ROLE OF ITGB1B IN ZEBRAFISH ISLET DEVELOPMENT AND FUNCTION

MASTER THESIS

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ABSTRACT

Multiple efforts to find a long-term treatment for Diabetes mellitus, such as transplantation of whole islets or β cells derived from pluripotent stem cells to restore β cell mass, failed to achieve the desired results. Particularly, the reduced longevity and function of the grafted β cells made it more difficult. This resulted in the investigation of the islets complex microenvironment. One important factor is the extracellular matrix (ECM) which provides a three-dimensional scaffold and contributes to the β cell proliferation, survival and function. This study aimed to investigate the islet ECM in the zebrafish model organism. Therefore a itgb1b−/− mutant was used to examine the importance of β1 integrins in the islet.

The glucose amount of the itgb1b deficient embryos was elevated and changed during development, indicating a dysregulated energy homeostasis. First investigations revealed that this defect was not caused by any histological or structural changes of the β, α and δ cells of the islet. Although the expression data of itgb1b suggest an involvement in blood vessel development, the results showed that the islet vessels were not affected in itgb1b−/− mutants. As the β cell activity was also not changed in the mutant animals the question for the root of the dysregulated glucose levels was still open. Upon investigation of the islet hormone expression it was found that somatostatin and glucagon were elevated in the itgb1b deficient animals. This led to the development of two, rather synergetic theories to explain the higher glucose levels in mutant embryos. First, stress is known to activate somatostatin and glucagon levels in mice. As the itgb1b−/− mutants lose their blood circulation and their tissue sort of collapses, one could argue that the embryos are in a very stressful situation. Through somatostatin, insulin secretion on fasting conditions could be impaired, accompanied by increased glucagon levels this could lead to a higher glucose level. Second, the loss of blood flow causes a deficient in nutrient delivery from the yolk. The mutant embryos therefore need to start their gluconeogenetic program earlier leading to a high production of glucose. But in the end it is hard to determine if these effects were caused by any defects in the islet or were just secondary effects of the systematic loss of itgb1b.

Even if this study couldn’t show the role of β1 integrins, the structure of the islet ECM in zebrafish embryos could be described. Further, the similarity between adult endocrinal vesicles and embryonic ones could be shown on an ultrastructural level. Future investigations of the vertebrate β1 integrin homologues itgb1a and itgb1b, could help to clear the picture. Understanding the ECM-islet interactions could produce a powerful tool to enhance the success of long-term treatment approaches for Diabetes.
1. INTRODUCTION

Loss or dysfunction of insulin producing β-cells results in chronic hyperglycemia and leads to a group of metabolic disorders called Diabetes mellitus. There are two main types: type 1 Diabetes (T1DM), caused by an autoimmune destruction of β-cells, and the more common type 2 Diabetes (T2DM), which develops through insulin secretory defects and insulin resistance. The chronic hyperglycemia can lead to damage, dysfunction or failure of different organs, resulting in e.g. blindness, kidney failure, nerve damage, stroke and heart attack. As of 2019 reportedly 463 million people suffer from Diabetes worldwide and according to World Health Organization (WHO), Diabetes was the seventh leading cause of death in 2016 with 1.6 million deaths. The only effective treatment for T1DM and late stage T2DM are daily insulin injections. Ongoing studies desire to develop approaches to transplant whole islets or β cells derived from pluripotent stem cells to restore β cell mass. However, these strategies showed only short lasting results due to reduced longevity and function of the grafted β cells, which need a complex microenvironment to survive. Within the pancreatic islet, β cells interact with other endocrinal cells, endothelial cells, neuronal projections and the extracellular matrix (ECM). The latter not only provides a three-dimensional scaffold to support the islet cells, but its components further contribute to β cell proliferation and survival. Understanding the interactions of the ECM components and islet cells could be a powerful tool to enhance the success of the new approaches to treat Diabetes.

1.1 Diabetes research in zebrafish

Although the first use of zebrafish (Danio rerio) in research dates back to the 1930s its popularity only started to rise with the big genetic screening of zebrafish in 1993. The easy hold, small size, fecundity, rapid development, transparency and ease of manipulation made the zebrafish a very attractive vertebrate development model. Due to the high degree of conservation in genetics, development and physiology between the zebrafish and humans, it further started to become a potent model organism for human diseases, such as diabetes. Both, pancreas morphogenesis and cellular architecture in zebrafish are similar to the mammalian pancreas. Additionally, the development and function of organ systems involved in glucose homeostasis (brain, liver, adipocyte tissues, skeletal muscle) are also conserved. The use of different transgenes expressed under specific promoters e.g. insulin, gives the amazing opportunity to follow the β cell fate in vivo. In combination with the ease of genetic manipulation, this renders the zebrafish a perfect vertebrate model to study Diabetes.
1.1 Pancreas development in zebrafish is highly conserved

Among vertebrates the pancreas development is very similar. At around 16 hours post fertilization (hpf) pancreas formation in zebrafish starts with the induction of pancreas specific transcription factors pdx1 and mnx1 in a few cells, in a right and left longitudinal row ventrally to the notochord\textsuperscript{12,13}. Those cells arrange to a single cell cluster termed the dorsal bud, visible at around 24 hpf\textsuperscript{14}. At around 34 hpf a second cluster, the ventral bud, starts to emerge from the gut and moves in direction of the dorsal bud, finally surrounding it (Fig. 1)\textsuperscript{15}. In contrast to mammals the dorsal bud of zebrafish gives only rise to endocrinal cells while the ventral bud forms all pancreatic tissues, including the entire exocrine part and the duct system\textsuperscript{15,16}. The ventral bud also gives rise to endocrine cells which contribute to the primary islet expansion and to all the secondary islets forming after embryogenesis\textsuperscript{15}.

1.2 Zebrafish as a Diabetes model

To establish a model of T1DM in zebrafish methods like pancreatectomy or inducible β cell ablation were used\textsuperscript{17}. For the latter, transgenic zebrafish lines with β cell specific expression of the bacterial nitroreductase (NTR) enzyme are exposed to metrodinazole. This NTR substrate is converted into a cytotoxic compound leading to β cell apoptosis\textsuperscript{18}. Due to the remarkable regenerative capacity of the zebrafish, however the β cell mass is restored once the metrodinazole is removed\textsuperscript{18}. For permanent β cell removal another transgenic model uses the expression of the cell-lethal diphtheria toxin α-chain under the control of the insulin promoter to completely ablate β cells. However, these fish display a severe growth retardation and fail to thrive due to the importance of insulin in stimulating growth\textsuperscript{19}. Although there is no robust model for T1DM in zebrafish, it is an attractive model to study the regenerative capacity of β cells\textsuperscript{19}.

![Fig. 1: Pancreas development in zebrafish. The dorsal bud emerges from the endoderm during early somite stages, around 24 hpf. The ventral bud on the other hand arises from the gut and moved to the dorsal bud to form the exocrine tissue surrounding the endocrinal part. Figure from Robin A. Kimmel, Dirk Meyer 2010\textsuperscript{124}](image-url)
For the generation of T2DM zebrafish models genetic and nutritional methods have been used. The latter includes either immersion in glucose solution or a high-fat diet, which is a major risk factor for T2DM. Overfeeding zebrafish quickly leads to insulin resistance, elevated fasting blood glucose and impaired glucose tolerance. However, returning to normal feeding can reverse the phenotype, indicating that the method is insufficient to lead to a dysfunction of glucose homeostasis. A different method induces insulin resistance in skeletal muscle by using dominant-negative IGF-1 receptor expression. Although it leads to insulin resistance in aged fish, it only affects fasting blood glucose in combination with overnutrition. A liver specific knockdown of the insulin receptor leads to a postprandial hyperglycemia but a fasting hypoglycemia, which can also be seen in mice and human liver insulin resistance. Due to slow progression of insulin resistance to glucose intolerance during age, these models can be helpful to find cooperating genes or metabolic conditions which accelerate the development of diabetes. There are several zebrafish models for another type of diabetes: maturity-onset diabetes of the young (MODY). This rare monogenic, autosomal dominant form is caused by a β cell dysfunction with the onset before 25 years. There are different forms of MODY caused by mutations in different genes of the glucose metabolism. The zebrafish hnf1ba mutant displays pancreas hypoplasia and reduced β cell numbers similar to MODY5. In neurod1 deficient zebrafish endocrine cell differentiation fails leading to increased free glucose levels in larvae, resembling MODY6. Mutations in the zebrafish pdx1 gene, like in MODY4, result in reduced β cell numbers, disrupted glucose homeostasis and sensitivity to over-nutrition. These MODY models display similar phenotypes observed in human patients and can be useful in vivo models to find new forms of therapy.

1.3 Pancreas cytoarchitecture

The pancreas is composed of an exocrine part, producing digestion enzymes, and an endocrine part organized in so called islets of Langerhans that are scattered throughout the exocrine tissue and make up only 1-2% of the organ. In mammals, reptiles and birds, islets are composed of five cell types: insulin (ins) expressing β cells, glucagon (gcg) expressing α cells, somatostatin (sst) expressing δ cells, ghrelin expressing ε cells and pancreatic polypeptide expressing PP cells. Regarding the cytoarchitecture of the islet, there are some notable differences between species. In mice and other rodents, the islet core is mainly formed by β cells, while the α cells build the most outer layer creating a mantle-like structure. Most of δ cells are located in the periphery, contributing to the mantle, or more internally and only a few are located in between. In contrast, the endocrinal cells of human and monkey islets are more dispersed, with β cells laying also in the periphery and α cells also in the center. It is suggested that
this islet structure allows the β-cells to respond also to low concentrations of glucose providing an advantage for primates. Islets in dogs and pigs display an intermediate cytoarchitecture, with some β cells also located in the periphery and some α cells occasionally occurring in the center. In zebrafish there is a slightly different organization of endocrine cells into one big islet, the principal islet, and smaller secondary islets which emerge later in development. Some scientists use the term Brockmann body as a synonym to the primary islet, although it defines more as the largest accumulation of islet tissue. However, if the principal islet is the largest accumulation, the terms can be used as synonyms. The zebrafish islet shows a similar architecture than mice islets, with β cells located in the center, α cells in the periphery and δ cells intermingled with the β cells in the core, in the intermediate space and also in the periphery (Fig. 2). While there are a few ghrelin expressing ε cells, it is not clear if PP-cells exist in the zebrafish islet. 

1.2 Regulation of blood Glucose levels by Insulin and Glucagon

The blood glucose level is regulated tightly through a network of hormones and neuropeptides released from the brain, liver, adipose and muscle tissue, intestine and pancreas. The ladder produces hormones with key roles in this regulation: insulin and glucagon. After a meal, when blood glucose levels are high, insulin is secreted from β cells. Upon binding to its receptors on muscle and adipose cells, insulin enables the uptake of glucose in these tissues, lowering blood glucose levels. On the other hand when the glucose level is low in between meals or during sleep, glucagon is secreted by α cells to promote

![Diagram of zebrafish islet structure](image)

**Fig. 2:** Islet structure in zebrafish. The primary islet of zebrafish is very similar to mice islets. The β cells are located in the center, surrounded by the α cells and δ cells intermingled with a further appearance in the periphery and in the core. The very few ε cells are located in the periphery. Figure modified from Mary D. Kinkel, Victoria E. Prince 2010.
hepatic and renal gluconeogenesis. Both, α and β cells are further regulated by paracrine actions of somatostatin and pancreatic polypeptide. This way insulin and glucagon balance the blood glucose level to keep glucose homeostasis. This kind of regulation requires the ability of α and β cells to somehow sense the blood glucose level. The molecular basis of glucose sensing in β cells of mammals is relatively well understood and starts with the uptake of exogenous glucose through the glucose transporter type 2 (GLUT2). The subsequent metabolism of glucose through glycolysis increases the intracellular ATP/ADP ratio leading to the closure of ATP-sensitive potassium channels ($K_{ATP}$). The resulting membrane depolarization opens the voltage-gated Ca$^{2+}$ channels leading to an increase of intracellular Ca$^{2+}$ finally triggering the secretion of insulin from stored granules (Fig. 3). While β cells are electrically inactive at basal glucose levels, α cells are electrically active resulting in the closure of most of its $K_{ATP}$ channels. This leads to the depolarization of the membrane potential and the opening of voltage gated Ca$^{2+}$ channels, resulting in the secretion of glucagon. Upon elevation of the blood glucose level more $K_{ATP}$ channels are closing leading to the inactivation of voltage gated Ca$^{2+}$ channels. Additionally, paracrine factors from β and δ cells are inhibiting the glucagon secretion.

![Figure 3: Ca$^{2+}$ dynamics in insulin secretion. Glucose enters the β cells through GLUT2 and is metabolized via glycolysis increasing the intracellular ATP/ADP ratio. Subsequently the $K_{ATP}$ channels close resulting in membrane depolarization leading to the opening of voltage-gated Ca$^{2+}$ channels. The following increase of intracellular Ca$^{2+}$ finally triggers the secretion of insulin from stored granules. Figure modified from Sergio Polakof et al. 2011.](image-url)
Recently it was shown that the molecular basis behind glucose sensing in adult and embryonic zebrafish β cells is very similar to mammals\textsuperscript{46,47}. This was demonstrated with the use of a transgenic line with insulin specific expression of the Ca\textsuperscript{2+}-sensitive dye GCaMP6s. Elevation of intracellular Ca\textsuperscript{2+} leads to a rapid increase in fluorescence of the dye, making it possible to observe the Ca\textsuperscript{2+} dynamics in β cells in vivo, adding another powerful tool to the box\textsuperscript{47}.

The glucose regulation in zebrafish changes during the first embryonic days. Upon exhaustion of the yolk-derived carbohydrates, usually between 4 and 5 days post fertilization (dpf), the zebrafish intents to achieve its glucose demand by starting the gluconeogenetic program and initiating the “feeding-to-fasting” switch\textsuperscript{48}. On low dietary carbohydrates pck1 transcription gets induced, producing the phosphoenolpyruvate carboxykinase (Fig. 4). The latter mediates the conversion of oxaloacetate to phosphoenolpyruvate and carbon dioxide, an important step in the gluconeogenesis. Pck1 is usually controlled by insulin but under fasting conditions it is controlled by glucagon, glucocorticoids and adrenaline\textsuperscript{49,50}.

1.4 Islet vascularization: The role beyond oxygen supply

Due to the endocrinal function of the cells, the islet is highly vascularized. The intra-islet capillaries display a fenestrated ultrastructure, allowing a close interaction with endocrinal cells and hence a rapid response to changing blood glucose levels\textsuperscript{6}. Additionally, the islet capillaries are thicker, denser and very tortuous\textsuperscript{51,52}. Blood vessels play a crucial role in pancreatic differentiation. Studies in mice showed that the embryonic aortic endothelial cells, which are in direct contact with the dorsal pancreatic bud, are able to induce and maintain the expression of the pancreatic transcription factor Pdx1 and Ptf1a\textsuperscript{53,54}. Disruption of those signals impairs

![Fig. 4: Fasting to feeding transition in zebrafish. Exhaustion of the yolk in zebrafish embryos leads to the start of the gluconeogenetic program, leading to higher transcription of pck1. Between 3 and 4,5 the glucose amount in the fish rises and then falls if the fish is not fed. modified from Gut et al\textsuperscript{48}](image-url)
pancreas development\textsuperscript{53}. The early pancreatic cells on the other hand, are producing vascular endothelial growth factor A (VEGF-A), leading to endothelial migration and proliferation\textsuperscript{55}. The recruited blood vessels provide further signals to regulate pancreas branching and differentiation\textsuperscript{56}. Loss of VEGF-A signaling in early β cells of mice results in defects in β cell proliferation and insulin secretion, leading to a disrupted glucose homeostasis\textsuperscript{55,57}. Overexpressing VEGF-A signaling in those newly formed β cells, however leads to hypervascularization, a disrupted islet formation and a reduction of β cells\textsuperscript{53,58}. VEGF-A signaling is not only critical during development, but further plays a role in normal β cell function in the adult pancreas. Reduction of VEGF-A expression in adult mouse β cells leads to a 10-fold decline in islet Vessels, a decreased islet innervation, reduced glucose tolerance and impaired insulin secretion\textsuperscript{59–61}. Conversely, overexpression of VEGF-A results in an increased islet vascularization and β cell loss in adult mice\textsuperscript{62}. These findings suggest that VEGF-A signaling underlies a critical range of action in the developing as well as in the adult pancreatic islets.

In zebrafish two homologues of VEGF-A exist, Vegfaa and Vegfab\textsuperscript{63}. Recently, it was shown that a double knockdown of vegfaa and vegfab results in a reduced islet vessel density during primary and secondary islet development\textsuperscript{64}. Ablation of Vegfaa/Vegfab expressing β-cells leads to a reduction of islet vessels and a revascularization upon regeneration of β cells\textsuperscript{64}. Blocking of VegfA signaling in the developed pancreas, shows a similar reduction in islet vessels, suggesting a continuous role to sustain islet vasculature\textsuperscript{64}. Zebrafish with reduced islet vasculature, display a decreased insulin mRNA expression, suggesting a similar role for VegfA signaling in insulin expression as in mice\textsuperscript{64,65}. However, no reduction of the β or α cells, was observed, indicating that both, Vegfaa and Vegfab, are crucial for islet vascularization but not required for β cell development\textsuperscript{64}. These results differ from the findings in mice, where VEGF-A inactivation leads to a decreased β cell proliferation in postnatal stages\textsuperscript{55,64}. These differences likely occur due to the fact that the main mechanism of embryonic β cell formation in zebrafish is neogenesis and not proliferation\textsuperscript{64,66,67}. Additionally, oxygen accessibility may contribute to the different results between the species. Mice relay on a functioning cardiovascular system to provide the tissues with oxygen. A hypoxic environment during murine endocrine cell differentiation, leads to a blunted endocrine differentiation\textsuperscript{64,68}. Due to the ex-utero development and their small size, zebrafish do not need a functioning oxygen transport for the first days of development, hence they do not experience hypoxia in an avascular environment\textsuperscript{64,69}. However it was demonstrated that the islet vascularization is important for the delivery of the optimal inductive glucose concentration for the maturation of β cells in
The gradually increased glucose concentration triggers the embryonic β cells to acquire and enhance their function through the activation of calcineurin/NFAT signaling. This mechanism was shown to be also conserved in mouse β cells maturation.

1.5 The extracellular matrix (ECM) of the islet: structure and composition

Both, human and mouse islets are surrounded by a peri-islet basement membrane (BM), which is a specialized ECM, that provides not only tissue integrity but also molecular signals that control cellular processes. This outer membrane separates the islet from the exocrine part of the pancreas. The endocrine cells are not able to form their own BM. Instead, they recruit endothelial cells through VEGF-A signaling, which then form a BM adjacent to β cells. Therefore, in mice only a vascular BM can be found within the mouse islet (Fig. 5). Contrary, in human islets the peri-islet BM coinvaginates with the islet blood vessels. This leads to the formation of a unique double BM, consisting of an inner vascular BM around the endothelial cells and an outer peri-islet BM (Fig. 5). Further, architectonic differences of the islet between mouse and human allow different interactions with the BM. In mice, manly β cells form the islet core, while the most outer layer is predominantly composed of α cells creating a mantle-like structure. As a result primarily the peripheral α cells are in contact with the peri-islet BM, while the β cells in the core are only in contact with the vascular BM. In contrast, the endocrinial cells in human islets are dispersed, with β cells laying also in the periphery. This allows all endocrinial cells, including β-cells to interact with the peri-islet BM. Regarding the zebrafish islet, there is currently no information on the ECM structure or composition.

Fig. 5: The ECM of the islet. Both human and mouse islets are surrounded by a per-islet BM (black). The endothelial cells don’t make their own BM, instead the endothelial cells form a BM adjacent to β cells. Contrary, in human the peri-islet BM coinvaginates with the blood vessels forming a unique double BM consisting of an inner vascular BM and an outer peri-islet BM. β cells are depicted in green, α cells in pink and δ cells in blue. Figure from Shannon E. Townsend, Maureen Gannon 2019.
The BM of mice and humans is composed of multiple ECM proteins, the most prominent being collagen, fibronectin, heparan sulfate and laminins.

1.5.1 Collagen
Collagens build a family of large triple helical proteins that are not only important for structural cell integrity but further play a role in a variety of cellular processes, such as differentiation, proliferation, migration and morphogenesis\textsuperscript{74,75}. There are 28 types of collagen known, about which type I, II, III, IV and V are the most common\textsuperscript{74}. In the islet Col-IV and Col-VI make up part of both, the peri-islet and vascular BM\textsuperscript{76,77}. While Col-IV is crucial for BM morphology and embryonic development, it was also shown to increase survival in cultured immortalized mouse and rat β cell lines and cultured isolated primary rat islets\textsuperscript{78–80}. The less characterized Col-VI is implicated with regulation of cell differentiation and autophagy in different tissues and was shown to be cytoprotective\textsuperscript{81}. Regarding the β cells, Col-VI was shown to enhance viability and increase oxygen consumption in vitro\textsuperscript{82}.

1.5.2 Fibronectin
Fibronectin is a heterodimeric glycoprotein dimer which is critical for cell adhesion, differentiation, growth and migration and thus indispensable for embryonic development\textsuperscript{83}. Although there is only one fibronectin gene, the final protein can show up to 20 known variants in humans. This variation arises due to alternative splicing of a single pre-mRNA\textsuperscript{83,84}. Fibronectin can not only be in contact with cells but are also able to bind to other ECM components such as collagen, gelatin, heparin and fibrin\textsuperscript{83}. Little is known about the role of fibronectin for the survival of β cells. Because fibronectin contains multiple protein-binding domains for growth factors like FGF and VEGF, it could be involved in the regulation of accessibility of mitogens and viability factors to β cells\textsuperscript{85}. Further, fibronectin was shown to lower apoptosis of β cells in vitro\textsuperscript{80}.

1.5.3 Heparan sulfate proteoglycans
Heparan sulfate proteoglycans (HSPGs) are glycoproteins that contain one or more covalently bound heparan sulfate chains, which are a type of glycosaminoglycans\textsuperscript{86}. There are 17 types of HSPGs known, classified in three groups according their location on membrane, ECM or secretory vesicle\textsuperscript{87}. HSPGs are able to bind growth factors, cytokines, chemokines and morphogens to protect them from proteolysis. This way HSPGs could affect cell migration and function\textsuperscript{87}. The role of HSPGs in islet biology is not well understood. The postnatally increased expression of HSPGs in islets, hints to an involvement in postnatal islet growth and maturation\textsuperscript{88}. This was also observed in a mouse model with β cell specific inactivation of
HSPG, which displayed a 75% reduction of β cell area and a reduced β proliferation one week after birth. Further, using an analog of heparan sulfate, heparin increased the viability of cultured β cells.

### 1.5.4 Laminin

Laminins form a family of glycoproteins of cross- or T-shaped heterotrimeres composed of one α, one β and one γ chain. There are five α, four β and three γ chains and the trimers are named according their chain composition. Laminins are crucial during embryonic development and are involved in cell adhesion, differentiation, migration and survival. During mouse pancreas development Laminin-111 seems to be the primary isoform in vitro. In mature islets, however laminin-111 seems to disappear and laminin-511 was shown to increase insulin secretion in cultured rat β cells. The latter was also found in human islets, next to laminin-411, which both were found to enhance β cell proliferation. Immortalized β cells or primary islets from rodents or human showed a prolonged β cell survival when cultured on laminin. It was even shown that human islets which were encapsulated in alginate containing laminin and Col-IV had a prolonged islet survival after transplantation.

### 1.6 The role of β1 Integrin in islet development and function

Integrins are a superfamily of transmembrane heterodimer receptors and the major mediators between the ECM and the cell. Their bi-directional signaling property enables them to integrate exterior and interior environments. Additionally, they are mediating cell-cell interactions and are involved in a wide range of cell processes including adhesion, differentiation, cell growth and migration. Integrins are composed of one α and one β subunit and are expressed on every cell, although their combination varies on different cell types. In humans, 24 unique heterodimers are known, whereby both subunits are determining ligand specificity. Such ligands can be e.g. fibronectin, laminin, collagen, fibrinogen or thrombospondins.

The exact Integrin composition in the islet is not fully clear yet, partially because the composition changes throughout development. The islets’ integrin composition further differs between research animals and humans, making it more complicated to gain a full picture. Of all integrins, β1 is not only the most studied, but also the most promiscuous, being able to dimerize multiple alpha subunits allowing it to bind different ECM components. β1 integrin is also expressed on β cells. Blocking β1 integrin through antibodies in cultured rat or human islets reduces adhesion and insulin secretion and increases apoptosis of β cells. Embryonic inactivation of β1 integrin in mice impairs beta-cell proliferation resulting in a significant reduction of the β cell area. In postnatal stages conditional
inactivation of \( \beta_1 \) integrin results in a similarly reduced beta cell mass, impaired glucose tolerance and insulin secretion\(^\text{106}\). Altogether these results suggest an important role for \( \beta_1 \) integrin in beta cell mass expansion, maintenance and function from embryogenesis to postnatal stages\(^\text{5}\). The molecular basis for the function of \( \beta_1 \) integrin lies in its mediation of intracellular signaling pathways upon receptor activation. Integrin stimulation activates focal adhesion kinase (FAK) which is involved in cell motility, cell growth and survival (Fig. 6)\(^\text{107,108}\). Along with paxillin and talin, FAK is also able to regulate intracellular actin dynamics and reorganization (Fig. 6) which is crucial to achieve exocytosis of the insulin vesicles stored in \( \beta \) cells\(^\text{107,109}\). In mice \( \beta \) cell specific deletion of FAK leads to a decreased beta cell mass and reduced insulin secretion resulting in impaired glucose tolerance\(^\text{108}\). The mutant mice further display reduced phosphorylation levels of AKT and ERK1/2\(^\text{108}\). AKT is involved in the regulation of cell growth, survival and proliferation\(^\text{110}\). Overexpression of constitutively active

**Fig. 6**: Integrin signaling. Integrins are the major mediators between the ECM and the cell enabling to integrate exterior and interior environments. They are composed of one \( \alpha \) and one \( \beta \) subunit and are involved in a wide range of cellular processes including adhesion, differentiation, cell growth and migration. Figure modified from Carl G. Gahmberg et al. 2010; David S. Harburger and David A. Calderwood 2009\(^\text{125,126}\).
AKT in β cells of mice results in increased β cell size and proliferation leading to an expansion of β cell mass\textsuperscript{111}. The AKT-induced activation of cyclin D1, cyclin D2, p21 and cyclin dependent kinase 4 leads to cell cycle progression, explaining the increased proliferation rates in β cells with constitutively active AKT\textsuperscript{111}.

ERK1/2 plays multiple roles in β cells, including mediation of cell proliferation, survival and modulation of the insulin expression to regulate the secretory demands of β cells\textsuperscript{112,113}. In pancreatic sections of humans with T2DM decreased phosphorylated FAK\textsubscript{Ser732} expression in β cells was observed, highlighting the importance of integrin activated FAK in β cell survival and function\textsuperscript{114}.

In zebrafish four homologs of β\textsubscript{1} integrin exist: itgb1a, itgb1b, itgb1b.1, itgb1b.2\textsuperscript{115}. While the first two are very similar to the β\textsubscript{1} integrin found in other vertebrates, the latter are unconventional subunits not described in other vertebrates so far\textsuperscript{115}. In situ hybridization studies of embryonic stages revealed that itgb1a, itgb1b and itgb1b.1 are maternally expressed during zygote, cleavage and blastula periods. At later developmental stages itgb1a is expressed in the epidermis, dorsal aorta, notochord, otic vesicle and in the heart\textsuperscript{115}. For itgb1b, the expression at 12hpf was observed in the somite and tail bud, at 24hpf in myotome borders and at 36hpf in the otic vesicle, pectoral fin epidermis, dorsal aorta and notochord. At 4dpf itgb1b expression was shown in branchial arches\textsuperscript{115}. Both itgb1a and itgb1b are expressed in the developing cardiovascular system suggesting their involvement in embryonic blood vessel and heart

**Fig. 7:** Mutation of itgb1b. By using a sgRNA a seven base pair deletion in Exon 3 was produced through non-homologous-end-joining DNA repair. This resulted in a frameshift creating a premature stop codon leading to a truncated protein as indicated by the lightning (A). The itgb1b\textsuperscript{+/-} mutants display a severe impairment in heart and brain development manifested in brain hemorrhage and heart edema (B) indicated by arrowheads. Figure A modified from ensamble.org\textsuperscript{116}.
At 12hpf itb1b.1 is expressed in the epidermis of the whole embryo and at later stages it can be seen in the petal fin, dorsal fin, the enveloping layer, the urogenital opening and slightly in the notochord. At 3dpf itgb1b.1 is expressed in the pharyngeal arches and intestinal epithelium. Contrary to the others, itgb1b.2 is not expressed in early stages but at 12hpf it can be slightly observed in the epidermis. At 36hpf it is also expressed in the urogenital opening and notochord and at 48hpf additionally in the otic vesicle and branchial arches. However, the integrin composition of the zebrafish islet is completely unknown and whether β1 integrins play a similar role as in rodents and humans is still unclear.

1.7 Aim of the study

Maintenance of an adequate blood glucose level relays on the proper secretion of different hormones like insulin, glucagon and somatostatin, produced by the islets of Langerhans. While the relevant cell types and hormones are well studied, especially the insulin secreting β cells, little is known about the complex interactions between them as well as the interaction with the extracellular matrix. As the importance of the islets’ microenvironment becomes more and more apparent, the Meyer lab decided to investigate the role of the ECM in islet development and function in zebrafish. As integrins are the major regulators between the ECM and the cell they are perfect to study the role of ECM in islet formation. Coincidentally, the group of assoz. Prof. Pia Aanstad recently created an Igtb1b−/− mutant which she kindly provided for the project. The mutant was created using the CRISPR/Cas9 system. Injection of a sgRNA and Cas9 mRNA resulted in seven base pair deletion in Exon 3 through non-homologous-end-joining DNA repair leading to a frameshift creating a premature stop codon (Fig. 7A). Embryos lacking itgb1b show a severe impairment in heart and brain development manifested in brain hemorrhage and heart edema (Fig. 7B) and eventually death at 5-6dpf. When Réka Lőrincz started the investigation, Réka Lőrincz used the Glucose Assay Kit from BioVision to measure whole embryo glucose levels at 5dpf. Mutant itgb1b−/− animals displayed almost a 2-fold increase in glucose levels, indicating a Diabetes like phenotype.

![Fig. 8: Glucose level of itgb1b−/− mutant. Réka Lőrincz used the Glucose Assay Kit from BioVision to measure whole embryo glucose levels at 5dpf. Mutant itgb1b−/− animals displayed almost a 2-fold increase in glucose levels, indicating a Diabetes like phenotype.](image-url)
she discovered that the glucose level of 5dpf itgb1b-/- embryos is almost doubled (Fig. 8) indicating a diabetic-like phenotype. The aim of this master thesis was to study the effects of the mutation in the itgb1b gene on the islet physiology. This included the investigation of the pancreas structure, the islet architecture and vascularization, the β cell function and hormone expression levels. Furthermore, the ultrastructure of the islet cells was investigated via Transmission electron microscopy (TEM). Understanding the role of Integrins in the zebrafish islet, gives a great advantage to study the interactions of the islet cells with the extracellular matrix in vivo.116
2. MATERIAL AND METHODS

2.1 Material

2.1.1 Fish lines

<table>
<thead>
<tr>
<th>Genotype</th>
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<td>Itgb1b-/+; ins: lynGCaMP6s; ins:H2B-RFP</td>
<td>5023</td>
</tr>
<tr>
<td>Itgb1b-/+; ins: cGCaMP6s</td>
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2.1.2 Oligodesoxynucleotides

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2.1.3 Buffers and solutions

Ridchardson staining solution

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<td>methylene blue</td>
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<tr>
<td>azur II</td>
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<tr>
<td>disodium tetraborate (borax)</td>
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<tr>
<td>ddH2O</td>
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</table>
Cacodylate buffer

INGREDIENTS  100 mL

2,14 g Cacodylate  2,14 g

Fill up to 100 mL with ddH$_2$O and adjust pH to 7,38 and store at 4°C

Resin

INGREDIENTS

| Embed 812 | 20 mL |
| DDASA   | 16 mL |
| NMA     | 8 mL  |
| BDMA    | 1,1-1,3 mL |

store at -20°C

2.2 Methods

2.2.1 Zebrafish maintenance

Developing zebrafish embryos were incubated at 28°C. Upon 5 dpf the embryos were transferred to mouse cages until they reached 3 months of age when they were transferred to fish tanks. If needed for imaging the developing embryos were treated with N-Phenylthiourea (PTU) to prevent pigmentation.

2.2.2 Isolation of genomic DNA

To assess the genotype of the adult fishes they were paralyzed with tricaine and a small part of the tail fin was cut using a scalpel. For embryos, either the tail or the whole animal was used to extract the DNA. The samples were incubated with 20-50 μL proteinase K lysis buffer at 55°C for 3h. The reaction was stopped 10 min at 99°C to inactivate the proteinase K enzyme and the DNA was stored at -20°C.

2.2.3 Genotyping

The DNA isolated form the tail fin of adults or from whole embryos was used to identify the homozygous or heterozygous itgb1b mutation. The used program is listed in Tab. 2.2.3.1. The PCR mix contained 2 μL Taq-Buffer (10x), 0,4 μL dNTPs (10 mM), 0,4 μL Itgb1b_geno_Fwd primer (100 μM), 0,4 μL Itgb1b_geno_Rev primer (100 μM), 2-4 μL DNA, 1,5 μL Taq DNA polymerase (the self-made polymerase was very low concentrated and we had to use a higher amount than usually) and the appropriate amount of ddH2O. The PCR product was digested for 2h at 65°C using TruIl in the following mix: 0,1μL Enzyme, 1 μL buffer R, 10 μL DNA and 8,9 μL ddH2O.
Either 10 or 20 larvae were pooled and 50 or 100 μL of Trizol was added. For homogenization, a syringe with a needle was used and the lysate was incubated 5 min at room temperature. 10 or 20 μL of Chloroform was added, samples were shaked for 15 sec and incubated for 2 min at room temperature. After centrifugation for 15 min at 12,000 rpm at 4°C, the clear supernatant was transferred into a fresh tube. Upon addition of 25 or 50 μL Isopropanol, the samples were incubated for 10 min at room temperature and centrifuged for 20 min at 12,000 rpm at 4°C. The supernatant was removed and the RNA pellet was washed once in 75% Ethanol, centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant was removed and the RNA pellet was air dried for 5-10 min at room temperature. To dissolve the RNA, 18 μL ddH2O was added and incubated at 55°C for 10 min. The quantity and quality of isolated DNA was evaluated by measuring the absorption of 1 μL of RNA at 260 and 280 nm, using the NanoDrop 2000 Spectrophotometer. To evaluate DNA contamination, 1 μL RNA was analyzed on a 1% agarose gel via gel electrophoresis. If DNA was found in the sample, 15 μL of the RNA were incubated with 2 μL DNase, 1 μL RiboLock, 2,5 μL DNase buffer (+MgCl2) and 4,5 μL ddH2O at 37°C for 30 min. To stop the reaction, 5 μL of 25 mM EDTA was added and incubated for 5 min at 65°C. To isolate the RNA again, 160 μL ddH2O, 20 μL 5 M NH4OAc and 600 μL 100% Ethanol were added and incubated at -20°C overnight or at -80°C for 1h. After centrifugation for 30 min at 13,000 rpm at 4°C, the RNA was washed twice in 75% Ethanol and the pellet was air dried for 5-10 min. The RNA was solved in 15 μL ddH2O. The RNA was again analyzed on a 1% agarose gel via gel electrophoresis and stored at -80°C until usage.

2.2.5 cDNA synthesis

Total cDNA was synthesized using the "Maxima First Strand cDNA Synthesis Kit" according the manufacturer's protocol. 1pg-5μg of RNA, 4 μL 5x Reaction mix, 2 μL maxima enzyme mix and the appropriate amount of ddH2O were incubated 10 min at 25°C, 30 min at 50°C and 5 min at 85°C. The cDNA was used directly for qPCR or stored at -20°C until usage.
2.2.6 Real Time Quantitative PCR (qPCR)

The generated cDNA was used to quantify the expression of itgb1b, glucagon, insulin, sst1.1, sst1.2 and sst2.2. The housekeeping gene ef1α was used as a reference. 0,5 μL of cDNA was mixed with 4 μL Fire-Pol EvaGreen qPCR mix and 5,5 μL ddH2O and amplified according the protocol in Tab. 2.2.6.1. Two biological and two technical replicas were performed. The results were analyzed using EXCEL.

<table>
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<td>Melt curve</td>
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Tab. 2.2.6.1: Program, used for the qPCR.

2.2.7 Glucose measurements

The whole larva glucose levels were measured using the Glucose Assay Kit from BioVision as described previously. Pools of 10 larvae where washed twice in ice cold PBS and euthanized on ice for 20 min. Embryos were collected in 200 μL ice cold autoclaved PBS, with 10 glass beads (0,5 mm) and homogenized using the Precellys24 bead homogenizer (four cycles of 20 s at 5000 rpm). After a 10-min centrifugation step at 10.000 rpm at 4°C, the supernatants were transferred into fresh tubes and centrifuged again for 5 min under the same conditions. The glucose assay was performed according the manufacturer's protocol. For the measurements, 10 μL of the samples were used and 2 biological and 2 technical replicates were measured.

2.2.8 Immunocytostainings

Larvae were collected at 3 or 4 dpf and fixed in 4% PFA (supplemented with 1% DMSO) for 1h at room temperature. After three washing steps with PBST for 5 min, the yolk was punctuated. The larvae were incubated in blocking buffer for at least 1h at room temperature. The primary antibody (1:200 in blocking buffer) was added overnight at 4°C on a rotor shaker. The larvae were washed 6 times for 15 min in PBST and incubated in blocking buffer for at least 1h at room temperature. The secondary antibody (1:500 in blocking buffer) was added overnight at 4°C on a rotor shaker. After 3 times 5 min washing in PBST, the larvae were incubated for 2-3h in 1:1000 DAPI in PBST, to stain the DNA. For imaging, the yolk was removed and the head and tail were cut off. The trunk was embedded in 1,2 % low melt agarose and the pancreas was imaged using the Zeiss Cell Observer SD. The images were analyzed using Fiji imageJ. The data were analyzed using Prism-GraphPad.
2.2.9 Whole-mount in situ hybridization (WISH)
Larvae were collected at 3 or 4 dpf and fixed in 4% PFA overnight at 4°C on a rotor shaker. After 3 x 5 min washing, the larvae were washed 3 x 5 min in 100% Methanol and stored for 30 min at -80°C or until further usage at -20°C. After washing twice 5 min in PBST, 1 mL Proteinase K buffer (10 μL/mL) was added for 20 min. The larvae were washed 2 x 5 min in PBST and fixed again in 4% PFA for 20 min at room temperature. After washing 1 x 5 min in PBST, 1 x 1 min in H2O and 1 x 5 min in PBST, the larvae were incubated in 200 μL Hyb+ at 68°C for 1h. The digoxigenin labelled antisense probe was added (1:200 in Hyb+) overnight at 68°C. The larvae were incubated in pre-warmed 1 mL Hyb- at 68°C for 20 min and subsequently washed 2 x 20 min in pre-warmed 1 mL 2xSSCT 50% Formamid at 68°C. After washing 1 x 20 min in pre-warmed 1 mL 2xSSCT 25% Formamid at 68°C, 2 x 20 min in pre-warmed 1mL 2xSSCT at 68°C, 2 x 30 min in pre-warmed 0,2xSSCT at 68°C and 1 x 5 min in PBST at room temperature, the larvae were incubated in 1 mL blocking buffer for 1h at room temperature. The α-Digoxigenin antibody was added (1:4000) overnight at 4°C on a rotor shaker. After washing 6 x 10 min in PBST and 3 x 5 min in NTMT buffer the larva were stained in 200 μL staining solution (3,5 μL/mL BCIP and 4,5 μL/mL NBT in NTMT buffer) for 20-120 min. The reaction was stopped with PBST and embryos were stored in Glycerol over night at room temperature. The yolk was removed and the exocrine or endocrine pancreas was imaged. The images were analyzed with Fiji imageJ and the data was processed using Prism-GraphPad.

2.2.10 Spontaneous β-cell activity
To measure the spontaneous activity of β cells, the 4 dpf embryos were paralyzed with tricaine and embedded in 1,2% low melt agarose. The larvae were imaged using confocal high-resolution time-laps microscopy with a time interval of 1s for 4 min. The number of reacting β cells was counted using Fiji imageJ and the data was analyzed with Prism-GraphPad.

2.2.11 Glucose stimulated insulin secretion (GSIS)
The 3 and 4 dpf larva where anaesthetized in tricaine to immobilize them. Subsequently they were embedded in 1,2% low melt agarose. To get direct access to the embryo, the agarose was carefully removed from the thorax region. A special injection setup, adjusted to the Zeiss Cell Observer SD, was used to inject ~1 nl of 0,5 M glucose into the blood vessels or next to the islet. The larvae were imaged using a 25x water immersion objective. Time laps images were performed with 1s time intervals for 2 min and embryos were injected after 20 sec. The fluorescent signals before, directly at and after the injection were measured using Fiji imageJ. The data was processed via Prism-GraphPad.
2.2.12 Transmission electron microscopy (TEM)

Embryos were fixed in 2% formaldehyde freshly prepared from PFA and 2,5% glutaraldehyde in 0,1 M cacodylate-buffer for 2 h on ice. The tail was cut using a razor blade and the larvae were fixed again at 4°C overnight. After washing twice in cacodylate-buffer and 4 x 30 min in cacodylate-buffer at room temperature, osmium tetroxide (1% in 0,05 M cacodylate-buffer) was added for 2h at room temperature under a laminar flow. The larvae were washed once shortly in cacodylate-buffer, 3 x 10 min in cacodylate-buffer at room temperature and incubated for 15 min in 50% acetone (in ddH₂O) at room temperature, 15 min in 75% acetone at room temperature and overnight in 90% acetone at 4°C. Larvae were further dehydrated 4 x 30 min in 100% acetone at room temperature and incubated overnight in 10% resin in acetone at room temperature. After incubation in 30% resin in acetone for 4h at room temperature, an incubation in 50% resin in acetone at room temperature overnight followed. The larvae were incubated at 70% resin in acetone at 4°C over the weekend, treated 2 x 3 h in 100% resin at room temperature and incubated overnight in 100% resin at room temperature. After treating with fresh 100% resin, the larvae were embedded in 100% EMBed812 resin and polymerized for at least 48h at 60°C. Embryos were cut either sagittal, or in cross sections using (2µm) using a Butler diamond knife (Diatome, Switzerland) and mounted on a glass slide. Semi-thin sections were stained according to Richardson for 30-60 sec at 60°C. Stained sections were examined and photographed with a Leica DM5000B microscope and a Leica DFC 450 camera and a Leica application suite 4.8 (Leica, Germany). The desired sections were popped up by sticking resin blocks using fresh resin (Fig. S2). After 24h of polymerization at 60°C, the blocks were removed by alternately dipping the slides in liquid nitrogen and warm ddH₂O. The blocks with the desired sections were polymerized for another 24 h at 60°C. Ultra-thin sections were cut with a Leica ultracut UCT (Leica, Austria), sections were mounted on copper grids and stained with lead citrate for 2 min and examined with a Zeiss Libra 120 Energy filter TEM (Zeiss, Germany). Images were recorded with a 2x2k high speed camera (Tröndle, Germany) and an Image SP software (Tröndle, Germany).
3. RESULTS

3.1 The elevated glucose level of itgb1b−/− mutants changes during development

As it gets more and more apparent that the microenvironment is crucial for the function of the islet, its ECM became a subject of interest in Diabetes research. The Meyer Lab recently found that 5dpf itgb1b−/− mutant embryos display an almost doubled glucose level compared to the itgb1b+/−;+/+ control embryos (Fig. 8-9), indicating a diabetes like phenotype. To get a picture of the glucose dynamics in the itgb1b−/− mutants, whole embryo glucose measurements were repeated with 3 and 4dpf embryos using the BioVision assay kit. While the glucose level of 3dpf itgb1b−/− embryos was elevated less than 0.5-fold compared to the itgb1b+/−;+/+ littermates, the glucose amount in 4dpf mutants was almost 3-fold higher (Fig. 9). The glucose level at 5dpf however was lower again. These results indicate that the glucose homeostasis in itgb1b−/− mutant embryos is dysregulated which manifests in elevated glucose levels that change during development.

3.2 Itgb1b−/− mutants display a normal islet structure and composition but tend to have a smaller exocrine pancreas

To find the root of the dysregulated glucose homeostasis in itgb1b−/− mutants the expression pattern of the pancreatic transcription factor ptf1a was analyzed via in situ hybridization on 4dpf embryos. The exocrine pancreas structure appeared smaller in itgb1b−/− mutants compared to the itgb1b+/−;+/+ control animals (Fig. 10A). The pixel area in the exocrine pancreas of a single plain of the recorded images was measured. The area tended to be smaller in mutants compared to wild type, although it was not significant (Fig. 10B). Zooming in, the islet structure and composition was investigated. Therefore embryos were fixed at 4dpf and antibody stainings

![Graph showing glucose levels](image)

**Fig. 9:** Glucose level of the itgb1b−/− mutant. The glucose level of 3dpf itgb1b−/− embryos was elevated less than 0.5-fold compared to itgb1b+/−;+/+ mutants. In 4dpf mutants the glucose level was increased to an almost 3-fold amount compared to the control. In the 5dpf embryos, measured by Réka Lorincz, the glucose level seemed to lower again to an almost 2-fold increased glucose level. (n=10 pooled embryos wt/mut for every timepoint, with two biological replicates)
against insulin, glucagon and somatostatin were performed. The general islet structure of the itgb1b−/− mutants appeared very similar to the islet structure of itgb1b+/+ littermates with β cells in the core, α cells on the periphery and δ cells in the intermediate space, with some in the periphery and in the core (Fig. 10C). To investigate the islet composition, the number of β, α and δ cells of the whole primary islet in 4dpf embryos was assessed by counting, using stack images of the islets. The number of the endocrine cells in itgb1b−/− mutants was not changed compared to the itgb1b+/+ littermates (Fig. 10D). These results indicated that the dysregulated

**Fig. 10:** Exocrine and endocrine structure in itgb1b−/− mutants. The exocrine pancreas structure appeared smaller in 4dpf itgb1b−/− mutants (A). The measured pixel area of the pancreas in 4dpf mutants tended to be smaller (n=4 wt/4mut), although it was not significant (B). The endocrine cells in the islet of the itgb1b−/− mutants were organized in a similar way than the ones of the itgb1b+/+ control. The β cells were located in the center, α cells on the periphery and δ cells in the intermediate state with some appearing in the center and in the periphery (C). The number of β, α and δ cells in itgb1b−/− mutants was similar to control animals (n=4 wt/4mut) (D).
Fig. 11: Islet vascularization in itgb1b−/− mutants. In 3dpf mutants the blood flow is extremely reduced compared to the itgb1b+/−/+ control animals indicated by arrowheads. In 4dpf mutants blood flow is lost as indicated by arrowheads (A). Islet vascularization in both 3 and 4dpf itgb1b−/− animals was normal compared to their littermates (B). The number of intra-islet endothelial cells is not changed in 3 (n=5wt/5mut) and 4dpf (n=5wt/5mut) itgb1b−/− mutants (C-D).
glucose homeostasis in itgb1b−/− mutants is not caused by changes in number or position of β, α or δ cells.

3.4 Islet vascularization is normal in itgb1b−/− mutants
The expression pattern of itgb1b suggests an involvement in vessel formation during development, which may also affect islet vascularization115. The blood flow of the itgb1b−/− mutants in general becomes extremely reduced between 2 and 3dpf and is almost non-existent in 4dpf embryos (Fig. 11A). To investigate the islet vascularization, transgenic wt fli:GFP fish were crossed with itgb1b+/− animals to generate itgb1b+/+;fli:GFP fish. Incross of the latter resulted in itgb1b−/− fish with green fluorescent blood vessels. The embryos were fixed at 3 and 4dpf and stained against the transcription factor islet1, expressed in endocrinal cells, and insulin to show the close interaction between endothelial cells and β cells mentioned above118. In general, islet vascularization appeared normal in both 3 and 4dpf itgb1b−/− mutants (Fig. 11B). Counting the intra-islet endothelial cells in 3 and 4 dpf islets confirmed that there was no change in islet vascularization in itgb1b−/− mutants (Fig. 11C-D), although the blood circulation was nearly absent.

3.5 Itgb1b−/− mutant islets display a normal β cell activity but a slightly altered endocrine hormone expression
Since no difference in islet composition and vascularization was observed, one could assume that the dysregulation of the glucose homeostasis in itgb1b−/− animals could root in a malfunction of β cells. The Meyer lab used the fact that endocrine cells show an increased intracellular calcium concentration when activated and established transgenic fishlines expressing the membrane-tagged fluorescent calcium-sensor ins:lynGCaMP6s47. Réka Lőrincz crossed the itgb1b+/− fish with wt ins:lynGCaMP6s;ins:H2B-RFP animals to get itgb1b+/+;ins:lynGCaMP6s;ins:H2B-RFP fishes. The latter where incrossed to get itgb1b−/−;ins:lynGCaMP6s;ins:H2B-RFP animals. To assess the β cell activity in fasting fish, 4dpf embryos were anaesthetized with tricaine, embedded in agarose and imaged for 4 min using confocal high-resolution time-laps microscopy. In general the fasting β cell activity in both itgb1b−/+ and itgb1b−/− fish was very low to none and no differences were observed (Fig. 12A). To test the β cell function upon glucose stimulation both 3 and 4 dpf embryos were injected with 0,5 M glucose. While in wild type embryos the glucose was injected directly into the blood vessels visible in Fig. 11A, the mutants were injected in close proximity to the islet due to the loss of blood flow, resulting in bad visualization of the vessels. The animals were anaesthetized with tricaine, embedded in agarose and imaged for 2 min using confocal high-resolution time-laps microscopy (Fig. 12B).
Fig. 12: Function of β cells. The fasting β cell activity in transgenic ins:lynGCaMP6s;ins:H2B-RFP itgb1b wild type (n=24) and mutant (n=25) animals was very low and no changes were observed between both (A). The embryos were embedded in agarose and injected with 0.5 M glucose (B). No differences in glucose stimulated insulin secretion (GSIS) were observed between itgb1b+/+;+/+(n=10) and itgb1b−/− animals (n=10) (C-F). The mRNA level of sst1.2 and sst2 was elevated significantly in 3dpf itgb1b−/− animals compared to itgb1b+/+;+/+ controls (n wt/mut=20 pooled embryos; 2 biological replicates). In 4dpf mutants they are even more elevated and additionally the mRNA levels of sst1.1 and glucagon are higher (n wt/mut=20 pooled embryos; 2 biological replicates) (G). Figure B modified from Lorincz et al. 2018.
After 20 sec the fish were injected with glucose and the maximum fluorescence signal prior, while and post injection was compared. Both, itgb1b+/-/+ control and itgb1b-/- mutant β cells reacted similar upon glucose injection (Fig. 12C-F). As the β cell function seemed normal in itgb1b-/- mutant animals, the expression of islet hormones was assessed via qPCR. Therefore

**Fig. 13:** Finding the islet structure. In semithin-sections of 7 and 6 dpf itgb1b+/-/+ the islet structure (indicated by arrows) was found closely after the gall bladder (indicated by arrowheads) (A-B). The mutant embryos displayed a very blown-up phenotype compared to their itgb1b+/-/+ littermates. Further, the exocrine structure appeared smaller in mutants (C-D). en = endocrine; ex = exocrine;
total RNA of 3 and 4dpf embryos was extracted and the expression of itgb1b, insulin, glucagon and all three somatostatin genes (sst1.1, sst1.2 and sst2) was assessed. At 3dpf both, sst1.2 and sst2 mRNA levels were elevated significantly in itgb1b-/- mutants compared to itgb1b+/-/+ controls (Fig. 12G). All three where even more elevated in 4dpf embryos and additionally glucagon and sst1.1 showed an increased expression (Fig. 12G). The expression of the elevated hormones was further investigated via in situ hybridization. A slightly darker stain of some cells indicated that the hormones were really expressed higher in the itgb1b-/- mutants (Fig. S1).

### 3.6 Islet ultrastructure

Even if the β cells work properly, differences in the ultrastructure of the islet blood vessels could possibly hinder the excretion of insulin in itgb1b-/- mutants. As the ultrastructure of the intra-islet vessels cannot be determined by a simple antibody staining it was assessed via electron microscopy. Therefore embryos were fixed at 3dpf and semithin-sections (2 μm) where cut either sagittal or in cross sections and mounted on glass slides. The sections were examined to find the islet structure using a Leica microscope. Because the appearance of the 3dpf embryonic islet structure was not known, it was difficult to find. Additionally the sections were full of dirt caused by the yolk (not shown). This led to the decision to investigate the islet structure at different stages. Therefore mutant and wild type embryos were fixed at the following stages: 3, 4, 5, 6 and 7 dpf for itgb1b+/-/+ and 3, 4 and 5 for itgb1b-/-.

The semithin-sections of itgb1b+-/- embryos were investigated from 7 to 4 dpf. In the 7dpf embryos, the islet structure was found closely behind the gall bladder (Fig. 13A and 14). As soon as the islet structure was found at 7dpf, it was easier to find it at 6, 5 and 4dpf (Fig. 13B-D, 14 and 15A).

![Fig. 14](image): Islet ultrastructure in zebrafish embryos. The extensive rough endoplasmic reticulum of the exocrine part (ex) of the pancreas leads to a higher electron density, making it appear darker compared to the endocrine part (en). Additionally, the hormone vesicles of the islet are visible as small dots and the zymogen granule of the exocrine pancreas are visible as bigger electron dense circles (*), making it easier to distinguish both parts.
The semithin-sections of 4 and 5 dpf itgb1b−/− mutants underline the severity of their phenotype as they almost seem to fall apart compared to their control littermates (Fig. 13C-D). This can also be observed in the electron microscopic images of the muscle cells (Fig. S4), which are losing contact to each other. Looking at the exocrine pancreas in the semi-thin sections of 4 and 5 dpf animals the structure appears smaller in itgb1b−/− mutants compared to the itgb1b−/+;+/+ controls (Fig. 13C-D), supporting the findings mentioned above. It can be observed that the exocrine pancreas only remains in contact with the intestine but loses contact to the other

Fig. 15: Ultrastructure of the 4dpf zebrafish islet. The exocrine (ex) part of the pancreas is recognizable by the highly electron dense zymogen granule (z) and its extensive rough endoplasmic reticulum (rER) which contributes to a high electron density making it appear darker (A-B). The endocrine (en) part is less electron dense and therefore appears brighter. In 4dpf the itgb1b−/− mutants appear similar to the itgb1b−/+;+/+ controls, although the exocrine part seems to be smaller in the mutants (A). In higher magnifications the hormone vesicles are clearly visible (v) and distinguishable, which can be seen in the schematic showing of the 4dpf islet ultrastructure (B). Around the islet there is a non-continuous peri-islet ECM (pECM) visible by electron dense dots. (B). Around the intra-islet blood vessels (iv) a similar non-continuous vesicular ECM (vECM) is visible (B). Additionally, the intra-islet blood vessels display a special fenestration (f) (A-B). n = nucleus
adjacent organs (Fig. 13C-D). The exocrine and endocrine cells themselves however, seem to be still in close contact to each other (Fig. 13C-D and Fig. 15A). On the ultrastructural level the exocrine and the endocrine part of the embryonic pancreas are well distinguishable and similar to adult structures (Fig. S3, 14 and 15). The exocrine pancreas is recognizable by its extensive rough endoplasmic reticulum which leads to a high electron density, making it appear

![Ultrastructure images of islets](image)

**Fig. 16:** Ultrastructure of the 5dpf islet. Around the islet a very thin non-continuous peri-islet ECM can be observed indicated by arrows (A). In mutants the cells seem to lose contact from the ECM indicated by arrowheads (A). Around the intra-islet blood vessels (arrowheads) a similar effect can be observed, with the ECM structure seemingly dissolving which leads to a deformation of the adjacent cell membranes indicated by asterisks (B). The islet blood vessels display a fenestration (arrowheads) in both mutants and controls (B). The glucagon vesicles (α) show a less dense halo with an eccentric electron dense core. Insulin granule (β) are moderately electron dense and the somatostatin vesicles (δ) are more electron dense (c).
Fig. 17: Ultrastructure of the 4dpf islet. The peri-islet ECM is very thin and non-continuous (arrowheads) (A). In the \textit{itgb1b} deficient embryos the cells loose contact, loosening the ECM (arrows) and deforming the adjacent cell membranes (asterisk) (A). The islet blood vessels look normal and also display fenestration mutants, indicated by arrows (B). The glucagon vesicles (α) show a less dense halo with an eccentric electron dense core. Insulin granule (β) are moderately electron dense and the somatostatin (δ) vesicles are more electron dense (c).
darker compared to the endocrine part (Fig. 14 and 15). Additionally, the highly electron dense zymogen granule mark the exocrine pancreas (Fig. 14 and 15). The endocrine part not only appears brighter, but its hormone vesicles are also visible as many small dots in the cells (Fig. 14 and 15A). In the 4dpf itgb1b−/− mutants, the islet structure appears similar to the control animals, but the exocrine part seems to be smaller in the mutants, showing again the reduction of the exocrine part in the itgb1b−/− mutants (Fig. 15A). Around the islet a very thin non-continuous ECM was detected in 4 and 5 dpf itgb1b−/+;+/+ animals (Fig. 15B, 16A and 17A). The cells adjacent to the peri-islet ECM start to loose contact to the ECM in 4 and 5 dpf itgb1b deficient animals, manifesting in a deformation of the cell membranes (Fig. 16A and 17A). In 4 and 5 dpf itgb1b−/−;+/+ embryos, the fenestration of islet vessels was clearly visible (Fig. 15, 16B and 17B). This fenestration still appeared in both 4 and 5 dpf itgb1b−/− mutants (Fig. 16B and 17B). Around the intra-islet vessels of 5dpf itgb1b−/−;+/+ embryos a thin non-continuous ECM was observed (Fig. 16B). Upon loss of itgb1b, the cells adjacent to the peri-islet ECM start to loose contact to the ECM, similar to the effect at the peri-islet ECM, deforming both, the vessel membrane and the adjacent endocrinal cell membrane (Fig. 16B). This observations and the fact that the endocrinal cells themselves appear still very close and in a wild type way (Fig. 13, 15B, 16C and 17C), indicates that like in mice and human the zebrafish endocrinal cells don’t produce their own ECM. Investigation of the embryonic α, β and δ cells showed that the vesicles in the corresponding endocrinal cells appeared similar to the vesicles found in adult endocrinal cells (Fig. S3B, 16C and 17C), recently described by the Meyer Lab (unpublished). Glucagon vesicles have an eccentrically located electron dense core surrounded by a less dense mantle or halo. While insulin granule appear spherical and moderately electron dense, the vesicles in δ cells are more electron dense and oval or roundish shaped (Fig. S3B, 16C and 17C). The vesicles in itgb1b−/− mutant embryos appeared wild-type like. In conclusion, the intra-islet blood vessels were still fenestrated and no evident defect in the itgb1b deficient endocrine cells could be detected.
4. DISCUSSION

Several studies recently pointed out the importance of the ECM-cell interactions in islet development and function. As there is no knowledge of such interactions in zebrafish, this study aimed to investigate whether β1 integrin plays a similar important role in the zebrafish islet as it does in mice or human islets. While there are four homologs of β1 integrin in zebrafish, only two of them are very similar to the β1 integrin found in other vertebrates: itgb1a and itgb1b. The group of assoz. Prof. Pia Aanstad recently generated a itgb1b-/- mutant which she kindly provided for the project. First investigations uncovered that the mutant embryos display a significantly higher glucose at 3dpf, even rising at 4dpf but then declining again in 5dpf, suggesting a dysregulation of the glucose metabolism. Therefore the physiology and histology of the islets deficient for itgb1b were examined. As the differences in the cytoarchitecture of the islet between species and the evolutionary change from rodents to dogs to monkeys and humans suggest a tight connection between islet structure and function, the composition of the itgb1b-/- mutant zebrafish islet was determined. Overall, the exocrine pancreas tended to be smaller in itgb1b-/- mutant embryos compared to the itgb1b+/+;+/+ littermates. The exocrine structure in mutants loses contact to all adjacent organs except the intestine. However, the exocrine cells themselves remain in close contact to each other, suggesting that itgb1b is not needed to form the exocrine pancreas and that its deformation is only a secondary effect.

Interrogation of the islets at 4dpf revealed that the structure and composition of α, β and δ cells in itgb1b-/- mutants is normal. The Meyer group recently described the ultrastructural appearance of α, β and δ cells and noticed that their respective vesicles display a special phenotype (not published). As the islet ultrastructure was investigated in 4 and 5dpf itgb1b-/-;+/+ embryos, it was found that the embryonic endocrine vesicles are similar to the ones in adult zebrafish. No differences in vesicle structure of 4 and 5 dpf itgb1b-/- mutants were found. These results suggest that itgb1b is not crucial for the development of α, β and δ cells in the zebrafish islets. The expression pattern of itgb1b implies an involvement in vessel development. As the islet vessels are important in development and function of β cells a defect in such could impact the glucose metabolism. However, the structure and number of intra-islet endocrinal cells was not changed in itgb1b-/- mutants. Further, the ultrastructure revealed that the fenestration of the islet vessels appears also in itgb1b deficient animals. Together this indicates that itgb1b is dispensable for islet vascularization during development.

Due to its important role in β cell development and function, the islet ECM was investigated. On an ultrastructural level a very thin non-continuous was observed around the islet in itgb1b-/-;+/+ embryos. A similar ECM was observed around the intra-islet blood vessels. Like in mice and
humans this was the only ECM found within the islet, suggesting that the endocrine cells of the zebrafish islet also don’t produce their own ECM. In itgb1b deficient embryos the adjacent cells to the peri-islet and vascular ECM are losing contact to the ECM deforming the cell membranes. This indicates that itgb1b is an important component in the islet-ECM interaction and therefore could play a role in β cell function. However, analyzation of the β cell function showed that there was no spontaneous activity and an appropriate glucose stimulated insulin secretion in itgb1b−/− mutants. Examination of the transcription levels of the islet hormones showed that the expression of all three somatostatin and the glucagon mRNA was elevated in itgb1b−/− mutants. Although these elevations were confirmed via in-situ-hybridization, the protein level was not assessed. The stress caused by the loss of blood circulation and the collapse of the tissue could lead to an elevation of adrenaline, which was shown to increase somatostatin levels in mices. Although the β cells of itgb1b−/− mutants are reacting normal upon glucose injections, this is only a hyperactivation and does not display the physiological function. The elevated somatostatin could lead to an inhibition of fasting insulin secretion, leading to a higher glucose level. Further, stress is also able to increase glucagon levels to prevent hypoglycemia. Together this could explain the dysregulated glucose metabolism in itgb1b−/− mutants, on an otherwise normal islet structure and function. During the experiments it was observed that the yolk of the itgb1b−/− mutants was declining less than in itgb1b+/−;+/+ animals (Fig. 7B). Upon exhaustion of the yolk-derived carbohydrates, usually between 4 and 5 dpf, the zebrafish intends to achieve its glucose demand by starting the gluconeogenetic program and initiating the “feeding-to-fasting” switch. As the blood flow in itgb1b−/− mutants is impaired it could interfere with the delivery of nutrients from the yolk, resulting in a deficient energy supply. This fasting condition could lead to an elevation of glucagon, activation of gluconeogenesis and initiation of the “feeding-to-fasting” switch earlier than in control animals. Looking at a time course in wild type embryos, it can be observed that the glucose amount initially increases from 3 to 5dpf and then falls in embryos which are never feed (Fig. 4) due to net depletion of gluconeogenetic substrates. Looking at the glucose measurements of itgb1b+/++;+/+ embryos from 3 to 5dpf, it can be observed that the glucose levels similarly rises (Fig. 9). In itgb1b−/− mutants the glucose amount rises faster but shows a similar pattern to the never fed wild type embryos (Fig. 9 and 4), supporting the idea that the feeding-to-fasting switch happens earlier in the itgb1b deficient embryos. Additionally to the stress-induced reduced insulin secretion, this could explain the elevated glucose level in the itgb1b−/− mutant embryos, which would also mean that the latter was only a secondary effect due to systematic loss of itgb1b.
In conclusion loss of itgb1b does not affect islet architecture and composition, ultrastructure, vascularization or β cell activity. Although the mutants display a higher blood glucose level and an elevated somatostatin and glucagon mRNA expression, it is difficult to determine if these effects are caused by loss of itgb1b specifically in the endocrinal cells. It is rather likely, that they are caused by the high systematic impact of itgb1b loss. The results obtained during this study suggest that itgb1b is not important for islet development and function. However, it should be considered that both β1 integrin homologues itgb1a and itgb1b could have redundant roles, as it was observed frequently in zebrafish. Even if this study couldn’t enlighten the role of β1 integrins, it was able to provide an insight in the ultrastructure of the embryonic islet ECM, intra-islet vessels and endocrinal vesicles in zebrafish.

4.6 Future Perspectives

In this study the systematic knockout of itgb1b was only used because it was available. For future studies on the role of β1 integrins, β cell specific knockouts should be used. Transcriptome analysis of adult zebrafish β cells indicate that itgb1a should be the gene of interest for future studies. The Meyer Lab currently works on RNA sequencing of β cells during islet development, which could help to find the target of interest in the ECM. Investigating the ECM-islet interaction in the zebrafish model could help understand the importance of such in vivo and during transplantations. Investigating the ECM-islet interaction in the zebrafish model could help understand the importance of such in vivo and during transplantations.
5. SUPPLEMENTARY

Fig. S1: Expression of islet hormones in itgb1b−/− mutants. The higher expression of sst1.2 and sst2 in 3dpf embryos was confirmed via in situ hybridization. In mutant 3dpf islets some cells showed a slightly darker staining compared to wild type endocrinal cells, indicated by arrowheads (A). In 4dpf embryos some glucagon, sst1.1, sst1.2 and sst2 expressing cells showed a similar darker staining indicated by arrowheads (B).
**Fig. S2**: Finding the islet structure. The semi-thin sections were mounted on microscope slides and stained. The sections were investigated via microscopy and the interesting slices were popped-up on a resin column again, before ultra-thin sections were made.

**Fig. S3**: The adult zebrafish islet. The endocrinal part (en) can be clearly distinguished from the exocrine part (ex) of the adult pancreas by its brighter appearance and the hormone vesicles which appear as many small dots (A). Additionally, the exocrine part displays highly electron dense zymogen granule (z), making it easier to distinguish the two parts (A). Insulin vesicles appear spherical and moderately electron dense, while the vesicles in δ cells are more electron dense. Glucagon vesicles have an eccentrically located electron dense core surrounded by a less dense mantle or halo (B).
Fig. S4: Muscle cells in \textit{itgb1b}\textsuperscript{-/-} mutants. The muscle cells start to lose contact to each other at 4dpf and get even more loose in the 5dpf \textit{itgb1b}\textsuperscript{-/-} mutants. This is also manifesting in holes between the cells which eventually fully lost contact (indicated by arrowheads).
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**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>T1DM</td>
<td>type 1 Diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>type 2 Diabetes mellitus</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ins</td>
<td>Insulin</td>
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<td>gcg</td>
<td>Glucagon</td>
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<tr>
<td>sst</td>
<td>Somatostatin</td>
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<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
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<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>AKT</td>
<td>protein kinase B</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>Itgb1</td>
<td>Integrin beta 1b</td>
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<td>Hpf</td>
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<td>Wt</td>
<td>wild type</td>
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<tr>
<td>GSIS</td>
<td>glucose stimulated insulin secretion</td>
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