Universitat de Lleida

The role of cyclin D3 in pancreatic β-cell metabolic fitness and viability in a cell cycle-independent manner. Implications in autoimmune diabetes

Noemí Alejandra Saavedra Ávila

THE ROLE OF CYCLIN D3 IN PANCREATIC β-CELL METABOLIC

FITNESS AND VIABILITY IN A CELL CYCLE-INDEPENDENT MANNER.

IMPLICATIONS IN AUTOIMMUNE DIABETES

Thesis by

Noemí Alejandra Saavedra Ávila

Scientific director

Concepción Mora Giral

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Biomedicine

University of Lleida

Lleida, Catalonia

2013

(Defended September, 2013)

To my parents, Erik, Ayax, Manuel and Daniel.

Not only my work, but my life is yours

CONTENT

x

ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune condition caused by the lymphocyte-mediated destruction of insulin-producing β cells in pancreatic islets. This thesis aims to unveil the final targets in the β cells responsible for the β cell loss caused by the lymphocytic attack. Microarray studies were performed to assess differentially expressed genes in islet endocrine cells as a consequence of insulitic infiltration by comparing the autoimmune-prone Non-Obese Diabetic (NOD) mouse model with its congenic, lymphocyte-free, NOD/SCID strain. Interestingly, we discovered that cyclin D3 (CcnD3) underwent downregulation in beta cells upon inflammatory insult in a dose-dependent manner, while the proliferative activity of beta cells downregulating CcnD3 was not altered. Moreover, NIT-1 cells stably overexpressing CcnD3 were protected from spontaneous apoptosis and from apoptosis induced by a pro-inflammatory environment provided by IL-1β. To demonstrate the causal link between CcnD3 repression and β cell death *in vivo*, we studied spontaneous diabetes onset in CcnD3 deficient NOD mice (NODCcnD3KO). NODCcnD3KO mice developed exacerbated diabetes compared to the wild type (WT) littermates (88% versus 61% respectively), and this fact was solely due to the CcnD3 deficiency in the β cells and not to an increased diabetogenicity of the NODCcnD3KO lymphocytic repertoire. However, diabetes exacerbation required the complicity of both CcnD3 deficiency and inflammation, since plain NOD/SCID CcnD3KO mice did not develop spontaneous diabetes.

CcnD3 also plays an essential role in pancreatic β-cell fitness, since pancreatic islets deficient in CcnD3 do not experience proper intracellular Ca^{2+} influx changes in response to different concentrations of glucose. This impairment, coupling glucose concentration in the extracellular milieu and intracellular increase in Ca^{2+} concentration, is not due to changes in Glut-2 expression levels between the CcnD3 KO and WT islets.

Moreover, we developed transgenic mice overexpressing cyclin D3 in pancreatic β cells (NOD/RIPCcnD3). These mice exhibit protection from autoimmune diabetes and NOD CcnD3KO mice are prevented from developing exacerbated diabetes.

RESUMEN

La diabetes tipo 1 (T1D) es una enfermedad autoinmune causada por la destrucción de las células β productoras de insulina en los islotes pancreáticos por parte de los linfocitos. En esta tesis pretendemos dar a conocer dianas moleculares de las células β responsables de pérdida de la célula β causadas por el ataque linfocitico. Desarrollamos un estudio de microarreglos en el cual encontramos genes expresados diferencialmente en células endócrinas del islote como consecuencia de la infiltración insulítica mediante la comparación de un modelo de ratón autoinmune susceptible No Obeso Diabético (NOD) con una cepa congénica libre de linfocitos NOD/SCID. Interesantemente, descubrimos que, la ciclina D3 (CcnD3) experimentó una disminución como consecuencia de un asalto inflamatorio de forma dosis dependiente, mientras que la actividad de proliferación de las células β no fue alterada. Además, células NIT-1 que sobreexpresan CcnD3 fueron protegidas de apoptosis espontánea y apoptosis inducida por un entorno proinflamatorio proporcionado por la IL-1β. Para demostrar la relación causal entre la represión de la CcnD3 y la muerte de las células β *in vivo*, hemos estudiado la aparición de la diabetes espontánea en ratones NOD deficientes en CcnD3 (NODCcnD3KO). Los ratones NODCcnD3KO desarrollan una diabetes exacerbada en comparación con sus compañeros de camada de tipo salvaje (WT) (88% contra el 61% respectivamente) y este hecho debido únicamente a la deficiencia de CcnD3 de las células β y no a un aumento de diabetogenicidad del repertorio linfocítico de los ratones NODCcnD3KO. Sin embargo, la exacerbación de la diabetes requiere la complicidad de ambos, la deficiencia de CcnD3 e inflamación, ya que los ratones NOD/SCIDCcnD3KO, los cuales carecen de linfocitos, no desarrollan diabetes espontánea.

CcnD3 tiene también un papel escencial en la fisiología de las células β del páncreas, ya que los islotes deficientes de CcnD3 no experimentan cambios apropiados en las concentraciones intracelulares de Ca^{2+} en respuesta a diferentes concentraciones de glucosa en el medio. Este deterioro en el acoplamiento de la concentración de glucosa en el medio extracelular y aumento de la concentración intracelular de Ca^{2+} no es debido al cambio del nivel de expresión del transportador de GLUT-2 entre islotes NODCcnD3 KO y NODWT. Por otro lado, hemos desarrollado ratones transgénicos que sobreexpresan la ciclina D3 en células β pancreáticas (NOD/RIPCcnD3). Estos ratones muestran protección contra la diabetes autoinmune y rescatan a ratones NOD CcnD3KO de desarrollar diabetes exacerbada.

RESUM

La diabetis tipus 1 (DM1) és una malaltia autoimmune causada per la destrucció de cèl·lules productores d'insulina β en els illots pancreàtics, aquesta destrucció és mediada per limfòcits. En aquesta tesi es pretén donar a conèixer les dianes moleculars de les cèl·lules β responsables de la mort de les cèl·lules β causada per l'atac limfocític. Es van realitzar assajos de microarrays per tal d'identificar els gens expressats diferencialment en les cèl·lules endocrines d'illots, com a conseqüència de la infiltració insulítica, mitjançant la comparació de cèl·lules endocrines d' illots provinents de ratolins NOD (Non Obese Diabetic)- model de ratolí predisposat genèticament a la diabetis autoimmune-, amb les provinents del seu homòleg lliure de limfòcits, NOD / SCID. Curiosament, vam descobrir que la ciclina D3 (CcnD3) es regula a la baixa a conseqüència de la inflamació d'una manera dosi-dependent, mentre que l'activitat proliferativa de les cèl·lules β no es veu alterada per aquesta disminució en l'expressió de ciclina D3. A més, les cèl·lules NIT-1 que sobreexpressen Ciclina D3 resten protegides de l'apoptosi espontània i de l'apoptosi induïda per un entorn proinflamatori proporcionat per IL-β. Per demostrar la relació de causalitat entre la repressió de CcnD3 repressió i la mort de les cèl·lules β *in vivo*, hem estudiat l'aparició de diabetis espontània en ratolins NOD deficients en CcnD3 (NODCcnD3KO). Els ratolins NODCcnD3KO desenvolupen diabetis exacerbada en comparació amb companys de llodrigada de tipus salvatge (WT) (88% enfront de 61% respectivament), i aquest fet deu únicament a la deficiència de CcnD3 a les cèl·lules β i no a un augment de diabetogenicitat del repertori limfocític dels ratolins NODCcnD3KO. No obstant això, l'exacerbació de la diabetis requereix de la complicitat de tots dos, la deficiència CcnD3 i la inflamació, ja que els ratolins NOD/SCIDCcnD3KO, els quals no tenen limfòcits, no desenvolupen diabetis espontània.

CcnD3 té també un paper essencial en la fisiologia les de cèl·lules β del pàncrees, ja que illots pancreàtics deficients en CcnD3 no experimenten canvis adients en les concentracions intracel·lulars de Ca^{2+} en resposta a concentracions canviants de glucosa en el medi, el qual denota una sensibilitat deficient a la glucosa en aquests illots. Aquest deteriorament d'acoblament concentració de glucosa en el medi extracel·lular i l'augment a la concentració intracel·lular de Ca^{2+} no és a causa dels canvis en els nivells d'expressió del transportador de glucosa GLUT-2 entre illots NODCcnD3 KO i NODWT.

D'altra banda, hem desenvolupat ratolins transgènics que sobreexpressen ciclina D3 en cèl·lules β pancreàtiques (NOD/RIPCcnD3). Aquests ratolins mostren protecció contra la diabetis autoimmune i rescaten ratolins NOD CcnD3KO de desenvolupar diabetis exacerbada.

1. *Acknowledgments*

Firstly, to Conchi Mora because she give me the opportunity to work in her lab, also for her constant support and supervision, because she let me imagine, say and feel that I could create. She contributed significantly to my development as a doctoral student and hopefully in the future as women in science. I always see in her a lot of human values, intelligence, understanding and effort specially because she never gave up despite the difficulties that science in Catalonia and Spain is now undergoing, thank you Conchi!.

To Joan Verdaguer, Didac Mauricio and Marta Hernández, because you have been a huge support of the work of the group and also for your advice.

I also want to thank the people in the lab, specially Upasana, Ester for being there and also for your company in the lab, because it was a pleasure working with you during this time. This is also to Anais, whose help has been very valuable.

People in the animal facilities, firstly Carmen Pinyol, José Antonio Moreno and especially to Marc Collado, Albert Ardevol, Jessica Pairada and Marcia Chávez because they always make an extra effort to keep the mice in optimal conditions.

To Prof. Richard A. Flavell, at the Dept. of Immunobiology at Yale University, because he give me the opportunity to work in his lab, and Dr. Dietmar Herndler, an exceptional fellow worker who offered valuable support during my stay at Yale.

To Peter Siscinski and Ewa Sicinska from the Dana Faber Cancer Center at Harvard University, because they kindly provided us with the CcnD3KO in the C57/BL6 background.

To Dr. Martine Roussel, at St. Jude´s Childrens Hospital, for provided us with the cDNA encoding for the murine cyclin D3 we used to generate transgenic founders and transfectants.

To Angel Nadal, Ivan, Alejandro and Bea and especially to Ana Ropero, because you received me in your lab at the University of Elche as one more fellow during the

time that I spent there, and taught me how do the experiments on islet sensitivity of glucose.

Yanis Toledano, Ayax Pérez, Alana Azevedo, Teresa Ramírez and Aitor Modol. Thank you all because you contributed with your two cents and good hopes in the work.

Xavier Gasull and Gerard in the University of Barcelona because you were always willing to work and collaborate with us. And also to Rosa Gasa, at the IDIPABS for her help with the rtPCR and good counselling.

To Cristobal Mezquita, Jovita Mezquita, Belén Mezquita and Montse Pau, for all the great advice, support and the time that you have invested, and the space you allowed me to use in your lab facilities.

Josep Esquerda, Jordi, Lidia Piedrafita, Inma Montoliu, Martha Hereu and Montse Ortega for their help and space when we were sharing the same lab.

To all Biomedicine 1 Building integrators for providing an enriching environment for science.

To Manuel Saavedra for the design of the thesis cover.

2. *List of illustrations*

Figure 1. T1D as a multifactorial disease.

Figure 2. Mechanism of glucose-stimulated insulin secretion

Figure 3. Regulation of glucose-stimulated insulin secretion by nutrients, hormones and neurotransmitters.

Figure 4. CDK-independent functions of cyclin D1

Figure 5. Pancreatic duct preparation to perform collagenase P infiltration of the pancreas.

Figure 6. Cyclin D3 protein expression is downregulated by islet infiltration in NOD mice in a dose-dependent manner, without affecting the proliferative activity of the β cells.

Figure 7. Generation of NODCcnD3KO mice

Figure 8. Downregulation of CcnD3 contributes to the development of T1D and insulitis without altering proliferation rates

Figure 9. CcnD3 downregulation in solely β pancreatic cells confers susceptibility to develop T1D independently of repertoire changes

Figure 10. Exacerbated diabetes in CcnD3KO mice requires leucocyte action

Figure 11. CcnD3 deficiency causes an impaired β-cell response to blood glucose response in an intraperitoneal glucose challenge

Figure 12. Generation of NOD mice overexpressing CcnD3 in β cells (NODRIPCcnD3).

Figure 13. CcnD3 overexpression delays diabetes onset in NOD mice.

3. *Nomenclature*

AC   Adenylate cyclase

ADP   Adenosine 5'-(trihydrogen diphosphate)

AIRE   Autoimmune Regulator

APC   Allophycocyanin

cAMP   3'-5'-cyclic Adenosine Monophosphate

AR   Androgen Receptor

ATP   Adenosine 5'-(tetrahydrogen triphosphate)

BRC   Breast Cancer

CaMKII   Calcium-Calmodulin-Dependent Protein Kinase II

Ccn   Cyclin

CD   Cluster of Differentiation

CDK   Cyclin-dependent Kinase

CDKi   Cyclin-dependent Kinase inhibitor

CRABP2   Cellular Retinoic Acid-Binding Protein 2

CREB   cAMP Response Element-Binding

CTL   Cytotoxic T lymphocytes

Cyc   Cyclin family proteins

DAISY   Diabetes Association In Support of Youth

DAPI   4, 6-Diamidino-2-Phenylindole

DAG   Diacilglicerol

DC   Dendritic Cell

DNA	Deoxyribonucleic Acid
Epac	Exchange protein activated by cAMP
ERα	Estrogen Receptor α
FITC	Fluorescein Isothiocyanate
FOX	Forkhead box
GAD	Glutamic Acid Decarboxilase
GDH	Glutamate Dehydrogenase
GEF	Guanine nucleotide Exchange Factor
GLUT	Glucose Transporter
GLP	Glucagon-Like Protein
GTP	Guanosine Triphosphate
GVHD	Graft Versus Host Disease
HDAC	Histone Deacetylases
HLA	Human Leukocyte Antigen
HNFα	hepatocyte Nuclear Factor 4α
HTZ	Heterozygous
IDDM	Insulin-Dependent Diabetes Mellitus
IDD	Insulin-Dependent Diabetes mellitus locus
IL	Interleukine
IFN	Interferon
IGRP	Islet-specific Glucose 6-phsphatase catalytic subunit-Related Protein
IGTT	Intraperitoneal Glucose Tolerance Test
iNOS	Inducible Nitric Oxide Synthase
INS	Insulin

IP3	Inositol 1,4,5-triphosphate
KO	Knockout
LN	Lymph Nodes
MAFA	Musculoaponeurotic Fifroblastoma oncogene homolog A
MAP-2	Microtubule-Associated Protein 2
MHC	Major Histocompatibility Complex
MyD88	Myeloid differentiation primary response 88 protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuroD1	Neurogenic Differentiation 1
NK	Natural Killer
NO	Nitric Oxide
NOD	Non-Obese Diabetic
PE	Phycoerythrin
PEPCK	Phosphoenolpyruvate Carboxykinase
PCR	Polymerase Chain Reaction
PDX-1	Pancreatic and duodenal homebox-1
PIP2	Phosphatidyl Inositol Biphosphate
PK	Protein Kinase
PLC	Phospholipase C
PPAR-γ	Peroxisome Proliferator-Activated Receptor γ
pRB	Retinoblastoma protein
RAR	Retinoic Acid Receptor
RHOA	Ras Homolog gene family, member A
RIP	Rat Insulin Promoter

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCID	Severe Combined Immunodeficiency
STAT5a	Signal Transducer and Activator of Transcription 5A
SUR-1	Sulphonylurea 1 Receptor
T1D	Type 1 Diabetes
TCA	Tricarboxilyc Acid cycle
TG	Transgenic
TGF	Transforming Growth Factor
Th	T helper
TNF	Tumor Necrosis Factor
Treg	T regulatory cell
UBASH3A	Ubiquitin Associated and SH3 domain containing A
UV	Ultraviolet
VAMP	Vesicle Associated Membrane Protein
VDCC	Voltage-Dependent Calcium Channel
WT	Wild type
ZnT	Zinc Transporter
+	Positive
-	Negative
-/-	Knockout
+/-	Heterozygous
+/+	Wild type

INTRODUCTION

4. *Introduction*

7.1 Type 1 diabetes (T1D)

Type 1 diabetes is a disease caused by organ-specific autoimmune destruction of pancreatic insulin-producing β cells in the islets of Langerhans leading to insulin deficiency and chronic hyperglycaemia(2) and numerous life-threatening complications including nephropaty, neuropathy and retinopathy.(3)

T1D is a multifactorial disease developing as a consequence of the combination of genetic predisposition and environmental factors.(2)
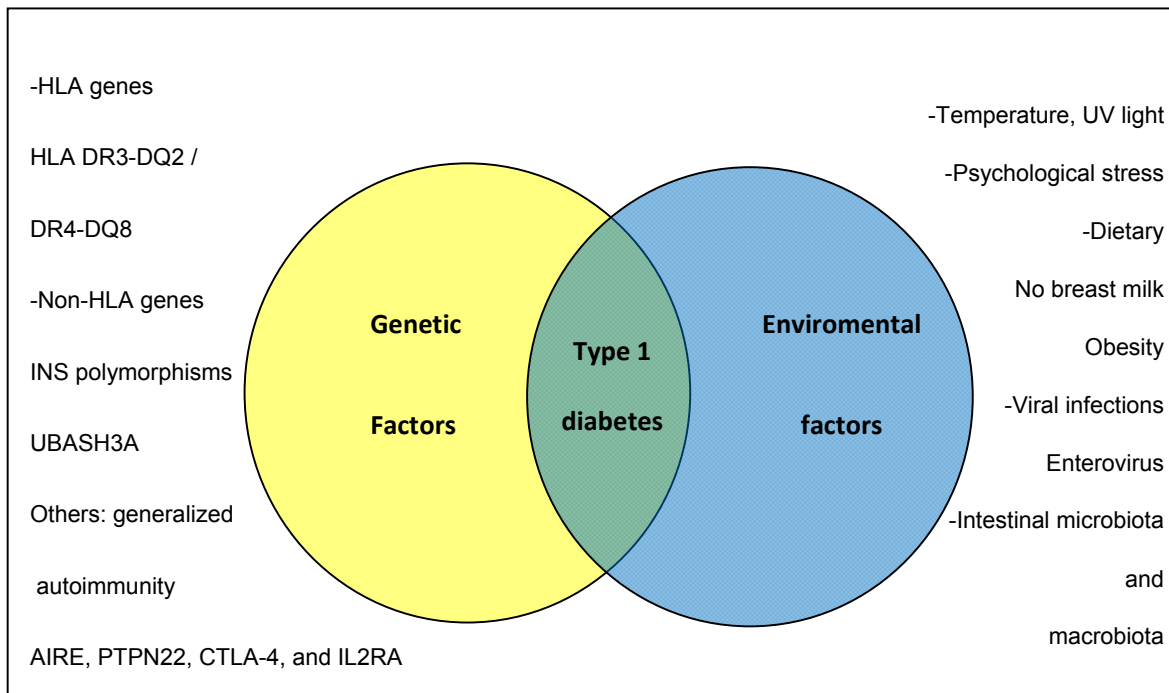
Figure 1. T1D as a multifactorial disease. T1D develops as a consequence of genetic factors and environmental factors. The most important genetic factors are related to the Major Histocompatibility Complex. In humans, some polymorphisms of class II loci of human leukocyte antigen (HLA) are the strongest genetic association with T1D. These include haplotype HLA-DR3-DQ2/DR4-DQ8. Other non-HLA genes, such as insulin polymorphisms or UBASH3A, and others that generate generalized autoimmunity have also been studied. Environmental factors like temperature, UV light, psychological stress, dietary, obesity, viral infections and intestinal microbiota and macrobiota are factors that contribute to the development of T1D.

7.1.1 Genetic factors

The most relevant genes related to T1D are located within the Major Histocompatibility Complex (MHC), which in humans is called the Human Leukocyte antigen (HLA) region is located to the chromosome 6p. The class I genes include HLA-A, HLA-B, HLA-C and class II genes, HLA-DP, HLA-DQ and HLA-DR each one encoding for both, the α and beta β. Nine chains of these genes are highly polymorphic and play a role in immune responses: HLA-DPA-1, HLA-DPB1, HLA-DQA1, HLA-DQA1, HLA-DQB1, HLA-DRB1 in the class II loci; and, HLA-A, HLA-B and HLA-C in the class I loci.

The high-risk HLA class II alleles exhibit the strongest genetic association with T1D and individuals with the HLA-DR3-DQ2/DR4-DQ8 haplotype have approximately a 20-fold higher risk of developing T1D than the general population[4]. Some haplotypes confer a moderately increased risk of T1D, while others seem to be protective[5]. Class I loci are not currently taken into account in risk models for T1D[6].

Regarding the non-HLA loci, they only confer a small additional risk compared to the strong effect of the HLA-DR or HLA-DQ loci[7]. The association of 20 genes with development of islet autoimmunity and T1D was tested among non-Hispanic white subjects with the high-risk HLA-DR, DQ haplotype in the Diabetes Association In Support of Youth (DAISY) cohort.

Some interesting variations are polymorphisms in:

- The associated ubiquitin and SH3 domain containing A (UBASH3A) gene, encodes for a suppressor molecule of T cell receptor signalling

- Protein tyrosine phosphatase nonreceptor type 22 (PTPN22) whose gene is located on chromosome 1p13 predicted the development of T1D haplotype when assessing the family history and presence of the HLA-DR3/4-DQB1*0302 haplotype.

- Polymorphisms in the insulin (INS) gene.

Although the effect of each individual gene is small, the combination of family history of T1D, the succeptibility haplotype and susceptibility variants of PTPN22, UBASH2 and INS increased the risk of islet autoimmunity 16-fold and that of T1D, 40-fold(8).

There are other genetic factors, not specifically related to T1D onset but rather to generalized autoimmunity. For instance, the Autoimmune Regulator (AIRE) that directs the expression of tissue-restricted antigens in the thymic medulla and the lymph node stromal cells. AIRE contributes substantially to the induction of immunological tolerance to self antigens. Mutations or lack of the expression of these genes can lead to an autoimmune condition.

In addition to the possible effects on the thymic selection, PTPN22 may affect the T-cell function in the periphery, as well as the function of the regulatory T cells and B lymphocytes(9, 10). Data emerging from both experimental and clinical studies support a role for B lymphocytes in T1D pathogenesis(11), and even C-peptide preservation has been reported following B-lymphocyte depletion in patients with new-onset diabetes(12). There are also reports of defects in B lymphocyte regulation(13).

Polymorphisms of the IL2RA gene, which encodes for the α chain of the interleukin-2 receptor (IL-2Rα, or CD25), modulate T1D risk(14). Functional studies of these polymorphisms include reduced levels of soluble IL-2 receptor(15), reduced Signal Transducer and Activator of Transcription 5A (STAT5a) responsiveness to IL-2 in antigen-experienced CD4$^+$ T cells, lower levels of the regulatory T-cell Foxp3 transcription factor, and reduced suppressive function of CD4$^+$CD25$^+$ regulatory T cells(15). IL-2 is a critical growth factor for lymphocytes and is important for the initiation of immune responses by promoting the proliferation and expansion of CD4$^+$ and CD8$^+$ T cells. IL-2 is also a key cytokine for immune homeostasis and for the development and function of CD4$^+$CD25$^+$ regulatory T cells (Treg)(16). The IL-2 gene is a susceptibility locus in Non-Obese Diabetogenic (NOD) mice, a mouse model of T1D in which abnormalities of the IL-2 pathway influence the development of

diabetes and the function of the regulatory T cells(17). In NOD mice, intra-islet CD4$^+$CD25$^+$ regulatory T cells express reduced amounts of IL2RA, which are associated with Treg increased apoptosis rates and disease development(18). Conversely, treatment of NOD mice with low doses of IL-2 corrects IL-2-dependent defects and reverses new-onset diabetes(19). Clinical trials with low-dose IL-2 treatment show benefits in two other immune-mediated conditions, graft vs. host disease (GVHD) and hepatitis C virus-induced vasculitis(20, 21). However the treatment with IL-2 and rapamycin of patients with T1D results in a temporary worsening of C-peptide secretion. Apparently, the IL-2 dose used was not low enough only to stimulate regulatory T cells, but also induced effector cell types(22).

7.1.2 Environmental factors.

There are many potential candidates, including environmental factors, that may contribute to the onset of T1D. These include climate, dietary factors (such as gluten and cow's milk), obesity, viral infections (particulary enterovirus) and intestinal microbiota and macrobiota.

7.1.2.1 Climate

Many of the countries with a high incidence of T1D are located close to the polar areas of the globe, both the North and the South Poles(23). Even within countries, latitude and seasonal shifts can make a difference: one Australian study, for example, found that T1D prevalence was three times higher in southern regions of that country than in northern areas(24). The hygiene hypothesis(1), suggests that vaccination and better sanitation, that result in a decrease in chronic parasitic infections, can impair the development of robust immune regulatory networks, favoring autoimmune inflammatory reactions.

Vitamin D, which is produced by the skin when exposed to sunlight, is a possible explanation for this pattern. In a study of 51 regions around the world(25), it was found that areas with lower levels of ultraviolet B radiation (the main source of

vitamin D in humans) had a higher incidence of T1D. Vitamin D deficiency appears to be a risk factor for type 1 diabetes, and vitamin D cannot be produced adequately by the skin during winter in areas close to the polar regions. Optimal vitamin D supplementation during early life has been shown to be protective.

7.1.2.2 Dietary factors

Dietary factors are related to the development of T1D. No breast milk uptake, the introduction of cereals (with or without gluten) before three months of age(26), a lower intake of omega-3 fatty acids and lower maternal consumption of vegetables and potatoes are associated with increased risk of early childhood islet autoimmunity(27). The "Accelerator Hypothesis" proposes that the rise in T1D, as well as type 2 diabetes (T2D), is related to increasing rates of childhood obesity and insulin resistance, and proposes that weight gain in both types of diabetes causes an increase in insulin resistance, which results in the weakening of glucose control. The rising blood glucose (glucotoxicity) accelerates β-cell apoptosis and promotes exposure to β-cell antigens, further accelerates β-cell destruction in a subset of individuals genetically predisposed to autoimmunity. The 'Accelerator Hypothesis' envisages an overlap between two types of diabetes. For this hypothesis, body mass is a key to the development and rising incidence of both types of diabetes, and the time frame will distinguish between 'types' of diabetes. Limiting weight gain, and with it, insulin resistance, could be the means of minimizing the development of both types of diabetes according to this hypothesis(28).

7.1.2.3 Viral infections

There is increasing evidence that viral infections are a factor in triggering the aetiology of T1D. To this effect, several viruses have been reported to have the capacity to induce or exacerbate T1D in both humans and mice. The most convincing evidence linking viral infection and autoimmunity comes from studies on enteroviruses, particularly Coxsackievirus. There is strong evidence associating coxsackievirus infection with T1D, presenting the current state of knowledge on the potential mechanism of coxsackievirus-mediated T1D(29).

7.1.2.4 Intestinal microbiota

The NOD mouse strain is an excellent model of spontaneous autoimmune disease and an important tool for dissecting tolerance mechanisms. The strength of this mouse strain is that it develops spontaneous autoimmune diabetes, which shares many similarities with the human autoimmune T1D, including the presence of pancreas-specific autoantibodies, autoreactive CD4$^+$ and CD8$^+$ T cells, and genetic linkage to the MHC class II risk haplotype. Because of the above, the NOD mouse model was used for the research work in this thesis.

The incidence of spontaneous T1D in non-obese diabetic (NOD) mice can be affected by the microbial environment in the animal housing facility or by exposure to microbial stimuli, such as injection with mycobacteria or various microbial products. Specific pathogen-free NOD mice lacking the myeloid differentiation primary response gene 88 (MyD88) protein (an adaptor for multiple innate immune receptors that recognize microbial stimuli) do not develop T1D. The effect is dependent on commensal microbes, because germ-free MyD88-negative NOD mice develop robust diabetes, whereas colonization of these germ-free MyD88-negative NOD mice with a defined microbial consortium (representing bacterial phyla normally present in the human gut) attenuates T1D. MyD88 deficiency changes the composition of the distal gut microbiota, and exposure to the microbiota of specific pathogen-free MyD88-negative NOD donors attenuates T1D in germ-free NOD recipients. Together, these findings indicate that interaction of the intestinal microbes with the innate immune system is a critical epigenetic factor modifying T1D predisposition(30).

7.1.2.5 Intestinal macrobiota: Helminths

The incidence of autoimmune type-1 diabetes is increasing in developed countries but is still relatively uncommon in the developing world. Autoimmune diabetes affects approximately 1 in 300 children and is a considerable burden for healthcare systems(31). This fact is consistent with the hygiene hypothesis and coincides with a

decrease in helminth infection(32). Helminth infections are a worldwide phenomenon but are becoming increasingly less frequent in developed countries(1).

Experimentally, helminths have been associated with protection against a number of autoimmune disorders, including inflammatory bowel disease and diabetes. Studies have now shown that chronic parasitic helminth infection in the non-obese diabetic (NOD) mouse, which spontaneously develops T1D, can dramatically reduce the onset of diabetes(31, 33-35). Components of the helminth-induced immune response, including both the cell surface and secreted molecules, have been proposed as potential agents that down-modulate the Th1-type inflammatory response that mediates β-cell destruction(36, 37). Diabetes is also prevented despite blockage of the Th2-type response(38). Moreover, there is a mechanism of helminth-induced Th2-independent control of T1D, which remains unclear. However, prevention requires IL-10(39).

7.1.3 Pathogenesis of T1DM

The etiopathogenesis of T1DM remains unclear, but clues to its origins and etiology can be gathered from studies of NOD mice and humans.

An initial hit, probably a physiological wave of β-cell apoptosis after weaning, that would prone autoreactive T cells (CD8$^+$ and CD4$^+$) in the pancreatic LN, and, in NOD mice, cellular debris that would not be properly cleaned can be the first step towards autoimmune diabetes. NOD macrophages are less efficient at engulfing apoptotic cells, leading to a defective clearance of apoptotic cells. This accumulation of dying cells can promote inflammatory responses(40). This same accumulation and its products can be related to the physiological apoptosis of pancreatic β cells in NOD mice(41). It has been suggested that the products released by dying cells initiate T1D development in this strain, possibly activating other cells of the innate immune system, such as DC.

Pathogenic autoreactive T cells, which infiltrate (insulitis) and destroy the pancreatic islets, are required for the onset of T1D. The activation of autoreactive T cells in

NOD mice is initiated by dendritic cells (DCs), presenting islet self-antigens in the draining pancreatic lymph nodes (LN) following a wave of β-cell death(42).

 Immune-mediated tissue damage results in additional shedding of islet antigens and epitope spreading in the autoreactive T cell response, leading to infiltration of the tissue by a varied population of autoreactive T cells that are nevertheless predominantly tissue-specific rather than recruited by bystander mechanisms(43).

CD8$^+$ and CD4$^+$ T cells can transfer disease in the NOD mouse model of T1D(44).

 T cells specific to islet self-antigens, including insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2 (IA2), zinc transporter 8 (ZnT8), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), have been found in the islets and peripheral blood of both NOD mice and T1D patients(45).

Diabetogenic CD8$^+$ T cells have a central role in the pathogenesis of T1D, as NOD mice deficient in CD8$^+$ T cells do not develop autoimmunity(46-48). These cells induce β-cell destruction during the effector stage of disease, and are essential in priming and expanding diabetogenic CD4$^+$ T cells. Effector CD8$^+$ T cells or cytotoxic T lymphocytes (CTLs) are important killers of human β cells(49, 50) by releasing cytotoxic granules with perforins and granzymes, via exocytosis, after direct contact with target cells(51). Perforins create holes in the plasma membrane of islet β cells, allowing cytotoxic serine proteases (granzymes) to enter and induce β-cell death by apoptotic and necrotic pathways(51). CTLs also exert a killing role by engaging the Fas receptor expressed on β cells with the Fas ligand (FasL) expressed on CTLs(52). CTLs play a key role, but do not account for all the β-cell destruction(52); NOD mice lacking MHC class I proteins still develop insulitis, but the incidence of this and hyperglycemia is significantly reduced suggesting that MHC class I-dependent cytotoxicity of the β-cells is a late event in the development of the disease(53). However this is not totally proved. In studies using a depleting α-CD8 antibody at two different times (the first at two to five weeks and the second, at seven weeks of age), after the first depletion, the NOD mice are protected against T1D and in the second, the NOD mice develop the disease similarly to the

controls(54). Also CD8$^+$ cells secrete IFN-γ and TNF-α to upregulate autoantigen presentation on DCs and enhance Fas and MHC class I expression in the β cells, augmenting T-cell mediated autoimmunity and promoting β-cell destruction(55).

CD4$^+$ T cells are also required to induce T1D pathogenesis through β-cell destruction but by different means than the CD8$^+$ T cells.

CD4$^+$ T cells play a critical role in T1D disease pathogenesis. NOD mice deficient in CD4$^+$ T cells are fully protected against the spontaneous onset of diabetes (56)(57). Only CD4$^+$ T cells can invade NOD pancreatic islets, while CD8$^+$ T cells do not enter islets unless CD4$^+$ T cells are also present(58-60). CD4$^+$ T cells act both early and late in T1D pathogenesis, as adoptive transfer of CD4$^+$ T cells precipitates the disease, and depletion of CD4$^+$ T cells protects against the onset of the disease(56). Autoreactive CD4$^+$ T cells synthesize pro-inflammatory cytokines, such as IFN-γ and TNF-α, for β-cell destruction. CD4$^+$ cells mediate β-cell death in a Fas-dependent manner, and the deficiency of CD4$^+$ cells blocks insulitis(61).

The synthesis of IFN-γ by diabetogenic T cells is an important contributor to the pathogenesis of the T1D(62). Blocking IFN-γ via specific antibodies(63) or soluble receptors(64) significantly reduces spontaneous T1D and prevents adoptive transfer of disease, while transgenic expression within the β cells exacerbates autoimmunity(65).

 TNF-α promotes cell adhesion by endothelial cell activation(66, 67), leukocyte homing(68), upregulation of MHC class I and II within the islet(69, 70) and activation of T cells and antigen presenting cells (APCs). Production of TNF-α by CD8$^+$ cells is directly cytotoxic to β-cells(71, 72). Like CD8$^+$ cells, CD4$^+$ T cells also produce TNF-α. Adoptive transfer of diabetogenic CD4$^+$ cells into a NOD mice deficient in TNF receptors 1 and 2 (TNFR1/2) significantly delayed T1D onset(72). The TNF-α of CD4$^+$ T cells activates DCs, natural killer (NK) cells and macrophages to promote β-cell destruction(73).

In addition to being produced by T cells, pro-inflammatory cytokines are induced by reactive oxygen species (ROS) via redox-dependent signaling pathways. ROS

(superoxide, hydrogen peroxide, hydroxyl radicals) influence a variety of essential processes including pro-inflammatory signalling, autophagy, chemotaxis, antigen cross-presentation, and immune modulation(74). ROS have an important role in eradicating pathogens, and high concentrations of ROS and decreased expression of antioxidants can induce oxidative stress states, which, in turn, propagate pathological tissue destruction in various autoimmune and inflammatory diseases such as T1D(75). A recent study reported that sera from T1D patients exhibited enhanced reactivity with hydroxyl radical-modified glutamic acid decarboxilase 65 (GAD65), an autoantigen in the T1D pathogenesis detected in 60% of patients diagnosed early(76, 77), and binding activity is more pronounced in T1D patients suffering diabetic complications (*e.g.* retinopathy and neuropathy). Hence, ROS may generate highly immunogenic neoepitopes that drive T1D pathogenesis(78). Acute fluctuations in extra- and intra-cellular ROS concentrations may have a function as intracellular signalling second messengers.

Other factors, as well as enzymes, are also related with the development of T1D in rodent islets. Exposure to IL-1β alone or combined with IFN-γ induces expression of inducible nitric oxide synthase (iNOS). Inhibition of iNOS or a deletion of the iNOS gene has been shown to be protective in animal models of T1D. Transplanting pancreatic islets deficient in iNOS (iNOS$^{-/-}$) would allow increased graft survival(79). Higher amounts of iNOS induce necrosis in the tissue, and lower amounts can induce apoptosis.

Studies on the pathogenesis of type-1 diabetes have mainly focused on the role of the immune system in the destruction of pancreatic β-cells. Lack of data on the cellular and molecular events at the β-cell level is caused by the inaccessibility of these cells during the development of the disease. Indirect information has been collected from isolated rodent and human islet cell preparations that were exposed to inflammatory conditions.β-cells rapidly die by necrosis because of toxic levels of oxidizing radicals or nitric oxide. They become progressively apoptotic after prolonged culture at low glucose concentrations or after exposure to pro-inflammatory cytokines. Their susceptibility to necrosis or apoptosis varies with their functional state and, thus, with the environmental conditions. A change in cellular

phenotype can alter their recognition of potentially cytotoxic agents and their defence mechanisms against cell death. β cells are not necessarily passive victims of a cytotoxic process but can actively collaborate in their own destruction(80).

7.2 *β-cell function*

β cells are responsible for secreting insulin in response to an increase in blood nutrient levels during the postprandial state. Glucose is the most important nutrient for insulin secretion (secretagoge). The process by which glucose promotes insulin secretion through β-cells requires its sensing and metabolism, a process called glucose-stimulated insulin secretion.

Glucose-stimulated insulin secretion is a biphasic and pulsatile process(81). The secretory pulses of beta-cells are associated with synchronous Ca^{2+} oscillations in response to the glucose stimulus(82), and they have been suggested to be coupled to glycolysis oscillations of the beta cell(83). Secretory pulses are also regulated and synchronized within the islet. Insulin and glucagon secretion show asynchronous patterns(81, 84), whereas somatostatin pulses are synchronized with insulin secretion(81).

Glucose-stimulated insulin secretion also shows a biphasic pattern. Shortly after glucose stimulus, a first burst of insulin secretion occurs, followed by a decrease in the rate of secretion. A second sustained phase of insulin secretion can be observed just after this decrease, which can continue for up to several hours, until euglycemia is reached(85) (Figure 2).

Although the mechanisms involved in the first phase of insulin secretion (termed the **triggering pathway**) are well understood, the mechanisms regulating the sustained second phase (or the **amplifying pathway**) have yet to be deciphered, and different players have been proposed to account for it. Notably, most of them are related to glucose metabolism inside the β-cell(86).
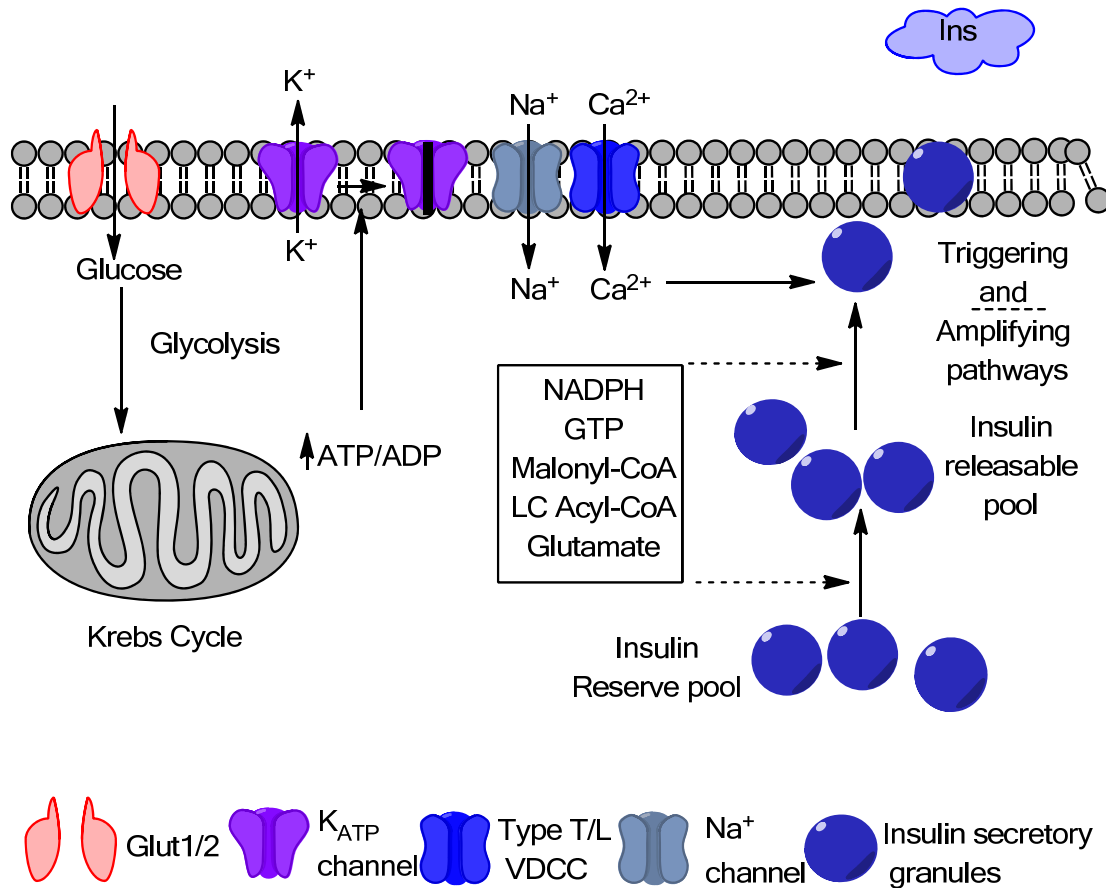
Figure 2. Mechanism of glucose-stimulated insulin secretion. Glucose enters the cell by glucose transporters (GLUT2 in rodents and humans, GLUT1 in humans) and enters glycolysis pathway, Krebs cycle and the respiratory chain, completing its oxidation and generating ADP, ATP. Increased levels of ATP close the ATP-sensitive K+ channel (K_{ATP}), allowing sodium (Na^+) entry without balance. These two events depolarize the membrane and open voltage-dependent T-type calcium (Ca^{2+}) and sodium (Na^+) channels. Na^+ and Ca^{2+} entry further and depolarize the membrane and L-type and perhaps other voltage-dependent calcium channels (VDCC) open. This activation increases intracellular Ca^{2+} ($[Ca^{2+}]_i$), which leads to the fusion of insulin-containing secretory granules with the plasma membrane and the first-phase insulin secretion (triggering pathway). A sustained second phase of insulin secretion happens when the granules from the readily releasable pool are converted to the immediately releasable pool, an ATP-dependent process termed "priming". Most of the signals involved in this process also come from glucose mitochondrial metabolism, comprising the amplifying pathways.

Is important to mention that the nervous system has a strong influence on insulin secretion, since insulin secretion is stimulated by parasympathetic nerves or their neurotransmitters, and inhibited by sympathetic nerves or their neurotransmitters. The islet autonomic nerves seem to be of physiological importance in mediating the insulin secretion, synchronizing the islets to function as a unit allowing oscillations of islet hormone secretion and optimizing islet hormone secretion during metabolic stress, e.g. hypoglycemia (an abnormally low level of glucose in blood) and neuroglycopenia (shortage of glucose in the brain). The autonomic nerves could also be involved in the islet adaptation to insulin resistance with possible implications for the development of glucose intolerance and Type-II (non-insulin-dependent) diabetes mellitus. Islet innervation, through the contribution of all branches of the autonomic nerves and several different neurotransmitters, is of importance for both the physiology and patho-physiology of the islets(87).

A recent study reported that the vagal nerve is involved in β-cell proliferation, the induction of endocrine progenitors and neogenesis of α- and β-cells. If the vagal nerve is blocked with atropine, proliferation of these cells is inhibited.(88).

The first phase of glucose-stimulated insulin secretion is a multi-step process that requires internalization and oxidation of glucose (Figure 3). Glucose enters the cells by facilitated diffusion mediated by glucose transporters (mainly GLUT-2 in rodents and humans, GLUT-1 in humans). Glucose is then phosphorylated by glucokinase to form glucose-6-phosphate initiating glycolisis (89) and hence its metabolism through glycolysis and oxidation in the Krebs cycle.

The generation of ATP by glycolysis, the Krebs cycle (or Tricarboxylic Acid (TCA) cycle) and the respiratory chain, leads to closure of the ATP-sensitive K^+ channel (K_{ATP}) (an hetero-octamer comprised of four subunits of the sulphonylurea 1 receptor (SUR1) and four subunits of the inwardly-rectifying K^+ channel Kir6.2)(90). The closure of the K_{ATP} channels permits the background sodium (Na^+) to enter without balance. These two events depolarize the membrane to a range that allows the opening of voltage-dependent T-type calcium (Ca^{2+}) and sodium (Na^+) channels.

Na^+ and Ca^{2+} entry further depolarizes the membrane and L-type and other voltage-dependent calcium channels (VDCC) may open. Their activation triggers action potentials that increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[91]. Together with calcium mobilized from intracellular stores, this increase in Ca^{2+} leads to the fusion of insulin-containing secretory granules with the plasma membrane and the release of insulin into the circulation[92]. Following glucose metabolism, the rate-limiting-step for the first phase lies in the rate of signal transduction between sensing the rise in $[Ca^{2+}]_i$ and the exocytosis of the immediately releasable granules[93].

The existence of a second phase of insulin secretion was first reported in the 1960s. Curry et al.[85] observed that total pancreas perfusion with glucose resulted in insulin release that showed an early and rapid increase 2 min after glucose infusion, peaking at 4 min.

A second or "slow" phase, characterized by an increasing rate of insulin secretion was sustained during the whole period of glucose perfusion within approximately 2 min. On the other hand, when the pancreas was perfused with tolbutamide, a sulfonylurea that blocks the potassium channels, only the first rapid release peak was observed, suggesting that this agent has no effect on insulinogenesis and/or the mechanism which gives rise to the second phase[85]. It was in the 1990s that evidence of mechanisms for glucose-stimulated insulin secretion independent of ionic action (i.e. K_{ATP} potassium channel activation) was found[94, 95]. Since then, the concept of a rapid first phase of glucose-stimulated insulin secretion, caused by a triggering pathway (or K_{ATP}-dependent mechanism), followed by a sustained second phase due to an amplifying pathway (or K_{ATP}-independent mechanism) has developed[96, 97].

Biphasic insulin secretion has been explained by the existence of different pools of insulin containing granules inside the beta cell[94, 98]. There is a reserve pool of granules located in the cytoplasm that accounts for approximately 94% of the total granules, and a releasable pool of granules that are docked to the plasma membrane. It has been suggested that in the docked granules there are two different subsets according to their ability to be released, the *readily-releasable pool*, and the

immediately-releasable pool. The granules from the immediately releasable pool are the first to be secreted in response to intracellular Ca^{2+} increase during the triggering pathway, leading to the first phase of insulin secretion. At the lowest point of secretion, in between the two phases, the granules from the readily-releasable pool are converted to the immediately-releasable pool, an ATP-dependent process termed "priming".

This priming has been suggested to be the rate-limiting step for exocytosis, and the target process for signals involved in the amplifying pathway that leads to the sustained second phase of insulin secretion(98) (Figure 2). Given the glucose-stimulated nature of biphasic insulin secretion and the ATP-dependence of priming, it is suggested that most of these signals are derived from glucose metabolism.

7.2.3 Regulation of β-cell function

Transcription factors in β cells act in a cooperative manner, forming transcriptional networks, to induce not only insulin expression, but also the expression of other genes involved in insulin gene regulation and insulin secretion, thus establishing and maintaining the β-cell phenotype and function. Some of these factors include Pancreatic and duodenal homeobox 1 (PDX-1), Hepatocyte Nuclear Factor 4α (HNF4α), Musculoaponeurotic Fibrosarcoma oncogene homolog A (MAFA), Forkhead box A2 (FOXA2) and Neurogenic differentiation (NeuroD1)(99).

PDX-1 is one of the most important transcription factors regulating the insulin gene transcription. This factor is determinant for pancreatic function. β-cell specific knockout studies show that when pdx1 is ablated in mice, β-cell function is impaired and mice exhibit a diabetic phenotype(100). Many of the target genes for pdx1 are crucial for glucose-induced insulin secretion, such as glucose transporter Glut-2(100), the insulin gene(101), and other transcription factors(100-103). PDX-1 also plays a role in the maintenance and proliferation of β cells(104). PDX-1 over-expression in diabetic mice (Irs2 knockout) is involved in β-cell mass recovery and helps to ameliorate glucose tolerance(105), whereas *pdx1* haploinsufficiency causes β-cell apoptosis(106). A decrease in PDX-1 has also been associated with apoptosis

and reduced expression of the antiapoptotic genes Bcl_{XL} and Bcl-2(107), defects in post-translational processing of insulin, repression of the Glucagon-like peptide receptor (GLP-1R) expression(108), glucotoxicity(109) and lipotoxicity(110, 111).

7.2.4 Metabolic factors and glucose-stimulated insulin secretion

An increase in the ATP/ADP ratio caused by glucose metabolism in β-cells is the mechanism by which the first phase of glucose-stimulated insulin secretion is triggered. However, glucose metabolism can also render a series of signals, or metabolic coupling factors, that may initiate and sustain the second phase of insulin secretion, presumably by favoring mobilization of the insulin granules form the reserve pool, and by replenishing the immediately-releasable pool of insulin granules. Some of these metabolic coupling factors participate in mitochondrial shuttles, involving NADPH, pyruvate, malate, citrate, isocitrate, acyl-CoAs, and glutamate(112).

There are also various signalling pathways that, when activated, may contribute to maintaining or increasing glucose-stimulated insulin secretion, for instance Calcium-Calmodulin-Dependent Protein Kinase II (CaMKII), Protein Kinase A (PKA), Protein Kinase C (PKC) and Protein Kinase G (PKG) pathways. Notably, most of the other insulin secretagogues, namely nutrients, hormones and neurotransmitters, also modulate insulin secretion by these pathways.

7.2.4.1 Mitochondrial signalling

The role of mitochondria in the second phase of glucose-induced insulin secretion has been established by several studies in cell lines and humans(112, 113). There is even evidence of an uncommon sub-form of diabetes, mitochondrial diabetes, where mutations in mitochondrial DNA cause pancreatic β-cell dysfunction(113).

Besides rendering the initial increase in the ATP/ADP ratio, mitochondrial metabolism and anaplerotic metabolites are also involved in sustaining the second phase of insulin secretion.

Pyruvate, the end-product of glycolysis, plays an important role in this process as it participates in several cycles whose final products constitute amplifying signals for insulin secretion. Particularly, NADPH, GTP, Malonyl-CoA, long-chain acyl-CoA, and glutamate have been suggested to sustain insulin secretion, although the exact mechanisms by which they exert these effects remain to be elucidated(112).

After entering the mitochondria, pyruvate may be either converted into Acetyl-CoA by pyruvate dehydrogenase, or carboxylated to oxalacetate by pyruvate carboxylase, and therefore enter the Krebs cycle or Tricarboxilic Acid Cycle (TCA) (Figure 3). Moreover, pyruvate carboxylase is highly expressed in the pancreatic islets, similarly to gluconeogenic tissues, but islets lack phosphoenolpyruvate carboxykinase (PEPCK), the first enzyme in the glyconeogenic pathway(114). In addition, several studies have correlated pyruvate carboxylation with insulin secretion(115-118).

Oxalacetate from pyruvate carboxylation may be converted to malate, exit the mitochondria, and be re-converted to pyruvate, producing NADPH (Pyruvate/malate cycle). Oxalacetate may also condense with acetyl-CoA to form citrate, which either continues in the TCA cycle, or exits the mitochondria, and is again converted into oxalacetate and acetyl-CoA by the ATPcitrate lyase (pyruvate/citrate cycle). Oxalacetate may re-enter the pyruvate/malate cycle, which will produce NADPH, while acetyl-CoA is carboxylated by Acetyl-CoA carboxylase and forms malonyl-CoA, the initial step of fatty-acid synthesis(112). As the pancreatic islet is not a lipogenic tissue, the fact that acetyl-CoA activity is high in this tissue may indicate that malonyl-CoA can also act as a metabolic coupling factor for insulin secretion(119).

Metabolites from the Krebs cycle can also exit the mitochondria and enter other cycles. Isocitrate, for example, is converted into α-ketoglutarate by the NADP-dependent isocitrate dehydrogenase, rendering NADPH. α-ketoglutarate may either re-enter the mitochondria to continue in the TCA cycle, or may be converted into glutamate by the glutamate dehydrogenase (GDH). Glutamate has been

suggested as another metabolic-coupling factor for insulin secretion, possibly by entering insulin secretory granules and promoting exocytosis(120).

Finally, GTP may be produced by an isoform of the succinyl-CoA synthetase, which catalyzes the conversion of succinyl-CoA into succinate in the TCA cycle. It has been suggested that GTP participates in insulin secretion. In β cells, suppression of GTP production by this pathway reduced glucose-induced insulin secretion, independently of changes in the NADPH or the ATP/ADP ratio(121).

7.2.4.2 Calcium signalling and CaMKII

As mentioned above, the glucose-stimulated insulin secretion is a Ca^{2+}-mediated process. The increase in cytosolic calcium inside the β-cell must be sensed and transduced in order to exert a secretory response. One of the candidate proteins involved in this transducing system is calcium-calmodulin-dependent protein kinase II (CaMKII). CaMKII activation has been correlated with glucose-stimulated insulin secretion. Besides being localized in the insulin secretory granules, CaMKII phosphorylates proteins involved in the secretory machinery, include synapsin I(122), microtubule-associated protein 2 MAP-2(123), Vesicle Associated Membrane Protein (VAMP)/ synaptobrevin(124) and others. Then, it is suggested that insulin release is modulated by CaMKII by mobilizing the secretory granules toward the cell membrane by MAP-2 phosphorylation and by potentially regulating the docking or priming mechanisms via VAMP and synapsin I protein phosphorylation. Since CaMKII remains active after glucose stimulation, it is suggested that it takes part in the mechanism of readily-releasable pool replenishment(125).

7.2.4.3 The G-protein coupled signalling pathways: PKA and PKC

The guanyl-nucleotide-binding (GTP) protein system, or G-protein coupled system, plays an important role in insulin secretion. In β cells, two G-protein regulated pathways, the Adenylate cyclase (AC)/Protein Kinase A (PKA), and the phospholipase C (PLC)/PKC pathways, modulate insulin secretion in response to nutrients and other peripheral signals(126).

Depending on the type of Gα subunit present, these signals will activate or inhibit Adenylate Cyclase (Gαs and Gαi subunits respectively). Gαq subunits are associated with the phosphatidyl inositol system.

When Adenylate Cyclase is activated in β cells, it converts ATP into cyclic AMP (cAMP), which in turn either activates the cAMP-dependent protein kinase (PKA) and the Rap guanine nucleotide exchange factor (GEF) 4; or exchanges protein directly activated by cAMP 2 (Epac2). PKA will phosphorylate several proteins, including L-type voltage-dependent calcium channels and proteins from the exocytotic machinery, increasing sustained insulin secretion(127). Epac2 has been shown to favor insulin secretion by increasing the size of the reserve pool and, facilitating the recruitment of the granules to the plasma membrane(128), mediating the pulsatility of insulin secretion(129), and binding to the sulfonylurea receptor (SUR1) subunit of the K_{ATP} channels(130). The insulin gene itself has cAMP response elements in its promoter that modulate insulin transcription in response to this nucleotide(131).

Therefore, ligands that increase the activity of adenylate cyclase and cAMP have a positive effect on insulin synthesis and secretion(132), while ligands that decrease adenylate cyclase activity affect insulin secretion negatively(133). Hormones and neurotransmitters mostly act on insulin secretion by this pathway.

Phospholipase C (PLC) is the other effector protein regulated by G-protein coupled receptors in β cells. PLC activation cleaves phosphoinositides into two second messengers, inositol 1,4,5-trisphosphate (IP3), involved in Ca^{2+} release from the endoplasmic reticulum, and DAG. DAG is involved in the activation of the Protein kinase C (PKC). PKC phosphorylates the K_{ATP} channels and the voltage-dependent Ca^{2+} channels and mobilizes the secretory vesicles(126), thus promoting insulin secretion. Both nutrients and neurotransmitters may act through PKC activation, albeit by different mechanisms. It has been proposed that nutrients may activate atypical isoforms of PKC (ζ, ι and µ) by a non-identified mechanism independent from DAG, while the typical isoforms (α, β, δ and ε) of Protein Kinase C (PKC) are activated by DAG(133).

7.2.4.4 The cGMP/PKG pathway

The cyclic GMP (cGMP) pathway is regulated basically by two factors: calcium and protein kinase G (PKG). Calcium increases the activity of calcium-dependent nitric oxide synthases, a key step in the synthesis of cGMP by soluble guanylyl cyclase (cGC). Calcium may also decrease cGMP synthesis by activating a calcium-dependent phosphodiesterase 1 (PDE1). On the other hand, protein kinase G (PKG), an enzyme activated by cGMP, may phoshporylate different targets and modulate intracellular calcium concentration, primarily closing K_{ATP} channels(134).

Although several studies have pointed to sGC and cGMP playing a role in insulin secretion(135, 136), a precise mechanism for action has yet to be elucidated for this pathway. A phosphorylation site for PKG has been identified in rat islets(133). This is likely the enzyme mediating cGMP actions in insulin secretion. It has also been shown that PKG activity is necessary to increase the ATP content in response to cGMP(137), and that glucose produces small increases in islet cGMP content(136, 138).

7.2.4.5 Other factors that can modulate insulin secretion

Insulin secretion in response to plasma glucose can be increased or decreased by several hormones, like insulin itself acting in an autocrine way, and neurotransmitters, via activation of their membrane receptors on β cells(139). The G protein receptors and adenylate cyclase pathway are responsible for mediating most of these effects.

The adenylate cyclase pathway may be activated by some neurotransmitters, like acetylcholine, and such hormones as Glucagon-like peptide-1 (GLP-1). GLP-1 is also an important factor for insulin synthesis and secretion, having a trophic effect on β cells as well(140). Other modulating pathways are activated in β cells in response to oxidative stress caused by high glucose levels, like the JNK pathway, which ablates insulin synthesis and interferes with its action(141).

Various studies have shown an autocrine role for insulin in β-cell function and survival(142-144). In this process, insulin binding to tyrosine-kinase insulin receptors located on the β-cell surface promotes insulin receptor autophosphorylation, regulating cell survival, proliferation, growth and nutrient metabolism, through engaged β-cell phosphorylation of different proteins (like Glycogen synthase kinase 3 (GSK-3), FOX-O and cAMP Response Element-Binding (CREB)(145). The insulin receptor may be activating MAP kinases ERK1/2, regulating growth, cellular differentiation and protein synthesis(146).

In human islets, insulin has a positive effect on insulin production at the transcriptional level, as well as on β-cell proliferation(147).

Glucagon is considered the contrarregulatory hormone of insulin, as its systemic actions are contrary to the ones exerted by insulin. Glucagon stimulates glucose production by gluconeogenic organs, such as the liver and kidney, glycogen degradation, and lipolysis. Paradoxically, it has been shown that glucagon stimulates insulin secretion both in rats(148) and humans(149). Glucagon induces a transient increase in plasma insulin up to glucagon concentrations of 1 mg, and this increase is seen before a rise in glucose levels is detected(149). There is evidence that the positive effect of glucagon on insulin secretion is mediated by the activation of glucagon receptors in β cells (148)(148), and this activation may increase cAMP levels, leading to the PKA pathway (**Figure 2)**.

Besides nutrients, neurohormonal signals, such as autonomic innervation can markedly modulate glucose-stimulated insulin secretion. Islets are thoroughly innervated by autonomic nerves, which contain an extensive variety of neuropeptide transmitters.

Increased sympathetic activity affects insulin secretion in situations of stress, exercise and trauma. Activation of parasympathetic nerves before and during feeding by smell, taste and the digestive tract, along with incretin hormones derived from the gut, are responsible for enhancing insulin response to meals.

Parasympathetic neurotransmitters that stimulate insulin secretion include acetylcholine, vasoactive intestinal polypeptide and gastrin-releasing polypeptide. Sympathetic neurotransmitters inhibit insulin release. These include norepinephrine,

galanin and neuropeptide Y. The enteroinsular axis, mediated by incretin hormones, explains why the insulin secretion in response to an ingested nutrient load is greater than when the same load is given parenterally. Gastrointestinal hormones such as gastric inhibitory peptide, glucagon-like peptide-1 and cholecystokinin, exert physiological relevant insulinotrophic effects(139) In particular, GLP-1 has attracted attention due to its potential role in the treatment of diabetes.

There are at least three potential sites where insulin can be modulated by hormones, peptides and neurotransmitters. Firstly, the ion channels that regulate membrane potential and calcium influx; secondly, the mobilization of intracellular calcium stores, mainly the endoplasmic reticulum, and therefore cytosolic calcium concentration; thirdly, the calcium sensibility of the contractile protein interactions that lead to the release of the insulin secretory granules(139). The two best-known targets of hormones, peptides and neurotransmitters within the β-cell, are related to adenylate cyclase and PLC.

Activation of adenylate cyclase in β-cell converts ATP into cAMP, which can activate PKA. PKA phosphorylates several proteins including VDCC, and proteins from the exocytotic machinery, increasing sustained insulin secretion(139).

PLC activation cleaves PIP2 in the membrane producing IP3, which in turn, inhibits calcium sequestration into the endoplasmic reticulum, while the adjacent cleavage product, diacylglycerol (DAG), activates PKC.

Similarly to the effects of the adenylate cyclase signalling pathway, the activation of PLC alters insulin secretion by mechanisms related to calcium sensitivity and protein phosphorilation(139).
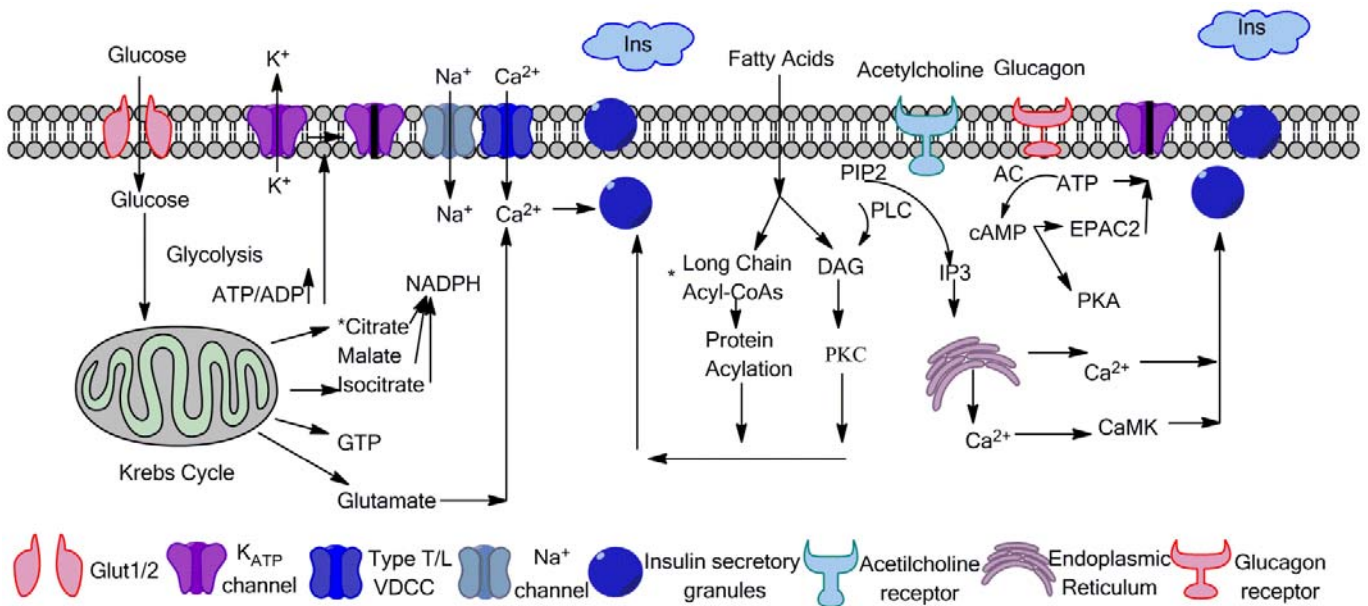
Figure 3. Regulation of glucose-stimulated insulin secretion by nutrients, hormones and neurotransmitters. Glucose-stimulated insulin secretion may be modulated by several mechanisms. Glucose metabolism increases the ATP/ADP ratio and closes ATP-sensitive potassium channels (K_{ATP}), depolarizing the membrane, opening voltage-dependent calcium channels (VDCC) and thus increasing intracellular calcium ($[Ca^{2+}]_i$). Glucose metabolism by the Krebs Cycle also generates a series of metabolic coupling factors that may initiate and sustain insulin secretion. These metabolic coupling factors participate in mitochondrial shuttles, involving NADPH, pyruvate, malate, citrate, isocitrate, acyl-CoAs, and glutamate. Signalling pathways that contribute to maintaining or increasing glucose-stimulated insulin secretion include PKA and PKC. Glucagon, glucagon-Like peptide 1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP) acts through the PKA pathway, while acetylcholine and cholecystokinine act through the PKC pathway. Fatty acids may contribute to insulin secretion through the PKC pathway through the formation of diacylglycerol (DAG) or through protein acylation. Aminoacids may stimulate insulin release by increasing ATP production from the Krebs Cycle, by membrane depolarization, or by participating in intracellular calcium increase. (PIP2: Phosphatidyl Inositol Biphosphate, IP3: inositol 1,4,5-trisphosphate).

7.3 Cell cycle

Every cell reproduces itself by perfoming an orderly sequence of events in which it duplicates its contents and then divides in two. This cycle of duplication and division is known as the cell cycle. To produce two genetically identical daughter cells, in eukaryotic cells, the DNA in each chromosome must first be faithfully replicated to produce two complete copies, and the replicated chromosomes must then be accurately segregated into the two daughter cells, so that each one receives a copy of the entire genome. Eucaryotic cells have evolved a complex network of regulatory proteins, known as the cell-cycle control system, that govern the progression through the cell cycle. The core of this system is an ordered series of biochemical switches that control the main events of cell cycle, including DNA replication and the segregation of the replicated chromosomes.

Cell growth (i.e. increase in cell mass) and proliferation (i.e. increase in cell number) are tightly controlled by growth factors in multicellular organisms. The presence of nutrients, cause growth factors to activate signalling cascades that trigger nutrient uptake and use(150).

Over the last few decades, major advances have been made in understanding the machinery controlling cell progression through the cell cycle, in particular identifying cell-cycle regulatory proteins. These proteins are divided into families comprising cyclins (cyc), cyclin-dependent kinases (CDKs), cyclin-dependent kinases inhibitors (CKIs), the pocket protein retinoblastoma family and the E2F family of transcription factors(150).

7.3.1 The family of cyclin proteins

The first approach to cyclin proteins was taken on 1976, the existence of nuclear antigens related to cell cycle that could lead to develop leukaemia being reported(151). It was in 1982 when a series of polypeptides, the relative proportion of

which was sensitive to shifts during cell proliferation and to transformation, were given the name of cyclins(152).

Cyclins are proteins that act as key controlling elements of the eukaryotic cell cycle. In mammalian cells, cyclins bind to CDKs and form complexes that are involved in the regulation of different cell cycle transitions: D-type cyclins (CcnD)-CDK4/6 complex for G1 progression, E-type cyclins (CcnE) - CDK2 for the G1-S transition, A-type Cyclins (CcnA)-CDK2 for S phase progression and CcnA/B-CDC2 for entry into M-phase. Cyclins have been related to human cancers, because of the major role they play in starting cell cycle progression(153, 154). In addition to these functions, cyclins are also involved in some metabolic processes not directly related to the cell cycle(155).

7.3.1.1 D-type Cyclins

There is a great deal of evidence describing the role of D-type cyclins in β pancreatic islets, most of which is available on CcnD1 Figure 4.(156) The function of D-type cyclins is currently under study, but many functions that have already been found.

7.3.1.1.1 CDK activation

The earliest known and best-understood function for D-type cyclins is to promote cell proliferation as a regulatory partner for either CDK4 or CDK6.(157) The activation of the CcnD–CDK4/6 complex initiates the release of the pRB‑dependent cell cycle-inhibitory 'brake' that governs cell cycle transitions during quiescence, senescence and differentiation.

Specific inhibition of CcnD–CDK4/6 complex formation through the induction of Cyclin-dependent kinase inhibitor 4A (INK4A) promotes RB‑dependent cell cycle arrest in some circumstances,(158) such as during senescence. The model in which pRB phosphorylation by CDKs, including CcnD–CDK4/6, promotes the activation of E2F‑responsive genes that are necessary for DNA synthesis, has been elaborated to take into account interactions between the pRB family of proteins and various members of the E2F family. The latter includes both activator and repressor proteins, as well as some atypical E2Fs that repress transcription independently of

pRB family members(159). CcnD1 is not necessary for the proliferation of cultured cells in the absence of functional pRB. This finding suggest that pRB is the principal substrate for CcnD1–CDK4, and presumably for other CcnD‑associated CDKs(158), at least in terms of this specific end point of CcnD1 action.

Recent studies have shown that CcnD–CDK4 has physiologically relevant substrates in addition to RB family members. These include binding sites for transcription factors, such as the transforming growth factor-β (TGFβ)-responsive transcriptional modulator Smad family member 3 (SMAD3)(160), members of the Runt-related transcription factor (RUNX) family(161, 162), GATA binding protein 4 (GATA4)(163) and the Myocyte enhancer factor 2 (MEF2) family(164), which are involved in the proliferation and differentiation of specific cell lineages; and Breast Cancer-1 gene (BRCA1)(165), which coordinates DNA damage repair, ubiquitylation and transcriptional regulation to maintain genomic stability.

 Other CDK4 targets have roles in processes that are linked to, and coordinated with, chromosomal DNA replication and segregation, such as centrosome duplication and separation(166), mitochondrial function(108, 167) and cell growth. CcnD–CDK4 regulation of cell growth occurs through effects on both protein synthesis(168) and ribosome biogenesis(169, 170).

CDK4 and CDK6 have overlapping, but not identical, substrate specificity *in vitro*(65). The cyclin component of the kinase complex also confers some substrate specificity (65), and functional differences between CcnD1 and CcnD2 have been identified(171).

Some CDK4/6 substrates take part in cellular processes that are less directly involved in cell cycle control, in particular cell motility, cell adhesion and cytoskeletal remodelling(172). Fibroblasts, epithelial cells and macrophages have increased adhesion and reduced migration in the absence of CcnD1(173-175), and thymocyte adhesion is decreased in the absence of either CDK4 or CcnD3(176). In fibroblasts and epithelial cells, the expression of a CcnD1 point mutant (K112E) that cannot activate CDK4 or CDK6 does not decrease adherence or enhance motility, unlike the wild-type protein(173, 174), indicating that these effects are dependent on CDK activity.

7.3.1.1.2 Non-catalytic functions.

Not all of the actions of CcnD–CDK4/6 depend on substrate phosphorylation.

One major non-catalytic function of the D‑type cyclins is transcriptional regulation. CcnD1 is tethered to the promoters of many genes during normal development, probably through interactions with various transcription factors(177). It also binds to regulators of histone acetylation and methylation(178-180), seeming to act as a bridge that links DNA-bound transcription factors with chromatin modifying enzymes and the transcriptional machinery in order to regulate cell proliferation and differentiation(177, 181, 182).

CcnD1‑responsive genes also include some that promote migration and invasion, such as thrombospondin and the Rho effector, ROCK2(175). CcnD–transcription factor interactions commonly occur through motifs in regions that are not shared by D‑type cyclins, indicating that there is specificity in the transcriptional effects of individual D‑type cyclins, although this has not been thoroughly tested, and, much of the published literature only focuses CcnD1. However, ectopic expression of each of the D‑type cyclins in hepatocytes leads to distinct transcriptional profiles(183). Multiple members of the steroid hormone receptor superfamily and their co-regulators interact with CcnD1(181, 182). Cyclin D1 regulates cell proliferation, growth and differentiation by binding representatives of several transcription factor families These include the nuclear hormone receptor family members, Estrogen Receptor-α (ERα), androgen receptor (AR) and peroxisome proliferator-activated receptor-γ (PPARγ) and their co-activators. CcnD1 enhances ERα activity, through interactions with ERα and its co-regulators. CcnD2 and CcnD3 interact poorly with ERα(184, 185). On the other hand, CcnD1 inhibits the activity of AR, thyroid hormone receptor-β and PPARγ, which has a crucial role in fatty acid metabolism, energy homeostasis and adipogenesis. Unlike CcnD1 and CcnD2, CcnD3 interacts with the cellular retinoic acid-binding protein 2 (CRABP2) and retinoic acid receptor-α (RARα)(186) but, like CcnD1, CcnD3 also inhibits AR and PPARγ(187, 188)(187, 189). Direct CcnD3–AR binding or CcnD3–CDK11 phosphorylation of AR have been implicated in fatty acid methabolism, energy homeostasis and adipogenesis(187, 189).

Another non-catalytic role of cyclin D is the sequestration of p21 and p27 by CcnD–CDK4/CDK6. The interaction with p21 and p27 has a key role in coordinating CDK activity during the G1 phase of the cell cycle, but may also be important for other end points. CcnD1 cannot promote migration following p27 knockdown, and p27 has effects on cell migration through Ras homolog gene family, member A (RHOA) and stathmin(190), suggesting that the CcnD1 association with p27 could contribute to the effect of CcnD1 on migration, independently of CDK4.

Similarly, CcnD1 interaction with p21 contributes to its emerging non-catalytic function in DNA repair. CcnD1 regulates the expression of genes that are involved in DNA replication and the DNA damage checkpoint, and it also interacts with a number of proteins that are involved in the DNA damage response(191, 192). By binding Breast Cancer type 2 protein (BRCA2) and the recombinase RAD51, CcnD1 facilitates the recruitment of RAD51 to sites of DNA damage and so promotes DNA repair by homologous recombination,(191, 192). Both the ability of CcnD1 to enhance the DNA damage response and the formation of RAD51 foci require p21(191, 193), suggesting that p21 may also be present in the CcnD1–RAD51–BRCA2 complex. Importantly, decreased CcnD1 expression, but not treatment with a CDK4/6 inhibitor, impairs DNA repair even in cells that lack pRB and so do not require CcnD1 for proliferation(192). In addition, both the CcnD1 K112E mutant and wild-type CcnD1 are able to restore an efficient response to DNA damage in cells lacking all three D-type cyclins(192). Thus, CcnD1 facilitation of DNA repair is independent of CDK4/6 activation, and, distinct from CcnD1 regulation of proliferation.

Many of the non-catalytic effects of the D‑type cyclins have been elucidated using cell culture models, and simultaneous knocking out of all three D‑type cyclins has a very similar phenotype to knocking out both *Cdk4* and *Cdk6*(194, 195), leading to questions about the degree to which non-catalytic effects contribute to the normal cellular functions of CcnD1. However, defects in retinal and mammary gland development in mice lacking CcnD1 are largely restored when the K112E CcnD1 point mutant is knocked in to the CcnD1 locus(196). Similarly, deletion of the gene encoding p27 (*Cdkn1b*) can rescue many of the developmental defects that are observed following the deletion of either the CcnD1 locus or Cdk4 locus respectively

(197, 198). Thus, the ability of the CcnD1–CDK4 complex to sequester p27 is required during development, but the kinase activity of this complex is not essential. This provides strong support for the idea that the non-catalytic functions of CcnD1, particularly its ability to sequester CDK inhibitors, are physiologically relevant.
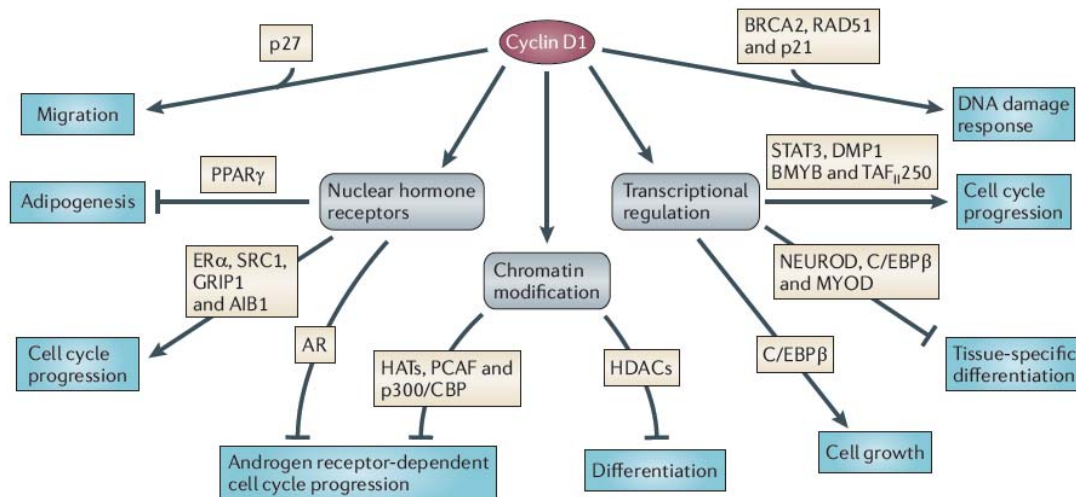
Figure 4. CDK-independent functions of cyclin D1. Although p21 and p27 are constituents of CcnD–cyclin-dependent kinase 4 (CDK4) or CDK6 complexes, CcnD1 can bind p21 or p27 independently of CDK4 or CDK6 binding, leading to effects on cell migration and the DNA damage response, respectively. It also has effects on response to DNA damage through interactions with RAD51 and BRCA2. CcnD1 regulates cell proliferation, cell growth and differentiation by binding representatives of several transcription factor families. These include nuclear hormone receptor family members (oestrogen receptor-α (ERα), androgen receptor (AR) and peroxisome proliferator-activated receptor-γ (PPARγ)) and their co-activators (SRC1, GRIP1 and AIB1), BMYB and the MYB-related transcription factor DMP1, as well as the helix–loop–helix transcription factors neurogenic differentiation factor 1 (NEUROD1), MYOD and C/EBPβ. In addition, CcnD1 binds chromatin-modifying enzymes, including histone acetyltransferases (HATs) such as P/CAF, p300/CBP and histone deacetylases (HDACs). More general effects on transcription can also result from cyclin D1 binding to TAFII250 (also known as TAF1), a subunit of the basal transcriptional machinery. (Figure taken from Musgrove, E.A. et al. *Nat. Rev. Cancer.* 2011). (156).

7.3.1.2 D-type cyclins and β pancreatic cells

Expression of D-type cyclins in pancreatic islets have been reported; CcnD1(199-202), CcnD2(200, 201, 203), CcnD3 in mice, CcnD1, CcnD2 in rats(204) and in humans, CcnD1(205) and CcnD3(206).

Overexpression of CcnD1 in β cells *in vivo* leads to islet hyperplasia without hypoglycemia, is not tumorigenic and does not result in diabetes(207).

CcnD1 and CcnD2 are essential for normal postnatal islet growth(200). β-cell proliferation, adult mass, and glucose tolerance were decreased in adult CcnD2 deficient (-/-) mice, causing intolerance to glucose that progressed to diabetes by 12 months of age. Although CcnD1 semi-deficient (+/-) mice never developed diabetes, life-threatening diabetes developed in 3-month-old CcnD1(-/+) CcnD2 (-/-) mice, as β-cell mass decreased after birth. It seems that CcnD1 and CcnD2 are also important for the regeneration of the β pancreatic mass(201).

 The Reg-Reg receptor system stimulates the PI(3)K/ATF-2/CcnD1 signalling pathway to induce β-cell regeneration(202).

Most of the CDK4(−/−) C57/BL6 mice develop diabetes mellitus by 6 weeks of age, associated with a severe deficiency of β cell mass observed in their pancreatic islets(198)

7.3.1.1.2 Cell cycle proteins in glucose metabolism

During the proliferation process, the cells have anabolic needs. Quiescent cells primarily metabolize glucose and do a full oxidation to carbon dioxide by glycolysis followed by TCA in the mitochondria. However, during proliferation, the cells have higher rates of lactate production derived from glycolysis, biosynthesis of lipids and other macromolecules(208). It seems that the rate of ATP production is lower, but it confers two advantages on the cell. First, when nutrient availability is not limited, high glycolytic fluxes can produce more ATP than the oxidative phosphorylation(209), and the second is that the glucose degradation in glycolysis

provides intermediates needed for anabolic biosynthesis. This glycolytic usage is exacerbated in cancer cells(210).

There is a cross talk between cell cycle and the metabolic control of the cell, and glucose metabolism is a key process the cell needs to control. In the thesis, we focus on the influence that D-type cyclins have on glucose homeostasis, specifically CcnD3, but there is a compilation that shows studies about the influence of cell-cycle proteins (inhibitors, cyclins, E2F1 and CDKs) on glucose metabolism.

7.3.1.1.2.1 Cyclins

Many studies in flies, mice, worms and plants have suggested that CcnD/CDK4 complexes function not only as cell-cycle regulators but also as key metabolic drivers. A first observation supporting this hypothesis came from phenotypic characterization of mice lacking either CcnD1 or CDK4, which were substantially smaller than wild type littermates(139, 211). The Cyclin D knockout mice models revealed that different members of the CcnD family can partially compensate for each other depending on the tissue expression pattern and their respective roles.

The study of the participation of D-type cyclins in cell growth and proliferation is, thus, complicated by the potential functional redundancy of the three D-type cyclins. However, some individual D-type cyclins show marked metabolic phenotypes, first demonstrated for CcnD2 in the pancreatic tissue. Another report shows that glucose infusion increases both the total protein abundance and nuclear localization of CcnD2 in islets(212).

The proliferation of β cells is a key mechanism for maintaining postnatal β-cell mass(213, 214) and it is the primary mechanism for β-cell regeneration(203, 215, 216). It is now well established that pancreatic β cells are able to replicate, although the origin of the newly formed islets remains controversial. CcnD2(-/-) mice showed decreased postnatal β-cell mass and disregulated glucose homeostasis, glucose intolerance as well as diabetes(203). Although CcnD1 (+/-) mice were normal, life-threatening diabetes developed in 3-month-old cyclin D1(+/-)D2(-/-) mice as β-cell mass decreased after birth(200). Hence, CcnD2 and CcnD1 are essential for β-cell expansion in adult mice. A deeper analysis of the data presented in one of these

studies(203) allows further interpretations. CcnD2(-/-) mice have fasting insulin levels similar to CcnD2(+/+) mice. However, upon glucose stimulation, the levels of insulin do not increase in CcnD2 while they double in CcnD2 (+/+) mice(203). This suggests that in addition to the control of β-cell mass, CcnD2 also participates in the control of the β-cell function and is fully consistent with the function of E2F1 in these cells.

7.3.1.1.2.2 CDKs

CDK4 is the major partner of D-type cyclins and the most marked phenotype of mice lacking CDK4 is reduced body size and insulin-dependent diabetes due to a severe deficiency in pancreatic β-cell mass(198, 211). Re-expression of CDK4 in β-cells of the CDK4(-/-) mice restores cell proliferation and normoglycemic condition. However, re-expression of CDK4 does not rescue small body size suggesting that this phenotype is not due to endocrine defects, secondary to decreased insulin levels, but rather in peripheric tissues. As regulation of cell growth is dependent on cell metabolism, this phenotype suggests that CDK4 participates in metabolic control.

CDK6 knockout mice do not display any metabolic phenotype, except that female mice are smaller(195). The mild phenotype of this knockout mice suggest that the functions of CDK6 are mainly compensated by CDK4, or by CDK2 which can interact with D-type cyclins in a CDK4/CDK6 double knockout background(195).

Both the proliferative and metabolic effects of insulin on β cells appear to be mediated by an increase in CDK4 activity and the subsequent E2F1 transcriptional activity. This further suggests that both cell proliferation and metabolic responses are intimately linked and regulated by the same upstream factors. Transgenic mice that specifically overexpress a constitutively-active form of Akt in β cells show higher β-cell mass and proliferation rate with increased β-cell size. Interestingly, these effects were abrogated when mice were bred in a CDK4(-/-) genetic background, demonstrating that AKT induces β-cell proliferation in a CDK4-dependent manner(217).

7.3.1.1.2.3 E2F1

Well-established E2F target genes include effectors of DNA replication, mitosis, DNA repair, apoptosis, differentiation and development(218, 219). Disruption of E2F1 also highlighted its role in the regulation of glucose homeostasis. E2F1(-/-) mice have decreased pancreatic size, as a result of impaired postnatal pancreatic growth. On the other hand, E2F1 is also highly expressed in non-proliferating pancreatic β cells, which suggests that besides controlling β-cell numbers, the protein has a role in pancreatic β-cell function(220). Indeed, it has been demonstrated both in vitro and in vivo that E2F1 directly regulates the expression of Kir6.2, a key component of the K_{ATP} channel involved in the regulation of glucose-induced insulin secretion in pancreatic β cells. Expression of Kir6.2 is lost in the pancreas of E2F(-/-) mice, resulting in insulin secretion defects in these mice. E2F1 transcriptional activity is regulated by glucose and insulin through the CDK4-dependent inactivation of the pRB protein(221). Interestingly, E2F1(-/-) mice are not diabetic. They have dramatically increased insulin sensitivity, secondary to decreased white adipose tissue. These effects are specific for E2F1, whereas the expansion of β cells can be compensated by E2F2. Consistently, E2F1/E2F2 double-mutant mice display insulin-deficient diabetes(222, 223).

7.3.1.1.2.4 Inhibitors

CDK5, an atypical CDK family member with no known cyclin partner has also been implicated in the regulation of insulin secretion. Mice lacking p35, a CDK5 activator, have increased insulin secretion in response to elevated glucose(224). Chemical inhibition of CDK5, or p35 deficiency, resulted in increased insulin secretion in β cells. The effects of p35 deficiency were mediated by CDK5, since inhibition of CDK5 had no effect on insulin secretion in p35(-/-) β cells. The closure of K_{ATP} channels in response to glucose stimulation of pancreatic β cells is followed by Ca^{2+} influx through the L-VDCC channels, a required event in the insulin secretion process. Interestingly, it was shown in this study that the L-VDCC channel was not inhibited in p35 (-/-) or CDK5-inhibited β cells(224). Furthermore, the authors

concluded that the α1C subunit of L-VDCC was a target for CDK5 by an inactivating phosphorylation. Two other reports have implicated CDK5 in the regulation of glucose homeostasis in adipocytes. Okada et al reported that CDK5 phosphorylation of TC10α (a Rho family GTPase) increases GLUT4 translocation, and hence glucose import, in adipocytes(225). GLUT4 is a glucose transporter whose activity is markedly regulated by insulin in muscle and adipose tissue cells(226). The translocation of GLUT4 was also increased by CDK5 phosphorylation of E-Syt1 (a 5C2- domain protein related to synaptotagmins). Phosphorylation of E-Syt1 leads to its increased association with GLUT4 and increased glucose uptake(227). Since CDK activity is regulated by CDK inhibitors, it is not surprising that CDK inhibitors also have a role in the control of glucose homeostasis.

In another study, insulin secretion and sensitivity did not change in p21(-/-) mice under a chow diet, but these mice displayed higher insulin resistance under a high-fat high-sucrose (HFHS) diet when compared to WT mice (228)(229). Deletion of p27 increased islet mass and insulin secretion and prevented hyperglycemia in diabetic mice models. Since the metabolic effects of p21 and p27 are most likely mediated by the inhibition of CDKs, differences in CDK activity due to selective p21 or p27 actions in these mice could explain these distinct phenotypes.

7.3.1.1.3 Cyclin D3

CcnD3 is a 292aa and approximately 42kDa protein mapped to chromosome band 6p21. This gene belongs to the D-type cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event(230). CcnD3 forms a complex with, and functions as a regulatory subunit of, CDK4 or CDK6 as the other D-type cyclins, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with, and be involved in, the phosphorylation of the tumor-suppressor protein pRb. The CDK4 activity associated with CcnD3 was reported to be necessary for cell-cycle progression through the G2 phase into mitosis after UV radiation. Several transcript variants encoding different

isoforms have been found for this gene and also pseudogenes(230)(231)(Sicinska et al., 2003)[231].

Not much has been reported about the independent cell-cycle role of CcnD3, but there is a growing number of redundant properties being discovered among D-type cyclins.

CB57/BL6 CcnD3(-/-) mice fail to undergo normal expansion of immature T lymphocytes and show greatly reduced susceptibility to T-cell malignancies triggered by specific oncogenic pathways(231).

 B-lymphocytes from CcnD2(-/-) mice can proliferate in response to anti-IgM and anti-CD40, but the time taken to enter the S-phase is longer than for the corresponding CcnD2(+/+) cells. This is due to the compensatory induction of CcnD3, but not CcnD1, which causes pRb phosphorylation on CDK4-specific sites. Loss of a D-type cyclin causes specific expression and functional compensation by another member of the family *in vivo* and provides a rationale for the presence of mature B-lymphocytes in CcnD2(-/-) mice(232)

Triple-mutant CcnD1(-/-), CcnD2(-/-), CcnD3(-/-) mice and double-mutant CcnD2(-/-), CcnD3(-/-) mice showed that CcnD3 was uniquely required for the development of pre-B cells. In contrast, the expression of the pre-B cell receptor and the activation of 'downstream' signalling pathways prevented proteasome-mediated degradation of CcnD3. CcnD3 has a key function in B-cell development by integrating cytokine and pre-B cell receptor-dependent signals to expand the pool of pre-B cells that have successfully rearranged immunoglobulin heavy chains(233).

RATIONALE AND

HYPOTHESIS

8. Rationale and hypothesis

Over the last few decades, important advances have been made in understanding the machinery controlling cell progression through the cell cycle, in particular identifying cell-cycle regulatory proteins. These proteins are divided into families comprising cyclins (cyc), cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKIs), the pocket-protein retinoblastoma family and the E2F family of transcription factors. During the G1 phase of the cell cycle there are several proteins that have been related to independent cell-cycle metabolic processes, like CcnDs, CDK4, CDK6 and the E2F family of transcription factors.

CcnD3 is the most important cyclin in the β-cell cycle in humans in spite of being the less-studied cyclin in mice. A priori since CcnD3 is a cell-cycle protein, it could be a target for preventing the development of T1D by slowing down β-cell death rate and promoting β-cell proliferation.

When we began this study, most of the previous work had been performed using *in vitro* models, such as insulinoma cell lines (NIT-1 or MIN-6) exposed to proinflamatory citokines. Thus, we became interested in gene expression changes in β cells *in vivo* as a consequence of leucocyte infiltration of islets.

We found that mRNA expression levels for a series of genes change during pancreatic islet infiltration (insulitis) in the autoimmune-prone non-obese diabetogenic (NOD) mouse genetic background, compared with the prone lymphocyte-free, because of a severe combined immunodeficiency in the same genetic background (NOD/SCID). Among these genes, CcnD3, a cell cycle protein that is downregulated, drew our attention.

The trigger for initial β-cell damage is not well known. However, β-cell death in an inflammatory environment may expose β-cell antigens to productive presentation and lead to autoimmune attack.

Therefore, bearing in mind that CcnD3, the key protein in human and mouse β cells is downregulated during the autoimmune attack against pancreatic β cells in the mouse model, we hypothesized that: CcnD3 downregulation causes a dramatic decrease in the number of CcnD3/CDK4 complexes, leading to a functional impaired β-cell replication response towards inflammatory stimulus.

If our hypothesis were correct, we could expect that a full CcnD3 deficiency would exacerbate diabetes, while its overexpression in β cells would lead to protection from diabetes in the NOD background, and this would be confirmed by *in vitro* models of either β cells or insulinoma cells submitted to a prinflamatory environment.

OBJECTIVES

9. <u>Objectives</u>

9.1 **Main goal**

Determine the causal relationship between cyclin D3 downregulation and T1D

onset in the NOD mouse model

9.2 **Specific goals**

9.2.1 Verify whether cyclin D3 mRNA downregulation is associated with a parallel Cyclin D3 protein downregulation in β cells *in vivo* and *in vitro.*

9.2.2 Determine whether cyclin D3 overexpression on the NIT-1 NOD insulinoma cell line:

 a) Protects these cells from cytokine induced apoptosis and/or necrosis

 b) Promotes cell proliferation.

9.2.3 Assess whether cyclin D3 deficiency in NOD mice exacerbates T1D

9.2.4 Assess whether cyclin D3 overexpression in NOD β cells protects these mice from T1D phenotyping NOD β cells overexpressing cyclin D3 (generation of NODCcnD3Tg mice)

9.2.5 Determine whether the restoration of cyclin D3 expression solely in β cells is enough to ameliorate the diabetic phenotype of CcnD3 NOD-deficient (NODCcnD3KO) mice.

9.2.6 Phenotyping the diabetogenicity of the immune repertoire for the strains used.

9.2.7 Phenotyping physiological competence of the NOD β cells either deficient or overexpressing CcnD3 compared to wild-type NOD β cells

MATERIALS AND

METHODS

10.   **Materials and methods**

All solutions and buffer compositions mentioned from here in bold, are included in the section **14.1 Buffer Preparation,** page 107.

10.1   Mice

The NOD colony originally derives from that of Chales A. Janeway, Jr. at the Yale University Mouse Facility.

The mice were kept in Specific Pathogen Free (SPF) conditions, in a dark-light 12h dark/light cycle and fed *ad libitum* using standard rodent diet chow (Panlab, Cornellà, Spain) in a microisolator cage with positive pressure of HEPA air at 21ºC.

The mice were sacrificed by cervical dislocation. For adoptive transfer experiments, inhaled anesthetic was used (Isoflurane; Abbot Laboratories, Madrid, Spain). All animal experimentation procedures performed in this work were overseen and approved by the Institutional Ethical Committee for Animal Experimentation at the University of Lleida (CEEA) in accordance with the European and U.S. Regulations on Animal Experimentation.

The personal in charge of the food and water supply or for carrying out any procedure that involved any contact with the mice always wore sterilized lab-overall clothes. Manipulation of the animals was done in a laminar flow hood. All the animal food, material and water in contact with these mice was sterilized by autoclaving.

Urine tests were performed routinely to check healthy levels of glucose with Medi-Test Glucose 3 (Macherey-Nagel, Düren, Germany). If there was an increase of gluose in urine and glycemic of more than 200mg/dL Glucocard test strips (Glucocard Gsensor, Arkray Inc., Shiga. Japan), the animals were diagnosed as diabetic and euthanized.

Mice carrying the SCID mutation were kept on Septrin antibiotic mixture (Sulfamethoxazole 1.2 g/l and trimethoprim 0.24g/l) on a week on-week off basis.

Except where otherwise stated, only female mice were used in the experiments described in the thesis.

10.2 Generation of the NOD/CcnD3 KO mice.

Mice homozygous for the deficiency null mutation in cyclin D3 were originally obtained in the C57BL/6 genetic background(231). Intensive backcrossing onto the NOD genetic background, and checking for the Idd markers as described(234) was performed to ensure the NOD genetic background. Genotyping for the Idd loci was performed by using PCR primers specific for the different loci that are polymorphic for the 129/Sv, C57BL/6, and NOD strains. The primers used were: Idd1 (H-2g^7; for Idd1 screening two sets of PCR primers were used, a and b: a, D17 Mit34; b, 5′-TGT CTT TTC TGT CAC CCT AGA ACA-3′, 3′-TAC ACC TCG TAG GGT CGG ACT TCT C-5′; the PCR product obtained by b primers (282 bp) was digested using ***Msp***I; the NOD allele is resistant to cleavage; Idd2, D9 Mit25; Idd3, D3Nds36; Idd4, D11 Mit 115, D11 Mit320; Idd5, D1 Mit24, D1 Mit26; Idd6, D6 Mit52; Idd7, D7 Mit20; Idd8, D14 Mit11; Idd9, D4 Mit59; Idd10, D3 Mit103; Idd11, D4 Mit202; Idd12, D14Nds3; Idd13, D2 Mit395; Idd14, D13 Mit61; and Idd15, D5 Mit48. PCR conditions were 35 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min. The PCR products were run in 4% agarose gels. We reached the 12th generation (N13), which was used for the experimental procedures. Genotyping of cyclin D3 gene targeting mutation was performed by PCR according to the literature(231).

10.3 Generation of the NOD/RIPCcnD3 transgenic mice.

We generated transgenic NOD mice by microinjection of the RIP-CcnD3-Eα construct into fertilized NOD oocytes (Taconic; NY, USA). The RIP-CcnD3-Eα construct consisted of the Rat Insulin Promoter 2 (RIP2) driving the murine cyclin D3 cDNA (the construct was a generous gift from Martine Roussel(235)). A fragment obtained by the double digestion of the mouse CCnD3 cDNA with EcoRI and XbaI restriction enzymes (between nucleotides 105 and 1130), followed by the intronic

sequence provided by the Eα fragment, consisting of the 3´region of the mouse MHC Class II gene I-E alpha[d] comprehending the restriction fragment by restriction digestion with AvaI and HindIII enzymes (between nucleotides 3635 and 5557). The progeny were analyzed for the insertion of the RIP CcnD3 transgene by PCR using the primers (Forward-RIP2: CAA GAC TCC AGG GAT TTG AGG GA; Reverse D3R1: GAC GCA GGA CAG GTA GCG ATC CAG) respectively. PCR conditions were 30 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min, and the PCR **product** size was 460bp. 5 different founder lines with germ-line transmission were obtained: 6876, 6877, 6880, 6889 and 6896. Expression of the transgene by these transgenic lines was assessed by intracellular staining of cyclin D3 by flow cytometry.

10.4 Generation of NODCcnD3KO/RIP CcnD3 mice.

NOD mice deficient in CcnD3 overexpressing CcnD3 on β cells were generated by intercrossing NOD/CcnD3 KO with NOD/ RIP CcnD3 transgenic mice (founder line 6896), and bringing the CcnD3 null mutation to homozygotig, while keeping the transgene in hemizigosity.

10.5 Generation of the different NOD/SCID strains

10.5.1 Genotyping of the SCID mutation in the different NOD strains.

NOD/SCID mice were purchased from The Jackson Lab (The Jackson Laboratories, Bar Harbor, ME, USA). The *scid* mutation was genotyped by using the PCR protocol recommended by the Jackson laboratory.(Forward-oIMR803: GGA AAA GAA TTG GTA TCC AC and Reverse-oIMR804: AGT TAT AAC AGC TGG GTT GGC)
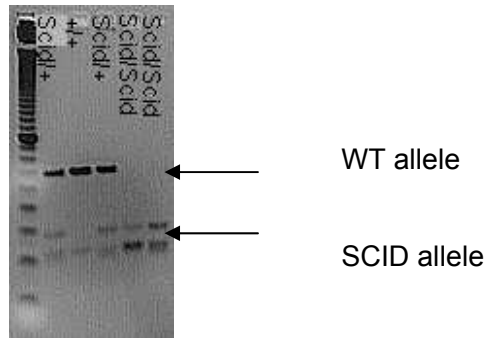
PCR was developed and AluI restriction enzyme was used at 37ºC Mutant=38bp, 28bp and 11bp; HTZ=68bp, 38pb 28bp and 11pb WT= 68bp and 11bp.

10.6 Assessment of diabetes.

Female mice from each strain were monitored weekly for the development of glycosuria with Medi-Test Glucose 3 (Macherey-Nagel, Düren, Germany) starting at 3 weeks of age in the case of natural history. In the case of adoptive transfer, recipient female mice were monitored once a week for glycosuria after adoptive transfer was performed. Diabetes was confirmed by measuring glycemia. Values over 200 mg/dL of glucose with the Glucocard test strips (Glucocard GSensor, Arkray Inc., Shiga. Japan) were considered to be a positive test for diabetes. Autoimmune diabetes were confirmed islet infiltration in tissues with Hematoxilin Eosine staining.

10.7 Adoptive transfer experiments.

Donors of total spleen cells were euthanized, and the spleens were extracted under a flow hood with autoclaved material. The total spleen cells were submitted to red blood-cell lysis by adding 900μL of pure water and immediately 100 μL of 10x PBS. The cells were washed with physiological saline solution 3 times and counted. The cells were transferred (10 million of cells per recipient/ 200μL) intravenously in physiological saline solution.

10.8 Intaperitoneal Glucose Tolerance Test (IGTT).

Before the test was performed, the mice were kept fasting overnight, for at least 16 hours. Before the intraperitoneal glucose tolerance test (IGTT), the mice were anaesthetized using sodium pentobarbital (60 mg/kg). The mice received an intraperitoneal injection of 2g glucose per kg body weight. Glycemia and insulinaemia were measured at 0, 15, 30, 60 and 120 min after the injection. Insulinemia was determined by mouse Insulin ELISA kit (Mercordia, Uppsala, Sweden).

10.9 Intravenous insulin tolerance test (IITT).

Before the test was carried out, the mice were kept fasting overnight for at least 16 hours. Before the intravenous insulin tolerance test (IITT), the mice were anaesthetized using sodium pentobarbital (60 mg/kg). 1 IU insulin per kg body weight (Regular Humulin; Lilly, Indianapolis, Ind. USA) was injected into the tail vein. Insulinemia and glycaemia was measured at 0, 15, 30, 45 and 60 min after the injection. Insulinemia and glycemia were determined as described above.

10.10 Immunohistochemical analysis of CcnD3 expression

The mice were euthanized, the pancreas and spleen were extracted and the tissue was immersed in 4% paraformaldehyde in **PBS** for 3 hrs. The tissue was dehydrated for 15 min in 70% ethanol 1h; and then, twice for 1h each time in 96% ethanol; three times, for 1h each time in 100% ethanol; and finally, 30 min in xylene. The tissue was then embedded in paraffin for 4h at 60ºC. Sections were cut 8-10µm thick, and placed the sections on Superfrost plus adhesion slides (Termo Scientific, Portsmouth, NH; USA). After the tissue sections were on the slides, they were kept overnight at 37ºC on a hot plate.

To deparaffinize the slides, they were immersed three times for 10 min each time in xylene; twice for 5 min each in 100% ethanol; twice for 5 min each in 60% ethanol and distilled water for at least 10 min.

The unmasking procedure was performed by immersing the samples in **citrate solution** inside a pressure cooker until it reached to its maximum pressure, keeping it on the heat for 5 min. The pressure cooker was taken off the heat for 15 min. to allow the samples to reach room temperature.

The slides were washed for 5 min in the **Tris solution** and then for 30 min in the **Permeabilization solution**, 5 min in the **Tris solution**, 1 hour in the **Blocking solution**, 5 min in the **Tris solution** and the tissues were incubated with the primary antibody overnight at 4ºC in a humidified chamber.

The slides were washed 3 times, 5 min each, in the Tris solution and the tissues were incubated with secondary antibody for at least 3 hours at 4ºC in a humidified chamber in **Blocking solution.** After that, the slides were washed 3 times for 5 min each time in the Tris solution. Slides were mounted with mowiol. Epipluorescence images were captured with a Leica DM R microscope (Leica Microsystems Gmbh, Wetzler, Germany).

First antibodies		
α_m-CcnD3	BD Pharmingen, clone 1, California, USA	1:50
polyclonal α_{GP}-insulin	Dako, California, USA	1:1000
Hoeschst 333421mg/mL	Sigma Aldrich, St. Louis, USA	1:200
Secondary antibodies		
:α_{GP}-IgG-Cy2	Jackson Immunoresearch laboratories, West Grove, PA, USA	1:500
αm-IgG-Cy3	Jackson Immunoresearch laboratories, West Grove, PA, USA	1:500

10.11 Immunohistochemical analysis of pancreatic infiltration.

The tissues were fixed by immersion in 4% paraformaldehyde in PBS for at least 6 to 8h.

Tissue dehydration: 15 min in 70% ethanol 1h; and then twice for 1h each in 96% ethanol; three times for 1h each in 100% ethanol; and finally, for 30 min in xylene. The tissues were then embedded in paraffin for 4h at 60ºC.

Tissues were then cut into sections 8-10µm thick, and placed on Super frost plus adhesion slides (Termo Scientific, Portsmouth, NH; USA). After the tissue sections are on the slide, the slides were dried at least overnight at 37ºC on a hot plate.

Deparaffinization of the slides: the procedure was performed by placing them three times for 10 min each time in xylene; twice for 5 min each in 100% ethanol; twice for 5 min each in 60% ethanol and in distilled water for at least 10 min.

The paraffin sections were counted and stained with Hematoxylin & Eosin (Sigma-Aldrich): 1 bath 25 min of hematoxylin, washed once with distilled water to remove the excess during 20 min, 10 sec in **Acid alcohol**, washed once with distilled water for 10 min and put in a bath of **Eosine solution** for 1-2 min. For the infiltration studies, the images were captured with a Leica DM R microscope (Leica Microsystems Gmbh, Wetzler, Germany). To assess pancreatic infiltration, a minimum of 4 mice per experimental group were used (a minimum of 100 of islets counted/group). The infiltration score was: 0, no infiltration; 1, pancreatic infiltration or peri-insulitis; insulitis invading less; 2, or more; 3 than half of the islet area. (BD PharMingen, California, USA)

10.12 Morphometric studies.

The pancreatic paraffin sections were stained for insulin (Dako, California, USA) and detected by a biotinylated secondary Ab (Biogenex, California, USA). FastRed chromogen (SIGMA Aldrich, St. Louis, USA) was used as the alkaline phosphatase substrate. Hematoxylin was used for islet counterstaining. The insulin area was quantified in a blinded fashion using optical microscopy (Leica, Wetzler, Germany) and ImageJ software (NIH, Bethesda, Maryland; USA) using a microscopic scale provided by the program. A minimum of 4 females were used per experimental group.

10.13 Pancreatic islet isolation.

The mice were euthanized by cervical dislocation. The islets were isolated by collagenase digestion, each pancreas was injected with 4 mL of collagenase P digestion solution (0.70 mg collagenase P/mL Hanks' Balance Salt Solution (HBSS)(Lonza; Belgium) + 0.1%BSA) through the pancreatic duct.
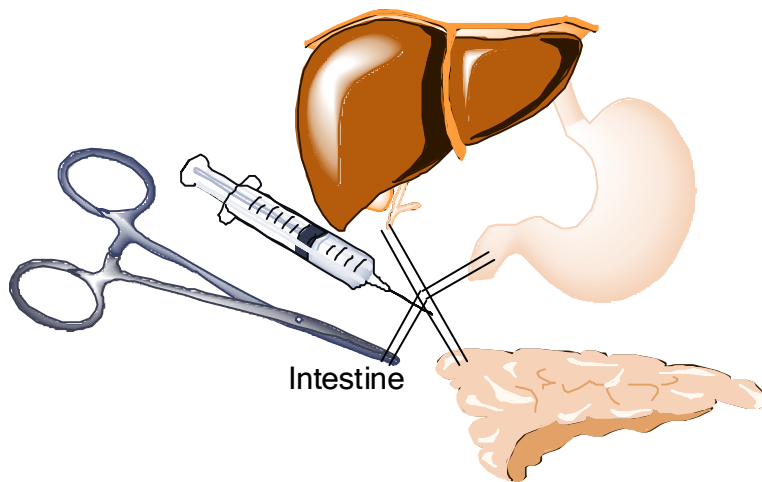
Figure 5. Pancreatic duct preparation to perform collagenase P infiltration of the pancreas. The pancreatic duct is found in the euthanized mice in the duodenum area, leakage of the colagenase P digestion solution is prevented by blocking the connection of the pancreatic duct-intestine with a Rochester haemostat

After the pancreases were injected with 3mL of collagenase P digestion solution, the tissues were incubated for 5 minutes at 37ºC, disaggregated mechanically with a Pasteur pipet and, digestion was stopped by adding 45mL of cold HBSS+ 0.1%BSA to the homogenate obtained. After digestion, this was kept in ice during the whole procedure.

The tubes stayed in the ice for 3 minutes and the lipid layer that remained on top was discarded. Individual islets were isolated by placing the disaggregated pancreas in Petri dishes and the islets were handpicked by pipetting them. The islets were

distinguished from exocrine pancreas by using black contrast as a background since the islets exhibit a characteristic white glare under a magnifying glass. The islets were handpicked twice with a micropipette under the magnifying glass.

10.14 Measuring islet insulin content.

To obtain a total islet protein extract, the islets were then immersed in 50μL **an acid–alcohol solution**, the extract was vortexed and left overnight at 4°C. The next day, the protein extracts were centrifugated and the insulin content was determined by Insulin ELISA kit (Mercordia, Uppsala, Sweden). The results are expressed as insulin content per 10 islets in a mL.

10.15 Islet cell staining for flow cytometry.

The isolated islets underwent trypsinization with 500 μL of trypsin/EDTA (Lonza, Verviers, Belgium) for 5 min at 37ºC to obtain a single cell suspension, and then, they were fixed with 1mL of cold methanol at -20ºC overnight prior to cellular staining. The cell suspension was centrifugated at 350xg 5 min, the supernatant was discarded and the cells were washed with 1mL of blocking solution and stained with the next antibodies in **Blocking solution** with 0.01% of triton X-100 (Sigma Aldrich, St. Louis, USA) choosing the staining with the antibodies:

First antibodies		
α_m- GLUT-2 biotinilated	R&D, Germany	1:100
α_m- Ki67 mouse	(Dako, California, USA	1:100
α_m- CcnD3 mouse	BD, Pharmingen; California, USA	1:100
PE-conjugated α_m-CD45	BD, Pharmingen; California, USA	1:100

Secondary antibodies or reagents		
and α_mIgG APC	Immunotools, Friesoythe; Germany	1:200
Streptavidin APC	Immunotools, Friesoythe; Germany	1:200

The cells were washed twice with blocking solution with 0.01% of triton X-100. The samples were acquired using the FACS-CantoII Flow Cell Cytometer (BD; California, USA).

10.16 Determination of early apoptotic and necrotic events by annexin V staining

The cells were stained for annexin V and either 1:200 7-aminoactinomycin D (7AAD; Calbiochem) or Propidium Iodide (PI) following the protocol of the Annexin V Detection kit (BD Biosciences; California, USA). Cells in suspension were incubated with 7-AAD or PI (1:200) and AV 1:100 in calcium buffer 1X included in the kit for 15 min at 37ºC in the dark. The samples were acquired using the FACS-CantoII Flow Cell Cytometer (BD; California, USA).

10.17 Determination of late apoptotic events by TUNEL staining

Tissues deparafinized as in 10.11 were incubated with a pool of TUNEL made with Tunel label mix and recombinant TdT (Roche, Penzberg, Germany) for 1 hour.

The slides were washed for 5 min in the **Tris solution** and then for 30 min in the **Permeabilization solution** followed by 5 min in the **Tris solution**, 1 hour in the **Blocking solution** and 5 min in the **Tris solution**. The tissues were incubated overnight with a primary antibody, polyclonal α_{GP}-insulin (Dako, California, USA), and Hoeschst 33342 1mg/mL (Sigma Aldrich, St. Louis, USA).

The slides were washed 3 times for 5 min each time in the Tris solution and the tissues were incubated with a secondary antibody in the **Blocking solution** of α-GP-Cy3 (Jackson Immunoresearch laboratories, West Grove, PA, USA) 1:500. After that, the slides were washed 3 times for 5 min each time in the **Tris solution**. The slides were mounted with mowiol. Epipluorescence images were captured with a Leica DM R microscope (Leica Microsystems Gmbh, Wetzler, Germany)

10.18 NIT-1 cell transfectants.

The NIT-1 NOD insulinoma cell line was stably transfected by electroporation with either the pBSKNeo plasmid containing the RIP CcnD3 transgene or the empty plasmid as a control. Stable transfectants were selected in DMEM media (Lonza, Verviers, Belgium) supplemented with 10% Fetal Calf Serum (Lonza, Verviers, Belgium), Gentamycin 1.21mM (0.058mg/mL) (BioSera, Boussens, France), β-mercaptoethanol (Invitrogen, Scotland), and G418 Sulphate 6 mM (0.4mg/mL) (PAA, Pasching, Austria). Cells from the flask were harvested with 1ml of trypsin/EDTA (Lonza, Verviers, Belgium). For cytokine-induced cell apoptosis, 100,000 cells per well were seeded in 12-well plates on day 0. On day 1, cytokines were added at a final concentration of 50U/mL for IL-1β (Prospect; Passaic County, NJ, USA) and 200U/mL for IFN-γ (Prospect; Passaic County, NJ, USA). On day 2, the cells were harvested mechanically and assayed for either annexin V staining, or for a proliferation of Ki67 staining.

10.19 Recording changes in intracellular calcium [Ca2+]i concentrations

Freshly-isolated islets of Langerhans were loaded with 5 mM Fura-2 AM DMSO (Invitrogen, Carlsbad, CA, USA) for at least 1 hour at room temperature. The islets were placed on a perfusion chamber mounted on the epifluorescence microscope (Zeiss, Axiovert 200, Gottingen, Germany). The perfusion chamber was mounted on the stage of the microscope and a poly-L-lysine–treated coverslip was placed on it for 10 min before starting the experiments. Small-sized islets were preferably used

for $[Ca^{2+}]_i$ measurements and the area of the islet was selected to measure these changes. The islets were then perfused at a rate of 1.5 ml/min with a modified Ringer solution containing (in mmol/l): 120 NaCl, 5 KCl, 25 NaHCO$_3$, 1.1 MgCl$_2$, and 2.5 CaCl$_2$, pH 7.35, then gassed with 95% O$_2$ and 5% CO$_2$. Ca^{2+}measurements were performed on individual islets with a Zeiss LSM 510 laser confocal microscope equipped with a 40× oil immersion objective (numerical aperture = 1.3). Calcium recordings in isolated cells were obtained by imaging intracellular calcium. Images were acquired every 2 s with an extended Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain) using a dual filter wheel (Sutter Instrument CO, CA, USA) equipped with 340 nm and 380 nm, 10 nm bandpass filters (Omega Optics, Madrid, Spain). Data were acquired using Aquacosmos software from Hamamatsu (Hamamatsu Photonics, Barcelona, Spain). Fluorescence changes were expressed as the ratio of fluorescence at 340 nm and 380 nm (F340/F380). The results were plotted and analyzed using commercially-available software (Sigmaplot, Jandel Scientific). The time interval used for the measurements was 10 min for each concentration of glucose and 25mM of K$^+$ for islets. The concentrations of glucose were 3mM, 8mM, 16mM and the solution for 25mM of KCl was done in 3mM of glucose.

RESULTS

11. Results

To identify genes differentially regulated in β cells by the inflammatory niche in NOD islets, pancreatic endocrine cells derived from either 11-week-old NOD non-diabetic females on the point of developing diabetes and the other from age-mated lymphocyte-free 11-week-old NODSCID female mice, which cannot develop T1D were compared. CcnD3 mRNA was found to be downregulated in the NOD endocrine cells to half of the expression levels found in the NODSCID as a consequence of pancreatic islet infiltration in the NOD female mice.

11.1 Cyclin D3 protein expression is downregulated by islet infiltration in NOD mice without affecting the proliferative activity of beta cells.

To prove that the amount of protein CcnD3 decreases during infiltration, islets were isolated from 11-week-old NOD, NODSCID and NODSCID mice that had received 10 million NOD total spleen cells intravenously (NODSCIDT) two weeks before. CcnD3 expression was measured by flow cytometry. The NOD expression of CcnD3 in the NOD β cells was lower than in the NODSCID mice, confirming the results obtained in the microarray experiment with mRNA (**Figure 6A**). The expression of CcnD3 for NODSCIDT is not statistically different to that found in NODSCID (**Figure 6A**). A comparison was perfomed using the Kruskal Wallis comparison test and the Mann-Whitney U as the statistical analysis. Lymphocyte infiltration in the NOD pancreatic islets could be causing downregulation of the expression of CcnD3 in the NOD mice. To prove the effect of infiltration on the expression of CcnD3, the NODSCID mice were given 0, 1, 3, 5 and 10 million of total spleen cells intravenously and two weeks later, the islets were isolated. We observed an inverse correlation between the CcnD3 expression and the number of total spleen cells transferred (**Figure 6B**). However, in spite of CcnD3 downregulation, the β cells did not exhibit impaired proliferation (**Figure 6C**). This may be due to the fact that the

function of CcnD3 is redundant with other CcnDs that are expressed in mouse islets and can compensate for CcnD3 repression in the proliferative activity of theβ cells(200-202).

In humans, D-type cyclins have a redundant role in β-cell proliferation(236). However, CcnD3 is responsible for the most robust rate of proliferation in adult human β cells(237) when complexed with CDK6, which is not expressed in mouse islets.

Downregulation of CcnD3 in a mouse model genetically prone to autoimmunity against β cells may point to a gene product requiring proper cell functioning. Therefore, we hypothesized that downregulation of CcnD3 precipitates T1D onset by compromising β-cell fitness in a cell-cycle independent manner.
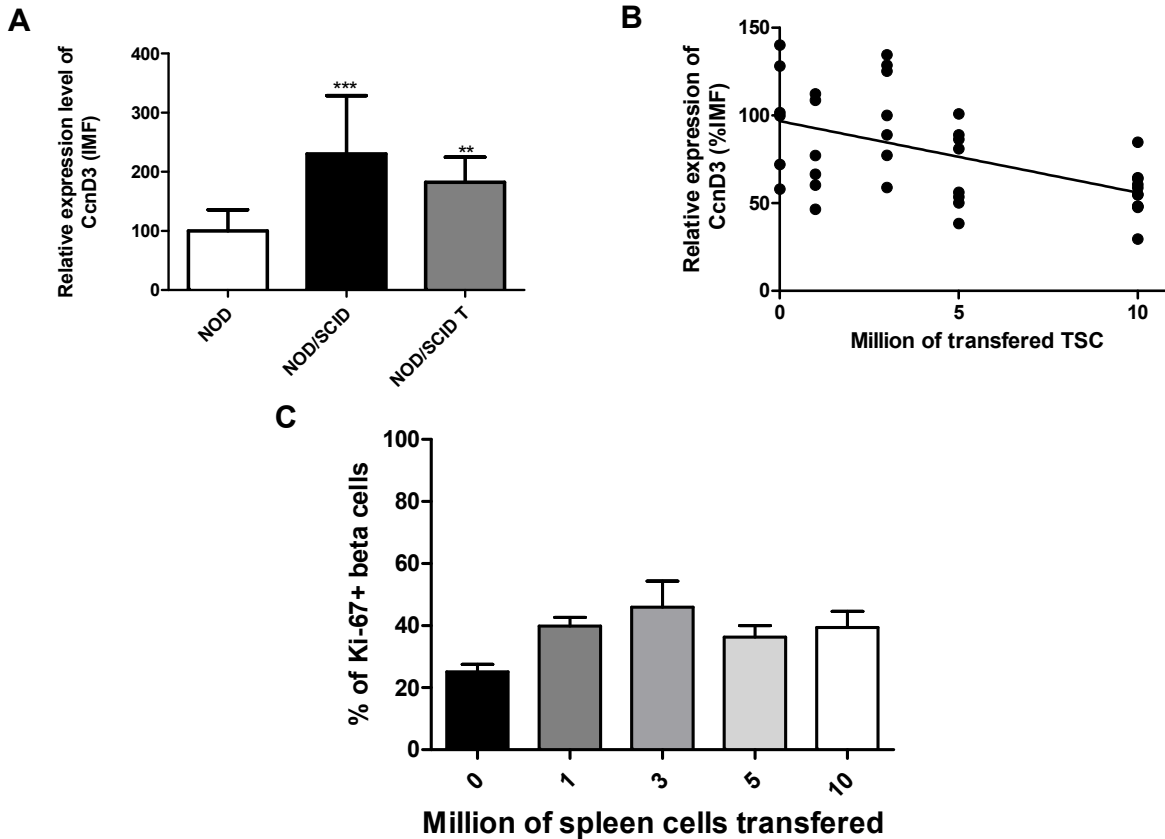
Figure 6. Cyclin D3 protein expression is downregulated by islet infiltration in NOD mice in a dose-dependent manner, without affecting the proliferative activity of the β cells. A. Pancreatic islets from either 11-week-old NOD female mice, 11-week-old NOD/SCID female mice, or 11-week-old NOD/SCID female mice which had been transferred with 10 million of NOD female total spleen cells at 9 weeks of age (NODSCID T) were extracted and disaggregated. Cyclin D3 staining IMF (Intensity of Median Fluorescence) was measured by flow cytometry on non-hematopoyetic CD45$^-$ Glut2$^+$ β cells. Comparisons were performed in relation to NOD results. **B.** Assessment of Cyclin D3 expression levels in beta cells from NOD/SCID females adoptively transferred with different amounts of total spleen cells (0, n=6; 1, n=6; 3, n=7; 5,n=8 and 10, n=11) two weeks before the islet extraction at 11 weeks old. Cyclin D3 staining IMF (Intensity of Median Fluorescence) was measured by flow cytometry on CD45$^-$Glut2$^+$ (β) cells (y=-4.64x+101.69 P=0.00046348). **C.** Ki67 (proliferation marker) expression levels in beta cells from 11-week-old NOD/SCID females adoptively transferred with different amounts of total spleen cells two weeks before the islet extraction, measured as a percentage of CD45$^-$ Glut-2$^+$ ki67$^+$ cells. Statistical analysis performed in **A** and **C** was Wilcoxon-Cox and Mann-Whitney U; (0, n=7; 1, n=7; 3, n=6; 5,n=4 and 10, n=9). ** p≤0,01, ***p≤0,001.

11.2 Generation of the NODCcnD3KO and NODSCIDCcnD3KO mice strains

Mice homozygous for the deficiency in cyclin D3 were originally obtained in the C57BL/6 genetic background(231). After intensive backcrossing into the NOD genetic background, checking for the NOD Idd (Idd1 to Idd15) T1D susceptibility loci, we reached the backcross 12th generation (N13). NOD female mice deficient in CcnD3 (NODCcnD3KO) and female littermates heterozygous for the null mutation (NODCcnD3HTZ), and wild-type female littermates (NODccnD3WT) were used for the experimental procedures performed in this thesis. To obtain immunodeficient NODSCIDCcnD3KO mice, NODCcnD3KO mice were outcrossed to NODSCID mice until the SCID mutation was incorporated into homozigosity, together with the CcnD3n null mutation.

The specificity of the α-mouse CcnD3 antibody was verified by using two techniques: flow cytometry (**Figure 7A**) and immunofluorescence (**Figure 7B**). In both cases, islets from 6-week-old NODCcnD3WT, NODCcnD3HTZ and NODCcnD3KO female mice were used.

CcnD3 expression levels are lower in both the cytoplasm and nucleus of NODCcnD3HTZ compared with NODccnD3WT and it is not expressed in either the cytoplasm or nucleus of the NODCcnD3KO mice. There are some islets in the NODCcnD3KO that have a lower expression of insulin, and, in some of them, it seems they are the same compared with the WT, but there is no statistical difference between them.

There are also phenotypical differences between the NODCcnD3KO and NODCcnD3WT littermates: a) the NODCcnD3KO mouse strain has a lower litter size (**Figre 7C**); b) a smaller size comparing with the CcnD3WT (**Figure 7 D**), c) at 11 weeks of age, the female NODCcnD3KO weighed less than the female WT littermates (**Figure 7E**); d) regarding pancreatic islets, the β cell area of 6-week-old female NODCcnD3KO mice is smaller than in their NODCcnD3WT female littermates and this is effect is insulitis-independent, because the size measured in the NODSCIDCcnD3KO is similar. The statistical analysis performed in A, C, E and F was the Wilcoxon-Cox and Mann-Whitney U; n=9. ** p≤0,01, ***p≤0,001.
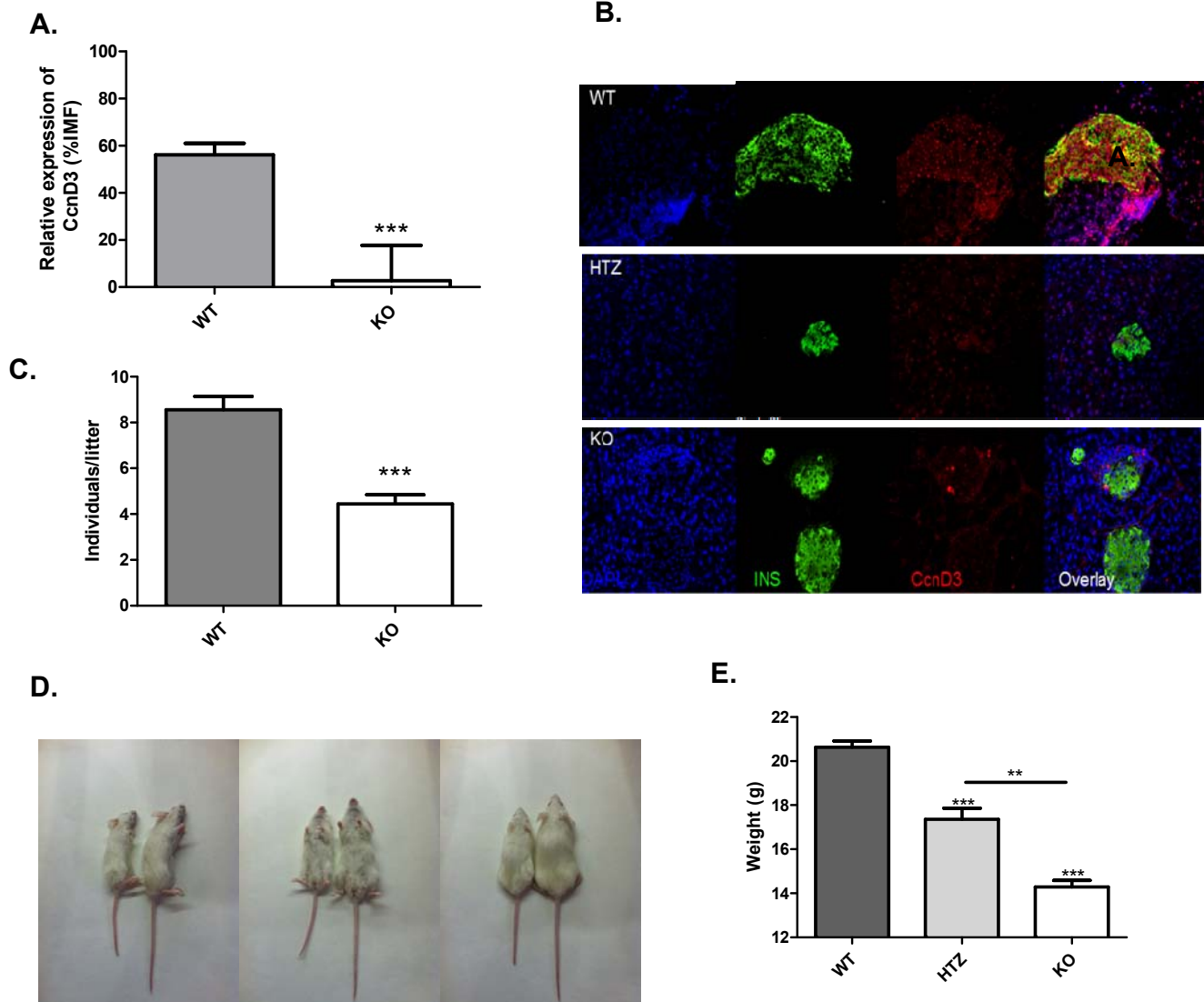
Figure 7. Generation of NODCcnD3KO mice. **A**. Pancreatic islets from 6-week-old NODCcnD3WT (n=24) and NODCcnD3KO (n=12) female mice, were extracted and disaggregated. Cyclin D3 staining IMF (Intensity of Median Fluorescence) was measured by flow cytometry on the CD45$^-$Glut2$^+$ β cells. The measurements were performed taking the IMF of the WT values as 50%. **B**. Pancreatic islet cell immunofluorescence labeled for insulin (green), CcnD3 (red) and nucleus (blue) of 6-week-old NODCcnD3WT, NODCcnD3HTZ and NODCcnD3KO females **C**. individuals per litter were counted (NODCcnD3WT n=38, NODCcnD3KO n=36) **D.** Picture of the size of the NODCcnD3WT and NODCcnD3KO **E**. The weight of mice (NODCcnD3WT n=23, NODCcnD3HTZ n=12, NODccnD3KO n=9) and **F**. The size of the β cell area was measured labelling insulin and taking photographs with an optical microscope and the measurement of the area was performed with the ImageJ software (NODCcnD3WT n=104, NODCcnD3HTZ n=5, NODCcnD3KO n=58, NODSCIDCcnD3WT n=101, NOD/SCIDCcnD3KO n=101). The statistical analysis performed in A, C, E and F. was the Wilcoxon-Cox and Mann-Whitney U; n=9. * p≤0,05, ** p≤0,01, ***p≤0,001. The statistical comparisons reflected

by stars on error bars refer to thw WT group; the stars on top of horizontal lines refer to comparison between the experimental groups ay both extremes of this line.

11.2.1 NODCcnD3KO mice exhibit exacerbated diabetes.

To demonstrate the causal link between cyclin D3 repression and β-cell death *in vivo*, we studied spontaneous diabetes onset in NOD female mice deficient for CcnD3 comparing to either female littermates, heterozygous for the mutation or WT, (**Figure 8A**).

 NODCcnD3KO develop accelerated T1D, since at 9 weeks of age, some of them had already developed diabetes, while none of the NODCcnD3HTZ or NODCcnD3WT strains started before 12 weeks of age. We observed that at 12 weeks of age, 43% of the NODCcnD3KO females had already developed the disease.

The age at which 50% of mice were diabetic was higher if the mice expressed more CcnD3 in the β cells: 13 weeks for -NODCcnD3KO, 18 weeks for-NODCcnD3HTZ, 21 weeks for-NODCcnD3WT showing that the expression of CcnD3 is important for the speed of the kinetics in T1D disease development.

 Moreover, the cumulative incidence of diabetes is higher in the NODCcnD3-deficient mouse strain with: 89.38%-NODCcnD3KO, 90.6%-NODCcnD3HTZ, 63.49%- NODCcnD3WT and 68.96%-NODCcnD3TG and this fact was due to the deficiency of CcnD3 in theβ cells.

Because NODCcnD3-deficient mice develop diabetes at an early age, pancreatic islet infiltration was assessed in 6-week-old female mice. NODCcnD3KO, NODCcnD3HTZ and NODCcnD3WT were scored for insulitis (**Figure 8B**). Insulitis was seen to be higher in the NODCcnD3KO and NODccnD3HTZ than in the NODCcnD3WT, as shown by the percentage of each kind of infiltration (**Figure 8C**).

Proliferation rates were measured by flow cytometry by assessing the percentage of CD45-Glut2+ki67+, and no differences in proliferation rates between different genotypes were found. The Mantel-Cox test and Man Withney U statistic analyses were performed (**Figure 8D**).
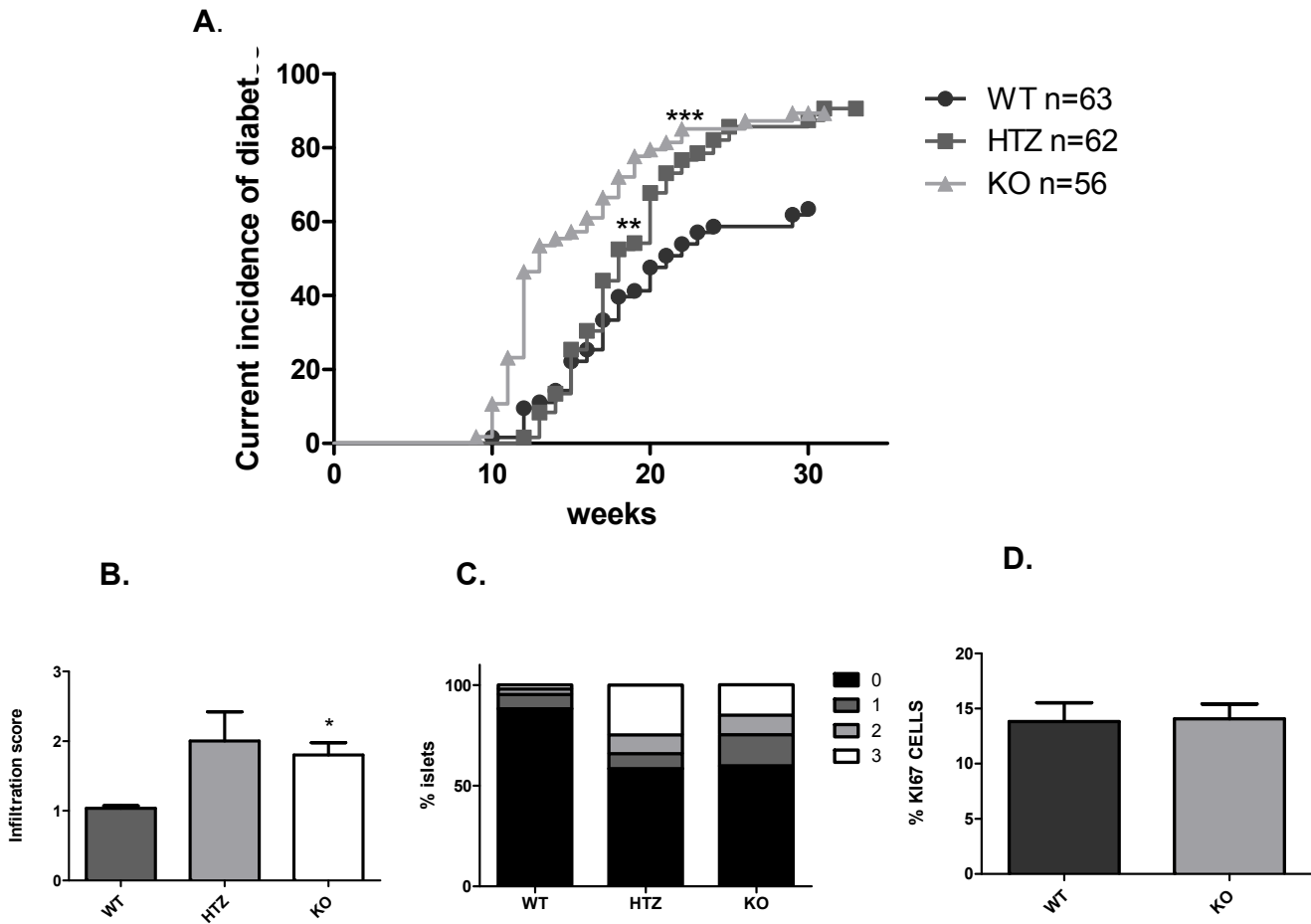
Figure 8. Downregulation of CcnD3 contributes to the development of T1D and insulitis without altering proliferation rates. A. Cumulative incidence of diabetes in NODCcnD3KO (n=63), NODCcnD3HTZ (n=62) and NODCcnD3WT (n=56) female littermates; **B**. Pancreas from 6-week-old female mice was embedded in paraffin and sections were stained for Hematoxilin-Eosin (n=4, for each experimental group: NODCcnD3KO, NODCcnD3HTZ and NODCcnD3WT), level of infiltration was counted, and **B**. scored taking 0 as non-insulitic; 1 as peri-insulitic; 2 intra-insular insulitis, less than 50% islet infiltration and 3, more than 50% of islet infiltration; **C**. the plot of the percentage of each kind of insulitis. **D**. proliferative activity of pancreatic β cells: islets were extracted from 6-week-old NODCcnD3WT and NODCcnD3KO female mice and trypsinized; the % of proliferative cells was plotted as CD45-Glut2+Ki67+. The statistical analysis performed in **A** was the Mantell–Cox and Logrank test and in **B** and **D**, the Wilcoxon-Cox and Mann Whitney U. * p≤0,05, ** p≤0,01, ***p≤0,001. All statistical comparisons performed took as a reference the WT group.

11.2.2 Exacerbated diabetes in NODCcnD3KO mice is not due to an alteration in the diabetogenicity of the immune repertoire

To prove that NOD female mice deficient in CcnD3 are more susceptible to develop diabetes solely because of the deficiency of CcnD3 in theβ cells, we did adoptive transfers of diabetogenic NOD spleen cells into 3- to 5-week-old female NOD/SCIDCcnD3KO, NOD/SCIDHTZ and NOD/SCIDWT mice. At 3-5 weeks of age, we transferred 10 million of total spleen cells from NOD female mice donors (>8 weeks) and monitored diabetes on a weekly basis for 16 weeks. In **Figure 9A,** the NOD/SCIDCcnD3KO-recipient mice show a higher susceptibility to develop diabetes than either the NOD/SCIDCcnD3 HTZ or the NOD/SCIDCcnD3WT. Thus, susceptibility to develop T1D seems to be inversely correlated to the amount of CcnD3 expressed in the β cells, the NODCcnD3HTZ exhibiting an intermediate phenotype between the NODCcnD3KO (more susceptibility) and the NODCcnD3WT.

To corroborate that the lymphocytes in all the experimental groups studied have the ability to generate the disease because they have an autoimmune repertoire, NODCcnD3KO, NODCcnD3HTZ and NODCcn3WT female mice (>8 weeks of age) were used as donors of 10 million of total spleen cells to be transferred into female recipient NOD/SCID mice (3-5 weeks of age). The incidence of diabetes was monitored weekly during 16 weeks. The results show that, independently of CcnD3 expression levels, the immune repertoire of all the experimental groups can generate diabetes (**Figure 9B**). Furthermore, spleen cells from the NODCcnD3WT female donors are more diabetogenic than those from either the NODCcnD3HTZ or NODCcnD3KO donors.

This observation supports the idea that CcnD3 expression in the β cells is responsible for exacerbated T1D in NODCcnD3KO and NODCcnD3HTZ female mice, rather than a more diabetogenic immune repertoire. Moreover, the insulitis

score is higher in the NODCcnD3KO mice, which implies that the islet niche in the NODCcnD3KO islets is influencing the insulitis score.

A.

B.
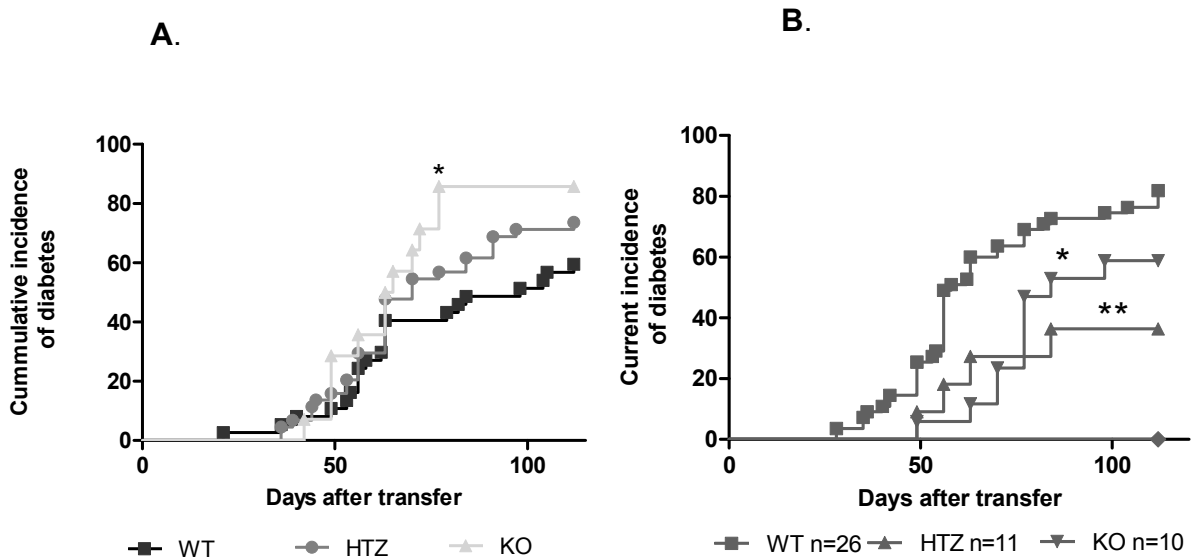
Figure 9. CcnD3 downregulation in solely β pancreatic cells generates susceptibility to develop T1D independently of repertoire changes. ***A.*** Cumulative diabetes incidence in female NOD/SCIDCcnD3KO (n=15), NOD/SCIDCcnD3HTZ (n=44) and NOD/SCIDCcnD3WT (n=37) mice of 3-5 weeks of age that were adoptively transferred with 10 million total spleen cells from female NODs older than 8 weeks of age. B. Female NOD/SCID mice of 3-5 weeks of age were adoptively transferred with 10 million total spleen cells from either NODCcnD3KO (n=10), NODCcnD3HTZ (n=11) or NODCcnD3WT (n=26) mice aged over 8 weeks. The statistics were performed in A and B using the Log-rank and Gehan-Breslow-Wilcoxon tests * p≤0,05, ** p≤0,01, ***p≤0,001. All comparisons are refered to the WT group.

11.2.3 Exacerbated diabetes in NODCcnD3KO mice requires the autoimmune attack

To assess whether the sole absence of CcnD3 is sufficient to promote β-cell death. NOD/SCIDCCnD3KO, NODCcnD3HTZ and NODCcnD3WT female mice were monitored weekly for the onset of spontaneous diabetes (**Figure 10**). Neither NOD/SCIDCcnD3KO, NOD/SCIDCcnD3HTZ nor NOD/SCIDCcnD3WT female mice developed spontaneous diabetes. Therefore, leucocyte action is required to induce β-cell death in NODCcnD3KO mice, showing that CcnD3 deficiency is not enough to promote β-cell death. This is confirmed by the phenotype of the CcnD3KO mice in the C57BL/6 genetic background for which diabetes did not develop spontaneously.
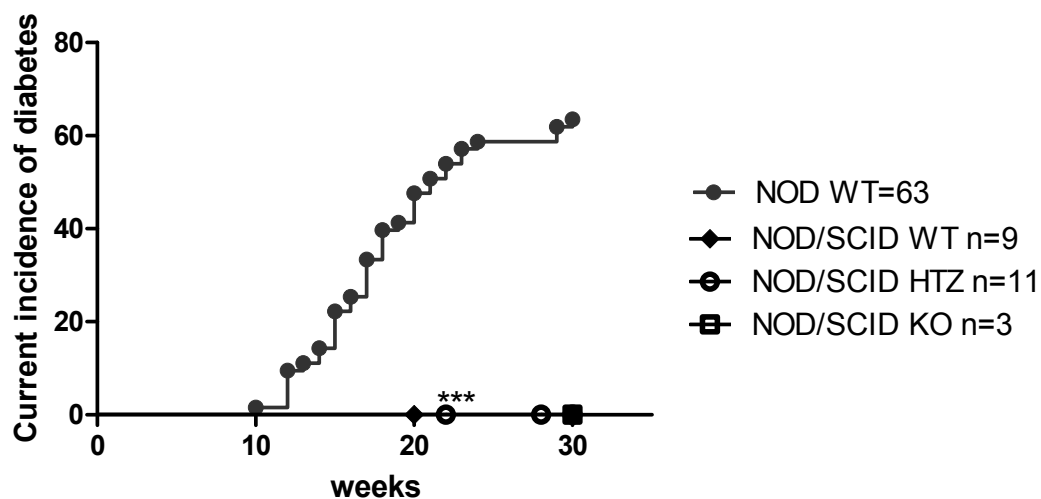
Figure 10. Exacerbated diabetes in CcnD3KO mice requires leucocyte action. Cumulative incidence of diabetes in NODCcnD3Wt (n=56), NOD/SCID CcnD3WT (n=9), NOD/SCIDHTZ (n=11) and NOD/SCIDCcnD3K0 (n=3). The statistics in A and B were performed with the Log-rank and Gehan-Breslow-Wilcoxon tests ***$p \leq 0,001$. All comparisons performed are refered to NODWT group.

11.2.4 CcnD3 deficiency in β-cell affects the response to blood glucose challenges in NOD mice

To assess β-cell fitness in NODCcnD3KO mice, we performed intraperitoneal glucose tolerance tests. We used 11-week-old NODCcnD3KO, NODCcnD3HTZ and NODCcnD3WT female mice littermates. After overnight fasting, we challenged them with 2g of glucose/kg of body weight intraperitoneally.

At first, NODCcnD3KO female mice seemed to have the same response to blood glucose (**Figure 11A**). NODCcnD3KO islets can even improve the response to glucose observed in NODCcnD3WT. However, at 11 weeks of age, 23% of the NODCcnD3KO female mice were already diabetic so the results were biased. Because of this, we have separated the results into two sets: prediabetic or normoglycemic mice, taking the glycemia values from the NODSCID mice, which are lymphocyte free and hence do not develop diabetes, as the control. The highest glycemic values in NODSCID mice when they were administered 2g of glucose/kg of body weight was 320mg/dL and it was reached after 30 min.

The glycemic response of the normoglycemic group is shown in **Figure 11B,** and that for the pre-diabetic in **Figure 11C,** where NODCCnD3KO and NODCcnD3WT were compared. As **Figure 11B** shows, the NODCcnD3KO mice and WT littermates responded similarly to glucose, and 60 minutes after the glucose stimulus, the NODCcnD3KO mice showed better glucose clearance than the WT mice and generated enough insulin to control the glycemia properly. On the other hand, severe islet infiltration in islets in pre-diabetic mice begin to provoke difficulties in the glycemic control 15 and 30 minutes after the glucose challenge , as it shown in **Figure 11C**.

In **Figure 11D**, glycemic values from pooled NODCcnD3HTZ (normoglycemic and prediabetic) are compared with NODCcnD3WT and at 30 min, as with pre-diabetic NODCcnD3KO mice, the response to glucose is deficient in NODCcnD3HTZ, showing that lower or absent CcnD3 expression levels causes a poorer glycemic control.
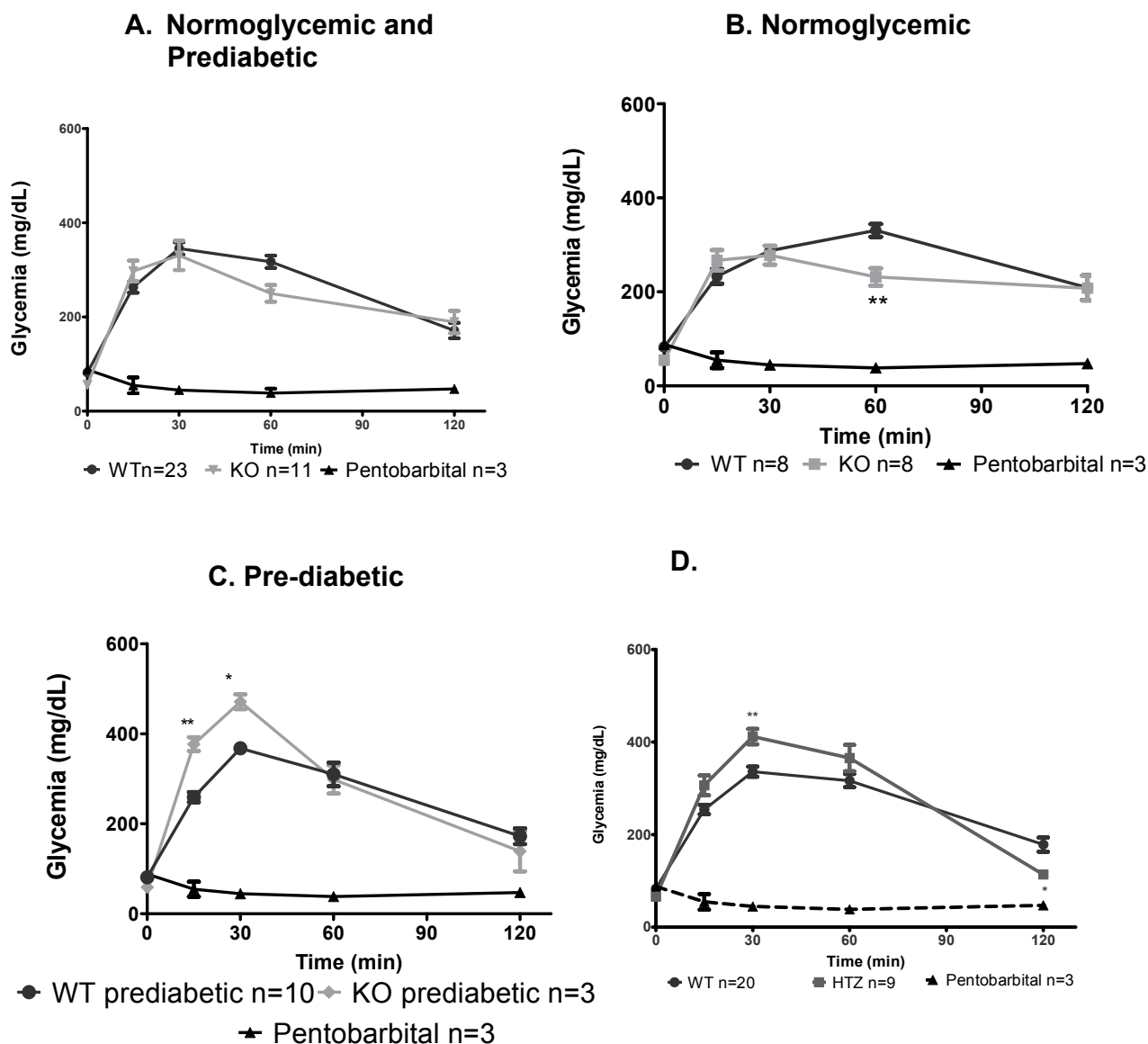
A. Normoglycemic and Prediabetic

B. Normoglycemic

C. Pre-diabetic

D.

Figure 11. CcnD3 deficiency causes an impaired β-cell response to blood glucose response in an intraperitoneal glucose challenge. 2g of intraperitoneal glucose per kg mouse body weight was administred intraperitoneally to 11-week-old NODCCnD3KO, NODCcnD3HTZ and NODCcnD3WT females and glycemia was measured. A. The results from NODCcnD3KO (n=11) and NODCcnD3WT (n=23) B. Results from the normoglycemic 11-week NODCcnD3KO (n=8) and NODccnD3WT (n=8) females; C. Pre-diabetic NODCcnD3KO and NODccnD3WT and D. Results from the total NODCcnD3HTZ and NODCcnD3WT mice. The statistical analysis to compare to the values obtained for the WT group was done with the two-way Anova and Bonferroni post tests. * p≤0,05, ** p≤0,01, ***p≤0,001.

11.3.1 Generation of NODRIPCcnD3 founder line 6896 and NOD/SCIDRIPCcnD3 6896 mice.

Transgenic NOD mice overexpressing CcnD3 in β cells were generated by microinjection of the RIP-CcnD3-Eα construct (**Figure 12A**) into fertilized NOD oocytes (Xenogen, now, Taconic; NY, USA). Six different founder lines were generated, one of which was lost during the SPF rederivation process. Expression of the RIP-CcnD3-Eα transgene was verified in the 5 different founder lines using 6-week-old NODCcnD3TG+ verified female mice and NODCcnD3TG- female littermates by flow cytometry (6876, 6877, 6880 and 6896) (**Figure 12B**). The only founder line to overexpress CcnD3 in the β cells was the founder line 6896. NOD/SCIDRIPCcnD3 6896 mice were also generated by intercrossing NODRIPCcnD3 with NOD/SCID mice until progeny homozygous for the SCID mutation and hemizigous for the transgene were obtained.

Immunofluorescence staining was also performed on the pancreatic reaction from 6-week-old NODCcnD3Tg+ or Tg- female mice (**Figure 12 C**) and we observed that, in both cases, CcnD3 is located in both the nucleus and cytoplasm of the β cells.

Regarding the macroscopic phenotype features of NODRIPCcnD36896 female mice, there are no significant differences between the NODCcnD3TG+ and NODCcnD3TG- littermates. NODRIPCcnD3TG+ and TG- have the same size and, at 11 weeks old, the females have the same body weight (**Figure 12D**). On the other hand, both groups have the same β cell area (**Figure 12E**).

A.

B.

C.

D.

E.
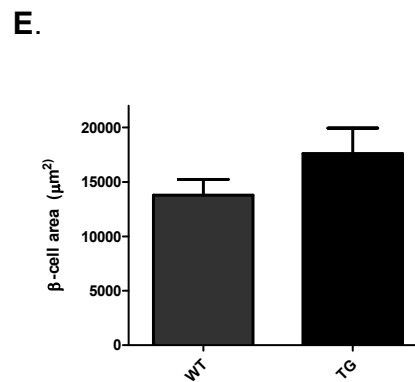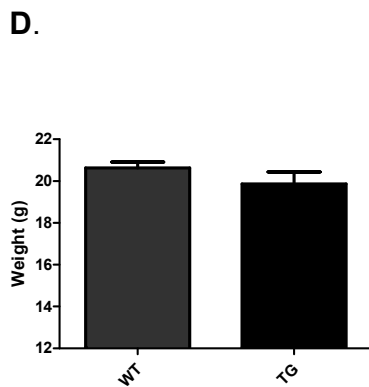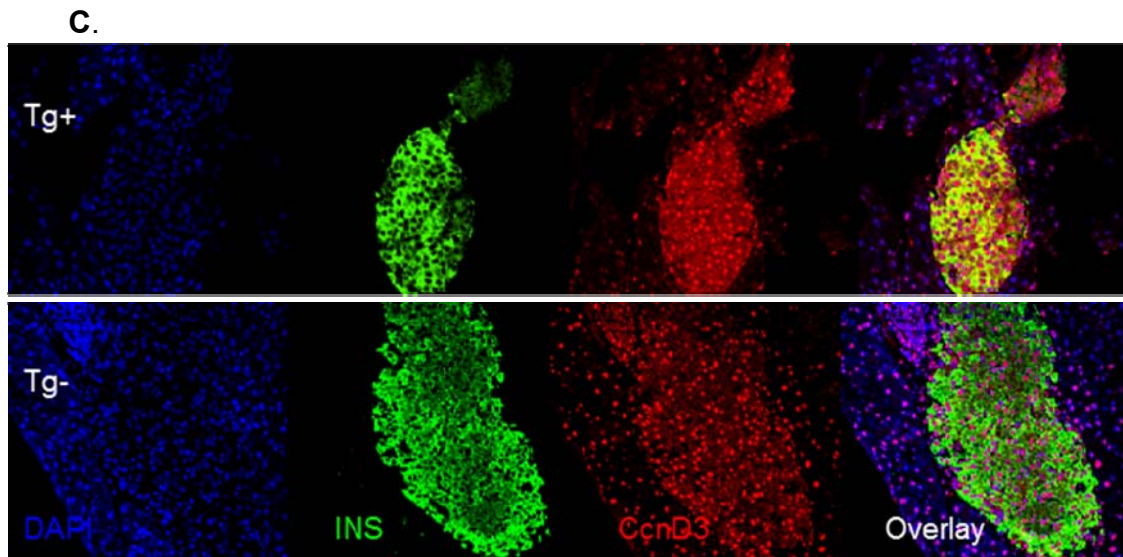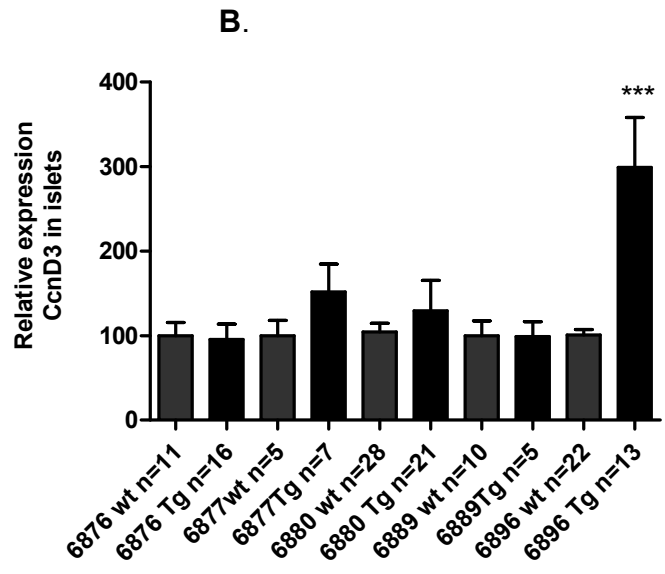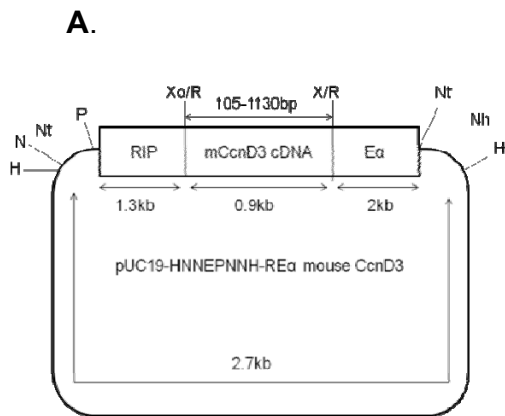
Figure 12. Generation of NOD mice overexpressing CcnD3 in β cells (NODRIPCcnD3). A.
Diagram showing β-cell specific CcnD3 construct NOD 6896 Tg strain construct: RIP-CcnD3-Eα, RIP,
Rat insulin promoter; Eα, 3´region of E alpha gene (I-E^d); CcnD3, mouse cDNA without endogenous
polyadenilation sequence; H, HindIII; N, NruI; Nh, NheI; Nt, NotI; P, PmeI; R, EcoRI; Xo, XhoI
restriction sites. The RIP-CcnD3-Eα homogene was escissed from the pUC19-backbone for
microinjection by Not I digestion **B**. Pancreatic islets from 6-week-old CcnD3 transgenic founder
female mice were extracted and diseggregated and trypsinized, fixed, permabilized and stained for
CcnD3. CcnD3 expression levels in the β cells were measured by Flow Cytometry selecting the
CD45$^-$Glut2$^+$ cell population as the β cells. The IMF (Intensity of Median Fluorescence) of the CcnD3
staining (6876Tg- n=11, 6876Tg+ n=16; 6877Tg- n=16, 6877Tg+ n=5; 6880Tg- n=28, 6880 n=21;
6889Tg- n=10, 6889Tg+ n=5; 6896Tg- n=22, 6896Tg+ n=13). **C**. Immunofluorescence staining on
pancreatic sections from either 6-week-old NODCcnD3TG+ and NODCcnD3TG- female littermates.
Insulin staining appears as green, CcnD3 as red and nucleus as blue. **D.** Body weight measurement
on 12-week-old female mice (NODCcnD3TG- n=23, NODCcnD3TG+ n=13) and **E**. Size of β cell area
was measured labelling insulin and photographs taken with an optical microscope. The area was
measured with ImageJ software (NODCcnD3TG- n=104, NODCcnD3TG+ n=94). Statistical analysis
was carried out on **B, D** and **E** with the Wilcoxon-Cox and Mann-Whitney U; n=9; ***p≤0,001. All
comparisons performed in **B** are refered to the WT group for each founder line; in **D** and **E** the
statistical comparisons refer to the WT group.

11.3.2 NODRIPCcnD3 6896 female mice are protected from T1D

As we have seen, loss of CcnD3 leads to an accelerated onset of T1D. To assess whether CcnD3 overexpression in the β cells delays T1D onset, we studied the spontaneous onset of diabetes in NODRIPCcnD3TG+ (founder line 6896) female mice compared to their transgenic negative female littermates, (**Figure 13A**). NODRIPCcnD3TG+ mice develop a delayed T1D, compared to transgenic negative littermates: the age at which 50% of mice are diabetic is delayed in mice expressing more CcnD3 in the β cells: for NODRIPCcnD3TG is 21 week old and NODRIPCcnD3TG+ is 25 weeks. These results, together with those obtained with NODCcnD3KO, show that CcnD3 is relevant for T1D kinetics, although the cumulative incidence of diabetes up to 30 weeks is not statistically different. The statistical analysis was carried out with the Log-rank and Gehan-Breslow-Wilcoxon tests.

Islet infiltration was examined in 6-week-old female NODRIPCcnD3TG-, NODRIPCcnD3TG+ mice (**Figure 13B**). No difference was detected in the insulitis score between the two groups.

We also assessed proliferation rates by flow cytometry in both groups; the % of proliferative cells was plotted as the % of CD45$^-$Glut2$^+$ki67$^+$ cells. There was not statistically difference between both experimental groups (**Figure 13C**). The Mantel-Cox and Man Withney U statistical analyses were performed.
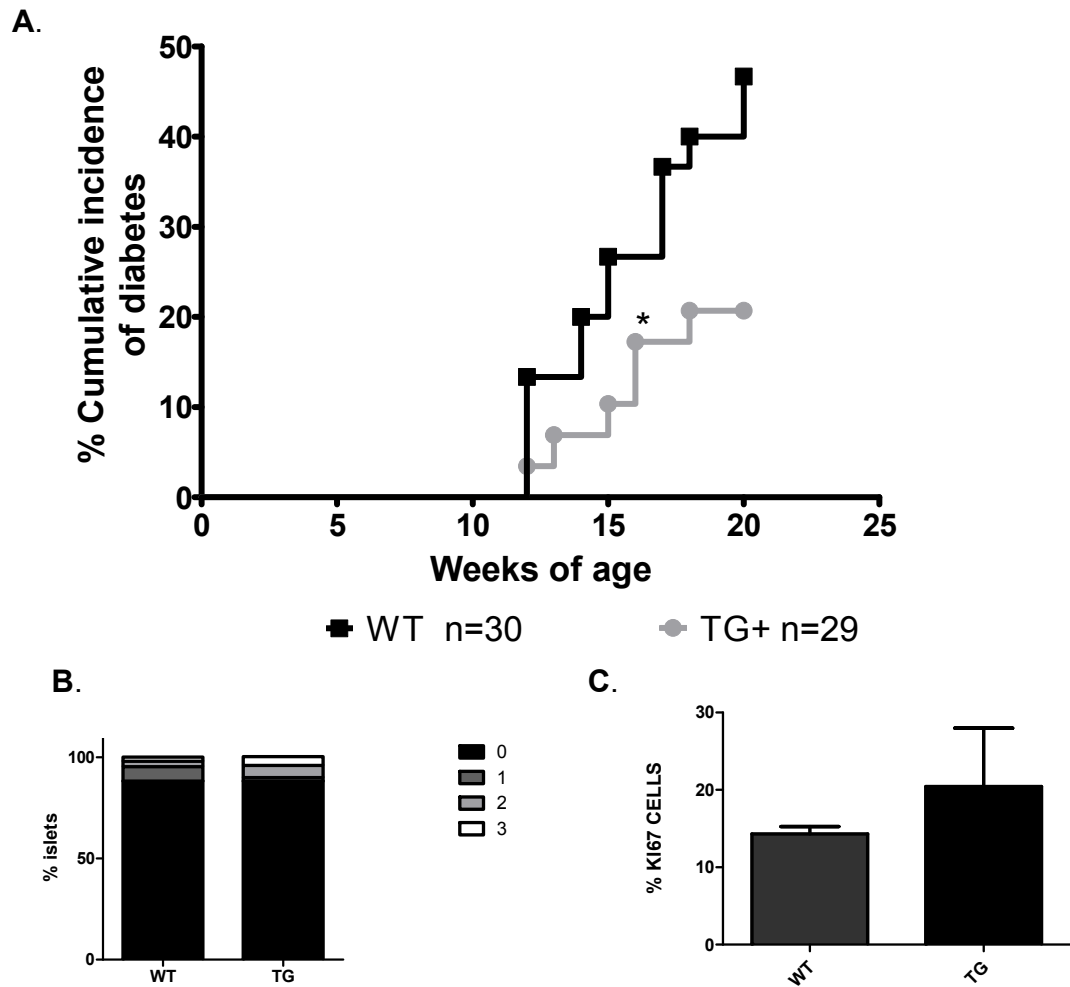
Figure 13. CcnD3 overexpression delays diabetes onset in NOD mice. A. Cumulative incidence of diabetes in NODRIPCcnD3TG+ (n=29) and NODRIPCcnD3TG- (n=30) female mice; **B**. The pancreas of 6-week-old NOD female mice was embedded in paraffin and sections were Hematoxilin-Eosin stained (n=4, each group of NODRIPCcnD3TG+ and NODRIPCcnD3TG-). The level of infiltration was quantified, taking 0 as preinsulitic; 1 as peri-insulitic; 2 intra-insular insulitis and 3 as complete islet destruction; the results are plotted as the % of each kind of insulitis. **C**. The pancreatic islets of 6-week-old NODRIPCcnD3TG- and NODRIPCcnD3TG+ were extracted, trypsinized and stained. The % of proliferative cells was plotted as $CD45^-Glut2^+Ki67^+$ cells. Statistical analysis performed **A**. Log-rank test and Gehan-Breslow-Wilcoxon test and, **B** and **C** Wilcoxon-Cox and Mann Whitney U. * $p \leq 0,05$, ** $p \leq 0,01$, ***$p \leq 0,001$. All statistical comparisons were performed taking the WT group values as reference.

11.3.2.1 NOD/SCIDRIPCcnD3 are not protected against adoptive transferred T1D.

NODCcnD3KO female mice are more susceptible to develop T1D, and NODRIPCcnD3TG+ mice are protected against it. To probe that this is due to expression changes in CcnD3 just in β cells, we performed adoptive transfer in NOD/SCIDRIPTG+, and NOD/SCIDRIPTG- females at 3-5 weeks of age with 10 million spleen cells, taking an NOD female mouse (>8 weeks) as a donor. The onset of diabetes was tested weekly for 16 weeks (**Figure 14A**). NOD/SCIDRIPTG+ mice had the same susceptibility to the disease as NOD/SCIDRIPTG-littermates, although a tendency to develop delayed disease could be observed (**Figure 14A**).

To test whether the immune repertoire from NODRIPCcnD3TG+ was altered, we used spleen cells from NODRIPCcnD3TG+ and NODRIPCcnD3TG- females (>8 weeks of age) to be transferred into NOD/SCID females (3-5 weeks of age). The incidence of diabetes was tested weekly for 16 consecutive weeks. The results show that the immune repertoire of all NOD mouse strains used, regardless of CcnD3 expression levels had the same ability to confer diabetes (**Figure 14B**).
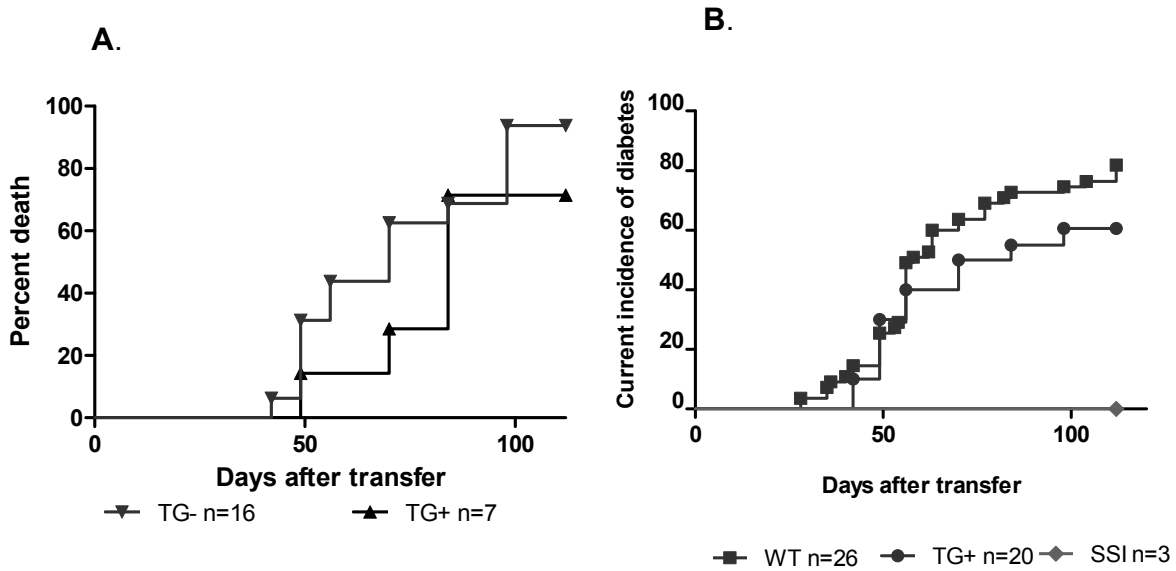
Figure 14. CcnD3 overexpression in β pancreatic cells protects from T1D. A. NOD/SCIDCcnD3TG (n=7) and NOD/SCIDTG- (n=16) female mice of 3-5 weeks of age were adoptive transferred with 10 million total spleen cells from female NOD aged over 8 weeks. **B**. Female NOD/SCID mice of 3-5 weeks were adoptive transferred with 10 million total spleen cells from different donors NODCcnD3TG and NODCcnD3TG- (n=20 and 26 respectively). The statistics were analysed in A and B with the Log-rank and Gehan-Breslow-Wilcoxon tests comparing to values obtained for the TG- group.

11.3.3 CcnD3 overexpression protects β cells from apoptosis

The comparison of the severity of the insulitic attack amongst NODCcnD3WT, NODCcnD3KO and NODRIPCcnD3TG⁺ female mice show that the level of expression of CcnD3 influences the severity of infiltration (**Figures 8B, 8C and 13B**). Therefore, we can presume that the higher incidence of diabetes observed in association to lower cyclin D3 expression levels, is a consequence of the autoimmune attack, (due to exacerbated insulitis and diabetes at low levels of cyclin D3 expression). Moreover NOD Antigen Presenting Cells (APCs) engulfing dead cells due to the insulitic assault in an inefficient fashion, will promote inflammation and attraction of phagocytes amplifying in this way the autoimmune response. To prove this, we performed TUNEL assay, counting the number of TUNEL-positive nuclei per insulin positive cells with each islet and, calculating the ratio of TUNEL-positive nuclei per total insulin positive area, having always a relationship between the number of apoptotic cells per β-cell area. As expected, the number of apoptotic cells per β-cell area (Apoptotic cells/mm^2) was higher in NODCcnD3KO than in NODCcnD3WT which, at the same time, was even higher than in NODRIPCcnD3TG⁺ female mice, (**Figure 15A**). The number of monocyte cells (CD11b⁺ cells/mm2) was the same in all three experimental groups(**Figure 15B**), but the number of activated macrophages (Mac-3⁺ cells/mm2) was higher in the islets of 6-week-old NODCcnD3KO female mice than in those from NODCcnD3WT and NODRIPCcnD3TG+ age, gender matched mice (**Figure 15C**).
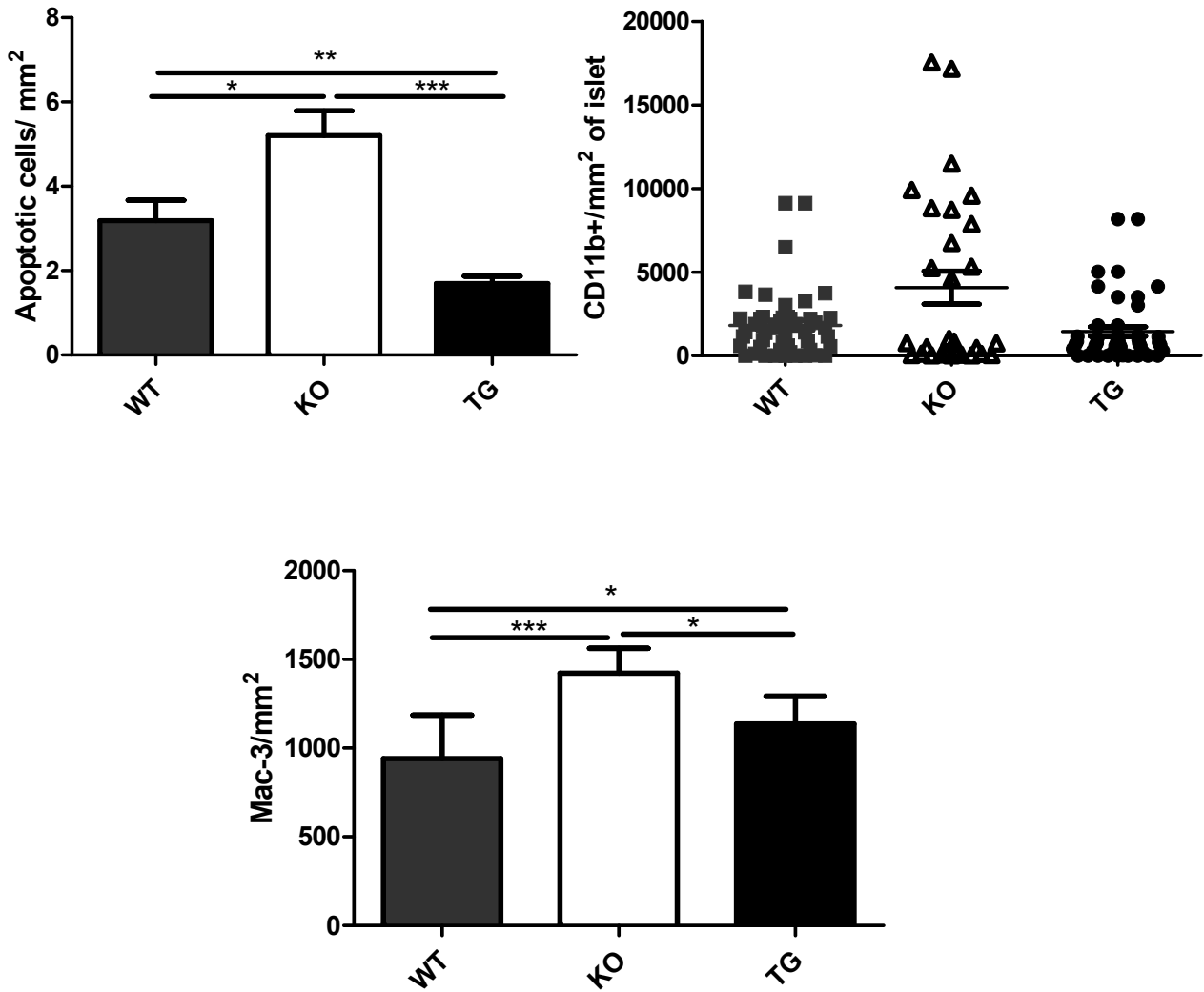
Figure 15. CcnD3 overexpression protects β cells from apoptosis. Immunofluorescence staining was carried out on pancreatic sections from 6-week-old NODCcnD3KO, NODRIPCcnD3TG+ and NODCcnD3WT females. **A**. The number of TUNEL-positive nuclei/insulin positive area was measured. **B**. The number of monocyte (CD11b+) per insulin area was measured and **C.**Number of activated macrophages (Mac-3) per insulin area were measured. A statistical analysis was carried out on **A**, **B** and **C** with the Wilcoxon-Cox and Mann Whitney U tests. * p≤0,01, ** p≤0,01, ***p≤0,001.

11.3.4 CcnD3 overexpression in NOD β-cell improves the response to blood glucose challenges

To determine if CcnD3 is relevant for the islet response to glucose challenges, we used 11-week-old NODRIPCcnD3TG+ and NODRIPCcnD3TG- females. After overnight fasting, we challenged them with 2g of glucose/kg of body weight intrapertoneally. NODCcnD3TG+ female mice as we seen have and improved glucose response at 15, 30 and 60 min after glucose challenge showing, as we have seen in the NODccnD3KO and HTZ female mice, that the expression of insulin confers not only β-cell protection, but also leads to an improved response to blood glucose challenges (**Figure 16).**
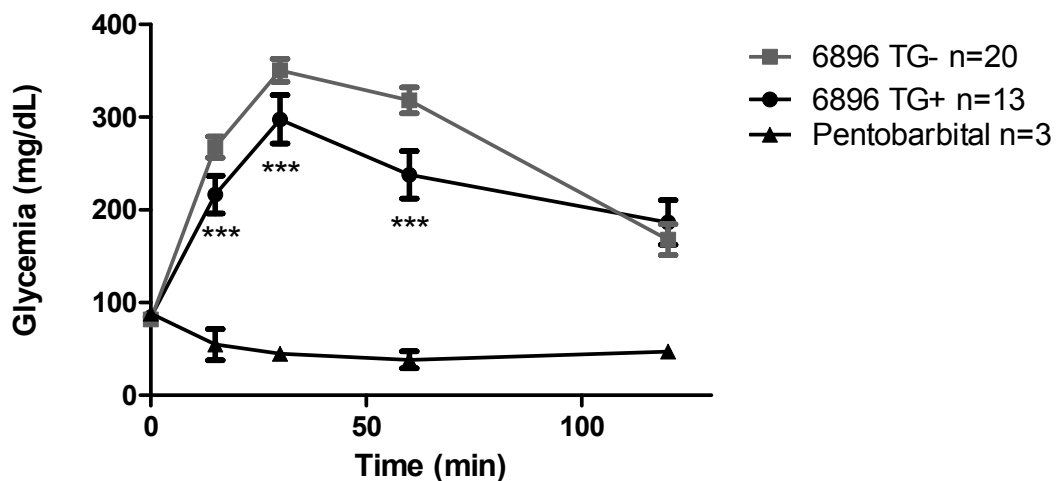
Figure 16. CcnD3 overexpression in β cells improves the response to blood glucose. 2g of glucose per kg of body weight was administered intraperitoneally to 11-week-old NODRIPCCnD3TG+ and NODRIPCcnD3TG- female mice; Glycemia was measured at 0, 15, 30, 60 and 120 min. NODCcnD3TG (n=13) and NODCcnD3WT (n=20) female mice. The statistical analysis was carried out with the two-way Anova and Bonferroni post tests. ***p≤0,001

11.4 Generation of NODCcnD3KO/ RIPCcnD36896 and NOD/SCIDCcnD3KO/ RIPCcnD3 6896 mice

To demonstrate the causal relationship between CcnD3 deficiency and β-cell death, in conjunction with the leucocyte assault we needed to prove that the sole restoration of CcnD3 expression in the β cells is enough to revert the exacerbated diabetic phenotype in NODCcnD3KO female mice. To this end intercrossed NODCcnD3KO mice and NODRIPCcnD36896TG+ mice to homozygozity for CcnD3 null mutation, while keeping the RIPCcnD3 transgene in hemizigozity.

We also obtained NOD/SCIDCcnD3KO/RIPCcnD36896TG+ by introducing the SCID mutation to homozigozity.

11.4.1 NOD/SCID CcnD3KO/ RIPCcnD3 6896 mice are protected from adoptively transferred diabetes

NODCcnD3KO/RIPCcnD3TG+ exhibit small body size like the NODCcnD3KO mice. The sole restoration of CcnD3 expression in the β-cells is not enough to restore the normal size (**Figure 17A)**.

The cumulative incidence of adoptive transfered (10 million spleen cells from 8-week-old NOD female mice donors) show that β cells from NODCcnD3KO/ RIPCcnD3TG+ recipient mice are protected from T1D (**Figure 17B**) compared with NODCcnD3KO/ RIPCcnD3TG- mice. These results confirm the causal relationship between CcnD3 and β cell survival.
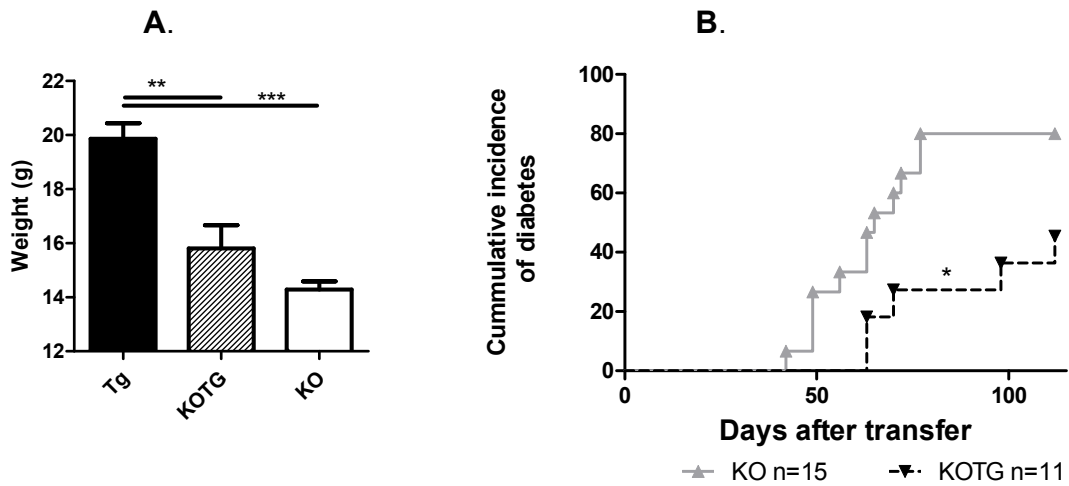
Figure 17. NOD mice deficient in CcnD3 overexpressing CcnD3 on β cells are protected from adoptive transferred diabetes. **A** Mean of body weight of 11-week-old NODRIPCcnD3TG (n=13), NODCcnD3KO/RIPCcnD3TG+ (n=5) and NODCcnD3KO/RIPCcnD3TG- (n=9) and NODCcnD3KO/RIPCcnD3TG- (n=15) littermate females. **B** Either 3-5 week old NOD/SCIDCcnD3KO/ RIPCcnD3TG+ (n=11) or NOD/SCIDCcnD3KO/RIPCcnD3TG- (n=15) were adoptively transferred with 10 million total spleen cells from NOD females aged over 8 weeks. The statistical analysis was done with the A Wilcoxon-Cox and Mann-Whitney U and B Log-rank and Gehan-Breslow-Wilcoxon tests. * p≤0,05, ** p≤0,01, ***p≤0,001

11.5 β pancreatic cell sensitivity to glucose challenges in relationship to CcnD3 expression levels

Pancreatic islets from 6-week-old NODRIPCcnD3TG+, NODCcnD3WT, NODCcnD3HTZ and NODCcnD3KO mice were extracted and loaded with 5 mM of Fura-2 AM, placed on a perfusion chamber, taking individual measurements of each islet mounted on the epifluorescence microscope in which was perfused with different glucose concentrations 3mM, 8mM, 16mM, and, at the end 25mM KCl in 3mM of glucose in a modified Ringer's solution. Each time interval measured was 10 min for each concentration of glucose (**Figure 18A**.) Similar results were obtained for NODRIPCcnD3TG+ and NODCcnD3WT islets on one hand; and for NODCcnD3HTZ and NODCcnD3KO islets on the other. **Figure 18A** shows a representative example of the Ca^{2+} response pattern from either NODCcnD3WT or NODCcnD3KO females, in which it can seen that at 8mM glucose NODCcnD3WT female islets respond at the wavelength ratio 340/380 which leads to an increase in the Area Under Curve (AUC) measurement, and thus oscillations, are marked at 8mM (**Figure 18B**) while in islets from NODCcnD3KO and NODCcnD3HTZ mice response is absent in most of the analyzed islets(**Figure 18C).** This impaired sentivity to glucose evhibited by islets from NODccnD3KO and NODCcnD3HTZ female mice is observed at 8mM glucose (**Figures 18B and 17C**). However, at 16mM, glucose only islets from NODCcnD3KO show impaired changes in the intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$, but not those from NODCcnD3HTZ female mice (**Figure 18D**). These changes in Ca^{2+} oscillations among the different strains are not due to impaired sensitivity of the K^+ channels and there is no difference between any of the strains (**Figure 18E**).

To see if these changes in $[Ca^{2+}]_i$ in CcnD3KO and CcnD3HTZ were due to changes in the expression levels of Glut-2, a glucose transporter expressed in the β pancreatic cells, in the β cells we measured Glut-2 expression. However, there is no difference in the expression of Glut-2 between the strains(**Figure 18F**).

We also checked the insulin islet content by ELISA using islets from the same mice that we had used to measure changes in intracellular Ca^{2+} levels in response to

glucose and we found no difference in the amount of insulin that the islets from different phenotypes had (**Figure17G**).

A.

A (ratio 340/380)

Time (sec)

3mM G
25mM K$^+$

16mM G

8mM G

3mM G

NODCcnD3WT

NODCcnD3KO

B.

8mM

AUC

TG n=18   WT n=20   HTZ n=30 **   KO n=18 *

C.

OSCILLATIONS 8mM

Oscillations/min

TG   WT   HTZ *   KO *

D.

16mM

AUC

TG   WT   HTZ   KO *

E.

K

AUC

TG   WT   HTZ   KO

F.

G.
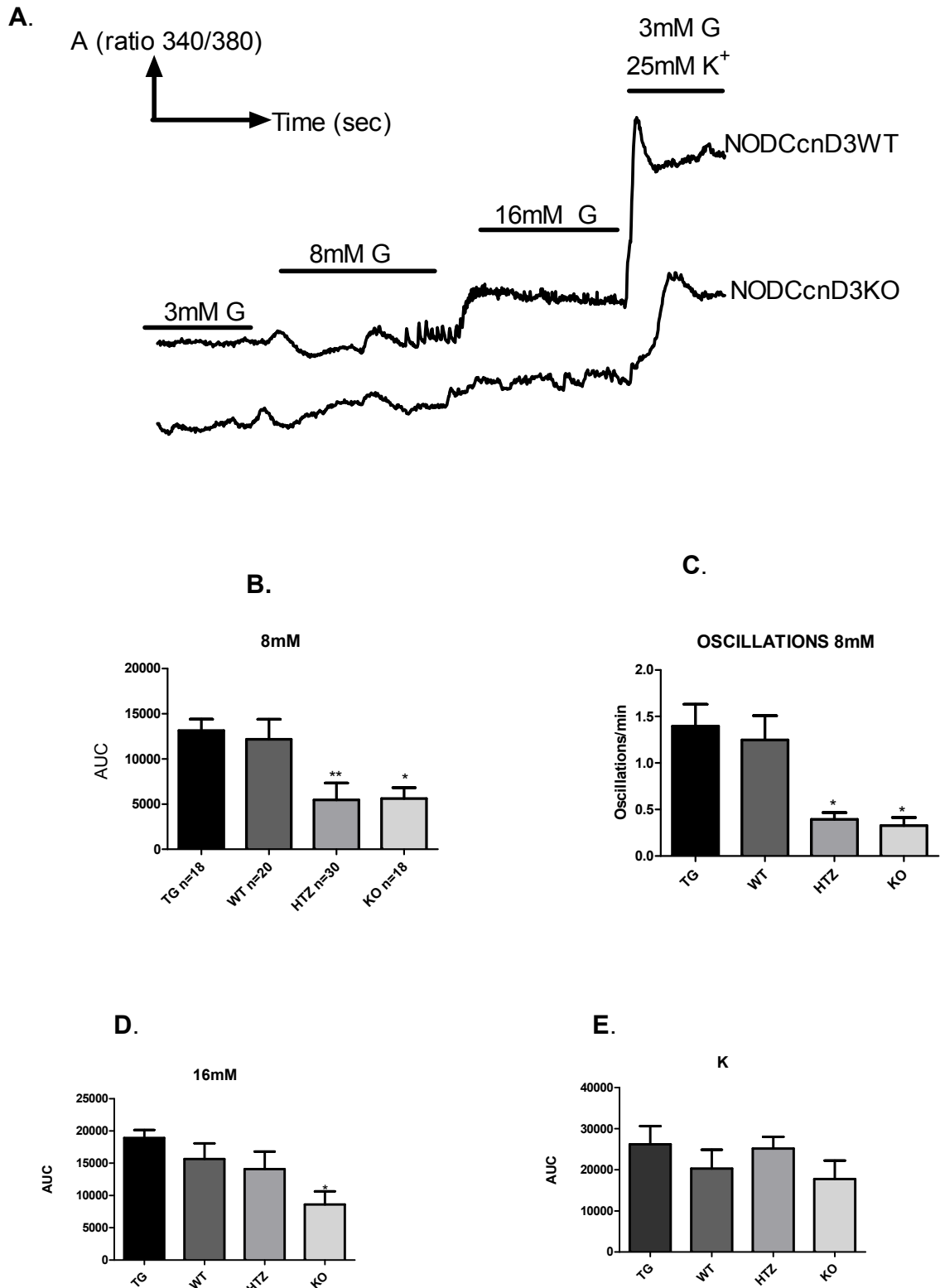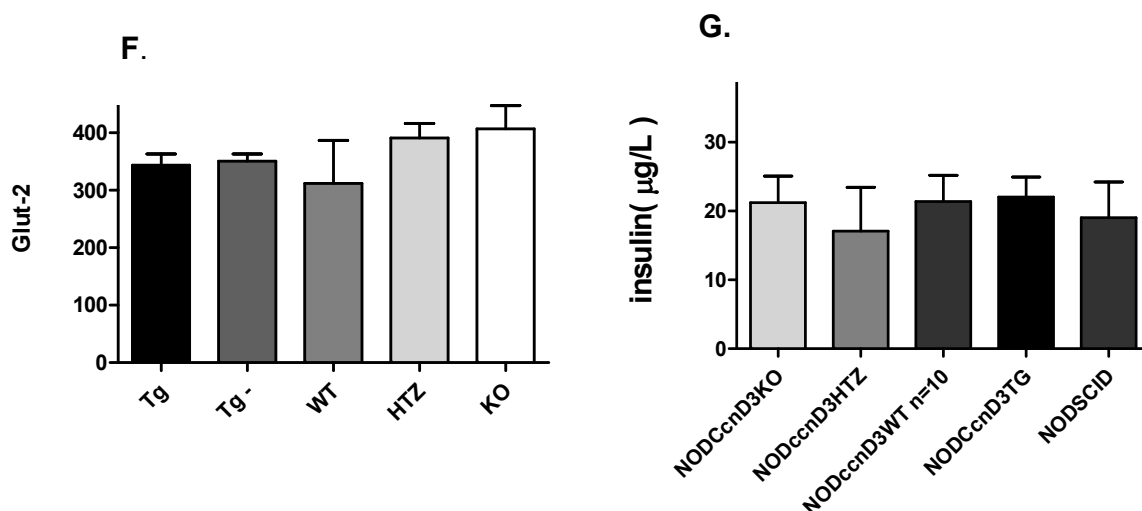
Figure 18. Downregulation of CcnD3 expression in β cells decreased sensitivity to islet response glucose challenges. Pancreatic islets from 6-week-old NODCcnD3WT(n=20), NODRIPCcnD3TG+ (n=18), NODCcnD3HTZ(n=30) and NODCcnD3KO(n=18) female mice were loaded with 5 mM Fura-2 AM. They were then placed in a perfusion chamber mounted on the epifluorescence microscope in which the islet was perfused with glucose concentrations of 3mM, 8mM, 16mM and 25mM KCl in 3mM of glucose in a modified Ringer solution. The time interval measured was 10 min for each concentration of glucose for each islet. **A** The application of 3mM, 8 mM, 16mM of glucose, and, 3mM of glucose with 25mM of K^+ elicited a intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$. **B**. Graph plots the Area Under Curve (AUC) for 5 min in response to 8mM of Glucose **C.** The frequency of $[Ca^{2+}]_i$ oscillations in pancreatic islets is shown **D.** Graph plotting AUC/5 min in response to 16mM **E.** Graph plotting AUC/5 min in response to 3mM of Glucose and 25mM of K^+ F. Assessment of the Glut-2 expression levels in pancreatic cells taking as a plot CD45⁻ cells by MFI **G**. ELISA Insulin content in a pool of 20 islets in a mL. The statistical analyses were carried out on B, C, D, E, F and G with the Wilcoxon-Cox and Mann-Whitney U tests. * p≤0,05, ** p≤0,01, ***p≤0,001. All statistical comparisons take WT group as control.

11.6 NIT-1 cells

11.6.1 NIT-1 cells overexpressing CcnD3 are protected from spontaneous and IL-1β-induced apoptosis

The NOD insulinoma cell line (NIT-1) was stably transfected by electroporation with either the pBSKNeo plasmid containing the rat insulin promoter driving the mouse CcnD3 cassette (RIP-CcnD3) transgene or the empty plasmid as the mock control insert (**Figure 19A**). Stable transfectants were selected in the DMEM media (Lonza, Verviers, Belgium) supplemented with 10% Fetal Calf Serum (Lonza, Verviers, Belgium), Gentamycin 1.21mM (0.058mg/mL) (BioSera, Boussens, France), β-mercaptoethanol (Invitrogen, Scotland), and G418 Sulphate 6 mM (0.4mg/mL) (PAA, Pasching, Austria). Cells from the flask were harvested with 1ml of trypsin/EDTA (Lonza, Verviers, Belgium). For cytokine-induced cell apoptosis, 100,000 cells per well were seeded in 12-well plates on day 0. On day 1, cytokines were added at a final concentration of 50U/mL for IL-1β (Prospect; Passaic County, NJ, USA) and 200U/mL for IFN-γ (Prospect; Passaic County, NJ, USA). On day 2, the cells were harvested mechanically and assayed for either annexin V staining or Ki67 proliferation. NIT-1 cells stably overexpressiong CcnD3 are protected against both spontaneous and IL-1β-induced apoptosis (**Figure 19B**), but not against IFN-γ-induced or IL-1β+IFN-γ induced apoptosis while no change in necrosis was observed (**Figure 19D**.) amongst the different genotypes studied.

The NIT-1 cells did not show either enhanced proliferative activity when overexpressing CcnD3 or impaired necrosis compared with mock-transfected cells (F**igure 19C**). Only IFN-γ alone, or in association with IL-1β, promotes NIT-1 cell replication regardless of CcnD3 overexpression.

For the experiments shown, the statistical analysis was done with the Wilcoxon-Cox and Mann-Whitney U tests and * shows differences with respective internal controls; _ * shows differences between same stimuli but different cell clones NIT-1 Neo and NIT-1 Neo/CcnD3 respectively; * p≤0,05, ** p≤0,01, ***p≤0,001.
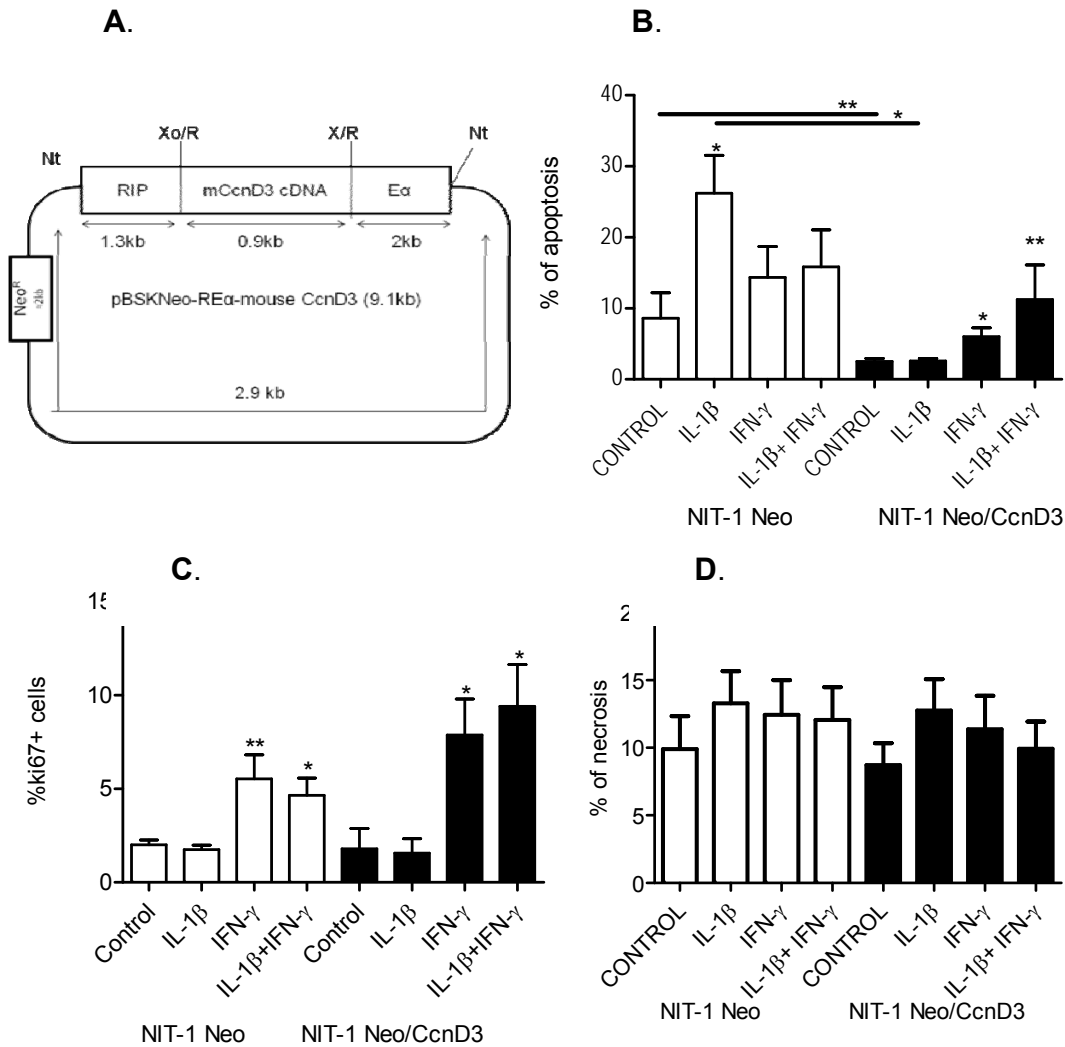
Figure 19. NIT-1 cells overexpressing CcnD3 are less succeptible to basal and IL-1β induced apoptosis. A. Map of the plasmid pBSKNeo containing the RIP-CcnD3 cassette used to stably transfect NIT-1 cells; H, HindIII; N, NruI; Nh, NheI; Nt, NotI; P, PmeI; R, EcoRI; Xo, XhoI restriction sites. **B**. The NIT-1 Neo cell line with either the empty CcnD3 construct or with the RIP-CcnD3 construct were cultured for 24h with DMEM in prescence or absence of IL-1β, IFN-γ or both; the % of early apoptosis was measured as AnnexinV⁺ PI⁻ cells in each experimental group **C.** Proliferation levels were measured as the % of Ki67⁺ cells in each group and **D**. Necrosis was plotted as the % of PI⁺ cells in each group. The statistical analysis was done with the Wilcoxon-Cox and Mann-Whitney U tests. * p≤0,05, ** p≤0,01, ***p≤0,001. Statistical comparisons are performed between treatments with each type or transfected cells, taking as reference the values obtained in the control group (no cytokines added); for comparisons between the same treatment in different types of cells, orizontal lines are used.

11.5.2 NIT-1 cells overexpressing CcnD3 are protected against NOD leucocyte-induced necrosis

Total spleen cells from non diabetic NOD females older than 8 weeks of age were co-cultured with NIT-1 cells for 24 hours in the absence of G418. Stable transfectants were selected in the DMEM media (Lonza, Verviers, Belgium) supplemented with 10% Fetal Calf Serum (Lonza, Verviers, Belgium), Gentamycin 1.21mM (0.058mg/mL) (BioSera, Boussens, France), β-mercaptoethanol (Invitrogen, Scotland), and G418 Sulphate 6 mM (0.4mg/mL) (PAA, Pasching, Austria). Cells from the flask were harvested with 1ml of trypsin/EDTA (Lonza, Verviers, Belgium). For leucocyte-induced cell apoptosis, 100,000 cells per well were seeded in 12-well plates on day 0. On day 1, the total spleen cells were added in different proportions (NIT-1: leucocyte ratio; 1:0,1:0.1; 1:0.5 and 1:1). On day 2, they were harvested mechanically (with cell scrappers) and assayed for either annexin V staining, or for proliferation Ki67 staining. The harvested cells were assayed for necrosis (**Figure20A**), apoptosis (**Figure 20B**) and proliferative activity (**Figure 20C**). The results show that at a low splenocyte ratio (1 NIT-:0.1 splenocyte), NIT-1 cells overexpressing CcnD3 are protected compared to NIT-1 Neo cells. However, no protection against spleen-cell induced apoptosis is observed in NIT-1 neo/CCnD3 compared with the NIT-1 Neo cells, suggesting that IFN-γ+ IL-1β (Figure 18A) is responsible for spleen cell induced NIT-1 cell apoptosis, and not IL-1β alone. CcnD3 overexpression does not alter the proliferative capacity of these cells. Four pooled experiments are shown. For the experiments shown, the statistical analysis was performed by using the Wilcoxon-Cox and Mann-Whitney U tests. * show differences with internal controls (i.e. no spleen cell); _ * shows differences between the same stimuli between different cell lines, NIT-1 Neo and NIT-1 Neo/CcnD3 respectively; * p≤0,05, ** p≤0,01, ***p≤0,001.
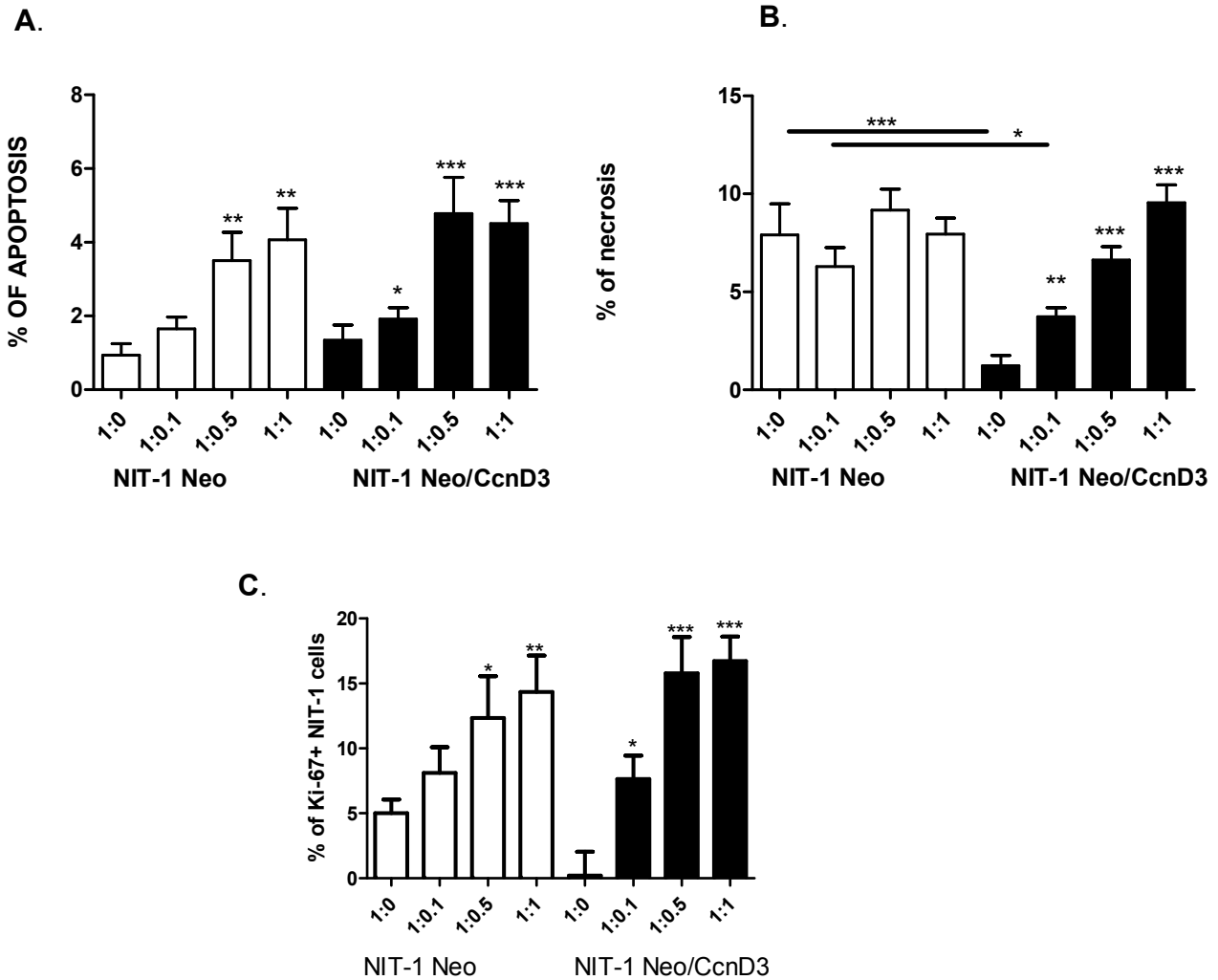
Figure 20. NIT-1 overexpressing CcnD3 cells are protected against necrosis. NIT-1 cells stably transfected with either the PBSKNeo plasmid or the PBSKNeo plasmid containing the RIP-CcnD3-Eα construct were cultured for 24h in DMEM with different ratios of total spleen cells from NOD female mice older than 8 weeks. The ratios shown state for the number of spleen cells per one NIT-1 cell (e.g. 1:0.1 means 0.1 spleen cells per 1 NIT-1 cell) **A.** % of apoptotic NIT-1 cells is plotted as % of CD45⁻ Glut2⁺ PI⁻ AnnexinV⁺ **B.** The % of Necrotic NIT-1 cells is plotted as % of CD45⁻ Glut2⁺ PI⁺ cells; and **C.** The % proliferation NIT-1 cells is plotted as CD45⁻ Glut-2⁺ Ki67⁺ cells (Ki67 is a cell proliferation marker). The statistical analysis used the Wilcoxon-Cox and Mann-Whitney U tests. * show differences with controls; _ * shows differences between same stimuli but different transfectants (NIT-1 Neo or NIT-1 Neo/CcnD3respectively); * p≤0,05, ** p≤0,01, ***p≤0,001.

12. Summary of results

To unveil the final targets in the beta cells responsible for the β cell loss caused by the lymphocytic attack that generates T1D, we performed microarray studies to assess differentially expressed genes in islet endocrine cells as a consequence of insulitic infiltration by comparing the Non-Obese Diabetic (NOD) autoimmune-prone mouse model with its congenic, lymphocyte-free, NOD/SCID strain. We discovered that CcnD3 underwent downregulation in the β cells upon inflammatory insult in a dose-dependent manner (**Figure 6**), while the proliferative activity of the beta cells downregulating CcnD3 was unchanged.

To demonstrate the causal link between CcnD3 repression and β cell death *in vivo*, we studied the spontaneous onset of diabetes in CcnD3-deficient NOD mice (NODCcnD3KO) (**Figures 7 and 8**). NODCcnD3KO mice developed exacerbated diabetes compared to wild-type (WT) littermates (88% versus 61% respectively), and this was solely due to the deficiency of CcnD3 in the β cells and not to a CcnD3 more diabetogenic lymphocytic repertoire in the NODCcnD3KO mice (**Figure 9**). However, diabetes exacerbation required the complicity of both CcnD3 deficiency and inflammation, since plain NOD/SCID CcnD3KO mice did not develop spontaneous diabetes (**Figure 10**).

CcnD3 overexpression in the β cells of transgenic mice delayed the onset of diabetes and protect against developing T1D (**Figures 13**) because the cells that overexpress CcnD3 are protected from apoptosis (**Figure 15**)**.**

Adoptively transferred T1D was not significantly impaided in NOD/SCID RIPCcnD3 TG+ recipient mice (**Figure 17**), although a certain tendency to be delayed could be observed **(Figure 14)**.

We have chosen 11-week-old female mice that were about to develop T1D because is near to the disease onset to be challenged with glucose.

The onset of T1D in NODCcnD3KO female mice happened at an earlier age (beginning at 9 weeks old) than the other strains under study (12 weeks). 23% of NODCcnD3KO mice already had diabetes showing that a normal comparison with the mice at this age is biased. Because of that, we decided to separate pre-diabetic mice of NODCcnD3KO and NODCcnD3WT and compare them. The blood glucose response of NODCcnD3KO mice was affected (**Figure 11C**). The same effect was shown by the NODCcnD3HTZ (**Figure 11D**) while the NODCcnD3TG+ strain had an improved glucose response (**Figure 16**).

CcnD3 also has an essential role in pancreatic β-cell fitness, since pancreatic islets deficient in CcnD3 do not experience proper changes in intracellular Ca^{2+} influx in response to a deficient sensitivity to glucose (**Figure 18)**. This impairment coupling glucose concentration in the extracellular milleau and an intracellular increase in Ca^{2+} concentration is not due to either sensitivity to K^+ or to changes Glut-2 expression levels between the CcnD3 KO and WT islets.

Finally, NIT-1 cells stably overexpressing CcnD3 are less susceptible to a basal and IL-1β induced apoptosis (**Figure 19**), and less susceptible to necrosis when the cells are in contact with lymphocytes (**Figure 20)**; no alteration in the proliferation rates were observed.

The mouse experimental groups generated for the work in this thesis were:

NODCcnD3KO

NODCcnD3HTZ

NODCcnD3WT

NOD/SCIDCcnD3KO

NOD/SCIDCcnD3HTZ

NOD/SCIDCcnD3WT

NODRIPCcnD3TG+

(6 different strains: 6876, 6877, 6880, 6889 and 6896; only 6896 in the TG+

littermate mice were used in the experiments of the thesis)

NODRIPCcnD3TG-

(6 different strains: 6876, 6877, 6880, 6889 and 6896; only 6896 TG- littermate

mice were used in the experiments of the thesis)

NOD/SCIDRIPCcnD3TG+

NOD/SCIDRIPCcnD3TG-

NODCcnD3KO/RIPCcnD3TG+

NODCcnD3KO/RIPCcnD3TG-

NOD/SCIDCcnD3KO/RIPCcnD3TG+

NOD/SCIDCcnD3KO/RIPCcnD3TG-

13. Discussion

Studies of the pathogenesis of T1D have mainly focused on the role of the immune system destruction of pancreatic β cells, but most lack data on the cellular and molecular events at the β-cell level. A change in cellular phenotype because of the change in the environment of the pancreatic islets can alter its recognition of potentially citotoxic agents and its defence mechanisms against cell death. β-cell are not necessarily passive victims of a cytotoxic process but can actively collaborate in the process of their own destruction. Basing on this idea, we performed microarray assays and we found CcnD3 underwent downregulation in β cells upon inflammatory insult and we proved that this happened in NOD mice in a dose-dependent manner without affecting the proliferative activity of the β cells.

Different strains of mice used in this study demonstrate that changes in CcnD3 levels of expression are related to changes in the severity of infiltration, susceptibility to death, and the response to glucose challenges. NOD mouse models show that if there is no expression of CcnD3, or it diminishes, there is a exacerbated spontaneous and adoptive transferred T1D, while the opposite happens when there is an overexpression of CcnD3. This, is not due to an alteration of the diabetogenicity of the immune repertoire and it that requires immune attack. We hypothesize that loss of CcnD3 enhances β cell apoptosis, leading to APC engulfing of autoantigens in a proinflamatory niche due to the reported inefficient scavenger activity of macrophages in the NOD strain, and promoting cellular autoimmune response. Also we have seen that there is the same number of monocytes inside the islets but the number of activated phagocytic macrophages is higher in islets from those mice lacking than in those that overexpress CcnD3, in agreement with our hypothesis.

β-cells are responsible for secreting insulin, a key hormone for regulating the glucose metabolism, and changes in expression of CcnD1, CcnD2 and cell-cycle proteins, lead to phenotype changes of pancreatic islets as hyperplasia. We were then interested in assessing whether CcnD3 is crucial for β-cell physiology, since, althought there are redundant roles for the 3 different D-type cyclins reported in

humans, CcnD3 yields the most robust rate of proliferation in pancreatic islets(237). In NODRIPCcnD3 6896 TG+ mice, there is no hyperplasia of pancreatic islets unlike the one exhibited by the CcnD1 transgenic mouse(207). However, loss of expression of CcnD3, causes a reduction in the size of the islets.When either CcnD3 or CDK4 expression levels one of the major kinase partners of D-type cyclins are reduced or lost, reduced body size is observed, however this is not due to endocrine defects.

There is a cross talk between the cell cycle and the metabolic control of the cell. The glucose metabolism is a key process the cell needs to control, and we have seen that if there is overexpression of CcnD3, there is an improvement in the response to blood glucose and also in the sensitivity of glucose by the β pancreatic islets. The opposite happens when there is a downregulation or loss of the expression of CcnD3. These changes are not due to a change in the activity of the K_{ATP} channels, and/or to downregulation in the expression of Glut-2. However, there is a report that shows that if an enzyme from glucolysis is overexpressed, the levels of expression of CcnD3 increase(238). There may be an alteration in the glycolisis or TCA activity levels that leads to a drop in the ATP generation and also diminish the CcnD3 expression in NOD WT infiltrated islets. Also CcnD3 deficiency diminishes the generation of the complex CcnD-CDK4/6 that limits the release of E2F1 that directly regulates the expression of Kir6.2, a key component of the K_{ATP} channel involved in the regulation of glucose-induced insulin secretion in pancreatic β cells(221). We have seen that the response to high concentrations of K^+ (25mM) and the K_{ATP} channels is similar in all experimental groups tested, regardless of CcnD3 expression levels. Maybe , the differences in between genotypes could be detectable on lower, more physiological, K^+ concentrations. However E2F1(-/-) mice are not diabetic. They have dramatically increased insulin sensitivity, secondary to decreased white adipose tissue. These effects are specific for E2F1, whereas the expansion of β-cells can be compensated by E2F2 (mainly released in the S phase of the cell cycle). E2F1/E2F2 double-mutant mice consistently display insulin deficient diabetes. It may be that redundancy of the cell-cycle proteins is one of the reasons why the results are not more dramatic.

Here we demonstrate that NIT-1 cells overexpressing CcnD3 are less susceptible to both, basal and IL-1β-induced apoptosis. It is noteworthy that cyclin D3 overexpression does not protect NIT-1 cells from residual apoptosis induced by IFN-γ although the apoptosis levels are not higher those achieved by mock-transfected NIT-1 cells in basal conditions. Anyhow, the extent to which IFN-γ is able to induce apoptosis in NIT-1 cells is very limited in comparison to that exhibited by IL-1β. These observations, in agreement with what has already been described, evidence that both cytokines signal by two different pathways to promote apoptosis, one of which, is severely negatively affected by cyclin D3, the one triggered by IL-1β, while the other, the one related to IFN-γ is not affected by changes in cyclin D3 expression levels. Although IFN-γ signals through the JAK2/STAT1 pathway, and IL-1β through the NFκB pathway, both pathways, however, can crosstalk through the MAPK kinase cascade(239, 240). Moreover, both, TNF-α and IFN-γ have been reported to potentiate IL-1β effects on β cells(240). Signaling through IL-1β receptor causes induction of Fas receptor, Caspase-1, iNOS, AP1 expression in beta cells amongst other genes(241) , and it has been reported to cause also β- cell dysfunction(242); while, signaling through IFN-γ receptor induces caspase genes, amongst many other genes(243), including iNOS, thus priming cells to be more sensitive to proapoptotic stimuli (244).

The mechanism accounting for CcnD3 protection against IL-1β induced apoptosis in NIT-1 cells is very probably due to some negative interference with either NFκB-mediated action, by acting on any/some of gene products encoded by its target genes, AP1-transcription factor, or with MAPK signaling. This would not be surprising, since CcnD3 has also been reported to bind certain transcription factors such as PPAR-γ, ATF-5(188, 245, 246) or nuclear receptors(187). CcnD3 potentiates the action of the ATF-5 (Activating Trancription Factor 5 alpha/beta) which is a transcription factor which binds to cAMP response element (CRE) existing in a number of promoters(245). It is interesting that ATF-5 transcriptional activity is enhanced by CcnD3, while CDK4 has the opposite effect on it (245).

There is a number of non-overlapping functions performed by CcnD1 and CcnD3 regardless of those being CDK-dependent or independent, which rely on transcription factor modulation: for example, CcnD3 enhances peroxisome proliferator-activated receptor-γ action through CDK-dependent mechanisms; while CcnD1 inhibits it in a CDK-independent mechanism(188, 247). Although not explored as thoroughly as CcnD1, CcnD3 seems to act preferentially on cAMP-regulated transcription while CcnD1 on nuclear-receptor regulated transcription(181, 182, 188, 245, 246).

CcnD3 has been described as a D-type cyclin associated with differentiation(248), for instance, it has been reported to participate in adipogenesis(188), muscle differentiation(249), and regulation of granulocyte differentiation(231, 250). Furthermore, direct interaction between CcnD3 and ERK3, an atypical mitogen activated kinase (MAPK) has been reported(251). ERK kinases are an important subgroup in the MAPK family, which are involved in IL-1β signaling in addition to NFκB. Since overexpression of ERK3 causes G1 arrest in several cell lines(252-254), and CcnD3 is involved is cell differentiation and interacts with ERK3, it has been suggested that the tandem ERK3/CcnD3 is restraining proliferation for the sake of differentiation.

Furthermore, CcnD3 associates with androgen AR in Prostate Cancer (PCa) cells and represses its ligand-dependent activation through CDK4-independent mechanisms, through a repressor motif that regulates ligand-induced intramolecular interactions. In addition CcnD3 can associate with HDAC3 and impede this way AR recruitment to endogenous target genes (187).

For all of the above mentioned potential points in which CcnD3 could intervene to protect β cells from apoptosis further work in this area need to be done to give insight into those CcnD3 partners involved in pancreatic β-cell fitness and viability. However this is beyond the scope of this work.

In the future, to find the signalling pathway and molecular partners that could explain the results that are shown in this thesis, is necessary. Some specific points, like the

function of the K_{ATP} channels, the levels of expression of Glut-2, and the amount of insulin, have been checked, but other remain to be explored.
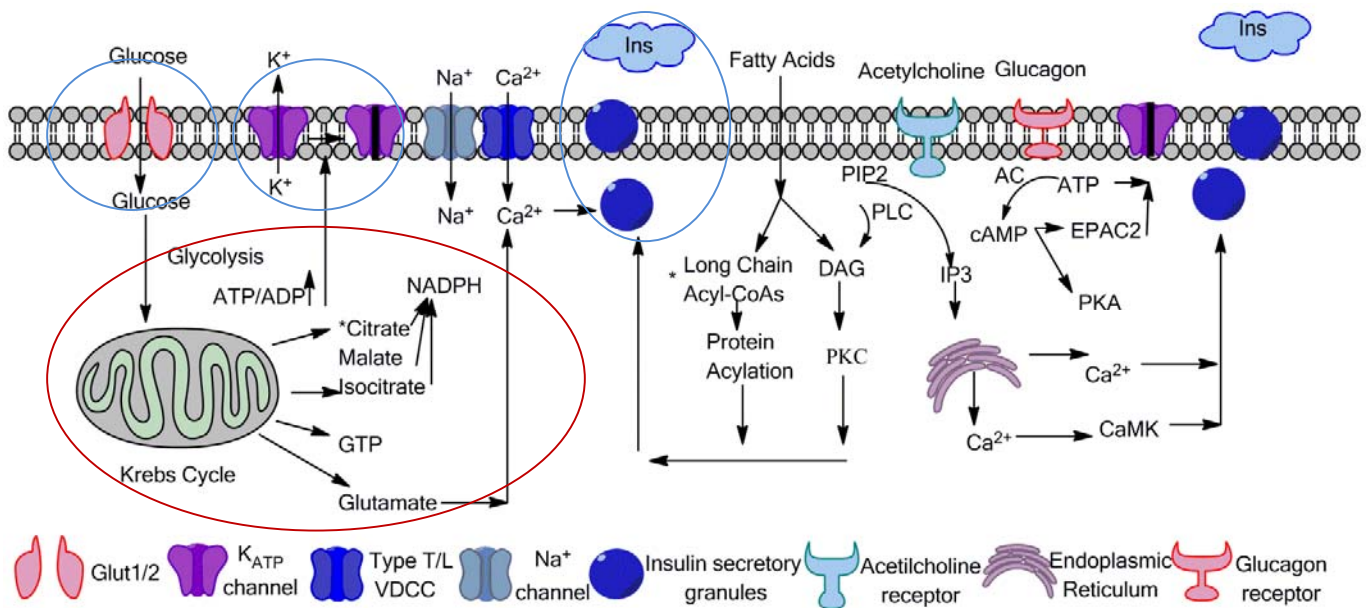
Figure 21. Search for the CcnD3 signalling pathway that contributes to an improvement response to blood glucose and sensitivity of glucose in β cells. Regulation of glucose-stimulated insulin secretion by nutrients, hormones and neurotransmitters can be influenced by the CcnD3 change of expression. The blue circles are clues about thesis explored information like Glut-2 expression, function of KATP channels and content of insulin in pancreatic isles in NOD mice and in a red circle literature results indicate as interesting targets that can be analyzed in the future by the group.

14. Conclusion

CcnD3 promotes pancreatic β-cell survival by protecting cells from inflammation, induced apoptosis in a cell cycle independent manner; and is required forβ-cell fitness in response to glucose challenges.

15. Supplementary information

15.1 Buffer preparation

<u>Citrate solution</u>

Sodium citrate tribasic dehydrate 99% $Na_3C_6H_5O_7$ 0.01M   2.60g/L

Adjust pH to 6

<u>TRIS solution</u>

TRIS $C_4H_{11}NO_3$ 12.1g/L

Adjust pH to 7.4

<u>Permeabilization solution</u>

TRIS solution   50mL

Triton X-100   500µL/50mL

<u>Blocking solution</u>

Permeablilization solution   50mL

BSA   1.5g/50mL

Serum blood of NOD   500µL

<u>Acid alcohol</u>

EtOH 80%/H_2O 250mL

HCl 37%/H_2O 3mL

<u>Tail lysis Buffer</u>

50mM Tris pH=8	6.055g
50mM KCl	3.72g
2.mM EDTA	0,93g
0.45% NP-40	4.5mL
0.45% Tween-20	4.5mL

Adjust the volume to 500mL an then solution is sterilized.

<u>Modified Ringer solution media</u>

120mM NaCl	7.01g
3mM KCl	0.0372g
25mM $NaHCO_3$	2,1g
21.1mM MgCl	0.233g
2.5mM $CaCl_2$	0.27g

Adjust to 1L

The pH of the solution is equilibrated gassing 95% O_2 and 5%CO_2 during the experiment. It is important to add $CaCl_2$ after the solution has some time been gassed for some time, otherwise $CaCl_2$ precipitates.

15.2 Forms

15.2.1 Glucose Tolerance Test

Pentobarbital concentration 60mg/kg

Original concentration of Dolethal (Vetoquinol, Spain) 0.2g Pentobarbital/mL

Dilution of Dolethal in physiological saline solution: 0.03mL Dolethal/mL

Glucose solution 5%

Weight (g)	Diluted Dolethal(mL)	Glucose solution (mL)
15	0.15	0.6
15.3	0.153	0.612
15.6	0.156	0.624
16	0.16	0.64
16.3	0.163	0.652
16.6	0.166	0.664
17	0.17	0.68
17.3	0.173	0.692
17.6	0.176	0.704
18	0.18	0.72
18.3	0.183	0.732
18.6	0.186	0.744
19	0.19	0.76

19.3	0.193	0.772
19.6	0.196	0.784
20	0.2	0.8
20.3	0.203	0.812
20.6	0.206	0.824
21	0.21	0.84
21.3	0.213	0.852
21.6	0.216	0.864
22	0.22	0.88
22.3	0.223	0.892
22.6	0.226	0.904
23	0.23	0.92
23.3	0.233	0.932
23.6	0.236	0.944
24	0.24	0.96
24.3	0.243	0.972
24.6	0.246	0.984
25	0.25	1
25.3	0.253	1.012
25.6	0.256	1.024
26	0.26	1.04

Mouse ID	Genotype	Weight (g)	Glycemia (mg/dL) t=0	15 min	30 min	60 min	120 min

15.2.2 Monitoring the spontaneous onset of diabetes

MONITORING DIABETES

OBSERVATION CAGE ID # _____ STRAIN: _____

RACK POSITION _____

LITTER ID# _____ DATE OF BIRTH (BD): _____

Mouse ID ↓ / Weeks of age →	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

15.2.3 Monitoring induced diabetes in Adoptive Transfers

MONITORING DIABETES

OBSERVATION CAGE ID #

TRANSFERRED WITH

LITTER ID#

STRAIN:

DATE OF BIRTH (BD):

Weeks of age Mouse ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

REFERENCES

15. References

1. Yazdanbakhsh, M., P.G. Kremsner and R. van Ree 2002. Allergy, parasites, and the hygiene hypothesis. Science. 296, 490-494.

2. Eisenbarth, G. S. 2007. Update in type 1 diabetes. J. Clin. Endocrinol. Metab. 92, 2403-2407.

3. Banting, F. G. and C.H. Best 2007. The internal secretion of the pancreas. 1922. Indian J. Med. Res. 125, 251-266.

4. Noble, J. A., A.M. Valdes, M. Cook, W. Klitz, G. Thomson and H.A. Erlich 1996. The role of HLA class II genes in insulin-dependent diabetes mellitus: Molecular analysis of 180 caucasian, multiplex families. Am. J. Hum. Genet. 59, 1134-1148.

5. Valdes, A. M., H.A. Erlich, J. Carlson, M. Varney, P.V. Moonsamy and J.A. Noble 2012. Use of class I and class II HLA loci for predicting age at onset of type 1 diabetes in multiple populations. Diabetologia. 55, 2394-2401.

6. Nokoff, N. and M. Rewers 2013. Pathogenesis of type 1 diabetes: Lessons from natural history studies of high-risk individuals. Ann. N. Y. Acad. Sci. 1281, 1-15.

7. Concannon, P., S.S. Rich and G.T. Nepom 2009. Genetics of type 1A diabetes. N. Engl. J. Med. 360, 1646-1654.

8. Steck, A. K., R. Wong, B. Wagner, K. Johnson, E. Liu, J. Romanos, C. Wijmenga, J.M. Norris, G.S. Eisenbarth and M.J. Rewers 2012. Effects of non-HLA gene polymorphisms on development of islet autoimmunity and type 1 diabetes in a population with high-risk HLA-DR,DQ genotypes. Diabetes. 61, 753-758.

9. Vang, T., M. Congia, M.D. Macis, L. Musumeci, V. Orru, P. Zavattari, K. Nika, L. Tautz, K. Tasken, F. Cucca, T. Mustelin and N. Bottini 2005. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. Nat. Genet. 37, 1317-1319.

10. Menard, L., D. Saadoun, I. Isnardi, Y.S. Ng, G. Meyers, C. Massad, C. Price, C. Abraham, R. Motaghedi, J.H. Buckner, P.K. Gregersen and E. Meffre 2011. The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. J. Clin. Invest. 121, 3635-3644.

11. O'Neill, S. K., E. Liu and J.C. Cambier 2009. Change you can B(cell)eive in: Recent progress confirms a critical role for B cells in type 1 diabetes. Curr. Opin. Endocrinol. Diabetes Obes. 16, 293-298.

12. Pescovitz, M. D., C.J. Greenbaum, H. Krause-Steinrauf, D.J. Becker, S.E. Gitelman, R. Goland, P.A. Gottlieb, J.B. Marks, P.F. McGee, A.M. Moran, P. Raskin, H. Rodriguez, D.A. Schatz, D. Wherrett, D.M. Wilson, J.M. Lachin, J.S. Skyler and Type 1 Diabetes TrialNet Anti-CD20 Study Group 2009. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N. Engl. J. Med. 361, 2143-2152.

13. Cox, S. L. and P.A. Silveira 2009. Emerging roles for B lymphocytes in type 1 diabetes. Expert Rev. Clin. Immunol. 5, 311-324.

14. Lowe, C. E., J.D. Cooper, T. Brusko, N.M. Walker, D.J. Smyth, R. Bailey, K. Bourget, V. Plagnol, S. Field, M. Atkinson, D.G. Clayton, L.S. Wicker and J.A. Todd 2007. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat. Genet. 39, 1074-1082.

15. Garg, G., J.R. Tyler, J.H. Yang, A.J. Cutler, K. Downes, M. Pekalski, G.L. Bell, S. Nutland, M. Peakman, J.A. Todd, L.S. Wicker and T.I. Tree 2012. Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T cell function. J. Immunol. 188, 4644-4653.

16. Malek, T. R. and I. Castro 2010. Interleukin-2 receptor signaling: At the interface between tolerance and immunity. Immunity. 33, 153-165.

17. Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V.E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S.L. Chen, R. Rosa, A.M. Cumiskey, D.V. Serreze, S. Gregory, J. Rogers, P.A. Lyons, B. Healy, L.J. Smink, J.A. Todd, L.B. Peterson, L.S. Wicker and P. Santamaria 2007. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. Nat. Genet. 39, 329-337.

18. Tang, Q., J.Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C.A. Piccirillo, B.L. Salomon and J.A. Bluestone 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. Immunity. 28, 687-697.

19. Grinberg-Bleyer, Y., A. Baeyens, S. You, R. Elhage, G. Fourcade, S. Gregoire, N. Cagnard, W. Carpentier, Q. Tang, J. Bluestone, L. Chatenoud, D. Klatzmann, B.L. Salomon and E. Piaggio 2010. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. J. Exp. Med. 207, 1871-1878.

20. Koreth, J., K. Matsuoka, H.T. Kim, S.M. McDonough, B. Bindra, E.P. Alyea 3rd, P. Armand, C. Cutler, V.T. Ho, N.S. Treister, D.C. Bienfang, S. Prasad, D. Tzachanis, R.M. Joyce, D.E. Avigan, J.H. Antin, J. Ritz and R.J. Soiffer 2011. Interleukin-2 and regulatory T cells in graft-versus-host disease. N. Engl. J. Med. 365, 2055-2066.

21. Saadoun, D., M. Rosenzwajg, F. Joly, A. Six, F. Carrat, V. Thibault, D. Sene, P. Cacoub and D. Klatzmann 2011. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. N. Engl. J. Med. 365, 2067-2077.

22. Long, S. A., M. Rieck, S. Sanda, J.B. Bollyky, P.L. Samuels, R. Goland, A. Ahmann, A. Rabinovitch, S. Aggarwal, D. Phippard, L.A. Turka, M.R. Ehlers, P.J. Bianchine, K.D. Boyle, S.A. Adah, J.A. Bluestone, J.H. Buckner, C.J. Greenbaum and Diabetes TrialNet and the Immune Tolerance Network 2012. Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments tregs yet transiently impairs beta-cell function. Diabetes. 61, 2340-2348.

23. Soltesz, G., C.C. Patterson, G. Dahlquist and EURODIAB Study Group 2007. Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology? Pediatr. Diabetes. 8 Suppl 6, 6-14.

24. Staples, J. A., A.L. Ponsonby, L.L. Lim and A.J. McMichael 2003. Ecologic analysis of some immune-related disorders, including type 1 diabetes, in australia: Latitude, regional ultraviolet radiation, and disease prevalence. Environ. Health Perspect. 111, 518-523.

25. Mohr, S. B., C.F. Garland, E.D. Gorham and F.C. Garland 2008. The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide. Diabetologia. 51, 1391-1398.

26. Norris, J. M., K. Barriga, G. Klingensmith, M. Hoffman, G.S. Eisenbarth, H.A. Erlich and M. Rewers 2003. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. JAMA. 290, 1713-1720.

27. TEDDY Study Group 2008. The environmental determinants of diabetes in the young (TEDDY) study. Ann. N. Y. Acad. Sci. 1150, 1-13.

28. Wilkin, T. J. 2001. The accelerator hypothesis: Weight gain as the missing link between type I and type II diabetes. Diabetologia. 44, 914-922.

29. Borchers, A. T., R. Uibo and M.E. Gershwin 2010. The geoepidemiology of type 1 diabetes. Autoimmun. Rev. 9, A355-65.

30. Wen, L., R.E. Ley, P.Y. Volchkov, P.B. Stranges, L. Avanesyan, A.C. Stonebraker, C. Hu, F.S. Wong, G.L. Szot, J.A. Bluestone, J.I. Gordon and A.V. Chervonsky 2008. Innate immunity and intestinal microbiota in the development of type 1 diabetes. Nature. 455, 1109-1113.

31. Saunders, K. A., T. Raine, A. Cooke and C.E. Lawrence 2007. Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. Infect. Immun. 75, 397-407.

32. Gale, E. A. 2002. The rise of childhood type 1 diabetes in the 20th century. Diabetes. 51, 3353-3361.

33. Zaccone, P., Z. Fehervari, F.M. Jones, S. Sidobre, M. Kronenberg, D.W. Dunne and A. Cooke 2003. Schistosoma mansoni antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. Eur. J. Immunol. 33, 1439-1449.

34. Cooke, A., P. Tonks, F.M. Jones, H. O'Shea, P. Hutchings, A.J. Fulford and D.W. Dunne 1999. Infection with schistosoma mansoni prevents insulin dependent diabetes mellitus in non-obese diabetic mice. Parasite Immunol. 21, 169-176.

35. Liu, Q., K. Sundar, P.K. Mishra, G. Mousavi, Z. Liu, A. Gaydo, F. Alem, D. Lagunoff, D. Bleich and W.C. Gause 2009. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. Infect. Immun. 77, 5347-5358.

36. Lehuen, A., J. Diana, P. Zaccone and A. Cooke 2010. Immune cell crosstalk in type 1 diabetes. Nat. Rev. Immunol. 10, 501-513.

37. Redondo, M. J., J. Jeffrey, P.R. Fain, G.S. Eisenbarth and T. Orban 2008. Concordance for islet autoimmunity among monozygotic twins. N. Engl. J. Med. 359, 2849-2850.

38. Hubner, M. P., Y. Shi, M.N. Torrero, E. Mueller, D. Larson, K. Soloviova, F. Gondorf, A. Hoerauf, K.E. Killoran, J.T. Stocker, S.J. Davies, K.V. Tarbell and E. Mitre 2012. Helminth protection against autoimmune diabetes in nonobese diabetic mice is independent of a type 2 immune shift and requires TGF-beta. J. Immunol. 188, 559-568.

39. Mishra, P. K., N. Patel, W. Wu, D. Bleich and W.C. Gause 2013. Prevention of type 1 diabetes through infection with an intestinal nematode parasite requires IL-10 in the absence of a Th2-type response. Mucosal Immunol. 6, 297-308.

40. O'Brien, B. A., Y. Huang, X. Geng, J.P. Dutz and D.T. Finegood 2002. Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. Diabetes. 51, 2481-2488.

41. Trudeau, J. D., J.P. Dutz, E. Arany, D.J. Hill, W.E. Fieldus and D.T. Finegood 2000. Neonatal beta-cell apoptosis: A trigger for autoimmune diabetes? Diabetes. 49, 1-7.

42. Turley, S., L. Poirot, M. Hattori, C. Benoist and D. Mathis 2003. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J. Exp. Med. 198, 1527-1537.

43. Lennon, G. P., M. Bettini, A.R. Burton, E. Vincent, P.Y. Arnold, P. Santamaria and D.A. Vignali 2009. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity. 31, 643-653.

44. Anderson, M. S. and J.A. Bluestone 2005. The NOD mouse: A model of immune dysregulation. Annu. Rev. Immunol. 23, 447-485.

45. Lieberman, S. M. and T.P. DiLorenzo 2003. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens. 62, 359-377.

46. Katz, J., C. Benoist and D. Mathis 1993. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur. J. Immunol. 23, 3358-3360.

47. Sumida, T., M. Furukawa, A. Sakamoto, T. Namekawa, T. Maeda, M. Zijlstra, I. Iwamoto, T. Koike, S. Yoshida and H. Tomioka 1994. Prevention of insulitis and diabetes in beta 2-microglobulin-deficient non-obese diabetic mice. Int. Immunol. 6, 1445-1449.

48. Serreze, D. V., E.H. Leiter, G.J. Christianson, D. Greiner and D.C. Roopenian 1994. Major histocompatibility complex class I-deficient NOD-B2mnull mice are diabetes and insulitis resistant. Diabetes. 43, 505-509.

49. Skowera, A., R.J. Ellis, R. Varela-Calvino, S. Arif, G.C. Huang, C. Van-Krinks, A. Zaremba, C. Rackham, J.S. Allen, T.I. Tree, M. Zhao, C.M. Dayan, A.K. Sewell, W.W. Unger, J.W. Drijfhout, F. Ossendorp, B.O. Roep and M. Peakman 2008. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J. Clin. Invest. 118, 3390-3402.

50. Willcox, A., S.J. Richardson, A.J. Bone, A.K. Foulis and N.G. Morgan 2009. Analysis of islet inflammation in human type 1 diabetes. Clin. Exp. Immunol. 155, 173-181.

51. Podack, E. R., D.M. Lowrey, M. Lichtenheld and A. Hameed 1988. Function of granule perforin and esterases in T cell-mediated reactions. components required for delivery of molecules to target cells. Ann. N. Y. Acad. Sci. 532, 292-302.

52. Savinov, A. Y., A. Tcherepanov, E.A. Green, R.A. Flavell and A.V. Chervonsky 2003. Contribution of fas to diabetes development. Proc. Natl. Acad. Sci. U. S. A. 100, 628-632.

53. Hamilton-Williams, E. E., S.E. Palmer, B. Charlton and R.M. Slattery 2003. Beta cell MHC class I is a late requirement for diabetes. Proc. Natl. Acad. Sci. U. S. A. 100, 6688-6693.

54. Wang, B., A. Gonzalez, C. Benoist and D. Mathis 1996. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. Eur. J. Immunol. 26, 1762-1769.

55. Santamaria, P. 2003. Effector lymphocytes in islet cell autoimmunity. Rev. Endocr Metab. Disord. 4, 271-280.

56. Shizuru, J. A., C. Taylor-Edwards, B.A. Banks, A.K. Gregory and C.G. Fathman 1988. Immunotherapy of the nonobese diabetic mouse: Treatment with an antibody to T-helper lymphocytes. Science. 240, 659-662.

57. Mora, C., F.S. Wong, C.H. Chang and R.A. Flavell 1999. Pancreatic infiltration but not diabetes occurs in the relative absence of MHC class II-restricted CD4 T cells: Studies using NOD/CIITA-deficient mice. J. Immunol. 162, 4576-4588.

58. Christianson, S. W., L.D. Shultz and E.H. Leiter 1993. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-thy-1a donors. Diabetes. 42, 44-55.

59. Thivolet, C., A. Bendelac, P. Bedossa, J.F. Bach and C. Carnaud 1991. CD8+ T cell homing to the pancreas in the nonobese diabetic mouse is CD4+ T cell-dependent. J. Immunol. 146, 85-88.

60. Yagi, H., M. Matsumoto, K. Kunimoto, J. Kawaguchi, S. Makino and M. Harada 1992. Analysis of the roles of CD4+ and CD8+ T cells in autoimmune diabetes of NOD mice using transfer to NOD athymic nude mice. Eur. J. Immunol. 22, 2387-2393.

61. Wen, L., E.A. Green, T. Stratmann, A. Panosa, R. Gomis, E.E. Eynon, R.A. Flavell, J.A. Mezquita and C. Mora 2011. In vivo diabetogenic action of CD4+ T lymphocytes requires fas expression and is independent of IL-1 and IL-18. Eur. J. Immunol. 41, 1344-1351.

62. Thomas, H. E., M.D. McKenzie, E. Angstetra, P.D. Campbell and T.W. Kay 2009. Beta cell apoptosis in diabetes. Apoptosis. 14, 1389-1404.

63. Campbell, I. L., T.W. Kay, L. Oxbrow and L.C. Harrison 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. J. Clin. Invest. 87, 739-742.

64. Nicoletti, F., P. Zaccone, R. Di Marco, M. Di Mauro, G. Magro, S. Grasso, L. Mughini, P. Meroni and G. Garotta 1996. The effects of a nonimmunogenic form of murine soluble interferon-gamma receptor on the development of autoimmune diabetes in the NOD mouse. Endocrinology. 137, 5567-5575.

65. Sarvetnick, N., J. Shizuru, D. Liggitt, L. Martin, B. McIntyre, A. Gregory, T. Parslow and T. Stewart 1990. Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. Nature. 346, 844-847.

66. Campbell, I. L., A. Cutri, D. Wilkinson, A.W. Boyd and L.C. Harrison 1989. Intercellular adhesion molecule 1 is induced on isolated endocrine islet cells by cytokines but not by reovirus infection. Proc. Natl. Acad. Sci. U. S. A. 86, 4282-4286.

67. Yagi, N., K. Yokono, K. Amano, M. Nagata, K. Tsukamoto, Y. Hasegawa, R. Yoneda, N. Okamoto, H. Moriyama and M. Miki 1995. Expression of intercellular adhesion molecule 1 on pancreatic beta-cells accelerates beta-cell destruction by cytotoxic T-cells in murine autoimmune diabetes. Diabetes. 44, 744-752.

68. Zhang, F., W. Yu, J.L. Hargrove, P. Greenspan, R.G. Dean, E.W. Taylor and D.K. Hartle 2002. Inhibition of TNF-alpha induced ICAM-1, VCAM-1 and E-selectin expression by selenium. Atherosclerosis. 161, 381-386.

69. Campbell, I. L., L. Oxbrow, J. West and L.C. Harrison 1988. Regulation of MHC protein expression in pancreatic beta-cells by interferon-gamma and tumor necrosis factor-alpha. Mol. Endocrinol. 2, 101-107.

70. David-Watine, B., A. Israel and P. Kourilsky 1990. The regulation and expression of MHC class I genes. Immunol. Today. 11, 286-292.

71. Stephens, L. A., H.E. Thomas, L. Ming, M. Grell, R. Darwiche, L. Volodin and T.W. Kay 1999. Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. Endocrinology. 140, 3219-3227.

72. Varanasi, V., L. Avanesyan, D.M. Schumann and A.V. Chervonsky 2012. Cytotoxic mechanisms employed by mouse T cells to destroy pancreatic beta-cells. Diabetes. 61, 2862-2870.

73. Padgett, L. E., K.A. Broniowska, P.A. Hansen, J.A. Corbett and H.M. Tse 2013. The role of reactive oxygen species and proinflammatory cytokines in type 1 diabetes pathogenesis. Ann. N. Y. Acad. Sci. 1281, 16-35.

74. Lam, G. Y., J. Huang and J.H. Brumell 2010. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. Semin. Immunopathol. 32, 415-430.

75. Haskins, K., B. Bradley, K. Powers, V. Fadok, S. Flores, X. Ling, S. Pugazhenthi, J. Reusch and J. Kench 2003. Oxidative stress in type 1 diabetes. Ann. N. Y. Acad. Sci. 1005, 43-54.

76. Bonifacio, E., S. Genovese, S. Braghi, E. Bazzigaluppi, V. Lampasona, P.J. Bingley, L. Rogge, M.R. Pastore, E. Bognetti and G.F. Bottazzo 1995. Islet autoantibody markers in IDDM: Risk assessment strategies yielding high sensitivity. Diabetologia. 38, 816-822.

77. Khan, M. W., S. Sherwani, W.A. Khan and R. Ali 2009. Characterization of hydroxyl radical modified GAD65: A potential autoantigen in type 1 diabetes. Autoimmunity. 42, 150-158.

78. Khan, M. W., K. Banga, S.N. Mashal and W.A. Khan 2011. Detection of autoantibodies against reactive oxygen species modified glutamic acid decarboxylase-65 in type 1 diabetes associated complications. BMC Immunol. 12, 19-2172-12-19.

79. Borjesson, A., A.K. Andersson and S. Sandler 2006. Survival of an islet allograft deficient in iNOS after implantation into diabetic NOD mice. Cell Transplant. 15, 769-775.

80. Pipeleers, D., A. Hoorens, M. Marichal-Pipeleers, M. Van de Casteele, L. Bouwens and Z. Ling 2001. Role of pancreatic beta-cells in the process of beta-cell death. Diabetes. 50 Suppl 1, S52-7.

81. Stagner, J. I., E. Samols and G.C. Weir 1980. Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration. J. Clin. Invest. 65, 939-942.

82. Bergsten, P., E. Grapengiesser, E. Gylfe, A. Tengholm and B. Hellman 1994. Synchronous oscillations of cytoplasmic $Ca2+$ and insulin release in glucose-stimulated pancreatic islets. J. Biol. Chem. 269, 8749-8753.

83. Kar, S. and D. Shankar Ray 2005. Sustained simultaneous glycolytic and insulin oscillations in beta-cells. J. Theor. Biol. 237, 58-66.

84. Grapengiesser, E., A. Salehi, S.S. Qader and B. Hellman 2006. Glucose induces glucagon release pulses antisynchronous with insulin and sensitive to purinoceptor inhibition. Endocrinology. 147, 3472-3477.

85. Curry, D. L., L.L. Bennett and G.M. Grodsky 1968. Dynamics of insulin secretion by the perfused rat pancreas. Endocrinology. 83, 572-584.

86. Henquin, J. C. 2000. Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes. 49, 1751-1760.

87. Ahren, B. 2000. Autonomic regulation of islet hormone secretion--implications for health and disease. Diabetologia. 43, 393-410.

88. Medina, A., S. Yamada, A. Hara, K. Hamamoto and I. Kojima 2013. Involvement of the parasympathetic nervous system in the initiation of regeneration of pancreatic beta-cells. Endocr. J.

89. Matschinsky, F. M. 1996. Banting lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes. 45, 223-241.

90. Aguilar-Bryan, L., J.P. Clement 4th, G. Gonzalez, K. Kunjilwar, A. Babenko and J. Bryan 1998. Toward understanding the assembly and structure of KATP channels. Physiol. Rev. 78, 227-245.

91. Hiriart, M. and L. Aguilar-Bryan 2008. Channel regulation of glucose sensing in the pancreatic beta-cell. Am. J. Physiol. Endocrinol. Metab. 295, E1298-306.

92. Rorsman, P. and E. Renstrom 2003. Insulin granule dynamics in pancreatic beta cells. Diabetologia. 46, 1029-1045.

93. Straub, S. G. and G.W. Sharp 2002. Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab. Res. Rev. 18, 451-463.

94. Aizawa, T. and M. Komatsu 2005. Rab27a: A new face in beta cell metabolism-secretion coupling. J. Clin. Invest. 115, 227-230.

95. Gembal, M., P. Gilon and J.C. Henquin 1992. Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. J. Clin. Invest. 89, 1288-1295.

96. Henquin, J. C. 2000. Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes. 49, 1751-1760.

97. Aizawa, T., Y. Sato and M. Komatsu 2002. Importance of nonionic signals for glucose-induced biphasic insulin secretion. Diabetes. 51 Suppl 1, S96-8.

98. Straub, S. G. and G.W. Sharp 2004. Hypothesis: One rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. Am. J. Physiol. Cell. Physiol. 287, C565-71.

99. Lazo-de-la-Vega-Monroy, M. L. and C. Fernandez-Mejia 2009. Transcription factors in the adult beta cell. Rev. Invest. Clin. 61, 428-446.

100. Ahlgren, U., J. Jonsson, L. Jonsson, K. Simu and H. Edlund 1998. Beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev. 12, 1763-1768.

101. Chakrabarti, S. K., J.C. James and R.G. Mirmira 2002. Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. importance of chromatin structure in directing promoter binding. J. Biol. Chem. 277, 13286-13293.

102. Raum, J. C., K. Gerrish, I. Artner, E. Henderson, M. Guo, L. Sussel, J.C. Schisler, C.B. Newgard and R. Stein 2006. FoxA2, Nkx2.2, and PDX-1 regulate islet beta-cell-specific mafA expression through conserved sequences located between base pairs -8118 and -7750 upstream from the transcription start site. Mol. Cell. Biol. 26, 5735-5743.

103. Thomas, H., K. Jaschkowitz, M. Bulman, T.M. Frayling, S.M. Mitchell, S. Roosen, A. Lingott-Frieg, C.J. Tack, S. Ellard, G.U. Ryffel and A.T. Hattersley 2001. A distant upstream promoter of the HNF-4alpha gene connects the transcription factors involved in maturity-onset diabetes of the young. Hum. Mol. Genet. 10, 2089-2097.

104. Holland, A. M., L.J. Gonez, G. Naselli, R.J. Macdonald and L.C. Harrison 2005. Conditional expression demonstrates the role of the homeodomain transcription factor Pdx1 in maintenance and regeneration of beta-cells in the adult pancreas. Diabetes. 54, 2586-2595.

105. Kushner, J. A., J. Ye, M. Schubert, D.J. Burks, M.A. Dow, C.L. Flint, S. Dutta, C.V. Wright, M.R. Montminy and M.F. White 2002. Pdx1 restores beta cell function in Irs2 knockout mice. J. Clin. Invest. 109, 1193-1201.

106. Kulkarni, R. N., U.S. Jhala, J.N. Winnay, S. Krajewski, M. Montminy and C.R. Kahn 2004. PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. J. Clin. Invest. 114, 828-836.

107. Johnson, J. D., E. Bernal-Mizrachi, E.U. Alejandro, Z. Han, T.B. Kalynyak, H. Li, J.L. Beith, J. Gross, G.L. Warnock, R.R. Townsend, M.A. Permutt and K.S. Polonsky 2006. Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome. Proc. Natl. Acad. Sci. U. S. A. 103, 19575-19580.

108. Wang, H., M. Iezzi, S. Theander, P.A. Antinozzi, B.R. Gauthier, P.A. Halban and C.B. Wollheim 2005. Suppression of pdx-1 perturbs proinsulin processing, insulin secretion and GLP-1 signalling in INS-1 cells. Diabetologia. 48, 720-731.

109. Olson, L. K., J.B. Redmon, H.C. Towle and R.P. Robertson 1993. Chronic exposure of HIT cells to high glucose concentrations paradoxically decreases insulin gene transcription and alters binding of insulin gene regulatory protein. J. Clin. Invest. 92, 514-519.

110. Gremlich, S., C. Bonny, G. Waeber and B. Thorens 1997. Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. J. Biol. Chem. 272, 30261-30269.

111. Hagman, D. K., L.B. Hays, S.D. Parazzoli and V. Poitout 2005. Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of langerhans. J. Biol. Chem. 280, 32413-32418.

112. Jitrapakdee, S., A. Wutthisathapornchai, J.C. Wallace and M.J. MacDonald 2010. Regulation of insulin secretion: Role of mitochondrial signalling. Diabetologia. 53, 1019-1032.

113. Maechler, P. and C.B. Wollheim 2001. Mitochondrial function in normal and diabetic beta-cells. Nature. 414, 807-812.

114. MacDonald, M. J. 1995. Influence of glucose on pyruvate carboxylase expression in pancreatic islets. Arch. Biochem. Biophys. 319, 128-132.

115. Han, J. and Y.Q. Liu 2010. Reduction of islet pyruvate carboxylase activity might be related to the development of type 2 diabetes mellitus in agouti-K mice. J. Endocrinol. 204, 143-152.

116. Hasan, N. M., M.J. Longacre, S.W. Stoker, T. Boonsaen, S. Jitrapakdee, M.A. Kendrick, J.C. Wallace and M.J. MacDonald 2008. Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-

mediated suppression of pyruvate carboxylase. J. Biol. Chem. 283, 28048-28059.

117. Lu, D., H. Mulder, P. Zhao, S.C. Burgess, M.V. Jensen, S. Kamzolova, C.B. Newgard and A.D. Sherry 2002. 13C NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS). Proc. Natl. Acad. Sci. U. S. A. 99, 2708-2713.

118. Xu, J., J. Han, Y.S. Long, P.N. Epstein and Y.Q. Liu 2008. The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta cells. Diabetologia. 51, 2022-2030.

119. Prentki, M., S. Vischer, M.C. Glennon, R. Regazzi, J.T. Deeney and B.E. Corkey 1992. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. J. Biol. Chem. 267, 5802-5810.

120. Maechler, P. and C.B. Wollheim 1999. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature. 402, 685-689.

121. Kibbey, R. G., R.L. Pongratz, A.J. Romanelli, C.B. Wollheim, G.W. Cline and G.I. Shulman 2007. Mitochondrial GTP regulates glucose-stimulated insulin secretion. Cell. Metab. 5, 253-264.

122. Matsumoto, K., K. Fukunaga, J. Miyazaki, M. Shichiri and E. Miyamoto 1995. Ca2+/calmodulin-dependent protein kinase II and synapsin I-like protein in mouse insulinoma MIN6 cells. Endocrinology. 136, 3784-3793.

123. Krueger, K. A., H. Bhatt, M. Landt and R.A. Easom 1997. Calcium-stimulated phosphorylation of MAP-2 in pancreatic betaTC3-cells is mediated by Ca2+/calmodulin-dependent kinase II. J. Biol. Chem. 272, 27464-27469.

124. Nielander, H. B., F. Onofri, F. Valtorta, G. Schiavo, C. Montecucco, P. Greengard and F. Benfenati 1995. Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous protein kinases. J. Neurochem. 65, 1712-1720.

125. Easom, R. A. 1999. CaM kinase II: A protein kinase with extraordinary talents germane to insulin exocytosis. Diabetes. 48, 675-684.

126. Doyle, M. E. and J.M. Egan 2003. Pharmacological agents that directly modulate insulin secretion. Pharmacol. Rev. 55, 105-131.

127. Ammala, C., F.M. Ashcroft and P. Rorsman 1993. Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells. Nature. 363, 356-358.

128. Shibasaki, T., H. Takahashi, T. Miki, Y. Sunaga, K. Matsumura, M. Yamanaka, C. Zhang, A. Tamamoto, T. Satoh, J. Miyazaki and S. Seino 2007. Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. Proc. Natl. Acad. Sci. U. S. A. 104, 19333-19338.

129. Idevall-Hagren, O., S. Barg, E. Gylfe and A. Tengholm 2010. cAMP mediators of pulsatile insulin secretion from glucose-stimulated single beta-cells. J. Biol. Chem. 285, 23007-23018.

130. Zhang, C. L., M. Katoh, T. Shibasaki, K. Minami, Y. Sunaga, H. Takahashi, N. Yokoi, M. Iwasaki, T. Miki and S. Seino 2009. The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. Science. 325, 607-610.

131. Melloul, D., S. Marshak and E. Cerasi 2002. Regulation of insulin gene transcription. Diabetologia. 45, 309-326.

132. Sharp, G. W. 1979. The adenylate cyclase-cyclic AMP system in islets of langerhans and its role in the control of insulin release. Diabetologia. 16, 287-296.

133. Jones, P. M. and S.J. Persaud 1998. Protein kinases, protein phosphorylation, and the regulation of insulin secretion from pancreatic beta-cells. Endocr. Rev. 19, 429-461.

134. Soria, B., I. Quesada, A.B. Ropero, J.A. Pertusa, F. Martin and A. Nadal 2004. Novel players in pancreatic islet signaling: From membrane receptors to nuclear channels. Diabetes. 53 Suppl 1, S86-91.

135. Russell, M. A. and N.G. Morgan 2010. Expression and functional roles of guanylate cyclase isoforms in BRIN-BD11 beta-cells. Islets. 2, 374-382.

136. Laychock, S. G., M.E. Modica and C.T. Cavanaugh 1991. L-arginine stimulates cyclic guanosine 3',5'-monophosphate formation in rat islets of langerhans and RINm5F insulinoma cells: Evidence for L-arginine:Nitric oxide synthase. Endocrinology. 129, 3043-3052.

137. Vilches-Flores, A., A.R. Tovar, A. Marin-Hernandez, A. Rojas-Ochoa and C. Fernandez-Mejia 2010. Biotin increases glucokinase expression via soluble

guanylate cyclase/protein kinase G, adenosine triphosphate production and autocrine action of insulin in pancreatic rat islets. J. Nutr. Biochem. 21, 606-612.

138. Schmidt, H. H., T.D. Warner, K. Ishii, H. Sheng and F. Murad 1992. Insulin secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. Science. 255, 721-723.

139. Flat, P. R. 2003. The hormonal and neural control of endocrine pancreatic function.

140. Baggio, L. L. and D.J. Drucker 2007. Biology of incretins: GLP-1 and GIP. Gastroenterology. 132, 2131-2157.

141. Kaneto, H., Y. Nakatani, D. Kawamori, T. Miyatsuka, T.A. Matsuoka, M. Matsuhisa and Y. Yamasaki 2006. Role of oxidative stress, endoplasmic reticulum stress, and c-jun N-terminal kinase in pancreatic beta-cell dysfunction and insulin resistance. Int. J. Biochem. Cell Biol. 38, 782-793.

142. Aikin, R., S. Hanley, D. Maysinger, M. Lipsett, M. Castellarin, S. Paraskevas and L. Rosenberg 2006. Autocrine insulin action activates akt and increases survival of isolated human islets. Diabetologia. 49, 2900-2909.

143. Navarro-Tableros, V., M.C. Sanchez-Soto, S. Garcia and M. Hiriart 2004. Autocrine regulation of single pancreatic beta-cell survival. Diabetes. 53, 2018-2023.

144. Xu, G. G. and P.L. Rothenberg 1998. Insulin receptor signaling in the beta-cell influences insulin gene expression and insulin content: Evidence for autocrine beta-cell regulation. Diabetes. 47, 1243-1252.

145. Song, G., G. Ouyang and S. Bao 2005. The activation of Akt/PKB signaling pathway and cell survival. J. Cell. Mol. Med. 9, 59-71.

146. Kahn, S. E., R.L. Hull and K.M. Utzschneider 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 444, 840-846.

147. Persaud, S. J., D. Muller and P.M. Jones 2008. Insulin signalling in islets. Biochem. Soc. Trans. 36, 290-293.

148. Kawai, K., C. Yokota, S. Ohashi, Y. Watanabe and K. Yamashita 1995. Evidence that glucagon stimulates insulin secretion through its own receptor in rats. Diabetologia. 38, 274-276.

149. Ahren, B., A. Nobin and B. Schersten 1987. Insulin and C-peptide secretory responses to glucagon in man: Studies on the dose-response relationships. Acta Med. Scand. 221, 185-190.

150. Aguilar, V. and L. Fajas 2010. Cycling through metabolism. EMBO Mol. Med. 2, 338-348.

151. Russell, A. R. and J.H. Pope 1976. Reactivity of antibody in acute myeloid leukaemia with proliferation-associated nuclear antigen(s). Clin. Exp. Immunol. 23, 83-90.

152. Bravo, R., S.J. Fey, J. Bellatin, P.M. Larsen and J.E. Celis 1982. Identification of a nuclear polypeptide ("cyclin") whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. Prog. Clin. Biol. Res. 85 Pt A, 235-248.

153. Smetana, K., F. Gyorkey, P.K. Chan, E. Tan and H. Busch 1983. Proliferating cell nuclear antigen (PCNA) and human malignant tumor nucleolar antigens (HMTNA) in nucleoli of human hematological malignancies. Blut. 46, 133-141.

154. Chan, P. K., R. Frakes, E.M. Tan, M.G. Brattain, K. Smetana and H. Busch 1983. Indirect immunofluorescence studies of proliferating cell nuclear antigen in nucleoli of human tumor and normal tissues. Cancer Res. 43, 3770-3777.

155. Stamatakos, M., V. Palla, I. Karaiskos, K. Xiromeritis, I. Alexiou, I. Pateras and K. Kontzoglou 2010. Cell cyclins: Triggering elements of cancer or not? World J. Surg. Oncol. 8, 111.

156. Musgrove, E. A., C.E. Caldon, J. Barraclough, A. Stone and R.L. Sutherland 2011. Cyclin D as a therapeutic target in cancer. Nat. Rev. Cancer. 11, 558-572.

157. Malumbres, M. and M. Barbacid 2009. Cell cycle, CDKs and cancer: A changing paradigm. Nat. Rev. Cancer. 9, 153-166.

158. Gil, J. and G. Peters 2006. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: All for one or one for all. Nat. Rev. Mol. Cell Biol. 7, 667-677.

159. Chen, H. Z., S.Y. Tsai and G. Leone 2009. Emerging roles of E2Fs in cancer: An exit from cell cycle control. Nat. Rev. Cancer. 9, 785-797.

160. Matsuura, I., N.G. Denissova, G. Wang, D. He, J. Long and F. Liu 2004. Cyclin-dependent kinases regulate the antiproliferative function of smads. Nature. 430, 226-231.

161. Shen, R., X. Wang, H. Drissi, F. Liu, R.J. O'Keefe and D. Chen 2006. Cyclin D1-cdk4 induce runx2 ubiquitination and degradation. J. Biol. Chem. 281, 16347-16353.

162. Zhang, L., F.B. Fried, H. Guo and A.D. Friedman 2008. Cyclin-dependent kinase phosphorylation of RUNX1/AML1 on 3 sites increases transactivation potency and stimulates cell proliferation. Blood. 111, 1193-1200.

163. Nakajima, K., M. Inagawa, C. Uchida, K. Okada, S. Tane, M. Kojima, M. Kubota, M. Noda, S. Ogawa, H. Shirato, M. Sato, R. Suzuki-Migishima, T. Hino, Y. Satoh, M. Kitagawa and T. Takeuchi 2011. Coordinated regulation of differentiation and proliferation of embryonic cardiomyocytes by a jumonji (Jarid2)-cyclin D1 pathway. Development. 138, 1771-1782.

164. Lazaro, J. B., P.J. Bailey and A.B. Lassar 2002. Cyclin D-cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation. Genes Dev. 16, 1792-1805.

165. Kehn, K., R. Berro, A. Alhaj, M.E. Bottazzi, W.I. Yeh, Z. Klase, R. Van Duyne, S. Fu and F. Kashanchi 2007. Functional consequences of cyclin D1/BRCA1 interaction in breast cancer cells. Oncogene. 26, 5060-5069.

166. Adon, A. M., X. Zeng, M.K. Harrison, S. Sannem, H. Kiyokawa, P. Kaldis and H.I. Saavedra 2010. Cdk2 and Cdk4 regulate the centrosome cycle and are critical mediators of centrosome amplification in p53-null cells. Mol. Cell. Biol. 30, 694-710.

167. Wang, C., Z. Li, Y. Lu, R. Du, S. Katiyar, J. Yang, M. Fu, J.E. Leader, A. Quong, P.M. Novikoff and R.G. Pestell 2006. Cyclin D1 repression of nuclear

respiratory factor 1 integrates nuclear DNA synthesis and mitochondrial function. Proc. Natl. Acad. Sci. U. S. A. 103, 11567-11572.

168. Zacharek, S. J., Y. Xiong and S.D. Shumway 2005. Negative regulation of TSC1-TSC2 by mammalian D-type cyclins. Cancer Res. 65, 11354-11360.

169. Aggarwal, P., L.P. Vaites, J.K. Kim, H. Mellert, B. Gurung, H. Nakagawa, M. Herlyn, X. Hua, A.K. Rustgi, S.B. McMahon and J.A. Diehl 2010. Nuclear cyclin D1/CDK4 kinase regulates CUL4 expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. Cancer. Cell. 18, 329-340.

170. Ren, J., Y. Wang, Y. Liang, Y. Zhang, S. Bao and Z. Xu 2010. Methylation of ribosomal protein S10 by protein-arginine methyltransferase 5 regulates ribosome biogenesis. J. Biol. Chem. 285, 12695-12705.

171. Baker, G. L., M.W. Landis and P.W. Hinds 2005. Multiple functions of D-type cyclins can antagonize pRb-mediated suppression of proliferation. Cell. Cycle. 4, 330-338.

172. Zhong, Z., W.S. Yeow, C. Zou, R. Wassell, C. Wang, R.G. Pestell, J.N. Quong and A.A. Quong 2010. Cyclin D1/cyclin-dependent kinase 4 interacts with filamin A and affects the migration and invasion potential of breast cancer cells. Cancer Res. 70, 2105-2114.

173. Li, Z., X. Jiao, C. Wang, X. Ju, Y. Lu, L. Yuan, M.P. Lisanti, S. Katiyar and R.G. Pestell 2006. Cyclin D1 induction of cellular migration requires p27(KIP1). Cancer Res. 66, 9986-9994.

174. Li, Z., C. Wang, X. Jiao, Y. Lu, M. Fu, A.A. Quong, C. Dye, J. Yang, M. Dai, X. Ju, X. Zhang, A. Li, P. Burbelo, E.R. Stanley and R.G. Pestell 2006. Cyclin D1 regulates cellular migration through the inhibition of thrombospondin 1 and ROCK signaling. Mol. Cell. Biol. 26, 4240-4256.

175. Neumeister, P., F.J. Pixley, Y. Xiong, H. Xie, K. Wu, A. Ashton, M. Cammer, A. Chan, M. Symons, E.R. Stanley and R.G. Pestell 2003. Cyclin D1 governs adhesion and motility of macrophages. Mol. Biol. Cell. 14, 2005-2015.

176. Chow, Y. H., X.D. Zhu, L. Liu, B.R. Schwartz, X.Z. Huang, J.M. Harlan and L.M. Schnapp 2010. Role of Cdk4 in lymphocyte function and allergen response. Cell. Cycle. 9, 4922-4930.

177. Bienvenu, F., S. Jirawatnotai, J.E. Elias, C.A. Meyer, K. Mizeracka, A. Marson, G.M. Frampton, M.F. Cole, D.T. Odom, J. Odajima, Y. Geng, A. Zagozdzon, M. Jecrois, R.A. Young, X.S. Liu, C.L. Cepko, S.P. Gygi and P. Sicinski 2010. Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen. Nature. 463, 374-378.

178. McMahon, C., T. Suthiphongchai, J. DiRenzo and M.E. Ewen 1999. P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc. Natl. Acad. Sci. U. S. A. 96, 5382-5387.

179. Reutens, A. T., M. Fu, C. Wang, C. Albanese, M.J. McPhaul, Z. Sun, S.P. Balk, O.A. Janne, J.J. Palvimo and R.G. Pestell 2001. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol. Endocrinol. 15, 797-811.

180. Fu, M., M. Rao, T. Bouras, C. Wang, K. Wu, X. Zhang, Z. Li, T.P. Yao and R.G. Pestell 2005. Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. J. Biol. Chem. 280, 16934-16941.

181. Coqueret, O. 2002. Linking cyclins to transcriptional control. Gene. 299, 35-55.

182. Fu, M., C. Wang, Z. Li, T. Sakamaki and R.G. Pestell 2004. Minireview: Cyclin D1: Normal and abnormal functions. Endocrinology. 145, 5439-5447.

183. Mullany, L. K., P. White, E.A. Hanse, C.J. Nelsen, M.M. Goggin, J.E. Mullany, C.K. Anttila, L.E. Greenbaum, K.H. Kaestner and J.H. Albrecht 2008. Distinct proliferative and transcriptional effects of the D-type cyclins in vivo. Cell. Cycle. 7, 2215-2224.

184. Zwijsen, R. M., E. Wientjens, R. Klompmaker, J. van der Sman, R. Bernards and R.J. Michalides 1997. CDK-independent activation of estrogen receptor by cyclin D1. Cell. 88, 405-415.

185. Neuman, E., M.H. Ladha, N. Lin, T.M. Upton, S.J. Miller, J. DiRenzo, R.G. Pestell, P.W. Hinds, S.F. Dowdy, M. Brown and M.E. Ewen 1997. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol. Cell. Biol. 17, 5338-5347.

186. Despouy, G., J.N. Bastie, S. Deshaies, N. Balitrand, A. Mazharian, C. Rochette-Egly, C. Chomienne and L. Delva 2003. Cyclin D3 is a cofactor of

retinoic acid receptors, modulating their activity in the presence of cellular retinoic acid-binding protein II. J. Biol. Chem. 278, 6355-6362.

187. Knudsen, K. E., W.K. Cavenee and K.C. Arden 1999. D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. Cancer Res. 59, 2297-2301.

188. Sarruf, D. A., I. Iankova, A. Abella, S. Assou, S. Miard and L. Fajas 2005. Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. Mol. Cell. Biol. 25, 9985-9995.

189. Zong, H., Y. Chi, Y. Wang, Y. Yang, L. Zhang, H. Chen, J. Jiang, Z. Li, Y. Hong, H. Wang, X. Yun and J. Gu 2007. Cyclin D3/CDK11p58 complex is involved in the repression of androgen receptor. Mol. Cell. Biol. 27, 7125-7142.

190. Besson, A., R.K. Assoian and J.M. Roberts 2004. Regulation of the cytoskeleton: An oncogenic function for CDK inhibitors? Nat. Rev. Cancer. 4, 948-955.

191. Li, Z., X. Jiao, C. Wang, L.A. Shirley, H. Elsaleh, O. Dahl, M. Wang, E. Soutoglou, E.S. Knudsen and R.G. Pestell 2010. Alternative cyclin D1 splice forms differentially regulate the DNA damage response. Cancer Res. 70, 8802-8811.

192. Jirawatnotai, S., Y. Hu, W. Michowski, J.E. Elias, L. Becks, F. Bienvenu, A. Zagozdzon, T. Goswami, Y.E. Wang, A.B. Clark, T.A. Kunkel, T. van Harn, B. Xia, M. Correll, J. Quackenbush, D.M. Livingston, S.P. Gygi and P. Sicinski 2011. A function for cyclin D1 in DNA repair uncovered by protein interactome analyses in human cancers. Nature. 474, 230-234.

193. Raderschall, E., A. Bazarov, J. Cao, R. Lurz, A. Smith, W. Mann, H.H. Ropers, J.M. Sedivy, E.I. Golub, E. Fritz and T. Haaf 2002. Formation of higher-order nuclear Rad51 structures is functionally linked to p21 expression and protection from DNA damage-induced apoptosis. J. Cell. Sci. 115, 153-164.

194. Kozar, K., M.A. Ciemerych, V.I. Rebel, H. Shigematsu, A. Zagozdzon, E. Sicinska, Y. Geng, Q. Yu, S. Bhattacharya, R.T. Bronson, K. Akashi and P. Sicinski 2004. Mouse development and cell proliferation in the absence of D-cyclins. Cell. 118, 477-491.

195. Malumbres, M., R. Sotillo, D. Santamaria, J. Galan, A. Cerezo, S. Ortega, P. Dubus and M. Barbacid 2004. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell. 118, 493-504.

196. Landis, M. W., B.S. Pawlyk, T. Li, P. Sicinski and P.W. Hinds 2006. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. Cancer. Cell. 9, 13-22.

197. Geng, Y., Q. Yu, E. Sicinska, M. Das, R.T. Bronson and P. Sicinski 2001. Deletion of the p27Kip1 gene restores normal development in cyclin D1-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 98, 194-199.

198. Tsutsui, T., B. Hesabi, D.S. Moons, P.P. Pandolfi, K.S. Hansel, A. Koff and H. Kiyokawa 1999. Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol. Cell. Biol. 19, 7011-7019.

199. Mares, J. and M. Welsh 1993. Expression of certain antiproliferative and growth-related genes in isolated mouse pancreatic islets: Analysis by polymerase chain reaction. Diabete Metab. 19, 315-320.

200. Kushner, J. A., M.A. Ciemerych, E. Sicinska, L.M. Wartschow, M. Teta, S.Y. Long, P. Sicinski and M.F. White 2005. Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. Mol. Cell. Biol. 25, 3752-3762.

201. Peshavaria, M., B.L. Larmie, J. Lausier, B. Satish, A. Habibovic, V. Roskens, K. Larock, B. Everill, J.L. Leahy and T.L. Jetton 2006. Regulation of pancreatic beta-cell regeneration in the normoglycemic 60% partial-pancreatectomy mouse. Diabetes. 55, 3289-3298.

202. Takasawa, S., T. Ikeda, T. Akiyama, K. Nata, K. Nakagawa, N.J. Shervani, N. Noguchi, S. Murakami-Kawaguchi, A. Yamauchi, I. Takahashi, T. Tomioka-Kumagai and H. Okamoto 2006. Cyclin D1 activation through ATF-2 in reg-induced pancreatic beta-cell regeneration. FEBS Lett. 580, 585-591.

203. Georgia, S. and A. Bhushan 2004. Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J. Clin. Invest. 114, 963-968.

204. Kauri, L. M., G.S. Wang, C. Patrick, M. Bareggi, D.J. Hill and F.W. Scott 2007. Increased islet neogenesis without increased islet mass precedes autoimmune attack in diabetes-prone rats. Lab. Invest. 87, 1240-1251.

205. Cozar-Castellano, I., K.K. Takane, R. Bottino, A.N. Balamurugan and A.F. Stewart 2004. Induction of beta-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. Diabetes. 53, 149-159.

206. Virgin, H. W. and J.A. Todd 2011. Metagenomics and personalized medicine. Cell. 147, 44-56.

207. Zhang, X., J.P. Gaspard, Y. Mizukami, J. Li, F. Graeme-Cook and D.C. Chung 2005. Overexpression of cyclin D1 in pancreatic beta-cells in vivo results in islet hyperplasia without hypoglycemia. Diabetes. 54, 712-719.

208. DeBerardinis, R. J., J.J. Lum, G. Hatzivassiliou and C.B. Thompson 2008. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell. Metab. 7, 11-20.

209. Guppy, M., E. Greiner and K. Brand 1993. The role of the crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. Eur. J. Biochem. 212, 95-99.

210. Vander Heiden, M. G., L.C. Cantley and C.B. Thompson 2009. Understanding the warburg effect: The metabolic requirements of cell proliferation. Science. 324, 1029-1033.

211. Rane, S. G., P. Dubus, R.V. Mettus, E.J. Galbreath, G. Boden, E.P. Reddy and M. Barbacid 1999. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat. Genet. 22, 44-52.

212. Alonso, L. C., T. Yokoe, P. Zhang, D.K. Scott, S.K. Kim, C.P. O'Donnell and A. Garcia-Ocana 2007. Glucose infusion in mice: A new model to induce beta-cell replication. Diabetes. 56, 1792-1801.

213. Kassem, S. A., I. Ariel, P.S. Thornton, I. Scheimberg and B. Glaser 2000. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes. 49, 1325-1333.

214. Meier, J. J., A.E. Butler, Y. Saisho, T. Monchamp, R. Galasso, A. Bhushan, R.A. Rizza and P.C. Butler 2008. Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes. 57, 1584-1594.

215. Dor, Y., J. Brown, O.I. Martinez and D.A. Melton 2004. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 429, 41-46.

216. Teta, M., M.M. Rankin, S.Y. Long, G.M. Stein and J.A. Kushner 2007. Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev. Cell. 12, 817-826.

217. Fatrai, S., L. Elghazi, N. Balcazar, C. Cras-Meneur, I. Krits, H. Kiyokawa and E. Bernal-Mizrachi 2006. Akt induces beta-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. Diabetes. 55, 318-325.

218. Dimova, D. K. and N.J. Dyson 2005. The E2F transcriptional network: Old acquaintances with new faces. Oncogene. 24, 2810-2826.

219. Dyson, N. 1998. The regulation of E2F by pRB-family proteins. Genes Dev. 12, 2245-2262.

220. Fajas, L., J.S. Annicotte, S. Miard, D. Sarruf, M. Watanabe and J. Auwerx 2004. Impaired pancreatic growth, beta cell mass, and beta cell function in E2F1 (-/-)mice. J. Clin. Invest. 113, 1288-1295.

221. Annicotte, J. S., E. Blanchet, C. Chavey, I. Iankova, S. Costes, S. Assou, J. Teyssier, S. Dalle, C. Sardet and L. Fajas 2009. The CDK4-pRB-E2F1 pathway controls insulin secretion. Nat. Cell Biol. 11, 1017-1023.

222. Iglesias, A., M. Murga, U. Laresgoiti, A. Skoudy, I. Bernales, A. Fullaondo, B. Moreno, J. Lloreta, S.J. Field, F.X. Real and A.M. Zubiaga 2004. Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. J. Clin. Invest. 113, 1398-1407.

223. Li, F. X., J.W. Zhu, J.S. Tessem, J. Beilke, M. Varella-Garcia, J. Jensen, C.J. Hogan and J. DeGregori 2003. The development of diabetes in E2f1/E2f2 mutant mice reveals important roles for bone marrow-derived cells in preventing islet cell loss. Proc. Natl. Acad. Sci. U. S. A. 100, 12935-12940.

224. Wei, F. Y., K. Nagashima, T. Ohshima, Y. Saheki, Y.F. Lu, M. Matsushita, Y. Yamada, K. Mikoshiba, Y. Seino, H. Matsui and K. Tomizawa 2005. Cdk5-dependent regulation of glucose-stimulated insulin secretion. Nat. Med. 11, 1104-1108.

225. Okada, S., E. Yamada, T. Saito, K. Ohshima, K. Hashimoto, M. Yamada, Y. Uehara, T. Tsuchiya, H. Shimizu, K. Tatei, T. Izumi, K. Yamauchi, S. Hisanaga, J.E. Pessin and M. Mori 2008. CDK5-dependent phosphorylation of the rho family GTPase TC10(alpha) regulates insulin-stimulated GLUT4 translocation. J. Biol. Chem. 283, 35455-35463.

226. Huang, S. and M.P. Czech 2007. The GLUT4 glucose transporter. Cell. Metab. 5, 237-252.

227. Lalioti, V., G. Muruais, A. Dinarina, J. van Damme, J. Vandekerckhove and I.V. Sandoval 2009. The atypical kinase Cdk5 is activated by insulin, regulates the association between GLUT4 and E-Syt1, and modulates glucose transport in 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. U. S. A. 106, 4249-4253.

228. Inoue, N., N. Yahagi, T. Yamamoto, M. Ishikawa, K. Watanabe, T. Matsuzaka, Y. Nakagawa, Y. Takeuchi, K. Kobayashi, A. Takahashi, H. Suzuki, A.H. Hasty, H. Toyoshima, N. Yamada and H. Shimano 2008. Cyclin-dependent kinase inhibitor, p21WAF1/CIP1, is involved in adipocyte differentiation and hypertrophy, linking to obesity, and insulin resistance. J. Biol. Chem. 283, 21220-21229.

229. Uchida, T., T. Nakamura, N. Hashimoto, T. Matsuda, K. Kotani, H. Sakaue, Y. Kido, Y. Hayashi, K.I. Nakayama, M.F. White and M. Kasuga 2005. Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. Nat. Med. 11, 175-182.

230. Motokura, T., H.F. Yi, H.M. Kronenberg, O.W. McBride and A. Arnold 1992. Assignment of the human cyclin D3 gene (CCND3) to chromosome 6p----q13. Cytogenet. Cell Genet. 61, 5-7.

231. Sicinska, E., I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A.A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer and P. Sicinski 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell. 4, 451-461.

232. Lam, E. W., J. Glassford, L. Banerji, N.S. Thomas, P. Sicinski and G.G. Klaus 2000. Cyclin D3 compensates for loss of cyclin D2 in mouse B-lymphocytes activated via the antigen receptor and CD40. J. Biol. Chem. 275, 3479-3484.

233. Cooper, A. B., C.M. Sawai, E. Sicinska, S.E. Powers, P. Sicinski, M.R. Clark and I. Aifantis 2006. A unique function for cyclin D3 in early B cell development. Nat. Immunol. 7, 489-497.

234. Mora, C., F.S. Wong, C.H. Chang and R.A. Flavell 1999. Pancreatic infiltration but not diabetes occurs in the relative absence of MHC class II-restricted CD4 T cells: Studies using NOD/CIITA-deficient mice. J. Immunol. 162, 4576-4588.

235. Inaba, T., H. Matsushime, M. Valentine, M.F. Roussel, C.J. Sherr and A.T. Look 1992. Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. Genomics. 13, 565-574.

236. Ciemerych, M. A., A.M. Kenney, E. Sicinska, I. Kalaszczynska, R.T. Bronson, D.H. Rowitch, H. Gardner and P. Sicinski 2002. Development of mice expressing a single D-type cyclin. Genes Dev. 16, 3277-3289.

237. Fiaschi-Taesch, N. M., F. Salim, J. Kleinberger, R. Troxell, I. Cozar-Castellano, K. Selk, E. Cherok, K.K. Takane, D.K. Scott and A.F. Stewart 2010. Induction of human beta-cell proliferation and engraftment using a single G1/S regulatory molecule, cdk6. Diabetes. 59, 1926-1936.

238. Yalcin, A., B.F. Clem, A. Simmons, A. Lane, K. Nelson, A.L. Clem, E. Brock, D. Siow, B. Wattenberg, S. Telang and J. Chesney 2009. Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. J. Biol. Chem. 284, 24223-24232.

239. Davis, R. J. 2000. Signal transduction by the JNK group of MAP kinases. Cell. 103, 239-252.

240. Andersen, N. A., C.M. Larsen and T. Mandrup-Poulsen 2000. TNFalpha and IFNgamma potentiate IL-1beta induced mitogen activated protein kinase activity in rat pancreatic islets of langerhans. Diabetologia. 43, 1389-1396.

241. Yamada, K., N. Takane-Gyotoku, X. Yuan, F. Ichikawa, C. Inada and K. Nonaka 1996. Mouse islet cell lysis mediated by interleukin-1-induced fas. Diabetologia. 39, 1306-1312.

242. Mandrup-Poulsen, T. 1996. The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia. 39, 1005-1029.

243. Stephanou, A., B.K. Brar, T.M. Scarabelli, A.K. Jonassen, D.M. Yellon, M.S. Marber, R.A. Knight and D.S. Latchman 2000. Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. J. Biol. Chem. 275, 10002-10008.

244. Cardozo, A. K., M. Kruhoffer, R. Leeman, T. Orntoft and D.L. Eizirik 2001. Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. Diabetes. 50, 909-920.

245. Wei, Y., J. Jiang, M. Sun, X. Chen, H. Wang and J. Gu 2006. ATF5 increases cisplatin-induced apoptosis through up-regulation of cyclin D3 transcription in HeLa cells. Biochem. Biophys. Res. Commun. 339, 591-596.

246. Liu, W., M. Sun, J. Jiang, X. Shen, Q. Sun, W. Liu, H. Shen and J. Gu 2004. Cyclin D3 interacts with human activating transcription factor 5 and potentiates its transcription activity. Biochem. Biophys. Res. Commun. 321, 954-960.

247. Wang, C., N. Pattabiraman, J.N. Zhou, M. Fu, T. Sakamaki, C. Albanese, Z. Li, K. Wu, J. Hulit, P. Neumeister, P.M. Novikoff, M. Brownlee, P.E. Scherer, J.G. Jones, K.D. Whitney, L.A. Donehower, E.L. Harris, T. Rohan, D.C. Johns and R.G. Pestell 2003. Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. Mol. Cell. Biol. 23, 6159-6173.

248. Bartkova, J., J. Lukas, M. Strauss and J. Bartek 1998. Cyclin D3: Requirement for G1/S transition and high abundance in quiescent tissues suggest a dual role in proliferation and differentiation. Oncogene. 17, 1027-1037.

249. Rao, S. S. and D.S. Kohtz 1995. Positive and negative regulation of D-type cyclin expression in skeletal myoblasts by basic fibroblast growth factor and transforming growth factor beta. A role for cyclin D1 in control of myoblast differentiation. J. Biol. Chem. 270, 4093-4100.

250. Kato, J. Y. and C.J. Sherr 1993. Inhibition of granulocyte differentiation by G1 cyclins D2 and D3 but not D1. Proc. Natl. Acad. Sci. U. S. A. 90, 11513-11517.

251. Sun, M., Y. Wei, L. Yao, J. Xie, X. Chen, H. Wang, J. Jiang and J. Gu 2006. Identification of extracellular signal-regulated kinase 3 as a new interaction partner of cyclin D3. Biochem. Biophys. Res. Commun. 340, 209-214.

252. Julien, C., P. Coulombe and S. Meloche 2003. Nuclear export of ERK3 by a CRM1-dependent mechanism regulates its inhibitory action on cell cycle progression. J. Biol. Chem. 278, 42615-42624.

253. Coulombe, P., G. Rodier, S. Pelletier, J. Pellerin and S. Meloche 2003. Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. Mol. Cell. Biol. 23, 4542-4558.

254. Crowe, D. L. 2004. Induction of p97MAPK expression regulates collagen mediated inhibition of proliferation and migration in human squamous cell carcinoma lines. Int. J. Oncol. 24, 1159-1163.