

**Pancreatic duodenum homeobox 1
(PDX-1) phosphorylation
in pancreatic β -cells**

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This thesis is dedicated to the memory of my grandfather, whose inspiration, support and love was always the drive of my study and will forever be remembered.

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Abstract

Pancreatic Duodenum Homeobox (PDX-1) is a homeodomain transcription factor and a key regulator of β -cell development and differentiation. In mice and humans, mutations of the *Pdx1* gene in β -cells have been linked to MODY and type 2 diabetes. Therefore, the regulation of PDX-1 transcriptional activity is very important.

Phosphorylation is known to be a pivotal regulatory mechanism for several transcription factors. Therefore in this study, I focused on the regulation of phosphorylation on PDX-1 in intact β -cells and investigated the potential signalling pathways involved in its regulation.

Firstly I showed that PDX-1 is phosphorylated under physiological conditions in pancreatic β -cell line. Additionally, recombinant PDX-1 is efficiently phosphorylated *in vitro* at Thr-152 in the homeodomain by the nutrient-regulated protein kinase, PASK. To explore the physiological relevance of this site I made dephospho- (T152A) and phosphomimetic (T152E) mutants of PDX-1. In INSr $\alpha\beta$ cells, a less differentiated cell line derived from INS-1 cells, wild-type PDX-1 and PDX-1^{T152A} were partially redistributed from the cytosol to the nucleus in response to elevated glucose concentrations (20 vs 3 mM). By contrast, PDX-1^{T152D} and PDX-1^{T152E} displayed enhanced nuclear immunoreactivity at low glucose concentrations, and underwent nuclear export as glucose concentrations were raised. PDX-1 binding to an oligonucleotide based on the consensus A3 binding site of the preproinsulin gene promoter was completely abolished by mutation of Thr-152 to an acidic amino acid. Furthermore, the PDX-1 phosphomimetic mutants failed to suppress preproglucagon gene expression in INSr $\alpha\beta$ cells, an action fully preserved in the T152A mutant. However, metabolic labelling of INS-1(832/13) cells with ³²Pi, as well as mass spectroscopic analysis, and the use of a phospho Thr-152-specific antibody, failed to reveal any clear evidence for phosphorylation at this site under a variety of *in vivo* conditions, including in cells over-expressing PASK. Thus, Thr-152 is not a major site of phosphorylation of PDX-1 in intact β -cells. However, a novel phosphorylation site, Ser-269, at the C-terminus of PDX-1 was identified by mass spectrometry. To explore the potential upstream regulatory kinase and the signalling pathway involved, I generated dephospho-(S269A) and phosphomimetic (S269E) mutant PDX-1 and a phospho-Ser-269-specific antibody. Candidate kinases were also identified using recombinant wild-type and mutant PDX-1 in *in vitro* phosphorylation experiments.

In this thesis I demonstrate that PDX-1 is phosphorylated under physiological conditions in β -cell lines. I have shown that Thr152 is not a major phosphorylation site of PDX-1 in intact cells and we also defined the novel *in vivo* phosphorylation site of PDX-1. Moreover, we reported that PDX-1 is phosphorylated *in vitro* by a number of kinases including Aurora, Rsk1, PKCa, JNK. The work presented in the thesis provides evidence that PDX-1 phosphorylation is important in its role as a transcription regulator in mature β -cells; moreover, it suggests several physiological signalling networks to investigate.

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Abbreviations

AICAR	5-amino-4-imidazolecarboxamide riboside
AMPK	AMP-activated protein kinase
ARNT	Vertebrate aryl hydrocarbon receptor nuclear translocator
BME	β-mercaptoethanol
DAPI	4', 6-diamidino-2-phenylindole
DPP4	dipeptidyl peptidase IV
DTT	dithiothreitol
EGFP	Enhanced fluorescent protein
EMSA	electrophoretic mobility shift assay
GFP	Green Fluorescent Protein
GK	glucokinase
GLP1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GSF	Glucose-sensitive factor
GSIS	Glucose-stimulated insulin secretion
GSK3	Glycogen synthase kinase 3
HBS	Hepes buffer saline
HEK	Human embryonic kidney
HIPK2	homeodomain-interacting protein kinase 2
HMG1Y	High-mobility group protein I
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HNF	hepatocyte nuclear factor
IAPP	Islet amyloid polypeptide
IAPP	Islet amyloid polypeptide
ID	International dollars

IDF	International Diabetes Federation
IDX-1	Islet/duodenum homeobox-1
IFN- γ	Interferon- γ
IL-1 β	Interleukin-1 β
IP	Immunoprecipitation
IPF-1	Insulin promoter factor-1
IPTG	Isopropyl- β -D-thiogalactopyranoside
IUF1	Insulin upstream factor-1
JNK	C-Jun N-terminal kinase
MAPK	mitogen-activated protein kinases
MKK6	MAP kinase kinase-6
MODY	Maturity-onset diabetes of the young
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal
NFATs	Nuclear factors of activated T-cells
NLS	Nuclear localization signal
PAS	Per-Arnt-Sim
PASK	Per-Arnt-Sim kinase
PCIF1	PDX-1 C-terminal interacting factor
PDX-1	Pancreatic duodenum homeobox-1
PER	Drosophila period clock protein
PGC-1	peroxisome proliferator-activated receptor γ coactivator 1
PI 3K	phosphatidylinositol 3-kinase
PKA	Protein kinase A
PPG	preproglucagon
R&D	Pharmaceutical research and development
SAPK2	Stress-activated protein kinase 2
SDM	Site Directed mutagenesis
SDS	Sodium monododecyl sulfate

SIM	Drosophila single-minded protein
STF-1	Somatostatin transactivating factor-1
TAE	Tris-acetate-EDTA
TBST	Tris-buffer saline with Tween 20
TE	Tris-EDTA
TEV	Tobacco Etch Virus
TGF β	Transforming growth factor
TNF- α	Tumor necrosis factor- α
ZMP	5-amino-4-imidazolecarboxamide ribotide

Publication

1. **R. AN**, G. da Silva Xavier, H-X. Hao, F. semplici, J. Rutter and G.A. Rutter, Phosphorylation by Per-Arnt-Sim (PAS) kinase may lead to nuclear exclusion and transcriptional repression of pancreatic duodenum homeobox-1 (PDX-1). *DIABETIC MED.* (2007) 24: 2-2
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1. Chapter 1

Introduction

1.1 Diabetes

1.1.1 What is Diabetes?

Diabetes is now taking its place as one of the main threats to human health in the 21st century¹. Diabetes is a chronic, potentially debilitating and often fatal disease, which is a condition primarily defined by abnormally high blood sugar levels (hyperglycaemia), giving rise to a risk of microvascular damage². There are two main forms of diabetes^{1, 2}. Type 1 diabetes, where insulin treatment was required for survival, is mainly due to autoimmune-mediated destruction of pancreatic β -cell islets, resulting in absolute insulin deficiency³. It is the most common chronic disease of children. Type 2 diabetes is characterized by insulin resistance and/or abnormal insulin secretion, where pancreatic insulin production continues, but may be insufficient for control of blood sugar levels³. The diabetes epidemic relates particularly to type 2 diabetes, accounting for more than 90% diabetes cases, and is taking place both in developed and developing nations^{1, 4}. Chronic elevation of blood glucose will eventually lead to tissue damage, with consequent and often serious disease, including the kidneys, eyes, peripheral nerves and vascular tree, sometimes even fatal. Indeed, diabetic complications are those aspects of the disease that are most feared linked to human suffering and disability, and the huge socio-economic costs through premature morbidity and mortality^{1, 5, 6}.

1.1.2 Diabetes affects everyone, at all ages.

Ageing, unhealthy diet, obesity and a sedentary lifestyle are some of the key factors that have conspired to create the current worldwide epidemic of type 2 diabetes. This is projected to occur because traditional lifestyles and dietary patterns, that

have sustained people over generations, are disappearing, and families are often forced to move into urban areas where diabetes is primarily concentrated^{7, 8}.

Ageing is an important factor for type 2 diabetes. This type typically occurs in people over the age of 40 and the 40-59 age group currently has the greatest number of persons with diabetes. Moreover, type 2 diabetes is now also affecting children and adolescents at an increasingly young age and to a greater extent, especially in those with a family history of diabetes^{9, 10}. Children as young as eight years of age are now affected. Type 2 diabetes has been reported in children from Japan, the United States, Pacific Islands, Hong Kong, Australia and the United Kingdom^{1, 10-13}. What is worse, though it is now becoming a global public health issue with potentially serious health outcomes, compared to that for adults, little information is available on type 2 diabetes incidence and prevalence in the young. What cannot be ignored, however is that, type 1 diabetes usually accounts for only a minority of the total burden of diabetes, but it is the predominant form of the disease in younger age groups in most developed countries. It is estimated that about 65,000 children aged under 15 years develop type 1 diabetes every year worldwide with an estimated overall annual increase of around 3% in the world¹⁴⁻¹⁷. The reason for this increase in the prevalence of type 1 diabetes remains largely obscure¹⁸.

1.1.3 Diabetes mellitus is a current and growing health problem.

The greatest challenge to our health systems all over the world in the 21st century might be how to develop effective responses to the growing burden of chronic diseases. Diabetes mellitus which will be referred to as Diabetes, one of the most important chronic diseases, has not been paid so much attention until recent decades, although been recognized since antiquity¹⁹.

Whilst absent 15 years ago, Diabetes treatment programmes have become common across Europe, and include medical and paramedical personnel training for the establishment of well-organized diabetic clinics, diabetes education programmes for patients, and so on²⁰. Pharmaceutical research and development (R&D) of antidiabetic agents has been holding top three of the most popular therapeutic areas in recent years and continually being promoted. In 2007, it showed an almost 15% rise in R&D over 2006²¹. Consequentially, new antidiabetic drugs with novel targets are introduced sequentially, such as the first glucagon-like peptide 1 (GLP1) analogue exenatide (Byetta; Amylin/Eli Lilly) approved in 2005 by the FDA, and the dipeptidyl peptidase IV (DPP4) inhibitors Sitagliptin phosphate (Januvia, Merck) in 2006^{7, 22}.

Vigorous scientific research continues to combat diabetes, and vastly expands understanding of the underpinnings leading to diabetes and its complications. The NIH (National Institutes of Health) of the United States, for example, spends \$1.037 billion on diabetes research²³. Almost all westernized countries have now established diabetes associations, and over 200 of them existing in more than 160 countries joined the IDF (International Diabetes Federation), a worldwide alliance, coming together to enhance the lives of people with diabetes everywhere, raising global awareness of diabetes, promoting appropriate diabetes care and prevention^{16, 17}.

Introduced in 1991 by the World Health Organization (WHO) and IDF, 14 November (Frederick Banting's birthday) was selected as World Diabetes Day in memory of Frederick Banting who, along with Charles Best, first conceived the idea which led to the discovery of insulin in 1922. It is celebrated worldwide every year, and had been made an official United Nations World Health Day²⁴.

One may be curious as to why diabetes is provoking so much concern from so many countries, health organizations, pharmaceutical companies and researchers currently.

In fact, diabetes is fast becoming the epidemic of the 21st century, creating serious problems not only for health and medicine but also for the global economy and society. There is an urgency to increase diabetes awareness worldwide⁸.

In 1985, there were an estimated 30 million people with diabetes worldwide. Today there are more than 246 million people with diabetes, or 6.0% in the adult population. In just over 20 years, there has been an amazing eight-fold increase, with the total so expected to rise to 380 million by 2025: a further 7 million people will develop diabetes each year, or two people every 10 seconds^{8, 16, 17, 25}. Nowadays, diabetes is forward distributed all over the world. For the adult (20-79) age group, the Western Pacific Region, with 67 million, and European Region, with 53 million, currently have the highest number of people with diabetes. However the prevalence rate of 4.2% for the Western Pacific Region is significantly lower than 8.3 % in the North American Region and 6.6% in the European Region, which will increase to 9.6% and 7.8% respectively by 2025¹⁷. The region with the highest prevalence of diabetes is Eastern Mediterranean and Middle East.

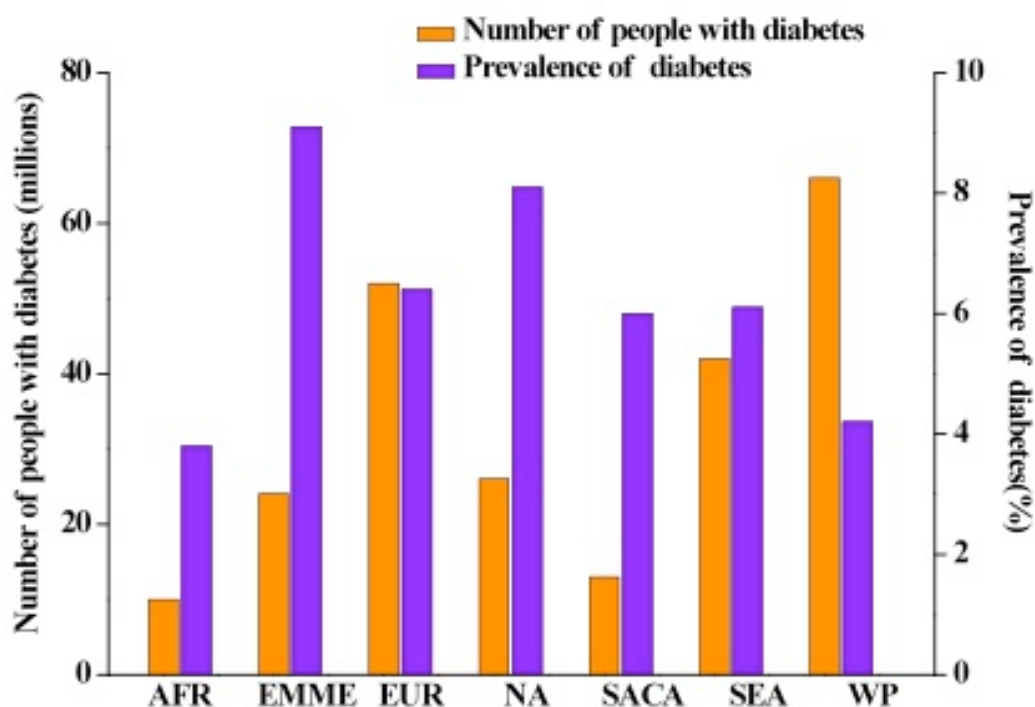


Fig.1.1. Number of people with diabetes and prevalence of diabetes (20-79 age group) by region 2007. AFR = Africa, EMME = Eastern Mediterranean and Middle East, EUR = Europe, NA = North America, SACA = South and Central America, SEA = South-East Asia, WP = Western Pacific¹⁷.

Figure was taken from Diabetes Atlas, third edition © International Diabetes Federation, 2006¹⁷

In 2007, the five countries with the largest numbers of people with diabetes were India (40.9 million), China (39.8 million), the United States (19.2 million), Russia (9.6 million) and Germany (7.4 million)^{16, 17, 25}. There are currently over 2.5 million people with diabetes in the United Kingdom and more than half a million people who have the condition but don't know it, as reported by Diabetes UK.

1.1.4 Impact on economy of the world

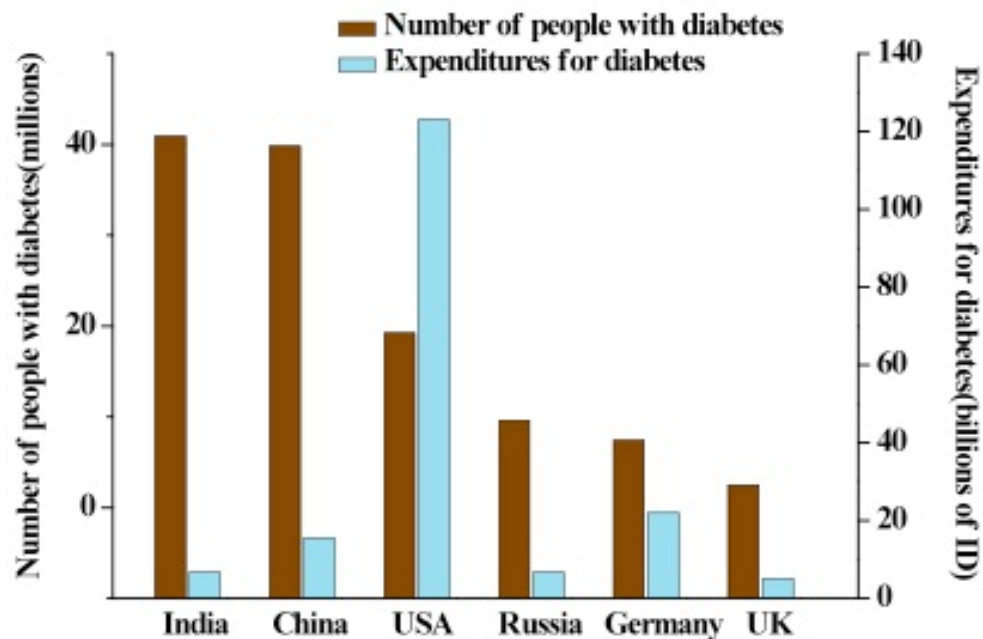


Fig. 1.2 Annual health expenditure for diabetes (ID) vs persons with diabetes in the five countries with the largest numbers of people with diabetes and in the United Kingdom in 2007. ID, international dollars. Figure was taken from American Diabetes Association. Economic costs of diabetes in the U.S. In 2007.^{5, 17}

The rapid increase in diabetes cases represents a considerable impact on the economy of the world. Its costs affect health services, national productivity, governments, insurance companies, as well as individuals and families^{5, 26}. The annual direct healthcare costs of diabetes worldwide, for people in the 20-79 age group, is estimated to be at least 153 billion international dollars, for blood sugar control, treating long-term complications, and so on. The costs will be between 213 billion and 396 billion international dollars in 2025, which would mean that the proportion of the world's healthcare budget being spent on diabetes care will be between 7% and 13% in high prevalence countries^{16, 17}. The largest costs of diabetes worldwide are its devastating effects on families and national economies.

People living with diabetes, and their families, feel the impact of diabetes most directly. People with diagnosed diabetes, on average, have medical expenditures that are about 2.3 times higher than what expenditures would be in the absence of diabetes⁵. In the poorest countries, people with diabetes and their families bear almost the entire cost of whatever medical care they can afford. Indirectly, diabetic retinopathy is the leading cause of vision loss in adults of working age in industrialized countries. The number of people in the United States with diagnosed diabetes now is the third in the world and the total cost of diabetes in 2007 is estimated at 174 billion international dollars, including 116 billion international dollars in excess medical expenditures and 58 billion in reduced national productivity. People with diagnosed diabetes incur average expenditures of 11,744 international dollars per year, of which 6,649 is attributed to diabetes⁵.

In addition to these quantified costs, diabetes imposes high intangible costs on society in terms of increased absenteeism, reduced quality of life, and the pain and suffering of people with diabetes, their families, and their friends^{5, 27}. Diabetes increases the likelihood that chronic disability prevents working, or in some cases limits employment opportunities. WHO estimates that diabetes as well as diabetes, associated heart disease and stroke together will cost about 333.6 billion international dollars in lost national income in India and 555.7 billion in China over the next 10 years, based on lost productivity, resulting primarily from premature death. Accounting for disability might double or triple these figures^{16, 17, 26, 28}.

1.1.5 Diabetes complications

It is estimated that the costs of diabetes complications, including visual impairment, kidney failure, heart disease, stroke and nerve damage, account for between 5% and 10% of total healthcare spending in the world^{16, 17}.

Diabetes is the main cause of partial vision loss and blindness in adults in developed countries. The prevalence of retinopathy is probably around 30% in type 2 diabetes, accounting for 5% of all cases of blindness globally. It is estimated that more than 2.5 million people worldwide are affected by diabetic retinopathy^{16, 17, 28}.

Diabetes is the leading cause of kidney disease (nephropathy) and is responsible for huge dialysis costs. About one third of all people with diabetes develop kidney disease and 10% to 20% of people with diabetes die of renal failure^{16, 17, 28}.

Diabetic nerve disease or neuropathy affects at least half of all people with diabetes.

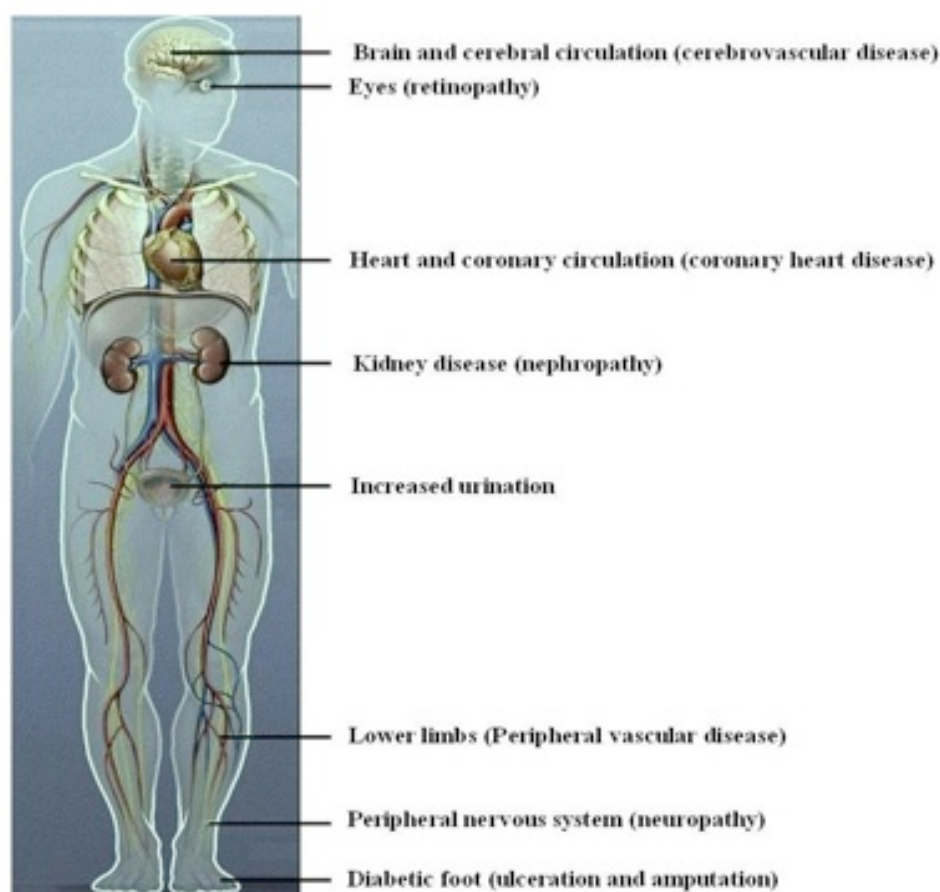


Fig.1.3. Diabetes complications^{16, 17}

Figure modified from the image (http://www.healthhype.com/wp-content/themes/healthblog-CT/topic/images/diabetes_complications.jpg)

This results in a loss of sensation in the feet or hands, pain in the foot and problems with the functioning of different parts of the body, leading to ulcers and possibly amputation^{16, 17, 28}.

Diabetes is the fourth leading cause of global death by disease. Every 10 seconds a person dies from diabetes-related diseases^{6, 29}. Cardiovascular disease is the major cause of death in diabetes, accounting for some 50% of all diabetes fatalities, and much disability. Their risk of heart disease is 2-4 times higher than those who do not have diabetes. On average, people with type 2 diabetes will die 5-10 years before people without diabetes, mostly due to cardiovascular disease^{16, 17, 28}. Additionally, people with diabetes are more prone to bacterial and fungal infections.

1.1.6 No cure for diabetes

Though still not fully defined, the mechanisms of the diabetic complications are likely to involve the toxic effects of high blood glucose levels, along with the impact of elevated blood pressure, abnormal lipid levels and both functional and structural abnormalities of small blood vessels. Diabetes has been treatable since insulin became medically available in 1920s³⁰. However, there is no cure for diabetes today³¹.

With appropriate medication, quality of care and good medical advice, diabetic patients should be able to lead an active and healthy life and reduce the risk of developing complications. Good diabetes control means keeping blood sugar levels as close to normal as possible, which can be achieved by a combination of controlled diet, physical exercise and medication. People with type 2 diabetes may require oral hypoglycaemic drugs to lower their blood sugar and some may need insulin injections at some point^{7, 31}. Treatment for type 2 diabetes typically begins with oral antidiabetic agents and a reduction in glycated haemoglobin A1c (HbA1c)-a reflection of glucose levels over the preceding 3 months — to below 7%

represents an elusive but key goal of therapy⁷. Metformin, the sulphonylureas and the thiazolidinediones were the most widely prescribed classes of non-insulin antidiabetic medications, whereas incretin-based therapies⁷ and some others are also approved as well as 503 candidates in development in 2008²¹. Metformin inhibits gluconeogenesis. Sulphonylureas sensitize pancreatic β -cells to produce insulin. Thiazolidinediones (TZDs) act by activating the peroxisome proliferator-activated receptor- γ (PPAR γ), which promotes adipocyte differentiation and improves insulin sensitivity. TZDs were also reported to increase the level of adiponectin³². Adiponectin was secreted by adipocytes and has been reported to increase fat oxidation and improve insulin sensitivity³³. Incretins are peptide hormones that stimulate insulin secretion, inhibit glucagon secretion and promote satiety after nutrient ingestion. Glucagon-like peptide 1 (GLP1) is the short-lived activity of native incretins which can be rapidly degraded by the dipeptidyl peptidase 4 (DPP4) enzyme. GLP1 analogues such as exenatide (Byetta; Amylin/Eli Lilly) and DPP4-selective inhibitors such as Sitagliptin (Januvia; Merck) are also applied to treat hyperglycaemia^{7, 22, 31}. However, with the controversy on some side effects of the marketed drugs (such as rosiglitazone, believed to slightly increase the risk of heart failure^{34, 35}) and insufficiency of even combination therapy, development of antidiabetic agents is limited^{7, 36, 37} and there is an urgent need to identify novel targets and develop new drugs to treat diabetes effectively.

1.2 INSULIN SECRETION AND BETA CELLS

As is discussed in the ‘diabetes’ section, Type 1 diabetes is caused by the failure of the body to secrete insulin due to autoimmune destruction of the pancreatic islet β -cells. Compared with type 1 diabetes, the cause of type 2 diabetes is more complicated, but provides more possibilities and pathways to study and potentially treat it. Type 2 diabetes is often associated with obesity and develops when chronic overnutrition conspires with genetic susceptibility to cause impaired insulin signaling (insulin resistance), as well as a relative insulin deficiency of non-autoimmune aetiology³⁸.

Insulin normally acts by regulating nutrient homeostasis through the stimulation of glucose uptake into peripheral tissues and by suppressing the release of stored lipids from adipose tissue. Therefore, any disorder of insulin secretion and action leads to multiple metabolic abnormalities in type 2 diabetes, including hyperglycaemia due to impaired insulin-stimulated glucose uptake and uncontrolled hepatic glucose production, and dyslipidaemia, which includes disrupted fatty acids homeostasis, triglycerides and lipoproteins metabolism. Chronic increases in circulating glucose and lipid levels can further worsen insulin secretion and action and cause other forms of tissue damage due to metabolic overload³⁸.

1.2.1 Regulation of insulin secretion in normal β -cells in islet of Langerhans

The endocrine pancreas plays a pivotal role in glucose homeostasis. The maintenance of plasma glucose within a narrow range of concentrations is critical and is a result of a balance between glucose storage and consumption. In higher animals the blood glucose level is controlled primarily by two antagonistic hormones, insulin and glucagon. Insulin stimulates glucose uptake and metabolism

in the peripheral tissue such as liver, muscle, and adipose tissue, whilst it also acts as an inhibitor of glucose release from the liver. In the fasting state, glucagon activates glycogenolysis and gluconeogenesis to maintain normoglycemia. In all the mammals, postprandial insulin secretion is regulated in a biphasic manner controlled by both nutritional and hormonal signals and the most important physiological regulator of insulin is glucose. Besides glucose, other secretagogues such as free fatty acids, amino acids or the incretin regulator glucagon-like peptide-1 (GLP-1) also works as potentiators but a threshold stimulatory level of glucose in the bloodstream is required for potentiation³⁸. Insulin is synthesized and stored in the β -cells in pancreatic islet of Langerhans where it is secreted from the pancreatic islet β -cells primarily in response to elevated blood concentrations of glucose (Fig. 1.4). Glucose stimulates insulin secretion by generating triggering and amplifying signals in β -cells^{39, 40}. Glucose is transported into the β -cells by facilitated diffusion through glucose transporters including GLUT2 and/or GLUT1⁴¹; increased extracellular glucose concentrations lead to elevated concentrations of glucose within the β -cell, which in turn leads to a rise in the ATP:ADP ratio and results in the closure of ATP-sensitive K^+ (K_{ATP}) channels. This shutdown of K_{ATP} channels leads to membrane depolarization, activation of the voltage-gated Ca^{2+} channels and an influx of extracellular calcium. The resulting increase in intracellular free Ca^{2+} is believed to trigger exocytosis of insulin-containing secretory granules⁴². There has also been evidence that the increased level of glucose within β -cells also appears to stimulate insulin secretion independently of changes in K_{ATP} channel activity such that glucose can trigger insulin release under conditions of fixed high $[Ca^{2+}]_c$ ⁴³.

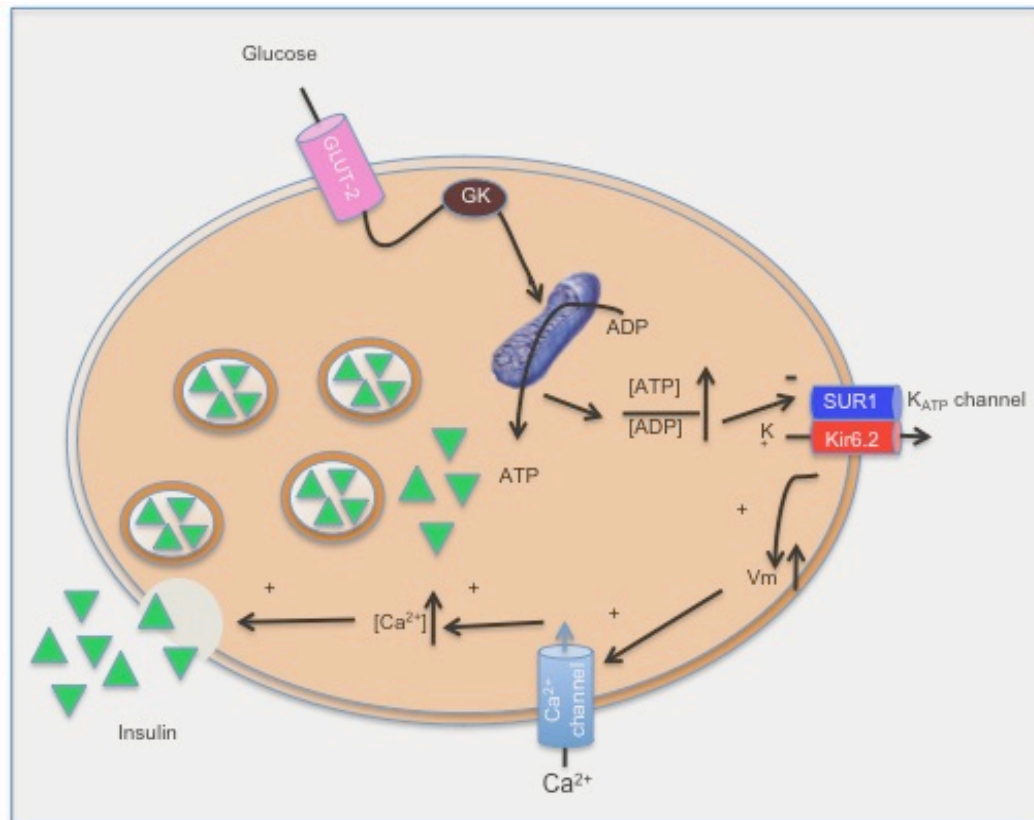


Fig. 1.4. K_{ATP} channels and insulin secretion in pancreatic β -cells. In pancreatic β -cells, glucose is shuttled in the cytosol by the glucose transporter GLUT2 and metabolized by glucokinase (GK) and mitochondria to produce ATP from ADP. Cytosolic ATP raises and ADP decreases. These changes cause closure of K_{ATP} channels by the cumulative effects of increased inhibition of Kir6.2 by ATP and decreased activation of SUR1 by MgADP. The ensuing depolarization elicits opening of voltage-dependent Ca²⁺ channels, Ca²⁺ entry, and Ca²⁺-initiated insulin granules exocytosis (Modified from Dr. Michel Vivaudou webpage, Institut de Biologie Structurale Jean-Pierre Ebel. www.ibs.fr).

1.2.2 β -cells and pancreatic transcription factors

The pancreatic β -cell is a highly specialized cell playing an irreplaceable role in regulating fuel metabolism in conjunction with the glucagon secreting α -cell and other cell secreted hormones to maintain the glucose homeostasis. The features of the β -cell are determined by a group of genes controlled by several pancreatic transcription factors which act as a network covering the whole life cycle of the β -cell from the differentiation, development, and maintenance until death⁴⁴. In brief, in early developmental stage, among these factors, pancreatic duodenum *homeobox1* (PDX-1)⁴⁵⁻⁴⁷ is crucial for defining the region of the primitive gut, which will later form the pancreas. Ngn3^{48, 49} expression will open up the endocrine lineage, and will later be joined by several other transcription factors such as PDX-1 (the second wave of expression), Pax4⁵⁰, NeuroD1/ β 2^{51, 52}, and MafA⁵³. In mature β -cells, the most important roles that these transcription factors play are in regulating insulin gene expression, and controlling some β -cell specific gene expression in order to maintain the normal function of β -cells. These transcription factors include PDX-1¹⁶, MafA⁵⁴, β 2⁵⁵ and Nkx2.2⁵⁶. During the survival stage of β -cell life, a number of transcription factors are also recruited to defend against the autoimmune-mediated attack and the toxic metabolic milieu. PDX-1¹⁶ helps maintain an adequate pool of healthy β -cell mass with its anti-apoptotic and proliferative activities which is also the case the Pax4^{57, 58}, Ngn3⁵⁸ is involved in the regulation of progenitor cells. There are also other transcription factors which are also highly involved and important for β -cell survival and proliferation including forkhead box O1 (FoxO1), nuclear factor of activated T-cells (NFAT), activating signal cointegrator-2 (Asc-2) and cAMP response element binding (CREB) family members⁵⁹.

1.3 Pancreatic Duodenum *Homeobox*-1

Various laboratories discovered and studied pancreatic duodenum *homeobox*-1 (PDX-1) in independent experiments in the early 1990's. The protein has been given different names in different studies: Somatostatin transactivating factor-1 (STF-1) ⁶⁰, Insulin promoter factor-1 (IPF-1) ⁶¹, islet/duodenum *homeobox*-1 (IDX-1) ⁶², insulin upstream factor-1 (IUF1) ⁶³, β cell-specific glucose-sensitive factor (GSF) ⁶⁴. According to the Human Genome Database Nomenclature Committee (University College, London), IPF-1 is used as the human gene encoding this protein and pancreatic duodenal *homeobox*-1 (*pdx*-1) was designated as the name for mice gene by the international Committee on Standardized Genetics Nomenclature ^{46, 65}. The PDX-1 protein in different species are highly homologous, eg, human PDX-1 shares 90% homology with hamster, 88% with rat, 87% with mouse.

1.3.1 Distribution and structure of PDX-1

The islets of Langerhans consist of four major types of cells (fig. 1.5). There are glucagon-expressing α -cells (15-20% of total islet cells), insulin-expressing β -cells (65-80%), somatostatin-producing δ -cells (3-10%) and pancreatic polypeptide-producing (PP) cells (3-5%) ⁶⁶.

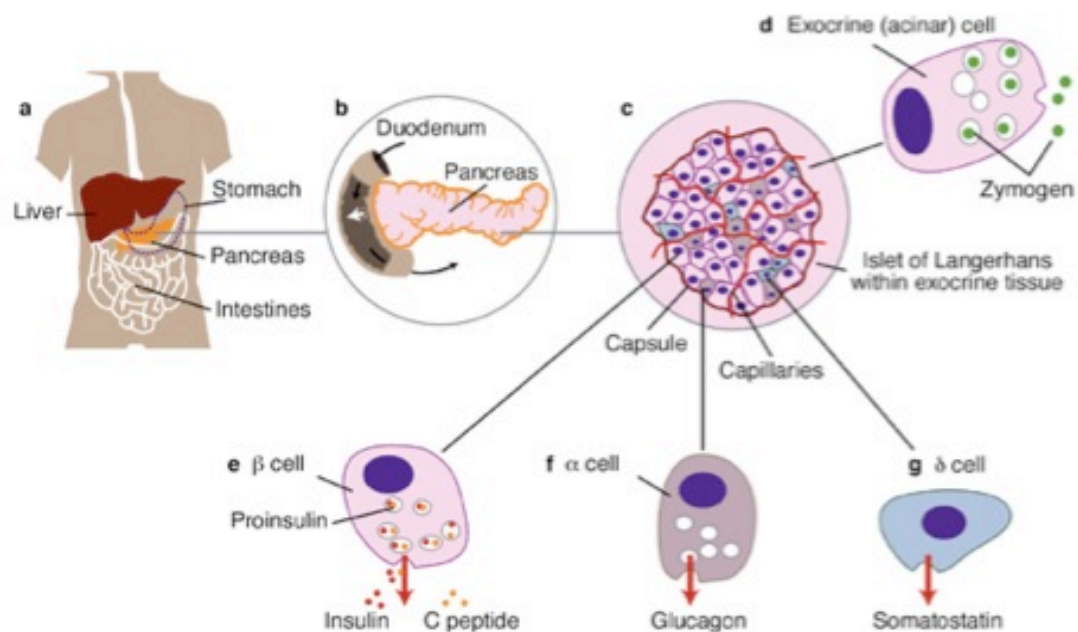


Fig. 1.5. Location of human β cells in the islets of the pancreas. (a) The pancreas is located in the upper abdomen, close to the liver and behind the stomach. (b) The pancreas secretes digestive enzymes via its duct into the duodenum. (c) An islet is a collection of endocrine cells supplied by capillaries. A thin fibrous capsule separates them from the surrounding (d) exocrine cells, which produce and secrete zymogen. The endocrine cells include: (e) β cells, which synthesise proinsulin, which is cleaved into insulin (stored in granules) and C peptide; (f) α cells, which secrete glucagon; and (g) δ cells, which secrete somatostatin. The hormones are under homeostatic control and are secreted into the bloodstream. The β cells form the islet core and the non- β cells (α , δ and pancreatic polypeptide-producing cells) form the islet mantle. The islets are vascularised by arterioles entering the islet core and the arterioles branch into a dense capillary network. The β cells are destroyed as a result of the autoimmune processes of type 1 diabetes, and can be replaced by islet cell transplantation. Disease processes (including malignancy) that result in the loss of pancreatic tissue can affect both the endocrine and exocrine functions of the organ (Taken from Expert Reviews in Molecular Medicine © 2000 Cambridge University Press).

In the adult pancreas, PDX-1 immunoreactivity is restricted to pancreatic β -cells and somatostatin-expressing δ -cells of the islets of Langerhans^{67, 68}. During rat central nervous system development, PDX-1 was also found expressed in neural forebrain cells. This finding provided the evidence for an extrapancreatic developmental function of PDX-1⁶⁹.

At the protein level, PDX-1 consists of 283 amino acids and has an estimated molecular weight of 31 kD⁶¹. The transcription factor contains an N-terminal transactivation domain, a *homeodomain* in the middle and a COOH-terminal domain of uncertain function (Fig.1.6). The proline-enriched N-terminal transactivational domain possesses three evolutionarily conserved subunits termed subdomain A (amino acid 13-22), subdomain B (amino acids 32-38), and subdomain C (amino acids 60-73)^{70 71}. The stimulatory properties of rat PDX-1 are very similar to the *Xenopus laevis* homeoprotein, XIHbox8^{72 71}, of which the N-terminal regions share 50% identity⁷³.

In PDX-1, an antenna-type pentapeptide (FPWMK) is present in the proline-rich region, close to the *homeodomain*. This pentapeptide motif mediates the PDX-1 heterodimerization with the DNA-binding protein termed PBX1 and interacts with a regulatory element of the somatostatin (SST) promoter^{74 75}.

In the central domain of the protein lies the antenapedia-like *homeodomain*, which is crucial for PDX-1 DNA-binding activity and protein-protein interactions. This *homeodomain* comprises three helices (amino acids 146-206) with proline-rich sequences flanked on both sides. In the third helix a seven amino acid-motif (RRMKWKK) conserved in human, mice and rats defines the nuclear localization signal (NLS)^{76 77}. A recent study by Kawamori *et al*⁷⁸ also reported a nuclear export signal (NES) at position 82-94 of the mouse PDX-1 protein.

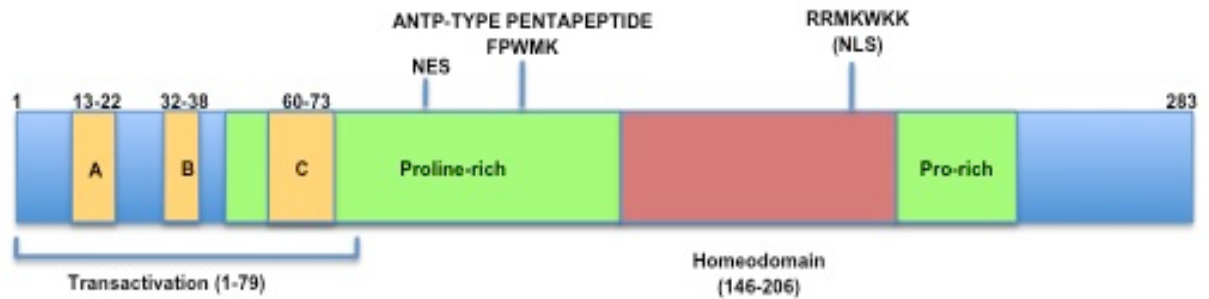


Fig. 1.6. Structure of PDX-1. The *homeodomain* of PDX-1 is flanked by two proline-rich regions. The NH₂-terminal activation domain comprises three sub-domains. A nuclear localisation signal (NLS) is present in helix three of the *homeodomain* and the pentapeptide motif that interacts with PBX1 located close to the *homeodomain*, whereas a nuclear export signal (NES) was proposed⁷⁸ at position 84-92 of mouse PDX-1 (Modified from McKinnon and Docherty, 2001).

1.3.2 PDX-1 regulates pancreatic morphogenesis

PDX-1 is of great importance in the development of the pancreas. The pancreas consists of three major types of cells: the digestive enzyme-producing exocrine acinar tissue; the endocrine cells which make up the islets of Langerhans and produce the nutrient homeostasis hormones, i.e. insulin, glucagon and somatostatin etc.; and the elaborately branched ductal tree⁷⁹.

Pancreatic development starts early during embryogenesis⁷⁹. In general, the pancreas arises from the endoderm as a dorsal and a ventral bud, which will finally grow to form the adult pancreas. For the mouse pancreas, there are three transitions during the development. On embryonic day 8 (e8), the dorsal and ventral buds are formed by the endodermal epithelium of the foregut. This process will last until e9.5. The buds will then grow into branching ducts and undifferentiated epithelium on e12.5, which is the first developmental transition. After this stage, the second

transition occurs. Hence, single endocrine cells are found among the undifferentiated epithelium and the clusters developed from the buds start to differentiate into endocrine and exocrine cells by e14. On e15 the dorsal and ventral clusters start to rotate and fuse, eventually forming a pancreas. The process takes about four days, up to e19. In the newly formed pancreas, islets of Langerhans are formed from the organized endocrine cells, which is the beginning of the third transition. During the third transition, the maturation of endocrine cells and the full nutrient responsiveness will be completed and this process will continue for two to three weeks after birth.

Different developmental stages involve the effects of a combination of specific transcription factors and signaling molecules. Considerable progress has been made in the understanding of the embryogenesis of pancreas, notably by identifying key genes controlling islet development. Most of these genes encode transcription factors such as Isl-1, PDX-1, BETA2/NeuroD1 Nkx2.2, Pax4 and Pax6^{80 81, 82}.

PDX-1 is the major regulator for the determination of the endocrine and exocrine cell population. It is found in murine foregut endoderm on e8.5 and is detected in both ventral and dorsal buds on e9.5^{47, 67}. After the first transition, PDX-1 continues to be expressed in the developing ductal tree during the second development transition. As the differentiation of the exocrine and endocrine cells occurs (e14-e15), PDX-1 expression starts to shift to the endocrine cells. On e13.5, PDX-1 still localizes with the exocrine cell-specific enzyme amylase. However, the expression is decreased dramatically by e16.5 and disappears in the adult murine exocrine pancreas. PDX-1 expression is predominantly restricted to the mature β -cells thereafter. In adult pancreas, besides β -cell expression, PDX-1 is also detected in somatostatin-producing δ -cells where it primarily regulates the expression of insulin and somatostatin expression⁷⁹. PDX-1 is also reported to be expressed in a very few glucagon-producing α -cells^{61, 62, 67, 83}.

1.3.3 Animal models of PDX-1 dysfunction

The role of PDX-1 in pancreas development and islet cell differentiation has been demonstrated using animal models.

Mice with targeted disruption of the *Pdx1* gene (*Pdx1*^{-/-}) were born with pancreas agenesis and abnormalities within specific regions of the duodenum^{45, 47}, homozygous null *Pdx1* mice die within days after birth⁴⁵. Interestingly, a few insulin-positive cells are detected during early pancreas development in mice without PDX-1 expression indicating the possible existence of PDX-1-negative insulin-expressing cells^{84, 85}.

PDX-1 expression also increases during pancreatic regeneration. Thus, in islets isolated 3-7 days after pancreatectomy the level of PDX-1 protein was much higher than control islets, indicating a possible up regulation of PDX-1 in the dividing ductal cells during pancreas regeneration⁸⁶. These findings highlight the potential role of PDX-1 as therapeutic target in pancreatic disorders.

1.3.4 PDX-1 and the regulation of gene expression

PDX-1 regulates a number of islet genes including insulin⁶¹, glucose transporter 2 (GLUT-2)⁸⁷, glucokinase⁸⁸ and islet amyloid polypeptide⁸⁹⁻⁹¹. Each of these is involved in maintaining β -cell identity and function. β -cell-specific inactivation of the mouse PDX-1 gene using the Cre-*lox* system results in reduced β -cell mass, loss of the β -cell phenotype, reduced insulin, and GLUT2 expression and eventually diabetes⁹².

The *homeodomain* of PDX-1 is very important for PDX-1 protein-protein interaction, by which PDX-1 interacts with other transcription factors to activate gene transcription in a tissue specific manner⁴⁶.

Serup *et al*⁸⁹ induced endogenous insulin mRNA expression by transfecting exogenous PDX-1 cDNA into transformed pancreatic α cells that contain islet-enriched factors. This measure failed to do so in fibroblasts which lack the PDX-1 interacting factors.

In exocrine acinar cell lines, the endogenous PDX-1 interacts with two three-amino acid loop extension (TALE) class *homeobox* proteins PBX1b and MRG1⁹³. This trimeric complex then activates the elastase promoter⁹³.

1.3.5 PDX-1 and insulin gene expression

Tight regulation of insulin synthesis and secretion is at the centre of glucose homeostasis. However, the regulation of this process involves a cooperative network comprising multiple factors including nutrients, hormonal stimuli, several transcription factors and even electrical activity (Ca^{2+} channels, ATP-sensitive potassium (K-ATP) channels) and other intracellular signaling pathways^{94, 95}.

In mice and rats there are two nonallelic insulin genes, insulin I and II. In human, and most of other animals, only carry one copy of the insulin gene exists. The human insulin gene is located on chromosome 11p 15.5 and consists of three exons and two introns⁹⁶.

Insulin gene expression is regulated substantially at the transcriptional level, i.e. through changes in insulin gene promoter activity. The insulin gene and its promoter sequences have been well conserved during evolution. The activation of insulin gene transcription depends on the binding and interaction of multiple nuclear factors⁹⁵ i.e. transcription factors at the enhancer region, which is located between -340 and -91 of the insulin gene⁹⁷. The human insulin promoter consists of five A elements (A1 to A5) and two E boxes (E1, E2) and except for the A2 element (GGAAAT), all the A boxes have the same core sequence TAAT which is

the motif for *homeodomain* protein recognition ⁹⁸.

By mutational analysis the rat insulin I promoter A3/A4 site, which corresponds to the A3 region in the human promoter, was found to be responsible for most of the insulin promoter activity ^{97, 99, 100}. The rat insulin I gene also contains two regulatory regions lying at: -104 to -112 and -233 to -241 ⁹⁹. They are now termed E1 and E2 boxes ⁹⁸ and bind to the β -cell specific heterodimeric protein complex IEF1. This consists of two basic helix-loop-helix proteins, E47 and BETA2/Neuro D1.

PDX-1 binds to the A3/A4 region of rat insulin I promoter and interacts with E47 and NeuroD1 thus binds to the adjacent E2 site to activate insulin gene transcription ¹⁰¹⁻¹⁰³. A multi-protein complex including E47, NeuroD1 and the high-mobility group protein I (HMG1Y) is formed by protein-protein interactions during which PDX-1 *homeodomain* acts as a protein-protein interaction domain ^{102, 103}. HMG1Y is an architectural protein that affects the conformation of DNA and is important in the assembly of nuclear pore complexes ¹⁰³. This complex acts as an activation group on the E2A3/4 mini-enhancer and activate transcription probably through further interaction with other potential coactivators like the p300 coactivator, which interacts directly with the N-terminal PDX-1 and activates the rat I insulin gene transcription synergistically ¹⁰⁴.

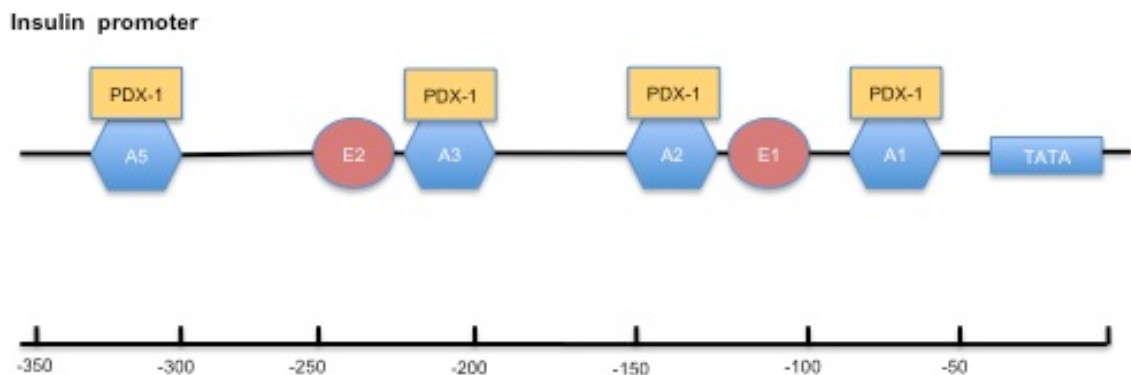


Fig. 1.7. Scheme of insulin promoter and PDX-1 binding sites (Modified from McKinnon and Docherty, 2001).

However important, the regulation of PDX-1 on insulin gene transcription is not the

only pathway regulating this process. In the rat insulin I gene, other *homeodomain* proteins can also activate the A/E mini-enhancer by binding to the A3/A4 boxes and co-operate with E47, such as Isl-I, Cdx-3, Lmx1.1, HNF1 α , and HMGY^{103, 105-107}. It has been reported¹⁰³ that the overall function of PDX-1 in forming the activation complex on the mini-enhancer of insulin promoter can be substituted by other *homeodomain* factors in the β -cells¹⁰⁸. As mentioned above, in β -cells with Cre-mediated deletion of PDX-1, insulin expression is still approved⁹², likewise, Kajimoto *et al* provided another example that in β -cells with reduced PDX-1 via anti-sense RNA, the insulin expression level was sustained¹⁰⁹, these examples may reflect insulin transcription being regulated by other *homeodomain* factors substituting for PDX-1. Conversely, overexpression of PDX-1 was reported to repress the insulin promoter activity^{64, 110}. This was possibly due to the fact that the overexpressed PDX-1 binds to other factors and inhibits the availability of other potential factors to form the transcription complex^{64, 110}.

1.3.6 PDX-1 and the regulation of other β -cell specific gene expression

1.3.6.1 PDX-1 and somatostatin gene expression

In mature pancreas, PDX-1 is expressed not only in β -cells but also in δ -cells where it regulates somatostatin gene transcription⁶². The somatostatin promoter region contains four *homeodomain* binding elements with the common TAAT motif which are termed SMS-UE-B, SMS-TAAT1, SMS-TAAT2, SMS-TAAT3, which function as the mini-enhancer⁶⁰.

PDX-1 binds to the SMS-UE-B (TSE-I) element, and cooperates with a heterodimeric protein complex containing two *homeodomain* factors, the *Drosophila melanogaster* extra-denticle PBX1 (the same *homeodomain* protein interacting with PDX-1 in acinar cells) and Prep1. The dimeric complex acts by

binding to the distal A-element of the somatostatin gene, upstream to the enhancer element ⁷⁵. The transcription factor complex consisting of PDX-1, PBX1 and Prep1 interacts and is stabilized through the interaction between PDX-1 and PBX1 involving the FPWMK motif that locates upstream of the PDX-1 *homeodomain* as discussed before in this Introduction ⁷⁴.

1.3.6.2 Glucose-transporter 2 (GLUT-2)

The membrane protein glucose transporter 2 (GLUT-2) works as a glucose diffusion facilitator through the plasma membranes in mammalian liver, small intestine, kidney, some limited areas of the brain and the pancreatic β -cells. In different tissues GLUT-2 gene is regulated under different factors ¹¹¹. The GLUT-2 expression has been shown to decrease in the β -cells in various diabetic animal models. Thus islet-derived insulin-secreting cells were shown to lose glucose responsiveness due to decreased levels of PDX-1 expression which further down regulates the intracellular level of GLUT-2 ¹¹². A repeated TAAT motif sequence was identified and was found conserved in both human and murine GLUT-2 promoters ⁸⁷. It has been proposed that PDX-1 binds this motif and mediate the transactivation of GLUT-2 gene transcription ⁸⁷. It has also been shown by Bonny *et al* that GLUT-2 gene transcription is also regulated by another islet-specific DNA-binding protein, GTIIa ¹¹².

1.3.6.3 Glucokinase

The glycolytic enzyme glucokinase (GK) is mostly expressed in pancreatic β -cells and hepatocytes ¹¹³ and plays a major role in glucose-stimulated insulin secretion. GK is the glucose sensor and controls the entry of glucose into the glycolytic pathway by regulating the transfer of phosphate from ATP to glucose to generate glucose-6-phosphate. In the liver, glucokinase controls the process of glucose storage as glycogen. Defects in the GK gene were shown to be responsible for one of the maturity-onset diabetes of the young (MODY2) ¹¹⁴⁻¹¹⁶. The human GK gene promoter region has been identified to contain multiple *cis*-acting elements,

including two crucial motifs: the palindrome structure hPal-1 and the insulin gene *cis* motif A element-like hUPE3⁸⁸. PDX-1 transactivates the GK gene transcription by binding the hUPE3 motif¹¹⁷. However, later evidence shows that by applying immunoblot in *pdx1* (+/-) mice, the expression of PDX-1 and GLUT2 was found reduced 68% and 55% compared to wild-type mice whereas GK expression was relatively unchanged.¹¹⁸

1.3.6.4 Islet amyloid polypeptide (IAPP)

IAPP is firstly isolated from the amyloid deposits from type 2 diabetic pancreas, as well as from the extracellular component of human insulinomas¹¹⁹. It is also known as amylin, a 37-amino acid peptide expressing in pancreatic β -cells and islet δ -cells. In β -cells it works by co-storing with insulin in the secretory granules and co-releasing with insulin in response to various secretagogues. IAPP is proposed to play a critical role in regulation of plasma glucose level in mammals due to its inhibitory effect on insulin secretion in isolated islets and its ability to neutralize the insulin action in peripheral tissues^{120, 121}. There are three A box-like sequences in the regulatory area of IAPP gene promoter^{89 90, 91} and it has been shown by Watada *et al* that all three A box-like elements could bind PDX-1 however only the AT1 or AT3 disruption shows an obvious reduction in IAPP gene promoter activity¹²². Additionally, overexpression of the two K-ATP channel sub-units SUR1 and Kir6.2 in the human β -cell line (NES2Y), which lacks PDX-1, and operational K-ATP channel shows that glucose induction of IAPP promoter activity requires PDX-1 and calcium influx¹²³.

1.3.7 Regulation of the *Pdx1* (*IPF1*) gene

The 6 kb human *IPF1* gene consisting of two exons is mapped to chromosome 13q12.1^{124, 125} while the mouse *Pdx1* gene is on chromosome 5¹²⁶ and the rat counterpart on chromosome 12¹²⁷. There are three consensus E-box motifs on the

Pdx1 promoter -190 to -95 region. It has been shown that deletion of the E2 box at -177 did not affect the *Pdx1* promoter activity. However, removal of the proximal E-box sequence at -104 completely abolishes PDX-1 expression in HIT-15 cells¹²⁴. It is also reported that this site binds to the ubiquitous bHLH/leucine zipper nuclear factor USF-1¹²⁴. Another E box at around -6 kb is identical to the insulin promoter E-box. Like the one in the insulin promoter, this E-box also binds the β -cell E-box transactivator-2 (BETA2/NeuroD1)/E47 heterodimer which in turn regulates PDX-1 expression in β -cells⁸².

Moreover, the endodermal transcription factor hepatocyte nuclear factor 3 β (HNF3 β ; alternative name: Forkhead box A2 (FoxA2)) binds to several sites on the *Pdx1* promoter^{82, 128-130}. As members of the HNF family are structurally related to histone H5 and can alter the nucleosomal structure, unfold the chromatin to provide access to promoter sequences for other transcription factors. Thus, this winged-helix family forkhead transcription factor may prime *Pdx1* gene expression by cooperating with other factors like BETA2/E47. Interestingly, PDX-1 is also able to bind at an HNF3 β sites with a TAAT sequence, which leads to the autoregulatory feedback mechanism of *Pdx1* expression¹²⁸. HNF3 β activity could be blocked by glucocorticoids, which in turn represses *Pdx1* expression. This could explain that finding that, *in vitro*, islet cell maturation is inhibited by glucocorticoids^{129, 131-133}.

It has also been demonstrated that there is an interaction between HNF1 α , HNF3 β and PDX-1 at a highly conserved region in both mice and human which lies at -2.5 kb upstream of the 5' flanking region of the *Pdx1/IPF1* gene^{128, 129}.

1.3.8 Nutrient and hormonal regulation of PDX-1

While the role of PDX-1 in pancreas development has been extensively studied, the post-translational regulation of PDX-1 is not as well understood. Glucose and a

number of other nutrients and some hormones have been shown to regulate PDX-1 at several levels, including protein content as well as nuclear localization.

1.3.8.1 Regulation of PDX-1 by phosphorylation

In a study to examine the role of IUF-1 (PDX-1) in glucose-stimulated insulin gene expression, Macfarlane *et al*⁶³ reported that, in isolated rat islets incubated at low glucose (3 mM) decreased the PDX-1 activity to ‘undetectable level’ in 6 h whereas at high glucose (20 mM) PDX-1 activity remained stable for 24 h. Moreover, the decrease in activity caused by low glucose was recoverable after 15 min at elevated glucose concentration. Interestingly, when islets were cultured for 4 h at low glucose concentrations together with phosphatase inhibitors, the decline of PDX-1 activity was prevented. These findings pointed out the importance of glucose in regulating PDX-1 activity and suggested that the regulation by glucose may be phosphorylation-dependent⁶³. Potential kinases triggering this event were investigated. Moreover, neither cyclic AMP-dependent protein kinase A nor protein kinase C could be involved.

Further study indicated that the glucose-stimulated PDX-1 DNA-binding activity (using oligonucleotides corresponding to regulatory region of the human insulin gene¹³⁴) and PDX-1-activated gene transcription were inhibited by a specific inhibitor of stress-activated protein kinase 2 (SAPK2, or, p38 mitogen-activated protein kinase, reactivating kinase, CSBP, and Mxi2), SB203580¹³⁵, which also inhibited the glucose triggered activation of MAPKAP-K2, an immediate downstream target of SAPK2. The protein kinase p38/SAPK2 is a member of the MAP kinase-related kinases stimulated by adverse stimuli such as heat, osmotic shock, ultraviolet light, DNA-damaging reagents and by proinflammatory cytokines made under stress condition¹³⁶. PDX-1 DNA-binding activity and PDX-1-dependent gene transcription are also stimulated by cellular stresses i.e. sodium arsenite and heat shock. Overexpression of p38/SAPK2 can also mimicked the glucose effect of stimulating PDX-1 DNA-binding activity and moreover, these

stimulations were also inhibited by SB 203580, which indicated that the p38/SAPK2 pathway is involved in regulating PDX-1 DNA-binding activity. Additionally, a role for phosphatidylinositol 3-kinase (PI 3-kinase) in glucose-stimulated PDX-1 DNA-binding activity is also proposed as the glucose stimulation was found inhibited by PI 3-kinase inhibitor wortmannin/LY294002 or the overexpression of dominant-negative form of the PI 3-kinase. Finally, the inhibition of PI3K did not affect the activation of MAPKAP-K2, PDX-1 DNA-binding and PDX-1-dependent gene transcription induced by arsenite in MIN6 cells, based on which a novel pathway that glucose induces the activation of SAPK2 was proposed¹³⁵.

However, later studies by Rafiq *et al*¹³⁷ later, which examined preproinsulin promoter (using pINS (260-60).*Luc* plasmid¹³⁸) activity in MIN6 β -cells treated with SB 203580 or overexpression of the upstream kinase MKK6 (MAP kinase kinase-6) or wild-type p38/SAPK2, reported failed to provide any evidence of a role for p38/SAPK2 in the context of insulin gene expression. Moreover, no sign of an effect of elevated glucose concentration on subsequently extracted p38/SAPK2 activity was observed. On the other hand, this study conferred a role of PI 3-kinase in the activatory effect of glucose in the promoter activity. Thus, overexpression of a constitutively-active form of PI3K (p110.CAAX), mimicked the activatory effect by glucose. Furthermore, the PI3K inhibitor LY 294002 or overexpression of a dominant-negative form of the p85 subunit lacking the p110-binding domain, both blocked activation of the insulin promoter by glucose. Hence, the conclusion drawn by the later study was that PI3K is 'necessary and sufficient' for the glucose-activated PPI gene transcription. Interestingly, work by da Silva Xavier *et al*¹³⁹ suggested that PI-3K is involved in a positive mechanism, through which glucose acts to stimulate insulin release and rebinding to its receptor in β -cells. Similar conclusions were also revealed by Leibiger, and colleagues¹⁴⁰. However, the downstream signaling pathway does not seem to involve p38/SAPK2 and remains unknown.

It was proposed that glucose stimulates the phosphorylation of PDX-1^{89, 141}, although the phosphorylation sites have yet to be mapped and the kinases (a putative PDX-1 kinase) involved identified.

In 2006, Boucher *et al*¹⁴² demonstrated that a minor portion of PDX-1 may be phosphorylated on serine 61 and/ or serine 66 in β -cells, as detected with a phospho-specific antibody. Oxidative stress, which is associated with the diabetic state, (i) increases IPF1/PDX-1 Ser61 and/or Ser66 phosphorylation and (ii) increases the degradation rate and decreases the half-life of IPF-1/PDX-1 protein. In addition, they provide evidence that GSK3 activity participates in oxidative stress-induced effects on β -cells. GSK3 has been found in insulin target tissues of diabetic mice and human with an increased activity^{143, 144} and inhibiting GSK3 using specific inhibitors or lithium chloride has been shown to improve the glucose metabolism in insulin resistant Zucker rats^{145, 146}. In the Boucher *et al*¹⁴² study, the authors also found that oxidative stress increased PDX-1 phosphorylation on either site, and increased the degradation of the protein, such that the half-life of the protein was decreased, and the phosphorylation is also modulated by GSK3-dependent mechanisms¹⁴². It has been reported that active canonical Wnt signaling decreases GSK3 activity¹⁴⁷ and β -cells are sensitive to the oxidative stress which increases with age. Thus a new mechanism to explain the declined levels of PDX-1 and resultant β -cell dysfunction during diabetes progression was proposed whereby canonical Wnt signaling counteracts age-related increase in oxidative stress by inhibiting GSK3 activity to rescue PDX-1 from protein degradation and in turn maintain normal β -cell function¹⁴².

In another later study by Boucher *et al*¹⁴⁸, it was reported that the *homeodomain-interacting protein kinase* (Hipk) 2, which was confirmed as expressed in both embryonic and adult pancreas, directly phosphorylates PDX-1 in the C-terminal domain. Moreover, active Hipk2 also increased PDX-1 transcriptional activity in these studies. Although the actual phosphorylation site needs to be identified, this mechanism was proposed to open up a new way through which PDX-1

transcriptional activity may be regulated to modulate pancreas development and/or β -cell function.

Kinase/pathway	Phosphorylation site(s)	Phosphorylation or kinase involved regulation on PDX-1	Ref
p38/SAPK2 and PI3K	UNKNOWN	P38/SAPK2 positively regulate PDX-1 DNA-binding activity and PI3K is involved in glucose-stimulated PDX-1 DNA-binding activity	Macfarlane et al, 1997
PI3K	UNKNOWN	PI3K is necessary and sufficient for the glucose-activated PPI gene transcription, and is involved in PDX-1 nuclear translocation	Rafiq et al, 2000
GSK3	S61, S66	oxidative stress increases PDX-1 phosphorylation on either site and further increases the degradation of the protein	Boucher et al, 2006
HIPK	C-terminus	Phosphorylation increases PDX-1 transcription activity	Boucher et al, 2008

Table 1.1. Review of studies on PDX-1 phosphorylation and references

1.3.8.2 PDX-1 subcellular localization: effects of glucose, insulin and oxidative stress

There appears to be a growing number of transcription factors that are controlled by a regulated nuclear translocation mechanism to control the expression of specific genes including signal transducers and activators of transcription, nuclear factors of IgkB cells, nuclear factors of activated T-cells (NFATs), p53, and steroid receptors¹⁴⁹. In the PDX-1 Structure section (Page 19) I discussed the fact that there is a nuclear localization signal lying on the third helix of PDX-1 *homeodomain*^{76, 77}.

It has indeed been demonstrated that glucose affects the intracellular distribution of PDX-1 in β -cells by different research groups^{138, 141}. Thus, it was first reported by Rafiq *et al*¹³⁸, using laser-scanning confocal microscopy, that *Pdx1.c-myc*, translocated from the nuclear periphery to nucleoplasm rapidly at elevated glucose concentration (3 mM to 30 mM). It was proposed that in cells exposed to 3 mM

glucose the endogenous PDX-1 might be tied to immobile anchors involving the microfilaments/microtubules beneath the nuclear membrane. However, it is noted that the PDX-1 fused to enhanced fluorescent protein (EGFP; 27 kDa) remained largely in the nucleoplasm at both 3 and 30 mM glucose and no sign of translocation was observed. This observation may indicate that the EGFP protein¹⁵⁰ blocked the interaction between PDX-1 and the binding site(s) located on or around the nuclear membrane. Nevertheless, in this study¹³⁸, Rafiq *et al* speculated that the localization to the nuclear periphery might be a general phenomenon for glucose-sensitive transcription factors such as, for example, the transcription factor Sp1¹⁵¹ which was found to interact with the nuclear pore protein p62 and is involved in the regulation by glucose of the fatty acid synthase promoter¹⁵². Another example is the β -cell-specific c-Jun interacting protein (JIP) homologue IB1¹⁵³ which regulates the glucose-sensitive GLUT-2 gene in β -cells. This transcription factor was also reported to be localized to the nuclear periphery¹⁵³.

As discussed in the previous “phosphorylation of PDX-1” Section (page 33), phosphorylation seems likely to be involved in regulation of PDX-1 by glucose. Rafiq *et al*^{137, 138} also proposed that a shift of PDX-1 from nuclear periphery to nucleoplasm at elevated [glucose] might be controlled by a phosphorylation event catalyzed by unknown kinase(s). However achieved, the authors speculated that the translocation of PDX-1 is very likely to affect the trans-activational capacity of PDX-1 in native β -cells exposed to high extracellular glucose concentrations.

In another study, Elrick and Docherty¹⁵⁴ also observed that PDX-1 shuttles between the nuclear periphery and the nucleoplasm in a phosphorylation-dependent manner in response to changes in extracellular glucose and insulin concentrations. Moreover, in this study, it was claimed that the translocation of PDX-1 from the nuclear periphery to the nucleoplasm stimulated by extracellular glucose or insulin is inhibited by the PI3-K inhibitor wortmannin and the p38/SAPK2 inhibitor SB203580 hence suggesting that the PI3K and p38/SAPK2 pathway is involved¹⁵⁴.

By contrast, Rafiq *et al*¹³⁷ reported that the movement of PDX-1 from the nuclear periphery to the nucleoplasm in response to glucose involved PI 3-kinase but not p38/SAPK2¹³⁷.

However, in both of the above studies the regulatory role of phosphorylation in the glucose stimulated PDX-1 translocation was agreed and also the PI3K was involved in this process. This left to be addressed which protein kinase(s) may be involved.

1.3.8.3 Role(s) of the N-terminal domain of PDX-1

The principal physiological regulator of insulin transcription in islet β cells is glucose¹⁵⁵. As discussed above, glucose regulates PDX-1 DNA-binding activity, phosphorylation and nuclear translocation. Moreover, it has been reported that glucose also directly stimulates the transactivation capacity of the PDX-1 N-terminal domain¹⁵⁵⁻¹⁵⁷.

Thus, Petersen *et al*¹⁵⁵ showed that glucose induces the transactivation capacity of PDX-1 by both affecting PDX-1 DNA-binding and also regulating the transactivation domain function. Another study by Shushan *et al*¹⁵⁶ explored the functional regions of the human PDX-1 protein by fusing it to the DNA-binding domain of the transcription factor Gal4. The N-terminal domain of the hPDX-1 turned out to be important for transactivation in transfected β TC6 and HeLa cells, as well as in transfected rat islets. Moreover, two regions, which are critical for PDX-1 transactivation properties, were also mapped within the N-terminus. Additionally, study by Peshavaria *et al*¹⁵⁷ also confirmed the important role of PDX-1 N-terminus in its transactivation activity to mediate glucose-inducible insulin gene transcription in islet β -cells.

1.3.8.4 Role(s) of the C-terminal domain of PDX-1

In contrast to the relatively well-studied functions of the N-terminus and the *homeodomain* of PDX-1, the role of the conserved C-terminus is less well defined. Mutations in the IPF-1 gene in human which affect the C-terminus of PDX-1 are associated with the development of type 2 diabetes¹⁵⁸⁻¹⁶⁰, and other findings indicate that the C-terminal domain may serve as both a repressor and an activator of PDX-1 function. Thus, the C-terminus may inhibit the synergy between PDX-1 and a second transcription factor, E47, on the preproinsulin promoter, whilst also containing a cryptic transactivation domain involved in regulating the somatostatin gene TAAT1 enhancer^{70, 101}. Stoffers and colleagues¹⁶⁰ have also identified a PDX-1 C-terminus interacting protein, termed PDX-1 C-terminal interacting factor (PCIF1) and suggested that PCIF1 may modulate PDX-1 function during pancreas development and/or differentiation.

Importantly, a recent study reported that HIPK2 is co-localized with PDX-1 both the developing and adult pancreas. Moreover, HIPK2 was reportedly able to modulate positively the transcriptional activity of PDX-1, possibly by phosphorylation of the C-terminal domain¹⁶¹.

1.3.9 Other mechanisms involved in regulating PDX-1

Besides glucose, several hormones such as the insulintropic hormone glucagon-like peptide-1 (GLP-1) and TGF β have been reported to regulate PDX-1 at the level of DNA-binding activity, transactivation potential or expression of the *Pdx1/IPF1* gene¹⁶²⁻¹⁶⁶.

GLP-1 was found to be able to stimulate insulin secretion and effectively lower the blood glucose levels¹⁶⁶. This hormone normally works through a G-protein coupled receptor linked to protein kinase A (PKA) and was found to stimulate Gal4:PDX-1 activity. Notably, the PDX-1 activation domain linked to the Gal4 DNA-binding

domain does not contain any phosphorylation site for PKA ¹⁶⁷, which suggests the regulatory effect of GLP-1 on glucose-dependent activity of PDX-1. Moreover, GLP-1 also increases the β -cell mass in diabetic animal models ¹⁶⁶, in a process which has been shown to be mediated by the transcription of PDX-1 ¹⁶⁶.

Finally, it has also been reported that increased fatty acid levels can down-regulate PDX-1 expression ¹⁶⁸. Isolated rat pancreatic islets exposed to palmitic acid expressed nearly 70% decrease in PDX-1 mRNA and protein expression. This treatment also resulted in a decrease of the PDX-1 DNA-binding activity to the promoter region of insulin to 65% and GLUT-2 to 40%, respectively ¹⁶⁸.

1.3.10 PDX-1 and diabetes

1.3.10.1 IPF-1 mutations in diabetes

Several mutations in the *Pdx1/IPF1* gene have been reported. These include frameshift and missense mutations ^{158, 169, 170} which lead to decreased binding activity of PDX-1 to the insulin promoter accompanied by reduced transcription of insulin in response to hyperglycemia *in vitro*.

PDX-1 was reported firstly to be associated with Type 2 diabetes in studies ¹⁶⁹ on a child patient born with pancreatic agenesis ¹⁶⁹ who was also found to be homozygous for an inactivating cytosine deletion in the protein coding sequence of *IPF1* (*pro63fsdelc*). This frameshift mutation generated a truncated protein that lacked a functional *homeodomain* necessary for DNA binding and nuclear localization. Members of the patient's family who carried the heterozygous mutation of the same allele exhibited an autosomal dominant early onset form of the type 2 diabetes (MODY) ^{171, 172}.

Maturity-onset diabetes of the young (MODY) is characterized by autosomal dominant inheritance and defects in insulin secretion, resulting in an early onset of non-insulin dependent diabetes mellitus, usually before 25 years of age and frequently in childhood or adolescence ^{171, 172}. There are six genes which are

associated with MODY: hepatocyte nuclear factor 4 α (MODY1), GK (MODY2), HNF1 α (MODY3), *Pdx1/IPF1* (MODY4), HNF1 β (MODY5), and BETA2 (MODY6). All these genes are expressed in pancreatic β -cells, and mutation of any of these genes leads to β -cell dysfunction and diabetes mellitus.

Macfarlane *et al*¹⁷³ identified three novel missense mutations (C18R, D76N and R197H) from patients with type 2 diabetes. These mutations were further demonstrated in a human β -cell line NES2Y to be able to decrease PDX-1 binding activity to the insulin promoter and in turn a declined insulin transcription in response to hyperglycemia.

Hani *et al*¹⁵⁸ identified three novel *IPF1* mutations; including two substitutions (Q59L and D76N) and an in-frame proline insertion (InsCCG243) in 192 French, non-MODY type 2 diabetic families. Functional transactivation assays were applied with these *IPF1* mutant isoforms in a pancreatic β -cell line transfected with a transcriptional reporter. Transfection with *IPF1* expression plasmids resulted in a significant inhibition of basal insulin promoter activity (stronger than with the InsCCG243 mutant)¹⁵⁸.

In a study aiming to examine the putative role of mutations in the *IPF1* gene in early-onset diabetes, Weng *et al*¹⁵⁹ reported two novel *IPF1* variants (G212R and P239Q) and the previously found D76N, in 115 Scandinavian families with at least two members with onset of diabetes younger than 40 years. They concluded that although mutations in the *IPF1* gene are rare in early- (3.5 %) and late- (2.7 %) onset Type 2 diabetes, they are functionally important and also occur in families with other MODY mutations¹⁵⁹.

1.3.10.2 PDX-1 and diabetes treatment

Transplantation of insulin-producing cells is still a potentially powerful tool for the treatment of type 1 and certain types of type 2 diabetes; however, donor supply

remains an obvious limitation. Meanwhile, progress based on regenerative medicine, aimed at converting adult cells into other cell types for tissue repair and regeneration through the molecular mechanisms of pancreatic islet engineering, could help to generate sufficient β -cells from embryonic stem cells and overcome this problem. Ferber *et al*¹⁷⁴ have reported that ectopic expression of PDX-1 in liver cells endowed the hepatic tissue with pancreatic β -cell characteristics and induced secretion of insulin from liver cells. This report indicated the capacity of PDX-1 to reprogram extrapancreatic tissue towards a β -cell phenotype. It thus became possible to study the self-neogenesis of islets in organs such as the liver, spleen, and pancreas through the introduction of PDX-1 and/or other related genes.

Very recently an important study by Zhou *et al*¹⁷⁵ again renewed the progress of treating diabetes via β -cell regeneration. Aiming to identify a way of turning one cell type into another in a controlled manner (“transdifferentiation”), by re-expressing key developmental regulators *in vivo*, Zhou *et al*¹⁷⁵ identified for the first time a specific combination of three transcription factors (Ngn3, PDX-1 and MafA) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β -cells¹⁷⁵. Remarkably, not only were the newly-induced β -cells indistinguishable from endogenous islet β -cells in size, shape and ultrastructure, they also expressed genes essential for β -cell function and could improve hyperglycaemia by remodeling local vasculature and secreting insulin. These exciting findings provide an example of cellular reprogramming using defined factors in an adult organ and may open up a new method for cell reprogramming without reversion to a pluripotent stem cell state.

1.4 Nutrient-responsive kinases

1.4.1 The metabolic ‘master switch’: AMPK

Imbalanced energy intake and energy expenditure underline various metabolic diseases such as obesity and type 2 diabetes. In general, systems that detect energy excess will shunt energy into storage and dissipate energy by increasing energy expenditure and consuming ATP. On the contrary, systems sensing energy deficits will increase fuel utilization in order to increase ATP production, decreasing carbohydrate oxidation in an effort to preserve glycogen stores.

Energy and nutrient sensors ultimately decided how cells respond to fuel substrates such as glucose, and further activate appropriate pathways^{176, 177}. Among the nutrient-responsive kinases, an important example is the 5' AMP-activated protein kinase, AMPK, a metabolic master switch¹⁷⁸ regulating a number of intracellular systems including glucose uptake, β -oxidation of fatty acids and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria¹⁷⁹⁻¹⁸². AMPK is conserved from yeast to human and plays an important role in cellular energy homeostasis¹⁷⁸. This kinase senses changes in the ratio (AMP:ATP) by binding both species in a competitive manner¹⁸³. Hence, the kinase is activated by increases in the concentration of AMP, which leads to the phosphorylation and differential regulation of a series of downstream targets that control anabolic and catabolic pathways¹⁸³. AMPK is believed to be activated by phosphorylation on Thr-172 by at least two upstream signaling pathways¹⁸⁴. In one case an increase of cellular AMP: ATP ratio caused by metabolic stress leads to the phosphorylation of AMPK by LKB1; alternatively phosphorylation at Thr-172 may be triggered by an increase in cytosolic free Ca^{2+} mediated by CaMKK β ¹⁸⁴. An adenosine analogue 5-amino-4-imidazolecarboxamide riboside (AICAR) was shown to be taken into cells via adenosine transporters¹⁸⁵ and further converted to 5-amino-4-

imidazolecarboxamide ribotide (ZMP)¹⁸⁶ which activates AMPK¹⁸⁷⁻¹⁹⁰.

AMPK is expressed in several tissues including liver, brain, skeletal muscle and pancreatic β -cells¹⁷⁸. AMPK is involved in regulating a number of downstream processes including the stimulation of hepatic and skeletal muscle fatty acid oxidation via phosphorylation and inactivation of acetyl-CoA carboxylase^{191, 192}, inhibition of cholesterol synthesis by phosphorylating and inactivating 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)¹⁹³, and modulation of insulin secretion by pancreatic β -cells¹⁷⁸.

Salt *et al*¹⁹⁴ firstly showed that stimulation of AMPK with AICAR stimulated the basal insulin secretion while inhibited the glucose-stimulated insulin secretion. This was later also found in rodent β -cell lines as well as in rodent and human islets¹⁹⁵⁻¹⁹⁷ using either the activator 5'-aminoimidazole carboxamide-4-ribonucleotide (AICAR) or an adenovirus expressing a constitutively active form of AMPK. It was reported that increases in glucose concentration within physiological range resulted in an inhibition of AMPK activity in both MIN6 cells¹⁹⁵ and in islets¹⁹⁶. The findings that both glucose-dependent changes in AMP: ATP ratio^{194, 195, 198} and the depolarization-stimulated free Ca^{2+} increase¹⁹⁹ regulate the AMPK activity and phosphorylation also indicated the involvement of the LKB1 and CaMKK β pathway in the stimulation of AMPK¹⁹⁹. Metformin is one of the most commonly prescribed oral antidiabetic agents worldwide which usually causes weight loss and reduced appetite. In 2001 Zhou G *et al*²⁰⁰ reported that the antidiabetic drug metformin stimulates peripheral AMPK and leads to reduced glucose production in the liver and reduced insulin resistance in the muscle.

1.4.2 The nutrient-sensing kinase: Per-Arnt-Sim Kinase

The per-arnt-sim domain or PAS kinase (also termed PASKIN), is another Ser/Thr kinase, distantly related to AMPK (it is phylogenetically closer to LKB1) ²⁰¹. It is one of a group of kinases containing PAS domains which regulate the function of many intracellular signaling pathways in response to both extrinsic and intrinsic stimuli ²⁰².

PAS is an acronym from the names of the proteins in which imperfect repeat sequences were first recognized ²⁰³: the *Drosophila* period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM) ²⁰⁴. The PAS domain is found in all kingdoms of life as a small regulatory module ²⁰². PAS domain-regulated histidine kinases are very common in prokaryotes and control a wide range of fundamental physiological processes, e.g. *Rhizobia* nitrogen fixation, via the oxygen-sensing protein *FixL* ²⁰⁵⁻²⁰⁷. The evolutionarily-conserved PAS kinase consists of two PAS domains and a canonical serine/threonine kinase domain ²⁰². PASK is conserved with orthologs in humans, flies, and yeast ²⁰². Evidence has been provided that phosphorylation of the activation loop within the catalytic domain of PASK is critical for kinase activity, analogous to the regulation of AMP kinase. Moreover, the enzymatic activity of PASK is reported to be subject to inhibition by the most N-terminal PAS domain, designated PAS-A. Finally, the PAS-A domain expressed separately is able to transinactivate its cognate kinase domain *in vitro* ²⁰².

As discussed by Rutter *et al* ²⁰⁸ in budding yeast gave evidence that PASK may be involved in the control of sugar metabolism and protein translation. It was later shown by da Silva Xavier *et al* ²⁰⁹ that mammalian PASK was rapidly activated in clonal β -cells and rat islets in response to elevated glucose concentrations both by reversible phosphorylation at Thr-152 in the “T-loop”, and through enhanced expression of the *PASK* gene. ²⁰⁹.

Importantly, by both transcriptional and post-transcriptional mechanisms, PASK activity was necessary for mediating the direct (intracellular) effect of glucose to stimulate transcription of the preproinsulin and PDX-1 genes²⁰⁹. Overexpression of wild-type PASK in pancreatic MIN6 β -cells resulted in marked increases in preproinsulin promoter activity at low glucose concentrations. Conversely, blockade of PASK function with a dominant negative form of the enzyme, with short interfering RNAs or by antibody microinjection into single cells blocked the induction of preproinsulin and *pdx-1* and other genes by glucose²⁰⁹. Notably, da Silva Xavier *et al* also found that regulation by PASK or glucose of both the preproinsulin and PDX-1 promoters was completely abrogated by deletion of PDX-1-binding sites²⁰⁹. As a result, it was proposed that PDX-1 and possibly MafA could very likely be the targets of PASK action²⁰⁹.

More recently, in order to understand the *in vivo* role of PASK in pancreatic β -cell function and energy homeostasis, Hao *et al*²¹⁰ studied the role of PASK in the pathogenesis of metabolic disease in PASK^{-/-} mice²¹¹. It was demonstrated that islets from PASK^{-/-} mice display defective glucose-stimulated insulin secretion although expression of preproinsulin was normal²¹⁰. PASK is thus required for normal β -cell insulin secretion and mice with PASK deletion exhibited resistance to the metabolic effect of a high-fat diet, including obesity, insulin resistance, and hepatic triglyceride accumulation. It was proposed that metabolic rate and energy expenditure are increased in PASK^{-/-} mice independently of the activity of AMPK, mammalian target of rapamycin (mTOR), or peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1). Hao *et al* also observed increased oxidative metabolism and ATP generation in cultured cells with PASK knocked down by RNAi. Taken together, it was hypothesized that PASK acts in a cell-autonomous manner to maintain cellular energy homeostasis and could be a potential therapeutic target for metabolic disease²¹².

The molecular mechanisms through which glucose regulates PASK activity, and by which activated PASK regulates β -cell function, remain undefined. However, the

finding that PASK^{-/-} mice displayed improved insulin sensitivity after a high-fat diet indicated that lowered levels of PASK in peripheral tissues may be protective against insulin resistance ²⁰¹. Interestingly, in 2007 Borter *et al* ²¹³ demonstrated that PASK/PASKIN expression is weak and insensitive to regulation by glucose in islets. However, unpublished studies in our laboratory involved screening the expression of PASK in islets from a variety of tissues, including human, and we demonstrated the clear presence of both PASK mRNA and protein, albeit at levels which are substantially lower than those in haemopoietic cells or testis, the main sites of PASK expression (GAR, G. daSilvaXavier, unpublished) ²⁰¹.

1.5 Background for work

There appears to be a growing number of transcription factors that function by a regulated nuclear translocation mechanism to control specific gene expression. These include signal transducers and activators of transcription (STATs), nuclear factors of IgkB cells (NF- κ B), nuclear factors of activated T-cells (NFATs), p53, and steroid receptors¹⁴⁹. By using immunocytochemistry and laser-scanning confocal microscopy, previous work in our laboratory has investigated the mechanisms by which PDX-1 may be activated by glucose. PDX-1 is associated with the nuclear periphery and the nuclear membrane region in cells incubated at low glucose concentrations^{137, 138}. Elevated glucose concentrations stimulated the transcriptional activity of a preproinsulin promoter reporter construct monitored in a single living MIN6 β -cell and caused a concomitant translocation of PDX-1 from the nuclear periphery into the nucleoplasm. We have therefore proposed that this shift may contribute to the transactivational capacity of PDX-1 in native β -cells exposed to high extracellular glucose concentrations¹³⁸.

It has been discussed previously in the Introduction (page 33) that phosphorylation of PDX-1 is important for the regulation of PDX-1 activity and that the regulation by glucose and potential kinases have been proposed to be involved in the upstream phosphorylation regulation of PDX-1. However, no direct evidence for PDX-1 phosphorylation in living cells has been provided so far using a well-validated biophysical approach such as mass spectrometry. Whether a specific kinase or kinases regulate PDX-1, and whether this phosphorylation regulates glucose-stimulated PDX-1 translocation, still remain unclear.

As discussed above, PASK was shown to be an important mediator of the effects of glucose on insulin and PDX-1 gene expression²⁰⁹. Therefore, PASK became a promising new upstream kinase candidate for PDX-1 regulation. However, given the complex regulating network surrounding PDX-1, there might also be other kinases involved. Hence, besides targeting potential kinase candidate, it is also an

interesting question to investigate the *in vivo* phosphorylation regions or residues on PDX-1 under physiological condition which could possibly provide more detailed information to help identify a kinase candidate.

Consequently, the overall aim of this thesis is to firstly investigate the phosphorylation of PDX-1 under physiological condition and investigate the *in vivo* phosphorylation sites, further focus on proposed potential upstream kinase (i.e. PASK) and understand the contribution of phosphorylation effect on PDX-1 function in pancreatic β -cells.

2. Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cell culture and reagents

INSr $\alpha\beta$ cells (kindly provided by Dr. Thomas Mandrup-Poulsen, Copenhagen) were maintained in RPMI-1640 medium containing 11.2 mM glucose, 10% FCS, streptomycin and penicillin, 50 μ M β -mercaptoethanol, 100 μ g/ml G-418 and 100 μ g/ml hygromycin-B in a humidified 5% CO₂ incubator at 37 °C. INS-1(832/13) cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). Human embryonic kidney (HEK) 293 cells²¹⁴ were grown in Dulbecco's modified Eagle's medium (Gibco Grand Island, NY, USA) containing 10% fetal bovine serum and penicillin and streptomycin. MIN6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco Grand Island, NY, USA) containing 15% fetal bovine serum and penicillin and streptomycin together with 0.25 μ M β -mercaptoethanol.

Mouse recombinant tissue necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin-1 β (IL-1 β) were from PreproTech EC (London, U.K.) and all chemical reagents are from Sigma unless specified. Anti-c-*myc* antibody was purchased from Roche, and rabbit polyclonal anti-PDX-1 antibody was generated as described¹³⁸. Bacterial culture media LB media and LB agar were purchased from Sigma.

Glycogen synthase kinase 3 (GSK3) and *homeodomain* interacting protein kinase 2 (HIPK2) were purchased from Upstate (Lake Placid, NY). CK1 δ isoform was prepared as previously described²¹⁵.

2.1.2 Plasmids and generation of the anti-pT152-PDX-1 and anti-pS269-PDX-1 antibodies

Plasmids pcDNA3.1-PDX-1-*c-myc* (wild-type, T152A, T152E and T152D) and HisMBPPDX-1-*c-myc* (wild-type) were generated by Dr. Huai-xiang Hao (University of Utah, USA).

Plasmids based on pcDNA3.1 (Invitrogen) and encoding wild-type human PASK bearing C-terminal *c-myc* and *his* epitope tags (pPASK.WT) or inactive PASK mutated at the ATP-binding site (K1028R; pPASK.KD) were provided by Prof. Jared Rutter (University of Utah, USA).

Polyclonal anti-phospho-PDX-1 antibodies against the region ¹⁴⁵EENKRTRpTAYTRAQ¹⁵⁸ (anti-pT152-PDX-1), or ²⁵⁹LPSGLSVpSPQPSSIAPLRPQ EPR²⁸⁴ (anti-pS269-PDX-1) of mouse PDX-1 were raised in rabbit by Pacific Immunology Inc, USA.

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Site Directed mutagenesis (SDM)

Complementary HPLC-purified oligonucleotide primers (approximately 30 nucleotides in length), containing the desired mutation flanked by the unmodified nucleotide sequence, were generated by Invitrogen UK. Primers were diluted to a concentration of 100 mM in distilled water prior to use. Amplification of the plasmid containing the cDNA of interest was performed by using QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene) mainly following the manufacturer's instruction with optimization. The *Pfu* polymerase extends the nucleotide chain by approximately 1000 nucleotides per minute. Extension times were calculated based on the size of the plasmid with 1½ minutes allowed for each 1000 nucleotides, ensuring the complete amplification of plasmid.

Following amplification, reaction product 10 µl was migrated on agarose gel to check the size of the product. The rest of template DNA was degraded by the addition of 1 µl of the restriction enzyme *DpnI*, which specifically recognizes and digests methylated template DNA, for 1 h at 37 °C. The reaction product (40 µl) was precipitated by adding 0.1 volume of 3 M NaAc pH 5.2, 2.5 volume of ice-cold 100% ethanol and 1 ml of glycogen. The mixture was precipitated at -80°C for 1 h. DNA was collected by centrifugation at 4°C for 20 min. at 13,000 rpm and the pellet was air-dried and resuspended in 8 µl dH₂O at 65°C for 10 min. Resuspended DNA 4 µl was introduced into XL10-Gold ultracompetent cells provided in the QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene). The obtained mutant DNA plasmids were sent for sequencing to further confirm (see section 2.2.1.7).

PDX-1 (S269A/E) site directed mutagenesis (SDM). pcDNA3.1-PDX-1-c-*myc* and HisMBPPDX-1-c-*myc* (S269A/E) mutation were made to prepare PDX-1

(S269A/E) mutant adenoviruses and recombinant protein in bacteria, respectively (for experiments described in Chapter 5).

The SDM primers used were:

PDX-1 S269A (Forward): 5'-ggccttagcgtggcgccacagccct-3'

PDX-1 S269A (Reverse): 5'-agggctgtggcgccacgctaaggcc-3'

PDX-1 S269E (Forward): 5'-gggccttagcgtggagccacagccctcc-3'

PDX-1 S269E (Reverse): 5'-ggagggctgtggctccacgctaaggccc-3'

2.2.1.2 Restriction digests

To enable the cloning of DNA fragments into plasmids, DNA was digested using specific endonucleases that identify unique restriction sites. Typically, restriction digests were performed using 0.5 µl of each restriction enzyme required in a 20 µl reaction mix containing a buffer suitable for the enzyme used. Reactions were allowed to proceed for 1 h (single enzyme digestion) or 2 h (double digestion) at 37°C, before the digested DNA was visualized on an agarose gel.

2.2.1.3 Ligation

The ligation of cloned DNA into expression vectors was performed using an insert-to-vector ratio of 3:1. Ligations were performed using a T4 DNA ligase (Invitrogen, UK) in its specific reaction buffer. Ligation reactions were typically performed for 16°C for 16 h, before the product was transformed into competent cells (see section 2.2.1.5).

2.2.1.4 Visualization of DNA on agarose gels

Visualization and separation of DNA was typically performed with 0.8% agarose gels. Molten agarose was generated by dissolving 0.7% (w/v) agarose in 1 x Tris-acetate-EDTA (TAE) buffer (diluted from 10 X stock (Sigma)) and heating in a microwave. Following cooling, 40 ng/ml ethidium bromide was added to the agarose and the gels poured using a gel mould with an appropriately sized comb. Gels were allowed to set and then submerged in 1 X TAE buffer in the gel tank. DNA samples in 6 X sample buffer along with appropriate DNA ladder (Promega, UK) were loaded into the wells of the gel and the gel run at 90 volts until adequate separation was achieved. DNA was visualized using Bio-Rad ChemiDoc XRS system with Quantity One software (Bio-Rad, Hertfordshire, UK). DNA concentrations were estimated by comparison to known DNA concentrations within the DNA ladder.

2.2.1.5 Estimation of the DNA concentration by Nano-Drop.

DNA concentration was determined by a Nano Drop Spectrophotometer (Labtech international). 0.5-1 μ l plasmid DNA was added onto the Nano Drop probe and the absorbance measured at 260 and 280 nm. The concentration was calculated as the extinction coefficient for DNA at 260 nm and compared to a distilled H₂O or Tris-EDTA (pH8.0; TE) buffer control.

2.2.1.6 Sequencing of DNA plasmids

DNA sequencing reactions were performed by GATC Biotech AG (Konstanz, Germany) or MWG Biotech (London, UK). Sequencing primers were generated by Invitrogen, UK. Sequence alignments were performed using online sequence alignment software Basic Local Alignment Search Tool provided by NCBI. The plasmids used throughout the thesis are detailed in Table 2.1.

2.2.1.7 Transformation of Top 10 electrical competent E.coli

Top 10 electrical competent *Escherichia coli* (Invitrogen, UK) cells were thawed on ice and mixed gently by flicking. The ligation product was transformed into 20 µl electrical competent cells in a 2.0 mm cuvette and mixed well without air bubbles. The mixture was electroporated at 2,500 V, 200 Ohms in an electroporator (Eppendorf). The transformation products were resuspended in 500 µl of LB-broth, and incubated at 37°C in shaking incubator for 1 h. After incubation, the bacteria were plated out onto 4 LB plates with selection antibiotics (Table 2.1), and were cultured at 37°C overnight (16-20 h).

2.2.1.8 Transformation of XL-1 blue super-competent E.coli

XL-1 blue super-competent cells (*Stratagene*, UK) were thawed on ice and mixed gently by flicking. 45 µl of cells were added to a pre-chilled rounded bottom falcon tube (BD science, UK), and 4 µl of ice-cold site-mutagenesis reaction product was mixed with the cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 30 s. and immediately placed on ice for 2 min. LB media (500 µl) was added to the cells and incubated for 1 h at 37°C with shaking. Cells (25 µl and 100 µl) were plated on to agar plates containing the relevant selection antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) for each specific plasmid and colonies grown at 37°C for 16 h.

2.2.1.9 RNA Isolation and Reverse-Transcription PCR

INSraβ cells²¹⁶ were cultured in six-well dishes and treated with 500 ng/ml doxycyclin or various viruses at an multiplicity of infection (MOI) of 100 plaque-forming units (pfu)/cell for 16-24 h. To isolate total mRNA, the monolayer was rapidly washed twice with PBS, pH 7.5, and scraped with a rubber spatula in to TRIZOL reagent (Invitrogen). Phenol extraction of the homogenate was performed as described by the manufacturer. Isopropyl alcohol precipitation of RNA was

performed at 20 °C overnight. The RNA was then washed with 75% ethanol and dissolved in nuclease-free water. DNase treatment was applied by using DNase-free kit (Ambion) and the absorbance of RNA at 260 nm and 280 nm was measured. The ratio of A260/A280 was between 1.8-2.1 and the concentration of sample RNA was calculated by A260. RNA 2 µg was converted into cDNA by reverse-transcription PCR as template for the downstream real-time PCR.

Real-time RT-PCR quantitative detection of target mRNA transcripts was carried out using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA). PCR reactions were performed in a total volume of 20 µl. SYBR green PCR master mix (10 µl; Applied Biosystems), 0.5 µl (final concentration 0.125 µM) of primers (forward and reverse), and 1.5 µl first-strand cDNA were mixed into 96-well PCR plates (Applied Biosystems). Primer sequences are as follows: cyclophilin A fwd, 5'-TATCTGCAC TGCCAAGACTGA-3'; cyclophilin A rev, 5'-CCACAATGCTCATGCCTTCTTT CA-3'; glucagon fwd, 5'-GATCATTTCCCAGCTT CCCAG-3'; glucagon rev, 5'-CTGGTAAAGGTCCCTTCAGC-3'²¹⁷. Data were analyzed using ABR PRIM SDS v1.3.1 (Applied Biosystems software). All samples were assayed in triplicate, and each real-time PCR experiment was repeated three times on three different occasions.

Table 2.1

Construct	Restriction sites used for ligation or recombination	Selection
pAdTrack-CMV	<i>HindIII</i> and <i>EcoRV</i> (to linearize the vector)	Kanamycin
pcDNA3-PDX-1-c-myc (Wild-type)	<i>HindIII</i> and <i>EcoRV</i> (to excise PDX-1 fragment)	Ampicillin
pcDNA3-PDX-1-c-myc (T152A/D/E)	<i>HindIII</i> and <i>EcoRV</i> (to excise PDX-1 fragment) <i>BsrG I</i> and <i>Xho I</i> (to excise PDX-1 fragment)	Ampicillin
pcDNA3-PDX-1-c-myc (S269A/E)	<i>HindIII</i> and <i>EcoRV</i> (to excise PDX-1 fragment)	Ampicillin
pAdTrack-PDX-1-c-myc (wild-type)	<i>HindIII</i> and <i>EcoRV</i> (for ligation) <i>PmeI</i> (for recombination with pAdEasy)	Kanamycin
pAdTrack-PDX-1-c-myc (T152A/D/E)	<i>HindIII</i> and <i>EcoRV</i> (for ligation) <i>PmeI</i> (for homologous recombination with pAdEasy)	Kanamycin
pAdTrack-PDX-1-c-myc (S269A/E)	<i>HindIII</i> and <i>EcoRV</i> (for ligation) <i>PmeI</i> (for homologous recombination with pAdEasy)	Kanamycin
pAdEasy-pAdTrack- PDX-1-c-myc (wild-type)	<i>PacI</i> (linearization before transfection into mammalian cells)	Kanamycin
pAdEasy-pAdTrack- PDX-1-c-myc (T152A/D/E)	<i>PacI</i> (linearization before transfection into mammalian cells)	Kanamycin
pAdEasy-pAdTrack- PDX-1-c-myc (S269A/E)	<i>PacI</i> (linearization before transfection into mammalian cells)	Kanamycin
HisMBPPDX-1-c-myc (wild-type)	<i>BsrG I</i> and <i>Xho I</i> (to excise wild-type PDX-1 fragment and replace with T152A mutant)	Ampicillin
HisMBPPDX-1-c-myc (T152A)	<i>BsrG I</i> and <i>Xho I</i> (for ligation with excised PDX-1 fragment from pcDNA3PDX-1 T152A)	Ampicillin
HisMBPPDX-1-c-myc (S269A)		Ampicillin

2.2.2 Cell and tissue work

2.2.2.1 Immunocytochemistry

INSr $\alpha\beta$ cells were infected with viruses expressing wild-type or mutant forms of PDX-1 at 100 MOI and cultured in medium containing 11.2 mM glucose for 24 h prior to treatment for 16 h in medium containing 3 mM glucose. Cells were then cultured at 3 or 20 mM glucose for 6 h. Cells were fixed and permeabilized with 4% (v/v) paraformaldehyde plus 0.2% Triton X-100¹³⁸. A monoclonal primary antibody against *c-myc* (Roche, 1:1000) was revealed with Alexa 568-conjugated goat-anti-mouse antibody (1:1000) in 0.2% (v/v) bovine serum albumin. Cells were washed with PBS between incubations. Cells were treated with 4', 6-diamidino-2-phenylindole (DAPI) staining and mounted on coverslips with Mowiol before analysis¹³⁸.

2.2.2.2 Confocal Imaging

Confocal Images were captured using a Leica SP2 upright laser scanning confocal microscope (X 63/1.32 oil-immersion lens) equipped with a krypton/argon laser (488 and 568 nm excitation lines) and a UV light. Images were analyzed off-line using VolocityTM 4.0 software.

2.2.2.3 Preparation of Nuclear Extracts

HEK293 cells cultured in T75 flasks were infected with adenoviruses expressing wild-type or mutant forms of PDX-1 at 100 MOI for 24 h and collected. These were then washed once with ice-cold PBS. The cell pellet was resuspended in 1 ml cold buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF)²¹⁸ by gentle pipeting. The cells were allowed to swell on ice for 15 min. and 50 μ l of 10% NP-40 was added and the resuspension was vortexed for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge at

13,200 rpm at 4 °C. The supernatant containing cytoplasm and RNA was discarded and the pellet was resuspended in 100 ml buffer (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF)²¹⁸ and the tube was vigorously rocked at 4°C for 15 min. on a shaking platform. The nuclear extract was centrifuged for 5 min. in a microcentrifuge at 13,200 rpm at 4°C. Protein concentrations were determined using the Pierce BCA protein Assay (Thermo scientific). Nuclear extracts were then divided into 20 µl aliquots and stored at -80 °C.

2.2.2.4 Ca²⁺-phosphate transfection

HEK293 cells were transfected using Ca²⁺-phosphate²¹⁹. HEK293 cells were grown to 70-90% confluency in 12-well plates or T25 flask. Total DNA (as indicated in different reaction) was mixed with 2.5 M CaCl₂ was added drop by drop to same volume of 2 X Hepes buffer saline (HBS) in a sterile tube while vortexing. The mixture was applied on top of the cells. Cells were harvested after 24-48 h after transfection for further immunoprecipitation or luciferase assay.

2.2.3 Protein expression and purification

2.2.3.1 Transformation of BL21 E.coli

BL21 cells (Invitrogen, UK) transformed using the same protocol as for XL-1 Blue super-competent cells.

2.2.3.2 Preparation of HisMBPPDX-1 mutants expressing DNA construct.

The HisMBP-PDX-1 (T152A) was constructed by replacing the PDX-1 fragment between restriction sites *Bsr*GI and *Xho*I in HisMBPPDX-1 (wild-type) with the same fragment containing T152A mutation site from pcDNA3-PDX-1 (T152A). A detailed plasmid map is shown in Fig. 2.1 with restriction sites in bold.

The HisMBPPDX-1 (S269A and S269E) was generated using site-directed mutagenesis kit (Stratagene, UK). The primers used and the method was as described in the previous section.

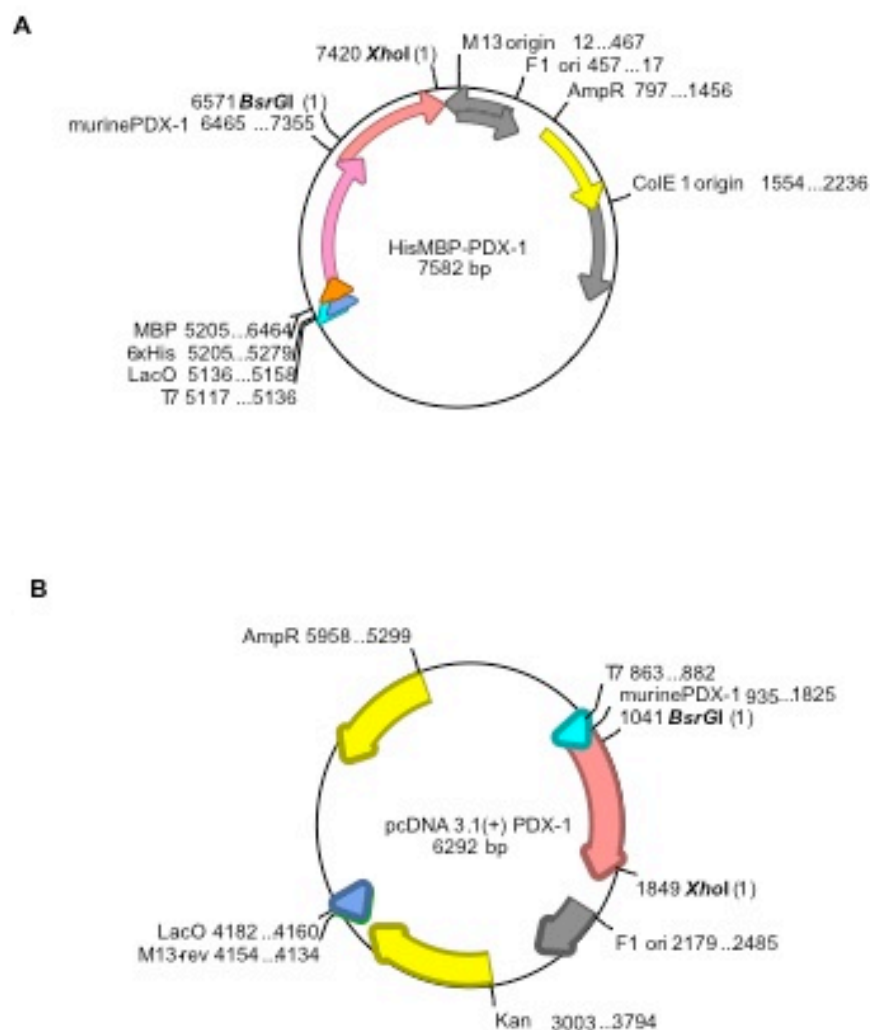


Figure 2.1. Illustration of HisMBPPDX-1 (WT) (A) and pcDNA3.1-PDX-1 (T152A) (B) DNA plasmid and restriction sites (*BsrG I* and *Xho I*) used in restriction digestion.

2.2.3.3 HisMBPPDX-1 protein production and purification

One colony was grown in 5 ml of LB broth containing 50 µg/ml ampicillin at 37°C for 16 h, which was then further inoculated into 500 ml LB containing 50 µg/ml ampicillin at 37°C until an O.D. of 0.4-0.6 at 600 nm was reached. 0.2 mM

Isopropyl- β -D-thiogalactopyranoside (IPTG) was added for induction and cells were cultured for another 16 h at 25°C. The induction process was illustrated in Fig. 2.2A. Cells were harvested at 5000 rpm for 10 min. at 4°C in a Sorvall centrifuge using an SLA-3000 rotor. The pellet was resuspended in ice-cold PBS and harvested at 5500 rpm for 10 min. at 4°C. The pellet was vortexed and resuspended in ice-cold lysis buffer (50 mM Tris pH 7.5; 150 mM NaCl; 5 mM MgCl₂; 1 mM β -mercaptoethanol (BME); 1 mg/ml lysosyme; 1X protease inhibitors (Roche)) and then kept on ice for 30 min. Tween was added to a final concentration of 0.2% and the sample was sonicated for 30 s and allowed to rest for 1 min. This procedure was repeated three times. The sonicate was then transferred to pre-chilled tubes and centrifuged at 100,000 X g for 1 h at 4°C. Ni²⁺-NTA beads (Qiagen) were washed once with 50 ml cold PBS and equilibrated with 50 ml ice-cold wash buffer (50 mM Tris pH 7.9; 500 mM NaCl; 5 mM MgCl₂; 1 mM BME; 0.2% Tween) twice and sedimented by centrifugation at 800 g for 5 min. at 4°C. Supernatant (100 μ l) after ultracentrifugation was kept as S100 whilst the rest was adjusted to 500 mM NaCl and added to the beads and incubated with rotation at 4°C for 1 h. The beads were sedimented by centrifugation at 3,000 rpm for 5 min. at 4°C. Flow through (FT; 100 μ l) was kept and the beads were washed twice with wash buffer and 100 μ l of the first wash was kept. The beads were then resuspended in 10 ml wash buffer and poured into a column (Biorad) and wash buffer allowed to run through.

Protein was eluted with increasing concentrations of imidazole (10, 50, 100, 150, 200, 250 mM imidazole, pH 8) in wash buffer. Each eluate (10 μ l), the S100, FT and column wash were separated on 12% SDS-PAGE and analyzed by coomassie blue staining. Eluate containing the protein product were dialysed for 16 h at 4°C in 2 L of dialysis buffer (50 mM Tris pH 7.9; 150 mM NaCl; 5 mM MgCl₂; 1 mM BME).

A representative SDS-PAGE gel with fractions of HisMBP-PDX-1 (wild-type) protein was shown in Fig. 2.2B. The band around 90 kD is HisMBP-PDX-1 protein.

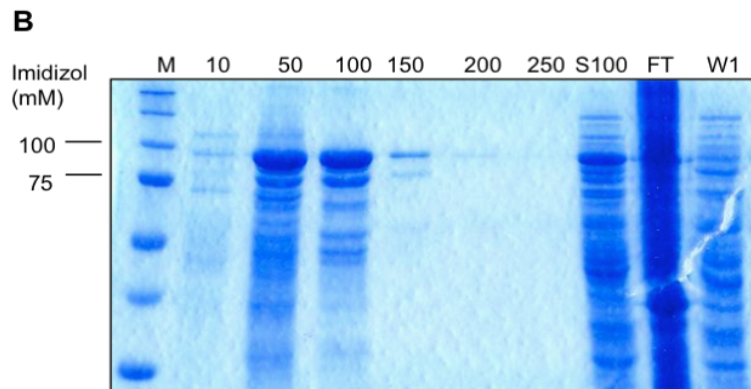
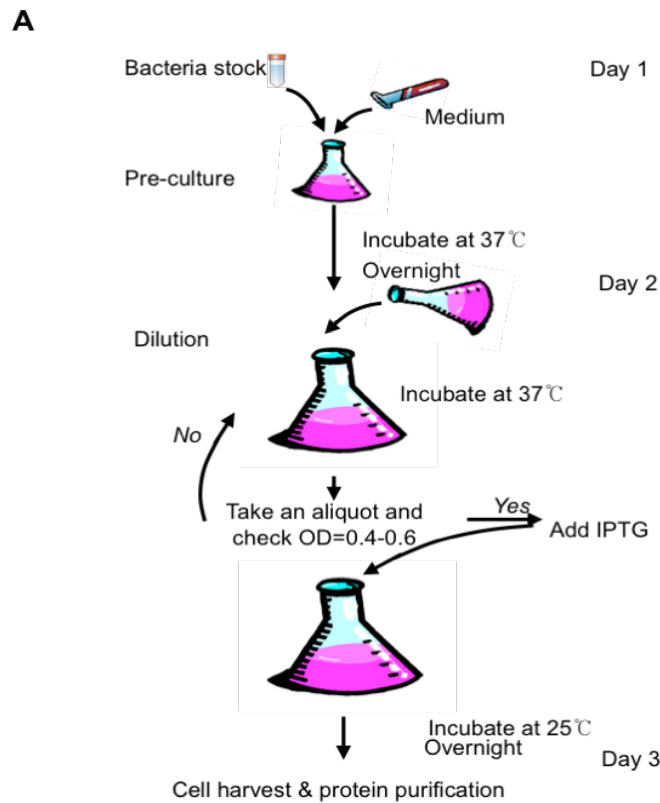


Fig.2.2. (A) Scheme illustrating the preparation HisMBP-PDX-1 recombinant protein. (B) Preparation of recombinant mouse MBP-PDX-1 (wild-type). Purification of HisMBP-PDX-1 (wild-type) recombinant protein. Protein was eluted with increasing concentrations of imidazole (as indicated) in wash buffer. 10 μ l of each eluate, the supernatant (S100), flow through (FT) and column

wash (W1) were separated on SDS-PAGE and analyzed by coomassie blue.

2.2.3.4 AcTEV protease cleavage time course

AcTEV Protease is an enhanced form of Tobacco Etch Virus (TEV) protease which recognizes the seven-amino-acid sequence: Glu-Asn-Leu-Tyr-Phe-Gln-Gly, present between the histidine tag and maltose-binding protein, and also between PDX-1 and maltose-binding protein (Fig. 2.3A). AcTEV protease cleaves between Gln and Gly with high specificity. In order to check the efficiency of the protease, a time course for protease digestion was carried out and samples of wild-type protein digestion product at 1, 2, 4, 6 h were collected and migrated on SDS-PAGE before coomassie analysis. Fusion protein (20 µg) was mixed with 20X Tobacco Etch Virus protease (TEV) Buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA), 1 mM dithiothreitol (DTT), and 1.0 µl AcTEV Protease (10 units) in a 150 µl reaction and incubated at 30°C for 6 h. Aliquots (30 µl) were collected at 1, 2, 4, and 6 h. These were mixed with 30 µl 2X SDS sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 1.4 M β-mercaptoethanol; 20% (v/v) glycerol; 0.01% bromophenol blue) and kept at -20°C until required. 40 µl of each sample was analyzed by SDS-PAGE and coomassie blue staining. A representative SDS-PAGE with wild-type PDX-1 protein is shown in Fig. 2.3B.

2.2.3.5 AcTEV protease cleavage and protein purification

Fusion protein was incubated with AcTEV Protease at 30°C for 6 h as described. Amylose beads (New England BioLab) and Ni beads were equilibrated with 1X cleavage buffer and poured onto columns. Cleaved recombinant protein was added to amylose beads column to remove maltose-binding protein, and Ni beads column to remove histidine tag and histidine-tagged AcTEV protease. The final eluate containing purified protein was collected. A representative SDS-PAGE with wild-type PDX-1 protein is shown in Fig. 2.3C.

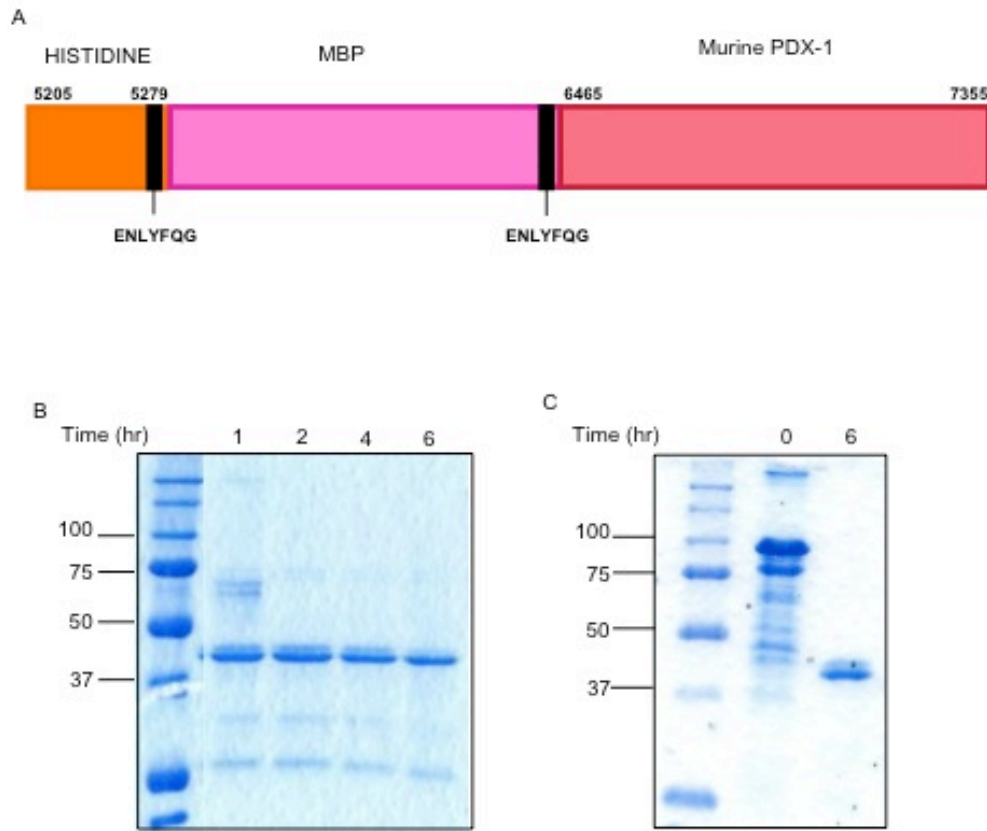


Figure 2.3. (A) The AcTEV cleavage sites (ENLYFQG) in recombinant HisMBPPDX-1 protein. (B) and (C) Cleavage and purification of PDX-1 recombinant protein (wild-type). (B) AcTEV Protease cleavage time course at 1, 2, 4, and 6 h. (C) AcTEV Protease cleavage for 6 h. Cleaved recombinant protein was added to amylase beads column to remove maltose-binding protein, and Ni beads column to remove histidine tag and histidine-tagged AcTEV protease. 5 ml of both HisMBPPDX-1 (wild-type) and cleaved PDX-1 (wild-type) protein were separated on SDS-PAGE and analyzed by coomassie blue staining.

2.2.3.6 Recombinant protein concentration

Recombinant protein was concentrated using vivaspin concentrators (Sartorius-stedim, UK) with a molecular weight cut off of 30,000 Da. Protein elutions were pooled and concentrated by centrifugation at 4,000 g, at 4°C, for the time required to reduce the pooled volume to approximately 0.5 ml. The protein concentration was measured with BCA assay kit (Pierce, Thermal Scientific, UK).

2.2.4 Adenovirus preparation

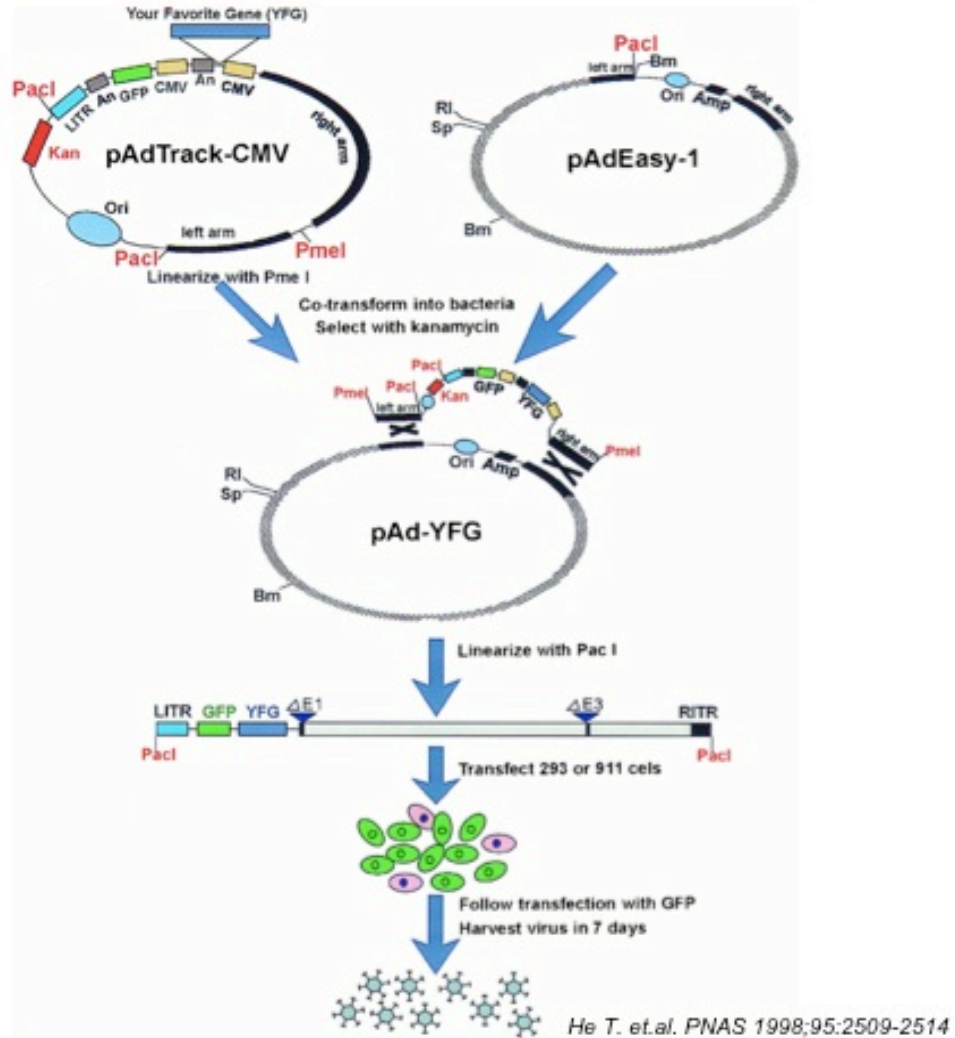


Fig.2.4. Schematic outline of the AdEasy system. The *Pdx-1* gene is first cloned into a shuttle vector, pAdTrack-CMV. The resultant plasmid is linearized by digesting with restriction endonuclease *PmeI*, and subsequently transformed into *E. coli* BJ5183 cells containing an adenoviral backbone plasmid, pAdEasy-1. Recombinants are selected for kanamycin resistance, and recombination was confirmed by multiple restriction endonuclease analyses. Finally, the linearized recombinant plasmid is transfected into adenovirus packaging cell lines, HEK 293 cells. Recombinant adenoviruses typically are generated within 7–10 days. The “left arm” and “right arm” represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector. The same strategy was applied to obtain mutant pAdEasy-CMV-PDX-1-c-myc plasmids.

2.2.4.1 Construction of pAdEasy-CMV-PDX-1-c-myc via homologous recombination in bacteria

A cDNA fragment encoding PDX-1 (wild-type or mutants) was excised from pcDNA3.1-PDX-1-c-myc using restriction enzymes *HindIII* and *EcoRV* as described in Table 2.1. The fragment was ligated into linearized vector pAdTrack-CMV under cytomegalovirus immediate-early gene promoter control with the same restriction enzymes. Restriction digest was as described above. pAdTrack-CMV-PDX-1 (wild-type or mutants)-c-myc (1 µg) were linearised with *PmeI*. The linearised plasmids were purified using Qiagen's PCR purification kit and resuspended in 60 µl of dH₂O. *PmeI*-digested shuttle plasmid (6 µl; 500 ng/µl) was transformed into 20 µl AdEasy-electrocompetent *E.coli* BJ5183 cells for homologous recombination with the pAdEasy-1 vector. Candidate clones were digested with *PacI* to verify proper recombination. With *PacI* digestion, two fragments (4.5 kb and 32 kb) were produced (Fig. 2.5C).

Plasmids miniprep was performed and the sizes of supercoiled plasmids were checked by running one-fifth of a miniprep on a 0.8% (w/v) agarose gel. The aim was to obtain bands of DNA similar to the pAdEasy vector. A pair of bands of 25 and ~40 kb were obtained. To further check the recombination, a restriction digest of the DNA from clones was performed with *PacI*. Candidate clones yielded a large fragment (near 30 kb), and a smaller fragment of 4.5 kb. Correct recombinant miniprep DNA 5 µl was re-transformed into Top 10 electrocompetent cells (Invitrogen). The plasmid was purified by Qiagen maxi-prep kit.

The representative agarose gels showing the wild-type and T152 mutants viral constructs were shown in Fig. 2.5.

Figure 2.5

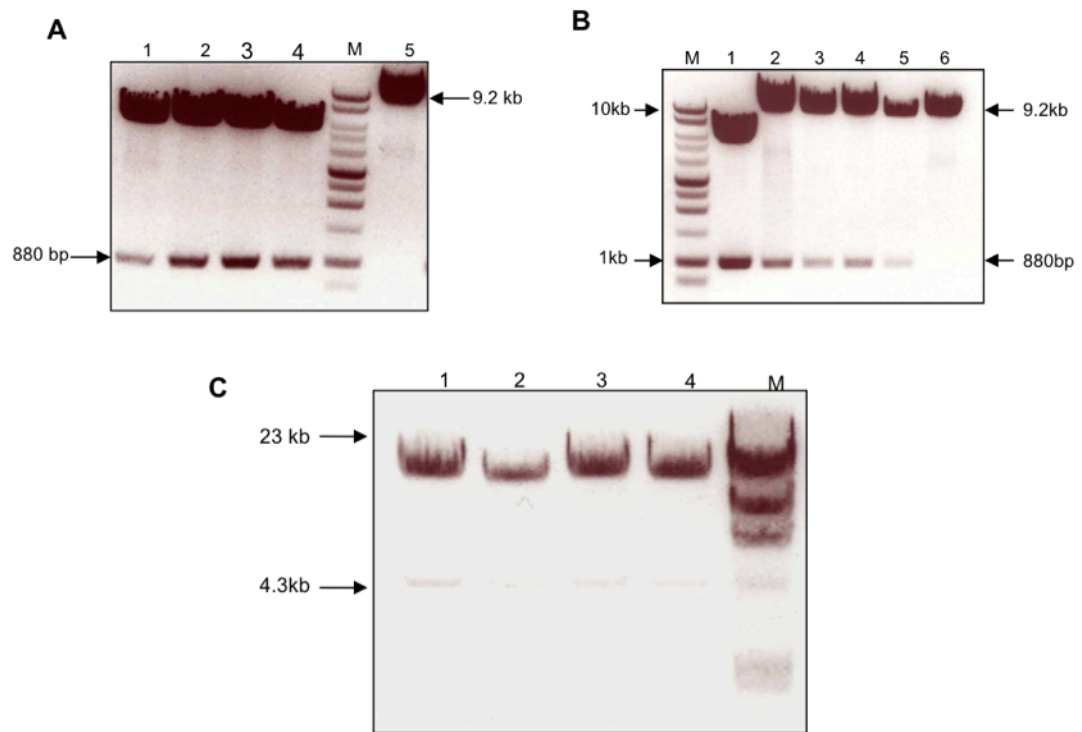


Fig.2.5. (A) Excision of PDX-1 cDNA and linearization of the viral vector. Restriction digest products by enzymes *Hind*III and *Eco*RV of wild-type and mutant forms of pcDNA3-PDX-1-c-myc. (lane 1: wild-type; lane 2: T152A mutant; lane 3: T152E mutant; lane 4: T152D mutant) and the viral vector pAdTrack-CMV (lane 5) were run alongside 1kb DNA marker. The lower band in lane 1-4 is corresponding to the size of PDX-1 cDNA (~880bp), and the band shown in lane 5 corresponds to linearised pAdTrack-CMV (9.2kb). **(B) Insertion of PDX-1 cDNA into viral vector pAdTrack-CMV.** Restriction analysis of pAdTrack-PDX-1-c-myc ligation products following *Hind*III and *Eco*RV restriction digest. The digestion products were run alongside 1kb DNA marker. The lower bands in lane 2-5 (lane 2: wild-type; lane 3: T152A mutant; lane 4: T152E mutant; lane 5: T152D mutant) corresponds to PDX-1 cDNA (~880bp) (Lane 1: pcDNA3-PDX-1-myc digestion); whilst the higher band in lane 2-5 corresponds to the viral vector (~9.2kb) which is the same as the linearised pAdTrack-CMV shown in lane 6. **(C) Recombinant adenoviral vector containing wild-type and mutant PDX-1 gene.** Restriction analysis of pAdEasy-CMV-PDX-1-c-myc following *Pac*I digestion. Wild-type and mutant PDX-1 recombinant digestion products (lane 1: wild-type; lane 2: T152A mutant; lane 3: T152E mutant; lane 4: T152D mutant) were run alongside the lambda DNA/*Hind*III marker (Sigma). The top band in lane 1-4 corresponds to ~30kb and the lower band corresponds to ~4.3kb, which suggesting the

recombinant viral plasmids containing wild-type and mutant forms of Pdx-1 gene were successfully obtained.

2.2.4.2 Viral production in HEK293 cells

Recombinant adenoviral plasmid pAdEasy-CMV-PDX-1-*c-myc* (10 µg) was digested with *PacI*, purified with Qiagen's PCR purification kit and resuspended in sterile H₂O. 8 µg of digested pAdEasy-CMV-PDX-1-*c-myc* was transfected into a T-25 flask of HEK 293 cells by using standard Lipofectamine (Invitrogen) transfection according to the Manufacturer's instructions. Transfection and viral production were monitored by Green Fluorescent Protein (GFP) expression by excitation with light at 488 nm microscope. Cells were extracted when 2-3 comets were formed (~7 days post-transfection), and the contents of the flask were transferred to a 70% confluent T-75 flask of HEK293 cells. Virus expression was evident at three days post infection.

HEK293 cells were harvested at 7–10 days post-transfection, then subjected to three cycles of freeze-thawing in liquid nitrogen and a 37 °C water bath, respectively. The extract was subjected to centrifugation at 13,000 rpm for 10 min. and the viral supernatant was either stored at -80 °C or used to infect more HEK293 cells to create viral stocks. The recombinant virus particles were purified by ultracentrifugation in a caesium chloride gradient. The same strategies were applied to mutant pAdEasy-CMV-PDX-1-*c-myc* plasmids to make viruses expressing mutant PDX-1-*c-myc*.

All the purified adenoviruses were then titrated by measuring green fluorescent protein (GFP) expression, as described below.

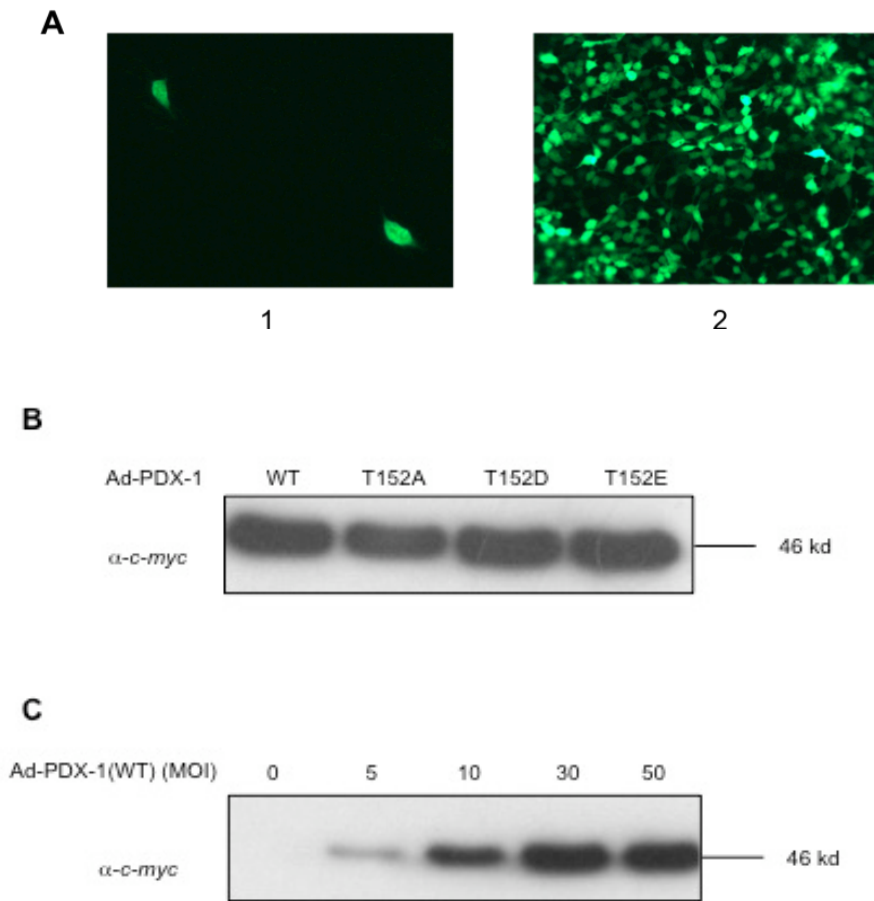


Fig.2.6. Adenovirus-mediated PDX-1-c-myc expression in mammalian cells (A) GFP expression in HEK 293 cells 24 h after transfection with pAdEasy-CMV-PDX-1-c-myc, and five days after infection with adenovirus at 100 MOI. (B) MIN6 cells were infected by indicated adenoviruses at 100 MOI for 24 h and 30 μ g whole cells lysate was separated on SDS-PAGE and analyzed with anti-c-myc antibody to recognize the viral PDX-1 expression. (C.) MIN6 cells were infected with wild-type PDX-1 at indicated MOI for 24 h and 30 μ g whole cell lysate was separated on SDS-PAGE and analyzed with anti-c-myc antibody to recognize the viral PDX-1 expression.

2.2.4.3 Titration of Adenoviruses

HEK293 cells were cultured in 24-well plates to 70% confluency. Then a serial dilution of the virus from the CsCl preparation was set up. Virus (2 µl) was added to 1ml of culture medium. This dilution (1.1 µl) was taken and added to 1ml of medium. Then, 100 µl of this dilution was taken and added into 900 µl of HEK medium. The mixture was well mixed and was taken 100 µl and added into 900 µl of HEK medium. This dilution was repeated three more times to give six separate concentrations. The medium from all the wells of the 24-well plates was removed and the first pair was added in 0.5 ml each of the most concentrated virus solution (2 µl in 1 ml medium). The next pair was added in 450 µl of the next most concentrated dilution and so on for the other wells. The first pair of well thus received 1 ml each and the next get 1×10^{-3} µl, then 1×10^{-4} µl, 1×10^{-5} µl, 1×10^{-6} µl, and finally 1×10^{-7} µl.

Cells were cultured overnight and then the next day GFP expression was monitored by fluorescence microscopy. Nearly all the cells from the first pair of wells were green. The next pair had quite a few (>50%) but there were successively fewer green cells with each dilution. The number of green cells was counted in the pair of wells that gives of the order of 10-100 green cells and was averaged. This is used to work out viral concentration (an average of 30 green cells in the 1×10^{-5} µl well is equivalent to 3×10^9 virus/ml).

The volume of virus required to infect cells could then be calculated using the following formula:

$$\text{Volume of Virus required (ml)} = \frac{\text{Number of cells} \times \text{MOI}}{\text{Viral concentration (pfu/ml)}}$$

MOI: Multiplicity of Infection

pfu : Plaque forming units

All the adenoviral plasmids used throughout this Thesis are detailed in Table 2.1 and all viruses were prepared using the same methods.

2.2.5 Protein Biochemistry

2.2.5.1 SDS-PAGE

Protein samples were denatured by the addition of Laemmli buffer ²²⁰ and boiling for 10 min. Protein were separated on SDS-PAGE gels comprising of a stacking gel (40% acrylamide 0.75 ml, 0.25 M Tris-HCl pH6.8 2.5 ml, 10% SDS 0.1 ml, dH₂O 6.55 ml, 10 ml TEMED and 10% APS 100 µl) and typically a 12% separating gel (40% acrylamide 3 ml, 1.5 M Tris-HCl pH8.8 2.5 ml, 10% SDS 0.1 ml, dH₂O 4.4 ml, 6.7 µl TEMED and 10% APS 33 µl). The protein was separated at 100 V. Proteins were then transferred onto PVDF membranes (GE Healthcare) using a BIO-RAD trans blot cell apparatus at 100 V for 1 h, or stained with Coomassie blue.

2.2.5.2 Western immuno-blotting

Proteins were analyzed immunologically after electrotransfer onto PVDF membranes which were blocked in 5% (M/V) nonfat dry milk in Tris-buffer saline with Tween 20 (TBST) for 1 h at 25 °C. Membranes were incubated with the indicated primary antibodies in TBST for 16 h at 4 °C followed by treatment with horseradish peroxidase-conjugated secondary antibodies. Blots were visualized with ECLTM western blotting system (GE Healthcare).

2.2.5.3 Stripping for reprobing western blots

Stripping buffer (20 ml SDS 10%, 12.5 ml Tris HCl pH 6.8 0.5 M, 67.5 ml ultra pure water, And 0.8 ml β-mercaptoethanol) was warmed to 50°C and added to a plastic bag together with the membrane. The bag was sealed and incubated at 50°C for up to 45 minutes with some agitation. The solution was disposed under fumehood and the membrane was rinsed under running water tap for 1 h. The PVDF membrane was rehydrated in methanol for 1 min. and washed in TBST for 5

min. and restart the blocking stage.

2.2.5.4 Densitometry

Coomassie blue stained gels or Western immuno-blots were analyzed and the band densities were determined using Quantity One software (Bio-Rad). All calculated densities were corrected for background using a blank region close to each defined band.

2.2.5.5 Electrophoretic Mobility Shift Assays

Single-stranded oligonucleotide probes were 5' end-labeled with [γ - ^{32}P] ATP using T4 polynucleotide kinase (Invitrogen). Labeled oligonucleotides were column-purified (Pharmacia) and annealed to an excess of complementary strand. Nuclear extracts (2 μg protein) were incubated with ^{32}P labeled probe for 30 min. at room temperature in electrophoretic mobility shift assay (EMSA) binding reaction buffer comprising 10 mM Hepes pH7.9, 60 mM KCl, 5 mM MgCl_2 , 5 mM DTT, 4 mM Spermidine, 1 mM EDTA, 1 μg of poly (dIdC)/poly (dI-dC) and 5% glycerol. 1 μl of PDX-1 antiserum (kindly provided by Dr. Chris Wright, Vanderbilt University) was added in supershift assays. Gel loading buffer was added and the mixtures were loaded on to a 20 X 20 cm non-denaturing 5% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 29:1), which was migrated in 0.5 X TBE (50 mM Tris, 50 mM boric acid and 1 mM EDTA) at 120-150 V for 3 to 4 h. The gel was dried and subjected to autoradiography. The oligonucleotide fragments were derived from the A3/A4⁹⁸ element of the mouse insulin gene promoter which is important in insulin gene transcriptional regulation and also contains a PDX-1 binding motif (TAAT). The following oligonucleotide probes were used (top strands shown): mouse I insulin promoter (A3/A4 element)^{98, 103}, 5' CTTATTAAGACTATAATAACCCTAAGACT A-3'²²¹.

2.2.5.6 Immunoprecipitation

Cells were washed twice with ice-cold PBS. Lysis buffer (750 μ l; 1% NP40; 50 mM NaCl; 1% sodium deoxycholate; 0.1% sodium monododecyl sulfate (SDS); 50 mM Tris-HCl pH7.5; 2 mM EDTA; 10 mM sodium phosphate; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 200 μ M sodium orthovanadate; 1x protease inhibitor cocktail solution and phosphatase inhibitor cocktail 1 & 2, Sigma) was added and the cells were collected by scraping. The mixture was transferred into a 1.5 ml centrifuge tube and was then placed on a rotating wheel at 4 °C for 30 min. to lyse the cells fully. The mixture was then centrifuged at 14,000 rpm for 5 min. to harvest the lysate (supernatant). Anti-c-*myc* antibody-conjugated beads (Santa-Cruz; 40 μ l) were added to the supernatant and lysis buffer was added to give a final volume of 1 ml in a 1.5 ml centrifuge tube, which was then rotated at 4 °C overnight. IP beads were then collected by centrifugation at 3,000 rpm at 4 °C for 30 s, and washed 5 X with lysis buffer. The immunoprecipitated protein was either used for further in vitro phosphorylation or separated on SDS-PAGE.

2.2.6 Statistical analysis

Data are given as means $\pm$ SEM for the number of experiments indicated in relative experiments. Comparisons were performed by two-tailed Student's *t*-test by using Microsoft ExcelTM. Two-tailed *t*-tests were used to determine whether the differences in indicated parameters between the two compared conditions were statistically significant. Bonferroni adjustment technique was used for multiple comparisons, and a *p*-value of 0.05 was considered as statistically significant.

3. Chapter 3

Phosphorylation of PDX-1 *in vitro* and in cloned β -cells

3.1 Introduction

In an earlier study investigating the role of IUF-1 (PDX-1) in mediating glucose-stimulated insulin gene expression, Macfarlane *et al*⁶³ reported that in isolated rat islets, low glucose (3 mM) incubation decreased the PDX-1 activity to 'undetectable levels' in 6 h by measuring PDX-1 DNA-binding activity, whereas at high glucose (20 mM) PDX-1 activity remain stable for 24 h. These findings also suggested that the changes of PDX-1 activity were phosphorylation-dependent⁶³.

Subsequent studies by several groups had examined the role of different kinases including the stress-activated protein kinase 2 (SAPK2)¹³⁵, PI3K^{135, 137}, glycogen synthase kinase 3 (GSK3)¹⁴² and the homeodomain-interacting protein kinase (Hipk) 2¹⁴⁸ to investigate potential regulatory effect of these kinases involved in PDX-1 regulation. Although it is controversial whether SAPK2 is involved in the pathway regulating PDX-1 DNA-binding activity^{135, 137}, independent studies demonstrated that PI3K may play an important role in PDX-1 transactivational activity regulation^{135, 137}. In 2006, Boucher *et al*¹⁴² demonstrated that a minor portion of PDX-1 is phosphorylated on serine 61 and/ or serine 66 in β -cells detected using a phospho-specific antibody technique. The phosphorylation is increased following proteasome inhibition, and it is also modulated by GSK3-dependent mechanisms¹⁴². In a later study it is reported that the C-terminal PDX-1 may also be regulated by phosphorylation as evidence showed that HIPK2 directly phosphorylates PDX-1 *in vitro* and increased the PDX-1 transcriptional activity¹⁴⁸.

PAS (Per-Arnt-Sim) domains regulate many intracellular signalling pathways in response to both extrinsic and intrinsic stimuli²⁰². First described as a nutrient-sensitive regulator in yeast²⁰², PAS domain regulated histidine kinase PASK (also

called PASKIN) was shown to be an important mediator of the effects of glucose on insulin and PDX-1 gene expression ²⁰⁹. Mice homozygous for the inactivation of PASK display normoglycemia on a normal chow diet, but become glucose intolerant on a high fat diet ²¹⁰ and islets isolated from these animals display defective glucose-stimulated insulin secretion.

In vitro phosphorylation studies performed in collaboration with Prof. Jared Rutter at University of Utah using recombinant PDX-1 and purified human PASK, showed that PDX-1 is efficiently phosphorylated *in vitro* at Thr-152 in the homeodomain by the nutrient-regulated kinase, PASK (Fig. 3.1). By applying phospho-site screening on the phosphorylated PDX-1 by PASK using mass spectrometry, a single site Thr-152 was detected to be phosphorylated. Not only does this make future experiment simpler and more promising, it may also suggest a potential interaction between PASK and PDX-1.

In this chapter, we sought to explore firstly whether Thr-152 is a specific phosphorylation site for PASK *in vitro*; additionally, we sought to study the phosphorylation of PDX-1 in living cells under physiological condition and moreover whether Thr-152, which was phosphorylated by PASK *in vitro* is a major phosphorylation site for PDX-1 in intact cells.

By applying *in vitro* phosphorylation screening of wild-type and T152A mutant PDX-1 by a series of Ser/Thr kinases, Thr-152 appeared to be a specific *in vitro* target only for PASK. We also showed for the first time that wild-type PDX-1 is phosphorylated under physiological condition using ³²Pi metabolic labeling in intact cells. However, T152A mutant PDX-1 was not able to abolish the phosphorylation in ³²Pi metabolic labeled living cells, indicating Thr-152 is probably not a major phosphorylation site for PDX-1 in living cells. Hence, whether Thr-152 is also phosphorylated in living cells by PASK need to be further studied using mass spectrometry.

3.2 Methods

3.2.1 *In vitro* phosphorylation of recombinant PDX-1 by PASK

HEK293 cells grown to 70% confluence in T-25 flasks were transfected overnight with 6 µg of plasmid encoding c-myc tagged-hPASK based in pcDNA3.1. DNA (6µg) in 225 µl ddH₂O was mixed with 25 µl 2.5 M CaCl₂. This mixture was then added drop by drop to 250 µl 2 X HBS in a sterile tube while vortexing. Empty expression plasmid (pcDNA3.1) was used to maintain a constant total amount of DNA among wells.

Cells were washed twice with ice-cold PBS. Lysis buffer (750 µl; 1% NP40; 50 mM NaCl; 1% sodium deoxycholate; 0.1% Sodium monododecyl sulfate (SDS); 50 mM Tris-HCl pH7.5; 2 mM EDTA; 10 mM Sodium Phosphate; 50 mM NaF; 1 mM Phenylmethylsulfonyl fluoride; 200 µM Sodium orthovanadate; 1X protease inhibitor cocktail solution and phosphatase inhibitor cocktail 1 & 2; Sigma) was added and the cells were collected by scraping. The mixture was transferred into a 1.5 ml centrifuge tube and was then placed on a rotating wheel at 4 °C for 30 min. to lyse the cells fully. The mixture was then centrifuged at 14,000 rpm for 5 min. to harvest the supernatant. C-myc antibody-conjugated beads (Santa-Cruz; 40 µl) were added to the supernatant and lysis buffer was added to give a final volume of 1ml. The supernatant was rotated at 4 °C overnight and the beads were collected by centrifugation at 3,000 rpm at 4 °C for 30 s, and washed 5 times with lysis buffer.

Immunoprecipitated PASK was washed twice in pre-kinase assay wash buffer (50 mM Tris-HCl; 100 mM NaCl and 10 mM MgCl₂, pH 7.4). The pellet was then resuspended in 60 µl kinase assay buffer (50 mM Tris-HCl pH 7.4; 10 mM MgCl₂;

0.2 mM ATP and 1mM DTT). The reaction mixture was preincubated at 30 °C for 1 h to autoactivate the kinase. [32 P] ATP (0.1 mM) and recombinant wild-type or T152A mutant PDX-1 protein (2 μ g) were added and mixed with the kinase and incubated at 30 °C for 1 h.

3.2.2 *In vitro* phosphorylation of recombinant PDX-1 by various kinases

Wild-type PDX-1 protein (1 μ g) or the mutant PDX-1 protein (2 μ g) were used in the assays. All kinases were active recombinant enzymes, either from commercial suppliers or from the University of Dundee. Each kinase was used at 50 ng per reaction, with the exception of the very active CaMKII (25 ng) and the less active Ark5 (100 ng) and SAPK2 (200 ng). The kinase buffer for PKC α was 10 mM HEPES pH 7.4, 10 mM 2-ME, 0.5 mM CaCl $_2$, 0.25 mg/ml phosphatidyl serine, 0.05 mg/ml diacylglycerol, 0.01% Triton X-100. CamKI was used in 20 mM Tris pH 7.5, 10 mM 2-ME, 1 mM CaCl $_2$, and 40 μ g/ml Calmodulin. CaMKII was activated by autophosphorylation in the presence of Ca $^{2+}$ /CaM and then used in 20 mM Tris pH 7.5, 10 mM 2-ME. All other kinases were used in 20 mM Tris pH 7.5, 10 mM 2-ME.

The kinase assays were carried out in duplicate at 30°C in the presence of 10 mM Mg-acetate and 0.1mM [γ 32 P]ATP. The [γ 32 P]ATP was freshly prepared for every set of experiments. The reactions were stopped by adding SDS-loading buffer and then analysed by SDS-PAGE, colloidal Coomassie staining and autoradiography. The reaction buffers for the kinetic study were supplemented with BSA in order to stabilize the kinases. The only exception was PKC α , because BSA inactivates PKC α . 1 μ g of recombinant wildtype (WT) PDX-1 was mixed with 0, 3, 10, 30, 100, 300, 1000 ng of SAPK2 (as indicated) and the kinase reactions were carried out at 30 °C. Time course was as indicated in the figures.

Recombinant wild-type (WT) PDX-1 (1µg) was mixed with 0, 3, 10, 30, 100, 300, 1000 ng of JNK1 (as indicated) and the kinase reactions were carried out at 30 °C. Initial rates were taken at different time points (as shown).

Recombinant wildtype (WT) PDX-1 (0 µg or 1 µg) was mixed with/ without 100 ng of JNK1 (as indicated) and the kinase reactions were carried out at 30 °C for 10 min or 60 min.

Time course of reactions for phosphorylation of substrates by other kinases were measured as described in the Figure legends.

I provided the recombinant wild-type (WT) and T152A PDX-1 and the *in vitro* phosphorylation kinase screening experiments and the following mass spectrometry phosphorylation site screening of JNK phosphorylated PDX-1 were done by Kinasource Ltd. Dundee, UK.

3.2.3 Mass spectrometry to identify sites on PDX-1 phosphorylated by JNK *in vitro*.

PDX-1 (5 µg) was incubated for 45 min. with 0.2 µg JNK as described above. The reaction was stopped with SDS and proteins were separated by SDS-PAGE. The PDX-1 band (~46 kD) was excised, destained, digested with trypsin. Phosphorylated peptides were analyzed by electrospray ionization mass spectrometry in both the positive and negative ion mode. Fragmentation of the phosphorylated species in the negative ion mode provides a “unique” daughter ion (m/z 79) which is characteristic of a phosphate group. A Pre79 precursor ion scan identifies those parent masses that yield the daughter ion with a m/z of 79 Da (PO_3^-). Given the known protein sequence, the peptide molecular weights obtained are sufficient to identify the phosphopeptides.

The PDX-1 sequence was inspected and some tryptic peptides, which may contain JNK sites, were predicted to be too large to be compatible with Pre79 parent ion scanning. In order to fragment PDX-1 further and generate peptides of compatible size, subsequent digestion was undertaken with chymotrypsin and the procedure was repeated.

I provided the recombinant wild-type (WT) and T152A PDX-1 and the *in vitro* phosphorylation kinase screening experiments and the mass spectrometry phosphorylation site screening of JNK phosphorylated PDX-1 were done by Kinasource Ltd. Dundee, UK.

3.2.4 Metabolic labeling with ^{32}P

INS-1(832/13) cells were grown to 70% confluence in T-75 flasks and infected for 24 h with viruses expressing wild-type or T152A mutant PDX-1 at an MOI of 100. After recovery for 24 h, cells were washed twice with phosphate-free DMEM medium (GIBCO) and then pre-incubated in the same medium for 1 h at 37 °C.

Cells were metabolically labeled for 4 h in phosphate-free medium containing 0.5 mCi/ml [^{32}P]-orthophosphate (Amersham, GE Healthcare) at 37 °C. Labeled cells were washed twice with ice-cold PBS and lysed for further immunoprecipitation. Lysis buffer and immunoprecipitation were as described above (Chapter 2, P81).

The IP beads were then collected by centrifugation at 3000 rpm at 4 °C for 30 s, and then washed 5 times with lysis buffer. The IP product was eluted with 30 µl sample buffer and boiled for 5 min. IP product was then migrated on a SDS-PAGE gel at 100V for 5 h and the gel was then silver stained and dried using a Bio-Rad Model 483 Slab Dryer at 80 °C for 1 h. Radioautography was then performed with Kodak BioMax MS Radioautography Film.

3.3 Results

3.3.1 *In vitro* phosphorylation of PDX-1 by PASK.

In preliminary studies, the *in vitro* phosphorylation of PDX-1 by PASK was examined in collaboration with Prof. Jared Rutter in University of Utah. PASK efficiently phosphorylated recombinant PDX-1 (Fig. 3.1) and a major phosphorylation site, Thr-152, was identified using mass spectrometry (Fig.3.5).

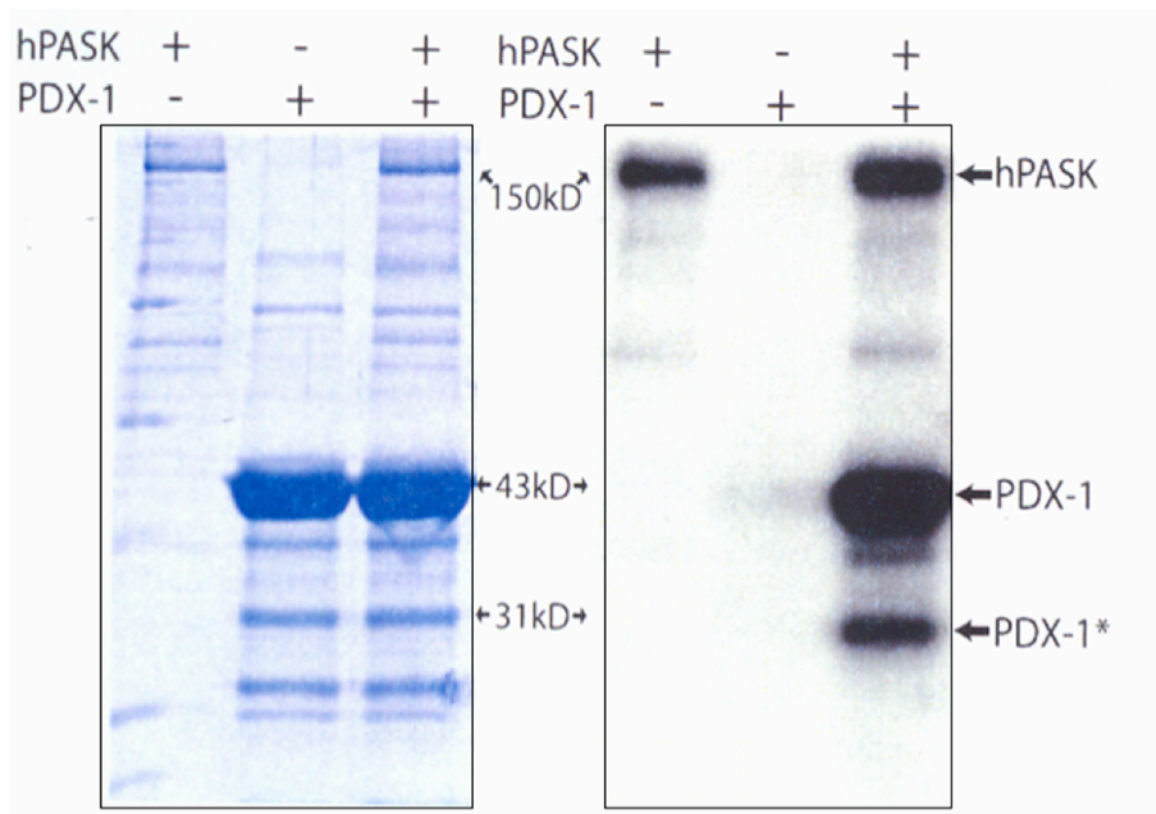


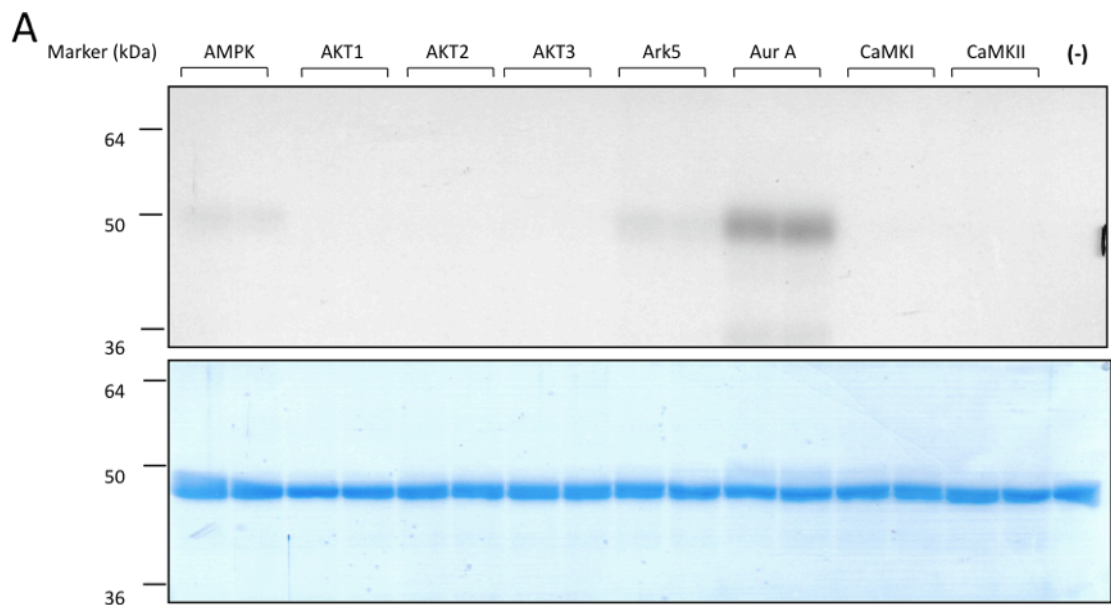
Fig. 3.1 PASK efficiently phosphorylates PDX-1 *in vitro*.

Purified human PASK, purified mouse PDX-1, or a combination were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP for 1 hour at 25°C. Proteins were then separated by SDS-PAGE and the gel visualized by Coomassie staining (left panel) or by autoradiography (right panel). Note: hPASK

autophosphorylation; PDX-1* is a C-terminal fragment of PDX-1. This experiment was done by Dr. Huai-xiang Hao, University of Utah.

3.3.2 Kinase screen for PDX-1 phosphorylation *in vitro*

We next determined whether PDX-1 may be a substrate for phosphorylation on Thr-152, or other sites, by other kinases. Thus, AMPK, AKT1, AKT2, AKT3, Ark5, Aur A, CaMKI, CaMKII, Erk2, SAPK2, JNK1, MSK1, PKA, PKC α , ROCK1, and Rsk1 were tested in *in vitro* phosphorylation assays in order to determine the potential involvement of other potential signaling pathways which may regulate PDX-1 function. As shown in Fig. 3.2A and 3.2B, JNK1, PKC α and Rsk1 phosphorylated PDX-1 efficiently and JNK1 and Rsk1 phosphorylated PDX-1 at more than one site. Moreover, PKC α also phosphorylated PDX-1 at one site. Aurora A and SAPK2 phosphorylated PDX-1, but only poorly. Additionally, there were very weak signals with AMPK, Ark5 and Erk2, which were not followed up.



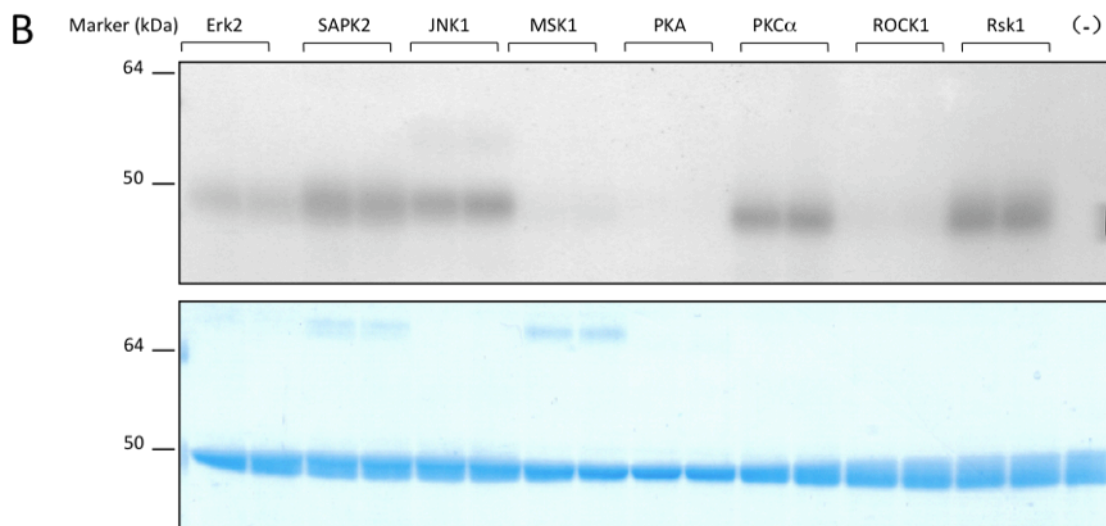
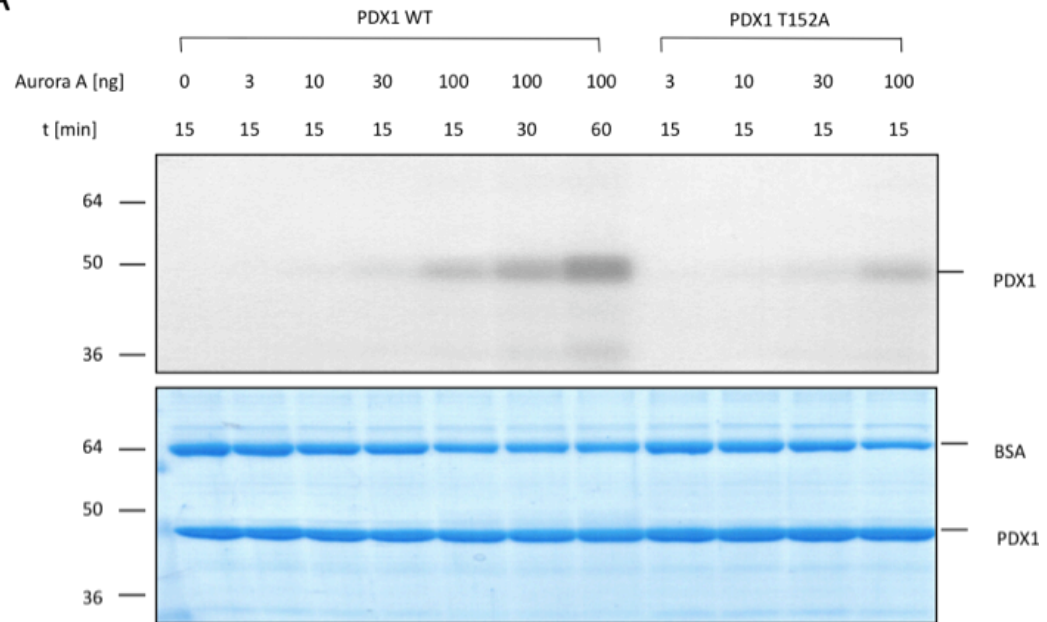


Fig. 3. 2. Kinase screen for PDX-1 phosphorylation *in vitro*

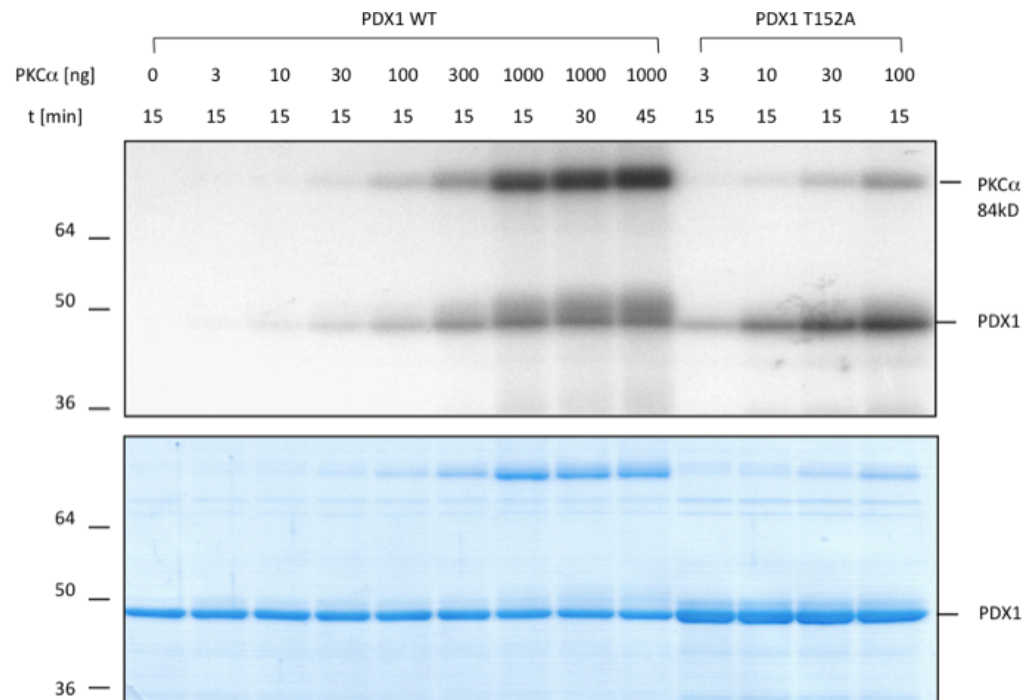
AMPK, AKT1, AKT2, AKT3, Ark5, Aur A, CaMKI, CaMKII, Erk2, SAPK2, JNK1, MSK1, PKA, PKC α , ROCK1, and Rsk1 were tested in *in vitro* phosphorylation assay in order to identify other potential pathways involved in PDX-1 regulation. Recombinant wildtype (WT) PDX-1 were provided by Rong AN and the *in vitro* phosphorylation kinase screening experiments were done by Kinasource Ltd. Dundee, UK.

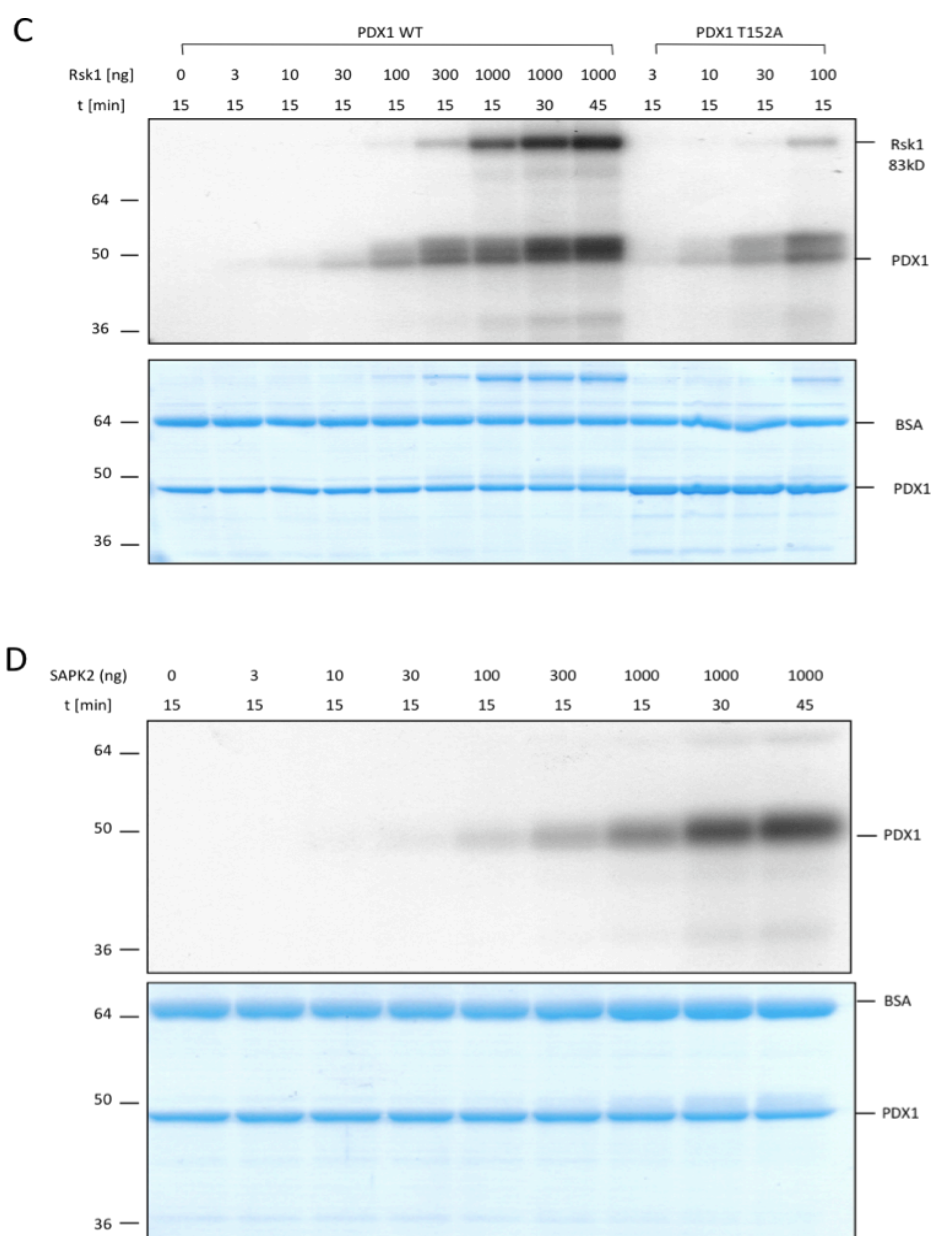
However despite of all the phosphorylation events detected for wild-type PDX-1 above, PDX-1 was not phosphorylated on Thr-152 *in vitro* by these kinases including Aurora, Rsk1, PKC α , c-Jun kinase (JNK). Nevertheless, these kinases phosphorylated PDX-1 *in vitro* at other, presently undefined, sites. In the cases of several of these kinases, including Rsk 1 and PKC α but not Aur A, a clear bandshift suggested multiple phosphorylation events (Fig. 3.3A-3.3D).

A



B





The phosphorylation with JNK1 was repeated by setting up the reactions with/without wild-type PDX-1 protein (Fig. 3.3E, 3.3F) and autoradiography demonstrated the existence of a labeled band in the reactions in the presence of PDX-1, while the signal increased as the incubation time was increased. These experiments confirmed that the phosphorylated protein was indeed PDX-1, and not autophosphorylated JNK1.

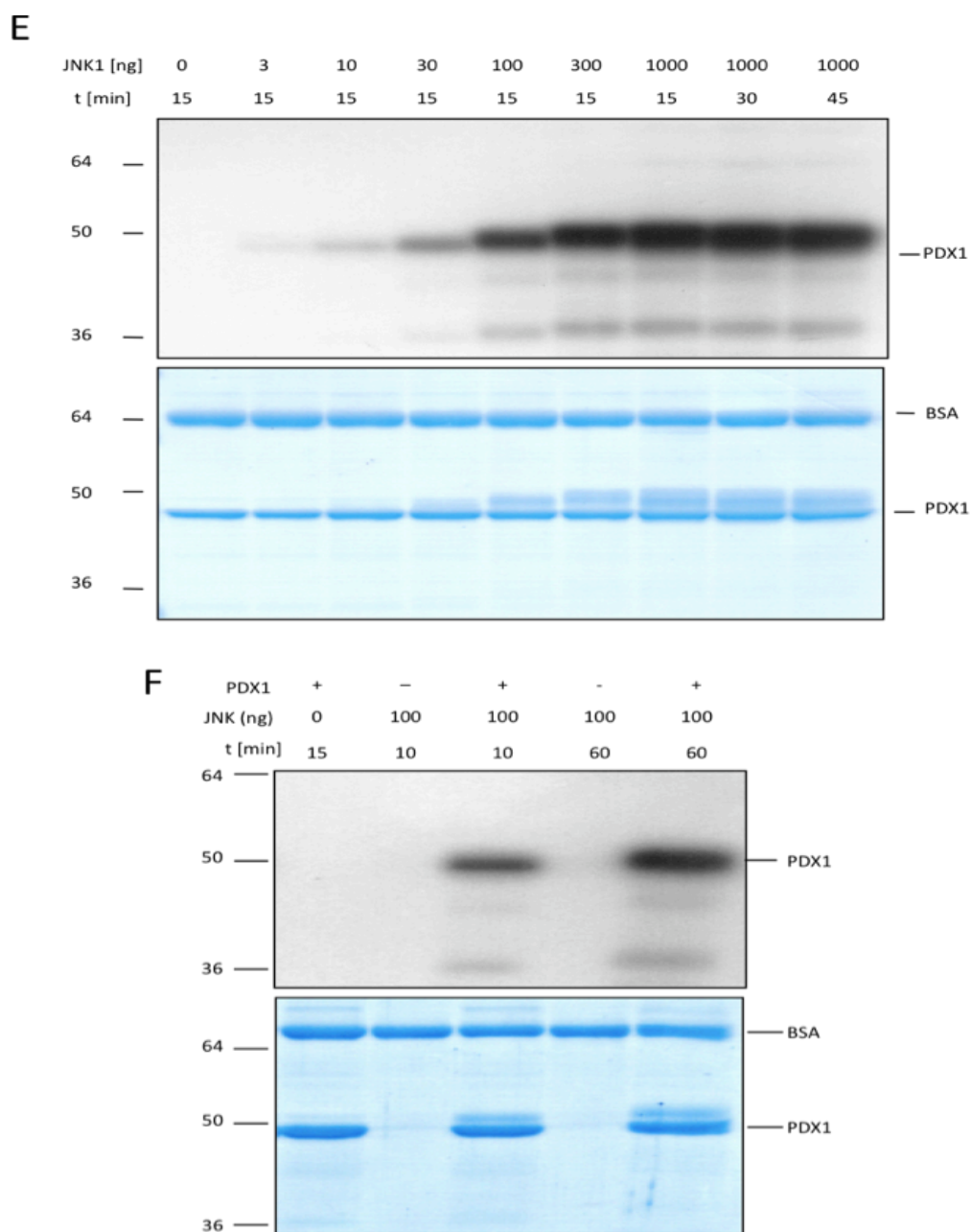


Fig. 3.3 *In vitro* phosphorylation of PDX-1 by Aurora A, PKC α , Rsk1, SAPK2 and JNK. Wild-type (WT) and the de-phosphorylation form mutant PDX-1 *in vitro* phosphorylation by (A) Auro A, (B) PKC α , (C) Rsk1, (D) SAPK2 and (E) JNK. (F) 0 μ g or 1 μ g of recombinant wild-type (WT) PDX-1 was mixed with/without 100 ng of JNK1. Reaction time course was indicated in figures. Recombinant wildtype (WT) and T152A PDX-1 were provided by Rong AN and

the *in vitro* phosphorylation kinase screening experiments were done by Kinasource Ltd. Dundee, UK.

3.3.3 Mass spectrometry phosphorylation site screening on recombinant PDX-1 phosphorylated by JNK *in vitro*

It was reported by Kawamori et al ⁷⁸ that PDX-1 translocates from the nuclei to the cytoplasm of pancreatic β -cells in response to oxidative stress in hamster β -cell-derived HIT-T15 cells and this oxidative stress-stimulated translocation is through the activation of the JNK pathway. However, the precise phosphorylation site(s) involved in the phosphorylation by JNK still remain unknown. In this chapter we showed that JNK phosphorylates PDX-1 *in vitro*, and we then applied phospho-site screening using mass spectrometry to identify the PDX-1 *in vitro* phosphorylation sites which could provide potential target(s) for further study in living cells.

Two major JNK1 sites on PDX-1 were mapped (Fig. 3.4). Ser66 was indicated to be phosphorylated from the Pre79 ion scan results of two peptides. One peak with m/z value of 602.3 represents L.EQGSPPDISPYEVPPL.A + Phospho (ST) and the other m/z 956.5 peak, which shows m/z 958.0 when searched using Mascot (the software search engine that uses mass spectrometry data to identify proteins from primary sequence databases; <http://www.matrixscience.com/>) represents F.TSSLGSLEQGSPPDISP.Y.E + Phospho (ST). Pre79 ion scan m/z 1286.7 peak (1287.67 in Mascot) represents R.SSGTIPSGGGGGGEEPEQDCAVTSGEEL.L + Phospho (ST) indicated that Thr-214 was phosphorylated.

Although the Pre79 ion scan results at 857.5 (858.7 in Mascot) represents R.SSGTIPSGGGGGGEEPEQDCAVTSGEEL.L + Phospho (ST) demonstrated phosphorylation on Ser-212, it is much more likely that Thr-214 is the phosphorylated site in this peptide as the tandem mass spectrometry (MS/MS) data could not separate the two. The Mascot also detected the Ser-269 site (m/z 868.1 and 651.3), but the peaks were almost invisible, indicating a very low level of

phosphorylation. Some tyrosine phosphorylation was also picked up by Mascot, but there were no corresponding peaks in the Pre79 ion scan spectrum.

Another major peak with m/z 802.4 represents -MNSEEQYYAATQL.Y + Acetyl (N-term); Oxidation (M). The other major Pre79 ion scan peaks with m/z of 630.3, 635.5, 644.4, 762.4, 773.4 (Na-adduct of 762.4), 802.4 and 1028.6 and the adducts were not identified (Fig.3.4).

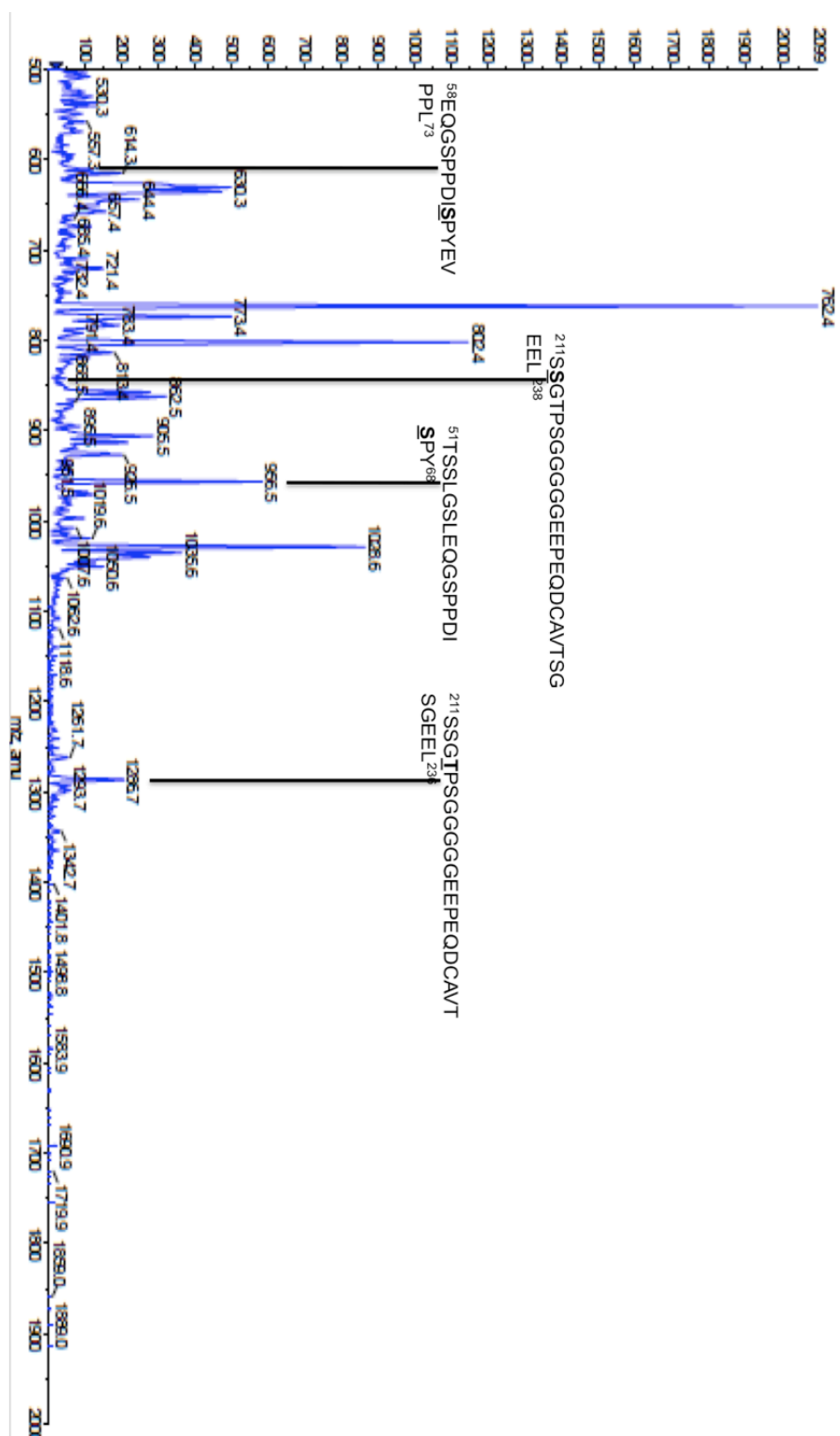


Figure 3.4. Mass spectrometry analysis of phosphorylation sites on recombinant PDX-1 generated by phosphorylation by JNK *in vitro*.

Pre79 ion spectrum of PDX-1 following phosphorylation by JNK1, and digestion with trypsin & chymotrypsin. PDX-1 (5 µg) was incubated for 45 min with 0.2 µg JNK. The reaction was stopped with SDS and separated by SDS-PAGE. The PDX-1 band was excised, destained, and digested with trypsin and chymotrypsin. Recombinant wildtype (WT) PDX-1 was provided by Rong AN and the *in vitro* phosphorylation kinase screening experiments and the mass spectrometry phosphorylation site screening of JNK phosphorylated PDX-1 were done by Kinasource Ltd. Dundee, UK.

3.3.4 *In vitro* phosphorylation of PDX-1 by PASK and generation of a phospho-Thr-152-specific antibody.

Human PASK was immuno-purified from HEK293 cells transfected with cDNA expressing human PASK. Recombinant wild-type or T152A PDX-1 protein was generated and purified as described in Chapter 2. PASK efficiently phosphorylated recombinant PDX-1 at one major phosphorylation site, Thr-152. Mutation of Thr-152 to Ala caused > 90 % suppression of ^{32}Pi incorporation from $[\gamma^{32}\text{-P}]$ ATP (Fig. 3.5). This experiment was conducted to verify the newly generated anti-phospho-T152 PDX-1 antibody. Parallel western blotting using phospho-specific antibody against T152 phosphorylation was carried out and the result clearly showed that PASK phosphorylated PDX-1 on Thr-152 *in vitro* and the anti-phospho-T152 PDX-1 antibody recognized the site with high specificity (Fig. 3.5).

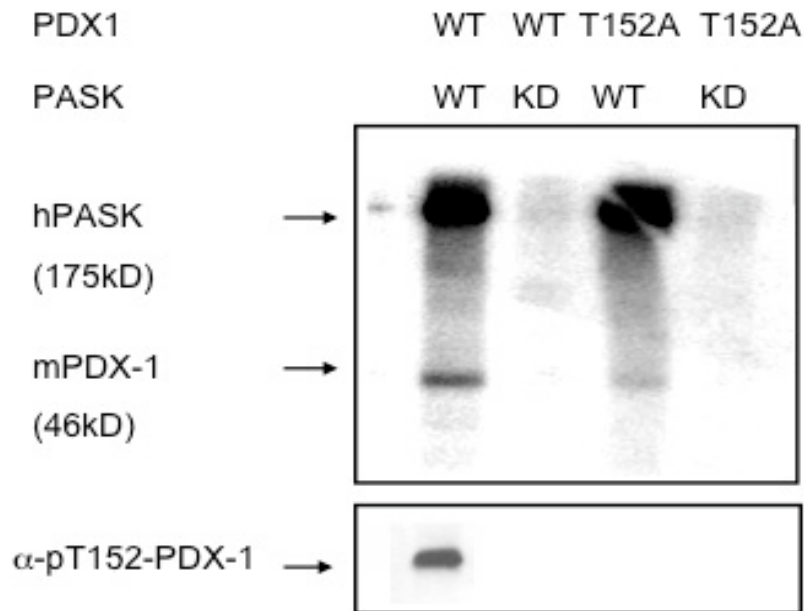


Fig. 3.5. PDX-1 is efficiently phosphorylated by PASK *in vitro*. Immunoprecipitated human PASK (both wild-type (WT) and kinase inactive forms (KD)) overexpressed in one T-25 flask HEK 293 cells, and 2 µg of purified *c-myc* tagged mouse PDX-1 (both wild-type and T152A mutant forms), were incubated with [γ - 32 P] ATP or non-radioactive ATP for 1 h at 30°C. Proteins were then migrated on SDS-PAGE gels and visualized by autoradiography. Western immuno-blot analyses were performed using the anti-phospho-T152-PDX-1 antibody on the parallel non-radioactive samples. The 175 kD band represents the autophosphorylated PASK. Wild-type (WT) and kinase inactive forms (KD) PASK were provided by Prof. Jared Rutter, University of Utah, USA (Chapter 2, Section 2.1.2).

3.3.5 Phosphorylation of PDX-1 at Thr-152 in intact INS-1(832/13) β -cells.

To determine whether Thr-152 represented a major phosphorylation site in living pancreatic β -cells, we next labeled INS-1(832/13) cells over-expressing wild-type or (de)phospho mimetic mutants mediated by adenoviral vectors (Chapter 2) with radio-labeled inorganic phosphate (32 Pi; Fig. 3.6). Incorporation of 32 P was clearly

evident in both the wild-type (Fig. 3.6A) and the Thr-152 Ala-mutated protein (Fig. 3.6B), while in the parallel experiment without ^{32}P i labeling the protein level of PDX-1 or GFP empty vector control was verified by western blotting. These data argue that the Thr-152 was unlikely to represent a major site of phosphorylation in intact cells, at least at 11.2 mM glucose.

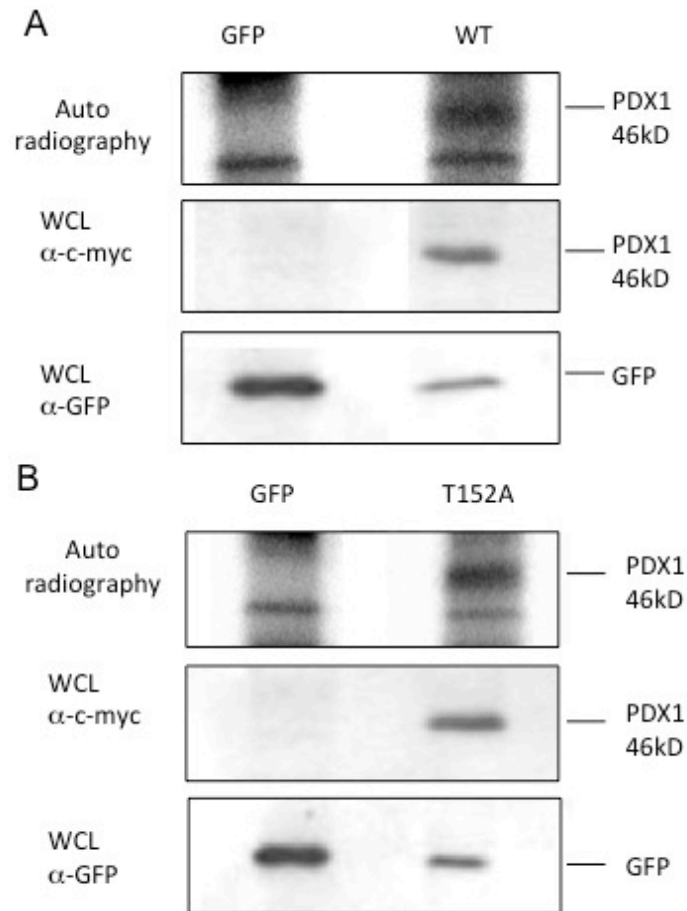


Fig. 3.6. Phosphorylation of PDX-1 in intact INS-1(832/13) cells.

INS-1(832/13) cells infected with wild-type (A) or T152A mutant (B) PDX-1-expressing viruses were then metabolically labeled and lysed. Immunoprecipitated samples were analysed by SDS-PAGE gel and radioautography was then performed. Parallel experiment without radiolabeling was also applied. PDX-1 or GFP expressing levels were examined using an anti-*c-myc* antibody (Roche) or anti-GFP antibody (Sigma) respectively. WCL: whole cell lysate.

3.4 Discussion

We show here that recombinant PDX-1 is efficiently phosphorylated *in vitro* at Thr-152 in the *homeodomain* by the nutrient-regulated kinase, PASK. Given the relatively low promiscuity of PASK towards a variety of substrates- PASK did not phosphorylate several other substrates tested, including the β -cell enriched transcription factors neuroD and MafA (personal communication, Prof. Jared Rutter, University of Utah), we speculated that this event may be of physiological significance in *transducing* intracellular signals generated by glucose to the β -cell nucleus.

Moreover, the site appeared to be specific for PASK as the T152A mutant PDX-1 recombinant protein showed no difference with wild-type PDX-1 in the *in vitro* phosphorylation by a series of kinases. This finding prompted us to investigate the phosphorylation of PDX-1 in living cells and, moreover, the role of Thr-152 phosphorylation on PDX-1 transactivational activity in living cells.

In order to study the physiological roles of Thr-152 phosphorylation in cells, adenoviruses expressing wild-type or Thr-152 (de) phospho- and phospho-mimetic forms of mutant were generated (as described in chapter 2) to over-express wild-type or mutant PDX-1 in living cells. Our novel finding obtained by metabolic labeling experiments with immuno-purified wild-type PDX-1, was of a direct phosphorylation signal, indicating that PDX-1 is phosphorylated under physiological conditions in living β -cells. This result suggests that reversible phosphorylation may regulate PDX-1 function as suggested in previous reports^{63, 135, 137, 142, 148, 154}. Our hypothesis, based on the unique Thr-152 phosphorylation site of PDX-1 specifically by PASK, was that the dephospho-form mutant on the site might very likely affect the phosphorylation status of PDX-1 in living cells if this

site represents the only, or one of a very few, phosphorylation sites. However, compared with the substantially decreased phospho-signal in recombinant T152A PDX-1 protein phosphorylated *in vitro*, the phosphorylation status of over-expressed T152A PDX-1 in β -cells (Fig. 3.6A) did not display a dramatic difference versus wild-type PDX-1 (Fig. 3.6B).

It has previously been reported that PDX-1 is phosphorylated at its N-terminus at S61 and S66 *via* a GSK-3-dependent pathway¹⁴². This finding suggests the possibility that additional kinases may phosphorylate PDX-1 at other sites. Here, we performed a series of *in vitro* phosphorylation reactions using recombinant PDX-1 and purified kinases in an effort to identify such kinases. Aurora, Rsk1, PKC α , JNK were all found to phosphorylate PDX-1 *in vitro*. Interestingly, it has been reported that induction of oxidative stress causes PDX-1 nuclear-cytoplasmic translocation via activation of JNK⁷⁸, although the phosphorylation site(s) have not been identified. Mass spectrometry was undertaken on recombinant PDX-1 phosphorylated by JNK *in vitro*, aiming to map the potential phosphorylation site(s) (Fig. 3.4). Although Thr-152 did not appear to be phosphorylated, the sequencing did pick up a few other novel phosphorylation sites (i.e. Thr-214 and, interestingly, Ser-66, as previously reported by Boucher *et al*¹⁴², though no evidence of phosphorylation at Ser-61¹⁴² by this kinase was obtained), which again, indicated that the phosphorylation by PASK or on Thr-152 may not be a major regulation for PDX-1 function in β -cells. Hence, in order to investigate the potential physiological role(s) for this phosphorylation event, more direct functional approaches are likely to be needed.

4. Chapter 4

**Phosphorylation of pancreatic
duodenum homeobox-1 (PDX-1) at
Threonine-152 is unlikely to mediate
nuclear uptake and DNA binding in
living β -cells.**

4.1 Introduction

In mature β -cells, PDX-1 is involved in the regulation of several key β -cell-specific genes which are critical for glucose-sensing and insulin synthesis (Chapter 1). Thus, PDX-1 was reported to bind to rat insulin I promoter A3/4 region and, by interacting with bHLH protein E47/Pan 1, binds to the E2 site to activate insulin gene transcription. In addition, PDX-1 has also been implicated in the regulation of the expression of the somatostatin, islet amyloid polypeptide (IAPP)^{90, 91}, glucokinase (GK)⁸⁸ and GLUT-2⁸⁷ genes. In mature β -cells, elevated glucose concentrations lead to the cytoplasmic nuclear-shuttling of PDX-1 which is accompanied by increased insulin promoter activity^{137, 138, 216, 222, 223}. This process may involve interaction of the *homeodomain* with the nuclear import protein, β -importin²²⁴. It has also been reported that changes in PDX-1 *homeodomain* DNA-binding activity may be involved in regulation of insulin promoter activity during nutrient-dependent regulation^{155, 222, 225}. Moreover, the *homeodomain* of PDX-1 plays a pivotal role in the recruitment of other transcription factors such as BETA2/Neuro D1, the E2A family proteins and coactivators including p300 and Bridge-1^{103, 104, 226, 227}.

Compared with the well-studied role for PDX-1 in pancreas development and β -cell function, less is known about its regulation, particularly at the post-translational level¹⁴². For many other transcription factors, including the steroid receptor superfamily²²⁸ and cytokine-activated 'STAT' (signal transducer and activator of transcription)²²⁹, a shift from the cytosol to the nucleoplasm is a major means of activation. Whether post-translational modifications of PDX-1, including phosphorylation, are involved in the nuclear uptake and activation of PDX-1 activity remains unclear.

In Chapter 3 we demonstrated that PDX-1 is phosphorylated under physiological condition in INS-1(832/13) cells. Moreover, we also have demonstrated that recombinant PDX-1 could be efficiently phosphorylated at a single residue, Thr-152, by purified PASK. Given the relatively low promiscuity of PASK towards a variety of substrates, the question we ask here is whether this phosphorylation event regulates the nuclear uptake of PDX-1 and may therefore contribute to changes in the transactivational capacity of PDX-1 in native β -cells exposed to high extracellular glucose concentrations ¹³⁸.

Here, we have generated phospho- and dephospho- mimetic PDX-1 mutants at Thr-152 to explore the likely effects of phosphorylation at this site on PDX-1 function, and examined the possible role(s) played by PASK in two β -cell systems, INS-1(832/13) cells and INSr $\alpha\beta$ cells ²¹⁶. The latter cells are INS-1-derived stable cell lines expressing PDX-1 under the control of the reverse tetracycline-dependent transactivator (rtTA). In INSr $\alpha\beta$ cells (though not in other α -cells and enteroendocrine cell lines) ²³⁰, PDX-1 potentially inhibites preproglucagon gene expression, allowing a switch from α -to β cell lineage.

4.2 Methods

4.2.1 Dispersed human islets preparation.

Human islets (produced by Prof. P. Johnsson, Department of Surgery, University of Oxford) were cultured in RPMI-1640 medium containing 11.2 mM glucose, 10% FCS, streptomycin and penicillin.

Human islets were washed twice with PBS and treated trypsin for 10 min at 37°C with shaking. The islets were sedimented by centrifugation at 1,000 rpm and homogenized in minimal volume of trypsin by pipeting. 15 ml islet medium was added to the homogenized cells to wash out the trypsin. Cells were then collected by centrifugation, resuspended and distributed onto coverslips treated with poly-lysine and covered with medium.

4.2.2 Transfection and Immunoprecipitation

MIN6 cells grown to 70% confluence in 6-well plate were transfected with *c-myc*-tagged pcDNA3.1-PDX-1 (wild-type or mutants) following standard lipofectamine transfection method. The transfected cells were prepared for immunocytochemistry and confocal imaging as described below. This was done by Dr. Gabriela da Silva Xavier.

HEK293 cells grown to 70% confluence in T-25 flasks were transfected by *c-myc*-tagged pcDNA3.1-hPASK plasmid (6 µg) for 16 h using Ca^{2+} -phosphate transfection. Immunoprecipitation follows the method described in Chapter 2.

4.2.3 Immunocytochemistry and Confocal Imaging

cDNA encoding *c-myc* tagged wild-type or T152 mutant forms of PDX-1 in plasmid pcDNA3.1 was transfected in MIN6 β -cells. Cells were cultured in medium containing 25 mM glucose for 24 h prior to treatment for 16 h in medium containing 3 mM glucose, and then medium containing 3 or 25 mM glucose for a further 6 h. Immunocytochemistry was performed as previously described in chapter 2.

INSr $\alpha\beta$ cells were infected with viruses encoding wild type or mutant forms of PDX-1, or virus overexpressing human PASK at an MOI of 100. Cells were cultured in medium containing 11.2 mM glucose for 24 h prior to treatment for 16 h in medium containing 3 mM glucose, and then medium containing 3 or 20 mM glucose for a further 6 h. Dispersed human islets on coverslips were cultured in medium containing 11.2 mM glucose for 24 h prior to treatment for 16 h in medium containing 3 mM glucose, and then medium containing 3 or 17 mM glucose for a further 6 h. Immunocytochemistry was performed as previously described in chapter 2.

Monoclonal primary antibody for PDX-1 against *c-myc* (Roche) was used at 1:1000, fluorophore-conjugated Alexa 568 goat-anti-mouse antibody was used at 1:1000. Monoclonal primary antibody against human PASK (provided by Prof. Jared Rutter, University of Utah) was used at 1:100, fluorophore-conjugated Alexa 568 anti-mouse antibody was used at 1:1000.

For double-primary antibody staining in dispersed human islets, primary antibody against PASK was used at 1:100 and mixed with either anti-insulin generated from

guinea pig (Invitrogen; 1:1000) or anti-PDX-1 generated from guinea pig (kindly provided by Prof. Chris Wright, Vanderbilt; 1:1000). Fluorophore-conjugated Alexa 488 anti-mouse antibody was used at 1:1000, Alexa 568 anti-guinea pig antibody was used at 1:1000.

Cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) to allow visualization of nuclei¹³⁸. Confocal Images were captured using a Leica SP2 upright laser scanning confocal microscope (X63/1.32 oil-immersion lens) equipped with a krypton/argon laser (488 and 568 nm excitation lines) and a UV light. Images were analyzed off-line using VolocityTM4.0 software.

Quantification of PDX-1 localization in MIN6 cells was performed by Dr. Gabriela da Silva Xavier using Image J software. Quantification of PDX-1 localization in INS $\alpha\beta$ cells was analyzed using VolocityTM offline software. ≥ 30 cells per coverslip were randomly selected, and PDX-1 nuclear to cytosol distribution ratio was analyzed (cytosol distribution = [whole cell distribution]- [nuclear distribution]). Experiments were carried out three times in triplicates. Representative pictures were shown.

4.2.4 Preparation of recombinant adenoviruses and viral infection

Recombinant adenoviruses expressing the wild-type (WT) and mutant forms of PDX-1 (T152A, T152E, T152D) were prepared using the AdEasy system with the WT and mutant forms of PDX-1 plasmids. The generation of adenoviruses was as described in²³¹ and in Chapter 2. INS $\alpha\beta$ cells were infected with various adenoviruses at 100 multiplicity of infection (MOI) for 5 h and maintained at 11.2 mM glucose for 24 h before subsequent experiments. The efficiency of adenovirus-mediated gene transfer was typically ~85%.

4.2.5 Molecular modeling

The crystal structure of the PDX-1 dimer bound to two double-stranded DNA duplexes (2H1K) was prepared for calculation in the following way: missing side-chains were remodelled as required; hydrogen atoms were added consistent with pH 7; the complex was soaked in a 10 Å layer of water which required 3829 water molecules. The complex was relaxed by 2000 cycles of conjugate gradient minimization. A phosphate group was built onto each Thr152 (residues A6 and B6 in 2H1K) and the phospho-PDX-1 complex relaxed with a further 2000 steps of conjugate gradient minimization. Graphics manipulations and energy minimizations were performed with InsightII (2005) and Discover (2.98) from Accelrys. The molecular modeling experiment was done by Dr. Richard Session, University of Bristol.

4.2.6 Sequencing and Mass Spectrometric Analysis of Phosphorylation sites mapping.

T-75 flasks (30) of INS-1(832/13) cells were infected at an MOI of 50 with adenoviruses expressing both wild-type PDX-1 and/or PASK for 48 h. Cells were then collected and lysed for immunoprecipitation as described above. The IP product was migrated on 10% (w/v) SDS-PAGE gels and stained with colloidal commassie blue (SimplyBlue SafeStain, Invitrogen).

In each case, an obtained 1D band with good intensity, by colloidal commassie staining, and migrating at 46 kD, was processed by in-gel digestion with Lys-C. The digested sample was then extracted and were taken through three stages of analysis using a 4000 QTRAP (Applied Biosystems) mass spectrometer. The stages are as follows: Identification of protein(s) by nLC-MS-MS; parents of -79 (PO3-) analyses to detect phosphorylated peptides; data interpretation including *de novo*

assignment of phosphorylation sites) and final reporting. Thr-152 phosphorylation was further detected using *OrbiTrap* by targeting site-of-interest-containing peptides²³².

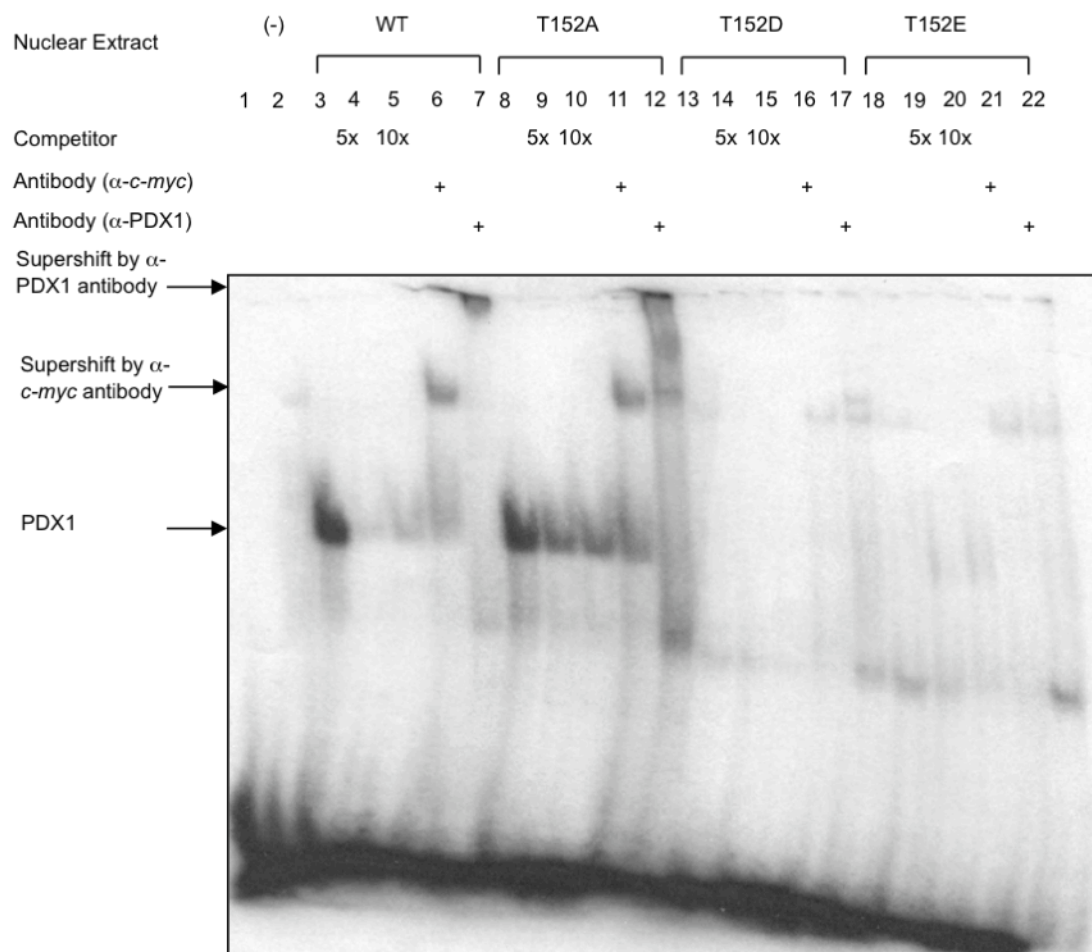
The mass spectrometry experiments were done by Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

4.3 RESULTS

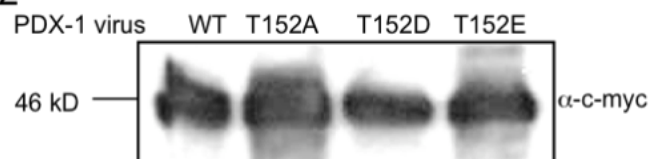
4.3.1 DNA-binding activity of PDX-1 phospho- and dephospho-mimetic mutants.

To explore the possible impact of Thr-152 phosphorylation on PDX-1 DNA binding, electrophoretic mobility shift assays were performed. These indicated that over-expressed wild-type or Thr-152 PDX-1 efficiently bound the oligonucleotide derived from the mouse preproinsulin promoter A3 element in a complex which was supershifted by anti- (*myc* or PDX-1) antibody, whereas PDX-1^{T152D} or PDX-1^{T152E} largely failed to demonstrate the formation of a complex which could be super-shifted (Fig. 4.1 A1).

A1



A2



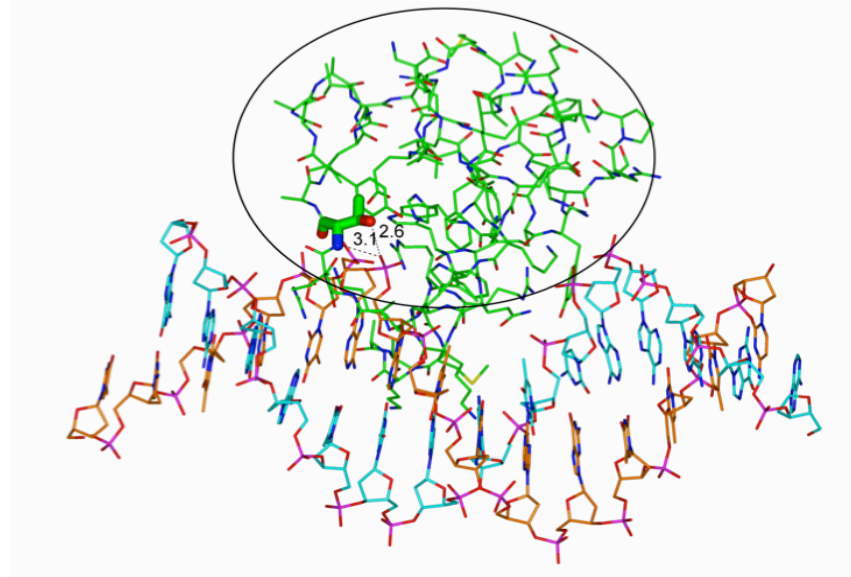
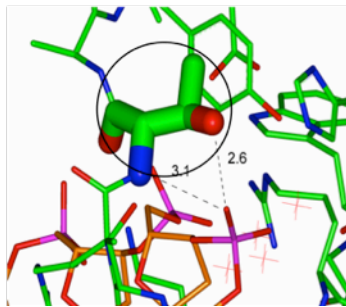
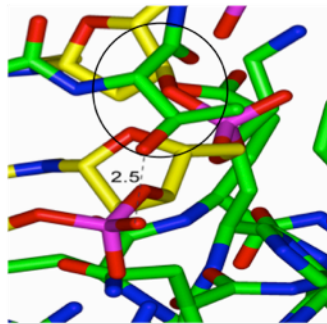
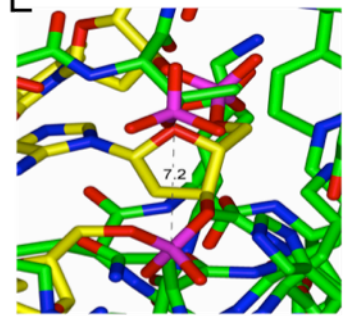
B**C****D****E**

Fig. 4.1. Dephospho- and phospho-mimetic mutants at Thr152 affect PDX-1 homeodomain DNA-binding capacity.

A1. Nuclear extracts from HEK293 cells transduced with adenoviruses to over-express wild-type or mutant forms of PDX-1 were incubated with a ^{32}P -labeled oligonucleotide corresponding to the mouse I insulin promoter (A3/A4 element), with or without 1 ml rabbit anti-PDX-1 serum for supershift assay. **A2.** 30 μg of nuclear lysates from HEK293 cells infected by indicated viruses were migrated on SDS-PAGE. The expression level of overexpressed PDX-1 was shown by immuno-blotting using anti-c-myc antibody. **B.** Structural mimicking of PDX-1 on DNA binding. The circled upper structure shows PDX-1 protein and the lower structure represents the bound DNA. Hydrogen bonds between Thr-152 and the DNA phosphate group are

shown as black dashed lines. **C.** The structure of PDX-1 DNA binding with the amplified Thr-152 residue circled in the middle. Hydrogen bonds between Thr-152 and the DNA phosphate group are shown as black dashed lines with the length of bonds. **D.** The circled free Thr-152, when minimized shows how the Thr-152-OH hydrogen bonds tightly to the lower DNA phosphate in purple with a bond length of 2.6 Å. **E.** The structure of Thr152-phosphate and DNA phosphate binding complex. When the Thr-152-phosphate is energy minimized, the structure of Thr-152 and DNA phosphate turn to repulsion with an increased bond length of 7.2 Å, given the constraints of the rest of the system (Molecular modeling was done by Dr. Richard Session, University of Bristol).

To explore the underlying molecular changes responsible for the loss of DNA binding in the phospho-T152-mimetic mutants, we also deployed molecular modeling of the Thr152-preproinsulin promoter A3 element DNA bound to the PDX-1 *homeodomain* protein. Incorporation of a phosphate group at Thr-152 was predicted by this approach to interfere with an interaction with the DNA phosphate backbone, likely to be important for the tight binding shown in Fig. 4.1B. Correspondingly, when the T152-phosphate was energy minimized (Fig. 4.1C), the structure of the T152-phosphate and DNA phosphate-binding complex changed from tight binding (Fig. 4.1D) to repulsion.

4.3.2 Subcellular localization of phospho- and dephospho-mimetic PDX-1 mutants.

cDNA encoding *c-myc* tagged wild-type or T152 mutant forms of PDX-1 in plasmid pcDNA3.1 was transfected to MIN6 β -cells. At low glucose concentrations (3 mM), both wild-type and T152A mutant PDX-1 remained very close to nuclear periphery and in the cytoplasm. At elevated glucose concentrations (30 mM) a translocation into the nucleoplasm was observed (Fig. 4.2). In contrast, T152D- and T152E-PDX-1 displayed a predominant cytosolic distribution at both low and high

glucose concentrations (Fig. 4.2)

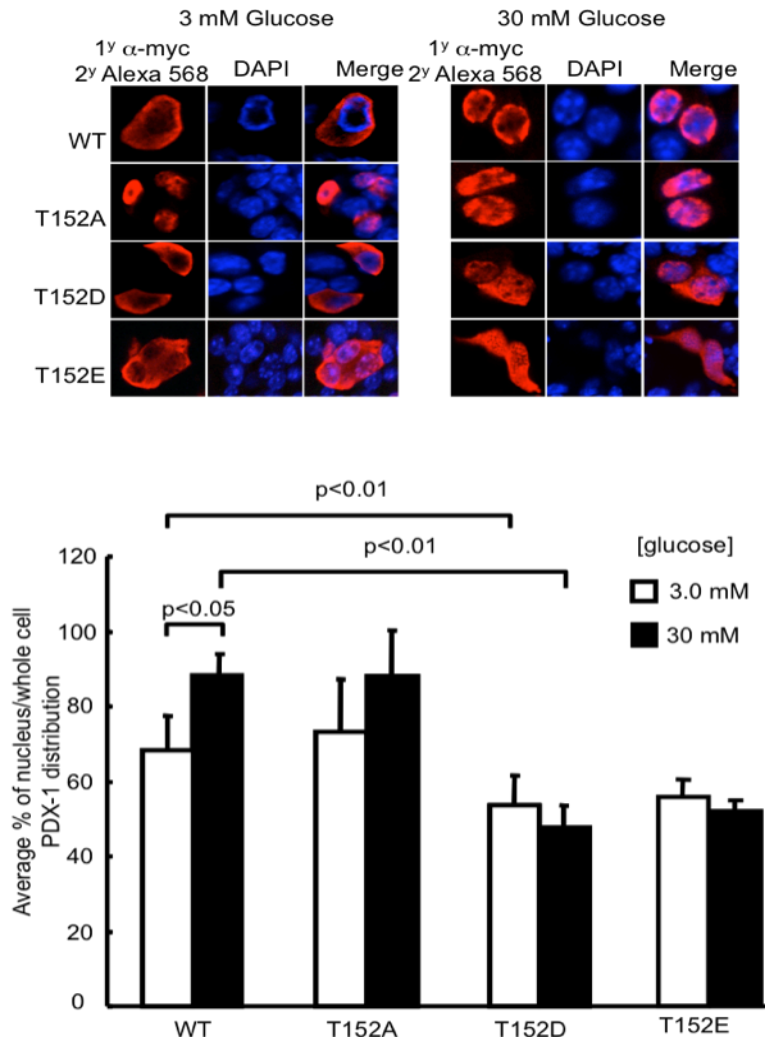


Fig. 4.2. Subcellular distribution of wild-type or mutant forms of PDX-1 examined by immunocytochemistry in MIN6 cells.

Wild-type or mutant forms of PDX-1 were induced in MIN6 cells by transfection with lipofectamine. Cells were cultured at 3mM glucose for 16 h and then treated with 3mM or 30mM glucose for 6 h. *c-Myc*-tagged PDX-1 was detected using anti-*c-myc* antibody (Roche) and Alexa 568 (Molecular Probe), and nuclear staining was achieved using DAPI (Immunocytochemistry were done by Dr. Gabriela da Silva Xavier; two-tailed students' t-test with bon-ferroni correction was used in statistical analysis).

We next expressed wild-type or mutant forms of PDX-1 in the INSra β cell line, a convenient model of a neuroendocrine precursor with properties of both α - and β cells²¹⁶. As previously described in MIN6 cells, elevated glucose concentrations led to a partial redistribution of c-myc-tagged PDX-1 from the cytosol to the nuclear compartment, an effect preserved in the T152A mutant (Fig. 4.3). By contrast, the acidic mutants of PDX-1 displayed lowered nuclear immunoreactivity at 3 mM glucose and a further decrease in the ratio of nuclear/cytosolic immuno-reactivity when the glucose concentration was raised to 20 mM.

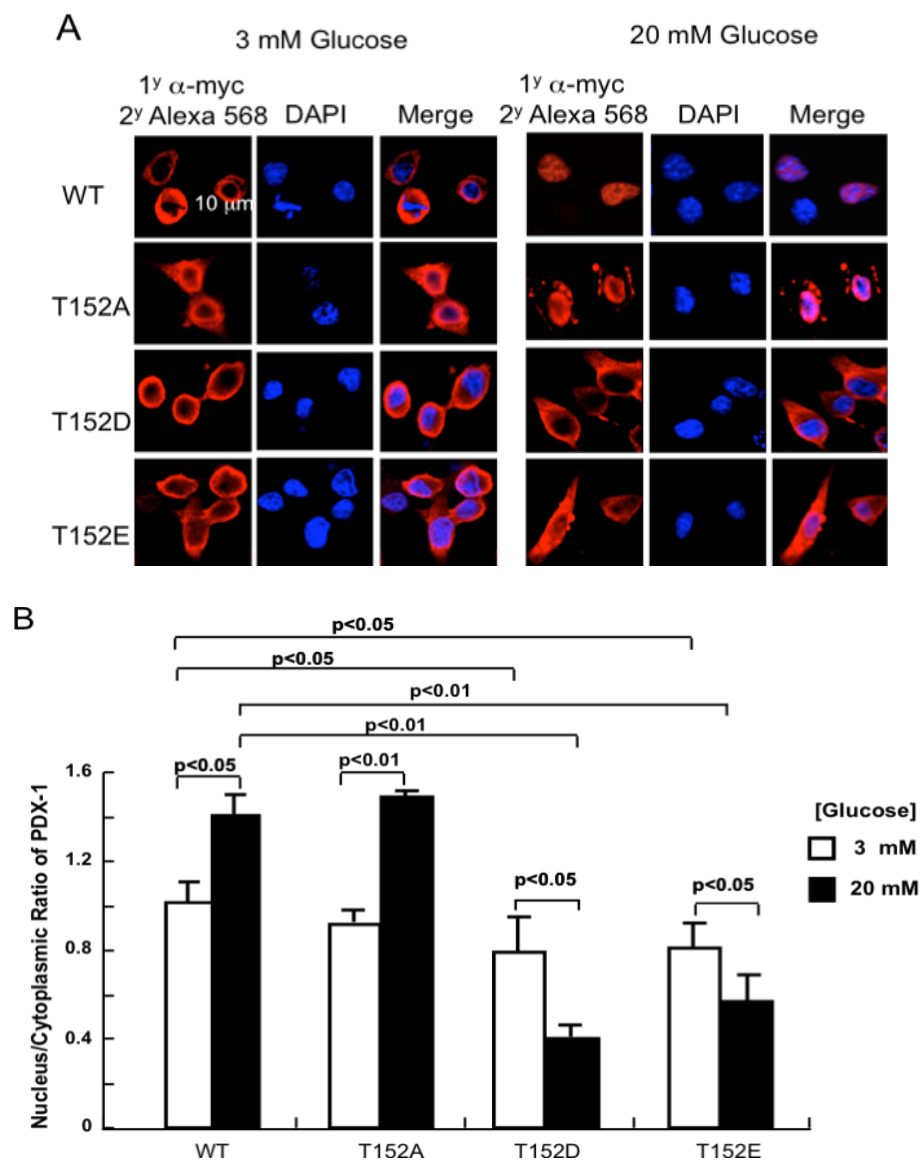


Fig. 4.3. Dephospho- and phospho-mimetic mutation of Thr-152 affects

PDX-1 nuclear-cytoplasmic distribution.

A. Immunocytochemical analysis of the subcellular distribution of PDX-1-c-*myc*. Wild-type, alanine, aspartic acid and glutamic acid mutant forms of PDX-1 were introduced into INSr $\alpha\beta$ cells by infection with the corresponding adenoviruses. **B.** The ratio of nuclear PDX-1 protein to cytosolic PDX-1 distributed in the cells was taken under different condition using VolocityTM 4.0 quantification software. (Mean $\pm$ SEM, $n = 3$; two-tailed student's *t*-test, Microsoft ExcelTM.)

4.3.3 Transcriptional activity of wild-type PDX-1 and of phospho- and dephospho-mimetic mutants.

To determine whether phosphorylation of PDX-1 at Thr-152 may affect the transcriptional activity of PDX-1 in intact cells, we over-expressed wild-type or mutant PDX-1 in INSr $\alpha\beta$ cells. Previous study demonstrated no obvious effect of over-expressed PDX-1 on insulin gene expression²¹⁶, whereas preproglucagon gene expression level was suppressed indicating an α - to β - cell lineage switch²¹⁶. We monitored the expression of preproglucagon (PPG) mRNA in INSr $\alpha\beta$ ²¹⁶. In the presence of doxycyclin and adenoviruses overexpressing wild-type or mutant PDX-1, these cells displayed an induction of mouse PDX-1 mRNA (Fig. 4.4A), whereas PDX-1 level in cells infected by adenoviruses overexpressing PASK or GFP remains the same with cells without any induction (Fig. 4.4A). Meanwhile, cells with overexpressed wild-type or T152A PDX-1 as well as doxycyclin-induced PDX-1 displayed decreased level of endogenous preproglucagon mRNA²¹⁶ (Fig. 4.4B, 4.4C); whereas cells with overexpressed PASK or GFP displayed no difference in preproglucagon mRNA expression compared with the control cells without any induction (Fig. 4.4B, 4.4C). Adenoviral transduction of the cells with wild-type or T152A mutant PDX-1 led to equally efficient suppression of PPG

mRNA. By contrast, PDX-1^{T152D} or PDX-1^{T152E} failed to alter PPG mRNA levels compared to infection with null adenovirus. Likewise, co-infection with both PASK-expressing viruses and PDX-1-expressing viruses failed to further affect the transcriptional activity (Fig. 4.5B, 4.5C).

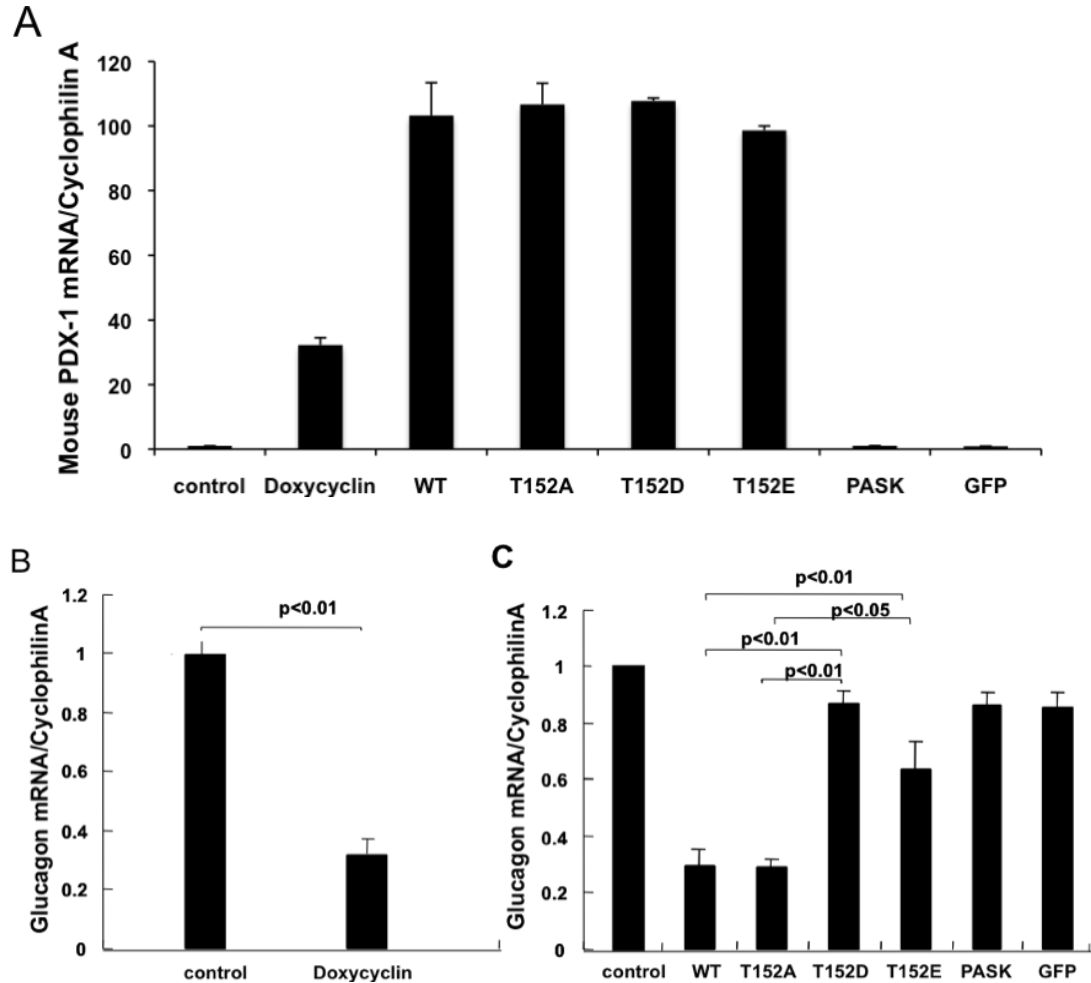


Fig. 4.4. Dephospho- and phospho-mimetic mutation of Thr-152 affects PDX-1 transcriptional activity.

A. INS $\alpha\beta$ cells were cultured in the presence of 500 ng/ml doxycyclin for 24 h or infected with wild-type or mutant forms of PDX-1 at 100 MOI for 24 h leading to increased levels of PDX-1 mRNA expression. **B.** INS $\alpha\beta$ cells were cultured in the presence of 500 ng/ml doxycyclin for 24 h leading to decreased levels of preproglucagon mRNA expression. **C.** RNA was

extracted from cells infected with wild-type or mutant forms of PDX-1 at 100 MOI for 24 h. Cyclophilin A, PDX-1 and glucagon gene expression were monitored by real time RT-PCR. (Mean $\pm$ SEM, $n=3$; two-tailed student's t -test, Microsoft ExcelTM.)

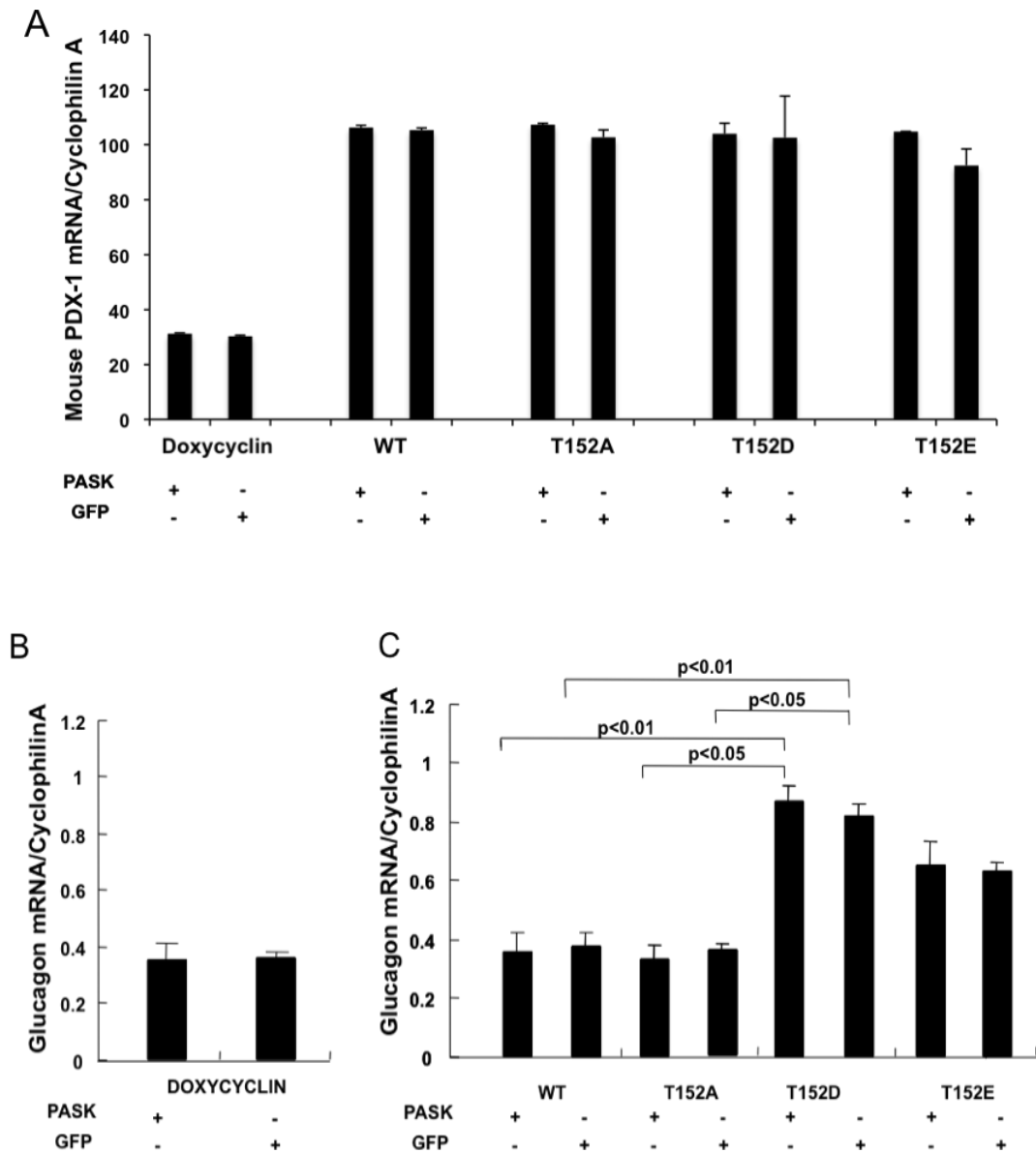


Fig. 4.5. Dephospho- and phospho-mimetic mutation of Thr-152 affects PDX-1 transcriptional activity.

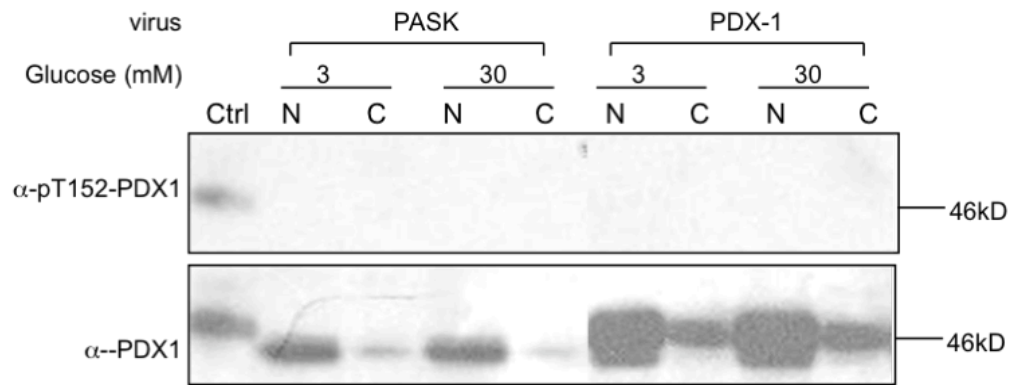
PASK or GFP viruses were added to the cells at MOI 100 treated under

the same conditions as **Fig. 4.4A**, for 24 h. RNA was extracted and relative PDX-1 (**A**) and glucagon (**B**) gene expression was determined by real time RT-PCR for the effect of the indicated mutant versus null (GFP) virus control. (Mean $\pm$ SEM, $n = 3$; two-tailed student's t -test, Microsoft ExcelTM.)

4.3.4 Phosphorylation of PDX-1 at Thr-152 in intact INS-1(832/13) cells.

In order to determine whether Thr-152 represented a major phosphorylation site for PDX-1 in living β -cells, we deployed in studies described in Chapter 3, metabolic labeling (Chapter 3, Fig. 3.6, P92). However, the incorporation of [³²Pi] in immunoprecipitated wild-type or T152A mutant PDX-1 was not different, indicating that Thr-152 is not likely to be a major phosphorylation site for PDX-1 in living β -cells or is one of many. To be able to look at the phosphorylation status of PDX-1 on Thr-152 in living cells, a phospho-specific antibody was raised (by Pacific Immunology) against the region ¹⁴⁵EENKRTRpTAYTRAQ¹⁵⁸. The phospho-Thr-152 antibody specifically recognized the recombinant wild-type PDX-1 protein phosphorylated by active PASK *in vitro* (Chapter 3, Fig. 3.5, P92). However, this antibody failed to recognize a specific band in either whole cell lysate (Fig. 4.6B), or cytoplasmic and nuclear extracts (Fig. 4.6A), despite detecting PDX-1 phosphorylated *in vitro* at Thr152 using purified PASK.

A



B

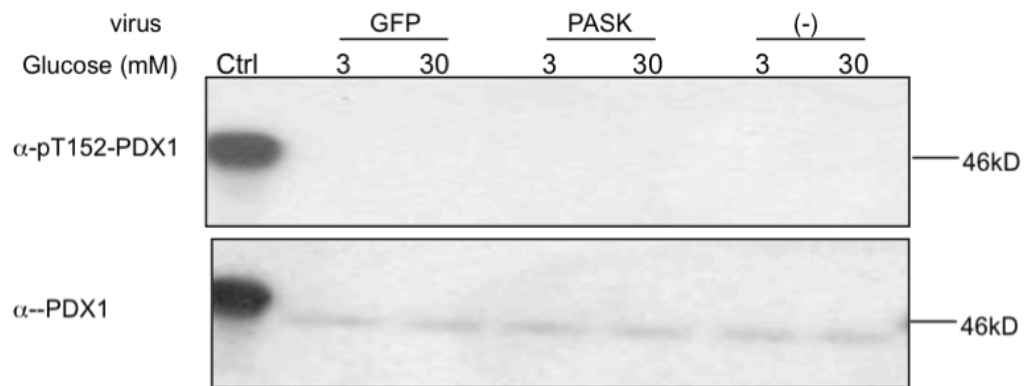
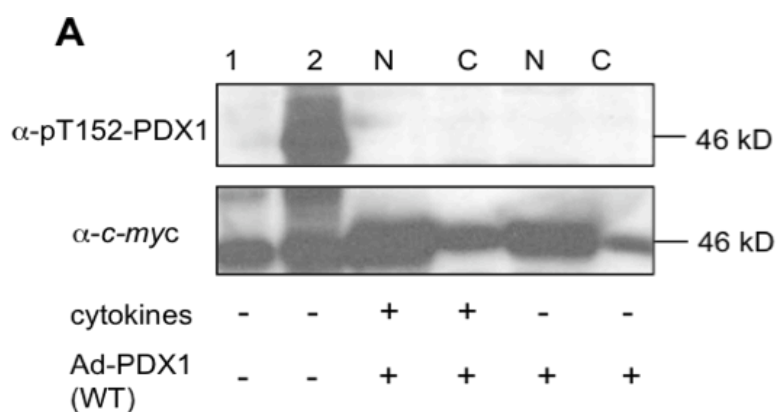


Fig. 4.6. Thr-152 phosphorylation in living β -cells (A) MIN6 infected with wild-type PDX-1 or PASK were cultured with 3 mM or 20 mM glucose for 6 h after 3 mM glucose for 16 h. Cytoplasmic (C)/ nucleic (N) extract were obtained; recombinant PDX-1 protein (Ctrl), phosphorylated *in vitro* by wild-type PASK (WT), was used as control. (B) MIN6 cells with/without overexpressed PASK/GFP as well as normal MIN6 cells were cultured with 3 mM or 20 mM glucose for 6 h after 3 mM glucose for 16 h. Whole cell lysates were obtained. In both cases, anti-phosphoT152-PDX-1 antibody and polyclonal rabbit anti-PDX-1 antibody were used in western blotting (representative blots were shown, $n=3$).

Given that unphosphorylated Thr-152 was shown to be important for maintaining normal PDX-1 function and DNA binding (Fig.s 4.1-4.5), we next explored the possibility of a phosphorylation event at this site under patho-physiological conditions, notably including the presence of the proinflammatory cytokines, tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin-1 β (IL-1 β) ²³³. However, no obvious sign of phosphorylation on Thr-152 was detected using anti-phospho-Thr-152 with the presence of cytokines (Fig. 4.7A and 4.7C). Likewise, co-infection with adenovirus expressing PASK also failed to affect phosphorylation of PDX-1 at Thr-152 (Fig. 4.7B).



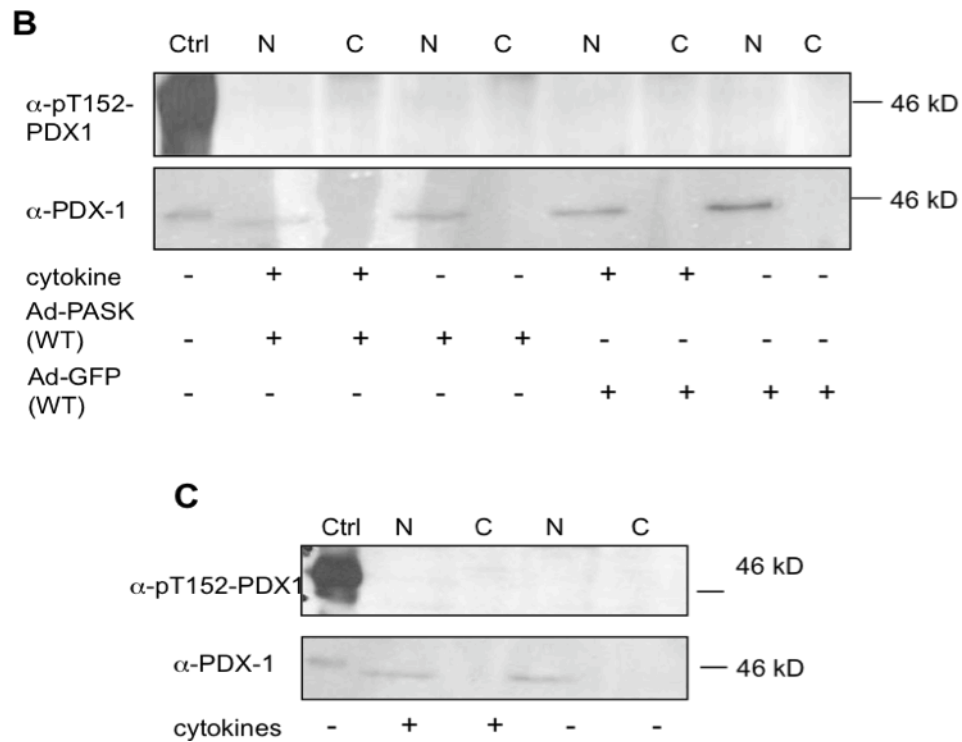


Fig. 4.7. PDX-1 is not phosphorylated at Thr-152 in intact INS-1(832/13) cells under a variety of conditions.

INS-1(832/13) cells infected with wild-type PDX-1 (A) or PASK/GFP (B) expressing viruses, cells without viruses (C) as controls. After 24 h, cells were treated with/without a cytokine mix containing TNF- α , IFN- γ and IL-1 β (PreproTech EC) for 6 h (51). Cytoplasmic (C)/nucleic (N) extracts were obtained; recombinant PDX-1 protein (Ctrl) phosphorylated *in vitro* by kinase inactive form PASK (DN) (lane 1, A) or wild-type PASK (WT) (lane 2, B and Ctrl in B, C) as control. Anti-phospho-PDX-1 antibody, anti *c-myc* antibody or polyclonal rabbit anti-PDX-1 antibody were used in western blotting respectively (representative blots were shown, $n=3$).

4.3.5 Mass spectrometry and phosphorylation site mapping

In an effort to confirm our findings concerning phosphorylation on Thr-152 in intact cells, we next performed mass spectrometry analysis of immunopurified PDX-1 from INS-1(832/13) cells cultured at 20 mM glucose co-overexpressed with both wild-type PDX-1 and PASK (Fig. 4.8). We used OrbiTrap, which traps ions with a specific mass to charge ratio and analyses mass with a high mass accuracy, high resolving power and a high dynamic range²³².

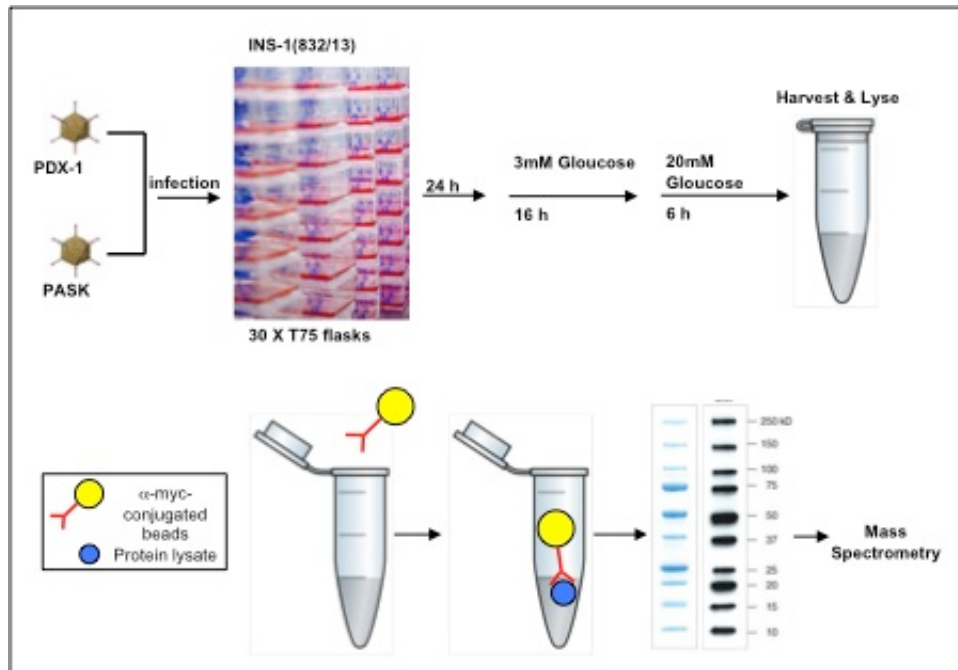


Fig. 4.8. Illustration of sample preparation for mass spectrometry sequencing.

T-75 flasks (30) of INS-1(832/13) cells were infected at an MOI of 50 with adenoviruses expressing wild-type PDX-1 and PASK for 48 h. Cells were then collected and lysed for immunoprecipitation using anti-*c-myc* antibody-conjugated beads (Santa Cruz). The IP product was migrated on 10% (w/v) SDS-PAGE gels and stained with colloidal comassie blue (SimplyBlue SafeStain, Invitrogen). Immuno-purified PDX-1 on SDS-PAGE was

provided by Rong AN. In-gel digestion and phosphorylation site mapping by mass spectrometry was done by Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

The results of the OrbiTrap analysis of sample PDX-1 after Lys-C digestion (Fig. 4.9) showed that the peptide sequence $^{149}\text{RTR}^{152}\text{TAYTRAQLLELEK}^{164}$, predicted to be released by Lys-C digestion which cleaves at K residues, was used with and without phosphate in order to generate an inclusion list of ions for the MS system. Both data sets, one with an inclusion list and one with no inclusion list used, were run against the PDX-1 sequence supplied for PDX-1 (Mascot Search provided by MATRIX SCIENCE). Orbi-Trap analysis of immuno-precipitated PDX-1 after Lys-C digestion indicated that the peptide $^{149}\text{RTR}^{152}\text{TAYTRAQLLELEK}^{164}$ was present in an un-phosphorylated form, but there was no evidence for the presence of the phosphorylated form.

PDX-1 peptide fragments sequence from LysC digestion		
LysC:/K-IP		
Frag#	Res#	Sequence
L1	1-15	(-)MNSEEQYYAATQLYK(D)
L2	16-124	(K)DPCAFQQRGVPVEFSANPPACLYMGRQPPPPPPQFTSSL GSLEQGSPPDISPYEVPLASDDPAGAHLLHHLPALGLAHP PPGPFNGTEPGGLEENRVQLPFPWMK(S)
L3	125-127	(K)STK(A)
L4	128-132	(K)AHAWK(G)
L5	133-148	(K)GQWAGGAYTAEPEENK(R)
L6	149-164	(K)RTR ¹⁵² TAYTRAQLLELEK(E)
L7	165-170	(K)EFLFNK(Y)
L8	171-192	(K)YISRP RRVELAVMLNLT ERHIK(I)
L9	193-201	(K)IWFQNR RMK(W
L10	202-203	) (K)WK(K)
L11	204-204	(K)K(E)
L12	205-208	(K)EEDK(K)
L13	209-209	(K)K(R)
L14	210-284	(K)RSSGTPSGGGGGEEPEQ DCAVTSGEELLAVPPLPPPG GAVPPGVPAAVREGLLPSGL SVSPQPSSIAPLRPQEPR(-)

Fig. 4.9. PDX-1 peptide fragments sequenced from LysC digestion

Peptide fragments sequence from mouse PDX-1 protein were predicted after LysC digestion which cleaves at the Lysine (K) residue. Fourteen peptides were expected among which Thr-152 is located in the middle of the L6 peptide as shown in the graph.

The data generated for PDX-1 for identity (ID) and phosphorylation site mapping analysis using precursor ion scanning of mass to charge ratio (m/z) 79 ($[PO_3]^-$) at 80 eV (Pre79; Fig. 4.10) - was also searched against the sequence supplied for PDX-1 (Mascot Search) and against the NCBI nr (all species). The ID analysis confirmed that PDX-1 was present. However the Pre79 analysis did not indicate any phosphorylated fragments (within detection limit of technique) from PDX-1 generated by Lys-C digestion (Fig. 4.10).

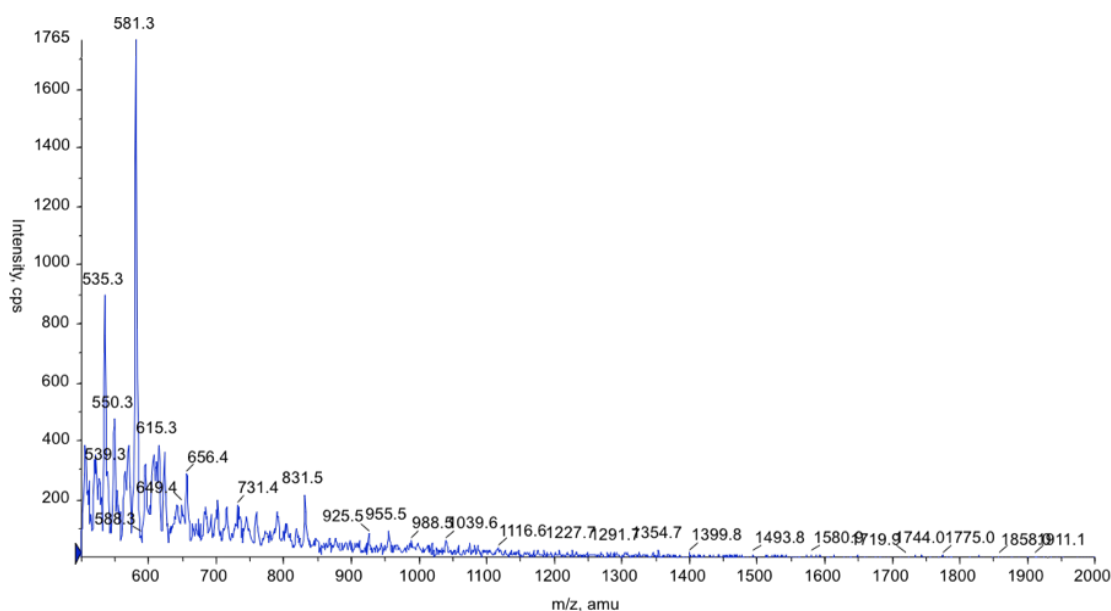


Fig. 4.10. Phosphorylation site mapping of PDX-1 by mass spectrometry.

Immuno-purified PDX-1 was migrated by SDS-PAGE and the 1D band with good intensity by colloidal coomassie staining was processed by in-gel digestion with Lys-C (as described in Material and Methods). The Pre79 (phosphorylation site mapping analysis) mass spectrum of PDX-1 peptides. m/z , mass-to-charge ratio. The data generated for PDX-1 for identity (ID) and phosphorylation site mapping analysis-using precursor ion scanning of mass to charge ratio (m/z) 79 ($[PO_3]^-$) at 80 eV (Pre79)- was also searched against the sequence supplied for PDX-1 (Mascot Search) and against the NCBI nr (all species). Immuno-purified PDX-1 on SDS-PAGE was provided by Rong AN. In-gel digestion and phosphorylation site mapping by mass spectrometry was done by Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

Samples from cells cultured under the same condition as above were also digested with trypsin, predicted to release a fragment $^{152}\text{TAYTR}^{156}$. However, this approach also failed to reveal evidence for the presence of phosphate at Thr-152 (data not

shown).

The mass spectrometry experiments were done in collaboration with Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

4.3.6 Subcellular localization of overexpressed PASK in INS $\alpha\beta$ cells.

PASK was previously reported in cloned β -cells²⁰² to display a cytosolic distribution, whereas PDX-1 mainly shuttles between nuclear periphery and nucleoplasm in normal β -cells under physiological conditions in response to elevated glucose concentrations¹³⁸. We therefore studied the effect of PASK on PDX-1 transcriptional activity and on preproglucagon mRNA level by over-expressing PASK and PDX-1 in INS $\alpha\beta$ cells (Fig. 4.4-4.5). The localization of over-expressed human PASK was also studied by immunocytochemistry using anti-human PASK antibody. In INS $\alpha\beta$ cells, as reported before in cloned β -cells²⁰², overexpressed PASK displayed a predominant cytosolic distribution without any shift into nuclear area at elevated glucose concentrations (Fig. 4.11)

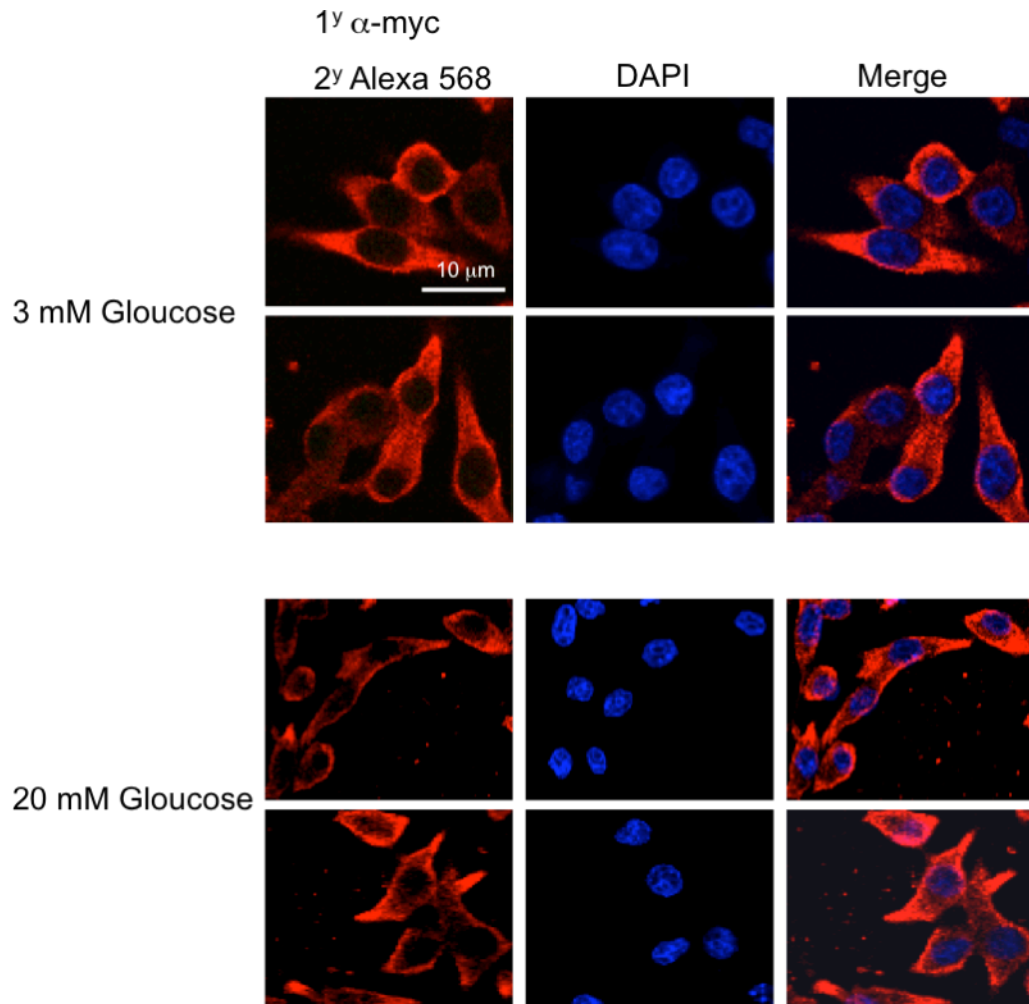


Fig. 4.11. Immunocytochemical analysis of the subcellular distribution of PASK.

INSrαβ cells were infected with hPASK-over-expressing-adenoviruse at 100 MOI for 24 h. Cells were cultured at 3 mM glucose for 16 h and then treated with 3 mM or 20 mM glucose for 6 h. c-*Myc*-tagged hPASK was detected using anti-PASK antibody and Alexa-568 (Molecular Probes). Nuclear staining was achieved using DAPI.

4.3.7 PASK is present in pancreatic β -cells from dispersed human islets.

A recent report by Borter *et al*²¹³ suggests that PASK (referred to as PASKIN) mRNA levels were high in testes. However these authors were unable to detect PASK mRNA in pancreas or in islet β -cells. However, in the research by Da Silva Xavier *et al*²⁰⁹, PASK was shown to be involved in regulating glucose-stimulated insulin gene expression in pancreatic β -cells and in a later study by Hao²¹⁰ using the same PASK knock-out mice as Borter *et al*²¹³, the mice exhibited impaired glucose-stimulated insulin secretion from isolated pancreatic islets, altered triglyceride storage in liver, and increased metabolic rate in skeletal muscle. A very important question for the present study was to explore whether or not PASK is expressed in pancreatic β -cells and whether the expression is regulated by glucose. Due to the unavailability of commercial anti-PASK antibody for rodent species, we chose to study the subcellular localization of PASK in dispersed human islets by immunocytochemistry using anti-human PASK antibody. By probing the dispersed pancreatic islets with an anti-insulin antibody, insulin-secreting β -cells were identified and PASK immunoreactivity was found in both the nuclear and cytoplasmic areas (Fig. 4.12A). To further confirm the localization of both PDX-1 and PASK in pancreatic β -cells, anti-PASK and anti-PDX-1 antibodies were used simultaneously. PASK and PDX-1 are both present in the nuclear area of β -cells (Fig. 4.12B). Taken together, these data provided evidence that PASK expression overlaps with PDX-1 expression in human pancreatic β -cells. However, no sign of translocation of PASK was observed in response to elevated glucose concentrations.

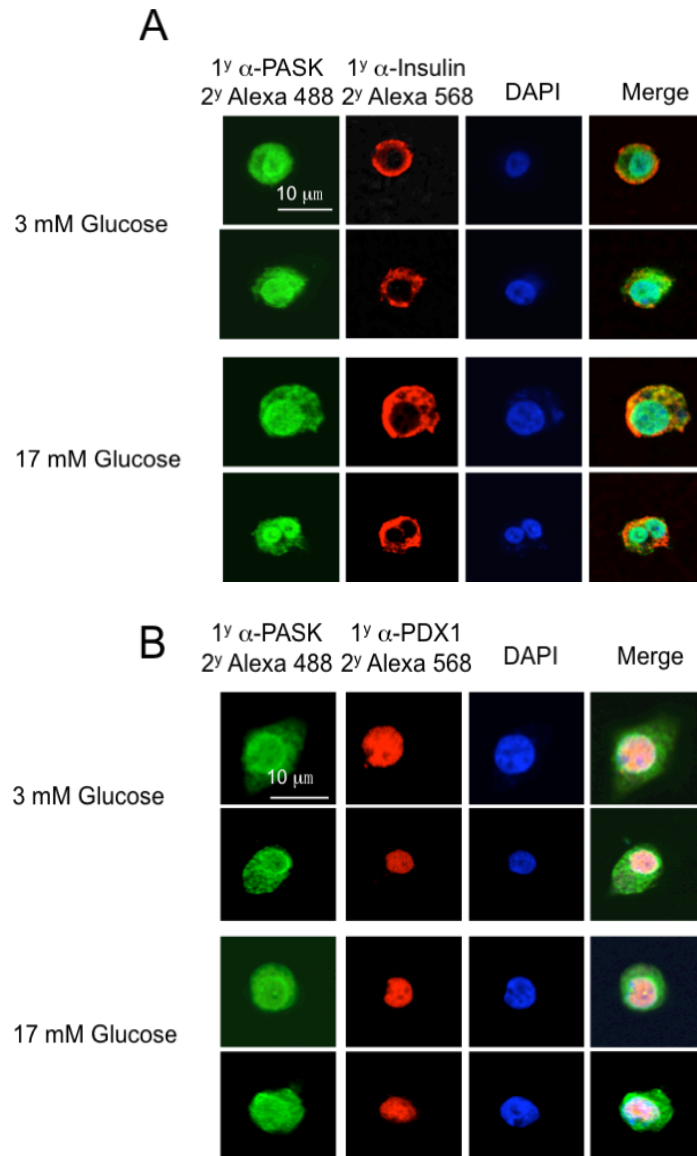


Fig. 4.12. PASK and PDX-1 co-localize in human pancreatic β -cells.

Dispersed human islets were cultured in RPMI-1640 medium containing 3 mM glucose for 16 h before subsequent incubation in media containing 3 or 17 mM glucose for 6 h. PASK in (A) and (B), was detected using anti-human (h) PASK antibody and revealed with Alexa 488-conjugated secondary antibody (Molecular Probes). In (A), insulin was detected to label b-cells and was probed with anti-insulin antibody (Roche) and Alexa 568-conjugated secondary antibody (Molecular Probes). In (B), PDX-1 was labeled using anti-PDX-1 antibody and Alexa 568-conjugated secondary antibody (Molecular Probes). In both (A) and (B), nuclear staining was achieved with DAPI.

4.4 DISCUSSION

We have shown in Chapter 3 that recombinant PDX-1 is efficiently phosphorylated *in vitro* at Thr-152 in the *homeodomain* by the nutrient-regulated kinase, PASK. Given the relatively low promiscuity of PASK towards a variety of substrates- PASK did not phosphorylate several other substrates tested, including the β -cell enriched transcription factors neuroD and MafA (personal communication with Prof. Jared Rutter, University of Utah), we speculated that this event may be physiological. In this Chapter we therefore deployed multiple approaches to explore the possibilities that Thr-152 may be phosphorylated in living β -cells, and that PASK may be the responsible PDX-1 kinase. This issue appeared particularly important given the profound effects, demonstrated here (Figs 4.1-4.5), of mimicking the phosphorylation at Thr-152 on the DNA binding and transactivation capacity of PDX-1, and on the ability of this factor to determine the lineage of a common α/β -cell precursor cell line.

In this Chapter, it was observed that mimicking phosphorylation on the potential site identified *in vitro* resulted in a potent inactivation of PDX-1 reflected in a near total loss of DNA-binding capacity and in the abilities of the factor to induce a cell lineage switch in INSr $\alpha\beta$ cells. Arguing against the view that PASK phosphorylates PDX-1 *in situ*, nuclear localization of the kinase in MIN6 β -cells was not previously been noted for over-expressed PASK²⁰⁹ (though studies of the endogenous protein in rodent cell lines were not possible due to the absence of an antibody with adequate specificity for immunocytochemical analyses). We now show, using direct and complementary approaches, that Thr-152 is unlikely to be significantly phosphorylated *in situ* under a variety of conditions, including those in which endogenous PASK is activated (elevated glucose concentrations) or after over-expression of the exogenous kinase. The present data thus indicate that Thr-152 is not a major site of phosphorylation of PDX-1 in living β -cells under most

conditions, and that phosphorylation at this site is not altered under conditions in which the nuclear accumulation of PDX-1 is clearly affected (i.e high versus low glucose concentrations).

What may underlie the apparent difference in the ability of PASK to phosphorylate PDX-1 at Thr-152 efficiently *in vitro*, but not in intact cells? The PDX-1 homeodomain is important for PDX-1 DNA binding ability and may play an even more pivotal role in protein-protein interaction between PDX-1 and other co-activators. One possibility, therefore, is that DNA-bound PDX-1 may provide a poor substrate for PASK at this site, owing to a direct competition/steric blockage by bound DNA or an associated protein-binding partner for PDX-1. On the other hand, as studies of the endogenous protein in rodent cell lines were not possible due to the absence of an antibody with adequate specificity for immunocytochemical analyses, alternatively, this difference might due to the fact that overexpressed, exogenous PASK and PDX-1 are differently localized in the cells. Indeed, as shown in Fig. 4.11, overexpressed human PASK exhibited a predominantly cytosolic distribution in INSr $\alpha\beta$ cells irrespective of the glucose concentration. Nonetheless, since PDX-1 shuttles dynamically from the cytosol to the nucleus^{138, 224}, it remains conceivable that the factor may interact with PASK in the cytosol. Overall, therefore, it seems most likely that PDX-1 is protected from phosphorylation by overexpressed PASK at Thr-152 by a combination of nuclear segregation, DNA binding and perhaps interactions with other binding partners.

However, since PDX-1 shuttles dynamically from the cytosol to the nucleus^{138, 224}, we also provided evidence that PASK is detected in both the cytosol and nuclear areas in dispersed human islets especially in pancreatic β -cells where PDX-1 was also detected in the nuclear area (Fig.12), suggesting possibilities that in primary human pancreatic β -cells, PDX-1 could indeed potentially interact with endogenous PASK.

We have evidence that PASK over-expression in MIN6 cells can increase preproinsulin and PDX-1 promoter activity ²⁰⁹. In our study, we looked at the regulation by PASK of PDX-1 transcriptional activity in INS $\alpha\beta$ cells in which the PDX-1 over-expression had undetectable effects on insulin gene expression. Instead, wild-type PDX-1 over-expression was shown to substantially decrease preproglucagon gene expression. However, co-overexpression of PASK and PDX-1 did not lead to any change in preproglucagon gene expression in these cells. Hence, regulation by PASK may act through more complicated pathways requiring engagement of other intracellular factors bridging between PASK and PDX-1 rather than a direct protein-protein interaction.

We conclude that the regulation by glucose of preproinsulin promoter activity, previously shown to require PASK activity ²⁰⁹, is unlikely to be achieved through direct phosphorylation of PDX-1 by this kinase. Future studies will be required to elucidate the mechanisms through which PASK acts in this signaling pathway.

5. Chapter 5

Glucose-dependent phosphorylation of PDX-1 at serine-269 in clonal pancreatic β -cells: identification of potential protein kinases, and impact on PDX-1 function

5.1 Introduction

In contrast to the relatively well-studied functions of the N-terminus and the *homeodomain* of PDX-1, the role of the conserved C-terminus is less known. Human mutations in the C-terminus of PDX-1 are associated with the development of type 2 diabetes¹⁵⁸⁻¹⁶⁰, and previous findings indicate that the C-terminal domain may serve as both a repressor and an activator of PDX-1 function. The C-terminus may thus inhibit the synergy between PDX-1 and a second transcription factor, E47 on the preproinsulin promoter, whilst also containing a cryptic transactivation domain involved in regulating the somatostatin gene TAAT1 enhancer^{70, 101}. Stoffers *et al*¹⁶⁰ identified the first PDX-1 C-terminus interacting protein-PCIF1 (PDX-1 C-terminal interacting factor) in the pancreas and suggested that PCIF1 may modulate PDX-1 function during normal pancreas development and/or differentiation.

Homeodomain-interacting protein kinase 2 (HIPK2) is an evolutionary conserved serine/threonine nuclear kinase belonging to the family of *homeodomain-interacting protein kinases* (HIPK) which includes HIPK1, HIPK2 and HIPK3 and also the more distantly related protein HIPK4.²³⁴

The HIPK family was initially discovered as a novel family of HIPKs that localize to nuclear speckles, and act as binding partners²³⁴ and transcriptional co-repressors for NK homeodomain transcription factors²³⁴. The NK homeodomain transcription factors were found to be important for embryonic development and organogenesis including development of the heart.

The kinase domains of HIPK1, 2 and 3 share >90% homology^{234, 235}. Mice deficient for either *Hipk2* or *Hipk1* alone are grossly normal, while doubly deficient mice die prenatally and show various abnormalities including defects in hematopoiesis and blood vessel formation^{235, 236}.

HIPK2 is the most highly characterized family member in the HIPKs family. It localizes mainly to subcellular nuclear area ²³⁵. HIPK2 contains an N-terminal kinase domain with a small ubiquitin-related modifier (SUMO) attachment site at lysine 25 ²³⁷⁻²³⁹. It also contains an interaction domain for the homeodomain transcription factors, and two PEST motifs (rich in Proline, Glutamic acid, Serine and Threonine) that overlap with a region allowing SUMO binding. These regions are flanked by an autoinhibitory domain and a C-terminal region that is rich in short repeats of S, Q or A. The entire sequence of the protein is well conserved among vertebrates, while the kinase domain is even found in *Drosophila melanogaster* and *Caenorhabditis elegans*.

HIPK2 is one of the kinases that simultaneously exert diverse biological functions ²⁴⁰. HIPK2 can be activated by DNA-damaging agents or morphogenic signals and consequently HIPK2-regulated gene expression programs trigger differentiation and development or alternatively, apoptosis ²⁴⁰. Biochemical and genetic studies have revealed an important role of HIPK2 for development ²⁴¹⁻²⁴⁴ and the regulation of cell death ^{240, 245-248}. HIPK2 regulates gene expression by phosphorylation of transcription factors and accessory components of the transcription machinery. As such, it interacts with, and modulates by phosphorylation, the function of homeodomain proteins including p53, Myb and CtBP1 ^{242, 247, 249-251}. The function of HIPK2 phosphorylation is dependent on the cell type and promoter context ²⁴⁰.

HIPK2 is regulated by a post-translational modification of an ubiquitin-like protein, SUMO-1, via covalent bonding to lysine 25 on HIPK2 ²³⁷⁻²³⁹. This is similar to the binding of SUMO-1 to PML and Sp100 ²⁵². The conjugation of SUMO-1 is thought to direct each of these proteins to nuclear bodies (NBs), which appear to play a role in autoimmunity and viral protection. HIPK2 is the first protein kinase found to be directed to nuclear bodies in response to ubiquitin-like modification ²⁵³.

A recent study reported that HIPK2 is expressed in the developing pancreatic

epithelium from embryonic day (e) 12 to e15 but that the expression becomes preferentially confined to pancreatic endocrine cells at later developmental stages. Moreover, HIPK2 was reportedly able to modulate positively the transcriptional activity of PDX-1, and HIPK2 directly phosphorylates the C-terminal portion of PDX-1¹⁶¹. These data not only provided evidence of a new mechanism by which IPF1/PDX1 transcriptional activity is regulated, but also lead to a novel potential pathway that could contribute to the study on the regulatory effect of PDX-1 C-terminus.

In this Chapter, we have identified a novel phosphorylation site in the C-terminal domain of PDX-1, Ser-269 via mass spectrometry analysis and an anti-phospho-Serine-specific antibody. This site is phosphorylated by HIPK2 *in vitro*, and the phosphorylation on S269 is regulated by glucose in MIN6 cells.

It has been reported in a previous study by Edlund, a minor proportion of PDX-1 is phosphorylated on S61 and S66, and that phosphorylation marks PDX-1 for degradation by a GSK3-dependent mechanism. *In vitro* phosphorylation studies undertaken here also provided evidence for a relationship between S269 phosphorylation and GSK3 phosphorylation at other sites in the regulation of PDX-1. However, mutation studies of indicated that S269 phosphorylation does not affect the localization, DNA-binding ability or transcriptional activity of PDX-1. Thus, our data provided the first *in vivo* phosphorylation site on PDX-1 that has been identified by mass spectrometry Phospho-site screening then provided evidence of the potential regulatory network in which S269 may be involved. This may shed light on signaling pathways by which the function of the PDX-1 C-terminus is regulated.

5.2 Methods

5.2.1 Sequencing and Mass Spectrometric Analysis of Phosphorylation sites

T-75 flasks (30) of INS-1(832/13) cells were infected at 50 MOI with adenovirus expressing wild-type PDX-1 for 48 h. Cells were then collected and lysed for immunoprecipitation as described (Chapter 2; P74). IP product was migrated on 10% (w/v) SDS-PAGE gels and stained with colloidal commassie blue (SimplyBlue SafeStain, Invitrogen). The 1D band with good intensity by colloidal commassie staining was processed by in-gel digestion with trypsin. The digested samples were then extracted and were taken through three stages of analysis using a 4000 QTRAP (Applied Biosystems) mass spectrometer. The stages were as follows: Identification of protein(s) by nLC-MS-MS; Parents of -79 (PO3-) analyses to detect phosphorylated peptides; data interpretation including *de novo* assignment of phosphorylation sites) and final reporting. Ser-152 phosphorylation was further detected using *OrbiTrap* by targeting site-of-interest containing peptide²³².

The mass spectrometry experiments were done by Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

5.2.2 Peptide competition assay for anti-phospho-S269-PDX-1 antibody.

INS-1(832/13) cells were infected at 50 MOI with adenoviruses expressing wild-type PDX-1 and PASK for 48 h. Cells were then collected and nuclear lysate was obtained as described in Chapter 2 (P58). Three identical lanes of the SDS-PAGE gel were loaded 30 µg each of the nuclear extract. Proteins were separated and transferred onto a PVDF membrane which was then blocked with 5% skimmed

milk in TBST as described for PCA assay. Anti-phospho-S269-PDX-1 antibody was slowly thawed on ice and diluted 1:100 in 5% BSA. The lyophilized phospho-/non-phospho peptides were slowly warmed to room temperature under desiccation. Each peptide was reconstituted to a concentration of 100 μ M using molecular biology-grade ultrapure water at room temperature. Peptide stock solution (2.66 μ M) and water control were prepared (sample (a): no peptide control; sample (b): non-phosphorylated peptide 2X stock; sample (c): phosphorylated peptide 2X stock) and 1 ml of the 2X antibody stock was added into each of the samples above. The mixture was incubated at 4°C for 24 h with gentle rocking. Samples were then centrifuged for 15 min. at 4°C in a microcentrifuge at 13,000 rpm to pellet any immune complexes. The supernatant was removed carefully. The pre-incubated antibody in each of the three samples was used onto the three identical strips for normal immunoblotting as described before in Chapter 2.

After the phospho-specific antibody blotting, the blots were stripped and re-blotted with anti-c-*myc* antibody.

5.2.3 Transient Transfections and Luciferase Assay

For immunocytochemistry, plasmids pAdTrack-PDX-1 wild-type, -S269A or -S269E were transfected into INSr α β cells with Lipofectamine 2000 according to the Manufacturer's instructions. Plasmids pcDNA3.1-PDX-1 wild-type and *p260Ins.Luc_{FF}*¹³⁸, S269A, S269E expression vectors were made using a site-directed mutagenesis kit (Stratagene) in pcDNA3.1 vector (Invitrogen). For luciferase assays, HEK293 cells were transfected using Ca²⁺-phosphate. HEK293 cells were grown to 70-90% confluency in 12-well plates. Total DNA (as indicated) was mixed with 2.5M CaCl₂ was added drop by drop to same volume of 2 X HBS in a sterile tube while vortexing. Empty expression plasmid was used to maintain a constant total amount of DNA among wells. Cells were harvested 48 h following

transfection and protein cells extraction for western blot analyses or for luciferase assays were carried out. For luciferase assays, the data are presented as firefly luciferase activity normalized to *Renilla* luciferase activity expressed from the cotransfected plasmid, pRL-TK (Promega).

5.2.4 *In vitro* phosphorylation of PDX-1 by HIPK2, CK1 or GSK3 β

In vitro phosphorylation was carried out following the manufacturer's instruction for active HIPK2 (Upstate). Purified wild-type or S269A PDX-1 (2 μ g) was mixed with the indicated amount of HIPK2 in kinase buffer (8 mM MOPS pH 7.0, 200 μ M EDTA, 10 μ M MgCl₂, 100 μ M ATP) with or without 2 μ Ci [γ -³²P] ATP. The reaction mixtures were incubated at 30 °C for 30 min. Reactions were stopped by adding Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2.2% SDS, 10% glycerol, 1 mM PMSF, 5% β -mercaptoethanol, 0.005% bromophenol blue). The samples were then separated on SDS-PAGE and analyzed by autoradiography (labeled samples) or western immuno-blotting (unlabeled samples) using the indicated antibodies.

PDX-1 (3 μ g, either wild type or S269A mutant) was firstly incubated at 37 °C in a medium (20 μ l) containing 10 mM Tris buffered MOPS pH 7.5, 20 mM magnesium acetate, 1 mM DTT, and 0.25 mM ATP in the presence of the phosphorylating kinase (1.7, 1.5 and 2 pmol for CK1 δ , GSK3 β and HIPK2, respectively). After 45 min., GSK3 β (1.5 pmol) was added (water in the controls) together with a new mixture of [γ -³³P] ATP (specific radioactivity 5000 cpm/pmol) and the incubation continued for 15 min. Samples were then blocked with Laemmli buffer and subjected to PAGE-SDS and western blotting. Nitrocellulose membranes were directly exposed to autoradiography and subsequently immunostained with antiPdx269 antibodies. Recombinant wild-type PDX-1 and S269A PDX-1 were

provided by Rong AN and the *in vitro* phosphorylation experiments by CK1 δ and GSK3 β were performed by Dr. Flavio Meggio and Prof. Lorenzo Pinna (University of Padova, Italy).

5.3 RESULTS

5.3.1 Mass spectrometry and phosphorylation site mapping

In an effort to identify the major phosphorylation sites on PDX-1 in intact β -cells, we scanned peptides released after trypsin digestion of the protein immunoprecipitated from MIN6 β -cells. Phosphorylation site mapping was then undertaken with a 4000 QTRAP LC/MS/MS system. The data generated for PDX-1 for identity (ID) and phosphorylation site mapping analysis - using precursor ion scanning of mass to charge ratio (m/z) 79 ([PO3]⁻) at 80 eV (Pre79) - were also searched against the sequence supplied for PDX-1 (Mascot Search) and against the NCBI nr (all species). Peptide 262LPSGLSV**p**SPQPSSIAPLRPQEPR284 was found to be phosphorylated on Ser269 (Fig. 5.1; bold; corresponding to Ser268 in the human sequence). Peptide 211SSGTPSGGGGGEEPEQDCAVTSGEELLAVPPLPPPGGAVPPGVPAAVR258 (Fig. 5.1) was also found to bear at least one phosphate group. However, the precise site of phosphorylation of this peptide was not investigated further.

Immuno-purified PDX-1 in SDS-PAGE was provided by Rong AN. In-gel digestion and the mass spectrometry experiments were done by Dr. Douglas Lamont in Fingerprint Ltd. University of Dundee.

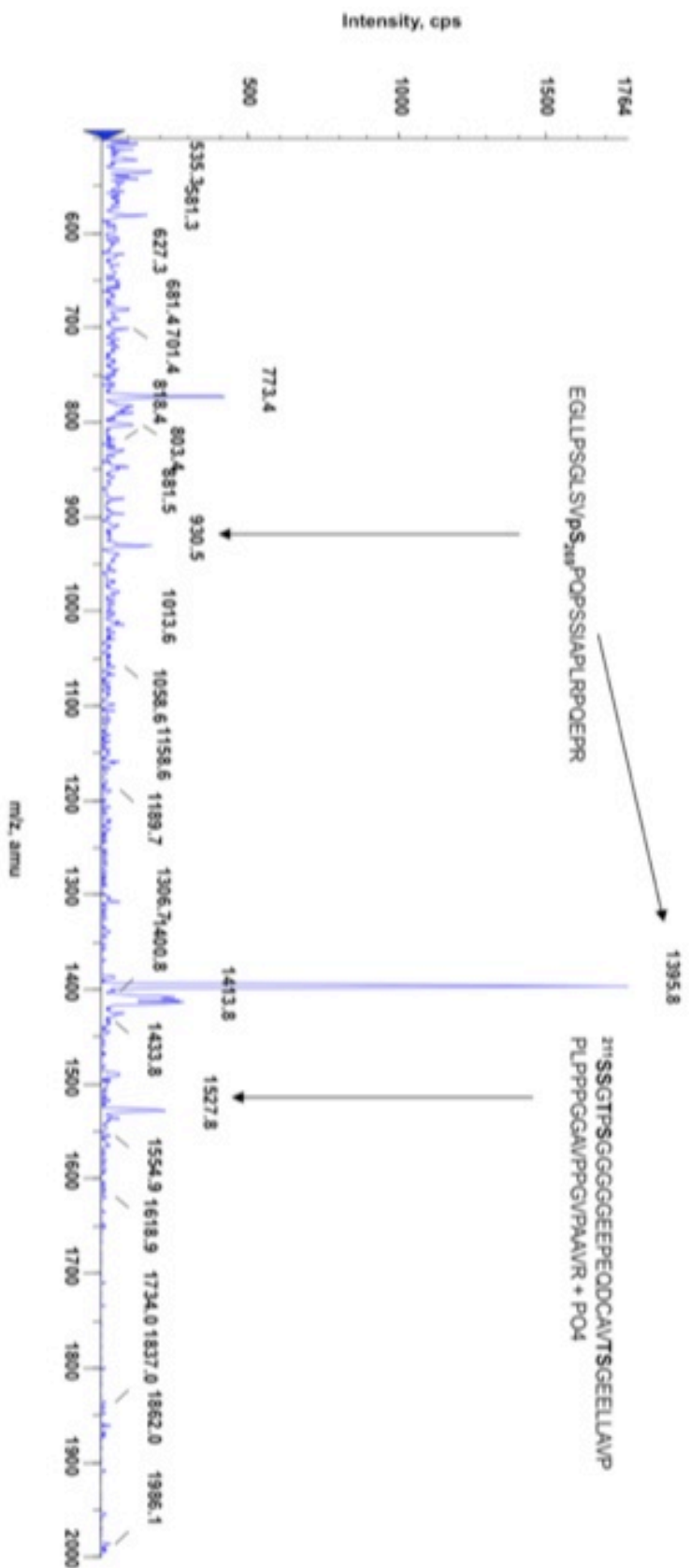


Fig. 5.1 Phosphorylation site mapping of PDX-1 by mass spectrometry. (A) phosphorylation site mapping of PDX-1 by mass spectrometry. The Pre79 (phosphorylation site mapping analysis) mass spectrum of PDX-1 peptides after trypsin digestion. m/z, mass-to-charge ratio. Peptide ²⁵⁶EGLLP²⁵⁶SGLSVp⁸SPQPSSIAPLRPQEP⁸R showing Mass/Charge (Masscot Report) at 1396.73/2+, 931.49/3+ and relevant Mass/Charge (Pre79 Scan) at 1395.8/2-, 930.5/3-was found phosphorylated on the indicated Serine site in bold with phosphate (Ser 269 in mouse PDX-1). A long peptide ²¹¹SSGTPSGGGGEEPEQDCAVTSGE²¹¹LLAVPLPPPGGA/PPGVPA²¹¹VR²⁵⁸ was detected been phosphorylated however without the actual site been identified.

5.3.2 Characterization of anti-phospho-S269-PDX-1 antibody using a peptide competition assay

The peptide competition assay aimed to confirm the specific reactivity of anti-peptide polyclonal phospho-specific antibodies. As the anti-phospho-S269-PDX-1 antibody was observed to reveal more than one band when probing in cell lysates, this assay provides a means of determining which band is specific for the phospho-specific antibody. Two experiments were run in parallel, one in which the phosphorylated form of the peptide immunogen was used and one with the non-phosphorylated form of the immunizing peptide. The immunoblotting experiment was run in duplicate; in one set including antibody pre-incubated with peptide, and another with a control antibody not pre-incubated with peptide. The signals in Fig. 5.2A (a) (water only, no peptide control) represent the maximum signal detected by the phospho-specific antibody. Signals obtained with sample (b) (non-phosphorylated peptide) and sample (c) (phosphorylated peptide) was compared to the signal from sample (a) to determine if the peptides compete for antigen binding. There was very little binding to the 46 kD band for sample (c) in which the antibody had been pre-incubated with excess of phosphorylated peptide. This suggests that the 46 kD band is the specific band for the anti-phosphoS269-PDX-1 antibody. Total protein level appear to be equal as indicated using anti-c-myc antibody in Fig. 5.2B.

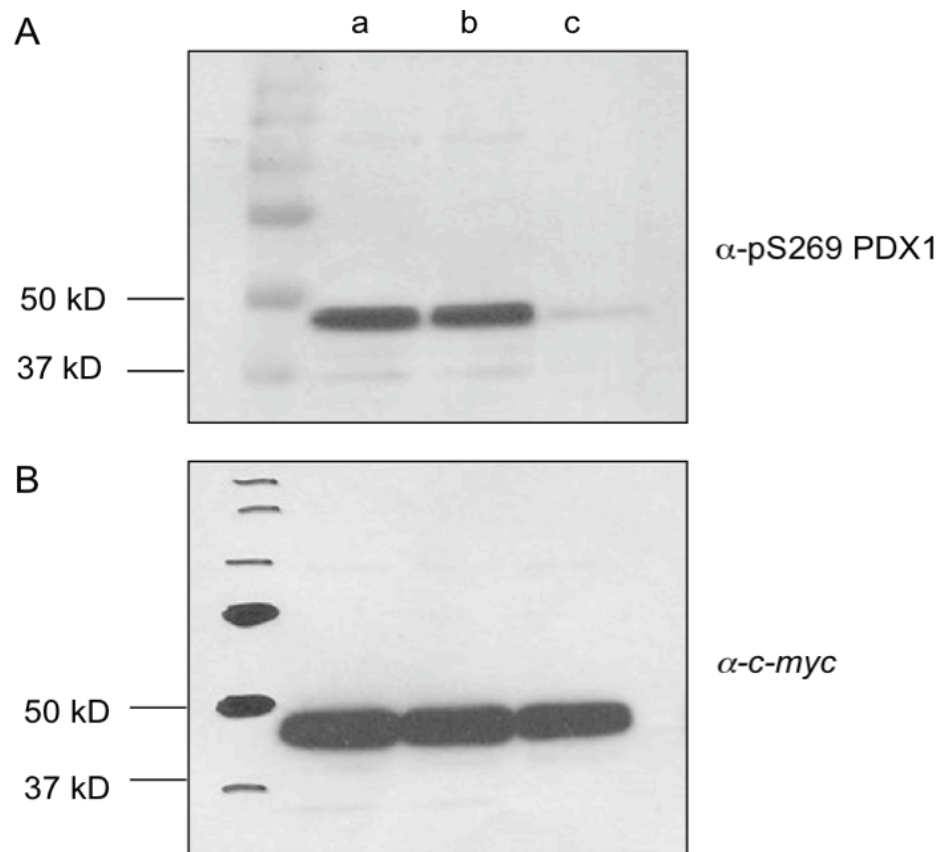


Fig. 5.2. Peptide competition assay for anti-phosphoS269-PDX-1 antibody.

Three identical strips a, b and c were immunoblotted by pre-incubated anti-phosphoS269-PDX-1 antibody with water, non-phosphopeptide and phosphopeptide respectively. The indicated band represents phosphoS269-PDX-1. Blots were then stripped and re-blotted by anti-*c-myc* antibody. The 46 kD band shows the whole *myc-tagged* PDX-1 level.

5.3.3 PDX-1 is phosphorylated on Ser-269 in living β -cells and by HIPK2 *in vitro*.

It has previously been reported¹⁶¹ that *homeodomain-interacting protein kinase 2* (HIPK2) directly phosphorylates the C-terminal domain of PDX-1 at undefined site(s) lying between aa215 and aa283¹⁶¹. Since HIPK2 is considered to be a proline-directed kinase¹⁶¹, and S269 is adjacent to a proline residue (see above and Fig. 5.1), we investigated whether S269 may be phosphorylated by HIPK2 *in vitro*. This possibility was examined using active HIPK2 and recombinant *c-myc*-tagged wild-type or S269A PDX-1 as substrates. Wild-type PDX-1 incorporated increasing amount of ³²P as the amount of active HIPK2 was increased, as expected (Fig. 5.3A). In contrast, incorporation of ³²P into recombinant S269A PDX-1 was hardly detectable at all levels of HIPK2 activity tested (Fig. 5.3A). In both cases, HIPK2 was also present as an autophosphorylated band. This was accompanied, in experiments performed with non-radioactive ATP, with the appearance of an immunoreactive band at ~46 kDa as revealed using an antiphospho-S269 PDX-1 antibody. As predicted, no immunoreactivity was apparent after treatment of the S269A mutant PDX-1 (Fig. 5.3B).

Whilst the increased signal detected with the anti-phospho-S269-specific PDX-1 antibody in the case of wild-type PDX-1 confirmed the above finding, residual incorporation of phosphate into S269A PDX-1 (Fig. 5.3A) suggested the existence of other sites for phosphorylation by this kinase. Indeed, and as revealed by mass spectrometry (Fig. 5.1), a long peptide, ²¹¹SSG²¹⁴TPSGGGGGEEPEQDCAVTSGEELLAVPPLPPPGGAVPPGVPAAVR was also detected as containing phosphate. Although the exact site of phosphorylation was not pursued further, we noted that Thr214, present in the above peptide, and followed by a proline residue, meets the criteria for phosphorylation by proline-directed kinases including HIPK2.

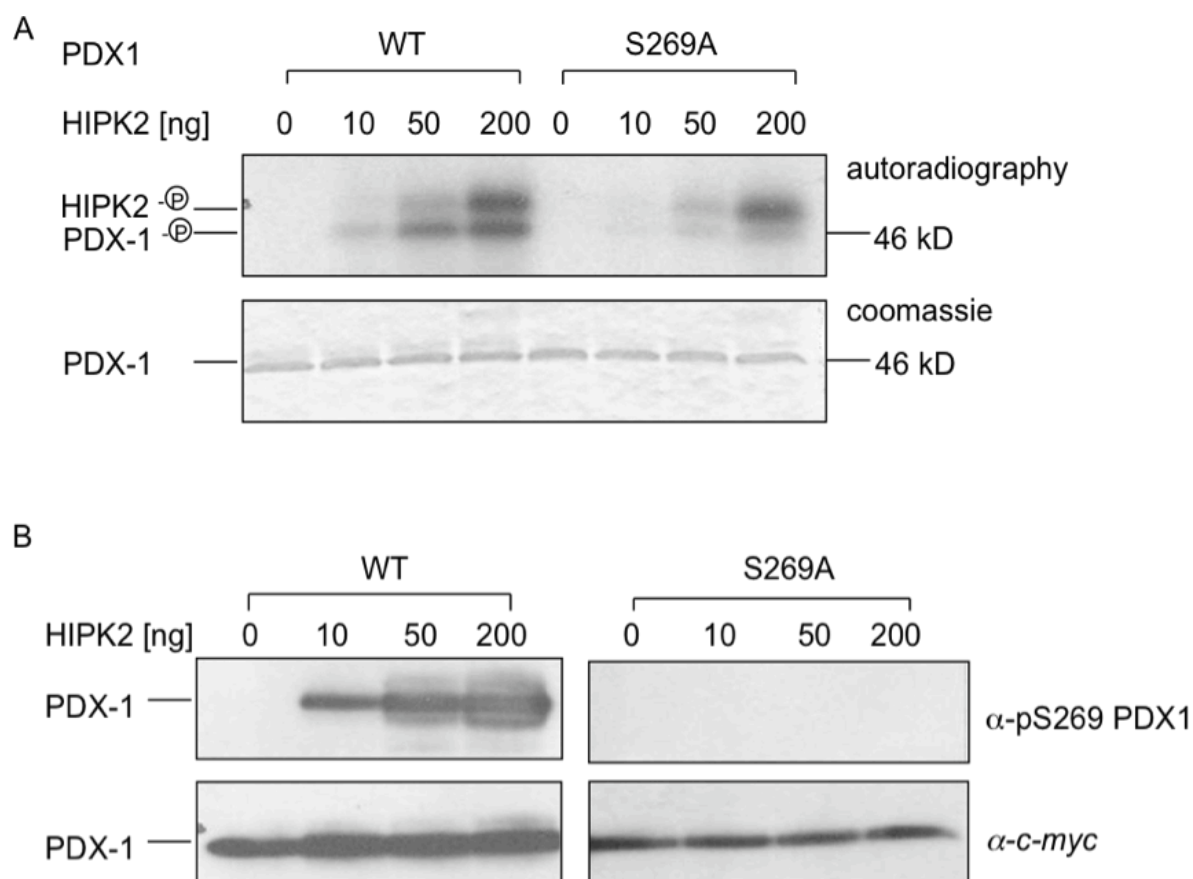


Fig. 5.3. PDX-1 is phosphorylated on Ser-269 in living b-cells and by HIPK2 *in vitro*. (A) Recombinant PDX-1-*myc* (2 μ g ; WT or S269A) were mixed with indicated amount of active HIPK2 in the presence of [γ - 32 P]ATP for 30 min. Autoradiography shows the radiolabeled PDX-1 and HIPK2. Coomassie staining shows the PDX-1 protein level. (B) Parallel experiment with the absence of [γ - 32 P]ATP was analyzed by western blot using indicated antibodies.

5.3.4 Ser-269 may also be phosphorylated by GSK3 β

It has previously been reported¹⁴² that a minor portion of PDX-1 is phosphorylated in the N-terminal domain at Ser61 and Ser66 and the phosphorylation is modulated by a GSK3-dependent mechanism. Hence, our data showing that Ser269 is phosphorylated by HIPK2 *in vitro* prompted us to explore the potential regulatory mechanisms controlling this phosphorylation event. We noted that Ser269 lies within the sequence²⁶⁹SPQP²⁷³S, corresponding to the consensus sequence for GSK3 phosphorylation (S/T-X-X-X-pS/T; pS/T represents the priming residue)^{254, 255}. We therefore conducted a series of *in vitro* phosphorylation trials to explore the phosphorylation of PDX-1 by GSK3. Since phosphorylation by GSK3 usually requires priming by phosphorylation of the downstream residue (Ser273 in the case of subsequent phosphorylation at Ser269) we studied phosphorylation in the presence of GSK3 alone, or in the additional presence of CK1 δ (often used to prime subsequent phosphorylation by GSK3^{256, 257}). CK1 δ phosphorylated PDX-1 on its own and the addition of GSK3 caused a highly reproducible increase (means $\pm$ SEM, 71 ± 14 %; $n = 3$, $p < 0.01$; two-tailed student's *t*-test, Microsoft ExcelTM) of the overall incorporation of ³³P (Fig. 5.4A). Moreover, when Ser269 was replaced by an alanine residue phosphorylation by CK1 δ and the increase of ³³P incorporation by addition of GSK3 β were significantly reduced, compared to wild-type (24 ± 15 %; $n = 3$; $p < 0.05$; two-tailed student's *t*-test, Microsoft ExcelTM; Fig. 5.4A) implicating Ser269 as a potential target of either CK1 δ or GSK3 β . However, this conclusion could not be confirmed using the anti-pSer269 PDX-1 specific antibody: the antibody failed to detect any signal in either case (Fig. 5.4A and see the Discussion).

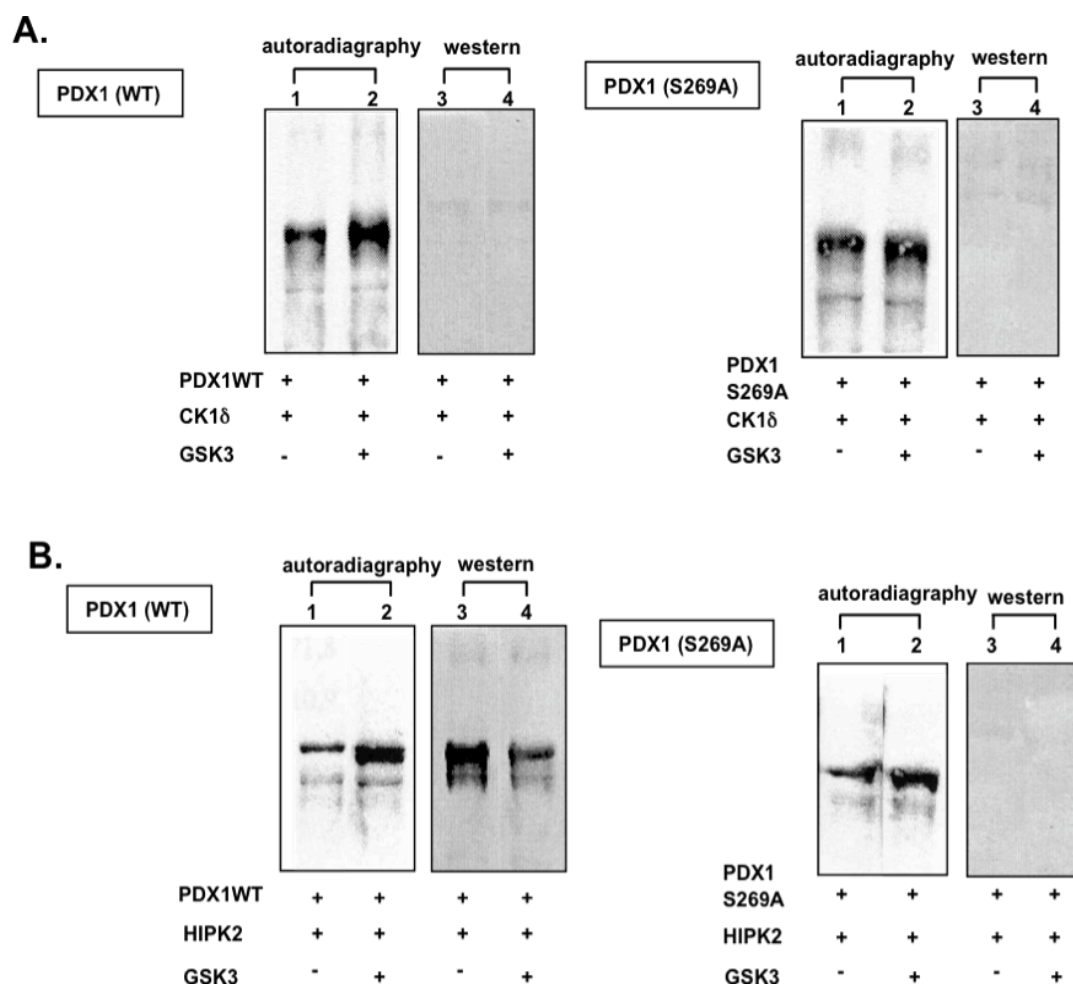


Fig. 5.4. Ser-269 may also be phosphorylated by GSK3.

PDX-1 either wild type or S269A mutant was firstly incubated with the phosphorylating kinase (CK1, GSK3 and HIPK2) before further reaction with GSK3/water. Samples were then blocked with Laemli buffer and subjected to PAGE-SDS and western blotting. Nitrocellulose membranes were directly exposed to autoradiography and subsequently immunostained with anti-pSer269 antibodies. (A) PDX-1 wild-type and S269A mutant phosphorylation by CK1δ and GSK3 and corresponding model. (B) PDX-1 wild-type and S269A mutant phosphorylation by HIPK2 and GSK3 and corresponding model. Recombinant wild-type PDX-1 and

S269A PDX-1 were provided by Rong AN and the *in vitro* phosphorylation experiments by CK1 δ and GSK3 β were performed by Dr. Flavio Meggio and Prof. Lorenzo Pinna (University of Padova, Italy). Statistical analysis shown in the text was done with the results from three independent experiments using two-tailed student's *t*-test, Microsoft ExcelTM.

As shown above (Fig. 5.3A) and in Fig. 4B, HIPK2 alone catalysed the incorporation of Pi into recombinant PDX-1, and this was accounted for by phosphorylation at Ser269 as revealed with the phospho-Ser269 specific PDX-1 antibody (Fig. 5.4B). Intriguingly, addition of GSK3 β besides HIPK2 also increased phospho-radiolabeling of PDX-1, while actually causing a decrease in the signal detected with the antiphosphoSer269-specific antibody (Fig. 5.4B, left panel). These data suggest that although HIPK2 and GSK3 β play a synergistic role in the phosphorylation of PDX-1, this hierarchical mechanism does not involve S269. Consistent with this, the S269A PDX-1 mutant undergoes synergistic phosphorylation by the combination of HIPK2 and GSK3 β similar to PDX-1 wild type (Fig. 5.4B). These results therefore indicate that there must be other residue(s) in PDX-1 which can be phosphorylated by HIPK2 *in vitro* besides S269, and that such a phosphorylation primes the subsequent intervention of GSK3 β .

5.3.5 Ser-269 phosphorylation is regulated by glucose in living β -cells (MIN6)

We next explored the possibility that phosphorylation of PDX-1 at Ser-269 may be regulated by glucose in living β -cell lines and may, at least potentially, contribute to the regulation of PDX-1 function by glucose. After treatment at low glucose concentrations (3 mM) for 16 h, we cultured MIN6 cells in medium containing 30 mM glucose for different times (Fig. 5.5A). Parallel control experiments, in which

the glucose concentration was maintained at 3 mM throughout, were also performed.

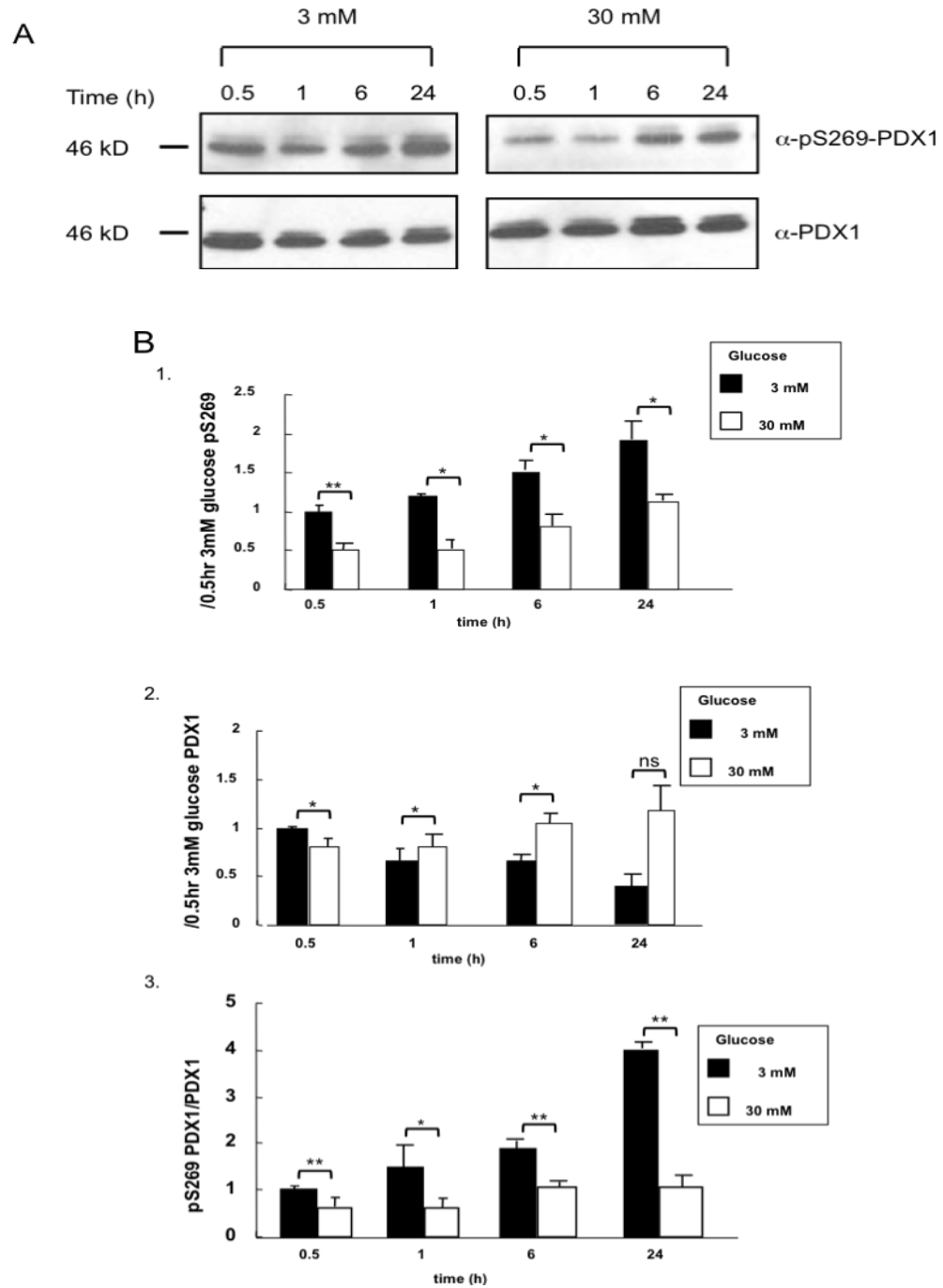


Fig. 5.5. Phosphorylation on S269 is regulated by glucose concentration in MIN6 cells.

(A) MIN6 cells were cultured in medium containing 3 mM glucose for 16 h and incubated with 3 or 30 mM glucose for indicated time. The same amount of nuclear lysate was separated on SDS-PAGE and gels were analyzed by western blot using anti-phospho-S269 antibody and anti-PDX-1 antibody. The graph is the representative one of three independent experiments. (B) The relative phospho-S269 PDX-1 (B1), PDX-1 (B2) expression level and the ratio of phospho-S269 PDX-1 to total PDX-1 level (B3) are shown in a graphic representation. Each condition was normalized to 3 mM for 0.5 h protein level. The results are the means $\pm$ SEM of three independent experiments. **, $p<0.01$; *, $p<0.05$; ns: non-significant (two-tailed student's *t*-test, Microsoft ExcelTM).

Both wild-type and mutant forms of PDX-1 at Ser-269 displayed a dominant nuclear area distribution at both low and high glucose concentration (Below; Fig. 5.6). Thus nuclear extracts from all conditions were separated on SDS-PAGE and analysed by immunoblotting using the antiphospho-S269 PDX-1 antibody.

At both low and high glucose concentrations, the phosphorylation of PDX-1 at Ser269 was increased with time (Fig. 5.5A and B1). However, as early as 30 min. after exposure to these glucose concentrations, cells maintained at 30 mM glucose displayed a markedly lower degree of phosphorylation at Ser269 in PDX-1 (Fig. 5.5B1), and this difference increased, with enhanced phosphorylation at Ser269, during the subsequent 24 h. Meanwhile, total PDX-1 level was examined using anti-PDX-1 antibody (Fig. 5.5A and B2). It displayed a gradual decrease in total PDX-1 immunoreactivity with time in cells maintained at 3 mM glucose (Fig. 5.5A and B2). By contrast, in cells maintained at 30 mM glucose, total PDX-1 level increased with time (Fig. 5.5A and B2).

In order to analyze the glucose regulation in the phosphorylation of PDX-1 at Ser269 independently from the changes of total PDX-1 level, ratio of phosphoSer269-PDX-1/total nuclear PDX-1 was taken and compared (Fig. 5.5B3). The normalized ratio (phosphoSer269-PDX-1/total nuclear PDX-1) increased

dramatically in cells maintained at 3 mM glucose during experimental time period (Fig. 5.5B3) whereas the ratio of phosphorylation at Ser269 levels to total nuclear PDX-1 was more stable at 30 mM glucose.

5.3.6 Phosphorylation at Ser269 affects the subcellular distribution of PDX-1 in MIN6 cells.

To explore the potential effect of PDX-1 of Ser269, MIN6 cells were infected with adenoviruses expressing *c-myc-tagged* wild-type, S269A or S269E mutant forms of PDX-1. Cells were then cultured for 6 h at either 3 mM or 30 mM glucose¹³⁸. Over-expressed PDX-1-*c-myc* was visualized in fixed and permeabilized cells, using anti-*c-myc* antibody. Both wild-type and S269A mutant PDX-1-*c-myc* were localized in the majority of cells to the nuclear periphery at low glucose concentration by co-labeling of the nucleoplasm with a DNA-binding stain (Fig. 5.6A and Fig. 5.6B). In the remaining cells, the over-expressed protein was localized to both the nuclear periphery and the nucleoplasm. No appreciable PDX-1-*c-myc* immunoreactivity was detectable in the cell cytosol. In MIN6 cells overexpressing wild-type or S269A mutant PDX-1, as previously reported in single MIN6 cells^{137, 138, 154, 224}, cells incubated for the same period in the presence of 30 mM glucose displayed largely nucleoplasmic localization indicating that high glucose causes a redistribution of PDX-1 towards the nucleoplasm²¹⁶ (Fig. 5.6A and Fig. 5.6B quantification). However, the phosphomimetic S269E mutant PDX-1 displayed a dramatically different localization pattern and response to high glucose, compared to wild-type or S269A mutant PDX-1. Thus, at low (3 mM) glucose concentrations, 15.6% of S269E mutant PDX-1 displayed a nuclear peripheral localization (Fig. 5.6B); whilst at 30 mM glucose, the percentage of nuclear peripheral localization of PDX-1-*c-myc* immunoreactivity was increased by around four times (64.2%; Fig. 5.6B). Thus, phosphorylation on Ser269 is likely to affect the subcellular localization of PDX-1 by hampering the PDX-1 translocation from

nuclear periphery to nucleoplasm induced by high glucose concentrations.

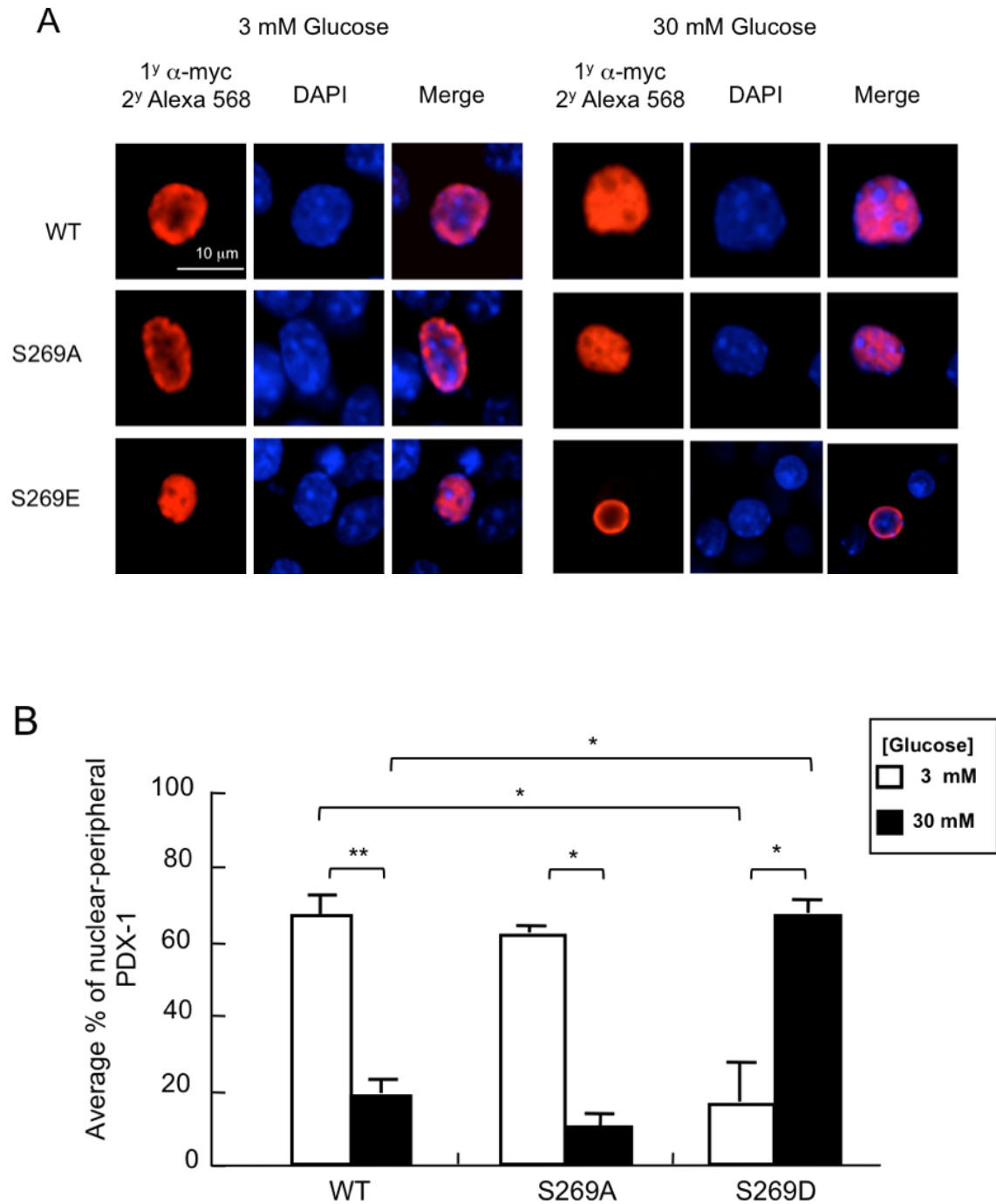


Fig. 5.6. Ser-269 phosphorylation and PDX-1 subcellular localization.

(A) MIN6 cells were infected by adenoviruses expressing Wild-type, S269A or S269E mutant PDX-1 at an MOI of 100 for 24 h. Cells were cultured at 3 mM glucose for 16 h and then treated with 3 mM or 30 mM glucose for 6 h. Anti-*c-myc* antibody (Roche) and Alexa-568 (Molecular Probes) were used to detect *c-myc tagged* PDX-1. Nuclear staining was achieved using DAPI. Confocal images were captured using a Leica SP2 upright laser scanning confocal microscope (x63/1.32 oil-immersion lens) equipped with a krypton/argon laser (488 and 568 nm excitation lines) and UV light. (B) 300-500 cells were analysed under each condition for quantification. The percentage of nuclear-peripheral PDX-1 protein distributed in the cells under different condition was taken using Volocity™ 4.0 quantification software. A graphic representation depicting the percentage of nuclear-peripheral PDX-1 at 3/30 mM glucose is shown. The results are the means±SEM of three independent experiments. **, $p<0.01$; *, $p<0.05$ (two-tailed student's *t*-test, Microsoft Excel™).

5.3.7 Phosphorylation on Ser269 does not affect the DNA-binding ability of PDX-1.

To explore the impact of Ser269 phosphorylation on PDX-1 DNA binding, a 30 bp fragment of the mouse insulin I gene promoter, containing a PDX-1 binding motif (TAAT)¹⁰³, was used in electrophoretic mobility shift assays (EMSA). In agreement with previous findings^{45, 61, 258, 259}, nuclear lysate from cells over-expressing wild-type PDX-1 showed strong binding to the oligonucleotide derived from this promoter element (Fig. 5.7). The binding was specific and was blocked efficiently with unlabeled probe (Fig.5.7). Binding was also super-shifted with anti-PDX-1 antibody. In nuclear extracts from PDX-1 S269A or PDX-1 S269E-expressing cells, the same binding band was evident and in each case this was susceptible to supershifting with anti-PDX-1 antibody. Thus, both phospho-mimetic

and dephospho-mimetic mutants of PDX-1 at Ser269 displayed similar DNA-binding activities compared to wild-type PDX-1, suggesting that phosphorylation at this site is unlikely to affect this parameter directly.

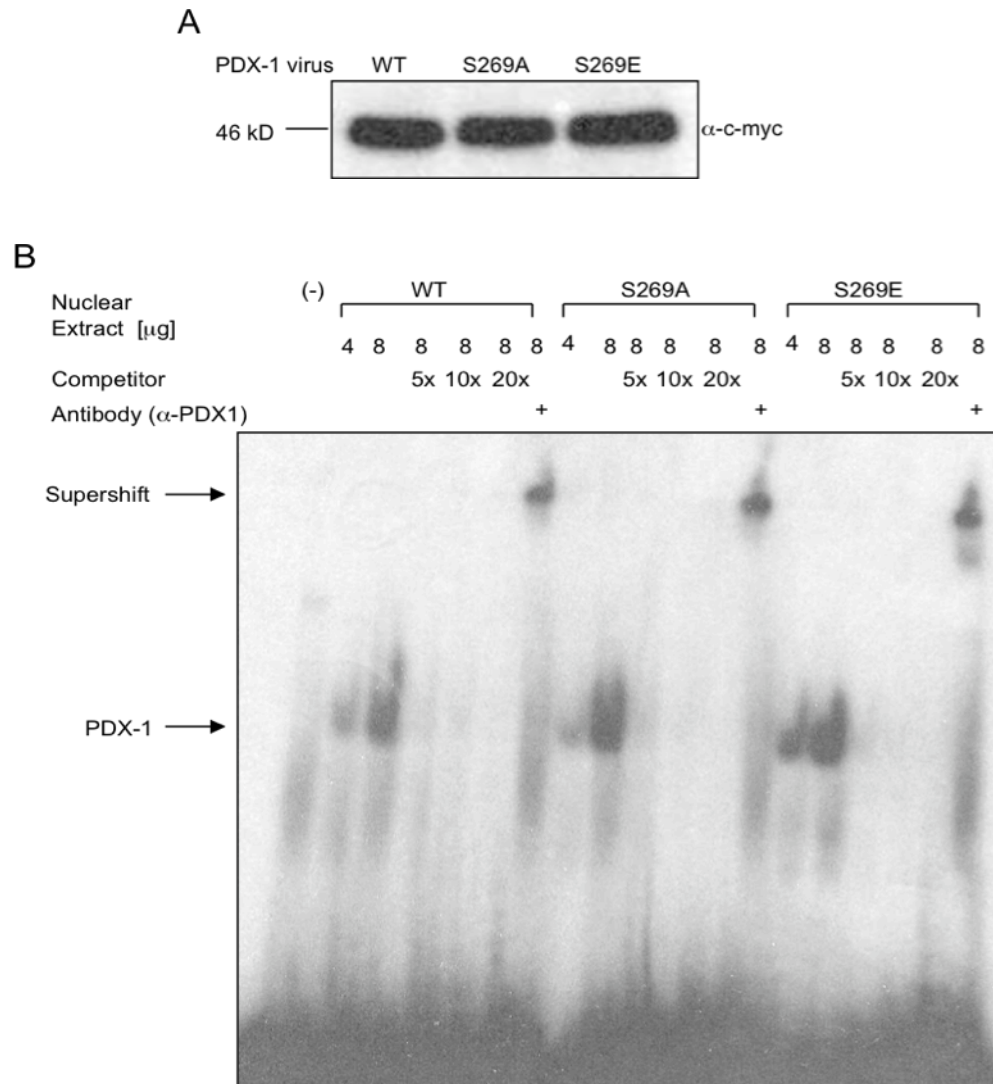


Fig. 5.7. Ser-269 phosphorylation and PDX-1 DNA-binding ability. **A.** 30 μ g of nuclear lysates from HEK293 cells infected by indicated viruses were migrated on SDS-PAGE. The expression level of overexpressed PDX-1 was shown by immuno-blotting using anti-c-myc antibody. **B.** The indicated amounts of nuclear extracts of HEK 293 cells with or without over-expressing wild-type or mutant forms of PDX-1 were incubated with

[γ -³²P] labeled oligonucleotide corresponding to mouse I insulin promoter (A3/A4 element) as indicated with/without 1 μ l PDX-1 antibody for supershift assay, or with 5X, 10X or 20X un-labeled oligonucleotides as binding competitor (Representative blot was shown; the experiments were performed three times independently).

5.3.8 Phosphorylation at Ser269 is unlikely to affect PDX-1 transcriptional activity or stability.

It has previously been reported that the C-terminal domain of PDX-1 may play an important role in controlling transcriptional activity ¹⁵⁹. We therefore investigated whether the dephospho- or phosphomimetic mutant forms of PDX-1, S269A and S269E respectively, displayed different transcriptional activity with respect to wild-type PDX-1.

We began by studying the ability of PDX-1 to affect cell lineage definition by monitoring the expression of glucagon mRNA in the INS $\alpha\beta$ cell line ²¹⁶. In these cells, doxycyclin induces mouse PDX-1 mRNA, and strongly decreases glucagon mRNA levels, reflecting a switch towards a β -cell lineage ²¹⁶. Adenoviral transduction of INS $\alpha\beta$ cells with wildtype, S269A or S269E mutant PDX-1 all led to similarly efficient suppression of glucagon mRNA expression compared with the effect of doxycyclin (Fig. 5.8A). Thus, the ability of the over-expressed dephospho- or phospho-mimetic forms of PDX-1 to affect lineage definition was not altered compared to wild type PDX-1 in this cell system.

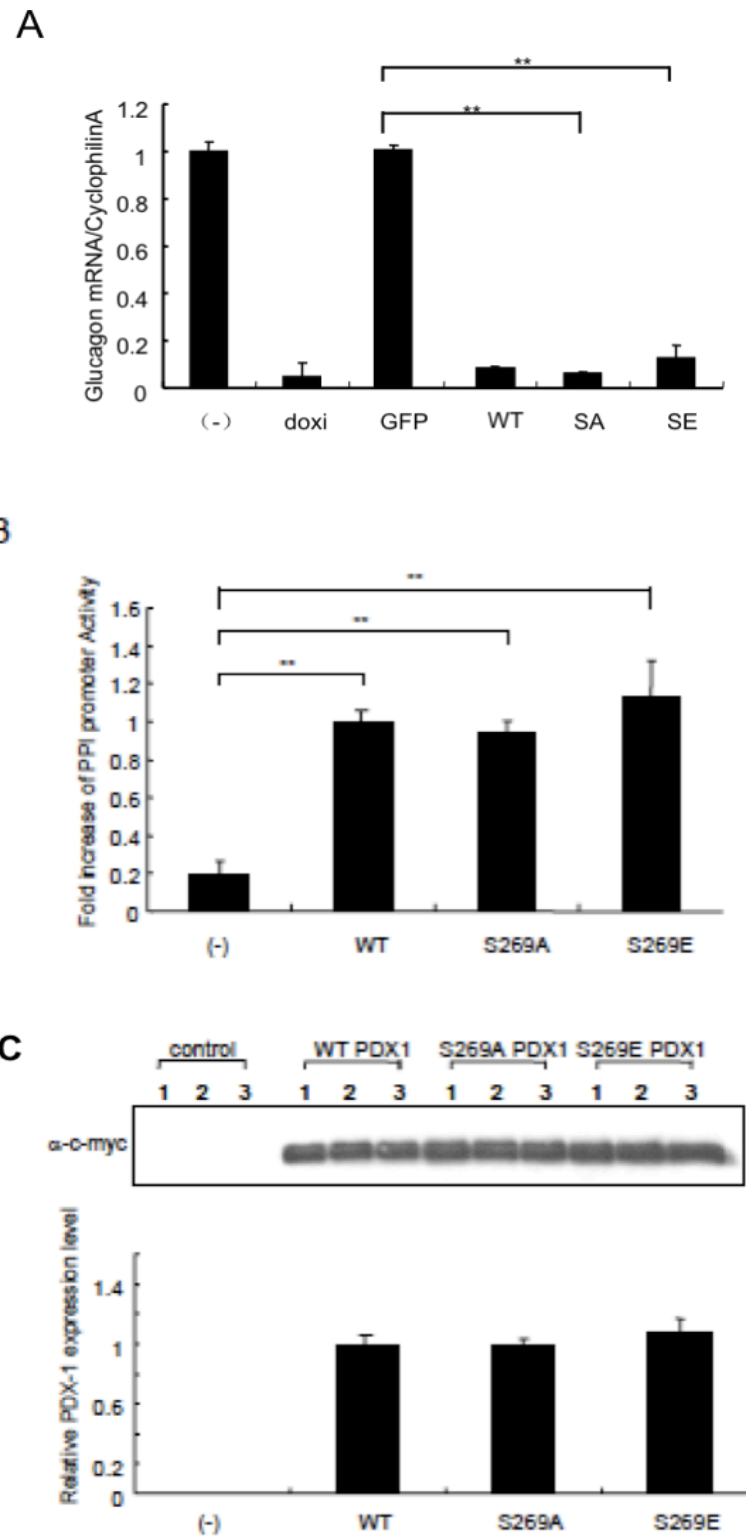


Fig. 5.8. Ser-269 phosphorylation and PDX-1 transcriptional activity.

Glucagons (A) gene expression in INS $\alpha\beta$ cells overexpression de- and phosphomimetic mutants on PDX-1. RNA was extracted from cells treated with 500 ng/ml doxycyclin or infected with wild-type or mutant PDX-1. Cyclophilin A and glucagon gene expression were monitored by real time RT-PCR. The relative glucagon gene expression level to cyclophilin A was shown in (A). (B) HEK 293 cells were transfected using Ca²⁺-phosphate with pcDNA3 empty vector or pcDNA3-PDX-1-*c-myc* WT or S269A/E mutant forms, *PPI promoter-luciferase*, and *Renilla-luciferase*. The results are the means $\pm$ SEM of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; two-tailed student's *t* test, Microsoft ExcelTM). (C) Equal amount of whole cell lysates from the luciferase assay were analysed by western blot using anti-*c-myc* antibody. The relative mean value of PDX-1 expression level was shown in a graphic representation.

We next explored the impact of mutations at Ser269 on the *transactivation* of the preproinsulin promoter using a luciferase promoter -reporter system in a heterologous cell line. Transfection of HEK293 cells with equivalent amounts plasmid encoding *myc*-tagged wild-type, S269A or S269E PDX-1 led to the same degree of induction of the human preproinsulin promoter, compared to cells transfected with empty vector only (Fig. 5.8B). Transfection efficiency in these experiments was verified by western (immuno-) blotting using anti-*c-myc* antibody; the levels of the three different forms of PDX-1 assessed in this way was similar.

In a preliminary experiment, we also tested whether the phosphorylation of PDX1 on Ser269 could affect PDX1 protein stability by pulse-chase analysis. MIN6 cells, infected either with PDX1 wild type or with the two Ser269 mutants, were pulse labeled for three hours with a mixture of [³⁵S] methionine and cysteine and then chased for 0, 2, 4, 8, 16 h in fresh medium containing an excess of unlabeled methionine and cysteine. Neither the phosphomimetic S269E nor the non-phosphorylatable S269A mutations displayed altered protein stability compared to the wild type (preliminary data by Dr. Francesca Simplici not shown).

5.4 DISCUSSION

The principal aim of the work described in this Chapter was to further elucidate the molecular mechanisms involved in the regulation by glucose and other stimuli of the nuclear uptake, DNA binding and *transactivation* capacities of PDX-1. We demonstrate firstly that Ser269 in the C-terminal domain of PDX-1 is phosphorylated in living β -cells. As far as we are aware, this represents the first description of a phosphorylation event on PDX-1 *in situ* achieved through the use of mass spectrometry and subsequently verified with a phosphospecific antibody. Whilst evidence has previously been obtained by others^{141, 142} and in chapter 3 that PDX-1 exists as a phosphoprotein in clonal β -cells, and that the incorporation of Pi may be modulated by glucose, the sites of phosphorylation were poorly defined. Of note, phosphorylation of PDX-1 at Ser61 and Ser66 was recently reported in MIN6 cells¹⁴² based on the abolition of a gel shift to a higher molecular weight species (43 kDa) presumed to represent a form phosphorylated at Ser66, and using phosphospecific antibodies. Oxidative stress in response to H₂O₂ was shown in the same study to increase phosphorylation at these sites, possibly via the activation of GSK3. However, the effects of glucose on the extent of phosphorylation at either site were not investigated in this earlier report, in which the action of glucose to promote nuclear uptake of PDX-1^{138, 154, 216, 260} was not observed. However, it must be emphasized that the extent of the change in cytoplasmic-nuclear localization of PDX-1 in response to altered glucose concentrations is relatively modest and may not have been apparent in the study by Boucher *et al*¹⁴² where the cytoplasmic *versus* nuclear distribution of the factor was not quantitated precisely at the level of individual cells. In the present studies, by contrast, we were unable to detect any direct evidence for significant phosphorylation of the corresponding peptide (bearing Ser61 and Ser66) under the conditions used. Likewise, whilst recombinant PDX-1 is avidly phosphorylated *in vitro* by the nutrient regulated kinase Per-Arnt-Sim (PAS) kinase²⁶⁰, we have been unable to demonstrate any occupancy at the site phosphorylated *in vitro* (Thr152) in intact cells (Chapter 4).

We also show here that the degree of phosphorylation of PDX-1 at Ser269 was decreased at elevated glucose concentrations and correlated inversely with PDX-1 levels. In a preliminary experiment testing whether the phosphorylation of PDX1 on Ser269 could affect PDX1 protein stability by pulse-chase analysis with MIN6 cells infected either with PDX1 wild type or with the two Ser269 mutants, neither the phosphomimetic S269E nor the non-phosphorylatable S269A mutations displayed altered protein stability compared to the wild type.

Wild type *c-myc*-tagged PDX1-expressing adenovirus was mutagenized to replace Ser269 either with an alanine, to produce a dephosphomimetic S269A mutant, or with a glutamic acid, producing a phosphomimetic S269E mutant. MIN6 cells were infected with either PDX1 wild type or the mutants and immunostained with anti-*c-myc* antibody. Whereas in response to an increase (3-30mM) in glucose concentration both the wild-type and the S269A mutant translocated from the nuclear periphery to the nucleoplasm, the phosphomimetic S269E mutant localized preferentially to the nuclear periphery at high glucose concentration. However, other functional analysis of the possible effects of phosphorylation at the site using (de) phosphomimetic mutants on transactivation of preproinsulin promoter activity or PDX-1 DNA-binding ability failed to reveal further differences between the transactivation capacity of the wild-type protein compared to mutants bearing A or E in place of S269. As the effect of PDX-1 mutants on preproinsulin promoter activity was studied in the non-beta-cell environment-HEK293 cells by plasmids-transfection, in order to obtain a definite conclusion, similar experiments in MIN6 β -cells will be a future direction.

What may be the role, if any, of GSK3 β in regulating PDX-1 function or stability? Whilst GSK β alone was unable to lead to the phosphorylation of PDX-1 (not shown), both wild-type and S269A PDX-1 were phosphorylated *in vitro* by CK1 δ .

Importantly, the apparent phosphorylation of wild-type PDX-1 was increased by the addition of GSK3, implying that the latter enzyme phosphorylates PDX-1 at a site “primed” by CK1δ. As proposed in Fig. 5.9B, CK1δ-catalysed phosphorylation of PDX-1 at Ser273 may render the sequence ²⁶⁹SPQP²⁷³S susceptible to subsequent phosphorylation by GSK3 at Ser269, now lying in a consensus site for GSK3 phosphorylation site. What is the evidence that CK1δ may, in fact, phosphorylate PDX-1 at Ser273? Although the sequence around Ser273 does not fit any known CK1δ consensus, it might be noted that CK1δ is often able to phosphorylate “atypical” targets, as reported by Marin *et al* for NF-AT4 ²⁶¹ and β-catenin ²⁶². Although direct confirmation of the above hypothesis was not provided by the use of an anti -phosphoSer269-specific antibody, it is possible that the close proximity of another phosphate residue on Ser273 would block the recognition of phospho-Ser269 by the anti-pSer269 phosphospecific antibody (Fig. 5.4A, and see Fig. 5.9B). This in fact was raised against the phosphopeptide...SV**pSPQPS**₂₇₃SI...including S₂₇₃ in its non-phosphorylated form (see Methods) and it is quite conceivable that the bis-phosphorylated sequence will escape recognition by a mono-phosphospecific antibody.

On the other hand, a priming effect of HIPK2 for phosphorylation by GSK3 (Fig. 5.4B) can be more easily reconciled with the observed data. Thus, if we assume that HIPK2 is a proline-directed kinase, then both Thr214 and Ser269 in the C-terminal domain of PDX-1 represent potential phosphorylation sites. Indeed we demonstrate directly (Fig. 5.3) that wild-type PDX-1 is phosphorylated by HIPK2 on Ser269, as well as at least one other site(s) (Fig. 5.3). However, Ser269 cannot prime phosphorylation by GSK3 as it lacks a Ser/Thr residue upstream, at the canonical n-4 position. Likewise Thr214, representing another appealing candidate as a potential HIPK2 site found by MS analysis in a phosphorylated peptide (see Fig. 5.1), lacks a S/T residue at position n-4.

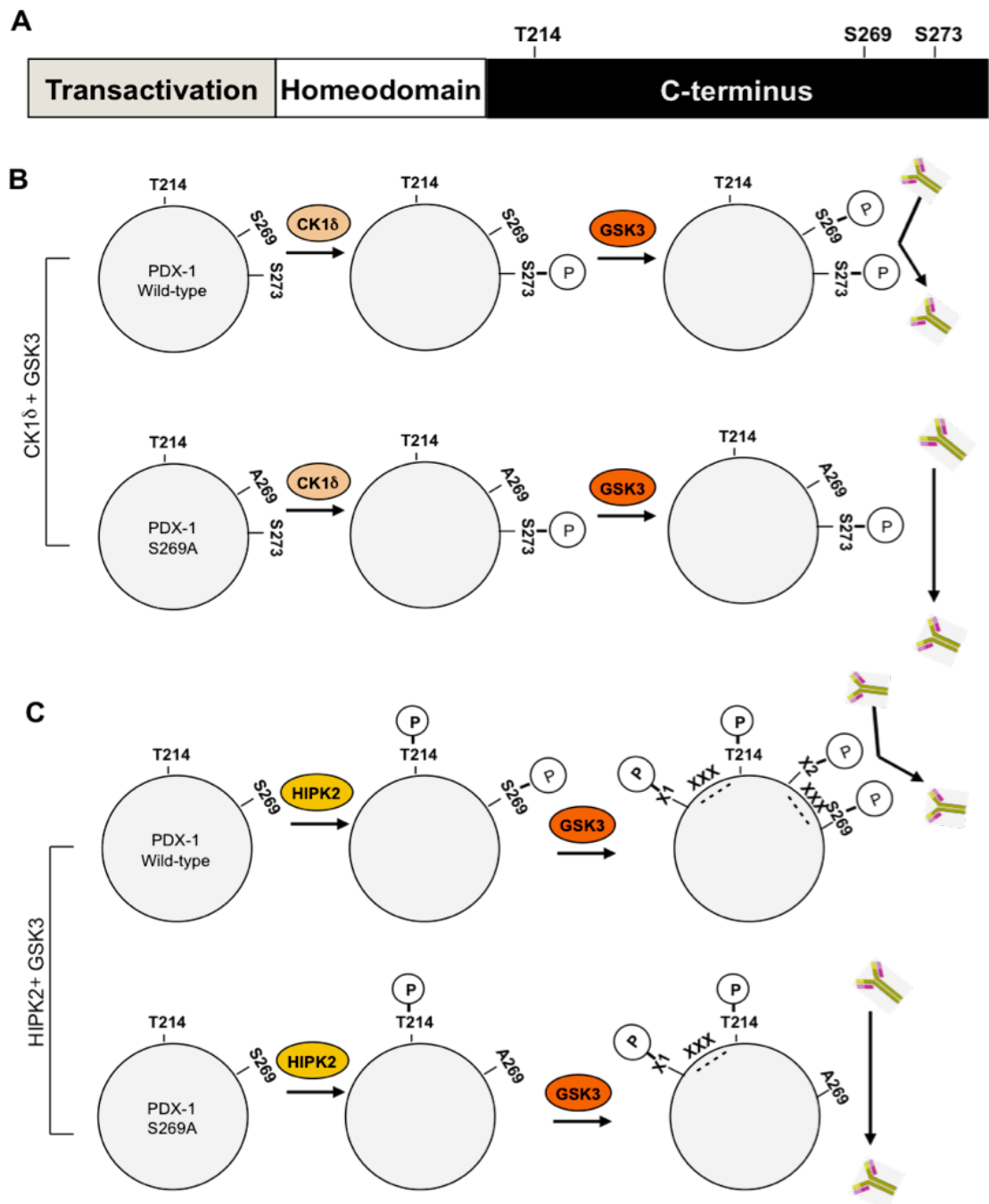


Fig. 5.9. Model of GSK3 regulation on Ser269 phosphorylation.

(A) Schematic diagram of PDX-1 structure and the potential phosphorylation sites involved in GSK3 phosphorylation. The black region represents PDX-1 C-terminus. (B) Model of synergistic phosphorylation by casein kinase I δ and GSK-3 on both wild-type PDX-1 and S269A mutant.

(C) Model of synergistic phosphorylation by HIPK2 and GSK-3 on both wild-type PDX-1 and S269A mutant. WT represents wild-type.

Moreover no S/T-x-x-x-S/T-P motif suitable for sequential phosphorylation by HIPK2 at the downstream residue, and then by GSK3 at the upstream can be found in the entire sequence of mouse PDX-1. We have to assume, therefore, that either phosphorylation by HIPK2 in this case is not specified by the S/T-P motif, or that the residue phosphorylated by HIPK2 primes the intervention of GSK3 at a position other than the usual one, i.e. n-4. Pertinent to this may be on the one hand the observation that the crucial dependence of HIPK2 targeting on a prolyl residue at position n+1 is only based on the observation that its phosphosites identified to date fulfill this feature ²⁶³ and we know that some Pro-directed protein kinases do tolerate the replacement of the crucial proline by a different residue; on the other hand, priming of GSK3 is sometimes determined by phosphoresidue(s) which are located far away in the primary structure from the target residue, as typically exemplified by Inhibitor-2 of protein phosphatase 1, whose crucially functional phosphorylation by GSK3 at T72 needs to be primed by CK2 which phosphorylates I-2 at several residues, mostly S86 and others even further away from T72 ²⁶⁴. It has been postulated that in these cases the consensus is “three-dimensionally” determined.

In summary, our data provide the first report of a phosphorylation event in PDX-1 in living β cells, as revealed through the use of mass spectrometry and confirmed by the generation of a phosphoserine-specific antibody. Moreover, we provide evidence that phosphorylation of the identified residue, Ser269, is dynamically regulated by glucose. Moreover, the phosphomimetic mutant S269E PDX-1 appeared to alter the reported PDX-1 translocation from nuclear periphery to nucleoplasm at elevated glucose. As it was mentioned in the introduction, Boucher *et al* reported that HIPK2 is expressed in the developing pancreatic epithelium from

embryonic day (e) 12 to e15 but that the expression becomes preferentially confined to pancreatic endocrine cells at later developmental stages. Moreover, HIPK2 was reportedly able to modulate positively the transcriptional activity of PDX-1, and HIPK2 directly phosphorylates the C-terminal portion of PDX-1¹⁶¹. The phosphorylation site(s) are not known, however, identification of phosphorylation sites on the few known HIPK2 targets suggest that HIPK2 may be a proline-directed serine/threonine kinase^{249, 265}. Analysis of PDX-1 sequence showed that Serine269-proline (Ser269), together with threonine214-proline (Thr214) were both potential phosphorylation sites on the C-terminus. In this Chapter, we reported that PDX-1 can be phosphorylated by HIPK2 on Ser269 *in vitro*. This finding indicated that that Ser269 is a strong candidate for HIPK2 phosphorylation and can help the future study on the phosphorylation of PDX-1 by HIPK2 in living cells.

We also provide evidence that phosphorylation of PDX-1 by HIPK2 and/or CK1δ may influence the upstream phosphorylation of PDX-1 by GSK3 in response to glucose, insulin or other factors, to mediate the stability of PDX-1. Future studies will be required to determine the roles, if any, of changes in the activity of these potential upstream kinases in regulating PDX-1 phosphorylation at Ser269 in living β-cells. Moreover, the extent to which phosphorylation of PDX-1 at Ser269 may influence the DNA binding or *transactivation* capacity of PDX-1 remains unclear, given our present findings using (de)phosphomimetic forms of the protein; experiments using more physiological levels of PDX-1 variants, which were not technically feasible in this study, will be necessary to address these questions in the future.

6. Chapter 6

General Discussion

6.1 Review of experimental findings

Pancreatic Duodenum *Homeobox* (PDX-1) is a *homeodomain* transcription factor and a key regulator of β -cell development and differentiation and plays a crucial role in β -cell specific gene expression. It is therefore critical to understand the regulation of PDX-1, especially the post-translational regulatory network. The work in this thesis investigated the phosphorylation of PDX-1 under physiological condition and directly demonstrated that PDX-1 is phosphorylated in living β -cells (Chapter 3). It also further explored the *in vivo* phosphorylation sites of PDX-1 and the regulatory effects of the phosphorylation on these sites on PDX-1 transcriptional activity (Chapter 4 and 5).

Additional kinases which may phosphorylate PDX-1 at various sites have been proposed by different groups^{63, 78, 137, 142, 148, 154}. We also performed a series of *in vitro* phosphorylation reactions using recombinant PDX-1 and purified kinases. Aurora, Rsk1, PKC α , JNK were all found to phosphorylate PDX-1 *in vitro* (Chapter 3). *In vitro* phosphorylation sites of PDX-1 by JNK were revealed (Thr-214 and Ser-66) using mass spectrometry sequencing (Chapter 3).

In an effort to investigate the signaling pathway involved in PDX-1 post-translational regulation, *in vitro* phosphorylation using recombinant PDX-1 and purified human PASK showed that PDX-1 is efficiently phosphorylated *in vitro* at Thr-152 in the *homeodomain* by the nutrient-regulated kinase, PASK (Chapter 3). Moreover, the site appeared to be specific for PASK as the T152A mutant PDX-1 recombinant protein showed no difference with wild-type PDX-1 in the *in vitro* phosphorylation by a series of kinases including Aurora, Rsk1, PKC α , JNK etc. (Chapter 3). This finding prompted us to investigate the phosphorylation of PDX-1 in living cells and the role of Thr-152 phosphorylation on the transactivational

capacity of PDX-1 in native β -cells exposed to high extracellular glucose concentrations (Chapter 4).

We demonstrated the first time that PDX-1 is phosphorylated under physiological condition in INS-1(832/13) cells (Section 3.3.5). The problem we tackled is what role does Thr-152 phosphorylation by PASK play in the post-translational regulation of PDX-1? We generated phospho- and dephospho- mimetic PDX-1 mutants to explore the likely effects of Thr-152 phosphorylation on PDX-1 function, and examined the possible role(s) played by PASK in two β -cell systems, INS-1(832/13) cells and INS $\alpha\beta$ cells²¹⁶. In the latter cell-type (though not in other α -cells and enteroendocrine cell lines)²³⁰, PDX-1 potentially inhibits preproglucagon gene expression, allowing a switch from α -to β -cell lineage.

It was observed that mimicking phosphorylation on Thr-152 resulted in a potent inactivation of PDX-1 reflected in a near total loss of DNA-binding capacity and in the ability of the factor to induce a cell lineage switch in INS $\alpha\beta$ cells. Arguing against the view that PASK phosphorylates PDX-1 *in situ*, nuclear localization of the kinase in MIN6 β -cells was not previously noted for over-expressed PASK²⁰⁹. We now show, using direct and complementary approaches, that Thr-152 is unlikely to be significantly phosphorylated *in situ* under a variety of conditions, including those in which endogenous PASK is activated (elevated glucose concentrations) or after over-expression of the exogenous kinase (Chapter 4). The present data thus indicate that Thr-152 is not a major site of phosphorylation of PDX-1 in living β -cells under most conditions, and that phosphorylation at this site is not altered under conditions in which the nuclear accumulation of PDX-1 is affected (ie high versus low glucose concentrations).

Additionally, we identified a novel phosphorylation site in the C-terminal domain of PDX-1, Ser-269, via mass spectrometry analysis and by using an anti-phosphoSerine-specific antibody (Chapter 5). This site is phosphorylated by HIPK2

in vitro, and the phosphorylation on S269 is regulated by glucose concentration in MIN6 cells. Moreover, the phosphomimetic mutant PDX-1 (S269E) appeared to alter the PDX-1 translocation from nuclear periphery to nucleoplasm at elevated glucose concentration. As reported in a previous study by Edlund a minor portion of PDX-1 is phosphorylated on S61 and S66, and the phosphorylation is modulated by a GSK3-dependent mechanism and marks the degradation of PDX-1. In this Thesis, the *in vitro* phosphorylation studies also provided potential possibilities of the relationship between the S269 phosphorylation, GSK3 phosphorylation and regulation of PDX-1 (Chapter 5). However, mutation of S269 phosphorylation did not affect the DNA-binding ability and transcriptional activity of PDX-1 (Chapter 5). Thus, we present the first *in vivo* phosphorylation site of PDX-1, identified from mass spectrometry phospho-site screening, and provide evidence of the potential regulation network the site could be involved in. This may shed light on the discovery of the signaling pathway which regulates the C-terminus of PDX-1.

6.2 Updated PDX-1 phosphorylation network

That PDX-1 transcriptional activity is regulated by glucose-dependent phosphorylation of the protein was first proposed by Macfarlane et al ⁶³. Potential kinases triggering this event were investigated in this early study ⁶³, and it was reported that neither cyclic AMP-dependent protein kinase A nor protein kinase C were involved.

Further study indicated that glucose-stimulated PDX-1 DNA-binding activity to the regulatory region of the human insulin gene¹³⁴, and PDX-1-activated gene transcription, were shown inhibited by a specific inhibitor of the stress-activated protein kinase 2 (SAPK2), SB203580 ¹³⁵, which also inhibited the glucose triggered activation of MAPKAP-K2, an immediate downstream target of SAPK2. As discussed on page 37 Elrick and Docherty ¹⁵⁴ observed that PDX-1 shuttles between the nuclear periphery and the nucleoplasm in a phosphorylation dependent manner in response to changes in extracellular glucose and insulin concentrations and the translocation of PDX-1 is inhibited by the PI3K inhibitor wortmannin and the p38/SAPK2 inhibitor SB 203580, suggesting that the PI3K and p38/SAPK2 pathways are involved ¹⁵⁴.

However, as reported by Rafiq et al ¹³⁷, in MIN6 β -cells, neither the inhibition of SAPK2 by SB 203580 nor overexpressing wild-type SAPK2 or its upstream kinase MKK6 (MAP kinase kinase-6) affected the preproinsulin promoter activity, PDX-1 transcriptional activity and PDX-1 subcellular translocation. Moreover, they failed to detect any regulation on extracted p38/SAPK2 activity by elevated glucose concentration. Meanwhile, this study did confirm that PI3K is ‘necessary and sufficient’ for the glucose-activated PPI gene transcription, however, whether or not the downstream signaling pathway involve p38/SAPK2 remains unknown.

P38/SAPK2, together with the extracellular signal-regulated kinases (ERK1, ERK2)²⁶⁶⁻²⁶⁸ and the c-Jun N-terminal kinases (JNKs), all belong to the serine/threonine-specific protein kinases, mitogen-activated protein (MAP) kinases. The JNKs are responsive to stress such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock and are involved in cell differentiation and apoptosis²⁶⁹. Kawamori et al⁷⁸ reported that PDX-1 translocates from the nuclei to the cytoplasm of pancreatic β -cells in response to oxidative stress in hamster β -cell-derived HIT-T15 cells and this oxidative stress-stimulated translocation is through the activation of the JNK pathway but the precise phosphorylation site(s) remain unknown.

In this Thesis, a series of kinases including ERK2, SAPK2 and JNK were tested by *in vitro* phosphorylation of PDX-1 (Chapter 3). The results suggested that both SAPK2 and ERK2 phosphorylate PDX-1 very poorly. However, JNK phosphorylated PDX-1 well *in vitro* and the phosphorylation sites, as revealed by mass spectrometry phosphorylation site screening on recombinant PDX-1 protein (Chapter 3), included Ser-66 and Thr-214. These findings provide direct evidence for JNK phosphorylation of PDX-1 *in vitro* and could account for the oxidative stress-induced PDX-1 translocation observed by Kawamori and colleagues⁷⁸, and are also potential candidate phosphorylation sites for JNK phosphorylation of PDX-1 in living cells.

Interestingly, Boucher et al¹⁴² demonstrated in 2006 using phospho-specific antibodies that a minor proportion of PDX-1 is phosphorylated on serine 61 and/or serine 66 in β -cells. Phosphorylation was increased following proteasome inhibition, and it is also modulated by a GSK3-dependent mechanism. GSK3 activity has been found to be increased in insulin target tissues from diabetic mice^{143, 144} and inhibiting GSK3 using specific inhibitors or lithium chloride improved glucose metabolism in insulin resistant Zucker rats^{145, 146}. Boucher et al also found

that oxidative stress increased PDX-1 phosphorylation on either site, and further increased the degradation of the protein, while the half-life of the protein is decreased. The phosphorylation was found to be modulated by oxidative stress in a GSK3-dependent manner. It has also been reported that active canonical Wnt signaling decreased GSK3 activity¹⁴⁷ and β -cells are sensitive to the oxidative stress which increases with age. Thus a new mechanism was proposed for the declined levels of PDX-1 and resultant β -cell dysfunction during diabetes progression in which the canonical Wnt signaling counteracts age-related increase in oxidative stress by inhibiting GSK3 activity to rescue PDX-1 from protein degradation and in turn maintain normal β -cell function¹⁴². It was discussed in Chapter 5 that a priming process is necessary for GSK3 phosphorylation, moreover the Kawamori et al⁷⁸ study together with our finding that Ser-66 is phosphorylated by JNK *in vitro* could indicate an intermediate role of the JNK pathway as a priming process for further GSK3 function during the oxidative stress-induced regulation of PDX-1.

In an attempt to identify potential upstream kinases for PDX-1 in pancreatic β -cells, we also studied the *in vitro* phosphorylation of PDX-1 by AMP-activated protein kinase (AMPK), and also Akt/PKB. However, neither of these candidates phosphorylated PDX-1 *in vitro*. Interestingly the nutrient-sensing kinase PASK efficiently phosphorylated PDX-1 on Thr-152, while not being able to phosphorylate other β -cell enriched transcription factors including NeuroD and MafA. However, studies using site-mutagenesis of Thr-152 in living β -cells indicated that the site is important for the DNA-binding, transcriptional activity and subcellular localization of PDX-1. Whether it is a physiological phosphorylation site of PDX-1 in normal β -cells remains unclear (Chapter 4).

Additionally, phosphorylation site screening by mass spectrometry helped us to identify a novel phosphorylation site on PDX-1 in living β -cells, S269. Interestingly, this was found to be a phosphorylation site for the homeodomain-interacting protein kinase-2 (HIPK2) *in vitro* (Chapter 5). The phosphorylation of

PDX-1 by HIPK2 on C-terminus was reported by Boucher et al recently ¹⁴⁸ but the exact phosphorylation sites were not identified. Our finding thus opens up a new avenue of investigation of mechanisms which regulate PDX-1's transcriptional activity, and also possibly pancreas development and/or β -cell function ¹⁴⁸.

In summary, as shown in Fig. 6.1, phosphorylation of PDX-1 involves a complex network which may involve several kinases and signalling pathways. We tackled the phosphorylation of PDX-1 based on previous results from others and from our group, involving PASK, p38/SAPK2, c-Jun N-terminal kinase, GSK3 and HIPK2. The findings in this Thesis substantially update our understanding of PDX-1 phosphorylation and the kinases involved, and lead us a further step on the way to unveiling the final regulatory signaling pathway(s) controlling PDX-1 phosphorylation.

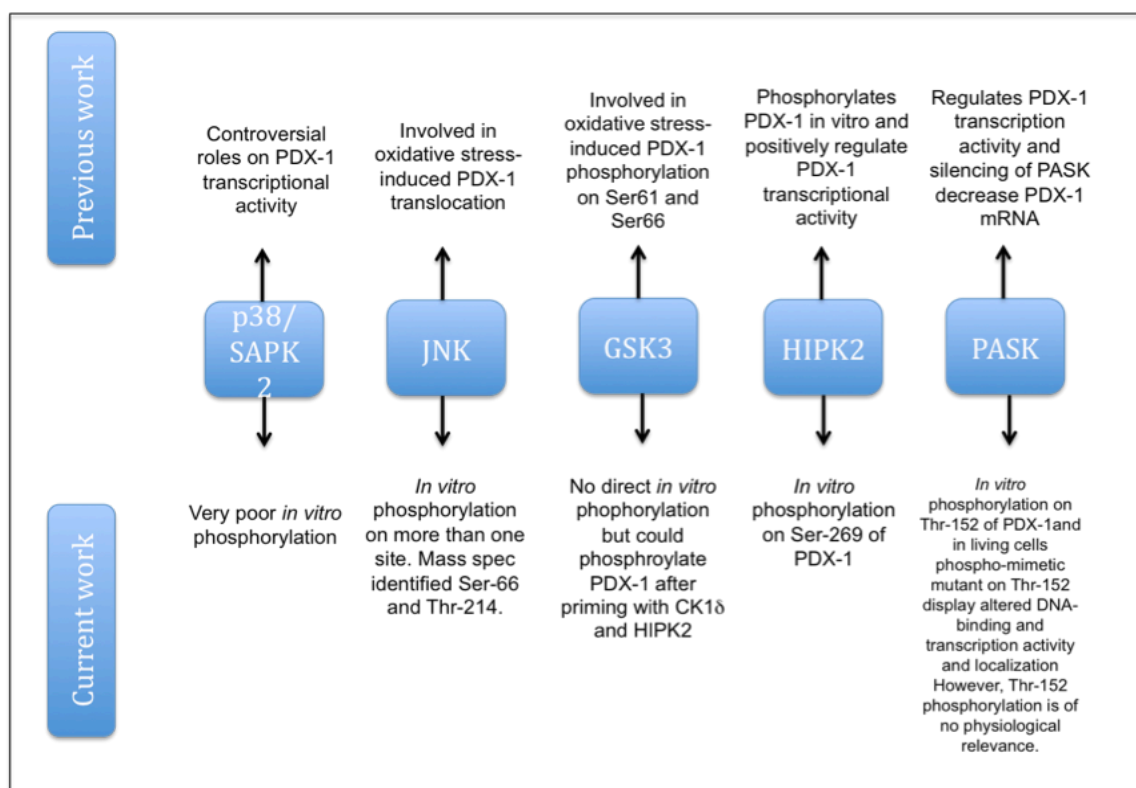


Fig. 6.1 Schematic summary of PDX-1 phosphorylation by kinases discussed in this Thesis.

6.3 Future perspectives

Despite the advances made in understanding PDX-1 phosphorylation in this Thesis, a number of questions still remain to be answered.

1. *Is PDX-1 a downstream target of PASK regulation in β -cells?* In the study of PDX-1 phosphorylation by PASK, Thr-152 was shown to be an *in vitro* phosphorylation site but with no physiological relevance in living β -cells. It has been reported by Da Silva Xavier *et al* ²⁰⁹ that silencing of PASK expression markedly decreased the accumulation of preproinsulin and PDX-1 mRNAs in response to high glucose. Additionally, regulation by PASK or glucose of both the preproinsulin and PDX-1 promoters was completely abrogated by deletion of PDX-1-binding sites, implicating PDX-1 and possibly MafA as targets of PASK action ²⁰⁹. Therefore, this could indicate other phosphorylation site(s) on PDX-1 by PASK if there is a direct interaction between PASK and PDX-1 in living cells or otherwise, as is proposed in ²⁰⁹, the downstream target of PASK could be MafA instead. However, the study by Hao *et al* in 2007 ²¹⁰ again made hypothesis more complicated as they found that the expression levels of several genes involved in insulin transcription and glucose-stimulated insulin secretion (GSIS), including PDX-1, MafA, GLUT2, and glucokinase, were also unchanged in PASK ^{-/-} islets ²¹⁰.
2. *Is phospho-Thr-152 less stable in vivo?* The results in Chapter 4 show that Thr-152 phosphorylation was not detected in living β -cells using either phospho-specific antibody against phosphorylation on Thr-152 or mass spectrometry phospho-site screening. However, the phosphorylation may bring about nuclear export and rapid degradation and thus it may be very difficult to detect phospho-Thr-152 using any approach.

Hence alternative mass spectrometry approach could be applied in the future. For example, the phosphoprotein peptides could be selected and concentrated using a phosphospecific surface (Surface-enhanced laser desorption ionization (SELDI)) or by immobilized metal ion affinity chromatography (IMAC) as well as by purifying the radioactive peptides and then using mass spectrometry to investigate the phospho-site. For sample preparation, protein lysate from cells cultured at low glucose conditions when the PDX-1-DNA binding is weaker could be used to obtain more efficient sequencing results.

3. *What is the subcellular localization of endogenous PASK in rodents?*

Overexpressed human PASK in rodent cloned β -cell lines was localized predominantly in the cytoplasm, as revealed by the anti-PASK antibody against human PASK due to the inavailability of anti-PASK antibody against rodent PASK. However, in dispersed human islets the endogenous PASK displayed universal distribution both in the cytosol and the nuclear area in β -cells. One explanation could be that endogenous PASK distribution between dispersed cells and intact islets is different; it has previously been reported by Pérez-Armendariz et al ²⁷⁰ that the behavior of cells in intact islets differs significantly from the behavior of dispersed cells when they were studying the electrical activity of pancreatic islets was studied using patch clamp. Therefore, more primary tissue work on human islets will be needed e.g. immunohistochemistry on intact human islets. Another explanation could be that overexpressed human PASK distributes differently in rodent cell lines in which case specific antibodies against rodent PASK for immunocytochemistry need to be generated.

4. *Is PDX-1 phosphorylated by HIPK2 or JNK at identified potential sites in living cells?* Potential phosphorylation sites of PDX-1 by HIPK2 and JNK

In vitro phosphorylations were identified, Ser-269 by HIPK2 and Ser-66, Thr-214 by JNK, respectively. In the study by Boucher *et al*¹⁴⁸, transfection of *Hipk2* siRNA reduced by approximately 50% the activity of the *insulin* promoter in MIN6 cells already 36 h post-transfection and HIPK2 was found positively modulate PDX-1 target genes in a PDX-1-dependent manner. What is the *in vivo* relevance of HIPK2 in related to PDX-1 phosphorylation on Ser269? In order to give a clearer answer, RNA interference approaches could be introduced to reduce HIPK2 expression and to examine the impact of siRNA mediated silencing of HIPK2 on PDX-1 phosphorylation on Ser269 in MIN6 cells.

The identification of the potential phosphorylation sites could lead to more specific phosphorylation study in living cells and the physiological relevance of the sites need to be investigated.

5. *What is the relationship between GSK-3 and phosphorylation of PDX-1 at Ser-269 in living cells?* In chapter 5, both wild-type and S269A PDX-1 were phosphorylated *in vitro* by CK1δ. Importantly, the apparent phosphorylation of wild-type PDX-1 was increased by the addition of GSK3, implying that the latter enzyme phosphorylates PDX-1 at a site “primed” by CK1δ. As discussed in Chapter 5, CK1δ-catalysed phosphorylation of PDX-1 at Ser273 may render the sequence ²⁶⁹SPQP²⁷³S susceptible to subsequent phosphorylation by GSK3 at Ser269, now lying in a consensus site for GSK3 phosphorylation site. However, anti-phosphoSer-269 PDX-1 antibody failed to detect the signal very likely because the close proximity of another phosphate residue on Ser273 would block the recognition of phospho-Ser269 by the anti-pSer269 phosphospecific antibody. Therefore, further study on the possible GSK3 phosphorylation on Ser-269 could be conducted with the help of phospho-specific antibody recognizing both phospho-Ser-269 and phospho-273. Additionally, the regulatory effect of

GSK3 on PDX-1 phosphorylation at Ser269 in living cells could be studied using GSK3 inhibitors and the anti-phospho-Ser-269-PDX-1 antibody.

6. *Studies in primary pancreatic islets.* In order to enhance the physiologic relevance of the studies, it is essential and interesting to validate the findings in this thesis in primary pancreatic islets in future study. For example, one future direction could be to study the glucose regulation on the phosphorylation of PDX-1 at Ser-269 using the phospho-specific antibody against phospho-Ser-269 PDX-1 in isolated primary murine pancreatic islets.

6.4 Overall conclusions

The findings in this Thesis bring new understanding on the role and regulation of PDX-1 phosphorylation. We have studied the phosphorylation sites of PDX-1 using mass spectrometry phospho-site mapping and by exploring the potential upstream kinases using *in vitro* phosphorylation. We focused initially on the novel *in vitro* phosphorylation site Thr-152 on PDX-1 by PASK and another phosphorylation site Ser-269 in living cells revealed by mass spectrometry. We investigated the role of phosphorylation on Thr-152 and Ser-269 in the regulation of this critical transcription factor by examining the transcriptional activity, subcellular localization etc. of PDX-1 mutants on these sites. Studies in living cells confirmed that PASK is not likely to phosphorylate PDX-1 on Thr-152 site under physiological conditions although phospho-mimetic mutant PDX-1 on this site showed a substantial loss of DNA-binding ability, increased cytosolic distribution at elevated glucose concentration and was able to increase preproglucagon gene expression in INS $\alpha\beta$ cells. We also show that the phosphorylation of PDX-1 at Ser269 was decreased at elevated glucose concentrations and correlated inversely with PDX-1 levels. Meanwhile it also appeared to alter PDX-1 nuclear uptake at elevated glucose concentration. However, further analysis of the possible effects of phosphorylation at the site using (de) phosphomimetic mutants failed to detect any differences between the transactivation capacity of the wild-type protein compared to S269A or S269E mutants. We found this site is phosphorylated by HIPK2 *in vitro* and we also provide evidence that *in vitro* phosphorylation of PDX-1 by HIPK2 and/or CK1 δ may influence the upstream phosphorylation of PDX-1 by GSK3 in response to glucose, insulin or other factors, to mediate the stability of PDX-1.

Finally, in order to further complete the networks of PDX-1 phosphorylation and to better understand the post-translational regulation of PDX-1, future questions on PDX-1 regulation by PASK, phosphorylation of PDX-1 by other upstream

candidate kinases (ie. JNK, HIPK2 and GSK-3) on proposed potential sites in living cells need to be answered as mentioned in the Future Perspective section.

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