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EFFECTS OF GLI-SIMILAR 3 KNOCKOUT MUTATIONS ON THE EXPRESSION OF INSULIN TRANSCRIPTION AND PANCREATIC ISLET DEVELOPMENT IN ZEBRAFISH

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**EFFECTS OF GLI-SIMILAR 3 KNOCKOUT MUTATIONS ON THE
EXPRESSION OF INSULIN TRANSCRIPTION AND PANCREATIC ISLET
DEVELOPMENT IN ZEBRAFISH**

A thesis
Presented to
The Faculty of the Department of Biological Sciences
Murray State University
Murray, Kentucky

In Partial Fulfillment
of the Requirements for the Degree
of Master of Science in Biology

By Dylan Hammrich
May 2017

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Abstract

Blood glucose homeostasis is a critical component in the physiological health of vertebrates. Regulation of blood glucose levels is the responsibility of the endocrine pancreas, which excretes hormones to the bloodstream in response to changes in glucose concentrations. The hormones excreted from the pancreas include insulin, which is secreted from the β cells of the pancreas and signals uptake of excess glucose by the peripheral tissues, and glucagon, which is secreted from the α cells and signals release of glucose to the bloodstream through gluconeogenesis in the liver. The Krüppel-like zinc finger protein Gli-Similar 3 (Glis3) is a transcription factor that has been shown to play a critical role in the development of β cells and transcription of the insulin gene.

Defects in the secretion and production of insulin are associated with the development metabolic diseases such as diabetes *mellitus*. Using genome wide association studies, GLIS3 has been identified as a risk locus for the development of both type 1 and type 2 diabetes *mellitus* and neonatal diabetes and hypothyroidism (NDH). Mice with ubiquitous knockouts of Glis3 exhibit phenotypes similar to that of humans, but conditional knockouts specific to the pancreas have produced mice with less severe phenotypes, leaving the role of Glis3 largely enigmatic.

The zebrafish (*Danio rerio*) has emerged as a powerful organism in studying pancreatic development due to its rapid development, short generation time, and transparent, externally developing embryos. The zebrafish pancreas is both morphologically and functionally comparable to that of humans, and the developmental programs that are responsible for pancreatic development appear to be highly conserved

between the two. Zebrafish also have the unique ability to regenerate their β cells after complete ablation without the need for insulin treatment. These factors have made zebrafish a powerful model for the study of pancreatic development and diabetes.

We found that expression of *glis3* mRNA begins after 14.5 hours post fertilization (hpf) in zebrafish. Zebrafish pancreas formation begins at approximately 24hpf, but whole mount *in situ* hybridization data shows that *glis3* expression is restricted to the zebrafish brain until approximately 48hpf, when expression can be visualized in the pancreas. During this time frame *glis3* expression levels increase, which coincide with the visualization of *glis3* in the zebrafish pancreata.

Finally, we found that reduction of *glis3* through a knockout mutation led to increased production of insulin and glucagon mRNA. Additionally, *glis3* heterozygous mutants exhibited a heightened ability to control blood glucose levels, with less variability of blood glucose levels between individuals under fasting, postprandial, and high fat diet conditions when compared to wildtype fish. After four months exposure to a high fat, high glucose diet, wildtype zebrafish expressed heightened levels of resting blood glucose, while heterozygous mutants showed no increase in blood glucose levels when compared to a normal diet.

Collectively, the results of this project provide novel information regarding the regulation of insulin in *glis3* mutant zebrafish. A decrease in *glis3* expression levels may increase the propensity for an organism to begin the compensatory mechanism for β cell mass expansion, giving insight into possible models for clinical therapies for the treatment of diabetes.

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Introduction

The Pancreas

The pancreas is a complex glandular organ located in the gut cavity that has multiple functions in vertebrate physiology. The pancreas is comprised of three distinct cell populations, including the acinar or exocrine cells, the pancreatic ductal cells, and the endocrine cells. These distinct cell populations give rise to the multiple functions of the pancreas. The acinar cells are responsible for the excretion of enzymes into the lumen of the small intestine, where they play a major role in the digestion of nutrients (Williams 2010). The ductal cells form the pancreatic duct, or duct of Wirsung, and connect the pancreas to the common bile duct and arise from the same bipotent trunk progenitor cells as the endocrine cells (Alpert et al. 1988, Solar et al. 2009). The endocrine cells of the pancreas are responsible for the excretion of hormones into the bloodstream, which play a role in maintaining blood glucose homeostasis. The endocrine cells provide approximately only 4% of the cell population of the pancreas in mice but are composed of a variety of cell types including α cells, β cells, ϵ cells, δ cells, and pancreatic polypeptide (PP) cells (Pelletier 1977, Andralojc et al. 2009). Each endocrine cell type is primarily responsible for the excretion of its own hormone: α cells secrete glucagon, β cells secrete insulin, ϵ cells secrete ghrelin, δ cells secrete somatostatin, and PP cells secrete pancreatic polypeptide. The endocrine cells of the pancreas are arranged into the islets of Langerhans, spherical clusters that are embedded into the exocrine tissues of the pancreas (Slack 1995). The primary structure of the islet consists of a majority of centrally located β cells with a periphery of α cells, indicating the importance of glucagon

and insulin in islet function (Fig. 1). These islets are closely associated with neurons in order to facilitate rapid response in blood glucose to changes in physiological conditions.

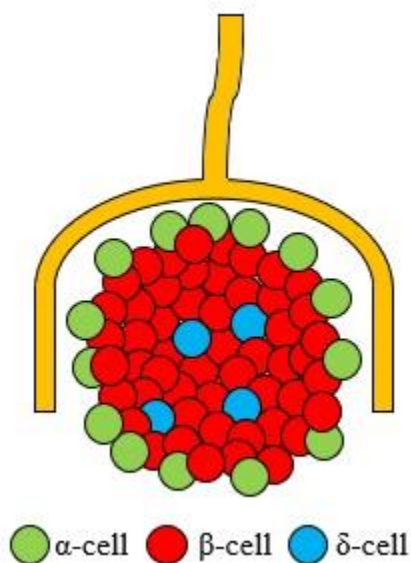


Figure 1. The structure of the primary islets of the pancreas. The central mass of the islet consists primarily of insulin-producing β cells (80% of islet mass) with interspersed δ cells (5% of islet mass). The periphery of the primary islet consists of glucagon producing α cells (20% of islet mass). Axons from nearby neurons surround islets to signal physiological conditions.

Organogenesis of the pancreas is a complex series of steps mediated by a number of transcription factors. Pancreatic endocrine and exocrine cells originate from a population of progenitor cells that arise during the differentiation of the gut endoderm. During the anterior to posterior patterning of the endoderm several transcriptional cues cause the posteriorization of the endoderm, including fibroblast growth factor (FGF) 4 and bone morphogenic protein (BMP) which specify foregut fate. (Deutsch et al. 2001, Rossi et al. 2001, Gu et al. 2002). Retinoic acid (RA) and the Wnt/ β -catenin pathway are also required in order to signal the posterior region of the gut endoderm. (McLin et al. 2007, Bayha et al. 2009). This anterior-posterior patterning of the gut endoderm gives rise to two separate epithelial buds on opposing sides of the foregut endoderm; one dorsally and one ventrally. These buds elongate alongside the prospective duodenum and

stomach and fuse together into a single organ as the gut endoderm rotates (Pictet et al. 1972).

Due to the formation of the pancreas as two convergent buds, the dorsal-ventral patterning that gives rise to these buds is extremely important. The presence of RA is used to signal in part for the dorsal bud formation, but evidence shows that close proximity to the endothelial cells of the fusing aorta is responsible for dorsal patterning (Lammert et al. 2001). Before the fusion of the dorsal aorta the presumptive dorsal bud of the pancreas is proximal to the notochord while it secretes activin- β and FGF 2, which suppress Sonic hedgehog (Shh) and lead to early dorsal patterning (Hebrok et al. 1998).

Proximity of the gut endoderm to the vitelline veins and lateral plate mesoderm (LPM) before fusion of the buds begins ventral patterning (Spooner et al. 1970, Lammert et al. 2001). Though less is known about the included pathways, signaling for the ventral pancreatic bud appears to be closely related to that of the dorsal bud. Dependence on external signals including activin- β , BMP, and RA seems to remain the same, though signals from the cardiac mesoderm which signal for dorsal development must be blocked (Lammert et al. 2001, Rossi et al. 2001). Expression of transformation growth factor (TGF) β has been shown to play an important role in blocking these dorsal patterning signals and leads to formation of the ventral bud (Wandozioch and Zaret 2009). Absence of FGFs and cardiogenic mesenchyme during development of the dorsal bud will lead to the formation of a second ventral bud, indicating that this may be the “default,” and additional signals are needed to cause the formation of the dorsal bud (Deutsch et al. 2001).

After the signal induction from the notochord and LPM rapid cellular proliferation will give rise to the forming dorsal and ventral buds, respectively. Pancreatic endocrine cells arise from the dorsal bud, which begin to form the primary islets of α and β cells. The ventral bud is responsible for formation of the acinar and ductal cells. As development continues the gut endoderm rotates and brings these two buds together where they begin to fuse (Golosow and Grobstein 1962). After fusion, the ventral bud of the pancreas will begin rapid proliferation of cells as branches begin to form. These branches will form the ducts used to transport digestive enzymes in the gut. The leading cells in these expanding branches excrete pancreatic transcription factor 1 (Ptf1a) and will give rise to the acinar cells after the enzymatic ducts form (Zeechin et al. 2003). Bipotent trunk progenitors follow the leading Ptf1a expressing tip cells, which are capable of forming both ductal and endocrine cells during the secondary transition, a developmental stage where pancreatic progenitor cells are rapidly and terminally differentiated into their final cell fates (Wang et al. 2005, Zhou et al. 2007).

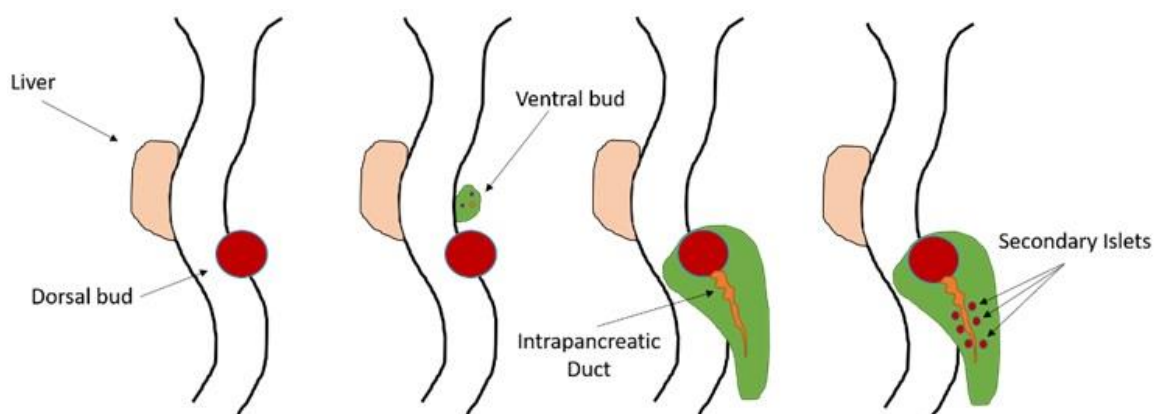


Figure 2. Formation of the pancreas from convergent buds in rodents. Signals from the notochord induce the dorsal bud, which gives rise to the α and β cells. Signals from the lateral plate mesoderm cause induction of the ventral bud, which gives rise to the acinar and ductal cells. Twisting of the gut endoderm causes the fusion of the dorsal and ventral bud. Rapid proliferation and branching of pancreatic progenitor cells form the pancreatic ducts and the secondary islets emerge.

Blood Glucose Homeostasis

In the mature organism, production and secretion of hormones from the α and β cells of the primary islets is highly regulated in order to maintain normal blood glucose levels. This rapid response system is imperative in maintaining a healthy physiological state. Insulin is expressed in response to high glucose levels when ATP generated during oxidative respiration depolarizes the plasma membrane. Ca^{++} ion channels open when the plasma membrane is depolarized, leading to the secretion of insulin (Braun et al. 2008). This secreted insulin then signals peripheral tissues to absorb glucose to use as energy or adipose tissue to convert glucose into fat for storage, leading to decreased blood glucose levels. When blood sugar levels decrease insulin is down regulated, and glucagon is secreted from the α cells of the primary islets into the bloodstream. This glucagon is absorbed by the liver stimulating gluconeogenesis. Glucose created during this process is released into the blood stream in order to normalize blood glucose levels.

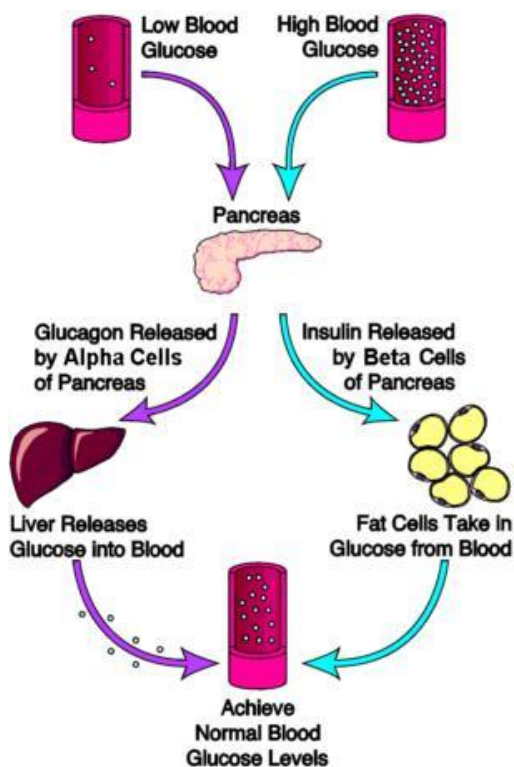


Figure 3. Schematic of pancreatic blood glucose homeostasis. At times of high blood glucose, insulin is released from β cells of the pancreas. Insulin signals periphery and adipose tissues to absorb glucose for energy and conversion into fat, decreasing blood glucose levels. At times of low blood glucose, α cells of the pancreas secrete glucagon, signaling for the liver to begin gluconeogenesis. This process forms glucose from fat, which is released to increase blood glucose levels.

Following a marked increase in blood glucose levels, ubiquitous and β cell specific activators bind to the 340 base pair (bp) promoter region located upstream of the insulin transcription start site (Ohneda et al. 2000, Melloul et al. 2002). The transcription factors pancreatic and duodenal homeobox-1 (Pdx 1), V-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MafA) and neurogenic differentiation 1 (NeuroD1) are among the transcription factors responsible for the expression of insulin in response to glucose levels in β cells (Armata et al. 2007, Kaneto et al. 2007, Kataoka 2007). As insulin is transcribed, it is stored in secretory vesicles in the cytoplasm to replenish insulin levels after secretion. In times of low blood glucose, the transcription factors responsible for the expression of insulin are down regulated through multiple mechanisms. Pdx 1 is controlled through a phosphorylation dependent manner which prevents its interaction with co-activators during times of low blood glucose (Mosley and Ozcan 2004). NeuroD1 localization is affected by glucose levels, and at low concentrations is located cytosolically, preventing its interaction with the insulin promoter region (Marcus et al. 1998). MafA is the insulin transcription factor most tightly controlled by glucose levels, and is only transcribed when cellular glucose levels rise above 10-25 mM concentrations (Sharma and Stein 1994).

Diabetes *mellitus* is characterized by β cell dysfunction and the inability to regulate blood glucose. Neonatal diabetes mellitus (NDM) and mature-onset diabetes of the young (MODY) are both monogenic forms of diabetes that affect children (Hattersley et al. 2009). In these forms of diabetes, a single gene mutation reduces the body's ability to express insulin. Type 1 diabetes *mellitus* (T1DM) is caused the inability of the pancreas to express insulin, due to an autoimmune-mediated destruction of β cells

(Goodison et al. 1992). In type 2 diabetes *mellitus* (T2DM), tissues peripheral to the pancreas become resistant to insulin, decreasing the efficiency of the body to regulate blood glucose levels (Taniguchi et al. 2006). Along with this, it has been shown that insulin resistance can be compounded by deteriorating function of β cells and decreased islet mass (Talchai et al. 2009). The molecular mechanisms underlying the development T1DM and T2DM are currently unclear. Both T1DM and T2DM have been associated with the development of multiple comorbidities, including heart disease, neuropathy, strokes, and kidney failure. The cost of treating the chronic symptoms of diabetes have steadily risen over the years, reaching as high as 245 billion dollars in the US alone in 2012 (Yang et al. 2013a).

Beta Cell Mass Expansion, Neogenesis

Understanding β cell mass regulation is a critical issue in determine the cause of T2DM. Formation of β cells in the primary islets of the dorsal bud from endocrine progenitor cells marks the beginning of insulin transcription and of blood glucose regulation in developing embryos (Rossi et al. 2001, Andralojc et al. 2009). As described above, this initial β cell population arises from the presumptive dorsal bud of the pancreas due to signals from the notochord and endothelial cells of the dorsal aorta. The secretion of FGF from the notochord drives the rapid proliferation of progenitor cells, giving rise to the presumptive islets by signaling for α and β cell fates (Elghazi et al. 2002). During the late gestational period of development, the population of β cells in the pancreas nearly doubles daily. This neogenesis from undifferentiated progenitor cells is assisted by the mitotic activity of the existing β cells, though only about 10% of the already existing β cells are in the mitotic state at any one time (Swenne 1982, Hellerstrom

and Swenne 1991). Neogenesis is the most rapid increase in β cell mass, and this time of fetal growth seems to be critical for determining the number of β cells in the adult pancreas. Despite the fact that β cell mass continues to expand after birth, embryos with severely decreased β cell mass during the late gestational period never catch up to their healthy counterparts and are more likely to develop glucose intolerance and diabetes *mellitus* (Simmons et al. 2001).

In mice, β cells mass expands through neogenesis from ductal cells surrounding the islet cells for approximately one week. After this time, the precursors that are responsible for neogenesis of β cells disappear (Bouwens et al. 1994, Solar et al. 2009). The generation of new β cells then becomes possible only through the mitotic proliferation of existing β cells, which occurs at a highly decreased rate when compared to late fetal growth (McEvoy and Madison 1980, Kaung 1994). In models where β cell proliferation was restricted during the late gestational period through genetic manipulation or through dietary restriction in the mother, it has been shown that mitotic proliferation of the existing β cell population is unable to compensate for the loss of the critical mass expansion from progenitor cells (Garofano et al. 1998, Simmons et al. 2001).

Beta Cell Mass Expansion, Compensation

Throughout adult life, β cell mass increases in order to compensate for the increased body mass of the individual (Montanya et al. 2000). This compensation is carried out through the same proliferation mechanism as postnatal growth, and because of this, the number of islets in the adult pancreas remains constant throughout the life cycle (Dor et al. 2004, Solar et al. 2009). Even though adult proliferation of β cells is relatively

slow, there is evidence of a compensation of β cell mass in response to excessive weight gain leading to obesity. In rodent models for obesity, there was shown to be a compensatory fourfold increase in β cell mass when compared to lean controls (Pick et al. 1998). The mechanisms of this compensatory mass expansion are currently unknown in mammals, as there does not appear to be an increase in β cell proliferation (Montanya et al. 2000), but may include neogenesis, slight increases in proliferation, or hypertrophy (Arystarkhova et al. 2013).

GLIS3

Krüppel-like zinc finger proteins make up one of the largest families of transcription factors, which is separated into subfamilies based on the number of zinc fingers motifs, sequence homology between the motifs, and the presence of certain transactivation domains (Williams et al. 1995, Poncelet et al. 1998). The Gli-similar 1-3 proteins (Glis 1-3) are a subfamily of the Krüppel-like zinc finger proteins similar to the Gli and Zic subfamilies (Kinzler et al. 1988, Lamar et al. 2001, Kim et al. 2003, 2005). The human gene *GLIS3* is mapped to chromosome 9p24.2 and spans 495 kb, coding for a protein that is 90 kDa. *GLIS3* contains nine exons and eight introns, sharing about 80% homology between the human *GLIS3* and the mouse *Glis3* genes (Kim et al. 2003, 2005). Homologs have also been identified in zebrafish (*glis3*, sharing 48-49% homology), as well as gleeful (*gfl*) and lame duck (*lmd*) in *Drosophila* (Duan et al. 2001, Furlong et al. 2001, ZeRuth et al. 2011) The *GLIS3* gene reportedly has several possible alternative transcripts, though the physiological function of any proteins generated by these transcripts is currently unknown (Senee et al. 2006).

The human GLIS3 protein contains a series of five Cys₂-His₂ zinc fingers motifs separated by the conserved amino acid sequence of T/SGEKPY/F, a sequence that is shared with the closely related Zic and Gli subfamilies (Agata et al. 1999, Kim et al. 2003). This domain spans from exons 2-4 of the gene and is used to interact with target DNA sequences (Kim 2003). The GLIS3 zinc fingers can bind at the Glis3 consensus sequence of 5'-(G/C)TGGGGGG(A/C)-3' (Beak et al. 2008) and the closely related Gli protein consensus sequence, although with much lower affinity (Lamar et al. 2001, Nakashima et al. 2002, Kim et al. 2003). The GLIS 3 gene also contains a transactivation domain on the C-terminal end that is critical in the activation of target genes (Kim et al. 2003, Beak et al. 2008) as well as an N-terminal conserved region (Fig. 4). The function of the N-terminal conserved region is still mostly unknown, but there is some evidence that it serves a role in protein-protein interaction. (Kim et al. 2003, ZeRuth et al. 2015).

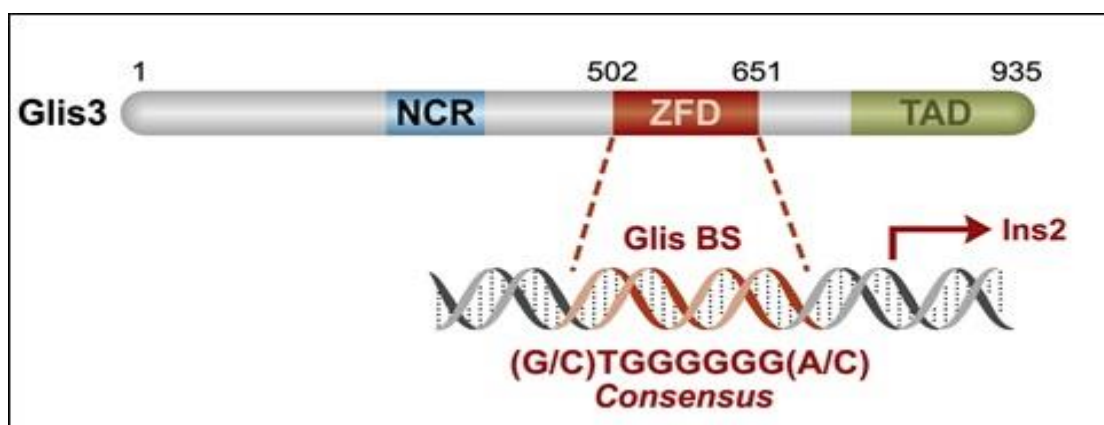


Figure 4. Map of the conserved regions in the Glis3 protein across homologs. Important regions include the N-terminal conserved region (NCR), zinc finger binding domain (ZFD), the C-terminal transactivation domain (TAD), and the Glis binding site (Glis BS). Numbers indicate amino acid positions. (Adapted from Lichti-Kaiser et al. 2012).

Glis3 Localization

During development, Glis3 expression is controlled both temporally and spatially. In mice, Glis3 RNA expression begins at E8.0 and can be seen in the pancreas, kidney, testes, lungs, brain, and eyes (Kim et al. 2003, Kang et al. 2009). Postnatally (E16.5-E18.5) Glis3 expression in the pancreas is restricted to the ductal cells and islet β cells (Senee et al. 2006, Kang et al. 2009). In the adult mouse, Glis3 expression continues in the pancreas, kidney, testes, retinas, brain, and lungs (Kim et al. 2003, Beak et al. 2007). Using EGFP-tagged Glis proteins it has been shown that these proteins are predominately expressed in the nuclei of exponentially growing cells (Kim et al. 2002, 2003, Zhang et al. 2002, Beak et al. 2008). While Glis3 contains nuclear location signals (NLS), mutation of these NLS, along with detailed studies of the mutations of individual zinc fingers (ZF) in the Glis3 ZFD have shown that the NLS are not required for the localization of Glis3 to the nucleus, but that mutation of ZF4 greatly decreases the accumulation of Glis3 in the nucleus (Kim et al. 2003, Zhang et al. 2002, Beak et al. 2008). Along with the nucleus, Glis3 accumulation can be measured in the primary cilium, much like the closely related Gli subfamily of proteins (Haycraft et al. 2005, Hashimoto et al. 2009). The primary cilia of cells are critical for the function of Shh- and Wnt- mediated signaling, and dysfunction of cilia leads to multiple ciliopathies (Oro 2007, Rohatgi et al. 2007, Veland et al. 2009).

Glis3-Associated Pathologies

Because of its early expression during development and the subcellular localization in the nucleus and primary cilia of rapidly proliferating cells, we know that Glis3 plays an important role in developing and adult cells. This is supported by the fact

that dysfunction in the expression and transcription of Glis3 in humans has been linked to the development of neonatal diabetes and hypothyroidism (NDH) and polycystic kidney disease (PKD) (Taha et al. 2003, Senee et al. 2006). NDH is characterized by hyperglycemia, hypoinsulemia, neonatal diabetes, and elevated thyroid stimulating hormone (Senee et al. 2006). NDH is also accompanied by multiple severe symptoms, including but not limited to cholestasis, hepatic fibrosis, congenital glaucoma, and facial deformities (Taha et al. 2003, Senee et al. 2006). PKD is characterized by the development of enlarged cysts in the kidneys, leading to kidney failure and death (Taha et al. 2003, Senee et al. 2006).

Glis3 knockout mice exhibit similar phenotypes of Glis3 mutant humans, including NDH, PKD, and small, misshapen primary islets in the pancreas (Kang et al. 2009, Watanabe et al. 2009). One identified target gene for Glis3 is insulin, indicating that Glis3 may play an important role in the development of the endocrine pancreas or in the differentiation of β cells (Kim et al. 2003, Senee et al. 2006, Kang et al. 2009, Yang et al. 2009). This is supported by the fact that a human genome-wide association study has identified GLSI3 as a risk locus for the development of both T1DM and T2DM (Barrett et al. 2009, Boesgaard et al. 2010, Dupuis et al. 2010).

Glis3-Mediated Gene Regulation

The mechanism through which Glis3 dysfunction leads to NDH and PKD are not fully understood, though the localization of Glis3 in the primary cilia gives indication in its role in the development of renal disease. For starters, NDH and PKD are both categorized as ciliopathies, which are caused by disruptions in the primary cilia (Saunier et al. 2005, Bisgrove and Yost 2006, Torres and Harris 2006). The primary cilia are

immotile, thread-like structures that extend from the cell into the extracellular matrix (Bisgrove and Yost 2006, Fliegauf et al. 2007, Berbari et al. 2009). Each cilium is composed of nine microtubule doublets that form an axoneme, surrounded by a continuation of the cellular plasma membrane (Bisgrove and Yost 2006, Berbari et al. 2009). A process called intraflagellar transport (IFT) is used to mediate the travel of ciliary proteins along the length of the axoneme (Rosenbaum and Witman 2002, Fliegauf et al. 2007, Gerdes et al., 2009). Because of the role of IFT in the transport of ciliary proteins into the primary cilia, the primary cilia functions as a chemo-, photo-, and mechano-sensor. The specialized plasmid membrane surrounding the primary cilia also gives it a role in the Shh- and Wnt- dependent signaling pathways (Oro 2007, Rohatgi et al. 2007, Corbit et al. 2008).

Because of the similarities in the Glis and Gli subfamilies of the Krüppel-like zinc finger proteins, it is hypothesized that Glis3 interacts with the primary cilia in a similar mechanism to the Shh/Gli3 pathway (Attanasio et al. 2007, Kang et al. 2009). A hypothetical model of Glis3 activation mirroring that of Gli3, has been suggested due to the similarities in subcellular localization. In this hypothetical model, activation of a membrane bound protein in the cilia activates IFT, leading to accumulation of Glis3 in the primary cilia (Haycraft et al. 2005, Kim et al. 2009, Kang et al. 2010). At this point activation of Glis3 may be dependent on phosphorylation or proteolytic processing. Following this processing and translocation back into the nucleus, Glis3 proteins can then repress or activate the transcription of target genes (Kim et al. 2002, Kang et al. 2009, Kang et al. 2010). More study is needed in order to identify the molecular components for each step, but initial studies have indicated that the Glis3 model appears to be highly

similar to that of the Shh/Gli3 model (Kim et al. 2002, Zhang et al. 2002, Hoskings et al. 2007).

Much like the molecular components of the proposed model for Glis3 activation, the mechanism by which Glis3 activates or repress transcription is not currently known. It has been shown that Glis3 works with multiple cofactors at the site of transcription in order to activate or repress target genes (ZeRuth et al. 2013). Glis3 was shown to be able to recruit Creb-Binding Protein (CBP/p300) (ZeRuth et al. 2013) in order to promote the transcription of insulin through protein-protein interactions with Pdx1, MafA, and NeuroD1 (Naya et al. 1997, Pashavaria et al. 1997, Qiu et al. 1998, Ohneda et al. 2000). Additionally, Glis3 has been shown to interact with WW domain-containing transcriptional regulator 1 (Wwtr1), which in turn interacts with various other transcriptional factors as either a coactivator or corepressor (Hong et al. 2005, Hong and Yaffe 2006, Kang et al. 2009).

Zebrafish as a Model Organism

In recent years, the zebrafish (*Danio rerio*) has begun to emerge as a powerful model for the study of pancreatic development. *Danio* have several advantages over the more commonly used mouse models, including rapid development, short generation time, and an amenability to genetic techniques (Streisinger et al. 1981). A single female can give rise to a clutch of 200-300 transparent, externally developing eggs at a time. The transparency of these eggs is important for the ability to efficiently visualize the developmental processes. Moreover, since zebrafish are reared in water, application of drugs is accomplished by simply placing the drugs in the water, as with tricaine-S anesthesia (Matthews and Varga 2012).

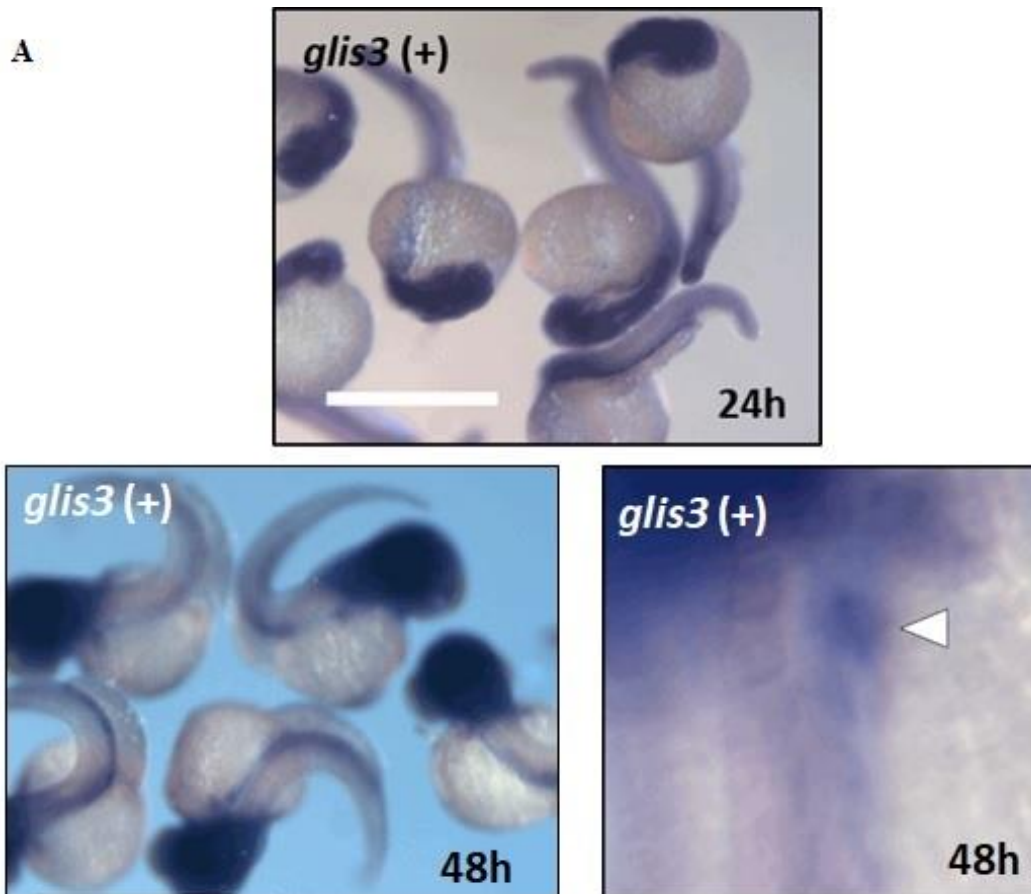
Importantly, the pancreatic development of the zebrafish appears to be highly conserved with that of humans and mice models (Pack et al. 1996, Argenton et al. 1996, 1997). RA signaling in the anterior endoderm specifies for a pancreatic fate, and Wnt/beta-catenin signaling has been shown to be responsible for tissue patterning, much like in mammals (Stafford and Prince 2002, Goessling et al. 2008). The cellular structure of the zebrafish pancreas matches that of mammalian models, with exocrine and ductal cells forming alongside the endocrine cells in two separate buds, one ventral and one dorsal (Argenton et al. 1999, Biemar et al. 2001, Devos et al. 2002, Pauls et al. 2007). Preliminary studies have shown that a fraction replicating β cells persist throughout the life of the adult fish (Yee et al. 2001, Pisharath et al. 2007). This can be related to the replication that is responsible for β cell mass expansion through adulthood in mammals (Kaung 1994, Teta et al. 2005). One unique feature that sets the zebrafish apart from rodent models is that ability to regenerate its β cells after complete ablation (Pisharath et al. 2007, Xu et al. 2008, Moss et al. 2009). The ability of zebrafish to regrow β cells after complete ablation indicates that adult zebrafish have a maintained population of pancreatic progenitor cells, something that seems to be lacking in mammals, as β cell mass expansion appears to be achieved through β cell proliferation alone (Bouwens et al. 1994, Solar et al. 2009). However, in zebrafish β cell mass expansion in response to nutrient excess stems primarily from neogenesis from postmitotic progenitor cells (Enrico et al. 2009). Understanding the mechanisms of β cell mass expansion in response to nutrient excess can be the first step to the development of clinical treatment for β cell dysfunction and glucose intolerance caused by obesity. By understanding the mechanism

through which β cell regeneration is achieved, it is possible that new clinical therapies for T2DM can be explored.

While pancreatic morphology and function appear to be highly conserved between mammals and zebrafish, there are key differences in pancreatic organogenesis. Neurogenin 3, a key transcription factor responsible for the differentiation of islet cells (Gu et al. 2002, Rukstalis and Habener 2009), has no known homologue in zebrafish. Endocrine cell differentiation in zebrafish appears to instead be under control of signaling from the transcription factor Sox9 (Huang et al. 2016). In addition to this, zebrafish pancreata form endocrine cells in scattered bilateral rows, that migrate to form a single islet during late-somitogenesis (Biemar et al. 2001). Migration of endocrine cells appear to be under the control of glypican knypek (*gpc4*) as loss of this gene function causes the failure of the bilateral endocrine primordia to merge (Biemar et al. 2001).

Due to a partial duplication of its genome, zebrafish tend to have multiple homologues of genes that are unique in mammals, making complete knockdown studies difficult in some cases (Force et al. 1999, Blader et al. 2004, Zecchin et al. 2007). However, there is only one *glis3* ortholog in the zebrafish genome on chromosome 10, which is responsible for encoding a 2,785 bp transcript (Howe et al. 2013, O'Hare et al. 2016). This transcript encodes for a protein that is 787 amino acids and has 49% homology with the human *Glis3* gene (Howe et al. 2013). This homology rises to more than 90% when comparing the critical zinc finger binding domains, and luciferase assays have been used to show that zebrafish *glis3* is capable of activating the human insulin gene (Howe et al. 2013, ZeRuth et al. 2015).

Preliminary studies of *glis3* localization and function have already been performed in zebrafish. Using oligonucleotide riboprobes, *glis3* mRNA localization was determined in a process called whole mount *in situ* hybridization. These synthetic riboprobes hybridize to the *glis3* transcript sequence and emit a fluorescent signal due to the digoxigenin-11-dUTP used during riboprobe synthesis (Fig. 5A). *glis3* expression has been measured in the brain and pancreas of developing zebrafish as early as 24hpf and 48hpf, respectively. Using a process called morpholino knockdown, *glis3* RNA translation was blocked using morpholino oligos. This process is becoming an advanced tool for silencing the translation, splicing, or ribosomal activity of target RNAs. By silencing *glis3* translation in developing embryos, a marked decrease in the number of insulin and glucagon producing cells is observed (Unpublished data, Fig. 5B).



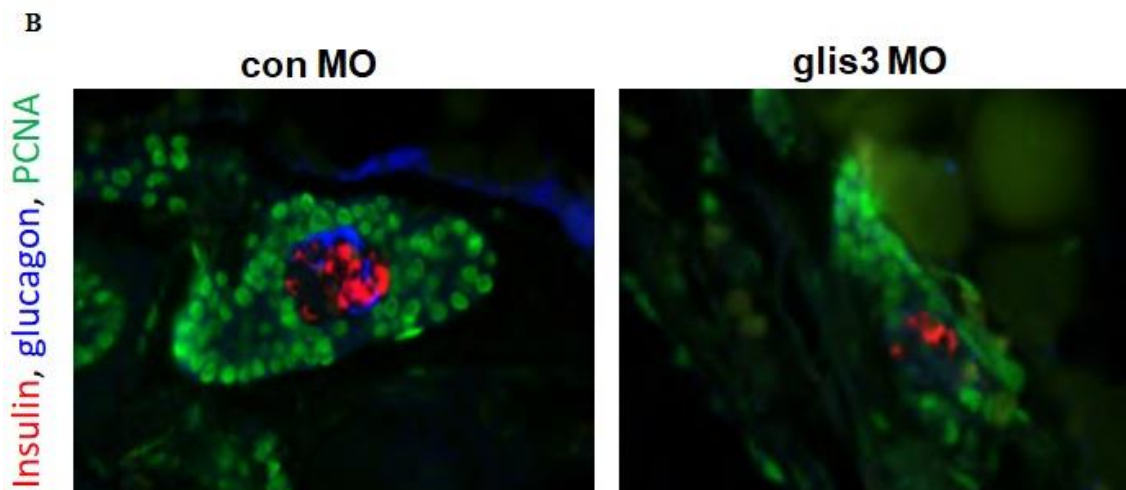


Figure 5. Preliminary analysis of localization and function of *glis3*. **A.** Whole mount *in situ* hybridization was used to determine the localization of *glis3* in developing zebrafish embryos. Fluorescently labeled digoxigenin-11-dUTPs are used to synthesize riboprobes that hybridize to *glis3* mRNA. *glis3* expression can be seen in the zebrafish brain at 24hpf, and in the developing pancreas as early as 48hpf. **B.** Morpholino oligos were used to silence *glis3* mRNA translation. Reduction of *glis3* expression due to morpholino knockdown was responsible for a reduction in insulin and glucagon expression in the developing zebrafish pancreas.

Knockout studies of *glis3* have been conducted in another small fish model called Medaka (*Oryzias latipes*). A transposon was found to be inserted in the fourth intron of *glis3*, resulting in a mutated zinc finger binding domain and a truncated protein. Medaka with *glis3* nonsense mutation exhibited the expected phenotype of PKD, including renal cyst formation and decreased urine flow rate (Hashimoto et al. 2009). However, unlike humans and mammals Medaka *glis3* mutants used showed none of the symptoms of a pancreatic phenotype, indicating a possible redundancy for *glis3* in pancreatic function in Medaka (Hashimoto et al. 2009). By using zebrafish as a model organism to study the effects of *glis3* mutation, it may be possible to develop models for the study of NDH, T1DM, and T2DM. By subjecting *glis3* mutant zebrafish to a high fat, high glucose diet, we hope to identify the effects of *glis3* mutations on the pancreatic islet mass expansion in response to nutrient excess. Identification of target genes in pancreatic endocrine cell

differentiation can eventually lead to generation of β cells from induced pluripotent stem cells (iPSCs) for use in β cell transplant. Identification of the mechanisms responsible for regeneration of β cells in zebrafish can lead to the use of regeneration as a clinical therapy in humans for the treatment of type 2 diabetes.

Material and Methods

Zebrafish Housing

The zebrafish were housed using a standard AQUANEERING aquatic housing system. Briefly, adult fish were housed in either 6.0 or 2.8 L tanks, containing a maximum of 30 or 14 fish, respectively (Vargesson 2007). These tanks are part of a five-rack continuous flow system maintained at a pH of 7.0 and a temperature of 27°C. The entire rack system is on a scheduled 14-hour light cycle to optimize zebrafish health and breeding (Lopez-Olmeda et al. 2006). Flowing water undergoes mechano-, UV-, and bio-filtration to remove particulates, microbial life, and ammonia. Half of the water in the system is replaced daily using a timed water exchange tank to supplement the filtration. Zebrafish are fed GEMMA Micro 300 at 5% of biomass once daily.

Zebrafish Lines

The wildtype zebrafish line used is the AB line, ordered from the Zebrafish International Resource Center (ZIRC). The mutant line of zebrafish used is a *glis3*^{+/-} heterozygote generated by ENU mutagenesis. Generation of mutants through ENU mutagenesis has been previously described (Kettleborough et al. 2013). Specifically, the heterozygous line is *glis3*^{+/*sa17645*}, and was purchased from ZIRC. This line of mutant fish contains a C/T mutation within exon 2 of the gene coding *glis3*, resulting in an early stop codon (Fig. 6). This early stop codon leads to a truncated protein containing only 47 amino acids that lacks the zinc finger binding domain, the transactivation domain, and the N-terminal conserved region of *glis3*. Since it lacks all the conserved regions of *glis3* that

have been identified as crucial for *glis3* function, it is expected that the protein produced will act as a sufficient knockout.

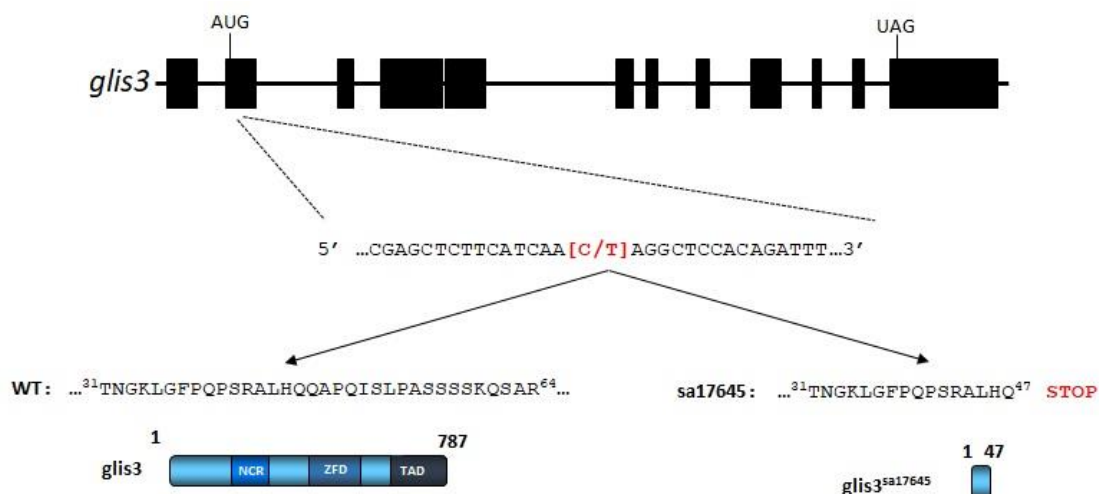


Figure 6. Schematic of the *glis3*^{+/sa17645} mutant. Shaded rectangles indicate exons of the *glis3* gene. Relative positions of the start and stop codons are indicated. The mutated region in exon 2 found in *glis3*^{sa17645} zebrafish is shown in detail. The wild type *glis3* product is comprised of 787 amino acids while the *glis3*^{sa17645} nonsense mutation prematurely truncates the protein at Gln47. NCR = N-terminal conserved region; ZFD = zinc-finger domain; TAD = transactivation domain.

Zebrafish Breeding

In order to obtain a complete *glis3* knockout model, the heterozygous *glis3*^{+/sa17645} mutants were intercrossed. Following the development of *glis3*^{sa17645/sa17645} males, these were then crossed with heterozygous *glis3*^{+/sa17645} females to increase the yield of *glis3*^{sa17645/sa17645} mutants. Zebrafish breeding protocol has been previously described (Naiadka and Clark 2012). Briefly, 1 female and 2-3 males are placed in a 1.0 L AQUANEERING crossing tank with a divider overnight. At the onset of the light cycle the divider is removed and zebrafish are allowed to spawn and fertilize the eggs, which fall through the slotted bottom of the inner tank. After spawning, the eggs are removed and placed in E2 media until hatched (approximately 5 dpf).

After hatching, larval fish are placed in a 1.8 L still water nursery tank at no more than 50 larval fish per tank (Vargesson 2007). Larval fish are fed concentrated ground paramecia until 9 dpf. Following 9 dpf, larval fish are transferred to the continuous flow system, and fed ZIRC Nursery Mix twice daily until 21 dpf. At 21 dpf the larval diet is switched to the standard GEMMA Micro food supplemented with ZIRC Nursery Mix once daily until 45 dpf, at which point the larval fish begin to follow the adult dietary protocol outlined above.

TaqMan SNP Genotyping Assays

TaqMan SNP genotyping assays were used to verify the genotype of offspring generated by $glis3^{+/sa17645}$ x $glis3^{+/sa17645}$ crosses. 90-day old fish were anesthetized using tricaine-S and tail clippings were taken. Anesthetization by tricaine has been previously described (Matthews and Varga 2012). Before tail clipping, zebrafish were weighed and measured to determine if a reduction or loss of $glis3$ during development effected phenotypic body size. After tail clipping, zebrafish were kept in isolation for ten days to allow their tail to regrow, and then were sorted into tanks with siblings that shared genotype. DNA from tail clippings was extracted using HotSHOT DNA extraction (Meeker et al. 2007).

Site directed mutagenesis was used to create the hypothesized C/T mutation in wildtype zebrafish DNA in order to use as a positive control for the TaqMan SNP genotyping assays. Wildtype $glis3$ was generated by PCR amplification using the $drglis3$ EcoRI IN and $drglis3$ BamHI OUT primers and inserted into the pCRII-TOPO plasmid. The $glis3$ -pCRII-TOPO plasmid was inserted into DH5 α cells grown on Amp⁺ LB

culture plates. Colonies were selected and cleaned using a plasmid miniprep kit (ThermoFisher) using the manufacturer's protocol.

Wildtype *glis3* DNA was then mutated using the *glis3* sa17645 C>T F and *glis3* sa17645 C>T R primers and PFU Turbo DNA polymerase. *glis3*-pCRII-TOPO plasmid DNA was incubated in the restriction enzyme Dpn I to remove unmutated DNA. Mutated DNA was then transfected into XL10-Gold competent cells and grown on AMP⁺ LB culture plates. Colonies were selected and cleaned using a plasmid miniprep kit (ThermoFisher) using the manufacturer's protocol. Mutated DNA was removed from the *glis3*-pCRII-TOPO plasmid using the restriction enzyme EcoRI. Mutation was verified by sequencing.

Primer Name	Primer Sequence (5' to 3')
drglis3 EcoRI IN	ATGCGAATTCAGCTCAGATATCTCCA
drglis3 BamHI OUT	GTCAGGATCCTCAGCCTTCAGTGAA
<i>glis3</i> sa17645 C>T F	CCGAGCTCTTCATCAATAGGCTCCACAGATTTC
<i>glis3</i> sa17645 C>T R	GAAATCTGTGGAGCCTATTGATGAAGAGCTCGG

Table 1. List of primers used for site directed mutagenesis of *glis3*.

Wildtype zebrafish DNA, heterozygous *glis3*^{+/sa17645} DNA, and the mutated *glis3*^{sa17645/sa17645} DNA were used as positive controls for genotyping the *glis3*^{+/sa17645} x *glis3*^{+/sa17645} cross offspring. TaqMan SNP genotyping assays use two probes that differ in sequence only at the SNP site. Each probe contains a covalent linked 5' reported dye and 3' quencher dye (Fig. 7). While the probe is intact, the quencher dye suppresses the signal of the reporter dye. Cleaving of the reporter and quencher dyes is accomplished by Taq polymerase if the probe and DNA are exactly complimentary. If the pairing is off by one nucleotide because of the SNP, then the probe is dislodged and the reported dye signal is

not received. By comparing the ratio of the signal received from the two reporter dyes, it is possible to determine the ratio of the genes expressed.

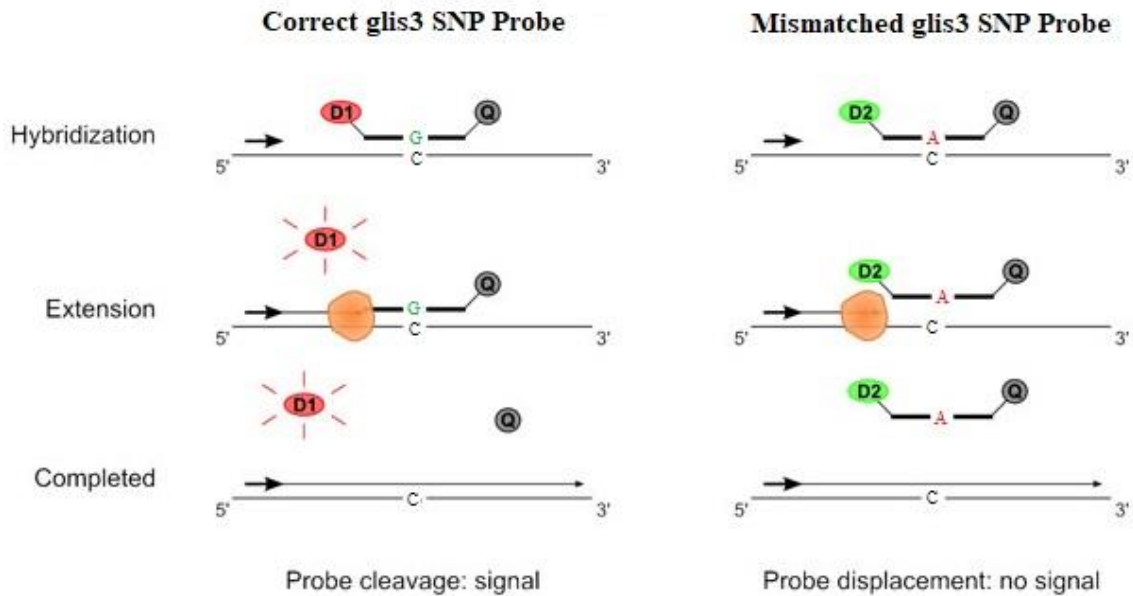


Figure 7. Schematic of the mechanism of TaqMan SNP genotyping assays. D1 and D2 are the reporter dyes for the separate probes. Q is the quencher dye, the orange circle is Taq polymerase, and the dark arrow are the forward primers. (Adapted from <http://www.applied-maths.com/applications/taqman-based-snp-genotyping>).

Blood Glucose Determination

The method for measuring blood glucose levels in zebrafish have been previously described (Eames et al. 2010). Briefly, fish are anesthetized in water at 0 °C, as tricaine-S anesthetization interferes with glucose levels (Eames et al. 2010, Matthews and Varga 2012). Fish are then decapitated, and blood glucose is measured using the FreeStyle Lite Blood Glucose and Monitoring System. Blood glucose measurements were taken for wildtype and $glis3^{+/sa17645}$ heterozygotes while fasting, post prandially, and following application of a high fat, high glucose diet.

To simulate a high fat diet (HFD) that leads to the onset of diabetes, 3 wildtype and 3 *glis3*^{+/sa17645} fish were submerged in 10.0 g of 99% D-glucose (Sigma-Aldrich) dissolved in 500 ml of system water. They were then fed 0.1 grams of powdered chicken egg yolk (Sigma-Aldrich) in supplement to their normal diet. This regiment was repeated 3 times a week for 4 months.

After the measurement of blood glucose levels, fasting and postprandial fish were dissected to determine the concentration of *glis3*, *insa*, *gcga*, and *gcgb* in the pancreas, kidney, brain, and gonads. Fish exposed to the HFD were fixed in 4% paraformaldehyde (PFA) and mounted in Paraplast X-TRA for sectioning to determine if reduction in *glis3* effected β cell mass expansion in response to nutrient excess.

Quantitative Reverse Transcriptase Real-Time PCR Analysis

RNA was isolated from wildtype eggs or wildtype and *glis3*^{+/sa17645} heterozygote organs using TRIzol RNA Extraction (Simms et al. 1993). Equal amounts of RNA were used to generate cDNA using a high capacity cDNA kit (Applied Biosystems). cDNA was then analyzed by quantitative real time PCR using the PowerUP SYBR green master mix (ThermoFisher Scientific). All qRT-PCR was performed using the Applied Biosystems 7500 real time PCR system. Primers used during RT-PCR are listed below (Table 2). *GapDH* and *eIF1A* were used to normalize RNA levels across different cell types and ages.

Immunohistochemistry and Microscopy

Zebrafish gut sections were fixed in 4% paraformaldehyde for 3 hours and washed in PBS. Tissue samples were then dehydrated with serial dilutions of ethanol in DEPC water with increasing amounts of ethanol. Tissue samples were infiltrated with

Gene Target	Primer Sequence (5' to 3')
<i>glis3</i>	F- ATGCGAATTCAGCTCAGATATCTCCA R- GTCAGGATCCTCAGCCTTCAGTGAA
<i>insa</i>	F- CTGTGTGGATCTCATCTGGT R- CTCTCTTCCTTATCAGCTCG
<i>gcga</i>	F- CGACAGCACAAGCACAGAGACAG R- GACGTTTGACAGAACCACCATTT
<i>gcgb</i>	F- GGAAAACGGCAGCCTTATGTCTG R- CGTGTCGGGACTCCACTCCTCT
<i>gapdh</i>	F- CGTCTGGTGACCCGTGCTGCTTT R – AGTGGAGGCTGGGATGATGTTCT

Table 2. List of primers used for RT-PCR.

Hemo-De and decreasing concentrations of ethanol. After infiltration, gut tissues were incubated at 65° C in 50% Hemo-De/50% Paraplast X-TRA (Sigma-Aldrich) for one hour, and then 100% Paraplast X-TRA at 65° C overnight. Tissue samples were then embedded in Paraplast X-TRA and allowed to cool overnight.

Paraplast blocks were sectioned into 20 um samples using the Spencer 820 Rotary Microtome and mounted on Superfrost Plus microscope slides (Fisherbrand). Mounted paraplast sections were allowed to dry at RT overnight. Sections were rehydrated in serial dilutions of ethanol with decreasing concentrations of alcohol. After rehydration of tissue sections, antigen retrieval was performed on slides preceding immune histochemical staining began. Both enzymatic (Shi et al. 1997) and heat-mediated antigen retrieval (Norton et al. 1994, von Wasielewski et al. 1994) were attempted.

For enzymatic antigen retrieval, slides were blotted dry and individual samples were enclosed with a hydrophobic barrier pap pen (Aqua Hold II). 100 ul of prewarmed 0.05% trypsin was applied to each sample, and then incubated at 37° C for 20 minutes. Samples were then rinsed with running water for three minutes. For heat-mediated antigen retrieval, slides were immersed in Sodium Citrate Buffer (10mM Sodium Citrate,

0.05% Tween 20, pH 6.0) heated to 100° C for 20 minutes. Samples were then rinsed with cold running tap water for 10 minutes.

Slides were rinsed in TBS plus 0.025% Triton X-100 with gentle agitation and blocked with 10% normal sheep serum with 1% bovine serum albumin (BSA) in TBS for 2 hours at RT. Slides were then drained and again enclosed with the hydrophobic barrier pen and incubated overnight at 4° C in 100 μ l of 7 μ g/ml Monoclonal Anti-Glucagon antibodies grown in mouse (Sigma) and 5 μ g/ml Rb pAb to insulin (Abcam) primary antibody diluted in TBS with 1% BSA. Slides were then rinsed in TBS 0.025% Triton with gentle agitation. Tissues were then stained with 100 μ l of 4 μ g/ml anti-rabbit AlexaFluor 594 secondary antibody (ThermoFisher Scientific) and 4 μ g/ml anti-mouse AlexaFluor 488 secondary antibody (ThermoFisher Scientific) for 1 hour at RT in the dark. Slides were then rinsed in TBS and mounted using Prolong Diamond anti-fade with DAPI (Life Technologies) and sealed with clear nail polish. Slides were observed using a Leica DMI8 fluorescence inverted microscope (Leica Microsystems) and images were captured using a DFC7000T cooled fluorescence camera and LAS X Expert software (Leica Microsystems).

Results

glis3 Expression Begins After 14.5 hpf in Wildtype Zebrafish

In order to determine when *glis3* RNA transcription begins in wildtype zebrafish, total RNA was harvested from zebrafish eggs every two hours during development, starting at 5 hpf when maternal RNA is degraded (Abrams and Mullins 2009). The total RNA was converted into cDNA and used in quantitative reverse transcriptase real-time PCR analysis (qRT-PCR) to determine *glis3* RNA expression levels. In addition, the expression of *insa* and *pdx 1* RNA levels were measured as positive controls, as it is known that *Pdx 1* expression begins at 13 hpf (Kimmel et al. 2011) and *insa* expression begins approximately 17 hpf (Papasani et al. 2006). Expression of *glis3* was shown to begin after 14.5 hpf (Fig. 8), as measured by a significant change ($p < 0.05$) in expression levels from 5 hpf, where the transition from maternal to zygotic RNA expression begins (Abrams and Mullins 2009). *Glis3* expression increased significantly again at E18.5 exhibiting a 3.5-fold increase in expression. During this experiment, the positive controls of *pdx 1* and *insa* both show transcription start times where they have been previously recorded, from 11-14.5 hpf and 16.5-18.5 hpf, respectively.

***glis3*^{sa17645} Zebrafish are Viable but Sex Determination May be Affected**

The *glis3*^{+/sa17645} mutants described above were intercrossed in an attempt to generate a line of zebrafish with a ubiquitous knockout of *glis3*. The genotype of offspring generated by this *glis3*^{+/sa17645} x *glis3*^{+/sa17645} cross was determined using TaqMan SNP Genotyping assays. Comparison of the ratio of magnitude of two separate reporter dyes was used to determine the ratio of genes expressed in the offspring genome

(Fig. 9). Each reporter dye was specific to a certain SNP in the region being examined, so homozygotes for either allele would show high expression of one probe and no expression of the other. Heterozygous *glis*^{3+/sa17645} fish showed near

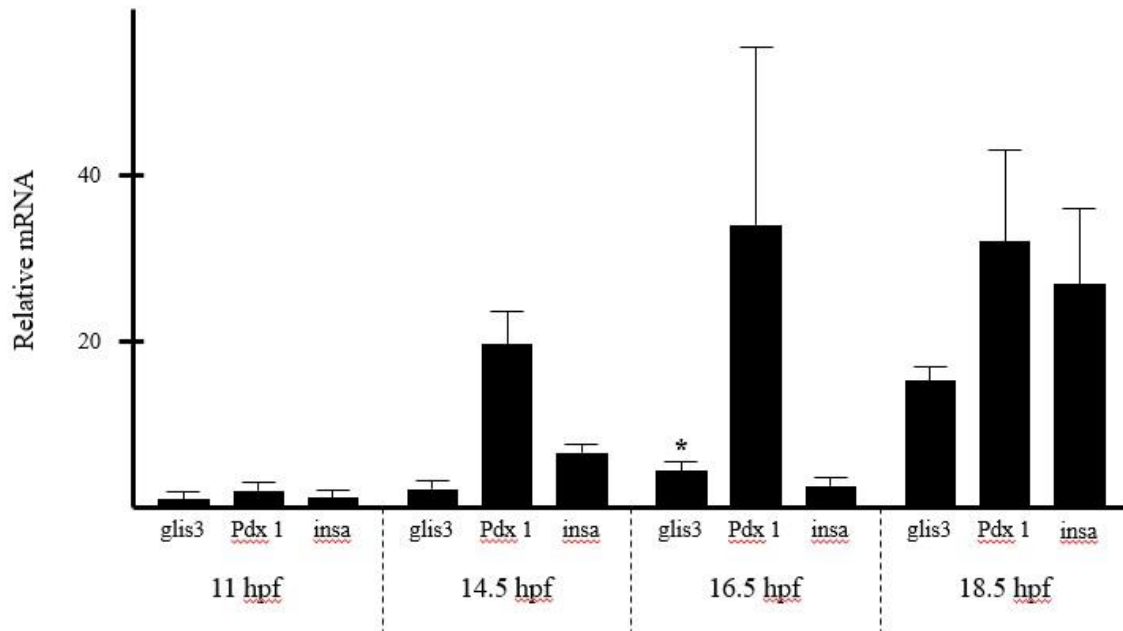


Figure 8. Expression of mRNA levels during development of zebrafish embryos. qRT-PCR was used along with PowerUP SYBR green to determine the beginning of RNA expression of multiple transcription factors. Pdx1 and insa are used as positives controls with expression beginning at 13 hpf and 17 hpf, respectively. *glis3* expression was shown to differ from time points without expression (represented by *) at 16.5 hpf ($p=0.017$), indicating that *glis3* RNA expression begins sometime after 14.5 hpf. All RNA levels were normalized against eIF1A expression levels.

equivalent expression of both probes. The genotypic and gender ratio of the performed crosses were examined for any abnormalities that may be caused by the lack of *glis3* expression during development (Table 3). The observed genotypic ratio was compared to the expected 1:2:1 ratio using the Chi Squared test (χ^2) and a χ^2 value of 1.628 was determined for the offspring genotypes. With two degrees of freedom, this means that the observed genotypic ratio is not statistically different from the hypothetical ratio ($p=0.443$). When comparing the gender ratio, a χ^2 value of 4.829 was obtained, which

indicates a significant difference in the expected gender ratio ($p=0.028$). However, until the line is further developed it is difficult to determine if this is a biologically significant difference or if difficulty in determining the sex of young adult zebrafish has skewed this data. Interestingly, the gender ratio is skewed more highly with reduction of *glis3*, while wildtype exhibit the expected 1:1 ratio. Unfortunately, the cause behind gender specification in zebrafish is complex (von Hofsten and Olsson 2005), so further research is needed in order to determine if the deletion of *glis3* may have an effect on the gender ratio of zebrafish.

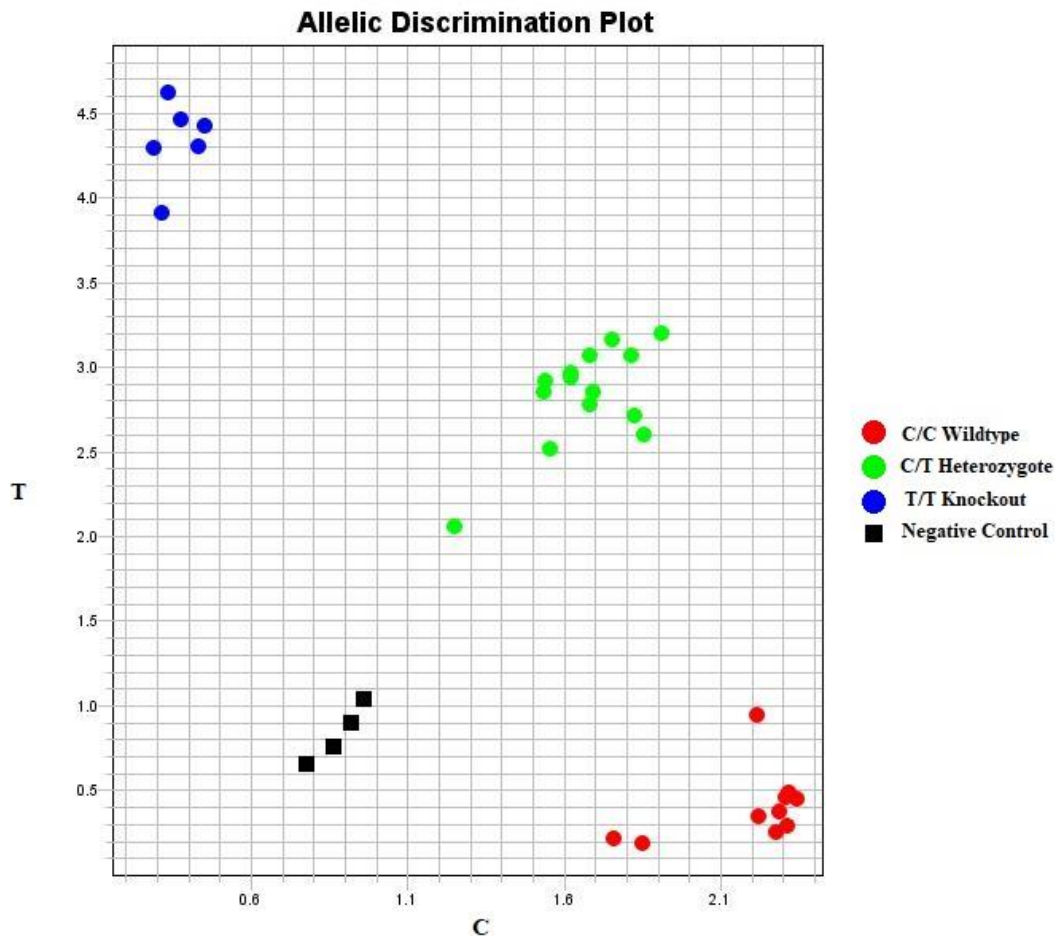


Figure 9. Determination of offspring genotype. A $glis3^{+/sa17645} \times glis3^{+/sa17645}$ cross was performed and DNA samples were taken using the HotSHOT DNA isolation method. Samples were run in duplicate using TaqMan SNP Genotyping assays to determine offspring genotype. Analysis of signaling dye ratio was done automatically using built in TaqMan Genotyper Software.

Gender	glis3 ^{+/+}	glis3 ^{+/sa17645}	glis3 ^{sa17645/sa17645}
Male	4	14	6
Female	4	7	0

Table 3. Examination of genotypic and gender ratios of offspring. A glis3^{+/sa17645} x glis3^{+/sa17645} cross was performed, resulting in 8 glis3^{+/+} offspring, 21 glis3^{+/sa17645} offspring, and 6 glis3^{sa17645/sa17645} offspring. This genotypic ratio is not significantly different from the expected model for heritable genes (p=0.443). The gender ratio is skewed toward males in the mutant models, with not knockout females, but with n=6 it is impossible to determine if this is significant until the line is further developed.

While zebrafish were anesthetized for genotyping, and before sacrificing fish to determine blood glucose levels, mass and body length were measured to determine if glis3 mutation had any effect of the development of zebrafish growth. In order to account for differences in the size of male and female zebrafish and differences in size due to age, overall growth is measured as body mass index (BMI), or mass/length in g/mm. Using the body size measurements of 63 fish with a mix of all three genotypes, it was found that the reduction or deletion of glis3 does not have any significant effect on the development of zebrafish in regard to overall BMI (Fig. 10). Both heterozygous (p=0.228) and glis3^{sa17645/sa17645} knockouts (p=0.068) show no significant deviation from average size of wildtype zebrafish. The borderline p-value of glis3^{sa17645/sa17645} knockouts when compared to wildtype zebrafish is likely due to the low sample size (n=6) of glis3 knockout BMI and will be further scrutinized once the line is further developed.

Reduction of glis3 May Increase Blood Glucose Regulation Efficiency

Wildtype and glis3^{+/sa17645} zebrafish had their blood glucose monitored under different dietary conditions in order to determine if a reduction in glis3 expression had any effect on blood glucose homeostasis (Fig. 11). Zebrafish were anesthetized in ice water as tricaine-S anesthetization interferes with glucose levels (Eames et al. 2010, Matthews and Varga 2012). Fish were then sacrificed, and blood glucose was measured

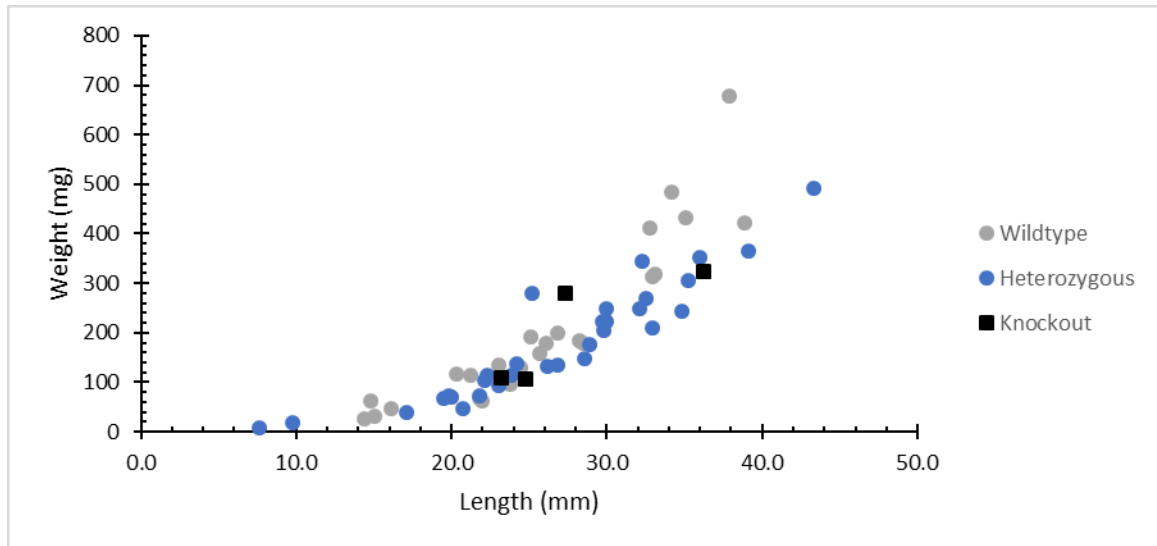


Figure 10. *glis3* reduction does not affect zebrafish body mass index. Wildtype zebrafish have an average BMI of $6.80 \times 10^{-3} \pm 3.95 \times 10^{-3}$ g/mm (n=24). Using a two-sided t-test, the average BMI of the *glis3*^{+/*sa17645*} and *glis3*^{*sa17645*/*sa17645*} were shown to not be significantly different (p=0.228 and 0.068, respectively).

using the FreeStyle Lite Blood Glucose and Monitoring System. Baseline glucose levels were taken in fasting zebrafish, and immediate glucose response was measured in postprandial zebrafish. After four months of exposure to the high fat/high glucose diet described previously, resting blood glucose was then measured in wildtype and heterozygous fish in order to measure their ability to regulate blood glucose in response to nutrient excess.

The control diet wildtype and *glis3*^{+/*sa17645*} showed a statistically significant difference in resting blood glucose levels (p=0.033). Neither the postprandial or HFD dietary groups show any significant difference between blood glucose levels of the wildtype and heterozygous fish, though in the case of the HFD fish, this is likely due to the high fluctuations in blood glucose levels in the WT fish (p=0.142). In the HFD case the heterozygous fish expressed less variability in blood glucose (relative standard

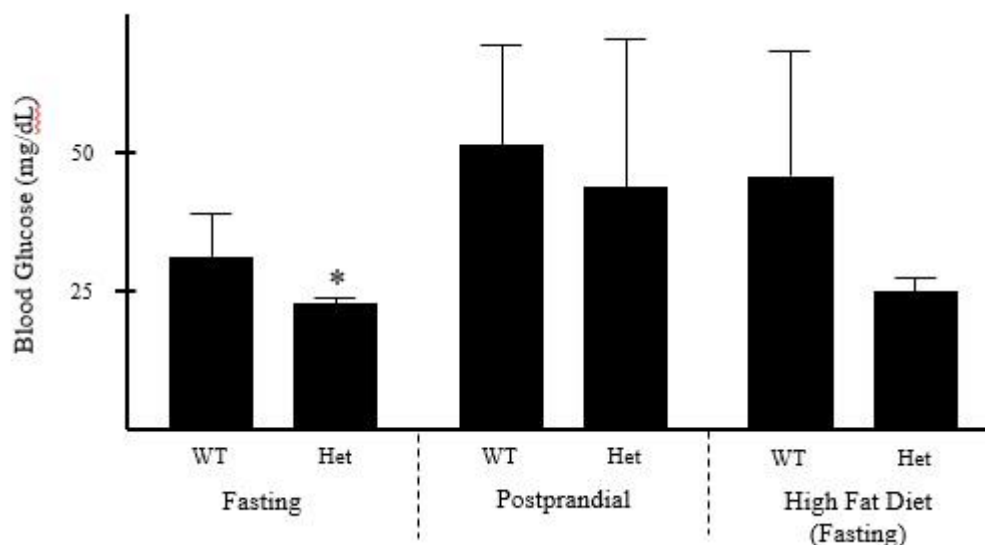


Figure 11. Blood glucose levels of fish with different dietary constraints. Blood glucose measurements were taken from fasting and postprandial control fish. Fasting heterozygotes exhibit lower resting blood glucose than wildtype counterparts ($p=0.033$). Fish exposed to a high fat diet (HFD) where then fasted and had resting blood glucose measurements take to determine if long term exposure to nutrient excess affected blood glucose homeostasis. There was no statistical significance in the difference in blood glucose levels between WT and heterozygous fish ($p=0.142$), though this is believed to be due to the high variability in in the WT fish, which were beginning to exhibit signs of T2DM. There was also no significant difference in the resting blood glucose levels of the control and HFD heterozygous fish ($p=0.354$). All blood glucose was measured using the FreeStyle Lite Blood Glucose and Monitoring System.

deviation = 0.10). Wildtype fish exposed to the HFD exhibit less efficient blood glucose regulation in comparison to wildtype controls, which may be an indicator for the onset of T2DM, while $glis3^{+/sa1645}$ fish showed no change in resting blood glucose levels ($p=0.354$).

$glis3$ Heterozygotes Express Increased Levels of Islet Hormones

RNA expression levels were measured in the pancreas and brain of wildtype and $glis3^{+/sa17645}$ heterozygotes under differing dietary constraints (Fig. 12). Total RNA was collected using TRIzol RNA extraction. Equal amounts of RNA were used to generate cDNA using a high capacity cDNA kit (Applied Biosystems). cDNA was then analyzed

by quantitative real time PCR. In both fasting and postprandial conditions, heterozygotes expressed increased levels of the primary islet hormone insulin ($p < 0.005$ and $p = 0.033$, respectively). Under postprandial condition, heterozygotes also displayed increased expression of glucagon ($p = 0.024$).

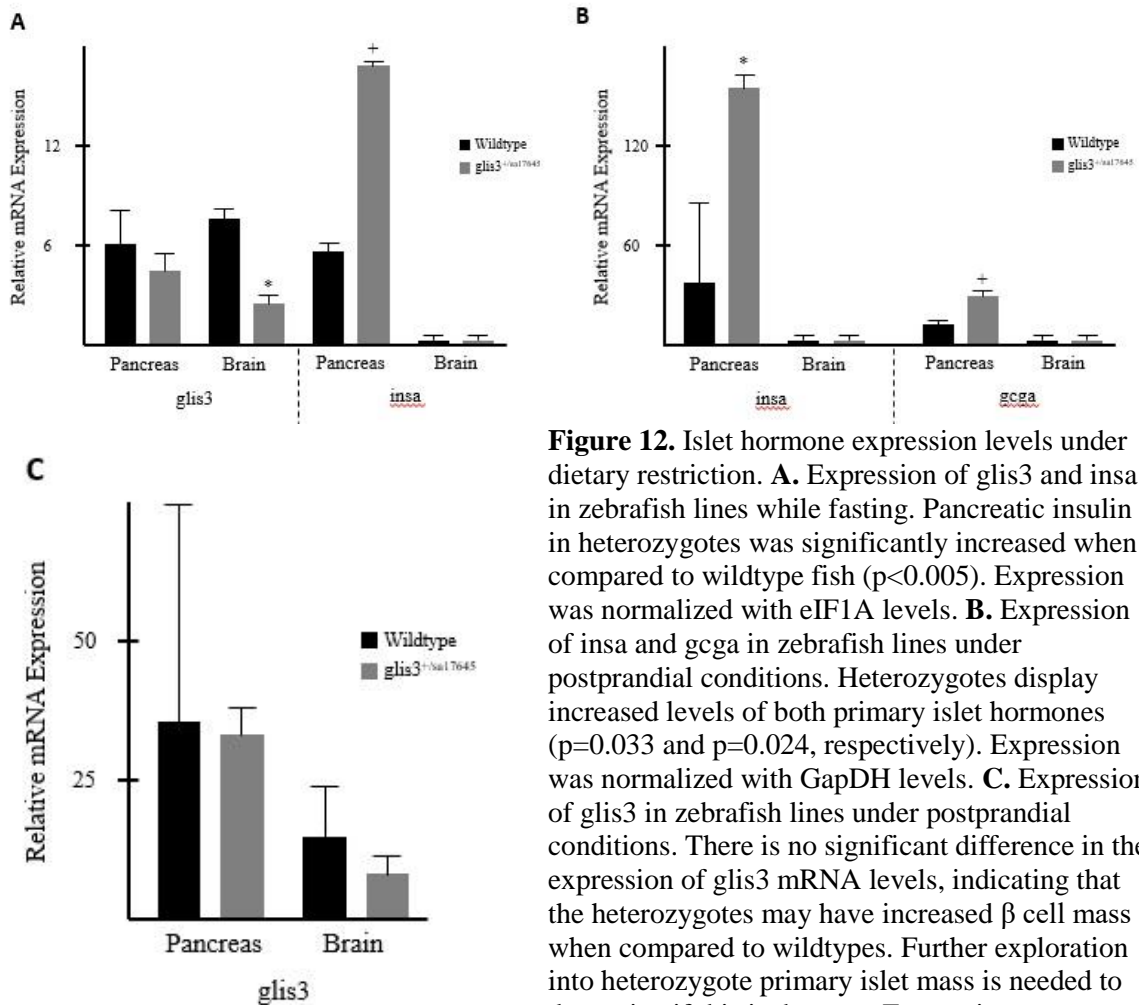
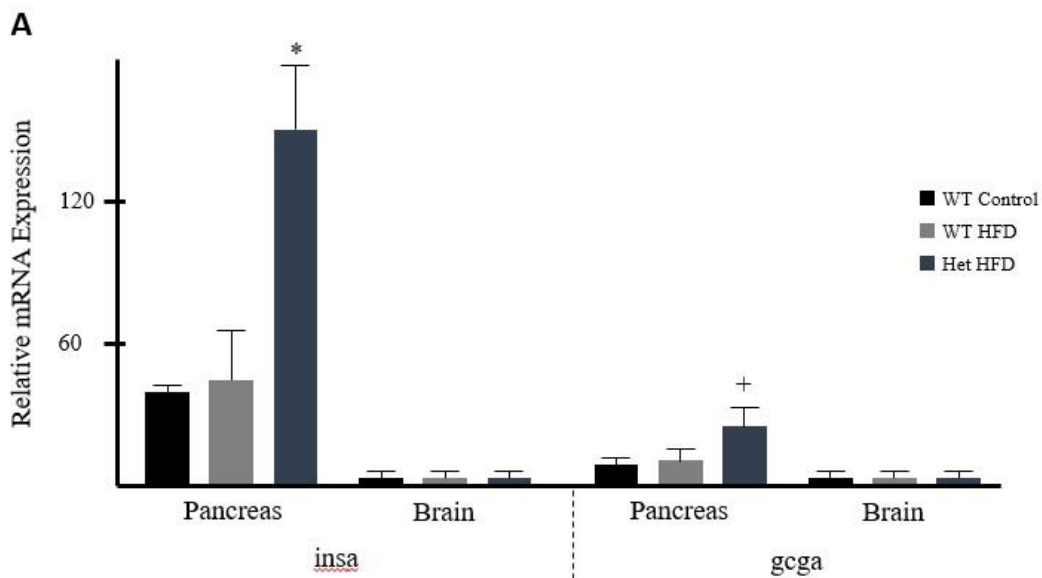


Figure 12. Islet hormone expression levels under dietary restriction. **A.** Expression of *glis3* and *insa* in zebrafish lines while fasting. Pancreatic insulin in heterozygotes was significantly increased when compared to wildtype fish ($p < 0.005$). Expression was normalized with eIF1A levels. **B.** Expression of *insa* and *gcga* in zebrafish lines under postprandial conditions. Heterozygotes display increased levels of both primary islet hormones ($p = 0.033$ and $p = 0.024$, respectively). Expression was normalized with GapDH levels. **C.** Expression of *glis3* in zebrafish lines under postprandial conditions. There is no significant difference in the expression of *glis3* mRNA levels, indicating that the heterozygotes may have increased β cell mass when compared to wildtypes. Further exploration into heterozygote primary islet mass is needed to determine if this is the case. Expression was normalized using GapDH expression levels.

Zebrafish Display Increased Islet Hormones in Response to HFD

Wildtype and *glis3*^{+sa17645} heterozygote fish were submerged in 0.1 M glucose and fed 0.1 g of powdered chicken egg yolk three times a week for four months in addition to their normal diet to simulate a high fat, high glucose diet. Blood glucose was

measured using the FreeStyle Lite Blood Glucose and Monitoring System (Fig. 11), and TRIzol RNA extraction was used along side RT-PCR to determine RNA expression levels in fish in response to the high fat diet (Fig. 13). Wildtype fish exposed to a high fat diet showed no change in RNA expression of *glis3* or *gcga* when compared to wildtypes under normal dietary conditions. Heterozygotes exposed to high fat diet displayed increased levels of *insa* ($p=0.007$) and *gcga* ($p=0.019$) when compared to wildtype controls (Fig. 13). Heterozygotes show the expected reduced expression of *glis3* in the brain ($p=0.013$), but do not express reduced *glis3* levels in the pancreas. The wildtype fish exposed to the high fat diet exhibit extremely reduced *glis3* expression in the brain, but this is likely due to the low sample size ($n=1$).



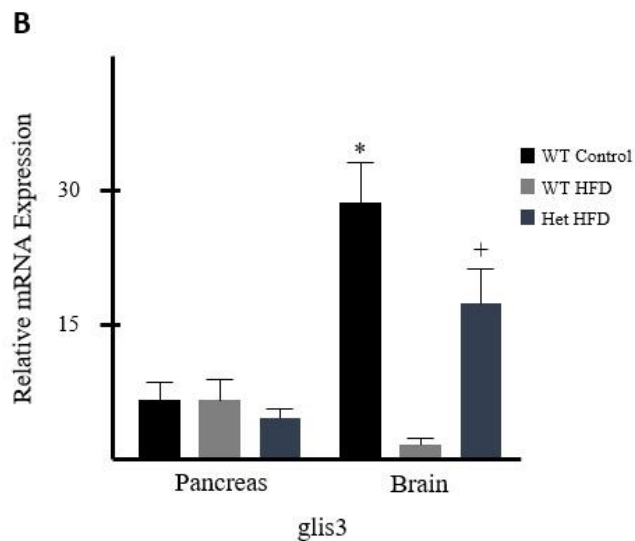


Figure 13. RNA expression levels in response to a high fat diet. **A.** Expression levels of *insa* and *gcga* in zebrafish. WT fish under normal dietary conditions were used as a control. *glis3*^{+/sa17645} fish exhibit increased *insa* ($p=0.007$) and *gcga* ($p=0.019$) expression. All RNA expression was normalized using *GapDH*. **B.** Expression of *glis3* was measured in fish in response to nutrient excess. Heterozygotes show the expected reduced *glis3* levels in the pancreas ($p=0.014$), but not in the pancreas ($p=0.064$). All RNA levels were controlled for using *GapDH*.

Discussion and Future Direction

These studies have shown that *glis3* expression levels in zebrafish have a significant impact on the expression levels of primary islet hormones. Zebrafish with decreased levels of *glis3* exhibit increased production of *insa* and *gcga* mRNA, and *glis3*^{+/sa17645} zebrafish exhibit an increased ability to regulate blood glucose. This is consistent with previous results that show that overexpression of *glis3* in β cells causes a reduction in insulin levels, but directly in contrast to the idea that Glis3 is responsible for promoting insulin transcription in mice and humans (Kim et al. 2003, Senee et al. 2006, Kang et al. 2009, Yang et al. 2009, Yang et al. 2013b). These data indicate that *glis3* might be acting as a repressor of insulin transcription in zebrafish, despite *glis3* acting as a potent activator of insulin transcription *in vitro* (Kang et al. 2009, Watanabe et al. 2009, Yang et al. 2009, Yang et al. 2013b).

The increased expression of primary islet hormones in *glis3* heterozygous zebrafish is indicative of *glis3* repression of *insa* and *gcga*, which agrees with reporter studies indicating *glis3* may act to inhibit insulin transcription. This may be due to *glis3* playing a role in the prevention of differentiation into α and β cells, thus loss of *glis3* might result in islet hyperplasia. This idea needs to be examined through immunohistochemical staining of islets in HFD challenged heterozygotes. Using whole mount *in situ* hybridization it can be shown that *glis3* expression does not begin in the pancreatic region until approximately 48 hpf, which is 24 hours after pancreas development begins in zebrafish. Zebrafish *glis3* expression begins after 14.5 hpf (Fig. 8), but early expression is restricted to the brain. The late expression of *gli3* in the

pancreas, coupled with the changes in islet hormone levels in adulthood, may indicate that *glis3* is required for stopping the signal that induces islet cell formation, or for controlling islet cell proliferation during β cell mass expansion.

Despite the fact that *Glis3* has been identified as a risk locus for the development of T1DM and T2DM in humans (Barrett et al. 2009, Boesgaard et al. 2010, Dupuis et al. 2010) Initial studies of the phenotype of *glis3*^{+/sa17645} and *glis3*^{sa17645/sa17645} fish indicate that loss of *glis3* is having no negative impact on physiological health. This is similar to Medaka *glis3* alternative splice isoform models, which displayed none of the symptoms of a pancreatic phenotype (Hashimoto et al. 2009). *Glis3* heterozygous and knockout fish have exhibited normal feeding and breeding patterns, and no difference in BMI has been detected. The only appreciable difference in heterozygote and wildtype fish is the gender ratio of their offspring. In the initial steps of producing a complete *glis3* knockout line, it has become apparent that reduction or loss of *glis3* may increase the likelihood of male progeny. Further exploration of the gender of the *glis3*^{+/sa17656} x *glis3*^{+/sa17656} line is needed to determine if this is biologically significant, or if this discrepancy is due to a low sample size. The mechanisms for zebrafish sex determination are currently unknown (von Hofsten and Olsson 2005), so an increased propensity towards male offspring in *glis3* mutants would be a novel discovery toward understanding these mechanisms.

Furthermore, although the sample size is too small to be conclusive, *glis3*^{+/sa1645} zebrafish seem to exhibit tighter regulation of blood glucose homeostasis than compared to wildtype fish (Fig. 11). In response to a high fat diet pancreatic *glis3*, *insulin*, and *gcga* expression levels in *glis3*^{+/sa17645} zebrafish increase compared to wildtypes, while blood glucose levels remain normal. The resting blood glucose of wildtype zebrafish exposed to

a high fat diet increases, while insulin expression levels remain constant. Since the wildtype zebrafish are exhibiting the early symptoms of T2DM while the heterozygous remain healthy, we again posit that the reduction in *glis3* may be aiding in the maintenance of healthy primary islets. Preliminary data indicates that *glis3* heterozygotes may be more likely to begin the routine mechanism for compensatory β cell mass expansion or exhibit an increased production of insulin in existing cells. However, since *glis3* is not expressed in the α cells of the pancreas, increased *gcga* production suggests an increase in islet mass. The lack of a significant change in *glis3* expression levels in the pancreas of heterozygous and wildtype zebrafish lines may also be indicative of an increase in primary islet mass, as an increase in the number of β cells would lead to increased *glis3* expression.

For a more in depth look at the role that *glis3* plays in pancreatic function and maintenance, the generation of the complete *glis3*^{sa17645/sa17645} knockout model will be continued. Analysis of *glis3*, *insa*, and *gcga* levels will be repeated for the *glis3* knockout model under fasting and postprandial condition to determine if the loss of *glis3* continues the trend of increased pancreatic hormone levels with decreased *glis3* levels. Knockout *glis3* mutants will be exposed to the high fat, high glucose diet and blood glucose and pancreatic hormone levels will be monitored to determine if loss of *glis3* makes zebrafish more likely to develop T2DM.

Collectively, these studies have indicated that zebrafish *glis3* may act as a repressor of insulin transcription. The increased likelihood of *glis3*^{+/sa17645} to undergo a compensatory mechanism for β cell mass expansion in response to nutrient excess may lead to the development of models for the study of T2DM. Investigation of pancreas

morphology and hormone expression in response to loss of *glis3* in environments of nutrient excess will be a continued focus of research in the future.

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Appendix

Recipes:

Embryo Media 0.5x E2

For 1.0 L

100x E2A	5 ml
500x E2B	1 ml
500x E2C	1 ml
0.1% w/v Methylene Blue	500 ul

Add reverse osmosis (RO) water to 900 ml. Adjust pH to 7.0-7.2 with concentrated HCl or NaOH. Add RO water to 1 L. Store at RT.

100x E2A

For 160 ml

NaCl	14 g
KCl	0.6 g
MgSO ₄	1.92 g
KH ₂ PO ₄	0.33 g
Na ₂ HPO ₄	0.11 g

Add Millipore water to a final volume of 160 ml. Stir to dissolve reagents. Sterilize by autoclaving. Stir overnight to dissolve any precipitation, store at 4° C.

500x E2B**For 200 ml**

CaCl₂ 11 g

Add Millipore water to a final volume of 200 ml. Stir to dissolve reagents. Sterilize by autoclaving. Aliquot in 20 ml portions. Store at -20° C.

500x E2C**For 200 ml**

NaHCO₃ 6 g

Add Millipore water to a final volume of 200 ml. Stir to dissolve reagents. Sterilize by autoclaving. Aliquot in 20 ml portions. Store at -20° C.

Luria Bertani (LB) Media**For 500 ml**

Tryptone 5 g

Yeast Extract 2.5 g

NaCl 5 g

Agar (if for plates) 7.5 g

Mix ingredients in 300 ml of water by stirring. Sterilize by autoclaving. Cool to ~55° C before adding antibiotic.

S.O.C Media**For 100 ml**

Tryptone	2 g
Yeast extract	0.5 g
NaCl	200 ul (5 M)
KCl	250 ul (1 M)
MgCl ₂	1 ml (1 M)
MgSO ₄	1 ml (1 M)
D-Glucose	2 ml (1 M)

Dissolve first 4 ingredients in 96 ml ddH₂O. Sterilize by autoclaving. Allow to cool to RT. Add last 3 ingredients by syringe filtration. Store at 4° C.

8% Paraformaldehyde**For 100 ml**

Paraformaldehyde	8 g
NaOH	600 ul (10 M)

Mix ingredients in 90 ml of water and stir until paraformaldehyde goes into solution without heating. After obtaining a clear solution, add 1 ml 6M HCl and adjust pH to 7.0. To prepare a working solution of 4% PFA, combine 50 ml 8% PFA, 10 ml of 10x PBS, and 40 ml DEPC water.

10x Phosphate Buffered Saline (PBS)**For 500 ml**

NaCl	40 g
KCl	1 g
Na ₂ HPO ₄	7.2 g
KH ₂ PO ₄	1.2 g

Dissolve reagents in 400 ml of ddH₂O. Adjust pH to 7.4 with HCl. q.s. to 500 ml with ddH₂O. Sterilize by autoclaving. Store at RT.

Trypsin Solution (0.05%)**For 10 ml**

Trypsin	0.05 g
CaCl ₂	0.1 g

Dissolve 0.05 g of trypsin in 10 ml of distilled water. Dissolve 0.1 g of CaCl₂ in a *separate* container of 10 ml of distilled water. Add 1 ml of the trypsin solution and 1 ml of the CaCl₂ solution to 8 ml of distilled water. Adjust pH to 7.8 with 1 M NaOH and store at 4° C.

10x Tris Buffered Saline (TBS)**For 500 ml**

Tris	12.1 g
NaCl	48.8 g

Dissolve salts in 400 ml of ddH₂O. Adjust pH to 7.6 with HCl. q.s. to 500 ml with ddH₂O. Sterilize by autoclaving. Store at RT.

Protocols:

HotSHOT DNA Extraction

1. Immerse tissue in 100 ul of 50 mM NaOH in a 1.5 ml microcentrifuge tube.
 - a. Poke a hole in the lid of the microcentrifuge tube.
2. Heat for 20 minutes at 95° C.
 - a. Shake tube after heating to dissociate tissue.
3. Chill tubes to 4° C on ice, then add 10 ul of 1 M Tris HCl pH 8.0.
4. Centrifuge for 10 minutes at 4° C at max speed.
 - a. DNA is in the supernatant after centrifuging.
5. Determine DNA concentration using a nanodrop.

TOPO TA Cloning

1. Equilibrate a water bath to 42° C.
2. Warm SOC broth and AMP⁺ LB culture plates to 37° C in an incubator.
3. Set up the TOPO cloning reaction:
 - a. 2 ul of PCR product DNA
 - b. 1 ul TOPO salt solution
 - c. 1 ul TOPO vector
 - d. 2 ul RNase free water
4. Incubate at RT for 5 minutes
5. Add 2 ul of the TOPO cloning reaction to 50 ul of DH5 α cells, mix gently.

6. Incubate on ice for 5 minutes.
7. Heat shock in the 42° C water bath for 30 seconds without shaking.
8. Place on ice, and add 250 ul of prewarmed SOC.
9. Shake tubes horizontally for 1 hour at 37° C.
10. Add 50 ul of the transformation to the prewarmed Amp⁺ LB plate and incubate overnight at 37° C.
11. Choose 3 to 4 colonies and incubate each in 3 ml of AMP⁺ LB broth at 37° C while rocking horizontally.
12. Purify amplified DNA using a plasmid miniprep kit.

Site Directed Mutagenesis

1. Dilute plasmid DNA (from TOPO TA cloning) to 50 ng/ul.
2. Set up PCR reaction:
 - a. 5 ul of 10x PFU buffer.
 - b. 1.25 ul of 100 ng/ul forward primer.
 - c. 1.25 ul of 100 ng/ul reverse primer.
 - d. 1 ul of dNTP mix.
 - e. 1 ul of 50 ng/ul plasmid DNA
 - f. RNase free water to a final volume of 50 ul.
3. Add 1 ul of PFU Turbo DNA polymerase
4. Run PCR cycle:
 - a. 1 cycle
 - i. 95° C for 30 seconds.
 - b. 12 cycles

- i. 95° C for 30 seconds.
 - ii. 55° C for 1 minute.
 - iii. 68° C for 5 minutes.
 - c. 1 cycle
 - i. 68° C for 10 minutes.
5. Add 1 ul of Dpn I restriction enzyme.
6. Gently mix the reaction, then spin down for one minute.
7. Incubate at 37° C for 1 hour.
8. Add 45 ul of XL10-Gold competent cells and 2 ul of β -mercaptoethanol to a separate, prechilled microcentrifuge tube.
 - a. Incubate on ice for 10 minutes, swirling every 2 minutes.
9. Add 2 ul of Dpn I treated DNA to the tube and incubate on ice for 30 minutes.
10. Transfect mutated DNA into the XL10-Gold competent cells (Steps 7-12 from TOPO TA Cloning protocol).

TaqMan SNP Genotyping Assay

1. Create a master mix using, for each sample:
 - a. 12.5 ul of 2x TaqMan Universal PCR Master Mix.
 - b. 1.25 ul of 20x SNP Genotyping Assay
 - c. 9.25 ul of RNase free water.
2. Load the master mix into an RT-PCR well plate. Each sample gets 23 ul of the master mix and 2 ul 25 ng/ul DNA.
3. Run the PCR cycle
 - a. 1 cycle

- i. 95° C for 10 minutes.
- b. 40 cycles
 - i. 92° C for 15 seconds.
 - ii. 60° C for 1 minute.

TRIzol RNA Extraction

1. Place ~30 eggs (or organ tissue) in a 1.5 ml sterile microcentrifuge tube.
2. Add 250 ul of TRIzol and lyse cells with a pestle.
3. Add 750 ul of TRIzol and incubate at RT for 5 minutes.
4. Add 200 ul of phenol:chloroform and gently invert for 15 seconds.
5. Incubate at RT for 2 minutes, then centrifuge at 12,000 x g for 15 minutes at 4° C.
6. Transfer the clear aqueous top layer to a new tube and add 500 ul of isopropanol.
7. Incubate at RT for 10 minutes.
8. Spin for 10 minutes at 12,000 x g and 4° C. Remove supernatant and wash in 1 ml of 75% ethanol.
9. Centrifuge for 5 minutes at 7,500 x g and 4° C. Remove ethanol and allow pellet to air dry for 10 minutes while inverted.
10. Resuspend RNA in 100 ul of RNase free water, store at -80° C.