



Gfi1 : a new target for diabetes therapy

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► To cite this version:

Tiziana Napolitano. Gfi1 : a new target for diabetes therapy. Human genetics. COMUE Université Côte d'Azur (2015 - 2019), 2017. English. NNT : 2017AZUR4141 . tel-03823179

HAL Id: tel-03823179

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Thèse de doctorat

Pour l'obtention du grade de

Docteur en Sciences de l'Université de Nice Sophia Antipolis

Discipline : Sciences de la Vie

Spécialité : Génétique et Développement

Présentée par

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Gfi1: a new target for diabetes therapy

Gfi1 : une nouvelle cible pour la thérapie du diabète

Soutenue publiquement le 19 décembre 2017

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Résumé

Le pancréas mature est constitué de deux types de tissus : le tissu exocrine, comprenant les cellules acinaires et les canaux pancréatiques, et le tissu endocrine. Les cellules acinaires sont dédiées à la synthèse d'enzymes digestives, qui sont collectées et acheminées via le réseau canalaire à travers l'organe entier. Les cellules endocrines sont organisées en groupes fortement vascularisés, nommés îlots de Langerhans, qui contiennent cinq sous-types cellulaires, les cellules α , β , δ , PP et ϵ sécrétant respectivement le glucagon, l'insuline, la somatostatine, le polypeptide pancréatique et la ghréline. L'approche génétique classique a permis de caractériser des facteurs bien connus et étudiés mais de plus en plus de recherches identifient des facteurs nouveaux impliqués dans le développement et la maturation du pancréas. Via des études *in silico*, nous avons accumulé des preuves suggérant un rôle pour *Gfi1* dans le développement et la spécification cellulaire du pancréas. Cette protéine, un facteur de transcription à doigt de zinc, a précédemment été impliquée dans l'hématopoïèse, le développement de l'oreille interne et la maintenance du phénotype des cellules intestinales. Dans cette étude, nous avons examiné le rôle de *Gfi1* dans le pancréas. Dans ce but, nous avons généré une lignée de souris transgénique permettant l'inactivation sélective de *Gfi1* dans le pancréas. Dans l'ensemble, nos observations suggèrent que *Gfi1* est nécessaire pour la maturation complète des cellules acinaires. De façon importante, nous avons démontré que l'ablation de *Gfi1* dans le pancréas est suffisante pour protéger les souris contre deux modèles d'induction du diabète.

Mots-clés

Gfi1 – Résistance diabète – Ghréline – Insuline – Cellules acinaires

Abstract

The mature pancreas consists of two main tissue types: the exocrine tissue, including acinar cells and a ductal tree, and the endocrine tissue. Acinar cells are dedicated to the synthesis of digestive enzymes, which are collected and conveyed to the duodenum by a ductal network running through the entire organ. Endocrine cells are organized into highly vascularized cell clusters, termed islets of Langerhans, which contain five cell subtypes, α -, β -, δ -, PP- and ϵ -cells secreting glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively. Classical genetic approaches have revealed much about individual factors regulating pancreatic development, however, we have yet to understand the regulatory network underlying pancreas formation and all the factors involved. Some of these factors are well known and studied but increasing researches revealed new and unknown factors involved in pancreas development and maturation. Among these, through *in silico* studies, we accumulated evidences suggesting a role of *Gfi1* in pancreas cell development and specification. This protein, a zinc finger transcription factor, had previously been implicated in hematopoiesis, inner ear cell development, and in the maintenance of intestinal cell phenotypes.

Here, we investigated the role of Gfi1 in the pancreas. Towards this goal, we generated a transgenic mouse line allowing *Gfi1* inactivation exclusively in the pancreas. Altogether, our observations suggest that Gfi1 is required for the full maturation of pancreatic acinar cells. Importantly, we demonstrated that the sole loss of *Gfi1* in the pancreas is sufficient to protect mice against two models of diabetes induction.

Keywords

Gfi1 – Diabetes resistance – Ghrelin – Insulin – Acinar cells

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Introduction

Preface

Over the last 4 years, I have been focusing my studies on a transcription factor termed *Gfi1*, in an effort to understand its role on rodent pancreas development and function. The pancreas is a digestive gland involved in the metabolism of all three macronutrients: proteins, lipids and carbohydrates. In the following pages, I will briefly guide the reader through the digestive process in order to highlight the crucial importance of pancreas activity in the digestive system.

In addition, the pancreas regulates glucose homeostasis by secreting insulin, glucagon and other endocrine hormones. Diabetes is a metabolic disease characterized by chronic hyperglycemia, due to either loss of insulin-producing cells or the development of severe insulin resistance. In the next chapter, the reader will find a description of all the pancreatic endocrine cells, the hormones they secrete and their physiological interplay, alongside with the classification, pathogenesis and current treatments of diabetes.

I strongly believe that a deep understanding of molecular mechanisms underlying pancreas development is the key to open new avenues for diabetes therapy. The data I will present in this manuscript convinced me that *Gfi1* is part of the complex interplay of transcription factors governing pancreas development. Therefore, a concise overview of the main steps of pancreas development and the physiological role of *Gfi1* in cell specification is presented.

The Nutrients: The Bricks of Life

Every living organism needs to assimilate nutrients to ensure its survival, growth and reproduction. Heterotrophic animals obtain nutrients by metabolizing carbonic food into simple molecules, which are subsequently converted into energy or integrated into cellular structures. Based on their requirement, nutrients are classified into macronutrients, which are needed in large amount to fuel the body, and micronutrients that are required in limited amount to produce enzymes, hormones and other substances crucial for proper growth and development^{1,2}. Macronutrients comprise carbohydrates, lipids, proteins and water whereas micronutrients include minerals and vitamins (**Figure 1**).

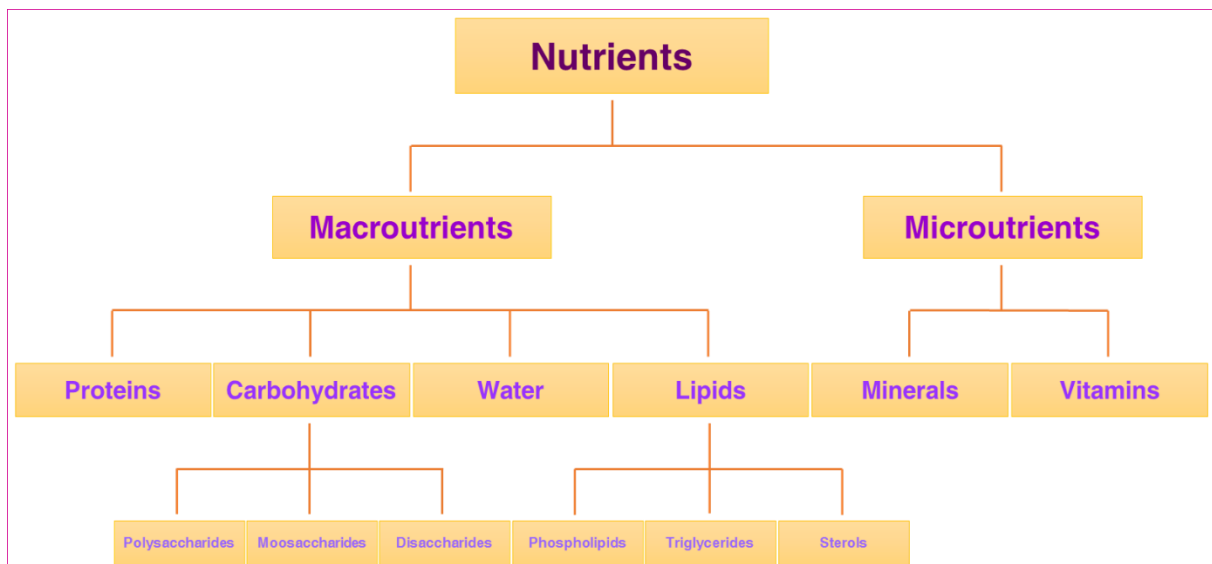


Figure 1. Schematic classification of nutrients

Carbohydrates represent the main source of energy for vertebrates and provide the major fuel for the central nervous system and muscular exercise³. They are found in fruits, grains, vegetables and milk products. At the chemical level, all carbohydrates are organized as ring structures and are always composed of the carbon, hydrogen, and oxygen elements. According to their molecular structures, carbohydrates are defined as simple or complex⁴.

Dietary sugars represent simple carbohydrates, containing one or two carbohydrate units, therefore termed mono and di-saccharides, respectively. Monosaccharides, like glucose,

fructose and galactose are rarely found in nature, as they normally constitute the molecular units for polymeric carbohydrates. Conversely, several disaccharides are common in nature, such as sucrose, maltose and lactose. These sugars are rapidly and efficiently absorbed upon being hydrolyzed by digestive enzymes into monosaccharides.

Polysaccharides represent complex carbohydrates composed from 10 up to several thousand monosaccharides molecules arranged in chains. Starch is the major form of storage of carbohydrates in plant seeds, it represents a primary source of glucose for all non-ruminant animals. Consisting of a large number of glucose units, it requires several steps of hydrolysis during the digestive process. Other plant polysaccharides, like cellulose and pectins, can be only fermented by bacteria but are not accessible to animal digestive enzymes. These belong to the dietary fibers: they are not assimilated by mono-gastric animals, but they play a major role in regulating gastric emptying and satiety⁵.

Lipids correspond to a heterogeneous group of compounds playing different roles: they represent an alternative and more concentrated source of energy when compared to carbohydrates, but they also serve as structural components of cell membranes, as well as substrates for signaling molecules synthesis⁶.

The major source of lipids for humans and animals are plant and animal triglycerides, membrane phospholipids and sterols. Triglycerides are made out of esters of glycerol and three molecules of fatty acids. Fatty acids consist of a hydrocarbon chain terminating with a carboxylic group and they are classified according to the number of double bonds they include. Saturated fatty acids (SFA) exclusively possess single bonds: short- and medium-chain fatty SFA (4 to 12 carbons in length) are more rapidly absorbed, while longer-chain SFAs (more than 14 carbons in length) require extensive hydrolyzing processes in the intestinal tract⁷.

Monounsaturated fatty acids and polyunsaturated fatty acids contain one or multiple double bonds, respectively. Omega(ω)-6 and omega(ω)-3 fatty acids are polyunsaturated fatty acids and they represent important components of cell membranes as well as precursors of signaling molecules. Linoleic acid, an ω -6 fatty acid, and alpha-linoleic, an ω -3 fatty acid, are essential nutrients for humans and many animals, as they cannot be synthesized by the body from other food components⁸.

Like triglycerides, phospholipids also consist of fatty acids esterified to a glycerol backbone; however, they generally contain only two molecules of fatty acids together with a modified hydrophilic phosphate group. Due to their amphipathic nature, they naturally aggregate into bilayers, constituting the main structural components of the cell membranes and lipoproteins. They further play a key role in intracellular pathways as they are precursors of membrane-derived second messengers⁹.

Sterols correspond to high molecular weight alcohols that naturally occur in both plants and animals. Cholesterol, the most common dietary sterol, is mainly found in fat tissues of animal origin. It is a crucial regulator of cell membrane fluidity and it is required for the normal development of the brain and nervous system^{10,11}. Furthermore, cholesterol serves as precursor for bile acids, steroid hormones and vitamin D.

Animals need proteins to grow, repair tissue damages and maintain a continuous nutritional homeostasis. This requirement is proportional to the growth rate and is therefore higher in pups, infants and pregnant and breast-feeding females^{12,13}. In mammals, proteins represent the most versatile class of molecules as they serve crucial functions in essentially all biological processes. Indeed, they form the main structures supporting cell architecture, division, and movement. Furthermore, proteins play key roles in the immune response, neurotransmission, gene regulation, and enzymatic catalysis.

Proteins consist of one or more chains of amino acids and the specific sequence of amino acids determines the tridimensional structure and therefore the function of each protein. The individual amino acid residues are linked together by peptide bonds: these covalent bonds must be hydrolyzed in the digestive system to allow the absorption of free amino acids in the bloodstream.

Plant and animal proteins contain 20 amino acids and they can be subdivided into essential, conditionally essential and dispensable amino acids¹⁴. The first group includes those amino acids that need to be supplied with food as they can be not synthesized *de novo*, their number varying among the species. The remaining amino acids can be produced by the body, but their synthesis might be limited to certain ages or to specific pathophysiological conditions. These amino acids are considered as 'conditional essential'. Finally, the dispensable amino acids do not need to be provided with the diet as they can be generated by transamination, starting from other amino acids.

Water is a vital nutrient, as it is the main constituents of cells, tissues and organs and it is virtually involved in all body functions¹⁵. Water plays a major structural role, as it is fundamental to maintain the shape and volume of eukaryotic cells and stabilize macromolecules conformation. From a metabolic point of view, water is involved in all the hydrolytic reactions of the other macronutrients and is the product of oxidative reactions of hydrogen-containing substrates in the body.

As the primary fluid of the body, water is an excellent solvent for ionic compounds and therefore represents the medium in which glucose, vitamins, minerals and amino acids are dissolved and delivered to the cells. It is also the main carrier of toxins and waste products from the cells toward the excretory system. In addition, whole-body thermoregulation critically depends on water: its high heat capacity limits internal temperature variations while evaporation

of water from the body surface efficiently induces heat loss. Finally, water is a potent lubricant. In combination with viscous molecules, it forms lubricating fluids for joints and it is present in saliva and mucous in the digestive, respiratory and genito-urinary tract. Surrounding specific sensitive structures as brain, eyes and spinal cord, water also offers a protection against shocks.

Micronutrients play a central role in metabolism and in the maintenance of tissue function. This heterogeneous class of nutrients includes organic molecules, termed vitamins, and inorganic ions, the minerals. These substances are not direct energy sources, but they control macronutrients metabolism and they are key regulators of several physiological functions¹⁶. Particularly, they act as cofactors or prosthetic groups for several enzymes and are therefore required for virtually all biochemical reactions. Besides being cofactors for enzymes, some vitamins are described as hormone-like molecules¹⁷. Hence, vitamin A and vitamin D are involved in the growth, differentiation, and maintenance in a broad variety of normal and malignant tissues¹⁷. Additionally, these vitamins together with some minerals, such as zinc, are among the nutrients that may directly influence gene expression.

Ultimately, micronutrients are considered a crucial component of a healthy diet as they display antioxidant properties and therefore they are required to prevent pathological conditions. Specifically, micronutrients prevent oxidative stress as they possess the ability to either directly quench the oxidant activity of free radicals or activate enzymes scavenging the products of oxidations¹⁸.

Turning food into nourishment: the digestive system

Converting food into nutrients requires a sequence of mechanical and chemical processes, termed digestion. When a meal is ingested, the physical processing of the food begins. While passing through the gastro-intestinal tract, complex molecules are progressively hydrolyzed

into simple nutrients to be finally absorbed into the blood stream. Once released in the general circulation, nutrients are assimilated by the cells and used as energy source or as structural subunits. Conversely, the indigestible waste products are eliminated from the organism in a process called egestion.

Overall, the digestive tract is strongly conserved among mammals, the major differences probably resulting from diet¹⁹. Given their shared omnivorous nature, humans and mice display strong similarities in their digestive systems. Main variations lie on the proportion between small and large intestine length, humans having evolved towards a smaller cecum and colon and relatively longer small intestine as compared to the murine system. However, in both, the gastrointestinal tract consists of five consecutive hollow organs: mouth, esophagus, stomach, small intestine and large intestine. A layer of smooth muscle propels food and liquids along the gastrointestinal tube, by promoting a series of wave-like contractions of the organ walls, termed peristalsis. This movement drives the bolus downward and mixes it with the digestive secretions, released by salivary glands, liver and pancreas (**Figure 2**).

Mouth

Digestion begins within the oral cavity. The major functions of the digestive oral phase are the mechanical disruption of food into smaller particles and bolus formation for swallowing.

All ingested solid food is processed by a series of masticatory cycles needed to break down, lubricate and soften food into a bolus ready to be swallowed. Mastication involves the action of the teeth, masticatory muscles, temporomandibular joint, and tongue. During this process food mixes with saliva, a watery substance secreted by the salivary glands²⁰. These exocrine glands consist of acinar cells producing lysozymes, immunoglobulin A, mucins, ptyalin (salivary amylase) and lipase. Saliva mediates taste perception and protects oral mucosa integrity by lubricating the mouth and clearing the buccal cavity from sugars, acids and bacteria.

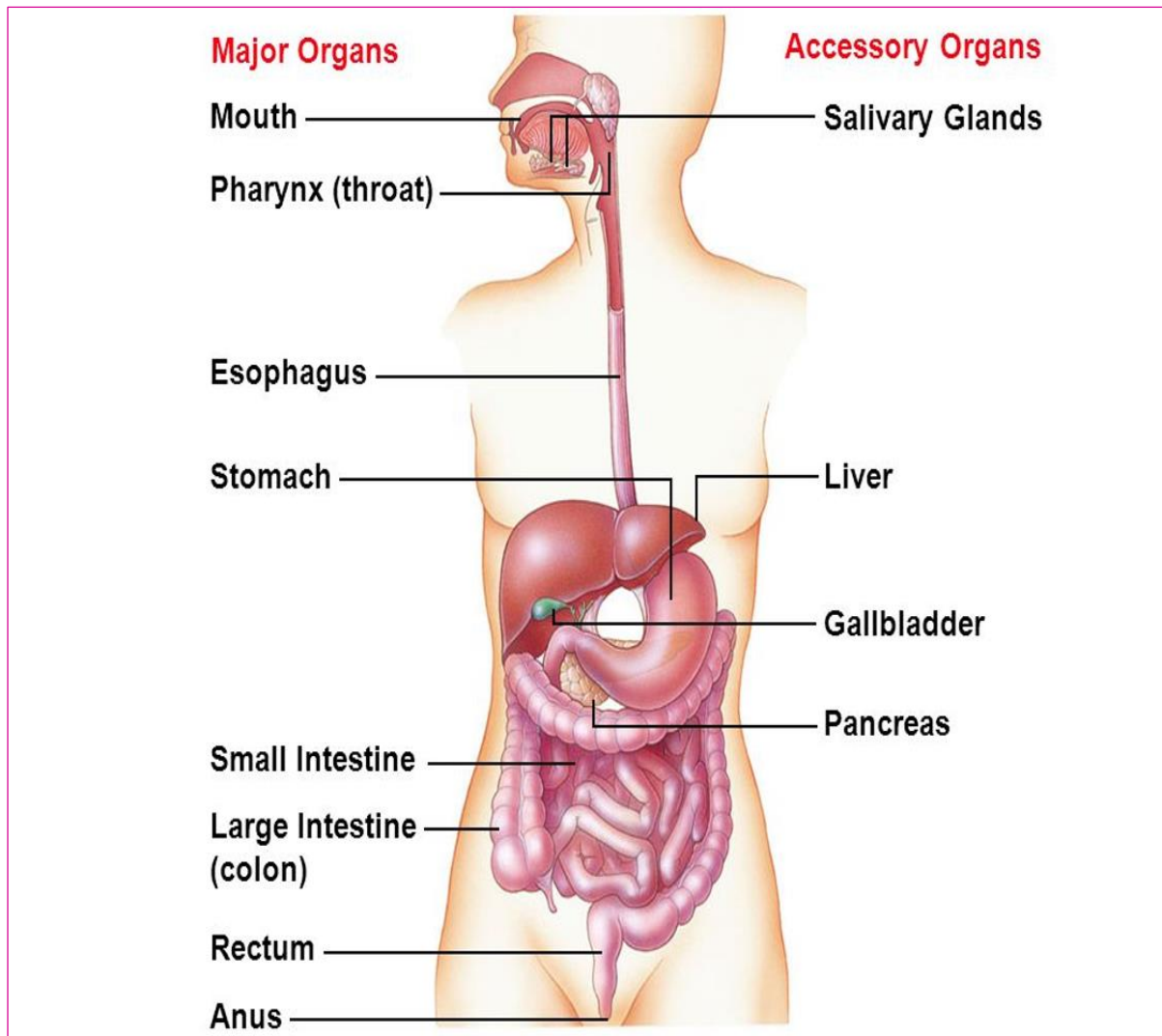


Figure 2. The human digestive system. The digestive system consists of the gastrointestinal tract and several accessory glands. The gastrointestinal tract spans from the oral cavity continuing through the pharynx, esophagus, stomach and intestines to the rectum and anus. Food is propelled along the length of the gastrointestinal tract by peristaltic movements of the muscular walls. The accessory glands include the salivary glands, the liver, the gallbladder and the pancreas. These secretory organs release digestive enzymes required for food processing into nutrients, suitable for intestinal absorption.

Furthermore, saliva contributes to digestion as it contains α -amylase, or ptyalin, an enzyme catalyzing the hydrolysis of carbohydrates into maltoses. Lingual lipase is another salivary enzyme: given its high stability at low pH, this protein acts in the stomach, mediating digestion of triglycerides into partial glycerides and free fatty acids²¹.

Esophagus

During deglutition, the bolus is conveyed into the pharynx and then in the esophagus upon closure of the epiglottis. The esophagus is a dynamic tube that drives food from the mouth to the stomach, where digestion continues. The wall of esophagus consists of three layers: mucosa, submucosa (connective tissue) and muscle fibers. Mucus released by the esophageal mucosa provides lubrication and eases the passage of food. Unlike humans, mice esophageal mucosa is keratinized to protect the epithelium against abrasive dietary components.

The human esophageal epithelium consists of a tough stratified squamous epithelium without keratin. A high turnover of epithelial cells and the secretions of submucosal glands defend human esophageal cells when exposed to harmful dietary substances²².

Peristaltic contractions of the muscular layer propel the bolus along the esophagus. Finally, the lower esophageal sphincter relaxes, allowing the food to pass in the stomach and then closes to prevent the stomach's gastric juices and contents from entering the esophagus.

Stomach

The stomach is a dilated part of the digestive tract, lying between the esophagus and the duodenum. Murine and human stomach is divided into three regions: the fundus, located on the uppermost region of the stomach, the body, corresponding to the large middle part and the pyloric part demarcating the end of the stomach and the beginning of the small intestine²³.

Like the esophagus, the stomach walls display a mucosal epithelium, a submucosa and a muscular layer. Peristaltic movements, generated by contraction of the muscular walls, mix food with enzymes and acids released by the mucosa. Resultantly, the bolus is partially digested by the physical and enzymatic degradation occurring in the stomach and is therefore converted into chyme.

The lumen of the stomach is covered by mucus-secreting cells, termed foveolar cells²⁴. These cells produce a thick, bicarbonate-rich mucus, which protects the mucosa against the highly corrosive acidity of the gastric juices. The epithelium of gastric mucosa contains invaginations called gastric pits, where the gastric glands empty their secretions. Gastric glands are composed of different exocrine cell types producing the gastric juice. The latter consists in a variable mixture of water, hydrochloric acid, mucus and enzymes. The acidity of the stomach inhibits salivary amylase and activates lingual lipase that begins the hydrolysis of the triglycerides. The low pH also triggers the conversion of pepsinogen into pepsin, a proteolytic enzyme secreted by the gastric glands and able to degrade proteins into small peptides. Furthermore, the gastric glands release the intrinsic factor, necessary for the absorption of the vitamin B₁₂ in the small intestine.

Scattered among the exocrine cells, the enteroendocrine cells regulate several aspects of digestive functions by releasing hormones into the general circulation. Particularly, the presence of food in the stomach stimulates the release of gastrin and histamine, these two hormones regulating the secretion of hydrochloric acid. Conversely, upon starvation, specific enteroendocrine cells secrete ghrelin, a potent orexigenic factor that stimulates food intake and body weight gain²⁵. Nutrient absorption in the stomach is negligible. The chyme requires passing into the intestine where it will be fully digested into nutrients, able to cross the intestinal epithelium and enter into the general circulation. Gastric emptying is regulated by both the stomach and the duodenum. The presence of chyme in the duodenum inhibits gastric secretion, preventing additional chyme from being released by the stomach before the duodenum is ready to process it.

Intestine

The chyme released from the stomach enters in the small intestine, where the digestion of organic macromolecules and absorption of nutrients predominantly occurs. The small

intestine can be subdivided in three functional segments: the duodenum, the jejunum and the ileum. In the duodenum, the chyme is mixed with the pancreatic enzymes and hepatic bile, while specialized glands present in the submucosa secrete alkaline mucus to neutralize the acid chyme. The digestion initiated in the duodenum further continues in the jejunum as does absorption. In the ileum, bile acids are re-absorbed to regenerate the bile stored in the gallbladder and several vitamins, including vitamin B₁₂, cross the intestinal epithelium in this region.

The wall of the small intestine is composed of the same layers typically found in the digestive tube: a muscle coating, allowing the descending movement of the food, a submucosa containing blood vessels and lymphatics, and an internal mucosa. However, the morphology of the intestinal epithelium is unique. Indeed, in this organ the mucosa forms hair-like vascularized projections, called villi, these structures tremendously increasing the surface of absorption in the intestine. Between the villi, the mucosa forms depressions termed crypts or intestinal glands.

Covering villi and crypts, the intestinal epithelium further expands the absorptive surface as it displays microvilli, cylindrical extensions on the apical surface that form the intestinal brush border²⁶. This specialized plasma membrane contains endopeptidases, able to catalyze the activation of the pancreatic trypsinogen into trypsin²⁷. Furthermore, brush border dipepsidases and disaccharidases are responsible for the last steps of carbohydrates and proteins digestion as they hydrolyze disaccharides and dipeptides into monosaccharides and amino acids, respectively.

The intestinal epithelium contains different cell subtypes. Particularly, the villi surface is constituted by absorptive cells, the enterocytes, and exocrine cells, termed goblet cells, secreting mucins for lubrication and protection of the intestinal epithelium²⁸. Scattered among these cells, the enteroendocrine cells release a variety of metabolism-regulating hormones,

including incretins that modulate pancreatic insulin secretion as well as cholecystokinin and secretin, regulating exocrine pancreatic activity. Additionally, in the crypts reside the Paneth exocrine cells releasing antimicrobial peptides, as well as proliferative stem cells that sustain the rapid turnover of the intestinal epithelium²⁹.

The chyme that passes from the small intestine into the large intestine mainly consists of water and undigested materials, the fibers. The large intestine is subdivided in caecum, colon and rectum and its primary functions are to complete absorption of nutrients and water, synthesize specific vitamins and form solid feces. The luminal surface of the large intestine lacks the villi of the small intestine, but it contains numerous glands mainly lined with enterocytes and goblet cells. The mucus secreted by the goblet cells protects the intestinal mucosa and eases the passage of the feces along the tube. In order to convert liquid chyme in semisolid feces, the enterocytes absorb water together with electrolytes like sodium and chloride.

The large intestinal epithelial cells do not secrete digestive enzymes, however chemical digestion is completed in this region of the intestinal tract and it relies on enzymes produced by the intestinal flora. This complex ecology of microorganisms is responsible for the fermentation of carbohydrates, like cellulose, that cannot be digested by the enzymes secreted by mammalian cells³⁰. Importantly, gut bacteria are a significant source of a number of vitamins, including vitamins B and K³¹.

Evolved to adapt to different dietary regimens, this region of the digestive system displays the highest level of variability among species. For instance, rodents possess a larger caecum with a greater fermentation capacity compared to humans, and do not have an appendix. Neither humans nor mice large intestinal epithelium is able to absorb vitamins B produced by the intestinal bacteria. However, rodents receive the benefit of microbial vitamins B production by consuming their feces, a behavior called coprophagy that localizes vitamins B in the duodenum

where they can be absorbed. Conversely, humans are obliged to rely mainly on dietary sources for these vital vitamins¹⁹.

Liver

The liver is the largest gland of the body exhibiting both endocrine and exocrine functions. As in humans, mouse liver displays four lobes; however, in humans, these lobes are separated by a band of connective tissue, while murine liver lobes are divided³². The liver has a dual source of blood supply: the hepatic artery, irrigating hepatic lobes with fully oxygenated blood from the heart, and the portal vein delivering the nutrients absorbed from the small intestine.

Liver cells are organized in clusters termed lobules³³. They represent the main architectural unit of liver parenchyma that contains hepatocytes, blood vessels, bile ducts and Kupffer cells. The complex arrangement between secreting cells with capillaries and bile ductal cells underlies both endocrine and exocrine functions of the hepatocytes. Hence, these highly polarized cells synthesize and release a wide range of proteins, coagulating factors and hormones in the general circulation. Simultaneously, hepatocytes are actively involved in lipid digestion as they secrete bile. This alkaline solution is a mixture of water, bile salts and phospholipids that are collected in the bile caniculi, located between two adjacent hepatocytes, and conveyed to the hepatic duct.

Stored and concentrated in the gallbladder, the bile is finally drained into the duodenum in order to emulsify lipids contained in the chyme. This process results in the formation of small amphipathic droplets that increase the surface area of activity for lipid-digesting enzymes and mediates fat absorption. Importantly, biliary secretion represents also the final step for the elimination of cholesterol and bilirubin, a toxic metabolite arising from the destruction of aged red cells.

Together with their secretory function, hepatocytes also play a key role in regulating metabolism, as they are able to store nutrients within their cytoplasm. During fasting, lipids are mobilized from the adipose tissue and are accumulated in the liver for energy synthesis. Conversely, hyperglycemia-induced insulin stimulation promotes the synthesis of glycogen, a polysaccharide representing the hepatic storage of glucose.

In addition to hepatocytes, endothelial cells and ductal cells, the liver is also composed of macrophages cells, termed Kupffer cells³⁴. These cells display a strong phagocytic activity, which protect the body from the entrance of pathogens thorough the alimentary canal.

Pancreas

The pancreas is an accessory digestive gland lying in the upper abdomen behind the stomach. It is usually described as a mixed gland, able to orchestrate endocrine and exocrine physiological responses due to the anatomical and functional interrelationship of its three main components: the islets of Langerhans, acini and ducts (**Figure 3**).

The exocrine pancreas

The exocrine pancreas accounts for about 98-99% of the total organ volume in adults of most mammalian species. This tissue is organized in small grape-like cell clusters, termed acini, each located at the terminal end of pancreatic ducts. Individual acinar cells have the shape of a truncated pyramid, arranged in groups around a central ductal lumen. These central ducts empty into progressively larger intercalated and collecting ducts that eventually join the main pancreatic duct. The pancreatic duct drains into the bile duct, which ultimately opens in the duodenum at the level of major duodenal papilla.

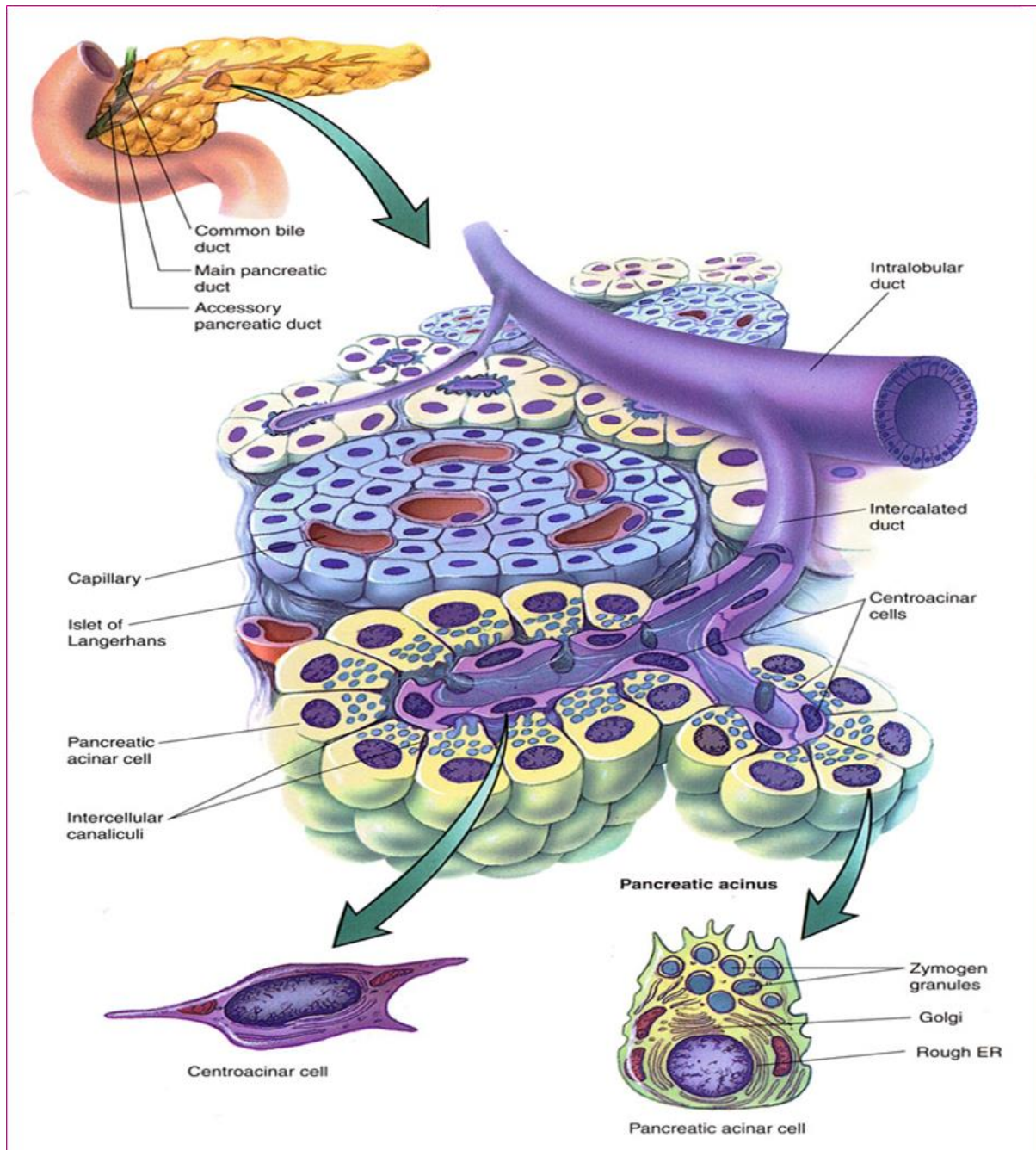


Figure 3. Human pancreas anatomy and histology. The pancreas lies within the abdominal cavity, posterior to the stomach and connected to the duodenum. The pancreas is a composite gland containing both exocrine and endocrine components. Acini, formed by zymogenic cells around a central lumen, are arranged in lobules. Each lobule has its own intercalated duct, and many intercalated ducts join to form intralobular ducts that drain into the main pancreatic duct. Scattered throughout the gland are found the islets of Langerhans, containing β -, α -, δ -, PP-, and ϵ - endocrine cells. Acinar cells are dedicated to the synthesis, storage and release of digestive enzymes, while endocrine cells secrete hormones involved in the regulation of glucose metabolism. Spindle-shaped centroacinar cells are present at the junction between the acinus and the ductal cells of the intercalated duct.

The acinar cells are specialized for the synthesis, storage and secretion of digestive enzymes. According to their substrates, these enzymes fall into four classes: proteases, lipases, amylases and nucleases, degrading dietary proteins, lipids, carbohydrates and nucleic acids, respectively. Importantly, protein-digesting enzymes are present in the acinar cytoplasm in their inactive form, the zymogens. This protective mechanism prevents the degradation of pancreatic cells, which would lead to tissue injuries and pancreatitis. Once released into the duodenal lumen, trypsinogen is cleaved by the brush border enterokinases and converted into an enzymatically active form, the trypsin^{35,36}. Consequently, this serine protease catalyzes the activation of other inactive pancreatic enzymes. Conversely, lipases and amylases accumulate in the acinar secretory granules in their active form, as they are not able to cause pancreatic tissue damage.

Although pancreatic acinar enzymes play a pivotal role in food digestion, they do not account for all of the processes needed to complete the breakdown and absorption of the nutrients in a meal. Thus, the final step in digestion of dietary carbohydrates and proteins occurs at the surface of small intestinal enterocytes, in the immediate vicinity of the carriers that transport the resulting monosaccharides and amino acids into the epithelial cells. Upon amylase-mediated hydrolysis of dietary starch and glycogen, maltose and short oligosaccharides are generated. As these molecules, together with dietary disaccharides, cannot be absorbed across the intestinal epithelium, brush border enzymes further digest them into single molecules of glucose³⁷. Likewise, the concerted action of pancreatic proteases generates a mixture of dipeptides and tripeptides that are further hydrolyzed by the aminopeptidase activity of the brush border membrane³⁸. The resulting free molecules of glucose and amino acids can be ultimately transported across the intestinal epithelium into the bloodstream. Finally, lipid digestion relies mainly on pancreatic enzymes, as lingual lipase contributes to fat degradation only to a limited extent. Pancreatic lipases hydrolyze triglyceride molecules to monoglycerides

and free fatty acids that can be absorbed by specific transporters on the surface of the small intestinal mucosa³⁹ (**Table 1**).

Nutrients	Site of digestion	Enzyme	Final product
Carbohydrates	Mouth	Salivary ptyalin	Monosaccharides
	Duodenum	Pancreatic amylase Int. disaccharidases	
Proteins	Stomach	Gastric pepsin	Amino acids
	Duodenum	Pancreatic trypsin Int. dipeptidases	
Lipids	Stomach	Salivary lipase	Glycerol
	Duodenum	Pancreatic lipase	Fatty acids

Table 1. Digestive processes of macronutrients

Following exocytosis, pancreatic digestive enzymes are released into the lumen of the acini to be conveyed into the duodenum through the pancreatic ductal system⁴⁰. The centroacinar cells constitute the beginning of the intercalated duct draining the acinus⁴¹. These cells are either considered as an extension of the most terminal ductal epithelium as it invaginates into the secretory acinus⁴², or alternatively as providing a fenestrated “cap” to the apical surface of acinar cells⁴³. Ductal cells not only deliver enzymes into the duodenum, but also actively secrete sodium bicarbonate in order to neutralize the acidic pH of the gastric contents delivered to the intestine from the stomach. This neutralizing activity of the ductal secretion is crucial, as pancreatic enzymes are effective only at neutral pH.

Acinar and ductal secretions are regulated by the parasympathetic nervous system and hormones⁴⁴. The entry of acidic chyme into the duodenum stimulates the release of secretin from the duodenal enteroendocrine S cells⁴⁵. Secretin, in turn, induces bicarbonate production from the duct cells, but it also has a direct effect on acinar cells and potentiates enzyme secretion. In the presence of proteins and fats in the duodenum, additional intestinal cells release

cholecystokinin, a potent stimulator of acinar activity⁴⁶. Pancreatic endocrine hormones also play a critical role in regulating exocrine pancreas functions. Indeed, insulin interacts with acinar cells and increases pancreatic enzyme synthesis and secretion⁴⁷. Conversely, pancreatic polypeptide (PP) and somatostatin appear to decrease secretin- and cholecystokinin -mediated protein release in a dose-dependent fashion^{48,49}.

Endocrine pancreas

Scattered throughout the pancreas parenchyma, pancreatic endocrine cells are organized in clusters termed islets of Langerhans. These cells are classified into insulin-secreting β -cells, glucagon secreting α -cells, somatostatin-secreting δ -cells and pancreatic polypeptide-secreting PP-cells, along with ghrelin-positive ϵ -cells that are mostly observed in late development. Interestingly, variations in the microarchitecture of islets between species have been observed. Indeed, while mouse islets display a more-defined architecture placing the β -cells in the core and non- β -cells at the mantle, human islets appear more unorganized having non- β -cells dispersed among β -cells. Human and rodent endocrine pancreas show also differences in cell proportions. While human islets contain approximately 50-70% of β -cells, 20-40% of α -cells and less than 5% of δ -cells, mouse islets consist of 60-80%, 10-20% and less than 5% of β -, α - and δ -cells respectively⁵⁰⁻⁵².

These specialized cells secrete hormones in the blood, which exert autocrine and paracrine actions within the pancreas. Therefore, the islets are highly vascularized, receiving 15% of pancreatic arterial blood flow despite composing only 2% of the pancreatic mass⁵³. The main role of the endocrine pancreas is to ensure a homeostatic control of the glycaemia by secreting insulin and glucagon. All vertebrates produce insulin and glucagon: the property of these hormones to maintain blood glucose level within a narrow physiological range, despite sporadic access to nutrients, has probably represented a survival advantage during evolution.

Insulin primary amino acid structure consist of two polypeptide chains connected by disulfide bonds, resulting from the cleavage of proinsulin by the action of prohormone convertases PC1/3 and PC2^{54,55}. When glycaemia increases, glucose enters in the β -cells across the GLUT2 transporters leading to an increase in intracellular ATP concentration. The resultant ATP binds and closes the ATP-dependent potassium channels, blocking potassium exit from the β -cells. Once the cells are depolarized, the L-type voltage-gated calcium channels are triggered, increasing the influx of calcium and thereby the intracellular calcium concentrations. Increased cytoplasmic calcium concentrations lead to the release of insulin from a pool of insulin-containing docked secretory vesicles⁵⁶⁻⁵⁸.

Once released in the general circulation, insulin mediates glucose uptake from target tissue, such as muscle, liver and adipocytes, upon recruitment on the plasma membrane of insulin-dependent glucose transporters. Consequently, the release of insulin in the fed state promotes the uptake of glucose by skeletal muscle and adipose tissue, leading to a storage of energy through glycogenesis and lipogenesis. Furthermore, insulin reduces new hepatic glucose output by preventing glycogenolysis and gluconeogenesis, the net effect of which is to maintain a normal circulating serum glucose levels while storing extra energy for use during later periods of fasting⁵⁹⁻⁶¹.

Insulin exerts its physiological functions by interacting with a specific tyrosine kinase receptor. The activation of the insulin receptor pathway leads to the tyrosine phosphorylation of a family of insulin receptor substrates (IRSs) that in turn activate intracellular signaling molecules. The main target of phosphorylated IRSs is phosphatidylinositol 3-kinase (PI3K). PI3K plays a major role in insulin function, mainly via the activation of the Akt/PKB cascade. PI3K/Akt pathway ultimately promotes glucose transporter type 4 (GLUT4) translocation to the plasma membrane, resulting in glucose uptake into insulin target tissues.

Together with glucose, other nutrients regulate insulin secretion. Oral protein intake, and the subsequent rise in serum amino acids, stimulates insulin release by direct β -cell stimulation. Lipids are also known to regulate pancreatic hormone release, but the precise pathways and molecules involved in the process remain unclear. Chronic elevation of fatty acids, however, increases basal insulin secretion levels but inhibits glucose-stimulated insulin secretion. Importantly, intestinal hormones, termed incretins, are also involved in facilitating insulin response to glucose and other nutrients⁶²⁻⁶⁵.

The tight control of energy utilization and storage by insulin is balanced by the counter-regulatory hormones glucagon, pancreatic polypeptide, cortisol, catecholamines, and growth hormone. Collectively, these counter-regulatory hormones act to promote glucose release from the liver through glycogenolysis and gluconeogenesis, and inhibit glucose storage during times of starvation or increased energy expenditure.

In states of decreasing blood glucose, pancreatic α -cells secrete glucagon in order to induce a rapid, yet transient, rise in blood glucose⁶⁶. This 29-amino acid peptide derive from the cleavage of proglucagon by prohormone convertase 2 (PCSK2)⁶⁷. High glucose concentrations inhibit glucagon release from pancreatic α -cells. Despite having an intrinsic mechanism that respond to glucose, α -cells synergize with extrinsic paracrine signals to regulate glucagon secretion under high glucose stimulation⁶⁸. Insulin is a potent inhibitor of glucagon secretion and glucagon gene transcription^{69,70}. During hyperglycemia, GABA (γ -Aminobutyric acid) is co-released with insulin from the pancreatic β -cells. This neurotransmitter has been shown to act as a suppressor of amino acid-stimulated glucagon release in the mouse and isolated α -cells via GABA_A receptors⁷¹.

Somatostatin, secreted by the islet δ -cells also inhibits glucagon release in isolated α -cells, as wells as in healthy and diabetic patients^{72,73}. Somatostatin exists as two isoforms, somatostatin-

14 and somatostatin-28, resulting from different cleavage of the prosomatostatin⁷⁴. Acting as a paracrine and endocrine peptide, the two isoforms of somatostatin exert a wide range of effects, by inhibiting the secretion of numerous hormones of the gastrointestinal tract, the nervous system and the pancreas⁷⁵.

Originally termed F cells, pancreatic polypeptide-secreting cells are currently referred to as PP-cells⁷⁶. PP is known to be part of a family of neuropeptide Y (NPY) and is initially synthesized as a precursor named pre-propancreatic polypeptide before further processing to produce the mature hormone⁷⁷⁻⁷⁹. The metabolic effects of PP are modest in rodents, including suppression of insulin and somatostatin secretion⁸⁰.

Abundant during pancreatic embryonic development, ghrelin-secreting ϵ -cells represent less than 1% of endocrine cells in adult mice and humans⁸¹⁻⁸³. Little is known about the role of ghrelin within the pancreas, but it has been suggested that ghrelin could play a paracrine/autocrine role in the regulation of β -cell survival and function⁸⁴. In this context, ghrelin has recently been reported to promote proliferation and cell growth and to inhibit apoptosis in a pancreatic β -cell line and human islets⁸⁴. This suggests that one function of ghrelin in the developing pancreas could be to promote cell growth and maturation locally.

Interestingly, *in vitro* studies demonstrated that ghrelin could inhibit β -cell apoptosis exposed to exogenous cytotoxic insults, such as exposition to interferon- γ /tumor necrosis factor- α (IFN- γ /TNF- α), doxorubicin as well as palmitate acids⁸⁴⁻⁸⁶. Additionally, ghrelin has been shown to protect newborn rats treated with streptozotocin against development of diabetes during adulthood. In this model, injection of exogenous ghrelin during the first week of life prevented the onset of diabetes and significantly reduced STZ-induced β -cell loss⁸⁷.

Importantly, ghrelin has been shown to inhibit glucose-stimulated insulin secretion in β -cell lines, islets of Langerhans and animal models⁸⁸. Accordingly, ghrelin receptor (GHS-R) antagonism, ghrelin immunoneutralization, and ghrelin-knockout mice display enhanced glucose-induced insulin release from perfused pancreas and isolated islets^{89,90}. Thus, pharmacological, immunological and genetic blockades of ghrelin in pancreatic islets, all markedly augment glucose-induced insulin release, showing that ghrelin physiologically restricts insulin release in rodents.

Diabetes Mellitus: The Black Plague of the 21st Century

Diabetes mellitus is a combination of heterogeneous metabolic disorders commonly presenting with episodes of hyperglycemia and glucose intolerance over a prolonged period of time. This chronic disease arises from defective insulin secretion, insulin action, or both⁹¹. Aberrant inactivation of insulin pathway leads to the disruption of the regulatory systems governing the storage and mobilization of metabolic fuels, including the catabolism and anabolism of carbohydrates, lipids and proteins.

Diabetes mellitus is one of the largest epidemics the world has faced, both in developed and developing nations. According to the World Health Organization, 1.5 million deaths were caused by diabetes mellitus in 2012, while in 2015 the International Diabetes Federation placed diabetes mellitus as one of the top three major worldwide causes of death from a chronic disease⁹².

Diabetics display an increased risk of cardiovascular, peripheral vascular, and cerebrovascular diseases. Consequently, the debilitating effects of diabetes mellitus encompass various organ failures, such as retinopathy, nephropathy, and neuropathy⁹³. If untreated, these pathological conditions can lead to severe complications, including blindness, nonfatal and fatal myocardial

infarction and gangrene of the feet, often necessitating amputation. Ultimately, these complications not only reduce both life expectancy and quality of life⁹⁴, but also pose an enormous economic burden with regards to health system costs⁹⁵.

The development or progression of the small-vessel complications of diabetes can be delayed or prevented by control of hyperglycemia. However, even very tight control of diabetes may lead to risks, such as hypoglycemia⁹⁶.

With chronic hyperglycemia as a common feature, diabetes mellitus is classified into three types, described hereafter.

Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by insulin deficiency that occurs as a consequence of the loss of pancreatic islet β -cells⁹⁷⁻¹⁰⁰.

Of all individuals with diabetes 10-15% are affected by T1DM, being type 2 diabetes mellitus (T2DM) the most common form. However, T1DM is one of the most common endocrine and metabolic conditions occurring in childhood, with more than 500,000 children currently living with this condition, globally¹⁰¹. The disease is most often diagnosed in children and adolescents, usually presenting with a classic trio of symptoms: polydipsia, polyphagia and polyuria⁹¹.

T1DM is a polygenic disease that is influenced by environmental factors. Genetic risk factors are necessary but not sufficient to develop the disease, as indicated by a concordance rate of only 30% for T1DM among monozygotic twins¹⁰². In a small subset of patients, no immune responses or autoantibodies are detected, and the cause of β -cell destruction is unknown. This idiopathic form of T1DM has a stronger genetic component⁹¹. Individuals with specific HLA genotypes, encoding MHC proteins, have an increased risk of developing T1DM¹⁰³⁻¹⁰⁵.

However, genetic polymorphisms throughout the entire genome contribute to risk, the majority of them being important genetic factors for the immune system^{106,107}.

Concomitantly, several environmental factors have been proposed as candidate etiological factors. Particularly, viral infections, the timing of the first introduction of food and gestational infections have been associated with an increased risk of T1DM¹⁰⁸⁻¹¹³.

As previously mentioned, T1DM is an autoimmune disease culminating with the destruction of the pancreatic β cells, characterized by islet cells inflammation and β -cell loss. The triggering event of β -cell autoimmunity is still unknown, but it is generally believed that the combined occurrence of a virus infection and an environmental exposure event might elicit the pathogenic immune response. Once activated, B cells interact with specific T cells as well as dendritic cells. Antigen presentation by B cells and dendritic cells drives the activation of T cells, capable of destroying β -cells, resulting in a progressive loss in insulin secretory function¹¹⁴. Curiously, it remains unclear why the volume and mass of non β -cells remains unaffected in T1DM patients, as the autoimmunity appears specific for insulin-secreting cells¹¹⁵.

The onset of β -cells autoimmunity is marked by the development of islet reactive autoantibodies. These autoantibodies are not thought to be pathogenic but serve as biomarkers of the development of the disease, as they appear many months or years before the symptoms emerge. The list of T1DM-associated antibodies has more than two dozens members; the most common target insulin, proteins anchored to the membrane of secretory vesicles, such as insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZNT8), and glutamic acid decarboxylase (GAD65), catalyzing the decarboxylation of glutamate to GABA¹¹⁶⁻¹¹⁸.

T1DM patients present the same microvascular complications than all diabetics. Microvascular failure predominantly affects the retina, peripheral nerves and renal glomeruli. These cells share

the inability to downregulate glucose uptake in the presence of increased levels of extracellular glucose. The pathogenic effects of hyperglycemia result from the overproduction of superoxide by mitochondria, which results in oxidative stress. Retinopathy in T1DM is characterized by impaired blood flow in the retinal vessels, and this stimulates a compensatory proliferation of retinal vessels. The new vessels are fragile and hyperpermeable, such features leading to hemorrhages and decrease in retinal perfusion which may ultimately cause blindness^{119,120}.

Macrovascular complications manifest predominantly as coronary heart disease, but also cerebrovascular disease and peripheral artery disease. Cognitive function may also be affected by long-term hyperglycemia. Genetic susceptibility and concomitant risk factors also contribute to the development of complications¹²¹.

Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is characterized by dysregulation of carbohydrate, lipid, and protein metabolism, resulting from impaired insulin secretion, insulin resistance or a combination of both. Of the three major types of diabetes, T2DM is far more common than either T1DM or gestational diabetes. It is associated to progressively impaired insulin secretion by pancreatic β -cells, usually within a background of pre-existing insulin resistance in skeletal muscle, liver and adipose tissue¹²². Overt hyperglycemia is usually preceded by prediabetes, a high-risk condition that predisposes individuals to T2DM development. Prediabetes is a metabolic condition characterized by any one of the following: impaired fasting glucose (IFG) levels, impaired glucose tolerance (IGT), or increased glycated hemoglobin A1c (HbA1c) levels. Individuals with IFG display fasting plasma glucose levels that are higher than normal but do not meet the criteria for the diagnosis of diabetes. They are characterized by hepatic insulin resistance and impaired early insulin secretion. Conversely, IGT is associated to insulin resistance in muscle and impaired late insulin secretion after a meal¹²³. The clinical presentation

of prediabetics and T2DM patients can vary considerably among individuals. At the time of diagnosis, many patients with T2DM are asymptomatic, whereas others present with severe hyperglycemia or even diabetic ketoacidosis.

T2DM is a major global public health concern. The International Diabetes Federation estimated that, in 2013, 382 million adults aged 20-70 years worldwide had T2DM, with 80% of those affected living in low- and middle-income countries. This number is expected to rise to 592 million by 2035¹²⁴.

Increasing adiposity, as reflected by higher BMI levels, is the single most important risk factor for T2DM, while physical inactivity is the main behavioral risk factor¹²⁵. Regardless the body weight, nutrition plays a key role in T2DM pathogenesis. High consumption of whole grains, green leafy vegetables, nuts and coffee and reduced intake of refined grains, red and processed meat, and sugar-sweetened beverages have a protective role against T2DM¹²⁶. Cigarette smoking and excessive alcohol consumption are significant risk factors for developing T2DM¹²⁷. Although genetics play an important part in the development of T2DM, the ongoing diabetes epidemic can be only explained by the epidemic of obesity¹²⁸.

T2DM clusters in families and is heritable. Genome-wide association studies have identified more than 100 common variants associated with T2DM¹²⁹. Notably, the majority of non-diabetic people carry risk variants for T2DM, as these genetic variants only have modest effects, increasing risk by 10–20%.

Insulin resistance is the earliest detectable abnormality in individuals who are likely to develop T2DM and it precedes the onset of the disease by many years^{122,123,130,131}. Insulin resistance can develop not only in muscles and liver^{122,132,133}, accounting for the majority of glucose disposal during hyperglycemia, but also in adipose^{134,135}, kidney¹³⁶, gastrointestinal tract¹³⁷,

vasculature¹³⁸ and brain tissues^{139,140}, and pancreatic β -cells¹⁴¹⁻¹⁴³. Altogether, impaired suppression of hepatic gluconeogenesis, muscular and hepatic insulin resistance, reduced insulin-independent glucose uptake^{144,145}, and excessive renal glucose reabsorption¹⁴⁶ contribute to postprandial hyperglycemia in T2DM. In addition, insulin resistance in the vascular endothelium impairs the vasodilating effects of the hormone, thereby further reducing not only its own delivery but also glucose distribution^{147,148}. Insulin resistance usually results from increased serine phosphorylation of insulin receptor substrate (IRS) proteins, which inhibits tyrosine phosphorylation^{149,150}. In some cases, serine phosphorylation also increases IRS degradation, further contributing to the insulin resistance¹⁵¹. Increased serine phosphorylation is a common consequence of ectopic lipid accumulation in muscle and liver¹⁵²⁻¹⁵⁶, typically occurring in obese individuals. Mitochondrial dysfunction and systemic inflammation frequently observed in liver, muscle and adipose tissue of T2DM subjects can also contribute to the phosphorylation of serine residues in IRS proteins^{157,158}. Finally, ER stress associated to obesity, hyperlipidemia, and T2DM can promote serine phosphorylation and reduce insulin-induced tyrosine phosphorylation of IRS factors, leading to insulin resistance¹⁵⁹. Persistent insulin resistance, together with a genetic predisposition, place stress on β -cells, leading to a failure of β -cell function and a progressive decline in insulin secretion^{122,123,133,160,161}.

As T1DM patients, individuals affected by T2DM suffer from microvascular complications, closely related to the severity and duration of hyperglycemia^{162,163}. Importantly, visceral obesity and hypertension are frequently associated with T2DM. Altogether, insulin resistance, hypertension, endothelial dysfunction, hypertriglyceridemia, obesity and activation of inflammatory pathways greatly increase the risk of macrovascular complications, such as myocardial infarction, stroke, peripheral vascular disease and congestive heart failure, and premature death¹⁶⁴.

Gestational diabetes mellitus

Pregnancy is normally associated with progressive insulin resistance, resulting from a combination of increased maternal adiposity and the insulin-desensitizing effects of placental hormones. Appearing near mid-pregnancy, it progresses through the third trimester to levels that approximate the insulin resistance observed in individuals with T2DM. Accordingly, pancreatic β -cells normally increase their insulin secretion to compensate for the insulin resistance underlying pregnancy¹⁶⁵. Gestational diabetes mellitus (GDM) is a form of hyperglycemia, resulting from an insulin supply that is inadequate to meet tissue demands for the regulation of glucose homeostasis. A small subset of women with GDM develops the same autoantibodies appearing in the circulation of T1DM patients¹⁶⁶⁻¹⁷⁰. In this particular type of GDM, inadequate insulin secretion results from autoimmune damage of pancreatic β -cells and it can rapidly lead to overt diabetes following pregnancy¹⁶⁹. The majority of women with GDM appears to have β -cell dysfunction that occurs on a background of chronic insulin resistance. After delivery, when the acquired insulin resistance of pregnancy abates, women who had GDM show on average a considerably greater insulin resistance than the general population^{165,171-175}. Long-term follow-up studies revealed that most, but not all, women with GDM progress to diabetes after pregnancy¹⁷⁶.

GDM is a risk factor of fetal morbidity and, if untreated, it can increase the risk of perinatal mortality¹⁷⁷. Importantly, the offspring of women with GDM are at increased risk of obesity, glucose intolerance and T2DM in late adolescence and young adulthood¹⁷⁶.

Current treatments

As previously mentioned, diabetic patients are exposed to various macrovascular and microvascular complications, all of which profoundly impacting on patients' mortality, morbidity and quality of life¹⁷⁸. Therefore, healthcare professionals and researchers are acting

cooperatively to manage or even reverse this hyperglycemia, without increasing the incidence of hypoglycemia.

Type 1 diabetes management

Currently, management of T1DM is focused on intensive insulin therapy with the goal of maintaining glucose levels as close to normal as possible while avoiding hypoglycemia¹⁷⁹. Frequent monitoring of blood glucose levels and carbohydrate counting of food are also required. Recombinant insulin analogues have mostly replaced recombinant human insulin formulations. They can be administered through subcutaneous injection or continuous subcutaneous insulin infusion using a pump worn 24 hours per day. Current insulin administration is designed to closely approximate the physiological insulin profile, in which the pancreas continuously secretes a small amount of insulin and produces larger amounts in response to a meal containing carbohydrates. Accordingly, a combination of long-acting and short-acting insulin analogues is now used in the form of multiple daily insulin injections.

Illness, pregnancy, exercise or excessive insulin administration can lead to hypoglycemia¹⁸⁰. Severe hypoglycemia results in a lack of glucose delivery to the brain and can also directly cause neuronal cell death¹⁸¹. Frequent episodes of hypoglycemia, due to insulin administration, can have negative consequences not only physically but also emotionally. Hypoglycemia anxiety is a common concern that can negatively affect glycemic control¹⁸².

Type 2 diabetes management

T2DM treatments aim to ensure glycemic control, in order to prevent microvascular complications^{162,163,183} and correct classic cardiovascular risk factors, including insulin resistance, to reduce the risks of macrovascular complications¹⁵². Because obesity and physical inactivity are associated with insulin resistance, lifestyle modification should be a basic component of all intervention programs^{184,185}. However, despite initial weight loss, most

patients regain weight over the subsequent 1-2 years¹⁸⁶⁻¹⁸⁸ and ~50% of obese individuals with prediabetes still progress to overt diabetes, regardless successful weight control¹⁸⁹. Therefore, achievement of durable glycemic control requires antidiabetic medications. Because no single medication reverses the multiple abnormalities, combination therapies are often adopted^{122,190-192}.

Metformin is the most commonly prescribed antidiabetic medication worldwide. It suppresses hepatic glucose production, leading to a reduction in fasting plasma glucose levels¹⁹³. However, metformin has no effect on β -cell functionality¹⁹⁴. Conversely, sulfonylureas augment insulin secretion, in order to overcome insulin resistance and decrease fasting plasma glucose levels^{122,194-196}. Neither of these drugs has a long-term protective effect on β -cell function; thus, their beneficial effects in glycaemia control are frequently limited over time.

Thiazolidinediones are insulin-sensitizing agents, able to enhance insulin action in skeletal and cardiac muscle, liver, and adipocytes^{122,152,197-199}. They also exert a potent effect on β -cells, stimulating and preserving insulin secretion²⁰⁰⁻²⁰². The combined insulin-sensitizing and β -cell stimulatory effects of thiazolidinediones explain their long-lasting action^{196,201,202}.

Incretin mimetics represent a relatively new and promising group of anti-diabetic medicaments. They induce pharmacological increase in plasma GLP1 levels, markedly augment insulin secretion and inhibit glucagon secretion²⁰³. The increase in the levels of plasma insulin and the decrease in those of glucagon effectively suppress hepatic glucose production and cause a durable reduction in fasting glycaemia²⁰⁴⁻²⁰⁶. Further, they promote weight loss, delay gastric emptying, correct endothelial dysfunction, reduce blood pressure, improve the plasma lipid profile and reduce C-reactive protein levels^{206,207}.

Another strategy to normalize glycaemia in T2DM patients is to slow the rate of carbohydrate absorption in the intestine. Alpha-glucosidase inhibitors, like acarbose, are oral anti-diabetic drugs able to prevent the digestion of carbohydrates. Importantly, these drugs have been shown to decrease the conversion of prediabetes to diabetes²⁰⁸.

Finally, if oral or injectable antidiabetic agents fail to normalize blood glucose levels, patients with T2DM can be treated with insulin, but large doses are often required²⁰⁹.

A future without diabetes

New avenues for treatment are currently in development and might, in combination with existing drugs, bring change and enhance our ability to control glycaemia in diabetics. Technological advances enabling broader implementation of smaller and better insulin pumps, and continuous glucose monitoring, have hastened progress towards the development of a true artificial pancreas.

Several efforts have been devoted to the identification of immunosuppressive agents that could preserve β -cell mass in symptomatic T1DM patients. Although single-compound studies dominate, several combination trials are in progress, along with studies investigating the efficacy of administering haematopoietic stem cells²¹⁰. The rationale to treat patients with haematopoietic stem cells is to induce T regulatory cells, which would supposedly dampen β -cell-targeted autoimmunity²¹¹. Likewise, agents that suppress inflammation, including IL-1 β blockers and salsalate (a potent inhibitor of NF- κ B), have shown some promise in improving glycemic control and β -cell function in T2DM trials²¹²⁻²¹⁴.

Islet transplantation normally results in a rapid glycaemia normalization and exogenous insulin independence for up to 5 years^{215,216}. However, islet transplantation faces many hurdles and is therefore suitable only for type 1 diabetic patients with serious complications²¹⁷. Nevertheless,

the development of encapsulated islets hold promises to reduce rejections and the requirement of lifelong immunosuppressant treatment²¹⁸.

Regenerative therapies based on the differentiation of embryonic stem cells and pluripotent stem cells that mimic natural healthy β -cells represent an intriguing option²¹⁹⁻²²³. Furthermore, the possibility to convert alternative pancreatic cells into insulin-secreting β -cells might become the next frontier for diabetes treatment²²⁴⁻²³³. These ambitious strategies imply a profound knowledge of the molecular mechanisms underlying pancreatic cells development, specification and differentiation.

Pancreas Development: A Multistep Journey

During embryogenesis, following fertilization, the zygote undergoes multiple cycles of mitotic divisions until the formation of the blastocyst. The blastocyst will implant into the uterine wall at which point the process of gastrulation begins. In vertebrates, gastrulation leads to the formation of three germ layers identified as ectoderm, mesoderm and endoderm, each layers giving rise to distinct organs. In mice, at embryonic day (E) 6-7.5, the endoderm initially appears as a flat single-cell layer epithelium which will then, 2 days later (E8.5), internalize and fold to form the primitive gut tube. This structure is the primitive precursor of both the respiratory and digestive systems.

The development of the vertebrate pancreas is a complex process comprising regionalization, cell differentiation, and morphogenesis²³⁴⁻²³⁶. Proper gut tube formation and pancreas organogenesis requires a series of permissive interactions with the neighboring mesodermal tissue. In particular, mesoderm-derived signaling molecules, including FGF, BMP, Wnt, Retinoic acid, Hedgehog and Notch, play crucial and stage-specific roles during endoderm patterning and pancreas specification^{237,238}.

In the mouse, the first sign of pancreas development appears at around embryonic day (E) 9.5 when the dorsal foregut thickens and evaginates into the surrounding mesenchyme at the foregut/midgut junction. Twelve hours later, the ventral bud begins to arise with a morphologically similar process^{235,239-241}. Once the ventral and dorsal domains are specified, a number of transcription factors become specifically expressed within the multipotent pancreatic progenitors (MPC) along the prospective pancreatic region. These early transcription factors, such as Pdx1, Ptf1a and Sox9, play multiple stage and lineage specific roles, as they are not only essential for pancreas specification, but also for maintaining the phenotype of differentiated cells²⁴²⁻²⁴⁶.

Primary transition

During the early phase of pancreas development, referred to as the primary transition²⁴¹, MPCs proliferate to rapidly increase the size of the organ (**Figure 4**).

Simultaneously, the pancreas develops into a highly branched tubular structure, forming multiple microlumens, which then coalesce to form a larger tubular network. At this phase of the development, occurring between E9.5 and E12.5, the first differentiated endocrine cells, mainly glucagon-expressing α -cells, begin to appear in the dorsal bud^{241,247-249}. Following the rotation of the gut tube, the ventral and dorsal buds come into proximity for their future fusion into a single organ. By E12.5, the pancreatic epithelium starts to undergo dramatic morphological changes leading to the formation of protrusions into the surrounding mesenchyme.

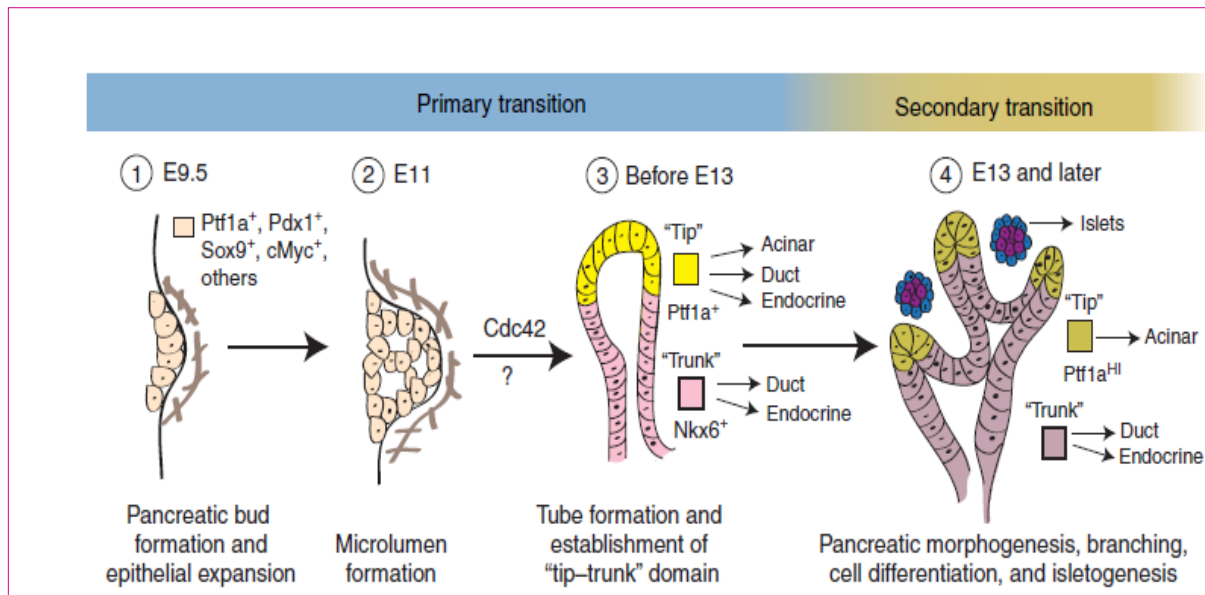


Figure 4. Primary and secondary transitions (from Benitez et al., 2012). Mouse pancreas development is characterized by two overlapping transitions from E9.5 to birth. MPCs proliferation induces the thickening of the foregut endoderm therefore leading to pancreatic bud emergence (1) and microlumen formation (2). The onset of the secondary transition is marked by the segregation of two domains known as tip/trunk compartmentalization with *Ptf1a* and *Nkx6.1* as key regulators (3). The bipotent “trunk” cells express *Nkx6.1* and give rise to endocrine and ductal cells while the multipotent “tip” domain contains MPCs capable of differentiating into acinar, ductal and endocrine cells, quickly restricted to acinar progenitors (4).

Secondary transition

Shortly after the primary transition, the nascent pancreatic buds have not yet acquired the macroscopic structure of the mature organ, nor have they formed the differentiated cell types necessary for pancreatic function. Instead, they are almost entirely composed of undifferentiated MPCs. The transition of non-committed MPCs into an organ with different cell compartments is a multistep process, of which the first stage is the segregation of a tip and trunk domain at the onset of the secondary transition²⁵⁰. Indeed, the trunk predominantly gives rise to endocrine and ductal cell lineages, whereas the tip cells are rapidly restricted to an acinar fate²⁵⁰⁻²⁵⁸.

The transcription factors *Nkx6.1/6.2* and *Ptf1a* act as master regulators during this process. During the primary transition, these factors are co-expressed in MPCs (**Figure 5**). However, at

the onset of branching morphogenesis at E12.5, *Ptf1a* becomes progressively restricted to peripheral tips of the epithelial branches, while *Hnf1 β* , *Nkx6.1* and *Nkx6.2* become progressively confined to trunks. Consequently, MPCs gradually convert into lineage-restricted cells. However, at E12.5 *Hnf1 β* ⁺ progenitor cells can still contribute to the islet, ductal and acinar lineages, although the proportion of acinar cells originating from *Hnf1 β* ⁺ progenitors is highly limited.

The commitment of a multipotent cell to an acinar or endocrine/ductal fate is determined by the mutually antagonistic action of *Nkx6* factors and *Ptf1a*. Indeed, in *Nkx6.1*-deficient mice, the *Ptf1a*-expressing domain expands through the trunk domain, with a concomitant decrease in endocrine precursor cells and an increase in acinar cells, by E15.5. In *Nkx6.1/Nkx6.2* double knockout pancreas, this phenotype is even more pronounced. Conversely, forced expression of either *Nkx6.1* or *Nkx6.2* throughout the epithelium at the onset of pancreas specification, greatly suppresses the number of *Ptf1a*⁺ cells, by E12.5 and *amylase*-expressing cells, by E14.5. Thus, the misexpression of either *Nkx6.1* or *Nkx6.2* in multipotent progenitors is sufficient to prevent the initiation of the acinar program²⁵⁹.

While pancreas development progresses, *Nkx6.1* and *Ptf1a* expression is maintained in adult β - and acinar cells, respectively. Conversely, *Nkx6.2* becomes transiently restricted to a few epithelial cells, being positive for either glucagon or amylase, and definitely disappears shortly before birth²⁶⁰.

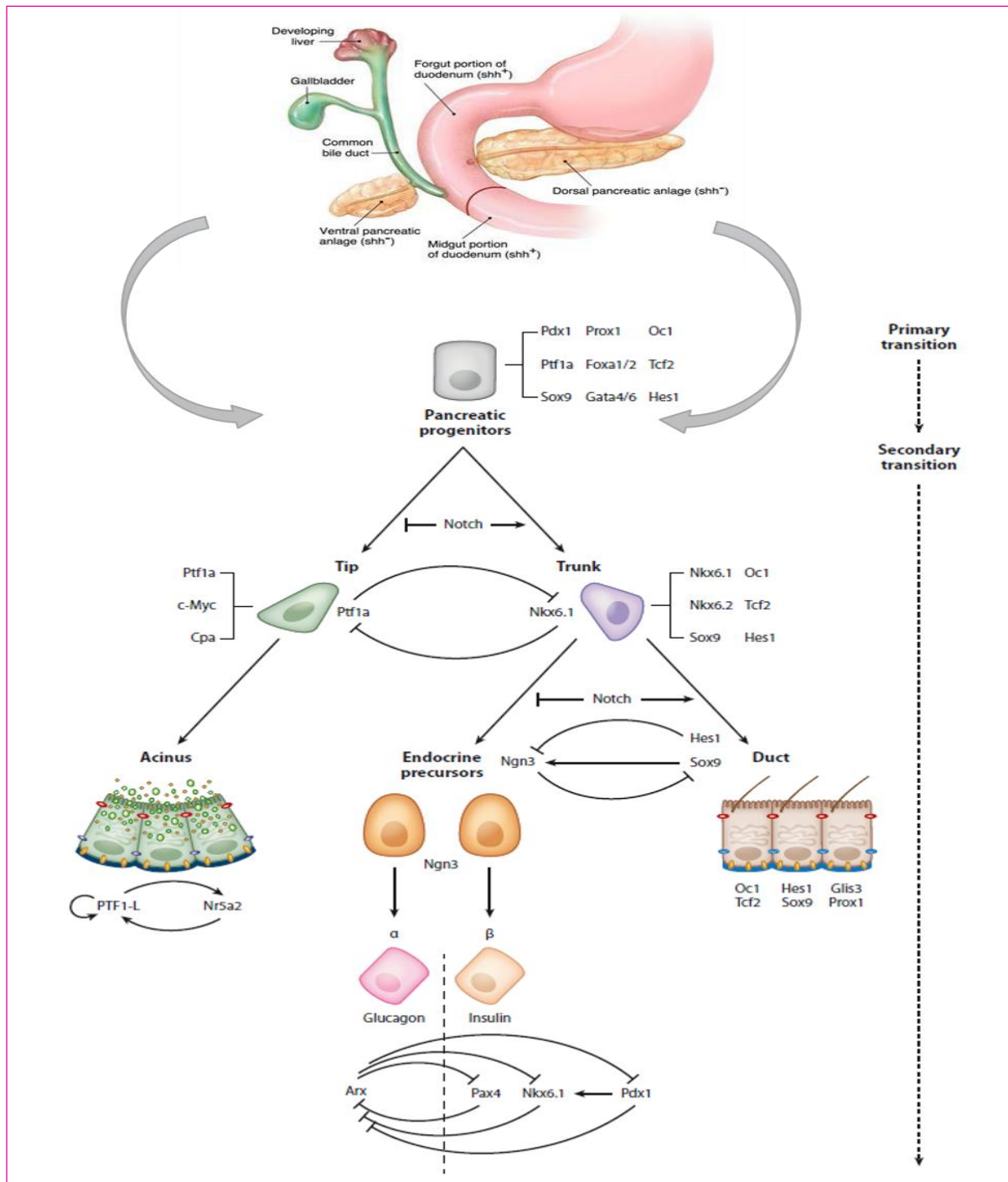


Figure 5. Simplified model of pancreatic cell fate determination (adapted from Shih et al., 2013). The mammalian pancreas arises from two evaginations in the posterior foregut, one dorsal and one ventral, which expand into the tail and head, respectively, of the mature organ. The first evidence of pancreas development is marked by the activation of a number of genes in multipotent pancreatic cells (MPCs), including Pdx1, Hnf1 β , Ptf1a, Mnx1 and Sox9. At the onset of the secondary transition, the tip/trunk segregation appears to depend on the Notch signaling pathway. A complex network of transcription factors orchestrates specification and development of acinar, ductal and endocrine cell lineages.

Together with a tip population of differentiating acinar cells expressing *Ptf1a*, but not *Hnf1 β* nor *Sox9*, and a trunk population expressing *Hnf1 β* and *Sox9*, but not *Ptf1a*, an intermediate population expressing *Ptf1a* and *Sox9*, but not *Hnf1 β* , has been identified at the tip/trunk junction²⁶¹. These cells appear around E14.5 and represent the initial manifestation of the centroacinar lineage.

Notch/Hes1 signaling pathway also plays an essential role in pancreatic cell fate determination. Activated throughout the entire epithelium in early MPCs^{251,257}, the Notch pathway becomes progressively silenced at the onset of the secondary transition. Specifically, *Hes1* expression is lost in endocrine progenitors as they activate *Ngn3* and delaminate from trunk epithelium²⁶², as well as in *Ptf1a*-expressing tip progenitors as they begin to initiate acinar cell differentiation^{251,257}. By E14.5, *Hes1* expression is largely confined to the trunk epithelium and by birth *Hes1* expression is entirely restricted to centroacinar and terminal duct cells, most of which also express *Sox9*^{246,251,257,263}. Functionally, Notch appears to effectively block both endocrine and acinar differentiation^{257,258,264-266}, by either repression of *Ngn3* expression or selective inhibition of the expression of specific *Ptf1a* target genes^{257,262,264,266-268}.

Development of the acinar lineage

As described above, acinar cells are polarized pyramidal-shaped cells with high secretory capacity. To accommodate their secretory burden, acini have numerous mitochondria, unusually extensive Golgi and endoplasmic reticulum, and prominent electron-dense zymogen granules²⁴¹.

Once confined in the tip compartment, *Ptf1a* drives acinar maturation by initiating and stabilizing a gene-regulatory network that drives acinar gene expression. During the primary transition, *Ptf1a* forms a complex with Rbp-jk²⁶⁹⁻²⁷¹. Together, these factors are responsible for the activation of *Rbp-jl*, an acinar-restricted paralog of *Rbp-jk*^{269,270}. As pancreatic progenitors

commit to an acinar fate, Rbp-jk is replaced by Rbp-jl. This switch is crucial for the direct activation of genes encoding hydrolytic enzymes, mitochondrial components, and exocytosis machinery, representing the hallmark components of functionally mature acini^{270,272}.

Similarly, *Nr5a2*, a direct target gene of *Ptf1a*, is necessary for acinar cell differentiation^{273,274}. *Nr5a2* activates the expression of genes encoding digestive zymogens and other acinar cell-specific genes²⁷³.

By E15.5, most tip cells have undergone acinar cell differentiation, and the subsequent expansion of the acinar domain is largely driven by cell proliferation. Acinar cells continue to differentiate, mature, and proliferate until weaning²⁷⁵. Adult acinar cells, however, show a low basal proliferative index and appear to have poor regenerative capacity^{275,276}. *Ptf1a* and *Mist1* are expressed in adult acini and they both induce the expression of the cell-cycle inhibitor *Cdkn1a* (p21)²⁷⁷. Thus, the expression of these transcription factors may contribute to the low proliferative index of adult acinar cells. Conversely, *Nr5a2*, *c-Myc* and β -*catenin* promote the proliferation of exocrine cells, driving acinar cell expansion²⁷⁸⁻²⁸³. Furthermore, acinar cells can adaptively expand in response to specific physiological demands, such as high protein diet, hyperphagia, pregnancy, and lactation. Moreover, the intestinal hormone cholecystokinin has been found to stimulate pancreatic acinar growth²⁸⁴.

Development of the ductal lineage

Whereas tip cells are fated to become acinar cells, the trunk domain is bipotential and produces ductal and endocrine cells^{251,252,254}. After the tip and trunk domains have separated at around E12.5, cells in the trunk undergo extensive morphogenetic changes to form a tridimensional network of tubules lined by a single layer of polarized epithelial cells^{247,249,285}. This network of tubules is referred to as the primitive ducts or progenitor cords, such epithelium giving rise to

pancreatic endocrine cells^{252,285}. During the secondary transition, a subset of cells in the progenitor cords initiate the expression of the transcription factor *Neurogenin3* (*Ngn3*), which marks the onset of endocrine cell differentiation²⁸⁶⁻²⁸⁹. Conversely, trunk epithelial cells that do not activate *Ngn3* eventually contribute to the ductal tree^{256,290,291}. Thus, the precise control of *Ngn3* induction is the key factor balancing the endocrine versus ductal cell fate decision. Many of the transcription factors (e.g., *Sox9*, *Tcf2*, *Onecut-1*, *Hes1*, *Prox1*, and *Glis3*) that segregate to the trunk domain during tip/trunk separation later become restricted to ducts and excluded from the endocrine compartment²⁹²⁻²⁹⁹. The precise role of these genes in ductal development and function is currently unknown.

Development of the endocrine lineage

Lineage tracing studies showed that all the mature pancreatic endocrine cells derive from a subset of epithelial cells that transiently express *Ngn3*. Upon *Ngn3* activation these endocrine progenitor cells delaminate from the ductal epithelium and aggregate in clusters called islets of Langerhans^{287,300,301}. Although individual endocrine precursors are unipotent, the pool of *Ngn3*⁺ cells generates all the five different endocrine cell types: glucagon-producing α -cells, insulin-producing β -cells, somatostatin-producing δ -cells, pancreatic polypeptide-producing PP-cells, and ghrelin-producing ϵ -cells. Notably, the timing of *Ngn3* expression determines the entry into a specific endocrine lineage: early *Ngn3*⁺ cells exclusively produce α -cells, while late *Ngn3*-expressing endocrine progenitors sequentially acquire the competence to give rise to β -cells, PP-cells and δ -cells at E11.5, E12.5 and E14.5, respectively²⁸⁸.

Interestingly, many transcription factors controlling ductal development, such as *Sox9*, *Tcf2*, *Glis3*, and *Onecut-1*, are also necessary for *Ngn3* induction, indicating dual roles for these factors in the development of both lineages^{293,294,299,302-305}. However, the selective expression of *Ngn3* in a subset of cells in the primitive ducts seems to rely on Notch/Hes1 pathway. Indeed,

genetic evidences suggest that bipotential ductal/endocrine progenitors must escape Notch signaling to initiate endocrine differentiation.

After the secondary transition, endocrine precursors gradually stop arising from the ductal epithelium^{251,252,254}. However, studies of pancreatic injury or genetically manipulated mouse models indicate that adult ducts retain the capacity to activate *Ngn3* and thus are competent to initiate, at least to some extent, endocrine programs^{252,306}.

While differentiating into hormone-producing cells, *Ngn3*⁺ endocrine precursors undergo dynamic changes in gene expression, resulting in the activation of specific transcription factors, which regulate many aspects of endocrine development. *NeuroD1*, *Islet1*, *IA-1*, *Rfx6* and *Nkx6.1* represent a group of transcription factors that control the proper formation of endocrine cells number by promoting progenitor cell survival and/or proliferation. Subsequently, additional factors such as *Pdx1*, *Pax4*, *Arx* and *Nkx2.2* drive the flux of endocrine progenitors towards a specific lineage allocation. Finally, *MafA*, *MafB*, *Foxa1* and *Foxa2* are required to control later aspects of endocrine maturation and physiological functionality³⁰⁷.

Interestingly, as with the mechanisms governing tip/trunk segregation, fate distinction between β - and α -cell precursors relies on mutual repression between opposing lineage determinants. *Arx* (aristaless-related homeobox) plays a key role in the determination of the α - and PP-cell lineages, later being restricted to mature glucagon-expressing cells where it is involved in maintaining their identity³⁰⁸. Indeed, *Arx* mutant mice display severe hypoglycemia caused by a loss of α -cells associated with a proportional increase in β - and δ -cell counts.

Conversely, *Pax4* is critical for β -cell determination and is exclusively expressed in β -cells in the adult pancreas. Accordingly, *Pax4*-deficient mice develop severe hyperglycemia and die shortly after birth, as a result of a profound deficiency of insulin-producing β -cells, concomitant

with an increase in α -cells number³⁰⁹. Loss-of-function experiments demonstrated that *Arx* and *Pax4* exhibit antagonistic roles in the specification of the endocrine subtype destinies through an inhibitory, cross-regulatory circuit that controls the transcriptional state of these two genes^{310,311}.

In our laboratory, we generated several mouse models misexpressing *Pax4* in embryonic or adult α -cells and notice a conversion of α -cells into β -like cells. Importantly, such conversion was associated with the re-expression of *Ngn3* in ductal cells and their differentiation into endocrine cells through a reawakening of the epithelial-to-mesenchymal transition (EMT). The newly formed α -cells being yet again converted into β -like cells upon α -cell-specific *Pax4* misexpression, such a cycle of conversion and neogenesis results in β -like cell hyperplasia over time. Strikingly, these β -like cells are functional and can revert the consequences of chemically-induced diabetes.

In order to identify novel genes involved in β -cells regeneration, we performed a transcriptomic screening in *Pax4* misexpressing animal models. Importantly, one of these studies suggested that *Gfi1* could play a role in pancreas cells development and function.

A New Player in Pancreas Development: *Gfi1*

Gfi1 is a transcriptional repressor involved in the development and cell determination in the bone marrow, peripheral nervous system and intestine³¹². *Gfi1* gene encodes a 55kDa nuclear protein containing six C-terminal C2-H2 zinc finger domains (DNA-binding) and an amino-terminal-located SNAG (*Snail* + *Slug*) transcriptional-repressor domain (**Figure 6**)^{313,314}. A mutation in the SNAG domain that replaces a proline at amino acid position 2 by alanine (P2A)

has been shown to completely abolish the activity of Gfi1 as a transcriptional repressor^{313,314}, suggesting that the SNAG domain constitutes a crucial element for the function of Gfi1.

SNAG zing finger proteins have been found in a wide array of tissues and are involved in the development of many organs. During developmental processes, they repress cell adhesion molecules and this ultimately results in epithelial to mesenchymal transition (EMT). As EMT is also involved in cancer pathogenesis, multiple members of the SNAG family participate in the progression or manifestation of a variety of tumors³¹⁵.

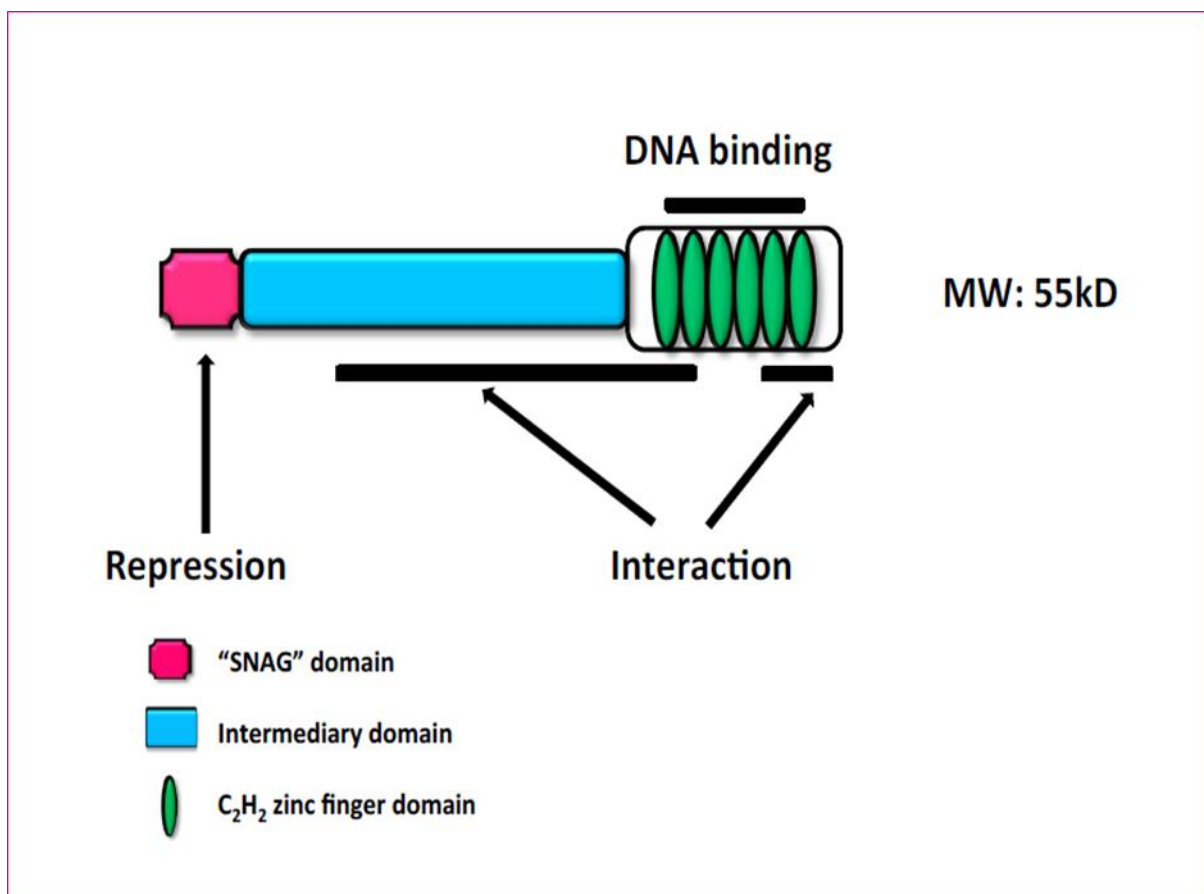


Figure 6. Schematic representation of Gfi1 protein structure. Both human and murine Gfi1 proteins consist of 422 amino acids and have a mass of 45297 Da. Gfi1 contains six c-terminal C₂H₂-type zinc-finger domains (depicted in green) and an N-terminal SNAG domain critical for its repressor activity (depicted in fuchsia). Zinc fingers 3 to 5 of Gfi1 recognize and bind to the DNA. Zinc fingers 1, 2 and 6 are required for the interaction with other proteins.

Gfi1 is a transcription repressor

Gfi1 specifically recognizes the DNA sequence TAAATCAC(A/T)GCA^{316,317}. Similar to most transcription factors, Gfi1 represses gene expression through a variety of mechanisms. The SNAG repressor domain can directly prevent the RNA polymerase holoenzyme II and associated proteins from binding to the TATA box, thereby inhibiting initiation of transcription. Alternatively, Gfi1 can mediate local chromatin modifications to conceal the TATA box from RNA polymerase II holoenzyme binding. Indeed, recent data indicate that Gfi1 interacts with the histone lysine methyltransferase G9a and recruits G9a and HDAC1 (histone deacetylase 1) to its target genes in order to repress transcription. SNAG-mediated repression can therefore be mediated by reversible chromatin changes, such as local histone deacetylation, and is unlikely to induce long-term silencing of target genes³¹⁵.

Physiological and clinical relevance

Gfi1 plays important biological roles in hematopoietic and neuronal cell development. Specifically, *Gfi1*-deficient mice not only lack neutrophils, but also have defective hematopoietic stem cell maintenance and developmental deficits in sensory neurons. Hematopoiesis is the process by which mature blood cells are produced from pluripotent hematopoietic stem cells (HSCs). Gfi1 plays a key role in this process, as it is an inhibitor of HSCs proliferation and is therefore considered as a tumor progression oncoprotein.

Blood cells with specialized functions, such as neutrophils, are derived from multipotent hematopoietic stem cells by a process that is tightly controlled by transcription factors³¹⁸.

Gfi1-deficient mice lack mature neutrophils and are especially susceptible to infections with Gram-positive bacteria^{319,320}. Corollary to this, severe congenital neutropenia or non-immune

chronic idiopathic neutropenia can be seen in patients with mutations in the *Gfi1* gene³¹². *Gfi1* plays a unique role in T cell activation: it is involved in transducing IL2-receptor signaling and IL-6-dependent T cell proliferation^{321,322}. In addition, *Gfi1* inhibits T-cell death, by directly inhibiting the expression of pro-apoptotic regulators³²³. Furthermore, *Gfi1* is required for early T cell development. Thus, *Gfi1* full knockout animals exhibit diminished thymic cellularity resulting from defects in differentiation and proliferation as well as increased cell death of early thymic cells³²⁴. In addition, these transgenic animals accumulate abnormal promyelocytes with monocytic characteristics, demonstrating that even if *Gfi1* is dispensable for macrophage development, it is nevertheless required for activated macrophage function³²⁵.

Finally, mice lacking *Gfi1* are deaf. *Gfi1* mRNA is expressed in both the central and peripheral nervous system as well as in many sensory epithelia, including the developing inner ear epithelia. Although *Gfi1*-deficient mice initially specify inner ear hair cells, these hair cells are disorganized in both the vestibule and cochlea. The outer hair cells of the cochlea are improperly innervated and display an aberrant neuronal markers expression pattern. Accordingly, *Gfi1* full knock out mice show behavioral defects consistent with hearing loss³²⁶.

Interestingly, *Gfi1* was found to have a role in intestinal cell lineage allocation. The intestinal crypt stem cells give rise to either enterocytes or secretory cells, this fate choice relying on Notch signaling^{327,328}. Particularly, Notch/Hes1 pathway drives progenitor cells toward an absorptive cell lineage fate at the expense of the secretory lineage. Conversely, atonal homolog 1 (*Atoh1*) expression is both required and sufficient for the secretory differentiation program³²⁹⁻³³¹. Thus, Notch pathway inhibition of the transcription factor *Atoh1* represents the crucial mechanism regulating cell fate choice. Once the cells are committed to a secretory fate, *Ngn3* was found required for enteroendocrine cells differentiation, while *Gfi1* regulates the Paneth and goblet cells specification, by inhibiting *Ngn3* expression^{332,333}. Accordingly, the deletion of

Gfi1 results in sporadic expression of *Ngn3* in a subset of cells belonging to Paneth and goblet lineages. If this aberrant expression of *Ngn3* occurs early in their differentiation, Paneth and goblet cells appear to undergo cellular reprogramming and to be driven to the enteroendocrine lineage³³⁴.

Mechanistically, the role of *Gfi1* in the pancreas is currently unknown. Recently, Qu et al. suggested a role of *Gfi1* in the specification of centroacinar cells. Describing a *Gfi1* full knock out mouse line, they reported that in the absence of *Gfi1* the pancreas fails to establish a centroacinar cell population. Lacking the interface between the acinar cells and the ductal tree, zymogen granules accumulate in the acinar tissue, leading to acinar tissue damage³³⁵.

In this study, we generated a conditional knockout model, where *Gfi1* was solely inactivated in the pancreas. The phenotypical characterization of this mouse line allowi us to shed a new light on the role of *Gfi1* in pancreatic cells development and function.

Materials and Methods

Animal Procedures

Animal care and experimental use were conducted according to the French ethical regulations. Mice were maintained on a 12-hour light/dark cycle and were allowed ad libitum access to water and food. Mice were weaned 21 days after birth and fed with either a standard diet (A03, SAFE) or high fat diet (60% fat of caloric intake, 260HF, SAFE – **Figure 7**).

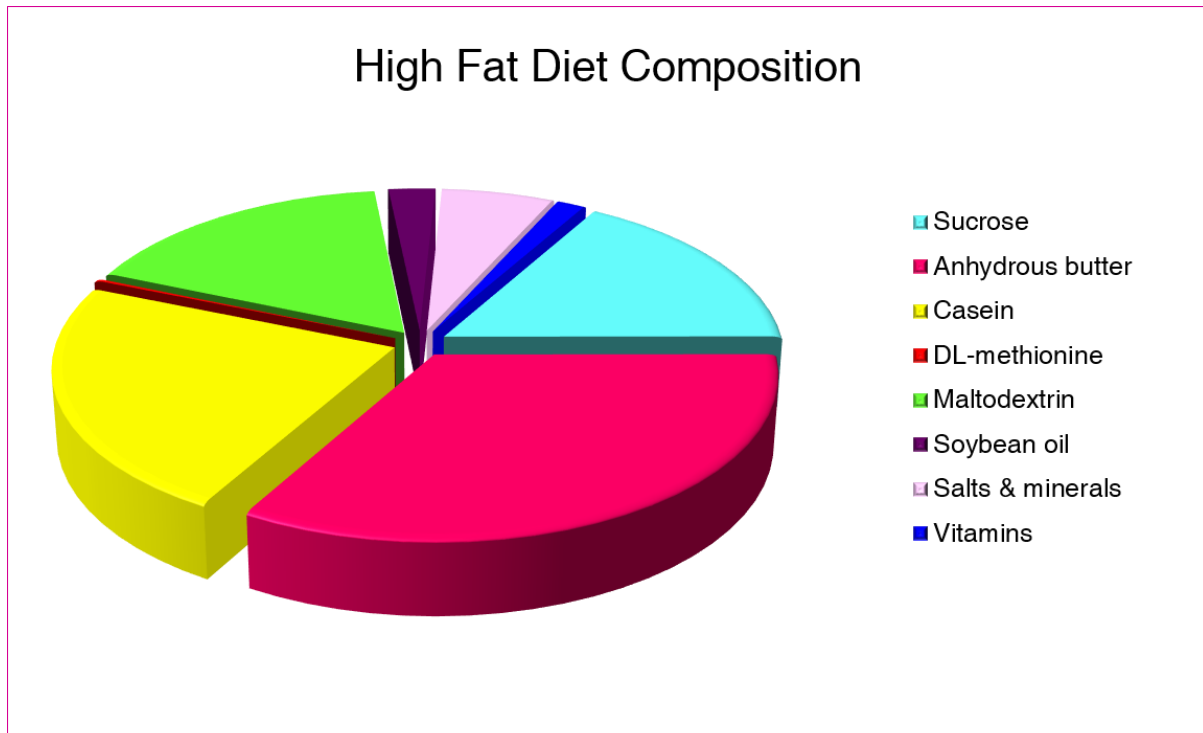


Figure 7. Nutrient composition of high-fat diet. For high fat feeding we selected a research diet (SAFE) contains 14.6% protein, 26.7% carbohydrate and 58.8% fat on a caloric basis. Carbohydrates mainly consist in sucrose and maltodextrins.

Transgenic mice

The complete loss of *Gfi1* is lethal, *Gfi1* full knockout animals developing severe immunodeficiency and dying shortly after birth. Aiming to characterize the effects of *Gfi1* ablation specifically in the pancreas from the early phases of development to adulthood and thereafter, a conditional knockout mouse line was generated. Pdx1Cre::*Gfi1*cko mouse line, expressing a truncated form of *Gfi1*, was obtained by crossing Pdx1-Cre mice with *Gfi1*cko animals^{336,337}. *Gfi1* floxed littermate animals were used as controls.

Gfi1Cre animals were crossed with ROSA- β -gal mice to establish the Gfi1Cre::RosaLac line, allowing the study of *Gfi1* expression pattern in the pancreas^{338,339}.

Mouse Genotyping

Biopsy samples of 2 mm diameter were collected by ear punch. Genomic DNA was extracted using the KAPA Express DNA Extraction Kit (KK7103, Kapa Biosystems). Briefly, each sample was incubated with 100 μ L of KAPA Express Extract lysis buffer (10 μ L 10X KAPA Express Extract Buffer; 2.0 μ L 1 U/ μ L c; 88 μ L PCR-grade water) for 10 min at 75°C, with vigorous shaking. Heat-inactivation of the thermostable KAPA Express Extract protease was achieved after incubation for 5 min at 95°C. Subsequently, samples were centrifuged at 14000 rpm for 1 min to pellet debris. The DNA-containing supernatant was transferred to a fresh tube and Polymerase Chain Reaction (PCR) was then performed to assess the genotype of the animals.

Gene	Primer Forward	Primer Reverse
Gfi1	CAGTCCGTGACCCTCCAGCAT	CTGGGAGTGCACTGCCTTGTGTT
Cre	ATGCTTCTGTCCGTTTGCCG	CCTGTTTTGCACGTTACCCG
Lac	TTGGCGTAAGTGAAGCGAC	AGCGGCTGATGTTGAACTG
Gfcre	GGG ATA ACG GAC CAG TTG	CCGAGGGGCGTTAGGATA

Table 2. Primer sequences used for genotyping

For standard PCR 1 μ L of genomic DNA were combined with 15 μ L of 2X GoTaq® G2 Hot Start Green Master Mix (M7423, Promega); 0.08 μ L of both forward and reverse primers (**Table 2**) and 13.84 μ L H₂O.

PCR results were analyzed by using the QIAxcel Advanced System (QIAGEN) and the QIAxcel DNA Screening Kit (929004, Qiagen).

Tolerance tests and blood glucose level measurements

For basal glycemia measurement, intraperitoneal glucose tolerance tests (IPGTT) and intraperitoneal insulin tolerance tests (IPITT), animals were fasted for 5 hours and injected intraperitoneally with 2g/kg of bodyweight of D-(+)-glucose (G7201, Sigma) or 0.75U/kg of insulin (Novo Nordisk A/S). Blood glucose levels were measured at the indicated time points post-injection with a ONETOUCH glucometer (Life Scan, Inc., CA).

Induction of streptozotocin-mediated diabetes

Streptozotocin is a glucosamine-nitrosourea compound, displaying cytotoxic effects, resulting from DNA and chromosomal damage. This compound is a structural analogue of the glucose and is therefore internalized specifically by pancreatic β -cells via the glucose transporter GLUT-2. To induce hyperglycemia, streptozotocin, (STZ; S0130, Sigma) was dissolved in 0.1M sodium citrate buffer (pH 4.5), and a single dose was administered intraperitoneally (130 mg/kg) within 10 min after dissolution. Diabetes progression was assessed by monitoring the blood glucose levels with a ONETOUCH glucometer (Life Scan, Inc., CA).

Proliferation assessment

To assess cellular proliferation, mice were treated with 5-bromo-2'-deoxyuridine, BrdU, a synthetic analogue of thymidine which is incorporated into DNA during the S phase (the phase of the cell cycle when the DNA is replicated). BrdU was dissolved in mice drinking water at a concentration of 1mg/ml. This solution was provided to mice in dark bottles to shield from light, changed every two days for 5 days prior to examination.

Gene Expression Analyses

To analyze gene level expression, pancreatic tissue was isolated from mice at different embryonic and adult stages. Fetuses and neonatal were sacrificed by decapitation with surgical scissors, while mice over 5 days of age were euthanized by cervical dislocation.

RNA extraction

For global pancreatic gene expression analysis, a small piece of pancreas was dissected and incubated in cold RNA later solution (AM7024, Fisher Scientific) at 4°C.

When RNA extraction from islets of Langerhans was required, 1mg/ml of collagenase (Sigma Aldrich) diluted in MEM medium (Sigma Aldrich) was injected into the main pancreatic duct and pancreatic tissue was incubated at 37°C for 10 minutes. Subsequently, islets were separated by crude tissue fractionation through a Histopaque (Sigma Aldrich) gradient.

The cell lines used and their respective culture media are listed in **Table 3**. Cells were cultured in 10cm petri dishes under conditions of 5% CO₂ and 37°C. Cell culture media were supplemented with 10% FCS, 50 U/ml Penicillin, 50 µg/ml Streptomycin, 200 µM L-Glutamine. Once grown to ~80% confluency, cells were collected by scraping, pelleted and stored at -20°C in lysis buffer.

Cell line	Description	Medium
MIN6	Mouse pancreatic β -cells	DMEM low glucose
INS-1	Rat pancreatic β -cells	RPMI-1640
266-6	Mouse pancreatic acinar cells	DMEM high glucose
Alpha-TC1	Mouse pancreatic α -cells	DMEM low glucose

Table 3. Immortalized cell lines used

All samples were homogenized in buffer (10 μ L of β -mercaptoethanol in 10mL of RLT buffer from Qiagen). Total RNA was therefore extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were determined using Agilent 2100 Bioanalyzer system (Agilent Technologies).

cDNA synthesis

10 μ L of H₂O containing 1 μ g of RNA was incubated with 1 μ L 10mM dNTP and 1 μ L 50mM oligodT, for 5' at 65°C. For cDNA synthesis, 10 μ L of DNA synthesis mix (2 μ L 10x RT buffer; 4 μ L 25 mM MgCl₂; 2 μ L 0.1 M DTT; 1 μ L Rnase out; 1 μ L Superscript III - INVITROGEN) was added to each sample and incubated for 50 min at 50°C, 5 min at 85°C and finally chilled on ice. Remaining RNA was ultimately degraded by incubation with 1 μ L of RNase H (INVITROGEN) for 20' and stored at -80°C.

qPCR analyses

Quantitative RT-qPCR reactions were carried out with the QuantiTect SYBR Green RT-PCR kit (Roche) and Qiagen-validated primer probes, according to the manufacturer's instructions. PCR amplifications were performed in a LightCycler 480 ® instrument (Roche Life Science). The program used for the RT-PCR is described in **Table 4**, the fluorescence being monitored during each amplification PCR cycle.

The housekeeping gene GAPDH was used as an internal control for normalization purposes. qPCR reactions were performed in tubes containing 5 μ L 2x SYBR Green Supermix, 0.5 μ L Primer Assay, 3 μ L H₂O and 1.5 μ L of 1/20 diluted cDNA.

Step	Temperature	Time	No of cycle
Pre-incubation	95°C	10 min	1
Amplification	Denaturation	95°C	10 sec
	Annealing	60°C	45 sec
	Elongation	72°C	1 sec
	Cooling	40°C	10 sec
	Melting curve	95°C	1
	Cooling	40°C	10 sec

Table 4. RT-qPCR program used

Pancreatic Tissue Processing and Sectioning

Paraffin embedding and microtome sectioning

Mice at different ages were euthanized as described above and pancreatic tissue was isolated and placed in cold PBS. Tissue was therefore fixed for 30 min at 4°C in Antigenfix (paraformaldehyde solution pH 7,2-7,4; Microm Microtech France), washed 5 x 20 min in cold PBS and incubated 1 hour in 0.86% saline. Following dehydration through a series of increasing ethanol dilutions (50%, 70%, 80%, 90%, and 100%), the pancreas was treated with isopropanol and toluene, a hydrophobic agent, to remove alcohol from the tissue. Finally, the organ was incubated in clean paraffin baths (1 hour, 3 hours and overnight) and embedded in paraffin in metal molds on a cold plate. Paraffin blocks were stored at 4°C prior to sectioning.

Finally, tissues were sectioned into 6µm slides, using a rotary microtome (Leica). Paraffin sections were placed on polarized glass slides (Fisher Scientific) and allowed to dry for 45 min at 42°C into a heating block and then kept at 37°C overnight. Slides were subsequently stored at 4°C until staining.

OCT embedding – Cryostat sectioning

For OCT embedding, pancreatic tissue was isolated and incubated for 30 min at 4°C in a fixative solution (1.35ml 37% paraformaldehyde; 400µl 25% glutaraldehyde; 100 µl 10X NP40; 5mL 10X PBS; H₂O to 50mL) and washed 6 x 20 min in cold PBS. To remove the water and protect the tissue from freezing damage, each sample was incubated overnight at 4°C in a 25% sucrose solution diluted in PBS. Finally, the organ was washed 2 x 1h in Jung freezing medium (Leica Biosystems) and embedded in plastic molds in Jung freezing medium on dry ice. Cryo blocks were stored at -80°C prior to sectioning.

Blocks were cut into 16 µm frozen sections using a Cryostat from Leica. Sections were therefore mounted on polarized glass slides (Fisher Scientific), dried for 45 min at 42°C into a heating block and stored at -80°C until staining.

Immunostaining of Pancreatic Sections

Immunohistochemistry on paraffin sections

Paraffin sections were deparaffinized by immersion in three washes of xylene for 3 minutes each. Subsequently, slides were rehydrated in decreasing ethanol dilutions (5 min in 2 x 95%; 5 min in 80%; 5 min in 60%; 5 min in 30%), and finally rinsed twice for 5 min in ddH₂O. To reveal epitopes that could have been masked during the tissue preparation, antigen retrieval was performed by boiling the tissue for 1 min at 140°C in 1.6L ddH₂O with 15mL Antigen Unmasking solution (Citric acid based pH6, Vector) in a pressure cooker. Slides were then transferred into tap water for approximately 30 min.

Tissue sections were then washed 3 x 5 min in PBS, kept in a blocking solution for 45 min at room temperature (PBS-FCS 10% - Gibco) to block any nonspecific binding of the antibodies and incubated overnight in a humid chamber at 4°C with primary antibodies (Table 3) diluted in PBS-FCS 10%.

Following three rinses in PBS for 5 minutes each, samples were incubated with appropriate secondary antibodies for 45 min at room temperature in a humid chamber.

Finally, slides were washed three times with PBS, dried, and mounted with coverslips and Vectashield HardSet Mounting Medium (Vector) with 4', 6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain.

Slides were kept at 4°C prior to observation. Pictures were processed using ZEISS Axioimager Z1 equipped with the appropriate filter sets and a monochrome camera, with Axiovision software from ZEISS.

Immunohistochemistry on cryosections

Cryosections were washed from the embedding Jung freezing medium three times for 15 minutes in PBS and blocked in a PBS-FCS 10% blocking solution for 45 min at room temperature in a humid chamber. Sections were then incubated overnight in a humid chamber at 4°C with primary antibodies (**Table 5**) diluted in PBS-FCS 10%. Antibodies were washed three times for 5 minutes in PBS before application of the appropriate secondary antibodies for 45 min at room temperature in a humid chamber.

For the X-gal staining, slides were washed three times in PBS for 15 minutes and incubated with an X-gal staining solution (625 µl 400mg/ml X-Gal, Invitrogen; 250µl 500mM K₃Fe(CN)₆, Sigma Aldrich; 500µl 250mM K₄Fe(CN)₆, Sigma Aldrich; 100µl 0.5M MgCl₂; 2.5ml 10XPBS) at 37°C overnight. Finally, slides were washed, dried, mounted and stored as described in the previous paragraph.

Antigens	Species	Dilutions	Provider
Insulin	Guinea Pig	1:500	DAKO
Glucagon	Rabbit	1:500	R&D systems
DBA		1:400	Vector
BrdU	Mouse	1:50	Roche
Amylase	Rabbit	1:100	Invitrogen
Ghrelin	Mouse	1:1500	Millipore
Mouse IgG	Chicken	1:1000	Invitrogen
Mouse IgG	Goat	1:1000	Invitrogen
Guinea Pig IgG	Donkey	1:1000	Jackson
Rabbit IgG	Donkey	1:1000	Invitrogen

Table 5. List of primary and secondary antibodies used for IHC

Quantification and data analysis

Quantification analyses were performed on pictures from Zeiss Axioimager Z1 with Axiovision software and an automatic mosaic acquisition system (Zeiss). The entire pancreata of at least 4 mice per genotype and per condition were serially cut in 6 μm thick sections and at least 5 pancreatic sections were processed for immunohistochemistry.

BrdU counting was assessed manually counting proliferative cells in the different pancreatic compartments. For insulin quantifications, widefield multi-channels tile scan images treatment and analysis were done using a Fiji³⁴⁰ home-made semi-automated macro. Briefly, the overall pancreas slice area was determined and measured using a low intensity based threshold on the insulin labelling channel. Islets were then segmented and counted on the insulin labelling, applying a high intensity threshold. The density of islets per pancreas section was obtained reporting the number of islets to the pancreatic area.

All values are reported as mean \pm SEM of data from at least 4 animals. Data were analyzed using GraphPrism 6 software. Normality was tested using D'Agostino-Pearson omnibus normality test and appropriate statistical tests were performed. Results are considered significant if $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

Blood Insulin Level Measurement

Mice were deeply anesthetized with isoflurane delivered in oxygen at a flow rate of 1l/min. Whole blood samples were collected from the retro-orbital sinus into K3EDTA blood collection tubes, using glass capillaries.

In order to measure basal insulinemia, blood samples were drawn after 5 hours of starvation. To assess glucose-stimulated insulin secretion level, an additional blood sampling was performed 20 minutes after an intraperitoneal injection of 2g/kg of bodyweight of D-(+)-glucose.

Whole-blood samples were cooled at once in iced water. Plasma was separated by centrifuging at 2000 g for 7 minutes at 4C°. The obtained plasma was transferred into pre-cooled tubes, promptly frozen in liquid nitrogen and finally stored at -80C°.

Elisa immunoassay

Plasma insulin concentration was measured using ELISA enzyme immunoassay (Mercodia, Uppsala, Sweden), following manufacturer's instructions. All reagents and samples were allowed to warm to room temperature before use. The optical density was read at 450 nm, using a spectrophotometer ELISA reader (Sunrise BasicTecan, Crailsheim, Germany), complemented by Tecan's Magellan data analysis software.

Insulin concentration was calculated using Microsoft excel. A calibrator curve was computed by plotting the absorbance values of each Calibrator, except for Calibrator 0, against the corresponding insulin concentration.

Electron Microscopy

For ultrastructural analyses of the pancreas, anesthetized mice were perfused transcardially with physiological serum in order to wash out the blood, then with 2% glutaraldehyde in 0.1 M cacodylate buffer. The pancreas was dissected, immersed in fixative for hours, rinsed in the same buffer, post-fixed for 2h in 1% osmium tetroxide in 0.1 M cacodylate buffer. Samples were then rinsed in distilled water, dehydrated in alcohols and lastly embedded in epoxy resin. Contrasted ultrathin sections (70 nm) were analyzed under a JEOL 1400 transmission electron microscope mounted with a Morada Olympus CCD camera.

For immunogold stainings, approximately 200 islets, isolated by collagenase (1mg/ml) digestion, were fixed with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) overnight at 4°C and were processed for ultracryomicrotomy according to a slightly modified Tokuyasu method³⁴¹. In brief, islets were spun down in 10% gelatin. After immersion in 2.3 M sucrose (in [pH 7.4], 0.1 M PB) overnight at 4°C, the samples were rapidly frozen in liquid nitrogen. Ultrathin (70 nm thick) cryosections were prepared with an ultracryomicrotome (Leica EMFCS, Austria) and mounted on formvar-coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA, USA).

Immunostainings were processed with an automated immunogold labeling system Leica EM IGL as following: the grids were incubated successively in PBS containing 50 mM NH₄Cl, PBS containing 1% BSA, PBS containing both anti-insulin and anti-glucagon primary antibodies diluted 1:1000 in 1% BSA for 1 h, PBS containing 0.1% BSA, PBS containing 1%

BSA and both 10 nm and 15 nm colloidal gold conjugated anti-guinea pig IgG and anti-mouse IgG, respectively, (BBInternational, Cardiff, UK), PBS containing 0.1% BSA for 5 min, PBS for 5 min twice. Finally, the samples were fixed for 10 min with 1% glutaraldehyde, rinsed in ddH₂O and were contrasted with a mixture of 1.8% methylcellulose and 0.3% uranyl acetate on ice. After air-drying, sections were examined under a JEOL 1400 transmission electron microscope.

Assessment of Insulin Receptor Activity

Mice were starved for 5 hours and sacrificed by cervical dislocation 30 minutes after intraperitoneal injection of 2g/kg of bodyweight of D-(+)-glucose. Tibialis anterior muscles were removed and immediately frozen in liquid nitrogen.

Western Blot

Frozen tissue was homogenized with a rotary tissue disruptor (QIAGEN) in ice-cold lysis buffer (25 mM Tris-Cl pH 7.4, 100mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P40, 1× protease inhibitor cocktail and 1× Phosphostop mix from Roche Diagnostics, Meylan, France). Protein concentration was evaluated by BCA assay according to manufacturer's recommendations (PIERCE, Thermo Scientific, France). Fifty micrograms proteins were blotted using SDS-PAGE basic protocol. Primary antibody incubation was performed overnight at 4 °C (polyclonal rabbit phospho-Akt and polyclonal rabbit anti-Akt, Cell Signaling). Primary antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse immunoglobulins (Promega, Charbonnières-les-Bains, France). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Merk-Millipore) and revealed with Amersham Imager 600. OD band intensities were evaluated using PcBAS Software.

Results

Gfi1 is expressed in the pancreas

Gfi1 has been suggested to play a role in pancreatic cells specification³³⁵. However, *Gfi1* expression and function in the pancreas has been poorly investigated. Aiming to characterize *Gfi1* expression pattern in the pancreas, total mRNA was extracted specifically from the pancreas at different developmental stages and adult ages. *Gfi1* mRNA was detectable by RT-qPCR during fetal development at embryonic (E) day 15.5, its expression levels remaining steady until birth (P0). During postnatal stages (between P0 and P6) and early adulthood (between 1m old and 3m old), *Gfi1* mRNA expression levels were found significantly increased in a stepwise fashion. Thereafter, *Gfi1* remained continuously and steadily expressed at all the stages analyzed spanning from 3 months to 12 months of age (**Figure 8**).

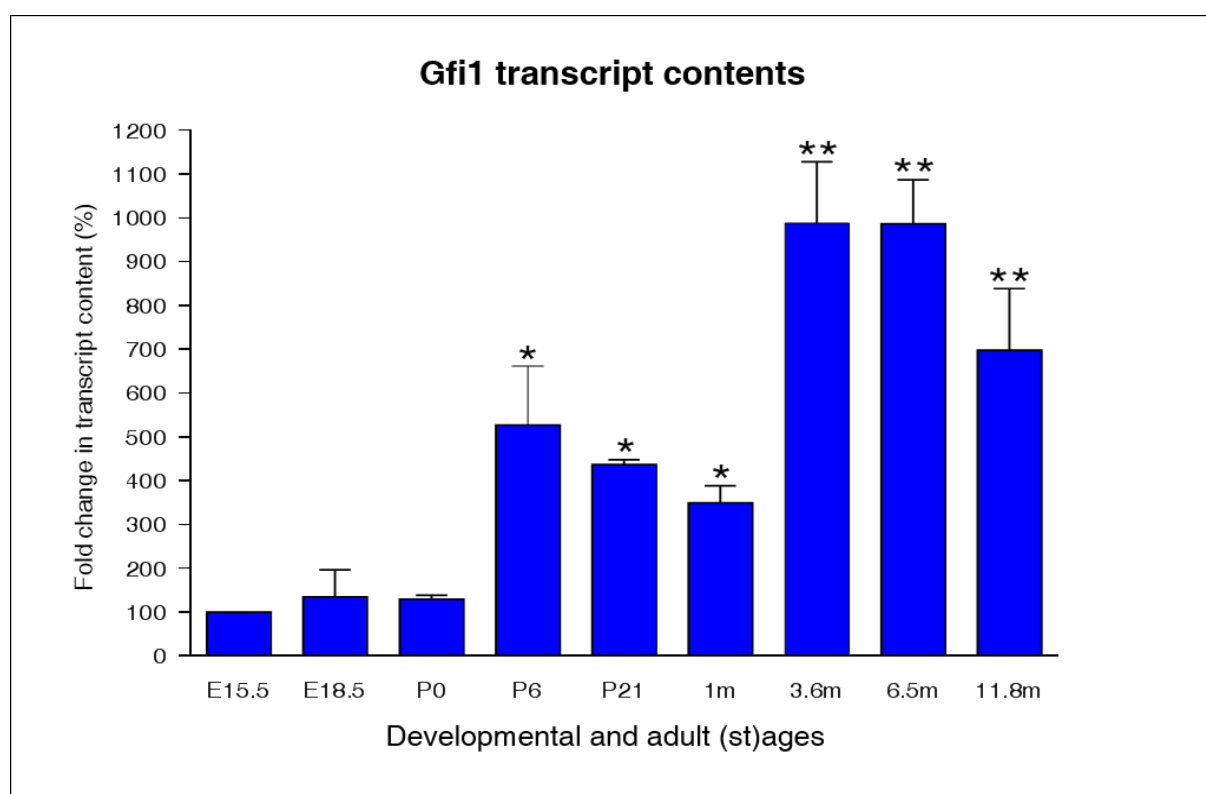


Figure 8. Gfi1 expression in the mouse pancreas. The expression levels of *Gfi1* were assessed by RT-qPCR in wild-type whole pancreata at different developmental and adult (st)ages. *Gfi1* expression was thereby detected as early as E15.5. *Gfi1* transcript content remained stable throughout embryonic development, while a significant increase was noted during the first days of life (from P0 to P6). A further increase was evidenced in adult pancreata between 1 and 3 months of age, *Gfi1* remaining continuously expressed until at least 12 months of age.

Antibodies specifically recognizing Gfi1 are not currently available³³⁴. Therefore, aiming to further define the expression pattern of *Gfi1* in mouse pancreas, we quantified *Gfi1* mRNA levels in four pancreatic mouse cell lines (**Figure 9A**): the 266-6 pancreatic acinar cell line, the alpha-tumor cell line (Alpha-TC1), the mouse insulinoma cell line (MIN6), and the mouse pancreatic β -cell line (INS-1). Furthermore, *Gfi1* transcript contents were assessed in samples extracted from adult whole pancreas and compared to isolated islets of Langerhans (**Figure 9B**). Importantly, *Gfi1* expression was detected only in 266-6 cells and whole pancreatic tissue, being negligible in endocrine cells and primary islet cells (**Figure 9A-B**). These data strongly suggested that *Gfi1* expression is largely excluded from pancreatic endocrine cells. To further investigate *Gfi1* expression pattern, a Gfi1-Cre mouse line³³⁹ (expressing the phage P1 *Cre Recombinase* under the control of the endogenous *Gfi1* promoter) was crossed with a ROSA26- β -gal mouse line³³⁸ (harboring a transgene containing the *Rosa26* promoter upstream of a loxP-Neomycin resistance-Stop-loxP- β -galactosidase cassette - **Figure 9C**). In the resulting Gfi1Cre::RosaLac animals, cells are permanently labelled once *Gfi1* expression is activated, all cells having once expressed *Gfi1* being permanently labelled. Thus, the spatiotemporal expression of β -galactosidase driven by *Gfi1* promoter was analyzed by X-gal staining. β -galactosidase activity was solely detected in the acinar compartment. Importantly, no activity was found in ductal nor islets of Langerhans cells (**Figure 9D-F**), demonstrating that the latter do not derive from cells having expressed *Gfi1*. Together, these results show that *Gfi1* expression in the pancreas encompasses the entire mouse lifespan and is specifically confined to acinar cells.

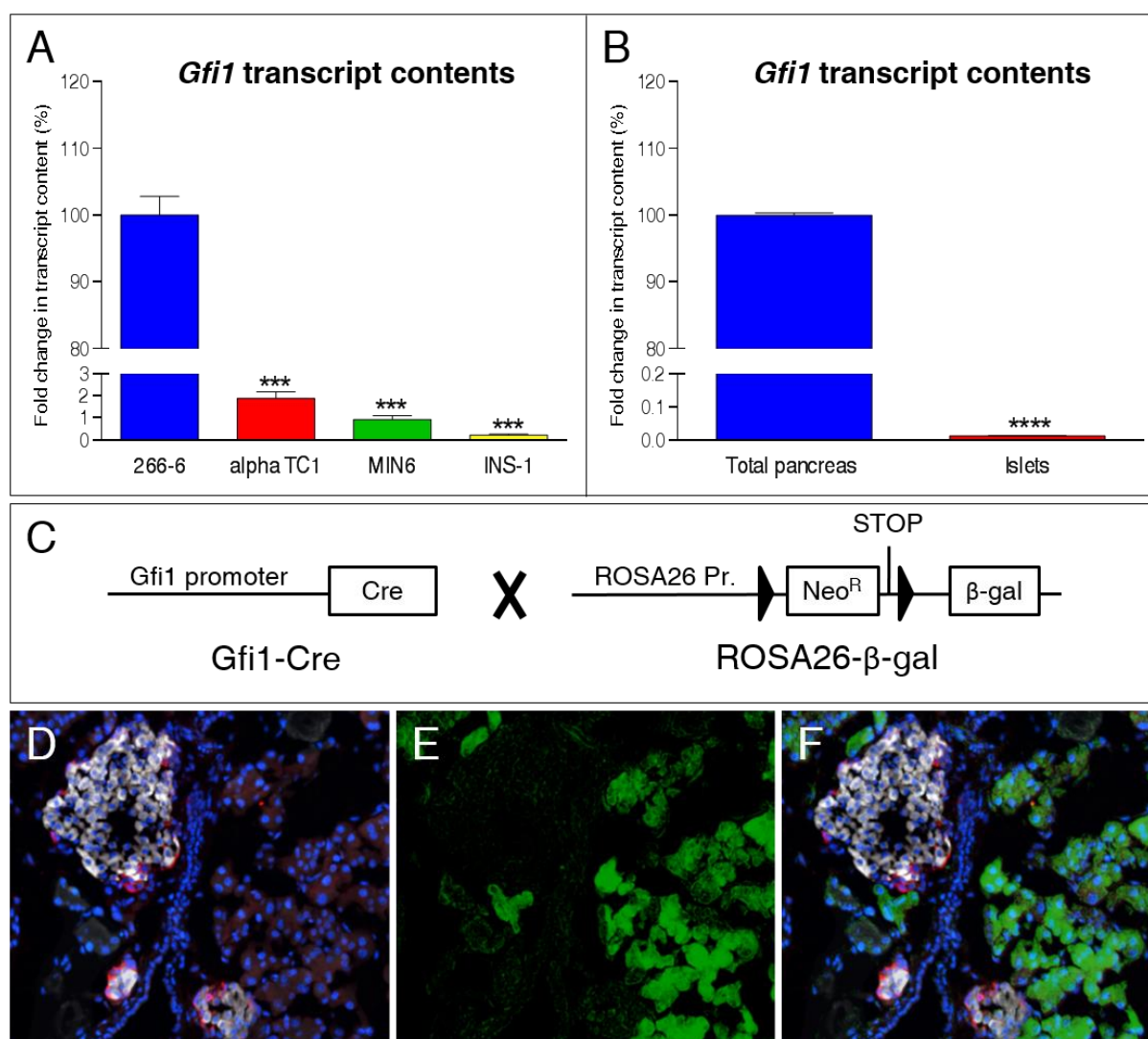


Figure 9. *Gfi1* expression in the mouse pancreas. *Gfi1* expression levels, as assessed by RT-qPCR, were found decreased by 98.1%, 99.07% and 99.77% respectively in alpha-TC1 α -cells, MIN6 and INS-1 β -cells, as compared to 266-6 acinar cells (**A**). Accordingly, *Gfi1* mRNA was hardly detected in isolated islets of Langerhans and was 99.98% decreased when compared with whole pancreas *Gfi1* mRNA (**B**). Due to the lack of antibodies specifically recognizing *Gfi1*, *Gfi1*-Cre animals (the *Cre* recombinase being knocked-in into the *Gfi1* locus) were crossed with the Rosa26- β -gal line (harboring a transgene composed of the ubiquitous *Rosa26* promoter upstream of a LoxP-STOP-LoxP cassette followed by the β -galactosidase *cDNA*). In the resulting *Gfi1*-Cre::Rosa26-Rosa- β -gal animals (**C**), *Gfi1* expression was assayed using X-gal staining (blue converted in green to increase the image readability). Interestingly, *Gfi1* was solely detected within the exocrine compartment, being excluded from the ductal tree and specifically expressed in acinar cells (**D-F**).

Pdx1Cre::Gfi1 Conditional Knockout Mouse Line Generation and analysis

The loss of *Gfi1* is lethal, *Gfi1* full knockout mice dying around weaning due to severe neutropenia³⁴². To study the role of *Gfi1* specifically in the pancreas, a conditional *Gfi1*

knockout mouse line was therefore generated. In order to target all pancreatic cells, Pdx1-Cre animals³³⁶ (harboring a transgene encompassing the *Pdx1* promoter driving the expression of the phage P1 Cre recombinase) were crossed with a Gfi1 conditional knock out mouse line³³⁷ (in which the 4th and 5th exon of the *Gfi1* gene are flanked by two LoxP sites – **Figure 10A**). In the resulting Pdx1Cre::Gfi1cko animals, the *Pdx1* promoter drives the expression of the *Cre recombinase* in all pancreatic cells. Once synthesized, the Cre recombinase induces the excision of the region encoding the Gfi1 DNA binding domain, consequently leading to *Gfi1* loss-of-function solely in the pancreas. Accordingly, *Gfi1* expression level measured by RT-qPCR from whole organ were found 99.8% decreased in *Gfi1* mutant pancreata compared to wild-type controls (**Figure 10B**).

Importantly, Pdx1Cre::Gfi1cko mice were found to be viable, fertile, with no premature death being observed. Similarly, *Gfi1* conditional knockout animals were normoglycemic. However, basal blood glucose levels were found significantly lower during adulthood at all ages tested (1 month, 3 months and 6 months of age) as compared to their age matched control counterparts (**Figure 10C**). To further investigate *Gfi1*-deficient pancreas functionality, an IPGTT was performed on 3 months old Pdx1Cre::Gfi1cko mice and age matched controls. Interestingly, *Gfi1* mutant mice displayed a significantly higher peak in blood glucose concentration 30 minutes after the injection of a high dose of glucose (**Figure 10D**). However, *Gfi1*-deficient mice were able to clear the blood out of glucose as efficiently as controls (**Figure 10D**). Altogether, these data show that the loss of *Gfi1* results in a slightly lowered basal glycemia and an impaired capability to react to a glucose challenge. However, the glucose clearance appears unaffected when comparing mutants to controls.

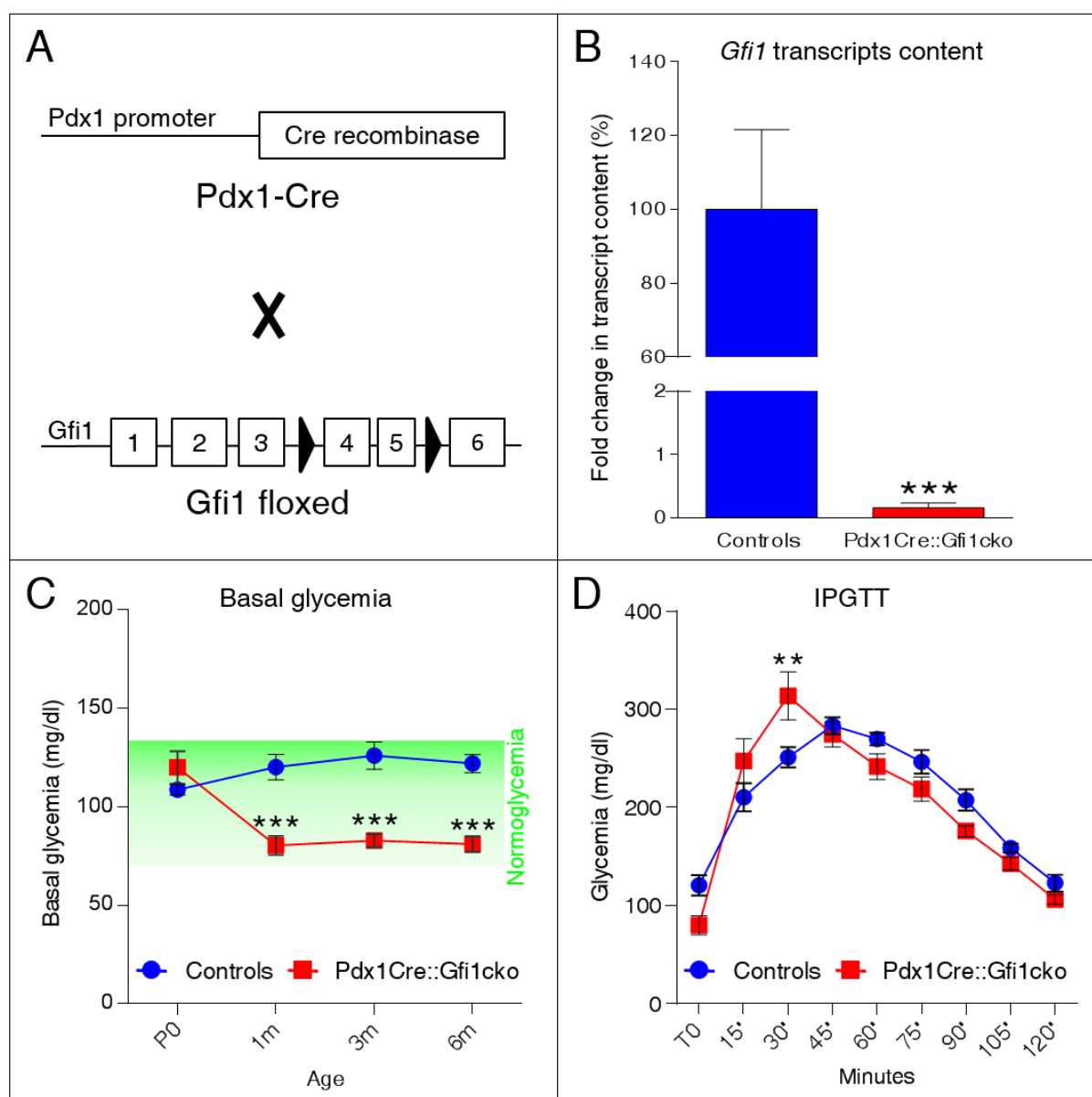


Figure 10. Generation and characterization of Pdx1Cre::Gfi1cko mouse line. Pdx1-Cre mice (harboring a transgene composed of the *Pdx1* promoter driving the expression of the *Cre recombinase*) were crossed with *Gfi1*cko animals (in which the 4th and 5th exon of the *Gfi1* gene are flanked by two *LoxP* sites). In the resulting Pdx1Cre::Gfi1cko double transgenics (**A**), the *Cre recombinase* mediates the excision of the sequence encoding the *Gfi1* DNA binding domain. A decrease of 99.8% in *Gfi1* expression levels was observed in Pdx1Cre::Gfi1cko compared to controls, confirming the efficiency of the strategy (**B**). The basal glycemia of Pdx1Cre::Gfi1cko was consistently found within normal ranges, but also significantly lower than age-match controls at different adult ages (**C**). Upon IPGTT, the glycemia at the starting point was accordingly lower in *Gfi1* mutant mice, these animals displaying a significantly higher peak in blood glucose concentration 30 minutes after the injection of a high dose of glucose (**D**). However, *Gfi1*-deficient mice were able to clear the blood out of glucose as efficiently as controls.

Loss of *Gfi1* has a Protective Role against Two Models of Induced Diabetes

As the loss of *Gfi1* is associated to a lower basal glycemia, we wondered whether the inactivation of *Gfi1* might be beneficial in a diabetic context. We therefore developed two models of induced diabetes.

High fat feeding is a widely used model of diabetes and it is associated to obesity, hyperinsulinaemia and altered glucose homeostasis³⁴³. In order to challenge Pdx1Cre::*Gfi1*cko animals, the normal chow containing 25% protein, 61% carbohydrate and 13% fat on a caloric basis was substituted at 1 month of age for a diet providing a substantial increase in the number of calories deriving from fat, 60% of energy being supplied by butter. Weight and basal glycemia were monitored on a weekly basis for a duration of 5 months. As expected, *Gfi1* mutants and age matched controls subjected to high fat diet developed an significant increase in body weight over time, doubling their mass within 12 weeks (**Figure 11A**). Concomitantly, control mice progressively developed the expected hyperglycemia throughout the treatment (**Figure 11B**). Strikingly, Pdx1Cre::*Gfi1*cko mice remained steadily normoglycemic despite such a 5-month long chronic high fat diet administration (**Figure 11B**). An IP-GTT performed 5 months following high fat diet initiation showed a glucose intolerance for control mice (**Figure 11C**), such animals failing to restore normoglycemia within 120 minutes following challenge. Importantly, Pdx1Cre::*Gfi1*cko that underwent a 5-month long high fat diet were able to efficiently respond to IP-GTT and therefore quickly clear glucose following a bolus (**Figure 11C**).

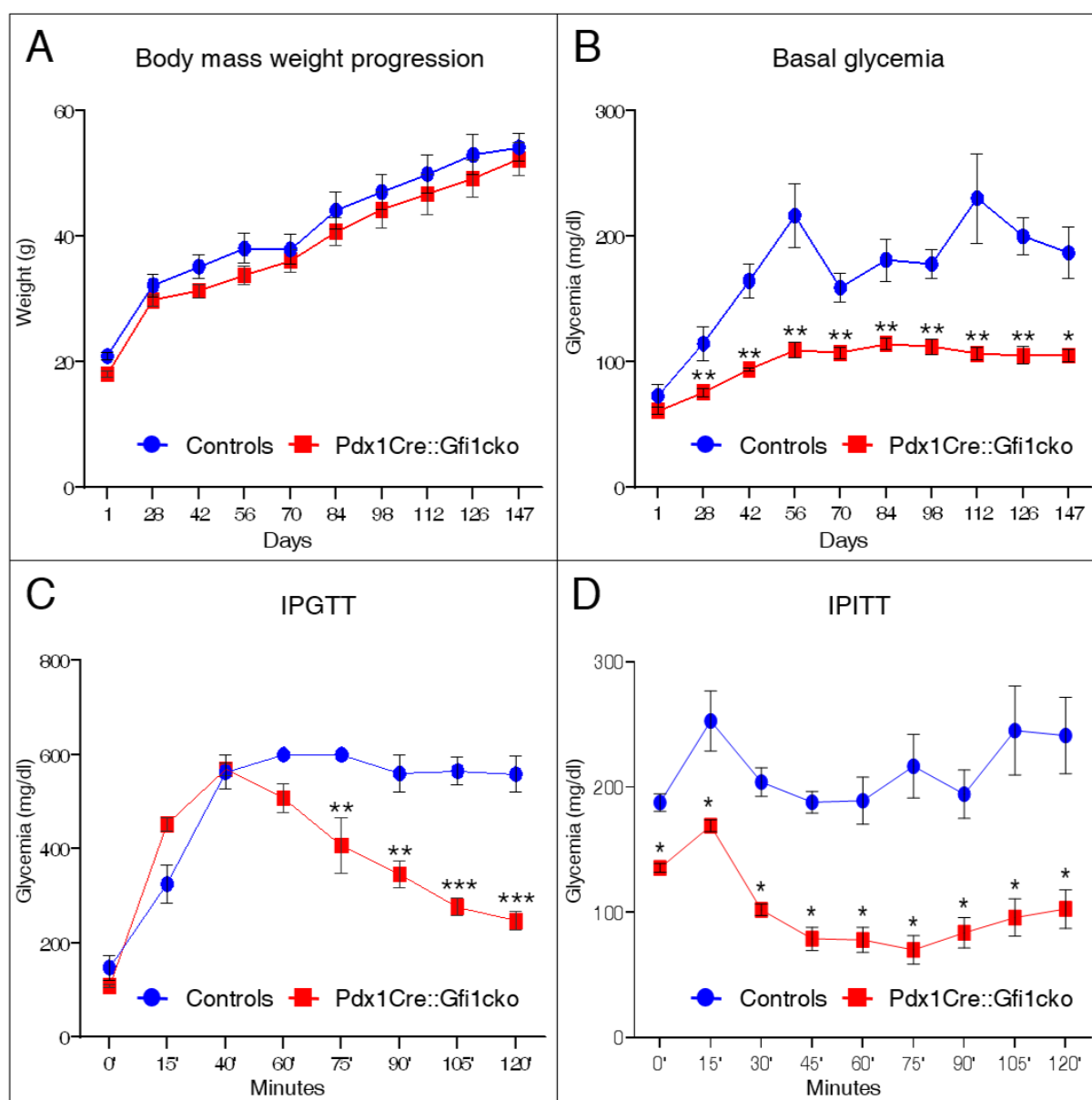


Figure 11. *Gfi1* conditional knockout mice are resistant to high fat diet-induced diabetes. Control and *Gfi1* mutant mice fed with HFD displayed a parallel increase in body weight over 5 months of weekly observation (A). Surprisingly, while control mice progressively developed hyperglycemia, *Gfi1* mutant mice remained normoglycemic throughout the entire treatment (B). Importantly, even after 150 days of this regimen, *Gfi1* mutants responded normally to IPGTT (C) and were not resistant to exogenous insulin (D).

Accordingly, long term high fat feeding induced insulin resistance in control mice, as assessed by IP-ITT (Figure 11D). Notably, *Gfi1* conditional knockout animals were still responsive to insulin, their glycemia promptly decreasing upon injection of exogenous insulin (Figure 11D).

Aiming to gain further insight into the mechanisms underlying the resistance to high fat diet-induced diabetes exhibited by *Gfi1* mutant mice, we made use of an additional diabetes experimental model using high-dose streptozotocin (STZ) injection. This glucosamine-nitrosourea compound is used clinically as a chemotherapeutic agent in the treatment of pancreatic β -cell carcinoma³⁴⁴. Causing specific β -cells necrosis, STZ is also employed to induce a diabetic phenotype in experimental mouse models³⁴⁵. To determine whether the lack of *Gfi1* was also beneficial in this diabetes model, we injected control and Pdx1Cre::Gfi1cko mice with a single high dose of STZ and monitored their glycemia daily. As predicted, control animals displayed a dramatic increase in blood glucose levels, few days following STZ administration and subsequent β -cell ablation (**Figure 12**). Remarkably, *Gfi1*-deficient mice were found to be resistant also to this model of diabetes, displaying an imperturbable normal glycemia for at least 50 days following STZ administration (**Figure 12**).

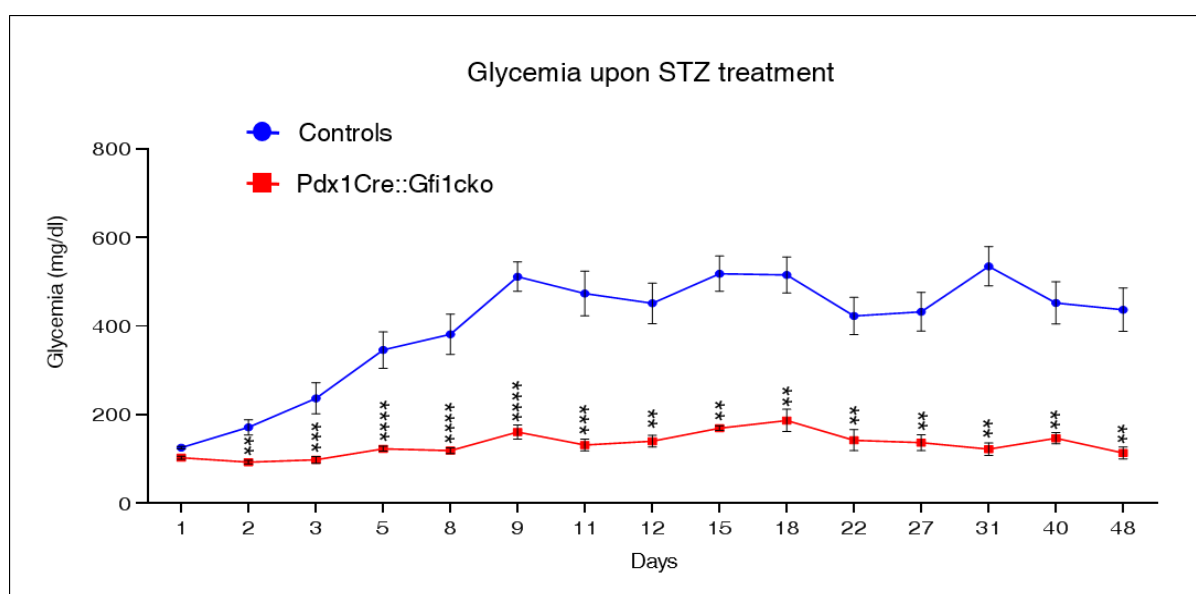


Figure 12. Pdx1Cre::Gfi1cko mice are protected against chemically-induced diabetes. Control and *Gfi1* mutant mice were injected with a single high dose of STZ and their basal glycemia was monitored daily for 7 weeks. Expectably, control animals rapidly developed a severe hyperglycemia. Impressively, Pdx1Cre:Gfi1cko animals remained steadily normoglycemic following STZ treatment.

After 8 weeks of observation, mice were sacrificed and their pancreata were isolated and processed into paraffin sections. Immunohistochemical analyses of control pancreatic samples highlighted the degenerative effect of STZ on β -cells, associated with a loss of insulin immunoreactivity compared to untreated controls (**Figure 13A-B**). Importantly, the β -cell mass and islets of Langerhans integrity were found unaffected by STZ cytotoxic activity in *Gfi1* conditional knockout pancreata (**Figure 13C**). Altogether, these findings indicate that the loss of *Gfi1* exerts a protective role against high fat diet-induced insulin resistance and glucose intolerance in mice. Similarly, mice lacking *Gfi1* are resistant to the β -cell cytotoxic effects of STZ and are therefore resistant to chemically-induced diabetes.

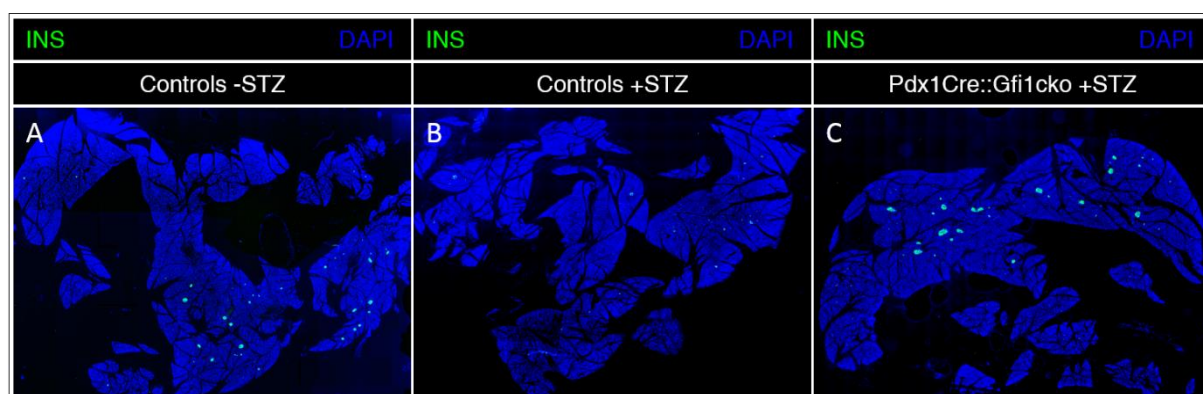


Figure 13. Pdx1Cre::Gfi1cko mice are protected against chemically-induced diabetes. Mice were sacrificed two months following STZ treatment and their pancreatic tissue was analyzed by insulin immunostaining. Control pancreatic sections were found largely devoid of insulin-labelled cells, further confirming the efficiency of STZ treatment (**A-B**). Astonishingly, the insulin⁺ area was found unaffected in *Gfi1* mutant pancreata, these samples displaying no defects in islets architecture and β -cell mass as compared to untreated control pancreata (**A, C**).

Gfi1 deficiency results in increased proliferation within the acinar compartment

Our results demonstrated that *Gfi1* is not expressed in the islets of Langerhans. However, the inactivation of *Gfi1* specifically in the pancreas is sufficient to protect rodents against two models of induced diabetes. To investigate the molecular link between a mutation in pancreatic acinar cells and the prevention of diabetes, the phenotype of Pdx1Cre::Gfi1cko was thoroughly investigated.

The macroscopical evaluation of pancreas size revealed an apparent increase in *Gfi1*-deficient pancreas mass compared to controls. The pancreas weight of mice at different ages was therefore assessed and normalized to the animal body weight. Importantly, *Gfi1* mutants displayed no difference in pancreas mass at birth, as compared with neonatal controls. Interestingly, the gland mass was found thereafter significantly and progressively increased during adulthood, with a 15%, 30% and 45% augmentation at 1 month, 3 months and 6 months of age, respectively (**Figure 14C**).

To determine the causes underlying Pdx1Cre::Gfi1cko pancreas hypertrophy, the proliferation rates of pancreatic cells were evaluated. To this aim, BrdU⁺ cells were detected and quantified by immunohistochemistry, following 5 days of BrdU supplementation. As expected, BrdU immunoreactive cells were rarely observed in 3 months old control pancreata, consistent with the poor proliferative capacity of adult pancreatic cells. Conversely, an impressive number of dividing cells was detected in pancreatic sections isolated from adult *Gfi1*-deficient mice, these cells being mainly located within the acinar compartment (**Figure 14A-B**). Quantitative analyses confirmed our observations, showing that the number of BrdU-labelled acinar cells was 4 times higher in Pdx1Cre::Gfi1cko compared to controls (**Figure 14D**). Of note, the number of proliferative ductal and endocrine cells was found unaffected by the loss of *Gfi1* expression. In summary, our results demonstrated that the *Gfi1* loss of function is associated with a strong increase in the rate of pancreatic acinar cell proliferation during adulthood, this hyper-proliferation ultimately leading to pancreas hypertrophy.

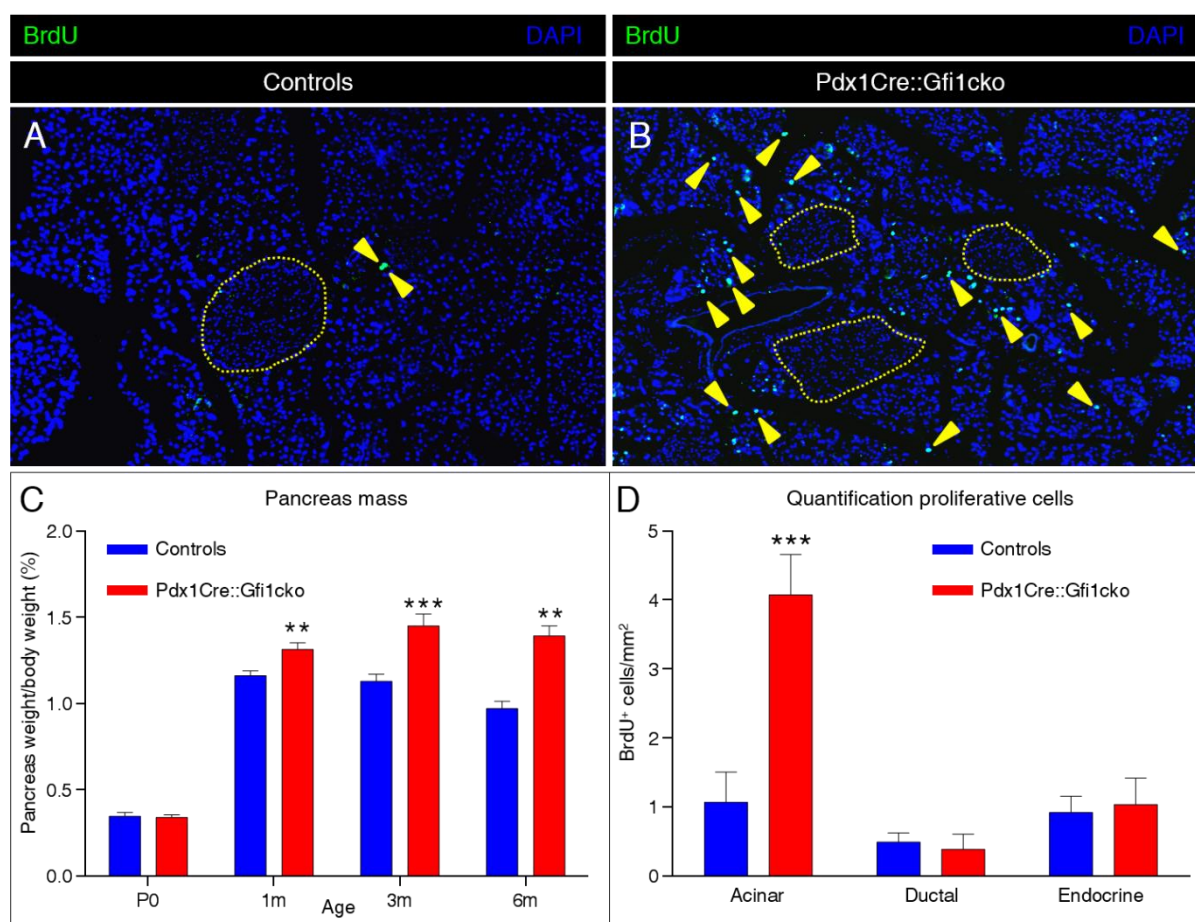


Figure 14. The loss of *Gfi1* is associated with a dramatic increase in proliferation rate within the acinar compartment. Pdx1Cre::Gfi1cko and controls were provided with BrdU for 5 days at 3 months of age. Subsequently, pancreatic sections were analyzed by BrdU immunostaining. Representative images of immunohistochemical assessment of BrdU⁺ cells highlight a dramatic increase in the proliferation rate of adult *Gfi1* mutant pancreatic cells (A-B). Accordingly, the mass of the pancreas normalized to the body weight was found progressively and significantly increased at all adult ages tested (C). Quantification analyses of BrdU-labelled cells in different pancreatic compartments revealed a significant 4-fold increase in dividing cell number in the acinar compartment, endocrine and ductal cells not showing any change in their proliferation rates.

Gfi1 conditional knockout mice display a strong decrease in *amylase* expression

To gain further insight in the phenotypical changes resulting from *Gfi1* deficiency, we scrupulously analyzed Pdx1Cre::Gfi1cko acinar cells morphology by electron microscopy. Acinar cells displayed the typical pyramidal, wedge-like shape in both Pdx1Cre::Gfi1cko adult mice and age-matched controls. Ultrastructural analyses showed the typical high content of zymogen granules in the apical pole of control pancreatic acinar cells (Figure 15A).

Interestingly, a massive decrease in secretory vesicles number was outlined in the cytosol of *Gfi1*-deficient pancreatic acinar cells as compared to controls (**Figure 15B**).

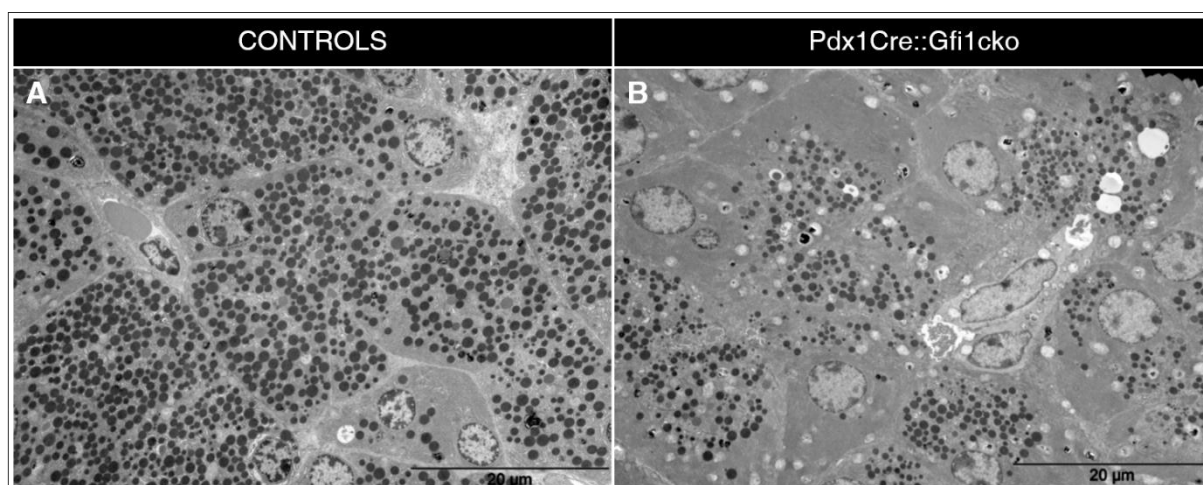


Figure 15. Electron microscopy analysis of the Pdx1Cre::Gfi1cko acinar compartment. Ultrastructural analyses showed a dramatic decrease in zymogen granule numbers when comparing Pdx1Cre::*FT1*cko acinar cells to controls using electron microscopy.

To further characterize these phenotypical alterations, we analyzed adult Pdx1Cre::Gfi1cko pancreatic sections by immunohistochemistry using antibodies raised against the acinar-specific enzyme, amylase. As expected amylase immunoreactivity was observed homogeneously in the control acinar compartment, surrounding the islets of Langerhans. Conversely, the majority of *Gfi1*-deficient acinar cells were found to be either negative or weakly positive for amylase, at 3 months of age (**Figure 16A-B**). To confirm these observations, the amount of *amylase* transcript was assessed by RT-qPCR at different developmental and adult (st)ages. As expected, *amylase* expression levels increased significantly from E15.5 throughout postnatal life of control animals up to 3 months of age. Importantly, *amylase* transcript contents also raised continuously throughout pre- and post-natal development, but these were consistently decreased compared to controls at all ages tested. Strikingly, a significant 78.3% decrease in *amylase* expression level was measured in 3 months old *Gfi1*-mutants (**Figure 16C**).

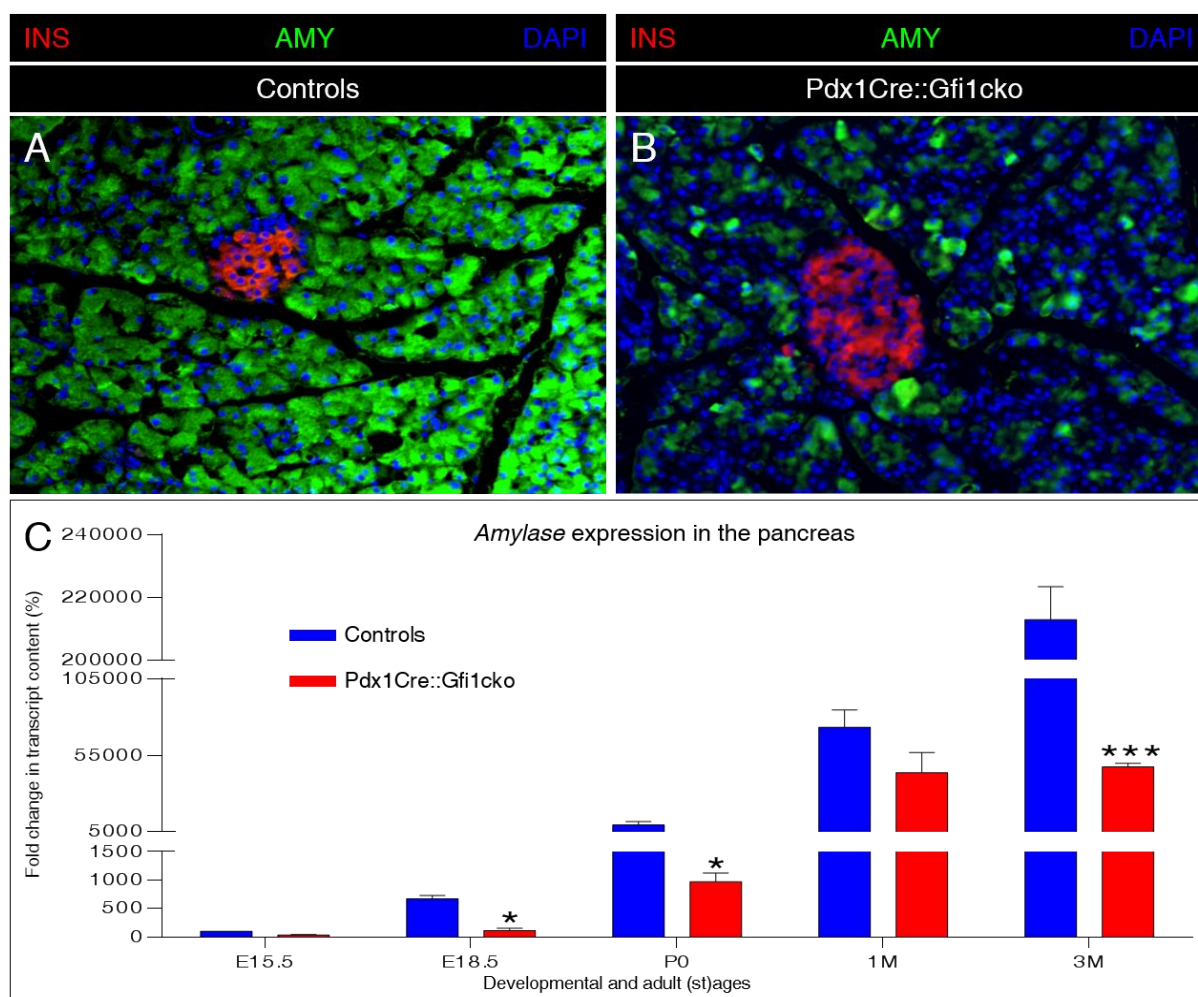


Figure 16. Amylase expression is strongly decreased in Pdx1Cre::Gfi1cko pancreas. Immunohistochemical characterization showed an impressive amount of acinar cell being negative or weakly positive for amylase in *Gfi1* mutants (A-B). Consistently, *amylase* expression measured by RT-qPCR from whole organ was found systematically decreased at all (st)ages tested. Impressively, at 3 months of age amylase mRNA levels were 78.3% decreased in *Gfi1* mutant pancreata compared to age-matched controls (C).

Together, these data demonstrate that Gfi1 activity in the pancreas is crucial for a physiological expression of *amylase* within the acinar cells.

Gfi1 mutants display an aberrant postnatal expression of Nkx6.2

To dissect the molecular pathway underlying Gfi1 activities in the pancreas, we set up a molecular screen in order to identify putative Gfi1 target genes. Toward this goal, we analyzed the pancreatic transcriptome extracted from *Gfi1*-deficient mice by RT-qPCR and assessed the expression level of all genes known for their involvement in pancreas development and

function. Using this approach, we found that *Nkx6.2* was abnormally expressed in *Pdx1Cre::Gfi1cko* mice. It is worth noting that *Nkx6.2* is normally exclusively expressed during embryonic development in the mouse pancreas. Accordingly, by means of RT-qPCR, *Nkx6.2* transcripts were solely detected at E15.5 in control pancreas, *Nkx6.2* being subsequently switched off (**Figure 17**). Importantly, *Pdx1Cre::Gfi1cko* pancreatic cells failed to inactivate *Nkx6.2* expression during the latest stages of embryonic development (**Figure 17**).

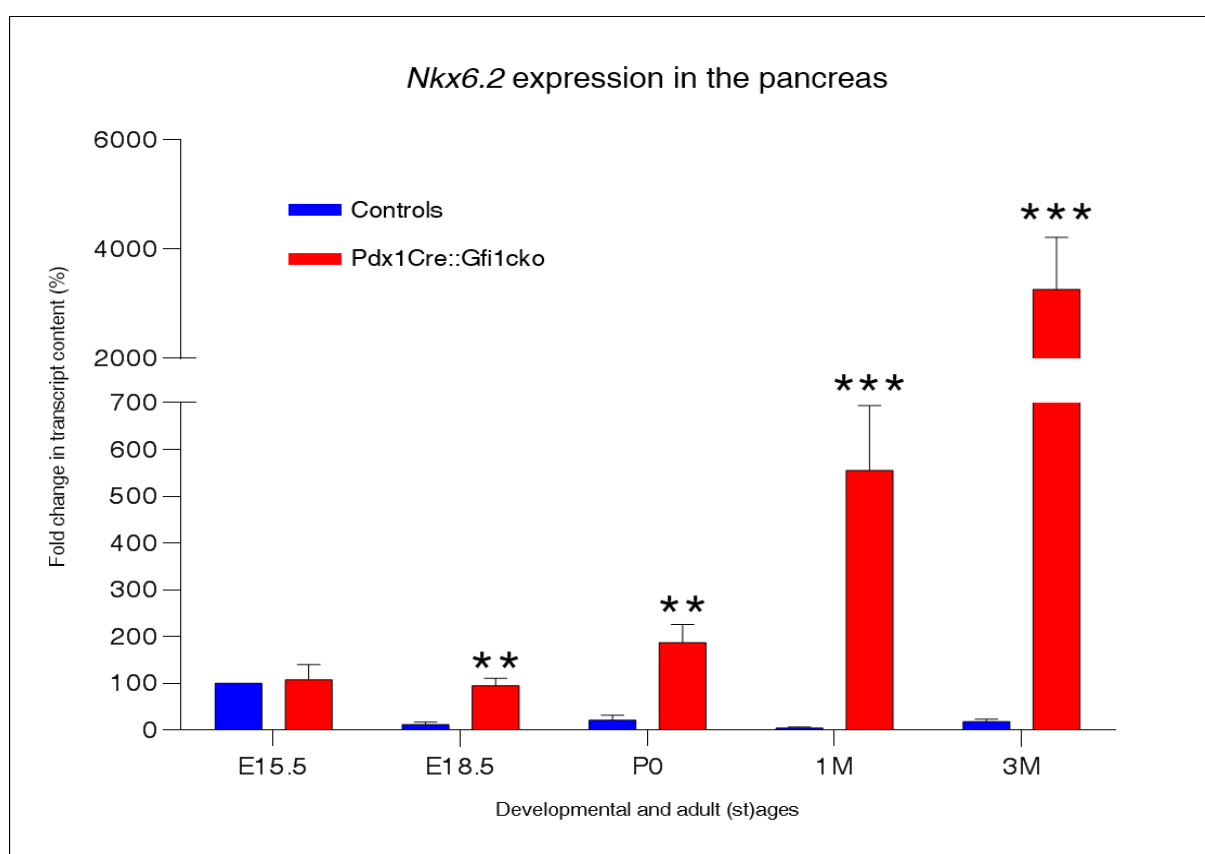


Figure 17. *Gfi1* is involved in the regulation of *Nkx6.2* expression in the pancreas. In order to dissect the *Gfi1* molecular pathway in the pancreas, we performed a screen targeting all the genes involved in pancreas development and function. We therefore identified *Nkx6.2* as a putative target of *Gfi1* in the pancreas. In control mice, *Nkx6.2* mRNA can be detected exclusively during embryonic development and its expression is extinguished by E18.5. Conversely, in *Pdx1Cre::Gfi1cko*, *Nkx6.2* remains permanently and increasingly expressed during the entire embryonic development and throughout adulthood.

Indeed, *Nkx6.2* transcript levels were not only maintained after E15.5, but they systematically increased throughout postnatal life of *Gfi1*-deficient mice. Accordingly, an impressive 37-fold increase in *Nkx6.2* expression was observed in adult *Gfi1* conditional knockout animals at 3

months of age. These data strongly suggest that *Gfi1* plays a key role in the regulation of *Nkx6.2* inactivation in the pancreas during intrauterine development.

Ectopic expression of *ghrelin* in the acinar cells of *Pdx1Cre::Gfi1cko* mice

Following a thorough characterization of the acinar compartment phenotype, additional immunohistochemical analyses were performed using antibodies directed against the pancreatic endocrine hormones. Insulin, glucagon, somatostatin and pancreatic polypeptide immunostaining confirmed the presence of all endocrine cell subtypes in both control and *Gfi1* mutant mice at 3 months of age (**Figure 18A-F**). The typical islet architecture, with a core of β -cells surrounded by α -cells, was observed in both *Gfi1*-expressing and *Gfi1*-deficient animals and the abundance of total β -cell mass was comparable between the two groups (**Figure 18G-I**).

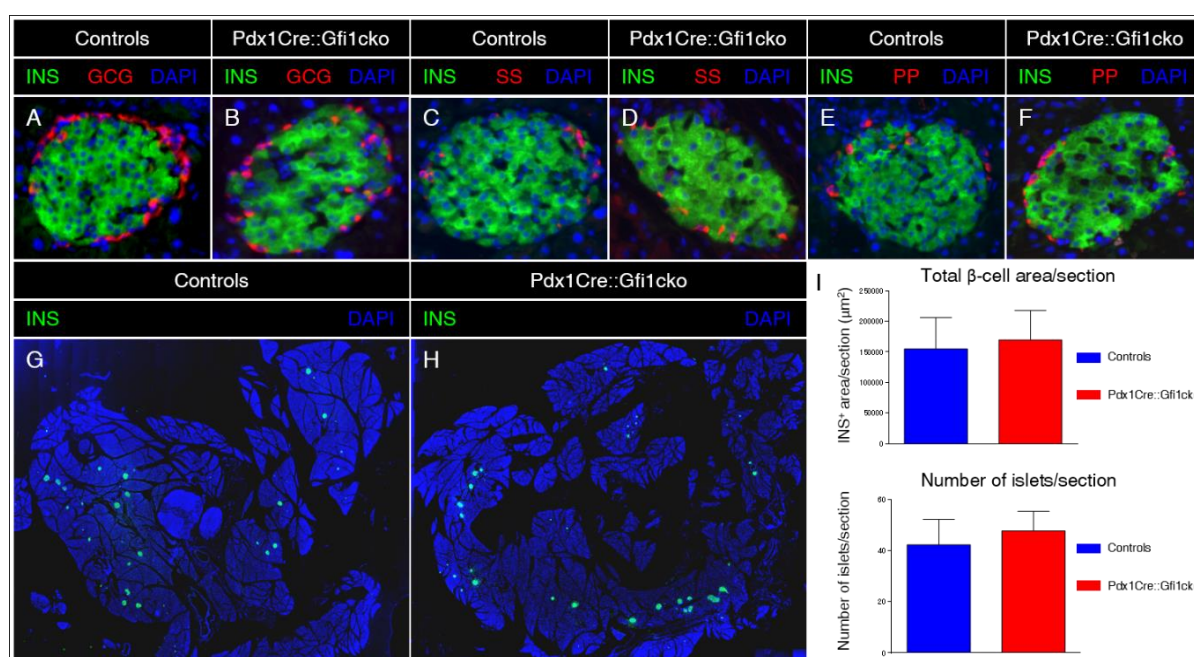


Figure 18. Characterization of the *Pdx1Cre::Gfi1cko* endocrine compartment. Immunohistochemical analyses confirmed the presence of insulin⁺ cells in the core of the islets of Langerhans surrounded by a mantle of glucagon-, somatostatin-, and PP-labelled cells in both controls and *Gfi1* mutants at 3 months of age (**A-F**). Examinations of insulin immunostaining on entire pancreatic sections did not reveal any significant differences in islets density and β -cell abundance comparing adult *Pdx1Cre::Gfi1cko* and age-matched controls (**G-H**). Quantitative analyses performed on 4 animals per each group corroborated these observations.

As expected, rare ghrelin-expressing cells were detected in the islets of Langerhans of both control and *Gfi1* knockout adult pancreatic tissue. Surprisingly, an impressive number of cells positive for ghrelin were detected within the acinar compartment of Pdx1Cre::Gfi1cko pancreas. A tridimensional reconstruction of serial sections labeled with ghrelin, insulin and the ductal marker DBA outlined the striking abundance of *ghrelin*-expressing acinar cells (**Figure 19A-B**). Intriguingly, ghrelin-labelled cells were found consistently negative for amylase and *vice versa* (**Figure 19C**). Yet again, a RT-qPCR approach was employed to confirm *ghrelin* misexpression in *Gfi1* knockout pancreata. According to previous observations³⁴⁶, *ghrelin* mRNA was detected in control samples exclusively during embryonic development with a peak at birth followed by a rapid decline within the first days of life. Conversely, *ghrelin* remained constitutively and increasingly expressed during adulthood in Pdx1Cre::Gfi1cko mice (**Figure 19D**).

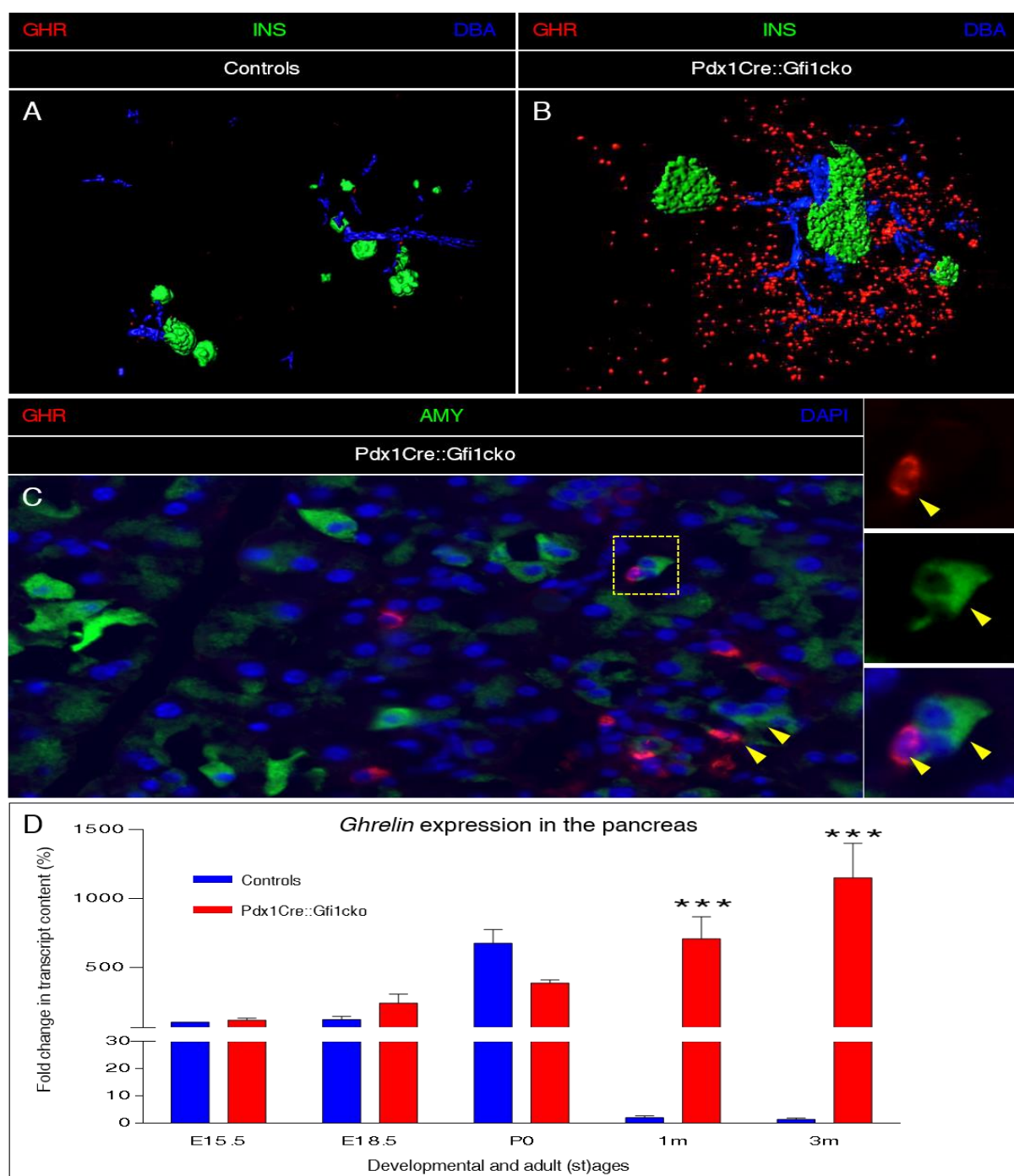


Figure 19. Pancreatic acinar cells ectopically express *ghrelin* in adult *Pdx1Cre::Gfi1cko*. A tridimensional reconstruction of serial sections labeled with ghrelin (in red), insulin (in green) and the ductal marker DBA (in blue) outlined the striking abundance of ghrelin-expressing cells within the acinar compartment of *Pdx1Cre::Gfi1cko* pancreata (A-B). Interestingly, co-immunostaining of *Gfi1*-deficient pancreatic sections failed to detect any cell co-expressing amylase and ghrelin (C). Quantitative evaluation of *ghrelin* expression by RT-qPCR confirmed that the inactivation of this gene does not occur around birth as seen in controls but remains permanently activated throughout adulthood (D).

As mentioned in the introduction, *ghrelin* is transiently expressed in the mouse pancreas during embryonic development by a population of endocrine cells, termed ϵ -cells. Aiming to identify and characterize ghrelin-labelled cells in Pdx1Cre::Gfi1cko, we performed a ghrelin immunogold staining and extensively analyzed the *Gfi1*-mutant acinar compartment by electron microscopy. Unexpectedly, this approach failed to detect a specific population of ghrelin-positive cells (**Figure 20A**). Instead, we discovered that pancreatic cells displaying an acinar cell ultrastructure misexpressed *ghrelin* within their secretory granules in adult Pdx1Cre::Gfi1cko animals (**Figure 20B**). Thus, these data showed that the loss of *Gfi1* results in ectopic expression of *ghrelin* in pancreatic acinar cells throughout adulthood. Importantly, amylase and ghrelin were found mutually exclusive, the cells failing to express amylase being found positive to ghrelin immunostaining and *vice versa*. Several studies highlighted the role of ghrelin in regulating glucose homeostasis, by modulating β -cell activity, proliferation and survival. To evaluate a putative paracrine effect on pancreatic endocrine cells exerted by ghrelin, we proceeded with our investigations focusing on β -cells functionality.

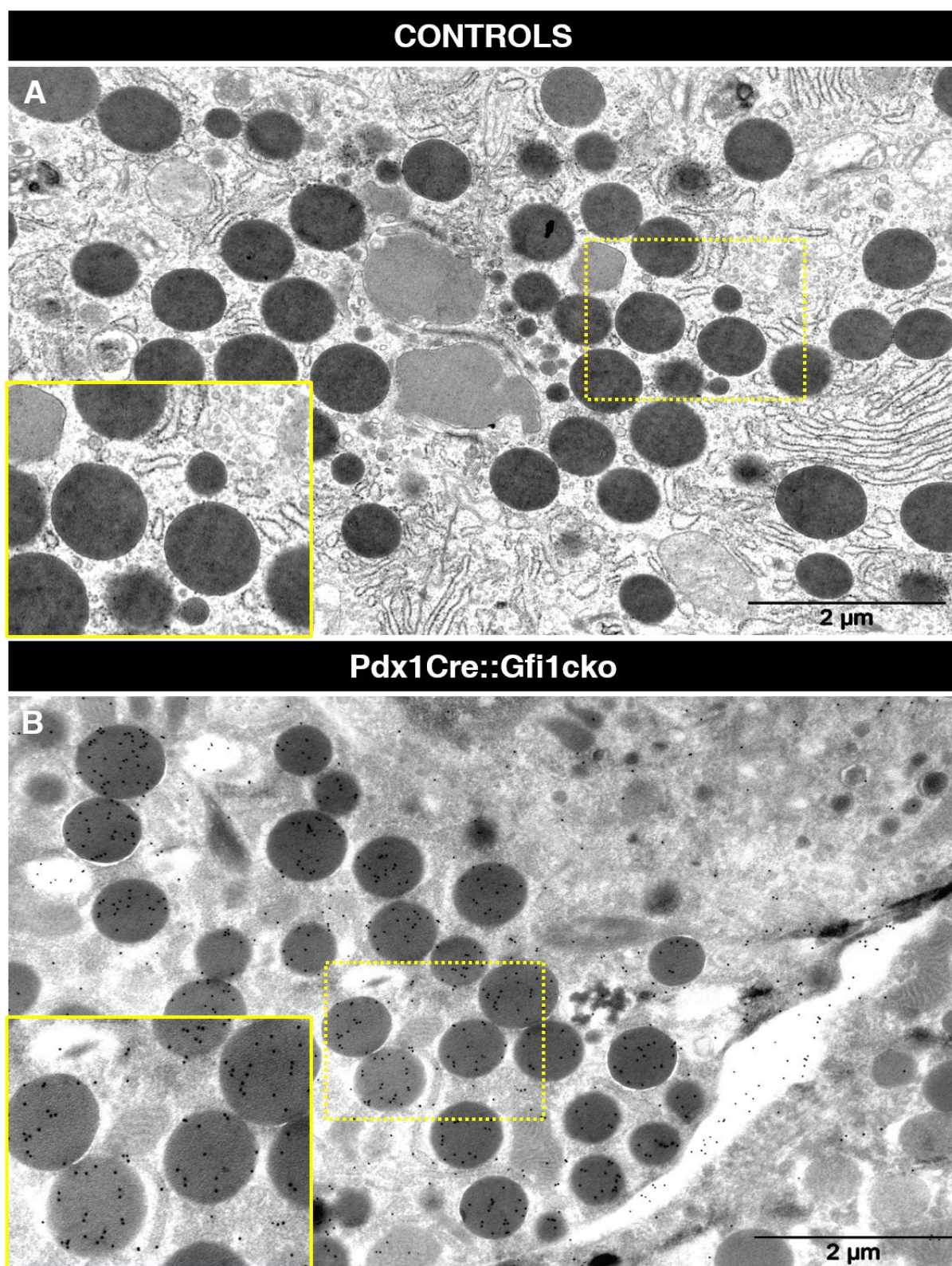


Figure 20. Pancreatic acinar cells express *ghrelin* in Pdx1Cre::Gfi1cko animals. Ghrelin was detected exclusively in *Gfi1* mutant pancreatic acinar cells as assessed by immunogold detection (A-B).

Gfi1 conditional knockout mice display blunted glucose-stimulated insulin secretion

Our results clearly demonstrated that Pdx1Cre::*Gfi1*cko mice express *ghrelin* in the acinar tissue throughout adulthood, while the endocrine compartment does not seem to be affected by the loss of *Gfi1*. Importantly, ghrelin has been shown to regulate β -cells activity by inhibiting insulin release both *in vivo* and *in vitro*⁸⁸. Consequently, aiming to investigate the effects of chronic ghrelin exposition on β -cells functionality, an evaluation of intracellular insulin content was performed using electron microscopy combined with insulin detection by immune-gold labeling. The ultrastructural analysis of isolated islets of Langerhans highlighted the presence of insulin-containing granules within the cytoplasm of both controls and *Gfi1* mutant β -cells (**Figure 21A-B**). However, the density of these vesicles and the overall amount of insulin content was found strongly decreased in *Gfi1* mutant β -cells compared to controls (**Figure 21A-B**), suggestive of a diminished capability to secrete insulin.

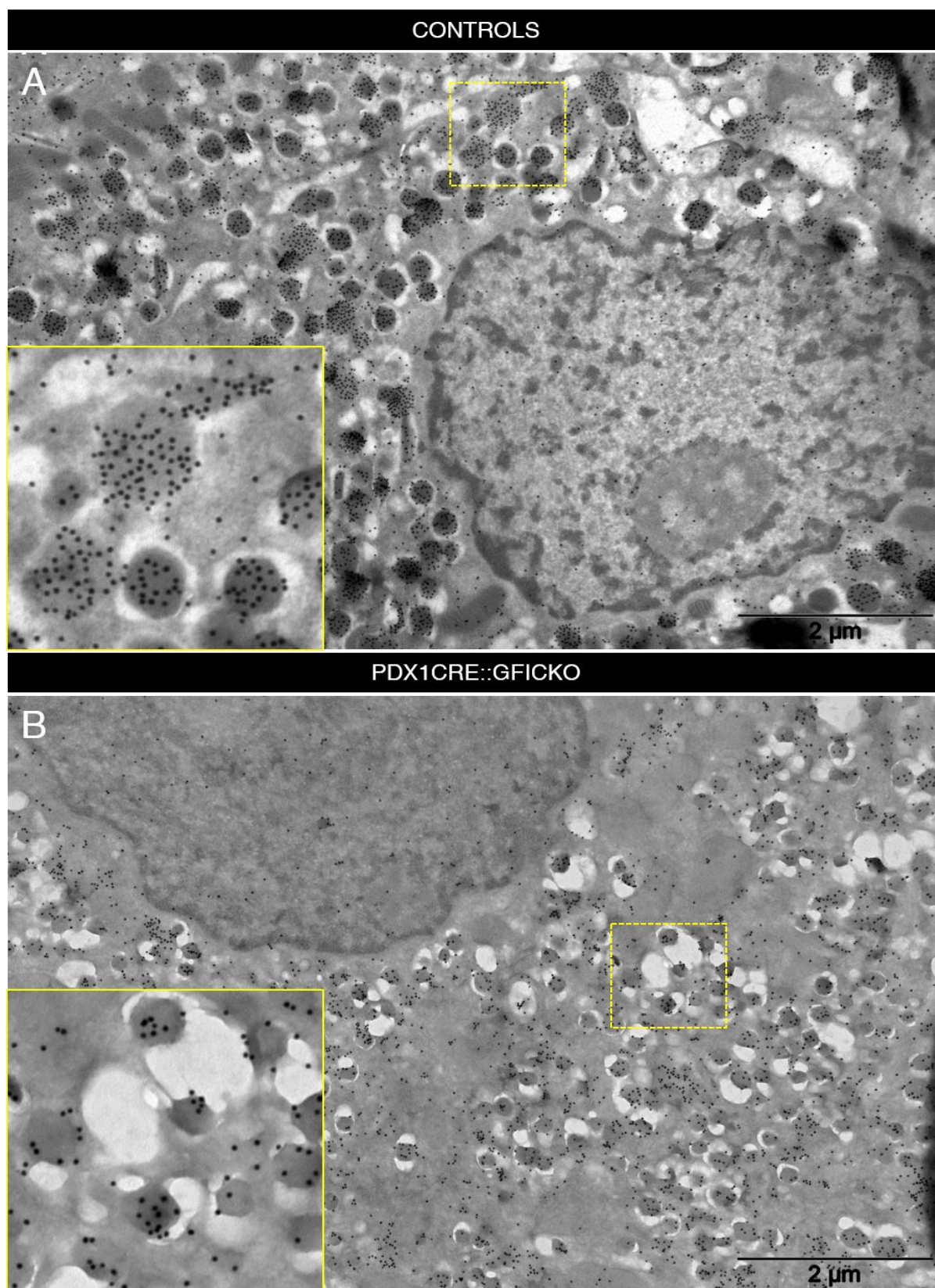


Figure 21. Ultrastructural analyses reveal a decreased in insulin content within *Gfil*-deficient β -cells. Insulin immunogold staining analyzed by electron microscopy showed a lower concentration of insulin within the secretory vesicles within the β -cells of *Gfil*-mutants (B) compared to controls (A).

We therefore proceeded with a functional assessment of β -cells measuring basal and glucose-stimulated insulinemia. To this aim, we collected blood samples upon starvation and 20 minutes after intraperitoneal injection of high concentration of glucose. Subsequently, plasma was separated and insulin concentrations were assessed by ELISA immunoassay. Importantly, while controls insulinemia predictably doubled upon glucose stimulation, Pdx1Cre::Gfi1cko blood insulin levels did not increase in response to glucose injection (**Figure 22A**).

Once released, insulin binds its specific tyrosine kinase receptor and triggers a cascade of phosphorylation events, leading to the recruitment of Akt on the plasma membrane. As phosphorylation of Akt mediates most of insulin's metabolic effects, we sought to analyze Akt activation in skeletal muscle. Toward this aim, we isolated tibialis anterior muscles 20 minutes following glucose administration and tissue lysates were subjected to western blot analyses using anti-phospho Akt (ser473) antibodies and anti-Akt antibodies. According to the lower level of insulin detected in the blood, the insulin receptor of Pdx1Cre::Gfi1cko knockout was tyrosine phosphorylated at a significantly lower extend compared to controls.

In summary, *Gfi1* conditional knockout mice displayed reduced intracellular insulin content within β -cells and decreased blood insulin concentration upon glucose stimulation. Consistently, the insulin receptor pathway in the muscle tissue was found significantly impaired following glucose administration. Thus, high concentrations of blood glucose failed to trigger hyperinsulinemia in Pdx1Cre::Gfi1cko. This permanent down-regulation of insulin signaling pathways appears crucial to prevent the development of insulin resistance when mice are challenged with long-term high-fat feeding.

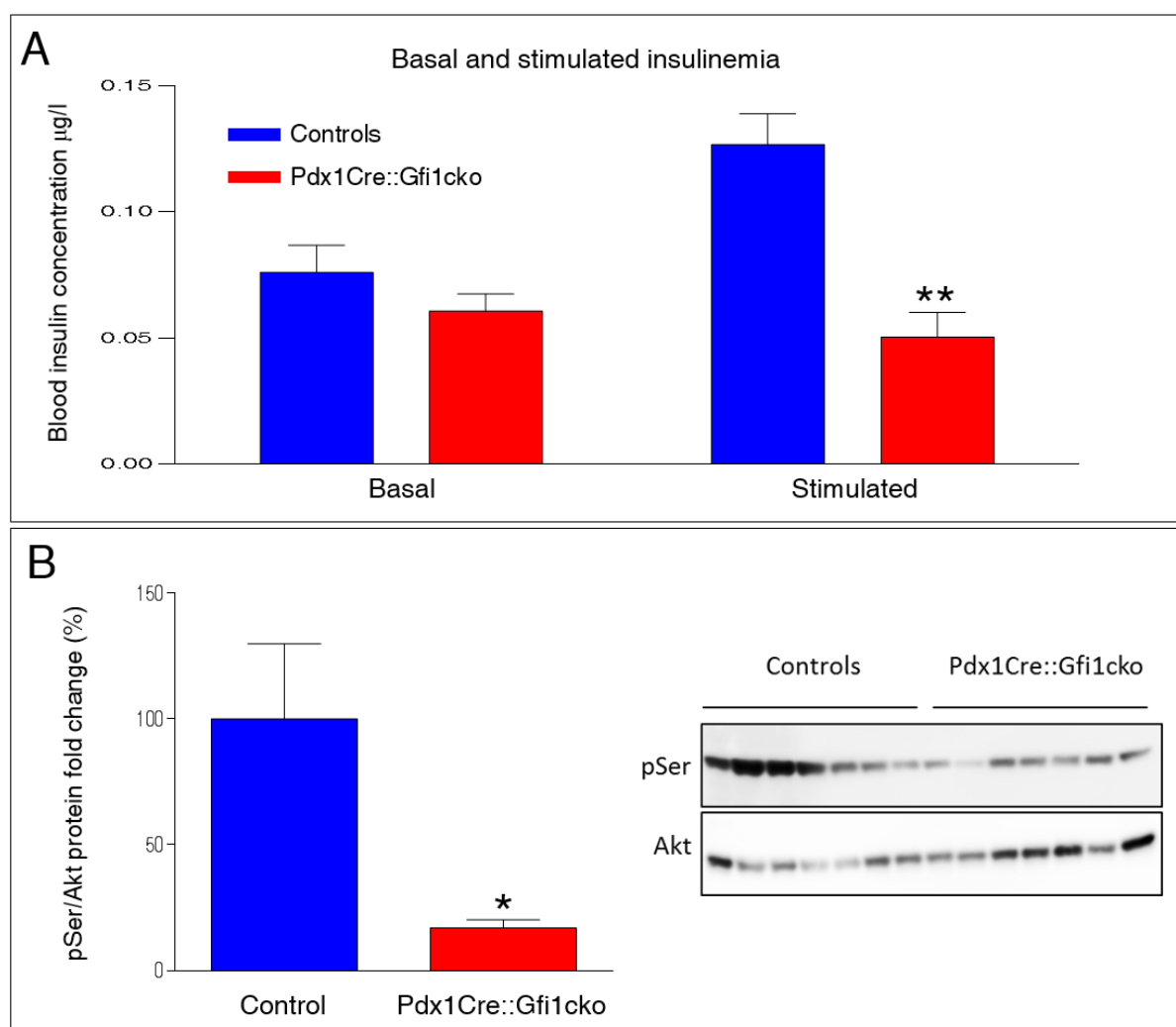


Figure 22. Insulin secretion is decreased in Pdx1Cre::Gfi1cko mice. Blood insulin concentration measurement by ELISA immunoassay showed a significant decrease in glucose-stimulated insulin secretion (**A**). Western-blot analyses, assessing the phosphorylation of Akt, demonstrated that the insulin receptor pathway is strongly down-regulated in *Gfi1*-deficient mice following injection with high-dose of glucose (**B**).

Discussion

Gfi1 conditional knockout animals are resistant to induced diabetes

The pancreas is frequently described as a mixed gland consisting of two compartments: the endocrine and the exocrine tissue. This distinction is not only histological but mainly physiological: while the islets of Langerhans are seen as the headquarters of glucose homeostasis, acinar cells are considered as factories secreting digestive enzymes. Consequently, as diabetes is a disease affecting β -cells, current research is mainly focused on endocrine cell specification, development and function, while the exocrine tissue is rather investigated in the context of pancreatitis and pancreatic tumors studies. Therefore, the role of the cross talk between acinar and endocrine cells in diabetes pathogenesis and progression has been poorly investigated.

In the present study, we identified *Gfi1* as a putative player in diabetes. This gene was shown to be expressed exclusively in the acinar compartment from the gestational development and throughout the entire mouse life. A Pdx1Cre::Gfi1cko mouse line was therefore generated and characterized with the aim to shed light on the physiological role of *Gfi1* in the pancreas. Importantly, these animals displayed a significantly and consistent lower basal glycemia during adulthood. These observations suggested that *Gfi1*, despite being excluded from the islets of Langerhans, is nonetheless involved in the regulation of glucose homeostasis. To confirm this hypothesis, we challenged *Gfi1*-deficient animals using two models of induced diabetes. Astonishingly, mice lacking *Gfi1* expression specifically in the pancreas were resistant to both high fat feeding- and chemically-induced diabetes. As loss of *Gfi1* did not affect longevity and fertility, fully characterizing the phenotype of *Gfi1* transgenics and dissecting the molecular mechanisms underlying *Gfi1* activities in the pancreas became extremely intriguing. We therefore thoroughly analyzed Pdx1Cre::Gfi1cko pancreatic tissue and gene expression.

Loss of *Gfi1* results in pancreas hypertrophy and decrease in *amylase* expression

Phenotypical analyses of *Pdx1Cre::Gfi1cko* pancreata revealed a dramatic increase in the number of dividing acinar cells, this hyperproliferation eventually leading to a pancreatic hypertrophy. Furthermore, *Gfi1* mutant acinar cells displayed a strongly affected expression of *amylase*, suggesting that these cells are not only hyperproliferative but have also functional defects. As described in the introduction, amylase is a digestive enzyme dedicated to the hydrolysis of carbohydrates into maltose and a mixture of short, branched chains of glucose molecules. These molecules are further cleaved by enzymes located on the luminal membranes of the small-intestine epithelial cells. Thus, amylase and intestinal disaccharidases play a major role in glucose absorption and their activity heavily affects the raise of blood glucose levels after a meal. Accordingly, amylase and/or disaccharidases have become molecular targets for the pharmacological treatment of diabetes. Indeed, it has been shown that inhibiting the enzymatic digestion of carbohydrates can reduce the rate of glucose absorption, delay gastric emptying, and improve post-prandial hyperglycemia. Nowadays, several compounds modulating amylase and/or disaccharidases activity, such as acarbose, miglitol and pramlintide³⁴⁷⁻³⁴⁹ are employed in diabetes management.

We believe that the decrease in *amylase* expression observed in the *Pdx1Cre::Gfi1cko* animal model is associated with carbohydrates absorption impairment. This hypothesis would ultimately explain the lower basal glycemia constantly observed in these mutants during adulthood. To verify this theory, we are currently using a global non-targeted metabolomics approach based on mass spectrometry³⁵⁰. This technique will allow us to draw a metabolomics profile of *Pdx1Cre::Gfi1cko* plasma and feces and thereby fully understand the impact of *amylase* expression defects on the ability of these animals to process and assimilate carbohydrates.

Pdx1Cre::Gfi1cko mice fail to inhibit *Nkx6.2* expression during embryonic development

The pancreas development is regulated by a hierarchy of transcription factors that specify progenitor cells toward alternative lineages and promote the genesis of mature pancreatic cells. During pancreas organogenesis, the expression of several transcription factors becomes gradually restricted to a specific pancreatic lineage and remains constitutively expressed during adulthood. Conversely, a subset of factors exhibits highly restricted temporal and spatial expression patterns and their inhibition is crucial to achieve and maintain pancreatic cells mature phenotype. Among these, *Nkx6.2* is a transcription factor involved in lineage specification early during development, its expression rapidly declining during late embryogenesis. Notably, the constitutive overexpression of *Nkx6.2* in pancreatic progenitors ablates acinar cells specification in the pancreas and is therefore lethal³⁵¹. Importantly, *Nkx6.2* appears normally expressed during fetal development in Pdx1Cre::Gfi1cko animals. However, the absence of *Gfi1* prevents the physiological inactivation of *Nkx6.2*, this gene remaining permanently and increasingly overexpressed after birth and beyond. Gfi1 therefore appears to play an important role in down-regulating *Nkx6.2* expression.

During terminal exocrine differentiation, acinar cells progressively acquire a mature secretory phenotype while they dramatically lower their proliferation rate. As described above, *Gfi1* mutant acinar cells are hyperproliferative during adulthood and their ability to synthesize amylase is considerably affected. As *Nkx6.2* is involved in exocrine pancreas development, it is conceivable that the sustained expression of *Nkx6.2* during adulthood causes the developmental defects we observed in *Gfi1*-deficient acinar cells. To prove this idea, we are presently crossing Pdx1Cre::Gfi1cko mice with *Nkx6.2*^{-/-} full knockout animals. The phenotypical characterization of the resulting Pdx1Cre::Gfi1cko:: *Nkx6.2*^{-/-} mouse line will

allow us to determine whether the loss of *Nkx6.2* in *Gfi1*-mutant acinar cells is sufficient to rescue their ability to express physiological levels of *amylase* and thereby normalize acinar cell proliferation rates.

In *Pdx1Cre::Gfi1cko* pancreata *ghrelin* is ectopically expressed in the acinar compartment

During perinatal pancreas development, *ghrelin* is transiently expressed in islet α -cells, where it co-localizes with glucagon³⁵². Furthermore, ghrelin is exclusively synthesized by a distinct population of pancreatic islet cells, the ϵ -cells³⁵³. In wild-type mice, pancreatic ghrelin expression rapidly declines after birth and is virtually extinguished at P10. Interestingly, an ectopic expression of *ghrelin* has been observed in instances where the inactivation of a transcription factor was preventing the specification of a particular pancreatic cell lineage. For instance, the targeted disruption of *Nkx2.2* in mice results in a complete lack of β -cells and reduced numbers of α - and PP-cells, these cells being replaced with ghrelin⁺ cells. Similarly, in *Pax4* full knockout animals, endocrine precursors fail to differentiate into insulin-producing cells and differentiate into glucagon/ghrelin co-expressing cells^{353,354}. In the present study, we showed that in the absence of *Gfi1*, a high proportion of acinar cells fail to express *amylase*, but instead misexpress *ghrelin*. We believe that such aberrant ghrelin expression is another manifestation of acinar cells immaturity, probably related yet again to the continued overexpression of *Nkx6.2* during adulthood. The immunohistochemical assessment of ghrelin-expressing cells in *Pdx1Cre::Gfi1cko::Nkx6.2^{-/-}* is planned in order to verify this hypothesis.

Notably, ghrelin has been shown to exert insulinostatic effects on β -cells, probably mediated by somatostatin release from δ -cells³⁵⁵. Accordingly, in *Pdx1Cre::Gfi1cko* mice, the overexpression of *ghrelin* is associated with decreased intracellular insulin content, blunted

glucose-stimulated insulin release, and inhibited activation of insulin receptor on skeletal muscles upon glucose stimulation.

Of note, high fat feeding commonly leads to hyperinsulinemia and hyperglycemia. Importantly, it has been shown that hyperinsulinemia *per se* can cause insulin resistance by downregulating insulin receptors and desensitizing post-receptor pathways³⁵⁶. Notably, we demonstrated that mice lacking *Gfi1* expression maintain the ability to respond to exogenous insulin even after long-term treatment with high fat regimen and therefore remain steadily normoglycemic. We suggest that constant ghrelin-mediated insulinostatic effect protects Pdx1Cre::Gfi1cko mice against the development of insulin resistance when fed with high fat diet, strongly ameliorating their glucose metabolism.

Furthermore, ghrelin has been demonstrated to protect β -cells against apoptosis when exposed to several cytotoxic molecules. Using HIT-T15 β -cell line and human islets, ghrelin has been shown to inhibit apoptotic events induced by serum starvation and treatment with cytokines, a major cause of β -cell loss in both type 1 diabetes and type 2 diabetes. Similarly, ghrelin was found to protect the INS-1 β -cell line against dexamethasone-induced apoptosis. The signaling pathway underlying the survival effects of ghrelin in β -cells has not been fully elucidated. However, ghrelin was suggested to elicit its survival potential by activating PI3K/AKT and MAPK pathways and inhibiting cytokine-induced nitric oxide (NO) production. Crucially, ghrelin treatment prevented diabetes development in STZ-treated neonatal rats. Therefore, the overexpression of ghrelin might also be the key to explain Pdx1Cre::Gfi1cko resistance against STZ-induced diabetes.

To fully understand the role of ghrelin in the insusceptibility of *Gfi1* mutants to become diabetics in our two models, we envisage treating these animals with the ghrelin receptor antagonist [D-Lys(3)]-GHRP-6. Particularly, we plan to determine whether the blockade of

ghrelin signalization pathways is able to increase glucose-stimulated insulin release and/or affect resistance against STZ-induced diabetes in Pdx1Cre::Gfi1cko animals.

In summary, our results show that *Gfi1* is expressed in the pancreatic acinar compartment. Intriguingly, Pdx1Cre::Gfi1cko rodents are resistant against high-fat diet- and STZ-induced diabetes. Thorough immunohistochemical, ultrastructural and gene expression analyses revealed a complex phenotype, characterized by several signs of immaturity of acinar cells. An unequivocal interpretation of this phenotype is not easy. However, our current data suggest the following model (**Figure 23**): Gfi1 is a transcription repressor that plays a crucial role in inhibiting *Nkx6.2* expression in the pancreas during fetal development. Sustained and increasing expression of *Nkx6.2* beyond birth hampers acinar cells full development, these cells failing to become terminally differentiated, fully functional, postmitotic cells. The resulting decrease in amylase synthesis affects intestinal carbohydrates processing and therefore glucose absorption in Pdx1Cre::Gfi1cko mice, these animals consequently displaying a consistent lower basal glycemia during adulthood. A further manifestation of acinar cell lack of maturity is the aberrant, ectopic expression of ghrelin in *Gfi1*-deficient pancreata. This hormone exerts a paracrine action on the pancreatic endocrine cells, protecting β -cells against STZ-induced apoptosis and inhibiting insulin secretion. This decrease in insulinemia ultimately prevents insulin resistance development upon high-fat diet, therefore preventing hyperglycemia onset.

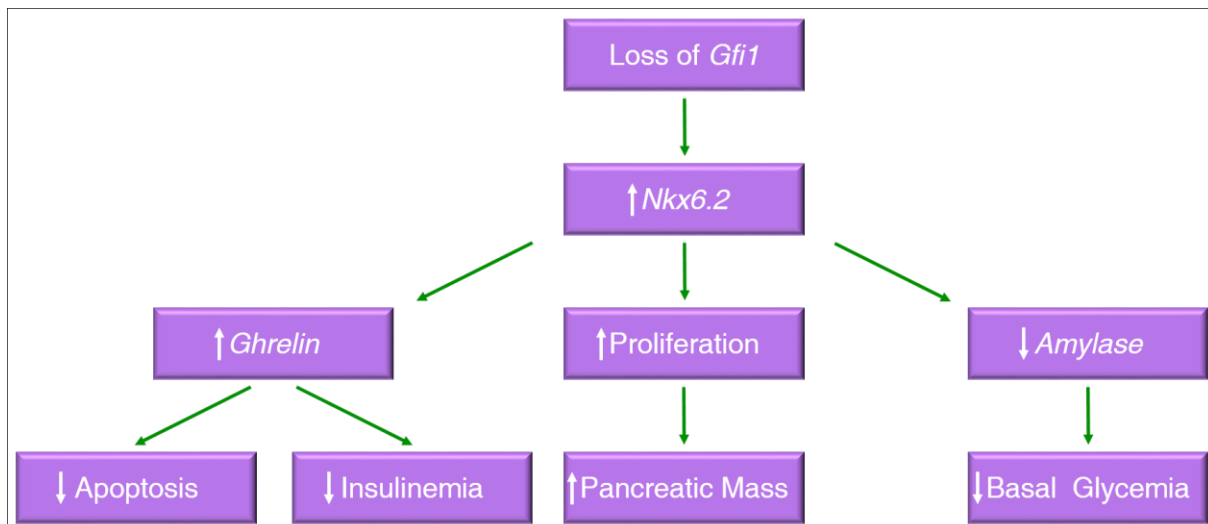


Figure 23. Pdx1Cre::Gfi1cko phenotype. In the absence of *Gfi1*, pancreatic *Nkx6.2* expression is not extinguished during fetal development. The resulting misexpression of *Nkx6.2* during late embryonic development and after birth affects full pancreatic acinar cells development. These cells maintain a high proliferation rate after weaning, leading to a progressive increase in the whole organ mass. Simultaneously, the majority of acinar cells fail to express *amylase* while they ectopically synthesize ghrelin. We suggest that the decrease in *amylase* expression leads to the impairment of carbohydrates digestion and processing in the intestinal tract. The consequent delay of glucose absorption would explain the levels of basal glycemia found consistently lower in Pdx1Cre::Gfi1cko animals compared to controls. Furthermore, blunted glucose-stimulated insulin secretion and resistance against STZ-induced apoptosis are proposed as the result of ghrelin paracrine effect on islet β -cells. According to this model, ghrelin insulinostatic effect regulates hyperinsulinemia and insulin resistance development upon high-fat feeding while protecting animals against chemically-induced diabetes.

The results described here suggest that the *Gfi1/Nkx6.2/ghrelin* pathway could represent an opportunity for management of type 1 and type 2 diabetes. A growing body of evidence suggest that ghrelin could suppress the release of insulin from the pancreatic islets³⁵⁷. However, the role of ghrelin in the pathogenesis and progression of diabetes is currently highly controversial. Dezaki et al. reported that *ghrelin* knockout mice displayed a lower basal glycemia upon short-term (4 weeks) high-fat diet treatment³⁵⁸. Similarly, *ghrelin* ablation has been shown to improve glucose homeostasis of leptin-deficient obese (*ob/ob*) mice³⁵⁹. However, exogenous ghrelin administration was found to increase glucose-stimulated incretin secretion and improve glucose handling in normal male mice³⁶⁰. In humans, low ghrelin concentrations have been associated to insulin resistance and type 2 diabetes. Such discrepancies are seemingly due to the vast

spectrum of ghrelin physiological actions. Beyond its ability to modulate insulin secretion, ghrelin stimulates the anabolizing growth hormone (GH) release from the anterior pituitary³⁶¹. Furthermore, it promotes food intake via centrally-regulated signal mechanisms, enhances gastric and intestinal motility and secretion³⁶², regulates satiety³⁶³ and modulates brown fat thermogenesis³⁶⁴. Thus, the generation of a Pdx1Cre::Gfi1cko presents new opportunities to study the role of ghrelin exclusively on pancreatic functions. As ghrelin is overexpressed ectopically in the exocrine compartment, it cannot be released into the general circulation thereby offering the possibility to evaluate the specific effects of ghrelin over-exposition on pancreatic cells, without interfering with other ghrelin target organs.

Targeting ghrelin pathway exclusively in the pancreas might be beneficial to protect obese patients against type 2 diabetes. Indeed, excessive dietary glycemic load is associated with frequent and powerful insulin spikes that could cause β -cell failure. The permanent modulation of insulin secretion mediated by ghrelin action could prevent the progressive exhaustion of β -cells upon chronic fuel surfeit. Additionally, the anti-apoptotic and anti-inflammatory effects of ghrelin on pancreatic insulin-producing cells could play a beneficial role in type 1 diabetes context, rendering β -cells more resistant to immune-destruction. The generation of mouse models overexpressing *ghrelin* specifically in pancreatic acinar cells would finally allow us to explore these possibilities.

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