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Role of the REST/RE-1 System in Beta-Cell Life, Function and Differentiation

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par

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Prof. Luc Tappy

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RESUME

Le diabète se caractérise par une déficience en insuline qui résulte d'une destruction des cellules bêta (β) pancréatiques sécrétant l'insuline (Type 1), ou à un défaut de sécrétion d'insuline qui peut être associé à la mort des cellules β (Type 2). La compréhension des mécanismes de néogenèse des cellules β, ainsi que l'identification de gènes impliqués dans leur survie et dans le contrôle de la sécrétion d'insuline est donc importante pour le traitement du diabète. Le facteur de transcription de type répresseur, RE-1 Silencing Transcription Factor (REST), contribue à la spécificité d'expression dans les neurones et les cellules β, d'un grand nombre de gènes portant son motif de fixation, le Repressor Element-1 (RE-1). Pour cela, REST est exprimé dans toutes les cellules, sauf dans les neurones et les cellules β. Afin d'identifier les gènes cibles de REST ainsi que leur fonction au sein de la cellule β, nous avons généré des souris transgéniques qui expriment REST spécifiquement dans ces cellules, sous la dépendance du promoteur de l'insuline (souris RIP-REST). Cette expression ectopique de REST a permis de diminuer l'expression des gènes contrôlés par REST, et d'en analyser les conséquences.

Nous avons montré que les souris RIP-REST étaient intolérantes au glucose et que ceci était du à un défaut de sécrétion d'insuline. Pour expliquer ce phénotype, nous avons mis en évidence le fait que des gènes cibles de REST codent pour des protéines importantes pour l'exocytose de l'insuline, comme SNAP25, Synaptotagmin (Syt) IX, Complexin II ou ICA512. De plus, nous avons découvert deux nouvelles cibles de REST impliquées dans la sécrétion d'insuline, Syt IV et Syt VII. Par la suite, nous avons démontré qu'une nouvelle lignée de souris RIP-REST étaient atteintes d'un diabète sévère à cause d'une perte massive des cellules β. La disparition de ces cellules a été expliquée par l'identification de gènes cibles de REST impliqués dans la survie des cellules β, comme Ib1, Irs2, Ica512 ou la Connexine36. De plus, nous avons découvert qu'une nouvelle cible, Cdk5r2, était aussi impliquée dans la survie des cellules β. Dans une dernière partie, nous suggérons, grâce à l'analyse de nouvelles souris transgéniques exprimant constitutivement REST dans les cellules progénitrices du pancréas embryonnaire, que REST empêche la formation des précurseurs de cellules endocrines ainsi que la différenciation de ces cellules. L'analyse de l'expression de REST au cours du développement embryonnaire du pancréas indique que la diminution de l'expression de REST conduit en partie, à l'induction d'un de ses gènes cible Myt1, qui favorise la formation de précurseurs endocrines. Nous proposons donc que le système REST/RE-1 est important pour la génération, la fonction et la survie des cellules β.

SUMMARY

Diabetes is characterized by insulin deficiency that results from the destruction of insulin-secreting pancreatic beta-cells (Type 1), or in part from beta-cell death and insulin secretion defects (Type 2). Therefore, understanding the mechanisms of beta cell neogenesis (to generate unlimited supply of beta cells for T1D transplantation) or identifying the specific genes that favors insulin secretion or beta-cell survival is of great importance for the management of diabetes. The transcriptional repressor RE-1 Silencing Transcription Factor (REST) restricts the expression of a large number of genes containing its binding element, called Repressor Element-1 (RE-1), to neurons and beta cells. To do so, REST is ubiquitously expressed but in neurons and beta cells. To identify these essential genes and their functional significance in beta cells, we have generated transgenic mice that express REST specifically in beta cells under the control of the rat insulin promoter (RIP-REST mice). This resulted in the repression of the RE-1-containing genes in beta cells, and we analyzed the consequences.

We first showed that RIP-REST mice were glucose-intolerant because of a defective insulin secretion. To explain this defect, we identified that a subset of the REST target genes were necessary for insulin exocytosis, such as Snap25, Synaptotagmin (Syt) IX, Complexin II, and Ica512, and we further demonstrated that among the identified REST targets, Syt IV and VII were also involved in insulin release. We next analyzed a novel RIP-REST mouse line that featured diabetes and we showed that this defect was due to a major loss of beta-cell mass. To explain this phenotype, we identified REST target genes that were involved in beta-cell survival, such as Ib1, Irs2, Ica512 and Connexin36, and revealed that another REST target, Cdk5r2 is also involved in beta-cell protection. In a third part, we finally suggest that REST may be important for pancreatic endocrine differentiation, since transgenic mice expressing constitutive REST in pancreatic multipotent progenitors show impaired formation of Ngn3-expressing endocrine-committed precursors, and impaired formation of differentiated endocrine cells. Mapping the pattern of REST expression in wild type animals indicates that it is expressed in multipotent progenitors to become then excluded from endocrine cells. Preliminary results suggest that a downregulation of REST would result in relieved expression of at least the Myt1 target, favoring subsequent acquisition of the endocrine competence by endocrine precursor cells.

Thus, we propose that the REST/RE-1 system is an important feature for beta-cell neogenesis, function and survival

ABBREVIATIONS

Ad-GFP	GFP-encoding adenovirus
Ad-REST	REST-encoding adenovirus
AKT/PKB	Thymoma viral proto-oncogene/protein kinase B
bHLH	Basic helix loop helix
bp	Base pair
Cdks	Cyclin-dependent kinases
CKIs	Cyclin-dependent kinase inhibitors
ChIP	Chromatin immunoprecipitation
Cx	Connexin
Dox	Doxycycline
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FPG	Fasting plasma glucose
GLP-1	Glucagon-like peptide-1
GSIS	Glucose-stimulated insulin secretion
HDACs	Histone deacetylases
HNF	Hepatocyte nuclear factor
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL1-β	Interleukin-1 beta
IFN-γ	Interferon gamma
iPSC	induced-pluripotent stem cell
IRS	Insulin receptor substrate
IPGTT	Intraperitoneal glucose tolerance test
ISH	*in situ* hybridization
JNK	c-Jun N-terminal kinase
KO	Knock-out
MAPK/SAPK	Mitogen-activated protein kinase/stress-activated protein kinase
MODY	Maturity onset diabetes of the young
mTOR	Mammalian target of rapamycin
Myt1	myelin transcription factor 1
NSC	Neural stem cell
PCR	Polymerase chain reaction
Pdx1	Pancreas and duodenum homeobox 1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PP	Pancreatic polypeptide
qPCR	Quantitative PCR
RE-1	Repressor element-1
REST	RE-1 silencing transcription factor
RIP II	Rat insulin II promoter
RNA	Ribonucleic acid
rTA	Reverse tetracycline transactivator
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
Syt	Synaptotagmin
TetO	Tetracyclin operator
TSA	Trichostatin A
tTA	Tetracycline-repressible transactivator
TF	Transcription factor
TNF-α	Tumor necrosis factor alpha

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GENERAL INTRODUCTION

1)Diabetes

Diabetes mellitus is one of the most common diseases globally. It is the fourth or fifth leading cause of death in most high-income countries and has reached epidemic proportions in many economically developing and newly industrialized nations. Estimations for the year 2010 indicate that approximately 285 million people worldwide have diabetes, resulting in a prevalence of the disease of 6.6 %. By 2030, some 438 million people, or 7.8% of the adult population, are projected to have diabetes. The medical and socio-economic burden of the disease is caused by the associated complications, which impose enormous strains on health-care systems. Long-term complications of diabetes include retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputations and cardiovascular symptoms and sexual dysfunction. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes. Cardiovascular morbidity in patients with type 2 diabetes is two to four times greater than that of non-diabetic people, responsible for 75% of death in diabetes patients. Diabetes is undoubtedly one of the most challenging health problems in the 21st century.

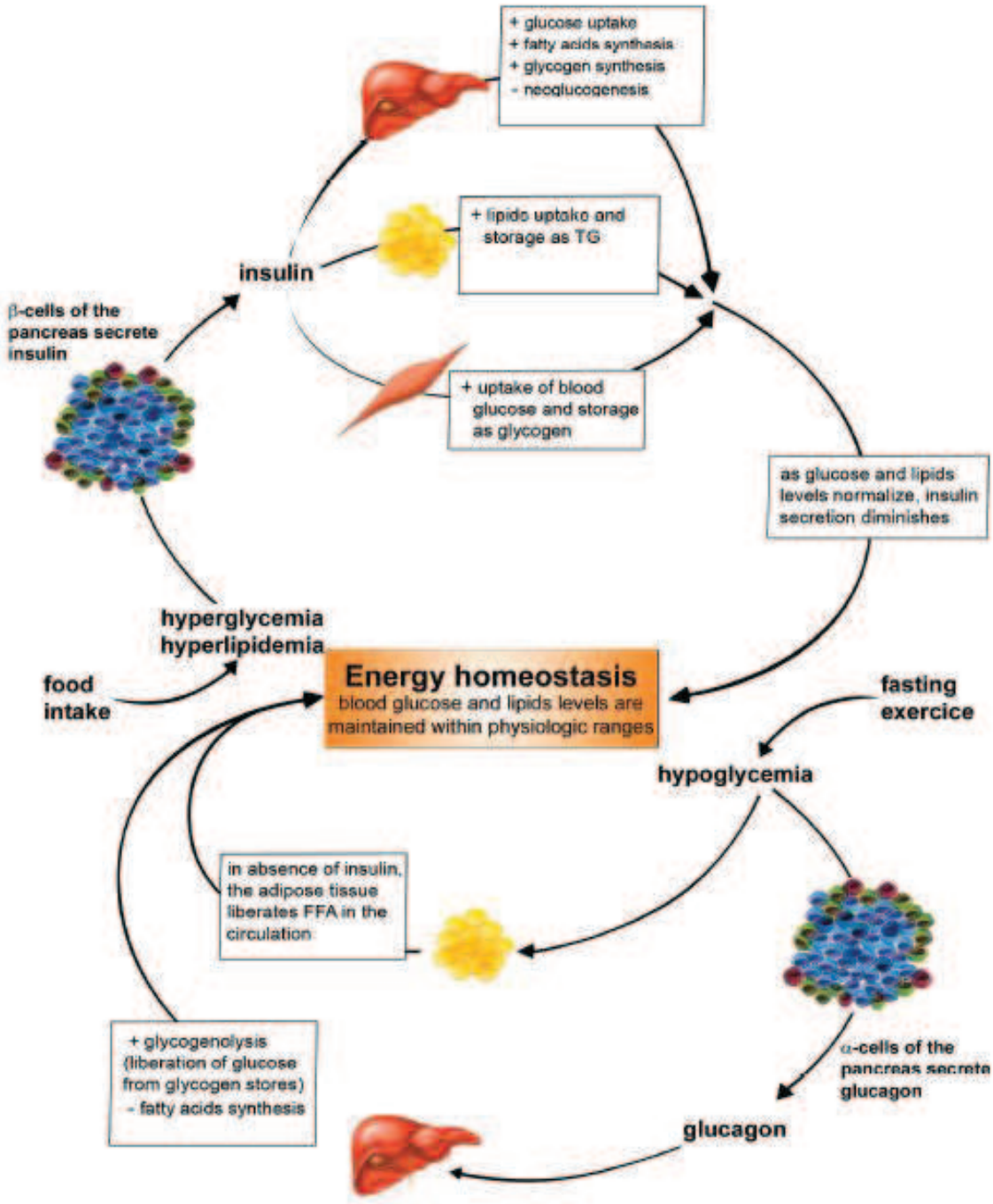
a) Definitions and description

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

Secreted by the beta cells of the pancreas, the overall action of insulin is to induce the storage of fuel as glycogen in the liver and skeletal muscles, or as triglycerides in the adipose tissue. To do so, insulin increases the glucose uptake by stimulating translocation of the glucose transporter GLUT4 and also triggers lipid absorption

through activation of the lipoprotein lipase. Thus, insulin stimulates glucose utilization in the peripheral tissues (glycolysis and glycogen synthesis), but also suppresses endogenous glucose production from the liver (glycogenolysis). Accordingly, insulin promotes synthesis of long-chain fatty acids in the liver (lipogenesis) and synthesis of triglycerides in adipose tissue (fatty acids esterification) while suppressing free (nonesterified) fatty acids (FFAs) release from the adipose tissue (lipolysis) (Scheme 1). Considering glucose homeostasis, the major part of glucose in the fasting state is produced by the liver through glycogenolysis and gluconeogenesis, and roughly half of it is used for brain glucose metabolism. The remainder is taken up by various tissues, mainly muscle and for a minor part adipose tissue. In this situation the main effect of the relatively low insulin levels is to restrain liver glucose production, which can be increased fourfold according to the needs in nutrients. After a meal, insulin is secreted in larger amounts and decreases liver glucose production even further and will lead to an enhancement of muscle and adipose tissue glucose uptake.

The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.



Scheme 1: Energy homeostasis

Energy homeostasis reflects the balance between the hypoglycemic action of insulin and the hyperglycemic action of diabetogenic (anti-insulin) hormones. After a meal, insulin release is stimulated, resulting in an increased glucose and lipid uptake by the liver and the peripheral tissues. Conversely, in the fasting state, plasma insulin levels are low and glucagon secretion is stimulated, resulting in an increased glucose release and lipid oxidation ensuring energy production. TG: triglyceride, FFA: free fatty acids. ***+****:stimulation, -:inhibition*

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome.

The vast majority of cases of diabetes fall into two broad etiopathogenetic categories (for a comprehensive list, see (2010)). In one category, type 1 diabetes (T1D), the cause is an absolute deficiency of insulin secretion. In the other much more prevalent category, type 2 diabetes (T2D), the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

Type 1 Diabetes

This form of diabetes, which accounts for only 5–10% of those with diabetes, previously known as insulin-dependent diabetes, or juvenile-onset diabetes, results from a cellular-mediated autoimmune destruction of the beta cells of the pancreas. People can present with pancreas inflammation (insulitis) for a long time (years in humans, months in rodent models) before it finally progresses to overt diabetes, and sometimes it never does. In diabetic patients, beta-cell mass is reduced by 70–80% at the time of diagnosis, so that in the last stage of evolution of the disease, there is little or no insulin secretion and diabetic people are dependent on external supply of insulin for survival.

Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life. Markers of the immune destruction of the beta cell include autoantibodies to insulin, to glutamic acid carboxylase GAD (GAD65), and to the tyrosine phosphatases IA-2 and IA-2β.

Type 2 Diabetes

This form of diabetes, which accounts for ~90–95% of those with diabetes, previously referred to as non–insulin-dependent diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive.

In T2D, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

The risk of developing this form of diabetes increases with age, obesity, unhealthy diets and lack of physical activity. Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance.

It is often associated with a strong genetic predisposition, more so than is the autoimmune form of T1D. However, the genetics of this form of diabetes are complex and not clearly defined.

MODY

Several forms of T2D, characterized by autosomal dominant transmission and early onset of hyperglycemia (generally before age 25 years), are associated with monogenic mutations leading to beta-cell dysfunction. They are referred to as maturity-onset diabetes of the young (MODY) and are due to impaired insulin secretion with no defects in insulin action. Abnormalities at six genetic loci on different chromosomes have been identified to date. MODY1, -3, -4, -5, -6 have been associated with mutations in genes coding for the transcription factors hepatocyte nuclear factor (HNF)-4α, HNF-1α, pancreas duodenum homeobox-1 (PDX1), HNF-1β, and NeuroD/BETA2, respectively. In MODY2, the mutated gene is that coding for the glucokinase, an enzyme that acts as a

glucose sensor in beta cells by catalyzing the rate-limiting step of glucose catabolism (for a review, see (Froguel, 2003)).

b) Clinical criteria

Diabetic people are diagnosed by measurement of plasma glucose in the fasting state or after a challenge with an oral glucose load. It is also possible to measure the levels of glycosylated haemoglobin (A1C), which is a widely used marker of chronic glycemia, reflecting average blood glucose levels over a 2- to 3-month period of time.

According to the ADA (Diagnosis and classification of diabetes mellitus, 2010) the clinical criteria establishing a person as suffering from diabetes mellitus are:

- Having A1C ≥ 6.5%.

or

- Having Fasting Plasma Glucose (FPG) ≥ 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.

or

- Having plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during 2h following an oral glucose tolerance test (OGTT).

or

- In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l).

In an intermediate group of individuals without clinical symptoms, glucose levels do not meet criteria for diabetes but are nevertheless sufficient to cause pathologic changes in various target tissues. These people were defined as having:

- Impaired fasting glucose (IFG) (FPG levels from 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)),

or

- Impaired glucose tolerance (IGT) (2-h values in the OGTT from 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)).

or

- A1C 5.7–6.4%

Individuals with IFG and/or IGT have been referred to as having pre-diabetes, indicating the relatively high risk for the future development of diabetes

c) Etiology

Type 2 Diabetes

Insulin resistance is the key primary defect underlying the development of T2D. It occurs when the biological effects of insulin are subnormal for both glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in liver (Dinneen et al., 1992).

It is strongly associated with obesity and is correlated with high triglycerides and FFA plasma levels (Paolisso et al., 1995), with elevated total cholesterol levels, seemingly normal or high levels of LDL and low HDL levels (Berneis et al., 2005), suggesting that diabetes is actually a dysregulation of fat metabolism as proposed by Mc Garry (McGarry, 1992) (see also (Shafrir and Raz, 2003). The adipocyte is the only cell that is specifically adapted to store large amounts of fatty acids, which it converts into triglycerides (TGs) before storage. When its buffering capacities are overwhelmed by consistent intake of fat above the rate of oxidation, the dietary fat must go elsewhere, and its ectopic deposition in other non-adipose tissues leads to insulin resistance and beta-cell dysfunction. Moreover, in obese subjects TGs are specifically stored in visceral or deep subcutaneous adipocytes, which have high basal lypolysis and are resistant to the ability of insulin to suppress lipolysis (as compared with subcutaneous adipocytes). This results in elevated release and increased flux of FFAs and TGs in the circulation, that have not only acute adverse effects on insulin sensitivity, but also in the longer term leads to accumulation of TGs in glucose-metabolizing tissues such as muscle, liver and beta cells (Raz et al., 2005).

Insulin resistance is also associated with a state of chronic low-grade inflammation, and it is now assumed that several mediators released from infiltrating macrophages in adipose tissue and from adipocytes themselves are involved in the development of insulin resistance (Hotamisligil, 2006, Rosen and Spiegelman, 2006). These products known as adipokines include leptin, adiponectin, resistin, as well as cytokines and chemokines, such as TNF and IL6. In addition to their effect on insulin signaling, the circulating adipose tissue factors strongly affect vascular endothelial function, linking the increased cardiovascular risk with insulin resistance.

Eventually, a vicious circle settles in, where metabolic abnormalities impair insulin secretion, which further aggravates metabolic perturbations, and so on.

In adipose tissue, the loss of insulin sensitivity results in the non-inhibition of the hormone sensitive lipase (HSL), which catalyzes the release of FFAs into the circulation. The generalized loss of insulin sensitivity stimulates the elevation of FFA blood levels, atherogenic lipoproteins particles and TGs, thus reinforcing the hyperlipidemia.

In the liver, excessive FFA oxidation results in stimulation of gluconeogenesis. Since endogenous glucose production by the liver is responsible for all of the glucose appearing in plasma in the fasting state, hepatic insulin resistance (with increased hepatic glucose production) is the driving force of hyperglycemia of T2D. Such a central role of hepatic insulin resistance in the pathogenesis of T2D has been demonstrated using conditional knock-out (KO) of the insulin receptor in mice. Only liver (Michael et al., 2000) and beta cell-specific (Kulkarni et al., 1999) KO became glucose intolerant whereas KO specific for muscle (Bruning et al., 1998) and fat cells (Bluher et al., 2003) did not.

In muscles, the first effect of FFA-induced insulin resistance is the restriction of cellular glucose entry, followed by reduction in its metabolism.

Thus, the conjunction of insulin resistance and hyperlipidemia leads to elevated plasma glucose levels (hyperglycemia), which have also been shown to promote reactive oxygen species (ROS) production, thereby further supporting the aggravation of insulin resistance. Eventually, the ROS accumulation starts to impact the physiology of several organs including the liver, the skeletal muscle and the adipose tissue, as well as the heart and the vascular endothelium, leading to the apparition of a variety of disorders, from diabetes to hypertension, atherosclerosis, and coronary artery disease (Robertson et al., 2004).

Type 1 Diabetes

T1D is an autoimmune disease resulting from specific destruction of the insulin-producing beta cells of the pancreas. It has two distinct phases: insulitis, when a mixed population of leukocytes invades the islets, and diabetes, when most beta cells have been killed off, and there is no longer sufficient insulin production to regulate blood glucose levels, resulting in hyperglycemia.

T1D is primarily a T-lymphocyte-mediated disease. Immunohistological analyses show that most leukocytes in the islet infiltrate are T cells. As a demonstration, disease does not develop in non-obese diabetic (NOD) mice that are genetically athymic or T lymphopenic, or were thymectomized at birth. Finally, diabetes can be transferred by injecting T cells from diseased donors into healthy NOD recipients (Matsumoto et al., 1993). T cells with diabetogenic properties fall into both the $CD4^+$ helper and the $CD8^+$ killer classes. They respond to several antigens that are specifically, but perhaps not uniquely, synthesized by pancreatic beta cells, including peptides derived from insulin, glutamic acid decarboxylase and the tyrosine phosphatases 1A-2 and 1A-2β (phogrin). At some point, probably induced by a wave of physiological beta-cell death that takes place perinatally, as observed in rodents (Scaglia et al., 1997), beta cell-derived antigens

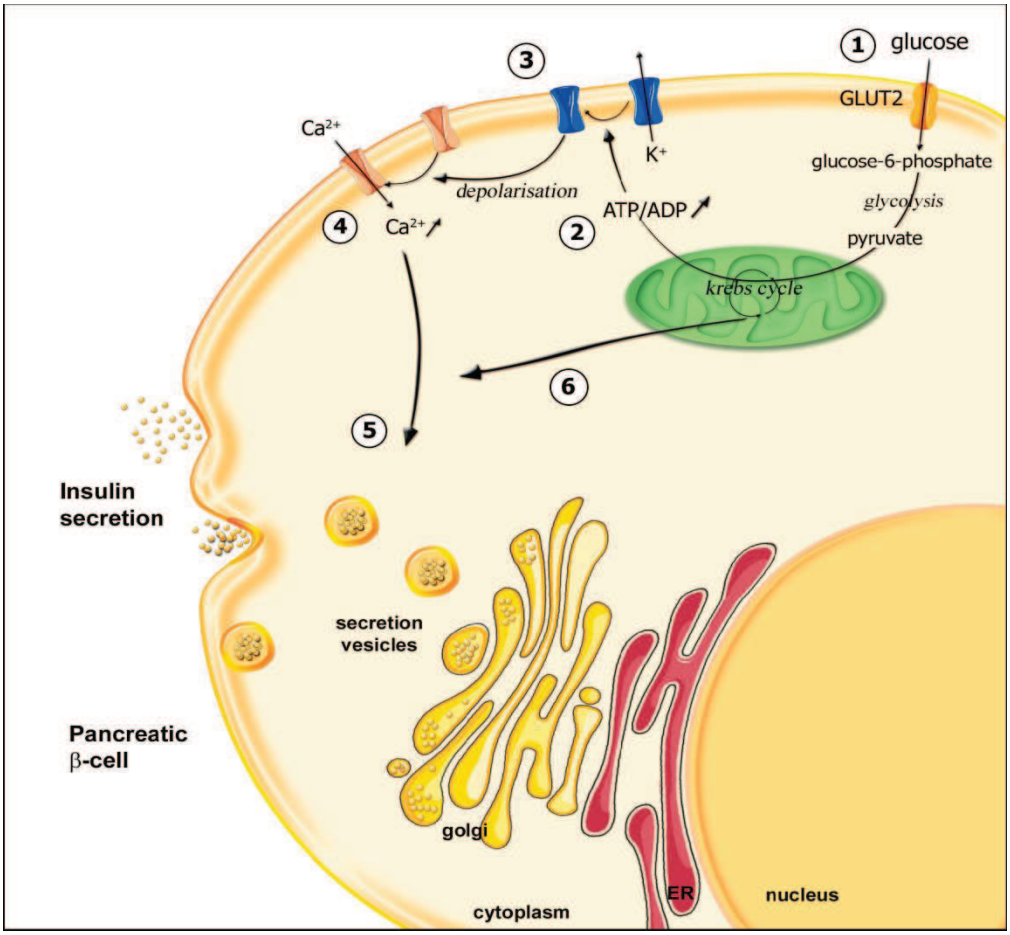
are taken up in the islets by antigen presenting cells (APCs) (probably dendritic cells). This induces maturation of the APCs and their migration to the immediately draining pancreatic lymph nodes. Here, they display the beta cell-derived antigens to naive beta cell-reactive T cells in circulation and activate them. As part of the activation process, the T cells acquire the ability to migrate through the tissues. In the islets, they re-encounter cognate antigen, become reactivated and are retained, thereby initiating insulitis (Mathis et al., 2001).

2) Beta-cell physiology

a) Insulin secretion

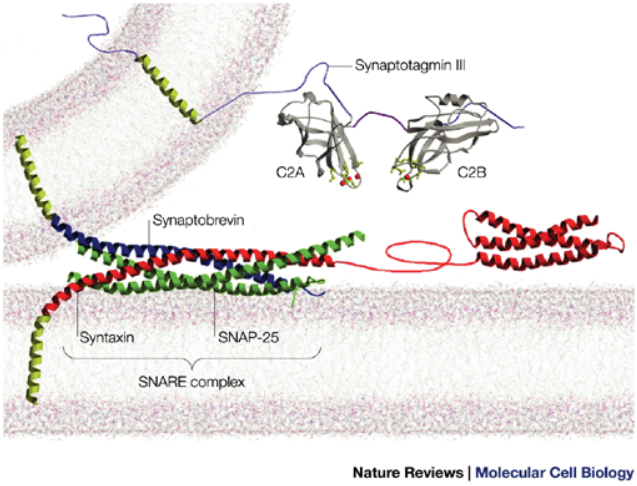
The main function of beta cells is to secrete insulin in response to an elevation of plasma glucose levels. Increased uptake of glucose by pancreatic beta cells is associated with an elevation in the intracellular ATP concentrations produced through glycolysis and Krebs cycle. This in turn conducts to a blockade of the ATP-sensitive K+ channels. The subsequent depolarization of the plasma membrane opens the voltage dependent calcium channels. Finally, the influx of Ca2+ in the cytoplasm triggers insulin secretion (Lang, 1999). Glucose triggers a biphasic insulin secretion, in which the Ca2+ signal is necessary but not sufficient for the full response. The first phase of insulin secretion occurs within 10 to 15 min of stimulation and corresponds to the KATP channel-dependent or “triggering” pathway. The second phase, which lasts up to 2 hours, corresponds to the KATP channel-independent “amplifying” or “potentiation” pathway, and is derived from mitochondrial metabolism (Henquin, 2000) (scheme 2). The second phase is known to require glucose metabolism and to be ATP-dependent, reflecting an energy requirement for the priming and docking of secretory vesicles at sites of exocytosis. Even if mitochondria-dependent nucleotides (GTP, ATP, cAMP or NADPH)

and metabolites (long-chain acyl-CoA and glutamate) have been proposed as coupling factors in this pathway (Maechler et al., 2006), the exact mechanisms are still in debate. To these temporally distinct phases are also associated distinct functional pools of insulin granules. The first phase of insulin release involves the rapid release, upon calcium influx, of a readily releasable pool of secretory vesicles docked at the plasma membrane. In contrast, the sustained second phase of insulin secretion not only involves the priming of granules located in the vicinity of the membrane, but also the translocation toward the plasma membrane of remotely located secretory granules (Rorsman and Renstrom, 2003).



Scheme 2: insulin stimulus-secretion coupling pathway.
Glucose enters beta cell through the transporter GLUT2 (1). Glucose metabolism generates an elevation of the ATP/ADP ratio (2), inducing closure of K+ channels (3). The subsequent depolarization triggers the entry of Ca2+ through voltage-gated calcium channels (4) and Ca2+ activates the fusion of the secretory granules containing insulin (5). In the amplifying pathway of insulin secretion, signals generated in mitochondria support the triggering pathway (6). Reproduced from Allagnat, F. PhD thesis, 2006.

Regulated exocytosis, which is the terminal step of this stimulus-secretion coupling pathway, ensures the fusion of secretory vesicles with the plasma membrane, and the subsequent release of their content. The exocytosis of insulin-containing large dense core vesicles (LDCV), or secretory granules, resembles that of neuronal synaptic vesicles (for reviews, (Gerber and Sudhof, 2002, Burgoyne and Morgan, 2003), and employs several common molecular determinants constituting the exocytotic machinery. These are the Soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNARE) complex (scheme 3), which encompasses the vesicular v-SNARE Synaptobrevin/VAMP and the plasma membrane t-SNAREs Syntaxin1 and SNAP25 (Wheeler et al., 1996), the cytosolic ATPase N-ethylmaleimide-sensitive-factor (NSF) and its cofactor α/β soluble-NSF-attachment protein(α/β SNAP) (Kiraly-Borri et al., 1996), the syntaxin chaperone Munc18-1 (Zhang et al., 2000), the small GTPases of the Rab3 family (Iezzi et al., 1999), and the Ca^{2+} sensor proteins of the Synaptotagmin (Syt) family (Gut et al., 2001). In particular, a direct implication in the control of insulin secretion has been established for the following members of the exocytotic machinery: SNAP25 (Sadoul et al., 1995), Syt II (Lang et al., 1997), Syt V and Syt IX (Iezzi et al., 2004), NSF (Vikman et al., 2003), Rab3a (Iezzi et al., 1999), Rab27a (Yi et al., 2002), Munc18-1 (Zhang et al., 2000) and many other. For reviews about their respective role, readers are referred to (Burgoyne and Morgan, 2003, Jahn et al., 2003, Bai and Chapman, 2004)



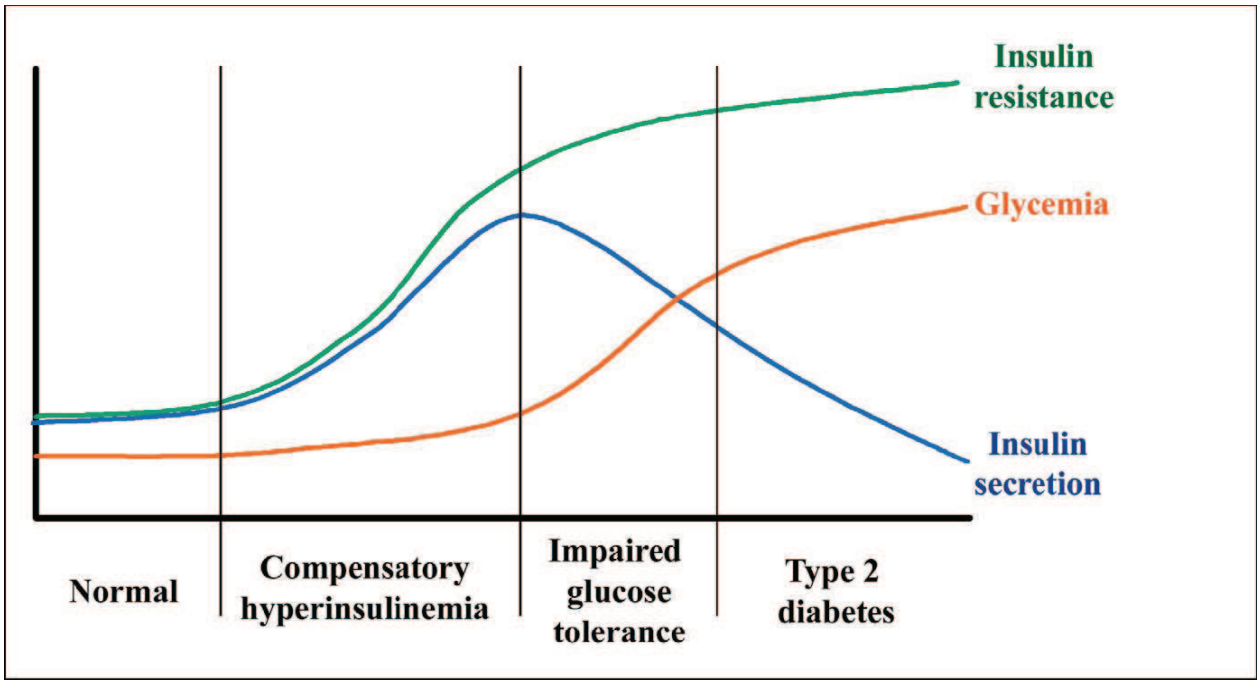
Scheme 3: Model depicting the synaptotagmin-SNARE complex during exocytosis
The core of the SNARE complex, constituted by the assembly of cytoplasmic domains of Syntaxin, SNAP25 and VAMP2 pulls the vesicle and plasma membrane together. Both Ca^{2+}-binding C2 domains of synaptotagmin are required for high affinity binding to the base of the SNARE complex. Reproduced from (Chapman, 2002).

The normal pancreatic beta cell is capable of adapting to changes in insulin action, i.e., a decrease in insulin action is accompanied by upregulation of insulin secretion (and vice versa). Beta-cell adaptation precludes development of diabetes in a large number of insulin-resistant subjects. When the adaptation of the beta cell is insufficient, the subjects will develop impaired glucose tolerance (IGT) or T2D. As shown in scheme 4, T2D development can be viewed has having three stages:

1. In order to maintain glucose homeostasis despite of insulin resistance, the beta cell secrete more insulin, this is the compensative hyper-insulinemia stage.

2. Subsequently, insulin resistance increases and beta cells do not manage anymore to secrete the ever-growing levels of insulin required to maintain normoglycemia. Progressively, insulin secretion declines, and hyperglycemia settles in, this is the impaired glucose tolerance stage.

3. The sustained hyperglycemia and hyperlipidemia lead to glucolipotoxicity, a phenomenon in which beta-cell secretion and survival functions are severely altered, resulting in the apparition of T2D (Prentki and Nolan, 2006).



Scheme 4: Etiology of type 2 diabetes

Environmental and genetic factors lead to the apparition of insulin resistance (green line), which is first balanced by hyperinsulinemia (blue line). When beta cells cannot compensate for insulin resistance, the glycemia (red line) augments and diabetes appears. Reproduced from Allagnat, F. PhD thesis, 2006.

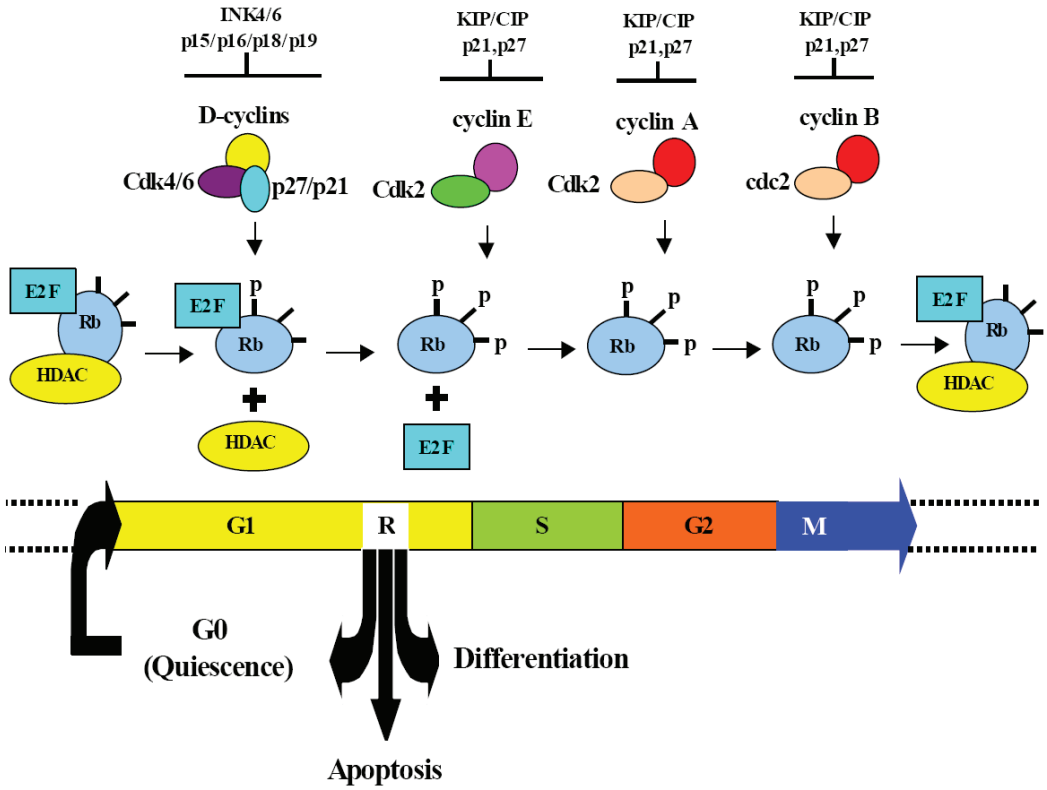
b) Beta-cell growth

General considerations

Pancreatic beta-cell mass is regulated by at least four independent mechanisms: beta-cell replication (i.e., the mitogenic division of existing beta cells), beta-cell size, beta-cell neogenesis (i.e., the emergence of ‘‘new beta cells’’ from pancreatic precursor stem cells), and beta-cell apoptosis. During pancreatic development, beta cells arise through neogenesis, while during late embryogenesis beta-cell mass is increased through a high rate of proliferation (approximately 10% per day in mice). In adulthood, then beta cells proliferate at a low rate (<1% of beta cells replicate per day in mice) and beta-cell size and mass stay relatively constant even if it may gradually decline with age (Ackermann and Gannon, 2007). However, beta-cell mass in the adult is plastic and during common physiological and pathological states in which insulin demand is increased (such as pregnancy, insulin resistance and obesity), beta cells can respond through several adaptations, including hyperplasia, hypertrophy, and increased insulin synthesis and secretion to adjust insulin supply to metabolic demand. For instance, in insulin-resistant states, pancreatic islets usually respond by increasing insulin secretion to maintain normoglycemia, a process termed beta-cell compensation. The mechanisms involved are not fully understood, but it is apparent from rodent studies that both expansion of the beta-cell mass and enhanced beta-cell function are important. Increased beta-cell mass has been observed in the pancreata of obese compared with lean nondiabetic subjects (Butler et al., 2003), although the fold increase (about 50%) is less than seen in rodents. In these conditions, and in mouse models of regeneration, the increase of beta-cell mass results predominantly from the proliferation of pre-existing beta cells rather than through neogenesis (Dor et al., 2004, Nir et al., 2007, Teta et al., 2007).

Cell cycle

The different capacities of beta cell to proliferate reflect the percentage of cells that are recruited to enter the cell cycle, which is controlled by the expression and function of three major classes of proteins: cyclins, cyclin-dependent kinases (Cdks), and cyclin-dependent kinase inhibitors (CKIs). Progression from G1 to S phase in the cell cycle requires phosphorylation of the retinoblastoma protein (Rb) by Cdk, complexed with a cyclin. This in turn releases E2F, which activates transcription of necessary cell cycle target genes (Scheme 5) (Cozar-Castellano et al., 2006, Heit et al., 2006b). Several genetic mouse models of cell cycle dysregulation impair postnatal beta-cell proliferation, and cause a progressive decrease in beta-cell mass, associated with a progressive glucose intolerant and diabetic phenotype.



Scheme 5: The mammalian cell cycle machinery

A typical cell cycle is divided into G1, S (where DNA synthesis occurs), G2 and M phases (where cells undergo mitosis). Every phase of the cell cycle is under regulatory influences of different cell cycle proteins: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). During the G1- to S-phase transition, cell cycle progression is regulated by cyclin/CDK complexes, which phosphorylate the retinoblastoma protein (Rb). Phosphorylation of Rb leads to E2F activation and initiation of a transcriptional program leading to S-phase. In non-proliferating cells, the transition to S-phase is blocked by INK4 (INhibitors of Kinase 4) and Kip/Cip proteins, which inhibit cyclin/CDK complex function (top of the scheme). Reproduced from (Rane and Reddy, 2000).

In the class of CDKs, Cdk4 and Cdk2 are expressed in beta cells. Global inactivation of Cdk4 in mice specifically affects endocrine cells within the pancreas, causing diabetes by 2 months of age (Rane et al., 1999). CDK activators cyclin D1 and cyclin D2 are both expressed in beta cells. Cyclin D1 KO mice exhibit normal islet cell size and number. However, cyclin D1 overexpression in mice has been found to increase beta-cell proliferation and mass *in vivo* (Zhang et al., 2005). In contrast, global deletion of cyclin D2, the predominant cyclin expressed in beta cells, impairs post natal beta-cell proliferation (Kushner et al., 2005). Regarding CKIs, $p16^{INK4a}$ (Ink4a) is thought to limit beta-cell proliferation by inhibiting Cdk4 (Serrano et al., 1993). Accordingly, Ink4a deficiency in mice permits increased beta-cell proliferation, supporting the conclusion that Ink4a constrains beta-cell proliferation in mice (Krishnamurthy et al., 2006). p27Kip1 is also particularly interesting because it is regulated by the insulin signaling pathway and is thought to be a major factor in the regulation of beta-cell mass. Mice lacking p27 show improved glucose tolerance with increased beta-cell mass and proliferation (Uchida et al., 2005).

Many growth factors have been implicated in increasing beta-cell mass through activation of the cell cycle (mostly occurring at the G1/S transition). For compensatory beta-cell mass expansion, increased nutrient supply in the blood is important as a stimulant, with considerable evidence for roles for both glucose and FFAs. Increased enteric nutrient supply also results in beta-cell mass expansion through increased glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) production from L cells in the intestine. Interestingly, many components of the beta-cell signaling pathways triggered by these growth factors are the same as elements in insulin signal transduction in peripheric tissues, including insulin/IGF receptor, insulin receptor substrate (IRS), Phosphatidylinositol 3-kinase (PI3K) and Thymoma viral proto-

oncogene/protein kinase B (AKT/PKB, later referred to as AKT) (Scheme 6) (Withers et al., 1999, Kulkarni, 2005).

Glucose

It has long been known that glucose is a potent beta-cell mitogen. In rodents, glucose infusion results in an approximately 50% increase in both beta-cell proliferation and mass, partially through anti-apoptotic effects (Paris et al., 2003, Alonso et al., 2007). Glucose stimulates several pathways playing an important role in growth signals in beta cells. These include insulin secretion and the subsequent autocrine insulin signaling via the insulin receptor (IR) (see below "insulin"), voltage-gated Ca2+ transients and the subsequent activation cascade of the cAMP-dependent protein kinase (PKA), the extracellular signal-regulated kinase (ERK1/2), and the transcription factor cAMP response element–binding protein (CREB) (Jhala et al., 2003, Costes et al., 2006, Heit et al., 2006a). Glucose also controls beta-cell size and proliferation through the mammalian target of rapamycin (mTOR) pathway, since it has been shown that glucose stimulation in beta-cell lines activates p70/ribosomal S6 kinase (p70/S6K), independently of IRS-2 and AKT (Dickson et al., 2001). p70/S6K and eukaryote initiation factor 4E-binding protein 1 (4EBP1) are two key regulators of protein translation, which are activated by mTOR (Scheme 6). Evidence for the importance of mTOR signaling on the modulation of beta-cell mass and proliferation, *in vivo,* comes with the observation that mice deficient for S6K displayed decreased beta-cell mass and hyperglycemia (Shima et al., 1998, Um et al., 2004). A recent publication also reported that the mTORC1 is a major regulator of beta-cell cycle progression by modulation of cyclin D2, D3, and Cdk4 activity (Balcazar et al., 2009).

Insulin

The role of insulin as a growth factor is supported by studies in beta-cell-specific insulin receptor KO mice (bIRKO), which display an age-dependent decrease in beta-cell mass (Kulkarni et al., 1999). Consistent with these data, insulin infusion stimulates beta-cell proliferation and increases beta-cell mass in rats, and costimulation with glucose augments this effect (Paris et al., 2003). Insulin and insulin like growth factor (IGF) signaling through insulin and IGF receptors leads to activation of the IRS2/PI3K/AKT and ERK1/2 pathways which are central in the induction of beta-cell survival and proliferation (Withers et al., 1999, Kulkarni, 2005, Elghazi et al., 2006) (Scheme 6).

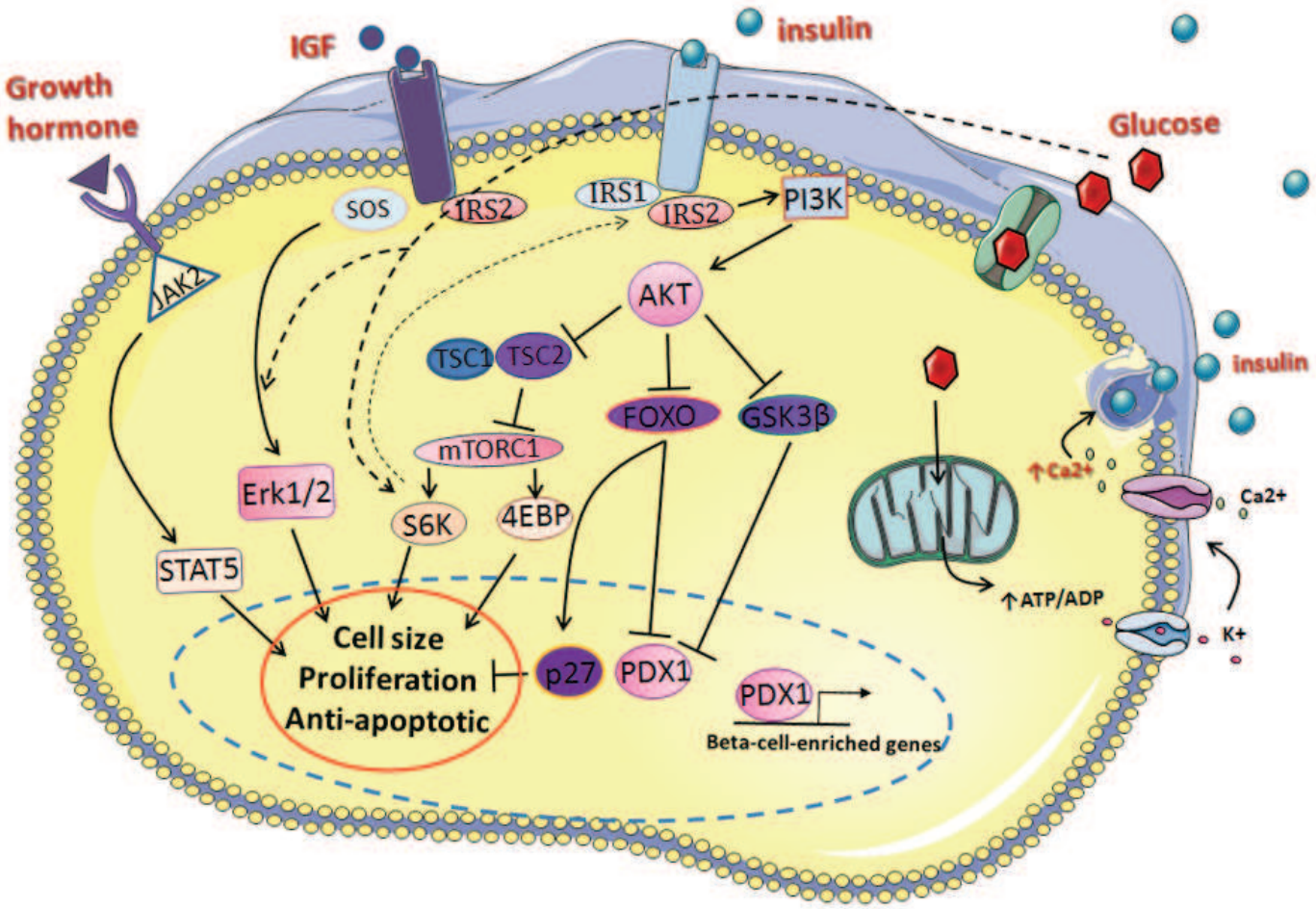
IRS2 is the most abundant and functionally important IRS family member in the pancreatic beta cell (Rhodes, 2005). Studies of mice lacking IRS2 further demonstrated the role of insulin in beta-cell proliferation. IRS2 KO mice are insulin resistant, and unlike IRS1 KO mice, IRS2 KO mice develop overt diabetes because of reduced beta-cell proliferation and increased apoptosis (Withers et al., 1998). Conversely increased IRS2 expression in beta cells prevents diabetes by inducing beta-cell proliferation and survival (Hennige et al., 2003). IRS2 signaling then results in activation of one of the critical mediators of beta-cell proliferation, AKT.

Indeed, overexpression of AKT α or β increases beta-cell mass by augmented proliferation, cell size and resistance to apoptosis (Cho et al., 2001, Tuttle et al., 2001). AKT-stimulated proliferation involves direct phosphorylation of p27Kip1, leading to its nuclear exclusion and increased beta-cell cycling (Uchida et al., 2005).

Another mechanism used for beta-cell cycle regulation by the insulin/AKT pathway is the AKT-mediated phosphorylation and inhibition of the forkhead transcription factor (FoxO) proteins and of glycogen synthase kinase 3β (GSK-3β). An inhibitory role of Foxo1 in beta-cell proliferation has been demonstrated with the misexpression of a constitutively nuclear FoxO1 in beta cells of insulin-resistant mice. This resulted in

reduced Pdx1 expression and inhibited compensatory beta-cell growth leading to diabetes (Nakae et al., 2002). Loss of one allele of Foxo1 in IRS2 KO mice was able to partially rescue the phenotype with a concomitant increase in Pdx1 expression levels (Kitamura et al., 2002). Similarly, loss of one allele of GSK-3β in IRS2 KO mice resulted in increased Pdx1 and reversed the reduced beta-cell proliferation and apoptosis, preserving beta-cell mass and preventing diabetes (Tanabe et al., 2008). Thus, beta-cell survival mediated by insulin/IGF signaling acts through inhibition of Foxo1 and GSK-3β, resulting in relieved function of Pdx1. Accordingly, insulin treatment was associated with the nuclear localization of Pdx1, and the pro-survival effects of insulin were largely absent in islets from Pdx1+/– mice, providing direct evidence that Pdx1 is a signaling target of insulin (Johnson et al., 2006).

Finally, it has also been demonstrated that activated AKT stimulates beta-cell proliferation through phosphorylation and inactivation of tuberous sclerosis complex (TSC) protein 2 (TSC2), an inhibitor of mTOR signaling (Manning et al., 2002) (Scheme 6).



Scheme 6: Signaling pathways activating beta-cell growth

Glucose mainly triggers beta-cell proliferation via the stimulation of insulin secretion and subsequent insulin-mediated autocrine signaling.

Insulin and IGF bind to insulin receptor and IGF receptor respectively. The two main branches of the IGF–insulin signal-transduction pathways are the MAPK and IRS2/AKT pathways, acting via serial activation of protein through phosphorylation. Upon receptor activation, IRS2 is phosphorylated and activates PI3K, which in turn, activates AKT. Phosphorylated AKT then inactivates GSK-3β and FOXO1, which both decrease Pdx1 expression at protein and transcription level respectively. AKT also inactivate TSC2, which results in mTOR activation. mTOR signaling leads to activation of S6K and 4E-BP, which upregulate protein synthesis and, in turn, positively influence beta-cell size. Note that in case of excessive glucose stimulation, S6K triggers a negative feedback loop onto IRS2, inhibiting beta-cell proliferation signaling (small dotted line).

Activated Erk-1/-2 can activate a downstream protein kinase, or directly translocate to the nucleus, promoting transcriptional activation of certain genes and potentially increasing beta-cell mitogenesis and survival.

Glucose activates Erk-1/-2 and S6K as well, independent of IRS2 (dotted arrows), and also stimulates beta-cell proliferation via the triggered increase in cytosolic Ca^{2+}.

Growth hormone binding to its receptor causes dimerization of JAK2 which subsequently activates STAT5, promoting its translocation into the nucleus to activate transcription of genes that potentially promote increased beta-cell mass.

Insulin like growth factors

IGFs are synthesized and secreted mainly from the liver but are also produced locally in almost every tissue, and circulate at much higher concentrations and with a longer half-life than insulin. Insulin and IGF-I bind to the insulin and IGF-I receptor respectively, but can each also cross-react with the other receptor. This makes it difficult to separate the specific effect of these peptides in beta-cell growth. IGF-I and IGF-II increase beta-cell proliferation in rat islets and beta-cell lines in vitro (Hogg et al., 1993). Glucose enhances IGF-I mediated proliferation of insulinoma cells in culture and this process is PI3K-dependent (Hugl et al., 1998). Overexpression of both IGF-I and II in beta cells of transgenic mice is associated with increased beta-cell proliferation, even if only IGF-II (Petrik et al., 1999) and not IGF-I (George et al., 2002) overexpressing mice had increased beta-cell mass.

Growth factors and incretins

Growth hormone, prolactin, GLP-1 and GIP, are known insulinotropic factors as well as having positive effects on pancreatic islet growth. Furthermore, their effects on endocrine pancreatic functions have been corroborated by the respective receptor-knock-out studies (Flamez et al., 1999, Freemark et al., 2002, Liu et al., 2004). Growth hormone and prolactin signaling via the Janus kinase 2 (JAK2)/Signal Transducer and Activator of Transcription 5 (STAT5) induce cyclin D2 expression (Friedrichsen et al., 2001, Friedrichsen et al., 2003) (Scheme 6). The gluco-incretin hormones GLP-1 and GIP have been shown to increase beta-cell proliferation and decrease beta-cell apoptosis induced by cytokines or by glucose and free fatty acids (Drucker, 2006). GLP-1 has previously been demonstrated to protect beta cells against apoptosis 1) by activating IRS2 and AKT via CREB activation (Hennige et al., 2003, Jhala et al., 2003), 2) by activating AKT through phosphorylation (Wang et al., 2004) and also 3) by

transactivating the epithelial growth factor receptor (EGFR) resulting in activation of AKT in a PI3K-dependent manner (Buteau et al., 2003). Additionally, EGFR-mediated signaling has been involved in postnatal beta-cell growth in mice (Miettinen et al., 2006).

3) Beta-cell failure

The normal pancreatic beta-cell response to an excess of caloric supply and obesity-associated insulin resistance is compensatory insulin hypersecretion in order to maintain normoglycemia. T2D only develops in subjects that are unable to sustain the beta-cell compensatory response. In a prospective cohort study, the follow-up of subjects developing T2D showed an early rise in insulin levels during the normoglycemic and prediabetic phases, keeping glycemia near normal despite the insulin resistance (beta-cell compensation). After this long-period of compensation is a stage of stable adaptation, when fasting glycemia surpasses the upper limit of 5.5 mM and when beta-cell mass and insulin secretion start to decrease (beta-cell failure). Eventually, there is a transient unstable period with a rapid rise of glucose reaching overt diabetes (Weir and Bonner-Weir, 2004, Tabak et al., 2009).

In type 2 diabetic subjects, divergent studies describe a beta-cell loss of 25 to 50%, when compared with control subjects matched for BMI (Butler et al., 2003, Rahier et al., 2008). In T1D there is a progressive decline over years in first-phase insulin secretion together with beta-cell mass, which is reduced by 70–80% at the time of diagnosis. Thus, both T1D and T2D are increasingly viewed as beta-cell mass defects-associated disorders, even if the triggering events (glucotoxicity, lipotoxicity, and inflammatory mediators) and the nature of the molecular effectors leading to beta-cell apoptosis in T2D are still in debate (Cnop et al., 2005, Donath et al., 2005).

The question of whether beta-cell mass loss or beta-cell dysfunction alone is sufficient or both are necessary for the development of hyperglycemia has been debated. It appears that neither alone is sufficient. Rather, for glucose to rise to the level at which diabetes would be diagnosed, defects in beta-cell mass and in beta-cell function are required (Kahn et al., 2009).

a) Type 1 diabetes

In the insulitis lesion in T1D, invading immune cells produce cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Predominant work in this field arise from Eizirik and Cnop's lab. They showed that IL-1β and/or TNF-α plus IFN-γ induce beta-cell apoptosis via the activation of beta-cell gene networks under the control of the transcription factors nuclear factor κB (NF-κB) and STAT-1. NF-κB activation leads to production of highly toxic free radical nitric oxide (NO) and chemokines and depletion of endoplasmic reticulum (ER) calcium. The execution of beta-cell death occurs through activation of mitogen-activated protein kinases (MAPKs), via triggering of ER stress and by the release of mitochondrial death signals (Cnop et al., 2005).

However, the involvement of NFκB in the triggering of anti- or pro-apoptotic signals in beta cells is still a matter of debate: previous work from Eizirik's lab showed that inhibition of NFκB activation by overexpression of a dominant negative IκB molecule, or "super-repressor", in beta cells prevents IL-1β+IFNγ-induced apoptosis, presumably by inhibiting NFκB-induced gene transcription of inducible nitric oxide synthase (iNOS) (Heimberg et al., 2001, Eldor et al., 2006). In contrast, *in vivo* inhibition of NFκB in beta cells of NOD mice reduces anti-apoptotic gene expression in beta cells, increases their susceptibility to apoptosis and increases the frequency of T1DM (Kim et al., 2007). However, *in vitro* array analysis performed by Eizirk's group has demonstrated that

NFκB induction by IL-1b and TNF-α has a pro-apoptotic role in beta cells (Ortis et al., 2008).

Pancreatic beta-cells exposed to IL-1β have an early and sustained increase in c-Jun N-terminal kinase (JNK) activity, a phenomenon potentiated by IFN-γ or TNF-α (Eizirik and Mandrup-Poulsen, 2001). Consistently, cell-permeable peptide inhibitors of JNK prevent cytokine-induced apoptosis in insulin-producing cells (Bonny et al., 2001). p38 MAPK and ERK are also activated by cytokines, and pharmacological inhibition of these MAPKs diminished cytokine-induced rat islet-cell death, possibly by attenuating transcriptional activation of iNOS (Saldeen et al., 2001).

Activation of iNOS leads to NO production, which is a triggering event for ER stress. Indeed, for the past few years a large amount of studies are pointing to ER stress as a major convergent mechanism for cytokines-mediated execution of beta-cell death, even if studies from Laybutt's lab contrast this idea (Akerfeldt et al., 2008). Also, ER stress implication in human T1D still needs to be confirmed (Cnop et al., 2008). Disruption of ER homeostasis triggers accumulation of unfolded proteins in the ER lumen and activation of a specific stress response, known as the unfolded protein response (UPR). Because of their high rate of protein synthesis, beta cells are particularly susceptible to ER stress. Upon cytokine treatment, activated NFκB increases iNOS expression, leading to massive NO formation. NO donors trigger an ER stress response in beta cells leading to CHOP expression and apoptosis (Oyadomari et al., 2001). Moreover, IL-1β + IFN-γ-mediated inhibition of SERCA2b expression in primary rat beta cells, via NF-κB activation and NO production, deplete ER Ca^{2+} stores leading to induction of ER stress (Cardozo et al., 2005). In case of prolonged and severe ER stress, the apoptosis program is activated and executed by the transcription factor CHOP, JNK and caspase-12. In particular, activation of JNK could lead to suppression of IRS/AKT signaling through

serine phosphorylation of IRS1 and 2 in beta cells, and inhibition of IRS/AKT signaling reduces survival signals and ultimately leads to apoptosis (Martinez et al., 2008).

b) Type 2 diabetes

Chronic exposure to elevated levels of glucose and free fatty acids (FFAs) causes beta-cell dysfunction and may induce beta-cell apoptosis in T2D. According to the report of Butler et al., the 40% and 63% of beta-cell loss observed in IFG and T2D obese subjects, respectively, compared with weight matched controls was associated with an increase in beta-cell death through apoptosis.

Glucotoxicity

It is important to note that while moderate increases in glucose levels induce beta-cell proliferation and survival, prolonged exposure of beta cells to significant elevations in blood glucose levels causes impaired proliferation and increased beta-cell failure and apoptosis in a way that sometimes counteract the positive effects of acute and transient hyperglycemia. For instance, while transient increase in extracellular glucose induces a significant activation of the CREB protein, through Ca2+ dependant accumulation of cAMP, a 24h incubation in high concentration of glucose reduces both activity and expression of CREB independently of Ca2+ (Costes et al., 2004). As well, the transcription of the insulin gene, which is stimulated by short term exposure to elevated glucose levels, is reduced after long-term exposure to high glucose concentrations (Poitout et al., 2006).

The effect of hyperglycemia on the beta-cell sensitivity to glucose is controversial. A first group of studies indicated that the absence of a glucose-induced rise in ATP production was probably due to hyperglycemia-induced expression of uncoupling protein 2 (UCP2) and was responsible for defective GSIS (Brownlee, 2003). These observations,

conceptualized as beta-cell "glucose desensitization," seem in contradiction with other studies, including that coming from Jonas' lab showing that beta cells exposed to hyperglycemia become more sensitive to glucose for the stimulation of mitochondrial metabolism, proinsulin biosynthesis, and insulin secretion. This would lead to maximal stimulation of triggering and amplifying pathways of GSIS at low glucose (a concept referred to as "glucose hypersensitization"). Therefore islets placed under conditions of prolonged hyperglycemia exhibit basal insulin hypersecretion but defective glucose stimulated insulin secretion (Khaldi et al., 2004).

Chronically increased glucose concentrations cause increased glucose metabolism through oxidative phosphorylation. This causes mitochondrial dysfunction and the production of reactive oxygen species (ROS) which adversely affect beta-cell function and survival (Tanaka et al., 1999). Beta cells have limited defense against excess ROS production due to low levels of ROS-detoxifying enzymes (Tiedge et al., 1997). Markers of oxidative stress are significantly higher in the islets of type 2 diabetic patients than of controls, and the levels of these markers correlate with the degree of impairment of glucose-stimulated insulin secretion (GSIS) (Tanaka et al., 2002). The mechanisms by which ROS decrease beta-cell mass and function are controversial, especially regarding the involvement of NF-κB. Studies from the group of Gordon weir's lab have shown that the generation of ROS will ultimately activate stress-induced pathways, including NF-kB and JNK (Kaneto et al., 2002). In contrast, work from the groups of Eizirik, Jonas and Henquin has specified that glucotoxicity results from an increase in beta-cell oxidative stress and subsequent JNK activation that is NF-kB and NO-independent (Elouil et al., 2005). Moreover, studies conducted in part in Eizirik and Cnop's lab have shown that high glucose do not induce or activate IL-1β expression in beta cells, and consequently induce neither NF-κB, nor iNOS (Welsh et al., 2005, Jorns et al., 2006). These observations argued against the unifying hypothesis that have originated in Donath and

Mandrup-poulsen's work to explain beta-cell death in both T1D and T2D by common mechanisms (Donath et al., 2005).

Other possible explanations for the beta-cell failure occurring before overt hyperglycemia include activation of the ER stress pathway (also in the context of lipotoxicity; see below) and long term increases of cytosolic Ca^{2+} concentration that may be pro-apoptotic and induce beta-cell dysfunction (Khaldi et al., 2004).

An additional event triggered by excess glucose is the increase in mTOR activity. This chronic activation results in serine phosphorylation of IRS2, which leads to its proteasomal degradation, and therefore results in a negative feedback downregulation of insulin signaling (Briaud et al., 2005), as opposed with the pro-survival effect of acute glucose stimulation (Cf. 2) beta-cell growth, scheme 6). Globally, over-stimulation of mTOR/S6K1 by glucose leads to inhibition of the pro-survival insulin/IGF/IRS2/AKT pathway and subsequent induction of the function of GSK3β and Foxo1, which ultimately reduces Pdx1 levels and increases the levels of the cell cycle inhibitor p27. Thus, mTOR signaling links chronic hyperglycemia to beta-cell insulin resistance, which leads to decreased beta-cell proliferation and increased beta-cell apoptosis. Moreover, this observation led to the suggestion that inhibition of mTORC1/S6K1 should improve beta-cell function and survival in T2DM. However, studies in S6K1 knockout mice and in mutant S6 knock-in mice demonstrate the central positive role of mTOR/S6K1 signaling in beta-cell growth and function. In summary, mTORC1/S6K1 has a dual role in the beta cell: it regulates beta-cell size on the one hand and on the other, inhibits insulin/IGF1 signaling, probably leading to increased apoptosis (Leibowitz et al., 2008).

Lipotoxicity

FFAs acutely stimulate insulin secretion, but prolonged exposure to high FFA levels reduces GSIS in rat islets (Zhou and Grill, 1994) and especially in individuals genetically predisposed to T2D (Kashyap et al., 2003). Increased FFAs also lead to impairment of expression of beta cell-enriched genes such as Pdx1, Glut2, MafA and insulin (Gremlich et al., 1997, Hagman et al., 2005). Another possible mechanism by which FFAs may impair beta-cell function involves the expression of UCP2 (Chan et al., 2001, Lameloise et al., 2001). Increased UCP2 helps to safely dissipate the elevated mitochondrial membrane potential and promotes fuel detoxification, because oxidation of these fuels becomes increasingly coupled to heat rather than ATP production. However, this occurs at the expense of ATP synthesis efficiency and consequently insulin secretion.

High levels of saturated FFAs also induce cell death by apoptosis (El-Assaad et al., 2003). Several mechanisms have been implicated, including ceramide formation (Kelpe et al., 2003). Palmitate, serves as a substrate for *de novo* synthesis of ceramide, which leads to activation of JNK with subsequent serine phosphorylation of IRS 1 and 2 and resulting decrease in anti-apoptotic AKT signaling and consequent increased apoptosis. Impaired insulin signaling also results in impaired insulin expression (Solinas et al., 2006). As discussed for the mechanisms of triggered beta-cell death during conditions of glucotoxicity, the involvement of NF-κB and NO in FFA-induced beta-cell death is controversial. Studies have indicated that ceramide formation would induce iNOS, resulting in NO-mediated beta-cell apoptosis (Shimabukuro et al., 1997). However, according to Cnop and colleagues, saturated FFA in the presence of low or high glucose induces beta-cell death in the absence of NF-κB activation and iNOS expression or NO production (Cnop et al., 2005).

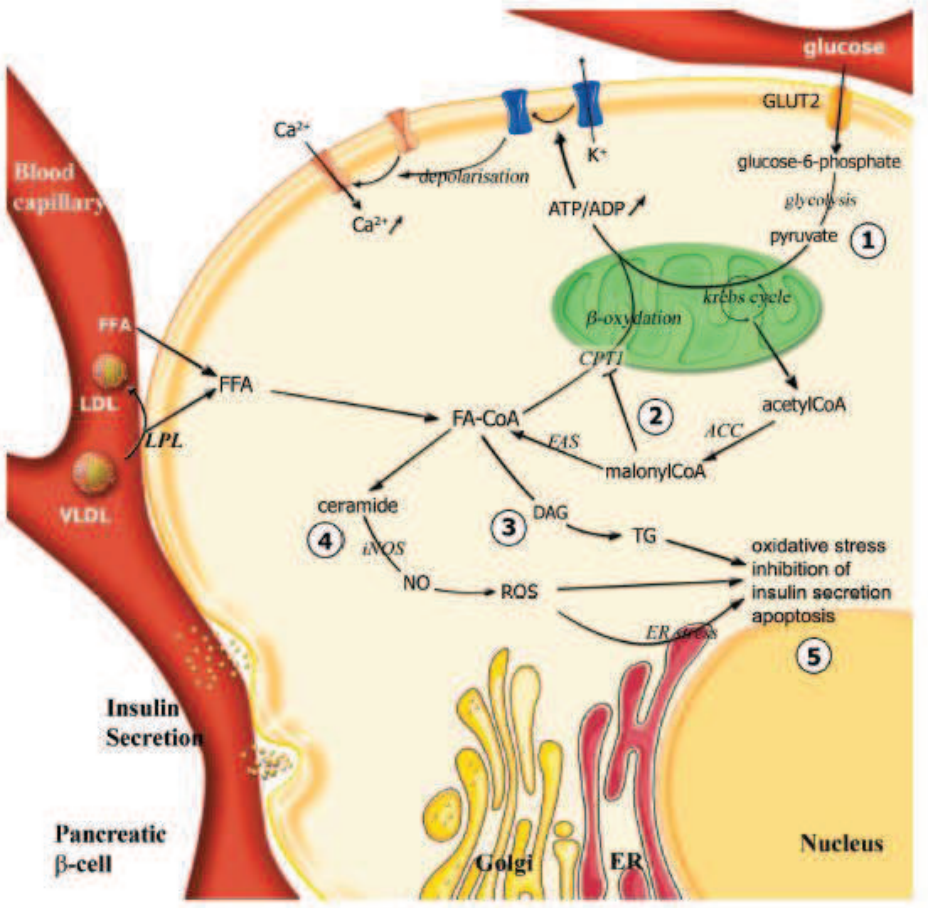
Recently, considerable evidence has been provided in support of a role for the UPR and ER stress in saturated fatty acid-induced cell death (Cunha et al., 2008, Gwiazda et al.,

2009) (reviewed in (Cnop et al., 2008)). Especially, ER stress in islets from type 2 diabetic individuals has been demonstrated by increased staining for ER chaperones and CHOP along with increased ER size (Laybutt et al., 2007, Marchetti et al., 2007). Saturated fatty acids such as palmitate induce ER stress through depletion of ER calcium stores and result in the activation of JNK in a NF-kB and NO-independent mechanism (Cnop et al., 2005).

Glucolipotoxicity

The concept of glucolipotoxicity, as described by Prentki's work (Prentki et al., 2002), has come with the observation that toxic actions of FFAs on tissues only become apparent in the context of hyperglycemia. In this view, hyperglycemia is a prerequisite for lipotoxicity to occur, and the term glucolipotoxicity, rather than lipotoxicity, is therefore more appropriate to describe deleterious effects of lipids on beta-cell function. The proposed mechanism for glucolipotoxicity is the inhibition of FFA oxidation by elevated glucose: in the presence of physiological glucose concentrations, FFAs are oxidized in mitochondria. Conversely, in the setting of hyperglycemia and elevated FFAs, glucose metabolism results in elevated levels of malonyl-CoA, a known inhibitor of carnitine palmitoyl transferase-1 (CPT1), enzyme responsible for the transport of long-chain fatty acyl-CoA (FA-CoA) into the mitochondria where β-oxidation occurs. The inhibition of CPT1 decreases fatty acid oxidation, which causes accumulation of elevated cytosolic long-chain acyl-CoA esters, generation of ceramide and lipid partitioning (Scheme 7). In summary, high glucose inhibits detoxification of excessive fat and promotes partitioning of FFA to toxic complex lipids. Thus, the combination of both nutrients is synergistically harmful and induces beta-cell dysfunction, inhibits insulin gene expression and causes apoptosis (Poitout and Robertson, 2008). For instance, cumulative damage from hyperglycemia, over-nutrition, and elevated FFA levels

overwhelm the ER of the beta cell, resulting in activation of the UPR and eventual apoptosis and beta-cell failure.



Scheme 7: Putative mechanism of glucolipotoxicity
In presence of high glucose concentrations (1), the increased production of malonyl-CoA results in inhibition of the carnitine-palmitoyl-tranferase-1 (CPT1)(2), enzyme responsible for the transport of fatty acyl-CoA (FA-CoA) into the mitochondria where they are β*-oxidized to produce energy. FA-CoA are converted into triglyceride (3) or ceramide (4). The accumulation of these products induces metabolic and oxidative stresses, leading to inhibition of insulin secretion and, eventually, apoptosis (5).*
FFA: free fatty acids; DAG: diacylglycerol; NO: nitric oxide; iNOS: inducible NO synthase; ROS: reactive oxygen species; ER: endoplasmic reticulum; LPL: lipoprotein lipase; LDL: low density lipoproteins; ACC: acetyl CoA carboxylase; FAS: fatty acid synthase

4) Regulation of transcription

a) General mechanisms of transcription

In higher eukaryotes, transcription initiation at promoters recognized by RNA polymerase II is brought about by concerted action of general transcription factors

(GTFs) and the RNA polymerase core enzyme. This assembly forms the pre-initiation complex (PIC), which initiates transcription and begin transcription elongation. Activators of transcription are often defined as sequence-specific DNA-binding proteins that stimulate transcription initiation or elongation. Many activators interact with proteins known as co-activators. Both can activate transcription by promoting the alteration of chromatin structure in the proximity of the promoter. Three classes of proteins are involved in this remodelling of chromatin: histone-modifying enzymes, chromatin-binding proteins, and ATP-dependent nucleosome-remodelling proteins. These changes in chromatin structure regulate transcription by altering the local accessibility of the DNA to transcription factors, RNA polymerase II, and other components of the PIC (Carey, 1998).

b) Repression

Transcriptional repression is of two types: general repression or gene-specific repression. General repression occurs when a repressor protein or complex either sequesters or modifies a central component of the PIC so that it is unavailable for transcription. In contrast, gene-specific repression occurs when the transcription of a particular gene or set of genes is controlled by the activity of gene-specific repressor or co-repressor.

In eukaryotes the mechanisms of gene-specific repression were first described as being either direct (active) or indirect (passive). Passive repressor proteins do not have intrinsic repressing activity or repression domain, and can mediate repression by two means: by competing with transcriptional activators for a common binding site (such as the inducible cAMP early repressor ICER (Molina et al., 1993)), or by the sequestration of an activator into an inactive complex (such as IkB). In contrast to passive, active

repressor proteins have an intrinsic repression activity that targets the chromatin organization of the genome.

However, over recent years, there has been a huge increase in the number of characterized repression mechanisms and furthermore, many repressors are now known to act via a combination of several mechanisms and the mechanism used by a particular repressor is often promoter dependent (such as the retinoblastoma protein). There are three major routes through which repressor proteins can downregulate specific genes: inhibition of the basal transcription machinery, ablation of activator function, and remodelling/compaction of chromatin.

Categories of repressor proteins are not easily defined and many examples of repressors do not fit into one or the other. In addition, there are a rapidly expanding number of 'context-dependent' transcription factors that bind DNA and are capable of positively or negatively regulating transcription depending on the context of their binding sites, the complement of protein interactions they can make and other environmental cues. A recent classification have divided repressor proteins into three main classes (Table 1) (Gaston and Jayaraman, 2003).

Class	Defining feature	Examples
Class I: DNA-binding proteins		
A	sequence-specific DNA binding	PRH, Eve, Krüppel, TGIF, Mad, IRF-2, RP58, E2F-6
B	methyl-CpG-binding proteins	MeCP2, MBD2
Class II: bind to DNA-binding proteins		
A	proteins (co-repressors) that bind to dedicated repressors	Dnmt3, MBD3, Tup1, Groucho
B	proteins that bind to context-dependent transcription factors	Groucho, CtBP, TGIF, NcoR, Rb, MDM2
Class III: other repressors		
A	bind to activators, co-activators, or PIC	IκB, Mot1, FIR, GAL80, E1A243R, Rb, PHO80,
B	post-translationally modify activators, co-activators or PIC	OGT, PHO80, CARM-1, Srb10, CCK-II

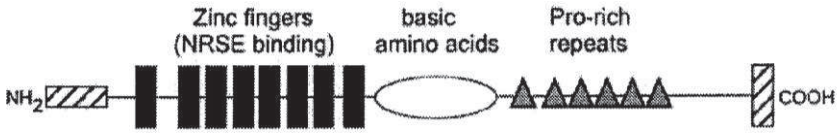
Table1: Classes of repressor proteins. *Reproduced from (Gaston and Jayaraman, 2003).*

The alteration of chromatin structure into a repressive state can involve different kinds of histone-modifying enzymes including histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone kinases, as well

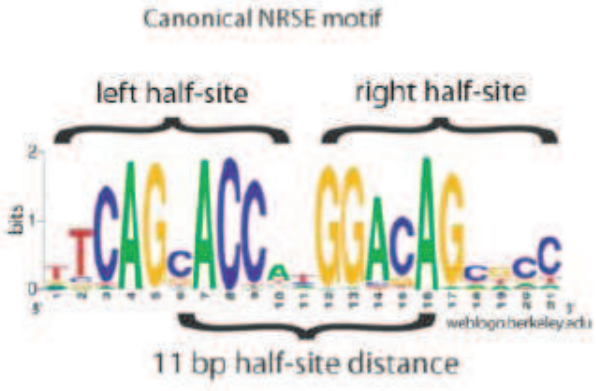
as enzymes that ubiquitinate histones. Transcription activation is generally associated with the acetylation of histones, whereas transcription repression is generally associated with their deacetylation.

5) The transcriptional repressor REST

Elucidation of the mechanisms that control neuronal gene expression is important for understanding how the brain develops or responds to insults. In this regard, a particular transcription factor, RE-1 silencing transcription factor (REST), have received considerable attention. REST is a nine zinc-finger transcription factor (Scheme 8) related to the GLI-Kruppel family which binds in target genes to a conserved 21-nucleotide element called RE-1 (Scheme 9) to inhibit their transcription.



Scheme 8: Schematic representation of the repressor element 1 (RE-1)-silencing transcription factor (REST) protein.
The nine zinc fingers are required for DNA binding to the RE-1. The two segments of the protein that have been defined to have repressor activity include the 83 N-terminal residues and the C-terminal zinc finger. Reproduced from (Jones and Meech, 1999).



Scheme 9: Canonical sequence of the RE-1
Ninety-three sequences known to bind REST were combined to form a position-specific scoring matrice (PSSM), here shown below a Weblogo recording the sequence conservation at each nucleotide position of this set. The height of each letter is proportional to its information content. Reproduced from (Johnson et al., 2007).

a) Identification

In 1995, Gail Mandel and David Anderson's groups have identified the protein that binds to the repressor element-1 (RE-1) present in the rat Scn2a2 gene (also known as Nav1.2) (Kraner et al., 1992), which has been referred to as neuron restrictive silencer element (NRSE) in the context of the rat Stmn2 gene (also known as SCG10) (Mori et al., 1992). This protein was therefore called REST, for RE-1 Silencing Transcription factor (Chong et al., 1995) and NRSF, for Neuron-Restrictive Silencer Factor (Schoenherr and Anderson, 1995). Because RE-1 sites had been mainly identified in genes involved in specific functions of terminally differentiated neurons (Table 2), REST was initially thought to function as a master repressor of neuronal genes.

Gene	Identification	Gene-Ontology	References
Scn2a1 (sodium channel, voltage-gated, type II, alpha1)	Gene reporter	Action potential Neurodevelopment	(Chong et al., 1995)
Stmn2 (SCG10)	Gene reporter	Neuronal growth Microtubule dynamics	(Schoenherr and Anderson, 1995)
Nrcam (neuronal-glia cell adhesion molecule)	Gene reporter	Cell adhesion Neurodevelopment	(Kallunki et al., 1995)
Syn1 (synapsin1)	Gene reporter	Neurotransmitter release	(Schoch et al., 1996)
Chrm4 (muscarinic acetylcholine receptor M4)	Gene reporter	Signaling pathway	(Wood et al., 1996)
Chrnb2 (nicotinic acetylcholine receptor, beta2)	Gene reporter	Dopaminergic synaptic transmission Action potential	(Bessis et al., 1997)
Chat (choline acetyltransferase)	Gene reporter	Neurotransmitter synthesis	(Lonnerberg et al., 1996)
L1cam (L1 cell adhesion molecule)	Gene reporter	Cell adhesion Neurodevelopment	(Kallunki et al., 1997)
Tubb3 (beta III-tubulin)	Endogenous (IHC)	Axon guidance Neurodifferentiation	(Chen et al., 1998)
Gria2 (glutamate receptor 2)	Gene reporter	Synaptic transmission	(Myers et al., 1998)
Grin1 (glutamate (NMDA) receptor subunit zeta-1)	Gene reporter	Synaptic transmission	(Bai et al., 1998)

Gabrg2 (GABA receptor subunit gamma-2)	Gene reporter	Synaptic transmission	(Mu and Burt, 1999)
Bdnf (brain-derived neurotrophic factor)	Gene reporter	Synaptic plasticity Neurodevelopment	(Timmusk et al., 1999)
Mapk8ip1 (islet-brain 1)	Gene reporter	Regulation of JNK pathway Beta-cell survival	(Abderrahmani et al., 2001)
Oprm1 (mu opioid receptor)	Gene reporter	Signaling pathway Behavior	(Andria and Simon, 2001)
Nppa (atrial natriuretic peptide)	Gene reporter Endogenous (NB)	cGMP biosynthesis Regulation of blood vessel size	(Kuwahara et al., 2001)
Crh (corticotrophin releasing hormone)	Gene reporter	Glucocorticoid biosynthesis Feeding behavior	(Seth and Majzoub, 2001)
Tac1 (protachykinin 1)	Gene reporter	Neuropeptide signaling Regulation of blood pressure	(Quinn et al., 2002)
Prlhr (prolactin releasing hormone receptor, GPR10)	Gene reporter	Signaling pathway Feeding behavior	(Kemp et al., 2002)
Syp (synaptophysin)	Gene reporter	Synaptic transmission	(Lietz et al., 2003)
Pax4 (paired homeobox 4)	Gene reporter	Beta-cell differentiation	(Kemp et al., 2003)
Htr1a (serotonin receptor 1A)	Gene reporter	Mood Feeding behavior	(Lemonde et al., 2004)
Vip (vaso-intestinal peptide)	Gene reporter	Neurodevelopment Immunity	(Hamelink et al., 2004)
P2ry4 (P2Y purinoreceptor 4)	Endogenous (RT-PCR/ChIP)	Signaling pathway	(Bruce et al., 2004)
Nptxr (neuronal pentraxin receptor)	Endogenous (RT-PCR/ChIP)	Signaling pathway	(Bruce et al., 2004)
Nrxn3 (neurexin III)	Endogenous (RT-PCR/ChIP)	Synaptic transmission	(Bruce et al., 2004)
Nefh (neurofilament)	Endogenous (RT-PCR/ChIP)	Cytoskeleton organization	(Bruce et al., 2004)
Grin2a (glutamate receptor 2A)	Endogenous (RT-PCR/ChIP)	Neurotransmission	(Bruce et al., 2004)
Grin2b (glutamate receptor 2B)	Gene reporter	Synaptic transmission Action potential	(Qiang et al., 2005)
Ntrk3 (neurotrophic tyrosine kinase, receptor type 3)	Gene reporter	Neurodevelopment	(Nakatani et al., 2005)
Calb1 (Calbindin)	Endogenous (qPCR)	Synaptic plasticity	(Ballas et al., 2005)
Syt4 (synaptotagmin IV)	Endogenous (qPCR)	Exocytosis	(Ballas et al., 2005)
Th (tyrosine hydoxylase)	Gene reporter	Dopamine biosynthesis Catecholamine biosynthesis	(Kim et al., 2006)

Trpc1 (transient receptor potential channel 1)	Endogenous (WB)	Calcium ion transport	(Ohba et al., 2006)
Nrp1 (neuropilin 1)	Endogenous (RT-PCR/WB)	Axon guidance Neurodevelopment	(Kurschat et al., 2006)
Hcn4 (hyperpolarization-activated cyclic nucleotide-gated K^+4)	Gene reporter	Potassium, sodium transport	(Kuratomi et al., 2007)
Uchl1 (ubiquitin carboxy-terminal hydrolase L1)	Endogenous (qPCR/ChIP)	Neuromuscular process axonogenesis	(Barrachina et al., 2007)
Bsx (brain specific homeobox)	Gene reporter	Neurodevelopment	(Park et al., 2007)
Cartpt (cocaine and amphetamine-regulated transcript protein)	Gene reporter Endogenous (RT-PCR/ChIP)	insulin secretion Feeding behavior	(Li et al., 2008)
Slc12a5 (K-Cl cotransporter 2)	Gene reporter Endogenous (IHC)	Synaptic transmission	(Yeo et al., 2009)
Kcnd3 (potassium voltage-gated channel D member 3)	Endogenous (qPCR/ChIP)	Neuropathic pain	(Uchida et al., 2010)
Pcdh (protocadherin)	Gene reporter Endogenous (RT-PCR)	Cell adhesion Neurodevelopment	(Tan et al., 2010)

Table 2: Comprehensive list of identified bona fide REST target genes
First column indicates the NCBI gene name annotation and common name between comas; Second column indicates the techniques used to demonstrate the REST-mediated transcriptional regulation (NB: Northern blot; WB: Western blot; IHC: immunohistochemistry; ChIP: chromatin immunoprecipitation); Third column indicates the GO terms associated with the gene according to NCBI.

When REST was discovered, its expression has been observed at high levels in most non-neuronal tissues, but not in neurons, confirming that the role of REST is to repress neuronal gene expression outside of the central nervous system (Chong et al., 1995, Schoenherr and Anderson, 1995). Using RNA *in situ* hybridization, the two groups also found that REST was expressed in neuronal progenitors, being then repressed as neurons differentiate and migrate to the marginal zone, suggesting an additional role of REST in neuronal differentiation. The simplistic scheme of REST action was thus dichotomized, according to the neuronal vs. non-neuronal cellular contexts: its expression in neuronal progenitors regulates the timing of differentiation, by preventing

from a precocious expression of genes related to terminal functions of differentiated neurons; in non-neuronal cells, REST ensures, through silencing of all RE-1-containing genes, that these neuronal traits-encoding genes be expressed specifically into neuron-related cell types. However, ever growing evidence indicates that the role and mechanisms through which REST acts are much more subtle than previously described.

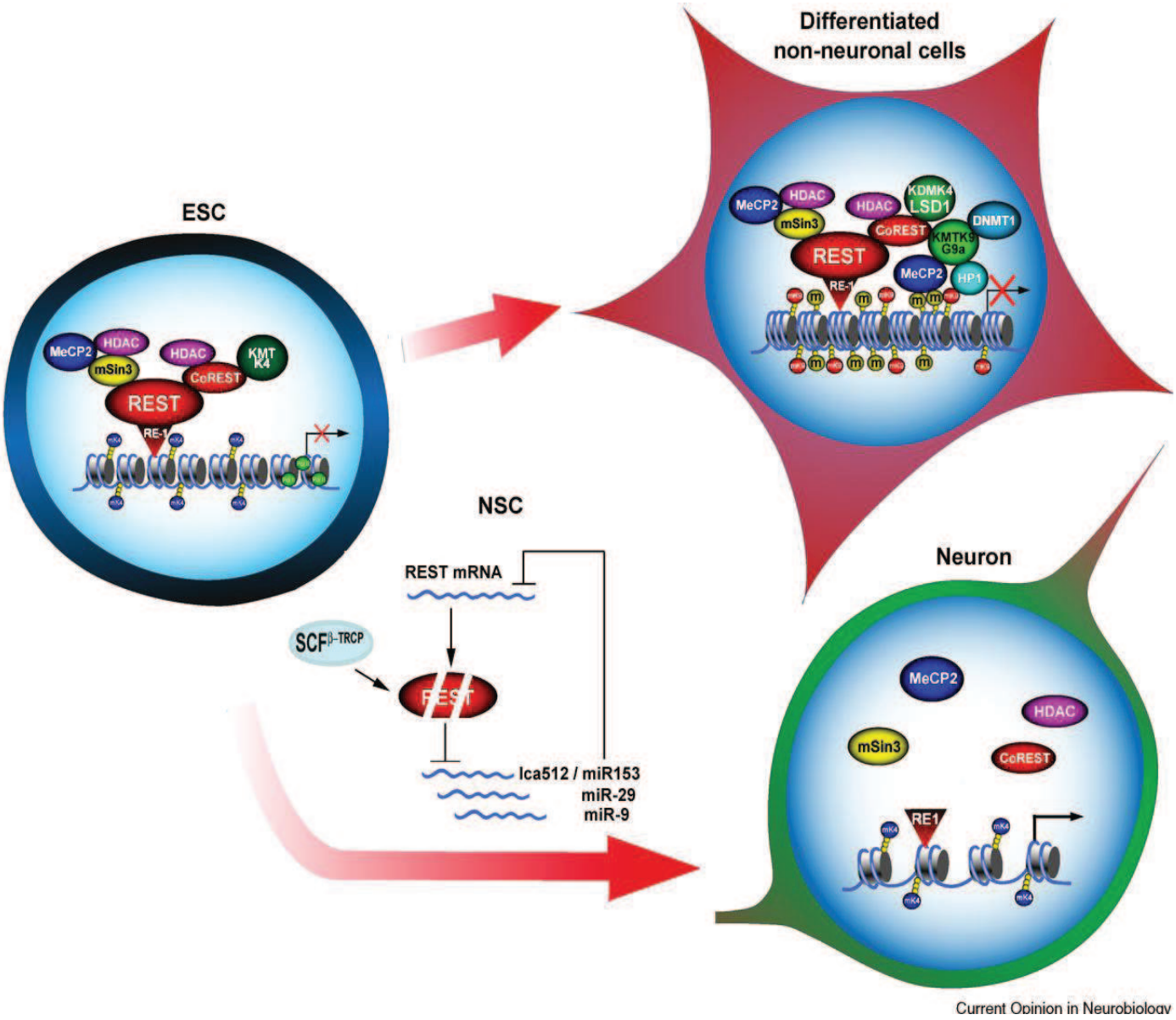
b) Mechanisms of repression

Several studies have suggested that the regulation of transcription mediated by REST is a selective process and involves distinct mechanisms, in a promoter- and cell-dependent fashion. This assumption first came out with the observation that in REST KO mice, the lack of REST did not lead to widespread reactivated expression of all RE-1-containing genes in non-neuronal tissues (Chen et al., 1998). Even if the proposed explanation for this lack of induction was the absence of appropriate activators (REST KO mice dye early in embryogenesis), other works have demonstrated that REST is not always required to control the expression of its target genes. For instance, using JTC-19 rat fibroblasts, the group of Noël J. Buckley identified differential modes of regulation of the expression of two REST targets, the muscarinic acetylcholine receptor M4 and the sodium channel Type II (Nav1.2) genes. The expression of Nav1.2 is repressed via REST recruitment (demonstrated by chromatin immuno precipitation (ChIP) and with relieved expression upon transfection of the dominant negative binding domain of REST) and does not involves histone deacetylase (as shown with stable Nav1.2 expression upon treatment with the inhibitor of HDAC, trichostatin A (TSA)) (Belyaev et al., 2004). In contrast, M4 expression in JTC-19 cells is repressed in a REST- and HDAC-independent mechanism, whereas its expression in PC12 neuronal cells can be repressed upon REST transduction and recruitment to the RE-1 binding motif (Wood et al., 2003, Belyaev et al., 2004). A striking conclusion from M4 (Wood et al., 2003) and Cx36 (Hohl and Thiel, 2005) gene

expression studies suggests that even if ectopic REST may be sufficient to down-regulate a specific target in neurons, REST is sometimes not required for the repression of the same target in non-neuronal cells. Using U373 glioma cells expressing REST, the authors of a third study from Buckley's lab have also shown that REST can be preferentially recruited to some but not all RE-1 sites in a particular cell type, resulting in differential regulation of gene expression. In this study, REST target genes were classified into different groups: those that are normally un-occupied by REST but can be repressed with higher REST concentration (suggesting a limitation in the binding affinity of the RE-1 and/or of the endogenous REST concentration), those that are occupied by REST but not repressed (suggesting a non-functional RE-1 site), those that are occupied and repressed by REST, and finally, those that appear to be un-occupied by REST but repressed anyway (suggesting additional mechanism of control of gene expression) (Bruce et al., 2004). Altogether, these studies concluded that REST differentially controls the expression of its target genes in a cell- and promoter-specific manner, and that this selectivity occurs through the recruitment of different co-repressors-containing complexes.

Gene repression by REST depends on the recruitment of multiple enzymatic corepressor complexes that modify chromatin to repress transcription. These include mSin3A (Huang et al., 1999, Grimes et al., 2000), which recruits histone deacetylases (HDACs), and CoREST (Andres et al., 1999), which also recruits HDACs in addition to the histone demethylase, LSD1 (Shi et al., 2004). The H3K9 methyltransferase G9a, heterochromatin protein 1 (HP1), and the methyl DNA binding protein MeCP2 are also associated with REST repression (Lunyak et al., 2002, Roopra et al., 2004). Deacetylation of core histones by HDACs leads to chromatin compaction, which becomes inaccessible to the transcriptional machinery. Dimethylation of lysine 9 of histone H3 (diMeK9H3) by G9a is usually associated with silencing and provides a high-affinity binding site for HP1, which

causes the formation of a compacted, transcriptionally inactive chromatin (heterochromatin). Mediator, a multiprotein complex that modulates the repressive activity of G9a has also been found associated with REST co-repressor complexes (Ding et al., 2008). In contrast to diMeK9H3, diMeK4H3 is a modification usually associated with transcriptionally active chromatin, and SMCX, that mediates K4H3 demethylation, is part of the REST repressor complex (Tahiliani et al., 2007). To explain the different mechanisms of REST-mediated repression, Ballas et al., from Mandel's lab, have identified, using ChIP experiments, the co-repressor complexes recruited by REST at different promoters in different cellular contexts (Ballas et al., 2005). They have analyzed differentiated non-neuronal cells, embryonic stem cells (ESC), neural stem cells (NSC) and differentiated neurons. The REST core complex constituted by REST, mSin3, CoREST, HDAC1/2 and MeCP2 was recruited to the analyzed RE-1 sites in every situation, except in cortical neurons where REST is absent. In non-neuronal cells, stable repression of RE-1-containing genes is brought about by high levels of G9a recruited to the chromatin and resulting diMeK9H3, and by a densely methylated DNA at CpG sites. In ESC, in contrast, RE-1-containing regions were characterized by a low methylation, an absence of diMeK9H3 associated with low levels of recruited G9a, and enriched di- and tri-methylated K4H3 (Scheme 10). The emerging picture is that REST can mediate both transient (i.e., during neural differentiation) and long term repression (i.e. in differentiated non-neuronal cells), by recruiting different complexes that will establish distinct epigenetic marks. Especially, the modifications directed in ESC maintain the chromatin in a repressed state which is nonetheless poised for subsequent activation (Ballas et al., 2005).



Scheme 10: ***Chromatin remodeling by differential REST-mediated recruitment of co-repressors.*** *In ES cells and differentiated non-neuronal cells, chromatin is relatively compact and neuronal genes are actively repressed by REST and its corepressors. However, the presence of KMT K4, which generates methylated lysine 4 on histone H3 (mK4, blue circles), and RNA polymerase II (Pol II, light green circles), renders stem cell chromatin permissive for very low basal transcription of neuronal genes (thin red cross). In contrast, neuronal genes are not expressed (thick red cross) in differentiated non-neuronal cells because the DNA around the RE1 sequence is methylated (m, yellow circles), thereby facilitating the binding of MeCP2 and other corepressors including the KDM K4, LSD1, which demethylates mK4, and the KMT K9, G9a, which methylates lysine 9 on histone H3 (mK9, red circles). During neuronal differentiation, REST is degraded by $SCF^{\beta\text{-}TRCP}$-mediated proteasomal degradation, leading to the relieved REST-mediated repression of miRNAs. This in turn, promotes a feed-forward loop onto REST mRNA, via miR-9-, miR-29- and miR-153-mediated transcriptional repression. REST corepressors also dissociate from RE1, resulting in a more loosely packaged chromatin and the expression of neuronal genes in mature neurons. Adapted from (Juliandi et al., 2010).*

c) REST regulon

To gain insight into the role of REST, bioinformatic analyses have been conducted by several independent labs to identify REST targets on the basis of DNA sequence alone. The first study was made by Anderson's lab, using a composite RE-1 consensus sequence to perform a simple search in GeneBank database for putative RE-1-containing genes (Schoenherr et al., 1996). They identified 25 genes, most of them being involved in specialized neuronal functions, including already known targets SCG10 (Schoenherr and Anderson, 1995), Nav1.2 (Chong et al., 1995), Synapsin I (Li et al., 1993) and BDNF (Timmusk et al., 1999). A second bioinformatic search in the human and murine genome has identified around 1000 putative target genes and suggested that a majority of them were considered as neuron-specific, while some putative targets appeared to be related to angiogenesis and chromatin remodeling (Lunyak et al., 2002). In 2004, Buckley's went a step forward with the generation of a database compiling the 1800 putative RE-1-containing sites identified, *in sillico*, in the human and mouse genomes (http://www.bioinformatics.leeds.ac.uk/cgi-bin/RE1db/nrse.cgi). The identified genes were associated with various aspects of neuronal function but also with very diverse functions such as transcriptional regulation or metabolism, suggesting that the role of REST is probably not limited to the control of neuronal-specific genes only (Bruce et al., 2004). In two recent *in sillico* analyses, based on a comparative analysis of RE-1 conservation during evolution, more than 800 sites containing RE-1 motifs were found in the human genome, a majority of which being associated with neuronal functions (Mortazavi et al., 2006, Wu and Xie, 2006). Their novel findings was that more than 10 of these predicted sites were located near miRNAs, many of them being considered as brain-specific (including miR9, miR124, miR132, miR135, miR139 and miR153). More importantly, by searching for predicted targets of these miRNAs, the authors indicated

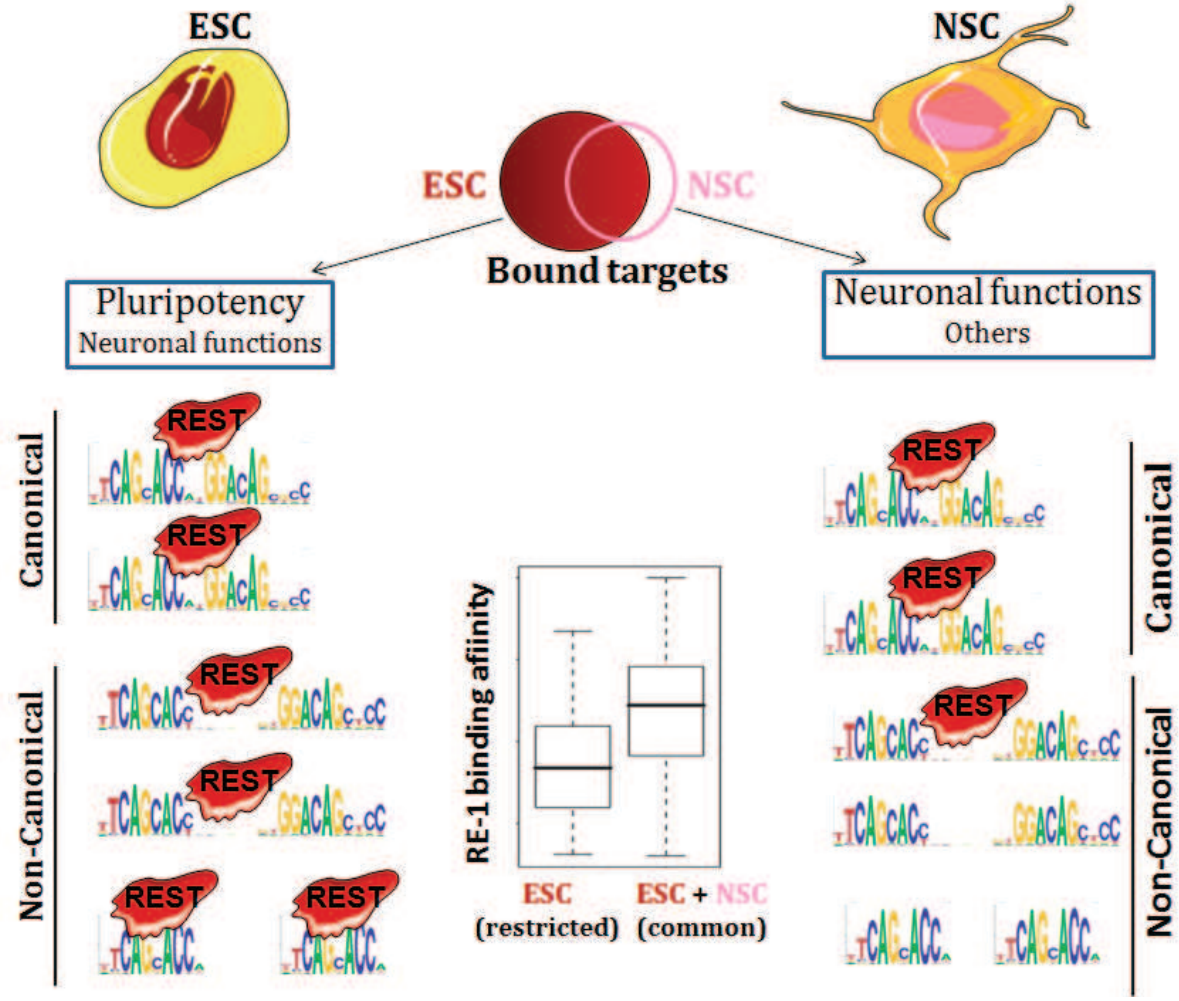
that CoREST is a prospective target of three of them, including miR-124a and miR-153, and that REST itself is a predicted target of miR-153 (Mortazavi et al., 2006, Wu and Xie, 2006) and of miR-9 and miR-29a (Wu and Xie, 2006). These data indicate a potential double-negative feedback loop between REST and brain-related miRNAs in controlling neuronal gene expression. This two-component system with mutual inhibition results in a more stable mechanism, where only one of the two components is active and stabilized in a particular state. When REST is silenced during neurodifferentiation, via $SCF^{\beta\text{-TRCP}}$-mediated proteasomal degradation (see d), e) cancer, and Chapter 3), this results in a feedforward loop directed onto its own expression, via the relieved expression of miRNA that targets REST mRNA for degradation (Mortazavi et al., 2006, Wu and Xie, 2006) (Scheme 10). A paper from Mandel's group confirmed that miR-124a is down-regulated by REST in non-neuronal cells and in neuronal progenitors, and further specified that REST and miR-124a have reciprocal activities. Whereas REST acts to repress the expression of neuronal genes, the role of the brain-specific miR-124a is, conversely, to decrease the expression of non-neuronal genes (Conaco et al., 2006). The combined actions of REST and miR-124a are thus supposed to maximize the contrast between neuronal and non-neuronal phenotypes. Collectively these reports suggest that REST action is wide. It directly down-regulates a large number of genes at the transcription level, and also indirectly activates the expression of other genes at the post-transcriptional level via the repression of miRNA expression.

The prediction of the set of genes that may be regulated by REST has greatly evolved with the apparition of wide scale- and deep-sequencing-based methodologies that allow increased sensitivity in the detection of low-level interactions (their loosed detection may have led to previous false conclusions in regards to the mechanisms brought about by REST). For instance, the serial analysis of chromatin occupancy (SACO) conducted on a murine kidney cell line by Otto et al., from Mandel's lab has provided several important

insights (Otto et al., 2007): 1) the location of RE-1 binding sites is not restricted to promoter regions, as 30% are found in the 5' part, 30% in the 3' part, 20% in introns and 4% in exons 2) novel RE-1 binding sites were identified, either expanded or compressed compared with the consensus RE-1, and even if they are sub-represented in the SACO experiment (30%), these non-canonical sites greatly increased the number of putative RE-1-containing sites found in the genome using *in sillico* analyses. Although some of these predicted target genes were found to be involved in non neuronal functions, including immune/inflammatory response and cell adhesion, a large network of targets is devoted to neurotransmission 3) using ChIP analysis of a large set of predicted target genes, the authors suggested that a majority of RE-1 sites are occupied by REST, *in vivo*, as opposed with previous conclusions describing the selective binding of REST to some but not all RE-1 (see above). However, the contribution of REST in maintaining stable repression still seemed to depend on each specific target. Recently also, the study from Stanton's and Buckley's labs presented a combination of ChIP with deep sequencing through paired-end tag (PET) technology to decipher the profiles of REST recruitment to the chromatin in ESC or NSC (Johnson et al., 2008). They identified 2460 RE-1 sites represented as PET clusters, a high number that could be accounted for by different existing combinations of RE-1 half sites, resulting in a variety of non-canonical RE-1-like binding motifs. Moreover, they observed that REST controls distinct transcriptional networks in each cell types, 50% of the targets being recruited in both ESC and NSC, 45% being restricted to ESC and very few being only specific to NSC. The majority of the common targets are devoted to neuronal functions, and even if such targets are also represented in the ESC-specific set, the authors have found that the ESC-specific network of REST recruitment is largely integrated into those of known inducers of pluripotency, Oct4, Nanog and Sox2 (see e) ES cells pluripotency). This study also specified that the targets restricted in ESC seemed to exhibit weaker sequence

conservation of the RE-1, meanwhile the targets that are common to ESC and NSC have higher quality RE-1 mediating higher levels of REST occupancy (Johnson et al., 2008) (Scheme 11). Finally, the mechanisms directing cell type- and target-specific regulation mediated by REST have been recently explained by the variations in the RE-1 DNA sequence itself, in a publication using ChIP in combination with microarray (ChIP-chip) (Bruce et al., 2009). By comparing chromatin occupancy, modifications, and gene expression in 8 different cell lines, the authors have observed that 1) the number of targets bound in a cell type reflects the level of REST protein 2) some RE-1 sites are commonly bound in all cell lines (common sites), while some are restricted to 2 to 6 cell lines (restricted sites) and others bound in a unique cell type (unique sites) 3) common sites show the highest levels of REST occupancy, followed by restricted and unique sites 4) common sites with high REST binding affinity resemble consensus RE-1, while weaker sites have some non-canonical variations (restricted sites), or show substantial degeneracy, resembling a RE-1 half site (unique sites) 5) restricted and unique target genes, when compared to high binders, are more likely to be already expressed at low levels, and are associated with both active and repressive epigenetic marks. This is characteristic of "bivalent" structure where rapid transcriptional responses are needed, and where genes need to be primed for expression. In conclusion, this study established that the level of REST protein and the sequence variations in the RE-1 motif direct a hierarchy of REST binding affinity to its target genes (Bruce et al., 2009) (Scheme 11). Moreover the weak binders, which show cell-type specific expression, are thought to represent the majority of REST target genes (it is of note that this rule may not be applicable to the study of Stanton and Buckley's lab exposed above, in which the different cell types (ESC and NSC) are actually related through differentiation). Collectively, these data suggest that REST repress distinct set of genes in different cell

types, contrasting with the view that REST is a global regulator in all non-neuronal tissues.



Scheme 11: REST regulon is determined by distinct recruitment profiles
The number of targets bound by REST is determined by the level of REST expression and by the RE-1 sequence. In ESC expressing high level of REST (left), a high number of targets are bound. Bound targets globally show weaker RE-1 binding affinities (middle; represented by a low RE-1 motif score (non-canonical motifs) and low ChIP counts) and are largely integrated into networks of pluripotency genes. In NSC, the lower REST levels dictate the lower number of bound targets (right). These bound targets show better RE-1 quality (canonical RE-1) and are mostly involved in neuronal functions. Thus, the number of bound targets decreases with neuronal differentiation, and the strong binders are likely to be commonly bound in different cell types. In this perspective, the NSC and differentiated non-neuronal cell profiles of REST recruitment are more similar to each other than to ESC, indicating an additional profile devoted to pluripotency in these cells.

d) Regulation of REST expression

Very few information has been gathered around the regulation of the REST gene itself. One paper has identified three different exons (A, B and C) in the 5'UTR of the mouse REST gene. Each exon can be alternatively spliced, leading to a differential regulation of REST gene expression through possible combinatorial arrangements of these promoters, together with the six enhancer and two repressor regions that are found associated with them. Promoter A seems to be the most efficient in directing REST expression in non-neuronal cells, whereas promoter B has the highest activity in neuronal cells (Koenigsberger et al., 2000). Using electroporation of stage 15-16 chick spinal cord, it has been shown that REST could be induced by the Wnt canonical pathway to participate to the proliferative state of neural progenitors. *In ovo* transfection of Wnt1 and β-catenin resulted in increased REST expression and this effect was mediated by a Tcf element found in the exon1A of REST, as demonstrated by Luciferase experiments (Nishihara et al., 2003). In the search for a stably induced repressor of the neuronal phenotype involved in differentiation and maintenance of astrocytes, a recent study has identified bone morphogenetic protein (BMP) 2 as an activator of REST expression. BMP2 treatment in NSC, resulting in astrocyte differentiation, led to induction of REST mRNA and protein, and was mediated by Smad1 binding to Smad binding elements found in the 5'UTR of REST gene (Kohyama et al., 2010). Concerning the extinction of REST expression, that is normally seen during neuronal differentiation (see Scheme 10, below, and Chapter 3), the Notch effector Hes-1 has been suggested as a possible candidate in a study of Waeber's group, using a model of Hes-1 ectopic expression in HeLa cells. ChIP experiment showed that Hes-1 was able to bind the REST promoter through an N element and to mediate a decrease in REST expression (Abderrahmani et al., 2005). It is also likely that REST itself exerts an autoregulatory feedback on its own

expression since two studies have identified RE-1 sequence in its gene. The ChIPSeq method of Wold and Myers (discussed in Chapter 3) has identified a non-canonical RE-1 motif 500 bp downstream of the REST transcription start site (TSS) (Johnson et al., 2007), and the ChIP-PET technology of Stanton and Buckley (see also Chapter 3) revealed 3 PET clusters associated to the REST gene. Moreover, the recruitment of REST to its own gene has been validated by conventional ChIP-qPCR (Johnson et al., 2008). In contrast, Ballas and Mandel's work did not observe REST occupancy on its own gene. However, they found that REST expression was induced in neurons upon TSA treatment, and that HDAC was recruited to a retinoic acid (RA) receptor element (RARE) 400 bp upstream of the REST TSS, together with mSin3A, CoREST, MeCP2, RA receptor and N-CoR (Ballas et al., 2005). They also have shown in this study that the first step in the clearance of REST that accompany neuronal differentiation, occurs via posttranslational degradation of REST protein. The protein responsible for this degradation has been identified as an E3 ubiquitin ligase, called $SCF^{\beta\text{-TRCP}}$ (Westbrook et al., 2008). Then, the emerging picture explaining the REST extinction involves miRNAs-mediated degradation of REST mRNA: REST proteasomal degradation may lead to a switch in the double-negative feedback loop between REST and brain-related miRNAs, which favors miR-153, miR-29a, and miR-9 expression. This in turn, leads to a feedforward loop to promote REST mRNA degradation, mediated by these miRNAs (Mortazavi et al., 2006, Wu and Xie, 2006) (Scheme 10).

e) Physiological role and implication in disease

According to the wide number of genes that REST may control in the whole organism, it is expected that any perturbation of REST expression (loss- or gain-of-function) in any tissue may lead to a number of cellular and physiological perturbations that may

account for certain diseases (for reviews, see (Majumder, 2006, Ooi and Wood, 2007, Gopalakrishnan, 2009)).

Neuronal differentiation

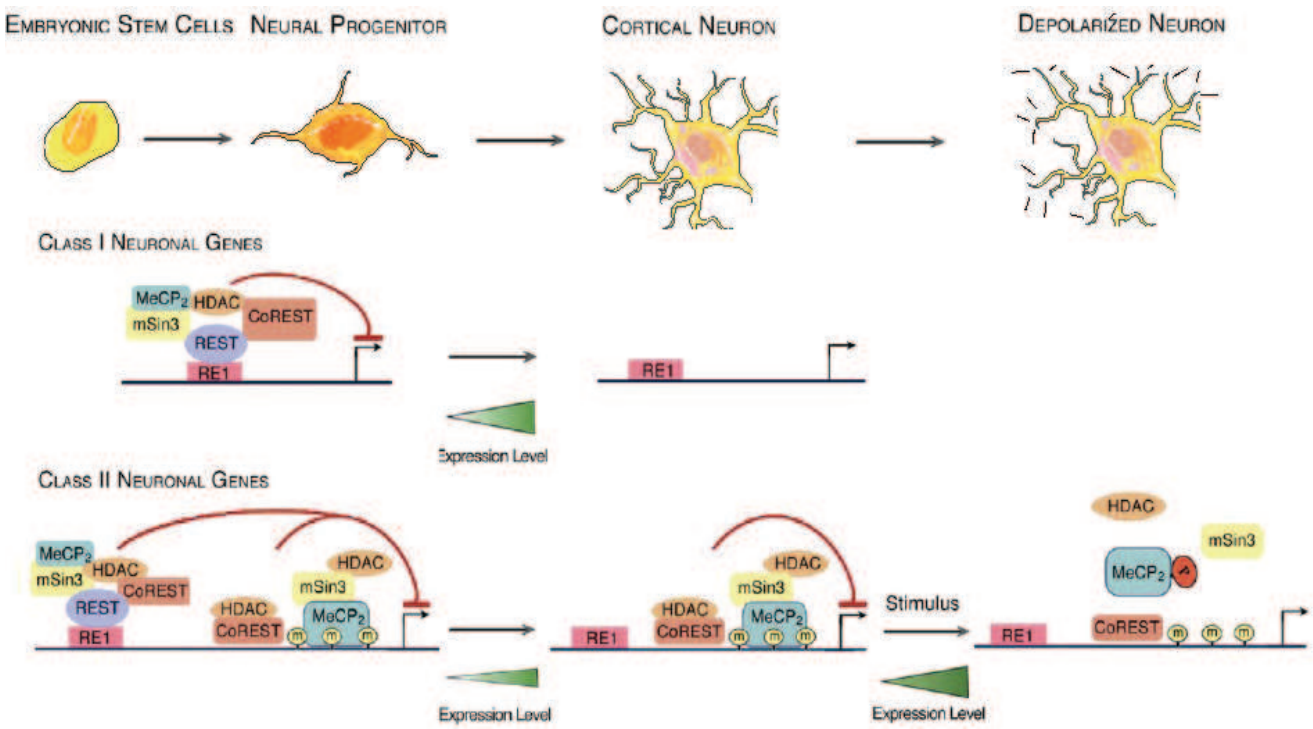
From its very discovery, REST was thought to function as a cellular brake for neurogenesis, as REST is expressed in neuronal progenitors but not in differentiated neurons (Chong et al., 1995, Schoenherr and Anderson, 1995). This role in neurodifferentiation is discussed in details in Chapter 3.

Neuronal cells plasticity

Even if it is accepted that REST expression is decreasing during neurodifferentiation (Chong et al., 1995, Schoenherr and Anderson, 1995, Ballas et al., 2005), REST protein (Calderone et al., 2003, Sun et al., 2005) and mRNA (Palm et al., 1998, Calderone et al., 2003, Kuwabara et al., 2004) have been detected in mature neurons, especially in those of the hippocampus. Alternative splicing of the REST mRNA is also occurring in these neurons and is conserved in mammals (Timmusk et al., 1999). Splice variants include REST4 which possess an inserted neuron-specific exon leading to translational frame shift and resulting in a truncated REST protein with either 4 zinc fingers (Palm et al., 1998, Timmusk et al., 1999).

The study of Palm et al., was one of the first to propose an implication of REST in neuronal physiological response. They have shown that a sustained activation of neuronal activity by kainate treatment in rat brain induced REST mRNA levels (Palm et al., 1998). Kainic acid treatment induces seizures that can be found in conditions of persisting hyper-excitability associated for instance with epilepsy. These defects are accompanied with changes in gene expression that mediate long-lasting or adaptive changes leading to synaptic reorganization. The authors suggested that prolonged activation of REST gene in response to enhanced neuronal activity may reflects either

neuroprotection, or synaptic plasticity through suppression of excessive expression of neuronal genes. Another study has involved REST in adaptive changes occurring after ischemia. REST induction in neurons of hippocampus after ischemic insult have been associated with a decrease in the expression of the AMPA receptor subunit GluR2 and increased cell death (Calderone et al., 2003). An elegant model for the involvement of REST corepressor complexes in mediating neuronal plasticity arose from the work of Mandel's group. In their paper deciphering the different transitions from ES to NSC and from NSC to mature cortical neurons (Ballas et al., 2005), they have described the gradual clearance of REST from its target genes, first, via posttranslational degradation of the protein and then via transcriptional repression of REST, leading to maximal activation of target genes belonging to class I, such as Nav1.2. For the class II genes, such as BDNF, they observed that methylated DNA at CpG sites bound an additional corepressor complex containing MeCP2 and CoREST, which persisted after differentiation, explaining low level of expression of these genes in differentiated neurons. However, after specific stimuli, such as membrane depolarization, while CoREST remains bound to the chromatin, MeCP2 is phosphorylated and dissociates from the chromatin together with HDAC and mSin3, resulting in dynamic activation of genes involved in neuronal plasticity (Scheme 12). Very recent studies from Mehler's group claiming that REST is expressed in every neuronal subtypes now suggest that REST participates in the specification and maintenance of these different neurons (Abrajano et al., 2009b) as well as in specification of glial cells and oligodendrocytes (Abrajano et al., 2009a). Considering the NSC-derived astroglial differentiation, the most convincing study has been made by studying BMP signaling (see d)). The authors have shown that BMP2 induces REST expression during NSC differentiation into astrocytes, and that even if REST expression in NSC is not able to drive astrocyte formation by its own, modulation of REST activity perturbs astrocytes integrity (Kohyama et al., 2010).



Scheme 12: Neuronal cell plasticity mediated by REST and co-repressor complexes
Two classes of RE1-containing genes are regulated differentially in postmitotic cortical neurons. For class I genes, the REST complex occupies the RE1 site and represses gene expression in neural progenitor/ES cells. Levels of expression are increased maximally as REST and its corepressors dissociate from the RE1 site in the transition to cortical neurons. For class II genes, in neural progenitor/ES cells, a REST repressor complex on the RE1 is accompanied by CoREST and MeCP2 co-repressor complexes residing on a site of mCpGs (yellow filled circles) in the promoter region. These genes are expressed to lower levels than class I genes (smaller green arrow) in cortical neurons. The transition to mature neurons is accompanied by loss of REST and corepressors from the RE1 site but persistence of the CoREST and MeCP2 repressor complexes at the mCpG site. Upon a specific stimulus, such as membrane depolarization, CoREST remains bound to chromatin, while MeCP2, together with mSin3 and HDAC, leaves the mCpG site of some genes, allowing higher levels of expression. Adapted from (Ballas et al., 2005).

ES cells pluripotency

REST has been implicated in the transcriptional regulatory networks that regulate ESC pluripotency, as REST is a target of known inducers of pluripotency Oct4, Sox2 and Nanog (Loh et al., 2006, Mikkelsen et al., 2007). The ChIP-PET analysis of Johnson et al., (see c)) has also indicated that the ESC-specific network of REST recruitment is largely integrated into those of Oct4, Nanog and Sox2 (Johnson et al., 2008). Their analysis showed that 20 to 30% of the REST targets in ESC were either bound by Oct4, Sox2 or Nanog, and that 107 are targets of all four factors, including REST itself and Nanog.

Therefore REST seems to have an extensive role in controlling gene network devoted to pluripotency, being part of an autoregulatory circuit (together with Oct4, Sox2 and Nanog) where every gene regulates its own expression and that of the others (Johnson et al., 2008). However, the direct evidence for an involvement of REST in maintenance of self-renewal and pluripotency of ESC is controversial. The group of Majumder (See their previous work in chapter 3) has been the first to propose that REST maintains pluripotency through the induction of self-renewal genes, using mouse ESC with heterozygous deletion of REST and ESC treated with REST-specific siRNA. They found in both conditions that REST loss-of-function resulted in loss of ESC self-renewal, associated with reduced levels in Oct4, Sox2, Nanog and c-Myc (Singh et al., 2008). Furthermore, they identified miR-21 as a key REST target responsible for this process, as they quantified that overexpression of miR-21 decreases self-renewal of ESC, in association with decreased expression of the markers cited above. The exit from self-renewal that was mediated by REST loss-of-function also predisposed the ESC to differentiate as they showed increased expression of various markers of the different germ layers (such as Mash1, Gata4, Ngn3 and Sox18). Two brief communications arose then from Fisher's lab (Jorgensen et al., 2009a) and Stanton's and Buckley's labs (Buckley et al., 2009) to contradict these conclusions. In the first, the authors used heterozygous and homozygous deletion of REST in ESC, as well as siRNA strategy to demonstrate that partial and full loss of REST protein did not induce, either decrease in the expression levels of pluripotency markers (Oct4, Sox2, Nanog), or increase in that of differentiating lineages markers (Jorgensen et al., 2009a). In the second, the authors used REST haplodeficient ESC to show that a 50% REST deficiency did not decrease expression of pluripotency markers, and did not increase, either expression of neuronal progenitors (Mash1, Ngn1), or that of miR-21, suggesting that miR-21 is not a target of REST (Buckley et al., 2009). To explain these discrepancies, the reply of Singh et al.,

(Buckley et al., 2009, Jorgensen et al., 2009a) pointed out at the differences in the various ESC lines used in each study (for instance, those used in Jorgensen's paper are not of wild type origin, but genetically modified with insertion in either Sox1, Sox2 or Oct4 genes), as well as the use of non-ES cell types in the studies of the genome-wide chromatin occupancy showing that miR-21 was not bound by REST (Johnson et al., 2007, Otto et al., 2007). The group of Fisher further published a study using ESC deleted for REST to confirm that REST was not required to repress the multiple lineage potential of ESC, as REST-deficient ESC still can express markers of the different germ layers in conditions of differentiation (Jorgensen et al., 2009b). Moreover, they showed that the lack of REST did not induce the expression of genes known to promote the early neural commitment (such as Ngn1 and 2, Mash1, Pax3, 6 and 7), but rather induce expression of neuron-specific REST target genes such as Secretogranin III (Scg3), SCG10-like protein (Stmn3), Cadherin (Celsr3) and Synaptophysin (Syp) in ESC. Lastly, another demonstration came from the study of Yamada et al., using ESC knocked-out for REST, ESC line with doxycycline-inducible Cre-mediated inactivation of REST and ESC line with inducible expression of REST (Yamada et al., 2010). ESC with deletion or conditional deletion of REST showed no difference in growth and morphology under self-renewal conditions, no difference in the expression of markers of pluripotency, and had the capacity to differentiate into the three germ layers. However under conditions of differentiation, loss of REST resulted in delayed repression of pluripotent genes, predominantly Nanog, together with increased markers of the primitive endoderm (such as Gata4 and 6 and Sox7). In contrast, forced expression of REST led to rapid differentiation and increased Gata6 expression. Together these results suggested, as mentioned in (Johnson et al., 2008), that REST is part of the Oct4-Sox2-Nanog regulatory network core circuitry, and that REST is necessary to promote the ESC early

differentiation towards the primitive endoderm via the breakdown of this core circuitry (Yamada et al., 2010).

Non-neuronal cells disorders

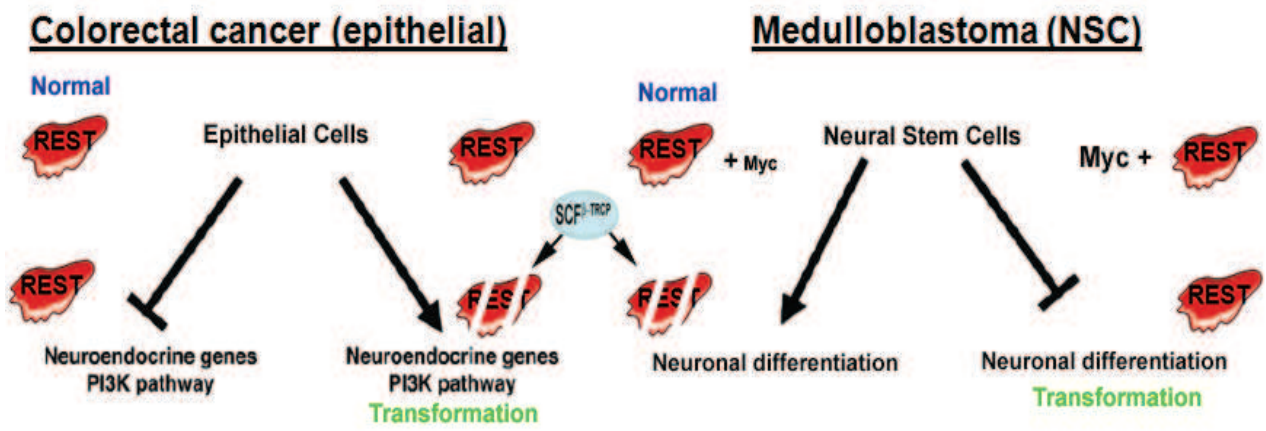
Among non-neuronal tissues, REST was found to repress the expression of multiple fetal cardiac genes in normal ventricular myocytes. Reduced REST expression in these cells led to reactivation of the fetal cardiac program and consequent cardiac dysfunction and arrhythmias in the hypertrophied heart (Kuwahara et al., 2003). REST loss of function has also been involved in the development of human neointimal hyperplasia, via the regulation of the expression of a critical potassium channel (K(Ca)3.1) that is important for the control of smooth muscle cells proliferation (Cheong et al., 2005). Finally, lower levels of REST in muscle fibers of patients with myotilinopathy has explained why these diseased muscles express neuron-specific genes, including the ubiquitin-carboxy-terminal hydrolase L1 (UCHL1) (Barrachina et al., 2007).

Cancer

A role for REST in tumor suppression has been revealed in cancers of epithelial origins. In the highly aggressive small cell-lung carcinomas, REST4 was observed at high levels in human biopsies and proposed to function as a dominant negative regulator of full length REST activity (Coulson et al., 2000). A confirmation that REST plays a role in the control of neuroendocrine phenotype acquisition in such tumors of epithelial origins was obtained in our lab using LNCaP prostatic cancer cell line (Tawadros et al., 2005). The tumor suppressor activity of REST was finally proven, using RNAi-based screen for tumor suppressor genes in human mammary epithelial cells that identified REST as a candidate (Westbrook et al., 2005). The group of Elledge has found that the REST gene was mutated in several primary colorectal cancer samples and that the resulting dominant negative form of REST caused a stimulated PI3K pathway and subsequent

transformation. They further showed, in another recent publication, that oncogenic transformation of human mammary epithelial cells is due to specific REST degradation by the E3 ubiquitin ligase $SCF^{\beta\text{-}TRCP}$, which is commonly overexpressed in human epithelial cancers (Westbrook et al., 2008) (Scheme 13).

In contrast, the group of Majumder has shown in many ways that REST has an oncogenic function in the context of medulloblastomas, which are supposed to arise from undifferentiated NSC of the cerebellum. First, they have found that REST is over-expressed in many human medulloblastomas samples (Lawinger et al., 2000). Second, they have demonstrated the direct implication of REST in these tumors, using a recombinant activator form of REST, REST-VP16, which led to the activation of REST target genes and blockade of tumorigenicity (Fuller et al., 2005). Third, they identified that the conditions for tumor transformation required NSC differentiation, and both c-Myc expression that favors increased proliferation, and REST expression that blocks differentiation or maintain "stemness" of the cells (Su et al., 2006). Thus in a context of epithelial tissue that normally express REST, abnormal lack of REST activity, which is to suppress tumor formation, leads to oncogenic transformation, such as in lung (Coulson et al., 2000), breast (Reddy et al., 2009) or colon cancer (Westbrook et al., 2005). In contrast, in a context of differentiating neuronal cells that extinguish REST, abnormal expression of REST will block differentiation and cause tumorigenesis (Scheme 13).



Scheme 13: Proposed mechanism for the tumor suppressor vs. oncogenic functions of REST
Left: *proposed tumor-suppressor function of REST in epithelial cells of the colon, breast, lung, and prostate. These cells normally show REST-mediated repression, and therefore repressed expression of neuronal genes and the genes required for activation of the PI3K pathway. In tumors, REST is mutated, or degraded by* $SCF^{\beta\text{-}TRCP}$*, causing activation of these genes and transformation of the epithelial cells (Westbrook et al., 2005).*
Right: *proposed oncogenic function of REST in neuronal cells. These cells normally loose the REST-mediated repressor activity and can therefore express neuronal genes and can differentiate. In medulloblastoma tumors, the abnormally maintained REST-mediated suppression of differentiation, together with elevated levels of c-Myc causing enhanced proliferation, leads to tumorigenesis (Su et al., 2006).*

Neurological disorders

REST gain-of-function has been involved in several human neuronal disorders. Down's syndrome (DS) is a mental retardation also called trisomy 21. Several REST target genes are down-regulated and REST expression is impaired in human DS NSC samples (Bahn et al., 2002), and in brain of DS mouse model in which DYRK1A dosage imbalance has been evoked as a triggering event (Lepagnol-Bestel et al., 2009). Recently, REST has also been involved in a neurodevelopmental disorder, the Rett syndrome (RTT). This disease is caused by a mutation in the gene encoding the co-repressor MeCP2, which binds mCpG to mediate transcriptional repression. Mandel's work showed that MeCP2 binds the REST promoter where it recruits a co-repressor complex to initiate REST extinction (Ballas and Mandel, 2005). In brains of MeCP2 deficient mice and Rett patients, REST protein was found to be increased and was associated with the decreased levels of BDNF normally observed in RTT (Abuhatzira et al., 2007). The group of Cattaneo has provided, through extensive analyses, an unexpected mechanism implying REST and its target

genes in the pathogenesis of Huntington disease (HD), a neurodegenerative disease caused by a mutation in the gene coding for huntingtin. In HD, decreased BDNF expression participates in the induction of neuronal degeneration (Zuccato et al., 2001), and wild type huntingtin has the capacity to activate BDNF. Zuccato et al., have demonstrated that in striatal neurons, this capacity occurs through cytoplasmic sequestering of REST by huntingtin. In cells, mouse model, and human brains with HD, mutated huntingtin is not able to interact with REST anymore and this leads to its accumulation in the nucleus where it mediates adverse effects through modifications in target genes expression (Zuccato et al., 2003, Zuccato et al., 2007). Finally, two recent studies have evoked the implication of REST in X-linked mental retardation since REST interacts with SMCX (Tahiliani et al., 2007) and Mediator (Ding et al., 2008), two complexes mediating histone modifications, that have been associated with this human disorder. Globally, perturbations in the activity of these two protein complexes impair REST-mediated regulation of neuronal target genes that have been either involved in mental retardation, epilepsy, autism, or schizophrenia.

Pancreatic beta cell physiology

In spite of different embryonic origins, pancreatic beta cells and neurons share a large number of similarities. Beta cells resemble neurons by being electrically excitable and by responding to hormonal stimuli and glucose by depolarization and exocytosis in a process similar to neurotransmitter release. At the molecular level, such similarities could be explained by overlapping patterns of gene expression between the two cell types. Indeed, beta cells are known to express different neuronal genes, such as enzymes implicated in the synthesis of neurotransmitters, receptors for growth factors and amino acids, neurofilaments, and hormones (Atouf et al., 1997), and also proteins involved in the machinery of exocytosis of synaptic vesicles (Burgoyne and Morgan, 2003), and

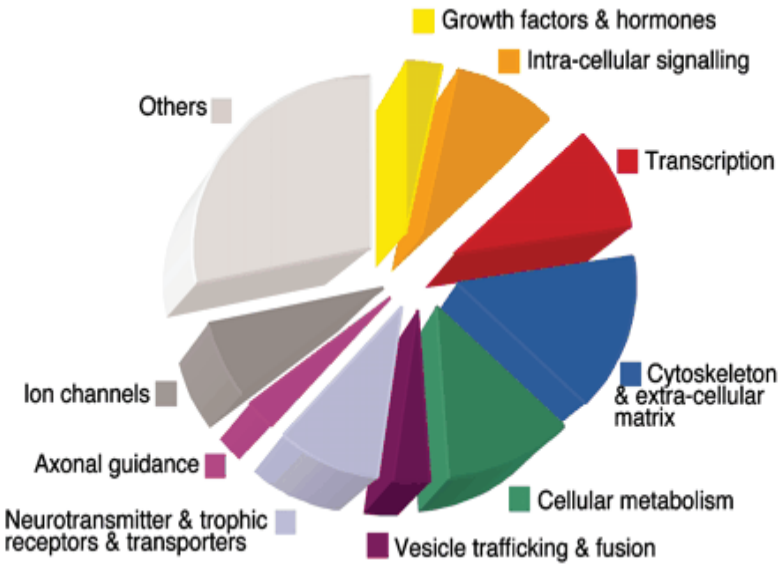
finally also certain transcription factors such as NeuroD/BETA2 (Naya et al., 1995). In 1997, the group of Scharfmann has specified that these common features could be explained by the presence, in both neurons and beta cells, of identical transactivators, but also by the absence of REST (Atouf et al., 1997). Especially, they have identified that several neuron-specific genes that are targets of REST were expressed in beta cells. These genes were the N-methyl-D-aspartate receptor, Nav1.2, dopamine β-hydroxylase, SCG10, SynapsinI, and the nicotinic acetylcholine receptor β2 subunit.

AIM OF THE PROJECT

The majority of reports identifying particular genes as *bona fide* REST targets have studied their role in relation to neuronal functions (Table 2). However, how this set of genes participates to beta-cell function and life remains to be established. In our department, we have shown that REST is responsible for the cell-specific expression of two genes that code for Connexin36 (Cx36) (Martin et al., 2003), and IB1/JIP-1 (Abderrahmani et al., 2001). Cx36 is a gap junction-forming protein participating to the control of insulin secretion via cell to cell communication (Le Gurun et al., 2003) and is also involved in the mechanism of beta cell survival (Klee et al., Submitted). IB1/JIP-1 is a scaffold protein protecting beta cells against apoptosis via the interaction with c-Jun N-terminal kinase (JNK) signaling pathway (Bonny et al., 2000, Haefliger et al., 2003) and has also been recently involved in the control of insulin secretion (Abderrahmani, personal communication). The first evidence that the whole set of REST target genes is important for beta-cell insulin secretion has been described in the βTC3 cell line after stable transfection of the repressor (Abderrahmani et al., 2004).

Given the multiplicity of RE-1-containing genes and their putative involvement in very diverse cellular functions (Cf. REST regulon and scheme 14), we expected to raise substantial relevant information by studying the REST/RE-1 system in insulin-secreting beta cells. Since REST is considered a key master negative regulator of neuroendocrine phenotype, its classification into the group of beta cells' forbidden genes appeared pertinent. In turn, the identification of as many as possible of its target genes, consequently classified into the group of beta-cell essential genes, would be of significant interest for the critical task of compiling a beta-cell signature. This strategy

could represent a starting point for answering to "What is a beta cell and can we improve it? (Schuit et al., 2005).



Scheme 14: Assignment of putative REST target genes to functional groups
Assignment has been performed with the ~1800 targets identified in sillico, which have been listed in the RE-1 database (http://www.bioinformatics.leeds.ac.uk/cgi-bin/RE1db/nrse.cgi). Reproduced from (Bruce et al., 2004).

Our objective was first to generate transgenic mice that would express REST specifically in beta cells, under the control of the rat insulin II (RIP II) promoter (RIP-REST mice). Then, we searched to identify in these transgenic animals what were the consequences of the decreased expression of RE-1-containing genes, in other terms, what is the functional significance of REST target genes, *in vivo*. The final goal of this project was to identify the *bona fide* REST target genes that would be responsible for the potential defects observed in RIP-REST mice, and to relate them to a specific function of beta cell. Particularly, we aimed at identifying new genes that are involved in the beta-cell features that participate to the development of diabetes: beta-cell function and survival.

RESULTS

Chapter 1: Functional significance of REST target genes in pancreatic beta cells

This work has been published in 2008 in Diabetologia.

Initiated by the Pr. Waeber and Dr. Abderrahmanni, this work aimed at identifying the functional consequences of an ectopic expression of the transcriptional repressor REST in pancreatic beta cells. To do so, we adopted an *in vivo* strategy by generating transgenic mice that express REST under the control of the rat insulin promoter II (RIP), the RIP-REST mice. Since REST has been first described as a repressor of neuronal genes, and since the majority of REST target genes identified at that time were mostly related to specialized neuronal function, the question that we asked to ourselves was:

"What is the functional significance of the REST target genes in pancreatic beta cells?"

Because RIP-REST mice are a model of REST gain-of-function but also a model of loss-of-function of RE-1-containing genes, we were able to define that, in beta cells, RE-1-containing genes serve insulin exocytosis.

This project has involved numerous interesting collaborations, with Pr. Maechler (Geneva), Pr. Meda (Geneva), Pr. Regazzi (Lausanne), Pr. Fukuda (Japan), and naturally, Pr. Waeber and Dr. Abderrahmanni.

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ARTICLE

Functional significance of repressor element 1 silencing transcription factor (REST) target genes in pancreatic beta cells

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Abstract
Aims/hypothesis The expression of several neuronal genes in pancreatic beta cells is due to the absence of the transcription factor repressor element 1 (RE-1) silencing transcription factor (REST). The identification of these traits and their functional significance in beta cells has only been partly elucidated. Herein, we investigated the biological consequences of a repression of REST target genes by expressing *REST* in beta cells.
Methods The effect of *REST* expression on glucose homeostasis, insulin content and release, and beta cell mass was analysed in transgenic mice selectively expressing *REST* in beta cells. Relevant target genes were identified in INS-1E and primary beta cells expressing *REST*.
Results Transgenic mice featuring a beta cell-targeted expression of *REST* exhibited glucose intolerance and reduced beta cell mass. In primary beta cells, REST repressed several proteins of the exocytotic machinery, including synaptosomal-associated protein (SNAP) 25, synaptotagmin (SYT) IV, SYT VII, SYT IX and complexin II; it impaired first and second phases of insulin secretion. Using RNA interference in INS-1E cells, we showed that SYT IV and SYT VII were implicated in the control of insulin release.
Conclusions/interpretation The data document the critical role of REST target genes in pancreatic beta cells. Specifically, we provide evidence that the downregulation of these genes is detrimental for the exocytosis of large dense core vesicles, thus contributing to beta cell dysfunction and impaired glucose homeostasis.

Keywords Exocytosis · Insulin secretion · RE-1 · RE-1 silencing transcription factor · REST · Synaptotagmins · Transgenic mice

Abbreviations

Ad-GFP	GFP-encoding adenovirus
Ad-REST	REST-encoding adenovirus
BCH	β-2-aminobicyclo [2.2.1] heptane-2-carboxylic acid
CX36	connexin 36
GFP	green fluorescent protein
hGH	human growth hormone
IBMX	3-isobutyl-1-methylxanthine
IPGTT	intraperitoneal glucose tolerance test
KRBH	KRB HEPES

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LDCV	large dense core vesicles
MUNC-18–1	UNC-18 homologue 1
NSF	*N*-ethylmaleimide-sensitive factor
RE-1	repressor element 1
REST	RE-1 silencing transcription factor
RIP II	rat insulin II promoter
siRNA	small interfering RNA
SNAP	synaptosomal-associated protein
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SREBP-1	sterol regulatory element-binding protein-1
SYT	synaptotagmin

Introduction

The transcription factor repressor element 1 (RE-1) silencing transcription factor (REST), also known as neuron-restrictive silencing factor, has been implicated in the control of glucose-induced insulin secretion in the βTC3 cell line [1]. This GLI-Kruppel zinc finger transcription factor was first described as a silencer of neuronal genes outside the central nervous system, since its expression is restricted to non-neuronal cells and undifferentiated neural progenitors, allowing genes encoding fundamental neuronal traits to be exclusively expressed in mature neurons [2, 3]. Target genes possess a 21 bp *cis* element called RE-1 or neuron restrictive silencer element, to which REST binds to inhibit expression. Initial studies indicated that many REST target genes contribute to synaptic plasticity/remodelling, inasmuch as REST regulates the expression of synaptic vesicle proteins [4], voltage-sensitive ion channels [2] and neurotransmitter receptors [5, 6]. However, increasing evidence suggests that the significance of the REST/RE-1 system is diverse in embryonic and adult cells and depends on the range of target genes that REST interacts with. Accordingly, a bioinformatic analysis recently revealed about 1,800 putative REST target genes within the human genome, with attributed roles ranging from transcriptional regulation through to metabolism and various aspects of neuronal function [7]. Previous reports have identified some of these target genes and their function in and outside the nervous system. Thus, it is now documented that REST target genes are involved in the reactivation of the fetal cardiac gene programme in hypertrophied and failing hearts [8], and modulate the vascular plasticity/remodelling of human neointimal hyperplasia [9].

Pancreatic beta cells, which lack REST [10], also express a number of REST target genes. However, it remains to be established how these genes participate in beta cell function. Investigating this molecular mechanism, we previously showed that in beta cells REST controls the expression of two neuron-specific genes that code for connexin 36 (CX36), a gap junction-forming protein participating in control of insulin secretion [11], and islet brain-1, a scaffold protein protecting beta cells against apoptosis via the c-Jun N-terminal kinase signalling pathway [12]. Since the whole set of REST target genes is involved in the control of insulin secretion in vitro [1], we have now examined the in vivo consequences of a gain of function of REST and have identified additional target genes that are significant to beta cell function. We show that transgenic mice specifically expressing *REST* in beta cells exhibit glucose intolerance and defective insulin release. We further demonstrate that *REST* expression affects both triggering and amplifying pathways of insulin secretion, by impairing the expression of proteins of the exocytotic machinery, including the hitherto neglected synaptotagmin (SYT) IV and VII. These data demonstrate the crucial role of REST targets in the control of insulin release and suggest that the downregulation of these genes may contribute to the pathophysiology of beta cell defects.

Methods

Transgenic mice Transgenic mice specifically expressing *REST* in the beta cells were obtained by pronuclear injection of C57Bl/6 zygotes with a SacII/XhoI fragment consisting of 660 bp of the rat insulin II promoter (RIP II) [13], separated from the full length human *REST* cDNA [12] by the rabbit β-globin intron (Fig. 1a). Three positive founder mice were successfully bred with C57BL/6 control mice to generate transgenic animals. Our institutional review committee for animal experiments approved all the procedures for mice care, surgery and animal killing.

Immunohistochemistry Pancreases were fixed in 4% (wt/vol.) paraformaldehyde, equilibrated overnight in 15% (wt/vol.) sucrose, embedded in 15% sucrose–7.5% (wt/vol.) gelatin and quickly frozen in methylbutane/liquid nitrogen. Cryosections were incubated overnight at 4°C with either polyclonal rabbit antibodies against human REST or polyclonal guinea pig antibodies against insulin. Primary antibodies were detected using appropriate fluorescein or rhodamine-conjugated antibodies. Sections were viewed on a fluorescence microscope (Leica, Nidau, Switzerland).

Electron microscopy Pancreas fragments were fixed in a 2.5% (vol./vol.) glutaraldehyde solution in 0.1 mol/l phosphate buffer (pH 7.4), postfixed in 1% (wt/vol.) osmium tetroxide in the same buffer, dehydrated and embedded in Epon [14]. Thin sections were examined in a Philips CM10 electron microscope (Philips, Eindhoven, the Netherlands).

Glucose tolerance and plasma insulin Male mice of 12 to 16 weeks were fasted for 14 h before blood samples were

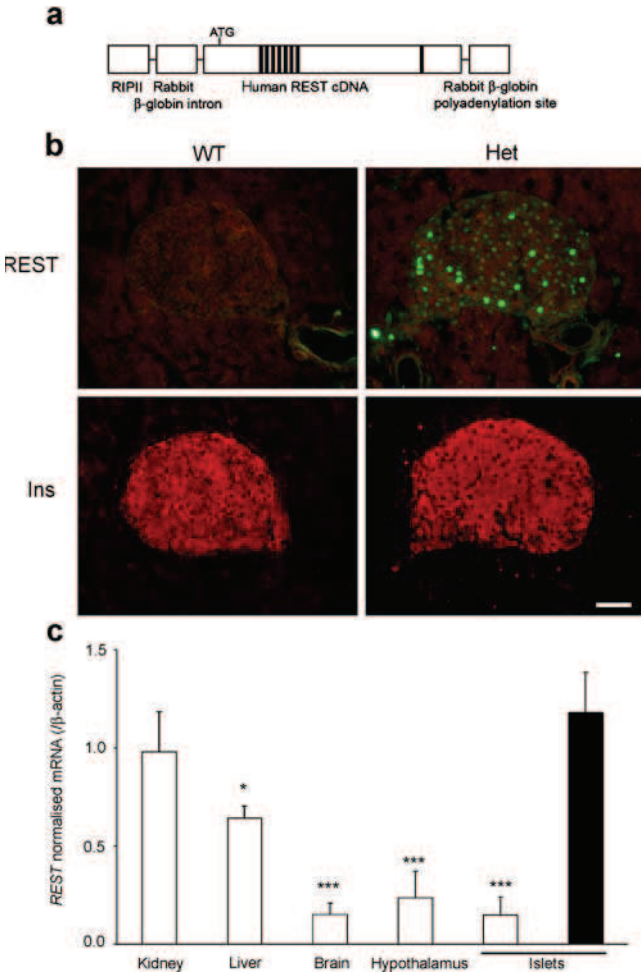

Fig. 1 Generation of transgenic mice expressing *REST* in beta cells. **a** The construct used to generate RIP–REST transgenic mice contained the full-length human *REST* cDNA, surrounded by rabbit β-globin intronic and polyadenylation sequences, and fused to the RIP II. Solid bars, the nine-zinc fingers; ATG, start codon of human *REST* cDNA. **b** RIP–REST mice featured morphologically normal pancreatic islets. Immunostaining using specific antibodies against human REST (in green) and insulin (in red) demonstrated that REST was specifically produced in the nucleus of beta cells of heterozygous (Het) RIP–REST but not wild-type (WT) mice. The corresponding insulin staining (Ins) is shown. Scale bar, 30 μm. **c** Quantitative RT-PCR analysis of mRNA levels of murine and human *REST* in RIP–REST mice. White columns show levels of endogenous murine *Rest.* The black column shows levels of the human *REST* transgene. No human *REST* expression was found outside pancreatic islets. Results are means±SEM of four independent experiments. *$p<0.05$, ***$p<0.001$ versus values for human *REST* mRNA levels

collected from the tail vein at 0 (fasting blood sample), 15, 30 and 120 min after an intraperitoneal injection of glucose (2 g/kg body weight as a 20% solution). Blood glucose levels were measured with a Glucometer (Bayer Healthcare, Zurich, Switzerland). Plasma insulin levels from the same time points were determined by ELISA (Mercodia, Uppsala, Sweden).

Cell line and mouse islet isolation The rat insulinoma cell line INS-1E was maintained in RPMI 1640 medium, as previously described [15]. Islets of Langerhans of adult C57BL/6 male mice, weighing 25 to 30 g, were isolated and cultured as previously described [15].

Insulin secretion INS-1E cells were seeded in 12-well plates and cultured for 48 h. Isolated islets were cultured overnight in non adherent dishes. Cells and islets were infected with adenoviruses as previously described [11], with a multiplicity of infection of 10 and 15, respectively. As judged by immunofluorescence and quantitative PCR detection of the *REST* transgene, this procedure typically resulted in the efficient transduction of 70 to 80% INS-1E cells and 30 to 40% primary islet cells. Insulin secretion of INS-1E cells and mouse islets perifusion were assessed in KRB–HEPES (KRBH) supplemented with the indicated stimuli, as published [16]. When 30 mmol/l KCl was added in the experiments testing diazoxide, the NaCl concentration of the medium was reduced to 105 mmol/l. Glucose, leucine, β-2-aminobicyclo [2.2.1] heptane-2-carboxylic acid (BCH), forskolin, 3-isobutyl-1-methylxanthine (IBMX) and diazoxide were purchased from Sigma (Fluka Chemie, Buchs, Switzerland).

Cell and mitochondrial membrane potentials INS1-E cell and mitochondrial membrane potentials were monitored using 100 nmol/l bis-oxonol and 10 μg/ml rhodamine-123 (Molecular Probes, Eugene, OR, USA), respectively, as previously described [17].

Calcium measurements Cytosolic calcium was monitored in INS-1E cells loaded for 90 min with 2 μmol/l Fura-2AM (Teflab, Austin, TX, USA) in KRBH at 37°C. Ratiometric measurements of Fura-2 fluorescence were performed in a plate-reader fluorimeter (Fluostar Optima; BMG Labtechnologies, Offenburg, Germany) with filters set at 340/380 nm for excitation and 510 nm for emission.

Chromatin immunoprecipitation assay INS-1E cells were cross-linked with 1% (wt/vol.) formaldehyde for 30 min and the reaction was stopped by addition of 0.125 mol/l glycine. Pelleted cells were lysed in an SDS buffer and submitted to sonication to obtain the desired chromatin length (~500 bp). Chromatin was precleared by addition of blocked protein A Sepharose (Amersham Bioscience Europe, Otelfingen, Switzerland) and the supernatants were immunoprecipitated overnight at 4°C with either polyclonal rabbit antibodies specific to human REST [11] or to irrelevant sterol regulatory element-binding protein-1 (SREBP-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein–antibody complexes were collected by addition of protein A Sepharose for 1 h and then washed. The protein–DNA complexes were eluted, treated with RNAseA and proteinase K and then the cross-links were reversed overnight at 65°C. DNA was submitted to PCR amplification of rat RE-1 flanking

sequences using specific primers (see Electronic supplementary material [ESM] Table 1).

RNA isolation and northern blotting RNA isolation and northern blot analysis were performed as previously described [15]. Transcripts levels were revealed using specific probes for rat *Cx36* (also known as *Gja9*), β-actin [11], rat *Munc18-1* (also known as *Stxbp1*), Syntaxin 1A and *Rab3a*. For the other genes, probes were obtained with cDNA from INS-1E cells or rat brain, which was amplified with specific primers (ESM Table 2).

Real-time RT-PCR Quantitative RT-PCR was performed using a kit (SYBR Premix Ex Taq PCR Kit TaKaRa; Axon Lab, Le Mont-sur-Lausanne, Switzerland) in a Lightcycler (Roche Diagnostics, Mannheim, Germany), as previously described [15]. cDNAs were amplified using specific primers (ESM Table 3).

Western blotting Western blots were performed as previously described [15]. Specific protein levels were revealed with polyclonal rabbit antibodies against: CX36 [11], synaptosomal-associated protein (SNAP) 25 (kindly provided by H. Hirling, EPFL, Lausanne, Switzerland) and SYT IV. Monoclonal antibodies against β-actin (Fluka Chemie) were used to normalise the signals.

SYT IV, SYT VII and REST silencing vectors Specific small interfering RNA (siRNA) were designed as follows: for rat *Syt4*, the sense target sequence 5′GAAGCACAAAGT GAAAACCA3′ was deduced from the reported mouse sequence [18]; for rat *Syt7* the sense sequence 5′TCAT CACCGTCAGCCTTAG3′ was selected according to a previous publication [19]; for *REST*, the sense sequence 5′ GGAACCTGTTGAGAAGGGA3′ was selected using the siRNA Target Finder (Ambion, Austin, TX, USA). Two complementary DNA fragments encoding the target sequence and separated from the reverse complement by a short spacer were synthesised by Microsynth (Balgach, Switzerland). The two fragments were annealed and cloned downstream of the H1-RNA promoter of the pSuper vector, using BglII–HindIII sites. BamHI–HindIII fragments containing the siRNA constructs were subcloned in a plasmid (pXGH) encoding human growth hormone (hGH).

Human growth hormone secretion INS-1E cells were transiently transfected with the pXGH vector to use hGH as a reporter for secretion or with the pXGH containing one of the specific siRNAs. At 72 h after transfection, secretion was assessed in KRBH supplemented with 20 mmol/l glucose, 10 μmol/l forskolin and 100 μmol/l IBMX, and measured as published [20].

Statistical analysis Data were expressed as mean±SD or SEM. Differences between means were assessed using Student's *t* test. Statistical significance was defined at a value of $p<0.05$, $p<0.01$ and $p<0.001$.

Results

Transgenic mice featuring a beta cell-targeted expression of REST exhibit reduced insulin content and altered tolerance to glucose To generate transgenic mice producing REST in pancreatic beta cells, we inserted a transgene consisting of the RIP II upstream of the full-length human *REST* cDNA (Fig. 1a). Examination of pancreas sections revealed morphologically normal islets in both wild-type and RIP–REST mice. However, in contrast to their control littermates, RIP–REST mice produced REST in the nuclei of the islets of Langerhans (Fig. 1b). Parallel immunostaining of insulin (Fig. 1b) confirmed that this expression was restricted to beta cells. Quantification of mRNA by real-time PCR revealed that levels of the human *REST* transgene achieved in the islets of RIP–REST mice were comparable to those of endogenous *Rest* in kidney. In contrast, no expression of the *REST* transgene was detected in the other tested organs (Fig. 1c). A much lower expression of the native murine *Rest* was detected in brain and islets, probably reflecting the non-neuroendocrine cells of these organs (Fig. 1c). Electron microscopy revealed normal distribution of the insulin-containing beta cells within the islets of both wild-type (Fig. 2a) and RIP–REST heterozygous littermates (Fig. 2b) and also normal appearance of these beta cells, which in both types of animals were packed with typical, dense-core and white halo secretory granules (Fig. 2c,d). Further morphological analyses showed that the numerical density of islets was similar in the pancreas of control and transgenic mice. However, the numerical density of beta cells, expressed as the ratio of total beta cell number to the total pancreas area, was reduced in RIP–REST mice (Table 1). Accordingly, the total insulin content of pancreas was twofold lower ($p<0.01$) in transgenic than in wild-type littermates (Table 1).

The ability of RIP–REST mice to regulate blood glucose levels was assessed in 3-month-old male animals during an intraperitoneal glucose tolerance test (IPGTT) (Fig. 3a). Compared with wild-type littermates, RIP–REST animals showed a significantly higher blood glucose concentration at 15 ($p<0.05$), 30 ($p<0.01$) and 60 min after glucose injection ($p<0.001$). Parallel measurements of plasma insulin levels revealed similar fasting levels in control and RIP–REST mice (Fig. 3b). However, the release of insulin in the latter was significantly reduced at 15 ($p<0.05$) and 30 min ($p<0.01$) after glucose challenging (Fig. 3b). These data show that RIP–REST mice have reduced levels of pancreatic

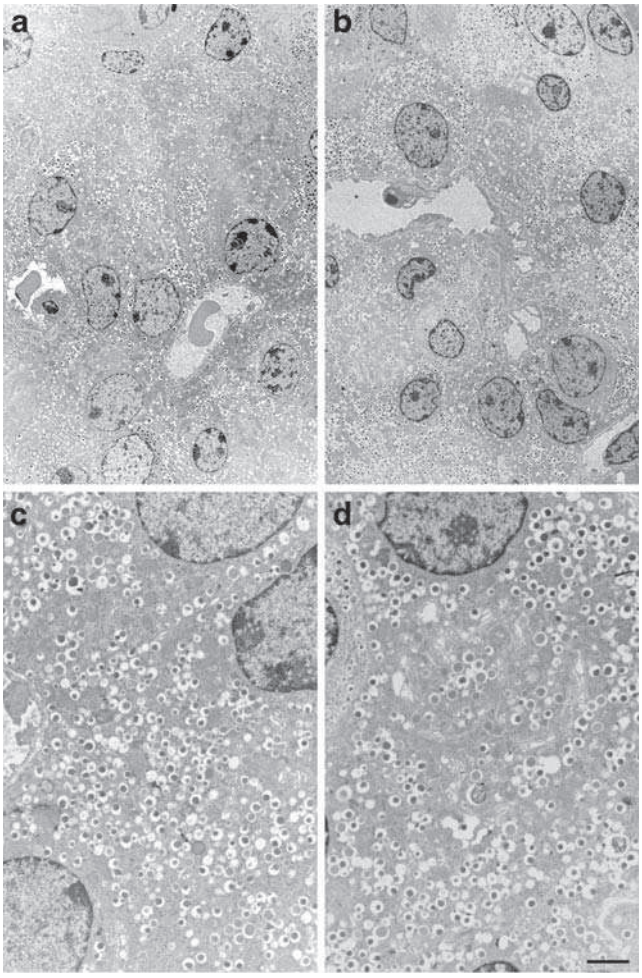

Fig. 2 RIP–REST mice feature ultrastructurally normal beta cells. Electron microscopy revealed normal organisation of beta cells within pancreatic islets of wild-type (**a**) and RIP–REST heterozygous littermates (**b**). Beta cells from wild-type (**c**) and RIP–REST mice (**d**) featured normal appearance and were packed with typical, dense-core and white halo secretory granules. Bar: 5 μm (**a**, **b**), 1.2 μm (**c**, **d**)

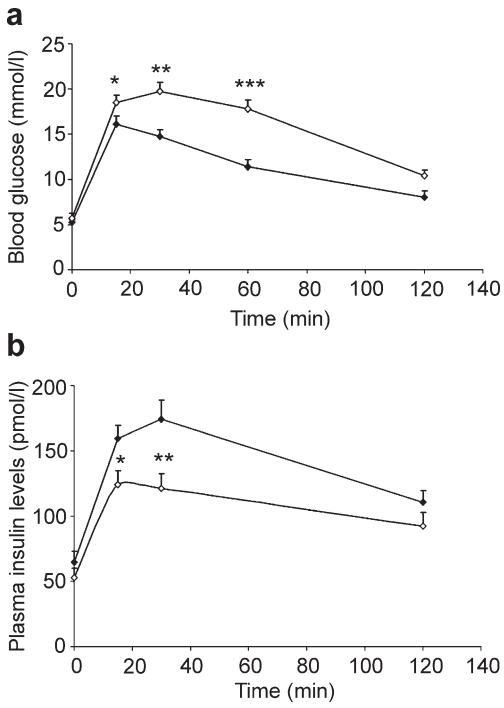

Fig. 3 RIP–REST mice are intolerant to glucose. **a** Blood glucose levels in 3-month-old wild-type (solid symbols) males (n=10) and heterozygous RIP–REST (open symbols) littermates (n=16) during an IPGTT. After 14 h fasting, blood samples were taken before (t=0) and after (t=15, 30, 60 and 120 min) intraperitoneal injection of glucose (2 g/kg). Results are mean±SEM. *p<0.05, **p<0.01, *** p<0.001. **b** Plasma insulin levels were measured in 3-month-old male wild-type (solid symbols) (n=6) and RIP–REST (open symbols) (n=6) mice. Blood samples were taken at 0, 15, 30 and 120 min of the IPGTT. Results are mean±SEM. *p<0.05, **p<0.01

insulin and are intolerant to glucose due to impaired secretory function of beta cells.

REST target genes are crucial for both first and second phases of insulin secretion To investigate this impairment, INS-1E-cells were infected with REST-encoding adenovirus (Ad-REST). Compared with control green fluorescent protein (GFP) transduction (Ad-GFP), ectopic *REST* expression did not alter insulin content (400±60 vs 423±39.6 pmol/l, in GFP and REST-transduced cells, respectively) or basal insulin secretion (ESM Fig. 1). In contrast, it reduced insulin release in response to stimulating concentrations of either glucose, KCl or leucine, compared with GFP-transduced cells (ESM Fig. 1a). These data show that the K_{ATP} channel-dependent pathway of insulin secretion, triggered by KCl, as

Table 1 Morphometric analysis of pancreas

Group	Pancreas weight (mg)	Insulin content (pmol/mg pancreas)	Islet numerical density (n per cm^2)	Beta cell numerical density (n per cm^2)
Wild-type	239±22	26±0.23	62.1±2.6	4,084±483
RIP–REST	228±13	13±0.28**	65.7±8.4	2,688±337*

Values are expressed as mean±SD
Number of mice per group: 4
*p<0.05, **p<0.01 versus WT

well as the K_{ATP} channel-independent pathway, triggered by the non-metabolisable leucine analogue BCH [21], were both decreased in cells expressing *REST*. In the diazoxide experiments, the cells, in presence of 250 μmol/l diazoxide and 30 mmol/l KCl, were stimulated with 2.5 mmol/l glucose and then with 15 mmol/l glucose. The second incubation at 15 mmol/l glucose, reflecting the amplifying effect of the sugar on insulin secretion [22], was significantly reduced in *REST*-expressing INS-1E cells (ESM Fig. 1a). Similar observations were made in REST and GFP-transduced mouse islets (ESM Fig. 1b), which also featured similar insulin contents (73.8±6.4 vs 66.9±5.4 pmol/l, in GFP and REST-transduced islets, respectively).

To ascertain whether REST affected both phases of insulin secretion, we perifused transduced mouse islets (Fig. 4a) and islets isolated from RIP–REST mice (Fig. 4b). Stimulation by 20 mmol/l glucose confirmed the REST-induced generalised impairment ($p<0.05$) of the stimulus–secretion coupling during both first (time points 20 to 27 min) and second phases (time points 28 to 44 min).

REST does not affect plasma and mitochondrial membrane potentials or intracellular levels of Ca^{2+} To investigate the intracellular signalling leading to altered insulin release of *REST*-expressing INS-1E cells, we first studied the hyperpolarisation of the mitochondrial membrane, which results from the activation of mitochondrial metabolism. This hyperpolarisation induced by glucose was not affected when cells were infected with Ad-REST (ESM Fig. 2a). We then evaluated the cell membrane depolarisation resulting from the ATP-dependent closure of K_{ATP} channels. INS-1E cells expressing *REST* depolarised normally under glucose stimulation (ESM Fig. 2b). Since membrane depolarisation triggers Ca^{2+} influx through voltage-gated Ca^{2+} channels, we measured the cytoplasmic levels of this cation. We found that the expression of *REST* did not affect the glucose-induced increase in intracellular Ca^{2+} (ESM Fig. 2c). These data suggest that REST does not alter the intracellular signalling that is activated by mitochondrial metabolism and leads to closure of K_{ATP} channels, cell membrane depolarisation and elevation of cytosolic Ca^{2+}.

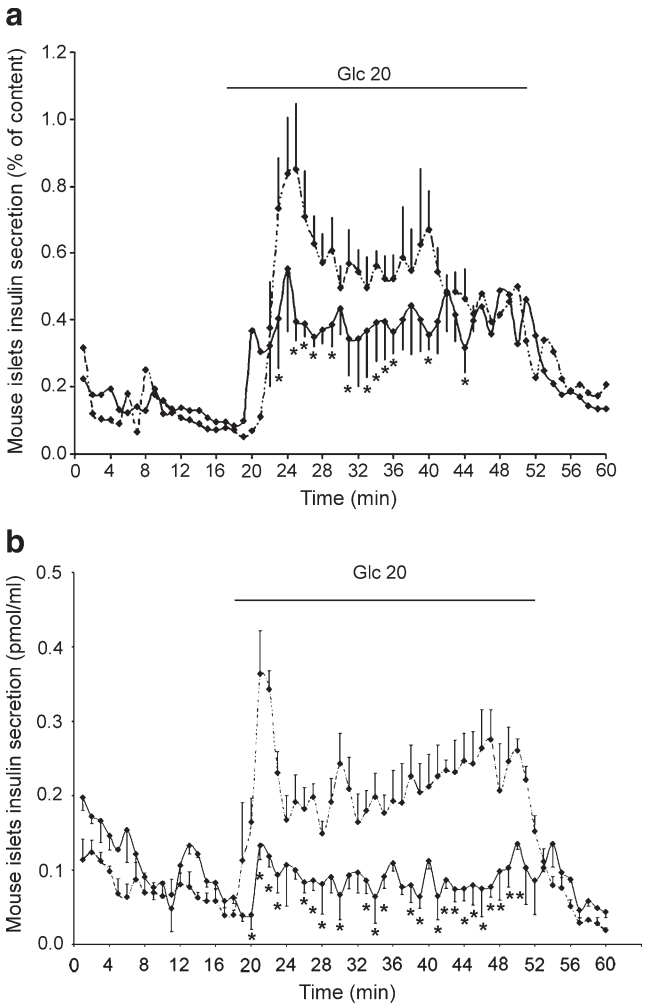

Fig. 4 Repression of REST target genes impairs the two phases of insulin release. **a** Insulin release of mouse islets transduced with Ad-GFP (dashed lines) and Ad-REST (solid lines) during perifusion experiments. After a 15 min period in presence of 2.5 mmol/l glucose, islets were stimulated for 30 min with 20 mmol/l glucose and then returned to 2.5 mmol/l glucose for 15 min. Values are means±SEM. of three independent experiments. *$p<0.05$ for corresponding time points. **b** Insulin release of islets isolated from wild-type (dashed lines) and RIP–REST (solid lines) mice during perifusion experiments. After a 15 min period in presence of 2.5 mmol/l glucose, islets were stimulated for 30 min with 20 mmol/l glucose and then returned to 2.5 mmol/l glucose for 15 min. Values are means±SEM of three independent experiments. *$p<0.05$, **$p<0.01$ for corresponding time points. Glc, glucose

Identification of functional RE-1 sequences We hypothesised that REST controls the expression of genes acting downstream of the elevation of intracellular Ca^{2+} and therefore selected, in a RE-1 database (http://www.bioinformatics.leeds.ac.uk/cgi-bin/RE1db/nrse.cgi) [7], the following putative targets: (1) the gene encoding the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein SNAP25, which contains two consecutive RE-1s, referred to as RE-1.1 and RE-1.2 [7]; (2) the genes encoding the members of the Ca^{2+} sensor synaptotagmin family SYT II, SYT IV, SYT VI, SYT VII, SYT IX; and (3) the genes coding for the *N*-ethylmaleimide-sensitive factor (NSF), for UNC-18 homologue 1 (MUNC-18-1) and for complexin II, which participate in the formation and stabilisation of the SNARE complex. The 21 bp human RE-1 sequences associated to these genes were compared with those of consensus RE-1 and of the known *CX36* RE-1 [11]. All sequences, except that of *MUNC18-1*, were closely homologous to the consensus one and displayed a typical core [23] consisting of two invariant domains (Fig. 5a).

The in situ binding activity of these RE-1s in *REST*-expressing INS-1E cells was assessed by a chromatin

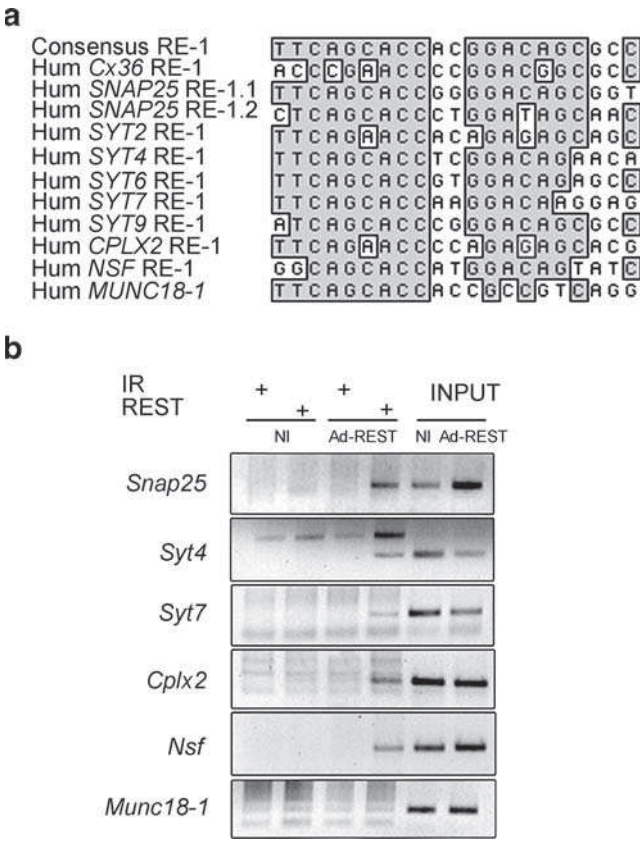

Fig. 5 Several genes coding for proteins of the exocytotic machinery have functional RE-1 binding sites. **a** Alignment of identified human (Hum) RE-1 sequences with consensus RE-1 and the functional Cx36 RE-1. The *SNAP25* gene contains two consecutive RE-1s referred to as RE-1.1 and RE-1.2. **b** Chromatin immunoprecipitation assay comparing wild-type (NI) and REST-transduced INS-1E cells (Ad-REST). PCR amplification of the indicated genes showed enrichment of the RE-1 flanking sequences in chromatin immunoprecipitated with anti-REST antibodies (REST) compared with irrelevant anti-SREBP-1 antibodies (IR). Non-precipitated chromatin (INPUT) is shown as control. Results are representative of three independent experiments

immunoprecipitation assay. The RE-1s of the *Snap25*, *Syt4*, *Syt7*, *Nsf and Cplx2* genes were immunoprecipitated with the antibodies against REST (Fig. 5b), confirming the functional properties of these RE-1 sequences. In contrast the RE-1 of *Munc18-1* was not enriched in the cells transduced for REST, consistent with the more variable sequence of this RE-1 motif (Fig. 5a).

REST represses a subset of genes implicated in exocytosis The effect of REST binding to RE-1s was assessed on several endogenous transcripts of INS-1E cells. A transcriptional repression was observed for the *Snap25*, *Syt4*, *Syt7*, *Syt9 and Cplx2* genes, but not for the genes coding for NSF and MUNC18-1 (Fig. 6a). The signal for the *Syt7* gene, which is subjected to alternative splicing [24], corresponds to the most abundant *Syt7a* splice variant. The negative regulation of *Cx36* [11] is shown as a control. The expression levels of several genes lacking RE-1, including those coding for t-

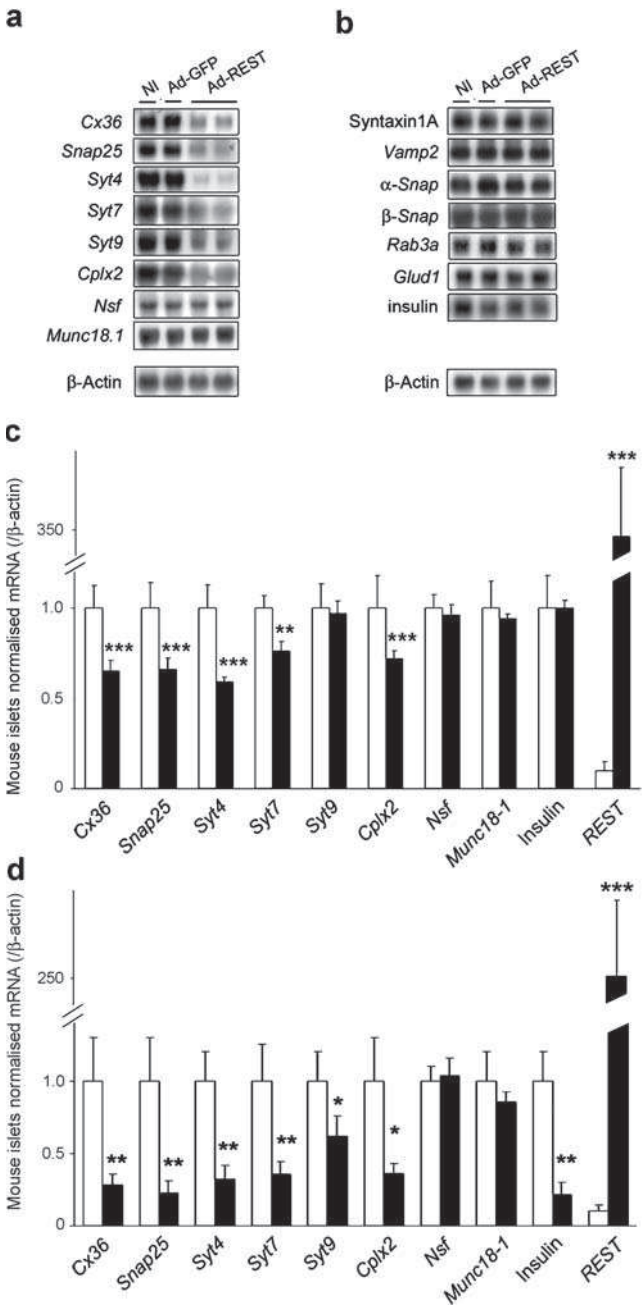

Fig. 6 REST downregulates the expression of several genes coding for proteins of the exocytotic machinery. **a**, **b** Northern blot of mRNA from wild-type (NI) and transduced (Ad-GFP, Ad-REST) INS-1E cells, displaying (**a**) the transcriptional downregulation of genes with identified RE-1 sequence and (**b**) the transcript levels of genes that lack RE-1 and which encode other proteins of the exocytotic machinery, insulin and mitochondrial *Glud1*. β-actin mRNA signals served as internal control. **c** Quantitative RT-PCR analysis of mRNA levels of putative target genes in mouse islets transduced with Ad-GFP (white columns) and Ad-REST (black columns). Results are mean ±SEM of four independent experiments. **d** Quantitative RT-PCR analysis of mRNA levels of putative REST target genes from islets of wild-type (white columns) (n=6) and RIP–REST (black columns) (n=10) mice. Results are mean±SEM. *p<0.05, **p<0.01, ***p<0.001

SNARE syntaxin 1A, v-SNARE vesicle-associated membrane protein 2 (VAMP2), α- and β-soluble NSF attachment protein, small GTP-binding protein RAB3A, member RAS oncogene family (RAB3A), *Glud1* gene encoding mitochondrial glutamate dehydrogenase (GDH), and insulin were not modulated by REST (Fig. 6b). Quantitative RT-PCR revealed a similar transcription pattern in islets transduced for REST (Fig. 6c) or isolated from RIP–REST mice (Fig. 6d). However, in contrast to the findings in transiently transduced INS-1E cells and islets, the constitutive expression of *REST* in islets from transgenic mice was also associated with lower levels of insulin mRNA (Fig. 6d). To assess whether these transcriptional changes affected the levels of the cognate proteins, we performed western blots with total extracts of transduced INS-1E cells (Fig. 7a) and of islets isolated from RIP–REST mice, where *REST* was declared as a 210 kDa immunoreactive band (Fig. 7b). In both cases, the presence of REST decreased the levels of SNAP25 and SYT IV proteins, detected as immunoreactive bands of 25 and 45 kDa, respectively. CX36 is shown as a positive control [11].

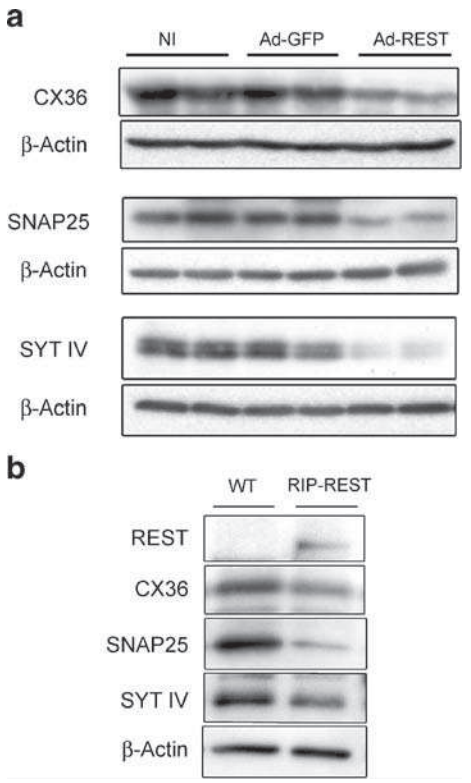

Fig. 7 REST downregulates the expression of selected protein. **a** Immunoblotting of total protein from wild-type (NI) and transduced (Ad-GFP, Ad-REST) INS-1E cells showed the decreased levels of SNAP-25 and SYT IV proteins in *REST*-expressing cells. CX36 levels are shown as a positive control. **b** Immunoblotting of total proteins from islets isolated from wild-type (WT) (n=3) and RIP–REST (n=3) mice. An immunoreactive band for REST (210 kDa) was found in RIP–REST but not in control islets. CX36, SNAP25 and SYT IV abundance were reduced in RIP–REST animals

SYT IV or SYT VII silencing inhibits hormone secretion To clarify the role of SYT IV and SYT VII in insulin secretion, we used a plasmid directing the expression of a specific siRNA and of hGH as a reporter gene. We compared the effect of three constructs, containing either a siRNA specific to the *Syt4* isoform (siRNA *Syt4*), to all *Syt7* splice variants (siRNA *Syt7*) or, as a negative control, to human *REST* (siRNA *REST*). The silencing efficiency of each construct was checked at the transcript levels of target genes fused to luciferase reporter gene (data not shown). The effect of SYT silencing on hGH secretion was assessed in transfected INS-1E cells, stimulated with a mixture of 15 mmol/l glucose, 10 μmol/l forskolin and 100 μmol/l IBMX (Fig. 8). Under these conditions, the control cells transfected with pXGH alone showed a fourfold increase in the release of hGH. The selective knockdown of either SYT IV or SYT VII did not significantly alter basal secretion, but inhibited hGH release by 60 and 50%, respectively (Fig. 8). The transfection of pXGH together with a plasmid coding for human REST, also resulted in a 40% decrease in the stimulated hGH secretion, whereas the transfection of siRNA *REST* did not alter hGH release. These data show that SYT IV and SYT VII contribute to the control of insulin release.

Discussion

Through its loss of function at different stages of neural differentiation, the transcriptional repressor REST plays a

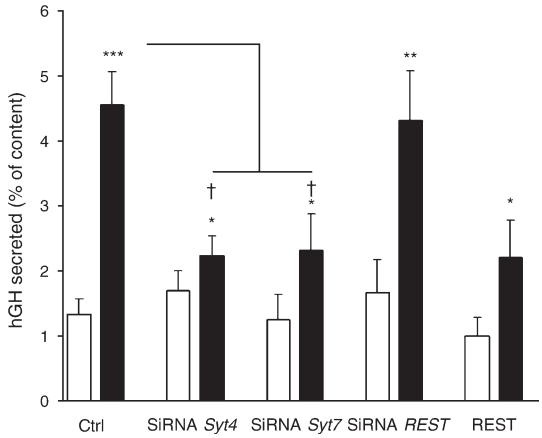

Fig. 8 SYT IV or SYT VII silencing inhibits hormone secretion. hGH release in INS-1E cells transiently transfected with a plasmid encoding hGH alone (Control [Ctrl]) or with a plasmid encoding hGH together with either the siRNA *Syt4*, the siRNA *Syt7* or the siRNA *REST*. Secretion was monitored under basal conditions (white columns) and after stimulation by 15 mmol/l glucose plus 10 μmol/l forskolin and 100 μmol/l IBMX (black columns). Silencing of either SYT isoforms or *REST* expression reduced the stimulated hGH release. Results are mean±SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus values of respective basal conditions; †p< 0.01 versus values of stimulated control condition

strategic role in the specification of the neuronal phenotype, [25]. The absence of REST from mature pancreatic beta cells also assigns to this cell type a specific pattern of genes, termed neuronal traits [10]. Recently, these traits have been implicated in the functioning of a transformed pancreatic beta cell line [1]. However, it has not been elucidated whether any of these genes is directly involved in primary beta cell function and if so, through which mechanism. In the present study, we show that the target genes of REST are crucial for proper function of adult primary beta cells, inasmuch as a gain of REST function impairs first and second phases of insulin secretion. Our data suggest that REST controls a step of the stimulus–secretion coupling pathway, which is downstream of the elevation of intracellular Ca^{2+} and common to both first and second phases of insulin secretion. We hypothesised that it may be related to vesicle trafficking and/or fusion. Accordingly, we found that REST repressed the expression of several genes coding for proteins of the exocytotic machinery, including SNAP25, SYT IV, SYT VII, SYT IX and complexin II. The exocytosis of insulin-containing LDCV resembles that of neuronal synaptic vesicles [26] and employs a similar broad set of proteins, each of which plays a precise role. In particular, a direct implication in the control of insulin secretion has been established for several members of the exocytotic machinery, including SNAP25 [27], NSF [28], MUNC18–1 [29] and both SYT V and SYT IX [30]. However, the role of SYT IV and SYT VII in neurons and pancreatic beta cells is still a matter of debate. In neuroendocrine PC12 cells, SYT IV has been identified as an essential component for the maturation of secretory granules [31]. This protein has also been reported to play a role in the stabilisation of the fusion pore and in the choice between kiss-and-run and full fusion events, for both synaptic vesicles of PC12 and LDCV of MIN-6 cells [32, 33]. In PC12 cells, SYT VII has also been implicated in the exocytosis of synaptic vesicle [19, 34, 35], whereas in beta cells this protein may be implicated in endocytotic traffic and insulin exocytosis [36, 37]. Using specific siRNAs, we confirm the recently published data on the role of SYT VII in insulin secretion [37] and show that SYT IV also plays a significant role in the control of insulin release from INS-1E cells.

The integrity of the exocytotic machinery is critical for glucose homeostasis, as observed in the Goto–Kakisaki rat model of type 2 diabetes and in type 2 diabetic patients, in both of which the expression of SNARE proteins is decreased [38, 39]. In view of the present knowledge on granule dynamics and distinct pools, it has been speculated that the proteins of the exocytotic machinery that are implicated in the rate-limiting priming of secretory granules are strong candidates for the triggering of beta cell dysfunctions associated with type 2 diabetes [40]. In the RIP–REST transgenic mice that specifically express *REST* in beta cells, expression of multiple REST target genes, including those coding for the major proteins controlling exocytosis, was decreased. This model provides a unique opportunity to assess, in vivo, the function of REST-dependent genes. We observed that these genes are critical for insulin secretion, inasmuch as their downregulation resulted in loss of glucose-dependent and glucose-independent insulin release to a degree sufficient to cause glucose intolerance. In these mice, the expression of *REST* did not alter the architecture of pancreatic islets, but induced a reduction in the number of beta cells. This effect is consistent with the anti-proliferative action of REST, previously reported in tumoral transformation [41] and human neointimal hyperplasia [9]. The reduced number of cells correlated with reduced expression of the insulin gene and lower pancreatic content of the hormone, which was not observed in INS1-E cells acutely transduced for REST. This lower rate of insulin production may be accounted for by the decreased insulin exocytosis rate and/or the long term REST-induced repression of key factors, such as the protein tyrosine phosphatase, receptor type, N (ICA512). Upon exocytosis, this LDCV-associated protein is cleaved and stimulates insulin synthesis through a retrograde pathway in order to adjust insulin production to its exocytosis [42]. The *Ica512* gene is a bona fide RE-1 containing REST target gene, whose expression was decreased in both *REST*-expressing INS-1E cells and islets of RIP–REST transgenic mice (data not shown).

According to recent data on the genome-wide chromatin occupancy mediated by REST in non neuroendocrine cells, new target genes implicated in different cell functions remain to be found and their effects evaluated [43, 44]. The multiplicity of these genes and their functional interactions make it necessary to perform comprehensive studies using gene array-based analyses of RIP–REST mice. Such studies should identify which of these genes is key to beta cell identity, insulin secretion and beta cell survival. Such a comprehensive analysis was beyond the scope of our study and will be the topic of future experiments.

In summary, using an innovative approach of ectopic *REST* expression in beta cells, we have identified several target genes that significantly contribute to the in vitro and in vivo control of glucose-stimulated insulin secretion. Our findings document the physiological importance of the native downregulation of REST in beta cells and possibly in other neuroendocrine cells [45] with a view to maintaining normal secretion by regulating the levels of key exocytotic proteins. Our findings point to the RIP–REST transgenic mice as a pertinent model for the identification of other REST target genes that may play a critical role in the pathophysiology of glucose intolerance and, possibly, of diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Supplementary data

Electronic supplementary material

ESM Table 1 *Specific primers for northern blot analysis*

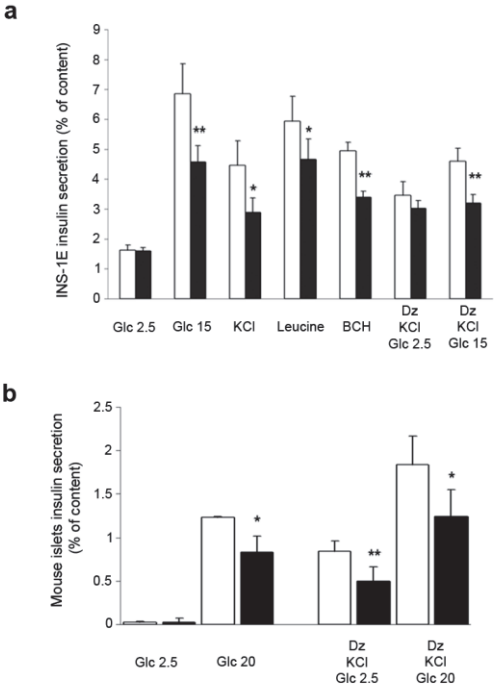
Gene	NCBI		
Snap25	NM_030991	TGATGAGTCCCTGGAAAGCACC	GGTTTTGTTGGAGTCAGCCTTCTC
Syt4	NM_031693	GAGAAGCGAGACCTCAATGG	AGACACATCAGATTTCGGTAGG
Syt7	NM_021659	GGTGAGAAGAAAGCCATCAAGTTGC	TGGAGTTGGCAGAGGGGTTG
Syt9	NM_019350	GGAGTTACATAGATAAGGTTCAGCC	CAGGACAATGACGGTGAGC
Cplx2	NM_053878	CAGTGGCTTAGACGGTTGC	GCTACTTGGGAGTGAAACAGG
Nsf	NM_021748	CAAGGAACTGGCTGTGGAGACTAAG	ACATCTTTGCGGCTGGTGG
Vamp2	NM_012663	CCATCATCCTCATCATCATCATCG	TTTGTTTCTGTGGGGTTTGC
α-Snap	NM_080585	ACAATCGCAGCCAAGCACC	GCAGGTCTTCCTCATCACCC
beta	NM_019632	ACAATCGCAGCCAAGCACC	CCCCTTGGATGGACTTCTTGATAC

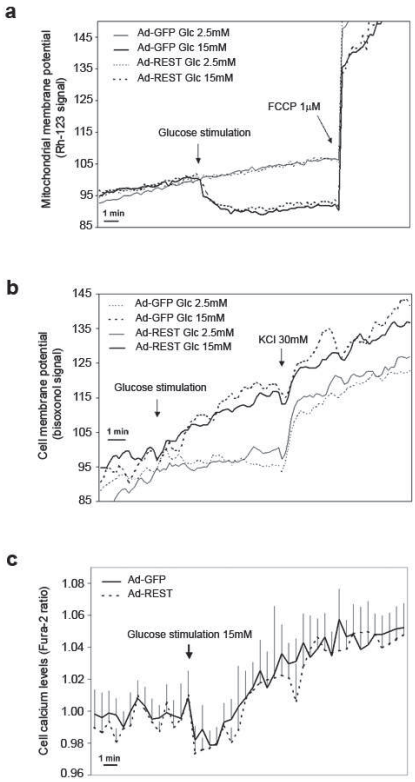
ESM Table 2 *Specific primers for real-time RT-PCR*

Gene	NCBI access	Sense primer (5′–3′)	Antisense primer (5′–3′)
Cx36	NM_010290	ATACAGGTGTGAATGAGGGAGGATG	TGGAGGGTGTTACAGATGAAA
Snap25	NM_011428	CGCAGGGTAACAAACGATG	GGAGGACAGCACAGCAGAA
Syt4	NM_009308	GGACAGAGCACGCAGAAA	TGTATGGAGGAGTCTTGTTGG
Syt7	NM_018801	ACCCGACAAGAAGCACA	GCTGAAACGGTCATAATCCA
Syt9	NM_016908	CTGGGTCCTGGCTACCATC	CCGGTCCAGCTCCTCTATTT
Cplx2	NM_009946	CAGTGGCTTAGACGGTTGCT	CCTCCTTCTTCTGTGCGTCT
Nsf	NM_008740	CATTTGCTTCTCGGGTGTTC	AGCCTCCTTTGCTCCTCTTC
Munc18	NM_009295	GCTGGATACAAAGCACTACCC	TCCTATCAGCACTTCCCACTT
Insulin	NM_008387	TGGCTTCTTCTACACACCCA	TCTAGTTGCAGTAGTTCTCCA
Rest	NM_011263	GGGATGTGTCTGGGAAGAAG	CCTGTTTGTCCGTCTGTGTG
REST	NM_005612	ACACCTGAAACACCACACCA	AACTTGAGTAAGGACAAAGTT

ESM Table 3 *Specific primers for chromatin immunoprecipitation assay*

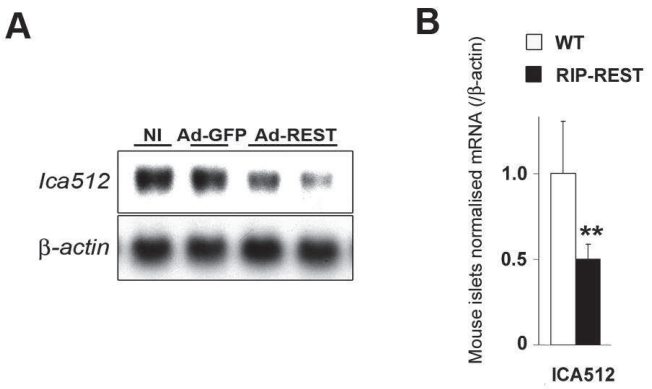
Gene	NCBI access	Sense primer (5′–3′)	Antisense primer (5′–3′)
Snap25	NM_030991	CGTCCCACAGACTTGATTTATG	CCCGATGACAGGATTTATTCT
Syt4	NM_031693	CCTTGTTTCAGGGTCCTTCC	GCTGTTCTCGTCTTCCCTTC
Syt7	NM_021659	CGTTCCAGTCTCTTGAGTCTTC	CCCTCTTGTGTGTTATTTCAC
Syt9	NM_019350	CAGGGCGTTCTCCTTTCTC	ACCCTTCTGGAGGTGCTGT
Cplx2	NM_053878	TCGAAGTTGTTCTCCAGGTTT	CGGCAGTGCGGTTTAGAG
Nsf	NM_021748	TTTAGTTTGAGTTCATGATCCT	ATTGGAGTCTCTGGTCCTCT
Munc18-1	NM_013038	CACCATTGAAGACAAACTGGA	AGGGTGCATGAAGGGAAAG





ESM Fig. 1 *REST target genes are crucial for proper beta cell insulin secretion.* ***a*** *Insulin release of INS-1E cells transduced with Ad-GFP (white columns) or Ad-REST (black columns). After incubation with 2.5 mmol/l glucose (Glc), cells were stimulated with either 15 mmol/l glucose, 30 mmol/l KCl, 20 mmol/l leucine plus 10 mmol/l glutamine or 20 mmol/l BCH plus 10 mmol/l glutamine. In experiments testing the K_{ATP} channel-independent pathway, cells were stimulated for 30 min with 30 mmol/l KCl and 250 μmol/l diazoxide (Dz), in the presence of 2.5 mmol/l glucose and then for another 30 min with the same medium containing 15 mmol/l glucose. Values are mean±SEM of four independent experiments.* ***b*** *Insulin release of mouse islets transduced with Ad-GFP (white columns) or Ad-REST (black columns), stimulated in static experiments with either 2.5 mmol/l or 20 mmol/l glucose, with or without diazoxide and KCl. Values are mean±SEM of four independent experiments. *p<0.05, ** p<0.01.*

ESM Fig. 2 *Repression of REST target genes does not alter plasma and mitochondrial membrane potentials or cytosolic Ca^{2+} levels.* ***a*** *The mitochondrial membrane potential was measured in Ad-REST and Ad-GFP-transduced INS-1E cells using rhodamine-123 fluorescence. Cells were stimulated with the indicated glucose (Glc) concentration for 10 min, before depolarisation was induced with 1 μmol/l protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Each trace is representative of five independent experiments.* ***b*** *The cell membrane potential was measured in transduced INS-1E cells using bisoxonol fluorescence. Cells were stimulated with the indicated glucose (Glc) concentrations for 10 min, before depolarisation was induced by 30 mmol/l KCl. Each trace is representative of four independent experiments.* ***c*** *Cytosolic calcium levels were measured in transduced INS-1E cells loaded with Fura-2. After 5 min at 2.5 mmol/l glucose, cells were challenged with 15 mmol/l glucose. Traces are mean±SEM of four independent experiments*



Additional Figure: *Ica 512 transcriptional downregulation by REST.* ***A****. Northern blot from INS-1E cells infected (Ad-GFP, Ad-REST) or not (NI).* ***B.*** *Quantitative RT-PCR analysis of mRNA from islets of wild type (WT) or RIP-REST mice. Values are mean±SEM of four independent experiments. ** p<0.0*

Chapter 2: Diabetic RIP-REST mice reveal the participation of REST target genes in beta-cell maintenance and survival

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This work has been submitted for publication in 2010.

This work has been achieved consecutively to the first report on RIP-REST mice. After the generation of transgenic mice, we obtained several RIP-REST founder mouse lines displaying different levels of REST transgene expression, and different phenotypes. Analysis of the glucose-intolerant RIP-REST mice indicated that, besides the implication of RE-1-containing genes in insulin secretion, a subset of these genes may be important for beta-cell maintenance, since RIP-REST mice featured reduced beta-cell mass. The observation that RIP-REST 5 mice were also glucose-intolerant led us to confirm our previous observation, while we definitely ascertained that REST target genes are important for beta-cell survival, since a third RIP-REST line featured diabetes due to dramatic loss of beta-cell mass.

This work reinforced the long-term ongoing collaboration that we had with Pr. Meda (Geneva). It also led to the first observations that REST may be important for beta-cell fate decision, and show the tendency to engage more extensive collaboration with Pr. Grapin-Botton (Lausanne), here represented by Dr. Gesina, to study the role of REST during pancreas embryonic development.

Abstract

Aim/hypothesis: Using transgenic RIP-REST mice, we have previously reported that REST target genes are essential to insulin exocytosis. Pancreatic beta cells from these mice featured defects in insulin secretion, as well as decreased mass, suggesting that a subset of the REST target genes is also crucial for beta-cell survival. The characterization of a novel RIP-REST founder mouse line featuring diabetes confirmed this hypothesis. Methods: Three different lines of RIP-REST mice were compared for REST transgene expression, glucose homeostasis, and insulin content. Quantitative assessment of the islet cell mass and insulin secretion were performed in the diabetic and control mice. New target genes were identified and their specific function assessed using INS1-E cells expressing REST or specific siRNA. Results: REST abundance in beta cells was inversely correlated with pancreatic insulin content in the different transgenic lines. High levels of transgenic REST expression were associated with beta-cell apoptosis after birth, which resulted in a major beta-cell loss in adult RIP-REST mice, and sustained hyperglycemia. Perinatal observations revealed that knock-down of REST target genes also impaired endocrine cell fate decisions. In INS1-E cells, screening for *bona fide* REST target genes identified several genes important for beta-cell survival, such as Irs2, and Ica512, but also pointed out at Cdk5r2 as a novel crucial gene for this process, as demonstrated by siRNA experiments. Conclusions/interpretations: Our data show that a subset of REST target genes is essential for beta-cell maintenance and survival. Specifically, we identified Cdk5r2 as a novel gene important for the protection of beta cells against cytotoxic attacks. The study calls for a comprehensive identification of other REST target genes.

Introduction

Type 1 (T1D) and type 2 (T2D) diabetes are characterized by an absolute or relative insulin deficiency, respectively. As observed in T1D (Kurrer et al., 1997), the loss of functional beta-cell mass in T2D patients occurs through beta-cell apoptosis (Butler et al., 2003). While the triggering events and the nature of the molecular effectors leading to diabetes-associated apoptosis are still disputed, some of the critical regulators of beta-cell survival have been identified (Reviewed in (Anderson et al., 2009)). Importantly, the search for intrinsic pro-survival factors has identified key proteins such as insulin receptor substrate 2 (IRS2) (Withers et al., 1999, Hennige et al., 2003), the anti apoptotic members of the BCL2 family: BCL2 (Allison et al., 2000, Saldeen, 2000) and BCL-XL (Carrington et al., 2009), islet brain 1 (IB1) (Bonny et al., 2000), islet cell antigen 512 (Ica512) (Mziaut et al., 2008), AKT/PKB (Datta et al., 1997) and many others.

Our incomplete knowledge of the mechanisms responsible for the unusual susceptibility of beta cells to metabolic stress and inflammation, imposes that specific positive regulators of beta-cell mass are identified. To better understand what is a beta cell and attempt to improving it under pathological situations (Schuit et al., 2005), we initiated a search for new beta cell-specific genes. Using transgenic mice expressing the transcriptional repressor REST specifically in beta cells (RIP-REST mice), we previously characterized the function of a wide group of genes that contains the REST binding motif, called Repressor Element 1 (RE-1) (Martin et al., 2008). REST is a zinc finger transcription factor which blocks the expression of neuroendocrine traits in all cell types but neurons and beta cells. Indeed, REST is commonly absent in mature insulin-producing cells and neurons (Chong et al., 1995, Schoenherr and Anderson, 1995, Atouf et al., 1997) but suppresses elsewhere the expression of the large set of RE-1-containing genes, thereby ensuring that their expression is specific to neuroendocrine cell types. Upon ectopic REST expression in the RIP-REST transgenic mice, REST target genes were

specifically silenced in beta cells. The resulting phenotype showed that, *in vivo*, RE-1-containing genes are crucial for proper beta-cell function (Martin et al., 2008). The observation that several *bona fide* REST target genes code for proteins that are key to exocytosis further substantiated our observations (Martin et al., 2008). Herein, we report the characterization of a novel line of RIP-REST founder mice which demonstrates that RE-1-containing genes are also essential to beta-cell survival. These mice featured diabetes as a consequence of a massive loss of beta cells through apoptosis. *In vitro* experiments with INS1-E cells transduced with REST-expressing adenoviral vector led to the identification of several genes critical for beta-cell survival, including Irs2, Ica512, and Cdk5r2 (p39). siRNA specific down-regulation of the latter activator increased the susceptibility of INS1-E cells to cytokine treatment, indicating that Cdk5r2 has an anti-apoptotic activity in beta cells.

Results

Diabetic RIP-REST mice feature hyperglycemia associated with loss of insulin secretion

We previously reported the characterization of a glucose-intolerant line (referred to as RIP-REST) featuring defects in insulin secretion as well as decreased insulin production (Martin et al., 2008). Mice from a novel line (referred to as diabetic RIP-REST) showed frank diabetes. These mice featured a glycemia of 23.6 ± 2.6 mmol/l in 4 weeks of age and increased until 3 months old at a maximum measurable value of 33 mmol/l, resulting in lethality after few months. Control littermates had a basal level of 9.2 ± 1 mmol/l (Fig. 1a). To assess the secretory function in diabetic mice, the pancreas of 4-5 month-old transgenic and wild type animals were perfused in situ. Infusion of 8.0 and 16.0 mmol/l glucose led to the typical increasing biphasic response of insulin secretion in control mice. In contrast, secretion of the hormone was barely detectable in response to glucose in the diabetic animals (Fig. 1b). The addition of 1nmol/l GLIP-1 to 8.0 mmol/l glucose largely potentiated insulin release from wild type mice, and to a much lesser extent also in diabetic RIP-REST mice (Fig. 1b).

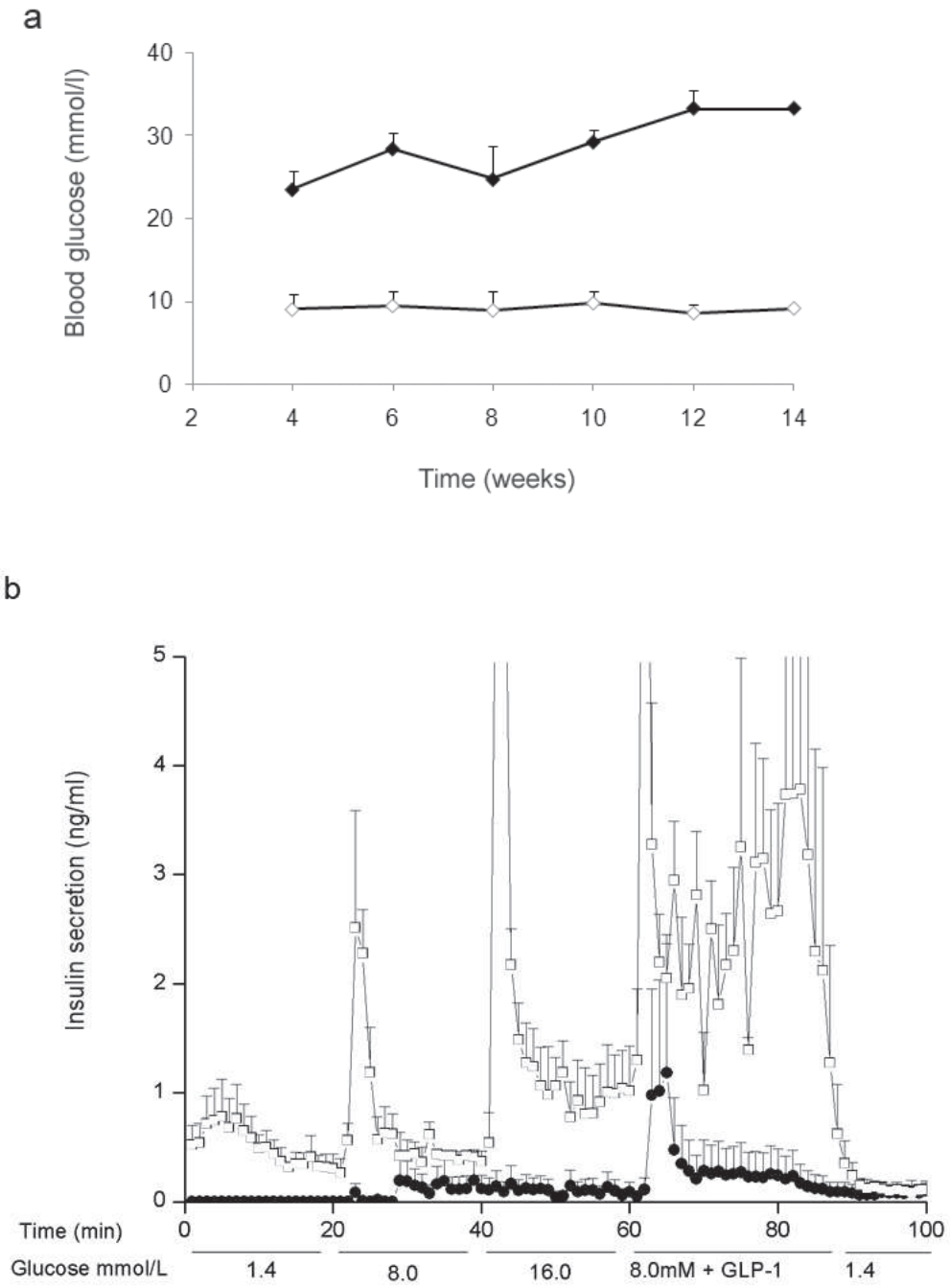
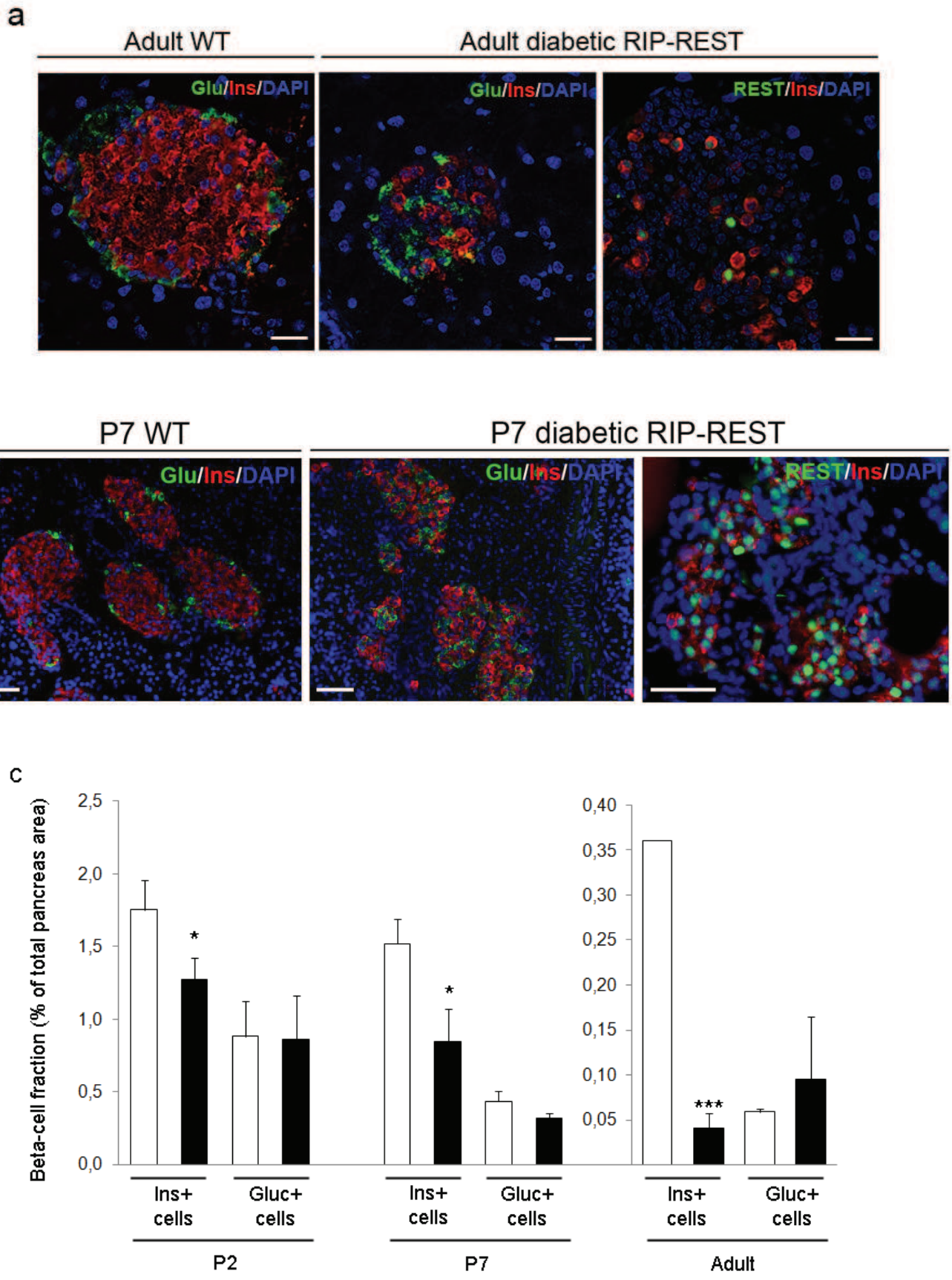


Fig. 1 Novel RIP-REST transgenic mice are hyperglycemic and show poor insulin secretion
a. Blood glucose levels were assessed at different ages in diabetic RIP-REST transgenic (dark diamonds; n=7) and wild type mice (open diamonds; n=5). Diabetic RIP-REST mice feature hyperglycemia from weaning onward.
b. Diabetic RIP-REST mice (black circles, n=3) and control littermates (open squares, n=4) were subjected to in situ pancreatic perfusion at 1.5 ml/min rate. After a 30-min equilibration period at basal 1.4 mmol/l glucose, the pancreas was perfused sequentially at different glucose concentrations, first at 1.4 mmol/l for 20 min, next at 8.0 mmol/l for 20 min, then at 16.0 mmol/l for 20 min, followed by a 30-min stimulation at 8.0 mmol/l plus 1 nmol/l GLP-1, and finally at 1.4 mmol/l for 15 min. Results are mean ± SD.

Loss of insulin secretion in diabetic mice is a result of a massive loss of beta cells

Confocal immunofluorescence for glucagon and insulin, performed on pancreas from 6 month-old mice, indicated that in contrast to the wild type islets showing numerous central beta cells and alpha cells at the periphery (Fig. 2a left panel), those of diabetic RIP-REST mice had much fewer beta cells and glucagon-positive cells scattered throughout the islets (Fig. 2a middle panel). Strikingly, we observed that only a small fraction of the surviving beta cells expressed the REST transgene (Fig. 2a right panel). When the same staining was performed on pancreas from 7 day-old mice (P7), a significant number of insulin positive cells was observed in diabetic mice (Fig. 2b middle panel), of which most expressed the *REST* transgene (Fig. 2b right panel). However, and in contrast to controls (Fig. 2b left panel), alpha cells were already distributed throughout the islets of diabetic mice (Fig. 2b middle panel). To better assess the time course of the beta cell loss occurring in diabetic mice, we quantified beta- and alpha-cell mass in P2, P7 and adult mice. Compared to control mice, we observed a gradual decrease of 30 ($p<0.05$), 45 ($p<0.05$), and 90% ($p<0.001$) in beta cells of P2, P7 and adult diabetic mice, respectively. In contrast, the alpha cell mass was statistically similar to that of controls at all ages (Fig. 2c). Strikingly, confocal microscopy also revealed a low frequency of double insulin and glucagon positive cells in adult diabetic RIP-REST mice, which was not observed in wild type mice (Fig. 2d).


a
Adult WT
Adult diabetic RIP-REST
Glu/Ins/DAPI
Glu/Ins/DAPI
REST/Ins/DAPI
b
P7 WT
P7 diabetic RIP-REST
Glu/Ins/DAPI
Glu/Ins/DAPI
REST/Ins/DAPI
c
Beta-cell fraction (% of total pancreas area)
2,5
2,0
1,5
1,0
0,5
0,0
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
*
*
***
Ins+ cells
Gluc+ cells
Ins+ cells
Gluc+ cells
Ins+ cells
Gluc+ cells
P2
P7
Adult

d

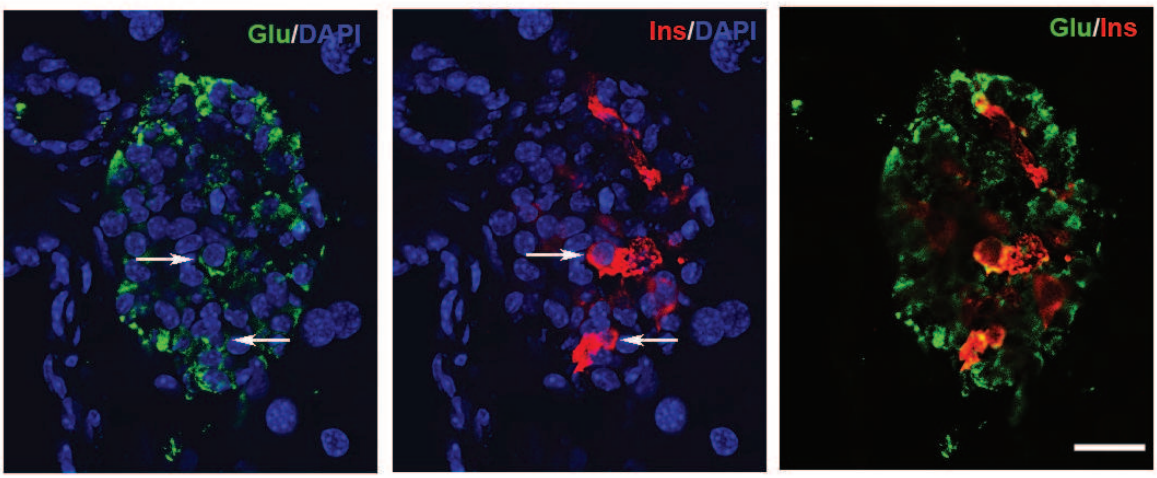


Fig. 2 REST expression is associated with a major loss of beta cell mass.
a. Immunostaining for insulin (Ins, red) and glucagon (Glu, green) reveals, when compared to wild type mice (WT, left panel), a major loss of insulin positive cells, within a low number of disorganized islets in pancreas of adult diabetic RIP-REST mice (middle panel). REST nuclear staining (green, right panel) indicates that only a few surviving beta cells still express the repressor. Blue staining is the DAPI labeling of nuclei. Scale bar, 25 µm.
b. Immunostaining for insulin (Ins, red) and glucagon (Glu, green) shows that, even if disorganized when compared to islets from wild type animals (WT, left panel), islets from diabetic RIP-REST mice at postnatal day 7 (P7) show a significant number of insulin positive cells (middle panel). REST nuclear staining (green, right panel) indicates that a majority of beta cells express the repressor. Blue staining is the DAPI labeling of nuclei. Scale bar, 50 µm.
*c. Quantification of beta (Ins+ cells) and alpha (Gluc+ cells) cell mass in P2, P7 and adult diabetic RIP-REST mice (black bars, n=3 each) and control littermates (white bars, n=3 each). A 30, 45 and 90% reduction in beta cell mass is observed in P2, P7, and adult diabetic RIP-REST mice, respectively, when compared with corresponding mass of controls. Results are mean ± SD. **P<0.01 versus values of control littermates.*
d. Confocal analysis of insulin (Ins, red) and glucagon (Glu, green) immunofluorescence shows scarce double insulin and glucagon expressing cells (arrows) in P2 pancreatic sections of diabetic RIP-REST mice. Blue staining is the DAPI labeling of nuclei. Scale bar, 25 µm.

REST expression is associated with beta cell loss through apoptosis

To investigate the postnatal beta-cell loss in diabetic RIP-REST mice, we first examined beta-cell proliferation in mice at a high proliferative stage (P2), using staining for PCNA. This marker of cell proliferation was observed at the same level in both control and transgenic mice (4.9 % ± 1.1 beta cells were $PCNA^+$ (79 out of 1598) vs. 6.2 % ± 0.26 (52 out of 833), respectively; p=0.1) (Fig. 3a). We next assessed beta-cell apoptosis in a stage where beta cell loss was high (P7), using TUNEL experiments. Whereas no apoptotic beta cells could be observed in control mice, we detected several in islets of P7 diabetic RIP-REST mice (Fig. 3b). These observations suggested that diabetes of transgenic mice mostly occurred because of the postnatal apoptosis of beta cells.

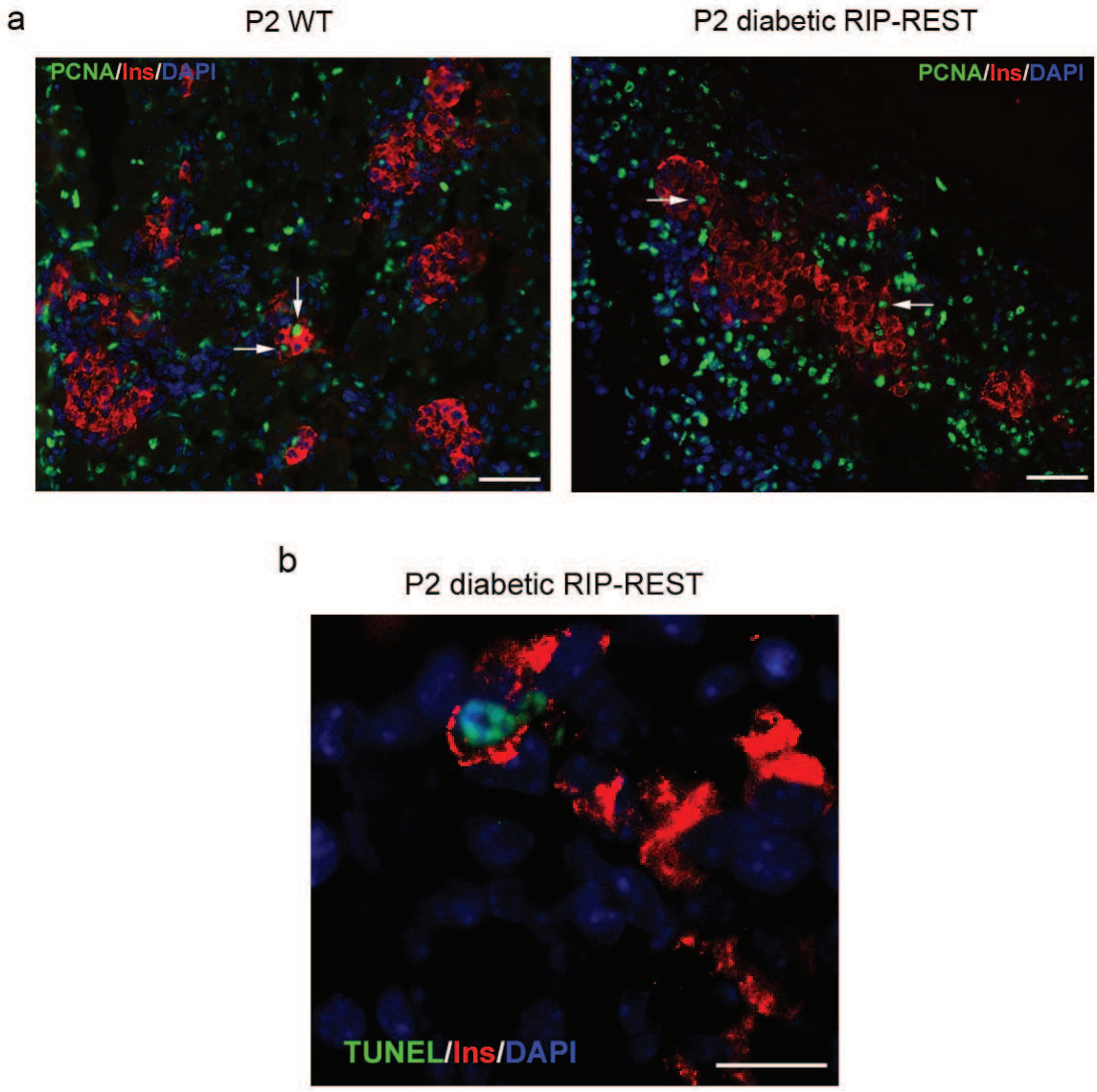


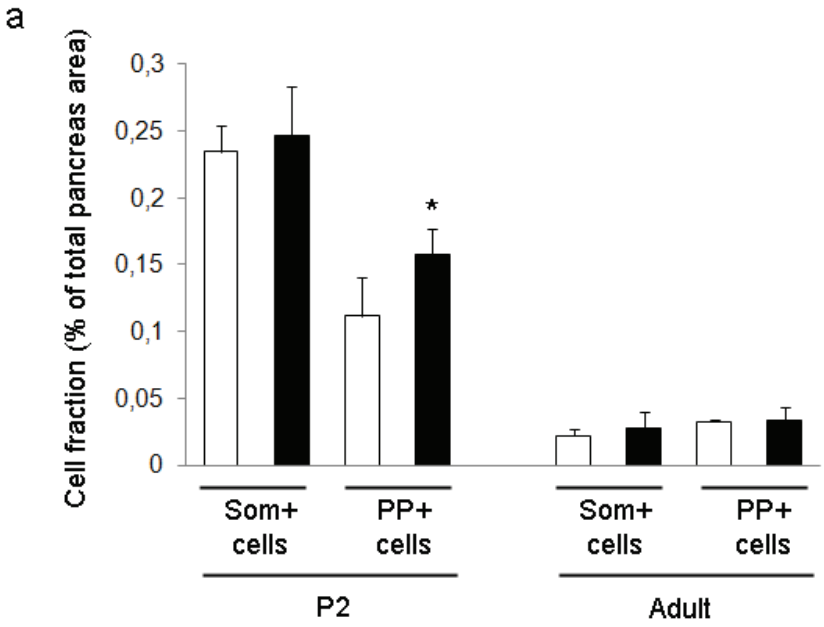
Fig. 3 Beta cell loss in diabetic RIP-REST mice occurs through apoptosis

a. Nuclear PCNA (green) and insulin (red) staining show similar levels of proliferating beta cells (arrows) in both wild type (WT, left panel) and diabetic RIP-REST mice (right panel) at P2. Blue staining is the DAPI labeling of nuclei. Scale bar, 50 μm.

b. Representative image of nuclear TUNEL (green) and insulin (red) staining showing apoptotic nuclei of beta cells in P7 sections of diabetic RIP-REST mice. Blue staining is the DAPI labeling of nuclei. Scale bar, 25 μm.

Increased PP cell mass in P2 diabetic RIP-REST mice suggests implication of RE-1-containing genes in beta-cell fate decision

We examined whether the loss of beta cells in the islets of diabetic RIP-REST mice would impact the other endocrine cell populations. We therefore quantified PP- and delta-cell mass after immunostaining for PP and somatostatin, respectively, when beta-cell mass reduction in diabetic mice was low (in P2 mice) and when it was maximal (in adult mice). Adult diabetic RIP-REST mice featured levels of PP- and delta-cell mass similar to those observed in control mice (Fig. 4a). In contrast, in presence of unchanged delta-cell fraction, P2 diabetic mice showed a 35 % increase in the PP-cell mass ($p<0.05$) as compared to wild type mice (Fig. 4a). Since at the same time point we noticed a few nuclei stained for REST that did not colocalize to insulin positive cells (data not shown), we stained sections for both REST and PP. We observed, using confocal microscopy, that 20% of the REST-stained nuclei co-localized with PP staining in the islets of diabetic mice (Fig. 4b upper panel). In the same animals, low numbers of PP cells were also stained for PDX1 (Fig. 4b lower panel). Together, these observations indicated that a fraction of developing beta cells, which were traced by REST and PDX1 staining, shifted towards a PP cell lineage in the P2 diabetic RIP-REST mice.



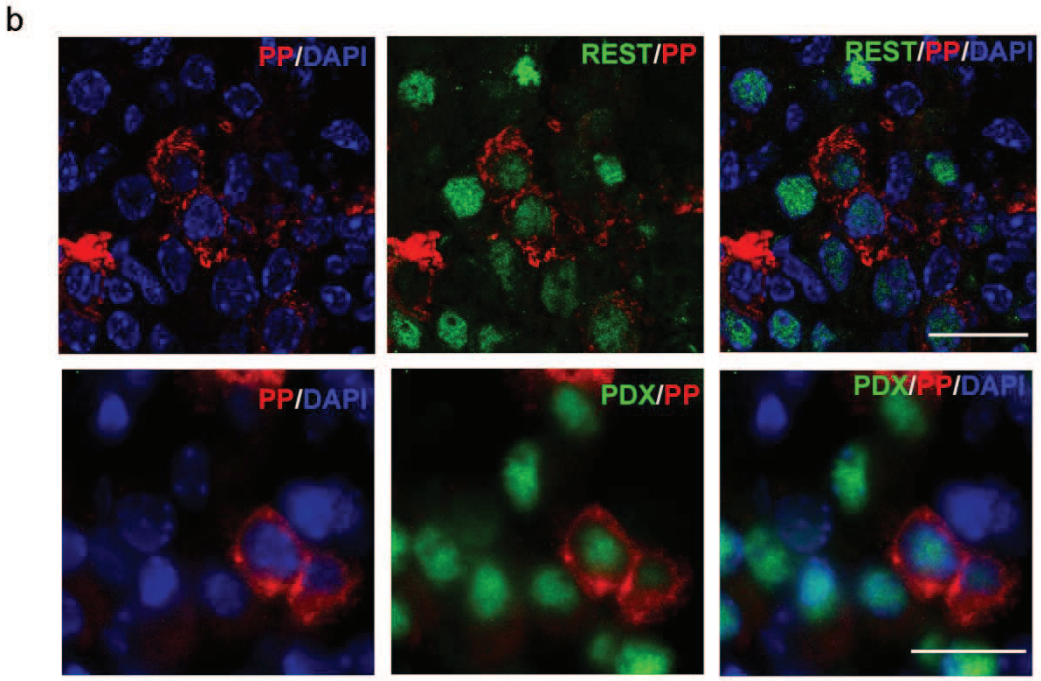
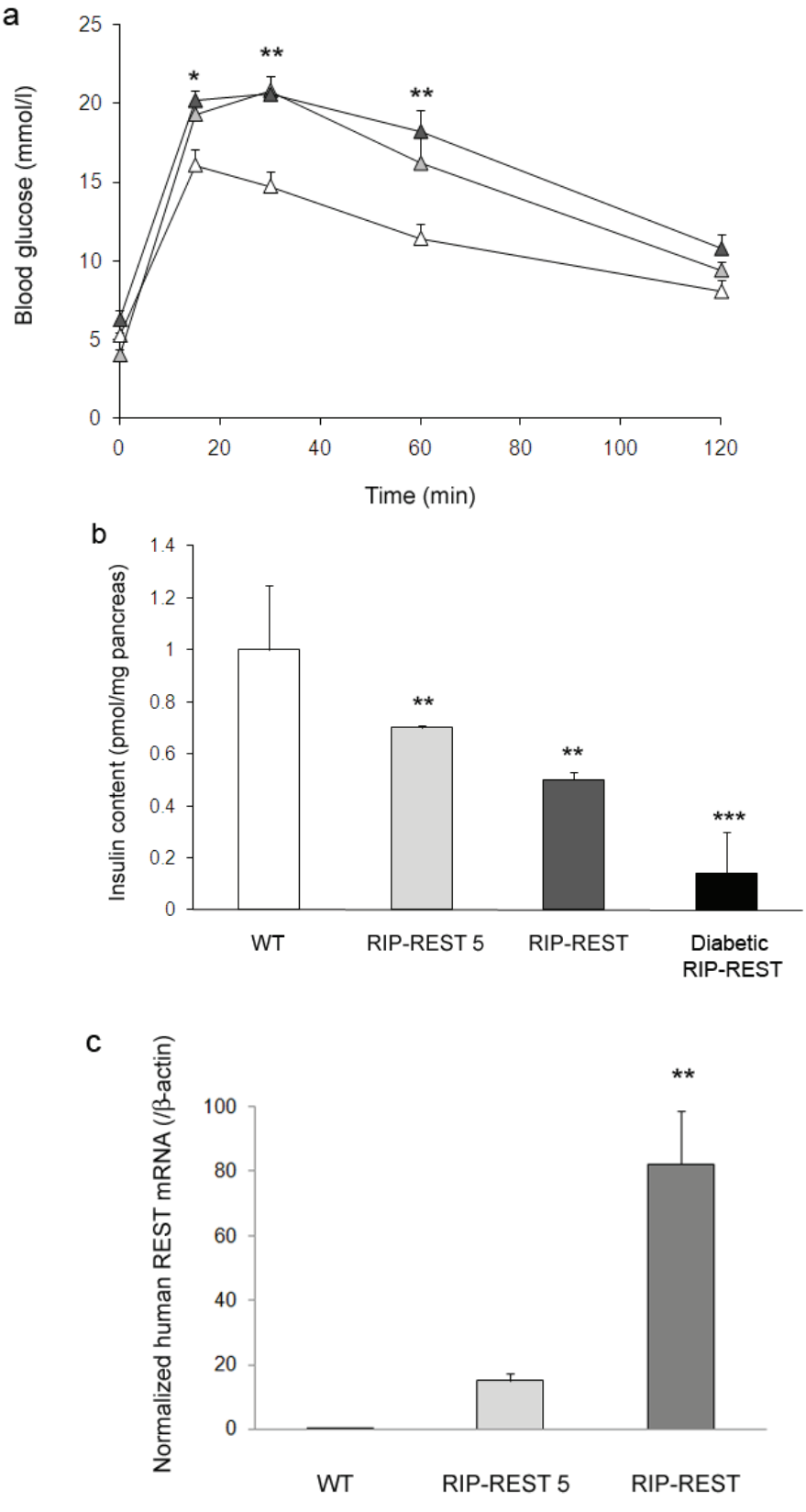


Fig. 4 Expression of RE-1-containing genes influences beta cell fate decision
*a. Quantification of delta (Som+ cells) and PP (PP+ cells) cell mass in P2 and adult diabetic RIP-REST mice (black bars, n=3 each) and control littermates (white bars, n=3 each). A 35% increase in PP cell mass is observed in P2 diabetic RIP-REST mice, when compared with corresponding mass of controls. Results are mean ± SD. *P<0.05 versus value of control littermates. Right panel shows representative images of somatostatin (green) and PP (red) staining in adult diabetic RIP-REST and control (WT) mice. Blue staining is the DAPI labeling of nuclei. Scale bar, 25 µm.*
b. Confocal analysis of REST (green) and PP (red) immunofluorescence in P2 pancreatic sections of diabetic RIP-REST mice (upper panel). REST staining allows following beta cell fate. REST and PP co-localization indicates that few beta cells shifted towards the PP phenotype. Confocal analysis of PDX1 (green) and PP (red) immunofluorescence in P2 pancreatic sections of diabetic RIP-REST mice (lower panel). A few PP cells express PDX1. Blue staining is the DAPI labeling of nuclei. Scale bar, 25 µm.

The level of REST expression inversely correlates with pancreatic insulin content in RIP-REST transgenic mice

We analyzed a third mouse line (referred to as RIP-REST 5) obtained after pronuclear injection of the RIP-REST transgene. As RIP-REST mice, those mice also featured glucose intolerance, as observed by IPGTT (Fig. 5a). Basal and glucose-stimulated blood glucose levels were similar in these two lines (Fig. 5a). However, measurement of pancreatic insulin content in each three transgenic line indicated that RIP-REST 5 mice featured a mild 30% decrease in insulin content, whereas RIP-REST mice had a 50% decrease, and diabetic RIP-REST mice a drastic 85% reduction, as compared to wild type mice (Fig. 5b). A qPCR analysis of islet mRNA from RIP-REST 5, RIP-REST, and wild

type mice (WT) indicated that REST transgene expression in RIP-REST animals was six fold higher than in RIP-REST 5 mice whereas, as expected, signal from wild type mice was null (Fig. 5c). As the line of diabetic RIP-REST mice could not be investigated by qPCR because of their low number of islets, we performed immunohistochemistry coupled with semi-quantitative peroxydase-based detection of REST protein. This revealed that REST abundance per beta cell was much higher in diabetic RIP-REST than in RIP-REST mice (Fig. 5d). REST protein level in the RIP-REST 5 mice was much lower than in RIP-REST mice, confirming the difference observed in transgene expression using qPCR (data not shown). These data demonstrated an inverse relationship between the levels of REST expression and insulin production and suggest that high levels of REST expression in beta cells were necessary and sufficient to induce diabetes in transgenic mice.


a
Blood glucose (mmol/l)
25
20
15
10
5
0
*
**
**
0
20
40
60
80
100
120
Time (min)
b
Insulin content (pmol/mg pancreas)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
***
WT
RIP-REST 5
RIP-REST
Diabetic RIP-REST
c
Normalized human REST mRNA (/β-actin)
100
80
60
40
20
0
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WT
RIP-REST 5
RIP-REST

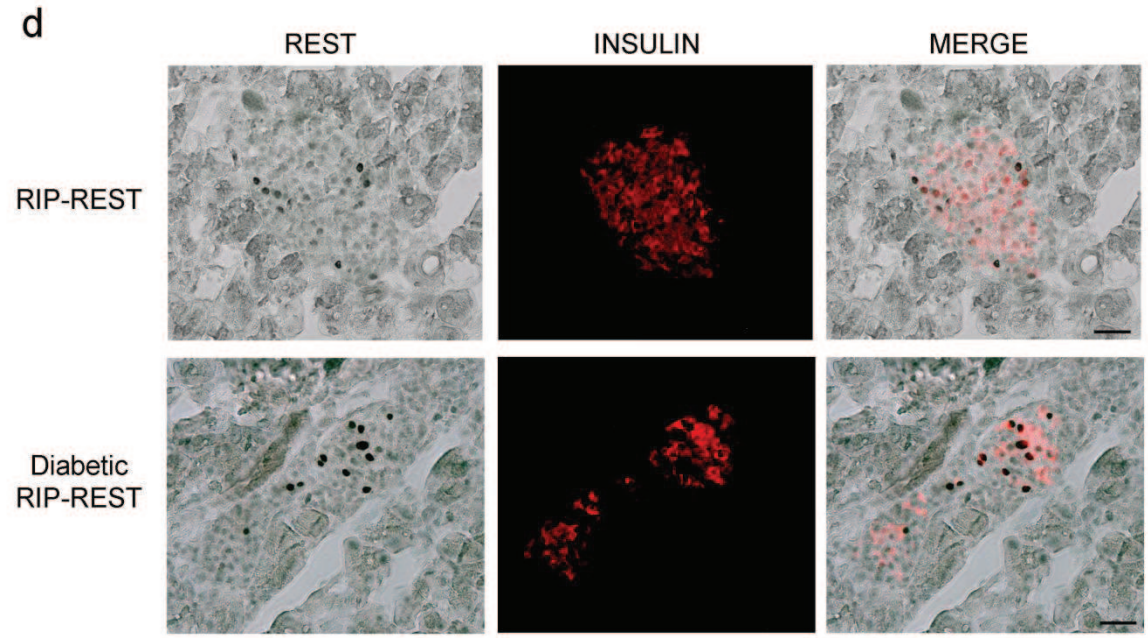

Fig. 5 Increasing REST level in beta cells leads to worsened glucose homeostasis
*a. Blood glucose levels in 6 month-old wild type (open triangles; n= 8), RIP-REST5 (gray triangles; n=8) and RIP-REST males (dark triangles; n=10) during an IPGTT. After a 14h fasting, blood samples were taken before (t=0) and after (t=15, 30, 60 and 120 min) intraperitoneal injection of glucose (2g/kg). Results are mean ± SEM. *P<0.05, **P<0.01.*
*b. Insulin content in pancreas of 5 month-old RIP-REST5 (light gray bar; n=8), RIP-REST (dark gray bar; n=6) and diabetic RIP-REST (black bar; n=6) transgenic mice reveal a 30, 50 and 85% reduction, respectively, when compared with wild type mice (white bars, n=6). Results are mean ± SD. **P<0.01, ***P<0.001 versus values of corresponding control littermates.*
*c. Quantitative RT-PCR analysis of human REST mRNA levels in islets of WT (white bar; n=5), RIP-REST5 (light gray bar; n=6) and RIP-REST (dark gray bar; n=6) mice. REST mRNA levels are six fold higher in RIP-REST than in RIP-REST5 animals. Results are mean ± SEM. **P<0.01.*
d. REST abundance in diabetic RIP-REST and RIP-REST mice. REST protein levels are revealed using specific antibodies against REST and AEC staining of peroxydase activity (nuclear black dots). Parallel immunostaining of insulin (red) and the merge shows colocalization of the two signals. Scale bar, 25 µm.

RE-1 database screening identifies several RE-1-containing genes involved in beta-cell survival

We screened a RE-1 database (http://www.bioinformatics.leeds.ac.uk/cgi-bin/RE1db/nrse.cgi) (Bruce et al., 2004) to identify RE-1-containing genes whose down-regulation could explain the apoptotic events responsible for the beta-cell loss occurring in the diabetic RIP-REST mice. We searched genes which should possess anti-apoptotic properties and with a good conservation of the RE-1 sequence. We selected 14 RE-1-containing genes that may be involved in growth signals transduction pathways (including members of the insulin-like growth factors pathway, PKB/AKT, and catenin isoforms), in transduction of apoptosis /proliferation (including mitogen-

activated protein kinase/stress-activated protein kinase) and in downstream mechanisms of mitogenesis (including cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors) (Fig. 6a).

We then used qPCR analysis to evaluate the expression of the corresponding mRNAs upon REST expression into INS-1E cells. These experiments showed that the majority of the selected candidates were not transcriptionally regulated by REST (data not shown). However, the expression of the genes coding for IRS2, PTPRN (also called ICA512), Cdk5 activator subunit 2, Cdk5r2 (also called p39), Nrg1, Ctnnd2 and Api5 were significantly reduced after expression of REST in INS-1E cells (Fig. 6b). While IRS2 and PTPRN are known to have anti-apoptotic effects (Hennige JCI 2003; Mziaut PNAS 2008), the latter four proteins are not (Fig. 6b).

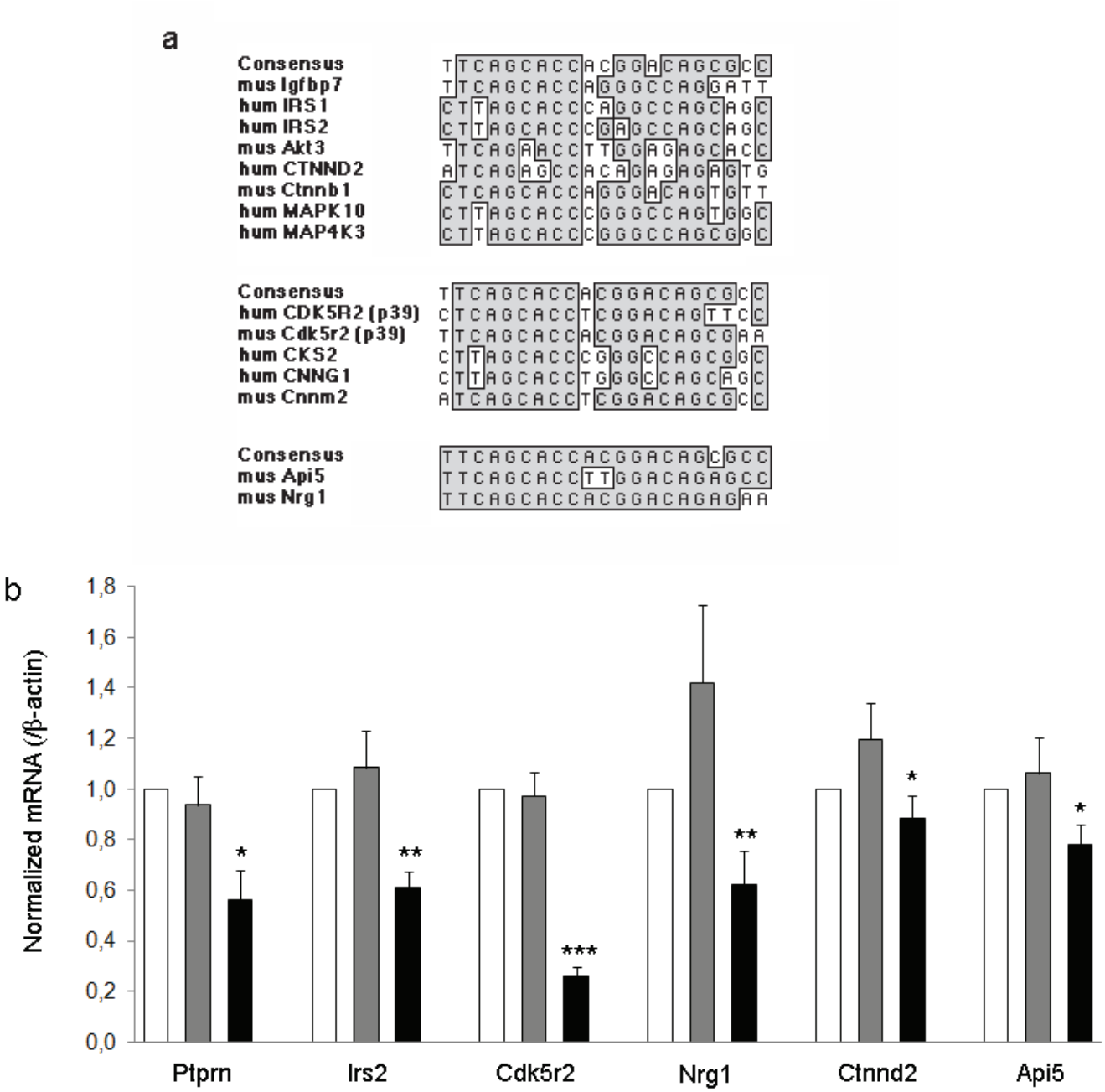

Fig. 6 Identification of RE-1-containing genes potentially involved in beta cell survival

a. Alignment of identified RE-1 sequences in human (hum) or murine (mus) genes with the consensus RE-1. Upper alignment identified members of the insulin-like growth factor transduction pathway: Igfbp7: insulin-like growth factor binding protein 7; IRS: insulin receptor substrate; Akt3: PKB gamma; CTNND2: delta2 catenin; Ctnnb1: beta catenin; MAPK10: JNK3; MAP4K3: MEK kinase kinase 3. Middle alignment identified members of the cyclin-related family of mitogenic factors: Cdk5r2: cyclin-dependent kinase 5 regulatory subunit 2; CKS2: CDC28 protein kinase regulatory subunit 2; CNNG1: cyclin G1: Cnnm2: cyclin M2. Lower alignment identified Api5: apoptosis inhibitor 5 and Nrg1: neuregulin1.

*b. Quantitative RT-PCR analysis of mRNA levels from control INS-1E cells (white bars) and INS-1E cells infected with GFP-expressing adenovirus (gray bars) or REST-expressing adenovirus (black bars). PTPRN: ICA512; IRS2: insulin receptor substrate2; Cdk5r2: cyclin-dependent kinase 5 regulatory subunit 2; Nrg1: neuregulin1; CTNND2: delta2 catenin; Api5: apoptosis inhibitor 5. Results are mean ± SEM of six independent experiments. *P<0.05, **P<0.01, ***P<0.001 versus INS-1E cells transduced with GFP.*

Specific siRNA silencing of Cdk5r2 increases INS-1E cells susceptibility to cytokine

We focused on Cdk5r2, a target gene which was the most potently regulated by REST expression, to assess its putative role in beta cell apoptosis. We selected, among three, a specific shRNA to the rat Cdk5r2 which efficiently silenced the target sequence of Cdk5r2 fused to the luciferase reporter gene (data not shown). Transfection of the corresponding siRNA into INS-1E cells demonstrated a 50% reduction in Cdk5r2 transcript level after 72 h (Fig. 7a). This change did not affect the levels of Cdk5r1 (also called p35), another activator of Cdk5 (Fig. 7a). We then evaluated the effect of silencing Cdk5r2 expression on the capacity of INS-1E cells to survive to a cytotoxic attack. 54 h after siRNA transfection, INS-1E cells were treated during 16 h with a mix of IL1-β, TNF-α and IFN-γ. Cdk5r2 silencing *per se* had no effect on INS-1E cells viability, as compared to non-transfected cells (Fig. 7b). However, it was sufficient to sensitize INS-1E cells to the cytokine-induced apoptosis, as we observed a 30% increase in cell death after cytokine treatment, as compared to cells transfected with control siRNA (Fig. 7b). These results indicate that Cdk5r2 contributes to protect beta-cell against cytokines.

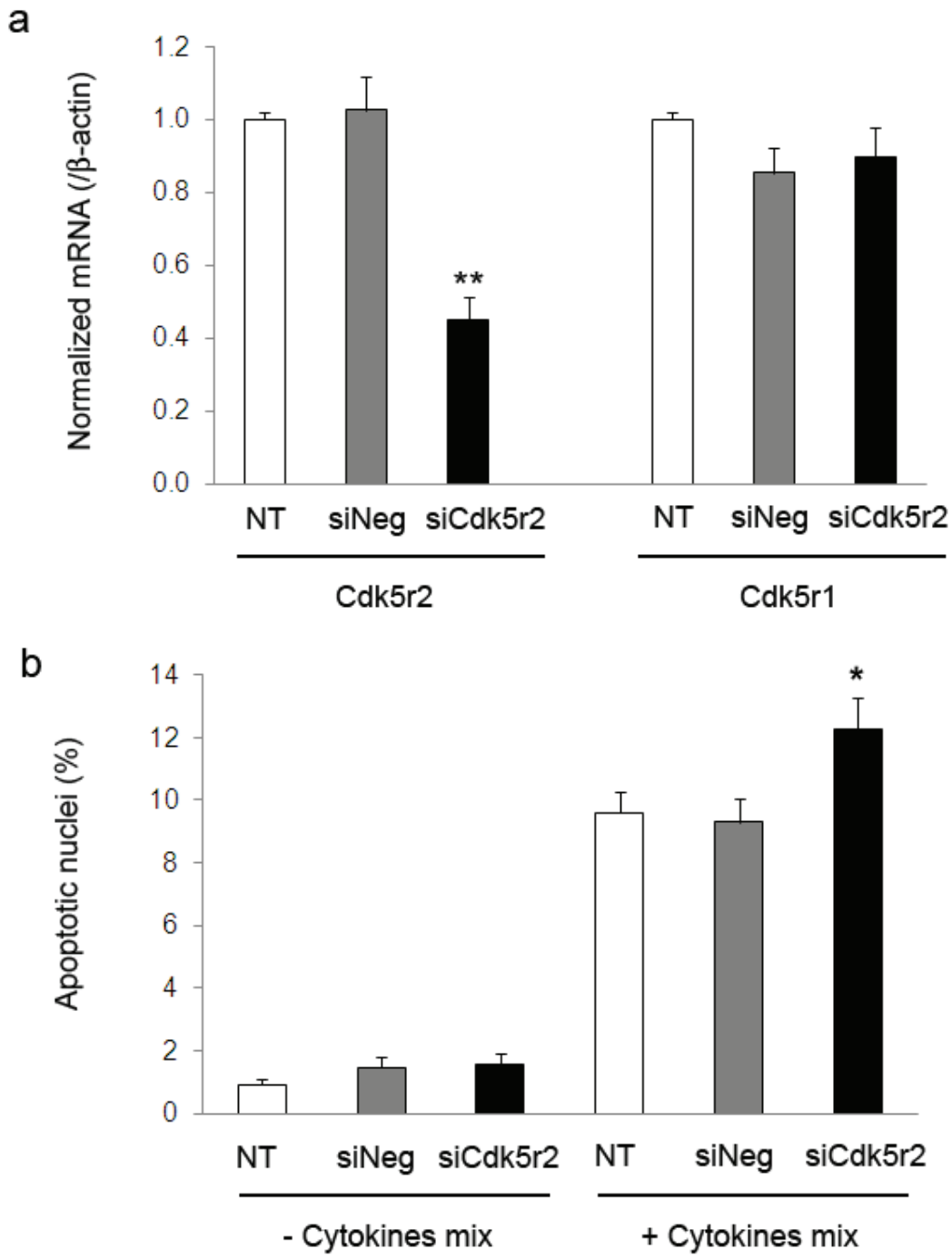

Fig. 7 Cdk5r2 protects beta cell against cytokines

*a. Quantitative RT-PCR analysis of Cdk5r2 and Cdk5r1 mRNA levels from control INS-1E cells (white bars) and INS-1E cells transfected with a negative siRNA (gray bars) or a siRNA against Cdk5r2 (black bars). Results are mean ± SD of three independent experiments. **P<0.01.*

*b. Quantification of apoptotic nuclei in control INS-1E cells (white bars) and INS-1E cells transfected with a negative siRNA (gray bars) or a siRNA against Cdk5r2 (black bars), treated (+) or not (-) with a mix of cytokines. Specific Cdk5r2 silencing induces increased susceptibility of INS-1E cells to cytokines. Results are mean ± SEM of six independent experiments. *P<0.05 versus controls.*

Discussion

Recent bioinformatic studies on the distribution of RE-1 binding sites in the genome, combined with analysis of chromatin occupancy mediated by REST, have revealed the existence of several hundred genes that are putative target genes of REST [(Bruce et al., 2004, Sun et al., 2005, Johnson et al., 2006, Mortazavi et al., 2006, Johnson et al., 2007, Otto et al., 2007). Despite this large output, the caveat of these studies is the lack of functional assessment of the candidate genes. The generation of transgenic mice expressing REST in beta cells represents a reliable system to identify the importance of some RE-1-containing genes in insulin-producing cells (Martin et al., 2008). Specifically, RIP-REST mice showed intolerance to glucose, which led to the identification of several REST target genes which are key to exocytosis of insulin-containing granules (Martin et al., 2008). These animals also showed a decrease in beta cell mass (Martin et al., 2008), suggesting that REST target genes were also involved in beta-cell survival. We now document this role in a RIP-REST mouse line which displays diabetes, as a result of a high level of REST expression, that was associated with a massive loss of beta cells. This massive loss is presumably due to downregulation of multiple REST target genes, whose number is likely to increase with REST levels. Indeed, transcription factor abundance is an important parameter influencing the activity of many, though not necessarily all cognate DNA binding motifs (Das et al., 2004). Furthermore, sequence variations in the RE-1 binding motif establish a hierarchy of binding affinity for REST to its target genes, indicating that genes bearing a weakly conserved RE-1 motif, have a suboptimal binding affinity for REST (Bruce et al., 2009). Thus, it is likely that more genes be down-regulated in the diabetic but not the glucose-intolerant RIP-REST transgenic mice, due to the higher expression of REST in the former animals. Several critical transcription factors belonging to the gene network that drives endocrine cell differentiation have been recently described as suboptimal REST target genes (Johnson et al., 2007).

Pancreatic endocrine differentiation requires the commitment of endocrine precursors into different endocrine cell lineages. Arx and Pax4, two important transcription factors that instruct endocrine cell fate decisions (Collombat et al., 2009) possess a RE-1 motif (data not shown). It is therefore possible that, by impairing the balance in the expression of these two opposing factors, REST shifts the differentiation pattern of beta cells towards that of PP cells, hence accounting for our observations in the diabetic RIP-REST mice. It has also been questioned whether a long-term *de novo* expression of REST is able to promote de-differentiation, via epigenetic reprogramming imposing an inactive state of chromatin, inversely to what is observed during neurodifferentiation (Ballas and Mandel, 2005). In this perspective, high levels of REST expression would be expected to result in alterations of the early differentiating insulin-producing cells, resulting in de-differentiation, rather than trans-differentiation of these cells. This is consistent with the increased volume density of PP cells which we observed in the diabetic RIP-REST, since PP-expressing cells are thought to represent embryonic precursors of at least some beta cells (Herrera, 2000). The REST-induced reprogramming is further supported by our finding that the GLUT2 levels were clearly reduced in the beta cells of the newborn diabetic mice (data not shown), suggesting their insufficient maturation.

The major consequence of high levels of REST expression is the death of beta cells. Several identified REST target genes code for proteins involved in beta cell survival/proliferation, such as IB1 (Bonny et al., 2000, Haefliger et al., 2003), Connexin36 (Klee et al., Submitted), IRS2 (Hennige et al., 2003) and ICA512 (Mziaut et al., 2008). Here, we discovered that Cdk5r2, also called p39 (Tang et al., 1995), is an additional, hitherto disregarded RE-1-containing gene that is essential for beta-cell survival. Cdk5r2, like Cdk5r1, is a neuron and beta cell-specific activator of the atypical kinase Cdk5. This kinase phosphorylates a large number of substrates, involved in a variety of neuronal and non-neuronal functions (reviewed in (Dhariwala and

Rajadhyaksha, 2008)). Conflicting reports have described the role of Cdk5 and of its activators in insulin secretion. Thus, whereas inhibition of Cdk5r1 activity was reported to promote insulin secretion (Wei et al., 2005), the association of Cdk5 to Cdk5r2, but not Cdk5r1, has also been shown to induce insulin release (Lilja et al., 2004). The role of Cdk5 in neuronal survival is also a matter of debate. Deregulation of Cdk5 activity by p25 has been involved in numerous neurodegenerative diseases (Patrick et al., 1999, Lee et al., 2000), while normal Cdk5 activity has been linked to neuronal survival (Li et al., 2002, Li et al., 2003). So far, the role of Cdk5 and Cdk5r2 in beta cell survival has not been explored. Here, we show that Cdk5r2 is down-regulated in INS-1E cells expressing REST. Moreover, we provide evidence that Cdk5r2 has anti-apoptotic activity in beta cells, inasmuch as siRNA-mediated Cdk5r2 silencing led to enhanced sensitivity of INS-1E cells to cytokine-induced apoptosis. It is noteworthy that in the adult RIP-REST diabetic mice, the surviving beta cells mostly lacked REST labeling. The finding is consistent with the existence of a beta-cell subpopulation with a genomic pattern that enables them to survive. We observed a few double insulin- and glucagon-expressing cells, suggesting that, as shown after pancreas injury (Thorel et al., 2010), some trans-differentiation of alpha to beta cells may occur in the adult diabetic RIP-REST mice. The different origin of these new beta cells may account for the resistant, REST-lacking cell subpopulation. In summary, the present findings extend our previous observations that ectopic expression of REST in beta cells unmasks this “disallowed” gene, which allow for novel insights into the specific traits that make a beta cell what it is (Schuit et al., 2005). Thus, we now document that high levels of REST make mice diabetic, due to the down-regulation of a subset of RE-1-containing genes that are known to contribute to β-cell survival. We further identify the hitherto neglected anti-apoptotic player Cdk5r2, which we show to be essential for beta-cell survival.

Materials and methods

Transgenic mice

Transgenic mice specifically expressing REST in beta cells were obtained by pronuclear injection of C57Bl/6 zygotes as previously described (Martin et al., 2008). Our institutional review committee for animal experiments approved all the procedures for mice care, surgery and euthanasia.

Pancreas perfusion and insulin secretion

Mice were anesthetized with sodium pentothal 100 mg/kg body wt i.p. and prepared for pancreas perfusion as previously described (Trimble et al., 1985). The pancreas was perfused at 37°C with modified Krebs-Ringer HEPES buffer supplemented with the indicated concentrations of glucose. The perfusion was maintained at 1.5 ml/min for mice. The pancreatic effluent of the first 30 min of perfusion with basal glucose (1.4 mmol/l) was not collected. After this equilibration period, the effluent was collected in 1-min fractions from a catheter placed in the portal vein. The insulin content of each fraction was determined by radioimmunoassay.

Immunohistochemistry and confocal microscopy

Pancreases were fixed in 4% (w/v) paraformaldehyde, equilibrated overnight in 15% (w/v) sucrose, embedded in 15% sucrose-15% (w/v) gelatin and quickly frozen in methylbutane/liquid nitrogen. Cryosections were permeabilized for 20 min in 0.1% Triton X-100, then blocked in 1.5% BSA in PBS for 30 min, and incubated overnight at 4°C with either polyclonal rabbit antibodies against human REST, glucagon (Dako, Baar, Switzerland, 1/500), somatostatin (Dako, 1/800), proliferating cell nuclear antigen (PCNA) (Dako, 1/200) or polyclonal guinea-pig antibodies against insulin (Zymed Lab. Inc., San Francisco, USA, 1/500) and pancreatic polypeptide (PP) (Linco Research Inc., Saint Charles, MO, USA, 1/1000). Primary antibodies were detected using appropriate fluorescein or rhodamine-conjugated antibodies. A supplementary antigen-retrieval treatment was required for anti-PCNA, which consisted of sub-boiling the slides in

citrate buffer (10 mM Na-citrate, pH 6) for 10 min before incubation with the blocking solution. Semi-quantitative immunodetection of REST was performed using secondary antibodies coupled to horseradish peroxidase from the rabbit Vectastain ABC kit, using 3-amino-9-ethylcarbazole (AEC) as substrate, according to the manufacturer's instructions (Vector Lab. Inc., Burlingame, CA, USA). Sections were viewed on either a Leica DM5500 fluorescence microscope or a Leica SP2 upright confocal microscope (Leica, Nidau, Switzerland). The relative area occupied by the different types of islet cells were measured on 16 sections taken throughout the entire pancreas and separated by at least 200 µm (adult animals), and 150 µm (newborn animals). Islet cells and total pancreas areas were measured using an ACECAD Professional graphic tablet connected to a Quantimet Leica 5001 (Leica, Cambridge Ltd, England) programmed for semiautomatic measurement of areas. TUNEL labeling was performed using the *in situ* cell death detection kit, according to the manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland).

Glucose tolerance test

Male mice of 12–16 weeks were fasted for 15 h before blood samples were collected from the tail vein at 0 (fasting blood sample), 15, 30 and 120 min after an intraperitoneal injection of glucose (2g/kg of body weight as a 20% solution). Blood glucose levels were measured with a Glucometer (Bayer AG Health Care, Switzerland).

Cell line and mouse islet isolation

The rat insulinoma cell line INS-1E was maintained in RPMI 1640 medium, as previously described (Martin et al., 2008). Islets of Langerhans of adult C57BL/6 male mice, weighing 25-30 g, were isolated and cultured as previously described (Martin et al., 2008).

RNA isolation and real time RT-PCR

RNA isolation was performed as previously described (Allagnat et al., 2005). Quantitative RT-PCR (qPCR) was performed using the SYBR Premix Ex Taq PCR Kit TaKaRa (Axon Lab, Switzerland) in a Lightcycler (Roche Diagnostics GmbH, Mannheim, Germany), as previously described (Allagnat et al., 2005). cDNAs were amplified using specific primers (supplementary material 1).

Apoptosis assay

Apoptosis was determined by scoring cells displaying nuclei stained with the DNA-binding dyes Hoechst 33342 (10 µg/ml; Molecular probes, Eugene, Oregon, USA), as visualized under an inverted fluorescence microscope.

siRNAs design and cell transfection

Specific shRNA against rat Cdk5r2 were selected using the siRNA Target Finder (Ambion, Austin, TX, USA). The silencing efficiency of each construct was checked at the transcript level using target genes fused to a luciferase reporter gene, as previously described (Martin et al., 2008). The siRNA corresponding to the best shRNA sequence and the negative siRNA were synthesized from Microsynth (Balgach, Switzerland). INS-1E cells were seeded in 12-well plates and incubated overnight in antibiotic-free medium. Negative or siRNA duplexes (60 pmol/ml) targeting Cdk5r2 were mixed with Lipofectamine2000 reagent according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). siRNA–lipofectamine complexes were added to the cells and incubated for 54 h. Cells were exposed to a cytokine cocktail (R&D Systems, Minneapolis, MN, USA) of rat IL-1β (0.5 ng/ml), mouse TNFα (1 ng/ml) and rat IFNγ (10 ng/ml) for 16 h.

Statistical analysis

Data were expressed as mean ± SD or SEM. Differences between means were assessed using Student's *t* test. Statistical significance was defined at a value of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

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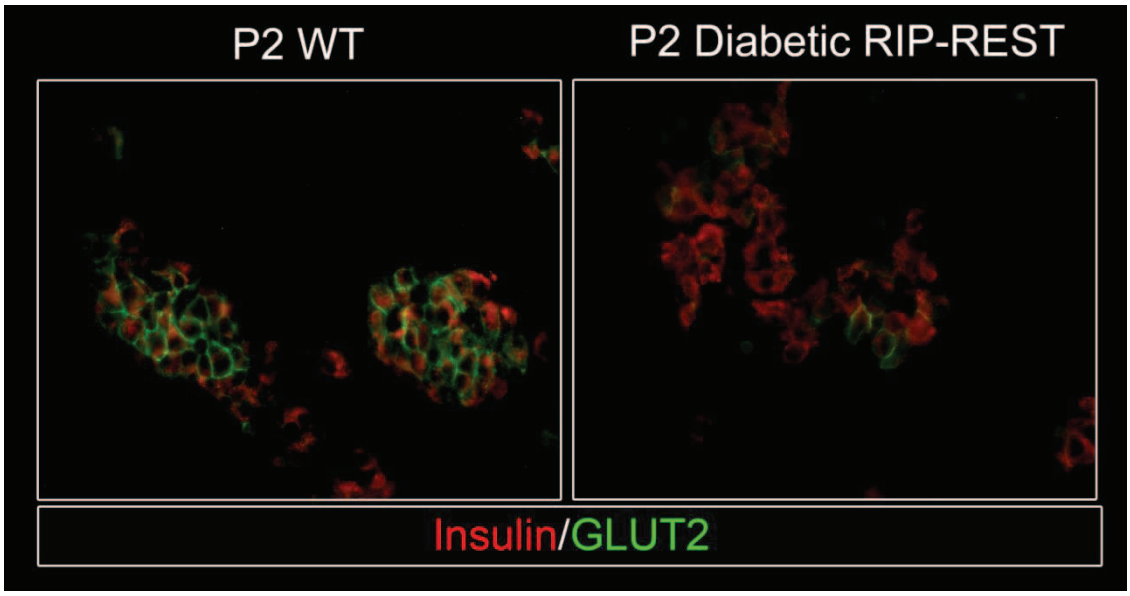
Supplementary data



Diabetic RIP-REST neonates show reduced levels of GLUT2 expression
Immunostaining for insulin (red) and for the glucose transporter GLUT2 (green) performed on pancreas from P2 mice show markedly decreased GLUT2 production in diabetic RIP-REST animals.

Gene	Identification	Gene-Ontology	Ref
Gjd2 (connexin36)	Endogenous (NB/WB)	Insulin secretion	(Martin et al., 2003)
Snap25 (synaptosomal associated protein 25)	Endogenous (qPCR/WB/ChIP)	Insulin exocytosis	(Martin et al., 2008)
Syt4 (synaptotagmin IV)	Endogenous (qPCR/WB/ChIP)	Insulin secretion	(Martin et al., 2008)
Syt7 (synaptotagmin VII)	Endogenous (qPCR/ChIP)	Insulin secretion	(Martin et al., 2008)
Syt9 (synaptotagmin IX)	Endogenous (qPCR)	Insulin exocytosis	(Martin et al., 2008)
Cplx2 (complexin II)	Endogenous (qPCR/ChIP)	Insulin secretion	(Martin et al., 2008)
Ptprn (Ica512)	Endogenous (qPCR)	Insulin exocytosis Beta-cell survival	(Martin et al., Submitted)
Irs2 (insulin receptor substrate 2)	Endogenous (qPCR)	Beta-cell survival	(Martin et al., Submitted)
Cdk5r2 (p39)	Endogenous (qPCR)	Beta-cell survival	(Martin et al., Submitted)
Myt1 (myelin transcription factor 1)	Endogenous (qPCR)	Beta-cell differentiation	Martin PhD thesis 2010

Table 3: Comprehensive list of our identified REST target genes related to beta-cell identity
First column indicates the NCBI gene annotation and common name between comas; Second column indicates the technique used to demonstrate the REST-mediated transcriptional regulation(NB: Northern blot; WB: Western blot; ChIP: chromatin immunoprecipitation); Third column indicates the GO terms of the gene according to NCBI.

Gene	Identification	Gene-Ontology
Ins2 (insulin)	Endogenous (qPCR/NB/RIA)	Glucose homeostasis
Nsf (N-ethylmaleimide-sensitive factor)	Endogenous (NB/ChIP)	Exocytosis
Stxbp1 (Munc18.1)	Endogenous (NB/ChIP)	Exocytosis
Stx1a (syntaxin1A)	Endogenous (NB)	Exocytosis
Vamp2 (vesicle-associated membrane protein 2)	Endogenous (NB)	Exocytosis
α/β-Snap (soluble NSF attachment protein)	Endogenous (NB)	Exocytosis
Rab3a	Endogenous (NB)	Exocytosis
Glud1 (glutamate dehydrogenase)	Endogenous (NB)	Insulin secretion
Slc2a2 (Glut2)	Endogenous (qPCR)	Glucose sensing
Pdx1 (pancreas and duodenum homeobox 1)	Endogenous (qPCR)	Pancreas development Insulin transcription
Akt3 (PKB gamma)	Endogenous (qPCR)	Signaling pathway
Ctnnb1 (Beta catenin)	Endogenous (qPCR)	Wnt signaling pathway
Ctnnd2 (delta 2 catenin)	Endogenous (qPCR)	Cell adhesion Signaling pathway
Mapk10 (JNK3)	Endogenous (qPCR)	JNK cascade
Cks2 (CDC28 protein kinase regulatory subunit 2)	Endogenous (qPCR)	Cell cycle
Map4k3 (MEK kinase kinase 3)	Endogenous (qPCR)	JNK cascade
Api5 (apoptosis inhibitor 5)	Endogenous (qPCR)	Anti apoptosis
Nrg1 (neuregulin1)	Endogenous (qPCR)	Neurodevelopment
Igfbp7 (IGF binding protein 7)	Endogenous (qPCR)	Regulation of cell growth
Cdk4 (cyclin-dependent kinase 4)	Endogenous (qPCR)	Beta-cell proliferation
Cdkn1b (p27)	Endogenous (qPCR)	Cell cycle inhibition
Bcl2l1 (BclX)	Endogenous (qPCR)	Anti apoptosis

Table 4: list of the genes identified as non-REST target genes

First column indicates the NCBI gene annotation and common name between comas; Second column indicates the technique used to demonstrate the lack of REST-mediated transcriptional regulation (NB: Northern blot; RIA: radio-immunoassay; ChIP: chromatin immunoprecipitation); Third column indicates the GO terms of the gene according to NCBI.

Chapter 3: Role of REST in pancreatic endocrine differentiation

This work has been done in parallel to that of the second chapter, to set up the basis of an extensive collaboration engaged with Pr. Grapin-Botton (Lausanne), in order to study the role of REST during pancreatic development.

Several observations indicate that, by analogy to its role during neurodifferentiation, REST may play an important role in pancreatic endocrine differentiation. Basically, the project has started with the development of an *in situ* hybridization protocol to map the pattern of REST expression during pancreatic development, and with the generation of transgenic mice to provide a model of inducible REST gain-of-function.

We are planning to carry on this collaborative project after the PhD, through a post-doctoral work achieved in Pr. Grapin-Botton's lab.

1) Beta-cell differentiation

a) New sources of beta cells

Regenerative medicine for the cure of diabetes in the form of islet transplantation has proven that replacement of a sufficient beta-cell mass can restore glucose homeostasis in T1D patients. However, the lack of high quality donor cells has prompted the search for alternative sources of beta cells to try to expand beta cells *in vitro* and generate an unlimited supply of beta cells for transplantation. Three strategies actually exist to find new sources of beta cells:1) to induce *de novo* beta-cell generation through oriented differentiation of multipotent embryonic stem cells (ESC) or of committed adult progenitor cells 2) to reprogram non-related, fully differentiated cell types, including through the generation of induced pluripotent stem cells (iPSC) 3) to expand existing beta cells (Scheme 1). For reviews, see (Borowiak and Melton, 2009)

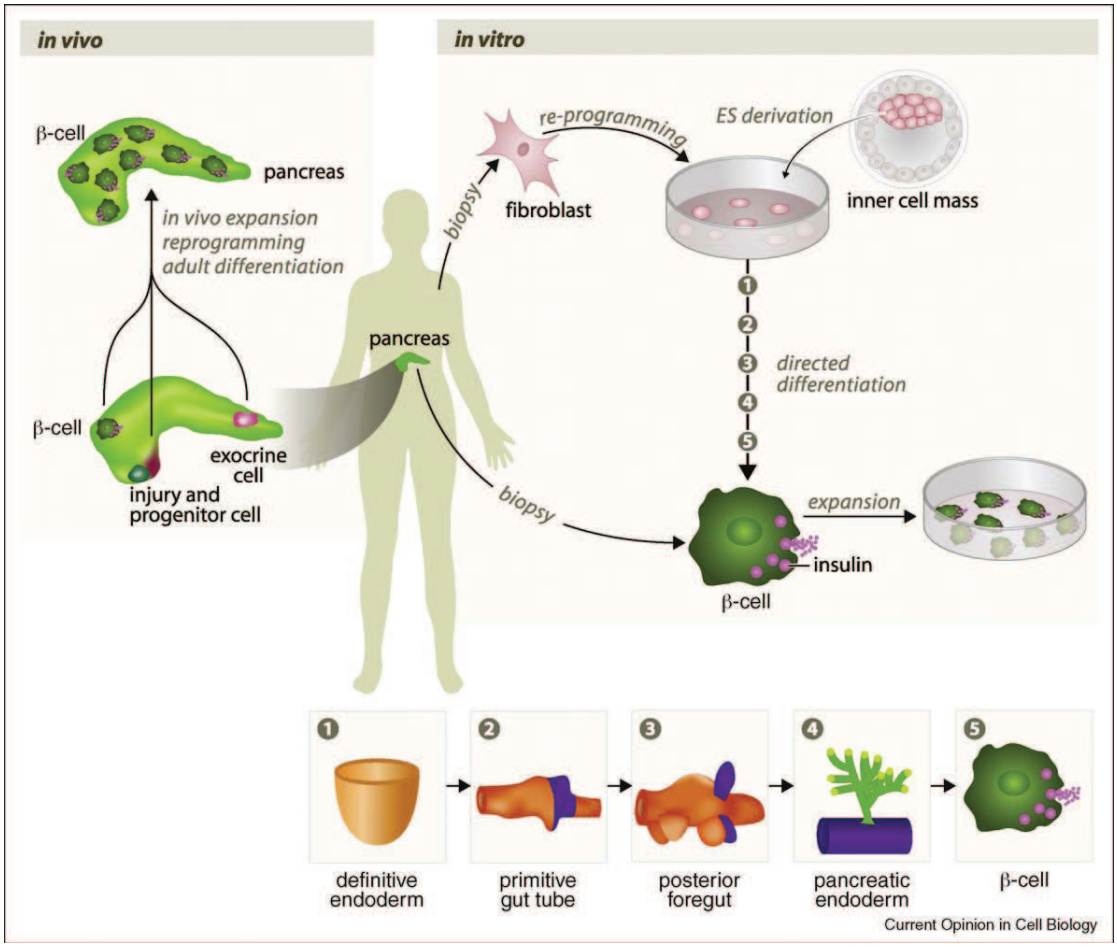
These tasks have been partly achieved (low-percentage of induced-differentiation and poor glucose-responsiveness, or uncontrolled tumorigenic proliferation are often encountered):

1) with the work done in the group of Baetge, where they have directed differentiation of human ESC, first towards definitive endoderm (D'Amour et al., 2005) and then towards insulin-secreting cells (Kroon et al., 2008), by using a combination of signaling molecules and compounds; with the paper from Heimberg's group in which, using a model of pancreatic duct ligation injury, they identified and were able to differentiate adult progenitor Ngn3-expressing cells into beta cells (Xu et al., 2008).

2) with the report of Melton's group, supporting *in vivo* transdifferentiation of acinar cells into beta cells, upon transduction of three crucial transcription factors (TFs)

directing endocrine differentiation, Pdx1, Ngn3 and MafA (Zhou et al., 2008); with the work of Collombat, from Mansouri's lab, where ectopic expression of Pax4 into alpha cells promoted their transdifferentiation into beta cells (Collombat et al., 2009); with the studies of Ferber (Ferber et al., 2000) or Yechoor (Yechoor et al., 2009) promoting hepatocytes transdifferentiation.

3) with the work of Dor's group suggesting that adult beta cells, upon partial loss of mass, are able to regenerate the normal beta-cell mass through increased proliferation (Nir et al., 2007).



Scheme 1: Strategies to generate new beta cells

Existing beta cells can be used for in vivo expansion after pancreas injury (see (Nir et al., 2007)). Other cell types, such as exocrine cells or alpha cells can be reprogrammed in vivo (see (Zhou et al., 2008) or (Collombat et al., 2009), respectively). Beta-cell neogenesis can also be achieved in vivo, following duct ligation (see (Xu et al., 2008)). An in vitro approach is to generate beta cells from ES cells (see (D'Amour et al., 2005, Kroon et al., 2008)). A more complex approach is based on the patient-specific cell reprogramming to generate iPS cells. Both in vitro strategies imply to recapitulate the different steps of differentiation to generate mature beta cells.

b) Pancreas differentiation

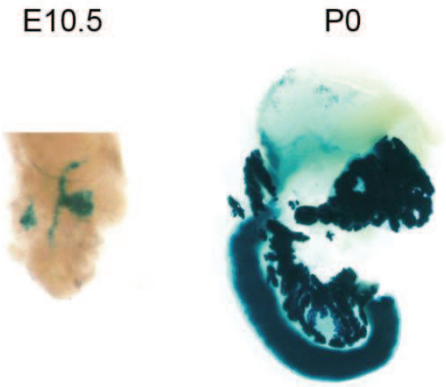
General considerations

To control the proper establishment of differentiation pathways leading to endocrine and specifically beta cells, there is an obvious need to identify and characterize the molecular determinants of this complicated process. Studying pancreas development turns it into a fascinating and frustrating organ as well, for many aspects: differentiation of the two main tissues in pancreas (endocrine and exocrine) involves combinatorial instructive extracellular signaling coming from different embryonic structures (to date, more than 10 families or sub-families of signaling molecules/pathways are described in the comprehensive review of Gittes, each pathway involving several isotypes (Gittes, 2009)). It also involves dynamic changes in expression levels (i.e. on/off) of numerous TFs (more than 25 TFs are now well characterized (Gittes, 2009)). Although it is possible to increase our knowledge in human development via the study of a variety of animal models, there is no *in vitro* cellular model that truly represents embryonic cell types. Complicating the task is the apparent lack of compartmentalization in the developing pancreas. Even if differentiated exocrine and endocrine cells are located in distinct regions of the embryonic pancreas (epithelium and mesenchyme, respectively), there is no such clear regional separation as in neuronal differentiation, between progenitors and differentiated cells, thus precluding from the possibility to isolate one or the other cell type. Even more complicated is the fact that, in embryogenesis, a particular cell type at a certain time will have a different fate than the same type considered later on. For instance, Ngn3-expressing cells, representing transient endocrine progenitors, are born continuously from embryonic day 9 (E9) till E17.5. However, their competence to differentiate into one or the other endocrine cell type differs in time, according to the different inductive milieu or intrinsic timing program (Johansson et al., 2007). Thus, this intricate pattern essentially imposes to perform

histological analyses to identify a particular cell type at a certain time (using co-labeling of a combination of markers), together with genetic modifications to assess the role of a particular TF at a certain time (cell type-specific and inducible KO and KI), or to assess the fate of a particular cell expressing a particular TF at a certain time (box 1).

Box 1. Lineage tracing

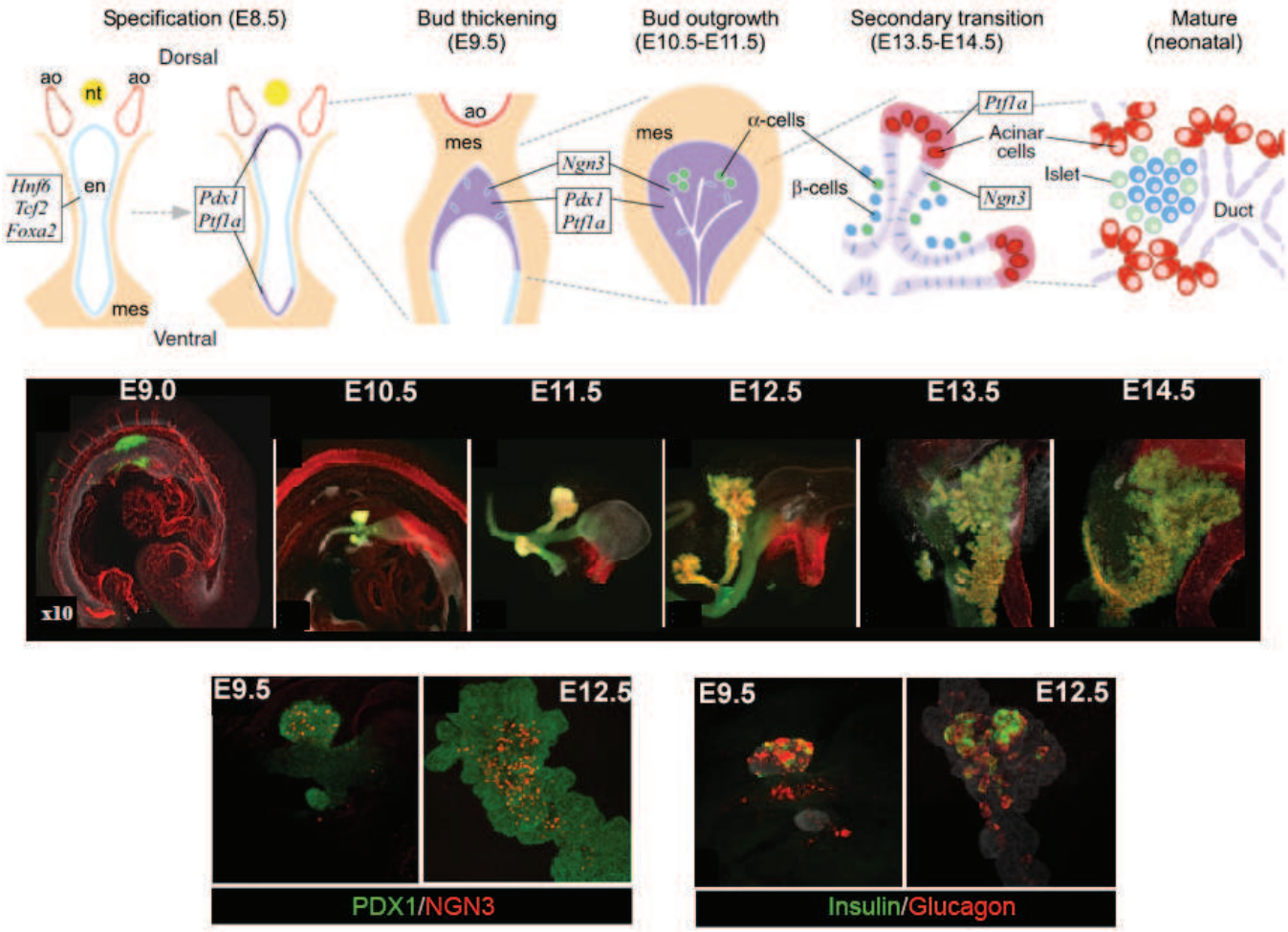
Genetic lineage-tracing commonly uses Cre recombinase, which can delete DNA segments that are flanked by *loxP* sites (so-called floxed). Mouse strains have been developed in which the ubiquitous expression of a reporter gene, such as *LacZ*, is prevented by a floxed sequence being placed between the promoter and the reporter. Cre-mediated deletion of this sequence results in heritable marking (with β-galactosidase substrate) of the Cre-expressing cell. When Cre expression is driven by a promoter that is active in progenitor cells, the descent of those cells in various classes of differentiated offspring can be traced.



In this example, a Pdx1-driven expression of Cre recombinase marks the progeny of Pdx1⁺ cells from E8.5 onwards. At E10.5, these cells are observed, after staining, in the pancreatic buds but also in the posterior stomach and proximal duodenum. Since the Cre-mediated chromosomal rearrangement confers an irreversible labeling to all cells deriving from those observed at E10.5, we can observe the same pattern of staining at birth, even if Pdx1 is only expressed in beta cells and in 20% of delta cell at this stage (Adapted from (Bonal and Herrera, 2008)).

Molecular mechanisms

Pancreas arises from the endoderm in the foregut region. The pre-pancreatic dorsal region receives instructive signals from the notochord and aortas, and the ventral region, from the lateral plate mesoderm and cardiogenic mesoderm. After these early interactions, the endoderm will be surrounded by and under the influence of the mesenchyme, which secretes permissive and instructive signals to influence the differentiation of the different pancreatic cell types. Two pancreatic lobes evaginate from the endoderm into the surrounding mesenchyme as dense epithelial buds, which subsequently expand, branch and differentiate to yield a fully functional organ system prior to birth (Scheme 2).



Scheme 2: Progression of pancreas development
Upper scheme: *Schematic cross-sections of an embryo in the foregut region. The pancreas is specified in the endoderm (en) upon signaling coming from notochord (nt) and aortas (ao). Pdx1 and Ptf1a expression marks the ventral and dorsal epithelium, which starts to bud out into the surrounding mesenchyme (mes). By E9.5, the pancreatic buds start to express Ngn3 in few cells of the epithelium, which differentiate into early alpha cells (first transition). Further growth and branching precedes the secondary transition (E13.5), which is marked by a massive differentiation of beta-cell and acinar cells, as well as by the progressive restriction of Pdx1 and Ptf1a expression to these respective cell types. Adapted from (Murtaugh, 2007).*
Middle panel: *Whole mount immunohistochemical (IHC) staining of mouse embryos at the indicated stages. The Pdx1 yellow-green staining shows the specified endodermal region that subsequently growth and branches. Adapted from (Jorgensen et al., 2007).*
Lower panel: *Whole mount IHC on E9.5 and E12.5 embryos show the pan-epithelium expression of Pdx1 (left, green) together with the centrally scattered Ngn3-expressing endocrine progenitors (left, red). These progenitor cells rapidly differentiate, mainly into insulin- (right, green) and glucagon- (right, red) expressing cells. Adapted from (Jorgensen et al., 2007).*

The specification of the pancreas begins at E8.5, upon the influence of the Cut-homeodomain TF hepatocyte nuclear factor6 (HNF6)/Onecut1 (Jacquemin et al., 2003) and homeodomain TF Hlxb9 (Harrison et al., 1999), both activating the homeodomain TF Pdx1 expression, and through the influence of Hex for the proper allocation of the

ventral bud (Bort et al., 2004). Although it expands by E10.5 to encompass the posterior stomach, duodenum and bile duct, early Pdx1 expression is a marker of the differentiating pancreatic epithelium. Genetic-lineage tracing showed that Pdx1^{+}(expressing) cells represent progenitors of all mature pancreatic cell types, including duct, islet and acinar cells (Gu et al., 2002). Moreover, the absolute requirement of Pdx1 for pancreas development has been confirmed with analysis of the Pdx1 null mutant mice created by Edlund's lab (Jonsson et al., 1994), which shows pancreas agenesis, even if limited growth a dorsal bud was observed (Ahlgren et al., 1996, Offield et al., 1996). A bHLH TF, Ptf1a, is co-expressed with Pdx1 in the early pancreas from E9. Ptf1a is also important for pancreas specification, as Ptf1a deficient mice present an aborted dorsal and very small ventral bud with no acinar cells and impaired endocrine differentiation (Krapp et al., 1998). Moreover, lineage tracing analysis indicated that all three mature lineages derive from Ptf1a^{+} cells (Kawaguchi et al., 2002). In the search for a master regulator of pancreas development, Pdx1 has proven to be necessary but not sufficient to induce ectopic pancreas formation (Grapin-Botton et al., 2001). However, cells that co-express Pdx1 and Ptf1a are instructed toward a pancreatic fate, as observed with misexpression studies in *Xenopus* (Afelik et al., 2006), and also as observed in Hes-1 mutant mice, where an ectopic pancreas originate in adjacent regions that normally express Pdx1 and re-express Ptf1a upon Hes-1 inactivation (Fukuda et al., 2006). Thus Pdx1 and Ptf1a mark the population of progenitor cells of the pancreas that will give rise to all differentiated cell lineages. The early expression of both TFs is pan-epithelial. By E15.5, Pdx1 expression starts to decrease in exocrine cells and progressively becomes restricted to beta cells, and conversely, the expression of Ptf1a becomes restricted to acinar cells.

The maintenance of the pool of multipotent progenitor cells is under the control of several factors. Notch signaling has been involved since mice deleted for the Notch

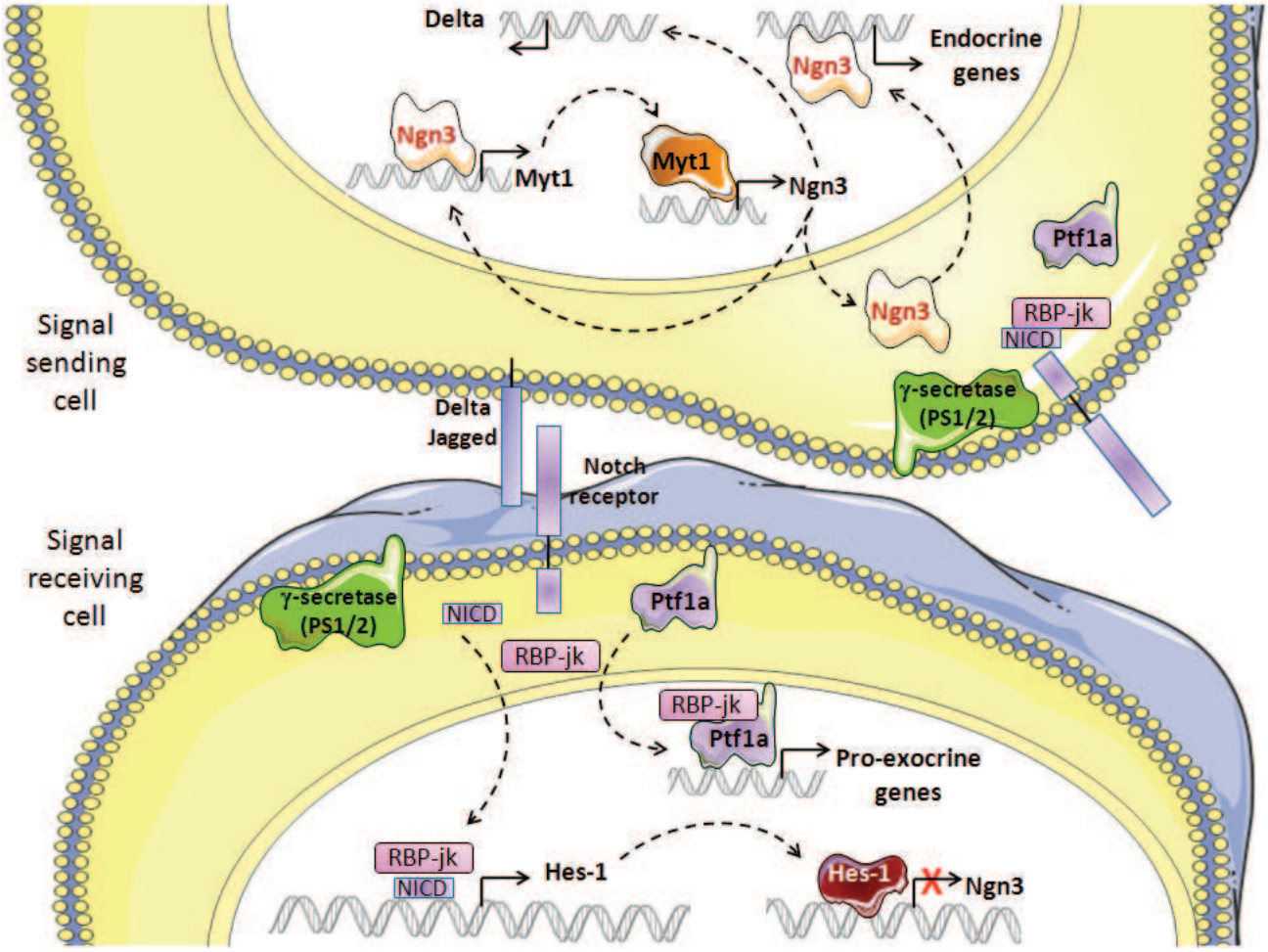
ligand Delta-like-1, for the intracellular mediator RBP-Jk (Apelqvist et al., 1999) or for the activated TF Hes1 (Jensen et al., 2000), all show pancreatic hypoplasia due to depletion of the progenitor pool, associated with premature differentiation of endocrine alpha cells. These observations, based on an early inactivation of Notch activity, suggested that Notch signaling maintains the pool of progenitor cells and also counteracts endocrine differentiation. This idea is also supported by the finding that Notch signaling blocks endocrine cell formation through Hes-1-mediated repression of the pro-endocrine gene Ngn3 (Lee et al., 2001). However, to examine the different actions that Notch signaling could bear at distinct developmental stages, the groups of Melton and Jensen directed sustained Notch activation in Pdx1-expressing cells. They finally found that Notch signaling inhibits endocrine as well as acinar differentiation at different stages of pancreas development (Hald et al., 2003, Murtaugh et al., 2003). Collectively, these studies indicate that the signaling mediated through activation of the Notch receptor expands the pool of progenitors in an uncommitted state by blocking their differentiation till after E12, around the secondary transition (see below). Another signaling molecule involved in this process, Fgf10 is secreted by the mesenchyme and is supposed to mediate its effect via activation of Notch (Miralles et al., 2006). Recently, the Sox9 TF has been suggested as a novel marker of undifferentiated multipotent progenitor. Sox9 co-localizes with Pdx1 in the progenitor domain until E14, and then is restricted to a proliferative and Notch-responsive subset of $Pdx1^+$ cells, and is absent from committed endocrine precursors or differentiated cells. Using mice inactivated for Sox9, the authors further showed that it stimulates the proliferation and survival of pluripotent progenitors via activation of Hes-1, since Sox9-deficient mice displayed pancreatic hypoplasia from E11.5, associated with decreased proliferation and decreased number of $Hes\text{-}1^+$ cells and increased apoptosis (Seymour et al., 2007).

The transitions in the pattern of Pdx1 and Ptf1a expression coincide with the overall conversion of progenitors to mature endocrine or acinar cells. This endocrine/acinar decision is also reflected in the dynamic expression of the bHLH TF neurogenin 3 (Ngn3). The expression of Ngn3 appears as early as E9 in a few Pdx1+ cells, peaks around E15.5, but is undetectable in the adult pancreas (Apelqvist et al., 1999, Gradwohl et al., 2000). Ngn3 inactivation resulted in lack of islet cells, showing that it is necessary for the specification of all pancreatic endocrine subtypes (Gradwohl et al., 2000). Moreover, Ngn3 gain-of-function studies indicated that it is necessary and sufficient to instruct endocrine differentiation (Apelqvist et al., 1999, Schwitzgebel et al., 2000, Grapin-Botton et al., 2001). The final deduction that Ngn3 specifically marks precursors of islet cells came from the lineage tracing performed in Melton's lab, indicating that all endocrine cell types derive from progenitors expressing Ngn3 (Gu et al., 2002).

The key role of Ngn3 necessitates tight control of its expression for proper endocrine differentiation, and several aspects underlying this regulation have been described: 1) the endocrine-committed precursor cells only transiently express Ngn3 in a shorter-than-48-hour time frame (Gu et al., 2002) 2) the number of Ngn3-expressing cells must be controlled to ensure a balance between islet cell differentiation and progenitor cell proliferation. This process is thought to occur via a mechanism of lateral inhibition mediated by Notch signaling (Apelqvist et al., 1999, Jensen et al., 2000), where cells secreting Notch ligands activate Notch signaling in the neighboring cells, subsequently inducing Hes-1 expression. Hes-1 will then repress Ngn3 expression (Lee et al., 2001), thereby precluding these cells to adopt an endocrine lineage. The scattered distribution of Ngn3+ cells within the epithelium reflects, in part, this mechanism of lateral inhibition. By analogy to studies of neuronal development (Kageyama et al., 2008), it was proposed that Ngn3 could mediate such lateral inhibition by upregulating the expression of Notch ligands, to prevent adjacent cells from activating Ngn3 expression.

However, work from Gu's lab has shown that genetically lowered Ngn3 production actually induces Ngn3 expression in a larger proportion of progenitor cells, but in spite of unchanged global expression levels of Notch ligands (Wang et al., 2010) 3) Ngn3 expression must reach a threshold level to trigger endocrine differentiation (Wang et al., 2008, Wang et al., 2010). It is probable that stochastic events or asymmetric cell division cause a subset of progenitor cells to reduce the strength of Notch signaling and raise Ngn3 level above a specific threshold level for islet cell specification and commitment. The zinc finger TF Myt1 has been involved in such a mechanism, in which a feed-forward loop between Myt1 and Ngn3 would ensure robust Ngn3 production (Wang et al., 2008). Thus, those cells could escape lateral inhibition and differentiate towards the endocrine fate, and not towards an acinar or duct fate as proposed in a context of low Ngn3-expressing progenitors (Wang et al., 2010). Recently, a paper from Permutt's lab came out to provide a model explaining how $Ngn3^+$ cells, originally thought to be committed to the endocrine fate, could actually be also directed towards an acinar fate through a non-canonical Notch pathway (Cras-Meneur et al., 2009). They found, using mutant mice for both presenilins (PS1 and 2, the catalytic core of the γ-secretase triggering Notch intra-cellular signaling via Notch receptor cleavage upon ligand binding), that γ-secretase activity on Notch2 was necessary to enhance the competitive advantage in selecting an endocrine rather than acinar fate in $Ngn3^+$ cells. The model is based on the finding that RBP-Jk, an intracellular mediator of Notch signaling, acts a cofactor of Ptf1a to promote acinar fate in a Notch-independent manner (Beres et al., 2006, Fujikura et al., 2007). Reinforcing the role of RBP-Jk over that of the receptor Notch in mediating pancreas differentiation was the observation that removal of both Notch1 and 2 still permitted formation of a functional pancreas whereas removal of RBP-Jk did not (Nakhai et al., 2008). Moreover Notch implication in the endocrine/acinar decision has finally proven to be transient, as constitutive Notch1 activity lead to both impaired endocrine and

acinar differentiation (Hald et al., 2003, Murtaugh et al., 2003). The model states that, in $Ngn3^+$ cells that also express Ptf1a and RBP-Jk, Ngn3 and Ptf1a compete to form a transcriptional complex with E12 proteins, and that Ptf1a needs RBP-Jk to do so. In normal $Ngn3^+$ precursor cells, γ-secretase activity generates a Notch intra-cellular domain that binds RBP-Jk, thereby sequestering it away from Ptf1a, ensuring the cell to competitively select the endocrine fate. Thus, the authors identified that 1) $Ngn3^+$ cells are bipotential 2) a stoichiometry for γ-secretase activity is needed to select the endocrine vs. acinar fate in cells expressing Ptf1a and Ngn3 3) this γ-secretase-mediated control occurs in a narrow developmental window since $Ngn3^+$ $Pax6^+$ cells already escape this control (Cras-Meneur et al., 2009). This model can also explain the impaired endocrine and acinar differentiation in the mice constitutively expressing Notch under Pdx1 control: Notch activity leads to enhanced Hes-1 expression and subsequent Ngn3 repression, but also leads to RBP-Jk sequestering thereby preventing Ptf1a activity (Scheme 3).



Scheme 3: Molecular mechanisms responsible for the endocrine/acinar cell fate decision
A proposed model relies on the lateral inhibition mediated by a signal-sending cell (upper cell), which presents Notch ligand (Delta, Jagged) in the extracellular space. Activation of the Notch receptor in the receiving cell (lower cell) leads to γ-secretase-mediated Notch cleavage, and release of a Notch intra-cellular domain (NICD), which will translocate into the nucleus to activate Hes-1 expression. Hes-1 is countering endocrine decision via repression of the pro-endocrine bHLH gene Ngn3. Ptf1a is an early marker of pancreatic epithelium which becomes then restricted to promote exocrine lineage. To do so, Ptf1a needs RBP-jk and is thus in competition with Notch signaling. In the signal-sending cell, Notch processing ensures the sequestering of RBP-jk away from Ptf1a, thereby favoring Ngn3 activity, to promote the expression of numerous endocrine TFs. To escape Notch inhibition and drive the endocrine program, Ngn3 must reach a threshold level of expression, which is promoted by the existence of a double feed-forward loop between Ngn3 and Myt1.

The way Ngn3^{+} cells then go through differentiation has been partly resolved. Ngn3 is known to control the expression of a cohort of genes that are important for endocrine differentiation including Arx (Collombat et al., 2003), Hlxb9 (Li et al., 1999), IA-1 (Gierl et al., 2006), NeuroD/beta2 (Naya et al., 1997), Nkx2.2 (Sussel et al., 1998), Nkx6.1, Nkx6.2 (Sander et al., 2000, Henseleit et al., 2005), Pax4 (Sosa-Pineda et al., 1997), and Pax6 (Sander et al., 1997, St-Onge et al., 1997). Subsequently, these transcription factors cooperatively activate the expression of endocrine hormones and other necessary genes

that are involved in islet cell development and maturation. Moreover, Ngn3 expression is turned off (probably through auto repression (Smith et al., 2004)) and cells exit the cell cycle and migrate from the epithelium toward the mesenchyme to aggregate into proto-islet structures. In the mouse, endocrine cells are produced through two phases. From E9–E13.5 (referred to as wave I endocrine differentiation or primary transition), glucagon expressing alpha cells account for the majority of the differentiated endocrine cells. After E13.5 (referred to as wave II endocrine differentiation or secondary transition), all endocrine cell types are produced in significant numbers. The wave I endocrine cells do not contribute significantly to the mature endocrine cell mass (Herrera, 2000), yet their production shares similar molecular mechanisms with the wave II cell production. For example, loss of Ngn3 function is reported to abolish both wave I and wave II endocrine differentiation (Gradwohl et al., 2000), although Pdx1 is not required for wave I α cell production at all (Offield et al., 1996).

In regards to the implication of TFs in directing endocrine cell fate decision, the works of Collombat from Mansouri's lab has provided great insights. Indeed, they have shown, using KO mice for Arx and Pax4 (Collombat et al., 2005) or mice with Arx (Collombat et al., 2007) or Pax4 (Collombat et al., 2009) misexpression, that both factors had opposing roles in driving endocrine differentiation, acting through an inhibitory cross-regulatory circuit to repress the expression of each other. Therefore, it appears that Arx expression instructs the progenitors towards α/PP lineages, whereas Pax4 favors the β/δ lineages. Importantly, Collombat et al., have revealed that Pax4 misexpression in alpha cells directs them towards a beta-cell fate (Collombat et al., 2009). In contrast, the mechanisms leading an endocrine-committed precursor to adopt one or the other endocrine lineage are unresolved. Nevertheless, the work from Grapin-Botton's lab has demonstrated that it proceeds in a temporal fashion, by using a transgenic "add-back" system of ngn3 expression at specific times in an Ngn3 null mutant background

(Johansson et al., 2007). They showed that early activation of Ngn3 almost exclusively induced glucagon$^+$ cells, while depleting the pool of pancreas progenitors. In contrast, from E11.5, Pdx1$^+$ progenitors become competent to differentiate into insulin$^+$ and PP$^+$ cells. Somatostatin$^+$ cells were generated from E14.5, while the competence to make glucagon$^+$ cells was dramatically decreased. The authors concluded that Ngn3$^+$ cells go through windows of competence to adopt the different endocrine lineages, that this mechanism occurs independently of mesenchyme, and that it is therefore possible to direct formation of insulin$^+$ cells *in vivo* (Johansson et al., 2007).

Finally, the dynamics of islet cell mass establishment connecting the pool of Ngn3-expressing progenitors to the different populations of hormone-expressing islets cells at birth have been described in a recent publication from Herrera's group (Desgraz and Herrera, 2009). To follow the fate of individual Ngn3$^+$ cells, they used an Ngn3-Cre-based mouse model of chromosomal translocation that allowed labeling of single Ngn3$^+$ cells at a clonal density (labeling of several cells, isolated from each others). The fate of a large numbers of Ngn3$^+$ cells has been followed at birth by co-localization of a specific endocrine hormone together with the tag. They unexpectedly found that 1) one Ngn3$^+$ cell differentiates without division to give one endocrine cell of a particular type at birth. This suggest that Ngn3$^+$ cells are non-proliferating and that they are unipotent 2) the definitive proportions of each endocrine lineage is thus determined at embryonic stage, by the corresponding fixed proportions of Ngn3$^+$ cells that are allocated into one or the other islet cell lineage. For instance, the greater number of Ngn3$^+$ cells that are committed to the beta cell lineage dictates the predominance of beta cells over the other endocrine lineages in adult mice 3) as previously reported, endocrine cells proliferation during embryonic and postnatal life is very low and the total number of islets remains constant during adult life (Desgraz and Herrera, 2009).

For more details, readers are referred to excellent reviews (Murtaugh, 2007, Bonal and Herrera, 2008, Gittes, 2009).

2) Role of REST in endocrine differentiation

Anderson's and Mandel's groups have identified REST in 1995. Using RNA *in situ* hybridization in the neural tube of mouse embryos aged 11.5 to 13.5 days, both studies right away suggested that REST functions as a cellular brake for neurogenesis, since REST was expressed in neuronal progenitors but not in differentiated neurons. Further confirmation for an essential role of REST during development came from the observation that mice lacking REST (REST-/-) die by E11.5. Although these mice appear normal until embryonic day 9.5, after this, widespread apoptotic death results in malformations in the developing nervous system and restricted growth. An apparent contradiction for this implication of REST in neuron formation is the fact that the constitutive expression of REST in developing spinal cord of stage 12-13 chicken embryos did not prevent overt neurogenesis (Paquette et al., 2000). In this study, newly generated neurons expressing exogenous REST had migrated from the ventricular to the marginal zone of the neural tube. It was sufficient, however, to disrupt neuronal gene expression and cause axon path finding errors, indicating a requirement of REST for proper development of neurons.

Despite the lack of direct evidence that *in vivo*, REST gain-of-function can prevent neuronal differentiation, or that REST loss-of-function results in precocious neuron formation, a wealth of different molecular analyses and models indicate an active role of REST-mediated transcriptional regulation in the process of neuronal differentiation. The work of Ballas et al., has correlated the decrease in REST concentration with the transition from ESC to NSC, and the repression of REST and clearance from chromatin of its targets with the transition from NSC to differentiated neurons (Ballas et al., 2005)

(Scheme 10, general introduction). Consistently, REST overexpression in NSC dramatically impaired neurons formation (Kohyama et al., 2010). A corollary to the model described by Ballas et al., has come from the observation that REST controls distinct transcriptional networks in ESC, NSC or mature neurons (Sun et al., 2005, Johnson et al., 2008). Thus, even if the profile of REST recruitment in ESC is partly related to neuronal function, a large set of genes that are recruited specifically in ESC is highly dedicated to pluripotency. In NSC, the REST recruitment profile is then largely enriched in genes involved in neuronal function, and the number of targets is decreased as compared with that in ESC, in line with the loss of developmental potential (Johnson et al., 2008). Thus, it seems that REST acts in a way to control and accompany the proper progress of each specific stage, towards differentiated neurons. Another unexpected model involving REST in neuronal differentiation came from the identification of a small modulatory dsRNA corresponding to the RE-1 motif that was expressed at low levels in NSC, and at high levels in NSC-derived hippocampal mature neurons. This RE-1 dsRNA has the capacity to interact with REST protein and/or the REST repressor complex to switch this repressor system into an activator. Thus, when this RE-1 dsRNA was introduced into NSC, it induced the expression of RE-1-containing genes such as SCG10, Synapsin I or Nav1.2, and consequently, triggered neuronal differentiation (Kuwabara et al., 2004). A group of elegant studies, conducted in the lab of Majumder, has also provided good evidence for an active role of REST in neurodifferentiation, using a recombinant form of REST in which both repressor domains were replaced with the activation domain of the herpes simplex virus protein VP16 (REST-VP16). Expression of REST-VP16 in NSC converted the cells into a mature neuronal phenotype showing physiological activities such as synaptic vesicle recycling and glutamate-induced calcium influx (Su et al., 2004). These results showed that direct activation of REST target genes in NSCs is sufficient to cause neuronal differentiation. Moreover, the expression of REST-

VP16 in myoblasts grown under muscle-differentiation conditions blocked their entry into the muscle-differentiation pathway, activated the REST target genes, and converted the myoblasts to a physiologically active neuronal phenotype (Watanabe et al., 2004). Thus, activation of REST target genes in myoblasts was sufficient to activate other terminal neuronal-differentiation genes that are not direct targets of REST, to override the muscle differentiation pathways, and convert the myoblasts to a physiologically active neuronal phenotype. Finally, a recent paper from the group of Elledge, which had previously studied the role of REST in cancer, has identified the protein responsible for the post-translational degradation of REST observed by Ballas et al., during neurodifferentiation. The authors have shown that this E3 ubiquitin ligase, $SCF^{\beta\text{-TRCP}}$, is induced during differentiation of ESC and promotes REST degradation (Scheme 10, General introduction). By using siRNA against $SCF^{\beta\text{-TRCP}}$ and REST, they further demonstrated that neurodifferentiation of ESC is dependent on both proteins. REST degradation or REST knock-down promotes neurodifferentiation, while the inability to degrade REST impairs neurodifferentiation (Westbrook et al., 2008).

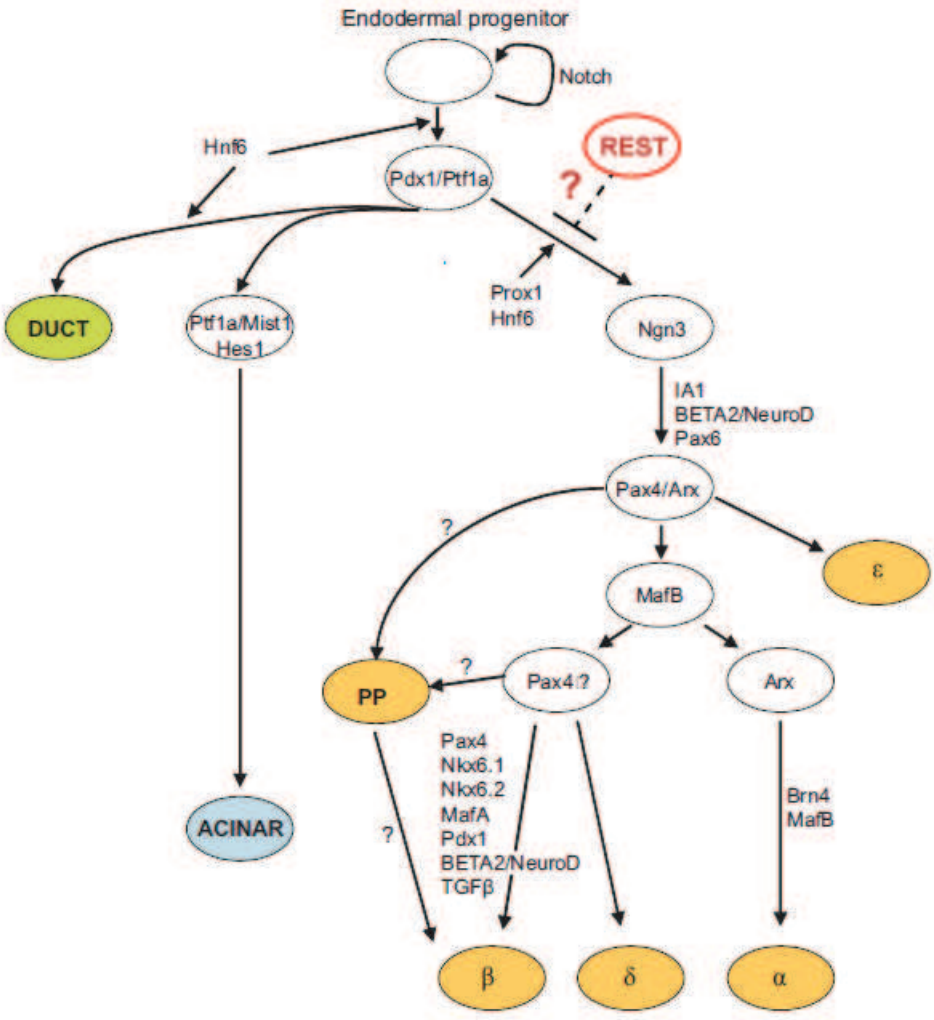
Aim of the project

By analogy to what has been described for the differentiation of neurons, we expected that REST would be expressed in the progenitor cells of pancreas, and that its gradual clearance from chromatin of crucial genes would lead to the dynamic relieve of expression of pro-endocrine genes, thereby triggering endocrine differentiation. Strong evidences that this holds true come from the ChIPSeq analysis from Myers and Wold (see Introduction 5) d) Regulation of REST transcription) performed with the Jurkat T cell line (Johnson et al., 2007). In this technique of ChIP coupled with ultra-high-throughput sequencing, the authors identified RE-1 sites that were bound in a suboptimal manner, which means that they were among the lowest ChIPSeq signals, indicative of a weak RE-1 binding motif, probably non-canonical. This group of suboptimal target genes bound by REST included a set of TFs known to be critical in the gene network driving islet cell differentiation: Neurod1/Beta2, Hnf4α, Hnf6/Onecut1, Ngn3 and Hes1. The authors suggested that their feature of weak binding affinity could be important for the progress of pancreas development, being released early from REST repression, thereby being involved in the establishment of a permissive state that helps the differentiation program to be launched.

A central question that remains in the comprehension of endocrine differentiation is to know what are the conditions required for a multipotent progenitor cell to acquire an endocrine-committed fate, and particularly, to begin express the pro-endocrine gene Ngn3 at a sufficient threshold level to initiate endocrine commitment. It has been proposed that stochastic events (Kaern et al., 2005), asymmetric cell division or modification by the Fringe molecules (Xu et al., 2006) may cause a subset of progenitors to reduce the strength of Notch signaling. This slight reduction in Notch pathway could be responsible for Ngn3 level to raise above a specific threshold level, that may involve

the feed-forward expression loop mediated by Myt1 (Wang et al., 2008), therefore allowing for islet cell commitment.

We were also interested in our study, to oppose to that question, the possibility that REST fulfils such a role in controlling the endocrine/acinar decision. Particularly it would be interesting, in relation to the hypothesis of Johnson et al cited above, to position REST into the regulatory gene network that instruct endocrine differentiation, and to verify whether REST action could play the role of a cell-autonomous switch that dictate Ngn3 expression and subsequent endocrine differentiation.



Scheme 4: Gene regulatory cascade in the progression of pancreatic development
Representation of the different cell lineages (arising as progeny of multipotent progenitors), which will give rise to the three different mature tissues, duct, acinar and endocrine (orange). The scheme depicts the sequential activation of key TFs that mark specific cell populations and drive the differentiation process. Before mapping REST expression to each of these intermediates, we ask whether REST is acting against, and at the top of the endocrine lineage.

To engage our study, we decided 1) to map the unresolved pattern of REST expression at different stages of pancreatic development. Especially, the objective was to know whether REST is expressed in multipotent progenitors, and whether it is excluded from endocrine-committed precursors 2) to generate a transgenic model of REST gain-of-function to assess whether REST is sufficient to block the formation of $Ngn3^{+}$ precursors and/or of differentiated endocrine cells 3) eventually, the aim was to generate a model of REST loss-of-function to ascertain whether REST is necessary to control proper timing of pancreas differentiation.

Results

REST pattern of expression is wide and partially maps that of multipotent Sox9⁺ cells in E14.5 pancreas

To detect the pattern of REST expression at different stages of pancreas development, we first tested available REST antibodies (our rabbit anti-human REST (Martin et al., 2003, Martin et al., 2008) using immunohistochemistry (IHC). However, these antibodies were neither efficient enough to detect endogenous levels of REST, nor very specific (as they labeled differentiated beta cells), especially in embryonic samples where the abundance of many different expressed TFs may lead to cross reactivity with common domains such as Zn finger binding domains. We therefore decided to perform *in situ* hybridization (ISH) to detect mouse REST mRNA, and tested several riboprobes using two protocols, starting with E14.5 pancreatic sections. The 4 different RNA probes, 500bp in length, and spanning different regions of REST mRNA were tested alone or in combinations, and in parallel with a positive control for Ngn3 mRNA detection. Commonly, ISH experiment rely on a succession of tricky treatments performed in RNase-free conditions (DEPC treatments, material sterilization), that aim at fixing (PFA) or permeabilizing (HCl) tissue, and increasing accessibility for the probe to the endogenous RNA (pKA-mediated protein hydrolysis), or increasing specificity (acetylation) before probe hybridization and detection. The simplest protocol (Braissant and Wahli, 1998) yielded no result as it is based on lack of fixation (that allows avoiding accessibility treatments without lowering sensitivity), what does not fit with embryonic material. The more "classical" protocol gave good results with the antisense (AS) Ngn3 probe and was adapted to tyramide-based signal amplification (TSA) associated with fluorescent detection (Fig. 1). We have selected a fluorescent detection system (as opposed to chromogenic system like NBT/BCIP) in order to be able to examine REST

signal at the cellular level, in parallel with co-IHC for cell type-specific markers (fluorescent signals does not mask each other, whereas superposition of chromogenic with fluorescent signals do). Hybridization with the different REST probes gave no signal, arguing probably for a problem of threshold of detection, as REST might be expressed at very low levels.

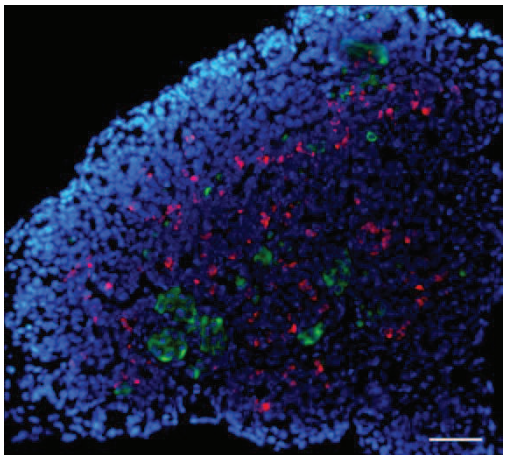


Figure 1: Tyramide signal amplification for in situ mRNA detection

Ngn3 mRNA fluorescent in situ hybridization (red) was paralleled with co-immunohistochemistry for Glucagon (green) on E14.5 mouse pancreas. Blue staining is the DAPI labeling of nuclei. Scale bar, 50 µm.

We therefore used a full length mouse REST RNA probe to increase the sensitivity of detection, and this resulted in a specific signal with AS probe (Fig. 2 middle and lower panel), as REST sense (S) probe gave no signal (Fig. 2 upper panel). Moreover, even if the signal generated by the AS probe was faint and not as well defined as that of Ngn3 (Fig. 1), the observed pattern was validated by the negative control represented by regions containing differentiated endocrine cells. Indeed, when the signals obtained for glucagon or insulin by IHC were superposed to that obtained for REST by ISH on the same section, this clearly showed, as expected, that both these endocrine regions lack REST signal (Fig. 2 middle and lower panel). We then searched to know whether REST was co-expressed together with Ngn3 in endocrine precursors. At the moment, we are unfortunately unable to perform double ISH for Ngn3 and REST mRNAs for technical reasons. Similarly, co-labeling for Ngn3 protein by IHC and for REST mRNA by ISH has proven negative, since REST ISH necessitates pKA treatment that lowers Ngn3 IHC sensitivity. Since our REST ISH signal is weak and that $Ngn3^{+}$ cells are scattered along the duct

epithelium as isolated cells, it is also unlikely that labeling consecutive sections will allow for a cellular resolution. Finally, our first aim was to assess whether REST was expressed in multipotent progenitors cells of the pancreas. To this aim, we paralleled ISH for REST and IHC for the marker of multipotent cells, Sox9, on two consecutives sections. This clearly indicated that the overall pattern of REST and Sox9 expression are overlapping, suggesting that REST is expressed in the multipotent progenitor cells of the developing pancreas (Fig. 3).

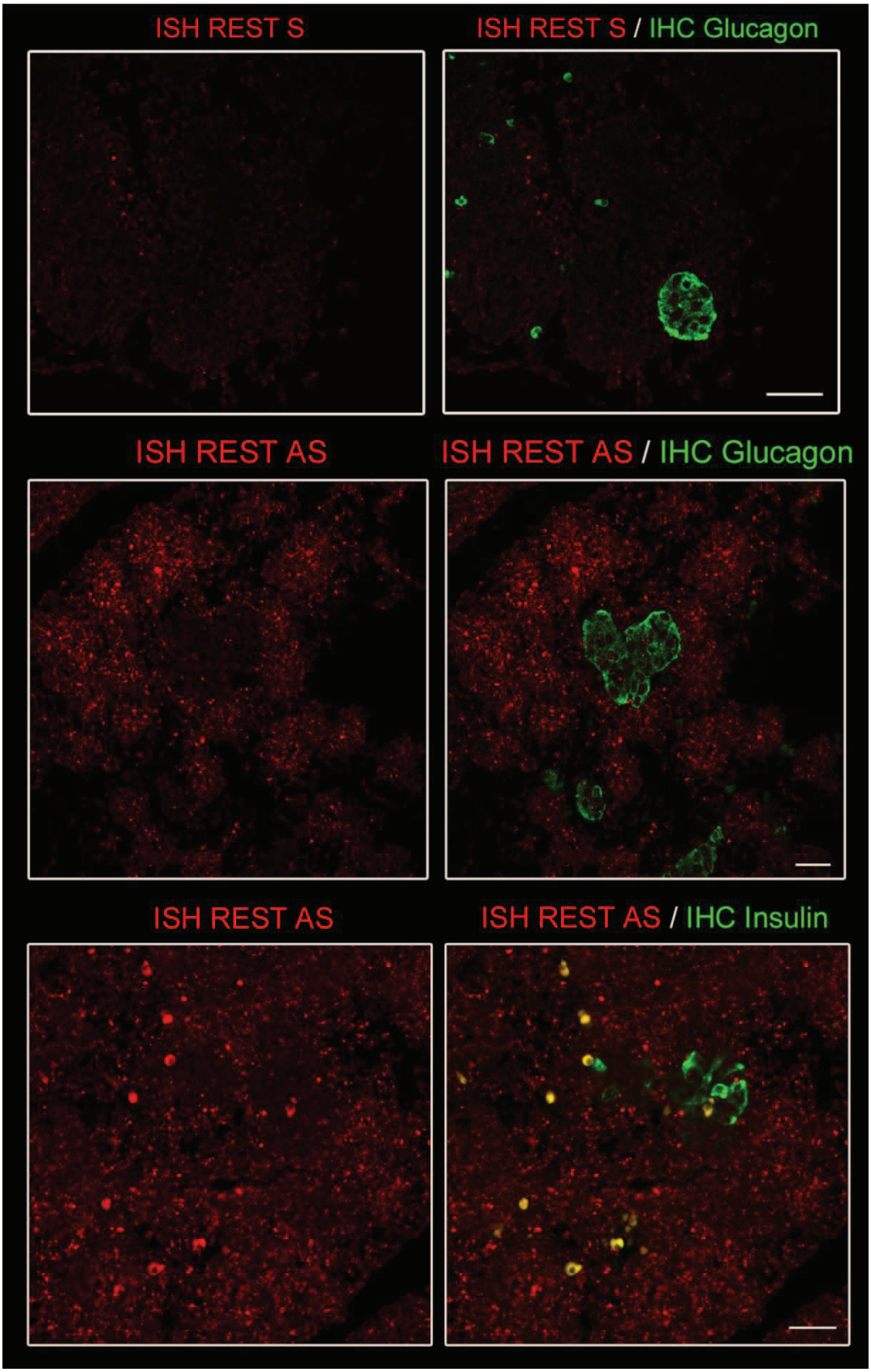


Figure 2: REST pattern of expression in E14.5 pancreatic epithelium

in situ hybridization (ISH) for REST(red) was performed with sense (S) (upper panel) or antisense (AS) probe (middle and lower panel), paralleled with co-immunohistochemistry (green) for glucagon (upper and middle panel) or insulin (lower panel) on E14.5 mouse pancreas. Scale bar, 25 µm.

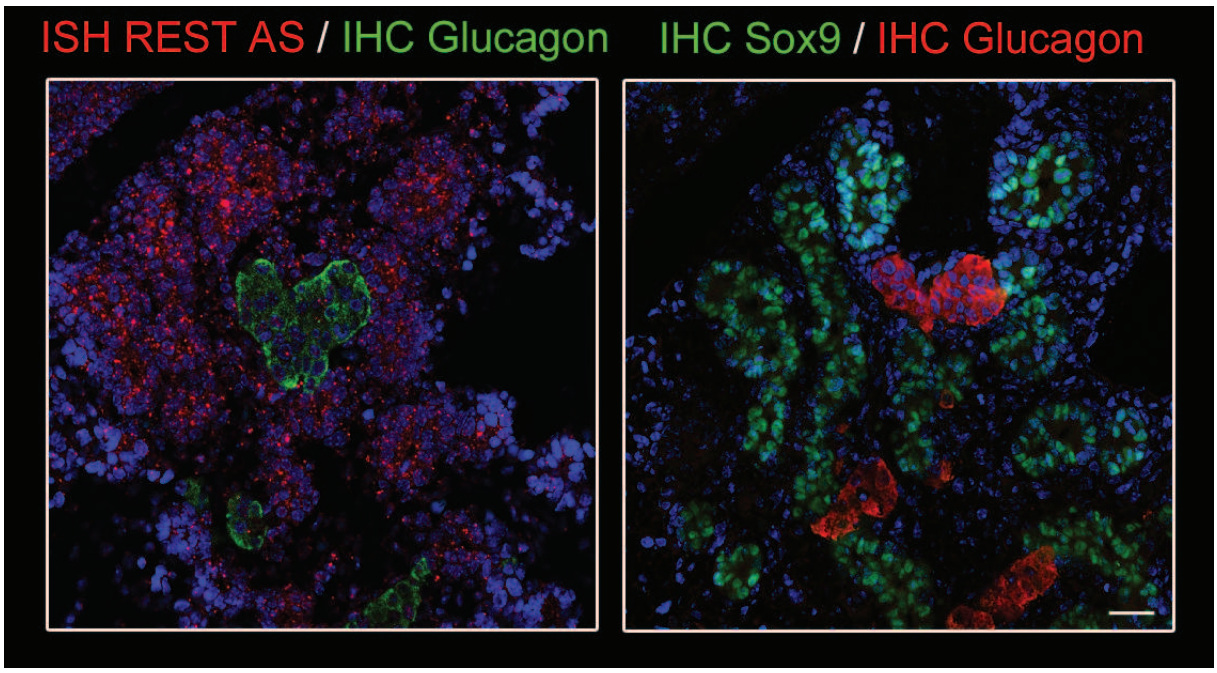


Figure 3: Comparison of REST (ISH) and Sox9 (IHC) pattern of expression in E14.5 pancreatic epithelium

in situ hybridization (ISH) for REST (red) was performed with antisense (AS) probe and paralleled with co-immunohistochemistry for glucagon (green) (left panel). Co-immunohistochemistry for the marker of multipotent progenitors, Sox9 (green) and for glucagon (red) (right panel). Blue staining is the DAPI labeling of nuclei. Scale bar, 25 µm.

Of note, qPCR experiments performed on mRNA from total pancreas at E14.5, also attested for REST widespread expression, as compared to REST expression in adult kidney and islets (Fig. 4).

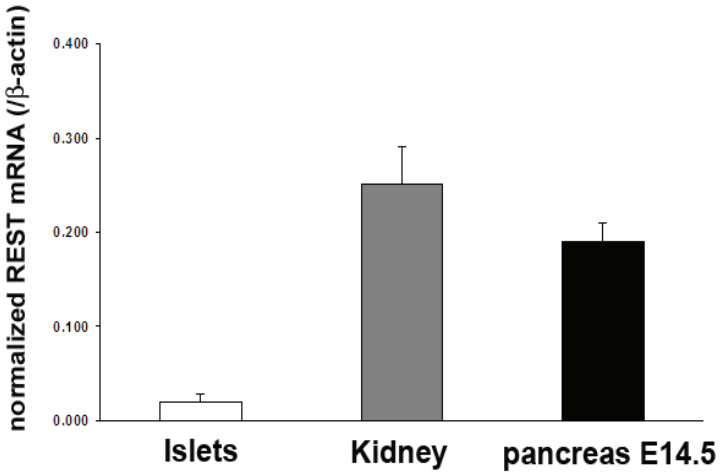


Figure 4: REST mRNA is detected in total pancreas at E14.5

qPCR analysis confirming that REST is expressed at substantial levels in E14.5 mouse pancreas, as compared to control levels in adult kidney. Islets are the negative control (the low expression level accounts for the non-endocrine cells in the islets). Results are mean ± SD.

Generation of Pdx1-driven tetracycline- activated REST transgene.

To study the role of REST during pancreas development, we first adopt a gain-of-function strategy in a way to express REST early in all the cells of the epithelium, and then, to assess whether this misexpression is sufficient to impair endocrine differentiation. We decided to take advantage of an inducible system that allows for the temporal regulation of REST at different stages of development. We have therefore sub-cloned a fragment containing the rabbit β-globin intronic sequence and human REST cDNA, which served for the generation of RIP-REST mice (Martin et al., 2008), downstream of a tetracycline operator (TetO) minimal promoter sequence (Fig. 5 A). The resulting plasmid was verified using REST cDNA sequencing and restriction analysis (Fig 5 B). The plasmid was then co-transfected into INS-1E cells, together with a plasmid encoding the reverse tetracycline transactivator (rTA; Tet-on system), and we verified, using IHC, that the presence of nuclear REST was actually dependent on doxycycline (Dox) (Fig. 5 C).

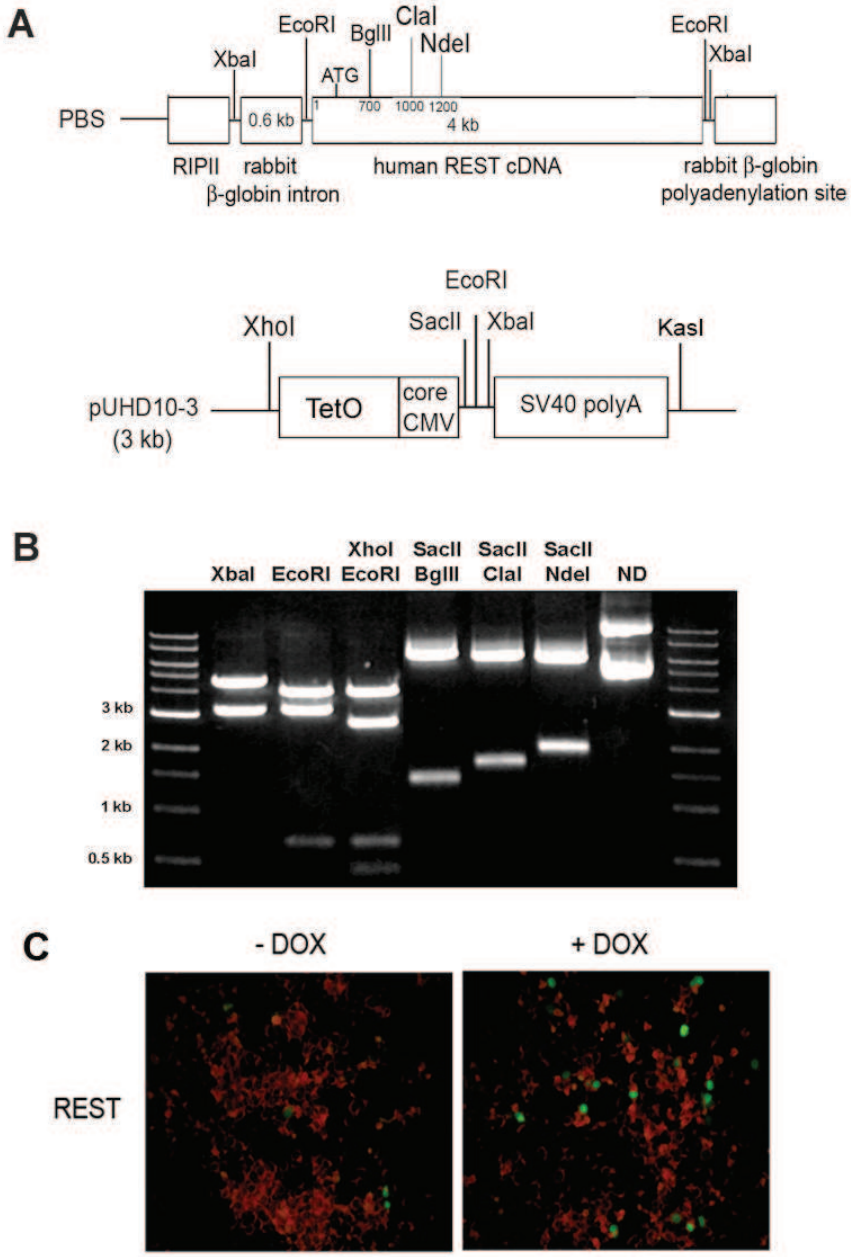


Figure 5: Construction of a plasmid coding for a tetracycline-regulatable human REST cDNA

A. schematic organization of the constructs used to generate TetO-REST mice. The XbaI fragment encompassing β-globin intron and human REST cDNA was subcloned into XbaI sites of pUHD10-3. The XhoI/KasI fragment was used to inject oocytes.
B. restriction pattern of the pUHD10-3 vector containing the REST transgene.
C. immunocytochemistry for REST(green) in INS-1E cells co-transfected with pUHD10-3 TetO-REST and a plasmid coding for the reverse tetracycline transactivator (rtTA) (Tet-on system) under the control of murine Pdx-1 promoter. Red labeling is the blue evans counterstain. DOX: 1µg/ml doxycycline, 24 h.

The XhoI-KasI fragment was then used to generate mice expressing tetracycline-inducible REST transgene (TetO-REST) by pronuclear injection in oocytes (Fig. 6A). The 6 founder mouse lines that we obtained were bred separately with another transgenic mice driving the expression of a tetracycline-repressible transactivator (tTA) under the control of the endogenous Pdx1 transcriptional regulatory sequence (Pdx1-tTA), that were kindly given by Pr. MacDonald (Hale et al., 2005) (Fig. 6B). Crossing the two different transgenic mice will result, in bigenic Pdx1-tTA/TetO-REST mice, in a Tet-off

regulatory scheme, in which REST expression is activated in the absence of Dox treatment. This model has the advantage to drive the transgene expression according to the endogenous pattern of Pdx1, but presents the inconvenient to knock-out one Pdx1 allele (KI for tTA/KO for Pdx1).

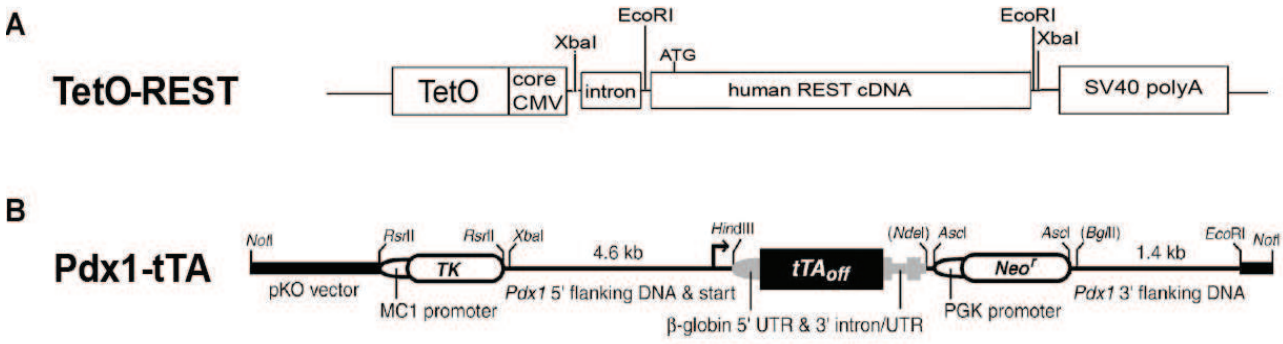


Figure 6: schematic representation of the transgenes used to derive bigenic mice with a Tet-off system to induce REST expression

A. Scheme of the TetO-regulatable REST transgene generated to derive the TetO-REST mice
B. Scheme of the KI/KO system placing the Tet-controlled transactivator (tTA)under the control of the endogenous regulatory region of Pdx1(Hale et al., 2005)

To check REST transgene activation, we crossed the 4 different transmitting TetO-REST founder lines with another transgenic mouse line expressing the rTA under the control of the RIP promoter (RIP-rTA, Tet-on system). The level of REST expression in the different founder lines was assessed using qPCR, after islet isolation from bigenic RIP-rTA/TetO-REST mice and 2 µg/ml Dox treatment for 24h, *in vitro* (Fig. 7A). Absent or very low signal in the untreated control islets (coming from the same animal than treated islets) attests for the low leakiness of the Dox system (Fig. 7A). The presence of REST protein was also verified using western blot after *in vivo* Dox treatment of bigenic animals for 15 days and islet isolation (Fig. 7B). Finally, efficiency of the Tet-off system was also verified with bigenic Pdx1-tTA/TetO-REST animals maintained under Dox treatment, *in utero*, and then during adulthood till the sacrifice for islet isolation (thereby ensuring that we avoid potential defects in beta cell formation upon REST

induction). Then, the islets were left in culture 24h without Dox and qPCR was performed (Fig. 7C). We concluded that the REST transgene is expressed in an inducible manner, *in vivo*, *in vitro*, and lead to the production of a REST protein at the expected size. Again, the TetO-REST control mice do not show any leakiness, as we did not detect any REST mRNA in their islets in the absence of Dox (Fig. 7C). For further analyses, we have used the line 1666 expressing the highest levels of REST transgene expression (Fig. 7A).

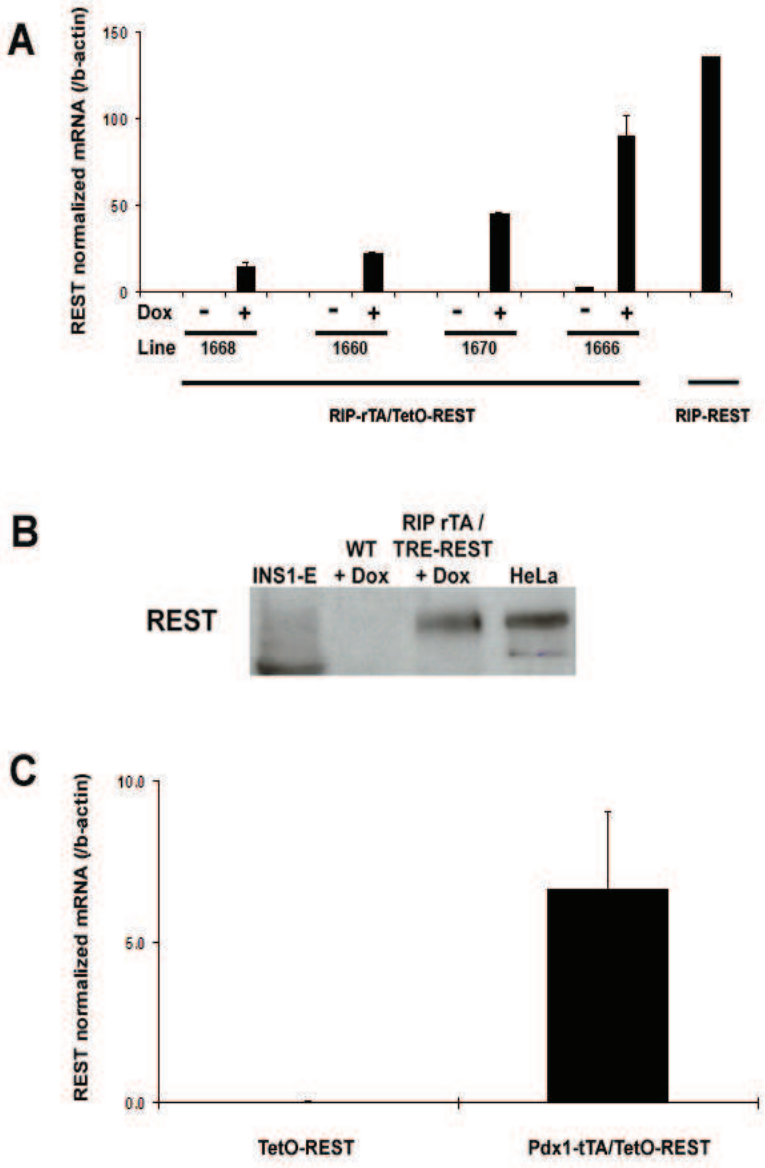
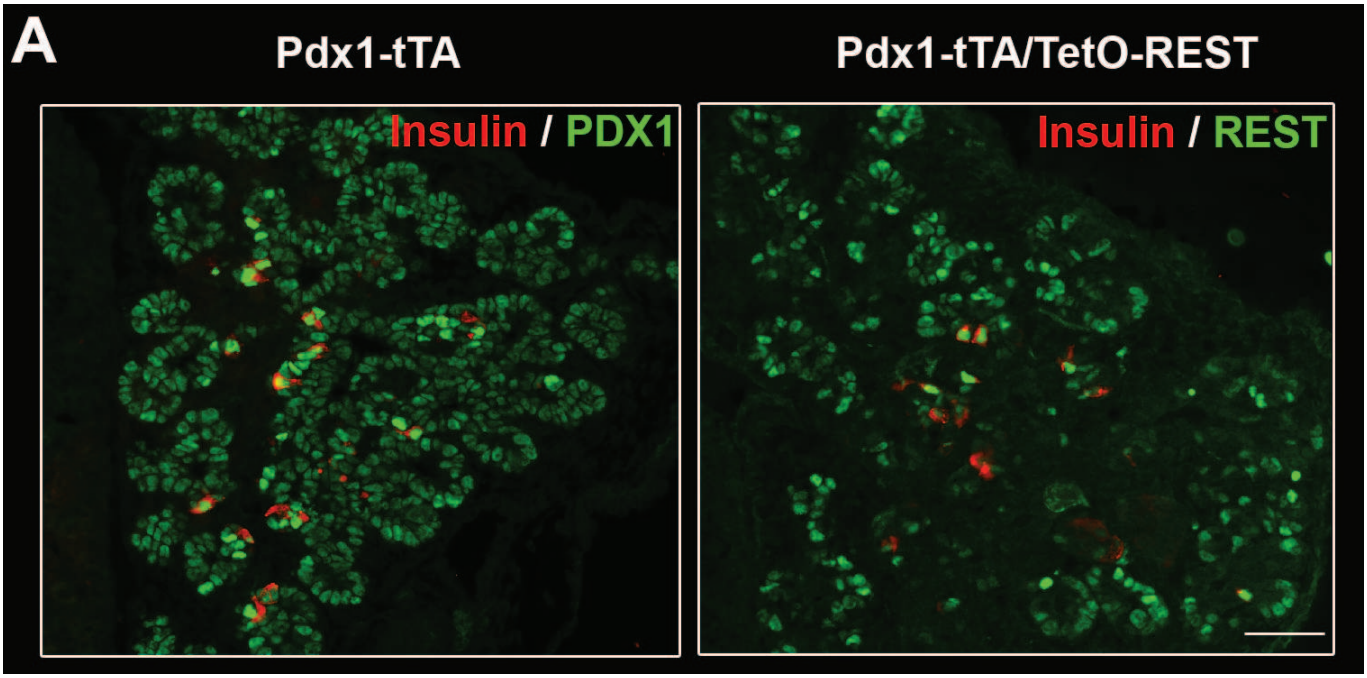


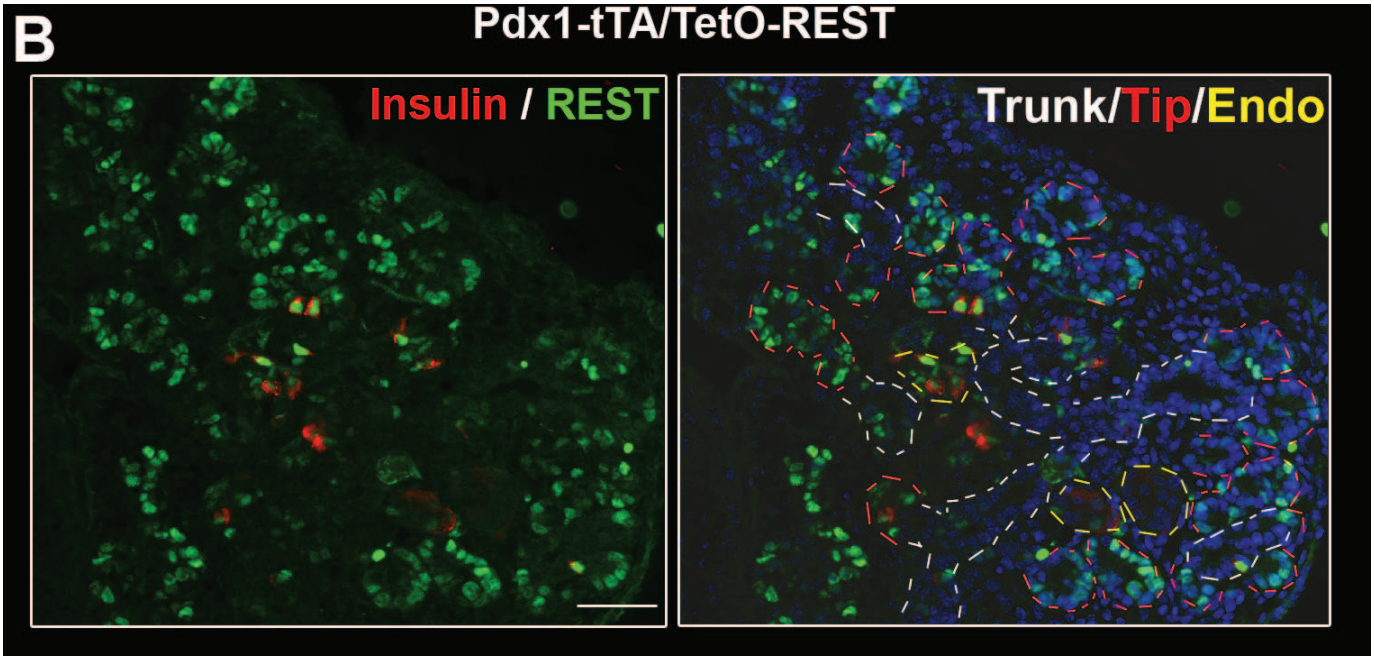
Figure 7: REST transgene expression in Tet-on and Tet-off systems

A. qPCR analysis using islets isolated from 2 month-old mice (n=5) from different lines bearing the Tet-on system (RIP-rTA); islets were incubated 24h with (+) or without (-) 2 µg/ml Dox after isolation. REST transgene level in RIP-REST animals is shown as control. Results are mean ± SD.
B. Western blot using islets isolated from 2 month-old mice bearing the Tet-on system (RIP-rTA) that have been treated with Dox (in food) during 15 days
C. qPCR analysis using islets isolated from 2 month-old mice bearing the Tet-off system (Pdx1-tTA), that have been treated with Dox in utero and during life. Islets were left 24h in culture without Dox for REST induction.

Forced expression of REST in Pdx1^{+} progenitor cells impairs formation of Ngn3^{+} endocrine-committed precursors and differentiation of endocrine cells

Since Pdx1 gene dosage is important for pancreas formation and islet architecture (Fujitani et al., 2006) and that Pdx1-tTA mice are similar to a Pdx1$^{+/-}$ genotype, we used them as controls rather than the TetO-REST mice. We first observed the pattern of REST transgene expression achieved in pancreas of E14.5 bigenic Pdx1-tTA/TetO-REST and control Pdx1-tTA mice, grown without Dox (Fig. 8 A). The pattern of Pdx1 expression in control mice was, as expected, pan-epithelial, with stronger expression in differentiated beta cells (Fig. 8 A, left picture). However, the pattern of REST expression in bigenic Pdx1-tTA/TetO-REST was not identical to that of Pdx1 in controls (note that our REST antibody ensures detection of REST transgene, but not of the low levels of endogenous protein). REST expression was higher in differentiated beta cells than in progenitors, but a large number of epithelial cells were not expressing the transgene, especially in the region that is central to the branching epithelium (Fig. 8 A, right picture). This region actually corresponds to the trunk of the branching (Fig. 8 B, right picture), which contains duct-like epithelial cells and where most Ngn3^{+} cells precisely originate (Fig. 8 C). It is noteworthy that we also have a heterogeneity in the levels of REST transgene expression between cells (some nuclei are strongly labeled and other not; compare with Pdx1 signal; Fig 8 A and B), alike seen in the intolerant RIP-REST mice (Martin et al., 2008). This discrepancy between the patterns of expression of the driver (Pdx1) and reporter (REST) gene was also observed at E16.5 (Fig. 8 D).


A
Pdx1-tTA
Pdx1-tTA/TetO-REST
Insulin / PDX1
Insulin / REST


B
Pdx1-tTA/TetO-REST
Insulin / REST
Trunk/Tip/Endo

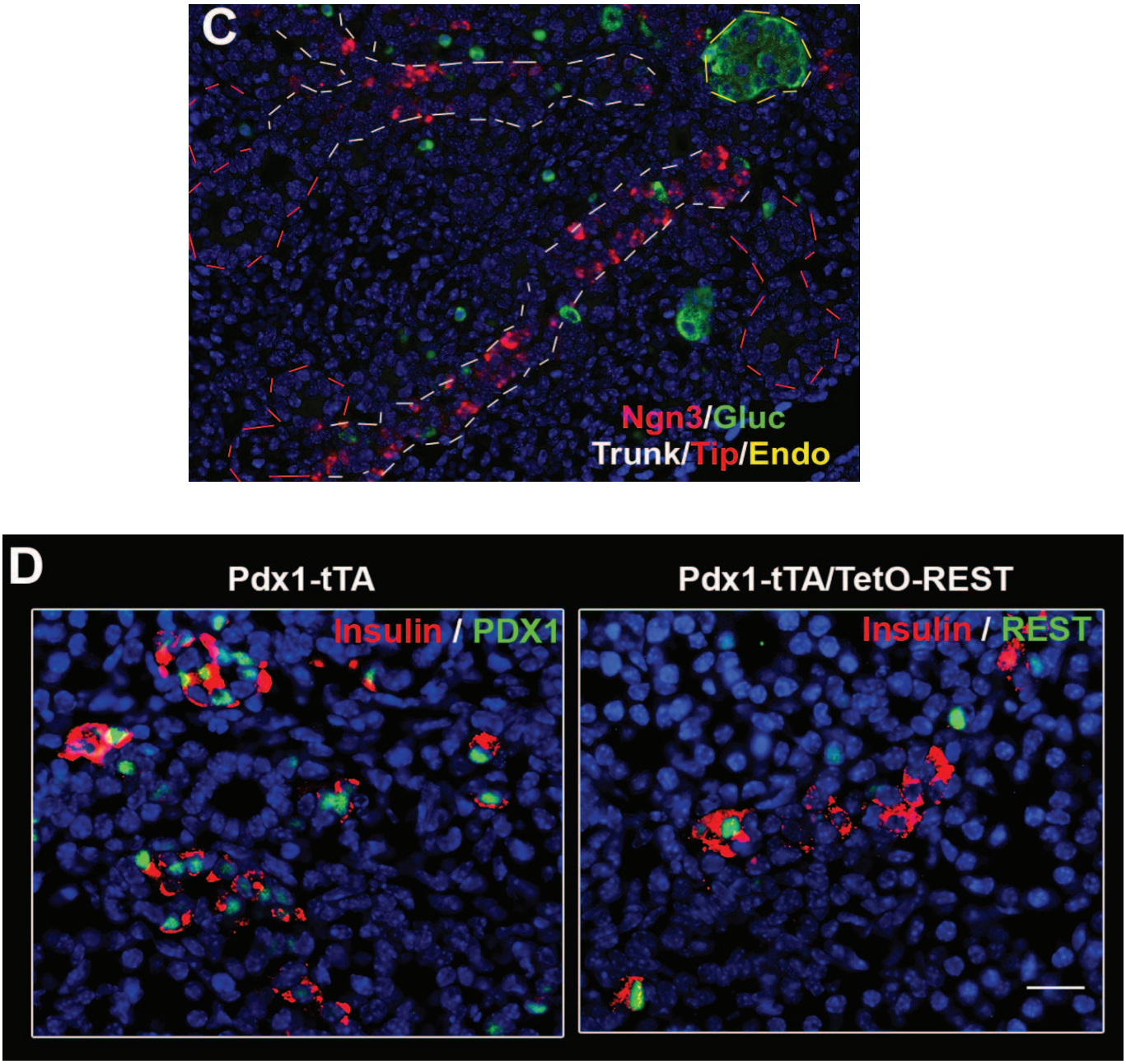


Figure 8: Patterns of expression of the inducer (Pdx1) and reporter (REST) gene in E14.5 and E16.5 pancreas

A IHC for Pdx1, REST (green) and insulin (red) were performed on E14.5 pancreas from Pdx1-tTA control and bigenic Pdx1-tTA/TetO-REST mice grown without Dox. Scale bar is 50 µm.
B. Same image as A., to which has been added the DAPI staining and dotted lines to delineate the different domains of the branching epithelium: trunk (white), tips (red), and endocrine (yellow).
C. ISH for Ngn3 (red) and co-IHC for glucagon (green) in E14 WT embryos. Dotted lines delineate the different domains of the branching epithelium: trunk (white), tips (red), and endocrine (yellow).
D. Same experiment as A performed with E16.5 embryos. Blue staining is the DAPI labeling of nuclei. Scale bar, 50 µm.

Despite the lack of efficiency of our system in directing REST expression in all the progenitor cells, we observed at E14.5 a significant 20% reduction in the number of $Ngn3^+$endocrine progenitors in Pdx1-tTA/TetO-REST, when REST was expressed from the beginning of Pdx1 expression (E8.5), as compared to the Pdx1-tTA control mice (Fig. 9). Moreover, whereas there was no difference in beta- and alpha-cell area by E14.5 and E16.5 (note that the variability in endocrine-cell mass at this stage is rather high), we observed at E18.5 a 20% and 30% decrease in beta- and alpha-cell area, respectively (Fig 10).

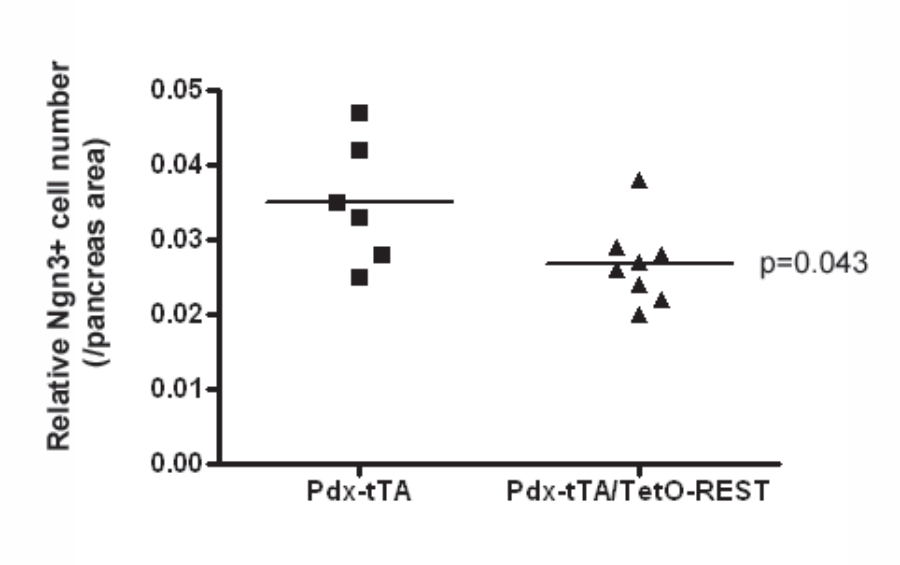


Figure 9: Quantification of the $Ngn3^+$ progenitor cells population

$Ngn3^+$ cell number was quantified in E14.5 pancreas from Pdx1-tTA control (n=6) and bigenic Pdx1-tTA/TetO-REST (n=8) mice grown without Dox, after IHC for Ngn3 and adjustment to the total pancreas area.

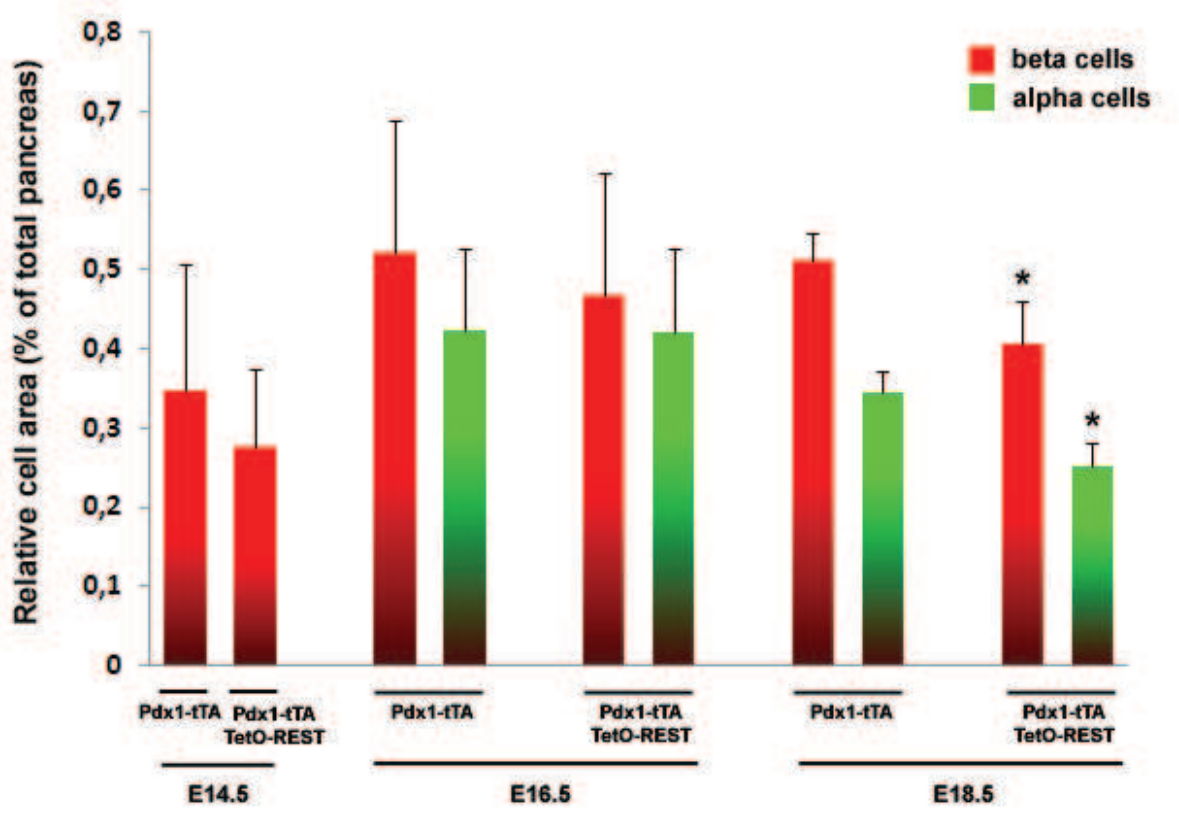


Figure 10: quantification of beta- and alpha-cell areas at E14.5, 16.5 and 18.5

*Beta- and alpha-cell areas were quantified at indicated time in pancreas from Pdx1-tTA control (n=4) and bigenic Pdx1-tTA/TetO-REST mice (n=4) grown without Dox. Results are mean ± SD. *P<0.05 versus values of control littermates.*

Identification of RE-1 motifs in crucial TFs of pancreas development. Myt1 is a bona fide REST target gene

The study of Johnson et al., has identified, using ChIPSeq analysis, RE-1 motifs in the chromatin of genes coding for known crucial TFs acting during pancreas development (Johnson et al., 2007). Importantly, these RE-1 sequences were bound by REST in a T cell line, even if their corresponding ChIPSeq signals were in the lowest range of intensity, indicative of a weak binding site. We retrieved and aligned the corresponding RE-1 motifs with the consensus (Fig. 11 A). Compared with the RE-1 sites found in genes involved in functions of terminally differentiated beta cells (Martin et al., 2008, Martin et al., Submitted), these new RE-1 have a lower level of conservation, that of Myt1 being

clearly non-canonical, as described by Otto et al., (Otto et al., 2007) or Johnson et al., (Johnson et al., 2008) (fig. 11 A). We have identified the location of some of these RE-1s into their cognate gene: that of Ngn3 resides 4020 bp after the gene in an antisense orientation, that of Hnf6/Onecut1 lies 6388 after the ATG in a sense orientation and that of Myt1 is in the 2nd intron, 6289 bp upstream of the ATG in a sense orientation. Importantly, Myt1, which is expressed in differentiated beta cells, is specifically downregulated upon REST expression in INS-1E cells (Fig. 11 B)

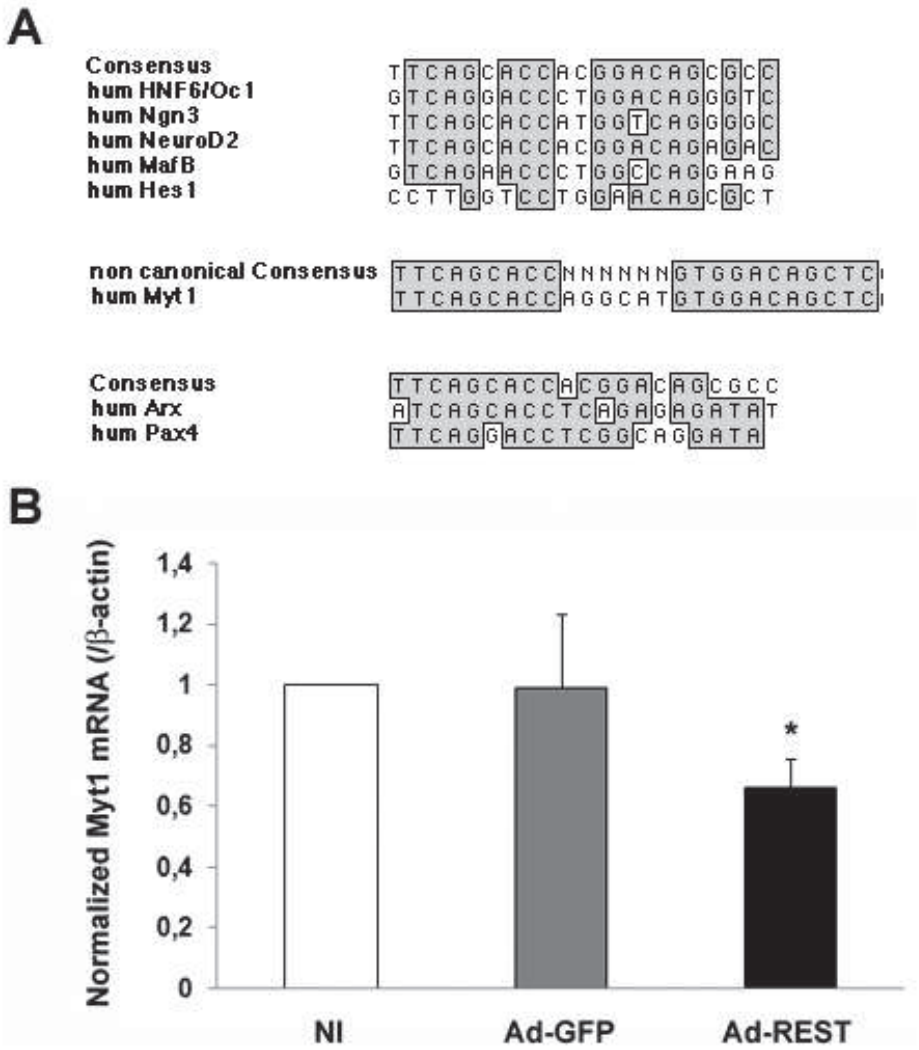


Figure 11: Identification of RE-1 motifs associated with crucial TFs such as Myt1

A. RE-1 sequences alignment.
*B. qPCR experiment showing the downregulation of Myt1 upon REST expression in INS-1E cells. NI: not infected; Ad-GFP: GFP-expressing adenovirus; Ad-REST: REST-expressing adenovirus. Results are mean ± SD. *P<0.05*

Discussion

Hes1, an effector of the Notch pathway, is a direct negative regulator of bHLH neuroendocrine determination genes such as Mash1 (Chen et al., 1997) or Ngn3 (Lee et al., 2001). Accordingly, Hes1 KO leads to premature neuronal differentiation in the brain (Ishibashi et al., 1995) and premature endocrine differentiation in the pancreas (Jensen 2000), and conversely, constitutive Hes1 expression in neuronal progenitors blocks their differentiation (Ishibashi et al., 1994). REST was first described as a direct negative regulator of terminal differentiation genes in neurons (Chong et al., 1995, Schoenherr and Anderson, 1995), and in pancreas (Atouf et al., 1997, Martin et al., 2008, Martin et al., Submitted). The observation that mice deficient for REST do not exhibit precocious neuronal differentiation (Chen et al., 1998), and that constitutive REST expression in spinal cord of chicken do not prevent neurodifferentiation (Paquette et al., 2000) led to the conclusion that REST, in contrast to Hes1, does not control the expression of regulatory genes. However, the authors did not exclude that the targeted mutation of REST in mice may lead to a hypomorphic REST protein, or that the early lethality in mutant mice precludes from studying pronounced neurogenesis, and that the levels and timing of REST expression achieved in the chick spinal cord was not carefully analyzed.

Now, it has been demonstrated that REST is expressed in ESC to participate to self-renewal or pluripotency (see Introduction 5) e) ES cells pluripotency), in neuronal progenitors to regulate the expression of a wider number of genes than previously accepted, not only related to terminal neuronal functions (see Introduction 5) c) “REST regulon”) and that REST activity is modified concordantly with neurodifferentiation (Ballas et al., 2005). More direct evidence suggesting an active role for REST in controlling the process of neuroendocrine differentiation is the finding that REST expression in NSC blocks neurons formation (Kohyama et al., 2010), that the mutant

REST activator REST-VP16 triggers neuron differentiation in NSC (Su et al., 2004), and finally, that in a non-neuronal cell line, REST binds some crucial TFs involved in pancreas differentiation (Johnson et al., 2007).

Altogether, these data call for the study of the, so far, neglected role of REST in pancreas development.

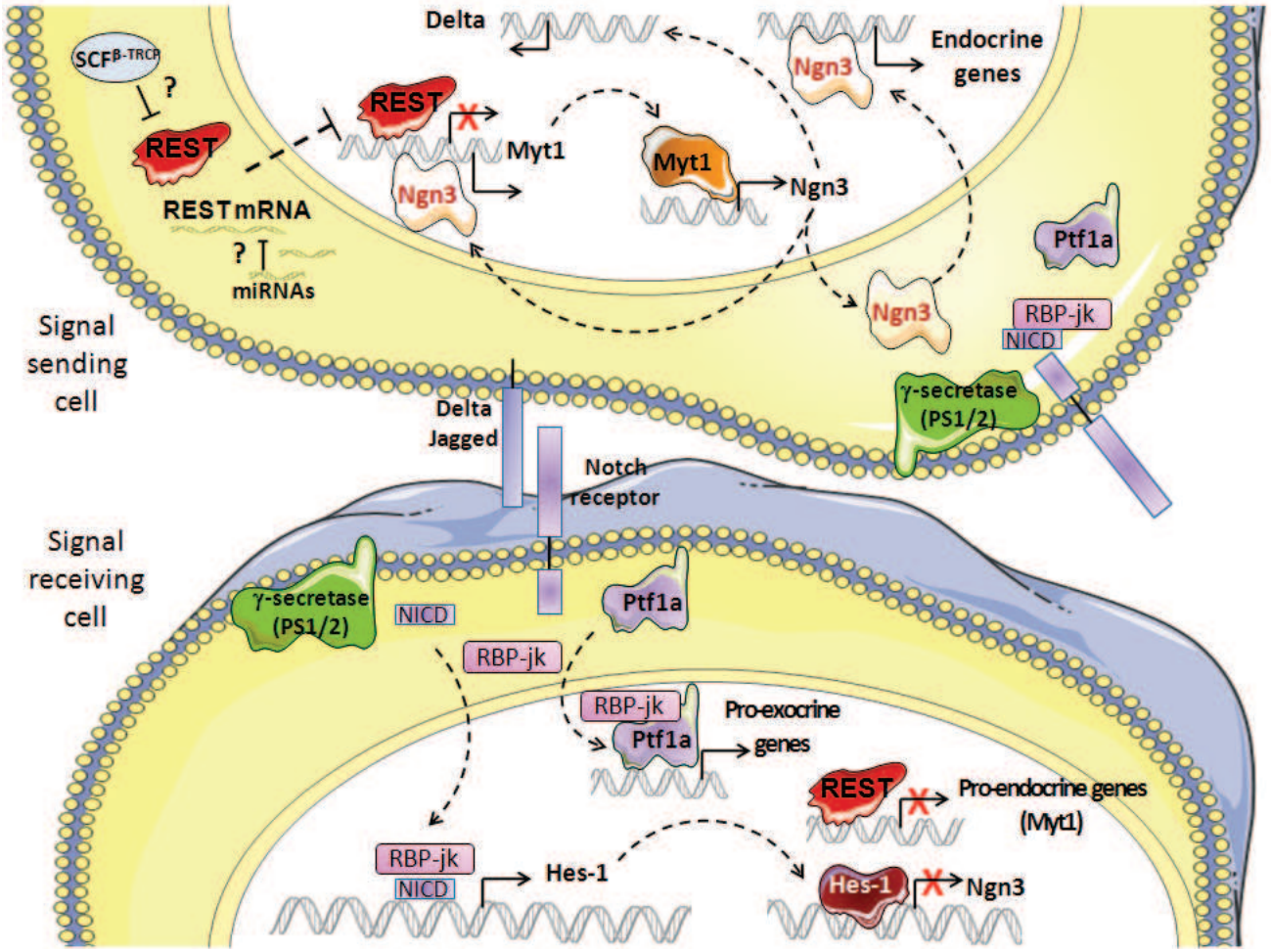
To our knowledge, we are the first to demonstrate, using *in situ* hybridization, that 1) REST is expressed in multipotent progenitors of the pancreas 2) REST expression, at some point in the differentiation process, becomes excluded from differentiated endocrine cells. Thus, we hypothesize that the pattern of REST expression in the developing pancreas underlies its activity and is similar to that in developing central nervous system: REST may be expressed in multipotent progenitors to control the proper timing of pancreas development, by preventing premature endocrine differentiation.

In our model of REST gain-of-function, the endogenous regulatory regions of Pdx1 drive the constitutive expression of REST in all the multipotent progenitor cells. Nevertheless, when its expression was directed from the beginning of Pdx1 activation (E8.5) onwards, we observed that the pattern of REST expression at E14.5 was only partially covering the epithelium. REST was strongly expressed in beta cells, and also found in the tips of the branching (corresponding to the population that will give rise to differentiated acinar cells), but almost lacking in the most centrally located trunks that contain endocrine progenitors (Fig. 8 B and D). It is likely that REST indirectly represses Pdx1 expression (Pdx1 is not a direct target of REST; see Chapter 2, supplementary data, table 4) and thus represses its own expression in a negative feedback loop. At this stage, high Pdx1 expression in beta cells and in the tips may be strong enough to direct REST transgene activation despite this negative control, meanwhile it may be too low in the trunk to drive detectable levels of REST (especially if considering that the Pdx1-tTA

background contains only one Pdx1 allele). It is noteworthy that in the RIP-REST mice, REST had also an indirect negative impact on insulin expression (Martin et al., 2008), and thus, on its own expression as well. We had suggested that this indirect effect occurred through direct repression of Ica512, a membrane protein that links insulin exocytosis with insulin synthesis in a positive feedback loop (Martin et al., 2008). We also suggest that this indirect negative feedback loop was responsible for the heterogeneity that we observed in the levels of REST expression per cell, some being REST ^{high} and other REST ^{low}: if REST inhibits its driver and thus its own expression (cells becoming REST ^{low}), the driver is consequently going up again and afterwards, REST also (cells becoming REST ^{high}), and so on. In the pancreas of E14.5 Pdx1-tTA/TetO-REST mice, this heterogeneity is visible in the tips, but not really in the trunks (Fig. 8 B).

Given the weakness of our system in directing strong and stable expression of REST, especially in the domain where Ngn3^{+} cells originate, it is yet stimulating to see that we have 20% less of these endocrine progenitors upon REST expression (Fig. 9). A fragment of the Ngn3 promoter has been suggested to recapitulate the full pattern and timing of Ngn3 expression (unpublished data; personal communication from Grapin-Botton A.). Knowing that the RE-1 motif associated with Ngn3 lies downstream of the Ngn3 gene and not in the promoter region, it is possible that Ngn3 is not a *bona fide* REST target gene. However, we have found that the RE-1 of Myt1, albeit non-canonical, mediates REST binding and transcriptional control in INS-1E cells (Fig. 11 B). The study of Johnson (Johnson et al., 2008) has specified that the location of a RE-1 motif near the transcriptional start site was globally indicative of a good functionality. In this perspective, we can hypothesize that the location of the RE-1 in the 5' part of the Myt1 gene, in a sense orientation is a good feature for mediating REST control. It is now increasingly believed that among the population of Ngn3^{+} cells, only those that are Ngn3 ^{high} are able to differentiate into endocrine lineages (Wang et al., 2008, Wang et al.,

2010). Moreover, it has been demonstrated that Myt1 is important to achieve this high level of Ngn3 expression via a feed-forward loop (Wang et al., 2008). Even if current research aims at deciphering the involvement of spatial cell arrangements to explain how some cells are becoming Ngn3 ^{high} and some Ngn3 ^{low}, we propose here, that REST may provide an intrinsic mechanism to precisely control this decision. The gradual decrease of REST activity, and thus, the gradual relieve of key target genes expression, such as Myt1, probably concomitantly with reduced canonical Notch signaling would be a triggering fact for endocrine fate decision (Scheme 5).



Scheme 5: Proposed mechanism involving REST in endocrine differentiation
REST actions have been added to the scheme 3 (introduction). Globally, REST represses pro-endocrine genes, including Myt1 (according to our observations), and may be several other crucial TFs involved in pancreas development, as proposed by Johnson et al., (Johnson et al., 2007). By analogy to what has been described in the process of neurodifferentiation, REST concentration could be decreased in certain cells (here in the upper cell) by either the action of the ubiquitin ligase SCF$^{\beta\text{-}TRCP}$, or via stochastic changes, including miRNAs-mediated repression. This could lead to relieved expression of crucial genes, such as Myt1, which would promote endocrine lineage selection, via upregulated Ngn3 expression.

The finding that REST may control several other key TFs, involved in different aspects of endocrine differentiation, suggests that it may intervene at several critical points. Several transcription factors thought to act relatively late in beta-cell specification, including Nkx2.2, Nkx6.1 and Hlxb9, are expressed in not only beta-cell precursors but also widely throughout the early progenitor population. This early expression may be an epiphenomenon, or it may represent the first stage of a progressive process by which $Ngn3^{+}$ cells acquire beta-cell differentiation competence. In liver development, by analogy, transcriptional activators of hepatocyte genes begin to modify the chromatin of their targets within multipotent progenitor cells, well before those cells have committed to a hepatocyte fate (Zaret, 2002). This raises the question of whether REST and/or its co-repressor complexes act in a similar way, via epigenetic chromatin modifications, to set up a permissive state that progressively predispose cells to adopt an endocrine fate.

A last observation that links REST with Ngn3 expression is the fact that REST is decreased in E10.5 pancreas constitutively expressing Ngn3 in a Ngn3 mutant background (add-back model, see introduction 1) b) "pancreas differentiation"), as compared with the mutant mice (Grapin-Botton, personal communication). This result indicates that Ngn3 downregulates REST expression either in a non-cell autonomous mechanism related to lateral inhibition or in a cell autonomous direct or indirect mechanism. Still, this inverse relationship between the expression of a pro-endocrine determination gene and REST suggests that REST has an anti-endocrine activity inside the developing pancreas.

Materials and methods

Embryonic pancreas preparation

Mice are mated at night and identification of a vaginal plug on the next morning defines noon as the embryonic day 0.5 (E0.5). At the desired stage, embryos are carefully dissected under binocular microscope, and pancreatic buds (usually taken out together with the stomach, spleen and duodenum) are fixed overnight at 4°C in 4% PFA. Samples are then washed, and immersed in phosphate buffer containing 15% sucrose for several days, so that tissues are soaked up with this cryo-preservant. Pancreas are finally incubated in phosphate buffer containing 7% gelatin and 15% sucrose at 37°C for 1h, and left at RT with this inclusion medium in small container. The solidified gelatin block containing pancreas is then quickly frozen in methylbutane immersed in dry ice-freezed EtOH 100%, and kept at -80°C.

In situ hybridization and co-immunohistochemistry

Tissues are cryo-sectionned and post-fixed 10 min with 4% PFA. Slides are washed twice in RNase free PBS, incubated 30 min in 0.3% H_2O_2 in PBS (to inhibit endogenous peroxydase), and treated with 0.2N HCL for 15 min before incubation 10 min with 2µg/ml proteinase K in PBS at 37°C. The reaction is stopped with 5 min incubation in fresh 0.2% glycine in PBS. Slides are washed twice in PBS, again post-fixed 10 min in 4% PFA, and washed twice in PBS. Slides are then treated twice with freshly prepared acetic anhydride in triethanolamine pH8, and finally washed twice in PBS and twice in 5X SSC before pre-hybridization. A solution of hybridization, containing 5XSSC pH4.5, 50% deionized formamide, blocking reagent (Roche) and yeast total RNA is added onto slides that are placed at 68°C in a humidified box during 1h. The buffer is replaced with hybridization buffer containing 1 µg/ml of denatured S or AS DIG-labeled RNA probe,

and the slides, protected with a plastic coverslip, are placed for more than 24h at 68°C for probe hybridization. After hybridization, there is no need to take care about RNase. Slides are washed once with 2XSSC pH4.5, three times with 2XSSC pH4.5, 50% formamide at 60°C, and finally once in TBS tween 0.1% (TBST). For probe detection, slides are blocked 30 min with 0.5% blocking reagent in TBST before incubation ON at 4°C with anti-DIG-peroxydase antibodies diluted 1/5000 in TBST. For tyramide signal amplification, a biotinylated tyramide (Perkin-elmer) was used diluted 1/500 in TBST for 20 min, and after washing, the complex was recognized by adding streptavidin-AlexaFluor 594 (Invitrogen) diluted 1/500 in TBST for 30 min.

To perform immunological detection on the same slides, primary antibodies diluted in PBS are added onto slides ON at 4°C, and after washing, is replaced by diluted secondary antibodies conjugated to appropriate fluorophore for 1h before washing, DAPI staining and mounting.

Quantitative PCR and western blots

qPCR and western blots were performed as described in Chap1 and Chap2.

Cell quantification

The relative area occupied by alpha or beta cells in the entire pancreas were measured after IHC every sixth (E14.5), seventh (E16.5) or eighth (E18.5) sections and normalized relative to the total epithelium area. Islet cells and total epithelium areas were measured using an ACECAD Professional graphic tablet connected to a Quantimet Leica 5001 (Leica, Cambridge Ltd, England) programmed for semiautomatic measurement of areas. Quantification of $Ngn3^{+}$ cell number was made by scoring the number of labeled nuclei over total epithelium area.

GENERAL CONCLUSIONS AND PERSPECTIVES

Very diverse studies conducted by groups using distinct modern biotechnologies (molecular biology, bioinformatic analyses, genome-wide analysis of chromatin occupancy, etc) have proven that the role of the REST/RE-1 system is complex, covering a large variety of functions (neuronal, pancreatic, cardiac, and vascular physiology, cancer, neurodegenerative diseases, stem cell pluripotency, etc).

Increasing our knowledge of REST action went through the identification (systematic or not) of its targets containing the RE-1 motif. Again, the emerging picture appeared complicated, inasmuch as REST is directly negatively controlling genes at the transcriptional level, as well as indirectly positively controlling genes at the post-transcriptional level (via miRNAs). The use of genome-wide analyses coupled with high throughput identification of bound sequences led to enhanced comprehension as well as complexity. We know, for instance, that RE-1 motifs exist in a variety of differential combinations of right and left domains, with distinct inter-space lengths, leading to canonical and non-canonical RE-1 of different binding affinities. It is also believed that the REST regulon is not a fixed entity and varies in a cellular- , temporal- and promoter-dependent fashion, and that the number of expected targets varies between around 40 (clearly identified) and more than 2000 (bound chromatins; *in sillico* identification). Two studies that substantially provide (speculative) knowledge and complexity have suggested that the regulatory network of REST in ESC seems to be integrated into those of other crucial TFs such as Oct4, Nanog and Sox2 (Johnson et al., 2008), and that in NSC, together with CoREST, REST integrates signals from different extracellular signaling pathways such as Wnt, BMP, Notch, FGF, controls the expression of many TFs, of epigenetic modifiers, and impacts many developmentally- and environmentally-

regulated cellular processes, being at “the nexus of an intricate circuitry” (Abrajano et al., 2009).

The complexity of this system is also represented in the number of epigenetic modifiers recruited by REST, and therefore in the different combinatorial mechanisms that REST uses to repress genes, sometimes in a target-dependent fashion.

Our studies aimed at clarifying the role of this REST/RE-1 system in both adult and embryonic endocrine pancreas.

Identification of RE-1-containing genes that are essential to differentiated beta cells

Chapter 1 and 2 show how, by directing the expression of REST specifically in beta cells of transgenic mice, we can better understand how beta cells work. REST is defined as a “forbidden” gene, as it is normally not expressed in differentiated beta cells. REST target genes are inversely considered to be essential traits for the acquisition of the beta cell phenotype. Our models directing ectopic expression of this forbidden gene has permitted 1) to define what were the physiological consequences of repressing the expression of RE-1-containing genes in beta cells *in vivo* 2) to identify, with the help of RE-1 databases, relevant RE-1-containing genes and to validate them as a *bona fide* REST target using an *in vitro* cell line model 3) to relate the selected candidates to the impaired function identified in 1).

Using this REST gain-of-function strategy, we have specified that the rationale for the expression of RE-1-containing genes in beta cells is to favor insulin secretion and beta-cell mass, inasmuch as these two features were impaired upon global repression of the set of REST target genes. Precisely, we were able to highlight RE-1-containing genes that were specifically involved in exocytosis, such as SNAP25, Synaptotagmin (Syt) IV, VII and IX, Complexin II and Ica512, and also involved in beta-cell survival, such as Cx36, IB1, IRS2, and Cdk5r2 (Martin et al., 2008, Martin et al., Submitted) (see the

comprehensive list, Chapter 2 Supplementary data, Table 3). These genes are acting in terminally differentiated beta cells and we specifically introduced the bias to select for genes that are strongly regulated by REST, thus bearing a canonical RE-1 motif with strong binding affinity, excluding therefore the putative low binders that could play a role earlier in beta cell formation. The novel findings in our studies was the identification of the hitherto neglected genes coding for Syt IV and for Cdk5r2, revealed to be important for insulin exocytosis and beta-cell survival, respectively (Martin et al., 2008, Martin et al., Submitted). Now more work needs to be done to really define the relevance of these proteins to beta cells.

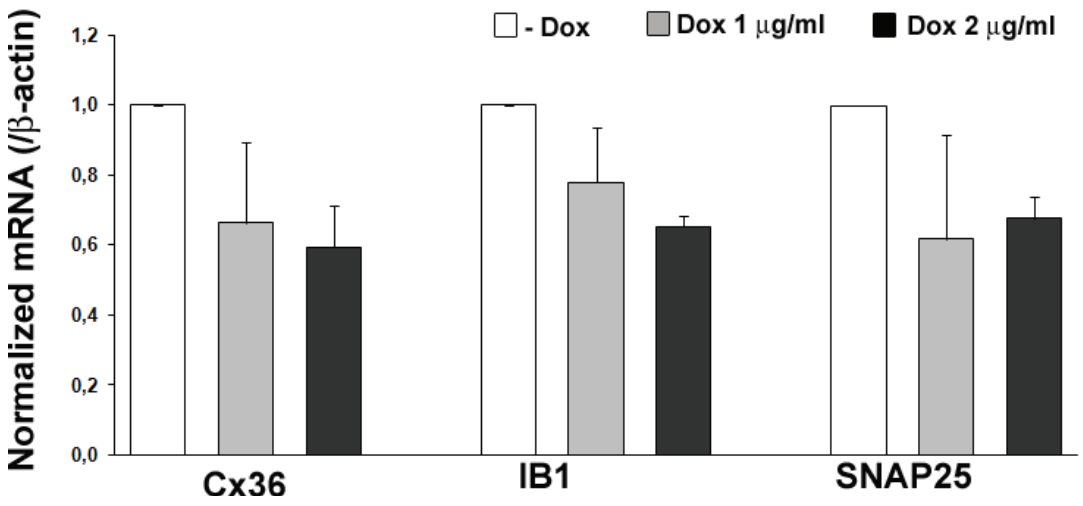
We further aim at performing a comprehensive study to build up a master list, representing a subset of the genes that make a beta cell what it is. This will be done in collaboration with the groups of Profs. F. Schuit in Leuwen (Belgium) and of P. Meda in Geneva (Switzerland).

This project involves:

1) the use of a new model of Tet on-inducible transgenic RIP-REST mice as a tool, recently generated and characterized in the lab 2) a gene array and statistical analysis 3) the validation of the selected target genes using rodent primary beta cells and siRNA approach combined with functional tests 4) the validation of the selected genes' relevance to human biology using human islets (provided by Prof. J. Kerr-Conte, Lille).

The mouse model is obtained by crossing RIP-rTA (Tet-on system; kindly provided by Prof. C. Widmann, Lausanne) with TetO-REST transgenic mice (See Chapter 3). Islets of bigenic mice are isolated and treated with Dox to induce REST expression during 24h, ensuring that we will detect only direct REST target genes. Preliminary results show that the system is functional. However, we suppose that the heterogeneity in REST expression from cell to cell that we previously observed ((Martin et al., 2008); Chap 3 discussion) is responsible for the variability and low degree of downregulation of known

target genes (Cf. below). We are therefore currently breeding bigenic RIP-rTA/TetO-REST mice that are homozygous for the TetO-REST locus, in an attempt to increase our output.



Preliminary results for the gene array analysis with inducible REST expression

qPCR using islets from bigenic RIP-rTA/TetO-REST, in vitro treated or not during 24h with 1 or 2μg/ml Dox.

The large number of expected additional REST target genes that remain to be identified suggests that we should generate, through our comprehensive analysis coupled with extensive validation, a highly relevant amount of information for the discovery of new beta cell-specific genes and pathways. Such findings should be of great interest for the beta-cell scientific community. This niche is also likely to provide new interesting pharmacological targets to enhance beta-cell survival and function for human therapies.

REST induction in differentiated beta cells

Dysfunctional REST has been proposed to explain several human disorders. For instance, REST loss-of-function has been involved in cardiac hypertrophy (Kuwahara et al., 2003), in neointimal hyperplasia of vascular smooth muscle cells (Cheong et al., 2005), or in colorectal cancer (Westbrook et al., 2005). Conversely, REST gain-of-

function has been reported in brain of RETT patients (Abuhatzira et al., 2007), in ischemic neurons (Calderone et al., 2003), or in brains of Huntington disease patients (Zuccato et al., 2003).

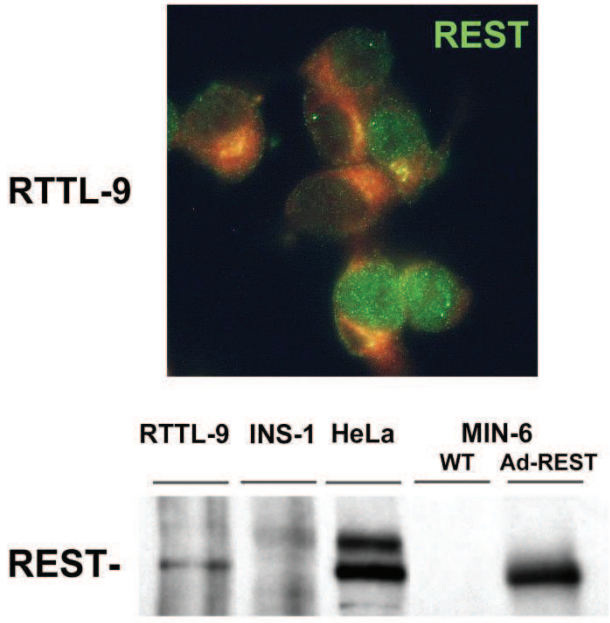
One of the most challenging questions that we had was whether REST induction was occurring in beta cells in certain disorders. According to our findings, and especially, given that the single expression of REST at high levels in beta cells can provoke diabetes (Martin et al., Submitted), or that its constitutive expression in embryonic pancreas may block beta cell formation (Chap 3), we expect that such an induction of REST expression would lead to tremendous defects in glucose homeostasis. To address this question, we propose to examine two possibilities.

1) In the class of monogenic forms of diabetes, about 39% of cases with permanent neonatal diabetes (PNDM) and 10% with MODY are of unknown genetic etiology. The majority of identified subtypes arise from a loss-of-function mutation in an important TF. However, we do not exclude the possibility that a mutation in the REST gene leading to a gain of activity could be responsible for such unexplained forms of monogenic diabetes. A search for mutations by sequencing the REST gene in diagnosed diabetic family relatives would help answering this.

2) We neither exclude the possibility that pathological conditions could lead to impaired repression of REST expression in beta cells. We naturally examined whether gluco-lipotoxic conditions were able to drive such a modification. Preliminary results obtained with islets from glucose-infused rats or with mouse islets treated with supra-physiological concentrations of glucose, palmitate or both did not reveal any modification in REST expression. Globally, good strategies to search for putative REST induction in beta cells include: looking for disorders impacting the expression of proteins that mediate REST extinction (such as retinoic acid receptor, MeCP2, CoREST (Ballas et al., 2005)); looking for disorders leading to REST neuronal induction, that can

be paralleled with pancreatic induction. In this perspective, several studies have reported a CNS-specific REST gain-of-function, in human disorders that are sometimes associated with diabetes, such as Huntington disease (Zuccato et al., 2003) or Rett syndrome (Abuhatzira et al., 2007).

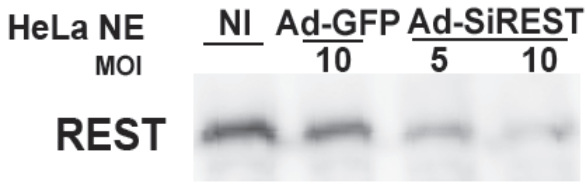
Such a beta-cell REST expression has never been reported and is still very speculative. Yet, we dispose in the lab of a human beta-cell line, called RTTL9, that was generated by the group of Prof. B. Thorens (CIG, Lausanne) and which exhibit poor glucose responsiveness. In fact, we have found that these cells probably do not secrete insulin because they express REST (Cf. below). This indicates that a particular remodeling, that may be consequent or inherent to the strategy used to immortalize these cells, has led to REST induction.



human RTTL-9 beta-cells express REST

Upper panel: IHC for REST shows REST protein in the nucleus of RTTL-9 beta cells
Lower panel: western blot showing REST protein in nuclear extracts from RTTL-9. Positive controls are HeLa and REST-transduced MIN-6 extracts; Negative controls are INS-1 and WT MIN-6 extracts.

We plan to try to improve RTTL-9 cells physiology by reducing REST levels or by counteracting REST activity, thanks to the adenovirus that we have generated expressing either a siRNA against human REST (see below) or the recombinant REST-VP16 (see next paragraph), respectively.



Adenovirus expressing siRNA against human REST are efficient

Western blot showing decreased REST production in nuclear extracts of HeLa cells infected 48h with Ad-siREST, as compared with Ad-GFP or native cells (NI).

Role of REST during endocrine cell lineage specification and differentiation

Since we discovered, in 1997, that the mechanism controlling part of the neuronal and pancreatic beta cells identity was identical, namely the absence of REST in these two cell types (Atouf et al., 1997), no one has explored the possibility that REST could be important for beta cell genesis as it is for neurogenesis.

By looking at the pattern of REST expression in WT mice at only one stage of development, we were able to define that REST is expressed in multipotent progenitors to become then excluded from endocrine differentiated domains. We further suggested, using transgenic mice, that constitutive REST expression in the progenitor domain impairs the commitment of progenitors to the endocrine lineage, and consequently, impairs the genesis of differentiated endocrine cells. We finally showed that this role of REST in endocrine cell fate decision may at least lie upstream of Ngn3, via the control of Myt1, which helps Ngn3-expressing cells to acquire the endocrine competence.

Future prospects will include:

1) the mapping of the pattern of REST expression in WT pancreas at different stages of development (E10.5, E12.5), using *in situ* hybridization (ISH).

2) the generation of a novel antibody raised against murine REST protein to confirm our previous observations obtained with ISH.

3) the quantification of $Ngn3^{+}$ cell population (and may be also of the global or per cell Ngn3 levels), and quantification of differentiated endocrine areas in Pdx1-tTA/TetO-REST mice that are homozygous for the TetO-REST locus. As homozygous mice for the

Pdx1-tTA locus are apancreatic and not viable, we thought that increasing REST gene dosage would perhaps improve our pattern of transgene expression, and thus, ascertain our conclusions on the role of REST during development.

If REST expression is improved with TetO-REST locus homozygosity, we can next use the conditional feature of our system with Dox treatment, which would allow assessing the specific requirements for REST at different stages of development. It would be interesting, as well, to assess the specific action of REST in different cell populations, by employing novel models (if available) that use the activity of specific markers to drive the expression of the repressible transactivator and hence, of REST.

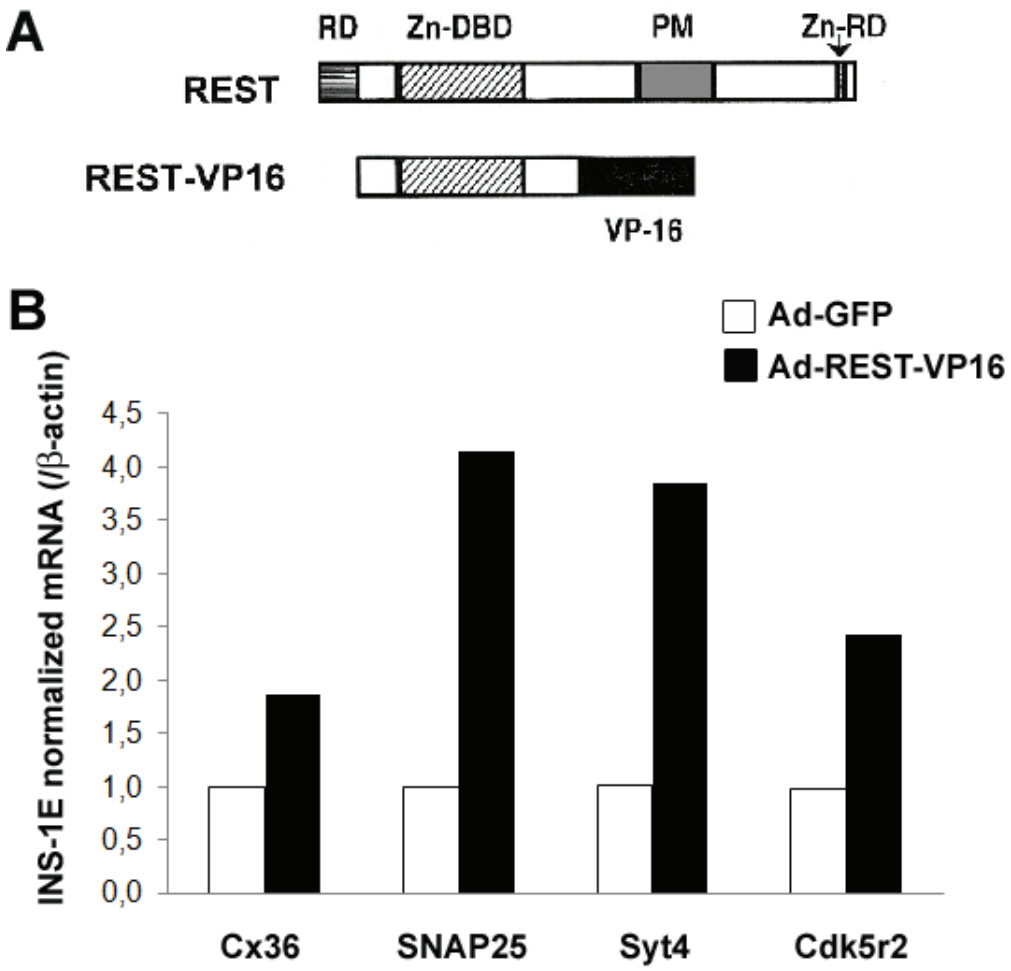
4) the quantification of $Sox9^{+}$ cell population upon constitutive REST expression to assess the role of REST in the maintenance or in the expansion of multipotent progenitor cells population.

5) the analysis of the chromatin occupancy mediated by REST in pancreas of WT mice at an early stage (through ChIPSeq analysis).

6) the development of REST loss-of-function studies.

To do so, we will develop two strategies:

One will take advantage of a recombinant dominant positive form of REST, in which both repressor domains have been replaced by activator domain of the herpes simplex virus protein VP16 (Immaneni et al., 2000) (see below, A). REST-VP16 counteracts REST activity and activates RE-1-containing genes (Lawinger et al., 2000). The REST-VP16 plasmid has been kindly provided by Sadhan Majumder from Texas, and we have produced adenoviruses expressing REST-VP16 and GFP (Ad-REST-VP16). Preliminary results show that a 24h infection of INS-1E cells with Ad-REST-VP16 is sufficient to increase the expression of several REST target genes (see below, B).



Ad-REST-VP16 is sufficient to activate transcription of RE-1-containing genes in INS-1E cells
A. Schematic organization of native REST gene and recombinant REST-VP16; RD: repressor domain, Zn-DBD: Zinc finger DNA binding domain.
B. qPCR showing increased expression of several REST target genes in INS-1E cells upon REST-VP16 transduction.

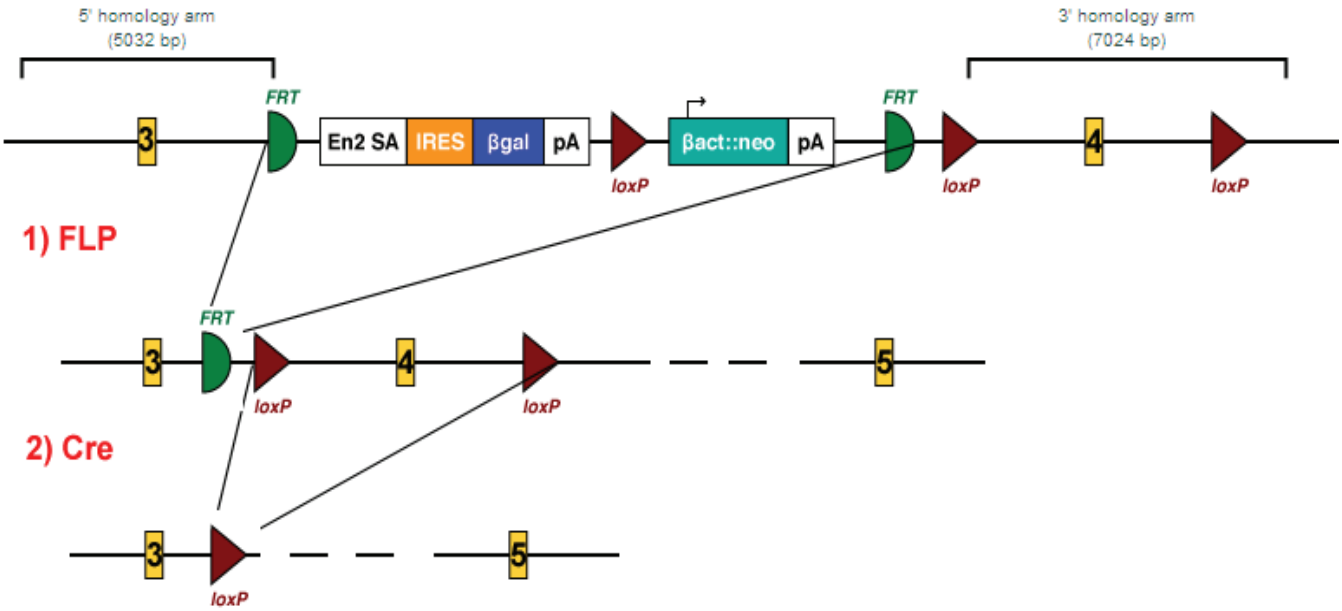
We are planning to infect E10.5 WT pancreatic explants with Ad-REST-VP16. If our previous hypothesis reveals true, we expect that the widespread blockade of REST-mediated repression and the concomitant activation of its pro-endocrine target genes may lead to precocious widespread commitment of progenitors towards the endocrine lineage. This will occur if the intrinsic mechanism brought about by REST to restrict endocrine cell fate decision (see Chapter 3) is not dependent on other signals or if the triggering of determination genes will be sufficient to bypass these restrictive signals.

The second loss-of-function strategy, that we have chosen to explore the role of REST during pancreas development, consists in generating conditional mutant mice for REST. We have actually bought from the European Conditional Mouse Mutagenesis (EUCOMM) consortium, two ES cell clones that are targeted for REST. The construct used to perform homologous recombination at the REST locus gives rise to a multipurpose knock-out /conditional allele (see below) (Testa et al., 2004). The knock-out is produced at the RNA processing level, with the insertion in an intron, of a cassette containing a splice acceptor (Engrailed-2 SA) that captures the RNA transcript. An efficient polyadenylation signal (pA) also truncates the transcript so that the gene is not transcribed downstream of the cassette. A second purpose provided by this cassette is to report the activity of the promoter via an IRES-mediated expression of the LacZ gene reporter. When inserted, this cassette provides a non-conditional knock-out. However, the surrounding Flippase recognition target (FRT) sites permit its removal upon Flippase-mediated recombination. The conditional knock-out feature is then brought about in the construct by the LoxP sites surrounding the exon 4 of murine REST gene (REST possess 3 exons in the 5' UTR, and the CDS starts with exon 4). Upon Cre-mediated recombination, exon 4 is deleted, leading to REST inactivation. Chimeras bearing the recombined REST allele are currently bred in our animal facility to select for those showing germ line transmission.

Planned experiments include: 1) analysis of heterozygous mice for REST expression reporting, using β-galactosidase activity, to correlate our previous ISH observations 2) mating heterozygous mice with FLP-expressing transgenic mice (available at the TAF, Lausanne) to generate heterozygous and then homozygous conditional REST mutant mice. These mice will then be crossed with Pdx1-Cre transgenic mice (available in Anne Grapin-Botton's lab) to generate an epithelial pancreatic-specific deletion of REST, in order to assess the necessity of REST activity for proper pancreatic development 3) it would also be conceivable to add a temporal component to our analysis, by using

transgenic mice expressing either a tamoxifen-inducible Cre recombinase (Pdx1-Cre-ER^{TM} (Gu et al., 2002)) or a triple cross between conditional REST mutant mice, the Pdx1-tTA mice, and a TetO-Cre mice (available in Pedro Herrera's lab, Geneva (Thorel et al., 2010)). Tamoxifen injection or Dox withdrawal provides the temporal condition, and Pdx1 promoter activity ensures tissue-specific deletion.

As exposed with the experiments using REST-VP16, we expect that REST inactivation will lead to a precocious endocrine differentiation, while there is so far, no strong indication that this will be accompanied with depletion of the pool of progenitors.



Strategy for the generation of conditional mutant mice for REST

Scheme showing the construct used to generate mouse ES cells targeted for REST (EUCOMM). The inserted cassette provides the inactivation of REST through REST mRNA extra-splicing (En2SA) and termination (pA), together with REST expression reporting (βgal). LoxP sites provides for the conditional KO system.
1) after crossing mutant mice with FLP-expressing mice, the cassette, surrounded with FRT, is deleted.
2) after crossing mutant mice with Cre-expressing mice, the first CDS exon (4) is deleted
A safety variation, in which only Cre is used results in exon deletion, while leaving in place the intronic knockout cassette.

As a final conclusion, we strongly believe that the REST/RE-1 system plays an important role in the fine tuning of proper progress of pancreatic development. We suggest positioning REST, in the regulatory cascade, upstream of the Ngn3 pro-endocrine bHLH determination gene. However, in front of the complexity of this system, we suggest that the emerging picture for the action of REST is far more complicated, and suggest that REST actually accompanies the developmental pathway in a default mechanism, thereby placing it sideways along this process.

We also suggest that the important role of REST in developmental gene networks relies on stochastic decisions. Negative feedback loops are known to promote systems stabilization, by minimizing fluctuations in genes expression. REST is under such negative feedback loops, via repression of miRNAs that target its own gene, or via its negative auto-regulation, thereby ensuring a fine tuning of its activity. Despite these stabilizing effects, negative auto-regulation has in contrast been proposed to results in oscillation of gene expression. It is of note that Hes1 autonomously promotes its oscillatory expression via such a negative auto-regulation (Hirata et al., 2002). It would be interesting to consider such a possibility for REST expression during development. Another way through which REST promotes stochasticity is through the control of Myt1, which is engaged in a positive feedback regulation with Ngn3. Relieving Myt1 expression and hence, promoting this positive feedback loop generates bistability, enabling that a distinct phenotypic subpopulation (endocrine-committed precursors) arise from a homogenous cell population (multipotent precursors). The REST-induced modifications of the chromatin may also contribute to set up stochastic variations in gene expression over time during pancreas development (to read about stochasticity, see (Kaern et al., 2005)).

We also finally underline the necessity to use *in vivo* models to study the REST/RE-1 system, particularly since it is acting through chromatin modifications. We therefore

strengthen the fact that our transgenic *in vivo* strategies represented innovative and efficient tools to gain insights into the REST/RE-1 system. Specifically, thanks to the commonly available inducible systems, we expect to generate soon, with the help of transcriptome-wide analysis, a huge amount of relevant information for the beta-cell scientific community. The expected findings will help us better understand what a beta cell is, and might as well allow us to help pre-diabetic compensating beta cells having a REST.

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