# PATTERNING THE EMBRYONIC BRAIN: MULTIPLE SIGNALS CONVERGE TO ESTABLISH SPATIAL IDENTITY WITHIN A NEUROMERIC FIELD



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Title Page

# Patterning the Embryonic Brain:

# Multiple signals converge to establish spatial identity within a neuromeric field

By

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# ABSTRACT

Precisely arranged signaling events are required to establish the immense complexity of the embryonic central nervous system. During gastrulation, as the organizing mesendoderm endows neural identity on the overlying ectoderm, it concurrently initiates differential specification along the anterior-posterior (AP) neuraxis by antagonizing caudalizing signals. Upon completion of the embryonic germ layer rearrangements of the late gastrulae, the prospective neural plate shows primitive AP identity, which must be extensively rearranged and refined during neurula and tadpole stages by organizing centers occurring in adjacent tissues (mesendoderm and non-neural ectoderm) as well as within both the dorsoventral (roof plate and floorplate) and AP (anterior neural ridge and isthmic organizer) plane of the neurectoderm. Signals along both axes establish a grid-like network of gene expression providing the spatial cues for a cell to adopt its precisely choreographed differentiation program. Perturbations to components of this network result in severely abnormal phenotypes, thereby providing information on their function. I have utilized this approach to determine the requirements of both a *Xenopus* Rel/NF-κB protein (Xrel3) and two novel components of the intracellular Wnt/β-Catenin signal transduction cascade (XPygo-2α/β) in morphogenesis of brain and optic vesicles. Xrel3 specifies both fore-midbrain and ventral identity of the nervous system by regulating *otx2* and *shh/gli1* gene expression, while both XPygo-2α and XPygo-2β mediate a late phase of Wnt signaling required to establish retinal and telencephalic domains of gene expression within the prospective forebrain.

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| | |
|---|---|
| ADE | Anterior Definitive Endoderm |
| ADMP | Anti-Dorsalizing-Morphogenic-Protein |
| AE | Anterior Endoderm |
| AER | Apical Ectodermal Ridge |
| ANR | Anterior Neural Ridge |
| AP | Anteroposterior |
| APC | Adenomatous Polyposis Coli |
| AVE | Anterior Visceral Endoderm |
| BCIP | 5-Bromo-4-Chloro-3-Indolyl-Phosphate |
| BMP | Bone Morphogenetic Protein |
| Cer | Cerberus |
| CK1 | Casein Kinase 1 |
| CNS | Central Nervous System |
| Dkk | Dickkopf |
| DV | Dorsoventral |
| EMSA | Electrophoretic Mobility Shift Assay |
| En-2 | Engrailed-2 |
| FGF | Fibroblast growth Factor |
| Frzb | Frizbee |
| GDF | Growth Differentiation Factor |
| GSK | Glycogen Synthase Kinase |
| IsO | Isthmic Organizer |
| LEF | Lymphoid Enhancer Binding Factor |
| LRP | LDL-Receptor Related Protein |
| MAB | Maleic Acid Buffer |
| MBT | Mid-Blastula Transition |
| MHB | Mid-Hindbrain |
| MO | Morpholino Oligonucleotide |
| murRRBE | Murine Rel Related Protein Binding Element |
| NAM | Normal Amphibian Medium |
| NBT | 4-Nitro Blue Tetrazolium Chloride |
| NHD | N-terminal Homology Domain |
| NLS | Nuclear Localization Sequence |
| PHD | Plant Homeodomain |
| Pygo | Pygopus |
| R85 | Lysine to Arginine (position 85) substitution of Rat GSK-3β |
| RA | Retinoic Acid |
| RH or RHD | Rel Homology Domain |
| SBE | Suprablastoporal Endoderm |
| Shh | Sonic Hedgehog |
| TA or TAD | Trans-Activation Domain |
| TCF | T-Cell Factor |

| | |
|---|---|
| TGF | Transforming Growth Factor |
| TrCP | β-Transducin Repeat Containing Protein |
| Wg | Wingless |
| Wnt | Wingless/Int-1 |
| Xbra | *Xenopus* Brachyury |
| Xnr | *Xenopus* Nodal Related |
| ZLI | Zona Limitans Intrathalamica |
| ZPA | Zone of Polarizing Activity |

# CO-AUTHORSHIP STATEMENT

Chapter 1:

This chapter was originally published in TheScientificWorldJournal (Lake and Kao, 2003a), as authored by B. Lake and edited by K. Kao. Collaborative efforts were involved in the design of the associated figures (Figures 1.1 and 1.2).

Chapter 2:

This research forms approximately 50% of an article published in Development (Lake et al., 2001). Design and identification of the research proposal through to data analysis was achieved with the assistance of K. Kao. The primary author for this manuscript was K. Kao, with B. Lake and R. Ford contributing equally as co-authors for the associated results and materials/methods sections.

Chapter 3:

This research was published in Developmental Biology (Lake and Kao, 2003b). Design and identification of the research proposal through analysis of the data and preparation of the manuscript was primarily accomplished by B. Lake. Guidance and editorial assistance was provided by K. Kao.

# CHAPTER 1:

## *Early Head Specification In Xenopus Laevis*

**Running Title:** *Xenopus* Head Development

## 1.1 ABSTRACT

In *Xenopus*, the progressive determination of the head is an extremely complex process involving the activation and localized antagonism of a number of interdependent intracellular signaling pathways including the Wingless/Int-1 (Wnt), Bone Morphogenetic Protein (BMP) and Nodal-related (Xnr) pathways. The sequence of events that specify the head are: dorsal-ventral polarization and head organizer specification in the blastula; gastrulation; neural induction; and patterning of the anterior-posterior and dorsal-ventral neuraxes. Wnt signaling is required for the specification of the dorsal side initially, but is then inhibited within the organizer once it has formed. Similarly, Wnt signaling is required along the length of the neural tube, but must be suppressed at its rostral end for normal brain development. Nodal signaling is also necessary for induction of the mesendoderm, but is subsequently suppressed in its dorsal-anterior extreme to specify head organizer. BMP signaling is required for ventral mesoderm and non-neural ectoderm, and must also be suppressed in the head organizer region and for the differentiation of the ventral midline of the neural tube. Thus, development of the head, and indeed the body plan in general, requires precisely timed and spatially restricted activation and repression of these signaling pathways.

## 1.2 AXIS SPECIFICATION AND ORGANIZER FORMATION

Understanding how cells adopt specific fates and become coordinated into an adult organism requires extensive manipulation of a developing and dynamic system. My interest within this broad arena of developmental biology has been inspired by an interest in understanding the processes that establish the human brain. However, because of the ethical unsuitability of direct studies and manipulations in humans, I have chosen to study embryos of the frog, *Xenopus laevis*, as a viable alternative for identifying the molecular events that shape the central nervous system.

*Xenopus laevis*, native to Southern Africa, was first popularized as a pregnancy test when it was found that these frogs ovulate after exposure to a hormone in human pregnancy urine (chorionic gonadotrophin). Their oviparous reproduction and induced ovulation made possible both *in vitro* fertilization and simultaneous culture of large numbers of embryos. These attributes, coupled with their large size and rapid development, facilitated extensive studies into early embryological events including blastula formation, establishment of the main germ layers and patterning of the basic vertebrate body plan. A major breakthrough for these studies was the demonstrated ability to introduce and express exogenous genetic sequences within early oocytes and embryos (Gurdon et al., 1971). This allowed gain and loss of function studies within a system that, due to a pseudotetraploidal makeup and long maturation time, made genetic experimental manipulations difficult. A further advancement came with the use of antisense technology (phosphorothioates and morpholinos) that could prevent maternal or zygotic protein expression and enable knockout mutant phenotypes (Weeks et al. 1991; Heasman et al., 1992; Heasman et al., 1994; Kofron et al., 1997; Heasman et al., 2000).

The highly documented developmental fate maps (Nakamura and Kishiyama, 1971; Keller, 1975; Keller, 1976; Cooke and Webber, 1985; Dale and Slack, 1987; Moody, 1987) and numerous studies into the biochemical pathways establishing these fates (outlined in upcoming sections), made *Xenopus* an excellent system to study the molecular events underlying anterior nervous system patterning. While early inductive events to establish the neural plate have been well characterized, events that proceed to elaborate this pattern have remained unclear. It is known, however, that the patterning of the ectoderm to form the brain is intimately dependent on events preceding it to form and pattern the mesodermal and endodermal germ layers. Therefore, regionalization of the brain has its earliest basis in the events that establish the basic body axes.

### 1.2.1 Establishment of the Dorsal-Ventral Body Axis

The head represents the most anterior and dorsal part of the body plan. In *Xenopus*, development of the body axes is dependent on the formation of Spemann's Organizer, which is set up by the intersection of two processes before gastrulation: dorsal-ventral polarization and mesendoderm induction (Harland and Gerhart, 1991; Nascone and Mercola, 1997; Nishita et al., 2000; Joubin and Stern, 2001). Establishment of dorso-ventral polarity occurs during the first cell cycle following fertilization, by the corticocytoplasmic rotation (Gerhart et al., 1989; Elinson and Holowacz, 1995). The rotation causes the displacement of vegetal pole cytoplasm to the dorsal vegetal side of the embryo, which activates intracellular Wingless/Int-1 (Wnt) signaling and subsequent translocation of β-Catenin in the dorsal vegetal region (Holowacz and Elinson, 1995;

Marikawa et al., 1997; Moon and Kimelman, 1998; Brown et al., 2000; Chan and Etkin, 2001). Thus, an early dorsal-to-ventral gradient of stabilized β-Catenin is generated which specifies the dorsal-ventral axis of the embryo. Stabilized β-Catenin molecules enter the nucleus and bind a transcriptional complex containing the co-regulator XTCF-3 associated with promoter elements of the target genes (Hamilton et al., 2001). At the onset of zygotic transcription at the mid-blastula transition (MBT), these complexes activate transcription of dorsal-specific genes such as the homeobox genes *siamois* and *twin*, both associated with the vegetal organizing Nieuwkoop Center, which in turn directly activate genes specific to both head and trunk inducing regions in the overlying marginal zone.

The mesendoderm is induced to form by a signal that emanates from the vegetal pole, which induces endoderm in vegetal cells and mesoderm in the overlying marginal zone cells (Kimelman and Griffin, 2000). This signal is initiated by the maternal T-box transcription factor, VegT (Zhang and King, 1996), which activates several TGF-β superfamily members, including the *Xenopus* Nodal-related (Xnr) proteins and Derrière (Kofron et al., 1999; Xanthos et al., 2001). Transduction of the Xnr-mediated signal to the nucleus involves pathways typical of SMAD-dependent TGF-β superfamily signaling (Hill, 2001; Whitman, 2001). In *Xenopus*, there is a proportional correlation between the strength of the SMAD signal with the concentration of stimulating ligand and consequent dorsal character of the induced tissue (Green and Smith, 1990; Gurdon and Bourillot, 2001; Bourillot et al., 2002). Thus, a higher concentration of ligand is associated with activation of dorsal-type genes. In the embryo, this requirement is met by the interaction

of β-Catenin with Xnr signaling which regulates the onset and possibly stability or intensity of the induction signal on the dorsal side (Mao et al., 2001a; Xanthos et al., 2002; Hashimoto-Partyka et al., 2003). Further, recent evidence demonstrates that the initiation of zygotic *Xenopus* Nodal expression is dependent upon early β-Catenin/TCF transcription occurring prior to the MBT (Yang et al., 2002b). The interaction of SMAD-dependent TGF-β signaling with Wnt signaling illustrates the existence of functional cross-talk to establish the Spemann Organizer and subsequently the dorso-ventral axis of the embryo.

### 1.2.2 Early Specification of the Organizer

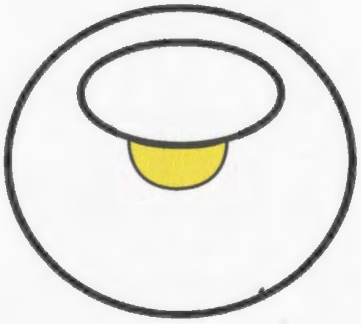
Early studies on the inductive properties of the organizer revealed that it is a non-homogeneous tissue with distinct head and trunk inducing regions (reviewed in Harland and Gerhart, 1997). This was first demonstrated this using heterotopic transplantation of dorsal blastopore lips to the ventral flank or blastocoel of host embryos, which led to the formation of conjoined twins (Spemann and Mangold, 1924; Spemann, 1927 as cited in Hamburger, 1988; Chan and Etkin, 2001). The supernumerary axis had differing anterior to posterior character depending on the time after the start of involution, with young lips inducing heads and older lips inducing tails. In 1952, Nieuwkoop proposed that differing head or trunk inducing regions within the organizer existed based on different anterior/posterior distributions of a posteriorizing agent (transformer) that altered the positional identity of neuroectoderm established by a general anterior neural inducer (activator) (Nieuwkoop, 1952).

Regionalization of the organizer has been detected as early as late blastula stages with the establishment of the head inducing region in a position vegetal to the trunk inducing region by the onset of gastrulation (Zoltewicz and Gerhart, 1997; Figure 1.1). This regionalization has its earliest basis in the differential activity of both mesendoderm inducing TGF-β superfamily signals and the β-Catenin effector Siamois. Siamois, expressed in dorsal vegetal cells (Lemaire et al., 1995) and to a lesser extent in marginal and animal cells (Ding et al., 1998), acts within a short temporal window during early blastula stages to induce spatial- and concentration-dependent expression of genes of both the head and trunk organizers (Kofron et al., 1999). Such heterogeneity subdivides the gastrula organizer into three main domains, two of which directly contribute to head formation: the anterior (deep) endoderm, fated to form liver and the prechordal endo-mesoderm, which gives rise to prechordal (head) mesoderm and pharyngeal endoderm. The third domain, the chordal mesoderm, induces trunk and is fated to form notochord. These domains are now fairly well characterized both morphologically and molecularly, with genetic profiles that are indicative of the associated inductive properties as well as prospective self-differentiation phenotype (Figures 1.1 and 1.2; reviewed in Harland and Gerhart, 1997; Niehrs, 1999; Chan and Etkin, 2001).

### 1.2.3 Formation of the Head Organizer Requires Signals That Originate From the Anterior Endoderm (AE)

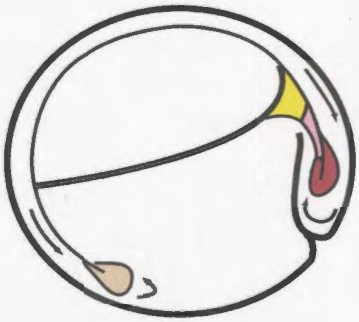
The AE is derived from deep vegetal cells localized to the floor of the blastocoel and is topologically equivalent to the mouse anterior visceral endoderm (AVE), the chick

**Figure 1.1. Differentiation of head organizer requires pregastrula interaction of the presumptive organizer with anterior endoderm.** This figure illustrates the displacement of prospective anterior endoderm from deep within the vegetal half core to a position adjacent to the presumptive organizer by the process of vegetal rotation. It also shows the relative positions of anterior endoderm, prechordal mesoderm and chordamesoderm and their movement during gastrulation along the dorsal midline.



Stage 8

Stage 9 (vegetal rotation)

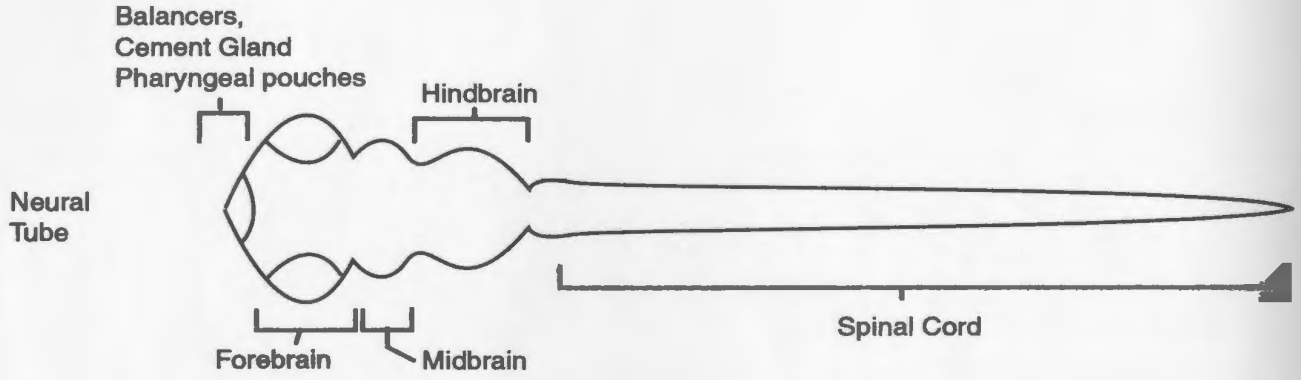


Stage 10.5

Stage 11

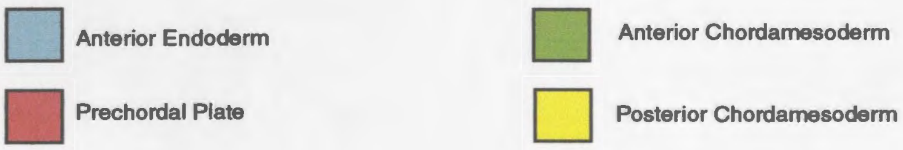
| Key to Tissues | FATE |
|---|---|
| Anterior Endoderm | Liver |
| Chordamesoderm | Notochord |
| Prechordal Endomesoderm | Head mesoderm and pharyngeal endoderm |
| Posterior Neurectoderm | Mid-hindbrain and Spinal cord |
| Anterior Neurectoderm | Eyes and Forebrain |
| Cement gland; Pharyngeal pouches | |
| Ventral Mesoderm | Blood |

**Figure 1.2. Expression patterns of secreted ligands and transcriptional regulators in the dorsal mesendoderm.** Also indicated are the associated overlying anterior-posterior identities specified in the central nervous system of *Xenopus*.





| | | FATE | | | |
|---|---|---|---|---|---|
| | | liver<br>Ventral fore<br>gut | head mesenchyme<br>pharyngeal endoderm | Notochord | Notochord |
| EXPRESSED GENES | Secreted Proteins | Cer, dkk1<br>frzb | dkk1, frzb1<br>noggin, follistatin | dkk1, frzb1, chordin,<br>noggin, follistatin | chordin<br>eFGF |
| | Transcriptional Regulators | xhex<br>xblimp1<br>siamois | gsc, otx2, Xlim1<br>Xanf1, Xblimp1,<br>XfhH1, siamois | | Xnot<br>Xbra |



hypoblast and zebrafish dorsal yolk syncytial layer, each with differing contributions to head formation (as reviewed in De Souza and Niehrs, 2000). The AE has been implicated in head induction after it was found to express the potent head inducer, Cerberus (Bouwmeester et al., 1996), as well as the fact that the AVE appears to be required for head induction in mouse. However, neither heterotopic transplantation nor ablation of the AE following the onset of gastrulation has demonstrated that it has distinct head inducing activity (Bouwmeester et al., 1996; Bradley et al., 1996; Schneider and Mercola, 1999), nor was it capable of neuralizing naïve ectoderm (Bouwmeester et al., 1996; Gamse and Sive, 2001). Quite possibly, the prospective AE functions prior to gastrulation, at a time when surgical manipulation is difficult. Consistent with this is the expression in the region, before gastrulation, of genes encoding inhibitors that are implicated in anterior specification, such as Cerberus (Cer), Hex, Dickkopf (Dkk-1) and Frizbee-1 (Frzb-1) (Figure 1.2, Table 1.1).

How then, does the AE influence head development? Following their establishment in centrally located deep endoderm, the Cerberus and Hex expressing prospective AE cells move from the central blastocoel floor to the dorsal side (Jones et al., 1999) via a pre-gastrula morphogenetic event, "vegetal rotation", a rearrangement of the vegetal cell mass that leads to expansion of the blastocoel floor and movement of the prospective AE in close apposition to the Organizer (Winklbauer and Schürfeld, 1999), where it becomes the AE proper (Figure 1.1). BMP antagonists from the organizer (Noggin and Chordin, Table 1.1) maintain Cerberus and Hex expression in the AE to perpetuate this anterior signal (Zorn et al., 1999).

**Table 1.1. Antagonistic Partners in Embryonic Patterning**

| *Extracellular Antagonist* | *Target(s)* | *Reference(s)* |
|---|---|---|
| Cerberus | BMP4, Xwnt8, Xnr1 | Nishita et al., 2000 |
| Frzb-1 | Xwnt8, Xwnt1 | Leyns et al., 1997; Wang et al., 1997a/b |
| Dickkopf-1 | Xwnt3a, Xwnt8 | Glinka et al., 1998; Kazanskaya et al., 2000 |
| WIF-1 | Xwnt8 | Hsieh et al., 1999 |
| Noggin | BMP4 | Zimmerman et al., 1996 |
| Follistatin | BMP2, BMP4, BMP7 | Iemura et al., 1998; Fainsod et al., 1997 |
| Chordin | BMP4 | Piccolo et al., 1996 |
| Xnr3 | BMP4 | Hansen et al., 1997 |

Early Nodal-related signaling is required for the establishment of head organizing genes within the prechordal endo-mesoderm, but subsequently must be antagonized by inhibitors, such as Cerberus, to enable head formation (Piccolo et al., 1999; Lee et al., 2001; Silva et al., 2003). Thus, the translocated anterior endoderm, through secretion of Cerberus, may titrate Nodal signals in prospective head organizer (prechordal endomesoderm), which in turn provides BMP inhibitors to maintain the AE. This is likely the first complementary antagonistic interaction in the generation of distinct head and trunk organizers (Fetka et al., 2000).

In *Xenopus*, the AE may be necessary, therefore, not directly for anterior neural induction, but for establishment of the head organizer through inhibition of trunk inducing activity. *Hex*, for instance, is expressed in the AE and can achieve this function non-autonomously through its ability to induce expression of *cerberus* in the AE (Jones et al., 1999; Zorn et al., 1999) that will act on adjacent prechordal mesendoderm. In addition, Hex can act autonomously by repressing trunk organizer markers, such as *goosecoid* and *chordin* (Brickman et al., 2000). A similar repression activity was described for the zinc finger transcriptional regulator, XBlimp1 (De Souza et al., 1999).

### 1.2.4 The Prechordal Endo-mesoderm as Head Organizer

Nestled between the anterior endoderm and chordamesoderm is the prechordal endo-mesoderm, a potent head inducing region (reviewed in Kiecker and Niehrs, 2001a). During gastrulation, the prechordal endo-mesoderm moves anteriorly beneath the prospective neurectoderm until it lies under the anterior neural plate and forms the

prechordal plate (Figure 1.1). Heterotypic grafting experiments in 1933 by Otto Mangold (as cited in Niehrs, 1999) and later ablation studies (Schneider and Mercola, 1999) indicated that the prechordal plate is absolutely required for inducing forebrain and eyes in the overlying neural plate. Mediating this role are several potent head inducers expressed in the prechordal plate (Figure 1.2) such as Dkk-1 and Frzb-1 (Leyns et al., 1997; Wang et al., 1997a; Glinka et al., 1998). While the chordamesoderm is primarily a trunk organizer, its signals are also required to specify caudal brain structures. Anterior chordamesoderm, as part of the head organizer, specifies the mid-hindbrain while posterior chordamesoderm, as part of the trunk organizer, specifies spinal cord (Niehrs, 1999).

### 1.2.5 Maintenance of Non-homogeneous Organizer

The maintenance of distinct head and trunk inducing centres requires a balance of Xnr, Wnt and BMP signals and their associated antagonists. As mentioned above, both Wnt and Xnr signaling are required early to cooperatively induce the head and trunk organizers, but subsequently, in conjunction with BMP signals, their antagonism by anterior endoderm is necessary to delimit the head organizer. This antagonism distinguishes head and trunk inducing regions and maintains head organizer identity. In fact, simultaneous BMP and Wnt inhibition is sufficient to convert mesoderm into prechordal plate while the coordinated inhibition of Nodal-related, BMP and Wnt proteins is required for complete organization of the head as shown by the trivalent inhibitor Cerberus (Glinka et al., 1997; Glinka et al., 1998; Piccolo et al., 1999; Silva et al., 2003).

Several Wnt antagonists are expressed within the head organizer itself (Figure 1.2). These are necessary for formation of head structures by inhibition of post-MBT ligand-dependent Wnt signaling, the second phase of Wnt signaling in the embryo (subsequent to Wnt signaling that establishes the Nieuwkoop center) which antagonizes organizer mesendoderm and posteriorizes the neurectoderm (reviewed in Niehrs, 1999). Thus, when Xwnt8 was overexpressed in embryos after the MBT, the embryos lacked heads and notochords but had over-represented somitic muscle (Christian and Moon, 1993).

Inhibition of Xwnt8 generated the opposite effect, whereby the embryos had enlarged heads and notochords at the expense of somitic muscle (Hoppler et al., 1996). The highest concentration of Wnt antagonists lies within the head organizer where their expression originates, indicating that the absence of Wnt signals in this region defines the head organizer, while a lower level of antagonism in the chordamesoderm is required to specify trunk organizer. In fact, Dkk-1 expression in anterior mesendoderm was found to be both necessary and sufficient for specification of the head organizer (Glinka et al., 1998; Kazanskaya et al., 2000). Wnt antagonism, therefore, is not only vital for maintaining a balance between non-organizer and organizer mesoderm, but also for maintaining the balance within the organizer between head and trunk inducing mesendoderm.

Recent evidence indicates that ventral BMP signaling, through activation of Xmsx1, suppresses the possibility of head structures developing ventrally by direct inhibition of Nodal-related induction of Cerberus, Hex and Dkk-1 (Yost, 1998), which are normally expressed in the anterior endoderm. BMP signaling also coordinately upregulates a pseudoreceptor, Bambi, which negatively regulates not only its own activity,

but also that of Nodal and Activin by preventing formation of activated TGF-β superfamily receptor complexes (Onichtchouk et al., 1999). Therefore, the ventral to dorsal gradient of BMP plays a role in maintaining the dorsal to ventral pattern of Xnr induced tissues to define and spatially restrict the Organizer, while antagonism of BMP signals by Organizer-derived inhibitors (Figure 1.2) is required to maintain both head and trunk structures.

Ventralizing BMPs, therefore, help maintain the correct proportions of Organizer and non-Organizer mesoderm, while other BMP family members play a role in Organizer specification and differentiation. For example, the persistence of distinct, abutting, organizing centers involves a BMP family member, ADMP or anti-dorsalizing-morphogenic-protein. ADMP is expressed in chordamesoderm and antagonizes prechordal markers in that tissue possibly through repression of anti-Wnts (Dosch and Niehrs, 2000). Nodals, Wnts and ADMP in the trunk organizer may inhibit head marker expression and promote trunk development while the head organizer secretes anti-Wnts (Cerberus, Dkk-1, Frzb-1, WIF-1, sFRP-1, 2) and anti-Nodal (Cerberus) to inhibit trunk while promoting head development (Dosch and Niehrs, 2000). Therefore, the coordinated action of BMPs, WNTs, Nodals and their associated antagonists define distinct head and trunk organizer regions by the onset of mesendoderm involution.

## 1.3 NEURAL INDUCTION

### 1.3.1 Pre-gastrula Specification

The majority of the neural plate is induced by signals derived from the organizer, except for ventral midline cells, also called the notoplate, which express the winged helix transcriptional regulator, *Xfd-12'* (Fetka et al., 2000). Notoplate cells are specified in the dorsal non-involuting marginal zone prior to induction of the prospective neural plate, possibly by Nodal-related ligands during mesendoderm induction. During pregastrula morphogenesis, at the same time during which the anterior endoderm is moved to the dorsal side by vegetal rotation, the animal hemisphere thins and stretches by epiboly, driving cells vegetally and translocating the future notoplate cells to the organizer in a domain abutting the prospective notochord (Fetka et al., 2000). These notoplate and notochord precursors are marked by their expression of *Xfd-12'* and *Xbra*, respectively, both of which are dependent on FGF signaling (Smith et al., 1991; Isaacs et al., 1994; Latinkie et al., 1997; Fetka et al., 2000). These tissues are also required for convergent extension movements during gastrulation that are regulated by Wnt signaling (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Fetka et al., 2000; Tada and Smith, 2000). At the onset of gastrulation, prospective notoplate cells undergo extensive mediolateral intercalation to bisect the basal neural plate anlage and position themselves along its ventral midline (Keller et al., 1992).

Induction of the remaining neural plate primordium occurs by vertical organizer-derived signals as the organizer migrates anteriorly beneath the prospective neural plate

during gastrulation. These signals neuralize the ectoderm through inhibition of the default epidermal fate by antagonizing extracellular BMP (reviewed in Weinstein and Hemmati-Brivanlou, 1999). However, ablation of the vertebrate organizer does not result in complete extinction of the neural plate (Harland, 2000), consistent with the observation that additional non-vertical processes acting through the plane continuous with the organizer and neural plate are involved in neural specification and patterning at a time prior to gastrulation (Doniach et al., 1992). Since dorsal ectoderm is already specified to form anterior neuroectoderm by late blastula stages, before the organizer has completely formed or undergone morphogenesis (Sharpe et al., 1987; London et al., 1988; Dixon and Kintner, 1989; Guthrie, 1991), it is possible that the planar signal acts through pregastrula suppression of BMP signaling in dorsal animal cap cells.

Recent studies have found that pregastrula asymmetry in the ectoderm results from dorsally stabilized β-Catenin established following fertilization. Firstly, ectopic Wnt signaling represses transcription of *bmp4* and concomitantly induces neural-specific markers in *Xenopus* ectoderm via a mechanism occurring outside the normal induction of the Wnt-responsive neuralizers, Xnr3 and Siamois (Baker et al., 1999). Thus, these pre-MBT Wnt signals actually repress *bmp* mRNA expression to establish a domain of prospective neuroectoderm in the dorsal animal hemisphere, possibly acting to increase sensitivity to neuralizing signals from the organizer. Secondly, dorsally localized β-Catenin activates early (blastula) expression of extracellular BMP antagonists such as Chordin, Noggin, Follistatin and Xnr3 in a "pre-organizer" region spanning the future organizer and possibly prospective neuroectoderm, independently of mesendoderm

induction (Wessely et al., 2001). This activation is most likely mediated by the early activity of Siamois (Kodjabachian and Lemaire, 2001). Thirdly, cleavage stage Wnt signaling establishes asymmetry in expression of the anti-neural homeodomain protein Dlx3, which becomes localized to ventral ectoderm while being repressed dorsally by early β-Catenin (Beanan et al., 2000). The exclusion of this anti-neural factor from prospective neuroectoderm further adds to the pro-neural bias of pre-gastrula β-Catenin activity, and strengthens the idea that the dorsal ectoderm is predisposed by the corticocytoplasmic rotation.

Therefore, the extracellular inactivation of BMP through planar signaling from the pre-organizer, in conjunction with β-Catenin-dependent intracellular depletion of *bmp* and *dlx3* transcripts, represent the earliest events in neural plate formation. Subsequently, Nodal-related signaling required for generation of the mesendoderm maintains expression of the BMP antagonists in the mature organizer. Their vertical secretion from mesendoderm to overlying prospective neurectoderm during gastrulation is required for maintenance and proper patterning of the pre-specified neural plate. These studies underscore the importance of the combined action of planar and vertical signaling from the organizer to generate a fully patterned nervous system (Brewster and Dahmane, 1999).

### 1.3.2 Neuralization

By the onset of gastrulation, limited anterior-posterior (AP) character is bestowed upon the prospective neuroectoderm (Gamse and Sive, 2001). However, the extensive complexity of the vertebrate nervous system is stabilized and organized by signals

emanating from underlying mesendoderm as it migrates and extends along the AP axis during gastrulation. The non-homogeneous nature of the organizer imparts positional character onto cells of the neural plate by establishing longitudinal and lateral domains of gene expression, which generate forebrain, midbrain, hindbrain and spinal cord (Gamse and Sive, 2001) (Figure 1.2).

Contraction of dorsal bottle cells that initiate upper blastopore lip formation triggers extensive morphogenetic rearrangement of tissues derived from each of the germ layers. AE is driven ahead of these involuting cells (Figure 1.1). In chick, this activity directs cell movements in the overlying ectodermal cells of the anterior-most neural plate that give rise to forebrain (Foley et al., 2000), physically segregating them from the caudalizing influence of the organizer (Foley and Stern, 2001). However, post-blastula ablation of *Xenopus* AE affected heart but not head formation (Schneider and Mercola, 1999), indicating a possible absence of further involvement of this tissue after organizer formation.

The AE may function alternatively in conjunction with the superficial epithelial cells (SBE-suprablastoporal endoderm) directly overlying the dorsal blastoporal lip (Smithers and Jones, 2002), which become internalized during gastrulation to form the anterior archenteron roof and subsequent epithelial lining of the gut (Keller, 1975; Shih and Keller, 1992). The SBE cells were found to transiently express XHex at the onset of gastrulation (Jones et al., 1999) and targeted morpholino knockout of XHex in animal cells generated severe anterior deletions (Smithers and Jones, 2002). Further, morpholino knockdown of Cerberus in the SBE demonstrated its requirement in this tissue for induction of anterior neural character when conjugated with dorsal ectodermal explants

(Silva et al., 2003). Since the SBE is the earliest group of cells to involute and co-migrate with the AE beneath the ectoderm during gastrulation, it may function in combination to impart or maintain anterior identity that has been induced or physically segregated from more posterior tissues by the AE. A similar function was proposed for the corresponding mouse tissue, the anterior definitive endoderm (ADE), whose maintenance properties were believed to compliment the inducing properties of the AVE (Acampora et al., 1995; Beddington and Robertson, 1999; Shawlot et al., 1999; Martinez-Barbera et al., 2000). This cooperative function of these two tissues may explain why extirpation studies failed to show any dependence of the AE on head formation.

Following the AE and SBE, the cells of the presumptive prechordal plate exhibit spreading behavior on the ceiling of the blastocoel to occupy a position beneath the future forebrain (Figure 1.1). Further posteriorly, the chordamesoderm undergoes extensive medio-lateral intercalation which extends the body plan along the AP axis and positions its anterior end beneath the mid-hindbrain and its posterior end beneath the spinal cord (Figure 1.1) (Keller, 1975; Keller, 1976; Keller and Tibbetts, 1989).

Neural induction results from inhibition of BMP signaling mediated via BMPs -2,-4,-7 and GDF6 within the prospective neurectoderm (Hawley et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Dale and Jones, 1999). The organizer provides the inhibitory signal by secreting BMP antagonists as it migrates anteriorly. Thus neural induction can be considered a developmental switch of ectoderm from an epidermal fate to the default neural fate resulting from active suppression of BMP signaling (reviewed in Weinstein and Hemmati-Brivanlou, 1999) and concomitant alteration of the gene expression profiles as shown using microarray analysis (Munoz-Sanjuan et al., 2002). The

organizer-mediated extracellular sequestration of BMPs is stronger along the ventral midline, creating a slight morphogen gradient with the lowest levels of BMP signaling medially, higher levels at the lateral most edges of the neural plate and the highest levels in flanking epidermis. Intermediate BMP signaling is required to specify tissues found at the epidermal-neural border such as the cement gland (Wilson et al., 1997) and neural crest (Marchant et al., 1998).

Inhibition of BMP signaling may not be the only requirement for neural induction. The existence of a constitutive low strength FGF signal in ectodermal cells suggests that it is required for anterior neural induction following BMP depletion. This pre-condition would otherwise be either overcome by high levels of BMP signal for an epidermal fate or act in concert with lower BMP levels for cement gland fate (Hongo et al., 1999).

### 1.3.3 Establishment of Anterior-Posterior Pattern

The induction of neuroectoderm is intertwined with its AP patterning such that by late gastrula a neural plate is determined with a defined AP axis. This process involves a set of events that originate within the late blastula and continues through gastrula stages to progressively define and redefine gene expression patterns that provide positional identity. The dorsal ectoderm at the blastula stage is already specified as an anterior domain, resulting from planar signaling from the "pre-organizer region" (Gamse and Sive, 2001). Once gastrulation is initiated, two domains of AP character are soon established within the presumptive neuroectoderm: an anterior domain expressing the pan-neural marker *opl* and the anterior-specific marker *otx2* (forebrain); and a domain that is

additionally expressing the more posterior marker *fkh5*, but not the hindbrain or spinal cord marker *hoxD1* (Gamse and Sive, 2001). At this stage, however, no stable neural determination has occurred, with presumptive neuroectoderm still capable of forming epidermis (Jacobson and Rutishauser, 1986; Jones and Woodland, 1989; Sive et al., 1989).

By mid-gastrula, the prospective neural plate has become determined (Sive et al., 1989; Sive et al., 1990; Saha and Grainger, 1992), and exhibits a more elaborate AP pattern of three expression domains: an anterior domain (*opl*, *otx2*), a middle domain (*opl*, *fkh5*) and a posterior domain (*opl*, *fkh5*, *hoxD1*) (Gamse and Sive, 2001). Additional positional markers expressed at these time-points further define these domains (Gamse and Sive, 2000) and demonstrate the sequential process of AP patterning that occurs concomitantly with neuralization. Differential exposure to quantitatively and qualitatively different secretory molecules derived from the underlying non-homogeneous organizer induces distinct spatial expression patterns of these positional markers while at the same time cumulatively inducing general pan-neural markers (Gamse and Sive, 2001).

### 1.3.4 Wnt Antagonism Imparts Anterior-Posterior Polarity to the Neuraxis

The transforming signals that provide AP polarity to the neuraxis primarily involve Wnt antagonists (Cer, Dkk-1, Frzb-1, sFRP-1, 2, WIF-1), which are expressed at high levels in the prechordal plate and anterior chordamesoderm (Figure 1.2) (Bouwmeester et al., 1996; Leyns et al., 1997; Wang et al., 1997a; Glinka et al., 1998; Hsieh et al., 1999). Differential exposure to these anterior signals during gastrulation and

upon final positioning under the neural plate generates a gradient of posteriorizing Wnt/β-Catenin signals in the overlying neuroectoderm (Kiecker and Niehrs, 2001b). By late gastrula the Wnt gradient specifies a rudimentary AP pattern within the neuraxis that becomes refined, possibly through secondary cell-cell interactions, during neurula stages (Kiecker and Niehrs, 2001b).

An absent or low level of Wnt signaling specifies the forebrain, while increasing levels of Wnts are required to specify increasingly posterior character. According to this model, the neural plate is innately fated to become anterior forebrain (telencephalon) and must be transformed by Wnt signals to posterior forebrain (diencephalon), midbrain, hindbrain and spinal cord. This process likely involves the interplay of multiple Wnt ligands and their associated antagonists. For instance, paraxial mesoderm derived Wnt8 (Christian and Moon, 1993; Bang et al., 1999) and chordamesodermal Wnt3a (McGrew et al., 1997), both exhibit long range signaling (Kiecker and Niehrs, 2001b) and so may diffuse from these sources to generate a gradient within the gastrula neural plate. In addition, several Wnts are expressed more widely within the dorsal gastrula ectoderm, including Wnt3a (McGrew et al., 1997), Wnt7b (Chang and Hemmati-Brivanlou, 1998) and Wnt8b (Cui et al., 1995), which can act as the posteriorizing signal.

Depletion of the numerous Wnt ligands potentially present in both the head organizer and overlying neurectoderm requires antagonists of differing specificities. Dkk-1, which is both necessary and sufficient for promoting prechordal plate development, binds different Wnt ligands than Frzb-1 (Kazanskaya et al., 2000). This differential affinity enables Dkk-1 to specify the anterior most endomesoderm, which subsequently

patterns the ventral fore-midbrain (Kazanskaya et al., 2000). It is for this reason that Dkk-1, unlike other Wnt antagonists such as Frzb-1, dnWnt8 and Cerberus, in conjunction with BMP inhibitors, induces secondary heads with two eyes rather than one (Bouwmeester et al., 1996; Glinka et al., 1997; Glinka et al., 1998). Once gastrulation has completed, the prechordal plate lies beneath the fore-midbrain where it specifies ventral fate. This is necessary for development of the ventral fore-midbrain cell types and cement gland, and the splitting of the eye field (Li et al., 1997). The importance of Dkk-1 in formation of ventral forebrain also supports the idea that Wnt signaling is involved not only in posteriorization but also dorsalization of the neural tube. Therefore, antagonism of Wnt signaling is required in the neuroectoderm to generate anterior ventral cell types, but must be preceded by proper specification of anterior endomesoderm endowed with the inductive properties of the head organizer.

While inhibition of canonical Wnt/β-Catenin signaling is necessary for specification of both the anterior neural plate and underlying endomesoderm, proper migration and positioning of the prechordal plate or anterior midline cells is also required. This involves a balance between non-canonical Wnt signaling (β-Catenin independent), possibly involving the Wnt 11 class of ligands (Wnts 4, 5a, 11) and their antagonists such as Crescent (Pera and De Robertis, 2000), also known as Frzb-2 (Bradley et al., 2000). In fact, overexpression of Crescent, normally expressed in deep endoderm and prechordal endomesoderm, leads to a loss of ventral brain structures and fusion of the eyes due to disrupted anterior progression of axial mesendoderm during gastrulation (Bradley et al., 2000; Pera and De Robertis, 2000). Crescent appears to regulate non-β-Catenin

dependent signaling by Wnts involved in cell morphogenetic events, implicating a role for Wnts and their antagonists not only in specification, but also migration of prechordal endomesoderm, both of which are necessary to establish a ventro-anterior brain phenotype.

### 1.3.5 Molecular Characteristics of Wnt Antagonists

The spatial restriction of the multiple Wnt ligands along the neuraxis involves antagonists derived from the anterior endoderm (Cerberus, Dkk-1), prechordal endomesoderm/anterior chordamesoderm (Frzb-1, Dkk-1, sFRP-1, 2, WIF-1) and paraxial presomitic mesoderm (WIF-1) (Bouwmeester et al., 1996; Leyns et al., 1997; Wang et al., 1997a; Glinka et al., 1998; Hsieh et al., 1999). The three primary head inducers, Cerberus, Frzb-1 and Dkk-1, each are structurally different, with overlapping yet distinct activities. Frzb-1 belongs to the frizzled-related protein (sFRP) family of secreted proteins that have a cysteine-rich domain (CRD), which is highly homologous to the Frizzled (Wnt-receptor) ligand binding domain (Rattner et al., 1997). This permits direct binding and sequestration of extracellular Wnt8 and Wnt1 proteins (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b). Similar binding also occurs with structurally dissimilar Cerberus and WIF-1, both shown to directly bind Wnt8 (Hsieh et al., 1999; Piccolo et al., 1999).

Dkk-1 is unique in that it shows a distinct mode of action through inhibitory interactions with a Wnt coreceptor, the LDL-receptor related protein (LRP 5/6) (Zorn, 2001). Activation of the intracellular canonical Wnt/β-Catenin is considered to involve Wnt binding to both the Frizzled receptor and membrane bound LRP 5/6 to form a

functional ligand-receptor-coreceptor complex (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; Mao et al., 2001a). Dkk proteins bind to the extracellular domain of LRP 5/6 with a higher affinity than Frizzled and blocks formation of active trimeric Wnt-Frizzled-LRP 5/6 complexes (Balfico et al., 2001; Mao et al., 2001b; Semenov et al., 2001). This unique mode of inhibition may account for the selective specificity of Dkk-1 for Wnt8 and Wnt3a (Glinka et al., 1998; Kazanskaya et al., 2000), recapitulating the importance of the inhibitors having different specificities to cooperatively antagonize multiple Wnt ligands.

### 1.3.6 Wnt Signaling Patterns the Brain

While Wnt signaling must be antagonized to allow proper specification of the forebrain and eyes, there is emerging evidence that components of this pathway are involved in its later patterning (Patapoutian and Reichardt, 2000; Kim et al., 2001). My findings, as described in chapter 3 (Lake and Kao, 2003b), show that the novel Wnt pathway components *Xenopus* Pygopus (XPygo)-2$\alpha$ and -2$\beta$, suggested to mediate Wnt transcription through chromatin remodeling (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), are differentially expressed within the developing brain; *Xpygo-2$\beta$* is expressed within the retinal field and *Xpygo-2$\alpha$* is expressed throughout the prospective brain and eyes. Antisense morpholino (MO) knockdown experiments demonstrated that these differentially expressed *Xpygo-2* isoforms establish unique expression domains of both Wnt-responsive and previously believed Wnt-independent patterning genes required for correct AP patterning of the fore-,

mid-, and hindbrain. Further, only a subset of Wnt regulated markers were affected by antisense knockdown of these isoforms, indicating that not all Wnt-responsive markers require XPygo-2 activity. Thus differential expression along the neural tube of multiple XPygo isoforms may provide additional means of AP patterning by Wnt signaling in addition to variable local Wnt ligand concentrations. This will be discussed further in chapter 3.

### 1.3.7 Non-Wnt Dependent Anterior-Posterior Neural Patterning

Wnt signaling is not the only means of posteriorization, as BMPs, FGFs and retinoic acid (RA) have been implicated in this process (Sasai and De Robertis, 1997; Gamse and Sive, 2000; Altmann and Brivanlou, 2001). Low doses of BMPs posteriorize neuroectoderm (Dale et al., 1992; Jones et al., 1992), but in a Wnt-dependent manner (Kazanskaya et al., 2000). Aside from induction, at low levels, of anterior neural fate coincident with BMP antagonism, organizer derived FGF signaling subsequently imparts anterior-posterior positional identity to neural tissue through a posteriorizing mechanism that is intimately tied to the Wnt/β-Catenin pathway (McGrew et al., 1997; Holowacz and Sokol, 1999; Kazanskaya et al., 2000; Domingos et al., 2001).

Independent of Wnt signaling (Kazanskaya et al., 2000), RA provides positional identity primarily to hindbrain and spinal cord (Blumberg et al., 1997; Kolm et al., 1997). The availability of bioactive retinoids involves the cooperative action of two enzymes, RALDH-2 and CYP26. RALDH-2 is present in the posterior mesoderm of a gastrula embryo with a sharp border of expression at the level of the first anterior somite (Chen et

al., 2001) and generates RA from retinaldehyde. On the other hand, CYP26, present in the gastrula prechordal mesoderm and overlying prospective anterior neural plate (Hollemann et al., 1998; De Roos et al., 1999), breaks down RA and may promote its clearance (White et al., 1996). Together these enzymes generate the appropriate boundaries of RA necessary to establish proper rhombomeric division of the hindbrain. The absence of RA in the anterior neural plate is required to promote appropriate development of this tissue through a mechanism of unliganded RAR-mediated repression of target genes (Koide et al., 2001).

A recent study has provided a model to tie together the posterior-promoting roles of Wnts, FGFs and RA to establish the proper neurectodermal AP pattern (Kudoh et al., 2002). Both FGFs and Wnts suppress anterior genes *otx2* and *cyp26* independently of RA and, through restriction of *cyp26* expression to rostral structures, promote posterior gene expression dependent on RA activity (Kudoh et al., 2002). Therefore, during gastrulation, Wnt and FGF signals were proposed to posteriorize neurectoderm through repression of the RA-suppressing enzyme (CYP26) and provide, through this anterior suppressing mechanism, the correct balance of RA-processing enzymes necessary to establish the AP pattern of RA-dependent neural markers.

### 1.2.8 Establishment of Dorsal-Ventral Pattern

Establishment of the Anterior-to-Posterior (AP) and Dorsal-to-Ventral (DV) neuraxes are independent events, with AP patterning intertwined with neural induction during and preceding gastrulation to establish AP fate at open plate stages. Signaling for

DV fate occurs later, after neural tube closure (reviewed in Altmann and Brivanlou, 2001). Signals from flanking, non-neural ectoderm and underlying mesendoderm establish dorsal to ventral oriented neuronal subtypes along the transversely segmented forebrain, midbrain, hindbrain and spinal cord. The epidermal signals, including BMP and Wnt ligands, initially induce lateral neural plate to give rise to neural crest and roof plate upon neural tube closure, the latter of which acts as a dorsal signaling center to generate a ventrally diffusing gradient of BMP signaling that, in conjunction with Wnt and FGF signals, specifies dorsal neuronal cell types (Lee and Jessell, 1999; Manzanares and Krumlauf, 2000; Altmann and Brivanlou, 2001; Wu et al., 2003).

The ventralizing signal derived from prechordal plate and notochord involves Sonic hedgehog (Shh; Ericson et al., 1995; Hynes et al., 1995; Martí et al., 1995a/b; Roelink et al., 1995; Wang et al., 1995; Chiang et al., 1996; Kohtz et al., 1998) and Nodal-related ligands (Muller et al., 2000; Rohr et al., 2001; Rastegar et al., 2002; Hayhurst and McConnell, 2003) that induce the ventral midline or floor plate. The floor plate subsequently generates a dorsally diffusing Shh gradient within the neural tube that acts as a spatial code for DV homeodomain gene expression needed to specify ventral neuronal progenitors (McMahon, 2000; Altmann and Brivanlou, 2001; Semenov et al., 2001). Work presented in chapter 2 (Lake et al., 2001) suggests that members of the Rel/NF-κB family of transcriptional regulators may be involved in ventral midline patterning. Ectopic expression of Xrel3 results in epidermal tumours that express ventral midline markers *shh* and *gli1* (Lake et al., 2001) and a dominant negative version of Xrel3 caused *shh* and *gli1* RNA-deficient embryos (chapter 2).

While the above paradigm is generally true for DV axial specification along the neural tube posterior to and including the midbrain, patterning of the forebrain seems to be more complex. BMP signaling dorsalizes the posterior CNS and cooperates with Shh to induce ventral fate within the forebrain (Dale et al., 1997). However, Shh retains a consistent role in specifying ventral identity, since it is shown in chapter 2 that a loss of *shh* expression by a Rel/NF-κB inhibitor generates ventral midline abnormalities such as the inability to split the initially single eye field to form two bilateral eyes (Lake et al., 2001), consistent with studies in mouse (Chiang et al., 1996; Hayhurst and McConnell, 2003).

Since the requirement for BMP signaling in the brain is restricted, its expression continues to be suppressed during neurula stages to establish discrete domains of activity that may further define expression boundaries of neural identity genes for specific populations of neuronal progenitor cells (Hartley et al., 2001). In support of this, several inhibitors of *bmp* transcription (BF1, Xiro, Geminin) are expressed in defined domains of the anterior CNS (Kroll et al., 1998; Mariani and Harland, 1998; Gomez-Skarmeta et al., 2001). These may restrict the suppressive activity of BMPs to defined domains that ultimately delineates the expression patterns of numerous neural markers. A similar process may also be involved for Wnt signaling, since gradients of Wnt receptors and antagonists are expressed in the developing mouse telencephalon (Kim et al., 2001) and sequential Wnt and FGF signaling has been shown to specify dorsal character in the chick telencephalon (Gunhaga et al., 2003).

The complexity of the forebrain requires numerous factors to establish its intricate pattern of neuronal populations. Consistently, a novel secreted glycoprotein, Tiarin, has been identified that is expressed in the non-neural ectoderm surrounding the anterior neural plate and acts to dorsalize the neural tube independently of Shh, BMP and Wnt signals (Tsuda et al., 2002). Therefore, multiple signaling pathways collide to pattern the vertebrate CNS along both the AP and DV neuraxes.

### 1.3.9 Secondary Brain Organizers

As described, early planar and vertical signals from the mesendoderm establish the basic AP pattern within the neurectoderm, defining broad territories of the forebrain, midbrain and hindbrain. However, as morphogenesis proceeds, the complex interconnected spatial and temporal neural proteome establishes transverse subdivisions of the forebrain into prosomeres and hindbrain into rhombomeres. In the forebrain, the superimposition on the established AP neuraxis of longitudinally aligned domains created by medial-lateral (ventral-dorsal) patterning generates a checkerboard or grid-like organization of the prosencephalic primordia (Bulfone et al., 1993; Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Hauptmann and Gerster, 2000). Refinement and additional complexity of this fundamental pattern requires planar signals from three secondary organizing centers formed at the junctions of specified territories. Cooperative cellular interactions at these boundaries are believed to produce molecular signals with unique inductive capabilities (Meinhardt, 1983).

The three main organizers present within the vertebrate central nervous system are: the anterior neural ridge (ANR), the zona limitans intrathalamica (ZLI) and the isthmic organizer (IsO). The ANR exists at the junction of the anterior neural plate and non-neural ectoderm (Couly and Le Douarin, 1988; Eagleson et al., 1995), encompassing the first row of neural plate cells in zebrafish (row 1), and is required for anterolateral gene expression within the prospective forebrain (Shimamura and Rubenstein, 1997; Houart et al., 1998; Shanmugalingam et al., 2000). The ANR in mice is characterized by its expression of FGF8 which initiates the expression of BF1 (Shimamura and Rubenstein, 1997), a transcriptional regulator required for growth and regional specification of the telencephalic and optic vesicles (Xuan et al., 1995). Interestingly, Wnt signaling may also play a role in this process since, as shown in chapter 3, *Xbf1* expression was found to be dependent on XPygo-2 within the anterior-neural plate (Lake and Kao, 2003b). This is consistent with recent studies showing coordinated actions of Wnt and FGF signaling in specifying dorsal telencephalic identity in the chick (Gunhaga et al., 2003).

While better characterized in mouse and zebrafish, the ANR recently has been found to play a conserved role in patterning and regionalizing the telencephalon and anterior-most diencephalon in *Xenopus* (Eagleson and Dempewolf, 2002), a region, as in mouse, encompassing neurectoderm anterior to the ZLI (Shimamura and Rubenstein, 1997). The ZLI, in mice, is positioned between dorsal (prosomere 2) and ventral (prosomere 3) thalamus (Rubenstein et al., 1994). This boundary was postulated to act as a morphogenetic barrier to segregate regions of different competence and prevent cell lineage mixing (Martinez et al., 1991; Marin and Puelles, 1994; Larsen et al., 2001) while also acting as a morphogenetic source since it expresses the secreted protein Shh

(Echelard et al., 1993; Puelles and Rubenstein, 1993; Bally-Cuif and Wassef, 1995; Martí et al., 1995a). In chapter 2, it is shown that anterior *shh* expression in *Xenopus* is dependent on the activity of Xrel3 which may localize Shh expression within the ZLI for a role in patterning neighboring forebrain tissues (Lake et al., 2001).

The most well characterized secondary organizer exists at the isthmic constriction between the mes- and metencephalon (mid-hindbrain boundary or MHB), with a well characterized function established in chick, mouse and zebrafish in patterning the midbrain and anterior hindbrain (cerebellum) from the diencephalon/midbrain boundary to and including the first rhombomere (reviewed in Alvarado-Mallart, 1993; Wassef and Joyner, 1997; Martinez, 2001; Rhinn and Brand, 2001). As such, transplantation experiments demonstrated the ability of the IsO to transform caudal prosencephalon to midbrain and rhombencephalon to cerebellum (Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994; Martinez et al., 1995).

Several genes, across multiple species, have been identified as expressed in and required for the development of the IsO (reviewed in Rhinn and Brand, 2001) including those encoding transcriptional regulators (En-1, En-2, Pax2, Pax5, Otx1, Otx2, Gbx2) and secreted proteins (Wnt1 and FGF8). Recent studies have also demonstrated the existence of similar genetic profiles with the associated interconnected dynamics and inductive capabilities in the *Xenopus* nervous system (Ristoratore et al., 1999; Glavic et al., 2002; Tour et al., 2002a; Tour et al., 2002b).

The position of the MHB boundary is established at the intersection of mutually repressive Otx2 and Gbx2 homeoprotein expression domains established during primary

neural induction (Hidalgo-Sanchez et al., 1999; Irving and Mason, 1999; Martinez et al., 1999; Katahira et al., 2000; Garda et al., 2001). Independently these proteins are required for development of the fore-midbrain and hindbrain, respectively (Acampora et al., 1995; Ang et al., 1996; Acampora et al., 1997; Wassarman et al., 1997). However, the convergence of their expression domains, starting at the late gastrula stage in *Xenopus*, enables expression of *Xfgf8* within the overlapping region (Glavic et al., 2002). In other species this is concomitant with or preceded by the independent expression of *pax2* and *wnt1* in a co-localized transverse band (Lun and Brand, 1998; Reifers et al., 1998). Through positive feedback with XGbx2 and negative feedback with XOtx2, XFGF8 propagates the formation of a sharp and exclusive *Xotx2/Xgbx2* boundary (Glavic et al., 2002) as in other vertebrate systems (Hidalgo-Sanchez et al., 1999; Liu et al., 1999a; Martinez et al., 1999; Garda et al., 2001; Liu and Joyner, 2001). This sharp boundary was postulated to maintain FGF8 expression and enable its activation and maintenance of IsO genes, such as *pax2*, *en-1*, *en-2*, and *wnt1* (Crossley et al., 1996; Liu et al., 1999a; Garda et al., 2001). Overexpression of either XOtx2 or XGbx2 generates a caudal or rostral shift, respectively, of the opposing marker's expression domain with the associated alteration of MHB-specific markers (Glavic et al., 2002) reminiscent of studies in mouse and zebrafish (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). Further, XOtx2 was found to be capable of independently inducing a large component of the MHB network with correct spatial positioning along the AP neuraxis dependent on mutually negative regulation with XGbx2 occurring at mid- to late gastrula stages (Tour et al., 2002a; Tour et al., 2002b). Interestingly, the expression of *Xotx2* was also shown in chapter 2 (Lake et

al., 2001) to be dependent on Rel/NF-κB, implicating a key role for the latter in not only specifying fore- midbrain but also the IsO.

Establishment of the abutting *Xotx2/Xgbx2* expression domains and resultant isthmic specification in *Xenopus* further requires the homeoprotein Xiro (Glavic et al., 2002). Xiro maintains *Xotx2* expression in the prospective midbrain by reciprocal activation at the gastrula stage before additionally activating *Xgbx2* expression at the late gastrula/early neurula stage in the future hindbrain (Glavic et al., 2002). Xiro is also necessary for XFGF8 induction at the isthmus and XFGF8 induced expression of *Xen-2* in the *Xotx2* expressing territory (Glavic et al., 2002). En-2 expression is required for growth and polarization of the mesencephalon for proper retinotectal map formation and is regulated by both FGF8 (Lee et al., 1997b; Liu and Joyner, 2001) and Wnt1 (Danielian and McMahon, 1996) mediated by XPygo-2α in *Xenopus* (chapter 3; Lake and Kao, 2003). Like FGF8, En-2 but not Wnt1 has the ability to ectopically induce the IsO genetic complement (Dickinson et al., 1994; Ristoratore et al., 1999). This activity is necessary for a later maintenance phase whereby the reciprocal associations between En-2, FGF8, Pax2, and Wnt1 are required for their continued expression (Reifers et al., 1998; Liu et al., 1999a; Shamim et al., 1999; Liu and Joyner, 2001).

Once established at the proper position, the IsO not only patterns both the midbrain and anterior hindbrain, but also acts as a mitogenic source to control the size and shape of the adjacent tissues through secretion of Wnt1 from the midbrain/*otx2* side and FGF8 from the hindbrain/*gbx2* side (reviewed in Martinez, 2001). Therefore, the complex interconnected genetic network required for the initiation and maintenance of

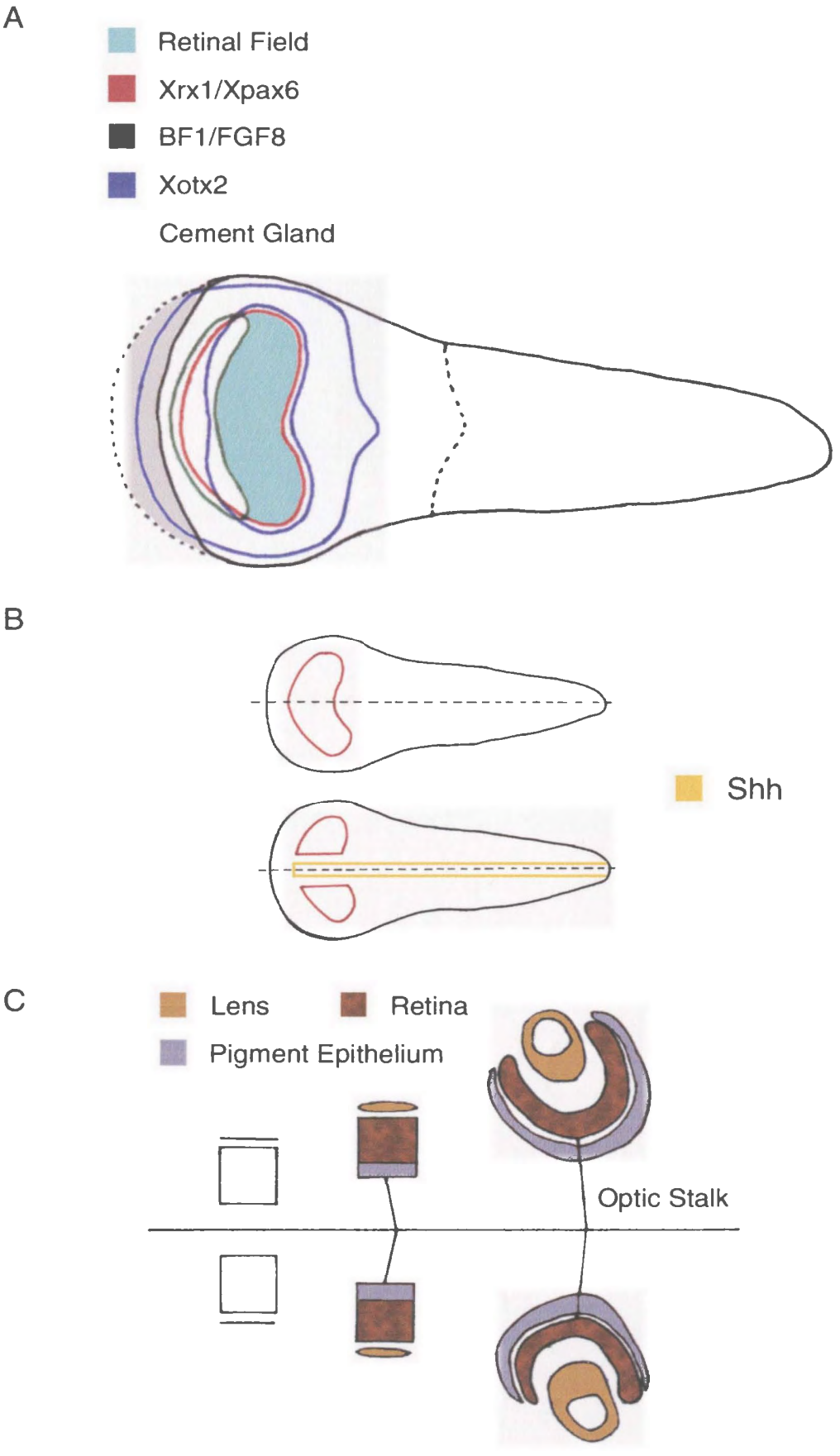
the IsO induces the cellular diversity of mid- and anterior hindbrain for development and outgrowth of higher order brain structures: the retinotectal map and cerebellum.

### 1.3.10 Formation of the Vertebrate Eye

One of the earliest regions specified from the anterior-most neurectoderm is the eye field, which evaginates from the prospective forebrain during folding of the neural plate to become the optic primordia and subsequently the optic vesicles (reviewed in Jean et al., 1998; Lupo et al., 2000; Chow and Lang, 2001). A complex genetic network set up during early induction of the neural plate establishes within its anterior-most region a continuous crescent shaped area predestined to form the bilayered optic cup (retinal pigmented epithelium and retina) laterally and the optic stalk (optic nerve) medially (Figure 1.3; Eagleson et al., 1995). Coincident with this is the induction of the lens placode by the optic vesicle from overlying ectoderm and its subsequent thickening and invagination to form the lens vesicle (Figure 1.3C). Numerous genes in *Xenopus*, among other species, were found to orchestrate this complex growth and morphogenetic process, including: *Xpax6*, *Xotx2*, *Xrx1*, *Xsix3* and *Xoptx2/six6* (reviewed in Lupo et al., 2000).

*pax6*, a paired class homeobox gene, was proposed to be the master eye gene expressed at late gastrula stages (Hirsch and Harris, 1997; Li et al., 1997) and capable of inducing ectopic eyes in *Xenopus* (Chow et al., 1999; Kenyon et al., 2001). However, mice deficient of *pax6* only exhibited severe eye abnormalities, with the loss of the lens and the formation of a malformed optic cup (Callaerts et al., 1997; Treisman, 1999). These studies indicated more of a role in imparting competence to non-neural ectoderm to

**Figure 1.3. Specification and morphogenesis of the eye.** A. Topological map of a molecular network specifying the retinal field (light green) of a mid-neurula *Xenopus* embryo as modified from Lupo et al., 2000. Indicated are the expression domains of *Xrx1/Xpax6* (red), *Xbf1/Fgf8* (green), *Xotx2* (between concentric blue limits only), and *XAG* (cement gland territory, gray). B. Initiation of *shh* expression (yellow) within the medial neural plate at mid- to late neurula stages will bisect the initially single eye field (red- *Xpax6/Xrx1*; top embryo) into two lateral eye fields (bottom embryo). C. Schematic representation of bilateral eye development (as modified from Wittbrodt et al., 2002). Signals from the midline which split the eye field (Shh) to specify optic stalk also establish proximal-distal identity within retinal primordia to specify territories fated to form retinal neurones (dark orange) and pigmented epithelium (gray). Extensive proliferation of the optic vesicle and invagination to form the optic cup concurrent with invagination of the lens vesicle (light orange) establishes the eye structure, with nerve axons projected along the optic stalk to the optic tectum of the midbrain.


A
Retinal Field
Xrx1/Xpax6
BF1/FGF8
Xotx2
Cement Gland
B
Shh
C
Lens
Retina
Pigment Epithelium
Optic Stalk

respond to optic vesicle signals in lens induction than a solitary role in initiating early eye development. Therefore, additional genes had to be involved. Both *Xsix3* and *Xoptx2/six6* are expressed within the early eye field overlapping that of *Xpax6*, with *Xsix3* expressed earlier and both shown in numerous species to be required for and capable of ectopically inducing eye formation (Kobayashi et al., 1998; Toy et al., 1998; Andreazzoli et al., 1999; Loosli et al., 1999; Wallis et al., 1999; Zuber et al., 1999; Bernier et al., 2000). *Xotx2* is also expressed within the prospective neurectoderm, initiated at earlier gastrula stages than *Xpax6*, and becomes expressed later in the developing retina (Acampora et al., 1995; Blitz and Cho, 1995; Matsuo et al., 1995; Kablar et al., 1996; Perron et al., 1998; Andreazzoli et al., 1999) with the capacity of inducing retinal fate (Kenyon et al., 2001). Unlike *Xotx2*, *Xrx1* becomes expressed later in gastrulation in a region overlapping *Xpax6*/*Xsix3* (Casarosa et al., 1997; Mathers et al., 1997) and actively represses *Xotx2* expression at early neurula stages to create almost completely mutually exclusive or complimentary expression domains (Andreazzoli et al., 1999). Like XPax6 and XOtx2, overexpression of XRx1 generates ectopic retinal tissue (Mathers et al., 1997; Andreazzoli et al., 1999; Kenyon et al., 2001).

The combined expression of these markers at early neurula stages regionalizes the neural plate into retina and diencephalic territories (region expressing *Xrx1*, *Xpax6*, *Xsix3* but not *Xbf1* and *Xotx2*) and the telencephalic territories (region expressing *Xrx1*, *Xbf1*, and *Xotx2*) (Figure 1.3A; Lupo et al., 2000). Studies in *Xenopus* have further implicated a role for Wnt signaling in early specification of the eye field through regulation of these markers, with the Frizzled 3 receptor (XFz3) capable of ectopically inducing *Xpax6*, *Xrx1* and *Xotx2* expression and eye formation (Rasmussen et al., 2001). Alternatively,

antagonism of XFz3 activity prevented eye formation (Rasmussen et al., 2001). Consistently, shown in chapter 3, the knockdown of an intracellular mediator of Wnt signaling, XPygo-2, generated eye-deficient phenotypes with the inhibition of *Xpax6* and *Xrx1* expression, but not *Xotx2* (Lake and Kao, 2003b).

Following the regional specification of the prospective eye from adjacent forebrain territories, subsequent events proceed to split this initially single field into two bilateral fields to restrict retinal development to the optic cups (Figure 1.3B; Li et al., 1997) coincident with proximal-distal patterning (McDonald et al., 1995; Hammerschmidt et al., 1996). This phase involves the morphogen Shh, expressed in the ventral midline of the neural tube, which regulates expression of paired-homeobox genes of the *pax*, *nkx*, *dbx* and *irx* families to establish DV polarity (McMahon, 2000; Altmann and Brivanlou, 2001; Semenov et al., 2001). Shh activates *nkx2.1/ nkx2.2* and *pax2* expression to promote ventral forebrain (diencephalon) and proximal eye (optic stalk) identity while concomitantly antagonizing *pax6* expression (retina, pigmented epithelium and lens) (Barth and Wilson, 1995; Ekker et al., 1995; McDonald et al., 1995; Chiang et al., 1996; Hammerschmidt et al., 1996; Zhang and Yang, 2001). As such, loss of Shh function in humans (Belloni et al., 1996; Roessler et al., 1996; Roessler et al., 1997; Nanni et al., 1999; Wallis and Muenke, 2000), mouse (Chiang et al., 1996; Hayhurst and McConnell, 2003), chick (Zhang and Yang, 2001) and possibly *Xenopus* (chapter 2, Lake et al., 2001) generates hypoteloric to cyclopic phenotypes characterized by the progressive loss of proximal eye structures (optic stalk), extension and possible fusion of retinas medially and the absence of ventral brain structures. Therefore, through regulation of markers involved in ocular tissue specification and dorsal-ventral patterning, Shh plays

a vital role in establishing the correct spatial position of eye and forebrain derivatives. Furthermore, it has been proposed in chick and *Xenopus* that, like in the neural tube, ventral Shh and dorsal BMP signals antagonistically regulate outgrowth and DV specification of the eye (Crossley et al., 2001; Zhang and Yang, 2001; Ohkubo et al., 2002; Sasagawa et al., 2002), with Shh expression potentially dependent on Rel/NF-κB (chapter 2; Lake et al., 2001) and Shh function potentially dependent on FGF signaling (Russell, 2003).

## 1.4 THESIS OVERVIEW

The Rel/NF-κB and Wnt/β-Catenin pathways are both intimately associated with embryonic development and oncogenesis. Recent studies have shown a direct association between these pathways, with antagonistic interactions between NF-κB and β-Catenin (Deng et al., 2002; Masui et al., 2002). The major hypothesis of this thesis is that the convergence of the neural patterning functions for both signaling cascades is required to establish the complex diversity of the vertebrate central nervous system. These studies outline the independent requirements for both pathways for a common developmental process, the patterning of the brain, as demonstrated through knockout and overexpression studies. Chapter 2 outlines the dependency of Rel/NF-κB target gene expression (*otx2, shh, gli1* and *frzb-1*) for anterior neural development. Each of these genes have established roles in patterning the brain, specifying both transverse (*otx2, frzb-1*) and longitudinal (*shh, gli1*) neuronal subdivisions. Chapter 3 describes the fundamental requirement of two intracellular Wnt pathway components, XPygo-2α and XPygo-2β, in formation of the fore-, mid-, and hindbrain. These proteins were found to play different spatial roles in brain patterning that were consistent with their unique patterns of message accumulation. While both proteins clearly mediated, in part, stereotypical Wnt-mediated posteriorization, they also appeared to perform additional functions in patterning the forebrain that challenged the dogma for Wnt-dependent AP patterning. In chapter 4, the analogous expression of hPygo-2 was examined to verify a conserved role in brain patterning. Further, hPygo-2 was expressed in a number of tissues requiring Wnt signaling for development, as well as tissues exhibiting susceptibility to cancer-causing

pathway mutations or deregulation. Finally, in chapter 5, a model for embryonic patterning of the central nervous system is proposed incorporating previous work with that presented in this thesis.

# CHAPTER 2:

## *Rel/NF-κB Is Required For Head Formation In Xenopus Laevis*

**Running Title:** *Xenopus rel3*

## 2.1 ABSTRACT

Several *Rel/NF-κB* genes have been identified in *Xenopus*. Only a few, however, including *Xrel2* (Tannahill and Slack, 1995) and *Xrel3* (Yang et. al., 1998) have been shown to possess spatially restricted expression patterns. *Xrel3* displays a particularly interesting expression pattern in the developing embryo (Yang et. al., 1998). *Xrel3* messages are present in cleavage and blastula equatorial cells, but accumulate after gastrulation in notochord and prospective brain tissues. These observations suggested that Xrel3 plays a role in pregastrula embryogenic events as well as in the development of the brain. As a member of the large Rel/NF-κB family of DNA-binding transcriptional regulators, Xrel3 was predicted to activate and/or repress the expression of neural patterning genes during morphogenesis of the neural tube. To address this potential function, a C-terminal truncated form of Xrel3, called Xrel3Δ58, was created which dimerized with and prevented the ability of wild-type Xrel3 to bind DNA. When overexpressed in embryos, Xrel3Δ58 prevented head formation, causing a loss of fore-midbrain structures and disorganized hindbrain tissue. Further, there was a fusion or loss of retinal tissues and the loss of expression of important forebrain (*Xotx)* and midline (*shh, gli1*) neural patterning genes, demonstrating their requirement for normal Rel/NF-κB activity in the prospective brain. The dependency for these markers to generate proper neural patterning along both the anterior-posterior (*Xotx2*) and dorsal-ventral (*shh, gli1*) axes demonstrated the importance of Rel/NF-κB signaling in establishing the correct 3-dimensional arrangement of neuronal precursors within the brain.

## 2.2 INTRODUCTION

### 2.2.1 The Rel/NF-κB Family

Rel/NF-κB transcriptional regulators enable rapid intracellular responses to physiological stimuli involved in development and differentiation, immunity, apoptosis, and oncogenesis (reviewed in Pahl, 1999; Perkins, 2000; Gilmore et al., 2002; Li and Verma, 2002; Bell et al., 2003). Their diverse cellular roles are manifested by the ability for multiple family members (Table 2.1) to form homo- or heterodimeric associations and bind cognate regulatory enhancers or κB elements (Chen and Ghosh, 1999) specific to a plethora of tissue-specific target genes (Chen et al., 1999b; Pahl, 1999).

All Rel/NF-κB family members contain a highly conserved N-terminal rel homology (RH) domain that allows them to bind DNA (Coleman et al., 1993; Toledano et al., 1993). This is achieved through two N-terminal β-sheet immunoglobulin folds that adopt a butterfly-like conformation and are thought to straddle DNA within its major groove (Ghosh et al., 1995; Müller et al., 1995; Cramer et al., 1997; Chen et al., 1998a/b). Also located at the C-terminus of the RH domain are sequences mediating homo- and heterophilic protein-protein interactions (Chen and Ghosh, 1999) that bring DNA binding residues from each dimer subunit in close apposition. This assembly of subunits forms a single DNA-binding region that confers both binding specificity and directionality of heterodimeric complexes for different κB elements (Verma et al., 1995; Ghosh et al., 1995).

**Table 2.1. Rel/NF-κB family members**

| Protein | Alternative Nomenclature | Gene | Organisms Found |
|---|---|---|---|
| **Class I:** | | | |
| p50 or p105 (NF-κB1) | p110, KBP1, EBP-1 | *nfkb1* | Human, Mouse |
| p52 or p100 (NF-κB2) | p50 or p97, p49 or p100, p55 or p98, Lyt10, H2TF1, Xp100 | *nfkb2, Xp100* | Human, Mouse, *Xenopus* |
| **Class II:** | | | |
| Rel | c-Rel | *rel* | Human, Mouse, Chicken |
| v-Rel | - | *v-rel* | Reticuloendotheliosis Virus Strain-T |
| RelA | p65, XrelA | *rela, XrelA* | Human, Mouse, *Xenopus* |
| RelB | I-Rel, XrelB | *relb, XrelB* | Mouse, *Xenopus* |
| dorsal | - | *dorsal* | *Drosophila* |
| Dif, Cif | *d*orsal-related *i*mmunity *f*actor *c*ecropia *i*mmunoresponsive *f*actor | *dif* | *Drosophila* |
| Xrel2 | - | *Xrel2* | *Xenopus laevis* |
| Xrel3 | - | *Xrel3* | *Xenopus laevis* |

(as per Blank et al., 1992; Siebenlist et al., 1994)

While the κB sequence depicts the type of Rel/NF-κB dimers that bind, there is redundancy that enables association with more than one dimer type. A recent study has demonstrated, however, that the exchange of dimers composed of different constituents and having differing activities at a single enhancer site can modulate the response a cell makes to Rel/NF-κB stimulation (Saccani et al., 2003). The differential upstream regulation of each dimer provided a means for sustained activation (Saccani et al., 2003). Therefore, further complexity than simple DNA binding by a dimer is provided by the inherent redundancy within this signaling pathway.

A distinguishing feature of the Rel/NF-κB family is the regulation of their subcellular localization and, as such, their post-translational activity (reviewed in Karin, 1999; Ghosh and Karin, 2002). In unstimulated cells, Rel/NF-κB is sequestered and maintained inactive in the cytoplasm by IκB inhibitors which bind and shield the nuclear localization sequences present within the RH domains of the dimer subunits. Extracellular stimuli trigger the phosphorylation and activation of IκB kinases (IKKs). These in turn phosphorylate IκB marking it for proteosomal degradation which unmasks the NLS and enables Rel/NF-κB proteins to migrate to the nucleus (May and Ghosh, 1998; Karin, 1999; Chen and Ghosh, 1999; Pahl, 1999).

While the RH domain characterizes the Rel/NF-κB family, individuality is determined by the variability of sequences C-terminal to this region. Family members are broadly divided on this basis into two classes, those having auto-inhibitory ankyrin repeats requiring proteolytic cleavage for activation (Class 1, Table 2.1), or those having a highly variable C-terminal trans-activation domain (TAD) (Class 2, Table 2.1). This

latter domain is typically a phosphorylation dependent serine-rich acidic region that mediates protein-protein interactions required to activate or repress transcription (Schmitz et al., 1994; Schmitz et al., 1995; Wang and Baldwin, 1998; Chen et al., 1999a; Fognani et al., 2000; Martin and Fresno, 2000; Ashburner et al., 2001; Martin et al., 2001).

Aside from a key role in modulating stress responses of a cell to environmental stimuli (Pahl et al., 1999; Li and Verma, 2002), Rel/NF-κB proteins are also involved in early embryonic patterning events. In *Drosophila*, specification of dorsal-ventral (DV) polarity requires the graded nuclear activity of the Dorsal morphogen along the prospective DV axis of the early embryo (Govind, 1999). This gradient is established by post-translational control over nuclear import of Dorsal by Cactus, a member of the IκB protein family. Orthologous upstream regulators of Dorsal can also activate secondary dorsal axes in *Xenopus* (Armstrong et al., 1998), demonstrating their potential conservation in DV patterning in vertebrates. However, mouse knockout mutants have, as yet, failed to implicate a conserved role for NFκB1, NFκB2, c-Rel, RelA or RelB (Table 2.1) in early embryonic body axis formation. In these studies NF-κB1, c-Rel and RelB are required for development of the immune system and RelA for the liver (reviewed in Attar et al., 1997; Gerondakis et al., 1999).

Several *Xenopus Rel/NF-κB* genes have been identified to date, including *XrelA* (*Xrel1*) (Kao & Hopwood, 1991; Richardson et al., 1994), *XrelB* (Suzuki et al., 1995), *Xrel2* (Tannahill & Wardle, 1995), *Xp100* and *Xp52* (Suzuki et al., 1998), as well as *Xrel3* (Yang et al., 1998). Studies on *Xenopus* embryos have suggested that Rel/NF-κB proteins may play a role in axial patterning of higher vertebrates (Kao and Lockwood, 1996;

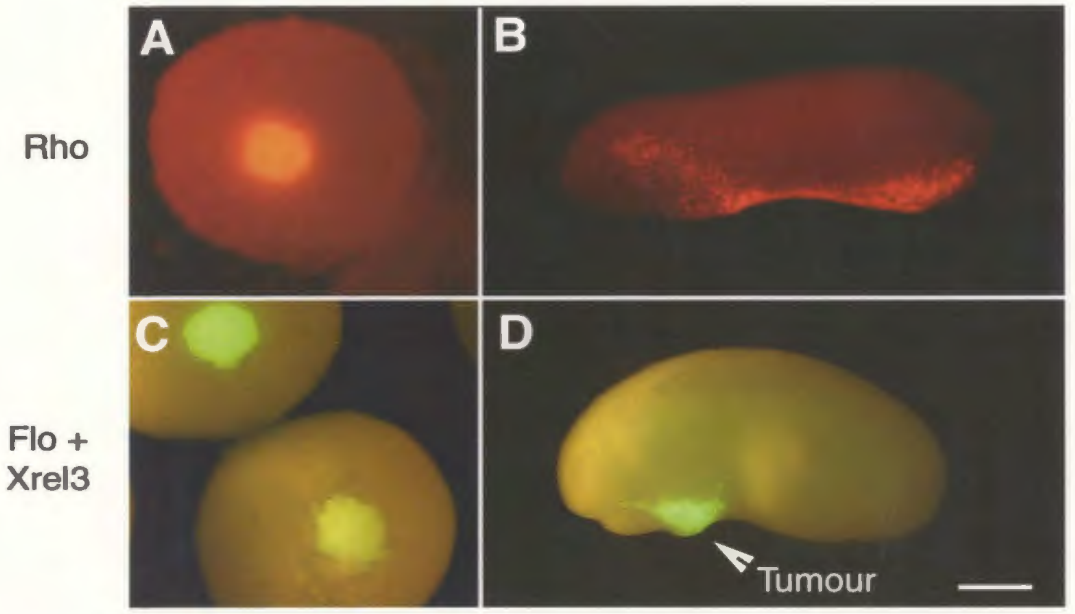
Armstrong et al., 1998). One potential candidate mediating this is Xrel3 (a Class 2 member, Table 2.1), whose messages are present in oocytes and early blastula embryos but not during gastrulation (Yang et al., 1998). After gastrulation, new messages accumulate in the notochord and anterior neural plate encompassing prospective forebrain, mid-hindbrain and otic placode of the early neurula stage embryos and the forebrain, dorsal mid-hindbrain and otocysts of later tadpole stage embryos (Yang et al., 1998). This expression pattern implicated an additional and later role than axial patterning in the development of the anterior nervous system.

Previous gain-of-function studies found that ectopic expression of *Xrel3* in the animal pole led to the development of abnormal epidermal growths in early neurula-stage embryos (Yang et al., 1998). Cells overexpressing Xrel3 appeared undifferentiated and became unable to migrate normally or contribute progeny to the epidermis later in development (Figure 2.1, K.R.K., unpublished observations). The cells continued to divide, forming masses on late gastrulae that remained on the flank of tailbud and early tadpole embryos before regressing during later tadpole stages. These tumours expressed neural patterning markers such as *otx2*, *shh* and *gli1* at the time of their normal endogenous expression (Lake et al., 2001) and closely resembled tumours resulting from overexpression of Gli1 (Dahmane et al., 1997).

### 2.2.2 Otx Family of Homeoproteins

The Otx proteins comprise a family of homeobox containing transcriptional regulators that are homologous to *Drosophila* orthodenticle (Otd). These proteins play

**Figure 2.1. Xrel3-expressing cells do not participate in normal ectodermal cell differentiation.** Animal pole cells were grafted into uninjected embryos from either embryos injected with Rhodamine (A,B) or *Xrel3* mRNA in conjunction with Fluorescein (C,D) (K.R.K., unpublished observations). Xrel3-expressing cells formed a tumourous mass and were unable to distribute progeny throughout the epidermis at tadpole stages (D, arrow) compared with control cells (B).


A
B
Rho
C
D
Flo +
Xrel3
Tumour

essential roles in mesendoderm specification and neurectodermal patterning (reviewed in Simeone et al., 2002). In *Xenopus*, *otx2* is initially expressed in the head organizer region of the mesendoderm where it confers head inducing activity by cooperatively regulating *cerberus* expression (Yamamoto et al., 2003) and repressing posterior identity (Andreazzoli et al., 1997) and posterior-type cell migratory behavior (Morgan et al., 1999). Subsequently, as shown in numerous species, *otx2* is expressed in the anterior-most region of the newly formed neural plate, where it plays a role in specifying prospective fore-midbrain and retinal tissues (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Gammill and Sive, 2001; Kenyon et al., 2001; Martinez-Morales et al., 2001; Martinez-Morales et al., 2003; Viczian et al., 2003). The rostral-most boundary of *otx2* expression also cooperates with intermediate levels of BMP signals at the neurectodermal/ectodermal border to induce the cement gland (reviewed in Wardle and Sive, 2003). Further, the caudal limit of *otx2* expression, as determined by antagonistic associations with the hindbrain marker *gbx2* during late gastrula and early neurula stages, defines the spatial position of the isthmic organizer for patterning of the mid-hindbrain (reviewed in chapter 1).

### 2.2.3 Shh/Gli Signaling Pathway

The Shh pathway involves the membrane bound proteins Patched (PTCH) and Smoothened (SMO) as well as the zinc-finger transcription factors Gli/Cubitus interruptus (reviewed in Ingham and McMahon, 2001; Koebernick and Pieler, 2002). Mutations in this pathway have been implicated in sporadic basal cell carcinomas, gliomas and

primitive neuroectodermal tumors of the central nervous system including medulloblastomas (Wolter et al., 1997; Ming et al., 1998; Taipale and Beachy, 2001; Ruiz i Altaba et al., 2002b). Over-representation of Shh or mutations of human *patched* is consistently associated with Nevoid Basal Cell Carcinoma Syndrome, one of the most common forms of human cancer (Hahn et al., 1996; Fan et al., 1997). These gain-of-function mutations have the common effect of increasing expression of the Shh transcriptional effector Gli1, predicted to be the primary oncogenic factor in both epidermal and neural tumors (Dahmane et al., 1997; Dahmane et al., 2001).

Normally, Shh is involved in morphogenesis, growth and patterning of numerous tissues, including the CNS, somatic and cardiac muscle and limbs (reviewed in Ingham and McMahon, 2001). Within the nervous system, Shh is further required for proliferation and survival of neuronal precursors, specification of ventral neurones and oligodendrocytes, control of axonal growth and morphogenesis, as well as the growth and patterning of the eye (reviewed in Chapter 1; Ingham and McMahon, 2001; Martí and Bovolenta, 2002; Ruiz i Altaba et al., 2002a). As such, Shh is expressed within a number of organizing centers in the embryo to cooperatively pattern the appropriate tissue fields in conjunction with additional secreted signaling factors such as FGFs and BMPs (Ye et al., 1998; Carl and Wittbrodt, 1999; Briscoe et al., 2000; Crossley et al., 2001; Martinez, 2001; Ohkubo et al., 2002).

### 2.2.4 Xrel3 regulates *otx2/shh/gli1* to pattern the frog brain

The normal post-gastrula expression pattern of *Xrel3* as well as its ability to activate the expression of *otx2*, *shh* and *gli1* ectopically in non-neural ectoderm suggested that it might also activate expression of these markers in the developing neurectoderm. To test this hypothesis, I designed an Xrel3 deletion construct (Xrel3Δ58) which antagonized wild-type Xrel3 DNA binding activity *in vitro*. The purpose for making this construct was to create a molecular antagonist that would interfere with the normal developmental function of endogenous Xrel3. Based on my predictions, Xrel3Δ58 would reduce in embryos expression of the markers Xrel3 activated in tumours. Consistently, when injected within the prospective nervous system, the inhibitory truncated protein depleted *otx2*, *shh* and *gli1* messages and caused the associated head abnormalities analogous of knockout mutants of each gene seen in other species. Therefore, this chapter presents evidence for a required role played by *Xenopus* Rel/NF-κB proteins in patterning and expansion of the central nervous system.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Oocytes and Embryos

Wild-type embryos were obtained and injected as described previously (Kao and Lockwood, 1996; Yang et al., 1998) and staged according to Nieuwkoop and Faber (1994). Capped, synthetic RNA was derived from the Ribomax Kit (Promega) and as described previously (Kreig and Melton, 1987). Embryos were injected with up to 1 ng of RNA on either side of the first cleavage plane of 2- or 4-cell stage embryos in either the animal pole or marginal zone. To distinguish the dorsal marginal zone, embryos were tilted within 40 minutes after fertilization, with sperm entry (ventral) sides facing towards gravity as described (Kao and Lockwood, 1996). Following cleavage, embryos were injected within the equatorial upward facing region to target dorsal derivatives, or rotated 180° for ventral derivatives.

### 2.3.2 Plasmids

The full length *Xrel3* coding sequence was previously cloned into pCS2+ (Yang et al., 1998). For Xrel3RHD and Xrel3Δ58 constructs the associated coding sequences were PCR amplified (for cloning primers see Table 2.2) from pCS2+*Xrel3* and ligated into either pCS2+ or pCS2+MT (gifts from Dave Turner). For myc-tagged Xrel3, the corresponding coding region was digested from pCS2+*Xrel3* and ligated in frame into pCS2+MT.

**Table 2.2. RT-PCR and Cloning Primer Sequences**

| Primer | Forward (5'-3') | Reverse (5'-3') | N* | Reference |
|---|---|---|---|---|
| *otx2* | CGGGATGGATTTGTTGCA | TTGAACCAGACCTGGACT | 25 | Pannese et al., 1995 |
| *frzb-1* | AGTAAGCCTACACATACAG GTTGG | GCAGACTCCTCTTCTGTCAT ATACGG | 25 | Wang et al., 1997 |
| *goosecoid* | GAGCAAAGTGGAGGAGGCA G | CCCACATCGTGGCACTGCTG | 25 | --- |
| *lim1* | ACTGACTTCTTCAGGAGATT TGG | GTTCCTCGCCTGTTGAGAGC | 30 | Glinka et al., 1997 |
| *blimp1* | AAGATTATGCAGAAAGGGA GGG | GAAAGGAGAAATACAGAGA AGGGG | 30 | De Souza et al., 1999 |
| *Dkk-1* | ACAAGTACCAACCTCTGGAT GC | ACAGGGACACAAATTCCGTT GC | 30 | Glinka et al., 1998 |
| *derriere* | TGGCAGAGTTGTGGCTATCA | CTATGGCTGCTATGGTTCCT T | 25 | Sun et al., 1999 |
| *calponin-H3* | CACCAATGGACCATTCCACC | GGTCGTAATGGCAATGTCGC C | 25 | Morgan et al., 1999 |
| *noggin* | GCTCTGATGGTCTTCTTGGG | CAGCATGAGCATTTGCACTC | 25 | --- |
| *hex* | TTCACCCTGCCTTCACCCAC CC | TTCTGCTCGGCGCTCAAACA CC | 30 | De Souza et al., 1999 |
| *cerberus* | GCTTGCAAAACCTTGCCCTT | CTGATGGAACAGAGATCTTG | 25 | Heasman et al., 2000 |
| *Xnr3* | CGAGTGCAAGAAGGTGGAC A | ATCTTCATGGGGACACAGG A | 30 | Agius et al., 2000 |
| *Xrel3* | GCTGAGCTGAGGATATGCC G | GCCTCGAGTTACTGCATCAC TTCTGAGGTC | 25 | --- |
| *H4* | CGGGATAACATTCAGGGTAT CACT | ATCCATGGCGGTAACTGTCT TCCT | 23 | Yang et al., 1998 |
| *Xrel3Δ58* | CGGAATTCCATGGCCGGTTT AAACG | GCCTCGAGTTACTGGGTGGT AACTAAATGG | --- | --- |
| *Xrel3RHD* | CGGAATTCCATGGCCGGTTT AAACG | GCCTCGAGTTACTGCATCAC TTCTGAGGTC | --- | --- |

*Number of annealing/elongation cycles used

### 2.3.3 Western Blot Analysis

RNA encoding myc-tagged Xrel3, Xrel3Δ58 and Xrel3RHD were injected at the 2-cell stage into animal pole cells. Embryos were subsequently macerated at stages 8, 10 and 13 in Triton Medium (10 mM Tris pH 7.5; 1% Triton-X 100; 10 mM EDTA; 0.002% Na-azide, Methionine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg/ml Nor-P-tosyl-L-lysine chloromethyl ketone). To reduce the interfering effects of yolk protein, the samples were extracted in 1,1,2-trichlorotrifluoroethane, acetone precipitated and resuspended in loading buffer (0.125 M Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 20% glycerol). Protein was then run on 7-10% SDS-PAGE, transferred to nitrocellulose membranes (Hybond-ECL™; Amersham) blotted with the anti-myc antibody (9E10 cell supernatant, purchased from DSHB, Iowa) and visualized using the associated enhanced chemiluminescence. Quantity of protein loaded was normalized using Biorad assay reagent.

### 2.3.4 RT-PCR Analysis

RNA was extracted from whole embryos (8-10 per injection group) using the Nucleospin RNA II Kit (Clontech Laboratories, Inc.) and reverse transcribed using MMLV reverse transcriptase (Promega). Primers used in the analysis are listed in Table 2.2. The cycling parameters used included: 4 minute denaturation at 94°; 45-60 second hybridization at 60° followed by equal duration of elongation at 72° and denaturation at 94° (total number of cycles varied by primer, see Table 2.2); lastly, a 45-60 second

annealing at 60° was proceeded by a 7-10 minute elongation at 72° before completion. All PCR products were electrophoresed on 1.5% agarose gels and photographed by a Chemiimager. Equal levels of cDNA used within the RT-PCR reactions were achieved by normalization to *histone (H4)* levels.

### 2.3.5 *In Vitro* Translation

Wild-type and truncated Xrel3 proteins were synthesized *in vitro* using the SP6 transcription/translation coupled rabbit reticulocyte lysate system (Promega). For protein used in immunoprecipitation: 17 μCi of [$^{35}$S]-methionine (1200 Ci/mmol; Mandel) and either 2 μg of pCS2+*mtXrel3* alone or 1 μg each of pCS2+*mtXrel3* and pCS2+*Xrel3* or pCS2+*mtXrel3* and pCS2+*Xrel3Δ58* to a total of 2 μg was added to the reticulocyte lysate to a total volume of 50 μl and incubated at 30°C for 90 minutes. For EMSAs involving cold κB competitive binding, 2 μg of pCS2+*Xrel3RHD* and pCS2+*Xrel3Δ58* were used for translation as above. However, for EMSAs involving competitive inhibition by Xrel3Δ58 of Xrel3 DNA binding, 1 μg of pCS2+ *Xrel3* or pCS2+*Xrel3Δ58* was used alone and 1 μg of pCS2+*Xrel3* was used in combination with either 0.5 μg, 1 μg or 2 μg of pCS2+*Xrel3Δ58*. These latter protein products were examined by SDS-PAGE and levels analyzed by spot densitometry (ChemiImager$^{tm}$4000; Alpha Innotech Corporation). Xrel3 protein levels were found to be consistent between individual and cotranslated samples while Xrel3Δ58 levels were approximately 0.6x, 0.9x and 1.3x that of Xrel3 in

the cotranslated samples or, adjusting for the proportional decrease in the number of methionines after deletion of the last 58 amino acids, 0.7x, 1.0x and 1.5x that of Xrel3.

### 2.3.6 Electrophoretic Mobility Shift Assays (EMSA)

Double stranded κB DNA elements (5 pmoles, Table 2.3) were forward labeled using T4 polynucleotide kinase (10 units; GibcoBRL) and 1 μCi/μl [$\gamma^{32}$P]-dATP (Mandel) in a total volume of 25 μl for 10-30 minutes at 37°C. Between 150,000 and 500,000 cpm of κB probe was incubated with 1 μl of *in vitro* translated protein for 20 minutes at room temperature according to Mavrothalassitis et al. (1990). Samples were then electrophoresed onto a 5% non-denaturing polyacrylamide mini-protein gel for 50 minutes (100V) at room temperature. Competition of protein binding to labeled κB sequences in testing specificity and affinity involved addition of labeled κB DNA as well as 2, 5, 10, 20, 100 or 1000 ng of unlabeled κB sequence to the protein/DNA binding mixture and proceeding as described.

### 2.3.7 Immunoprecipitation

Xrel3 fused with the myc epitope was either translated alone or cotranslated with Xrel3 or Xrel3Δ58. To demonstrate dimeric protein complexes, 10 μl of the *in vitro* translated proteins were covalently linked by incubating with glutaraldehyde, used in numerous studies to test for potential oligomerization (Wang and Lemon, 1993; Rossini and Camellini, 1994; Antoshechkin et al., 1997; Raab-Graham and Vandenberg, 1998;

**Table 2.3. Rel/NF-κB enhancer sequences**

| κB Site | Sequence | | | Binding Specificity | |
|---|---|---|---|---|---|
| | | | | Xrel3 | XrelA |
| Consensus* | | **GGGPNNppCC** | | | |
| κB-pd | GCAG | **GGGAATTCCC** | CT | YES | YES |
| 1/2 κB-pd | GCAG | **GGGAA** | CT | NO | NO |
| *MIRRBE* | GCTG | CA**GAAA**G**T**A**C** | | YES | NO |
| *murRRBE* | GGAT | A**GGAAA**G**T**A**C** | | YES | NO |
| *HIV*-κB | GCTG | **GGGACTTTCC** | AG | YES | YES |
| *MIHIV*-κB | GCTG | **GGGACTT**G**CC** | AG | NO | NO |
| *Dorsal* | GTTTT | **GGGAAATCC**A | GAAG | YES | YES |

Note: P=purine; p=pyrimidine
*Consensus sequences are in highlighted in bold

Morrow et al., 2000; Hayman et al., 2001), in 8 mM potassium phosphate buffer (pH 8) for 1 hour at room temperature. Cross-linked protein dimers were then incubated overnight at 4°C with 20 µl mouse monoclonal anti-myc antibody 9E10 cell supernatant, purchased from DSHB (Iowa), in 1 ml triton medium (10 mM Tris pH 7.5; 1% Triton-X 100; 10 mM EDTA; 0.002% Na-azide) with 20 mM methionine and 5 µl of 0.2 M PMSF. Antibody-antigen complexes were immunoprecipitated with Protein A-Sepharose beads (Pharmacia) then washed 3x with triton medium/20 mM methionine then 2x with 150 mM NaCl. Immunoprecipitate was boiled for 4 minutes in 0.125 M Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 20% glycerol and run on SDS-PAGE along with pre-stained molecular weight standards (BioRad). All gels were visualized and recorded using a Cyclone (Canberra-Packard) phosphorimager.

## 2.4 RESULTS

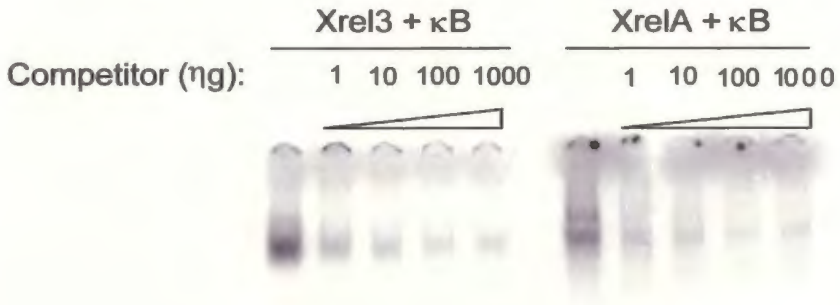
### 2.4.1 Xrel3 Binds κB DNA Enhancer Sequences

Rel/NF-κB family members bind differentially to cognate enhancer (κB) sites that vary in sequence from a perfect palindrome (κB-pd, Table 2.3; Chen and Ghosh, 1999). In order to determine the binding characteristics of *Xenopus* Rel proteins, I performed a series of *in vitro* DNA binding assays (EMSAs). For these analyses, I used DNA binding sites that were previously used to analyze the binding properties of Rel/NF-κB proteins from other species (Hansen et al., 1994). Like these other family members, the *Xenopus* proteins, Xrel3 and XrelA, bound most efficiently to the idealized κB-pd sequence (Figure 2.2), which was blocked by unlabeled sequences (Figure 2.2A). In addition, variations or deletions within this primary DNA sequence either abrogated or reduced binding of both proteins (Figure 2.2B). While both Xrel3 and XrelA bound to the *Dorsal* κB sequence, only Xrel3 bound the *murine rel related protein binding element* (*murRRBE*) (Figure 2.2B), present in the *urokinase plasminogen activator* (*uPA*) promoter (Hansen et al., 1994). Alteration of *murRRBE* to give *m1RRBE* resulted in a slight reduction in Xrel3 binding (Figure 2.2B). A similar alteration eliminated binding ability of mammalian NF-κB (p50/RelA) and c-Rel-RelA complexes (Hansen et al., 1994), suggesting that Xrel3 has less restricted binding specificity than these other proteins.

Both Xrel3 and XrelA formed specific associations with the *HIV* κB that were abrogated by a single base pair alteration (*m1HIV* κB; Figure 2.2B). Similar binding has

**Figure 2.2. Xrel3 binds differentially to κB enhancer sequences.** A. Comparative EMSAs showing Xrel3 and XrelA binding to the perfect palindromic κB sequence (κB-pd). Specificity of the protein/DNA interactions was demonstrated through addition of unlabeled κB-pd sequences (competitor) to the binding reactions at the concentrations indicated (1-1000 ng). Incomplete competition may be representative of either the affinity of Xrel3/XrelA to the DNA or of the existence of low level non-specific associations. B and C. EMSAs demonstrating the binding affinities of Xrel3 and XrelA, respectively, to half of the κB-pd sequence as well as the *Dorsal*, *m1RRBE* (*murine rel related binding element 1*), mutated *m1RRBE* (*murRRBE*), *HIV* and mutated *HIV* (*m1HIV*) κB sequences (Hansen et al., 1994). Unbound and labelled probe migrates at the bottom margin of the gel.

A.




B.


C.


Free Probe

been shown for c-Rel-RelA complexes but not for NF-κB, which retained the ability to bind even after the alteration (Hansen et al., 1994). Therefore, consistent with other Rel/NF-κB proteins, Xrel3 binds with different sequence-dependent affinities to a distinct subset of known κB binding sites. These results demonstrate Xrel3's potential to specifically activate or repress transcription of a unique subset of genes during embryogenesis.

### 2.4.2 Xrel3 DNA-Binding Can be Antagonized by Xrel3Δ58

To understand the normal function of Xrel3 in embryonic development, deletion mutants were created that either eliminated or truncated the putative C-terminal transactivation domain (Figure 2.3). Deletion of the entire TA domain did not affect the DNA binding potential (Figure 2.4A), consistent with the demonstrated DNA binding activities of similar, naturally existing proteins, p50 and p52 (Table 2.1; Cramer et al., 1997; Huang et al., 1997; Chen et al., 1998a). However, successive C-terminal deletions within the region corresponding to TADs identified in RelA and c-Rel (Bull et al., 1990; Schmitz et al., 1994; Schmitz et al., 1995; Martin et al., 2001) indicated that the C-terminal 58 amino acids are required for Xrel3/κB-pd associations (Figure 2.4A).

When co-translated *in vitro*, Xrel3Δ58 reduced the ability, in a concentration-dependent manner, of Xrel3 to bind DNA (Figure 2.4B). Also, *in vitro* co-translated myc-tagged Xrel3 and untagged Xrel3Δ58 (Figure 2.5A) co-precipitated using an anti-myc antibody (Figure 2.5B), demonstrating the ability for Xrel3 and Xrel3Δ58 to form dimers.

**Figure 2.3. Maps of Xrel3 constructs.** Wild-type Xrel3 consists of a rel-homology domain (stippled box) and a C-terminal domain (hatched box) with transactivation motifs (TA) at the extreme C-terminus. The 58 C-terminal residues are deleted in Xrel3Δ58, which includes the putative TA-region. Xrel3 was fused with a human cMyc-epitope by subcloning the Xrel3 coding region into pCS2+mt, which encodes six copies of the epitope and is recognized by the 9E10 antibody.

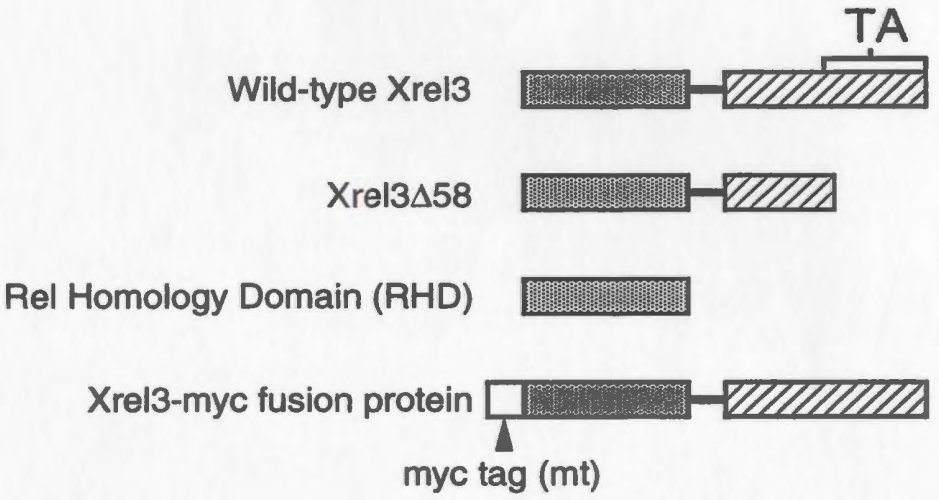
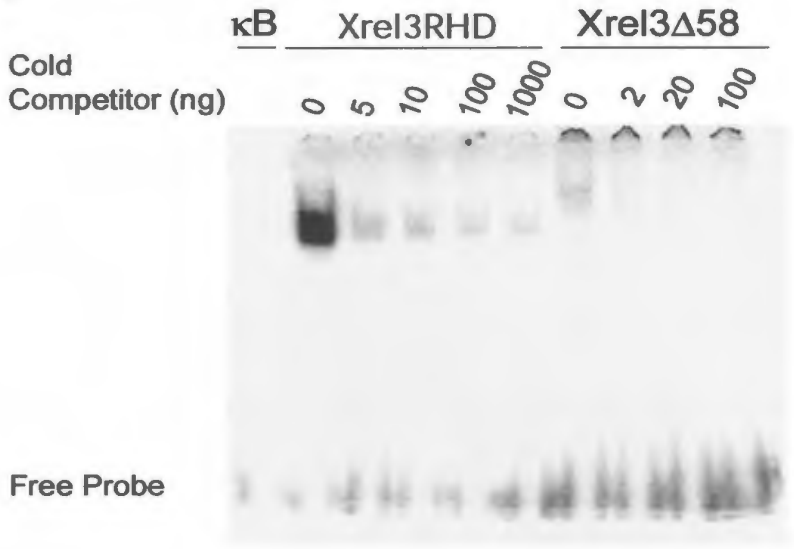

TA
Wild-type Xrel3
Xrel3Δ58
Rel Homology Domain (RHD)
Xrel3-myc fusion protein
myc tag (mt)

**Figure 2.4. Xrel3Δ58 competitively inhibits binding by Xrel3 to the κB enhancer sequence.** (A) Xrel3Δ58 binds the perfect κB element very weakly in an EMSA reaction as compared with the RHD of Xrel3 alone. Binding specificity is shown using increasing quantities of unlabeled κB sequence (cold competitor). Much less competitor is required to eliminate Xrel3Δ58 binding (2 ng) as compared with Xrel3RHD (1000 ng). (B) Equal amounts of Xrel3 (lanes 2-4) were co-translated with increasing proportions of Xrel3Δ58 (0-1.3x) and used in an EMSA with the κB perfect palindrome as a probe. Free probe (lane 1) is not retarded in the gel and Xrel3Δ58 alone (lane 6) binds the palindrome weakly. This assay was repeated three times over two independent experiments.

A.


κB
Xrel3RHD
Xrel3Δ58
Cold
Competitor (ng)
0
5
10
100
1000
0
2
20
100
Free Probe

B.

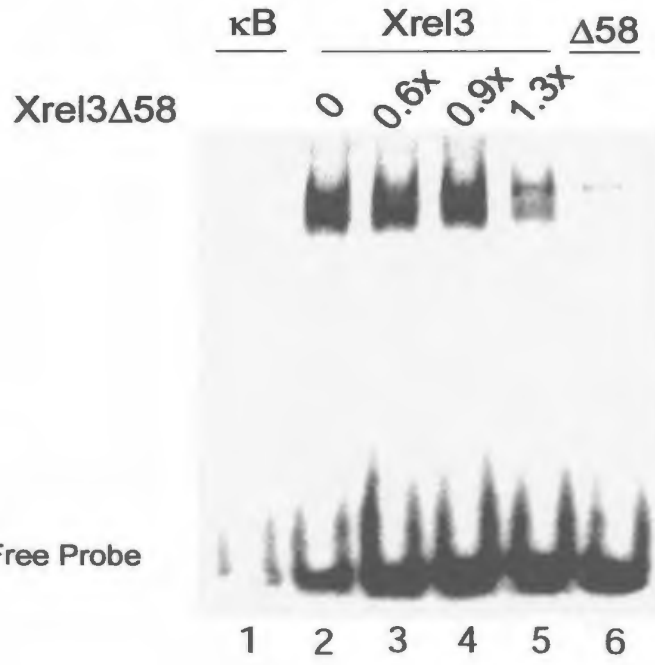
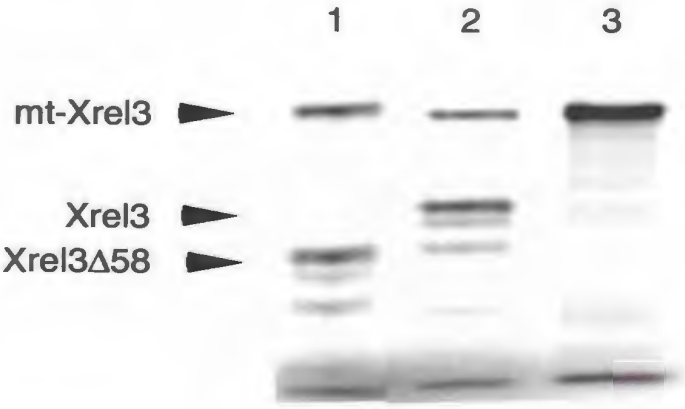
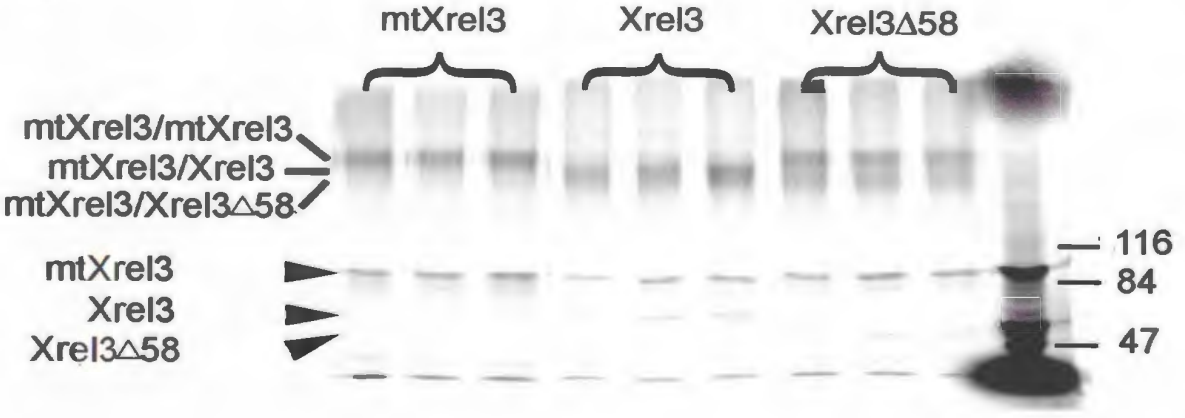

κB
Xrel3
Δ58
Xrel3Δ58
0
0.6x
0.9x
1.3x
Free Probe
1
2
3
4
5
6

**Figure 2.5. Xrel3Δ58 competitive inhibition occurs through dimerization with full length Xrel3.** (A) Xrel3-myc fusion protein (mt-Xrel3) was co-translated with Xrel3 (lane 2) or Xrel3Δ58 (lane 1) and resolved by SDS-PAGE. Arrows indicate full-length translation products. (B) mt-Xrel3 alone or covalently cross-linked to co-translated Xrel3 or Xrel3Δ58 using 0.005%, 0.01% and 0.015% glutaraldehyde (left to right), was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography. Sample in lane on extreme right is myc-tagged Xrel3 co-translated *in vitro* with Xrel3Δ58. The top bands represent covalently linked myc-tagged Xrel3 (mtXrel3/mtXrel3), myc-tagged Xrel3 dimerized with wild-type Xrel3 (mtXrel3/Xrel3) or myc-tagged Xrel3 dimerized with Xrel3Δ58 (mtXrel3/Xrel3Δ58). The lower bands represent unlinked monomeric subunits (mtXrel3, Xrel3 or Xrel3Δ58) that co-immunoprecipitated with mtXrel3.

A.


1
2
3
mt-Xrel3
Xrel3
Xrel3Δ58

B.


mtXrel3
Xrel3
Xrel3Δ58
mtXrel3/mtXrel3
mtXrel3/Xrel3
mtXrel3/Xrel3Δ58
mtXrel3
Xrel3
Xrel3Δ58
116
84
47

Therefore, I concluded that Xrel3Δ58 can act in a dominant inhibitory fashion by dimerizing with Xrel3 and preventing its ability to bind cognate DNA.

### 2.4.3 Xrel3 Is Required For Head Formation

Based on previous biochemical evidence, I wanted to determine what developmental phenotypes, if any, would result from overexpression of a protein that could interfere with endogenous, wild-type Xrel3. Embryos were therefore microinjected with mRNA encoding Xrel3Δ58 and examined for their development. Translation of 0.25 ng of *mtXrel3RHD*, *mtXrel3Δ58* and *mtXrel3* RNA in embryos was assayed by western blot analysis (Figure 2.6). Each of the corresponding proteins was expressed up to early neurula stages.

Injection of *Xrel3Δ58* mRNA into the animal pole of embryos at the two-cell stage caused them to develop anterior neural plate abnormalities arising at the late neurula stage. These defects manifested into head malformations at the tadpole stage (Figure 2.7B). The abnormal phenotypes (Table 2.4) ranged from normal (grade I) to reduced eyes (grade II), anopthalmy or synopthlamy and microcephaly (no eyes or fused eyes and small head, grades III and IV) to acephaly (no heads, grade V). While these defects could be partially rescued by co-injection of up to 0.25 ng of wild-type *Xrel3* RNA, the rate of recovery of normal structures was relatively low: only 57% of the embryos injected with 0.5 ng *Xrel3Δ58* had head defects and there was only a 30% rescue of the normal phenotype by the highest concentration of *Xrel3* RNA (Table 2.5, Figure 2.8).

**Figure 2.6. Xrel3 constructs are stably expressed *in vivo*.** Western analysis of whole protein lysates from embryos injected with 250 pg *mtXrel3*, *mtXrelRHD* and *mtXrel3Δ58* RNA using the 9E10 monoclonal myc antibody. Exogenous protein levels were analysed from blastulae (st. 8), gastrulae (st. 11) and neurulae (st. 13). Equal levels of protein extracts were loaded as determined by Biorad assays.

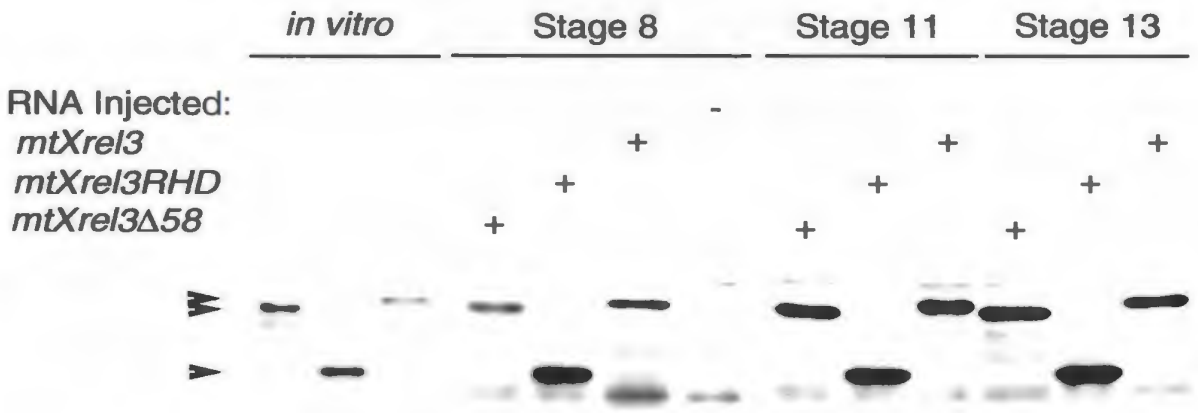
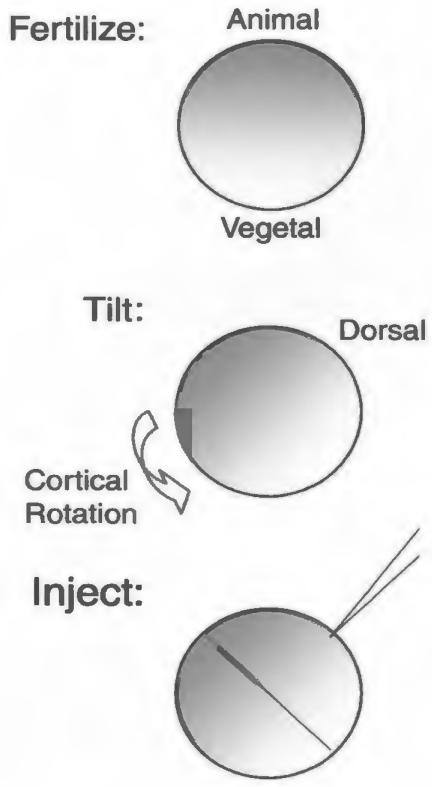

in vitro
Stage 8
Stage 11
Stage 13
RNA Injected:
mtXrel3
mtXrel3RHD
mtXrel3Δ58
-
+
+
+
+
+
+
+
+

**Figure 2.7. Overexpression of a dominant negative Xrel3 construct causes anterior-defective embryos.** A. Embryos were either injected into the animal pole or, to more efficiently target dorsal structures, were tilted within 45 minutes post-fertilization to force dorsal-ventral axis formation along the gravitational plane and injected in both dorsal blastomeres at the two-cell stage. B. Embryos that were injected at the two-cell stage with varying concentrations (0.25 to 1.0 ng) of *Xrel3Δ58* RNA showed varying degrees of phenotypic alterations as compared with normal embryos (I) when assessed at the tadpole stage. Defects ranged from mildly reduced eyes, forebrain and cement gland (II) to more significant reduction or loss of these structures (III), microencephaly, cyclopia (IV) and anencephaly (V). Scale bar =0.5 mm.

A.


Fertilize:
Animal
Vegetal
Tilt:
Dorsal
Cortical
Rotation
Inject:

B.

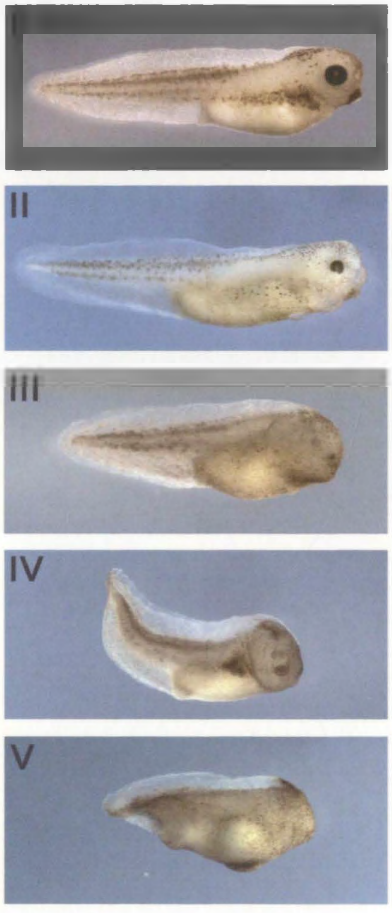

I
II
III
IV
V

**Table 2.4. Characteristic Xrel3Δ58 Defects (Figure 2.7)**

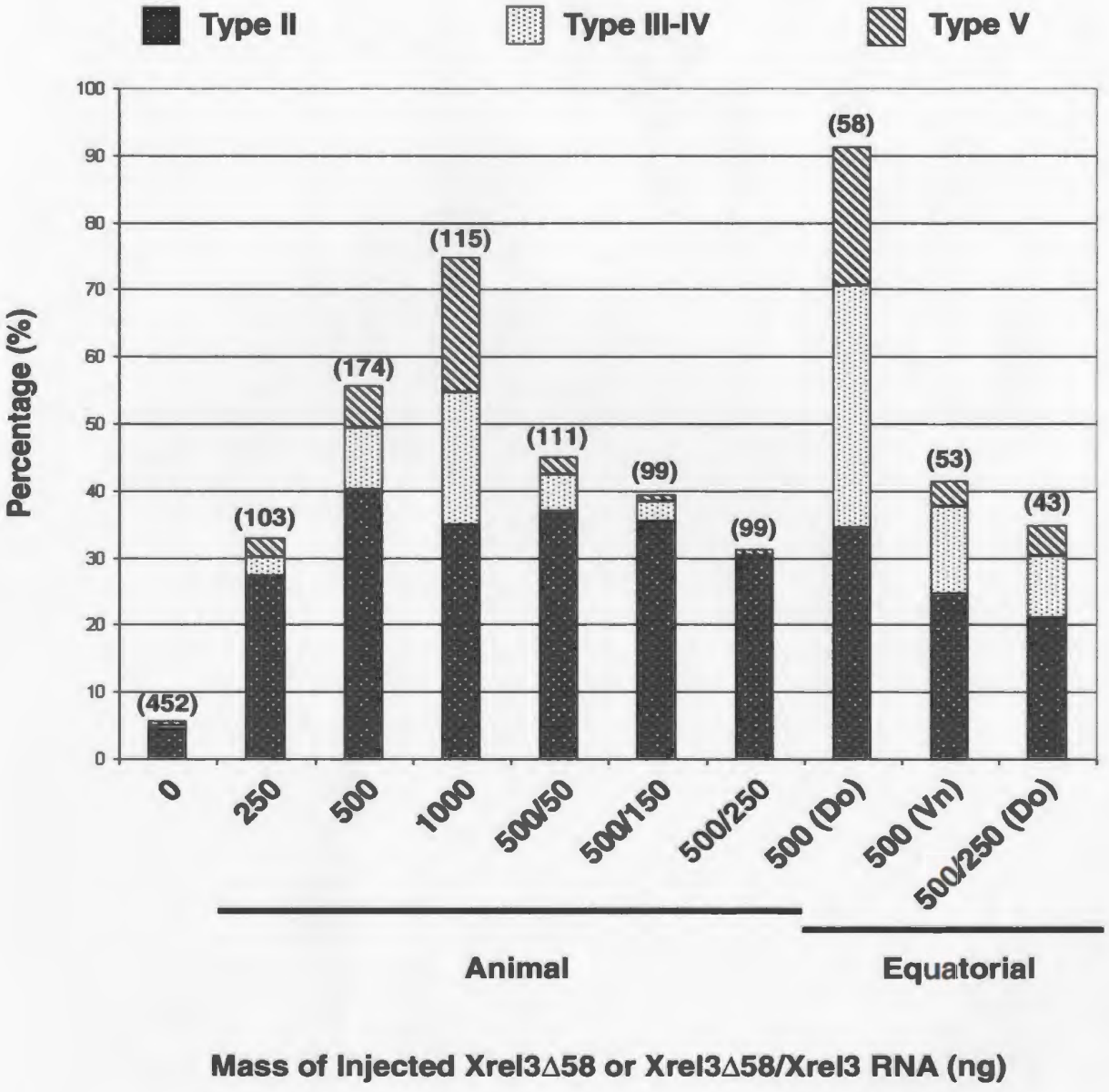
| | |
|---|---|
| **Type I** | Normal phenotype |
| **Type II** | Eyes and cement gland reduced in size |
| **Type III** | Anopthalmy (no eyes), significantly reduced cement glands and microencephaly (small head) |
| **Type IV** | Synopthlamy (fused eyes or cyclopia) |
| **Type V** | Acephaly (no heads) |

**Table 2.5. Distribution of phenotypes (Table 2.4) caused by Xrel3Δ58 overexpression**

| Picograms of Injected mRNA | | | Number (percentage) of Embryos | | | | |
|---|---|---|---|---|---|---|---|
| Xrel3 Δ58 | Xrel3 | N | I (normal) | II (Cyclopia) | III and IV | V (acephaly) | Total |
| 0 | 0 | 10 | 426(94) | 20(4) | 2(1) | 1(1) | 452 |
| 250 | 0 | 4 | 69(67) | 28(27) | 3(3) | 3(3) | 103 |
| 500 | 0 | 6 | 77(44) | 70(40) | 16(10) | 11(6) | 174 |
| 1000 | 0 | 4 | 29(25) | 40(35) | 23(20) | 23(20) | 115 |
| 500 | 50 | 3 | 61(55) | 41(37) | 6(5) | 3(3) | 111 |
| 500 | 150 | 3 | 60(61) | 35(35) | 3(3) | 1(1) | 99 |
| 500 | 250 | 3 | 68(69) | 30(30) | 0(0) | 1(1) | 99 |
| 500* | 0 | 4 | 4(6) | 21(36) | 21(36) | 12(21) | 58 |
| 500¥ | 0 | 4 | 31(58) | 13(25) | 7(13) | 2(4) | 53 |
| 500* | 250* | 3 | 28(65) | 9(21) | 4(9) | 2(5) | 43 |

N=Number of Experiments
*Dorsal Injection Site
¥Ventral Injection Site

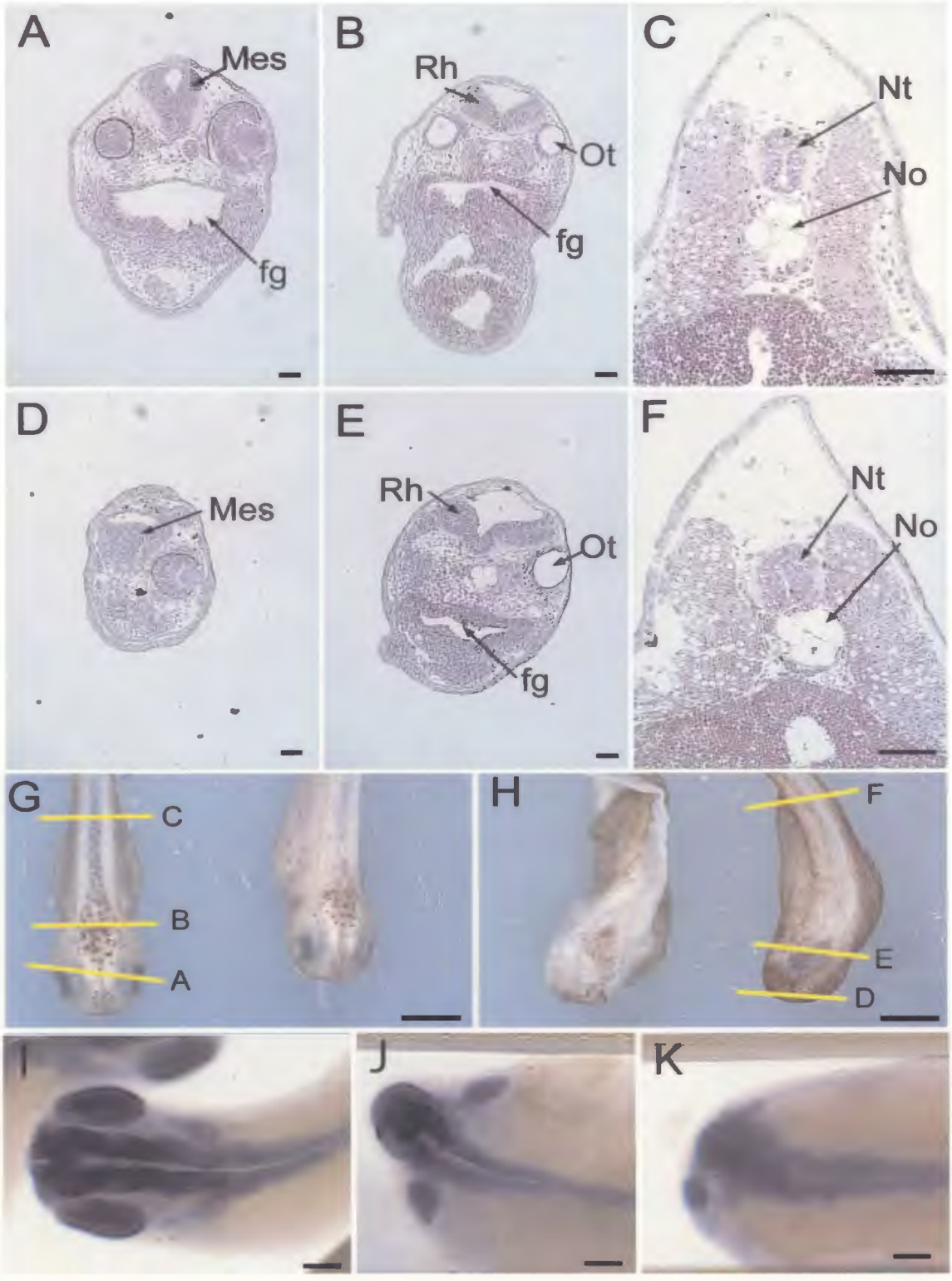
**Figure 2.8. The percentages of anterior defective embryos combined from several experiments as shown in a histogram.** Dead embryos and embryos that failed to gastrulate were not included in these analyses. Defects as categorized in Figure 2.6 are shown following injection into both the animal and equatorial regions of two-cell stage embryos. The bars showing reduction of defects in *Xrel3Δ58* RNA (500 pg) injected embryos by co-injection of wild-type *Xrel3* RNA (50, 150 or 250 pg) are indicated. Numbers at tops of bars indicate total number of embryos scored. Vn= ventral injection, Do=dorsal injection.


Type II
Type III-IV
Type V
Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
(452)
(103)
(174)
(115)
(111)
(99)
(99)
(58)
(53)
(43)
0
250
500
1000
500/50
500/150
500/250
500 (Do)
500 (Vn)
500/250 (Do)
Animal
Equatorial
Mass of Injected Xrel3Δ58 or Xrel3Δ58/Xrel3 RNA (ng)

The frequency of abnormalities was increased to 94% when I injected 0.5 ng *Xrel3Δ58* RNA into the dorsal marginal zone (Figure 2.7A, Figure 2.8, Table 2.5), a region approximately mapped to the prospective neural plate (Keller, 1975; Lane and Sheets, 2000). Only 40% of the embryos injected on the opposite, ventral side developed anterior abnormalities and the majority of these scored as low grade (II) (Figure 2.8, Table 2.5). Rescue of dorsally targeted Xrel3Δ58 defects was also more pronounced, with 0.25 ng of *Xrel3* RNA resulting in a significant reduction of the number and severity of the defects (35% of predominantly grade II) (Figure 2.8; Table 2.5). Therefore, the injection of a dominant inhibitory *Xrel3* message on the dorsal side of the embryo more effectively targeted the head to generate severe and specific anterior depletions.

To address the types of tissue disrupted in these mutants, histological sections of the more severe phenotypes were analyzed (Figure 2.9A-F). The embryos had normal appearing notochords and spinal cords (Figures 2.9E,F). However, rhombencephalic (hindbrain) development, while disorganized, was over represented (Figure 2.9E). The mutant embryos exhibited differing severity of anterior truncations (Figure 2.9I-K) with reduced mesencephala (midbrains) and severely disorganized or absent diencephalic, eye and prosencephalic (forebrain) structures (Figure 2.9D). If present, the eyes were deeply embedded and cyclopic. Also, the ventral-anterior most derivative of the neurectoderm, the cement gland, was partially or completely deleted. Unlike the optic vesicles and cement gland, the otocysts appeared unaffected (Figure 2.9E), but were shifted anteriorly with progressive anterior deficiencies (Figure 2.9I-K).

**Figure 2.9. Histological analysis of head-defective embryos.** Control (A,B,C) and *Xrel3Δ58* RNA injected embryos (D,E,F) with clear head-defective phenotypes were serially sectioned and examined at comparable levels as determined by anatomical landmarks. Embryo sections are through the mesencephalon (mes) or midbrain (A,D), rhombencephalon (Rh) or hindbrain (B,E), and trunk (C,F). fg, foregut; Ot, Otocyst; Nt, neural tube; No, notochord. Dorsal views of control (G) and Xrel3Δ58 (H) embryos are shown before sectioning with level of section indicated by yellow bars. Letters next to yellow bars correspond to histological sections in A to F. (I-K) Early tadpole embryos processed for whole mount RNA *in situ* hybridization using nrp-1 as a neural marker. An unaffected embryo (I) has a full complement of brain, eyes, otocysts and spinal cord while there is progressive loss of anterior structure and organization in type II (J) and V (K) embryo. Scale bars for A-F=0.1 mm, G and H=0.5 mm and I-K = 0.25 mm.


A
Mes
fg
B
Rh
Ot
fg
C
Nt
No
D
Mes
E
Rh
Ot
fg
F
Nt
No
G
C
B
A
H
F
E
D
I
J
K

The apparent over-representation of the hindbrain, with coincident anterior depletion, suggests the expansion of the former at the expense of the latter, indicating that the anterior most neural tissue within these embryos failed to develop into neural elements rostral to the hindbrain.

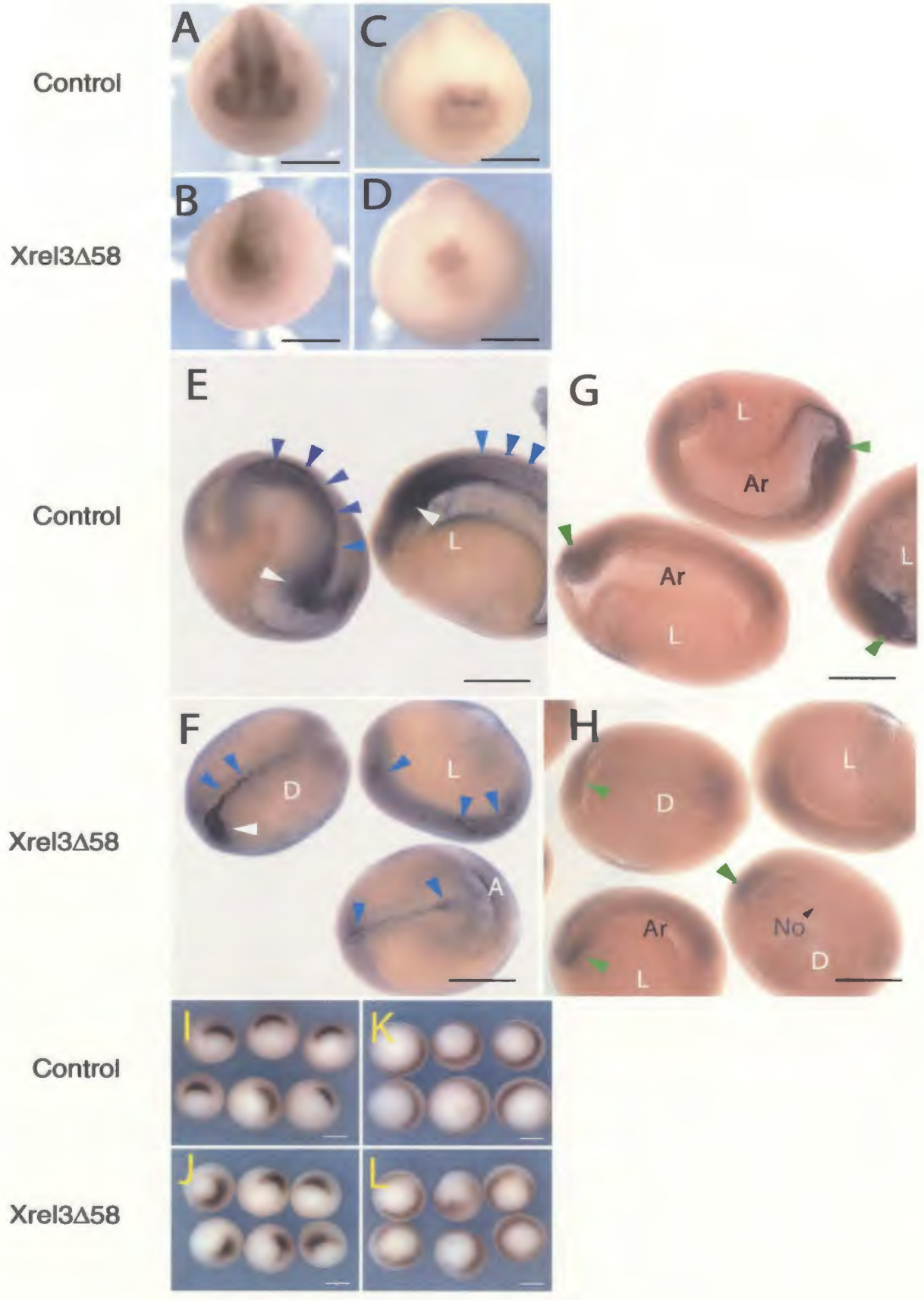
### 2.4.4 Xrel3 Patterns the Brain

To more finely analyze the effect of Xrel3Δ58 on development, mRNA expression of a variety of neural specific molecular markers was determined using whole mount *in situ* hybridization. For instance, the expression of the general pan-neural marker *nrp-1* (Figure 2.10A) was reduced and disorganized in the anterior most neural plate at mid-neurula stages (Figure 2.10B) resulting in a rostral shift of caudal structures. This finding confirms initial histological analysis as observed at the tadpole stage (Figure 2.9J,K).

The rostral neural transformation and ventral midline (cyclopia) defects resulting from overexpression of dominant inhibitory Xrel3Δ58 were similar to those observed in mice following knockout of Otx2 or Shh function (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Chiang et al., 1996). Since both of these markers were upregulated in Xrel3 induced tumours (Lake et al., 2001), their expression was also analyzed in *Xrel3Δ58*-injected embryos (Figure 2.10).

At the mid-neurula stage, *Xotx2* is expressed primarily within the midbrain and anterior neural ridge (Figure 2.10C; Eagleson and Dempewolf, 2002). However, in Xrel3Δ58-expressing embryos, there is a loss of its expression in both these territories (Figure 2.10D). Since the cement gland is derived from the ANR region, it is not

**Figure 2.10. Anterior-defective embryos have disrupted expression patterns of neural patterning markers in neurulae but normal expression of mesodermal markers at gastrulation.** Control (A,C,E,G,I,K) embryos are compared against *Xrel3Δ58* (B,D,F,H,J,L) injected embryos. Stage 15 embryos were stained for *nrp-1* (A,B), *otx-2* (C,D), *shh* (E,F) and *gli1* (G,H). Stage 10-10.5 embryos were stained for *chordin* (I,J) and *Xbra* (K,L) and viewed from the vegetal pole. Blue arrowheads in E and F indicate specific expression (dark blue-black stain) of *shh* in floor-plate and white arrows indicate expression of *shh* in prospective brain. Similarly, green arrowheads in G and H indicate expression in prospective brain of *gli1*. White letters indicate perspective views of embryos: L, lateral; D, dorsal; A; anterior. Embryos in A-D are shown at anterior end. Ar= archenteron and No= notochord. Scale bars=0.5 mm.


Control
Xrel3Δ58
Control
Xrel3Δ58
Control
Xrel3Δ58
A
C
B
D
E
L
G
L
Ar
Ar
L
L
F
D
L
A
H
L
D
Ar
L
No
D
I
K
J
L

surprising that Xrel3Δ58 embryos have deficiencies in this glandular tissue at later stages (Figure 2.7B).

Expression of *shh* within the ventral midline along the entire length of the neural plate is required to establish ventral identity (Figure 2.10E; Martí et al., 1995b; Ekker et al., 1995; Lee et al., 1997a). However, this expression was significantly reduced in Xrel3Δ58 embryos, with anterior expression being more significantly affected than posterior expression (Figure 2.10F). While the loss of anterior expression is consistent with the head abnormalities observed (Figure 2.7B), the reduced posterior expression did not translate into visible posterior neural or axial abnormalities (Figure 2.7B, Figure 2.9F). Therefore, the reduction of *shh* expression posteriorly was not significant enough to cause permanent patterning defects, perhaps due to recovered expression at later stages. However, it is possible that slight defects exist that are not apparently obvious at the stages in which I examined the embryos. The absence of any posterior abnormalities could be definitively proven using assays that identify specific ventral neuronal subtypes.

Like *shh*, *gli1* is normally expressed ventrally along the length of the neural tube (Figure 2.10G). Its role is to mediate the Shh response (Lee et al., 1997a) and in *Xrel3Δ58*-injected embryos (Figure 2.10H) its expression was significantly reduced, especially within the brain.

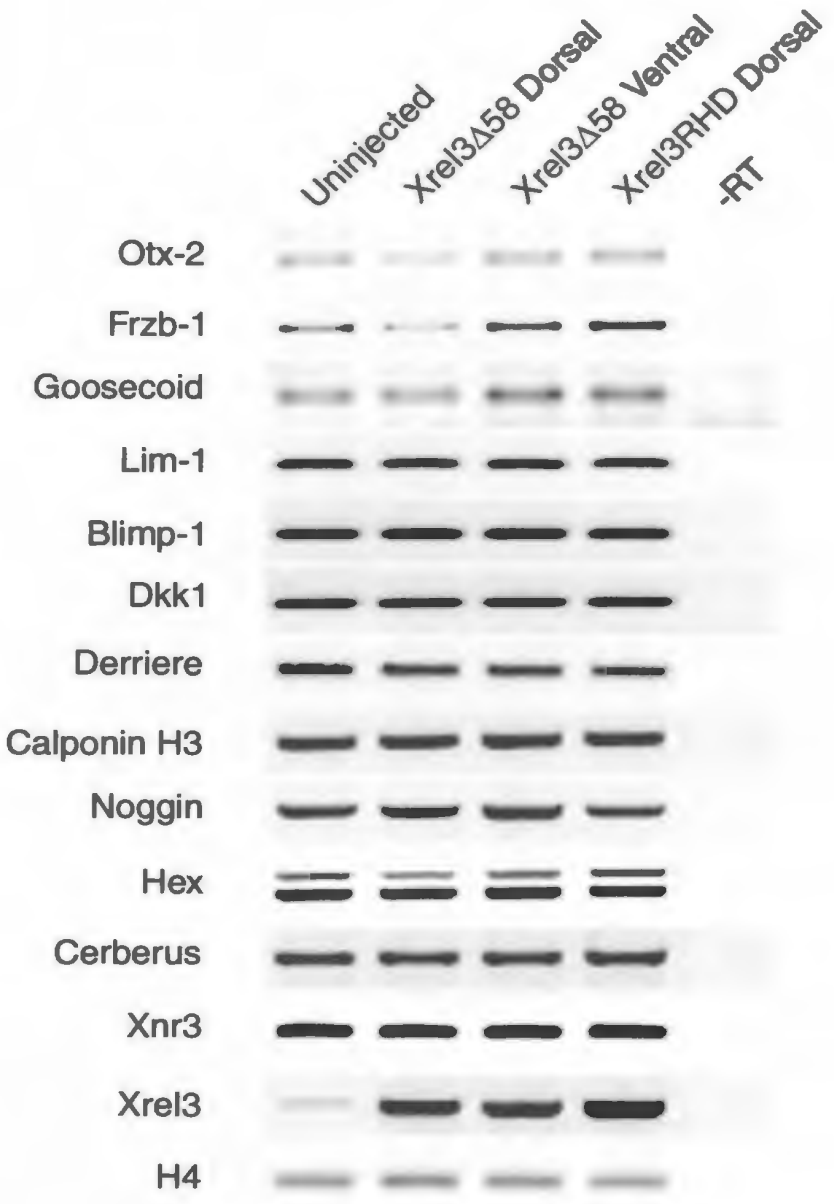
Therefore, overexpression studies (Lake et al., 2001) in conjunction with these observed dominant inhibitory effects demonstrate the dependency of *otx2*, *shh* and *gli1* expression on *Xenopus* Rel/NF-κB activity.

### 2.4.5 Xrel3Δ58 Does Not Block Organizer Formation

While the defects observed were consistent with Otx2 and Shh knockouts in other species, they also might be interpreted as abnormalities associated with incomplete specification of the inducing mesendoderm (Fredieu et al., 1997; Li et al., 1997). My evidence has suggested that *shh* depletion in the floorplate of the prospective brain may originate from disruption in the anterior mesendoderm or as a secondary event resulting from an earlier disruption in the proper formation of the organizer. It seems unlikely Xrel3Δ58 disrupted dorsoventral axial patterning or mesendoderm induction, since the embryos gastrulated normally with complete blastopore closure. However, Xrel3Δ58 could nonetheless have modified these events. Therefore a more extensive analysis of organizer marker expression at the onset of gastrulation was undertaken.

*In situ* analysis of organizer markers showed relatively normal expression of the trunk-inducing marker *chordin*, expressed in the dorsal lip of gastrulae (Figure 2.10I,J; Sasai et al., 1994), and the pan-mesodermal marker *Xbra* (Figure 2.10K,L; Smith et al., 1991). To further analyze the potential effect of Xrel3Δ58 overexpression, I also examined the expression of a variety of mesendoderm-specific markers (shown in Figure 1.2) using RT-PCR analysis of *Xrel3Δ58*-injected gastrulae (Figure 2.11). These assays indicated normal expression of the BMP antagonist *noggin* (Smith and Harland, 1992), which is expressed in the trunk organizer; the ventroposterior mesodermal marker *derrière* (Sun et al., 1999); and the head organizer markers *goosecoid* (Cho et al., 1991), *lim-1* (Taira et al., 1992), *blimp-1* (de Souza et al., 1999), *dkk-1* (Glinka et al., 1998), *hex* (Jones et al., 1999), *cerberus* (Bouwmeester et al., 1996), and *Xnr3* (Smith et al., 1995),

**Figure 2.11. Xrel3Δ58 does not antagonize organizer formation.** Embryos were tilted following fertilization and injected with 1000 pg *Xrel3Δ58* RNA within the two dorsal or ventral blastomeres at the two-cell stage or with *Xrel3RHD* RNA (1000 pg) dorsally. RNA was extracted from gastrula stage embryos (st. 10-10.5) and assayed through RT-PCR analysis for any reduction in organizer marker expression. Only *otx2* and *frzb-1* expression was found to decrease following Xrel3Δ58 expression. This experiment was repeated twice to confirm reproducibility of results, and embryos from each experiment were allowed to develop to tadpole stages to ensure effectiveness of *Xrel3Δ58* RNA injections. As such, Xrel3RHD was found to have no effect on embryo development. Levels of cDNA used were standardized using *histone (H4)* levels. –RT: negative control, uninjected without reverse transcriptase.


Uninjected
Xrel3Δ58 Dorsal
Xrel3Δ58 Ventral
Xrel3RHD Dorsal
-RT
Otx-2
Frzb-1
Goosecoid
Lim-1
Blimp-1
Dkk1
Derriere
Calponin H3
Noggin
Hex
Cerberus
Xnr3
Xrel3
H4

some of which are also expressed within the anterior endoderm (Figure 1.2). However, the head organizing Wnt antagonist Frzb-1 (Leyns et al., 1997; Wang et al., 1997a) was reduced at the RNA level by Xrel3Δ58 (Figure 2.11), indicating that this contributed to the anterior defective phenotype. However, because of its highly localized expression, it is unlikely that reduction in *frzb-1* expression generated the global reduction of *shh* and *gli1* found in *Xrel3Δ58*-injected embryos. Therefore, these results further verify that *shh* and *gli1* are downstream targets of Xrel3.

The expression of *otx2* was reduced in *Xrel3Δ58*-injected gastrula (Figure 2.11), indicating a possible reduction of expression in the mesendoderm prior to the observed later reduction in neurectoderm (Figure 2.10D). It is unlikely, however, that Xrel3Δ58 generated deficiencies of *otx2* in the mesendoderm since its downstream targets within this tissue, *cerberus* (Yamamoto et al., 2003) and *calponin H3* (Morgan et al., 1999), were unaffected (Figure 2.11). Therefore, Xrel3 mediated activation of *otx2* may be specific to ectodermal derivatives coincident with the earliest onset of *otx2* expression within the prospective brain. This conclusion is predicted based on several observations, including the severe disruption of *otx2* expression by Xrel3Δ58 in the anterior neurectoderm (Figure 2.10) with associated head abnormalities, as well as the absence of any significant effect on mesendodermal derivatives or direct gene targets within this tissue. Further examination of the spatial distribution of *otx2* messages at the gastrula stage is required for definitive conclusions on the tissue-specificity of Xrel3Δ58 actions in this regard.

My results suggest that Xrel3Δ58 generated head abnormalities specifically by targeting the expression of neurectodermal patterning genes (*Xotx2*, *gli1*, *shh*) and certain underlying inducing genes (*shh*, *frzb-1*). These latter markers were found to be reduced in the absence of any global or non-specific disruption of early organizer specification. Increased accuracy in the analysis of gene expression levels could be obtained by using more quantitative RT-PCR techniques (e.g. measurement of incorporated radiolabeled nucleotides). However, irrespective of this, these experiments demonstrate that Xrel3 establishes spatial identity within the anterior nervous system by regulating the expression of key patterning genes within both the neurectoderm and mesendoderm.

## 2.5 DISCUSSION

### 2.5.1 Rel/NF-κB in Neural Patterning

Emerging evidence has implicated complex roles for vertebrate NF-κB proteins in regulating neural development, survival and dysfunction in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (reviewed in Denk et al., 2000). However, the knockout of individual family members has provided limited information for the definitive role these proteins play in embryonic CNS development (reviewed in Attar et al., 1997; Gerondakis et al., 1999), due perhaps to functional redundancy between the numerous Rel/NF-κB proteins. The results of this study demonstrate a requirement for *Xenopus* Rel/NF-κB protein activity in patterning the anterior-most neural tissue by regulated expression of markers that not only specify spatial identity, but also consequently generate secondary organizing centers that further refine the pre-established AP and DV neuraxes.

Previous expression studies (Yang et al., 1998) in conjunction with the present functional study allowed me to develop a spatiotemporal model for the involvement of Xrel3 in early embryonic development. *Xrel3* is expressed within the pregastrula marginal zone, placing it in the correct location for possible initiation of *frzb-1* expression in the head organizer and *otx2* expression in the anterior neurectoderm (Figures 2.10 and 2.11). The incomplete knockdown of these markers, however, may be reflected in the inefficiency of Xrel3Δ58 to target maternally derived Xrel3. Maternal *Xrel3* messages (Yang et al., 1998) are likely translated and associated into dimeric complexes before exogenous *Xrel3Δ58* RNA is introduced. Therefore, the pre-gastrula requirement for

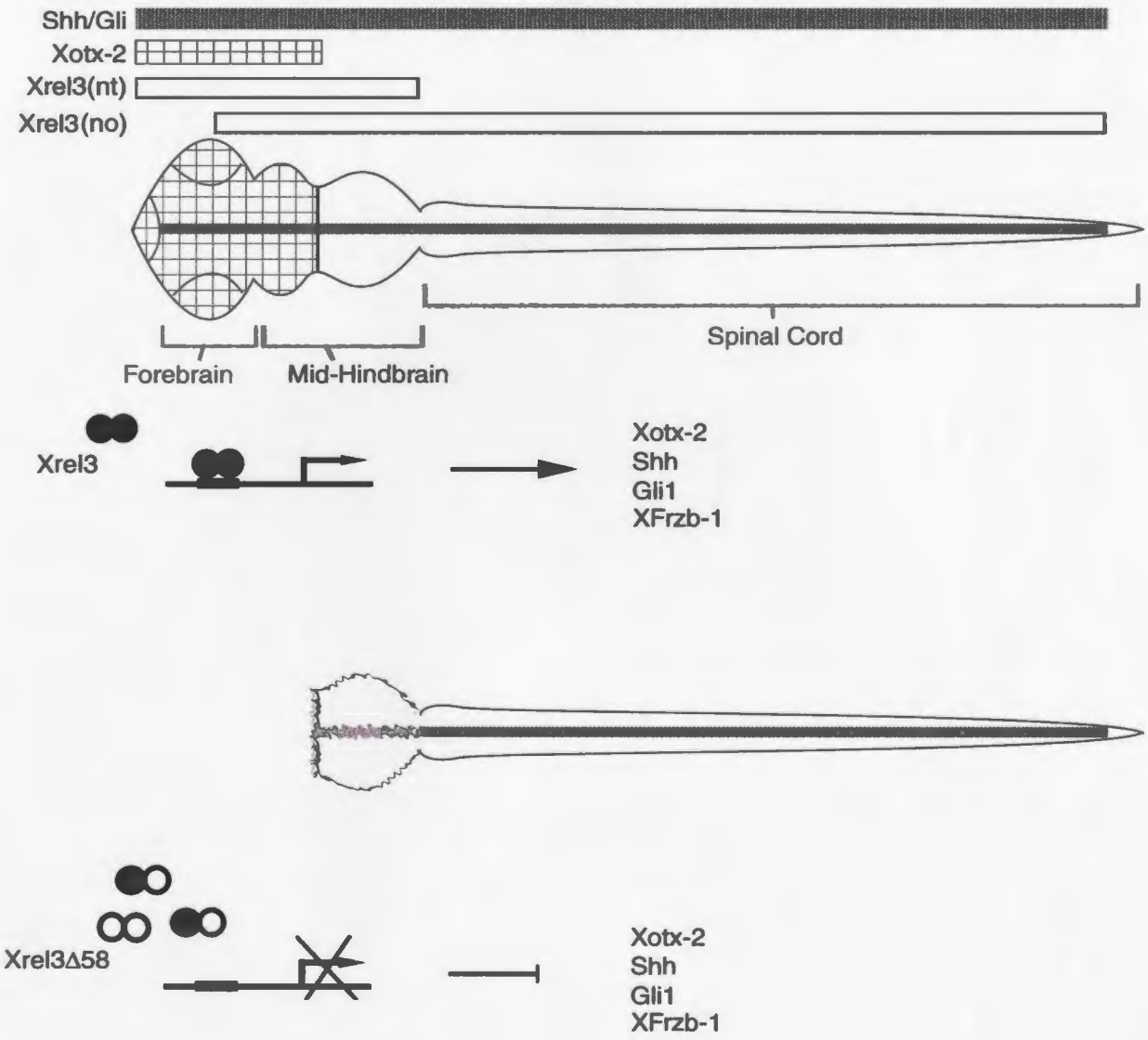
Xrel3 may be more efficiently analyzed using antisense technologies in oocytes (Weeks et al. 1991; Heasman et al., 1992; Heasman et al., 1994; Kofron et al., 1997).

*Xrel3* message levels decline by gastrula stages and new zygotic messages accumulate in neurulae in the anterior-most neurectoderm fated to form the primary brain vesicles that give rise to the fore-, mid- and hindbrain (Figure 2.12; Yang et al., 1998). Analysis of Xrel3 protein levels would be required to determine more accurately the actual Xrel3 expression pattern. However, based on this distribution of *Xrel3* transcripts it seems that *otx2* expression must be initiated by Xrel3 established mainly from maternal stores and maintained later in the future fore- midbrain by zygotic Xrel3.

The window during late gastrula/early neurula stages in which *Xrel3* is not expressed suggests that there is likely an alternative mode for regulating *otx2* expression active during this period. Indeed, the initially broad expression pattern of *otx2* is gradually restricted more anteriorly at this time by Gbx2 to specify the isthmic organizer (Glavic et al., 2002). The observed drop in *Xrel3* expression may be an essential component of this process. Xrel3 expressed at later neurula stages would then be expected to reinforce repositioned *otx2* expression in the fore-midbrain region (Figure 2.12). Further analysis of the temporal requirement for Xrel3 in Otx2 expression (and possible Gbx2 repression) is required to confirm this hypothesis.

*Xrel3* is also expressed in the notochord (Yang et al., 1998) where it may initiate expression of *shh* at mid-neurula stages (Figure 2.10; Ekker et al., 1995). Shh from the mesendoderm diffuses to the overlying neurectoderm to induce its own expression within the floor plate where it acts as a morphogenetic source to pattern ventral neuronal

**Figure 2.12. Model of Xrel3Δ58 inhibitory activity in *Xenopus* embryos.** Xrel3 expressed in the chordamesoderm and anterior-most neurectoderm fated to form fore-, mid- and hindbrain establishes the correct spatial expression of *otx-2*, *shh* and *gli1* expression within the nervous system. This may further require Xrel3-dependent expression of XFrzb-1 to antagonise Wnt activity in the anterior neurectoderm. Targeted expression of Xrel3Δ58 to the head region of developing embryos enables its dimerization with and antagonism of endogenous Xrel3 proteins within this region. The resultant inability of Xrel3 to bind DNA and regulate target promoter activity either directly or indirectly prevents proper expression of *otx-2*, *shh*, *gli1* and *Xfrzb-1* and the loss of tissues normally specified by these genes.


Shh/Gli
Xotx-2
Xrel3(nt)
Xrel3(no)
Spinal Cord
Forebrain
Mid-Hindbrain
Xrel3
Xotx-2
Shh
Gli1
XFrzb-1
Xrel3Δ58
Xotx-2
Shh
Gli1
XFrzb-1

populations (Ericson et al., 1995; Hynes et al., 1995; Martí et al., 1995a/b; Roelink et al., 1995; Wang et al., 1995; Chiang et al., 1996; Kohtz et al., 1998), in part through induction of its effector Gli1 (Lee et al., 1997a). The more significant loss of *shh/gli1* expression in the brain following *Xrel3Δ58* injection may result from a greater dependency of this tissue on Xrel3 activity.

Alternatively, it is possible that the anterior-posterior differences in loss of *shh/gli* expression may simply be a reflection of differential amounts of injected *Xrel3Δ58* in the dorso-anterior region. In fact, injection on either side of the dorsal meridian at the two- to four-cell stage targets chordamesodermal/neurectodermal cells that, through convergent extension movements during gastrulation, arise more anteriorly within the notochord/neural plate. Cells derived more laterally would receive less *Xrel3Δ58* RNA and become incorporated into more posterior positions of the prospective notochord/neural field (Lane and Sheets, 2000). As such, due to the nature of the localized dorsal injections, more anterior derivatives would inherit the highest concentrations of antagonizing Xrel3Δ58 activity, and would be expected to show the greatest reduction in target gene expression. If this were correct, I would expect ventral or lateral injections to generate more posterior than anterior depletions, a possibility that has yet to be tested.

Irrespective of the derivative tissue, Xrel3 appears to be required upstream of Shh expression in the floorplate where anteriorly, in other species, it is normally required for specification of ventral neural identity in the fore- and midbrain (Ericson et al., 1995; Hynes et al., 1995; Wang et al., 1995; Chiang et al., 1996; Kohtz et al., 1998). Indeed, double heterozygous knockouts of *otx2* and *hnf-3β* in mice (Jin et al., 2001) developed

compound defects of anterior ventral structures and holoprosencephaly, due to a loss of Shh signaling from the ventral telencephalon (Jin et al., 2001). These findings suggested that regulated expression of Shh was dependent on overlapping Otx2 and HNF-3β expression domains initially in the ventral midline of forebrain and midbrain and subsequently only within ventral midbrain (Jin et al., 2001). In fact, HNF-3β, while typically downstream of Shh signaling (Sasaki et al., 1997), regulates Shh expression (Echelard et al., 1993; Sasaki and Hogan, 1994; Chang et al., 1997) and forms direct associations with Otx2 (Nakano et al., 2000). Further examination into a potential role for Xrel3 in regulating *hnf-3β* expression is required to determine whether this is a possible intermediate step in activating *shh* expression in the brain.

Following the initial specification of fore-midbrain identity and potential induction of Shh expression within this region, Otx2 was found to further provide AP and DV spatial identity by limiting FGF8 expression to the IsO and Shh expression to the ventral floor-plate region of the midbrain (Puelles et al., 2003). However, Shh does become expressed more dorsally within the forebrain as part of the ZLI (Echelard et al., 1993; Puelles and Rubenstein, 1993; Bally-Cuif and Wassef, 1995; Martí et al., 1995a), possibly though Rel/NF-κB dependent activity, since all anterior staining for *shh* is lost in Xrel3Δ58 expressing embryos. Therefore, regulation of these markers by Xrel3 is more complex than simple direct transcription, with different temporal and spatial requirements coupled with the potential for initially agonistic then antagonistic feedback between Otx2 and Shh expression in the brain.

### 2.5.2. Dominant Interference

Confirmation of the proposed model of Xrel3 regulation (Figure 2.12) comes from embryos exhibiting interference of this activity. By dimerizing with and antagonizing wildtype Xrel3, I postulated that Xrel3Δ58, when expressed in embryos, inhibited endogenous Xrel3 activity within both head mesendoderm and neurectoderm. The underlying mechanism of this interference, with disrupted DNA binding by a C-terminally truncated Xrel3 protein, is unsurprising for two reasons:

**1.** Rel/NF-κB protein activity is regulated at multiple levels that only in part include the IκB kinase network. DNA binding to regulate transcription is also dependent on the phosphorylation status of the protein, variations in the protein's primary sequence that determine which κB sites are bound, interacting proteins (including dimer composition) and tertiary conformational changes (reviewed in Chen and Ghosh, 1999). Therefore, alterations in the primary sequence may influence any number of these regulatory functions.

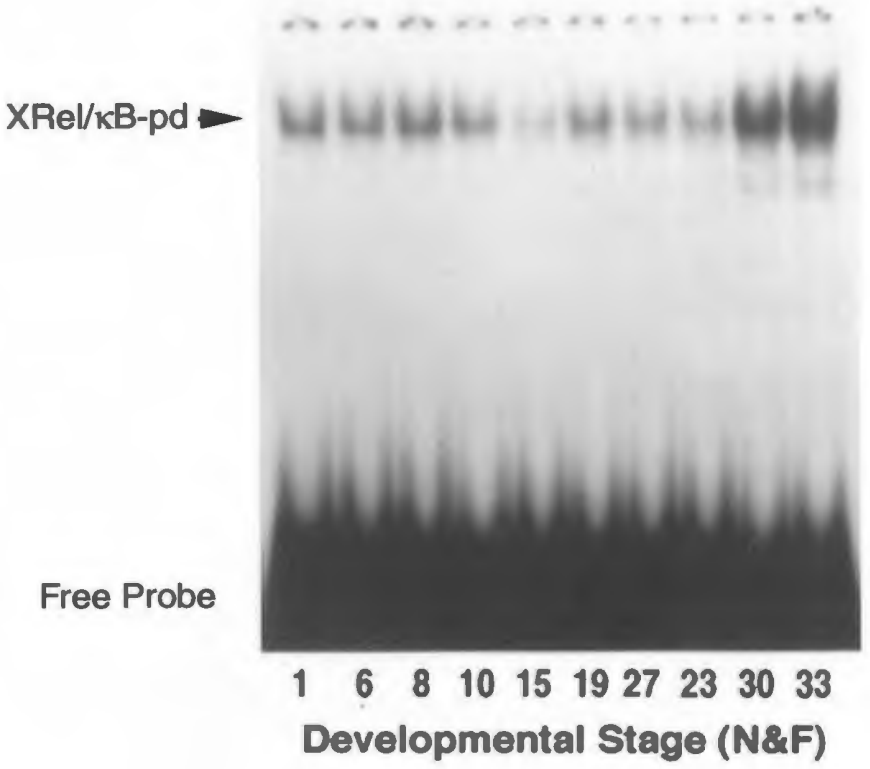
**2.** Direct regulatory associations between the RH and TA domains have been demonstrated for numerous Rel/NF-κB proteins. For instance, intramolecular interactions of RelA termini mask TAD sites that associate with the transcriptional co-activator CBP/p300 (Zhong et al., 1998). The Dorsal morphogen was also found to naturally regulate its own transcriptional activation and repression functions through inhibitory associations between its RH and TA domains; specific mutations within the RH domain were capable of enhancing these associations and completely eliminated all transcriptional regulatory activity (Jia et al., 2002). Furthermore, alteration of the

phosphorylation status of the C-terminal TAD of c-Rel by mutation resulting in conversion of specific serine residues to alanine was capable of generating dominant negative versions that blocked Rel/NF-κB reporter activity (Martin et al., 2001).

Therefore, based on these studies, the tertiary structural conformation of Xrel3 likely allows intramolecular C-terminal and N-terminal interactions that are required for its normal activity. According to this, DNA binding by the RH domain would be influenced and possibly regulated by residues present within the TA domain.

However, the potential for Xrel3 proteins to associate with other family members to regulate transcription from different enhancer sequences complicates inferences made about the target specificity of Xrel3Δ58. The possibility remains that Xrel3Δ58 may dimerize not only with endogenous Xrel3 but also with XrelA, an association predicted to exist *in vitro* (B.B.L., unpublished observations), or other identified Rel/NF-κB family members (Table 2.1). In fact, XrelA and Xp100 were both found to be expressed in dorsal structures, including the central nervous system of neurula and tadpole embryos (Kao and Hopwood, 1991; Bearer, 1994; Suzuki et al., 1998). In addition, while the efficiency of Xrel3Δ58 to antagonize Xrel3 DNA binding was clearly demonstrated *in vitro*, it has yet to be shown *in vivo*. Protein isolated from early embryos binds the κB-pd sequence in a temporal pattern resembling *Xrel3* RNA expression (Figure 2.13, K.R.K, unpublished observations), so injection of *Xrel3Δ58* would be predicted to abrogate, primarily, the post-gastrula phase of this binding activity. Alternatively, it would be interesting to identify the proteins bound and antagonized by Xrel3Δ58 using, for instance, Yeast 2-

**Figure 2.13. Perfect κB palindromic binding pattern during early *Xenopus* development.** Nuclear extracts from various embryonic stages (N&F: Nieuwkoop and Faber, 1994) were used within an EMSA using the κB-pd (K.R.K, unpublished observations). Bandshift occurred prior to and following gastrulation, with re-emergence occurring at mid neurula stages. This closely resembles the pattern of *Xrel3* message accumulation (Yang et al., 1998).


XRel/κB-pd
Free Probe
1 6 8 10 15 19 27 23 30 33
Developmental Stage (N&F)

hybrid assays, or by mass spectrometric analysis of immunoprecipitated complexes. Therefore, further analyses could definitively identify the target of Xrel3Δ58 and, as such, the required Rel/NF-κB complex that patterns the embryonic brain.

Another more direct way to identify the normal endogenous role of Xrel3 alone could be achieved through morpholino knockdown studies as employed in chapter 3 against XPygo-2 (Lake and Kao, 2003b). This would allow specific targeted depletion of Xrel3 protein to validate the results obtained by Xrel3Δ58 and identify individual contributions to the patterning of the embryonic nervous system.

### 2.5.3 Regulation of Neural Patterning by Xrel3-mediated Expression of *shh* and *otx2*

The graded ventromedial brain defects in *Xrel3Δ58*-injected *Xenopus* embryos are reminiscent of those exhibited by *shh* mutations in mouse (Chiang et al., 1996; Hayhurst and McConnell, 2003) and holoprosencephaly in humans (Belloni et al., 1996; Roessler et al., 1996; Roessler et al., 1997; Nanni et al., 1999; Wallis and Muenke, 2000). Shh depleted chick mutants also exhibited an overall reduction in head size similar to that observed in *Xrel3Δ58*-injected *Xenopus* embryos, due largely to the death of migratory craniofacial neural crest cells (Ahlgren and Bronner-Fraser, 1999; Britto et al., 2002). This experimental result might imply that Xrel3 in frogs plays a role in neural crest development. In fact, the neural crest marker *twist* was upregulated by ectopic Xrel3 expression (Lake et al., 2001) predicting that its reduction would be found in *Xrel3Δ58*-injected embryos.

The ventral hindbrain of Xrel3Δ58 embryos was also expanded, with an enlarged brain vesicle creating a gap visible from the dorsal anterior view of the head (Figure 2.9E, H). This observation is consistent with the recently identified role of Shh in expansion of dorsal neuroepithelium, with Shh depletion resulting in abnormal folding and overall collapse of the brain vesicles due to reduced proliferation dorsally and increased cell death (Dahmane and Ruiz I Altaba, 1999; Dahmane et al., 2001; Britto et al., 2002; Ishibashi and McMahon, 2002; Lai et al., 2003).

*Xrel3Δ58*-injected embryos also developed anterior to posterior transformations characteristic of *otx2* homo- or heterozygote knockout mice (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). *Xenopus* defects additionally included loss of cement gland tissue, which is dependent on Otx2 activity (Gammill and Sive, 2001). This suggests that Xrel3 may be involved in cement gland formation by regulating Otx2 expression. Xrel3Δ58 embryos also exhibited a more severe phenotype having either absent eyes or a deeply set single eye and the absence of the majority of head structures. All of these phenotypic alterations were also observed following *otx2* ablation in mice (Matsuo et al., 1995; Hide et al., 2002), consistent with studies demonstrating that Otx2 is expressed within the cephalic mesenchyme and neural crest cells and correspondingly plays a critical role in craniofacial development (Kimura et al., 1997). Further, Otx2 is initially expressed in the retinal field and was shown to be required for retinal development (Acampora et al., 1995; Blitz and Cho, 1995; Matsuo et al., 1995; Kablar et al., 1996; Perron et al., 1998; Andreazzoli et al., 1999; Kenyon et al., 2001).

These observations are further consistent with the model by which the Otx2 transcriptional regulator and the Shh pathway exist, possibly in an interdependent fashion, downstream of Rel/NF-κB in patterning the brain. Xrel3 both induces their expression ectopically in non-neural ectoderm (Lake et al., 2001) and is required for their endogenous expression in the developing nervous system (Figure 2.10). It is possible that Xrel3 induces and maintains their expression initially and through association with other regulatory molecules, their expression domains become refined. Since ectopically induced expression of these markers by Xrel3 only occurs at their normal time of endogenous expression (Lake et al., 2001), the correct temporal and spatial patterns of expression are further restricted by additional factors that regulate ectodermal competence to respond to Xrel3. Therefore, I suggest that both restricted competence *and* downstream regulatory networks enable broadly expressed Xrel3 to induce the more spatially restricted expression of its downstream targets.

### 2.5.4 Congenital Birth Defects and Mitogenesis

While Rel/NF-κB has yet to be linked with defects associated with aberrant encephalospinal development, this study demonstrates the important roles these proteins play in regulating known embryologically relevant genes. One possibility for the failure to form a link to congenital defects in mouse knockout studies may be the redundancy of function and extensive dimerization exhibited by Rel/NF-κB proteins. This may reflect an evolutionary adaptation to counteract potentially disastrous single mutations which occur in other systems, such as the frog. The ability to generate embryos exhibiting Holoprosencephaly provides a novel system to study the potential molecular components

of a disease found in 1/16000 live births and highly associated with prenatal mortality in humans, being identified as the cause of 1/250 aborted conceptuses (Cohen, 1989; Muenke et al., 1994).

The activation of *shh* and *gli1* expression in Xrel3 induced tumors (Lake et al., 2001) also indicates a possible mitogenic role for Rel/NF-κB by activating the Shh pathway and subsequent enhanced Gli1 expression (Dahmane et al., 1997). Rel/NF-κB pathway components, associated with normal epidermal development and differentiation, become deregulated in skin pathologies involving proliferation that range from psoriasis to carcinogenesis (Bell et al., 2003). Several studies have implicated Shh and its downstream affecter Gli1 in basal cell carcinomas of the skin (Hahn et al., 1996; Fan et al., 1997; Wolter et al., 1997; Taipale and Beachy, 2001; Ruiz i Altaba et al., 2002b) and in non-neural ectodermal tumors in *Xenopus* embryos (Lake et al., 2001; Dahmane et al., 1997). My studies provide a link between Rel/NF-κB and the Shh pathway, and therefore implicate their potential interconnected role in skin malignancy. Furthermore, since Rel/NF-κB activation is an intracellular response for skin cells to mutagenizing environmental factors (e.g UV; Bell et al., 2003); it remains possible that epidermal damage can progress into a malignant state through Rel/NF-κB induction of Shh/Gli1 overexpression.

The Shh pathway is not only involved in cell proliferation of skin cell progenitors, but also other types of epithelial stem cells including those of the developing nervous system, from invertebrates (Shyamala and Bhat, 2002) to vertebrates (Hynes et al., 1997; Jensen and Wallace, 1997; Parisi and Lin, 1998; Ahlgren and Bronner-Fraser, 1999; Fan and Khavari, 1999; Matise and Joyner, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999; Britto et al., 2000; Dahmane et al., 2001; Britto et al., 2002). Following an initial requirement in patterning the dorsal-ventral axes of the brain, Shh becomes expressed in

dorsal CNS cortical structures, including the cerebral cortex (forebrain), optic tectum (midbrain) and cerebellar cortex (hindbrain) (Traiffort et al., 1999) where it is required for proliferation of dorsal neuronal progenitors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechlser-Reya and Scott, 1999; Dahmane et al., 2001; Britto et al., 2002). A similar role has been proposed for the mouse retina (Jensen and Wallace, 1997). As such, the overexpression of Gli1 in the developing frog CNS caused increased proliferation and hyperplasia (Dahmane et al., 2001). Therefore, my studies demonstrate the potential for Rel/NF-κB to regulate cellular proliferation within the embryonic nervous system by activation of Shh/Gli1. Correspondingly, abnormal activation in adult cells of Rel/NF-κB might be an instigating factor in certain neurectodermal malignancies that are associated with aberrant Shh/Gli1 activity, a possibility that requires further investigation.

My results indicate that Rel/NF-κB signaling, either directly or indirectly in *Xenopus* is required for both the specification and expansion of central nervous system neuronal populations for proper formation of the head. A perturbation of this activity, and consequent downstream gene expression, as I have shown, results in the loss and disorganization of head structures (Figure 2.12).

# CHAPTER 3:

## *Pygopus is Required for Embryonic Brain Patterning in Xenopus*

Running Title: *Xenopus Pygopus*

## 3.1 ABSTRACT

This chapter outlines the identification of two *Xenopus* mRNAs that encode proteins homologous to a component of the Wnt/β-Catenin transcriptional machinery known as Pygopus. The predicted proteins encoded by both mRNAs (XPygo-2α and XPygo-2β) share the same structural properties with human Pygo-2, but with XPygo-2β lacking 21 N-terminal residues. *Xpygo-2α* messages accumulate in the prospective anterior neural plate after gastrulation and then are localized to the nervous system, rostral to and including the hindbrain. *Xpygo-2β* mRNA is expressed in oocytes and early embryos but declines in level before and during gastrulation. In late neurula, *Xpygo-2β* mRNA is restricted to the retinal field, including eye primordia and prospective forebrain. A C-terminal truncated mutant of XPygo-2 containing the N-terminal Homology Domain (NHD) caused both axis duplication when injected at the 2-cell stage and inhibition of anterior neural development when injected in the prospective head, mimicking the previously described effects of Wnt-signaling activators. Inhibition of XPygo-2α and XPygo-2β by injection of gene-specific antisense morpholino oligonucleotides into prospective anterior neurectoderm caused brain defects that were prevented by co-injection of *Xpygo-2* mRNA. Both XPygo-2α and XPygo-2β morpholinos reduced the eye and forebrain markers *Xrx1*, *Xpax6* and *Xbf1*, while the XPygo-2α morpholino also eliminated expression of the mid-hindbrain marker En-2. The differential expression and regulatory activities of XPygo-2α/β in rostral neural tissue indicate that they represent essential components of a novel mechanism for Wnt signaling in regionalization of the brain.

## 3.2 INTRODUCTION

The Wnt proteins belong to a large family of secreted glycoproteins that derive their name from the *Drosophila* morphogen Wingless (Wg, Cabrera et al., 1987; Rijsewijk et al., 1987) and the mouse oncogene *int-1* (known as *wnt-1*) (Nusse and Varmus, 1982; van Ooyen and Nusse, 1984). Members of this multigene family (currently about 19 in human and mouse, 16 in *Xenopus*) are involved in myriad processes including limb development, CNS patterning, somitogenesis, lymphopoiesis and establishment of the primary body axis (Cadigan and Nusse, 1997; Moon et al., 1997; Gradl et al., 1999; Moon et al., 2002; van de Wetering et al., 2002; Lake and Kao, 2003a). This critical role in growth and differentiation enables activating mutations within this pathway to contribute to cancer (Huelsken et al., 2001; Taipale and Beachy, 2001).

Wnt control of cell fate involves multiple signaling pathways, the best characterized of which is that of the canonical Wnt-1/Wg class, in which ligand-dependent subcellular localization and stability of the transcriptional co-activator, β-Catenin, is regulated by an elaborate network of transducers (Martinez Arias et al., 1999; Sharpe et al., 2001). Wnt ligands bind the frizzled family of receptors (Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997) with concomitant binding of the LRP5/6 transmembrane proteins (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; Mao et al., 2001). These ligand-receptor-coreceptor complexes activate intracellular Dishevelled (Yanagawa et al., 1995) which inhibits a cytoplasmic β-Catenin destruction complex (Noordermeer et al., 1994; Kishida et al., 1999; Lee et al., 1999; Peters et al., 1999; Smalley et al., 1999; Itoh et al., 2000), composed of the scaffolding proteins Axin

and Adenomatous Polyposis Coli (APC) and the serine/threonine kinase, Glycogen Synthase Kinase-3β (GSK-3β). The deregulation of cytoplasmic β-Catenin destruction allows it to accumulate in the nucleus where it interacts with TCF/LEF transcription factors (Behrens et al., 1996; Molenaar et al., 1996; van Noort and Clevers, 2002) to activate Wnt responsive genes (Brannon et al., 1997; Brunner et al., 1997; McKendry et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). Conversely, the absence of pathway stimulation results in cytosolic β-Catenin destruction, allowing TCF/LEF interaction with co-repressors to inhibit Wnt target gene transcription (Cavallo et al., 1998; Roose et al., 1998; Brannon et al., 1999).

Localization of β-Catenin to dorsal nuclei of *Xenopus* cleavage stage embryos, establishes the dorsal organizing centre required for body axis formation (Schneider et al., 1996; Moon and Kimelman, 1998; Brown et al., 2000; Chan and Etkin, 2001). As such, ventral activation by ectopically expressed activators of Wnt signaling generates body axis duplications (McMahon and Moon, 1989; Moon, 1993; Cui et al., 1995; Dominguez et al., 1995; Guger and Gumbiner, 1995; He et al., 1995; Pierce and Kimelman, 1995; Sokol et al., 1995; Yang-Snyder et al., 1996; Vleminckx et al., 1997; Yost et al., 1998; Liu et al., 1999b), providing a useful bioassay to identify novel pathway components and to establish their place within the signaling hierarchy (reviewed in Moon and Kimelman, 1998; Gradl et al., 1999).

Following dorsal axis formation, a second phase of ligand-dependent Wnt signaling occurs whereby Wnts promote ventral-type mesoderm while antagonizing Spemann Organizer mesoderm (reviewed in Niehrs, 1999). Thus, extracellular Wnt

ligand inactivation is required to differentiate head from trunk Organizer (Glinka et al., 1997; Glinka et al., 1998; Piccolo et al., 1999). The non-homogeneous Organizer establishes longitudinal and lateral domains of gene expression in the overlying neural plate, which generate forebrain, midbrain, hindbrain and spinal cord (Gamse and Sive, 2001). Low levels of Wnt signaling in anterior neural plate, by exposure to Wnt antagonists expressed at high levels in the prechordal- and anterior chordamesoderm, specify the forebrain, while increasing levels of Wnts specify increasingly posterior character (Kiecker and Niehrs, 2001).

While Wnt inhibition during gastrulation is required for early brain development, recent evidence suggests that they are, interestingly, also required for a later phase of brain patterning (Patapoutian and Reichard, 2000; Kim et al., 2001; Gunhaga et al., 2003). For instance, β-Catenin antisense morpholinos or *Xgsk-3β* RNA injected into prospective neurectoderm caused eye and brain malformations (Itoh et al., 1995; Pierce and Kimelman, 1996; Heasman et al., 2000). Wnt1 also has an established role in specification and function of the isthmic organizer (reviewed in Lake and Kao 2003a; Chapter 1) and as such the development of the midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). This is accomplished, in part, through regulating the expression of En-1/En-2 in the MHB region (Hemmati-Brivanlou and Harland, 1989; McMahon and Bradley, 1990; McMahon et al., 1992; McGrew et al., 1999).

In addition, multiple components of the canonical Wnt pathway are expressed in the forebrain including *Xwnts - 2B, -3A, -8B* (Wolda and Moon, 1992; Wolda et al., 1993;

Cui et al., 1995; Landesman and Sokol, 1997), *Xfzs -2, -3, -5, -7* (Shi et al., 1998; Deardorff and Klein, 1999; Wheeler and Hoppler, 1999; Sumanas and Ekker, 2001) *Xtcf-3, lef-1, and Xtcf-4* (Molenaar et al., 1998; Konig et al., 2000). Also, the XFz3 receptor promotes ectopic eye formation and its inhibition suppresses normal eye development (Rasmussen et al., 2001). Wnt signaling is also required for eye development in *Drosophila*, where it establishes the equatorial/polar axis of the retinal epithelium (Wehrli and Tomlinson, 1998).

This chapter describes the identification of two components of the *Xenopus* Wnt signaling pathway, XPygo-2α and XPygo-2β, which are orthologues of human Pygopus-2 (Kramps et al., 2002; Thompson et al., 2002). *pygopus* was first discovered in a screen for suppressors of an eye phenotype in *Drosophila* as a segment polarity gene necessary for transmission of the Wg signal (Kramps et al., 2002). Mutations within this gene generated an additional legless phenotype, inspiring its name (a pygopus is a legless lizard) (Kramps et al., 2002). Pygopus' role in Wnt signaling is to associate in the nucleus with β-Catenin/Armadillo, through the adaptor legless/BCL9, as a necessary step for TCF/LEF-1 mediated transcription (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002; Townsley et al., 2003). XPygo-2 has been recently identified by Belenkaya et al. (2002) as an important component of the dorsalizing Wnt signal in *Xenopus* axis formation by maternal mRNA depletion. My work furthers these studies to report the existence of two isoforms of *Xpygo-2* that exhibit distinct embryonic expression patterns and functions consistent with an additional role in anterior neural patterning. These

results support my hypothesis that XPygo-2α and XPygo-2β cooperatively pattern the frog brain as part of a late, post-gastrula, phase of Wnt signaling.

## 3.3 MATERIALS AND METHODS

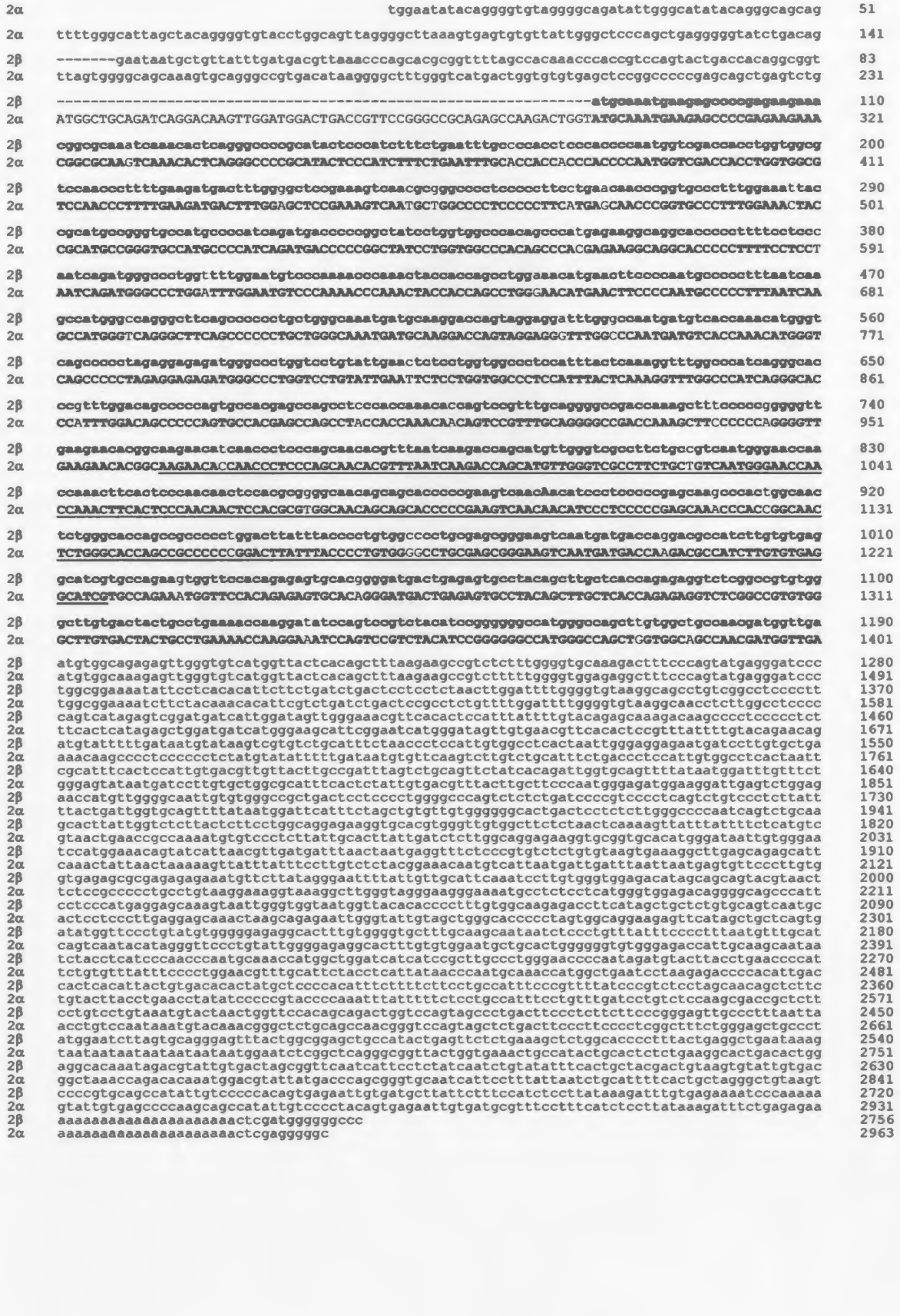
### 3.3.1 Library Screening

Screening of a *Xenopus* stage 10 lambda uniZap II cDNA library (gift from Michael King) was performed using randomly labeled PCR fragments (Prime-a-Gene®, Promega) constituting the 964-1227 bp region of full length *Xpygo-2α* (Figure 3.1). Hybridization of nitrocellulose membranes (Protran™, Schleicher and Schuell) was carried out at 42°C in a formaldehyde (50%) based buffer. Membranes were washed under low stringency (2X SSPE, 0.5% SDS at 45°C). Positive plaques were eluted and re-screened twice under the above conditions. cDNA sequences from individual cross-hybridizing clones were recovered in pBluescript using R408 helper phage (Stratagene). The two isolated clones, *Xpygo-2α* (NCBI Accession number AY141128) and *Xpygo-2β* (NCBI Accession number AY141129) were completely sequenced twice (Hospital for Sick Children, Toronto).

### 3.3.2 Oocytes and Embryos

Wild-type embryos were obtained and injected as described previously (Kao and Lockwood, 1996; Yang et al., 1998) and staged according to Nieuwkoop and Faber (1994). Oocytes were surgically dissected from the ovaries and manually defolliculated in Ca2+/Mg2+ free OR (Opresko, 1991). Dorsal and ventral blastomeres were distinguished based on pigmentation differences and asymmetric cleavage at the 4-cell stage. Both blastomeres (ventral or dorsal; vegetal or animal) on either side of the first cleavage plane

**Figure 3.1. Comparison of *Xpygo-2α* and *Xpygo-2β* complete cDNA sequences.** Sequence identity between the two isoforms is represented in bold-face and the region corresponding to the *hpygo-2* probe used in the library screening for these two Xenopus orthologues is underlined. Start and stop of translation occurs at beginning and end of bold-face region, respectively. Aside from an additional 63 base pairs of coding sequence for *Xpygo-2α*, the coding sequences show very little deviation.



were injected at the 8-cell stage with either 9.2 nl or 4.6 nl to give 10 ng total of synthetic capped mRNA per embryo or up to 40 ng total of either XPygo-2α or XPygo-2β morpholinos (with or without 1 ng each of *Xpygo-2α* and *Xpygo-2β* or 2 ng *Xpygo-2β NHD* RNA). Defects were analysed at tadpole stages (st. 33-35) with disruptions to eye development assessed by abnormalities in quantity and morphology of the retina pigmented epithelium-derived black pigmentation.

UV ventralized embryos were generated by exposure of de-jellied embryos approximately 30 minutes post-fertilization to varying degrees of UV radiation (30-45 seconds) to generate extremely axis deficient embryos (Kao and Danilchik, 1991). For axis rescue experiments, embryos were subsequently injected at the 1-2 cell stage with 10 ng of synthetic mRNA within the vegetal marginal zone.

For animal cap experiments, embryos were injected at the 2-cell stage with or without *noggin* RNA (125 pg) and XPygo-2α MO (40 ng) or control MO (40 ng). For rescue of marker expression, *Xpygo-2α* RNA (2 ng) was also injected. Animal caps were dissected at stage 8 in normal amphibian medium (NAM; Slack, 1984) and cultured overnight in half-strength NAM supplemented with BSA until stage 20-25. RNA was then obtained as described below for RT-PCR analysis.

### 3.3.3 Plasmids

*Xpygo-2α* and *Xpygo-2β* expression constructs were made by PCR amplification of coding sequences (see Table 3.1 for primer sequences) from full length cDNA and ligation into pCS2+ or pCS2+NLS (gifts from Dave Turner) to generate pCS2+*Xpygo-2α*

**Table 3.1. Primer and Morpholino (MO) sequences.**

| Primer | Forward Sequence (5' to 3') | Reverse Sequence (5'to 3') | N* |
|---|---|---|---|
| *Xpygo-2α/β* | GAACAACCCGGTGCCCTTTGG | GGAGGGATGTTGTTGACTTCGG | 25 |
| *Xpygo-2α* | CCCTCGAGGGTGTCCCTCTTATTGCAC | AAGGCCTTGGAGATGAAAGGAAACGC | 30 |
| *Xpygo-2β* | GTCCAGTACTGACCACAGGCGG | CGGGGGAGGGATGTTGTTGA | 30 |
| *Xrx1* | GAACACCTCAAGGGTCCTC | CTGAAATGAGCCCAGGACGC | 30 |
| *Xpax6* | CCGGAACTCATGCAGAACAGTCACAGCGGTG | CCGCTCGAGTTACTGTAATCTTGGCCAGTACTG | 30 |
| *Xsnail-1* | GCACATCCGGAGCCACACG | CGTCGGAATGGGTCTGCAG | 30 |
| *Xchordin* | AACTGCCAGGACTGGATGGT | GGCAGGATTTAGAGTTGCTTC | 25 |

| Morpholino | Antisense Sequence (5'to 3') |
|---|---|
| XPygo-2α | AGCCATCAGACTCAGCTGCTCGGG |
| XPygo-2β | CCGCCTGTGGTCAGTACTGGACGGT |
| Control | CCTCTTACCTCAGTTACAATTTATA |

*Number of annealing/elongation cycles

and pCS2+*Xpygo-2β*. The *Xpygo-2 PHD* plasmid was created by cloning an EcoRI (internal) /XhoI (vector) restriction fragment of pCS2+*Xpygo-2α* into pCS2+NLS. The *Xpygo-2α NHD* construct was generated by sub-cloning the N-terminal EcoRI fragment of *Xpygo-2α* into pCS2+. pCS2+*Xpygo-2β NHD* was constructed by deleting a SmaI (internal)/XhoI (vector) restriction fragment from pCS2+*Xpygo-2β* . A pCS2+noggin expression construct was generated by cloning a HindIII/EcoRI restriction fragment from full-length cDNA (*noggin* pGEM5 a3; gift of W. C. Smith) into the pCS2+ vector.

### 3.3.4 RNA Synthesis

Synthetic RNA was made using NotI linearized templates (for CS2+ plasmids) and transcription with Sp6 RNA polymerase (Krieg and Melton, 1987). *GSK-3β* and *R85* constructs (gifts from Isabel Dominguez) were linearized with SacI and transcribed with T7 polymerase (Krieg and Melton, 1987). Axis duplication activity using RNA encoding the R85 construct of Glycogen Synthase Kinase-3β, which has a lysine to arginine substitution at position 85, was previously described (Dominguez et al., 1995; Kao and Lockwood, 1996). All synthetic mRNAs were found to translate efficiently *in vitro* using a cell-free rabbit reticulocyte system (Promega).

### 3.3.5 RT-PCR Analysis

Embryos (n=8) or animal caps (n=15-20) injected with various *Xpygo-2* constructs or morpholinos were obtained at either stage 10 or stage 20 and RNA was extracted using

the Nucleospin RNA II Kit (Clontech Laboratories, Inc.). Reverse transcribed RNA was subsequently amplified as described (Lake et al., 2001), using oligonucleotide primers previously reported for *engrailed-2* (*en-2*; 30 cycles; Hemmati-Brivanlou et al., 1991), *Xbf1* (25 cycles; Bourguignon et al., 1998), *Xotx-2* (25 cycles; Pannese et al., 1995), *XAG* (25 cycles; Sive and Bradley, 1996), *krox-20* (25 cycles; Hemmati-Brivanlou et al., 1994) *hoxB9* (30 cycles; Sharpe et al., 1987), *H4* (23 cycles; Yang et al., 1998), *siamois* (30 cycles; Lemaire et al., 1995) and listed in Table 3.1.

For temporal expression analysis of *Xpygo-2α/β*, RNA was prepared as described for Northern analysis below. Spatial distribution of messages was determined through dissection of stage 8, 9 or 10 embryos (n=24) into animal, vegetal and equatorial sections. Stage 10 embryos (n=24) were also dissected into dorsal and ventral sections using the blastopore lip for orientation. RNA was processed for RT-PCR as described above.

### 3.3.6 Northern Analysis

Total RNA from oocytes and embryos (n=16) was extracted using the Nucleospin RNA II Kit (Clontech Laboratories, Inc.) and processed for Northern analysis as described (Kao and Hopwood, 1991). Equal levels of RNA were run onto a formaldehyde gel, transferred to GeneScreen™ hybridization membrane (DUPONT) and UV crosslinked. A random primed (Prime-a-Gene®, Promega) PCR fragment (537-1173 bp) of *Xpygo-2α* was hybridized at 65°C using ExpressHyb (Clontech Laboratories, Inc.) and blots washed at high stringency (0.5X SSC and 0.5% SDS at 55°C). Blots were re-probed (45-55°C) using *Xenopus histone H4* (Gift from Kim Mowry).

### 3.3.7 Whole Mount *in situ* Hybridization

Templates for probes were made by subcloning PCR-amplified sequences common to both *Xpygo-2α* (nucleotides 964-1227 and 295-1309) and *Xpygo-2β* (nucleotides 749-1011 and 80-1094) into pBluescript (Stratagene). A *Xpygo-2β* specific template was amplified from the 5' non-coding region of *Xpygo-2β* (nucleotides 1-87). Templates were linearized with XbaI and transcribed with T7 polymerase. Albino embryos were fixed in MEMFA (Harland, 1992) and hybridized with digoxigenin- and fluorescein- linked sense and antisense probes and stained by NBT/BCIP as described (Harland, 1991, modified by Sagerström et al., 1996). To reduce background staining due to GC-rich content of *Xpygo-2*, hybridization was done at 65°C and all washes were at 65-68°C. Embryos were dehydrated in ethanol and cleared in Murray's solution (1:2 benzyl alcohol and benzyl benzoate).

### 3.3.8 Whole Mount Immunohistochemistry

Embryos were fixed in MEMFA, washed in maleic acid buffer (MAB; pH 7.5), incubated in 0.1 M $K_2Cr_20_7$ in 5% acetic acid for 30', followed by three 5' washes in MAB and bleached in 5% $H_20_2$/MAB under white light. After rinsing in MAB, embryos were blocked for 60' in 2% blocking reagent/MAB (Roche) and incubated overnight at 4°C in the appropriate primary antibody diluted as follows: 1:1000 for 12-101 (developed by J. Brockes) and 1:500 for 4D9 (Patel et al., 1989, developed by C. Goodman) both from Developmental Studies Hybridoma Bank, U. of Iowa, and 1:5 for 2G9 (gift of E. A.

Jones). Embryos were washed for five hours in MAB and incubated at 4°C overnight in a 1:1000 dilution of alkaline phosphatase conjugated goat anti-mouse IgG in MAB (Cedar Lane Laboratories Ltd.). Embryos were washed in MAB for 24 hrs with multiple buffer changes then twice for 5 min in alkaline phosphatase buffer (AP: 0.1 M Tris HCL ph 9.5, 0.1 M NaCl. 50 mM $MgCL_2$). Staining was performed in AP buffer containing NBT and BCIP and stopped in MEMFA. Embryos were then destained in methanol for approximately 12 hrs before being cleared in Murray's solution.

### 3.3.9 Antisense Morpholinos

Antisense morpholino oligonucleotides (MO, Genetools, LLC) were designed to complement the 5' noncoding regions of *Xpygo-2α* and *Xpygo-2β* (Table 3.1). For visualization within the embryos, the XPygo-2α MO was linked to fluorescein (green fluorescence) and XPygo-2β MO to lissamine (red fluorescence). The control MO (Table 3.1), linked with fluorescein (green fluorescence), was a random sequence (designed by Genetools, LLC) of equal length to the anti-Pygo MO's, used to ensure specificity of XPygo-2 MO interactions. Translation inhibition efficiency as well as target specificity were tested *in vitro* using the transcription/translation coupled cell-free system (Promega) using as templates full-length *Xpygo-2α* and *Xpygo-2β* cDNA sequences (in pBluescript) or an equivalent quantity of synthetic mRNA lacking complimentary sequences to the morpholinos, as used in rescue experiments.

## 3.4 RESULTS

### 3.4.1 Isolation of the *Xenopus* Orthologues of Human *pygopus-2*

An assay for sequences differentially regulated by Xrel3 (Lake et al., 2001) provided PCR fragments with homology to *human (h)pygo-2*, which were used to screen a *Xenopus* stage 10 cDNA library to obtain the corresponding full-length orthologues. Two clones were isolated, *Xpygo-2α* and *Xpygo-2β*, both of which shared coding sequences, but with variable untranslated regions (Figure 3.1). In addition, *Xpygo-2α* had an extra 63 bp of 5' coding sequence when compared to *Xpygo-2β* . In a comparison of the predicted translation products with the Pygopus proteins encoded by the human genome, both exhibited the highest overall sequence identity (68%) with hPygo-2 as compared with hPygo-1 (36%) (Figure 3.2). Therefore, these *Xenopus* proteins most likely represent true orthologues of hPygo-2 and not hPygo-1.

All Pygopus proteins share two common domains, a 50 amino acid stretch within the N-terminus referred to as the N-terminal homology domain (NHD) or N box and the C-terminal plant homeodomain (PHD) (Kramps et al., 2002; Thompson et al., 2002). The PHD is a zinc finger-like domain with a Cys4-His-Cys3 consensus present within certain chromatin remodeling-type transcriptional regulators (Aasland et al., 1995). Both the NHD and PHD domains are present within XPygo-2α and -2β (Figure 3.2), as well as the conserved N-terminal putative nuclear localization sequence in all Pygopus proteins (Figure 3.2; Kramps et al., 2002; Thompson et al., 2002). The *hpygo-2* gene (1q23.1) is organized into three exons capable of generating alternative splice variants that resemble

**Figure 3.2. Comparison of predicted XPygo-2α/β protein sequences with hPygo-2** (NCBI Accession number: AAL91371). Sequence identity between the three proteins is in bold-face. The conserved putative nuclear localization sequence, (KKRRK) is double-underlined, while the C-terminal PHD is single-underlined and the NHD or N-Box is indicated by broken underline.

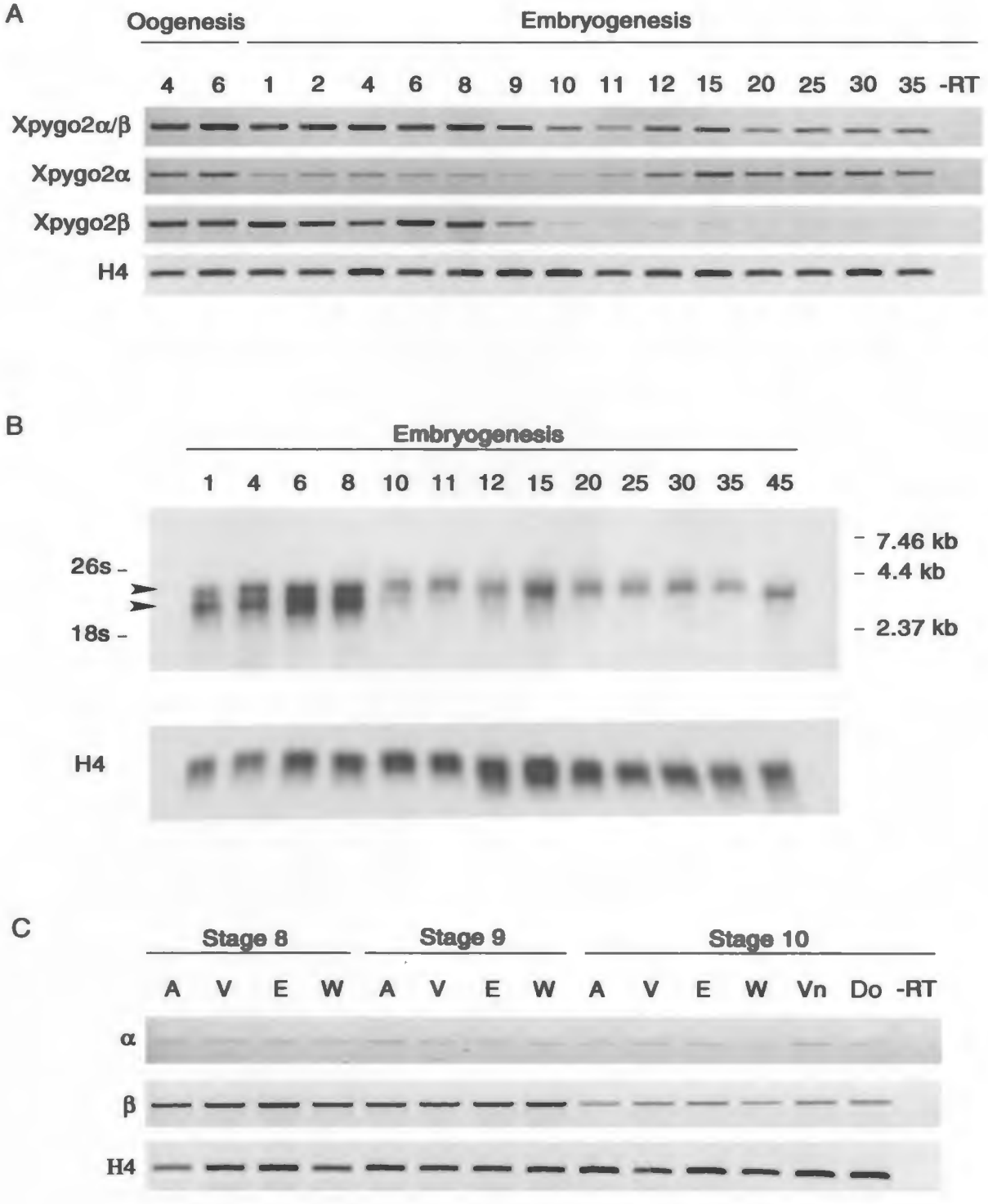

hPygo-2 MAASAPPPPDKLEGGGGPAPPPAPPSTGRKQGKAGLQMKSPEKKRRKSNTQGPAY 55
Xpygo-2α MAA----------DQDKDGLTVPGRRAKTGMQMKSPEKKRRKSNTQGPAY 40
Xpygo-2β ----------MQMKSPEKKRRKSNTQGPAY 20
hPygo-2 SHLTEFAPPPTPMVDHLVASNPFEDDFGAPKVGVAAPPFLGSPVPFGGFRVQGGMAGQVP 115
Xpygo-2α SHLSEFAPPPTPMVDHLVASNPFEDDFGAPKVNAGPSPFMSNPVPFGNYRMPGAMPHQMT 100
Xpygo-2β SHLSEFAPPPTPMVDHLVASNPFEDDFGAPKVNAGPSPFLNNPVPFGNYRMPGAMPHQMT 80
hPygo-2 PGYSTGGGGGPQPLRRQPPPFPPNPMGPAFNMPPQGPGYPPPGNMNFPSQPFNQPLGQNF 175
Xpygo-2α PGYP----GGPQPTRRQAPPFPPNQMGP--GMS-QNPNYHQPGNMNFPNAPFNQAMGQGF 153
Xpygo-2β PGYP----GGPQPMRRQAPPFPPNQMGPGFGMS-QNPNYHQPGNMNFPNAPFNQAMGQGF 135
hPygo-2 SPPSGQMMPGPVGGFGPMISPTMGQPPRAELGPPSLSQRFAQPGAP--FGPS--PL-QRP 231
Xpygo-2α SPPAGQMMQGPVGGFGPMMSPNMGQPPRGEMGPGPVLNSPGGPPFTQRFGPSGHPFGQPP 213
Xpygo-2β SPPAGQMMQGPVGGFGPMMSPNMGQPPRGEMGPGPVLNSPGGPPFTQRFGPSGHPFGQPP 195
hPygo-2 GQGLPSLPPNTSPFPGPDPGFPGPGGEDGGKPLNPPAS-TAFPQEPHSGSPAAAVNGNQP 291
Xpygo-2α -VPRASLPPNNSPFAGADQSFP-PGVEEHGKNTNPP-SNT-FNQDQHVGSPS-AVNGNQP 275
Xpygo-2β -VPRASLPPNTSPFAGADQSFP-PGVEEHGKNINPP-SNT-FNQDQHVGSPS-AVNGNQP 250
hPygo-2 SFPPNSSGRGGG-TPDANSLAPPGKAGGGSGPQPPPGLVYPCGACRSEVNDDQDAILCEA 350
Xpygo-2α NFTPNNSTRGNSSTPEVNNIPPPSKPTGNSGHQPPPGLIYPCGACERFVNDDQDAILCEA 335
Xpygo-2β NFTPNNSTRGNSSTPEVNNIPPPSKPTGNSGHQPPPGLIYPCGPCERFVNDDQDAILCEA 310
hPygo-2 SCQKWFHRECTGMTESAYSLLTREBSAVWACDYCLKTKEIQSVYIRGAMGQLVAANDG* 406
Xpygo-2α SCQKWFHRECTGMTESAYGLLTTEASAVWACDLCLKTKEIQSVYIR-EMGQLVAANDG* 389
Xpygo-2β SCQKWFHRECTGMTESAYSLLTREVSAVWACDYCLKTKDIQSVYIRGAMGQLVAANDG* 368

the two *Xpygo-2α/β* clones. Because of the high sequence identity between *Xpygo-2α* and *-2β* coding regions, it is possible that, like *hpygo-2,* the *Xenopus* cDNAs represent two different splice variants of the same *Xpygo-2* gene.

### 3.4.2 *Xenopus pygopus-2* Isoforms are Differentially Expressed During Early Development

Whether the two *Xpygo*-2 mRNA isoforms represent true spliced alternatives or whether they are derived from separate alloalleles, their differential patterns of expression suggest that they have distinct roles in development. To determine the temporal pattern of *Xpygo-2* message accumulation during early *Xenopus* embryogenesis, semi-quantitative RT-PCR was performed on total RNA extracted from staged oocytes and embryos (Figure 3.3A and B) using oligonucleotide primers that were specific to either *Xpygo-2α* or *Xpygo-2β* transcripts. *Xpygo-2α*-specific primers amplified sequences in oocytes but at reduced levels after fertilization and in all stages of pregastrula and gastrula development. The levels increased steadily during neurulation (stage 12) and in subsequent stages (Figure 3.3A). Unlike *Xpygo-2α*, specific amplification of *Xpygo-2β* sequences indicated that *Xpygo-2β* transcripts were only present at high levels maternally in both oocytes and cleavage stage embryos, but rapidly diminished at late blastula stages just prior to gastrulation and remained at low levels thereafter (Figure 3.3A). Parallel results were obtained using a probe which hybridized to both transcripts resolved by Northern analysis (Figure 3.3B). Thus *Xpygo-2α* and *-2β* exhibit distinctly different temporal patterns of mRNA expression in development.

**Figure 3.3. *Xpygo-2α/β* exhibit distinct expression patterns during *Xenopus* development.** (A) RNA from 16 oocytes or embryos were analyzed by RT-PCR using primers specific to both messages (*Xpygo-2α/β*) and either *Xpygo-2α* or *Xpygo-2β*. (B) Northern blot of staged embryo RNA probed for both *Xpygo-2* isoforms (top arrow, *-2α*, bottom arrow, *-2β*). The 28S and 18S ribosomal RNAs as well as an RNA ladder are indicated. (C). Whole Embryos (W) as well as animal (A), vegetal (V), equatorial (E), ventral (Vn) and dorsal (Do) sections were analysed by RT-PCR using primers specific to *Xpygo-2α* or *Xpygo-2β* at stages 8, 9 and 10. Stages of development are indicated at the top of each figure (Nieuwkoop and Faber, 1994). Levels of cDNA used or RNA loaded were standardized using *histone (H4)* levels. –RT: negative control, without reverse transcriptase.


A
Oogenesis
Embryogenesis
4 6 1 2 4 6 8 9 10 11 12 15 20 25 30 35 -RT
Xpygo2α/β
Xpygo2α
Xpygo2β
H4
B
Embryogenesis
1 4 6 8 10 11 12 15 20 25 30 35 45
26s
18s
7.46 kb
4.4 kb
2.37 kb
H4
C
Stage 8
Stage 9
Stage 10
A V E W A V E W A V E W Vn Do -RT
α
β
H4

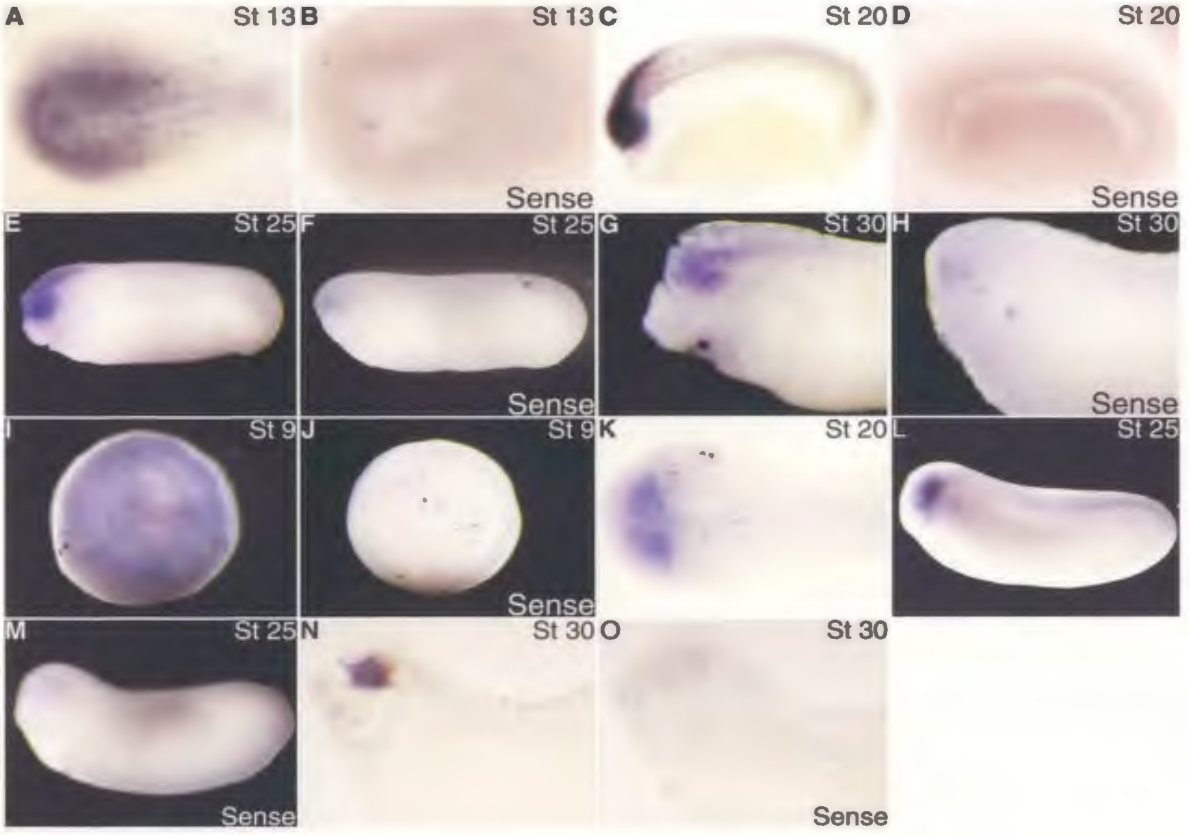
To determine the spatial location of *Xpygo-2* messages during pre-gastrula stages, embryos were dissected into animal, vegetal and equatorial sections at stages 8, 9 and 10 or ventral and dorsal sections at stage 10. Primers specific for *Xpygo-2α* or *Xpygo-2β* demonstrated ubiquitous expression for both, but with low levels of *Xpygo-2α* for all stages examined and a high level of *Xpygo-2β* in early blastulae that globally declined prior to gastrulation (Figure 3.3C).

### 3.4.3 *Xpygo-2α* and *Xpygo-2β* mRNAs have Distinct Patterns of Expression in the Developing Brain and Eyes

Whole mount *in situ* hybridization was used to determine the spatial distribution of *Xpygo-2* messages. Transcript accumulation in embryos was detected by staining of hybridized probes. Staining for both *Xpygo-2α* and *–2β* was observed at early open neural plate stages predominantly within the anterior neurectoderm (Figure 3.4A) and by late neurula stages within the anterior-most neural tube fated to form the brain (Figure 3.4C). This trend for anterior-most message accumulation was observed through to tailbud (Figure 3.4E) and tadpole (Figure 3.4G) stages, with messages becoming restricted to the fore-, mid- and hindbrain, as well as the optic cup.

An *in situ* probe specific to the 5' untranslated region of *Xpygo-2β* was used to determine if this isoform was differentially expressed. Staining for *Xpygo-2β* transcripts was even throughout the embryos at the late blastula stage (Figure 3.4I) but became localized to a band of anterior neural tissue encompassing the prospective retinal field (Figure 3.4K), which gives rise to eyes and forebrain (Eagleson and Harris, 1990;

**Figure 3.4. *Xpygo-2α/β* are differentially expressed within the brain.** Whole mount *in situ* hybridization analysis of staged embryos using probes complementary to both transcripts (A-H) or specific to *Xpygo-2β* mRNA (I-O), or sense probes, where indicated (B,D,F,H,J,M,O). Specific hybridization is indicated by purple staining reaction. All embryos are shown with anterior to the left. *Xpygo-2* mRNA is found early (St 13) in the anterior neural plate (A, dorsal view). By stage 20 and at least until stage 25, messages localized within the anterior neural tube, including prospective brain and eyes (C and E, lateral views) and in the tadpole, in the mid-hindbrain, forebrain and eyes (G, lateral view). *Xpygo-2β* transcripts are present throughout blastulae (I, animal pole view), but are restricted to the retinal field at stage 20 (K, dorsal view) and in derivatives of this region (forebrain and eyes) at stage 25 (L, lateral view) and stage 30 (N, lateral view).


A
St 13
B
St 13
Sense
C
St 20
D
St 20
Sense
E
St 25
F
St 25
Sense
G
St 30
H
St 30
Sense
I
St 9
J
St 9
Sense
K
St 20
L
St 25
M
St 25
Sense
N
St 30
O
St 30
Sense

Eagleson et al., 1995; Li et al., 1997). Structures arising from this region continued to stain for *Xpygo-2β* in subsequent tailbud (Figure 3.4L) and tadpole (Figure 3.4N) embryos, predominantly restricted to the eyes. These results demonstrate that both *Xpygo-2α* and *Xpygo-2β* are expressed in the brain, but the domain of *Xpygo-2β* is restricted more anteriorly to the forebrain, eyes and possibly midbrain.

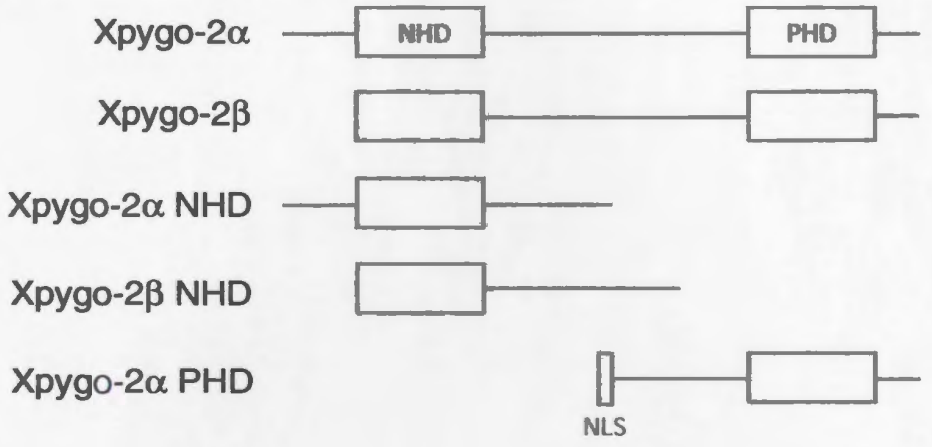
### 3.4.4 The XPygo-2 NHD Can Mediate the Dorsalizing Wnt Signal

Activation of the canonical Wnt pathway ventrally in early embryos using many different components of the signal transduction cascade induces an ectopic secondary axis (Moon and Kimelman, 1998). Conversely, its antagonism dorsally through depletion of β-Catenin or expression of dominant negative TCF, inhibits dorso-anterior structures leading to a ventralized fate (Molenaar et al., 1996; Wylie et al., 1996; Heasman et al., 2000). On the other hand, ectopic activation of the Wnt pathway within dorsal vegetal cells results in the loss of signals required for inducing anterior neural plate derivatives, since formation of the head organizer is dependent on the antagonism of Wnt signaling (Christian and Moon, 1993; Fredieu et al., 1997; Glinka et al., 1997, Glinka et al., 1998; Piccolo et al., 1999).

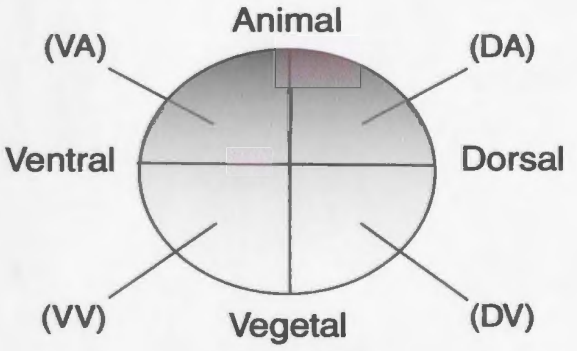
To determine the function of XPygo-2 in dorsal-ventral axis formation, RNA encoding wild-type and mutant versions of XPygo-2α/β (Figure 3.5A) was injected into different regions of early cleavage stage embryos (Figure 3.5B). Unfortunately, neither overexpression of XPygo-2α nor XPygo-2β had any affect on *Xenopus* development.

**Figure 3.5. Targeted overexpression of various XPygo-2 constructs.** (A) The constructs used for subsequent injection experiments encoded wild-type XPygo-2α and -2β, XPygo-2α NHD (amino acids 1-194), XPygo-2β NHD (amino acids 1-216) , and XPygo-2α PHD (amino acids 194-389) fused to a strong nuclear localization sequence (NLS). (B) Injection experiments were performed at the 8-cell stage. For targeted expression within ventral mesendodermal derivatives, synthetic RNA was injected into both ventral vegetal blastomeres (VV). To target neural plate and epidermis, RNA or morpholinos were injected into either dorsal (DA) or ventral (VA) animal blastomeres, respectively.

A


Xpygo-2α
NHD
PHD
Xpygo-2β
Xpygo-2α NHD
Xpygo-2β NHD
Xpygo-2α PHD
NLS

B

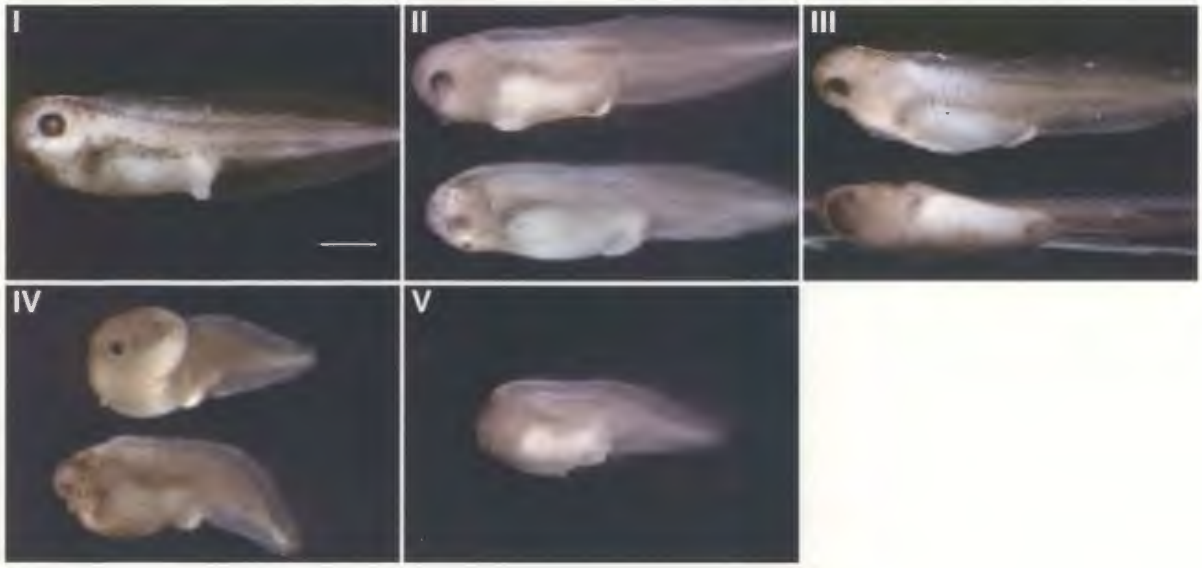

Animal
(VA)
(DA)
Ventral
Dorsal
(VV)
Vegetal
(DV)

This is not unexpected as Pygopus is believed to act within a complex with β-Catenin, and as such, its activation of Wnt target gene expression would be limited by nuclear β-Catenin levels (Kramps et al., 2002; Thompson et al., 2002). Therefore, the effect of overexpressing the N- and C-terminal functional domains was examined, since they may interfere with or modify the normal function of endogenous XPygo-2 or may have activity on their own. As such two mutant variants were generated to encode either the N-terminal half of XPygo-2α containing the NHD domain or the C-terminal half containing the PHD domain (Figure 3.5A). To ensure that the PHD domain could gain access to the nucleus it was cloned within the expression vector (pCS2+NLS) downstream and within frame of a strong nuclear localization signal (NLS).

To target the prospective dorsal organizing center, I injected mRNA into dorsal vegetal blastomeres at the 8-cell stage (Figure 3.5B). Injection of 10 ng of *Xpygo-2α PHD* RNA, like that encoding wild-type protein, had no effect on axial patterning in *Xenopus* embryos (Figure 3.6; Table 3.2). However, dorsal vegetal injection of *Xpygo-2α NHD* RNA resulted in progressive loss of dorsal anterior-most structures (Figure 3.6; Table 3.1). The phenotypes ranged from slightly disrupted and ventrally displaced eyes (Type II) to fusion of eyes ventrally (cyclopia, Type III), microencephaly (Type IV) and loss of all head structures (anencephaly, Type V). These defects resembled those associated with ventral midline abnormalities resultant from disruption or loss of head organizing prechordal mesendoderm (Li et al., 1997) and are consistent with those observed following misexpression of XWnt-8 or exposure to lithium ions (Fredieu et al., 1997). Therefore, the XPygo-2α NHD mutant appears to activate Wnt signaling.

**Figure 3.6. XPygo-2α NHD overexpression in dorsal mesoderm generated head truncations resembling ectopic Wnt activation.** (A). Injection of *Xpygo-2α NHD* RNA (10 ng) within the dorsal vegetal blastomeres at the 8-cell stage generated anterior-defective embryos having progressive abnormalities that ranged from normal (Type I) to slightly disrupted and ventrally displaced eyes (Type II) to fused eyes or cyclopia (Type III, top embryo - lateral view, bottom embryo - ventral view), microencephaly (Type IV) and anencephaly (Type V). (B). The percentage of each type of head malformation shown in (A) over 5 experiments is shown in a histogram. Numbers at the tops of the bars indicate total number of embryos scored, while numbers at the bottom represent site of injection (Figure 3.5B). Embryos that failed to gastrulate properly (17-uninjected; 25-αNHD DV; 19-αNHD VV; 19-PHD VV; 18-αWT DV) were excluded from this analysis.

A


I
II
III
IV
V

B

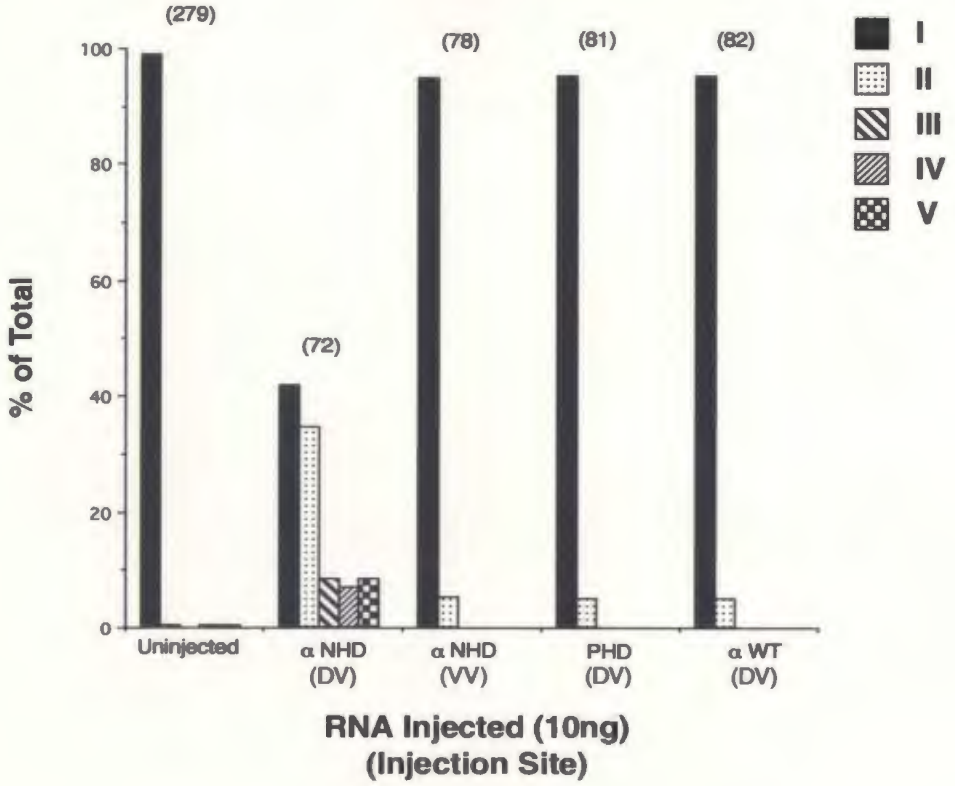

(279)
(72)
(78)
(81)
(82)
100
80
60
40
20
0
% of Total
I
II
III
IV
V
Uninjected
α NHD (DV)
α NHD (VV)
PHD (DV)
α WT (DV)
RNA Injected (10ng)
(Injection Site)

**Table 3.2. Distribution of phenotypes caused by XPygo-2α/β activator mutants and Morpholino knockout**

| Treatment | Injection Site (Fig.3.5) | Number (percentage) of Phenotypes (Fig. 3.6) I | II | III | IV | V | ∑ |
|---|---|---|---|---|---|---|---|
| Uninjected | | 276(98.9) | 1(0.4) | 0 | 1(0.4) | 1(0.4) | 279 |
| *Xpygo-2α NHD*[*] | DV | 30(41.7) | 25(34.7) | 6(8.3) | 5(6.9) | 6(8.3) | 72 |
| *Xpygo-2α NHD*[*] | VV | 74(94.9) | 4(5.1) | 0 | 0 | 0 | 78 |
| *Xpygo-2 PHD*[*] | DV | 77(95.1) | 4(4.9) | 0 | 0 | 0 | 81 |
| *Xpygo-2α*[*] | DV | 78(95.1) | 4(4.9) | 0 | 0 | 0 | 82 |

| | | Normal | Eye Defects (Fig. 3.8) | ∑ |
|---|---|---|---|---|
| Uninjected | | 276(98.9) | 3(1.1) | 279 |
| *Xpygo-2α NHD*[*] | DA | 20(18.5) | 88(81.5) | 108 |
| *Xpygo-2β NHD*[*] | DA | 18(20.9) | 68(79.1) | 86 |
| *Xpygo-2α NHD*[*] | VA | 63(95.5) | 3(4.5) | 66 |
| *Xpygo-2 PHD*[*] | DA | 67(100) | 0 | 67 |
| *Xpygo-2α*[*] | DA | 55(96.5) | 2(3.5) | 57 |

| | | | Eye Defects (Fig. 3.11) | | | |
|---|---|---|---|---|---|---|
| | | Normal | Bilateral | Unilateral | Total | ∑ |
| Uninjected | | 111(99.1) | 1(0.9) | 0 | 1(0.9) | 112 |
| Control Mo[**] | DA | 57(90.5) | 0 | 6(9.5) | 6(9.5) | 63 |
| α Mo[**] | DA | 2(3.7) | 21(38.9) | 31(57.4) | 52(96.3) | 54 |
| β Mo[**] | DA | 6(10.9) | 31(56.4) | 18(32.7) | 49(89.1) | 55 |
| α /β Mo[**] | DA | 1(1.4) | 37(51.4) | 34(47.2) | 71(98.6) | 72 |
| α /β Mo[**] + *α/β*[Ŧ] | DA | 26(53.1) | 4(8.2) | 19(38.8) | 23(46.9) | 49 |
| α /β Mo[**]+ *NHD*[Ŧ] | DA | 1(2.9) | 14(41.2) | 19(55.9) | 33(97.1) | 34 |

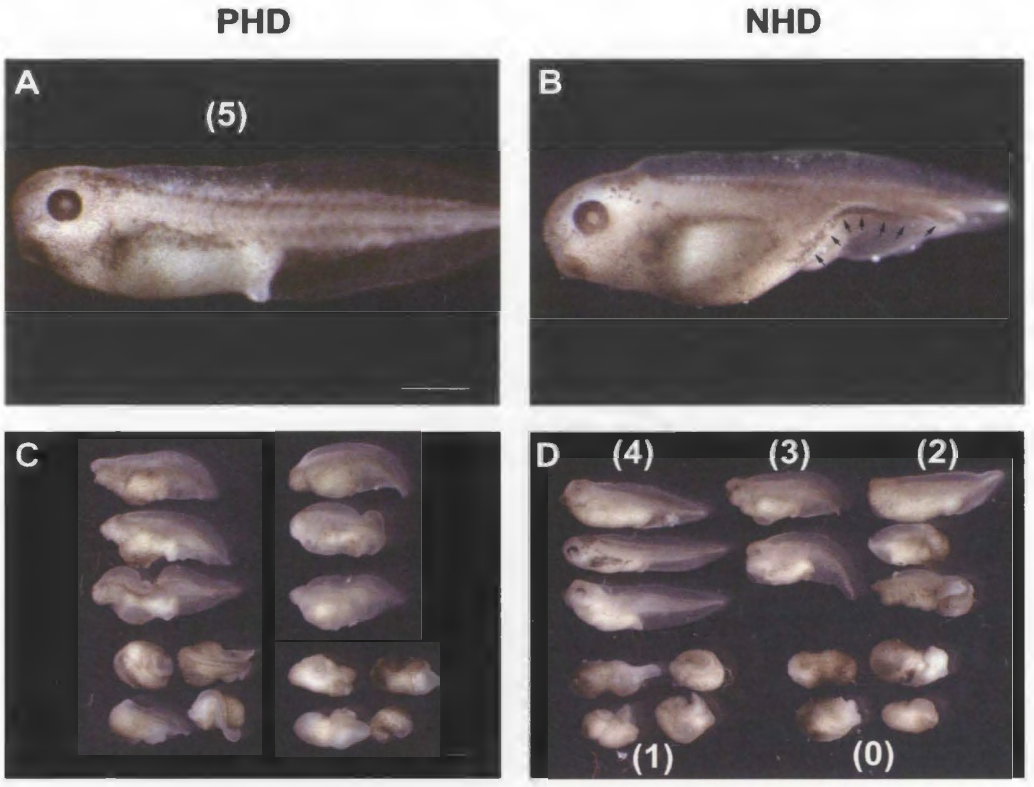
[*]Injected a total of 10 ng per embryo.
[**]Injected a total of 40 ng per embryo.
[Ŧ]Injected a total of 2 ng of rescuing RNA (1 ng each of *Xpygo-2α* and *Xpygo-2β* or 2 ng *Xpygo-2β NHD*).

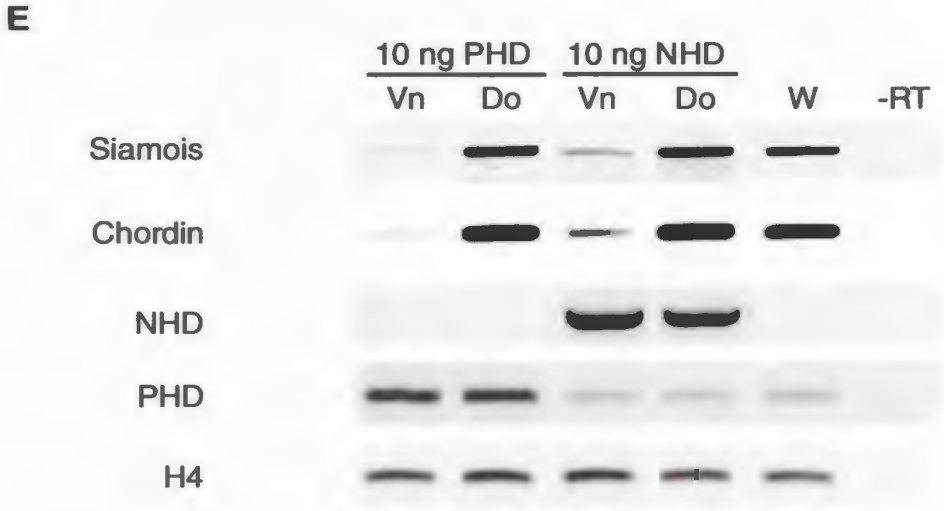
Overexpression of XPygo-2α NHD within the ventral vegetal region did not affect normal axial development, but did induce partial secondary axes in approximately 15% (12/78) of the embryos injected (Figure 3.7B), coincident with a slight increase in expression of the dorsal markers *siamois* (Lemaire et al., 1995) and *chordin* (Sasai et al., 1994) on the ventral side (Figure 3.7E). Also, injection of *Xpygo-2α NHD* RNA rescued the formation of partial axes in UV irradiated embryos, with an increase in the proportion of embryos containing head structures (DAI 3-4) from 1/70 (*Xpygo-2 PHD* injected; Figure 3.7C) to 17/65 (*Xpygo-2α NHD* injected; Figure 3.7D), coincident with an average DAI (dorsoanterior index, Kao and Elinson, 1988) shift from 0.66 to 1.46, respectively. These observations were consistent with maternal XPygo-2 depletion studies using antisense phosphorothioate oligonucleotides (Belenkaya et al., 2002), suggesting that the NHD had weak Wnt-activation properties. Increasing or decreasing the size of the NHD domain failed to increase the truncation mutant's potency (data not shown).

### 3.4.5 The XPygo-2 NHD Can Mediate the Anterior Neural-Suppressing Wnt Signal

Wnt signaling plays a vital role in establishing the proper AP character of the neuraxis by its ability to posteriorize neurectoderm (reviewed in Niehrs, 1999). As such, extracellular Wnt ligands must be antagonized for specification of the anterior most neural plate fated to form forebrain and eyes (Glinka et al., 1997; Glinka et al., 1998; Piccolo et al., 1999). Ectopic activation of Wnt signaling within prospective anterior neurectodermal cells generates anterior malformations, ranging from mild disruption to

**Figure 3.7. XPygo-2 NHD induces partial secondary axes when expressed ventrally and rescues dorsal axial structures in UV ventralized embryos.** While XPygo-2α PHD had no effect on axis formation, injection of *Xpygo-2α NHD* RNA within ventral vegetal (VV) blastomeres at the 8-cell stage generated partial axial duplications (B, arrows). UV treated embryos were classified according to the dorsoanterior index (Kao and Elinson, 1988) where a DAI of (5) is normal and a DAI of (0) is devoid of all dorsal structures. Dorsal axial perturbations could be partially rescued by XPygo-2α NHD (D) but not XPygo-2α PHD (C), with a shift in the number of embryos having head structures (DAI 3-4) from 1/70 (*Xpygo-2α PHD* injected) to 17/65 (*Xpygo-2α NHD* injected), coincident with an average DAI shift from 0.66 to 1.46, respectively. Representative phenotypes are shown (C-D). Scale bar: 1 mm. (E) Embryos that had been injected with 10 ng of either *Xpygo-2α PHD* or *Xpygo-2α NHD* RNA into ventral vegetal (VV) blastomeres at the 8-cell stage were dissected into dorsal (Do) and ventral (Vn) halves at stage 10. RT-PCR analysis showed increased levels of *siamois* and *chordin* ventrally in *Xpygo-2α NHD* containing cells as compared to *Xpygo-2α PHD* containing cells. Amounts of cDNA used were normalized to *histone (H4)* levels. –RT: negative control, without reverse transcriptase.


PHD
NHD
A
(5)
B
C
D
(4)
(3)
(2)
(1)
(0)


E
10 ng PHD
10 ng NHD
Vn
Do
Vn
Do
W
-RT
Siamois
Chordin
NHD
PHD
H4

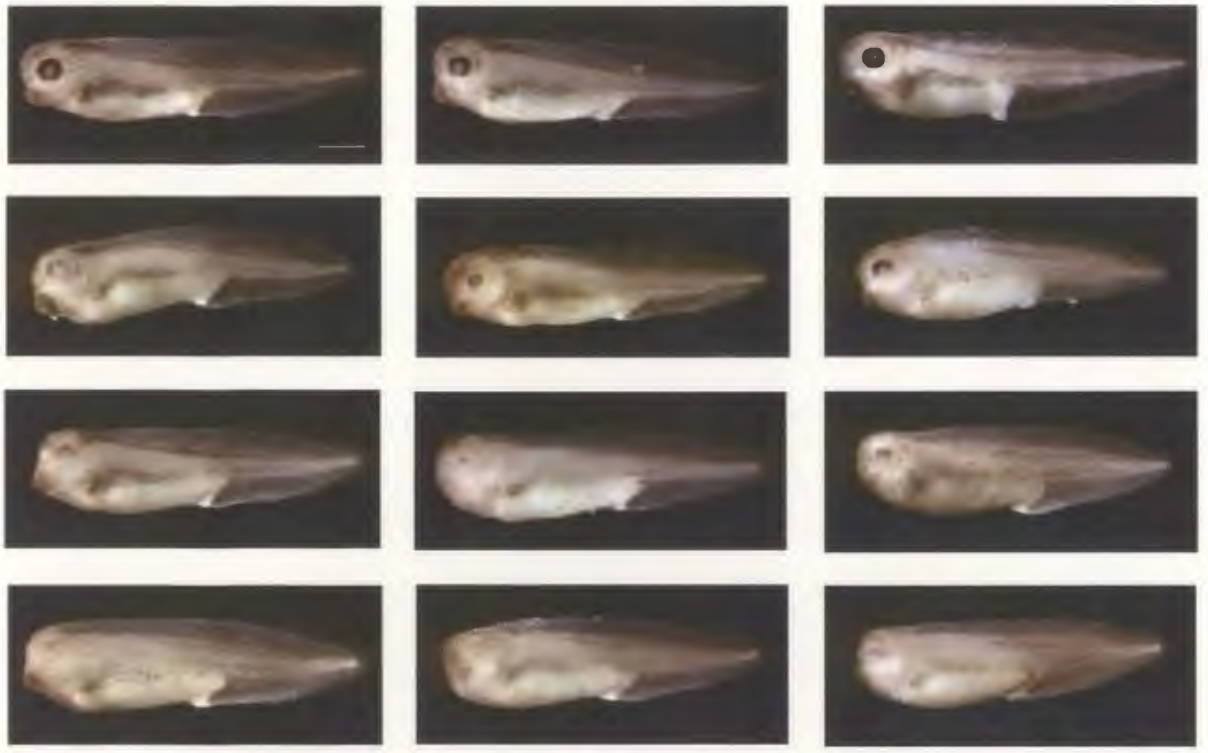
complete loss of forebrain and eyes (Yamaguchi and Shinagawa, 1989; Christian and Moon, 1993; Fredieu et al., 1997; Darken and Wilson, 2001).

The axis-promoting activity of the NHD, when overexpressed in early ventral vegetal cells, suggested that the XPygo-2α NHD might mimic the effects of Wnt pathway activators when expressed within the anterior neural plate. To target these cells, *Xpygo-2α NHD* RNA was injected into dorsal animal cells at the 8-cell stage (Figure 3.5B). The majority of injected embryos (81.5%) had defects in one or both eyes ranging from ventral defects to reduced or almost complete loss of pigmented epithelium (Table 3.2, Figure 3.8). While *Xpygo-2α PHD* and wild-type *Xpygo-2α* RNA had no effect on normal development (Table 3.2, Figure 3.8), *Xpygo-2α NHD* RNA caused similar defects to those caused by expression of the GSK-3β dominant mutant Wnt activator, R85 (Dominguez et al., 1995), both through phenotypic alterations of the head and eyes (Figure 3.8A) and the brain (Figure 3.9) as shown by immunostaining with the pan-neural antibody 2G9 (Jones and Woodland, 1989).

Both the *Xpygo-2α NHD* RNA-injected and *R85* RNA-injected embryos showed disrupted forebrain tissues (Figure 3.9E, F, I, J, arrows). In contrast, embryos injected with *gsk-3β* RNA (Wnt pathway inhibitor), while exhibiting similar eye defects (Figure 3.9A), showed relatively normal, if not expanded, staining of the anterior-most neural tissue, with more significant disorganization of the diencephalon-midbrain region (Figure 3.9M, N, arrow). All embryos showed normal staining of skeletal muscle with the 12/101 antibody (Figure 3.9C, G, K, O), demonstrating the specificity of the defects to anterior

**Figure 3.8. XPygo-2α/β NHD overexpression in dorsal ectoderm generates anterior defects similar to aberrant Wnt signaling.** (A) 10 ng of *Xpygo-2α/β NHD* RNA injected within the dorsal animal blastomeres at the 8-cell stage caused eye defects of differing degrees characterized by loss of ventral structures and reduced size. Top panels represent embryos having normal morphology, and lower panels represent increasing degrees of eye abnormalities. These defects were compared to those caused by overexpression, within the same region, of a dominant activating mutant GSK-3β (R85) and wild-type GSK-3β. Scale bar: 1 mm. (B) The percentage of *Xpygo-2 NHD* injected embryos having eye defects shown in (A) was compiled over 4-5 experiments and shown in a histogram. Numbers at the tops of the bars indicate total number of embryos scored, while numbers at the bottom refer to injection site (as shown in Figure 3.5B). Embryos that failed to gastrulate properly (17-uninjected; 22-αNHD DA; 0-αNHD VA; 0-PHD DA; 1-αWT DA) were excluded from this analysis.

A.


NHD
R85
GSK

B.

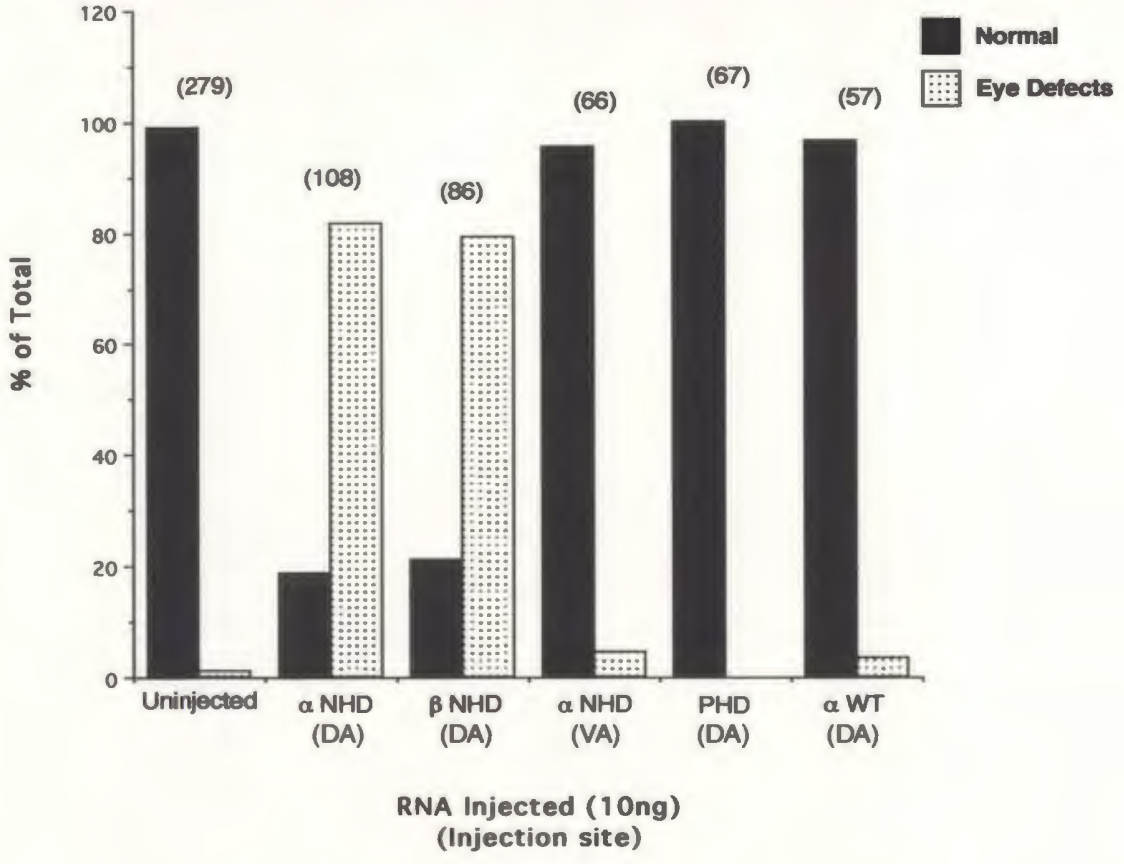
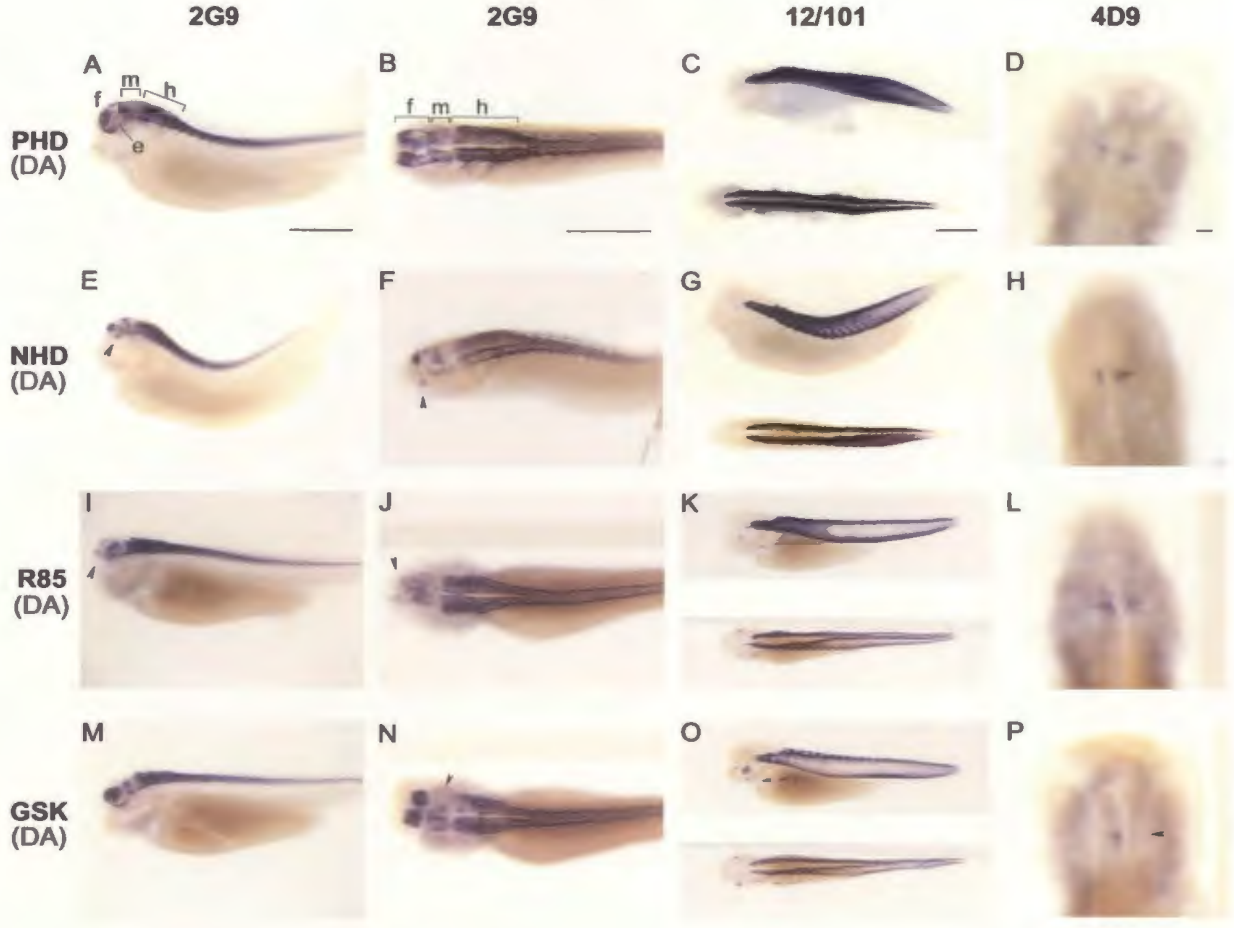

120
100
80
60
40
20
0
% of Total
Normal
Eye Defects
(279)
(108)
(86)
(66)
(67)
(57)
Uninjected
α NHD (DA)
β NHD (DA)
α NHD (VA)
PHD (DA)
α WT (DA)
RNA Injected (10ng)
(Injection site)

**Figure 3.9. XPygo-2 NHD acts as a Wnt pathway activator to suppress anterior neurectoderm.** Embryos were injected in dorsal animal blastomeres (DA) with 10 ng of *Xpygo-2α PHD* RNA (control) (A-D), *Xpygo-2α/β NHD* RNA (E-H), dominant Wnt activator *R85* (I-L) or *gsk-3β* (M-P). Embryos were processed at stage 35 using pan-neural (2G9; A, E, I, M, lateral view; B, F, J, N, dorsal view), skeletal muscle (12/101; top, lateral view; bottom, dorsal view) and Engrailed-2 (4D9, dorsal view) antibodies. 2G9 stained the anterior forebrain (f), the boundary between forebrain and midbrain (m), hindbrain (h) and spinal cord. The eye (e) was not stained at this stage. The NHD (E, F) and R85 (I, J), unlike GSK (M, N), disrupted anterior most staining of the forebrain (arrows). GSK disrupted or depleted midbrain staining (N, arrow). 12/101 staining indicated that all embryos developed normal somitic muscle (C, G, K, O). 4D9 staining showed normal or expanded Engrailed-2 localization in embryos injected with NHD (H) and R85 (L) as compared to PHD (D) controls. GSK injection however, reduced expression of En-2 (P, arrow). Scale bars: 1mm (A, B, and C); 0.1 mm (D).


2G9
2G9
12/101
4D9
A
B
C
D
f
m
h
e
f m h
PHD
(DA)
E
F
G
H
NHD
(DA)
I
J
K
L
R85
(DA)
M
N
O
P
GSK
(DA)

nervous system, consistent with that shown by others (Itoh et al., 1995; Friedieu et al., 1997).

Expression of the Wnt-responsive mid-hindbrain marker Engrailed-2 (En-2, Hemmati-Brivanlou and Harland, 1989; McMahon and Bradley, 1990; McGrew et al., 1999) detected using the 4D9 antibody (Patel et al., 1989) was slightly expanded in both *Xpygo-2α NHD* and *R85*-injected embryos (Figure 3.9H and L) when compared to *Xpygo-2α PHD*-injected embryos (Figure 3.9D). *gsk-3β*-injected embryos had little or no staining, with some embryos lacking En-2 completely and others showing loss of localized En-2 protein (Figure 3.9P, arrow) only on the side of the embryo that had the defective eye and diencephalon-midbrain region (Figure 3.9N, arrow). Therefore, like R85, XPygo-2α NHD acts as a Wnt signaling activator to suppress anterior neurectoderm. This behaviour of NHD is consistent with the demonstrated role for Pygopus as a transcriptional activator (Belenkaya et al., 2002) and implies that this activity lies within its NHD-containing half of XPygo-2.

### 3.4.6 XPygo-2α and XPygo2-β are Required for Correct Brain Patterning

Ectopic activation of the posteriorizing Wnt response by XPygo-2 NHD within the anterior neurectoderm helps place XPygo-2 within the Wnt signaling hierarchy. To specifically address the requirement for XPygo-2, we designed fluorescent antisense morpholino oligonucleotides (MO) that were specific to the 5'-noncoding regions of either *Xpygo-2α* or *Xpygo-2β*. Both of the XPygo-2α and -2β MOs efficiently inhibited translation of their corresponding messages *in vitro*, but were unable to block translation

of mRNA synthesized from templates of *Xpygo-2* coding regions lacking sequences complementary to the MOs (Figure 3.10). Neither MO blocked translation of the other isoform, further confirming their target specificities (Figure 3.10).

Each MO was injected either alone or in combination into two dorsal blastomeres at the 8-cell stage (Figure 3.11). Injection of 40 ng of the XPygo-2α MO generated severe anterior head and eye defects (52/54; Table 3.2; Figure 3.11M and N), while 40 ng of the XPygo-2β MO generated only eye defects ranging from ventral disruption to almost complete loss of pigmented epithelium (49/55; Table 3.2; Figure 3.11O and P). However, combining 20 ng of each morpholino generated eye deficits and anterior deletions (71/72; Table 3.2; Figure 3.11A-I ) with greater severity than for either MO alone (β mo- 8/13 normal; α Mo- 6/13 normal). Injection of up to 40 ng of control MO had no effect on normal development with only slight abnormalities in a minority of embryos (57/63 normal; Table 3.2; Figure 3.11Q-T). These experiments indicate that the additive depletion of both isoforms results in more severe anterior-defective phenotypes than with either alone. The effects of the MOs were highly specific since co-injection of as little as 1 ng each of synthetic *Xpygo-2α* and *Xpygo-2β* RNA rescued the double MO-injected embryos (Table 3.2; Figure 3.11J-L), with a 47.6% drop in the percentage of embryos displaying the abnormal phenotype.

Embryos immunostained for neural tissue (2G9) had disorganized to severe deletion of anterior neural tissues rostral to and including the hindbrain as a result of combined XPygo-2α and -2β MOs (Figure 3.12A and B, arrows), all of which was rescued with synthetic *Xpygo-2* RNA (Figure 3.12D, E). To dissect the individual roles of

**Figure 3.10. Xpygo-2α and Xpygo-2β morpholinos (MO) specifically block translation of their target messages.** *In vitro* translation of *Xpygo-2α* or *-2β* full length cDNA constructs was blocked in a graded fashion (200-600 nM MO) by the corresponding MO sequence, but not by the highest concentration of Control MO (600nM). In addition, neither MO could block translation from synthetic mRNA used in rescue experiments that lacked corresponding complimentary sequences (*). The additional translation product from *Xpygo-2α* mRNA of similar molecular weight to XPygo-2β most likely represents the alternative start of translation site provided by the *Xpygo-2β* start codon, an *in vitro* artifact.

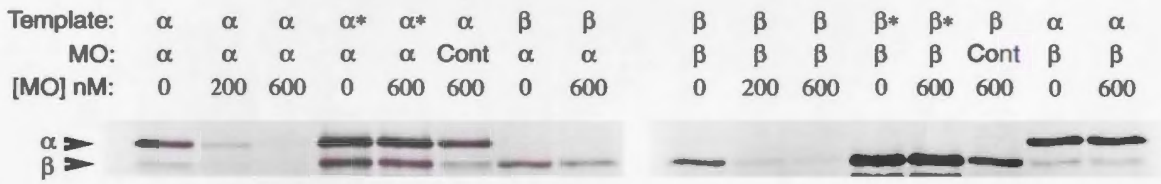

Template: α α α α* α* α β β β β β β* β* β α α
MO: α α α α α Cont α α β β β β β Cont β β
[MO] nM: 0 200 600 0 600 600 0 600 0 200 600 0 600 600 0 600
α
β

**Figure 3.11. XPygo-2α and XPygo-2β antisense morpholinos (MO) cause severe head abnormalities.** (A,D,G,J) Head-defective embryos resulting from injection at the 8-cell stage (DA) with a mixture of 20 ng each of both XPygo-2 MOs and their corresponding fluorescence views optimized for Lissamine (red fluorescence, B,E,H,K) to reveal the -2α MO, and fluorescein (yellow-green fluorescence, C,F,I,L) to reveal the -2β MO. Note the association of MO fluorescence with the head defects seen in the dorsal view (D-F). The co-injection of 1 ng each of *Xpygo-2α/β* RNA not targeted by the MOs (J,K,L) rescued head structures even in the presence of both morpholinos (K,L). XPygo-2α MO alone (M,N) caused severe head abnormalities including reduced or absent eye pigmented epithelium and displaced or expanded cement glands (M, arrow). XPygo-2β MO (40 ng per embryo; O,P) generated only eye deficits that ranged from reduction and ventral deficits to an almost complete loss. Control MO (green fluorescence; 40 ng per embryo; Q R, lateral view; S, T, dorsal view) did not affect development. Scale bars: 1 mm.

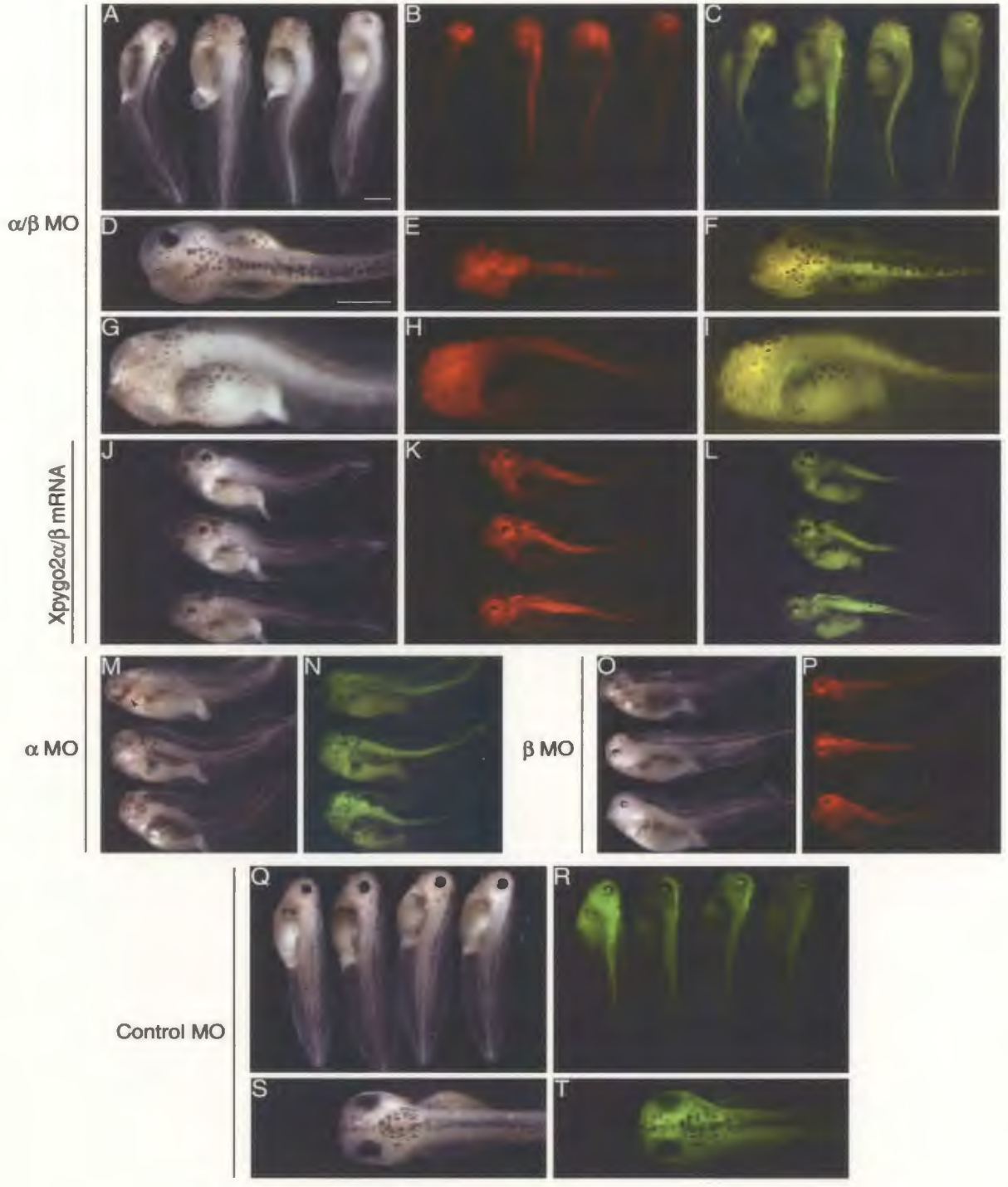
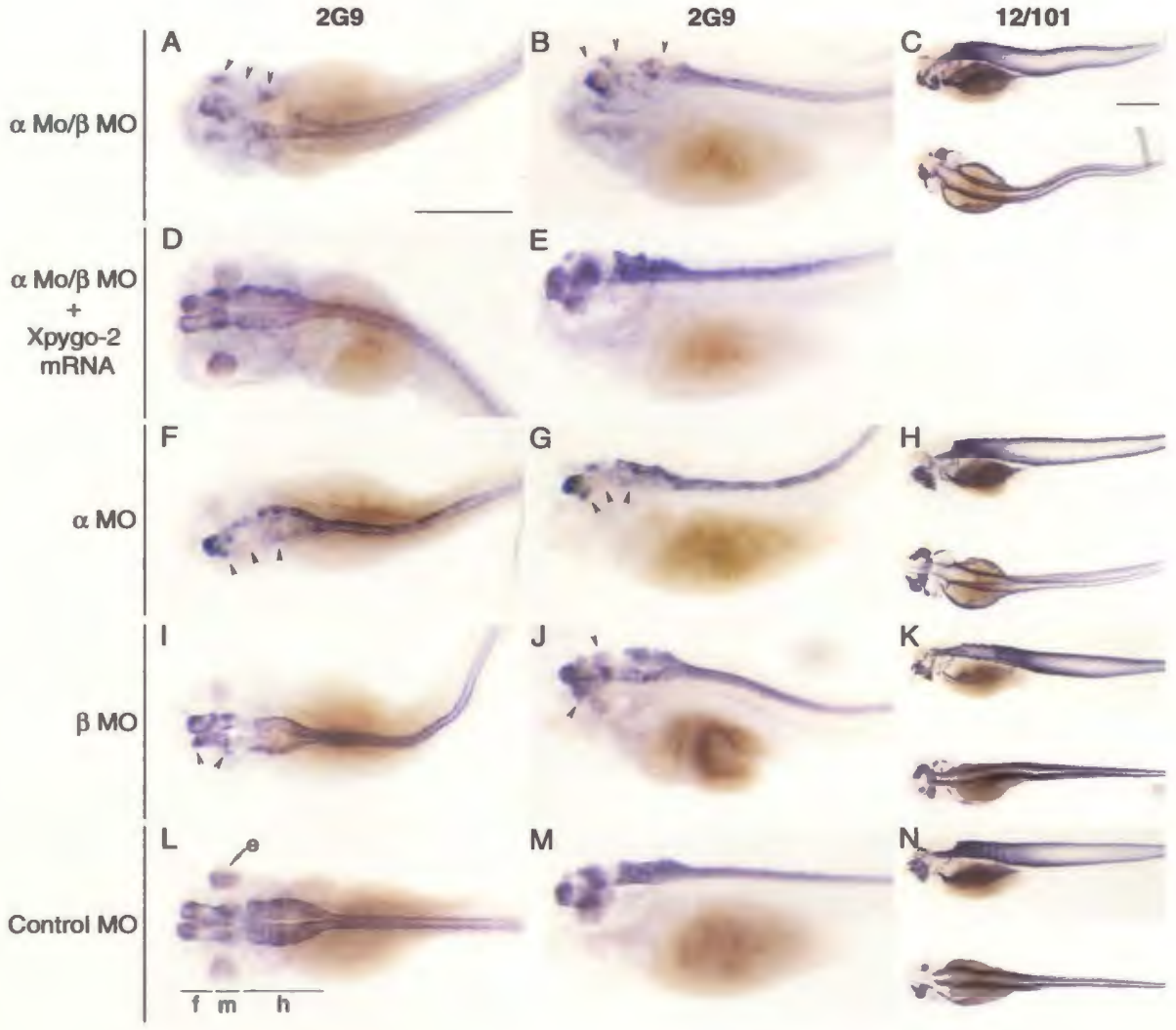

A
B
C
α/β MO
D
E
F
G
H
I
Xpygo2α/β mRNA
J
K
L
α MO
M
N
β MO
O
P
Control MO
Q
R
S
T

**Figure 3.12. XPygo-2α/β MOs inhibit brain development.** Immunostaining of tadpoles (st. 43) co-injected with XPygo-2α MO and XPygo-2β MO (20 ng each) using the pan-neural antibody 2G9 (A, dorsal view; B, lateral view) indicated anterior neural truncations with loss of retinal tissue as well as fore-, mid- and hindbrain defects (arrows). These structures were rescued by co-injection of *Xpygo-2α/β* RNA (1 ng each; D, dorsal view; E, lateral view). XPygo-2α MO alone (40 ng) generated severe neural deficits rostral to and including the hindbrain (F, dorsal view; G, lateral view; arrows), while XPygo-2β MO alone (40 ng) primarily disrupted and reduced neural tissue rostral to and including the midbrain (I, dorsal view; J, lateral view; arrows). Control MO (40 ng) injected embryos showed normal 2G9 immunostaining of the eyes (e), forebrain (f), midbrain (m), hindbrain (h) and spinal cord. Undisrupted 12/101 staining indicated that all embryos developed normal muscle (C, H, K and N; top, lateral; bottom, dorsal). Scale bars: 1 mm.

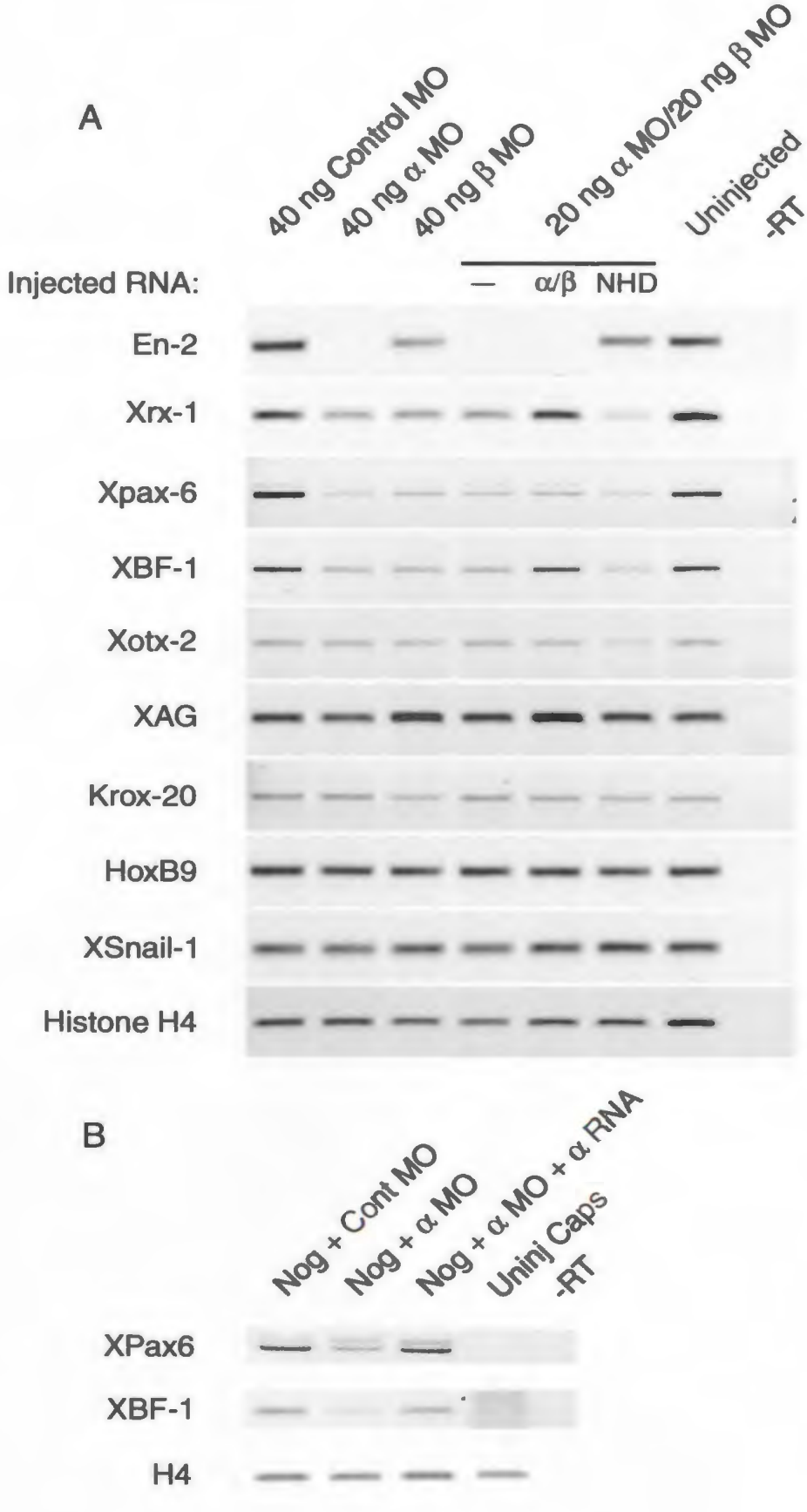

2G9
2G9
12/101
A
B
C
α Mo/β MO
D
E
α Mo/β MO
+
Xpygo-2
mRNA
F
G
H
α MO
I
J
K
β MO
L
M
N
Control MO
e
f
m
h

XPygo-2α and XPygo-2β, embryos injected individually with 40 ng of each MO were analyzed for the types of neural tissue disrupted in comparison with 40 ng of Control MO (Figure 3.12L and M). XPygo-2α MO caused severe disruptions to the fore-, mid- and hindbrain regions in addition to severe reduction to loss of eye tissue (Figure 3.12F and G, arrows), while the XPygo-2β MO primarily caused fore- to midbrain and eye reductions (Figure 3.12I, J). Both MO effects were restricted to anterior neural structures and were not extended to muscle tissues as shown by 12/101 immunostaining (Figure 3.12C, H, K and N).

Further examination of neural marker expression using RT-PCR analysis of embryo mRNA at the tailbud stage (st. 23) demonstrated the specific effect of each MO to anterior brain tissues (Figure 3.13A). Neither the posterior neural marker *hoxB9* (Sharpe et al., 1987) nor the neural crest marker *Xsnail-1* (Smith et al., 1992) showed reduction in level of expression as a result of injection of both MOs in combination or individually, even though the MOs were present along the entire anterior-posterior neuraxis (Figure 3.11). Also unaffected was the hindbrain marker *krox-20* (Bradley et al., 1993), indicating that while the MOs generated severe hindbrain defects, this hindbrain patterning gene is not regulated by XPygo-2. Thus, there must be other essential untested hindbrain patterning genes with XPygo-2-dependent activities.

The XPygo-2α MO and to a lesser extent the XPygo-2β MO, each at 40 ng caused significant reduction in En-2 expression (Figure 3.11). However, combining 20 ng of each MO eliminated En-2 expression, indicating that En-2 is most likely dependent on

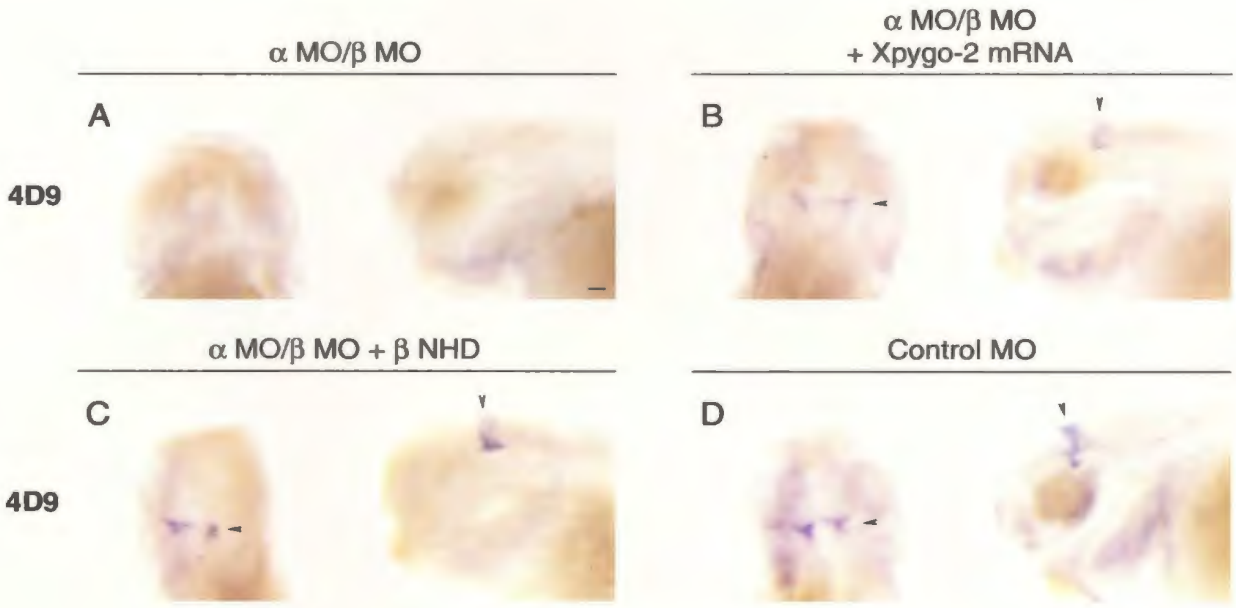
**Figure 3.13. XPygo-2α/β MOs reversibly reduce RNA expression of a subset of brain markers.** RT-PCR analysis of tailbud (st. 22-23) embryos (A) or *noggin* (125 pg) injected animal caps (B) co-injected with Control MO (40 ng), XPygo-2α MO (40 ng), XPygo-2β MO (40 ng) or combined XPygo-2α MO/XPygo-2β MO (20 ng each) with or without 2 ng of rescuing RNA (1 ng each *Xpygo-2α/Xpygo-2β*, 2 ng *Xpygo-2β NHD*, or 2 ng *Xpygo-2α*). This experiment (A) was repeated four times to confirm reproducibility of results. Levels of cDNA used were standardized using *histone (H4)* levels. –RT: negative control, without reverse transcriptase.


A
40 ng Control MO
40 ng α MO
40 ng β MO
20 ng α MO/20 ng β MO
Uninjected
-RT
Injected RNA:
—
α/β
NHD
En-2
Xrx-1
Xpax-6
XBF-1
Xotx-2
XAG
Krox-20
HoxB9
XSnail-1
Histone H4
B
Nog + Cont MO
Nog + α MO
Nog + α MO + α RNA
Uninj Caps
-RT
XPax6
XBF-1
H4

both isoforms of XPygo-2, but with a greater dependence on XPygo-2α. This is consistent with the severe observed loss of midbrain tissues at the tadpole stage caused by injection of both the XPygo-2α and the XPygo-2β MO together or the XPygo-2α MO alone (Figure 3.12, 3.14A), and only a slight disruption by XPygo-2β MO alone (Figure 3.12). These results further implicate that the caudal limit of *Xpygo-2β* messages likely exists at the MHB junction.

The retinal field markers *Xrx1* (Casarosa et al., 1997; Mathers et al., 1997) and *Xpax6* (Hirsch and Harris, 1997; Li et al., 1997) as well as the forebrain marker *Xbf1* (Bourguignon et al., 1998), but not the fore-midbrain marker *Xotx2* (Blitz and Cho, 1995; Kablar et al., 1996; Perron et al., 1998; Andreazzoli et al., 1999) were all reduced by MO injection (Figure 3.13A). Further, full induction of *Xbf1* and *Xpax6* were prevented by XPygo-2α MO in neural-induced animal caps (Figure 3.13B), demonstrating the specificity of the defects to the neural and not inducing tissue. While the disruption of anterior-most neural tissue (Figure 3.12) may represent a reprogramming of anterior to posterior tissue, it appears in fact to reflect the loss of cells normally constituting the eye-forebrain field. This likely results from the loss of XRx1 which was previously found to generate deletions of the telencephalon, eye vesicles and ventral diencephalon not only as a result of the loss of its functions in anterior specification and proliferation, but also the loss of a role in cell survival, since anterior-most neural plate cells actually underwent apoptosis (Andreazzoli et al., 1999; Andreazzoli et al., 2003). A similar role in mitogenesis of the telencephalic and optic territories has been demonstrated for XBF1 (Bourguignon et al., 1998).

**Figure 3.14. XPygo-2α/β MOs reversibly eliminated En-2 protein expression.** Embryos injected with XPygo-2α MO/XPygo-2β Mo (20 ng each) with or without rescuing RNA (2 ng) were fixed (st. 35) and immunostained for En-2 using the 4D9 monoclonal antibody (A, B and C; right – dorsal view, left – lateral view). Normal En-2 staining can be seen at the mid-hindbrain junction of Control MO (40 ng) injected embryos (D, arrows), but is lost in XPygo-2α MO/XPygo-2β MO injected embryos (A). Co-injection of *Xpygo-2α/β* RNA rescues limited expression (B, arrows), while co-injection of *Xpygo-2β NHD* RNA rescues full expression (C, arrows) of En-2. Scale bar: 0.1 mm.


α MO/β MO
α MO/β MO
+ Xpygo-2 mRNA
A
B
4D9
α MO/β MO + β NHD
Control MO
C
D
4D9

The level of expression of the cement gland marker *XAG* (Sive et al., 1989; Sive and Bradley, 1996) was unaffected by the XPygo-2 MOs, even though *XAG* is normally negatively regulated by Wnt signaling, since β-Catenin MOs greatly expanded its expression and associated cement gland territory (Heasman et al., 2000). While the lack of enhancement of XAG expression levels is consistent with normal appearing cement glands at this stage, by tadpole stages there does appear to be some expansion of cement glands in XPygo-2α MO injected embryos only (Figure 3.11M, arrow), with the embryos typically having darkly pigmented cells resembling those of the cement gland diffusely overlying the reduced or absent eye regions.

Co-injection of the MO's with full-length *Xpygo-2α* and *-2β* mRNA rescued the normal expression of retina and forebrain markers *Xrx1* and *Xbf1* but not of *Xpax6* or the Wnt-responsive *en-2* at this stage (st. 23). There was, however, limited recovery of En-2 protein by full-length *Xpygo-2* mRNA at later tadpole stages as shown by 4D9 immunostaining (Figure 3.14B). This discrepancy is most likely due to delayed onset, or reduced level of induction of these markers. This inability to fully recover the proper expression of all the anterior neural markers by *Xpygo-2* RNA accounts for the observed incomplete rescue (Table 3.2). More significantly, however, co-injection of 2 ng *Xpygo-2β NHD* RNA restored *en-2* RNA expression at the tailbud (Figure 3.13A), and tadpole (Figure 3.14C) stages with En-2 protein expressed at levels comparable to those of Control MO injected embryos (Figure 3.14D). These results further confirm the XPygo-2 NHD domain as a Wnt activator, and demonstrate the requirement for both isoforms of XPygo-2 in brain patterning.

## 3.5 DISCUSSION

### 3.5.1 XPygo-2α/β Are Components of the *Xenopus* Wnt Signal Transduction Pathway

Wnt proteins control numerous cell fate decisions by assembling β-Catenin/TCF/LEF-1 complexes in the nucleus to activate Wnt target gene transcription. Upon activation, β-Catenin binds nuclear TCF and may recruit the basal transcriptional complex to the promoter possibly via the TATA binding protein TBP (Hecht et al., 1999) or the TBP associating protein TIP49 (Bauer et al., 1998). It may also recruit co-activators such as p300/CBP (Hecht et al., 2000; Takemaru and Moon, 2000) or Brg-1 (Barker et al., 2001). Further dissection of these complexes will be necessary to fully understand the nuclear aspect of Wnt/β-Catenin signaling.

Since Pygopus is required for β-Catenin-dependent transcription (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002) and PHD-containing proteins are associated with chromatin-remodeling activity (Aasland et al., 1995; Jacobson and Pillus, 1999), Pygopus was postulated to mediate access of TCF or β-Catenin/Arm to chromatin (Thompson et al., 2002). However, the demonstration that Pygopus can activate transcription when fused to the Gal4 DNA-binding domain suggests it does more than simply facilitate access to chromatin (Belenkaya et al., 2002). In my study, the XPygo-2α NHD promoted partial dorsal axis development when expressed within the ventral vegetal region or in UV ventralized embryos and prevented anterior-most neurectoderm formation when injected in the dorsal animal region, consistent with other studies on Wnt pathway activation (Yamaguchi and Shinagawa, 1989; Christian and

Moon, 1993; Fredieu et al., 1997; Moon and Kimelman, 1998; Darken and Wilson, 2001). These results suggest an additional level of Wnt pathway activation that can be achieved by Pygopus via the NHD domain.

XPygo-2 NHD proteins were also able to induce the Wnt-responsive mid-hindbrain marker En-2 more strongly in XPygo-2α/β depleted embryos than in normal embryos, requiring significantly less injected RNA to generate a profound increase in En-2 expression. Thus, in order to have a hyperactivation effect in normal embryos, XPygo-2 NHD proteins may need to overcome already associated endogenous canonical Wnt transcription complexes. This would explain the low potency of the NHD as an activator in the absence of XPygo-2 MOs, with secondary axes induced of small size and in a low proportion of embryos. Additionally, there was only a slight coincident increase of *siamois* and *chordin* expression observed on the ventral side following *Xpygo-2 NHD* RNA injection. As such, it would be expected that the XPygo-2 NHD would have a more significant effect in maternally depleted embryos. However, neither morpholino was able to knock down maternal XPygo-2α/β protein levels as evidenced by reporter assays and phenotypic analyses (not shown).

The mechanism by which the NHD mutants activate Wnt responses poses an interesting question: how can the loss of the β-Catenin-associating domain (PHD) allow for possible chromatin-mediated target gene transcription? This would imply that the model by which TCF-bound β-Catenin recruits Pygopus to the promoter is not so simple. In the absence of Wnt signaling, XPygo may already be assembled within a chromatin associated transcriptional complex, but negatively regulated by its PHD domain either

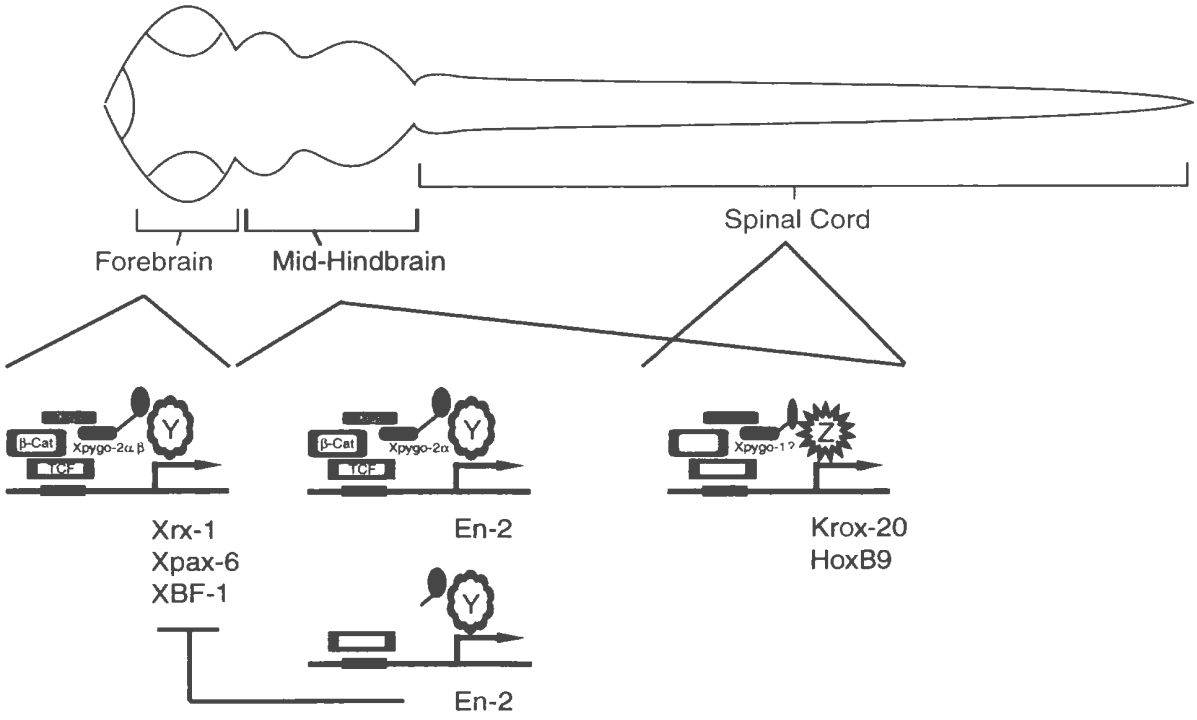
directly or through additional PHD interacting proteins. The binding by β-Catenin would then be required to either displace these regulatory proteins or alter Pygopus protein conformation. In these experiments, overexpression of the NHD was effectively equivalent to deletion of the PHD, allowing deregulation of the protein and activation of target genes (Figure 3.15).

### 3.5.2 XPygo-2α and XPygo-2β Cooperatively Pattern the *Xenopus* Embryonic Brain

While the experiments on the effect of NHD overexpression on dorsal axis induction and anterior neural reduction placed XPygo-2α/β within the Wnt signaling cascade, they could not definitively demonstrate the normal function of *Xpygo-2* in *Xenopus* embryogenesis. Antisense MOs were designed, therefore, to block *Xpygo-2*-specific translation. The combined expression studies and MO depletions provided a more complete picture of the types of Wnt inductive or patterning events that are mediated by XPygo-2 proteins.

Multidimensional Wnt signaling carries out multiple functions for patterning events within clonal populations of cells at different times in development. Not unexpectedly, both *Xpygo-2α* and *-2β* are widely spatio-temporally expressed in early *Xenopus* embryogenesis. The early expression of *Xpygo-2β* (and even *Xpygo-2α*) coupled with the dorsal-axis inducing properties of XPygo-2 NHD and the ventralizing capability of antisense XPygo-2 phosphorothioated oligonucleotides (Belenkaya et al., 2002) confirm that XPygo is required for early axial patterning and organizer formation.

**Figure 3.15. Summary figure.** XPygo proteins are differentially expressed along the neural plate to mediate multiple Wnt patterning events. Within the prospective forebrain XPygo-2α/β expression represents the onset of a Wnt-dependent phase to regulate, directly or indirectly, the transcription of *Xrx1*, *Xpax6*, and *Xbf1*. Within the mid-hindbrain XPygo-2α mediates the caudalizing Wnt signal for specification of the isthmic organizer. Both XPygo-2α and XPygo-2β potentially relay the canonical Wnt signal through associations with some downstream protein(s) (Y). Deletion of the PHD domain may permit deregulated associations of the NHD with (Y) independent of β-catenin to activate transcription of target genes. However, the full complement of Wnt/β-catenin regulated transcription requires additional XPygo protein(s), such as any putative hPygo-1 orthologues. These likely associate with different downstream effectors (Z) to regulate transcription from a different subset of promoter elements (*krox-20*, *hoxB9*).


Spinal Cord
Forebrain
Mid-Hindbrain
β-Cat
Xpygo-2α β
TCF
Y
β-Cat
Xpygo-2α
TCF
Y
Xpygo-1?
Z
Xrx-1
Xpax-6
XBF-1
En-2
Krox-20
HoxB9
Y
En-2

Subsequently, during gastrula stages, a morphogen gradient of active Wnt signaling establishes A-P patterning in the neuraxis (Niehrs, 1999; Kiecker and Niehrs, 2001). However, the expression patterns I observed fail to implicate XPygo-2 in neural posteriorization since both isoforms showed reduced expression at this time. *Xpygo-2α* RNA started accumulating at neural plate stages more predominantly in the anterior neurectoderm, consistent with an anteriorizing and not a posteriorizing role. Furthermore, MO depletion of XPygo-2 failed to alter phenotypes or marker expression of posterior neural tissues including spinal cord and neural crest, even though the MOs were found throughout the central nervous system.

The initiation of zygotic *Xpygo-2α* expression in the anterior neural plate is concomitant with the earliest specification of the eye or retinal field (Saha and Grainger, 1992; Perron and Harris, 1999). By late neurula stages, *Xpygo-2α* messages continue to accumulate within the retinal field, overlapping the first detectable zygotic *Xpygo-2β* messages. This observation demonstrates that specification of this anterior-most neural tissue requires XPygo-2α and XPygo-2β activity, which was confirmed by the rescue of expression of certain retinal field genes in XPygo-2 morpholino-injected embryos.

XPygo-2α/β morpholinos did not, however, affect *Xotx2* expression which is normally within the fore- and midbrain and developing retina (Blitz and Cho, 1995; Kablar et al., 1996; Perron et al., 1998; Andreazzoli et al., 1999). While *Xotx2* expression was expanded by a β-Catenin morpholino (Heasman et al., 2000), suggesting that it is regulated by β-Catenin, it is not surprising that it was not affected by XPygo-2 morpholinos since *Xotx2* is expressed during gastrulation, a time when *Xpygo-2* is not.

These observations, therefore, indicate that XPygo-2 proteins regulate the expression of a subset of retinal and forebrain patterning genes and that β-Catenin regulates some genes independently of XPygo-2.

Unlike *Xpygo-2β*, *Xpygo-2α* messages are not restricted to the eye field, but extend posteriorly to encompass the entire brain. This places it within the proper spatial location to mediate the posteriorizing role of Wnt/β-Catenin within the mid-hindbrain for induction of *en-2* and *krox-20* expression (Hemmati-Brivanlou and Harland, 1989; McMahon and Bradley, 1990; Bradley et al., 1993; McGrew et al., 1999; Kiecker and Niehrs, 2001). Partial regulatory effects of XPygo-2β on En-2, however, imply that its expression domain extends into midbrain territories to cooperate with XPygo-2α in expression of this marker. While the -2α morpholino disrupted neural tissues extending as far posteriorly as the hindbrain (Figure 3.9), only *en-2* but not *krox-20* expression was eliminated. Since *krox-20* expression has been shown to be dependent on Wnt signaling (Kiecker and Niehrs, 2001), these results suggest that additional *Xenopus* Pygopus proteins may be involved to mediate its expression. Therefore, a gradient of Wnt/β-Catenin signaling is not the only mechanism involved in anteroposterior neuraxial patterning. Differential spatial distributions of the XPygo transcriptional mediators may also alter the cellular competency to the same activated Wnt/β-Catenin pathway.

Precedence for differential spatio-temporal requirements of XPygo comes from evidence that both the pre-MBT axial specification and post-MBT mesodermal and ectodermal patterning involves the same canonical pathway with activation of β-Catenin/XTCF-3 (Fredieu et al., 1997; McGrew et al., 1999; Heasman et al., 2000;

Darken and Wilson, 2001). As such, there is a stage-specific change in cellular competence that occurs within the nucleus, at the level of the target gene promoter and possibly the XTCF-3 protein (Darken and Wilson, 2001; Hamilton et al., 2001).

Work presented in this chapter demonstrates that differentially localized XPygo proteins may provide a novel mechanism by which cells acquire different spatial or temporal competencies to the same intracellular Wnt signaling cascade during development. I postulate that the response a cell makes to canonical Wnt stimuli, including target genes activated, depends on the type of Pygopus protein present within the β-Catenin/TCF complex. While both XPygo-2α and XPygo-2β morpholinos reduced the same forebrain and retinal markers, they did diverge in their ability to regulate posterior markers. However, I cannot rule out the possibility that this could be due simply to their differential expression patterns rather than unique transcriptional activation abilities. It is possible that XPygo-2β may further uniquely regulate markers not analyzed, or may simply act through its restricted expression pattern to enhance a general XPygo-2 signal within certain tissues. This latter model would allow different marker expression patterns based upon the local concentration of XPygo-2 proteins.

### 3.5.3 Model for Xpygo-Dependent Patterning of the Central Nervous System

The ability of XPygo-2 knockdown to eliminate both mid-hindbrain (*en-2*) and forebrain (e.g. *Xbf1*, *Xrx1*, *Xpax6*) gene expression, leads to the proposal of a potential role for wild-type XPygo-2 in mediating a Wnt-dependent genetic network controlling fore-midbrain patterning. This is consistent with recent studies in chick showing a change

in competence of the neural plate to Wnt signals, from an early phase necessitating the absence of Wnts for specification of the forebrain to a later phase requiring Wnts in order to establish dorsoventral identity within the telencephalon (Gunhaga et al., 2003). Therefore, XPygo-dependent transcription may pattern the central nervous system by establishing not only different spatial (Figure 3.15), but also different temporal competencies to Wnt signals.

Within the prospective forebrain during gastrula stages, Wnt antagonists from the prechordal endomesoderm generate a Wnt free zone preventing nuclear accumulation of β-Catenin (reviewed in Chapter 1; Lake and Kao, 2003a). Increasing Wnt activity along the AP neuraxis during this period specifies progressively more posterior identity. The absence of Xpygo-2 expression indicates that this occurs through associations of β-Catenin with an alternative XPygo protein, potentially an hPygo-1 orthologue (Figure 3.15).

At the end of gastrulation (stage ~12), XPygo-2 becomes expressed as a necessary step in establishing telencephalic and retinal genetic networks. Therefore, I propose that XPygo-2α renders the anterior neural plate competent to express *Xrx1*, *Xpax6* and *Xbf1* in response to Wnt signals from adjacent non-neural ectoderm (Figure 3.15). Maintenance of these expression domains may further require XPygo-2β expressed at later neurula stages. More posteriorly, XPygo-2α (and XPygo-2β) will also enable expression of En-2 in response to Wnt1 signals from the IsO for specification of midbrain identity (Figure 3.15; Hemmati-Brivanlou and Harland, 1989; McMahon and Bradley, 1990; Danielian and McMahon, 1996; McGrew et al., 1999).

The NHD-specific downstream regulatory proteins (Y and Z, Figure 3.15) are fundamental components of my model. In the brain, XPygo-2α/β likely regulates expression through bridging the canonical Wnt machinery to the protein or protein complex (Y), possibly involved in chromatin remodeling. Deletion of the PHD domain would permit deregulated associations of the NHD domain with (Y). Since the marker showing the greatest sensitivity to XPygo-2 disruption was En-2, it is not surprising that this marker was upregulated to a greater extent. This activation of posterior neural identity would result in the expected rostral transformation to more caudal identity (Yamaguchi and Shinagawa, 1989; Christian and Moon, 1993; Fredieu et al., 1997; Darken and Wilson, 2001), thereby suppressing any induction of telencephalic or retinal markers and generating the observed forebrain malformations (Figures 3.8 and 3.9). In fact, this effect can be mimicked by overexpression of En-2 alone (Ristoratore et al., 1999), indicating that any ectopic activation of more anterior markers by the NHD would likely have been overpowered by this caudalizing signal. The finding that NHD constructs from both XPygo-2α and -2β could interchangeably generate this effect (Figure 3.8, Table 3.2) further confirms their joint requirement, at some level, for En-2 expression.

Further analyses must be undertaken to identify potential hindbrain targets of XPygo-2 dependent Wnt signaling. In addition, since the XPygo-2α MO did not generate a complete loss of Wnt regulated posterior neural markers, hPygo-1 orthologues likely exist to mediate transcription at these promoters, possibly by bridging associations with an alternative set of downstream proteins (Z; Figure 3.15). Alternatively, the observed

disruption of hindbrain structure may have been, in part, a secondary consequence of disrupting Wnt dependent IsO specification or function, loss of which generates hindbrain abnormalities (McMahon and Bradley, 1990; Thomas and Capecchi, 1990).

According to my model, the type of Pygo protein available at a promoter determines the cell's competency to respond to Wnt/β-Catenin signals. In addition, the type of promoter regulated is ultimately dependent on the type of NHD-specific downstream effector (Y or Z) involved. Extensive experimental analyses must be undertaken to test this hypothesis, including the cloning and functional analysis of XPygo-1, identification of the Y, Z proteins/complexes and the empirical demonstration of Wnt-induced forebrain marker expression.

# CHAPTER 4:

## *hPygo-2 is differentially expressed in the adult brain*

Running Title: hPygo-2

## 4.1 ABSTRACT

Pygopus proteins represent newly discovered components of the Wnt/β-Catenin signaling cascade, with putative functions in bridging β-Catenin transcriptional complexes to chromatin remodeling machinery for transcription from target promoters. My studies presented in chapter 3 demonstrated the different spatial requirements of XPygo proteins during embryonic development of the frog. In this chapter, human tissues were screened to determine if this differential expression pattern is conserved and to verify a primarily neural requirement for Pygo-2 orthologues. Consistent with the expression studies in *Xenopus*, *hpygo-2* encoded messages and proteins were found to accumulate within brain structures previously shown to involve Wnt signaling during development, maintenance or oncogenesis.

## 4.2 INTRODUCTION

Wnt/β-Catenin signaling is intrinsically associated with cell fate decisions in regulating growth and differentiation events, with a prominent role in the expansion of predefined cells (reviewed in Clevers, 2002). As such, pathway constituent mutations have been linked to a number of human cancers derived from colorectal, breast, ovarian, and neuroectodermal tissues (reviewed in Polakis, 2000; Brown, 2001; Taipale and Beachy, 2001; Lustig and Behrens, 2003).

Stereotypical Wnt signaling ultimately relies on the intracellular shuffling of β-Catenin between the cytosol and nucleus. This is regulated by Wnt ligand-receptor interactions that balance β-Catenin's association with either a cytoplasmic destruction complex or nuclear transcriptional regulators of the TCF/LEF-1 family (reviewed in Lustig and Behrens, 2003; Chapter 3). In the absence of extracellular stimulation of Wnt receptors or Frizzleds, β-Catenin remains bound and phosphorylated by the Axin-APC-GSK-3β-CK1 containing multiprotein complex, triggering ubiquitination by β-TrCP for proteosomal degradation. Binding of the Wnt glycoproteins to both Frizzled receptors and LRP 5/6 co-receptors enables cytosolic Dishevelled to release β-Catenin from its antagonistic protein interactions. With renewed stability, β-Catenin migrates to the nucleus and associates with HMG-box containing TCF proteins, while displacing co-repressors that actively prevented target gene expression in Wnt inactive cells. As mentioned in chapter 3, β-Catenin/TCF nuclear complexes activate transcription through an association, mediated by the adaptor Legless/BCL9, with Pygopus proteins.

Wnt signaling plays a pivotal role in the establishment and organization of the embryonic central nervous system, with roles in proliferation of neural precursors (Dickinson et al., 1994; Ikeya et al., 1997; Megason and McMahon, 2002), establishment of defined cell adhesion molecule expression domains (Shimamura et al., 1994), synaptogenesis (reviewed in Salinas, 2003) and possible suppression of apoptosis (Zhang et al., 2001; Zhang et al., 1998). As such, transduction cascade dysfunctions are associated with developmental anomalies including schizophrenia (Beasley et al., 2002; Kozlovsky et al., 2002) and neurodegenerative disorders such as Alzheimer's disease (Zhang et al., 1998). In addition, activating β-Catenin mutations are associated with oncogenic dysfunctions putatively due to aberrant activation of normal cell expansion programs. For instance, such mutations are associated with pituitary adenomas (Semba et al., 2001; Howng et al., 2002), potentially by upregulating Pitx2 (Kioussi et al., 2002) and neural crest-derived melanomas (Rubinfeld et al., 1997), by upregulating Microphthalmia-associated Transcription Factor (MITF, Widlund et al., 2002).

The most comprehensive characterization of early embryonic Wnt function comes from studies in the frog, where Wnt signaling establishes the neural organizing tissue, segregates it into head and trunk inducing divisions, pre-specifies neurectoderm through BMP antagonism and defines both the AP and DV neuraxes through graded intracellular signaling (reviewed in Lake and Kao, 2003a). These processes appear to be conserved to some extent in mammals (Yamaguchi, 2001), with Wnt8c overexpression generating posterior axis duplications and fore-, midbrain deletions in transgenic mouse embryos (Popperl et al., 1997).

Wnts additionally have a conserved requirement in late patterning events in the mammalian nervous system to establish dorsal posterior neural identity along the spinal cord (Muroyama et al., 2002). As in *Xenopus* (chapter 3) and chick (Gunhaga et al., 2003), this late patterning also appears to encompass the brain, including the forebrain. *wnts3a, -5a* and *-2b* are expressed within the embryonic cerebral cortex at the boundary between the hippocampus and choroid plexus known as the cortical hem (Grove et al., 1998), and *wnt8B* in human and mouse embryos is expressed within the developing hippocampus, the dorsal thalamus and the mammillary and retromammillary regions of the posterior hypothalamus (Lako et al., 1998). The importance of Wnt signaling in development of these forebrain tissues requires fine spatial regulation of intracellular activity of the pathway, as indicated by the existence of gradients of Wnt receptors and Wnt antagonists within the developing mouse telencephalon (Augustine et al., 2001; Kim et al., 2001).

The fundamental role for Wnt signaling in CNS development ultimately is derived from mutant phenotypes. Disruption of Wnt1 in mouse generates deletions of the midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Wnt3a mutant mice exhibit hippocampal deletions (Lee et al., 2000), due to reduction in proliferation of hippocampal cell precursors normally directed through β-Catenin/TCF dependent transcription (Galceran et al., 2000). Both *wnt1* and *wnt3a* are expressed along the dorsal midline of the neural tube with redundant functions demonstrated in double mutant mice which exhibit, compared with single mutants, a further reduction of the posterior diencephalon, anterior hindbrain, spinal ganglia and neural crest derivatives (Ikeya et al., 1997). *wnts -7a, -7b* and *-3a* are expressed in the hindbrain in a region of the

developing postnatal cerebellum (Salinas et al., 1994; Lucas and Salinas, 1997). Here they play a role in axonal remodeling and synaptic differentiation, as demonstrated for Wnt7a (Hall et al., 2000), as well as maintaining subsequent cerebellar viability and integrity through the Frizzled-4 receptor (Wang et al., 2001).

This chapter outlines the novel expression analysis of hPygo-2 in normal adult human tissues through both dot blot and Western blot analysis. I propose that Pygo-2 proteins mediate a number of the above mentioned Wnt functions in the brain. Consistently, *hpygo-2* encoded messages and protein accumulated within regions wherein Wnt signaling is implicated in precursor proliferation, patterning, synaptogenesis and maintenance, including the hippocampus, pituitary and cerebellum. These results confirm a conserved requirement for Pygo-2 proteins in the anterior nervous system from the early embryo to the adult.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Dot Blot Analysis

I.M.A.G.E. Consortium (LLNL) cDNA clones of human *pygo-2* (CloneIDs: 41570072 and 3627860) were obtained (Incyte Genomics Inc.). Probe templates were generated against 3'untranslated sequences (BglII/SalI) or 5'untranslated sequences (F: CTGGGCTGCCCCTGACACCC; R: CCGGCCTTGCCCTGCTTCC ) and random primed (Promega). Radiolabeled probes were used to screen a human multiple tissue expression (MTE™) array (Clontech) with hybridization occurring at 65°C in ExpressHyb (Clontech). Densitometric analysis of the associated blots gave relative expression levels of tissues examined. These semi-quantitative values were converted into a scale from 1 to 4, with values falling below half points being rounded down and values at or higher than half points being rounded up (e.g. $<1.5 = 1$; $\geq 1.5 = 2$).

### 4.3.2 Western Blot Analysis

Protein Medleys™ (BD Biosciences) from human tissue samples were obtained with known concentrations. For western analysis, 25-50μg of protein was run on 10% SDS-PAGE, transferred to nitrocellulose membranes (Hybond-ECL™; Amersham) and visualized using the associated enhanced chemiluminescence. Relative protein levels for most tissues (except heart) was shown using a monoclonal anti-β-actin antibody (Sigma), with equivalent levels loaded confirmed using Red Ponceau staining of western membranes (Sigma).

Primary antibodies used for immunoblotting included: hPygo-2 rabbit polyclonal (created and characterized by P. Andrews, unpublished observations), β-Catenin goat polyclonal (Santa Cruz), anti-phospho-GSK-3α/β (Cell Signaling Technology).

## 4.4 RESULTS

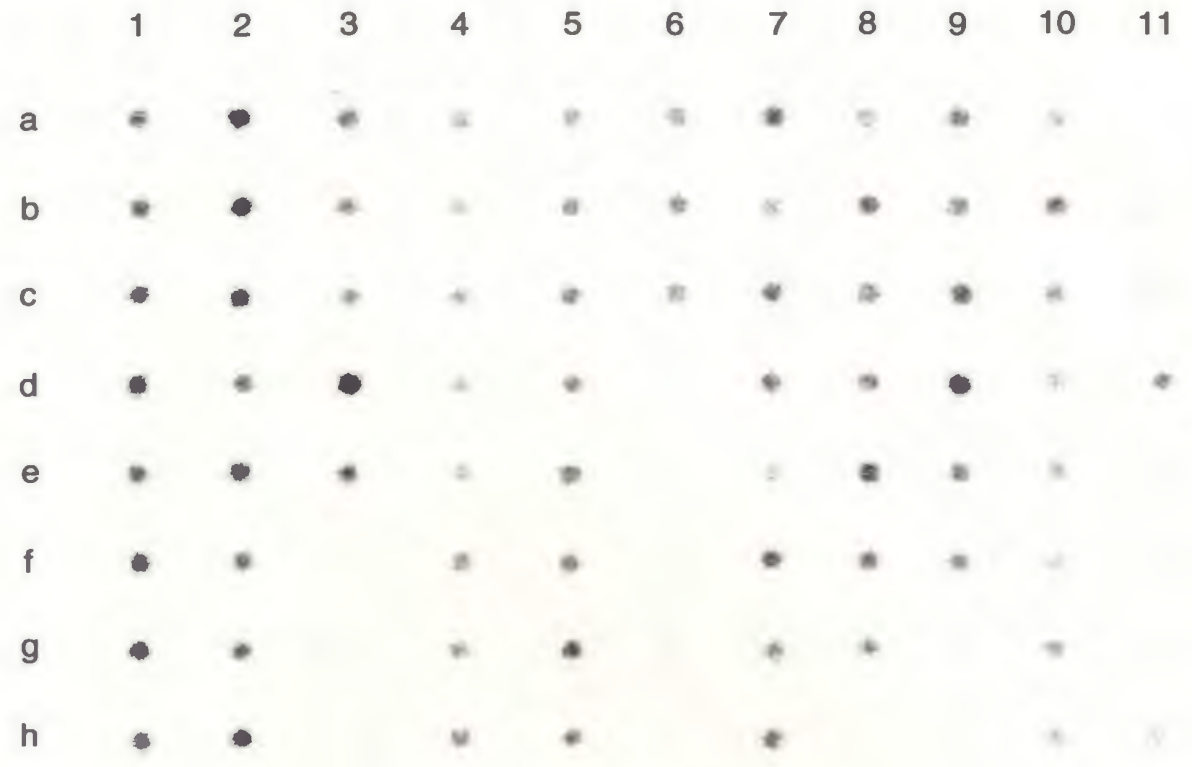
### 4.4.1 *hpygo-2* mRNA is Expressed in Multiple Tissues With Highest Levels in the Human Brain

The normal expression of *hpygo-2* was analyzed in multiple human tissues (Figure 4.1) using probes against non-coding sequences. Messages were expressed in most of the tissues tested, with a low level of background non-specific binding as indicated by the human and prokaryotic DNA controls. This non-specificity likely results from short repeat sequences present within the probes used, as well as the high GC content of the genetic sequence. However, semi-quantitative comparison (Table 4.1) showed higher levels in brain tissues, reproductive tissues (prostate) and glandular tissues (adrenal gland and thyroid gland).

The spatial distribution of messages in brain tissues paralleled what I found in *Xenopus laevis* embryos (Lake and Kao, 2003b), with higher expression levels in brain structures and lower levels in the spinal cord. This observation indicates a continued function of Pygo-2 proteins in the brain following their earlier role in development and specification (Lake and Kao, 2003b). *hpygo-2* messages were detected within the telencephalon at high levels in the parietal and temporal lobes of the cerebral cortex, the paracentral gyrus, corpus callosum, caudate nucleus and the putamen. However, medial levels were detected in frontal and occipital lobes, as well as the hippocampus and low levels of messages accumulated in the amygdala. Message distribution within the diencephalon was low in the thalamus but high in the pituitary gland. *hpygo-2* transcripts were also detected at moderate levels in the mesencephalon (midbrain) in the substantia

**Figure 4.1. *hpygo-2* mRNA expression analysis.** A. Dot Blot showing the levels of *hpygo-2* messages on a human multiple tissue expression (MTE™) array (Clonetech) with the identity of the tissues assayed shown in B.

A.




B.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| a | whole brain | cerebellum, left | substantia nigra | heart | esophagus | colon, transverse | kidney | lung | liver | leukemia, HL-60 | yeast total RNA |
| b | cerebral cortex | cerebellum, right | accumbens nucleus | aorta | stomach | colon, desending | skeletal muscle | placenta | pancreas | HeLa S3 | yeast tRNA |
| c | frontal lobe | corpus callosum | thalamus | atrium, left | duodenum | rectum | spleen | bladder | adrenal gland | leukemia, K-562 | *E. coli* rRNA |
| d | parietal lobe | amygdala | pituitary gland | atrium, right | jejunum | | thymus | uterus | thyroid gland | leukemia, MOLT-4 | *E. coli* DNA |
| e | occipital lobe | caudate nucleus | spinal cord | ventricle, left | ileum | | peripheral blood leukocyte | prostate | salivary gland | Burkitt's lymphoma, Raji | Poly r(A) |
| f | temporal lobe | hippo-campus | | ventricle, right | ilocecum | | lymph node | testis | mammary gland | Burkitt's lymphoma, Daudi | human $C_0t$ -1 DNA |
| g | p. g.* of cerebral cortex | medulla oblongata | | inter-ventricular septum | appendix | | bone morrow | ovary | | colorectal adeno-carcinoma, SW480 | human DNA 100 ng |
| h | pons | putamen | | apex of the heart | colon, ascending | | trachea | | | lung carcinoma, A549 | human DNA 500 ng |

* paracentral gyrus

**Table 4.1. *hpygo-2* mRNA Levels By Dot Blot Analysis**

| Central Nervous System | | Reproductive System | |
|---|---|---|---|
| Whole Brain | ++ | Placenta | ++ |
| Cerebral Cortex | ++ | Uterus | + |
| Frontal Lobe | ++ | Prostate | +++ |
| Parietal Lobe | +++ | Testis | ++ |
| Occipital Lobe | ++ | Ovary | + |
| Temporal Lobe | +++ | **Glandular Tissue** | |
| Paracentral Gyrus of Cerebral Cortex | +++ | Liver | ++ |
| Pituitary Gland | ++++ | Pancreas | + |
| Pons | ++ | Adrenal Gland | +++ |
| Cerebellum, Left | +++ | Thyroid Gland | +++ |
| Cerebellum, Right | ++++ | Salivary Gland | + |
| Corpus Callosum | +++ | Mammary Gland | + |
| Amygdala | + | **Cell Lines** | |
| Caudate Nucleus | +++ | Leukemia HL-60 | + |
| Hippocampus | ++ | HeLa S3 | + |
| Medulla Oblongata | ++ | Leukemia, K-562 | + |
| Putamen | +++ | Leukemia, MOLT-4 | + |
| Substantia Nigra | ++ | Burkitt's Lymphoma, Raji | + |
| Accumbens Nucleus | + | Burkitt's Lymphoma, Daudi | + |
| Thalamus | + | Colorectal Adenocarcinoma, SW480 | + |
| Spinal Cord | ++ | Lung Carcinoma, A549 | + |
| **Cardiac Tissues** | | **Immune System** | |
| Heart | + | Spleen | ++ |
| Aorta | + | Thymus | ++ |
| Atrium, Left | + | Peripheral Blood Leukocyte | + |
| Atrium, Right | + | Lymph Node | ++ |
| Ventricle, Left | + | Bone Marrow | + |
| Ventricle, Right | + | **Other** | |
| Interventricular Septum | + | Kidney | ++ |
| Apex of Heart | + | Skeletal Muscle | + |
| Esophagus | + | Trachea | ++ |
| **Gastro-Intestinal System** | | Lung | + |
| Stomach | + | Bladder | + |
| Duodenum | + | **Controls** | |
| Jejunum | + | Yeast Total RNA | - |
| Ileum | ++ | Yeast tRNA | - |
| Ilocecum | ++ | E. coli rRNA | - |
| Appendix | ++ | E. coli DNA | + |
| Colon, Ascending | + | Poly r(A) | - |
| Colon, Transverse | + | Human Cot-1 DNA | - |
| Colon, Descending | + | Human DNA 100ng | - |
| Rectum | + | Human DNA 500 ng | + |

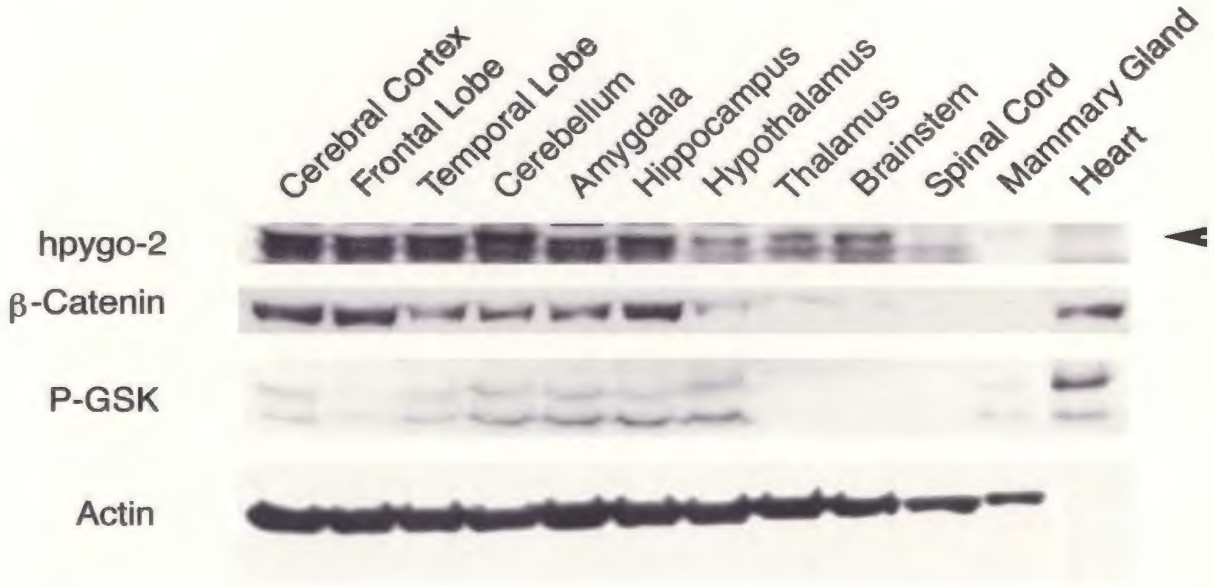
nigra. High levels were found in the metencephalon (anterior hindbrain) in the cerebellum, however this demarked the posterior-most boundary of *hpygo-2* messages in the brain, with low levels detected in the pons and fewer in the medulla oblongata of the myelencephalon or brainstem (posterior hindbrain).

### 4.4.2 hPygo-2 protein is expressed in neural structures anterior to the spinal cord

To determine if the mRNA levels accurately predict protein expression within the anterior neural structures, total protein from a subset of the tissues analyzed was screened with a rabbit polyclonal antibody directed against hPygo-2 (Figure 4.2). hPygo-2 protein levels were high within all brain tissues examined except the hypothalamus and thalamus of the diencephalon and the brainstem, all of which showed lower levels of expression as demonstrated with dot blot analysis. Also consistent with message levels was the lack of protein expressed in the spinal cord, mammary gland or heart. Comparison of hPygo-2 protein levels with that of β-Catenin showed an overlap in the majority of the tissues examined, but with little to no expression of β-Catenin in tissues having reduced hPygo-2 expression (hypothalamus, thalamus, brainstem) and a high level of β-Catenin expression within the heart, which did not express hPygo-2 (Figure 4.2).

The state of Wnt pathway activity was analyzed by detection of phosphorylated GSK-3α/β (active state). Within the brain, the co-expression of β-Catenin and hPygo-2 was associated with active Wnt signaling in all tissues except the frontal lobe, which

**Figure 4.2. hPygo-2 protein expression analysis.** Protein extracts from various different human tissues (BD Biosciences) were screened on a western blot using a polyclonal rabbit anti-hPygo-2 antibody. For comparison, blots were probed with anti-β-Catenin goat polyclonal and anti-phosphorylated GSK-3α/β (P-GSK) antibodies. Loading of relatively even levels of protein was verified both by Red Ponceau staining and blotting with an anti-β-Actin antibody.


Cerebral Cortex
Frontal Lobe
Temporal Lobe
Cerebellum
Amygdala
Hippocampus
Hypothalamus
Thalamus
Brainstem
Spinal Cord
Mammary Gland
Heart
hpygo-2
β-Catenin
P-GSK
Actin

showed little to no detection of either phosphorylated GSK-3 isoform (Figure 4.2). Outside of the brain, however, co-expression of β-Catenin and hPygo-2 was not reflected in the phosphorylation state of GSK, since neither was expressed within the mammary gland and only β-Catenin was expressed in the heart. These tissues demonstrate the diversity of the pathway, with activity in the mammary gland possibly reflecting non-canonical signal transduction and activity in the heart likely reflecting the utilization of additional hPygo proteins (hPygo-1) by β-Catenin to mediate the canonical Wnt signal.

## 4.5 DISCUSSION

The diversity of Wnt pathway constituents in promoting cellular events from growth and differentiation to adhesion increases the need to identify isolated components suitable as therapeutic targets for individual cellular programs, such as proliferation. This demand was potentially met with the recent discovery of the Pygopus family of proteins (Kramps et al., 2002; Thompson et al., 2002; Lake and Kao, 2003b), currently representing the lowest identified point in the pathway. Indeed, my current expression studies indicate that hPygo-2 activity is not ubiquitous and may be required for only a limited set of tissue-specific processes, consistent with orthologous isoforms in *Xenopus laevis* (chapter 3; Lake and Kao, 2003b).

Both messenger RNA and protein levels of *hpygo-2* are differentially expressed in adult human tissues, with a high level localized to fore-, mid- and hindbrain structures, reproductive tissues (prostate) and glandular tissues (adrenal and thyroid). Interestingly, tissues expressing *hpygo-2* at relatively higher levels were also associated with malignancies involving activating Wnt pathway mutations, including the cerebellum (Dahmen et al., 2001; Howng et al., 2002; Baeza et al., 2003), pituitary (Semba et al., 2001; Howng et al., 2002), thyroid (Garcia-Rostan et al., 2001; Ishigaki et al., 2002) and prostate (Chesire et al., 2000; Chesire et al., 2002; Sharma et al., 2002; Yang et al., 2002a; de la Taille et al., 2003). Wnt signaling has further been implicated in the embryonic development of these same structures, including regions of the cortex such as the hippocampus (Galceran et al., 2000; Lee et al., 2000) and major axonal tracts such as thalamocortical, corticothalamic and nigrostriatal tracts, the anterior commissure and corpus callosum (Wang et al., 2002). Wnt signaling is also involved in cerebellar

(McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Hall et al., 2000; Brault et al., 2001; Wang et al., 2001), pituitary (Douglas et al., 2001; Kioussi et al., 2002), adrenal gland (Eberhart and Argani, 2001; Heikkila et al., 2002; Gummow et al., 2003) and prostate (Truica et al., 2000; Chesire et al., 2002) development. Low levels of *hpygo-2* mRNA were also observed in kidney, placenta and thymus; tissues developmentally dependent on Wnt signaling (Eberhart and Argani, 2001, Staal and Clevers, 2003), implying possible involvement in their embryonic patterning or later maintenance.

Therefore, while correlative, my observations indicate that hPygo-2 may be involved in Wnt/β-Catenin associated normal and/or abnormal molecular processes of the brain (including cortex, cerebellum and pituitary), prostate, thyroid and adrenal gland. In addition, these findings may identify tissues expressing hPygo-2 that may have a potential susceptibility to activating Wnt mutations for oncogenesis through β-Catenin/hPygo-2. Alternatively, tissues known to have susceptibility to oncogenic canonical Wnt signals that do not express hPygo-2 may require hPygo-1. However, this requires further investigation, including characterization of both normal and abnormal hPygo-1/hPygo-2 expression. In addition, any developmental significance of either hPygo protein requires extensive analysis of embryonic expression patterns.

# CHAPTER 5:

## *Summary: A Model for Embryonic Brain Patterning in Xenopus*

## 5.1 MULTIPLE SIGNALS PATTERN THE CNS

Throughout embryogenesis, multifaceted developmental programs that regulate growth and differentiation of histogenically distinct structures tend to utilize common molecular components. Among these are the Rel/NF-κB, Shh, BMP, FGF and Wnt signaling cascades, each having potent effects on cellular behavior in multiple contexts to regulate the expansion and organization of cellular precursors. As reviewed in chapter 1, these growth factors are extensively involved throughout neurectodermal morphogenesis to establish both the DV and AP neuraxes.

The accumulated evidence from both my studies and from other laboratories indicate that specification of the numerous neuronal constituents of the CNS requires the generation of a grid-like expression pattern of genetic factors. The induction of this pattern is coordinated by multiple signaling centers that specify the primary subdivisions of the brain. This provides the framework for the extensive expansion and morphogenesis required to convert a sheet of neurectoderm into the complex 3-dimensional brain vesicles.

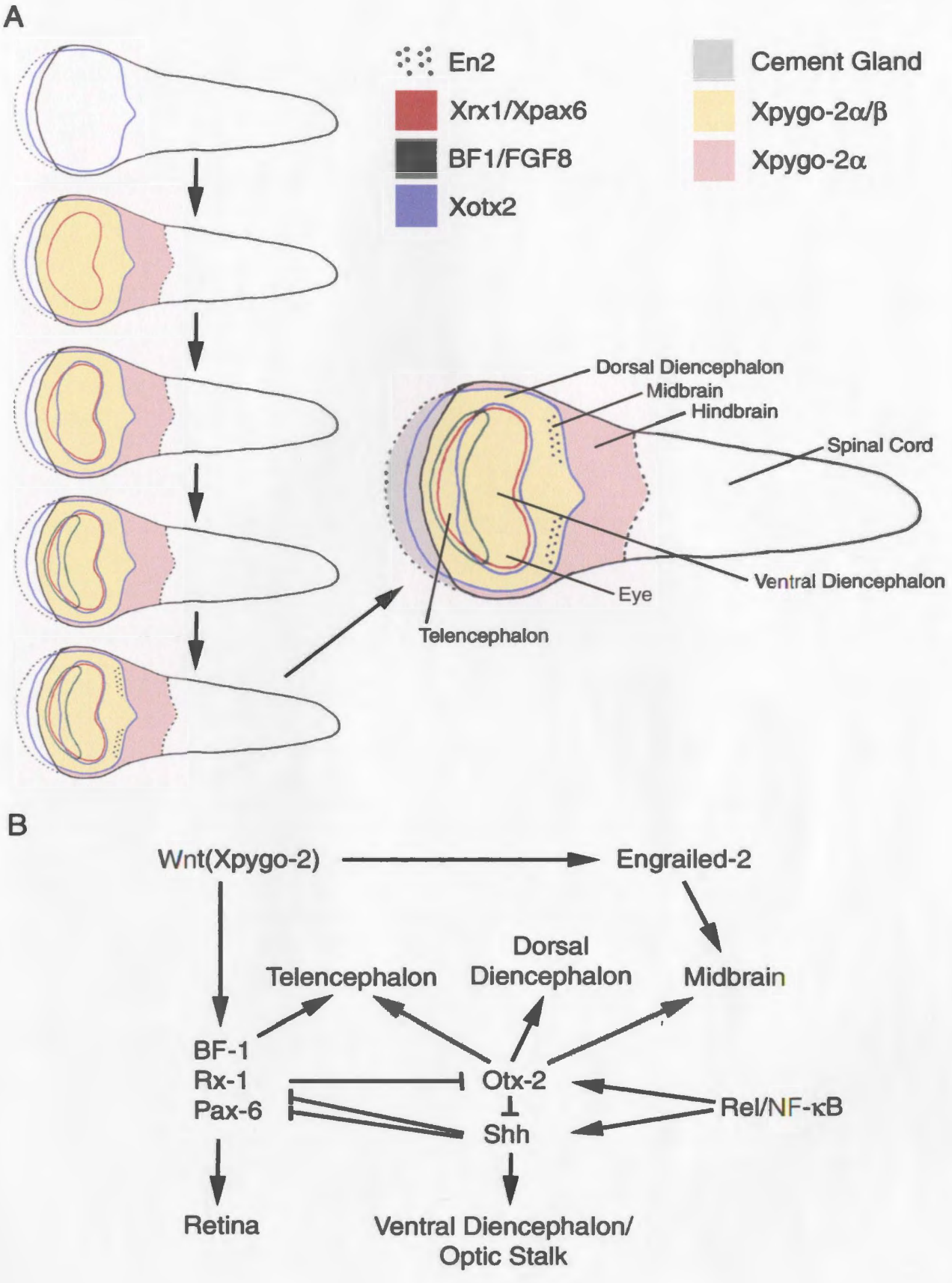
Basic medial-lateral identity has its earliest basis in neural plate formation during gastrulation. Ectodermal cells overlying the organizer and expressing *Xfd-12'* undergo extensive medial-lateral convergence extension which bisects the field of more anteriorly positioned cells (Keller et al., 1992; Fetka et al., 2000). As such, based on proximity to the organizer within the prospective neural field prior to gastrulation, more proximal (posterior) cells (notoplate) will populate the floorplate, and progressively more distant (anterior) cells will compose concentrically arranged ventral (basal) and dorsal (alar) fated rings by open neural plate stages. Differential expression of markers along these

longitudinal domains generate the medial-lateral or ventral-dorsal divisions of the CNS. Such longitudinal identity is derived from signals emanating first from mesendoderm (anti-BMPs, Nodals, Shh) and the adjacent non-neural ectoderm (Wnts, BMPs) at open neural plate stages.

Later, following neurulation, the mesendoderm (Nodals, Shh, anti-BMPs) in conjunction with both a dorsal signaling centre or roof plate (BMPs, Wnts) and ventral signaling centre or floorplate (Shh, Nodals) in the neural tube continue to define and refine the DV pattern of neuronal precursors (reviewed in chapter 1; Lake and Kao, 2003a). The non-homogeneous nature of the organizer also endows differential anterior-posterior character onto the neuraxis, enabling transversely bisecting organizers to form at the juxtaposition of distinctly specified domains. As such the ANR (which expresses FGF8) at the anterior-most limit of the neurectoderm and the IsO (FGF8, Wnt1) at the mid-hindbrain junction (the boundary of Otx2/Gbx2 expression) form and pattern adjacent forebrain and mid-hindbrain tissues, respectively (reviewed in chapter 1; Lake and Kao, 2003a).

The means by which these signaling centers are established and by which they direct the growth and differentiation of the brain to generate its immensely complex structure remains poorly understood. This thesis provides insight into the processes by which key neural patterning genes are choreographed during embryonic brain development (summarized in Figure 5.1). *Xenopus* Rel/NF-κB is necessary for the expression of *otx2* within the anterior neurectoderm as a prerequisite of retinal and fore-midbrain specification and positioning of the IsO (reviewed in chapter 1; Lake and Kao,

**Figure 5.1. Model of the genetic networks subdividing the neural plate.** A. Progressive molecular maps to generate a mid-neurula *Xenopus* embryo (around stage 15) as modified from Lupo et al., 2000. Areas within the coloured lines represent regions of expression. *Xotx2* (blue) becomes expressed first encompassing the fore-midbrain. Then concomitant with *Xpygo-2* (*Xpygo-2α/β* - yellow; *Xpygo-2α* alone - pink) expression, *Xrx1 and Xpax6* (red) become expressed. XRx1 represses *Xotx2* expression to exclude it from the retinal field. At the same time *Xbf1/Fgf8* (green) becomes expressed. Finally, a little later *en-2* (spots) becomes expressed. The fates of the resulting subdivisions at the mid-neurula stage are indicated with the anterior most limit of the neural plate marked by the cement gland (gray). Note that the ventral diencephalon becomes specified from the region indicated only following Shh-dependent repression of *Xpax6/Xrx1* expression during subsequent stages. B. An overview of the interconnected network of events required to specify the tissues indicated in A. See text for further discussion and references.


A
En2
Xrx1/Xpax6
BF1/FGF8
Xotx2
Cement Gland
Xpygo-2α/β
Xpygo-2α
Dorsal Diencephalon
Midbrain
Hindbrain
Spinal Cord
Ventral Diencephalon
Eye
Telencephalon
B
Wnt(Xpygo-2)
Engrailed-2
Telencephalon
Dorsal Diencephalon
Midbrain
BF-1
Rx-1
Pax-6
Otx-2
Shh
Rel/NF-κB
Retina
Ventral Diencephalon/
Optic Stalk

2003a). While *otx2* expression is not required for proper specification of the ANR, it *is* required to respond to signals from this organizer to specify the telencephalon and permit FGF8 induced expression of *bf1* at the end of gastrulation (stage 12) (Eagleson and Dempewolf, 2002; Tian et al., 2002). However, components of the Wnt signaling cascade, XPygo-2α/β, that also become expressed at this same time, are required for *bf1* expression. These findings indicate that the combined action of Rel/NF-κB, FGF and Wnt signals are required for induction of telencephalic gene expression.

Concomitant with *bf1* expression is the expression of *Xpax6* and *Xrx1* (Casarosa et al., 1997; Hirsch and Harris, 1997; Li et al., 1997; Mathers et al., 1997), both dependent on XPygo-2 (Figure 3.13). Xrx1 normally represses *otx2* expression in the prospective eye field to delimit *otx2* to telencephalic primordia (Andreazzoli et al., 1999; Lupo et al., 2000; Eagleson and Dempewolf, 2002). Subsequently, specification of the ventral diencephalon and splitting of the eye field (*Xpax6/Xrx1* expression) involves induction (requiring Xrel3) of *shh* expression in the ventral midline or floorplate, as initiated, presumably, by underlying Shh-expressing mesendoderm cells, at mid-neurula stages (stage 15; Figure 2.10E; Martí et al., 1995b; Ekker et al., 1995; Lee et al., 1997a). By late neurula (stage 18) the *Xpax6/Xrx1* expressing retinal field is segregated into two bilaterally displaced retinal vesicles (Figure 1.3; Li et al., 1997). While initiation of *shh* expression may initially involve Otx2 (Jin et al., 2001), it subsequently becomes negatively regulated by Otx2 and restricted ventrally first within the fore-midbrain then later in the midbrain only (Puelles et al., 2003). Anterior-lateral cells expressing *otx2*, in

the absence of FGF8 and presumably Shh, are fated for dorsal diencephalic character (Eagleson and Dempewolf, 2002).

At the posterior limit of *otx2* expression, antagonistic feedback with Gbx2 generates a sharp boundary at which Wnt1 and FGF8 become expressed (Glavic et al., 2002; Tour et al., 2002a/b). Wnt1 acts within the Otx2 expressing cells rostral to the IsO to induce En-2 expression (stages 13-15; Glavic et al., 2002), mediated by XPygo-2 (chapter 3) and found to be dependent on FGF8 (Danielian and McMahon, 1996; Lee et al., 1997b; Liu and Joyner, 2001; Glavic et al., 2002). The region of overlapping *otx2* and *en-2* expression will form the midbrain which becomes organized by FGF8 signals from the IsO (Lee et al., 1997b; Liu and Joyner, 2001; Eagleson and Dempewolf, 2002). It is interesting to note that both forebrain (BF1) and midbrain (En-2) markers that are activated by FGF8 require XPygo-2 protein activity in *Xenopus*, implying a dependency on both FGF and Wnt signaling to pattern these tissues.

## 5.2 DEVELOPMENT OF TELENCEPHALIC AND OPTIC PRIMORDIA

### 5.2.1. Wnt-Dependent Patterning Events

Localized expansion of the brain is required to generate its ultimate overall structure and complexity. The telencephalic fields are derived from a relatively small domain encompassing the anterior-most concentric ring of neurectoderm at neural plate stages that becomes the antero-marginal neural folds (Couly and Le Douarin, 1988; Eagleson and Harris, 1990; Eagleson et al., 1995). Therefore, extensive cellular proliferation and morphogenesis is required to generate the enlarged telencephalic vesicle

derivatives (cerebral cortex and basal ganglia), a process that occurs at later stages than eye vesicle evagination (Figure 1.3).

While antagonism of Wnt signaling is a necessary step in telencephalon specification during neural plate stages (Kiecker and Niehrs, 2001b; Nordstrom et al., 2002; Houart et al., 2002), chapter 3 demonstrates that components of this pathway, XPygo-2α and XPygo-2β, are required for the expression of *Xenopus bf1*. These components must therefore promote both proliferation at high concentrations of *bf1* expression and neurogenesis at low concentrations (Bourguignon et al., 1998) for growth and differentiation of the telencephalic and optic vesicles. Wnts also play roles in mitogenesis and dorsal specification of the telencephalon later in development in part through direct activation, in conjunction with BMPs, of *emx2* expression (Theil et al., 2002).

To account for this apparent contradictory role of Wnts in forebrain patterning, recent studies in chick have proposed a switch in competence after the early Wnt-independent phase of specification of prospective telencephali to a phase requiring Wnt signals to block ventral and induce dorsal identity (Gunhaga et al., 2003). Consistent with this scenario, several Wnts become expressed within the forebrain of mice (Patapoutian and Reichard, 2000; Kim et al., 2001; Coyle-Rink et al., 2002), chicks (Hollyday et al., 1995) and frogs (Wolda and Moon, 1992; Wolda et al., 1993; Cui et al., 1995; Landesman and Sokol, 1997).

It has been proposed (Gunhaga et al., 2003) that prospective telencephalic cells at the open neural plate stage are intrinsically ventral in identity by exposure to

mesendodermal signals such as provided by Shh (Gunhaga et al., 2000). However, at neural fold stages the lateral and prospective dorsal cells are exposed to Wnt (Wnt1 and Wnt4) and BMP signals from adjacent non-neural ectoderm, while medial or prospective ventral telencephalic cells are exposed to ANR derived FGF8. Wnt blocks the potential ventral telencephalic fate and through induction of markers such as Pax6, specifies dorsal telencephalic cells. Following neural tube closure, Wnt8b becomes expressed in the dorsal telencephalic cells and FGF8 expands into the dorsal midline for coordinated roles with Wnt and BMP signals in regulating dorsal telencephalic and midline fate (Gunhaga et al., 2003).

Results in *Xenopus* (chapter 3), showing the loss of both *Xpax6* and *Xbf1* in the neural tube following depletion of XPygo-2 suggest that the process described above may be conserved in frog forebrain morphogenesis. These results also further strengthen the existence of a biphasic requirement for Wnt signaling in specification of this tissue, with the later phase occurring at the end of gastrulation (stage ~12) dependent on the expression of XPygo-2α/β isoforms. The early phase of Wnt-induced caudalization likely reflects a role in regulating retinoic acid dependent neural posteriorization through CYP26 restriction anteriorly (Kudoh et al., 2002). Therefore, expression of XPygo-2 in the anterior neurectoderm can be considered the molecular switch in cellular competence to Wnt signals that enables the transition between these two phases.

### 5.2.2. Forebrain Patterning by a Conserved Mechanism Involved in the Developing Limb

To understand the extensive molecular processes involved in outgrowth of telencephalic and optic primordia in mouse and chick, recent studies have drawn comparisons between genetic networks involved in the morphogenesis of the limb and prosencephalon (Crossley et al., 2001; Ohkubo et al., 2002). FGF8 is initially expressed within the ANR which overlaps the rostral prosencephalon fated to form the rostromedial telencephalon and later becomes expressed in the optic vesicles (Crossley et al., 1996; Shanmugalingam et al., 2000; Xu et al., 2000; Crossley et al., 2001), likely due to an influx of migrating ANR cells into this region as shown in *Xenopus* (Eagleson et al., 1995). These expression domains become juxtaposed with that of Shh and BMP in the ventral and dorsal domains, respectively, of the telencephalon and optic stalk (Crossley et al., 2001; Ohkubo et al., 2002).

This tripartite signaling center resembles that in the limb bud, with FGF expression in the apical ectodermal ridge (AER), BMP expression in the mesenchyme and Shh in the zone of polarizing activity (ZPA), regulating the pattern and expansion of a population of mesenchymal cells known as the progress zone (reviewed in Capdevila and Izpisua Belmonte, 2001; Niswander, 2002; Wolpert, 2002; Panman and Zeller, 2003). The activity of this center requires interdependent regulation of each signaling molecule's expression. This involves negative regulation between Shh and BMP in addition to maintainance of Fgf8 expression by Shh and repression by BMP (Buckland et al., 1998; Pizette and Niswander, 1999; Zuniga et al., 1999; Sun et al., 2000; Krauss et al., 2001).

These interdynamics are also found in the telencephalon (Golden et al., 1999; Anderson et al., 2002; Ohkubo et al., 2002).

Consistent with a conservation of mitogenic and morphogenetic activities between the limb and forebrain, Wnt signals were found to be required at multiple levels of limb development including a role in dorsoventral patterning (Kawakami et al., 2000; Kawakami et al., 2001; Chen and Johnson, 2002; Church and Francis-West, 2002; Barrow et al., 2003; Soshnikova et al., 2003) reminiscent of that found in the prosencephalon (Theil et al., 2002; Gunhaga et al., 2003; Lake and Kao, 2003b). Similarly, Rel/NF-κB, when antagonized, causes aberrant development and reduced Shh expression in the limb (Bushdid et al., 1998; Kanegae et al., 1998) and, as I have shown, in the brain (Chapter 2). Therefore, the results presented in my thesis imply the conservation of Rel/NF-κB regulated expression of Shh between these two developmental paradigms. They further support the hypothesis that coordinated behavior of numerous signaling pathways generates distinct structures based on the temporal and spatial differences of their combined activities.

## 6.1 REFERENCES

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