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Frk/Shb Signalling in Pancreatic Beta-cells

Roles in Islet Function, Beta-cell Development and Survival as Implicated in Mouse Knockout Models

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Abstract
Åkerblom, B. 2009. Frk/Shb Signalling in Pancreatic Beta-cells. Roles in Islet Function, Beta-cell Development and Survival as Implicated in Mouse Knockout Models. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 426. 58 pp. Uppsala. 978-91-554-7429-4.

The adaptor protein Shb and the non-receptor tyrosine kinase Frk have been implicated in intracellular signalling in insulin-producing beta cells. In this thesis, knockout mice are used to further elucidate the role of Shb and Frk for beta cell number, cytokine-induced cell death, and glucose homeostasis. In addition, the effect of Shb deficiency upon tumour growth is studied in a mouse model of endogenous tumourigenesis.

Previously, overexpression of Frk has been associated with increased beta cell replication, and increased susceptibility to cytokine induced beta cell destruction. To test whether Frk has a non-redundant role in regulating beta cell mass, beta cell number in Frk-/- mice was assessed at different stages of life. The results showed that Frk is involved in regulating beta cell number during embryonal and early postnatal life, but is probably redundant in the adult.

An earlier study had suggested that Shb participates in cytokine-induced beta cell death, a model of autoimmune diabetes. To test this further, Shb-/- islets were exposed to cytokines, or to an ER-stress inducing agent. Shb knockout islets exhibited decreased cell death, and this effect appeared to be independent of NO, JNK, p38 MAP kinase, FAK and c-Abl, but may involve an augmented induction of Hsp70.

Furthermore, glucose homeostasis in Shb-/- mice was impaired, with elevated basal blood sugar concentration and reduced glucose-induced insulin secretion.

Previously Shb deficient mice had showed an impaired ability to sustain growth of implanted tumour cells, due to reduced angiogenesis. Tumour growth and angiogenesis were here assessed in an inheritable tumour model. Shb deficient mice exhibited fewer tumours, and reduced vessel density in small tumours, indicating impaired angiogenesis. However, a few large tumours developed in Shb-/- mice, suggesting that tumours can escape the angiogenic restriction caused by the absence of Shb.

Keywords: Frk, Shb, beta cell, beta cell death, islets of Langerhans, glucose homeostasis, insulin secretion, angiogenesis, VEGF, RIP-Tag2

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To my parents

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Åkerblom B, Annerén C, Welsh M. (2007) A role of Frk in regulation of embryonal pancreatic beta cell formation. *Molecular and Cellular Endocrinology 270: 73–78*

II Mokhtari D*, Åkerblom B*, Mehmeti I, Wang X, Funa NS, Olerud J, Lenzen S, Welsh N and Welsh M. Reduced cell death and increased Hsp70 expression in response to cytokine exposure in islets of Langerhans isolated from Shb knockout mice. *Submitted*

III Åkerblom B, Calounova G, Mokhtari D, Jansson L, Welsh M. Impaired glucose homeostasis in Shb-/- mice. *Manuscript*

IV Åkerblom B, Zang GX, Calounova G, Welsh M. Absence of the Shb adapter protein leads to a reduced tumour incidence in the oncogenic RIP-Tag2 mouse strain. *Manuscript*

*Shared contribution as first author.

Contents

Abbreviations

ANOVA	Analysis of variance
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCL	B cell lymphoma associated protein
CD	Cluster of differentiation
CPA	Cyclopiazonic acid
C_T	Cycle threshold
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
E15.5	Embryonal day 15.5
EGF	Epidermal growth factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
Frk	Fyn-related kinase
G1	Growth phase 1 of the cell cycle
GLP1	Glucagon-like peptide-1
GLUT	Glucose transporter
HES1	Hairy and enhancer of split 1
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor
HSP	Heat shock protein
IκB	Inhibitor kappa B
IFN	Interferon
IGF1	Insulin-like growth factor
IL-1	Interleukin-1
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	kiloDalton
Km	the Michaelis constant in Michaelis-Menten kinetics

MAPK	Mitogen activated kinase
mM	millimolar
MMP	matrix metalloproteinase
NFκB	Nuclear factor kappa beta
NGF	Nerve growth factor
NGN3	Neurogenin 3
NO	Nitric oxide
PBS	phosphate buffered saline
PC12	Pheochromocytoma 12,
PDGFR	Platelet-derived growth factor receptor
PDX1	Pancreatic and duodenal homeobox 1
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
pRB	Retinoblastoma protein
PTB	Phosphotyrosine binding
RIP	Rat insulin promotor
RNA	Ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S	Synthesis phase in the cell cycle
SDS	Sodium Dodecyl sulphate
SDS-Page	SDS-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shb	Please see page 31
siRNA	short interfering RNA
STAT	Signal Transducers and Activator of Transcription
T3	Triiodothyronine
Tag	T-antigen
TNF	Tumour necrosis factor
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction

The pancreas is a gland with two different functional components. The majority of the pancreas consists of exocrine acini emptying their product, the pancreatic juice, into a ductal tree discharging its content in the duodenum. Interspersed in the exocrine mass, there are highly vascularized endocrine islets of Langerhans emptying their contents into the blood stream.

The endocrine islets are made up by four to five different endocrine cell types, α, β, δ, PP, ε, producing the hormones, glucagon, insulin, somatostatin pancreatic polypeptide and ghrelin. The β-cells constitute the major part of the endocrine cells in the islets of Langerhans comprising 65%, with α-cells, δ-cells, PP-cells, and ε-cells 20%, 10%, 5%, and <1%, respectively. Islets also contain endothelial cells supporting the endocrine cells with basal lamina, nerve endings, and occasionally macrophages/dendritic cells and fibroblasts. A thin connective tissue capsule surrounds each islet, separating it from the exocrine acini. The islets of Langerhans play an essential role in the regulation of blood glucose homeostasis and body energy metabolism.

Blood glucose homeostasis

(references to this section [1-5])

Energy metabolism

The body needs energy and nutrients for sustaining organ and tissue functions, and in the long run, for the survival of the individual. This requirement may vary with activity level, but is never zero. Body intake of energy and nutrients is not constant and normally matched to the demand, thus the body needs to be able to store energy and nutrients when these are abundant, and then to utilize these stores during fasting conditions to sustain organ and tissue function.

Glucose is the preferred source of energy in the body, while triglycerides (fat) constitute the main bulk of the energy stores. Glucose may be stored in the form of glycogen, a highly branched glucose polymer, or metabolised and used for triglyceride synthesis for energy storage. Skeletal muscle and the liver contain most of the glycogen stored in the body, while triglycerides are mainly stored in the adipose tissue. Some tissues as central nervous system, renal medulla and red blood cells depend on glucose for energy, whereas other tissues in addition to glucose may use fatty acids for energy production.

The metabolism of carbohydrates, amino acids and lipids in the body is governed by the action of opposing hormones, i.e. while anabolic processes are stimulated, katabolic processes are generally inhibited and vice versa, the ratio between different hormones deciding the net metabolic outcome. Insulin is the main anabolic hormone, while several hormones act opposing to insulin, e.g. glucagon, catecholamine, glucocorticoids.

During fasting conditions, when the insulin concentration in the blood is low, the effects of the actions of glucagon, catecholamines, glucocorticoids etc dominate, promoting use of energy stores. In adipose tissue lipolysis is stimulated, leading to release of free fatty acids (FFA) and glycerol. Skeletal muscle relies mainly on glycogen stores and fatty acids for energy production. Proteinolysis is favoured, releasing amino acids into the blood. The liver maintains the blood glucose concentration during fasting, by releasing glucose from glycogen stores and/or by de novo glucose synthesis (gluconeogenesis) from substrates like lactate, glycerol and some amino acids.

Following a meal, nutrients are appearing in abundance after uptake in the intestines, and this causes the insulin concentration in the blood to increase. This rise in the insulin concentration inhibits the effects of the "fasting hormones" and promotes energy storage and anabolic processes. Insulin causes the liver to stop glycogenolysis/gluconeogenesis and to start glycogen synthesis. Insulin also promotes liver formation of lipid transporting lipoproteins, thereby facilitating lipid uptake in peripheral tissues. In skeletal muscle insulin promotes glucose uptake, use in metabolism and storage as glycogen. Insulin also inhibits proteinolysis and promotes protein synthesis. In adipose tissue lipolysis is inhibited, glucose and triglyceride uptake is stimulated, and lipid synthesis promoted, leading to increased storage of triglycerides.

Blood glucose regulation

The blood glucose concentration is held within narrow limits, normally ~5.5mM, but can be one mM lower after an overnight fast, and 2-3 mM higher for 1-2 hours following a meal. After several days of fasting, the blood glucose concentration is stabilized at 3-4 mM. Several cell types participate in keeping the blood glucose concentration within these values. Pancreatic beta cells respond to an elevated glucose concentration by secreting insulin and thus promoting glucose uptake and metabolism. Pancreatic alpha cells and neurons in the brain react to hypoglycaemia, leading to rapidly rising blood glucagon and catecholamines concentrations, stimulating liver glucose release.

Cellular uptake of glucose is facilitated by a family of glucose transporter proteins (GLUTs). Most cells in the body express glucose transporters 1 and 3. These have a low Km (~1mM), and thus transport glucose at a nearly constant rate at physiological blood glucose concentrations. GLUT2 has a high Km

(~15-20mM) and is expressed by beta cells and the liver. The high Km makes glucose uptake to increase significantly with rising blood glucose concentrations. A fourth glucose transporter, GLUT4 (Km~5mM), is expressed in skeletal muscle and adipose tissue. This transporter is stored in intracellular vesicles, but upon insulin stimulation it is translocated to the plasma membrane, increasing cellular glucose uptake 10-40 fold. Thus, following a meal, when glucose is abundant, the blood glucose concentration is mainly dependent on glucose uptake by liver, skeletal muscle and adipose tissue.

When glucose enters the cell it is rapidly phosphorylated to glucose-6-phosphate by hexokinase. This often commits glucose to enter metabolic pathways and also prevents glucose from leaving the cell. Hexokinase is inhibited by its end-product, thus glucose uptake is in this way dependent on glucose-6-phosphate metabolism. In addition, glucokinase, is present in liver, beta cells and hypothalamus. Glucokinase has a much higher half-saturated concentration (~8mM) than hexokinase, and this makes its activity to change within the important range of 4-10mM blood glucose. Furthermore glucokinase is not, like hexokinase, inhibited by its end-product. These properties make glucokinase a glucose sensor, which increases the glucose-6-phosphate concentration as a response to elevated blood glucose concentration, in the tissues where it is expressed.

In beta cells, glucose metabolism rapidly increases when the blood glucose concentration rises above 5.5mM. This leads to increased intracellular levels of ATP. The beta cell membrane potential is regulated by K^+channels, through which K^+ ions leave the cell. These K^+channels are sensitive to high levels of ATP, and ATP-binding causes closure of the channel. As a consequence the plasma membrane depolarizes, which in turn opens voltage-sensitive Ca^{2+} channels, letting extracellular Ca^{2+} into the cell. The rapidly rising intracellular Ca^{2+} concentration affects several signalling pathways and ultimately leads to insulin granulae exocytosis.

Beta cell insulin secretion in response to elevated blood glucose is bi-phasic. First there is an initial burst of insulin secretion for 5-10 min, then insulin secretion falls to a lower level which is maintained/slowly raised as long as stimulation continues. The beta cells release insulin in coordinated short pulses that possibly serve to maintain recipient cell receptor sensitivity. The pulsatile changes in insulin concentrations are most prominent in islets and in the liver, which is first to receive pancreatic blood via the portal vein. Also, insulin and glucagon is released in asynchrony, which may have importance for liver glucose metabolism.

In addition to glucose, amino acids and acute high concentrations of long chain fatty acids stimulate insulin secretion. There are also several other hormones that modulate beta cell insulin release. Acetylcholine from parasympathetic innervation, incretins from gastrointestinal endocrine cells, and glucagon all potentiate glucose stimulated insulin secretion. Somatostatin from islet delta cells, epinephrine from adrenal medulla, and

norepinephrine from sympathetic innervation all inhibit beta cell insulin secretion. In contrast glucagon secretion is stimulated by epinephrine, norepinehrine and hypoglycaemia, while inhibited by insulin, somatostatin and free fatty acids.

Diabetes mellitus

In diabetes mellitus (DM) insulin is absent or present in insufficient amounts. In Type 1 DM, this is caused by autoimmune destruction of the beta cells. In Type 2 DM, the etiology is more unclear, probably the cause is a defect in the beta cell function and/or decreased insulin sensitivity in peripheral tissues, leading to a situation where insulin production is not sufficient to meet the demand. In either type, the shortage of insulin causes a dysregulation of glucose metabolism in the body, and its severity depends on the degree of insulin deficiency. In general, metabolic processes normally inhibited by insulin become enhanced. The shift in insulin/glucagon ratio causes increased liver gluconeogenesis. In adipose tissue lipolysis is promoted, leading to a release of glycerol and free fatty acids. Skeletal muscle uses fatty acids for energy metabolism, instead of glucose. The increased glucose production and the decreased utilization of glucose cause the elevated blood glucose concentrations, which is the hallmark of the disease.

The pancreas - development and after birth

Pancreas development I - morphology

The embryonal pancreas starts to form from the foregut endoderm at embryonal day 9.5 (E9.5) in the mouse (for reviews, see [6, 7]). The pancreas initiates at two sites, a dorsal bud located in proximity of the aorta and a ventral bud located at the bile duct-duodenal junction and close to the vitelline veins. The buds are at this stage compact epithelial invaginations. As these form, there is an expansion of the surrounding mesenchyme. The epithelium of the buds then branch in different directions, thus forming digitations into the mesenchyme.

Endocrine cells, mostly glucagon-producing cells, single-lying or in groups, are present in the pancreatic epithelial buds from E9.5 to E13.5. Between approximately E10.5 and E13.5 the pancreatic epithelium expands and continues to branch, without any major changes in cell morphology or differentiation state.

Around E13.5, the differentiation of mature pancreatic endocrine cells starts, while the expansion of the pancreatic epithelium continues. The precursors of mature endocrine cells appear in a scattered manner in the

pancreatic epithelium. The endocrine cells form small groups protruding out from the main pancreatic epithelium into the mesenchyme.

Beginning at day E15.5, other mature components of the pancreas, ducts and acini may be seen. Due to rotation of stomach and duodenum, the two pancreatic buds become positioned next to each other and begin to fuse. As the pancreas continues to grow, the maturation of the pancreatic epithelium into ducts and acini continues, meanwhile from E18.5 the buds of endocrine cells start to enlarge and incorporate blood vessels and leave the pancreatic epithelium to form islets, although maintaining contact with small ducts.

At birth, (around 20 days), the pancreas consists of loosely packed exocrine acini and ducts, with the endocrine cells situated in islets or found in buds still connected to the ducts. The proportion of endocrine cells adjacent to ducts and acini is quite high at birth, but this decreases rapidly, as the exocrine tissue expands more extensively than the endocrine tissue after birth. Studies in rats have shown that the pancreas continues to grow as the animal increases in weight [8].

In the adult rat, the pancreas forms many lobes. The pancreas is regarded as consisting of two parts, the duodenal part, from the stomach-duodenal junction further along the duodenum, and the “spleenic” part, stretching from the stomach to the spleen. The two parts are very similar in morphology with some exceptions, e.g. the duodenal parts contain more pancreatic peptide-producing PP-cells, while the “spleenic” part is richer in glucagon-producing alpha cells [9, 10]. In the adult pancreas, the islets of Langerhans is by far the dominating site of endocrine cells, but single endocrine cells scattered in the duct epithelium can be found, as well as small buds of endocrine cells protruding from ducts. In addition, many islets of Langerhans seem to be in contact with small ducts. If this has any functional significance is however debated [11].

Pancreas development II – regulating factors

Many transcription factors and signaling molecules have been found to specify and govern the development of the pancreas and its endocrine and exocrine components. The transcription factors Ipf1/Pdx1 [12, 13] and Ptf1a [14] are both required for the formation and growth of pancreatic buds and absence of either leads to pancreatic agenesis. Later on Ipf1/Pdx1 becomes restricted to endocrine beta cells and necessary for function and identity of these cells. Ptf1a is later restricted to and expressed by acinar cells.

Signaling from the pancreatic mesenchyme is necessary for proper differentiation of the pancreatic epithelium [15]. For example, absence of FGF10 signalling from the mesenchyme causes an early arrest in pancreatic growth and branching [16]. Endocrine/exocrine cell fate decision during pancreatic differentiation involves Notch signaling [17]. At this stage, certain epithelial cells express the receptor Notch and its ligand

Delta/Jagged. Upon cell-cell contacts, in certain cells, Delta expression will prevail causing signalling to the neighboring Notch cell, which becomes activated. This causes a response that involves Hes-1 downregulation and NGN3 activation. Cells that up-regulate NGN3 become endocrine progenitors [18]. This scheme of events may have other components that influence the process. Particularly FGF10 is thought to interfere at Notch/Delta signaling step by maintaining a high rate of proliferation among these endocrine precursors. However, other factors may regulate as well.

The endocrine cell fate mechanism is not fully elucidated. Inactivation of transcription factors Pax4, Arx, and Pax6, all lead to absence of a specific endocrine cell types at birth. Pax4-/- mice lack beta cells [19], while Arx null [20] and Pax6 null mice [21] have no alpha cells. The proper growth and development of the pancreas also requires ErbB signaling, since absence of ErbB-1 results in retarded pancreatic growth and beta cell differentiation [22]. EGF or Betacellulin signaling through ErbB-1 appear to promote beta cell differentiation and neuregulin-4 through ErbB-4 promotes delta cell differentiation [23], all at the expense of alpha cell differentiation.

Endocrine pancreas growth and maintenance through life

The life cycle of pancreatic endocrine cells, in particular the beta cells, has been the subject of many studies. The beta cell mass is regarded as being determined by (1) beta cell replication rate, (2) beta cell size, (3) beta cell neogenesis from precursors and (4) beta cell death. The first three options increase the beta cell mass and the fourth reduces it. The contribution of each factor has been extensively investigated in the young rat [24, 25].

Replication rate of beta cells is high at birth but declines with increasing age. The beta cell mass is unchanged from day 2-20, while both body and pancreatic weight increase during the same time. Beta cell death rate is higher during 2-20 days than in the adult, peaking at days 13-17. Between 20 and 30 days, there is an increased growth of the pancreas and along with this, a burst of islet neogenesis. The cell death preceding this is thought to occur due to remodeling of the organ, preparing for the future growth. The pancreas continues to increase in weight as the animal grows. With increasing age the islets of Langerhans enlarge, but are still found in various sizes [26].

Beta cell turnover in the adult rodent has been estimated to be 2-3% per day [27]. This would, theoretically allow for a total replenishment of the beta cell compartment in about one month. Since the beta cell mass does not double each month, the beta cell death rate is likely to be of the same proportion.

It is debated whether beta cell neogenesis contributes to the beta cell mass in adult life. One study has suggested that self-replication of beta cells inside islets of Langerhans is the only mechanism in use during adult life [28]. However, upon duct ligation, significant contribution of beta cells through

neogenesis was observed [29]. In any case, it is apparent that beta cells continue to replicate throughout life, although at a slow rate.

Beta cell replication is responsive to numerous factors in vitro and in vivo (reviewed in [30]). The physiologically foremost significant factor appears to be hyperglycemia. However, hormones like growth hormone and prolactin, HGF, GLP1 and others have all been proposed to play a role in this context. Of particular interest is that insulin/IGF-1 may control beta cell replication and survival, since insulin will become deficient in Type 1 DM. Physiological or pathophysiological manipulation may also influence the beta cell proliferation rate, as seen after partial pancreatectomy, duct ligation and during pregnancy.

Beta cell death in type 1 diabetes

Beta cell destruction in type 1 diabetes is mediated by islet infiltrating immune cells. The cause of the autoimmune response against beta cells is largely unknown, but might be initiated by environmental factors such as beta cell toxins, nutritional components, stress, metabolic overload or viral infection [31, 32]. It is generally accepted that beta cell damage and death is accomplished through multiple ways of action: through direct contact with T-killer cells, by free radicals generated by immune cells or by pro-inflammatory cytokines activating cell surface receptors on beta cells. Of these cytokines, IL-1β, IFN-γ and TNFα, are probably directly involved in induction of beta cell death. Stimulation of isolated islets, in vitro, with a combination of IL-1β, IFN-γ leads to beta cell death through apoptosis and necrosis [32-34].

Multiple intracellular signalling pathways are activated upon stimulation with cytokines, of these many have been associated with beta cell death, but their net effects are dependent on the strength and duration of the stimuli. Inhibition of signalling through NFκB, JAK-STAT1 or the stress-associated MAPkinases JNK and p38, has all been shown to reduce cytokine-mediated cell death in insulin-producing cells [35-39]. IL-1β mediated activation of NFκB, leads to transcription of inducible nitric oxide synthase iNOS and subsequent NO-production. IFN-γ–STAT1 activation further enhance iNOS transcription [40]. High NO levels are deleterious to the beta cell, with multiple effects upon ER and mitochondria function, leading to caspase activation and subsequent apoptosis [41-44]. While this role of NO in islet cell death is true for rodent islets, human islets do not seem to exhibit the same sensitivity for NO, maybe explained by higher scavenger content and increased expression of the heat shock protein 70 [45, 46].

The unfolded protein response

The unfolded protein response (UPR) (reviewed in [47, 48]), is activated when endoplasmatic reticulum (ER) homeostasis is disturbed, “ER-stress”. This occurs for example if the protein inflow exceeds the capacity of ER chaperone molecules, causing unfolded or misfolded proteins to accumulate within the ER. ER-stress may also be induced by glucose deprivation or perturbed ER Ca^{2+} concentration, both affecting chaperone activity, and thus ER protein processing capacity. Cells, like the pancreatic beta cells, with high protein production for secretory purposes, are particularly sensitive to ER-stress.

The ER-bound heat shock protein BiP/Hsp70-5, is a major ER-chaperone and has a key function in the initiation of the UPR. During homeostatic conditions, ER BiP content exceeds the chaperone need, and some BiP proteins occupy the luminal part of three intermembrane UPR-activators, PERK, IRE-1 and ATF-6, keeping these in an inactive state. If unfolded proteins accumulate, this will deplete BiP content freeing PERK, IRE-1 and ATF-6. Activated PERK phosphorylates the α-subunit of translation initiation factor eIF2, causing an attenuation of translation, and thus a reduced inflow of proteins into the ER. Free IRE-1 dimerizes and becomes activated, and is then able to activate the XBP1 transcription factor by processing the XBP1 mRNA. ATF-6 will free of BiP, translocate to the Golgi, where it is cleaved into a 50kDa active transcription factor. Both XBP-1 and ATF-6 go into the nucleus and activate transcription of several stress genes, among these chaperones, BiP, proteins in the ER associated protein degradation machinery, to deal with the ER-overload.

If the ER-stress is prolonged and the response does not ameliorate the ER-stress, pro-apoptotic mechanisms are promoted. The transcription factor ATF-4, which is activated by PERK, and ATF-6, induce transcription of CHOP, which represses transcription of the anti-apoptotic protein Bcl-2. This causes a shift in the balance between anti-apoptotic and pro-apoptotic Bcl-2 family members, ultimately causing mitochondrial release of cytochrome c, apoptosome formation and activation of the caspase cascade. IRE-1 is also involved in pro-apoptotic pathways by interaction with TRAF2 and ASK1, causing JNK and p38 activation, which may promote transcription of Fas ligand, respectively CHOP. When secreted, Fas ligand, may activate death receptors, with subsequent activation of the caspase cascade. IRE-1 sequestering of TRAF2 also allows clustering of membrane bound procaspase 12, promoting caspase activation. Other pathways than those described here also contribute to ER-stress induced cell death.

Heat shock proteins (Hsp)

Heat shock proteins [49-51] are a group of protein families of different sizes, originally found to be induced in response to a transient heat shock. Induction of these proteins mediated an increased cellular tolerance against a subsequent more severe heat shock. Among the heat shock proteins, the 70kDa heat shock protein family is of cardinal importance, and highly conserved between different organisms. Hsp70s are chaperones, proteins that are able to bind unfolded or partly unfolded proteins and put them back into shape, thus restoring protein function and avoiding protein aggregation and degradation. This action is ATP-dependent, where ATP-binding and hydrolyses drives Hsp70 substrate binding and release. Besides being induced by various stress stimuli, Hsp70 chaperones perform housekeeping duties e. g. preventing unintentional protein aggregation, dissociation of protein complexes, refolding of unfolded proteins and support protein shape during translocation between different cellular compartments.

There are several Hsp70 isoforms, where Hsp70-1 is the stress inducible variant. BiP/Hsp70-5, and Grp75/Hsp70-9 are constitutively expressed and reside in the ER and mitochondria respectively. Hsc70 is a non-stress inducible, constitutively expressed variant of Hsp70. Hsp70 induction is mediated by heat shock transcription factors (HSFs), which are constitutively expressed but kept latent during normal conditions. Upon stress stimuli these are activated, to concentrate in the nucleus inducing Hsp70 transcription. Induction of Hsp70 has a protective effect against cell death, and Hsp70 upregulation is common in malign tumour cells, and correlates with poor prognosis and resistance to anti-cancer drug therapy. One part of the survival effect of Hsp70 during various stress stimuli, is probably by Hsp70 mediated stabilization of lysosomal membranes, preventing escape of lysosomal hydrolases into the cytosol.

The RIP-Tag2 mouse – a mouse model of pancreatic islet carcinogenesis

The RIP-Tag2 transgenic mouse was generated by Douglas Hanahan in 1985, by expressing a DNA fusion construct consisting of rat insulin promotor with the large T region from the polyoma virus SV40 [52]. The RIP-Tag2 mice were found to die at around 10-12 weeks of age, and autopsy revealed that the pancreas contained multiple red nodules. Examination of the nodules showed that they were solid adenomas, consisting almost entirely of insulin-producing cells, thus making hypoglycaemia due to hyperinsulinemia a likely cause of death. By supplying RIP-Tag2 mice with sugar in their drinking water, life could be prolonged by several weeks. The adenomas only rarely become invasive or seeded off metastases, and

developed in a time-predictable manner. This last characteristic, together with the relatively rapid appearance of tumours has made the RIP-Tag2 mouse a useful model for studying tumour development.

The large T-antigen, the protein that promotes tumour development in the RIP-Tag2 mouse, is a multi-functional protein [53]. Its viral function is to serve as replication initiating factor for viral replication, and in addition it interacts with host cell proteins to promote S-phase cell cycle entry, which is necessary for replication of the virus genome. For example, large T-antigen binds to pRB, causing release of E2F transcription factors, which stimulates the cell to enter into S-phase. It also sequesters the p53 protein, inhibiting p53's function in cell cycle and DNA damage control.

Expression of large T-antigen in the RIP-Tag2 mouse follows that of the insulin gene, starting during the later half of mouse embryonal development [54], and is then expressed in all beta cells throughout life [52].

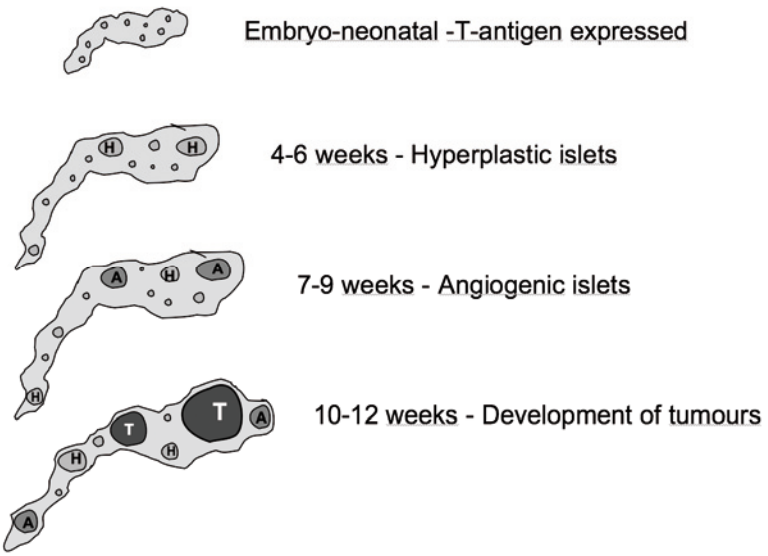

Figure 1. Pancreatic tumour development in the RIP-Tag2 mouse, adapted from Hager&Hanahan 1999 [55]

The early start point of expression makes the large T-antigen to be regarded by the immune system, as a self-antigen, and as such it does not provoke an immune response. Despite the early start point of T-antigen expression, islet cells appear normal until 4-5 weeks of age, when some islets start to show signs of hyperplasia/dysplasia. As time proceeds, more and more islets become hyperplastic, and by 7-9 weeks a subset of these hyperplastic islets exhibit pathological angiogenesis with dilated, tortuous blood vessels and often microhaemorrhages. Some of these angiogenic islets will then have developed into tumours by 10-12 weeks. At the end of 12 weeks, it has been

estimated that 50-70% of all islets are hyperplastic, about 10% are angiogenic and 2-4% have developed into tumours [55].

Proliferation and apoptosis during tumour development in the RIP-Tag2 model have been thoroughly investigated [56]. In normal islets, proliferation and apoptotic rates are very low, and T-antigen-expressing but non-hyperplastic beta cells in the RIP-Tag2 mouse exhibit comparable rates. As islets become hyperplastic the proliferation rate increases to about 10% of the islet cell population. Angiogenic islets then exhibit 2-fold higher proliferation, however, the proliferation in tumours is then not further elevated, but is comparable with the level of proliferation in angiogenic islets. Apoptosis on the other hand follows a different pattern. The apoptotic rate in hyperplastic islets is about 1%, in angiogenic islets 2-3%, but then apoptotic rates are lower in tumours, and decline with increasing tumour size.

Insulin-like growth factor II (IGF-II) has been shown to be induced in hyperplastic islets and expression correlates with the mitotic index, suggesting a role in inducing cell proliferation. Rip-Tag2-IGF-II null mice exhibit a very reduced tumour burden. Interestingly, cell proliferation rates were unchanged, while apoptosis in tumours was increased 5-fold [55, 57], indicating that IGF-II is a survival factor for tumour cells. The anti-apoptotic *bcl2*-family member Bcl_{XL} has been found to be upregulated in RIP-Tag2 tumours. RIP7- Bcl_{XL}-RIP-Tag2 mice exhibit an elevated tumour incidence, but unchanged incidence of angiogenic islets, despite decreased apoptosis in both hyperplastic and angiogenic islets [56]. Down-regulation of apoptosis thus marks tumour expansion from angiogenic islets.

Another factor enabling tumour expansion is the induction of angiogenesis. In normal mouse islets of Langerhans, the endothelial cells plays an essential role by providing beta cells with basal lamina, which in addition to physical support, also contains components that sustain insulin gene transcription and beta cell replication [58, 59]. Thus, in the RIP-Tag model, angiogenesis is likely essential in the role of providing tumour cells with stroma, in addition to nutrients and oxygen. Angiogenesis inhibitors given to RIP-Tag2 mice with developed tumours were able to reduce the tumour burden. In treated tumours, apoptotic endothelial cells could be observed, but also apoptotic tumour cells, situated in proximity to blood vessels, supporting a hypoxia/nutrient-independent tumour cell death [60]. Several studies have shown that angiogenesis inhibitor-caused restriction of tumour growth, is due to increased tumour cell apoptosis, rather than reduced tumour cell proliferation.

The induction of pathological angiogenesis, or “angiogenic switch”, in the RIP-Tag2 model, has been found to be highly dependent on VEGF-signalling. RIP-Tag2 mice with beta-cell specific ablation of VEGF-A, display only scarce angiogenic islets [61], likewise treatment between 5-10 weeks of age with VEGFR2 inhibitor SU5416, strongly reduced of the number of angiogenic islets [62]. VEGF-A is highly expressed in normal

islets and also throughout tumourigenesis [61, 63], The matrix metalloproteinase-9 has been shown to be a inducer of the angiogenetic switch, by liberating VEGF that is sequestered in the extracellular matrix. Inhibition of MMP-9 by molecular inhibitor or genetic ablation reduces the number of angiogenic islets [62]. Invading neutrophils and rim-associated macrophages have been shown to be a source of MMP-9, thus playing a role in the angiogenic switch mechanism [64]. The secreted factor Bv8, has been suggested to be involved in mobilization of myeloid cells to hyperplastic/angiogenic islets [65].

Inhibition of angiogenesis may induce changes in tumour behaviour, resulting in less responsiveness to the treatment. Late stage tumours treated with anti-VEGFR2 antibody upregulated FGF-signalling to sustain angiogenesis [66].

Table 1. Criteria for histological classification of RIP-Tag2 tumours, adapted from Lopez&Hanahan 2002 [67].

Stage	Lesion size Ø	Characteristics
"Normal" T-antigen positive islet	<0.20 mm	Morphology as normal islets
Hyperplastic islets	0.2-0.5 mm	Increased nuclear density/disorganized islet structure
Angiogenic islets	<1.0 mm	Dilated, abnormal vessels, micro-haemorrhage often present
Tumour	1-10 mm	Dilated, abnormal vessels, well defined border
Invasive carcinoma	1-10 mm	As previous with regions of tumour cells invading surrounding tissue.

Summaries of the previous studies of Frk and Shb

The non-receptor tyrosine kinase Frk

Frk is a Src-like kinase that is expressed in various cell types such as mammary gland, intestine, pancreas, liver, lung and kidney. Expression is however, lacking in tissues such as heart, brain or spleen. It shares with other Src-family members a similar structure, consisting of an N-terminal unique domain, an SH3- and an SH2-domain, a kinase domain and a short C-terminal regulatory tail. The corresponding rodent homologues of the human Frk were originally named rat GTK (Gastrointestinal tyrosine kinase) or mouse BSK/IYK (Beta-cell Src-like kinase or Intestinal tyrosine kinase). Tyrosines 394, 497 and 504 have all shown to exert regulatory roles where 504 corresponds to the C-terminal regulatory tyrosine 527 in Src and 394 to the kinase activation loop tyrosine 416 in Src. Rodent Frk may have partial myristoylation signal and a putative bipartite nuclear localization signal. Human Frk has been shown to bind by its SH3-domain to pRB [68]. Mouse Frk binds to and phosphorylates Shb [69], an adapter protein that is involved in signalling downstream of several tyrosine kinase receptors, e.g. FGFR1, VEGFR2, PDGFR. (Shb will be described in detail in the next section). Rat Frk has been reported to be downstream of HGF/c-Met signalling [70]. Frk expression experiments in cell lines and transgenic mice have shown changes in cell growth [68, 71, 72] and cell survival [72-74]. Frk expression in neural PC12 cells induced cell differentiation, seen as neurite outgrowth through Shb-FAK-CRKII mediated Rap1 activation [72]. Together, this implies a role for Frk in various important cellular processes.

Antiproliferative effects of Frk

Some studies indicate a role of Frk as a tumour suppressor gene. It has for instance been shown that overexpression of murine or human Frk in NIH3T3 fibroblasts, RINm5F insulinoma and MCF4 or BT474 breast carcinoma cells, significantly reduces cell growth. A possible mechanism for this may be envisaged through the association with the retinoblastoma tumour susceptibility gene product pRB [68]. Overexpression of wild-type Frk showed binding of Frk to pRB during G1 and S phases of the cell cycle. Binding of Frk to pRB did not inhibit pRB phosphorylation, but bound Frk retained its kinase properties. The reduced proliferation effect has been shown to be dependent on Frk's kinase domain [75]. The subcellular localization of Frk may also play a role in this context. Interestingly, Frk overexpression, leads to elevated levels of other cell cycle inhibitory proteins. For example, levels of RB2/p130 are increased in FrkY497/504F expressing NIH3T3 cells and elevated levels of the cell cycle inhibitor p27Kip1, have also been observed in FrkY504F and FrkY497/504F expressing RINm5F cells [76]. Frk has also been shown to induce G1 arrest

in a tumour cell line that lacks pRB [75], suggesting that Frk pRB interaction may not be necessary for Frk's growth reducing properties.

Mice with genetically altered Frk expression, and effects upon pancreatic beta cells.

The findings that insulin-producing RINm5F cells expressing FrkY504F cells exhibited increased cell death upon treatment with cytotoxic cytokines [76], and Frk overexpression in PC12 cells induced cell differentiation [72], did motivate the generation of a beta cell specific Frk transgenic mouse, to address the role of Frk in beta cell survival and differentiation [73]. The RIP-FrkY504F mouse displayed a slightly larger pancreas/bodyweight, with 1.6 fold increase in the relative beta cell area, without signs of beta cell hypertrophy. Following a 60% partial pancreatectomy, RIP-FrkY504F mice displayed increased beta cell proliferation, 2.3 fold vs. pancreatectomy treated wild-type mice [77]. RIP-FrkY504F mice performed similar to wild-type upon intravenous blood glucose tolerance test, but isolated RIP-FrkY504F islets displayed a relatively higher glucose stimulated insulin release. Insulin content of transgenic islets did not differ from wild-type islets [73]. Given a sub-diabetogenic dose of beta cell toxin streptozotocin, RIP-FrkY504F mice displayed a more severely impaired glucose tolerance when challenged with an intraperitoneal glucose tolerance test [77]. Isolated transgenic islets exhibited increased islets cell death upon stimulation with cytotoxic cytokines, while cell death during basal condition was unaltered. Male RIP-FrkY504F mice, maintained in single mouse cages developed glucose intolerance at 4-6 months of age, an effect that correlated with reduced islet blood flow [78]. Interestingly, in humans, a single nucleotide polymorphism in Frk has been associated with overweight/obesity [79], which is a known risk factor for diabetes.

The Frk knockout mouse was generated by Chandrasekharan et al [80] to test the role of Frk as a tumour suppressor gene in epithelial tissues. However Frk-/- mice show no signs of increased tumour incidence. Frk-/- mice are viable, born at Mendelian frequency and display no major phenotype. A moderate up-regulation of the c-Src content in the intestine and decreased levels of circulating thyroid hormone T3 has been reported [80]. In addition, in vitro islet glucose stimulated insulin release and islet insulin content was similar to that of wild-type islets [74]. Isolated Frk-/- islets were found to display decreased cell death upon exposure to cytotoxic cytokines, and siRNA mediated Frk knockdown of wild-type islet yielded similar results [74].

Taken together, the results from RIP-FrkY504F and Frk-/- argues for an involvement of Frk in cytokine induced beta cell death since Frk overexpression elevated beta cell death upon cytokine exposure while absence of Frk had an protective effect.

The adapter protein Shb

Adapter proteins are proteins that participate in cellular signalling, but lack intrinsic enzymatic or signalling capacity. Adapter proteins instead act as linkers between other signalling proteins, and participate in building short-lived signalling protein complexes following for example the activation of a receptor tyrosine kinase. By controlling the pattern of recruitment of downstream signalling proteins upon receptor kinase activation, adapter proteins may influence the cellular response to an extracellular signal. Adapter proteins contain protein-interaction domains that recognize and interact with specific amino-acid sequences in other proteins, and these domains and their specificity largely decides the binding partners of an adapter protein.

Shb is an adapter protein, consisting of a N-terminal proline-rich domain, a middle PTB-domain, putative phosphotyrosine residues and a C-terminal SH2-domain. The proline rich domain enables constitutive interaction with Src homology 3 domain containing proteins, while the PTB and the SH2-domains mediate activation-dependent binding to phosphorylated tyrosine residue in other proteins, in an amino acid sequence specific manner. The tyrosine phosphorylation sites in Shb may allow other PTB or SH2-containing proteins to interact with Shb. Shb was identified in 1994 [81] as a result of a screening for serum inducible genes in an insulin producing cell line, however Shb was found to be ubiquitously expressed. Shb expression may also be influenced by growth factors [82]. Shb has been shown to be a versatile component in different intracellular signalling pathways leading to cellular responses such as proliferation, differentiation, cell motility or cell death, depending on the context (this will be further addressed in detail below). Many proteins have been identified that interact with Shb [82]. Src, Eps8, PLC-γ, Grb2, p85PI3kinase, JAK1/3 and c-Abl [83] have been shown to interact with Shb's N-terminal domain, while LAT, VAV and FAK associates with the PTB domain. Shb's SH2-domain binds activated tyrosine kinase receptors like PDGFR, FGFR-1 and VEGFR-2 [84] and also the T-cell receptor and the IL-2 receptor. To this date Shb has been the subject in about 35 published scientific articles, including two reviews.

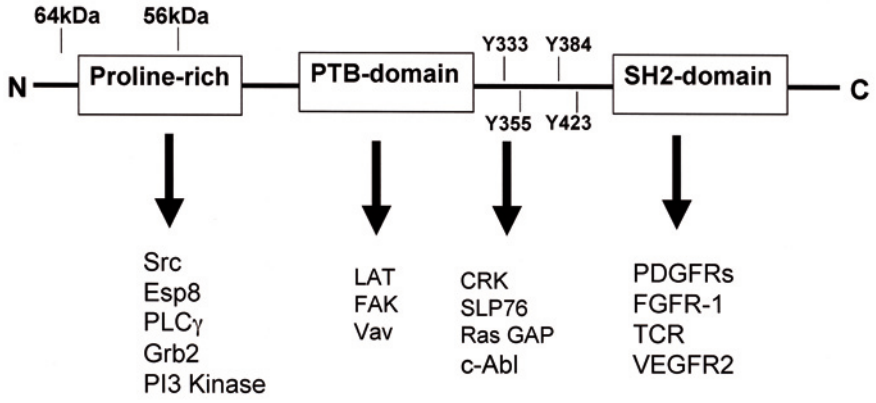

Figure 2. The adaptor protein Shb

Shb and cell death

The first indication of Shb being involved in signalling leading to cell death, came in 1996 when it was found that NIH3T3 fibroblasts overexpressing Shb, exhibited increased apoptosis when cultured in 1% serum [85]. Next, a study investigating the effect on endothelial cells of the endogenous angiogenesis inhibitor angiostatin, showed that transfection with Shb cDNA strongly enhanced the apoptotic response to angiostatin in primary bovine adrenal cortex capillary endothelial cells, while Shb alone exerted a more modest increase in apoptosis [86]. Following this, isolated islets of Langerhans from beta cell specific Shb transgenic mice (RIP-Shb), showed elevated apoptosis after culture in 1% serum, and this was further enhanced by presence of cytotoxic cytokines [87]. Similar results were seen with insulin producing RINm5F cells overexpressing Shb, in low serum culture and nicotinamide addition [87]. Moreover, RIP-Shb mice given the beta cell toxin streptozotocin, exhibited more pronounced beta cell damage, than islets from streptozotocin treated wild-type mice [77]. Following studies showed that Shb transfected porcine aortic endothelial cells exhibited increased apoptosis [88] and that Shb overexpressing immortalized brain endothelial cells had increased apoptotic rate when cultured in low serum [89]. The mechanisms behind this effect of Shb overexpression remain largely unknown. However, one study has showed that Shb may interact with and activate c-Abl [83]. The c-Abl kinase may induce cell death in response to DNA-damage, oxidative stress or a failed unfolded protein response (ER-stress). Shb knockdown in betaTC6 cells led to reduced c-Abl activation, and reduced cell death upon exposure to the genotoxic agent cisplatin or the ER-stress inducing agent tunicamycin. Another study [90] has strengthened the argument for c-Abl as a possible mediator of Shb

overexpression induced cell death. PC3 prostate cancer cells overexpressing Shb exhibited an elevated apoptotic response to 2-methoxyestradiol (2-ME), together with a tendency to increased apoptosis in untreated cells. This was accompanied by increased basal c-Abl activity and a stronger c-Abl phosphorylation upon 2-ME exposure in the Shb overexpressing cells.

Shb in endothelial cells

First, Shb's role in endothelial cells was coupled to the anti-angiogenic response to endogenous angiogenesis inhibitors, since Shb overexpression enhanced angiostatin induced endothelial cell apoptosis [86] and endostatin-mediated inhibition of endothelial cell tube formation in vitro [88]. Then, experiments linked Shb to regulation of endothelial cell cytoskeleton in the context of endothelial cell spreading and tube formation, in response to angiogenic growth factor stimulation. Shb was found to bind to and relay signals from activated FGFR-1 [91] and VEGFR-2 [84] in endothelial cells. Upon FGF-2 stimulation of FGFR-1, Shb mediated proliferative signals through Shp2-FRS2-Grb2-Sos-Ras-Raf activation of MAPK, and cytoskeletal rearrangements in concert with Src and FAK in murine immortalized brain endothelial cells [89, 91, 92]. FGF-2 induced tube formation, was reduced in cells transfected with a mutant Shb, lacking a functional SH2-domain [89]. Similarly, following VEGF-A mediated VEGFR-2 activation, Shb stimulated PI3kinase activity, and through interactions with Src and FAK, promoted formation of focal adhesions and cell migration in porcine aortic endothelial cells. Shb knockdown led to decreased VEGF induced: FAK activation, focal adhesion formation and cell migration [84]. Also, SVR angiosarcoma cells, transfected with short hairpin RNA targeting Shb, showed reduced FAK activity and changes in cell morphology, with an increased proportion elongated cells. There was no effect upon cell death during basal conditions but Shb knockdown increased apoptosis induced by the unspecific kinase inhibitor staurosporine and DNA-damaging agent cisplatin.[93].

Shb and insulin producing cells

The role of Shb in insulin-producing cells was first addressed in 1999 by engineering of a transgenic mouse, expressing Shb under the control of the rat insulin promotor 1. The RIP-Shb mouse was found to exhibit an increased beta cell mass and an accelerated return to normoglycemia following glucose challenge. Isolated transgenic islets also displayed an increased glucose-stimulated insulin secretion without showing increased insulin content [87]. Further analysis of transgenic islets and Shb overexpressing RINm5F cells showed that Shb interacts with insulin receptor substrates 1 and 2 following insulin stimulation, and this promotes FAK-association and PI3Kinase and ERK activation, thereby possibly leading to pro-proliferative and anti-apoptotic signalling [69, 94]. In line

with this, RIP-Shb mice exhibited increased β-cell replication following partial pancreatectomy [77]. However, as mentioned previously, Shb overexpression also made insulin-producing cells more sensitive to stressful conditions and cytotoxic compounds [77, 87].

Shb and PC12 cell differentiation

The rat pheocromocytoma cell line PC12 [95] may be maintained undifferentiated and in proliferation, but addition of growth factors like NGF, FGF, or the neurotransmitter PACAP, results in stopped proliferation and neurite outgrowth [96]. PC12 cells have become a useful model for studying signalling leading to neuronal differentiation. Testing the role of Shb in PC12 differentiation was motivated by the fact that many of the growth factors receptors affecting PC12 differentiation are receptor tyrosine kinases. Shb was found to be expressed in PC12 cells, and stimulation with NGF, FGF or EGF all induced elevated Shb protein levels to different degrees. Shb overexpressing PC12 cells exhibited enhanced neurite outgrowth upon stimuli with NGF or FGF or EGF, however PC12 cells expressing the SH2-domain inactive mutant ShbR522K, displayed less NGF-induced neurite outgrowth, compared to mock-transfected PC12 cells [97]. Subsequent studies [72, 98] revealed that Shb-mediated PC12 neurite outgrowth was Rap1-dependent, since inhibition of Rap1 activity, by transfection with a Rap1 binding inhibitory peptide or Rap1-GAP, blocked the Shb-enhanced neurite outgrowth. Tyrosine phosphorylated Shb mediated Rap1 activation by binding to CRKII, leading to CRKII-recruitment of C3G, a guanine-exchange factor for Rap1, resulting in Rap1 activation.

Shb and differentiation of mouse embryonic stem cells

Embryonic stem cells (ES-cells) comprise a source of potentially renewable cells and tissues for transplantation therapy. ES-cell differentiation into embryoid bodies (EBs) recapitulates fairly well events of early embryogenesis, but with an obvious lack of organization and development of an embryo. Nevertheless, ES-cells have been used for studying signalling events promoting differentiation of e.g. vasculogenesis, haematopoiesis, cardiac myocytes, neural cells.

The role of Shb in ES-cell differentiation has been the subject of five studies, utilizing ES-cells overexpressing Shb (wtShb), ES-cells overexpressing Shb with mutant functionally inactivated SH2-domain (ShbR522K) or ES-cells with Shb gene knock-out (Shb-/-).

The first study [99] addressed Shb's role in ES-cell differentiation in general. Microarray analysis showed that wtShb ES-cells exhibited changes in expression in 16 of the genes examined, whereas ShbR522K ES-cells exhibited 128 genes with changed expression, with the majority decreased. Gene expression of markers for tissues derived from ectoderm, mesoderm and endoderm was assessed in EBs differentiated for three weeks in

suspension culture. Gene expression in wtShb EBs did not differ from control, but ShbR522K EBs exhibited decreased gene expression of endoderm derived pancreas associated markers. In addition, ShbR522K EBs showed impaired cavitation, an early differentiation step that resembles the process of gastrulation in the embryo. This was caused by reduced expression of basal lamina proteins laminin-1 and collagen-IV in ShbR522K EBs. Expression of these proteins at this stage of EB-differentiation has been coupled to FGF-signalling [100].

The second study [101] investigated possible involvement of Shb in vasculogenesis and angiogenesis in EBs grown in attached cultures. It was found that wtShb EBs had more vascular structures, whereas ShbR522K EBs failed to produce blood vessels. Further analysis suggested that wtShb promotes expansion of endothelial/haematopoietic precursor cells; endothelial cells and enhance vessel formation. ShbR522K did not negatively affect the number of endothelial cells but impaired vessel formation. Gene expression of haematopoietic markers Tal1 and CD41 was increased in wtShb EBs, suggesting that Shb overexpression also promotes early haematopoietic differentiation.

Signalling from blood vessels plays a prominent role in initiation of pancreatic bud formation in the foregut endoderm [102] and later for beta cell development [59] and functionality [58]. Since Shb in the previous study promoted endothelial cell differentiation and blood vessel formation, and the RIP-Shb mouse exhibits increased beta cell area at birth, the third study aimed to investigate whether Shb overexpression could positively influence differentiation of insulin-producing cells from ES-cells in vitro [103]. Shb overexpressing ES-cells differentiated in attached culture for 21 days exhibited a 6-fold increase in the number of Pdx1-positive cells, and elevated Pdx1 and insulin gene expression. Pdx1-positive cells were found close to vascular structures in the embryoid bodies. Stimulation with VEGF increased Pdx1-gene expression in control EBs but could not further increase Pdx1-gene expression in wtShb EBs

The fourth study [104], addressed mesoderm differentiation in Shb-/- EBs, since Shb overexpression promoted vessel formation and haematopoietic gene expression. The vasculature and the haematopoietic cells of the early embryo are of mesoderm cell origin, and share also a common precursor, the hemangioblast. Shb-/- EBs exhibited a delayed down regulation of early mesoderm marker Brachyury, and reduced expression at early time points of haematopoietic markers Tal1, CD41 and other mesoderm-associated markers cardiac actin and VEGFR2. Vasculogenesis and angiogenesis was not different in Shb-/- EBs compared to control EBs, but when cultured in presence of VEGF, the VEGF-stimulatory effect upon vessel growth and formation was reduced in Shb-/- EBs. CD41 expression was found to be upregulated at day 12 in Shb-/- EBs, but this was probably

due to a delayed differentiation as Shb-/- EBs gave rise to fewer blood cell colonies in a haematopoietic cell colony assay.

The fifth study [105] addressed Shb and endoderm differentiation. While ectoderm and mesoderm differentiation are common in EBs, endoderm differentiation is more rare and also difficult to distinguish from visceral endoderm (VE). VE is an extraembryonal epithelium that expresses many proteins that are associated with definitive endoderm derived tissues of the embryo proper. It was found that 5 days old ShbR522K EBs displayed elevated gene expression and protein staining of the definitive endoderm marker Cxcr4 [106]. After 24 days of differentiation in attached culture, ShbR522K also displayed elevated FGFR1 and FGF2 expression. FGF-signalling plays an important role in patterning the foregut endoderm, affecting cell fate [107, 108]. Activin A on the other hand has been shown to promote endoderm differentiation in EBs [109]. ShbR522K EBs cultured with Activin A, exhibited after 24 days of differentiation, elevated expression of genes associated with fetal liver: HNF1a, HNF4a, HNF6, alpha-fetoprotein, albumin. Staining for HNF1a and albumin, confirmed increased amounts of positive cells in ShbR522K EBs. Addition of an FGFR1-inhibitor (SU5402) during culture abolished HNF1a and alpha-fetoprotein gene expression. In summary, ShbR522K and Activin A promoted endoderm differentiation towards a liver cell identity, in an FGFR-signalling dependent manner.

Frk and Shb interactions

Overexpression of Shb or Frk, in PC12 cells and in insulin-producing cells yielded similar results. Therefore it was not unexpected that Shb was found to be phosphorylated in FrkY504F-overexpressing PC12-cells [72], and that FrkY504F RINm5F-cells also exhibited increased Shb phosphorylation following insulin stimulation or in 10% serum [69]. In both models, increased Shb-FAK association accompanied the increase of Shb phosphorylation. When co-transfected in COS-7 cells, Shb and Frk showed strong association. Co-transfection also resulted in strong phosphorylation of Shb, whereas this was not seen when Shb was transfected alone, showing that Shb is a substrate for Frk [69]. One may speculate that Frk when overexpressed in these models exerts a role in Shb signalling, similar to the role of Src in Shb signalling in endothelial cells**.**

The Shb knockout mouse

The Shb-/- mouse was generated “in group” by Vitek Kriz et al [110]. The Shb-/- mouse is viable on a mixed genetic background (FVB/C56Bl/6/129sv) and shows no major abnormalities. However the transmission rate of the Shb-allele is distorted, due to an increased malformation rate and intrauterine death of Shb-/- embryos, and to preferential ovulation of Shb null allele oocytes. The Shb-allele has been bred onto C57Bl/6 background, but no

Shb-/- offspring has been born, the cause for this remains to be elucidated. The Shb-/- mouse has also been shown to display vascular abnormalities, with abnormal endothelial cell morphology in capillaries, in liver and heart muscle. Basal vascular permeability is increased in many organs in the Shb-/- mouse, while VEGF-induced vascular permeability is decreased. A matrigel plug assay revealed impaired in vivo angiogenesis in the Shb-/- mouse. In addition, tumour cells implanted into Shb+/- mice, exhibited perturbed growth, which correlated with decreased angiogenesis [111].

The name Shb

The name Shb has been consistently used for the gene and protein. There have not been any alternative abbreviations for mouse or human Shb. While the abbreviation Shb has been constant, the description of the abbreviation has evolved during the years passed. In the first study published the abbreviation Shb was explained by "The deduced protein was called Shb since the cDNA was initially identified in the betaTC-1 cell line and found to contain a Src homology 2 (SH2) domain close to its C-terminus" [81]. In a review 2003, Shb was described as "an abbreviation of SH2-protein of Beta-cells" [82]. Presently 2008, there exist two different descriptions of the abbreviation Shb. The human Shb gene and product is described as "Src homology 2 domain containing adaptor protein B" (NCBI GeneID: 6461), while the mouse homologue is called "Src homology 2 domain-containing transforming protein B" (NCBI GeneID: 230126). This is of use to know when searching gene array or proteonomic databases for information about Shb.

Aims

In this thesis gene knockout mice were used to shed light upon the functional significance of the non-receptor tyrosine kinase Frk and the adapter protein Shb. The specific aims of this thesis were to:

- Investigate if the non-receptor tyrosine kinase Frk has a role in regulating the pancreatic endocrine beta cell number. (Paper I)

- Assess whether absence of the adaptor protein Shb elicits a protective effect against cytokine induced cell death, and if so, to elucidate the underlying mechanism(s). (Paper II)

- Address blood glucose homeostasis and beta cell function in Shb deficient mice. (Paper III)

- Study tumour development on Shb null background in an inheritable tumour model. (Paper IV)

Methods

Animals

The Frk-/- mouse, generated by Chandrasekharan et al [80], was originally of C57Bl/6J/129Sv/Ev mix. For the experiments in paper I, mice had been bred for 4–10 generations with C57Bl/Ks mice. Wild-type or Frk+/- littermates were used as controls.

The Shb-/- mouse was generated "in group" by Vitek Kriz et al [110]. The Shb – allele was maintained on a mix of FVB/N, C57Bl/6, and 129/SvJ strains by breeding Shb +/- pairs. For experiments in paper II and III, Shb +/+ (wild-type) and Shb -/- breeding pairs were set up from littermates and their offspring were then used for experimentation.

RIP-Tag2 [52, 112] mice on C57Bl/6 background, were kindly provided to us by Dr Kristian Pietras (LICR, Karolinska Institutet Stockholm, Sweden). To obtain RIP-Tag2 mice lacking the Shb gene, two Male RIP-Tag2 siblings were first mated with four Shb-/- females. From their litters, Shb+/-RT2 positive mice were selected and mated with Shb+/- mice of the same generation, forming seven breeding pairs/triplets. Their offspring was used for experiments in paper IV. RIP-Tag2 bearing mice were supplied with sucrose 5-10% in their drinking water, beginning at 10 weeks of age, to counteract symptoms of hypoglycemia caused by the developing insulinomas.

All animal experimentation was approved by the local animal ethics committee at Uppsala University

Genotyping of mice

Mice were earmarked at 3 weeks of age, and the piece of ear was used for genotyping (paper I-IV). DNA was extracted by DirectPCR Lysis Reagent (Ear) (#401-E, Viogen Biotech Inc, Los Angeles, CA, USA). PCR was performed with TaKaRa LA Taq (#RR002A, Takara Shuzo CO. LTD, Japan).

Blood glucose tolerance test

Blood glucose tolerance test (paper I, III) was initiated between 08.15-08.45 AM. Mice had before and during the test free access to food. Mice subjected to testing were given a single dose of 250µl 30% w/v D-glucose intravenously. Blood was withdrawn from the tail, prior to injection and then at 10, 30, 60 and 120min after injection. Blood glucose was measured with Freestyle mini system (Abbot, TheraSense Inc, Alameda, CA, USA).

Insulin sensitivity test

Mice were given an intraperitoneal (ip) injection with insulin analogue NovoRapid 1.6U/kg body weight (Novo Nordisk, Bagsværd, Denmark) (paper III). Blood glucose was determined on blood samples from the tail, before injection and 15, 30, 45, 60, 75, 90, 120 and 180min later using the Freestyle mini system. The animals had free access to food before the insulin injection, and were transferred to new cages without food. Following the 120min measurement, food was resupplied.

Blood sample collecting

(Paper III) To determinate fasting blood glucose concentration, blood was withdrawn from the tip of the tail, following an overnight fast and analysed using the Freestyle mini system. For serum insulin analysis, randomly fed mice were bled retroorbitally by an experienced lab technician. Approximately 0.5ml blood was withdrawn and allowed to clot in room temperature. Subsequently, samples were centrifuged, and serum was collected and frozen in -20°C.

Perfusion of pancreas

(Paper III) Mice were anaesthetized with an intraperitoneal injection of Avertin, 0.02ml/g body weight. (a 2.5% (vol/vol) saline solution, of 10g of 97% 2,2,2-tribromoethanol (Sigma-Aldrich) in 10 ml of 2-methyl-2-butanol (Kemila, Stockholm, Sweden).) Pancreas and duodenum were isolated as described in [113] and subsequently perfused with Krebs-Ringer-bicarbonate-Hepes Buffert (KRBH) supplemented with 2% Dextran 70 (Amersham Biosciences AB, Uppsala, Sweden) and 2% bovine serum albumin fraction V (MP Biomedicals, Eschwege, Germany). Perfusion media and time intervals were in the following order: 2.8mM D-glucose in KRBH for 15min, 16.7mM D-glucose in KRBH for 20min, 2.8mM

D-glucose in KRBH for 15min, 10mM arginine & 5mM D-glucose for 10min, 2.8mM D-glucose in KRBH for 15min. Perfusion was performed with a constant rate of 1ml/min, with perfusion media kept at 37°C and continuously gassed with O_2/CO_2 (95:5). Perfusion medium was collected and stored at -20°C prior to analysis of insulin content.

Blood flow analyses

(Paper III) This method is described in [114], but in brief, the animals were anesthetized with a single ip injection of Avertin, 0.02ml/g body weight, and placed supine on a 37°C operation table. Polyethylene catheters were inserted into the ascending aorta through the right carotid artery and into the right femoral artery. A pressure tranducer (BP Amp, PowerLab/8SP, Chart v4.0 software, all from AD Instruments) was coupled to the catheter in the aorta, allowing continuous monitoring of mean arterial pressure. After 5 min of stable blood pressure, 1.5-2x105 10µm Ø black microspheres (E-Z TRAC, cat#409-010, Interactive Medical Technologies Ltd, CA, USA) in 0.15-0.17ml saline, were injected through the carotid catheter into the aorta. A free flow arterial blood sample was collected from the femoral artery catheter, starting 5s prior to the microsphere injection and continuing for 60s. This reference sample was weighed to determine exact withdrawal rate (ml/min) for each experiment. The animals were killed by cervical dislocation. The pancreas and both adrenal glands were removed, weighed, and squashed between microscopy slides, and frozen in -20°C [16]. Pieces of the colon, duodenum, kidney were treated in the same manner. The microspheres in the reference blood sample and in each organ/tissue were then determined by counting in a stereomicroscope. The blood flow values were calculated as $Q_{org} = Q_{ref} \times N_{org}/N_{ref}$, where Q_{ref} is organ blood flow (ml/min), Q_{ref} is withdrawal rate of reference blood sample (ml/min), N_{org} is the number of spheres present in the organ/tissue and N_{ref} is the number of microspheres present in the reference blood sample. Islet blood flow% is the number of spheres in islets divided by number of spheres in the pancreas.

Measurement of vascular permeability and vascular perfusion in tumours

(Paper IV) To address tumour vascular permeability, mice were given an intravenous injection with FITC-conjugated dextran. (100mg/kg body weight, Fluka Analytical, fluorescein isothiocyanate dextran 70.000 conjugate, #46945 Sigma-Aldrich). After 2 hours animals were sacrificed, pancreata were collected and frozen in liquid nitrogen. Photos of frozen

sections were taken with a fluorescence microscope, 200x magnification, and the number of vascular leakage/field was counted on multiple fields.

To measure tumour vascular perfusion, mice were injected intravenously with 50µg fluorescein tomato lectin (#FL-1171, Vector Laboratories Inc, Burlingame CA, USA), and sacrificed after 30min. Pancreata were collected and treated as described above. Frozen sections were stained for endothelial cell marker CD31, and the fraction FITC-positive CD31 fluorescence was determined on multiple fields.

Islet isolation

(Paper II, III) Animals were killed and pancreata were removed and put into Hanks solution (#991750, SVA, Uppsala, Sweden). Pancreata were cut into pieces with scissors and subjected to collagenase (3mg/3ml Hanks/pancreas, Collagenase A, 10-154 121, Roche) digestion for 20min 37°C, on shaking waterbath, then washed twice with wash buffer (Ringer-acetate, Fresenius-Kabi, Uppsala, Sweden, supplemented with 4.5mM $NaHCO_3$, 2.5mM glucose and penicillin-streptomycin), and suspended in Hanks. Islets were handpicked under a stereo microscope and kept in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine and penicillin-streptomycin.

Islet insulin release

(Paper II) For insulin release, doublets of 10 islets, either untreated or stimulated with 50 U/ml IL-1β + 1000 U/ml IFN-γ for 18 h, were transferred to Krebs-Ringer-bicarbonate-Hepes Buffert (KRBH) supplemented with 0.2% bovine serum albumin (BSA) and 1.7mM glucose, and incubated in 37°C, 95% O_2, 5% CO_2 atmosphere for 60 min. The islet medium was then collected and replaced with KRBH, 0.2% BSA, 16.7mM glucose. Islets were incubated for another 60 min, after that medium and islets were separately collected. Islets were transferred to 200µl deionized water and sonicated. 50µl of the islet homogenate was mixed with 125µl acid-ethanol, (0.18M HCl in 95% [vol/vol] ethanol), for determination of insulin content by ELISA. The rest of the islet homogenate was used for DNA measurements by fluorophotometry (PicoGreen dsDNA Quantitation kit; Molecular Probes, Eugene, OR, USA).

In vitro treatment of islets and evaluation of cell viability

(Paper II) Islets were isolated, precultured and left untreated or incubated with cytokines (50 U/ml IL-1β and 1000 U/ml IFN-γ. for 18 hours or 10μM CPA for 8 hours. Islet viability was determined by staining the cells with propidium iodide (Sigma) (20μg/ml) and bisbenzimide (Hoechst) (5μg/ml) for 10min at 37°C. After careful washing the cells were analyzed by fluorescence microscopy. Total number of cells, as well as propidium iodide positive cells, were counted by investigators not aware of sample identity. For experiments using SB203580 and STI-571 viability was determined after staining with bisbenzimide + propidium iodide by quantifying red and blue colours on photographs using Adobe Photoshop.

Nitrite measurement

(Paper II) As an indicator of NO formation, nitrite concentration in islet culture media was measured using Griess reagent. The nitrate concentration was determined by spectrophotometry at λ 546nm, against a standard curve of sodium nitrate.

Determination of insulin concentration

(Paper II, III) Insulin concentration in samples was measured by ELISA, (Rat insulin ELISA 10-1124, Mercodia, Uppsala Sweden) according to manufacturer's instructions.

RNA-isolation

(Paper II, III) Total RNA was prepared by using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase digestion with RNase-Free DNase set (Qiagen), according to the manufacturer's descriptions. For RNA isolation, 30 islets/sample were handpicked and briefly washed in PBS and then lyzed and homogenized. RNA yield was determined with a spectrophotometer at λ 260nm.

Quantitative real-time RT-PCR

(Paper II, III) One-step quantitative real-time RT-PCR was performed with QuantiTect™ SYBR® Green RT-PCR-kit (Qiagen) on a LightCycler™ real-

time PCR machine (Roche Diagnostics, Mannheim, Germany). The PCR conditions were according to kit supplier's instructions. Cycle threshold (C_T) values were determined with the LightCycler Software v3.5. Gene expressing were normalized by substracting corresponding house-keeping gene (beta-actin) C_T-value. Statistical comparisons were made on normalized C_T-values.

Histology

Fixation and embedding

Preparations of tissue sections for histological staining and analysis was performed as follows. *For paraffin sections,* (paper I, III) tissues were fixed in 4% paraformaldehyde or 10% formalin, dehydrated in increasing % of ethanol, then xylen and embedded in paraffin. 5µm sections were mounted on polylysine-coated glass slides (Menzel Gläser).

For freeze sections, (paper IV) tissues were either directly frozen in liquid nitrogen cooled 2-methyl-butan, or fixed in 4% paraformaldehyde in +4°C over night, infiltrated with sucrose and frozen in OCT-compound (Sakura) on a metal surface embedded in dry ice. 7µm sections were cut on a cryostat microtome and mounted on Superfrost Plus glass slides (Menzel Gläser).

For transmission electron microscopy, (paper III, IV) 1mm^3 tissue pieces were fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.15M sodium cacodylate buffer, then dehydrated and embedded in Epon812. Ultrathin (50nm) sections were contrasted with uranylacetate and lead citrate.

Stainings

(Paper I, III, IV) Tissue sections were immunostained for detection of proteins of interest. Anti-insulin antibody was used as a marker for pancreatic beta cells, anti-glucagon antibody as a marker for pancreatic alpha cells, anti-CD31 antibody as a marker for blood vessel endothelial cells, anti-Ki67 antibody for proliferating cells and anti-cleaved caspase 3 antibody as a marker for cells in progress of apoptosis. For amplification of signal, fluorescent-dye conjugated secondary antibodies were used, or biotinylated secondary antibodies in combination with either avidin-biotin-horseradish peroxidase and diaminobenzidine, or avidin-biotin-alkaline phosphatase and Vector® Red (Vector Laboratories). DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclei in fluorescent stainings, hematoxylin was used as counterstain in non-fluorescent stainings.

Morphometry

All morphometric measurements were done on blinded photos/sections. Adobe Photoshop 6.0 with Image Processing Toolkit v3.0 was used to determine: stained area relative to total tissue section area, on photos of pancreatic tissue sections (paper I, III). ImageJ v1.38 was used to assess lesion dimensions on photos of RIP-Tag2 positive pancreatic tissue sections (paper IV). Blood vessel density was determined on photos by grid overlay and counting percentage of grid intersections positive for CD31-staining. Percentage of proliferating cells or apoptotic cells were counted as number of positive cells divided by total number of cells in the same field or tissue of interest (paper I, II, IV).

Immunoblotting

(Paper II) Cells were washed with ice cold PBS and directly lysed in SDS-sample buffer, boiled for 5 min and separated on SDS-PAGE. Proteins were electrophoretically transferred to Hybond-P filters (GE Healthcare). Filters were blocked in 5% BSA for one hour, after which they were probed with primary antibody. Horseradish peroxidase-linked secondary antibody thereafter were used. The immunodetection was performed as described for the ECL® immunoblotting detection system (Amersham Biosciences) and using the Kodak Imagestation 4000MM. The intensities of the bands were quantified by densitometric scanning using Kodak Digital Science ID software (Eastman Kodak, Rochester, NY, USA).

Statistical analysis

Data in this thesis are presented as means +/- Standard error of the mean (SEM). For normal distributed data, comparisons between two groups were made with unpaired two-sample Student's t-test, and comparisons between more than two group with ANOVA.

All statistical analyses were done using SigmaStat® (SPSS Inc.), and p-values, less than 0.05 were considered significant. (Paper I-IV)

Results and discussion

Paper I

A role of Frk in regulation of embryonal pancreatic beta cell formation.
Molecular and Cellular Endocrinology 270: 73–78

Previously, a transgenic mouse overexpressing a constitutively active form of Frk under control of the rat insulin promotor (RIP-FrkY504F) had been generated and analysed [73, 77]. In addition to increased sensitivity to beta cell toxin streptozotocin *in vivo* and increased islet cell death upon exposure with cytotoxic cytokines *in vitro*, this transgenic mouse also displayed an 1.6 fold increased beta cell area at 3 months of age and 2.3 fold increased beta cell replication following a 60% partial pancreatectomy; suggesting a dual role of Frk in life and death of pancreatic beta cells. A Frk-/- mouse [80], however, was reported to be healthy and not to display any histological abnormalities in epithelial organs with known Frk expression. Interestingly, isolated islets from Frk-/- mice, and Frk-siRNA treated wild-type islets, displayed reduced islet cell death when exposed to cytokines [74], suggesting a non-redundant role of Frk in beta cells. The aim of this paper was to address whether Frk also has role for pancreatic endocrine mass and function, by assessing these parameters in the Frk-/- mouse.

To begin with, relative beta cell area in adult mice was addressed by insulin staining of pancreatic sections from 2.5 months wild-type and Frk-/- mice. The relative insulin area (insulin positive area / total pancreatic area) in Frk-/- was very similar to that of wild-type, indicating that absence of Frk does not affect beta cell mass in the adult mouse. However, this did not exclude the possibility of a more rapid beta cell turnover in the Frk-/-. To investigate this, pancreatic sections from 3-5 months old Frk-/- and wild-type mice, were stained with antibodies against cell proliferation marker Ki67 and insulin. This showed that the beta cell proliferation in Frk-/- was not significantly altered when compared to wild-type. To address the beta cell function, Frk-/- mice were subjected to a blood glucose tolerance test, but exhibited no impairments in glucose handling. In addition, the blood glucose concentration in randomly fed Frk-/- mice was not different from that of wild-type. Furthermore, there was no difference in body weight, pancreas weight or pancreas/body weight in 3-5 months Frk-/- mice, when compared to control. Together this pointed to the fact that Frk, at least during normal conditions, is redundant for beta cell mass and beta cell function in the adult mouse.

To address whether Frk was of importance for pancreatic endocrine cell differentiation and beta cell mass during embryogenesis and at birth, pancreata from embryonal day 15.5 (E15.5) and day one post birth were collected for histological evaluation. Surprisingly, the relative beta cell area in Frk-/- mice at E15.5 was decreased, 1/3 of that in the wild-type, and the glucagon positive area appeared to be increased although this was not statistically significant (p=0.15). The combined area of insulin and glucagon positive cells was almost identical in wild-type and Frk-/- mice. The relative beta cell area in Frk-/- mice on day one post birth was also found to be reduced, 70% of that in the wild-type. Due to shortage of wild-type mice, further comparison was performed using Frk+/- mice as control to Frk-/- mice. There was no difference in relative glucagon area between Frk+/- and Frk-/- mice. In addition there was no difference in body weight, pancreas weight or pancreas/body weight at day one post birth, indicating that the effect caused by absence of Frk was restricted to the endocrine compartment of the pancreas.

Islets from the RIP-FrkY504F mouse showed a trend towards increased basal IRS-protein tyrosine phosphorylation, and perturbed insulin induced stimulation of IRS2. Furthermore, RINm5F-cells overexpressing FrkY504F, exhibited increased basal IRS2, PI3K and ERK activation, and impaired ability of insulin to stimulate further activation of IRS2, PI3K, AKT and ERK [69]. Thus it was suggested that Frk might participate in IRS2-mediated signalling. Pancreatic or beta-cell specific deletion of IRS2 leads to impaired glucose tolerance, decreased glucose stimulated insulin secretion and reduced beta cell mass in the adult mouse [115, 116]. However the Frk-/- mouse did not exhibit similar changes, arguing against such a role of Frk in adult mouse beta cells. It is not likely that alterations in IRS-mediated signalling are the cause of the reduced beta cell mass seen in E15.5 and day one post birth in Frk-/- mice, since global or beta cell specific genetic ablation of components in insulin/IGF mediated signalling, have shown essentially normal embryonic pancreatic endocrine development and beta cell mass at birth [115, 117, 118].

In summary, this paper shows that Frk is involved in regulating the beta cell number during embryonal and early postnatal life in mice. The mechanism remains unclear but may reside at a step subsequent to endocrine specification.

Paper II

Reduced cell death and increased Hsp70 expression in response to cytokine exposure in islets of Langerhans isolated from Shb knockout mice, *Submitted*

The adaptor protein Shb may be involved in beta cell death since islets from the rat insulin promoter Shb transgenic mouse exhibit increased sensitivity to cytotoxic cytokines *in vitro* [87], as well as increased damage *in vivo* after a single dose of the beta cell toxin streptozotocin [77]. Shb is a downstream target and binding partner of the non-tyrosine kinase Frk [69], which has been implicated in cytokine induced beta cell destruction [73, 74]. The aim of this paper was to assess whether absence of Shb protected pancreatic islets against cytokine induced cell death and if so, to elucidate the underlying mechanism(s).

Exposure to cytotoxic cytokines has adverse effects upon beta cell function and survival in vitro [119]. To assess the sensitivity of Shb-/- islets to cytokine exposure, wild-type and Shb-/- islets were incubated for 18 hours with or without a combination of IL-1β + IFN-γ. Wild-type islets exposed to cytokines displayed reduced glucose stimulated insulin secretion, and cytokine-treated Shb-/- islets showed a similar reduction though not statistically significant. Surprisingly, the glucose stimulated insulin secretion of Shb-/- islets was reduced compared to wild-type islets, in the absence of cytokines, while no differences in islet DNA or insulin content could be assessed. Islet cell viability was determined by bisbenzimide-propidium iodide staining. Shb-/- islets were found to exhibit lower percentage apoptotic/necrotic cells, compared to wild-type islets after exposure to IL-1β + IFN-γ, thus absence of Shb has a protective effect against cytokine induced cell death. This is in line with previous result with Frk-/- islets [74]; further linking Frk and Shb in the beta cell cytotoxic response.

IL-1β + IFN-γ induces expression of inducible nitric oxide synthase with subsequent production of nitric oxide (NO), through activation of the NFκB pathway [120, 121]. NO has been suggested to be an effector in cytokine induced islet cell death [122]. However there was no difference in NO production, measured as nitrite concentration in culture medium after cytokine incubation, between wild-type and Shb-/- islets. In addition IκB phosphorylation, which is an early marker of NFκB pathway activation, did not differ between cytokine exposed wild-type and Shb-/- islets, suggesting that Shb mediated death is not linked to production of NO.

The ER-stress and the unfolded protein response (UPR) have been suggested to be involved in cytokine induced beta cell death [41, 42]. To test if absence of Shb protected islet cells against ER-stress-induced cell death, Shb-/- and wild-type islets were incubated with cyclopiazonic acid (CPA). CPA induces ER-stress and the UPR by inhibiting the action of SERCA, sarcoendoplasmic Ca^{2+} ATPase, perturbing ER Ca^{2+} homeostasis. Quantitative

RT-Real time PCR analysis of early and late UPR-markers BiP respectively CHOP, showed that CPA-incubation induced the unfolded protein response in similar manner in Shb-/- and wild-type islets. CPA-incubation lead to increased cell death in wild-type islets, but not in Shb-/- islets. Previously, Shb knockdown in betaTC6 cells was shown to have a protective effect against tunicamycin-induced ER-stress cell death [83]. The present results support the notion that Shb is involved in ER-stress/UPR-mediated islet cell death.

The stress associated MAP kinases, JNK and p38 have previously been implicated in cytokine-induced cell death [35, 36, 123]. Therefore JNK and p38 activation in Shb-/- was determined by western blot analysis. JNK was similarly phosphorylated in wild-type and Shb-/- islets exposed to cytokines. p38 phosphorylation in Shb-/- islets was decreased in unstimulated and cytokine stimulated islets. Treatment of islets with a p38 inhibitor did however not level out the differences in cytokine-induced cell death between wild-type and Shb-/- islets, arguing against reduced p38 activation as a cause of the protective effect of Shb deficiency in cytokine induced islet cell death.

Inhibition of the c-Abl kinase suppresses beta cell death induced by streptozotocin, NO or cytotoxic cytokines [124] and knock-down of Shb affects c-Abl activity and reduces genotoxic or ER-stress induced cell death [83]. Therefore, Shb mediated cell death has been suggested to be c-Abl-coupled, nonetheless, incubation with the c-Abl, c-kit, and PDGFR inhibitor STI-571, failed to change the difference in cytokine-induced islet cell death between wild-type and Shb-/- islets, contradicting the previous hypothesis.

The focal adhesion kinase (FAK) has in other cell types been implicated in Shb-mediated cell death [93], but western blot analysis of FAK-phosphorylation revealed no differences in FAK activation in unstimulated or cytokine-stimulated islets.

Heat-shock proteins may protect cells from various stress stimuli [49]. It has been shown that prolonged exposure with IL-1β increases islet content of Hsp70 [125], and liposomal delivery of Hsp70 into islets exert a protective effect against cytotoxic cytokines upon beta cell insulin secretion and insulin mRNA content [126]. Western blot analyses revealed a marked increase in Hsp70 content in Shb-/- islets following cytokine stimulation compared to wild-type islets. Elevated induction of Hsp70 is thus a candidate for explaining the reduced cytokine induced cell death in Shb deficient islets.

In summary, Shb-/- islets show a decreased secretion of insulin in response to glucose and a reduced susceptibility to cytokine and UPR stress toxicity. The mechanisms appear independent of nitric oxide, JNK, p38, FAK and c-Abl activation but could involve augmented Hsp70 induction under these conditions.

Paper III

Impaired glucose homeostasis in Shb-/- mice. ***Manuscript***

The aim of paper III was to assess blood glucose homeostasis of Shb deficient mice. The motives behind this were threefold: Firstly, the RIP-Shb transgenic mouse exhibits an increased beta cell mass and an accelerated return to normoglycemia following glucose challenge. Isolated transgenic islets also display increased glucose-stimulated insulin secretion without any associated increased insulin content [87]. Secondly, the Shb-/- mouse exhibits abnormalities in the endothelial ultrastructure in capillary blood vessels, alterations in vascular permeability and an impaired ability to support implanted tumour cells with blood vessels [111]. This is of interest since it has been reported that impaired islet vascularization/vascular function may have adverse effects upon insulin secretion and glucose homeostasis [127-129]. Thirdly, in paper II, we found that isolated Shb-/- islets exhibit reduced glucose-stimulated insulin secretion.

To test beta cell function in Shb-/- mice in vivo, randomly fed wild-type and Shb-/- mice were subjected to an intravenous glucose tolerance test. Shb-/- mice handled the glucose challenge similar to wild-type mice, although they exhibited a somewhat higher blood sugar concentration before and 120 min after the glucose challenge. Furthermore, there was a trend towards an elevated blood glucose concentration at 60 min after glucose injection. Partly to exclude the possibility that this reflected an altered food intake both wild-type and Shb-/- mice were starved over night and the blood glucose concentration was measured the following morning. Fasting blood glucose concentration in the Shb-/- mouse was elevated akin to that in the randomly fed condition. Since the liver is the main determinant of the basal blood glucose concentration during fasting, this could be a causative factor in the Shb-/- mouse. To address if these blood glucose changes were due to altered systemic insulin concentration, serum insulin was determined. However, no differences in serum insulin concentration could be assessed between randomly fed wild-type and Shb-/- mice. To investigate the peripheral insulin sensitivity, wild-type and Shb-/- mice were given an intraperitoneal insulin injection. In line with previous observations blood glucose concentration in Shb-/- mice was elevated prior to injection. The insulin challenge decreased blood glucose concentrations in both groups in a similar manner, although the initial difference between wild-type and Shb-/- blood glucose concentrations remained during the test. This suggests that Shb deficiency does not impair glucose uptake in the main insulin sensitive tissues: skeletal muscle and adipose tissue. To address whether the observed alteration in blood sugar concentration could be caused by a decreased number of beta cells, both islet mass and beta cell area were investigated in 3 months and 7-11 months old Shb-/- and wild-type mice, respectively. However, these investigations revealed no differences.

Isolated Shb-/- islets exhibit reduced glucose stimulated insulin secretion in vitro [130]. To assess insulin secretion ex vivo, whole pancreata were perfused with different glucose concentrations or arginine. Insulin secretion in response to 16.7mM glucose was impaired in Shb-/- pancreata, showing a blunted first-phase insulin secretion and the total amount of insulin released during 16.7mM glucose was reduced with 40% in Shb-/- pancreata. There was also a trend to decreased insulin secretion in response to arginine ($p=0.11$).

Since the insulin secretion from the Shb-/- pancreas was impaired, it was of interest to assess if Shb deficiency leads to alterations in gene expression of genes important for beta cell function. RNA was prepared from isolated islets and gene expression was assessed by quantitative real time RT-PCR. Analysis showed upregulation of the beta cell transcription factor Pdx1, whereas no differences in gene expression downstream of Pdx1 (insulin, Glut2, glukokinase) could be observed. Expression of transcription factors Hnf1α and Hnf3β, that may regulate expression of Pdx1, was not altered, and in addition the upregulation of Pdx1 disappeared after culture of the islets. Gene expression of VEGF-A, and the endothelial cell markers CD31 and VEGFR2 was also analysed to investigate if there were signs of alterations in beta cell–endothelial cell interactions. Expression of CD31 and VEGFR2 in Shb-/- islets was similar to wild-type islets, whereas expression of VEGF-A was found to be reduced by 50% in Shb deficient islets.

Reduced islet VEGF-A expression has previously been reported to lead to changes in islet endothelial cell morphology, with loss of fenestrations, increased number of caveolae, thickened basement membrane, as well as an overall reduced islet vascular density [127-129]. In comparison, the endothelial cell morphology in Shb-/- islets displayed increased amount of cytoplasm when compared to wild-type islet endothelial cells, but there appeared to be no alterations in the amount of fenestrations or caveolae. There was, however, a reduced islet capillary density of in Shb-/- islets. To address whether the changed islet microvasculature affected islet basal blood perfusion and thus could be involved in the impaired insulin secretory response, mice were injected with microspheres to measure blood flow. The number of microspheres in pancreas or in the islets was similar in the wild-type and Shb-/- mice. Thus the altered capillary density and endothelial cell morphology appear to not affect basal blood flow through the Shb-/- islets or pancreas.

In summary, Shb deficiency is associated with somewhat elevated basal blood glucose concentration, impaired glucose-stimulated insulin secretion, and altered islet microvascular morphology.

Paper IV

Absence of the Shb adapter protein leads to a reduced tumour incidence in the oncogenic RIP-Tag2 mouse strain. ***Manuscript***

Experiments in endothelial cell lines have implicated Shb in endothelial cell cytoskeletal rearrangement, tube formation, cell migration and survival [84, 86, 88, 89, 91, 92]. Knockdown of Shb also increased cell death in angiosarcoma cells treated with cytotoxic agents [93]. Our group has recently reported [111] that Shb-/- mice display abnormal endothelial morphology in liver sinusoids and heart capillaries, increased basal vascular permeability in skin, heart and kidney, and reduced angiogenesis in subcutaneously implanted VEGF-A and FGF-2 containing matrigel plugs. In addition retarded tumour growth was observed in Shb+/- mice on C57Bl/6 background in two different transplantable tumour models, with decreased vascularization and tumour cell proliferation. Thus Shb-mediated signalling may be a potential target in anticancer therapy. The aim in paper IV was to extend the investigation of tumour growth in absence of Shb, using an inheritable tumour model.

RIP-Tag2 mice express the tumourigenic large T-antigen from the SV40 virus under the control of the rat insulin promoter, and develop multiple insulinomas in a time-reproducible manner. RIP-Tag2 (RT) mice were bred with Shb-/- mice to introduce the RT-transgene onto the Shb-/- background. Shb+/+RT, Shb+/-RT and Shb-/-RT mice were used in the study. First tumour burden (total volume) and lesion frequency were assessed in all genotypes at 12 weeks of age, a timepoint where multiple tumours have arisen in the RT-model. Tumour burden in Shb+/-RT and Shb-/-RT mice was not different from that of Shb+/+RT mice, however Shb+/-RT and Shb-/-RT mice exhibited a similar reduction in lesion frequency. Further analysis revealed this to be due to a reduction in the number of lesions with a size corresponding to early small tumours, Ø 1.0-1.5mm [25], in Shb+/-RT and Shb-/-RT mice. In comparison, the number of large tumours (Ø>2.0mm) that make up the bulk of the tumour burden was not significantly altered. This suggests that absence of Shb may have different effects at different stages of tumourigenesis and/or possibly elicit adaptations in tumour cell behaviour.

The reduction in the number of small tumours prompted investigation of the vasculature in Shb-/-RT tumours. Small and large tumours were stained with CD31 to visualize the microvasculature. The endothelial cell proportion in small Shb-/-RT tumours was reduced to ¾ of corresponding Shb+/+RT tumours, indicating a lower vessel density in small Shb-/-RT tumours. There was no difference in endothelial proportion in large tumours between Shb-/-RT and Shb+/+RT. Electron micrographs of tumour capillary vessels showed endothelial cells with increased cytoplasm in Shb-/-RT tumours, in line with what previously been observed in the Shb-/- mouse [111]. There

were also fewer capillaries in Shb-/-RT showing fenestrae, compared to Shb+/+RT tumour capillaries. The percentage of perfused tumour vessels was addressed by injection of blood vessel binding FITC-conjugated tomato lectin and subsequent fluorescent microscopy with CD31-staining of endothelial cells. Tumour vascular permeability was tested by injection with FITC-conjugated dextran. Shb+/-RT mice were used in these experiments because of lack of Shb-/-RT mice. Due to a low number of observations, no statistical comparisons have yet been possible, but the data exhibit a trend to increased percentage of perfused vessels and mildly increased vessel permeability in Shb+/-RT tumours. In a study where VEGFR2 inhibitory antibody was administrated in the RT2-model, it was found that the suppressive antiangiogenic effect was transient and that tumour hypoxia eventually induced expression of other angiogenic factors, thereby permitting continued tumour growth [66]. This may explain the observed presence of large tumours in the Shb knockout mice. Presumably, these have exercised means to overcome the restrictions imposed by Shb deficiency to expand their microvasculature, allowing tumour progression.

Expansion of tumour cell mass is dependent of the balance between tumour cell proliferation and tumour cell death. In the RIP-Tag2 model, small and large tumours exhibit similar cell proliferation rate. By contrast, large tumours display less apoptosis than small tumours, suggesting that downregulation of apoptosis is the deciding factor necessary for tumour mass expansion [56] in RT transgenic mice.

Shb has previously been implicated in cell death, since overexpression of Shb cause increased apoptosis during stressful conditions in fibroblasts [85], endothelial cells [86, 88] and insulinoma cells [87]. Shb has also been reported to interact with and regulate the activity of the c-Abl kinase, which may induce cell death in response to DNA-damage, oxidative stress, or a failed unfolded protein response [83]. To address whether absence of Shb may have a direct effect on tumour cell apoptosis, thus exerting an influence on tumour expansion, tumours from Shb+/+RT and Shb-/-RT were stained for cleaved caspase 3, a marker of apoptosis. No difference in apoptotic rate could be assessed in small tumours, but large Shb-/-RT tumours showed a reduction in apoptosis compared with large Shb+/+RT tumours. Based on these observations, it is not likely that absence of Shb exerts a distinct effect upon tumour cell apoptosis. The reduction in apoptosis in large Shb null tumours may reflect the relatively improved vascularization in these tumours, although it cannot be excluded that absence of Shb might be beneficial for cell survival in far progressed tumours.

To assess cell proliferation, small and large tumours were stained for the cell cycle associated antigen Ki67. Cell proliferation, seen as % of Ki67-positive cells, did as expected not differ between small and large Shb+/+RT tumours, but surprisingly cell proliferation in large Shb-/-RT tumours was moderately increased when compared with small Shb-/-RT tumours. This

difference may be explained by the relative improvement of tumour vascularization, as compared with the wild-type situation.

In summary, Shb deficiency restricts tumour expansion in an inheritable tumour model, most likely by reduced angiogenesis. However, the observations also suggest that a subset of tumours may evade this restriction, and elucidation of this mechanism could be of future interest.

Conclusions

The main conclusions of this thesis are:

- Frk is involved in regulating beta cell number during embryonal and early postnatal life, but it is probably redundant for beta cell number and function in the adult mouse, at least under normal conditions.

- Shb deficiency exerts a protective effect against cytokine or UPR induced cell death. This effect appears independent of NO, JNK, p38MAPkinase, FAK and c-Abl, and may involve augmented upregulation of Hsp70.

- Shb deficiency is associated with somewhat elevated basal blood glucose concentration, altered islet microvascular morphology and impaired insulin secretion in response to glucose.

- Tumour growth in an inheritable tumour model is restricted on Shb deficient background, most likely caused by reduced angiogenesis.

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