A New Functional Zebrafish Gastrointestinal Motility Assay

By

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Abstract

Gastrointestinal (GI) motility is the coordinated contractions of smooth muscles resulting in mixing and propulsion of material through the GI tract. GI motility is influenced by smooth muscle, enteric neurons, and Interstitial cells of Cajal (ICC). New model systems for GI motility are needed because regulation of motility is poorly understood, and the number of drugs which can assist in GI motility disorders is insufficient. The Kit receptor is required for ICC development and maintenance in mammalian model systems and in humans. Mutations in the Kit receptor are associated with GI motility disorders. The objective of these experiments was to determine motility patterns in wildtype zebrafish and compare them to motility patterns in the zebrafish *kita* null mutant, *sparse* (spa^{b5}). Contraction frequency was measured from 7 minute digital video recordings of anesthetized larvae immobilized in 1.2% agar. Contraction frequency averaged 0.586 ± 0.222 contractions per minute in wildtype larvae at 7 dpf, and 0.329 ± 0.158 contractions per minute in 7 dpf *sparse.* The apparent contraction intensity was also scored, with a score from 0, no contractions, to 2, complete occlusion of the lumen. Contraction intensity averaged 1.250 ± 0.444 in wildtype larvae and 1.619 ± 0.498 in *sparse* larvae at 7 dpf. At 11 dpf contraction intensity decreased to 1.000 ± 0.000 and 1.125 ± 0.354, respectively. A functional motility assay was developed to quantify GI motility that contributes to propulsive movement of intestinal contents. Larvae were fed FITC labeled microspheres, washed, and fluorescence intensity of the GI tract was digitally imaged. Larvae were placed in clean system water, and re-imaged after 24 hours. Fluorescence intensities were normalized against initial intensity of ingested microspheres at 7 dpf in the intestinal bulb of the anterior GI tract. At 7 dpf the change in fluorescence intensity in the anterior GI tract decreased by 0.371 ± 0.030 and 0.119 ± 0.021 in the posterior GI tract of wildtype larvae. At 11 dpf fluorescence intensity decreased to 0.455 ±

0.056 in the anterior GI tract, and 0.258 ± 0.046 in the posterior GI tract. Peritoneal injection of the ACK-2 anti-Kit antibody results in distention and an increased volume in the GI tract (Figure 12) (Maeda, Yamagata et al. 1992). These effects result from inactivation of Kit function by ACK-2. The size of the GI tract in *sparse* larvae appeared to be larger compared to wildtype, suggesting that the null *kita* mutant resulted in a phenotype similar to the ACK-2 effects on mice. We measured the area of a longitudinal cross section of wildtype and *sparse* mutant larvae at 7 dpf. The average area of the GI tract in wildtype larvae was 4491 ± 754 and 5532 ± 880 in *sparse* larvae. Fluorescence spectroscopy was used to quantify the fluorescence intensity. Total fluorescence emission averaged 62,630 ± 23,780 counts per second immediately after ingestion in 11 dpf larvae. One day later, total fluorescence intensity averaged 53,277 ± 6,333 counts per second, a 15% decrease. 5-hydroxytryptamine (5-HT), a prokinetic agent, was used to validate the assay. One day after loading, the total fluorescence intensity decreased after application of 5-HT to 86,820 ± 21,850. 11 dpf zebrafish larvae ingest fluorescent labeled microspheres, and spectroscopic analysis showed reduced fluorescence intensity one day after loading. Functional studies showed a reduced contraction frequency and an increase in surface area between *sparse* mutant and wildtype larvae. The decrease in fluorescence intensity in wildtype larvae suggests aboral movement of microspheres, out of the posterior GI tract.

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Specific Goals of the Research

The purpose of this study is to develop novel and enhance preexisting methods to study gastrointestinal (GI) motility in zebrafish larvae. This study resulted in the development of three functional GI motility assays. The contraction frequency assay was used to determine if the rate of propulsive contractions differed between the zebrafish *kita*-null mutant, *sparse*, and wildtype zebrafish larvae. The fluorescence assay was used to evaluate differences in gastric emptying resulting from propulsive contractions in larvae at different developmental stages in wildtype larvae. The spectroscopy assay measured differences in fluorescence emissions between untreated wildtype larvae and wildtype larvae treated with a prokinetic agent.

Introduction

The function of the gastrointestinal (GI) tract is to breakdown food and extract nutrients and energy then expel the remaining material as waste. Each part of the digestive tract performs a different function during this process; therefore material must move through the tract in an oral to anal fashion for proper digestion. The transit of food, along with contractions that mix food, are defined as gastrointestinal motility.

From an anatomical perspective the human GI tract is a collection of concentric cylinders consisting of four distinct tissue layers. Moving outward from the lumen are the mucosa, submucosa, muscularis, and serosa. This thesis will focus on the muscularis, which can be further broken down into concentric layers of circular smooth muscle, myenteric plexus (a collection of ganglia) and longitudinal smooth muscle. Interstitial cells of Cajal (ICC) are primarily located in the myenteric plexus region, and at lower densities throughout smooth muscle. ICC are derived from smooth muscle and are pacemaker cells that generate slow waves through the GI tract. The collective effort of ICC, enteric neurons, and smooth muscle result in GI motility.

Common GI Motility Disorders

The importance of GI motility can be demonstrated by diseases that result in GI dysmotility. Four diseases of this kind are Hirschsprung's disease, intestinal pseudo-obstruction, diabetic

gastroparesis, and post-operative ileus. Hirschsprung's disease is a potentially fatal disorder first described in 1911 by Harald Hirschsprung and affects 1 in 5000 infants and children (Dasgupta and Langer 2004). This disease arises from the absence of ganglia in portions of the large bowel, which causes the lumen to continuously contract, and thus prevents material from passing through. In addition to ganglia, a decreased number of ICC have been observed in patients with Hirschsprung's disease when compared to normal individuals (Dasgupta and Langer 2004). Common treatment for this condition is surgical removal of the intestinal segment lacking ganglia, however, even after surgery up to 70% of patients have continued difficulties passing stool (Rose 1998).

Intestinal pseudo-obstruction, sometimes called chronic intestinal pseudo-obstruction (CIP), is a disease in which peristalsis is halted in parts of the intestine yet no physical obstruction, or lesion is present. It has been suggested that CIP could result from an absence of ICC (Jain, Moussa et al. 2003). There are two forms of CIP; the first form presents itself at or near birth, and a second form develops, in post-surgical circumstances or through administration of drugs that affect GI motility (Jain, Moussa et al. 2003). Therapy for this disease is limited to treatment of the symptoms. Abdominal pain can be alleviated with tricyclic antidepressants, constipation can be treated with suppositories and/or enemas, and antibiotics are prescribed for bacterial infections (Rose 1998). In severe cases removal of part or all of the intestine or intestinal transplantation may be necessary (Rose 1998). Such extreme procedures can drastically decrease the patient's quality of life.

Delayed gastric emptying resulting from diabetes (diabetic gastroparesis) is reported in 50% of diabetic patients, approximately 10.4 million adults and children (CDC 2005; Med 2007). Changes in blood glucose level can affect GI motility by either delaying or accelerating gastric emptying. The number of neurons in the myenteric plexus of the stomach and colon are reduced in the streptozotocin-induced diabetes rat model (Rayner and Horowitz 2006). Additionally, a decrease in ICC is associated with the onset of diabetes. A reduction in the number of ICC is also seen in humans with type 1 diabetes (Smith DS 2003). Treatment for diabetic gastroparesis is limited to the relief of symptoms via medications but more commonly, adjustment of the patient's diet.

Post-operative ileus (POI) is a temporary impairment of GI motility, which is typically associated with abdominal surgery and less commonly with other types of non-abdominal surgeries (Person and Wexner 2006). It has been suggested this condition may result from abnormalities in neural reflexes (Kehlet and Holte 2001). The illness typically transpires post surgery and treatments are required to lessen complications associated with these issues. Treatments for POI are limited to prokinetic agents and surgical procedures such as laparoscopy (Kehlet and Holte 2001). Patients suffering from the POI have longer recovery times then those who do not have surgical complications (Person and Wexner 2006). In addition to being unpleasant for the patient, POI leads to increased health care costs due to extended hospital stays.

Those of us fortunate enough not to suffer from such severe disorders most likely have experienced some kind of abdominal pain or discomfort associated with GI dysmotility. Common disorders affecting millions of people across the nation include irritable bowel disease (IBD), inflammatory bowel syndrome (IBS), constipation, bloating, and heartburn. These diseases are often caused by a variety of factors and overlap with other functional diseases sometimes making diagnosis and treatment difficult.

Treatment Options for Intestinal Dysmotility in Humans

Prokinetic agents are pharmaceuticals that increase the rate of gastric emptying. Examples of prokinetic agents currently on the market include cisapride, Gleevec, metoclopramide, and domperidone. Metoclopramide is a dopamine D_1 and D_2 receptor antagonist, meaning it blocks the action of dopamine in the GI tract (Tonini 1996). Preventing dopamine from binding to its receptor in the GI tract is important in promoting GI motility. Significant amounts of dopamine are located in the walls of the GI tract; this neurotransmitter is one of the molecules capable of inhibiting GI motility (Tonini 1996). In addition to impeding the action of dopamine, metoclopramide also facilitates the release of acetylcholine in the myenteric plexus, which aids in both its prokinetic and antiemetic (preventing nausea and vomiting) actions (Tonini 1996). Unfortunately, metoclopramide is only effective for short-term treatment because tolerance develops with extended use (Tonini 1996). Adverse effects such as anxiety, insomnia, agitation and mood swings occur in as many as 20% of patients (Tonini 1996), all of which result from metoclopramides ability to cross the blood brain barrier.

Domperidone is another prokinetic and antiemetic agent which blocks the release of dopamine and promotes the release of acetylcholine, similar in action to metoclopramide. However, domperidone specifically antagonizes the dopamine D_2 receptor (Tonini 1996). This drug has never been approved by the FDA for use in the United States, but is in wide use in Europe and in Canada (da Silva and Knoppert 2004). Domperidone has few side effects; however, it has been known to promote lactation in women that have not recently given birth, and irregular menstruation (da Silva and Knoppert 2004). Within the last three years intravenous administration of domperidone has been linked to serious heart problems including cardiac arrhythmia, cardiac arrest, and even sudden death (FDA 2004). This resulted in its removal from the market in several countries (FDA 2004).

Cisapride is among the newer prokinetic agents on the market. The mechanism of action of cisapride differs from previous prokinetic agents in that it specifically stimulates the release of acetylcholine in the ENS and does not counteract the effects of dopamine (Tonini 1996). Adverse side effects included minor discomforts such as headaches and cramping. As cisapride prescriptions increased, the FDA announced that a significant number of patients taking the medication for several years were at a much greater risk for heart rhythm disorder (FDA 2000). The risk of cardiac arrhythmia was further increased in patients who were taking additional medications in addition to cisapride (FDA 2000).

Gleevec (spelled Glivec in Europe) was approved for the treatment of gastrointestinal stromal tumors (GIST) by the Food and Drug Administration (FDA) in February 2002 (FDA 2002). GISTs develop in the muscular wall and nervous system of the GI tract. Gleevec specifically targets tyrosine kinase receptors which mediate cell differentiation and growth (Trent and Benjamin 2006). Cells of GIST express c-Kit, and therefore treatment with Gleevec blocks GIST cell growth (Trent and Benjamin 2006). C-Kit is also expressed on the surface of ICC, the pacemaker cell of the GI tract, located in the muscularis. Because the drug targets c-Kit it has the potential to be a therapy for GI motility disorders associated with ICC. Side effects range from lower limb edema (54%-76%), to muscular cramping (28%-62%), and in a small percentage hemorrhaging (19%) (Corporation 2007). The survival rate of patients with GIST increased to 80% with Gleevec treatment (Corporation 2007).

Rational for a Zebrafish-Based Model for GI Motility

The zebrafish, *Dario rerio*, is an ideal candidate to study gastrointestinal motility for several reasons. A single female can produce upwards of 150 eggs every two weeks or less. The resulting fertilized embryos develop externally and are optically transparent, enabling direct *in vivo* observation of GI muscular contractions in addition to visceral organs such as the heart and kidney (Figure 1). Larvae hatch from the chorion between 48 and 72 hours post fertilization (hpf) and remain transparent for the first few weeks of life. Zebrafish are also attractive from an economic stand point; they are smaller and cheaper to take care of, in comparison to the leading vertebrate model organism, mice. Up to 50,000 zebrafish can be maintained in the same area that would house only a few hundred mice (Horn 2003). In addition, a larger sample size of

zebrafish develops quickly and thus allows for the collection of statistically meaningful data more often.

Zebrafish are more similar to humans than most people think. They are teleosts, meaning they are a vertebrate, similar to us. Vertebrates have more genes in common with each other than non-vertebrates and therefore their genomes are more similar. This gives the zebrafish an advantage in research over other model organisms such as the fruit fly (*Drosophila melanogaster*) or nematode (*Caenorhabditis. elegans*). The zebrafish genome is currently being sequenced at the Sanger Institute in the United Kingdom and is predicted to be completed by the end of 2008 (http://www.sanger.ac.uk/Projects/D_rerio/). The GI tract of zebrafish and humans are anatomically similar, and zebrafish development is rapid. A single celled embryo develop into tiny fish with a brain, heart, and circulating blood within 24 hours (Horn 2003). At 5 days post fertilization (dpf) zebrafish have a functioning GI tract and are able to eat (Holmberg, Schwerte et al. 2003).

Researchers have found several similarities between the human GI tract and that of the zebrafish. As in humans, the zebrafish GI tract develops as concentric cylindrical layers of tissue however, it consists of three layers, mucosa, muscularis, and serosa, instead of the four found in humans (Wallace, Akhter et al. 2005). Smooth circular muscle, enteric neurons, and longitudinal muscle are present within the muscularis (Wallace, Akhter et al. 2005). Spontaneous contractions are observed in zebrafish before and after the onset of feeding (Holmberg, Schwerte et al. 2004).

ICC have yet to be identified in zebrafish, but papers have been published suggesting their existence (Rich, Leddon et al. 2007).

Kit is a proto-oncogene, a gene involved in cell signaling, growth or regulation that if mutated could result in cancer. Kit codes for a receptor tyrosine kinase, a class of proteins that play an important role in cell growth and differentiation. Kit plays a vital role in hematopoiesis signaling, a kit null mutation is lethal in humans and in mice because blood will not develop. Kit is also important in melanocyte development, the mouse model used to study Kit, White spotting (W/Wv), is so named because these mice have abnormalities affecting the color of their coat in addition to irregular intestinal pace-making and hematopoiesis (Maeda, Yamagata et al. 1992). The zebrafish mutant *sparse* exhibits similar abnormalities to the W/Wv mouse model. *Sparse* mutants have altered stripe patterns which have been mapped to the *kita* gene (Hultman, Bahary et al. 2007). Two orthologs for the *kit* gene have been identified in the zebrafish, *kita* and *kitb* (Hultman, Bahary et al. 2007).

Genes and resulting proteins are commonly given the same name. To distinguish a gene from a protein in literature it is common for the gene to be written in italics. When the same protein is present across species the capitalization of the first, all letters, or none of the letters may change. For example, in humans KIT is written in all capital letter, in mice and zebrafish, only the first letter is capitalized.

This thesis will further focus on the development of a functional assay to quantify gastrointestinal motility in the zebrafish larvae. These assays will be used to determine the role of *kita* on GI motility by comparing GI function between wildtype and *sparse* mutants. We hypothesize that kit a plays a role in the development of ICC within the zebrafish GI tract. Preliminary observations of GI motility in *sparse* mutant larvae show erratic and poorly organized contractions. Mutations in the *kit* gene in the mouse model are associated with the lack of ICC and irregular, unorganized GI motility patterns (Hirst and Edwards 2004). Functional data generated from these experiments will be correlated with morphological and molecular data collected in our laboratory and will contribute to the overall goal of developing a zebrafish based model for GI motility.

Methods

Aquaculture and Husbandry

The single most important parameter for zebrafish health and fecundity is water. Appropriate water chemistry must be maintained for a colony to be successful. Zebrafish originate from the freshwater lakes and streams in the Indian subcontinent ranging in temperature from 18 to 24°C. Many laboratories successfully raise zebrafish at a variety of levels of salinity and pH. Our lab aimed to maintain disease-free fish that reach maturity quickly, and have reliable and high fecundity. Protocols were obtained from two common zebrafish laboratory guides, The Zebrafish Book and Zebrafish: A Practical Approach (Nüsslein-Volhard and Dahm 2002). System water, the water used for housing adult zebrafish, was prepared using reverse osmosis water with 240 mg of Instant Ocean salts per liter (this value has since been increased to 720 mg, as higher salinity increases fecundity) resulting in a salinity of approximately 450 ppm. Optimal pH was 7.2 and was reached by adding 75 mg/L sodium bicarbonate ($NaHCO_3$), and adjusting pH with NaOH or HCl as needed. The temperature of the system was kept at 28°C.

Understanding the nitrogen cycle is vital to maintaining a healthy fish colony. When a new colony is established, water quickly becomes polluted with nitrogen waste in the form of ammonia (NH_3) stemming from uneaten food and fish waste. Denitrifying bacteria metabolize nitrogen from NH_3 to nitrites (NO_2^-) which are further broken down to nitrates (NO_3^-), the least bio-toxic form of nitrogen. Nitrates are removed by water

changes. Approximately 20% of the system water in our tanks was changed daily. Circadian rhythms are also important for a healthy zebrafish colony and regular day/night cycles are fundamental for reproductive success. Our lab used the common cycle of 14 hours of light and 10 hours of dark.

Nutrition is important to maintain fecundity. Common practice is to feed live food, this is widely reported to give high fecundity. Fish were fed live brine shrimp or cyclopeeze (Argent, Redmond, WA) three times daily, morning, afternoon and evening. Brine shrimp (*Artemia*), (Argent, Redmond, WA) were cultured using a cylindrical cone and bubbler. Water to grow shrimp was prepared by dissolving 66 g of Instant Ocean in 2 L of deionized water and pH was adjusted to 8.6. Approximately 1 g of cysts were added to shrimp water and incubated with continuous illumination at room temperature for 48 hours. Fish were administered an amount of food that all was consumed within 5 minutes.

Adults were bred in the morning shortly after lights were turned on. Two males and three females were typically placed in each breeding tank. Eggs were checked for and collected every 30 minutes. Fertilized eggs were transferred to embryo water (E3). A 10X stock solution of E3 was prepared using 86 g NaCl, 3.8 g KCl, 14.5 g $CaCl_2 \bullet 2H_2O$, 24.5 g $MgSO_4 \bullet 7H_2O$ dissolved in 500 ml deionized water (Nüsslein-Volhard and Dahm 2002). E3 was diluted to 1X using deionized water and the pH was adjusted to approximately 7.2. A small amount of methylene blue was added to E3 to discourage

fungal growth; however, this practice was suspended after we learned that the peak fluorescence emissions of methylene blue and FITC (fluorescein isothiocyanate) were not well separated.

Embryos were kept in beakers of E3 approximately one third full, beakers and embryos were placed in water baths held at a constant temperature of 28.5°C. It is important to keep the surface area-to-water volume ratio high so that dissolved oxygen levels remain high, or larvae will not survive. Beakers were cleaned and one-half of the water volume was changed daily. At 7 dpf, larvae were fed Hatchfry encapsulation, grade 0, H0 (Argent, Redmond, WA) and transferred to system water. At 11 dpf, brine shrimp were introduced to the larvae's diet.

Functional GI Motility Assays

Functional GI motility refers to the muscular contractions of GI smooth muscle that contribute to mixing and propulsion of luminal contents. Standard protocols have been developed to measure gastric emptying resulting from functional motility in the mouse model. In one such experiment, phenol red is combined with methylcellulose and added to food. Mice are deprived of food then gavaged a phenol red meal, the rate of GI transit can be determined upon subsequent sacrifice (Suchitra, Dkhar et al. 2003). Similar assays have not been developed for the zebrafish model. GI motility has been reported as contraction frequency in zebrafish larvae, but it is not clear whether these contractions contribute to propulsion or to mixing functions of the GI tract, or whether they are completely spontaneous and disorganized. The objective of this thesis is to develop a set

of assays that will quantify propulsive motility in the zebrafish. Three separate assays were developed; contraction frequency, fluorescence loading, and spectrophotometer-based. Assay methodology and rational for development are described in the following sections.

Contraction Frequency Assay to describe GI motility patterns

GI muscular contractions can be directly observed in zebrafish larvae beginning at 4 dpf. Spontaneous contraction of GI smooth muscles occurs at a frequency of 1 contraction per minute beginning at 4 or 5 dpf, and does not change with development (Holmberg, Schwerte et al. 2004). We decided to repeat these experiments under our laboratory conditions, and extend the time points to determine whether spontaneous GI contractions changed as larvae developed beyond 6 dpf.

Larvae were removed from their beakers and transferred in a minimal amount of solution to a beaker containing a mixture of system water and blue dye (McCormick). Larvae spontaneously ingested system water and dye, which aided in visualization of the GI tract lumen. Blue dye displayed no apparent ill effects on the zebrafish larvae's health. Larvae were kept in the solution for 1 hour to allow sufficient dye loading, and were rinsed three times in beakers of clean system water. Tricane/mesab (17.2 mg/100 ml) was added to the final beaker of system water to anesthetize larvae before video recording.

Video records of the larvae GI tract were collected to document muscular contractions. Movement of larvae during recordings was minimized using a 1.2% agar (Sigma, St. Louis, MO) solution with embryo medium (E3). The agar solution was held at constant temperature, 42°C, in a circulating water bath, along with several glass slides and narrow orifice glass pipettes (Westerfield 1993). Once anesthetized, larvae were individually transferred to the agar solution and placed on a warm dry slide. Agar in combination with anesthetic effectively immobilized larvae and prevented drift during filming. An optimal view of the GI tract resulted when larvae were oriented laterally. Larvae blood flow was easily observed and was therefore used to monitor health. If blood flow slowed appreciably then the larvae was not used in the experiment. One common problem was agar-drying. Therefore, one drop of E3 containing tricaine anesthetic was placed on the agar to keep the larvae anesthetized during filming and to prevent the agar from drying out. GI contractions were filmed at 4X magnification for 7 minutes with a Canon Optura Xi digital video camera mounted to a Nikon inverted microscope. When filming was completed, larvae were carefully released from the agar and returned to system water (Westerfield 1993). Analog footage was converted to a digital format with Pinnacle Studio AV/DV software. Experiments were performed on 6 dpf, 7 dpf, and 11 dpf larvae using wildtype, *sparse*, and *colourless* mutants.

Digital videos were reviewed manually for contraction frequency (number of contractions per minute) and total number of contractions per 7 minute recording. Contraction

intensity was determined visually by scoring the contractions using 0 for no contractions, 1 for modest contraction, and 2 for the strongest contraction which resulted in complete occlusion of the lumen. The position where contractions were graded was important because more frequent and disorganized contractions were observed in the intestinal bulb (proximal gut), versus the mid and anterior portions of the GI tract. Our data were collected for GI contractions beginning immediately posterior to the intestinal bulb, traveling towards the anus. GI contractions in the zebrafish are slow, occurring about once per minute, and are therefore extremely easy to miss. However, speeding up the digitized video to five times the normal speed made it much easier to identify contractions, and allowed distinction between incomplete and complete contractions that fully propagated to the anus. Data extracted from these videos was exported to Microsoft Excel for further analysis. A minimum of 20 separate fish were examined for each time point; the mean and standard deviation were then calculated. Differences between samples were determined using the un-paired student's t test and P values < 0.05 were considered statistically significant.

Fluorescence Assay to evaluate differences in gastric emptying

Zebrafish embryos are naturally transparent, a feature persisting for the first few weeks of their lives. Essential organs such as the heart, brain, and GI tract can be directly observed developing and functioning *in vivo*. FITC labeled microspheres (Fluka, Item#: 06296) are an ideal material to load larvae with because they make it easier to see the GI tract when illuminated with the proper wavelength of light. A series of fluorescent images

containing wildtype and *sparse* mutant larvae were collected with fixed imaging parameters at different developmental stages, subsequent to loading with fluorescent microspheres. We expected that propulsive GI contractions would decrease fluorescence intensity as fluorescent micro spheres are propelled through the GI tract, and eliminated. Therefore, a decrease in fluorescent intensity over time will reflect propulsive GI motility. Fluorescence intensity was measured in specific proximal and anterior regions of the GI tract immediately after washing loaded larvae, and after larvae had 24 hours to process the labeled microspheres. Measurements were taken at two developmental time points, 7 dpf and 11 dpf. We hypothesize that gastric emptying resulting from propulsive contractions will correlate with larvae development. The *sparse* mutant lack a functional *kita* gene, which has been predicted to play a role in ICC development. ICC are GI pacemaker cells, and deficiencies in Kit function have been correlated to deficiencies in ICC and GI motility in mice and in humans. Therefore, we hypothesize that propulsive GI motility will be deficient in the *sparse* mutant larvae.

Larvae were removed from system water and transferred, in minimal volume, to a beaker containing E3 and FITC labeled microspheres at a concentration of 1mg/ml. FITC is a common fluorescent probe that absorbs light at approximately 488 nm and emits light at 520 nm. Larvae were held in the solution for 1 hour, and were rinsed three times in beakers of clean system water to remove microspheres that were not confined to the GI tract. Tricane/mesab was added to the final beaker of system water making a 1X anesthetic. Larvae were oriented laterally and fluorescent images of the GI tract were collected at 4X and 10X magnification. Post-imaging, each larvae was placed in

individually labeled wells of a 24-well plate and kept in a room at 28°C for 24 hours. The following day larvae were re-imaged to assess whether there was a decrease in florescence intensity of FITC resulting from evacuation of microspheres from the GI tract. Following image collection, larvae were returned to system water. These experiments were preformed on both wildtype and *sparse* mutants.

All specimens were examined using an Olympus BX51 microscope. Images were captured with a Spot RT digital camera (Diagnostic Instruments). Image Pro Plus (software version 5.0; Media Cybernetics) was used for image analyses. Collection parameters were held constant throughout, ensuring image intensity could be directly compared between samples. Exposure time, binning, and gain were standardized to 500 ms, 2x2, and 10, respectively. A basic working knowledge of each of these parameters, and digital imaging in general, is important during acquisition of images and extraction of data. Digital images are comprised of pixels, or X-Y positions, and each position has a brightness (intensity) value. We collected monochrome images, and therefore each pixel is associated with a grayscale value ranging form 0 (no light) to 2^{12}, or 4096 (maximum exposure = very bright). Exposure time is the amount of time an image is subjected to light, thus the longer an image is exposed, the brighter it will be. Pixel intensity is directly related to exposure time, therefore the longer a pixel is exposed to light the more intense the pixel will be.

The process of binning involves adjustment of the size of each pixel in the image. Accumulation of electrons on a pixel is important since the number of elections increase the brightness of the image increases. Binning can be selected in increments such as 1x1, 2x2 and 4x4. A binning of 1x1 uses a single pixel to collect photoelectrons and results in the best resolution. However, 1x1 binning requires the longest exposure time. A binning of 2x2 uses 4 pixels to collect photoelectrons and thus significantly decreases exposure time but results in an image at ½ resolution when compared to an image with 1x1 binning. (http://www.ccd.com/ccd103.html).

Gain can be thought of as a range of grayscale. Small gain numbers, for example a gain of 1, will result in a greater difference between black and white. A gain of 20 yields a minimal range between black and white resulting in an image with less contrast. Increasing the gain value is extremely useful for dim images, enabling visualization of relevant structures; however, this reduces image contrast. These parameters are related as gain will affect exposure time. For example, a large gain number reduces exposure time.

After reviewing numerous videos it became obvious that different regions of the larvae GI tract had different contraction frequency, intensity, and coordination. Pixel intensities in two locations in the GI tract were measured. The first was in the anterior GI tract, 7 cm from the nose; the second was in the posterior GI tract 14 cm from the nose. This separation can be converted into a distance measured in pixels. At 4X magnification the separation between these two points of interest was approximately 43,887 pixels; at 10X

magnification the separation was approximately 109,718 pixels. The mean pixel density for each area of interest was measured using a fixed area ellipse. The next day larvae were re-imaged and the difference in mean pixel density was calculated for the anterior and posterior GI tract.

In addition to measuring the change in intensity of FITC labeled microspheres in the GI tract, the total area of the GI tract was also calculated. Studies in the W/Wv mouse model have shown that gastrointestinal volume increases when Kit signaling is blocked with an anti-Kit antibody. We observed a larger then normal gastrointestinal area in *sparse* larvae which may suggest that events similar to those seen in the W/Wv mouse model experiment are taking place. To measure the size of the GI tract in zebrafish larvae we used tools within Image Pro Plus software (Media Cyberbetics) to outline and calculate the area.

Spectroscopy Assay to measure propulsive GI contractions

Spectrophotometers measure the intensity of light transmitted through or emitted by a fluid. In our experiments this methodology allowed for measurement of the total fluorescence emission of ingested rhodamine-labeled microspheres (Fluka Item#: 90518). We expected to see a decline in fluorescence emissions over 24 hours in zebrafish larvae. A decrease in fluorescence emissions would result from the removal of fluorescent microspheres due to propulsive GI motility. Experiments were preformed on both 7 dpf and 11 dpf larvae incubated in a solution of E3 and rhodamine-labeled microspheres (1

mg/ml), washed three times in fresh E3, and anesthetized using the procedure analogous to that followed in fluorescence assay.

Larvae were separated into two groups, the first contained five larvae that were manually homogenized in a micro-centrifuge style tissue masher. The supernatant was removed and fluorescence emissions were measured. The second group was kept overnight at approximately 28°C to allow for excretion of the microspheres. Post-incubation, five larvae from this group were washed and processed identically to the first group. A second time point was measured for 11 dpf larvae. The experiment was also performed on wildtype larvae treated with 5-hydroxytryptamine (5-HT) (10-5 mg/ml), a prokinetic neurotransmitter commonly found in the GI tract (Furness 2006). This assay was fundamentally different compared to the fluorescence assay, in that groups of larvae were assayed as opposed to individual larvae. This practice was necessary in order to increase the fluorescence signal, because individual larvae do not eliminate enough beads in 24 hours for a significant decline in fluorescence intensity to occur.

Results

Contraction Frequency Analysis

The greatest advantage of the zebrafish model system is the opportunity to directly visualize organ function in intact, unperturbed zebrafish larvae. Contractions of GI muscle occur at low frequency, about 1 per minute, and are relatively easy to see using a microscope with 10X magnification. GI motility was recorded to video tape using a standard digital video camcorder mounted to the microscope, and digitized to the computer hard drive. Digitized video recordings were evaluated for the number of contractions per 7 minutes, and the number of contractions per minute was calculated. A *t* test was preformed to determine the significance of the data, if the *P* value was less than 0.05 the difference was considered significant. Propagating contractions appeared to differ in contraction intensity. Apparently powerful contractions completely occluded the lumen, and apparently weaker contractions did not. Therefore the apparent contraction intensity was graded with a score of 0 for no contractions, 1 for modest contraction, and 2 for contractions resulting in complete occlusion of the lumen.

Twenty wildtype larvae were filmed at 7 dpf and contraction frequency averaged 0.586 ± 0.222 contractions per minute. The contraction frequency was measured in 11 dpf larvae to determine if GI motility changes with development at this stage. At 11 dpf contraction frequency was 0.393 ± 0.071 contractions per minute. Although it appears contraction frequency decreased by ~20% (Table 1, Figure 2), the results were not significant (*P*

value = 0.104). It is interesting that the variability was reduced at 11 dpf compared to 7 dpf.

The role of the *kita* gene on GI motility, and the development of GI motility, was examined using the *sparse* mutant larvae, a *kita* null mutant. GI motility was filmed in 7 dpf and 11 dpf *sparse* larvae and contraction frequency was determined. Average contraction frequency in 7 dpf *sparse* larvae was, 0.329 ± 0.158 contractions per minute, a 26% reduction compared to wildtype 7 dpf larvae (Table 2, Figure 3). A *t* test revealed this to be significant with a *P* value of 0.0288. Contraction frequency increased to 0.714 ± 0.241 contractions per minute in 11 dpf *sparse* mutants, compared to 11 dpf wildtype larvae. A *t* test revealed this to be significant with a *P* value of 1.70E-05 (Table 3, Figure 4). Comparing contraction frequency in 7 dpf and 11 dpf *sparse* mutant larvae shows a ~50% increase between 7 dpf and 11 dpf (Table 4, Figure 5). This data was also significant with a *P* value of 1.70E-05.

Contraction intensity was scored on a scale from 0 (weak) to 2 (strong) to quantify differences in the contraction appearance. Apparent contraction intensity decreased in wildtype larvae from 1.250 ± 0.444 at 7 dpf to 1.000 ± 0.000 in 11 dpf larvae (Table, 5, Figure 6). Apparent contraction intensity also decreased as *sparse* mutant larvae developed. Contraction intensity in 7 dpf *sparse* larvae was 1.619 ± 0.498 that decreased to 1.125 ± 0.354 at 11 dpf (Table 8, Figure 9). Comparing wildtype and *sparse* larvae

shows that contraction intensity was greater in both 7 and 11 dpf *sparse* when compared to age-matched wildtype larvae (Table 6, Figure 7, Table 7, Figure 8).

Contraction frequency and intensity was also measured in the *colourless* mutant, a line of zebrafish that lacks an enteric nervous system. Measurements were taken at 6 dpf with the concern that the larvae would not survive to 7 dpf. Sixteen larvae were included in the study, however contractions were only observed in 8. Contraction frequency averaged 0.393 ± 0.183 per minute at 6 dpf. Contraction intensity averaged 1.000 ± 0.000.

Fluorescence Analysis

The fluorescence assay, attempts to measure the movement of a marker substance through the GI tract. FITC labeled microspheres were added to the zebrafish medium, and larvae spontaneously swallowed the microspheres. Larvae were subsequently washed, and returned to clean media, anesthetized, and imaged. Care was taken to keep all imaging parameters that affect 'brightness' constant so that image intensity could be compared. Exposure time, binning, and gain were fixed at 500 ms, 2x2, and 10, respectively. An area of interest was defined in the anterior and posterior GI tract, and the mean pixel density was measured using Image Pro Plus software version 5.0. Larvae were imaged immediately after loading, and after 24 hours. Fluorescence intensities were normalized against initial intensity of ingested microspheres at 7 dpf in the intestinal bulb of the anterior GI tract (Figure 10). This was the brightest portion of the GI tract and we reasoned that larvae that ingest many microspheres would pass many

microspheres into the posterior gut. Conversely larvae that ingested fewer microspheres were expected to have a smaller drop in fluorescence intensity. Differences in bead ingestion in the intestinal bulb resulted in an average pixel intensity ranging from 407 to 3245 in a defined region of interest (an ellipse) that was placed over the intestinal bulb.

Fluorescence intensities in the anterior and posterior GI tract were measured in wildtype and *sparse* larvae. Fluorescence intensity in the anterior GI tract of 7 dpf wildtype larvae decreased by 0.371 ± 0.030. Fluorescence intensity in the posterior GI tract decreased by 0.119 ± 0.021 . Changes in fluorescence intensity were also measured in 11 dpf larvae. At 11 dpf fluorescence intensity 0.455 ± 0.056 in the anterior GI tract, and 0.258 ± 0.046 in the posterior GI tract (Table 9, Figure 11).

Peritoneal injection of the ACK-2 anti-Kit antibody results in distention and an increased volume in the GI tract (Figure 12) (Maeda, Yamagata et al. 1992). These effects result from inactivation of Kit function by ACK-2. The size of the GI tract in *sparse* larvae appeared to be larger compared to wildtype, suggesting that the *kita* null mutant resulted in a phenotype similar to the ACK-2 effects on mice. We measured the area of a longitudinal cross section of wildtype and *sparse* mutant larvae at 7 dpf. The average area of the GI tract in wildtype larvae was 4491 ± 754 and 5532 ± 880 in *sparse* larvae (Table 10, Figure 13).

Spectroscopy Analysis

A third assay was developed to determine the functional outcome of propulsive GI motility. Zebrafish larvae were loaded with rhodamine labeled microspheres and separated into two groups. Total fluorescence emission was immediately measured in one group, and the second group was incubated overnight, and total fluorescence emission was measured. Propulsive motility should eliminate some microspheres, and therefore the decrease in fluorescence intensity should be proportional to propulsive motility. Fluorescence emission from the microspheres was measured at a wavelength of 530 nm using a steady-state fluorimeter.

Total fluorescence emission of ingested rhodamine labeled microspheres from 7 dpf wildtype larvae averaged 112,475 ± 21,919 counts per second. One day later, total emissions averaged 55,788 ± 56,687 counts per second, a decrease of 56,687 (Table 11, Figure 14). Fluorescent emissions averaged 62,630 ± 23,780 counts per second immediately after ingestion at 11 dpf. One day later, total average fluorescence emissions declined to 53,277 ± 6,333 counts per second, a decrease of 9,353 (Table 12, Figure 14).

Pharmacological agents which promote propulsive GI motility should result in elimination of more ingested microspheres compared to control experiments. Total fluorescence emission was 56,243 ± 12,638 counts per second in 5-HT treated larvae. Compared to untreated larvae (112,475 ± 21,919 counts per second) fluorescence

emission dropped 56,232 (Table 11, Figure 14). At 11 dpf the average fluorescent emission of 5-HT treated larvae was 86,820 ± 21,850 counts per second and decreased to 40,626 ± 4,754 counts per second.

Discussion

Gastrointestinal motility is coordinated muscular contractions that mix and propel luminal contents in an oral to anal fashion. Coordination is controlled by three cell types enteric neurons, ICC, and smooth muscle. Information is sent from enteric neurons to ICC which regulates the information sent to smooth muscle to produce coordinated GI motility. Kit is a tyrosine kinase receptor found specifically on the surface of ICC in the GI tract. These receptors are commonly used to identify ICC via immunohistochemistry. Kit is required for both the development and function of ICC. When kit is lesioned ICC disappear, and a loss of coordinated motility occurs (Sanders 2006).

The objective for this thesis was to develop an assay to quantify GI motility in the zebrafish. The primary advantage of the zebrafish model system is that the larvae are optically transparent, allowing direct visualization of the GI tract in the intact organism. Therefore, it is possible to observe muscular contractions in the zebrafish GI tract while simultaneously tracking movement of intestinal contents. This is not possible in any other model system unless x-rays are used. Although it is straightforward to watch movement of intestinal contents, a method to quantify the movement is unavailable. This assay is the first step to determine if the zebrafish is a suitable organism to a study GI motility.

Current GI motility assays in model systems, and in humans measure the functional outcome of GI motility, or measure the processes that propel the intestinal contents. A description of current GI motility assays will help determine what needs to be done for the zebrafish model. In mice, GI transit is commonly measured using the phenol red method. Mice are gavaged a meal containing phenol red and allowed to process the food for a given period of time. Once the allotted time has passed, a mouse is sacrificed and its GI tract removed. The GI tract is separated into distinct regions, homogenized, treated with acids and bases and centrifuged. The absorbance of the supernatant is measured and correlated with the concentration of phenol red. The location of phenol red relates to propulsive contractions and gastric emptying (Suchitra, Dkhar et al. 2003). In humans GI motility is measured with electrical activity in the enteric nervous system, or pressure recordings in different regions of the GI tract. For example, electrogastrography or EGG records electrical activity from the ENS in the GI tract, manometry measures pressure applied by sphincters at different locations in the GI tract, and gamma camera scintigraphy, which involves the tracking of radioactive isotopes as they move through the GI tract (Kim, Myung et al. 2000). These methods measure the mechanisms that propel intestinal contents or the movement of the intestinal contents, but not both. It is possible that an increase in activity of the enteric nervous system will increase the GI muscular contractions but may not result in an increased rate of movement of the intestinal contents. Therefore, an optimal GI motility assay would simultaneously measure the frequency of muscular contractions and the propulsion of intestinal contents.

Motility in zebrafish larvae is currently reported by measuring contraction frequency. Movement of intestinal contents has not been reported to date. Researchers in Sweden recorded contraction frequency in zebrafish larvae, from 3dpf to 8dpf, in the presence and absence of ENS neurotransmitters (Holmberg, Schwerte et al. 2003). Video recordings of the GI tract were collected and a single frame was extracted each second. The number of contractions per minute was determined. Since these were the only reports on zebrafish GI motility we decided to repeat their experiments as a starting point. Their results were extended to later developmental time points because ICC may develop after 8 dpf. We also measured contraction frequency in the *sparse* mutant because it lacks a functional *kita* gene that may play an important role in ICC development. We developed two separate assays to determine the movement of intestinal contents in the zebrafish because none currently exists. One assay involves loading fluorescent microspheres in zebrafish larvae and tracking intestinal movement of the microspheres over time using fluorescence microscopy. The second method also loads larvae with fluorescent microspheres and quantifies total fluorescence emission using spectroscopy. This assay measures the total fluorescence emission which decreases with time, as propulsive motility eliminates microspheres. These measurements were performed 7 dpf and 11 dpf wildtype larvae, and *sparse* mutant larvae.

Contraction Frequency Assay

Spontaneous contractions in GI muscle are first observed at 3 dpf, and become consistent at 4 dpf. Holmberg et al. report that GI contractions occur at a frequency of 1.02 ± 0.24

cycles per minute at 4 dpf, and 0.97 ± 0.19 between 6 and 8 dpf (Holmberg, Schwerte et al. 2003). We have observed that GI motility becomes coordinated at 7 dpf in most larvae, and is highly consistent at 11 dpf. Therefore, we believe ICC, enteric neurons, and smooth muscle to begin to function together near 7 dpf. Contraction frequency at 7 dpf was 0.59 ± 0.22 contractions per minute and decreased at 11 dpf to 0.39 ± 0.07 contractions per minute. The intensity of each contraction appeared to be different at different time points. This might influence propulsive motility. For example, weak contractions would be ineffective, and stronger contractions would be more propulsive. The strength of the contraction was scored from 0, meaning no occlusion of the GI tract to 2, meaning complete occlusion. The average intensity of contractions in wildtype larvae at 7 dpf was 1.25 ± 0.44 and 0.67 ± 0.52 at 11 dpf.

Contraction frequency was decreased when compared to Holmberg et al. (Holmberg, Schwerte et al. 2003). There are several possible reasons for this difference. Identifying GI contractions from video recordings is somewhat arbitrary, and it is possible that a GI contraction was more rigorously defined in these experiments compared to Holmberg. Our definition required at least partial occlusion of the GI lumen beginning just posterior to the intestinal bulb and traveling, uninterrupted, the entire length of the GI tract. It is also possible that the differences between our data and Holmberg's results from typical biological variability and small sample sizes. Holmberg used 8 larvae between 6 dpf and 8 dpf, and we used 20 larvae at 7 dpf. Environmental differences, fish health, and age have a direct effect on the viability of offspring, and also may influence GI motility. A constant temperature of 28.5°C is the standard temperature for zebrafish care and

development of zebrafish zygotes changes with temperature. A difference in one degree in either direction will change the rate of development. Experiments for these studies were performed at room temperature, Holmberg et al. did not report temperature. Finally, it is possible that zebrafish strains differ slightly in GI motility. We used wild type AB and gold larvae, whereas Holmberg used Hamilton zebrafish. No differences were observed between wild type AB or gold larvae. Hamilton zebrafish are unavailable.

Contraction frequency decreased in 11 dpf compared to 7 dpf larvae. It is likely that development of connections between enteric neurons, ICC, and smooth muscle accounts for increased coordination of GI muscular contractions. It is possible that each individual contraction may be more productive at propelling intestinal contents at 11 dpf as a result of better coordination. Therefore, fewer GI contractions are necessary to move intestinal contents aborally. Physiological control of GI motility may also explain decreased motility in older larvae. For example, motility differs in the fed and fasted states in humans. During the fasted state the human GI tract displays few contractions, but each individual contraction is highly organized. The contractions, called migrating motor complexes, provide a housekeeping function removing undigested debris from the GI tract and keeping area specific bacteria in its appropriate place (Karila and Holmgren 1995). A similar mechanism in the zebrafish could explain the lower frequency contractions in 11 dpf larvae compared to 7 dpf, but can not explain lower intensity contractions at 11 dpf.

During assay development the length of video recording was important. Previous work by Holmberg reported contraction frequency during 3 minute recordings (Holmberg, Schwerte et al. 2003). Initial experiments for this thesis also used 3 minute recordings and variability in the data was an issue. Variability can result from biological differences between larvae or from experimental error. The degree of biological variability was completely unknown, and our goal was to reduce experimental error. Clearly longer recording times will reduce experimental error, but more memory is required for longer recordings. Contraction frequency was slow, about 1 per minute, and therefore contractions were never missed during video recordings (approximately 50 frames per second). However, collecting 3 minutes of data resulted in between 2 and 4 contractions or 0.66 to 1.33 contractions per min. Doubling the collection time period to 7 minutes reduced variability, resulting in between 6 and 8 contractions per 7 minute recording, or 0.86 to 1.14 contractions per min. From a qualitative perspective this degree of experimental error was acceptable, and variability was considered to be biological.

Fluorescence Assay

The fluorescence assay measured the elimination of fluorescent microspheres from the GI tract of zebrafish larvae with respect to time. An assay of this kind was necessary because contraction frequency does not measure the movement of luminal contents. For example, a higher contraction frequency will increase propulsion of intestinal contents if the contractions are coordinated. Alternatively, an increased contraction frequency that is not coordinated may serve to mix intestinal contents. This assay was designed to directly

measure movement of the intestinal contents. Larvae spontaneously ingested fluorescent microspheres from the incubation medium to differing degrees. This was evident in fluorescent images collected of the loaded larvae. Therefore each image was normalized against the brightest fluorescence intensity in the intestinal bulb. Variability may be a result of the rate of development of different zebrafish. Overall the intensity of fluorescent microspheres decreased in both the anterior and posterior GI tract indicating propulsive motility. From 7 dpf to 8 dpf the fluorescence intensity in the intestinal bulb of wildtype larvae decreased from 1 to 0.758. In the posterior GI tract, fluorescence intensity decreased from 0.338 to 0.328.

A decline in both regions of the GI tract was expected. The decrease in fluorescence intensity suggests larvae are successfully removing microspheres from their GI tract with propulsive coordinated contractions. The decrease in intensity in the posterior GI tract was small, and this was also expected. Fluorescence intensity increases or decreases depending on the number of microspheres present. The posterior GI tract has a significantly smaller volume then the anterior GI tract and holds fewer microspheres. Since we are starting with a smaller number of microspheres in the posterior GI tract we expect to see a smaller change in fluorescence intensity.

Spectroscopy Assay

Changes in fluorescence intensity indicated propulsive movement but a measure of overall GI function was lacking. Therefore the spectrometry assay was developed to

measure intestinal emptying due to propulsion of luminal contents. Total fluorescence emission of ingested microspheres at 7 dpf averaged 112,475 ± 21,919 counts per second. The next day, total emissions averaged 55,788 ± 56,687 counts per second, a decrease of 50% (Table 9, Figure 11). At 11 dpf fluorescent emissions averaged 62,630 ± 23,780 counts per second. The following day fluorescence emissions dropped to 53,277 ± 6,333 counts per second, a decrease of 15%. The decrease in fluorescent emission is consistent with coordinated GI muscular contractions. The smaller fluorescence emission intensity observed in 11 dpf larvae was consistent with our measure of decreased contraction intensity and frequency in 11 dpf compared to 7 dpf larvae. For these experiments 7 dpf larvae were not fed (feeding typically begins on day 6 or day 7, after the yolk has been depleted). 11 dpf larvae were fasted overnight prior to the experiment. These conditions could have resulted in relatively poor health of the larvae, and less robust GI contractions. Protocols for raising larvae have been modified for more recent experiments and include twice daily feeding (compared to once per day), and do not fast larvae overnight before experiments. Larvae appear to be healthier but more experiments would be necessary to verify this observation.

The spectroscopy assay requires a high signal to noise ratio. Fluorescence excels in this property, a very small amount of fluorescent microspheres can be measured. An ideal marker for GI transit will be well separated from the naturally occurring fluorescence of the zebrafish larvae, and will be a stronger signal. After comparing several fluorophores and measuring the fluorescence emission of larvae, rhodamine was selected. The peak emission of rhodamine is 530 nm, well separated from background emissions that peak

near 350 nm. In addition, rhodamine has a high quantum yield which is important because of the small volume of the zebrafish GI tract.

5-hydroxytryptamine, also known as 5-HT or serotonin, is a neurotransmitter found in abundance in the GI tract and stimulates GI motility (Furness 2006). We applied an exogenous source of 5-HT zebrafish larvae and observed a significant increase in gastric emptying as seen in humans and mammalian model organisms (Bulbring and Lin 1958). These data validate the spectroscopic assay because 5-HT acts as a prokinetic in zebrafish larvae, similar to humans.

Sparse Comparison

Sparse is a line of zebrafish with a mutation in the *kita* gene. Kit is a proto-oncogene that codes for a receptor tyrosine kinase. In humans and mammals, Kit is expressed on the surface of ICC and is commonly used as a marker for their identification. ICC are required for proper GI motility and serve a pacemaker function that regulates contraction frequency and also as an intermediate cell, integrating and relaying information from enteric neurons to smooth muscle (Hirst and Edwards 2004). Zebrafish with a mutation in the *kita* gene display altered patterning of pigments and low fecundity (Parichy, Rawls et al. 1999). Video recordings showed irregular motility patterns in *sparse* larvae compared to wildtype. Contraction frequency was reduced in 7 dpf when compared to wildtype, but was increased in 11 dpf compared to wildtype. It is not clear why

contraction frequency was increased in 11 dpf *sparse* mutant larvae, but may relate to an altered distribution of ICC resulting from an inactive kita protein.

We attempted to measure the change in fluorescence intensity in *sparse* larvae at the same time points as recorded in wildtype. We successfully measured the change in fluorescence intensity of 7 dpf larvae over 24 hours however, measuring the change in fluorescence in 11 dpf proved to be difficult. Initial measurements were taken on 11 dpf *sparse* larvae but rarely did larvae survive the 24 hours until the next measurement could be taken. Because of this, the difference between the two time points could not be measured. There are several reasons for why *sparse* larvae did not survive. Homozygous adult *sparse* are notoriously difficult to breed and produce a relatively low number of fertilized embryos whose health is questionable. To increase the chances of obtaining homozygous *sparse* embryos heterozygous adults are bred. This method is reliable to producing homozygous *sparse* larvae however the health of homozygous larvae is still uncertain. Both wildtype and *sparse* larvae used in this study were fasted, and if *sparse* larvae were weak to begin with this factor may have contributed to the low survival rate.

The *white spotted mouse* (W/Wv) is a mouse model with a mutation similar to the *sparse* mutant affecting the *kit* gene. Maede et al. reported an increase in the volume of the GI tract of W/Wv mice with the application of an anti-Kit antibody (Maeda, Yamagata et al. 1992). We used the two dimensional fluorescence image and compared changes in fluorescence intensity and area of the GI tract. The gastrointestinal area of *sparse* larvae

was found to be ~20% larger then wildtype larvae of the same age. An increase in area of the GI tract in *sparse* mutants shows that *kita* function plays a role in GI smooth muscle tone, similar to the role of the *kit* gene in the mouse.

Recently, an ortholog to the *kita* gene, *kitb*, has been identified in zebrafish (Mellgren and Johnson 2005). The role of *kitb* is unknown, as well as the expression pattern. Work by our lab shows both *kita* and *kitb* mRNA expression in wildtype GI tissues (Rich, Leddon et al. 2007). Immunohistochemistry reveals expression of *kitb* in adult *sparse* mutants (unpublished observations). These data suggest the both *kita* and *kitb* play a role in ICC development, and show that *kitb* is sufficient to support ICC in *sparse* mutants.

Data from these assays contribute answers to a much larger question, what is the role of *kita* in zebrafish GI motility? We know that in humans KIT is expressed on the surface of ICC, and ICC are required for normal GI motility. We also know that Kit is required for normal GI motility in animal model systems such as *xenopus* (frogs) and mice (Hirst and Edwards 2004; Miyamoto-Kikuta and Komuro 2007). Now our question is, is *kita* required for normal GI motility in zebrafish? We have shown *kita* mRNA and kit protein expression in the GI tract of adult zebrafish and zebrafish larvae (Rich, Leddon et al. 2007). Kit positive cells can be found sandwiched between layers of longitudinal and circular smooth muscle in the zebrafish GI tract; the same location where ICC are found in humans and model organisms. The presence of *kita* mRNA supports functional data suggesting the role for ICC in coordinated GI motility in the zebrafish.

The development of the zebrafish as a model organism for GI motility holds great potential for high-throughput screens and drug discovery. We have shown the presence of *kita* and *kitb* mRNA and protein in the zebrafish GI tract, and have determined that kita plays an important role in the development of coordinated GI motility. Therefore, the zebrafish displays the three components that determine GI propulsive motility: enteric neurons, ICC, and smooth muscle cells. Experiments conducted for this thesis have contributed to the development of a GI motility assay for the zebrafish. Zebrafish larvae are the only model organisms where GI motility and throughput of intestinal contents can be observed in an undisturbed, intact setting. Tissue covering the abdominal wall of zebrafish larvae is thin and permeable to a variety of chemical and biological agents, or drugs can absorbed through the gills (Holmberg, Schwerte et al. 2003). Therefore, the effects of drugs on GI motility, and even on regional changes in motility may be observed. For example, a drug may increase contraction frequency but not in a coordinated manner, and would therefore be expected to produce dysmotility symptoms.

Future Directions

The contraction frequency assay has evolved into an assay which utilizes spatiotemporal mapping, a technique we use to spatially track the movement of luminal contents resulting from contraction of GI smooth muscles that propagate. This is a powerful analytical tool that quantifies the regularity of GI muscular contractions. It is not possible to watch muscular contractions in real time from a video and determine the

regular contractions frequency with accuracy. The spatialtemporal maps from 11 dpf larvae show that contraction frequency is highly regular. In addition, we began experiments with Gleevec, a new drug designed to treat gastrointestinal stromal tumors (GIST) which arises when the Kit gene is mutated (Hornick and Fletcher 2007). Gleevec works by inactivating KIT receptors. Larvae treated with Gleevec displayed GI dysmotility a result that further supports to our labs goal of developing the zebrafish as a model system for human GI motility.

The next steps in this research include identifying the specific roles of *kita* and *kitb* in zebrafish. Since no *kitb* mutant exists a morpholino will be injected into wildtype larvae. Morpholinos are small pieces of synthetic DNA that do not degrade and will bind to a segment of DNA and temporarily prevent it from being transcribed. A time course for *kita* and for *kitb* expression in the GI tract will help to determine the physiological role for these genes as well as the role of ICC in zebrafish GI motility.

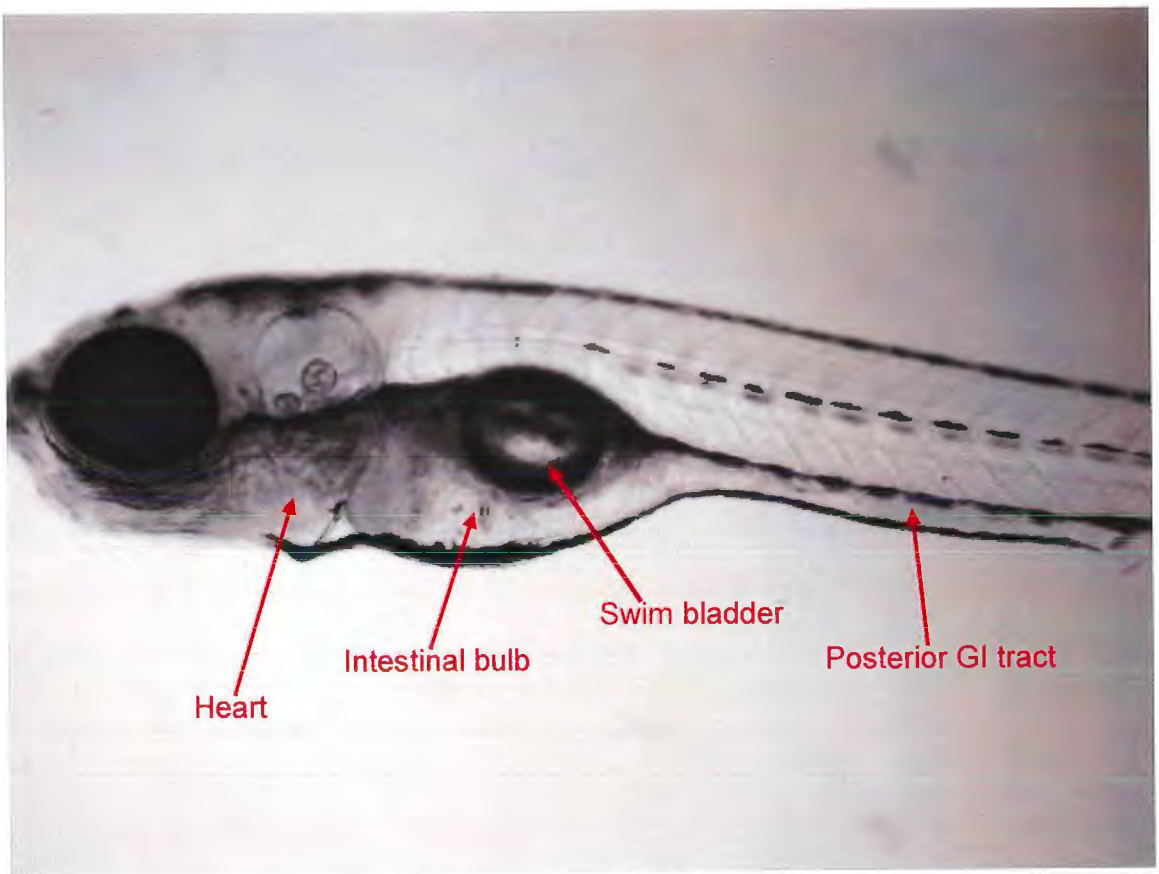

Figure 1. 7 dpf wildtype zebrafish larvae view at 10X magnification.

Table 1

Contraction Frequency in Wildtype Larvae (contractions/min)		
	7 dpf	11 dpf
Wildtype	0.586 ± 0.222	0.393 ± 0.071

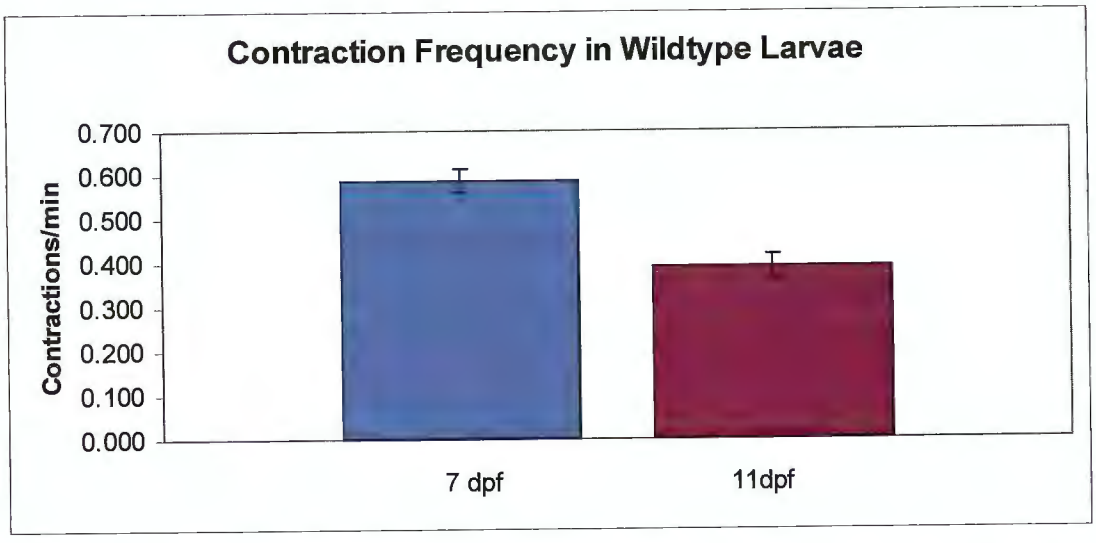

Figure 2. Contraction frequency was greater in 7 dpf wildtype larvae than in 11 dpf wildtype larvae. Contractions were manually counted from 7 minute digitized video recordings of 7 dpf and 11 dpf wildtype larvae mounted in 1.2% agar.

Table 2

Contraction Frequency in 7 dpf Wildtype and *Sparse* Larvae (contractions/min)	
Wildtype	0.586 ± 0.222
Sparse	0.329 ± 0.158

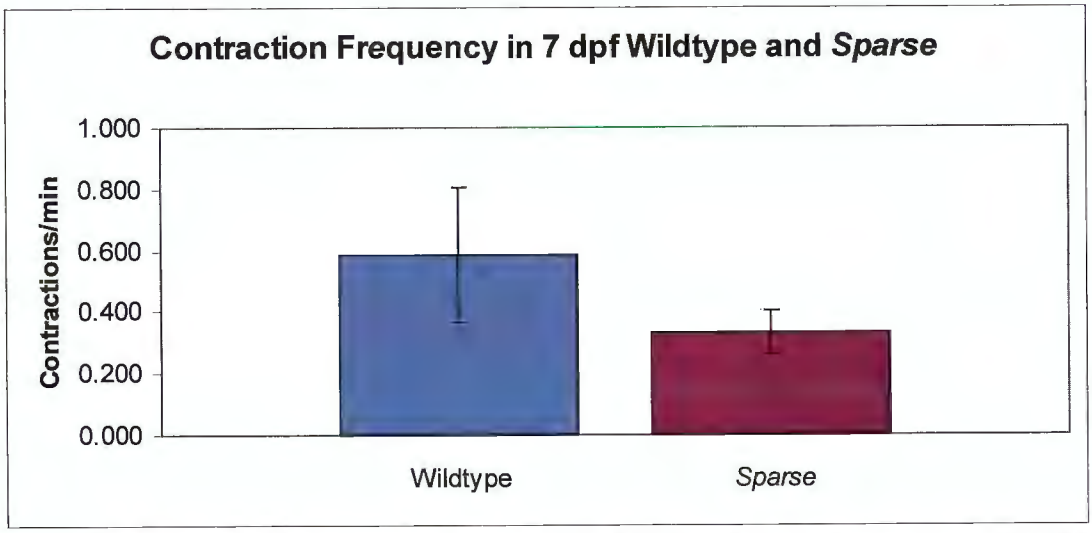

Figure 3. Contraction frequency was greater in 7 dpf wildtype larvae compared to 7 dpf *sparse* mutant larvae.

Table 3

Contraction Frequency in 11 dpf Wildtype and *Sparse* Larvae (contractions/min)	
Wildtype	0.262 ± 0.071
Sparse	0.714 ± 0.241

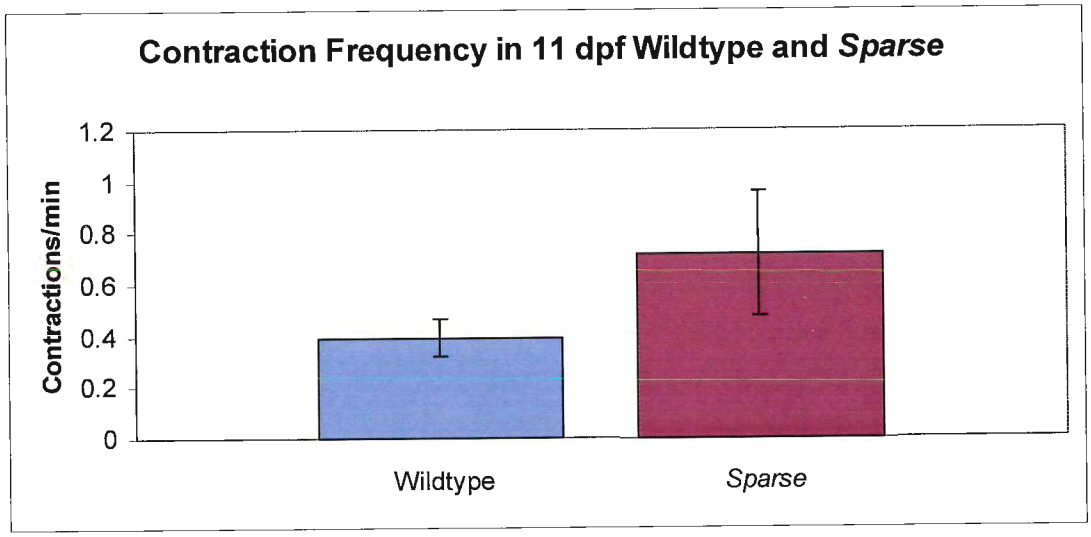

Figure 4. Contraction frequency was greater in 11 dpf *sparse* compared to 11 dpf wildtype larvae.

Table 4

Contraction Frequency in 7 dpf and 11 dpf *Sparse* Larvae (contractions/min)	
7 dpf	0.329 ± 0.125
11 dpf	0.714 ± 0.241

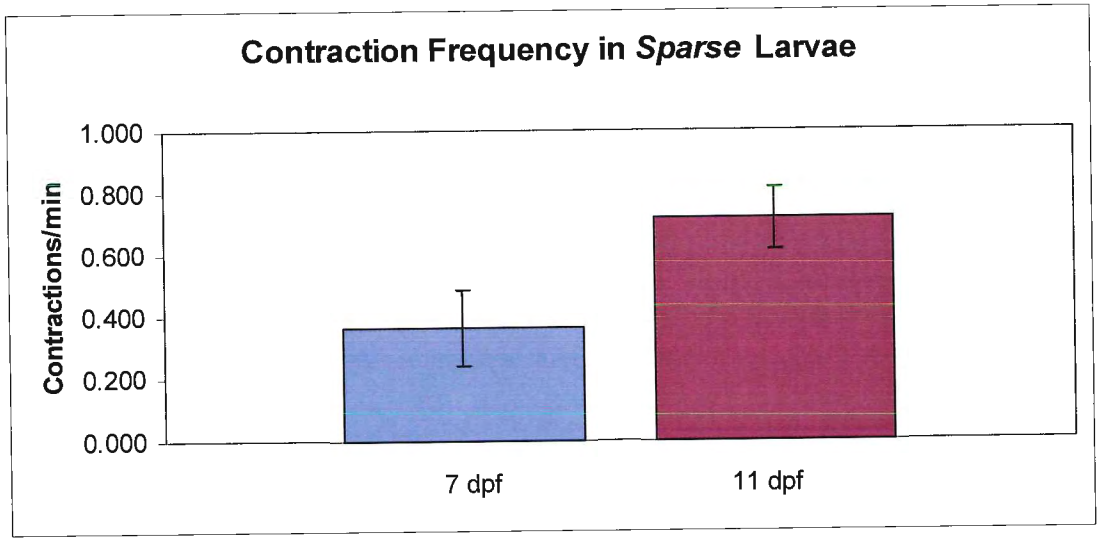

Figure 5. Contraction frequency was greater in 11 dpf compared to 7 dpf *sparse* larvae.

Table 5

Contraction Intensity in 7 dpf and 11 dpf Wildtype Larvae	
7 dpf	1.250 ± 0.444
11 dpf	1.000 ± 0.000

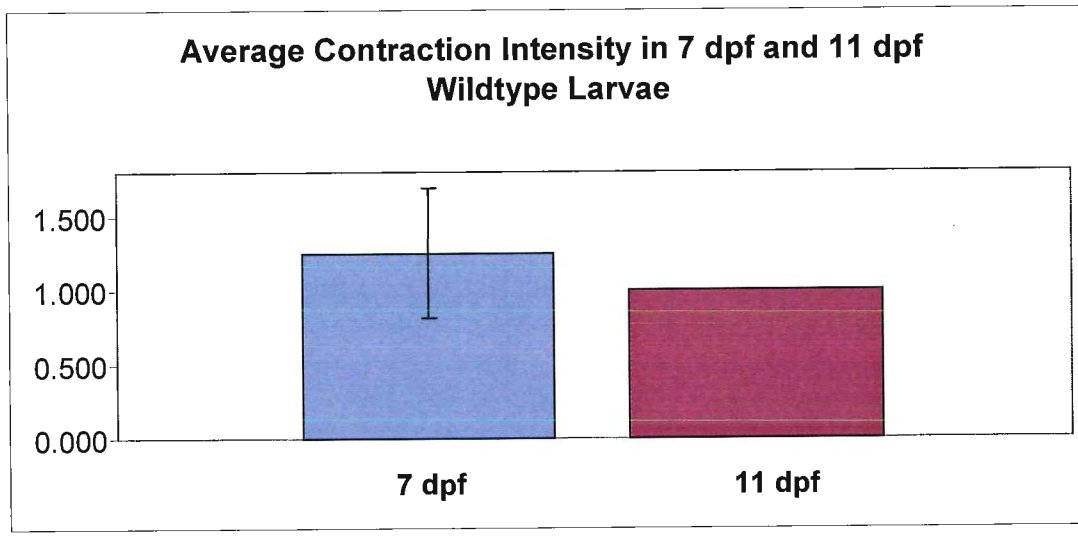

Figure 6. Contraction intensity was decreased in 7 dpf wildtype compared to 11 dpf wildtype larvae.

Table 6

Contraction Intensity in 7 dpf Wildtype and 7 dpf *Sparse* Larvae	
Wildtype	1.250 ± 0.444
Sparse	1.619 ± 0.498

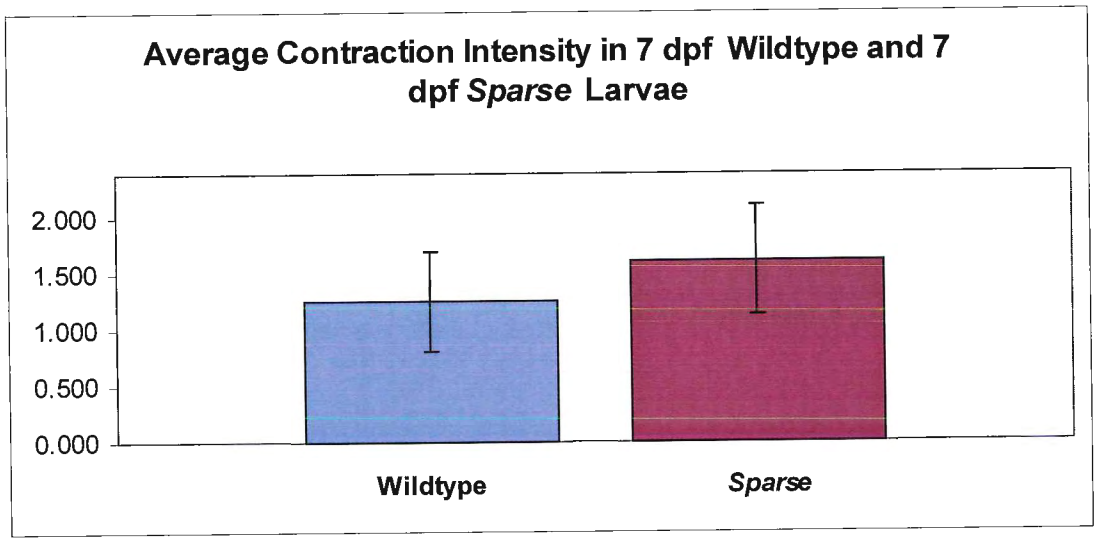

Figure 7. Contraction intensity was greater in *sparse* larvae at 7 dpf compared to wildtype larvae at 7 dpf.

Table 8

Contraction Intensity in 7 dpf and 11 dpf *Sparse* Larvae	
7 dpf	1.619 ± 0.498
11 dpf	1.125 ± 0.354

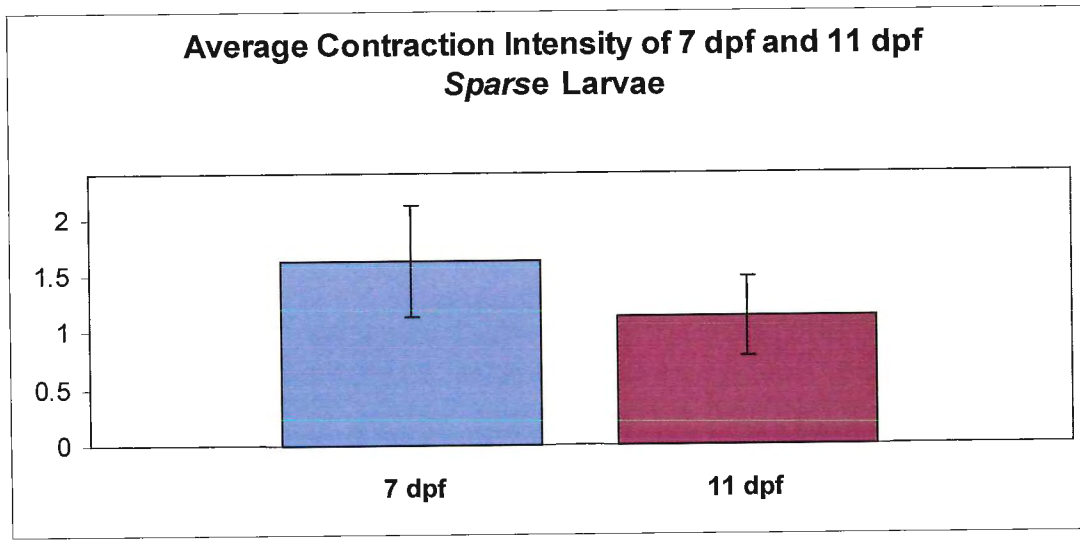

Figure 9. Contraction intensity in 7 dpf *sparse* larvae compared to 11 dpf *sparse* larvae.

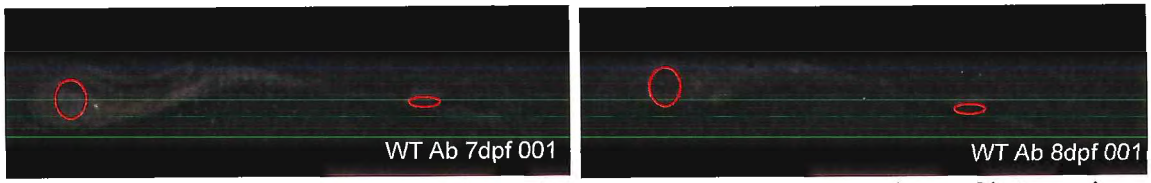

Figure 10. Changes in fluorescence intensity after 24 hours in two regions of interest in wildtype zebrafish larvae.

Table 9

Change in Fluorescence Intensity in Wildtype Larvae		
	7 dpf/8 dpf	11 dpf/12 dpf
Anterior	0.371 ± 0.030	0.455 ± 0.056
Posterior	0.119 ± 0.021	0.258 ± 0.046

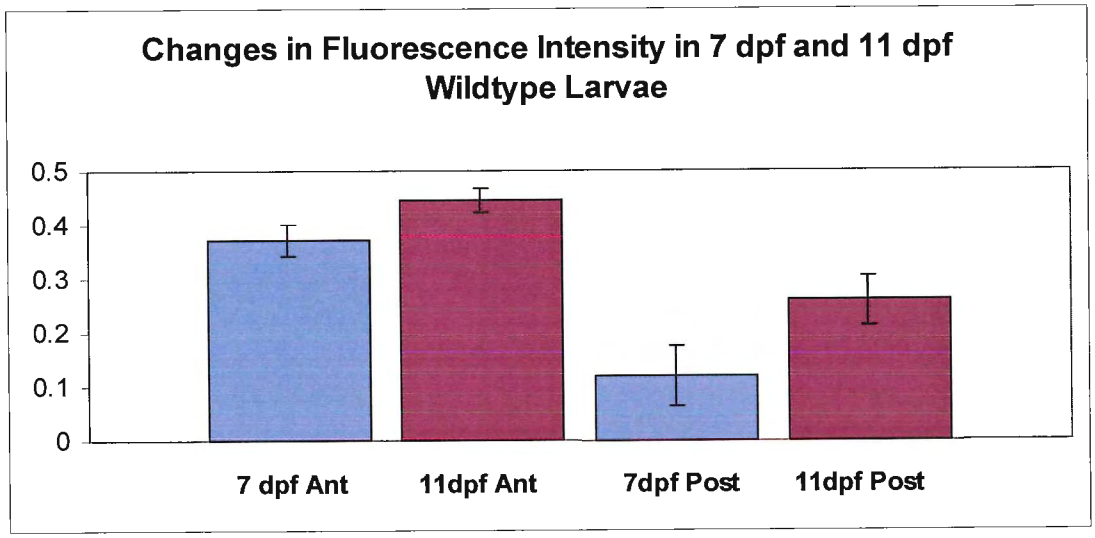

Figure 11. Changes in fluorescence intensity of wildtype zebrafish larvae after a 24 hour period. In 11 dpf larvae the change in fluorescence intensity is greater in the anterior and posterior GI tract then at 7 dpf larvae.

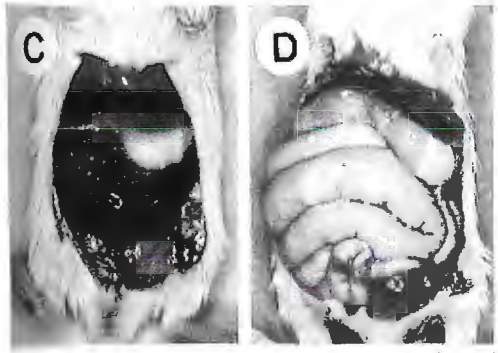

Figure 12. Mouse C was exposed to ACK4, a rat anti-mouse monoclonal antibody raised against c-kit that is non-inactivating. Mouse D was exposed to ACK2 a rat anti-mouse antibody specific for c-kit that inactivates c-kit (Maeda, Yamagata et al. 1992).

Table 10

Average Intestinal Area of 7 dpf Zebrafish Larvae	
Wildtype	4491 ± 754
Sparse	5532 ± 880

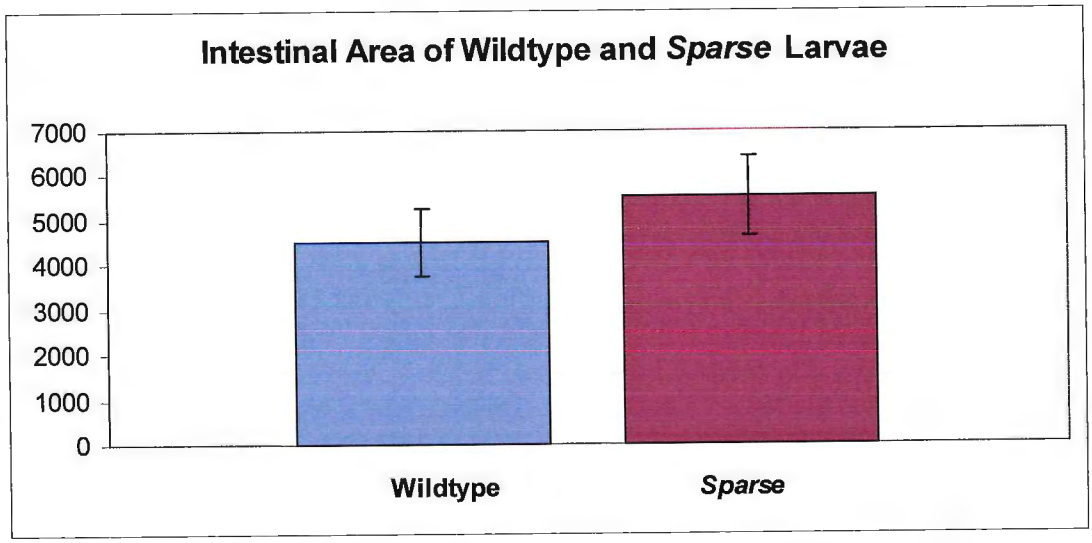

Figure 13. Intestinal area in *sparse* larvae was greater then wildtype at 7 dpf.

Table 11

Fluorescence Intensity of Rhodamine Labeled Microspheres Localized to the GI tract in Wildtype Zebrafish (counts/second)			
	7 dpf	8 dpf	Change in Intensity
Blank	71793 ± 10379	73294 ± 7226	-1501
Control	112475 ± 21919	55788 ± 13125	56687
5 HT	-	56243 ± 12638	56232

Table 12

Fluorescence Intensity of Rhodamine Labeled Microspheres Localized to the GI tract in Wildtype Zebrafish (counts/second)			
	11 dpf	12 dpf	Change in Intensity
Blank	51309 ± 10669	63252 ± 12219	-11943
Control	62630 ± 23780	53277 ± 6333	9353
5 HT	86820 ± 21850	40626 ± 4754	46194

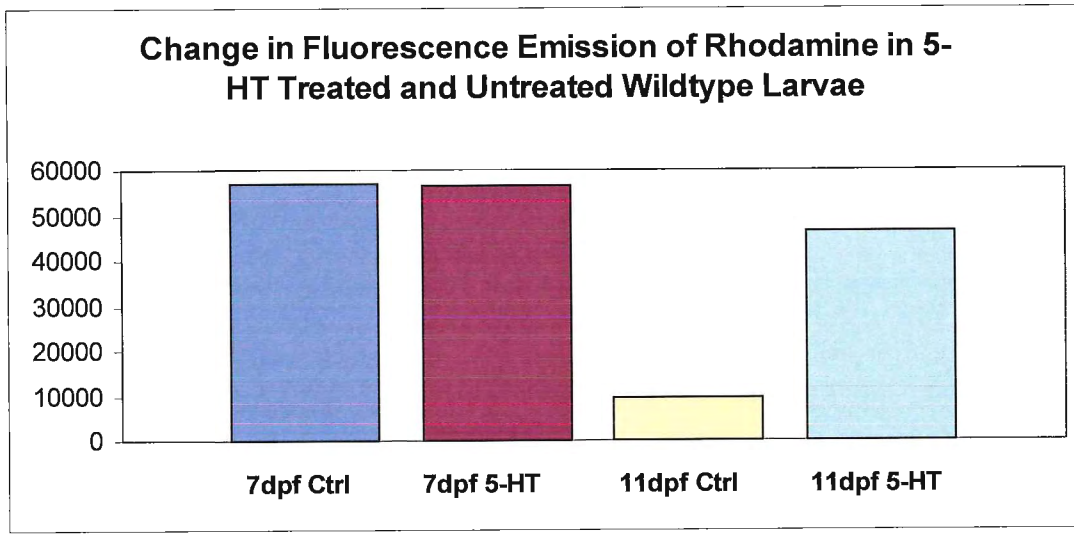

Figure 14. From 7 dpf to 8 dpf the changes in fluorescence emissions from control larvae and larvae treated with the prokinetic 5-HT were approximately the same. From 11 dpf to 12 dpf the change in fluorescent emission of larvae treated with 5-HT was much greater then control larvae.

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Addendum

Distinguishing proteins and genes in humans, mice, and zebrafish in literature

Genes and resulting proteins are commonly given the same name. To distinguish gene from protein in literature it is common for the gene to be written in italics.

When the same protein is present across species the capitalization of the first, all, or none of the letters may change.

Example: kit

The kit protein	Humans-	KIT
	Mice-	Kit
	Zebrafish-	kit
The kit gene	Humans-	*Kit*
	Mice-	*Kit*
	Zebrafish-	*kit*

Brine Shrimp Culture

1. Shrimp Water- In a 2L flask add 1L diH_2O, 66g Instant Ocean, and stir bar. Mix contents on stir plate at a medium speed.
2. When salt is dissolved add one more liter of diH_2O for a final volume of 2L.
3. pH the solution to 8.6. Add extremely small/diluted volumes of HCl or $NaHCO_3$ to adjust the pH.
4. Make sure stop cock to brine shrimp hatchery is closed and add ~1L of shrimp water to the hatchery.
5. Weigh out ~1g of brine shrimp cysts and add to hatchery.
6. Place cap on top and insert plastic straw. The top of the straw should fit into the hole at the base of the hatchery so cysts do not get stuck in the crevice.
7. Plug in the bubbler and turn on light. The light should to be on at all times, angle the light to give the hatchery the greatest amount of exposure. However, the light should not be positioned so close as to melt the plastic or to heat the water above room temperature, this could kill the shrimp.
8. Label the hatchery with a piece of tape with the date the culture was started.
9. After 48 hours the shrimp are ready to be fed. Unplug the bubbler and allow the culture to settle for ~10 minutes.
10. Place a small beaker under the stop cock and remove ~20ml. This is to remove unhatched cysts that have sunk to the bottom. Discard this volume.
11. Place a shrimp filter in a reservoir under the stop cock and remove ~1L from the culture. Discard the liquid.
12. Wash the brine shrimp caught by the filter with system water 3 times. Leave the shrimp/filter in the reservoir of system water after the 3rd wash.
13. Larvae that are 9-11 dpf need only a few drops of shrimp. Larvae that are 12 dpf to 1 month can eat one to two droppers full of brine shrimp. Fish in tanks should get one pipette full per 10 fish.
14. When all the culture is gone rinse hatchery, tubing and plastic top with tap water and begin a new culture.

*Refer to The Zebrafish Book (blue book) pg. 3.8.

Fish Breeding and Embryo Retrieval

Optimal breeding for zebrafish occurs between months 7 and 18. Zebrafish breed in the morning hours just after first light.

Overnight Breeding

1. The day before you want to breed, the fish should be fed live food several times.
2. The day previous to breeding, place 2 male and 3 females in a breeder tank just before you leave. (Males tend to be smaller and more torpedo shaped, females have a rounder abdomen)
3. The next morning remove the dividers from the breeder tanks.
4. Check the water approximately every 20 minutes for eggs. Look very carefully, eggs are very small and colorless. It can help to raise the tank and look through the bottom if you are unsure if you have eggs.
5. If the fish have laid eggs transfer the eggs to an appropriately labeled beaker with 200 ml of E3 and place the beaker in the incubator.
6. After about an hour return the fish to the appropriate tanks.

Larvae Care

Approximately ½ the total water volume in larvae beakers needs to be changed daily.

1. For the 7 days larvae should be in E3. To remove ½ the water volume, take a clean fish beaker off the shelf and attempt to pour ½ the E3 out of the beaker without dumping out any larvae. If larvae are poured into the new beaker use a pipet to put them back in the original beaker.
2. Remove any debris and dead larvae from the beaker with the larvae and discard. Transfer the larvae to a new clean beaker containing ~50 ml of E3.
3. After 7 dpf a similar routine is followed however, E3 is replaced with system water and the larvae are fed H0.
4. Between 9 dpf and 11 dpf the larvae can begin to be fed brine shrimp.

PTU and Methylene Blue

*PTU can be used in small amounts to hinder the development of melanocytes in larvae. Melanocytes can impede the view of the GI tract when capturing video or still images.

Methylene blue can be used as a fungicide in embryo media but unfortunately it auto fluoresces. Larvae that may be used in any type of fluorescence assay or used for any immunohistochemical experiment SHOULD NOT BE EXPOSED TO METHYLENE BLUE. In addition, larvae received from an outside source such as zfin are commonly shipped in methylene blue. Upon arrival larvae should be immediately removed from their shipping containers and rinsed several times with E3.

*PTU is toxic, you should always handle PTU in the hood and always wear gloves. Glassware that contained any amount of PTU should be rinsed several time before placing it on the bench to be washed.

Agar Mounting

To keep larvae from floating away during imaging or filming

Mounting

1. Make a 1.2% stock solution of agar: Dissolve 1.2 g of agar in 98.8 ml 1X E3 on a hot plate, do not boil. Maintain heat until agar is completely dissolved, it may take several minutes. Divide agar into 15 ml falcon tubes with 5-10 ml in each, label and date. Store in the refrigerator for future use.

2. For use, remove a tube of agar from the refrigerator, loosen cap and microwave for ~10 seconds. Remove and mix with pipette. Continue to microwave and mix the agar in 3-5 seconds intervals until agar is fluid. Keep the agar in a beaker of water in a water bath at a constant temperature of 42°C to keep it liquid.

3. Place extra glass slides and glass pipettes in the beaker to keep warm.

4. Place the larvae to be mounted in anesthetic for 30 seconds or until it stops moving. If the larvae is not anesthetized after 30 seconds the anesthetic may have expired, in this case discard the anesthetic and make fresh.

5. Remove a glass slide from the water bath and dry completely. Remove glass pipette from water bath and pick up larvae in minimal amount of anesthetic and place in agar.

6. Transfer the larvae in the agar to the slide; **larvae must be oriented laterally**. If it is not on its side quickly add a few drops of agar directly above the larvae and attempt to reposition it. The larvae should be in a drop of agar the size of quarter. Replace agar in water bath.

7. Once the agar has hardened add a few of drops of anesthetic to keep the larvae anesthetized and to keep the agar from drying out.

8. To remove the larvae use a blade to make a V-shaped cut with the point of the V pointing at the larvae's nose.

9. Hold a pair of closed tweezers at the V-cut and gently, without hitting the larvae push them into the agar.

10. Carefully open the tweezers, this will release the larvae from the agar.

11. When the larvae is free it can be removed with a pipette and E3.

*If the larvae has been in a solution other then E3/system water is should be rinsed in a beaker of E3/system water before placed in the anesthetic.

**Refer to The Zebrafish Book (blue book) pg. 4.3

Contraction Frequency

Data Acquisition

1. Place larvae (7 dpf or 11 dpf) in a beaker containing 50 ml system water and 200 ul of blue or green dye. This makes it easier to differentiate the lumen of the GI tract from the rest of the fish. Put the beaker in the warm water bath (set to 28.5°C) for approximate 1 hour.
2. During the incubation

   -Obtain two 250 ml beakers and one 100 ml beaker. Fill the two 250 ml beakers with system water, these beaker will serve as wash baths to rinse off excess dye. Fill the 100 ml beaker with 24 ml of system water and add 1 ml of 25X tricane/mesab to make a working strength anesthetic solution.

   -Obtain a fourth 250 ml beaker, fill it with water and place it in a circulating hot water bath set to 42°C. Once the desired water temperature has been reached, place several slides and short glass pipettes in the beaker.

   -Remove two tubes of agar from the refrigerator, uncap them and place them in a plastic beaker. Reheat the agar according to agar mounting protocol. When the agar is completely fluid it can be put in the hot water bath along with the slides and pipettes.

   -Retrieve a video tape to record contraction frequency. In pencil write the date type of larvae and the age of the larvae to be recorded. Place the tape in the camera (on the under side of the camera a lever can be pushed to eject and load the tape).

   -Carefully screw microscope adapter to lens of video camera (located on the left-hand side of the microscope). Replace adapter with video camera now attached to microscope. Place a jack stand under the camera for support.

3. After the 1 hour incubation removed the larvae in a minimal amount of dye and transfer to the first system water rinsing bath. When all larvae are in the first bath transfer them to the second bath. All larvae will remain in this bath until they are ready to anesthetized and filmed.
4. Remove one fish from the second bath and transfer in a minimal amount of solution to the 1X anesthetic. Leave the larvae in here for approximate 30 seconds or until the larvae stops moving. If the larvae continues to move after 1 minute the anesthetic may have expired, in this case put it back in the second

system water bath. Discard the anesthetic and make fresh anesthetic (see solution recipes).

5. Once anesthetized, immobilize the larvae in agar and mount on a slide, refer to agar mounting protocol.
6. Turn on microscope light source (located underneath microscope to lower left). When larvae is properly oriented on its side, place the on slide on inverted microscope stage and view under 4X magnification. View the larvae first through the ocular lenses. Make sure blood flow in the larvae is good; this indicates the larvae is healthy. If the larvae doesn't not have good blood flow, humanly discard it. If the larvae did not up take the dye you should still count contractions. Increase the magnification to 10X and reposition and refocus if necessary.
7. Turn on video camera by rotating a disk near the eye piece (refer to the operators manual for detailed procedures on how to operate the camera). Open LCD screen and center the larvae so the posterior intestinal bulb through the anus is in view. The focus may need to be adjusted again.
8. Press the record button on the video camera. Say the date, type of larvae (example- Spa or Wt), its age, and the number fish you are recording. Continue recording for 7 minutes. All the information you stated should be recorded in your notebook, in addition you should record the information you wrote on the video tape, the date it was fertilized, the time you began recording and the time you stop recording. Any other observation you make or any different treatment the larvae may have received that could affect physiological function should also be addressed.
9. After you have acquired 7 minutes of video, remove the larvae from the agar and replace it in the original beaker it came from (refer to agar mounting protocol for removal instruction).
10. Repeat these steps for all larvae.

Fluorescence Assay

Measurements for this assay are taken at two time points (7dpf and 11dpf) separated by 24 hours.

1. Loaded 7 dpf or 11 dpf larvae with FITC labeled microspheres, 1 mg of microspheres per ml of E3, for approximately 1 hour (see recipes).

2. While the larvae are incubating the microscope can be prepared for use.
   A. 4 pieces of equipment need to be turned on to capture fluorescent images
      -Computer

      -Olympus fluorescence box

      -Camera

      -Microscope's transmitted light source

      4X objective should be turned to face the specimen.

   B. Four filters are used in this experiment. The first filter located just above the objective lens should pulled out from the BFPI position to the U-DICT position. A second filter labeled U-AN should be pulled out to the first position, pull the filter until you feel/hear a "click". The third filter has a wire attached to it and is located toward the back of the microscope. This filter should be pulled to the out position. If it is not in the out position your images will look striated.
   C. The filter wheel located just above the light source (at the base of the microscope) should be rotated so the white dot is located to the left of DIC20.
   D. Open ImagePro Plus, the software used to acquire images. Double click the icon on the desktop. A window will appear giving you options of the version of ImagesPro you can launch, choose complete.

3. After the 1 hour incubation all larvae are removed in a minimal amount of fluorescent solution and transferred to beaker containing approximately 200 ml of system water to remove any fluorescent beads that are not in the GI tract. Next, transfer the larvae into a second rinse bath of clean system. Larvae will remain here until they are ready to be anesthetized and imaged.

4. Larvae are imaged one at a time. When you are ready to image transfer a larvae to a beaker of 1X MESAB, this will also serve as a third and final rinse. Leave the larvae in the anesthetic for 30 seconds or until it is fully anesthetized. *If the

larvae is not anesthetized after 1 minute the MESAB may have expired. In this case, make a new 1X solution, if this still does not work make a new 25X stock (see recipes).

5. Once fully anesthetized transfer larvae in a small drop of anesthetic to a slide. **It is imperative that the larvae is positioned on its side**. A larvae is on its side when you look directly above is and only one eye can be seen, the other one is hidden by the one closest to you. It is unlikely you will properly orient the larvae on your first try. If this is the case, the larvae can be gently nudged with a plastic pipet, a small drop of water can be released on top of the larvae, or the larvae can be picked up again and repositioned.

6. When the larvae is properly oriented transfer it slowly and carefully to the microscope stage. Look at the larvae through the ocular lens, make sure the larvae's blood flow is good. If its not the fish should not be imaged.

7. In ImagePro, click on the icon that looks like a video camera, this will bring up the Qlmaging Digital Camera window. At the bottom of the new window is a button labeled "more>>", click this button, the window will expand. At the bottom of the expansion is a drop down menu labeled setting, select Stacey Hess, this will automatically set the gain to 10 and binning to 2x2 (Note: you should do a quick check of the gain and binning to make sure no one has accidentally changed them).

8. Focus the larvae under transmitted light first. To do this click "auto exposure" in the Qlmaging digital camera window, the software will determine the optimal exposure time, next click preview. A new window will pop up feeding you continuous footage of the larvae. Focus the GI tract from the beginning of the intestinal bulb to the anus. Use first the course then the fine adjustment knobs, click stop.

9. Rotate the filter wheel, located in between the fluorescence switch and the objective lenses, to "4". Turn off the transmitted light source and shift the fluorescence lever, the black horizontal switch located just above the objective lenses to the right, from the black circle to the white circle. When the larvae is illuminated with an intense blue light, click "snap" in the Qlmaging digital camera window. Now, the monitor will display a window which shows an image of the GI tract of the larvae. If the image is to dark the exposure time wasn't long enough and should be increased. If parts or all of the GI tract are white, the exposure time was to long and should be decreased until there is little to no white in the GI tract. Every time a new set of larvae is imaged a new folder is created and larvae images are given a systematic file name. The file name reflects the

type of larvae and its age, kind of light the image was taken with [transmitted (tl) or fluorescent (fl)], exposure time, and a number to designate one larvae from another. For example, the first image you take is of a 7 dpf Ab wildtype larvae, you exposed the it to fluorescent light for 500 milliseconds therefore when you save it the file name is: 7dpf Ab fl expo 500ms 001. If the same larvae was auto exposed with transmitted light the file name would be 7dpf Ab tl 001. In addition the larvae number and exposure time should be recorded in your laboratory notebook so they can be referred to the next day so all conditions can be the same for the same larvae.

Because the fluorescence assay is paired between days 7 and 8 larvae need to be kept separate, to do this a 24 well plate is used. After the first larvae has been imaged it is removed from the slide and is transferred to the first well of the plate along with a few milliliters of fresh system water. The second larvae is transferred to the second well and so on and so forth.

When all larvae have been imaged all instruments should be turned off and the to the microscope room pulled shut. The larvae in the welled plate and brought to the fish room to be kept at a warmer temperature overnight.

The next day larvae do not need to be reloaded but the same steps should be taken to prepare the microscope, at least fifteen minutes should be allowed for the instruments to warm up but before anything is imaged.

When re-imaging under the same conditions with they were imaged under on the initial day, including length of exposure to fluorescent.

Solution Recipes

Embryo Media (E3)

Make 500 ml of a 60X stock solution

1. Mix 86g NaCl, 3.8g KCl, 14.5g $CaCl_2*2H_2O$, 24.5g $MgSO_4*7H_2O$ in 500ml diH_2O. Parafilm, label and store in fridge.
2. Dilute 32 ml of 60X stock in diH2O to make up 2 L of 1X E3. The solution should be pHed to 7.0-7.4.

*Refer to purple Zebrafish book, Nüsslein-Volhard & Dahm, pg. 22.

1.2% Agar (for mounting larvae)

1. Add 1.2 g agar to 100 ml 1X E3. Add stir bar, heat and mix gently. Place a cover on the beaker to prevent to much evaporation.
2. When agar is completely dissolved aliquot 10 ml into 15 ml falcon tubes. Cap, label and store in refrigerator.

*Refer to The Zebrafish Book (blue book), Monte Westerfield Institute of Neuroscience University of Oregon, pg. 10.1.

System Water

To 40 L of diH_2O add 96 g Instant Ocean, 1 g $NaHCO_3$ and 8 ml of 0.1M HCl. The final pH should be between 7.1 and 7.5.

Dyed System Water

Mix 50 ml of system water with 200 ul of McCormack food dye (blue or green).

Kit-Like Immunoreactivity in the Zebrafish Gastrointestinal Tract Reveals Putative ICC†

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Gastrointestinal (GI) motility results from the coordinated actions of enteric neurons, interstitial cells of Cajal (ICC), and smooth muscle cells. The GI tract of the zebrafish has a cellular anatomy that is essentially similar to humans. Although enteric nerves and smooth muscle cells have been described, it is unknown if ICC are present in the zebrafish. Immunohistochemistry and PCR were used determine expression for the zebrafish Kit orthologue in the zebrafish gastrointestinal tract. Cells displaying Kit-like immunoreactivity were identified in the muscular layers of the adult zebrafish gastrointestinal tract. Two layers of Kit-positive cells were identified, one with multipolar cells located between the longitudinal and circular smooth muscle layers and one with simple bipolar cells located deep in the circular muscle layer. Primers specifically designed to amplify mRNA coding for two zebrafish *kit* genes, *kita* and *kitb*, and two *kit* ligands, *kitla* and *kitlb*, amplified the expected transcript from total RNA isolated from zebrafish GI tissues. The *Sparse* mutant, a *kita* null mutant, showed reduced contraction frequency and increased size of the GI tract indicating a functional role for *kita*. These data establish the presence of a cellular network with Kit-like immunoreactivity in the myenteric plexus region of the zebrafish GI tract, adjacent to enteric neurons. Expression of *kita* and *kitb*, and the ligands *kitla* and *kitlb*, were verified in the adult GI tract. The anatomical arrangement of the Kit-positive cells strongly suggests that they are ICC. *Developmental Dynamics 236:903–911, 2007.* © 2007 Wiley-Liss, Inc.

Key words: Kit immunohistochemistry; zebrafish; interstitial cells of Cajal (ICC); gastrointestinal tract motility

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INTRODUCTION

Gastrointestinal (GI) motility is primarily mediated by complex interactions between enteric neurons, interstitial cells of Cajal (ICC), and smooth muscle cells. These cell types have been identified and characterized in many vertebrate model systems including the mouse, guinea pig, rat, dog, chick, and human (Faussone-Pellegrini and Thuneberg, 1999; Komuro, 1999; Sanders et al., 1999; Young, 1999). It is now widely understood that ICC play a vital role in regulating GI motility. ICC generate a pacing signal that drives smooth muscle, mediates neuronal input to smooth muscle, and establishes a smooth muscle membrane potential gradient across the thickness of the circular smooth muscle layer (Huizinga et al., 1995; Farrugia et al., 2003; Strege et al., 2003; Ward et al., 2004; Sanders et al., 2006). Disturbances in ICC distribution have been correlated with GI dysmotilities in animal model systems, and in humans (He et al., 2000, 2001; Huizinga et al., 2001; Lyford et al., 2002; Sanders et al., 2006). The zebrafish is a well-established model system for studies on development, and is an important model system for human disease (Dodd et al., 2000; Goldsmith, 2004). Zebrafish larvae are transparent, which allows direct observation of organ function in the intact organism. The GI tract is functional and is apparently fully formed at 5 days post fertilization (dpf). Mus-

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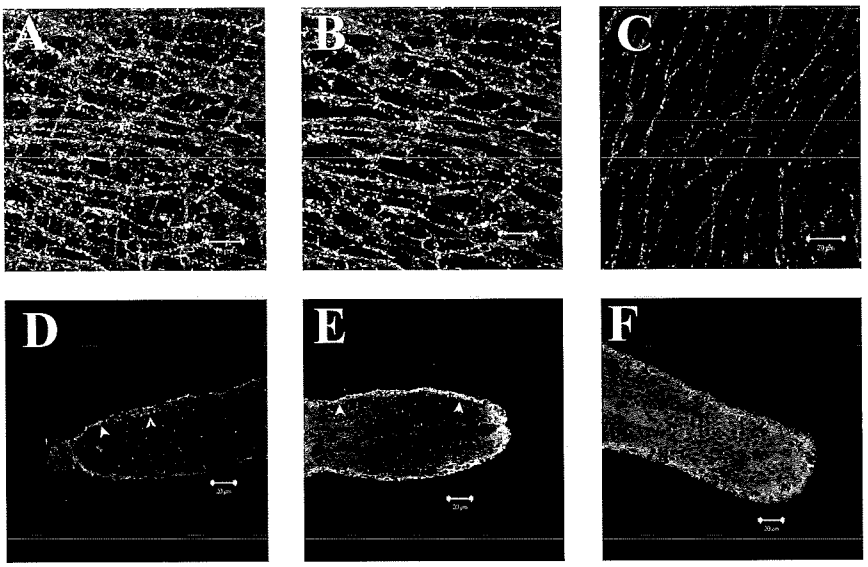

Fig. 1. Anti-Kit rabbit polyclonal antibody identifies putative ICC networks in the adult zebrafish GI tract. Full thickness confocal stack reconstruction of an adult zebrafish GI whole mount preparation immunostained using rabbit anti-Kit polyclonal antibody (**A**). Total thickness of reconstructed stack ≈20 μm. Reconstruction of a partial thickness stack (≈8-μm-thick) shows a kit-positive network in the myenteric plexus region with stellate-shaped, highly branching cells (**B**). A second distinct network of slender bipolar cells oriented parallel to the circular smooth muscle cells was located closer to the submucosal border and is shown in a partial stack reconstruction of a 6-μm-thick region (**C**). Kit-like immunoreactivity was observed in the GI tract of zebrafish larvae beginning at 7 dpf. Single confocal sections approximately 0.6-μm-thick of mid-saggital sections from the posterior end of the larvae GI tract show branching cells with slender processes in the tunica muscularis (**D**, arrows). Background staining of the mucosal layer prevented full-thickness stack reconstruction. The density of Kit-positive cells was increased at 11 dpf (**E**), and a network of Kit-positive cells was observed at 20 dpf (**F**). Scale bars = 20 μm.

clonal antibody that has been widely used to identify mouse ICC also specifically identified cellular networks in acetone-fixed adult and larvae zebrafish GI tissues (data not shown).

Two types of Kit-positive cells are observed at high magnification: one branching cell with prominent nuclei (black arrow) and thinner bipolar cells (Fig. 2A). The anatomical position of these cells was determined in transverse sections of adult zebrafish GI tissues. Hematoxilyn and eosin stained transverse sections show that the outer longitudinal and inner circular smooth muscle layers are approximately 2 and 4–5 cell layers thick, respectively (Fig. 2B). Kit-like immunoreactivity was consistently observed in two distinct layers of cells in transverse sections of adult GI tissues (Fig. 2C). The outer layer of cells with Kit-like immunoreactivity appeared more dense and continuous, and a second thinner layer of Kit-positive cells was observed closer to the lumen. The inner layer was discontinuous and oriented in parallel with the circular smooth muscle cells. Both layers of Kit-positive cells were observed in anterior, mid, and posterior intestinal segments. The anatomical position of Kit-positive cells was also determined using transverse sections of zebrafish larvae. A single continuous layer of Kit-positive cells within the tunica muscularis of the GI tract was observed in 13 dpf larvae (Fig. 2D).

The relative anatomical positions of Kit-positive cells, enteric neurons, and smooth muscle cells in the zebrafish GI tract was examined further for comparison with established mammalian model systems. A pan-neuronal antibody, anti HuC/D, was used to identify cell bodies, and anti-acetylated α-tubulin antibody was used to identify neural processes. Composite images resulting from dual labeling experiments with whole mounted tissue show that cells displaying Kit-like immunoreactivity are in the same area as neuronal cell processes, labeled with acetylated α-tubulin antibody (Fig. 3A). Composite images of transverse sections show Kit-positive cells and neural cell processes are located in the myenteric plexus region (Fig. 3B). Similarly, dual labeling with anti Hu C/D and anti-Kit antibody shows neural cell bodies located near Kit-positive cells in whole mounted tissues, and in the myenteric plexus region in transverse sections (Fig. 3C and D). Enteric nerves were only occasionally observed to extend deep into the circular muscle layer near the thin inner layer of Kit-positive cells (data not shown). Dual labeling with anti-SM22 antibody to detect smooth muscle cells and anti-c-Kit antibody identified longitudinal and circular smooth muscle layers, and Kit-positive cells located between the layers in the whole mounted tissue (Fig. 3E). Smooth muscle cells were identified using an anti-Desmin antibody in transverse sections. Desmin is an intermediate cytoskeleton filament protein expressed by GI smooth muscles. Composite images of transverse sections dual labeled with anti-Desmin and anti-Kit antibody show one dense layer of Kit-positive cells positioned between the longitudinal and circular muscle layers and a second thin layer of Kit-positive cells near the innermost circular smooth muscle cells (arrowhead, Fig. 3F).

Expression of the zebrafish orthologs of mammalian c-Kit was verified by determining mRNA expression for the *kita* and *kitb* genes within GI tissues. Reverse transcriptase PCR was performed on cDNA prepared from total RNA isolated from adult zebrafish GI tissues. The presence of mRNA for two known orthologs of the c-Kit receptor, *kita* and *kitb*, as well as orthologues of c-Kit ligand, *kitla* and *kitlb*, were determined using specific primer sets for each gene, and for β-actin as a positive control (see Table 1). Primers were intron-spanning to rule out the possibility of genomic contamination. Products of the expected size for *kita, kitb, kitla*, and *kitlb* were amplified (Fig. 4). The identity of the bands was confirmed by sequencing. These data show for the first time the presence of mRNA encoding *kita, kitb*, and the ligands for these receptors, *kitla* and *kitlb*, in the zebrafish GI tract.

Development of ICC requires functional c-Kit signaling during embryogenesis in the mouse model, and also to

TABLE 1. Oligonucleotide Primer Sequences

Target	Sequence	Expected size (bp)
kita (AF 153446)	F: GTT ATC CCA CTC CTC AGA TCA AGT R: TCA CAG CTA CAG TCATCA CAG TGT	564
kitb (DQ072166)	F: GGG AGG AAT CAC CAT CAG AA R: CTC AGG TGG AAA TCGTGG TT	234
kital (AY929068)	F: CACAGTTGCTGCCTATTCCA R: GGTGAGGAGCCACCTGAGAT	580
Kitbl (AY929069)	F: GGC TGC ATT TGA ACC TGT ATC C R: GTG TCT GCA CAC CCTAAA GAA TCC	542
β actin (BC067566)	F: GAT ACG GAT CCA GAC ATC AGG GTG TCA TGG TTG GTA R: GAT ACA AGC TTA TAGCAG AGC TTC TCC TTG ATG	580

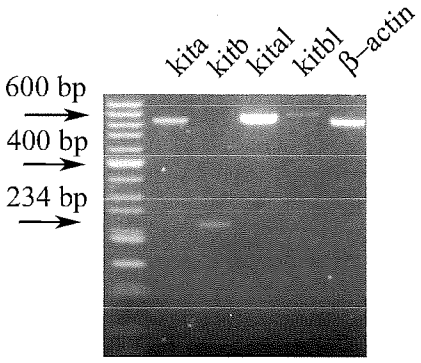

Fig. 4. RT-PCR amplifies the zebrafish Kit receptor tyrosine kinase *kita* and *kitb*, and Steel factor, *kitla* and *kitlb*, from isolated adult GI tract. Amplification products are shown for primer sets designed for each gene (Table 1), and for β-actin. Products of correct size for each primer set were observed and confirmed by sequencing.

Sparse mutants was compared to wild0type larvae (Maeda et al., 1992; Torihashi et al., 1999). *Sparse* and wild-type larvae were incubated for 1 hr in media containing FITC-labeled dextran, anesthetized, and washed. The lumen of the GI tract was observed using fluorescence microscopy. The cross-sectional area of the lumen was outlined from fluorescent images and measured using Image Pro Plus software (Media Cybernetics, version 5.0). The size of the GI tract in 7 dpf *Sparse* mutants was larger compared to 7 dpf wild-type larvae, 12,331 ± 467 μm^2 and 14,985 ± 483 μm^2, respectively (mean ± standard error, n = 20 wild-type, 12 *Sparse*, $P \leq 0.05$). Adult *Sparse* zebrafish consistently exhibited a distended GI tract, which was most apparent in the intestinal bulb (data not shown). Cells displaying Kit-like immunoreactivity were observed in *Sparse* mutant larvae and in adult GI tissues, with a similar density as wild-type zebrafish (data not shown).

DISCUSSION

Results presented here show that anti-Kit antibodies identify a network of cells located between the circular and longitudinal muscle layers of the adult zebrafish GI tract and a separate, second group of cells in a region analogous to the boundary between the inner and outer circular muscle in the mouse. Expression of mRNA encoding for the two zebrafish orthologues of mammalian *Kit, kita*, and *kitb*, as well as two orthologues for mammalian *Kit* ligand, *kitla* and *kitlb*, was confirmed in adult zebrafish GI tissues. The Kit-positive cells were distributed in the myenteric plexus region near enteric neurons. A reduction in contraction frequency and an increase in size of the GI tract were observed in 7 dpf *Sparse* mutant larvae, which exhibit a null *kita* allele. These data are consistent with expression of zebrafish orthologs to the mammalian *c-Kit* receptor tyrosine kinase, *kita* and *kitb*, in the zebrafish GI tract. Furthermore, the data strongly indicate the presence of ICC in the zebrafish GI tract, which are distributed similarly to mammalian ICC, thus establishing the zebrafish as a suitable model system for mammalian GI motility.

Expression of *kita* and *kitb* was not detected previously in zebrafish larvae at 4 dpf using in situ hybridization techniques (Parichy et al., 1999; Mellgren and Johnson, 2005). Our data confirm this result. Kit-like immunoreactivity was not detected in 5-dpf larvae, and was first detected at 7 dpf. It is likely that expression of *kita* and *kitb* occurs developmentally later then 4 dpf, or that mRNA levels in the 4-dpf GI tract occur at very low and not detectable levels by in situ hybridization techniques. Functional data show that spontaneous contractions of the GI tract that develop near 4 dpf are poorly organized (Holmberg et al., 2004). Coordinated contractions, which are better organized and contribute to propulsive motility movements, develop gradually between 5 and 7 dpf, consistent with the time course of appearance of Kit immunoreactivity (personal observation). The role of ICC on intestinal transit has been well characterized in the mouse model and mutant mice lacking ICC exhibit spontaneous but poorly organized contractions and delayed intestinal transit (Torihashi et al., 1995, 1997; Ward et al., 1995). The poorly organized contractions in early zebrafish development are, therefore, similar to the ICC-deficient mutant mouse model. The morphological data from these studies are consistent with the presence of myenteric ICC and deep muscular plexus ICC, similar to the mouse small intestine. However, no obvious differences were observed in the distribution of Kit-positive cells in anterior, mid, or posterior regions of the zebrafish GI tract, whereas in the mouse model deep muscular plexus ICC distribution differs regionally along the GI tract. The functional role of Kit-positive cells in the zebrafish GI tract, as well as the relationship between Kit-positive cells and enteric neurons, remains to be explored.

spontaneous, rhythmic contractions, transmit electrical activity along the long axis of the GI tract, and coordinate enteric neurotransmission similar to mouse ICC.

EXPERIMENTAL PROCEDURES

Aquaculture

Wild-type and *Sparse* mutant zebrafish (ZFIN ID 960809-7 and 980202-7) were obtained from the Zebrafish international resource center and maintained according to standard guidelines in accordance with IACUC guidelines (Westerfield, 1993). Wild-type long-finned golds (Scientific Hatcheries, Huntington Beach, CA) were also used for some preliminary experiments and no differences in immunohistochemical staining were observed when comparing strains (data not shown). Fish were maintained at 28°C in system water comprised of deionized water containing 240 mg/L Instant Ocean salts and 75 mg/L $NaHCO_3$ with 20% system water change each day (pH was adjusted to ≈ 7.2, conductivity ≈450 PPM). Zebrafish were fed 3 times daily, alternating Cyclopeeze (Argent, Redmond, WA) with live brine shrimp, and maintained on a 14-hr/10-hr light/dark cycle. Crosses were performed in the morning, and embryos were maintained in embryo medium in 400-ml beakers kept in a water bath set to 28°C. Larvae were fed hatchfry encapsulation, grade 0, beginning at 7 dpf, and live brine shrimp after 11 dpf (Argent, Redmond, WA).

Immunohistochemistry

Adult and larvae zebrafish were anesthetized in system water containing MS222 (3-aminobenzioc acid ethyl ester, Sigma Chemical Co., St Louis, MO) and sacrificed for immunohistochemistry. Intact larvae and freshly dissected adult GI tissues were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (Fisher) with pH adjusted between 7.3 and 7.4 for a minimum of 2 hr, but not longer than overnight. Immunostaining using the ACK2 antibody requires acetone fixation, and for these experiments larvae and tissues were fixed for 15 min in ice-cold acetone. Fixed tissues were washed 4 times in PBS containing 0.02% sodium azide. Larvae were carefully dissected such that the GI tract was separated from the body so that the head remained attached to the GI tract. Although time-consuming, removing the GI tract in this manner allowed direct access for antibody-antigen binding, producing consistent immunostaining. Nonspecific binding of primary antibody to tissues was minimized by incubation for at least 1 hr at 4°C in blocking solution comprised of 10% normal donkey serum (NDS, Chemicon) and phosphate buffered saline containing 0.02% sodium azide, 0.1% Triton-X-100, and 0.05% -Tween 20 (PBS-TT). Primary antibodies were diluted in PBS-TT containing 5% normal donkey serum and were applied for 24–48 hr at 4°C on an orbital platform. After washing 4 times in PBS-TT, tissues were incubated with appropriate secondary antibody conjugated to a fluorescent marker and diluted in PBS-TT containing 2.5% normal donkey serum for 24 hr at 4°C on an orbital platform. Nonspecific immunoreactivity was assessed by immunostaining tissues or larvae in an identical manner but with the primary antibody omitted. The optimal concentration for each primary and secondary antibody was determined using serial dilutions. Antibodies were applied simultaneously during dual labeling experiments, followed by a PBS-TT wash and the simultaneous application of two appropriate secondary antibodies. Tissues were washed with PBS-TT and mounted on glass slides using Slow Fade medium (Invitrogen).

Paraffin-embedded sectioned tissues.

Adult fish were anesthetized in MS 222, decapitated posterior to the gills, tails were removed to aid fixative penetration and were immersed in freshly prepared 4% paraformaldehyde overnight at 4°C on an orbital platform. The GI tract was subsequently dissected and washed with PBS followed by 70% ethanol. Tissues were dehydrated and paraffin embedded using a Tissue-Tek VIP E150 processor with 1-hr infiltration steps at 70% (1 step), 95% (2 steps), 100% (2 steps) ethanol, followed by xylene (3 steps) and paraffin infiltration (30 min, 4 steps) comprising a 12-hr schedule. GI tissues were sectioned into anterior, mid, and posterior portions and embedded in a paraffin block oriented for transverse sections. One piece of paraformaldehyde-fixed mouse GI tract (small intestine) was included in each block to serve as a positive control for immunostaining. Paraffin blocks were stored at 4°C until sectioning. Specimen blocks were trimmed, soaked in ice water for 30 min, and sectioned at either 4 or 8 μm using a Reichart-Jung microtome. Sections were placed on slides (Superfrost plus, Fischer), baked at 56°C for 1 hr, and stored at −20°C until de-paraffinization.

Slides with sectioned tissues were placed on a 56°C warming tray for 5 min, and then a xylene bath for de-paraffinization (2 steps, 5 min). Tissue sections for hematoxylin and eosin staining were rehydrated using a graded alcohol series; 100% (2 steps, 3 min), 95% (2 steps, 3 min), followed by dIH_2O (1 step, 10 min). Tissue sections used for immunohistochemistry were similarly rehydrated followed by PBS-TT (1 step, 10 min). Preliminary immunostaining experiments using anti-Kit antibody resulted in a weak signal, and therefore antigen retrieval techniques were utilized to enhance specific immunoreactivity. Slides were immersed in antigen retrieval solution (Abcam) at 100°C for 20 min., allowed to cool to room temperature in the same solution, and rinsed in PBS-TT. Immunostaining was performed at room temperature. Tissue sections were incubated in blocking solution (1 hr), primary antibody solution (1 hr), and secondary antibody solution (1 hr). Optimal dilutions were determined for each primary and secondary antibody. Wash steps using PBS-TT were performed after the primary and secondary antibody incubations. Sections were cover slipped after application of Slowfade (Invitrogen).

Fluorescence and transmitted light imaging.

Tissues were examined with conventional light and fluorescence microscopy using an Olympus BX51 microscope equipped with an Optiscan z-axis controller (Prior Scientific) and supported on an anti-vibration table

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