A GENOMIC SCREEN FOR ZIC1 TARGET GENES

IN NEURAL DEVELOPMENT

by

Shuzhao Li

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2006

APPROVAL

of a thesis submitted by

Shuzhao Li

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Dr. Christa Merzdorf

Approved for the Department of Cell Biology and Neuroscience

Dr. Gwen Jacobs

Approved for the Division of Graduate Education

Dr. Joseph Fedock

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Shuzhao Li

July 2006

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

The transcription factor Zic1 plays important roles in patterning the neural plate in early vertebrate development. A crucial step toward understanding the mechanisms of Zic1 function is to identify the downstream target genes. We misexpressed Zic1 in animal caps from Xenopus embryos, and screened for differential gene expression with DNA microarrays. Candidate genes from these microarray data were further validated by quantitative PCR. Through this study, eleven genes have been shown to be directly upregulated by Zic1. One of the direct targets of Zic1 is a novel gene that we have named *Xfeb*. *Xfeb* is expressed in the presumptive hindbrain region during neurula stages and in somite tissues later in development. Xfeb represses the hindbrain gene *hoxB1* and the anterior neural gene *otx2*, suggesting that Xfeb is involved in regionalizing the neural plate, possibly by ensuring a posterior expression limit for *otx2*.

INTRODUCTION

Early neural development has been proposed as a two-step model, with neural induction as the first step, followed by initiative patterning of the resulting neural plate (Sasai and De Robertis, 1997). For neural induction, besides inhibition of BMP (bone morphogenetic protein) signaling, roles of positive signals, such as FGFs (fibroblast growth factors), Wnts and IGFs (insulin-like growth factors, Pera *et al*. 2001), have been suggested in recent studies (reviewed in Harland, 2000; Bainter *et al*, 2001; Munoz-Sanjuan and Brivanlou, 2002. Stern, 2002; Bally-Cuif and Hammerschmidt, 2003). Many effector genes, supposingly linkers between neural induction and initial patterning of nervous system, have entered the picture in the last few years. These include zinc-finger transcription factors Gli, Zic, XSIP1/ZEB2, Kheper; winged helix family XBF; homeodomain proteins Iroquois; HMG-domain proteins Sox; several basic helix-loop-helix (Atonal, Achaete-scute and Olig) factors; POU2; Geminin, etc. (Sasai, 1998; Bainter *et al*, 2001; Bally-Cuif and Hammerschmidt, 2003) However, the patterning step might well be interconnected with neural induction by sharing genetic circuits, since distinct regions of the nervous system to become are already discernable at gastrulation stage, and many of these effector genes are already expressed by then and show neural inducing activities.

Zic genes encode C2H2 zinc finger transcription factors, homologous to *odd-paired* in fruit fly. At least five *zic* genes have been are found in human, mouse and frog. A few

zic orthologs have been recently identified in protochordates (Gostling and Shimeld, 2003; Yamada *et al*., 2003; Wada and Saiga, 2002; Imai *et al*., 2002; Satou *et al*, 2002). Zic genes are expressed widely in early neural domains in all chordates; while in vertebrates they have specific dorsal expression during neuralation. This corresponds to their proposed roles in patterning more complex neural structures and inventing a novel structure of neural crest (Gostling and Shimeld, 2003).

zic1 plays a very early role in establishing different regions of the future nervous system. *zic1* is expressed at the lateral edges of the neural plate and in the dorsal neural tube. In particular, *zic1* is expressed in midbrain and hindbrain regions during early neurula stages and later extends along the dorsal spinal cord (Kuo *et al.*, 1998; Mizuseki *et al*., 1998; Nakata *et al*., 1998). Studies from several model organisms suggest important roles for *zic1* in patterning the neural plate, in formation of the neural crest, and in cerebellar development (Aruga *et al*., 1998; Kuo *et al*., 1998; Mizuseki *et al*., 1998; Nakata *et al*., 1998; Grinblat and Sive, 2001; Sasai *et al*., 2001; Aruga *et al*., 2002a; Sato *et al*., 2005).

One possible activity of zic1 is the inhibition of premature neuronal differentiation (Aruga *et al*., 2002b; Ebert *et al*., 2003). Accordingly, a targeted deletion of *zic1* in mice (*zic1-/-*) results in hypoplasia of the cerebellum, a derivative of the anterior hindbrain region (Aruga *et al*., 1998; Ogura *et al*., 2001). Further, a deletion of ZIC1 and ZIC4 was recently shown to be the cause of Dandy-Walker malformation in humans, a common cerebellar defect affecting 1/5,000 births, which is characterized by hypoplasia of the

cerebellar vermis. A similar phenotype develops in heterozygous *zic1+/-*, *zic4+/-* mice (Grinberg *et al*., 2004). Although *zic1* is expressed in the developed cerebellum (Aruga *et al*., 1994; Yokota *et al*., 1996), this abnormality is likely due to premature differentiation of cells in the hindbrain region prior to cerebellum formation.

Currently, the molecular mechanisms of *zic1* in patterning of the neural plate are largely unknown. Studies in Xenopus have shown that *zic1* induces the dorsal neural tube marker *pax3*, the midbrain/hindbrain boundary marker *en-2*, and the neural crest marker *slug*, along with several *wnt* genes (Kuo *et al* 1998; Nakata *et al.*, 1998; Mizuseki *et al*., 1998; Merzdorf and Sive, submitted). In a screen for genes regulated by *zic1* in mouse cerebella, the cerebellum-specific gene *dorz-1* was identified (Hoshino et al., 2003). However, these data tell little about the regulatory cascades initiated by zic1 and the relative positions of these genes in those cascades. Zic1 directly inhibits expression of the proneural gene *Math1* (Ebert et al., 2003), confirming that *zic1* may act in inhibiting neuronal differentiation.

It is of great interest to elucidate the mechanisms of neural development by identifying *zic1* downstream target genes. To this end, we used DNA micoarrays to conduct a screen at the genomic level in *Xenopus laevis*.

GENOMIC SCREEN WITH DNA MICROARRAYS

Experimental Design

Xenopus laevis, the African clawed frog, has been an excellent model for studying early neural development. Animal caps are naïve ectodermal tissue from the animal pole of *Xenopus* embryos. When isolated from the embryos, these animal caps develop to epidermal tissue by default. But they can be "induced" to become neural by inhibiting BMP signaling pathway. As neural genes are not expressed in animal caps in the first place, this system is suitable to study regulatory relation of neural genes. We used this system to overexpress Zic1 and compare the subsequent gene expression profile against controls that overexpress β-globin.

In order to control the timing of Zic1 function, we used a *zic1GR* construct that fuse zic1 with human glucocorticoid receptor binding domain. This fusion protein only enters nucleus and exerts its function at the presence of dexamethasone (dex). A translation inhibitor, cycloheximide (CHX), can be applied to cultured animal caps. In this scenario, because protein synthesis is inhibited, only immediate transcription events will be under investigation.

Synthetic mRNA was injected into *Xenopus* embryos at two-cell stage. Animal caps were dissected at stage 9 and cultured in 0.5X MBS. CHX was applied at the equivalent of stage 12 and dex was added in 30 minutes after. The animal caps were cultured for three more hours and total RNA was extracted from them. Each batch of RNA was

checked by PCR for the presence of genomic DNA and small portions from each batch were subjected to RT-PCR with muscle actin primers to ensure the absence of mesoderm. In the case of using spotted cDNA arrays, the experimental and control RNAs were labeled with Cy3 and Cy5 through reverse transcription; the resulting cDNA probes were then mixed and hybridized to duplicate arrays (Fig. 2.1.1). In the case of using Affymetrix genechips, RNAs were reverse transcribed and labeled cRNAs were made from cDNAs; samples were loaded on different genechips.

Zic1 is not among the earliest genes expressed in embryos and functions in a fairly complex environment. We use co-injected *noggin* to sensitize animal caps, which seems to bring up a high background too, because *noggin* injection also induces *zic1*. On top of this, several factors compounded the challenge here. In spite of our search, we could not find an immediate downstream gene to serve as a positive control before this experiment. The dissection of animal caps is a very laborious procedure, and the microarrays required a significant amount of RNA materials. Contamination from mesoderm happens easily and that trashes a whole batch of samples. Although we tried to control the timing of experiments, embryonic developments are very dynamic in nature and that added more noises into the system, because gene expressions cannot be really synchronized when the profiles are measured. Another problem is that *Xenopus laevis* has a tetraploid genome: more complexity in gene expression and regulation. For the same reason, the frog genomic sequencing was carried out with *Xenopus tropicalis*. Lack of genomic information also brought more difficulty in analyzing the array data later.

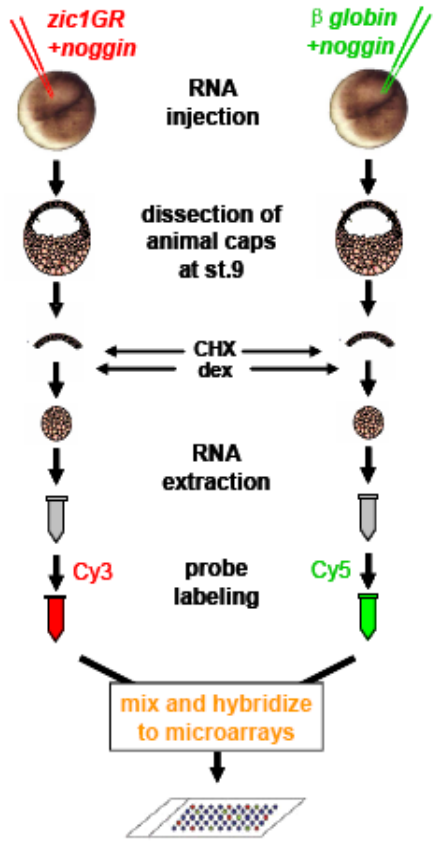

Fig. 2.1.1. Schematic of cDNA microarray experiment.
zic1GR mRNA or *β-globin* control mRNA was injected into both blastomeres of 2-cell *Xenopus* embryos. 1 pg of *noggin* was co-injected to neuralize the explant tissue. Animal caps were dissected at late blastula stage and cultured in saline. In order to identify genes directly regulated by *zic1*, CHX was added to the animal caps at the equivalent of stage 12 to inhibit protein synthesis. Dex was added 30 mins later to induce activity of *zic1GR*. Animal caps were harvested after an additional 3 hours of culture and RNA was extracted. Experimental and control samples were labeled with Cy3 or Cy5 during reverse transcription; the resulting cDNA probes were then mixed and hybridized to spotted cDNA arrays. **zic1GR:** hormone-inducible construct of *zic1*; GR is the human glucocorticoid receptor binding domain. **CHX:** cycloheximide, a translation inhibitor. **Dex**: dexamethasone, used to induce activity of Zic1GR.

Results from Spotted cDNA Arrays with CHX

This experiment was done in collaboration with Cho lab at UC Irvine. RNA samples were generated from animal caps overexpressing zic1 or β-globin, with CHX. The

hybridization was performed on duplicate miroarrays at UCI, Feb. 2004.

After background subtraction, the data were processed by a modified t-test (Baldi and Long, 2001). 2,450 spots were selected with p-value <0.01. When further cut by |fold change|>2, we had 163 upregulated clones and 435 downregulated clones. Their p-values were not much differentiated, probably because the statistical power was rather limited with duplicate arrays.

The top candidates from this experiment are listed in Table 2.2.1. The clone ID inherits a system from Japan's NIBB database. We reorganize them with NCBI's Unigene system. Further studies on these genes are discussed in section 3.4.

Table 2.2.1 Top candidates from spotted cDNA array experiment. These genes were selected by fold change (4X for upregulation and 8X for downregulation). Repetitive and false clones were removed. This list includes 23 upregulated genes (from 35 clones) and 28 downregulated genes (from 40 clones). N = not determinated.

Clone ID	log2-transformed ratio_Average	Bayes. p-value	Gene/description	in house name	Unigene
XL082h18	4.94	1.48E-04	myoD		
XL071c06	4.91	7.45E-03	Chaperonin subunit 2 (beta)	cct2	Xl.7645
XL073h19	4.57	6.00E-03	similar to PI3P binding protein-2	pbp2	Xl.25498
XL092o10	4.50	8.07E-03	similar to apoptosis response protein	par4	Xl.12484
XL088m01	3.90	5.71E-03	Acetylcholine receptor protein,alpha-1B chain precursor	ARPa	Xl.1117
XL086j24	3.34	2.46E-04	snail		
XL092b09	3.27	6.46E-03	similar to Caveolin-1	cav1	Xl.22006
XL087a21	3.07	6.88E-03	no hit	nieA	N
XL092a21	2.57	9.38E-03	Hypothetical protein MGC53111	hupa	Xl.23093
XL070a21	2.52	7.58E-03	transcribed locus	clau	Xl.13961
XL090n01	2.34	7.37E-03	nbx		Xl.25851
XL093a09	2.32	1.43E-03	transcribed locus	feeh	Xl.34026
XL087b17	2.30	4.93E-03	a ubiquitin-like protein	ulys	Xl.47769
XL105p17	2.16	8.95E-03	transcribed locus	-	Xl.2399

Table 2.2.1 - Continued

XL093l08	2.15	4.90E-03	XFD-11		N
XL068p07	2.14	3.48E-04	no info	-	N
XL106h17	2.12	3.73E-04	sox18		Xl.11958
XL090o12	2.05	2.07E-06	feb		Xl.15703
XL100p03	2.05	1.83E-06	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	-	Xl.12560
XL070i22	2.01	7.72E-03	Karyopherin alpha 2		Xl.1819
XL086o07	2.01	8.73E-03	TIMP-2		Xl.26350
XL080p04	2.01	8.82E-03	Similar to RNA, U transporter 1	-	Xl.4398
XL096k05	2.00	6.55E-03	SHB containing protein	-	Xl.12634
Clone ID	log2-transformed ratio_Average	Bayes. p-value	Gene/description	in house name	Unigene
XL0c2h08	-5.66	1.50E-05	Xnr-2		Xl.1038
XL079g16	-5.09	2.46E-08	transcribed locus	Psrepair	Xl.9606
XL0c2h12	-5.04	1.68E-05	Xnr-4		Xl.378
XL058n03	-4.90	1.95E-04	similar to Stromal interaction molecule 1 precursor	STM	Xl.10078
XL068b23	-4.02	5.37E-06	MGC83747, Type I inositol-1,4,5-trisphosphate 5-phosphatase	CEL	Xl.8191
XL059m10	-3.94	1.52E-03	weakly similar to angiopoietin-like 1 precursor	AGI	Xl.24094
XL0c2j04	-3.90	2.60E-04	noggin		Xl.834
XL059k04	-3.76	1.92E-04	transcribed locus	KIA	Xl.16216
XL0c2a20	-3.69	7.73E-04	pax-6		Xl.647
XL0c2b02	-3.67	4.50E-03	cardiac actin		Xl.1115
XL064p23	-3.66	1.22E-03	unknown, weakly aligned to chick ChEST976i21	VNS	N
XL062n10	-3.58	5.96E-03	muscle aetylcholine receptor alpha subunit	MARa	Xl.1117
XL0c2e20	-3.56	1.40E-07	Xhish1		Xl.806
XL065e21	-3.55	6.90E-03	MGC64592, similar to phosphorylase	PNPH	Xl.16206
XL095n19	-3.54	1.28E-05	transcribed locus	PCA	Xl.13287
XL0c2b03	-3.50	9.00E-04	mox2		Xl.1502
XL058c24	-3.40	9.20E-04	Dihydrolipoamide S-succinyltransferase	Dlst	Xl.6474
XL065d24	-3.33	2.78E-03	similar to EGF-containing fibulin-like extracellular matrix protein 2	-	?
XL060o01	-3.32	4.59E-03	hiting X.laevis U7 snRNA genes	McDan	N
XL0c2h15	-3.24	5.78E-04	Xnr-6		Xl.76
XL0c2i18	-3.22	3.56E-05	fkh-related		
XL065d21	-3.16	3.58E-06	transcribed locus	LUN	Xl.11931
XL0c2j08	-3.13	1.39E-04	hbox4		

Table 2.2.1 - Continued

XL0c2a11	-3.10	1.15E-04	gsc		Xl.801
XL064h24	-3.10	9.79E-06	Hypothetical protein MGC68907	AXR	Xl.34
XL0c2e21	-3.04	7.27E-08	cDNA clone 29C6-2	Xl.215	Xl.215
XL059e19	-3.03	7.16E-04	similar to arginyl-tRNA synthetase	-	Xl.45187
XL0c2c09	-2.99	5.65E-05	xtwn		Xl.390

End of Table 2.2.1

Affymetrix Genechips: Early Response without CHX

After Xenopus genechips became available from Affymetrix, we continued our screen with these commercial chips. In this experiment, RNA samples were generated from animal caps overexpressing zic1 plus noggin or β-globin plus noggin, without CHX, so that we could include both direct and indirect gene targets in our screen. Hybridization was performed in biological duplicates (four chips in total) at MSU's core genomic facility, Aug. 2004.

The result from this experiment was expected to overlap partly with the earlier cDNA microarrays, as this experiment looked for both direct and indirect target genes of Zic1. Since the validation of the first set of data was hindered by many false positives, we actually compared these two sets of data and selected several overlapping genes to check with QPCR.

After we reran the +CHX experiment with genechips in April, 2006, this -CHX 2004 set of data was reanalyzed and compared to the newer data. Table 2.3.1 lists the top candidates from this -CHX 2004 experiment. Two methods were used in data analysis:

one by Genespring ® (selected genes made up the list “RMA4”, 289 genes), and one at probe levels by myself (list “sli4”, 552 genes). Table 2.3.1 is the combined result of both methods. Priority was given to consistency across all chips. The explanation of data processing is in section 2.5.

Similarly, the +CHX 2006 data give a “RMA5” list by Genespring, and a “sli6” list by my method.

Table 2.3.1 Top candidates from Affymetrix experiment without CHX, 20 genes. If sli6=1, the corresponding gene is also selected in Affymetrix experiment with CHX (April, 2006). If RMA4=1, the corresponding gene is also selected by Genespring. Full list is in file nochx2004.xls.

sli6	RMA4	SL score	Affy ID	Annotation
1	1	-8.425	Xl.2565.2.S1_at	gb:CB559825 /DB_XREF=gi:29479355 /DB_XREF=AGENCOURT_12922336 /CLONE=IMAGE:4030965 /TID=Xl.2565.2 /CNT=108 /FEA=EST /TIER=Stack /STK=77 /UG=Xl.2565 /UG_TITLE=Xenopus laevis, Similar to alpha-Tubulin at 84B, clone MGC:53359 IMAGE:5571121, mRNA, complete cds
1	1	-7.25	Xl.24486.1.A1_at	gb:CB561087 /DB_XREF=gi:29480617 /DB_XREF=AGENCOURT_13329097 /CLONE=IMAGE:6879469 /TID=Xl.24486.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.24486 /UG_TITLE=ESTs, Weakly similar to A36056 tumor-associated antigen CO-029 - human (H.sapiens)
1	1	-7.15	Xl.26274.1.A1_at	gb:BE189559 /DB_XREF=gi:9729909 /DB_XREF=db67b12.x1 /CLONE=IMAGE:3378047 /TID=Xl.26274.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.26274 /UG_TITLE=ESTs
1	1	-5.8458	Xl.1136.1.S1_at	gb:BG160459 /DB_XREF=gi:12694378 /DB_XREF=daa26b08.y1 /CLONE=IMAGE:4057287 /TID=Xl.1136.1 /CNT=2 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.1136 /UG_TITLE=X.laevis beta-2-globin mRNA
1	1	-4.4134	Xl.17153.1.A1_at	gb:BM261069 /DB_XREF=gi:17924109 /DB_XREF=dag33e08.x3 /CLONE=IMAGE:4783214 /TID=Xl.17153.1 /CNT=1 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.17153 /UG_TITLE=ESTs
0	1	-4.1501	Xl.586.1.S1_at	gb:AF146088.1 /DB_XREF=gi:6665659 /GEN=ESR-7 /TID=Xl.586.1 /CNT=7 /FEA=FLmRNA /TIER=FL /STK=2 /UG=Xl.586 /DEF=Xenopus laevis enhancer of split related protein-7 (ESR-7) mRNA, complete cds. /PROD=enhancer of split related protein-7 /FL=gb:AF146088.1
0	1	-3.6014	Xl.19741.1.S1_at	gb:BQ398596 /DB_XREF=gi:21086283 /DB_XREF=NISC_mo09b08.x1 /CLONE=IMAGE:5278790 /TID=Xl.19741.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.19741 /UG_TITLE=ESTs, Moderately similar to hypothetical protein DKFZp564K0822 (Homo sapiens) (H.sapiens)
1	1	-3.3357	Xl.946.1.S1_at	gb:AB030904.1 /DB_XREF=gi:6691470 /TID=Xl.946.1 /CNT=3 /FEA=FLmRNA /TIER=FL /STK=2 /UG=Xl.946 /DEF=Xenopus laevis mRNA for thimet oligopeptidase, complete cds. /PROD=thimet oligopeptidase /FL=gb:AB030904.1

Table 2.3.1 - Continued

1	1	-3.3007	Xl.17670.1.A1_at	gb:BG811162 /DB_XREF=gi:14182142 /DB_XREF=daf41b08.x1 /CLONE=IMAGE:4740831 /TID=Xl.17670.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.17670 /UG_TITLE=ESTs, Weakly similar to contactin 5; neural adhesion molecule (Homo sapiens) (H.sapiens)
1	0	3.3011	Xl.22458.1.A1_at	gb:BF072333 /DB_XREF=gi:10848972 /DB_XREF=db55e12.x1 /CLONE=IMAGE:3302446 /TID=Xl.22458.1 /CNT=1 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.22458 /UG_TITLE=ESTs
0	1	3.4209	Xl.23515.1.S1_at	gb:BC042293.1 /DB_XREF=gi:27503422 /TID=Xl.23515.1 /CNT=10 /FEA=FLmRNA /TIER=FL /STK=4 /UG=Xl.23515 /DEF=Xenopus laevis, Similar to claudin 1, clone MGC:53308 IMAGE:5570517, mRNA, complete cds. /PROD=Similar to claudin 1 /FL=gb:BC042293.1
1	0	3.4689	Xl.3549.1.S2_at	gb:L35764.1 /DB_XREF=gi:603944 /TID=Xl.3549.1 /CNT=40 /FEA=FLmRNA /TIER=FL /STK=4 /UG=Xl.3549 /DEF=Xenopus laevis chordin mRNA, complete cds. /PROD=chordin /FL=gb:L35764.1
0	1	3.5086	Xl.16116.1.A1_at	gb:BF611924 /DB_XREF=gi:11782058 /DB_XREF=df13b02.y1 /CLONE=IMAGE:3556947 /TID=Xl.16116.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.16116 /UG_TITLE=ESTs
1	0	3.5512	Xl.16667.1.A1_at	gb:BM172514 /DB_XREF=gi:17312077 /DB_XREF=imageqc_5_2001smz444bdff41.x1 /CLONE=IMAGE:4964128 /TID=Xl.16667.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=4 /UG=Xl.16667 /UG_TITLE=ESTs, Weakly similar to guanylate binding protein 4 (Homo sapiens) (H.sapiens)
0	0	3.761	Xl.24860.1.A1_at	gb:BF047438 /DB_XREF=gi:10765941 /DB_XREF=dc87d09.x1 /CLONE=IMAGE:3404081 /TID=Xl.24860.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.24860 /UG_TITLE=ESTs
1	1	3.9338	Xl.5071.1.A1_at	gb:BE505236 /DB_XREF=gi:9708767 /DB_XREF=dc19h06.x1 /CLONE=IMAGE:3397595 /TID=Xl.5071.1 /CNT=3 /FEA=EST /TIER=ConsEnd /STK=3 /UG=Xl.5071 /UG_TITLE=ESTs, Weakly similar to JC7190 D-2-hydroxy-acid dehydrogenase (EC 1.1.99.6) - human (H.sapiens)
1	0	3.9777	Xl.13537.1.A1_at	gb:BJ054042 /DB_XREF=gi:17499048 /DB_XREF=BJ054042 /CLONE=XL049b08 /TID=Xl.13537.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.13537 /UG_TITLE=ESTs
0	1	4.1574	Xl.2784.1.S1_at	gb:M96729.1 /DB_XREF=gi:214585 /TID=Xl.2784.1 /CNT=14 /FEA=FLmRNA /TIER=FL /STK=3 /UG=Xl.2784 /UG_TITLE=X.laevis mRNA for metallothionein /DEF=Xenopus laevis (clones pXlMT1 and pXlMT10) metallothionein mRNA, complete cds. /FL=gb:M96729.1
1	0	4.4913	Xl.1003.1.S1_at	gb:U28370.1 /DB_XREF=gi:1002540 /GEN=XANF-2 /TID=Xl.1003.1 /CNT=1 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.1003 /DEF=Xenopus laevis homeobox protein (XANF-2) mRNA, complete cds. /PROD=homeobox protein /FL=gb:U28370.1
1	1	5.575	Xl.13309.1.S1_at	gb:X53450.1 /DB_XREF=gi:65256 /TID=Xl.13309.1 /CNT=28 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.13309 /UG_TITLE=Xenopus laevis mRNA homologous to Drosophila Snail gene /DEF=Xenopus laevis mRNA homologous to Drosophila Snail gene.

End of Table 2.3.1.

Affymetrix Genechips: Early Response with CHX

This was the rerun of experiment with CHX on Affymetrix genechips. Hybridization was performed with biological triplicates at MSU, April 2006. Since we had identified Xfeb and snail as Zic1 targets in previous studies, these two genes were used as positive controls in preparation of new samples.

Two methods were used in data analysis: one by Genespring ® (selected genes made up the list "RMA5", 292 genes), and one at probe levels by myself (list "sli6", 656 genes). Table 2.4.1 lists the top candidates from this experiment, as the combined result of both methods. Priority was given to consistency across all chips. This table also took into consideration of comparisons to other data. The explanation of data processing is in section 2.5.

Table 2.4.1. Top candidates from Affy experiment with CHX, 28 genes. If sli4=1, the corresponding gene is also selected in Affy experiment without CHX (Aug, 2004). If RMA5=1, the corresponding gene is also selected by Genespring. Full list is in file chx2006.xls.

sli4	RMA5	SL score	Affy ID	Annotation
1	1	-8.7444	Xl.2565.2.S1_at	gb:CB559825 /DB_XREF=gi:29479355 /DB_XREF=AGENCOURT_12922336 /CLONE=IMAGE:4030965 /TID=Xl.2565.2 /CNT=108 /FEA=EST /TIER=Stack /STK=77 /UG=Xl.2565 /UG_TITLE=Xenopus laevis, Similar to alpha-Tubulin at 84B, clone MGC:53359 IMAGE:5571121, mRNA, complete cds
1	1	-4.0784	Xl.10555.1.A1_at	gb:BE679068 /DB_XREF=gi:10061375 /DB_XREF=df79c02.x1 /CLONE=IMAGE:3745538 /TID=Xl.10555.1 /CNT=1 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.10555 /UG_TITLE=EST
1	0	-3.6721	Xl.2565.4.S1_x_at	gb:BG810694 /DB_XREF=gi:14181674 /DB_XREF=daa63d11.x2 /CLONE=IMAGE:4060484 /TID=Xl.2565.4 /CNT=7 /FEA=EST /TIER=Stack /STK=7 /UG=Xl.2565 /UG_TITLE=Xenopus laevis, Similar to alpha-Tubulin at 84B, clone MGC:53359 IMAGE:5571121, mRNA, complete cds

Table 2.4.1 - Continued

1	0	-3.6394	Xl.26274.1.A1_at	gb:BE189559 /DB_XREF=gi:9729909 /DB_XREF=db67b12.x1 /CLONE=IMAGE:3378047 /TID=Xl.26274.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.26274 /UG_TITLE=ESTs
1	0	-3.6185	Xl.13472.1.S1_at	gb:BJ099031 /DB_XREF=gi:17601144 /DB_XREF=BJ099031 /CLONE=XL148n21 /TID=Xl.13472.1 /CNT=6 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.13472 /UG_TITLE=ESTs
1	0	-3.4272	Xl.1136.1.S1_at	gb:BG160459 /DB_XREF=gi:12694378 /DB_XREF=daa26b08.y1 /CLONE=IMAGE:4057287 /TID=Xl.1136.1 /CNT=2 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.1136 /UG_TITLE=X.laevis beta-2-globin mRNA
0	0	-3.2417	Xl.13415.1.A1_at	gb:BJ092954 /DB_XREF=gi:17592309 /DB_XREF=BJ092954 /CLONE=XL057p08 /TID=Xl.13415.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.13415 /UG_TITLE=ESTs
0	0	-2.8887	Xl.2060.1.A1_x_at	gb:BJ055271 /DB_XREF=gi:17423255 /DB_XREF=BJ055271 /CLONE=XL009a10 /TID=Xl.2060.1 /CNT=4 /FEA=EST /TIER=ConsEnd /STK=4 /UG=Xl.2060 /UG_TITLE=ESTs, Weakly similar to Y226_HUMAN HYPOTHETICAL PROTEIN KIAA0226 (H.sapiens)
0	1	-2.3453	Xl.12938.1.A1_at	gb:BJ092938 /DB_XREF=gi:17592287 /DB_XREF=BJ092938 /CLONE=XL057o14 /TID=Xl.12938.1 /CNT=3 /FEA=EST /TIER=ConsEnd /STK=2 /UG=Xl.12938 /UG_TITLE=ESTs
1	1	1.9888	Xl.12160.1.S1_at	gb:BC046942.1 /DB_XREF=gi:28422662 /TID=Xl.12160.1 /CNT=12 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.12160 /DEF=Xenopus laevis, Similar to RIKEN cDNA 4930415M08 gene, clone MGC:52580 IMAGE:5570286, mRNA, complete cds. /PROD=Similar to RIKEN cDNA 4930415M08 gene /FL=gb:BC046942.1
1	1	2.0098	Xl.10868.1.S1_at	gb:AB080019.1 /DB_XREF=gi:27884296 /GEN=xgalectin-VIa /TID=Xl.10868.1 /CNT=23 /FEA=FLmRNA /TIER=FL+Stack /STK=10 /UG=Xl.10868 /DEF=Xenopus laevis mRNA for galectin family xgalectin-VIa, complete cds. /PROD=galectin family xgalectin-VIa /FL=gb:AB080019.1
1	1	2.1855	Xl.373.1.S1_at	gb:U75996.1 /DB_XREF=gi:1743868 /TID=Xl.373.1 /CNT=5 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.373 /DEF=Xenopus laevis eomesodermin mRNA, complete cds. /PROD=eomesodermin /FL=gb:U75996.1
1	1	2.5387	Xl.1003.1.S1_at	gb:U28370.1 /DB_XREF=gi:1002540 /GEN=XANF-2 /TID=Xl.1003.1 /CNT=1 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.1003 /DEF=Xenopus laevis homeobox protein (XANF-2) mRNA, complete cds. /PROD=homeobox protein /FL=gb:U28370.1
1	1	2.5844	Xl.182.1.S1_at	gb:AJ242680.1 /DB_XREF=gi:5042352 /GEN=xfd-13 /TID=Xl.182.1 /CNT=5 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.182 /DEF=Xenopus laevis mRNA for XFD-13 protein. /PROD=XFD-13 protein
0	0	2.7484	Xl.3150.1.S1_at	gb:AB049354.1 /DB_XREF=gi:10336598 /GEN=xFRP /TID=Xl.3150.1 /CNT=34 /FEA=FLmRNA /TIER=FL+Stack /STK=12 /UG=Xl.3150 /DEF=Xenopus laevis xFRP mRNA for follistatin-related protein, complete cds. /PROD=follistatin-related protein /FL=gb:AB049354.1
0	1	2.7708	Xl.9271.1.S1_x_at	gb:AJ009285.1 /DB_XREF=gi:3821761 /TID=Xl.9271.1 /CNT=1 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.9271 /UG_TITLE=Xenopus laevis cDNA clone 11A10 /DEF=Xenopus laevis cDNA clone 11A10.

Table 2.4.1 - Continued

0	0	2.8307	Xl.9271.1.S1_at	gb:AJ009285.1 /DB_XREF=gi:3821761 /TID=Xl.9271.1 /CNT=1 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.9271 /UG_TITLE=Xenopus laevis cDNA clone 11A10 /DEF=Xenopus laevis cDNA clone 11A10.
0	1	3.1221	Xl.11405.1.S1_a_at	gb:BC044063.1 /DB_XREF=gi:28277238 /TID=Xl.11405.1 /CNT=342 /FEA=FLmRNA /TIER=FL+Stack /STK=98 /UG=Xl.11405 /DEF=Xenopus laevis, clone MGC:52615 IMAGE:4681241, mRNA, complete cds. /PROD=Unknown (protein for MGC:52615) /FL=gb:BC044063.1
0	0	3.1255	Xl.639.1.S1_at	gb:AF253504.1 /DB_XREF=gi:7689274 /TID=Xl.639.1 /CNT=1 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.639 /DEF=Xenopus laevis homeodomain protein dbx mRNA, complete cds. /PROD=homeodomain protein dbx /FL=gb:AF253504.1
1	1	3.2556	Xl.1249.2.S1_a_at	gb:M31117.1 /DB_XREF=gi:214589 /TID=Xl.1249.2 /CNT=1 /FEA=FLmRNA /TIER=FL /STK=1 /UG=Xl.1249 /UG_TITLE=X.laevis MyoD1 homologue (mf1) gene (expressed prior to somite formation) mRNA, complete cds /DEF=X.laevis MyoD1 homologue (mf11) gene (expressed prior to somite formation) mRNA, complete cds. /FL=gb:M31117.1
1	1	3.3382	Xl.13309.1.S1_at	gb:X53450.1 /DB_XREF=gi:65256 /TID=Xl.13309.1 /CNT=28 /FEA=mRNA /TIER=ConsEnd /STK=0 /UG=Xl.13309 /UG_TITLE=Xenopus laevis mRNA homologous to Drosophila Snail gene /DEF=Xenopus laevis mRNA homologous to Drosophila Snail gene.
1	1	3.3889	Xl.15703.1.A1_at	gb:BJ045643 /DB_XREF=gi:17370251 /DB_XREF=BJ045643 /CLONE=XL004n03 /TID=Xl.15703.1 /CNT=10 /FEA=EST /TIER=Stack /STK=6 /UG=Xl.15703 /UG_TITLE=ESTs, Weakly similar to 17kD fetal brain protein (Homo sapiens) (H.sapiens)
1	0	3.5632	Xl.5813.1.S1_at	gb:BJ048791 /DB_XREF=gi:17409207 /DB_XREF=BJ048791 /CLONE=XL022a03 /TID=Xl.5813.1 /CNT=23 /FEA=EST /TIER=Stack /STK=6 /UG=Xl.5813 /UG_TITLE=ESTs
1	1	3.9175	Xl.9933.1.A1_at	gb:BG731538 /DB_XREF=gi:14016613 /DB_XREF=dac26f11.x1 /CLONE=IMAGE:4408101 /TID=Xl.9933.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.9933 /UG_TITLE=ESTs, Weakly similar to blood vessel epicardial substance; bves protein (Homo sapiens) (H.sapiens)
0	0	3.9472	Xl.13925.1.A1_at	gb:BJ091281 /DB_XREF=gi:17589860 /DB_XREF=BJ091281 /CLONE=XL094k20 /TID=Xl.13925.1 /CNT=2 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.13925 /UG_TITLE=ESTs, Weakly similar to AXN2_HUMAN Axin 2 (Axis inhibition protein 2) (Conductin) (Axin-like protein) (Axil) (H.sapiens)
0	1	3.9914	Xl.194.1.S1_at	gb:D83712.1 /DB_XREF=gi:1213588 /TID=Xl.194.1 /CNT=18 /FEA=FLmRNA /TIER=FL /STK=3 /UG=Xl.194 /DEF=Xenopus laevis mRNA for Prostaglandin D Synthase, complete cds. /PROD=Prostaglandin D Synthase /FL=gb:D83712.1
1	1	4.8726	Xl.11302.1.A1_at	gb:BG439728 /DB_XREF=gi:13349378 /DB_XREF=dab26c11.x1 /CLONE=IMAGE:4175612 /TID=Xl.11302.1 /CNT=1 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.11302 /UG_TITLE=ESTs, Weakly similar to A56666 glutathione transferase (EC 2.5.1.18) alpha-2 (validated) - human (H.sapiens)
0	1	6.4442	Xl.22603.1.A1_at	gb:AW766683 /DB_XREF=gi:7698676 /DB_XREF=da66g05.x1 /CLONE=IMAGE:3199928 /TID=Xl.22603.1 /CNT=1 /FEA=EST /TIER=ConsEnd /STK=1 /UG=Xl.22603 /UG_TITLE=ESTs

End of Table 2.4.1.

Data Processing and Cross Comparisons

Our spotted array experiment had only duplicates. The subsequent analysis showed a high rate of false positives. In order to gain sufficient statistical power, we redid the experiment with triplicate Affymetrix genechips.

A software suite, Genespring ® , is available at MSU's core facility. Our data from all genechips (with and without CHX) went through ANOVA analysis implemented in Genespring (by Kate McInnery). 289 genes (RMA4 list) were selected from the data without CHX, and 292 genes (RMA5 list) were selected from the data with CHX (in Merzdorf Microarray (1).xls).

As Genespring uses data at the gene level, I also analyzed these genechip data at probe level. Probe level comparison seems to offer more reliable results, introducing less bias against low-abundance genes. It worked as:

1) pairwise comparison between samples and controls at probe level. For the 4 chips from the experiment without CHX, this yields 4 comparison results (sample1 vs control1, sample1 vs control2, sample2 vs control1, sample2 vs control2). For the 6 chips from the experiment with CHX, this yields 9 comparison results. This step was performed with Affymetrix GCOS suite (Liu *et al.*, 2002).

2) filtering for the significant results only (fold change >2 and p-value < 0.01).

3) combine all the (4 or 9, respectively) comparisons with a SL score:

$SL\,score = \sum R_k * \log_2(p_k) / \sum \log_2(p_k)$, k=1...4 (...9) ; where R is log(fold change) and p is p-value. It should be noted that in Affymetrix GCOS, the p-values for downregulation were given as (*1 - p*) instead.

4) sort all genes according to their SL score.

This method was implemented and documented in a Matlab script (processing.m).

I selected 552 genes (sli4 list) from the experiment without CHX, among which 88 genes overlap with Genespring's RMA4 list (saved in nochx2004.xls). Similarly, 656 genes (sli6 list) were selected from the experiment with CHX, among which 124 genes overlap with Genespring's RMA5 list (saved in chx2006.xls).

A comparison was also done between the experiment without CHX and that with CHX, and 98 genes were found in common (chx2006.xls, sheet3).

In the analysis in Genespring, one experimental sample in +CHX experiment was excluded because it was suspected to be an outlier comparing the other two experimental chips. All chips were included in my probe-level analysis. Since high priority was given to the p-values in my process, my method is robust to outliers. In fact, my lists contain the very top candidates from the Genespring's lists while give more recommendations.

Since the spotted cDNA array used a different format, comparison to the genechip data was only possible by converting its clone IDs into Unigene numbers. Since it's a slow process, we only compared the top 51 genes from spotted array to the sli6 list. 6 genes were found in common (Cho2004chx-sli6.xls).

Materials and Methods

The cDNA microarrays used in this study were constructed from a Xenopus tailbud library, containing 21,504 sequenced clones (Tran et al., 2002). zic1-induced and uninduced samples were used as experimental and reference samples, respectively.

Genechips ® and related reagents were purchased from Affymetrix Inc.

200 pg zic1GR RNA or b-globin control RNA were co-injected with 1 pg noggin RNA into both blastomeres of 2-cell Xenopus embryos. Ectodermal explants (animal caps) were dissected at stage 9 and cultured in 0.5x MBS (Modified Barth's Saline). In order to limit our screen to direct targets of zic1, 10 µg/ml cycloheximide (Sigma) was added to the animal caps at the equivalent of stage 12 to inhibit protein synthesis. 10 µM dexamethasone (Sigma) was added 30 min later to induce activity of zic1GR. Animal caps were harvested after an additional 3 hours of culture and total RNA was extracted. We have been using protease K and phenol/chloroform to extract RNA. Although this method has good yield, the resulting RNA tends to be fragmented. Hence, it is a suboptimal method and alternative (TRI reagents, etc.) should be explored.

Each batch of RNA was checked by PCR for the presence of genomic DNA and small portions from each batch were subjected to RT-PCR with *muscle actin* primers to ensure the absence of mesoderm.

For spotted cDNA arrays, 40 µg of experimental and control RNAs were labeled with Cy3 or Cy5, respectively, during reverse transcription; the resulting cDNA probes were then mixed and hybridized to duplicate spotted cDNA arrays. Hybridization results were scanned and processed by GeneData Expressionist (Genedata). A modified t-test was applied (Baldi and Long, 2001) to analyze the resulting data.

For Affymetrix genechips, over 5 µg of total RNA was used for preparing each target, according to one-cycle protocol from the manufacturer. Samples and controls were

loaded on different chips. Totally 10 genechips were used in this study (four in -CHX 2004 experiment, six in +CHX 2006 experiment). Scanned data were read through Affymetrix GCOS software, and further processed with Genespring and custom methods (section 2.5).

SCREEN RESULTS AND ANALYSIS

Sequence Analysis

The clones on Cho's cDNA array mostly carry short EST sequences, and the annotations were somewhat dated. To investigate candidate genes, several measures were taken:

1) Converting the original ID (mostly from NIBB, http://xenopus.nibb.ac.jp/) to Unigene numbers. Repetitive or false clones were removed.
2) Finding a best representative sequence for each gene. Priority was given to the 3' end.
3) Confirming ORF when possible. We encountered NIBB sequences that were deposited as negative strands.
4) Selecting optimal regions for PCR or cloning.

2-4) also applied to genes identified on Affymetrix genechips.

Cloning and *in situ* Hybridizations

In situ hybridization was planned to find out the expression domain of relevant genes and to compare them with *zic1* expression. For some previously studied genes, clones were requested from other investigators. For novel genes, we used a TA cloning strategy to clone part of the transcripts. *In situ* probes were made from these subcloned regions.

Undergraduate students Cindi Japhet and Chandra Denherder took part of this route

in the second half of 2004. Below is a list of the genes they worked on. Most of these *in situ*s failed. AGI and MARa had interesting *in situ* staining, and were included in our poster at SDB meeting 2005. None of these genes was confirmed in subsequent QPCR analysis.

Table 3.2.1 Genes tested by in situ hybridization.
This list was based on the spotted cDNA array results.

ID	log2(fold change)	Gene/description	in house name
XL071c06	4.91	Chaperonin subunit 2 (beta)	cct2
XL073h19	4.57	similar to PI3P binding protein-2	pbp2
XL092o10	4.50	similar to apoptosis response protein	par4
XL088m01	3.90	Acetylcholine receptor protein,alpha-1B chain precursor	ARPa
XL087a21	3.07	no hit	nieA
XL079g16	-5.09	transcribed locus	Psrepair
XL058n03	-4.90	similar to Stromal interaction molecule 1 precursor	STM
XL059m10	-3.94	weakly similar to angiopoietin-like 1 precursor	AGI
XL064p23	-3.66	unknown, weakly aligned to chick ChEST976i21	VNS
XL062n10	-3.58	muscle aetylcholine receptor alpha subunit	MARa
XL065d21	-3.16	transcribed locus	LUN
XL064h24	-3.10	Hypothetical protein MGC68907	AXR
XL0c2e21	-3.04	cDNA clone 29C6-2	Xl.215

End of Table 3.2.1

Confirmation with PCR: Technical Development

PCR technology has been often used in animal cap assays. However, such studies usually look at gene expressions in committed or differentiated cells, where the differential expression is significant. In our study, animal caps had only three hours to respond to overexpressing genes. The coinjected noggin also caused substantial

background. As a result, it turned out later that the difference of gene expression between samples and controls was often just that of a few PCR cycles. So it was impossible to validate the array results with regular PCR. Without prior knowledge of the gene abundance, and given the conditions in our lab, radioactive PCR was not up to the job either. Fluorescent real-time PCR was necessary to study the candidate genes.

We were able to use RotorGene 3000 system at MSU's core genomic facility. Home-spun QPCR recipes were tested with a number of genes with little success. Confusions were also caused by unusually high melting points of DNA in these solutions. We later used commercial reagents from Eurogentec, and focused on a small number of genes that overlapped between cDNA array data and the first set of Affymetrix genechip data.

We ordered many PCR primers based on previously published primer sequences. Most turned out not to work with Sybr Green QPCR. In fact, the most important factor of all is the quality of primers in Sybr Green QPCR. Without commercial software, we tweaked the free web-based Primer3 to design reasonable primers for QPCR. It is also important to get a best sequence for a gene. Primers targeting the 3' end are preferred.

Limited by time and resources, we didn't optimize QPCR reactions for many a genes. Since we only need comparative quantification, we used the simplest while probably most robust method of quantification, the ΔΔCt method. At the time of our experiments, this method was not included in RotorGene software. I implemented it into a Matlab script (ct.m).

We used *EF1a* as an internal control to normalize QPCR results. Another gene, *ODC*, was also tested and yielded similar results. These genes are suboptimal as

quantification controls because their transcripts are too abundant. Better control genes should be sought in future studies. We used three or more biological replicates in these QPCR reactions. When resources allow, triplicates for PCR are highly recommended.

Confirmation with PCR: Results

Our initial efforts were targeted at the top list from the cDNA microarray experiment, which turned out to have many false positives (Table 3.4.1). We happened to have performed a new experiment (-CHX) with Affymetrix genechips. So we focused later on several genes that overlap with results from the new experiment.

Table 3.4.1. 49 genes tested with PCR, 5 confirmed (Y). PCR was performed on animal cap samples +CHX. N = No change. Blank cell indicates that QPCR is not completely working with existing primers at current condition.

ID	log2(fold change)	Gene/description	in house name	PCR
XL082h18	4.94	myoD		
XL071c06	4.91	Chaperonin subunit 2 (beta)	cct2	N
XL073h19	4.57	similar to PI3P binding protein-2	pbp2	
XL092o10	4.50	similar to apoptosis response protein	par4	N
XL088m01	3.90	Acetylcholine receptor protein,alpha-1B chain precursor	ARPa	
XL086j24	3.34	snail		Y
XL092b09	3.27	similar to Caveolin-1	cav1	
XL087a21	3.07	no hit	nieA	
XL092a21	2.57	Hypothetical protein MGC53111	hupa	
XL070a21	2.52	transcribed locus	clau	
XL090n01	2.34	nbx		Y
XL093a09	2.32	transcribed locus	feeh	
XL087b17	2.30	a ubiquitin-like protein	ulys	N
XL106h17	2.12	sox18		Y
XL090o12	2.05	feb		Y
XL0c2h08	-5.66	Xnr-2		
XL079g16	-5.09	transcribed locus	Psrepair	N
XL0c2h12	-5.04	Xnr-4		
XL058n03	-4.90	similar to Stromal interaction molecule 1 precursor	STM	
XL068b23	-4.02	MGC83747, Type I inositol-1,4,5-trisphosphate 5-phosphatase	CEL	N
XL059m10	-3.94	weakly similar to angiopoietin-like 1 precursor	AGI	

Table 3.4.1 - Continued

XL059k04	-3.76	transcribed locus	KIA	
XL064p23	-3.66	unknown, weakly aligned to chick ChEST976i21	VNS	
XL062n10	-3.58	muscle aetylcholine receptor alpha subunit	MARa	
XL065e21	-3.55	MGC64592, similar to phosphorylase	PNPH	N
XL095n19	-3.54	transcribed locus	PCA	
XL0c2b03	-3.50	mox2		
XL058c24	-3.40	Dihydrolipoamide S-succinyltransferase	Dlst	
XL060o01	-3.32	hiting X.laevis U7 snRNA genes	McDan	
XL0c2h15	-3.24	Xnr-6		
XL0c2i18	-3.22	fkh-related		
XL065d21	-3.16	transcribed locus	LUN	
XL0c2a11	-3.10	gsc		
XL064h24	-3.10	Hypothetical protein MGC68907	AXR	
XL0c2e21	-3.04	cDNA clone 29C6-2	Xl.215	N
XL0c2c09	-2.99	xtwn		
XL0c2b12	-2.41	lim-1		
XL110i20	1.04	hairy2		
XL0c2b14	-1.11	follistatin		
XL097h17	1.69	HRT		
XL083a23	1.00	HMG-X		
XL0c2i02	-2.60	tak1		
XL0c2f20	-2.60	tob		
XL0c2d19	1.56	Xanf-1		
XL066n17	1.27	zic5		Y
Xl.13537	3.98		Ane	N
Xl.9137	2.2		Apl	N
Xl.5071	3.94		Ease	N
Xl.2784	4.16		mt-A	N

End of Table 3.4.1.

Several genes confirmed in Table 3.4.1 were further shown as direct target of Zic1 (Fig. 3.4.1). Among these genes, *nbx* was previously suggested to be a Zic1 targets (Kurata and Ueno, 2003); *Xfeb* is a novel gene that will be discussed in depth in Chapter 5; *snail* was also shown to be abolished in whole embryos by dominant interfering *zic1* (DNzicD, Fig. 5.1.2D).

After we redid the CHX+ experiment with six more Affymetrix genechips, we were able to select candidate genes more efficiently (genes10chips2006.xls). Table 3.4.2 shows QPCR performed on 16 genes, and six have been confirmed.

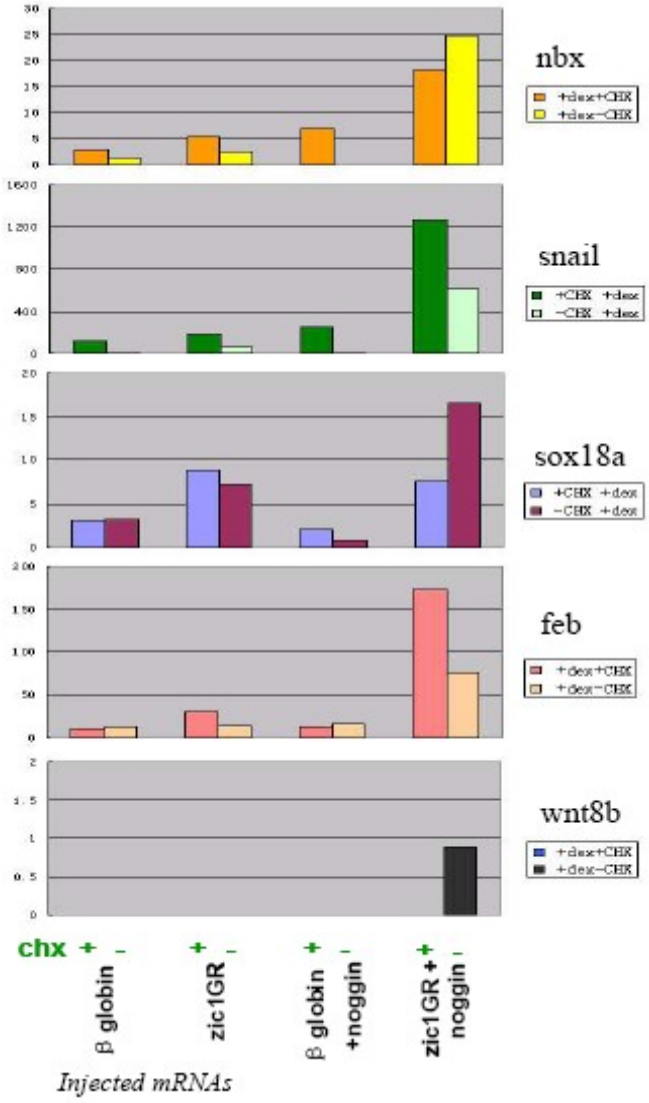

Figure 3.4.1 Several direct targets of Zic1.

Embryo injections and animal cap isolations were performed as in Fig. 2.11. The following samples were added: 1) injection of β-*globin* or *zic1GR* alone; 2) samples collected with and without CHX treatment. Quantitative RT-PCR was carried out on a Rotorgene 3000 platform and all data were normalized against EF1α expression levels. Comparable data were obtained in at least three independent experiments and representative data are shown here (Y axes represent relative transcript levels). The variation between assays was below 20%. *Nbx*, *snail*, *sox18a* and *feb* are direct targets of *zic1* because they are induced both in the presence and in the absence of CHX. For comparison, *wnt8b* is an indirect downstream target of *zic1*because *wnt8b* is induced only in the absence of CHX.

Table 3.4.2. With data from more genechips,16 genes were tested, 6 confirmed (UP). PCR was performed on animal cap samples +CHX. NC = No change; ? = further investigation is needed. The QPCR data for these genes are included in QPCR/Real-time-PCR4/2006-05-17(1).xls, 2006-05-24(1).xls.

SL score	Affy ID	major genebank entry	gene description	QPCR
2.5387	Xl.1003.1.S1_at	U28370.1	anf2-A: Homeobox protein (XANF-2)	UP
-4.0784	Xl.10555.1.A1_at	BE679068	retired, see UniGene Xl.55107	NC
2.0098	Xl.10868.1.S1_at	BC077627.1	xgalectin-Via, gal6a	UP
1.9888	Xl.12160.1.S1_at	BC046942.1	Trinucleotide repeat containing 4 (tnrc4-prov)	up?
3.2556	Xl.1249.2.S1_a_at	BC041190.1	myoD	UP
3.5086	Xl.16116.1.A1_at	CF285831.1	retired, see UniGene Xl.51335, similar to Leucine-zipper-like transcriptional regulator 1 (LZTR-1)	NC
-3.3007	Xl.17670.1.A1_at	BC072368.1	Similar to contactin 6; neural adhesion molecule; ctt6prov	NC
2.5844	Xl.182.1.S1_at	AJ242680.1	XFD-13 protein	?
6.4442	Xl.22603.1.A1_at	AW766683.1	retired, see UniGene Xl.50886, transcribed locus	?
-8.7444	Xl.2565.2.S1_at	BC044001.1	Similar to alpha-Tubulin at 84B	NC
-7.15	Xl.26274.1.A1_at	BC070664.1	Hypothetical protein MGC82269	NC
2.1855	Xl.373.1.S1_at	U75996.1	Eomesodermin, a key early gene in Xenopus mesoderm differentiation	UP
3.1255	Xl.639.1.S1_at	AF253504	dbx-A: Homeodomain protein dbx, Xdbx inhibits neuronal differentiation in the developing embryo	UP
2.8307	Xl.9271.1.S1_at	AJ009285.1	esr10-A: Enhancer of split related 10	?
1.7155	Xl.8933.1.A1_at	BG554194	Transcribed locus	UP
2.0261	Xl.22006	BC070672	cav-1	NC?

End of Table 3.4.2.

Detailed data for the confirmed genes are listed in Table 3.4.3.

Table 3.4.3. Normalized QPCR data for six confirmed genes.
p-values of upregulation were computed by paired t-test.

genes	Relative transcript abundance in Controls/Samples								p-value of upregulation
	C1	S1	C2	S2	C3	S3	beta3	opl15	
gal6a	19.533	132.55	13.073	139.01	49.029	103.05	8.3908	143.69	1.67E-04
myoD	3.6007	631.93	19.999	615.53	30.492	605.04	152.14	483.39	3.22E-05
eomes	1.5753	6.2824	1.1646	6.6931	0.27184	7.5859	1.0701	6.7876	5.34E-06
Xanf2	296.31	1988.6	578.31	1706.6	235.21	2049.7	437.44	1847.5	8.45E-06
dbx	4.8416	34.033	10.475	28.4	6.8029	32.072	0.99035	37.884	6.61E-05
Xl.8933	33.689	137.42	30.256	140.85	23.451	147.66	27.196	143.91	2.65E-08

Discussion

This genomic screen had many difficulties as discussed in section 2.1. Most of the work was done with very limited resources. Good QPCR assays were crucial in this project. The validation of array data by whole mount *in situ* hybridizations, although taking along a lot of cloning, did not contribute in results. High rate of false positives seemed to be inevitable with duplicate arrays. Because of the complexity of these developmental experiments, at least triplicate arrays were required to select candidates with a reasonable confidence.

Through my work, eleven genes have been confirmed to be upregulated by Zic1: *snail, nbx, Xfeb, zic5, Xgalectin6a; myoD, eomesodermin, sox18; Xanf-2, dbx*; and *Xl.8933*.

Among these, *Xl.8933* is a gene that has not been previously studied. At the time of writing, it has 11 EST sequences in NCBI database, and is associated with TIGR *Xenopus* gene index TC290335. Since these EST sequences are relatively short and have no clear open reading frames, no information was obtained by sequence searches; neither through *tropicalis* genome. However, with these EST sequences, it would be straightforward to isolate this gene by PCR and obtain the missing sequence. *Xfeb* was also a novel *Xenopus* gene. We characterized this gene and found it is expressed in the developing hindbrain region. More findings on *Xfeb* are reported in Chapter 5.

MyoD and *eomesodermin* are potent mesodermal genes. *MyoD* is a key factor in

muscle development. It has been found in our lab that *zic1* is also expressed in chick dorsomedial somites and overlaps partially with the expression of *myoD* (Sun Rhodes and Merzdorf, 2006). *Eomesodermin* is one of the earliest genes activated in the mesodermal lineage, and is essential for mesodermal formation (Ryan *et al*., 1996; Showell *et al*., 2004). Its expression starts in early gastrula and gets confined after mid-neurula. The ectopic expression of *eomesodermin* in animal caps induces other mesodermal genes *Xwnt8*, *Xbra*, *chd* and *Mix.1* (Ryan *et al*., 1996). This implies that *zic1* might have a role in mesodermal development. Further evidences were added in a recent study, where *Xenopus sox18*, another Zic1 target identified in this study, was shown to be essential for cardiogenesis (Zhang *et al.*, 2005). In the referred study, the authors could not differentiate the functions of *sox18* from *sox7*, and proposed they have redundant roles. It will be interesting to examine *sox7* in our study too. The authors also showed *sox18* activated *snail* and *eomesodermin* in animal caps, two other direct *zic1* targets identified in our study. It is difficult to tell from that experiment what developmental stage these regulations occur in. The data from our microarrays point to the isoform of *sox18a*, which is also expressed quite later than *sox18b* (Hasegawa *et al.*, 2002). This is certain room to analyze these relations further.

Galectins are proteins that bind to β-galactoside-containing carbohydrate moeties of glycoconjugates (Shoji *et al*., 2003). Galectin family has been previously reported to be involved in embryonic development. *Xenopus* β-galactoside-binding lectins were proposed to be important for the development of neural crest cells and other tissues

(Frunchak *et al*., 1993; Evanson and Milos, 1996). Although these lectins have not be specifically identified, our finding of *Xgalectin6a* as a Zic1 target evokes interesting questions along this line.

Our data indicate that *nbx*, *snail* and *zic5* are directly regulated by *zic1*, while *slug* is indirectly regulated (Johnson and Merzdorf, unpublished). Published *in situ* hybridization results show that the expression domains of *zic1*, *nbx*, *snail*, *zic5* and *slug* overlap. It has been reported that *snail* is upstream of *slug* (Aybar *et al*., 2003), *zic5* activates *snail* and *slug* (Nakata *et al*., 2000), *nbx* induces *slug* and is regulated by *zic5* (Kurata and Ueno, 2003). Combined, these data suggest pathways in neural crest development (Fig. 3.5.1). Whether *Xgalectin6a* is involved in this plot remains to be found out.

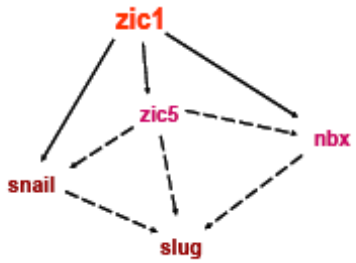

Fig. 3.5.1 Emerging regulatory relationships in neural crest development.

Xenopus Xanf-2 and *Xanf-1* (Zaraisky *et al.*, 1992; Mathers *et al.*, 1995; Zaraisky *et al*., 1995; Kazanskaya *et al*., 1997; Ermakova *et al*., 1999) are very close in both nucleotide and amino acid sequences. Although findings have been reported for them separately, these two genes cannot be possibly distinguished by in situ hybridizations. It is also a question whether this is true for PCR. Since *Xenopus tropicalis* genome seems to contain only a single *anf* gene, *Xanf-1* and *Xanf-2* are likely to be tetraploid copies of a

same gene. The expression of *Xanf* overlaps with *zic1* since late gastrula stages. But later in neurula stages, *Xanf* expression overlaps more with *zic2* in stead of *zic1*. As *zic2* presumably function more anterior to *zic1*, and *Xanf* is also involved in anterior development, the question arises whether the overexpression of Zic1 in our experiments affected Zic2 target genes. Such regulations may be among a whole array of gene cascades following neural induction.

Xdbx is a homeodomain containing gene that is expressed in posterior hindbrain and spinal cord at neurula stages. Its expression domain overlaps largely with *Xash3*, and supposedly with *Xfeb*, given also the relative positioning to *en-2* and *krox20* (Gershon *et al.*, 2000). Hence, *zic1*, *Xfeb* and *Xdbx* seem to be all expressed in the medially located undifferentiated cells.

Xdbx has been reported to inhibit neuronal differentiation in a Notch1-independent pathway (Gershon *et al.*, 2000). Z*ic1* also has an important role in inhibiting neuronal differentiation, which was proposed to be linked to Notch signaling (Aruga *et al*, 2002; Aruga 2004). Our finding indicates that X*dbx* is a target of Zic1; this proposes *Xdbx* could be one of the mechanisms that Zic1 inhibits neuronal differentiation. On the other hand, how Zic1 function is related to Notch signaling remains to be clarified.

We have not been able to identify a gene that is downregulated by Zic1. Several possible reasons are: such genes are not explicitly expressed in animal caps *per se*, therefore no way to detect; hybridization signals are low for repressed genes, so they are difficult to be selected; Zic1 might act mostly as a transcription activator.

Overall, we have identified a number of highly interesting Zic1 target genes in this genomic screen. These regulations and the inspired future studies will largely enhance our understanding of neural development. However, the pitfall of overexpression should be born in our mind. Since the DNA binding domain of Zic1 is similar to some other zinc finger transcription factors, it is also possible that genes were induced in this experiment while they are actually regulated by other TFs. Would *myoD* and *eomesodermin* be actually regulated by Gli family proteins? Precautions should be taken in further investigation of these findings.

Materials and Methods

Injections, animal cap isolation and treatment were carried out as done for microarray experiments.

Total RNA was extracted from animal caps or embryos with protease K and phenol/chloroform, treated with DNase and reverse transcribed with oligo-dT$_{16}$. (Fig. 3.6.1) Samples without addition of reverse transcriptase served as -RT controls to check for the presence of genomic DNA. In animal cap assays, all samples were also checked for the presence of mesoderm with *muscle actin* primers. Conventional PCR was carried out with *Taq* polymerase (Invitrogen) and PCR products separated on polyacrylamide gels. Real-time PCR was performed with a Sybr Green master mix (Eurogentec) on a Rotorgene 3000 platform. PCR products were further examined by melt curve analysis and gel electrophoresis. Data were quantified by the ΔΔCt method. For animal cap

samples, gene expression levels were normalized against *EF-1α* expression levels from the same RNA pool (normalization with *ODC* levels produced similar results).

All primers used in this study are included in an Excel file (Primers_SL.xls).

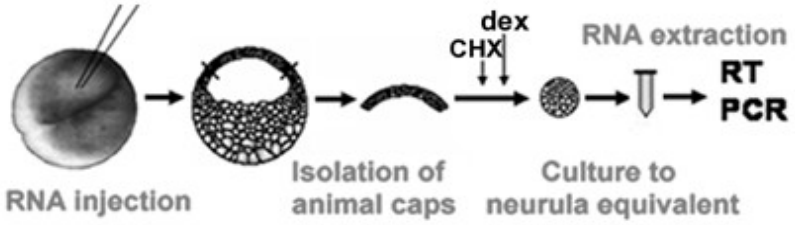

Fig. 3.6.1. Schematic of RT-PCR assays.

COMPUTATIONAL ANALYSIS OF GENE EXPRESSION

This chapter gives a brief review on some of the computational tools applicable in this type of genomic study, and perspectives on possible future directions.

Studying Promoters of Confirmed Genes

The ultimate test of a target gene of a transcription factor is the binding of this transcription factor to its promoter. This could be an important research direction for the target genes we identified from this screen. Knowledge on these gene promoters will also help understand how the regulations are exerted, and promote further experimental investigations.

However, to map the short promoter regions out of dozens of KB on the genome could be very challenging. Toward this goal, computational tools are indispensable and become fairly powerful. Since all the genes directly regulated by Zic1 should have Zic1 binding sites on their promoters, these promoters should share the same binding motifs. A computational tool could search candidate sequences for shared motifs to identify potential Zic1 binding sites. An excellent program is MEME, exemplified in Figure 4.1.1.

Further more, because promoters are likely to be more conserved than nonfunctional sequences across species, searching in conserved genomic regions helps to improve the chance of success. Identification of conserved non-coding sequences has been a goal of

several computational tools, including that in VISTA project (Figure 4.1.2). Once the Zic1 binding sites are mapped computationally, experimental assays (gel-shift or reporter assays) can be performed to validate the results.

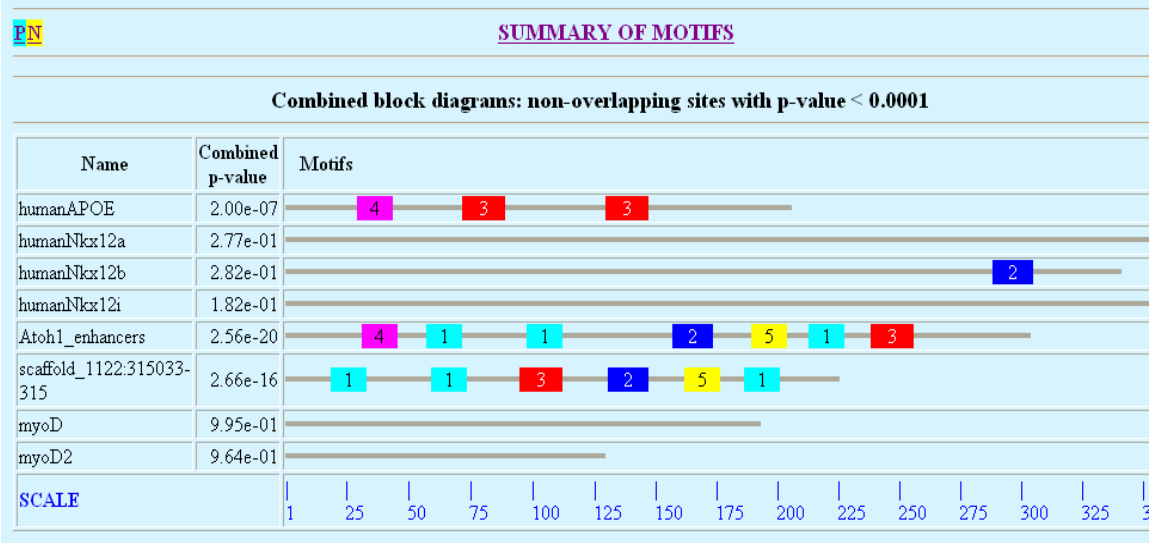

Fig. 4.1.1. An example output of MEME, identifying shared motifs among several sequences. Red blocks = GCTCCCCGGGGAGCTG, a published Zic1 binding site in Math1 promoter. http://meme.sdsc.edu.

Evolutionary View of Zic1 Functions

With knowing a number of Zic1 direct targets, an important question to ask is how these gene regulations evolved. This may help understand how important, new evolutionary features (*e.g.* neural crest) arose.

Zic1 is a well-conserved gene in bilateria, with homologues found in *D. melanogaster* and *C. elegans*. One way to address the question to examine the presence of both the orthologues of Zic1 targets and the binding sites of Zic1 in representative genomes. As some of these Zic1 target genes will represent new features in evolution, the

results shall shed light on the evolution of such new features.

For example, both neural crest and extensive brain structure are novel features of vertebrates. Several neural crest genes including *zic* and *snail* have been studied to address the evolution of neural crest (Shimeld and Holland, 2000). The results showed these genes were already expressed in comparable patterns before the evolution of vertebrates. We have identified that *Xenopus snail* is a direct target of Zic1. Hence, to determine the presence of Zic binding site on *snail* promoter will be an important approach to further our understanding of neural crest evolution. (we have also found another zic1 target *nbx* that is also involved in neural crest development.) Similarly, we also found a novel hindbrain gene, *Xfeb*, as zic1 target, the evolution of *zic/feb* regulation may help understand brain evolution.

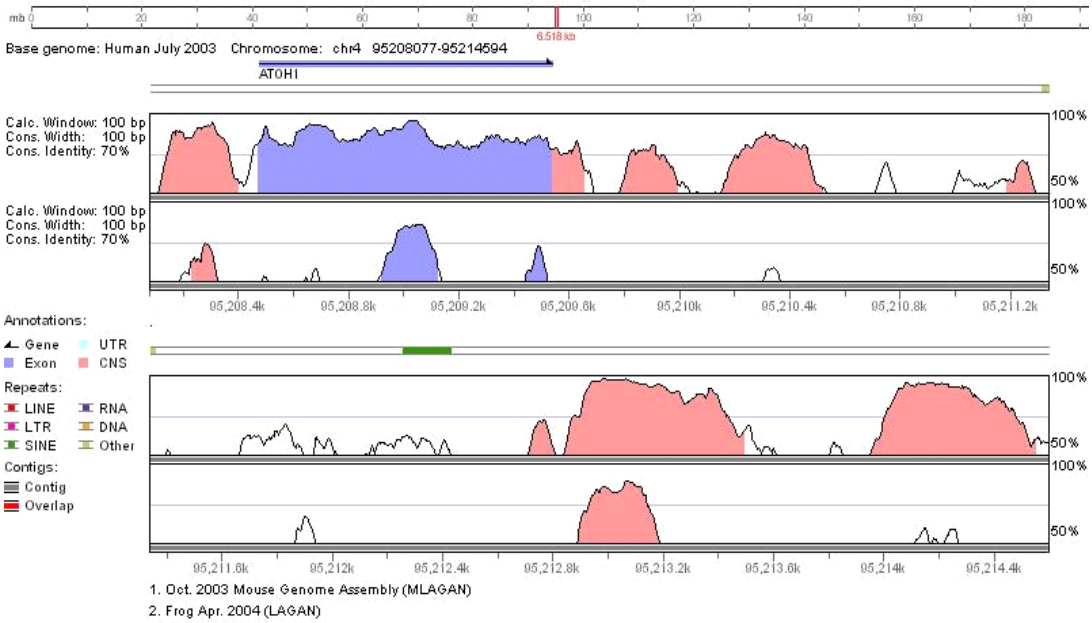

Fig. 4.1.2. Identification of conserved non-coding regions through comparative genomics: an example from VISTA, http://genome.lbl.gov/vista/index.shtml.

On the other hand, the spectrum of genes regulated by *zic1* reveals the function of this transcription factor, which changed and expanded during evolution. So overall, this study could reveal interesting insights of evolving important new features along with transcriptional regulation.

Regulators of *zic1*

Similar techniques can be used to look for binding sites in *zic1* promoter, therefore gaining information on upstream regulators of *zic1*. Fig. 4.3.1 shows an example to examine promoter regions of human and mouse zic1. Conserved regions were identified first, then searched against known TF binding sequences (from Transfac database).

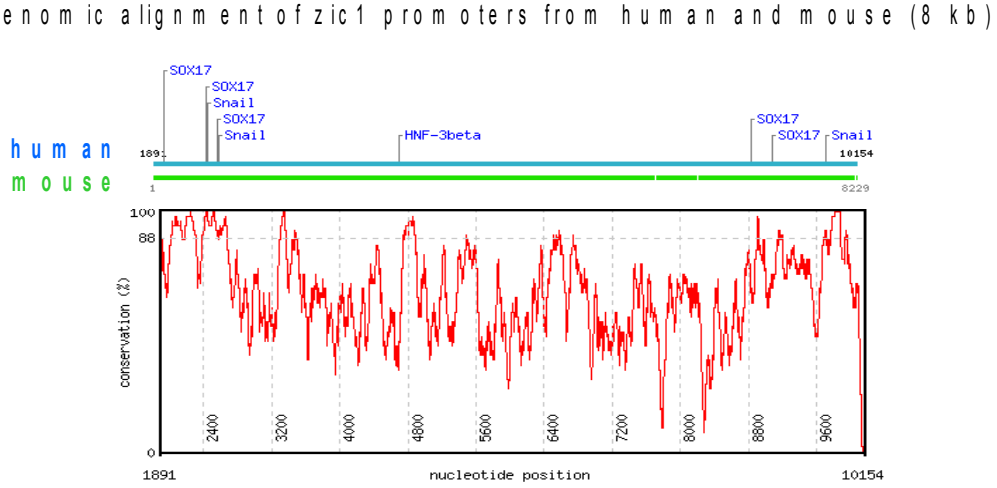

Fig. 4.3.1. An example of identifying TF binding site through phylogenetic footprinting. ConSite, http://www.phylofoot.org/.

XFEB AS A NOVEL TARGET OF ZIC1

Major contents in this chapter are presented in a paper in press (Li *et al*., 2006).

Xfeb Is a Direct Target of Zic1

Several lines of evidences suggest that *Xfeb* is a direct target of Zic1.

We first demonstrated this by QPCR (Fig. 5.1.1, A; Fig. 3.4.1). Conventional PCR also showed *Xfeb* is activated by full-length *Zic1* (Fig. 5.1.1, B).

In order to investigate whether *zic1* is able to induce ectopic expression of *Xfeb* in whole embryos, we misexpressed three *zic1* constructs: RNA for full length *zic1*, *zic1GR*, or *zic1ΔC* (where the domain C-terminal to the zinc fingers had been deleted; Kuo et al., 1998) was injected into one cell of two-cell albino *Xenopus* embryos. *lacZ* RNA was co-injected as tracer. The embryos injected with zic1GR were treated with dexamethasone at mid-gastrula stage 11. Embryos were harvested at neurula stage 17 and the expression of *Xfeb* was assayed by whole mount in situ hybridization. *zic1ΔC* induced significant ectopic *Xfeb* expression (67%, n=69), both in the epidermal ectoderm (Fig. 5.1.2A) and in the neural ectoderm (Fig. 5.1.2B). A similar effect was found in embryos injected with *zic1GR*, although we did not find any significant *Xfeb* induction in full length *zic1* injected embryos. The induction of *Xfeb* by *zic1ΔC* and *zic1GR* is probably due to the increased activity of both constructs (Kuo et al., 1998). It is interesting that these zic1

constructs were able to induce ectopic *Xfeb* expression at great distance from its normal expression domain both in the neural ectoderm and in the epidermal ectoderm. The latter is consistent with our finding that zic1GR activates *Xfeb* expression in non-neuralized animal caps (Fig. 3.4.1). Overall, these data show that *Xfeb* is upregulated by *zic1* in whole embryos.

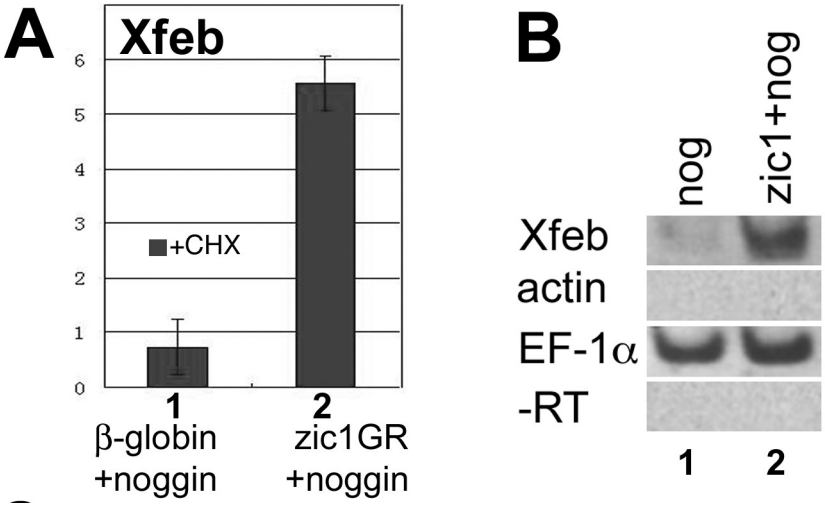

Figure 5.1.1. Xfeb is activated by Zic1. (A) Collective QPCR data from 11 experiments. The samples were generated same as for microarrays. Lane 1 shows the Xfeb transcript level in control samples and lane 2 shows Xfeb level in Zic1 overexpressing samples. Xfeb is upregulated by Zic1GR. The original data are included in QPCR/Real-time-PCR3/2005-07-15working.xls, sheet6. (B) Xfeb is induced by full-length zic1 in animal caps. The assay was performed as described in section 3.6, but injected with 1 pg of noggin (lane 1) or 1 pg noggin in combination of 200 pg zic1 RNA (lane 2). Xfeb is clearly induced by zic1. EF-1 α was used as loading control. No muscle actin or genomic DNA was found in the assay.

To further investigate whether *Xfeb* expression is dependent on *zic1*, we injected RNAs of *DNzic1D*, a dominant interfering form of *zic1*, and *lacZ* were coinjected into one cell of two-cell albino embryos. These embryos were then fixed at stage 14, stained for *lacZ* expression by X-gal and *Xfeb* by in situ hybridization. On the injected side,

endogenous expression of *Xfeb* is demolished by injected *DNzic1D* (Fig. 5.1.2C). This exhibits that *Xfeb* expression is dependent on zic1, further ascertaining *Xfeb* is a downstream target of *zic1*. Fig. 5.1.2D shows that *DNzic1D* has the same effect on *snail*.

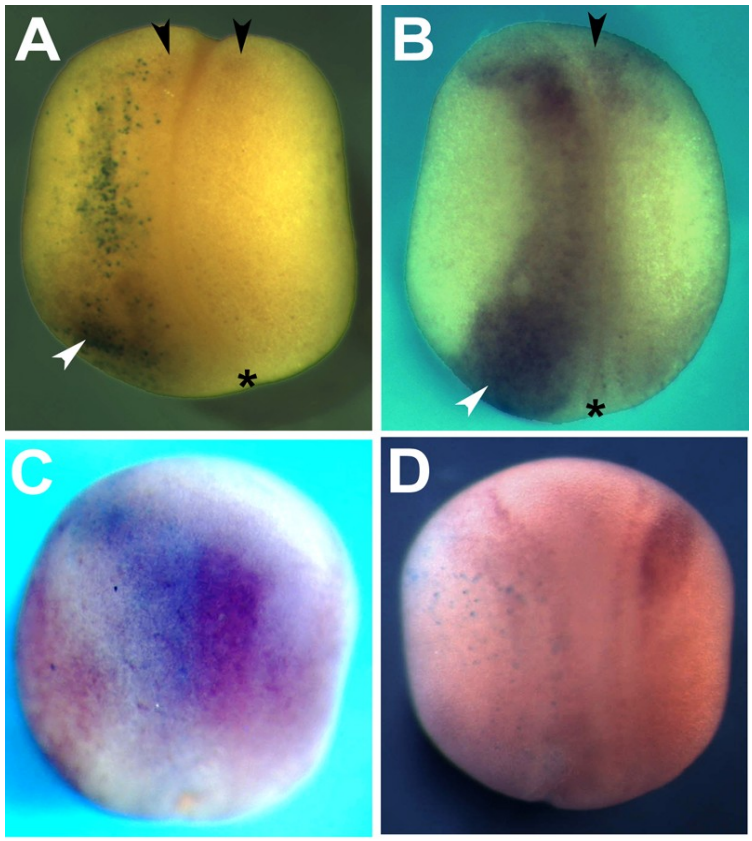

Fig. 5.1.2. Zic1 regulates Xfeb in whole embryos. (A, B) Injected zic1ΔC induce ectopic expression of Xfeb (white arrowheads). (C) Dominant interfering form of zic1, DNzic1D, diminishes Xfeb expression (left side with blue staining of co-injected lacZ). (D) DNzic1D also diminishes expression of snail, another direct target identified in this study.

Xfeb Contains Five SCP Domains and May be a Serine Protease

Among the differentially expressed genes was a previously uncharacterized gene that we have named *Xfeb* (covered by UniGene cluster Xl.15703) due to its homology to a

human protein that is referred to as “17 kD fetal brain protein” in NCBI data bases (NP_071738), since an early EST for this gene was derived from human fetal brain (AA339686) (Adams et al., 1995). Similar sequences from *Xenopus laevis* are found under another UniGene cluster, Xl.32643, which apparently represents the second Xfeb copy in the tetraploid *Xenopus* genome.

Xfeb encodes a protein of 919 amino acids, containing five repeated SCP domains (Fig. 5.2.1A). Sequence alignments show that all five SCP-like domains are very similar to each other (Fig. 5.2.1B). A single Xfeb gene is present in the *Xenopus tropicalis* genome, located on scaffold_667:565792-578997 (version 4.1). This protein also contains five tandem SCP domains and its predicted peptide sequence is 84% identical to that in *Xenopus laevis*. In other species, Xfeb homologues with one or multiple SCP domains are found. Hypothetical proteins containing either two or three tandem SCP domains are present in zebrafish (AAH56553 or XP_690132), sea urchin (XP_783519), honeybee (XP_624623) and Schistosoma (AAW25499). The zebrafish protein shows greater than 50% identity to Xfeb.

Xfeb belongs to the pathogenesis-related (PR) protein superfamily. While the proteins in this superfamily are evolutionarily related through their SCP domains, they are involved in diverse processes, including sperm maturation, gamete fusion, pathogenesis, and venom toxicity. The only two known Xenopus proteins that contain SCP domains are Allurin, a sperm chemoattractant (Olson et al., 2001), and a CRISP protein (Schambony et al., 2003), each with one SCP domain. However, Xfeb shows greater similarity to its

homologues in other species than it does to these *Xenopus* proteins.

The human homologue of Xfeb is the "17 kD fetal brain" protein, which has also been named GAPR-1 (Golgi-associated PR-1; Eberle *et al.*, 2002) at chromosomal location C9orf19 (Eisenberg *et al*., 2002). GAPR-1 contains 154 amino acids and a single SCP domain. It shares 46% identity (aligned as shown in Fig. 1B; the percentage is computed over the whole length of GAPR-1) overall with Xfeb at the amino acid level (Fig. 5.2.1B; NP_071738). The human and rat Xfeb homologues are expressed in various adult tissues, including lung, spleen, leukocytes, and uterus (Eberle *et al*., 2002; Eisenberg *et al*., 2002). The rat Xfeb homologue is also detected in embryonic tissues, although the embryonic stage was not specified, nor the identity of the embryonic tissues that were utilized (Eberle *et al*., 2002).

With the notable exception of GAPR-1, proteins that contain SCP domains are typically extracellular. In contrast, GAPR-1 is a myristoylated cytosolic protein that is associated with the outer membrane of the Golgi complex (Eberle *et al*., 2002). The cytosolic location of GAPR-1 is consistent with the apparent lack of a signal sequence in the Xenopus Xfeb protein, suggesting that Xfeb is also a cytosolic protein. Further, we have been unable to identify a consensus N-terminal myristoylation sequence for Xfeb. Interestingly, the activity of GAPR-1 has been suggested to be dependent upon dimerization (Serrano *et al.*, 2004). In this regard, the oligomeric structure of GAPR-1 may mirror the tandem SCP domains of Xfeb and its multidomain homologues.

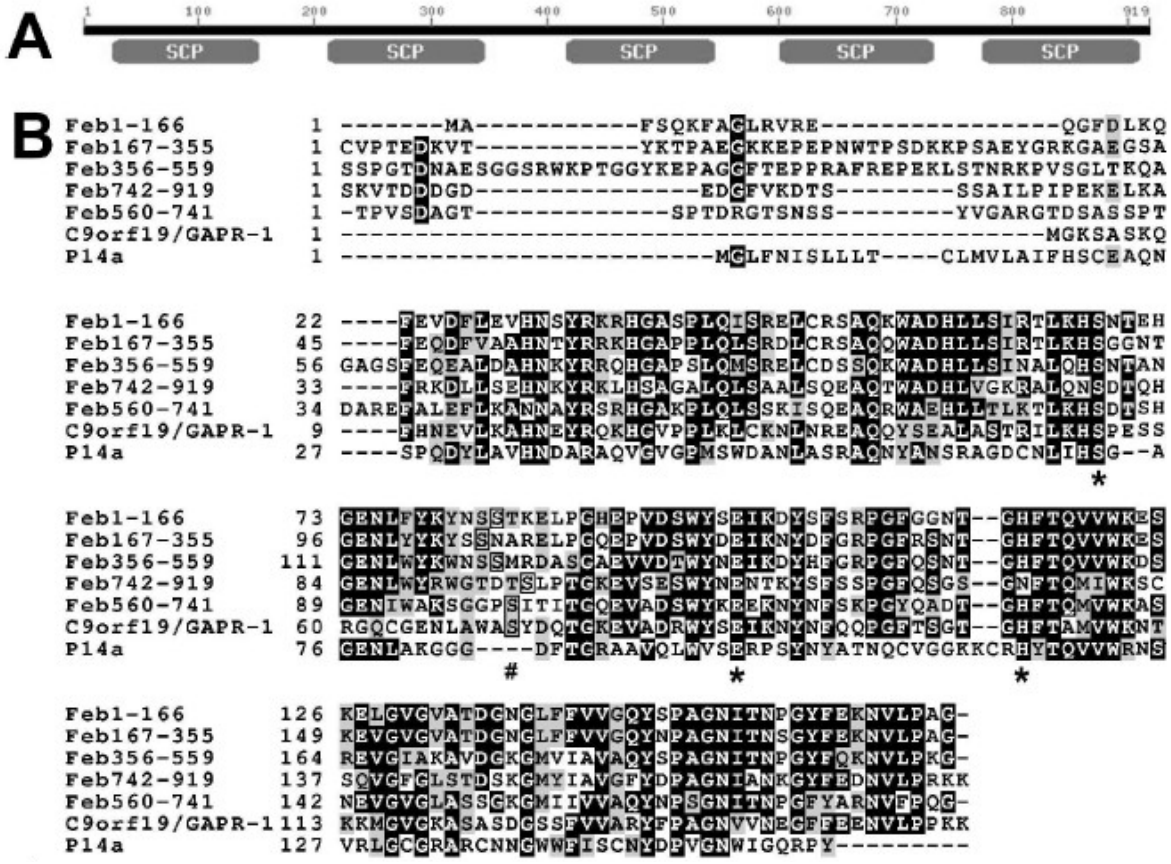

Figure 5.2.1. Xfeb contains five tandem SCP-like domains that may have serine protease activity. A) Schematic distribution of five SCP domains within the Xfeb protein. B) The *Xfeb* sequence was manually divided into its five constituent SCP domains. They were then aligned to each other, to the human Xfeb homolog C9orf19/GAPR-1, and to another SCP domain protein (P14a) that has a determined structure (NCBI accession number for consensus SCP domain: cd00168). Starred residues (Ser, Glu, His) constitute a putative serine protease active site commonly found in this protein family. Since four of the five Xfeb SCP domains contain these residues, Xfeb may act as a serine protease. The structure of GAPR-1 suggests Ser71 (marked by #) as an alternative active site serine. The five SCP domains of Xfeb contain serines (boxed) +/- two residues on either side of Ser71 in GAPR-1. NCBI accession numbers: *Xenopus* Xfeb, AAH84924; human C9orf19/GAPR-1, NP_071738; P14a, P04284.

The SCP domains in several proteins are thought to contain serine protease activity. Tex31 from snail venom contains an SCP domain and was recently shown to possess protease activity (Milne *et al.*, 2003). Structure-based homology modeling of Tex31 (Milne *et al.*, 2003) based upon the structure of the SCP domains of the P14a (Fernandez

et al., 1997) and Ves v 5 (Henriksen *et al*., 2001) proteins suggests a catalytic triad (Ser, Glu and His) of putative active site residues (Fig. 5.2.1B). Since four of the five SCP domains in Xfeb contain these conserved residues, Xfeb may also possess serine protease activity. Structural studies of GAPR-1 suggest an alternative active site serine (Serrano *et al*., 2004). This serine residue may also be conserved in the five SCP domains of Xfeb, although minor adjustments are required to bring them into alignment (Fig. 5.2.1B).

In an attempt to find the subcellular location of Xfeb proteins, we generated a Myc-tagged construct by cloning Xfeb into a pCS2+MT plasmid. However, the fusion protein failed to express. A different tagging strategy may be needed to investigate this issue.

Xfeb is Expressed in the Developing Hindbrain Region

We determined the expression of *Xfeb* by RT-PCR in whole *Xenopus* embryos at stages throughout early development. The quantitative PCR data show that *Xfeb* expression begins at embryonic stage 10. *Xfeb* expression levels rapidly rise and continue throughout neurula stages (Fig. 5.3.1). In comparison, *zic1* expression begins at stage 9 (Kuo et al., 1998). Thus, *Xfeb* is expressed during gastrula and neurula stages, shortly after *zic1* expression begins.

The expression domains of *Xfeb* were determined by whole-mount in situ hybridization. Through neurula stages, *Xfeb* is expressed in the hindbrain region. It starts in a relatively broad domain encompassing the lateral sides of the neural plate at stage 13

to 15 (Fig. 5.3.2A, B). At stage 17, *Xfeb* expression is more defined in the region that will become the dorsal neural tube (Fig. 5.3.2C). Double in situ hybridization with probes for *Xfeb* and the midbrain/hindbrain markers *en-2* and *wnt1* shows that *Xfeb* expression in the hindbrain extends up to the midbrain/hindbrain boundary (Fig. 5.3.2E-G).

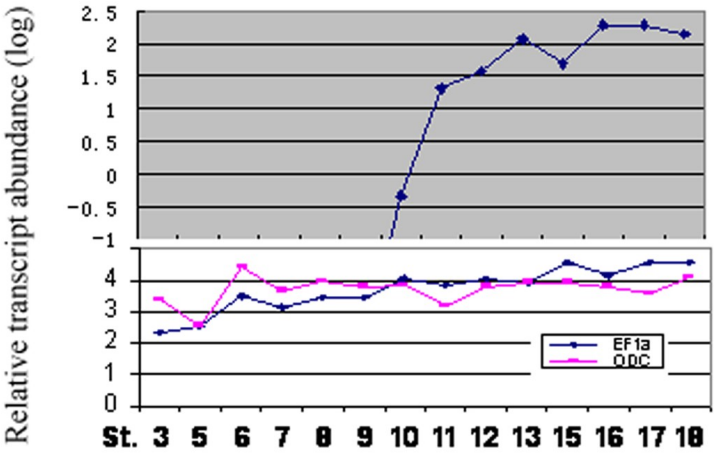

Fig. 5.3.1. A developmental profile of Xfeb expression was generated by quantitative RT-PCR using RNA from staged *Xenopus* embryos (top). *EF-1a* and *ODC* were used as loading controls (bottom). Y axes represent the relative abundance of transcripts in log scale. *Xfeb* expression begins at early gastrula stage 10 and continues throughout neurula stages.

Since at this stage, *zic1* is strongly expressed throughout the midbrain and the hindbrain (Fig. 5.3.2D and Kuo et al., 1998), *Xfeb* expression overlaps extensively with zic1 expression in the hindbrain. The overlap between the expression domains of these two genes is consistent with our finding that *Xfeb* expression is activated by zic1. By stage 20 (Fig. 5.3.2H), *Xfeb* is expressed broadly in the midbrain and hindbrain and in the region of the dorsomedial somites. Interestingly, *zic1* is also expressed in dorsomedial somite tissues (Nagai et al., 1997; Nakata et al., 1998; Aruga et al., 2002b) (Sun Rhodes

and Merzdorf, in press). At stage 23 (Fig. 5.3.2I), *Xfeb* is expressed in the dorsal midbrain and hindbrain. It is also faintly expressed in chevron shapes in the trunk (Fig. 5.3.2I, J). Since trunk neural crest cells in Xenopus do not begin their migration until stage 24 (Sadaghiani and Thiebaud, 1987; Collazo et al., 1993), this expression of *Xfeb* in the trunk is probably within the somites.

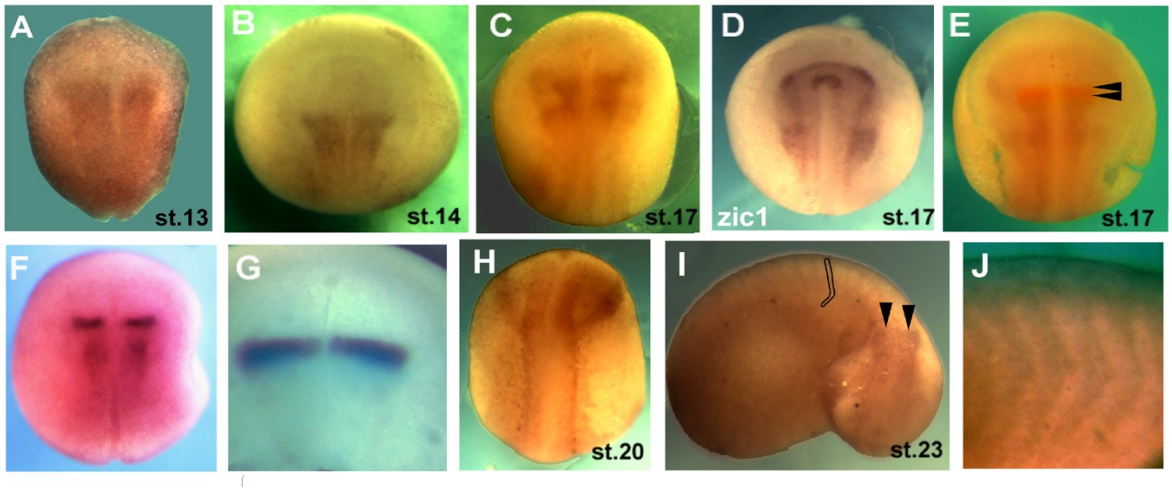

Fig. 5.3.2. Spatial expression of Xfeb. Whole mount *in situ* hybridization with *Xfeb* and *zic1*, *en-2* and *wnt1* probes. Dorsal views of *Xfeb* expression at stage 13 (A), at stage 14 (B) and at stage 17 (C). At stage 17, *Xfeb* expression overlaps extensively with *zic1* expression (D) in hindbrain regions. (E-G) *Xfeb* is expressed in the hindbrain region up to the midbrain/hindbrain boundary. At stage 17, double *in situ* hybridization shows that *Xfeb* expression (brown) is adjacent to that of *en-2* (orange, arrowheads, E); while with a small gap to the anterior bands of wnt1 expression (dark purple, F). Since wnt1 expression (purple, G) is slightly anterior to that of en-2 (blue, G), Xfeb is expressed up to but not overlapping with en-2. At stage 20 (H), *Xfeb* is expressed in a broad area in the midbrain and hindbrain and lateral to the neural tube. At stage 23, *Xfeb* is expressed in the midbrain and hindbrain region (arrowheads, I) and in a very faint chevron pattern in the trunk (one of these domains outlined in I, and detailed in J).

In early neurula embryos, when the boundary between otx2 and gbx2 has not been fully established, we observed a gap between the expression of Xfeb and wnt1. As embryos develop older, this gap closes up (Fig. 5.3.3).

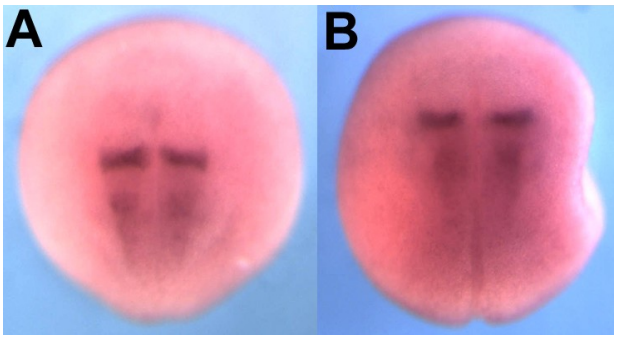

Fig. 5.3.3. The gap between Xfeb and wnt1 closes up as embryos age. Double in situ with wnt1 (bands on top, purple?) and Xfeb (strips below, brown). (A) stage 15. (B) stage 17.

Xfeb Represses *otx2* and *hoxB1*

The expression of *Xfeb* in hindbrain regions suggested that it may play a role in neural patterning. To further investigate Xfeb activities, we misexpressed Xfeb in animal caps and assayed for a range of neural markers by RT-PCR. *Xfeb* RNA, noggin RNA, or their combination were injected into both blastomeres of 2-cell embryos and animal caps were assayed by RT-PCR. Most neural markers tested showed no change in expression levels (*Xanf-1, en-2, krox20, gbx2, fgf8, hoxB1, hoxD1, hoxB9, NCAM, N-tubulin*, and several *wnt* genes; not shown). However, misexpression of *Xfeb* resulted in repression of the anterior neural gene otx2 (Fig. 5.4.1A). *noggin* is known to induce neural tissue of anterior character (Lamb et al., 1993; McGrew et al., 1995). Therefore, the anterior neural gene *otx2* is expressed in noggin-induced animal caps (lane 2). The induction of *otx2* by noggin is inhibited by co-expression of *Xfeb* in these animal caps (lane 4). In order to test whether Xfeb is able to inhibit otx2 expression in whole embryos, *Xfeb* RNA was injected into one side of 2-cell albino embryos. These embryos were assayed for otx2 expression

by in situ hybridization. *otx2* expression is clearly reduced on the injected side (Fig. 5.4.1B; 69%, n=64). Thus, *Xfeb* is able to interfere with expression of the otx2 gene.

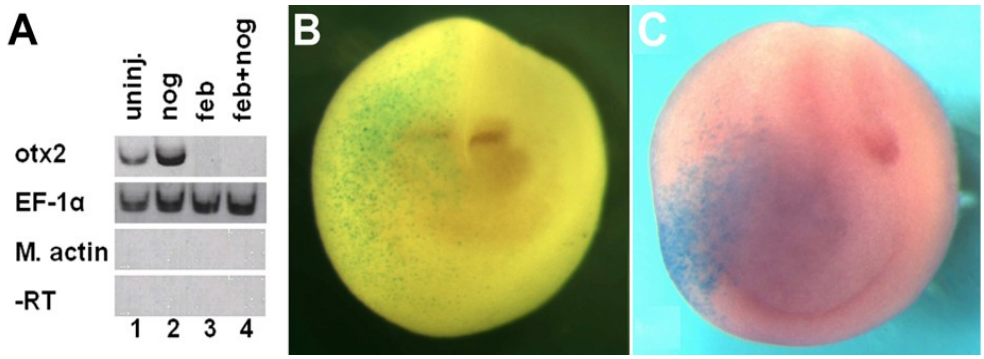

Fig. 5.4.1. Xfeb represses expression of the anterior neural gene otx2 and hindbrain gene hoxB1. (A) *Xfeb* represses *otx2* expression in animal cap assays. Two-cell embryos were injected with 1 pg *noggin* RNA (lane 2), 500 pg *Xfeb* RNA (lane 3), or their combination (lane 4). Animal caps were dissected at stage 9 and cultured to equivalent of stage 23. RT-PCR analysis demonstrated that *otx2* was induced by *noggin* (lane 2), but repressed by co-injection of *Xfeb* (lane 4). Uninjected animal caps were used as controls (lane 1). Muscle actin primers verified the absence of mesoderm; -RT samples control for genomic DNA. (B) *Xfeb* represses *otx2* expression in whole embryos. Two-cell albino embryos were co-injected into one cell with 250 pg *Xfeb* RNA and 25 pg *lacZ* RNA. Embryos were fixed at stage 17 and stained for b-galactosidase (blue) and by *in situ* hybridization for *otx2* (brown). *otx2* expression is significantly reduced on the injected (left) side. (C) Xfeb represses hoxB1 expression on the injected (left) side. Embryos were prepared as in (B).

The position of the midbrain/hindbrain boundary is largely determined by mutual repression between the transcription factors *otx2* and *gbx2* (Rhinn and Brand, 2001), with expression of *otx2* in the forebrain and midbrain, and *gbx2* in the hindbrain. The expression boundary between *otx2* and *gbx2* is established before we are able to detect specific *Xfeb* expression in the hindbrain region. Thus, *Xfeb* probably does not play a role in establishment of the midbrain/hindbrain boundary, but it might play a role in maintaining the posterior expression boundary of *otx2*. Should Xfeb indeed be an active protease, it might proteolize an upstream factor that is involved in regulating expression

of *otx2*. Thus, Xfeb activity might represent a safeguard to ensure proper regionalization at the midbrain/hindbrain boundary.

We also used in situ hybridization to investigate a few other genes in Xfeb overexpressing embryos. While Xfeb had no effect on *gbx2*, *krox20*, *nbx* and *hoxD1*, it repressed *hoxB1* in a number of embryos (Fig. 5.4.1C). What's more puzzling is the effect of Xfeb on *snail* expression (Fig. 5.4.2). Some *snail* expression was abolished by overexpressed Xfeb, while some was distorted. The mechanism behind this observation awaits for further investigation.

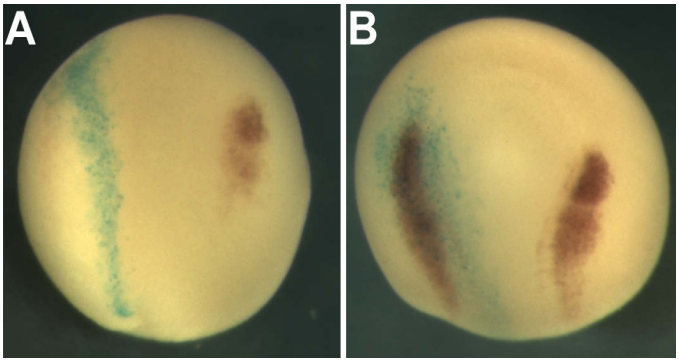

Fig. 5.4.2. Xfeb affects snail expression. Embryos were prepared as in Fig. 5.4.1B and stained for snail expression, which is abolished in some embryos (A) and distorted in some others (B).

Discussion

In conclusion, Xfeb is a direct target of zic1 that is upregulated by *zic1* at a time when the hindbrain is regionalized. Xfeb is likely to modulate signaling pathways that regulate the expression of *otx2*, and may play a role in maintenance of the midbrain/hindbrain boundary. Therefore, lack of Xfeb expression might contribute to the

hindbrain defects resulting from mutations in zic1 (Aruga et al., 1998; Ogura et al., 2001; Grinberg et al., 2004). Further studies of Xfeb will elucidate the mechanisms by which Xfeb modulates the expression of *otx2* and may provide further insight into the hindbrain defects that are observed in *zic1* mutants. The effects of Xfeb on *hoxB1* and *snail* may reflect its function in different developmental contexts and invite future investigations.

Materials and Methods

Microinjection and in vitro transcription: *Xenopus laevis* embryos were obtained and microinjected as previously described (Kuo *et al*., 1998). RNA was synthesized from full length *zic1*, *zic1ΔC* (zic1 lacking part of the C-terminal domain) and *zic1GR* constructs in pCS2+ as previously described (Kuo et al., 1998; Merzdorf and Sive, submitted). *zic1GR* is a fusion construct of the zic1 coding region with the human glucocorticoid receptor binding domain; the resulting fusion protein only enters the nucleus in the presence of dexamethasone (Kuo et al., 1998). A full length clone of Xenopus laevis Xfeb (BC084924) was isolated by the I.M.A.G.E. Consortium and obtained through Open Biosystems in the pCMV.SPORT6 vector. Xfeb sense RNA was synthesized by digesting the Xfeb-containing pCMV.SPORT6 plasmid with ScaI and transcribing with SP6 polymerase (NEB). Other sense RNAs were synthesized as published: *noggin* (Smith and Harland, 1992), b-*globin* (Krieg and Melton, 1984), and *lacZ* as in (Gammill and Sive, 1997).

RT-PCR analysis: Injections, treatments and animal cap isolations were carried out

as described above (Fig. 2A) and in the figure legends. Total RNA was extracted from animal caps or embryos and reverse transcribed as in Kuo et al. (1998). Samples without addition of reverse transcriptase served as -RT controls to check for the presence of genomic DNA. In animal cap assays, all samples were also checked for the presence of mesoderm with muscle actin primers. Conventional PCR was carried out with Taq polymerase (Invitrogen) and PCR products separated on polyacrylamide gels. Real-time PCR was performed with a Sybr Green master mix (Eurogentec) on a Rotorgene 3000 platform. PCR products were further examined by melt curve analysis and gel electrophoresis. Data were quantified by the *ΔΔ*Ct method. For animal cap samples, gene expression levels were normalized against *EF-1a* expression levels from the same RNA pool (normalization with *ODC* levels produced similar results). In order to generate the developmental expression profile for Xfeb, each PCR reaction started with materials from an equivalent volume of embryos; both *EF-1a* and *ODC* were used as loading controls and no normalization was applied.

Whole mount in situ hybridization and probe synthesis: Whole mount in situ hybridization on albino embryos was carried out as described in (Harland, 1991). In some cases, embryos were sliced open to help buffer exchange and substrate penetration. One cell of 2-cell embryos was injected with 100 pg of zic1GR or *zic1Δ*C RNA, or 200 pg of *DNzic1D* or full length *zic1* RNA (also attempted at 500 pg), or with 250 pg *Xfeb* RNA, together with 25 pg *lacZ* RNA as tracer. ß-galactosidase staining was performed as in (Kolm and Sive, 1995) and the alkaline phosphatase substrate NBT/BCIP (Sigma) was

used for color detection. Double *in situ* hybridization was performed as for Fig. 5.3.2E: a digoxygenin-labeled *Xfeb* probe (stained with NBT/BCIP, Sigma) and a FITC-labeled *en-2* probe (stained with INT/BCIP, Boehringer); Fig. 5.3.2F: a digoxygenin-labeled Xfeb probe (stained with NBT/BCIP) and a FITC-labeled *wnt1* probe (stained with BM purple, Roche); Fig. 5.3.2G: a digoxygenin-labeled *en-2* probe (stained with INT/BCIP) and a FITC-labeled *wnt1* probe (stained with BM purple). Xfeb antisense RNA probe was synthesized from the Xfeb-containing pCMV.SPORT6 plasmid by StuI digestion and T7 polymerase transcription (NEB). Other antisense RNA probes were synthesized as previously described: *en-2* (Hemmati-Brivanlou and Harland, 1989) and *otx2* (Gammill and Sive, 2000).

REFERENCES CITED

1. Adams MD, Kerlavage AR, Fleischmann RD, Fuldner RA, Bult CJ, Lee NH, Kirkness EF, Weinstock KG, Gocayne JD, White O, et al. 1995. Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. Nature 377:3-174.

2. Aruga J, Inoue T, Hoshino J, Mikoshiba K. 2002a. Zic2 controls cerebellar development in cooperation with Zic1. J Neurosci 22:218-225.

3. Aruga J, Minowa O, Yaginuma H, Kuno J, Nagai T, Noda T, Mikoshiba K. 1998. Mouse Zic1 is involved in cerebellar development. J Neurosci 18:284-293.

4. Aruga J, Tohmonda T, Homma S, Mikoshiba K. 2002b. Zic1 promotes the expansion of dorsal neural progenitors in spinal cord by inhibiting neuronal differentiation. Dev Biol 244:329-341.

5. Aruga J, Yokota N, Hashimoto M, Furuichi T, Fukuda M, Mikoshiba K. 1994. A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. J Neurochem 63:1880-1890.

6. Bainter JJ, Boos A, Kroll KL. Neural induction takes a transcriptional twist. Dev Dyn. 2001 Nov;222(3):315-27.

7. Baker JC, Beddington RS, Harland RM. Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. Genes Dev. 1999;13(23):3149-59.

8. Baldi P, Long AD. 2001. A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. Bioinformatics 17:509-519.

9. Bally-Cuif L, Hammerschmidt M. Induction and patterning of neuronal development, and its connection to cell cycle control. Curr Opin Neurobiol. 2003 Feb;13(1):16-25.

10. Collazo A, Bronner-Fraser M, Fraser SE. 1993. Vital dye labelling of Xenopus laevis trunk neural crest reveals multipotency and novel pathways of migration. Development 118:363-376.

11. Eberle HB, Serrano RL, Fullekrug J, Schlosser A, Lehmann WD, Lottspeich F, Kaloyanova D, Wieland FT, Helms JB. 2002. Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex. J Cell Sci 115:827-838.

12. Ebert PJ, Timmer JR, Nakada Y, Helms AW, Parab PB, Liu Y, Hunsaker TL, Johnson JE. 2003. Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. Development 130:1949-1959.

13. Eisenberg I, Barash M, Kahan T, Mitrani-Rosenbaum S. 2002. Cloning and characterization of a human novel gene C9orf19 encoding a conserved putative protein with an SCP-like extracellular protein domain. Gene 293:141-148.

14. Ermakova GV, Alexandrova EM, Kazanskaya OV, Vasiliev OL, Smith MW, Zaraisky AG. The homeobox gene, Xanf-1, can control both neural differentiation and patterning in the presumptive anterior neurectoderm of the Xenopus laevis embryo. Development. 1999 Oct;126(20):4513-23.

15. Evanson JE, Milos NC. A monoclonal antibody against neural crest-stage Xenopus laevis lectin perturbs craniofacial development of Xenopus. J Craniofac Genet Dev Biol. 1996 Apr-Jun;16(2):74-93.

16. Fernandez C, Szyperski T, Bruyere T, Ramage P, Mosinger E, Wuthrich K. 1997. NMR solution structure of the pathogenesis-related protein P14a. J Mol Biol 266:576-593.

17. Frunchak YN, Martha GN, McFadden KD, Milos NC. Alterations of heart development in Xenopus laevis by galactoside-bindinglectin or its sugar hapten inhibitor. Anat Embryol (Berl). 1993 Mar;187(3):299-316.

18. Gammill LS, Sive H. 1997. Identification of otx2 target genes and restrictions in ectodermal competence during Xenopus cement gland formation. Development 124:471-481.

19. Gammill LS, Sive H. 2000. Coincidence of otx2 and BMP4 signaling correlates with Xenopus cement gland formation. Mech Dev 92:217-226.

20. Gershon AA, Rudnick J, Kalam L, Zimmerman K. The homeodomain-containing gene Xdbx inhibits neuronal differentiation in the developing embryo. Development. 2000 Jul;127(13):2945-54.

21. Gostling NJ, Shimeld SM. Protochordate Zic genes define primitive somite compartments and highlight molecular changes underlying neural crest evolution. Evol Dev. 2003 Mar-Apr;5(2):136-44.

22. Grinberg I, Northrup H, Ardinger H, Prasad C, Dobyns WB, Millen KJ. 2004. Heterozygous deletion of the linked genes ZIC1 and ZIC4 is involved in Dandy-Walker malformation. Nat Genet 36:1053-1055.

23. Grinblat Y, Sive H. 2001. zic Gene expression marks anteroposterior pattern in the presumptive neurectoderm of the zebrafish gastrula. Dev Dyn 222:688-693.

24. Hansson EM, Lendahl U, Chapman G. 2004. Notch signaling in development and disease. Semin Cancer Biol 14:320-328.

25. Harland R. Neural induction. Curr Opin Genet Dev. 2000. 10(4):357-62.

26. Harland RM. 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol 36:685-695.

27. Hemmati-Brivanlou AH, Harland RM. 1989. Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early Xenopus embryos. Development 106:611-617.

28. Henriksen A, King TP, Mirza O, Monsalve RI, Meno K, Ipsen H, Larsen JN, Gajhede M, Spangfort MD. 2001. Major venom allergen of yellow jackets, Ves v 5: structural characterization of a pathogenesis-related protein superfamily. Proteins 45:438-448.

29. Hoshino J, Aruga J, Ishiguro A, Mikoshiba K. 2003. Dorz1, a novel gene expressed in differentiating cerebellar granule neurons, is down-regulated in Zic1-deficient mouse. Brain Res Mol Brain Res 120:57-64.

30. Imai KS, Satou Y, Satoh N. Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in Ciona savignyi embryos. Development. 2002 Jun;129(11):2723-32.

31. Kazanskaya OV, Severtzova EA, Barth KA, Ermakova GV, Lukyanov SA, Benyumov AO, Pannese M, Boncinelli E, Wilson SW, Zaraisky AG. Anf: a novel class of vertebrate homeobox genes expressed at the anterior end of the main embryonic axis. Gene. 1997 Oct 24;200(1-2):25-34.

32. Kolm PJ, Sive HL. 1995. Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. Dev Biol 167:34-49.

33. Krieg PA, Melton DA. 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res 12:7057-7070.

34. Kuo JS, Patel M, Gamse J, Merzdorf C, Liu X, Apekin V, Sive H. 1998. Opl: a zinc finger protein that regulates neural determination and patterning in Xenopus. Development 125:2867-2882.

35. Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM. 1993. Neural induction by the secreted polypeptide noggin. Science 262:713-718.

36. Li S, Shin Y, Cho WYK Merzdorf CM. 2006. The Xfeb gene is a direct target of zic1 and is expressed in the hindbrain. Developmental Dynamics, in press.

37. Liu, W. M., Mei, R., Di, X., Ryder, T. B., Hubbell, E., Dee, S., Webster, T. A., Harrington, C. A., Ho, M. H., Baid, J., and Smeekens, S. P. (2002) Analysis of high density expression microarrays with signed-rank call algorithms. Bioinformatics, 18, 1593–9.

38. Mathers PH, Miller A, Doniach T, Dirksen ML, Jamrich M. Initiation of anterior head-specific gene expression in uncommitted ectoderm of Xenopus laevis by ammonium chloride. Dev Biol. 1995 Oct;171(2):641-54.

39. McGrew LL, Lai CJ, Moon RT. 1995. Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. Dev Biol 172:337-342.

40. Milne TJ, Abbenante G, Tyndall JD, Halliday J, Lewis RJ. 2003. Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. J Biol Chem 278:31105-31110.

41. Mizuseki K, Kishi M, Matsui M, Nakanishi S, Sasai Y. 1998. Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development 125:579-587.

42. Muñoz-Sanjuán I and Brivanlou AH. Neural induction, the default model and embryonic stem cells. Nature Review Neuroscience. 3:271-280, 2002.

43. Nagai T, Aruga J, Takada S, Gunther T, Sporle R, Schughart K, Mikoshiba K. 1997. The expression of the mouse Zic1, Zic2, and Zic3 gene suggests an essential role for Zic genes in body pattern formation. Dev Biol 182:299-313.

44. Nakata K, Nagai T, Aruga J, Mikoshiba K. 1998. Xenopus Zic family and its role in neural and neural crest development. Mech Dev 75:43-51.

45. Ogura H, Aruga J, Mikoshiba K. 2001. Behavioral abnormalities of Zic1 and Zic2 mutant mice: implications as models for human neurological disorders. Behav Genet 31:317-324.

46. Olson JH, Xiang X, Ziegert T, Kittelson A, Rawls A, Bieber AL, Chandler DE. 2001. Allurin, a 21-kDa sperm chemoattractant from Xenopus egg jelly, is related to mammalian sperm-binding proteins. Proc Natl Acad Sci U S A 98:11205-11210.

47. Pera EM, Wessely O, Li SY, De Robertis EM. Neural and head induction by insulin-like growth factor signals. Dev Cell. 2001 Nov;1(5):655-65.

48. Rhinn M, Brand M. 2001. The midbrain--hindbrain boundary organizer. Curr Opin Neurobiol 11:34-42.

49. Sadaghiani B, Thiebaud CH. 1987. Neural crest development in the Xenopus laevis embryo, studied by interspecific transplantation and scanning electron microscopy. Dev Biol 124:91-110.

50. Salero E, Perez-Sen R, Aruga J, Gimenez C, Zafra F. 2001. Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. J Biol Chem 276:1881-1888.

51. Sasai Y. Identifying the missing links: genes that connect neural induction and primary neurogenesis in vertebrate embryos. Neuron. 1998 Sep;21(3):455-8.

52. Sasai Y, De Robertis EM. Ectodermal patterning in vertebrate embryos. Dev Biol. 1997 Feb 1;182(1):5-20.

53. Sasai N, Mizuseki K, Sasai Y. 2001. Requirement of FoxD3-class signaling for neural crest determination in Xenopus. Development 128:2525-2536.

54. Sato T, Sasai N, Sasai Y. 2005. Neural crest determination by co-activation of Pax3 and Zic1 genes in Xenopus ectoderm. Development 132:2355-2363.

55. Schambony A, Hefele JA, Gentzel M, Wilm M, Wedlich D. 2003. A homologue of cysteine-rich secretory proteins induces premature degradation of vitelline envelopes and hatching of Xenopus laevis embryos. Mech Dev 120:937-948.

56. Serrano RL, Kuhn A, Hendricks A, Helms JB, Sinning I, Groves MR. 2004. Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism. J Mol Biol 339:173-183.

57. Shimeld SM, Holland PW. Vertebrate innovations. Proc Natl Acad Sci U S A. 2000 Apr 25;97(9):4449-52.

58. Shin Y, Kitayama A, Koide T, Peiffer DA, Mochii M, Liao A, Ueno N, Cho KW. 2005. Identification of neural genes using Xenopus DNA microarrays. Dev Dyn 232:432-444.

59. Shoji H, Nishi N, Hirashima M, Nakamura T. Characterization of the Xenopus galectin family. Three structurally different types as in mammals and regulated expression during embryogenesis. J Biol Chem. 2003 Apr 4;278(14):12285-93.

60. Smith WC, Harland RM. 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell 70:829-840.

61. Stern CD. Induction and initial patterning of the nervous system - the chick embryo enters the scene. Curr Opin Genet Dev. 2002 Aug;12(4):447-51.

62. Stutz F, Spohr G. 1986. Isolation and characterization of sarcomeric actin genes expressed in Xenopus laevis embryos. J Mol Biol 187:349-361.

63. Sun Rhodes LS, Merzdorf CS. The zic1 gene is expressed in chick somites but not in migratory neural crest. Gene Expr Patterns. 6(5): 539-545.

64. Tran PH, Peiffer DA, Shin Y, Meek LM, Brody JP, Cho KW. 2002. Microarray optimizations: increasing spot accuracy and automated identification of true microarray signals. Nucleic Acids Res 30:e54.

65. Wada S, Saiga H. HrzicN, a new Zic family gene of ascidians, plays essential roles in the neural tube and notochord development. Development. 2002 Dec;129(24):5597-608.

66. Yamada L, Kobayashi K, Degnan B, Satoh N, Satou Y. A genomewide survey of developmentally relevant genes in Ciona intestinalis. IV. Genes for HMG transcriptional regulators, bZip and GATA/Gli/Zic/Snail. Dev Genes Evol. 2003 Jun;213(5-6):245-53.

67. Yokota N, Aruga J, Takai S, Yamada K, Hamazaki M, Iwase T, Sugimura H, Mikoshiba K. 1996. Predominant expression of human zic in cerebellar granule cell lineage and medulloblastoma. Cancer Res 56:377-383.

68. Zaraisky AG, Ecochard V, Kazanskaya OV, Lukyanov SA, Fesenko IV, Duprat AM. The homeobox-containing gene XANF-1 may control development of the Spemann organizer. Development. 1995 Nov;121(11):3839-47.

69. Zaraisky AG, Lukyanov SA, Vasiliev OL, Smirnov YV, Belyavsky AV, Kazanskaya OV. A novel homeobox gene expressed in the anterior neural plate of the Xenopus embryo. Dev Biol. 1992 Aug;152(2):373-82.

70. Zhang C, Basta T, Klymkowsky MW. SOX7 and SOX18 are essential for cardiogenesis in Xenopus. Dev Dyn. 2005 Dec;234(4):878-91.