ABSTRACT

MAXIMILIAN J. KLEIN

Investigating the Early Developmental Expression of Lysosomal Enzymes in Zebrafish

(Under the Direction of RICHARD STEET, PH.D)

Proper breakdown of molecules within the lysosomal compartment is necessary to maintain the normal function of cells and their surrounding environment. The importance of this process in human health is stressed by a growing number of genetic diseases that involve defects in the proteins and enzymes responsible for this task. These diseases (termed lysosomal storage disorders or LSDs) have a diverse etiology, encompassing defects in individual acid hydrolases, metabolite transporters and enzymes that aid in targeting hydrolases to this organelle. Collectively, LSDs are one of the most frequently occurring genetic diseases affecting children in the U.S, with an estimated incidence of 1 in every 5000-7000 live births. Using zebrafish as a model system for developmental studies has many advantages which include but are not limited to large-scale experiment replication, early developmental genetic manipulation via the use of morpholinos, and microscopic phenotypical analysis in the early stages of embryogenesis. Surprisingly, there is little known regarding the early expression of lysosomal enzymes in zebrafish as well as overall yolk biology. To better gauge which lysosomal enzymes are best suited for morpholino based gene knockdown, developmental expression and regulation of zebrafish lysosomal enzymes were elucidated for enzymes that are well characterized in human disease. Several enzymes were then selectively targeted for morpholino-based knockdown, and the phenotypical and biochemical effects were analyzed. Additionally, the nature of several of these zebrafish lysosomal enzymes was better characterized by establishing pH activity profiles, yolk deposition, and percent mannose 6-phosphorylation, which yielded some novel findings in zebrafish lysosomal biology.

INDEX WORDS: lysosome, lysosomal storage disorder, acid hydrolase, embryogenesis, morpholino, mannose 6-phosphorylation

INVESTIGATING THE EARLY DEVELOPMENTAL EXPRESSION OF LYSOSOMAL ENZYMES IN ZEBRAFISH

by

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CHAPTER 1
INTRODUCTION AND BACKGROUND

Biological Function of the Lysosome

Cellular components including glycoproteins, glycolipids and even organelles are constantly being degraded and re-synthesized in an effort to maintain homeostasis. Much of the degradation and recycling of these cellular components takes place in the hydrolytic organelle known as the lysosome. The lysosome is involved in several digestive processes including endocytosis and phagocytosis (where cell surface proteins and foreign invaders, respectively, are taken up from outside the cell) and autophagy (where the cell degrades its own components).[1] The lysosome contains over 50 soluble acid hydrolases that work to perform the final degradation of macromolecules such as glycoproteins, glycosaminoglycans, and glycosphingolipids.[2] The hydrolases of the lysosome include several cathepsin proteases and a host of monosaccharide- and linkage-specific glycosidases. The lysosome's internal environment is unique because it maintains an acidic pH of about 4.5-5 while the majority of the cell has a slightly basic pH of about 7.2. Most of the lysosomal enzymes have evolved to work best at acidic pH, although there are some hydrolases that retain significant activity at neutral pH. Aside from hydrolytic enzymes, the lysosome contains many proteins that are involved in the transport and recycling of metabolites out of the lysosome for use in biosynthetic processes as well as several proteins that help to maintain the structural integrity of this organelle and prevent the leakage of potentially damaging enzymes into the intracellular environment.[2] The lysosomal enzymes are synthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus to be sorted and sent, through endosomes, to the lysosome itself. These

enzymes typically contain several N-linked oligosaccharides that play important roles in their proper folding and targeting (see below).[3] Furthermore, most of the enzymes are synthesized as proenzymes and require the removal of their propeptides prior to becoming enzymatically active. This latency is critical to avoid the enzymes from acting on other glycoproteins prior to their delivery to the lysosome.

Mannose 6-Phosphate Targeting Pathway

The primary way in which acid hydrolases are trafficked to the lysosome is through the mannose 6-phosphate (M6P) targeting pathway. Most soluble lysosomal enzymes are selectively recognized by uridine diphosphate (UDP)-N-acetylglucosamine lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (PTase). This enzyme initiates a two-step biosynthetic process that results in the addition of phosphomannosyl residues on certain asparagines-linked oligosaccharides of the enzymes.[4]

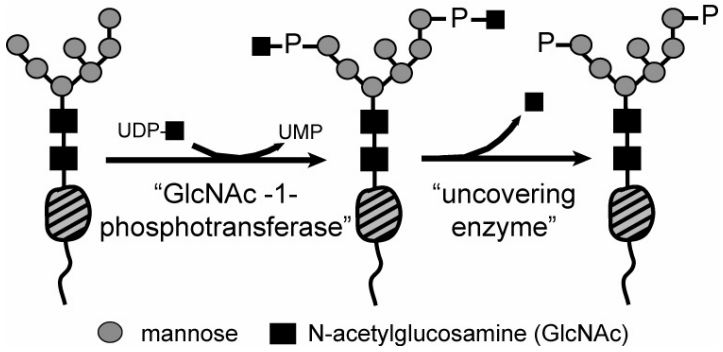

Figure 1. The two-step biosynthesis of mannose 6-phosphate residues on lysosomal enzymes

The PTase enzyme catalyzes the first step in forming M6P residues in the Golgi to tag lysosomal enzymes.[2] The second event that occurs is the removal of an N-acetylglucosamine (GlcNAc)-1-phosphate residue that cover the phosphates by an "uncovering enzyme" (UCE) which leads to the exposure of the M6P biomarker.[5] M6P receptors, found at the *trans*-Golgi network, bind to

these M6P-modified enzymes and are responsible for targeting them to the lysosome via clathrin-coated vesicles.

After reaching the lysosome, the M6P recognition marker is removed. Recently, it was also shown that the enzyme that is responsible for removal of the phosphate residue once the hydrolase reaches the lysosome is uteroferrin or acid phosphatase 5 (Acp5). Analysis of mice that are deficient in Acp5 revealed that there were highly elevated levels of M6P-modified glycoproteins in tissues that normally express Acp5.[6] It was suggested that the high levels of M6P came as a result from a failure to dephosphorylate lysosomal proteins via Acp5. These results point toward the central role for Acp5 in the removal of M6P residues.

While the M6P pathway is the most evolutionarily advanced targeting mechanism currently known, some enzymes are still targeted to the lysosomes by other mechanisms independent of the canonical M6P-mediated pathway. The alternative routes may follow an intracellular, direct pathway to the lysosome or indirect trafficking mechanisms mediated by secretion and recapture and/or direct transfer between proximal cells.[7,8] The molecular components of intracellular M6P-independent trafficking have not been elucidated, nor is it known how this pathway varies between tissue and cell types as well as species specificity. The hypothesis of such M6P independent pathways came largely as a result of many experiments that studied the specifics of the ML-II disease, in which the M6P targeting pathway is non-functional.[9]

Lysosomal Storage Disorders

Proper breakdown of molecules within the lysosomal compartment is necessary to maintain the normal function of cells and their surrounding environment, but when this process malfunctions, a wide array of abnormalities results. The importance of this process in human

health is stressed by a growing number of genetic diseases that involve defects in the proteins and enzymes responsible for this task. These diseases, termed lysosomal storage disorders (LSDs), have a diverse etiology, encompassing defects in individual acid hydrolases, metabolite transporters and enzymes that aid in targeting hydrolases to the lysosome. Collectively, LSDs are one of the most frequently occurring genetic diseases affecting children in the U.S, with an estimated incidence of 1 in every 5000-7000 live births.[2] Depending on the specific type of LSD, affected children can exhibit defects in the skeletal, neurological, cardiac and ocular systems that are apparent at or shortly after birth.[10]

Mucolipidosis II: A Unique Lysosomal Storage Disorder

ML-II is a rare autosomal recessive disorder in which affected individuals are deficient in the PTase enzyme, and as a consequence several lysosomal enzymes have severely decreased mannose phosphorylation and cannot make it to the lysosome.[4] Although clinically similar to several forms of mucopolysaccharidosis (MPS), ML-II is biochemically unique, as it results from an error in trafficking of enzymes to the lysosomes instead of the deficiency of a particular LE. ML-II leads to hypersecretion of lysosomal enzymes from cells and accumulation of material in lysosomal compartments, which further leads to associated phenotypic characteristics such as abnormal skeletal development, restricted joint movement, psychomotor retardation, craniofacial abnormalities, cardiac defects and frequent upper respiratory infections.[11] Studying ML-II led to the finding that even without a functioning PTase enzymes, certain lysosomal enzymes were still present at high levels within the lysosome, and thus led to the proposal that there are M6P independent pathways for lysosomal enzyme sorting.[12] Experiments on a disease such as ML-II clearly showed that cellular abnormalities in lysosomal enzyme trafficking can provide clues to understanding the mechanisms behind normal cellular processes.

The Challenges of Defining Lysosomal Storage Disorder Pathogenesis

Despite advances in uncovering the genetic basis for these disorders, surprisingly little is known about the underlying mechanisms that lead to disease pathology. Many researchers have begun to appreciate mechanisms other than lysosomal storage are likely relevant to the pathology of several tissues including the brain.[10] Understanding the pathogenesis of ML-II is particularly challenging since many enzymes are M6P-modified and can be affected when this recognition marker is lost. Another hurdle in understanding ML-II pathogenesis is the lack of animal models that are suitable for the analysis of early developmental events. The overall goal of this research is to investigate the pathogenesis of lysosomal storage disorders using the zebrafish system. The zebrafish model has been developed in order to address the limitations presented by other model systems (i.e. mice or rats), such as the inability to study developmental processes due to *in utero* gestation. A zebrafish model of the severe LSD, mucolipidosis II, or I-cell disease, was recently developed using an antisense morpholino-based knockdown strategy. These mutant zebrafish exhibit many features that are similar to human patients, including a pronounced motility defect as well as craniofacial cartilage and cardiac abnormalities.[7] Experiments in this model have begun to yield important clues into the cellular and molecular mechanisms that lead to the abnormal cartilage development associated with this disorder. One intriguing finding that arose during the initial characterization of this model was the fact that significant levels of mannose 6-phosphorylated glycosidases were detected in ML-II embryos despite robust inhibition of PTase activity.[13] One mechanism to account for this observation is that M6P-modified enzymes maternally deposited in the yolk (and therefore not sensitive to loss of PTase activity induced by the morpholino injection) were masking the decreases in mannose phosphorylation levels within the embryo.

Loss of β-Glucuronidase in Zebrafish Embryos Does Not Result in Any Obvious Phenotypes

In an effort to model MPS disorders in zebrafish and extend the utility of this experimental organism, studies were conducted to find out whether the lysosomal enzyme β-glucuronidase (GUSB) was required for normal embryonic development in zebrafish. Enzyme activity experiments showed that a splice-blocking morpholino designed to inhibit GUSB activity effectively reduced the activity of this enzyme to only 3-5% of control. Furthermore, the morpholino remained effective past the fifth day of embryonic development. Despite this highly efficient knockdown of GUSB activity, there were no noticeable phenotypes displayed in the affected zebrafish. After testing various aspects of our protocol to make sure that the GUSB enzyme was not being destroyed in a preparatory step, we began to consider why the zebrafish do not appear to need GUSB in early developmental processes. One possibility is that the substrates normally degrading by GUSB were not present in large enough amounts to cause lysosomal storage, even in the absence of GUSB. It is also possible that high-level expression of GUSB does not occur until later in development. To resolve this difficulty and address the possibility that glycosidases are in fact deposited in the yolk of the embryo, we undertook an in-depth analysis of the regulation and enzymatic properties of lysosomal hydrolases in the zebrafish yolk and embryo. We sought to gain a more fundamental understanding of the zebrafish lysosomal enzyme biology which would not only help our entire lab, but the zebrafish community as a whole. In summary, our results uncovered several unique features of the lysosomal biochemistry of this marine organism.

CHAPTER 2
MATERIALS AND METHODS

Materials. Wild-type zebrafish were obtained from Fish 2U (Gibson, FL) and maintained using standard protocols. Eggs were collected and incubated at 25°C in a 0.30x Danieu's solution with methylene blue. When appropriate, 0.003% 1-phenyl-2-thiourea was added to the growth media to prevent pigmentation. Human skin fibroblasts were obtained from ATCC and maintained in a 37˚C incubator with 5% CO_2. All zebrafish brains were dissected from adult wild type zebrafish and stored at -80°C until needed. When yolks were collected for experimentation, development was arrested within one hour post fertilization. Morpholinos and a mispaired control were ordered from Gene Tools, LLC in 1.0mM stock solution. Morpholinos were diluted and stored in a -20°C until the day of use.

Injection of Morpholinos. WT eggs were injected by using a Picospritzer III injection device. Needles for the injection set-up were pulled using glass without filaments and a Nanishige needle puller. Morpholinos were injected in concentrations ranging from approximately 0.1 ng to 2.0 ng. In an effort to ensure that our observed phenotypes and decreased enzyme activity was caused by the morpholino's eliminating translation of the GUSB mRNA instead of a adverse off-target effects resulting from too much morpholino, an antisense morpholino was injected into WT fish at identical concentrations as a control. After morpholino injections, the WT fish were stored in a 25°C incubator in ranges of one to five days. The fish kept longer than four days were fed a small amount of rotifers twice daily. After observing the morpholino-injected fish against WT fish, they were subsequently analyzed for total protein and total lysosomal enzyme

activity. Before removing yolks, the fish were anesthetized with a 20x tricaine solution. After applying anesthesia, the yolks were suctioned off with a glass pipette when testing early developing embryos.

Enzyme Activity Measurements. Biochemical analysis of enzymatic activity was done with fluorescent 4-methylumbelliferone (4MU) substrates for each respective enzyme. Samples of WT Fish 2U zebrafish embryos were collected in quantities of at least 20, yolks in quantities of at least 10, and brains in quantities of at least 2. For experiments testing embryo enzymatic activity, yolks were removed prior to analysis. For brain experiments, adult WT zebrafish were fully anesthetized with 20x tricaine and then dissected. Collected brain tissue was washed with phosphate buffered saline to remove any contaminating blood. All samples were frozen on dry ice and put in a -80ºC storage unit if not immediately used. Samples were standardized to total amount of protein using a Thermo Scientific Micro BCA Protein Assay Kit. If the experiment was testing for a percentage of the highest or total activity within a sample, no protein standardization was necessary. Samples were homogenized via sonication in a Triton X-100 containing buffer solution. All enzyme assays were carried out in the tested enzyme's respective citrate buffer solution so that the reaction was run at the enzyme's optimum pH. Each enzyme assay was run with a minimum of at least 5µg of sample and an excess of the respective 4MU substrate. The 4MU substrates were prepared by first solubilizing them in dimethyl sulfoxide. Reactions were performed in 50 mmol/L citrate buffer (pH 4.5) and 0.5% Triton X-100 containing 3 mmol/L of the respective substrate in Eppendorf tubes in a 37ºC water bath for at least 1-2 hours. These enzyme activity reactions were quenched with a high pH sodium carbonate stop-buffer solution and then read using a Turner Biosystems 380 fluorometric absorbance reader that reads the wavelengths at an ultraviolet excitation of 380 and an emission

of 450. The resulting fluorometric units were then converted into activity readings in units of (nmol activity/mg protein/hours of reaction). For pH profile activities, reactions were carried out in McIlvaine's citric acid/dihydrogen sodium phosphate buffer system. Enzymes tested for included β-glucuronidase (ß-GlcA; GUSB), β–glucosidase (β-Glu; GBA), β–hexosaminidase (β-Hex), β-galactosidase (β-Gal), α-mannosidase (α-Man), α–glucosidase (α-Glu; GAA), α-iduronidase (α-Idu), and α-aspartylglucosaminidase (AGA).

M6P Receptor Column Chromatography. Percent of mannose phosphorylation for selected enzymes (β-Gal, α-Man, GAA, α-Gal) in the zebrafish brain, embryo, and yolk was calculated by chromatography with a mannose 6-phosphate receptor affinity column given to our lab by Dr. Peter Lobel. Collected samples were frozen immediately on dry ice if not directly used. A lysate was prepared via short pulses of sonication in a citrate buffer solution. A 200µL lystate was applied to the column with an additional 400µL of column buffer to yield a 600µL collected fraction. Column buffer (50 mM imidazole/HCl, pH 6.5, 150mM NaCl, 0.05% Triton X-100, 5 mM EDTA) was used to elute the unbound fractions while column buffer containing 5 mM M6P was used to elute the bound fractions. Eluted fractions were used in our standard enzyme assay protocol to calculate the percentage of M6P and non-M6P activity relative to the total activity.

CHAPTER 3
RESULTS

Lysosomal Enzyme Activity During Embryonic Zebrafish Development

We first investigated the expression and activity of several representative lysosomal enzymes during the first seven days of development. Zebrafish embryos were collected at each day of development, deyolked to remove contamination of maternally deposited hydrolases and activity measured in protein-normalized lysates. The results of this analysis are shown in **Figure 2**. Several distinct patterns were apparent that can be portrayed in connection to the type of material on which the specific lysosomal enzyme acts. The lysosomal enzymes that were tested generally cover three different types of macromolecule degradation: glycoprotein (α-Man, β-Gal, β-Hex), glycosaminoglycan (α-Ido, GUSB), and glycogen (GAA). Importantly, both ß-Gal and ß-Hex also play a role in the degradation of specific types of glycosaminoglycans. The three glycoprotein-degrading enzymes are shown below first in **Figure 2**. All three enzymes (α-Man, β-Gal, β-Hex) are present in significant amounts beginning at the first day of embryonic development. The upregulation of α-Man is noticeably slower than β-Gal and β-Hex, however, at the seventh day of development the relative percent increase of activity is comparable. β-Gal and β-Hex are upregulated in a more linear fashion with respect to time, with β-Hex reaching a plateau the fastest at 5 days.

The activity graphs of the glycosaminoglycan-degrading enzymes, α-Ido, GUSB, are shown below next in **Figure 2**. Both enzymes follow similar patterns in increase of activity. There is little activity present of both α-Ido, GUSB at day one, and a peak activity measurement is seen at day five, after which it declines. The activity for glycogen degrading GAA is shown

below last in **Figure 2**. GAA is unique in that the activity remains fairly constant from day one until day six, until it peaks at day seven. This may reflect the necessity for glycogen utilization in the muscle of the embryo throughout its early development.

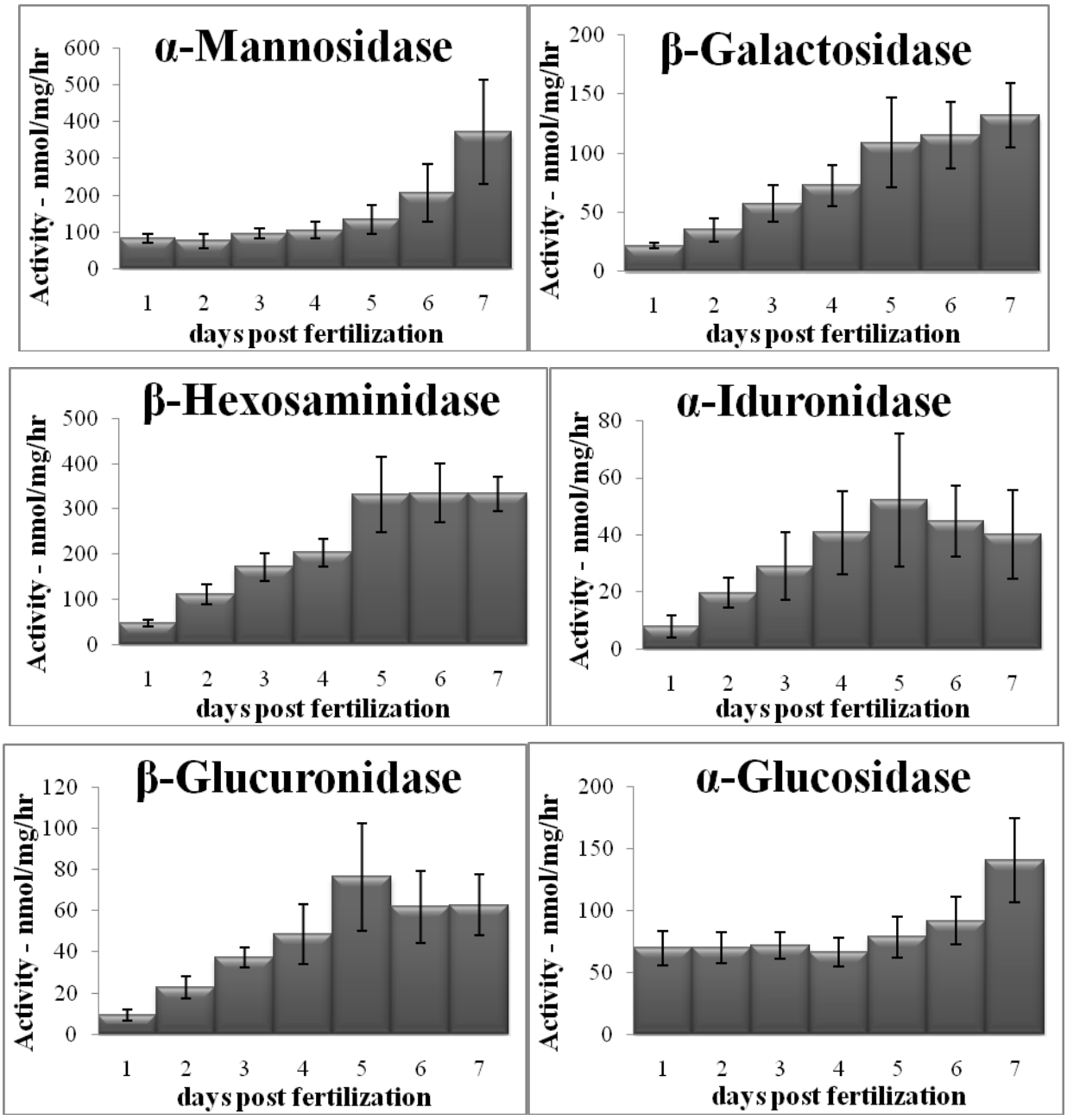

Figure 2. α-Mannosidase**,** β-Galactosidase, β-Hexosaminidase, α-Iduronidase, β-Glucuronidase, and α-Glucosidase activity in wild-type zebrafish embryos during the first 7 days of embryonic development. Yolks were removed prior to each activity assay. All samples were normalized to total protein. The data represents the average of three independent experiments; error bars indicate +/- one standard deviation.

Selective Deposition of Certain Hydrolases into the Yolk

We next asked whether certain lysosomal enzymes were deposited into the zebrafish eggs. In an effort to minimize the contribution of enzyme activity from the developing zygote, activity was measured in yolk lysates prepared from eggs not more than one hour post-fertilization. Below in **Figure 3**, it can be clearly seen that certain lysosomal enzymes (α-Man, β-Gal, and GAA) are maternally deposited into the yolk in substantial amounts. Even though all the lysosomal enzymes tested show significant activity levels in developing embryonic ZF tissue, only these three enzymes were found to be present in the yolk. Two of the enzymes present in the yolk are predominantly glycoprotein-degrading enzymes (α-Man and β-Gal) while the third enzyme is a glycogen-degrading enzyme (GAA).

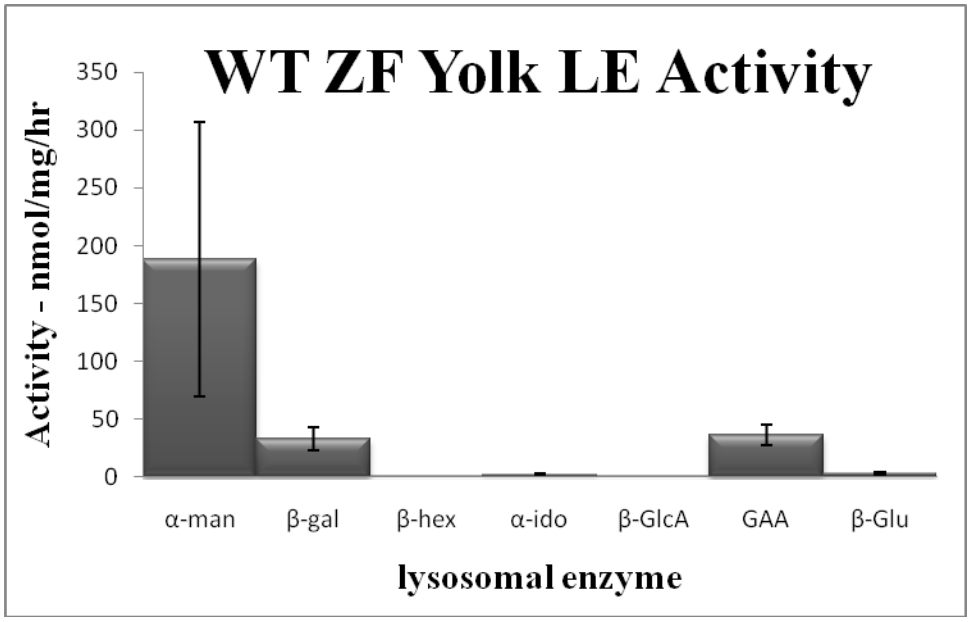

Figure 3. Activity of various lysosomal hydrolases present in wild type zebrafish yolks. Yolks were collected and prepared approximately 1 hr. post fertilization. All samples were normalized to total protein. The data represents the average of three independent experiments; error bars indicate standard deviation.

Levels of Mannose 6-Phosphorylation of Zebrafish Lysosomal Glycosidases

To better understand the extent to which the yolk-deposited lysosomal enzymes from are mannose 6-phosphorylated, wild type ZF yolks were collected and run over the M6P affinity column. α-Man, β-Gal, and GAA were individually assayed to determine percent of mannose 6-phosphorylation. **Table 1** shows that while there are roughly equal amounts of α-Man and β-Gal from ZF yolk unbound and bound to the M6P column, 100% of the GAA activity is consistently unbound to the column. A cell culture line of human fibroblasts was also tested as a control.

Table 1. Percent of total enzymatic activity unbound and bound to a mannose 6-phosphate receptor affinity column in wild-type zebrafish yolk.

Enzyme	% Unbound to Column	% Bound to Column	Standard Deviation
β-Galactosidase	46.0	54.0	5.39
α-Galactosidase	23.2	76.8	-
α-Mannosidase	43.8	56.2	8.04
α-Glucosidase	100	0	0

Table 2. Percent of total enzymatic activity unbound and bound to a mannose 6-phosphate receptor affinity column in wild-type zebrafish brain.

Enzyme	% Unbound to Column	% Bound to Column
β-Galactosidase	88.4	11.6
α-Galactosidase	91.4	8.6
α-Glucosidase	100	0

pH profiles of Yolk and Brain Glycosidases

In order to better understand the nature of the yolk-deposited LE activities, α-Man, β-Gal, and GAA from ZF yolks and brains were tested across a pH profile. Human fibroblasts were used again as a control. α-Man and GAA followed the activity curves of human fibroblasts very closely, however, β-Gal showed an interesting neutral activity in the ZF brains. **Figures 4, 5, and 6** show the comparisons of ZF brain activity against human fibroblasts. Overall, the LE enzymes show a typical pH activity curve, except that the peak activity for the lysosomal enzymes is in the acidic range.

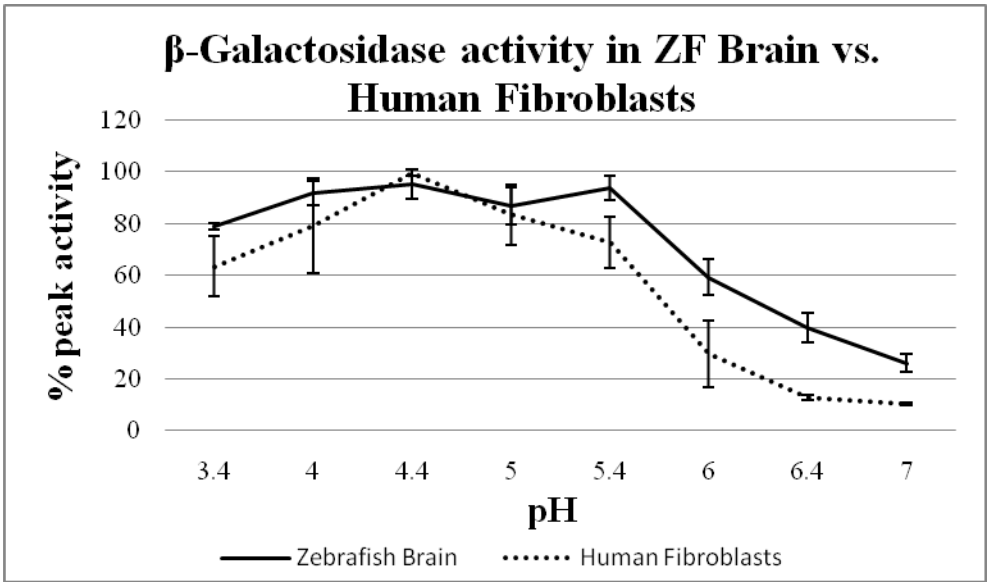

Figure 4. β-Galactosidase enzyme activity in WT ZF brains and human fibroblasts was calculated over a pH curve. Brains were collected from adult ZF and normalized to total protein. Activity is plotted as a percentage of the peak activity within the pH range. The data represents the average of at least three independent experiments; error bars indicate +/- one standard deviation.

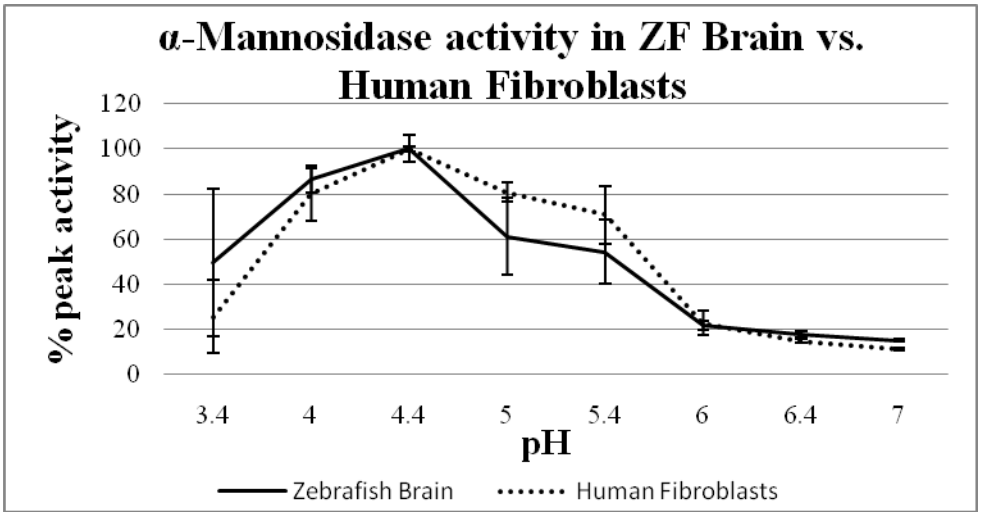

Figure 5. α-Mannosidase enzyme activity in WT ZF brains and human fibroblasts was calculated over a pH curve. Brains were collected from adult ZF and normalized to total protein. Activity is plotted as a percentage of the peak activity within the pH range. The data represents the average of at least three independent experiments; error bars indicate +/- one standard deviation.

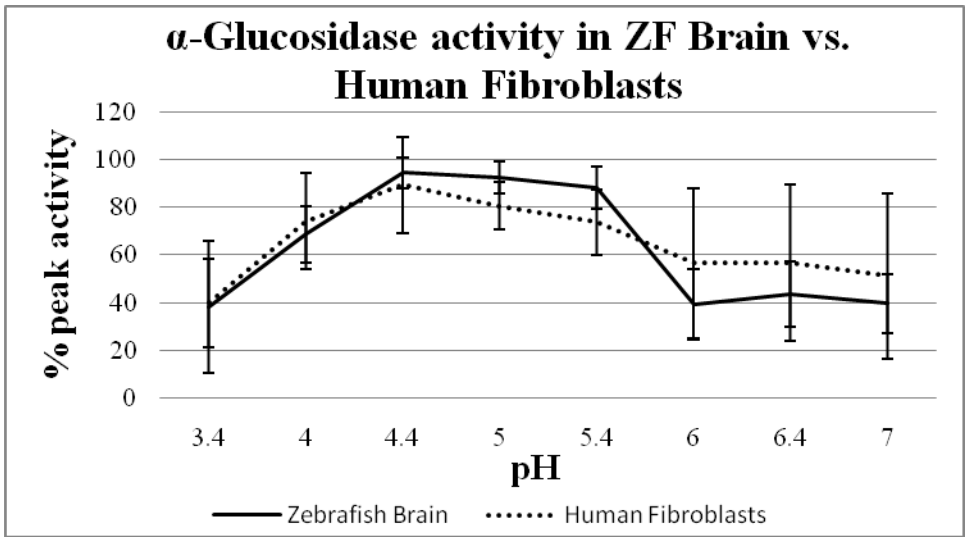

Figure 6. Acid α-Glucosidase enzyme activity in WT ZF brains and human fibroblasts was calculated over a pH curve. Brains were collected from adult ZF and normalized to total protein. Activity is plotted as a percentage of the peak activity within the pH range. The data represents the average of at least three independent experiments; error bars indicate +/- one standard deviation.

Potential Neutral pH β-Galactosidase Activity Discovered in Zebrafish Brain Tissue

The consistent finding of a neutral β-Gal activity in the ZF brain (of approximately 30% of the peak activity), led us to investigate the specific nature of this novel activity. Other ZF sections, including the yolk, trunk, and heart, were tested for β-Gal activity across a pH scale. The results are shown in **Figure 7**. As shown, there is some neutral ß-Gal activity in both the trunk and heart of the zebrafish (but not the yolk). These results suggest that this novel activity may be present in other tissues, although it is most prominently noted in the brain.

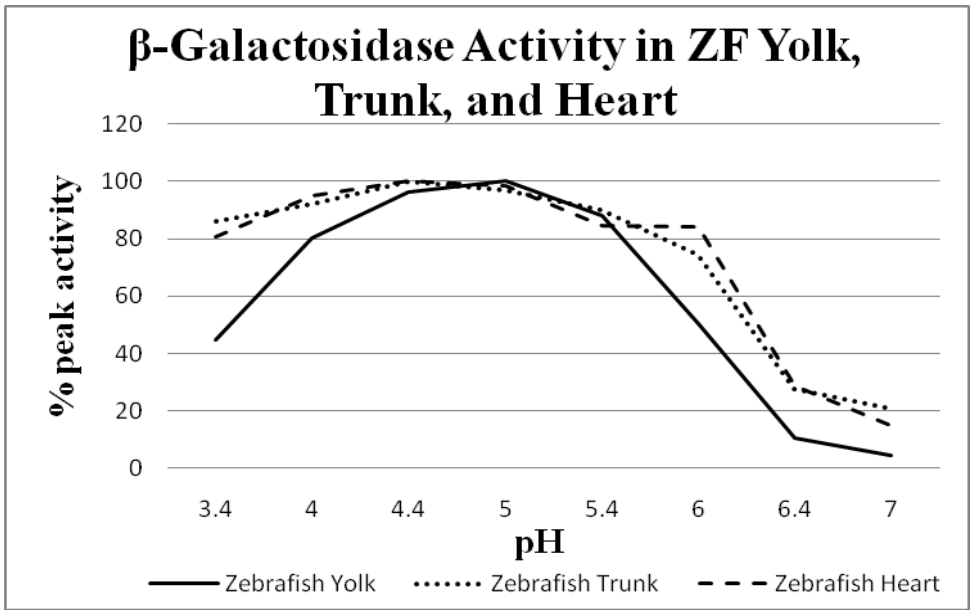

Figure 7. % of peak β-Galactosidase activity for wild-type zebrafish yolk, trunk, and heart sections. All samples were collected from adult wild-type zebrafish and normalized to total protein.

In an effort to fractionate the lysosomal ß-Gal activity from the novel neutral ß-Gal activity, ZF brain lysates were run over the M6P column, and the fractions were tested to see whether or not this neutral β-Gal activity might contain M6P residues. **Figure 8** shows that the neutral β-Gal activity is primarily unbound to the M6P column, and therefore does not appear to contain M6P residues. It is important to note that the unbound fractions likely contain lysosomal

ß-Gal activity that no longer bears M6P residues. Nonetheless, these results raise the possibility that this enzyme may act within the extracellular environment to modify galactose-containing oligosaccharides.

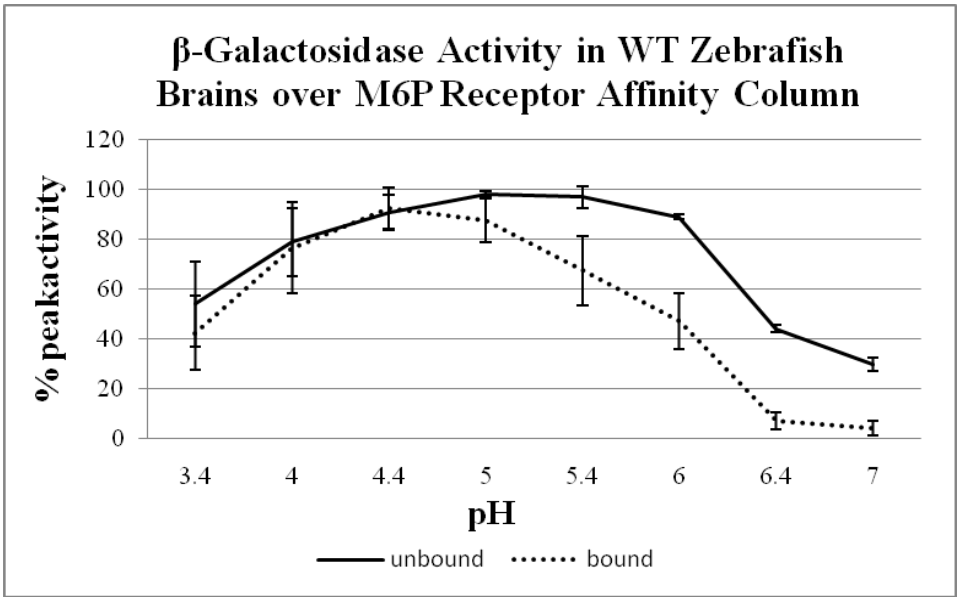

Figure 8. % of wild-type zebrafish β-Galactosidase activity unbound and bound to a M6P affinity column. Brains were collected from adult wild-type zebrafish and normalized to total protein. Activity is plotted as a percentage of the peak activity within the pH range. The data represents the average of three independent experiments; error bars indicate +/- one standard deviation.

In addition to β-Gal, the pH profile activity of α-Galactosidase (α-Gal) was also tested to deduce whether or not it had the same type of residual neutral activity as its β-Gal counterpart. **Figure 9** shows that α-Gal does not possess the same residual neutral activity in either WT ZF yolk or brain. The β-Gal neutral activity is therefore novel to the enzyme. Future experiments will be designed to identify this novel ß-Gal activity and investigate its function in zebrafish development and brain structure.

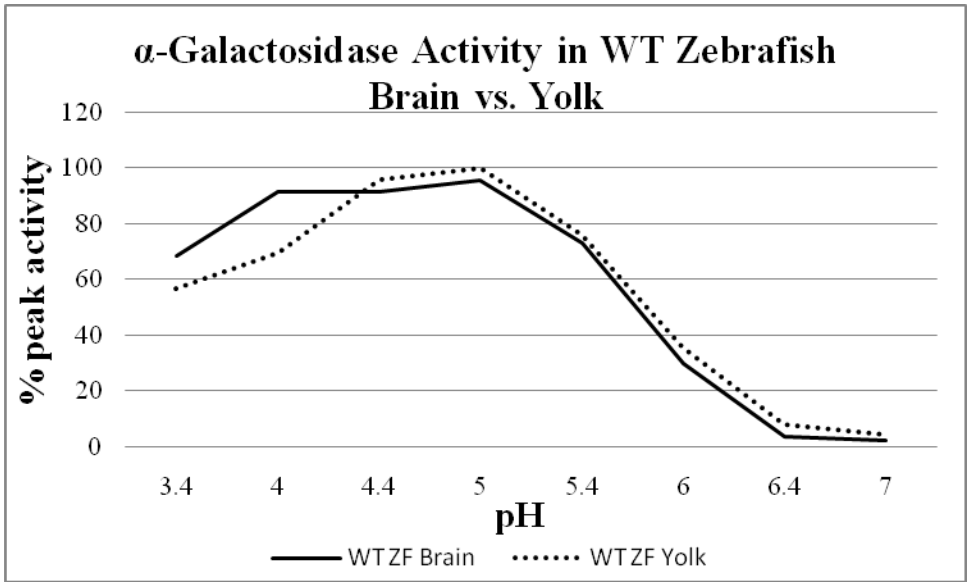

Figure 9. α-Galactosidase activity measured in WT ZF brain and yolk over a pH curve. Brains were collected from adult WT ZF and normalized to total protein. Activity is plotted as a percentage of the peak activity within the pH range. The data represents a mean of two separate experiments.

Acp5 Activity Levels in Zebrafish Brain and Yolk

Finally, Acp5 activity levels for WT ZF brain and yolk were tested against human fibroblasts with and without the presence of tartrate, a phosphatase inhibitor used to eliminate any non-Acp5 specific activity. Analyzing the Acp5 activity levels in WT ZF brain and yolk revealed a substantial difference. In all experiments, there was a lower relative Acp5 activity level in yolk when compared to brain tissue (~10-fold lower), which may account for the high levels of mannose 6-phosphorylation in the yolk and the somewhat lower levels in brain tissue.

CHAPTER 4
DISCUSSION

Glycosaminoglycan vs. Glycoprotein Degradation in Zebrafish

The goal of our initial investigations was to create an effective mucopolysaccharidosis VII (MPS VII) zebrafish with the use of a morpholino-based knockdown strategy. Although the morpholino worked with surprising effectiveness, there were no noticeable phenotypes in the resulting β-GlcA deficient mutant zebrafish. This puzzling observation led us to ask more fundamental questions in zebrafish lysosomal biology. Specifically, our desire to understand the regulation of zebrafish lysosomal enzymes with respect to time led us to characterize the expression of lysosomal enzymes across the first seven days of zebrafish embryonic development. Clearly shown in **Figure 2** is that enzymes involved in the degradation of glycosaminoglycans and glycoproteins are not maximally expressed until approximately day five post fertilization. After day 5 of post fertilization development, there is necessary glycosaminoglycan turnover and thus, the expression of enzyme such as β-GlcA (GUSB) increases. It seems possible that the reason why β-GlcA expression does not increase substantially until day 5 of post fertilization development is because the zebrafish simply does not turnover a large amount of glycosaminoglycans until after day 5. It follows that if there are no substrates to be degraded, there will be no storage in the event of enzyme depletion and thus no noticeable phenotypes.

In contrast, glycogen degrading enzymes are present in significant quantities beginning at day one of embryonic development. These patterns of zebrafish lysosomal enzymatic expression show that there is a particular sequence of upregulation depending on what type of degradation

the cell needs. Therefore, until approximately day five of embryonic development very little glycosaminoglycan turnover is occurring within the tissue, and since morpholino-based knockdown is most effective until the fifth day of development, the disorders of glycosaminoglycan degrading enzymes are not suitable for this type of enzyme depletion study. Conversely, because it was shown that glycogen degrading enzymes are present in substantial concentrations earlier in development, depletion of this enzyme would be hypothesized to yield interesting phenotypes. Overall, the results of our time course investigation provide significant clues in understanding the nature of glycoprotein and glycogen metabolism in embryonic zebrafish development.

Functions of Glycosidases in Yolk

The yolk of developing embryonic zebrafish is present for approximately the first five or six days post fertilization. During this time the yolk size decreases as the fish embryo size increases. It is reasonable to believe that the yolk deposited by the mother contains all the necessary components for early development, and these essential nutrients are being rapidly taken up and utilized by the growing zebrafish embryo. Little information, however, is actually known about yolk biology. The yolk contains glycoproteins that presumably serve as fuel for the developing embryo, but how exactly this material is transferred from the yolk to the embryo and to what extent is unclear. Measuring the lysosomal enzyme activity in one-hour post fertilized yolks has given insight into the type of digestive properties that are crucial for the yolk's functions. **Figure 3** shows β-Gal, α-Man, and GAA are all deposited in the yolk in significant amounts, while β-Hex, α-Id, and β-GlcA, are entirely absent. From this, it is thought that there is active glycoprotein and glycogen degradation in yolk beginning the moment the egg is laid.

Because the zebrafish does not begin to utilize external food sources until after day 5 post fertilization, it is very likely that the yolk is providing the developing embryo with glucose and other sugars via glycogen and its degrading enzymes. This idea of glycogen degradation within the yolk is consistent with the large quantities of acid α-glucosidase that were found deposited into the yolk. It is not clear, however, whether the lysosomal enzymes themselves are active while remaining in the yolk, or whether they are being transported to the embryonic tissues. If in fact the lysosomal enzymes are crossing the barrier between the yolk and the embryo, then it would be reasonable to conclude that morpholino-based knockdown of a lysosomal enzyme that is maternally deposited into the yolk will always be ineffective. Whether or not the enzymes are crossing from the yolk to the embryo has not been fully investigated, but regardless of the potential transport of lysosomal enzymes, there still appears to be evident glycosidase activity within the yolk itself. Additionally, α-Man may be important for degrading glycosylated portions of the main yolk protein, i.e. vitellogenin (X. Fan, R. Steet, unpublished data). There is no information so far, however, regarding β-Gal activity in the yolk. An important question to further investigate is whether or not there are glycolipid substrates in the yolk, and at what point is their degradation necessary. Additionally, determining if there are lysosomal organelle-like structures within the yolk where these lysosomal enzymes function and the extent to which these organelle-like granules are activated are other important questions for better understanding yolk biology. In sum, the discovery that there is evident lysosomal function within the yolk prompted further investigation regarding the nature of these enzymes compared to other tissues.

Differences in % Mannose Phosphorylation of Glycosidases

In human cells, most soluble lysosomal enzymes are highly mannose phosphorylated. As less evolved mammalian animal models are analyzed, however, there is less reliance on the M6P

pathway and more use of uncharacterized independent pathways. The zebrafish appear to utilize both the M6P dependent and independent pathways. **Table 1** shows how the reliance on the M6P pathway can vary within zebrafish for each enzyme. For the yolk-deposited enzymes, both β-Gal and α-Man have similar distributions of M6P dependent and independent reliance, while GAA is unequivocally M6P independent. It is interesting to see the extent to which percent mannose 6-phosphorylation of different enzymes differs between the species. In humans, GAA is a mannose 6-phorphorylated enzyme, and in an evolutionary view, it is reasonable to believe that shortly after zebrafish, mannose phosphorylation of GAA may have occurred. Zebrafish GAA has fewer N-linked glycans than the human GAA, suggesting that the increase in glycans parallels the acquisition of mannose 6-phosphate residues. It would be interesting to look at the mannose phosphorylation of GAA across an evolutionary timeline between zebrafish and humans to see exactly where the additional sequences for adding mannose 6-phosphate residues were acquired. Even though the M6P pathway requires a large energy investment, its targeting efficiency is generally high. In the case of a mutation such as the one that occurs in ML-II, where the M6P pathway is effectively eliminated, it is no surprise that such drastic phenotypic abnormalities result due to the heavy reliance on this pathway. However, there are several tissues that are not affected likely due to the presence of M6P-independent sorting mechanisms. It would be of interest to determine which pathway GAA utilizes in zebrafish to target to the lysosome.

When compared to yolk lysosomal enzymatic activity, the brain had significantly lower levels. It has been hypothesized that there are lower levels of mannose 6-phosphorylation in the brain because such extracellular activity is quite harmful, and that the M6P pathway exists as a last resort mechanism in case some lysosomal enzymes leak out of the cells by mistake. If the

enzymes that leak out are mannose 6-phosphorylated, then the M6P pathway will allow them to be quickly taken up by the cell and resent to lysosome. In order to investigate the possible cause for different relative levels of mannose 6-phosphorylation of enzymes, Acp5 levels were established for both brain and yolk lysosomal enzymes. As expected, the Acp5 activity levels in the yolk are low while the mannose 6-phosphorylation is high, and conversely, Acp5 activity levels in brain tissue is high while the mannose 6-phosphorylation is low. Attempts to knockdown Acp5 activity through the use of two separate morpholinos were unsuccessful, however. In an attempt to further investigate the nature of these yolk-deposited enzyme's activities, the level of enzymatic activity over a pH range was characterized.

Neutral β-Galactosidase Activity in Zebrafish Brain

From the pH activity graphs of zebrafish brain versus human fibroblasts in **Figures 4, 5, and 6**, α-Man activity levels hold pretty close between zebrafish and humans, but β-Gal and GAA show interesting neutral pH activity. For GAA, this neutral activity is likely due to ER glucosidases in the brain. These enzymes would function best at the neutral pH of this compartment. However for β-Gal this residual activity of approximately 30% was unexpected. Through separate experiments, the neutral β-Gal activity was shown to be unique to zebrafish, somewhat brain-specific (zebrafish heart and trunk β-Gal activity showed less of this neutral activity and yolk showed very little – **Figure 7**), and non-phosphorylated (**Figure 8**). Further biochemical experiments involving ion-exchange and size exclusion column chromatography and will be carried out in an attempt to separate the hypothesized two β-galactosidase activities.

This neutral, brain β-Gal activity has not before been observed and zebrafish, so its function is not characterized. Presumably, because of its neutral activity, this novel enzyme may act on substrates within the extracellular space. There could some degree of glycoprotein

degradation outside the lysosome critical for brain metabolism. It seems understandable that because this neutral β-Gal activity functions outside of the lysosome, it would not use the M6P targeting pathway, and is thus why the neutral activity was entirely in the unbound fraction when running the zebrafish brain over the M6P column. Further investigation into the exact purpose and/or functions of this novel enzyme could yield deeper insight into the digestive functions of the cell outside the lysosome.

Final Perspectives on the Use of Zebrafish for Modeling Lysosomal Storage Disorders

One of the main issues preventing the advancement of knowledge in the pathologies of lysosomal storage disorders stems from the inability to study these disorders in a convenience animal model. Many murine and feline models have been developed; however, these models involve *in utero* gestation, which significantly inhibits studying the developmental processes. Modeling a disease such as a lysosomal storage disorder in zebrafish is effective not only because gestation takes place outside the uterus, but also because germination happens rapidly. Comparatively, zebrafish show us in weeks what might take several months in other organisms. Zebrafish can also produce hundreds of embryos on a daily basis, allowing for more experiment replications, and thus more accurate data averages in the end.

Modeling a lysosomal storage disorder in zebrafish by using a morpholino based strategy is effective for many reasons, but also gives several difficulties. And as far as convenience with the zebrafish reproductive cycle goes, morpholinos have been shown to be effective tools for testing genetic interactions *in vivo*, which conversely would not be useful in studying development in feline and murine models.[14] While using morpholinos for gene regulation is extremely cost effective, many times the targeted protein is simply not a good candidate because of its point of upregulation. For instance, it was shown in **Figure 2** that glycosaminoglycan

degrading enzymes do not become significantly expressed until approximately the fifth day of development, which is precisely the point at which the morpholino becomes too diluted within the cells. Further, because the morpholino only knockdowns endogenous enzyme production and not the activity of the enzyme that is contributed maternally, the morpholino's effectiveness is questionable for enzymes such as β-Gal, α-Man, and GAA that are deposited into the yolk. However, when the morpholino-based knockdown is successful and effective, as in the case of the established ML-II zebrafish model, many critical aspects of the etiologies of lysosomal storage disorders can be investigated that would be impossible in any other animal model. These unique insights in the molecular understanding of lysosomal storage disorders made possible by zebrafish provide an excellent groundwork for the evaluation of new therapeutic possibilities.

CHAPTER 5
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