THE *XDBX* GENE IS A DIRECT TARGET OF ZIC1 IN EARLY NEURULA STAGE

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In both vertebrates and invertebrates homeobox genes encode transcriptional regulators that act to specify spatial domains within the developing embryo. In the early vertebrate embryo, the development of the nervous system depends upon striking a balance between differentiating neurons and neural progenitors (Gershon *et al.,* 2000). The eventual fate of these progenitors is in part linked to the timing of their differentiation as neuronal subtypes during development. The temporal regulation of neuronal differentiation and the maintenance of a pool of neural progenitors, therefore, insure that the diversity of neuronal cell fates is generated during nervous system development (Lu *et al.,* 1992).

More than 20 mammalian Antennapedia class I homeobox genes are expressed in the developing central nervous system (CNS). Their expression patterns are spatially restricted along both the anteroposterior and dorsoventral axis. The spatial restrictions within the dorsoventral axis of the CNS are limited and often extend over many cell layers. These genes generally have different anterior boundaries of expression that are never anterior to the hindbrain (Lu *et al.,* 1992).

The establishing of correct dorsal-ventral patterning in spinal cord development is a key process in producing a functional spinal cord. Discrete domains of spinal progenitors express a code of transcription factors, and then produce moto-, inter-, and sensory-neurons. Studies in a variety of vertebrate species have described the extrinsic and intrinsic cues necessary for establishing the progenitor domains. For example, *dbx* genes encode a family of homeodomain transcription factors that define an intermediate spinal progenitor domain (Lu *et al.,* 1992). This gene family has multiple functions in spinal cord development. In *Xenops laevis, Xdbx* inhibits neurogenesis by regulating *Xash3* expression at neural plate stages (Gershon *et al.,* 2000), whereas in mouse and chick, studies have shown that *dbx1-2* are necessary in spinal cord development for the production of V0-V1 interneurons (Pierani *et al.,* 2001), radial glia, astrocytes, and oligodendrocytes (Fogarty *et al.,* 2005). The *Xenopus* embryo provides a useful system for studying the factors that maintain this balance as undifferentiated progenitors and differentiating neurons are maintained in a stereotypic, regionalized pattern within the neural plate (Hartenstein, 1993).

zic genes encode C2H2 zinc finger transcription factors, homologous to odd-paired in *Drosophila*. At least five *zic* genes have been found in human, mouse and frog. A few *zic* orthologs have been identified in protochordates (Aruga, 2004). *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).

The *zic1* gene plays a role in the development of the midbrain-hindbrain boundary (MHB) (Merzdorf, 2007). *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). 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 (Kuo *et al.,* 1998; Aruga *et al.,* 2002; Sato *et al.,* 2005).

The objective of this study was to achieve more understanding for the molecular role of both *zic1* and *Xdbx* genes in neural development, it is of a great interest to identify whether *Xdbx* is a direct target of *zic1* or not. Previously, we used *Xenopus laevis* Affymetrix microarrays (Cornish *et al.,* 2009) to find the direct targets of *zic1*. In this study we describe the results from comparable experiments using reverse transcriptase PCR (RT-PCR), real-time PCR instead and *in situ* hybridization in whole embryos to analyze the expression of *Xdbx* gene.

MATERIALS AND METHODS

In vitro transcription

RNA was synthesized from *zic1GR* and *dnzic1* constructs in pCS2 plasmid as previously described (Kuo *et al.,* 1998; Merzdorf and Sive, 2006). *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 (dex) (Kuo *et al.*, 1998). The dominant interfering *zic1* construct (*dnzic1*) comprises the zinc finger domain and the C-terminus of *zic1* (Merzdorf and Sive, 2006).

Microinjection and RNA isolation

Xenopus laevis embryos were injected at the 2-cell stage into both blastomeres with a total of 200 pg *zic1GR* and 1 pg noggin to neuralize the animal caps (ectodermal explants) or with 200 pg *β-globin* and 1 pg *noggin* for controls (Li *et al.,* 2006). Animal caps were dissected at stage 9 and cultured. When the caps reached the equivalent of stage 12, they were treated with 10 μg/ml cycloheximide (C-7698 Sigma, St Louis, MO) to prevent further protein synthesis and 30 min later with 10 μM dex (D-4902 Sigma St Louis, MO) to activate pre-formed *zic1GR*. After a further 3 h in culture, the animal caps

were harvested and total RNA was isolated according to Li *et al.* (2006). RNA concentration was determined by measuring absorbances at 260 nm and 280 nm on a GeneQuant II spectrophotometer and quality was checked using an Agilent bioanalyzer. Small samples of each batch of RNA were tested by RT-PCR with muscle actin primers to ensure that mesoderm was not present and minus RT (–RT) samples were used to confirm that genomic DNA had been removed.

RT-PCR and real-*time PCR analysis*

Injections, treatments, and animal cap isolations were carried out as previously described. 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 ensure the absense of genomic DNA. For 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 were 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 EF1-α expression levels from the same cDNA pool. EF1-α was used as loading control. The following primers were used: muscle *actin* (Stutz and Spohr, 1986); *EF1-α* (Gammill and Sive, 1997); *Xdbx*-forward: 5'-GAGCAGACACTACCCACCAAA-3', and *Xdbx*-reverse: 5'-GGAGTTAAAAGGGGAGGAAGG-3'.

Whole mount in situ hybridization

The *Xdbx* clone was linearized with *BglII* and transcribed with T7 to generate a 1.6 kb antisense probe for *Xdbx* (Gershon *et al.,* 2000). Whole mount *in situ* hybridization on albino embryos was carried out as described by Harland (1991). *Xdbx* expression was determined in uninjected embryos and in embryos that were injected in one cell at the 2-cell stage with 100 pg *dnzic1* RNA (Merzdorf and Sive, 2006) together with 25 pg *lacZ* RNA as tracer. β-galactosidase staining was performed as in Kolm and Sive (1995). This preceded *in situ* hybridization using a digoxygenin-labeled *Xdbx* antisense RNA probe, an antidigoxigen-AP antibody (#1093274, Roche Diagnostics, Mannheim, Germany) and the alkaline phosphatase substrate NBT/BCIP (B1911 Sigma, St.Louis, MO) for color detection. *Xdbx* antisense RNA probe was synthesized from a *BglII* digestion of the pGEM3 plasmid containing *Xdbx*, transcribed with T7 polymerase (Gershon *et al.,* 2000). *zic1* probe was obtained as in (Kuo *et al.,* 1998).

RESULTS AND DISCUSSION

Xdbx is directly upregulated by zic1

We were interested in determining if *Xdbx* expression required Zic1 tran-

scription factor for its induction. Animal caps were isolated from injected embryos at late blastula stage 9 and were treated with CHX to prevent further protein synthesis and dex to activate pre-formed *zic1GR* as described above (all control animal caps were also treated with CHX and dex). Animal caps were harvested after an additional 3 hr of culture and each sample was subjected to RT-PCR and quantitative real-time RT-PCR analysis.

In this study, we confirmed that *Xdbx* expression is upregulated by *zic1GR* in the presence of CHX. Using RT-PCR analysis, the averages of seven independent experiments demonstrated that animal caps taken from control embryos injected with *β-globin* plus *noggin* RNAs showed basal levels of *Xdbx* expression, while caps taken from *zic1GR* plus *noggin*-injected embryos showed a significant increase in *Xdbx* expression (Fig. 1). In real-time PCR analysis, the approximately 26.7-fold change of *Xdbx* expression level by *Zic1GR* in the presence of CHX was found. These results suggest that *Xdbx* is a direct target of the transcription factor Zic1, meaning that *zic1* is necessary for the induction of *Xdbx* expression.

Expression pattern of Xdbx at the neurula stage

The whole-mount *in situ* hybridization was used at stage 15 (neurula stage) to determine the expression patterns of *Xdbx* (Fig. 2A). Within anterior regions, *Xdbx* is expressed at the midline of the mediolateral axis of the neuroectoderm. Based on the fate map of the *Xenopus* neural plate, this region will later give rise to diencephalic derivatives (Eagleson and Harris, 1990). *Xdbx* is also expressed in longitudinal stripes that first define the middle of the mediolateral axis of the neural plate and subsequently define the midpoint of the dorsoventral axis of the neural tube (Fig. 1A). Expression of the *Xdbx* gene initiates at the neural plate stage of development and appears neural specific (Gershon *et al.,* 2000). At early stages, *Xdbx* expression is regionally restricted both within anterior and posterior domains of the embryo. This regional expression is shared with murine *dbx* and zebrafish *hlx1* (Fjose *et al.,* 1994; Lu *et al.,* 1992) as well as with more divergent members of this family, including murine *dbx2* and chicken *ChoxE* (Rangini *et al.,* 1991; Shoji *et al.,* 1996). The transient expression of *Xdbx* within ventricular zone progenitors in the frog precludes tracking the differentiated fate of the *Xdbx*-expressing progenitors. However, previous work in both mouse and chick suggests that *dbx1* and *dbx2* progenitors are bound for an interneuron fate (Matise *et al.,* 1999).

Zic1 is required for the induction of Xdbx gene expression

In order to examine whether Zic1 protein is required for *Xdbx* expression, we injected embryos with a dominant interfering construct of *zic1* where the N-terminal domain of *zic1* had been deleted (*dnzic1*; Merzdorf and Sive, 2006). At neurula stages, endogenous *Xdbx* expres-

sion is highly reduced on the injected side (Fig. 2B). Although *dnzic1* retains the *zic1*-specific C-terminal domain, it is likely that this construct interferes with the activities of other *zic* genes family members. In addition *Xdbx* expression overlaps with *zic1* expression (Fig. 2A and 2C). Thus, these data indicate that *Xdbx* expression is dependent on regulation by Zic1 protein and supports our finding that *Xdbx* is a direct target of Zic1 transcription factor in neurula stage embryos.

SUMMARY

Homeobox gens play an important role in the embryonic development of both vertebrates and invertebrates. They encode transcriptional factors that act to specify spatial domains within the developing embryo. *dbx* genes encode a family of homeodomain transcription factors that define an intermediate spinal progenitor domain. This gene family has multiple functions in spinal cord development. *zic* genes encode zinc finger transcription factors, homologous to odd-paired in *Drosophila*. The *zic1* gene plays a role in the development of the midbrain-hindbrain boundary (MHB). *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. In this study *Xenops laevis dbx* (*Xdbx)* proved to be a direct downstream target of Zic1 transcription factor using RT-PCR, Real-time PCR and *in situ* hybridization. *Xdbx* is expressed in a bilaterally symmetric stripe that lies at the middle of the mediolateral axis of the neural plate. This expression overlaps the expression domain of *zic1* gene, indicating that *Xdbx* is upregulated by Zic1 transcription factor.

REFERENCES

Aruga, J. (2004). The role of zic genes in neural development. Mol. Cell. Neurosci., 26: 205-221.

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

Cornish, E. J., S. M. Hassan, J. D. Martin, S. Li and C. S. Merzdorf (2009). A microbarry screen for direct targets of Zic1 identifies an aquaporin gene, aqp-3b, expressed in the neural fold. Dev. Dyn., 238: 1179-1194.

Eagleson, G. W. and W. A. Harris (1990). Mapping of the presumptive brain regions in the neural plate of *Xenopus laevis*. J. Neurobiol., 21: 427-440.

Fjose, A., J. C. Izpisua-Belmonte, C. Fromental-Ramain and D. Duboule (1994). Expression of the zebrafish gene hlx-1 in the prechordal plate and during CNS development. Development, 120: 71-81.

Fogarty, M., W. D. Richardson, N. Kessaris (2005). A subset of oligoden-

drocytes generated from radial glia in the dorsal spinal cord. Development, 132: 1951-1959.

Gershon, A. A., J. Rudnick, L. Kalam and K. Zimmerman (2000).The homeodomain-containing gene Xdbx inhibits neuronal differentiation in the developing embryo. Development, 127: 2945-2954.

Harland, R. M. (1991). *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol., 36: 685-695.

Hartenstein, V. (1993). Early pattern of neuronal differentiation in the Xenopus embryonic brainstem and spinal cord. J. Comp. Neurol., 328: 213-231.

Kolm, P. J. and H. L. Sive (1995). Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. Dev. Biol., 167: 34-49.

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

Li, S., Y. Shin, K. W.Cho, and C. S. Merzdorf (2006). The Xfeb Gene is directly upregulated by Zic1 during Early neural development. Dev. Dyn., 235: 2817-2827.

Lu, S., L. D. Bogarad, M. T. Murtha and F. H. Ruddle (1992). Expression pattern of a murine homeobox gene, Dbx, displays extreme spatial restriction in embryonic forebrain and spinal cord. Proc. Natl Acad. Sci. USA, 89: 8053-8057.

Matise, M., M. Lustig, T. Sakurai, M. Grumet, and A. Joyner (1999). Ventral midline cells are required for the local control of commissural axon guidance in the mouse spinal cord. Development, 126: 3649-3659.

Merzdorf, C. S. and H. L. Sive (2006). The zic1 gene is an activator of Wnt signaling. Int J. Dev. Biol., 50: 611-617.

Merzdorf, C. S. (2007). Emerging Roles for zic genes in early development. Dev. Dyn., 236: 922-940.

Pierani, A., L. Moran-Rivard, M. J. Sunshine, D. R. Littman, M. Goulding, T. M. Jessell (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron, 29: 367-384.

Rangini, Z., A. Ben-Yehuda, E. Shapira, Y. Gruenbaum and A. Fainsod (1991). CHox E, a chicken homeogene of the H2.O type exhibits dorsoventral restriction in the proliferating region of the spinal cord. Mech. Dev., 35: 13-24.

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

Shoji, H., T. Ito, Y. Wakamatsu, N. Hayasaka, , K. Ohsaki, M. Oyanagi, R. Kominami, H. Kondoh, and N. Takahashi (1996). Regionalized expression of the Dbx family homeobox genes in the embryonic CNS of the mouse. Mech. Dev., 56: 25-39.

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

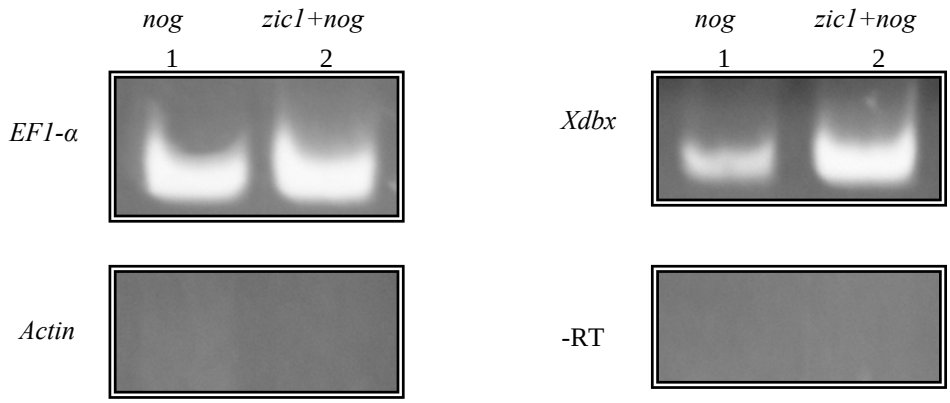
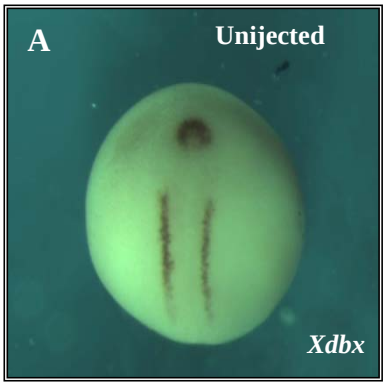
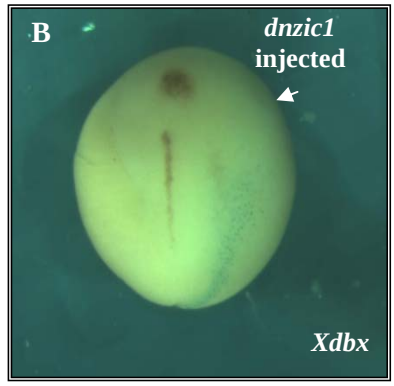


Fig. (1): RT-PCR analysis show that *Xdbx* is induced by full-length *zic1*. Basal level of *Xdbx* expression was induced by *noggin* alone as a control (lane 1), but it is strongly induced by full-length *zic1* plus *noggin* (lane 2). No *actin* expression or genomic DNA (-RT control) was found.





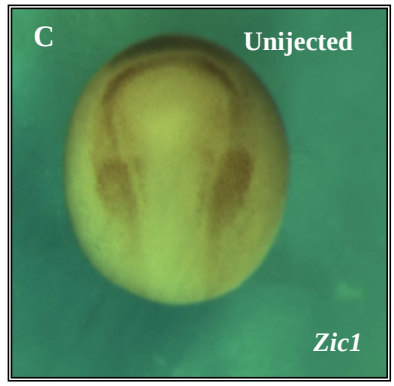


Fig. (2): *Xdbx* and *zic1 in situ* hybridizations at stage 15 (neurula stage). (A): Dorsal view for uninjected embryo, *Xdbx* is expressed at the midline of the mediolateral axis of the neuroectoderm, *Xdbx* is also expressed in longitudinal stripes that first define the middle of the mediolateral axis of the neural plate, (B): Dorsal view for *dnzic1*-injected embryo, endogenous *Xdbx* expression is highly reduced on the injected side (white arrow head) and (C): Dorsal view for *zic1 in situ* hybridization in uninjected embryo.