Exploring the Mechanisms Involved in Paxillin Amerlioration of Dystrophies in Zebrafish

Margaret E. Pasquarella
University of Maine

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EXPLORING THE MECHANISMS INVOLVED IN PAXILLIN AMELIORATION OF DYSTROPHIES IN ZEBRAFISH

by

Margaret E. Pasquarella

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Zoology)

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Advisory Committee:
Clarissa A. Henry, Associate Professor of Biological Sciences, Advisor
Michelle Goody, Postdoctoral Research Associate in Biological Sciences
Roger Sher, Assistant Professor of Biochemistry and Microbiology
Len Kass, Associate Professor of Biological Sciences
Mimi Killinger, Adjunct Assistant Professor in Honors (History)
Abstract

Muscle cells must anchor to their environment, the extracellular matrix (ECM), in order to function properly. Muscular dystrophy occurs when muscle cells cannot anchor to the ECM because specific protein complexes, such as the dystroglycan complex, are disrupted. Previously published studies have shown that overexpressing Paxillin can reduce dystrophy in \textit{dag1} deficient embryos. The aim of this study is to determine which domains of Paxillin are necessary to rescue dystrophy by overexpressing Paxillin with the LD2 or LD4 domains knocked out in dystrophic embryos. However, disruption of \textit{Dag1} via the previous method stopped producing the muscular dystrophy phenotype, so ethanol (EtOH) treatment was used to induce dystrophy. It was determined that this is not a sufficient method of inducing dystrophy for this study. The EtOH treatment was inconsistent, and Paxillin overexpression was unable to rescue dystrophy in EtOH treated zebrafish. Furthermore, the dystrophy seen in EtOH treated embryos was much more severe than that seen in typical muscular dystrophy. Although this experiment could not be used to determine the necessity of the LD domains of Paxillin, it did give insight into the toxicity of EtOH. This information will be useful as we move on using different techniques.
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Introduction

Cells are the building blocks of our body and it is critical to our survival that they function properly, but most cells cannot do this if they are not anchored to their environment (the extracellular matrix or ECM). This ECM is the substance that lies between cells and contains many proteins that act as a point of attachment for cells. This requirement for attaching to the ECM is especially true of muscle cells, which function by continuously contracting and relaxing; exhibiting a force on their environment. Thus, muscle cells cannot function in voluntary movement of the body on their own and must have some component to anchor them. This component is the myotendinous junction (MTJ), where muscles anchor to tendons. The MTJ is rich in extracellular matrix. During zebrafish early embryonic muscle development small precursor cells elongate to the MTJ and then bind to ECM proteins at the MTJ as an anchor.

Two of the major types of macromolecules found within the ECM are the proteoglycans, a gel like substance that fills the interstitial space between the proteins, and the fibrous proteins, mostly collagens, elastins, fibronectins, and laminins. Many of these adhesion proteins are found within a subunit of the ECM called the basement membrane. Of particular importance is laminin, which is a cross-shaped protein found within the basal lamina of the basement membrane. It is involved in binding the intracellular actin cytoskeleton of the muscle cell to the basement membrane of the MTJ. This attachment can either occur through laminin binding to transmembrane integrin proteins of the cell, or through laminin binding to the dystroglycan complex (DGC).
The DGC is a transmembrane protein complex consisting of alpha and beta subunits. The intracellular β-dystroglycan subunit lies within the membrane and binds to a rod-shaped protein called dystrophin in the cytosol of the cell.\textsuperscript{[5]} Dystrophin anchors β-dystroglycan to actin within the cytoskeleton. The α-dystroglycan subunit binds to β-dystroglycan within the membrane, but also has a region that lies outside of the membrane and links to laminin. The LG4-5 tail region of laminin binds to the highly glycosylated α-dystroglycan subunit while the alpha-1 LN at the top of laminin binds to type IV collagen in the BM.\textsuperscript{[6]}

There are multiple pathologies that result from a disruption of this attachment. This is the case in many of the congenital muscular dystrophies (CMD) including Duchenne (DMD) and Becker muscular dystrophies, which have a mutation in dystrophin. These are x-linked recessive diseases that appear most frequently in young males.\textsuperscript{[7]} The major symptom is muscle wasting, which can become so severe that it limits lung and cardiac function. Children are typically diagnosed between three and five years of age, and in the case of DMD generally have a short life expectancy of about 25 years. Becker muscular dystrophy is caused by a different dystrophin allele, is milder, and occurs less frequently.\textsuperscript{[5]}

In both variations of the disease the mutation on the X chromosome is for the intracellular protein dystrophin.\textsuperscript{[5]} When this is altered or missing β-dystroglycan is unable to anchor to the actin cytoskeleton. With use, the muscle cell-BM attachment and/or the muscle membrane tear, and the muscle cells recoil and degenerate. This results in atrophy of the muscle that worsens over time, generally causing significant reduction
in muscle mass. The severity varies depending on the individual and on the type of dystrophy, but in many cases their life is severely impaired.

An excellent model for studying muscular dystrophies is the zebrafish. Zebrafish have external fertilization, which makes them ideal compared to other model organisms, such as mice, that gestate in utero. This form of reproduction allows us to view and manipulate early stages of development beginning at fertilization. Zebrafish also have translucent bodies, making visibility of muscle segmentation distinctly clear even under a simple dissecting microscope. Above all of this, the zebrafish share 70% of the same DNA as humans [8] and display the same phenotypes for congenital muscular dystrophies. This includes conserved sequences for dystrophin and dystroglycan across humans and zebrafish.

Recent studies have used zebrafish as a model to study the relationship between muscle cells and the MTJ as a way to treat dystrophy. The Henry lab has shown how activating the Nrk2b pathway can result in increased polymerization of laminin at the MTJ and improved muscle cell attachment. This involved supplementing exogenous NAD+ precursors to Dagl morphants, which are embryos that lack dystroglycan and express symptoms of a type of muscular dystrophy known as dystroglycanopathy. This treatment was successful in improving both muscle health and motility [9]. Paxillin, which is a cell adhesion molecule found downstream of the NAD+ pathway, was also discovered to contribute to a reduction in muscle fiber detachments when the gene for it was overexpressed in Dagl morphants. Dagl morphant embryos showed improved muscle health as well as increased organization of laminin at the MTJ. Paxillin overexpression did not, however, rescue motility [9].
This elicited some curiosity in how Paxillin is acting within the cell; specifically how it is contributing to laminin polymerization. This is the central question behind this research. Paxillin is a cell adhesion molecule that is believed to be involved in multiple signaling pathways. There are several domains within Paxillin, several of which act as docking sites for other proteins \(^{[10]}\). One such example is with the integrins, but it is unclear what the mechanisms are behind this relationship. The study done by Goody et al. displayed a correlation between increased Paxillin and increased polymerization and organization of laminin at the MTJ. Understanding the mechanisms behind this could potentially lead to new opportunities in treatment of muscular dystrophy.

One way to glean this information would be to look at each domain of Paxillin and determine which ones are contributing to its ability to ameliorate dystrophy. This could be done by overexpressing Paxillin with specific domains knocked out in a transgenic embryo and looking to see if dystrophy is still rescued. Once it is determined which domains are necessary it would be possible to look at the proteins that interact with those domains. With this knowledge, we could look downstream of the signaling pathways of those proteins to come up with a mechanism. Thus, the purpose of this thesis is to investigate domains of Paxillin that play a critical role in ameliorating muscular dystrophy.
Materials and Methods

Zebrafish Husbandry:

Zebrafish were set up for natural spawning in a facility that is kept on a 16-hour light/8-hour dark cycle. The zebrafish lines of interest that were used for this study were transgenic for overexpression of Paxillin. This process involves inserting a transgene (such as Paxillin with a domain knocked out) into the single celled embryonic stage. The transgene is intended to be inserted wherever there are transposable elements within the genome. However, it is not a perfect system and the transgene does not always get inserted. Furthermore, in order to be passed on to the next generation the transgene must be inserted into an autosome. It is not guaranteed that this process will be successful, thus it is necessary to cross male and female pairs and identify the embryos as carriers of the transgene. The inserted Paxillin transgene is tagged with green fluorescent protein (GFP), which fluoresces under a UV microscope. This is used to identify carriers.

Once a male and female pair has been identified as carriers of the transgene they are moved into a new tank. The offspring that also carry the transgene can then be reared and breed within a few months. The first part of this thesis was to identify the carriers of the transgene that would be used for further studies. Eggs from these identified parents were collected in the morning and kept in embryo rearing medium (ERM) in a 28°C incubator.

Investigating Different Ethanol (EtOH) Dosages

Preliminary research was required to determine the dilution of EtOH that was sufficient for inducing fiber detachment in the embryos. Embryos were reared in different
solutions of varying dilutions of ERM:EtOH across two different lines of Paxillin transgenics: HS Paxillin GFP (green fluorescent protein) and β-actin Paxillin GFP.

The β-actin Paxillin embryos were divided into four groups: 0% EtOH, 1.5% EtOH, 1.75% EtOH, and 2% EtOH. To make each solution 200 proof EtOH stock solution was diluted in ERM. Embryos were reared in pure 1x ERM immediately following egg collection and were transferred to the EtOH solution at 24hpf (hours post fertilization). The EtOH solution was changed with fresh solution daily and dead embryos and empty chorions were removed. The embryos were fixed at 72hpf. All the embryos fluoresced positive for GFP, thus there were no wild type controls for this trial.

There was a much lower yield of HS Paxillin embryos than β-actin Paxillin, so it was only possible to use two groups. These were 1.75% EtOH heat-shocked embryos, and 1.75% EtOH non heat-shocked embryos. Heat-shocking is necessary to activate the overexpression of Paxillin. 24 hpf embryos were heat-shocked in an incubator at 42°C for one hour and 30 minutes. Since there was such a low yield of embryos there were no 0% EtOH controls.

Investigating the Necessity of the Paxillin LD2 Domain in Ameliorating Dystrophy:

The HS Paxillin (-LD2) GFP transgenics overexpress Paxillin with the LD2 domain knocked out. They were divided into four groups: 0% EtOH heat shocked, 0% EtOH no heat-shock (controls), 2% EtOH heat-shocked, and 2% EtOH no heat-shock. To make each solution 200 proof EtOH stock solution was diluted in ERM. Embryos were reared in pure 1x ERM immediately following egg collection. The embryos were heat-shocked at 42°C for one hour and 30 minutes, and then immediately transferred to the 2% EtOH solution. The EtOH solution was changed daily and dead embryos and empty
chorions were removed. The embryos were fixed at 72hpf. Not all of the heat-shocked embryos fluoresced positive for GFP under a fluorescent light, thus some controls were also built into the trial.

**Investigating Paxillin Overexpression with LD2 or LD4 Domain Knock-outs:**

Three lines of Paxillin transgenics were used for this trial: HS Paxillin GFP, HS Paxillin (-LD2) GFP, and HS Paxillin (-LD4) GFP. Each line was divided into two groups: 0% EtOH heat-shocked and 2% EtOH heat-shocked. The embryos were heat-shocked at 42°C for one hour and 30 minutes, and then transferred to the 2% EtOH solution. This solution was made by diluting 200 proof EtOH stock solution in 1x ERM. The EtOH solution was changed daily and dead embryos and empty chorions were removed. The embryos were fixed at 72hpf.

**Immunofluorescent Staining:**

The embryos are contained within a thin membrane called the chorion at fertilization, but most break out of this by 72hpf. The chorion was manually removed with forceps for those that had not, and the embryos were added to tubes in groups of 10-15. This prevents the embryos from fixating in a curled position, and makes imaging much cleaner. The embryos were fixed in 4% paraformaldehyde (PFA) for four hours at room temperature on a rocker, or overnight at 4°C. This was followed by rinsing five times for five minutes each in PBS-0.1% Tween20 (PBS-Tw) on a rocker. The embryos were then permeabilized in PBS-2% Triton (PBS-Tx) solution for one and a half hours on a rocker.

Immediately following permeabilization 20µL of 1:20 dilution of Phalloidin: Alexa fluor 546: PBS-Tw were added to each tube. The tubes were kept under foil from
this point forth since phalloidin degrades under light. The embryos were phalloidin stained either from 2-4 hours at room temperature on a rocker, or overnight at 4°C. They were then rinsed in PBS-Tw for five times for five minutes each.

**Imaging:**

It is necessary to remove the yolk from the embryos and sever the head before attempting to image them as this will facilitate clear images by allowing the embryos to lie flat. The yolk was removed in 1% PBS with small dissecting pins. The embryos were then mounted on a slide in 20% PBS/80% glycerol. The embryos were imaged with a Zeiss Axio Imager Z1 microscope with a Zeiss ApoTome attachment. Images were taken at the 20x objective with five frame averaging.

Z-stack images were obtained at 0.55µm thick slices with several filters. A rhodamine filter was used to view the F-actin in the muscle cells that were phalloidin stained and depicted in red. The green staining is showing the GFP tag on Paxillin with the LD2 domain knocked out.
Results

In order to determine which domains of Paxillin are important to ameliorate dystrophy we need a zebrafish model of muscular dystrophy. Unfortunately, around the time I began this research, the standard Henry lab technique for generating zebrafish with muscular dystrophy stopped working (we used morpholinos and the company changed its purification methods rendering them more useful for cell culture experiments but more toxic in zebrafish). Thus, I needed a new way to generate dystrophy. I turned towards using EtOH exposure as a way to induce dystrophy in the zebrafish. Most of the current research on EtOH has been on pre-muscle development for the purpose of studying fetal alcohol syndrome. The Henry Lab has been interested in the effects of EtOH in post-muscle development. Preliminary studies have shown that adding EtOH after 24hpf can induce dystrophy in wildtype zebrafish. This will be a useful technique for studying dystrophy since EtOH is both easy to use and readily available.

Dystrophy can then be measured by the number of embryos that display fiber detachments as well as the overall percent of myotomes that have fiber detachments in each embryo. These measurements would then be indicative of the ability of Paxillin to ameliorate dystrophy. Comparisons can be made between zebrafish with different domains knocked out as well as to zebrafish that are expressing the complete Paxillin sequence.

Overview of Transgenics and Treatments:

We have multiple controls for these experiments. β-actin Paxillin zebrafish express Paxillin from a constitutive (always active) promoter. Alternatively, HS promoter allows us to control when during muscle development Paxillin is turned on by heat
shocking the embryos. However, we also need a group of zebrafish transgenic for HS Paxillin that are controls and are not heat shocked. We will refer to these as non-heat shocked Paxillins.

**Investigating Different EtOH Dosages:**

Before we could begin to test the efficacy of Paxillin with different domains knocked out we had to determine what dose of EtOH would be sufficient to induce dystrophy. The β-actin Paxillin control group that was not treated with EtOH showed no adverse muscle development, as expected. The β-actins treated with 1.5% EtOH also had healthy muscle with no fiber detachments (fig 4). The 1.75% EtOH treated β-actins however had 17.6% occurrence of dystrophy across all embryos at n = 28. The HS Paxillin embryos that were heat-shocked and treated with the same 1.75 % EtOH dose only had a 3% occurrence of dystrophy at n = 32. This result was initially exciting, but then analyzing the other control group, the non-heat shocked Paxillins at 1.75% EtOH, showed normal muscle development with no fiber detachments. Thus, because the non-heat shock Paxillin controls did not show dystrophy it is difficult to make any conclusions from this experiment.

The β-actin Paxillins that were treated with a 2% EtOH dilution also had a 17.6% occurrence of dystrophy across all embryos at n = 28 (fig 2). Embryos from this group that did not have fiber detachments displayed abnormally curvy muscle cells (fig 3). Although the β-actin Paxillins displayed more dystrophy with EtOH treatment than the HS Paxillins, the Henry Lab only has transgenics with different domains of Paxillin knocked out with the HS promoter. Since all of the subsequent studies would involve domain knock outs it was decided to use a 2% EtOH dilution for future studies since
1.75% EtOH treatment had too low of an occurrence of dystrophy in the HS Paxillins. Figure 4 shows a complete comparison.

**Investigating the Necessity of the Paxillin LD2 Domain in Ameliorating Dystrophy:**

One of the domains of Paxillin that we predicted might play a role in rescuing dystrophy is the LD2 domain. I was interested in this domain because it has been recognized as acting as a docking site for focal adhesion kinase (FAK).\(^{[13]}\) Thus, if Paxillin is unable to rescue dystrophy with the LD2 domain knocked out that would indicate that FAK may be involved downstream of the signaling pathway. Unfortunately, this experiment is also difficult to interpret because of contamination of the dishes. We have found that sometimes contamination can cause aberrant development that is not specific to the treatment/mutation we are trying to study.

At 24hpf all the petri dishes had contamination. Most were in the control dishes but some were in the EtOH treated dishes as well. This may affect the results. The non-heat shocked HS Paxillin embryos with no EtOH treatment acted as the controls for this experiment. As expected, both the wild type and the fluorescing (signifying they are transgenic) embryos had no fiber detachments (fig 5) since they were not treated with EtOH. The heat shocked HS Paxillin had a 6% occurrence of dystrophy across all embryos at n = 18. This 6% is a bit misleading because it is all due to the fact that one embryo had fiber detachments on four myotomes (fig 6). This outlier could have occurred because the heat shock treatment done at 42 °C for 1.5 hours, which could be too severe. One other reason could be that a number of the genes that promote muscle cell adhesion to laminin are maternally expressed.\(^{[11]}\) Thus, if the mother does not happen to deposit enough RNA and proteins in the eggs they will be more likely to show
dystrophy. Some other unknown event may have resulted in dystrophy in this embryo as well, but regardless of the reason for the outlier, 17/18 embryos had normal muscle development.

The next part of this experiment was to heat shock the HS Paxillin (-LD2) embryos that have the LD2 domain knocked out and compare them to HS Paxillin embryos with full length Paxillin. The heat-shocked HS Paxillin (-LD2) GFP embryos that were treated with 2% EtOH and did not fluoresce, meaning they weren’t overexpressing Paxillin with the LD2 domain knocked-out, had a 52% occurrence of dystrophy across all embryos at n = 25 (fig 7). The average number of myotomes with fiber detachments was 3.76 myotomes (fig 8). This average was low since it takes into account all the embryos in the group that had no fiber detachments, however many of the affected embryos had extremely severe dystrophy with as many as 19 myotomes with dystrophy (fig 9). We do not understand the etiology of this variation, but some potential causes of this variability will be considered in the discussion.

The experimental embryos are those that express HS Paxillin (– LD2) and fluoresced, indicating that they are GFP positive and transgenic. If dystrophy is still rescued with the LD2 domain knocked out, then that domain is not necessary for amelioration of dystrophy. Unfortunately, in this experiment, there were only two embryos that fluoresced. The heat-shocked HS Paxillin (-LD2) GFP embryos that were treated with 2% EtOH and fluoresced had a 50% occurrence of dystrophy n = 2. The one embryo with fiber detachments shown in figure 10 (red showing F-actin and green showing GFP) had the same severity of dystrophy as the non-fluorescing group had. This embryo had 19 myotomes with fiber detachments.
Investigating Paxillin Overexpression with LD2 or LD4 Domain Knock-outs:

Another domain of Paxillin that we predicted might play a role in rescuing dystrophy is the LD4 domain. Fibroblasts that express the LD4 domain have been associated with poor focal adhesion, thus we predict that knocking out this domain may actually make Paxillin more efficient in ameliorating dystrophy. This domain is also a docking site for FAK, just like the LD2 domain, and has the potential for downstream signaling.\[13\] This experiment also retested 2% EtOH treatment on the HS Paxillin (-LD2) and HS Paxillin embryos. However, at 72hpf both the HS Paxillin (-LD4) and HS Paxillin (-LD2) embryos that were treated with 2% EtOH were dead. These embryos had been alive at 48hpf when the EtOH solution was changed. Since the breeding zebrafish with the Paxillin LD4 domain knock out were so young and still growing, we were unable to obtain another spawn from them. Thus, no further testing with the LD4 knock out was completed in this study.

The HS Paxillin embryos with 2% EtOH survived and were used for further analysis. The heat shocked HS Paxillin controls with no EtOH treatment had no fiber detachments at \( n = 10 \) embryos (fig 11). The heat shocked HS Paxillin embryos that were treated with 2% EtOH and fluoresced (were transgenics) had a 60% occurrence of dystrophy across all embryos at \( n = 10 \). Those that had fiber detachments appeared to be just as severe as the HS Paxillin (-LD2) embryos from the previous experiment (fig 12). A comparison of the occurrence of dystrophy for both groups is shown in figure 13. All of the EtOH treated embryos from each experiment had the majority of the fiber detachments occur nearer to the head region than the tail (fig 14). These embryos also exhibit very short and wide myotomes. The embryos from the EtOH treated HS Paxillin
embryos with the domain knock outs also appeared short and stumpy when viewed under a dissecting microscope. Control embryos did not express this phenotype, implicating this happening in response to the EtOH treatment. Potential reasons for these differences will be discussed further in the discussion.
Discussion

The results from these experiments are not conclusive since every experiment had something go wrong – not enough controls, not enough experimental embryos, contamination, etc. However, these experiments do give insight into the dilution of EtOH that is sufficient to induce dystrophy as well as allude to some interesting differences between the different Paxillin transgenic lines.

Investigating Different EtOH Dosages:

There was a difference of 14.6% in the responses of the β-actin Paxillins and the HS Paxillins to 1.5% EtOH. This discrepancy across the lines makes studying the ability of overexpression of Paxillin difficult using EtOH. More surprisingly, the non-heat shocked HS Paxillins at 1.5% EtOH having seemingly healthy muscle suggests an inconsistency with the EtOH treatment since this was the same dose on the same line. This could be due to the heat shock process facilitating fiber detachment by stressing the muscle. The Henry Lab recently determined that the temperature of 42°C may be much higher than the level the embryos can withstand before muscle formation is affected. A shorter exposure time or a lower temperature may be required to avoid malformations.

Investigating the Necessity of the LD2 Domain in Ameliorating Dystrophy:

The heat-shocked HS Paxillin (-LD2) GFP embryos that were treated with 2% EtOH that both fluorescing and non-fluorescing (were not transgenic) showed extreme dystrophy. Not only did they have an unusually high amount of fiber detachments, but most of the dystrophy was seen anteriorly more frequently than posteriorly. Under normal circumstances fiber detachments are more numerous in the tail region around myotomes 10-15. These embryos with 2% EtOH treatment had fiber detachments
occurring more frequently in the anterior region around myotomes 5-10. This could be occurring in response to the age of the muscle cells. Zebrafish embryos develop in a head to tail fashion, thus the muscle cells in the tail are much younger than the muscle cells near the head. Perhaps the older muscle cells are more sensitive to the EtOH treatment than the younger ones.

The contamination of the dishes must also be regarded when trying to determine the cause of the unusually severe dystrophy. The Henry Lab has observed that contamination in the dishes generally results in curled embryos after hatching from the chorion. In most cases motility is negatively affected. The control groups for this experiment, heat shocked 0% EtOH and non-heat shocked 0% EtOH, had straight, healthy looking bodies before fixing at 72hpf. Some of the EtOH treated embryos had curled or stumpy bodies, but this has also been observed with EtOH treatment when there was no contamination present. However, the EtOH treatment may contribute to the contaminating microorganisms thriving better than in ERM solution alone and result in severe dystrophy. This is only conjecture and cannot be validated without further testing.

Only two embryos fluoresced (were transgenic) which is too small of a sample to be able to glean any information from. Furthermore, although the transgenic HS Paxillin (-LD2) did not rescue dystrophy this cannot be linked to a lack of an LD2 domain since the full length HS Paxillin embryos also could not rescue dystrophy with EtOH treatment. Thus, domain knock outs cannot be studied using EtOH treatment as a means to induce dystrophy. This is more evident from the results of the next section.

Investigating Paxillin Overexpression with LD2 or LD4 Domain Knock-outs:
It was very unfortunate that all of the HS Paxillin embryos with the LD2 and LD4 domains knocked out died since a comparison of the two had the potential to yield interesting results. However, since the HS Paxillins that survived and had full length Paxillin had a 60% occurrence of dystrophy for 10 embryos, it is evident that Paxillin is unable to rescue dystrophy at all that is caused by EtOH. Thus, different methods will have to be implemented in future studies to study the different domains of Paxillin and amelioration of dystrophy.

Here I will hypothesize some of the reasons behind the trends we observed (if one temporarily ignores the caveats of the experiments). The major issue with using EtOH treatment was that it was extremely inconsistent. A dose that was sufficient to induce phenotypic symptoms of muscular dystrophy in one experiment would annihilate whole dishes of embryos when it was used for another. This suggests that EtOH is overall toxic to the zebrafish embryos. Because of this, there is a limit to the amount of EtOH they can withstand, but the sensitivity to EtOH may vary depending on the clutch.

One possible reason for this variation in response to the treatment may be timing of hatching from the chorion. The chorion is a thin membrane that envelops and protects developing embryos until about 48-72hpf when they hatch. The embryos that perished in the HS Paxillin experiments with 2% EtOH were alive at 48hpf and dead when checked at 72hpf. It is possible that the chorion is non-permeable or semipermeable to EtOH, thus the embryos were exposed to greater concentrations of EtOH after hatching. Since there is a lot of variability in the timing of hatching, this may have resulted in the variability of the effects of EtOH. The embryos that hatched later may have had a shorter exposure time to EtOH and had severe dystrophy, such as the heat shocked HS Paxillin (---
LD2) GFP embryo seen in figure 9. Alternatively, the embryos that hatched earlier would have had a longer exposure time to EtOH and died from the toxicity. This is only a hypothesis and would require further testing.

There is also potentially a difference in the sensitivity of the different lines of Paxillin transgenics. In the last experiment that was conducted, the HS Paxillin (-LD2) and the HS Paxillin (-LD4) embryos died while the HS Paxillin embryos survived. This may suggest that the embryos are more sensitive to EtOH treatments when the LD2 and LD4 domains are knocked out. However, I was identifying transgenic embryos by looking for fluorescing MTJs after they were fixed during the imaging process, so it is not certain whether all the embryos in those dishes were transgenic since they died and degraded before they could be imaged.

There is also the issue that Paxillin was unable to rescue dystrophic embryos in these experiments. Previous studies that used overexpression of Paxillin to ameliorate dystrophy used morpholinos that targeted dystroglycan to induce dystrophy. Since EtOH treated fish could not be rescued with Paxillin overexpression, it appears it is working via some other pathway. Additional evidence of this is seen in the body shape of the embryos that perished and in some of those that survived EtOH treatment. These embryos had very short, stumpy bodies with wide myotomes (fig 12). These embryos look very similar to laminin mutants that are missing laminin-111.

If EtOH is affecting laminin and not dystroglycan (which we previously targeted with MOs) this may explain why Paxillin cannot help ameliorate dystrophy. Previous studies associated overexpression of Paxillin with an increased polymerization of laminin at the MTJ, which improved cell anchorage to the basement membrane. If EtOH
directly or indirectly disrupts laminin to a greater extent, then Paxillin overexpression might not be sufficient to ameliorate dystrophy. However, there are some inconsistencies in that not all embryos displayed the same stumpy tail phenotype. It was, like all other aspects of EtOH treatment, varied per each trial. The HS Paxillin (-LD2) embryos and the HS Paxillin embryos from the EtOH dosage experiment did not display this phenotype (fig 9).

We cannot make any conclusive hypotheses about the LD domains of Paxillin because the EtOH treatment was so unreliable. However, the results do give some inclinations about the potential sensitivity of these domains that we can keep in mind going forward with a different approach. These experiments have also given rise to some new hypotheses about EtOH treatment that could guide further studies.
Figures

**Figure 1.** 72hpf β-actin Paxillin treated with a 1.75% dilution of EtOH. Fiber detachments are present. This embryo also has damage to the muscle cells that was inflicted during the deyolking process.

**Figure 2.** 72hpf β-actin Paxillin treated with 2% EtOH. Muscle cells appear wavy and fiber detachments are present.
Figure 3. 72hpf β–actin Paxillin treated with 2% EtOH. Although there are no visible fiber detachments, the muscle cells appear wavy.

Figure 4. Graph of the percent of HS Paxillin and β-actin Paxillin embryos with dystrophy at 72hpf. This was merely preliminary data that was lacking controls, however it does suggest an inconsistency with EtOH treatment both across different Paxillin transgenics lines as well as within the same lines at the same dose.
Figure 5. 72hpf Non-heat shocked Paxillin embryo 0% EtOH, myotome 10. There are no fiber detachments and the muscle cells appear straight and uniform.

Figure 6. Heat-shocked HS Paxillin 0% EtOH, myotome 5. There are a large amount of fiber detachments on four myotomes.
Figure 7. Graph of the percent of HS Paxillin (-LD2) GFP embryos with dystrophy at 72hpf. This graph takes into consideration the average percent of dystrophy across all embryos in each group. There was one outlier in the HS Paxillin 0% EtOH group that had dystrophy, resulting in a 6% average that misrepresents the group.

Figure 8. Graph of the average number of myotomes with dystrophy for HS Paxillin (-LD2) embryos at 72hpf. This graph takes into account the average number of myotomes all of the embryos in each group had. The HS Paxillin 2% EtOH that fluoresced is misleading because there were only two embryos (one with dystrophy on 19 myotomes and one with no dystrophy).
Figure 9. 72hpf heat shocked HS Paxillin (-LD2) GFP non-fluorescing embryo with 2% EtOH treatment. The image starts at myotome five. The dystrophy is so severe that is nearly impossible to see myotome boundaries.
Figure 10. 72hpf heat shocked HS Paxillin (-LD2) GFP fluorescing embryo with 2% EtOH treatment. The image stars at myotome five. In image A The red staining depicts F-actin and the green depicts GFP. The embryo was curled, so the image cuts off at the top. Image B is the phalloidin stain in grey scale. There are severe muscle detachments.
Figure 11. 72hpf heat shocked HS Paxillin control with no EtOH treatment, myotome 10. Muscle development appears healthy and there is no evidence of fiber detachments.
Figure 12. 72hpf heat shocked HS Paxillin fluorescing (transgenic) embryo with 2% EtOH treatment, myotome 8. Image A shows GFP (demonstrating the embryo is a transgenic) in green and F-actin in Red. Image B shows only the red phalloidin staining for F-actin so fiber detachments are more visible.
Figure 13. Graph of the percent of 72hpf HS Paxillin embryos with dystrophy n = 10. 60% of the group that was treated with 2% EtOH had fiber detachments. None of the embryos that were not treated with EtOH had fiber detachments.
Figure 14. 72hpf heat shocked HS Paxillin fluorescing (transgenic) embryo with 2% EtOH treatment, myotome 15. Image A shows GFP (demonstrating the embryo is a transgenic) in green and F-actin in Red. Image B shows only the red phalloidin staining for F-actin so fiber detachments are more visible. This is the same embryo from figure 14 imaged in the tail region to demonstrate that fiber detachments occur more frequently anteriorly than posteriorly with EtOH treatment.
References


Appendix

All procedures involving zebrafish (*Danio rerio*) described here are reviewed and approved by the IACUC at the University of Maine
Author’s Biography

Maggie is from New Milford, Connecticut where she has spent more time working with wolves and handfeeding sharks than any sane person ever should. She is in the 4+ Master’s program at the University of Maine for a degree in Zoology. After graduation the rest is unknown, although she hopes to continue working in research. Her ultimate life goal is to own a husky, or three, and name one Balto. She spends her free time drawing the Beatles, wildlife, and the occasional dragon. She intends to somehow combine her love for art and science in her future career.