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Role of xARX2 in Xenopus brain development.

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ROLE OF *xARX2* IN *XENOPUS* BRAIN DEVELOPMENT

By

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ABSTRACT

Vertebrate brain development is a multi-step process involving a tight regulation of gene expression. Homeobox genes of the *paired-like* family have been shown to play prominent roles in directing forebrain regionalization and patterning. The *aristaless*-related homeobox gene (*Arx*) has been shown to regulate proliferation in the mouse forebrain and mutations in human *ARX* lead to a spectrum of cognitive disorders. To identify the role that *Arx* plays during amphibian development we characterized the spatial and temporal expression of a *Xenopus Arx* homolog, *xArx2*. *xArx2* is present as a maternal transcript and its initial expression detectable by whole-mount *in situ* hybridization occurs briefly during late blastula in the dorsal region of the embryo. *xArx2* is detected throughout neurulation in the anterior neural plate and is found within the presumptive forebrain territory and in the somites during tailbud stages. Early tadpoles show expression of *xArx2* within the floor plate of the anterior spinal cord, in the ventral and lateral telencephalon, and in the lateral diencephalon. To further understand the functional role that *xArx2* may play during *Xenopus* embryogenesis, we established the consequences of *xArx2* misexpression both phenotypically and on various marker genes. Ectopic *xArx2* expression expands the forebrain territory and impairs eye development. Inhibiting xArx2 translation by means of antisense morpholino oligonucleotides (MO) results in embryos with shrunken forebrains and impaired craniofacial structures. Both gain- and loss-of-function mutants display extensions of pigmented retinal epithilium towards the midline, microcephaly, and fused telencephali or underdeveloped forebrains. The forebrain markers *XBF-1* and *xArx* were up-regulated in *xArx2*-injected embryos and reduced in *xArx2*-MO-injected embryos. Additionally, the expression domains of

posterior brain markers, *xGbx2a* and *xKrox20*, were shifted caudally in embryos misexpressing *xArx2*. The expression domains of several other general brain/eye markers were affected following *xArx2* misexpression. Our findings suggest a role for *xArx2* in forebrain patterning in *Xenopus*.

For my Grandparents, my parents, my sister and Mihael.

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TABLE OF CONTENTS

LIST OF FIGURES

PAGE

LIST OF TABLES

LIST OF ABBREVIATIONS

ANR – anterior neural ridge

AP – anterior-posterior

BMP – Bone Morphogenic Protein

CMO – control morpholino oligonucleotides

CNS – central nervous system

DIG – digoxygenin

DV – dorsal-ventral

FGF – fibroblast growth factor

GFP – *Green Fluorescent Protein*

H&E - hematoxylin and eosin

HOM-C – homeotic complex

HOM/Hox genes – homeotic genes

IsO – isthmic organizer

MBS - Modified Bart's Saline

MHB – midbrain-hindbrain boundary

MO – antisense morpholino oligonucleotides

P1-P3 – prosomere 1 - prosomere 3

RPE – extension of retinal pigmented epithelium

RT-PCR – reverse transcriptase – polymerase chain reaction

SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

SHH – Sonic hedgehog

TUNEL - Terminal dUTP nick end labeling

UTR – untranslated region

UV - ultraviolet

XLAG - X-linked lissencephaly with abnormal genitalia

ZLI - zona limitans intrathalamica

CHAPTER 1

GENERAL INTRODUCTION

Chapter Summary

In vertebrates, the progressive differentiation of the mature brain from the anterior neural plate is extremely complex. Overall morphology is attributable to regional patterning along both the dorsal-ventral and the anterior-posterior axes, and is regulated by transcription factors and signaling molecules. Once the major segments of fore-, mid-, and hindbrain are established, the brain subdivides further giving rise to discrete functional units, or neuromeres, along the neuroaxis. A *paired-like* homeobox transcription factor, *Arx,* has been reported to express in the anterior brain and possibly to play a role in neural proliferation.

INTRODUCTION

Understanding the complexity of brain regionalization and patterning represents a major focus for scientists in the field of developmental neurobiology. The premise of much of this research involves first identifying the individual molecular components involved in this process and then determining their complex interactions. The initial step in forming a central nervous system (CNS) in vertebrates is the demarcation of the neural plate, a thickened area of ectoderm along the dorsal midline of the gastrula stage embryo. At the end of gastrulation the lateral boundaries of the neural plate, the neural folds, begin to merge inward. They enclose a progressively deepening groove, until they fuse apically to form the neural tube. The neural tube, which is then covered by ectoderm, is composed of dorsal, ventral and two lateral laminae, commonly referred to as the roof,

floor and lateral plates, respectively (reviewed by Colas and Schoenwolf, 2001). Migrant neural crest cells emerge from the dorsal boundary between the surface ectoderm and the neural tube. Cranial neural crest goes on to contribute to the formation of much facial bone, musculature, tongue, and throat (Mayor and Aybar, 2001; Helms and Schneider, 2003). The anterior end of the neural tube balloons markedly, producing a series of swellings that form the anlagen of forebrain, midbrain and hindbrain regions, while the narrow, posterior part of the tube forms the spinal cord. Continued subdivision of this neuroaxis produces small segment-like bulges called neuromeres. Within each region a large diversity of neuronal cell types differentiate at stereotypical intervals to generated segments with distinct identities in terms of morphology, molecular markers, and cellular properties (Lumsden and Krumlauf, 1996; Rubenstein *et al.*, 1998). It has become apparent that the CNS of all vertebrate species share many features in common and diverge only slightly from a common plan of organization. Transcriptional cues and regional signals must be tightly regulated to establish the proper patterning of the anteroposterior (AP) and dorsoventral (DV) axes throughout the developing CNS, however, the entire spectrum of the players involved and how they exert their function has yet to be fully elucidated.

The South African clawed frog, *Xenopus laevis*, provides a favourable model system for the study of vertebrate development. Unlike mammalian embryos, amphibian embryos are comparatively large, they develop externally, and they are available in large numbers throughout the full year. Although it takes about a year and a half for a fertilized egg to become a sexually mature adult, its initial development occurs rapidly, so that the embryonic stages of cleavage, gastrulation, and initiation of the nervous system take less

than 24 hours. The swimming embryo hatches from its jelly coat on the third day post-fertilization and becomes a feeding tadpole within one week. A major benefit provided by *Xenopus* embryos for the analysis of development is the opportunity that they afford investigators to conduct *in vivo* gain- and loss-of function studies by way of microinjecting molecules into the eggs or cells of the early embryo. Advantageously, the tadpole is relatively transparent with a few pigment cells, allowing decent visualization of its internal organs. Thus, many phenotypic outcomes resulting from these studies can be observed in intact tadpoles. Although not exploited in our studies, this model also allows for surgical procedures, such as transplants, explants and recombinants, to be conducted with relative ease. Furthermore, there are well characterized fate maps of the anterior neural plate (Eagleson and Harris, 1990; Rubenstein *et al.*, 1998) and the anterior neural ridge (Eagleson *et al.*, 1995) for this organism.

Much of our current knowledge of the events leading to neurulation is based on studies in *Xenopus.* Briefly, the process of neural induction converts part of the embryonic ectodermal cells into neural precursors through the inhibition of signals that would otherwise induce an epidermal fate. In *Xenopus*, inductive events commence prior to gastrulation when signals from the Nieuwkoop Center specify the above mesoderm to form an organizing center (Spemann's Organizer) and to produce signals which cause the ectoderm to take on a neural fate (for reviews see Harland, 2000; Wilson and Edlund, 2001; Bainter *et al.*, 2001).

REGIONALIZATION OF THE NEUROAXIS

Once the neural plate is established, it rolls up and forms the neural tube (reviewed by Colas and Schoenwolf, 2001). Patterning of the neural tube along the DV axis is dependent upon the relative amounts of dorsalizing factors of the Bone Morphogenic Protein (BMP) family from the non-neural ectoderm (Barth *et al.*, 1999; Lee and Jessel, 1999) and ventralizing factors of the sonic hedgehog (SHH) family, which are produced by the notochord and the floor plate (Echelard, 1993; reviewed by Marti and Bovolenta, 2002). Patterning along the AP axis, however, is currently under intense scrutiny. In *Xenopus*, spatiotemporal expression analysis of genes in the presumptive neuroectoderm implies that neural patterning along the AP axis has already begun at the early gastrula stage (reviewed by Gamse and Sive, 2000). One of the earliest brain patterning genes known to be expressed is *Otx2*, a homeobox gene which is detected at early to mid gastrula stages and specifies the anterior part of the future brain: a region that gives rise to the forebrain and midbrain (Blitz and Cho, 1995; Pannese *et al.*, 1995). In *Xenopus,* microinjection of synthetic *xOtx2* RNA results in an abnormal reduction in the size of tail and trunk structures, and in the appearance of a second cement gland, a transient structure of the embryonic head (Blitz and Cho, 1995; Pannese *et al.*, 1995). Mouse $Otx2^{-/-}$ mutants die early in embryogenesis and lack the rostral neuroectoderm fated to become forebrain, midbrain and rostral hindbrain (Acampora *et al.*, 1995). At early neurula stages another homeobox gene, *xGbx2*, appears to specify the region posterior to the *xOtx2* domain (von Bubnoff et al., 1996) and this orientation has also been shown to occur in chick and mouse (Broccoli *et al.*, 1999; Millet *et al.*, 1999). Thus, the future brain region seems to be divided into the anterior and posterior portions at these

early stages. Over the past decade many new players have been identified both upstream and downstream of organizer activity during vertebrate neural plate formation.

Regionalization of the neural tube within the CNS is the basis for the structure of the mature brain. The brain is comprised of three major divisions: the prosencephalon (forebrain) which delineates rostrocaudally into the tel- and diencephalon; the mesencephalon (midbrain); and the rhombencephalnon (hindbrain), which comprises the met- and myelencephalon (rostral to caudal, respectively).

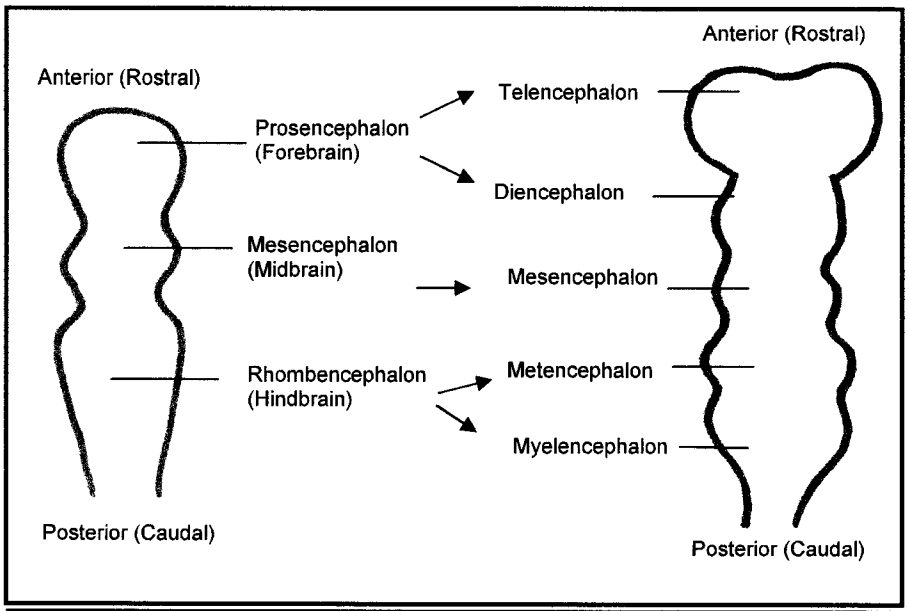

Figure 1. Schematic representation of the different segments within the developing neural tube (dorsal view).

In mammals, the telencephalon forms the cerebral hemispheres while the diencephalon forms the thalamic and hypothalamic regions. A model of early regionalization which has gained much support is the two-signal or activation/transformation model of Nieuwkoop and colleagues (reviewed in Foley and Stern, 2001). They hypothesized that development of the full range of neural subdivisions arises via a two-step process. Activating signals from early-involuting mesoderm are thought to induce a default state of

anterior neural differentiation, which is then modified to a more posterior character in a graded fashion by transforming signals from later-involuting mesoderm. The molecular identity of the transforming gradient remains elusive, however a number of candidate posteriorizing signals have been proposed, notably fibroblast growth factors (FGFs), retinoic acid and members of the Wnt family (Foley and Stern, 2001 and references therein). In mammals, antagonists of posteriorizing factors may reside in the anterior visceral endoderm, which acts to protect the prospective forebrain cells from caudalizing signals (Kimura *et al.*, 2000). Anterior fates including the telencephalon are missing in embryos carrying mutations in several genes which are expressed in the anterior visceral endoderm (Acampora *et al.*, 1995; Shawlot and Behringer, 1995).

While primary neural induction and fundamental AP or DV patterning of the early neural tube are due to the activity of the "primary organizer" (Spemann and Mangold, 1924), emerging data reveals that the regionalization of neuroepithelial areas are brought one step further through morphogenetic controlling processes that arise from "secondary organizers" located in specific regions along the developing neural primordium. An organizer is thought to establish gradually within a tissue by a series of steps: first a field is specified and then boundaries are defined (Meinhardt, 1983). Once a boundary differentiates two fields, co-operative cell–cell interactions can eventually produce signaling molecules that activate specific differentiation programs in adjacent cells. Secondary organizers located at: the isthmus, which is found at the midbrain-hindbrain boundary (MHB); at the anterior neural ridge (ANR), which is located at the boundary between the non-neural ectoderm and the prospective forebrain; and at the zona limitans intrathalamica (ZLI) have all been reported (reviewed by Echevarria *et al.*, 2003).

Secondary Organizers

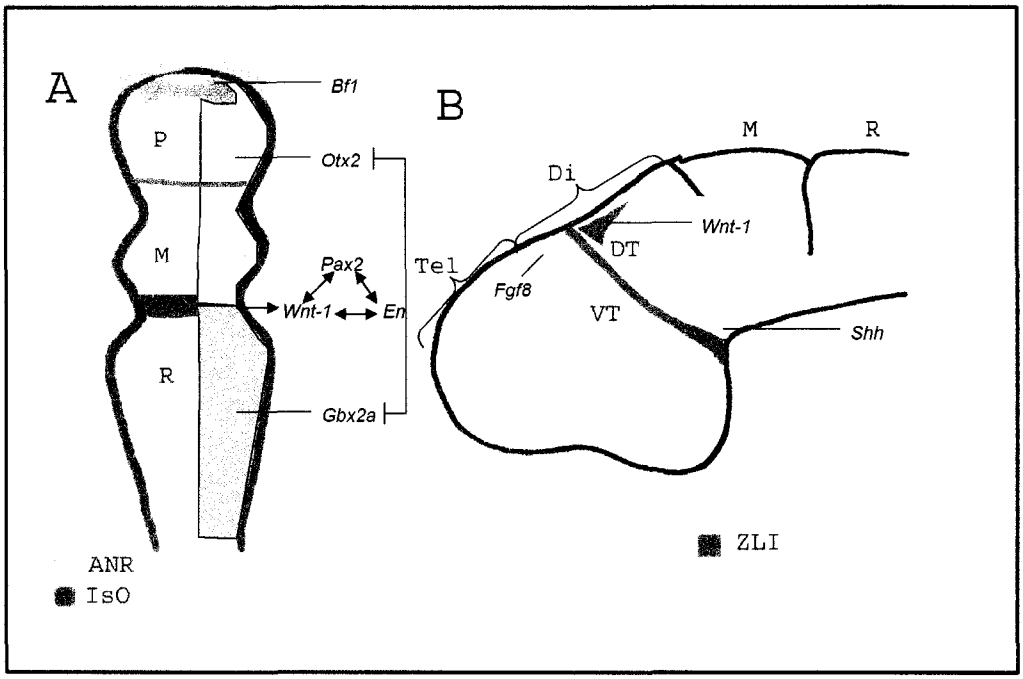

Figure 2. Schematic representation of the location of regionalization centers and neighbouring gene expression patterns within the vertebrate brain. Dorsal view (A) of the early neural tube and lateral view (B) of the forebrain are shown. Abbrevieations: ANR: anterior neural ridge; IsO: isthmus organizer; ZLI: zona limitans intrathalamica; P: prosencephalon; M: mesencephalon, R: rhombencephalon; Tel: telencephalon; Di: diencephalon; DT: dorsal thalamus; VT: ventral thalamus. Adapted from Echevarria *et al.*, 2003; Wurst and Bally-Cuif, 2001.

The best understood secondary organizer is located at the constriction between the midbrain and hindbrain, called the isthmus, which controls anterior hindbrain and midbrain regionalization. Activity at the isthmus organizer (IsO) has been found in all vertebrate species studied, and was first studied in the avian embryo. Isthmic tissue grafts transform chick caudal forebrain into an ectopic midbrain with rostrocaudal polarity similar to the endogenous counterpart (reviewed by Echevarria *et al.*, 2003). This switch of fate is reflected by induced ectopic expression of the homeobox gene *engrailed* (*En*), which is specific to the isthmus, in the host neural tube around the graft (Martinez *et al.*, 1995). Complete removal of the isthmus coincides with the loss of the entire

mesencephalon and metencephalon: the isthmic territory is both necessary and sufficient for the development of the mes-metencephalic domain.

A complex spatio-temporal pattern of gene expression including *Otx2*, *Gbx2*, *Pax2*, *En*, *Wnt-1*, and *Fgf8*, has been reported to occur in the IsO (reviewed by Wurst and Bally-Cuif, 2001). All of these genes are first expressed in the prospective MHB region at late gastrula to neurula stages in *Xenopus*, suggesting that these genes act in concert to mediate positioning of the isthmus. Briefly, the interface between the caudal *Otx2* and the rostral *Gbx2* expression domains is thought to position the IsO, and these genes have been shown to inhibit each other (Martinez-Barbera *et al.*, 2001; Katahira *et al.*, 2000; Millet *et al.*, 1999). This is a fundamental event for the stabilization of the autoregulative loop of *En-1*, *Wnt-1*, and *Pax2* expression (reviewed by Martinez, 2001). Mutant mice or zebrafish lacking either of these genes do not develop the isthmo-cerebellar complex and *Gbx2* null mutants lack a cerebellum and exhibit caudal expansion of the midbrain.

Another local signaling centre resides in the anterior neural ridge, which lies at the junction between the most rostral part of the neural plate and the non-neural ectoderm. This region is necessary for the maintenance of forebrain identity (Shimamura and Rubenstein, 1997). *Brain factor-1* (*BF-1*) encodes a winged-helix transcription factor required for regionalization and growth of the telencephalic and optic vesicles. Mice mutant for *BF-1* have small telencephalons and lack expression of a basal telencephalic marker, *Dlx2* (Xuan *et al.*, 1995). Excision of the anterior neural ridge has been shown to eliminate expression of *BF-1* in neural plate explants (Shimamura and Rubenstein, 1997). Moreover, transplantation of the anterior neural ridge cells from zebrafish into more caudal regions of the neural plate induces the expression of *Nkx2.1* and *Emx*, genes

typically expressed in the telencephalon (Houart *et al.*, 1998). One proposed role of the anterior neural ridge is to regulate the subdivision of the anterior neural plate into telencephalic, optic, and diencephalic domains (Wilson and Rubenstein, 2000).

An important mediator of anterior neural ridge and isthmic organizing properties is the signaling molecule fibroblast growth factor 8 (FGF8), which exerts its function in a concentration dependent manner (Eagleson and Dempewolf, 2002; Lee *et al.*, 1997). *Fgf8* is expressed in early anterior neural ridge cells and has been shown to be crucial for the specification of the anterior areas of the forebrain: FGF8-soaked beads can rescue *BF-1* expression subsequent to elimination of the anterior neural ridge (Shimamura and Rubenstein, 1997). Fibroblast growth factors secreted from the isthmus are required to direct the ordered growth and regionalization of the midbrain and anterior hindbrain. Studies in chick have shown that insertion of Fgf8-soaked beads into the prospective diencephalon results in ectopic isthmic markers in the host (reviewed by Wurst and Bally-Cuif, 2001).

Evidence for the existence of another organizing center which resides in the zona limitans intrathalamica, a structure that separates the dorsal and ventral thalami, has begun to emerge (Larsen *et al.*, 2001; reviewed by Echevarria *et al.*, 2003). Several secreted factors express in the dorsal region of the zona limitans intrathalamica, specifically *Shh*, whose expression is flanked by *Wnt-1* caudally and *Fgf8* rostrally, and which are thought to control proliferation, regionalization and polarity within the diencephalic segments of the forebrain (Echevarria *et al.*, 2003).

Complexity of the Vertebrate Forebrain

The forebrain itself comprises one of the most intricate structures of the vertebrate brain and consists of anatomically and functionally distinct domains patterned along their anterior-posterior and dorsal-ventral axes (reviewed by Rubenstein *et al.*, 1998). The hippocampus, thalamus, hypothalamus, olfactory bulbs, and eyes are some of the structures that are derived from or within the forebrain. For example, optic vesicles are among the earliest morphological features of the forebrain to appear. The vesicles bulge laterally from the neural tube at the presumptive diencephalon and remain connected to the diencephalon territory by optic stalks. When the vesicles make contact with the overlying ectoderm, the ectoderm is induced to thicken to form lens placodes. This in turn induces invagination of the optic vesicles to form a double-walled optic cup. These layers differentiate and the outer layer forms pigmented retina while cells of the inner layer proliferate into the neuronal types that constitute the neural retina. The optic stalk becomes the optic nerve.

Since complex structures derive from the prosencephalon, it is of great interest to understand how this regionalization and patterning is regulated. For the past decade Rubenstein and colleagues have been developing the prosomeric model for the vertebrate forebrain in order to lend a framework for comparative research and to help to categorize topologically numerous molecular expression patterns (Rubenstein et al., 1994; Puelles and Rubenstein., 2003). They suggest that like rhombomeres in the hindbrain, the forebrain consists of segments termed prosomeres. The prosomeric model divides the forebrain into the defined segments (prosomeres) and DV wedges in all vertebrates (schematically shown in Figure 3). Under this paradigm, the diencephalon is first

subdivided into three prosomeres, P1-P3 in a caudal to rostral manner: P1 corresponds with the area that comprises the presumptive pretectum, P2 contains the presumptive dorsal thalamus, and P3 is associated with the presumptive ventral thalamus (Puelles and Rubenstein, 2003). The rest of the forebrain, or the secondary prosencephalon, is divided into the hypothalamus and the telencephalon, which itself is divided into pallial (dorsal) and subpallial (ventral) compartments (Bachy *et al.*, 2002).

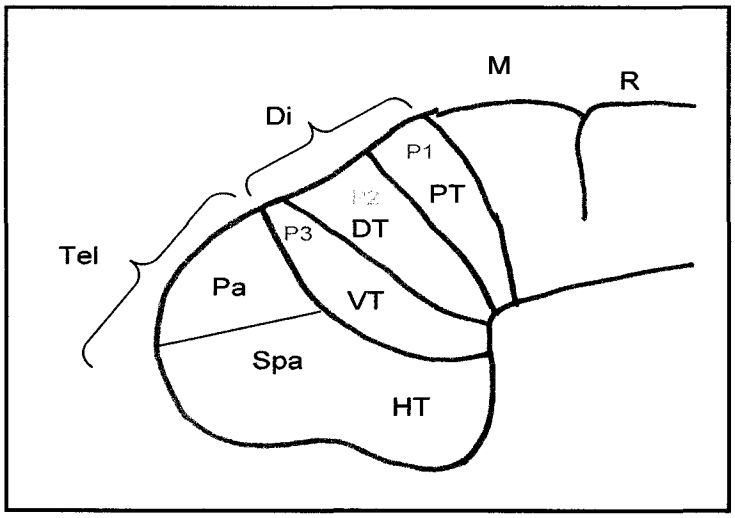

Figure 3. Schematic representations of main divisions within the vertebrate forebrain shown from a lateral view. Abbreviations: R: rhomencephalon; M: mesencephalon; Di: diencephalons; Tel: secondary telencephalon; P: prosomere; PT: pretectum; DT: dorsal thalamus; VT: ventral thalamus; HT: hypothalamus; Pa: pallium; Spa: Subpallium. Adapted from Bachy *et al.*, 2002.

The telecephalic regions appear to be maintained through cross-regulatory interactions among transcription factors. While the *Hox* genes have been shown to predominate in establishing the hindbrain, it is becoming evident that other homeobox genes play important roles in the establishment of more anterior regions of the CNS.

HOMEOBOX GENES

Presently, many of the genes found to play prominent roles in vertebrate neural development have been found to be transcriptional regulators belonging to the super class of genes known as homeobox genes. These genes encode a well-conserved 60 amino acid domain known as the homeodomain. It specifies a helix-turn-helix-motif which is involved in DNA binding. Target specificity of homeodomain proteins strongly depends on the amino acid composition of the homeodomain, in particular on the identity of the residue on position 50, and the presence of other DNA binding domains within the protein (Gehring *et al.*, 1994).

Homeobox genes were first identified in *Drosophila* and were found to exert key developmental functions throughout the Metazoa (Martinez and Amemiya, 2002). They have been shown to specify segmental identity and positional information along the antero-posterior axis by regulating expression patterns of target genes in a temporal, spatial, and tissue-specific manner. There are over twenty major classes of homeobox-containing genes arrayed in clusters or dispersed separately throughout the genome (Gehring *et al.*, 1994). Among all families of homeobox-containing genes, particular attention has been given to the class of clustered homeotic (HOM/Hox) genes. In *Drosophila* there are two linked homeotic gene complexes, the *Antennapedia* and the *Bithorax* complex. The chromosome region containing both complexes is often referred to as the homeotic complex (HOM-C). HOM-C genes display spatio-temporal colinearity, meaning that they are arranged in the same general order on the chromosome as their order and timing of appearance along the anteroposterior body axis, (Krumlauf, 1992). In mammals and chicks, the *Hox* genes are organized in four chromosomal

complexes, termed *Hox A, B, C, D,* and the genes in each of these complexes not only show striking molecular homology to the homeotic genes in *Drosophila*, but also spatio-temporal colinearity (Duboule and Morata, 1994). A feature of some homeotic genes is that gain-of and loss-of function mutations result in homeotic transformations, whereby one structure is transformed into the likeliness of another. A classical example of this phenomenon is the mutation in the *Drosophila* gene *Antennapedia* which leads the cells of the antennal imaginal discs to form leg structures that protrude from the head in the place of antennae (Kaufman *et al.*, 1990). In vertebrates, both misexpressions and null mutations of *Hox* genes have produced structural deletions and homeotic transformations similar to those previously seen in flies (Gehring, 1993).

Although *Hox* genes are not expressed anterior to the hindbrain, many members of nonclustered homoebox genes express in these areas. A growing number of genes belonging to the *paired-like* homeobox gene family display restricted expression patterns in anterior regions of the developing neural tube suggesting a role for this family in patterning anterior structures of the CNS.

***Paired*-like Genes**

The *paired* genes represent a large group of homeodomain proteins which contain a *paired* type homeodomain first described in the *Drosophila* gene *paired.* Dimerization and cooperative DNA binding modulates target specificity of these genes (Wilson et al., 1993). Three subclasses of *paired* genes have been defined according to the residue at position 50 of the homeodomain (reviewed by Galliot *et al.*, 1999). *Pax* genes encode a homeodomain with a serine residue at position 50 as well as a second DNA binding-domain, the *paired* domain. The other two groups, which lack the *paired* domain, are

categorized as either K50 or Q50 *paired-like* genes, and contain a lysine or a glutamine residue at position 50 within their respective homeodomains. Phylogenetic analysis of the *paired* homeodomains has revealed 18 distinct gene families: 3 *Pax*-type (*Pax*-3/7, *Pax-6*, *Pax-4*), 3 K50 *paired-like* (*Otx, Ptx, Gooscoid*), and 12 Q50 *paired-like* (*aristaless, Ceh-10, Rx, Unc-4, Cartl, Otp, Arix, Prx, Og12, Anf, Mix, Siamois*) (Galliot *et al.*, 1999). A myriad of genes from several of these classes are emerging as regulators of critical events during anterior development, and regulate elaboration of the developing forebrain.

Aristaless-related Genes

Recently, *aristaless*-related homeobox genes have been shown to act as important regulators during critical events in vertebrate embryogenesis. Members of this gene family contain *paired-like* homeodomains with high homology to *aristaless*. *Aristaless* was originally discovered in *Drosophila* where embryonic expression is important for the ontogeny of specific head segments and specifies the distal tip of imaginal discs (Schneitz *et al.*, 1993; Campbell and Tomlinson, 1998). A structural feature shared by all *aristaless*-related genes is a conserved 17 amino acid-sequence located near the C-terminus, termed the *aristaless*, OAR (Otx, Arx, Rx), FACE or C-peptide domain. The C-peptide domain is likely to have a similar molecular function in all *aristaless*-related proteins, but this function is poorly understood. Several studies have shown that it is involved in modulating the DNA binding and transcriptional activities of the protein in which it is contained (Amendt *et al.*, 1999; Brower *et al.*, 2003). An activation domain function in the Otp protein was proposed originally (Simeone et al., 1994), however more recent *in vitro* and *in vivo* studies have suggested that the C-peptide domain acts as a

molecular switch to attenuate transcriptional activation (Amendt *et al.*, 1999; Norris and Kern, 2001; Brower *et al.*, 2003). In the *aristaless*-related protein Pitx2 the C-peptide domain modulates transcriptional activity by inter- and intramolecular interactions. Binding of the transcription factor Pit1 to the C-peptide domain facilitates Pitx2 DNA binding and transcriptional activation, presumably by unmasking an activation domain in a region N-terminal from the homeodomain (Amendt *et al.*, 1999).

Vertebrate *Aristaless*-related genes can be divided into three groups based on similarities in gene structure as well as expression patterns and functional data. Group-I-*aristaless*-related genes, which include *Alx3/4, CartI, and Prx1/2/3*, are linked to functions in the development of the craniofacial and appendicular skeleton and are expressed in neural crest-derived mesenchyme (Beverdam and Meijlink, 2001). *Prx1* and *Prx2* are required for correct skeletogenesis of the skull, face, limbs and vertebral column by controlling cell proliferation (Ten Berge *et al.*, 2001). Other members of this group have been shown to regulate apoptosis. *Cart1* mutants have an anterior neural tube closure defect that appears to result from increased cell death in the mesenchyme surrounding the forebrain vesicles (Zhao *et al.*, 1996), and aberrant apoptosis in an outgrowing frontonasal process is the reputed cause of severe nasal clefting observed in Alx3/4 double mutant mice (Beverdam *et al.*, 2001).

Group-II-*aristaless*-related genes, including *Drg11, Arx, Chx10, Otp* and *Rx*, are expressed predominantly in the central and/or peripheral nervous system and have been reported to play crucial roles in the specification of these organs. *Drg11* is required for the formation of spatio-temporally appropriate projections from nociceptive sensory neurons to their central targets in the dorsal horn of the spinal cord (Chen, 2001). *Rx* is

expressed in the anterior neural plate, in regions which gives rise to the eye and forebrain (Casarosa *et al.*, 1997; Mathers *et al.*, 1997). Loss-of-function *Rx* mutations result in severe early eye phenotypes in addition to defects in forebrain development (Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999).

Group-III-*aristaless*-related genes include the *Pitx* family whose members each encode K50 *paired-like* homeodomains. *Pitx1* was first identified for its role in activating pro-opiomelanocortin gene transcription in anterior pituitary corticotrophes (Lamonerie *et al.*, 1996) and has since been reported to play be involved in anterior patterning as well as hindlimb specification (Lanctot *et al.*, 1997; Szeto *et al.*, 1999; Chang *et al.*, 2001). *Pitx2* plays a role in regulating both craniofacial development and left-right asymmetry (Lui *et al.*, 2001) and *Pitx3* plays a prevalent role in lens development (Rieger *et al.*, 2001; KhosrowShahian, unpublished).

ARX

The Q50 *paired-like* homeobox gene *Arx* (*a*ristaless-related homeobox gene) is a recent addition to the group-II-*aristaless*-related genes. It was first isolated in mouse and zebrafish in an attempt to characterize homeobox genes expressed within the vertebrate CNS (Miura *et al.*, 1997). *Arx* shows striking similarity to the *Drosophila aristaless* gene and contains two conserved domains, the homeodomain and the C-peptide domain. In zebrafish *Arx* first expresses at 10 h in the presumptive diencephalon and later restricts to band-like domains that border the telencephalon-diencephalon boundary. *Arx* is also detected in the ventral thalamus and hypothalamus (Miura *et al.*, 1997). Mouse *Arx* is initially detected at E9 in the dorsal telencephalon, anterior diencephalon and in the isthmus. Strong expression ensues in the dorsal telencephalon, particularly in the

ganglionic eminence and the ventral thalamus (Miura *et al.*, 1997). *Arx* is also expressed in the somites and the floor plate in both mice and zebrafish. Based on the expression patterns in these organisms it was predicted that *Arx* may be required for neuronal differentiation in the ganglionic eminence and ventral thalamus and may play a role in differentiation of the dorsal telencephalon. *Arx* deficient mice die shortly after birth and have smaller brains with thin embryonic cortexes caused by decreased proliferation of neuroepithelial cells (Kitamura *et al.*, 2002). Loss of *Arx* also results in defective interneural migration.

The human *Arx* gene (*ARX*) was identified through an in silico search for candidate CNS developmental genes within a defined region of the X chromosome (Bienvenu *et al.*, 2002; Ohira *et al.*, 2002). *ARX* consists of five exons and has been mapped to the distal part of the short arm on the X chromosome, at Xp22.13 (Stromme *et al.*, 2002). Murine *Arx* has been mapped to a syntenic region of the X chromosome indicating that *ARX* and *Arx* are orthologues (Ohira *et al.*, 2002). Northern blot analysis shows that *ARX* expressses higher in human fetal brain than in adult brain (Ohira *et al.*, 2002). Strong expression in adult heart, skeletal muscle, liver and pancreas were reported (Ohira *et al.*, 2002; Bienvenu *et al.*, 2002). *In situ* hybridization analysis of human fetal brain sections revealed that *ARX* is expressed predominantly in the germinal matrix of the ganglionic eminence, and ventricular zone of the telencephalon. Weaker signals were detected in the cortical plate, caudate nucleus, putamen, globus pallidus, substantia nigra, cigulate, and hippocampus (Ohira *et al.*, 2002).

ARX has been identified as the causative locus for Partington syndrome, X-linked West syndrome, and other syndromic and non-specific forms of Mental Retardation

associated with epilepsy and dystonia (Stromme *et al.*, 2002; Bienvenue *et al.*, 2002; Scheffer *et al.*, 2002; Hirose and Mitsudome, 2003). Clinical data from over 50 mentally retarded patients, alone or in conjunction with other abnormalities, revealed a co-segregation of various mutations in the *ARX* gene with affected individuals (reviewed by Stromme *et al.*, 2002). *Arx* mutant patients with the aforementioned diseases were not reported to display brain abnormalities in diagnostic images, however, *ARX* mutations have been found in patients with brain cysts (Stromme *et al.*, 2003) and with X-linked lissencephaly with abnormal genitalia (XLAG) (Kitamura *et al.*, 2002; Uyanik *et al.*, 2003). XLAG is a brain malformation resulting from abnormal neuronal migration and is characterized by the absence of normal convolutions in the cerebral cortex, resulting in a smooth brain, and in some cases an abnormally small head (microcephaly) (Bonneau *et al.*, 2002). Recently, *ARX* mutations were found in patients with hydranencephaly, a rare condition in which the brain's cerebral hemispheres are absent and replaced by sacs filled with cerebrospinal fluid (Kato *et al.*, 2004). *ARX* is also associated with Proud syndrome, which is a form of mental retardation with agenesis of the corpus callosum (Kato *et al.*, 2004). Thus, it appears that the pleiotropic effects of *ARX* are associated with functional disorders with and without cerebral malformations. A variety of distinct mutations in *ARX* have been reported including conservative and nonconservative missense mutations within and outside of the homeodomain, in frame insertions and duplications in GC rich regions resulting in an expansion of polyalanine tracts, and premature termination mutations. The frequency of mutation types between syndromes involving cerebral abnormalities and those in which the brain appeared normal in diagnostic images were compared revealing that premature truncation mutations were more prevalent in

malformation syndromes while polyalanine expansions were more common in patients with normal brain morphology (Kato *et al.*, 2004). Although extensive examination of genotype-phenotype correlations have been conducted, less analyses have been performed to functionally characterize *Arx in vivo*. Since *ARX* appears to play an important role in human cognitive function, studies using model vertebrates are essential to understand the role that this protein plays during neural development.

DESCRIPTION OF THE PROJECT

The main objective of this thesis was to gain further insight into forebrain development and the role that *Arx* may be playing in this process. The model organism *Xenopus laevis* allows us to examine the spatial and temporal expression profiles of *Arx* during early embryogenesis. To characterize the functional role of *xArx*, *in vivo* gain- and loss-of-function studies were conducted by means of microinjecting synthetic RNAs or antisense morpholino oligonucleotides directly into early cleavage stage *Xenopus* embryos. Since human *ARX* mutations are associated with a range of neurological and cerebral malformation disorders, and since *Arx* homologues express predominantly in the developing rostral brain, we decided to focus our attention on the neural aspects resulting from *xArx* misregulation, although several other malformations also arise. Finally, to get a better understanding of the morphological effects induced by *xArx* misexpression, we analyzed markers of specific brain territories for positional identities. These studies also provided insight into the putative targets of endogenous *xArx*.

CHAPTER 2

xArx2 Expresses in an Evolutionarily Conserved Pattern

Chapter Summary

Xenopus laevis xArx2 encodes a conceptual protein of 528 amino acids belonging to the Q50 *paired-like* subclass of homeodomain proteins. xArx2 shows high homology to other reported Arx sequences from mouse, zebrafish, humans, and another *Xenopus* Arx, xArx, at the amino acid level. There is complete identity within the homeodomain, and the C-peptide domain among all Arx sequences.

XArx2 is first detected by RT-PCR as a maternal transcript and continues to express throughout gastrulation. It is substantially up-regulated at early neurula and expression continues to increase to early tadpole stage. The earliest expression of *xArx2* detectable by whole-mount *in situ* hybridization is at blastula stage, where it is faintly expressed on the dorsal side of the embryo. Expression is not detected again until early neurula stages in the anterior neural plate, in the region which gives rise to the prosencephalon. Throughout tailbud stages expression of *xArx2* is restricted to the presumptive forebrain territory and resides in the telencephalon and diencephalon at tadpole stages. *XArx2* is also transcribed in the somites and floor plate. In two particulars the expression domain differs since described family member, *xArx*, does not express either in blastomeres or in somites.

INTRODUCTION

The elaborate morphology of the vertebrate brain arises from an intricate regionalization process, which specifies unique domains within the neural plate, linked to tightly regulated proliferation and differentiation processes of the neural tissue therein. An approach to understanding the complex molecular mechanisms involved in patterning the vertebrate brain involves studying the expression patterns of various transcription factors throughout early embryogenesis. In an effort to identify novel transcription factors in the developing mouse brain, the *aristaless*-related homeobox gene, *Arx*, was isolated and subsequently cloned in zebrafish (Miura *et al.*, 1997). *Arx* expresses in the forebrain and floor plate of the developing central nervous system in each of these vertebrates as well as in the somites. *Arx* also expresses in the presumptive cortex of fetal mice. Subsequent analysis on human tissues demonstrate that *ARX* is also expressed in neuronal precursor cells in the human brain, with stronger levels of expression in fetal brain compared to adult brain (Ohira *et al.*, 2002). This is consistent with the effects of *ARX* mutations on human brain and cognitive anomalies (Stromme *et al.*, 2002; Kato *et al.*, 2004).

We sought to characterize the spatiotemporal expression of *Arx* in *Xenopus*. Throughout the course of our studies another *Xenopus Arx* homologue, *xArx*, was identified and shown to express in the forebrain (El-Hodiri, 2003). We have demonstrated that our *Arx* homologue, *xArx2*, is structurally conserved among vertebrates and also expresses in the developing amphibian forebrain. Except for the detection of *xArx2* in dorsal blastomeres and in the somites, the two *xArx* transcripts display an otherwise identical expression profile.

MATERIALS AND METHODS

Sequence Analysis

ClustalW was used to generate an amino acid sequence alignment of *Xenopus* Arx2 with known homologues from other organisms (human, mouse, and zebrafish) and with the previously published *Xenopus Arx* sequence (El-Hodiri, 2003).

Embryo Preparation

Xenopus eggs were obtained by injecting adult females with 500U of human chorionic gonadotropin (Chorulon; Intervet) into the dorsal lymph sac. Embryos were fertilized, dejellied in 2% cysteine solution (pH 8.0), and cultured as previously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (1967).

RT-PCR

Embryos were reared in 0.1 X MBS at 12°C, 17°C, or room temperature until all of the desired developmental stages had been achieved. Poly $(A)^+$ RNA purifications from ten pooled embryos of each developmental stage were performed in parallel using oligo dT-polystyrene beads (Sigma DMN-10). mRNA equivalent to one embryo was utilized for first strand cDNA synthesis in the presence of RNasin (Promega) using reverse transcriptase according to the manufacturer's instructions (Omniscript, Qiagen). A reaction using RNA obtained from a stage 21 embryo was used in the absence of reverse transcriptase to serve as a control for contamination during subsequent amplification. One fifth of the reactions were employed as templates for amplifications. Primers were designed to specifically amplify *xArx2* rather than the previously published sequence (El-Hodiri, 2003). PCR conditions were determined empirically to establish

the linear range of amplification for *xArx2* and reactions were conducted using a thermo-stable Taq polymerase in 10 mM Tris (pH 9.0), 50mM KCL, 0.1% Triton X-100, 3 mM MgCl2, 0.2 mM dNTPs, 0.1 mM [^{32}P]dCTP, and 1uM of each primer (*xArx2* – 5'-CCGCACTGGACTCTGCT-3' and 5'-ACACTTCTTTGCCGGTGC-3'; *Ef1-α* – 5'-CAGATTGGTGCTGGATATG-3' and 5'-ACTGCCTTGATGACTCCTA-3'). All amplifications were preceded by a 4 minute denaturation step at 94°C, then immediately cycled 29 times at 94°C for 45 seconds, 57°C for 1 minute, and 72°C for 45 seconds. One tenth of each reaction was electrophoresed on 4% polyacrylamide in 0.5 x TBE and visualized by autoradiography.

Whole-Mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to Harland (Harland, 1991). Embryos at various stages were fixed in MEMPFA overnight at 4°C and stored in 100% methanol at -20°C. Digoxygenin (DIG)-UTP (Roche) labeled sense and antisense riboprobes for *xArx2* were generated from full length linearized templates using T3 and T7 RNA polymerase, respectively. An antisense riboprobe for *xArx* was generated similarly using T3 RNA polymerase and linearized template (kindly provided by El-Hodiri). Hybridization was detected using an alkaline phosphatase-coupled anti-DIG antibody (Roche, dilution 1:2000). Alkaline phosphatase staining was developed with NBT/BCIP (Roche). Embryos were placed in a clearing solution (2:1 benzyl benzoate:benzyl alcohol) to view internal structures.

Sections

Embryological sections were prepared by embedding the *in situ* hybridization stained embryos in 5% agarose. Vertical sections were made 30 μm thick with a vibratome (Leica VT1000S).

RESULTS

xArx2 Shows High Sequence Homology to *Arx* in Other Vertebrates

The sequence of x*Arx2* encodes a conceptual open reading frame encoding a protein of 528 amino acids (Fig. 1). It contains a Glutamine at position 50 of its homeodomain. The sequence also encodes an octapeptide domain and a nuclear localization sequence near the N-terminus, and a C-peptide (aristaless) domain at the C-terminus. Alignment of the predicted Arx amino acid sequences among vertebrates revealed a high degree of homology between Arx2 and homologues from human, mouse, and zebrafish (Fig. 1). It was found that *xArx2* and *xArx* (GenBank accession numbers AY519474 and AY130460, respectively) share 90% similarity at both the nucleotide and the amino acid levels. Both *Xenopus* Arx homologues share 67% identity with the mouse and zebrafish proteins (GenBank accession numbers O35085 and O42115, respectively). However, xArx2 was found to be marginally more similar at the amino acid level to human Arx (GenBank accession number NP_6206) than was xArx (68% versus 66%). All vertebrate Arx sequences analyzed were identical in the octapeptide domain, the nuclear localization sequence, the homeodomain, and the C-peptide domain.

Figure 1. Amino acid sequence alignments of Arx homologues. The deduced amino acid sequences from *Xenopus Arx2* (xArx2) and *Xenopus Arx* (xArx)(GenBank accession numbers AY519474 and AY130460, respectively) were compared to the Arx amino acid sequences from human (hARX), mouse (mArx), and zebrafish (zArx) (GenBank accession numbers NP_6206; O35085; and O42115, respectively). Identities are shown as asterisks, while dashes represent gaps introduced to maximize sequence alignments. The octapeptide domain, nuclear localization sequence (NLS), homeodomain, and aristaless domain are identical in all species, and their homeodomains contain a characteristic Glutamine at position 50. There are 47 amino acid differences between xArx2 and xArx, indicated in red.

1

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                                                  Octapeptide
hARX          MSNQYQEEGCSERPECKSKSPTLLSSYCIDSILGRRSPCKMRLLGAAQSLPAPLTSRADP 60
mArx          MSNQYQEEGCSERPECKSKSPTLLSSYCIDSILGRRSPCKMRLLGAAQSLPAPLASRADQ 60
zArx          MSSQYDDD-SRDRSECKSKSPTVLSSYCIDSILGRRSPCKVRQLG-AQSLPA--PVRPDH 56
xArx2         MSSHYQEE-TSERSECKSKSPTLLSSYCIDSILGRRSPCKMRLLG-AQSLPS-LAHRLDP 57
xArx          MSSHYQQE-TSERSECKSKSPTLLSSYCIDSILGRRSPCKMRLLG-ASSLPS-LAHRLDP 57
              **  *       * ******** ***************** * *  * * *     * *
                                  NLS
hARX          EKAVQGSPKSSSAPFEAELHLPPKLRRLYGPGGGRLLQGAAAAAAAAAAAAAAAA-ATATA 119
mArx          EKAMQGSPKSSSAPFEAELHLPPKLRRLYGPGGGRLLQGAAAAAAAAAAAAAAAATATGTA 120
zArx          EMTTEVTSKEN--SFDSDMHLPPKLRRLYGPGG----------------------KYLD 91
xArx2         DKTLEGSPKGN-ATFEADLHLPPKLRRLYGPVG----------------GGRILQSSA 100
xArx          DKTLEGSPKGN-ATFEADLHLPPKLRRLYGPVG----------------GGRILQSSA 100
                      *     *    ************ *

hARX          GPRGEAPPPPPPTARPGERPDGAGAAAAAAAAAAAAWDTLKISQAPQVSISRSKSYRENG 179
mArx          GPRGEVPPPPPPAARPGERQDSAG--AVAAAAAAAAWDTLKISQAPQVSISRSKSYRENG 178
zArx          SGRG---------------FHEHLEKGERERLLDQACESLKISQAPQVSISRSKSYRENA 136
xArx2         DVRSGVPRS--------EKTDRILLLGGT-GENSWDTHSLKISQAPQVSISRSKSYRENS 151
xArx          DNRSGVPRS--------EKTDRILLLGGSAGENSWDTHSLKISQAPQVSISRSKSYRENS 152
                *                                      ********************

hARX          APFVPPPPALDELGGPGGVTHPEERLGVAGGPGSAPAAGGGTGTEDDEEELLEDEEDEDE 239
mArx          APFVPPPPALDELSGPGGVAHPEERLSAASGPGSAPAAGGGTGAEDDEEELLEDEEDEEE 238
zArx          PFS-------------------------------------------------QSDEGQ 145
xArx2         AP------LLREETSPELMQQSQQQTQNSSNNACTSLQDRLGALSQSP---RDEEDDDE 202
xArx          AP------LLREESSPELLQQSQQQT-QSSTTACTSLQDRLGALSQSP---RDEEDDDE 203
                                                                    *

hARX          EEELLEDDEEELLEDDARALLKEPRRCPVAATG---AVAAAAAAAVATEGGELSPKEELL 296
mArx          EEELLEDDDEELLEDDARALLKEPRRCSVATTGTVAAAAAAAAAAVATEGGELSPKEELL 298
zArx          SPEHMAQELVELSTLKFEEDVVKEEACGD---------------------NSLSPKDEES 184
xArx2         TEEEEEEEEESSKQHNSSNPNRGHLSDQQSPSFQSQTQSSPGCGTD-AELSPKEELM 261
xArx          PEEEEEEEEESSKQHNSSNPNRGHLSDQQSPSFQSQ--QTSPGCGTD-AELSPKEELM 260
                *       *                                        **** *
                                                  Homeodomoain
hARX          LHPEDAEGKDGEDSVCLSAGSDSEEGLLKRKQRRYRTTFTSYQLEELERAFQKTHYPDVF 356
mArx          LHPEDAEGKDGEDSVCLSAGSDSEEGLLKRKQRRYRTTFTSYQLEELERAFQKTHYPDVF 358
zArx          LH-NDGDVKDGEDSVCLSAGSDSEEGMLKRKQRRYRTTFTSYQLEELERAFQKTHYPDVF 243
xArx2         LHSSDADGKDGESSVCLSAGSDSEEGMLKRKQRRYRTTFTSYQLEELERAFQKTHYPDVF 321
xArx          LHSSDADGKDGEESSVCLSAGSDSEEGMLKRKQRRYRTTFTSYQLEELERAFQKTHYPDVF 320
              **  *   **** ************ ********************************

hARX          TREELAMRLDLTEARVQVWFQNRRAKWRKREKAGAQTHPPGLPFPGPLSATHPLSPYLDA 416
mArx          TREELAMRLDLTEARVQVWFQNRRAKWRKREKAGAQTHPPGLPFPGPLSATHPLSPYLDA 418
zArx          TREELAMRLDLTEARVQVWFQNRRAKWRKREKAGVQAHPTGLPFPGPLAAAHPLSHYLEG 303
xArx2         TREELAMRLDLTEARVQVWFQNRRAKWRKREKAGAQTHPPGLPFPGPLSAGHPLGPYLDA 381
xArx          TREELAMRLDLTEARVQVWFQNRRAKWRKREKAGAQTHPPGLPFPGPLSAGHPLGPYLDA 380
              ********************************** * *  ******** * ***  **

hARX          SPFPPH-HPALDSAWTAAAAAAAAAFPSLP-PPPGSASLPPSGAPLGLSTFLGAAVFRHP 474
mArx          SPFPPH-HPALDSAWTAAAAAAAAAFPSLP-PPPGSASLPPSGAPLGLSTFLGAAVFRHP 476
zArx          GPFPPHPHPALESAWT-AAAAAAAAFPGLA-PPPNSSALPP-ATPLGLGTFLGTAMFRHP 360
xArx2         SPFPPH-HPALDSAWTAAAAAAAAAFPSLPPPPGSAALPPGGSPLGLSTFLGAAVFRHP 440
xArx          SPFPPH-HPALDSAWTAAAAAAAAAFPSLPPPPGSAALPPGGSPLGLSTFLGAAVFRHP 439
               ***** **** **** ********** *  *** *  ***   **** **** * ****

hARX          AFISPAFGRLFSTMAPLTSASTAAALLRQPTPAVEGAVA-SGALADP----ATAAADRRA 529
mArx          AFISPAFGRLFSTMAPLTSASTAAALLRQPTPAVEGAVA-SGALADP----ATAAADRRA 531
zArx          AFIGPTFGRLFSSMGPLTSASTAAALLRQTAPPVESPVQPSAALPEPPSSSSSTAADRRA 420
xArx2         AFISPAFGRLFSTMGPLTSASTAAALLRQPNPAVESAVQ-SSGLSDP----TAAADRRA 495
xArx          AFISPAFGRLFSTMAPLTSASTAAALLRQPNPAVESAVQ-LSGLSDP----TAAADRRA 494
              *** * ****** * **************  * **  *     *  *       ******
                Aristaless
hARX          SSIAALRLKAKEHAAQLTQLNILPGTSTGKEVC 562
mArx          SSIAALRLKAKEHAAQLTQLNILPGTSTGKEVC 564
zArx          SSIAALRLKAKEHSAQLTQLNILPSGTAGKEVC 453
xArx2         SSIAALRLKAKEHAAQLTQLNIIPGNSTGKEVC 528
xArx          SSIAALRLKAKEHAAQLTQLNIIPGNNTGKEVC 527
              ************* ******** *    *****
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Spatial and Temporal Expression of *xArx2*

The temporal expression profile of *xArx2* during early *Xenopus* development was analyzed by RT-PCR (Fig. 2). *xArx2* was detectable in unfertilized embryos (stage 0) and remained present at moderate levels up until the end of gastrulation. Just following the onset of neurulation, the expression level at stage 14 was markedly increased. The expression of *xArx2* continued to increase throughout neurulation into tailbud stages, at which time regionalization within the neural tube is occurring to produce the specific segments of the mature brain. *Ef1-α* is a constitutively expressed housekeeping gene and was used as a loading and reaction control. It was amplified in relatively equal amounts in all of the developmental stages assayed.

The spatiotemporal expression of *xArx2* in *Xenopus* was analyzed by whole-mount *in situ* hybridization (Fig. 3). The transcript is initially visualized at blastula stage (Fig. 3A) on the prospective dorsal side of the embryo and was not again detectable until stage 14 (Fig. 3B), where it is observed as a pair of bands straddling the anterior neural plate and persists throughout neurulation (Fig 3C). This site later develops into portions of the prospective telencephalon and diencephalon (Eagleson and Harris, 1990). During early tailbud stages (Fig. 3D, E) *xArx2* is detected in the prosencephalon, or presumptive forebrain area, and in the somites. At late tailbud stages (Fig. 3F) *xArx2* is transcribed in the procencephalon and expresses strongly in the telencephalon and diencephalon (Fig. 3G), as well as in the floor plate (Fig. 3H) at early tadpole stages. There was no staining observed with the sense probe at any of the stages.

Figure 2. Temporal expression of *xArx2* analyzed by RT-PCR. *xArx2* is detectable as a maternal transcript and expresses throughout gastrulation. Significant up-regulation is observed at early neurula and expression continues to increase throughout tailbud stages. Elongation factor 1-alpha (Ef1-α) is shown as a control.

2

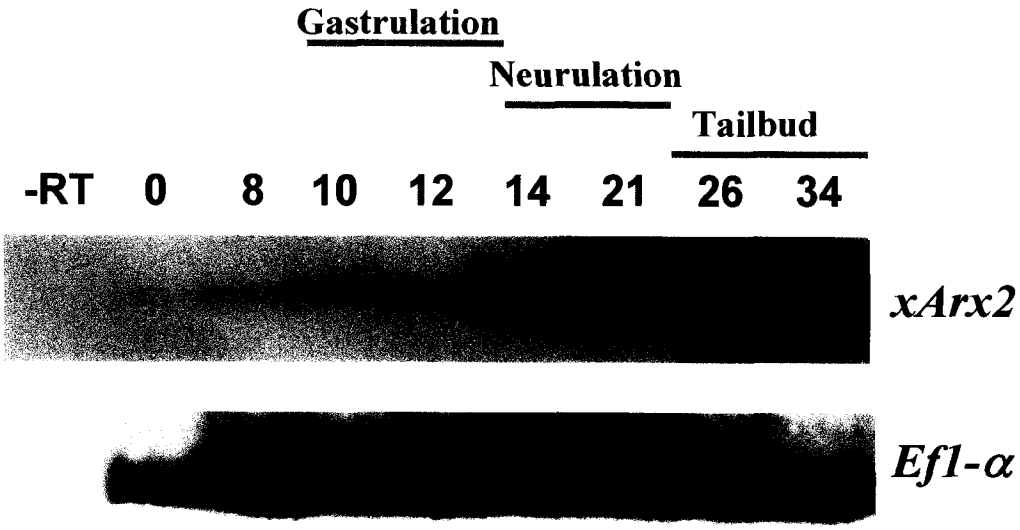
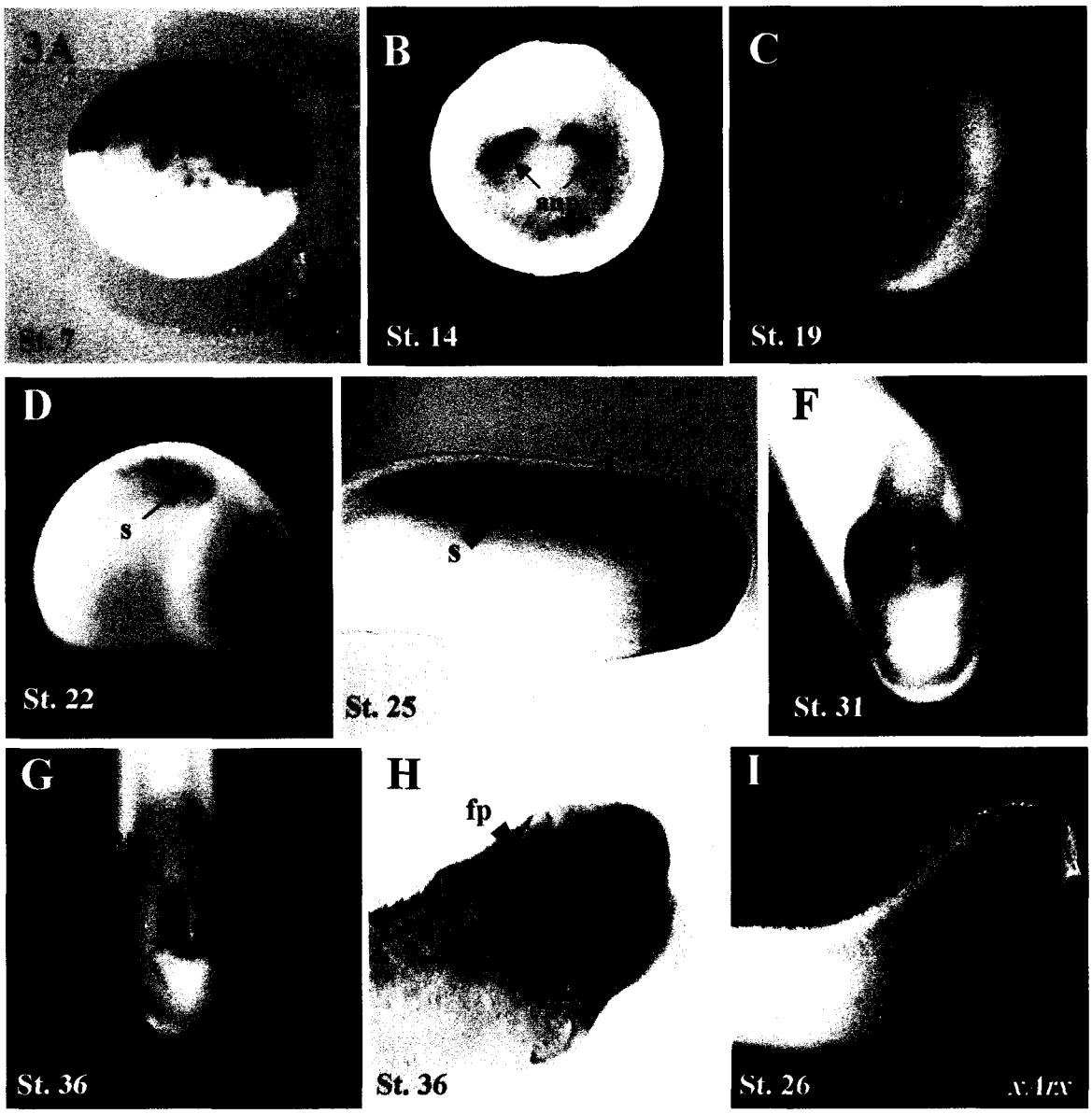
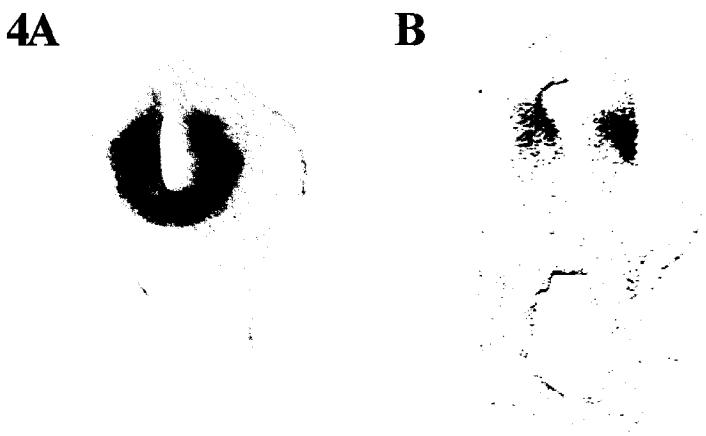


Figure 3. Spatial expression of *xArx2* analyzed by whole-mount *in situ* hybridization. At blastula stage (A) *xArx2* is observed in dorsal blastomeres. Anterior views are shown for neurula stage embryos (B,C); tailbud stage embryos are seen laterally from the right side with a slight anterior perspective (D,E), and from an anterior view (F); (G) anterior view for a stage 36 tadpole; (H) lateral view from the right side of a stage 36 tadpole in clearing solution (2:1 benzyl benzoate:benzyl alcohol); (I) right lateral view of a stage 26 embryo probed for *xArx* expression. Abbreviations: anp, anterior neural plate; pf, presumptive forebrain; s, somites; fp, floor plate, di, diencephalon; tel, telencephalon.

Figure 4. Histological examination of *xArx2* expression. Transverse sections of a stage 36 tadpole, subjected to whole-mount *in situ* hybridization using a *xArx2* antisense riboprobe, were made 30μm thick. *xArx2* expresses in the lateral and ventral telencephalon (A) and in the lateral diencephalon (B).





In order to assess the variations in expression between *xArx2* and *xArx*, *in situ* hybridization was repeated in parallel for both *xArx* riboprobes. Expressions are identical in the anterior region at many developmental stages, however, expression of *xArx* was not observed at blastula stage nor was it detected in the somites (compare Figs. 3I with 3E).

Analysis of sectioned tadpoles subjected to *in situ* hybridization reveals that in anterior sections *xArx2* expresses in the ventral and lateral telencephalon (Fig. 4A). At the level of the eyes, *xArx2* is restricted to the lateral diencephalon (Fig. 4B).

DISCUSSION

xArx2 contains two highly conserved domains, a *paired-like* Q50 homeodomain and an aristaless domain, both of which show complete homology to previously described human, mouse, and zebrafish *Arx* sequences (Stromme *et al.*, 2002; Miura *et al.*, 1997). Recently, another *Xenopus Arx* has been cloned (El-Hodiri *et al.*, 2003) which is observed to contain several differences to the *Xenopus Arx2* characterized in this study. Sequence comparison between the two revealed that they are 10% dissimilar with regard to their amino acid content in regions scattered throughout the protein. It is likely that *xArx2* and *xArx* represent alternate *Arx* family members that have diverged slightly since the ancestral *Xenopus laevis* genome underwent duplication to become pseudotetraploid. At the amino acid level the two *Xenopus* Arx homologues show the same degree of similarity to the zebrafish and mouse homologues, 67% in either case. Interestingly, xArx2 shows a slightly higher degree of similarity to human ARX than does xArx (68 versus 66 percent). This may indicate that the function of xArx2 more closely resembles that of the human homologue.

Complete homology is observed between each species within the octapeptide domain, the nuclear localization domain, the homeodomain, and the C-peptide domain. The fact that these domains remain identical from frogs to humans, suggests that they are essential to proper protein function. More specifically, the highly conserved homeodomain, which is required for DNA binding specificity, suggests that Arx proteins bind to highly conserved regulatory sequences within their target genes.

xArx2 Expression Suggests it May Perform a Role in Forebrain Development

xArx2 was first detectable at low levels as a maternal transcript by RT-PCR. Its expression remained moderate throughout gastrulation. Just proceeding the onset of neurulation, the expression of *xArx2* dramatically increased and continued to do so up until early tadpole stages. Similarly, *xArx2* expression analyzed by *in situ* hybridization revealed an initial period of expression in blastula stage embryos. Although it was detected throughout gastrulation by RT-PCR, no staining was observed by *in situ* hybridization at these stages. Expression did, however, reappear in *in situ* wholemounts at stage 14 where it strongly expressed bilaterally along the mediolaterial anterior neural plate. This coincides with the timing of the dramatic increase in expression observed by RT-PCR as well as the stage in which the neural plate is formed and commences to establish the neural tube (Nieuwkoop and Faber, 1967). Expression increased in the anterior neural plate and its derivatives throughout neurulation and tailbud stages and occupied the floor plate and the procencephalon, specifically the ventral and lateral telencephalon and lateral diencephalon, during early tadpole stages. These data are consistent with RT-PCR data. The lack of detection during gastrulation by *in situ* hybridization may reflect the differential sensitivity of these two methods of analysis.

Our findings augment those of El-Hodiri *et al.* (2003) as the anterior expression domains of both *xArx* transcripts were identical in several stages analyzed; however, we observed that *xArx2* is also strongly expressed in the somites during tailbud stages. Somites give rise to cells that form vertebrae and other tissues including skeletal muscle and the dermal layers of skin. Interestingly, *ARX* has been reported to express strongly in human skeletal muscle (Ohira *et al.*, 2002). This may indicate that *xArx2* performs a critical function during somitogenesis, such as maintaining segmentation of somites, however, disorders of this nature have not yet been reported to associate with mutations in *ARX.*

Consistent with the expression patterns we observed, it has been shown that *Arx* expresses in similar structures and at similar times throughout development in other vertebrates. In mouse *Arx* first expresses at E9 in the dorsal telencephalon, anterior diencephalon, the isthmus, and the floor plate. Expression remains persistent in the dorsal telencephalon (presumptive cerebral cortex), ganglionic eminence and ventral thalamus. Expression in the somites was also detected (Miura *et al.*, 1997). Zebrafish *Arx* was initially detected at 10 h in the region of the presumptive diencephalon, and is temporarily expressed in the caudal telencephalon at 12 h. By 40 h the expression of *zArx* is restricted to telencephalic and diencephalic bands, along the telencephalon/diencephalon boundary, and the hypothalamus. Zebrafish *Arx* expression in the floor plate and the somites was also observed (Miura *et al.*, 1997). Human *ARX* has been reported to express in neuronal precursors in the germinal matrix of the ganglionic eminence and in the ventricular zone of the telencephalon in fetal tissue (Ohira *et al.*, 2002).

Since *xArx2* expresses in the anterior neural plate during neurulation, and its expression remains confined in derivatives of this territory, it is likely that it plays a crucial and evolutionarily conserved role in the establishment of the forebrain in *Xenopus*. Moreover, the timing of its expression, detectable by both RT-PCR and *in situ* hybridization, suggests that *xArx2* may possibly be playing this role very early in development, indeed well before neural territories have been specified.

The restricted pattern of expression within the presumptive forebrain and prosencephalon of *Xenopus* has been reported for a variety of other forebrain transcription factors, such as *Otx*, *Dlx, Pax*, and *BF-1* genes (Simeone *et al.*, 1994; Price *et al.*, 1991; Chalepakis *et al.*, 1993; Xuan *et al.*, 1995). These genes have been shown to contain specific expression boundaries within the telencephalic neuroepithelium and are believed to establish positional identity leading to patterning of the CNS. From the expression profile we observed for *xArx2* we surmise that it plays an integral role in the development of the *Xenopus* forebrain possibly as early as in the establishment of the neural plate. Additionally, its restricted expression presents *xArx2* as a useful marker for forebrain development in *Xenopus*.

CHAPTER 3

xArx2 Plays a Role in Forebrain Development During *Xenopus* Embryogenesis

Chapter Summary

Here we report the effects caused by gain- and loss-of-function of the *Xenopus Arx* homologue, *xArx2*. Overexpression studies reveal that ectopic *xArx2* expression leads to several anterior defects including enlarged forebrain, reduced eye, microcephaly, and extension of the pigmented retina. Translational knockdown of xArx2, using antisense morpholino (MO) oligonucleotides targeted to complement the 5'UTR of *xArx2*, results in embryos with a reduced forebrain territory, as well as microcephaly and retarded craniofacial development. The specificity of the *xArx2*-MO was confirmed by *in vitro* translation experiments, which showed that translation from the related *xArx* locus was likely unaffected by the MO used in our studies. We also report here the effects of *xArx2* misexpression on various brain and eye marker genes. With the exception of its effects on the forebrain markers *XBF-1* and *xArx*, ectopic *xArx2* expression results in a reduced expression level in all other markers analyzed. Inhibiting *xArx2* causes a similar reduction in all markers with the exception of *xOtx2* and *xRx1*, which increases in xArx2-MO-injected embryos.

INTRODUCTION

Inductive interactions are fundamental to the formation of all brain structures and several recent studies provide evidence for the involvement of signaling molecules of the *Bmp* and *Wnt* gene families in patterning the dorsal forebrain (Furuta et al., 1997; Golden

et al., 1999; Lee et al., 2000). Signaling molecules play a paramount role in the establishment and maintenance of cell identities within the CNS, amongst which numerous transcription factors also play a pivotal role (Gomez-Skarmeta, 1999; Theil *et al.*, 1999; Lake and Kao, 2003; Bach *et al.*, 2003). Transcription factors can regulate aspects of cell migration, proliferation, and differentiation (Caric *et al.*, 1997; Hardcastle, Z., and Papalopulu, 2000; Talamillo *et al.*, 2002). For example, loss of function of the homeobox transcription factor *Pax6* in mice affects both radial and tangential migration of neuronal precursors (Talamillo *et al.*, 2002).

Although the *ARX* sequence has been extensively examined in analysis of mutant pedigrees (Bienvenue *et al.*, 2002; Scheffer *et al.*, 2002; Stromme *et al.*, 2002; Kato *et al.*, 2004), functional studies still remain limited. Since vertebrate *Arx* genes are similar in sequence and expression, analysis of loss-of-function mutants provides useful information about the potential roles that this protein plays during early development. *Arx* knockout mice have been shown to manifest defects in neuroblast proliferation as well as selective abnormalities in gamma-aminobutyric acid-ergic interneuron migration (Kitamura *et al.*, 2002). Mutant mice had smaller brains and died shortly after birth. *Xenopus laevis* has been exploited extensively as an organism for the study of embryonic development, and offers several advantages. Gain and loss-of-function studies can be conducted through microinjection of synthetic capped mRNA or antisense morpholino oligonucleotides, respectively, directly into the embryos.

It has been shown that *Arx* expresses in the developing rostral forebrain in *Xenopus* (El-Hodiri *et al.*, 2003; Wolanski *et al.*, unpublished), however, to date no functional studies have been conducted with *Arx* in this organism. In order to understand

the putative role of *Arx* in embryonic brain development, it is of particular relevance to define its functional contribution to the different tissues where it is expressed. We sought to characterize the effects of misexpression of *Arx2* in *Xenopus* through the injection of synthetic capped *xArx2* mRNA or *xArx2*-antisense morpholino oligonucleotides (*xArx2*-MO) into embryos at the two-cell stage. We show that ectopic *Arx2* expression results in an expansion of the forebrain region and that loss-of-function results in a reduction of this domain. Several other anterior phenotypes resulted from *xArx2* misexpression, such as craniofacial under development, and brain and eye abnormalities. We further show that loss of function phenotypes are attributed to the specific translational knockdown of *xArx2*, as the morpholino oligonucleotides used had no effect on *xArx* translation *in vitro*. Finally, we used a panel of seven genes (*XBF-1, xGbx2a, xKrox20, xArx, xOtx2, xRx1, xPax2,* and, *xPax6*), representative of a broad range of markers for positional identity in the developing brain and eye fields, to assess the role of *xArx2*. Examination of marker gene transcription following the ectopic expression of *xArx2* reveals that forebrain markers are up-regulated while markers of more caudal regions of the brain are posteriorized and reduced. Specifically, ectopic *xArx2* expression expanded *XBF-1* domains and elevated the levels of *xArx* in the forebrain, while the mid- and hindbrain markers *xGbx2a*, and *xKrox20*, respectively, were expressed in more posterior territories than normal and their expression levels were reduced or diminished. Furthermore, *xOtx2*, *xRx1*, and *xPax2*, whose functions involve regulating events in both brain and eye development, were reduced in embryos ectopically expressing *xArx2*. Conversely, morpholino studies showed that reduced xArx2 levels coincided with a reduction in *XBF-*

1 and *xArx* and an increase in *xOtx2* and *xRx1* expression levels, however, the levels of *xGbx2a, xKrox20,* and *xPax2* were reduced.

MATERIALS AND METHODS

Embryo Preparation

Xenopus eggs were obtained by injecting adult females with 500U of human chorionic gonadotropin (Chorulon; Intervet) into the dorsal lymph sac. Embryos were fertilized, dejellied in 2% cysteine solution (pH 8.0), and cultured as previously described (Drysdale and Elinson, 1991) in 0.1X Modified Bart's Saline (MBS). Once embryos had reached the two-cell stage they were transferred to 1.5% Ficoll-400 (Sigma) in 0.3 x MBS for injections.

Microinjection

An *xArx2* expression construct was derived using Vent polymerase (New England Biolabs) and primers (forward 5'-GAAGGCCTGCAGCCCAGCATTGA-3'; reverse 5'-GCTCTAGACTGCATAAAAGTTACACTC-3') which bracketed the open reading frame and possessed restriction sites for *StuI* and *XbaI*, respectively, to facilitate insertion into pCS2-. Synthetic capped mRNA of *xArx2* and *Green Fluorescent Protein* (*GFP*) was made from linearized template using mMessage Machine (Ambion) driven by SP6 and T3 promoters, respectively. Capped mRNAs were resuspended in nuclease free water and coinjected into the animal pole of embryos at the 2-cell stage with a Drummond nanoinjector. Concentrations of the *xArx2* capped mRNA ranged from 100 pg to 800 pg. 400 pg of *GFP* capped mRNA was used for coinjections and 800pg for control injections. Injection volumes never exceeded 4.6 nl. Injected embryos were

cultured in 1.5% Ficoll-400 in 0.3 x MBS at 12°C overnight. The solution was subsequently changed to 0.1 x MBS and embryos were reared at 17°C until they reached early tailbud stage. At this stage embryos were separated on the basis of side of injection, which was determined by *GFP* fluorescence under UV light, and grown up at room temperature until they reached stage 46, at which point phenotypic analysis was performed. The uninjected side served as a contralateral control.

Loss-of-function assays were conducted similarly using fluoresceinated *xArx2* antisense morpholino oligonucleotides (Gene Tools) designed according to parameters recommended by the company and selected to specifically target the 5'UTR of *xArx2* (5'-TGCTGGGCTGCAGGACTGTGTCGGT-3'). Concentrations of 6, 9, and 18 ng were injected into one balstomere at the two-cell stage. Fluoresceinated control morpholino oligonucleotides which represented a random sequence (5'-CCTCCTACCTCATTACAATTTATA-3') were injected at a concentration of 20 ng.

Histology

Embryological sections, stained with Hoechst or hematoxylin and eosin (H&E) were prepared in order to better visualize the overall brain morphology involved with the gain-of and loss-of function of *xArx2*. Stage 46 tadpoles, that had been injected with 400 pg of *xArx2* mRNA or 18 ng of *xArx2*-MO, and which showed slight forebrain defects, were fixed in MEMPFA for two hours at room temperature. For Hoechst staining, fixed tadpoles were subsequently dehydrated to 100% methanol gradually, removed to Hoechst 33258 (5 μg/ml) for one hour, gradually rehydrated to water, then embedded in 5% agarose. Tadpoles were then sectioned vertically, 30 μm thick on a vibratome (Leica VT 1000S) and visualized under filtered UV light.

For H&E staining, injected tadpoles were sectioned prior to staining. Fixed tadpoles were dehydrated through an ethanol-xylene series, embedded in paraplast, and 20 μm horizontal sections were cut using a microtome (Spencer 820). Sections were deparaffinized, stained with H&E, and visualized by light microscopy.

***In vitro* protein synthesis**

To test the specificity of the *xArx2*-MO used in these studies, an *in vitro* translation approach was utilized. A construct containing a *xArx2* morpholino-equivalent site was created into the pCS2-Myc vector. The oligonucleotides (5'-GATCCACCGAGACAGTCCTGCAGCCCAGCA-3' and 5'-CGTGCTGGGCTGCAGGACTGTGTCGGAG-3'), which contained restriction sites for *BamHI* and *ClaI*, respectively , and which complemented the *xArx2* morpholino oligonucleotide used in our loss-of-function studies, were used to create the site. One microgram of each oligonucleotide was first annealed, by gradually heating them to 90°C then allowing them to cool slowly back to 37°C. Phosphate groups were added to the ends of the annealed oligonucleotides using T4 polynucleotide kinase (Promega) and they were then ligated into the *BamHI* and *ClaI* sites of pCS2-myc. A second construct, containing a *xArx* morpholino-equivalent site was created in the *BamHI* and *ClaI* sites of pCS2-myc in a similar manner using the oligonucleotides (5'-GATCTTGAGACAGTCCGGAGCTCAGCATTG-3' and 5'-CGCAATGCTGAGCTCCGGACTGTCTCAA-3'). Subsequently, *xArx2* was subcloned into these constructs. It was amplified using Vent polymerase (New England Biolabs), and the primers (forward 5'-GAAGGCCTATGAGCGGCCACTACCAA-3' and reverse 5'-GCTCTAGACTGCATAAAAGTTACACTC-3'), which contained restriction sites for

StuI and *XbaI*, respectively, to facilitate directional insertion into each of the vectors, as well as into the unmodified pCS2-myc vector. Synthetic capped RNA was made from linear templates using mMessage Machine (Ambion) driven by a SP6 promoter for the following *xArx2*-containing constructs: pCS2-myc, pCS2-myc with the *xArx2*-MO-equivalent site, and pCS2-myc with the *xArx*-MO-equivalent site. *In vitro* protein translations, using ^{35}S, were performed according to the manufacturer's protocol (Retic Lysate, Ambion), in the presence and absence of 18ng of *xArx2*-MO using 1 μg of each mRNA template. Additionally, 20 ng of control morpholino was added to one of the reactions, and 1 ug of *GFP* mRNA was utilized as an internal control in each reaction in order to equate levels of protein syntheses. Protein products were run out on a 10% polyacrylamide gel, and visualized by autoradiography.

Whole-mount *in situ* hybridization

To examine the putative effects of *xArx2* misexpression on various brain and eye marker genes, embryos, injected with either synthetic capped *xArx2* (600 pg) and *GFP* (400 pg) mRNA or *xArx2* morpholino oligonucleotides (18 ng), were subject to whole mount *in situ* hybridization, performed according to Harland (Harland, 1991). Digoxygenin (DIG)-UTP (Roche) antisense riboprobes were generated from linearized templates for each of the marker genes. Hybridization was detected using an alkaline phosphatase-coupled anti-DIG antibody (Roche, dilution 1:2000). Alkaline phosphatase staining was developed with NBT/BCIP (Roche) or BM Purple (Roche). The side of injection was predetermined prior to fixation, on the basis of fluorescence of the injected side under UV light, and the uninjected side was used as a contralateral control. All of the constructs used, with the exception of *xGbx2a*, were obtained as gifts: *XBF-1* (N.

Papalopulu), *xKrox20* (D. Wilkinson), *xArx* (H. El-Hodiri), *xOtx2* (I. Blitz), *xRx1* (G. Barsacchi), *xPax2* (N. Heller), and *xPax6* (W. Harris). *XGbx2a* was amplified from a whole embryo cDNA library using primers (forward 5'-CGGAATTCAGGCTTCATTTGACTCTCAG-3' and reverse 5'-AAGGCCTGAACATTTCAAGGTCTTGC-3') which contained *StuI* and *XbaI* restriction sites, respectively, to facilitate directional insertion into pCS2-.

RESULTS

Misexpression of *xArx2* Results in Anterior Defects

In order to gain insight into the endogenous function of *xArx2* we performed gain- and loss-of-function studies by microinjecting either synthetic capped *xArx2* plus *GFP* mRNA or antisense *xArx2*-Morpholino oligonucleotides (MO) into one blastomere at the two-cell stage. Since embryos were injected at the two-cell stage, the contralateral side served as a control. Misexpression of *xArx2* resulted in several distinct and reproducible anterior abnormalities, specifically in the forebrain and developing eye (Fig. 1).

The majority of morphological effects of *xArx2* overexpression appeared to be dose-dependent until the RNA injected reached 400 pg (summarized in Table 1). Beyond this dose (at 600 and 800 pg) survival rate to swimming tadpole stage dramatically declined, and head structures were severely impaired and barely recognizable. Anterior expansion of the telencephalic region was commonly observed (Fig. 1A). Another frequent phenotype observed in injected embryos was microcephaly, characterized by a reduced head circumference (Fig. 1B) and an underdeveloped or mispositioned forebrain (Fig. 1C arrowhead). Other forebrain abnormalities, collectively referred to as "fused

brain", occured in which the anterior most region of the forebrain appeared completely fused or where the telencephalon failed to form two distinct lobes, giving a flattened overall appearance at the rostral end. These phenotypes were often associated with a reduction in the brain, head and/or a complete fusion of the two eye fields resulting in cyclopia. Furthermore, a low percent (1%) of tadpoles had brain outgrowths at high doses, characterized by ectopic tissue which extended from the forebrain (Fig. 1I arrowhead). Embryos injected with GFP displayed no anterior abnormalities.

Several classes of phenotypes associated with the developing eyes also resulted from ectopic *xArx2* expression. Tadpoles were observed with reduced (Fig. 1D) and absent (Fig. 1E, arrowhead) eye structures. Additionally, extension of retinal pigmented epithelium (RPE) towards the midline (Fig. 1F) and pigmentation along the optic nerve (Fig. 1G) occurred. The severity of the effects increased, and phenotypes often compounded at doses of 400 pg and higher. The most severe phenotypes were gross perturbations of the anterior head and brain with more medially located, or oftentimes ventrally fused, retinas (Fig. 1H).

Inhibition of xArx2 Translation Affects the Developing Forebrain

We analyzed the effect of loss-of-function of *xArx2* by means of antisense morpholino oligonucleotide-mediated translational knockdown (summarized in Table 2). This commonly resulted in a reduction of the telencephalon, both mediolatarally and caudally (Fig. 1J), as well as asymmetrical, or underdeveloped head structures on the injected side of the embryo (Fig. 1K). Microcephaly was also observed (Fig. 1L). Embryos injected with the control morpholino oligonucleotides displayed a slight reduction (2% of embryos) in the eye, but otherwise developed normally.

Table 1. Percentage of phenotypes observed in embryos injected with *xArx2* or *GFP* capped mRNA .

	Dose of *xArx2* mRNA injected					Control
Phenotypes	80 pg	200 pg	400 pg	600 pg	800 pg	GFP (800pg)
Normal	76%	70%	67%	54%	56%	100%
Enlarged forebrain	5%	8%	10.5%	27%	22.5%	0%
Microcephaly	8%	11%	12%	5%	7.5%	0%
Reduced eye	1.5%	5%	8%	7%	4%	0%
Absent eye	0%	1%	1%	1%	2%	0%
Pigmented optic nerve	5%	4%	2%	0%	0%	0%
RPE	7%	3%	5%	3.5%	6%	0%
Fused eyes	0%	0%	1%	3.5%	0%	0%
Fused brain	4%	7%	6%	8.5%	2%	0%
TOTAL NUMBER	115	119	95	82	53	189

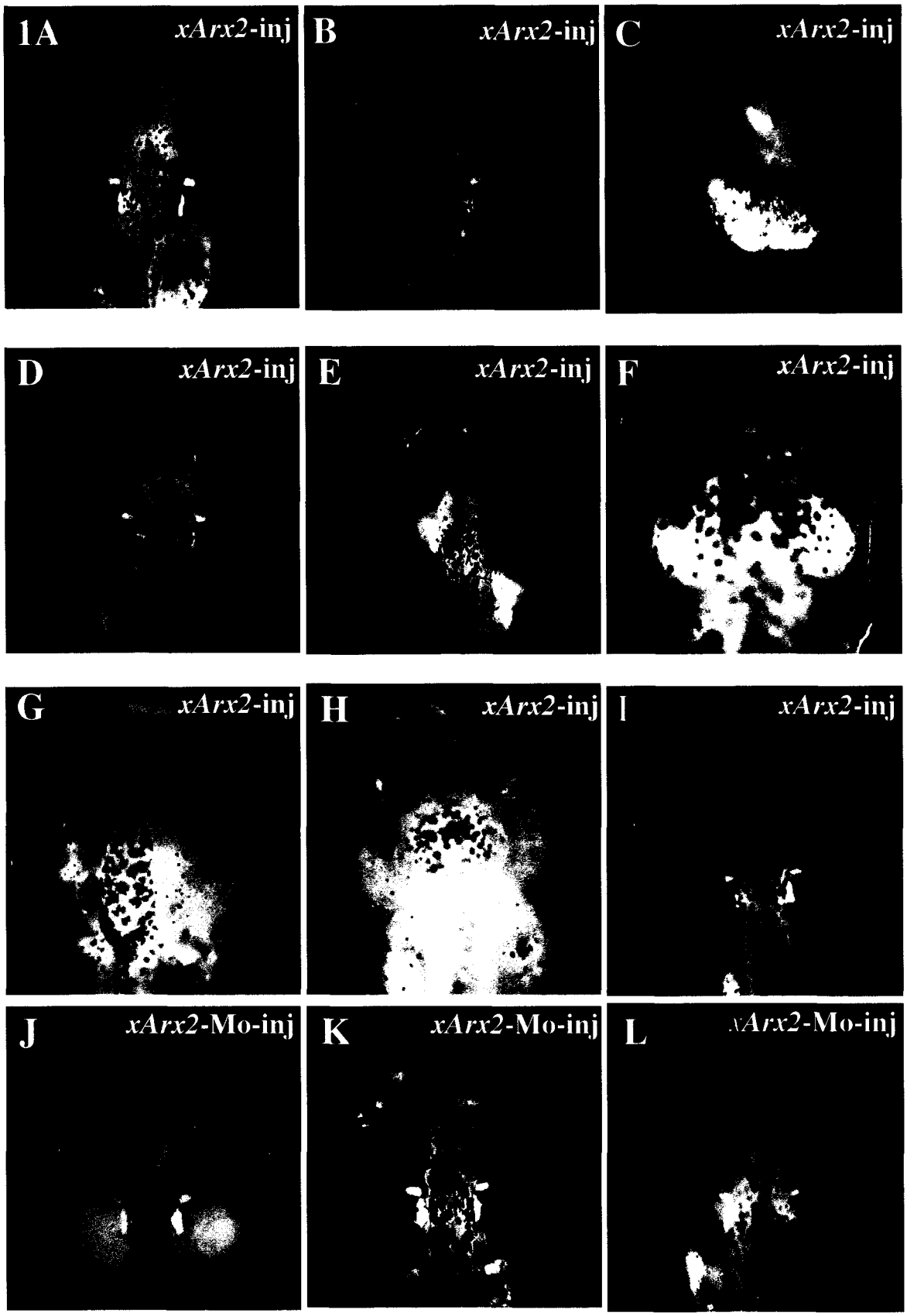
Note: These data summarize the results of ectopic *xArx2* expression in stage 46 tadpoles shown in Figure 1. Various doses of synthetic *xArx2* mRNA was co-injected with *GFP* mRNA (400 pg) into one blasomere at the 2-cell stage. The percentage of tadpoles arising with the indicated phenotypes, described in the text, and the total number of embryos assayed for each dose is indicated. RPE refers to extensions of pigmented retinal epithelium. As deformities sometimes compound, column percentages will not sum to 100.

Table 2. Percentage of phenotypes observed in embryos injected with *xArx2*-morpholino oligonucleotides (MO) or control morpholino oligonucleotides (CMO).

	Dose of *xArx2*-MO injected			
Phenotypes	6 ng	9 ng	18 ng	CMO(20 ng)
Normal	79%	73%	50%	98%
Reduced forebrain	6%	8%	16%	0%
Microcephaly	3%	3%	8%	0%
Underdeveloped head	7.5%	5%	8%	0%
Reduced eye	4.5%	6%	16%	2%
Absent eye	0%	0%	0%	0%
RPE	1.5%	1.5%	3%	0%
Fused eyes	0%	0%	1.5%	0%
Fused brain	6%	9%	12%	0%
TOTAL NUMBER	66	64	72	84

Note: These data summarize the results of inhibition of Arx2 expression in stage 46 tadpoles shown in Figure 1. Various doses of fluorescein-tagged *xArx2*-morpholino antisense oligonucleotides (*xArx2*-MO) were injected into one blasomere at the 2-cell stage. Control morpholino oligonucleotides (CMO-20 ng) were injected similarly and served as an injection control. The percentage of tadpoles arising with the indicated phenotypes, described in the text, and the total number of embryos assayed for each dose is indicated. RPE refers to extensions of pigmented retinal epithelium. As deformities sometimes compound, column percentages will not sum to 100.

Figure 1. Misexpressing *xArx2* causes anterior abnormalities. Dorsal view of stage 46 tadpoles injected with *xArx2* (A-I) or *xArx2* antisense morpholino oligonucleotides (J-L) in one blastomere at the two-cell stage. Over-expression of *xArx2* yielded several brain and eye abnormalities: expansions of the forebrain (A arrowhead), microcephaly (B), mispositioned forebrains (C arrohead), reduced (D arrowhead) and absent (E arrowhead) eyes, extension of pigmented retinal epithelium towards the midline (F), pigmentation along the optic nerve (G), centrally located eyes that fuse laterally (H), and brain outgrowths (I). Inhibition of *xArx2* results in a reduction of the forebrain (J arrowhead) and interferes with proper head development (K, L).



Histological Examination of Tadpoles Reveals Forebrain Size Abnormalities

In order to better examine the forebrain region in tadpoles misexpressing *xArx2*, horizontal sections were made on selected embryos following Hoechst or preceding Hematoxylin and eosin (H&E) staining. At this level, a striking expansion of the forebrain territory on the side of the embryo injected with *xArx2*, as compared to the control, was observed (Fig. 2A, C). Conversely, the forebrain on the injected side of the embryo appeared substantially reduced in *Arx2*-MO-injected embryos (Fig. 2B, D).

xArx2*-Morpholino Specifically Blocks Translation *in vitro

To confirm the specificity of the morpholino oligonucleotides used in our studies, we assayed levels of *in vitro* translated ^{35}S-labelled products using constructs which contained the open reading frame of *xArx2* fused to morpholino-equivalent sites as templates (Fig. 3). Protein translation was inhibited in a construct which contained the *Arx2*-MO-equivalent site when *Arx2*-MO was introduced. Moreover, translational levels were unaffected by *Arx2*-MO in a construct which contained the other *Arx*-MO-equivalent site. The standard control morpholino oligonucleotides had no effect on *xArx2* translation and neither morpholino impaired translational levels of the GFP protein.

Interfering With Proper *xArx2* Function Results in Laterality Defects

Sonic hedgehog (*Shh*) has been shown to play a prominent role in establishing left-right body asymmetry in vertebrates (Levin *et al.*, 1997; Meyers and Martin, 1999; Schilling *et al.*, 1999). Misexpressing *xArx2* induced anterior deformities, such as cyclopia, reminiscent of those observed with a disruption of *Shh* signalling (reviewed by Roessler and Muenke, 2001). This directed us to investigate its putative role in features of left-right asymmetry. We examined the developed heart and gut in stage 46 tadpoles.

Figure 2. Histological examination reveals that *xArx2* affects forebrain size. Horizontal brain sections of stage 46 tadpoles injected with *xArx2* RNA or *xArx2*-Morpholino oligonucleotides in one blastomere at the two-cell stage. (A,B) Tadpoles were stained with Hoechst, embedded in agarose, and sectioned 30 μm thick. (C,D) Tadpoles were paraffin embedded, sectioned 20 μm thick, and stained with hematoxylin and eosin. Forebrain expansion (A,C) and reduction (B,D) is observed (arrowheads) with over-expression and inhibition, respectively.

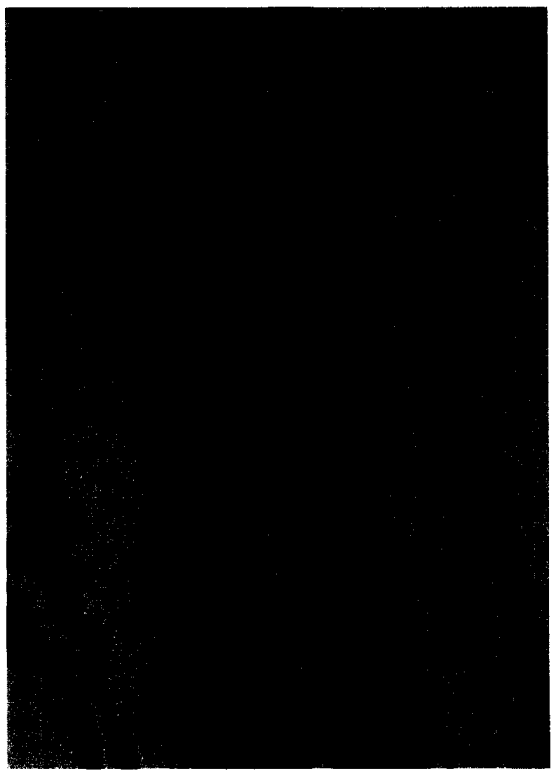
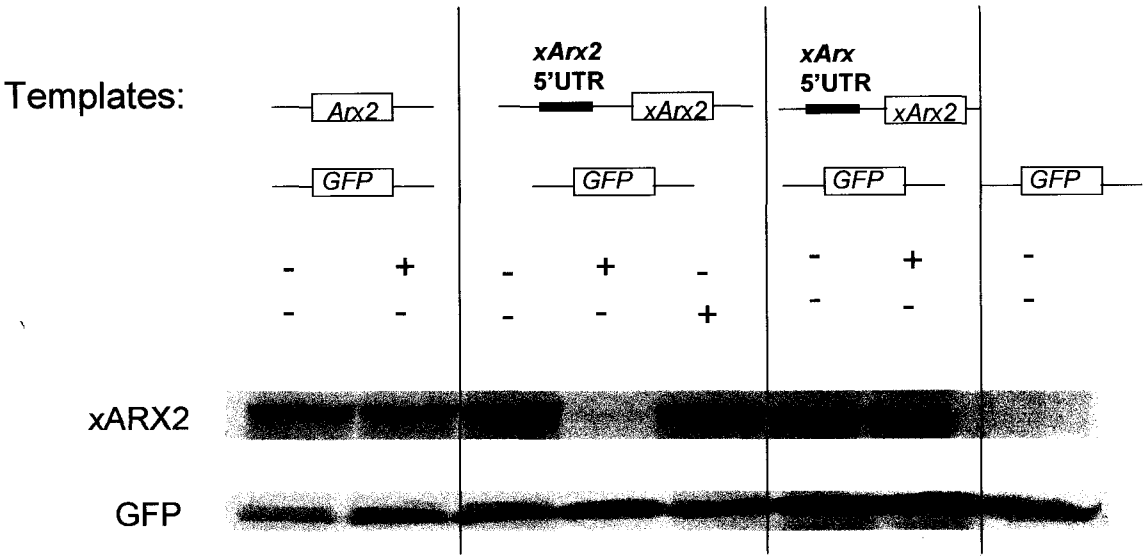


Figure 3. Antisense morpholino oligonucleotides inhibit xArx2 translation *in vitro.* The specificity of *xArx2* antisense morpholino oligonucleotides (*xArx2*-MO) was examined *in vitro* (Retic Lysate, Ambion) employing 1 ug of synthetic capped mRNA (Ambion) created using linearized templates (constructs indicated above) with and without morpholino-equivalent sites contained within a portion of the *xArx2* 5'UTR or *xArx* 5'UTR. Radiolabelled products were electrophoresed on SDS-PAGE gels and visualized using autoradiography. Translation is inhibited by *xArx2*-MO (18ng) in the presence of the *xArx2* 5'UTR but not the *xArx* 5'UTR nor in the absence of either 5'UTR. 20 ng of control morpholino oligonucleotides (CMO) did not affect translation. GFP was cotranslated with the *xArx2*-containing constructs to serve as an internal reaction control.

3



These structures both undergo asymmetric looping during normal development. In the normal frog heart the ventricle is situated on the left side, with the outflow tract looping to the right, and the gut coils counterclockwise. We observed a low (1-6%) frequency of reversed looping in both of these organs among *xArx2*-injected and *xArx2*-MO-injected embryos, but not in *GFP*-injected control embryos. Heart and gut reversals could arise in tandem or separately. Since laterality was not the focus of this project, further studies of gut and heart development were not pursued.

xArx2 Misexpression Alters the Expression of Genes Expressed in the Brain and Eye

In order to gain a better appreciation of the effects of misexpression of *xArx2* on the developing *Xenopus* brain, we decided to examine the effects on marker genes of the brain and eye territories. We employed whole mount *in situ* hybridization, using eight different markers as probes, on embryos injected into one blastomere with either *xArx2* capped mRNA (400 pg) or *xArx2*-MO (18 ng) at the two-cell stage. We examined the effects on both early, early to mid-neurula stage, and late, late tailbud stage, embryos. The uninjected side of the embryo was used as a contralateral control to the effects of microinjection and represented the endogenous expression of each marker in a normal embryo. Ectopic and inhibited *xArx2* expression resulted in several differences in expression of the various marker genes.

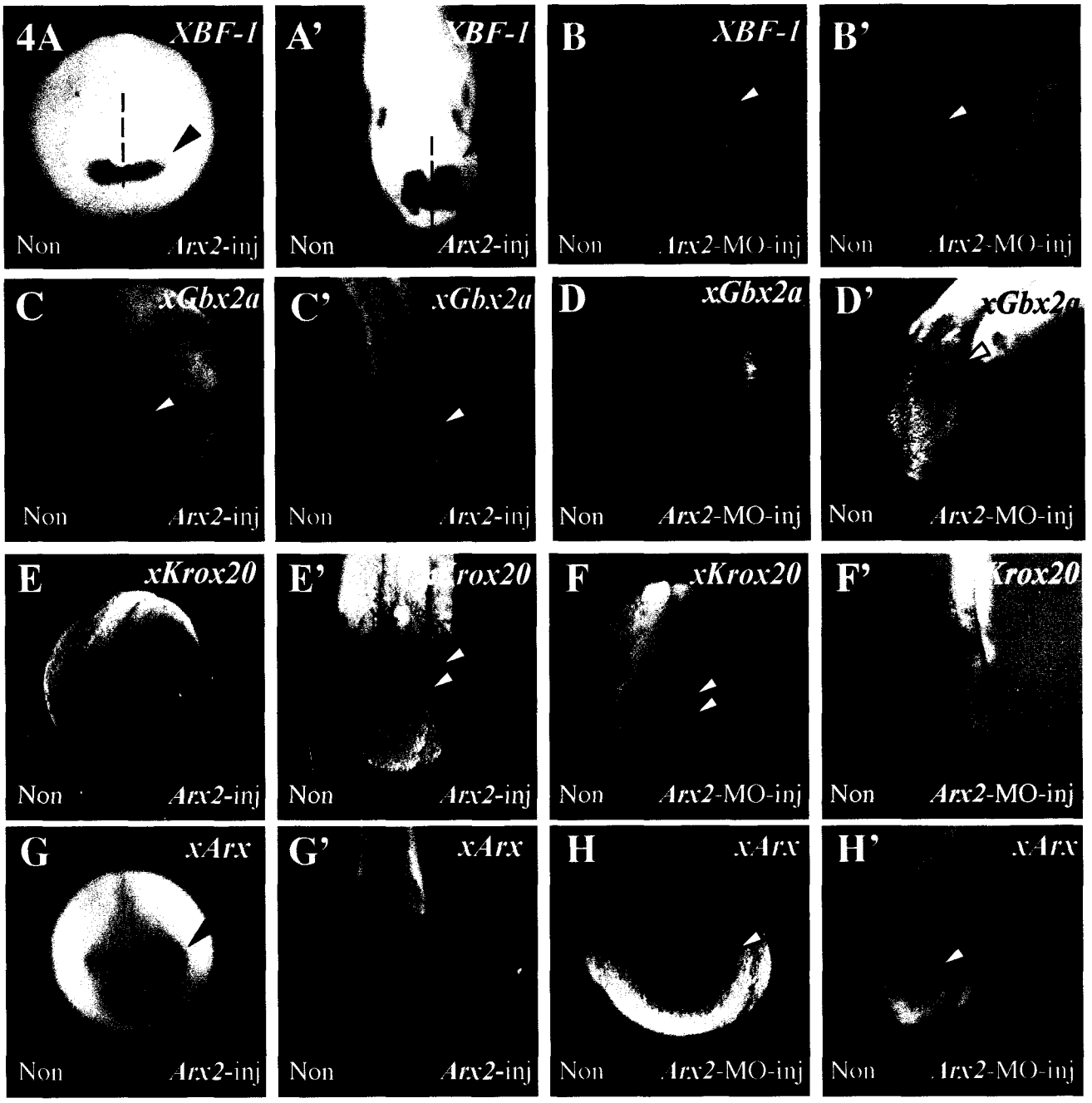
We first examined the overall brain morphology using markers of the fore-, mid-, and hindbrain (Fig. 4). *XBF-1* marks the telencephalic territory of *Xenopus* embryos (Papalopulu and Kintner, 1996). As a result of ectopic *xArx2* expression, the *XBF-1* domain of both early and late embryos (Fig. 4A, A', respectively) slightly expanded laterally. Although not much change was observed in early embryos injected with *xArx2*-

MO (Fig. 4B), a reduction was apparent in the late tailbud stage (Fig. 4B'). *XGbx2a* has an anterior expression border in the region of the midbrain-hindbrain boundary (von Bubnoff *et al.*, 1996). Ectopic *xArx2* reduced the early expression of *xGbx2a* (Fig. 4C) and posteriorized its later expression (Fig. 4C'). This posteriorization of the *xGbx2a* expression domain was also observed in *xArx2*-MO-injected embryos (Fig. 4D, D'), and late embryos also showed a reduction in the expression level of *xGbx2a.* Analysis of the hindbrain marker *xKrox20* (Bradley *et al.*, 1993; Seitanidou *et al.*, 1997), revealed that ectopic *xArx2* expression posteriorized its expression in early-stage embryos (Fig. 4E), while in late-stage embryos, the *xKrox20* expression domains in the hindbrain were significantly reduced (Fig. 4E'). This reduction in expression was also observed in early embryos following translational inhibition of *xArx2* (Fig. 4F), but expression levels appeared to normalize by tailbud stages (Fig. 4F').

We were also interested in the effects that misregulation of *xArx2* had on the homologous transcript *xArx* (El-Hodiri *et al.*, 2003), as it represents an alternate forebrain marker, and analysis could provide insight into the different roles of the two genes. We noticed that ectopic *xArx2* up-regulated the level of *xArx* expression both early and late (Fig. 4G, G') and conversely, its inhibition down-regulated *xArx* expression early (Fig. 4H) and late (Fig. 4H').

Since we also observed that the misexpression of *xArx2* had an effect on eye formation, we examined its effects on genes that play a role in patterning of both the eye and the brain (Fig. 5). Analysis of *xOtx2*, a head specific gene (Pannese *et al.*, 1995), showed that ectopic *xArx2* expression caused reduced expression in early stage embryos (Fig. 5A). Later *Arx2* diminished *xOtx2* expression in the eye, while the expression in the

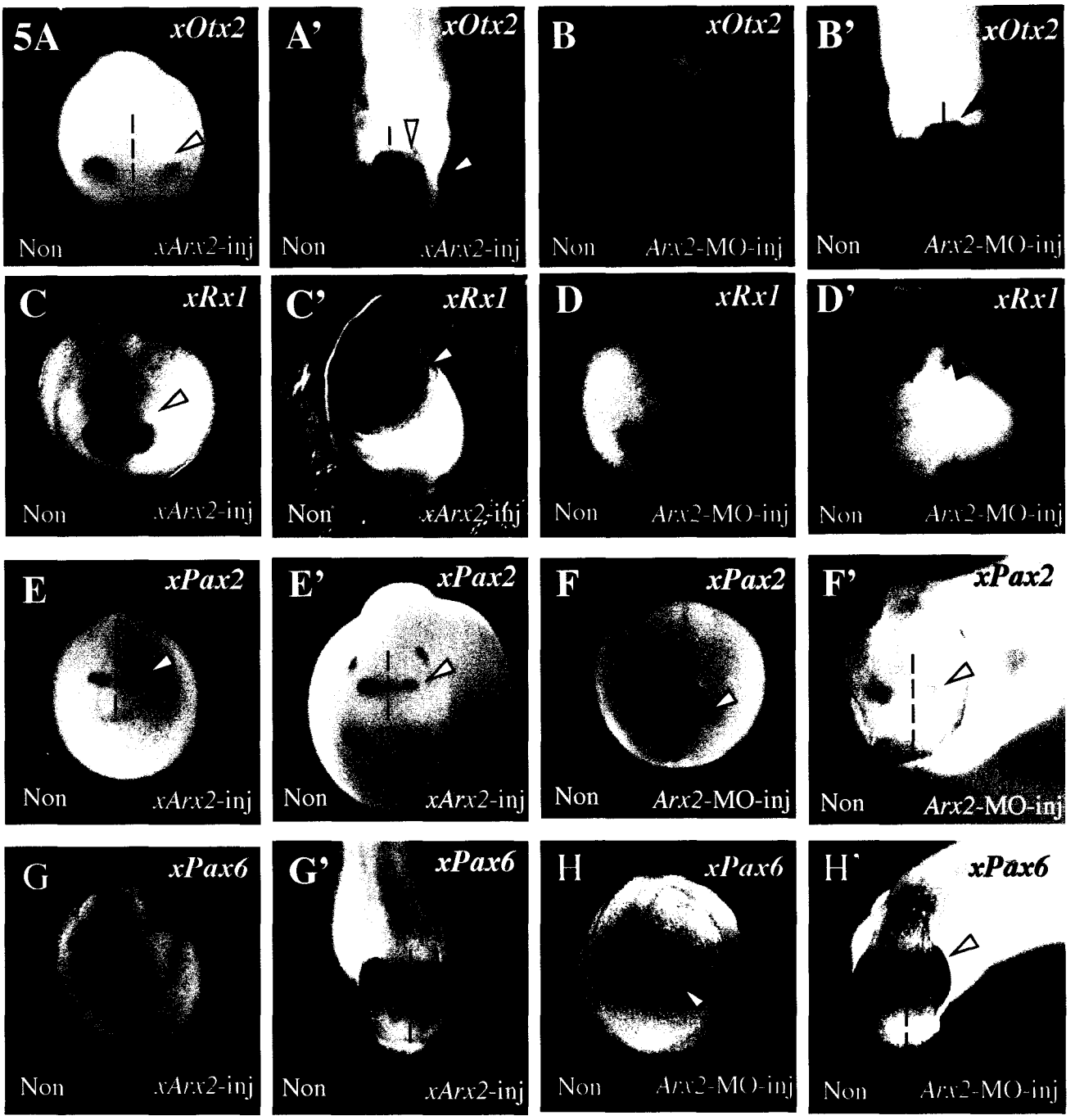
Figure 4. The effect of misexpression of *xArx2* on fore- (*XBF-1* and *xArx*), mid- (*xGbx2a*), and hindbrain (*xKrox20*) markers analyzed by *in situ* hybridization. The un-injected side of the embryos was used as a contralateral control in the comparison of the gene expression changes (arrowheads) induced by *xArx2* mRNA or *xArx2* antisense morpholino oligonucleotides (*xArx2*-MO). The expression patterns of these genes in *xArx2*-injected (400 pg) embryos at both early (A, C, E, G) and late (A', C', E', G') developmental stages are compared with embryos injected with *xArx2*-MO (18 ng) both early (B, D, F, H) and late (B', D', F', H'). Anterior (A, A', B, B, C, D', E, G, G', H, H') and dorsal (C', D', E', F, F') views are shown. Black arrowheads indicate enhanced or expanded expression, white arrowheads denote reduced or diminished expression, and open arrowhead signify a shift in the expression domain.



brain was reduced at the posterior boundary and expanded laterally (Fig. 5A'). Inhibition of *xArx2* translation resulted in an expansion of the *xOtx2* expression domain early (Fig. 5B) and up-regulated *xOtx2* levels late in the eye (Fig. 5B'). Similar effects were observed with *xRx1*. Ectopic *xArx2* expression resulted in a reduction in the early and late expression levels of *xRx1* (Fig. 5C, C'), while *xArx2*-MO induced the opposite effects, as the *xRx1* expression levels were marginally up-regulated both in early and late stage embryos (Fig. 5D, D'). *Pax2* marks the presumptive midbrain-hindbrain region (Rowitch and McMahon, 1995) and is an important regulator of optic stalk morphogenesis (Torres *et al.*, 1996). In both *xArx2*- and *xArx2*-MO-injected embryos, slight reductions in *xPax2* expression levels were observed in this domain (Fig. 5E, E', F, F'), however there was no effect on *xPax2* expression in its other domains (Fig. 5E'). Preliminary findings using the forebrain/eye marker *Pax6* (Hirsch and Harris, 1997) revealed that a small percentage of *xArx2*-injected embryos showed a slight expansion of *xPax6* in late neurula stage embryos contrasted to a slight reduction in its expression level in embryos injected with *xArx2*-MO at similar stages. Neither injection caused any significant change in *xPax6* expression in tailbud stage embryos, although a few embryos injected with *xArx2*-MO showed a slight reduction in *xPax6* in the eye field (Fig. 5 H').

Overall, gain-of-function of *xArx2* led to an increase in the forebrain markers, *xArx* and *XBF-1,* a decrease and/or posteriorization in the mid-hind and hindbrain markers, *xGbx2a* and *xKrox20*, respectively, and a reduction in *xOtx2, xRx1*, and *xPax2,* which are involved in general brain and eye development. Loss-of-*xArx2* function led to a decrease in the fore- mid-hind and hindbrain markers, as well as in *xPax2*, while elevated levels of expression of the brain/eye markers, *xOtx2* and *xRx1* were observed.

Figure 5. The effect of misexpression of *xArx2* on the brain/eye markers *xOtx2, xRx1, xPax2,* and *xPax6* analyzed by *in situ* hybridization. The un-injected side of the embryos was used as a contralateral control in the comparison of the gene expression changes (arrowheads) induced by *xArx2* mRNA or *xArx2* antisense morpholino oligonucleotides (*xArx2*-MO). The expression patters of these genes in *xArx2*-injected (400 pg) embryos both at early (A, C, E, G) and late (A', C', E', G') developmental stages are shown from an anterior perspective. Anterior views of embryos injected with *xArx2*-MO (18 ng) at the early (B, D, F, H) and late (B', D', F', H') stages are displayed. Black arrowheads indicate increased or expanded expression while white arrowheads denote reduced or diminished expression.



DISCUSSION

This study describes an investigation on *in vivo* misexpression of the homeobox gene, *xArx2*, in *Xenopus*. *xArx* has been shown to express in the developing rostral forebrain (El-Hodiri *et al.*, 2003; Chapter 2 of the present study). The major findings here are that *xArx2* overexpression results in an expansion of the forebrain territory as well as a disruption of normal eye development. Knocking down *xArx2* translation by means of *xArx2*-antisense morpholino oligonucleotides (MO) results in: a reduction of the forebrain, impaired head development on the side of injection, and anomalous development of the eyes. Additionally, both gain- and loss-of-function of *xArx2* perturbs anterior development, such that the rostral brain does not fully form and reductions in the head circumference often arise. Presumably, over expression of *xArx2* yields circumstances in which both *Arx* and *Arx2* target genes are precociously affected. By contrast, the morpholino-induced knockdown presumably affects only those genes normally targeted by *Arx2*, as its specificity was demonstrated *in vitro*. This may explain why the phenotypes observed in *xArx2*-MO-injected embryos were less severe than those resulting when *xArx2* was ectopically expressed. In addition, the two *xArx* genes most likely possess some overlapping functions and endogenous xArx can partially compensate for the loss of Arx2.

The phenotypic effects appear to be, for the most part, dose dependent as frequencies increased with increasing amounts of *xArx2* mRNA or *xArx2*-MO, however, some variability was observed. For instance, at high doses of *xArx2* the frequency of microcephaly and "fused brain" is less than that observed at lower doses (7.5% and 1.5% at 800 pg compared to 12% and 6%, respectively, at 400 pg). This is probably a result of

the high mortality rate of higher dosed tadpoles, where phenotypes compounded, and embryos with severe defects did not survive long enough to be analyzed. Thus, the recorded frequencies at higher doses may not be a true reflection of the actual percentage of embryos with such defects. The occurrence of pigmentation along the optic nerve, which reflected ectopic pigmented retinal tissue, did not appear to correlate with injected doses. However, since elevated amounts of *xArx2* resulted in an increasing frequency and severity of other eye abnormalities, such as medial extensions of pigmented retinal epithelium (RPE) toward the midline and fused eyes, the optic nerve was indistinguishable or even absent in these tadpoles. Finally, some variability in the phenotypes observed following the injection *xArx2* synthetic mRNA may be attributed to the varied distribution and stability of transcript within the embryo. As *GFP*-injected embryos developed normally, the possibility of RNA toxicity can be ruled out.

Misexpression of *xArx2* Results in Cephalic Anomalies

A striking phenotype that appears to be specific to the misexpression of *xArx2* is its effect on forebrain size, where an expansion of this territory is elicited among embryos in which *xArx2* was ectopically expressed, and conversely, reduced in tadpoles in which *xArx2* translation was impaired. Any substantial change in brain size requires a change in the number of neurons and their supporting elements in the brain. Defects in expansion of precursor cell populations can arise as a result of abnormal cell death, premature differentiation and exit from the cell cycle, or reduced mitogenesis. Thus, ectopic *xArx2* expression may regulate the forebrain cell population either by increasing proliferation of neuroectoderm, or by reducing apoptosis in these cells. Although our analyses did not address such possibilities, simple experiments to resolve these theories can be performed.

Analysis of potential differences in patterns of apoptotic cells, marked by TUNEL staining, in wild-type and mutant embryos would reveal if *xArx2* is acting through an apoptotis pathway. Additionally, the rate of mitotic activity can be compared *in vivo* using BrdU labeling on *xArx2*-injected, *xArx2*-MO-injected, and control embryos.

Similar forebrain phenotypes have been reported with misexpression of other transcription factors which express in the anterior brain. Mice carrying a null mutation in the *Gli3* gene show disruption in dorsal telencephalon development (Theil *et al.*, 1999), which correlates with a loss of *Emx1* and *Emx2* expression. *Emx* genes express in the telencephalon of mouse, chick, turtle and frog embryos during development (Fernandez *et al.*, 1998). *Emx2* is required for normal growth and maturation of the hippocampus (Tole et al., 2000) and *Emx2* homozygous null mutant mice have a reduced hippocampus and neocortex. It is possible that *Arx* may interact in a cascade with Gli3 and Emx2 to regulate dorsal telencephalon development. Since *xArx2* perturbation occasionally induced laterality defects, and since *Gli3* plays a role in left-right asymmetry, the relationship between *xArx2* and *Gli3* is worth pursuing in future studies.

As some of the cells of the neuroepithelium differentiate into neurons and glial cells, others will remain in a proliferative state to establish the pool of neural progenitors. The vertebrate forebrain is notably expanded in size compared to its more posterior counterparts. This is thought to result as a consequence of delayed neurogenesis in the anterior portion of the neural plate to allow for prolonged proliferation (Papalopulu and Kintner, 1996). Several genes which regulate such events, such as *Xanf-1* (Zaraisky et al., 1995), *XBF-1* (Papalopulu and Kintner, 1996), and *xRx1* (Casarosa *et al.*, 1997), have been isolated in *Xenopus* and their misregulations give rise to similar forebrain

malformations as observed in this study (Ermakova *et al.*, 1999; Bourguignon *et al.*, 1998; Andreazzoli *et al.*, 2003). *Xanf-1* expression is restricted to the anterior neural ectoderm as early as the midgastrula stage (Zaraisky et al., 1995) and controls not only early patterning of the forebrain primordium, but also the initial steps of neural commitment of embryonic ectoderm (Ermakova *et al.*, 1999). Ectopic *Xanf-1* can expand the neural plate at the expense of non-neural ectoderm, which later manifests in brain outgrowths in tadpoles, and suppresses terminal differentiation of primary neurons (Ermakova *et al.*, 1999). The expression domain of *XBF-1* lies in the most anterior border of the neural plate during late gastrula and restricts to the neuroepithelial cells of the telencephalon during tadpole stages in *Xenopus* and ectopic *XBF-1* expression has been shown to expand the neuroectoderm (Bourguignon *et al.*, 1998; Hardcastle and Papalopulu, 2000). *XBF-1* controls formation of the telencephalic primordium through mechanisms affecting both proliferation and differentiation (Papalopulu and Kintner, 1996; Bourguignon *et al.*, 1998; Hardcastle and Papalopulu, 2000; Hanashima *et al.*, 2002). *XBF-1* prevents anterior neural plate cells from undergoing early neuronal differentiation in a dose-dependendent manner (Bourguignon *et al,* 1998). At high doses of *XBF-1*, neuronal differentiation is suppressed, as analyzed by *N-tubulin*, a marker of differentiated neurons. Lower doses of *XBF-1* result in induced neuronal differentiation in competent ectoderm. Concentration-dependent proliferation of *XBF-1* has also been shown where a low dose inhibits ectodermal proliferation while high doses of *XBF-1* promote proliferation of neuroctodermal cells through the inhibition of the cell cycle inhibitor $p27^{XIC1}$ (Hardcastle and Papalopulu, 2000). Mouse embryos lacking *BF-1* die at

birth with hypoplasia of the cerebral hemispheres due to premature neuronal differentiation and lack of proliferation within the forebrain (Xuan et al., 1995).

A candidate gene for the regulation of *XBF-1*, whose expression domain covers the entire proliferative region of the anterior neural plate is, *xRx1* (Andreazzoli *et al.*, 2003). Ectopic *xRx1* expression expands the expression domain of *XBF-1* into the lateroanterior border of the neural plate and inhibits *N-tubulin* expression (Andreazzoli *et al.*, 1999; Andreazzoli *et al.*, 2003). Furthermore, *xRx1* was also shown to control proliferation through $p27^{XIC1}$ (Andreazzoli *et al.*, 2003). Since ectopic expression of *xArx2* results in disproportionately large forebrains, it is possible that *xArx2* acts in parallel or hierarchical pathways with these genes to control the timing of neurogenesis and/or the status of proliferation.

Interestingly, β-catenin, which lies downstream of the *Wnt* signaling pathway and plays a role in establishing the initial DV axis prior to gastrulation, has also been implicated in the regulation of cerebral cortical size by controlling the generation of neural precursor cells. Mice expressing high levels of a stabilized β-catenin transgene in neural precursors develop enlarged brains with expanded precursor populations (Chenn and Walsh, 2002; Chenn and Walsh, 2003). Possibly, *xArx2* acts through the *Wnt* signaling pathway to maintain the level of neural precursors in the forebrain.

xArx2 May be Required for Proper Eye Development

Ectopic *xArx2* expression was also observed to disrupt eye development. Gain- and loss-of-function of *xArx2* causes reduced or missing eyes. It is not likely that *xArx2* is involved in eye patterning directly as it is not expressed in the eye fields throughout their development. However, it may indirectly influence the proper patterning of the eye

field through its effect during diencephalon development. Vertebrate eye formation is a complex process which involves early specification of the prospective eye territory, inductive events, patterning along the coordinate body axes, and regional specification (reviewed by Lupo *et al.*, 2000). The prospective eyes develop from optic vesicles, which invaginate to form optic cups from the diencephalic walls. Thus, improper patterning of the diencephalic territory at this region would disrupt proper eye development. As *xArx2* normally expresses in the diencephalon, its misregulation of may result in altered patterning of this forebrain region. *XArx2* may induce a caudal-to-rostral transformation whereby the telencephalon is expanded at the expense of the diencephalon. This rostralization may alter the expression of genes involved in eye development and could account for some of the observed eye defects attributable as a secondary effect of *xArx2* misexpression. Similar to the eye morphological effects of misexpression of *xArx2*, mutation of *Pax2* results in ectopic extension of the pigmented retina into the optic nerve and abnormal differentiation of the optic nerve (Torres *et al.*, 1996). This raises the possibility that altered expression of *xArx2* may directly or indirectly cause misregulation of *Pax2*.

Holoprosencephaly-like Features Result From Severe Misregulation of *xArx2*

Holoprosencephaly is one of the most common anomalies of the developing forebrain in humans, and is caused by the failure of the prosencephalon to sufficiently divide into the double lobes of the cerebral hemispheres (Roessler and Muenke, 2001). The result is a single-lobed brain structure and severe skull and facial defects. Clinical manifestations of Holoprosencephaly are variable and extend from closely spaced eyes to a failure of separation of the eye field and forebrain that is associated with cyclopia. *Sonic Hedgehog* (*Shh*) signaling is thought to play a causative role in this disorder

through the mediation by different Gli proteins (Nanni *et al.*, 1999; Roessler *et al.*, 2003). Loss of function of *Shh* activity causes cyclopia in mice, humans and zebrafish (reviewed in Roessler and Muenke, 2001). Holoprosencephaly-like features were observed in both our gain-of and loss-of function mutants. The anterior brains of these tadpoles, which were categorized phenotypically as "fused-brain" mutants, lacked distinct telencephalic lobes. This was often associated with more midline-displaced or even fused eyes. These data may suggest that *xArx2* impinges upon SHH signaling, either directly or by disrupting other genes involved in the pathway.

Laterality Defects May be Associated With Abrogated *Arx2* Function

In addition to anterior-posterior and dorsal-ventral body axes, vertebrates develop left-right asymmetries. A vast amount of research has been put into understanding the molecular mechanishms regulating body asymmetry. Asymmetrically expressing signaling molecules, including *Shh*, *nodal*, and *lefty*, as well as the class-III *aristaless*-related gene *Pitx2* have been implicated as key regulators of sidedness in *Xenopus* (reviewed by Bisgrove *et al.*, 2003). We found that some of the embryos misexpressing *xArx2* displayed reversals in proper heart and gut looping. Although misexpression does not always result in reversal or randomization (looping with equal probability to either the left or to the right), the fact that these anomalies were absent from control embryos suggests that misexpression of *xArx2* interrupts the signals necessary for establishing laterality. This may not be a direct effect of *xArx2* but rather a secondary effect. However, it is interesting to note that other *Aristaless*-related genes, namely *Pitx2* and *Pitx3,* have been reported to influence left-right asymmetry of internal organs (Ryan *et al.*, 1998; Campione *et al.*, 1999; KhosrowShahian *et al.*, unpublished). This may imply

that the asymmetry irregularities observed in our mutant embryos are non-specific effects, whereby similarities in the homeodomain amino acid sequence result in a mimicking of the effects reported in related genes when *xArx2* is ectopically expressed. However, since we also observed these defects in *xArx2*-MO-injected embryos, this explanation is unlikely, and *xArx2* may be playing a role in regulating genes involved in establishing laterality.

We found that *xArx2* restricts its embryonic expression to the developing forebrain. Subtle anatomical differences exist between the left and right hemispheres of the brain and components of the Nodal pathway are expressed on the left side of the zebrafish diencephalon briefly during embryogenesis (reviewed by Halpern *et al*, 2003). Laterality within the cortex has been implicated in certain aspects of cognitive dysfunction (Herbert *et al.*, 2002). The asymmetries in the heart and gut that we observed in mutant adpoles misexpressing indicates that *xArx2* may normally be involved in establishing laterality within the brain by impinging upon a network of genes that enjoy conserved utility in directing laterality elsewhere.

Brain Regionalization is Perturbed in Embryos Misexpressing *xArx2*

We used a panel of eight genes (*XBF-1, xGbx2a, xKrox20, xArx, xOtx2, xRx1, xPax2,* and, *xPax6*), representative of a broad range of markers of positional identity in the developing brain and eye fields, in order to obtain a more thorough assessment of the suggested role of *xArx2*.

Misexpression of *xArx2* Alters fore-, mid- and hindbrain markers

To further examine the effects of misexpression of *xArx2* on the forebrain, we looked at two forebrain markers, *XBF-1* and the *xArx2* homologous gene, *xArx*. *XArx2*

misexpression had a similar effect on both of these markers. Ectopic *xArx2* expression increased the level of *xArx* expression and expanded the *XBF-1* expression domains, while inhibiton of *xArx2* translation via *xArx2*-MO reduced the level of expression of both *XBF-1* and *xArx.* The expansion of the *XBF-1* domain in x*Arx2*-injected embryos may result from *xArx2* acting to up-regulate *XBF-1*, thereby promoting proliferation. Alternatively, the observed expansion of the *XBF-1* domain could be a secondary result of an increase in the progenitor cell population where it normally expresses, thus implying that *xArx2* plays a role specifying proliferation status and cell fate.

The effect that misexpression of *xArx2* had upon *xArx* may indicate that *xArx2* normally impinges upon *xArx* to activate transcription of target genes or that the *xArx* genes autoregulate and *xArx2* is mimicking an *Arx* effect ectopically. This observation could have also resulted from a *xArx2*-induced expansion in the cell population normally expressing *xArx.*

The effect of *xArx2* misexpression on posterior regions of the brain was analyzed using the markers *xGbx2a* and *xKrox20*, which endogenously express in the mid-hindbrain and hindbrain, respectively. We found that these markers were both posteriorized and reduced or diminished in *xArx2*-injected embryos. Since the expression domains do not overlap with that of *xArx2*, direct regulation of *xGbx2a* and *xKrox20* by *xArx2* is unlikely. Thus, a change of the expression patterns following a deformation of brain patterning is a simpler and more plausible explanation for the coherent shifts in the expression patterns of these genes. Since temporal and spatial attributes of brain specification are linked but poorly understood, it is unclear whether mid/hindbrain differentiation is orthographically posteriorized or delayed and inhibited temporally. As

embryos were observed to have an expanded forebrain later in development, it is possible that more posterior regions of the brain may be physically displaced caudally as a result of over-proliferation of cells in more anterior regions. Furthermore, the fact that similar effects on these markers were observed following inhibition of xArx2 suggests that proper specification of the anterior region of the brain by *xArx2* may be required to maintain positional identities of more posterior domains. Thus, the lack of a fully differentiated forebrain may to lead to delayed or retarded differentiation of mid- and hindbrain tissues.

xArx2 Misexpression Causes Irregularities in General Brain/Eye Markers

Ectopic *xArx2* expression had the effect of reducing the levels of expression of genes which play a role in eye development. *XOtx2* is a homeobox gene involved in patterning the body axis and in specifying anterior regions and their spatial relationship with trunk structures (Pannese *et al.*, 1995). It transcription encompasses the fore- and midbrain regions, as well as the eye (Blitz and Cho, 1995). Mice deficient in *Otx2* lack eyes and $Otx2^{-/-}$ mice lack forebrain and midbrain (Acampora *et al.*, 1995; Matsuo *et al.*, 1995). It has also been suggested that *Otx2* potentiates the functional interaction among eye field transcription factors (Zuber *et al.*, 2003). *XRx1* is a *paired-like* homeobox gene required for normal eye and brain development, and mice lacking functional *Rx* do not develop eyes (Mathers *et al.*, 1997). *XPax*2 marks the midbrain-hindbrain territory (Rowitch and McMahon, 1995) and *xPax6* marks the forebrain and the eyes (Hirsch and Harris, 1997).

Both early and late expressions of *xOtx2*, *xRx1*, and *xPax2* were decreased in *xArx2*-injected embryos. Conversely, expression levels of *xOtx2* and *xRx1* were

increased in *xArx2*-MO-injected embryos, while the level of *xPax2* was again reduced. Our preliminary analysis on the resultant *xPax6* expression revealed a slight increase in its early expression domain in *xArx2*-injected embryos and a slight reduction in the early *xPax6* expression in *xArx2*-MO-injected embryos. Interpreting these results in light of the observed morphological effects of *xArx2* misexpresssion is challenging, however, we speculate that *xArx2* acts indirectly to initiate a regulatory cascade that affects remote brain/eye marker genes.

It appears that many factors must be taken into account in attempting to understand the overall effect that *xArx2* has on markers of the brain and eye. For example, a mutual inhibtion between *XGbx2* and *Otx2* has been shown to determine the placement of the midbrain-hindbrain boundary and to thereby establish *Pax2* expression levels (Tour *et al.*, 2002a; Tour *et al.*, 2002b). Thus, if *xArx2* has a direct or indirect effect on one of these genes, it will in turn alter the expression of the others. Therefore, we suggest that the overall effects on the eye and brain marker genes caused by the misexpression of *xArx2* is due to mispatterning, or even respecification, of the anterior brain regions, resulting in a cascade of morphological and genetic misregulation. Alternatively, since *xArx2* misexpression affected the levels of genes found in the isthmic organizer, it may be effecting partitioning at the isthmus.

Arx Function May be Conserved Among Vertebrates

Mutations in human *ARX* generate a wide range of phenotypes including X-linked infantile spasms, Partington syndrome, characterized by mental retardation, ataxia, and dystonia, and various forms of mental retardation (Stromme *et al.*, 2002; Kitamura *et al.*, 2002). The first functional studies of *Arx* were conducted using mouse knockouts, which

resulted in developmental abnormalities of the brain and testis similar to with human XLAG (Kitamura *et al.*, 2002). Due to these effects, it is thought that *Arx* regulates genes involved in the differentiation of structures required for cognitive development by promoting neuronal proliferation, and may also play a role in neuronal migration (Ohira *et al.*, 2002; Bienvenu *et al.*, 2002). We conclude that *xArx2* plays a crucial role in regulating forebrain patterning. Whether it does so by regulating mechanisms pertaining to cell differentiation, neuronal migration, or cellular proliferation still remains to be elucidated.

CHAPTER 4

Directions for Future Study

Our goal was to establish the spatial and temporal expression pattern of a second *Xenopus Arx*, *xArx2*, during embryogenesis and to determine the consequences of its misexpression both phyenotypically and on various marker genes. These studies left us with the following findings which we have summarized in greater detail within chapters two and three:

1. *xArx2* encodes a conserved *aristaless*-related homeobox transcription factor.
2. *xArx2* is transcribed throughout embryogenesis with peak levels occuring at the onset of neurulation.
3. Spatial expression of *xArx2* is restricted to the developing forebrain, floor plate, and somites.
4. Ectopic *xArx2* expression leads to anterior defects including an expanded forebrain territory while inhibition of *xArx2* translation results in a reduction in this domain.
5. Misexpression of *xArx2* results in posterior shifts in more caudal brain markers and up-regulates forebrain markers.

FUTURE PROSPECTS

Although the findings of the present study implicate *xArx2* as a regulator of forebrain development in *Xenopus*, several key experiments remain to be conducted in order to determine the precise function(s) of this gene during *Xenopus* development. The focus of this thesis was to characterize *xArx2* in *Xenopus*, however another *Arx* homologue has been reported in this organism (El-Hodiri, 2003). While our studies focused on the role of *xArx2*, we did not tease out the differences between the two *xArx* genes beyond their spatial expression patterns and the effects of 'knockdown' of one of

them. Comparisons of loss-of-function mutants generated using MO targeted specifically against the 5'UTR of *xArx* with those induced by *xArx2*-MO may provide insight into the functional differences between the two *xArx* genes. Furthermore, as loss of *Arx* function results in mice that die shortly after birth (Kitamura *et al.*, 2002), utilizing both *xArx*-MOs will determine the necessity of *Arx* in *Xenopus*. In addition, to test the specirficities of the morpholino oligonucleotides, *in vivo* rescue experiments should be performed, by co-injecting combinations of *xArx*-MOs with *Arx* synthetic mRNAs to determine if *xArx* function can be restored.

A principle finding of our studies is that misregulation of *xArx2* affects the size of the forebrain, where ectopic *xArx2* expression expands this region, both laterally and anteriorly, and inhibition of xArx2 translation causes a reduction in the anterior brain. These findings suggest that *xArx2* may be playing a role in neuroepithelial proliferation, suppression of neuronal differentiation, or both. As these possible functions of *xArx2* remain to be addressed, BrdU pulse labeling and TUNEL staining, as discussed in chapter three, would elucidate the involvement of *xArx2* in cell proliferation and rule out the possibility that the observed increase in forebrain size was due to reduced apoptosis. As it is known that, in normal development, neuronal differentiation in anterior neural plate is delayed in comparison with the posterior neural plate (Papalopulu and Kintner, 1996), and since *xArx2* expresses early in the anterior domain of the neural plate, one of its putative functions may be to suppress neuronal differentiation during neurulation. To address this, wild type embryos and embryos misexpressing *xArx2* can be stained for the presence of *N-tubulin*, a marker of differentiated neurons, and compared.

There are also several neurologically unrelated functions that *Arx* may regulate that were out of the realm of the present study but that should still be addressed. As we, and others (Miuria, *et al.*, 1997), detected *Arx* in the somites, and since *ARX* has been reported to express strongly in human skeletal muscle (Ohira *et al.*, 2002), its role during somitogenesis and myogenesis needs to be addressed. Furthermore, there is evidence that *Arx* may play a role in endocrine cell specification in the pancreas. Subsequent to the initial expression studies on *Arx* in mice Collombat *et al.* (2003) reported a previously unnoticed expression of *Arx* in the mouse pancreas at the onset of pancreatic bud evagination which later becomes restricted to the islets of Langerhans. Loss-of-function mutant mice die 2 days after birth with severe hypoglycemia and an absence in α cells (Collombat *et al.*, 2003). The authors propose that a mutual inhibition of Arx and Pax4 during endocrine development is required to generate normal islets of Langerhans. During embryogenesis, the pancreas arises from dorsal and ventral evaginations of the foregut that will subsequently fuse into a single organ. Interestingly, pancreatic expression of *ARX* has also been reported in humans, as detected by Northern blot analysis (Bienvenue *et al.*, 2002). Although we did not detect any *xArx2* expression in the developing pancreas, our *in situ* hybridization analysis was limited to below stage 36 of embryogenesis. Future studies should examine the expression of *xArx2* at stages 36 and beyond to determine if it is at all expressed in the pancreas in *Xenopus.* Additionally, histological examination from *in situ* hybridizations of *xArx2* during early embryogenesis of the pancreatic anlagen may reveal some level of expression therein. Should these studies prove promising, characterization of *xArx2* during pancreatic development is another avenue of *xArx2* function that will need to be explored.

As *Arx* is a transcription factor, it presumably acts by regulating the expression of target genes. Moreover, many homeobox transcription factors exert their specificity through dimerization, which suggest that an interaction between *Arx* and other proteins is required for proper transcriptional regulation. Both of these factors still need to be addressed in order to completely understand the role of *Arx* in forebrain patterning. It is currently unclear where in a molecular cascade *xArx2* lies with respect to other genes involved in forebrain specification. RNA subtractions or microarray analysis of RNA from embryos injected with *xArx2* capped mRNA or xArx2-MO compared to RNA from control embryos could reveal potential downstream targets of *xArx2*. Candidate genes identified in these screen, which appear to be up-regulated from embryos overexpressing *xArx2*, can be subsequently analyzed using the *Xenopus* animal cap assay to determine if the effect is direct. Also, *in situ* hybridization can be performed using the candidate genes as probes following the misregulation of *xArx2* for any gene which appeared up- or down-regulated on the array. Finally, to screen for potential interacting proteins of *xArx2* the yeast two-hybrid assay can be employed. Results from these and the aforementioned studies will provide insight into the pathogenesis of the disorders associated with mutations in *ARX*.

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PUBLICATIONS

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ABSTRACTS/POSTERS

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