COVER SHEET

TITLE: Going from embryo to adult: How zebrafish *zic* genes are regulated during development

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ABSTRACT

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This project aims to further our understanding of developmental genetics by identifying unknown transcription factor binding sites that play an important role in regulating *zic2a,* a gene required for proper neural development in vertebrates. To do this, transgenic zebrafish were created that have various versions of the *zic2a* gene coupled with green fluorescent protein coding sequence. The transgenes differ by a single mutation in one of the several evolutionarily conserved sequences in the enhancer region, which are thought to be transcription factor binding sites. By comparing the expression patterns of the green fluorescent protein among the transgenic zebrafish I identify sites that are required for *zic2a* transcription during development.

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Going from embryo to adult: How zebrafish *zic* genes are regulated during development

Introduction to Current Topic

An extremely interesting question in biology today is: what is it that makes organisms structured the way that they are? If everything starts out as one single cell, how do organisms end up with such an elaborate and extremely well defined body plan? Much work has been done with fruit flies with a focus on this question, and as a result we now know that very early in development intricate expression patterns of transcription factors (TFs) result in the activation and inhibition of genes important for development (See Figure 1). These activated genes are themselves TFs and can in turn activate or inhibit other genes, which will eventually lead to more boundaries or simply an increase in boundary definition. These cascades of transcription lay a blueprint for many aspects of the adult form.

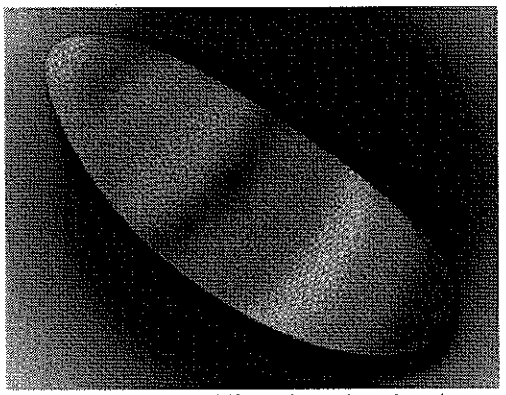

Figure 1. A Drosophila embryo is colored to show the expression of early gene regulators (Berkeley Lab, 2008).

The question that I examine is: what genes at what time in which areas of a zebrafish regulate the TF gene *zic2a*? The primary goal of this research project is to further our understanding of developmental genetics by identifying unknown TF binding sites. I have chosen to study *zic2a* because its function has been shown to be important

for proper neural development in vertebrates, its regulation has yet to be fully understood and it is the primary focus of the Grinblat lab where I am currently working.

Zic genes are the vertebrate homolog of the drosophila odd-paired gene. They have been shown to promote cell proliferation and also have critical roles in neural tissues, neural crest formation, left-right axis patterning, somite development, cerebellum formation, and muscle and skeletal development (Merzdorf, 2007). *Zic* gene products have zinc finger domains similar to those of Gli proteins which are known transcriptional mediators of the important developmental pathway, hedgehog signaling (Aruga, 2004). In many cases the malfunction of a *zic* gene results in severe abnormalities during development. For example, the deletion of *zic1* in mice results in cerebral and axial skeletal malformations (Aruga, 2004), and *zic2* knock-down mice acquire holoprosencephaly, a birth defect that is characterized by forebrain abnormalities (Nagai et al., 2000).

Many new zic related discoveries have recently come out of the Grinblat lab, some of which I have had the privilege of working on. In one project that I helped with *zic2a* was shown to have a patterning role in the prethalamus and function independently of Hh signaling the diencephalon (Sanek and Grinblat, 2008). It was also determined that *zic2a* predicts areas of dorsolateral hinge-point bending during cranial neural tube formation, and that *zic* genes act downstream of Wnt signaling to control cytoskeletal organization during neurulation (Nyholm et al., 2009). Finally, it was shown that *zic2a* patterns the forebrain through modulation of hedgehog-activated gene expression (Sanek et al., 2009).

There are a number of reasons zebrafish is used as a model organism for vertebrate development. Of these, two important reasons are that zebrafish have externally fertilized translucent embryos, as well as a rapid life cycle. Together, these aspects greatly facilitate the observation of development.

Experimental Methods

Determining Possible Transcription Factor Binding Sites

To figure out which TFs are controlling *zic2a* expression the enhancer region of the *zic2a* gene was compared to other organism's *zic2a* enhancer region to look for evolutionarily conserved sites. This step was preformed by a post-doctoral researcher in the Grinblat lab, Aaron Taylor. An enhancer region is a section of DNA that does not code for a protein product, but instead contains sequences called *cis*-regulatory elements that TFs will recognize and bind to. When a TF binds to a cis-regulatory element it either encourages or discourages the binding of transcriptional machinery to the promoter region of the gene, leading to either gene expression or repression. In zebrafish one important zic2a enhancer has already been sequenced, so it was possible to compare it to other organism's *zic2a* enhancer regions. By searching for evolutionarily conserved sequences in this region, it was possible to identify areas that are likely important *cis*-regulatory elements.

Creating a Vector for the Enhancer/Promoter Region

The promoter together with the enhancer region of the *zic2a* gene was given to me by Aaron Taylor. These sequences were originally acquired by first isolating wild type zebrafish DNA and then running a PCR reaction using primers that only bind to the edges of this region of DNA. I added restriction enzyme sites to the edges of this enhancer-

promoter region, and inserted this isolated piece of zebrafish DNA into an *E. coli* plasmid. Additional plasmids were also made that contain a mutation in one suspected TF binding site. This was done using site-directed mutagenesis (Zaret et al. 1990). A computer algorithm has identified families of TFs that could possibly bind to the *cis*-regulatory elements that I have mutated (See Figure 2).

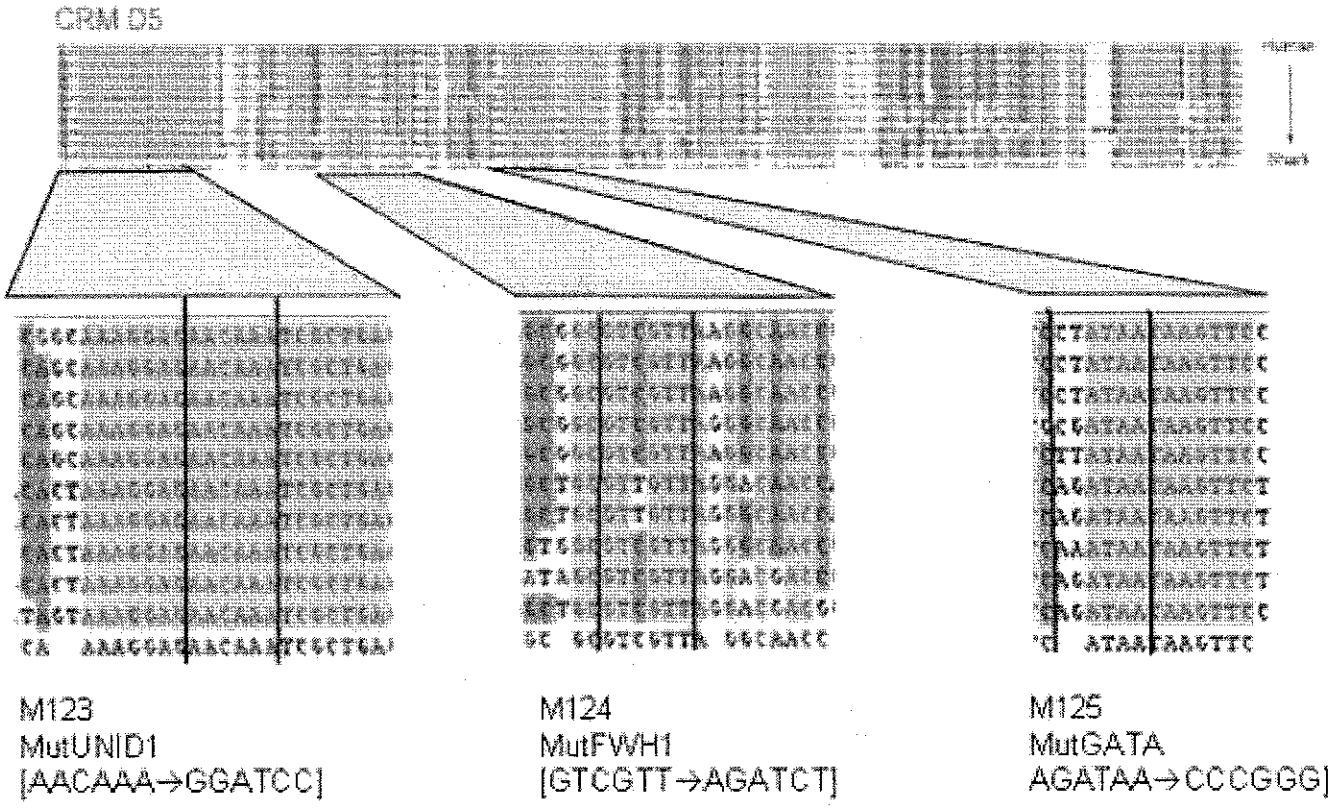

Figure 2. At the top is shown a sequence alignment of the incomplete version of the *zic2a* enhancer that was used. Expanded and outlined are the three suspected cis-regulatory elements that I mutated. At the bottom of the figure the call number, suspected site identity and nucleotides changed are listed.

Finally, the reporter gene, green fluorescent protein (GFP), was inserted into the plasmid immediately following the zebrafish DNA (See Figure 3). To confirm that the constructs were properly formed they were sequenced using a dideoxy nucleotide sequencing reaction. I was then able to use the bacteria to replicate either normal zebrafish DNA or DNA that is normal except for one mutated *cis*-regulatory element.

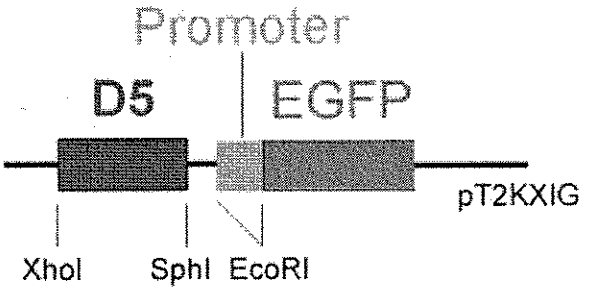

Figure 3. The construct that was created is shown. The D5 enhancer is either normal or contains one mutation in a suspected cis-regulatory element.

Injecting the Constructs

I injected the isolated constructs along with transposase mRNA into zebrafish embryos at the one-cell stage. The transgene is flanked by Tol2 transposon sites. These sites are targets for Tol2 transposase which is capable of splicing the transgene out of the injected plasmid and inserting it randomly into the zebrafish genome. When the embryos are injected at this stage the transposase mRNA gets translated into protein and facilitates integration of the transgene into the genome. The construct then gets incorporated into the germ line such that in the next generation it will be present in all cells of the embryo (Stuart et al., 1990). I screened the injected (F0) and next-generation (F1) developing embryos for successful DNA uptake by analyzing GFP expression using a fluorescent microscope (See Figure 4).

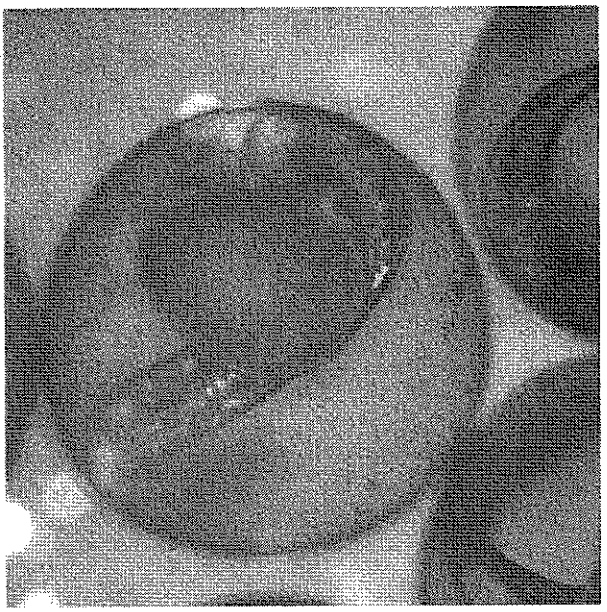

Figure 4. An embryo that was previously injected is shown. GFP is present throughout the embryo.

Results

Visualizing the Result of *Cis*-Regulatory Sequence Mutation

The injected F0 embryos were grown to adulthood and F1 transgenic carriers were identified among their progeny. This allowed the injected transgene to be stably incorporated into the germline. The fish take 3-6 months to mature, so I did not have time to analyze the transgenic fish that I created. Instead, I analyzed two different mutations in transgenic lines created in the Grinblat lab by Kyle Webert, another undergraduate, in collaboration with Aaron Taylor (See Figure 5). The GFP expression patterns for these

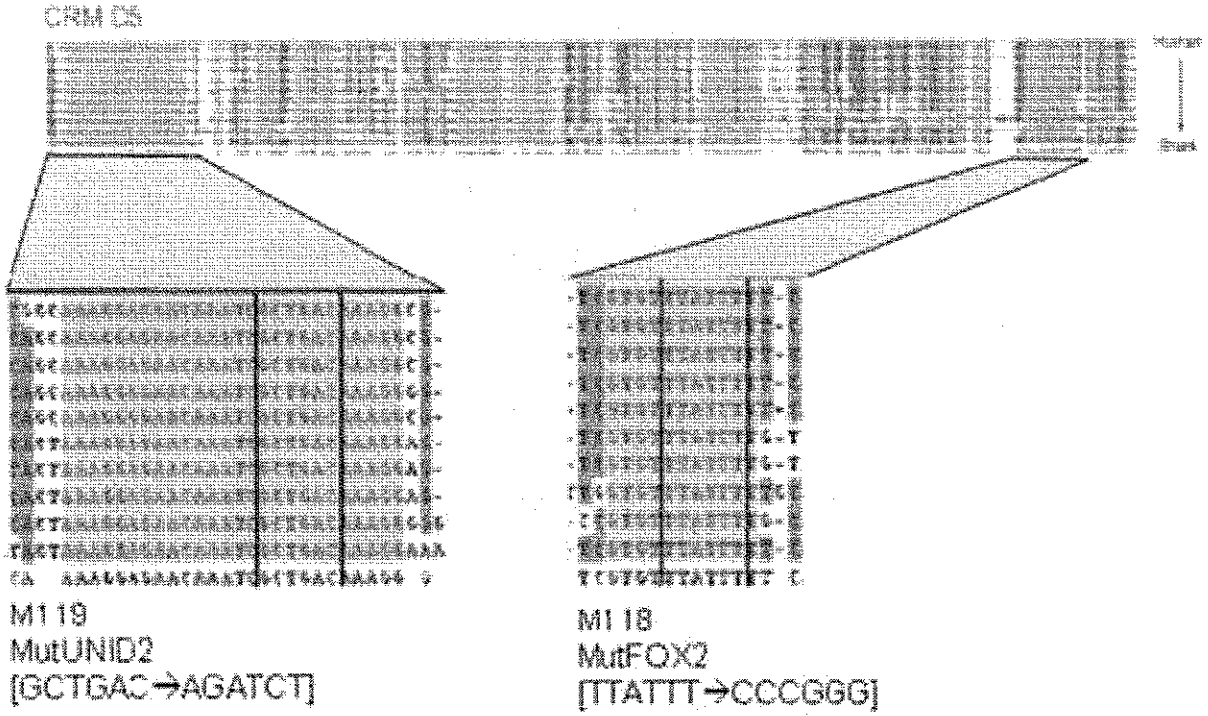

Figure 5. The suspected cis-regulatory elements that I analyzed are shown here. Call number, suspected site identity and the nucleotides changed are listed at the bottom of the figure.

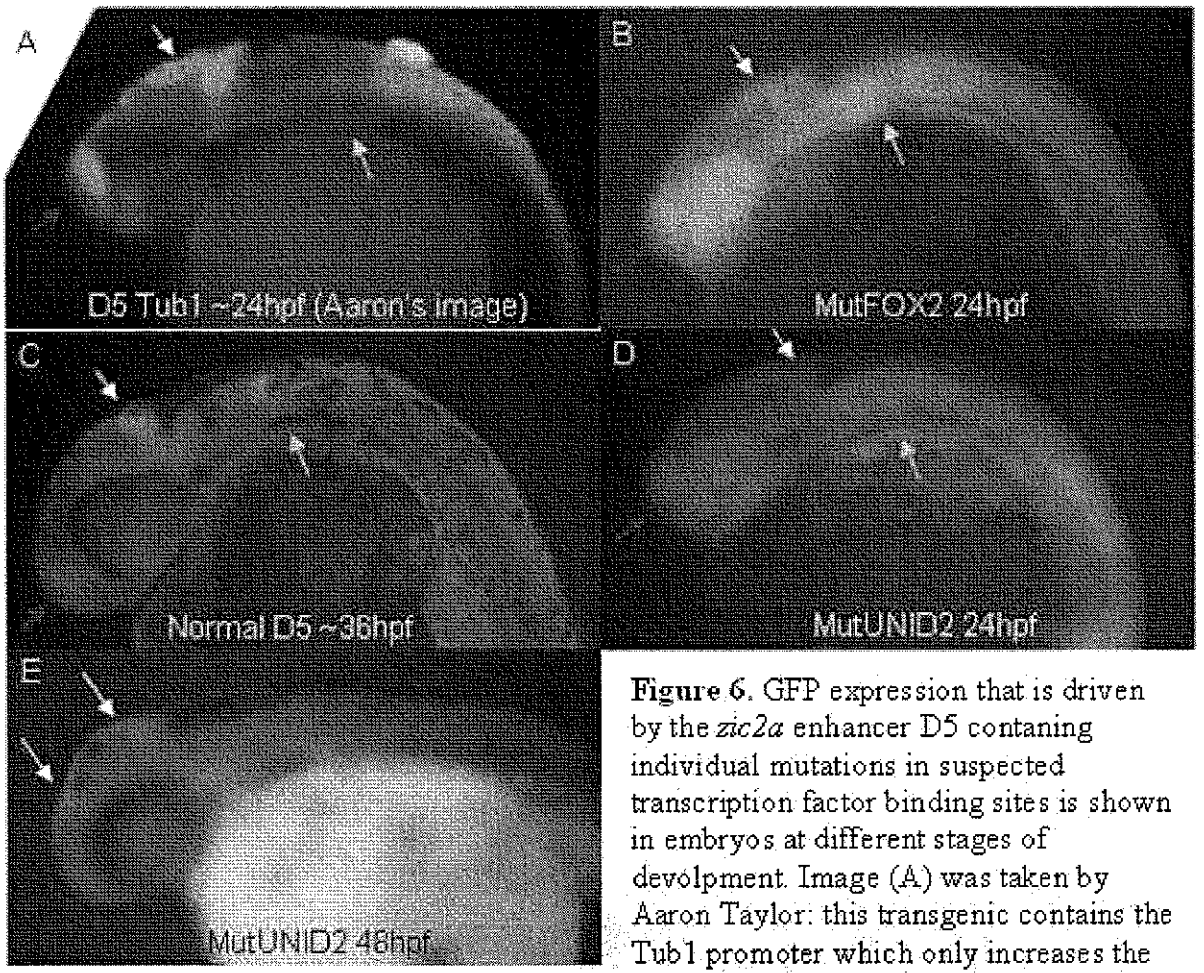

Figure 6. GFP expression that is driven by the *zic2a* enhancer D5 contaning individual mutations in suspected transcription factor binding sites is shown in embryos at different stages of devolpment. Image (A) was taken by Aaron Taylor: this transgenic contains the Tub1 promoter which only increases the level of GFP expression, not the area in which it is expressed. Images (A) and (C) both show embryos with an unmutated D5 enhancer. In the FOX2 mutant expression is lost in the dorsal posterior region (Image B). The UNID2 mutant shows complete loss of expression at 24hpf with returning expression at 48hpf (Images D and E).

embryos are shown in Figure 6.

With the expression data shown in figure 6 along with the sequence data from figure 5, it is possible to determine which potential TF binding sites are important for *zic2a* transcription. This can be done by comparing GFP expression driven by mutated *zic2a* enhancers to GFP expression driven by the wildtype (unchanged) enhancer. If the embryos injected with an enhancer containing a mutated cis-regulatory element show an expansion or reduction of GFP transcription relative to embryos that were injected with a normal enhancer, it can be concluded that a TF that normally binds to this site within the zic2a enhancer is required for normal expression of the zic2a gene

Conclusions

From looking at the images in figure 6 it can be determined that UNID2 is a binding site for a transcription activator that is under temporal control and is very important for the expression of zic2a. The FOX2 site also appears to be the binding site for a transcription activator. Without this TF binding site GFP expression is lost in the dorsal posterior region of the embryo. Therefore, that TF must exist in that region of the embryo, and it also must play a less important role in the anterior portion of the embryo.

Future Directions

The constructs that I created should be analyzed in more depth in the future. Furthermore, it would be useful to analyze the embryos at different stages of development. It is highly likely that there will be differences in the timing and spatial expression of the reporter gene during the development of the embryo. This information would be very useful in identifying the TF that is failing to bind to the mutated enhancer. To more clearly visualize the GFP transcript in situ hybridization should be used. An in situ hybridization will detect specific mRNA sequences transcribed from a gene of interest by using a complimentary RNA probe that can be detected by an antibody (See Figure 7).

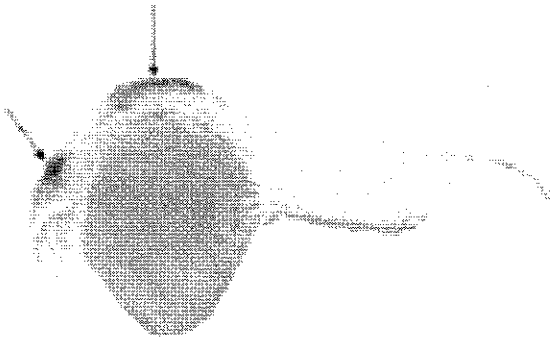

Figure 7. A zebrafish embryo has been injected with a construct similar to the constructs I created. The reporter GFP mRNA has been labeled by in situ hybridization (See arrows). (Taylor and Grinblat, unpublished)

By scanning the expression patterns of other known TF genes for an expression pattern that matches an area of expansion or repression of GFP transcription, strong inferences can be made regarding the identity of the TF that normally binds to the

predicted cis-regulatory elements analyzed here. This could be done to test the suggestions made by the computer algorithm about the identities of the TF binding sites.

It is also possible to show that a TF is physically binding to a *cis*-regulatory element using an in vitro mobility shift assay or an in vivo chromosomal immunoprecipitation assay (ChIP). A mobility shift assay measures the speed at which DNA can move through a gel in either the presence or absence of a probable DNA binding protein. If the protein is able to bind to the DNA its mobility in a gel will be reduced. This can easily be visualized using gel electrophoresis. A ChIP assay precipitates a DNA binding protein of interest and then analyzes the DNA that is bound to it. This is done by crosslinking the DNA with the DNA binding proteins in vivo, breaking apart the DNA, and then precipitating the protein of interest using a specific antibody. The DNA that is bound to the protein is then sequenced to determine if it corresponds to the predicted DNA binding region. If a ChIP assay is preformed on a transgenic zebrafish containing the construct with the normal *zic2a* enhancer, the results could be compared to a second ChIP assay on a zebrafish containing the mutated enhancer. If the second assay fails to isolate the mutated enhancer region, it is very likely that the given mutation actually disrupted the binding site for that TF.

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