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Characterization of the principal and secondary islets during pancreatic development in zebrafish with a CRISPR-mediated Glis3 knockout

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Murray State University Honors College
HONORS THESIS
Certificate of Approval

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April 2023

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Characterization of the principal and secondary islets during pancreatic development in zebrafish with a CRISPR-mediated Glis3 knockout

Submitted in partial fulfillment of the requirements
for the Murray State University Honors Diploma

Caleb Harsin

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Abstract

The Krüppel-like transcription factor, Gli-similar 3 (Glis3) has been implicated in several human pathologies including neonatal diabetes, congenital hypothyroidism, and polycystic kidney disease. Numerous genome-wide association studies (GWAS) have additionally identified Glis3 as a risk locus for the development of both type 1 and type 2 diabetes *mellitus*. Our previous data suggest possible roles for Glis3 in endocrine pancreas specification in mice, but despite its clinical significance, much remains unknown about the role(s) Glis3 plays during development. To elucidate Glis3 gene function, a CRISPR-mediated knockout line of zebrafish was developed by deleting a segment of the Glis3 coding region resulting in a non-functional protein product. Founder fish were subsequently outcrossed to give rise to offspring that are heterozygous for the knockout mutation. In this study, fish heterozygous for the Glis3 deletion were crossed to produce zebrafish of varying genotypes, including those lacking a functional copy of the Glis3 gene. Genotypes were determined by PCR using tail biopsies and primers that flank the deleted region of the Glis3 locus. At select time points in development, the resulting embryos were analyzed via fluorescence imaging to determine the effect of the Glis3 knockout on embryonic pancreatic development. Analysis of the knockout embryos demonstrated that development of the principal islet remained largely unaffected by the absence of Glis3. In addition to fluorescence microscopy, immunohistochemistry was employed to further characterize the development of the secondary islets. In a similar fashion to mice, the knockout zebrafish displayed a phenotype of polycystic kidney disease that was visible by 30 dpf.

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Introduction

Diabetes *mellitus* (DM) is a disease characterized by insulin deficiency, insulin resistance, or both conditions together. The prevalence of DM has reached an alarming level, especially within the United States. As of 2019, 37.3 million people in the country have DM (11.3%) while another 96 million (38.0%) have less severe symptoms which confer a susceptibility called pre-diabetes (CDC). DM has adverse effects on both quality of life and mortality rates, with major risk factors for macrovascular disease (cardiovascular disease) and microvascular disease (retinopathy, neuropathy, and kidney disease) (Cole & Florez 2020). While the term DM alludes to a single homogenous condition, reality instead reveals a broad spectrum of dysfunctions that fall under this umbrella term. Most notably, DM can be divided into type 1 diabetes (T1DM) and type 2 diabetes (T2DM). Worldwide, T2DM covers a heterogeneous collection of pathologies and composes up to 95% of all diabetes diagnoses (Cole & Florez 2020). Other common DM variants include monogenic diabetes (MODY), gestational diabetes, and neonatal diabetes.

T1DM is characterized by an absence of pancreatic beta cells, which are responsible for releasing insulin in response to elevated levels of blood glucose. The lack of beta cells is attributed to an autoimmune response in the case of T1DM. Hyperglycemic conditions ensue due to the lack of insulin production and release. T2DM is a condition that is not as easily defined as T1DM due to the complex interplay between genetic, lifestyle, and environmental factors. Mainly, T2DM is characterized by insulin resistance at key tissues, such as the liver, skeletal muscle, and adipocytes (Wang et al. 2015). Insulin resistance occurs when the target tissues fail to respond to insulin by taking in available glucose in the bloodstream even though the hormone

may be present in the bloodstream. In addition to resistance, T2DM has also been associated with pancreatic beta cell deficiency or dysfunction (Ashcroft & Rorsman 2012).

In the fight against DM, the central role of the beta cell demands a proper understanding of how it is specified and functions. In humans, the rate of proliferation of the beta cell is significantly reduced compared to other important structures; at the height of cell division, only 1 to 3% of beta cells are actively dividing in comparison to a normal rate of 20 to 30% in other tissues (Wang et al. 2015). The slow rate of beta cell replication is notable because beta cell deficiency plays a role in T2DM pathology, the most common form of the disease. It is possible that beta cell mass is decreased up to 60% in the T2DM population, which explains the lack of glucose-induced insulin release (Butler et al. 2003). In reflection of the importance of the beta cell, this study considers a protein related to both pancreatic development and DM.

Gli-Similar 3 (Glis3) is a Kruppel-like zinc finger protein transcription factor with a role in pancreatic development (Beak et al. 2008). Glis3 is related to the Gli and Zic protein subfamilies, which are related via the conserved zinc finger domain (ZFD) that consists of five Cys_2-His_2-type zinc finger motifs (Kim et al. 2003). During embryonic development, Glis3 is expressed in various tissues, including the kidney, pancreas, thymus, and brain (Kim et al. 2003). In addition to expression in the developing pancreas, Glis3 has been identified as a risk locus in both T1DM and T2DM via genome-wide association studies (Barrett et al. 2009, Rees et al. 2011). In mice, Glis3 has been shown to positively regulate insulin expression, further linking this gene to diabetes pathology. Specifically, Glis3-mediated activation of the insulin gene is synergistic with other important pancreatic factors, such as Neurod1, Mafa, and Pdx1 (ZeRuth et al. 2013). Glis3 carries out its function as a transcription factor via a transactivation domain located at the carboxyl terminus while the amino terminus serves as a location for negative

regulation of Glis3 activity; moreover, all zinc finger domains are necessary for proper DNA-binding and subsequent activation (Beak et al. 2008).

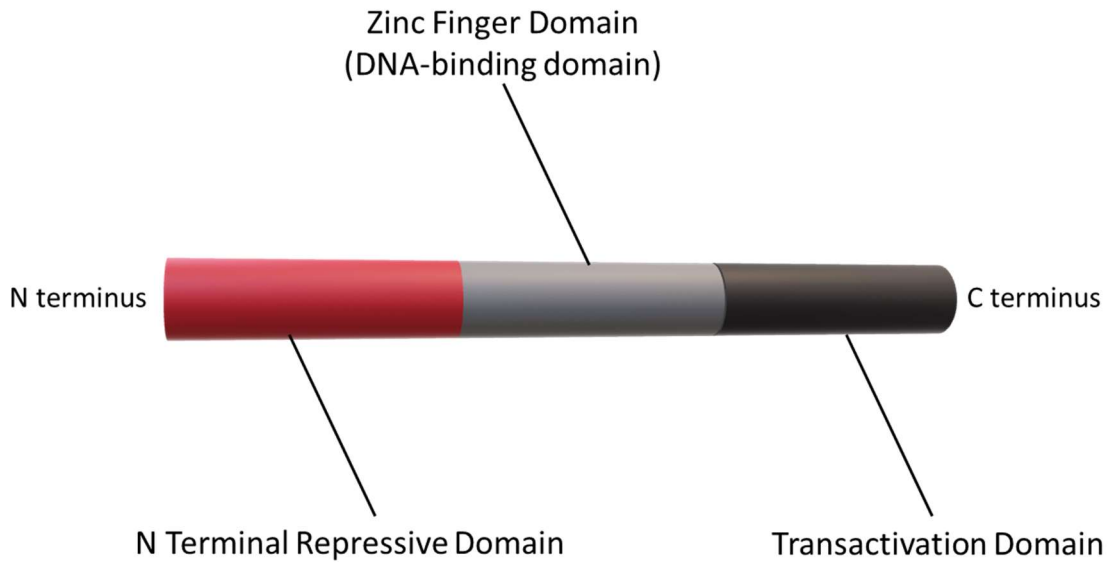

Figure 1. Functional domains of Glis3. Both the C terminal transactivation domain and the Zinc Finger domain are crucial to the function of Glis3 as a transcription factor. The N terminal domain allows for meta regulation of Glis3.

While Glis3 has been shown to serve as a transcription factor for positive regulation of insulin, it may also serve a role in pancreatic beta cell specification and maturation. In mice, the onset of Glis3 expression coincides with the secondary transition in pancreatic development (Kang et al. 2009). The secondary transition is a period marked by the differentiation of acinar, ductal, and endocrine cells, which are the three major cell fates of the mature pancreas (Scoville & Jetten 2021). Specifically, Glis3 is first expressed in a population of bipotent progenitor cells in the trunk of the developing organ. These bipotent cells have the ability to differentiate into either ductal or endocrine cells. The ductal cells continue to express Glis3 through maturation as

do the beta cells of the endocrine lineage (Kang et al. 2009). Glis3 expression in the mature pancreatic beta cell along with its role as a transcriptional activator of insulin has led to investigation into its possible role in diabetes pathology.

While the characterization of Glis3 function in mice has allowed for the development of a neonatal diabetes model (Kang et al. 2009), there are certain challenges inherent to using the mouse in this fashion. First, mouse development occurs internally, making early visualization techniques difficult to employ. The process of organogenesis also takes much longer when compared to other model organisms. For these reasons, this study focuses on characterizing the function of glis3 in zebrafish, a model organism equipped to overcome the aforementioned challenges. Zebrafish are fertilized and develop externally. To further aid in visualization, the embryos are naturally transparent; at 24 hours post fertilization (hpf), treatment with phenylthiourea (PTU) ensures that the embryos will remain transparent throughout development. PTU inhibits the migration of melanocytes, which localizes pigment development to the dorsal midline, allowing for easy observation of organogenesis. Besides transparency, the zebrafish offers further advantages. Large clutch sizes and quick organ system development lead to efficient experimental analysis. Thus, in order to take advantage of the zebrafish as a model organism, this study characterizes the role of glis3 in comparison to the previously elucidated functions in mice.

Pancreatic development in zebrafish is first detectable at 14 hpf, with a specific population of cells in the endoderm expressing Pdx1, a pancreas specific factor (Kim & Hebrok 2001). Then, in a similar fashion to mammals, two pancreatic buds will form in different locations. The dorsal bud emerges at 24 hpf and contains only endocrine cells (Tiso et al. 2009). The dorsal bud will give rise to the principal islet, which is a collection of endocrine cells in the

mature pancreas. By 35 hpf, the ventral bud emerges from the pancreatic primordia; this bud contains cells of exocrine, ductal, and endocrine lineages (Matsuda 2018). The ventral bud will engulf the dorsal bud by 48 hpf during the rotation of the gut (Field et al. 2003). From this point, the body of the pancreas is formed, allowing for both proliferation and maturation of the various cell lineages. Secondary islets, which contain mature hormone-secreting endocrine cells, differentiate during the secondary transition after fusion. The cells of the secondary islets come from a population of cells in the ventral bud that are negative for Ptf1a but have active Notch signaling (Parsons et al. 2009). The Ptf1a- /Notch+ cells have the capability of becoming either ductal or endocrine cells and are thus bipotent (Figure 2). After the bipotent population is specified, the continuation of active Notch signaling leads to a ductal cell fate while the discontinuation of Notch signaling allows for an endocrine cell fate in the secondary islets (Matsuda 2018). The secondary islets contain both alpha cells, which secrete glucagon, and beta cells, which secrete insulin. While the principal islet is large in comparison to the secondary islets, it is the beta cells of the secondary islets that are responsible for the maintenance of blood glucose homeostasis in the adult organism.

Understanding that the pancreatic beta cell has a paramount role in DM pathology and that Glis3 has been shown to upregulate insulin in the mouse, a CRISPR-mediated glis3 knockout has been developed in the zebrafish. The absence of the gene product and the potential deficits in development have allowed for the characterization of glis3 function in the normal zebrafish embryo. After comparison with the already established role of Glis3 in mice and humans, the conclusions stemming from the glis3 knockout will determine how the zebrafish can be best used to develop solutions for DM.

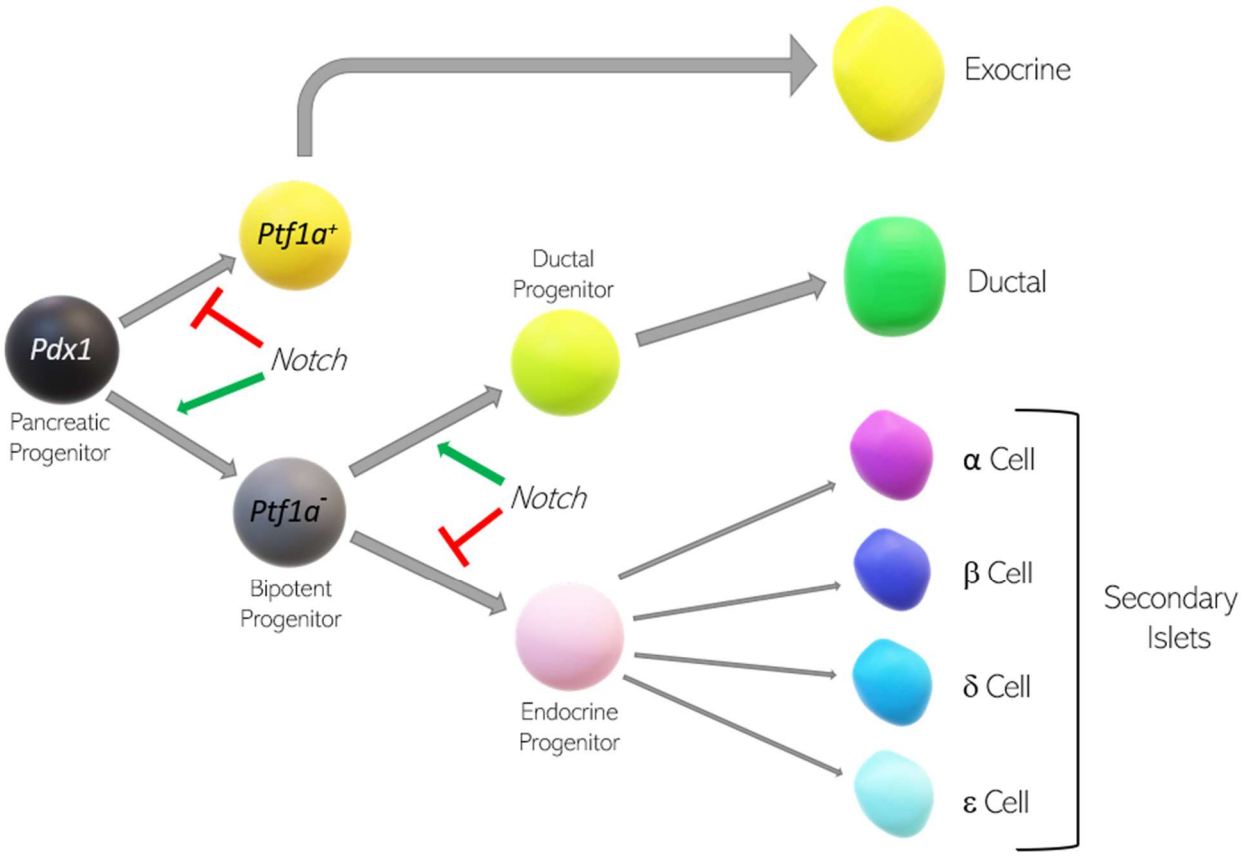

Figure 2. Stepwise specification of pancreatic cells in zebrafish. Pancreatic beta cells form originally from pancreatic progenitor cells that are negative for Ptf1a, an exocrine specific factor. Bipotent progenitor cells form from pancreatic progenitor cells in the presence of active Notch signaling. Then, endocrine progenitors form once Notch signaling is discontinued. The beta cells differentiate from the endocrine progenitor cells.

Methods

Zebrafish Husbandry

Transgenic jh2 zebrafish (Tg(ins:mCherry)jh2) were used for all experiments. These fish contain a transgenic insertion of the mCherry ORF under the control of the insulin proximal promoter and were purchased from the Zebrafish International Resource Center (ZIRC). Fish were maintained in an animal facility containing a 7-rack continuous flow system with 14 h/10 h light/dark cycles controlled through automation. Water quality is maintained daily and kept at 27° C with a pH 7.4-7.8. Zebrafish were fed GEMMA micro 300 daily.

Imaging

Fish were imaged using a Leica MC165FC fluorescence stereo microscope or a Leica DMi8 inverted fluorescence microscope. Images were captured and processed using Leica LAS X software.

Whole Mount in-situ Hybridization

Embryos were fixed at the indicated time points in 4% paraformaldehyde (PFA). The fixed embryos were dehydrated in methanol and stored at -20°C until use. The embryos were serially rehydrated with increasing concentration of phosphate-buffered saline/1% Tween 20 (PBST) and then treated with proteinase K at 20 µg/mL for 20 minutes. The fish were re-fixed in 4% PFA for 20 minutes at room temperature (RT), washed in PBST three times, and incubated in pre-hybe, rinsing twice. The embryos were split into two groups and each group was incubated in hybe at 65°C for 24 hours. After 24 hours, the hybe was replaced with fresh hybe containing glis3 sense or antisense probes at 0.75 ng/µL. The samples were incubated at 65°C for 24 hours.

After 24 hours, the vials were serially washed in saline sodium citrate/Tween 20 (SSCT) with increasing concentrations of PBST. After washes, the embryos were blocked at 4°C for 24 hours in 10% sheep serum containing 0.5% bovine serum albumin (BSA). Lastly, the fish were rinsed with PBST for 10 minutes and sequentially washed with PBST2 (2% Tween 20) in well plates, all at RT. The embryos were treated with 4:1 staining buffer:BM Purple and developed overnight. Once the stain was sufficiently developed, the development was stopped using a PBS rinse, fixing in PFA at 4°C for 1 hour, a final PBS-RNAse rinse and stored in methanol at -20°C. The treated zebrafish were imaged under bright field microscopy.

Chemical Treatment of Embryos

To inhibit pigmentation, embryos were treated with 200 μM phenylthiourea (PTU) in E3 medium at 24 hpf. For the stimulation of precocious secondary islet formation, embryos were maintained in E3 media containing 100 μM of the γ secretase inhibitor tert-Butyl (S)-{(2S)-2-[2-(3,5-difluorophenyl)acetamido]propanamido}phenylacetate (DAPT) from 4 dpf through 7 dpf with daily media changes.

Genotyping

To determine the genotype of the offspring of the fish heterozygous for the glis3 knockout, a genotyping was performed with either tail clippings or head samples depending on the age of the embryo. First, the tissue samples were placed in 50 μL of 50 nM NaOH. Then, the samples were heated at 95°C for ten minutes and allowed to cool to RT. The samples were buffered with 5 μL of 1M Tris HCl (pH=8.0). Once pH was stabilized, the samples were

centrifuged for 3 minutes at 21,000 rcf. The resulting supernatant solution was transferred to a new vessel, completing the extraction of genomic DNA.

To determine whether the sample contained the glis3 knockout, PCR was performed using the extracted genomic DNA. Primers that flank the targeted knockout in exon 4 were used to amplify a region of DNA that is 311 bp in length. A successful glis3 knockout deletes 169 bp from this section, so an amplicon featuring the knockout is 142 bp in length. After amplifying the specified region using PCR, the product was analyzed by gel electrophoresis. The samples were then separated based on the presence of solely the larger amplicon (homozygous wild type), both the larger and smaller amplicon (heterozygous), or only the smaller amplicon (homozygous knockout).

CRISPR/Cas9 Gene Editing

Two single guide RNA (sgRNA) molecules were generated that target exon 4 of the zGlis3 locus by cloning two 20 bp fragments corresponding to the targeted regions into pDR274 (Hwang et al. 2013) using BsaI to generate pDR274-EX3A and pDR274-EX3B. DR274 was a gift from Keith Joung (Addgene plasmid # 42250 ; http://n2t.net/addgene: 42250 ; RRID:Addgene_42250) The resulting constructs were then linearized using DraI and sgRNAs were produced *in vitro* using a MEGAshortscript T7 kit and 75 µM of linearized template. The efficiency of each sgRNA was optimized by cleaving a PCR amplicon encoding the full length 2.8 kb zGlis3 cDNA with a 10:10:1 molar ratio of sgRNA:Cas9:target DNA providing maximal cleavage for both sgRNA.

To mutate the zGlis3 locus in the zebrafish genome, 3.75 µg of TrueCut Cas9 v2 (ThermoFisher Scientific) was combined with 22.5 pmol of each sgRNA and incubated at 37°C

for 10 min to generate ribonucleoprotein complexes. Cas9/sgRNA complexes were then injected into zebrafish embryos at the unicellular stage. Control embryos were prepared following the same injection process but were injected with sgRNA without the Cas9 protein. At 90 dpf, when the zebrafish were large enough to sustain a tissue dissection, a genotyping was performed to determine the efficacy of the injection. The most distal end of the tail was cleaved and digested for genomic DNA as described above.

Whole Mount Immunohistochemistry

Embryos were fixed at the indicated time points in 4% paraformaldehyde (PFA) for 1 h at RT, washed in PBS, and permeabilized in PBS containing 0.5% Triton-X 100 (PBX) for an additional hour at RT. Permeabilized embryos were blocked overnight at 4° C in 0.5% PBX containing 5% sheep serum, 0.5% BSA, and 1% DMSO. After 18 hours, 7 ug/ml of mouse monoclonal anti-glucagon antibody (Sigma Aldrich) was added and the samples were incubated for 48 h at 4° C. Following washes in 0.2% PBX, the embryos were incubated in anti-mouse AlexaFluor 488 secondary antibody for 3 h at RT, washed, and imaged.

Results

Development of a CRISPR-mediated glis3 knockout line

To better characterize the function of glis3 in the zebrafish embryo during normal pancreatic development, a glis3 knockout model was engineered using the CRISPR/Cas9 gene editing technique. Adapting the bacterial mechanism for the present purpose, two guide RNA molecules were designed with the ability to anneal at sites targeted for excision. The guide RNA molecules were allowed to complex with the Cas9 protein, which contains endonuclease activity. Together, the sgRNA/Cas9 complexes were guided via base-pairing specificity to two predetermined locations marked for cutting within the exon 4 region of the glis3 gene (Figure 3). The technique excised a 169 bp region in exon 4, which introduced an early stop codon in the processed mRNA transcript. Thus, when the gene is transcribed and translated, the protein product lacks both a functional DNA-binding domain and a transactivation domain, rendering the product nonfunctional.

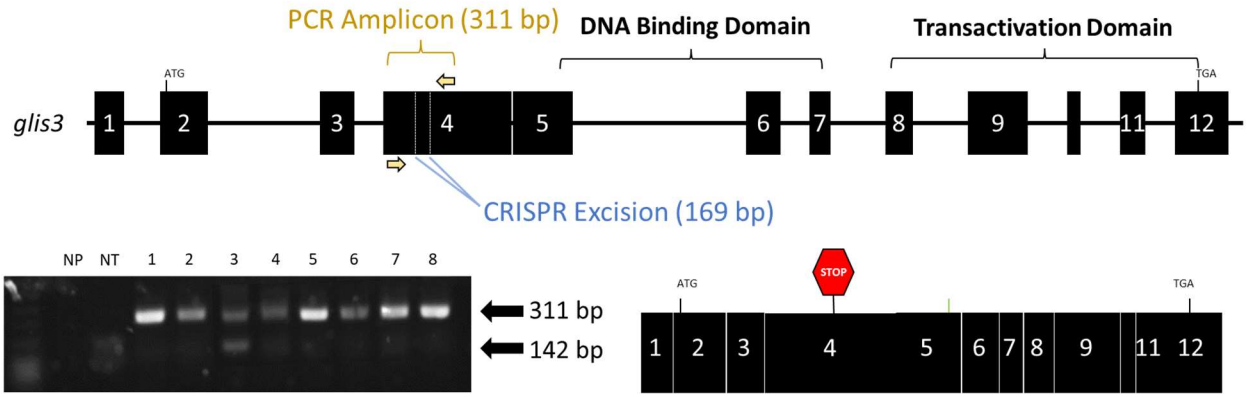

Figure 3. CRISPR-mediated knockout of glis3 in zebrafish. The 169 bp excision introduced a stop codon in exon 4 of the processed mRNA transcript (right), leading to the production of a nonfunctional protein. An agarose gel was used to validate the knockout (left), which was identified by the presence of a shorter amplicon that lacked the excised genetic material.

A prominent phenotype associated with the glis3 knockout is polycystic kidney disease, which has been previously observed in humans and in Glis3 knockout mice (Senee et al 2006; Kang et al. 2009). The founder zebrafish of the glis3 knockout line demonstrated the same cystic kidneys, which were observable by 30 dpf. The founder had a lifespan of six months with cause of death still unspecified. The cysts most commonly appeared posterior to the pectoral fins, but in some cases will extend anteriorly toward the head (Figure 4).

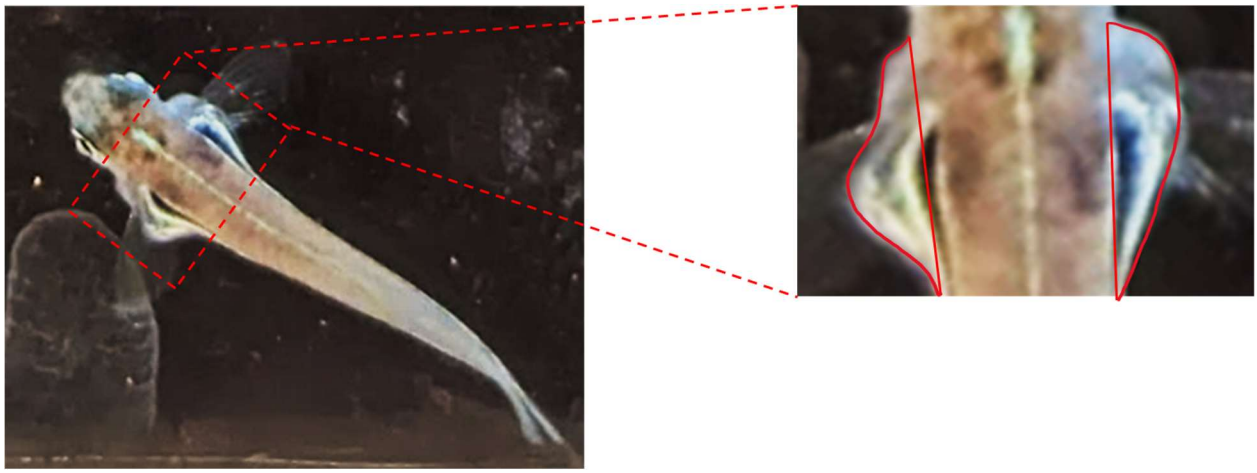

Figure 4. Polycystic kidney disease (PKD) phenotype, outlined in red, shown in a glis3 homozygous knockout fish. This image was captured at 90 dpf while performing a genotyping on reproductively mature offspring of the heterozygous zebrafish. The polycystic kidneys (right) can be either bilateral or unilateral.

The glis3 knockout was also investigated to determine whether the inheritance pattern was Mendelian. After genotyping multiple clutches of offspring from the heterozygotes, the frequency of the homozygous knockout was calculated to be .24; the homozygous wild types and heterozygotes presented with frequencies of .26 and .50 respectively (n=182). Since a normal heterozygous cross gives a 1:2:1 ratio, the glis3 knockout gene demonstrates a Mendelian pattern of inheritance. A body mass index was determined by calculating a ratio of length (cm) to weight (g). The resulting index described whether growth was affected by the glis3 deficiency. The

knockout fish displayed a mean body mass index of 23.2±7.7 cm/g; n=6. The homozygous wild type fish had a mean body mass index of 18.11±5.9 cm/g; n=5. The body mass indices of the wild type and homozygous knockout fish were not significantly different ($p<0.72$). Overall, the body mass index suggests that the glis3 knockout fish are not affected with respect to overall body mass development during the first 90 dpf.

Principal islet development in glis3 knockout fish

The offspring of the zebrafish were analyzed via fluorescent microscopy to determine whether the development of the endocrine pancreas was affected by the knockout of glis3 (Figure 5a,b). The CRISPR-mediated knockout was developed in jh2TG zebrafish. In this construct, the mCherry open reading frame is driven by the *insa* proximal promoter, so red fluorescence is produced wherever insulin is expressed, which allows for the specific visualization of pancreatic beta cells. Before microscopic imaging was performed, the fish were euthanized and set aside for genotyping.

The fluorescence imaging revealed that the development of the principal islet is largely unaffected by the absence of glis3. There was not a significant difference in either the intensity or the morphology of the red signal between the knockout and wild type genotypes (Figure 5c). To ensure that the results were representative, three rounds of fluorescence imaging were performed on three different clutches of embryos. Though the collection of wild type embryos contains the largest and most intense signal for beta cells within the principal islet, the variance between this signal and the knockout signals is not consistently replicable, nor is it outside the realm of normal variance in zebrafish development.

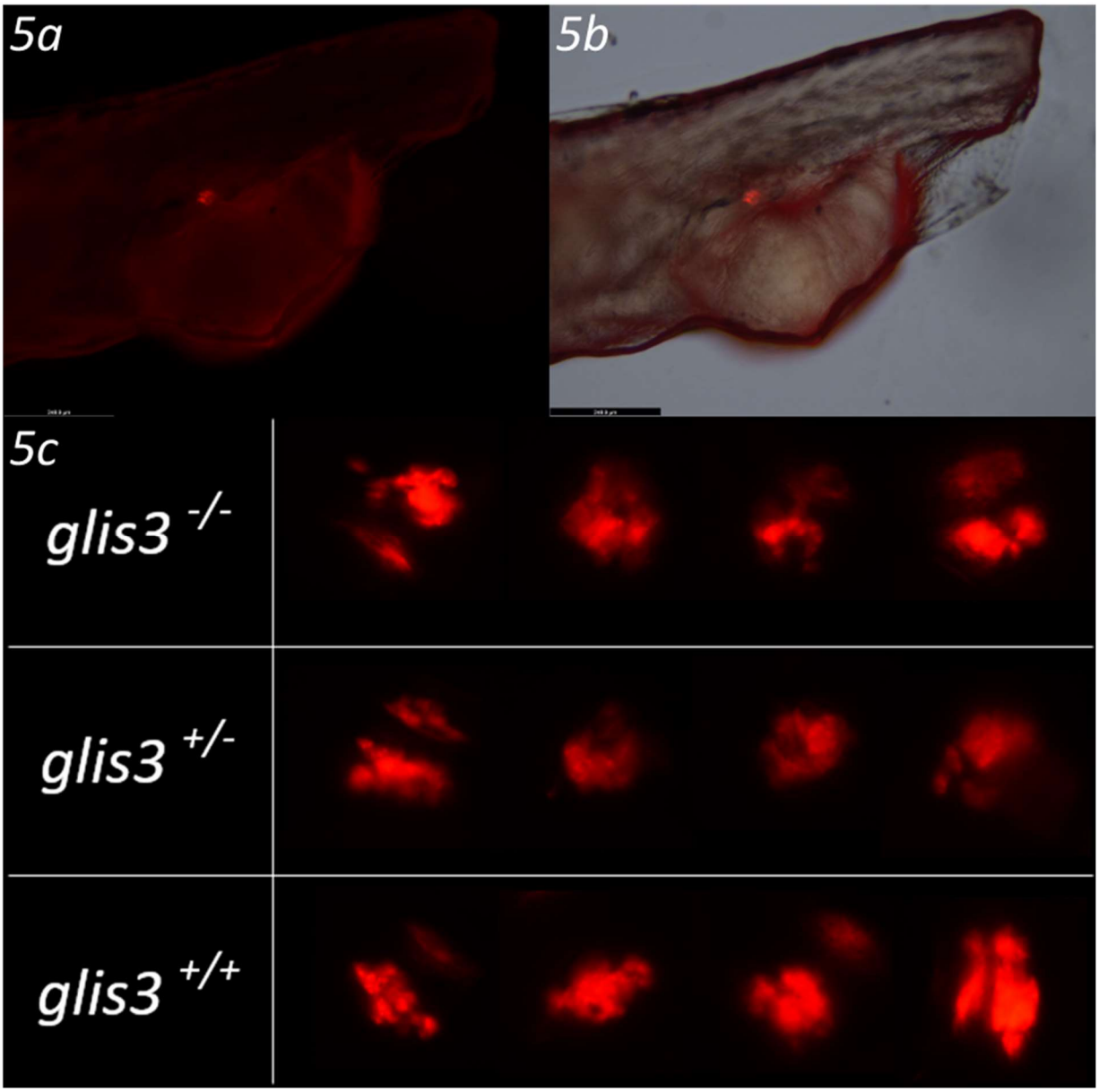

Figure 5. Fluorescent imaging of the principal islet. The observed red signal originates from a red fluorescent protein whose production is driven by the insa proximal promoter. The bright field and fluorescent images (5a & 5b) demonstrate the location of the principal islet signal relative to the rest of the organism. To compare signals more efficiently, the principal islet signals were isolated and grouped according to the corresponding genotype (5c).

Development of secondary islets in glis3 knockout fish

The development of the secondary islets is especially important because these groups of endocrine cells are responsible for regulating blood glucose homeostasis in the adult zebrafish. During normal pancreagenesis, the secondary islets form during the secondary transition. The secondary transition is a period of differentiation of all three cell types in the pancreas, including satellite populations of endocrine cells termed the secondary islets. The transition coincides with the onset of feeding, which features a switch from dependence on the yolk for nutrition to external food sources. Also, for endocrine progenitor cells to form from bipotent progenitor cells, the previously active notch signaling must be discontinued, lest a ductal progenitor cell form (Figure 2).

To stimulate the early formation of secondary islets, two strategies were employed. First, a clutch of embryos was treated with DAPT from 4 to 7 dpf. DAPT is a potent inhibitor of notch signaling that helps to specify bipotent progenitor cells as endocrine progenitor cells. The premature appearance of suitable populations of endocrine progenitor cells caused secondary islets to form earlier than normal. In addition to the DAPT treatment, the zebrafish were fed twice during the same three-day period. The methodology demonstrates a synergistic stimulation of secondary islets via the inhibition of notch signaling in combination with the onset of feeding.

At 7 dpf, the fish were euthanized, genotyped, and prepared for analysis via immunohistochemistry (IHC). The immunohistochemical analysis featured the usage of an antibody for glucagon, allowing for the simultaneous visualization of both the alpha and beta cells of the endocrine pancreas. In this way, the secondary islets were identified via the colocalization of the red and green fluorescent signals (Figure 6).

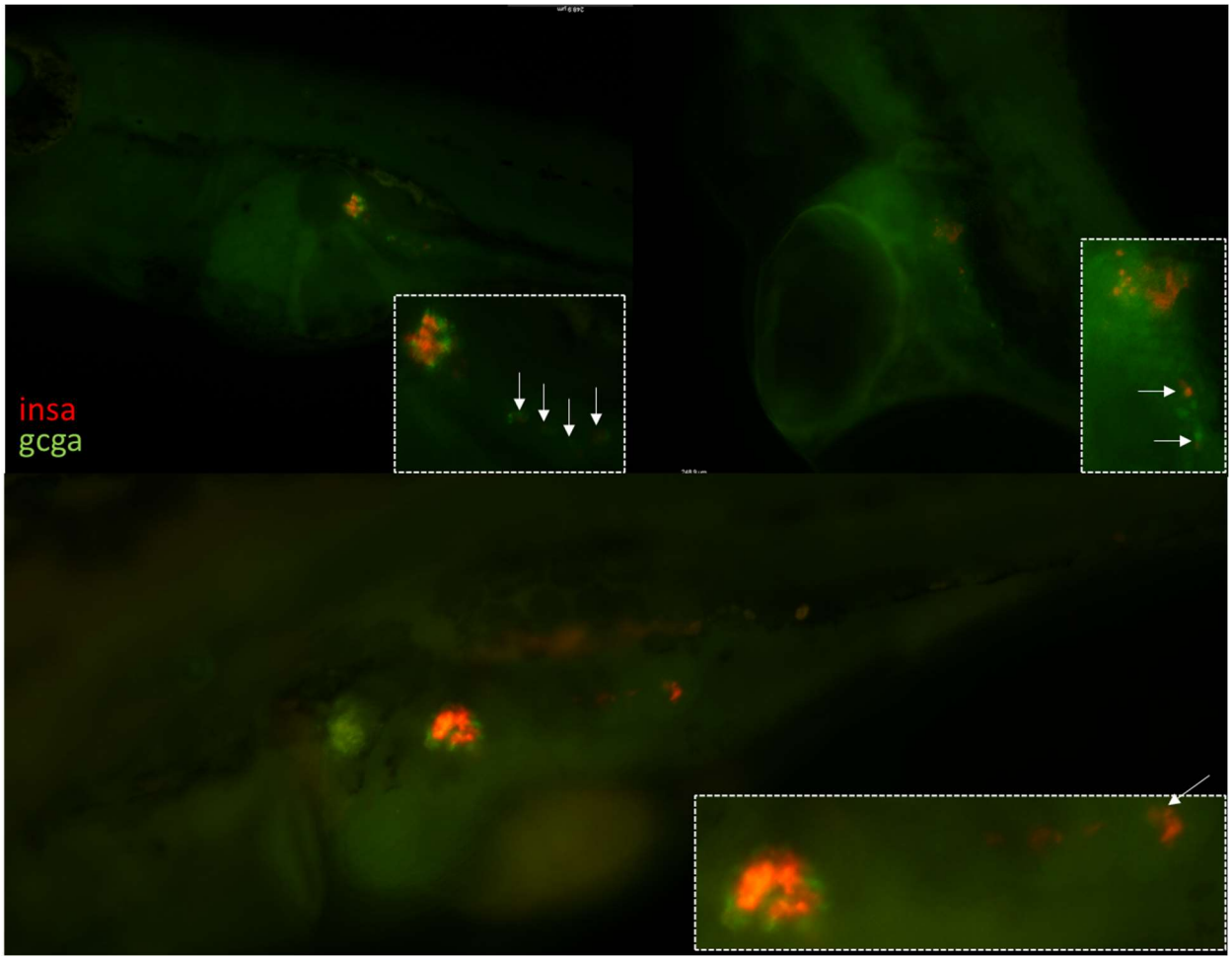

Figure 6. Fluorescent imaging of the secondary islets with immunohistochemistry. The red signal denotes insulin produced in the beta cells while the green signal marks glucagon produced in the alpha cells of the endocrine pancreas. The white arrows highlight the secondary islets, which form as satellite populations of endocrine cells along the gut tube away from the principal islet. The colocalization of the red and green signals helps to identify the comparatively small secondary islets. The above pancreatic signals correspond to fish with at least one functioning copy of Glis3.

Regrettably, the protocol for the immunohistochemical analysis required a troubleshooting process, which prevented the analysis of all the possible genotypes. Consequently, only homozygous wild type and heterozygous fish were analyzed via this method. In the future, homozygous glis3 knockout fish will be imaged using the colocalization technique

to determine whether secondary islets form properly in the absence of the functional Glis3 protein. The fluorescence imaging following the IHC was able to accurately identify the presence of secondary islets as evidenced by the small cell populations showing both glucagon and insulin expression.

Discussion

When Glis3 was knocked out in mice, the offspring developed polycystic kidney disease (Kang et al. 2009). Likewise, loss of glis3 in zebrafish also resulted in offspring that developed PKD between 7 and 30 dpf. Interestingly, genotyping analysis identified fish that lack glis3 expression but show no obvious signs of polycystic kidneys; accordingly, the PKD phenotype may display incomplete penetrance. Histological analysis will be performed to determine whether PKD is evident in these individuals. The mechanism by which loss of glis3 contributes to PKD is not known. Given the abnormal development of the kidneys, it is possible that the glis3 deficiency is affecting cell polarity in the context of the proximal tubules and collecting duct. Further analysis is required to elucidate how glis3 may modulate other factors that play a role in cell polarity and attachment in the kidney.

When compared with fish with functional copies of glis3, the knockout fish did not display any significant differences in body mass index. As a result, glucose utilization may remain unaffected in the knockout animals. If the beta cells were compromised either in function or number, the resulting blood glucose dysregulation would most likely affect the overall growth of the fish. Logically, the fish would display a decreased body mass index due to the inability of the peripheral tissues to properly utilize glucose as an energy source for tissue growth. However, the lack of significant difference between the body mass indices of the three genotypes suggests that beta cell function remains unaffected in the absence of glis3 expression.

Further supporting the finding that glis3 expression does not affect the initial function of the beta cell is the imaging of the principal islet. Analysis of the fluorescent imaging between the various genotypes shows that there is no difference in the pancreatic signals with respect to intensity or morphology. The seemingly unaffected presence of beta cells in the knockout shows

that a glis3 deficiency does not affect the prevalence or the function of insulin-producing cells in the large principal islet. Since factors like Neurod1 and Pdx1 are commonly expressed in cells of the principal islet, the images further suggest that glis3 is not heavily involved in the regulation of these two genes. To confirm without ambiguity whether these factors are affected by a glis3 knockout, RT-PCR will be employed to quantify the expression of these factors in the absence of glis3 expression. Since the main function of glis3 is that of a transcription factor, RNA-sequencing will also be performed using knockout embryos to identify target genes for glis3 regulation.

While the formation of the principal islet is necessary for proper pancreatic development, the secondary islets are also important because of their role in regulating blood glucose homeostasis in the adult organism. The secondary islets are small satellite populations and thus require a different visualization technique for proper identification and characterization. In addition to the red fluorescent signal for insulin, the secondary islets were described using the green fluorescent signal conferred by glucagon expression. Glucagon, which is produced in the alpha cells of the endocrine pancreas, was marked using immunohistochemistry and an antibody that binds specifically to glucagon. The colocalization of glucagon and insulin provides a more accurate technique for the description of smaller populations of endocrine cells. Due to time constraints, this technique was not applied to knockout embryos. Once employed with knockout embryos, this protocol will determine if glis3 has a differential role in the secondary islets in comparison with the principal islet. Given the distinct purposes of the two kinds of islets in the mature individual, it is possible that the respective specification and maturation mechanisms involve different regulators. It remains possible that glis3 may not adversely affect the specification of the islet but may be required for its expansion in response to nutrient excess.

Challenging glis3 knockout fish to a high fat diet (HFD) may provide insight into whether loss of glis3 expression increases susceptibility to T2DM.

Conclusion

When glis3 was knocked out in zebrafish embryos, polycystic kidney disease (PKD) was observed in individuals lacking any functional copy of the transcription factor. The normal body mass index of the homozygous knockout fish and the unchanged red fluorescent beta cell signals both suggest that glis3 does not play an important role in the development of the principal islet of the pancreas. Further analysis of the glis3 knockout in zebrafish will serve a myriad of purposes, chief among them being the role of glis3 in the maturation and responsiveness of the secondary islets. This role will be elucidated using immunohistochemistry along with insulin fluorescence. RNA sequencing and RT-PCR analysis of the glis3 knockout fish will direct further investigation as to which factors glis3 regulates and in which regions this regulation heavily influences development. This continued characterization will also contribute to the comparison of the roles of Glis3 in mice, zebrafish, and humans. Any further discovery regarding the function of this gene will inform the use of zebrafish as both a model for development and pathology with respect to humans.

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