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DEVELOPMENTAL EXPRESSION PATTERN OF THE *zgc:154061* GENE IN
ZEBRAFISH

A Thesis

By

BRANTLEY SUSAN GRAHAM

Submitted to the Graduate School

Appalachian State University

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Department of Biology

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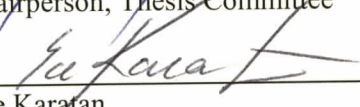
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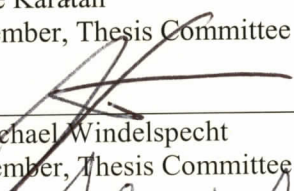
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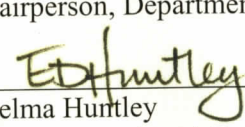
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ABSTRACT

DEVELOPMENTAL EXPRESSION PATTERN OF THE *zgc:154061* GENE IN
ZEBRAFISH

(December 2009)

Brantley Susan Graham, B.S. Clemson University

DVM, Tuskegee University

M.S., Appalachian State University

Thesis Chairperson: Ted Zerucha

The *zgc:154061* gene has been identified in the genomes of all vertebrates examined to date. It is organized in a convergently transcribed orientation relative to *Meis2* (*meis2.2* in zebrafish) and is also in the region of at least one Highly Conserved Non-coding Element (HCNE) hypothesized to be involved in controlling *Meis2* and/or *zgc:154061* expression. This conformation and organization is highly conserved amongst all vertebrates. The conservation of this genomic proximity of *zgc:154061*, *Meis2* and the HCNEs over the hundreds of millions of years of evolution of vertebrates indicates that the components of this region of synteny are linked and serve an important function. I describe here the temporal expression of *zgc:154061* using quantitative real time PCR and *in situ* hybridization.

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INTRODUCTION

The *Meis* genes belong to the Three Amino Acid Loop Extension (TALE) class of the transcription factor superfamily of homeobox genes. Homeobox genes are identified by a conserved 180-bp region (the homeobox) that codes for the translation of a 60 amino acid domain known as the homeodomain. The homeodomain is a DNA-binding domain made up of three α -helices with a loop separating the first and second α -helices and the second and third α -helices forming a helix-turn-helix motif. Homeobox genes have been identified in all eukaryotic organisms and code for transcription factors that regulate target genes by binding to specific nucleotide sequences in their *cis*-regulatory elements. This large superfamily of homeobox genes is broken down into several classes including the TALE class of which *Meis* genes are a member. The TALE class derives its name from a three amino acid loop extension in the region between helix 1 and helix 2 of their homeodomains. It has become increasingly clear that homeodomain proteins rarely bind to DNA *cis*-regulatory elements as monomers since one homeobox protein alone has low affinity for binding DNA (Geerts *et al.*, 2005). Instead, these proteins often work by forming complexes, recruiting other homeodomain proteins to form a complex with a much higher binding affinity (reviewed in Holland, 2001; Geerts *et al.*, 2005). The members of the TALE class of homeodomain proteins are known to be important in forming complexes with other homeodomain proteins and because of this, are often referred to as being cofactors for other homeodomains. *Meis* proteins thus generally work as parts of complexes with other homeobox gene products,

including the Hox proteins. *Hox* genes are the most well-known of the homeobox genes. They are arranged in uninterrupted physical clusters and are highly conserved in all animals (reviewed in Holland, 2001; Santini *et al.*, 2003). *Hox* clusters consist of anterior, central and posterior genes and are responsible for embryological development and patterning along the anterior-posterior axis in all bilaterally symmetrical organisms. They accomplish this by regulating the expression of other developmentally important genes. The organization of *Hox* clusters is closely associated with their expression. *Hox* genes exhibit both spatial and temporal colinearity. This means that *Hox* genes are expressed along the anterior-posterior axis in the same order that they appear in the *Hox* cluster. Genes located in the 3' position of the cluster are expressed more anteriorly and earlier in development than genes at the 5' position which are expressed more posteriorly and at later stages of development (reviewed in Holland, 2001).

All vertebrate model organisms investigated have at least three functional *Meis* genes. Teleost fish such as zebrafish have undergone a 'fish-specific' genome duplication event that took place early in the history of all bony, ray-finned fish 300 to 450 million years ago (Taylor *et al.*, 2001; Prohaska *et al.*, 2004; Hoegg *et al.*, 2007; Prince *et al.*, 1998; Amores *et al.*, 1998). Due to this event, zebrafish have duplicates of many genes including *Meis2*. The two zebrafish *meis2* genes (*meis2.1* and *meis2.2*) have been previously characterized and their expression patterns have been studied using whole-mount *in situ* hybridization analysis. In zebrafish, *meis2* transcripts are found in distinct regions of the central nervous system with the strongest expression in the hindbrain. Later expression is seen in the isthmus, along the spinal cord, in the developing retina and in the pharyngeal arches (Biemar *et al.*, 2001; Zerucha *et al.*, 2001; Rohrschneider *et al.*, 2007).

The most well documented function of Meis proteins is as a cofactor that forms a complex with Pbx and Hox proteins. Hox alone has relatively low binding affinity for DNA, but in combination with Meis and Pbx, the Hox protein has increased specificity and binding affinity for certain DNA sequences. Meis thus acts in concert with other gene products, but appears to also have some independent effects on gene expression. For example, A Pbx/Hox protein complex without Meis results in a different expression pattern than a Hox/Pbx/Meis complex. Zebrafish Pbx/Hox complexes without Meis also result in severe deficits in hindbrain development in rhombomeres 3 and 4 (Waskiewicz, 2001). Furthermore, the expression of *Meis2* overlaps the expression of *Hox* genes in the posterior hindbrain, but *Meis* is expressed further anteriorly than *Hox* alone. This implies some additional *Hox*-independent function of *Meis2* (Zerucha *et al.*, 2001; Choe *et al.*, 2002). The interactions between *Meis* and *Pbx* are complex and not completely understood. There is a conserved region within the *Meis* which codes for a domain known as the Meinox domain that has been associated with *Pbx* binding (Fig. 1). The M1 region of this Meinox domain does not appear to be involved in Pbx binding and may be responsible for the more anterior expression pattern of *Hox* genes when they are complexed with *Meis* genes (Choe *et al.*, 2002).

```

meis2.1  MLMAQRYEDLAHYGGGMDGVVPTSMYGDPHAPRPLPQVHHLNHGPPPHANQHYGAHAPH
meis2.2  --MAORYDELAHYGG-MDGVG----MYGDPHAPRPLPOVHHLNHGPPLHASQHYGTHAPH

meis2.1  SI-MPSSMGSAVNDALKRDKDQIYGHPLFPLLALVFEKCELATCTPREPGVAGGDVCSSE
meis2.2  PNMPTSMGSAVNDVLKRDKDQIYGHPLFPLLALVFEKCELATCTPREPGVAGGDVCSSE

meis2.1  SFNEDIAVFAKQIRAEKPLFSSNPELDNLMIQSIQVLRFHLLLELEKVHELCDNFCHRYIS
meis2.2  SFNEDIAVFAKQVRAEKPLFSSNPELYNLMIQAIQVLRFHLLLELEKVHELCDNFCHRYIS

meis2.1  CLKGKMPIDLVIDERDG-CKSDFDLDSGSSTNLADHNPASWRDMDDAHSTPSVGTGPGSS
meis2.2  CLKGKMPIDLVIDERDGSSKSDHEELSGSSTNLADHNPASWRDHDDATSTHSAGTGPSS

meis2.1  GGHASQSGDNSSELGDGLDNSLSPGTGDED--DQDKKRQKKRGI FPKVATNIMRAWLFQ
meis2.2  GGLASQSGDNSSEQDGLDNSVASPGTGDDDDDPDKDKKRQKKRGI FPKVATNIMRAWLFQ

meis2.1  HLTHYPSEEQKKQLAQDTGLTNLQVNNWFINARRRIVQPMIDQSNRA-----VSQGT
meis2.2  HLTHYPSEEQKKQLAQDTGLTILQVNNWFINARRRIVQPMIDQSNRAGFLLDPSVQGA

meis2.1  AYS PDGQPMGGFVLDGQQHMGLRPGGPMGGMGMNMGMDGQWHYM
meis2.2  AYSPEGQPMGSFVLDGQQHMGIRPAGPMSGMGMNMGMDGQWHYM

```

Figure 1. Meis and MEINOX domain. Amino acid sequence of the two zebrafish *meis2* homologs with the MEINOX domain (red) and homeodomain (yellow) sequences highlighted

Overexpression of *Meis* has also been shown to increase endogenous *Pbx* protein levels *in vivo* and *Meis* alone or in combination with *Hox* may work to stabilize *Pbx* proteins in zebrafish (Waskiewicz *et al.* 2001).

The TALE class homeobox genes tend to exhibit a relatively high rate of alternative mRNA splicing. These splice variants are well conserved, suggesting that these variants have evolved functional differences. One example of this higher rate of alternative splicing is seen in *MEIS2* in humans which has four different full length splice variants. These splice variants likely recruit different types of proteins to the DNA binding complex, resulting in varying activity during transcription. Most of these splice variants have positive regulatory effects, but some variants have been found to negatively regulate *Meis2* function by binding

competitively for the same recognition sites (Yang *et al.*, 2000; Geerts *et al.*, 2005). Each splice variant shows a different expression pattern in mice: *Meis2c* and *d* are the major forms expressed in the female genital tract while *Meis2a* and *b* are the major forms in the brain (Oulad-Abdelghani *et al.*, 1997).

Meis genes are linked to many forms of cancer. The most notable is in leukemia, where the gene was first identified in mice (*Meis* was named for Myeloid ecotropic viral integration site). *Meis* is active in the development and proliferation of neural tube cells during development and disruption of this regulation results in tumor like cell masses in the area. *Meis2* has also been shown to be upregulated in many types of tumor cells including metastatic pancreatic endocrine neoplasms as well as lung adenosarcomas (Geerts *et al.*, 2005).

While it is known where *Meis2* is expressed during development and many of its molecular functions are understood, little is known of how its transcription is regulated. Four downstream Highly Conserved Non-coding Elements HCNEs have been identified in the Zerucha lab (Appalachian State University) and are being investigated as possible regulatory elements for this gene. All four elements are highly conserved in tetrapods, including human, mouse and chicken, however only one of these elements has been identified in zebrafish and pufferfish to date (Zerucha, unpublished data, Appalachian State University). All of these elements are located within introns of the *zgc:154061* gene. This organization has been conserved in the genomes of all vertebrate species examined, thus *zgc:154061* has remained in close proximity to *Meis2* through hundreds of millions of years of evolution. This implies that the organization of the two genes and the HCNEs have been maintained due to selective pressure.

As genomes evolve, point mutations and genome rearrangement are the two most common changes observed. The most dramatic of these changes is rearrangement. The exact mechanism by which rearrangements occur is still undetermined; however two main theories have been proposed to explain this process of genome evolution. The first theory is that areas of synteny between two species are randomly distributed within the genome (Nadeau *et al.*, 1984). The second theory is the “fragile breakage” theory that says there are certain areas of the genome predisposed to breakage. These same areas break each time, leading to large areas of synteny between species (Pevzner *et al.*, 2003; Peng *et al.*, 2006). These large areas of synteny exist primarily around developmental genes. The organization of this area of synteny consists of a developmental gene flanked on either side by gene deserts that contain HCNEs. These HCNE regions are very highly conserved between species, suggesting a strong evolutionary requirement for these areas to remain unchanged. Many HCNEs activate expression in the same pattern in different model organisms, indicating that these HCNEs are being bound by the same transcription factors and are likely serving a similar function in different species (de la Calle-Mustienes *et al.*, 2005).

Comparative genomics has allowed extensive study of the areas of conserved synteny between species and has helped narrow the search for regulatory elements. If a large block of synteny is found between two species, it is likely that the regulatory mechanisms for the gene at the center of the block are found within the conserved area. The points known as *cis*-regulatory elements are regions of DNA that regulate the expression of genes on the same strand. These *cis*-regulatory elements generally remain in close proximity to the genes they act on; this is likely at least one of the reasons for these evolutionary constraints (Ahituv *et al.*, 2005). Despite the pressure for regulatory mechanisms to remain close to the genes they

control, regulatory elements for a gene can be found at great distances upstream or downstream from the target and even located within the introns of other genes. One example involves the expression of the Sonic Hedgehog (*Shh*) gene in *Drosophila melanogaster*. *Shh* plays important roles throughout the development of all animals including limb development in tetrapods and fin development in teleosts. A *Shh* cis-regulatory element, ZRS, which directs *Shh* expression to the developing limb and that is well conserved across vertebrate species has been located 1Mb away from the *Shh* gene within intron 5 of the *Lmbr1* gene. The zebrafish ZRS contains specific nucleotide differences relative to tetrapods, presumably the result of point mutations that have been demonstrated to cause polydactyly in vertebrates, hinting at the molecular changes that may have lead to the evolution of tetrapod digits from fin rays (Lettice *et al.*, 2003).

Zebrafish are a powerful model organism for studying genome evolution. The lineage that gave rise to zebrafish underwent a total genome duplication event after branching from the tetrapod lineage and before the divergence of the pufferfish approximately 320-350 million years ago. This makes the genome of zebrafish more closely related to tetrapods than cartilaginous fish (Volf, 2005). Having duplicates of all genes reduces evolutionary pressure on this family to conserve the original order and function of the genome. This has allowed some genes to be deleted or evolve new or modified functions over the course of time. It is thought that this genetic diversity has allowed the teleosts to evolve from one common ancestor to the approximately 23,600 different species of teleost fish known today. The extent of diversity within the teleost family suggests that there may have also been some additional independent gene duplications within different species of the teleost family (Venkatesh, 2003).

The duplicate genome of teleosts has allowed them more flexibility than other vertebrates in the order and function of individual genes. Duplicate copies of genes, including their regulatory elements, have been deleted or modified by gain or loss of function. These duplicate genes and their regulatory elements have one of three fates: nonfunctionalization, neofunctionalization or subfunctionalization. In nonfunctionalization, the duplicate redundant gene accumulates mutations that make it nonfunctional. These nonfunctional genes have been completely or partially deleted over the course of evolution and often their remnants can still be found in the genome as pseudogenes. In neofunctionalization, one of the gene duplicates has mutated in a way that gives it an entirely new function that becomes necessary or advantageous to the animal. In subfunctionalization, the two duplicate copies each accumulate mutation resulting in each of them assuming a complementary portion of the actions of the original gene. The fact that each copy has a unique function ensures that it is maintained in the genome (Force *et al.*, 1999).

HCNEs under investigation as possible regulatory elements controlling *Meis2* are found within the introns of the *Meis2* linked gene *zgc:154061*. The exact relationship between *Meis2*, *zgc:154061* and the HCNEs is not understood, but this type of gene configuration has been described in other developmental genes. A pattern of Genomic Regulatory Blocks (GRB) has been found to be highly conserved between mammal and teleost chromosomal segments. These GRBs consist of one developmental regulatory target gene surrounded by HCNEs either upstream or downstream and functionally unrelated “bystander” genes that are not involved in the regulatory network of the target gene (Kikuta *et al.*, 2007).

The location of regulatory elements within the introns of other genes is a common pattern in humans and mice, but is complicated in zebrafish by their duplicate genome. GRBs between humans and mice are generally completely conserved. The genome duplication event in the zebrafish lineage initially resulted in two copies of every gene and HCNE. This relieved biological pressure to strictly conserve the organization on both copies of the genome, allowing evolutionary changes that have resulted in the deletion of multiple gene copies (nonfunctionalization). In zebrafish, often the “bystander” genes have been completely or partially deleted on one copy while genes and HCNEs involved in direct regulation of the target are maintained on both copies of the genome (de la Calle-Mustienes *et al.*, 2005; Kikuta *et al.*, 2007). If a group of HCNEs are completely conserved on both copies and found within a gene that is completely conserved on both copies, it would be reasonable to suspect that the HCNEs and the associated gene are directly involved in target regulation. A more common theme seems to be HCNEs preserved on both copies of the genome, embedded within a bystander gene on one copy and standing alone on the other copy. In this case, the HCNEs may be controlling the target, but the bystander gene is probably unrelated (Carvajal *et al.*, 2001; Kikuta *et al.*, 2007).

Several examples of GRBs have been documented in the literature. Orthopedia (*OTP*) is a homeobox gene responsible for cell migration, proliferation and differentiation of the neuroendocrine system. The human *OTP* locus is spanned by many HCNEs, the most distal of which are found upstream within the introns of a neighboring gene, *AP3B1*. Zebrafish have two *OTP* orthologs, *otp* and XP_683186.1. The GRB containing *otp* retains the HCNEs found in the bystander gene *AP3B1* in humans, but *AP3B1* has been completely deleted from the GRB (Acampora *et al.*, 1999; Kikuta *et al.*, 2007). Another example

involves the *Mrf4* and *Myf5* genes, which are muscle specific transcription factors involved in skeletal myogenesis in the vertebrate embryo. The expression of these two genes is linked. If *Mrf4* expression is blocked, the embryo will develop normally. If *Myf5* is not expressed, muscle development will be delayed, but *Mrf4* will compensate and the embryo will develop normally. This link between the two genes is also seen in zebrafish. In mice, the connection has been identified as a common regulatory element for both *Mrf4* and *Myf5* found within the introns of an unrelated neighboring gene (Carvajal *et al.*, 2001; Hinitz *et al.*, 2008).

OBJECTIVES

The first step in determining the relationship between *zgc:15406*, *Meis2* and the HCNEs is to characterize the expression of *zgc:15406*. To characterize *zgc:15406* expression, I used two main tools : Quantitative Real Time PCR (qRT-PCR) and *in situ* hybridization. qRT-PCR provided the relative transcript copy number of *zgc:15406* at each level of development and *in situ* hybridization showed the areas of the developing embryo where *zgc:15406* was expressed. Using these tools together gave me a spatial and temporal expression pattern for *zgc:15406*.

An overlapping *in situ* expression pattern between *zgc:15406* and *meis2* in combination with the conservation of the two genes in close proximity throughout evolution would indicate that the two genes might be linked.

MATERIALS AND METHODS

Zebrafish Husbandry

Embryos were collected using pair-wise breeding of adult AB line fish housed in an enclosed Z-Mod (Aquatic Habitats) system and maintained at 28°C on a 14h light and 10h dark cycle. Embryos were allowed to develop in a 28°C incubator until they reached the desired developmental stage. 30 to 100 embryos were removed from the incubator at the desired stage (represented as hours post fertilization, hpf). All experiments involving zebrafish were approved by the Appalachian State University Institutional Animal Care and Use Committee (IACUC). The IACUC approval date for this project is January 30, 2009.

RNA Extraction and cDNA Preparation

Prior to RNA extraction, reverse transcription or RNA gel electrophoresis, all surfaces and equipment used were cleaned with RNase zap spray (Sigma, St. Louis, MO, R2020-250ml) or a dilute bleach solution. All glassware was baked and water was treated with Diethyl pyrocarbonate (DEPC) (Sigma, St. Louis, MO, 016K3726) following product recommendations. All tips and tubes were RNase free and sterilized prior to use.

RNA extraction with TRIzol (Invitrogen, Carlsbad, CA, 15596-026) was accomplished following the protocol recommended by Invitrogen as described by Leung and Dowling (Leung *et al.*, 2005). 30 to 100 embryos at the same stage of

development were homogenized in TRIzol reagent using a sterile plastic mini mortar (Kontes, NJ , 749520-0090). The product recommendations for extraction of RNA from small volumes of tissue were followed. Each sample was homogenized in 800 μ L of TRIzol. The sample was incubated for 5 minutes at room temperature to permit complete dissociation of complexes. Chloroform was added at a rate of 0.2 ml per 1 ml of TRIzol used and the tube shaken vigorously by hand for 15 seconds and incubated at room temperature for 2-3 minutes. The sample was centrifuged at 12,000x g for 15 min at 4°C to separate the organic and aqueous phases. The upper aqueous phase was removed and placed into a fresh microcentrifuge tube. 5 μ L of RNase-free glycogen (Invitrogen, Carlsbad, CA, 10814-010) was added as a carrier to the aqueous phase. To mix and reduce viscosity, the sample was passed twice through a 26 gauge needle and syringe. Isopropyl alcohol was added at 0.5 ml per 1 ml of TRIzol reagent used for the initial homogenization to precipitate the RNA. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitated to form a gel like pellet. Supernatant was removed and the pellet washed by adding 75% ethanol at 1ml per 1ml of TRIzol used for the original homogenization. The sample was vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. Ethanol was removed, preserving the pellet and the wash was repeated with another 800 μ L 75% ethanol. After centrifugation and removal of the second round of ethanol, the pellet was allowed to dry at room temperature for 30 minutes to 1 hour. The dry pellet was resuspended in RNase free water by passing the solution several times through a pipette tip and incubating for 10 minutes at 60°C. RNA samples were stored at -80°C.

RNA samples were evaluated for concentration and purity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). The samples were also run on

a denaturing gel to determine purity of the sample. Each sample loaded into the gel contained 5 μL of RNA in RNase free water, 12 μL formamide (Sigma, St. Louis, MO, F9037-100ml) (60% of sample), 2 μL 10X loading dye (10% of sample) and 1 μL EtBr (10mg/ml). By denaturing each individual sample, I was able to run the gels using a regular 1.2% agarose gel run in TAE buffer. Prior to loading, each sample was heated for 5 minutes at 65°C and then immediately chilled on ice for 5 minutes.

RNA samples were reverse transcribed into cDNA using Applied Biosystems (AB) High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, 4304437) following product recommendations. Samples were thawed over ice and vortexed to resuspend the sample. 1 μg of the RNA sample was placed into a PCR tube. 4 μL of the High Capacity RNA-to-cDNA Master Mix was added and RNase free water to a final volume of 20 μL . The sample was put into a thermal cycler machine for one cycle (25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes, hold at 4°C). The cDNA was immediately used in a qRT-PCR reaction or stored at -80°C.

Quantitative Real Time Polymerase Chain Reaction

Relative Quantitative Real Time PCR was utilized to measure the expression levels of the *zgc: 154061* target gene relative to the expression of an endogenous control. The endogenous control used was *β -actin* which is involved in cytoskeletal structure and has been demonstrated to be present at near constant levels in all tissue throughout zebrafish development (McCurley *et al.*, 2008). Total RNA was extracted at 0, 4, 8, 12, 16, 20, 24, 72 and 120 hpf. RNA extractions were taken from two separate sets of embryos and each reaction was performed in triplicate.

Primer and probe sets for the gene of interest and the internal control were designed on Primer Express software and ordered from Operon (Huntsville, AL) (Fig. 2).

zgc:154061 Forward (5'-GCAGACGCACTTCACACATCTC-3'),

zgc:154061 Reverse (5' – TGCGCTTCATTCTCTTCTGGTA-3'),

zgc:154061 probe (5'- FAM-CGCGTCCACTCTGCTGAGCATCTTC-TAMRA-3'),

β -actin Forward (5' – GCTGTTTTCCCCTCCATTGTTG-3'),

β -actin Reverse (5' – TTTCTGTCCCATGCCAACCAT-3'),

β -actin Probe (5'- FAM-CCCAGACATCAGGGAGTG-TAMRA-3').

Figure 2. Sequences for Primers and TaqMan Probes. Forward and reverse primers and TaqMan probes sequences for the target gene and the endogenous control

Primers and probes were optimized using the protocol provided with the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, 4304437) (PremierBiosoft 2008). Gene sequences and location of primer and probes are shown in Figures 3 and 4.

A standard curve was performed on the primer/ probe sets for the gene of interest and the internal control using a cDNA gradient and following the TaqMan Universal PCR Master Mix Protocol. The standard curve measures the efficiency of the primers and probes at detecting the target using different known levels of cDNA template (Applied Biosystems, 2002; Larionov *et al.*, 2005; University of Chicago, 2009).

Zebrafish *zgc:154061*

gtcgcgtgtgctgcagaactggagcctgtacagtgtattggctgtttgtgttcagagttgtgaaataggacgcagtgccaggc
ggcagcggccacaggaacaggtgaacaggtgaataatctgaggatctgcagggcagaatacaggagcatcagcaggtttgtgg
agcagctgcgtccacacggcagtgcatgaagaccctgcagacgcacttcacacatctcccgcgtccactctgctgagcatctt
ctcccaggagtaccagaagagaatgaagcgcagtatggccagacatcactctcctgaggtgctcagagtgattatcagaggtat
cgagatgaagctgagaccagagccacagaaccactgctgctggagctcgttaaccaggtggatctatctccagctcttttgctc
gtctgatgctggagtgttctagaggagcgaacgcttcagtcctccagacaagtctcaacaacatgctgcgtgagccgtatt
taattccagatctggtgttagccaagcacatcgcagcagtgacacagtaaatgactgctgttatggaccgctggtcagctgcatcaa
catgccatcggctggaacatgaagacactctgagagacaaactcagagagaggaacctgctgttttagatgagaatcagctgc
gggtcaaggatacagaaaaacccggacatcctggagtgccgatcgtgttgatggccacatcgttcactggatcgaga
gtaaagcttcattggagatgatcacagtcacaacacatactgaacgagcagttctggagctactgcaacaggtttgtccgggt
ctggtcactactggtcggctcactcagagctggactgccagcgggagcggggatcctgctgaaggacggctccccacg
gacatcagcagcctgtgtcgggacccagcgtgaggacggggcgtttcagtgatggatccggaagcaatttaagctt
tcctgcagccagagactctgacaaacatgtgctgtaactccatctgaacctctgtgtgagtggtgtgtgtgtgtgtgtgtgtgt
gt
actcattgaaactgcacagtaaatgcaagttttgtctggtgtttggccagatctgtgaactcttaataaagaagcatttttgatg
tgactgaatgtagtgtttcagaggtgatgtctaaagtgaagtgagttttaattagagcaggctgaa taatctgccagtgggg
gaagcagaataatctatgtgtaaaggaaaaagccagtgatttccctcaccctggcggattattctgctgttttgaggtaggac
tctcttttttggtcgttatttctgtaacaacactgtattttatccctgtctataaaatgcttctgatttaagaacgttcagatatttac
caaaaacaagcgaactcttaagaatacgcagtttactcgtgtactaatatttgaaatctttgcatgtaattacaattttccgtt
aataattacacctctcttacaccttaaccctcttaaccttcccatatcactaaacctgtcaacaaccaaccccgatcccagatt
aatagcaccataactgttctgcaatgattataaacaggaggagtacattgtgctgatgttttgatggtagtattgtgactcaataa
agtgtatgattgtgtacatgctgatatggaataaataaagagtattctcgtaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 3. Sequence of *zgc:154061*. Forward primer (blue), reverse primer (yellow) and TaqMan probe (pink) are highlighted. Start codon is highlighted in green and stop codon in red.

Zebrafish β -Actin

1 ggcacgagag atcttcactc ccctgttca caataaccta ctaatacaca gccatggatg
61 aggaaatcgc tgccttgggc gttgacaacg gctccggtat gtgcaaagcc ggttttgctg
121 gagatgatgc cctcgtgct gtttcccct ccattgttgg acga^{cccaga catcagggag}
181 ^{tgatggttg catgggacag} aaagactcct atgtgggaga tgaggctcag agcaagagag
241 gtatcctgac cctcaatac ccattgagc acggtattgt gaccaactgg gatgacatgg
301 agaagatctg gcatcacacc ttctacaatg agtccctgtg tgcccctgag gagcacctg
361 tcgtgctcac tgaggctccc ctgaatccca aagccaacag agagaagatg acacagatca
421 tgttcgagac ctcaacacc cctgccatgt atgtggccat ccaggctgtg ctctctctgt
481 acgcttctgg tcgtactact ggtatttga tggactctgg tgatggtgtg acccacaccg
541 tgcccatcta tgagggttac gctcttccc atgccatcct gcgtctggat ctagtgtg
601 gtgacctgac agactacctg atgaagatcc tgaccgagcg tggctacagc ttaccacca
661 cagccgaaag agaaattgct cgtgacatca aggagaagct gtgctatgtg gccctggact
721 tcgagcagga gatgggaacc gctgcctctt ctctctcct ggagaagagc tatgagctgc
781 ctgacgttca ggtcatcacc atcggcaatg agcgttccg ttgccccgag gctctctcc
841 agccttctt cctgggtatg gaatctgctg gtatccacga gaccacctc aactccatca
901 tgaagtgcga cgtggacatc cgtaaggacc tgtatgcaa cacagtctg tctggaggtg
961 ccaccatgta cctggcatt gctgaccgta tgcagaagga aatcacctct ctgtctctt
1021 ccaccatgaa gatcaagatc atgtctccc ctgagcgcga atactccgct tggatcgggtg
1081 gctccatctt ggctccctg tccacctcc agcagatgtg gatcagcaag caggagtacg
1141 atgagtctgg cccatccatc gttcacagga agtgcttcta aacagaactg ttgccacct
1201 aaatggccta gcaatgagat taaacgaac gaccaaccta aactctcga cagaacaaga
1261 tgacatcagc atggcttctg ctctgtatgg cgcattgact caggatgagg aaactggcaa
1321 agggaggtag ttgtctaaca ggggagagct tccccgaga ggacaacaat gtacattct
1381 tttagtcatc ccagaagcgt ttaccacttt gcctctctca caatggcgt ccatgacct
1441 tttgtatag tttttatgt aaattatgta ctgatacat tgttttctt tttgtactc
1501 agccttaaac ttggcccagt ttgtattgt tgcaatgagg ggaaagctt accctttaa
1561 aagtgaagat cttgcaggac ttcctaggg tatgtgaata agggatgtcc cttgaaaatg
1621 taagccaggg tctctgta cactgacaag tcaacccaaa taaaactgtc acatgtaaaa
1681 ccaaaaaaaaa aaaaaaaaa

Figure 4. Sequence of β -actin. Forward primer (blue), reverse primer (yellow) and TaqMan probe (pink) are highlighted.

A working solution was created containing 12.5 μ L TaqMan Universal PCR Master Mix, 900 nM forward primer, 900 nM reverse primer, 250 nM probe, enough DEPC-treated water to bring the volume to 25 μ L per reaction. Each sample was performed in triplicate and each triplicate sample was mixed in one 1.7 ml microcentrifuge tube. After preparing the working

solution, 80 μ L were placed into a microcentrifuge tube and 4 μ L of cDNA were added to the tube (50 ng/ μ L). The sample was mixed well and briefly centrifuged. The same pipette tip was used to put 25 μ L of the sample into each of three wells on the microamp optical 96 well reaction plate (Applied Biosystems, Foster City, CA, N801-0560). Each time point was tested for both the gene of interest and the endogenous control. After all samples were loaded into the 96 well plate, the top of the plate was sealed with optical film (Applied Biosystems, Foster City, CA, 4313663). The plate was loaded into an Applied Biosystems 7300 real-time PCR System and run using the following program: 50°C for 2 minutes; 95°C for 10 minutes; 95°C for 15 seconds, 60°C for 1 minute repeated for 40 cycles. Results were interpreted using the $2^{-\Delta\Delta C_t}$ Method (Livak *et al.*, 2001). Thresholds were determined manually and used to interpret all data. The thresholds used were 0.062948 for β -actin and 0.040564 for *zgc:15406*. All results were calibrated to an adult male zebrafish. All reactions were performed in triplicate using two separate RNA extractions (Livak *et al.*, 2001; Ambion, 2008).

***In situ* Hybridization**

In situ hybridization with a DIG-labeled riboprobe was performed to determine the regions of the developing embryo where *zgc:15406* is expressed. Embryos were allowed to develop in an incubator to the desired developmental stage (2 hours post fertilization (hpf), 8hpf, 24hpf, and 48hpf). Embryos older than 24hpf were dechorionated manually prior to fixation. Embryos less than 24hpf were fixed prior to dechorionation. All embryos were fixed overnight on a shaker table at 4°C in 4% paraformaldehyde (PFA). The embryos were then washed twice for 5 minutes in PBS and dehydrated with a 5 minute wash in a 50% methanol

/ 50% PBS solution at room temperature. This was followed by two 5 minute washes in 100% methanol. Embryos were stored in fresh methanol at -20°C for at least 1 hour before using.

The full length 1914-bp cDNA clone of *zgc:15406* was isolated by the Zebrafish Gene Collection and was made available through Open Biosystems (Clone ID: 8334609, accession: BC124527). This full length clone, in the Express Vector System, was purchased from Open Biosystems. An antisense DIG labeled riboprobe was synthesized using T7 RNA polymerase after linearizing the plasmid with NotI.

Methanol stored embryos at 2, 8, 24 and 48 hpf were rehydrated by washing in solutions of gradually decreasing methanol and increasing in PBS. Washes included one 5 minute wash in 75% methanol / 25% PBS , one 5 minute wash in 50% methanol 50% PBS , one 5 minute wash in 25% methanol / 75% PBS, three 5 minute washes in 100% PBT.

Embryos were treated with 20 µg/ml Proteinase K in PBT solution for different lengths of time depending on their stage of development: 2hpf and 8hpf were not treated; 24hpf embryos were digested for 30 seconds and; 48 hour embryos were digested for 1 minute. Proteinase K digests were followed by two quick PBT washes. Embryos were refixed for 20 minutes in 4% PFA on a shaker table. Embryos were then washed 4 times for 5 minutes each in PBT.

Embryos were incubated for 5 hours in prehybridization buffer (50% formamide, 5xSSC (pH 7.0), 500 µg/ml torula yeast RNA, 50 µg/ml heparin, 0.1% Tween 20 in RO H₂O and then pH was adjusted to 6.345 with 9mM acetic acid and buffer was heated to 70°C prior to incubation) at 70°C and then hybridized overnight at 70°C in prehybridization buffer with

DIG labeled RNA probe. All stages were incubated with 2 μ l antisense DIG labeled RNA probe in 988 μ l prehybridization buffer.

Embryos were washed in a series of washes increasing in SSC and decreasing in prehybridization buffer: one 10 minute wash in 75% prehybridization buffer / 25% 2x SSC at 65°C; one 10 minute wash in 50% prehybridization buffer / 50% 2x SSC; one 10 minute wash in 25% prehybridization buffer / 75% 2x SSC; one 10 minute wash in 100% 2x SSC and; two 30 minute washes in 0.2x SSC were performed. A series of washes were then performed at room temperature in increasing PBT and decreasing SSC: One 5 minute wash in 75% 0.2x SSC/ 25% PBT; one 5 minute wash in 50% 0.2x SSC/ 50% PBT; one 5 minute wash in 25% 0.2x SSC/ 75% PBT and; one 5 minute wash in PBT.

Embryos were blocked in 5% sheep serum (600 μ l of Stock BSA (100 mg/ml) in 30 ml of PBT in a 50ml conical. 9.5 ml of the BSA/PBT was placed in a 15 ml conical and 500 μ l of Stock Sheep Serum was added) for 1 hour at room temperature on a shaker table. Embryos were then incubated in a 1:2000 dilution of anti-DIG antibody (750units/ml) in BSA/PBT at room temperature for 3 hours. Antibody was cleared with six 15 minute washes in PBT at room temperature. Embryos were equilibrated with three 5 minute washes in NTMT (0.1 M Tris-HCl pH 9.5, 50mM MgCl₂, 0.1M NaCl, 0.1% Tween20).

In a 15 ml conical, 2.5ml of NTMT, 8.75ul of 5-Bromo-4Chloro-3Indolyl Phosphate (BCIP), 11.25ul of Nitro Blue Tetrazolium (NBT) were mixed and embryos were stained from 15 minutes to several hours depending on expression level in the dark on a shaker table. Once desired level of stain was reached (1 hour and 45 minutes for 0 and 48 hours and overnight for other stages), embryos were rinsed twice for 5 minutes in 5mM EDTA in PBS (Thisse and Thisse, 2008).

RESULTS

Identification of *zgc:154061*

In zebrafish, the *zgc:154061* gene is located downstream of the *meis2.2* gene on chromosome 17 and the two genes are separated by approximately 10.9-Kb (Fig. 5) as determined by examining the publicly available zebrafish genome data.

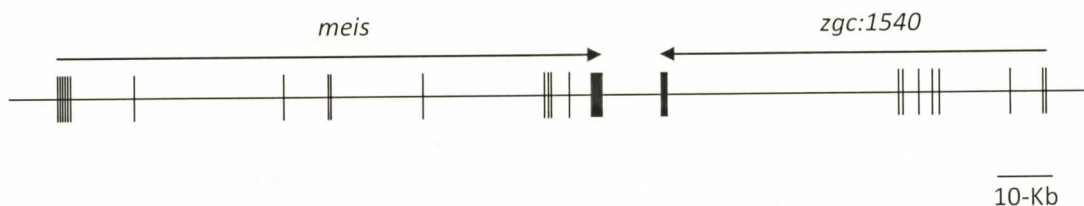


Figure 5. Genomic organization of *zgc:154061*. Shown with respect to *meis2.2* in zebrafish. Vertical lines/ boxes represent exons. Arrows indicate the direction of transcription.

zgc:154061 is convergently transcribed relative to *meis2.2* to produce a transcript of 1914-bp in length that encodes an open reading frame predicted to produce a protein 300 amino acids in length. (Fig. 6).

zgc:154061

MDAVSGGSATGTGEQVNNLRICRAEYRSISRFEQLRPTRQCMKTLQTHFTHLPASTLLSIFSQE
YQKRMKRSMARHHSPEVLRVYYQRYRDEAETRATEPLLELANQVDLSPALLARLMLECFLEERN
ASVPSRQVLNNMLREPYLIPDLVLAKHIEQCTVNDCCYGPLVDCIKHAIGLEHEDTLRDKLRERN
LSFLDENQLRVKGYDKTPDIILEVPIAVDGHIVHWIESKASFGDDHSHNTYLNEQFWSYCNRFGP
GLVIYWFGFISELDCQRERGIILLKDGFPDISSLCAGPQR

Figure 6. Translated amino acid sequence of *zgc:154061*.

Predicted orthologs of *zgc:154061* have been identified in all vertebrates examined and these orthologs are present in the same convergently transcribed orientation directly adjacent to that species' *Meis2* ortholog.

qRT-PCR expression of *zgc:154061*

In order to determine expression levels of *zgc:154061* throughout embryonic development, Quantitative Real Time PCR (qRT-PCR) was conducted on total RNA extractions from sets of 30 to 100 embryos from every 4 hours of development until the end of the first day and then every 24 hours of development for the next 3 days of development. Total RNA was converted to cDNA and qRT-PCR determined the levels of expression of *zgc:154061* in comparison to the levels of the endogenous control, β -*actin*. All results were calibrated to expression levels in an adult male zebrafish (Fig. 7).

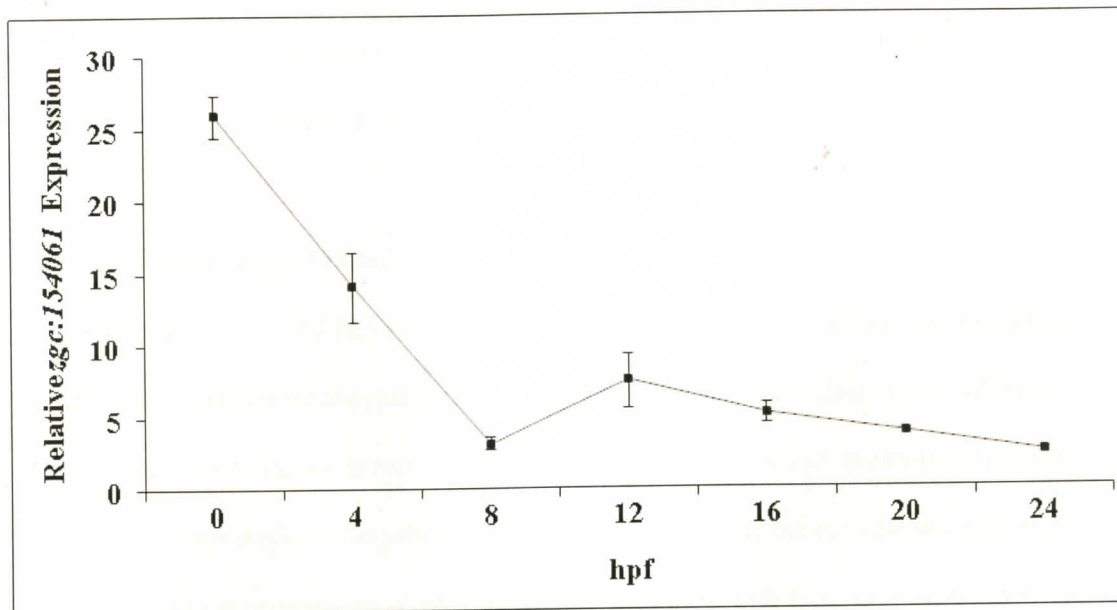


Figure 7. Quantitative real-time PCR analysis of *zgc:154061*. Expression of *zgc:154061* is relative to that of the endogenous control β -actin at each developmental stage. Results were calibrated to the expression in an adult male zebrafish using the $2^{-\Delta\Delta C_t}$ method. Error bars reflect the standard error of the mean for each sample. Each value of the graph is significantly different from the neighboring values as determined by t-test ($p < 0.05$).

The quantity of transcripts of *zgc:154061* steadily decreased from their maximum level at the earliest stages of development to barely detectable levels at 8hpf. This expression pattern is consistent with a maternal transcript that is present in the zygote and that gradually degrades over time while not being replenished by new transcriptional activity. After the maternal mRNA is degraded, there is a slight increase in the expression levels of *zgc:154061* at 12 hpf. This transcript is actively produced by the developing embryo and suggests a secondary function of *zgc:154061* after its initial role in early development.

zgc:154061 levels decrease slightly after 12hpf until 24hpf. At 24hpf, *zgc:154061* levels are low, but the transcript is still being actively produced. This low level of expression was detected at all stages examined for the remainder of development.

In situ* expression of *zgc:154061

At 2hpf, *zgc:154061* has a uniform expression in all developing cells of the embryo (Fig. 8a). As supported by the quantitative real time PCR data, this ubiquitous expression is maintained but in decreasing levels until 8hpf. After 8hpf expression increases again but at this and subsequent stages is largely restricted to the developing neural tube and eye at 24hpf (Fig. 8b). By 48hpf, expression is more anteriorly restricted with faint expression throughout the forebrain and anterior hindbrain. The strongest expression at 48hpf is found in the branchial arches (Fig. 8c).

This pattern of ubiquitous early expression that precedes activation of the zygotic genome at the mid-blastula transition (2.75hpf) is consistent with a maternal transcript present in all cells of the embryo during early development. The later neural expression of *zgc:154061* suggests this gene may be serving a second function in the developing eye and neural tube.

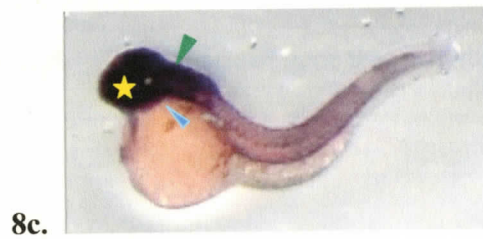
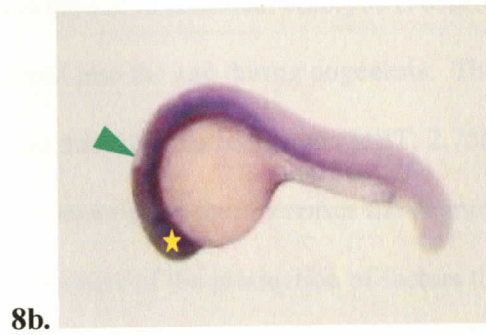


Figure 8. Whole-mount *in situ* hybridization analysis of *zgc:154061* expression.

Embryos are shown as whole mounts with the anterior to the left. 8a (2hpf), 8b (24hpf) and 8c (48 hpf). Eye (☆), Midbrain- hindbrain boundary (▼), Branchial arches (▲)

DISCUSSION

Early embryonic development in zebrafish embryos is controlled by maternally synthesized mRNA incorporated into the egg during oogenesis. The zygotic genome is transcriptionally silent until the mid-blastula transition (MBT, 2.75hpf). After MBT, the zygotic genome begins active transcription and increases the degradation of maternally inherited mRNA presumably because of the production of factors that break down maternal transcripts (Streisinger *et al.*, 1981; Wolpert, 2002; Ferg *et al.*, 2007). In this study I have shown that *zgc:154061* is a maternally transcribed gene that is highly expressed in all cells of the embryo until the MBT.

An important control mechanism in early embryonic development is the activity of maternally synthesized microRNAs (miRNAs). miRNAs are a family of small regulatory non-coding RNAs that act as negative regulatory factors post-transcriptionally (Chen *et al.*, 2005). Most miRNA are expressed in a highly tissue-specific manner during segmentation and later stages but they are universally expressed in early development, suggesting a unique function in early embryonic development, possibly in maintaining tissue identity (Wienholds *et al.*, 2005). All miRNA activity is maternally derived until the zygote begins producing zygotic miRNA during the blastula period (4hpf) (Chen *et al.*, 2005).

My qRT-PCR results are consistent with the expression pattern of a maternally expressed gene. Transcript levels are highest at 0hpf and have decreased by almost half by the time of the next reading at 4hpf. During this time interval, the embryo reached the mid-blastula transition and began transcribing its own genome, producing products that break down maternal transcripts. At the next time point, 8hpf, the embryo has begun to produce microRNAs that target maternal transcripts and expression levels decrease again by half to reach the lowest *zgc:154061* transcript levels at 8hpf.

My *in situ* results also indicate a maternally expressed gene with ubiquitous expression in all cells of the embryo at 2hpf. The next *in situ* time point is 24hpf, which takes place after the observed increase in real time expression at 12hpf. All *zgc:154061* expression at this point is therefore due to embryonically transcribed *zgc:154061* and indicates a secondary function of *zgc:154061* at later stages of development and in the adult zebrafish. The location of expression suggests that the secondary function is involved in the development of the neural tube. Further studies will be required to define the exact location of expression and determine a possible function .

Orthologs of *zgc:154061* have been identified in all vertebrates examined and its genomic organization of being adjacent to *Meis2* has been maintained in all vertebrates (Carpenter and Zerucha personal communication). One possible explanation for this conserved gene linkage is that *Meis2* and *zgc:154061* share *cis*-regulatory elements. If this hypothesis is true, the transcripts of *Meis2* and *zgc:154061* would be predicated to exhibit overlapping patterns of expression. There is some overlap between the expression pattern of *zgc:154061* and the expression pattern reported for *meis2.2*, primarily in the anterior neural tube and brain at 24 and 48 hours as well as in the branchial arches (Waskiewicz, 2001;

Thisse and Thisse, 2008). It is also possible that the HCNEs located within the introns of *zgc:154061* regulate the expression of *Meis2* with no direct effects on *zgc:154061* expression. To investigate this question, it will be interesting to examine the expression of *zgc:154061* and its relationship to *Meis2* and the HCNEs within the introns of *zgc:154061*. In addition, while my studies represent a first step in characterizing the novel *zgc:154061* gene by trying to glean some hints of its function by determining when and where it is active much work remains to elucidate the function of this gene during early development as well as later during neural development. It will be interesting to determine more of the function of this gene by making use of transgenic studies to overexpress the gene by injecting transcripts for it into developing embryos, or conversely to remove its function using morpholino knockdown experiments in zebrafish.

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BIOGRAPHICAL SKETCH

Brantley Susan Graham was born in the great state of South Carolina in 1974. She grew up in and attended school in a town with two stoplights. She graduated from Clemson University with a BS in Animal Science and went on to attend Tuskegee University School of Veterinary Medicine which seemed like a good idea at the time. After graduating with a DVM in 2000, she worked as a small animal practitioner for one year in a clinic run by the devil incarnate. She then worked as a Veterinary Medical Officer with the United States Department of Agriculture trying to convince grown people that they had to actually show up at work to get paid. In 2007 she opted to learn some new skills by pursuing a Master's degree in Biology at Appalachian State University. She received an MS in Biology in 2009 and now uses her new molecular skills to genotype mice at the University of Kentucky where she is paid poorly but has excellent benefits. Dr. Graham plans to remain employed so that she can continue to make payments on the \$75,000.00 worth of student loans she has accrued on this adventure.

