Intestinal GATA-4 mediates regulation of lipid homeostasis *in vivo*

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Dissertation

Intestinal GATA-4 mediates regulation of lipid homeostasis *in vivo*

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To nature, and the continuous human endeavor of understanding it

Declaration

I hereby declare that this thesis consists my original conception and own work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Date,

Signature

Please note that parts of this thesis have been published and/or are under scrutiny for future publications.

Loss of intestinal GATA4 prevents diet-induced obesity and promotes insulin

sensitivity in mice.

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This work is dedicated to

My Beloved, Late Father

Mr. Vasant Purushottam Patankar

PREFACE

The work presented in this thesis was supported by the PhD program 'Molecular Medicine', Medical University of Graz, Austria, through the academic years 2007 – 2010. The study was performed in its entirety at the Institute of Molecular biology and Biochemistry, Medical University of Graz, Austria.

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ABSTRACT

Transcriptional regulation of small intestinal gene expression controls plasma total cholesterol (TC) and triglyceride (TG) levels, which are major determinants of metabolic diseases. GATA-4, a zinc finger domain transcription factor, is critical for jejunal identity and intestinal GATA-4 deficiency leads to a jejuno-ileal transition. Although intestinal GATA-4 ablation is known to misregulate jejunal gene expression, its pathophysiological impact on various components of metabolic syndrome remains unknown.

Here, we used intestine specific GATA-4 knockout mice (GATA4iKO) to dissect the contribution of GATA-4 on obesity development. We challenged adult GATA4iKO and control littermates with a Western-type diet (WTD) for 20 weeks. Our findings show that WTD-fed GATA4iKO mice are resistant to diet induced obesity. Accordingly, plasma TG and TC levels are markedly decreased. Intestinal lipid absorption in GATA4iKO mice was strongly reduced whereas luminal lipolysis was unaffected. GATA4iKO mice displayed a greater GLP-1 release on normal chow and even after long term challenge with WTD and remained glucose sensitive. Given a higher GLP-1 release we also found a reduced gastric emptying in these mice. On the other hand GATA4iKO mice showed attenuation in Gip release upon fat load leading to a suppression of lipid absorption via chylomicrons. These mice also showed higher glucose stimulated insulin release upon oral fat load which contributes to their reduced absorption of dietary fat. GATA4iKO mice also showed lower accumulation of lipids in the liver and exhibited a healthier gene expression profile compared to controls for lipogenic and steatotic potential.

In summary, our findings show that the absence of intestinal GATA4 has a beneficial effect on decreasing intestinal lipid absorption causing resistance to hyperlipidemia, obesity and non-alcoholic fatty liver disease. In addition, the increase in GLP-1 release in GATA4iKO mice shows that these mice have an improved ability to counter insulin resistance after feeding a WTD.

Zusammenfassung

Die transkriptionelle Regulierung der Genexpression des Dünndarms ist mitentscheidend für die Kontrolle von Plasma Total-Cholesterin (TC) und Plasma Triglyzerid (TG) Konzentrationen, welche als wichtige Faktoren für metabolische Erkrankungen gelten. GATA-4, ein Zinkfinger Domänen Transkriptionsfaktor, ist kritisch für die Identität des Jejunum, denn die Defizienz von GATA-4 im Darm führt zu einer Umwandlung von Jejunum zu Ileum. Obwohl bekannt ist, dass das Fehlen von GATA-4 zu einer Deregulierung der Genexpression des Jejunum führt, ist die pathophysiologische Konsequenz auf verschiedene Komponenten des metabolischen Syndroms nach wie vor unbekannt.

In dieser Studie verwendeten wir darmspezifische GATA-4 knockout Mäuse (GATA4iKO) um herauszufinden, welche Rolle GATA-4 in der Entwicklung von Fettleibigkeit spielt. Wir fütterten adulte GATA4iKO und die entsprechenden Kontrollen mit einer „Western-type diet“ (WTD) für 20 Wochen. Unsere Ergebnisse zeigen, dass WTD-gefütterte GATAiKO-Mäuse resistent gegenüber einer futterinduzierten Fettleibigkeit sind. Dementsprechend stellten wir auch stark erniedrigte TG und TC Levels fest. Die intestinale Fettabsorption in GATA4iKO war stark reduziert, wobei die luminale Lipolyse keine Änderungen vorwies. GATAiKO Mäuse zeigten eine erhöhte Freisetzung von GLP-1, wenn sie mit normalen Futter gefüttert wurden, aber darüber hinaus auch nach dem Füttern mit WTD, wobei sie hierbei glukosesensitiv blieben. Entsprechend der erhöhten GLP-1 Freisetzung, konnten wir auch ein verspätetes Entleeren des Magens feststellen. Weiters zeigten GATAiKO Mäuse eine verspätete Freisetzung von Gip nach Gabe eines Fettbolus, was zu einer erniedrigten Lipidabsorption via Chylomikronen führt. Weiters zeigten diese Mäuse auch eine erhöhte Freisetzung von glukosestimulierter Insulinfreisetzung nach der Gabe eine Fettbolus, was ebenfalls zu der erniedrigten Fettabsorption dieser Mäuse beiträgt. GATAiKO Mäuse weisen auch eine erniedrigte Akkumulierung von Fett in der Leber auf und zeigen ein gesünderes Genexpressionprofil verglichen mit den Kontrollmäusen in Bezug auf das lipogene und steatotische Potential.

Zusammengefasst zeigen unsere Ergebnisse, dass das Fehlen von GATA4 im Darm zu dem positiven Effekt der Erniedrigung der intestinalen Fettabsorption führt, was schließlich in einer Resistenz gegenüber Hyperlipidämie, Fettleibigkeit und eine Nicht-Alkohol induziertern Fettleber, resultiert. Darüber hinaus zeigt die erhöhte Freisetzung von GLP-1 in GATA4iKO Mäusen, dass diese Mäuse leichter eine Insulinresistenz nach einer Fütterung mit WTD entgegentreten können.

CONTENTS

I. Introduction:

1.0 *Short overview of intestinal biology*

The intestine has evolved into a complex multi-cellular organ that affects important physiological processes. The adult intestine is patterned in an antero-posterior axis and forms the core of the bodies' nutrient ingestion, assimilation and absorption system. The developing intestine is segmented into anatomically distinguishable regions along its longitudinal axis. These segments are reinforced into distinct parts which are the duodenum, jejunum and ileum of the small intestine and the ascending, transverse and the descending colon of the large intestine. This patterning is imparted during development and is maintained throughout the adult lifespan. In mice, the intestine undergoes self-renewal every 3 days for goblet and absorptive enterocytes and within 5 days the entire epithelial sheet is renewed (5 days; 7 days respectively in case of humans). Thus new enterocytes constantly originate from the resident stem cells at the crypt base and from the cells in the proliferating compartment, then differentiate and finally are sloughed as an end result of controlled apoptosis at the tip of villi. Apoptosis at the tip of each villus is primarily thought of as an outcome of division pressure exerted from the penultimate cell at the tip.

However, understanding of the mechanisms that lead to the induction of apoptosis at the tip villus is vague. The anatomical organization of the intestine greatly increases available surface area by projecting multi-cellular villi into the lumen in order to maximize nutrient absorption. Each villus is composed of various specialized cell types such as the enterocytes (absorptive lineage), goblet cells (secretory lineage), paneth cells (lysozyme positive crypt base cells), enteroendocrine cells (L cells, K cells etc. endocrine lineage) and intestinal stem cells (crypt base self renewing cells). Differentiation of these cell types is maintained through strict patterns of gene expression which in turn depend on differential transcription factor activities. Spatial patterning for any given intestinal part reflects predominantly in two forms (a) cell type composition and (b) physiological diversification, each of which mutually compliment and sustain. Understanding of the mechanisms of many modern day ailments originating from nutrient imbalances, commonly termed as the metabolic syndrome, has been

restricted to the site of their pathological outcomes. Work in recent years indicates that these ailments share a causal relationship to metabolic de-regulation in other organs such as the intestine and not just in those exhibiting pathological effects.

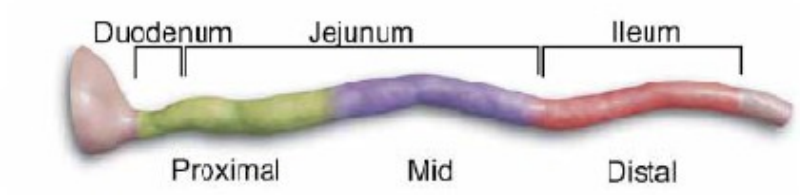

Illustration 1: Schematic representation of the three anatomical regions of the small intestine in the antero-posterior axis and general segments namely proximal, medial and distal.

1.1 *Embryonic development and differentiation of intestine*

Intestine is an endoderm derived organ. Cells of the visceral endoderm from the anterior and posterior sides fuse as a single primitive gut tube of undifferentiated striated epithelial cells at embryonic day (E) 8.5. Starting at E9.5 till E14.5 appears a rapid proliferation of the epithelial cells and the gut tube undergoes considerable lengthening. At E14.5, the interactions of the mesoderm and visceral endoderm enable organ specification which is marked by the transition of the stratified epithelia into a single sheet/layer of columnar epithelial cells. During this time, the length of the embryonic gut can be divided into 3 distinct parts, the fore-gut, mid-gut and the hind-gut. Development of these parts would lead to the adult stomach, small intestine and colon, respectively. From E14.5 to E19.5, each of these undergoes a process of cyto-differentiation starting proximally and proceeding distally in a wave-like manner. Intestinal villi are formed as projections into the gut lumen, due to the division pressures of the underlying mesenchyme and its interactions with epithelial cells. At this stage nascent villi protrude as finger-like projections and are interspersed with proliferating epithelial cells. Completion of maturation in the murine intestine happens in postnatal life. Lineage allocation occurs and differentiated cell types such as the

absorptive enterocyte, goblet cells, paneth cells and enteroendocrine cells are formed.

1.2 *Intestinal physiology and transcription*

1.2.1 Small intestinal absorptive physiology

The small intestine is the site for uptake, assimilation and absorption of dietary nutrients. Intestinal absorptive enterocytes have evolved elaborate and intricate mechanisms that ensure optimum absorption of nutrients. The process involves the breakdown of complex macromolecules into simple monomers in the intestinal lumen. Enterocytes express various surface transporters which assist in the uptake of molecules, the intracellular transport proteins bind to and transport the respective nutrients and finally on the basolateral surface, exit into the lymph to be circulated throughout the body. This general scheme is true for absorption of several dietary molecules including carbohydrates, lipids and proteins. The specifics of intestinal lipid absorption are discussed here. Dietary lipids are first emulsified in the stomach acid to form the lipid containing oil phase in the chyme. As the chyme enters the duodenum, it forms a viscous-isotropic phase in which the triglycerides and complex lipids are broken down with the help of lipases. The source for the pancreatic lipases is the secretion from the exocrine pancreas released into the duodenum at the "*papilla vateri*" where it meets the bile coming from the common bile duct. The third phase is the formation of micelles in which the bile acids solubilize the monoacylglycerol, cholesterol and free fatty acids and make them available for uptake into the enterocytes. Lipid and cholesterol absorption follows a second order kinetics and hence is a facilitated process requiring the activity of transporter proteins. Several transporters are important for the uptake process such as Cd36 antigen/long chain fatty acid translocase (Cd36/FAT) and scavenger receptor class B, member 1 (Scarb1) for fatty acids and Niemann-Pick C1-like protein 1 (Npc1l1) for cholesterol. Similarly, many proteins contribute in the intracellular trafficking of lipids and cholesterol through organelles and vesicles and facilitate their transport. Finally, the free fatty acids (FFA) and cholesterol are esterified, packed into pre-chylomicrons in the ER which mature in the golgi and are exported across the basolateral surface of the

enterocytes into the lymph. The process requires several transporters such as members of the fatty acid binding (Fabp) family and fatty acid transport protein (Fatp) family. Specific isoforms of each family are expressed along the small intestine and this leads to selectivity of different intestinal parts for different lipid species.

1.2.1 (a) Intestinal lipid absorption:

Evolutionarily, lipid uptake and storage is a process of prime importance since it ensures the supply one of a molecule with the highest calorie content. To ensure its optimum absorption, a number of physico-chemical as well as biological determinants have evolved to play important roles in the absorption of dietary lipids.

Before translocation from the intestinal lumen into the enterocytes can occur, dietary lipids must undergo a number of physicochemical alterations. This is achieved in a sequence of events called the intraluminal phase of lipid digestion and absorption, including:

emulsification of dietary lipid

lipolysis

solubilization (micelles, vesicles)

translocation of lipolytic products across the enterocyte membrane

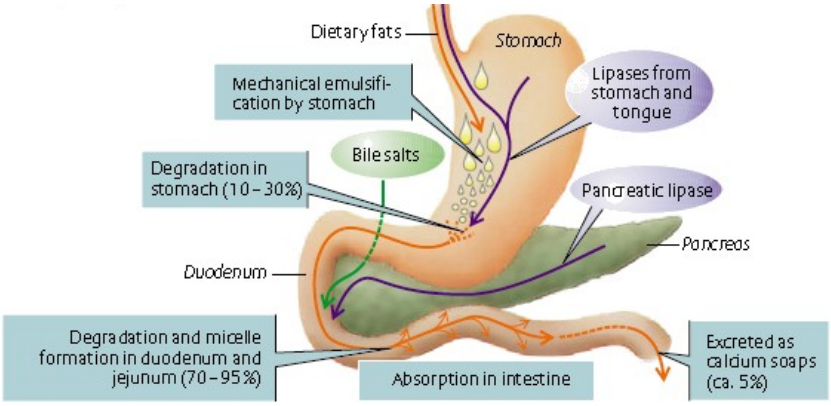

Illustration 2: Schematic representation of various steps involved in the digestion of lipids in the stomach and proximal small intestine showing the contribution that various parts of the proximal alimentary tract play in lipid

absorption. *Adopted from: Color Atlas of Physiology, Stefan Silbernagel, Thieme publishing group*

Fatty acids are taken up by absorptive enterocytes involving both protein-mediated and protein-independent processes. Dietary fats consist of a wide array of polar and nonpolar lipids (21, 23). Polar lipids, which are insoluble in water, are cholesteryl esters, hydrocarbons, and carotene.

Polar lipids are sub-divided into:

(a) Immiscible non-swelling amphiphiles that form a stable monolayer in aqueous solutions. E.g. triacylglycerols (TG), diacylglycerols (DG), non-ionized long chain fatty acids (LCFA), unesterified cholesterol and fat soluble vitamins A, D, E, and K.

(b) Immiscible swelling amphiphiles that form stable monolayers as well as laminated water-lipid structures or liquid crystals; in aqueous solutions. E.g. monoacylglycerols (MG), ionized fatty acids (FA), and phospholipids (PL).

(c) Miscible amphiphiles that possess strong polar head groups rendering these molecules soluble under aqueous conditions at low concentrations. This allows formation of both unstable monolayers as well as micelles. E.g. Sodium salts of long chain fatty acids and bile salts.

TG is a dominant dietary fat and contributes 90–95% of the energy derived from dietary lipids. Dietary fats also consist of PL, sterols (predominantly cholesterol), and several other lipids (e.g. fat-soluble vitamins). Phosphatidylcholine comprises a predominant portion of dietary PL derived mostly from bile (10–20 g/day in humans) but also from dietary sources (1–2 g/day). The dietary sterols consist of cholesterol (0.5g/day animal origin) and β-sitosterol (major plant sterol). Although β-sitosterol accounts for 25% of dietary sterols, it is not absorbed by humans under physiological conditions. The absence of biliary components due to medical conditions such as cholestasis differentially affects the solubilization and hence absorption of lipid classes due to the resulting ectopic interaction with water. The first step of dietary fat digestion starts in the stomach with mechanical emulsification followed by the partial TG hydrolysis

caused due to the secretion of gastric lipase. This results in the formation of the two TG lipolytic products DG and FFA. However, the gastric lipase is unable to hydrolyze PL and cholesterol esters, but its activity is important for efficient lipolysis of 10 to 30% of TG in the stomach (15, 23, 75). The completion of TG digestion takes place in the lumen of the duodenum by the activity of pancreatic lipase, which cleaves the fatty acid chains on sn-1 and sn-3 position of TG molecules in a hydrolytic reaction, releasing 2-MG and two molecules of FFA (15, 75). Pancreatic lipase is abundantly present in the pancreatic juice secreted by the exocrine pancreas. Thus, severe pancreatic insufficiency manifests clinically in the form of lipid malabsorption syndrome. The formation of micelles occurs in duodenal lumen when the bile salts emulsify dietary lipids. After the formation of bile acid-lipid micelles TG is no longer accessible to the pancreatic lipase and hence the pancreatic lipase requires a cofactor called pancreatic co-lipase-1 for its complete activity. The binding of pancreatic co-lipase-1 to the micelle/water interface facilitates the docking of pancreatic lipase with the active site over the TG molecules. Digestion of PL requires the activity of phospholipase A2 secreted from the exocrine pancreas and takes place entirely in the lumen of the duodenum.

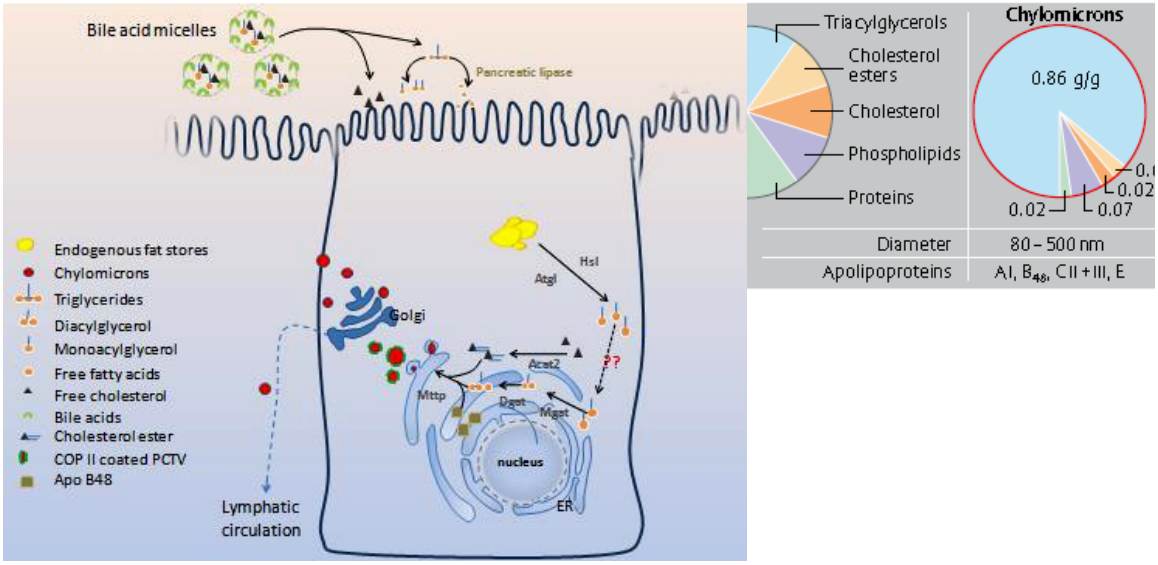

Illustration 3: Cartoon depicting the enterocytic mechanisms leading to the uptake and absorption of dietary lipids along with an inlay showing percent distribution of various lipid classes in mature chylomicrons particles.

In the presence of bile salts phospholipase A2 requires calcium as a cofactor for its activity. It hydrolyzes PL at the sn-2 position, resulting in the release of FFA and lyso-phosphatidylcholine as end products of hydrolysis. Most of dietary cholesterol is present in the form of free cholesterol. Only 10-15% occurs in form of CE. CE are hydrolyzed in the duodenum into free cholesterol by the activity of pancreatic cholesterol ester hydrolase (CEH), an enzyme secreted by the exocrine pancreas. CEH is a multi-functional enzyme which hydrolyzes not only CE but also TG on sn-1, sn-2 and sn-3 position, PL on its sn-1 and sn-2 position and lapidated-vitamin esters (78, 79). The activity of this enzyme is greatly enhanced by the presence of bile salts. The transport of cholesterol from luminal dietary contents into enterocytes lining the gut lumen depends directly on the emulsification and micellar solubilization brought about by detergent-like properties of biliary lipids and the products of dietary lipid lipolysis (61). Biliary phosphatidylcholine is of central importance in this process and cholesterol cannot be effectively solubilized in bile without the presence of biliary phosphatidylcholine (22). Thus, successful absorption of intestinal cholesterol relies on adequate amounts of PL in the gut lumen. Although adequate amounts of PL are required for the normal absorption and successful lymphatic transport of cholesterol, an excess of luminal PL lead to the suppression of cholesterol absorption (95, 96, 111).

1.2.1 (b) GATA-4 and intestinal gene expression of lipid transporters:

Apart form spatio-temporal control, intestinal gene expression is also sensitive to dietary insults, colonizing microflora and gut homing lymphocytes and other immune cells. Of all these factors the ones most relevant here are spatial and dietary changes in gene expression. Both these factors account for physiological adaptation of the small intestine to nutrient overload. Expression of some of the most important lipid transporters is spatially restricted along the small intestinal axis. Ones critical for lipid uptake and absorption are predominantly expressed in the proximal part, whereas those involved in the uptake and transport of bile acids are found in the terminal part. For example *Fabp-1* (liver fatty acid binding

protein) and *Cd36*, are expressed in the duodenum and jejunum (proximal small intestine) (37, 77, 87). On the other hand, Asbt (apical sodium dependant bile acid transporter) and Fabp6 (ileal fatty acid binding protein) are restricted in expression to the ileum (distal small intestine) (5, 76). Spatial restriction is brought about by a combinatorial network of transcription factors and co-factors which activate promoters in distinct spatially defined manner. The scheme in illustration 4 depicts interaction of HNF and GATA family transcription factors in regulating endoderm specific transcription.

The adult duodenal-ileal axis is maintained through spatio-temporal gradients of transcription factors, which persist throughout the adult life span. These include Gata4, Gata5, Gata6, Hnf1α, Cdx1, Cdx2, Pdx1 and the CCAAT displacement protein (CDP)/Cux (45, 103). Several of these factors regulate expression of genes leading to physiological diversity of the three parts of the small intestine, the duodenum, jejunum and the ileum. However, their study in adulthood has been impeded due to embryonic lethal phenotypes of their respective knockouts. So far six members have been assigned to the GATA family of zinc finger-domain transcription factors that bind to the conserved promoter motif 'WGATAR', identified as the GATA consensus site (81). GATA-4 was identified as a gene encoding a zinc finger domain transcription factor involved in development and organogenesis. However, its mRNA and protein are also expressed in adult tissues including heart, adrenal glands, and small intestine (19, 105). Embryonic lethality in GATA-4 knockout mice is known to arise mainly from defective heart tube formation and extraembryonic endoderm differentiation (84, 118). In the small intestine, GATA-4 mRNA expression is restricted to the duodenum and jejunum, and the expression is undetectable in the terminal ileum (45). This expression pattern is conserved across several vertebrate species including humans (17). GATA-4 is expressed throughout the length of the crypt-villous axis in epithelial cells (16, 17). GATA-4 controls the expression of several absorptive enterocyte genes among them are *Lph*, *sucrase isomaltase*, fatty acid binding protein 1 (*Fabp-1*) and ATP-binding cassette sub-family G member 5 and 8 (*Abcg5 &8*). GATA-4 has previously been reported to control the expression of

genes that are important for carbohydrate, lipid and sterol homeostasis and insulin secretion including sucrase isomaltase (Sis), lactase (Lct) and Fabp1 (17, 93, 99, 116). Some of these genes are themselves expressed in distinct antero-posterior gradients with similar expression patterns as Gata4 (37, 77, 87). Even though GATA-4 has been implicated in regulating physiologically relevant intestinal genes, few studies have described its role in regulating adult intestinal physiology so far.

Bosse et al. generated viable intestine-specific inducible Gata4 knockout mice and showed that in the jejunum, expression of jejunal markers such as Fabp1 and Lct is downregulated whereas that of ileal markers such as solute carrier family 10, member 2 (Slc10a2) and Fabp6 is upregulated suggesting the jejuno-ileal transition (17). The jejunum of these mice acquired ileal characters. Using a Villin-Cre approach, Battle et al. generated intestine specific Gata4 knockout (GATA4iKO) mice (7). The authors reported that 47% of ileal gene set is ectopically expressed in jejunum of GATA4iKO male mice with only a modest decline (approx. 20%) in jejunal lipid uptake and absorption and no effect on glucose absorption. GATA4iKO male mice showed a decreased jejunal expression of several genes involved in lipid uptake and transport, such as Cd36 and Scarb1 (7). Both Cd36 and Scarb1 have been implicated in controlling TG absorption in the small intestine (87, 112). Additionally, it was shown that due to the induction of nuclear receptor subfamily 1, group H, member 4 (Nr1h4 or FXR) several bile acid co-transporters such as Asbt, organic solute transporter alpha (Ost-α) and organic solute transporter beta (Ost-ß) were expressed in the jejunum of GATA4iKO mice (7). An ectopic expression of bile acid transporters in the jejunum would imply an increased bile acid absorption leading to an altered bile acid pool size contributing to decreased lipid absorption. GATA4iKO mice showed a significant decrease in body weight during suckling, but had similar body weight after weaning (7). Whether these effects on body weight persist throughout the adult lifespan contributing to decreased absorption has not been studied. As shown by Bosse et al. GATA-4 promotes jejunal identity and repress ileal identity (17). Such changes in tissue identities or homeosis observed in case

of intestine specific GATA4iKO mice impacts cell type composition as well as tissue physiology. Therefore the crypt-villus length in the knockout jejunum is smaller and resembles that of the ileum (7, 17). Number of goblet cells in the ileum is normally higher when compared to the jejunum, but GATA4 knockout jejunum shows an increase in number of goblet cells which parallels an ileal phenotype (7, 17). The observed jejuno-ileal transition closely resembles the bariatric surgical procedure of ileal interposition.

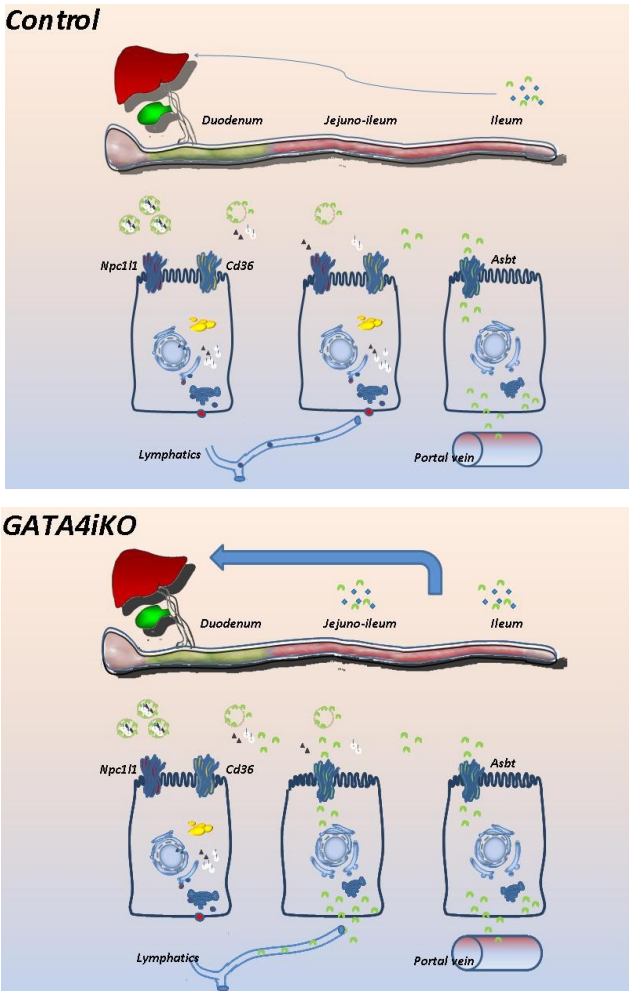

Illustration 4: Jejuno-ileal homeosis is induced due to the lack of intestinal GATA-4. Comparative shift in lipid and bile acid absorption along with release of some entero-endocrine molecules in GATA4iKO mice is shown.

Kohli et al. recently reported that in rats that had undergone ileal interposition surgery, the cycling of bile acids was increased, and these rats were protected from obesity associated co-morbidities (67). As shown in illustration 7 above, the expression of several important lipid transporters is altered due to the absence of intestinal GATA-4. The jejuno-ileal transition also causes a proximal shift in the expression of distal genes such as genes expressed normally in the ileum e.g. Asbt, Ostα and Ostß (the baso-lateral bile acid efflux transporters), Fabp6 etc. The proximal expression of these genes implies that bile acids which would be normally reabsorbed in the terminal ileum are now reabsorbed more proximally since at least three of the constituent genes which make up the pathway of bile acid reabsorption are now ectopically expressed in the jejunum. Thus, it would be expected that micellar bile acids from the lumen would be ectopically taken up by the jejunal enterocytes, a process mediated by Asbt. The next component is Fabp6 which shows specificity for binding bile acids and thus it could facilitate their intracellular trafficking. Finally, these bile acids would have to be put downstream into the portal vein for their homeostatic delivery into the liver. This would occur at the baso-lateral surface of the jejunal enterocytes, a process carried out by the Osta and Ostb transporters. Thus, the entire machinery necessary for the trans-cellular transport of bile acids is expressed in the jejuna of GATA4iKO mice and one might speculate that this leads to a premature collapse of the bile acid-lipid micelles.

This would eventually lead to malabsorption of lipids owing to the loss of emulsification and solubilization afforded by bile acids implying that the activity of enzymes which are dependent on bile acids for a supply of co-factors would be compromised in the intestine of GATA-4 mice. These include the pancreatic lipase and the pancreatic CEH. One might expect that this compromise in activity might add to the lipid malabsorption phenotype of GATA4iKO mice. Additional corollaries that might be drawn from these observations are that since the change is predominantly enterocytic (given that villin expression is restricted to enterocytes), the underlying anatomical structures such as the mesenteric lymphatics and capillaries supplying the portal vein would still be unchanged.

Thus, it would be expected that considerable amounts of the absorbed bile acids would enter into the peripheral circulation using the lymphatic architecture of the jejunum.

1.2.2 Intestinal transcription factors

Several transcription factors are known to regulate gene expression in the intestine. With respect to this great attention has been given to the promoters of genes, the expression of which are known to be temporally “hardwired”. E.g. the promoter of the *lactase/lactase phlorizin hydrolase* (*Lph*), the expression of which begins at the postnatal feeding transition has been carefully dissected. Several transcription factors have been identified to play a crucial role in the expression of spatio-temporally restricted genes. Many of these are not only important for proper gastrointestinal organogenesis but continue to be expressed in adulthood. Table 1 shows a summary of some of the important intestinal transcription factors in their distribution in the antero-posterior axis of the intestine. Notably, GATA-4, GATA-5, HNF-1alpha, HNF-1beta and HNF-3beta/Foxa2 are highly expressed in the jejunum of the small intestine indicating their role in governing gene expression in the jejunum.

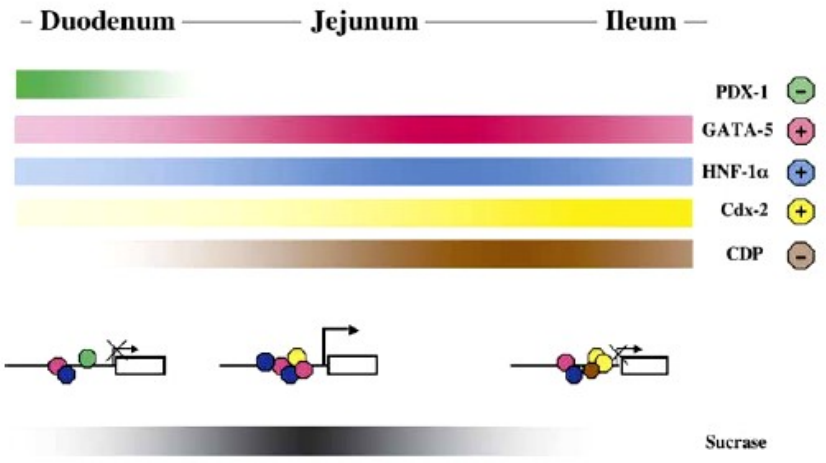

Illustration 5: Schematic representation adopted from Fang *et. al* (45) showing combinatorial expression patterns of various transcription factors in the proximo-distal axis of the small intestine involved in control of the Sucrase promoter.

Table 1

Gene Name	Duodenum	Jejunum	Ileum
Cdx1	low	medium	highest
Cdx2	none	none	highest
Foxa1	low	low	highest
Foxa2	low	highest	highest
GATA-4	low	highest	low
GATA-5	low	highest	highest
GATA-6	medium	medium	medium
HNF-1alpha	low	highest	highest
HNF-1beta	low	highest	highest
HNF-4alpha	medium	medium	medium
Klf-4& 5	low	low	highest
Nkx2.2 & 2.3	medium	medium	medium
Math-1	medium	medium	medium

Table 1: List of important intestinal transcription factors: Above listed transcription factors are expressed in a station-temporally regulated manner. Differences in the expression patterns in the three parts of the small intestine, along the antero-posterior axis has been indicated.

Cdx1 and Cdx2: The caudal-related homeobox transcription factors (Cdx) were identified as intestine specific transcription factors. *Cdx1* (caudal-related homeodomain 1) was first identified in normal adult and embryonic mouse intestine (98, 107, 109). As shown in Table 1, Cdx family members show high levels of expression in the posterior intestine compared to the proximal intestine, with highest levels in the colon. Cdx1 is localized predominantly in undifferentiated crypts cells (8). This 37-kd protein transcription factor was localized by immunohistochemistry to the epithelial nuclei of intestinal mucosa in normal adult human and mouse colon and small intestine (29, 83, 98). Cdx1 has been hypothesized to regulate intestinal proliferation, differentiation and development (8, 83, 107). It also contributes in maintaining the differentiation of adult intestinal epithelial cells (8, 107). Cdx1 also functions in intestinal metaplasia of human stomach and esophagus, as suggested by strong nuclear localization of CDX1 in cases of Barrett's esophagus with intestinal metaplasia and in human gastric mucosa with intestinal metaplasia (65). Cdx1 is known to regulate the expression of intestinal alkaline phosphatase gene and is also known to repress transcriptional activation mediated by the beta-catenin/T-cell

factor complex. It also regulates the expression of important intestinal differentiation markers such as cytokeratin 20 (25). However, Cdx1 knockout mice proceed normally in the development of the intestine indicating that Cdx2 might compensate for the loss of Cdx1 during early intestinal morphogenesis (14). Cdx2 is expressed at high levels in differentiated enterocytes. Cdx2 controls the expression of several genes including transporters such as multidrug resistance protein 1 (Abcb1b), adhesion molecules such as liver-intestine type cadherin, secretory glycol-proteins such as mucin-2 (Muc2) as well as genes involved in nutrient absorption such as sucrase isomaltase (Sus) and lactase phlorizin hydrolase (Lph) (57, 70, 82). Thus Cdx2 plays diverse roles in regulating intestinal function.

Foxa1 and Foxa2: The forkhead family transcription factors Foxa1 and Foxa2 are expressed in the definitive endoderm during embryonic development and continue to be expressed in many adult tissues of endodermal origin such as pancreas, liver, stomach, and intestine (11). Both Foxa1 and Foxa2 are important intestinal determinants of cell type composition since they regulate differentiation of goblet and enteroendocrine cells (119). They are expressed early in the developing intestine where they participate in governing the onset of transcriptional programs important for development and differentiation (11). Both of these factors have been shown to directly bind to the mucin-2 promoter and transactivate it (113).

GATA-4, 5, 6: The GATA family of transcription factors is expressed in several tissues and is important for cellular differentiation. The GATA factors are generally divided into two subgroups, GATA-1, 2, 3 and GATA-4, 5, 6 based on their spatial expression patterns (20). GATA-1, 2, 3 are expressed in hematopoietic cells, whereas GATA-4, 5, 6 are expressed in the endodermal tissues (20). “GATA” refers to the conserved DNA binding motif that the GATA factors recognize. All members contain a pair of zinc finger domains, the N and C terminal activation domains and a nuclear localization signal. GATA factors 4/5/6 have been implicated in the development of heart and endodermal derivatives in vertebrates (20, 100). In Xenopus embryos, GATA-4, 5 and 6 were shown to mediate TGF-beta maintenance of endodermal gene expression (1). In that,

GATA-4 is known to be important for cardiac specification and germline knockout of GATA-4 leads to lethality due to the failure of extra-embryonic endoderm differentiation and defective heart tube formation (20, 100). SNPs in the human GATA-4 gene have been linked to congenital heart defects (48). On the other hand, using a zebrafish model system, GATA-5 and GATA-6 were shown to be functionally redundant for the specification of cardiomyocytes (59). Moreover, GATA-5 knockout mouse has no apparent embryonic phenotype, thereby questioning the importance of this gene for vertebrate development (85). GATA-6 was identified to be upstream to other endoderm specific transcription factors such as Sox17, HNF1beta and Nkx2 (1). Apart from their roles in controlling early embryogenesis, GATA factors have also been implicated in regulating several disease conditions in adulthood. GATA-6 for example promotes colon cancer cell invasion by regulating urokinase plasminogen activator gene expression (9). A plethora of studies have implicated the involvement of GATA-4 & 6 in the regulation of pathological cardiac hypertrophy. GATA-4 has been implicated in the induction of other members of the GATA family, importantly GATA-6. Given that, GATA-4 upregulation elevates common targets of GATA-4 and GATA-6, it has been speculated that GATA4 induces these genes via induction of GATA6 (69, 86).

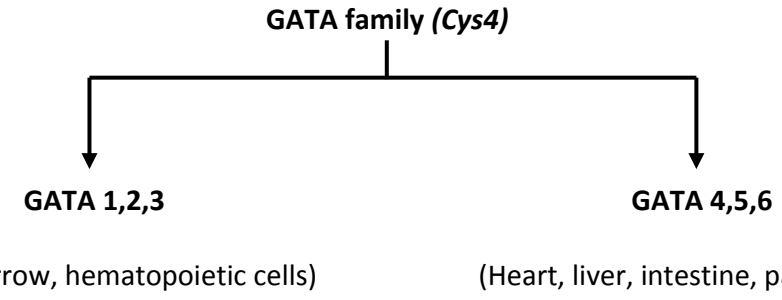

Illustration 6: The GATA family of transcription factors categorized based on their expression patterns encountered in various tissues.

HNF1alpha & beta: The broad family of hepatocyte nuclear factors (HNFs) is a group of phylogenetically unrelated transcription factors which regulate transcription of a diverse gene sets. These genes include clotting factors, transporters and enzymes involved in glucose metabolism, cholesterol and fatty acid transport and metabolism (4). Members of the HNF1 subfamily, also

abbreviated *Tcf* for T-cell factor, are POU-homeodomain DNA binding transcription factors that act as homodimers. Both Hnf1alpha and beta were identified as liver enriched factors but were soon shown to be expressed by other endodermal tissues such as intestine and kidney (33). Human Hnf1aplha and beta have been implicated in the development of maturity onset diabetes of the young (MODY) and so have been named MODY3 and MODY5 respectively (4). Both HNF1alpha and beta are expressed in entero-endocrine cells along the gastrointestinal tract (33). The expression of HNF1beta, however, predominated in the colon compared to HNF1alpha (33). Both Hnf1alpha and beta can heterodimerize with other endodermal factors (52). For ex: HNF-1alpha regulates the human sodium-glucose co-transporter gene, SGLT1, by heterodimerizing with Sp1. Transcriptional transactivation of HNF1a is potentiated via the interaction of several factors including GATA-4, Cdx-2 as well as HNF4a (10).

HNF4alpha: HNF4α is a liver enriched transcription factor. It is also expressed in developing endodermal tissues such as intestine and pancreas (39). It has been implicated in the control of proliferation, morphogenesis, differentiation and detoxification (72). It was shown that HNF4a is transcriptionally repressed in tissue undergoing carcinogenic transformation. The role of HNF4α has been extensively studied in hepatocytes and pancreatic β-cells, and HNF4α is also regarded as a key regulator of intestinal epithelial cell differentiation. Apart from its role in intestinal tissue homeostasis, HNF4a is important for metabolic flexibility of enterocytes based on dietary insults. For ex HNF4a regulates the alcohol induced downregulation of intestinal tight junction genes where levels of HNF4a itself are transcriptionally repressed upon alcohol consumption (120). Apart from the regulation of barrier function HNF4a also controls the expression of several genes involved in enterocyte lipid handling (18, 74, 92). Temporal regulation of both apolipoprotein AIV (ApoAIV) and microsomal triglyceride transfer protein (MTP) in response to dietary lipids in newborn pig enterocytes was induced at least in part through HNF4a mediated regulation (74). Also expression levels of HNFa mRNA and its target genes were enhanced in intestinal cell lines treated with oleic acid (74). Differentiation dependent upregulation of intestinal alkaline phosphatase is also regulated by HNF4a by

direct binding to the alkaline phosphatase promoter (89). Finally, HNF4a has been shown to co-operate and enhance the HNF1a mediated transcriptional activation of downstream genes (41).

Klf4&5: The Krüppel-like factors (KLFs) are conserved zinc finger domain transcription factors that regulate epithelial proliferation, differentiation, development and apoptosis (46, 64). Klf's were reported earlier as gut enriched factors but later studies confirmed their expression in other organs. KLF4 as well as KLF5 are highly expressed in epithelial tissues. In intestinal epithelium expression of KLF4 is restricted to differentiated epithelia at the villus margin where it maintains their differentiated state and inhibits cell growth (97, 108). KLF5, on the other hand is expressed in the proliferating intestinal crypt base cells where it promotes cellular proliferation (108). Klf-4 has also been reported as an important factor governing the expression of early intestinal marker genes such as the intestinal alkaline phosphatase (iap) (56). Klf4 is an inhibitor of Wnt signaling by its ability to directly interact with ß-catenin and thereby inhibiting the p300/CBP recruitment (43). Thus mice lacking intestinal Klf4 have an altered epithelial homeostasis as exemplified by reduced expression of *iap* as well as *ephrin* B. Another effect of the lack of Klf-4 was the upward displacement of intestinal Paneth cells to the upper crypt region (50). Given its ability to counter proliferative signals induced by Wnt, Klf4 negatively affects cellular proliferation and thus acts as a tumor suppressive gene in vivo (49). Consequently, it has been reported that Klf4 expression is repressed in several cancers (26, 28, 51). Studies in mice lacking intestinal Klf4 revealed that it also plays an important role in intestinal cellular differentiation and that intestine specific Klf-4 knockout mice have a 90% reduction in the number of colonic goblet cells (50). All these features make Klf4 an important transcription factor for the maintenance of intestinal homeostasis.

Nkx2.2 & 2.3: NKX2 are homeodomain containing transcription factors which belong to the NKX family. They are implicated in cell type specification, differentiation and proliferation (101). These factors are expressed in several tissues and organs and function in concert with other transcription factor partners to both activate and repress downstream genes in a combinatorial manner.

Nkx2.2 is expressed in intestinal crypts, whereas Nkx2.3 is expressed in gut mesenchyme of embryonic and adult mice (101). Nkx2.2 regulates cell lineage commitment among the intestinal enteroendocrine population; in the Nkx2.2 knockout mice, several sub-types of the enteroendocrine cell populations are absent or reduced whereas the number of ghrelin-producing cells is enhanced (35). However, populations of remaining cells including the paneth, goblet and absorptive enterocytes seems to be unaffected by loss of Nkx2.2 (35). Disruption of Nkx2.3 leads to a retarded growth of the intestinal villi, although in surviving adults a massive expansion of the crypt cells is seen (91). However these mice show differentiated cell types in intestinal epithelium, suggesting that Nkx2.3 is not essential for cell lineage allocation or migration dependent differentiation (91). In the cardiac muscle Nkx family members have been reported to interact with other transcription factors such as GATA-4 (40).

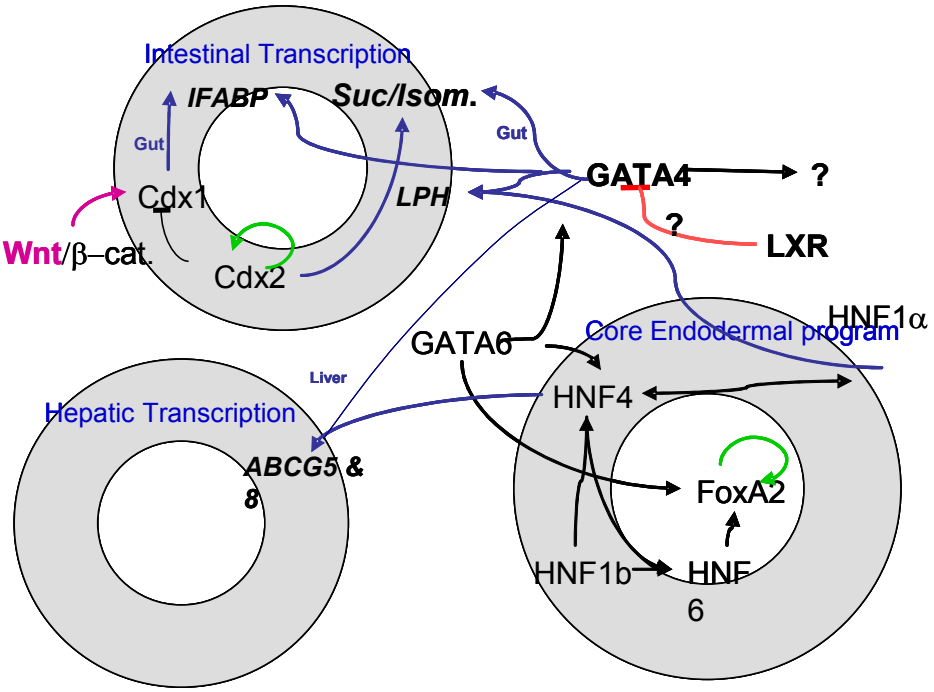

Illustration 7: Transcription factor networks in endodermal tissues: Role of HNF and GATA family transcription factors in regulation of the core-endodermal program. Co-operation of GATA and HNF family members, in controlling gene expression in the liver and small intestine (grey circles).

1.3 *The metabolic syndrome and role of the intestine*

1.3.1 Metabolic syndrome its symptoms and defining features

The metabolic syndrome, also known as syndrome X or insulin resistance syndrome is the co-occurrence of a group of predisposing risk factors that increase the risk for coronary artery disease, stroke and type 2 diabetes. The hallmark of these predisposing risk factors is an overall metabolic deregulation on an organ scale encompassing hormonal, biochemical as well as inflammatory deregulation. The defining features according to the definition by the world health organization are presence of either type II diabetes, impaired glucose tolerance, impaired fasting glucose or insulin resistance, along with any two of the following:

(a) Blood pressure: ≥ 140/90 mmHg
(b) Dyslipidemia: triglycerides (TG): ≥ 1.695 mmol/L and high-density lipoprotein cholesterol (HDL-C) ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)
(c) Central obesity: waist:hip ratio > 0.90 (male); > 0.85 (female), or body mass index > 30 kg/m2
(d) Microalbuminuria: urinary albumin excretion ratio ≥ 20 µg/min or albumin:creatinine ratio ≥ 30 mg/g

With the common incidence of obesity as a complicating feature in most cases of metabolic syndrome, measuring central obesity (defined as waist circumference with ethnicity specific values) has become centrally important to the diagnosis of metabolic syndrome. The presence of these features results in an increased risk of developing coronary artery disease or cardiac failure resulting in death.

The mechanisms and complex pathways involved in metabolic syndrome have not yet been completely unraveled. The pathophysiology is extremely complex, involving multiple organs and has been only partially elucidated. Further complications arise due to age, obesity, sedentary life style, stress and degree of insulin resistance. Apart from these factors, genetic predisposition, endocrine

disorders and excessive caloric intake also complicate the degree and extent of physiological deregulation and pathological damage.

1.3.2 Obesity and metabolic syndrome

Although the metabolic syndrome has been correlated with insulin resistance and obesity, it is not certain whether they are the cause of the metabolic syndrome or a consequence of a more far-reaching metabolic derangement (80). Pathophysiological analysis has identified elevated levels of a number of inflammatory markers including C-reactive protein, fibrinogen, interleukin 6 (IL–6), tumor necrosis factor-alpha (TNFα) and others (47). These pro-inflammatory factors point to the involvement of the immune system as a contributing factor for the observed pathological damage leading to fibrosis as seen in case of the progression of non-alcoholic fatty liver disease (NAFLD) into liver fibrosis (47).

High incidence of obesity in cases of metabolic syndrome also point to the involvement of adipocytes as a contributing factor. The adipocytes which store fat coming from dietary sources or from hepatic lipogenesis can undergo up to a 5 fold increase in their size to accommodate the increasing demand for storage. However, in doing so, they loose their plasticity and potential for self-renewal resulting in a condition called adipocytes hypertrophy. Occasionally, when lipid laden, hypertrophic adipocytes undergo apoptosis, they invite macrophages to phagocytose and clear out the dying cell. Obesity thus induces higher recruitment of macrophages within the growing fat depots where they release pro-inflammatory cytokines such as TNFα triggering adipocyte insulin resistance via the TNFα receptor.

Development of visceral fat is common, which leads to increased plasma levels of a number of other important determinants of adipose tissue function including adipokines such as leptin, adiponectin, resistin and PAI-1 (3, 71). An elegant example about how these adipose tissue hormonal queues regulate complex metabolic outcomes is of leptin and its receptor. Study involving mice lacking the gene for the adipokine leptin showed that they develop overt obesity and insulin

resistance. Further work showed that leptin circulates at levels proportional to body fat. Also mice that lack the receptor for leptin, which is expressed on the hypothalamus, are also obese and insulin resistant. Leptin was thus identified as an adiposity signal which reports to the hypothalamus which in turn regulates food intake and energy expenditure to regulate body weight. Feeding, increase in plasma glucose concentration and post-prandial insulin are factors which lead to adipocytes leptin release(42, 63, 115).

1.3.3 Insulin resistance and metabolic syndrome

Another complicating component of the metabolic syndrome is insulin resistance (IR). High plasma levels of insulin and glucose due to IR are a major component of the metabolic syndrome. It is described as a complete or partial loss in the ability of organs to recognize and reconcile signaling abilities of insulin. Inherently, IR in case of metabolic syndrome is a mechanism involving the insulin receptor and downstream signaling effectors unlike type I diabetes which is characterized by the loss of insulin production itself. However, several factors influence the overall insulin sensitivity of organs and their deregulation also leads to pathophysiological complications encountered in the metabolic syndrome. Prolonged IR leads to overproduction of insulin from the pancreatic beta cells. Consequent to IR, plasma glucose levels remain high in turn causing gluco-toxicity to the pancreatic beta cells and causing apoptotic death of these cells at late stages.

The derangement in insulin action eventually leads to the deregulation of several hormones which depend on insulin action for their regulation. This overall collapse in endocrine relay leads to further complications in the ability of the hypothalamus to regulate energy intake and expenditure. IR on the other hand does not remain exclusively a gluco-deregulatory syndrome. The lack of insulin sensitivity in adipose and liver tissue leads to dyslipidemia associated with IR since these tissues not only start taking up lower amounts of circulating lipids but also hydrolyze endogenous lipid stores and exporting them as FFA into the circulation (80). Liver and intestine also loose their ability to suppress the post-

prandial release of TRLs in response to insulin leading to excessive circulating lipids and causing dyslipidemia (24). The complex link between obesity and insulin resistance although still not completely understood has been addressed in few studies. A complex interaction between elevated FFA and inflammatory cytokines seen in obesity activating Protein Kinase C (PKC) isoform theta has been reported. PKC Theta inhibits Insulin Receptor Substrate (IRS) activation thereby preventing insulin-mediated glucose up-take (102). But studying individual pathophysiological outcomes such as obesity and insulin resistance does not deal with the root cause of the development of the metabolic syndrome. For this purpose we need to look at organs which directly deal with the nutrient handling absorption and assimilation, namely the intestine.

1.3.4 Intestinal contribution to the metabolic syndrome

Possible derangements in intestinal homeostasis have for long been unnoticed since they are not among the prime pathophysiological outcomes of the metabolic syndrome. But the intestine is known to play a crucial role in nutrition sensing, absorption and assimilation. The intestine releases several hormones in response to various dietary components which in turn regulate other hormones as well as metabolic and biochemical activities or other organs. The secretion of these peptides varies in the antero-posterior axis. Some of these are secreted exclusively by the anterior small intestine such as cholecystokinin (CCK), from the duodenum whereas others such as glucagon like peptide 1 (GLP-1) are secreted from the distal ileum. A compliment of these peptides also regulate food intake and hence are called satiation peptides, others such as glucose dependent insulinotrophic polypeptide (Gip) and GLP-1 act as incretin hormones (gastro-intestinal signals that lead to insulin release from the beta cells).

These peptides also act on enterocytes of the intestine and regulate absorptive processes foe example Gip regulates the formation and release of chylomicrons in absorptive enterocytes. These peptide hormones thus regulate the metabolic functions of diverse organ systems. Most of the intestinal hormone/peptides are released by the intestinal entero-endocrine cells in response to the luminal

nutritional content with differing potencies for different nutrient classes. Their release is proportional to the caloric load for example GLP-1 and peptide YY (PYY) are secreted by distal intestinal L-cells with the highest macronutrient potency for dietary lipids greater than that of carbohydrates greater than that of proteins(34).

Under IR, it has been shown that intestinal TRL output is enhanced due to the loss of insulin sensitivity. This contributes to the post-prandial overproduction of chylomicron particles from the intestine and dyslipidemia. Chylomicrons are the largest of all lipoproteins and are rich in TG. Thus, an insulin resistant intestine would lead to an increment of peripheral lipid deposition and would increase the risk of cardiovascular disease.

Apart from the expression of insulin receptor, enterocytes also express leptin receptor and respond to adipocytes leptin release. Leptin potentiates gastro intestinal (GI) satiation signals both by enhancing gut-peptide secretion for example, GLP1 release from distal-intestinal L-cells and/or by heightening vagal-afferent responsiveness to gut peptides for example, to CCK from proximal intestinal I-cells. Thus, under conditions of GI leptin resistance, the intestinal satiation mechanisms also fail to assist hypothalamic regulation of energy intake.

Therefore, the GI tract especially the small intestine plays a crucial role in regulating various steps which when deregulated lead to the development of metabolic syndrome.

1.4 *Aims of thesis*

The introduction describes how the patterning in the small intestine leads to the regulation of specific sets of genes in a spatio-temporally restricted manner. This puts the regulators of transcription such as transcription factors into the limelight for identifying mechanisms leading to disease specific transcriptional deregulation. It also poses an interesting scenario where the inability of the distal intestine to absorb lipids is coupled to the lack of expression of important transporters involved in the transport and uptake of lipids. The effect of inhibiting

transcriptional activity in an intestine specific manner is therefore exciting for the understanding of spatial gene expression and its impact on physiological alteration in the small intestine. We hypothesize that the lack of a homeotic transcription factor GATA-4 that leads to posteriorization of the anterior intestine would mimic the bariatric surgical procedure of ileal interpositioning where the ileum is placed more proximally leading to an earlier release of factors regulating nutrient overload.

Specific Aim 1:

To study the impact of intestinal GATA-4 deficiency on plasma lipid and lipoprotein levels and whole body lipid deposition.

Specific Aim 2:

To study the expression levels of intestinal transporters linked to the uptake and absorption of dietary lipids, in the intestines of mice lacking intestinal GATA-4.

Specific Aim 3:

To study the impact of intestinal GATA-4 deficiency on the development of diet induced obesity.

Specific Aim 4:

To determine the effect of ectopic overproduction and or earlier release of posterior intestinal endocrine factors on diet induced diabetes mellitus.

II. Materials and Methods:

2.0 *Animals and diets*

Generation of viable Gata4loxP (Gata4tm1Sad), Gata4null (Gata4tm1Eno), and VilCre (Tg(Vil-cre)997Gum) mice has been described previously (29, 32, 49). Homozygous GATA4LoxP mice were bred with the heterozygous GATA4nullVilCre mice to generate GATA4LoxP/nullVilCre intestine-specific knockouts (GATA4iKO) and GATA4LoxP/+ VilCre controls. All experiments were performed on male mice. Mice had free access to food and water under a 12-hour light/12-hour dark cycle in a temperature-controlled environment. Individually housed knockout and control littermates (n = 10) were switched to a western type diet (WTD) or continued on a normal chow diet (11.9% caloric intake from fat, Ssniff®, Soest, Germany) at 6 weeks of age. WTD contained 21% (wt/wt) crude fat and 0.15% (wt/wt) cholesterol and was ≈ 4.53 kcal/g (42% of calories from crude fat, 15% from protein, and 43% from carbohydrate; Ssniff®, Soest, Germany). Mice were weighed weekly for a period of 20 weeks.

2.1 *Biochemical analysis*

Blood was collected through the tail vein and plasma was used for further determinations. Triglycerides (TG), total cholesterol (TC) and free fatty acids (FFA) were assayed in fed and fasted plasma using enzymatic kits (DiaSys, Holzheim, Germany; Wako Chemicals GmbH, Neuss, Germany) according to manufacturer's protocol. Plasma lipoproteins were separated using the Pharmacia P-500 fast protein liquid chromatography (FPLC) system and a Superose 6 column (Amersham Biosciences, Piscataway, NJ). Plasma was pooled from 5 mice and TG concentrations in lipoprotein fractions were measured enzymatically. Plasma apolipoprotein profiles were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by Coomassie blue staining. 1µl freshly isolated plasma from fasted male mice was loaded onto a gradient polyacrylamide gel (4% - 15%) for separation followed by an overnight Coomassie blue staining. Measurement of

plasma bile acid levels was performed using the DiaSys Ecoline kit (DiaSys, diagnostic systems GmbH, Holzheim, Germany).

Enzyme linked immunoassays (ELISA) were performed using kits for insulin and leptin (Crystal Chem, Inc., Downers Grove, IL), adiponectin (R&D Systems, Inc., Minneapolis, MN) and glucose-dependent insulinotropic polypeptide (Gip) (Millipore Corp., Inc., Billerica, MA) in control and GATA4iKO mice (n = 5). Glucagon like peptide-1 (GLP-1) was measured using radioimmunoassay (Linco Research, St. Charles, MI) on both dietary regimens (n = 4). Blood glucose levels were measured using Glucometer Accu-check-active (Roche diagnostics, Palo Alto, CA).

2.2 *GLP-1 and Gip release assay*

For release assays of GLP-1 and Gip, overnight fasted mice were gavaged with 200 µl corn oil (98% triglyceride) and blood was drawn at various time points for analysis. Alternatively, to study the effect of increased GLP-1 on glucose-stimulated insulin release, overnight fasted mice were gavaged with 200 µl corn oil (98% triglyceride). After 30 min, the mice were challenged with an oral glucose bolus (2 g/kg body weight). Plasma samples were drawn at various time points and subsequently insulin levels were measured.

2.3 *Absorption of dietary fat and assay for luminal lipolysis*

We assessed dietary fat absorption using radioactive (^{3}H)-triolein. Mice (n = 6) fasted 10 h were intra-peritoneally injected with the lipase inhibitor Tyloxapol (500 mg/kg in PBS) to prevent peripheral lipolysis. Following 30 min after injection, blood was collected from the tail vein before (0 h) and 1 h, 2 h and 4 h after an oral gavage of 1 µCi (^{3}H)-triolein in 200 µl corn oil per mouse. Radioactive counts were measured by liquid scintillation counting and expressed as cpm per ml plasma. Luminal lipolysis was assayed in GATA4iKO and control mice as previously described (8). Briefly, 1 µCi (^{3}H)-triolein and 1 µCi (^{14}C)-oleate were simultaneously gavaged to fasted mice (n = 4), and levels of both tracers were measured in plasma drawn at 0 h (before gavage) and 1 h, 2 h, 4 h, and 6 h

(after gavage) by liquid scintillation counting. Radioactive counts were presented as percent of dose per ml plasma.

2.4 *Histological staining*

5µm sections were prepared from formalin-fixed, paraffin-embedded gonadal white adipose tissues from GATA4iKO and control mice (n = 3) and subjected to hematoxylin and eosin (H&E) staining using standard procedures.

2.5 *Tissue distribution and fecal output of dietary fat*

Uptake and incorporation of TG was studied in duodenum, jejunum, ileum, liver, white adipose tissue and feces. Non-fasted mice on chow (n = 4) were gavaged with 1 µCi (^{3}H)-triolein in 200 µl of corn oil. Four hours post-gavage, 1 cm segments of the duodenum (1cm from pylorus), jejunum (1cm of medial) and ileum (1cm pre-cecum) were excised and flushed in 0.5 mM sodium taurocholate in PBS, and feces were collected. Tissues were weighed and solubilized in 1 ml of 1 N KOH at 65°C overnight, and feces were dried and pulverized. Radioactivity was measured by liquid scintillation counting and expressed as radioactive counts percent of dose per gram organ or feces. Additionally, the feces of both chow diet- and WTD-fed mice (n = 7) collected daily over a 4-day period were dried, pulverized and subjected to Folch extraction (19) with slight modifications. Briefly, tissues were weighed and homogenized and lipids were extracted in chloroform-methanol (2:1) in a volume 20 times the weight of the sample. Lipid extracts were then solubilized in freshly prepared 0.2% Triton X-100 in chloroform, dried under nitrogen and resuspended in water. TG and FFA were measured enzymatically.

2.6 *Fecal fat balance*

2.6 (a) 72-hour fecal fat balance:

72-hour fecal fat balance was measured as described previously (50) with slight modifications. Mouse WTD and fecal pellets collected over 72 h were weighed, freeze-dried and homogenized. Samples were hydrolyzed, derivatized and

measured using Electron impact-gas chromatography-mass spectrometry (EI-GC-MS)

2.6 (b) Preparation of trimethylsilyl-ether derivatives:

Pre-weighed lyophilized material was transferred to Pyrex tubes and extracted according to the Folch procedure in the presence of 100 µg stearic-d35-acid (C18:0-d35, Isotec) as an internal standard (19). For quantification of FFA in mouse feces, 10 µl of 1000 µl lipid extract were converted to the corresponding trimethylsilyl (Pierce, Rockford IL, US) ether derivatives in 100 µl MSTFA-solution (ABCR, Karlsruhe, Germany), (MSTFA, containing 1% TMCS, in pyridine (2/1, v/v)) for 60 min at 25°C, and stored at -20°C until GC-MS analysis. To quantify total amounts of fatty acids in the diet, esterified fatty acids were hydrolyzed in 0.5 M NaOH at 90°C for 60 min, neutralized with 0.5 M HCl, extracted according to the Folch procedure (19) and derivatized as mentioned above.

2.6 (c) EI-GC-MS:

A Thermo Scientific Trace GC coupled to a DSQII mass spectrometer was used. The GC was fitted with a SGE BPX5 capillary column (15 m, I.D: 0.25mm inner diameter, 0.25µm methyl silicone film coating). The injector was operated in the splitless mode at 180°C. Helium was used as carrier gas at a flow rate of 1 ml/min. Initial column temperature was 80°C for 2 min, followed by an increase of 30°C/min to 170°C, an isothermal hold of 2 min, a second increase at 30°C/min to 230°C, an isothermal hold for 3 min and increased by 20°C/min to 300°C with a hold for 3 min. The transfer line was kept at 310°C and the ion source was 200°C. Electron impact spectra were recorded with electron energy of 70 eV, and an emission current of 100 µA. All samples were monitored either in full scan mode or using selected ion recording (SIR). SIR was performed at m/z = 313 (C16:0; RT:8.97 min), m/z = 341 (C18:0; RT:10.01 min), m/z = 376 (C18:0-d35; RT:9.83 min), m/z = 339 (C18:1; RT:9.84 min), m/z = 337 (C18:2; RT:9.82 min). Quantitation was performed by peak area comparison along with a standard calibration curve consisting of unlabeled primary fatty acid standards (Sigma, St Louis, MO, USA) mixed with the stable isotope labeled standard. All solvents and

reagents of analytical grade were from (Sigma, St Louis, MO, USA) and Roth (Vienna, Austria). Absorption of four major dietary fatty acids (palmitate, stearate, oleate, linoleate) was determined as a difference between the amount of individual fatty acids ingested and excreted into feces over 72 h.

The measure was expressed as:

$$\% \text{ of total fat absorption} = \frac{\text{Fat intake (g day-1)} - \text{Fecal fat output (g day-1)} \times 100}{\text{Fat intake (g day-1)}}$$

Total fat absorption was quantified as the difference in fecal loss of the four major fatty acids from the total amount ingested.

2.7 *RNA isolation and quantitative real-time PCR*

Total RNA from tissues of mice kept on WTD (n = 5) and on chow (n = 5) was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Two microgram of total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed on a Roche LightCycler 480 (Roche Diagnostics, Palo Alto, CA) using the QuantiFastTM SYBR® Green PCR Kit (Qiagen, Valencia, CA). Samples were run in triplicates for each experiment with Cyclophilin A as an internal control. For analyzing the expression profiles and associated statistical parameters the public domain program Relative Expression Software Tool – REST 2008 (http://www.gene-quantification.com/download.html) was used (37). Primer sequences are available upon request.

2.8 *SDS-polyacrylamide gel electrophoresis and Western blot analysis*

To determine the levels of total Akt, phospho-Akt (Ser 473), total IRS-1, phospho-IRS-1 (Ser 307) and beta-actin as loading control, SDS-PAGE followed by Western blotting was performed on tissue lysates prepared in RIPA buffer from control and GATA4iKO livers. Proteins from the lysate were separated on a 10%SDS-PAGE denaturing gel. Antibodies against indicated proteins were obtained from Cell Signaling technology, USA.

2.9 *In vivo MR imaging for body fat*

Mice were anesthetized with Isoflurane according to the guidelines of the local steering committee. MR images were acquired at a 3T MRI system (Siemens Tim-Trio, Erlangen, Germany) with an 8-channel multipurpose coil (Noras MRI products, Hoechenberg, Germany) to maximize signal-to-noise ratio. For fat-measurements two T1 weighted 2D Turbo Spin Echo (TSE) sequences one with spectral selective fat suppression and one without were carried out. The following imaging parameters were used: TR, 800ms; TE, 37ms; TF, 5ms. A field of view 90x45mm with a 384x192 matrix provided an in-plane resolution of 230x230µm with a slice thickness of 1.2mm. 12 excitations were averaged. Post-processing: For the fat-quantification the image without fat saturation was divided by its fat saturated counterpart in order to cancel signal-variations coming from coil non-uniformities. The ratio between the images was then interpreted as (fat + water)/water. Each pixel with a ratio of >1 was chosen for volume summation to get the collective fat volume over all images. This value was then multiplied with the mean fat-fraction: (1 – water / (fat + water)) to get the fat-volume. All experiments have been approved by the Committee for Genetic Engineering and Animal Experimentation at the Austrian Federal Ministry of Science and Research (Vienna, Austria) and by the Ethics Committee for Animal Experiments of the Medical University of Graz.

2.10 *Oral glucose tolerance test*

The oral glucose tolerance test was performed as described previously (4, 18). Briefly, blood was drawn through the tail vein from controls and GATA4iKO, mice (n = 5) fasted for 6 h before gavage (0 min) and 15, 30, 60 and 120 min after an oral gavage of 2 g D-glucose/kg body weight. Blood glucose levels were measured immediately using Glucometer Accu-check-active (Roche Diagnostics, Palo Alto, CA).

2.11 *Gastric emptying*

Liquid gastric emptying was assessed in both genotypes as previously described (6, 31, 47). Briefly, mice fasted overnight were challenged with oral gavage of

200 µl (0.6 g/l) phenol red (3.5 mg/kg) in a glucose solution (6.5 mg/kg). Mice were sacrificed by cervical dislocation after 30 min. stomachs were collected in 0.1 N NaOH and homogenized. The homogenate was centrifuged at 8500 rpm for 15 min. 500 µl supernatant was treated with 50 µl TCA (20%) and centrifuged as above. Finally supernatants were treated with 0.5 N NaOH (1:1 v/v) for maximum color development. Phenol red concentration in the mixture was then measured at 560 nM. Percent gastric emptying was calculated as

% Gastric Emptying = 1 – (amount of phenol red in the stomach ⁄ average amount recovered from control stomach) * 100

2.12 *Measurement of hepatic VLDL release*

Hepatic release of VLDL was measured in overnight fasted control and GATA4iKO mice without prior exposure to dietary insults. Mice received an intra-venous injection of 500mg/kg body weight Tyloxapol (Sigma, St Louis, MO, USA) through the tail vein. Blood was drawn via retro-orbital puncture in EDTA vials at 1, 2, 4 & 6 h. post injection and plasma was isolated by centrifugation at 1,500 rpm, 4°C for 20 min. Plasma TG were measured enzymatically as described previously.

2.13 *Statistical analysis*

Plasma biochemical parameters in non-fasted and fasted states were compared independently. For body weights and plasma parameters, controls and GATA4iKO mice on chow and WTD were compared using two parameter analysis of variance (Two-way ANOVA) followed by Bonferroni posttest. For oral glucose tolerance tests, differences between control and GATA4iKO groups were compared using the Two-way ANOVA with repeated measures followed by a Bonferroni posttest. For absorption studies, intergroup differences were calculated using two-tailed, unpaired Student's t test, p values < 0.05 were considered significant.

2.14 *General Molecular Biological techniques, buffers and recipes*

2.14.1 Diethyl pyrocarbonate (DEPC) water

Four hundred µl DEPC was mixed with 400 ml ddH2O and thereafter kept under the hood overnight and autoclaved.

2.14.2 Western blotting buffers

(a) Lysis buffer:

One M dithiotheritol, 1 mM EDTA, 0.1 M KH2PO4, 1:2000 protease cocktail inhibitor (Sigma) and 0.25 M sacharose were mixed and pH 7 was adjusted with KOH.

(b) 10X running buffer:

150.1g glycin, 10 g SDS and 30.3 g Tris were adjusted to 1000 ml ddH2O.

(c) Separating gel buffer (Buffer 1)

Four ml of 10% SDS, 18,2 g Tris and 80 ml ddH2O were mixed and pH 8.8 was adjusted up to 100 ml with ddH2O.

(d) Stacking gel buffer (Buffer 2)

Six g Tris was dissolved in 90 ml ddH2O. pH 6.8 was adjusted with HCl and the volume was adjusted up to 100 ml with ddH2O.

(e) SDS sample buffer

2.15 g SDS ,0.76 g Tris and 45 ml ddH2O were mixed and pH 6.8 was adjusted with HCl. Thereafter, 10 ml glycerol (80 %) and a small quantity of bromophenol blue were added.

(f) 10X transfer buffer

One mg/ml EDTA, 30 g glycin and 12.1 g Tris were mixed in 1000 ml ddH2O.

(g) 10X washing buffer (TBS)

Ninety g NaCl, 5 g Tween 20, 100 ml of 1 M Tris-HCl (pH 7.4) were mixed in 1000 ml ddH2O.

(h) Separating gel

Reagents	**10% SDS-gel**
Acrylamide	2866.25 µl
Buffer 1	2712.5 µl
ddH2O	5171.25 µl
10 % SDS	100µl
TEMED	4.4 µl
10% APS	7.6 µl
Final volume	87804.4 µl

(i) Stacking gel

Reagents	**4% SDS-gel**
Acrylamide	326 µl
Buffer 2	500 µl
ddH2O	1650 µl
10 % SDS	21.5µl
TEMED	1.25 µl
10% APS	19 µl
Final volume	2517.75 µl

2.14.3 cDNA preparation

cDNA was prepared from RNA by reverse transcription. Two µg RNA in a final volume of 10 µl were reverse transcribed by using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, USA). High-Capacity cDNA Reverse transcription kit's master mix composition is as follows:

Component	Volume (µl)/reaction
10 X Buffer	2
25 X dNTP Mix (100 mM)	0.8
10 X RT Random Primers	2
Multiscribe reverse transcriptase	1
RNase inhibitor	0.7
Nuclease-free water	3.5
2 µg RNA/10 µl water	10
Total volume / reaction	20

Thermo-cycler conditions for reverse transcription

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 s	∞

2.14.4 Real Time PCR

cDNA was diluted 1:50 in ddH2O. Three µl of diluted cDNA and 1 µl from each primer (forward and reverse) were mixed with Quantifast Sybr Green PCR kit (Qiagen, Hilden, Germany). Quantitative real time PCR was performed on an ABI prism 7900 real time PCR instrument (Applied Biosystems, Vienna, Austria) and the data are displayed as expression ratios of target genes normalized to the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) for cells and cyclophilin A for tissues as internal reference in each sample. Quantitative real-time PCR data were analyzed by the 2-ΔΔCt method (Livak & Schmittgen, 2001)

2.14.5 Histology Protocols

(a) H&E staining

Incubate slides (Formalin-Fixed Paraffin-Embedded or cryo-fixed) for 30 minutes at 65°C & follow the following steps in ascending order:

1. 15 min Xylol
2. 2 min 100%, 70%, 50% ethanol each
3. 5 sec in aqua bidestillata
4. 3 min modified Haematoxylin :1g Haematoxylin in 1000ml distilled water, 0.2g Sodium iodide and 50g Potassium alum
5. 3 min under tap water
6. 2 min Eosin (0,1% solution), before first use put a few drops of 100% glacial acetic acid
7. Wash for 2 min each in 90% and 100% ethanol
8. Briefly rinse in Butylacetate for color differentiation
9. Mount using suitable mounting medium (glycerin or water based).

(b) Oil Red O Staining Protocol

Stock solution:

0,5 g Oil Red (Merck)

100 ml Isopropanol (Fluka)

Store for 24 hours at room temperature

Working solution:

60 ml stock solution

40 ml distilled water

Store for 24 hours at room temperature

Filter the working solution before use. Follow the following steps in ascending order.

1. Immerse slides in 60 % Isopropanol (in distilled water) for 5 min.
2. Transfer in coupling jars with working solution for 10 min.

3. Treat with 60 % Isopropanol (in distilled water) for 5 sec (for tissue sections only).
4. Wash in distilled water for 5 sec.
5. Counter stain nuclei in modified Haematoxylin for 5 min.
6. Wash under tap water for 5 min.
7. Mount using suitable mounting medium (water based).

III. Results

3.0 *(a) GATA4iKO mice exhibit decreased plasma lipid levels*

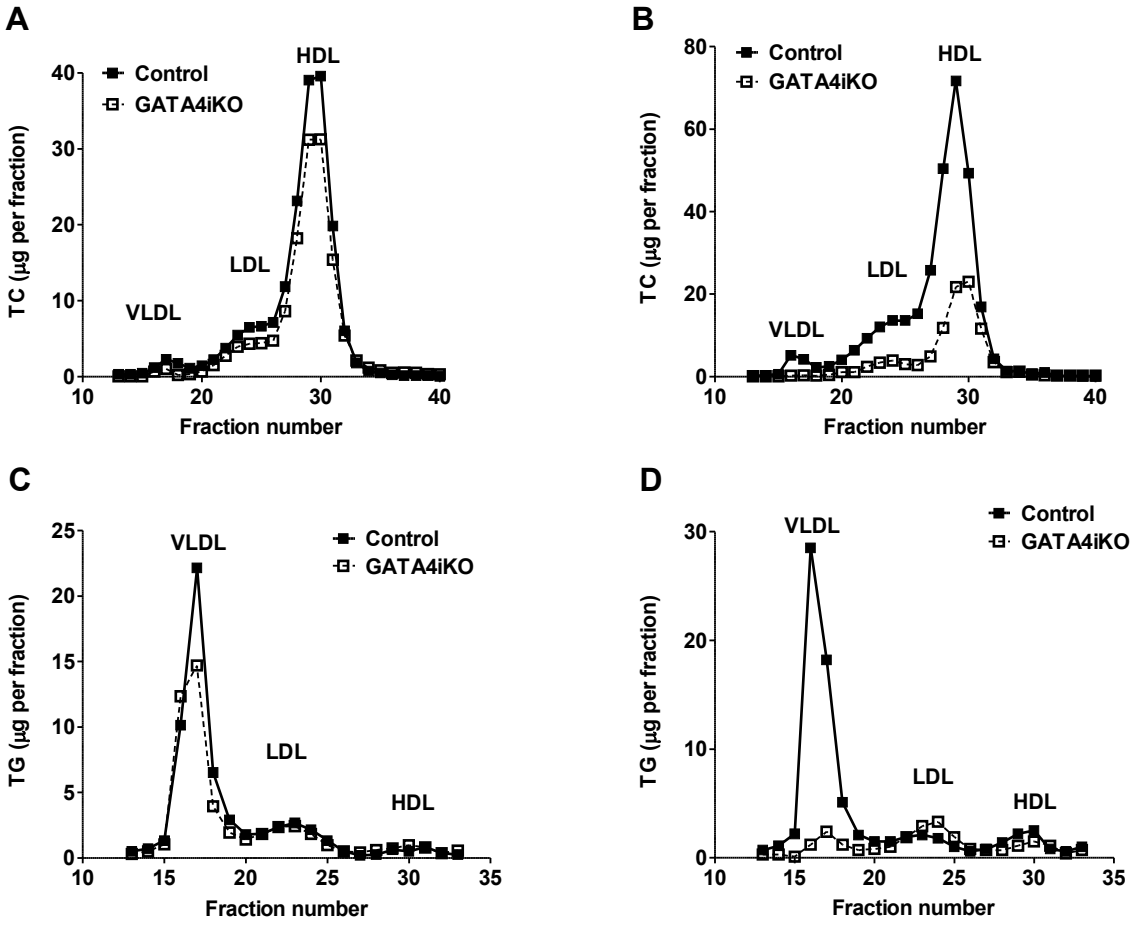

Figure 1

Plasma TC concentrations were significantly decreased on chow diet in both fasted (49%) and non-fasted (57%) states (Table 1). On WTD, GATA4iKO mice showed 43% and 70% less plasma TC in fasted and non-fasted states, respectively, when compared with controls. Lower levels of plasma cholesterol on a chow diet accounted for decreased HDL-cholesterol fraction in GATA4iKO mice (Fig. 1A). Decrease in HDL-cholesterol was more pronounced in mice fed WTD (Fig. 1B). GATA4iKO mice fed chow diet showed slight but not significant decrease in plasma TG concentration in both fasted and non-fasted states ($p = 0.0546$). On WTD, GATA4iKO mice showed 23% and 60% decrease in plasma TG concentrations in the fasted and non-fasted states, respectively, when

compared to controls (Table 1). The lower plasma TG levels were mainly due to decreased VLDL/chylomicron-TG levels in GATA4iKO mice (Fig. 1C & D).

Fasted	Chow		WTD	
	Control	GATA4iKO	Control	GATA4iKO
TG (mg/dl)	55.78 ± 11.47	45.12 ± 8.01	110.06 ± 6.51	85.27 ± 12.60 **
TC (mg/dl)	149.48 ± 13.60	75.25 ± 37.41*	232.76 ± 35.55	132.30 ± 24.52 ***
FFA (mmol/ml)	1.67 ± 0.40	1.38 ± 0.31	1.33 ± 0.05	1.19 ± 0.06
Glucose (mg/dl)	97.5 ± 7.59	88.25 ± 8.66	114.5 ± 25.09	114.75 ± 12.58

Non-fasted	Chow		WTD	
	Control	GATA4iKO	Control	GATA4iKO
TG (mg/dl)	63.36 ± 11.86	41.68 ± 8.27	209.69 ± 41.23	84.36 ± 11.49 ***
TC (mg/dl)	178.00 ± 39.85	95.07 ± 22.28**	391.75 ± 59.91	119.93 ± 28.53 ***
FFA (mmol/ml)	0.81 ± 0.18	0.64 ± 0.11*	0.83 ± 0.09	0.51 ± 0.12 ***
Glucose (mg/dl)	98.43 ± 19.89	108.56 ± 22.34	158.45 ± 21.78	126.65 ± 22.24*

Table 1 *Plasma levels (Mean, SD) of TG, TC, FFA and glucose of chow diet (n = 5) and WTD (n = 8) fed control and GATA4iKO mice in the fasted and non-fasted state.*

WTD, western-type diet; TG, triglycerides, TC, total cholesterol; FFA, free fatty acids; *p<0.05; **p<0.01; ***p<0.001 vs. control.

Non-fasted GATA4iKO mice had significantly lower plasma FFA concentrations in both chow diet and WTD-fed groups compared to control mice (Table 1). Plasma glucose levels in non-fasted mice were significantly reduced only in GATA4iKO mice fed WTD (Table 1). In the fasted state, plasma FFA and glucose levels were comparable on both chow diet and WTD (Table 1). FPLC and data analysis for plasma lipoprotein fractionation was performed by A. Ibovnik.

3.0 *(b) GATA4iKO mice have lower plasma ApoB100 and B48 levels*

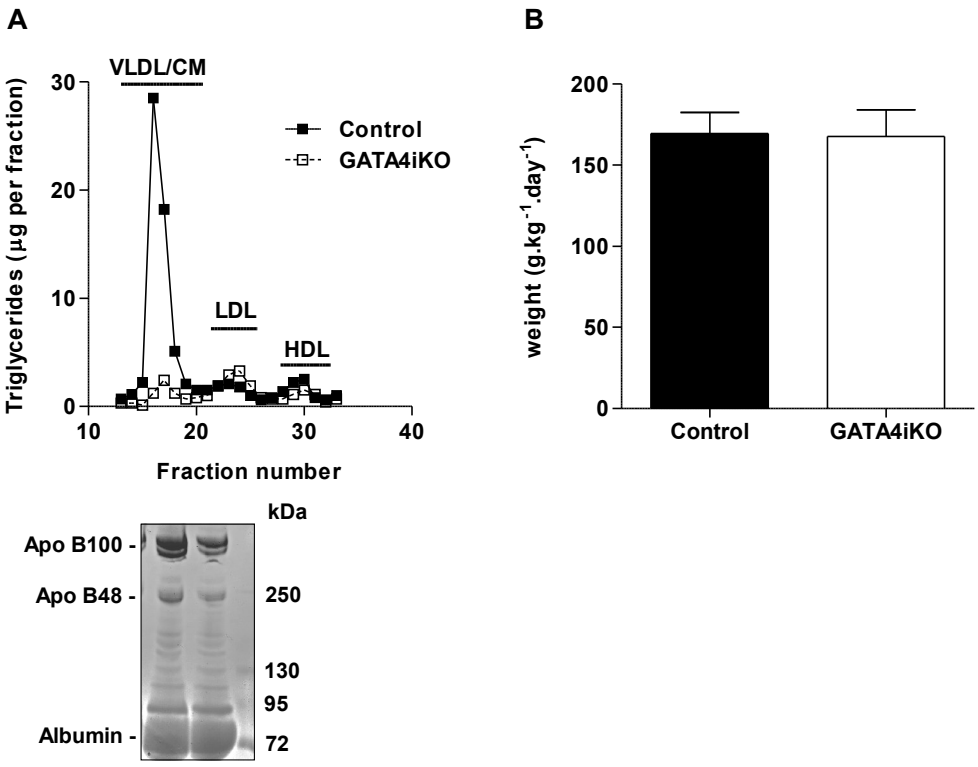

Figure 2

On a WTD, GATA4iKO mice showed decreased levels of plasma ApoB100 and ApoB48 (bottom inset Fig. 2A). This finding was in good correlation with decreased VLDL/CM TG levels in WTD fed GATA4iKO when compared to control mice (Fig. 2A). The food consumption was monitored over a 72 hour period where balance food was weighed each day and was found comparable in both groups (Fig. 2B).

3.1 *GATA4iKO mice exhibit impeded TG absorption*

To investigate the underlying cause of reduced TG levels in plasma of GATA4iKO mice, we examined intestinal absorption and uptake of dietary fat using a radiolabelled tracer method. After oral administration of corn oil containing (^{3}H)-triolein, a delayed accumulation of lipids in plasma of GATA4iKO mice was observed (Fig. 3A). After the oral gavage the radioactive counts were

measured in plasma where GATA4iKO mice showed a consistently lower accumulation of radioactivity at 1, 2, and 4 hours. Radioactive counts in the plasma of GATA4iKO mice at the 2 h time point were 43.5 % of that in control group whereas at the 4 h time point the plasma counts in GATA4iKO mice fell to 52.0% that of controls.

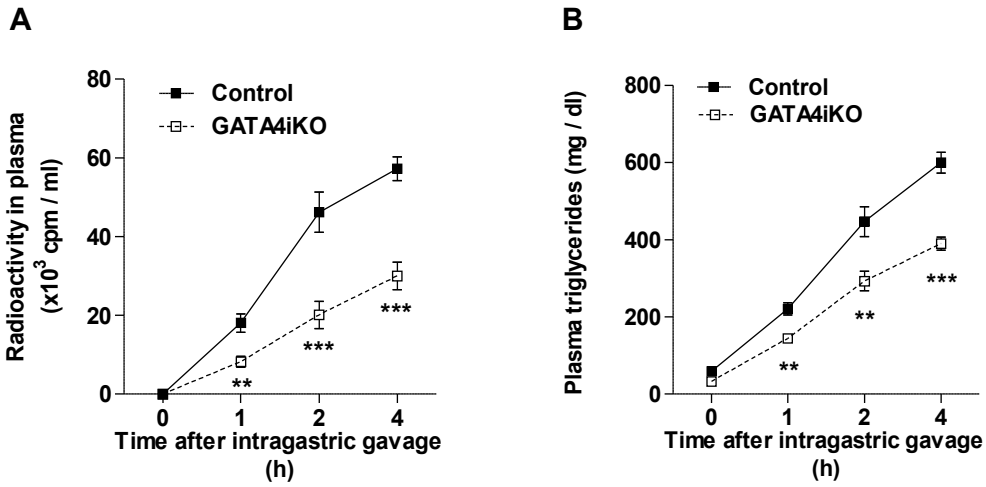

Figure 3

This decrease in the radioactivity was also reflected in the overall plasma TG levels monitored at the same time points. Plasma TG in GATA4iKO mice fell to 64.45% of controls at 2 h time point whereas to 65.0% at the 4h time point (Fig. 3B).

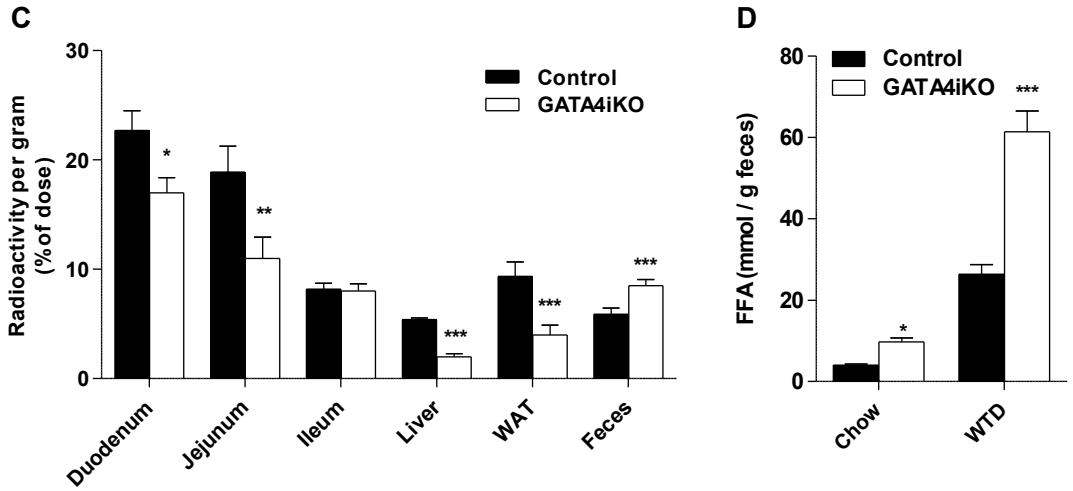

Figure 3

GATA4iKO mice also showed a 77.27% decrease of (^{3}H) counts in the duodenum and 61.11% decrease in the jejunum but no detectable decrease was observed in the ileum suggesting a compromised enterocytic lipid uptake into the proximal small intestine compared to controls (Fig. 3C). The fecal excretion of (^{3}H) was 60% higher in GATA4iKO mice compared to that of controls (Fig. 3D), confirming a higher fecal output of dietary fat which could not be absorbed owing to the reduced intestinal absorption. In addition, we found a 37% decrease in (^{3}H) counts in the liver and a 42% decrease in (^{3}H) counts in the white adipose tissue (gonadal fat pads) (Fig. 3C), which provides additional evidence for reduced intestinal absorption and a consequential reduction in incorporation into systemic lipid depots. GATA4iKO mice fed WTD also exhibited very high levels of fecal excretion of FFA (2.32 fold of control) (Fig. 3D), supporting decreased lipid uptake. The fecal excretion of FFA on normal chow was also 2.4 fold higher in GATA4iKO mice when compared to controls. However, fecal extracts of both controls as well as GATA4iKO mice when subjected to enzymatic measurements of TG, yielded no quantifiable outcomes supporting the complete luminal digestion of TG and a comparable luminal lipolytic activity between GATA4iKO and control mice (data not shown).

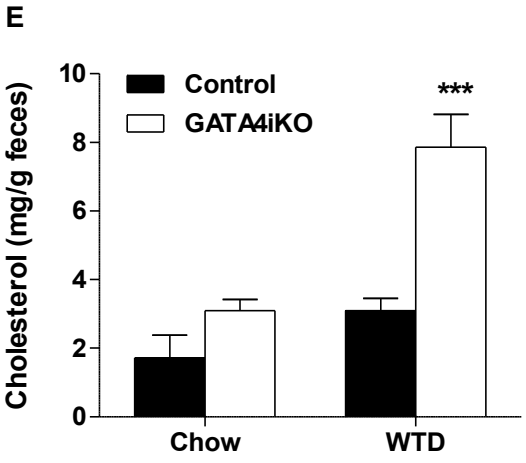

Figure 3

Additionally, the levels of fecal neutral sterols as determined enzymatically from fecal lipid extracts were 2.54 fold higher in GATA4iKO mice compared to control mice on WTD (Fig. 3E).

As an additional measure of dietary lipid malabsorption, we estimated the 72-hour fecal fat balance which revealed that GATA4iKO mice have a defective absorption of three of the four major dietary FFA species. Over a period of 72 hours, GATA4iKO mice showed a net reduction of 21.85% in their ability to absorb dietary FFA (Fig. 4A). The overall reduction was calculated as a summation of individual free fatty acids tested. Out of palmitate, stearate, oleate and linoleate, palmitate was the most affected FFA species, followed by stearate, oleate and linoleate respectively. Levels of linoleate were also found to be decreased in GATA4iKO mice but did not reach statistical significance.

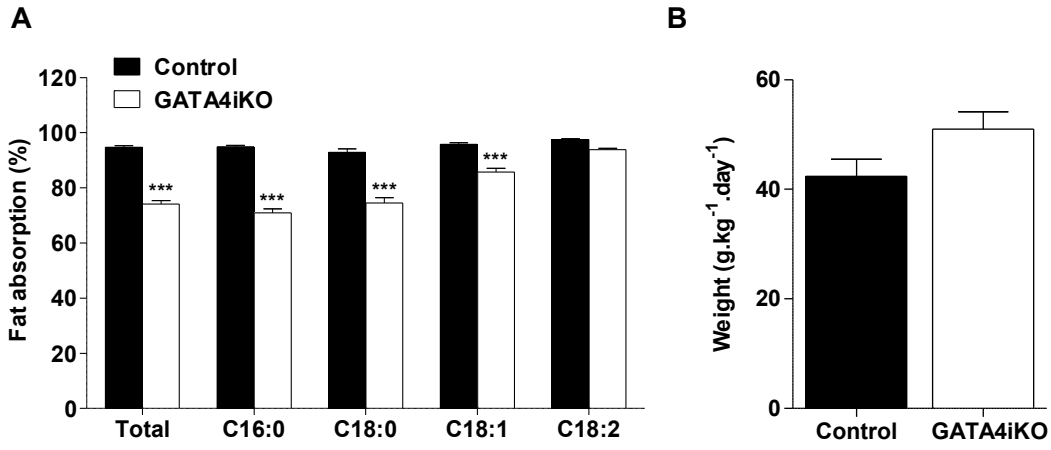

Figure 4

Palmitic acid (16:0) was decreased by 30.2%, oleic acid (18:1) by 25.5% and stearic acid (18:0) by 14.3% (Fig. 4A). Overall fecal weights tended to be higher in GATA4iKO mice compared to controls, but the differences lacked statistical significance (Fig. 4B). Measurement of 72h fat absorption was performed in collaboration with Prof. W. Sattler and Mag. A. Uellen, Institute of Molecular Biology and Biochemistry, Medical University of Graz.

3.2 *GATA4iKO mice have unchanged luminal lipolysis*

As stated previously, enzymatic measurements of TG from fecal extracts did not yield measureable results hinting that luminal lipolysis is not affected in GATA4iKO mice. To methodically test whether differential rate of luminal lipolysis contributes to the observed lipid malabsorption in GATA4iKo mice, a dual isotope gavage of TG and FFA was performed. Mice were gavaged with a

combination of (^{3}H)-triolein and (^{14}C)-oleate in corn oil followed by measurement of radioactive counts in the plasma at various time points. Absorption of both (^{3}H)-triolein as well as of (^{14}C)-oleate was equally compromised in GATA4iKO mice. As a result luminal lipolysis was found intact in GATA4iKO mice.

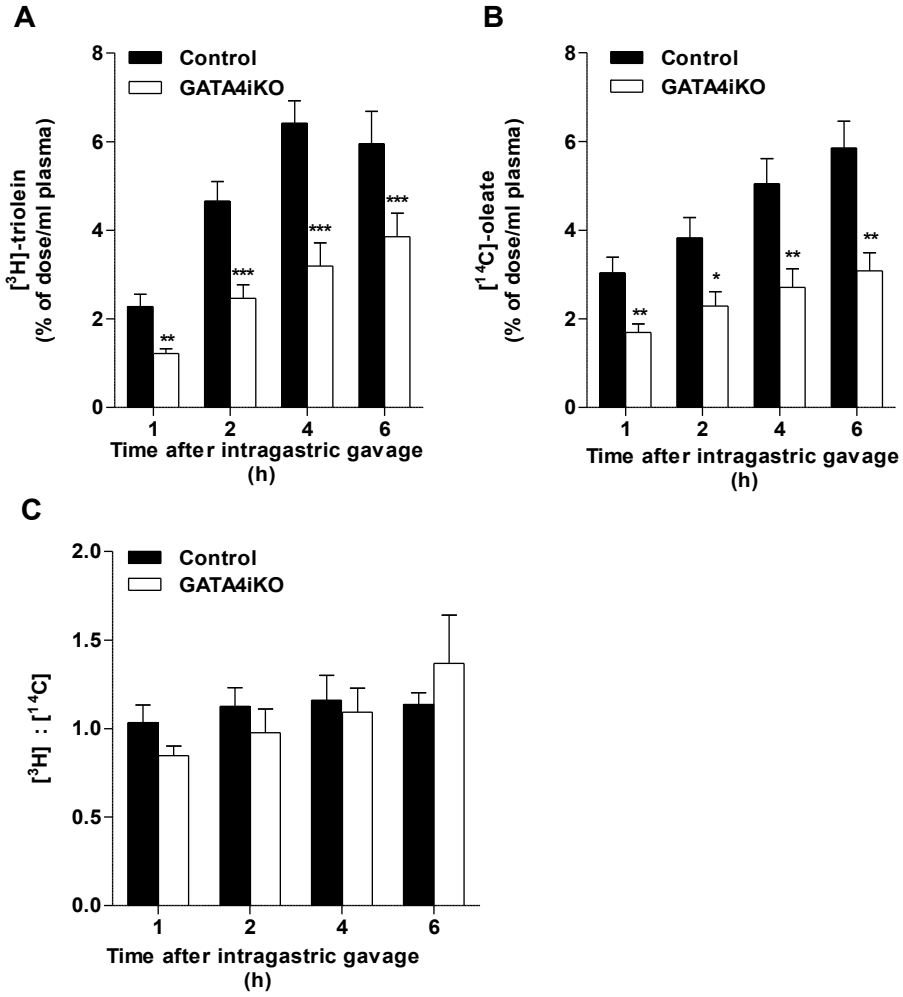

Figure 5

At the 4 hour time point the absorption of (^{3}H)-triolein was 2.06 fold compromised (Fig. 5A) which was comparable to the 1.80 fold reduction in (^{14}C)-oleate (Fig. 5B) at the same time point for GATA4iKO mice. This resulted in a comparable (^{3}H):(^{14}C) ratio (Fig. 5C) between GATA4iKO and control mice confirming that absorption of both triolein as well as oleate is affected to a similar degree in

GATA4iKO mice. This result shows that changes in luminal lipolysis rate do not contribute to the lipid malabsorption in GATA4iKO mice.

3.3 *GATA4iKO mice have altered intestinal expression of several lipid metabolic genes*

To further understand the causal mechanism leading to reduced absorption of lipids in the small intestine of GATA4iK mice, we measured the expression of genes involved in lipid absorption in the proximal small intestine. Intestinal expression of key genes involved in lipid uptake, transport and absorption was found to be downregulated in GATA4iKO mice. To study this we performed quantitative real-time PCR analysis independently from the duodenum and jejunum obtained from GATA4iKO and control mice fed both chow diet and WTD.

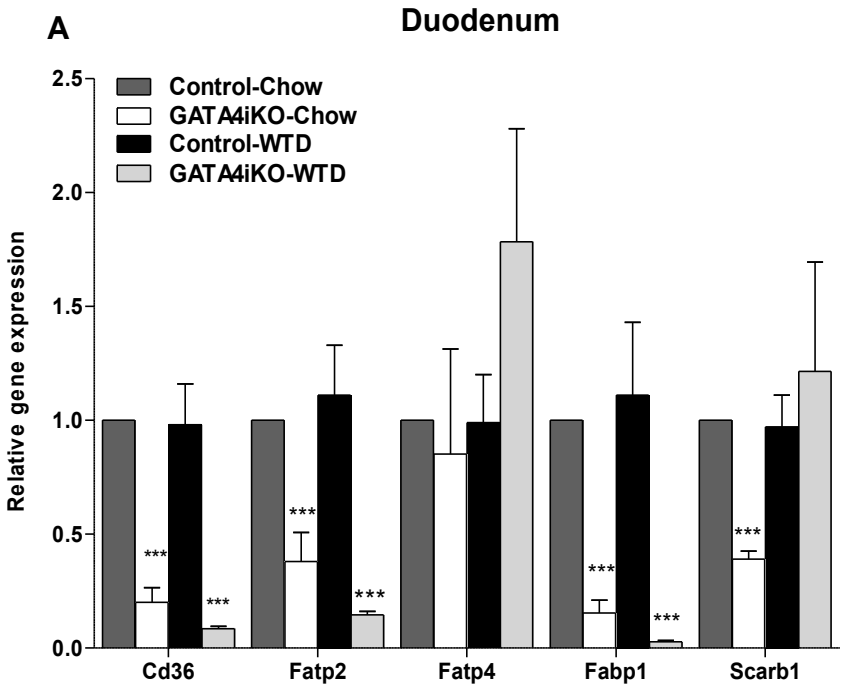

Figure 6

This analysis revealed downregulation of Cd36, fatty acid transport protein 2 (Fatp2), and Fabp1 in duodenum and jejunum of GATA4iKO compared to control mice on both diets (Fig. 6A & B). Relative expression levels of the key apical lipid transporter CD36/FAT were severely compromised (11-fold on WTD and 5

fold on chow diet in the duodenum and 30-fold on chow diet and 25-fold on WTD in the jejunum) in GATA4iKO mice suggesting a possible role of GATA4 in directly or indirectly regulating the transcription of this transporter.

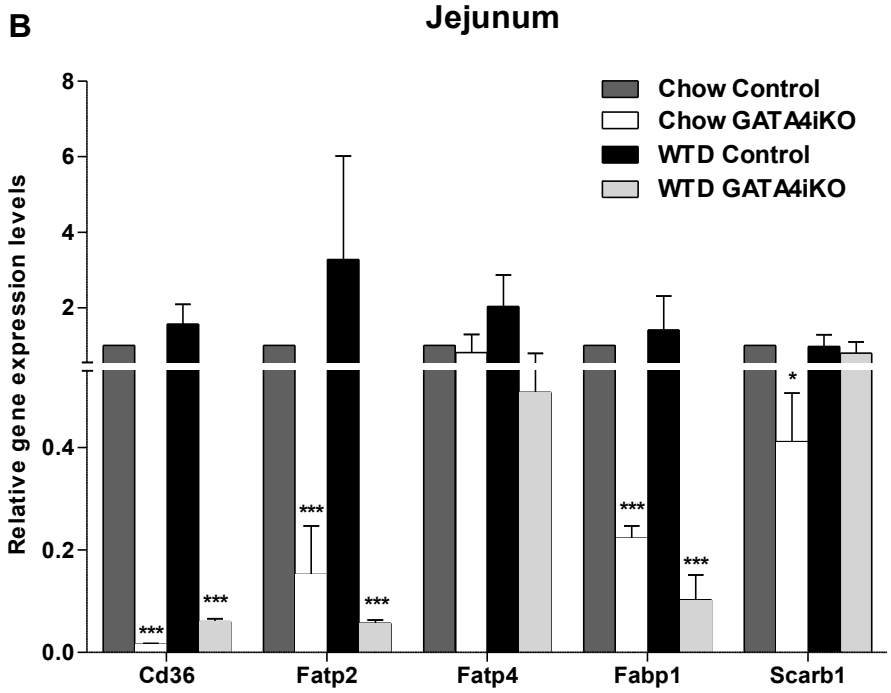

Figure 6

Expression levels of Scarb1 were decreased on chow diet (duodenum 33-fold and jejunum 2.3-fold) in the of GATA4iKO mice but were found to be comparable to control mice when fed a WTD (Fig. 6A & B).

Among proteins mediating intracellular transport of fatty acids the levels of both Fabp1 (4.5-fold chow and 13.6 fold WTD in the jejunum and 6.5-fold chow and 41.1-fold WTD in duodenum) and Fatp2 (6.5 fold chow and 57.0-fold WTD in the jejunum and 2.7-fold chow and 7.6-fold WTD in the duodenum) were downregulated, expression of Fatp4 however remained similar in both genotypes on wither diet in both the duodenum as well as the jejunum (Fig. 6A & B).

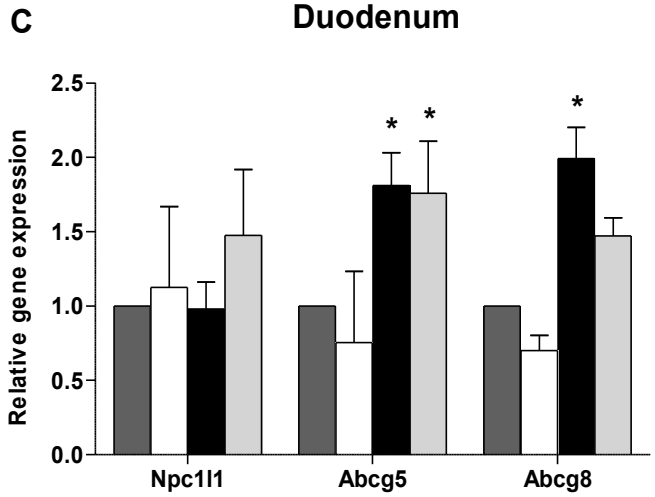

Figure 6

Although plasma cholesterol levels of GATA4iKO mice were significantly reduced on both diets (Table 1), mRNA expression of the cholesterol transporter Niemann-Pick C1 like 1 (Npc1l1) in the duodenum and jejunum was comparable to that of controls fed either diet (Fig. 6C & D).

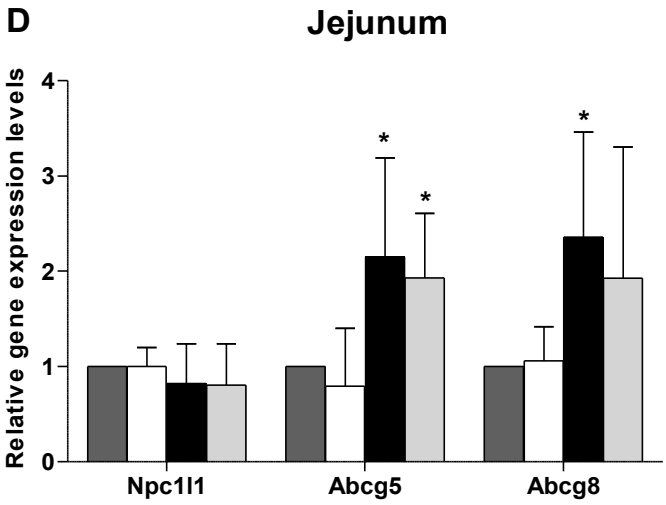

Figure 6

In accordance with previous studies (45, 51), we also detected significant increase in the expression levels of ATP-binding cassette transporter (ABC) G5 (Abcg5) and G8 (Abcg8) upon WTD in both genotypes (Fig. 4B & D) (Fig.6C & D). To test whether the liver contributes to the observed reduction in plasma

cholesterol levels in GATA4iKO mice, we checked the expression levels of Hmg Co-A reductase, a rate-limiting enzyme of the cholesterol biosynthetic pathway.

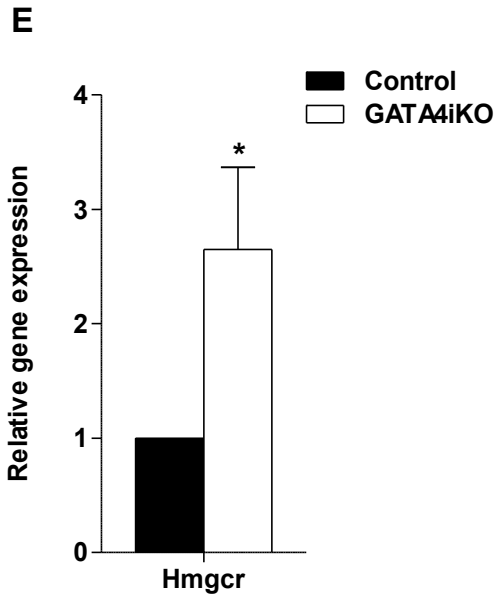

Figure 6

The mRNA expression of hepatic HmgCoA reductase was 2.8-fold upregulated in the GATA4iKO mice compared to control mice (Fig. 4F). This indicated that hepatic cholesterol biosynthesis could be normal to enhanced in the livers of GATA4iKO mice probably as a compensatory mechanism for reduced intestinal uptake.

To control for the lack of intestinal GATA4 we also measured the expression levels of small intestinal regional markers Fabp-1 (proximal) and sodium/bile acid co-transporter (Asbt) (distal). Expression of Asbt was induced in the jejunum of GATA4iKO mice and was comparable to that of control ileum (Fig. 4E).

The expression of jejunal Fabp-1 in GATA4iKO mice was decreased compared to control jejunum (Fig. 4F).

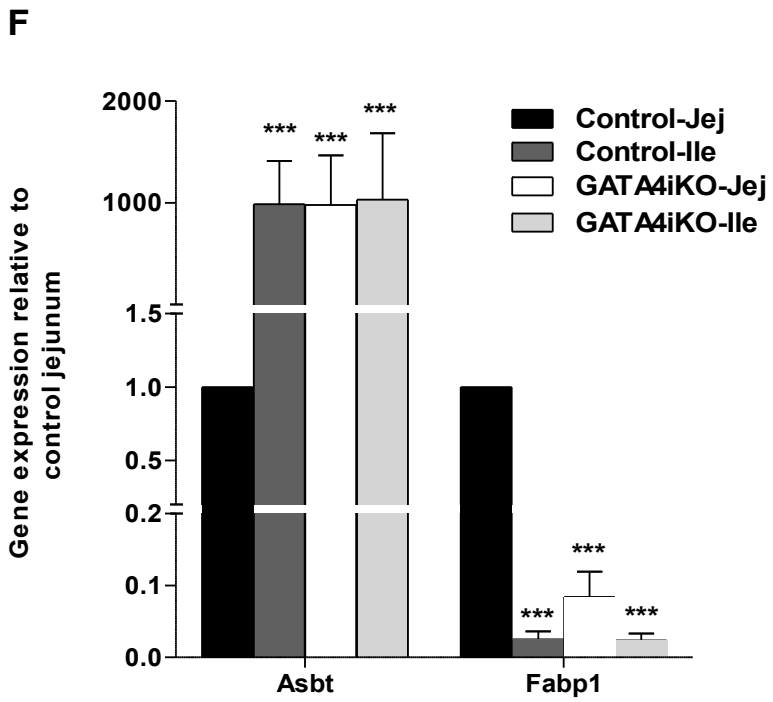

Figure 6

These results confirmed the previously reported jejuno-ileal homeosis in GATA4iKO mice.

3.4 *GATA4iKO mice are resistant to diet-induced obesity*

Next, we examined the impact of intestinal GATA4 deficiency on body and adipose tissue mass in mice fed WTD over a 20-week period. We found that GATA4iKO mice weighed less from the beginning of the feeding study. This difference was even more pronounced during WTD feeding, as GATA4iKO mice lost rather than gained weight in the initial 2 to 3 weeks of WTD feeding compared to controls (Fig. 5A).

Differences in body weights between controls and GATA4iKO mice reached significance at week 4. Over the feeding study, percent body weights of control mice continued to rise at an average rate of 0.97 per week whereas those of GATA4iKO mice rose at a rate of 0.66 per week.

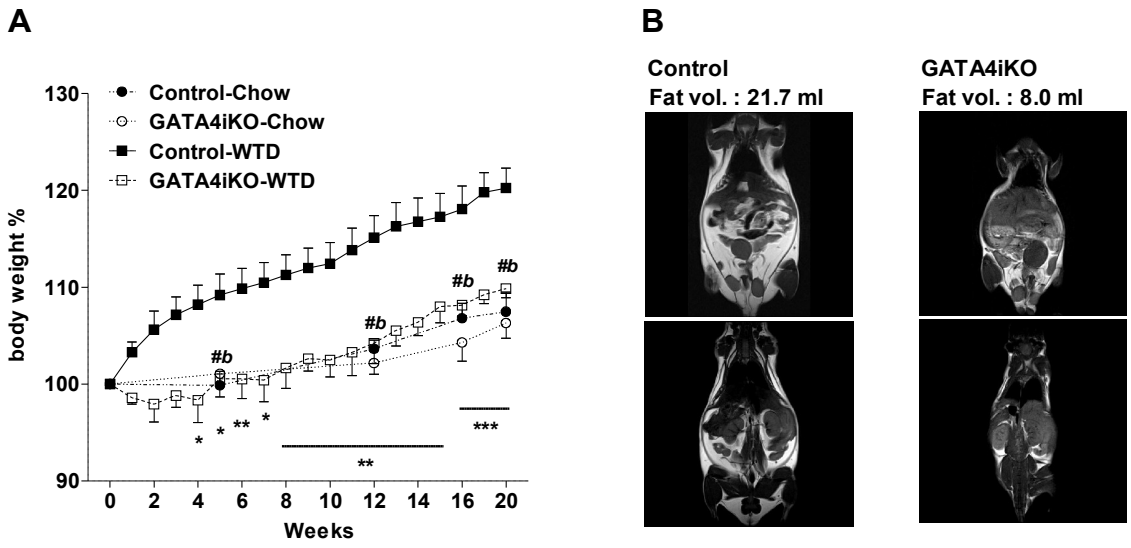

Figure 7

Finally, at the 20th week of WTD feeding, the GATA4iKO mice weighed on an average 10 ± 1.5 grams lesser than control mice (Fig. 7A). The decrease in weight gain was attributed to lower adipose tissue mass in GATA4iKO mice (Table 2). Liver, heart, spleen and brain showed comparable weights in both groups.

Organ	Control	GATA4iKO
Liver	4.87 ± 0.72	5.68 ± 1.28
WAT	4.19 ± 0.46	1.40 ± 0.42 ***
BAT	0.85 ± 0.20	0.57 ± 0.11 *
Heart	0.66 ± 0.11	0.55 ± 0.08
Spleen	0.32 ± 0.07	0.25 ± 0.09
Kidney	0.55 ± 0.12	0.60 ± 0.16
Brain	1.37 ± 0.27	1.15 ± 0.12
Mean body weight (g)	40.45 ± 3.28	32.7 ± 4.36

Table 2 *Organ weights in grams of WTD-fed control and GATA4iKO mice. Results are expressed relative to total body weight (Mean, SD) of 9 mice per group.* WAT, gonadal white adipose tissue; BAT, intra-scapular brown adipose tissue; *p<0.05; ***p<0.001 vs. control.To monitor body composition with regard to adipose tissue mass, GATA4iKO and their control littermates were subjected to T1-weighted magnetic resonance imaging. Figure 7B shows representative

coronal sections of epidermal, retroperitoneal, gonadal and perirenal fat pads of control and GATA4iKO mice in the 18th week of WTD feeding. GATA4iKO mice exhibited a 2.7-fold decrease in the fat volume when compared to control (8.0 ml vs. 21.7 ml). Overall reduction in body fat of GATA4iKO mice was calculated by subtracting the summation of the fat content in the gonadal (top) and peri-renal (bottom) sections. This difference between GATA4iKO and controls was found to be 2.6 fold (Fig. 7B). The magnetic resonance imaging was performed in collaboration with Prof. R. Stollberger and Mag. C Diwoky, Institute of Medical Engineering, Graz University of Technology.

3.5 *GATA4iKO mice retain adipocyte size as well as adipose tissue function upon WTD*

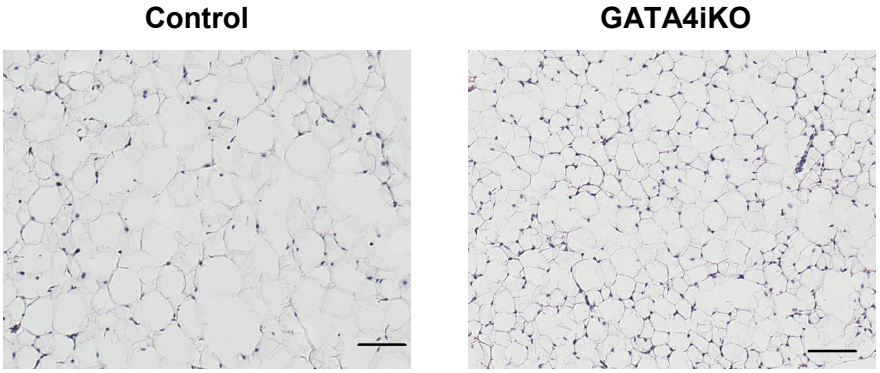

Figure 8

Adipocytes from the gonadal fat pads of GATA4iKO mice fed WTD retained a smaller size (Fig. 8) and showed an intact regulation of two important adipokines, leptin and adiponectin when compared to control littermates (Fig. 9A & B). Adipocyte hypertrophy is co-associated with the loss in the ability to regulate adipokine release in response to external queues. GATA4iKO littermates were able to retain a smaller adipocytes size which corresponded with better ability to regulate leptin and adiponectin release. Fasting plasma leptin concentrations of WTD fed GATA4iKO mice (Fig. 9A) were as low as chow fed mice. On WTD, GATA4iKO mice showed 7.5-fold lower fasted control leptin levels compared to control mice. Levels of adiponectin (Fig. 9B), were higher in GATA4iKO mice fed either diet compared to WTD fed control mice.

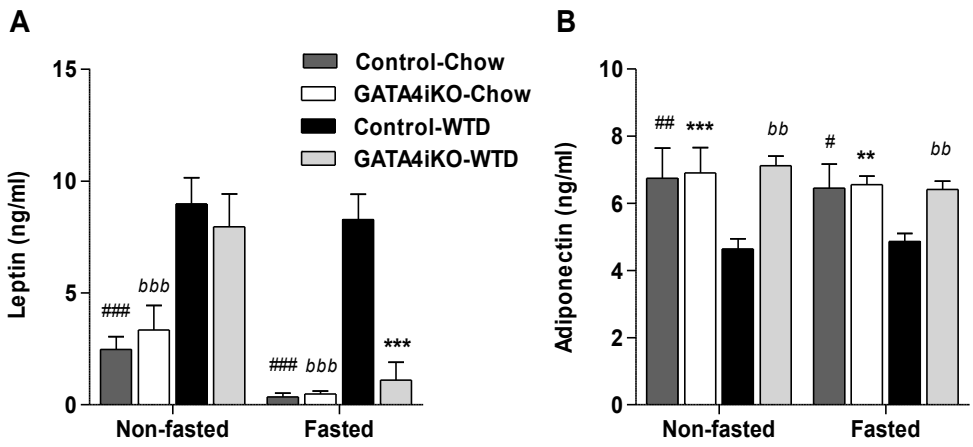

Figure 9

Especially on a WTD, fed levels were 1.5-fold higher and fasted levels were 1.3-fold higher in GATA4iKO littermates. Adipocyte histology was performed in collaboration with Prof. G. Hoefler, Institute of Pathology, Medical University of Graz.

3.6 *GATA4iKO mice are glucose-tolerant and insulin-sensitive on WTD*

To determine whether resistance to obesity was accompanied by increased insulin sensitivity, we measured plasma insulin levels and performed oral glucose tolerance test during the 18th week of WTD feeding.

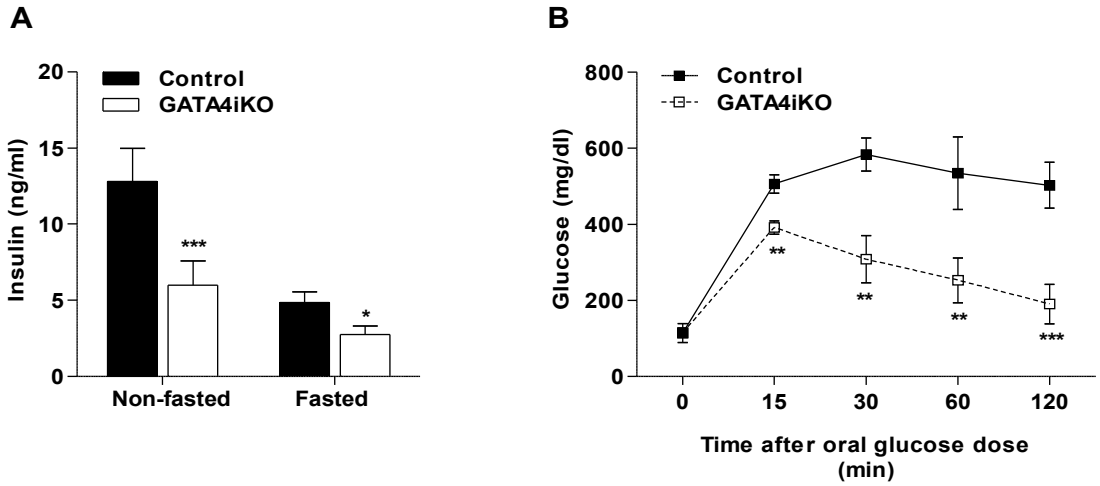

Figure 10

GATA4iKO mice remained eu-insulinemic in both non-fasted and fasted states (Fig. 10A) and showed higher glucose tolerance compared to controls (Fig. 10B). The levels of insulin in GATA4iKO mice were 2.1 fold and 1.77 fold lower in fed and fasted states respectively, when compared to control mice.

Plasma glucose levels were significantly lower (1.3-fold) at 15 minutes post oral glucose gavage and kept lower at an average rate of 0.87 mg/dl per minute in knockout compared to a rate of 0.49 mg/dl per minute in controls after the 30 minute time point (Fig. 10B). Hence, peripheral glucose uptake in GATA4iKO mice was about 0.38 mg/dl per minute higher than that of control litter mates.

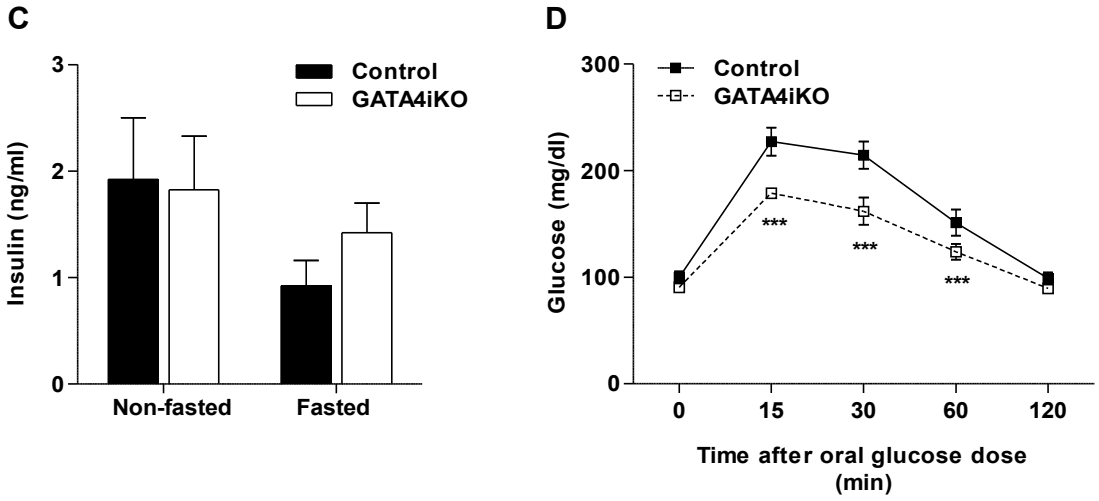

Figure 10

In chow diet fed mice, insulin levels in both groups were comparable in fasted and non-fasted states (Fig. 10C). However, GATA4iKO mice showed improved glucose tolerance even upon chow diet feeding (Fig. 10D).

3.7 GATA4iKO mice show higher GLP-1 levels

Next, we assayed the release of GLP-1 in mice under both dietary conditions. GATA4iKO mice fed either diet had increased release of GLP-1 (Fig. 11A and 11B).

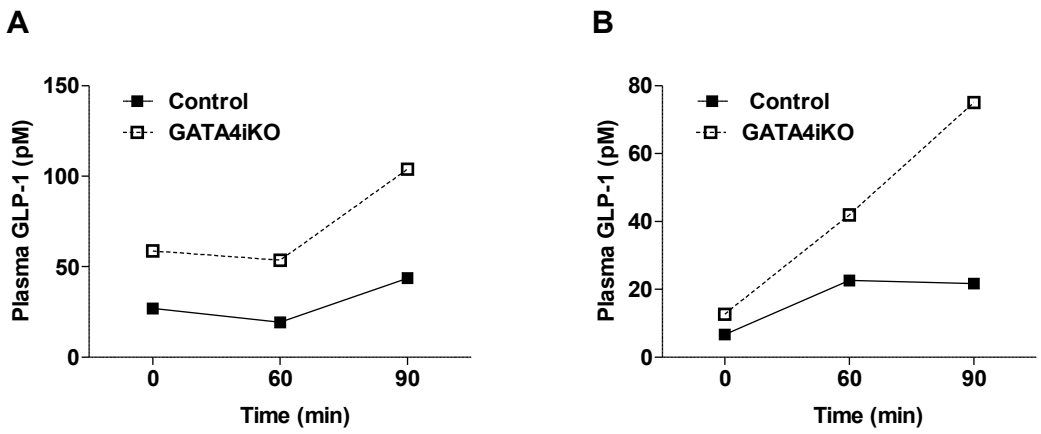

Figure 11

Measurement of GLP-1 levels was a contribution by Prof. A. Heinemann at the institute of experimental and clinical pharmacology, medical university of Graz.

3.8 *GATA4iKO mice show lower Gip release*

Consequent to lower lipid absorption and less plasma ApoB100 & B48 we investigated the release of Gip upon oral fat load since Gip secretion in response to triolein is required for chylomicron formation. The release of Gip in response to fat load was attenuated to 33% (30 min time point) in GATA4iKO mice (Fig. 12A), indicating that entero-endocrine factors are also subject to posteriorization which might influence the malabsorption phenotype.

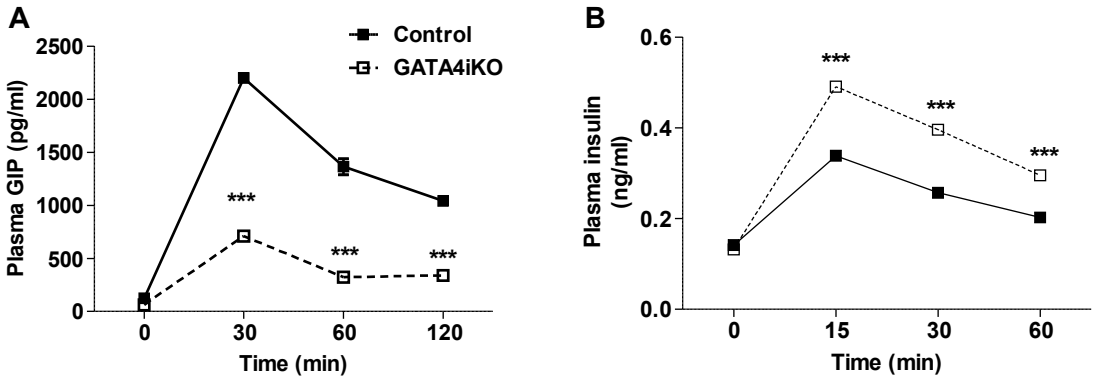

Figure 12

Owing to the incretin effect of GLP-1 we addressed whether GATA4iKO mice show an increased glucose-stimulated insulin release upon fat load. Insulin also inhibits release of triglyceride-rich lipoproteins and thus a higher or a quicker insulin peak would essentially assist in abrogating the release of chylomicrons and hence contribute to lower lipid absorption.

Although overall insulin levels were low due to overnight fasting, 25% increase in the peak levels of glucose-stimulated insulin release after a fat load in GATA4iKO mice were observed (Fig. 12B).

3.9 *GATA4iKO mice exhibit lower gastric emptying*

Given the increased GLP-1 levels, even at baseline (Fig. 11B), we investigated whether GATA4iKO mice showed lower levels of gastric emptying, a well known physiological effect of GLP-1.

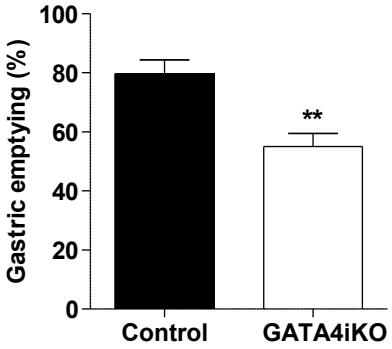

Figure 13

Percent liquid gastric emptying as investigated by the dye retention assay, was reduced by 24% in GATA4iKO mice compared to controls (Fig. 13).

3.10 *Plasma bile acid levels are higher in GATA4iKO mice*

Further we investigated the levels of circulating bile acids in the plasma of GATA4iKO and control mice. We found a highly significant increase in the levels of circulating bile acids in the plasma of GATA4iKO mice compared to control littermates. This observation indicates that the jejuno-ileal homeosis in the GATA4iKO mice leading to ectopic expression of the bile acid uptake and

transport pathway leads to higher absorption of bile acids which then enter the systemic circulation.

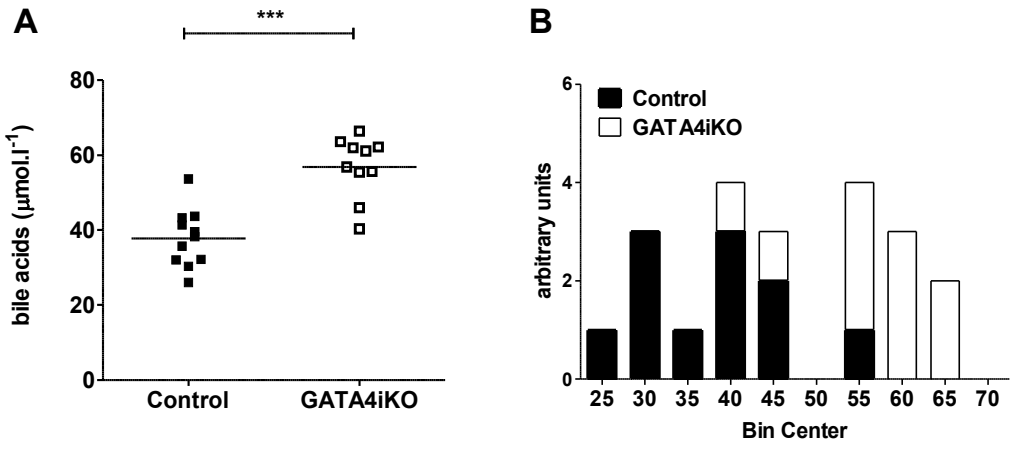

Figure 14

Plotting the histogram for frequency distribution also indicated that GATA4iKO mice fall in the higher bin quintile. Plasma bile acid levels for controls averaged at 37.8 µmol/L plasma whereas those for GATA4iKO mice averaged at 56.91 µmol/L liter plasma (Fig. 14A & B).

3.11 *GATA4iKO mice accumulate less hepatic lipids*

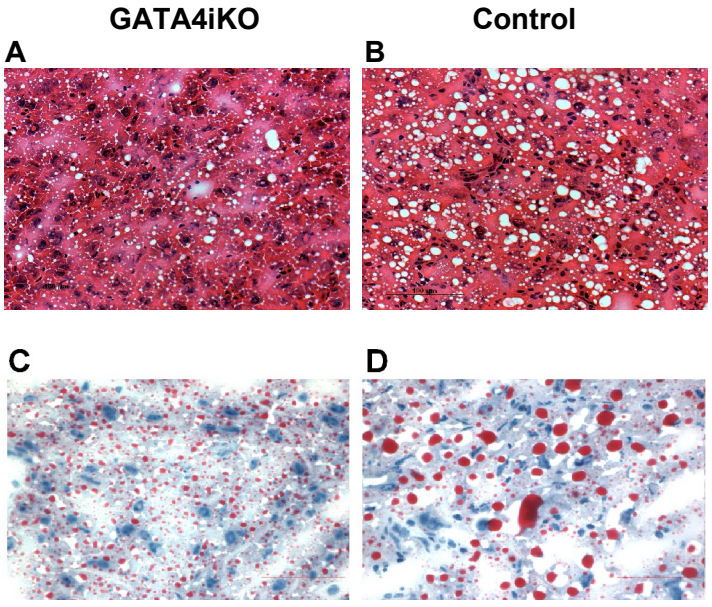

Figure 15

An assessment of hepatic lipid storage revealed that GATA4iKO mice had lower levels of hepatic lipids compared to control mice as revealed by Oil Red O staining for neutral lipids (Fig. 15C & D). H&E staining also revealed characteristic pathology associated with fatty liver in control mice which was found to be lower in case of GATA4iKO mice (Fig. 15A & B).

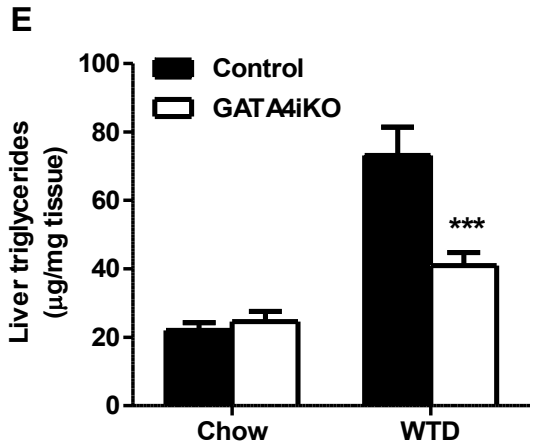

Figure 15

We also assessed the triglyceride levels in liver lipid extracts from of GATA4iKO and control littermates and found lower hepatic triglycerides in GATA4iKO mice compared to controls after 20 weeks of western type diet but levels were comparable on a normal chow diet (Fig. 15E). Triglyceride levels in WTD fed GATA4iKO mice averaged at 41 µg/mg tissue whereas those for controls were 73.12 µg/mg tissue (Fig. 15E).

When expressed as a percent of tissue weight, these values would represent 4.1% and 7.3% of liver weight for GATA4iKO and control mice, respectively (Fig. 15E). Oil Red O and H & E staining of the liver sections was a contribution of Prof. G. Hoefler, Institute of Pathology, Medical University of Graz.

3.12 *WTD fed GATA4iKO livers retain their ability to activate Akt*

Next, we measured the level of Akt phosphorylation. Akt is an important determinant in the development of fatty liver. In cases of insulin resistance and diabetes Akt phosphorylation does not occur efficiently, leading to the diabetes

associated postprandal-dyslipidemia arising mainly due the lack of insulin mediated suppression of VLDL release from the liver.

Western blot analysis from liver samples of control and GATA4iKO mice revealed that GATA4iKO mice were able to efficiently phosphorylate hepatic Akt as indicated in lanes 1,2 & 3 whereas control mice had comparable total Akt levels but very low levels of Akt phosphorylation shown in lanes 4,5 & 6 (Fig. 16).

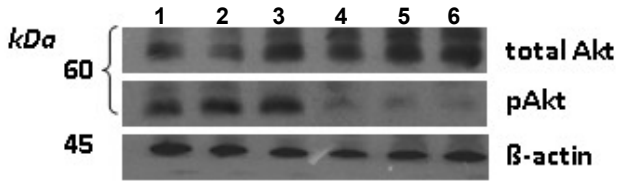

Figure 16

Thus the observed increase in insulin sensitivity reflected by the enhanced GLP-1 secretion in GATA4iKO mice translates to a lower hepatic lipid accumulation possibly via an intact phosphorylation of Akt (Fig. 16).

3.13 *GATA4iKO livers have lower VLDL release rate*

An assessment of hepatic VLDL release revealed that GATA4iKO mice had a lower release of VLDL in the absence of dietary insults.

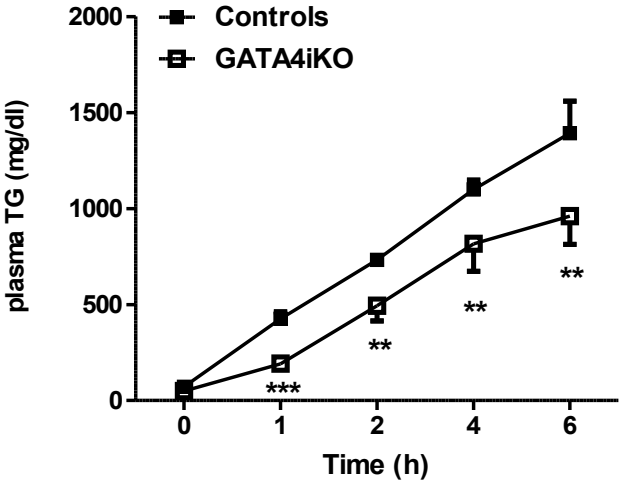

Figure 17

VLDL release in control mice averaged at 148.9 mg/h/kg body weight whereas that for GATA4iKO mice averaged at 107.7 mg/h/kg body weight (Fig. 17).

3.14 *WTD fed GATA4iKO livers show lower lipogenic and fibrotic gene expression*

Measurement of hepatic gene expression revealed that GATA4iKO mice had downregulated lipogenic and fibrotic gene expression (Fig. 18A & B). Out of seven genes involved in lipogenesis four were downregulated (Gpat1, 2.0-fold; FAS 2.8-fold; SCD-1, 2.0-fold and CD36 4.4-fold.). Dgat1, Mttp and Agpat1 had comparable levels of expression in both groups (Fig. 18A).

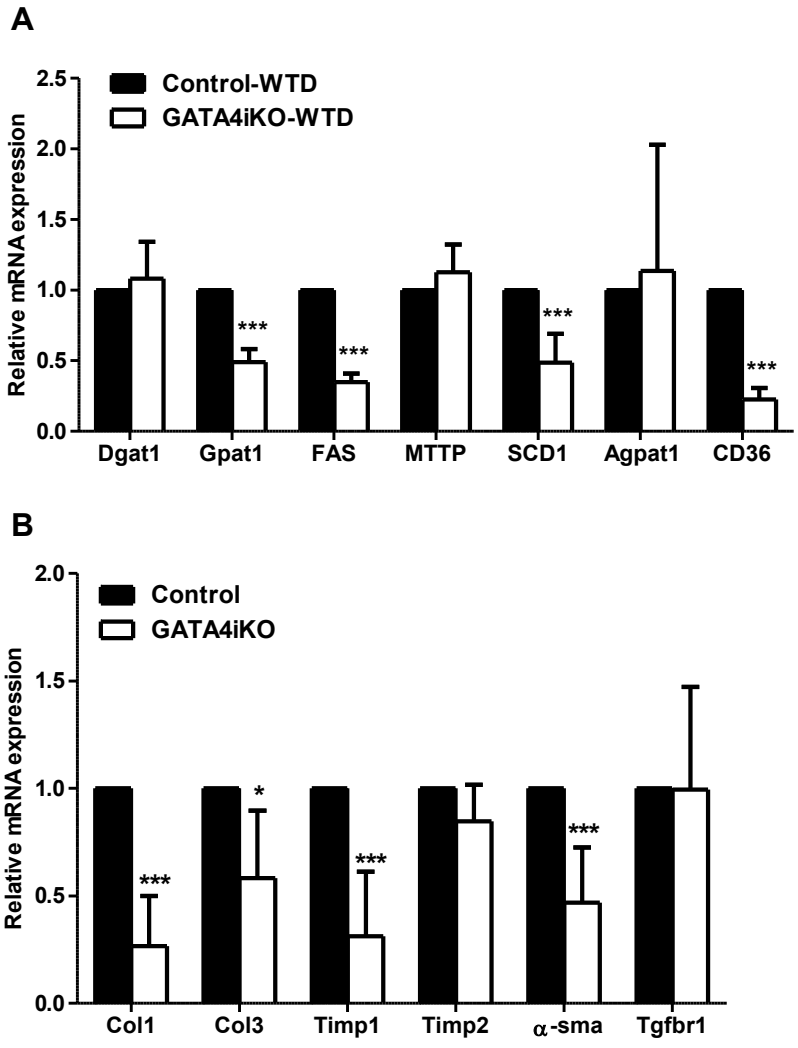

Figure 18

Fibrotic gene expression was also attenuated in livers of GATA4iKO mice. Out of six genes measured four genes involved in liver fibrosis were downregulated (Col1, 3.8-fold; Col3, 1.7-fold; Timp1, 3.2-fold; α-Sma, 2.1-fold). Other than these genes Timp2 and Tgfbr1 were also measured but had comparable values in both the groups (Fig. 18B).

IV. Discussion:

Dietary lipids are known to be predominantly absorbed by the proximal (duodenum and jejunum) small intestine, whereas the terminal part (ileum) is mainly involved in bile acid re-absorption (88, 94). We have shown that the posteriorization of the small intestine caused by the lack of intestinal GATA-4 has a beneficial effect of lowering plasma TG by reducing intestinal absorption and promoting resistance to diet-induced obesity. Over a 72-hour period GATA4iKO mice excreted greater amounts of dietary fatty acids implying that the underlying cause of obesity resistance is their inability to absorb dietary lipids. The lack of GATA-4 had profound effects on the expression of several genes involved in intestinal lipid uptake and transport. This included the downregulation of Cd36, Fatp2 and Fabp1, genes involved in fatty acid and cholesterol uptake and/or transport (38, 44, 53, 77, 87). The expression of all transporters with the exception of Scarb1 was downregulated on both WTD and chow diet. Surprisingly, in WTD-fed GATA4iKO mice Scarb1 mRNA levels were similar to controls although its expression on chow diet was downregulated. WTD is known to increase the levels of intestinal Scarb1 mRNA and protein in mice and this contributes to the regulation of trans-intestinal cholesterol excretion (TICE) (114). This would imply that there exists a pathway regulated by dietary lipids which can override the lack of GATA4 and regulate Scarb1 expression.

Apart from the reduced plasma TG concentrations, GATA4iKO mice also have reduced plasma TC levels. However, the expression of the well-established cholesterol importer Npc1l1 was unchanged on both dietary regimens and genotypes. Several cases based on our observations might explain the decrease in plasma TC levels in GATA4iKO mice. i) Downregulation of CD36: It is important to note the downregulation of Cd36 in GATA4iKO mice since a number of studies have proposed that Cd36 is a putative cholesterol importer (77, 94, 112). It was recently shown that Cd36 regulates cholesterol uptake into the proximal but not distal intestine (87). The ileal expansion observed in GATA4iKO mice lends further support for Cd36 as an important component for cholesterol uptake and or transport in the proximal intestine. ii) Increased TICE in GATA4iKO

mice: Our observation of increased fecal excretion of neutral sterols in GATA4iKO mice on WTD lead to the possibility of enhanced TICE as a probable cause of reduced plasma TC levels. iii) Decreased cholesterol synthesis rate: Given the upregulated HmgCo-A reductase mRNA in the livers of GATA4iKO mice, a lower cholesterol synthesis would be improbable to account for the drastic reduction in the plasma TC concentration. Our results taken together with the previous report from Battle et al. (7) confirm that the most likely cause of the reduced plasma TC levels in GATA4iKO mice is the lower intestinal absorption owing to reduced expression of Cd36.

Another arguable cause for general reduction in intestinal lipid absorption of TG and TC is the defective luminal lipolysis or improper bile release. Experiments involving co-administration of triolein and (^{14}C)-oleate revealed that luminal lipolytic activities were comparable between GATA4iKO and control mice. Therefore, differences in activity of luminal lipases or formation of micelles do not account for the observed reduction in lipid absorption in GATA4iKO mice. In a recent study, mice with a conditional deletion of GATA4 in the intestine were shown to have a normal bile acid pool size. Except for enhanced retention of tauro-β-muricholic acid, these mice did not show other differences in bile composition (12). These observations support that changes in luminal lipolysis do not account for the reduced lipid absorption seen in GATA4iKO mice.

The reduced TG absorption of GATA4iKO mice effectively translated into a long term obesity resistance. Plasma leptin is an indicator of body fat, and its circulating levels are suppressed upon fasting. Inability to lower plasma leptin level upon fasting is associated with obesity and is termed as peripheral leptin resistance (42, 54, 63). WTD-fed GATA4iKO mice failed to develop peripheral leptin resistance and exhibited lower levels of fasting plasma leptin reflecting an intact physiological regulation of leptin in these mice. Consequences of long term WTD-feeding not only include hyperlipidemia and obesity but also other adverse effects like insulin resistance and type II diabetes, all of which are components of the metabolic syndrome. Adiponectin produced from white adipose tissue is

known to suppress metabolic derangements including obesity and glucose intolerance. It exerts an anti-diabetic and anti-atherogenic effect and plays an important role in the regulation of lipid and glucose metabolism. Adiponectin levels are inversely proportional to body fat (3, 71). In accordance, plasma adiponectin levels were higher in GATA4iKO mice compared to controls indicating increased insulin sensitivity and supporting the lower adipose tissue mass.

As described previously, a mechanism leading to a partial jejuno-ileal homeosis is operative in GATA4iKO mice (7, 17). This phenotype closely resembles ileal-interposition, a form of bariatric surgery. In rats subjected to ileal interposition obesity related co-morbidities decrease (67). The interposed ileum adapts to surgical transposition but retains its ability to absorb bile acids and also the ability to release GLP-1, an ileal entero-endocrine peptide (30, 67). GLP-1 release is potentiated by the presence of dietary lipids (110). GLP-1 mainly enhances peripheral insulin sensitivity and stimulates glucose dependent insulin release from the pancreatic ß-cells (62, 90). We thus hypothesized that the ileal expansion in GATA4iKO mice would cause an increase in GLP-1. Concurrently, in GATA4iKO mice, GLP-1 release was elevated which reflected in a preserved glucose tolerance on both diets. Increased GLP-1 release in GATA4iKO mice was coupled with higher glucose-stimulated insulin release upon oral fat load. Insulin itself is known to inhibit the synthesis of TG rich lipoproteins such as chylomicrons. Thus, the increased insulin release might be seen as an additional mechanism for decreased lipid absorption. Other physiological effects of GLP-1 include inhibition of gastric emptying. Consequently, GATA4iKO mice showed a slower liquid gastric emptying, which could explain at least in part, the TG malabsorption.

Following ileal interposition surgery, the levels of Gip, a proximal intestinal entero-endocrine peptide, were shown to be decreased (30). Gip potentiates chylomicron production (104). Accordingly, we found that lack of intestinal GATA4iKO also led to decreased levels of circulating Gip. Gip release, upon oral

fat load was also lower in GATA4iKO mice. These findings point to a possibility of lower Gip being an additional mechanism for the lower TG absorption in GATA4iKO mice. In a canine model, Gip was shown to increase peripheral fat deposition by enhanced chylomicron clearance (117). In another study it was found that Gip activates lipoprotein lipase on adipocytes and thus enhances lipid deposition (66). Therefore, lower Gip levels imply reduced adiposity as encountered in the GATA4iKO mice.

Apart from these beneficial effects, WTD-fed GATA4iKO mice also accumulated lower amounts of hepatic TG than control mice which suggest that intestinal elimination of GATA4 impacts hepatic lipid homeostasis. As a consequence of this a decrease in hepatic VLDL release in GATA4iKO mice was also observed. Expression levels of hepatic lipogenic genes were also found to be downregulated in GATA4iKO mice revealing a lower lipogenesis. This could be explained on the basis of the preserved insulin sensitivity in GATA4iKO mice compared to controls. Insulin is known to inhibit clearance of TG-rich particles by inhibiting their secretion (27). As a result intracellular TG concentrations remain high and in turn suppress further synthesis of lipids through de novo lipogenesis. Moreover, diabetic individuals or individuals experiencing hepatic insulin resistance develop fatty liver and NAFLD. Thus, treatment modes that lead to the promotion of insulin sensitive states are considered as therapeutic strategies not only for diabetes mellitus but also for NAFLD associated with diabetes. Although the insulin sensitivity could be a possible explanation for lower hepatic lipid storage, it cannot fully account for the downregulation of key lipogenic genes such stearoyl Co-A desaturase (Scd-1). This could be due to the action of GLP-1 which was found to be elevated in GATA4iKO mice on both dietary regimens. GLP-1 receptor agonist exendin-4 has been reported to reverse hepatic steatosis in *ob/ob* mice (36). In fact, GLP-1 receptor agonists are of strategic importance for the treatment of type 2-diabetes mellitus (58). The ileal enterocytes as well as entero-endocrine cells release several peptide hormones in response to dietary stimuli. We confirmed as reported previously that an expansion of the ileal domain in GATA4iKO mice lead to an increase in the production of some of

these ileal-derived peptide hormones, FGF15 in particular (7, 32). FGF15 is sensed by the liver through FGFR4 comprising an entero-hepatic regulatory loop (60). Also the human homologue of FGF-15, FGF-19 was shown to inhibit hepatic fatty acid synthesis (13). Although the lipogenic potential of insulin cannot be underscored, this study also showed that FGF-19 overrides the ability of insulin to suppress hepatic fatty acid synthesis and the expression of key lipogenic enzymes (13). The enhanced levels of FGF-15 in GATA4iKO mice might lead to lower hepatic TG levels in GATA4iKO mice. Thus, enhanced secretion of FGF-15 in GATA4iKO mice could be one of the reasons for lower hepatic lipid accumulation. Higher levels of GLP-1 could be among the other possible reasons for lower hepatic TG. GLP-1 receptor agonist, Exendin-4 is known to reverse the hepatic steatosis in ob/ob mice (36). GLP-1 is also known to suppress hepatic lipogenesis via activation of the AMPK pathway (106). GLP-1 inhibits hepatic fat accumulation and also nutrient-induced hepatic proinflammatory responses which curbs the progression to hepatic steatosis (106).

In addition, GATA4iKO mice on long term WTD had phosphorylated Akt2/protein kinase B at much higher levels compared to controls. Akt2 has been implicated as an indispensible component of mediating hepatic lipid accumulation in response to insulin signaling and also known to stimulate VLDL release (55, 73). Contrary to the action of dietary lipids, dietary glucose along with insulin induces de novo lipogenesis (DNL) and promotes lipid storage. During insulin resistant states hepatocytes loose their ability to regulate the outcomes of insulin challenge. This deregulation, on one hand, leads to an over-production instead of suppression, of VLDL and hence post -prandal hyperlipidemia. On the other hand, the condition still stimulates greater DNL instead of a suppression of DNL as would be expected. As we have shown, GATA4iKO mice have severe reduction in the absorption of dietary lipids along with enhanced secretion of hormones which influence hepatic DNL. Thus, data regarding the role of insulin in non-alcoholic liver disease is still inadequate, possibly because different pathways dominate in different nutritional states, according to status of non-

esterified fatty acids delivery to the liver, either hepatic DNL or VLDL assembly and secretion becomes the rate-determining step. This is especially relevant in case of GATA4iKO mice since WTD is rich in dietary lipids, the malabsorption of lipids in the intestine of GATA4iKO mice leads to a disproportionate uptake of glucose vs. lipids, altering the nutritional states.

As previously stated, the jejuno-ileal transition observed in GATA4 mice closely resembles the bariatric surgical procedure of ileal interposition. Long term effects that ileal interposition surgery might have on other organs have not been elucidated. The observations of lower hepatic lipid accumulation hint towards a possible protective outcome of such a surgical procedure. Whether these observations translate into clinical practice is so far unclear. However, this warrants further investigation which is out of the scope of the present work.

Based on our data we conclude that intestinal lack of GATA4 could alleviate at least three components of the metabolic syndrome; diet induced obesity, type 2-diabetes mellitus and non-alcoholic fatty liver disease. Given the cholesterol lowering effects of intestinal GATA4 knockout, it would be interesting to investigate its ability to reduce atherosclerosis susceptibility. Although direct clinical data are limited, increasing number of studies represent ileal-interposition surgery as a novel and effective mode for treatment of type 2-diabetes mellitus (2, 6, 31, 68). In this respect, GATA4iKO mice are an interesting model not only as they recapitulate the effects of ileal-interpositioning but also since they provide a potential target for drug discovery.

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