LOCALIZATION OF UFD2A TRANSCRIPTS IN ADULT AND FRY ZEBRAFISH

By

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LOCALIZATION OF UFD2A TRANSCRIPTS

IN ADULT AND FRY

ZEBRAFISH

An Undergraduate Honors Project Presented

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To

The Department of Biology

Approved:

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DEDICATION

TO

SARAH SPINETTE

IN TOKEN OF MY GRATITUDE
FOR HER CONSTANT SUPPORT AND AID
ALONG WITH THE BIOLOGY DEPARTMENT FACULTY
FOR NUTURING MY CURIOSITY

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ABSTRACT

Critical inquiry concerning biological pathways is crucial when attempting to understand how living systems function. Like a jigsaw puzzle, pieces must be successively investigated and then combined to comprehend the larger picture. Examining the spacio-temporal expression of the three alternative splice forms of Ufd2a, an enzyme involved in protein degradation, may only be one piece towards understanding its novel roles at the cellular level, but may help to envision the grand scheme, specifically in muscle development. Using zebrafish as a model organism, *in situ* hybridization techniques were employed on tissue sections to investigate where and when Ufd2a is expressed. After much optimization of existing protocols, it could not be determined if Ufd2a was expressed in skeletal muscle, as results were inconclusive. Further studies must be conducted in order to learn more about the three splice form's expression patterns in zebrafish muscle tissue.

INTRODUCTION

Laboratory research employing scientific experimentation is essential to the acquisition of knowledge and understanding of complex biological systems. Generally, analysis of such systems is first accomplished by examining and inquiring about a particular component of the system. Once a series of relevant observations are made, a set of hypotheses can be generated, and investigation can commence.

One such system that has been under scrupulous investigation is the process of cellular differentiation and development in eukaryotes. Differentiation and development progress by way of intracellular and extracellular signals, including cell-to-cell signaling, genetic regulation, protein turnover, and programmed cell death. Countless signals operate via protein-protein interactions, and many signals have multiple pathways, and as a consequence, multiple affects. Examples of molecular mechanisms which contribute to the functional diversity that is required in order for an organism to survive include posttranscriptional and posttranslational modifications of mRNA and proteins, respectively, which may entail alternative splicing, phosphorylation, acetylation, and

ubiquitylation. Therefore, research concentrated on regulatory mechanisms, such as alternative splicing and patterns of expression, is a key component to understanding how organisms develop and survive at the cellular level.

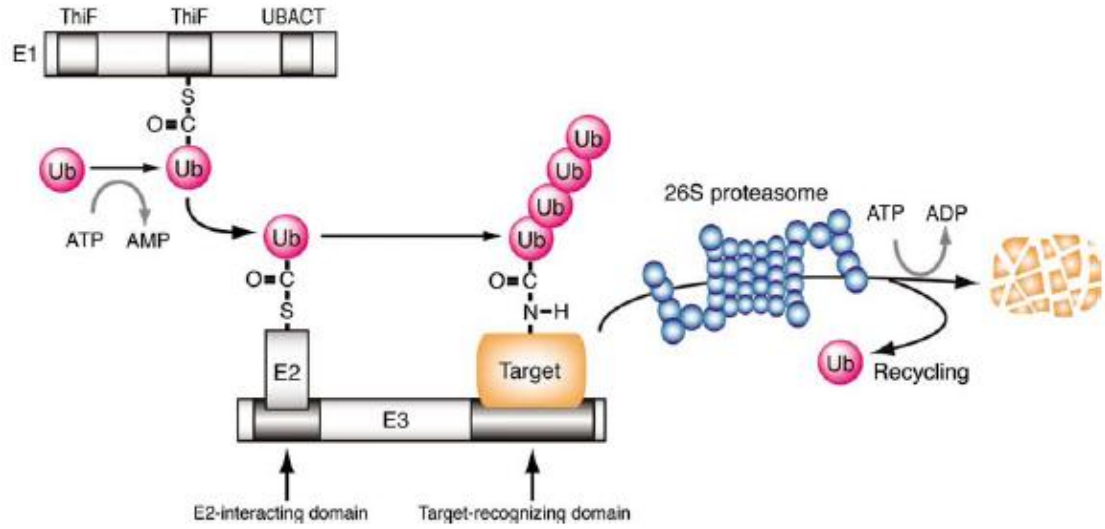
Studying such pathways and mechanisms will increase our understanding of cellular growth, maintenance and repair, and this information can be applied clinically with regard to regenerative therapies and treatment of degenerative diseases. Using a multitude of different techniques, the spacio-temporal expression and function of Ufd2a, an alternatively spliced ubiquitylating enzyme which has been implicated in cell differentiation and development, has been studied.

Ubiquitin Proteasome System.

Protein degradation is one essential molecular mechanism which controls organismal development, from conception to death. Normally, degradation will proceed in one of two ways: lysosomal degradation or via the ubiquitin-proteasome system (UPS). Lysosomes are usually responsible for degrading proteins and other molecules that have been taken into the cell by the process of endocytosis. The UPS is hence a more specific and specialized way in which eukaryotic cells regulate the process of protein degradation.

Ubiquitin is a seventy-six amino acid protein present in all eukaryotes that is commonly used to identify proteins that have been marked by the cell for degradation. Numerous reasons for a cell to utilize the ubiquitin-proteasome pathway in order to degrade a specific protein include destroying proteins that are misfolded or denatured, decreasing the concentration of a particular enzyme or signaling protein, inhibition of selected enzymes, or removal of an inhibitor on an enzyme.[1] The specificity of the UPS is

Figure 1. The UPS system involves the ATP-dependent E1 activation of a ubiquitin moiety which is then transferred to an E2. This enzyme in turn binds with an E3, which is already bound to its target protein, and transfers successive ubiquitins onto the protein to be degraded.[8]



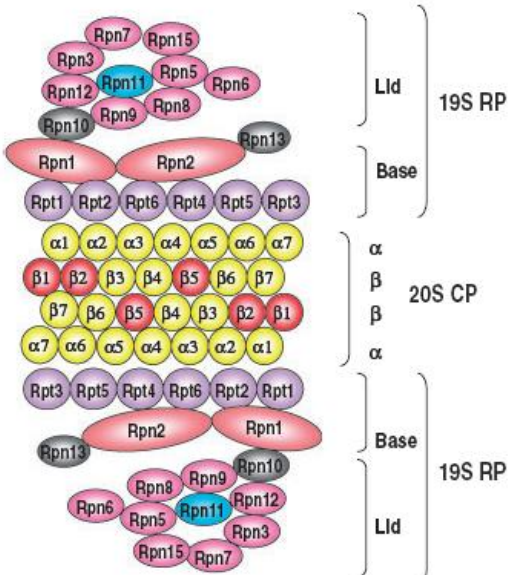


Figure 2. Diagram showing the basic structure of the proteosome, including the RPs and CP.[4]

thus critical for the regulation of processes and signaling pathways.

The system involves a specific and orderly cascade of protein interactions and may be localized to the cytosolic rough endoplasmic reticulum in the case of misfolded or denatured proteins or found throughout the cytoplasm of the cell (Fig. 1). Activation of the ubiquitin-proteasome pathway begins when a ubiquitin-activating enzyme, or an E1, utilizes an ATP molecule to activate a single ubiquitin unit. The ubiquitin is then transferred to an E2, or ubiquitin-conjugating enzyme. The E2 relocates its ubiquitin via a peptide bond formed between the carboxyl terminus of the ubiquitin moiety and a lysine residue of the target protein, which had been selected for degradation by a ubiquitin ligase, or E3.[1] The entire process is repeated until the selected protein has been polyubiquitinated, usually

conjugated to many (but at least four) ubiquitin units. Unlike polyubiquitinated proteins, which are recognized by the proteasome for fragmentation, monoubiquitination of proteins may result in activation or inhibition of an enzyme or transcription factor in a manner similar to phosphorylation. Once polyubiquitylation has led to the target molecule being tagged, it is shuttled to the proteasome for degradation.[2]

The Proteasome.

The proteasome is a 2.5 megadalton barrel-shaped superstructure that is responsible for recognizing and digesting polyubiquitinated proteins. It is composed of thirty-three subunits: four seven-membered ring subunits stacked one atop the other and a lidded opening at each end (Fig. 2). The central domain is named the core particle (CP) and contains the proteolytic active sites that are responsible for cleaving peptide-peptide bonds of the tagged protein. The regulatory particles (RP) are found at the top and base of the structure and contain the ATPases responsible for ensuring that the correct incoming protein is being unfolded as it enters the cavity. Within the lid component of the proteasome is a metalloprotease, Rpn11, which is responsible for deubiquitination of the protein. The detached ubiquitins and the digested protein's peptides, which are cleaved into about seven to eight residue peptides, can be recycled by the cell, making the UPS system time and energy efficient. The importance of the ubiquitin-proteasome system is magnanimous in that it is the major player in protein turnover within a cell. [3,4]

Protein Turnover.

Protein turnover is crucial to the development and survival of an organism. Proteins often signal for changes within a cell to take place, and their concentrations and temporal expression help determine the function of a given cell by controlling

transcriptional activation. Many genes, and as a result their respective proteins, are solely expressed at a set period in development and may be localized to a particular body region or tissue. Misexpression, such as a complete lack of expression or overexpression, particularly in the wrong cell type, can lead to many problems for the organism, ranging from deformity to death.

All proteins have a characteristic half-life, a given amount of time in which the amount of that protein in the cell is approximately decreased by a half. Intricate mechanisms exist to ensure that these proteins are tagged with ubiquitin and shuttled to the proteasome in a correct and timely manner. For example, the mitotic cell cycle is enormously dependent on the specific timing of cyclin protein turnover to allow the cell to progress into the subsequent stages of the process. Other proteins that are habitually degraded via the UPS pathway include transcription factors, tumor suppressors, and membrane proteins. Proliferation of cells via mitosis allows for growth of the organism and tissue repair, and without the correct sequence of mitotic events, development cannot occur correctly. As with cell proliferation, normal development of an organism also requires that specific cells in the embryo undergo apoptosis, or programmed cell death.[5]

Apoptosis.

Apoptosis requires the synchronized processing of proteins resulting in the coordinated self-induced termination of the cell. In the case of organismal development, cells may undergo the process simply because they were programmed to do so upon reaching a certain point in growth. In this instance, certain trophic factors are required for a cell to remain alive, and if those signals are inhibited, apoptosis may result.[2] Outside of development, in the case of the adult organism, for example, apoptosis may be

due to other factors, such as infection by a foreign pathogen. Failure of a cell to commit programmed cell death may lead to uncontrolled cell proliferation and tumorous growth. Because apoptosis is a controlled mechanism, unlike necrosis[A], proteins within the cell must be disposed of in an orderly fashion. As follows, the UPS comes in handy in helping to regulate these processes and the correct regulation and function of ubiquitylating proteins is crucial for organismal development and survival.

Ufd2.

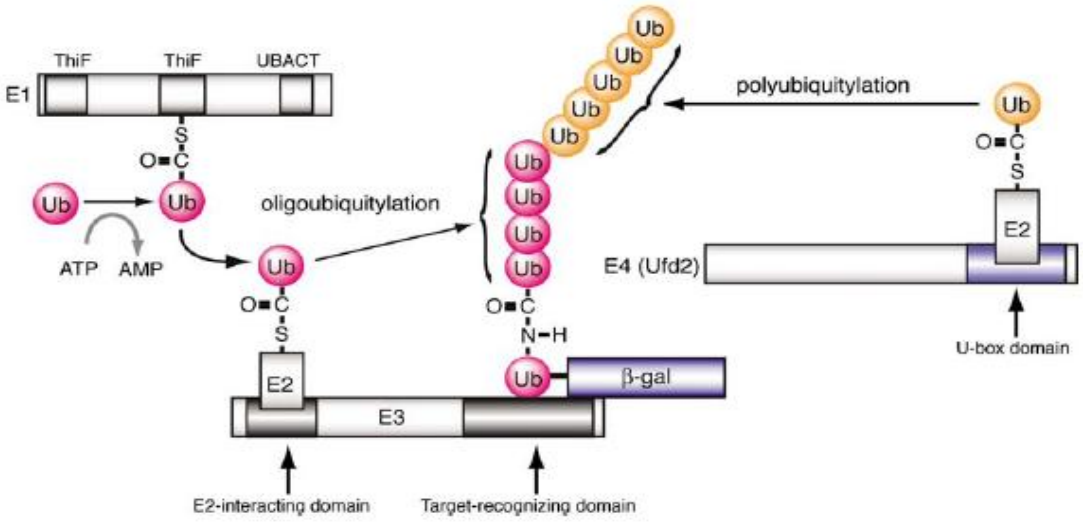
Ufd2 is an incompletely characterized protein that is involved in the aforementioned cellular mechanisms. It was named **U**biquitin **F**usion **D**egradation protein based on its essential role in ubiquitin-mediated protein degradation when it was first discovered in yeast (designated as Ufd2p). The enzyme has been found to have two mammalian orthologues, E4A, and Ufd2a (also known as E4B), which has been shown to possess E3/E4 capabilities. E3 ligases are known to belong to one of three families, having a RING-finger, HECT, or U-box domain. Ufd2 contains a U-box domain, structurally similar to RING-fingers, on its C-terminal end, and is about seventy-six amino acids in length and is extremely well conserved in eukaryotes. The N-terminus of the protein, however, is only conserved in vertebrates and has been shown to be a novel regulatory domain. Phosphorylation within the N-terminus by Cdk1, especially hyper-phosphorylation during mitosis, severely limits Ufd2a's ubiquitylating capability. Also, Ufd2a is known to be controlled within the N-terminus by degradation by apoptotic proteases such as caspase 3 and 6 and granzyme B. This particular region of the protein has been termed the MPAC (Mitotically Phosphorylated, Apoptotically Cleaved) domain,

[A] Necrosis is the unregulated and rather sloppy death of a cell due to physical trauma or damage, releasing its contents to the external environment and resulting in an inflammatory response.

and its presence may promote protein-protein interactions that are necessary for involvement in the UPS. It is known that the activity of the U-box depends upon the N-terminal domain, as cleavage or cdk1-mediated hyperphosphorylation of this region results in loss of ubiquitylating function. Interestingly, i*n vitro* knockdown of the gene causes mitotic cell arrest at the metaphase-anaphase changeover. Ufd2 is somewhat unique in that its catalytic activity is due to its U-box domain, which is conserved in eukaryotes, and ΔU-box mutants display no ubiquitylating action.[6,7,8]

The E4s are categorized as a specific type of E3 in which they are responsible for recognizing oligoubiquitylated proteins and further extending the ubiquitin chain (Fig. 3).[8] Substrates of Ufd2a remain largely uncharacterized; however, it is known that they include ataxin-3, securin, and FEZ1.[9]

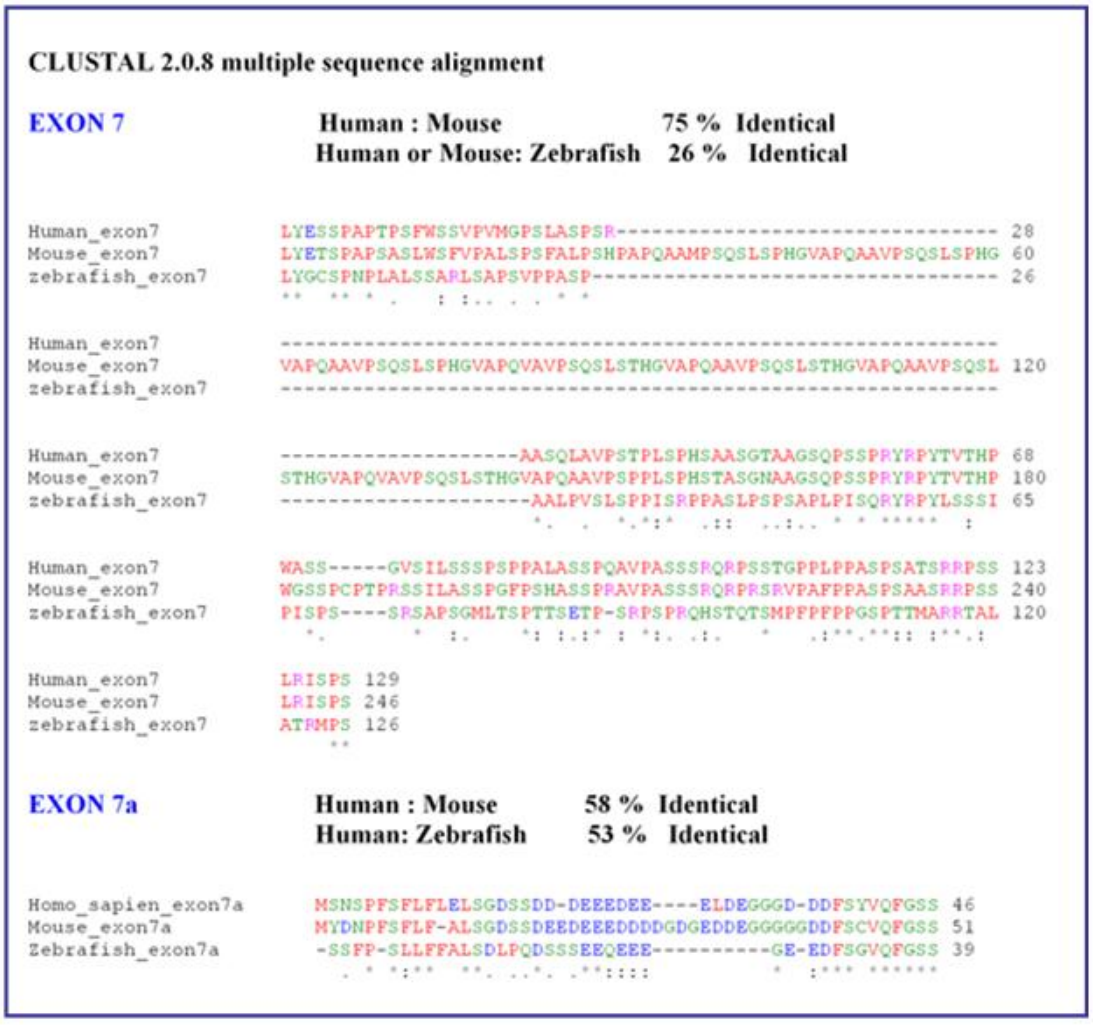
Figure 3. The UPS system in light of E4 enzymes. Thus the previous diagrammatic scheme of ubiquitylation has been modified, as E4s recognize and extend ubiquitin moieties onto oligoubiquitylated target proteins.[8]



Remarkably, there is a general trend seen with genomic studies in which more evolved organisms do not possess vastly greater quantities of functional genes than less evolved organisms. This suggests that differential splicing of transcripts serves as a considerable source of genetic diversity and is essential for correct development and proper functioning of the organism. In mammalian cells, including humans, Ufd2a exists in three isoforms due to differential splicing (Figure 4): ubiquitous form I (exons 1-6 and 8-27; 1,173 residues), form II (exons 1-6, 7, and 8-27; 1,302 residues), and form III (exons 1-6, 7 and 7a, and 8-27; 1,353 residues). While most tissues in the body express Ufd2aI uniquely, striated muscle tissue expresses the three different isoforms during different stages of cell differentiation. Ufd2aI is found solely in undifferentiated myoblasts, while Ufd2aII is found in differentiating and fusing myoblasts. In fused myotubes, mature skeletal muscle and cardiomyocytes, Ufd2aIII is expressed.

Figure 4. The three alternative Ufd2a splice forms showing phosphorylation sites, apoptotic protease cleavage sites, U-box domains, and the tissue-specific location of each form. Ufd2aI is found in satellite cells and myoblasts. Ufd2aII, containing exon 7, is found mainly in myoblasts and differentiating muscle cells. Ufd2aIII, containing exons 7 and 7a, is found in mature myotubes.

Figure 5. Sequence alignment of exons 7 and 7a comparing human, mouse, and zebrafish sequences. Note the blue region of exon 7a is highly acidic.



The Ufd2a zebrafish orthologue is located on chromosome 23, and in contrast to several other E3 ligases examined, is highly conserved (Ufd2aI has an 80% identical amino acid sequence to human Ufd2aI), suggesting its entire sequence is important within both organisms, not just its catalytic U-box region. The amino acid sequence of exon 7 is similar to the N-terminal domain of Ufd2a in that it contains many phosphorylation sites, which are characterized by numerous serine, threonine, and proline residues (Figure 5). Its sequence is 26% identical between human and zebrafish. Exon

7a is unique in that it is highly acidic in nature, a feature which seems to be well-conserved in vertebrates. Conservation between the two species is 53% identical.

Knockout/down Experiments.

It is interesting to note that in a study performed by Kaneko-Oshikawa et al., Ufd2a$^{-/-}$ knockout mice were found to have complications involving cardiac development, triggering massive cardiomyocyte apoptosis, killing the organism before birth. Heterozygotes survived, however they displayed brain defects.[10] In addition, when the mouse myoblast C2C12 cell line were treated with miRNA against exon 7 or morpholino oligonucleotides designed to block the expression of Ufd2aII and III, abhorrent differentiation was observed. In particular, myoblasts did not efficiently fuse to form multi-nucleated myotubes (Spinette et al., personal correspondence)[B]. These data suggest that Ufd2a is necessary for cardiomyocyte cell differentiation and muscle development.[6]

Myogenesis.

Patterns of muscle tissue development begin very early on in embryonic development. Teleosts, or ray-finned fish, differ from vertebrates in that they posses multiply innervated fast fibers, the majority being of the white type. Fish also possess pink fibers, which are a combination of red and white fibers and have properties analogous to fast oxidative fibers.[11] Skeletal muscle myogenesis can be categorized into three main series of events: determination, proliferation, and terminal differentiation and assembly.[12] Developing muscle is arranged into discrete V-shaped regions of muscle tissue, called somites. One type of teleost fish, *Danio rerio,* has thirty-two somites running down the dorsal axis, which are derived from mesodermal cells, and upon

[B]microRNAs, or miRNAs, are single-stranded RNA sequences which can bind to complementary transcripts within cells, thereby knocking down translation.

development give rise to myoblasts. Myoblasts will migrate to their predetermined location based on positive and negative feedback, and through complex intracellular communication, align and fuse into syncytia, forming multinucleated myotubes.[2] There are two types of myoblasts: slow-twitch myoblasts, which develop first, are under the regulatory control of Hedgehog and give rise to superficial muscle tissue, and fast-twitch myoblasts, which are found deeper within the tissue.[13,14] One of the first regulatory mechanisms in myogenesis is the expression of MyoD, a basic helix-loop-helix transcription factor that works to coordinate the process by activating other transcription

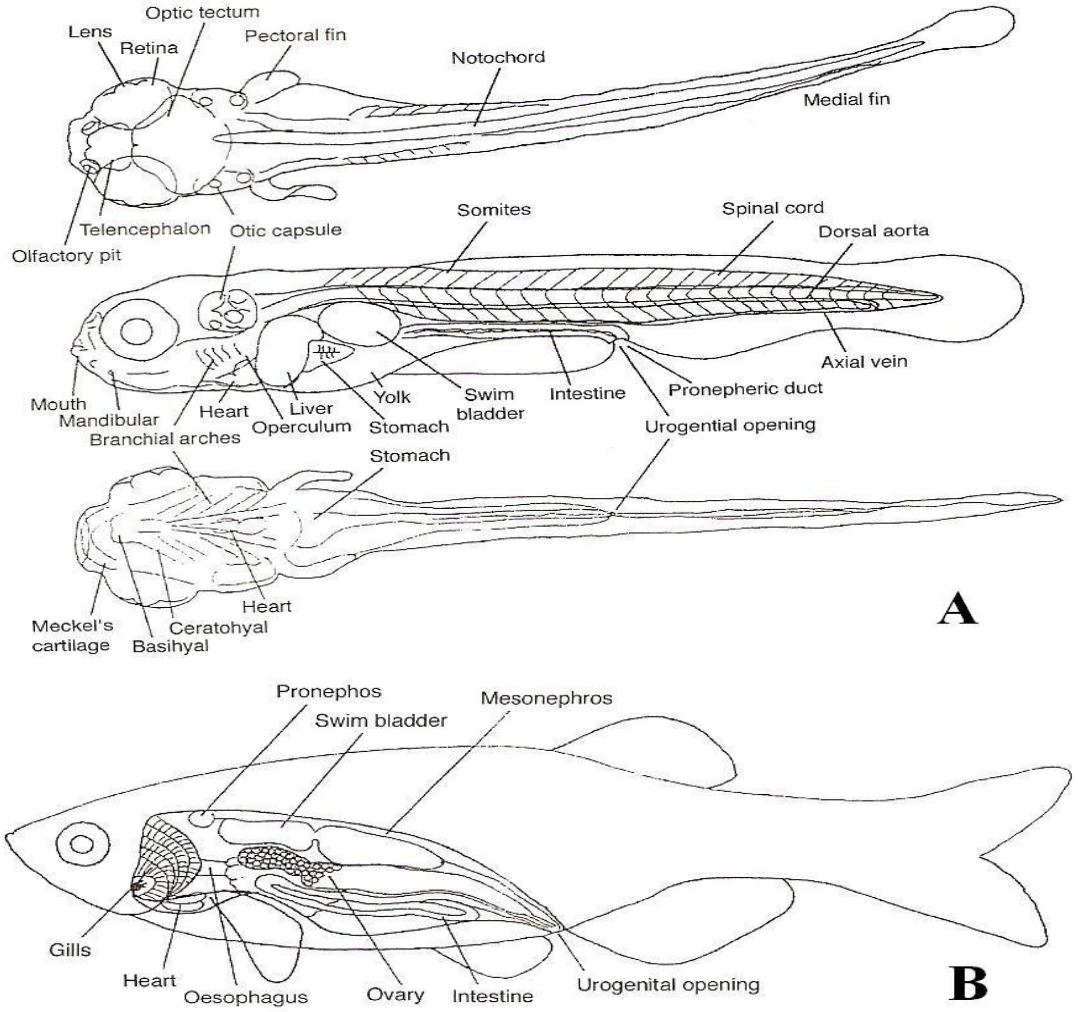


Figure 6. Zebrafish anatomy. (A) Anatomical rendering of early larvae viewed dorsally or laterally. (B) Adult zebrafish viewed at lateral position.[18]

factors and altering chromatin structure, thereby initiating muscle development. Those mononuclear myoblasts that do not receive the signals to differentiate into myotubes remain as satellite cells. Satellite cell populations, located on the periphery of myotubes, are inherently quiescent, and proliferate in order to fuse with existing myofibers as well as to replenish themselves.[15,16,17]

Zebrafish Background.

The zebrafish, *Danio rerio*, has been well studied and characterized since the early nineteenth century and therefore makes an excellent scientific model organism. These teleosts are native to northern and central India and are included in the minnow family. Their natural environment is in freshwater rivers and streams. Anatomically, they have a single dorsal fin and a toothless pharyngeal jaw which is located at the back of the throat and helps them break up their food (Fig. 6; Supplemental Figures). A special feature that these organisms possess includes a Weberian apparatus, which consists of ligaments and bones that connect the swim bladder with the inner ear. The hearts of these organisms are composed of a single atrium and single ventricle with four chambers in total. While skeletal muscle composes the majority of the musculature, smooth muscle lines the guts and vasculature. Adults are those that are greater than ninety days post-fertilization, are generally

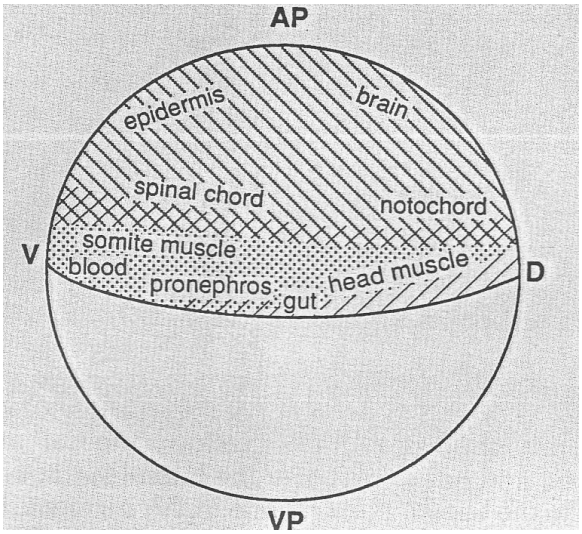


Figure 7. Zebrafish fate map detailing embryonic origins of various prominent tissues. [21]

about three centimeters long, and have a life span of approximately two years. Sexual maturity is reached at about ten to twelve weeks post-fertilization. Individuals have five alternative black and blue stripes lengthwise, while the rest of the body is of a silver or yellow coloring. Males are generally thinner, have more yellow pigmentation, especially on their bellies, and can be observed in the tank to chase females. Females are larger with more silver pigmentation and have a fuller belly, especially when full with eggs.[18]

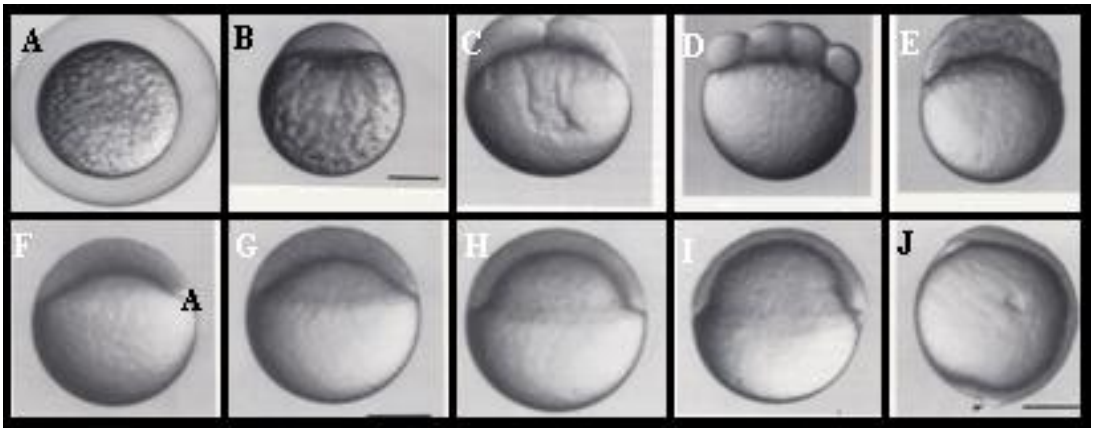
Zebrafish as a Model Organism.

There are numerous reasons why zebrafish are very useful to scientists as an *in vivo* model organism. They are well-characterized, relatively easy to maintain, easy to manipulate, are inexpensive, and a single spawning can produce upwards of two hundred fertilized eggs. A copious amount of mutants exists, and genetic databases are easily accessible. Before twenty hours post-fertilization, embryos can be treated with PTU (1-phenyl-2-thiourea) which prevents pigmentation, and this is advantageous with regard to certain experimental techniques which utilize detection of a signal via visualization. While they are not mammals, the two classes share many similar anatomical and developmental patterns (Fig. 7). Growth and development of the organism, and in particular the process of myogenesis, can be easily observed as they develop external to the mother. While zebrafish are not mammals, the two classes share many similar anatomical and developmental patterns.[19]

Zebrafish Development.

Developmentally, cytoplasm begins to stream towards one pole of the egg, raising the blastodisc and dividing the organism from the yolk (the animal and vegetal poles,

Figure 8. Sequential stages of embryonic zebrafish development. (A) Zygote, 0hpf. (B) 1-cell stage, 0.2hpf. (C) 2-cell stage, 0.75hpf. (D) 8-cell stage, 1.25hpf. (E) 256-cell stage, 2.5hpf. (F) Dome stage, 4.3hpf. (G) 30% epiboly, 4.7hpf. (H) 50% epiboly, 5.3hpf. (I) Shield stage, 6hpf. (J) Bud stage, 10hpf.[20]



respectively) (Fig. 8).[20] The first cell cleavage begins at about forty minutes post-fertilization, and subsequent cleavages occur at about fifteen minute intervals up to the 1,024 cell stage. At this point in time, cellular divisions become nonsynchronous and the synthesis of new RNA transcripts begins.[21] At about the same time point that gastrulation begins (5 hpf), myogenesis commences with the expression of initial transcription factors such as *myod* and *myf*5.[22] Larvae are ambulatory at the one day stage, with progressive muscular development.[23]

Rationale.

Biology in a way examines the emergent properties of life: scientific research provides novel insight into how molecular mechanisms work to form viable cells, which in turn form properly functioning tissues, followed by organs, organ systems, and finally the complete organism. The increasing amount of knowledge that is discovered at each of these levels of the organism helps connect the unknown gaps so that a complete picture of how life is sustained can be obtained. This new information can be applied

clinically and medicinally to help improve the quality of life and generate innovative methods of treatment. Zebrafish can be used as a model organism to ultimately study human diseases. Using *Danios* to study the spacio-temporal expression of Ufd2a and its mechanism of action *in vivo* are just one part of the puzzle, but will be important to our ultimate understanding of organismal development, muscle repair, and regenerative therapies using stem cells.

MATERIALS AND METHODS

Overview. The experimental technique referred to as *in situ* hybridization (ISH) has been performed to identify which splice forms of zebrafish Ufd2a are present at different points in development and if they are tissue-specific. ISH is the technique of using an RNA probe that has a complementary sequence to the target mRNA transcript in order to localize it within a cell. The probe can be labeled with a DIG-tag, and once hybridized to the correct mRNA sequence, can be detected by an anti-DIG antibody. This antibody is conjugated to alkaline phosphatase, which acts enzymatically on its substrate to produce a visual signal. This procedure is generally efficient and can be used to identify spacio-temporal expression of genes in whole-mounts and tissue sections. However, protocols must be optimized depending on the probe specifications and the tissue being used in the experiments.[24]

Zebrafish care, embryo collection, and rearing. Zebrafish were kept according to standard IACUC regulations. Tank temperatures were maintained at about 28°C, were monitored daily along with pH at a range of about 7.3-7.7, and fish were kept on a 14:10 hour light:dark cycle. Tank water was conditioned with 3 grams Instant Ocean, (Marineland Labs) and 1 gram sodium bicarbonate per 20 liters reverse osmosis water.

Diet consisted of a variety of sources, including dry pellets, flakes, and live artemia. In order to collect embryos, sexually mature fish were placed at night in breeding tanks with small slits which allowed eggs to pass into the lower compartment. When tank lights turned on, fish were cued to spawn, usually within the first half hour. Embryos were then collected using a standard tea strainer and placed in embryo media (0.06g Instant Ocean, Marineland Labs, 25µL methylene blue stock at 10g/L, 1L dH_2O) in a 30°C incubator. Nonviable embryos and fry were removed daily via pipetting to avoid passing infections. If fry were to be reared, water quality had to be routinely monitored and replaced as necessary. When fry began swimming freely, about 4-5 days post-fertilization (dpf), they were fed by sprinkling very small amounts of powder food (Hatchfry Encapsulation, Grade 0, Argent Chemical Laboratories) over the water surface twice daily. At about day 10, water removed from the adult fish tanks, approximately 50mL, was added to the fry water generally every other day to acclimate them to tank water. At about 15 dpf, fry were transferred to a small (2 gallon) tank which had no filter or water turnover. Water quality in this tank had to be carefully monitored and changed out for fresh water when necessary. Dead fry were removed daily. Feeding with powder continued, and at approximately 1 month, they were fed a mixture of powder and live artemia. At about 2 months, now juveniles, they were then transferred into a larger 10 gallon tank and fed dry pellets (Aquatic Habitats). At about 3 months, sexually mature young adults were moved into the recirculation benchtop, which was kept on constant water flow under the same conditions as the tank systems.

Fish preparation and paraffin embedding. Zebrafish raised to about 10-15 dpf (as above) were treated with 1:20 tricaine in water (ethyl 3-aminobenzoate, 4mg/mL,

Sigma), which over-anesthetized the fish. Next, they were fixed in 4% PFA in PBS overnight at 4°C. Fry were dehydrated through a series of solution changes for 30 minutes each with gentle rocking: 50% EtOH, 70% EtOH, 90% EtOH, 100% EtOH, fresh 100% EtOH, 50% EtOH/50% xylene, 100% xylene, 50% xylene/50% paraffin, 100% paraffin overnight, changing out paraffin two times. When moving into paraffin, fish were transferred to glass containers with no lid and kept in a 62-65°C oven. Fish were embedded using standard metal molds and paraffin was allowed to solidify.

APES slide preparation. Standard glass microscope slides were treated as follows: slides were cleaned with 50% acetic acid/50% hot water, rinsed with dH_2O, air dried in dust-free hood, soaked in 2% APES (3-aminopropyltriethoxysilane, Sigma-Aldrich) in acetone for 30 seconds, rinsed briefly in acetone then water, dried in a hood and stored in a slide box.

Sectioning. Paraffin blocks were first prepared by shaping the blocks into a trapezoid to give maximal structural stability during sectioning. Serial sections were cut at a 10-12µm thickness using the American Optical Model 820 microtome and a resharpenable metal blade. Sections were floated in 57°C water bath for several minutes to flatten, then 'scooped' from underneath onto coated slides ensuring that sections clung to slides. Prepared slides were dried overnight at 48°C on slide warmer.

Histological hematoxylin and eosin staining. Sections taken at 10µm thickness as described above on poly-L-lysine (Polysciences, Inc.) or APES coated glass slides were used. Slides were treated as follows: 3 minutes xylene, 5 minutes fresh xylene, 2 minutes 100% EtOH, 2 minutes 95% EtOH, 1 minute running water, 3 minutes Harris Hematoxylin (Fischer), 1 minute running water, 1 minute 1% hydrochloric acid in 70%

EtOH, 1 minute running water, 0.2% ammonium hydroxide in water, 1 minute running water, 30 seconds alcoholic eosin Y (Sigma), 1 minute 30% EtOH, 45 seconds 95% EtOH, 1 minute 100% EtOH, 1 minute xylene, and 1 minute fresh xylene. Slides were mounted with glass coverslips and mounting medium (Permount, Fischer).

Probe synthesis. Plasmids contained short 500 to 1000bp fragments or skeletal muscle sequences: smyHC1 (slow muscle heavy chain), acta1 (alpha actin), MyoD or Ufd2a. Ufd2a probes were: exon 6/8, exon 7, or exon 7/7a, and were linearized and transcribed with T7, T3, or SP6 polymerase (Ambion) in the presence of DIG-labeling mix (Roche). DIG-labeled RNA probes were then isolated from the reaction using the RNeasy miniprep kit (Quiagen) and their lengths confirmed by gel electrophoresis. DIG-labeled locked nucleic acid (LNA) probes specific for Ufd2aII (short sequence spanning the junction of exon 7 and exon 8) and polyT positive control LNA probes were purchased from Exiquon.

In situ hybridization experiments. *In situ* hybridization was carried out in the following manner: Slides were submerged for 15 minutes each in: 100% xylene twice, 100% EtOH twice, 70% EtOH, 50% EtOH, and PBS, followed by proteinase K solution treatment (10µg/mL in PBS) for 10 minutes at 37°C. Slides were postfixed in 4% PFA/PBS for 20 minutes at RT, briefly rinsed with active DEPC-H_2O, then washed 5 minutes in DEPC-treated PBS. Acetylation was then performed for 10 minutes at 37°C in 0.25% acetic anhydride (Sigma) in 0.1M triethanolamine hydrochloride (Sigma), pH 8.0, followed by washing for 5 minutes at RT in PBT. Slides were prehybridized for at least 1 hour at 65°C in hybridization buffer (2x SSC, 50% formamide, 1x Denhardt's solution, 10% dextran sulfate, and 250µg/mL yeast tRNA). Probes were linearized at

70°C during last ten minutes. Slides with parafilm coverslips were treated with hybridization buffer with salmon sperm DNA (1:100) and probe (1:200) at 65°C overnight in slide mailers placed in lidded box with dampened Kim wipes. Slides were washed for 30 minutes in 2x SSC at RT, followed by 1 hour in 2x SSC at 65°C then 1 hour in 0.1x SSC at 65°C. Next they were incubated for 5 minutes at RT in detection buffer (0.1M Tris pH 9.5, 50mM $MgCl_2$, 0.1M NaCl, 0.1% Tween-20), followed by incubation for 1-2 hours in blocking buffer (20% lamb serum, 2% BMB(Roche), 60% maleic acid buffer). Overnight incubation with anti-DIG-AP Fab fragment antibody (Roche) diluted 1:2000 in blocking buffer with parafilm coverslips. Incubated 4x for 20 minutes each in detection buffer. Passed through dH_2O with 0.1% Tween-20 and 1mM levamisole (tetramisole hydrochloride, Acros Organics), then stained overnight in NBT/BCIP (Roche). When staining was completed, slides were washed in PBS 4x for 5 minutes each, then mounted with aqueous mounting medium (AquaMount, Fischer) and glass coverslips. Pictures were taken with the Carl Zeiss stereoscope and AxioCam digital camera with and accompanying Axiovision software.

RESULTS

In previous studies total mRNA was extracted from 7dpf zebrafish embryos and RT-PCR was performed using primers designed specifically for amplification of the region between exons 6 and 8 (Fig. 9A). The bulk of the cDNA product generated was only from exons 6/8 (313bp). However, there was a faint band running at 811bp, as well as a very faint product running at about 590bp, presumably also containing exons 7/7a or only exon 7, respectively. These data confirmed the presence of the three alternative splice forms in zebrafish. With that known, examination of temporal expression in

Danios was necessary in order to assess when these alternative splice forms might first become important for the development of this organism. It was determined from RT-PCR experiments using RNA extracted from whole embryos from 2-72hpf (Figs. 9B and C and data not shown) that the fish progressively expressed form II, and congruent with its disappearance form III was expressed (Spinette, S., personal communication).

Based on the tissue-specific expression of the alternative splice forms of Ufd2a during models of muscle regeneration in adult myoblasts, it was hypothesized that larval

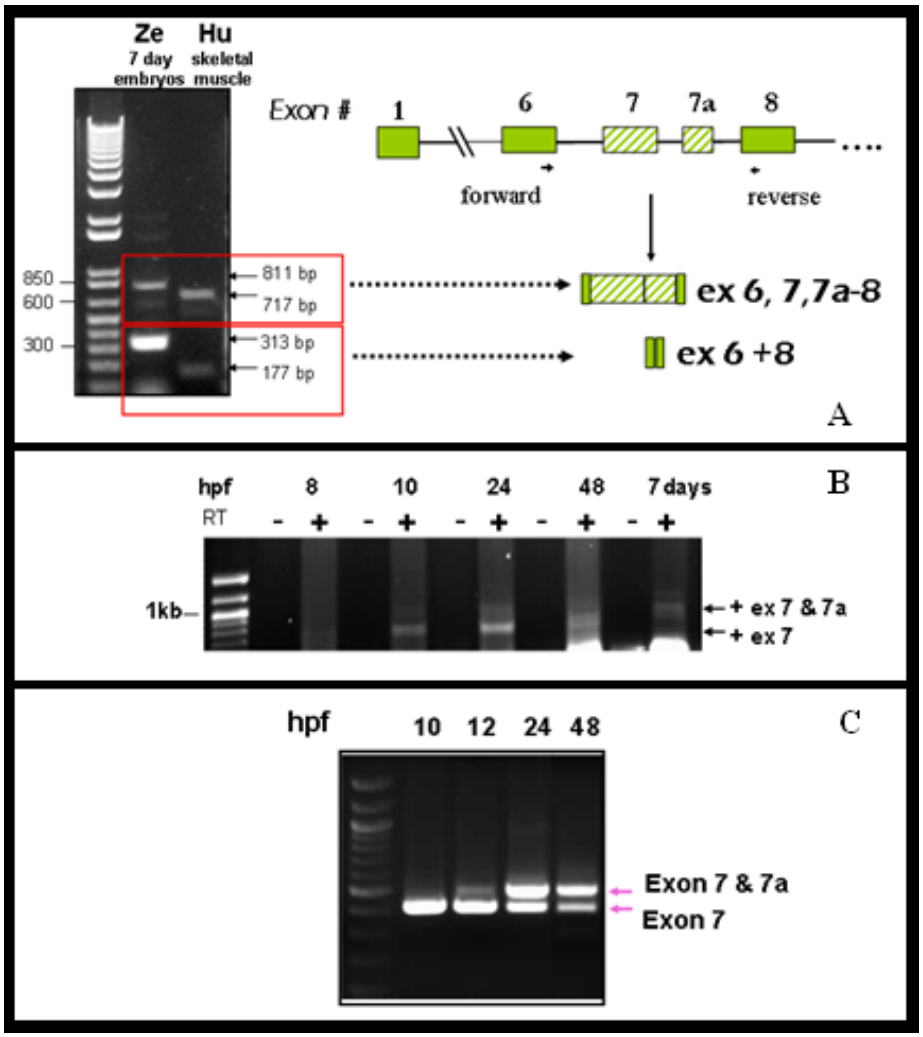
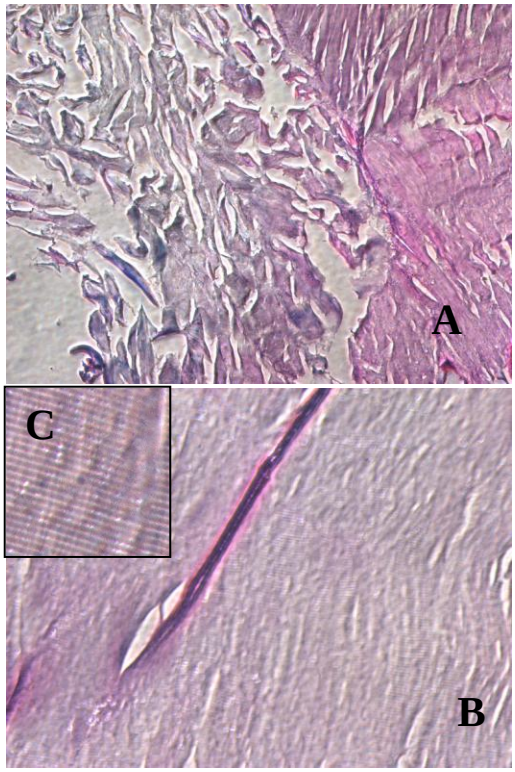


Figure 9. Temporal expression of the three alternative Ufd2a splice forms. (A) RT-PCR of 7dpf zebrafish embryos compared to that of human skeletal muscle. Lower bands correspond to exons 6 and 8, while higher running bands correspond to exons 6, 7, 7a, and 8. (B) RT-PCR of Ze cDNA shows that at 8hpf neither Ufd2aII nor III are present. However, at 10, 24, and 48dpf organisms manufacture Ufd2aII (exon 7), while embryos at 24 and 48hpf and 7dpf have Ufd2aIII (exons 7 and 7a). (C) Nested PCR analysis shows the diminution of form II and augmentation of form III as development of zebrafish progresses.

and adult zebrafish sections would show expression of Ufd2aI, II and/or III in cardiac and skeletal muscle depending on the time point during development. It was determined, therefore, that *in situ* hybridization would provide a good tool for testing this hypothesis.

In situ was first tried on adult zebrafish cryosections, which required a cryostat, or frozen microtome, and a tissue freezing medium to embed the specimen in a frozen block. Fish were segmented into a head, midbody, and tail region to make sectioning more manageable. During sectioning, the tissue sections often ripped and did not come off the blade in a single smooth slice (Fig. 10A). Chattering, in which the blade cut unevenly in a single section, was also a problem.

Figure 10. Adult muscle cryosections with eosin staining. (A) Highly degraded whole tissue, which was often observed. (B) A fairly continuous sheet of dissected tissue. (C) Inset showing skeletal muscle striation.

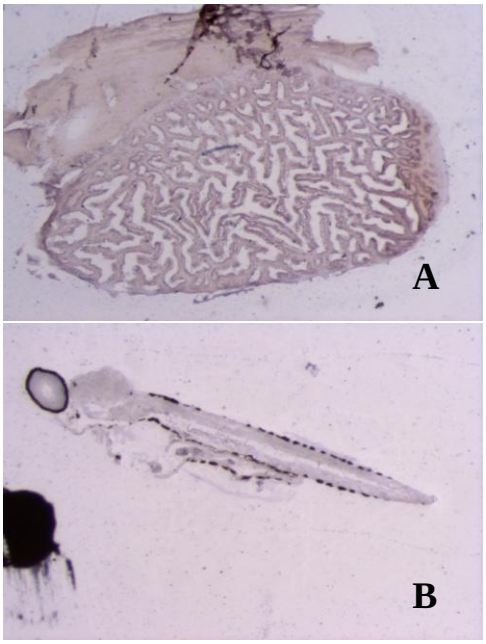


Therefore, scales and skin were removed to decrease the friction the blade encountered while cutting. Additionally, organs such as heart, brain, spleen, and skeletal muscle were removed from the fish and sectioned individually so that the blade only had a single tissue to cut through. These precautions produced a slight improvement in tissue preservation (Fig. 10B), demonstrated, for example, by the observance of sarcomeric striations with eosin staining (Fig. 10C). However, the tissue was also found to be damaged by freezing and thawing of the blocks. Curling of sections was also observed, which

was due to differential temperatures between the tissue and the blade. The sectioned tissue was often found to be degraded and dislodged from the slides during the *in situ* experiment. Therefore, because the frozen tissue sections were found to be impractical for this application, we next attempted to produce paraffin sections of the zebrafish.

Paraffin is a wax that is used in a manner similar to the frozen blocks. Tissue must be prepared first, however, by dehydrating through a series of graded ethanol steps followed by a stepwise transition into the wax via xylene, which is partially miscible in both ethanol and paraffin. The same type of blade used for the cryosections was employed, however the microtome used was different in that it did not produce frozen sections. Because the wax was less rigid than the frozen blocks and because the wax permeates the specimen's tissues, the sections were easier to process and handle. Again, adult zebrafish had their scales and skins removed and were cut into three body sections.

Figure 11. Paraffin sections. (A) Dissected adult brain section. (B) Fry showing early skin pigmentation patterns as black specks.



EDTA (ethylenediaminetetraacetic acid) which acts as a chelating agent to sequester metal ions, was used on adults to soften bone, thereby facilitating sectioning. Tissues were also dissected from the fish (Fig. 11A). Unfortunately, on most slides, parts of the tissue still separated from itself. Thus, it appeared that adult fish were just too large to cut through cleanly. Therefore, zebrafish fry were next used.

In order to perform ISH on fry, they first had to be raised. Raising fish proved to be a

challenge because embryos and early fry are delicate and are highly susceptible to infection. Fry raised to about 12dpf were chosen because younger fry were not developed enough and older fry had significant pigmentation, which can be difficult to discern from ISH signals (Fig. 11B). However, once fry were successfully raised, they could be used in experiments. Because the fry are much smaller, they were much easier to cut through because bone had not yet ossified and there was less of a chance for tissue separation. Also, multiple fry could be placed into the same paraffin block, increasing the number of sections per slide. Fixation of the fish was imperative because it cross-linked proteins, preserving the tissue. In a subset of experiments it was found that overnight treatment in 4% PFA in PBS produced better sections than 10% formamide, as the formamide severely degraded tissues.

Once paraffin blocks were made they had to be sectioned. The quality of the sections was affected by a number of variables, including blade angle, blade sharpness, section thickness, and block and blade temperature differences. Maximizing the quality of the sections was accomplished by adjusting the blade angle, sharpening or changing the blade, increasing the slice thickness, and cooling the blade with a refrigerant. Once sectioned, the ribbons had to be mounted onto the glass slides. However, because the tissue was not flat with the glass's surface, the majority of the tissue did not remain on the slides during the experiments. To remedy this problem, paraffin sections were first floated in a 57°C water bath to smoothen out the tissue. Once they were mounted onto the slides, they were dried overnight on a 48°C slide warmer, which helped to further flatten out the sections. Also, a comparison of the relative strength of tissue adhesion on APES slides compared to poly-L-lysine slides showed that the tissue remained adherent

to the APES coated slides more than to the standard poly-L-lysine slides. Thus, tissue adhesion to the slides was vastly improved using the two aforementioned techniques (data not shown).

Once, the quality of the tissue sections was suitable, *in situ* was performed using several positive control DIG-labeled riboprobes. SmyHC, which is expected to result in staining exclusively in skeletal muscle, showed that the protocol worked properly and could be used as a guide with regard to where to expect Ufd2a-specific staining

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Figure 12. ISH on juvenile zebrafish paraffin sections. (A, B, C) Sagittal sections of 17dpf fry with smyHC probe, where A and B are full body sections and C represents the cranial structure. (D) Coronal cranial sections of 8dpf fry probed with smyHC. (E) Sagittal section of 8dpf fry probed with exon 7. (F) Sagittal section of 8dpf fry probed with smyHC.

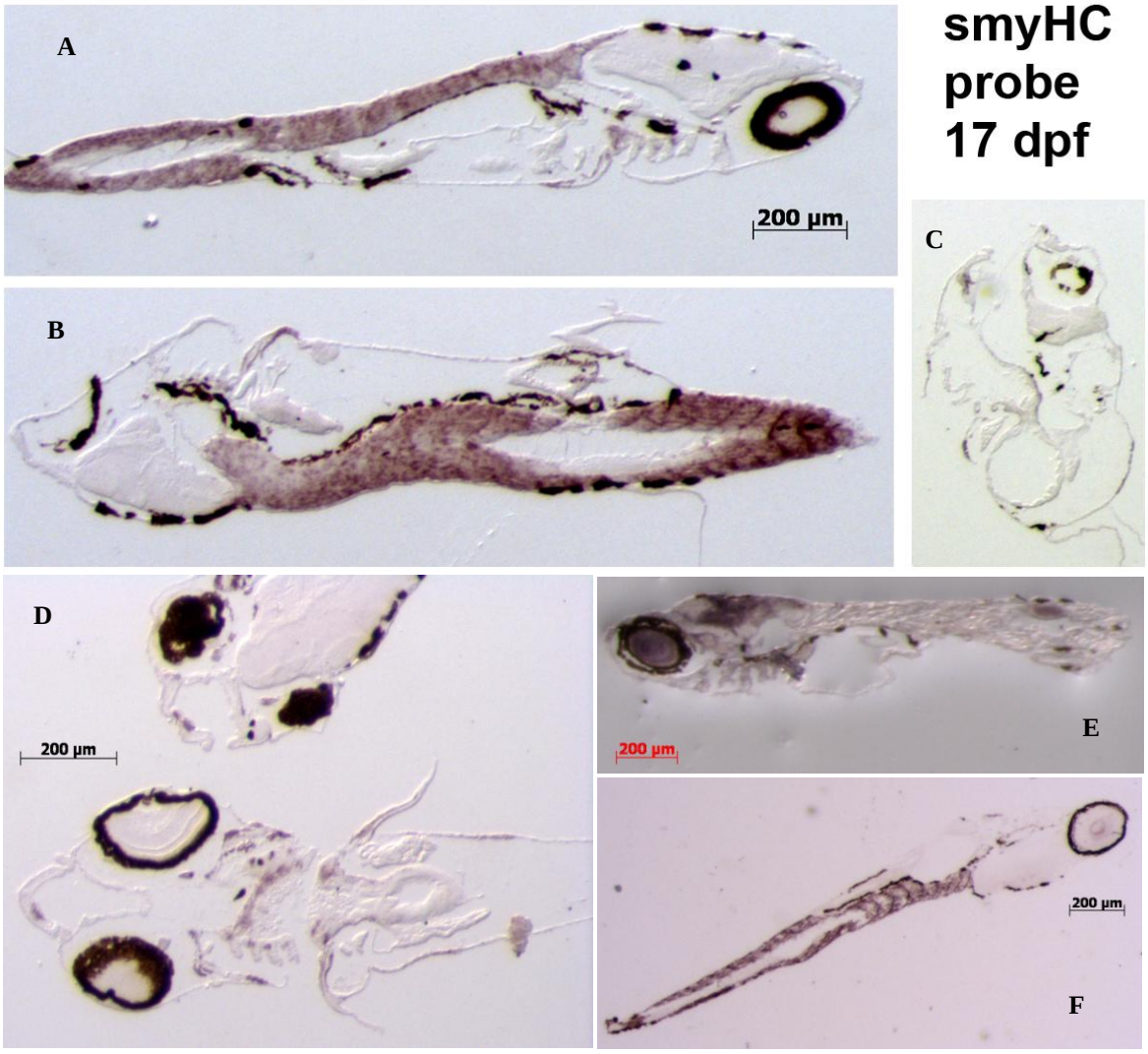
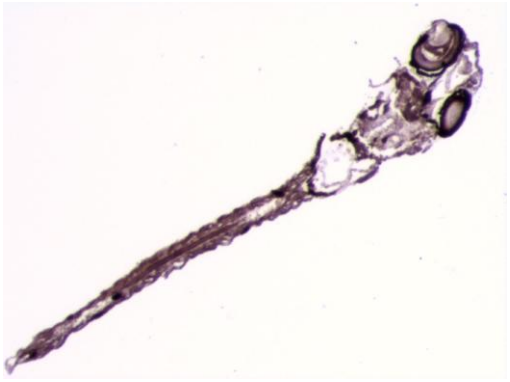


Figure 13. Paraffin section of fry showing non-specificity of probe, as staining was observed ubiquitously.



(Figure 12). Unfortunately, when the Ufd2a-specific riboprobes were then tested, the overall results were inconclusive in defending our hypothesis that larval and adult zebrafish would express Ufd2aI, II and or III in cardiac and skeletal muscle depending on the time point during development as much unspecific and inconsistent staining was observed (Fig. 13). Interestingly, hybridization with exon 7 probe did show staining only in the region of the brain while skeletal muscle remained unstained (Fig. 12E). This may suggest that Ufd2a has novel roles in neural tissue as well. However, these results are inconclusive since the quality of all of the Ufd2a specific probes was not verified in other experiments (see discussion below).

DISCUSSION

While there are numerous published protocols utilizing different laboratory techniques, they may require extensive modification in order to produce the desired level of signal to noise ratio, and they depend greatly on the type and age of the sample being examined. While *in situ* protocols for use on whole mount zebrafish embryos are widely available, it was found that detailed protocols for using this technique on fry or adult sections were difficult to come by, and each protocol that was found varied greatly from the others. Therefore, a detailed protocol had to be developed through varying iterations of the experiment in order to attempt to optimize it for this particular application. Thus, before our hypothesis could be rigorously tested, much trial and error had to be performed, and many technical challenges had to be resolved.

During the optimization process, several key variables were identified which were critical to tissue section quality, including fixation method, type of section, age of fish, and tissue adherence. Overall, our experiments determined that fixation in 4% PFA was preferable compared to 10% formamide and paraffin sections retained desirable tissue preservation compared to cryosections. In addition, adult fish were found to be too difficult to cut (possibly explaining the lack of protocols found in the literature using adult fish), so that zebrafish fry of about 12dpf were preferred. Finally, more complete tissue adherence was obtained using APES rather than poly-lysine coated glass slides, and pre-warming and flattening the freshly cut sections by floating them on top of a water bath was also critical. We noted that the incubation temperature used during the *in situ* experiment may be another factor affecting retention of the tissue on the slide, though this was not rigorously or systematically tested.

It was concluded that continuation of this project would require further optimization with regard to the protocol used. Because it was difficult at times to discern between staining and pigmentation, treatment of embryos with PTU to reduce pigmentation would be helpful. Furthermore, changing the concentrations of solutions and the duration of treatment could be optimized by changing these variables incrementally. Specifically, proteinase K treatment should be optimized, since treating the tissue for too short a duration does not permeabilize the cells enough, while too long a duration can degrade tissue. While ISH experimentation was conducted on whole-mount fry, it seemed to be that the probe had difficulty penetrating the whole organism, as absolutely no staining was observed. If this technique was to be further tested,

permeabilization methods would need to be explored considerably. Additionally, experimental probes would need to be enhanced.

Control ISH probes such as MyoD, smyHC1, and acta1 were shown to be specific for muscle tissue, as seen in both whole mount zebrafish embryos at early time points (data not shown) and in tissue sections (above data). However, our experimental Ufd2a probes did not show any specificity in whole mount embryos (data not shown) nor tissue sections (Fig. 12). Consequently it can be concluded that these probes most likely did not recognize Ufd2a transcripts within the cells. Additional measures that could be taken to give robust and specific results include modifying the probes' sequences and lengths so as to increase specificity and binding.

Other studies performed on human cell lines found that Ufd2, regulated by phosphorylation, is a necessary component in mitosis. Knockdown of gene expression using siRNA targeted against Ufd2 resulted in mitotic arrest at the metaphase-anaphase transition, suggesting that this enzyme is necessary for cellular differentiation and development.[25] This, along with other evidence, suggests that Ufd2aI should be present in all dividing cells since it is critical to mitosis. RT-PCR experiments performed on adult zebrafish tissue RNA showed that Ufd2a II and III appear to be muscle specific (Fig. 14A) in a pattern similar to adult mammals. In addition, upon induction of differentiation using C2C12 mouse myoblast cultures, form II production followed rapidly by form II production is evident. This further suggests a role for the alternative splice forms of Ufd2a in development and differentiation of muscle cells (Fig. 14B). Therefore, we still hypothesize that with specific probes for the three isoforms of zebrafish Ufd2a, we would find Ufd2aI in all dividing cells of the fry while we would

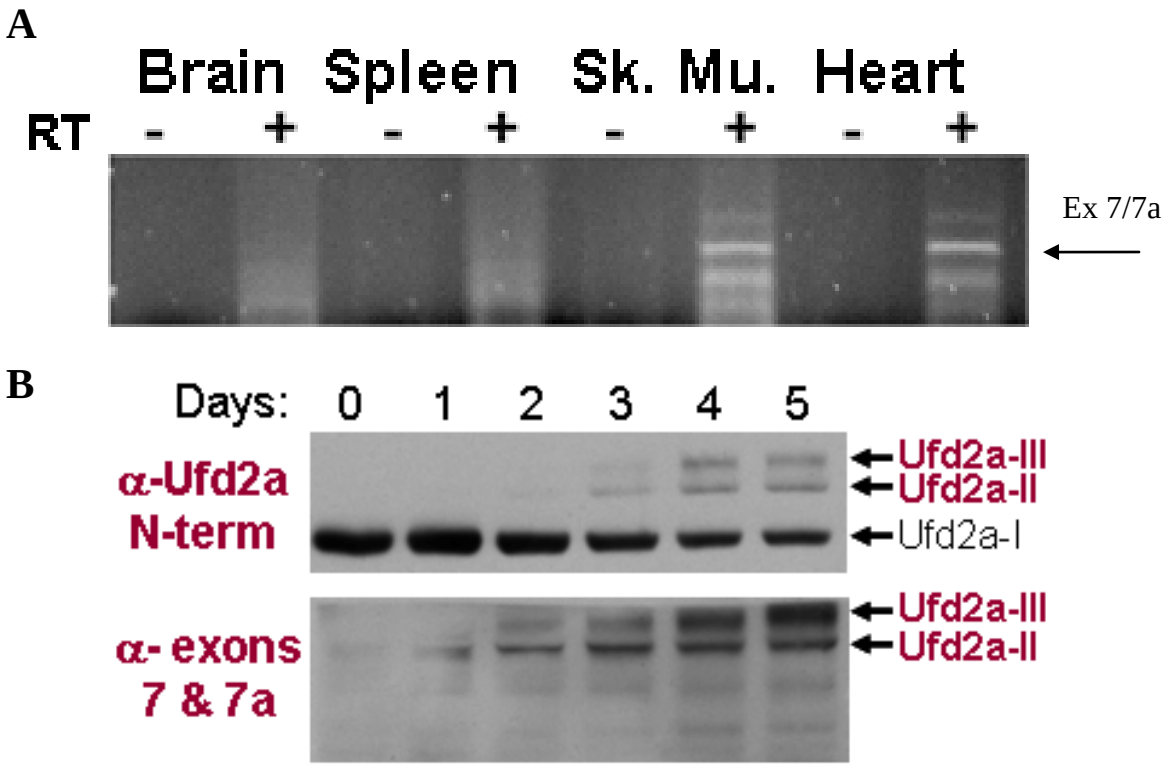


Figure 14. Muscle tissue specificity of Ufd2. (A) Gel electrophoresis of RT-PCR products using primers 7 forward and 7a reverse show that exon 7/7a is present only in skeletal muscle in adult zebrafish. (B) Western blot showing expression of forms II and III in C2C12 cells upon differentiation.

find Ufd2aII only in the maturing muscle cells and Ufd2aIII in the mature muscle fibers of the somites and heart.

There is still much to be learned about Ufd2, including the function and spacio-temporal expression patterns of the alternative splice forms, their substrates, and their specific roles in development and differentiation. Novel roles of this protein and its isoforms may be discovered as more experimentation is performed. For instance, Ufd2 has been identified as an autoantigen in the autoimmune disease scleroderma. Findings like this suggest Ufd2's importance within cells and knowledge of its role in biological pathways may contribute to developing medicinal treatment for such diseases as

scleroderma. In addition, determining the significance of the alternative spicing of Ufd2a during differentiation may provide insights into how E3 ligase activity or substrate binding may be regulated by the presence of novel protein domains.

Finally, I would like to again emphasize that biological research is unequivocally essential for elucidating the biological mechanisms that are involved in causing diseases. Once the root of the issue has been identified, further investigation into treatment options and the development of therapies and therapeutic drugs can commence.

SUPPLEMENTAL FIGURES

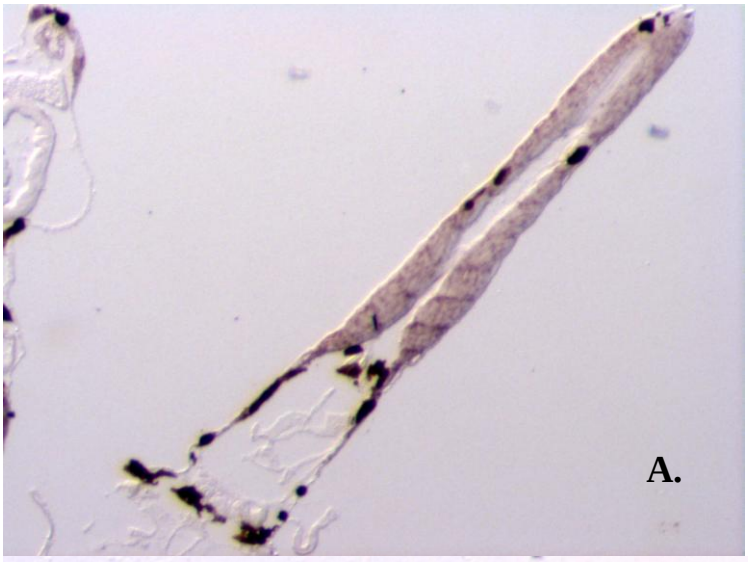
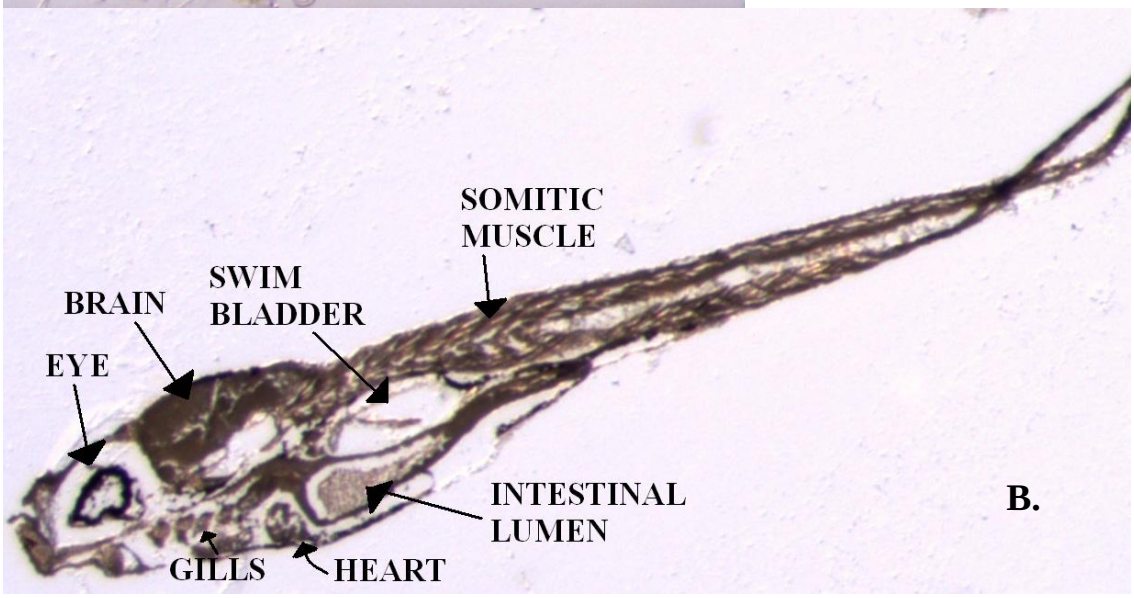
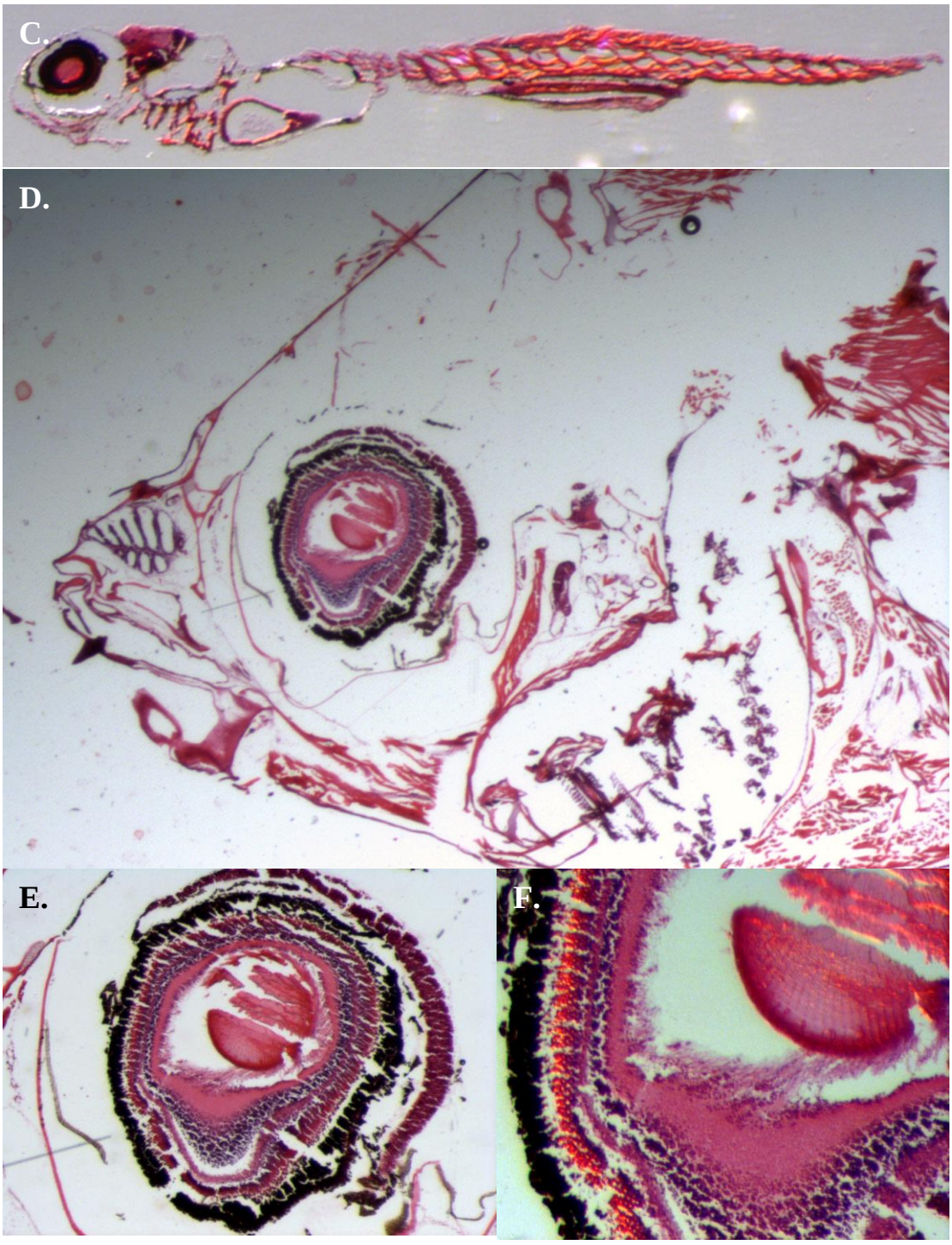


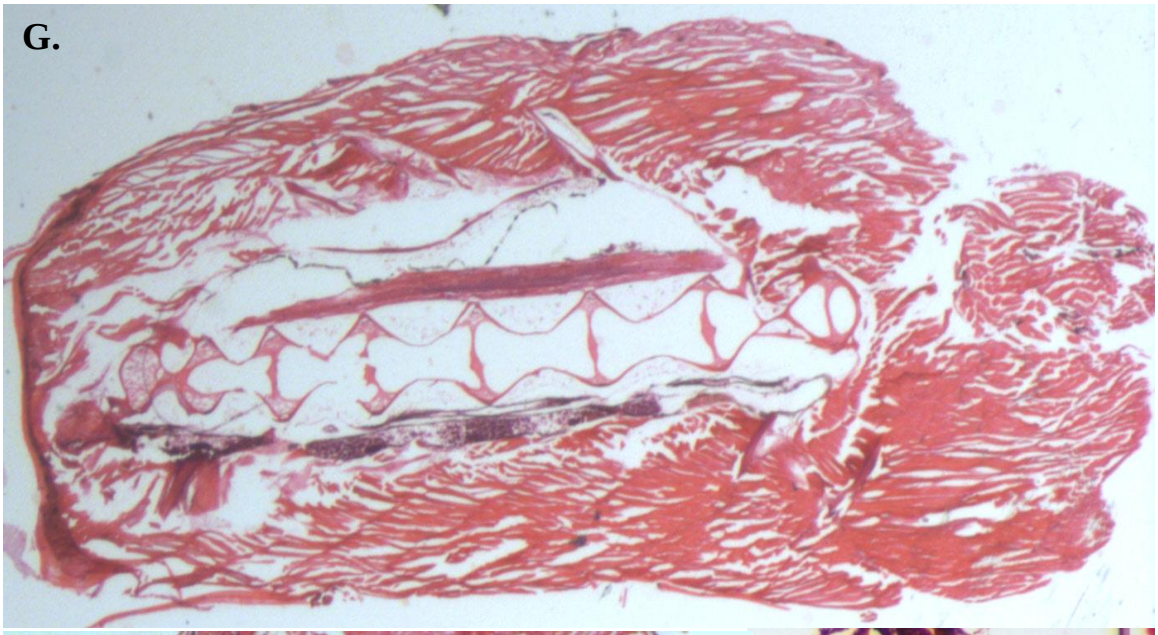
Fig. A
ISH using smyHC on 17dpf zebrafish fry. Staining is localized only to the skeletal muscle.

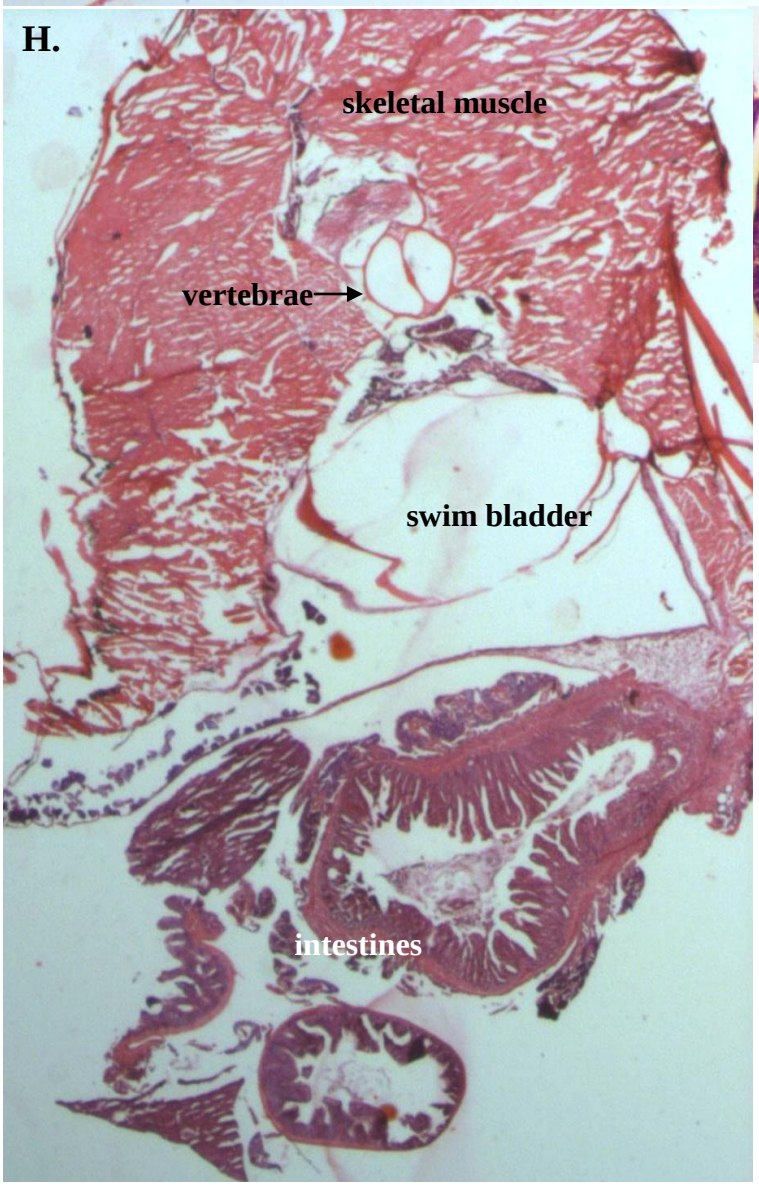
Fig. B
Paraffin tissue section showing fry anatomy.

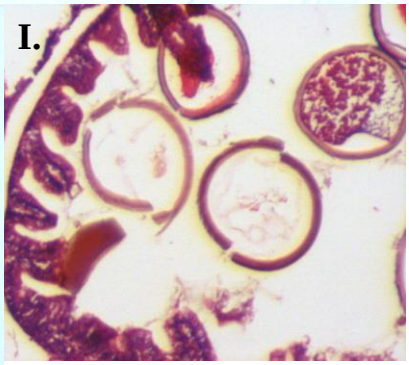




H & E staining of zebrafish tissue sections. Fig. C- Fry. **Fig. D-** Adult head. **Fig. E-** Adult eye. **Fig. F-** Close-up of adult eye.





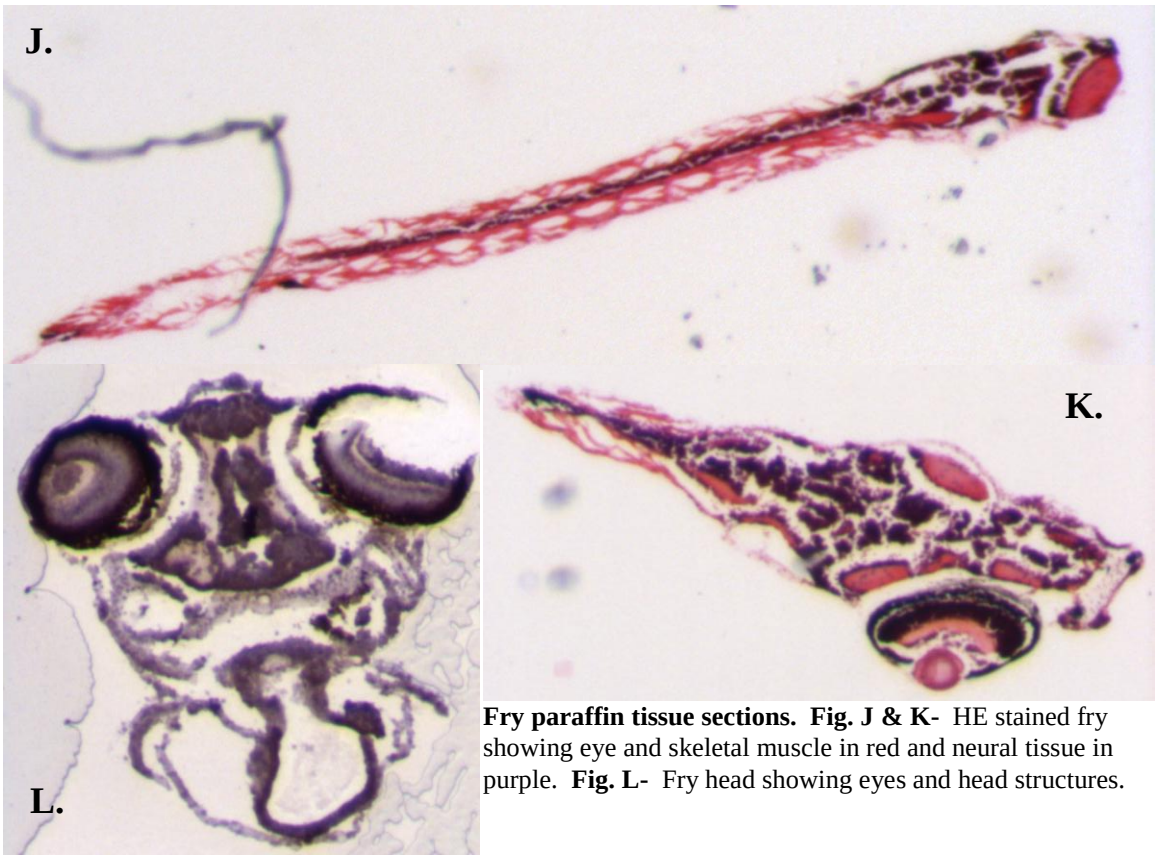


H & E staining of adult zebrafish tissue sections.

Fig. G- Sagittal section showing skeletal muscle and spinal column, including vertebrae and spinal cord.

Fig. H- Transverse section showing skeletal muscle, vertebral column, swim bladder, and intestines.

Fig. I- Close-up of intestines showing intestinal epithelia and artemia eggs within the lumen.



Fry paraffin tissue sections. Fig. J & K- HE stained fry showing eye and skeletal muscle in red and neural tissue in purple. **Fig. L-** Fry head showing eyes and head structures.

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