and all translated regions are represented in the dark gray. The two aphakia deletions (ak1, ak2) are shown to remove part of the promoter region and the first exon I-l,b. The alternative exon Im for muscle is represented in the region covering the first intron. **B.** The protein structure of Pitx3. The two highly conserved motifs are represented in red –the homeodomain and in green- the OAR domain. Also, the two possible nuclear localization signals are shown. Maps are not drawn to scale.

2.3.2. *Pitx3* expression and mutations

Pitx3 was initially characterized in mouse after being linked to the aphakia mutation where it plays a significant role in lens development (Semina et al., 1997). Later, other areas of expression and other phenotypes related to misexpressed *Pitx3* have added to the picture. With very small inconsistencies, such as expression in the pituitary gland of the frog and fish (Dutta et al., 2005; Pommereit et al., 2001), *Pitx3* seems to display conserved expression and function in all species it was studied.

Human

Based on the high degree of homology in the Pitx3 sequences of various species, combined with the phenotypical analysis of the *PITX3* perturbation, it can be inferred that *PITX3* has a similar expression pattern with that of the other vertebrates: lens, midbrain dopaminergic neurons and muscle (Semina et al., 1998; Semina et al., 1997; Smidt et al., 1997), while the pituitary expression appears to have been lost in mammals.

Three mutations occur in the human *PITX3* and cause ocular phenotypes, ranging from congenital total cataracts, congenital posterior polar cataracts (CPP4), and anterior segmental mesenchymal dysgenesis (ASMD). ASMD is a dominant syndrome, consisting of a heterogeneous set of findings including aniridia, abnormal development of the anterior ocular structures, iris hypoplasia and central corneal leukoma, which is referred to as Peter's anomaly (Gould and John, 2002; Hittner et al., 1982). A 17-bp duplication in exon4 (G219fs) results in a frameshift mutation causing the C-terminal third of the gene to be abnormally translated leading to the ASMD and CPP4 phenotypes. (Semina et al., 1998) A serine to aspargine substitution (S13N) was identified in the N-terminal end of the PITX3 protein and was found to be responsible for congenital total cataract with a high incidence of glaucoma at an early age (Berry et al., 2004; Semina et al., 1998; Summers et al., 2008). A third mutation characterized by a single nucleotide deletion in the C-terminal end of the protein (G217fs) results in a truncated form of the protein and elicits various phenotypes in a dose-dependent manner: heterozygotes show congenital polar cataract (CPP4) with few cases of associated ASMD (Addison et al., 2005; Berry et al., 2004), while homozygous patients exhibit severe microphtalmia and central nervous system pathology, with mental retardation and locomotor symptoms resembling advanced

Parkinson's disease (Bidinost et al., 2006). Unlike other Pitx family members, which mutate primarily in the homeodomain, there are no reported mutations in the binding domain of human PITX3, probably due to a severe phenotype which could cause embryonic lethality.

Two single nucleotide polymorphisms (SNPs), located in intron1 and exon3, have been reported to increase the incidence of early onset Parkinson's disease in a large sample of patients, probably since *PITX3* assures the survival of dopaminergic neurons and these SNPs enhance the risk of early neuronal degeneration (Bergman et al., 2008; Le et al., 2009).

Mouse

The murine embryonic *Pitx3* expression in lens starts around the late lens placode stage and continues in all phases characterizing the development of the lens. It was detected in the detaching lens vesicle and later in the epithelial cells and the fiber cells and has continuous expression at low levels throughout adult life (Semina et al., 2000). *Pitx3* also expresses in the eye muscle and eyelids (Semina et al., 1997). During midbrain development, *Pitx3* expresses in the dopaminergic neurons pertaining to the ventral tegmental area (VTA) and *substantia nigra compacta* (SNc) of the ventral mesenchephalon (Smidt et al., 1997) and where it expresses throughout adult life (Nunes et al., 2003). *Pitx3* is also present in all skeletal muscle groups, in both the developing embryo and the adult mouse. During myogenesis it is first detected in the differentiated myotome and in all muscle masses of both fore- and hindlimbs and the forming abdominal wall (Coulon et al., 2007; L'Honore et al., 2007). Aside for the three major

areas of expression, the mouse *Pitx3* is also found in the head, sternum and vertebral mesenchyme, cranio-facial muscles and tongue (Semina et al., 1997).

Aphakia, a spontaneous recessive phenotype, characterized by small eyes lacking lenses and is caused by mutations in the *Pitx3* locus (Semina et al., 2000). Mutant mice are blind due to abnormalities in a wide range of ocular structures; the lens, the iris, the pupil and the anterior chamber are absent, the retina is folded and the eyelids are often closed in adults (Rieger et al., 2001). *Aphakia* is associated with a double deletion occurring in the upstream region of *Pitx3* – a minor deletion of 625bp, about 2.5kb upstream of the transcriptional start site (Semina et al., 2000), and a major deletion of 1.4kb, which eliminates part of the putative promoter, the untranslated exon1-l,b and a portion of the first intron (Rieger et al., 2001). The persistence of very low levels of the *Pitx3* transcript in whole embryo (about 5% of wild-type) (Rieger et al., 2001), even when the major deletion removes a big part of the gene, is likely due to the alternative muscle-specific promoter, that allows for *Pitx3* expression to be maintained in the skeletal muscles (Coulon et al., 2007). The absence of *Pitx3* transcript noticed in the midbrain of the *ak* mutant is probably responsible for the degeneration of the primary dopaminergic neurons associated with *aphakia* (Hwang et al., 2003). Interestingly, the reported dyskinesia and behavioral changes were not conformant with the dramatic loss of dopaminergic neurons in the *substantia nigra* of the midbrain, likely due to the fact that the remainder of the dopamine production pathway is maintained in a relatively normal functioning condition (Smidt et al., 2004; van den Munckhof et al., 2003).

A second spontaneous mutation (eyeless-*eyl*) with recessive transmission has been described in mice with lens and brain phenotype very similar to *aphakia* (Rosemann

et al., 2009). It is the first murine mutation occurring in the coding sequence, more specifically a guanine nucleotide insertion at position 416 of the cDNA, resulting in a shifted open reading frame (Rosemann et al., 2009). The consequent Pitx3 protein maintains an intact homeodomain but loses the OAR motif and subsequently its normal functions (Rosemann et al., 2009). In addition to microphtalmia or anophtalmia and the loss of dopaminergic neurons, homozygotes for this mutation exhibit liver steatosis, reduced locomotor activity and increased pain sensitivity (Rosemann et al., 2009). Unlike the *ak* mice, where the muscle promoter is still intact and therefore the muscular activity is relatively unaffected, the *eyl* mutants show a defective Pitx3 protein in all expressing tissues (Rosemann et al., 2009).

Additional to these two spontaneous murine mutations, three other *Pitx3* targeted mutations have been reported: two were knock-in reporter mutants, one for GFP (Zhao et al., 2004) and one for tau-lacZ (Vives et al., 2008). Both reporters recapitulate the expression of *Pitx3* and could be used for the visualization of nigrostriatal neurons and the dopaminergic pathways both *in vivo* and in ESC cultures. A conditional *Pitx3* knock-out mutant designed to target the third exon, which encodes part of the homeodomain, results in a mutant protein that loses function in lens, brain and muscles (L'Honore et al., 2007). The null mutants were viable and fertile although they display abnormalities in the eye and in a specific subpopulation of midbrain neurons very similar to *ak* mice, while the muscular development and morphology were maintained in a relatively normal range (L'Honore et al., 2007).

Zebrafish

pitx3 expresses at very low levels in zebrafish around the end of gastrulation, where it is restricted to a crescent shaped area at the anterior end of the embryo (Zilinski et al., 2005). Levels increase by late tailbud stages and are maintained during adulthood in the eye and at much lower levels in the internal organs (liver, intestine, pancreas, heart, kidneys) (Shi et al., 2005). Throughout the segmentation stages expression progressively restricts to the anterior pituitary, the lens and olfactory placodes and the diencephalon (Filippi et al., 2007; Zilinski et al., 2005). Later, it disappears from the olfactory placode and expresses strongly in a population of cells around the lens equator, the pituitary, the ventral diencephalon and the mesenchymal cells specifying the presumptive first brachial arch (Dutta et al., 2005). During the late stages, transcripts have also been detected in the mouth cartilage, brachial arches, pectoral fins and trunk musculature; however the expression is not maintained in adults in these tissues (Shi et al., 2005).

Morpholino mediated translational knockdown of *pitx3* results in phenotypes with a severity proportional to the amount of morpholino injected. The morphants have small eyes with lens and retina abnormalities, deficient jaw and pectoral fin development and deviated body axis. Subsequent to *pitx3* knock-down, the lens epithelial and fiber cells fail to undergo complete differentiation and growth - the epithelial cells display a disorganized array of actin fibers, while the fiber cells retain their nuclei and fail to elongate properly (Shi et al., 2005). Although the lens phenotype in *aphakia* is more severe than the one in zebrafish, the cell death and failed neuronal differentiation occurring in the retina results in a bleaker pathology than the retinal folding seen in *ak* mice (Shi et al., 2005). However, since there are no reported retinal defects in

mammalian *Pitx3* mutants and zebrafish *pitx3* expression could not be detected in the retinal layers, it is safe to presume that the morphant retinal phenotype is a secondary result of aberrant lens development (Shi et al., 2005). A dopaminergic phenotype with extensive neuronal apoptosis was initially reported to be a consequence of morpholino-mediated knock-down of *pitx3*, however phenotypical rescue by co-injection of p53-morpholino hints towards non-specific neuronal death with no immediate implication of direct *pitx3* action (Filippi et al., 2007).

Frog

In Xenopus laevis, zygotic transcription commences around stage 8.5, as the mid-blastula transition nears completion. Very low levels of *Xenopus laevis pitx3* are first detected by RT-PCR shortly before the beginning of gastrulation. The expression gradually increases to reach a steady status during neurulation, when the transcript becomes detectable by *in situ* hybridization (ISH) (Khosrowshahian et al., 2005). These stages encompass the periods during which lens forming competence (pre-gastrulation through gastrulation), bias (neurulation), commitment and differentiation (early through late organogenesis) occur (Khosrowshahian et al., 2005). Moreover, *pitx3* expresses in mesodermal derivatives such as lateral plate mesoderm, pre-somitic mesoderm, and in somites as these latter arise in periodic fashion from stage 18 through 42 (KhosrowShahian et al, 2005; Smoczer et al, In Press; chapter 2). Elsewhere, the spatial expression pattern is similar to the ones previously described in other studied organisms, with the exception that *pitx3* is expressed in the frog pituitary. *pitx3* starts by being present early in the pituitary anlage, the stomodeal-hypophyseal anlage, in the eye field

and the prechordal plate during the neurulation stage. During the tailbud stage, *pitx3* transcript is detected in the pituitary, head mesenchyme, lateral plate mesoderm, otic vesicles, lower jaw and somites (Khosrowshahian et al., 2005; Pommereit et al., 2001), while in the late stages of embryonic development, *pitx3* expresses in the coiling heart and gut (Khosrowshahian et al., 2005). *pitx3* plays an important role in lens development and is expressed at high levels in all studied organisms (Khosrowshahian et al., 2005; Semina et al., 1998). In *Xenopus* it is identified in most phases of lens development, starting with the presumptive lens ectoderm, lens placodes, lens vesicle and finally in the anterior epithelial layer, with the exception of the primary fiber cells of the fully developed lens (Pommereit et al., 2001).

Both *pitx3* overexpression and morpholino-induced knock-down in *Xenopus laevis* have similar outcomes and they are also comparable to the phenotypes documented in the zebrafish morphants. *pitx3* misexpression results in craniofacial abnormalities, defective lens development and retinal expansion towards the midline at the expense of the diencephalon. Midline defects account for a bent dorsal axis and the occasional cyclopia, reported as dose-dependent defects associated with abnormal *pitx3* expression (Khosrowshahian et al., 2005).

2.3.3. *Pitx3* interactions and functions

Midbrain differentiation

The majority of dopaminergic neurons (DA) are located in the midbrain in two areas: the *Substantia Nigra compacta* (SNc) and the Ventral Tegmental Area (VTA) (Wallen and Perlmann, 2003). During mammalian embryonic development, the DA

precursors migrate from the neuroepithelium to the presumptive midbrain, where neuronal induction is initiated by *sonic hedgehog* (*Shh*) – a diffusible molecule secreted in the floor plate, and by the activity of *fibroblast growth factor 8* (*Fgf8*) – secreted in the isthmus (midbrain-hindbrain boundary) (Hynes et al., 1995a; Hynes et al., 1995b; Ye et al., 1998). *Fgf8* activates the expression of *Lmx1a* and *Lmx1b*, the earliest markers of DA neuronal differentiation, which in turn induce the expression of *Wnt1*, a gene that promotes neurogenesis by increasing the proliferation of the neuronal precursors (Chung et al., 2009). These factors are also responsible for initiating the exit of the subset of neurons from the cell cycle, with the concomitant expression of the transcriptional factors responsible for terminal DA differentiation: *Pitx3* and *Nurr1* (Andersson et al., 2006). These proteins might work in cooperation to ensure the dopaminergic phenotype by regulating *tyrosine hydroxylase (TH)*, the rate-limiting enzyme for dopamine synthesis (Jacobs et al., 2009). Pitx3 plays also an important role in the survival of the DA, with the *ak* phenotype characterized by marked cell death of the *TH* expressing neurons from the SNc and to a lesser extent from the VTA (van den Munckhof et al., 2003). Although both areas of the midbrain express *Pitx3*, they have different dependencies upon *Pitx3* and therefore are affected differently by mutations in this gene (Smits et al., 2006). Pitx3 also supports mesDA throughout the adult life as part of a tightly regulated network responsible for the maintenance and survival of this subcategory of midbrain neurons (Li et al., 2009).

Lmx1a and *Lmx1b*, genes encoding members of the LIM homeodomain family of transcription factors, specify the mesDA neurons and have a spatial and temporal expression partially overlapping with that of *Pitx3*. Loss-of-function experiments for

Lmx1b result in a complete absence of *Pitx3* in TH+ neurons and a dramatic loss of dopaminergic neurons (Smidt et al., 2000). *Wnt1* knock-out, as well as a mutation of the *Lmx1a* gene, have outcomes identical to *Lmx1b* loss with respect to *Pitx3* expression in the dopaminergic neurons (Prakash et al., 2006). Both *LIM* genes work cooperatively on the promoter of their target sharing redundant functions. They directly bind tothe promoter of *Pitx3*, creating a Wnt1-Lmbx1a/b-Pitx3 pathway that is responsible for neuronal differentiation (Chung et al., 2009). The interaction between the *lmxb1* and *pitx3* genes seems to be conserved in zebrafish, where *lmxb1* knock-down results in low levels of *pitx3* transcript in the diencephalon, and where none of the players is expressed in the dopaminergic neurons (Filippi et al., 2007).

Another regulator of *Pitx3* is FoxP1, a transcription factor belonging to the forkhead winged-helix domain family, mainly involved in the development and differentiation of immunity cells, but also recognized as a marker for the midbrain dopaminergic neurons (Carlsson and Mahlapuu, 2002; Hu et al., 2006; Shi et al., 2008). High expression levels of *FoxP1* are found in the intermediate zone cells of the ventral-most region of the mesenchephalon, the area of nascent dopaminergic neurons. Over-expression of *FoxP1* in mouse embryonic stem cells induces the ectopic formation of *tyrosine hydroxylase (TH)*+/*Pitx3*+ cells, followed by neuronal differentiation (Andersson et al., 2006; Parmar and Li, 2007). FoxP1 is also capable of inducing the expression of *Pitx3* in both TH- neuronal and non-neuronal cells, therefore it can be inferred that FoxP1 regulates *Pitx3* independent of the dopaminergic phenotype. Two high-affinity binding sites in the *Pitx3* promoter were shown to recruit FoxP1 and thus promote transcriptional activation (Konstantoulas et al., 2010). Also, FoxP1 changes the chromatin signature at

the *Pitx3* locus from bivalent, characterized by simultaneous presence of both active and inactive histone markers, to significantly higher levels of active chromatin markers, thus heightening the active transcriptional profile of *Pitx3* (Konstantoulas et al., 2010).

While the relationship between *Pitx3* and *Nurr1* is somewhat controversial, as to whether they synergistically collaborate in regulating downstream genes, or they have a hierarchical relation, there is a general consensus regarding their role in the terminal differentiation and maturation of the midbrain neurons (Cazorla et al., 2000; Messmer et al., 2007; Smidt et al., 1997). *Nurr1* is a member of the nuclear receptor family that precedes temporally the expression of *Pitx3* in the DA neurons by about one developmental stage (Smits et al., 2003) and the knock-out mice exhibit progressive loss of TH+ neurons in both VTA and SNc, with only a transient expression of *Pitx3* before complete disappearance (Saucedo-Cardenas et al., 1998). Nurr1 directly activates *Pitx3* by specificaly binding to its promoter (Volpicelli et al., 2012), while Pitx3 acts as a key regulator of the Nurr1-mediated transcription through protein-protein interaction (Jacobs et al., 2009; Martinat et al., 2006). Both Nurr1 and Pitx3 are known to interact with the PSF co-repressor and form a complex that binds to the promoters of Nurr1 target genes (Jacobs et al., 2009). By default Nurr1 is maintained in an inactive state by the presence of SMRT, a co-repressor known to bind to unliganded nuclear receptors (Nishihara et al., 2004) and to exert its repressive activity by recruitment of histone deacetylases (HDAC) (Guenther et al., 2001). When Pitx3 binds to the PSF-Nurr1 complex, it mimics the effect of a ligand on the nuclear receptor and results in the dissociation of SMRT/HDAC from the Nurr1 transcriptional complex with subsequent activation of the target genes (Jacobs et al., 2009).

The major gene targeted by Pitx3 is *TH*, the rate-limiting enzyme in dopamine biosynthesis and a marker for dopaminergic neurons (Fig. 1.4). Both *TH* and *Pitx3* are expressed in the VTA and *SNc* dopaminergic neurons with a different hierarchical relationship in the two midbrain regions. In the VTA both *Pitx3* and *tyrosine hydroxylase* express simultaneously and *TH* expression is unaffected by the absence of Pitx3. By contrast, in the *SNc*, expression of *Pitx3* precedes the *TH* and the neurons in this area fail to produce *TH* as a consequence of the Pitx3 knock-out (Maxwell et al., 2005; van den Munckhof et al., 2003). The appropriate conclusion is that the presence of *TH* is dependent upon the expression of Pitx3 only in the *SNc*. In tissue cultures, Pitx3 is capable of recognition, direct binding and modulation of the *TH* promoter, and the subsequent response is strongly influenced by the co-factors present in the tested cellular environment (Messmer et al., 2007). While Pitx3 has the capability of acting alone on the *TH* promoter (Lebel et al., 2001), Nurr1 can cooperate with it to enhance its transcriptional activity (Cazorla et al., 2000). Other findings show that once bound to the bicoid site on the *TH* promoter, Pitx3 recruits the co-activator complex MTA1/ DJ1 through direct interaction with MTA1. Subsequently, the complex releases the HDAC2 (histone deacetylase 2) which maintains it in a repressed state and recruits the RNA pol II to initiate the activation of the *TH* production (Reddy et al., 2011).

Vesicular monoamino transporter 2 (*VMAT2*) and *dopamine transporter* (*DAT*) are genes involved in dopamine storage and reuptake in the synaptic vesicles of the dopaminergic neurons and are drastically down-regulated in *ak* mouse and also in the adult midbrain of mice lacking *Pitx3* (Hwang et al., 2009). Not only is the synthesis of dopamine required for normal DA differentiation, the re-uptake and transportation of this

neurotransmitter is equally important. These two processes are also under the control of Pitx3, which directly regulates the genes responsible for these processes, *VMAT2* and *DAT* (Hwang et al., 2009) (Fig. 1.4). Both the Nurr1 null mouse and the *ak* mouse show drastically reduced levels of both *VMAT2* and *DAT* and it was shown that Pitx3 modulates the potency of the Nurr1 transcriptional activity on these genes (Hwang et al., 2009; Jacobs et al., 2009; Smits et al., 2003).

Brain derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) are key factors involved in the process of protection and survival of dopaminergic neurons (Lin et al., 1993; Seroogy et al., 1994). Reduced levels of these neurotrophic factors in the *substantia nigra* of the midbrain have been observed in Parkinson's disease models (Gash et al., 1996). Since Pitx3 knock-out mouse exhibits phenotypes similar to Parkinson's disease, with a drastically decreased number of the *SNc* neurons that also fail to express *tyrosine hydroxylase* (Smidt et al., 2004), the question of Pitx3 regulating *BDNF* and *GDNF* in that region was raised. Although studies have shown that Pitx3 up-regulates both factors in both neuronal cells and astrocytes (Peng et al., 2007; Yang et al., 2008), *in vivo Pitx3* is activated by GDNF via NF-κB mediated signaling and subsequently, regulates the transcription of BDNF during both embryogenesis and adulthood (Peng et al., 2011) (Fig.1.4). *Pitx3* loss results in a drastic decrease of BDNF in MesDA neurons, with consequently impaired protection response to neurotoxins and increased cell death (Yang et al., 2008), however exogenous application of BDNF rescues the survival of the *Pitx3-/-* neurons (Peng et al., 2011).

ADH2 is a gene encoding for *aldehyde dehydrogenase 2*, an enzyme necessary to convert retinol into retinoic acid (RA) and it was shown to be expressed in the ventral

SNc and VTA of the midbrain, the exact regions where neurons disappear in the *ak* mutant mice (Niederreither et al., 2002a). RA is necessary for neuronal patterning and terminal differentiation and is detected in the midbrain during both the early embryonic stages and as well as during adulthood. Since it follows the same temporal and spatial expression as *ADH2* it can be inferred that *ADH2*-dependant RA production is important for the dopaminergic neurons (McCaffery et al., 2003; Niederreither et al., 2002b). *ADH2* expressing neurons have been differentiated *in vitro* and *in vivo* after transplantation in the mouse striatum as a result of transgenic expression of *Pitx3* in ES cells (Chung et al., 2005). Pitx3 is binding to the proximal promoter of *ADH2* and directly inducing its transcription, while exogenous administration of RA results in an increase of TH-expressing neurons and rescue of the defects induced by *Pitx3* null mutation (Jacobs et al., 2007) (Fig.1.4). However, not all genes downstream of Pitx3 recover post-treatment with RA: while TH increases as a result of exogenous RA, VMAT2, DAT and Adh2 remain largely unaffected, suggesting RA-dependant and -independent modes of regulation for Pitx3 (Jacobs et al., 2011).

While the pathway established for the development and regulation of the midbrain includes a cascade of many well characterized transcription factors, post-translational modifications are also known to play a role in the process of specification, differentiation and maintenance of the mesencephalic dopaminergic neurons. A miRNA precursor, *miR-133b* is expressed in the dopaminergic neurons of both rodents and humans and exhibits significantly diminished levels in Parkinson's patients (Kim et al., 2007). While in *ak* mice the expression of *miR-133b* drastically decreases, *Pitx3* overexpression in ES cells results in transcriptional activation of the microRNA, due to direct activity upon its

promoter (Kim et al., 2007). Furthermore, the 3'-UTR region of *Pitx3* possesses a direct target of *miR-133b* completing a negative regulatory feed-back loop: Pitx3 induces the activity of *miR-133b*, which in response suppresses the *Pitx3* activity at the post-transcriptional level (Kim et al., 2007). Since the levels of expressed *Pitx3* have major consequences in the maintenance of the dopaminergic neurons, a microRNA feed-back regulatory loop likely fine-tunes this circuit, with Pitx3 activating the locus for *miR-133b* microRNA and in turn being subsequently regulated by it (Kim et al., 2007) (Fig. 1.4). This system ensures an increase in the stability of the Pitx3 response and action to specific triggers.

Pitx3 is part of the complex network of gene responsible for the differentiation, survival and maintenance of the midbrain dopaminergic neurons and its role is evident by the Parkinson's-like symptoms associated with its mutation.

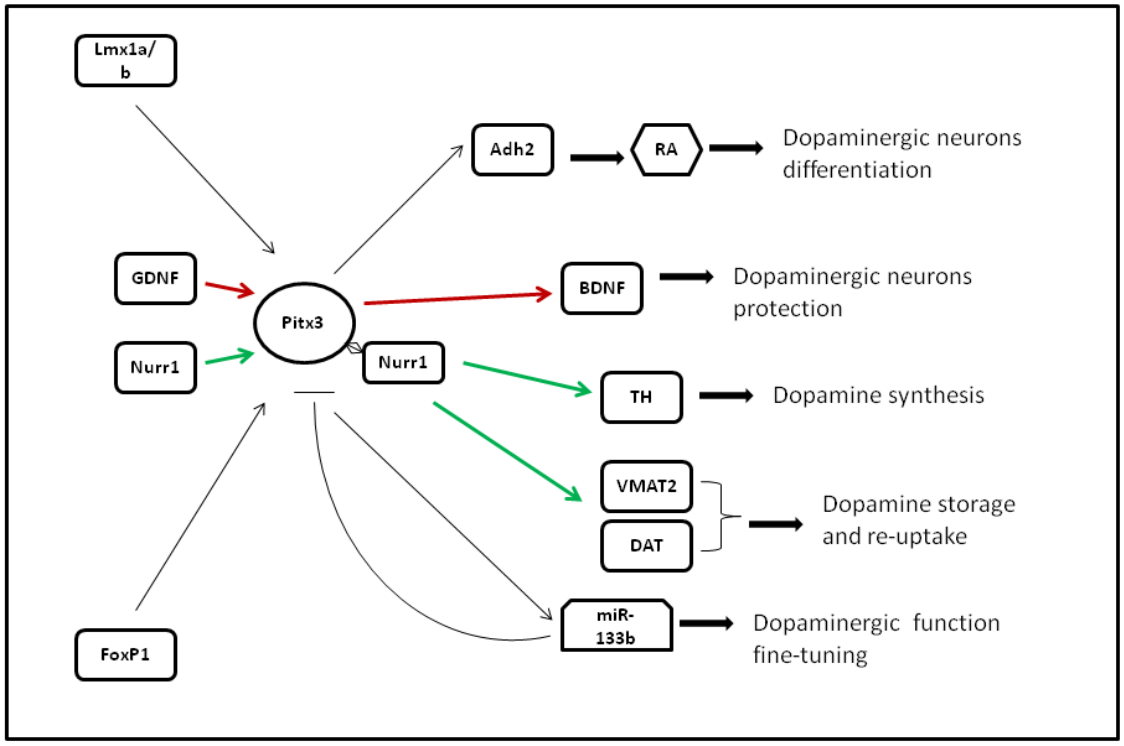

Figure 1.3. The position and role of Pitx3 in the gene regulatory network governing the differentiation and survival of the dopaminergic neurons. Adapted from (Li et al., 2009)

Eye development

Pitx3 plays a key role at different levels responsible for the regulation of the eye development in most species. The development of the lens is a process that begins with the formation of lens placode by juxtaposition of the optic vesicle with the surfacing ectoderm. This is followed by a progressive invagination of the lens cup and finally, closure and detachment from the ectoderm which results in the formation of the lens vesicle. The epithelial cells residing in the anterior equator of the lens vesicle begin to proliferate and subsequently differentiate into lens fibers to fill the lens cavity. The differentiation process includes elongation, loss of nuclei, cell cycle arrest and the expression of *crystallins* – the fiber specific genes (McAvoy et al., 1999).

The expression of *Pitx3* is consistently reported during lens development in several vertebrate species; however there are subtle differences in the expression pattern and role between mammals and lower organisms. In amphibians and fish *pitx3* expresses early in the presumptive lens ectoderm during the lens induction phase and it gradually restricts to the epithelial layer of the lens anterior equator during the differentiation stage. Also, knocking-down *Pitx3* results in morphants with both lens and retina defects (Khosrowshahian et al., 2005; Shi et al., 2005). Mouse *Pitx3* is first detected during the late lens placode stage and mostly during the lens fiber differentiation stages. The mouse mutants exhibit only lens defects, while their retinas are largely unaffected (Semina et al., 1997).

The exact position of *Pitx3* in the gene pathways that regulate the development of the lens in various models is still unknown, but the functional involvement with other players has been explored. *Pitx3* is operating through at least two different pathways: a widely general pathway for fiber cell differentiation and growth, and for maintenance of lens transparency and a pathway specific to only a few species, where perturbed lens development interferes with the induction of normal retina.

The observed lens abnormalities in *ak* mouse were thought to be a consequence of the deletion of the binding sites for L-maf and AP-2alpha, two transcription factors responsible for lens differentiation, in the *Pitx3* promoter (Semina et al., 2000). However, latest reports show that AP-2alpha is not capable of recognizing the binding site on the *Pitx3* promoter and does not affect its expression in the lens, leaving L-Maf as a possible candidate for *Pitx3* regulation (Munster, 2005; Pontoriero et al., 2008) (Fig. 1.5).

Pax6, the eye master homeobox gene responsible for proper eye development, overlaps in expression domain with *Pitx3*, however their relationship needs to be evaluated more in depth. *Pitx3* expression is downregulated in *Pax6* heterozygous mice (Chauhan et al., 2002a; Chauhan et al., 2002b) and Pax6 protein is capable of directly binding to and repressing *Pitx3* in cell culture (Munster, 2005). However, there is also a line of evidence for Pitx3 influencing the expression of *Pax6*. The *ak* mice and the *pitx3*-morphant frogs show altered *Pax6* expression, but since the lens morphology is severly distorted in both cases it is hard to evaluate the influence of Pitx3 on *Pax6* without acknowledging the possibility of the changes being indirectly due to the rudimentary lenses (Grimm et al., 1998; Khosrowshahian et al., 2005). Also, knowing that homeodomain proteins have a tendency to heterodimerize we can hypothesize a synergistic relationship between these two paired-related genes in regulating common targets (Fig. 1.5).

FoxE3/Lens1 encodes a forkhead domain transcription factor conserved in vertebrates, which plays a role in the lens development process: a mutation of the gene is responsible for anterior segmental dysgenesis (ASMD) and cataracts in humans (Semina et al., 2001). Mouse *FoxE3* and *Pitx3* share a similar expression pattern, with *FoxE3* preceding *Pitx3* in the lens placode, while at later stage both are present in the anterior lens epithelium and the primary fiber cells (Brownell et al., 2000; Semina et al., 1997). Both *ak* and *FoxE3* knock-out mice have comparable phenotypes with small or absent lenses and persistent attachment to the surface ectoderm, however superposition of both mutations does not aggravate the previously existing phenotype (Medina-Martinez et al., 2009; Semina et al., 2000). Since *FoxE3* expression is lost in *ak* mice, it can be inferred

that Pitx3 is necessary for the expression of *FoxE3* through a direct interaction, however this has not been experimentally evaluated yet (Ho et al., 2009). This presumption is supported by the work done in zebrafish, where *pitx3* morphants fail to express *foxE3*, while *foxE3* morphants have normal levels of *pitx3* (Shi et al., 2006). *FoxE3* is responsible for regulating the proliferative activity of the epithelial cells through the inhibition of *p27Kip1* and *p57Kip2* (Ho et al., 2009) (Fig. 1.5). Both these genes are known cell cycle suppressors and are ectopically activated in the anterior lens epithelium of the Pitx3 null mouse, explaining the drastic decrease in the number of proliferative cells in the anterior epithelial lens (Ho et al., 2009). This inhibition of proliferation is combined with an increase in apoptotic activity in the lens epithelium which accounts for the small lenses in the absence of *Pitx3* (Medina-Martinez et al., 2009).

MIP/Aquaporin O is a highly conserved gene expressed in the lens fibers of major vertebrates. It acts as a water channel and adhesion molecule to preserve the transparency of the lens (Chepelinsky, 2009). With an expression pattern in mouse and zebrafish overlapping with that of *pitx,* and with mutations in the *MIP/Aqp0* gene resulting in lens phenotypes, it readily identifies as a likely downstream target for Pitx3. The first 500bp promoter sequence upstream of the transcriptional start site of this gene possesses two bicoid-related binding sites (Sorokina et al., 2011). Pitx3 physically binds to these evolutionary conserved bicoid sites in human cell lines, while functional analysis of the binding activity showed Pitx3- specific transactivation of the promoter (Sorokina et al., 2011). Zebrafish *pitx3* morphants also exhibit alteration of expression in the *mip1* gene in the early stages of lens development. Therefore, *MIP/Aqp0* is a direct transcriptional

target of Pitx3 in different species and *Pitx3* mutations are likely to produce a lens phenotype through this interaction (Sorokina et al., 2011) (Fig. 1.5).

A fine balance between the proliferative state of the epithelial cells and the differentiation of the fiber cells has to be maintained for the development of a functional lens. *Pitx3* acts as an important player in the regulation the fiber cell differentiation by controlling the spatial and temporal expression of the *crystallins*, which are the markers of a normal terminal differentiation process. Reduced protein and transcript levels of α and β-*crystallin* have been noted in *ak* mice and zebrafish morphants and are probably responsible for the high rate of fiber cell death, given the known anti-apoptotic role of these genes (Grimm et al., 1998; Medina-Martinez et al., 2009; Morozov and Wawrousek, 2006). Pitx3 could regulate the *crystallins* either directly, indirectly by controlling one of the two *crystallin* activators, *Prox1* and *L-Maf,* or by cooperation with *Pax6*, a known *crystallin* inhibitor (Cui et al., 2004; Ho et al., 2009; Medina-Martinez et al., 2009) (Fig. 1.5). Pitx3 is also responsible for the maintenance of lens transparency through the regulation of another major component of the differentiated fiber cells, the *MIP/AQP0* gene, however there are still unknowns regarding the integrative mechanism of the fiber organization to ensure appropriate transparency and diffraction (Sorokina et al., 2011) (Fig. 1.5).

Both zebrafish and *Xenopus pitx3*-morphants exhibit malformed retinas, however since *pitx3* expression has not been detected in either fish or frog retina, it is likely that the retina phenotype is a secondary inductive effect of the lens abnormalities resulting from *pitx3* misexpression (Khosrowshahian et al., 2005; Shi et al., 2005). Unlike the laminated multilayered wild-type retinas, the zebrafish morphant retinas lack lamination

and organization with a significant number of pyknotic nuclei in the cells of all layers, indicative of cell death (Shi et al., 2005). In amphibians, the retina phenotype appears even more severe, with complete absence of differentiated retina structures, phenotype that is completely rescueable by grafting normal competent pre-lens ectoderm (Khosrowshahian et al., 2005). This supports the presumption that retina defects are a secondary effect of the damaged lens and it is interesting to determine the mechanism that allows mammalian retinas to evade this influence.

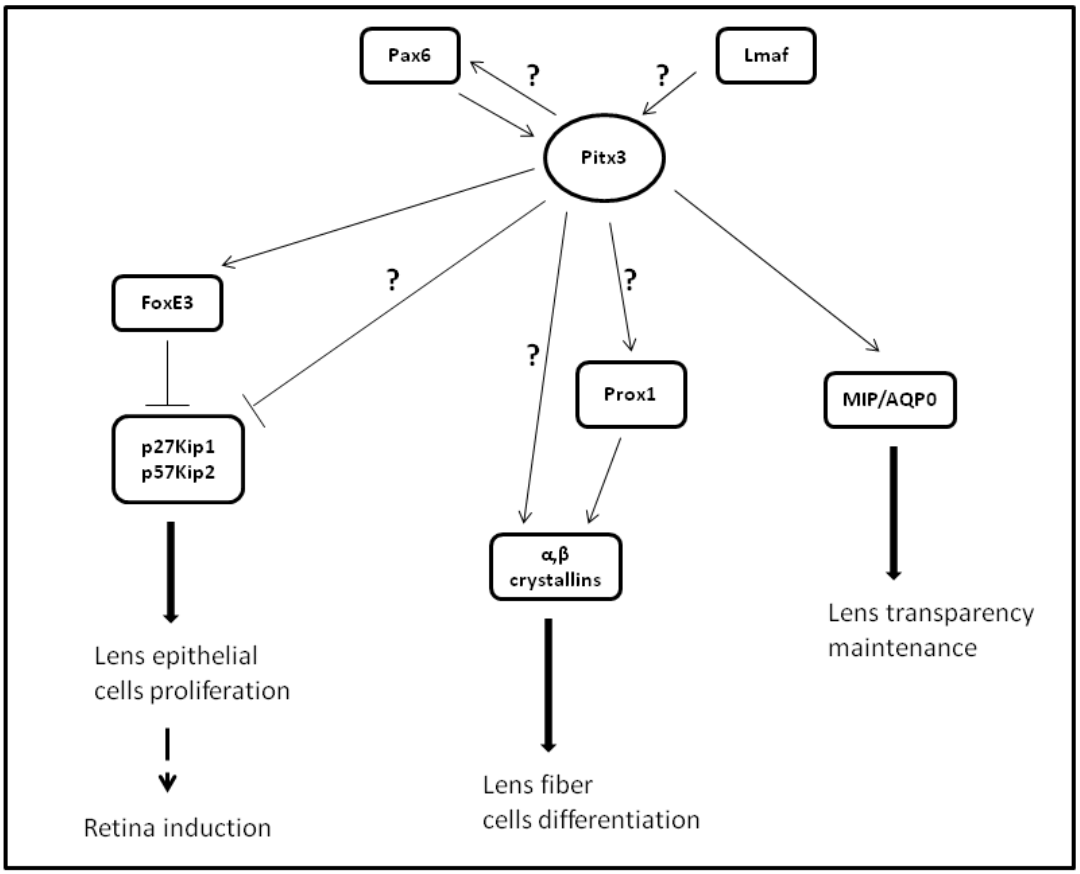

Figure 1.4. Pitx3 and its role in the cascade responsible for lens development. Adapted from (Ho et al., 2009)

Myogenesis

The third major network in which *Pitx3* operates is the one responsible for the skeletal muscle development. Skeletal muscles originate from the dermomyotome, the dorso-lateral compartment of the mature somites. The primary progenitor muscles cells express *Pax3* and *Pax7* and obtain their final identity as myoblasts when they start the myogenic differentiation program, initiated by *Pax3* and *Pax7* downregulation and concomitant expression of the myogenic regulatory factors (*Mrf4*, *Myf5*, *MyoD*). Eventually the myoblasts irreversibly arrest their division and aggregate to form multinucleated myotubes (Buckingham, 2001).

Pitx3 is expressed in the somites and the trunk and limb muscle groups in a conserved fashion, from zebrafish to *Xenopus* and mouse (Pommereit et al., 2001; Shi et al., 2005; Zhao et al., 2004). While the specific cell expression and function in muscle development have not yet been researched in amphibians and fish, initial steps towards defining the role and regulation of *Pitx3* during the mouse myogenic differentiation program have been made. Murine *Pitx3* is first expressed in the differentiated myoblasts, which form the myotome and the developing muscle masses of the fore- and hindlimb and is maintained in most adult myotubes (Coulon et al., 2007). Since no muscle abnormalities have been reported in *Pitx3* knock-out mice, despite the complete loss of the muscle transcript, the position of *Pitx3* in the myogenic regulatory network has been assessed. *Pitx3* is initially detected in the myoblasts and later in the myotubes, while its paralog, *Pitx2* is expressed earlier in the multipotent muscle progenitors and is gradually replaced by *Pitx3* by the time the myotube organization is definitive (L'Honore et al., 2007) (Fig. 1.6). *Pitx2* expression is enhanced and persistent in the already differentiated

muscle of the *Pitx3* knock-out mice (L'Honore et al., 2007), and conversely, *Pitx3* expresses to compensate in the muscle progenitors of *Pitx2* null mice (Lozano-Velasco et al., 2011), suggesting the presence of a compensatory mechanism set into place to guarantee the maintenance of at least one of the *Pitx* transcription factors during the myogenic program (L'Honore et al., 2007). Since the wild-type expression of these two family members is very well defined to certain stages of muscle development, it seems likely for *Pitx2* to operate in the pathway controlled by *Pax3* and *Pax7* to maintain the proliferative status of the myogenic precursors (Lozano-Velasco et al., 2011), while *Pitx3* operates in the pathway governed by the muscle regulatory factors, more specifically *MyoD*, to induce and maintain the final myogenic differentiation (Coulon et al., 2007) (Fig. 1.6).

MyoD and *Pitx3* are co-expressed in myotome and the forelimb muscles (Coulon et al., 2007) and several E-boxes, the MRFs-specific binding sites, have been found in the *Pitx3* muscle-specific promoter (Blais et al., 2005). High levels of *MyoD* present in differentiated muscle cell lines result in induction of the *Pitx3* reporter gene (Coulon et al., 2007). *MyoD* directly binds to a specific region upstream of the exon1-m on the *Pitx3* promoter and directs transcriptional activation to assist the differentiation program (Coulon et al., 2007). Since the overexpression of *Pitx3* in myoblasts results in a drastic decrease of *MyoD* expression (Lozano-Velasco et al.), *Pitx3* and *MyoD* are likely to operate in a feed-back mechanism to initiate and maintain the myogenic differentiation program. Pitx family members and dimerized bHLH transcription factors are known to physically interact to control transcription of downstream genes (Poulin et al., 2000). Therefore, a synergistic interaction between the *MRFs* (bHLH factors) and *Pitx3* could be

responsible for the activation of muscle-specific genes such as *myogenin*, and structural proteins like fast and slow troponins (L'Honore et al., 2007).

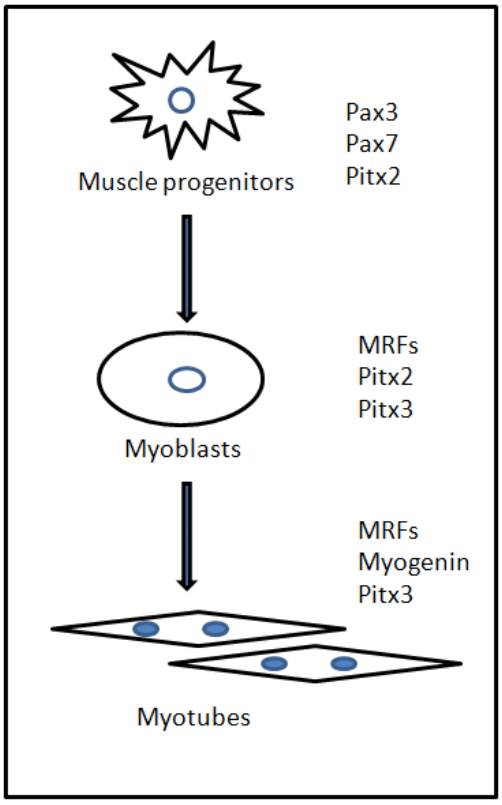

Figure 1.5. The sequential expression of Pitx2 and Pitx3 during the muscle development process. Adapted from (L'Honore et al., 2007)

Other functions

Unlike mouse and humans where the pituitary expression is restricted to *Pitx1* and *Pitx2*, in zebrafish and *Xenopus* the anterior pituitary also expresses *pitx3*. The adenohypophysis (AH) in fish and amphibians originates in the placode located between the ectodermal midline and the anterior hypothalamus. *pitx3* is expressed in a crescent-shaped anterior area during gastrulation defining a domain of progenitors for both lens and pituitary lineages (Dutta et al., 2005). *sonic hedgehog* (*shh*) sets a gradient that

allows for *pitx3* to specify the early pituitary placode, while inhibiting its expression in the presumptive lens ectoderm, to prevent lens differentiation (Dutta et al., 2005). *shh* inhibition results in the absence of *pitx3* in the pituitary pre-placode with blocked AH development, while its expression in the lens placode is largely extended to the midline, resulting in fused lenses (Varga et al., 2001; Zilinski et al., 2005). Given that *pitx3* morphants lack the expression of the early AH marker *lim3*, the main characteristic of the murine *Pitx1* and *Pitx2* double mutants, it is safe to assume that *pitx3* in fish has assumed their functions in the development of the anterior pituitary (Dutta et al., 2005; Pogoda and Hammerschmidt, 2007). Also, the relationship with *shh*, a midline-specific gene combined with the findings that *Xenopus pitx3* morphants exhibit a similar phenotype of cyclopia at the expense of diencephalon, make *pitx3* a possible candidate in the gene nexus that maintains the midline identity (Khosrowshahian et al., 2005).

3. Aim of research

Aside from the known phenotypes generated by a mutated *Pitx3* in different species, a morpholino-mediated knock-down of *pitx3* in *Xenopus laevis* had a few outcomes that have not been characterized to date (Smoczer et al, In Press, Chapter 2). The lack of *pitx3* resulted in a bent dorsal axis, probably due to defective somitogenesis and myogenesis, and consequently the replacement of the sigmoidal swimming of the tadpoles by uncontrolled twitching. I also observed inverted looping of the heart and gut, most likely a consequence of anomalous laterality and midline set-up. My quest was to determine co-players for *pitx3* in the networks that govern these developmental processes and we began by employing a microarray experiment to allow us to find genes influenced

by the absence of *pitx3* at embryonic stages 19 and 27. This generated a large data-set of putative targets and the most affected genes that shared a common expression pattern with *pitx3* were chosen for further investigation (Hooker et al., 2012, Chapter 3). The potential interactants for our gene were subsequently assessed for changes in expression by RT-PCR and ISH, and we detected trends of action for *pitx3*. Key players in the eye develoment, such as *pax6*, *crybb1*/*βB1-Crystallin*, genes that patterns the midbrain-hidbrain boundary, such as *hes7.1*, and a large number of genes that play a role in both the segmentation clock (hes4/*hairy2b*, hes7.2/*esr-4*, and ripply2.1/*stripy*) and the positional identity of the dorsal axis (*hoxA11*) confirm the developmental processes where *pitx3* operates.

The curved dorsal axis and twitching phenotype, described also in the zebrafish morphants (Shi et al., 2005), was corroborated with the presence of the large number of genes in the microarray responsible for precise segmentation and inferred a role for *pitx3* in the somitogenesis and myogenesis processes. Also, the expression of *pitx3* in the lateral plate mesoderm (Pommereit et al., 2001) combined with the defects in the rotation of the heart and gut suggests that *pitx3* might be involved in the correct set-up of the laterality cascade. The second part of my research focuses on finding a position for *pitx3* in these complex genetic networks.

The most accurate way of characterizing a transcription factor is by finding its direct targets and the most common approach for this is employing a reporter assay. All reporter assays utilized to date take an average reading of the reporter output in a heterogenous population of cells and are insensitive to slight changes in gene expression. My quest was to generate a more precise technique that allows for the selection of a

homogenous population, expressing both the transcription factor and reporter gene driven by the test-promoter, in which the reporter output can be measured more accurately. The third part of my work focuses on the development and calibration of this system on the known Pitx3-*TH* interaction, with subsequent testing on new putative direct target genes. The gene that was most dramatically affected by the *pitx3* knock-down, *crybb1*, was the obvious choice as a possible direct target for pitx3.

The general objective of this work is to shed a light on new roles for pitx3 during the *Xenopus* embryogenesis and to find direct and indirect targets for this transcription factor in the complex web of genes that govern various developmental processes.

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CHAPTER II

THE *XENOPUS* HOMEOBOX GENE *PITX3* IMPINGES UPON SOMITOGENESIS AND LATERALITY

1. 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 (Semina et al. 1997; Smidt et al. 1997; Smidt et al. 2004). In mice, *Pitx3* has been identified as the causative locus for *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 *Pitx3* has been tied solely to *substantia nigra* deficits, autosomal dominant mesenchymal dygenesis, 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 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 *Pitx3* expression in frog embryos (Khosrowshahian et al. 2005) we frequently observed craniofacial, and midline phenotypes reminiscent of *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, *Pitx3* and morpholino (*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 *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, *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 *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.

2. 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 mN KCL; 1 mM $MgSO_4$; 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 ^{o}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 CCTCTATTTGTTAAATCCTTCCTGC; mis-match control CCaCaATTTcTTAAATCCTTCgTcC; 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^{o} 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 (5ug/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^{o}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 14um 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 Cuture 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 2.5ug/ml Amphotericin B at 37^{o}C in a humidified 5% CO2 incubator. HEK293 cells were transfected either with pCINeo/IRES-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-equivalents on either side of section in both pre- and post-somitic regions. Ten specimens were assessed.

3. 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 2.1; Figs 2.1, 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 2.1a - control injected with 1b - *Pitx3MO* injected, and Fig 2.3). 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 2.1b, 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 2.1). 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.

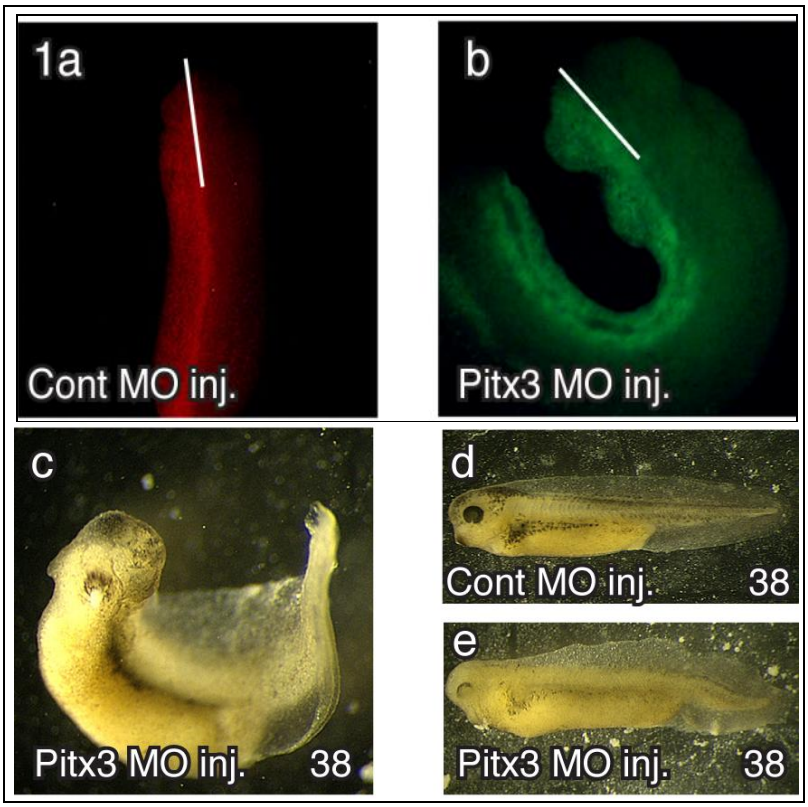

Figure 2.1: **Effect of morpholinos on dorsal axis differentiation.** Embryos injected unilaterally on the left at the two-cell stage with control morpholino (Cont MO) develop normally or with a mild dorsal curve **(a)** while those injected with Pitx3 MO reflex dramatically inward on the side of injection **(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.

	Treatment				
	Cont MO (20ng)	**Pitx3 MO (20ng)**	**Pitx3 MO (15ng)**	**Pitx3 MO (11.5 ng)**	**Pitx3 MO (11.5 ng) + Pitx3 RNA (100 pg)**
Bent Axis	19%	76%	38%	57%	26%
Suppressed Rotation/Depressed Somitogenesis	0%	100%	100%	84%	60%

Table 2.1: Effect of unilateral injection of morpholino upon dorsal axis patterning.

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 2.2).

	Injected Transcript (Injected at 2 cell stage)			Injected Morpholino (Injected at 1 cell stage)		
Phenotype	**Pitx3 left side (300pg)**	**Pitx3 right side (300)pg**	**GFP (300pg)**	**Pitx3MO (15 ng)**	**Pitx3MO (18 ng)**	**Control (18ng)**
Aberrant Heart Looping	31% (15%)	22% (7%	0%	47% (22%)	49% (25%)	0%
Aberrant Gut Looping	38% (18%)	31% (14%)	4%	71% (3%)	53% (10%)	4%
Complete Situs inversus (all visceral organs inverted)	12%	6%	0%	12%	12%	2%
n	206	217	80	186	51	120

Table 2.2: 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.

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

2.2a), 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 2.2b-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.

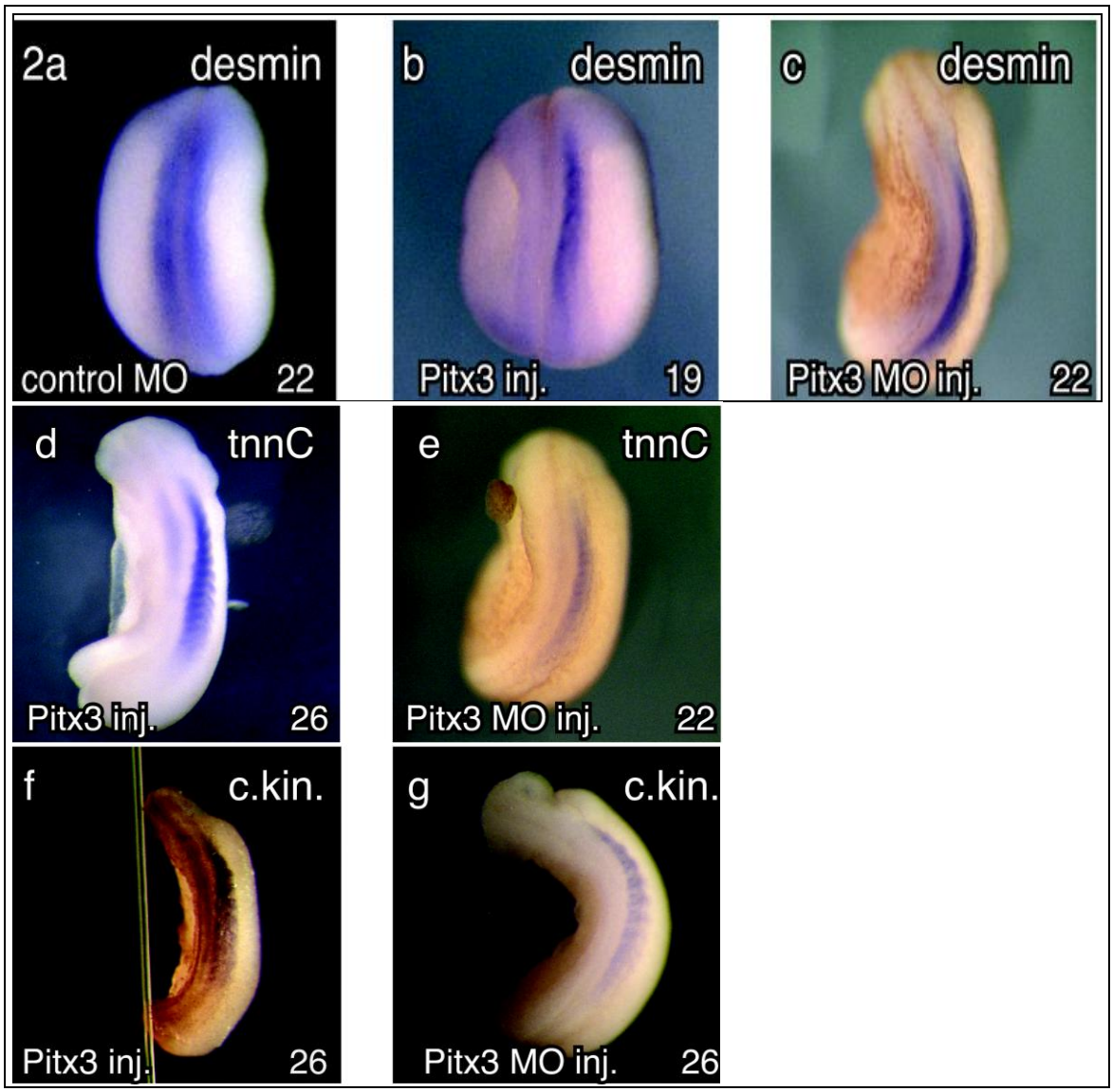

Figure 2.2: Effect of *Pitx3* perturbation upon myogenic/somite markers. *Pitx3* ectopic mRNA expression as well as *Pitx3 MO* inhibit expression of the early myogenic marker *desmin* (compare a to b, c), as well as the fast and slow skeletal markers *troponin C* (d, e), and *creatine kinase* (f, g). Dorsal view of left injected whole embryos with head oriented to the top. All embryos have been unilaterally injected on the left side.

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 2.3a 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 2.3e). 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 2.4). Apoptosis is not the cause of retarded somitogenesis.

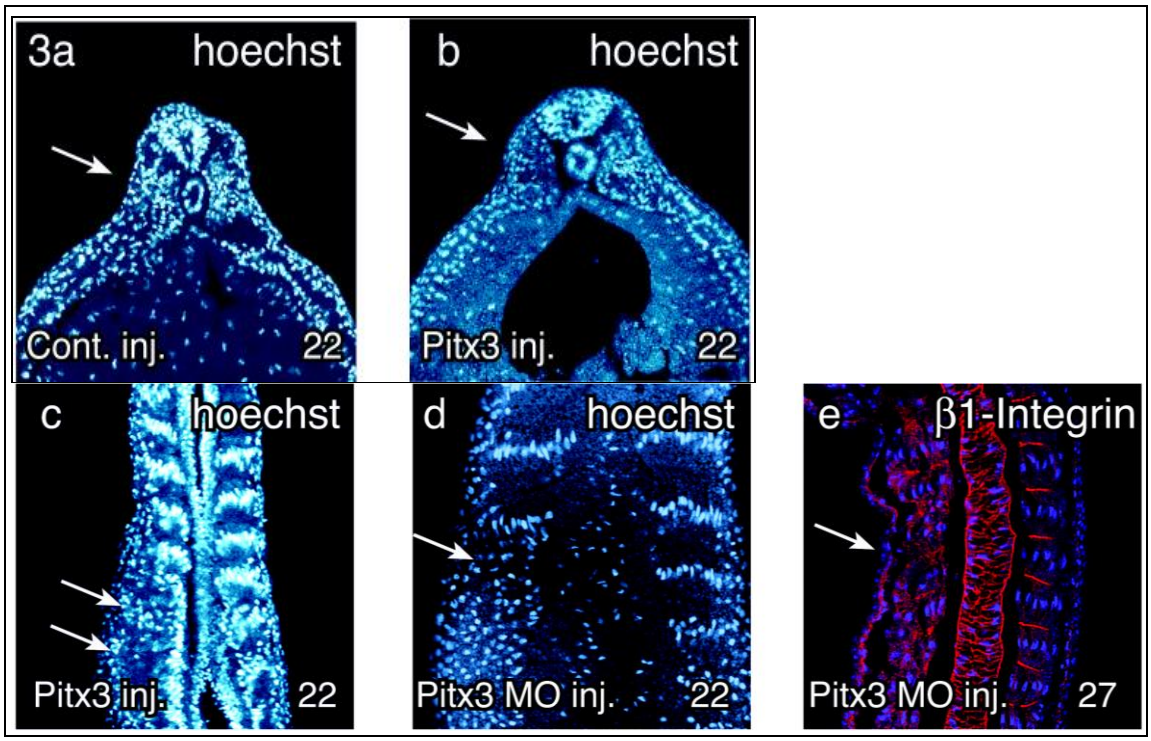

Figure 2.3: Effect of Pitx3 perturbation upon somite formation. *Pitx3* ectopic mRNA expression as well as *Pitx3 MO* inhibit the normal assembly of cells into somites indicated in Hoechst-stained coronal sections. Compare controls to left injected *GFP* mRNA and *Pitx3* mRNA embryos (compare **a** to **b**). In coronal sections (top is rostral), both mRNA and morpholino 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 (20x) (**e**) where nuclei are stained with Hoechst (blue) and inter somitic borders are indicated by b1-integrin staining (red).

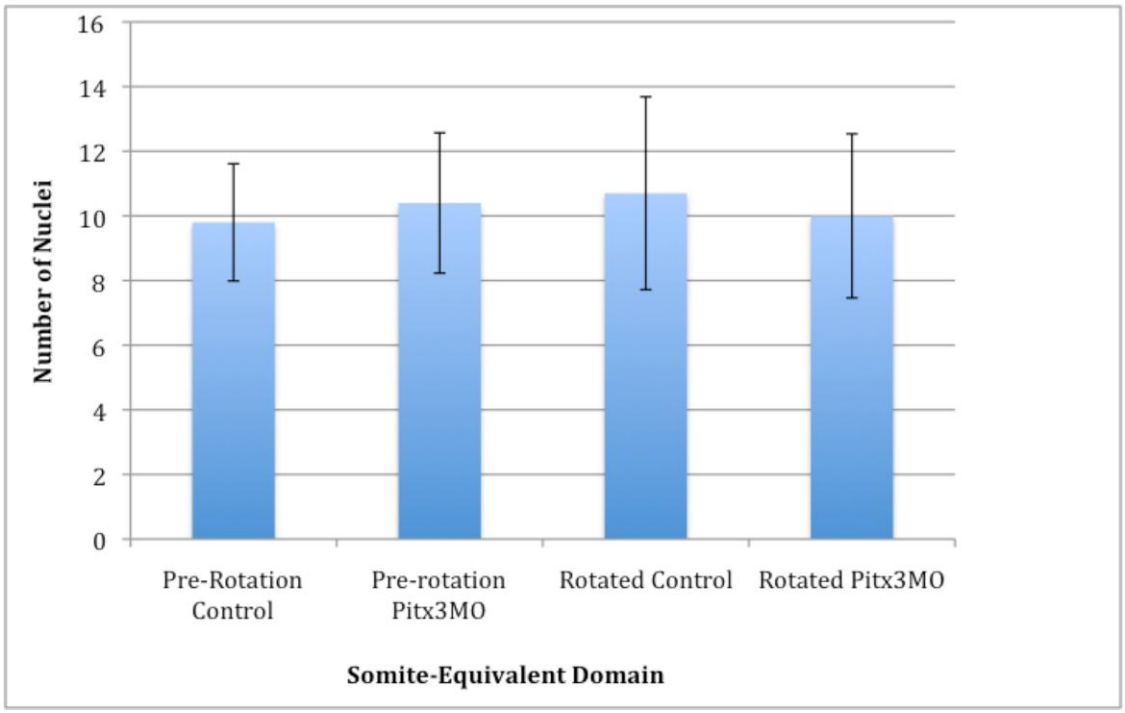

Figure 2.4: Graph comparing pre- and post somitic nuclear counts on either side of unilaterally injected *Pitx3 MO* embryos. Axially paired counts were made using coronally sectioned embryos stained with the nuclear stain Hoechst. There is no significant difference in cell number between control and *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.

Pitx3 is expressed just prior to gastrulation – the image in Fig 2.5a 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 2.5b). 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* mis-regulation, and in the case of *Shh*, both *Pitx3*-engrailed repressor as well as antisense morpholino injection have a similar inhibitory effect (Figs 2.5 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).

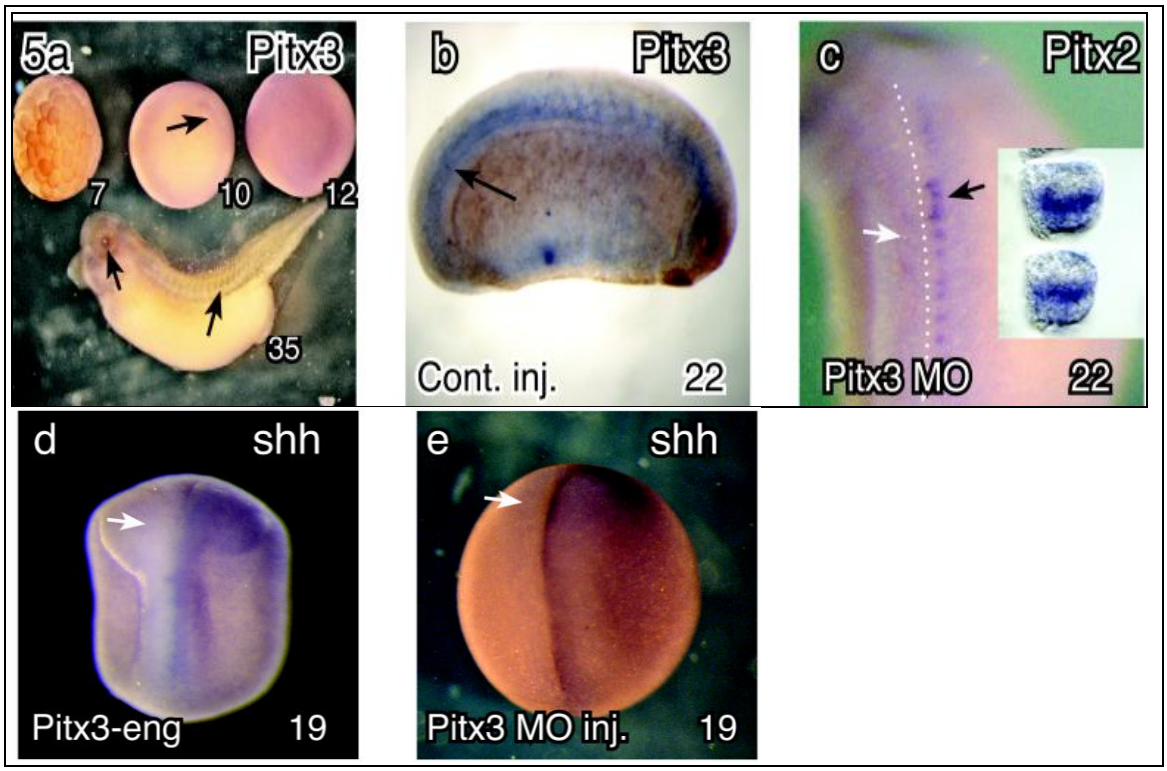

Figure 2.5: ***Pitx3*** **is detectable by in situ hybridization in early stages commencing just before gastrulation and through stages 10 and 12 (a)**. These four embryos were processed in the same vial, and the specificity of staining in the stage 35 embryo (lens and somites) as well as low background serves to indicate the legitimacy of staining at the earlier stages. *Pitx3* expression is expressed in re-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).**

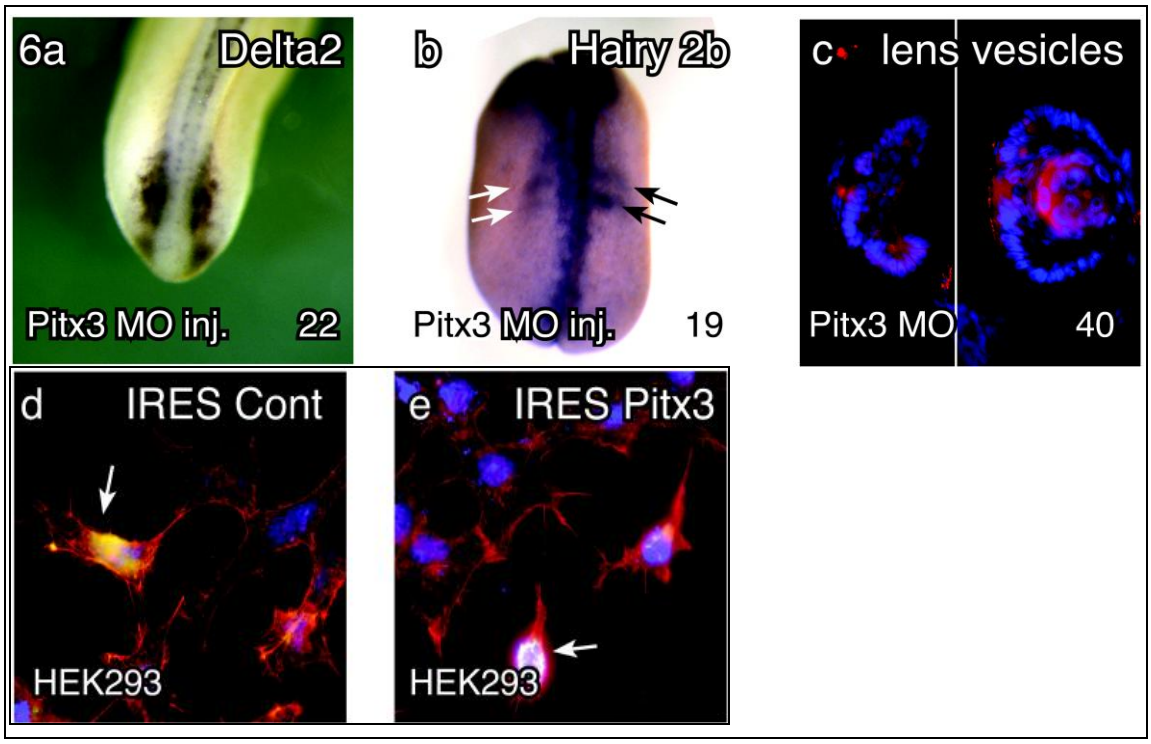

Figure 2.6: *Pitx3MO* has mixed effects upon the segmentation clock, and alters patterns of tissue differentiation. The characteristic periodic expression patterns of the upstream element of the clock (*Delta2*) were unaffected, while the downstream efector (*Hairy2b/Hes4*) was reduced or abolished by *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.

Perturbation of *Pitx3* activity in both embryos and tissue culture suggests that *Pitx3* plays a role in mediating cytoskeletal architecture (Fig 2.3 e and 2.6c, 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 2.6 d, e).

4. 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 them 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 serving 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 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.

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CHAPTER III

MICROARRAY BASED IDENTIFICATION OF PITX3 TARGETS DURING *XENOPUS* EMBRYOGENESIS

1. 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., 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.

2. Results and Discussion

2.1 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 3.1).

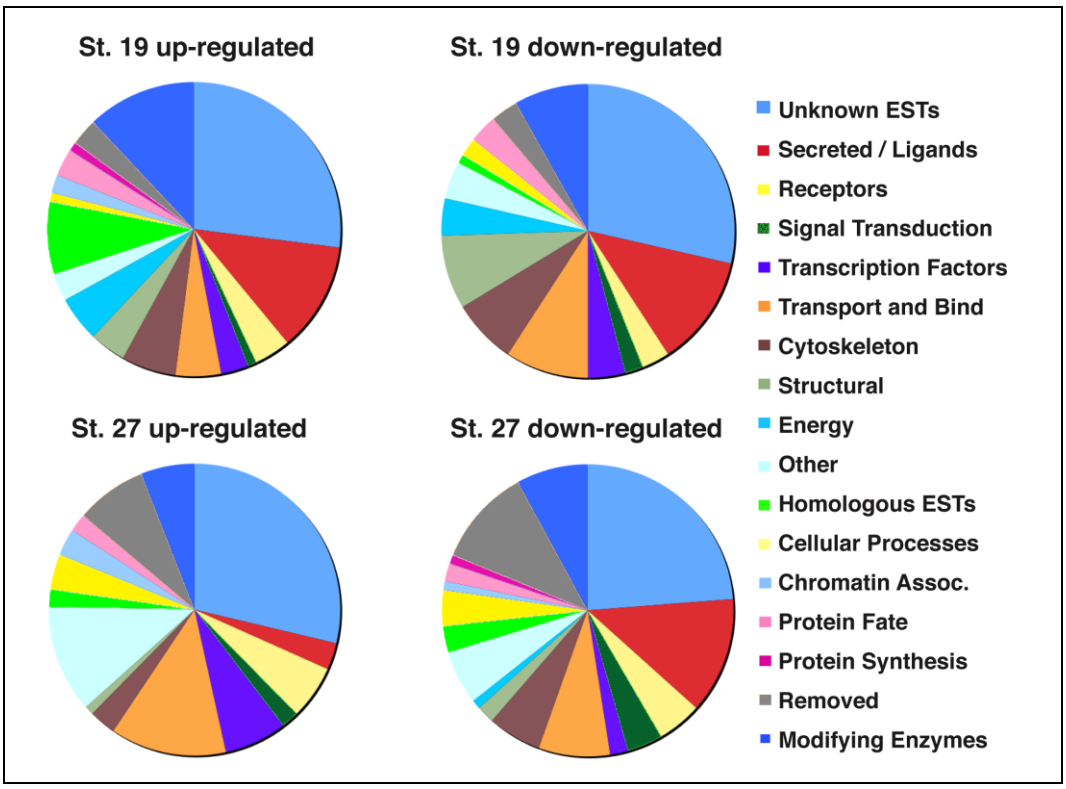

Figure 3.1: Microarray data represented according to putative gene function. The 100 most up- and down-regulated transcripts affected by *Pitx3*-morpholino-mediated knockdown were categorized by sequence analysis for stages 19 and 27 of *X. laevis* embryonic development. Colors correspond to functional groups in the legend (right).

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.

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 3.1). We then looked deeper into the data set to see if genes in the same signaling pathway or developmental process were similarly affected (Table 3.2). 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.

Gene ID	UniGene ID	Gene Highest BLASTn Hit (Xenopus laevis)	Microarray Ratio	Coincident Pathway Inductive Secondary	RT-PCR Confirms	Change in ISH expression pattern	BBE sites in 5kb of 5'UTR
xGsc	Xl.801	Goosecoid (gsc)	2.328 (19) 4.743 (27)* 2.267 (27)*	√	No	N/A	15
Bix4	Xl.399	homeobox protein BIX4 (bix4)	0.363 (27)	√	No	N/A	N/A
xLim1	Xl.21652	EST - LIM class homeodomain protein (Lim5/Lhx5) (lhx1)(Xlim-2B)	2.046 (27)	√ √	No	Yes	5
xVent2	Xl.37	VENT homeobox 2, gene 2 (ventx2.2) Xom protein	0.406 (27)	√ √ √ √	Yes	Ambiguous	17
xPax6	Xl.647	Paired box 6 (pax6-b)	0.226 (19) 0.477 (27)	√ √	No (19) Yes (27)	Yes	11
XL-Maf	Xl.767	neural retina leucine zipper (nrl) bZIP transcription factor L-Maf (maf)	0.357 (19)	√	No	N/A	19
Crybb1	Xl.21502	Beta B1-crystallin (Crybb1)	0.325 (27)	√	Yes (27)	Yes	13
xHey1	Xl.15572	EST - Moderately similar to transcription factor HES-7.1-B (XHR1)	0.211 (27)	√ √	Yes	Yes	N/A
xSpr1	Xl.17379	Sp5 transcription factor (sp5) Sp1-like zinc-finger protein XSPR-1	0.441 (19)	√	No	Ambiguous	N/A
XeFGF	Xl.1181	fibroblast growth factor 4B (fgf4-b) XeFGF(ii) embryonic fibroblast growth factor	0.406 (19)	√	No	N/A	5
xRXRa	Xl.877	retinoid X receptor, alpha (rxra)	0.319 (19)	√	No	N/A	1
Stripy	Xl.9206	Ledgerline (Stripy)	2.014 (19)	√ √ √	Yes (19)	Ambiguous	20
Hes4	Xl.25977	basic-helix-	0.299 (27)	√ √	Yes (27)	Yes (27)	7

		loop-helix transcription factor hairy2b (hairy2)		√			
Hes7	Xl.15142	EST - Highly similar to Xenopus laevis Esr-4	0.381 (27)	√ √	No (27)	Yes	16
xObscnl	Xl.13958	EST - Weakly similar to obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	6.211 (19)	√	No (19)	Yes (27)	N/A
xHoxA11	Xl.266	Homeobox A11 (HoxA11)	0.423 (27)	√	No	No	10
xSpr2	Xl.2755.1	Sp1-like zinc-finger protein XSPR-2 GLI family zinc finger 1, gene 2 (gli1.2)	0.396 (27)	√	No	Yes	N/A
xRbp4l	Xl.17576	EST Weakly similar to RET_B Human plasma retinol-binding protein precursor (PRBP)	6.164 (19) 4.429 (27)	√	Yes	N/A	N/A
xGalectinIX	Xl.15089	EST - Fish-egg lectin-like isoform 1	0.154 (19) 0.246 (27)	√ √	No (19) Yes (27)	N/A	N/A
xBaz2b	Xl.19899	EST - Moderately similar to bromodomain adjacent to zinc finger domain, 2B	0.400 (19)	√ √ √	No	Yes	N/A
xRdh16	Xl.5553	EST - retinol dehydrogenase 16 (all-trans) (rdh16)	6.288 (19) 2.758 (27)	√ √	No	N/A	N/A

Table 3.1: **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

Gene ID	*UniGene ID*	*Gene* *Highest BLASTn Hit (Xenopus laevis)*	*Microarray Ratio*
Rax1	Xl.186	Retina and anterior neural fold homeobox (Rax-a)	2.148 (19)
βB3-crystallin	Xl.26355	Crystallin, beta B3 (crybb3)	0.366 (27)
γ-crystallin-like	Xl.23710	Transcribed locus, strongly similar to NP_001087320.1 crystallin, gamma A (X. laevis)	0.140 (19)
γB-crystallin	Xl.21441	Crystallin, gamma B (crygb)	0.298 (19)
βB3-crystallin-like	Xl.26349	69% similar to beta-crystallin B3 (H. sapiens)	3.800 (27)
βA4-crystallin	Xl.19126 (retired) replaced Xl.67080	Crystallin, beta 4 (cryba4)	0.223 (19) 2.741 (27)
βB1-crystallin-like	Xl.1337	Transcribed locus, strongly similar to XP_002938264.1 predicted: beta-crystallin B1-like (X. tropicalis)	2.122 (27)
Tbx4	Xl.21543	T-box 4 (tbx4)	0.436 (27)* 0.237 (27)*
Tbx5	Xl.529	T-box 5 (tbx5-b)	0.432 (27)
HoxA10	Xl.21639	Homeobox A10 (hoxa10)	0.373 (27)
HoxA13	Xl.21581	Homeobox A13 (hoxa13)	2.337 (27)
Galectin I	Xl.747	Lectin, galactoside-binding, soluble, 1 (lgals1)	2.264 (19) 0.436 (27)
Galectin IIa	Xl.17371	Galectin family xgalectin-IIa (xgalectin-IIa)	0.291 (19)
Galectin IIb	Xl.21879	Galectin 4 (lgals4-a)	2.367 (19)
Galectin IIIa	Xl.15364	Lectin, galactoside-binding, soluble, 9c (lgals9c-a)	0.414 (27)
Galectin IIIb	Xl.21878	Lectin, galactoside-binding, soluble, 9c (lgals9c-b)	0.304 (19)

Table 3.2: **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 *Stripy*) 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 3.1). 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.

2.2 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. 3.2-7), brain (Fig 3.8), somite (Fig 3.9), and tailbud (Fig 3.10).

2.2.1 Eye development

Among other domains*, Vent2* (a.k.a. *Ventx2*) is expressed in the dorsal retina (Fig 3.2F, 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 3.2H, I, J).

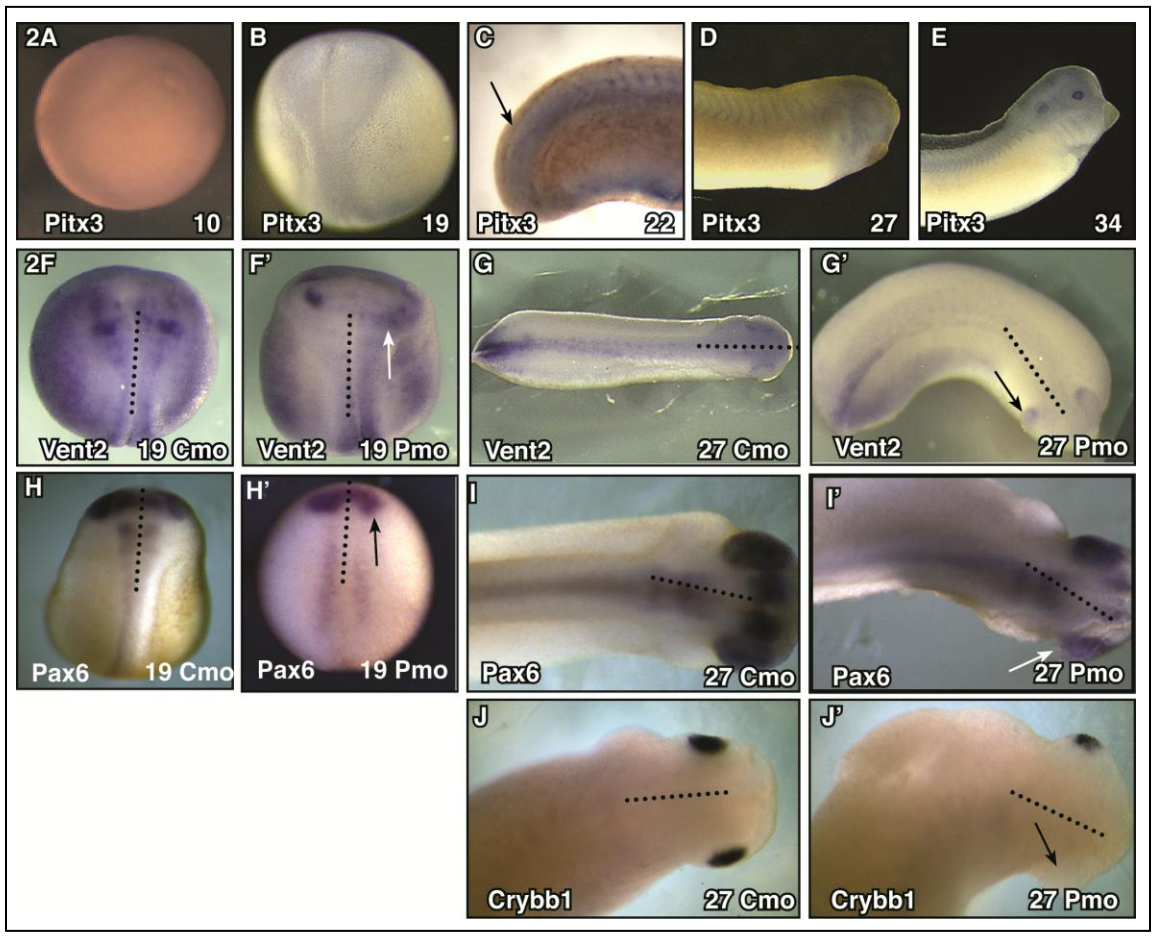

Figure 3.2: *In situ* hybridization analysis for putative targets of Pitx3 involved in eye development. Visual comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or *Pitx3*-morpholino (Pmo) embryos and their untreated contralateral control. **A-E: *Pitx3*** expression 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 detactable throughout neural ridge, while at stage 22, the gene is expressed in a cleared specimen where an arrow indicates pre-somitic 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 (F' white arrow) and at stage 27 (G' 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 (H' black arrow) and 27 (I' white arrow). **J-J': *Crybb1*** shows drastic loss of expression in the eye vesicle on the Pmo treated side of stage 27 embryo (J') and no difference caused by Cmo treatment (J). Dotted line represents the midline of the embryo, separating injected right side from contra-lateral left side control.

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 it 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 *γ- 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 the microarray also underwent significant fractional change, albeit at less spectacular levels, namely : *γ crystallin* (0.14), *γ B crystallin* (0.3), *β B3 crystallin* (3.8), *β A3 crystallin* (2.74), and species weakly similar to human *β B1 crystallin* (2.12), and *β 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 3.3B). *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 3.3C-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 3.3B). Consistent with the microarray and RT-PCR data, the gene undergoes up-regulation as a consequence of Pitx3 knockdown (Fig 3.3F). 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 show the possibility of acting downstream of *Pitx3* in lens developmental pathways, where both genes are expressed.

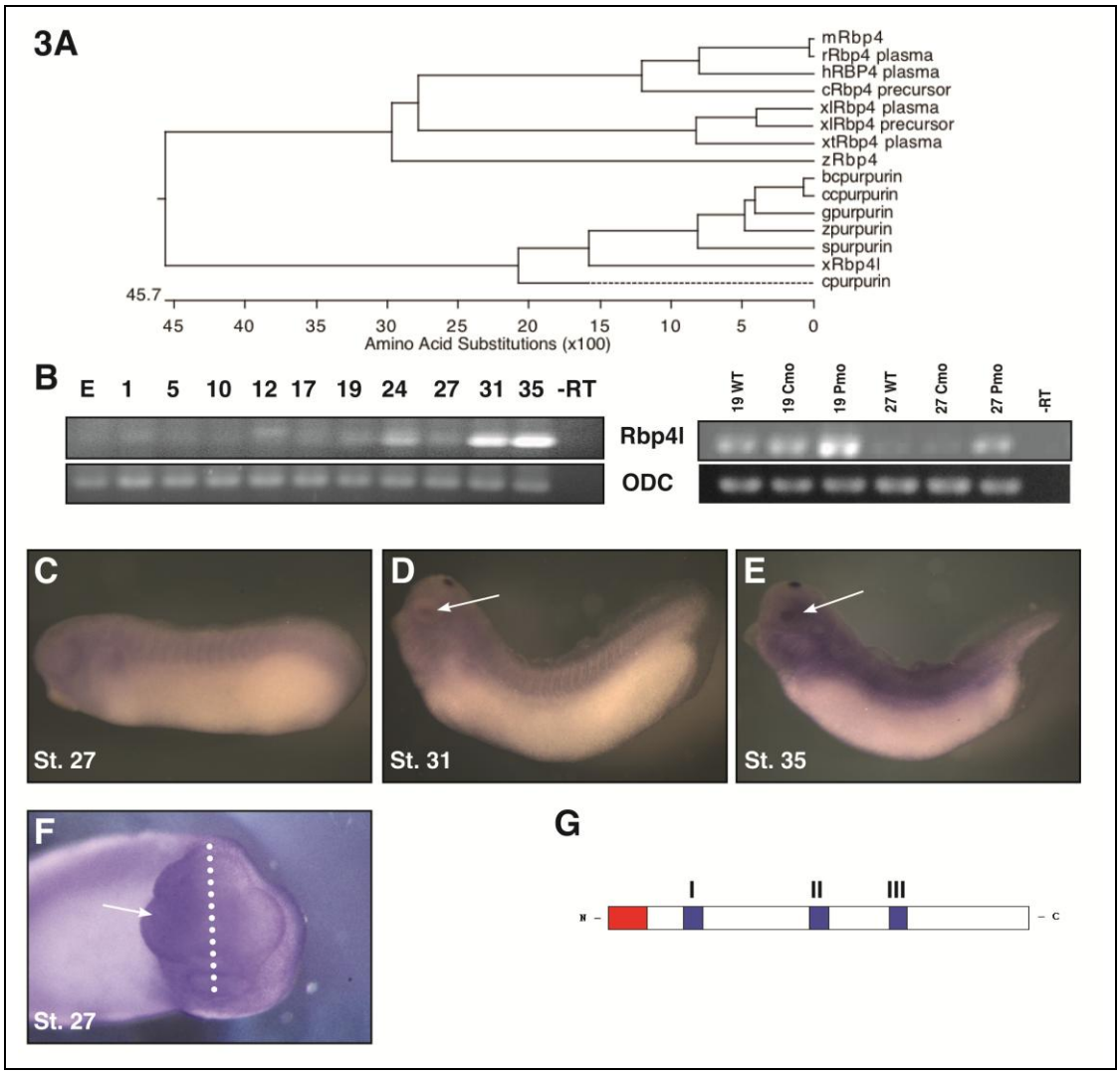

Figure 3.3: Characterization of a novel transcript, *Rbp4l*, in *X. laevis*. **A:** Protein alignment showing distinct groups between retinol binding proteins and purpurin family members. **B:** Temporal expression of *Rbp4l* throughout embryonic stages of development, showing slight detection at stages 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*), xlRb4 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).

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 3.4). We identify this sequence as a *Galectin IX* (Genbank Accession JN975639). It is related to the tectonin family that encode 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).

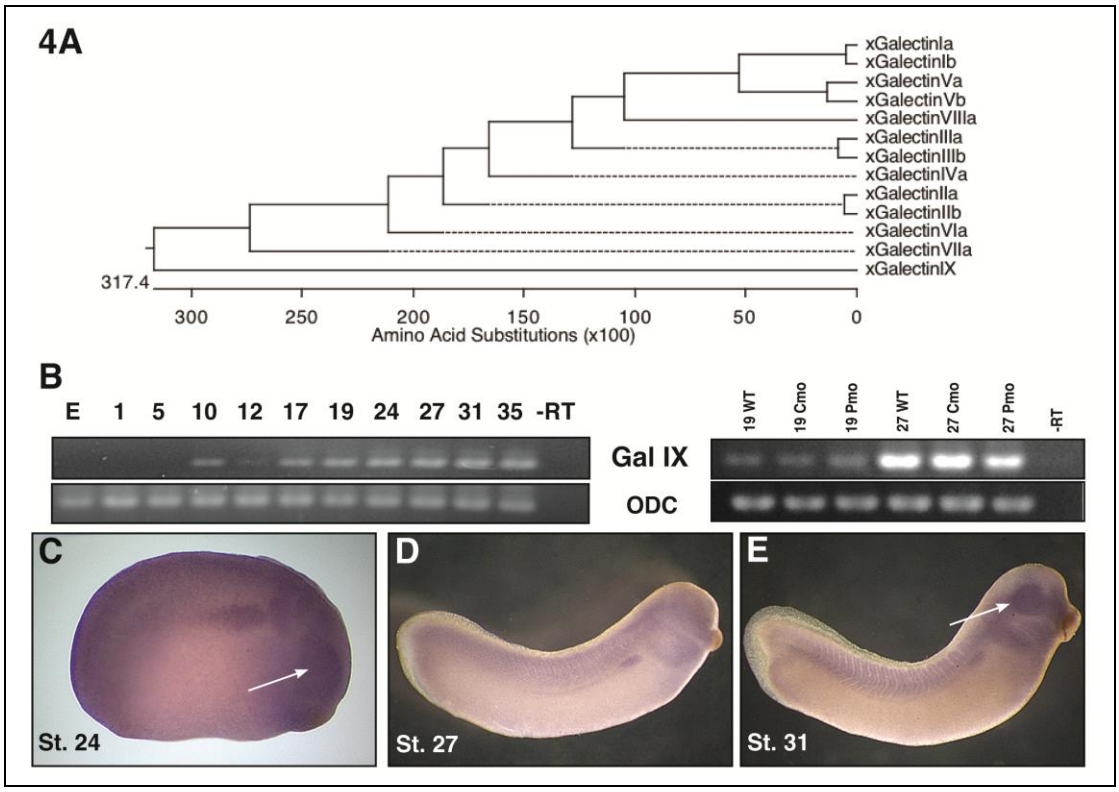

Figure 3.4: Characterization of a novel transcript *Galectin IX* in *X. laevis*.
A: Protein alignment showing amino acid similarities between *Xenopus* Galectin family members. **B:** Temporal expression of *Galectin IX* throughout embryonic stages of development, shows expression beginning at gastrulation (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, xGalectinIIIa AB060971, xGalectinIIIb AB080017, xGalectinIVa AB060972, xGalectinVa M88105, xGalectinVb AB080018, xGalectinVia AB080019, xGalectinVIIa AB080020, xGalectinVIIIa AB080021, xGalectinIX BJ056659.

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 3.4). 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 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 3.4C-E). Expression begins at gastrulation, fades and then increases gradually beginning at neurulation (Fig 3.4B). Curiously, RT-PCR for microarray confirmation (Fig 3.3B) 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 3.5). 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 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 3.5B) 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 3.5C-E). If this gene is a homolog of human 11-cis *RDH*, the expression in the eye would support a conserved functional role.

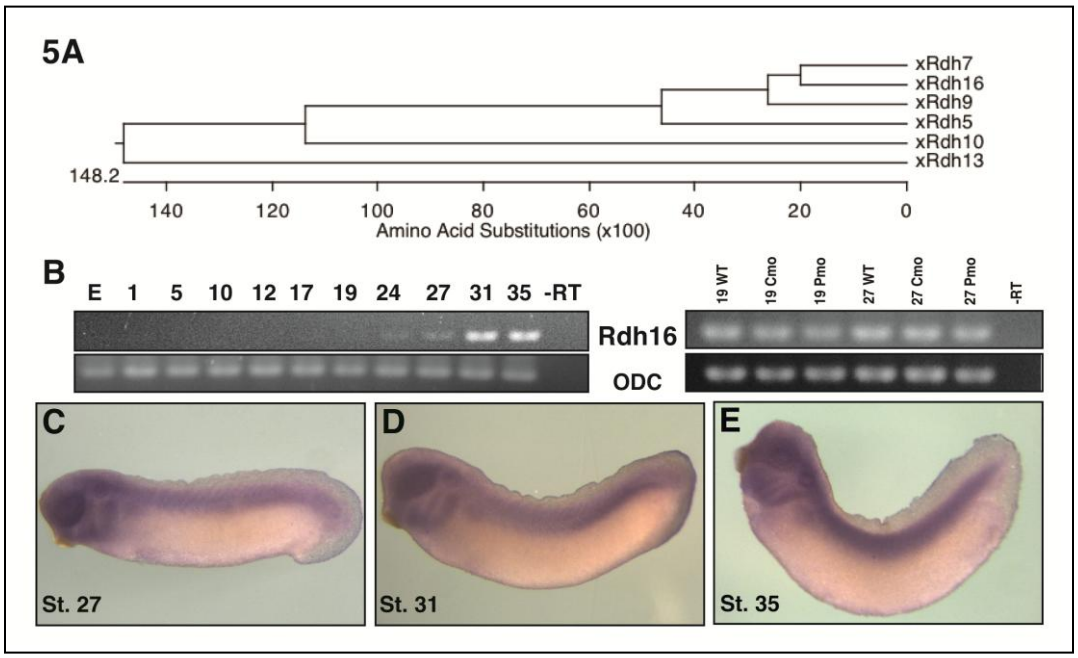

Figure 3.5: Characterization of a novel transcript, *Rdh16*, in *X. laevis*. A: Protein alignment showing amino acid similarities between *Xenopus* retinol dehydrogenase (rdh) family members. **B:** Temporal expression of *Rdh16* throughout embryonic stages of development shows faint expression beginning at stage 24 and 27, then increasing at stages 31 and 35. B 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.

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 3.6A), and a chromatin remodeling gene *Baz2b* (Figs 3.6B, 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.

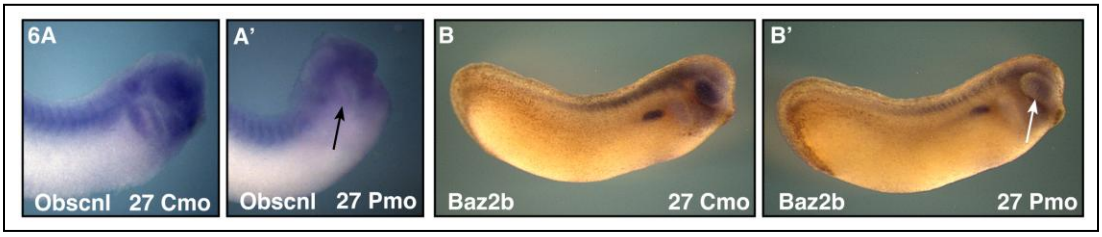

Fig 3.6: *In situ* hybridization analysis for putative Pitx3 target genes *Obscnl* and *Baz2b*. Visual comparisons of gene expression patterns between control-morpholino (Cmo) and *Pitx3*-morpholino (Pmo) right side-injected embryos. *Obscnl* shows a loss of expression in the branchial arches (black 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).

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 3.7F). 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 3.4F). 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 3.7B). Its spatial expression pattern, initially quite diffuse (not shown), condenses around the developing eye and pronephric structures during tailbud stages (Fig 3.7C-E).

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).

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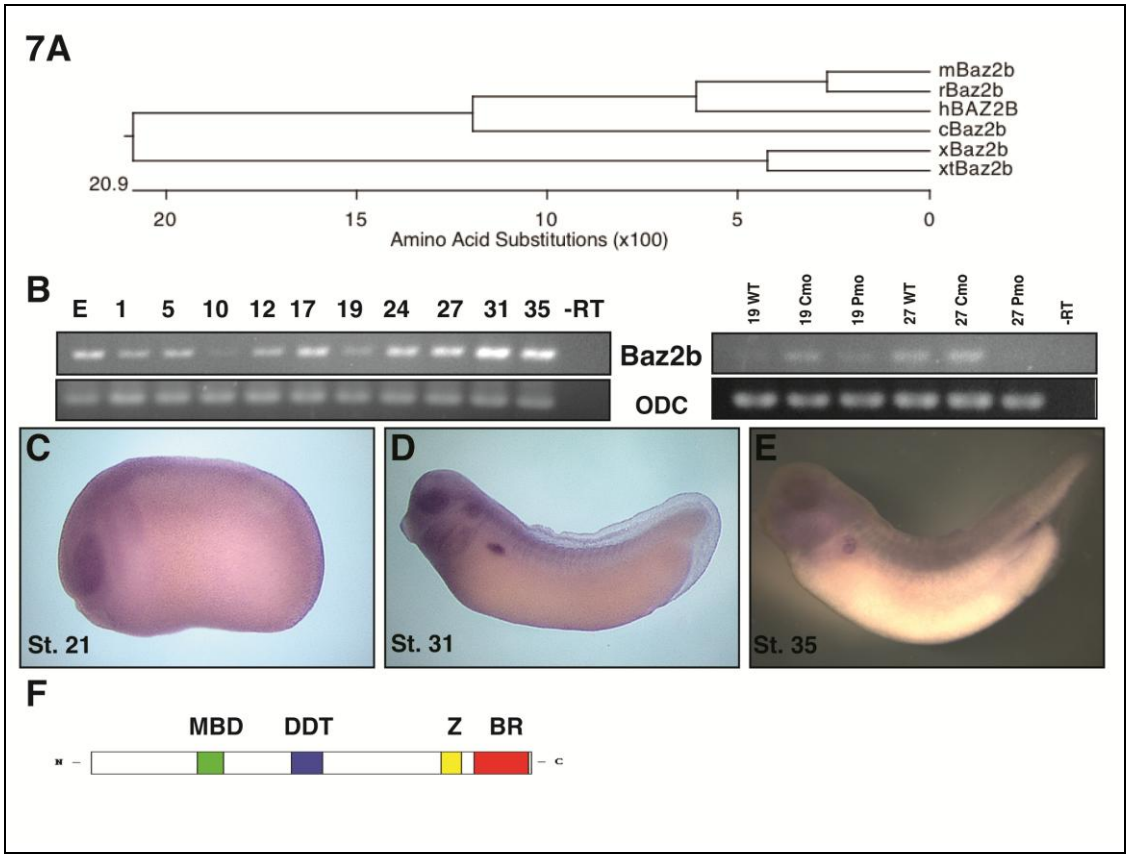

Figure 3.7: Characterization of a novel transcript, *Baz2b*, in *X. laevis*. **A:** Protein alignment showing amino acid similarities between *Baz2B* homologs across organisms. **B:** Temporal expression of *Baz2b* throughout embryonic stages of development show expression as a maternal transcript in 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*).

2.2.2 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 3.8).

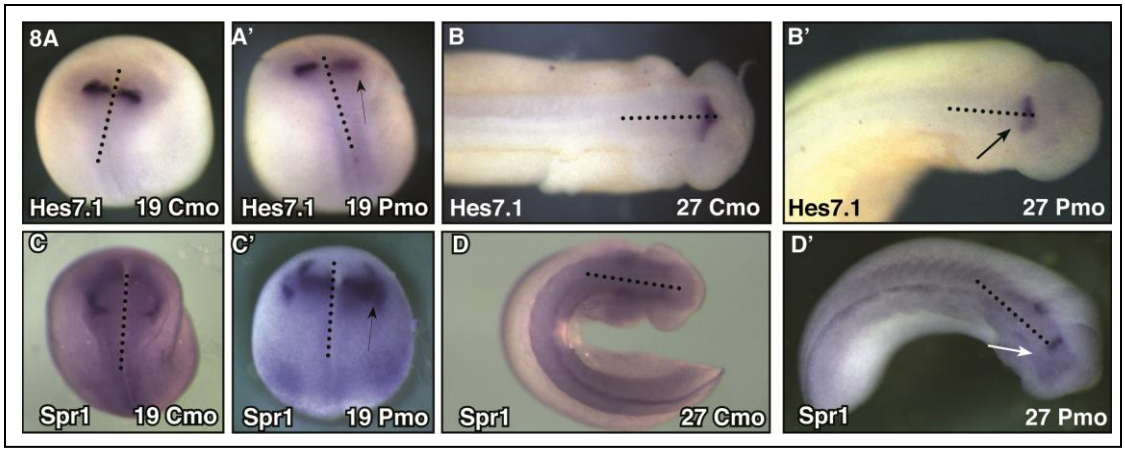

Figure 3.8: *In situ* hybridization analysis for putative brain targets of Pitx3. Comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or *Pitx3*-morpholino (Pmo) embryos and their untreated contra-lateral control. **A-B': *Hes7.1*** at stage 19 shows decreased 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.

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 3.2). 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).

2.2.3 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 somitogeneisis. 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 3.9), 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 mediates 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., 2006). 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.

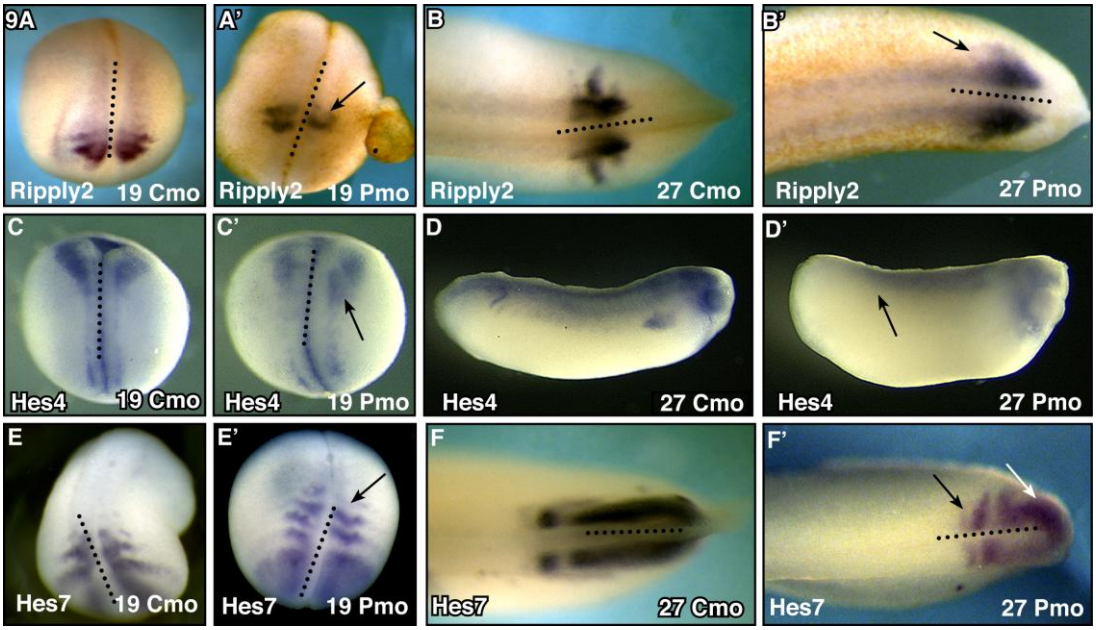

Figure 3.9: *In situ* hybridization analysis for putative segmentation targets of Pitx3. Visual comparisons of gene expression patterns between right-side injected control-morpholino (Cmo) or *Pitx3*-morpholino (Pmo) embryos and contralateral control. **A-B': *Ripply2*** expression, showing as two stripes 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.

Both *eFGF* and *RXRα* 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 *RXRα* 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 3.10), it might be worth re-examining their failed candidacy at targets.

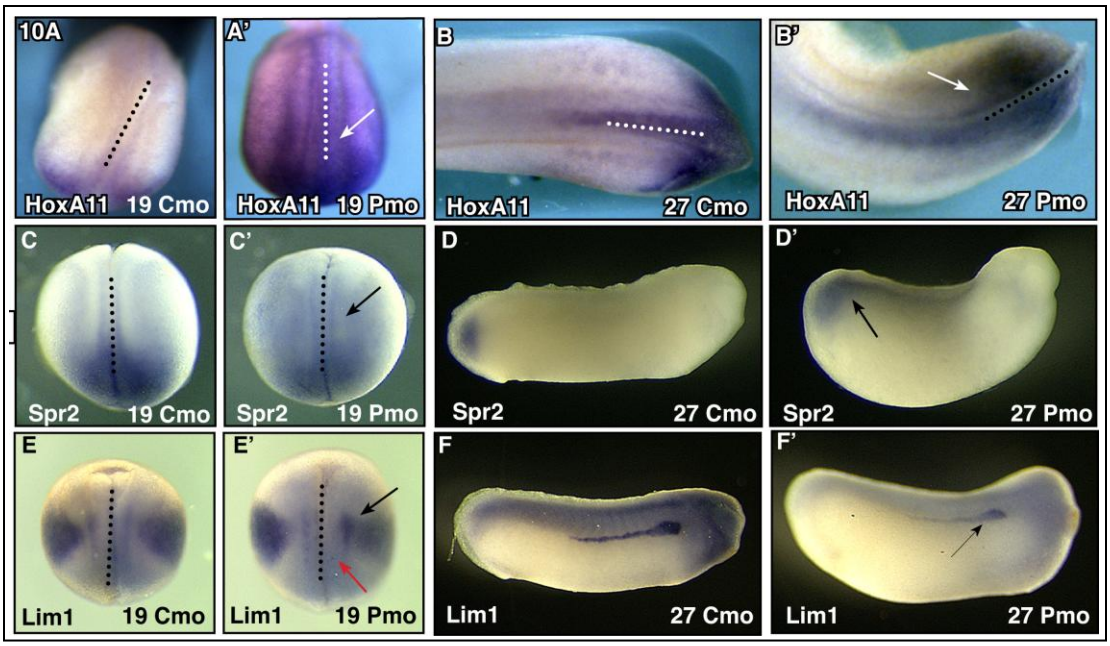

Figure 3.10: *In situ* hybridization analysis for putative tailbud targets of Pitx3. A-B': *HoxA11* shows decreased posterior expression in the tailbud region (white arrows) of Pmo embryos at stages 19 (A') and 27 (B');**C-D': *Spr2*** displays a broader and larger domain of expression (black arrows) 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.

Spr2 and *HoxA11* are affected by *Pitx3* mis-regulation (Fig 3.10). *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.

2.2.4 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).

2.4 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.

3. Experimental procedures

3.1 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^{o} C for at least 1 hr to allow healing before being removed and allowed to develop at 12^{o} C in 0.1 X MBS.

3.2 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.

3.3 RT-PCR

cDNA was made using Omniscript reverse transcriptase (Qiagen) and Oligo(dT)$_{18}$ primers (Sigma) from 1ug 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 outline in Table 3.3.

Gene	Primers	Size	Anneal Temp	GenBank	Reference
ODC	Sense: 5' - GTC AAT GAT GGA GTG TAT G - 3' Antisense: 5' - TCC ATT CCG CTC TCC TGA - 3'	385bp	57^{o}C		XenBase
Lim1	Sense: 5' - CCG ACA CAT AAG GGA GCA GC - 3' Antisense: 5' - CTG GTG GGT GTG ACA AAT GG - 3'	573bp	60^{o}C	X63889	Homemade
Spr1	Sense: 5' - CCA GGT ACA AGT CCT ACT GA - 3' Antisense: 5' - GAG TGC CAC CTC AAA TGA GC - 3'	752bp	54^{o}C	AY062264	Ossipova et al., 2002

Spr2	Sense: 5’ - CAA ACT GTT GCC TCT CAT GAG - 3’ Antisense: 5’ - CAC TTA CAC CTC CGG CAG CGC - 3’	380bp	54°C	AY062263	Ossipova et al., 2002
Vent2	Sense: 5’ - GCT TTC TCC TCG GTT GAA TG - 3’ Antisense: 5’ - TCT CCT TCA GGG GCT GTA GA - 3’	461bp	57°C	X98454	Homemade
Hes4	Sense: 5’ - GCA CGA ACG AAG TCA CAC GA - 3’ Antisense: 5’ - GCT GGG TTG GGA ATG AGG AAA G - 3’	297bp	65°C	AF139914	Homemade
Hes7.1	Sense: 5’- TGT AAT GTG CTC AAA TGG CG - 3’ Antisense: 5’ - TCC GTC AGC CCT ACA AAG AC – 3’	336bp	54°C	BJ088128	Homemade
Obscnl	Sense: 5’ – ACA GTA TGG TTC ACA GCC – 3’ Antisense: 5’ – CAG TTG GCA CAT CAA TCC AG – 3’	283bp	57°C	BJ085487	Homemade
Gsc	Sense: 5’ - ACA ACT GGA AGC ACT GGA - 3’ Antisense: 5’ - TCT TAT TCC AGA GGA ACC - 3’	279bp	52°C	M81481	XenBase
RXRα	Sense: 5’ - AAG ATA CTT GAG GCG GAG CA - 3’ Antisense: 5’ - TTC GGG GTA TTT CTG TTT GC - 3’	531bp	54°C	L11446	Homemade
L-Maf	Sense: 5’ - CTT GCT CCT CCT CAA TCT CTG G - 3’ Antisense: 5’ -CCG ACA AAG GCG AAA GCT GGT G - 3’	331bp	54°C	AF202059	Ishibashi and Yasuda, 2001
eFGF	Sense: 5’ - TTA CCG GAC GGA AGG ATA - 3’ Antisense: 5’ - CCT CGA TTC GTA AGC GTT - 3’	222bp	56°C	X62594	Kroll Lab
Bix4	Sense: 5’ - CAG AAC AGG AGA TCA AAA GC - 3’ Antisense: 5’ - CGG GTA GGT ACT AGA TGC TG - 3’	414bp	54°C	AF079562	Homemade
Hes7	Sense: 5’ – TGT TGG CTT GAA AGG TTT GT - 3’ Antisense: 5’ – CTC AAA ATG TGT CAT AAT CCA - 3’	394bp	60°C	BJ058661	Homemade
Ripply2	Sense: 5’ – ATG GAG CCG AAT CAA CAG C - 3’ Antisense: 5’ – TGT CTT CCT CTT CAG AGT CA - 3’	352bp	57°C	AB073615	Homemade
Crybb1	Sense: 5’ – CGT GGT GAG ATG TTT ATC CTG GAG - 3’ Antisense: 5’ – CCT TCT GGT GCC ATT GAT TGT CTC - 3’	394bp	60°C	CD303346	Homemade
Pax6	Sense: 5’ – GCA ACC TGG CGA GCG ATA AGC - 3’ Antisense: 5’ –CCT GCC GTC TCT GGT TCC GTA GTT - 3’	448bp	56°C	U77532	Zuber, M.E. et al., 2003
HoxA11	Sense: 5’ – AAT CCC TCC AAT GTC TAC CAC C - 3’ Antisense: 5’ – CTG GTA TTT GGT ATA CGG GCA C – 3’	363bp	56°C	AJ319668	Slack, J.M.W. et al. 2001
Rbp4l	Sense: 5’ – AGA TGC AAT GCT CAG TCC T – 3’ Antisense: 5’ – GCG GGA GAA TAT AAT AGA ATA – 3’	432bp	54°C	CD362061	Homemade

Galectin IX	Sense: 5' – CCC GTG CCT GGT ATT TCA – 3' Antisense : 5' – ACC TGG CTG GAG TGA ACA – 3'	448bp	55°C	BJ056659	Homemade
Baz2b	Sense: 5' – AAG ATG ATG ATG AGG ACG A – 3' Antisense: 5' – CCA TTT TAG CCT GCT GTT TC – 3'	837bp	55°C	BQ400337	Homemade
Rdh16	Sense: 5' – CTG CGA CTC TGG GTT TGG A – 3' Antisense: 5' – TCA TAG CCG GCA GAG TAG – 3'	750bp	57°C	BG514525	Homemade

Table 3.3: Parameters and primer sequences used in RT-PCR experiments.

3.4 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.

3.5 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.

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CHAPTER IV

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

1. 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). In mammals, 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 (Maxwell et al., 2005; Smidt et al., 2004; van den Munckhof et al., 2003). *Pitx3* also expresses in developing somites, lens placode, and in forming lens pit (Semina et al., 1998; Smidt et al., 2004; Smidt et al., 1997). 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 dygenesis 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 (Khosrowshahian et al., 2005; Pommereit et al., 2001; Smoczer et al., 2012).

Murine *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 transcription of *miR-133b* and miR-133b in turn represses 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.

Murine Pitx3 is known to directly regulate *tyrosine hydroxylase* expression (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 protein 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 fibers) (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.), 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 both 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, namely *lhx1*, *gsc*, *nodal5* and *crybb1* 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 asymmetry. The 4 newly identified putative target genes possess between 4 to 13 *Pitx3* binding motifs.

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 (for example see Beachy et al., 1993)would not be easily discerned using standard assays.

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-*GFP* IRES unit that is bicistronically linked to the cDNA for *pitx3* (input); the other houses CMV-*HcRed1* in opposite orientation to a test-promoter driven reporter, *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 *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 with the range at which GFP does not accumulate and fluoresce more than pitx3 is detectable on Western blots.

We can confirm three new direct targets for pitx3 and show that the factor acts as either an activator or repressor, contingent upon the context of its environment, including the promoter at hand. Translated pitx3 represses both crybb1, a lens-specific

differentation marker and *nodal5*, an early inducer of mesendoderm formation, while it activates *lhx1*, a factor present early in the Spemann organizer and later in the pronephric kidney (Taira 1992). Based upon our preliminary slate of putative signaling targets, our suspicion is that *pitx3* plays a heretofore uncharacterized role during gastrulation – previous work has indicated that it expresses in fish hypoblast (Dutta et al., 2005), and somewhere in *Xenopus* pre-gastrula (Khosrowshahian et al., 2005; Smoczer et al., 2012).

2. Materials and methods

Plasmid constructs

Expression plasmid (pPitx3-IRES-GFP). The p*itx3* 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 occurrs in the murine 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 was produced through site-directed mutagenesis, by mutating the leucine into a proline at the inter-helix hinge position 39 of the pitx3 homeodomain sequence (L99P).

Reporter plasmid. The pCS2-HcRED1 vector was generated through PCR-amplification of the HcRED1 sequence from pCAG-HcRed1 (Addgene 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 subcloned 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). I thereby also introducing a *KpnI* site (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 (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 (small case) utilizing mutated oligonucleotides to introduce an *NcoI* restriction site (bold) into the bicoid-motif (underlined) (GTGCTTAATGGTTTA → GTGCT**ccATGG**TTTA).

nodal5 promoter and mutant (pHcRed/Xnr5-DsRed). The *nodal 5* promoter was PCR-amplified using adaptors for *KpnI* and *BamHI* using *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 (small case) was used to create the *nodal5* mutant promoter situated at the bicoid motif (underlined), introducing a novel *SalI* site (bold) (TGAAGTAAGCTTCTG→TGAA**GTcgaC**TTCTG).

gsc promoter (pHcRed/xGsc-DsRed_The *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 *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.

Crybb1 promoter and mutants (pHcRed/crybb1-DsRed) The *crybb1*reporter cassette was generated by cloning the 3.5kb *SacI/ApaI* digested promoter out of the *X. laevis crybb1* promoter (kindly gifted by Dr. H. Kondoh) into the multiple cloning site of pDsRED-express-N1. The transcription cassette was PCR amplified, cloned into the *PvuII* site of the pCS2-HcRED1 and selected for a reporter cassette inserted in reverse orientation to the CMV-HcRed1. *Crybb1*mutant A was generated by deleting the last 750bp containing six binding site with restriction enzymes *BlpI* and *SpeI*. *Crybb1*mutants B and C were produced by site-directed mutagenesis (small case) using mutated primers to introduce new *EcoRV* and *HindIII* restriction sites (bold) into the bicoid motif (underlined) respectively (GTACTGCATTATCAA → GTACTGC**gaTAT**CAA and TTAAAACATTATTTC → TTAA**AAgcTT**ATTTC).

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.

The SK-N-BE(2)c neuroblastoma cell line (kind gift from Dr. L.Porter) was cultured under standard conditions in DMEM/Ham's F-12 (Sigma-Aldrich) supplemented with 2mM L-Glutamine (invitrogen) and 10% fetal bovine serum (Invitrogen), and Penicillin-Streptomycin (Sigma-Aldrich).

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 were 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.

SK-N-BE(2)c cells were transfected using METAFECTENE EASY+ (Biontex Laboratories Gmbh) as described by the manufacturer. The lipoplexes, representing a combination of 1.5ml 1xbuffer, 42ul transfection reagent and 42ug DNA (30ug:12ug reporter plasmid: expression plasmid), were added to a cell suspension of 7x10^5 cells/ml the day of splitting. The cells were washed with Hank's and media was replaced about 7 hours post-transfection to minimize the autofluorescence caused by the transfection reagent. The experiment was run through the flow cytometer 48 hours after transfection, following extensive washes with PBS.

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. 1^0 Rabbit Antibody: PAS 3131/3132), 47kDa a-actin 1:10,000 (Sigma 1^0 Rabbit Antibody: A2066), 27kDa eGFP 1:5000 (Torrey Pines Biolabs Inc. 1^0 Rabbit Antibody: TP401), Chemicon International 2^0 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 4.1 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.

Channel Detector	**Fluorescent Protein**	**Colour**	**Excitation Peak (lmax)**	**Emission Peak (lmax)**	**Filter**	**Voltage**	**Gain**
FL1	eGFP [F64L/S65T]	Red-shifted Green	490nm	510nm	525BP	329	1.0
FL2	DsRED-Express	Red-orange	557nm	579nm	575BP	332	1.0
FL5	HcRED-1	Far-red	588nm	618nm	640LP	500	1.0

Table 4.1. 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 statictical 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. 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 $p<0.05$.

3. Results

3.1 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 *pitx3* and *GFP* (Fig.4.1A) and simultaneously produces two proteins from a single mRNA transcript (Trouet et al., 1997). A corresponding pitx3 binding mutant was constructed by inserting a mutated form of pitx3 as the first coding sequence of the bicistronic unit. The point mutation within the DNA-binding homeodomain was modeled after one described for *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). This mutation encodes a L99P substitution that is situated between helices II and III of the homeodomain. The second vector harbours the promoter reporter and a transfection calibration fluor (Fig.4.1B). Mutants were also generated for promoters to serve as

specificity controls by prohibiting pitx3 binding: *TH* mutant (-350bp from ATG: TAATCC →TAccCC), *lhx1* mutant (-709bp from ATG: TAATGG →TccaTGG), *nodal5* mutant (-94bp from ATG: TAAGCT →TcgaCT), *crybb1* mutant (-1156bp from ATG: ACATTA →AgcTTA).

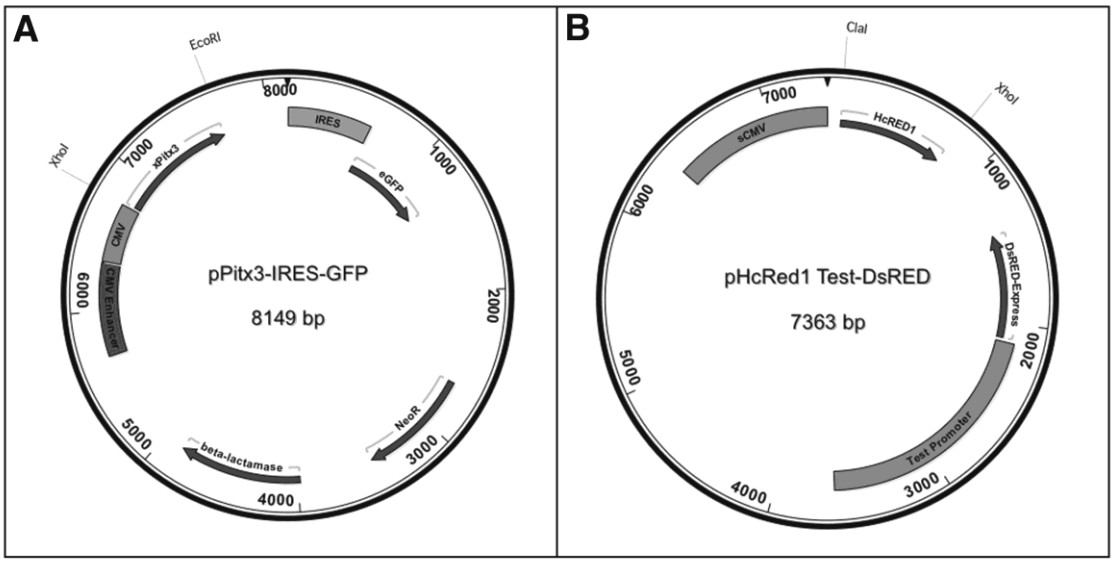

Figure 4.1: Expression and reporter plasmids. **A.** Expression plasmid with pitx3 bicistronically linked to GFP. **B.** Reporter plasmid with the reporter gene DsRed-express driven by the tested promoter, cloned in opposite orientation from the transfection control gene HcRed1 driven constitutively by CMV.

3.2 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 are 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 revealed a strong and consistent correlation between the two proteins across all concentrations (Fig. 4.2A).

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 (Fig. 4.2B) 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.

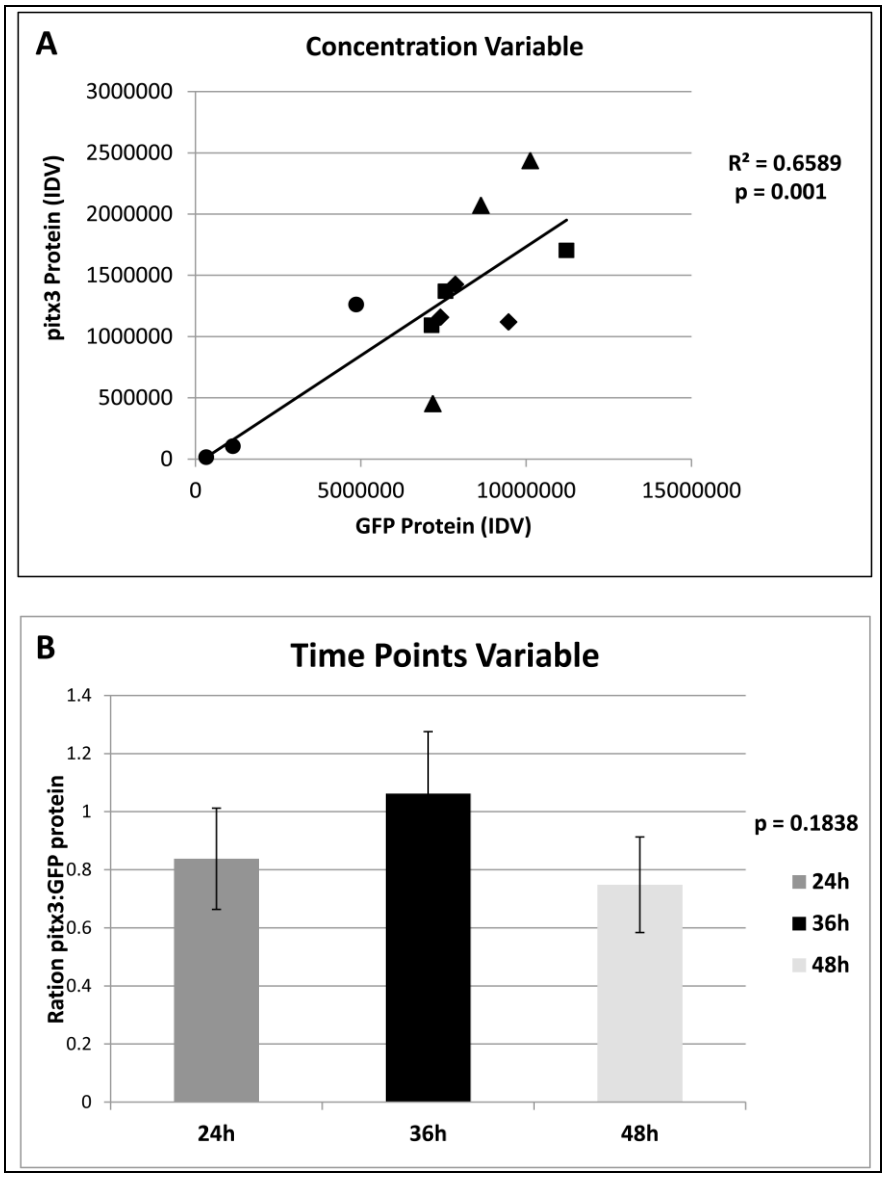

Figure 4.2: Correlation between the pitx3 and GFP proteins. The proteins were assayed by Western blotting and the amount of each protein was assessed as the optical density of the respective band. **A.** Regression analysis to correlate the levels of pitx3 and GFP proteins in cells transfected with different concentrations of expression vector: ● = 5.48ug; ▲ = 7.31ug**;** ♦ = 9.75ug; ■ =13ug. **B.** Ratios between the levels of Pitx3 and GFP protein in cells transfected with a set concentration of expression vector and analyzed at 24 (medium gray), 36 (black) and 48 hours (light gray)post-transfection

3.3 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 (Fig. 4.3A and B).

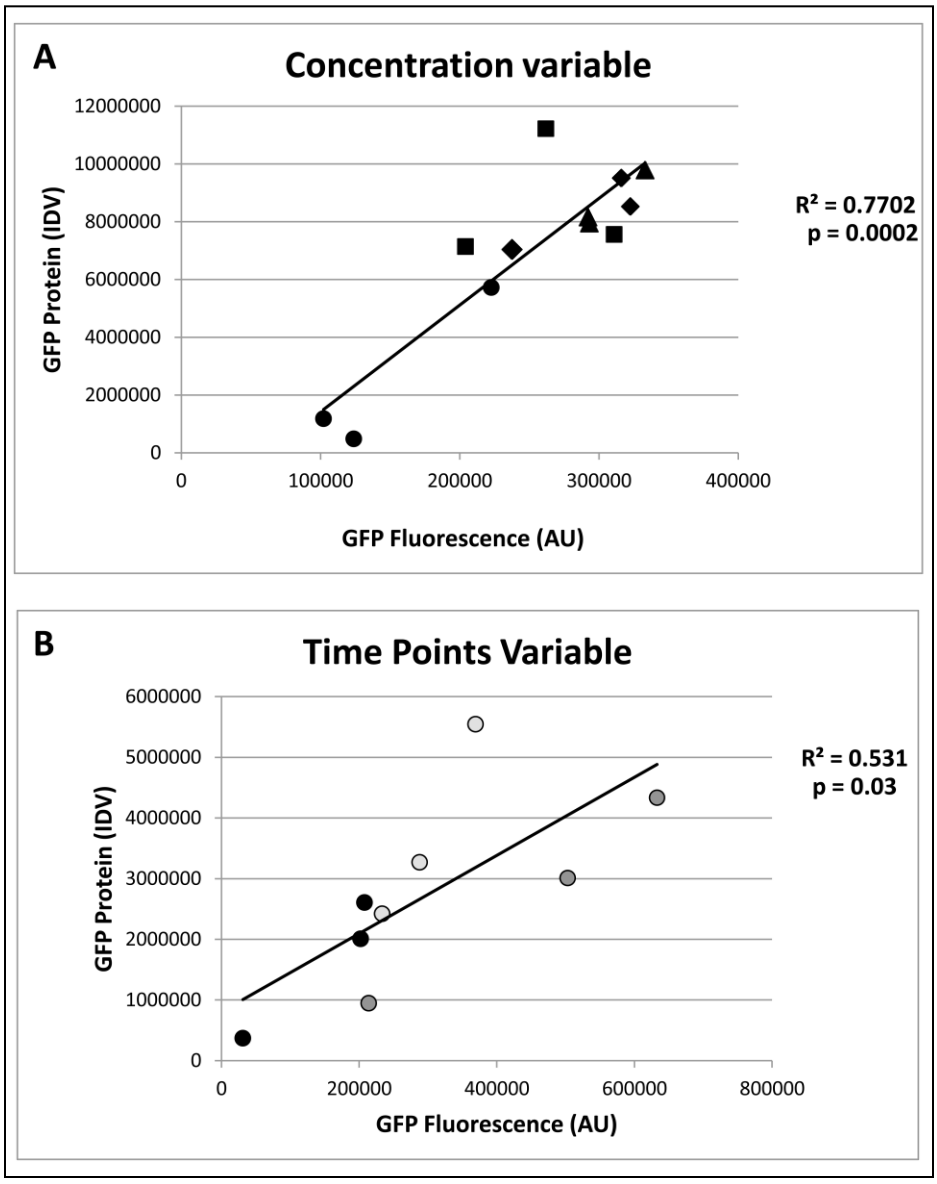

Figure 4.3: Correlation between eGFP protein detected by Western blot and eGFP fluorescence monitored by flowcytometry. The eGFP protein levels were determined by Western blotting and evaluated as the optical density of the band on the blot. A percentage of the total cells were used to detect the fluorescence using flow cytometry. **A.** Regression analysis to correlate eGFP protein levels and eGFP fluorescence in cells transfected with 4 decreasing concentrations of expression vector by 1.3 fold: ● = 5.48ug; ▲ = 7.31ug**;** ♦ = 9.75ug; ■ =13ug.**B.** Linear regression between the eGFP protein and fluorescence in cells transfected with equal concentrations of expression vector and evaluated at 3 different times post-transfection: 24h = medium gray, 36h = black, 48h =light gray.

3.4 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 4.1). 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 (Fig. 4.4A 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 (Fig.4.4C) and this co-expressing population was plotted on a FL2 histogram to collect the total background DsRed fluorescence that was subsequently subtracted from each experimental data set (Fig. 4.4D).

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.

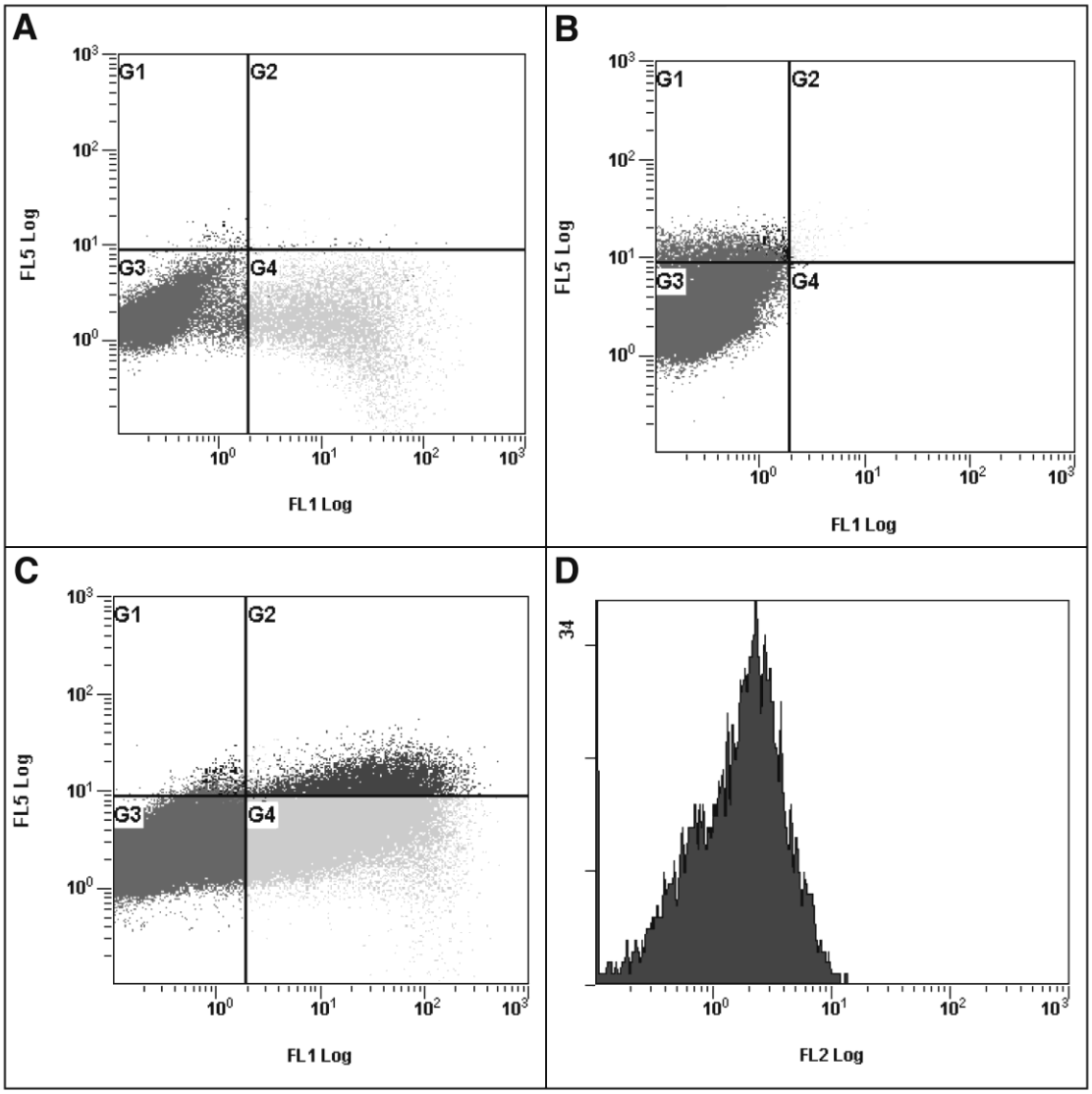

Figure 4.4: **Flow cytometer set-up to detect the three fluors in the new reporter assay.** A. Gate set-up for eGFP in FL1 for cells transfected with the pCI-Neo/IRES-eGFP control vector. B. Cells transfected with pCS2-HcRed1 control plasmid, recorded in FL5 and gated for HcRed1 expression. C. Gated population of 10,000 cells expressing both eGFP and HcRed1. D. Histogram of DsRed output in FL2 for the population of eGFP and HcRed co-expressing cells.

3.5 Calibration utilizing the previously characterized effect of Pitx3 upon the murine *Tyrosine hydroxylase* promoter.

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 share 100% identity.

The first experiment was conducted in HEK293 cells, 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 leaves expression unchanged (Fig. 4.5A). Conversely, by site-directed mutagenesis we mutated the known pitx3 binding site (TAATCC →TAccCC) 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 ((Fig. 4.5B). 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 (Fig. 4.5C).

To address the possibility of pitx3 acting as either an activator and repressor on the same promoter depending on the cellular context, we replicated the experiment in a second cell line, the human neuroblastoma SK-N-BE(2)c. Although the relationship between pitx3 and TH in these cells has not been reported to date, we hypothesized that pitx3 will serve as an activator. As expected, we determined a 30% increase in the TH output in the pitx3 transfected sample, while both the homeodomain and the binding site mutants reporter levels were indistinguishable from wild-type (Fig.4.5D)

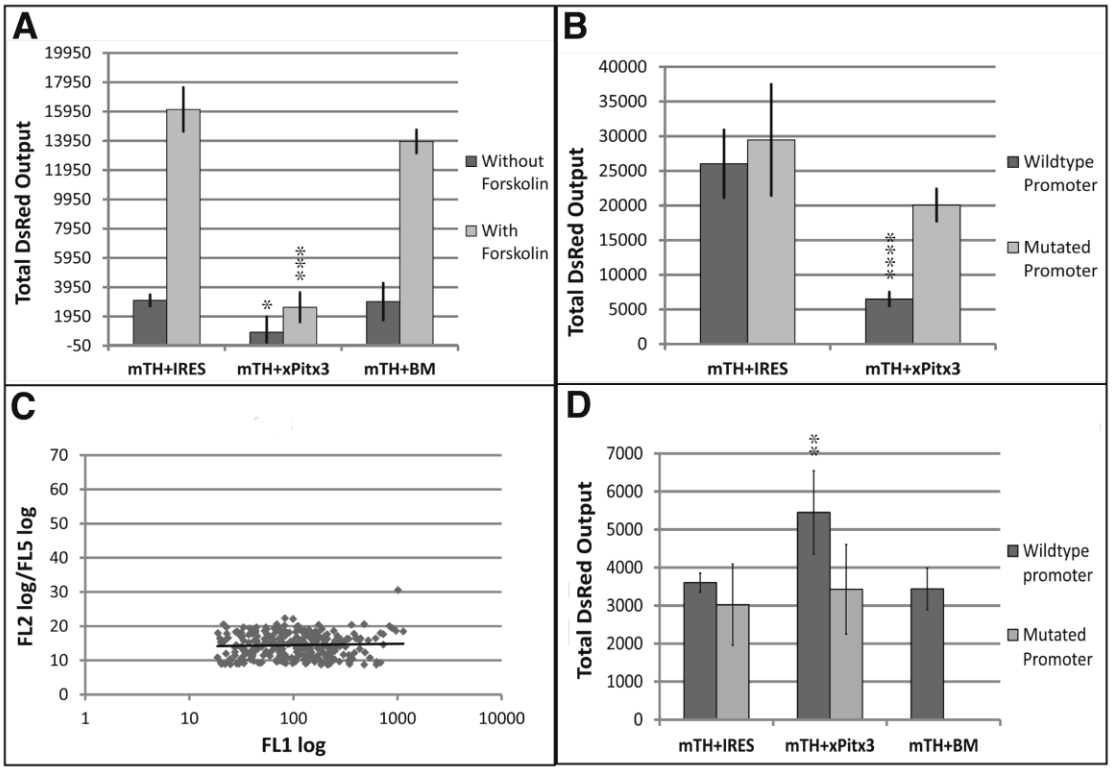

Figure 4.5: Calibration of the new technique using the known xPitx3 – *tyrosine hydroylase* (TH) interaction. **A.** pitx3 represses *TH* in HEK293 cells, under both basal and forskolin-treated conditions. The pitx3 binding mutant (BM) restores the constitutive expression of *TH*. **B.** The mutant for the known pitx3 binding site on the TH promoter prevents the repressive activity of pitx3 on *TH* in forskolin-treated HEK293 cells.**C.** Transcription factor cooperativity assessed in HEK293 cells expressing all three fluors, by plotting the normalized DsRed output to the GFP input and determining the generated trendline. D. pitx3 activates *TH* in SK-N-BE(2)c cells, while both the pitx3 binding mutant and the binding site mutant on the *TH* promoter restore the basal *TH* activity. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (****) $p<0.0001$

3.6 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 *βB1-crys* 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 (Fig.4.6C), while *crybb1* is inhibited in the HEK293 cell context (Fib.4.7B). 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 (Fig.4.6B). *gsc* showed no significant transcriptional regulation by pitx3 in this environment (Fig.4.6D). To determine the site responsible for pitx3 binding in each targeted promoter, we started by searching the ENSEMBL.org datebase 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 in all three organisms were mutated by site-directed mutagenesis and assessed for where pitx3 binding effects (Fig.4.6A and 4.7A). 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 (Fig.4.8).

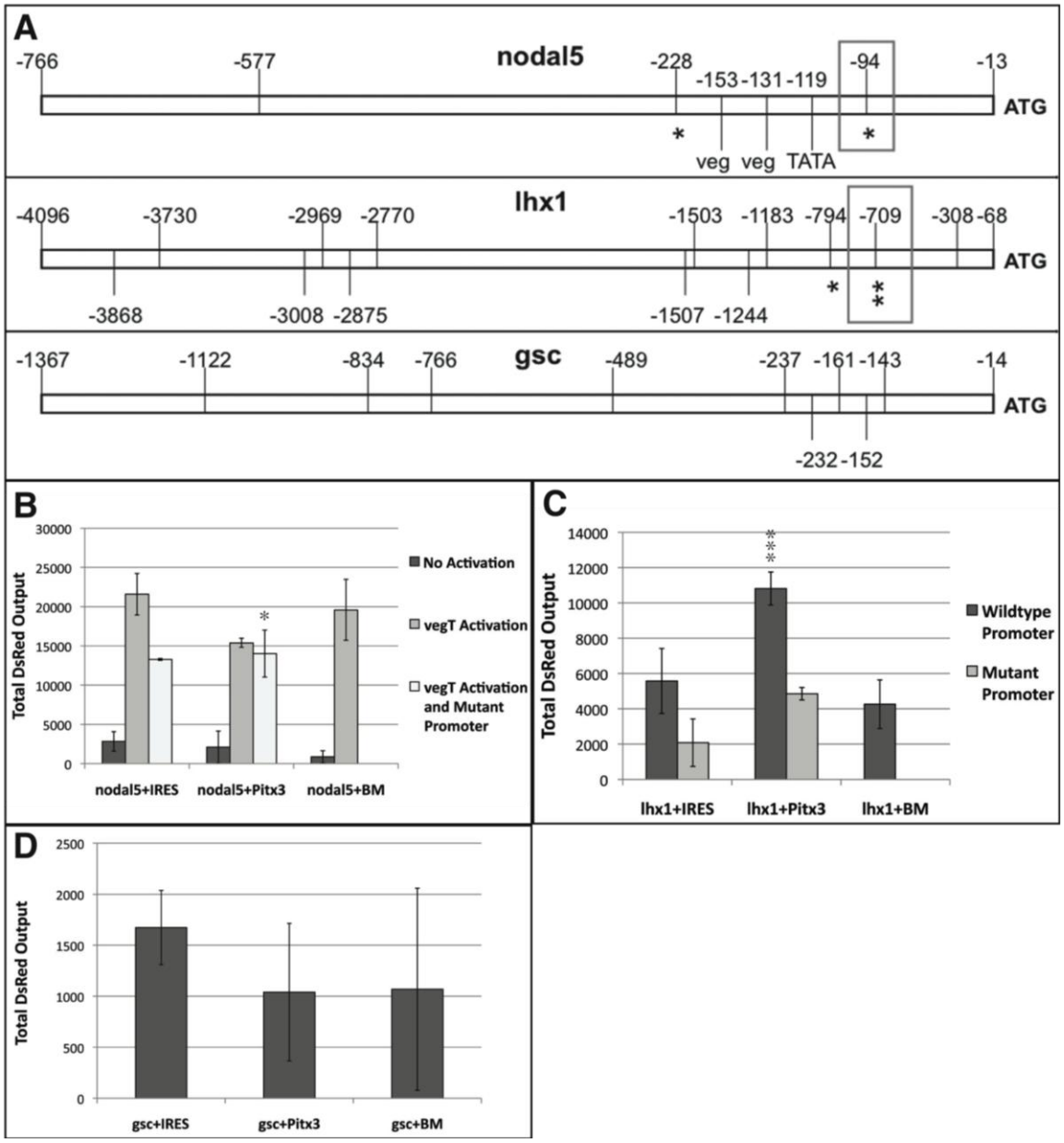

Figure 4.6: New pitx3 targets in early embryonic development. **A.** Diagramatic representation of the tested promoters with the location of the putative pitx3 binding sites. Promoter sequences for *X.laevis*, *X.tropicalis* and *D.rerio* were aligned using the MULAN software and conserved binding sites 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 putative pitx3 binding sites. The p-values for effects in graphs B and C are (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

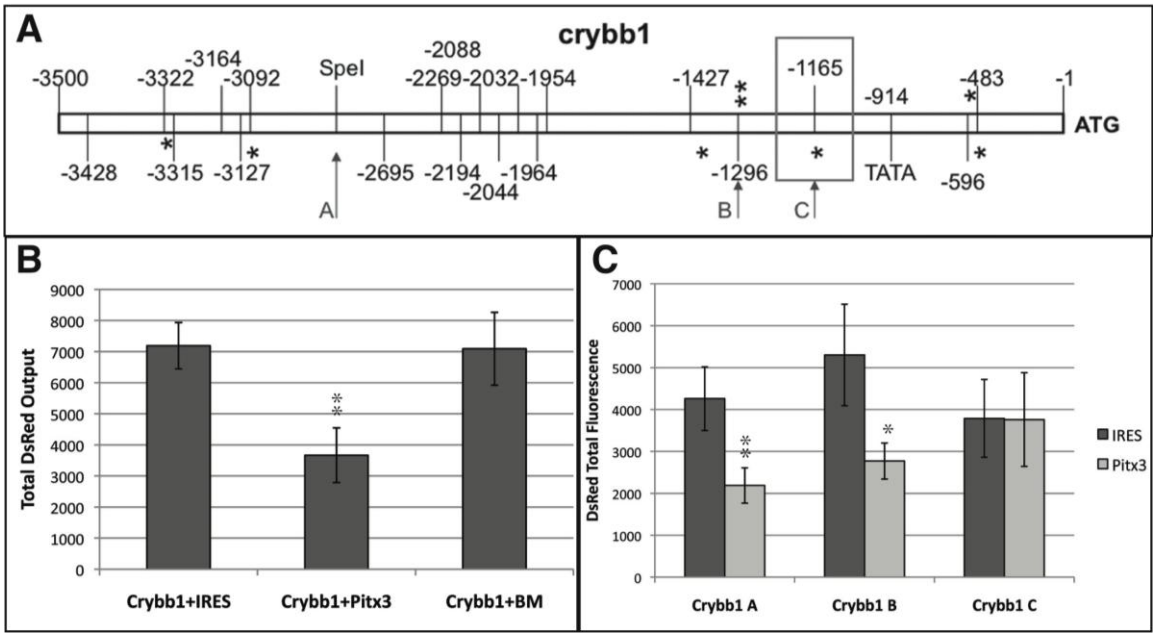

Figure 4.7: New pitx3 target during lens development. A. *crybb1* promoter represented as a diagram with the location of both conserved and non-conserved possible pitx3 binding sites. The position of the deletion (SpeI) and site-directed mutagenesis mutants is also shown (arrows). **B**. pitx3 inhibits 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. The p-values for effects in graphs B and C are (*) $p<0.05$, (**) $p<0.01$.

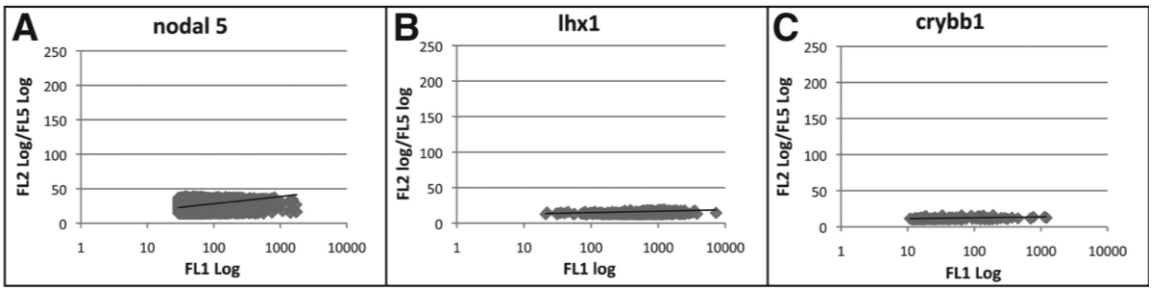

Figure 4.8: Cooperative mode of action for pitx3 on the new targets Target promoter fluorescence output (FL2) was normalized against promoter availability (FL5), and plotted against pitx3/GFP presence (FL1) for promoters The major conclusion is that Pitx3 does not act in a cooperative way on the tested promoters *nodal5* (A), *lhx1* (B) and *crybb1*(C). The absence of hyperbolic curves suggests a linear all-or-nothing response.

4. 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 titres 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 (Fig. 4.2, 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 is facilitated by delivering quantitative data regarding transcription factor concentrations (input) and candidate promoter reporter activity (output) (Fig.4.4).

To calibrate the specificity and sensitivity of the newly developed method, we tested the interaction between Pitx3 and the *tyrosine hydoxylase* 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 to test the pitx3-TH relationship in two different cell

lines. In the non-neuronal HEK293 cells line the interaction has been previously analyzed by luciferase assay where Pitx3 is known to inhibit the *TH* transcription (Cazorla et al., 2000). Although this interaction has not reported in the neuroblastoma line SK-N-BE(2)c, these cells have the same enzymatic makeup as the SY-SH5Y cells (Ciccarone et al., 1989), where Pitx3 slightly activates *TH* (Reddy et al., 2011).

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) (Fig.4.5A). The 30% activation of TH by pitx3 in the SK-N-Be(2)c cells (Fig. 4.5D) matches the results reported in SY-SH5Y (Reddy et al., 2011). 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 (Fig.4.5B). Finally, we further confirmed specificity by showing that the mutated homeodomain cannot induce transcriptional change. 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 motifs 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 time-frame 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 (Fig.4.6A, 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 (Cirio et al., 2011; Taira et al., 1992). 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 (Fig.6A, 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* (Mochizuki et al., 2000; Taira et al., 1992). Using our reporter assay to test 1.4kb of the *gsc* promoter, we observed no changes in HEK293 cells that can be ascribed to the presence of pitx3, despite 9 putative homeodomain binding sites (Fig.4.6A, 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) 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 *βB1-crys* 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 for the ATG, in close proximity to the *pax6* and *prox1* binding sites (Mizuno et al., 2005), abolishes repression by pitx3 (Fig.4.7).

The described reporter assay is unique in that it has the potential to reveal information regarding cooperativity of transcription factors upon tested promoters. 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 of murine Pitx2 (Saadi et al., 2003) and cooperative regulation of transcription in *ultrabithorax* 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 36 bp and to head-to-head sites separated by only 3 bp (Yuan et al., 1999). Since none of our tested promoters have neighboring binding sites that meet this criteria, the lack of observable cooperativity in our tested promoters is perhaps not surprising (Fig.4.8).

Making use of different fluorescent proteins, spread on 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.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis I explore new phenotypes observed in *Xenopus pitx3* morphants and identify new direct and indirect downstream targets for pitx3. My ultimate goal is to characterize the gene regulatory networks (GRNs) for developmental processes in which pitx3 plays a role, and to thereby separate the direct effects of the gene from the indirect consequences of its perturbation upon inductive and morphogenic pathways. .

A gene regulatory network represents the gene circuit that defines the timing and specific outcomes for a developmental process. These networks are generally deeply layered and hierarchical, employing sub-circuits responsible for individual tasks. Some of these sub-circuits are very malleable and can be used in various developmental contexts, while others are less flexible and are responsible for similar biological functions wherever they are deployed. The terminal periphery of a GRN tends to be shallow with only a small number of transactions that activate differentiation genes and thereby the cellular type specified early in the network hierarchy (Davidson, 2010; Levine and Davidson, 2005).

Changes in network structure have outcomes that are dependent upon the hierarchical operating level of the altered gene. In the big scheme of a developmental process, the hierarchical position of a gene is sometimes more important than its identity, since its connections are critical for its function. Mutations of key regulatory genes that intersect multiple sub-circuits and that are subject to feedback from other players can yield dramatic morphological changes with possible catastrophic outcome (Hanks et al.,

1995). However, advantageous mutations in these regulatory genes can also have a major evolutionary impact and drive morphological innovations responsible for differences between species (Carroll, 1995). Meanwhile, changes in effector genes are generally non-detrimental, affecting only the module in which they operate and resulting in a change in the type of cells they specify (Davidson, 2010; Davidson and Erwin, 2006; Davidson and Erwin, 2010; Nowick and Stubbs, 2010).

Elucidating a GRN requires identification of the involved players and their relationships in a developmental process by methods of prediction and authentication. Usually, perturbation of a regulatory gene generates a cascade response that can be assessed both phenotypically and by the anomalous effect it propagates upon downstream linkages. In general, a high-throughput assay such as a microarray generates a list of genes perturbed by a specific regulator. The steps necessary to elucidate a GRN involve assessment of: overlapping spatial and temporal expression among the involved factors; a change in a downstream partner's expression commensurate with proximity to the mutated transcription factor; and in the case of direct linkages, physical binding of the TF to the *cis*-regulatory sequence of the target gene. A more global view of a network is generated by mining the literature for data that hopefully predicts nodes that can be authenticated experimentally (Levine and Davidson, 2005; Li and Davidson, 2009).

While trying to situate pitx3 in previously established networks responsible for the embryonic development of amphibians, a few major questions were raised by my research. My goal was to determine approximate positions for pitx3 in GRNs that are responsible for patterning different organs. These positions are not always predictable

due to non-linear dynamics or redundant functionality in the nodes (Hanks et al., 1995; Wurst et al., 1994).

Following the general rules for building a GRN, I started by correlating the morphology of the *pitx3* morphants' phenotypes with the genes responsive to pitx3 perturbation in the microarray experiment. Those genes that were confirmed by means of *in situ* hybridization and RT-PCR, and were most likely to serve as direct targets for pitx3 contributed valuable information to partnering nodes for pitx3. The rest permitted me to refine a more concrete role in likely downstream pathways for *pitx3*. There are a few developmental processes where *pitx3* is indisputably a key player with an already established role - and we are adding to that knowledge, while also contributing pathways that have not been previously described. Since *pitx3* functions in other developmental processes, for example in the midbrain where the pathways are well understood both in frog and other organisms, the focus of this study centers upon the new additions to knowledge.

1. GRNs defining developmental processes where pitx3 plays a role

1.1 Gastrulation

While *pitx3* is first detectable in mammals around the somitogenesis stage, in frog and zebrafish it is first expressed much earlier at mid-gastrula stages. This raises the question of what role it could be playing at this stage (Dutta et al., 2005; Khosrowshahian et al., 2005). The anomalous expression of a subset of gastrulation markers, namely *nodal5*, *gsc*, *lhx1*, suggests that pitx3 functions in this process, however it is interesting to speculate the reason behind its apparent loss of function in mammalian gastrulation.

Possibly pitx3 activity has not been detected in mammalian gastrula simply because it is expressed at levels undetectable by *in situ* hybridization and it has not been tested by RT-PCR yet. Alternatively, amphibians and fish might deploy slightly different pre-gastrulation cues. During gastrulation, complex cell movements are strictly coordinated by strict genetic signals that establish the three germinal layers of the embryo. While the GRN responsible for this process is generally well conserved, some differences in early patterning have been reported across species (Davidson and Erwin, 2006). A significant point of departure could be the positioning of pitx3 directly upstream of both *nodal5* and *lhx1* in frogs, thus explaining the change in their expression and consequently also of *gsc* in pitx3 morphants (Luxardi et al., 2010; Mochizuki et al., 2000) (Fig.5.1).

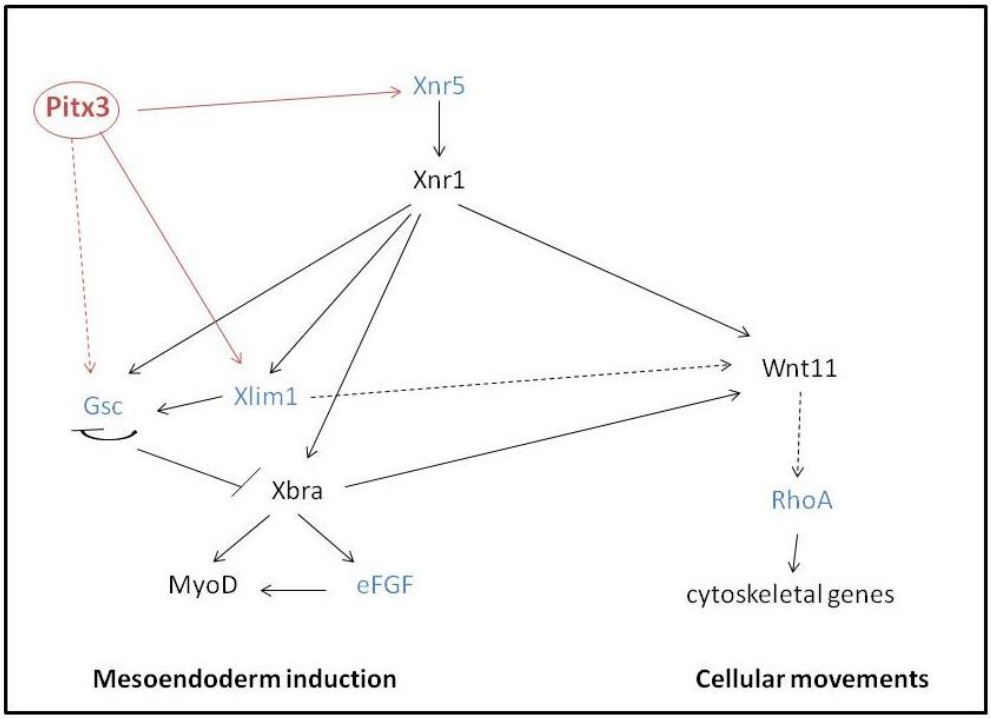

Figure 5.1. Graphic representation of a general molecular mechanism responsible for gastrulation. Genes represented in blue are found in the microarray data, while the red arrows signify direct or indirect interactions tested in this research.

It will be interesting to investigate the molecular signals responsible for the morphogenetic rearrangements that characterize convergent extension during this process and to determine if the absence of pitx3 in *Xenopus* perturbs cellular organization (Wallingford et al., 2002; Wyczalkowski et al., 2012). *RhoA* is a gene that operates downstream of *Wnt11* in the planar cell polarity pathways (PCP) required for correct convergent extension (Wallingford et al., 2002) (Fig.1), and it shows a significant fold-change in the microarray data. More investigations are necessary to confirm the change in spatial and temporal expression and to determine if it is due to direct interaction or if it represents an indirect result of pitx3 is regulating *nodal5 (Xnr5)*. Morphant dorsal lip explants plated onto blastocoel roof matrix could be used to differentiate signalling from cell polarity and migration effects (Shi et al., 1987).

Since formation of the axes and the germinal layers are inextricably linked, the potential for a modulator of early gastrulation to exert multiple downstream effects would be large. If the modulating gene redeploys again at later stages, then separating direct from indirect and early from late effects presents significant challenges.

1.2 Eye development

pitx3 expression and function in eye development is well documented and is highly conserved in all organisms, with the few exceptions of the retinal defects presented in frog and zebrafish *pitx3* morphants (Khosrowshahian et al., 2005; Shi et al., 2005). This unique phenotype requires a closer examination to determine if pitx3 protein is necessary for retina induction.

From my present studies, not a lot of information can be added to this network; however a GRN is never definitive unless all the direct interactions between nodes are known. Here we confirmed the suspicion that *βB1-crystallin* is directly regulated by pitx3, subsequently proving that this transcription factor is responsible for the terminal differentiation of lens fibers and the maintenance of their transparency. This could have interesting implications for the understanding and treatment of cataracts. It will be worth examining the signalling hierarchies and cooperation necessary to regulate the *crybb1* promoter: both *l-maf* and *prox1* serve as activators, whereas *pax6* acts as repressor (Chen et al., 2001; Cui et al., 2004; Duncan et al., 1998) (Fig.5.2). The relationship between *pitx3* and *pax6* remains controversial and needs a close dissection of the developmental stages where they are influencing each other. For example, does this influence occur by direct cooperation on the target promoter or by indirect cues deriving from other genes that they each regulate? Coimmunoprecipitation in combination with mass-spectroscopy characterization could prove fruitful in assessing the degree of interaction occuring as these factors collaborate or compete to activate specific targets.

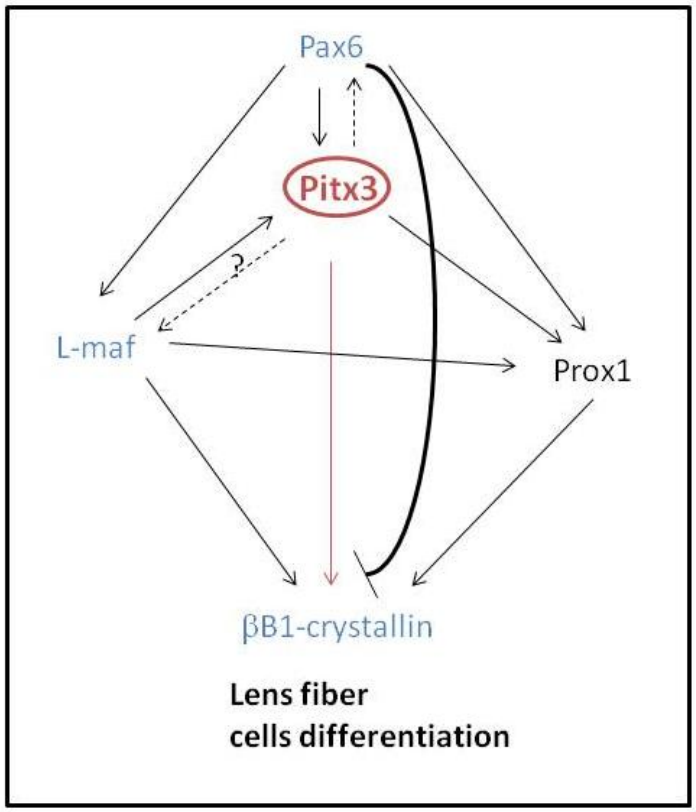

Figure 5.2. Graphic representation of the lens fiber differentiation pathway. Genes represented in blue are found in the microarray data, while the red arrow signifies a direct interaction demonstrated in this research.

Similar to the zebrafish phenotype (Shi et al., 2005), *Xenopus pitx3* morphants exhibit abnormal cellular adhesion in the lens vesicle with an absence of the concentric organization of fiber cells and remnants of undifferentiated cells. We suspect that *chd2* could be one of genes responsible for this disarray, since the murine *cdh2* mutant lens closely resembles the aphakia phenotype (Pontoriero, 2008; Pontoriero et al., 2008). The relationship between pitx3 and pax6 can also shed light upon the involvement of *pitx3* in these events since pax6 directly regulates *β1-integrin* in the lens to ensure the maintenance of the normal fiber cells organization (Duncan et al., 2000) (Fig.5.4). *cdh2(N-cadherin)*and a few members of the *integrin* family *(α3-integrin, α5-integrin,*

β2-integrin, β6-integrin) are part of the microarray data and they all possess putative binding motifs for pitx3 in their promoters. The next step would be to assess whether their temporal and spatial expression changes if pitx3 is absent and to employ a reporter assay and promoter analysis for each. This would illuminate the possible role that *pitx3* plays in the cytoskeletal regulation pathway.

1.3 Segmentation and myogenesis

A specific role for *pitx3* in muscle development has already been demonstrated in mouse, where it is responsible for the differentiation of myoblasts into myotubes (Coulon et al., 2007; L'Honore et al., 2007), however its function in *Xenopus* myogenesis has not yet been described. Given the unusual segmentation mechanism that produces somites in amphibians (Hamilton, 1969), we suspected that *pitx3* might play a distinct role in frog metamerism and muscle development. While mouse *Pitx3* mutants do not exhibit any muscle anomalies, presumably due to a compensatory mechanism involving Pitx2 (L'Honore et al., 2007), *Xenopus* and zebrafish *pitx3* morphants display a bent dorsal axis and difficulties swimming due to defective somitogenesis (Shi et al., 2005) (Chapter 2).

In the quest to determine the cause for axis curvature in *pitx3*-MO injected embryos, we used markers for different components of the somitogenesis process and determined that *pitx3* plays a role in the pathway that controls cytoskeletal rearrangements during cell rotation and in the terminal differentiation of the myotome. *Hoechst* staining revealed aberrant nuclear rotation, and staining against *β1-integrin* distribution revealed a lack of cellular cohesion in somites on the injected side. This led to defective intersomitic boundary formation. The fast muscle specific antibody 12/101

exposed a reduced size as well as disorganization of myotome on the injected side: the somitic metamerism was hardly detectable (Appendix B, Fig.1C and D).

As expected, key components of the segmentation clock, *delta2* and *notch* are unaltered, while some of their downstream targets, *hes4* (*hairy2b)*, *hes7.2* and Ripply2 (*ledgerline*/*stripy)* shift expression rostrally, presumably due to a shift in the segmentation front (Appendix B, Fig.1C, C' and D) (Fig.5.3). The front is established at the intersection between the anterior expression of retinoic acid (RA) and the posterior expression of *fgf* (Fig.5.3). The segmentation clock can act at this front to position the segmental boundary and thereby to initiate budding of a new somite from the presomitic mesoderm (Pourquie, 2001). Since Pitx3 operates upstream of RA in the murine midbrain (Jacobs et al., 2007), the anteriorized behaviour of the wave front can be explained by decreased levels of RA in the *pitx3* morphants (Duester, 2007; Moreno and Kintner, 2004). It will be interesting to test whether the treatment with exogenous RA resolves the shift in the expression of the segmentation genes or whether pitx3 affects their expression in a RA-independent manner (Jacobs et al., 2011). Similarly, a plethora of RA and retinoid receptor agonists and antagonists could be employed for an exquisite dissection of this pathway (Collop et al., 2006). These experiments will also answer the question of whether the effect of pitx3 on the *Hox* genes, responsible for positional identity, is direct or mediated by RA (Kessel and Gruss, 1991) (Fig.3).

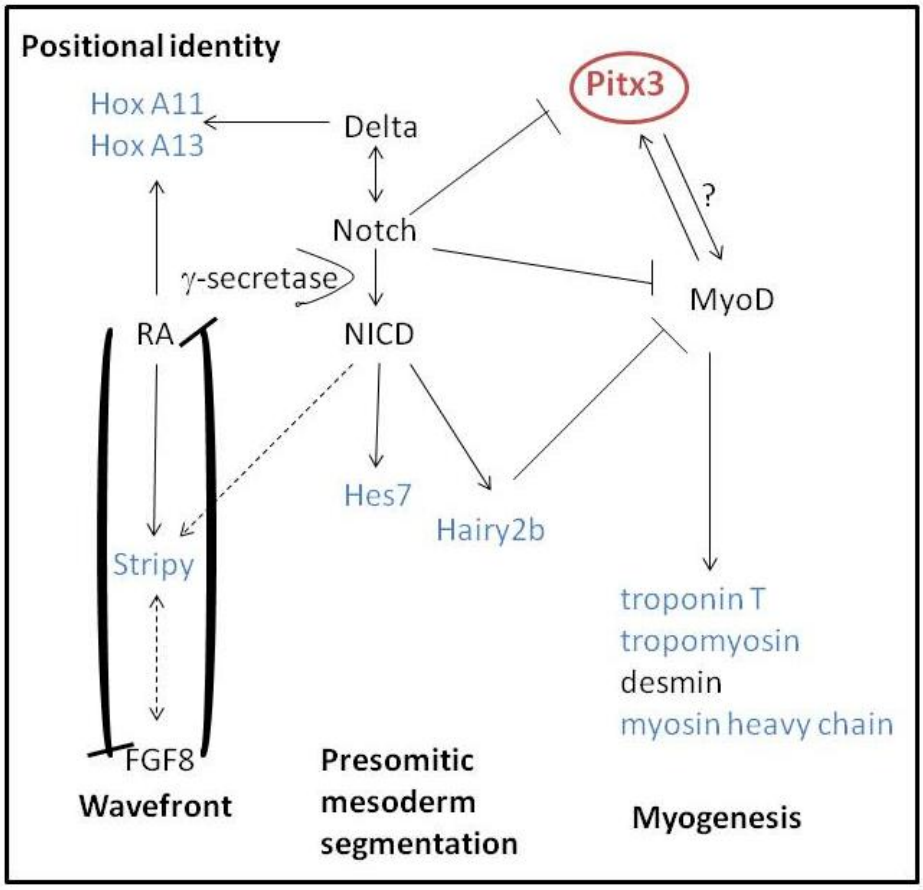

Figure 5.3. Graphic representation of the interacting pathways responsible for somitogenesis and myotomal differentiation. Genes represented in blue are found in the microarray data.

Following indirect disruption of *notch* signalling by treatment of embryos with the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) we observed increased *pitx3* expression in the somites and in the immature mesoderm of the tailbud region where *pitx3* expresses at very low levels, observed only in cleared specimens (Appendix B, Fig.1D, D', E and E'). Notch inhibits *Pitx2* expression in the murine lateral plate mesoderm (Sakano et al., 2010) and given its known interaction with a Pitx3 paralog, I can speculate that *notch* also restricts the expression of *pitx3* to formed somites. *Notch* is a known repressor of myogenic

differentiation (Shawber et al., 1996) and therefore inhibition of *pitx3* expression in the presomitic mesoderm likely alters the timing for the onset of the myogenic program. *Pitx3* is directly regulated by MyoD in mouse (Coulon et al., 2007) and also it reciprocally regulates *myod* expression in frog (Appendix B, Fig.2A, A' and C) suggesting a tight level of control during muscle specification and differentiation. This reciprocal interaction may also indicate a possible collaboration on the downstream promoters where myoD needs a resident factor to gain access to the target sequences (Berkes and Tapscott, 2005). The myogenic markers assessed and downregulated in *pitx3* morphants are more likely a result of this latter collaboration (Fig.5.3). Both *desmin* and *troponin C* are regulated by myoD during zebrafish myogenesis (Maves et al., 2007), while *troponin C* and *creatine kinase* are also a targets of mimecan (Tasheva et al., 2004a), a small leucine-rich proteogylcan (SLRP) that is directly regulated by pitx3 (Tasheva et al., 2004b).

The cell rotations that precede the separation of the newly formed somite from the presomitic mesoderm involve changes in cell shape, motility and adhesion (Wilson et al., 1989). The lack of cohesion during rotation could be a result of inhibited *cdh2*, a cell-cell adhesion molecule that appears to be downregulated by *pitx3* perturbation in the microarray and RT-PCR data. *Cdh2(N-cadherin)* is required for cellular adhesion during rotation and boundary formation and a phenotype that is produced by a dominant-negative form of it is very similar to the *pitx3* phenotype (Giacomello et al., 2002). Members of the *integrin* family are associated with a tightly orchestrated tissue morphogenesis and disruption of this signal results in chaotic somite formation through

aberrant cell-cell adhesion and *notch* induced segmentation cues (Marsden and DeSimone, 2003; Rallis et al., 2010) (Fig.5.4).

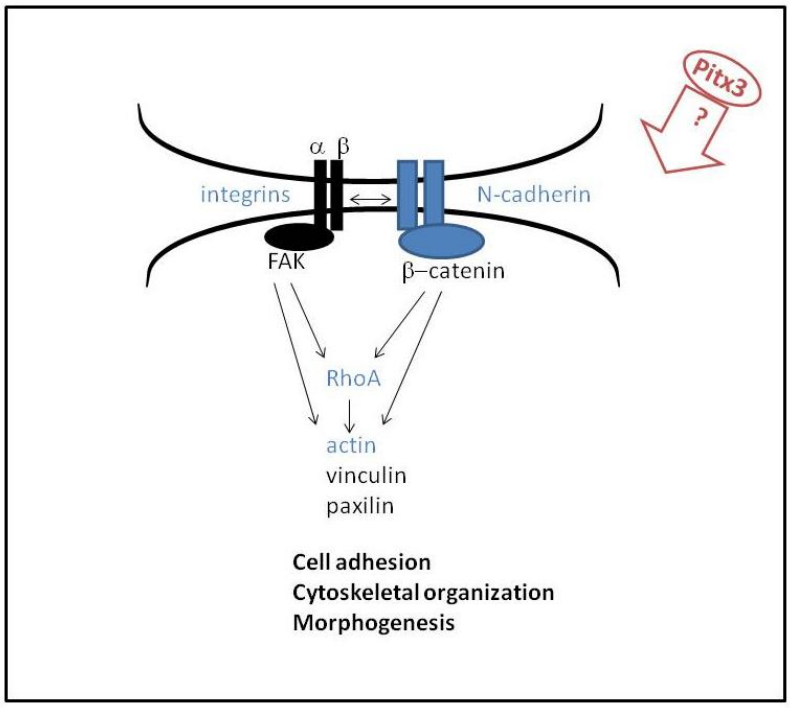

Figure 5.4. Graphic representation of the GRN sub-circuit responsible for cellular adhesion and cytoskeletal integrity. Genes represented in blue are found in the microarray data.

The list of microarray genes responsible for myogenic differentiation (*titin*, *calmodulin*, *myosin heavy chains*) and cytoskeletal reconfiguration (*integrins*, *cdh2*) is large and more genes need to be confirmed for a more concrete positioning of *pitx3* in the somitogenesis network. Genes from this large list could be tested for a change in expression due to *pitx3* knock-down. For those genes that show a drastic response, direct regulation by pitx3 protein can be assessed via reporter assays.

1.4 Heart and gut rotation

A surprising phenotype that arose in response to *pitx3* knock-down is the aberrant looping of heart and gut. First assumptions of this being an artifact of additional repression of *pitx2*, the paralog responsible for the left-right asymmetry and proper rotation of these organs (Schweickert et al., 2000) were easily dismissed since two different sets of morpholinos targeting a non-conserved region in the 5'-UTR of *pitx3* resulted in identical outcomes. However, the next question that needs to be asked is whether this anomaly is the result of early defective specification of the laterality pathway or whether the differentiated cell movements necessary to sustain the looping are prevented from acting under normal parameters.

The left-right patterning of the embryo is established early during gastrulation within the embryonic organizer (node) and the cues for laterality are transmitted to the left lateral plate mesoderm where *nodal*, *lefty* (*antivin)* and *pitx2* are asymmetrically expressed (Campione et al., 1999; Ryan et al., 1998) (Fig.5.5). For proper lateralization, expression of the midline marker *Shh* is necessary in mammals (Bisgrove et al., 2003). Abnormal *shh* expression infrog and zebrafish *pitx3* morphants can explain the complete *situs inversus* phenotype which is characteristic of perturbation of the earliest steps of the laterality signalling pathway reported in other organisms (Bisgrove et al., 2003). Given the large number of putative bicoid binding sites in the *shh* promoter it is interesting to test the possibility of direct interaction between *pitx3* and *shh*. This might account for the abnormal expression of *shh* downstream genes, such as *nodal*-related genes and *Pitx2,* in *pitx3* morphants.

Both heart and gut looping involve cell shape changes to cumulatively direct the bending and rotation movements that characterize these processes (Wyczalkowski et al., 2012). As seen in eye and somites, *pitx3* is part of genetic system that influences cytoskeletal modifications and any *pitx* paralog can target the expression in the gut of *shroom3*, a gene that regulates actin-based cytoskeletal organization and works cooperatively with *chd2(N-cadherin)* to regulate cellular rearrangement during gut morphogenesis (Chung et al., 2010; Plageman et al., 2011).

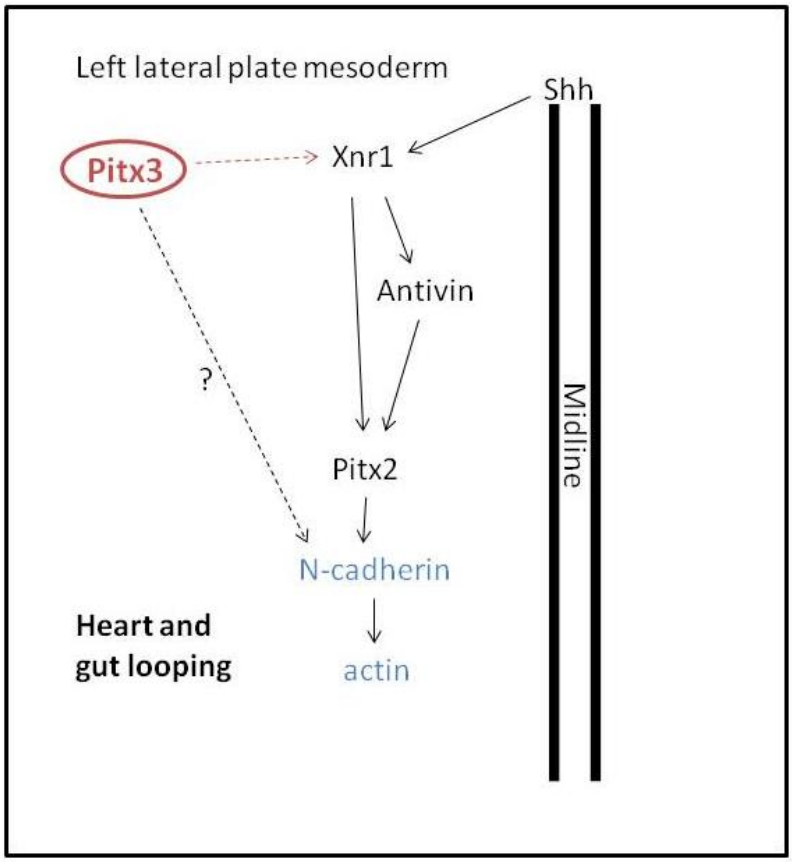

Figure 5.5. Graphic representation of a generalized laterality pathway responsible for the correct heart and gut looping. Genes represented in blue are found in the microarray data.

Is aberrant looping just a result of randomized laterality cues or are the mechanical changes to cells also responsible for the rotation problems? It is necessary to compare the phenotypes generated by mutating the genes in the laterality pathway with the one in *pitx3* knockdown embryos and to employ complementation or rescue studies to approximate the level at which *pitx3* operates. Also, changes in expression of these genes following knock-down of *pitx3* can be investigated and employing heart and gut specific antibodies would allow for the exact characterization of the aberrations present in cell morphology.

2. Evolutionary view on pitx3 function

From an evolutionary stand-point, the discovery in fly and tunicates of a *pitx* gene is evidence for the existence of this family before the divergence between the invertebrates and vertebrates (Christiaen et al., 2002; Vorbruggen et al., 1997). However, the lack of an apparent morphological phenotype resulting from gain- or loss of function of the fly *pitx* gene suggests that the common ancestor was not a player in patterning process of the embryonic body (Vorbruggen et al., 1997). An evolutionary assessment of the *Pitx3* gene reveals that the human and mouse orthologs are strikingly divergent from those in non-mammalian vertebrates with respect to expression in the pituitary (Angotzi et al., 2008) (Fig.5.6).

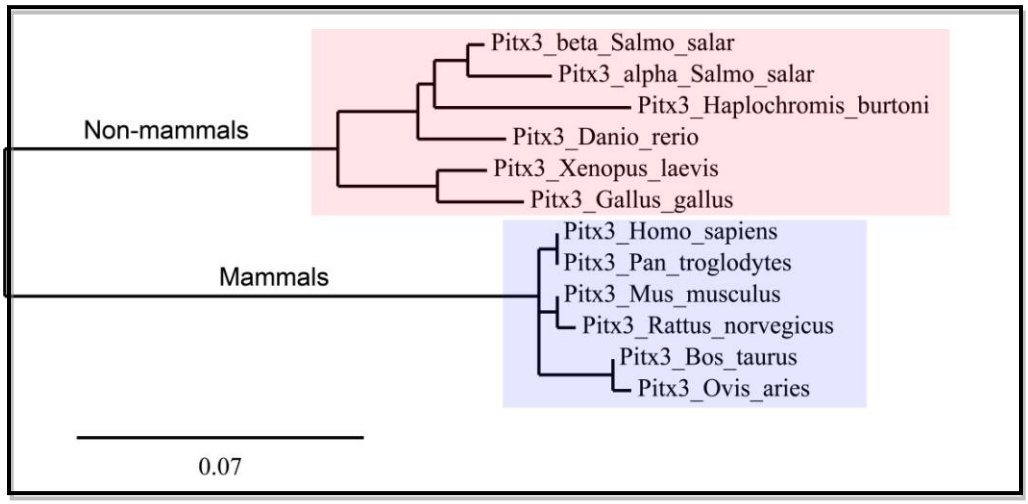

Figure 5.6. Phylogenetic tree for Pitx3 protein underlines the clear divergences between mammalian and non-mammalian organisms. *Danio rerio* Pitx3 AAT68296; *Bos Taurus* Pitx3 DAA14908; Xenopus *laevis* Pitx3 AAI70394; *Mus musculus* Pitx3 AAB87830; *Homo sapiens* Pitx3 NP_005020; *Rattus norvegicus* Pitx3 NP_062120; *Ovis aries* Pitx3 CBA 10131; *Gallus gallus* Pitx3 XP_421631; *Pan troglodytes* Pitx3 XP_521591; *Haplochromis burtoni* Pitx3 ACZ51351; Salmo salar Pitx3 alpha ABW37418; Salmo salar Pitx3 beta ABW37419.

pitx3 in lower vertebrates is found in a genomic region that exhibits greater plasticity comparative to mammals. The region of synteny extends over only 200kb in amphibians and fish, with a lot of genomic reshuffling occurring in the vicinity of the *pitx3* gene (Jaszczyszyn et al., 2007). This signifies weaker constrains upon the regulatory regions surrounding the gene, possibly allowing for less restrictive expression and comparatively relaxed conservation between species (Jaszczyszyn et al., 2007). Moreover, the protein structure of Pitx3 is very similar in all studied organisms, with almost identical homeodomain and OAR motifs, and with very similar expression patterns. However, the C-terminal region between the HD and the OAR in *Xenopus* and zebrafish pitx3 is more similar to pitx1 and pitx2 than to the human and mouse Pitx3 sequences, indicating that a relatively recent change in this region may account for the loss of Pitx3 expression and function in mammalian pituitaries (Smits et al., 2006). I speculate that while *pitx3* assumes more general roles, that somewhat overlap with those

of *pitx2* in non-mammals, following divergence in mammals, the role of the *Pitx* genes became more specialized. *Pitx3* appears to be confined to the late stages of tissue differentiation, while *Pitx2* holds greater sway during early patterning and organogenesis.

3. General conclusions

1. While expression of *pitx3* is first observed during gastrulation in amphibians and fish (Dutta et al., 2005; Khosrowshahian et al., 2005), in mouse and humans the expression is first detected during organogenesis (Semina et al., 1997). *Pitx3* expression is restricted to brain, lens and somites in mammals (Semina et al., 1998; Semina et al., 2000; Smidt et al., 2004), while in the lower vertebrates it can be detected in additional regions such as the pituitary, head mesenchyme, jaw, lateral plate mesoderm and heart (Dutta et al., 2005; Khosrowshahian et al., 2005; Pommereit et al., 2001). Also, interestingly, in amphibians and fish *pitx3* also expresses earlier during lens and somite development, suggesting the possibility of a more foundational role in these developmental processes.

2. *pitx3* appears to be key regulator in various GRNs in lower vertebrates, while in higher vertebrates it seems to have redirected the strategic role of central coordinator towards a more peripheric position, where its responsibility lies primarily in the terminal differentiation program. Since cellular differentiation is usually characterized by an arrested cell cycle, an intriguing question is whether *pitx3* is the factor responsible for halting proliferation or if its expression only becomes present in the cells that are ready for terminal differentiation. *pitx3* also appears to be a player in the sub-circuit that employs *cdh2(N-cadherin)* and *integrins* and that is utilized by various developmental

processes to establish cellular adhesion and cytoskeletal integrity (Fig.5.4). It remains to be determined if *pitx3* operates in a conserved pathway that dictates cell morphology or if different interactants are required for each specific process. The common theme of *cdh2* sensitivity in the development of all aforementioned processes combined with the similarities between the phenotypical outcomes for both *pitx3* and *cdh2* knockdown, makes it worth-while to test the possibility of direct relationship between these genes.

3. *Pitx3* mutants in mice and humans exhibit dosage dependant phenotypes that are never lethal, indicating a more peripheral role for *Pitx3* in mammals. While heterozygotes show no morphological defects, homozygotes will show a defective differentiation of the lens fibers and mesDA (Semina et al., 1998; Semina et al., 2000). Xenopus and zebrafish *pitx3* morphants also display dose-dependent penetration of the morphological defects; however the phenotypes are generally more dramatic, with high mortality rates during gastrulation. In lens and somites, the absence of pitx3 has also more drastic results – the lens phenotype is accompanied by retinal defects (Khosrowshahian et al., 2005; Shi et al., 2005), and the somitic phenotype is represented by a bent dorsal axis that induces difficulties in swimming. Interestingly, in these organs, *pitx3* seems to have a very complex relationship with the "master regulators" of the respective processes. *Pitx3* is directly regulated by both Pax6 (Munster, 2005) and MyoD (Coulon et al., 2007), while it concomitantly induces changes in their expression either by feed-back regulation or cooperation on common target promoters.

Taking into account this new data, it will be of high interest to determine the cause for the evolutionary loss-of-function and repositioning of pitx3 inside some well-conserved developmental networks, and the mechanism by which this occurred in the

course of evolution. Scarce information regarding post-translational regulation by miRNA and epigenetic control of *pitx3* indicates that a more attentive approach is necessary to investigate these regulatory mechanisms in pitx3-associated developmental processes.

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APPENDIX A

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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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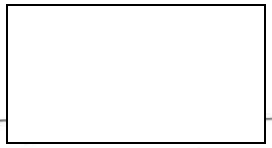
Signature: 

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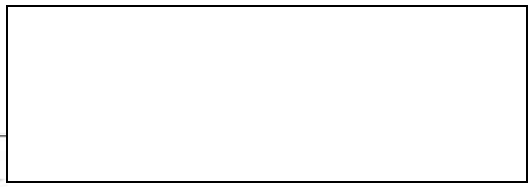
Signature: 

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Signature: _____

Date: April 18/12

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TD Waterhouse Tower
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M5K 1A1

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

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

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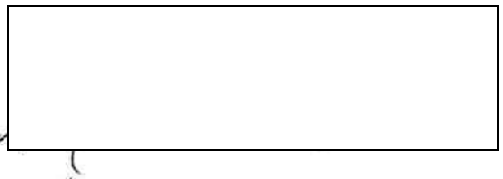
Signature: 

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Windsor, ON
N8R 1A5

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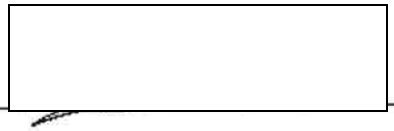
Signature: 

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Department of Biological Sciences
University of Windsor
401 Sunset Avenue
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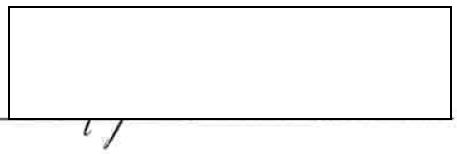
Signature: 

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

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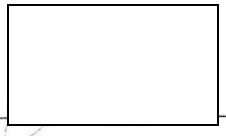
Signature: 

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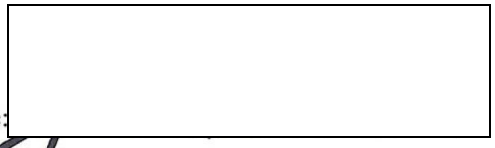
Signature: 

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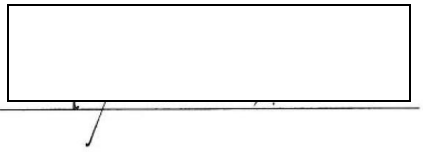
Signature: 

Date: 9 July 2012

APPENDIX B

ADDITIONAL DATA FOR CHAPTER II

The bent dorsal axis phenotype in *pitx3* morphants raised the question of a novel role for this gene in *Xenopus* somitogenesis. Although the molecular pathways for segmentation are relatively conserved, in frog somitogenesis and myogenesis are unique in that: somitic cells undergo rotation before budding off; somites do not epithelialise; and they are formed primarily of myotome (Hamilton, 1969). While a role for *pitx3* has been confirmed in Chapter 2, additional data was collected in order to gain more fulsome understanding. The results were not unambiguous, however they contributed to the general view of the role that *Pitx3* plays in this process and certain possibilities were ruled out.

Given the strong *pitx3* expression in formed somites (Fig.1A), combined with the bent axis phenotype, the level of action for *pitx3* in somitogenesis had to be analyzed. In Chapter 2 we demonstrated using nuclear counts that defective somitogenesis in *pitx3* morphants is not the result of excessive cell death or proliferation in somitic cells on the injected side. I confirmed this conclusion by employing TUNEL assay (terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling) to visually assess patterns of cell death. The pattern of TUNEL staining is conformant with the wild-type (Hensey and Gautier, 1998), with a low cell death evident only in the developing tailbud; the somites are largely unaffected (Fig.1B). To examine the effect of *pitx3* morpholino on the patterning and morphology of somites, I used the skeletal muscle specific antibody 12/101 (Kintner and Brockes, 1984). Both in whole-mounts and in

sections we observed weaker staining on the injected side, with disorganized and significantly reduced somites (Fig. 1C and D). This suggests that *pitx3* plays some role in normal muscular differentiation and its absence results in disrupted myogenesis.

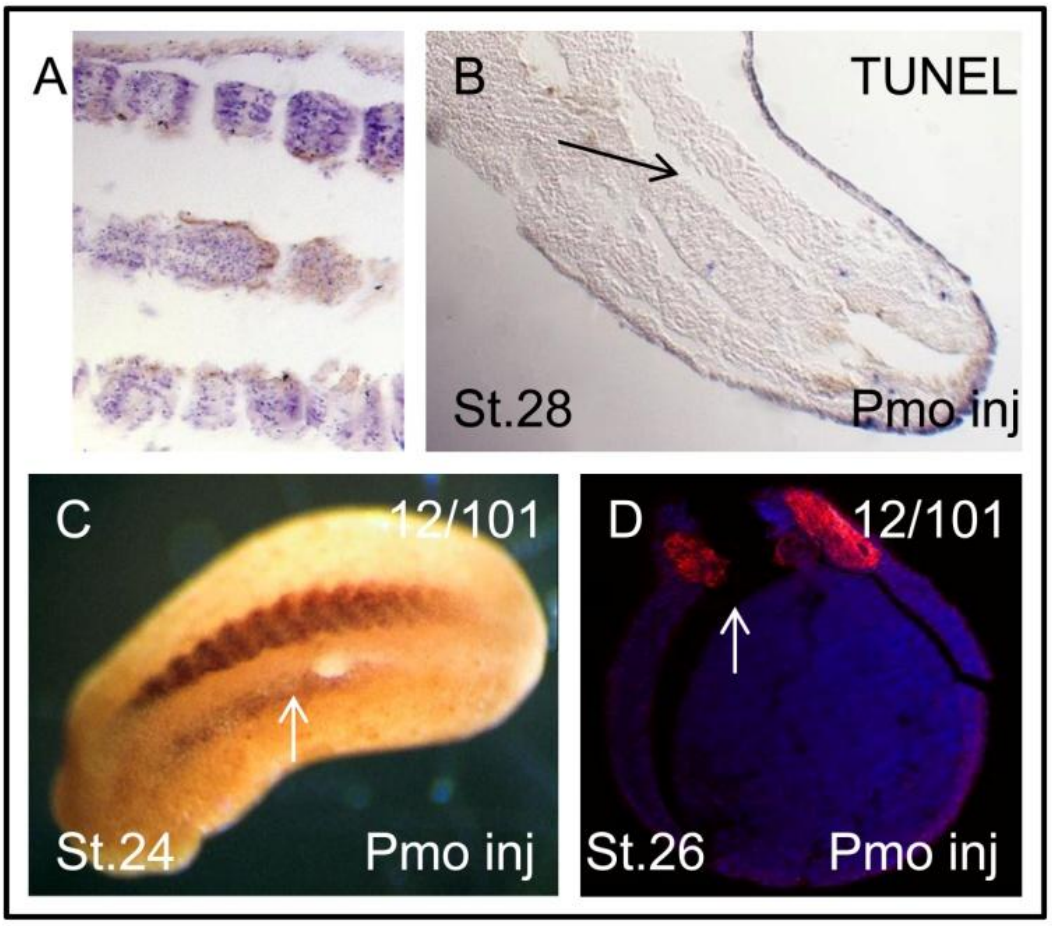


Figure1. A. Section of somites in embryo stained for *pitx3* by *in situ* hybridization. **B.** Cell death assessed in the somites of stage 28 *pitx3* morphant embryo, with the injection side indicated by the arrow. **C.** Dorsal view of stage 24 embryo with *pitx3* morpholino and stained with the 12/101-DAB antibodies. **D.** Transversal section of morphant embryo stained with 12/101-Cy3 antibodies.

To investigate if Pitx3 disturbs either the segmentation or the myogenic signalling pathways, we analyzed the expression pattern of two major players in these pathways, *notch* and *myoD*. While the spatial and temporal expression of *notch* remains unchanged in morphants (Fig. 2B, B' and C), *myoD* expression strongly decreases in the absence of Pitx3 (Fig. 2A, A' and C), suggesting a positive control of this factor. Since MyoD binds on the murine *Pitx3* promoter and directly regulates it (Coulon et al., 2007) a closer investigation of the interaction between MyoD and Pitx3 will be necessary.

While *notch* expression is unaltered by the pitx3 depletion, chemical inhibition of notch by the γ-secretase inhibitor DAPT **(**N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) results in a stronger expression of *pitx3* in the myotome and extended expression in the presomitic mesoderm (Fig. 2 D, D'and C, C'). I speculate that *motch* prohibits the differentiation program to occur prematurely in the presomitic mesoderm, while restricting the differentiating role of *pitx3* to the mature somites.

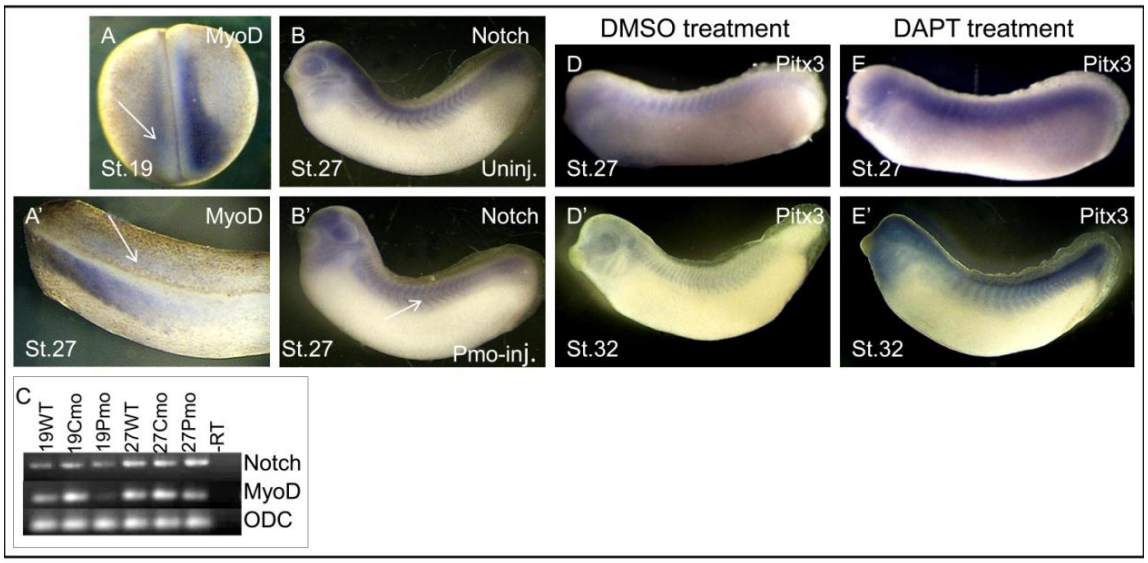
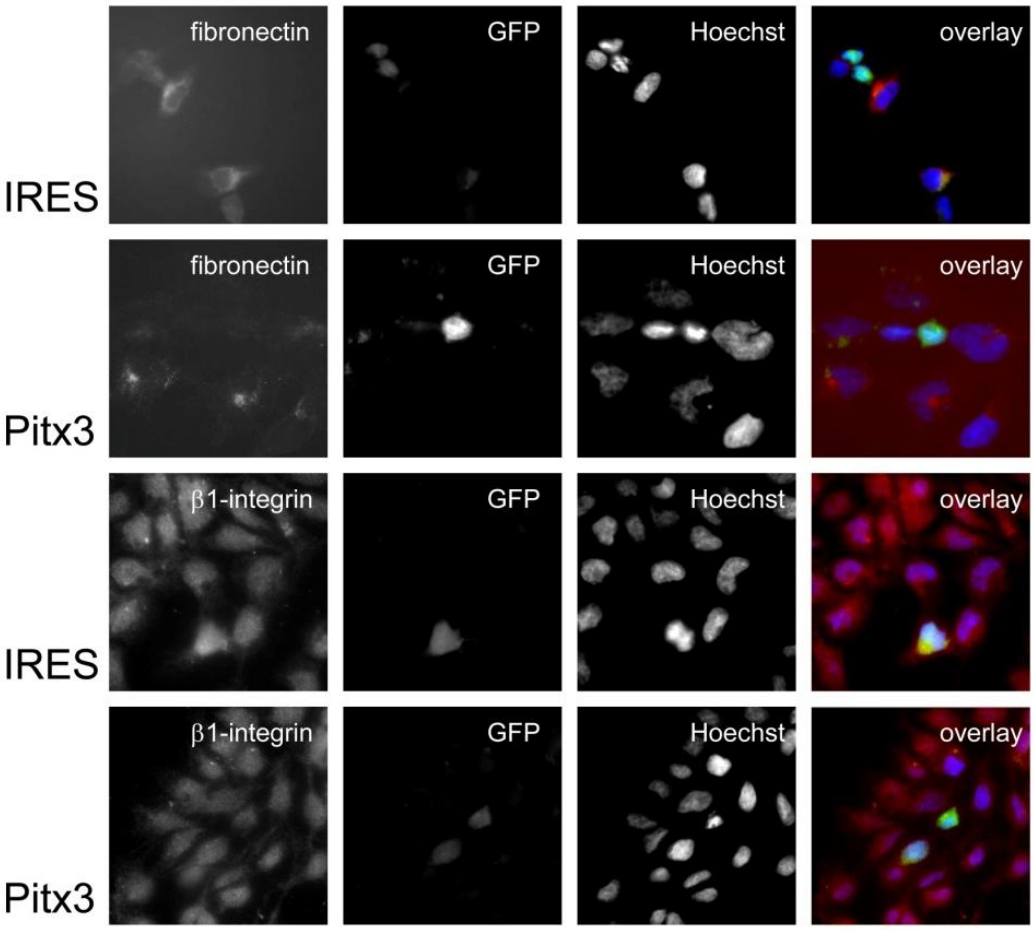


Figure 2. A, A'. Dorsal view of stage 19 and 27 morphants stained for *myoD* by *in situ* hybridization (arrow points to the side of injection). **B, B'.** *In situ* hybridization of *notch* – lateral view of unilaterally injected embryos. **C.** RT-PCR for *notch* and *myoD* at stages 19 and 27. **D, D' and E, E'.** *pitx3 in situ* hybridization on embryos treated with DMSO and DAPT respectively at stages 27 and 32.

To further investigate the cellular adhesion defects observed in the somites of embryos stained for β1-integrin and the suspected role of *pitx3* in cytoskeletal organization I employed an array of antibodies against various players in these processes. Given the changes observed in HEK293 cells expressing *pitx3* and stained with phalloidin (Chapter II), I decided to determine if *pitx3* controls other areas of cytoskeletal regulation. Antibodies against β1-integrin and its ligand fibronectin were used to confirm the abnormalities observed in embryos, however no changes were detectable when overexpressing *pitx3* in cells. Vinculin, a protein that links β1-integrin to actin and control focal adhesion, appears similarly unaffected by *pitx3* as is β-tubulin, a protein responsible for microtubules formation (Fig.3). Since the defects observed in the somites were the result of *pitx3* knock-down and here I report the effects of *pitx3* overexpression, I speculate that the necessity of *pitx3* in the cytoskeletal integrity is dose-dependent or it operates with interactants that are as yet unknown.

While the role of *pitx3* in the *Xenopus* somitogenesis is indubitable, more experiments are required in order to indentify its specific function and exact co-players.


fibronectin
GFP
Hoechst
overlay
IRES
fibronectin
GFP
Hoechst
overlay
Pitx3
β1-integrin
GFP
Hoechst
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IRES
β1-integrin
GFP
Hoechst
overlay
Pitx3

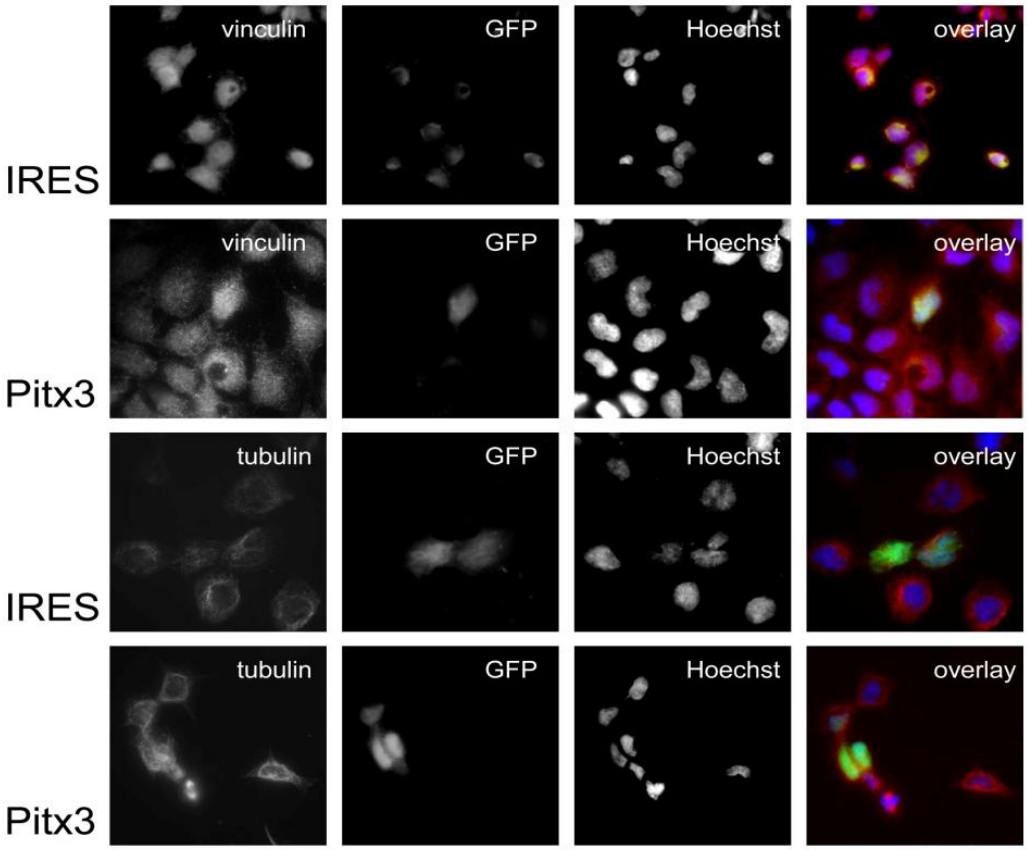


Figure 3. Immunocytochemistry on HEK293 cells transfected with either an IRES-GFP control or a Pitx3-IRES-GFP vector and stained for various antibodies. Separate images were collected for GFP, Hoechst and the respective antibody and images were merged using the Northern Eclipse software.

Materials and methods

Riboprobe synthesis. Antisense DIG-labeled riboprobes for *pitx3*, *notch* and *myoD* were generated by digesting the respective plasmids with *PvuII*, *ClaI* or *BamHI* and transcribing with SP6 or T3 RNA polymerase. Probes were visualized using anti-DIG antibody coupled to alkaline phosphatise (Roche 1:2000) and BM Purple (Roche). Stained embryos were paraffin wax embedded and sectioned at 35-um thickness.

TUNEL staining. The whole-mount TUNEL staining was carried out following the protocol previously described (Hensey and Gauthier 1997). Stained embryos were cleared in benzyl benzoate/benzyl alcohol 2:1 and subsequently wax embedded and sectioned at 15-um thickeness.

Whole-mount immunostaining. Embryos fixed in MEMPFA were incubated with muscle specific antibody 12/101 (DSHB, 1:1000) and with anti-mouse secondary horseradish peroxidase-conjugated antibody (Chemicon 1:250) or with anti-mouse Cy3 secondary antibody (Chemicon 1:200). Diaminobenzidine (DAB) was used as HRP substrate for color reaction. Wax embedded embryos were sectioned at 15-um thickness.

RT-PCR. cDNA was made using Omniscript reverse transcriptase (Qiagen) and Oligo(dT)$_{18}$ primers (Sigma) from 1ug total RNA isolated at stages 19 and 27 using Trizol (Invitrogen). RT-PCR was performed using corresponding primers at appropriate annealing temperatures (*myoD* (57C) F 5'-AGCTCCAACTGCTCCGACGGCATGAA-3', R 5'-AGGAGA GAATCCAGTTGATGGAAACA-3'; *notch* (57C) F 5'-GCTGTGAAGGCGATGTGAACGAG-3', R 5'-CGTCAAACCCAGGAGGGCATTT-3')

DAPT treatments DAPT **(**N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma-Aldrich) was disolved in DMSO at the stock concentration of 100 uM. Embryos at stages 9-10 were treated with DAPT diluted at 4uM in MBS and grown to the developmental stages of interested. For controls, the appropriate volume of DMSO was added to MBS and embryos were grown in identical conditions to their counterparts.

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 and were transfected either with pCINeo/IRES-GFP vector or pCINeo/ pitx3-IRES-GFP vector using the polyethylenimine method. 48 hours post-transfection, HEK293 cells were fixed with 3.7% PFAand following primary antibodies were used: anti-fibronectin (1:100), anti-β1-integrin (1:20), anti-vinculin (1:100) and anti-b-tubulin (1:100) (all from DSHB). After washing, cells were incubated with anti-mouse Cy5 secondary antibody (Chemicon, 1:200). Nuclei were stained with Hoechst 33285 (1:1000) followed by microscopy analysis.

References

Coulon, V., et al., 2007. A muscle-specific promoter directs Pitx3 gene expression in skeletal muscle cells. J Biol Chem. 282**,** 33192-200.

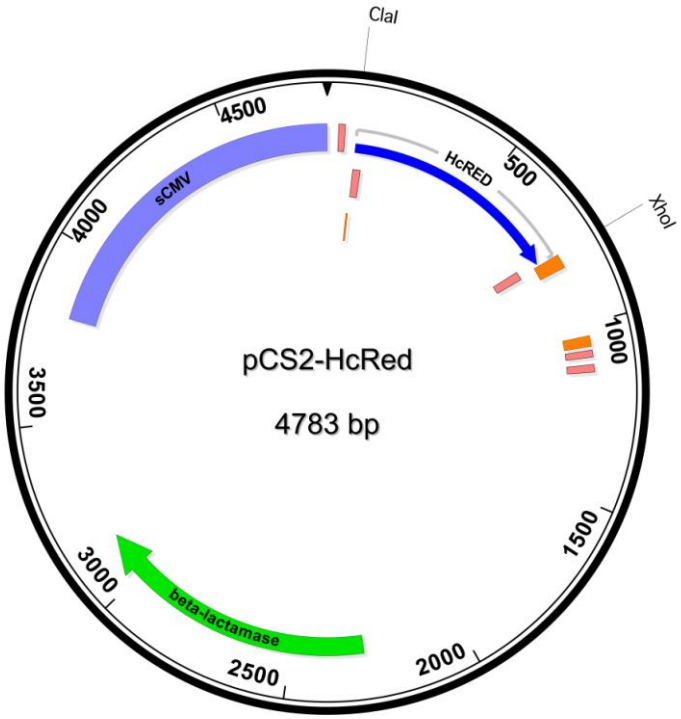
Hamilton, L., 1969. The formation of somites in Xenopus. J Embryol Exp Morphol. 22**,** 253-64.

Hensey, C., Gautier, J., 1998. Programmed cell death during Xenopus development: a spatio-temporal analysis. Dev Biol. 203**,** 36-48.

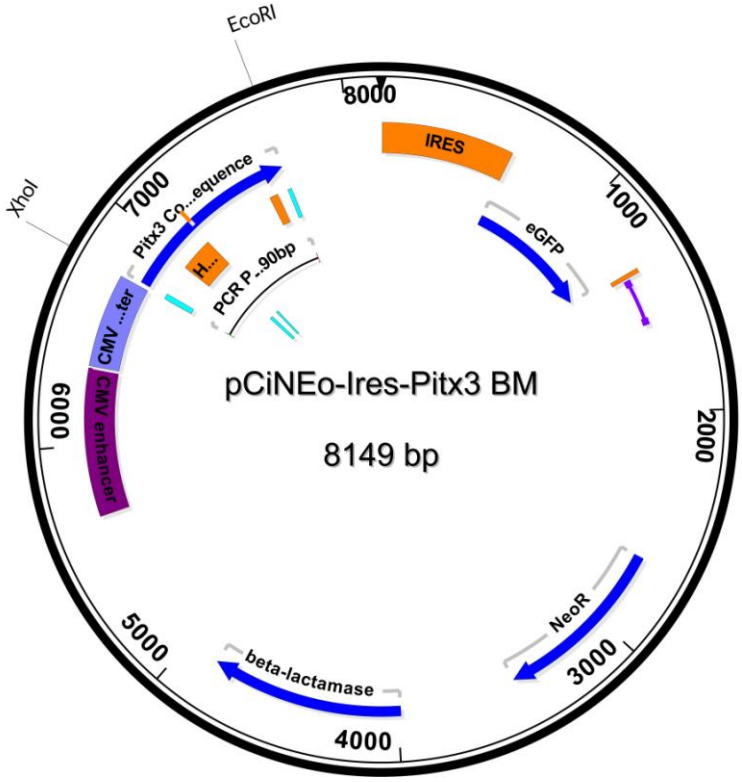
Kintner, C. R., Brockes, J. P., 1984. Monoclonal antibodies identify blastemal cells derived from dedifferentiating limb regeneration. Nature. 308**,** 67-9.

APPENDIX C

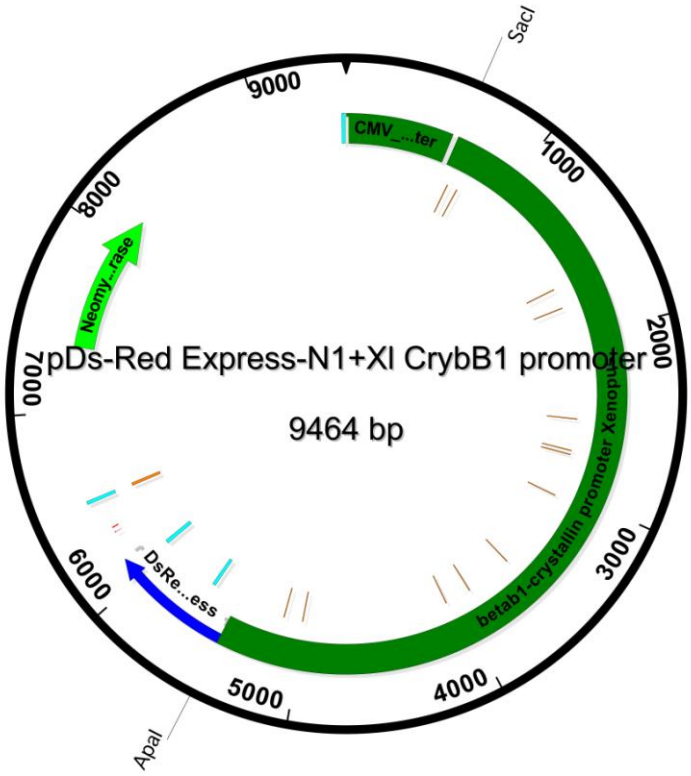
PLASMID MAPS



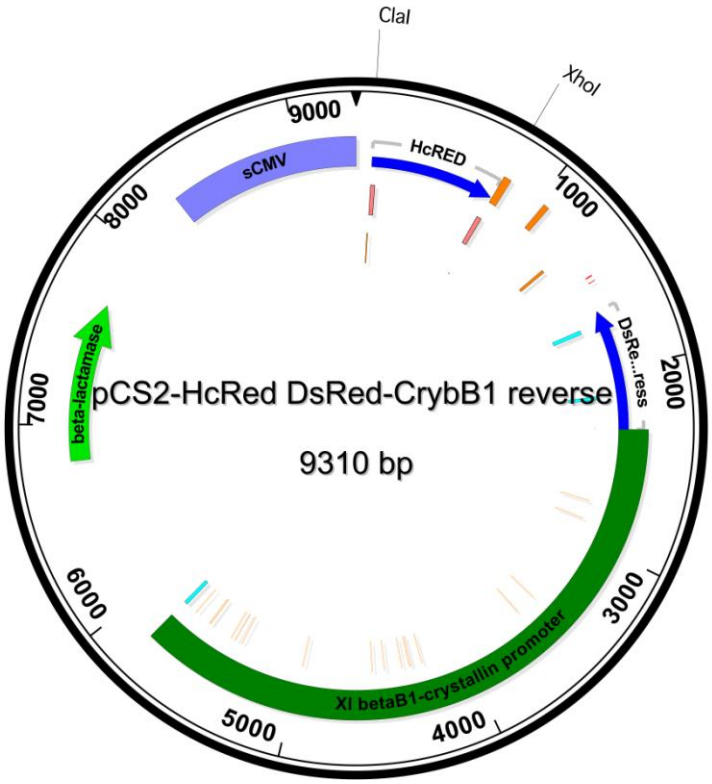
pCS2-HcRed. The pCS2-HcRed1 vector was generated by PCR-amplifying the HcRed1 coding sequence out of pCAG-HcRed1 (Addgene collection) using the following primers harboring adapters: F 5'-CC*ATCGAT*GGATCCTGAGCGGCCTGCTGAA-3'with *ClaI* adapter and R 5'-CCG*CTCGAG*CGGGCTTCAGTTGGCCTTC-3' with *XhoI* adapter, and subsequent ligation into the *ClaI*/*XhoI* sites of the pCS2 vector.



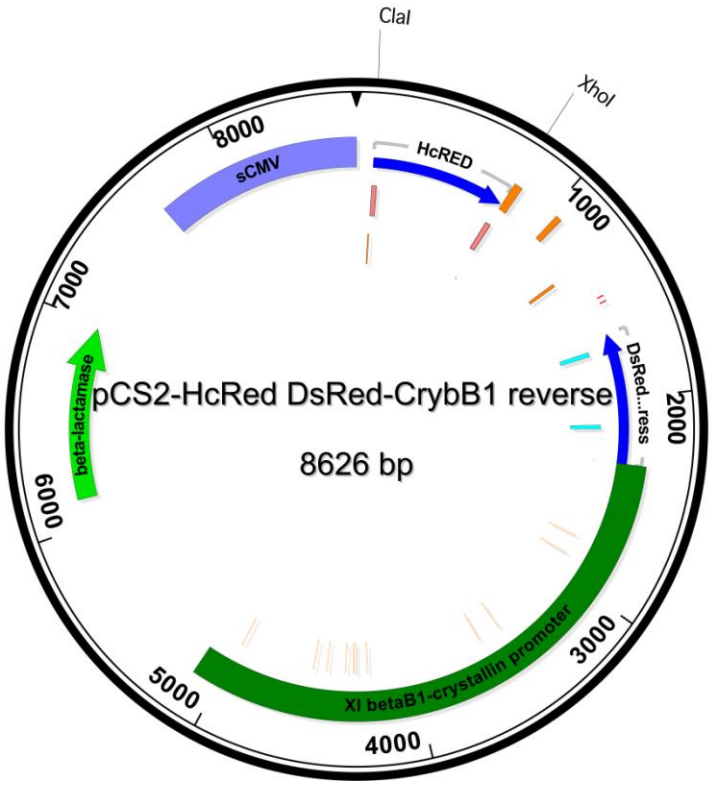
pCiNeo-IRES-Pitx3 BM The Pitx3 coding sequence containing the T->C mutation was PCR-amplified of the homemade pBSSK-Pitx3 BM plasmid using the following primers harboring adapters: F 5'-CCG*CTCGAG*CTGTTGCCACATGGATTTCAATCT-3'with *XhoI* adapter and R 5'-CGGAATTCCGTCCTTCATACTGGCCGATCCA-3' with *EcoRI* adapter, and subsequent ligation into the *XhoI*/*EcoRI* sites of the pCiNeo-IRES-GFP vector (kindly gifted by Dr. J. Eggermont).



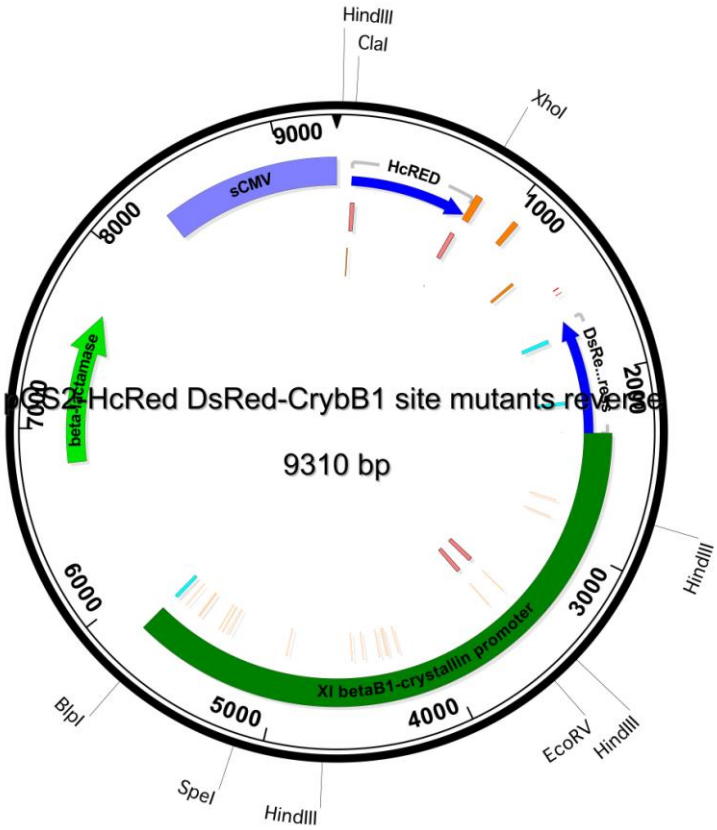
pDsRED-XlCrybB1 The CrybB1 reporter cassette was generated by cloning the 3.5kb *SacI/ApaI* digested promoter out of the *X. laevis* Crybb1 promoter plasmid (kindly gifted by Dr. H. Kondoh) into the multiple cloning site of the pDsRED-express-N1 vector.



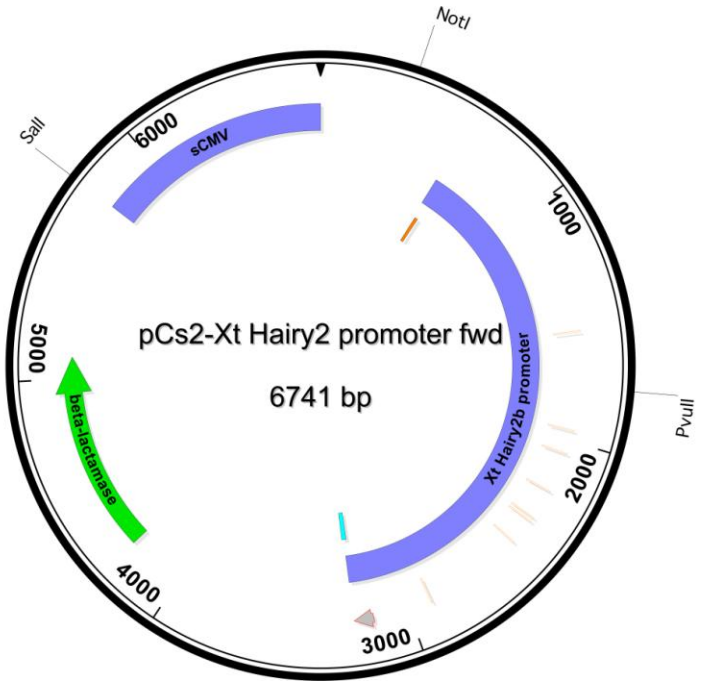
pCS2-HcRED/CrybB1-DsRED The CrybB1-DsRED cassette was PCR amplified using the following primers: F 5'-ACAATCAGAAGGTACAAGGCCC-3' and R 5'-CCCTATCTCGGTCTATTC-3', blunt cloned into the *PvuII* site of the pCS2-HcRED1 vector and selected for a reporter cassette inserted in reverse orientation to the CMV-HcRed1.



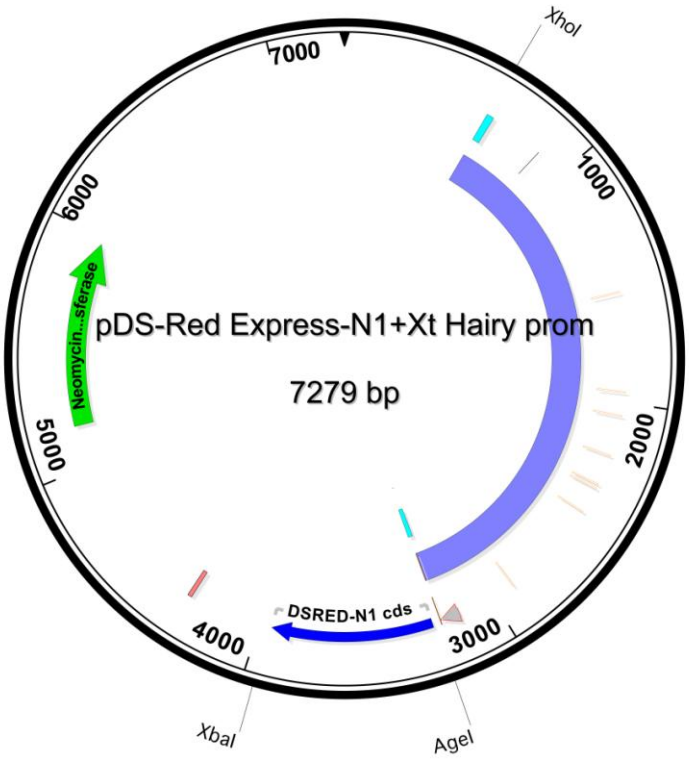
pCS2-HcRED/CrybB1-DsRED mutant A Mutant A was generated by deleting the last 750bp containing six binding sites out of the pCS2-HcRED/CrybB1-DsRED vector, using the restriction enzymes *BlpI* and *SpeI*.



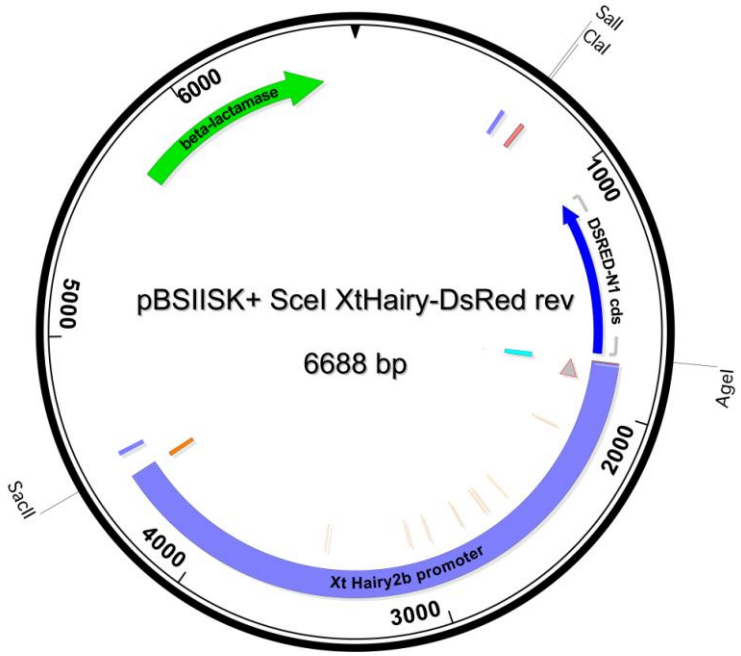
pCS2-HcRED/CrybB1-DsRED mutants B and C Mutants B and C were produced by site-directed mutagenesis using mutated primers to introduce new *EcoRV* and *HindIII* restriction sites respectively (GTACTGCATTATCAA → GTACTGCgaTATCAA and TTAAAACATTATTTC → TTAAAAgcTTATTTC).



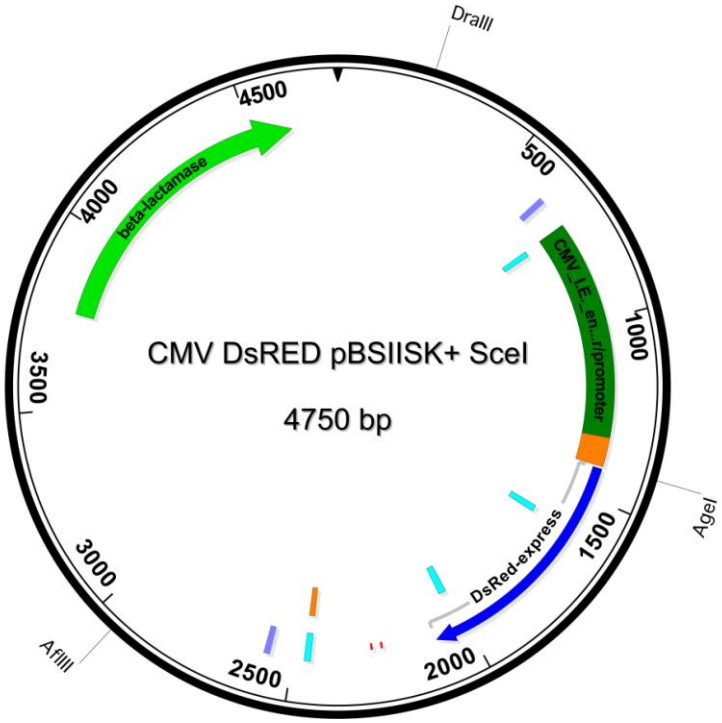
pCS2-Xt Hairy2 The *Hairy2* promoter was PCR-amplified from *Xenopus tropicalis* genomic DNA using the primers: F 5'-TTACCGAGGGAATGCACTC-3' and R 5'-GGGGTACCCCATCAGTCTTGCATATTCC-3' and blunt-cloned into the *PvuII* site of the pCS2 vector.



pDsRED-XtHairy The Xt Hairy reporter cassette was generated by PCR amplifying the XtHairy2 promoter from the pCS2-Hairy plasmid using the following primers: F 5'-CCG*CTCGAG*CGGTTACCGAGGGGAATGCACTC-3' with *XhoI* adapter and R 5'-GGGGTACCCCATCAGTCTTGCATATTCC-3'and cloning into the *XhoI* and and *SmaI* sites of the pDsRED-ExpressN1 vector (Clonetech)



pBSSK+SceI/ XtHairy-DsRED The XtHairy-DsRED cassette was PCR-amplified from the pDsRED-XtHairy vector using the following primers: F 5'-TTACCGAGGGAATGCACTC-3' and R R 5'-CCCTATCTCGGTCTATTC-3' and blunt cloned into the *EcoRV* site of the pBSSK+SceI vector (kind gift from Dr. T. Pieler)



pBSSK+SceI/ CMV-DsRED The CMV-DsRED cassette was PCR-amplified from the pDsRED-XtHairy vector using the following primers: F 5'-GATAACCGTATTACCGCC-3' and R R 5'-CCCTATCTCGGTCTATTC-3' and blunt cloned into the *EcoRV* site of the pBSSK+SceI vector (kind gift from Dr. T. Pieler)

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Publications:
1. Smoczer* C., Hooker* L., Brode S., Wolanski M., KhosrowShahian F., Crawford M.J. (2012) The *Xenopus* Homeobox Gene *Pitx3* Impinges Upon Somitogenesis and Laterality. Submitted to *Biochemistry and Cell Biology* BCB 2012-0057
2. Hooker L., Smoczer C., KhosrowShahian F., Wolanski M., Crawford M.J. (2012) Microarray Based Identification of Pitx3 Targets During *Xenopus* Embryogenesis. Submitted to *Developmental Dynamics* DVDY-12-0073
3. Smoczer C., Hooker L., Sachani S., Crawford M.J. (2012) Microinjection manipulations in the elucidation of *Xenopus* brain development. *Methods in Molecular Biology*. Methods in Brain Development, Springer Verlag

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