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Regulation and Function of the *dlx* Genes in the Zebrafish Forebrain

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Regulation and Function of the *dlx* Genes in the Zebrafish Forebrain

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Thesis is submitted as a partial fulfillment of the Doctor of Philosophy program in Biology

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This Thesis is dedicated in loving memory of my Grampie, Cecil Rae Lewis, who provided me with great inspiration throughout my studies. I can only hope to be as loved and respected by my family and peers as he was. I know he wouldn't have understood anything written in this thesis but would have been proud of me no matter what I did.

"Our real teacher has been and still is the embryo, who is, incidentally, the only teacher who is always right." Viktor Hamburger (1900-2001).

Statement of Contribution

The thesis, "Regulation and function of the *dlx* genes in the zebrafish forebrain" is composed of three related studies, all of which were collaborative efforts.

The first study, "The relationship between *dlx* and *gad1* expression indicates highly conserved genetic pathways in the zebrafish forebrain", was a collaborative effort with Dr. Charles Kimmel's laboratory. I cloned the *dlx* regulatory elements into a modified reporter vector that I have generated. I injected and screened the transgenic zebrafish with these constructs. Jared Talbot and I carried out triple fluorescent *in situ* hybridizations on embryonic zebrafish and imaged them. Dr. Melanie Debiais-Thibaud and I carried out standard *in situ* hybridizations and immunohistochemistry on sections. Melanie and I analyzed the data and wrote the manuscript. Dr. Marc Ekker reviewed this study.

The second study, "Functional conservation of a forebrain enhancer from the elephant shark (*Callorhinchus milii*) in zebrafish and mice" was performed as a collaborative effort with Dr. Byrappa Venkatesh's laboratory. In this study, Kyle Martin identified *dlx* specific regulatory regions in the elephant shark genome by BLAST search. Boon-Hui Tay screened a BAC library for *dlx* positive clones. Dr. Bryappa Venkatesh provided elephant shark genomic DNA and the *dlx* positive BAC clones. Kyle Martin and I generated the transgene constructs containing the forebrain enhancers and the reporter genes. I injected and screened the transgenic zebrafish while Adrianna Gambarotta generated the transgenic mice. I genotyped, performed *lacZ* staining, and sectioned transgenic mouse embryos. I also cryosectioned and performed immunohistochemistry on the transgenic zebrafish lines. Dr. Melanie-Debiais Thibaud and myself carried out *in situ*

hybridization analysis of transgenic zebrafish. Melanie performed bioinformatic analysis on the BAC and regulatory elements. Melanie, Dr. Marc Ekker, and I, collaboratively analyzed the data and wrote the manuscript.

The third study, "A conserved gene regulatory network controls the differentiation of GABAergic interneurons in the zebrafish prethalamus". In this study I injected the morpholino embryos and collected them at appropriate stages. Jared Talbot and I carried out fluorescent *in situ* hybridizations on embryos and imaging. Dr. Melanie Debiais-Thibaud and I performed standard *in situ* hybridization analysis. I wrote the article and Dr. Marc Ekker corrected it.

Abstract

The *Dlx* genes encode a family of transcription factors important to the development of the vertebrate forebrain. The expression of the *Dlx* genes is controlled by a number of highly conserved regulatory elements, which have been extensively studied in the mouse. The *Dlx* genes have been shown to have highly overlapping expression during the development of the telencephalon/diencephalon and play a role in GABAergic interneuron differentiation and in the regulation of the *Gad* genes, responsible for GABA synthesis. Using fluorescent *in situ* hybridizations, we show the *dlx* genes are expressed in highly overlapping domains within the zebrafish forebrain and appear to follow a conserved pathway involved in the differentiation of GABAergic interneurons. We have identified one of the *dlx* regulatory elements, URE2, in the three major branches of jawed vertebrates and tested the orthologous sequences for regulatory function. These regulatory sequences have similar activity in the forebrain of mice and zebrafish. Transgenic and *dlx* expression data indicate there may be similar genetic cascades controlling *Dlx* expression in the mammalian and teleost forebrain. To elucidate the genetic cascade regulating the expression of the *dlx* and *gad1* genes in zebrafish, we knocked down the function of *ascl1a*, a *Mash1* ortholog known to regulate *Dlx* expression in the mouse. Knockdown of *ascl1a* function resulted in a loss of *dlx* and *gad1* expression in the prethalamus. We also impaired the function of the *dlx* genes in the zebrafish forebrain. Double knockdown of the *dlx1a/2a* genes resulted in a loss of *gad1* expression in the prethalamus, but not in the telencephalon, in contrast to observations made in the mouse. We propose the *ascl1a* and *dlx* genes are involved in an

evolutionarily conserved genetic cascade controlling GABAergic interneuron differentiation in the prethalamus.

Résumé

Les gènes *Dlx* codent pour une famille de facteurs de transcription qui jouent un rôle important au cours du développement du cerveau antérieur des vertébrés. L'expression des gènes *Dlx* est contrôlée par des éléments de régulation très conservés et caractérisés principalement chez la souris. Les gènes *Dlx* sont exprimés avec des profils chevauchants au cours du développement du télencéphale et du diencéphale. Ils jouent un rôle dans la différenciation des interneurones GABAergiques et dans la régulation des gènes *Gad* essentiels à la synthèse du GABA. A l'aide de l'hybridation *in situ* en fluorescence, nous avons montré que les gènes *dlx* sont exprimés de manière chevauchante dans le cerveau antérieur du poisson-zèbre, *Danio rerio*, et semblent participer à la voie de différenciation des interneurones GABAergiques. Nous avons identifié un des éléments de régulation des gènes *Dlx*, URE2, dans trois branches majeures des vertébrés et testé les éléments orthologues pour leur fonction régulatrice. Les éléments ont des profils d'activité semblables dans le cerveau antérieur de la souris et du *Danio*. Nos données d'expression et en transgénèse suggèrent l'existence de cascades génétiques semblables contrôlant l'expression des *Dlx* dans le cerveau des poissons téléostéens et des mammifères. Pour mieux comprendre les cascades génétiques qui contrôlent les gènes *dlx* et *gad1* chez le *Danio*, nous avons causé la perte de fonction d'*ascl1a*, un orthologue de *Mash1*, gène connu comme régulateur de l'expression des *Dlx* chez la souris. L'inactivation d'*ascl1a* à l'aide de morpholinos a causé une baisse d'expression des *dlx* et de *gad1* dans le pré-thalamus. Nous avons aussi inactivé la fonction des gènes *dlx* dans le cerveau antérieur du *Danio*. L'inactivation combinée de *dxl1a* et de *dlx2a* a causé la perte d'expression de *gad1* dans le pré-thalamus mais pas

dans le télencéphale, un résultat qui contraste avec les observations faites chez la souris. Nous proposons que les gènes *ascl1a* et *dlx* participent à une cascade génétique conservée responsable du contrôle de la différenciation des interneurones GABAergiques dans le pré-thalamus.

Table of Contents

List of Figures and Tables

List of Abbreviations

AER: Apical ectodermal ridge

A/P: Anterior and posterior

ANB: Anterior border of the neural plate

bHLH: Basic-helix-loop-helix

BMP: Bone morphogenic protein

bp: base pairs

CmURE2: URE2 *cis*-regulatory element ortholog from the elephant shark

CNC: Cranial neural crest

CNE: Conserved non-coding elements

CNS: Central nervous system

CRE: *cis*-regulatory elements

Dll: Distal-less

Dlx: *Distal-less* related mouse ortholog

dlx: *Distal-less* related zebrafish ortholog

dlx2bDRE: *dlx2b* downstream regulatory element

DrURE2: URE2 *cis*-regulatory element ortholog from the zebrafish

D/V: Dorsal-ventral

EGFP: Enhanced green fluorescent protein

FGF: Fibroblast growth factor

GABA: γ-aminobutyric acid

Gad: *glutamic acid decarboxylase*

GFP: Green fluorescent protein

GRN: Gene regulatory network

Hh: hedgehog signalling

Hpf: Hours post-fertilization

HS: Hybridization solution

IG: Intergenic

kb: Kilobases

LacZ: β-galactosidase

MAR: Matrix attachment region

mbl: *masterblind*

MmURE2: URE2 *cis*-regulatory element ortholog from the mouse

MO: Morphants

PD: Proximodistal

PNS: Peripheral nervous system

REST: RE-1 silencing factor

sFRP: Secreted frizzled related protein

SHFM: Split hand/foot malformations

Shh: *Sonic Hedgehog*

TDO: Tricho-Dento-Osseous

TF: Transcription factors

Tg: Transgenic

URE: Upstream Regulatory Element

ZLI: Zona limitans intrathalamica

3R: Three rounds of whole genome duplication

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While everyone in the Ekker lab certainly had a hand in my project, whether it is help with experiments or useful discussions, they are too numerous to thank individually. With this is mind I would like to thank certain people:

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relevant scientific issues and is solely responsible for my exploration into evolutionary research, has opened my eyes to the relevance of the field. Each of you, and our ridiculous conversations, will be what I remember when thinking back on the early days in the lab.

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My family, Mom, Dad, Karissa, Corey, and Grammie, for always supporting me and never questioning my reason for pursuing science. I would also like to thank them for never pushing me to do something I didn't want to do and always wanting the best for me.

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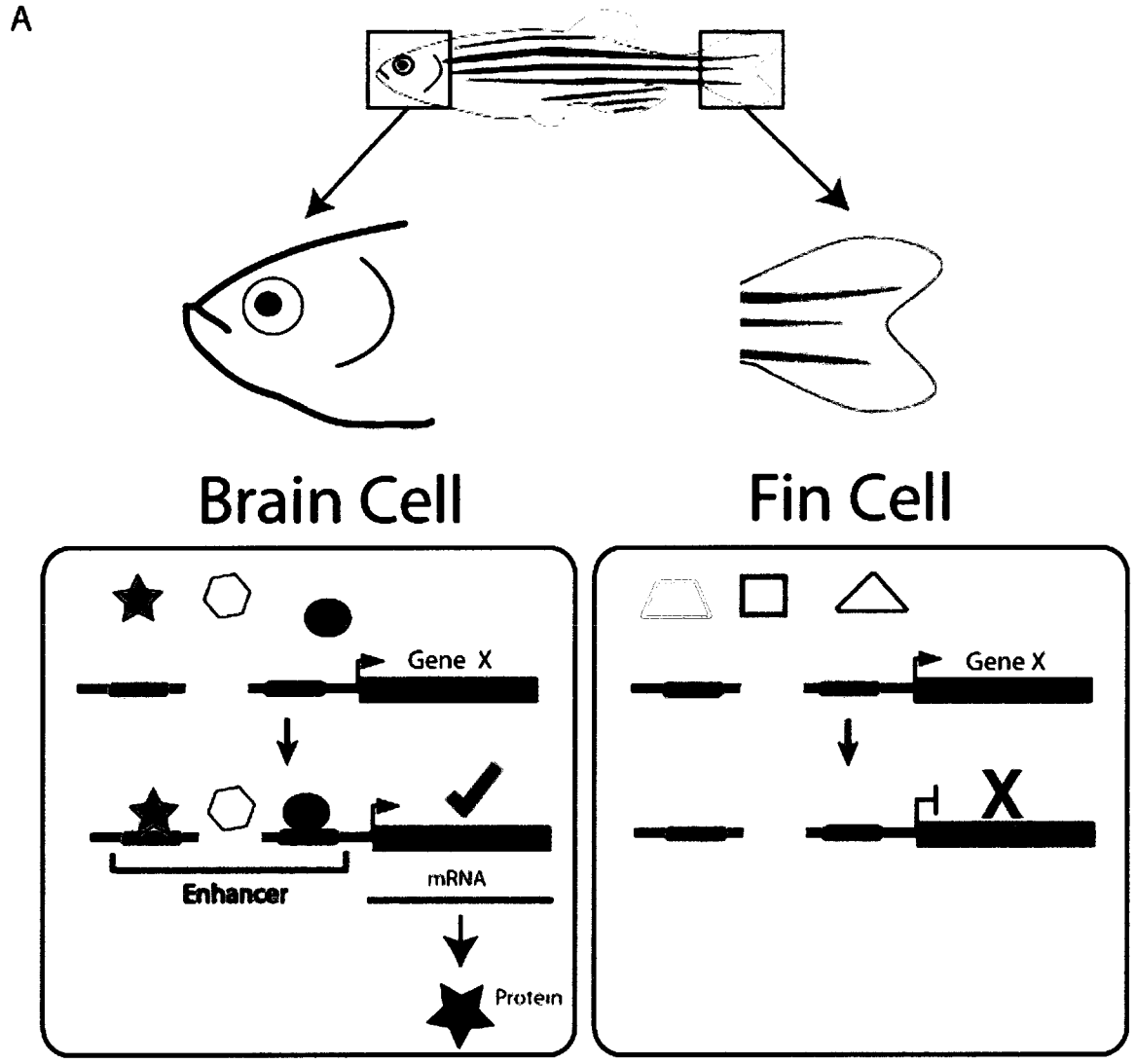
1. Introduction

1.1. Gene Regulatory Networks

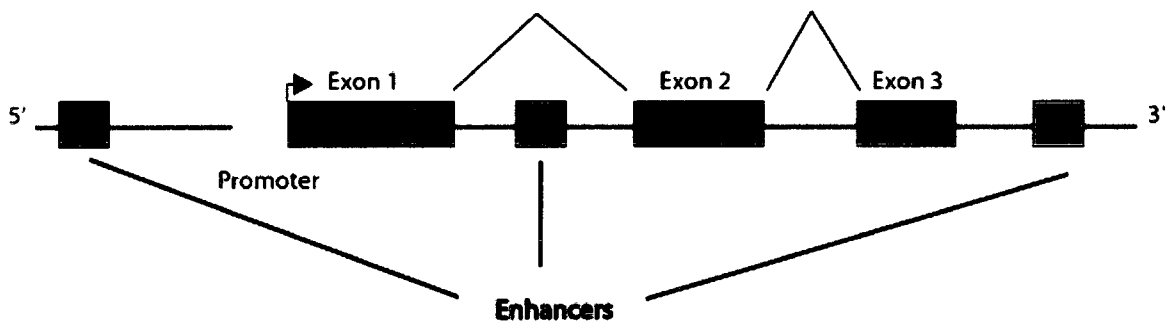
Vertebrate development requires a number of carefully coordinated signals to properly control gene expression, thus leading to restricted cell proliferation, differentiation, and migration. Gene regulatory networks (GRNs) are made up of dynamic interactions between combinations of transcription factors (TFs) and *cis*-regulatory elements (CREs) found within the genome (for reviews see: Levine and Davidson, 2005; Davidson and Levine, 2008). These regulatory sequences are comprised of a number of clustered transcription factor binding sites, and when bound they have the ability to affect the transcription of specific genes (for reviews see: Smale, 2001; Kulkarni and Arnosti, 2003; Kadonaga, 2004; Panne, 2008) (Figure 1.1A). The overall levels and timing of gene expression are conferred by the cumulative contributions of multiple TFs on possibly multiple regulatory regions (Gray and Levine, 1994; Kel et al. 1995; Arnosti and Kulkarni, 1997). The genes regulated by this process during development often encode TFs that will play a role in the regulation of other transcription factor genes downstream. In turn, each downstream gene may play a role in many diverse developmental processes (Bolouri and Davidson, 2003; Sandmann et al. 2007) (for review see: Shirasaki and Pfaff, 2002). Therefore, these distinct regulatory steps will form a cascade by which an initial signal will be amplified by the successive rounds of gene expression (Britten and Davidson, 1969). During development, cells will differentiate to a specific fate usually in correlation with the distinct combination of TFs they express. The GRNs involved in the differentiation of the endoderm and mesoderm

Figure 1.1. Regulatory elements are composed of a number of distinct transcription factor binding sites allowing them to impart tissue-specific expression of a target gene. A) Tissue-specific expression of a gene occurs when the correct combination of transcription factors is present. Transcription factors will specifically bind DNA regulatory sequences based on their DNA-binding preferences (blue, yellow, and green boxes). In this example, the brain cell contains the proper combination of transcription factors to bind to regulatory sequences, whereas the fin cell does not. Transcription factor binding increases the frequency of transcription initiation at the promoter, leading to higher mRNA and protein (star) production within the cell. The green checkmark indicates the presence of transcription, where the red X indicates no transcription. B) A gene structure and the location of regulatory elements. A schematic of gene structure with exons (blue), a promoter (yellow), and enhancers (green, blue, red) are depicted. The enhancers can be found upstream, within an intron, or downstream, with respect to the promoter.

A


Brain Cell
Fin Cell
Gene X
Gene X
X
Enhancer
mRNA
Protein

B


Exon 1
Exon 2
Exon 3
5'
3'
Promoter
Enhancers

in the sea urchin embryo may be the best-studied example (for reviews see: Davidson et al. 2002; Oliveri and Davidson, 2004; Davidson and Levine, 2008). Overall, the understanding of transcriptional regulation is still under developed. However, identifying the roles of important regulatory genes and how they interact within the GRNs will allow for a better understanding of the distinct developmental processes in vertebrates.

1.1.1. Regulatory elements in the genome

The genome contains a number of regions that play a crucial role in the proper spatial-temporal expression of genes during development. These regulatory regions often affect transcription in one of two ways: 1) binding TFs and interacting with the transcriptional machinery (Ferreri et al. 1994; Metz et al. 1994; MacDonald et al. 1995; Schmitz et al. 1995) or 2) affecting the chromatin structure and limiting the availability of gene regulatory regions to transcription factors/basal transcriptional machinery (for reviews see: Wallace and Felsenfeld, 2007; Bartkuhn and Renkawitz, 2008).

Promoters are immediately proximal to the transcription start sites, unlike other regulatory elements. Promoters may contain a number of typical motifs, such as: CAAT, TATA, or GC boxes; however, these are not required for promoter function (Ohler et al. 2002). The most prevalent is the TATA box which is found in ~35% of core promoters (Kutach and Kadonaga, 2000). The "core" promoter is defined as the 100 basepairs (bp) flanking the transcription start site and functions as the recognition site for the basal transcription apparatus, including RNA Polymerase II (for reviews see: Smale, 2001; Butler and Kadonaga, 2002). In practice, a core promoter is often not sufficient to drive the proper spatial and temporal expression of the associated gene. The rate of

transcription initiation at a basal promoter is greatly influenced by a number of signals provided by additional regulatory sequences found in the surrounding genomic environment (for review see: Michel, 2009).

Enhancer elements are non-coding elements found within the genome that activate transcription in a temporal and spatially restricted manner. These elements can be found upstream, downstream, or within introns of genes and can act independent of orientation with respect to their target promoter (Fig 1.1B)(Banarji et al. 1981; Arnone and Davidson, 1997). Enhancers are made up of a number of TF binding sites, which only impart their regulatory activity on a promoter if the correct combinations of TFs are present (for reviews see: Smale and Kadonga, 2003; Arnosti and Kulkarni, 2005). If the correct factors are not present in the cellular environment, or blocked from binding the DNA, the enhancer will remain inactive. One well-studied example in zebrafish is the *sonic hedgehog* gene locus (*shh*). This gene encodes a secreted peptide that controls a wide range of developmental processes. Expression of this gene in incorrect tissues has been implicated in the development of tumors, and can also change cell fates by acting as a morphogen in different cell types (for reviews see: Ingham and McMahon, 2001; McMahon et al. 2003). Thus, tight control of s*hh* gene expression is necessary for normal embryonic development. Several enhancers have been identified within the *shh* locus, each controlling expression in a specific tissue. These distinct enhancers combine to recapitulate most of the endogenous *shh* gene expression, indicating each element is responsible for a specific part of *shh* gene expression domains (Müller et al. 1999; Ertzer et al. 2007). Furthermore, unlike promoter elements, enhancers can be found large

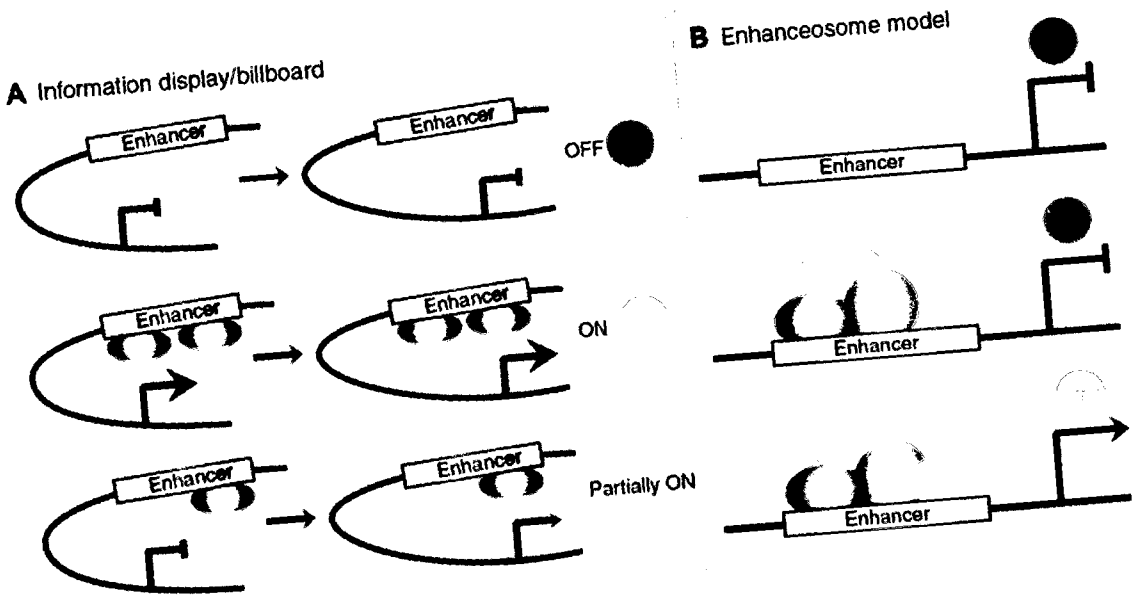
distances (~ one megabase) away from the transcription start sites illustrated by a few of the aforementioned enhancers in the *Shh* locus (Jeong et al. 2006b; Sagai et al. 2009).

The fundamental question surrounding CREs is how they affect transcription on a molecular level. Currently, there are two proposed mechanisms in which enhancers function: 1) billboard or type 1 enhancers are unstructured, with varying organization of the TF binding sites. These enhancers will not function as a whole, rather as a series of individual signals each independently affecting transcription of the target gene (Arnosti et al. 1996; Kulkarni and Arnosti, 2003) (Figure 1.2A); 2) The enhanceosome model hypothesizes that transcription factors and basal transcriptional machinery are arranged in an organized structure called the enhanceosome that will cooperate to affect transcription (Thanos and Maniatis, 1995; Panne et al. 2007) (Figure 1.2B).

Silencers are regulatory regions displaying an overall negative effect on transcription (Ogbourne and Analtais, 1998). As an example, the RE-1 silencing factor (REST) functions as a transcriptional repressor of neural specific genes in non-neural tissues (Schoenherr and Anderson, 1995; Chen et al. 1998). REST functions primarily by binding a regulatory element and affecting the chromatin structure via histone acetylation, but other repressors can have more direct effects on transcription (Zheng et al. 2009; Murai et al. 2004).

Insulator elements are an interesting class of regulatory element as they may have a role in enhancer-promoter interactions and modifying the chromatin environment (for review see: West and Fraser, 2005). This is in contrast to enhancers and silencers that

Figure 1.2. Information display/billboard and enhanceosome models of regulatory element function. A) In the information display/billboard model, the basal transcriptional machinery comes in contact with transcription factors with different activities. In the presence of only repressors (orange) there is no transcription of the target gene. With the binding of only activators (purple) there will be an upregulation in transcription of the target gene. A combination of activators and repressors will lead to an intermediate level of target gene activity. B) In the enhanceosome model, the regulatory element is only functional when many transcription factors come together to form a complex (purple, blue, green and pink). If a repressor (orange) blocks binding of one transcription factor, or if one component of the complex is absent, there will be no target gene expression. Green circle, high transcription level; yellow circle, moderate transcription level; red circle, no transcription. Adapted from Borok et al. 2010.


A Information display/billboard
Enhancer
Enhancer
OFF
Enhancer
Enhancer
ON
Enhancer
Enhancer
Partially ON
B Enhanceosome model
Enhancer
Enhancer
Enhancer

may act large genomic distances away from the promoter. For example, the "Homie" element in *Drosophila* blocks short-range enhancer-promoter interactions yet it facilitates long-range interactions over several megabases (Fujioka et al. 2009). Enhancers have been called "promiscuous" elements when they are not specific to any one promoter, and thus their actions must be restricted to prevent activation of non-target genes (West and Fraser, 2005). This is theoretically done by insulator elements, which interfere with enhancer-promoter interactions when placed between the two elements, yet have no effect when placed on either side. One example is the Chicken β-globin HS4 insulator element that is dependent on the binding of CCCTC binding factors (Bell et al. 1999; Farrell et al. 2002). Gene transcription can be silenced by heterochromatin, which can affect the transcription of neighboring genes (Grewal and Moazed, 2003; Ghirlando et al. 2004). Insulator elements will protect the locus by acting as a barrier to invasion by heterochromatin, although the exact mechanisms for this function have not yet been uncovered (West et al, 2002). Insulators have also been used to protect transgenes from chromosomal silencing and position effects in transgenic assays (for review see: Recillas-Targa et al. 2004).

Matrix attachment regions (MARs) are specific regions of the genome that are able to mediate the interaction of chromatin loops with the nuclear matrix (Laemmli et al. 1992). They are involved in chromatin remodeling and also in activating transcription or blocking improper transcription of genes by position effects (Allen et al. 2000; Bode et al. 2000). When the mouse and human genomes were compared, 11% of the non-coding conserved elements were MARs and their genomic locations may be associated with other CREs (Glazko et al. 2003).

1.1.2. Identification of regulatory elements by comparative genomics

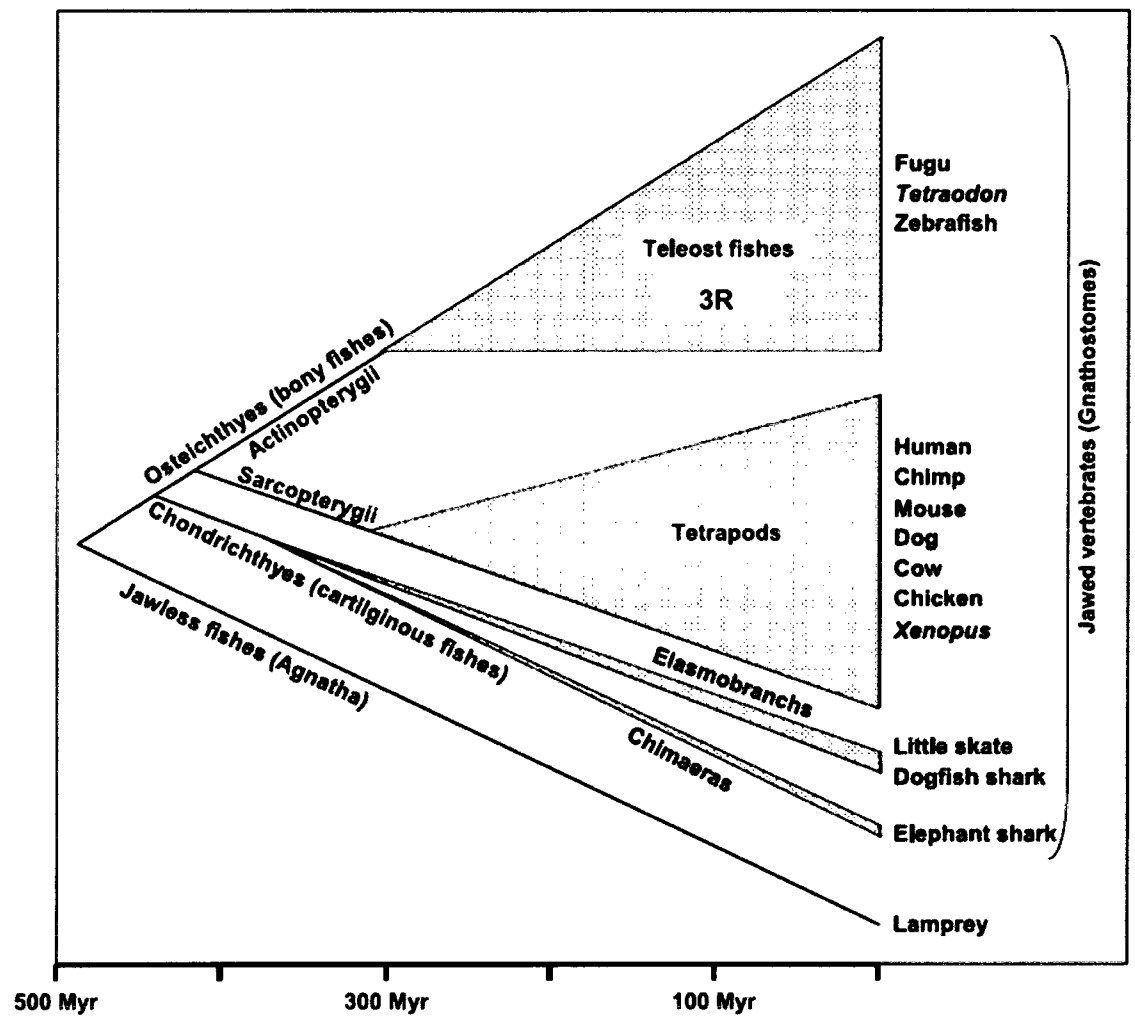
Identifying regulatory regions in the genome was first based on the candidate approach and systematic deletion of regions thought to contain functional elements in transgene constructs. With whole-genome sequencing projects, comparative genomics is now a powerful approach to identifying conserved sequences in the human genome by comparison with distantly related vertebrates. Comparisons of human and mouse genomes estimate that 5% of the human genome is under selective pressure (Waterston et al. 2002). Only 1-2% of the human genome is coding leaving ~3% unaccounted for (Lander et al. 2001). A large proportion of conserved sequences could be non-coding RNA, as Cawley et al. (2002) proposed there are as many non-coding RNA sequences as there are protein coding RNA genes. Comparative genomics is based on the assumption that functionally significant regions in the genome will evolve more slowly than non-functional regions, allowing for selective pressure (Kimura, 1983). Mutations in functional elements are thought to be deleterious and will be selected against, putting further selective pressure on the sequences (Hardison, 2000; Sumiyama et al. 2001). However, comparisons of genomes allowed for the identification of conserved non-coding elements (CNEs) and showed these often function as CREs (Pennacchio et al. 2006).

Improved bioinformatics programs and sequencing of whole genomes have allowed genome wide comparisons to be carried out between orthologous loci of one or more related species, a technique referred to as "Phylogenetic footprinting". The genomic areas around developmental genes are good candidates for phylogenetic footprinting as

they are often highly conserved in their coding regions, frequently carry out similar cellular functions, and are expressed in homologous tissues in distantly related phylogenetic groups (for review see: Dickmeis and Müller, 2005). The latter point indicates that CREs may also be conserved in structure and function (Dickmeis and Müller, 2005). The identification of CREs has been carried out in a number of species, both closely and distantly related with respect to evolutionary time. Some regions in the mouse and human genomes show high levels of conservation although no regulatory function can be determined, thus revealing that phylogenetic footprinting at this level potentially identifies more sequences than those actually playing a regulatory role (Wasserman et al. 2000; Tautz, 2000). The high degree of sequence conservation is due to the close evolutionary relationship of mouse and human (~60 million years) combined with the slow rate of divergence for neutrally evolving regions (between 0.1 and 0.5% per million years) (Gottgens et al. 2000). Thus, to increase the efficiency in detecting functionally conserved elements, comparisons of more distantly related vertebrate genomes have been required. Comparing distantly related genomes allows for the identification of CNEs between human and other vertebrate genomes such as: chicken (Ahituv et al. 2005), fish (Bagheri-Fam et al. 2001; Shin et al. 2005), and lamprey (McEwen et al. 2009).

While there are a number of vertebrate genomes available, a select few are of particular interest due to their phylogenetic position in vertebrate evolution. The teleost fish are the most diverse class of vertebrate species and shared a common ancestor with mammals, approximately 430 million years ago (Figure 1.3)(Powers, 1991). The teleost lineage is also of interest due to the presence of additional genes in the genome, as a

Figure 1.3. A phylogenetic tree of the vertebrates. This tree depicts the divergence of the common ancestors throughout vertebrate evolution. Within the tree, the four major groups of vertebrates are listed underneath the branch and examples of each group are listed and on the far right: the agnathans (lamprey), the chondrichthyes (elephant shark), the tetrapods (mouse and human), and the teleosts (zebrafish). The 3R indicates an additional genome duplication event specific to the teleost lineage. The x-axis depicts the evolutionary time since the divergence on the common ancestors (based on fossil records). Adapted from Venkatesh et al. 2007.


Fugu
Tetraodon
Zebrafish
Teleost fishes
3R
Osteichthyes (bony fishes)
Actinopterygii
Sarcopterygii
Tetrapods
Human
Chimp
Mouse
Dog
Cow
Chicken
Xenopus
Chondrichthyes (cartilginous fishes)
Elasmobranchs
Little skate
Dogfish shark
Chimaeras
Elephant shark
Jawless fishes (Agnatha)
Lamprey
Jawed vertebrates (Gnathostomes)
500 Myr
300 Myr
100 Myr

consequence of at least one additional genome duplication event before their diversification (Amores et al. 1998). The additional genome duplication event may have altered the selective constraints on these CREs. Thus the teleost lineage provides an interesting model for comparisons between genomes and to study the evolution of CRE function after genome duplication. One species of teleost that is advantageous for phylogenetic footprinting is the pufferfish, *Takifugu rubripes* (genome size is 390 Megabases) (for review see: Venkatesh and Yap, 2005). The pufferfish genome is particularly useful for comparative genomics due to its small size and association with loss of neutral DNA, which leaves the remaining non-coding DNA enriched for potential *cis*-regulatory elements (Brenner et al. 1993). Despite having a reduction in the size of its genome, the pufferfish still contains a similar complement of genes with conserved synteny compared to mammals (for review see: Elgar et al. 1996). The zebrafish is another teleost that has been useful for the identification of *cis*-regulatory elements, not only in identifying them but also in testing their function (see section: 1.1.3).

CNEs identified in mammals and teleosts have not been identified in non-vertebrate species, including urochordates, which are thought to be the closest relatives to the vertebrates (Woolfe et al. 2005; Blair and Hedges, 2005; Delsuc et al. 2006). Invertebrates are not lacking CREs; however, they possess their own CRE networks that are similarly clustered around developmental genes (Glazov et al. 2005; Davidson, 2006; Vavouri et al. 2007). Within vertebrates, the cartilaginous fishes (chondrichthyans) occupy an interesting phylogenetic position as the sister group to bony vertebrates (that include teleosts and tetrapods) (Figure 1.3). Similarly, tetrapods and cartilaginous fishes

have not experienced additional genome duplication like the teleosts. The elephant shark (*Callorhinchus milii*) was selected as a representative species for cartilaginous fishes due to its relatively small genome of 910 megabases. The availability of a low coverage chondrichthyan genome (~1.4×) fascilitated the identification of CNEs when compared to teleosts and mammals, indicating that these sequences are present in all jawed vertebrates (bony and cartilaginous vertebrates) (Venkatesh et al. 2007). Investigation of the sea lamprey, *Petromyzon marinus*, was the next logical step to address where in vertebrate evolution CNEs originated, as they represent the sister-group to jawed vertebrates (Figure 1.3). Lampreys, along with hagfish, make up the jawless fishes (agnathans) that diverged from jawed vertebrates (gnathostomes) ~550 to 650 million years ago (Kumar and Hedges, 1998; Blair and Hedges, 2005). Draft sequences from the genome of *Petromyzon marinus* have been used to identify CNEs when compared with other vertebrates' genomes (McEwen et al. 2009).

1.1.3. Testing *cis*-regulatory elements for function

While the identification of CNEs may be challenging, testing these elements for functional relevance is key to determining their role in the control of gene expression. In general, the organisms used as models for genome-wide comparisons lack certain key characteristics necessary for testing the function of the CNEs (e.g. elephant shark or lamprey). That is, there is no possibility of testing these CNEs in a homologous developmental context due to the inability of collecting embryos or the lack of molecular methods to introduce transgenes. The mouse and zebrafish are commonly used to characterize the functions of CREs (Zerucha et al. 2000; Ghanem et al. 2003; Jarinova et

al. 2008). These tests are based on the assumption that endogenous TFs will bind to the sites found within these regulatory regions in the same fashion as on their endogenous targets. Generally, CNEs are tested for "enhancer" activity, in which they are placed in an artificial context with a minimal promoter and reporter gene, such as enhanced green fluorescent protein (EGFP) or β-galactosidase (*lacZ*). These reporter constructs are injected into the embryo that will be screened for tissue-specific up-regulation of the reporter gene. Often the resulting reporter gene expression pattern will be compared to the endogenous expression of the gene that the CRE is associated with. These injected embryos can be used to establish transgenic lines, in which the transgene will be integrated into the genome of all cells in the embryo. This generally leads to a more consistent and accurate assay of function. However, generating transgenic animals can be laborious and time consuming, which has led some researchers to explore transient expression, where the primary injected embryo is analyzed (Müller et al. 1997; Müller et al. 1999; Woolfe et al. 2005; McEwen et al. 2009). This method is particularly suitable to zebrafish embryos as they are easily obtained, microinjected, and their transparent developmental stages allow for more than one stage of development to be studied.

A second approach to assay function was carried out by Ahituv et al. (2007) who deleted four CREs from the genome of the mouse. These four CREs were selected due to previous data indicating the genes in which these CREs potentially function have strong phenotypes when mutated. In addition, they are all 100% conserved (or ultraconserved) in over 200bp in humans, mice, and rats (Bejerano et al. 2004). Surprisingly, mice carrying these deletions were viable, phenotypically normal, and mostly exhibited unaltered gene expression. This study indicates that although these sequences are highly

conserved and their enhancer activity is observed in transgenesis assays, their loss does not necessarily have an effect on viability (Ahituv et al. 2007).

1.1.4. Evolution of *cis*-regulatory elements

Assaying the function of CREs from different species is a way of identifying distinct differences in the genetic cascades that will control the development of the embryo. Comparisons of distantly related vertebrate genomes could uncover potential CREs fundamental to vertebrate development and life. A group of 25 CNEs from the pufferfish genome were tested in transgenic zebrafish assays, and 23 of these showed enhancer activity in one or more tissues (Woolfe et al. 2005). A large number of CNEs from human-pufferfish comparisons resulted in uncovering functional human enhancers when tested in transgenic mice (Pennacchio et al. 2006). Sequence conservation between orthologous CREs often correlate with similar functions in homologous tissues (Uemura et al. 2005; Jarinova et al. 2008). However, although enhancer function is retained, discrepancies between the domains of activity of mouse and zebrafish CNEs have been reported for the *Shh* locus (Jeong and Epstein, 2003; Ertzer et al. 2007). These studies indicate that the TFs in orthologous genetic cascades may be similar in distantly related vertebrates, and may be able to activate orthologous CREs.

Understanding when the functional CREs appeared in the genome will allow for a better understanding of how GRNs evolved during vertebrate evolution. Identification of a number of duplicated CNEs in vertebrates indicates that these elements were present in the genome prior to the two whole genome duplication events that happened approximately 600 million years ago, before vertebrate diversification (Vavouri et al.

2006). Large scale comparison of the conserved non-coding regions between the elephant shark and the human and/or zebrafish genomes has shown that more CNEs are shared between human and elephant shark than between human and zebrafish (Venkatesh et al. 2006; Venkatesh et al. 2007). These data are consistent with the observation of greater conservation in gene synteny between human and elephant shark genomes than between human and zebrafish genomes (Venkatesh et al. 2007). It may seem counterintuitive given that mammals share a more recent common ancestor with teleost fishes than with cartilaginous fishes. This apparent paradox likely results from the 'fish-specific' whole genome duplication event that occurred before the divergence of the teleosts, and led to loss or modification of CNEs and high level of genome re-organization in this group (Venkatesh et al. 2007; Ravi et al. 2009). A detailed analysis of the conservation of CNEs associated with the *Hox* clusters in the elephant shark, human and fugu yielded various hypotheses on a possible correlation between the level of sequence conservation of vertebrate CREs and their functional variation (Ravi et al. 2009). Analyses of these CNEs showed that they initially evolved at a rapid pace, but remained almost unchanged in the last 500 million years (McEwen et al. 2006). However, the potential functional divergence of the tetrapod, teleost, and cartilaginous fish CNEs has never been tested. Identifying CNEs in the lamprey, the most basal vertebrate group, provides insight into how vertebrate CNEs evolve and when they became fixed in the genome. From an analysis of 13 human loci for homology with the lamprey genome, ~70% of known CNEs in these regions were identified (McEwen et al. 2009). While the total length of the sequence matches is much lower than for other gnathostomes, and overall sequence conservation is lower than for fish and mouse comparisons, some CREs taken from the

human and lamprey genomes were able to drive similar expression in zebrafish embryos (McEwen et al. 2009). Identifying the role and function of lamprey CREs will assist in depicting the vertebrate wide conserved regulatory networks and thus building the genetic cascades from the most fundamental basis of vertebrate development.

1.1.5. Mechanisms of CRE evolution

CREs are modular in nature, made up of a number of TF binding sites within the overall conserved sequences (Small et al. 1992; Kurchhamer et al. 1996; Poitras et al. 2007) (for review see: Arnosti and Kulkarni, 2005). Tissue specific expression is a result of a number of TFs coming together in the correct combination to undertake the correct regulatory function (Panne et al. 2008). Addition or loss of these binding sites can lead to altered regulatory function and expression of the target gene (Gompel et al. 2005; Jeong et al. 2006a). As an example, five highly conserved CREs active in the notochord of the zebrafish share two similar motifs that are necessary for notochord expression. Both motifs are necessary for proper function of the CRE in the notochord; however, their position, distance, and orientation within the CREs are variable (Rastegar et al. 2008). This study suggests the arrangement of the TF binding sites is not crucial in the CREs being conserved throughout evolution (Rastegar et al. 2008). High levels of conservation do not always infer function (Pennacchio et al. 2006; Elgar and Vavouri, 2008); conversely, the lack of conservation does not eliminate the possibility of function. Teleost specific CNEs for the zebrafish *ret* locus and mammal specific CNEs in the human RET locus both directed *ret*-specific expression in transgenic zebrafish despite having no sequence conservation observed between the two CNE groups (Fisher et al. 2006a). Both

conserved and non-conserved regions around the zebrafish *phox2b* gene had regulatory function (McGaughey et al. 2008). These studies indicate that many regulatory sequences may not be detected based on sequence comparisons possibly due to TF binding site shuffling within the CRE to the point where homology is no longer identifiable.

The evolution of the CRE sequences lead to altered expression of the target gene, or changes in the genetic cascade. They often occur in one of four different ways: 1) loss of TF binding sites in CREs by mutation (Jeong et al. 2006a); 2) mutations in existing CREs will allow for new TFs to bind, potentially leading to interactions within new genetic cascades or different expression patterns for genes (Stone and Wray, 2001; Gompel et al. 2005); 3) remodeling of CREs by changing the number, affinity, or organization of TF binding sites resulting in a change in expression pattern or overall quantitative expression of the affected gene (Zinzen et al. 2006); 4) transposable elements are integrated in the genome and function as regulatory elements. There are many transposable elements that have integrated next to developmental genes and can function as CREs (Bejerano et al. 2006). These changes in CREs can alter the way in which GRNs regulate developmental genes and therefore alter their spatial-temporal expression and the genes that they can interact with. Due to these changes, there can be new evolutionary alterations, such as modification of body/organ patterning, which may be the driving force behind the vast morphological differences between vertebrates, and potentially invertebrates (for review see: Carroll, 2008).

1.2. Overview of the central nervous system

The vertebrate nervous system is comprised of billions of cells, with thousands of different cell types, making this arguably the most complex organ system in the embryo. There are two major cell types: neurons, which can send and receive signals as electrochemical impulses, and glial cells that are involved in the support and protection of neurons within the nervous system (Purves, 2001). The central nervous system (CNS) contains the brain and spinal cord, whereas the peripheral nervous system (PNS) relays information to and from the CNS. The development of the nervous system is an incredibly dynamic and complex process, in which we are still uncovering the exact molecular cues that go into making a functional nervous system. One such question is: how are these many different types of neurons specified, giving each their own specific function within the nervous system? Studies of the molecular mechanisms behind neuronal specification have shown that there is no one individual cue, but rather many involved in a combinatorial cascade (for reviews see: Bertrand et al. 2002; Allan and Thor, 2003; Guillemot, 2007).

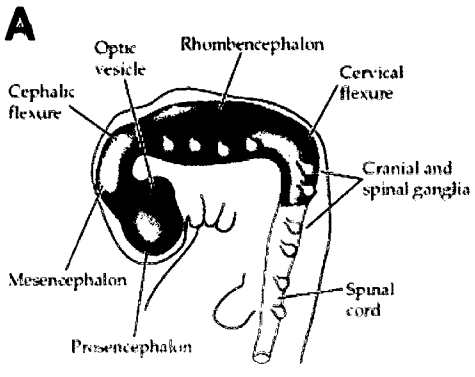
1.2.1. Organization of the embryonic forebrain

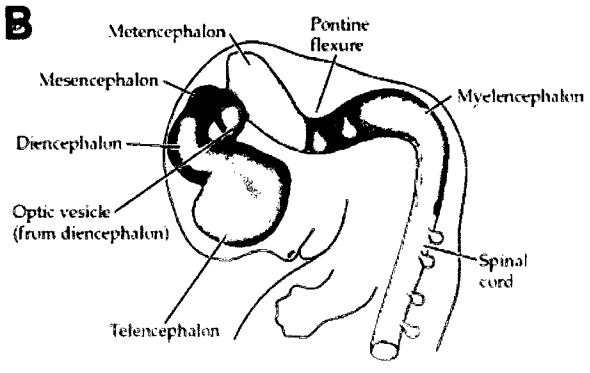
During gastrulation, cellular movements bring groups of cells together, allowing them to regionalize through interactions in a process called induction. The chordamesoderm cells induce the overlying dorsal ectoderm to differentiate into the neural plate. This neural plate will roll into the neural tube, the primordium of the CNS by a process called neurulation (Gilbert, 2000). From the neural tube will later form the brain anteriorly and the spinal cord posteriorly, along with the neural pituitary, motor

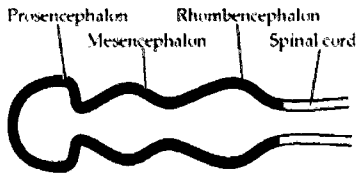
neurons and the retina in specific areas along the anterior-posterior axis (for reviews see: Smith and Schoenwolf, 1997; Rubenstein and Beachy, 1998). The embryonic brain will itself be regionalized into three subdivisions anterior to posterior: prosencephalon, mesencephalon and the rhombencephalon, respectively (Figure 1.4A). These subdivisions will further divide during development and lead to a total of five subdivisions. The prosencephalon will be divided into two domains, the telencephalon and diencephalon, and the rhombencephalon into the metencephalon and the mylencephalon (Figure 1.4B). Each of these embryonic structures will further develop into adult structures with distinct cellular composition, physiological characteristics, and function (Gilbert, 2000)(for review see: Wilson and Edlund, 2001; Wilson and Houart, 2004). I will focus on the telencephalon and diencephalon of the zebrafish, as they are the structures relevant to my research.

The vertebrate forebrain, composed of the telencephalon and diencephalon, is derived from the anterior neuroectoderm. Morphological boundaries within the developing forebrain often coincide with the boundaries of expression of specific molecular markers at different developmental stages (Puelles, 2001; Kiecker and Lumsden, 2005). The telencephalon is divided into two compartments, the pallium and subpallium, each distinguished by expression of genes involved in neurogenesis (e.g. *neurogenin1* in the pallium; *zash1a* in the subpallium) (Figure 1.5) (Wullimann and Mueller, 2004: Osario et al. 2010). In addition, genes playing a role in the differentiation of glutamatergic (e.g. *tbr1* in the pallium) or GABAergic (e.g. *dlx* genes in the subpallium) neurons can identify the boundary between the pallium and subpallium (Mueller et al. 2008). The pallium is the dorsal most division of the telencephalon that

Figure 1.4. The organization and development of the mammalian brain. A) Schematic of the neural tube during early developmental stages divided into the three primary vesicles: the prosencephalon, the mesencephalon, and the rhombencephalon. B) These primary vesicles will divide further as development progresses to form the five secondary vesicles: the prosencephalon will be divided into two domains, the telencephalon and diencephalon, and the rhombencephalon into the metencephalon and the myelencephalon. These vesicles are best viewed as longitudinal sections located directly below each view. Adapted from Purves et al. 2001.


A
Optic vesicle
Rhombencephalon
Cervical flexure
Cephalic flexure
Cranial and spinal ganglia
Mesencephalon
Spinal cord
Prosencephalon


B
Metencephalon
Pontine flexure
Mesencephalon
Myelencephalon
Diencephalon
Optic vesicle (from diencephalon)
Spinal cord
Telencephalon


Prosencephalon
Rhombencephalon
Mesencephalon
Spinal cord

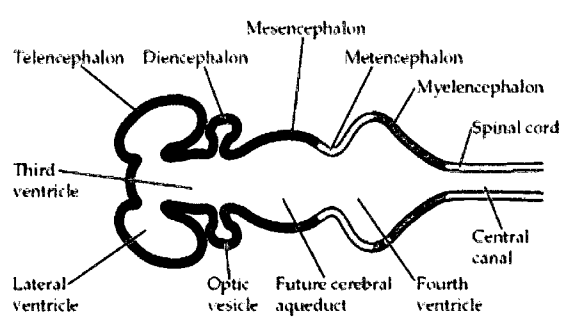
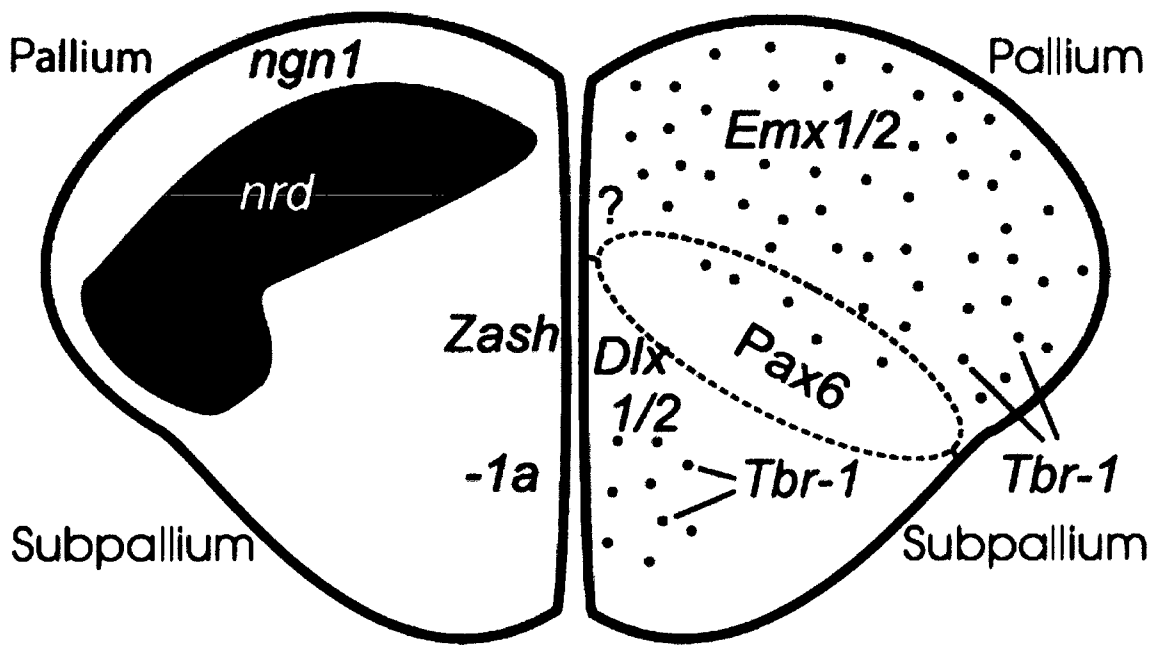

Mesencephalon
Telencephalon
Diencephalon
Metencephalon
Myelencephalon
Spinal cord
Third ventricle
Central canal
Lateral ventricle
Optic vesicle
Future cerebral aqueduct
Fourth ventricle

Figure 1.5. A cross section of the zebrafish telencephalon at 3 days post fertilization (dpf). The telencephalon has two domains: the ventral domain named the subpallium, and the dorsal most pallium. Each domain expresses a unique set of regulatory genes, such as subpallium expression of *Zash1a* or *Dlx1/2* or pallium expression of *Tbr1* or *neurogenin1* (*ngn1*). There are other genes that serve to define these domains such as, *Emx1/2*, *Pax6*, and *neurod* (*nrd*). Adapted from Wullimann and Mueller, 2004.

Early Teleostean Telencephalon

3dpf



bHLH Genes **Regulatory Genes**

will give rise to the neocortex in mammals, and is thought to function as the center for memory, learning, language, and consciousness (Striedter, 2005). The subpallium is a source of interneurons that will follow a discrete migratory route to the pallium (Anderson et al. 1997b; Marin et al. 2000; Mueller et al. 2008) (see section: 1.2.5.).

The diencephalon has been shown to possess a segmental organization that can be defined based on anatomical, molecular, and functional data (Figdor and Stern, 1993; Puelles and Rubenstein, 2003). The diencephalon contains the prethalamus (or ventral thalamus), thalamus (or dorsal thalamus), epithalamus, pretectum, and hypothalamus (Puelles and Rubenstein, 2003). The prethalamus and thalamus are separated by the zona limitans intrathalamica (ZLI), an embryonic signaling center (Scholpp et al. 2006; Scholpp et al. 2007). Distinct zones of the diencephalon can also be distinguished by expression of particular genes: the ZLI is marked by the expression of *shh*; ventral thalamus expresses *dlx1a, dlx2a* or *lhx5* (Scholpp et al. 2006; Akimenko et al. 1994).

1.2.2. Regionalization of the forebrain

Specification of the brain boundaries along the anterior-posterior (A/P) and dorsal-ventral (D/V) axis is due to the combined activities of many signaling molecules and transcription factors (for reviews see: Wilson and Houart, 2004; Rhinn et al. 2006). Once induced to a neural fate, the anterior-most part of the neural tissue acquires rudimentary segmentation by avoiding caudalizing factors, allowing it to maintain anterior properties. Caudalizing factors such as: Fibroblast growth factors (FGFs), Wnts, Hedgehog (Hh), and Bone morphogenic proteins (BMPs) are sequestered away from the

anterior neural tissue by localized expression of themselves or agonists (Kudoh et al. 2002) (for reviews: Fuccillo et al. 2006; Bertrand and Dahmane, 2006; Schier, 2001; Bally-Cuif and Hammerschmidt, 2003). The regions protected from caudalizing factors will become the prospective forebrain (for review see: Wilson and Houart, 2004). As a result of the anterior neural plate being regionalized, signaling centers or "organizers" will control gene expression and lead to the establishment of the distinct forebrain domains. I will focus on the literature describing the regionalization of the zebrafish forebrain, and supplement this with mouse or other vertebrate data where appropriate.

1.2.2.1. Regionalization of the telencephalon

With regards to regionalization of the forebrain, the development of the telencephalon is the most studied and therefore best understood (for reviews see: Wilson and Rubenstein, 2000; Wilson and Houart, 2004; Rhinn et al. 2006). The regionalization of the telencephalon requires a number of transcription factors and signaling molecules that are refined by "organizers". An organizer is an area that will play a role in the patterning of the embryo by providing TFs and signaling molecules to the surrounding tissues (Wilson and Houart, 2004). One such organizer is the anterior border of the neural plate (ANB) that promotes the expression of genes specific to the telencephalon (Rubenstein et al. 1998; Wilson and Houart, 2004). The proper regionalization of the forebrain requires suppression of Wnt signaling by the secretion of Wnt antagonists. A secreted frizzled related protein (sFRP), Tlc, can restore proper telencephalic gene expression in embryos lacking the ANB (Houart et al. 2002). The sFRPs are thought to antagonize Wnt activity, and they share the ability to induce telencephalic gene expression with other Wnt agonists (Uren et al. 2000; Houart et al. 2002). Consistent with

this, zebrafish mutants for Wnt repressors, *Tcf3* or *Axin1*, lack a telencephalon (Kim et al. 2000; Heisenberg et al. 2001). The *Axin1* mutant, *masterblind* (*mbl*), also lacks eyes and has an expansion of diencephalic gene expression (Masai et al. 1997). A transcriptional activator of Wnts is β-catenin, and its expression is found in a gradient low rostrally to high caudally, with high Wnt activity leading to the expression of diencephalic markers and reduced telencephalic markers (Kiecker and Niehrs, 2001; Nordstrom et al. 2002; Braun et al. 2003). Knockdown of *wnt8b* leads to a slight increase in telencephalon size and can induce telencephalic fate in the anterior neural ectoderm in $mbl^{-/-}$ mutants. Therefore, within the prospective forebrain, the telencephalon is specified in areas of low Wnt activity, whereas the diencephalic fates are promoted by increased Wnt activity (Wilson and Houart, 2004).

The ANB also expresses *Fgf3* and *Fgf8*, signaling molecules induced by Wnt agonists. Fgfs play a prominent role in patterning of the telencephalon, as reduced Fgf activity results in midline defects and changes in proliferation and apoptosis (Shanmugalingam et al. 2000; Walshe and Mason, 2003; Storm et al. 2003). In the telencephalon of mice, Fgfs may repress *Emx2* expression and play a role in D/V patterning of the cortex (Crossley et al. 2001; Garel et al. 2003; Storm et al. 2003). Knockdown of *emx3* expression in zebrafish leads to reduced expression of markers specific to the dorsal telencephalon; however, knockdown of *emx1* or *emx2* had little effect on the development of the telencephalon (Viktorin et al. 2009). A number of basic-helix-loop-helix (bHLH) transcription factors (*e.g. Mash1* and *Neurogenin1/2* (*Ngn1/Ngn2)*) specify D/V identity of the mouse (and possibly the zebrafish) telencephalon. These transcription factors have opposite effects as *Mash1* is expressed in

the ventral telencephalon and will specify ventral progenitors, while *Ngn1/Ngn2* are expressed dorsally and will specify dorsal progenitors (Ma et al. 1997; Casarosa et al. 1999; Parras et al. 2002; Fode et al. 2000; Nieto et al. 2001; Wullimann and Mueller, 2002; Osorio et al. 2010). In mice, defects in Shh signaling result in the loss of ventral telencephalic structures and markers, such as *Nkx2.1* and *Dlx2* (Chiang et al. 1996). Ectopic Bmp expression represses ventral telencephalic markers and imparts the maintenance of dorsal markers, leading to decreased proliferation and increased apoptosis (Furuta et al. 1997; Golden et al. 1999). Alternatively, ectopic expression of *Shh* can induce a ventral phenotype within the telencephalon and repress the expression of dorsal markers in mice and zebrafish (e.g. *Emx1* and *Tbr1*) (Kohtz et al. 1998; Gaiano et al. 1999; Corbin et al. 2000).

1.2.2.2. Regionalization of the diencephalon

The regionalization of the diencephalon is not well understood in comparison to the telencephalon. However, there is increasing interest in elucidating the mechanisms behind the development of the distinct subdivisions in the diencephalon. The ZLI is an important signalling center, which will separate the rostral (telencephalon) from the caudal (diencephalon) in the forebrain (Braun et al. 2003; Kiecker and Lumsden, 2004). In the zebrafish, expression of *otx1l/2* and *irx1b* genes is necessary to the formation of the ZLI, and leads to mis-specification of the pretectum and ventral thalamus (Schlopp et al. 2007). The ZLI expresses *Hh* genes, which are upstream of *fgfs*, and are involved in the growth and development of the thalamus and prethalamus in zebrafish and mice (Ishibashi and McMahon, 2002; Hashimoto-Torii et al. 2003; Schlopp et al. 2006). Knockdown of *fgf18* leads to defects in the ventral telencephalon and diencephalon, and

may play a role in the cross talk between Fgf and Hh signaling (Miyake et al. 2005). The transcription factor Foxg1 is also downstream of Shh and induces ventral identity within the telencephalon and inhibits Wnt signaling through direct transcriptional repression of Wnt ligands. The Wnt/ β-catenin signaling in the pallium is also *Shh* independent, thus *foxg1* controls the boundary between the telencephalon and basal diencephalon (Danesin et al. 2009). The Zic2a factor is downstream of Shh signaling as well, and plays a role in the modulation of Hh signaling in the diencephalon by regulating downstream genes (Sanek and Grinblat, 2008; Sanek et al. 2009).

The Wnt signaling pathway has also been linked to the development of the diencephalon. In the mouse, targeted loss of Six3 function in the prospective forebrain leads to increased Wnt activity and increased diencephalic specification (Lagutin et al. 2003). Reduced *wnt* expression in the diencephalon by knockdown of *fez1* results in loss of the ventral thalamus and an expansion of the ZLI (Jeong et al. 2007). Secondly, overexpression of *fez1* can restore the expression of telencephalon markers in *wnt* repressor mutants, showing *fez1* is important for the regionalization of not only the diencephalon but potentially the telencephalon and hypothalamus as well (Jeong et al. 2007). Wnt8b is an upstream activator of *lef1* expression and both are required for the development of the posterior hypothalamus (Lee et al. 2006). Knockdown of Wnt/β-catenin signaling promotes the hypothalamic fate in midline tissues instead of the floorplate, indicating this pathway plays a role in the specification of the hypothalamus (Kapsimali et al. 2004). Similarly, loss of *Msx1* in mice downregulates *Wnt1* expression and is required for midline and dorsal diencephalon patterning (Bach et al. 2003). Wnt/β–catenin signaling also regulates the Nodal pathway in the patterning of left-right

asymmetries in the zebrafish epithalamus (Carl et al. 2007). Zebrafish lacking Nodal have a telencephalon, however the hypothalamus is lost (Rohr et al. 2000). The knockdown of *zic1* causes loss of Nodal and Hh signaling in the ventral diencephalon and leads to cyclopia. There is also an increased expression of retinoic acid (RA) in the forebrain that leads to decreased expression of genes involved in dorsal BMP signaling (Maurus and Harris, 2009). In conclusion, there are a number of signaling molecules and transcription factors involved in the regionalization of the diencephalon, acting in parallel and potentially overlapping pathways.

1.2.3. Neural specification

In parallel to the patterning of the neural ectoderm into distinct domains, neurogenesis is initiated and subsets of neural ectoderm cells are specified to be neural progenitors. For example, neural progenitors are born from the pseudostratified epithelium in the ventricles of the forebrain (Rakic, 1972). These neural progenitors will undergo asymmetric divisions to renew themselves and by cell type commitment produce one of the primary neural cell types: neurons, astrocytes, or oligodendrocytes (for reviews see: Caviness and Takahashi, 1995; Guillemot, 2007). "Neural factors" are expressed in neural progenitors and regulate their commitment to one neural fate. For example, *Mash1* expression controls the commitment to the neural fate and is therefore called a proneural gene (Nieto et al. 2001; Parras et al. 2002), whereas *Olig2* expression specifies the oligodendritic fate of progenitors (Zhou and Anderson, 2002). These factors are involved in determining specific fates by regulating the expression of their target genes, thereby controlling sequential temporal expression of genes leading to terminal fate specification (Sharma et al. 1998; Baumgardt et al. 2007; Sugimori et al. 2007). Proneural genes also

control the arrest of progenitor divisions and the migration of newborn neurons from the progenitor zones, where they will terminally differentiate (Farah et al. 2000; Hand et al. 2005; Berninger et al. 2007). The final fate of these neural cells lies in the cumulative action of the spatial and temporal gene expression that control downstream genetic programs. During my thesis research, I have studied the *dlx* genes in zebrafish and how they are involved in the specific genetic cascade involved in the differentiation of neural progenitors to a GABAergic fate.

1.2.4. GABAergic interneurons

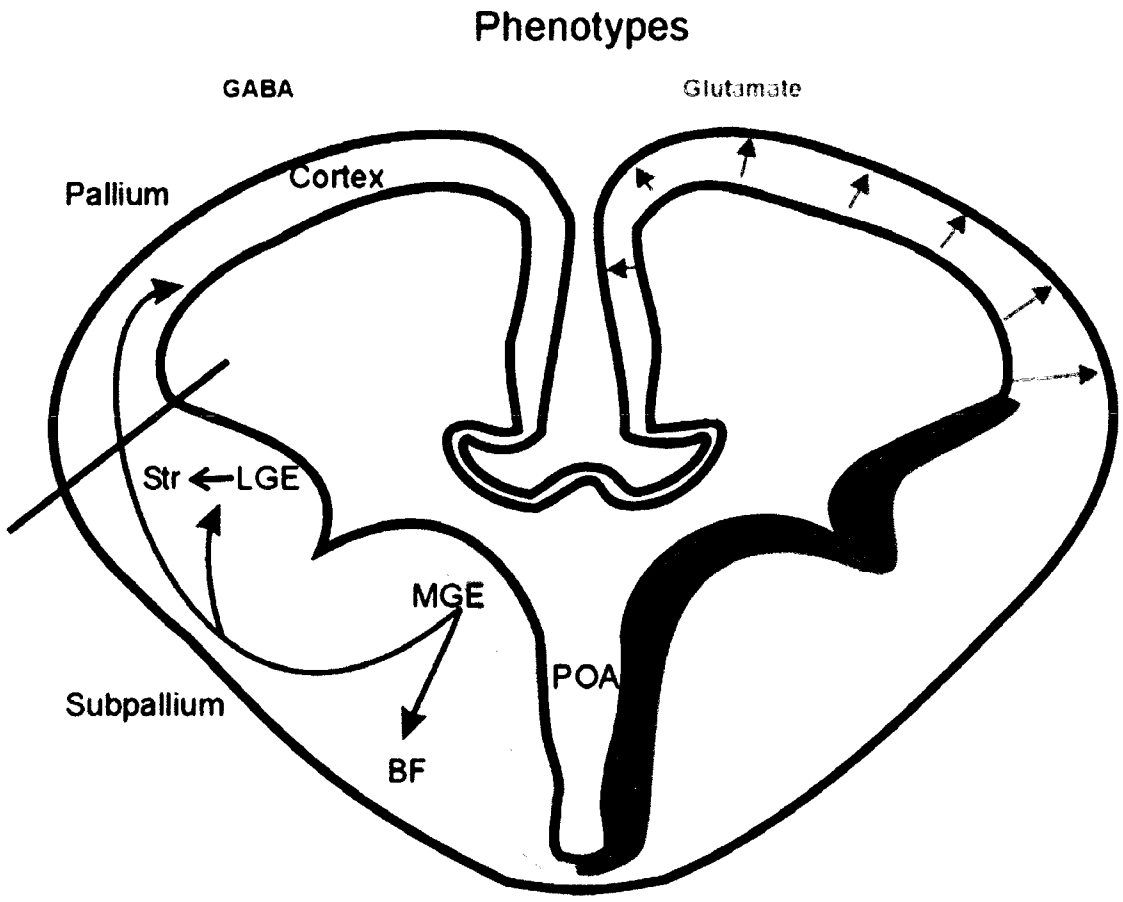
The neurotransmitter γ-aminobutyric acid (GABA) predominantly has an inhibitory function in the brain (Iversen et al. 1971; Houser et al. 1984). Interneurons or local circuit neurons expressing GABA are most commonly found in the cerebral cortex and will inhibit the function of pyramidal (excitatory) cells (for review see: DeFelipe and Farinas, 1992). The enzyme(s) responsible for the production of GABA in GABAergic interneurons are encded by the *glutamic acid decarboxylase* genes (*gad1* or *gad67* and *gad2* or *gad65*), which vary in cellular localization and function (Erlander et al. 1991; Bu et al. 1992; Feldblum et al. 1993; Esclapez et al. 1994). The *Gad* genes also appear to be regulated by different mechanisms as suggested by differences in their regulatory regions (Szabo et al. 1996; Pinal et al. 1997; Yanagawa et al. 1997). In the zebrafish there are at least two *gad* genes, and possibly a third *gad* gene resulting from the duplication of the *gad1* gene through the teleost genome duplication, although its expression and characterization has yet to be determined (Martin et al. 1998; Bosma et al. 1999). Expression of the *gad1/2* genes is found in the zebrafish telencephalon, diencephalon,

and spinal cord, all areas positive for GABA (Martin et al. 1998). Different types of GABAergic interneurons can be classified by morphology, anatomy, electrophysiology, and molecular properties (for review see: Markram et al. 2004). GABAergic interneurons found in the cortex are primarily born within the ventral proliferative zones of the telencephalon; however, they must follow a distinct migratory route to make their proper connections in the cortex (Anderson et al. 1997b; Lavdas et al. 1999; Wichterle et al. 1999; Pleasure et al. 2000; Wichterle et al. 2001; Stühmer et al. 2002b).

1.2.5. Neuronal migration in the forebrain

The correct position of neurons, by way of directed migration, is crucial for proper brain function. There are two major modes of migration in the CNS: radial migration, in which cells migrate from the progenitor zone towards the surface of the brain following the radial orientation of the neural tube, and tangential migration, in which cells migrate orthogonally to the direction of radial migration (Figure 1.6)(for reviews see: Marin and Rubenstein, 2003; Ayala et al. 2007). Radial migration is considered the most significant mode of neuronal migration in the telencephalon, as 80%-90% of cortical neurons will migrate from the proliferative zone in the dorsal telencephalon to the cortex (Ayala et al. 2007). These neurons will migrate along the radial glial fibers to reach their final destination in the cortex, where they differentiate into glutamatergic (excitatory) neurons (Edmondson and Hatten, 1987; Noctor et al. 2001) (for review see: Kriegstein and Noctor, 2004). Radial migration is principally seen in highly stratified structures in the CNS such as the spinal cord, cerebellum, and thalamus (for review see: Marin and Rubenstein, 2003).

Figure 1.6. A cross section of the mammalian telencephalon depicting the two major modes of neuronal migration. On the left, γ-amino butyric acid (GABA) expressing interneurons (inhibitory) are born in the medial ganglionic eminence (MGE) and will migrate tangentially (red arrows) to the cortex. On the right, the glutaminergic projection neurons (excitatory) are migrating radially (blue arrows) in the cortex. LGE, lateral ganglionic eminence; POA, pre-optic area; BF, basal forebrain; str, striatum. The black bar separates the pallial and subpallial boundary. Adapted from Wullimann, 2009.


Phenotypes
GABA
Glutamate
Pallium
Cortex
Str ←LGE
MGE
Subpallium
BF
POA

Although radial migration has been identified as the major migratory mode, there is a second population of neurons migrating tangentially (for review see: Marin and Rubenstein, 2001a; Marin and Rubenstein, 2003). This population of cells is largely composed of GABAergic interneurons that originate in the subpallial domain of the telencephalon and will comprise 20% of neurons in the cortex (Anderson et al. 1997b; Lavdas et al. 1999; Wichterle et al. 2001; Stühmer et al. 2002b). These migrating cells originate from multiple sites within the subpallium, including the ganglionic eminences, and move toward the neocortex and hippocampus (de Carlos et al. 1996; Anderson et al. 1997b; Tamamaki et al. 2001; Wichertle et al. 1999; Anderson et al. 2002; Nery et al. 2002). The preoptic area, found in the diencephalon, has been shown to be an additional source of migrating GABAergic interneurons to the cortex (Gelman et al. 2009). Migration of GABAergic interneurons from the subpallium to the pallium has been observed in mammals, such as rodents and humans, as well as birds and teleost fish indicating these migrational events may be evolutionarily conserved among vertebrates (Anderson et al. 1997b; Tuorto et al. 2003; Metin et al. 2007; Mione et al. 2008; Rodriguez-Moldes, 2009).

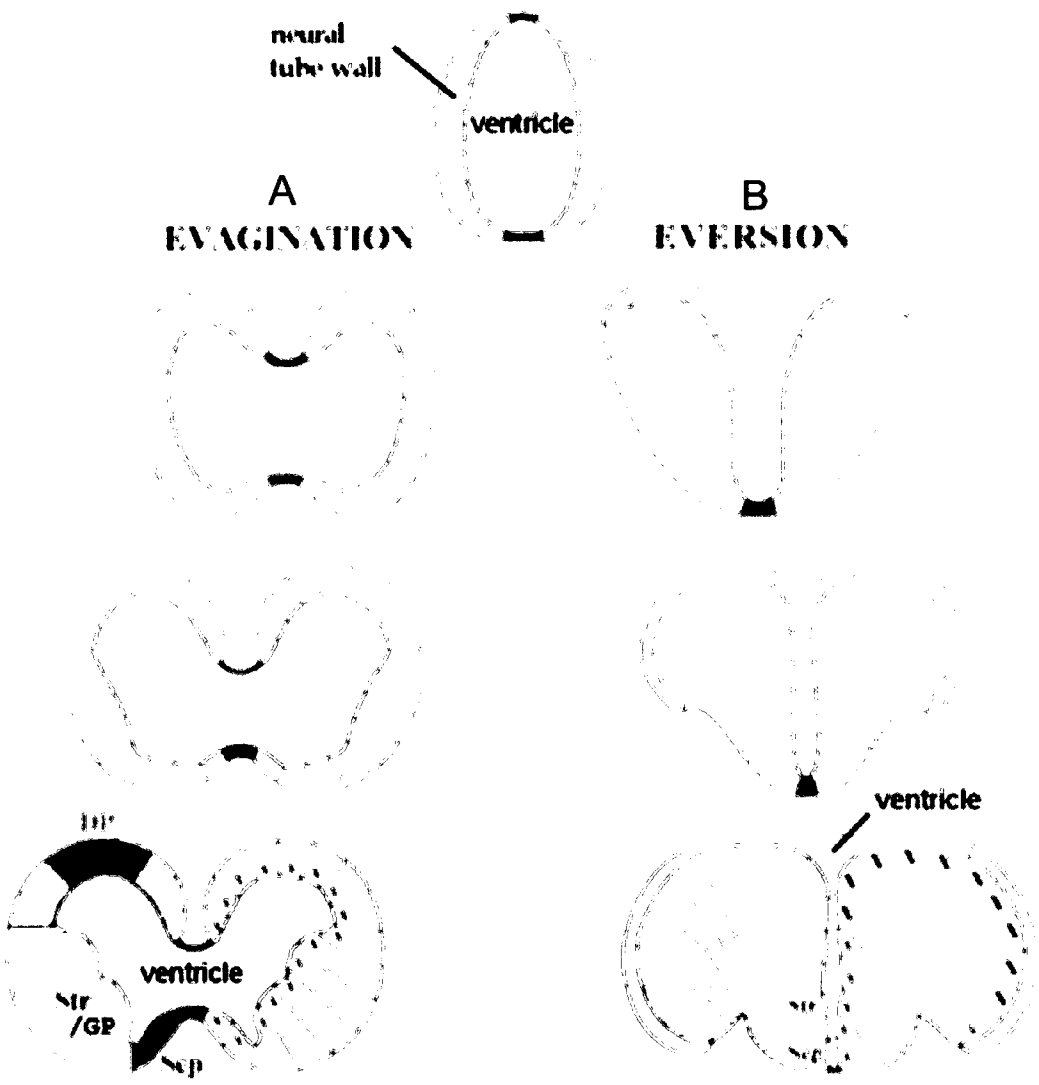
Tangential migration may not be limited to the telencephalon, as there is evidence of both radial and tangential migration in the developing thalamus (Golden et al. 1997; Redies et al. 2000; Ortino et al. 2003). These neurons originate in the reticular nucleus, a part of the ventral thalamus, and will migrate tangentially to the dorsal thalamus (Ortino et al. 2003). The cells migrating from the reticular nucleus, both radially and tangentially, express GABA while the tangentially migrating cells express calretinin and calbindin in

the dorsal thalamus (Ortino et al. 2003). The homeoprotein Otx2 may play a role in the migration of the GABA positive cells to the dorsal thalamus (Inverardi et al. 2007).

1.2.6. Zebrafish forebrain vs. Mammalian forebrain

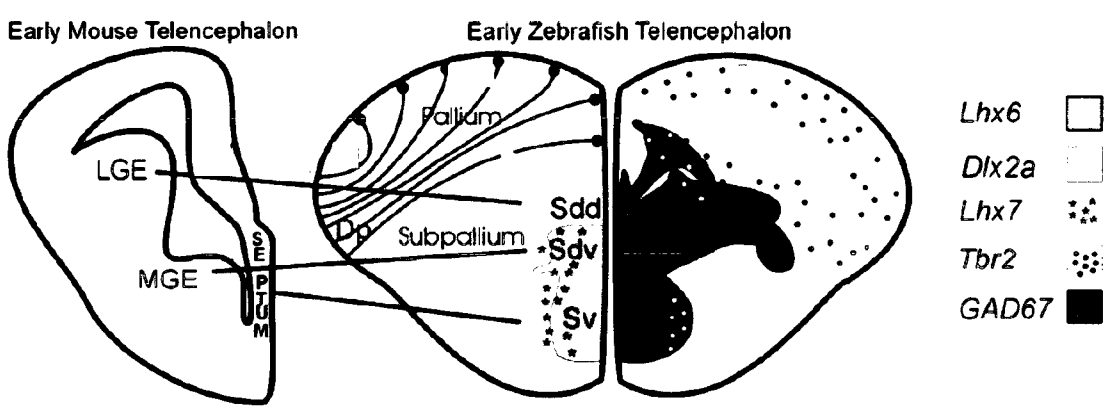
Although the first steps of neural tube development are similar between all vertebrates, the adult structures can vary greatly in morphology and possibly function (for reviews see: Rubenstein et al. 1998; Puelles and Rubenstein, 2001). Identifying homologies between distinct morphological structures found in distantly related vertebrates is challenging. However, by studying the expression of common developmental genes and molecular markers, homology between divergent structures in different vertebrate groups can be inferred. There have been attempts to compare embryonic forebrain development in teleost (e.g. zebrafish) and mammals (e.g. mouse) via proliferation and neurogenesis markers and gene expression data (Mueller et al. 2006; Mueller et al. 2008)(for reviews see: Wullimann and Puelles, 1999; Mueller and Wullimann, 2009). These comparisons are difficult as the modes of telencephalon morphogenesis differ between teleosts and all other vertebrates (Figure 1.7) (Nieuwenhuys and Meek, 1990). The general vertebrate mode of telencephalic development is evagination. That is, the telencephalon develops as two telencephalic hemispheres enclosing a central ventricle. On the contrary, in teleosts, eversion is characterized by the pallium rolling out laterally, leaving the ventricular surface on the outside of the pallium (Niewenhuys and Meek, 1990). Due to these differences Wullimann and Mueller have proposed the "Partial eversion hypothesis" to explain the

Figure 1.7. A schematic depicting the two different modes of telencephalic development from the neural tube. On the left (A), is evagination occurring in all vertebrates except ray-finned fishes. The telencephalon develops as two telencephalic hemispheres enclosing a central ventricle. On the right (B), the telencephalon of ray-finned fishes, a group that includes teleosts, will develop by eversion. Eversion is characterized by the pallium rolling out laterally, leaving the ventricular surface (proliferative) on the outside of the pallium (marked with an x). DP, dorsal pallium; GP, globus pallidus; LP, lateral pallium; MP, medial pallium; Sep, septum; Str, striatum; VP, ventral pallium. Adapted from Niewenhuys and Meek, 1990.


neural
tube wall
ventricle
A
EVAGINATION
B
EVERSION
ventricle
ventricle

different telencephalic topography between the teleosts and other vertebrates (for reviews see: Wullimann, 2009; Mueller and Wullimann, 2009). However, this model is not universally accepted, as there are several other hypotheses attempting to explain the organization of the teleost telencephalon (for review see: Braford, 2009). Despite this fundamental difference in telencephalon development between these two groups, comparative studies have uncovered a number of anatomical homologies between zebrafish and mice (e.g. basal ganglia and pallium). By using the expression of marker genes known to be associated with distinct regions of the mammalian forebrain (e.g. *ascl1a* and *dlx2a*), regional homologies were proposed between the zebrafish and mouse (Mueller et al. 2008) (Figure 1.8). These homologies are also found in the expression of neurotransmitters as well as in neuronal migration pathways (Wullimann and Rink, 2002; Mueller et al. 2006). For example, the expression of GABA in specific areas of the zebrafish forebrain, at two and three days post-fertilization, resembles the mouse forebrain at embryonic stage E12.5-13.5 (Katarova et al. 2000; Mueller et al. 2006). These data, along with data from other amniote species, lead to the proposal of a phylotypic stage of neurogenesis, which is similar in all vertebrates (Mueller et al. 2006). Although there are fundamental differences between teleost and mammalian telencephalic development, the molecular organization is highly conserved between these two groups, potentially due to conserved genetic mechanisms.

Figure 1.8. Comparison on the mouse and zebrafish telencephalon during early development. The proposed homologous areas in the telencephalon are partially based on gene expression data (depicted by lines between structures). The regulatory genes used in the comparison are shown as different patterns (legend on right). LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Sdd, dorsal subdivision of dorsal subpallium (striatum); Sdv, ventral subdivision of dorsal subpallium; Sv, ventral subpallium (septum). Adapted from Wullimann and Mueller, 2009.


Early Mouse Telencephalon
Early Zebrafish Telencephalon
LGE
MGE
SEPTUM
Pallium
Dp
Subpallium
Sdd
Sdv
Sv
Lhx6
Dlx2a
Lhx7
Tbr2
GAD67

1.3. The *Dlx* Genes

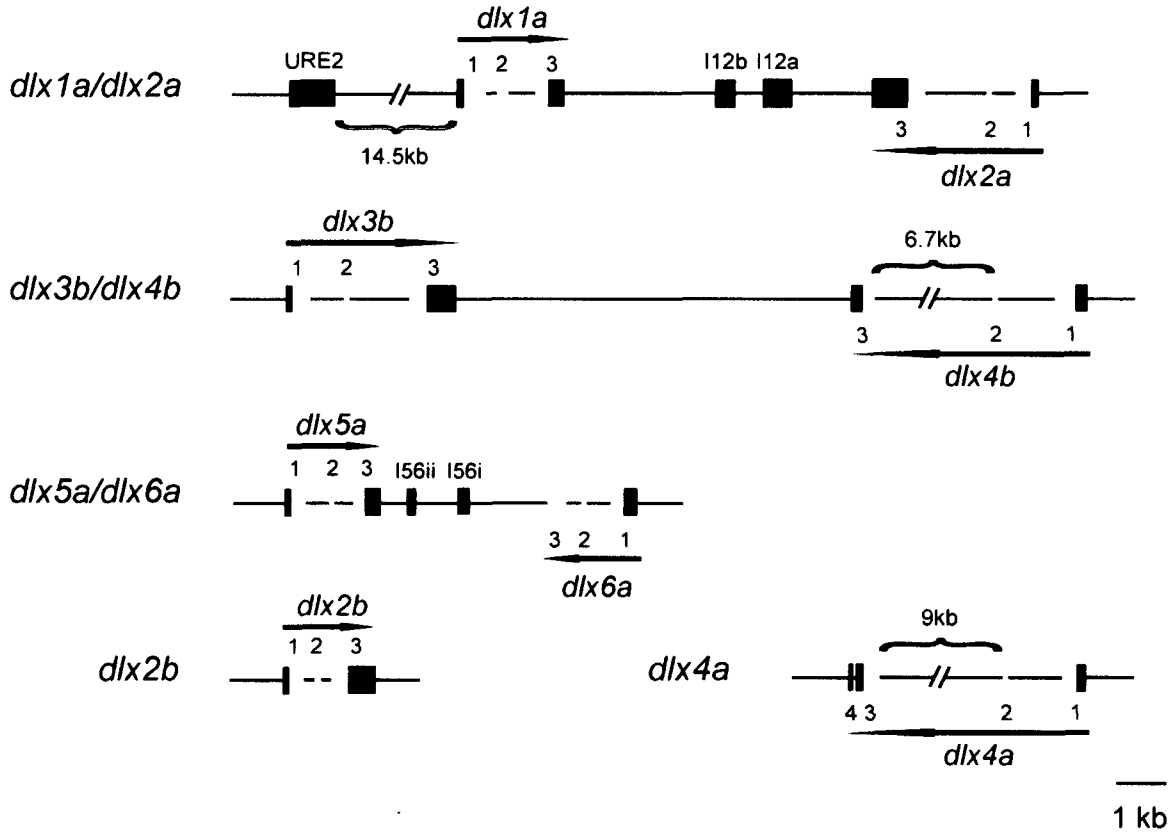
1.3.1. *Drosophila Distal-less* Gene

The *Distal-less (Dll)* gene encodes a homeodomain transcription factor required for proximodistal (PD) patterning during *Drosophila* limb development. As the name indicates, *Dll* is required for distal limb specification as null mutants die during larval stages from the lack of rudimentary larval limbs (Cohen et al. 1998; Cohen and Jurgens, 1989; Gorfinkiel et al. 1997; Wu and Cohen, 1999). The limbs are not the only tissue where a *Dll* gene functions, as it is also important for the PD development of the *Drosophila* antenna, which subsequently acts as the ears and nose of the fly (Cohen et al. 1989; Dong et al. 2000). Interestingly, the orthologous vertebrate *Dlx* genes have been shown to play a role in patterning of homologous tissues to the ears, nose, and mouth (mandible and maxilla) (Akimenko et al. 1994; Zhao et al. 1994; Yang et al. 1998; Acampora et al. 1999; Depew et al. 1999; Solomon and Fritz, 2002; Beverdam et al. 2002; Depew et al. 2002; Polley et al. 2006; Jeong et al. 2008; Esterberg and Fritz, 2009). While *Dll* is crucial for proper distal appendage development, it also plays a role in components of the PNS such as, larval antennal, maxillary, and labial sense organs (Cohen and Jurgens, 1989).

1.3.2. Genomic Organization

Contrary to the insects, vertebrates have several *distal-less* related (*Dlx*) genes. The *Dlx* genes of vertebrates encode homeobox transcription factors, each containing a highly conserved homeodomain consisting of 61 amino acids (Liu et al. 1997). The sequences found in the homeodomain and carboxyl terminus allow for the *Dlx* genes to

Figure 1.9. The organization of the zebrafish *dlx* genes. The *dlx1a*/*dlx2a*, *dlx3b*/*dlx4b*, and *dlx5a*/*dlx6a* genere are physically linked in a bigene arrangement. The *dlx2b* and *dlx4a* genes are found as a single gene. The *dlx1a*/*dlx2a* genes have a number of highly conserved regulatory elements: Upstream regulatory element 2 (URE2), I12b, and I12a, the latter two found in the shared intergenic region. The *dlx5a*/*dlx6a* have two intergenic regulatory elements named I56i and I56ii. Exons are numbered (black= untranslated region; white= coding). Regulatory sequences are in blue (*dlx1a*/*dlx2a*) and red (*dlx5a*/*dlx6a*).


dlx1a
dlx1a/dlx2a
URE2
1 2 3
I12b I12a
3 2 1
14.5kb
dlx2a
dlx3b
6.7kb
dlx3b/dlx4b
1 2 3
3 2 1
dlx4b
dlx5a
1 2 3 I56ii I56i
dlx5a/dlx6a
3 2 1
dlx6a
dlx2b
1 2 3
dlx2b
9kb
dlx4a
4 3 2 1
dlx4a
1 kb

be grouped into two clades: *Dlx1/4/6* and *Dlx2/3/5* (Stock et al. 1996). The functional significance of these two groups has yet to be determined. Six of the *Dlx* genes in vertebrates are organized in convergently transcribed bigene clusters, each consisting of one member from each clade: *Dlx1/Dlx2, Dlx3/Dlx4,* and *Dlx5/Dlx6* and each linked to a *Hox* cluster on separate chromosomes (McGuinness et al. 1996; Nakamura et al. 1996; Stock et al. 1996). Two additional *dlx* genes have been identified in zebrafish, named *dlx2b* and *dlx4a*, formerly *dlx5* and *dlx8* respectively (Figure 1.9) (Stock et al. 1996). In zebrafish, like the six mammalian orthologs, six of the eight *dlx* genes are found in three convergently transcribed pairs. However the additional genes, *dlx2b* and *dlx4a*, are not found in the bigene arrangement (Stock et al. 1996).

1.3.3. *Dlx* evolution

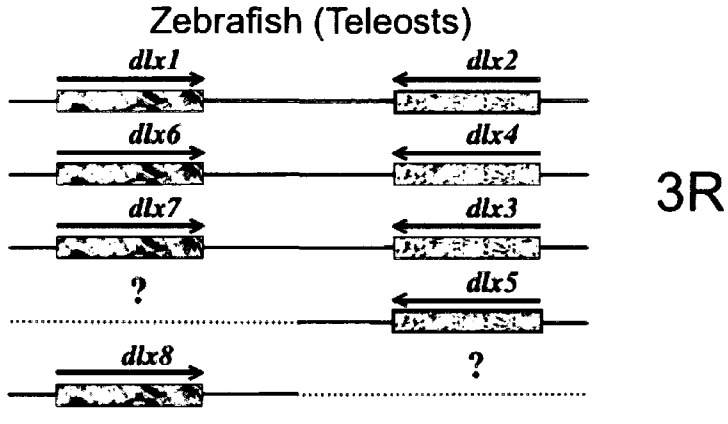
Invertebrates, such as *Drosophila*, have one *Dll* gene in their genome. The Amphioxus, a primitive chordate, possesses a single *Dll* gene while the ascidian *Ciona intestinalis* has three *Dll* genes in its genome (Holland et al. 1996; Caracciolo et al. 2000). The *C. intestinalis Dll* genes are found in a tail-to-tail bigene cluster and linked to *Hox* genes (Irvine et al. 2007). This suggests a scenario where the ancestral *Dll* gene from Amphioxus was linked to a *Hox* cluster and duplicated in tandem to form the bigene arrangement. The presence of the third *Dll* gene may have been a result of an additional tandem duplication event specific to this lineage, as this gene is not linked to another *Dll* or *Hox* gene (Irvine et al. 2007). The *Dlx* genes have also been studied in cyclostomes, more specifically lamprey. The lamprey has six *Dlx* genes, two of which are found in a bigene cluster (Neidert et al. 2001; Myojin et al. 2001; Kuraku et al. 2009). Mammals,

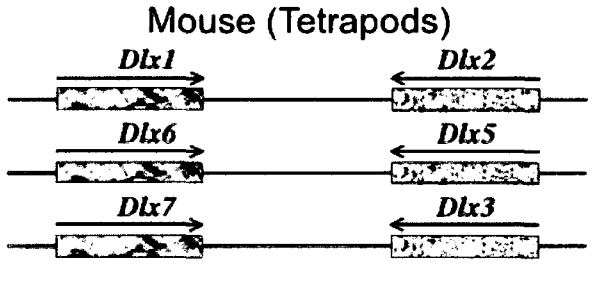
such as humans and mice, have six *Dlx* genes found in the bigene arrangement, as mentioned above (Price et al. 1991; Robinson et al. 1991; Robinson and Mahon, 1994; Simeone et al. 1994; Weiss et al. 1994; Scherer et al. 1995; Nakamura et al. 1995; Stock et al. 1996). The increased number of *Dlx* genes compared to primitive chordates and their conserved genomic organization leads to further expansion of the previous hypothesis, where the tandem duplication event was followed by two successive genome duplication events and a subsequent loss of one bigene pair, resulting in a total of six *Dlx* genes (Zerucha and Ekker, 2000; Sumiyama et al. 2003). The *Dlx* genes are termed *Dlx1-Dlx6*, with *Dll* being most closely related to *Dlx1* (Stock et al., 1996). In the teleost lineage, additional genome duplication may have occurred after the divergence of the lineage that would give rise to mammals. This event lead to additional *hox* clusters, and the two additional *dlx* genes named *dlx2b* and *dlx4a* in zebrafish (Figure 1.10) (Amores et al. 1998).

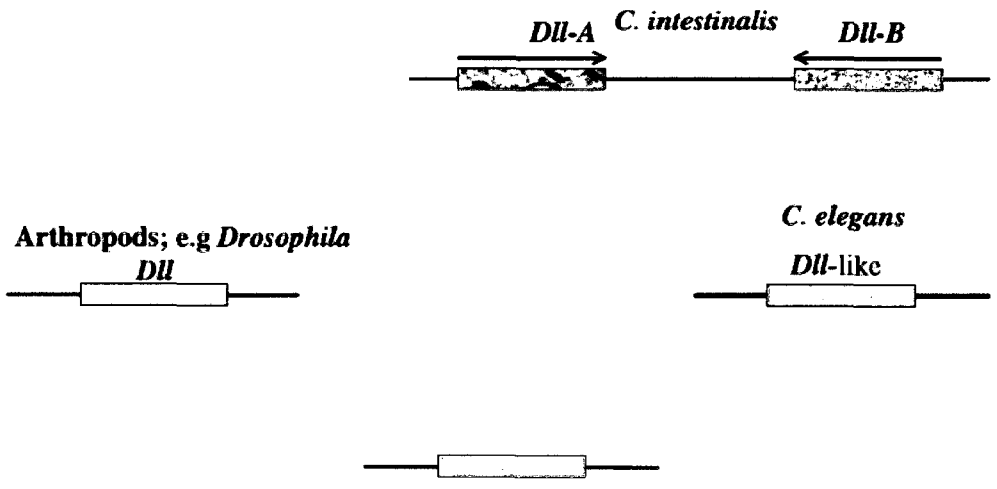
1.3.4. *Dlx* Gene Expression in Vertebrates

The *Dlx* genes are transcription factors important for the proper development and survival of the embryo. These genes begin to be expressed at mid-gestational stages in ectodermal derivatives, such as the neural tube and surface ectoderm, and continue into adulthood (Dollé et al. 1992; Bulfone et al. 1993; Akimenko et al. 1994; Liu et al. 1997; Yang et al. 1998; Eisenstat et al. 1999). The *Dlx* genes are expressed in a number of tissues and organs such as: the forebrain, a subset of migrating Canial Neural Crest (CNC) cells, in the branchial arches, limbs/fins, sensory organs, bone and cartilage. The expression of the *Dlx* genes within these tissues is complex; however, there are two

Figure 1. 10. A proposed model depicting the evolution of the *Dll/Dlx* genes. The invertebrates such as arthropods have a single *Dll* gene and the *Ciona intestinalis* has *Dlx* genes arranged in a bigene. Two whole rounds of genome duplication events (2R) results in the mouse having six *Dlx* genes, three convergently transcribed bigene pairs. At least one additional duplication event (3R) leads to two additional *dlx* genes in zebrafish, for a total of eight. However, the additional two genes are not found in the bigene arrangement. The arrow points to increasing evolutionary time from the basal hypothetical common ancestor. Old nomenclature-new nomenclature: *dlx4* is *dlx5a*, *dlx7* is *dlx4b*, *dlx5* is *dlx2b*, and *dlx8* is *dlx4a*. Adapted from Zerucha and Ekker, 2000.


Zebrafish (Teleosts)
dlx1
dlx2
dlx6
dlx4
dlx7
dlx3
?
dlx5
dlx8
?
3R


Mouse (Tetrapods)
Dlx1
Dlx2
Dlx6
Dlx5
Dlx7
Dlx3
2R


Dll-A
C. intestinalis
Dll-B
Arthropods; e.g Drosophila
Dll
C. elegans
Dll-like
Hypothetical common ancestor to nematode,
arthropod and vertebrate Distal-less genes

general characteristics that should be noted: 1) their expression is nested and follows a distinct sequential spatial-temporal expression pattern along the proximal distal (proximo-distal) axis of the embryo, specifically in the forebrain and branchial arches (Liu et al. 1997; Qui et al. 1997; Eisenstat et al. 1999) and 2) expression of genes found within the same bigene pair is highly overlapping with one another, but also with other members of the *Dlx* gene family (Ellies et al. 1997b; Quint et al. 2000). This high degree of overlapping expression between physically linked pairs is most probably due to their genomic organization, with a short intergenic region between each gene (<12kb) (Quint et al. 2000; Zerucha et al. 2000) leading to the possibility of shared regulatory regions. However, there are exceptions to this, as distinct differences in *Dlx* expression have been noted in the migrating CNC cells and forebrain (Bulfone et al. 1993; Akimenko et al. 1994; Liu et al. 1997).

1.3.4.1. Neural Crest Cells and Branchial Arches

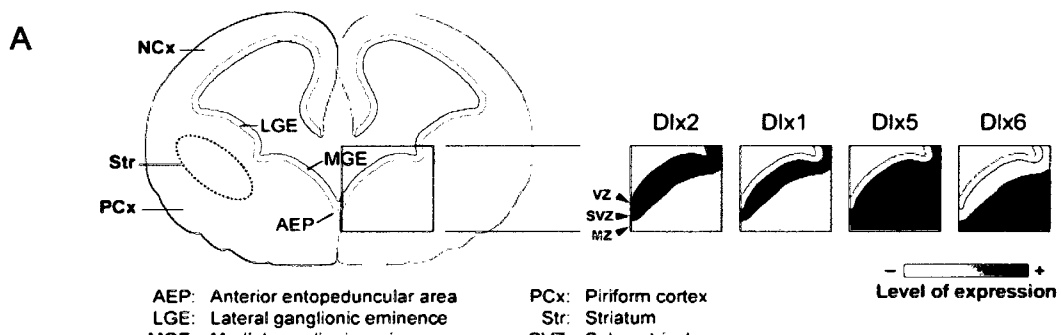
CNC cells are destined to form several tissues in the embryo such as bone, cartilage, cranial ganglia, enteric neurons, and muscles of the branchial arches (Noden, 1988; Langille, 1993). The *Dlx2* (*dlx2a*) genes are uniquely expressed in the CNC cells both in mouse and in zebrafish, as the expression of the physically linked *Dlx1* is not present (Bulfone et al. 1993; Akimenko et al. 1994; Robinson and Mahon, 1994). The branchial (pharyngeal) arches are a series of arches in the walls of the mouth cavity and pharynx that correspond to the gill arches (Graham et al., 2005). The first and second branchial arches go on to form the upper (maxilla) and lower (mandible) jaws, teeth, and the inner ear (Köntges and Lumsden, 1996). All of the zebrafish *dlx* genes are expressed in the branchial arches during development, except *dlx2b* (Akimenko et al. 1994; Ellies et

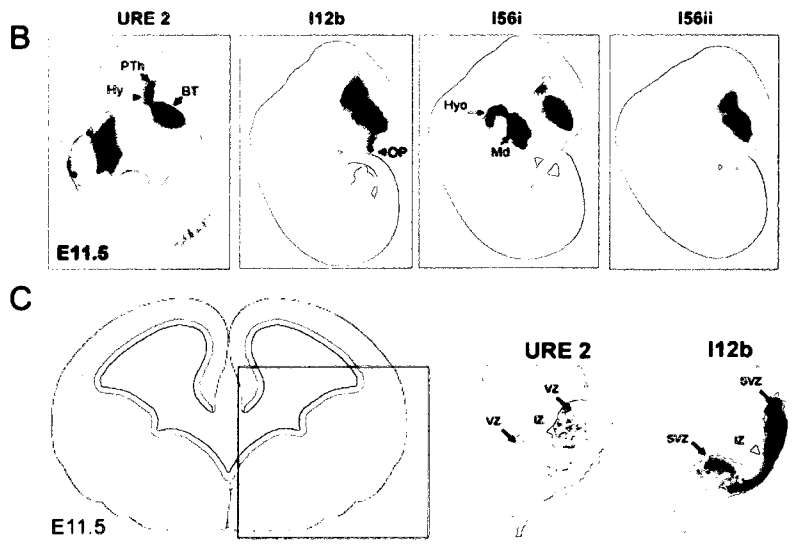
al. 1997b). This expression becomes restricted to the craniofacial cartilage before condensation (Ellies et al. 1997a). In the mouse, all six *Dlx* genes are expressed in the branchial arches in a complex and overlapping pattern (Qui et al. 1997; Depew et al. 2002). The *Dlx1* and *Dlx2* genes are expressed in the proximo-distal axis of the first branchial arch, *Dlx5* and *Dlx6* are expressed more distally only in the mandibular component of the first arch, and *Dlx3* and *Dlx4* are restricted to a narrow domain in the most distal position of the first arch. This nested expression domain is termed the "*Dlx* code" in branchial arch patterning and the functional significance of this is still being investigated (Depew et al. 2002). The overlapping expression patterns indicate redundant and distinct functions of the *Dlx* genes, confirmed by *Dlx* mutant experiments (see section: 1.3.5.).

1.3.4.2. Forebrain

Five of the eight *dlx* genes are expressed in the forebrain of the zebrafish: *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* (Akimenko et al. 1994; Ellies et al. 1997b). The four orthologous genes from mammals are expressed in the forebrain of the mouse: *Dlx1, Dlx2, Dlx5,* and *Dlx6* (Liu et al. 1997; Yang et al. 1998; Anderson et al. 1997a; Eisenstat et al. 1999). The expression of the *Dlx* genes is found within two domains of the forebrain: the telencephalon and diencephalon. These domains in the zebrafish (teleost) and mouse (mammalian) forebrains appear to be homologous to one another based on proliferation, gene expression, and neurotransmitter expression data (see review: Mueller and Wullimann, 2009). These expression domains are also found in: birds, turtles, frogs, and lamprey (Fernandez et al. 1998; Puelles et al. 2000; Myojin et al. 2001; Neidert et al. 2001). The *Dlx* genes are expressed in a spatial-temporal pattern, starting during the mid-

Figure 1.11. The expression of the *Dlx* genes is closely mimicked by the activity of the *Dlx* forebrain regulatory elements. A) A schematic of the expression of the *Dlx* genes in the mouse forebrain. The *Dlx* genes each have a distinct spatial expression domain within the telencephalon: *Dlx2* is highly expressed in the most immature cells in the ventricular zone (VZ), where *Dlx1* and *Dlx5* will follow in the sub-ventricular zone (SVZ), and the *Dlx6* gene is expressed in the most differentiated neurons of the mantle zone (MZ). B) The activity of the four *Dlx* regulatory elements, URE2, I12b, I56i, and I56ii, in transverse sections of the forebrain in the E11.5 mouse embryo. C) Transverse sections of the telencephalon indicate these specific regulatory elements will recapitulate the spatial differences of *Dlx* expression. Arrows show the boundary of regulatory element activity. Adapted from Ghanem et al. 2007 and Ghanem et al. 2008.


A
NCx
LGE
MGE
Str
PCx
AEP
Dlx2
Dlx1
Dlx5
Dlx6
VZ
SVZ
MZ
Level of expression
AEP: Anterior entopeduncular area
LGE: Lateral ganglionic eminence
MGE: Medial ganglionic eminence
NCx: Neocortex
PCx: Piriform cortex
Str: Striatum
SVZ: Subventricular zone
VZ: Ventricular zone


B
URE 2
I12b
I56i
I56ii
PTh
Hy
BT
OP
Hyo
Md
E11.5
BT: Basal telencephalon
Hy: Hypothalamus
Hyo: Hyoid arch
Md: Mandibular arch
OP: Olfactory placodes
PTh: Prethalamus

C
E11.5
URE 2
I12b
I56i
I56ii
VZ
IZ
SVZ
MZ

gestational stages of mouse development, beginning with *Dlx2* in the ventricular epithelium, followed by *Dlx1*, *Dlx5*, and then *Dlx6* in further differentiated neurons (Figure1.11A) (Liu et al. 1997: Eisenstat et al. 1999). This cascade of expression in the *Dlx* genes has been proposed to be evolutionarily conserved in zebrafish; however, a direct comparison between *dlx* paralogs was previously not available (Zerucha et al. 2000). I will show in Chapter 2 that the distinct spatial relationship between the *dlx* genes in the telencephalon fits the model that was previously suggested by Liu et al. (1997).

The expression of the *Dlx3* and *Dlx4* genes were thought to be absent from the CNS, more specifically the forebrain, of vertebrates (Akimenko et al. 1994; Robinson and Mahon, 1994; Pera and Kessel, 1999). However, a report has identified expression of *Dlx3* in chicken in the ventral forebrain in a similar pattern to *Dlx5* (Zhu and Bendall, 2006).

1.3.4.3. Limbs/Fins

All eight *dlx* genes are expressed in the median fin fold and apical ectodermal cells of the developing zebrafish fins (Akimenko et al. 1994). This expression may be homologous to the expression of all six *Dlx* genes in tetrapod mammals in the apical ectodermal ridge (AER) of the limb buds (Dollé et al. 1992; Morasso et al. 1995). Although there is no evolutionary relationship between insect appendages and mammalian limbs, this expression may be conserved from the *Dll* gene.

1.3.4.4. Other Tissues

The expression of the *dlx* genes begins with *dlx3b* and *dlx4b* during gastrulation in cells extending bilaterally on each side of the midline in zebrafish (Akimenko et al. 1994; Ellies et al. 1997b). This expression will later be restricted to the prospective

olfactory and otic placodes (Ekker et al. 1992; Akimenko et al. 1994; Ellies et al. 1997b; Solomon and Fritz, 2002). This expression will later be joined by *dlx5a* and *dlx6a* in the developing olfactory placodes and inner ear (Ekker et al. 1992; Akimenko et al. 1994; Ellies et al. 1997b). Expression of the *Dlx* genes in the above tissues is also found in the mouse (Robinson and Mahon, 1994; Qui et al. 1997; Yang et al. 1998; Acampora et al. 1999; Depew et al. 1999). The *Dlx* genes are also expressed in the genital eminence (Porteus et al. 1994), teeth (Thomas et al. 1997; Depew et al. 2002), zebrafish pharyngeal teeth (Borday-Birraux et al. 2006; Jackman et al. 2006), hematopoietic cells (Shimamoto et al. 1997), immune cells (Sunwoo et al. 2008), and retina (Dollé et al. 1992; Eisenstat et al. 1999, de Melo et al. 2003). Zebrafish *dlx* genes are expressed in the visceral skeleton but not the bones of the braincase (Verreidjt et al. 2006). *Dlx5* is expressed in all bones, including the base and roof of the skull, prior to osteoblast differentiation and disappears upon the formation of fully mature osteocytes in mice (Acampora et al. 1999).

1.3.5. *Dlx* function

The *Dlx* genes encode transcription factors involved in the regulation of target downstream genes. Expression, knockout, or knockdown experiments are critical in assaying the function of the *Dlx* genes. Genetic mutations have primarily been carried out in mice, as there is no simple method for targeted mutations in the zebrafish. The most prominent method to assay the functions of the *dlx* genes in zebrafish is the use of knockdown technologies, such as morpholino oligonucleotides.

1.3.5.1. Limb Development

Although the *Dlx* genes are widely expressed in the AER of the developing limb bud, there is no phenotype associated with a single *Dlx* mutant to date (Qui et al. 1995; Qui et al. 1997; Acampora et al. 1999; Depew et al. 1999). This may be due to the highly overlapping expression of the *Dlx* genes in this tissue and to functional redundancy. Thus, the *Dlx* gene family is highly conserved in the homeodomain and other protein regions and can possibly compensate for the loss of a single *Dlx* gene. This scenario is not unique to the limbs and has been noticed in other tissues such as the forebrain and branchial arches (see below). However there is, strong evidence that implicates the *Dlx* genes in limb development. Genetic analysis of families suffering from Split hand/foot malformations (SHFM) identified the DLX5 and DLX6 loci as candidate genes for the disease (Sherer et al. 1994). *Dlx5*$^{-/-}$/*Dlx6*$^{-/-}$ mice show bilateral ectrodactyly, a phenotype consistent with the phenotype of SHFM (Robledo et al. 2002; Merlo et al. 2002). Conversely, *Dlx1*$^{-/-}$ /*Dlx2*$^{-/-}$ mutants have normal limbs, however *Dlx2*$^{-/-}$ /*Dlx5*$^{-/-}$ mice do show severe defects in the distal limbs (Panganiban and Rubenstein, 2002). The *Dlx5* and *Dlx6* genes have also been implicated in elbow joint formation, as they are specifically expressed in cells which will form this joint in the chick (Ferrari et al. 2006).

1.3.5.2. Blood

Dlx4 is expressed in bone marrow cells and knockdown of this gene leads to inefficiencies in plating and increases apoptosis (Shimamoto et al. 1997). *Dlx1* has been shown to play a role in the regulation of the TGF-β superfamily in hematopoietic differentiation during blood production (Chiba et al. 2003). Natural killer cells are lymphocytes that develop from precursors in the bone marrow. In immature natural killer

cells *Dlx1*, *Dlx2*, and *Dlx3* are expressed until maturation where their expression is lost (Sunwoo et al. 2008).

1.3.5.3. Bone

Dlx5$^{-/-}$ mice show defects in osteogenesis, suggesting a role in osteoblast differentiation and bone formation. *Dlx5*$^{-/-}$ */Dlx6*$^{-/-}$ mutant mice show severe defects in the axial and appendicular skeleton early in development (Robledo et al. 2002). These defects were first identified by a kinked tail, exencephaly due to cartilage loss in the frontonasal, supraoccipital, and rostral temporal areas of the skull, and retardation of endochondral ossification (Robledo et al. 2002). In culture, *Dlx2* and *Dlx6* will stimulate osteoblast differentiation which could explain the relatively mild bone phenotypes of *Dlx5*$^{-/-}$ when compared to *Dlx5*$^{-/-}$ */Dlx6*$^{-/-}$ double mutants (Li et al. 2008). In addition to the previous study, tissue-specific misexpression of a *Dlx5* allele in immature and differentiating chondrocytes results in accelerated mineralization and hypertrophy throughout the endochondral skeleton (Zhu and Bendall, 2009). *Dlx5*$^{-/-}$*/Dlx6*$^{-/-}$ mice have chondrogenesis defects, which are partially rescued by the hemizygous allele expressing *Dlx5*; however, complete rescue required the rescue allele in the homozygous state. This study suggests that not only is *Dlx5* functionally equivalent to *Dlx6* in chondrocytes, but also the quantitative levels of DLX5 or DLX6 activity are necessary for proper development of specific elements of the endochondral skeleton (Zhu and Bendall, 2009). Mutations in DLX3 result in Tricho-Dento-Osseous (TDO) syndrome, which is an autosomal dominant disorder, characterized by abnormalities in teeth, hair, and increased bone density (Price et al. 1998; Haldeman et al. 2004). Targeted expression of a mutated version of *Dlx3*, containing a four-bp deletion identical to TDO individuals, showed

increased bone formation markers and osteoblasts in mice suggesting a role in osteoclast differentiation and bone resorption (Choi et al. 2009).

1.3.5.4. Sensory organs

Knockdown of both *dlx3b* and *dlx4b* in zebrafish results in reduced otic and olfactory placodes, with *dlx3b* being sufficient for normal development (Solomon and Fritz, 2002). Similar studies show that *dlx3b* and *dlx4b* are necessary for specifying the fates of rohon-beard sensory neurons and trigeminal placodes (Kaji and Artinger, 2004). Also, *dlx2a* has been shown to be necessary for the proper development of the trigeminal ganglia in zebrafish (Sperber et al. 2008). Mice lacking *Dlx5* have a reduction in the size of the olfactory epithelium, and olfactory neurons fail to innervate the olfactory bulb (Long et al. 2003; Levi et al. 2003). The inner ear is also affected in these mutants, as there are defects in the semi-circular canals (Acampora et al. 1999). In the retina, $Dlx1^{-/-}/Dlx2^{-/-}$ mice show a reduced ganglionic cell layer, due to increased apoptosis (de Melo et al. 2005). This phenotype suggests that *Dlx1/Dlx2* gene function is necessary for terminal differentiation of retinal gangionic cell progenitors (de Melo et al. 2005).

1.3.5.5. Branchial Arches

The complex expression of the *Dlx* genes within the developing branchial arches is a model for better understanding both the redundant and unique functions of the *Dlx* genes. $Dlx1^{-/-}$, $Dlx2^{-/-}$, or $Dlx1^{-/-}/Dlx2^{-/-}$ mutant mice showed defects in the skeleton of the upper jaw derived from proximal positions in the first arch, with slight alterations in the distal structures and mandible (Qiu et al. 1995; Qiu et al. 1997; Depew et al. 2005). These defects are less severe distally in the arches, as the expression of other *Dlx* genes such as

Dlx3/Dlx4 and *Dlx5/Dlx6* may be able to compensate for the loss (Qiu et al. 1995; Qui et al. 1997; Depew et al. 1999). Contrarily, $Dlx5^{-/-}$ mice exhibit dysmorphic mandibles, bones of the inner ear, and teeth (Depew et al. 1999). The $Dlx5^{-/-}/Dlx6^{-/-}$ mutant mice show a much more prominent phenotype, resulting in a homeotic transformation of the upper jaw into the lower jaw (Beverdam et al. 2002; Depew et al. 2002). Molecular markers have confirmed these defects in branchial arch development; sets of dorsally expressed genes that require *Dlx1* and *Dlx2* for expression expand ventrally upon the loss of *Dlx5* and *Dlx6* (Jeong et al. 2008). Taken together, these results show that the expression of the *Dlx* genes provides regional specification cues for the first branchial arch; *Dlx1/Dlx2* are necessary for proper development of the maxilla, where as *Dlx5/Dlx6* are necessary for mandibular specification. Similarly in zebrafish, the loss of *dlx1a* and/or *dlx2a* results in reduced and dysmorphic branchial arch elements indicating their involvement in the specification of proximal elements within the first two branchial arches (Sperber et al. 2008). Consistent with this model, in zebrafish *dlx3b* and *dlx5a* are redundantly required for patterning specifically within more distal domains (Walker et al. 2007). Finally, knockdowns of *dlx2a*, *dlx2b*, *dlx3b*, and *dlx5a* resulted in altered pharyngeal tooth morphology in the zebrafish (Jackman and Stock, 2008).

1.3.5.6. Forebrain

The *Dlx* genes have partially overlapping expression in the mouse developing telencephalon: *Dlx1* and *Dlx2* are expressed in the most immature cells located in the proliferative zone in the ventricular zone; *Dlx1*, *Dlx2* and *Dlx5* is expressed in subventricular zone and and *Dlx6* is expressed laterally in postmitotic migrating and differentiating neurons in the mantle zone (Liu et al. 1997; Anderson et al. 1999;

Eisenstat et al. 1999, Yun et al. 2003). While the *dlx* genes of zebrafish are also expressed in the forebrain, there is currently no experimental data reported on their functions in this tissue. Outlined below is the literature from mutant mice experiments, as this provides the most useful current evidence regarding the function of the *Dlx* genes.

Within the forebrain, the *Dlx* genes are highly co-expressed with *Gad* genes, which encode the enzyme responsible for the production of GABA (Soghomonian et al. 1998). The *Dlx* genes are required for the differentiation and migration of most GABAergic neurons, GABAergic projection and GABAergic interneurons, in the telencephalon (Anderson et al. 1997a; Anderson et al. 1997b; Stühmer et al. 2002a; Stühmer et al. 2002b). This close relationship indicates the *Dlx* genes are important for the development of neurons that use GABA as their neurotransmitter. This was confirmed when ectopic expression of *Dlx2* or *Dlx5* in brain slice cultures led to neurons taking on the GABA phenotype (Anderson et al. 1999; Stühmer et al. 2002a). The function of the *Dlx* genes during forebrain development has principally been studied in mouse knockouts (Qui et al. 1995; Qui et al. 1997; Anderson et al. 1997a; Anderson et al. 1997b; Acampora et al. 1999). Mice lacking $Dlx1^{-/-}/Dlx2^{-/-}$ show a major block in neurogenesis and differentiation of late-born striatial projection neurons that fail to migrate and accumulate within the proliferative zone (Anderson et al. 1997a; Marin et al. 2000). This deficiency in differentiation affects the number of GABAergic projection neurons, but also GABAergic, dopaminergic, and cholinergic interneurons (Qui et al. 1995; Anderson et al. 1997a; Pleasure et al. 2000; Marin et al. 2000; Anderson et al. 2001). These losses are caused by reduced neuronal differentiation, but also the lack of migration of immature interneurons from the basal telencephalon to the cerebral cortex

and olfactory bulb (Anderson et al. 1997b; Anderson et al. 1999; Bulfone et al. 1998; Anderson et al. 2001; Pleasure et al. 2000; Marin and Rubenstein, 2001; Long et al. 2007). This loss of tangential migration may be caused by increased neurite length, identifying a role of the *Dlx* genes in repression of axon and dentrite growth (Cobos et al. 2007). Within the thalamus, the *Dlx1* and *Dlx2* genes may play a role in the disturbed patterning of thalamocortical projections to the neocortex (Garel et al. 2002). *Dlx1*$^{-/-}$ /*Dlx2*$^{-/-}$ mice show a loss of tyrosine hydroxylase expression in the ventral thalamus implicating the *Dlx* gene function in the differentiation of dopaminergic neurons (Andrews et al. 2003). The *Dlx1* and *Dlx2* genes have also been shown to play a role in determining the neuronal or oligodendrocyte fate in the mouse ventral telencephalon The *Dlx1* and *Dlx2* genes promote the neuronal fate by repressing Olig2 expression in neuronal progenitors (Petryniak et al. 2007).

The expression of *Dlx5* and *Dlx6* is almost completely absent in *Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$ mice, suggesting the *Dlx1* and *Dlx2* genes are genetically upstream and play a role in the regulation of *Dlx5* and *Dlx6* expression in the forebrain (Anderson et al. 1997a; Zerucha et al. 2000; Stühmer et al. 2002b). This cross-regulation leads to a difficult situation where the phenotypes associated with the loss of *Dlx1* and *Dlx2* cannot be uncoupled from the loss of *Dlx5* and *Dlx6*. Therefore, it is not yet possible to assign functional properties to the *Dlx1* and *Dlx2* genes alone. Unfortunately, the forebrain phenotype of *Dlx5*$^{-/-}$/*Dlx6*$^{-/-}$ mice cannot be assayed due to exencephaly (Robledo et al. 2002). Heterozygous mutants are phenotypically normal, while single mutants for *Dlx1*, *Dlx2*, and *Dlx5* have mild phenotypes associated with this loss. *Dlx1*$^{-/-}$ mice have a loss of postnatal neocortical and hippocampal interneurons, resulting in epilepsy and an

increased fear response (Cobos et al. 2005; Mao et al. 2009). *Dlx2*$^{-/-}$ mutants show normal morphology and histology of the embryonic forebrain, however they have reduced numbers of TH in the olfactory bulbs (Qiu et al. 1995). There are no abnormalities identified in the forebrain of the *Dlx5*$^{-/-}$ mice (Acampora et al. 1999). In summary, the *Dlx* genes have largely redundant functions within the forebrain; however, they each may have distinct functions based on the subtle phenotypes of single mutants.

1.3.6. *Dlx* gene regulation

1.3.6.1. *cis*-Regulation of the *Dlx* genes

The highly overlapping expression of physically linked *Dlx* genes and the presence of a short intergenic domain lead to the hypothesis that this region may be enriched with regulatory elements responsible for the overlapping expression patterns (Ellies et al. 1997b). Orthologous *Dlx* genes also have highly similar expression patterns, indicating these regulatory regions may have been present before the expansion of the *Dlx* genes in vertebrates. The first evidence of gene regulatory sequences within the *Dlx* intergenic regions were two *dlx5a/dlx6a* (previously *dlx4/dlx6*) regulatory sequences, highly conserved between zebrafish and mice, named I56i (400 bp, 83%) and I56ii (300bp, 85%) (Figure 1.9)(Zerucha et al. 2000). When tested for regulatory function in transgenic mouse assays the two zebrafish sequences, I56i and I56ii, activated reporter expression in the forebrain and olfactory placodes (Figure 1.11B)(Zerucha et al. 2000). The orthologous mouse I56i element also drove reporter expression in highly similar domains in mice: forebrain and olfactory placodes, and also the branchial arches and AER. Within the forebrain, a 1.4kb intergenic region from *dlx5a/dlx6a* drove reporter

gene expression in the forebrain of mice and zebrafish, faithfully mimicking endogenous *Dlx5* (*dlx5a*) and *Dlx6* (*dlx6a*) expression (Zerucha et al. 2000). This heterologous transgenic experiment suggests not only are the functions of these distinct elements conserved, but also the upstream GRN controlling their activity may be conserved in distantly related vertebrates as well (Zerucha et al. 2000).

The intergenic region between *Dlx1* and *Dlx2* also contains highly conserved *cis*-acting regulatory elements (Figure 1.9)(Ghanem et al. 2003). To identify these *cis*-acting regulatory elements, the sequence of the intergenic region separating *Dlx1* and *Dlx*2 was examined and compared between five distantly related vertebrate species: human, mouse, zebrafish, and two species of pufferfish, *Takifugu rubripes* and *Spheroides nephelus.* A small number of sequences were highly conserved in all five species. Within the *Dlx1* and *Dlx2* intergenic region, there are two sequences identified named I12a (550bp) and I12b (450 bp) (Ghanem et al. 2003). In transgenic mice experiments, the I12b enhancer sequence targets reporter gene expression to the forebrain (Figure 1.11B), while the I12a enhancer targets expression to a subset of mesenchymal cells of the first two branchial arches (Ghanem et al. 2003, Park et al. 2004). Both I12a and I12b recapitulate endogenous *Dlx1* and *Dlx2* expression in the forebrain and partially in the branchial arches (Ghanem et al. 2003). The activities of I12b, I56i and I56ii are highly similar in the mouse forebrain despite very limited sequence similarities between these enhancers (Ghanem et al. 2003). There was however a small sequence (<100 bp) with similarities identified with two putative *Dlx* binding sites within it (Ghanem et al. 2003). A fragment of I56i, containing the two *Dlx* binding sites, had been previously tested in transgenic mouse assays and drove reporter expression similarly to the complete I56i element in the

forebrain, suggesting this region is sufficient for forebrain activity of these elements (Zerucha et al. 2000).

Dlx regulatory sequences are not only found within the intergenic domains as several have been identified upstream of the *Dlx* transcriptional start sites (Thomas et al. 2000; Verzi et al. 2006; Ghanem et al. 2007; Jackman and Stock, 2008). Analysis of the upstream region of *Dlx1* identified two additional regulatory elements named Upstream Regulatory Element (URE) 1 and 2, or URE1 and URE2, respectively. The URE1 sequence has activity only in the retina (unpublished observations), a tissue known to express *Dlx1* and *Dlx2* (Dollé et al. 1992; Eisenstat et al. 1999, de Melo et al. 2003). The URE2 was active in the forebrain, similar to the three other forebrain enhancers (mentioned above) (Figure 1.11B). Comparison of the I12b, URE2, and I56i elements displayed distinct spatial-temporal differences in the forebrain and marked unique and overlapping neuronal populations based on co-labelling with molecular markers (Ghanem et al. 2007; Potter et al. 2009). These three enhancers mark interneuron progenitors from the ganglionic eminences; however, the fourth forebrain enhancer, I56ii, showed activity only in post-mitotic projection neurons (Ghanem et al. 2007; Ghanem et al. 2008). Thus, there has been success in identifying a higher order of *Dlx* regulatory element activity due to their activity in distinct neuronal populations, potentially mimicking the distinct differences of *Dlx* expression in the forebrain (Figure 1. 11C) (Liu et al. 1997; Eisenstat et al. 1999). Other regions that may be responsible for domains of *Dlx* expression have been identified. The 3.8kb *5' flanking region* region from *Dlx2* drives reporter expression in the epithelium of the first branchial arch (Thomas et al. 2000). A region upstream of *Dlx6* drives reporter expression in the first and second branchial arches close to

endogenous *Dlx5/Dlx6* expression (Verzi et al. 2006). Jackman and Stock (2008) identified the 4.1kb promoter region from *dlx2b* with the ability to drive reporter expression specifically in the pharyngeal teeth.

Similar to the *Dlx1/Dlx2* and *Dlx5/Dlx6* intergenic regions, there were five potential regulatory elements identified in the *Dlx3/Dlx4* intergenic region of mouse and human (between ~180 to ~350bp) (Sumiyama et al. 2002). One of these elements can be identified in the zebrafish *dlx3b/dlx4b* intergenic domain (M. Ekker, unpublished observations). In addition to intergenic regulatory elements, there were three elements identified: two upstream of *Dlx3* and one upstream of *Dlx*4 (Sumiyama et al. 2002). A large 79kb transgene was inserted into the first exon of *Dlx3*, and recapitulated endogenous expression at embryonic stages in the limb buds and first and second visceral arches (Sumiyama et al. 2003). Upon deletion of 4kb in the large transgene, reporter activity is lost in the visceral arches and this is attributed to the removal of a distinct element, I37-2. Upstream of the mouse and *Xenopus Dlx3* gene there are enhancers which drive reporter expression in the surface ectoderm (Morrasso et al. 1994; Morasso et al. 1995; Park and Morasso, 1999). These data suggest that the complex expression of *Dlx3* and *Dlx4* is the result of several distinct regulatory regions located in their genomic locus.

1.3.6.2. DLX targets

The *Dlx* genes encode important developmental transcription factors, which bind to DNA in a sequence specific manner. A consensus binding site has been identified for the *Xenopus Dlx3*, (A/C/G) TAATT (G/A) (C/G), which can also be bound by other *Dlx* proteins *in vitro* (Feledy et al. 1998). The *Dlx* genes will typically function by

recognizing this consensus sequence and activating transcription of a nearby gene, shown by their ability to activate artificial and endogenous regulatory elements (Feledy et al. 1999; Masuda et al. 2001; Zerucha et al. 2000; Morasso et al. 1996; Stühmer et al. 2002b). The DLX proteins can homodimerize and heterodimerize with other factors, such as Msx and Dlxin1, affecting their abilities to activate transcription (Zhang et al. 1997; Masuda et al. 2001). However, DLX proteins can also potentially act as repressors (Le et al. 2007).

1.3.6.3. Auto- and Cross- Regulation of the *Dlx* genes

The highly overlapping and sometimes nested expression of the *Dlx* genes indicates that they may regulate the expression of one another. Loss of *Dlx1* and *Dlx2* expression results in an almost complete loss of *Dlx5* and *Dlx6* expression in the forebrain (Anderson et al. 1997a). This is probably due to the loss of activity of the regulatory elements found within the *Dlx5/Dlx6* intergenic region in $Dlx1^{-/-}/Dlx2^{-/-}$ mice (Zerucha et al. 2000; Stühmer et al. 2002b). More specifically, sequence comparisons identified two potential *Dlx* binding sites in the I12b, I56i, and I56ii regulatory elements (Zerucha et al. 2000; Ghanem et al. 2003). The I56i enhancer can be activated and bound by a number of *Dlx* proteins using *in vitro* assays (Zerucha et al. 2000; Stühmer et al. 2002b). DLX1 and DLX2 proteins also directly bind this enhancer, although this differs in the newborn retina and striatum (Zhou et al. 2004). Cross-regulation of the *Dlx* genes has been proposed for the nested expression of *Dlx5/Dlx6* and *Dlx3/Dlx4* in the developing branchial arches (Sumiyama et al. 2003). Previously, *in vitro* evidence suggested an auto-regulatory loop between in the *Dlx1/Dlx2* loci, as Dlx2 could bind and activate the I12b enhancer (Poitras et al. 2007). Recently, *in vivo* evidence has also been

provided for auto-regulation of the *Dlx* genes as both URE2 and I12b are direct targets of DLX1 and DLX2 and require *Dlx1/Dlx2* expression for proper activity (Potter et al. 2009). Fitting this model, DLX2 has been shown to be necessary for the proper expression of *Dlx1* in the telencephalon (Eisenstat et al. 1999). Also, $Dlx1^{-/-}/2^{-/-}$ mice have reduced activity of I12b and URE2 in the forebrain, suggesting auto-regulatory mechanisms controlling *Dlx1* and *Dlx2* expression (Potter et al. 2009). These data provide evidence that the *Dlx* genes not only regulate one another but also their own expression.

1.3.6.4. Upstream factors

Signaling molecules play a crucial role in the patterning of the embryo; however, their role in the expression of the *Dlx* genes is still vague. BMPs and FGFs are necessary for the proper expression of *Dlx1* and *Dlx2* in the mesenchyme of the first branchial arch (Bei and Maas, 1998; Tucker et al. 1998). FGF8 induces *Dlx2* expression in the ectomesenchyme but will repress its expression in the epithelium when induced by BMP4 (Thesleff and Sharpe, 1997; Thomas et al. 2000). Consistent with these findings, BMP4 exhibits an overall negative effect on the activity of the *Dlx* regulatory elements I12a and I56i in the mesenchyme of the branchial arches and inhibits the positive effects of FGFs (Park et al. 2004). Knockdown of *dlx3b/dlx4b* in zebrafish result in reduced expression of a BMP agonist and affects the development of the preplacodal region (Solomon and Fritz, 2009). Loss of BMP and FGF signaling, and their regulation of *dlx* expression, has been linked to the loss of oral teeth in zebrafish (Jackman and Stock, 2006). Endothelin-1 (ET-1) regulates *Dlx* expression in the branchial arches of mice and zebrafish. Zebrafish and mice mutants for ET-1, or its receptor, show a loss of *Dlx* expression in the branchial

arches (Miller et al. 2000; Clouthier et al. 2000). A loss of ET-1 results in decreased I56i enhancer activity, which corresponds with the loss of *Dlx6* (Charite et al. 2001; Park et al. 2004). Ectopic expression of *Shh* leads to ectopic expression of *Dlx2* and forebrain deformities (Gaiano et al. 1999; Khotz et al. 2001). Retinoic acid treatment of zebrafish embryos resulted in a loss of *dlx* expression and craniofacial dysmorphologies (Ellies et al. 1997a).

A number of transcription factors have been implicated in playing a role in the proper expression of the *Dlx* genes, but few have proven to have direct effects. Mash1 induces the expression of *Dlx1* gene when ectopically expressed in the cerebral cortex and directly binds the I12b enhancer (Fode et al. 2000; Poitras et al. 2007). Also, based on biochemical and bioinformatic analysis of the I12b enhancer, Nkx2.1, Msx1/2, and Meis1/2 are candidates to regulate *Dlx* expression (Poitras et al. 2007). In culture, Meis2 and Islet1 activate the I56ii enhancer (Ghanem et al. 2008). Regulation of both the *Dlx5* and *Dlx6* promoter regions by p63 has shown that p63 is upstream of the *Dlx5/Dlx6* genes and suggests a possible explanation for SHFM (Lo Iacono et al. 2008). Msx1 is required for maintenance of *Dlx2* expression in the branchial arch mesenchyme (Bei and Maas, 1998).

The master regulatory gene, *All-1*, may have the ability to regulate all three *Dlx* pairs in mammals as the expression of *Dlx2*, *Dlx3*, *Dlx4*, *Dlx5*, and *Dlx6* is lost in human acute leukemias (Ferrari et al. 2003). Finally, a long non-coding RNA named *Evf-2* regulates the *Dlx* genes. *Evf-2* has been shown to cooperate with DLX2 and increase the activity of *Dlx5/Dlx6* enhancers (Feng et al. 2006). *Evf-2* recruits DLX2 and Mecp2 to the *Dlx5/Dlx6* regulatory regions (Bond et al. 2009). Interestingly, *Evf-2* is a transcript

synthesized from the intergenic region between *Dlx5/Dlx6* and contains a small piece of the I56i enhancer, a region previously thought to be non-coding (Feng et al. 2006). Chromatin structure has been shown to be important for *Dlx2* expression, along with neurogenesis in the mouse telencephalic ventricular zones. *Dlx2* is a direct target of MLL1, which will modify the chromatin state around *Dlx2* by methylation (Lim et al. 2009).

1.3.6.5. Downstream targets

Until recently, the targets of the *Dlx* genes in the forebrain have been elusive; however, many downstream genes affected by the loss of *Dlx1* and *Dlx2* in the olfactory bulb, basal ganglia, and striatum have been identified by large candidate screens in *Dlx* mutants (Long et al. 2007; Long et al. 2009a; Long et al. 2009b). Microarray data on the ventricular zones of $Dlx1^{-/-}/Dlx2^{-/-}$ mice has also identified numerous genes regulated by *Dlx* that may play a role in neurite growth and maturation (Cobos et al. 2007). I will outline the downstream genes with additional molecular data or particular relevance to my project.

The *Dlx* genes have been shown to be involved in the development of many tissues and organs, often studied is their role in the development of GABAergic interneurons. The ectopic expression of *Dlx in vitro* leads to ectopic expression *Gad65* and *Gad67* (Stühmer et al. 2002a). In $Dlx1^{-/-}/Dlx2^{-/-}$ mice the expression of *Gad67* is reduced in the forebrain (Long et al. 2009a; Long et al. 2009b). Also, there have been reports of *Dlx* activating reporter expression *in vitro* from a *Gad65* enhancer element (Panganiban and Rubenstein, 2002). The Dlx genes can also act as negative regulators, as Dlx1 and Dlx2 bind to the *Neuropilin-2* promoter region and repress its expression (Le et

al. 2007). Nrp-2 is a receptor for semaphorins, guidance cues that play a role in the sorting of tangentially migrating interneurons from the basal ganglia to the cortex (Marin et al. 2001b). In *Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$ mutant mice, there is ectopic expression of *Nrp-2* in the subventricular zone (SVZ) and may play a role in blocking migration of interneurons from the SVZ to the neocortex (Marin et al. 2001b; Le et al. 2007). Further to this, *Aristaless* (*Arx*) is known to play a role in tangential migration of GABAergic interneurons and is a direct target of DLX2 (Colasante et al. 2008). These results indicate the *Dlx* genes play a direct role in GABAergic interneuron differentiation and migration.

The *Dlx* genes also play a role in the development, either directly or indirectly, of other tissues via numerous downstream targets. In the branchial arches, similar large-scale analysis has been done to better understand the downstream targets of *Dlx1*/*Dlx2* and *Dlx5*/*Dlx6* (Jeong et al. 2008). In this study, 20 new candidate downstream genes were identified of which one, *Gbx2*, has an enhancer activated by DLX5 (Jeong et al. 2008). In the zebrafish, knockdown of *dlx2a* reduced the expression of neural crest markers, *crestin* and *sox9*, and other branchial arch markers (Sperber et al. 2009). Dlx2 binds the Trkb promoter during mouse retina development, indicating an involvement in the survival of retinal ganglionic cells (de Melo et al. 2008). In hematopoietic cells DLX4 activates the expression of GATA1 and MYC (Shimamoto et al. 1997). DLX5 has been shown to have several target genes involved in bone formation, such as, osteocalcin, collagen 1A1, and bone sialoprotein (Dodig et al. 1996; Ryoo et al. 1997; Newberry et al. 1998; Benson et al. 2000). In total, the number of DLX targets currently identified is limited; however, there is potential to identify many more with new technologies such as, CHIP-seq and affinity purification.

Statement of Inquiry

It has been proposed that changes in the expression or functions of regulatory genes in the embryo are responsible for the vast morphological diversity present in vertebrates. All vertebrates surveyed to date have *Dlx* genes, which encode transcription factors important for the development of a number of embryonic structures such as the forebrain, branchial arches, and limbs. The *Dlx* genes are expressed in a specific spatial-temporal pattern and play a role in the differentiation of GABAergic interneurons in the mouse forebrain. The proper expression of the *Dlx* genes is controlled by a number of regulatory elements identified in the surrounding genome. These regulatory elements are highly conserved in sequence between distantly related vertebrates, some even ultraconserved, indicating they have been under strong selective pressure during vertebrate evolution. This strong selection may be due to an evolutionarily conserved genetic cascade present in all vertebrates that will interact with these regulatory elements. Overall, the understanding of the genetic cascades controlling the development of many vertebrate tissues is still undeveloped; however, studying pieces of these cascades will provide clues into the similarities or differences between the distinct developmental processes of vertebrates.

Within my PhD project I have addressed several unresolved issues regarding the regulation and function of the zebrafish *dlx* genes: i) Does sequence conservation of regulatory elements indicate similar function in distantly related vertebrates? ii) Does *dlx* expression follow a spatial-temporal cascade in zebrafish forebrain? iii) Are the gene regulatory cascades involved in *dlx* regulation conserved between distantly related

vertebrates? iv) What is the function of the zebrafish *dlx* genes in the development of the forebrain?

I have addressed these questions in my doctoral thesis. The studies done in Chapters Two and Three specifically address the first and second questions. We have generated transgenic zebrafish, containing regulatory elements orthologous to those identified in mice, and studied their activities with respect to endogenous *dlx* expression. Using fluorescent *in situ* hybridization and immunohistochemistry, we have shown that the *dlx* genes are expressed in highly overlapping domains within the forebrain and appear to follow a conserved pathway of neuronal differentiation. By genome comparisons, we have identified one of the highly conserved *dlx* regulatory elements in the three major branches of jawed vertebrates. We tested the orthologous sequences from the elephant shark, zebrafish, and mouse genomes for regulatory function in transgenic mouse and zebrafish. The similar activity of orthologous regulatory sequences, in heterologous and homologous transgenic backgrounds, coupled with *dlx* expression data indicates there may be similar genetic cascades controlling *dlx* expression in mammals and teleosts. These transgenic studies also provide insight into the evolution of regulatory mechanisms in vertebrates and how changes in *cis*-regulatory elements translate into changes in their regulatory activity.

Both studies in Chapters Two and Three begin to address question iii. However, in the fourth chapter, we look at *ascl1a*, whose mouse orthologs was identified as one upstream regulator of *Dlx* expression in the forebrain. We knocked down the *ascl1a* transcription factor by morpholino oligonucleotides (MO) in zebrafish embryos and concluded it is necessary for proper *dlx* expression in the forebrain and for GABAergic

interneuron differentiation in the prethalamus. In Chapter Four, we also addressed question iv: *dlx* knockdown experiments enabled me to show that the *dlx* genes regulate one another in an evolutionarily conserved cross-regulatory mechanism. The *dlx* genes are involved in GABAergic interneuron development in the prethalamus but not in the telencephalon, a result that is not consistent with the mouse. Therefore, we speculate that the genetic cascades controlling GABAergic interneuron development may be conserved in the prethalamus but divergent in the mouse and zebrafish telencephalon.

2. The relationship between *dlx* and *gad1* expression indicates highly conserved genetic pathways in the zebrafish forebrain

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Key Words: *dlx*, Forebrain; Transgenesis; Zebrafish; Regulatory elements; Fluorescent in situ hybridization

Abstract

The *Dlx* genes encode a family of transcription factors important to the development of the vertebrate forebrain. These genes have very similar expression domains during the development of the telencephalon in mouse and play a role in GABAergic interneuron differentiation. We have used triple fluorescent *in situ* hybridization to study the relative expression domains of the *dlx* and *gad1* genes in the zebrafish telencephalon and diencephalon. We also generated transgenic zebrafish with regulatory elements from the zebrafish *dlx1a/2a* locus. The zebrafish *dlx* regulatory elements recapitulated *dlx* expression in the forebrain and mimicked the relationship between the expression of the *dlx* genes and *gad1*. Finally, we show that a putative enhancer located downstream of *dlx2b* can also activate reporter gene expression in a tissue specific manner similar to endogenous *dlx2b* expression. Our results indicate the *dlx* genes are regulated by an evolutionarily conserved genetic pathway and may play a role in GABAergic interneuron differentiation in the zebrafish forebrain.

2.1 INTRODUCTION

The *Dlx* genes encode homeodomain containing transcription factors involved in the development of the mammalian forebrain (Anderson et al. 1997a; Anderson et al. 1997b; Marin et al. 2000; Pleasure et al. 2000). They have partially overlapping expression in the developing telencephalon of the mouse and follow a distinct spatial and temporal expression pattern: *Dlx1* and *Dlx2* are expressed in the ventricular zone and subventricular zone; *Dlx5* is expressed in the ventricular zone and *Dlx6* is expressed laterally in the mantle zone (Liu et al. 1997; Anderson et al. 1999; Eisenstat et al. 1999;

Yun et al. 2003). *Dlx* gene expression highly overlaps with that of *glutamic acid decarboxylases* (*Gads*), the enzymes responsible for the synthesis of γ-amino butyric acid (GABA). Furthermore, DLX proteins can induce *Gad* gene expression *in vitro* and *in vivo* (Anderson et al. 1999; Stühmer et al. 2002; Yun et al. 2003). *Dlx1*$^{-/-}$/*Dlx2*$^{-/-}$ mutant mice show a loss of GABAergic interneuron differentiation in the ventral telencephalon, further implicating the *Dlx* genes in the differentiation of GABAergic interneurons (Anderson et al. 1997a).

Vertebrate *Dlx* genes are typically organized in convergently transcribed bigene pairs, separated by a short intergenic region usually less than 10kb (Zerucha et al. 2000; Sumiyama et al. 2002; Ghanem et al. 2003). Zebrafish have eight *dlx* genes of which six show similar bigene genomic arrangements to the mouse: *dlx1a*/*dlx2a*; *dlx3b*/*4b*; and *dlx5a*/*6a* clusters orthologous to *Dlx1*/*2*; *Dlx3*/*4*; *Dlx5*/*6,* respectively (Quint et al. 2000). Two additional genes, *dlx2b* and *dlx4a*, are thought to be duplicates of ancestral *Dlx2* and *Dlx4,* respectively, after the teleost specific genome duplication event (Amores et al. 1998). The expression domains of two *Dlx* genes comprising a given bigene pair are very similar, both in mice and zebrafish, most likely due to shared regulatory regions (Liu et al. 1997; Eisenstat et al. 1999; Ellies et al. 1997). Regulatory regions have been identified both upstream of the transcription start sites and within the intergenic domains of a given bigene pair (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007). The zebrafish *dlx* genes share similar genomic arrangements to the mouse, including the presence of the regulatory elements I12a and I12b in the *dlx1a*/*2a* intergenic region, Upstream regulatory element 2 (URE2) upstream of *dlx1a*, I56i and I56ii between *dlx5a* and *dlx6a* (Zerucha et al. 2000; Ghanem et al. 2003). Five *dlx* genes (all but *dlx3b*, *dlx4b* and *dlx4a*) are

expressed in the zebrafish forebrain with very similar expression domains in the telencephalon and diencephalon (Akimenko et al. 1994; Ellies et al. 1997). In particular zebrafish *dlx1a* and *dlx2a* have been proposed to mark cells closer to the ventricular wall than the *dlx5a* and *dlx6a* genes, reminiscent of the spatial-temporal succession of *Dlx* gene expression pattern in mouse (Zerucha et al. 2000). However, the precise spatial relationship between different *dlx* expression domains and *gad1* gene expression has not been determined.

In this study, we describe in detail the extent to which zebrafish *dlx* and *gad1* expression patterns overlap in the developing forebrain. The *gad1* gene, orthologous to *Gad67* in mammals, is used as a marker for neuronal differentiation. The spatial-temporal expression pattern described in the mouse telencephalon appears to be similar in the zebrafish, with *dlx1a* and *dlx2a* being expressed in the ventricular zone and *dlx5a*, *dlx6a* and *gad1* expressed in more lateral differentiated cells. We tested whether the conserved regions linked to the *dlx1a/2a* cluster of the zebrafish have regulatory functions consistent to previously reported data in the mouse forebrain, by designing reporter constructs containing conserved sequences from the *dlx1a/2a* locus. We also utilized a previously described transgenic line, *Tg(dlx5a/6aIG:GFP)*, showing these regulatory regions are sufficient to mimic the endogenous nested expression patterns of the *dlx* genes in the zebrafish forebrain (Zerucha et al. 2000). We further explored the regulation of the *dlx2b* gene, paralogous to the *dlx2a* gene in the zebrafish. A small domain containing two putative *dlx* binding sites had been identified downstream of *dlx2b* (Ghanem et al. 2003), denoted *dlx2b* downstream regulatory element (*dlx2b*DRE). The

forebrain expression of *dlx2b* is at least partially recapitulated by reporter gene expression when under the regulation of *dlx2b*DRE.

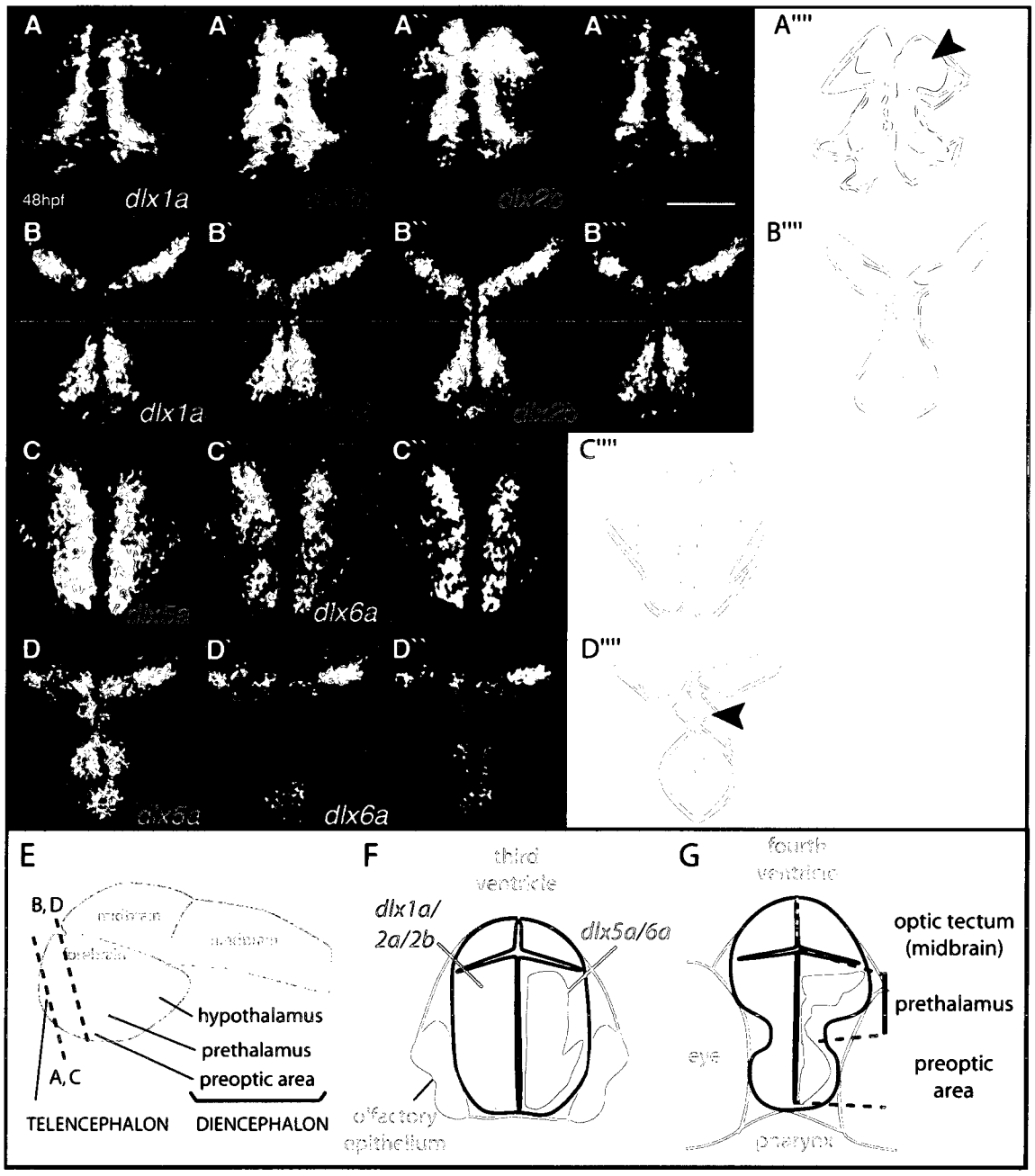
2.2 RESULTS

2.2.1 Partially overlapping expression domains of *dlx* and *gad1* genes in the zebrafish forebrain

The zebrafish *dlx* genes from a bigene pair have been proposed to have highly overlapping expression domains within the forebrain (Zerucha et al. 2000). Currently there are no antibodies against specific zebrafish Dlx proteins except Dlx3b, therefore we relied on fluorescent RNA *in situ* hybridization to determine the extent to which the *dlx* genes expression domains overlap. We examined the *dlx* expression in the zebrafish forebrain from 24 hours post-fertilization (hpf) until 48 hpf. At 48 hpf, the forebrain is beginning to undergo secondary neurogenesis, and *dlx* and *gad1* are expressed in the forebrain (Akimenko et al. 1994; Ellies et al. 1997; Martin et al. 1998). As observed by confocal imaging, the *dlx1a*, *dlx2a*, and the paralogous *dlx2b* genes are expressed in very similar domains in the subpallium of the zebrafish telencephalon at 48hpf (Fig. 2.1A-A''' and delimited by solid lines in Fig. 2.1A''''). Within this domain, the three genes are expressed close to the ventricle, an area shown to be proliferative (Mueller and Wullimann, 2003). There may be quantitative differences within these domains as *dlx2b* appears to be more strongly expressed in the most dorsal domain of the subpallium (Fig. 2.1A'' and colored zone in A''''), while the *dlx2a* domain of expression is extended ventrally compared to the other two genes (see colored zone in Fig. 2.1A''''). The three *dlx* genes also show virtually identical expression domains in the diencephalon, more

Figure 2.1. Comparable expression domains for genes from the same *dlx* cluster.

Single z transversal sections of triple fluorescent *in situ* hybridization shows *dlx1a*, *dlx2a*, and *dlx2b* expression domains at 48hpf in the telencephalon (A-A''') and diencephalon (B-B'''), and *dlx5a* and *dlx6a* expression domains at the same levels (as defined on E), on respectively C-C''' and D-D''', dorsal is up. Comprehensive schematics showing the overlap of *dlx* expression domains are shown on A'''', B'''', C'''' and D'''': solid lines define the limit of detected expression, zones of higher expression is colored when applicable. Limits of expression domains are comparable for *dlx1a*, *dlx2a* and *dlx2b* but *dlx2b* specifically displays a more intense zone of expression in a dorsal-lateral domain (arrow on A''''). These three genes all show higher levels of expression in the ventricular zone. Limits of expression domains for *dlx5a* and *dlx6a* are mainly comparable although *dlx6a* was not significantly detected in the ventral domain of the prethalamus (arrowhead on D''''). Shared expression domains between clustered genes (and their paralogs) are mapped on a schematic of a brain section at the level of the telencephalon (F) and diencephalon (G): *dlx1a/2a/2b* in blue and *dlx5a/6a* in red. Scale bar= 100 μm

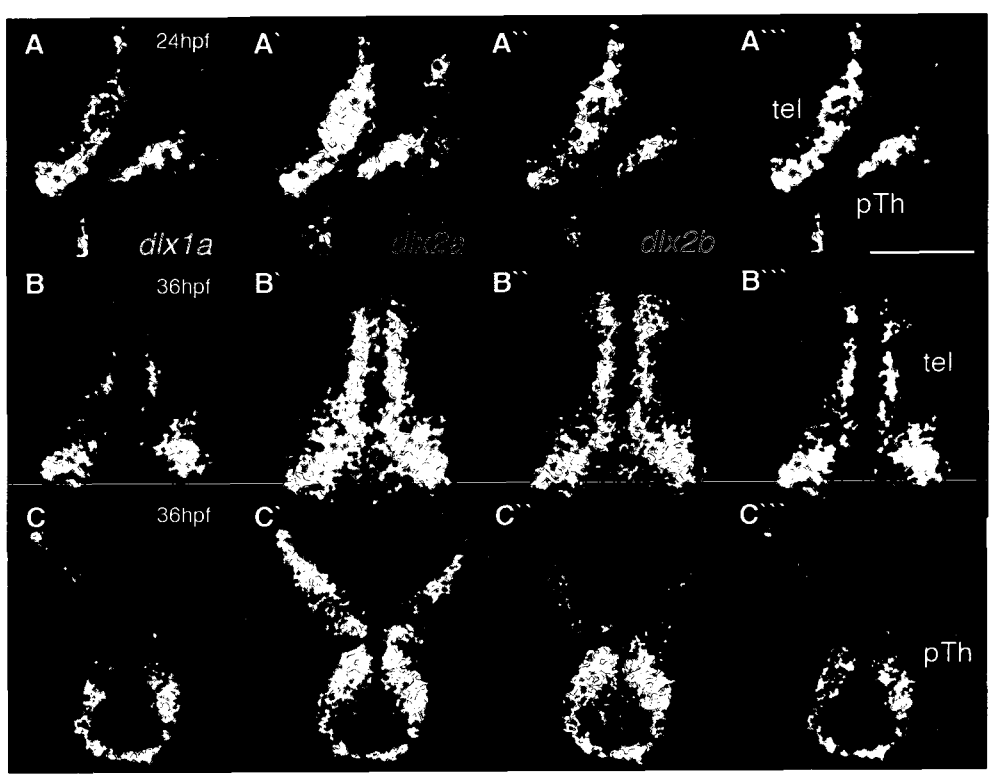

A
A`
A``
A```
A''''
48hpf
dlx1a
dlx2a
dlx2b
B
B`
B``
B```
B''''
dlx1a
dlx2a
dlx2b
C
C`
C``
C''''
dlx5a
dlx6a
D
D`
D``
D''''
dlx5a
dlx6a
E
B, D
midbrain
hindbrain
prethalamus
hypothalamus
prethalamus
preoptic area
A, C
TELENCEPHALON
DIENCEPHALON
F
third ventricle
dlx1a/ 2a/2b
dlx5a/6a
olfactory epithelium
G
fourth ventricle
optic tectum (midbrain)
prethalamus
eye
preoptic area
pharynx

specifically in the prethalamus and preoptic area (Fig. 2.1B-B'''), with an apparently higher level of expression for *dlx1a* and *dlx2b* in the dorsal part of the prethalamus compared to the rest of their expression domains (Fig. 2.1B'''').

The *dlx5a* and *dlx6a* genes display very similar expression domains in the subpallial telencephalon at 48hpf (Fig. 2.1C-C'') although *dlx6a* is highly expressed only in the dorsal-most part of the telencephalon. Both genes are expressed outside of the ventricular zone (Fig. 2.1F). In the diencephalon, transcripts of *dlx5a/6a* are localized throughout the prethalamus and preoptic area but *dlx6a* transcripts are strongly detected only in the dorsal-most part of the prethalamus (Fig. 2.1D). The expression limits of the *dlx1a/2a/2b* and *dlx5a/6a* genes appear to be, overall, very similar in the diencephalon (Fig. 2.1G). The highly overlapping domains of *dlx* forebrain expression are also observed at 24 and 36hpf (Suppl. 2.1 and Suppl. 2.2).

Consistent with previous reports, we find that *dlx2a* is transcribed in the ventricular zone of the zebrafish forebrain, where *zash1a* (*ascl1a*), *gad1*, and GABA expression can also be detected (Wullimann and Mueller, 2002; Mueller et al. 2006; Mueller et al. 2008). Based on the results presented in Fig. 2.1, it appears that expression of a gene in a *dlx* bigene cluster is representative of the second gene in the cluster. To further compare the expression domains of zebrafish *dlx* genes in the forebrain with areas of GABAergic interneuron differentiation, we studied the relative expression domains of one *dlx* gene from each bigene pair, *dlx1a* and *dlx5a*, alongside the specification marker *gad1*. The *dlx1a*, *dlx5a*, and *gad1* genes are expressed in very comparable domains within the telencephalon and diencephalon at 24hpf (Fig. 2.2A). Beginning at 36hpf and

Supplementary figure 2.1. *dlx1a*, *dlx2a*, *dlx2b* show similar expression in the forebrain at 24 and 36hpf. po, preoptic area; pTh, ventral thalamus; tel, telencephalon. Scale bar= 100 μm


A
24hpf
A`
A``
A```
tel
pTh
dlx1a
dlx2a
dlx2b
B
36hpf
B`
B``
B```
tel
C
36hpf
C`
C``
C```
pTh

Supplementary figure 2.2. The expression of *dlx5a*, *dlx6a,* and *gad1* expression overlaps in the forebrain at 24 and 48hpf. B```) Expression of *dlx5a* and *dlx6a* share overlapping domains with *gad1* expression on the lateral boundary. C``) *gad1* expression is found throught the ventral thalamus at 36hpf. po, preoptic area; pTh, prethalamus; tel, telencephalon. Scale bar= 100 μm

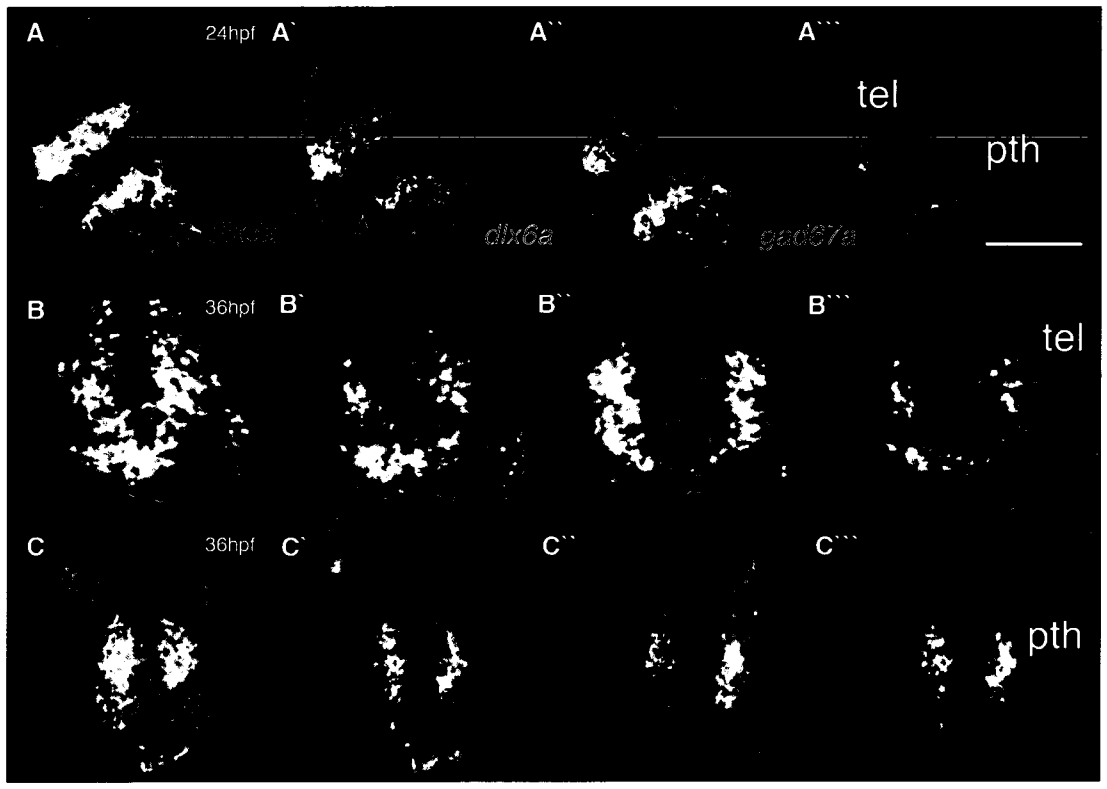
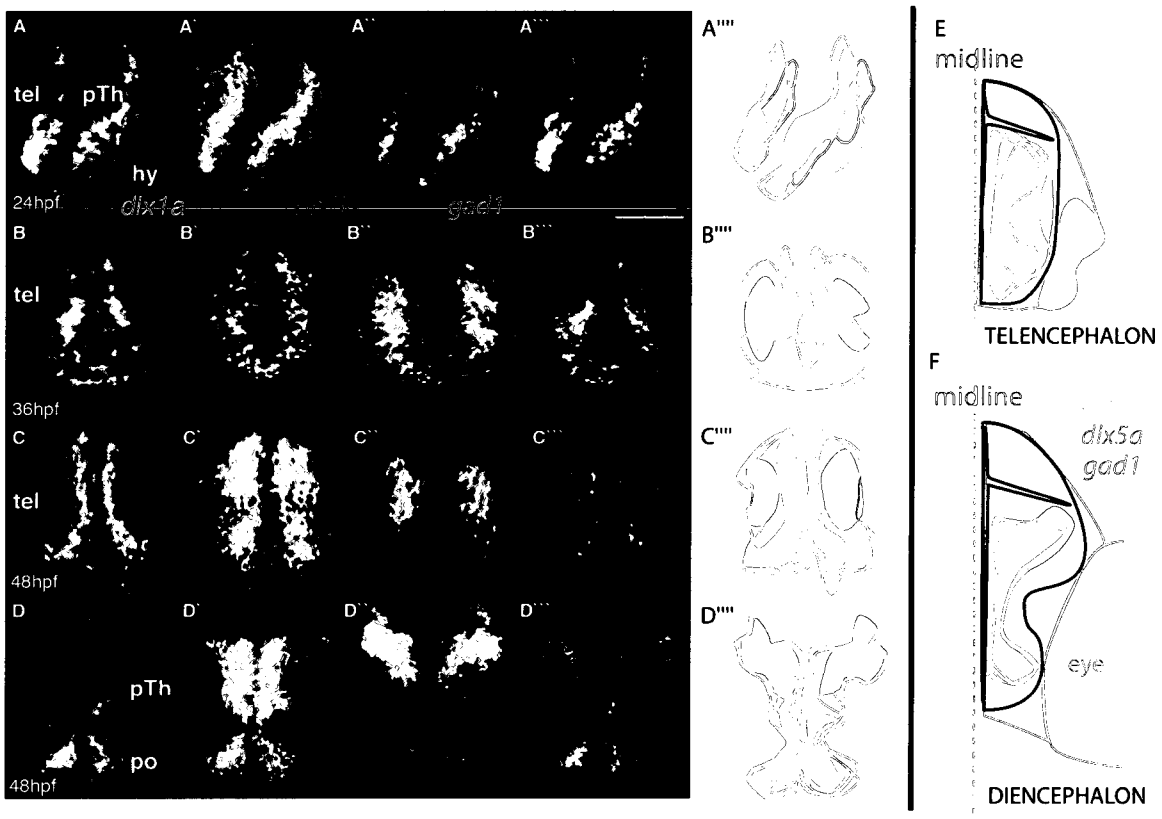

A
24hpf
A`
A``
A```
tel
pth
dlx6a
gad67a
B
36hpf
B`
B``
B```
tel
C
36hpf
C`
C``
C```
pth

Figure 2.2. Expression domains of *dlx* genes with respect to *gad1* expression.

Single z sections of triple fluorescent *in situ* hybridization for *dlx1a*, *dlx5a*, and *gad1* in the telencephalon at 24hpf (A-A'', sagittal section, anterior is on the left), 36hpf (B-B'', transversal section in the telencephalon, dorsal is up), and 48hpf (C-C'', transversal section in the telencephalon; D-D'', transversal section in the diencephalon; dorsal is up) with a colored merge of all three channels on A''', B''', C''' and D'''. For each section level, a schematic is given to localize the limits of each expression domain (solid line) and a domain of higher level of expression is represented as a colored surface. Panel E gives a schematical representation of the partially nested expression domains in the telencephalon (left half) and diencephalon (right half) at 48hpf, after data shown on panels C-C''' and D-D''': the proximal (ventricular) domain where only *dlx1a* is detected is shown green and the lateral domain where *gad1* only is detected is colored red. hy, hypothalamus; po, preoptic area; pTh, prethalamus; tel, telencephalon. Scale bar= 100 μm

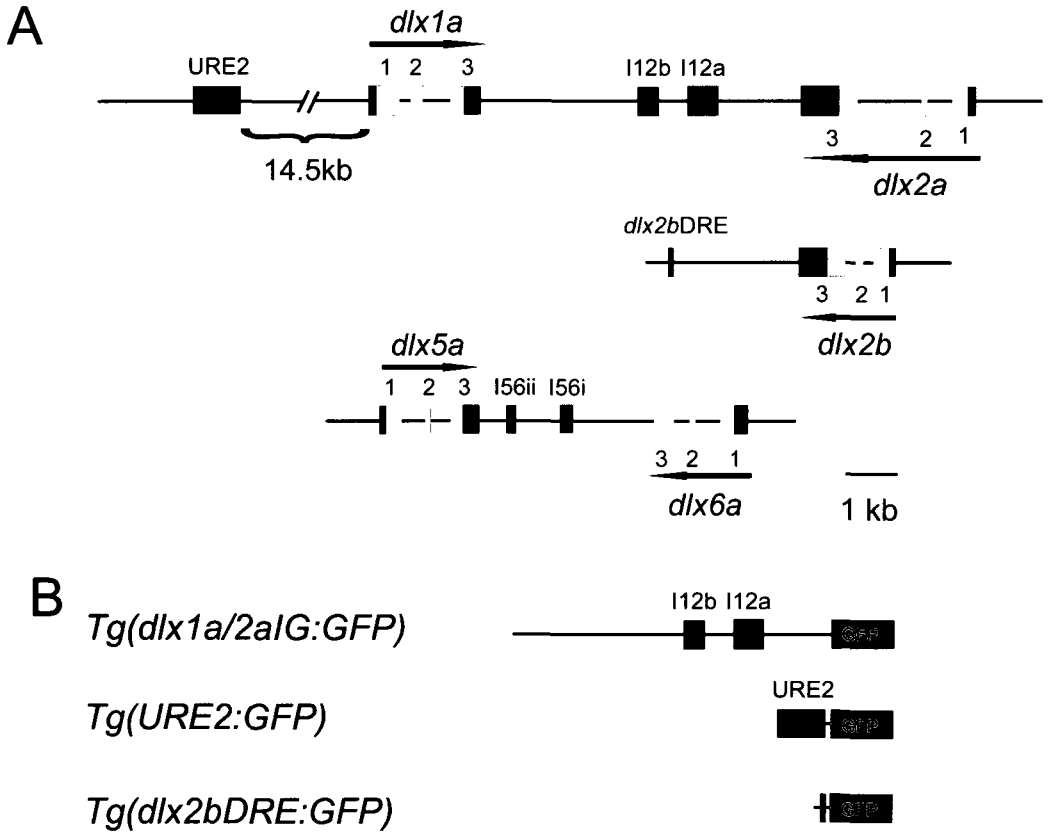

A
A`
A``
A```
tel
pTh
hy
24hpf
dlx1a
gad1
B
B`
B``
B```
tel
36hpf
C
C`
C``
C```
tel
48hpf
D
D`
D``
D```
pTh
po
48hpf
A''''
B''''
C''''
D''''
E
midline
TELENCEPHALON
F
midline
dlx5a
gad1
eye
DIENCEPHALON

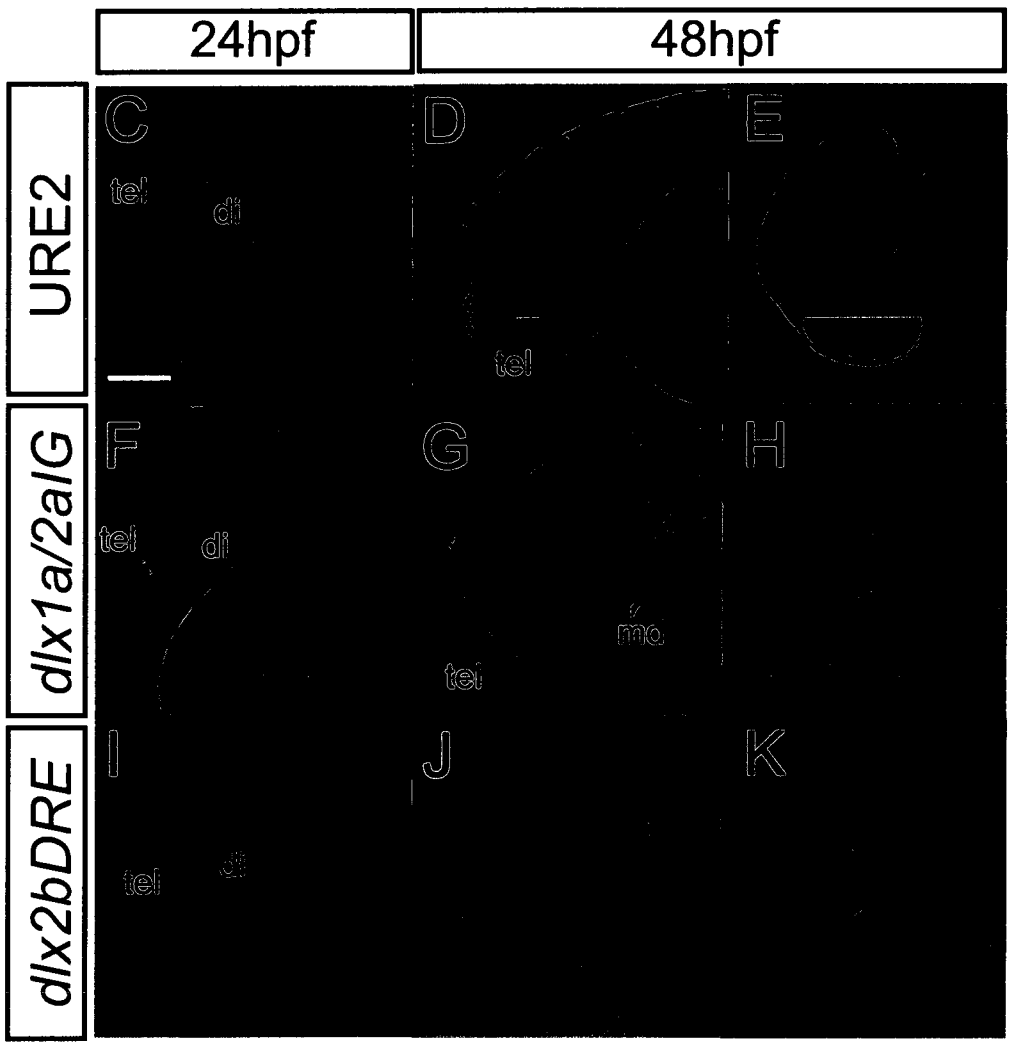
continuing at least until 48hpf, overt differences are observed between *dlx1a*, *dlx5a*, and *gad1* expression patterns within the telencephalon (Fig. 2.2B and C and see schematic on Fig. 2.2E). The medial limit of *dlx1a* expression was found at the level of the ventricle, while the medial limit of expression for both *dlx5a* and *gad1* was found more lateral. As for the lateral limits of *dlx5a* and *gad1* expression domains, these are found outside of the *dlx1a* expression domains. In addition, relatively stronger expression of *dlx1a* is detected close to the ventricle while stronger expression of *gad1* is located in the lateral part of the telencephalon (see Fig. 2.2C'''', and Fig. 2.2E). In the diencephalon, the *dlx1a*, *dlx5a*, and *gad1* expression domains are very comparable in the preoptic area and prethalamus (Fig. 2.2D), although the limit of *gad1* expression is found more dorsal than that of the *dlx* genes. The levels of transcripts are not homogenous within the *dlx1a* and *gad1* domains, with *dlx1a* weakly expressed and *gad1* highly expressed in the dorsal-most domain of the prethalamus and vice versa in the preoptic area (Fig. 2.2D'''').

2.2.2 The activity of zebrafish *dlx* enhancers is similar to their mouse counterparts

The I12a and I12b enhancers, located in the mouse *Dlx1/2* intergenic region, have regulatory functions when tested in transgenic mice, with only I12b displaying forebrain activity (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007). The mouse URE2 enhancer is located upstream of *Dlx1* and drives expression in a number of tissues, one of which is the developing forebrain (Ghanem et al. 2007). To test if orthologous sequences from the zebrafish genome have regulatory function in the zebrafish, we designed two reporter constructs containing these genomic sequences (Fig. 2.3A, B). We selected 6kb of the *dlx1a/2a* intergenic region, including I12a and I12b, and 900bp encompassing URE2. These sequences were cloned upstream of a β-globin minimal

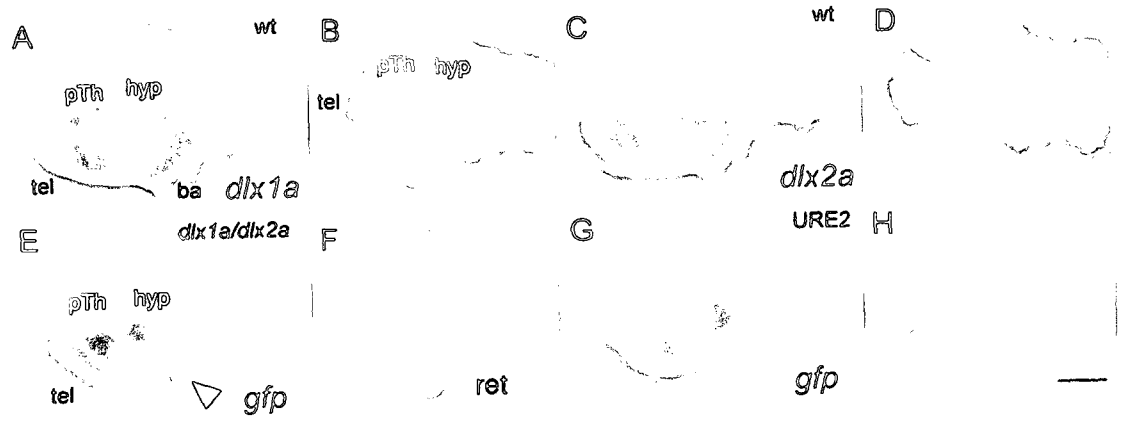
Figure 2.3. Regulatory elements of *dlx1a/2a* and *dlx2b* drive reporter gene expression in the zebrafish forebrain and branchial arches. (A) The *dlx* genes are typically organized in convergently transcribed bigene pairs (*dlx1a* with *dlx2a*; *dlx5a* with *dlx6a*), while *dlx2b* is found as a single gene. Exons are numbered in white and UTRs in black. Intergenic regulatory elements shown as blue (URE2 and I12b) and red boxes (I56i and I56ii) and *dlx2b*DRE in purple. (B) Schematic of the transgene constructs (containing the β-globin minimal promoter (grey) linked to GFP (green)) used to generate transgenic zebrafish. The *Tg(dlx1a/dlx2a:GFP)* construct contains the 6kb of the intergenic region between *dlx1a* and *dlx2a*, the *Tg(URE2dlx1a/2a:GFP)* contains the URE2 enhancer fragment and the *Tg(dlx2bDRE:GFP)* containing 200 base pairs downstream of *dlx2b*. The activity of the transgene constructs in the forebrain at 24hpf and 48hpf (C-E) *Tg(URE2dlx1a/2a:GFP)* transgene (F-H) *Tg(dlx1a/dlx2a:GFP)* transgene (I-K) *Tg(dlx2bDRE:GFP)*. tel, telencephalon; di, diencephalon; md, mandibular portion of branchial arches. Scale bar= 100 μm


A
dlx1a
URE2
1 2 3
I12b I12a
3 2 1
14.5kb
dlx2a
dlx2bDRE
3 2 1
dlx2b
dlx5a
1 2 3 I56ii I56i
3 2 1
dlx6a
1 kb
B
Tg(dlx1a/2aIG:GFP)
I12b I12a
Tg(URE2:GFP)
URE2
Tg(dlx2bDRE:GFP)


24hpf
48hpf
dlx2bDRE
dlx1a/2aIG
URE2
C
D
E
F
G
H
I
J
K
tel
di
tel
tel
di
mo
tel
tel
di

promoter associated with the coding sequences of GFP to produce the *Tg(dlx1a/2aIG:GFP)* and the *Tg(URE2dlx1a/2a:GFP)* reporter transgenes, respectively. Embryos harboring the *Tg(URE2dlx1a/2a:GFP)* transgene show GFP expression in the telencephalon and diencephalon starting at approximately 24hpf (Fig. 2.3C). GFP expression can be detected in the telencephalon and diencephalon from 24hpf until at least 72hpf (Fig. 2.3D and E). Expression of GFP in the *Tg(URE2dlx1a/2a:GFP)* line can also be detected in the mesenchyme of the visceral arches at 96hpf (data not shown). The *Tg(dlx1a/2aIG:GFP)* embryos show GFP expression in domains highly similar to those observed in *Tg(URE2dlx1a/2a:GFP)* embryos with fluorescence in the telencephalon and diencephalon starting at 24 hpf (Fig. 2.3F). Transgene expression can also be detected in the jaw region starting at approximately 48hpf (Fig. 2.3G). The overall forebrain GFP expression of *Tg(dlx1a/2aIG:GFP)* and *Tg(URE2dlx1a/2a:GFP)* lines closely mimics the endogenous forebrain expression of the *dlx1a* and *dlx2a* genes (Fig. 2.4). However, the transgenes are only active in small domains within the branchial arches and not at all in the olfactory or otic placodes, other areas of known *dlx1a/2a* expression (Akimenko et al. 1994; Ellies et al. 1997). The *Tg(dlx1a/2aIG:GFP)* transgene also targets GFP expression in the retina at 48hpf, an area where expression of *dlx1a* or *dlx2a* has not been previously reported (Fig. 2.4F).

Figure 2.4. The *dlx* regulatory elements recapitulate endogenous *dlx* expression in the forebrain of the zebrafish at 48hpf. Whole mount *in situ* with probes against *dlx1a* (A, B), *dlx2a* (C, D) and *gfp* (E,H) at 48hpf. E, F) *Tg(dlx1a/dlx2a:GFP)* is active in the telencephalon, ventral thalamus, and hypothalamus. There is also activity in the pharyngeal arches (arrowhead), which recapitulates a small region of endogenous *dlx* arch expression. G, H) URE2 activity closely resembles the activity of *Tg(dlx1a/dlx2a:GFP)* in the telencephalon, ventral thalamus, and hypothalamus and mimics *dlx* expression in these tissues. tel, telencephalon; pTh, prethalamus; hyp, hypothalamus; di, diencephalon; md, mandible; ba, branchial arches. Scale bar= 100 μm


A
wt
pTh
hyp
tel
ba
dlx1a
B
C
wt
D
pTh
hyp
tel
dlx2a
E
dlx1a/dlx2a
F
G
URE2
H
pTh
hyp
tel
gfp
ret
gfp

2.2.3 *dlx* enhancer activity closely mimics endogenous *dlx* expression in the telencephalon

To determine if the *dlx* regulatory elements are active in cells that express Gad proteins, we co-labeled transverse sections of the zebrafish forebrain with antibodies against GFP and Gad proteins (Fig. 2.5). At 48hpf, *Tg(dlx1a/2aIG:GFP)* reporter expression is located close to the ventricle and dorsally in the telencephalon but Gad proteins are localized more laterally (Fig. 2.5A). Very few cells are co-expressing Gad and GFP and those rare cells are located in the dorsolateral domain of the telencephalon (Fig. 2.5A). Expression of the *Tg(URE2dlx1a/2a:GFP)* reporter transgene is detected close to the ventricle and ventrally in the subpallium of the telencephalon, with only very few cells co-expressing GFP and Gad, again located in a dorsolateral domain (Fig. 2.5B). To assess the activity of *dlx5a/6a* regulatory elements in the developing zebrafish forebrain we utilized the *Tg(dlx5a/6aIG:GFP)* transgenic line containing the two enhancers, I56i and I56ii, that recapitulate endogenous *dlx5a/6a* expression (Zerucha et al. 2000; Mione et al. 2008). The *Tg(dlx5a/6aIG:GFP)* embryos show reporter expression in the telencephalon and diencephalon of zebrafish embryos and most Gad positive cells co-express GFP (Fig. 2.5C, data not shown) (Mione et al. 2008). These data suggest that the enhancers associated with the *dlx1a/2a* bigene cluster drive expression in the ventricular zone of the subpallium, while the enhancers associated with the *dlx5a/6a* bigene cluster drive expression in more lateral cells expressing Gad proteins. The expression domains described for GFP in transgenic lines are therefore consistent with the spatially restricted expression of the *dlx* genes (Fig. 2.6).

Figure 2.5. Immunolocalization of GFP and Gad proteins on transverse cryosections of the telencephalon at 48hpf. Expression domain of EGFP (A, B, C) and Gad proteins (A', B', C') is detected in the three transgenic lines, a merge is presented on panels A'', B'' and C'' to show overlap (yellow arrows). An interpretative schematic of a hemi-section is presented in each case on panels A''', B''' and C'''. Scale bars: 50 μm

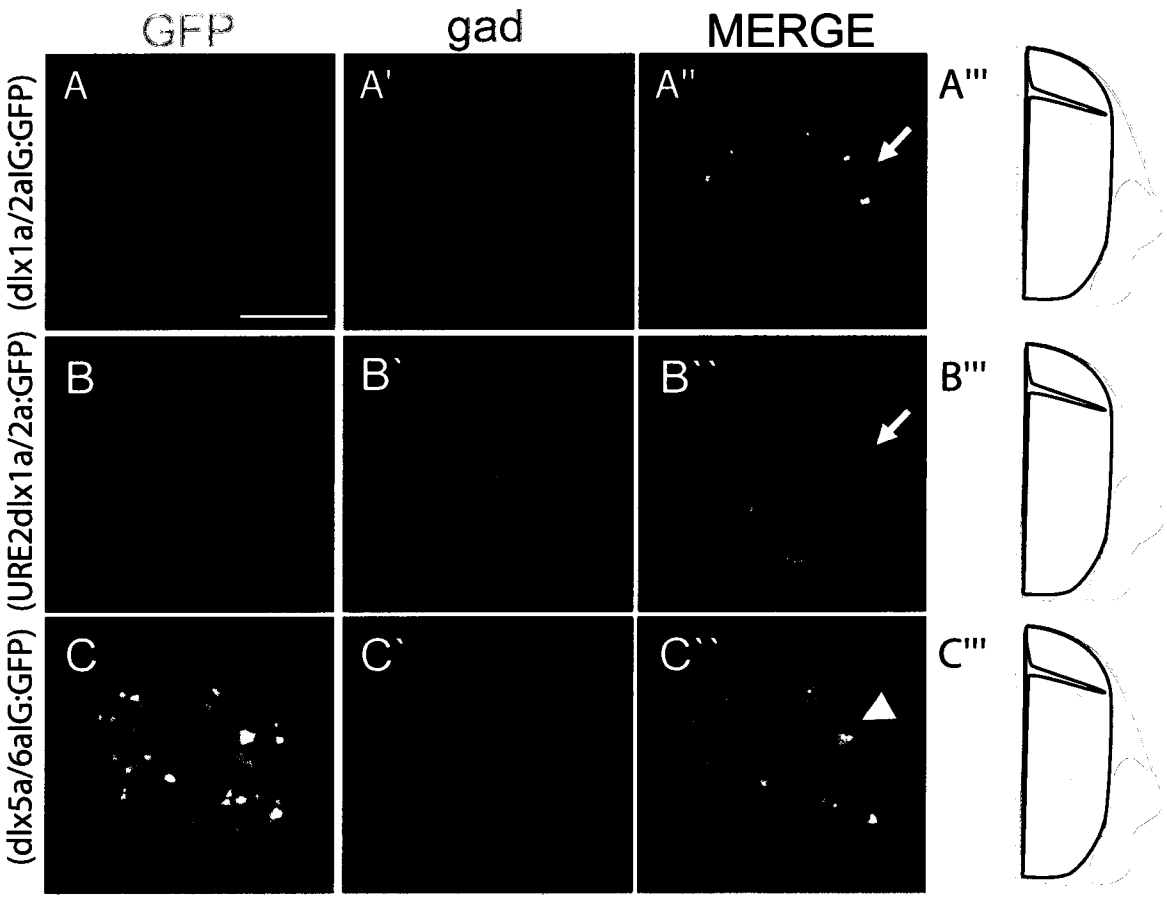

GFP
gad
MERGE
A
A'
A''
A'''
B
B`
B``
B'''
C
C`
C``
C'''
(dlx1a/2alG:GFP)
(URE2dlx1a/2a:GFP)
(dlx5a/6alG:GFP)

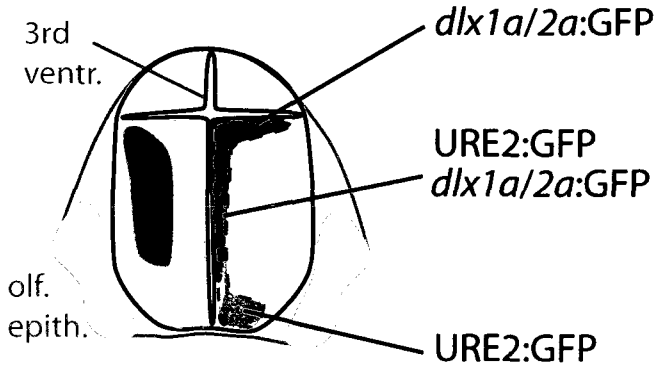
Figure 2. 6. Schematic showing the overlap of *dlx* and *gad* expression (left) and the activity of *dlx* regulatory elements (right) in the zebrafish forebrain.

dlx1a/2a/2b

dlx1a/2a/2b/5a/6a

dlx5a/6a

dlx5a/6a gad67a



2.2.4 Co-expression of paralogous genes and insights into the regulation of zebrafish *dlx2b* expression

The I12b and URE2 enhancers appear to be sufficient to recapitulate expression of *dlx1a/dlx2a* in the forebrain. The paralogous gene to *dlx2a*, *dlx2b,* is also expressed in similar domains of the forebrain (Fig. 2.1). These highly overlapping domains of expression for *dlx1a*, *dlx2a*, and *dlx2b* suggest the existence of a regulatory sequence at the *dlx2b* locus that would be similar, in sequence and/or in function, to those described for the *dlx1a/2a* locus. Phylogenetic footprinting of the *dlx2b* locus identified a 60bp sequence located in the 3' region of the gene and showing similarity to other *dlx* enhancers, including two putative *dlx* transcription factor-binding sites (Ghanem et al. 2003). We amplified a 200 bp region downstream of *dlx2b*, including these 60bp, that we named *dlx2b* Downstream Regulatory Element (*dlx2b*DRE), and placed it in a β-globin:GFP transgene which was tested in primary transgenic zebrafish embryos (Fig. 2.3B). This transgene targets GFP expression in the telencephalon and diencephalon of 84% (77/92) of primary injected zebrafish at 24hpf (Fig. 2.3I). This expression persists until at least 48hpf in the telencephalon and diencephalon and closely resembles that observed in the *Tg(URE2dlx1a/2a:GFP)* and *Tg(dlx1a/2aIG:GFP)* transgenic lines (Fig. 2.3J and K).

2.3 DISCUSSION

2.3.1 Conserved activity of *dlx* regulatory elements between zebrafish and mouse

Several conserved regulatory elements have been identified that are sufficient to mimic many aspects of endogenous *Dlx* expression in the mouse (Zerucha et al. 2000;

Ghanem et al. 2003; Ghanem et al. 2007). Similarly, the distinct expression domains of the *dlx* genes in the forebrain seem to be completely recapitulated by orthologous zebrafish enhancers. Therefore, our results show that zebrafish and mouse regulatory elements not only show strong sequence conservation, but also are sufficient to drive similar expression in homologous tissues (forebrain and pharyngeal arches) indicating their activity has been conserved throughout vertebrate evolution. The expression domains of the *Tg(URE2dlx1a/2a:GFP)* and *Tg(dlx1a/2aIG:GFP)* transgenes are similar in the forebrain, although the former shows an extended expression domain in the ventral subpallium while the latter has an extended expression domain in the dorsal subpallium. Heterologous transgenic experiments have indicated that zebrafish *dlx5a/6a* regulatory sequences are functional in the mouse forebrain, mimicking the endogenous *Dlx* gene expression patterns (Zerucha et al. 2000; Stühmer et al. 2002). Consistent with these results, the URE2 element from distantly related vertebrates has conserved function in the forebrain of zebrafish and mice (R.B.M., M.D.T, and M.E., unpublished observations). Taken altogether, the high levels of conservation of the sequence and regulatory function strongly suggest that the genetic cascades upstream of the *Dlx* genes are conserved between distantly related vertebrates.

2.3.2 Spatial-temporal expression of *dlx* and *gad* genes is conserved between mouse and zebrafish

The *Dlx* genes have been shown to play an important role in the differentiation and migration of GABAergic interneurons in mice (Anderson et al. 1997a; Anderson et al. 1997b; Stühmer et al. 2002a; Stühmer et al. 2002b). The *Dlx1/2* genes are expressed in immature neurons and *Dlx5/6* in more mature neurons, eventually differentiating into

Gad positive interneurons (Liu et al. 1997; Eisenstat et al.1999; Stühmer et al. 2002b; Yun et al. 2003). Previous studies of gene expression and cell proliferation revealed similarities in the topological relationships of mouse and zebrafish telencephalon (For reviews see: Wullimann and Mueller, 2004; Mueller and Wullimann, 2009; Wullimann, 2009). Thus, the ventricular (proximal) zone is a site of proliferation and expresses *dlx2a* (Mueller et al. 2002; Mueller et al. 2008). The expression domain of *dlx5a* (and by extension, *dlx6a*) is shifted laterally compared to *dlx1a* (*dlx2a*), and overlaps partially with the more lateral of *gad1* expression domain. These relative expression patterns can be indicative of a temporal sequence of gene expression (*dlx1a/2a* →*dlx5a/6a*→ *gad1*) in cells moving from the proliferative zone to more lateral regions of the telencephalon and may be related to the various stages of GABAergic neuron differentiation. This apparent movement of cells from the ventricular zone to more lateral domains may be due to tangential and/or radial migration, as both modes of migration have been previously identified in the zebrafish telencephalon (Mueller et al. 2006; Mueller et al. 2008; Mione et al. 2008). As the domains of enhancer activity are consistent with gene expression data (Fig. 2.6), transgenic zebrafish lines may be useful to isolate neural progenitors from the forebrain at different stages of GABAergic interneuron differentiation.

2.3.3 Conserved genetic mechanisms leads to the highly overlapping expression domains of *dlx1a/2a* and *dlx2b*

The two *Dlx* genes of a bigene pair have highly overlapping expression domains that have been suggested to be due to shared regulatory regions (Ellies et al. 1997). In this study, we report the very similar expression domains of *dlx1a*, *dlx2a* and *dlx2b* in the forebrain starting at 24hpf and continuing until at least 48hpf. We explored the possibility

that *dlx2b* expression was controlled by sequences resembling *dlx1a/2a* enhancers, such as URE2 or I12b. Sequence comparisons of the forebrain enhancers (I12b, I56i, and I56ii) to the *dlx2b* locus had identified a 60bp sequence containing two putative *dlx* binding sites located downstream of *dlx2b* (Ghanem et al. 2003). Given the position of this element, it could be paralogous to the I12b sequence located between *dlx1a* and *dlx2a*, but would have accumulated mutations since the whole-genome duplication event specific to teleosts. Here we show that this *dlx2b*DRE region is sufficient to drive reporter gene expression in the forebrain, with patterns similar to those observed with the *dlx1a/2a* transgenes, suggesting that similar regulatory mechanisms may be involved in *dlx2b* and *dlx1a/2a* expression. The *dlx2b*DRE forebrain activity may also be attributable to the presence of the two *dlx* binding sites within a conserved 60bp region. A region from the I56i enhancer containing two *Dlx* binding sites was sufficient to drive forebrain expression of a reporter gene in transgenic mice (Zerucha et al. 2000; Ghanem et al. 2003). The conservation of the *dlx* binding sites is suggestive of potential cross-regulation of *dlx2b* expression by *dlx1a* and/or *dlx2a*, as the mouse Dlx2 protein has been shown to bind directly to DNA sequences organized in a similar context (Zerucha et al. 2000; Zhou et al. 2004; Potter et al. 2009). Cross-regulation between *dlx1a/dlx2a* and *dlx2b* via the *dlx2b*DRE would explain the strong overlap in expression. Overall, the highly overlapping expression of the *dlx1a/2a* bigene pair with the unlinked *dlx2b* is suggestive of similar gene regulation mechanisms at the two loci.

In conclusion, we have described the spatial overlap between *dlx* bigene pairs and *gad1* expression in the embryonic zebrafish forebrain. The *dlx1a/2a* genes are expressed close to the ventricle, *dlx5a/6a* are expressed throughout the subpallium, and *gad1* is

expressed in the lateral most regions consistent with the hypothesis that *dlx* genes play a role in GABAergic interneuron differentiation in the zebrafish forebrain, similarly to what is known in the mouse. The *dlx* regulatory elements have conserved function in the zebrafish and mouse forebrain, and mimic the spatial relationship between *dlx* and *gad1* within the zebrafish telencephalon. These results support the idea that genetic pathways controlling *dlx* gene expression and GABAergic interneuron development, in the forebrain of mammals and teleosts, may be conserved over approximately 430 million years of evolution.

2.4. Experimental Procedures

2.4.1 Zebrafish Husbandry

Wild type zebrafish were raised according to standard procedures (Westerfield, 2000). Embryos were staged in hours post-fertilization (hpf) according to specific criteria outlined by Kimmel et al. 1995. All experiments were carried out in accordance with animal care guidelines provided by the Canadian Council on Animal Care and the University of Ottawa animal care committees approved all protocols.

2.4.2. *dlx* transgene constructs

The *dlx1a/2aIG*:GFP transgene construct is made up of a six kilobase (kb) *HindIII-HindIII* region from the *dlx1a/2a* bigene intergenic (IG) locus containing the two identified enhancers I12a and I12b (Ellies et al. 1997b; Ghanem et al. 2003). This region was cloned into a modified SP72 vector (Promega, Madison, WI) containing a human β-globin minimal promoter and green fluorescent protein (GFP) cassette and has no activity in zebrafish on its own. The URE2*dlx1a/2a*:GFP transgene construct contains 900 bp of a region 5` to the *dlx1a* transcriptional start site (Ghanem et al. 2008). This region was

PCR amplified using the primers 5` GCAAAGCACAGAATTATTCT 3` and 5` CTTTTAGGGTTTTTGTTCGGA 3`. This 900 bp fragment was cloned into a second modified SP72 vector containing the *β*-globin:GFP cassette, and Tol2 transposase recognition sites.

2.4.3. Generating and visualization of transgenic zebrafish

Transgenic *dlx1a/2aIG*:GFP zebrafish lines were generated as described in Amsterdam *et al.* 1995. The *dlx1a/2aIG*:GFP construct was injected at 200ng/µl in standard DNA microinjection buffer (0.2 mM KCl, 0.1% phenol red). The URE2*dlx1a/2a*:GFP construct was co-injected at 50 ng/µl with 50 ng/µl of tol2 *transposase* mRNA following standard procedures as described by (Fisher et al. 2006b). Approximately 200 zebrafish larvae expressing GFP were obtained for each construct and retained as founders for transgenesis. Founder transgenic zebrafish were intercrossed and at least 100 embryos for each pair was screened by GFP fluorescence at 24-48 hpf. At least two independent transgenic lines were generated for both *dlx1a/2aIG*:GFP and URE2*dlx1a/2a*:GFP constructs and yielded identical results. GFP positive F1 embryos were raised to adulthood and intercrossed to establish stable transgenic lines. Screening for GFP positive embryos was done on a Nikon NBZ 1500 dissecting microscope and imaging was done with a Nikon DXM 1200C digital camera.

2.4.4 *in situ* hybridizations

Whole mount mRNA *in situ* hybridizations were done as described in Thisse and Thisse (1998). The antisense mRNA probes were labeled with digoxygenin-11-UTP (Roche, 11277073910) and synthesized from cDNA clones: *dlx1a* (Ellies et al. 1997b), *dlx2a* (Akimenko et al. 1994), *dlx2b* (Ellies et al. 1997b), *dlx5a* (Akimenko et al. 1994),

dlx6a (Ellies et al. 1997b), *gad1* (Mueller et al. 2008). The GFP probe was synthesized as complementary to the sequence amplified by the primers 5` AAGGGCGAGGAGCTGTTCAC 3` and 5` GAACTCCAGCAGGACCATGT 3` then cloned into the pDrive vector (Qiagen, Valencia, CA).

2.4.5. Fluorescent *in situ* hybridizations

Probes for fluorescent *in situ* hybridizations were synthesized using standard protocols. The *dlx1a* probe was synthesized using Digoxigenin-11-UTP, *dlx2a* and *dlx5a* probes were synthesized using the Fluorescein labeling mix (Roche, 11685619910), and the *dlx2b* and *gad1* probes were labeled with DNP-11-UTP (Perkin Elmer, NEL555001EA). This protocol was adapted by J.C.T from Welton et al. 2006 and Jowett and Yan, 1996. Embryos were brought through a methanol/PBST (1X PBS, 0.25% tween-20) series (75%, 50%, 25%) and 4 X PBST washes at 5 min per wash. Embryos at different embryonic stages were digested with proteinase K (1µg/ml in PBST) at room temperature for the following lengths: 24hpf for 10 min, 36hpf for 30 min, and 48hpf for 45 min. Embryos were post-fixed in 4% PFA for 20 min and washed 5 X 5mins in PBST. Embryos were incubated with 1 ml pre-hybridization solution (50% formamide, 5X SSC, 100 µg/ml yeast RNA, 50 µg/ml Heparin, 0.25% tween-20, Citric acid to pH 6.0) for 2-4 hours at 65 °C. The pre-hybridiaztion solution was replaced with 200 µl pre-hybridization solution plus 200ng of each RNA probe and incubated overnight at 65 °C. The probes are removed and the embryos are washed 5 min in hybridization solution (HS; 50% formamide, 5X SSC, 0.25% tween-20), 5 min 75% HS/2X SSC, 5 min 50% HS/2X SSC, 5 min 25% HS/2X SSC, 5 min 2X SSC (2X SSC, 0.25% tween-20), and 3X 20 min 0.2X SSC with 0.25% tween-20. All washes were done at 65 °C.

2.4.6. Detection of fluorescein labeled probe

Detection of fluorescein and digoxigenin labeled probes were done with the TSA Cyanide 3 and Fluorescein system (Perkin Elmer, NEL753001KT). While detection of DNP probes was done with the TSA Cyandide 5 system (Perkin Elmer, NEL745001KT). Embryos are washed 2 X PBST at room temperature and placed in 2% hydrogen peroxide in PBST on a shaker for 1 hour. Embryos were washed 4 X 5 min in TNT (0.1 M Tris-HCl pH=7.5, 0.15 M NaCl, 0.5% tween-20) and blocked four hours in 500 µl TBSTB (TNT with 0.5% Perkin-Elmer blocking powder). The blocking solution was replaced with 500 µl of 1:5000 anti-fluorescein-POD (Invitrogen, A-21253) in TBSTB and rocked overnight at 4 °C. Embryos were washed 8 X in TNT at room temperature over the course of 2 hours. Embryos were washed 5 min in 50 µl of Perkin-Elmer amplification dilutent and replaced with 50 µl of Tyr-Fluorescein in amplification dilutent (1:50). All steps from here were carried out in the dark. The tubes were placed on a shaker for 1 hour and washed 2 X in TNT. To remove peroxidase activity embryos were washed for one hour in 2% hydrogen peroxide/TNT.

2.4.7. Detection of digoxigenin probes

Embryos were washed 4 X 5 min in TNT and blocked for 4 hours with 500 µl TBSTB. The block was replaced with 500 µl 1:1000 anti-DIG-POD (Roche, 1207733) in TBSTB and rocked overnight at 4 °C. Embryos were washed 8 X in TNT at room temperature over the course of 2 hours. Embryos were washed 5 min in 50 µl of Perkin-Elmer amplification dilutent and replaced with 50 µl of Tyr-Cy3 in amplification dilutent (1:50). All steps from here were carried out in the dark. The tubes were placed on a

shaker for 1 hour and washed 2 X in TNT. To remove peroxidase activity embryos were washed for one hour in 2% hydrogen peroxide/TNT.

2.4.8. Detection of DNP labeled probes

Embryos were washed 4 X 5 min in TNT and blocked for 4 hours with 500 µl TBSTB. The block was replaced with 500 µl 1:200 anti-DNP-POD (Perkin Elmer, NEL747A001KT) in TBSTB and rocked overnight at 4 °C. Embryos were washed 8 X in TNT at room temperature over the course of 2 hours. Embryos were washed 5 min in 50 µl of Perkin-Elmer amplification dilutent and replaced with 50 µl of Tyr-Cy5 in amplification dilutent (1:50). All steps from here were carried out in the dark. The tubes were placed on a shaker for 1 hour and washed 2 X in TNT. To remove peroxidase activity embryos were washed for one hour in 2% hydrogen peroxide/TNT. Embryos were washed 4 X 5 min in TNT and stored in 1% PFA/TNT at 4 °C until use.

2.4.9. Imaging of fluorescent *in situ* hybridizations

Embryos were placed on glass slides and positioned under coverslips for confocal imaging. Confocal z-stacks were obtained by using a Zeiss LSM5 PASCAL (Carl Zeiss, Germany) and excitation lasers were at 488 (Fluorescein), 543 nm (Cy3), 633 nm (Cy5). Channels were acquired sequentially to avoid cross talk between the different filters. LSM Image Manager initially processed the z stacks and Volocity LE software (Improvision) was utilized to slice in regions of interest.

2.4.10. Immunohistochemisty on zebrafish forebrain sections

Zebrafish embryos were collected and fixed using 4% PFA overnight, washed in PBS, and placed in 30% sucrose in PBS to equilibrate. Embryos were then embedded in Shandon cryomatrix (Thermo Scientific, 2860051) and frozen at -20 °C. Cryosections

were done on a Leica CM1850 (Leica Microsystems, Weltzar, Germany) at a thickness of 10 μm and stored at -20 °C until use. Sections were washed 3X in PBS 0.1% Triton X (PBST) for 10 minutes and blocked for 2 hours in 2% BSA in PBST. The sections were incubated with the primary antibody (see below) overnight in a humid chamber at 4 °C. After removal of the primary antibody with 3 X PBST washes, the appropriate secondary antibody was incubated on the slides for 2 hours at room temperature. After the final washes, the slides were mounted using Aquatex mounting media (VWR, 65036-62). Signals were visualized on a Nikon Eclipse E3600 stereomicroscope for both fluorescent stains. The following primary antibodies were used in this study: Rabbit anti-GFP (1:1000, Invitrogen, A-11122); Mouse anti-glutamic acid decarboxylase (BioMol International, GC3108). The following secondary antibodies were used in this study: Goat anti-rabbit Alexa Fluor488 (1:300, Invitrogen, A11008); Goat anti-mouse Alexa Fluor594 (1:300, Invitrogen, A11005).

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3. Functional conservation of a forebrain enhancer from the elephant shark (*Callorhinchus milii*) in zebrafish and mice.

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Abstract

The phylogenetic position of the elephant shark (*Callorhinchus milii*) is particularly relevant to study the evolution of genes and gene regulation in vertebrates. Here we examine the evolution of *Dlx* homeobox gene regulation during vertebrate embryonic development with a particular focus on the forebrain. We first identified the elephant shark sequence orthologous to the URE2 *cis*-regulatory element of the mouse *Dlx1/Dlx2* locus (herein named CmURE2). We then conducted a comparative study of the sequence and enhancer activity of CmURE2 with that of orthologous regulatory sequences from zebrafish and mouse. The CmURE2 sequence shows a high percentage of identity with its mouse and zebrafish counterparts but is overall more similar to mouse URE2 (MmURE2) than to zebrafish URE2 (DrURE2). In transgenic zebrafish and mouse embryos, CmURE2 displayed enhancer activity in the forebrain that overlapped with that of DrURE2 and MmURE2. However, we detected notable differences in the activity of the three sequences in the diencephalon. Outside of the forebrain, CmURE2 shows enhancer activity in areas such as the pharyngeal arches and dorsal root ganglia where its' counterparts are also active. Our transgenic assays show that part of the URE2 enhancer activity is conserved throughout jawed vertebrates but also that new characteristics have evolved in the different groups. Our study demonstrates that the elephant shark is a useful outgroup to study the evolution of regulatory mechanisms in vertebrates and to address how changes in the sequence of *cis*-regulatory elements translate into changes in their regulatory activity.

3. 1. Introduction

Changes in gene expression patterns, via changes in *cis*-regulatory elements or in the *trans*-acting factors binding to these elements have largely contributed to the development of novel morphological structures during evolution (Prud'homme et al. 2007). The high degree of conservation in the coding region of genes necessary to establish the animal body plan has been extensively documented. The growing wealth of metazoan genome sequence data has also provided evidence for the conservation of sequences outside the coding regions of genes, the Conserved Non-coding Elements (CNEs) that tend to be located close to developmental genes (Siepel et al. 2005; Sandelin et al. 2004). However, whether the conservation of CNE sequence is necessary for any conservation of CNE regulatory activity remains debateable. Recent studies have identified regulatory sequences with very little sequence conservation that have the ability to activate transcription in highly similar tissues (Fisher et al. 2006; Visel et al. 2008; McEwen et al. 2009; Weirauch and Hughes, 2009). Furthermore, highly conserved regulatory sequences can drive transcription in highly divergent patterns (Blader et al. 2004; Navratilova et al. Navratilova et al. 2009a, b). Therefore, it remains challenging to predict regulatory activity based solely upon sequence similarity, or vice versa. Currently, a great effort has been made in systematically characterizing the CNEs in the mouse genome allowing for comparison with other model and non-model organisms.

Dlx homeobox genes of vertebrates are involved in the development of the forebrain, visceral arches, sensory organs, and limbs (for review see: Panganiban and Rubenstein, 2002). They are organized as three convergently transcribed bigene clusters, present in most jawed vertebrates: *Dlx1/Dlx2*, *Dlx3/Dlx4* and *Dlx5/Dlx6* (Ellies et al.

1997b). The three bigene clusters most likely originate from the duplications of an ancestral bigene cluster occurring as whole genome duplication events that accompanied vertebrate evolution (Stock et al. 1996; Amores et al. 1998; Dehal and Boore, 2005). A similar bigene arrangement of *Dlx* genes has been reported in the ascidian *Ciona intestinalis* (Caracciolo et al. 2000), suggesting that the ancestral bigene existed prior to vertebrate radiation. The bigene organization of *Dlx* genes appears to be important for the concerted expression of the two genes within each cluster as *cis*-regulatory elements (CREs) have been identified in the relatively short (~3-15kb) intergenic regions separating the two genes of each cluster (Zerucha et al. 2000; Ghanem et al. 2003).

Of the six *Dlx* genes found in tetrapod vertebrates, the *Dlx1/Dlx2* and *Dlx5/Dlx6* clusters are involved in forebrain development. We have previously reported three CREs from the intergenic regions of these two bigene clusters in mouse: I12b from the *Dlx1/Dlx2* locus and I56i and I56ii from the *Dlx5/Dlx6* locus (Zerucha et al. 2000; Ghanem et al. 2003). However, CREs regulating *Dlx* expression can also be found outside the intergenic region and we reported one such CRE named Upstream regulatory element 2 (URE2), located approximately 12 kb upstream of the mouse *Dlx1* gene (Ghanem et al. 2007). We have previously shown some degree of conservation in the function of *Dlx* CREs between mouse and zebrafish but it is not clear when these CREs and their associated regulatory mechanisms originated during vertebrate evolution (Zerucha et al. 2000; Ghanem et al. 2003).

As a cartilaginous fish, the elephant shark (*Callorhinchus milii*) occupies an interesting phylogenetic position as the sister group to bony fishes and tetrapods. Its relatively small genome of 910 megabases and the availability of a low coverage genome

(~1.4×) make this species a useful cartilaginous fish model to examine conservation of CREs (Venkatesh et al. 2007). Large scale comparison of the conserved non-coding regions between the elephant shark and the human and/or zebrafish genomes has shown that much more CNEs are shared between human and elephant shark than between human and zebrafish (Venkatesh et al. 2006; Venkatesh et al. 2007). These data are consistent with the observation of greater conservation in gene synteny between human and elephant shark genomes than between human and zebrafish genomes (Venkatesh et al. 2007). This may seem counterintuitive given that mammals share a more recent common ancestor with teleost fishes than with cartilaginous fishes. However this situation likely results from the 'fish-specific' whole genome duplication event that occurred before the teleost radiation and led to loss or modification of CNEs and a high level of genome re-organization in this group (Venkatesh et al. 2007; Ravi et al. 2009). A detailed analysis of the conservation of CNEs associated with the *Hox* clusters in the elephant shark, human and fugu yielded various hypotheses on a possible correlation between the level of sequence conservation of vertebrate CNEs and their functional variation (Ravi et al. 2009).

Here, we report the identification of an elephant shark sequence orthologous to the conserved regulatory element URE2 associated with *Dlx1* and *Dlx2* genes. Sequence comparisons show a high level of conservation within gnathostomes, with higher similarity between elephant shark and mouse URE2 than between elephant shark and zebrafish URE2. Here we show that the enhancer activity of the elephant shark URE2 (CmURE2) in transgenic mouse and zebrafish is highly similar to that of its orthologous mouse and zebrafish counterparts in transgenic assays. In addition, CmURE2 shows more

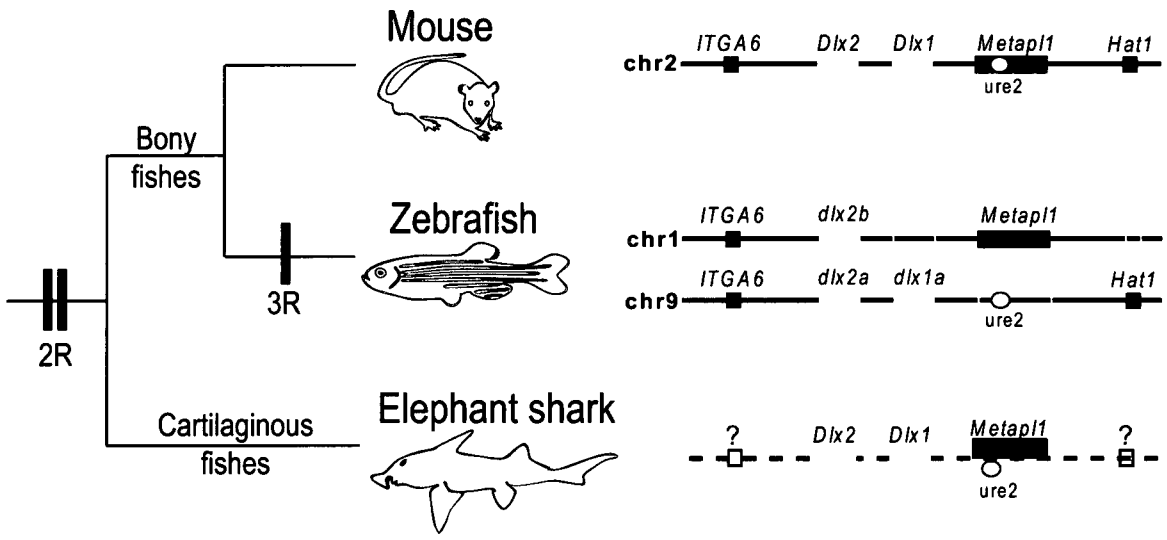
similarity in sequence and function to the orthologous mouse sequence than to the zebrafish sequence, in agreement with the hypothesis of additional genome and gene regulation remodelling subsequent to the teleost specific genome duplication.

3.2. Results

3.2.1. Sequence and synteny conservation near the *Dlx1/Dlx2* bigene cluster

The *Dlx* genes of most tetrapod vertebrates described thus far are organized as three *Dlx* bigene clusters. As a result of the whole genome duplication event occurring in their ancestors, several other *dlx* genes have been found in teleosts; (i) an additional *dlx1a/dlx2a* bigene cluster located on chromosome 9, and (ii) a *dlx2*-related gene, *dlx2b*, located on chromosome 1, which is not physically linked to a *dlx1*-like gene (Stock et al. 1996; Amores et al. 1998). Conserved synteny between the *dlx*-containing regions of zebrafish chromosomes 1 and 9 supports the idea that *dlx1a/dlx2a* and *dlx2b* arose from the duplication of a large chromosomal region, followed by the loss of the *dlx1*-like gene from the b cluster. Furthermore, the synteny is also conserved with a region of mouse chromosome 2 that contains the nearby genes *ITGA6*, *Metapl1* and *Hat1* (Figure 3.1). The presence of CREs within these genomic regions may contribute to the conservation of these synteny blocks (Engstrom et al. 2007, 2008; Kikuta et al. 2007). Interestingly, in the mouse, the MmURE2 *cis*-acting regulatory sequence, previously reported to be involved in *Dlx1/Dlx2* regulation and located 12 kb upstream of *Dlx1*, falls within the sixth intron of the *Metapl1* gene (Ghanem et al. 2007). In zebrafish, DrURE2 is located in a similar position upstream of the *dlx1a* gene but the unique *metapl1* ortholog is found in the corresponding position on chromosome 1 (Figure 3.1). We did not find any URE2-like sequence on zebrafish chromosome 1 which rules out the possibility of a URE2

Figure 3.1. The *Dlx1/Dlx2* bigene cluster in vertebrate evolution. Vertebrate phylogeny indicates the hypothesized position of the three genome duplication events (2R, 3R). The chromosomal neighbourhood of *Dlx1/Dlx2* in mouse and zebrafish presents the relative position of flanking genes and of URE2. In the mouse, URE2 falls within intron 6 of the *Metapl1* gene. Synteny information is not yet available for the elephant shark although the presence of the *Dlx1* and *Dlx2* genes on the same BAC clones as URE2 has been confirmed. Drawings are not to scale.


Mouse
Bony
fishes
Zebrafish
3R
2R
Cartilaginous
fishes
Elephant shark
chr2
ITGA6
Dlx2
Dlx1
Metapl1
Hat1
ure2
chr1
ITGA6
dlx2b
Metapl1
chr9
ITGA6
dlx2a
dlx1a
Hat1
ure2
?
Dlx2
Dlx1
Metapl1
?
ure2

enhancer–like sequence acting on the *metapl1* gene and suggests that the remaining *dlx2b* gene is not under the regulation of a URE2 sequence, except if the sequence has been highly remodeled after the duplication while still keeping its function. This, as well as loss of other regulatory elements found associated with the *dlx1a-dlx2a* bigene cluster (Ghanem et al. 2003), may account for the reduced domains of expression in comparison to *dlx2a* (Akimenko et al. 1994; Mueller et al. 2008).

To investigate the corresponding genomic region in a cartilaginous fish species, we searched the sequence of the elephant shark genome for *Dlx*- and URE2-like sequences. We found independent reads including putative Cm*Dlx1*, Cm*Dlx2*, Cm*Metapl1*, CmURE2 as well as CmI12a, a second *Dlx1/Dlx2* regulatory element. After screening of a BAC library for the putative Cm*Dlx1* sequence, we isolated a BAC clone from which we could also PCR-amplify Cm*Dlx2*, the two putative enhancer sequences CmURE2 and CmI12a, as well as exons 9 and 10 of the Cm*Metapl1* gene (Figure 3.1).

We produced an alignment of the elephant shark, mouse and zebrafish URE2 sequences (1017bp, Figure 3.2), half of which could be aligned with no ambiguity (517bp). The CmURE2 sequence closely resembles its mouse and zebrafish counterparts (Figure 3.2) with 85% identity between MmURE2 and CmURE2, 75% identity between MmURE2 and DrURE2, and 73% identity between DrURE2 and CmURE2. We then aligned the orthologous URE2 sequences extracted from the Ensembl Genome Browser (release 56; http://uswest.ensembl.org) for three other tetrapod species (a frog, *Xenopus tropicalis*; a lizard, *Anolis carolinensis*; a bird, *Gallus gallus*) and three other teleost species (the medaka, *Oryzias latipes*; and two pufferfish, *Takifugu rubripes* and *Tetraodon nigroviridis*) (Suppl. Figure 3.1). Again, the elephant shark sequence was

Figure 3.2. Sequence alignment of the elephant shark (Cm), mouse (Mm) and zebrafish (Dr) URE2 sequences. Alignable regions are highlighted grey, identical sequences (>10bp) between all three species are boxed, and the primers used to amplify these sequences are highlighted orange.


MmURE2
CmURE2
DrURE2

Supplemental figure 3.1. Jawed vertebrate URE2 sequences alignment and similarity. A. Alignment of URE2 sequences from tetrapods (Mm: *Mus musculus*; Gg: *Gallus gallus*; Ac: *Anolis carolensis*; Xt: *Xenopus tropicalis*) and teleosts (Dr: *Danio rerio*; Ol: *Oryzias latipes*; Tn: *Tetraodon nigroviridis*; Tr : *Takifugu rubripes*) with elephant shark (Cm) URE2 sequence highlighted grey. B. Percentage of identity between two sequences after gap exclusion in the previous alignment.

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55         65         75         85         95
MmURE2           GCAGAGAACA CTGGATTCTT ATTCAGGCAT TCTGTGGA-G -TTCTGCATT CATGGCTGTG TCTAAAGGGC ATGTCAGCCT TT--GATTCT CTCTGAGAGG
GgURE2           GCACAGAACA CTAGATTCTT ATTCAAGCAT TCTATCGA-G C-TCTGCATT CATGGCTGTG TCTAAAGGGC ATGTCAGCCT TT--GATTCT CTCTGAGAGG
AcURE2           GCACCGAAGA CGAGATTCTT ATTCAAGCAT TCTCCTGA-G CA-CTGCATT CATGGCTGTG TCTAAAGGGC ATGTCAGCCT TT--GATTCT CTCTGAGAGG
XtURE2           GCAGAGTGGA GCAGATTCTT ATTTAGGCAT TCTATGGA-G C-TCTGCATT CATAGCTGTG TCTAAAGGGC ATGTCAGCCT TT--GATTCC TTCTGAGAGG
CmURE2           AAAAGCTCCA --GAATTCTT ATTCAAGCAT TCTATCGA-G CAGC-GCATT CATGACTGTG TCTAAAGGGC ATGTCAGTCT TTT-GATTCA CTTTGAGAGG
DrURE2           GCAAAGCACA --GAATTATT CTCCCTGCCT TCTATCAA-G CTCCTGCATT CAGCAGCGCG TCTGAAGGGC GCGTCAGTCC GCCTGCCTCT CTCAGAGAGG
OlURE2           GCAAAGAACA --AAATTATT CCTCAAGCTT TCTATCAAAG CCTCTGCATT CATGGCAAAG TCTAAAGGGC ATGTCAGTCC TCCTGCC--T CTTAGAGAGG
TnURE2           GCAAGGAAGA --AAATTATT CCTCCAGCTT CCTATTGA-G CTTCTGTATT CACGGCCAAG TCTAAAGGCC ATGTCAGTCC TCCTGCC--T CTCAGAGAGG
TrURE2           GCAAAGAACA --AAATTATT CCTCAAGCTT TCTATCAA-G CTTCTGCATT CACGGCCAAG TCTAAAGGGC ATGTCAGTCC TCCTGCC--T CTGAGAGAGG

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                   105        115        125        135        145        155        165        175        185        195
MmURE2           TAATTATCC- TTTTCCTGTC ACGGAACAAC AAATGATAGC TAACTACAGA GGCACATTTG CAGTAGTCAC A-TTCATCAA CTGCAGAAAA AAAAAA-TTC
GgURE2           TAATTATCC- TTTTCCTGTC ACGGAACAAC AAATGATAGC TAACTACAGA GGCACATTTG CAGTAGTCAC A-TTCATCAA CTGCAGAA-A AAAAAA-TTC
AcURE2           TAATTATCC- TTTTCCTGTC ACGGAACAAC AAATGATAGC TAACTACAGA GGCACATTTG CAGTAGTCAC A-TTCATCAA CTGCAGGGGA AAAAAA-TTC
XtURE2           TAATTATCC- TTTTCCTGTC ACGGAACAAC AAATGATAGC TAACTACACA AGCGCATTTG CGGTAGTCAC A-TTCATCAA CTGCAGGGAA AAAAAA-TTC
CmURE2           TAATTATCC- TTTTCCTGTC ACTGAACAAC AAATGATAGC TAACT---GA GGCACATTTG CAGTAGTTGC A-TTCATCAA TTGCTA---A AAAAAA-TTG
DrURE2           TAATTATCCT TTTTCCTGTC ACAGGGCTCC AAATGATCTC TAACCACTGG GGAGCTTTGT TAGGGGCTCA GCTTTGGCAA TAGGAAGTCG AAAGAGCTTT
OlURE2           TAATTATCC- TTTTCCTGTC AGGGAATAAC AAATGATAGT CAACTATGGG GGTGCAGTTT CAGGAGCTGA AATTTGTTGA TAGGAAGGAG AAGGAGGTTT
TnURE2           TAATTATSCC TTTTCCTGTC AGAGGACAAC AAATGATCGT CGACGAC-GG GGCGCATTTT CTGGAGCTGA AATTTGTCAG TTGCTAGAAA AAGAGGGTTT
TrURE2           TAATTATCC- TTTTCCTGTC AGAGACCAAC AAATGATCGC CAACTAT-GG GGC--ATTTT CTGGAGCTGA A-TTTGTCAG TTGGGAGAAA AAGGGGATTT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245        255        265        275        285        295
MmURE2           AATTTAATTG TACAACACAG CTGCACATGG GCTTTC---- ---GAGC--T TC---TGTTG TTCTCCCTGC ---------- ---------- ----------
GgURE2           AATTTAATTG TGCAACACAG CTGCACATAG GCTTTTT--- ---GAGCATT TC---TGTTG TTCTCCCTGT ---------- ---------- ----------
AcURE2           AATTTAATTG TACAACACAG CTGCACATAG GCTTTTT--- ---GAGCATT TC---TGTTG TTCTCTCTGT ---------- ---------- ----------
XtURE2           AGTTTAATTG TACAACCCAG CTGCACATGA GCCTTT---- ---CAGCATA TC---TGTTG TTCTCTCTTT ---------- ---------- ----------
CmURE2           AATTTAATTG TGTAACACAG CTGCATGTCA GCTTTTAT-- ---CA-CATC TC---TCTTG TACTGTGTGT ---------- ---------- ----------
DrURE2           AATTT---TC AGA----CAG CTGCACATGT ACTTTCATC- --GCGGCATT ATAAANCCAT ATAGCGGTCA TCNCTAC--- ---------- ----------
OlURE2           AATTT---TC TGG----CAG CTGCACATCT ACTCTTATCT GGGTTTTTTT TTTGGTCAGT TTTTTGCCTT GTGCAG---- ---------- ----------
TnURE2           AATT---TTC TGTTTC-CCA CTGGTCAT-T TCTT-CTTTT TTTTCCTTTT TCTCCACACT CTCAGTCTGC CGGAG----- ---------- -------CAC
TrURE2           AATT---TTC TGG----CAG CTGTACATCT ACTTTCATGT TTTTCCCCCC ACTGGTCATT TCTTGTTTTT TGGGGGGGGG GTTCCCCCAC TATCCTGCAC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
                   305        315        325        335        345        355        365        375        385        395
MmURE2           ---------- --------CT TGCTAGTCCT CCCT------ CCAGATCTAT TTTT------ -TAAACTTTT TTTT---TTC TGGTTATTTT TTCCCCT---
GgURE2           ---------- --------CT TGCTATTCCT CCCT------ CCAGATCTAT TTTT------ -TAAACTTTT TTT------C TGGTTATTTT TTCCCCC---
AcURE2           ---------- --------CT TGCTATTCCT CCCTA----- CCAGATCTAT TTTT------ -TAAACTTTT TTTTCCTTCC TGCTTATTTT TCCCTCTCCC
XtURE2           ---------- --------CT TCTTA-CACT CCTT------ CCTGGTCTTT TTTTTTTTCC CTAAACTTTT ---CCCC-CT TGATTATTTT TTTTCCC---
CmURE2           ---------- --------CT GGTTCTCTTT CCCT------ CTATATATCC ATTTTCTTAT T--------- --------CC ATCTTGTTTT TTTTTCT---
DrURE2           -GAGCGCACG CGCGCACTTT TGTTTGTGAC GTGGGGCCGC GCGCGCGCGC CTGCCTGTGC ACGGCCTCCC CGCATTAGCA TATTTATTTA TAGACTTACA
OlURE2           --AGCAGTGC TG-------- -TTTGTTTAC GTGTGCGAGC TTGTGAGGGT GTATTTATGT G------CCC TGCATTAACA TGCCTATTTA TAATCCTTCA
TnURE2           AGAGCGCTCC TGGGCCATTT TTTTGTG--C CTGTGAA-GT CTCTGAGGGT GTATTTATGC GA-----TCC TACATTAACA TACCTATTTA TAGTCCTACA
TrURE2           AGAGAGCT-C TGCCGGGGCT GTTTGTGCGC CTGTGAA-GC CTCTGAGAGT GTATTTATGT G------CCC TACATTAACA TACCTATTTA TAGTCCTACA
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                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                    405         415         425         435         445         455         465         475         485         495
MmURE2           ---------- ---TTTTTTG TCTC-----T TCTTCCATTT TTACTCTCTG ----TACTTT CTTGTTAAAG TAATTTTCCT TTGTGGCTCT CGTTCTTTTT
GgURE2           ---------- ----TTTTTG TCTC-----T TCTTCCATTT TTACTCTCTG ----TACTTT CTTGTTAAAG TAATTTTCCT TTGTGGCTCT CATTCTTTTT
AcURE2           CCTCCCCCCT CTTTTTTTTG TCTC-----T TCTTCCATTT TTACTCTCTG ----TACTTT CTTGTTAAAG TAATTTTCCT TTGTGGCTCT CATTCTTCTC
XtURE2           ---------- ----ATTTTG TCTA-----T TCCTCCATTT TTTTTTTTTA CTC-TACTTT CTTGTTAAAG TAATTTTCCT TTGTGGCTCC TTTTTTTTTC
CmURE2           ---------- -----CTTTA CTCT------ CTCTCCCTCT CTCCCTCTCT C---TCCTTT CCTGTTAAAG TAATTTTCCT TTGTGAGTCT CATTCTT---
DrURE2           CTTTTCTTAC AGTCATTTTT GCGCAA---- ---------- --CAATTTT- CACACTCGAC ATTGTCTGTG TAATTTCCCT TTCAGTGTCT CTCTGTTTTC
OlURE2           CTTT-CTTAC AGTCATCTTT TTT------T TTCTGTTGT- GACCATTTCA CAGATTATAT ATTGTCCAAG TAATTTCCCT TTCAGCATCT GTGTTTTTTA
TnURE2           CTTT-CTTAC ATTCATCTTT TTTTTTTTTT TTTCTGTTGT GACCATTTCA CAGATTATAT ATTGTCCGAG TAATTTCCCT TTCAGCATCT GTGTTTTTTA
TrURE2           CTTT-CTTAC AGTCATCTTT TT-------- TTTCTGTTGT GACCATTTCA CAGATTATAT ATTGTCCAAG TAATTTCCCT TTCAGCATCT GTGTTTTTTA

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                    505         515         525         535         545         555         565         575         585         595
MmURE2           TCC------- -----CCATT ----GAAGGC TATGAATGTA G--AAAATTA TCACAATTAC TCATATAATT GAGCC-TCTT TGTAGCAAGT ACGACTCCAG
GgURE2           TCC------- -----CCATT ----GAAGGC TATGAATGTA G--AAAATTA TCACAATTAC TCATATAATT GAGCC-TCTT TGTAGCAAGT GCAACTCCAG
AcURE2           CCCCCC---- -CCCCCCATT ----GAAGAC TATGAATGTA G--AAAATTA TCACAATTAC TCATATAATT GAGCC-TCTT TGTAGCAAGT GCAACTCCAG
XtURE2           TCTCTTGCTT CTCCCCCATT ----GAAGGA TATGAATGTA G--AAAATTA TCACAATTAC TCATATAATT GGGCC-TCTT TGTAGCAAGT GCAACTCCTC
CmURE2           ---------- -----GATCA AGTAAAGGTC CATGAATGGA CT--AAATTA TCACAATTAC TCATATAATT GAACCTCCTT TGTAGCAAGT GCAACTCCAC
DrURE2           TCCTCTCTTT CCTTCCCTTT CAGAAAGAGC CATGAATGGA TGGAAAATTA TCACAATTAC TCACATAATT GAGCCGTCTT TGTGGCAAGT GCAGCTCGAC
OlURE2           C--------- ------CTTT CAGAGAAAGC CGTGAATGGA GGGGAAATTA TCACAATTAC TCATATAATT GAGCCGTCTT TGTAGCAAGT GCGGCTTCAC
TnURE2           C--------- ------CTTT CAGAGAAAGG AGTGAATGGA GGG-AAATTA TCACAATTAC TCATATAATT GAGCCGTCTT TGTAGCAAG- GCAGCTTCAC
TrURE2           C--------- ------CTTT CAGCGAAAGG AGTGAATGGA GGGAAAATTA TCACAATTAC TCATATAATT GAGCCGTCTT TGTAGCAAGT GCAGCTTCAC

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                    605         615         625         635         645         655         665         675         685         695
MmURE2           TAGCCT---T TCTCCATCA- TGAAAATGGT TTCATTATA- GGGTTTTTCA TATTCCCTGA CACCATCTAC -ACAGAGGAG CAAGCGTGCA GATGAGATG'
GgURE2           TAGCCT---T TCTCCATCA- TGAAAATGGT TTCATTATA- GGGTTTTTCA TATTCTCTGA CACCATCTAC -ACAGAGGAA CAGGCGTGCA GATGAGATG'
AcURE2           TAGCCT---T TCTCCATCA- TGAAAATGGT TTCATTATA- GGGTTTTTCA TATTCTCTGA CACCATCTAC -ACAGAGGAA CAGGCGTGCA GATGAGATG'
XtURE2           TAGCCT---T TCTCCTTCA- TGAAAATGGT TTCATTATA- GGGTTTTTCA TATTCCCTGA CACCATCTAC -ACAGAGGAA CAGGCGTGCA GATGAGATG'
CmURE2           TAGCCT---T TTTCCATCA- TGAAACTGCT TTCATTATAT G-TTTTTTCA TATTCTCTGA CACCACGCGC -ACACAGAAG CAGGCGTGCA GATGAGATG
DrURE2           GAGCCC--TT TTTCCATCAG CCAAAATGGT TTCATTATAG GGG-TTTTCA TATTCCCTGA CACGGGGCGC AGGGGGGGCC GCGGTGTGCG CAGGAGTCG
OlURE2           TAGCCCCTTT TTTCCATCAA CCAAAATGGT TTCATTATAT GGGTTTTTCA TATTCCCTGA CACAGGGCTC CGCTGAGGGC CTGGCGCGTG GATGAGATG
TnURE2           TAGCCCC-TT TTTCCATCAG CCAAAATGGT TTCATTATAT GGGTTTTTCA TATTCTCTGA CACAGGGCTC TGCTGAGGGC CTGGCGCGTG GATGAGATG
TrURE2           TAGCCCCCTT TTTCCATCAG CCAAAATGGT TTCATTATAT GGGTTTTTCA TATTCCCTGA CACAGGGCTC TGCTGAGGGC CTGGCGCGTG GATGAGATG

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                    705         715         725         735         745         755         765         775         785         795
MmURE2           GCT--GGGAA CAGGCTAGAT CAGTAAGGTC ACAGTAGGAA TAATTAGCTC TGCTATGGAA AGAGCATCCA G-GCCTTTTA CTG------C TACATAAAT
GgURE2           ACT--AGGAA CAGGGTAGAT CAGTAAGGTC ACAGTAGGAA TAATTAGCTC TGCTATGGAA AGAGCATCTA G-GCCTTTTA CTG------C TACATAAAT
AcURE2           ACT--AGGAA TAGGGTAGAT CAGGAAGGTC ACAGTGGGAA TAATTAGCTC TGCTATGGAA AGAGCATCTA G-GCCTTTTA CTG------C TACATAAAT
XtURE2           ACT--CGGAA CAGTGCAGAT CAGCGAGGTC ACAGTAGGAA TAATTAGCTT TGCTATGGAA AGAGCTTGTA G-GCCTTTTA CCG------C TACATAAAT
CmURE2           AGGGGAGGAA CATTACAGAT CAACGAGGTC ACAGTGGGAG TAATTAGCTG TGGTATGGAA AGAGCATCCA G--CCTTTTC TG------C GGTAGAGAT
DrURE2           TGG--GGGAG AAGGGTGGAT CATTAAGGTC ACAGTGGGGA TAATTATCTT GACTATGGAA AGAGCATCCA GCACCTTTTT TCTCTTCCCC GCCACAAAA
OlURE2           AGG--ATGAA CAGAGTGAAT CATTAAGGTC ACAGTAGGAA TAATTATCCC AGCTATGGAA AGAGCATCTA AAGCCTTTTT TCTC--CCCC GGCACAAAT
TnURE2           AGG--ATGAA CAGAGTGAAT CATTAAGGTC ACAGTAGGAA TAATTATCCC AGCTATGGAA AGAGCATC-A G--CCTTTAT TCTC--CCCC AGCACAAAT
TrURE2           AGG--ATGAA CAGAGTGAAT CATTAAGGTC ACAGTAGGAA TAATTATCCC AGCTATGGAA AGAGCATC-A G--CCTTTAT TCTC--CCCC AGCACAAAT
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                 805        815        825        835        845        855        865        875        885        895
lmURE2   TACTGTCCGT GGCTTTTAGC CACAAA--AA AAC-TTACTA ACAAATGGAG CTCCCGCCTA CTACTTTGA- ---------- ------AAAA AAGATTTGTA
gURE2    TACTGTCCAT GGCTTTTAGT CACAAA--AA AAC-TTACTA ACAAATGGAG CTCCCGCCTA CTACTTTGA- ---------- ------AAAA AAGATTTGTA
cURE2    TACTGTCCGT GGCTTTTAGT CACACAAAAA AACCTCACTA ACAAATGGAG CTCCCGCCTA CTACTTTGA- ---------- ------AAAA AAGATTTGTA
tURE2    TACTGTCCGT GGCTTTTAGT CACAGA-AAA AAC-TTACTA ACAAATGGAA CTCCCGCCTA CTACTTTGA- ---------- ------AAAA AAAATTTGTA
mURE2    TACAATCCAC GGCTTTCAGT CACA---AAG AAC-TTAGTA ACAAATGGAG CTCCCGCCCA TTGCTTTAA- ---------- ------AAAA AAGATTTGTA
rURE2    CACCGTCCAT GGCCTTTCGG CACA---AAG AAC-TTGGAA ATAAATGGAG CTTCCACCTA CAACTTATTC ACAAA----- ----GAGAGA GAGACTTGTA
lURE2    CAATGTCCAC GGCTTTTAGT CACA---AAG AAC-TTTGTT ACAAATGGAG CTTCCACCTT CTACTTATAC AAAGAAGAGA GGGGGAGAGC AAGACTTGTA
nURE2    CAACGTCCAC GGCT------ -------TAG AAC-TTTGTT ACAAATGGAG TTTCCACCTT CCACTTATAC AAAGACGAGA GGGGGAGAGC AAGACTTGTA
rURE2    CAATGTCCAT GGCT------ -------TAG AAC-TTTGTT ACAAATGGAG TTTCCACCTT CCACTTATAC AAAGACGAGA GGGGGAGAGC AAGACTTGTA

                 905        915        925        935        945        955        965        975        985        995
lmURE2   TCAACACTAC AATTTTCCAT CATTAAGACT AATAACACAG A-GCCTAGTA TACATCAAGG GGAATAAAAA G-AAAAATCT CACATTCAAG TGGTGG-CTG
gURE2    TCAACACTAC AATTTTCCAT CATTAAGACT AATAACACAG A-GCCTAGTA TACATCAAGG GGAATAAAAA G-AAAAATCT CACATTCAAG TGGCGG-CTG
cURE2    TCAACACTAC AATTTTCCAT CATTAAGACT AATAACACAG A-GCCTAGTA TACATCAAGG GGAATAAAAA G-AAAAATCT CACATTCAAG TGGCGGACTG
tURE2    TCAACACTAC AATTTTCTAT CATTAAGACT AATAACACAG A-GCCTGCTA TACATCAAGA GGAATGAAAA G-AAAAATCT CACATTCAAG TGGCGG-CTG
mURE2    TCAACACTAC AATTTTCCAT CATTAAGACT AATAATGGAG A-GCTCGGTA TGCATCAAGG GGAACAAAA- --AAATATCT CACATTCAAG TGGCTG-CTA
rURE2    TCAACATGAC AATTTTCCAT TATTAGGACT AATGGCGGGC T--GCCTTAG GAGATCAAGG GGAG------ ---AGGGGGA CGCGTTCAGG TGT-GC-TGG
lURE2    TCAACATGAC AATTTTCCAT CATTAAGACT AATGACAGGC ACAGACTGTG GGGATCAAGG GGAGCACAAA A--AAAGAGG CGCATTCAGG TGTTGG-CTG
nURE2    TCAACATGAC AATTTTCCAT CATTAAGACT AATGACAGGC GCAGACTGTG GGGATCAAGG GGAGCACAAA AA-AGAGAGG TGCATTCAGG TGTTGG-CTG
rURE2    TCAACATGAC AATTTTCCAT CATTAAGACT AATGACAGGC ACAGACTGTG GGGATCAAGG GGAGCACAAA AAAAGAGAGG CGCATTCAGG TGTTGG-CTG

                1005       1015       1025       1035       1045       1055       1065       1075       1085       1095
MmURE2   GGCGCTGACC TTTGTTCCC- CCTTTTTGTA TACGA----- ---------- ---------- ---CTTAACT CTTT-ACAAA AAAGAGCCAC ACGCCAC
GgURE2   GGTGCTGACC TTTGTTCCC- TTTTTTTGTG TTCAA----- ---------- ---------- ---CTTAACT CTTT-ACAAA AAGGAGCCAC ACGCCAC
AcURE2   GGTGCTGACC TTTGTTCCCC TTTTTTTGTG TACGA----- ---------- ---------- ---CTTAACT CTTT-ACAAA AAGGAGCTAC ACGCCAC
XtURE2   GGTGCTGACC TTTGTTC--- TTTTTTTGTG TATGTGTGTG TGAGTGTGCG CGTGTGTGTG TGTATGCACG ATTTCACAAA AAAGACCCAC ACAGCAT
CmURE2   GGTTCCAACC CTCCTTCCC- --TTTTTGTC TGCGA----- ---------- ---------- ---GTTAACT CTTTACCATA AACCAGCAGA CTGCACA
DrURE2   AGTACTGACC TCTCCCCCGT CT---TTGTT TGGGA----- ---------- ---------- ---CTTAACT CCGA-ACAAA AACCCTA--A AGGGGAT
OlURE2   AGTGCTGACC TCCTTCCTGT CT---TTGTT GGAGA----- ---------- ---------- ---CTTAACT CTTA-ACAAA AAC--TAAAA AGGGT-T
TnURE2   AGTGCTAACC TCCTTCCTGT CT---TTGTT GGAGA----- ---------- ---------- ---CTTAACT CTTA-ACAAA AAC--TAAAA AGGGGGT
TrURE2   AGTGCTGACC TCCTTCCTGT CT---TTGTT GGAGA----- ---------- ---------- ---CTTAACT CTTA-ACAAA AAC--TAAAA AGGGGGT
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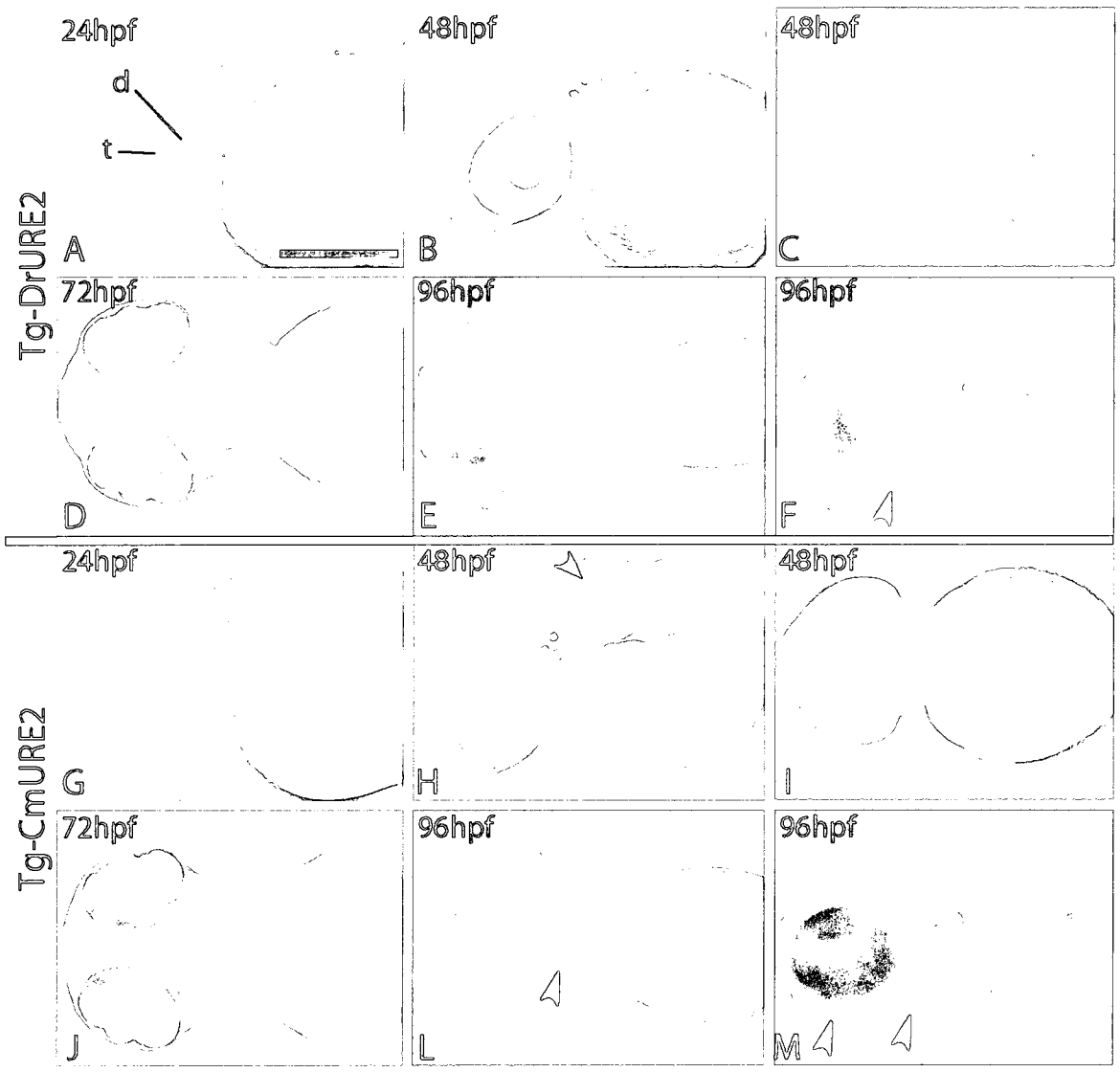
	Tetrapods					Teleosts			
	MmURE2	GgURE2	AcURE2	XtURE2	CmURE2	DrURE2	OlURE2	TnURE2	TrURE2
MmURE2	na	0.97	0.96	0.91	0.83	0.70	0.76	0.73	0.75
GgURE2		na	0.98	0.92	0.84	0.70	0.77	0.75	0.77
AcURE2			na	0.91	0.84	0.70	0.76	0.74	0.75
XtURE2				na	0.80	0.66	0.73	0.71	0.73
CmURE2	0.83	0.84	0.84	0.80	na	0.68	0.74	0.71	0.73
DrURE2						na	0.80	0.78	0.81
OlURE2							na	0.93	0.96
TnURE2								na	0.96

significantly (t-test; $p<0.05$) more similar to tetrapod sequences (mean: 82.5%) than to teleost sequences (mean: 71.5%). When testing for relative substitution rates with the elephant shark as an outgroup, the null hypothesis of equivalent substitution rates could be confidently rejected ($p=0$) when comparing zebrafish and mouse URE2 sequences or medaka and *Xenopus* URE2. In both cases a higher substitution rates was obtained in teleosts compared to tetrapods. These results strongly suggest that the elephant shark sequence is more similar to tetrapod sequences than to teleost sequences, most probably due to higher mutation rates in the latter clade.

3.2.2. The elephant shark URE2 sequence acts as a forebrain regulatory element in transgenic zebrafish and mice.

To determine if the CmURE2 sequence can act as a regulatory element and to compare its activity with its zebrafish and mouse counterparts, we prepared a series of reporter constructs in which URE2 sequences are placed upstream of a cassette containing a β-*globin* minimal promoter and either the *GFP* or *lacZ* reporter genes. The resulting constructs were tested in both transgenic (Tg) zebrafish and mice. In zebrafish, the DrURE2 sequence drove GFP expression in the telencephalon and diencephalon starting at around 24 hpf (Figure 3.3 A-C). This expression was observed in two independent lines of transgenic zebrafish and persisted (Figure 3.3 D and E) until 96 hpf, a time where GFP expression was also seen weakly in the pharyngeal arches (Figure 3.3E and F). Similarly, the CmURE2 sequence targeted GFP expression to the forebrain of zebrafish embryos and larvae from 24hpf until at least 96 hpf (Figure 3.3 G and L) with pharyngeal arch expression of the reporter transgene observed at the later time points (Figure 3.3 K and L). Overall, examination of live embryos indicated that the spatial

Figure 3.3. Expression of URE2-GFP reporter transgenes in zebrafish. Panels A, B, F, G, H, M are lateral views, anterior to the left and dorsal to the top; panels C, D, E, I, J, and L are ventral views. t: telencephalon; d: diencephalon; arrowhead: visceral arch muscles. White arrowheads indicate pharyngeal arch expression and the black arrowhead indicates somite expression. Scale bar: 250μm.


Tg-DrURE2
Tg-CmURE2
24hpf
d
t
A
48hpf
B
48hpf
C
72hpf
D
96hpf
E
96hpf
F
24hpf
G
48hpf
H
48hpf
I
72hpf
J
96hpf
L
96hpf
M

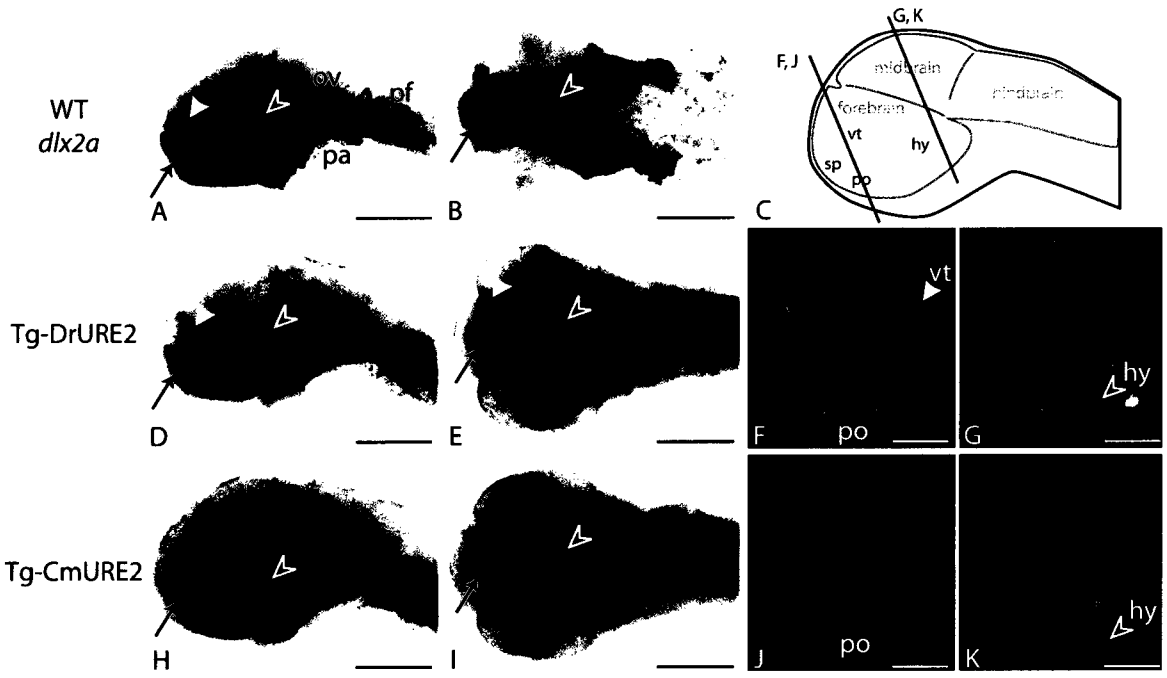
distribution of the GFP protein was generally similar with both constructs, suggesting similar activities for the elephant shark and zebrafish sequences in the brain and visceral arches (Table 3.1). One site of Tg-CmURE2 activity that is not observed with Tg-DrURE2 and is not consistent with endogenous *dlx1a* or *dlx2a* expression is the somites at 2dpf until at least 5dpf (Figure 3.3H, black arrowhead).

We next examined forebrain URE2 activity in greater details and compared it with endogenous expression of the zebrafish *dlx1a/2a* genes. The endogenous *dlx* expression domains correspond to the subpallium of the telencephalon (Figure 3.4 A-C) and to specific parts of the diencephalon (preoptic area, prethalamus, and hypothalamus (Figure 3.4 C) (Mueller et al. 2008). Comparative *in situ* hybridization analysis of the GFP transcripts identifies highly comparable expression of endogenous *dlx2a* (Figure 3.4 A-C) and GFP in Tg-DrURE2 embryos (Figure 3.4 D-E). However differences in transgene expression could be pointed in the Tg-CmURE2 line with no apparent detection of GFP expression in the prethalamus as well as very restricted expression in the hypothalamus (Figure 3.4 H and I). Anti-GFP immunohistochemistry on sections of transgenic embryos confirmed that the prethalamus expression was completely absent from Tg-CmURE2 embryos (Figure 3.4 J), while expression in the pre-optic area is comparable between Tg-CmURE2 and Tg-DrURE2 (Figure 3.4F and J). More posteriorly in the hypothalamus, the transgene was expressed only in a restricted lateral domain in the Tg-CmURE2 transgenic line, while GFP expression was wider in the Tg-DrURE2 line (Figure 3.4 G and K). Immuno-localization of the GFP in these two lines also allowed us to detect GFP in the muscles associated to the visceral arches, as well as muscles in the tail (data not shown), consistent with the fluorescence patterns described

Table 3.1. Summary of URE2 reporter gene expression patterns. Summary of reporter gene expression patterns under regulation by the zebrafish, elephant shark or mouse URE2 sequences (respectively Tg-DrURE2, Tg-CmURE2, Tg-MmURE2) in zebrafish embryos staged from 1 to 4 days post-fertilization (dpf) in case of stable transgenic lines, 2 to 4 dpf in case of primary transgenic expression (Tg-MmURE2) and in mouse embryos aged E11.5 (one stable transgenic line for Tg-MmURE2, primary transgenic expression for Tg-DrURE2 and Tg-CmURE2).

	Tg-DrURE2					Tg-CmURE2					Tg-MmURE2	
	1dpf	2dpf	3dpf	4dpf	E11.5	1dpf	2dpf	3dpf	4dpf	E11.5	2-4dpf	E11.5
Telencephalon	+	+	+	+	+	+	+	+	+	+	+	+
Diencephalon	+	+	+	+	+	+	+	+	+	+	+	+
Visceral arches	-	-	+	+	-	-	-	+	+	-	+	+
Dorsal root ganglia	na	na	na	na	-	na	na	na	na	+	na	+
Somite muscles	-	-	-	-	na	-	+	+	+	na	na	na
Fin/limb buds	-	-	-	-	-	-	-	-	-	-	na	+

Figure 3.4. Expression of URE2-GFP reporter constructs in the brain of 48 hpf zebrafish. Expression patterns obtained by in situ hybridization using a *dlx2a* cDNA probe in wild-type embryos (A, B) or a *GFP* probe in Tg-DrURE2 (D, E) and in Tg-CmURE2 embryos (H, I). Immunolocalization of GFP proteins on sectioned embryos of the Tg-DrURE2 (F, G) and Tg-CmURE2 (J, K). Expression in the telencephalon is comparable for the endogenous gene and the two transgenes (black arrow in A, B, D, E, H, I). Expression in the dorsal domain of the prethalamus (white arrowhead) in Tg-DrURE2 for *gfp* mRNA (D, E) and GFP proteins (F) is not observed in Tg-CmURE2 (H-K). Expression of GFP in the hypothalamus (black arrowhead) was restricted to lateral cells in Tg-CmURE2 (H, I, K) compared to Tg-DrURE2 (D, E, G). Panels A, D, H are lateral views, B, E, I are ventral views. Plan for the transversal sections presented in F-G and J-K are localized on the scheme in panel C. Blue domains in the scheme are the forebrain expression domains described for *dlx* genes: the telencephalic domain being the subpallium (sb, black arrow); the diencephalic domains being the preoptic area (po), prethalamus (vt, white arrowhead) and hypothalamus (hy, black arrowhead). Scale bars: A, B, D, E, H, I, 250µm; F, G, J, K, 50µm.


WT
dlx2a
ov
pf
pa
A
B
C
G, K
F, J
midbrain
forebrain
hindbrain
vt
hy
sp
po
Tg-DrURE2
D
E
F
po
vt
G
hy
Tg-CmURE2
H
I
J
po
K
hy

in Figure 3.3. Examination of primary transgenic zebrafish obtained with a similar construct containing the mouse MmURE2 sequence indicates that it behaves similarly to its elephant shark and zebrafish counterparts with expression in the telencephalic and diencephalic domains (Suppl. Figure 3.2). Notably, the transgene could also be detected in visceral arches at 5dpf, in a pattern similar to that observed for Tg-DrURE2 and Tg-CmURE2 (Suppl. Figure 3.2 C).

Similar constructs using *LacZ* as the reporter gene were tested in primary transgenic mouse embryos at E11.5. The three URE2 enhancers had very similar activities in the forebrain (Figure 3.5; Table 3.1). All three URE2 sequences targeted reporter expression to the telencephalon and diencephalon (Figure 3.5 A-C). Forebrain expression of the reporter constructs was observed in 4/5 and 4/4 primary transgenic embryos obtained with *CmURE2-lacZ* and *DrURE2-lacZ*, respectively (Suppl. Figure 3.3). Outside the forebrain, the mouse and elephant shark URE2 sequences showed more similarities in their activities compared to zebrafish URE2: both CmURE2 and MmURE2 could target expression to the dorsal root ganglia in primary transgenic embryos (CmURE2-lacZ, n=1/5; Suppl. Figure 3.3) or in two independent transgenic lines (MmURE2-lacZ, Figure 3.5 A and Ghanem et al., 2007). The DrURE2-lacZ transgene was never expressed in the dorsal root ganglia (n=4). The mouse URE2 element was the only one able to target expression to the branchial arches and to the apical ectodermal ridge of limb buds.

Supplemental figure 3.2. Primary transgenic zebrafish embryos with *gfp* expressed under MmURE2 sequence. GFP fluorescence could be detected in the forebrain (fb) of primary transgenic zebrafish at 2dpf (A) and 3dpf (B) after injection of the construct. GFP fluorescence was also detected in the visceral arches (va) of 4dpf old embryos after injection (C).

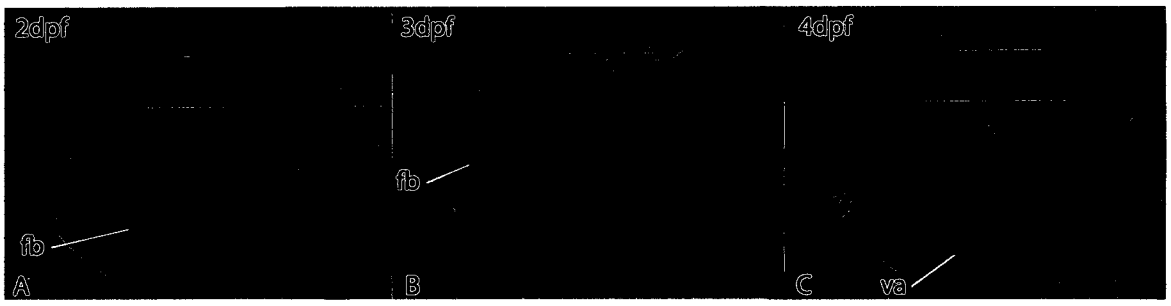
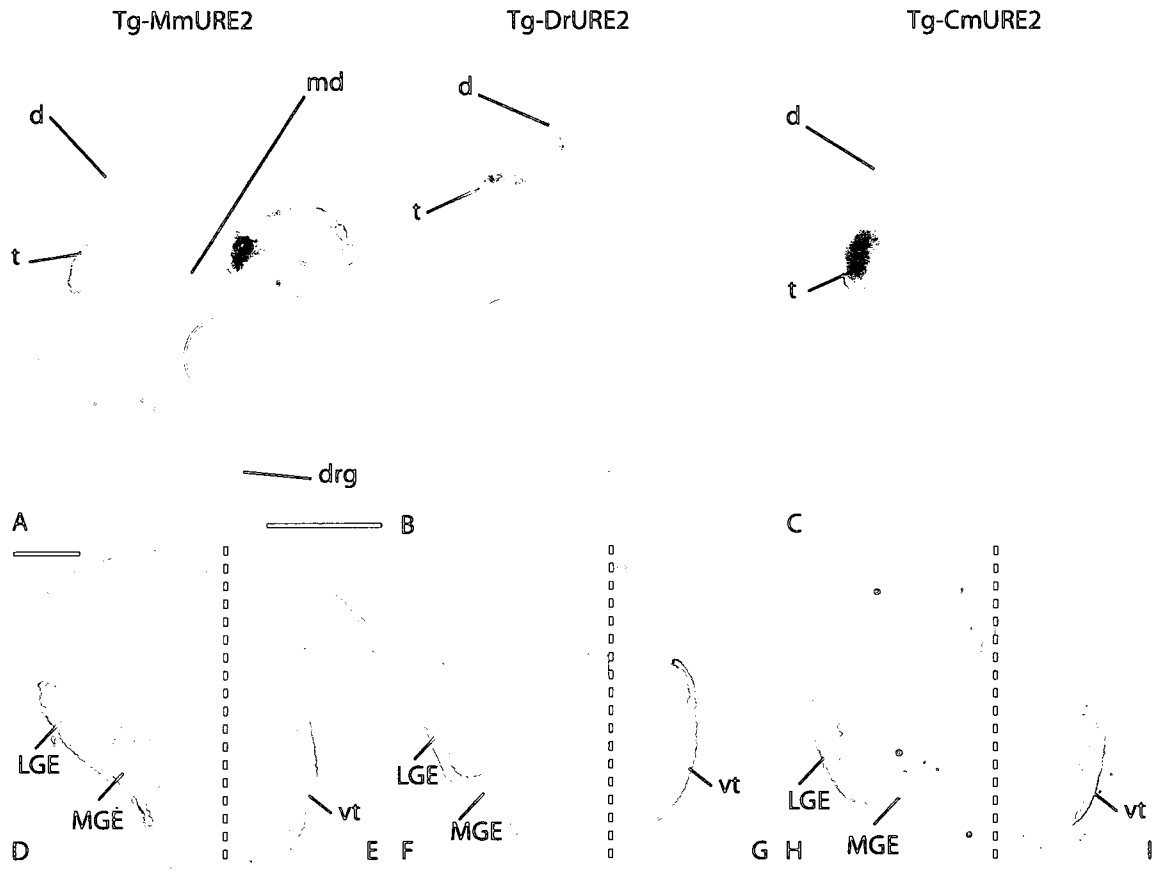
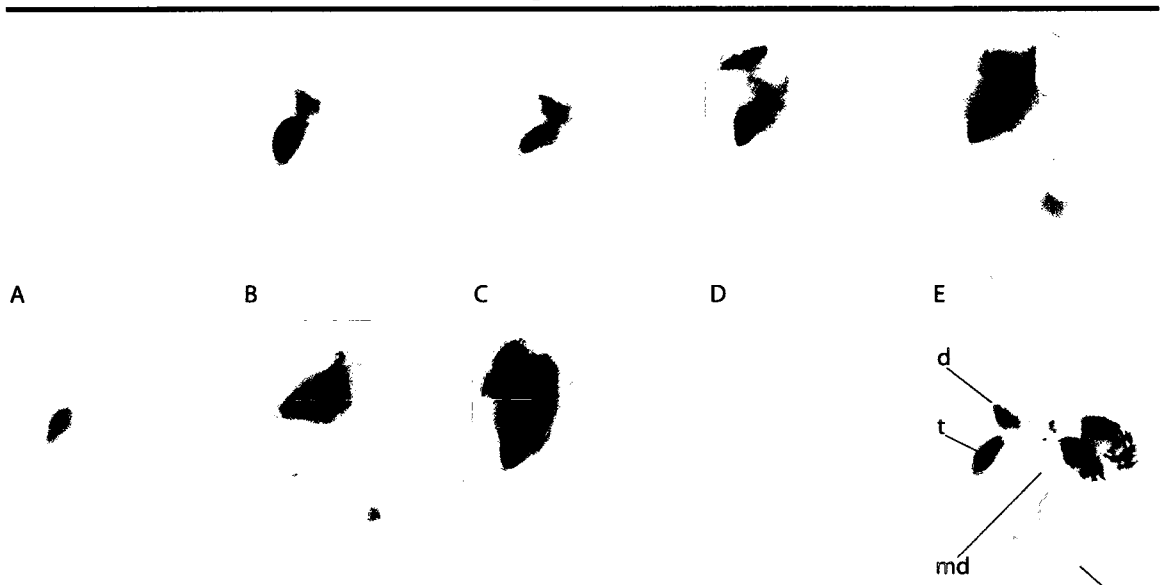

2dpf
3dpf
4dpf
fb
fb
fb
va
A
B
C

Figure 3.5. Expression of URE2-lacZ reporter constructs in E11.5 mouse embryos. Lateral views of an E11.5 embryo from a Tg-MmURE2 transgenic line (A) and primary transgenic embryos for Tg-DrURE2 (B), or Tg-CmURE2 (C). Dissected brains were sectioned at the level of the telencephalon (D, F, H) and diencephalon (E, G, I) and detailed expression of the transgene was found comparable for Tg-MmURE2 (D, E), Tg-DrURE2 (F, G) Tg-CmURE2 (H, I), or. Panels D-J are halves of transversal sections, dorsal is up, left side is shown in D, F, H, right side in E, G, I, sagittal plan figured in dashed line. d: diencephalon; drg: dorsal root ganglia; t: telencephalon; vt, prethalamus. Scale bars: 1mm in A-C, 500μm in D-I.


Tg-MmURE2
Tg-DrURE2
Tg-CmURE2
d
md
t
d
t
d
t
drg
A
B
C
LGE
MGE
D
vt
LGE
MGE
E F
vt
LGE
MGE
G H
vt
I

Supplemental figure 3.3. Primary transgenic mice with *LacZ* expressed under the control of orthologous URE2. CmURE2 (A-E), DrURE2 (F-H) sequences to be compared with *LacZ* expression in a stable transgenic line under MmURE2 regulation (I), at E11.5. d: diencephalon, drg: dorsal root ganglia, md: mandibular arch, t: telencephalon.


Tg-CmURE2
A
B
C
D
E
d
t
md
drg
F
G
H
I
Tg-DrURE2
Tg-MmURE2

3.3. Discussion

3.3.1. Locus and sequence conservation among jawed vertebrates

In this study we identify a conserved regulatory region associated with *Dlx* genes in the elephant shark. Conserved synteny could not be strictly determined because the elephant shark genome is not assembled. However, the sequence identified as CmURE2 is located on the BAC clone containing the elephant shark *Dlx1*, *Dlx2* and *Metapl1* genes, similar to what is observed in all other jawed vertebrates for which genomic data are available (Ensembl Release 56). The putative conservation of the bigene cluster organisation between bony vertebrates and cartilaginous fish is consistent with the hypothesis that an ancestral chordate bigene cluster has been duplicated twice before the radiation of jawed vertebrates (Stock, 2005; Niedert et al. 2001). The identification of a URE2 sequence in vicinity of the elephant shark *Dlx1* and *Dlx2* genes also indicates this locus was linked to these genes in the ancestor of all jawed vertebrates. A search for sequence orthologous to this URE2 enhancer in species outside of jawed vertebrates did not yield any significant hit with the BLASTn tool from the NCBI sequence browser (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the BLAT tool in the UCSC genome browser (http://genome.ucsc.edu/) on the lamprey (*Petromyzon marinus*), tunicate (*Ciona intestinalis*) or lancelet (*Branchiostoma floridae*) genomes, even though *Dlx* genes have been identified in these organisms and bigene *Dlx* tandems are present in lamprey and tunicate (Holland et al. 1996; Caracciolo et al. 2000; Niedert et al. 2001). The high level of URE2 sequence conservation observed in jawed vertebrates suggests that it has evolved under high evolutionary constraints and that its enhancer activity likely emerged in the jawed vertebrate ancestor, after divergence of the cyclostomes, i.e., more than 400

Myrs ago. Interestingly, this reflects the trend observed for a number of other vertebrate CNEs (McEwen et al. 2009).

3.3.2. Enhancer activity, conservation and variation

The most prominent expression domain of a transgene under the regulation of MmURE2 or DrURE2 has been shown to be the forebrain, more precisely in the ventral telencephalon and diencephalon, in agreement with the pattern of endogenous expression of *Dlx1* and *Dlx2* genes (Ghanem et al. 2003). Both zebrafish transgenic lines obtained with DrURE2 and CmURE2 driving expression of GFP produced comparable expression patterns, suggesting the regulatory activity of these orthologous sequences in developing forebrain and visceral arches is likely to be conserved. This overall conservation of the enhancer activity along with a high level of identity between the two sequences, implicates conservation of transcription binding sites that allows the CmURE2 to be active in the zebrafish and mouse developing brain. The results obtained with the DrURE2 transgenic zebrafish lines show that the activity of this enhancer recapitulates part of the *dlx1a/dlx2a* endogenous expression pattern in the forebrain. Similarly MmURE2 transgenic mouse lines recapitulate endogenous *Dlx1/Dlx2* expression in this domain (Ghanem et al. 2007). Thus, the conservation of URE2 regulatory sequences correlates with conservation in its activity.

However, some differences could be identified in the diencephalic expression domains (prethalamus and hypothalamus) between the two transgenic lines. These discrepancies suggest that while the overall activity is conserved, the URE2 enhancer also shows some degree of modularity across the vertebrate phylogeny. Accordingly, the

differences between CmURE2 and DrURE2 sequences could account for the differential expression pattern in the prethalamus and hypothalamus between different vertebrate species whereas these sequence differences do not modify the enhancer activity in the telencephalon. In turn, these results support the idea that these two *Dlx* gene expression domains (telencephalon and diencephalon), even though regulated through a unique functional URE2 enhancer, are perhaps the result of two distinct genetic pathways.

The DrURE2 and CmURE2 sequences also drive expression in the developing telencephalon and diencephalon of transgenic mice, a pattern comparable to the endogenous MmURE2 enhancer. Again, this highlights the conservation of the regulatory cascade leading to *Dlx* gene expression in the developing forebrain of mice. In this species, the function of URE2 in the forebrain seems to be completely conserved despite variation in the sequences, which highly contrasts with our results from transgenic assays in zebrafish where expression in the diencephalon seems to be sensitive to sequence variations. Our results suggest that a distinct genetic pathway is specifically involved in teleost diencephalon development that would not be shared with mouse. This new pathway could have emerged after the additional genome duplication event that occurred before teleosts radiated, which seeded many paralogous developmental genes that could eventually have been co-opted (or recruited) as new independent upstream signals interacting with the zebrafish URE2 enhancer. However, we cannot rule out the possibility that subtle changes in zebrafish transcription factor binding specificity may account for the apparent divergence of the CmURE2 enhancer function in the zebrafish forebrain, compared to the mouse forebrain.

The URE2 sequences studied here are also able to drive expression in the developing branchial arches. More specifically, the MmURE2 can drive expression in the hyoid arch mesenchyme in transgenic mice, while DrURE2 and CmURE2 did not produce any branchial signal in our primary transgenic embryos. DrURE2 and CmURE2, and possibly MmURE2, were able to drive GFP expression in muscles of the growing mandible and of the posterior-most visceral arches in transgenic zebrafish. The fluorescence pattern obtained in zebrafish visceral arches (mandible and branchial arches) suggest expression in the muscles associated with the arches, rather than expression in the chondrogenic mesenchyme, where the *dlx* genes are known to be transcribed (Verreijdt et al. 2006). This expression pattern is unlikely to be an insertion artefact because it could be observed in both DrURE2 and CmURE2 transgenic lines. GFP expression in arches muscles could thus be the result of endogenous URE2 activity that was not reported in previous studies on *dlx* gene expression patterns (Verreijdt et al. 2006). Alternatively, detection of GFP in these muscle cells could be the result of long GFP stability in cell lineages deriving from cells where *dlx* genes are endogenously transcribed.

Lineage-specific modifications may account for the differences observed in the branchial arches between mouse and zebrafish, such as the mammal-specific loss or teleost-specific gain of upstream signal targeting in the visceral arch mesenchyme. These hypotheses could be tested by biochemical and molecular techniques comparing the ability of different activators from the different lineages to enhance expression in response to these signals on the orthologous CREs. In this respect, it will be interesting to characterize the various transcription factors that interact with the MmURE2 sequence and contribute to expression in the brain or visceral arches. In mouse assays, the Tg-

MmURE2 and Tg-CmURE2 sequences were able to consistently drive expression not only in the developing brain, but also in the dorsal root ganglia. None of the Tg-DrURE2 primary embryos (n=4) had expression in these structures, suggesting that MmURE2 and CmURE2 share some enhancer activity that has been lost by DrURE2 consistent with absence of *dlx1a/dlx2a* expression in dorsal root ganglia.

3.3.3. URE2 evolution in vertebrates

As no expression data is available from the elephant shark, we cannot correlate the CmURE2 enhancer activity to the endogenous *Dlx1* and *Dlx2* expression patterns. It is therefore difficult to propose an overview of the evolution of the URE2 enhancer in vertebrates. However, our results show that the genomic organization of the *Dlx1/Dlx2* bigene cluster with a URE2 sequence in the vicinity is conserved amongst all jawed vertebrates. The three URE2 sequences coming either from a cartilaginous fish (the elephant shark), a teleost fish (the zebrafish), or a tetrapod (the mouse) are able to drive expression in the forebrain with apparent complete robustness. These results highlight the strong selective constraint that may have acted against the modification of the regulatory sequences and the *trans*-activating protein domains, which interact with these enhancers, during jawed vertebrate evolution. However our results also suggest that URE2 enhancer activity in visceral arches and diencephalon is only partially conserved and has accumulated evolutionary modifications leading to variations from one organism to another. In particular, the lack of regulatory activity of CmURE2 and DrURE2 in the visceral arches of the mouse could be the result of lineage-specific sequence modification in transcription factor binding sites during tetrapod evolution, possibly leading to

modifications of the regulatory cascade involving the URE2-*Dlx1*-*Dlx2* module in the branchial arches.

3.4. Conclusions

As a chondrychtyan, the elephant shark provides a useful model to carry out comparative studies with jawed vertebrates to evaluate the relative contributions of the changes that took place in coding sequences and in *cis*-regulatory elements and that led to morphological innovations such as the tripartite brain and branchial arches of jawed vertebrates. The use of the elephant shark had been limited to comparative DNA sequence analysis (Ravi et al. 2009). Here, we have shown that *cis*-regulatory elements from the elephant shark can be successfully tested in teleost and tetrapod experimental models. Whereas transgenes with elephant shark *cis*-regulatory elements cannot yet be tested endogenously as transgenesis in this species has yet to be developed, it may be possible to obtain gene expression data in elephant shark for comparative purposes. Such expression studies would further increase the usefulness of the elephant shark in evolutionary developmental biology as a outgroup of bony vertebrates (zebrafish and mouse) showing not only a conserved genome structre but also, as highlighted here, gene regulatory mechanisms.

3.5. Experimental Procedures

3.5.1. Sequence identification and manipulation

The sequence of the previously identified regulatory sequence URE2 from the mouse was blasted against the 1.4× coverage survey-sequencing data of the elephant shark genome (http://esharkgenome.imcb.a-star.edu.sg/). One significant hit allowed us to identify the homologous sequence to the mouse URE2 (MmURE2) in the elephant

shark genome (CmURE2). A BAC from the elephant shark genome library (IMCB_Eshark BAC library) was isolated by 3-step PCR screening of the pooled BAC DNA using primers for the elephant shark *Dlx1* gene (5'-CTCCTCTCCCTTTCAGCAGCAG-3' and 5'-ATTACCTGTGTCTGTGTGAGTCC-3'). This BAC was used as a template for PCR with primers designed for *Dlx2* gene (5'-GAGAAATGCCGACAGATCAGCTC-3' and 5'-CCACCATAGGCTGATGTTGTATG-3') and the CmURE2 enhancer (5' AAAGCTCCAGAATTCTTATTCA-3' and 5'-GTCTGCTGGTTTATGGTAAAG-3') and the *Metapl* gene (exons 9/10: 5'-GCTCGAACTGGGCTGATCTA-3' and 5'-TGGACAGCAATTTCCAATGA-3': exon 7: 5'-AATGGACTGCAAGTTTGCCC-3' and 5'-GCAGCCCTTATCCAGTAGAA-3') that were hypothesized to be in a region of conserved synteny with the URE2 sequence in other vertebrate genomes (Figure 3.1).

Orthologous URE2 sequences were retrieved from the Ensembl genome browser (release 56) by blast with the zebrafish URE2 sequence (DrURE2) against the genome of seven other species: mouse, *Mus musculus*; chicken, *Gallus gallus*; anole lizard, *Anolis carolinensis*; xenopus, *Xenopus tropicalis*; medaka, *Oryzias latipes*; fugu *Takifugu rubripes*; Tetraodon *Tetraodon nigroviridis*. The sequences were first aligned with ClustalW implemented in BioEdit and the alignment was then refined by eye (total 1097 nucleotidic sites, see Suppl. Figure 1) (Thompson et al. 1994). In a zebrafish/mouse/elephant shark sequences comparison, we defined by eye the unambiguously aligned regions within the alignment of the three sequences (see Figure 2, final 578bp). Percentages of identity were calculated and conserved regions were identified by BioEdit software (minimum segment length, 10bp; gaps limited to 2 by

segment and only 2 consecutive gaps allowed). In the comparisons of all nine species, only positions with gaps were excluded (final 705bp) before the percentages of identity were calculated between two sequences or before relative substitution rate tests were evaluated with the MEGA software (Kumar et al. 1994).

3.5.2. Transgene constructs

For transgenic zebrafish, the URE2 element was inserted into the multiple cloning site of a vector that contained a β-globin minimal promoter-GFP cassette. The URE2 sequence is located immediately upstream of the β-globin-GFP fragment and the resulting URE2-β-globin-GFP DNA fragment is flanked at both ends by Tol2 recombinase recognition sequences (Fisher et al. 2006b). For transgenic mice, the URE2 element was inserted into the multiple cloning site of the p1230 construct (Yee and Rigby, 1993). Microinjection of transgene constructs into fertilized mouse eggs and production of transgenic mice were carried out as previously described (Zerucha et al. 2000). For the production of transgenic zebrafish, approximately 125 ng of a tol2 transposase mRNA synthesized *in vitro* with 50ng/ml of DNA construct was co-injected along with the transgene constructs into fertilized zebrafish embryos at the one-cell stage. At least two independent lines of transgenic zebrafish were produced, unless otherwise indicated.

3.5.3. Animals

Zebrafish were raised at 28° C under a 14:10 hour light-dark cycle as previously described (Westerfield, 1995). All animal manipulations were preformed according to guidelines from the Canadian Council for Animal Care.

3.5.4. *in situ* hybridization

Zebrafish for RNA *in situ* hybridizations and fluorescence imaging were treated with 0.0015% 1-phenyl 2-thiourea (PTU) to inhibit melanogenesis. Whole mount in situ hybridizations were carried out following previously described protocol with digoxygenin-labelled cRNA probes synthesized on previously described templates *dlx2a* (Akimenko et al. 1994) and *gfp* (Dorsky et al. 2002) (Thisse et al. 1993). Coloration was achieved with Nitro-Blue Tetrazolium Chloride (NBT) and 5-Bromo-4-Chloro-3'Indolyphosphate P-Toluidine Salt (BCIP) solution.

3.5.5. Microscopy and Immunohistochemistry

Whole mount images were taken on a Nikon NBZ 1500 dissecting microscope with a Nikon DXM 1200C digital camera. Immunohistochemistry was carried out as previously described (Chapter 2, Ghanem et al. 2003), on cryosections at a thickness of 10 μm. Primary antibody: Rabbit anti-GFP (1:1000, Invitrogen, A-11122). Secondary antibodies: Goat anti-rabbit Alexa Fluor488 (1:300, Invitrogen, A11008). Signals were visualized on a Nikon Eclipse E3600 stereomicroscope for fluorescent stains.

Authors' contributions

RBM contributed to the production of constructs, transgenic mice and zebrafish lines/primary embryos, to the analysis of transgenic lines and to the writing of the manuscript; MDT contributed to sequence analyses, transgenic animal analyses and to the writing of the manuscript; KM contributed to the production of gene constructs and transgenic zebrafish lines; LP contributed to the production of constructs used in

transgenesis and of the mouse transgenic line; BHT screened and identified the elephant shark BAC clone; BV provided access to elephant shark sequences and to BAC clones, contributed to the analyses of the data and to the writing of the manuscript; ME contributed to the design of the study, to the analyses of the data and to the writing of the manuscript.

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4. A conserved gene regulatory network controls the differentiation of GABAergic interneurons in the zebrafish prethalamus

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Abstract

In this study, we have shown that *ascl1a*, the *dlx* genes, and *gad1* are part of a gene regulatory network (GRNs) involved in the differentiation of GABAergic interneurons in the zebrafish prethalamus. Expression of *ascl1a* partly overlaps with *dlx1a* partly in the telencephalon and diencephalon early in forebrain development. The loss of Ascl1a function results in a loss of *dlx* regulatory element activity and *dlx* expression in the prethalamus, and subsequent losses of *dlx5a* and *gad1* expression. The cross-regulation between bigene pairs is intact in the zebrafish forebrain and loss of Dlx1a and Dlx2a function, not Dlx5a and Dlx6a, results in a loss of *gad1* expression in the prethalamus. The *ascl1a* and *dlx* genes are involved in a conserved GRN necessary for the proper differentiation of GABAergic interneurons in the prethalamus, however this pathway may have diverged between mammals and teleosts in the telencephalon.

4.1. Introduction

Vertebrate development requires a number of carefully coordinated signals in order to properly control gene expression, thus leading to restricted cell proliferation, differentiation, and migration. Gene regulatory networks (GRNs) are made up of dynamic interactions between combinations of transcription factors and regulatory elements found within the genome (for reviews see: Levine and Davidson, 2005; Davidson and Levine, 2008). These regulatory sequences are made up of a number of clustered transcription factor binding sites, and when bound they have the ability to affect transcription of specific genes (for reviews see: Kulkarni and Arnosti, 2003; Kadonaga et al. 2004; Panne et al. 2008). The overall levels and timing of gene expression are

conferred by the cumulative contributions of multiple transcription factors on possibly multiple regulatory regions. The genes regulated by this process during development often encode transcription factors that will play a role in the regulation of other transcription factor genes located downstream in the GRN.

The proper development of the forebrain requires a vast number of regulatory genes that control critical developmental processes, such as patterning and neurogenesis. One class of regulatory genes is the basic helix-loop-helix (bHLH) transcription factors, which are thought to play important roles in the GRNs controlling neurogenesis (for reviews see: Bertrand et al. 2002; Allan and Thor, 2003)(Gohlke et al. 2008). Mash1 is one such bHLH transcription factors expressed in proliferating neural precursors in the subpallial telencephalon and prethalamus (Lo et al. 1991; Guillemot and Joyner, 1993; Porteus et al. 1994; Yun et al. 2002; Andrews et al. 2003). The expression of *Mash1* overlaps with *Dlx* expression in the forebrain, specifically in proliferating progenitor cells located in the telencephalon and prethalamus (Porteus et al. 1994; Yun et al. 2002; Andrews et al. 2003). *Mash1* mutants have defects in neural specification and the timing of differentiation in the ventral forebrain, including premature and potentially increased expression of the *Dlx* and *Gad1* (*Gad67*) genes in the ventral telencephalon (Casarosa et al. 1999; Horton et al. 1999: Yun et al. 2002). Ectopic expression of *Mash1* leads to *Gad1* expression in the mouse dorsal telencephalon, indicating *Mash1* plays a role in GABAergic interneuron development (Fode et al. 2000). One of the zebrafish *Mash1* orthologs, *ascl1a*, is expressed in the forebrain, including the subpallial telencephalon and prethalamus of the zebrafish forebrain, strongly correlating with *Mash1* expression in the mouse forebrain (Allende and Weinberg, 1994; Wullimann and Mueller, 2002).

Knockdown of Aslc1a function in the zebrafish affects the development of the pituitary gland, neurogenesis in the epiphysis, and regeneration in the retina (Cau and Wilson, 2003; Herzog et al. 2004; Pogoda et al. 2006; Fausett et al. 2008).

The *Dlx* genes encode are homeodomain transcription factors expressed in the ganglionic eminences of the telencephalon, prethalamus, and hypothalamus in mouse (Liu et al. 1997; Yang et al. 1998; Anderson et al. 1997a; Eisenstat et al. 1999). More specifically, four *Dlx* genes are expressed in the forebrain of the mouse: *Dlx1, Dlx2, Dlx5,* and *Dlx6* (Liu et al. 1997; Yang et al. 1998; Anderson et al. 1997a; Eisenstat et al. 1999), while five orthologous *dlx* genes are expressed in the forebrain of the zebrafish: *dlx1a*, *dlx2a*, *dlx5a*, *dlx6a*, and *dlx2b* (Akimenko et al. 1994; Ellies et al. 1997b). The *Dlx* genes are expressed in highly overlapping but also distinct domains within the forebrain, often correlating with neuronal differentiation (Liu et al. 1997; Eisenstat et al. 1999; MacDonald et al. unpublished observations). The *Dlx* genes are highly co-expressed with the two *glutamic acid decarboxylase* (*Gad*) genes in the forebrain, which encode the enzymes responsible for the production of γ-amino butyric acid (GABA) (Stühmer et al. 2002a; Yun et al. 2003; MacDonald et al. unpublished observations). Functional studies have shown that the *Dlx* genes are required for the differentiation and migration of most GABAergic neurons in the telencephalon (Anderson et al. 1997a; Anderson et al. 1997b; Stühmer et al. 2002a; Stühmer et al. 2002b). The zebrafish *dlx* genes are involved in branchial arch and sensory placode development; however, their function in the forebrain has not previously been determined (Solomon and Fritz, 2002; Kaji and Artinger, 2004; Walker et al. 2007; Jackman and Stock, 2008; Sperber et al. 2008).

The proper expression of the *Dlx* genes in mouse and zebrafish embryos is due to a number of conserved enhancers found in the surrounding genome. Two enhancers from the *Dlx1/Dlx2* bigene pair, upstream regulatory element 2 (URE2) and I12b, and two from the *Dlx5/Dlx6* bigene pair, I56i and I56ii, are active in the forebrain (Zerucha et al. 2000; Ghanem et al. 2003; Ghanem et al. 2007; Potter et al. 2009). The orthologous regulatory elements from the zebrafish *dlx* bigene clusters are also active in the developing forebrain (Zerucha et al. 2000; Ghanem et al. 2003; MacDonald et al. unpublished observations). MASH1 activates and directly binds to the I12b regulatory element suggesting it plays a role in the regulation of the *Dlx* genes (Poitras et al. 2007). Orthologous *dlx* regulatory elements from the zebrafish, mouse, and elephant shark drive similar reporter gene expression in the telencephalon and diencephalon in transgenic mouse and zebrafish assays, indicating highly conserved GRNs control the expression of the *dlx* genes in the forebrain (Zerucha et al. 2000; Stühmer et al. 2002b; MacDonald et al. unpublished observations).

To identify the role of *ascl1a* and the *dlx* genes in the GRN involved in the differentiation of GABAergic interneurons in the zebrafish forebrain, we have systematically knocked down their functions and assayed the effects on downstream targets. We show there is substantial overlap of *ascl1a*, *dlx*, and *gad1* expression leading to the possibility that these genes genetically interact. Loss of Ascl1a function leads to a reduction of *dlx* and *gad1* expression in the prethalamus, which is mimicked by reporter gene expression under the control *dlx* regulatory elements. Knockdown of individual *Dlx* gene function individually results in no visible consequences on *gad1* expression, indicating functional redundancy between the *dlx* genes. However, double knockdown of

dlx1a and *dlx2a* results in a loss of *gad1* expression in the prethalamus but not the telencephalon, similar to the knockdown of *ascl1a*. The single or double knockdown of *dlx5a* and *dlx6a* does not result in modification of *gad1* expression, indicating they do not play a direct regulatory role on this gene. Our results show that the *ascl1a* gene regulates *dlx* genes necessary for proper *gad1* expression in the prethalamus of the zebrafish. These genes are thus part of a GRN involved in early forebrain development and conserved among bony vertebrates.

4.2. Results

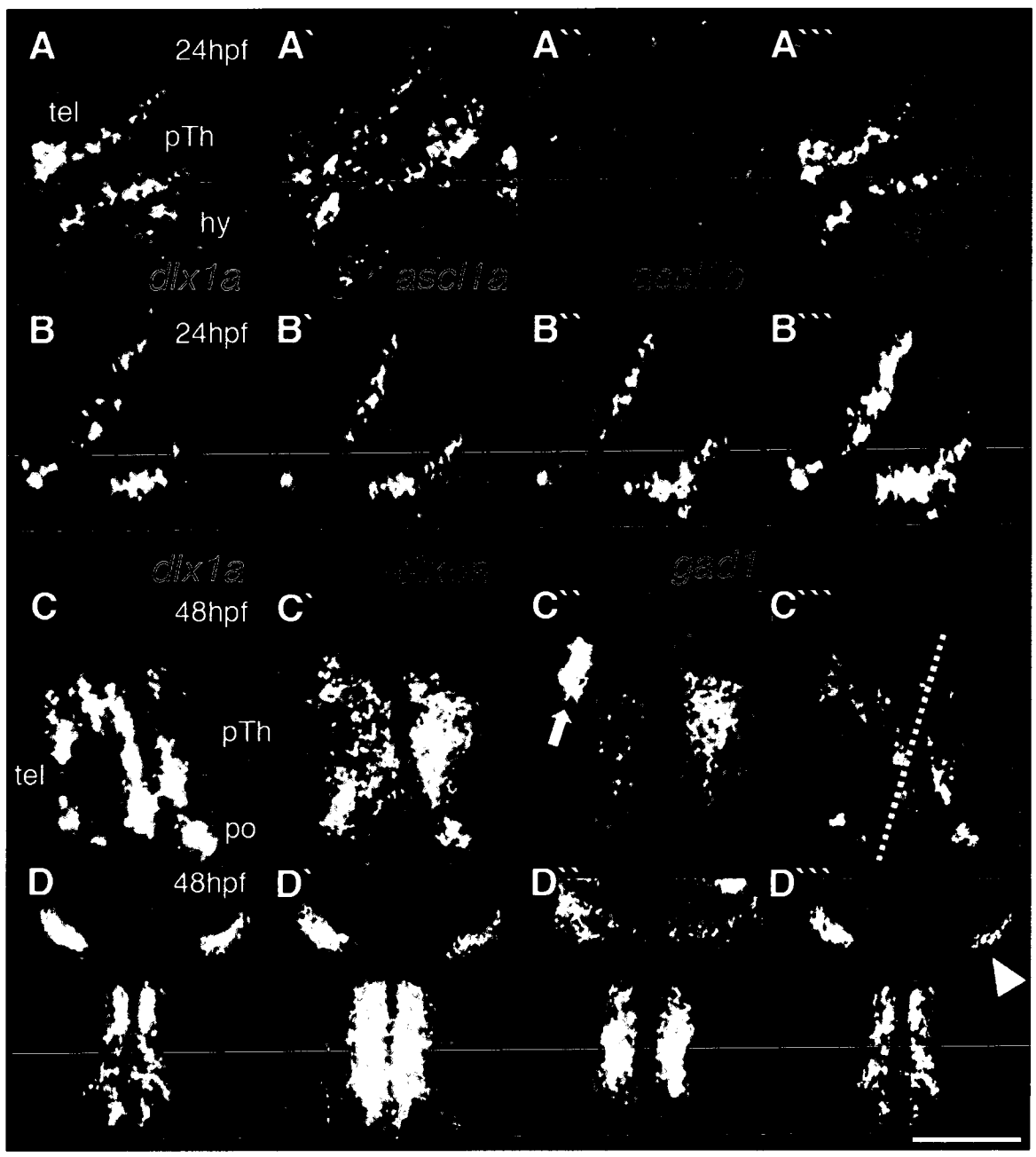
4.2.1. Co-expression of *ascl1a*, *dlx*, and *gad1* supports genetic interactions in the forebrain

The overlapping expression of *Mash1*, *Dlx*, and *Gad* genes indicates these genes may genetically interact during mouse forebrain development (Porteus et al. 1994; Yun et al. 2002; Andrews et al. 2003). *Mash1*$^{-/-}$ mice have premature expression of *Dlx* in the ganglionic eminences (Casarosa et al. 1999; Horton et al. 1999: Yun et al. 2002) and MASH1 proteins have been shown to activate and directly bind to a *Dlx1/Dlx2* regulatory element (Poitras et al. 2007). We utilized triple fluorescent *in situ* hybridizations to determine if their zebrafish orthologs also show overlapping expression domains in the forebrain. The expression of *ascl1a* begins in the prospective forebrain at 10 hours post fertilization (hpf) and lasts until 72hpf (Allende and Weinberg, 1994). Shortly after the onset of *ascl1a* expression, the *dlx1a/dlx2a* genes are expressed starting at 13hpf in the prospective forebrain (Akimenko et al. 1994; Ellies et al. 1997b). At 24 hpf *dlx1a* is expressed in the telencephalon and two domains of the diencephalon, the prethalamus (or

ventral thalamus) and the hypothalamus (Figure 4.1A). We have previously shown that *dlx* genes found within one bigene cluster arrangement have identical expression domains in the forebrain, and therefore make the assumption that the expression of one *dlx* gene represents the expression of the other (e.g. *dlx1a* and *dlx2a*; *dlx5a* and *dlx6a*) (MacDonald et al. unpublished observations). At 24hpf, expression of *ascl1a* is detected in the telencephalon and prethalamus, and partially overlaps with the *dlx1a* expression domain (Figure 4.1A). The *ascl1b* gene is paralogous to *ascl1a*; however, each has unique and overlapping expression domains with the central nervous system, including the forebrain (Allende and Weinberg, 1994). At 24hpf, little expression of *ascl1b* was detected in the forebrain without co-expression with *dlx1a* or *ascl1a,* indicating *aslc1b* probably does not interact with the *dlx* genes at this stage in the forebrain (Figure 4.1A'').

The *Dlx* genes are expressed in very similar domains within the developing forebrain of mice and zebrafish (Liu et al. 1997; Anderson et al. 1997a; Eisenstat et al. 1999; Akimenko et al. 1994; Ellies et al. 1997b; Zerucha et al. 2000; MacDonald et al. unpublished observations). At 24hpf, the expression domains of *dlx5a* and *gad1* are both highly overlapping with *dlx1a* expression in the telencephalon, prethalamus, and hypothalamus (Figure 4.1B). Expression of *dlx1a*, *dlx5a*, and *gad1* remains highly overlapping at 48hpf in the telencephalon (Figure 4.1C). However, there is an area of intense staining for *gad1* in the dorsal region of the telencephalon potentially corresponding to GABAergic interneurons that will migrate into the pallium starting at approximately 72 hpf (Figure 4.1C''')(Mione et al. 2008). Within the preoptic area *dlx1a* and *dlx5a* are co-expressed, but *gad1* is virtually absent from this area (Figure 4.1C). The

Figure 4.1. The expression domains of the *ascl1a*, *dlx*, and *gad1* genes overlap in the forebrain of the embryonic zebrafish. Single z sections of triple fluorescent *in situ* hybridizations. (A) *dlx1a* and *ascl1a* are co-expressed throughout the telencephalon and prethalamus at 24hpf, while *ascl1b* is not co-expressed at this stage. (B) The *dlx1a*, *dlx5a*, and *gad1* genes are co-expressed in the telencephalon, prethalamus, and hypothalamus at 24hpf. (C) The *dlx1a*, *dlx5a*, and *gad1* genes continue to be co-expressed in the telencephalon and prethalamus at 48hpf. *gad1* expression is increased in the dorsal tip of the telencephalon (arrow). The plane of section for (D) is shown as a dotted line in C'''. (D) Cross-section showing co-expression of *dlx1a*, *dlx5a*, and *gad1* in the prethalamus (arrowhead). hy, hypothalamus; po, preoptic area; pTh, prethalamus; tel, telencephalon. Scale bar= 100 μm.


A
24hpf
A`
A``
A```
tel
pTh
hy
dlx1a
ascl1a
ascl1b
B
24hpf
B`
B``
B```
dlx1a
gad1
C
48hpf
C`
C``
C```
pTh
tel
po
D
48hpf
D`
D``
D```

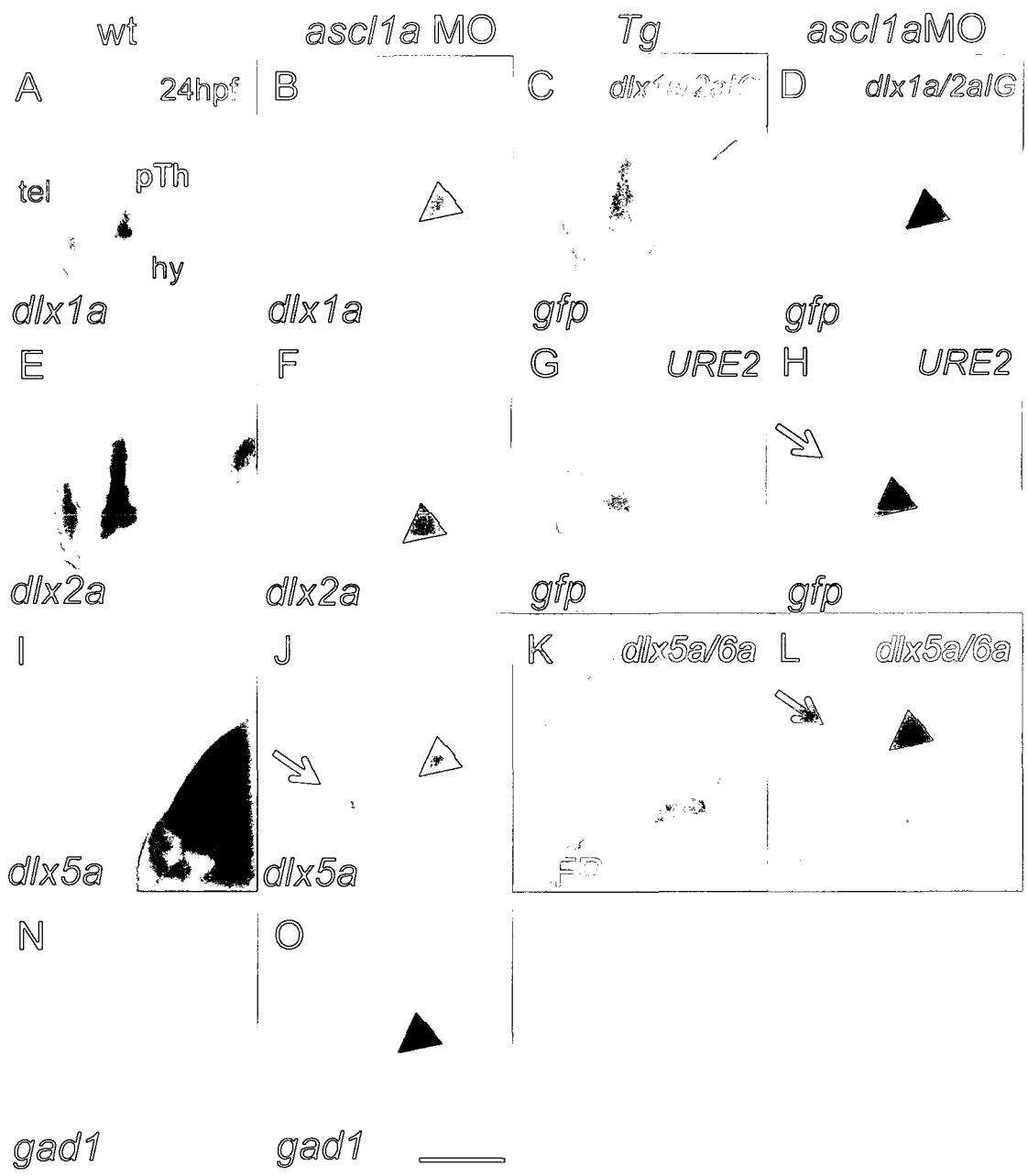
dlx1a, dlx5a, and gad1 genes are all co-expressed in the prethalamus (Figure 4.1C and D). These closely related expression domains within the forebrain are similar to observations made in mouse, suggesting that the genetic cascade controlling *gad1* expression in the zebrafish forebrain may also be similar.

4.2.2. Knockdown of *ascl1a* reveals a role in the regulation and expression of the *dlx* and *gad1* genes in the prethalamus

To determine the role of *ascl1a* in the regulation of the *dlx* genes and *gad1* expression, we knocked down Ascl1a activity with a translation blocking morpholino (MO) (Cau and Wilson, 2003). Embryos injected with *ascl1a*-MO (morphants) were examined at 24hpf, a stage when the genes of interest are expressed and the distinct regions of the forebrain are evident. In the *ascl1a* morphants there is a loss of *dlx1a* and *dlx2a* in the ventral domain of the prethalamus (Figure 4.2A, B, E, F). Additionally, *dlx5a* expression is reduced in the prethalamus of *ascl1a* morphants in a pattern similar to the modified expression of *dlx1a* and *dlx2a* (Figure 4.2I and J). A slight reduction of *dlx1a*, *dlx2a*, and *dlx5a* expression in the telencephalon is also possible but difficult to ascertain by *in situ* hybridization. Additionally, *gad1* is also reduced in the prethalamus of *ascl1a* morphants, suggesting that the loss of the *dlx* genes may play a role in the differentiation of *gad1* expressing cells (Figure 4.2N and O).

We then tested whether the knockdown of *ascl1a* affects the regulation of the *dlx* genes by controlling the activity of *dlx* regulatory elements. To address this, we knocked down Ascl1a function in the transgenic lines: *Tg(dlx1a/2aIG:GFP)*, *Tg(URE2dlx1a/2a:GFP)*, and *Tg(dlx5a/6a:GFP)*, which express *gfp* under the control of

Figure 4.2. *ascl1a* function is required for proper regulation and expression of the *dlx* genes in the forebrain at 24hpf. (A, B, E, F) *dlx1a* and *dlx2a* expression is reduced in the prethalamus (arrowheads), but is less affected in the telencephalon in *ascl1a* morphants. (C,D) The *Tg(dlx1a/2aIG:GFP)* has reduced *gfp* expression in the prethalamus, but the telencephalon appears relatively unaffected in morphants. (G,H) The *Tg(URE2dlx2a/2a:GFP)* has reduced *gfp* expression in the telencephalon (arrow) and prethalamus. (I, J, K, L) The expression of *dlx5a* is reduced in the telencephalon and prethalamus, consistent with the loss of GFP expression in the *Tg(dlx5a/6a:GFP)* transgenic line. (M, N) *gad1* expression is similary unaffected in the telencephalon, but is reduced in the prethalamus. hy, hypothalamus; pTh, prethalamus; tel, telenecphalon. Scale bar= 100 μm


wt
ascl1a MO
Tg
ascl1aMO
A
24hpf
B
C
dlx1a/2aIG
D
dlx1a/2aIG
tel
pTh
hy
dlx1a
dlx1a
gfp
gfp
E
F
G
URE2
H
URE2
dlx2a
dlx2a
gfp
gfp
I
J
K
dlx5a/6a
L
dlx5a/6a
dlx5a
dlx5a
N
O
gad1
gad1

specific *dlx* regulatory regions and recapitulate endogenous *dlx* expression (Zerucha et al. 2000; Mione et al. 2007; MacDonald et al. unpublished). Both the *Tg(dlx1a/2aIG:GFP)* and *Tg(URE2dlx1a/2a:GFP)* lines injected with *ascl1a* MO show reduced activity in the prethalamus consistent with the loss of *dlx1a* and *dlx2a* expression in this domain (Figure 4.2 A-H). The expression of *gfp* in *Tg(URE2dlx1a/2a:GFP)* is also reduced in the telencephalon indicating *ascl1a* is also necessary for proper regulation of *dlx1a* and/or *dlx2a* in the telencephalon (Figure 4.2G and H). The *Tg(dlx5a/6a:GFP) ascl1a* morphants showed loss of GFP expression in the prethalamus, while expression in the telencephalon may be reduced but is still detectable. In total, our morphant data supports the hypothesis that *ascl1a* acts as an upstream regulator of the *dlx1a, dlx2a, dlx5a* and *gad1* genes in the embryonic prethalamus.

4.2.3. Knockdown of *dlx1a/dlx2a* results in the loss of *gad1* expression in the prethalamus

The co-expression of the *dlx* genes and *gad1* in the forebrain suggests a possible role of *dlx* in the regulation of *gad1* expression. In addition, ectopic expression of the *Dlx* genes in mice leads to expression of *Gad* genes, *Gad1* and *Gad2*, in telencephalon slice cultures (Anderson et al. 1999; Stühmer et al. 2002a). Furthermore, $Dlx1^{-/-}/Dlx2^{-/-}$ mice show a loss of *Gad1* expression in the olfactory bulb and the ventral telencephalon (Anderson et al. 1997a; Anderson et al. 1997b; Bulfone et al. 1998). To assay the function of the *dlx* genes on *gad1* expression in the zebrafish forebrain at 48hpf, we used translation and splice blocking *dlx* MOs against *dlx1a*, *dlx2a*, *dlx5a*, and *dlx6a* (see

Supplementary Figure 4.1. Single knockdowns of *dlx* genes do not affect *gad1* expression in the forebrain. A) The wild type expression of the *gad1* gene in the zebrafish forebrain at 48 hpf. The knockdowns of *dlx1a* (B), *dlx2a* (C), *dlx2b* (D), *dlx5a* (E), or *dlx6a* (F) do not alter *gad1* expression.

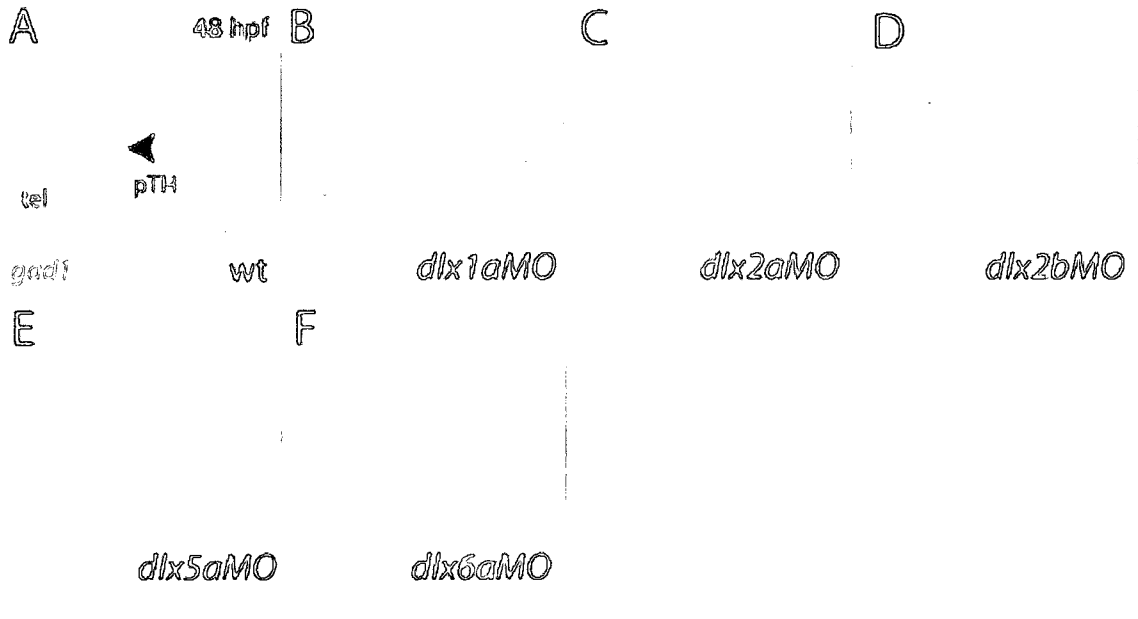

A
48 hpf
B
C
D
pTH
tel
gad1
wt
dlx1aMO
dlx2aMO
dlx2bMO
E
F
dlx5aMO
dlx6aMO

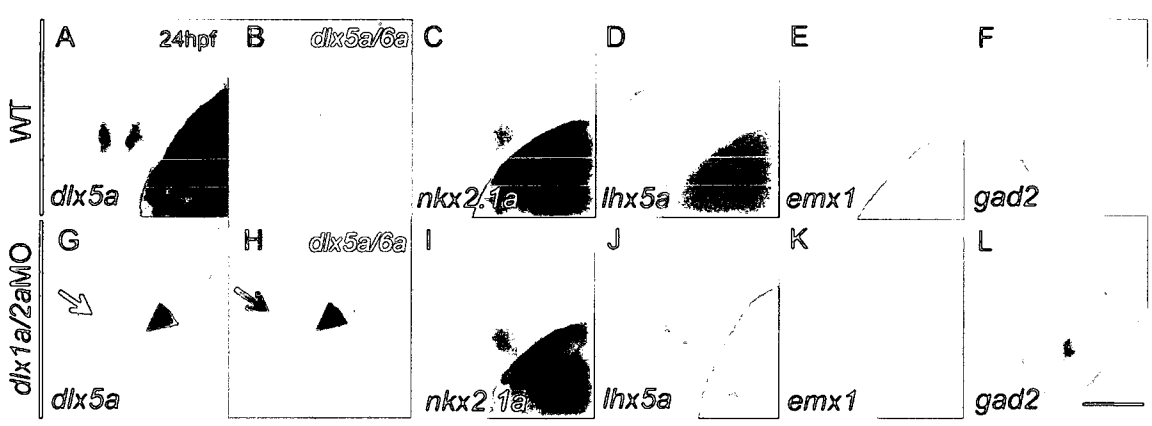
Figure 4.3. The expression of *gad1* is lost in the prethalamus but not the telencephalon in *dlx1a/dlx2a* morphants at 48hpf. (A) Expression of *gad1* in wild type embryos is present in the telencephalon and prethalamus (arrowhead). (B) Double morpholino knockdown of *dlx1a* and *dlx2a* results in a loss of prethalamus *gad1* expression (arrow). Dorsal views of wild type (C) and *dlx1a/dlx2a* morphants (D) showing the loss of *gad1* expression in the prethalamus. (E) Double knockdown of *dlx5a/6a* does not result in the loss of *gad1* in prethalamus or telencephalon. (F) Triple knockdown of *dlx1a/dlx2a/dlx2b* resulted in a loss of *gad1* expression in the prethalamus, mimicking the loss in *dlx1a/dlx2a* morphants. Arrowheads= wild type expression of *gad1* in the prethalamus; Arrows= prethalamus loss of *gad1* expression. tel, telencephalon; pTh, prethalamus. Scale bar= 100μm

A
48 hpf
B
C
wt
pTH
tel
D
gad1
wt
dlx1a/2aMO
E
F
dlx5a/6aMO
dlx1a/2a/2bMO

material and methods - Sperber et al. 2008). Single *dlx* morphants had no effect on *gad1* expression in the telencephalon or prethalamus at 48hpf (Suppl. 4.1). However, knockdown of *dlx1a* and *dlx2a* in double MO injections resulted in a loss of prethalamic *gad1* expression at 24 (data not shown) and 48 hpf (Figure 4.3A-D). The loss of *dlx5a* and *dlx6a* had no effect on *gad1* expression in the forebrain at 24hpf (data not shown) or 48hpf (Figure 4.3E). Additionally, triple knockdown of the paralog *dlx2b*, and *dlx1a* and *dlx2a* did not increase the severity of *gad1* loss in the prethalamus nor result in any additional loss in the telencephalon (Figure 4.3F). Therefore, *dlx1a* and *dlx2a* are necessary for proper expression of *gad1* in the prethalamus but not the telencephalon.

The *Dlx* genes also act in auto- and cross- regulation between the distinct bigene pairs by direct binding of Dlx transcription factors to their regulatory elements (Zerucha et al. 2000; Zhou et al. 2004; Poitras et al. 2007; Bond et al. 2009; Potter et al. 2009). Specifically in the forebrain, $Dlx1^{-/-}/Dlx2^{-/-}$ mice have a large reduction in *Dlx5* and *Dlx6* expression in the telencephalic ventricular and subventricular zones due to the loss of regulatory activity of intergenic enhancers (Anderson et al. 1997a; Zerucha et al. 2000). However, it is currently unknown if this cross-regulation of the *Dlx* genes also applies to the mouse diencephalon. To test whether the *dlx1a* and *dlx2a* genes play a role in the regulation of *dlx5a* and *dlx6a*, we injected MOs and looked into *dlx5a* expression and the activity of *Tg(dlx5a/6aIG:GFP)*. There is no loss of *dlx5a* expression in single *dlx1a* or *dlx2a* morphant embryos (data not shown). In double MO injected embryos, there is a severe reduction of both telencephalic and prethalamic expression of *dlx5a*, consistent with the loss of GFP expression in *Tg(dlx5a/6aIG:GFP)*(Figure 4.4A-D). To confirm that the observed differences in expression were not due to alteration of the anatomy of the

Figure 4.4. Knockdown of *dlx1a/dlx2a* identifies cross-regulatory interactions between the *dlx* bigene pairs. (A, B, G, H) Double knockdown of *dlx1a/dlx2a* results in reduced regulatory activity of *Tg(dlx5a/6a:GFP)* and expression of *dlx5a* in the telencephalon and prethalamus, similar to *ascl1a* morphants (arrow, arrowhead). The observed defects in the prethalamus are not due to loss of mis-patterning of the structures as (C, I) *nkx2.1a*, (E, K) *emx1*, and (D, J) *lhx5a* makers are unaffected in *dlx1a/dlx2a* morphants. (F, L) The *dlx1a/dlx2a* morphants show a loss of *gad1* expression but not *gad2* in the forebrain. Arrow, loss of expression in the telencephalon; arrowhead, loss of expression in the prethalamus. Scale bar= 100 μm


WT
dlx1a/2aMO
A
24hpf
B
dlx5a/6a
C
D
E
F
dlx5a
nkx2.1a
lhx5a
emx1
gad2
G
H
dlx5a/6a
I
J
K
L
dlx5a
nkx2.1a
lhx5a
emx1
gad2

telencephalon or prethalamus, we verified the expression of marker genes such as *lhx5a*, *nkx2.1a*, and *emx1* and their expression domains were unchanged after the knockdown of *dlx1a* and *dlx2a* (Figure 4.4. C-E, I-K). The *gad2* gene is also expressed in the telencephalon and prethalamus, in a pattern very similar to *gad1*, and therefore could also be regulated by the *dlx* genes (Martin et al. 1998). However, *dlx1a* and *dlx2a* MO knockdowns have no effect on the expression of *gad2* at 24hpf or 48hpf (data not shown), indicating the *gad1* and *gad2* genes are regulated by distinct genetic pathways in the zebrafish (Figure 4.4F and L).

4.3. Discussion

4.3.1. *ascl1a* is necessary for the proper regulation and expression of the *dlx* and *gad1* genes

In this report, we show that *ascl1a* is co-expressed with *dlx1a*, which will also be co-expressed with *dlx5a* and *gad1* in the forebrain, reminiscent of the expression of the mouse orthologs (Porteus et al. 1994; Yun et al. 2002; Yun et al. 2002; Andrews et al. 2003; Stühmer et al. 2002a). The similar expression of the *dlx* genes and *gad1* in homologous tissues suggests the GRNs necessary for their expression described in mouse may be conserved in teleosts. In the mouse, *Mash1* is required for the generation of early born neurons in the subcortical telencephalon, where *Dlx* genes play a role in late neurogenesis (Casarosa et al. 1999; Horton et al. 1999; Anderson et al. 1997a). The gene expression data, coupled with their relationship during neurogenesis in the forebrain, suggests that *Mash1* is upstream of *Dlx*. Knockdown of the *Mash1* ortholog, *ascl1a*, resulted in the loss of *dlx1a*/*dlx2a* and *dlx5a* expression in the prethalamus, and possible

reduced expression domain in the telencephalon, showing that *ascl1a* is involved in the regulation of *dlx* genes, and genetically upstream of them. The loss of Ascl1a function resulted in a loss of *dlx1a/dlx2a* in the prethalamus, as well as a loss of *dlx5a* and *gad1* in the same region. Additionally, MO knockdown of both *dlx1a* and *dlx2a* mimics this loss of *dlx5a* and *gad1* in the prethalamus. We therefore attribute the loss of *dlx5a* and *gad1* expression in *ascl1a* morphants to the loss of proper *dlx1a/dlx2a* expression in the prethalamus. Therefore, our data suggest that *ascl1a* regulates *dlx1a/dlx2a* expression, which will then regulate *dlx5a/dlx6a* and *gad1* expression. The results of *dlx5a/6a* morpholino injections suggest that the modification of *gad1* expression in *dlx1a/2a* morphants is not dependent on proper *dlx5a/6a* expression.

Interestingly *Mash1*$^{-/-}$ mice have neurogeneis and differentiation defects, including premature expression of *Dlx* and *Gad* in the ventricular zone of the telencephalon (Horton et al. 1999; Casarosa et al. 1999). These data suggest that *Mash1* acts as a negative regulator of *Dlx* expression. However, mutagenesis of a MASH1 binding site in a *Dlx1/2* enhancer, I12b, will lead to a reduced activity of this element in the forebrain (Poitras et al. 2007), suggesting MASH1 as a transcriptional activator. Our data suggest that the *Mash1* ortholog, *ascl1a*, is a positive regulator on *dlx* transcription in the prethalamus but has no affect in the telencephalon. It is however difficult to rule out precocious expression of *dlx* within the zebrafish telencephalon with *in situ* hybridizations but may be possible with confocal microscopy. Overall, our data supports the hypothesis that *Mash1* (*ascl1a*) is a positive regulator of *Dlx* expression in the forebrain.

4.3.2. Loss of *dlx* expression in *ascl1a* morphants is consistent with the loss of *dlx* regulatory activity

In mammals, the DLX proteins can directly bind to their own regulatory elements and others from different bigene pairs, thus playing a role in the regulation of their own expression and in the regulation of their paralogs (Zerucha et al. 2000; Zhou et al. 2004; Bond et al. 2009; Potter et al. 2009). The loss of DLX binding to the regulatory elements results in the loss of transgene activity and *Dlx* expression in the forebrain in mammals (Zerucha et al. 2000; Poitras et al. 2007). To explore this issue in the zebrafish, we tested the effect of *ascl1a* and *dlx* morpholino injections on transgenic zebrafish where the reporter gene (*gfp*) expression is under the control of zebrafish *dlx* enhancer sequences. The *Tg(dlx1a/2aIG:GFP)* contains the I12b element and is active in telencephalon and prethalamus, similar to orthologous mouse sequences (Ghanem et al. 2003; Ghanem et al. 2007; Poitras et al. 2008; MacDonald et al. in prep). This element contains a highly conserved bHLH-binding site (E box) that was shown to physically interact with the MASH1 protein resulting in activation *in vitro* (Poitras et al. 2007). Mutagenesis of the MASH1 binding site resulted in a reduction of enhancer function in the telencephalon and prethalamus in mouse embryos (Poitras et al. 2007). In *ascl1a* morphants, transgene expression is severely reduced in the prethalamus at 24hpf. The loss of *Tg(dlx1a/2aIG:GFP)* regulatory activity in *ascl1a* morphants is most likely due to the loss of *ascl1a* binding to I12b, but it is noted that the telencephalic expression of the transgene does not seem to be severely affected by the lack of Ascl1a protein (discussed below). Knockdown of *ascl1a* also leads to a reduction in the activity of the *Tg(URE2dlx1a/2a:GFP)* in the telencephalon and diencephalon. This strong loss of

URE2 activity is not consistent with the observed loss of *dlx1a/dlx2a* expression in the diencephalon but relatively unchanged expression in the telencephalon. The loss of URE2 regulatory activity may be masked by the remaining activity of the I12b enhancer in the telencephalon, as these elements are potentially shared by *dlx1a* and *dlx2a* (Ellies et al. 1997b). Notably, the URE2 regulatory element does not contain an E box (data not shown), suggesting that Ascl1a may not directly bind to this sequence. This data supports the hypothesis of the presence of two distinct and redundant genetic pathways regulating *dlx* expression in the forebrain through the URE2 enhancer.

4.3.3. Dlx1a/Dlx2a function during prethalamus development in the zebrafish

The *Dlx* genes have largely redundant functions within the mouse forebrain, as single mutants of *Dlx1*, *Dlx2*, and *Dlx5* have only mild phenotypes (Qiu et al. 1995; Acampora et al. 1999; Cobos et al. 2005; Mao et al. 2009). In this report we show that single knockdowns of *dlx* genes in the zebrafish had no effect on *gad1* expression while double knockdown resulted in the loss of *gad1* expression in the prethalamus, making *dlx* protein redundancy in the forebrain a characteristic shared by mammals and zebrafish. In the mouse, $Dlx1^{-/-}/Dlx2^{-/-}$ mutants show a major block in neurogenesis and differentiation resulting in a loss of *Gad1/2* expression in the ventral telencephalon (Anderson et al. 1997a; Marin et al. 2000). Our double MO knockdown of *dlx1a/dlx2a* did not result in the loss of *gad1* expression in the zebrafish telencephalon. However, there was a loss of *gad1* expression in the prethalamus, consistent with the knockdown of *ascl1a*. Knockdown of *dlx2b*, a gene highly co-expressed with *dlx1a* and *dlx2a* (MacDonald et al. unpublished observations) and paralogous to *dlx2a*, did not have any *gad1* phenotype

alone or in triple morpholino knockdowns. This suggests that *dlx2b* is not sufficient to rescue the *gad1* phenotype in the prethalamus and may not be functionally redundant with the *dlx1a* or *dlx2a* genes. These results also show that the lack of an effect of the *dlx1a/dlx2a* morpholinos on *gad1* expression in the telencephalon is not due to a putative redundant function of *dlx2b*. The lack of a *gad1* phenotype in the telencephalon in morphants, coupled with the loss in the prethalamus, leads us to two conclusions: i) The genetic pathways regulating *gad1* expression in the telencephalon have diverged between mammals and teleosts; ii) The *dlx1a/dlx2a* genes are necessary for proper *dlx5a* expression via cross-regulatory interactions and control *gad1* expression in the prethalamus (discussed below). The knockdown of *dlx1a/dlx2a* leads to a reduction of *dlx5a* expression in the telencephalon and prethalamus, consistent with the loss of the *Tg(dlx5a/6a:GFP)* activity, indicating that the cross regulatory interactions from one bigene to another are conserved in the zebrafish forebrain.

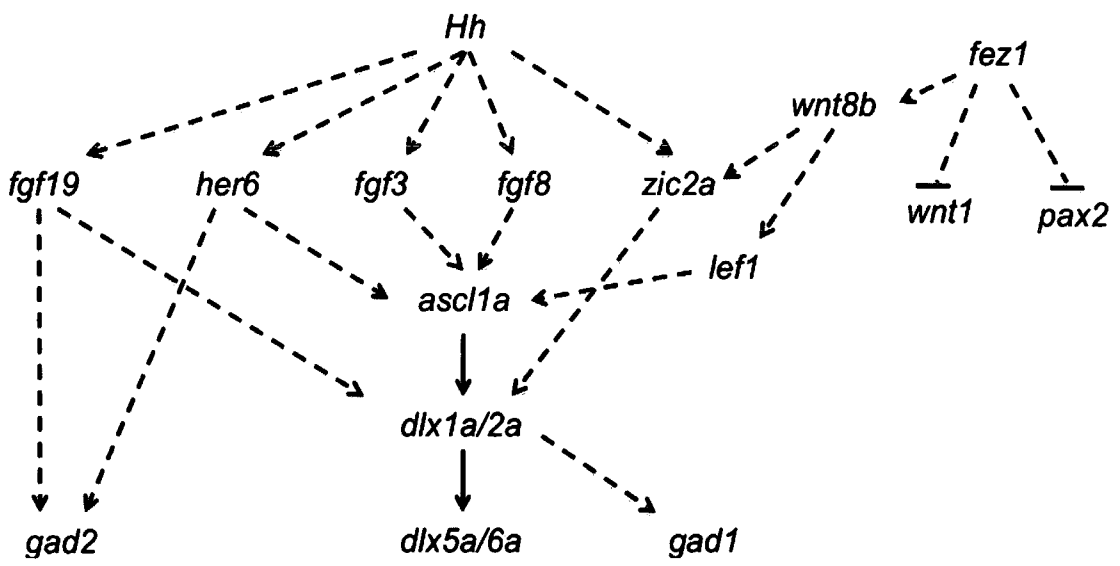
The knockdown of *dlx5a* and *dlx6a* did not result in a chage of *gad1* expression in the forebrain indicating the specific loss of *gad1* in the prethalamus is due to the loss of *dlx1a/dlx2a* activity and not the corresponding loss of *dlx5a/dlx6a*. Unfortunately, the $Dlx5^{-/-}/Dlx6^{-/-}$ mice forebrain phenotype cannot be assayed due to exencephaly (Robledo et al. 2002). However, $Dlx1^{-/-}/Dlx2^{-/-}$ mice also have defects in the tangential migration of GABAergic interneurons to the cortex (Anderson et al. 1997b; Marin et al. 2000). Our *in situ* data are not sufficient to infer defects in neuronal migration but it is possible that *dlx5a/dlx6a*, or *dlx1a/dlx2a* are involved in this process. The loss of specific markers in the prethalamus is not due to increased cell death or loss of the anatomical structure as specific molecular markers for this forebrain region are unaltered in *dlx* morphants. The

knockdown of *dlx* genes did not affect the expression of *gad2*, indicating that distinct genetic pathways regulate the *gad1* and *gad2* genes, possibly due to their 5` flanking regions (Szabo et al. 1996; Pinal et al. 1997; Yanagawa et al. 1997). Contrary to this finding, several factors such as *fgf3/8* and *her6* were shown to be necessary for the proper expression of *gad1* and *gad2* in the prethalamus, indicating that these genes can be regulated by similar factors (Miyake et al. 2005; Scholpp et al. 2009). The *dlx* morphants did not affect the expression of *gad1* or *gad2* in the telencephalon; however, they may alter the specific fate of the GABAergic interneurons that can be assessed by specific molecular markers (for review see: Markram et al. 2008).

4.3.4. Wnt and Hedgehog signaling act in distinct parallel genetic cascades to regulate the *dlx* genes in the zebrafish forebrain

We have shown that *ascl1a* and the *dlx* genes play a role in the specification of GABAergic interneurons in the prethalamus. However, it is still unclear how these genes fit into the much larger GRN involved in the proper patterning of the diencephalon (Figure 4.5). The area located between the prethalamus and the dorsal thalamus is the zona limitans intrathalamica (ZLI) that acts as a major signaling center for the development of the diencephalon (Shimamura et al. 1995; Scholpp et al. 2006). The ZLI express hedgehog molecules *sonic hedgehog* (*shh*) and *tiggy winkle hedgehog* (*twhh*) in the zebrafish (Krauss et al. 1993; Ekker et al. 1995). Both *shh* and *twhh* are necessary for the differentiation of the prethalamus, and subsequently the expression of *dlx2a* (Scholpp et al. 2006). Interestingly, consistent with our results, the loss of Hedgehog expression results in the complete loss of *dlx2a* expression in the prethalamus but unaffected *dlx2a*

Figure 4.5. Proposed gene regulatory network controlling *gad1* expression in the prethalamus. The model is based on our results as well as published studies (Jeong et al. 2007; Lee et al. 2006; Minyake et al. 2005; Miyake et al. 2005; Pogoda et al. 2006; Sanek and Grinblat, 2008; Scholpp et al. 2006; Shinya et al. 2001; Varga et al. 2001; Walshe and Mason, 2003.


Hh
fez1
wnt8b
fgf19
her6
fgf3
fgf8
zic2a
wnt1
pax2
lef1
ascl1a
dlx1a/2a
gad2
dlx5a/6a
gad1

expression in the telencephalon (Varga et al. 2001; Miyake et al. 2005; Scholpp et al. 2006). Hedgehog signaling appears to be upstream of Fibroblast growth factors (Fgfs) in both the mouse and zebrafish forebrains (Ishibashi and McMahon, 2002; Miyake et al. 2005). Knockdown of two *Fgf* genes, *fgf3* and *fgf8*, results in the loss of *dlx2a* expression in the zebrafish ventral forebrain (Shinya et al. 2001; Walshe and Mason, 2003; Minyake et al. 2005). Knockdown of these genes also results in the loss of *gad1* expression specifically in the prethalamus; however, knockdown of *fgf19* results in the complete loss of *gad1* and *gad2* expression in the telencephalon and prethalamus (Miyake et al. 2005). It has been suggested that *ascl1a* acts downstream of *fgf3* in the diencephalon and mediates the differentiation of the zebrafish adenohypophysis (Pogoda et al. 2006), leading to the possibility that this pathway is intact in the prethalamus. Furthermore, overexpression of *Fgf8* in the mouse thalamus induces ectopic *Mash1* and *Gad1* expression (Kataoka and Shimogori, 2008), identifying the *Fgfs* as regulators of *Mash1* (or *ascl1a*), *Dlx*, and *gad1/gad2* gene expression in the forebrain. Finally, knockdown of *her6* results in the loss of *ascl1a* expression, the loss of *Tg(dlx5a/6a:GFP)* activity, and *gad1/gad2* expression in the prethalamus (Scholpp et al. 2009). We have shown that this loss of *Tg(dlx5a/6a:GFP)* activity in the prethalamus is most likely due to the subsequent loss of Dlx1a/Dlx2a function; however, this data strongly supports the described genetic cascade which we describe is regulated by *her6*.

Wnt signaling may also play a role in determining diencephalic fates (Masai et al. 1997; Kiecker and Niehrs, 2001; Nordstrom et al. 2002; Braun et al. 2003). Overexpression of *fez1*, a repressor of wnts, leads to an expansion of the ZLI and loss of prethalamus marker expression, including *dlx2a* (Jeong et al. 2007). Within the

hypothalamus, knockdown of *wnt8b* or *lef1* results in loss of *ascl1a* in progenitors and subsequently *dlx2a* in postmitotic neurons (Lee et al. 2006). Wnt signaling activates *zic2a* transcription, which is necessary for *dlx2a* expression in the prethalamus and does not function through the Hedgehog pathway (Sanek and Grinblat, 2008). This suggests the Wnt and Hedgehog signaling cascades are distinct but function in parallel in the development of the prethalamus. One such factor that may regulate *gad* expression in the zebrafish telencephalon is *foxg1*, by acting to integrate Wnt and Shh signaling. Morpholino knockdown of *foxg1* leads to the complete absence of *dlx2a* expression in the telencephalon, yet unaffected in the prethalamus and hypothalamus (Danesin et al. 2009). Therefore, we can speculate that the loss of *dlx2a* expression may also correspond to the loss of *dlx1a* expression, resulting in the loss of *gad1* (and/or *gad2*) in the telencephalon.

Large-scale analysis of genes downstream of *Dlx1/Dlx2* and *Mash1*, indicates that these genes function in unique and distinct genetic pathways to regulate GABAergic neuron differentiation in the developing olfactory bulb, striatum, and basal ganglia in the mouse forebrain (Long et al. 2007; Long et al. 2009a; Long et al. 2009b). While these studies have identified a large number of downstream affected genes in the telencephalon, the prethalamus has yet to be studied in depth. We have shown that the *ascl1a* gene is necessary for proper regulation and expression of the *dlx* genes that will control *gad1* expression in the zebrafish prethalamus. Interestingly, neither the *ascl1a* nor *dlx* genes appear to be necessary for GABAergic interneuron development in the telencephalon, despite highly similar expression in the mouse and zebrafish. This data suggests that although the expression of the *dlx* genes, and their potential upstream regulators, are expressed in homologous tissues their function in the regulation of *gad1* in

the telencephalon appears to be altered. Therefore, the *ascl1a* and *dlx* genes are involved in a conserved GRN necessary for the proper differentiation of GABAergic interneurons in the prethalamus; however, this pathway appears to have diverged between mammals and teleosts in the telencephalon.

4.4. Experimental Procedures

4.4.1. Fish strains and staging

Embryos were obtained and housed by standard procedures described in Westerfield (2000). The following transgenic zebrafish lines were used in this study: *Tg(dlx1a/2aIG:GFP)* (MacDonald, unpublished), *Tg(URE2dlx1a/2a:GFP)* (MacDonald, unpublished), and *Tg(dlx5a/6a:GFP)* (Zerucha et al. 2000). All developmental stages are reported in hours post-fertilization (hpf) as previously described (Kimmel et al. 1994). All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by institutional animal care committees.

4.4.2. Morpholino injections

Morpholino oligonucleotides (MO) were injected into one-cell stage wild type or transgenic zebrafish embryos at concentrations ranging from 2-4 ng/µl. The following translation blocking morpholinos were used: *dlx1a* (Sperber et al. 2007), *dlx2a* (Sperber et al. 2007), *dlx2b* (Jackman and Stock, 2006), *dlx5a* (Walker et al. 2007), *dlx6a* (5'TGGTCATCATCAAATTTTCTGCTTT3'), and the *ashl1a*$^{5'UTR}$ MO (Cau and Wilson, 2003) was kindly provided by Dr. S. Wilson. Splice blocking MOs for *dlx5a* and

dlx6a were kindly provided by Dr. Chuck Kimmel, and were used to confirm the translation blocking MO phenotypes.

4.4.3. *in situ* hybridizations

Whole mount mRNA *in situ* hybridizations were carried out as described in Thisse and Thisse (1998). The antisense mRNA probes were labeled with digoxygenin-11-UTP (Roche, 11277073910) and synthesized from cDNA clones: *dlx1a* (Ellies et al. 1997b), *dlx2a* (Akimenko et al. 1994), *dlx5a* (Akimenko et al. 1994), *dlx6a* (Ellies et al. 1997), *dlx2b* (Ellies et al. 1997b), *gad2* (Martin et al. 1998), *gad1* (Mueller et al. 2008), *ascl1a* (Cau et al. 2000), *nkx2.1a* (Rohr et al. 2001), *emx1* (Morita et al. 1995), *lhx5a* (Toyama et al. 1995), *olig2* (Park et al. 2002), and *gfp* (MacDonald et al., Chapter 2). After hybridization, embryos were post-fixed in 4% PFA for 20 minutes and cleared overnight in glycerol.

Fluorescent RNA *in situ* hybridization was carried out with a protocol modified from those described previously (Jowett and Yan, 1996; Welten et al., 2006). DNP labeled probes were revealed with tyr-Cy5, dig labeled probes were revealed using tyr-Cy3, and fluorescein labeled probes were revealed with tyr-fluorescein (available from Perkin-Elmer). The full tissue labeling protocols can be found in Chapter 2 and online: http://wiki.zfin.org/display/prot/Triple+Fluorescent+In+Situ.

For imaging, embryos were placed on glass slides and positioned under coverslips for confocal imaging. Confocal z-stacks were obtained by using a Zeiss LSM5 PASCAL confocal laser scope (Carl Zeiss, Germany) and excitation lasers were at 488 (Fluorescein), 543 nm (Cy3), and 633 nm (Cy5). Channels were acquired sequentially to

avoid cross talk between the different filters. LSM Image Manager initially processed the z stacks and Volocity LE software (Improvision) was utilized to visualize regions of interest.

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Conclusions and suggestions for future work

The *Dlx* genes are homeodomain containing transcription factors important for the development of many embryonic structures such as the forebrain, branchial arches, and limbs. The expression of these genes in the forebrain of the mouse is controlled by a number of highly conserved regulatory elements that are active in overlapping but distinct domains. Within the forebrain, the *Dlx* genes are involved in a genetic cascade crucial for the proper differentiation and migration of GABAergic interneurons, the major class of inhibitory interneurons in the cortex. In my PhD thesis, I have studied the regulation and function of the zebrafish *dlx* genes in the developing forebrain and thus shed some light on the genetic cascade involving the *Dlx* genes during vertebrate evolution. I have contributed to the understanding of the evolution of regulatory elements through the comparative analysis of the function of orthologous regulatory elements from the elephant shark, zebrafish and mouse in heterologous and homologous transgenic assays. I have also shown that the *dlx* and *gad1* genes are expressed in a spatial pattern within the zebrafish forebrain reminiscent of the situation in mouse, suggesting a role for *dlx* genes in the differentiation of GABAergic interneurons through conserved regulatory mechanisms. Finally, I have shown that the genetic cascades controlling *dlx* and *gad1* expression are conserved in the zebrafish prethalamus but possibly divergent in the telencephalon when compared to mouse data. I have discussed my results in each chapter (see sections: 2.3, 3.3, and 4.3); therefore, I will focus on work that will be done in the future to expand on my findings.

Despite my contribution to the understanding of regulatory element evolution and the genetic cascades controlling GABAergic interneuron differentiation, there are many

avenues remaining that need to be explored. I will discuss some of the pertinent questions that remain unanswered that will be the goals of future work.

Functional comparisons of orthologous regulatory elements provides insights into the evolution of gene regulatory networks

I have shown that the URE2 *Dlx* regulatory element from the three groups of jawed vertebrates retained function in the forebrain, and possibly branchial arches, in distantly related jawed vertebrates. This activity is not only due to the necessary transcription factors being expressed in homologous tissues in the mouse and zebrafish, but also to the ability of these regulatory elements to retain binding sites for these factors despite long evolutionary divergence times. These upstream transcription factors and the regulatory elements are the components of gene regulatory networks (GRNs), indicating the GRN which controls the expression of *Dlx* in the brain and branchial arches may be conserved in jawed vertebrates. However, the functional conservation of URE2 may not be representative of the numerous conserved regulatory elements found in distantly related genomes. Therefore, we could utilize comparisons in homologous and heterologous transgenic experiments of the other *Dlx* regulatory elements, but also regulatory elements that have been extensively studied in the mouse and zebrafish (e.g. *neurogenin1* and *sonic hedgehog* enhancers) (Blader et al. 2003; Müller et al. 1999; Jeong et al. 2003; Jeong et al. 2006b; Ertzer et al. 2007). By studying multiple regulatory elements and how they have evolved in distantly related vertebrates, not only on the sequence but at functional levels, we can begin to understand the evolution of regulatory elements and the gene regulatory networks (GRNs) that they are involved in.

The highly overlapping expression of *dlx* bigene pairs provide insight into the significance of the bigene arrangement

I have shown that the expression of *dlx* genes of a particular bigene pair extensively overlaps in the zebrafish forebrain, a scenario consistent with that seen in the mouse (Liu et al. 1997; Eisenstat et al. 1999). The forebrain is not the only tissue where *Dlx* expression overlaps as there is overlapping expression both within bigene pairs but also with other *Dlx* family members in the branchial arches, apical ectodermal ridge of limb buds, and sensory placodes (Dollé et al. 1992; Akimenko et al. 1994; Morasso et al. 1995; Qui et al. 1997; Depew et al. 2002). This suggests two specific scenarios for the co-expression observed between distinct *dlx* bigene pairs (1) but also within a bigene pair (2).

(1) Co-expression of different members of the gene family is a consequence of shared genetic mechanisms regulating their expression. This scenario is consistent with the manner in which these bigene clusters could hypothetically originated from the duplication of an ancestral chordate cluster. The ancestral cluster would contain regulatory elements that would be duplicated along with the *Dlx* genes. The function of these ancestral regulatory elements may have been conserved over vertebrate evolutionary time, although poor sequence conservation does not allow for identification of similarities between these conserved regions in vertebrate *Dlx* clusters. However, another mechanism that might explain co-expression of the *Dlx* family members is cross-regulatory interactions between the *Dlx* genes as previously shown in the mouse (Anderson et al. 1997a; Zerucha et al. 2000; Stühmer et al. 2002b; Zhou et al. 2004). I

have shown this mechanism to be conserved in the forebrain of the zebrafish. Therefore, these cross-regulatory interactions may be responsible for the highly overlapping expression of the *Dlx* genes in other tissues.

(2) Highly overlapping expression domains in a bigene cluster are due to shared regulatory regions found in close proximity to the transcription start sites of both genes. This scenario has been proposed by Ellies et al. (1997b) as the bigene pairs are separated by short intergenic regions that contain many of the identified *Dlx* regulatory elements. However, my data does not help determine each regulatory element's contribution to overall *Dlx* expression (i.e. will each enhancer activate transcription of one or both *Dlx* genes in a bigene pair?). To address this question, a large transgene construct containing a *Dlx* bigene pair (e.g. *Dlx1* and *Dlx2*) and their regulatory sequences has been generated in the laboratory. Different reporter genes were cloned in frame with the *Dlx1* and *Dlx2* genes. Currently these constructs are being injected for mouse and zebrafish transgenesis. Once an established transgenic line is obtained, systematically deleting each regulatory element will allow for the contribution of each regulatory element on specific gene transcription to be assayed. Alternatively, studying enhancer-promoter interactions by chromosome conformation capture (3C) can identify interactions between genomic regions (Simonis et al. 2007), thus allowing for the identification of which regulatory element will physically interact with individual *Dlx* promoters. Understanding the dynamics of *Dlx* gene regulation within a particular bigene will not only allow for better overall understanding of *Dlx* regulation by highly conserved sequences but also the evolutionary significance of this bigene arrangement.

Identifying upstream signals responsible for *Dlx* regulatory element activity

I have shown that the expression of the *dlx* genes is regulated in the zebrafish forebrain by the *Mash1* ortholog, *ascl1a*, consistent with the situation in the mouse forebrain. The expression pattern of *ascl1a* is comparable in the mouse and zebrafish, potentially resulting in the expression of the *Dlx* genes in homologous forebrain domains in the distantly related vertebrates. Therefore, it appears the interactions between specific transcription factors and *Dlx* regulatory elements are conserved throughout the bony fish lineage. However the number of direct upstream transcription factors currently known to control the expression of the *Dlx* genes is limited. Ongoing studies in the laboratory are aimed at identifying distinct binding sites for transcription factors within *Dlx* regulatory elements, leading to the identification of upstream regulators. Molecular methods such as DNAseI footprinting and chromatin immunoprecipitation have been successful in identifying upstream activators of *Dlx* genes by their interactions with their specific regulatory elements (Zhou et al. 2004; Poitras et al. 2007; Potter et al. 2009). Utilizing these methods, the transcription factors interacting with the *Dlx* regulatory elements can be identified. By the comparison of orthologous regulatory elements, differences (or similarities) in the combination of transcription factor binding sites may explain the characteristics of each regulatory element's function. Furthermore, once upstream regulators of *Dlx* are identified, we can begin to expand on our knowledge of the GRN controlling *Dlx* expression and how these genetic interactions play a role in the overall expression of *Dlx* genes in a variety of tissues throughout development.

In addition, there may be *Dlx* regulatory elements yet to be discovered, as there are currently no regulatory elements active in the migrating neural crest cells or that fully

recapitulate *Dlx* expression in the branchial arches. These elements may potentially be located outside of the genomic regions examined previously, which only encompass the *Dlx* bigene pairs. Several enhancers of *Sonic Hedgehog* have been identified at greater than one megabase from the transcription start site (Lettice et al. 2003; Sagai et al. 2009). Therefore, it is not likely that a similar situation occurs for the regulation of the *Dlx* genes.

In conclusion, understanding the upstream regulation of the *Dlx* genes and their function in controlling the expression of downstream targets allows for a better understanding of their role in the GRNs involved in vertebrate forebrain development. Studying this GRN can provide insight in to a number of vertebrate developmental processes and may provide the basis for understanding human disease, as *Dlx* gene mutations or mis-expression have been associated with Autism (Hamilton et al. 2005), Schizophrenia, and Epilepsy (Cobos et al. 2005), among others. Finally, understanding the expression of distinct transcription factors and how they interact with gene regulatory elements will provide insight into the GRNs common amongst all jawed vertebrates and how they have evolved in the distinct lineages. Comparisons of these GRNs between distantly related vertebrates will allow for the identification of the underlying mechanisms responsible for the vast morphological differences throughout the vertebrate lineage.

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