ABSTRACT

Transgenic Zebrafish That Carry Inducible *zic2a* Fusion Proteins

Zic genes encode proteins that contain zinc fingers used to bind DNA and modify transcription of target genes. Several *zic* genes have been identified that are critical in regulating nervous system development in a wide variety of organisms. We have chosen to study *zic2a* because its transcription activity has not been well defined. The goal of this study was to begin determining the regulatory nature of *zic2a* in the zebrafish (*Danio rerio*) central nervous system. To do this, three constructs were assembled that, when induced, over expressed constitutively activating, constitutively repressing, or wild type *zic2a* function. These constructs were further modified and then used to develop three stable transgenic zebrafish lines, which allow conditional over-expression of *zic2a* in zebrafish embryos. These transgenic, *zic2a*-expressing zebrafish will be used in further studies to characterize the function of *zic2a* in the developing nervous system.

Daniel Matson/Genetics
Author Name/Major

Daniel R. Matson
Author Signature

5/15/2007
Date

Yevgeny Grinblat
Mentor Name/Department

Yevgeny Grinblat
Mentor Signature

COVER SHEET

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AUTHOR'S NAME:_Daniel R. Matson_____

MAJOR: _Genetics_____

DEPARTMENT: _Genetics_____

MENTOR: _Dr. Yevgenya Grinblat_____

DEPARTMENT:_Zoology_____

MENTOR(2): _____

DEPARTMENT(2):_____

YEAR: _2007_____

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Transgenic Zebrafish That Carry Inducible *zic2a* Fusion Proteins

Daniel R. Matson

Introduction

Starting at conception, the growth of an animal from a single cell to its mature form is largely controlled by the animal's genes. This is particularly amazing when one considers the extremely complex structures, such as the vertebrate brain, which must be routinely assembled during the development of an organism. One particular family of genes, known as the *zic* family, is essential for early mammalian neural development. *zic* genes encode proteins which contain five highly conserved C_2H_2 class zinc finger domains. These zinc finger domains allow *ZIC* proteins to bind DNA and act as transcription factors to regulate the activity of other genes. *zic* genes are found in numerous animals, including mouse (*Mus musculus*), fruit fly (*Drosophila melanogaster*), and zebrafish (*Danio rerio)* (Grinblat and Sive, 2001, Toyama *et al*., 2004). Additionally, *zic* homologues have been identified in cephalochordates, urochordates, and nematodes (Aruga, 2004). The presence of *zic* genes in a wide variety of animals suggests that their roles in neural development may be evolutionarily conserved.

There are currently seven known *zic* genes in zebrafish and relatively little is known about how they function as trans factors to modulate gene transcription. *zic2a* is one family member that is interesting, because it is known to be critical to the process of nervous system formation. Knocking down zebrafish *zic2a* is sufficient to inhibit neural proliferation (Nyholm et al., 2007), while overexpression of zic2a promotes neural development (Dodou, 2004). A detailed understanding of where *zic2a* is expressed in the

nervous system, its regulation by endogenous activators or repressors, and its transcriptional targets is required to clarify the mechanisms by which it functions.

This study will provide new insight into genetically based developmental disorders, especially those related to central nervous system development. Mutations in *ZIC* genes have been linked to several developmental defects in humans. Notably, specific mutations in the human *ZIC2* gene are associated with holoprosencephaly, a birth defect characterized by defects in face and brain structure (Grinberg and Millen, 2005, Brown *et al*., 1998, Thomas *et al*., 2003). In a broader sense, this project will lead to improved knowledge of developmental biology and the various processes by which genes and their encoded proteins bring about the formation of complex neural structures.

Traditionally, one of the most popular methods to study the role of a gene has been to modify the gene and measure what effects this altered gene has on the organism. With this in mind, our original approach involved the construction of three different vectors, each containing a different heat-shock inducible *zic2a* fusion (Figure 1). The three *zic2a* fusions represented wild-type *zic2a*, *zic2a* fused to a universal activator, and *zic2a* fused to a universal repressor. These constructs were micro-injected into one-cell-stage zebrafish embryos and expression of the *zic2a* fusions was activated by heat-shock during the first 24 hours of development.

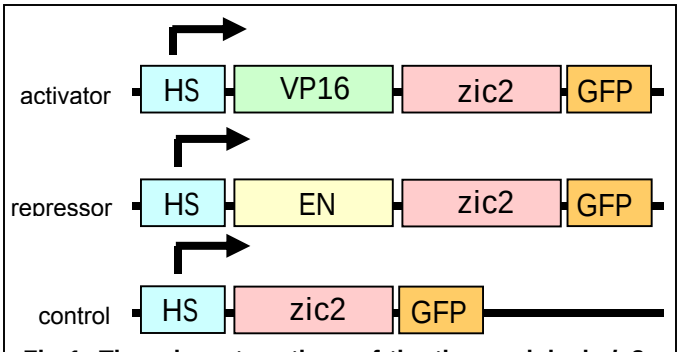

Fig 1: The relevant portions of the three original *zic2a* fusion constructs. HS is the heat shock promoter, which will activate transcription of the following sequence at 37°C.

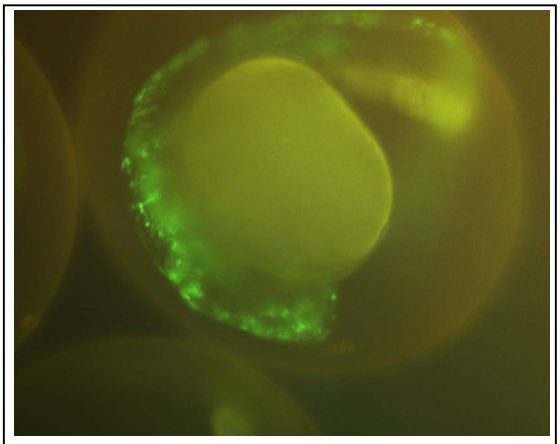

Figure 2: *zic2a* fusion injected embryos with mosaic post-heat-shock GFP expression.

Although neural development of some of the injected embryos 12 hours after heat-shock varied significantly from controls, expression of the *zic2a* fusions (visualized by a co-translated eGFP marker) was mosaic throughout the embryo and the developing central nervous system (Figure 2). This mosaic expression was a result of diffusion of the injected construct during the numerous cell divisions that occur during development. Despite preliminary evidence which provided some insight into the general activating or repressing properties of *Zic2a*, it was impossible to draw meaningful conclusions from this method, primarily because comparisons between the three *zic2a* fusions could not be made since each embryo expressed each vector in different cells.

In order to conduct a meaningful study of *Zic2a* function, generation of zebrafish with ubiquitous *zic2a* fusion expression was necessary. To this end, this study aimed to develop three stable transgenic zebrafish lines, which would allow conditional, ubiquitous over-expression of three *zic2a* fusions in embryos. Moreoever, unlike the previously assembled fusions, a *Gal4/UAS* system would be utilized to induce transcription of each *zic2a* fusion. This decision followed an earlier experiment, in which direct HS-*zic* fusions were injected with the goal of raising stable transgenics, but no transgenic fish were recovered. This suggested that the direct fusions were somewhat

toxic, due to low-level "leaky" transcription from the HS promoter. To circumvent this toxicity, the indirect Gal4/UAS system was invoked in this study.

Methods

Three DNA constructs (Figure 3) were created using the same basic procedure and injected into one-cell-stage zebrafish embryos. Each construct included a *zic2a* fusion portion, capable of either constitutively activating, constitutively repressing, or carrying out wild type *zic2a* transcriptional activity.

Synthesis of these constructs began with PCR amplification of three different inserts from the three original *zic2a* fusion constructs, all previously constructed by the Grinblat Laboratory (Figure 1). Custom primers were used, which added an XbaI restriction site to the 5' end of the amplified inserts and allowed for conservation of a pre-existing AgeI site near the 3' terminus. Digestion of the XbaI and AgeI sites enabled ligation of each insert into the vector.

A member of the Bleeding Heart family of vectors, pBH-UAS-mcs-YFP (Figure 3 and

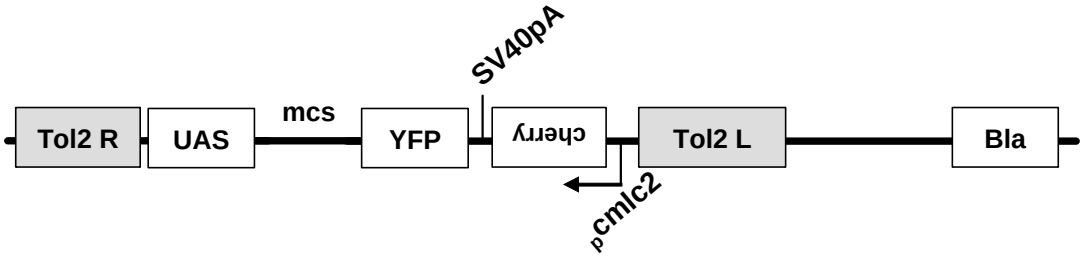

Fig 3: The relevant portions of the bleeding heart vector. Tol2 R and Tol2 L are tol2 transposon sites. UAS is a Gal4-driven enhancer element. mcs represents a multiple cloning region. YFP is a reporter of transcriptional activity. Cherry is a reporter of integration and is driven by a heart specific $_{p}$cmlc2 promoter. ßla is a ß-lactamase resistance gene.

courtesy Nonet laboratory) was utilized in the assembly of all three constructs. The pBH-UAS-mcs-YFP vector contains a number of pertinent regions: 1. A UAS enhancer element, which induces expression of the *zic2a* fusion region when bound by Gal4 protein; 2. A yellow fluorescent protein (YFP) sequence is located in frame downstream of the *zic2a* fusion and functions as a reporter of *zic2a* fusion translation. Downstream of this YFP region is a SV40 polyA region, containing multiple transcription stop sites. Following this SV40 polyA is *cherry*, a reporter which codes for a red-colored protein. Transcription of *cherry* is driven by a $_{p}$*cmcl2* cardiac-specific promoter. Both *cherry* and the $_{p}$*cmcl2* cardiac-specific promoter are in the opposite orientation of the region UAS-containing region. Flanking this entire region are two *tol2* transposon sites. A β-lacatamase resistance gene is also present on each construct outside of the transposable region which facilitates selection during cloning.

A double-digest of restriction sites NheI and AgeI, both present within the multiple

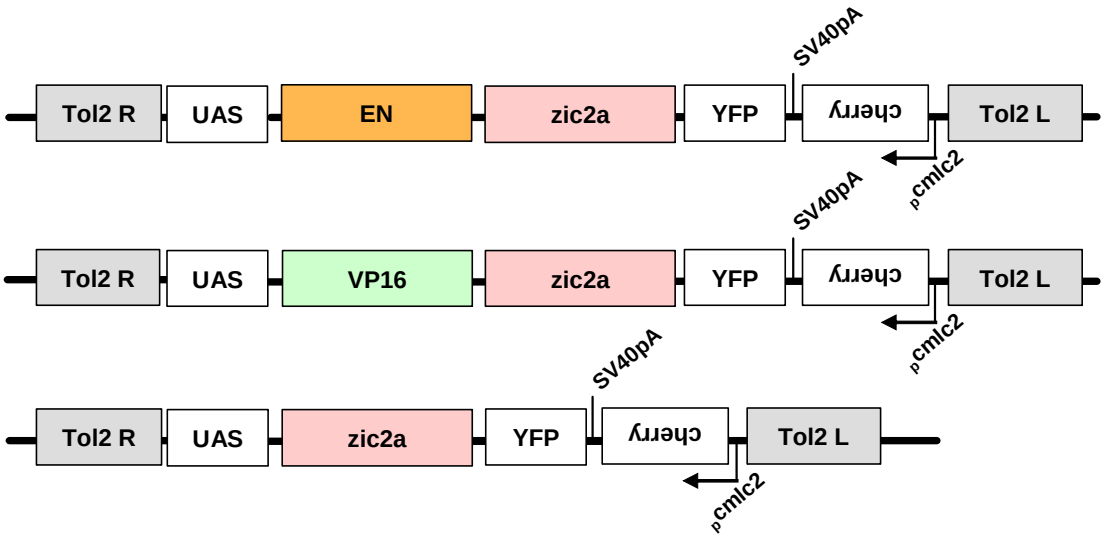

Fig 4: The relevant portions of the three proposed constructs. Tol2 R and Tol2 L are tol2 transposon sites. UAS is a Gal4-driven enhancer element. YFP is a reporter of transcriptional activity. Cherry is a reporter of integration and is driven by a heart specific $_{p}$cmlc2 promoter. βla is a β-lactamase resistance gene. **On the three inserts**: the zic2 gene is the gene under investigation. The VP16 sequence is a universal activator. Engrailed is a universal repressor.

cloning site, allowed for ligation of each insert into the vector and formation of the final three constructs (Figure 4). Digested XbaI and NheI sites create compatible overhanging ends. All ligations were verified by sequencing and restriction digests.

In the near future, each of the three completed constructs will be injected into one-cell-stage zebrafish embryos along with tol2 transposase mRNA. Screening the putative transgenics for the presence of a red-colored heart, brought about by cardiac-specific transcription of cherry, will allow us to identify those zebrafish embryos in which integration was successful.

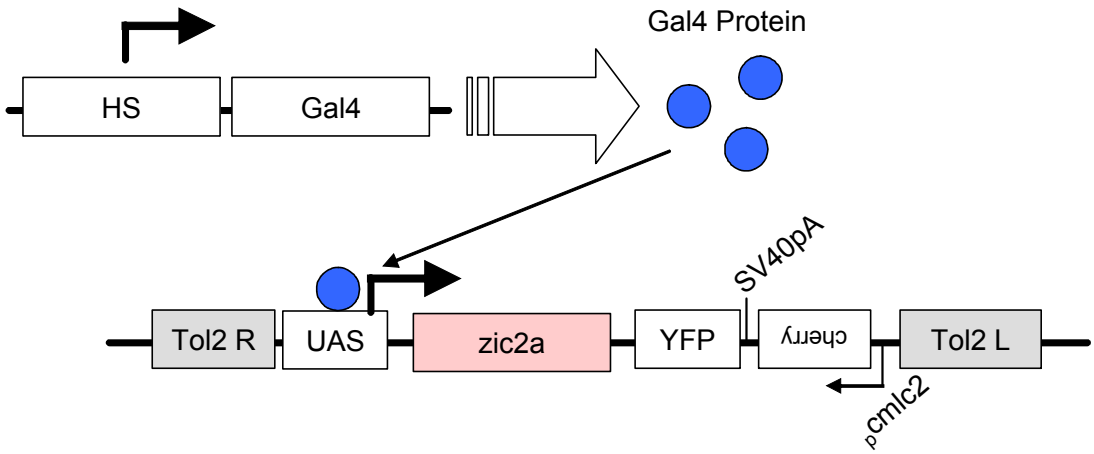

Fig 5: Simplified diagram detailing regulation of the *zic2a* fusion in transgenic zebrafish containing the inducible HS-Gal4 fusion. Heat-shock induced Gal4 expression produces Gal4 protein, which bind to the UAS site and induce expression of the *zic2a*-containing region.

These transgenic zebrafish will be raised to sexual maturity and crossed with a pre-existing transgenic zebrafish line containing *Gal4* under control of the HS promoter. In this line, temperature-induced activation of the HS promoter drives expression of *Gal4*. The progeny of this cross will contain fish with both the HS-*Gal4* fusion and the UAS-*zic2a*-fusion-YFP region (Figure 5). In this final transgenic zebrafish line, heat-shock

will drive expression of *Gal4*, creating *GAL4* protein. *GAL4* will bind to the UAS site and induce expression of the downstream *zic2a* fusions, along with YFP.

Discussion

Transgenic zebrafish carrying inducible *zic2a* fusion constructs will serve as an excellent model for use in a variety of future studies to characterize the function of *zic2a* in the developing nervous system. The growth of the central nervous system, the formation of neurons, and transcription of key genes will be analyzed following induction. Data will be compared between the three different transgenic lines, as well as to control, wild-type zebrafish. Specifically, data gathered from the activity of the universally activating and universally repressing *zic2a* fusions will be compared to that of the *zic2a* alone fusion. For instance, using these fish lines, an experiment could be proposed to study *dlx2*, a well-conserved gene known to be involved in zebrafish neurogenesis, which could be either a direct or indirect target of *Zic2a*. In this approach, the expression of *dlx2* in the developing zebrafish forebrain would be monitored following the expression of each fusion. If both the VP16-*zic2a* fusion and the *zic2a* alone fusion produced an expansion of *dlx2* expression, but the EN-*zic2a* fusion produced a reduction in *dlx2*, it would serve as evidence that *zic2a* naturally activates *dlx2* expression during that particular developmental stage. Comparisons such as these will allow us to determine if *Zic2a* functions as an activator or a repressor for a particular phenotype.

After the general activating or repressing characteristics of *zic2a* are determined, further studies will aim to determine novel genes that are targeted by *Zic2a* and how their

transcription is modulated.

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