Cyclin D3 and CDK11 partnership in pancreatic beta cell homeostasis in autoimmune diabetes. Studies on the NOD mouse model

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CYCLIN D3 AND CDK11 PARTNERSHIP IN PANCREATIC BETA CELL HOMEOSTASIS IN AUTOIMMUNE DIABETES. STUDIES ON THE NOD MOUSE MODEL

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Doctoral Thesis

Scientific Director

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"The truth may be puzzling. It may take some work to grapple with. It may be counterintuitive. It may contradict deeply held prejudices. It may not be consonant with what we desperately want to be true. But our preferences do not determine what's true. We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth—never there, just closer and closer, always finding vast new oceans of undiscovered possibilities. Cleverly designed experiments are the key."

Carl Sagan

To my parents

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Upasana

Abbreviations

AR	Androgen Receptor
ATP	Adenosine-5'-triphosphate
bp	Base pair
BRCA 2	Breast cancer 2
$[Ca2^{+}]i$	Cytoplasmic free $Ca2^{+}$ concentration
CaMKII	Calcium-Calmodulin-Dependent Protein Kinase II
CBP	p300/cAMP response element-binding protein-binding protein
Ccn	Cyclin
CcnD1	Cyclin D1
CcnD2	Cyclin D2
CcnD3	Cyclin D3
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
CNS	Central Nervous System
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
Cyc	Cyclin family proteins
DAISY	Diabetes Autoimmunity Study in the Young
DAPI	4, 6-diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
E2F	Transcription Factors in Eukaryotes
ER α	Estrogen Receptor α
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAD65	Glutamic acid decarboxilase 65
GADA	Glutamic acid decarboxylase
GATA4	Globin transcription factor 4
GDH	Glutamate dehydrogenase
GLUT	Glucose transporter
HAT	Histone Acetyltransferase
HDACs	Histone Deacetylase
HFHS	High fat high sucrose
HLA	Human leukocyte antigen

HM	Hemideficient
HNF-4α	Hepatocyte nuclear factor 4 alpha
HTZ	Heterozygous
IAA	Insulin autoantibodies
IDDM	Insulin-dependent diabetes mellitus
IDD	Insulin-dependent diabetes mellitus locus
IL	Interleukine
IF	Immunoflourescence
IFN	Interferon
IL-2	Interleukin-2
iNOS	Inducible nitric oxide synthase
INS	Insulin
IRS	Insulin receptor substrates
i.v.	Intra venous
IVGTT	Intravenous glucose tolerance test
K_{ATP}	ATP-sensitive K^+ channels
kDa	Kilo Dalton
Kb	Kilobase
KO	Knockout
LB	Lysogeny Broth
LN	Lymph Nodes
LPS	Lipopolysaccharide
L-VDCC	Voltage dependent calcium channels
MafA	Musculoaponeurotic Fibrosarcoma Oncogene Homolog A (Avian)
MHC	Main histocompatibility complex
MODY	Maturity-onset diabetes of the young
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene (88)
MyoD	Myogenic differentiation
Neg	Negative
NeuroD	Neurogenic differentiation
NK	Natural killer
NO	Nitric oxide
NOD	Non-obese diabetic
PE	Phycoerythrin
PCR	Polymerase Chain Reaction
P/CAF	p300/CBP-associated factor
PDX-1	Pancreatic and duodenal homeobox 1

PI	Propidium Iodide
PK	Protein Kinase
Pos	Positive
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
pRB	Retioblastoma protein
PTPN22	Protein tyrosine phosphatase nonreceptor type 22 gene
PVDF	Polyvinylidene difluoride
RAD51	RAD51 recombinase
RB	Retioblastoma
RIP	Rat insulin promoter
RNA	Ribonucleic acid
ROS	Reactive oxygen species / Radicle Oxygen Species
RT	Room Temperature
rT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Runx2	Runt-Related gene 2
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	Specific Pathogen Free
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
T1DM	Type 1 diabetes mellitus
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline Tween
TCA	Tricarboxylic Acid cycle
TG	Transgenic
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSC	Tuberous Sclerosis
TSC 2	Tuberous Sclerosis 2
UV	Ultraviolet
UVR	Ultra-Violent Radiation
VDCC	Voltage-dependent calcium channel
WB	Western Blot
WT	Wild type
β	Beta
Δ	Delta

γ	Gamma
ω	Omega
+	Positive
-	Negative
-/-	Knockout
+/-	Heterozygous
+/+	Wild type
Δ/+	Heterozygous for CDK11 gene

Abstract

3. ABSTRACT

Cyclin D3 and CDK11 are downregulated in pancreatic islet endocrine cells during the autoimmune attack progression in autoimmune-prone NOD (Non-obese diabetic) mouse strain. D-type cyclins are crucial in order to connect mitogenic signals with the Rb/E2F pathway, which regulates transcription of factors involved in further cell cycle progression. CDK11, protein-kinase PITSLRE, exhibits two gene products: p58 and p110 (p130 in mouse) in humans. CDK11p110 regulates transcription and RNA splicing. CDK11p110 is expressed in all cell cycle phases, while CDK11p58 is only expressed during mitosis (G2/M) and is essential in apoptosis. The interaction between CDK11p58 and cyclin D3 has been reported and it represses certain nuclear receptors action. This observation may suggest that in pancreatic beta cells simultaneous downregulation of cyclin D3 and CDK11 may obey to a coordinated regulation of both molecules. In this thesis we have studied whether there is a causal relationship between coordinated cyclin D3 and CDK11 downregulation and type 1 diabetes *in vitro* and *in vivo.*

a) *In vitro* approach:

NIT-1 NOD insulinoma cell line stably co-tranfected with cyclin D3 and/or CDK11p58, were submitted to IL-1β and IFNγ induced apoptosis, and apoptosis susceptibility measured by Annexin V staining.

b) *In vivo* approach:

1) Generation of NOD mice deficient cyclin D3 and hemideficient CDK11 (i.e. CDK11(Δ/+) CcnD3(-/-),

2) Generation of NOD mice overexpressing cyclin D3 just in β cells with the hemideficiency in CDK11 (i.e. CDK11(Δ/+) CcnD3Tg+ or -).

In 1 and 2 we monitored the incidence of spontaneous diabetes as well as performed morphometric analysis of islets which allowed us to determine the relevance of CDK11 and cyclin D3 in proliferation and survival of beta cells, and therefore evaluate possible targets to design therapeutic intervention points in T1D.

3) Generation of NOD/SCID mice overexpressing cyclin D3 just in β cells with the hemideficiency in CDK11 (i.e. NODSCIDCDK11(Δ/+)CcnD3Tg+ or NODSCID CDK11(Δ/+)CcnD3Tg-).This experimental approach will address whether CDK11 and cyclin D3 act synergistically. In the mice overexpressing cyclin D3 and/or hemideficient in CDK11, NOD mice should be less susceptible to spontaneous diabetes onset than transgenic negative, and NOD/SCID mice should be less susceptible to adoptively transferred diabetes by diabetogenic lymphocytes.

The outcome of our research will allow us to establish whether cyclin D3 and CDK11 are molecular targets in type1 diabetes.

RESUMEN

Ciclina D3 y CDK11 están regulados a la baja en las células endocrinas del islote pancreático durante la progresión ataque autoinmune en la cepa de ratones NOD predispuesta genéticamente a la autoinmunidad. Los ciclinas de tipo D son cruciales para conectar las señales mitogénicas con la vía Rb/E2F, la cual regula la transcripción de factores implicados en la progresión del ciclo celular. El gen que codifica para la CDK11, la proteína-quinasa PITSLRE, tiene dos productos génicos en humanos: p58 y p110 (p130 en el ratón). CDK11p110 regula la transcripción y procesameinto del ARN. CDK11p110 se expresa en todas las fases del ciclo celular, mientras que CDK11p58 sólo se expresa durante la mitosis (G2 / M) y está implicada en procesos apoptóticos. La interacción entre CDK11p58 y ciclina D3 reprime cierta acción de algunos receptores nucleare, por ejemplo, afecta negativamente a la actividad transcripcional del receptor de andrógenos. Esta observación puede sugerir que en las células beta del páncreas la regulación a la baja simultánea de ciclina D3 y CDK11 puede obedecer a una regulación coordinada de ambas moléculas. En esta tesis se ha estudiado *in vitro* e *in vivo* si existe una relación causal entre la regulación a la baja coordinada de ciclina D3 y CDK11 y la aparición de la diabetes tipo 1,

a) Enfoque *in vitro*:

Obtención de líneas celulares NIT- 1 NOD de insulinoma establemente co- ransfectadas con ciclina D3 y / o CDK11p58, fueron sometidas a la acción de las citoquinas por IL- 1β y IFNγ para inducir apoptosis. Se midió la susceptibilidad a apoptosis por tinción con Anexina V.

b) Enfoque *in vivo*:

1) Generación de ratones NOD deficientes en ciclina D3 y hemideficientes en CDK11 (es decir, CDK11(Δ/+) CcnD3 (- /-) ,

2) Generación de ratones NOD que sobreexpresan ciclina D3 en las células β-pancreáticas y son hemideficientes en CDK11 (es deci, CDK11(Δ /+) CcnD3tg+ o -).

En 1 y 2 hemos monitorizado la incidencia de diabetes espontánea, así como realizado análisis morfométricos de los islotes que nos permitan determinar la relevancia de CDK11 y ciclina D3 en la proliferación y la supervivencia de las células beta, y por lo tanto, diseñar posibles puntos de intervención terapéutica en la T1D.

3) Generación de ratones NOD / SCID que sobreexpresan ciclina D3 en la celula β y son hemideficientes en CDK11 (es decir, NODSCIDCDK11(Δ/+)CcnD3Tg+ or NODSCID CDK11(Δ/+)CcnD3Tg-). Este enfoque experimental abordó si CDK11 y ciclina D3 actúan sinérgicamente. Los ratones NOD que sobreexpresan ciclina D3 en las células β-pancreáticas y / o hemideficientes en CDK11 deben ser menos susceptibles a la aparición de la diabetes espontánea, y los ratones NOD / SCID lo deben ser a la menos a la diabetes transferida adoptivamente por los linfoticos diabetogénicos.

El resultado de nuestra investigación nos permitirá establecer ciclina D3 y CDK11 como blancos moleculares en la diabetes tipo 1.

RESUM

La ciclina D3 i CDK11 estan regulades a la baixa en les cèl·lules endocrines de l'illot pancreàtic durant la progressió atac autoimmune en la soca de ratolins NOD propensos a la diabetis autoimmune. Les ciclines de tipus D són crucials per connectar senyals mitogèniques amb la via Rb/E2F , que regula la transcripció de factors implicats en la progressió del cicle cel · lular . El gen que codifica per a la CDK11 , proteïna-quinasa PITSLRE, té dos productes gènics en humans: p58 i p110 (p130 al ratolí). LaCDK11p110 regula la transcripció i processament del 'ARN. La CDK11p110 s'expressa en totes les fases del cicle cel·lular , mentre que la CDK11p58 només s'expressa durant la mitosi (G2 / M) i participa en processos apoptòtics . La interacció entre CDK11p58 i ciclina D3 reprimeix certa acció d'alguns receptors nuclears, per exemple afecta negativament l'activitat transcripcional del receptor d'andròg ens . Aquesta observació pot suggerir que en les cèl·lules beta del pàncrees la regulació a la baixa simultània de ciclina D3 i CDK11 pot obeir a una regulació coordinada de les dues molècules . En aquesta tesi s'ha estudiat si hi ha una relació causal entre la regulació a la baixa coordinada de ciclina D3 i CDK11 i l'aparició de la diabetis tipus 1 , *in vitro* i *in vivo* .

a) Aproximació *in vitro*:

La línia cel·lular NIT -1 d d'insulinoma NOD va ser establement co- transfectada amb ciclina D3 i / o CDK11p58 , i va ser sotmesa als a les citocines IL-β i IFNγ. Es va mesurar la susceptibilitat a apoptosi per tinció amb anexina V.

b) Aproximacions *in vivo*:

1) Generació de ratolins NOD deficients en ciclina D3 i hemideficients en CDK11 (és a dir CDK11(Δ/+)CcnD3(-/-) ,

2) Generació de ratolins NOD que sobreexpressen ciclina D3 a les cèl·lules β–pancreàtiques i són hemideficients en CDK11 (és a dir CDK11(Δ/+) CcnD3tg+ or -).

En 1 i 2 hem monitoritzat la incidència de diabetis espontània, així com realitzat anàlisis morfomètriques dels illots que ens permetin determinar la rellevància de CDK11 i ciclina

D3 en la proliferació i la supervivència de les cèl·lules beta, i per tant, donar la possibilitat de dissenyar punts potencials d'intervenció terapèutica a la T1D.

3) Generació de ratolins NOD / SCID que sobreexpressin del ciclina D3 a les cel·lules β–pancreàtiques i són hemideficients en CDK11 (és a dir NODSCIDCDK11(Δ/+) CcnD3Tg+ or NODSCIDCDK11(Δ/+) CcnD3Tg-). Aquest enfocament experimental vol abordar si la CDK11 i la ciclina D3 actuen sinèrgicament. Els ratolins NOD que sobreexpressen la ciclina D3 a les cèl·lules β–pancreàtiques i / o hemideficients en CDK11 haurien ha de ser menys susceptibles a desenvolupar diabetis espontània, i els ratolins NOD/SCID a la diabetis adoptivament transferida pels limfòtis diabetogénics.

El resultat de la nostra recerca ens permetrà establir si la ciclina D3 i la CDK11 podran ser blancs moleculars en la diabetis tipus 1 .

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Introduction

5. INTRODUCTION

5.1 Autoimmune diseases

The immune system in certain individuals loses the capability to discriminate between self and nonself, leading to an immune attack upon the host. This condition is called autoimmunity, which can generate a number of chronic debilitating diseases [1]. Thus, autoimmunity is considered to result from failure to establish self-tolerance.

5.1.1 Causes of autoimmune diseases

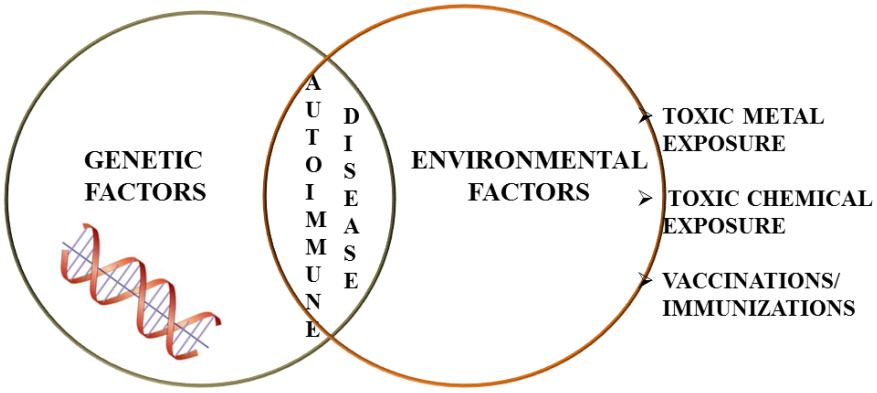


Figure 1. Causes of Autoimmune Disease. *The autoimmune disease is caused by a combination of genetic and the environmental factors* [2].

5.1.2 Mechanism involved in generating T-cell mediated autoimmune diseases

There are a variety of mechanisms proposed for T-cell mediated development of autoimmune diseases. Not only a single step but rather a multiple number of events are required for the onset of autoimmunity. The breakdown of mechanisms responsible for self tolerance causes autoimmunity. The immune tolerance can be of central (in thymus and in bone marrow) and peripheral tolerance [2].

It is briefly explained in the following **Figure 2.**

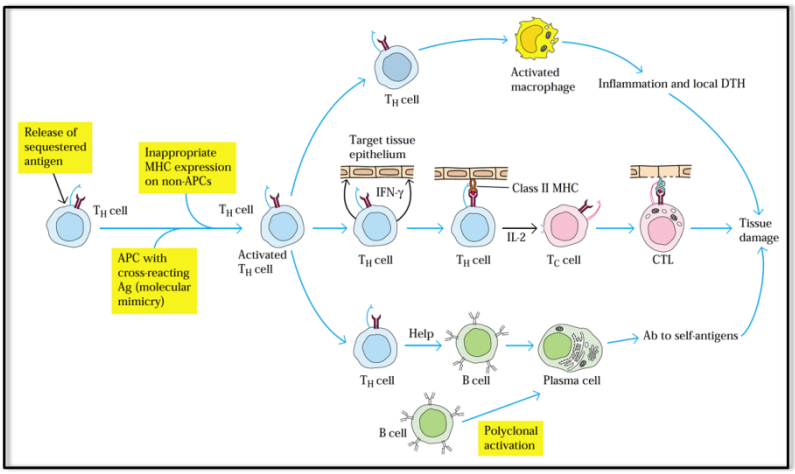


Figure 2. Proposed mechanisms for inducing autoimmune responses. *Normal thymic selection removes most of some self-reactive Th cells; alterations in this process may release to circulation an abnormal number of autorreactive T cells. Activation of these selfreactive T cells in various ways, as well as polyclonal activation of B cells, is thought to induce an autoimmune response, in this case resulting in tissue damage. [Adapted from V. Kumar et al., 1989, Annu. Rev. Immunol. 7:657]*[1, 3]

5.1.3 Types of autoimmune diseases

Variations in the symptoms of autoimmunity depend on which tissues and organs are under attack [1]. Depending upon that, there are a number of types of autoimmune diseases (**Figure3**) [2].

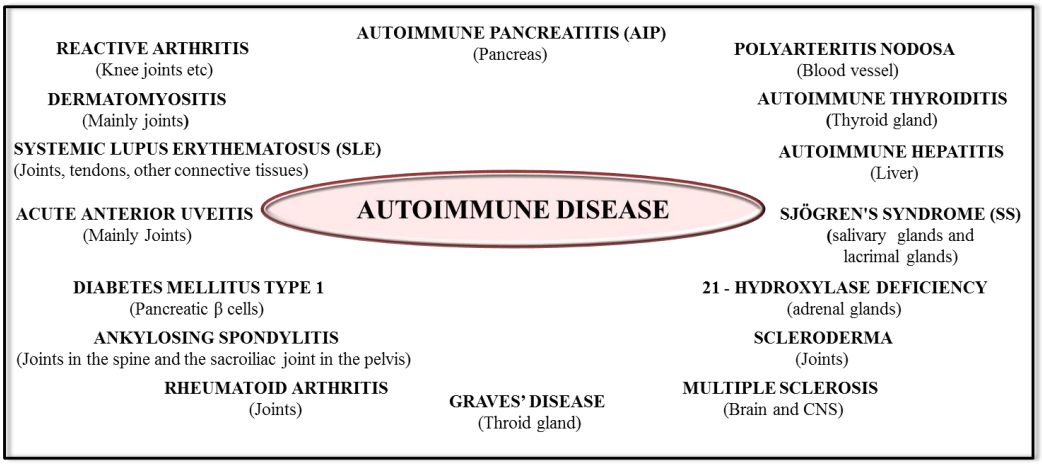


Figure 3. Types of Autoimmune Diseases. *Examples of various types of autoimmune disease* [2].

5.2 Type 1 Diabetes (T1D)

Our research work is focused in Type 1 Diabetes (T1D). T1D is an autoimmune disease which is caused by the selective destruction of the insulin producing β pancreatic cells and that results in severe hyperglycemia. This is mediated by the autoimmune attack of T lumphocytes towards β cells [4]. T1D represents 5-10% of all the cases of diabetes [5].

Symptoms:

- Polyuria (frequent urination),
- Polydipsia (increased thirst),
- Polyphagia (increased hunger),
- Weight loss. [6]

The peak age of T1D onset is at 12 years and it usually occurs before 40 years[5, 7]. The global annual incidence varies from 1 per 100,000 in Asia to 14 per 100,000 in USA and more than 30 per 100,000 in Scandinavia [8].

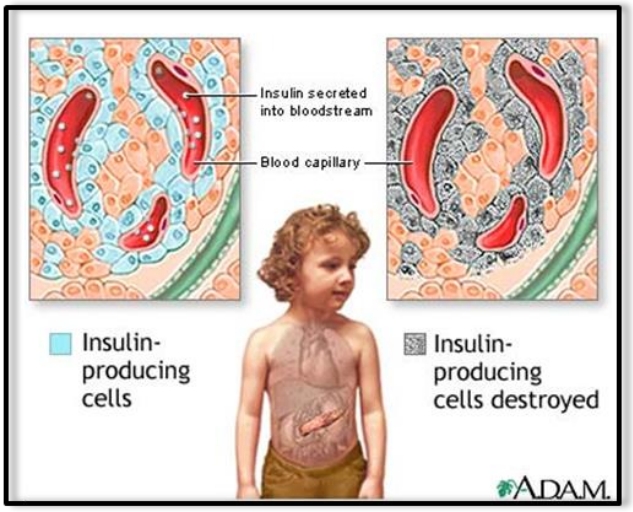


Figure 4. *Type I diabetes occurs when the insulin producing β pancreatic cells are destroyed by the body's own immune system. [Adapted from http://health.allrefer.com]*[9]

5.2.1 Factors responsible for the incidence of T1D

T1D shows a complex mode of inheritance, with disease susceptibility caused both by genetic and by environmental components [7]. Despite the fact that T1D is strongly

influenced by genetic factors, it does not fit any pattern of inheritance and is contemplated as a complex, multifactorial disease [10].

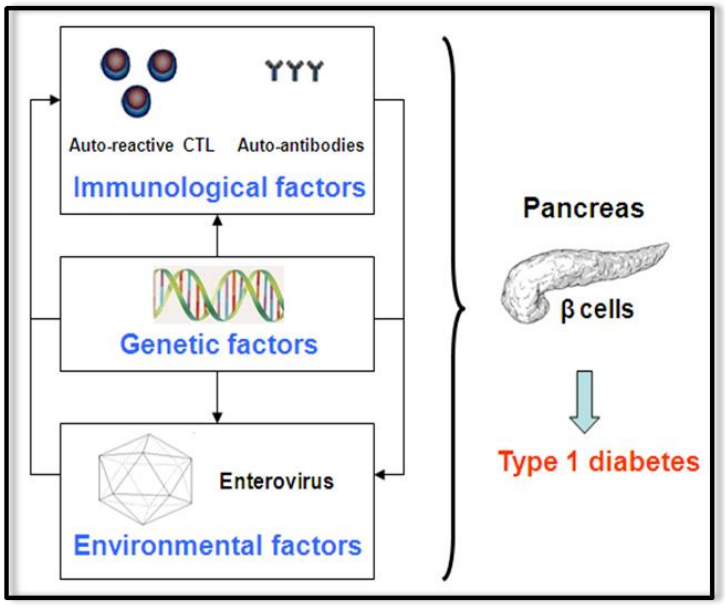


Figure 5. Type 1 diabetes and environmental factors. *T1D is the result of interactions between immunological, genetic, and environmental factors, especially viruses in humans mainly represented by enteroviruses. [Adapted from D. Hober, Enteroviral Pathogenesis of Type 1 Diabetes, Discov Med 10(51):151-160, August 2010]*[5]

5.2.1.1 Genetic Factors

Many components related to the natural history and pathogenesis of T1D has been reviewed in detail.

- Inheritance and Family History:

Inheritance or genetics plays an important role in the onset of T1D is proved by the fact that the risk of developing the disease in general population is only 0.4% while the siblings have 15% more risk of having the disease than the normal population [11, 12]. The disease concordance rates in monozygotic twins are about 30-50% whereas in case of dizygotic twins are about 10 % [13, 14]. It has also been reported that the offspring of diabetic mothers are at a lesser risk (approximately 6%) than the offspring of the diabetic fathers (approximately 12%) [12, 15].

The studies of genetic factors responsible for the onset of T1D mainly have focused on some candidate genes. All of the 4 well-established risk loci, including HLA (Human leukocyte antigen), INS (Insulin), CTLA4 (Cytotoxic T-lymphocyte-associated protein 4), and PTPN22 (Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)) were identified in candidate gene association studies.

- HLA: The most important gene associated with T1D is the HLA class II loci, HLA DRB1 and HLA-DQB1 on chromosome 6p21. DR3 and DR4 haplotypes are the most important contributors to T1D susceptibility that account for 30%–50% of genetic T1D risk [12, 16, 17]. DQ8 haplotype is also another major contributor [12]. The Aspartic acid in position 57 (Asp57) of the DQβ chain is very important and is also associated with T1D onset [18, 19]. A haplotype is "a linked set of genes associated with one haploid genome" [*Adapted from Janeway & Travers, 1994*] [20]. This term is connected with the genes from the Major Histocompatibility Complex (MHC). Usually the individuals inherit the alleles encoded by the closely linked loci as two sets, one from each parent and these alleles are the haplotype[1].Certain other alleles like HLA-DPB1, DRB1, DQA1 and DQB1 loci are strongly implicated in T1D risk [12,17,21-23]. Some HLA-A, HLA-B and HLA-C alleles belonging to HLA class I loci are the other allelomorphisms associated to T1D milderly compared to HLA classII [12] .

Non-MHC Genetic Factors: The Non- MHC Genetic factors include:

Table 1. Non-MHC Genetic Factors associated with Type 1 Diabetes

Non-MHC Genetic Factors	Chromosome Position	Relation to T1D	Ref
INS GENE (Insulin gene)	11p15.5	(i) Confers about 10% of the genetic susceptibility to T1D.	23, 24
		(ii) The INS gene also showed polymorphism. Of all the polymorphism VNTR refers to the association of haplotypes of the region with T1D.	25-28
		(iii) INS genotype is associated with Insulin autoantibody which is also important in the progression of the disease	24
		(iv) Insulin has been suggested as the	29

		primary autoantigen in the NOD mouse model of T1D. This is because in the thymus, decrease expression of insulin is associated with the risk of the disease.	
PTPN22 Gene (Protein tyrosine phosphatase, non-receptor type 22)	1p13	(i) The PTPN22 allele is expressed in T cells and prevents spontaneous T cell activation. Particularly the regulatory T cell repertoire is also affected by the PTPN22 risk allele, thereby further contributing to the emergence of autoimmune diseases. (ii) PTPN22 risk allele allows the accumulation of large numbers of autoreactive mature naive B cells in the periphery. This will help in the development of autoimmune syndromes by capturing and presenting the self-antigens to T cells. Hence it is associated with T1D.	12, 30 30 31
CTLA4 Gene (Cytotoxic T-lymphocyte-associated protein 4)	2q33	(i) The CTLA4 Gene is associated with T1D. (ii) The CTLA4 gene encodes a molecule that functions as a negative regulator of T-cell activation. The G allele of the first exon (Ala17Thr) has been associated with T1D.	12,17, 32
IL2R Gene (Interleukin-2 receptor)	10p15	(i) IL-2 is a critical growth factor for lymphocytes and is important for the initiation of immune responses by promoting differentiation and expansion of $CD4^+$ and $CD8^+$ T cells. (ii) IL-2 is also a key cytokine to immune homeostasis and $CD4^+CD25^+$ T cells are completely dependent on exogenous IL-2 for its growth. (iii) Regulatory T cells (Treg cells) controls the progression of diabetes. Blockage of IL-2 activity might disrupt the T cell development leading to diabetes. (iv) Polymorphisms linked to IL-2, IL 2Rα, and IL-2Rβ are also linked with T1D.	25 33-37 35,36, 38,39 33,40

	(v) Rapamycin/IL-2 therapy study was done in T1D patients, which resulted in transient β-cell dysfunction despite an increase in Tregs. This was done as it is known that Rapamycin/interleukin-2 (IL-2) combination treatment of NOD mice effectively treats autoimmune diabetes.	41

5.2.1.2 Environmental Factors

The environmental factors are of great importance with respect to the increased incidence of T1D. The major environmental factors being pursued are included in the chart below:

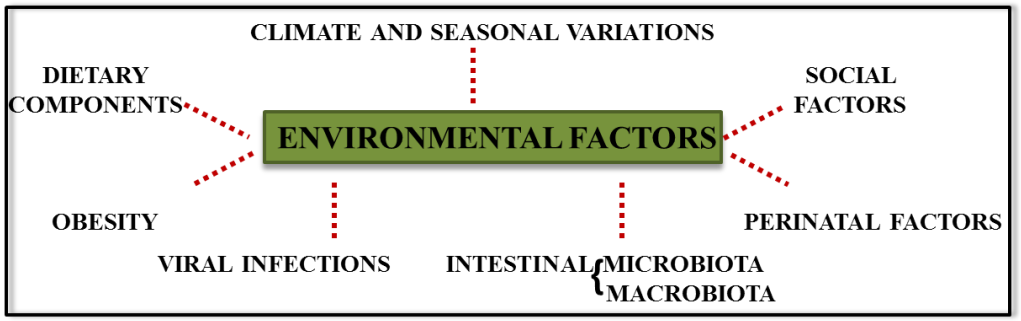


Figure 6. The Environmental Factors associated with Type 1 Diabetes.

5.2.1.2. A. Climate and Seasonal variations

The incidence of T1D also varies according to the climatic changes through different latitudes. The highest incidence rate is found in European populations or in populations of European origin like USA, Canada, Australia, and New Zealand that is in countries located close to the south and the North pole [42, 43] **(Figure 7)**. Moreover, a study done in Australia found that the prevalence of T1D in South Australia is higher than in North Australia. Here, the latitudinal and seasonal variations were involved in these observations [44, 45]. Another environmental factor contributing to T1D is the amount of daylight and sunshine hours, most importantly in Northern Europe, where the incidence of T1D is the highest in the world [45, 46]. It has been proposed that around 90% of plasma vitamin D in humans is produced endogenously when the skin is exposed to the Ultra-Violent Radiation (UVR) from the sun, therefore Northern latitudes would have less vitamin D supply. Also vitamin D deficiency is observed in Australian populations and in Northern Europe [44, 45, 47]. Hence,

these studies have indicated that the lack of oral vitamin D could be involved in T1D onset which is also related to the climate and the season [44, 45, 48]. Vitamin D supplementation showed less risk in developing T1D in the first year of life in EURODIAB study, however it did not gave consistent results subsequently [49].

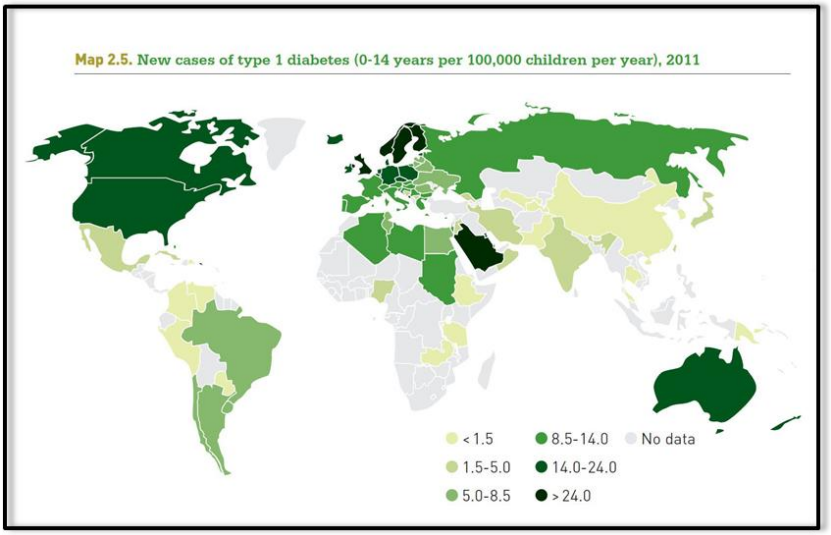


Figure 7. Map of published incidence rates (per 100 000 children) of type 1 diabetes in children. *[Adapted from Diabetes Atlas, 5th Edition 2012]* [50]

5.2.1.2. B. Dietary Components

The T1DM is characterized by a preclinical phase in which environmental exposure, such as food, can contribute to the development of the autoimmune process of pancreatic β-cells destruction. The dietary compounds which are related to the development of T1D are:

- Introduction of cow's milk in childhood [45, 51, 52].
  Conversely breast milk seemed to protect from T1D. This could be due to the fact that breast milk has cytokines and growth factors promoting the maturation of intestinal mucosa helping in the development of the immune system [53-55].
- Introduction of cereals with gluten or other cereal derived proteins before three months after birth have been implicated as potential triggering antigens in T1D [45, 53, 55-57].

- Low intake of Vitamin D and E could trigger T1D onset as they promote the secretion of anti-inflammatory cytokines and improve the antioxidant defense system respectively [53, 58, 59].
- Lower consumption of ω-3 fatty acids and greater consumption of ω-6 fatty acids can increase the incidence of T1D [53].

5.2.1.2. C. Obesity

Physical inactivity and overfeeding results in obesity. According to the Accelator Hypothesis postulated by Wilkins, weight gain causes increased insulin resistance in younger children leading to high blood glucose levels exhausting β-cells and accelerating their loss [53, 60]. The hypothesis states that weight and BMI increase are inversely related to age at diagnosis of T1D [53, 61-66].

5.2.1.2. D. Viral Infections

Viral infections can be considered as one of the environmental factors involved in the aetiology of T1D for more than 100 years.

- Viruses can directly damage the pancreatic β cells. Viruses like enteroviruses are capable of inducing diabetes [53, 67].
- Coxsackie virus is related to T1D onset [48, 53]. Since reduced insulin secretion and inflammation of the islet mediated by the attack of natural killer (NK) cells has been observed in islets infected with Coxsackie B4 enterovirus [53, 68-70].

5.2.1.2. E. Intestinal Microbiota and Macrobiota

The Intestinal Microbiota and macrobiota in the ecosystem of the intestine can affect the incidence of spontaneous T1D in nonobese diabetic (NOD) mice. The NOD mouse is an important model for T1D and exhibit a susceptibility to spontaneous development of autoimmune insulin dependent diabetes mellitus. The animals exposed to microbial stimuli, such as injection with mycobacteria or various microbial products can interfere the disease onset [71-73]. The Specific Pathogen free (SPF) NOD mice donot have activated MyD88 (Myeloid differentiation primary response gene (88)) and they donot develop T1D. MyD88 adapter protein recognizes microbial stimuli. MyD88 is correlated with the gut microbiota

and hence SPF NOD mice having inactive MyD88 with the exposure to a defined microbial consortium mitigate T1D. Thus the innate immune system has interractions going with the intestinal microbiota[7.]

The macrobiota community such as the helminth parasites is related to T1D. Helminthes like *Trichinella spiralis*, *Heligmosomoides polygyrus*, or *Schistosoma mansoni* not only reduces the lymphoid infiltration in the islets but also decreases the rate of T1D, whenever it is inoculated in the NOD mice [74-76].

5.2.1.2. F. Perinatal Factors

Certain factors taking place during the perinatal period are important in the incidence of T1D. Amongst them we find:

- Any kind of infections during pregnancy i.e. intrauterine infections could be affecting the incidence of the disease [53].
- Birth weight can be another important factor as newborn's body weight higher than 4kg can increase a 10% risk in developing T1D [53, 77].
- Caesarean section is also associated with T1D [51, 78, 79].

5.2.1.2. G. Social Factors

Social factors include maternal age, immune diseases (eg. allergy), lifestyle changes which are related to type 1diabetes. According to the "Hygiene Hypothesis" formulated in 1870, the improved hygiene and living conditions decreases the infections in children leading to increasing risk for autoimmune diseases like T1DM [53, 80].

5.2.2 Pathogenesis of Type 1 Diabetes

The pathogenesis of T1D is very well explained by the modern model which integrates the roles for genetics, immunology, and environment [81].

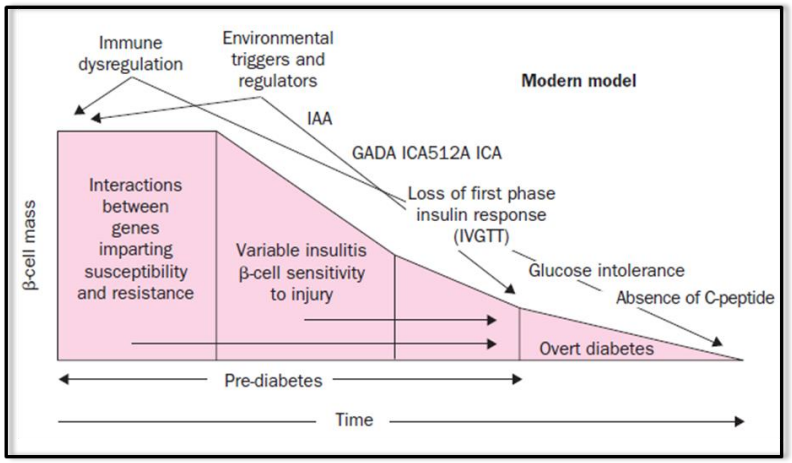


Figure 8. Model of the pathogenesis and natural history of type 1 diabetes. *IVGTT=intravenous glucose tolerance test. IAA=insulin autoantibodies. GADA=glutamic acid decarboxylase. [Adapted from Atkinson and Eisenbarth, 2001]*[81]

The modern model of pathogenesis explains that there is some sort of genetic susceptibility that allows an interaction between the environment and the immune system which leads to an inflammatory condition. This inflammation damages the β cells and makes them even more susceptible to injury. Autoantibodies develop in this process are autoantibodies against insulin (IAA), glutamic acid decarboxylase autoantibodies (GADA), or islet cell autoantibodies (ICA). Eventually there is further loss of islet-cell function so that the first phase of insulin secretion is decreased. There is a progressive loss of islet-cell function, decreasing insulin secretion, the person becomes glucose intolerant, and eventually overt diabetes occurs [81] (**Figure 8**).

The presence of autoantibodies takes place usually between 9 months and 3 years and they are related to the development of T1D [81]. IAA (Insulin autoantibodies) the first and then GADA (Glutamic acid decarboxylase) autoantibodies are present in the infants [81]. The three major islet autoantigens related to T1D are: insulin, glutamic acid decarboxylase (in particular GADA65), and ICA512/IA2A (see **Figure 8**) [81, 82-84.] The presence of other islet

cell antibodies in the absence of these three autoantibodies is associated with a very low risk to diabetes progression [81].

Another model for T1D (in the mid-1980s) combined all the three factors for the pathogenesis of the disease -autoantibody, genetic, and metabolic markers of T1D. It stated that β cell autoimmunity occurred when a genetically susceptible individual having an accurate and fixed number of β cells are exposed to any environmental trigger. This helped to the development of activated autoreactive T cells capable of destroying β cells and islet reactive autoantibodies promoting a progressive loss in insulin secretory function [85, 86].

Both, the CD4 and the CD8 T cells participate in destroying the β cell mass [87, 88]. In NOD mice, the $CD4^+$ T cells are able to transfer the disease from a diabetic donor induce pathogenicity via the proinflammatory cytokine into a NOD/SCID recipient and Fas-induced apoptosis of β cells [89, 90-92]. Effector $CD8^+$ T (CTLs) cells kill β cells by releasing cytotoxic granules, containing perforins which create holes in the plasma membrane of islet cells, allowing cytotoxic serine proteases present in the granules such as granzymes to enter and induce cell death by apoptotic and necrotic pathways [91, 93]. CTLs can also kill β cells, providing Fas ligand (FasL) on their surface, which engages Fas upregulated on β cells, by T cell action thereby initiating apoptosis [91, 92, 94]. Moreover it has been stated that T1D is a Th-1 response mediated process. So, most of the autoreactive T-cell clones produce a Th-1 associated cytokine profile mainly IFN-γ [95, 96]. The two most important effector of T1D pathogenesis are IFN-γ, IL-1β and TNFα [91]. $CD8^+$ T cells and TH-1 $CD4^+$ T cells secrete IFN-γ, and macrophages secrete IL-1β to enhance autoantigen presentation on dendritic cells (DCs) and Fas and MHC class I expression on β cells, thereby augmenting T cell–mediated autoimmunity and promoting β-cell destruction [97]. Hence, there is an interplay going on between the pro and the anti-inflammatory cytokines which determine the fate of the pancreatic β cells.

In addition to the proinflammatory cytokines, ROS (Radicle Oxygen Species) (superoxide, hydrogen peroxide, hydroxyl radicals), a group of highly reactive free radical and non-radical molecules, also play an important role in the pathogenesis of the disease [98, 99]. A recent study found out that the sera of T1D patients exhibited enhanced reactivity to

hydroxyl radical–modified glutamic acid decarboxylase 65 (GAD65), a putative autoantigen in T1D pathogenesis detected in 60% of newly diagnosed patients [91, 100, 101] .

Much has been learned in the last 40 years regarding the pathogenesis and natural history of T1D [85]. T1D has become perhaps one of the most intensively studied autoimmune diseases. A major motivation driving research efforts in these areas is the belief that such gains will result in a means to prevent as well as to reverse the disease [85, 102] . A long-term goal is the development of effective therapy for the prevention of this predictable disorder. Presently it has become very much essential to achieve the clinical targets to preserve health as these newer pathways could enter clinical trials [103].

Table 2. A brief comparison between autoimmune diabetes in NOD mice and men *[Adapted from B.O. Roep, 2003]* [95]

	HUMAN	**NOD MICE**
Name	Type 1 diabetes mellitus	(exp.) autoimmune diabetes
Genetic predisposition	multigenetic trait	multigenetic trait
IDDM-1/idd1	multiple (DR3, DR4, DQ2, DQ8)	one allele (I-Ag7)
Environmental influence	probable	yes
Endogenous retrovirus	?	yes
Incidence	0.25–0.40%	>80%
Gender bias	no	female
Defective peripheral immunoregulation	yes	yes
T-cell driven insulitis	mild	severe
Periinsulitis	no	yes
Lymphocyte infiltrates in other tissues	rarely	always
Disease transmissible with BMT	yes	yes
B lymphocytes required	no	yes
Humoral reactivity to β cells	GAD65, IA-2,insulin,ICA	insulin
Autoantigens	GAD65, IA-2,insulin, p38	GAD65, IA-2,insulin, p38.
Delayed onset with immunosuppression	yes	yes
Successful intervention studies	?	multiple (A-Z)

5.3 Pancreatic β cells

Pancreatic β cells are one of the four major types of cells that are present in the pancreatic islets and they are responsible for the storage and the release of insulin. It is found that adult humans have approximately 2 million islets, which is about 2% by weight of the pancreas [104]. And in rodents, each islet is composed of about 70–80% of β-cells [104]. An individual β cell contains about 13,000 insulin granules [105]. Any variation in the functioning of the β cells will have a deep impact on glucose balance i.e. Hypoglycemia: due to excessive secretion of insulin and hyperglycemia and diabetes: due to insufficient secretion of insulin [106].

5.3.1 Function of the pancreatic β cells

The primary function of the mammalian islet β-cell is to secrete appropriate amounts of insulin, the body's blood glucose-lowering hormone, in response to hormones, nutrients and nervous stimuli [104, 105]. Glucose is the most important nutrient and the prime modulator for insulin secretion, and the process by which insulin is secreted in response to the changing concentrations in glucose is known as glucose stimulated insulin secretion [105, 107] (see **Figure 9**).

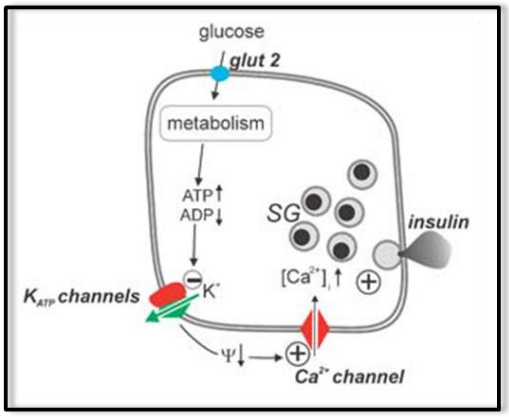


Figure 9. "Stimulus-secretion coupling in pancreatic beta cell".

Abbreviations used: glut2, glucose transporter; KATP channels, ATP-regulated K+ channels; Ψ, membrane potential; SG, secretory granules. The + and – signs denote stimulation and inhibition, respectively, whereas the arrows (↑, ↓) indicate an increase or decrease of the indicated parameter. [Adapted from Rorsma and Renström, 2003][105]

Figure 9 depicts the importance of 2 different ion channels which are responsible for insulin secretion from the β pancreatic cells [105]. And they are-

1. ATP-regulated K^+-channels (K_{ATP}-channels) and

2. Voltage-gated Ca^{2+}- channels.

The pathway proceeds in the following way as described in **Figure 9**:

a. At low glucose level K_{ATP}-channels become active.

b. Negative charge is generated when there is an efflux of positively charged K^+ inside the cell through these channels.

c. Negative membrane potential is generated.

d. Glucose then enters the β cell via the Glut2 transporter which leads to glucose metabolism with the release of ATP.

f. It results in closure of the K_{ATP}-channels that leads to depolarization of the membrane.

h. The K_{ATP}-channels becomes almost inhibited at insulin-releasing glucose concentrations.

i. The opening of voltage-gated Ca^{2+}-channels then take place results in the increase in $[Ca^{2+}]_i$. This triggers the exocytosis of the insulin granules.

j. Moreover insulin granule exocytosis are related to the L-type Ca^{2+}-channels [105, 108,109].

Glucose stimulated insulin secretion involves the 2 main pathways:

(i) Triggering and
(ii) Amplifying signals. [106]

5.3.1.1 Triggering Pathway

The mechanism of how cytoplasmic Ca^{2+} serves as triggering signal in glucose-induced insulin secretion is as follows:

i. Entry of glucose in the β cells by facilitated diffusion through Glut-2.

ii. Oxidation of glucose by glycolysis and Tricarboxylic Acid (TCA) cycle and as a consequence ATP modulation [106, 110-112].

iii. The rise in ATP to-ADP ratio in β-cells.

iv. This leads to the closure of K_{ATP} (ATP-sensitive K^+ channels) channels [106, 113,114]. K^+ channels are tetramers of a complex of two proteins: a high-affinity sulfonylurea receptor (SUR1) which mediates the opening action of Mg^{2+}-ADP and, an inwardly rectifying K^+ channel (Kir 6.2) sensitive to the closing action by ATP [106].

v. Lead to plasmamembrane depolarization.

vi. This depolarization when reaches to the threshold of activation of voltage-operated Ca^{2+} channels, the Ca^{2+} channels opens up resulting in the influx of Ca^{2+} into the β cells [106, 115, 116].

vii. Rise in $[Ca^{2+}]i$. Increase in $[Ca^{2+}]_i$ (intracellular Ca^{2+}) can trigger the insulin secretion [106, 117] .

viii. Activation of the exocytotic machinery. Hence, increased $[Ca^{2+}]$ triggers exocytosis of insulin granules releasing this hormone into circulation [106].

In the β cells pyruvate is the main product of glycolysis. High proportion of glucose-derived carbon enters the mitochondria in the form of pyruvate, the main substrate for both pyruvate dehydrogenase and pyruvate carboxylase, and finally enters in the TCA cycle [118]. Electron transport from the TCA cycle to the respiratory chain is mediated by NADH and $FADH_2$ which promotes the generation of ATP. This ATP is exported then to the cytosol. Thus, the ATP: ADP ratio increase causes depolarization of the plasma membrane by the closure of ATP-sensitive K^+ channels (K_{ATP}) , followed by activation of voltage-dependent calcium channels (VDCCs) in the plasma membrane, and finally leading to glucose-stimulated insulin secretion [118, 119-122].

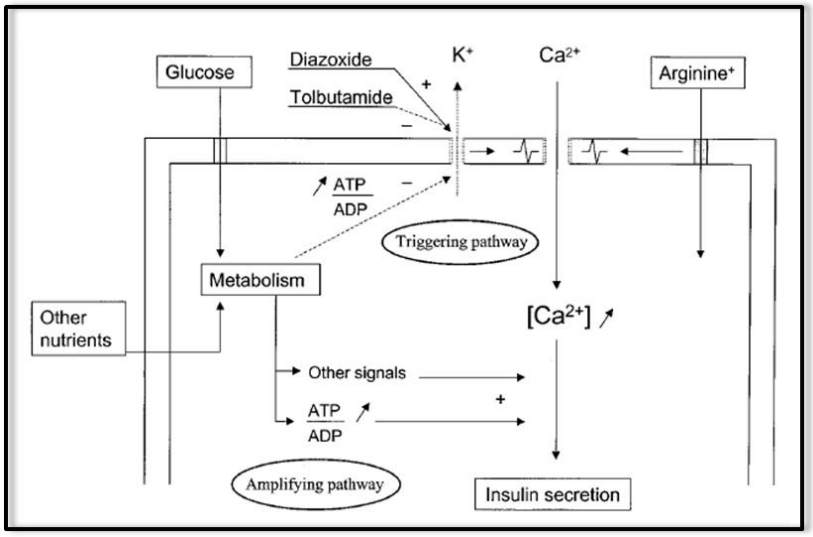


Figure 10. The triggering and amplifying pathways of the stimulation of insulin secretion by glucose. *+, stimulation;–, inhibition. [Adapted from J.C. Henquin, 2000]*[106]

5.3.1.2 Amplifying Pathway

The Amplifying pathway involves glucose, [K_{ATP}] channels and insulin secretion. They are categorized in 2 important conditions:

- When K_{ATP} channels cannot be closed :
  The β cell cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ is elevated and clamped by a depolarization with a high concentration of extracellular K^+ in the presence of diazoxide which opens the K_{ATP} channels and thus allows insulin secretion [106, 123].

- When K_{ATP} channels are completely closed:
  The β cell cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ is elevated and clamped by a depolarization with a high concentration of sulfonylurea which closes the K_{ATP} channels in the presence of diazoxide and thus allows insulin secretion [106].

Glucose induces and increases insulin secretion in a concentration-dependent manner. This Amplifying pathway is activated by all metabolized nutrients which requires glucose metabolism. This amplifying pathway of glucose is also independent of phospholipase C–protein kinase C pathway, protein kinase A and doesnot implicate long chain acryl-CoAs

[106, 124-126]. ATP-to-ADP changes are involved in this process. The amplification pathway consists of an increase in efficacy of Ca^{2+} on exocytosis of insulin granules.

5.3.2 Mitochondrial signaling related to glucose stimulated insulin secretion

The mitochondria are the main source of energy, which is released in the form of ATP and is required for the essential cellular functions like the maintenance of transmembrane ion gradients, protein synthesis and vesicular transport [118]. In the pancreatic β cell, mitochondria play a key role in regulating the glucose-induced insulin secretion [127], not only by providing energy in the form of ATP to support insulin secretion but also by synthesizing metabolites which can perform its role both intra and extramitochondrially, as factors that helps in glucose sensing to the exocytosis of the insulin granule [121].

Moreover, from mitochondrial metabolism derivatives of the β-cell, glutamate have been suggested which directly triggers insulin exocytosis [127, 128]. Glutamate is formed in the mitochondria from α-ketoglutarate, a TCA-cycle intermediate, by glutamate dehydrogenase [127, 129].

The mtGTP (mitochondrial GTP) has an important role to control insulin secretion [122]. Glucose generates GTP which is formed in the mitochondria (mtGTP) and is capable of initiating insulin exocytosis in a Ca^{2+}-independent fashion [118, 130-132].

"**Mitochondrial Diabetes**" is a phenomenon which is caused due to a point mutation and deletion of the mitochondrial-encoding transfer RNA genes in humans [121, 133]. These mutations severely affect mitochondrial protein synthesis, which results in the diminishing number of β cells and insulin secretion [121,133].

5.3.3 Calcium signaling related to glucose stimulated insulin secretion

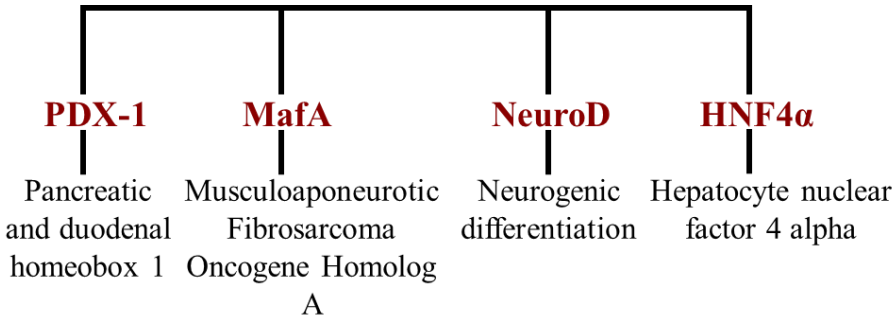
Calcium (Ca^{2+}) is the common denominator for the hormonal, neuronal and metabolic influences regulating the exocytosis process of the islet hormones [134, 135]. Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) regulates insulin secretion from the pancreatic β cells [136,137]. In the β-cell, Calmodulin is the principal Ca^{2+} binding protein and CaM kinase is also the primary mediator of actions of Ca^{2+} [138,139]. In some articles it has also

been suggested that CaM kinase II regulates insulin secretion via phosphorylation of synapsin I-like protein [140].

The Ca^{2+}/calmodulin-dependent protein kinase activity is associated with the cytoskeleton of pancreatic islet cells which is related to glucose-regulated insulin-secretion[141, 142, 143.]

5.3.4 Transcription factors directing pancreatic development and β-cell differentiation

Various transcription factors are important for the development of the pancreas, including the differentiation and function of β cells. Maintenance of these functional mature β-cells is imperative for ensuring glucose homeostasis. The transcription factor regulates a number of genes. This network of transcription factors directs the overall expression of these β-cell genes [144-148]. Some of these transcription factors include: [149,150]



Among them Pancreatic and duodenal homeobox 1(Pdx-1) also known as STF1, IDX1, and IPF1 is one of the most important transcription factors in the pancreas mainly in the β cells[144, 151-153]. Autosomal forms of early-onset diabetes (maturity-onset diabetes of the young [MODY]) is caused when there are mutations in Pdx1 or upstream hepatocyte nuclear factors (HNF) [154].

a) Pdx-1 interacts with the insulin signaling system regulating the function of insulin and it is a critical regulator of β cell plasticity for the maintenance of glucose homeostasis[144, 155-159]. Pdx-1 mediates the antiapoptotic effects of insulin and in turn Pdx-1 thus, helps to regulate the islet apoptosis [159].
b) Pdx-1 regulates the promoter of glucose transporter gene, Glut-2 [144, 155,160,161].
c) Pdx-1 helps the regulation of islet amyloid polypeptide (IAPP), a β cell specific gene [144, 155, 161- 163].

d) Pdx-1 regulates glucagon expression [144,155,161, 164].

e) Pdx1 plays an important role in the activation of its own gene Pdx1 gene [165].

f) Pdx1 induces the glucokinase gene [144, 166, 167].

g) The Irs2–/– (Insulin receptor substrates) mice have β-cell mass reduced with increase in islet apoptosis [154,159,168,169]. In case of these Irs2–/– mice the haploinsufficiency for Pdx1 causes diabetes [154]. But, the overexpression of Pdx1 restores β cell mass and function in Irs2–/– mice and helps to boost up the glucose tolerance throughout life [154].

The other genes like the MafA (Musculoaponeurotic Fibrosarcoma Oncogene Homolog A (Avian)), Neuro D (Neurogenic differentiation), HNF4α (Hepatocyte nuclear factor 4 alpha) are also important in maintaining the function of β cells [149, 150,170].

5.3.5 Apoptosis and β pancreatic cells

Eukaryotic cell death is typically discussed dichotomously as:

Table 3. Apoptosis vs Necrosis [171,172]

	Apoptosis	Necrosis
Definition:	➢ Active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation.	➢ Passive, accidental cell death caused by ischemic, chemical, physical, or thermal cell injury.
Effect:	➢ Beneficial	➢ Detrimental
Process:	➢ Membrane blebbing i.e. budding off membrane- bound apoptotic bodies containing organelles and nuclear fragments, cell shrinkage, chromatin condensation, nuclear fragmentation and chromosomal DNA fragmentation.	➢ Loss of selective permeability of the cell membrane, hypoxia which causes ATP depletion, metabolic collapse, cell swelling and rupture leading to inflammation.
Result:	➢ Can prevent tumor formation.	➢ Necrosis results in inflammation, which could become chronic.

It is proposed that programmed cell death or apoptosis is the cause of increased loss of β-cell mass in autoimmune diabetes in rodents and probably also in humans [171].

(i) Apoptosis by FAS Dependent pathway.
(ii) Apoptosis by FAS Independent pathway.

There is considerable experimental evidence supporting the involvement of pro-apoptotic pathway initiated by stimulation of CD95 (Fas) through its ligand CD95L (FasL) in initiating and achieving β cell death in NOD mice [173-175]. Moerover, β cell apoptosis may be also caused by the induction of FAS on β cells by T cell infiltration. IL-1β and IFN-γ regulate FAS expression on β cells [92, 171].

Secondly, the Fas-independent mechanisms causing β cell apoptosis mediated by pro-inflammatory cytokines IL-1β and IFN-γ[176-178].

The cytokine IL-1β alone is being intensively studied for its implication in the induction of apoptosis and necrosis in the β cell [171,179-182]. Functional inhibition of β cell, DNA fragmentation and apoptosis caused by IL-1 β are independent of iNOS (inducible Nitric Oxide Synthase), while DNA single strand breakage and necrosis is mostly due to to iNOS induction by IL-1β [171, 180,183,184]. Recent studies using mice deficient in iNOS (iNOS-/-), showed that induction of Fas on the surface of islets by IL-1 β is independent of iNOS while the combination of different human cytokines cause apoptosis independent of iNOS[179, 185, 186]. However, recent work including ours show that, despite the potential of IL-1β as a cytotoxic agent for β cell *in vitro*, the absence of signaling by this cytokine in vivo does not abrogate the development of diabetes in NOD mice, nor has profound effects on the kinetics of the same [92,187,188]. For example the elimination of Caspase 1 (Interleukin-1-Converting Enzyme (ICE)), responsible for cleavage of the mature cytokine from the pro-cytokine, does not affect the onset of diabetes in NOD mice [187]. Moreover, despite the demonstrated evidence of toxicity of interleukin 1 on the β cell has shown that the absence of interleukin-1 receptor (IL-1R) in NOD mice has an effect only delaying the appearance of disease, but not on the cumulative incidence of the same [188]. These results show that different mechanisms cytotoxic and pro-apoptotic *in vivo* that can supply the deficiency of IL-1 β.

It is hypothesized that CD8 would be responsible for initial β cell damage and final disease exacerbation and release of the auto-antigenic cell content of the first β damaged cells in a

proinflammatory environment would cause the activation of autoreactive $CD4^+$T cells [189]. $CD4^+$ T lymphocytes are the primary effector cells causing the destruction of pancreatic β in a Fas dependent fashion[190]. Activated macrophages secrete IL-1 β, which together with IFN-γ and TNFα, cause severe alterations in β-cell function and compromise their viability, causing its death. Apoptosis mainly results from the activation of caspase 3 [191]. The cytokines IL-1α, IL-1β and IFN-γ secreted by macrophages and T cells, respectively, seem to "mark" β cells to make them susceptible to Fas-mediated apoptosis [192]. IFN-γ action on β cell involves the transcription factor STAT-1 (signal transducer and activator of transcription 1) while signaling via the IL-1 β, IL-1RI, and TNFα, TNFRI , is mainly done through the transcription factor NF κ B and MAPK [171]. Both signaling currents interact through the MAPK by activating Raf by JAK (Janus Kinases tyrosine) pathway.

The effects caused by pro-inflammatory cytokines in the β cell include:

(i) activation and translocation of transcription factors to the nucleus,
(ii) induction of transcription of target genes (iNOS, Fas, ICE, c-myc, etc.) [193],
(iii) post-translational modification of proteins (phosphorylation state / dephosphorylation)[171] .

Experimental approaches have been made to address the differential expression of genes (genomic analysis) due to the action of proinflammatory cytokines in the β cell or islets [193]. Some molecules have been identified to undergo changes in the β cells or islets resulting from *in vitro* incubation with IL-1 β and / or IFN-γ [193]. In certain reports it is mentioned that the combination of three cytokines (IL-1β, TNFα, and IFN-γ) was not only able to induce apoptotic cell death in invitro culture but also in special doses can be able to induce DNA strand breaks [194].

All of the above points to a scenario in which there are multiple mechanisms involved in the induction of beta cell death. But late goals remain to be discovered, which probably reside the confluence of different pathways marked by different agents involved in the induction of cell death. Intervention in such sites, it should have a definite impact on the development of autoimmune diabetes.

5.4 An approach towards the treatment of Type 1 Diabetes

5.4.1 Treatment with Insulin

Insulin was discovered in the year 1921 [81]. Insulin has the capability to induce β-cell rest and has antiapoptotic effect on β-cells which contributes in preventing the disease [171].

Benefits: The availability of rapid-acting insulins has also contributed to the greater use of insulin as a therapeutic approach towards T1D.

5.4.2 Regeneration of β cells

It is hypothesized that most people with long-standing T1D have some remnant β cells, despite ongoing autoimmunity and glucose toxicity implies that concomitant new β cell formation must be occurring [195,196]. Hence, the regeneration of β cells constitutes another target for the therapy of T1D. There are many studies done in order to regenerate β pancreatic cells. The well-known studies points to:

(i) In the adult life or after pancreatectomy in mice, the new β cells are generated from the pre-existing β-cells by its replication and not from the pluripotent stem cells [195, 197].

(ii) A single, murine, adult pancreatic precursor exists that can be differentiated into cells with the characteristics of islet β cells [195,198] (neogenesis or transdifferentitation).

5.4.3 Transplantation

Pancreatic Transplantation: Pancreatic transplantation has been a successful therapeutic approach for many years [195, 199, 200]. But it requires continuous immunosuppression to prevent rejection and also to block recurrent autoimmune islet destruction [81, 195, 201]. Also the immunosuppressive regimens for pancreas transplantation such as mycophenolate

mofetil make pancreas transplantation more successful for patients with diabetes [81].

Limitations: The limitation for pancreatic transplantation is the availability of donor organs [195].

Islet Transplantation: An alternative option to pancreatic transplantation is the islet transplantation to the liver via portal vein through the catheter [81,195]. With the help of the Edmonton Group and Edmonton protocol the use of modified immunosuppressive regimen like daclizumab, sirolimus, and tacrolimus and islets from more than 1 donor pancreas per recipient, success rates were 80% in 1 year and 20% in 5 years have been reported [81, 195, 202-204] . Hence, transplantation showed that autoimmune and alloimmune islet destruction can be overcome, and that isolated islets can cure diabetes.

Limitations: The donor islets are in short supply as more than one donor pancreas is needed for each patient [81, 195].

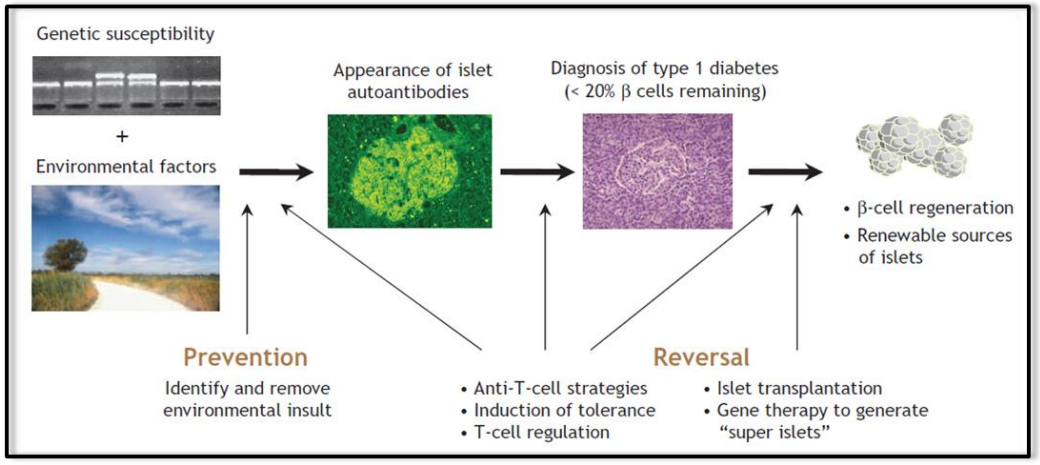


Figure 11. "Potential targets for therapeutic intervention of type 1 diabetes".
(i) to prevent initiation of autoimmunity
(ii) to reverse the effects of ongoing autoimmunity coupled with β-cell regeneration
[Adapted from Kathleen M. Gillespie, 2006] [195]

5.5 Cell Cycle

The cell cycle is an ordered ubiquitous complex process involved in cell growth and proliferation with organism development. It is an ordered set of events resulting in duplication or division into two daughter cells. In eukaryotic cells, at first the DNA in each chromosome divides and produces two identical copies and the replicated chromosomes are segregated into two identical daughter cells [205, 206]. The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and the production of two daughter cells [205].The cell passes through a number of phases. At first is the G1 phase for DNA synthesis and the G2 phase where the cell gets ready for mitosis [205].

Over the last 10 years or so, major advances have been made in understanding the machinery controlling cell cycle progression, mainly in analyzing cell cycle regulatory proteins. The two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine the cell's progress through the cell cycle. The other group of proteins comprehends cyclin dependent kinases inhibitors (CKIs), the pocket protein retinoblastoma family and the E2F family of transcription factors [207].

The entry into cell cycle in mammal eukaryotic cells is coordinated by D (delta)-type cyclins: D1, D2 and D3. D-type of cyclins interacts with the corresponding cyclin-dependent kinases, CDK4 and CDK6, to form a holoenzimatic complex, which activity triggers cell cycle progression through G1 phase towards the S phase (see **Figure 12**).

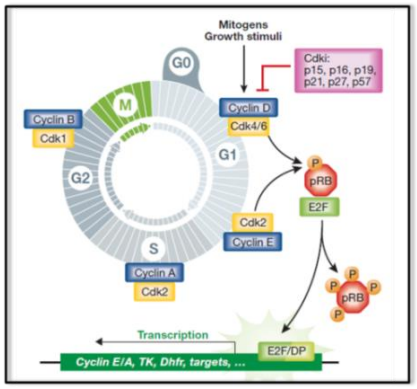


Figure 12. Molecular Regulation of cell cycle. *[Adapted from V. Aguilar & L. Fajas, 2010]* [207]

5.5.1 Cyclin and CDK proteins

5.5.1.1 Cyclins

The cyclins were first discovered by Timothy Hunt and his colleagues in the early 1980's as oscillating proteins that drive the entry into mitosis [208-210]. They were named cyclins because the levels of these proteins vary periodically during the cell cycle which means that the synthesis of this protein drove cells into mitosis and its degradation allowed cells to finish one cell cycle and begin the next [210] (see **Figure 13**). They were first discovered in Sea urchins, Arbacia, and later were discovered in other species [211].

One of the major functions of these proteins is to regulate the activity of their catalytic binding partners, the cyclin-dependent kinases (CDKs). The detailed function of the cyclins is illustrated in Table 4.

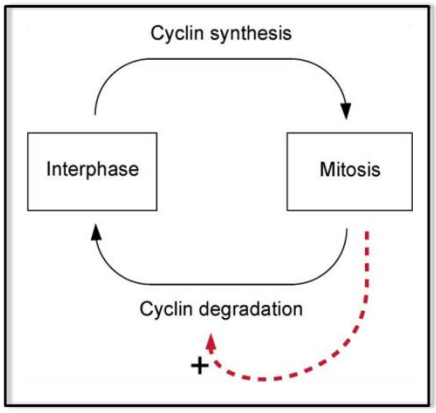


Figure 13. The Original Cyclin-Based Model for the Cell Cycle. *The simplest possible model for the cell cycle based on the discovery of cyclin. [Adapted from Andrew W. Murray, 2004]*[208]

5.5.1.2 CDK

The CDKs are subunits of a large family of heterodimeric serine/threonine protein kinases that are involved in controlling progression through the cell cycle.

During the Cold Spring Harbor Symposium on the Cell Cycle in 1991, a group of interested scientists proposed that members of the kinase family whose activity depended on

association with some cyclin-like regulatory subunit should be called cyclin-dependent kinases (CDKs) [212].

Thus by now 29 Cyclins and more than 20 CDKs have been identified in mammalian cells [209, 213].

In mammalian cells, cyclins bind to CDK and form complexes that are involved in regulation of different cell cycle phases:

Table 4. Importance and Functions of the Cyclin-CDK complexes [208, 214,215]

CYCLIN-CDK COMPLEX	IMPORTANCE	FUNCTION OF CYCLINS
Cyclin D-CDK4/6	G1 progression	Cyclin D helps in cell growth and proliferation.
Cyclin E-CDK2	G1-S transition	Cyclin E supports DNA replication and centrosome duplication.
Cyclin A-CDK2	S phase progression	Cyclin A supports DNA replication, centrosome duplication and mitosis.
Cyclin A/B-CDC2 or CDK1	Entry into M-phase	Cyclin A supports DNA replication, centrosome duplication and mitosis. Cyclin B supports mitosis alone.

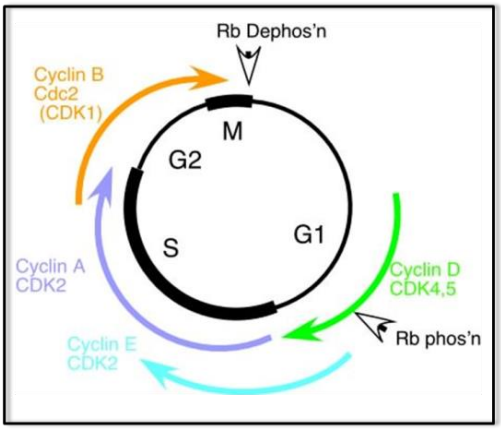


Figure 14. "Dynamic regulation of cyclin levels throughout the cell cycle". *The cyclins bind to the cyclin-dependent protein kinase subunit (CDK), activating it and promoting cell cycle progression. The specific cyclin-CDK complexes which are responsible for directing*

progression during G1, S, or G2/M phase of cell cycle are described. [Adapted from D. Pinheiro & C.Sunkel, 2012] [215]

5.5.1.3 D Type Cyclins

D-type cyclins (cyclins D1, D2, and D3) are components of the core cell cycle machinery. Entry into the cell cycle (G1 → S) in eukaryotic cells is coordinated by mammalian D-type cyclins. The 3 most important D type cyclins are: cyclin D1, D2 and D3. All the three encode 33–36kDa proteins that share an average of 57% identity over the entire coding region and 78% identity in the N-terminal ‘‘Cyclin box’’ [216].

5.5.1.3.1 Cyclin D1, D2 and D3

Cyclin D1, D2 and D3

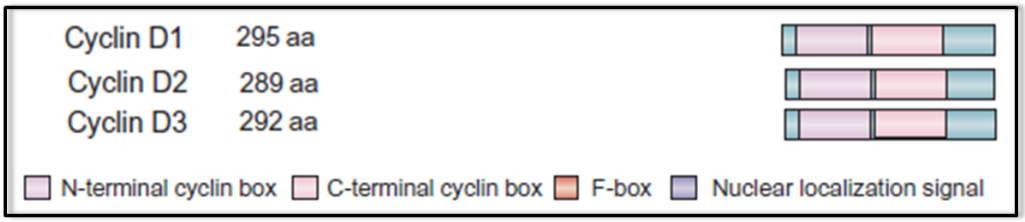


Figure 15. The mammalian protein Cyclin D1, D2 and D3. *The complete amino acid sequences of the human proteins were alligned using CLUSTAL software* [217, 218]*. The number of amino acids (aa) deduced from the nucleotide sequence and the principal structural domains are indicated for each protein. [Adapted from M. Malumbres & M. Barbacid 2005]*[218]

5.5.1.3.1.1 CDK-dependent roles of D-type cyclins

The regulation of cell cycle is controlled by CDKs in association with their key regulatory partners, the cyclins, CDK inhibitors INK, Cip/Kip family together with the tumor suppressor retinoblastoma (Rb) protein [219,220]. The D-type cyclins interact with the corresponding cyclin dependent kinase, (CDK4 and CDK6), to form an holoenzymatic complex, the activity of which causes cell progression through the G1 phase of the cell cycle into S phase. D type cyclins are essential for connecting to mitogenic signals to Rb/E2F pathway, which regulates the transcription of factors involved in the subsequent

cell cycle progression [209,221] (see **Figure 16**). At first, the cyclin D CDK4/CDK6 promotes the phosphorylation of pRb (retinoblastoma tumor suppressor protein) protein and, hence, its partial release from E2F, leading to the transcription of factors important for DNA synthesis [222] and, promotes the entry of cells into S phase. The E2Fs are a large family of transcription factors consisting of one or more DNA binding domains (DBDs) that bind target promoters and regulate their expression [223-225]. Cyclin D / CDK (4/6) holoenzimatic complexes do not restrict their action to cell cycle progression, but seem to have a key-role in metabolic control, eg. insulin secretion by the pancreatic β cell [226]. Moreover, it has been suggested that cell cycle progression is dependent on the control that the Cyclin D / CDK (4/6) holoenzimatic active complex exerts on the cell metabolism [207]. The existence of three D-type cyclins could be due to their different tissue expression patterns and their expressions varies considerably among different cell types therefore having essential functions in particular tissues [209, 227]. The pRB-related pocket proteins are p107 and p130, and they directly associate with E2Fs and can be co-recruited to E2F-responsive promoters to inhibit gene expression induced by active E2F [225, 228, 229]. Besides the pRB- family members, recent studies show that cyclinD-CDK4 complexes have other substrates for instance:

i. Smads contain potential phosphorylation sites for CDK. Smad 3 is a good substrate for CDK4 and also it is phosphorylated by CDK4 and CDK6 [230].
ii. Cyclin D1/CDK4 complex helps in the phosphorylation of BRCA1, a multifunctional tumor suppressor protein and coordinates DNA damage repair [231].
iii. TSC (Tuberous Sclerosis 2) is the cell growth regulator and acts as a cyclin D-binding protein. Cyclin D1–CDK4/6 coexpression in cultured cells leads to increased phosphorylation of both TSC2 and TSC1, and promotes the phosphorylation of the mTOR substrates, 4E-BP1 and S6K1, two key effectors of cell growth that are negatively regulated by the TSC1-TSC2 complex.
iv. Phosphoylation and futher degradation of Runx2 (runt-related gene 2) is brought about by the cyclin D1–CDK4 complex in an ubiquitin-proteasome-dependent manner. Runx2 is involved in the activation of genes encoding osteoblast and chondrocyte-specific proteins [219].

v. CDK4/6 is involved in the regulation of GATA4 (Globin transcription factor 4). Cyclin D1 activates CDK4, which in turn inhibits the differentiation of cardiomyocytes by degradation of GATA4 [232].

vi. The suppression of skeletal muscle differentiation in proliferating myoblasts is carried out by cyclin D–CDK4 complex [233].

vii. Cyclin D1 deficiency causes increased mitochondrial size and activity. This was further enhanced by cyclin D1 in a CDK-dependent manner [234].

These are some important functions of the cyclins related to CDK activities.

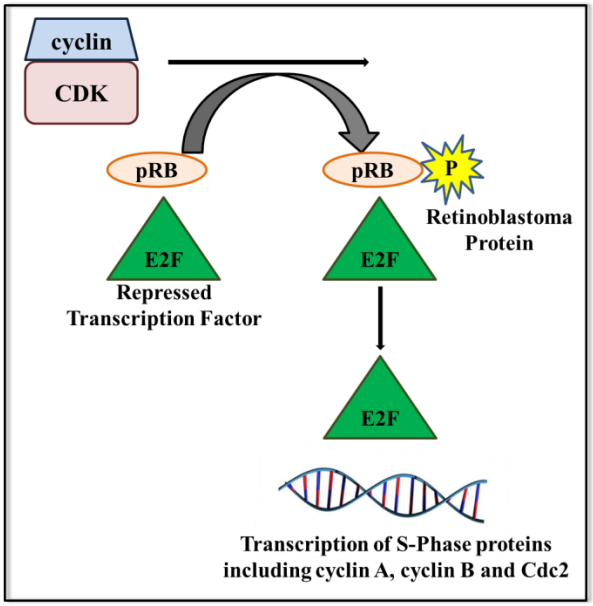


Figure 16. Rb/E2F pathway. *Cyclin D binds to CDK4/or CDK6. Cyclin/CDK complex phosphorylate the pRB repressor proteins (Retinoblastoma Proteins). The phosphorylated pRb dissociates from E2F Transcription Factor which in turn is activated and induces transcription of S-Phase proteins including cyclin A, cyclin B and Cdc2*[239].

5.5.1.3.1.2 CDK-independent roles of D-type cyclins

In addition to its CDK-binding function, a body of evidence indicates that D-type cyclins have CDK-independent activities[235-238]. The CDK independent activities for the 3 D-type cyclins are:

- Cyclin D1 is associated with transcription as it forms physical associations with more than 30 transcription factors or transcriptional coregulators [235,236, 239-241].
- D-type cyclins regulate the activity of some nuclear receptors –
  i. It is a positive regulator of Erα (Estrogen Receptor α) -mediated transcription [235,242-246]. It associates with p300/CBP (p300/cAMP response element-binding protein-binding protein)-associated factor (P/CAF) and potentiates activation of ERα [235,245] Cyclin D2.
  ii. Cyclin D3 interacts poorly with Erα [242, 247].
  iii. Cyclin D1 and D3 regulate the Androgen receptor (AR) activity[235, 248-251]. (Cyclin D1 repressesAR- hormone dependent signaling in a P/CAF-dependent manner [235,249]. Cyclin D3 also inhibits the androgen receptor [248].
  iv. PPARγ is a ligand-activated transcription factor and plays a critical role in fatty acid metabolism, energy homeostasis, and adipogenesis [235, 252, 253]. (Cyclin D3 interacts with the peroxisome proliferator-activated receptor gamma (PPARγ) and thus acts as an important factor governing adipogenesis and obesity [254]. Cyclin D1 also binds to thyroid hormone receptor and PPARγ in cultured cells [235].
  v. Both basal and ligand-dependent transactivation of nuclear receptors is regulated by cyclin D1 [235,242-244, 248-252, 255].
- It has been stated that cyclin D1 may be also associated with the regulation of myoblast differentiation by interfering with the MyoD CDK4 interaction, which normally disrupts the ability of MyoD (Myogenic differentiation) to induce myogenesis [235,239].
- TATA-box binding protein-associated factor (II) 250 is associated with cyclin D1. This causes the suppression of pRb-mediated inhibition of TATA-box binding protein-associated factor (II)250 kinase activity [235,256,257]. pRb activity is impaired when there is a deletion of the N-terminal 20 amino acids of cyclin D1. But this doesnot affect cyclin D1 transforming ability which suggested that these domains may be are involved in cyclin D1 transformation [235,236, 258]. Cyclin D1 regulates the expression of genes that are involved in DNA replication and the DNA damage checkpoint. By binding BRCA2 and the recombinase RAD51, CyclinD1 facilitates

the recruitment of RAD51 to sites of DNA damage and DNA repair mediated by homologous recombination [259,260].

- Cyclin D1/ and D3 physically associates with transcriptional factors or coactivators including HATs (Histone Acetyltransferase) and HDACs (Histone Deacetylase) to regulate transcription and epigenetic changes [261].
- Cyclin D1 acts as a regulator of metabolism and cell growth evidenced by the fact that Cyclin D1(-/-) mice have small body size, hepatic steatosis, hypoplastic retinopathy, and defective mammary development during pregnancy [209, 235,262,263].

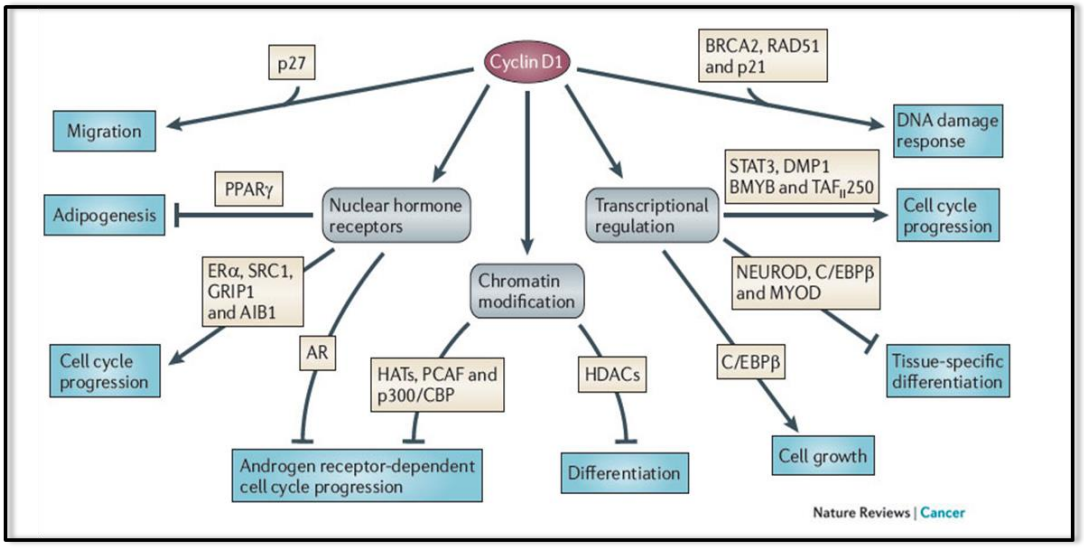


Figure 17. "CDK-independent functions of cyclin D1". *Cyclin D1 helps in cell duplication, growth, differentiation, cell cycle progression through the interaction and binding with several transcriptional factors. [Adapted from E. A. Musgrove, 2011]*[264]

5.5.1.3.1.3 Cyclin D3

Cyclin D3 is a soluble protein belonging to the subfamily of D-type cyclins. It consists of 292 amino acids and approximately 32 kDa molecular weight. . This is an unstable protein, a half-life of approximately 30 hours in mammals.

Its location can be nuclear or cytoplasmic.

Figure 18. The mammalian protein Cyclin D3. *The complete amino acid sequence of the human protein was alligned using CLUSTAL software* [217,218]*. The number of amino acids (aa) deduced from the nucleotide sequence and the principal structural domains are indicated. [Adapted from M. Malumbres & M. Barbacid 2005]* [218]

The Role of Cyclin D3 in Cell Cycle

D type cyclins are necessary for entry into cell cycle G1 / S phase by transmitting signals to mitogenic pathway marked by the pair consisting of the retinoblastoma protein (Rb) and the transcription factor E2F1, which is inactive when bound to hypophosphorylated retinoblastoma protein. Cyclin D3 also enhances the action of the transcription factor ATF5 (Activating Factor Transcrition 5-alpha/beta) [265]. ATF-5 is an activator of transcription factor that binds to the cAMP response element (CRE) present in different promoters [265]. Interestingly, the transcriptional activity of ATF-5 is increased by cyclin D3, whereas CDK4 has the opposite effect on it [265]. D-type cyclins have dual role in its activity: (i) firstly it is involved in the cell cycle progression of CDK-dependent manner and (ii) involved in CDK-independent transcriptional activation. This duality has been described in both: cyclin D1 and D3, respectively [254, 265, 266]. Hence, cyclin D3 can interact with different kinds of transcription factors and play an important role in the regulation of transcription [267]. In turn, cyclin D3 becomes an activated target of the transcription factor E2F1, when it has been activated by the cyclin D/CDK4 holoenzimatic complex [268]. It has also been detected a physical interaction between cyclin D3 and ERK3, a highly unstable protein and a mitogen-activated kinase (MAPK).

It is also interesting to note that cyclin D3, and not cyclin D1, is highly expressed in human pancreatic cancers [269], while both D1 and D3 are associated with the progression of pancreatic adenocarcinomas [269]. The promoter of cyclin D3 presents binding sequences of such factors GATA, NF-κ B, ATF, E2F, etc. [270], NF-κB transcription factor being linked to action mediated by cytokines such as IL-1β and TNFα [171].

We have generated NOD mice deficient in cyclin D3, in collaboration with Dr. Peter Sicinski (Dana Farber Cancer Institute, Harvard, MA, USA), these mice developed exacerbated diabetes compared with wild type littermates (as seen in our article Ávilaet.al see Publication). Therefore, cyclin D3 protects against diabetes and is a candidate molecular target therapy.

5.5.1.4 CDK11

The CDKs are the catalytic subunits of a large family of heterodimeric serine/threonine protein kinases. Their characterized members are involved in controlling progression through the cell cycle [218] (see **Figure 19**).

The CDK11, belongs to the family of p34cdc2 kinases, and comprises of a group of PITSLRE protein-kinases which is ubiquitously expressed in both mouse and human. 10 different CDK11 isoforms with their molecular weights varying from 46 to 110 kDa has been cloned from the eukaryotic cells [271, 272]. But, CDK11 protein has two main gene products:

(i) p58 and p130 in mouse
(ii) p58 and p110 in humans.

Figure 19. The mammalian protein CDK11 . *The complete amino acid sequences of the human proteins were alligned using CLUSTAL software [217,218]. [Adapted from M. Malumbres & M. Barbacid, 2005]* [218]

- In Mouse, CDK11p110 and CDK11p58 are encoded by a single gene - **cdc2l**.
- In Humans, CDK11p110 and CDK11p58 are encoded by two genes in humans –

Table 5: Different products of CDK11, its location and functions.

	SIZE	LOCATION	FUNCTION	REFERENCE
CDK11p58	58kDa	Expressed only in the mitosis stage (G2/M) [273, 274].	(i) The actions of CDK11p58 is fundamental in mitosis, since it maintains the cohesion of the sister chromatids. (ii) CDK11p58 is involved in cytokinesis, bipolar spindle assembly, centrosome maturation and the completion of mitosis. (iii) CDK11p58 is related to apoptosis in various cell lines. It downregulates the anti-apoptotic protein Bcl-2. (iv) CDK11p58 represses estrogen receptor α (Erα) (related to transcription) function and negatively regulates cell growth.	275 276, 277 278,279 280
CDK11p110	110 kDa	Expressed throughout the cell cycle [273,274]	(i) CDK11p110 is associated with the cyclin L regulatory subunit, and several factors of "splicing" or pre-mRNA processing and regulates the transcription and processing pre-mRNA in proliferating cells. (ii) CDK11p110 plays an important role in the regulation of RNA transcript processing in addition to affecting RNA transcript production. (iii)Cdk11p110 isoform interacts with pre-mRNA splicing factors RNSP1 and 9G8, RNA polymerase II (RNAP II),and casein kinase 2 (CK2). Thus it links the two processes: transcription and splicing.	281,282 277, 282 283-285 273,282

CDK11p46/ p60	46kDa 60kDa	Both CDK11p58 and CDK11p110 in response to pro-apoptotic stimuli yields two fragments: P60: N-terminal region, part of the regulatory kinase P46: C region - terminal containing the catalytic domain.	(i) Mediates the amplification of apoptotic processes.	286-288

Table 6. Representative mouse models carrying gene-targeted CDK alleles

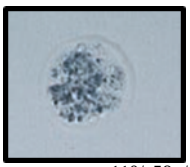
KINASE	GENOTYPE	PHENOTYPE	PICTURES (of fixed Blastocysts)
CDK11	CDK11 $^{p110/p58\ +/+}$	Mice develop normally	 CDK11 $^{p110/p58\ +/+}$
CDK11	CDK11 $^{p110/p58\ +/-}$	Mice are viable and appear to develop normally.	 CDK11 $^{p110/p58\ +/-}$
CDK11	CDK11 $^{p110/p58\ -/-}$	Embryonic lethality in peri-implantation embryos accompanied by mitotic aberrations. Embryonic cell death was due to apoptosis	 CDK11 $^{p110/p58\ -/-}$

Figure 20: CDK11p110/ p58-/- blastocyst cells fails to proliferate normally. The fixed blastocysts were stained with anti-BrdU antibody. *[Adapted from Tongyuan Li et al, 2004]*[276]

5.5.1.5 D type Cyclins, CDKs and β pancreatic cells

The β cell mass plays an important role in determining the amount of insulin that is secreted to maintain the body's glucose levels within a narrow range (around 5mM).

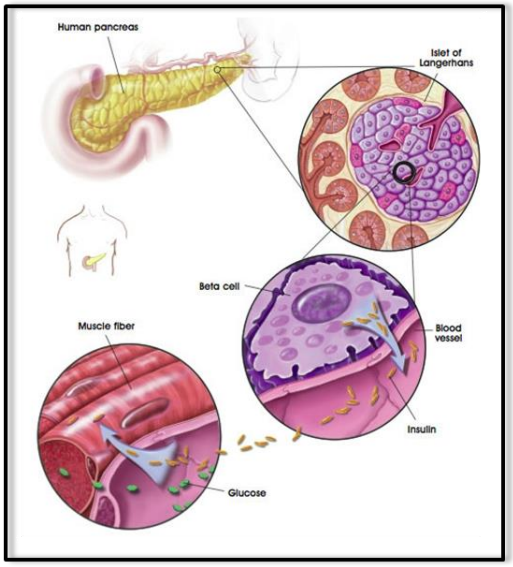


Figure 21. Insulin Production in the Human Pancreas. *The diagram shows the location of the pancreas in the abdomen beside the duodenum, the cross section of the pancreas shows the islet of Langerhans enclosing the insulin secreting β cell. [Adapted from Terese Winslow, Lydia Kibiuk, 2001]* [289].

A balance between β cell generation and death is required to maintain β cell mass homeostasis.

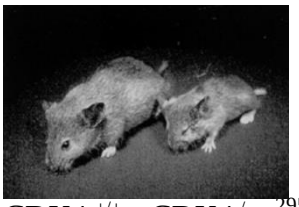
Expression of the D type cyclins and the CDKs in the pancreatic β cells has been reported before (Table 7).

Table 7. Occurence of Cyclins and CDKs.

	ORGANISM	REFERENCE
Cyclin D1	Mice, Rat, Human	290
Cyclin D2	Mice, Rat	291,292
Cyclin D3	Mice, Human	291,293
CDK4	Mice, Human	294,295,296
CDK6	Human	294

Table 8. Relationship of Cyclins and CDKs with β pancreatic cells and their importance in Type 1 Diabetes.

Cyclins and CDKs	Relation with β cells and Importance in diabetes		Reference
Cyclin D1	(i) Overexpression of CyclinD1 in β cells *in vivo* results in islet hyperplasia without hypo-glycemia, it is not tumorigenic and does not result in diabetes [297]. (ii) It can even induce cell proliferation in rat and human islets and is important for normal postnatal islet growth [290, 261,298]. Thus, cyclin D1 may have potential as a novel therapy to stimulate β-cell replication.	In CyclinD1$^{+/-}$ mice: Donot develop diabetes. [261]	261, 290, 297, 298
Cyclin D2	(i) Cyclin D2 is one of the most important among the cyclins that causes β cell growth [261]. (ii) Cyclin D2 is involved in promoting β-cell proliferation throughout the adulthood of mice [292,298].	In CyclinD2$^{-/-}$ mice: Develop severe diabetes by 12 weeks due to inadequate β cell mass & altered β cell replication. Shows deregulated glucose homeostasis as well as glucose intolerance.	298,299 299
Cyclin D1 & Cyclin D2	(i) Both cyclin D1 and cyclin D2 are very important cyclins for postnatal islet growth [299]. (ii) Both cyclin D1 and cyclin D2 are essential for the regeneration of the β pancreatic mass [298].	In CyclinD1$^{+/-}$ Cyclin D2$^{-/-}$ Mice: Show profound defects in glucose tolerance, impaired islet growth resulting in very small islets with few β cells. Hence, develop severe diabetes. In CcnD1$^{-/-}$CcnD2$^{-/-}$ Mice: They die before or soon after birth.	299 299

Cyclin D3	(i) In mice, but not in humans, cyclin D3 is expressed in less amount than cyclin D1 and cyclin D2 in the pancreatic islets. (ii) They are important for β cell growth in absence of cyclin D2 [261].	Cyclin $D3^{-/-}$ Mice: They fail to undergo normal expansion of immatureT lymphocytes. (Our group) $CcnD2^{-/-}$ $CcnD3^{+/-}$ Mice: Slow progression to diabetes.	[300] [261]
CDK4	(i) CDK4 plays an important role in the postnatal development of the pancreatic islets [301, 295, 290] (ii) Our group has shown that CDK4 has an important role in NOD mice, and is also expressed in healthy human β cells.	$CDK4^{-/-}$ Mice: Has defective β cell proliferation, degeneration of islets or forming hyperplastic islets and develop severe diabetes.  $CDK4^{+/+}$ $CDK4^{-/-}$ [295]	[292,299, 301, 295, 302, 303]
CDK6-Cyclin D1	(i) Helps in the proliferation of the human β cells around ~9fold increase [290,294].		

5.5.1.6 CDK and D-type cyclins: Their role in Glucose Metabolism

Glucose is the body's fuel. Glucose metabolism involves two different pathways: Anaerobic and Aerobic. The anaerobic process occurs in the cytoplasm. The aerobic cycle takes place in the mitochondria and results in ATP production. As the name implies, it requires oxygen. Glucose metabolism takes place differently in quiescent cells and proliferating cells. In case of quiescent cells glucose is converted to pyruvate which is then oxidized in the TCA cycle releasing energy in the form of ATP. They can oxidize other substrates like amino acids and fatty acids obtained from either the environment or the degradation of cellular macromolecules. In proliferating cells have higher rates of glycolysis derived lactate production, biosynthesis of lipids and other macromolecules [304]. Metabolism is always related to cell cycle and that is explained in the above Table 8,

which suggests that CDK4, cyclin D1 and cyclin D2 are all related to cell proliferation. And, since the regulation of cell growth is directly related to metabolism, all of these proteins are related to the metabolic control of the cells. Normal glucose level homeostasis is achieved by the coordinated secretion of insulin and glucagon, produced by β and α cells in pancreatic islets, respectively [305, 306].

Cyclin D/CDK4 complex not only plays an important role in cell cycle but also act as a metabolic driver. Evidences are made in mice, worms, flies and plants. Cell proliferation and growth depends on cell metabolism (**Figure 22**) [207]. β cell proliferation is a key mechanism to maintain postnatal β-cell mass [207, 307,308], and it is the primary mechanism for β-cell regeneration [197,299,309].

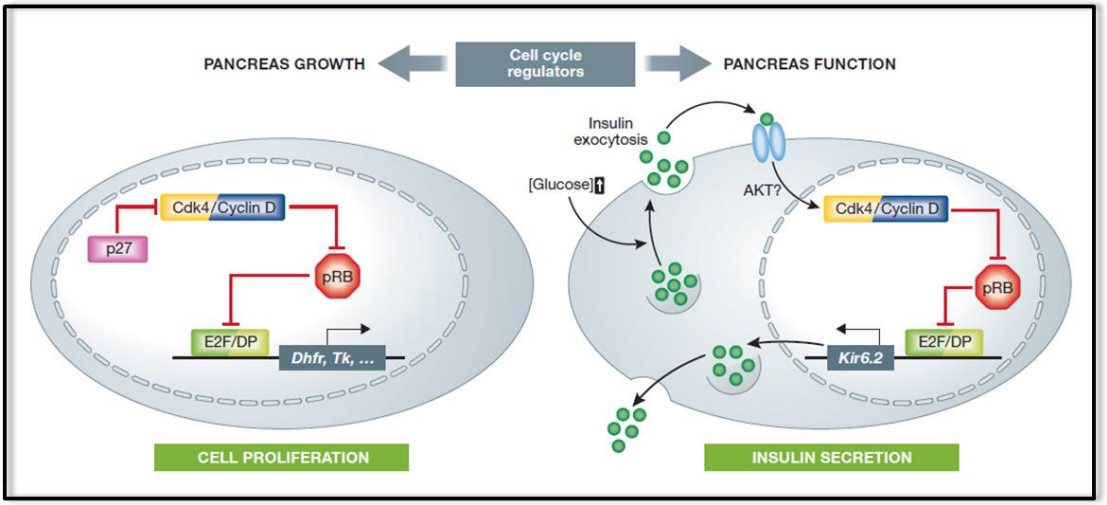


Figure 22. "Schematic representation of the dual role of cell cycle regulators in β cell growth and fitness." *The figure depicts the pancreatic growth by cell proliferation and pancreatic function by insulin secretion via the cdk-pRB-E2F pathway. [Adapted from V. Aguilar & L. Fajas, 2010]*[207]

The E2F activity controls the transcription of a group of genes that encode proteins important for cell cycle progression [305, 310]. Beside this, E2f1 functions in the regulation of glucose homeostasis [207]. E2f1-/- mice have decreased pancreatic size, as a result of impaired postnatal pancreatic growth. E2F1 protein controls both β-cell number and function [226, 311]. E2F1 directly regulates Kir6.2 expression. Kir6.2 is a key component of the K_{ATP} channel involved in the regulation of glucose-induced insulin secretion in pancreatic

β-cells [207,226]. It was also seen that Kir6.2 expression is regulated at the promoter level by the CDK4–pRB–E2F1 pathway. Kir6.2 expression is lost in E2f1-/- mice leading to defects in insulin secretion in mice. But, E2F1-/- mice do not develop diabetes but have dramatically increased insulin sensitivity. The E2f1/E2f2 double mutant mice displayed insulin dependent diabetes [207, 305,312].

The mTOR pathway is central for the transduction of nutrient availability signals [207, 313]. Strikingly, the metabolic phenotype of E2F1-/- mice is reminiscent of the phenotype of mice carrying inactivating mutations in the mTOR substrate S6K1[207, 314, 315]. Both E2F1-/- and S6K1-/- mice show impaired metabolism in pancreatic β-cells, adipose tissue, muscle and likely in other tissues with metabolic functions. Hence it suggests a cross talk between the mTOR-S6K and cdk4-RB-E2F1 pathways and mTOR-S6K pathway controls metabolic processes, at least in part through regulation of the cdk4-pRB-E2F1 activity [207].

CDK5 has also been implicated in the regulation of insulin secretion. It is a member of CDK family with no known cyclin partner. Mice lacking the CDK5 activator p35 show increased insulin secretion in response to elevated glucose and its chemical inhibition resulted in increased insulin secretion in isolated β-cells [207, 316]. 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^{++} influx through the L-VDCC (Voltage dependent calcium channels) channels, a required event in the insulin secretion process. It was also found that the L-VDCC channel was not inhibited in p35-/- or CDK5-inhibited β-cells [316]. Hence it was concluded that the α1C subunit of L-VDCC was a phosphorylation-inactivating target of CDK5. Reports suggested also that CDK5 is involved in the regulation of glucose homeostasis in adipocytes. It was reported that CDK5 phosphorylation of TC10alpha (a Rho family GTPase) increases GLUT4 (glucose transporter whose activity is markedly regulated by insulin in muscle and adipose tissue cells) translocation and hence glucose import in adipocytes [207,317,318]. GLUT4 translocation was also increased by CDK5 phosphorylation of E-Syt1 (a 5C2- domain protein related to synaptotagmins). E-Syt1 phosphorylation leads to its increased association with GLUT4 and increased glucose uptake [207,319].

Since CDK activity is regulated by CDK inhibitors, it is not surprising that these proteins also have a role in the control of glucose homeostasis. p21-/- , a Cip/Kip inhibitor mice under chow diet showed no change in insulin sensitivity and secretion whereas these mice displayed improved insulin resistance under high fat high sucrose (HFHS) diet when compared to WT mice [207,320]. In contrast, p27 was shown to participate in β-cell mass determination [207,321]. p27 gene deletion caused increased islet mass and insulin secretion, and hyperglycemia prevention 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 in these mice could explain the distinct phenotypes [207].

5.5.1.7 Cyclin D3 and CDK11: How are they related?

It has been suggested cyclin D3, specifically interacts with the G2/M CDK, p58PITSLRE, and that resulted in enhanced kinase activity of p58PITSLRE. Moreover the kinase activity of the p58 is also regulated by cyclin D3 [322].

Table 9. Relationship between cyclin D3 and CDK11p58.

	SITE OF INTERACTION	FUNCTION	REFERENCE
Androgen receptor (AR), a member of the nuclear receptor Family.		Cyclin D32/CDK11p58 holoenzyme kinase complex act as a negative regulator of AR. The cyclin D3/ CDK11p58 signaling pathway might participate in the regulation of AR-dependent physiological and patho-logical activities in male reproductive systems.	[323] YEAR: 2007
Spinal cord	Neurons and glial cells of the damaged spinal cord. Mainly Nuclear.	The expression and interaction of CDK11p58 and cyclin D3 is important in dissecting cellular and molecular mechanisms of spinal cord injury.	[324] YEAR: 2008
Schwann cells	Nuclear region	Cyclin D3/CDK11p58 complex plays an important role in Schwann cells proliferation and apoposis induced by Lipopolysaccharide (LPS).	[325] YEAR: 2010

Therefore, cyclin D3 functioned not only in G1 phase as a regulatory subunit of CDK4 and 6 but also in G2/M phase as a partner of CDK11p58 during cell cycle progression.

Using the Microarray technology our group has identified two genes, cyclin D3 and CDK11, which experience downregulation in pancreatic islet endocrine cells during the autoimmune attack progression. As our group has already shown that cyclin D3 alone can protect from diabetes (Ávila et al., See Publication), in this thesis I addressed whether the cyclin D3 and CDK11 partnership is relevant for T1D onset.

5.6 Brief Description of our model of choice for our work

5.6.1 *IN VIVO* MODEL

5.6.1.1 NON OBESE DIABETIC MOUSE (NOD)

The NOD mouse emerged in Japan about 30 years ago around 1980 and was originally found from an outbred ICR mouse [326,327]. The NOD mice develop spontaneous autoimmune diabetes. At first, the mononuclear infiltrates surrounds the pancreatic islet called peri-insulitis which progresses further and invades the whole of the islet known as pancreatic infiltration, a chacteristic feature of T1D. This is followed by the destruction of the insulin-producing β cells of the pancreas which leads to T1D [326,327,328]. In pancreatic infiltrates $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, dendritic cells, and macrophages are found. T cells are essential for the pathogenesis of T1D. [175,326,329].

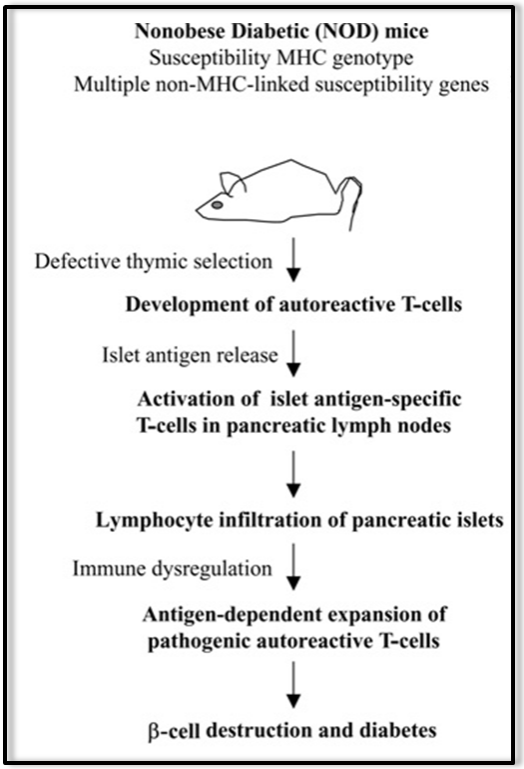


Figure 23. "Evolution of the diabetogenic autoimmune response in spontaneous model of autoimmune diabetes". *[Adapted from Y. Yang & P. Santamaria, 2006]*[330]

The female mice develop diabetes faster than the male mice at around 12 to 14 weeks of age. At early 5 weeks of age female mice develops early insulitis. The male mice develops later. But at 10 and 14 weeks the female NOD mice exhibit more pronounced inflammatory changes in the islets. This may be because of the late regulatory events in the male mice that controls the disease progression [326,327].

Our NOD colony is kept in the Specific Pathogen Free Zone (SPF) i.e. free of pathogens as the frequency of the disease increases significanty reaching nearly 100% in the germ free environment. However the incidence of the disease decreases if the NOD mice are infected with the pathogens like bacteria, virus or certain parasites [25,331].

Hence the female NOD mouse has been chosen as the model of choice in our work.

5.6.1.2 NOD/SCID MOUSE

A NOD strain was developed congenic for the severe combined immunodeficiency (scid) mutation. This mouse is the NOD/SCID mouse [334]. NOD-SCID/SCID mouse are diabetes resistant as they lack the functional T and B lymphocytes because of the PRKC mutation [332, 333]. Diabetes can be adoptively transferred by either T lymphocytes or bone marrow [332, 334,335]. So, we are using the NOD/SCID mouse to observe the occurrence of adoptive transferred diabetes by spleen cells from NOD donor (**Figure 24**).

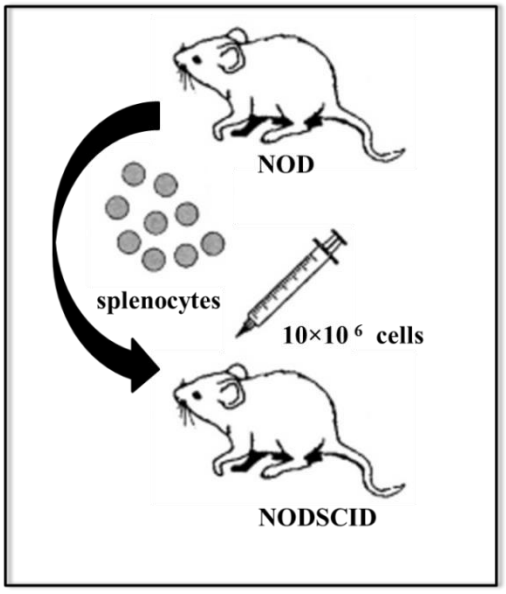


Figure 24. Adoptive transfer of splenocytes from NOD to NOD/SCID

5.6.2 *IN VITRO* MODEL:

5.6.2.1 Nit-1 insulinoma cell line

NIT-1 cells are derived from the spontaneously diabetic nonobese diabetic (NOD) mouse and are characterised by glucose- responsive insulin secretion and ultrastructural features of differentiated mouse beta cells [336-338].

NIT-1 cell is a pancreatic beta insulinoma cell line which bears the characteristic feature of the pancreatic beta cell having high insulin content [339]. NIT-1 cell is derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic β cells [339].

Hypothesis

6. HYPOTHESIS

The working hypothesis of the present study is that pancreatic β cell mass homeostasis requires the interaction between CDK11 and Cyclin D3 in pancreatic beta cell.

Over the last 10 years or so, major advances have been made in understanding the machinery controlling cell cycle progression, mainly in analyzing cell cycle regulatory proteins. The cell cycle proteins are the proteins that mainly control the progression of cells through the cell cycle.

The mRNA expression of several genes changes during pancreatic islet infiltration in the NOD mice prior to T1D in comparison to the lymphocyte-free NOD/SCID mouse model. Among these genes cyclin D3 and CDK11 are both downregulated.

We hypothesize in our present work that-

i. Cyclin D3 and CDK11 are required for β cell mass homeostasis and they interact in pancreatic β cell.

ii. CDK11 downregulation affects negatively cell cycle completion (CDK11p58 related to apoptosis and expressed in mitotic stage of cell division), and transcription of suitable genes (CDK11p130) in proliferating β cells as well as, impeding β cell mass recovery due to the autoimmune attack.

Objectives

7. OBJECTIVES

The experimental goals to test our hypothesis are the following:

7.1 Main Goal

Assess the relative expression levels between cyclin D3 and Cdk11 required to keep β cell mass homeostasis. That is, in other words to determine the role of cyclin D3 and CDK11, respectively, in pancreatic β cell mass metabolic and cell homeostasis.

7.2 Specific Goals

To study the interaction between cyclin D3 and CDK11 in pancreatic β cells *in vitro* and *in vivo*.

- ***IN VITRO***

  Determine whether cyclin D3 and/or CDK11p58 overexpression on NIT-1 NOD insulinoma cell line

  i. Aggravates or protects these cells from cytokine induced apoptosis and/or necrosis.
  ii. Promotes cell proliferation.

- ***IN VIVO***

  i. To determine whether in the CDK11(Δ/+) CcnD3(-/-) mice i.e. in the mice having deficiency of cyclin D3 and hemideficiency of CDK11 the onset of diabetes is faster than in CDK11(+/+) CcnD3(-/-) mice.
  ii. To assess whether in CDK11(Δ/+) CcnD3Tg- mice diabetes onset is faster than the mice with CDK11(Δ/+) CcnD3Tg+.
  iii. To confirm the interaction between cyclin D3 and CDK11 in the pancreatic β cells.

Materials

8. MATERIALS

8.1 TABLE 10. MATERIALS FOR *IN VIVO* AND *IN VITRO* TECHNIQUES

COMPANY	PRODUCT
BD (Becton, Dickinson and Company)	Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) Bacto Agar Bacto Tryptone Bacto Yeast Extract Propidium iodide staining solution
Biontex (San Diego, USA)	Metafectene® Pro
Biorad Laboratories(CA,USA)	30% Acrylamide/Bis solution 29:1
BioSera (Boussens, France)	Gentamycin 1.21mM Penicillin (100X) Glutamin (100X
Corning (USA)	Cryotubes
CTL	DPX mountant for microscopy
eBioscience (San Diego, CA, USA)	Protein A Anti-Rabbit IgG bead slurry
Falcon(BD Labware , USA)	Serological pipet (different volume) Plates 100X 20 mm 6 well plates for cell culture 96 well plates for cell culture 24 well plates for cell culture
Fermentas	PageRuler™ Prestained Protein Ladder Plus
Fisher Scientific (Madrid ,Spain)	Centrifuge tubes (different volume)
Fluka(Sigma Aldrich) (St.Louis ,MO, USA)	β-Mercaptothanol Chlorophorm
G.E. healthcare (Buckinghamshire,UK)	X-ray film
GIBCO-Invitrogen(Carlsbad ,USA)	Trypsin EDTA SYBR safe DNA gel stain β-Mercaptoethanol
Invitrogen (Scotland)	Proteinase K Trizol Reagent β-mercaptoethanol Cell scrapers
KLeinipath Good(s) in pathology (Netherlands)	Microscopic slides
Life Technologies	Sybr safe DNA gel stain
Lonza (Verviers, Belgium)	Dulbecco's Modified Eagle Medium

	(DMEM) media Dulbecco's Phosphate Buffer Saline (DPBS) Hanks' Balanced Salt Solution (HBSS) Fetal bovine serum (FBS) Trypsin-EDTA
Millipore (Bedford, MA, U.S.A)	PVDF membrane
NESTLE SVELTESSE	Non fat dry milk
PAA (Pasching, Austria)	G418 Sulphate
Panreac (Barcelona, Spain)	Ethanol (100%) Ethanol(96%) Hydrochloric acid (37% HCl) Isopropanol Methanol
Prospec (Passaic County, NJ, USA)	IL-1β cytokine IFN-γ cytokine
Panreac (Barcelona, Spain)	Ethanol (100%) Ethanol(96%) Hydrochloric acid (37% HCl) Isopropanol Methanol
Roche (Germany)	Collagenase- P
Santa cruz biotechnology, inc.	Protease Inhibitor cocktail tablets
SERVA Electrophoresis GmbH (Heidelberg,Germany)	TRIS
Sigma Aldrich (St.Louis ,MO, USA)	2-Propanol Albumin ,Bovine,96-99% Agarose Ammonium Persulfate Ampicilln Brilliant blue r Bromphenol blue sodium salt Calcium chloride Coomassie blue DMSO (Dimethyl Sulfoxide) EDTA Eosin FastRed Chromogen Ficoll Formamide Glycerol Glycine Harris hematoxylin HEPES

	Imidazole Magnesium chloride Mowiol Paraformaldehyde Potassium phosphate dibasic acs reagent Potassium phosphate monobasic acs reagent Saccharose SDS Sodium azide Sodium bicarbonate reagent Sodium chloride Sodium citrate dihydrate Sodium hydroxide pellets Sodium phosphate dibasic Sodium phosphate monobasic Sodium pyhrophosphate tetrabasic decahydrate TBE (10X) N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED) TRIS Triton X-100 Trizma Base Tween 20
Thermo Scientific	Pierce ECL Western blotting substrate SuperSignal West Femto maxximum sensitivity substrate DNA Ladder 100bp DNA Ladder 1kb λ DNA Marker dATP 100mM dCTP 100mM dGTP 100mM dTTP 100mM DEPC Treated Water ECO LADDER

8.2 TABLE 11. PCR PRIMER Sequences

Primers used for the genotyping of mice

Primer	Sequence	Size of the PCR product	Annealing Temp
Cyclin D3 KO			
D3A	GAA CGT TGT GAC GTA GGA GC	400bp	60ºC
D3G	TCC ATC CTG CGA TGG CTC AC		
D3N3	TGC TGT CCA TCT GCA CGA GA		
Cyclin D3 Tg			
RIP2	Forward: CAA GAC TCC AGG GAT TTG AGG GA	460bp	60ºC
D3R	Reverse: GAC GCA GGA CAG GTA GCG ATC CAG		
CDK11			
Mp70	GAG ATA CTC TTT ACA TGC CAA CC	320bp	60ºC
Cprev	CAA GAG AAG CCT GAG CAA ATAG		
SCID			
OIMR 803	GGA AAA GAA TTG GTA TCC AC	38bp, 28bp and 11bp	53ºC
OIMR 804	AGT TAT AAC AGC TGG GTT GGC		

Primers used for rT-PCR in cell lines

Primer	Sequence	Size of the PCR product	Annealing Temp
CDK11			
CDK11 clof	Forward: GAT TAA CAA GAT TTT CAA GGA CCT GGG TAC TCC TAG	1400bp	62ºC
Ealb1	Reverse: CGG CGT TCT ACA ACA TTG CG		
CDK11 clof	Forward: GAT TAA CAA GAT TTT CAA GGA CCT GGG TAC TCC TAG	500bp	62ºC
E alpha	Reverse: GCG GAA GAG GTG ATC GTC CCT C		

Housekeeping gene			
HPRT1	Forward: GTT GGA TAC AGG CCA GAC TTT GTT G	400bp	62ºC
HPRT2	Reverse: GAG GGT AGG CTG GCC TAT AGG CT		

8.3 TABLE 12. ANTIBODIES

Flow Cytometry			
Primary antibody	**Dilutions**	**Clone**	**Company**
anti-mouse GLUT-2 biotinylated	1:100	205115	R & D Systems, Germany
Purified Mouse anti-CyclinD3	1:100	1/Cyclin D3	BD Pharmingen, clone 1, California, USA
Monoclonal Rat anti-mouse Ki-67	1:100	TEC-3	Dako, California, USA
Annexin V FITC-conjugated	5 ul in 195ul of buffer	1-65874X (Comp No.)	BD Biosciences
PE-conjugated anti-mouse CD45	1:100	HI30	BD, Pharmingen; California, USA
Propidium Iodide	10ul in 190 ul of buffer	51-66211E (Comp No)	BD Biosciences
Secondary antibody		**Catalogue No.**	
CF™405S	1:200	SCJ4600013	Sigma-Aldrich, St.Louis ,MO, USA
Streptavidin-APC	1:200	31274246	Immunotools, Germany
Alkaline phosphatase staining (AP)			
Primary antibody		**Catalogue No.**	**Company**
Polyclonal Guineapig anti-Insulin	1:1000	A0564	Dako, California, USA
Secondary antibody			**Company**
Biotinylated secondaryAb	1 drop ready	QA900-9L	Biogenex

	to use			(California, USA)
Alkaline Phosphatase Substrate	1 drop ready to use	QA900-9L		Biogenex (California, USA)
Immunofluorescence				
Primary antibody				**Company**
Polyclonal Guineapig anti-Insulin	1:500	A0564		Dako (California, USA)
Hoeschst 33342 1mg/mL	1:200	B 2261 (Product No.)		Sigma Aldrich (St. Louis, USA)
Secondary antibody				
anti-guineapig Cy-2	1:500	706-225-148		Jackson Immunoresearch laboratories (West Grove, PA, USA)
Western Blot				
Primary antibody	**Molecular weight (KDa)**	**Dilutions**		**Company**
anti-rabbit PIC CDK11	58 & 130 kDa	1:100		a gift from Dr. Jill Lahti, USA.
β-Actin	42kDa	1:2000		Sigma
Secondary antibody			**Cat no.**	
Goat anti-rabbit HRP conjugated		1:1000	1858415	Pierce, Thermo Scientific
Goat anti-mouse HRP conjugated		1:2000	1858413	Pierce, Thermo Scientific

Methods

9. METHODS

9.1 *IN VITRO*

9.1.1 Cell culture

A NIT-1 pancreatic beta insulinoma cell line was generously provided by Dr. J. Verdaguer. NIT-1 cell is a pancreatic β cell line which is derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic β cells [339].

All operations of the cell culture were carried out under strict aseptic conditions. Cells were cultured in a CO_2 incubator under a humid atmosphere at 37°C, supplied with 5% CO_2. The cells were cultured in DMEM medium with L-glutamine and was supplemented (Table 13) and pre-warmed to 37°C.

Table 13: Composition of DMEM medium

	Volume	**Final Concentration**
DMEM Medium (22.7mM glucose and L-glutamine)	500ml	
Fetal bovine serum	50ml	10%
Penicillin-Streptomycin (100X)	5ml	-
L-Glutamine (100X)	5ml	2mM
β-mercaptoethanol	50µl	1.75mM

9.1.1.1 Trypsinization of adherent cells

In order to allow subculture or harvesting, cultured cells were trypsinized under the commonly used procedure for trypsinization of cells in a monolayer culture. Briefly after reaching an adequate confluency, cells were rinsed with warm phosphate buffer saline (PBS, pH 7.4, 37ºC) to eliminate traces of serum from the medium, and then incubated with 0.25% trypsin EDTA solution for 5 mins at 37ºC. Trypsinization was stopped by dilution with serum supplemented high glucose DMEM medium and the resuspended cells were centrifuged at 1500 rpm 5 minutes RT. The supernatant was discarded and the cell pellet was resuspended in 10 ml of fresh medium. From this cell suspension the cells were

counted and subsequently seeded in adequate flasks and dishes depending on experimental design (around 2-5×10^6 cells for stock and 10^5- 2×10^5 cells for experiment).

9.1.1.2 Cryopreservation of cells

Cells were grown until approximately 80% confluency and fresh growth medium was added 24 hours before freezing. For freezing, the cells were trypsinized as previously described. After centrifugation the cell pellet was resuspended in special freezing medium composed of Fetal Bovine Serum supplemented with 10% DMSO. The cell suspension was aseptically aliquoted into sterile cryovials which were placed in Styrofoam container. Cells underwent process of gradual freezing (48 hours at -80ºC) to avoid formation of intracellular crystals and then were transferred to a liquid nitrogen freezer for long time storage.

9.1.1.3 Thawing of cultured cells (Cell recovery)

Thawing of cells was done in a 37ºC water bath by gentle agitation for approximately 1-2 minutes. As soon as the content was thawed, the cryovial was removed from the water bath and decontaminated by spraying with 70% ethanol. The vial content was transferred to a centrifuge tube containing 9 ml of complete culture medium and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended with complete medium and dispensed into a 75 cm culture flask for further propagation.

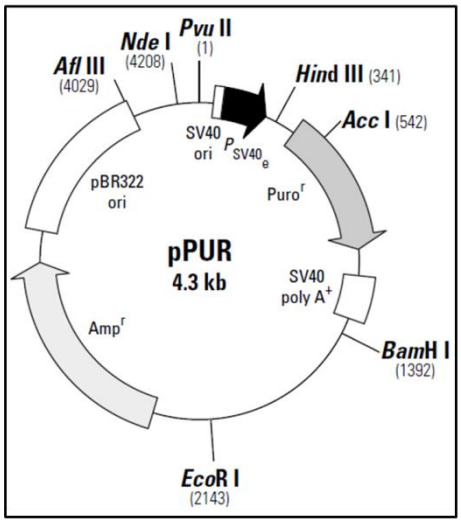
9.1.2 Subcloning of either CD11p58 or cyclin D3 for transfection

We performed two types of subcloning in order to transfect NIT-1 cells.

a) The cDNAs of either mouse CDK11p58 or mouse cyclin D3 were subcloned under the control Rat Insulin Promoter (RIP) in a plasmid derived from pBluescript SK (Stratagene) named pBSK-NEO, which confers resistence to Neomycine (Figure 25 D & E) . As a negative control, cells were tranfected with the vector pBSK-NEO with no cDNA insert. Eα is a genomic sequence from the I-Eα^d gene [340].

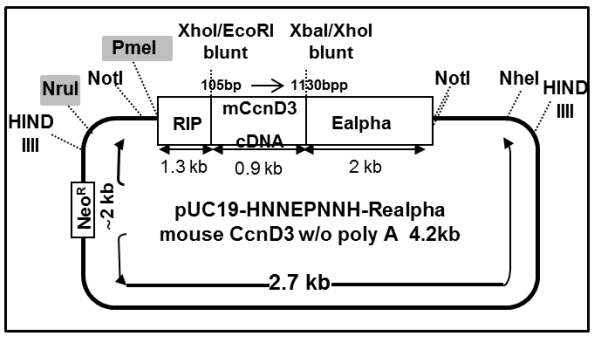
b) The respective cDNAs of CDK11p58 and cyclin D3 were subcloned under the Rat Insulin Promoter (RIP) in a vector that confers resistance to puromycin (pPUR, InvitroGen) (Figure 25 F & G). As a negative control, cells were tranfected with the vector pPUR (Figure 25A) without a cDNA insert.

A



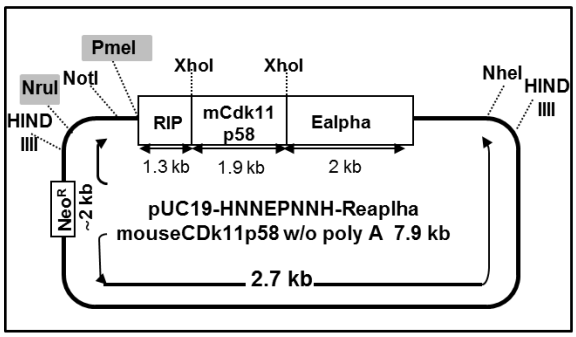
pPUR vector

B



pUC19 cyclin D3

C



pUC19 CDK11p58

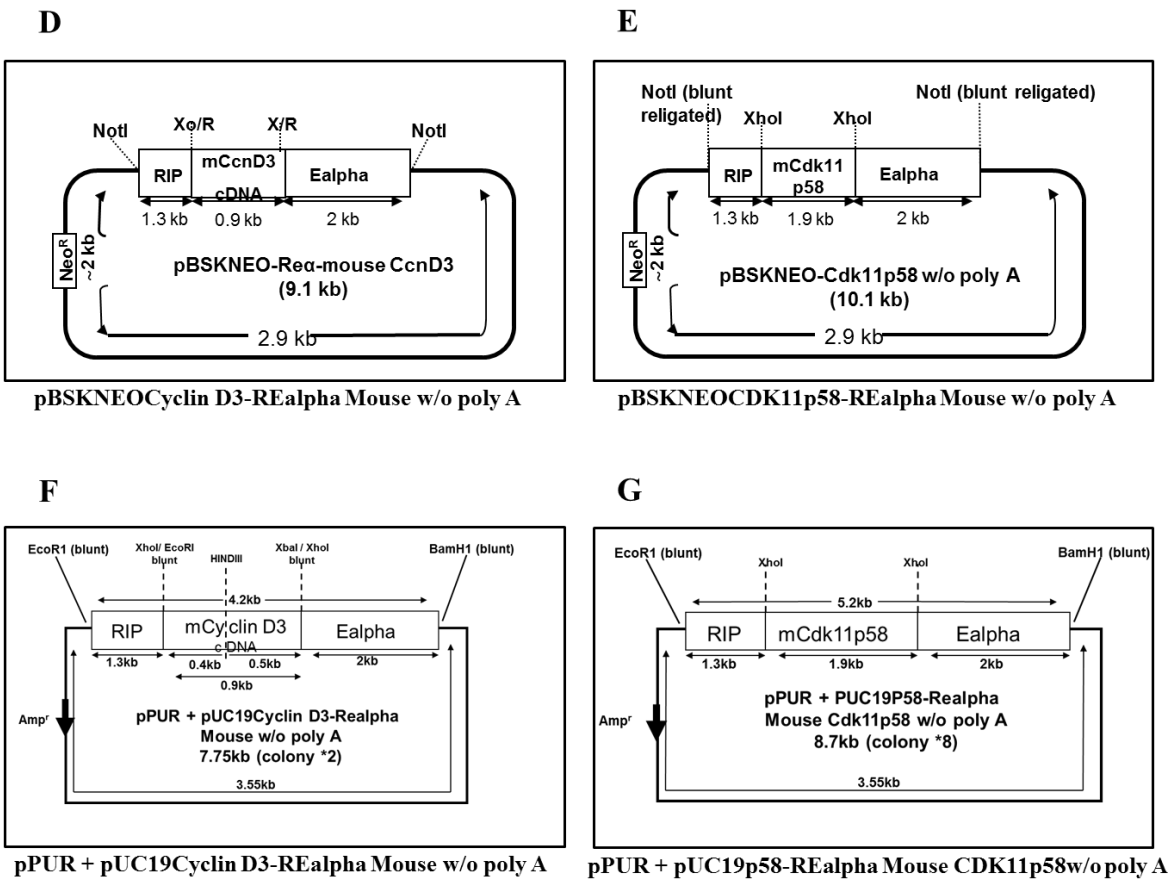


Figure 25: Maps of the vectors, the inserts and the plasmid DNA used for transfection in the NIT-1 cells. A. *Map of pPUR vector. pPUR is a selection vector that confers puromycin resistance to eukaryotic cells.* ***B*** *&* ***C.*** *Map of the pUC19RIPcyclinD3 and pUC19RIPCDK11p58 used vectors, from which either the RIPCcnD3Eα or RIPCDK11p58Eα were released to be subcloned into the pPUR vectors for NIT-1 transfection experiments.* ***D, E, F*** *&* ***G.*** *Map of the pBSKNeoCcnD3, pBSKNeoCDK11p58, pPURCcnD3 and pPURCDK11p58 constructs transfected into the cells.*

Table 14. List of single and the double constructs transfected into the NIT-1 cells. *The following are the 7 types of transfections done into NIT-1 cells. All cDNAs were driven under the control of the RIP promoter.*

TYPE OF CONSTRUCTS	NAMES
SINGLE CONSTRUCTS	pBSKNEO (empty vector)
	pBSKNEO CcnD3
	pBSKNEO p58
DOUBLE CONSTRUCTS	pBSKNEO + pPUR (empty vectors)
	pBSKNEO CcnD3 + pPUR p58
	pBSKNEO p58 + pPUR CcnD3
	pBSKNEO CcnD3 + pPUR (empty)
	(empty) pBSKNEO + pPUR CcnD3
	pBSKNEO p58 + pPUR (empty)
	(empty) pBSKNEO + pPUR p58

9.1.3 Bacterial Transformation

9.1.3.1 Obtaining competent cells

A pre-inoculum of 10ml of LB from a single colony was made from a plane plate (kept at -80ºC glycerol) and grown overnight at 37ºC in shaking conditions. 250ml of SOB together with 1ml of the preculture was inoculated and grown at 37ºC in stirring condition until OD_{600} nm of 0.3 was reached. The culture was then transferred to 250ml Nalgene tubes precooled and then was incubated in ice for 10-15minutes. They were centrifuged at 950-1300g at 4ºC for 10 minutes and supernatant was removed. The cells were then resuspended in 33ml of chilled TfBI (see Annex I, Section Molecular Biology, G.2 Reagents for competent cell preparation) (prechilled in ice) and incubated in ice for 15-30 minutes. The cells were again centrifuged at 950-1300g at 4ºC for 15 minutes. After removing the supernatant the cells were resuspended in 8ml of TfBII chilled (prechilled in ice) and incubated in ice for 15minutes (see Annex I, Section Molecular Biology, G.2 Reagents for competent cell preparation). The cells were aliquoted in 100µl by volume in an eppendorf and frozen in liquid nitrogen and then finally stored at -80ºC.

9.1.3.2 Bacterial Transformation

Transformation is the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s).

At DAY 1: At first, competent E.coli cells were taken from –80 ºC. For each transforming procedure of DNA vector into bacteria 100 ul of competent cells (1 aliquot) was used. To thaw the competent cells they were incubated in ice for 10 min. and then 1ul of the circular DNA (plasmid) was introduced in it. The tube containing both, the DNA with the competent cells was first kept in ice for 20 minutes and then a heat-shock treatment was given by placing the tube it in water bath at 42ºC for 30 seconds. And then, the tube was placed again immediately in ice for 2 minutes to reduce damage to the E.coli cells. 1 ml of LB (with no added antibiotic) was added to the tube which was incubated for 1 hour at 37ºC in shaking conditions. About 100µl of the resulting culture was spread on the LB plates (with appropriate antibiotic added –Ampicillin in this case). The culture was allowed to grow overnight at 37ºC.

At DAY 2: One colony was picked up and was allowed to grow in the LB medium with antibiotic Ampicillin (for high copy number we used 100ml of LB medium + 100 µl Ampicillin (final concentration-50µg/ml). The culture was allowed to grow for 16 hours in shaking condition at 37ºC for future miniprep purification.

At DAY 3: Maxiprep (Invitrogen)
Maxiprep is a large scale purification of plasmid from a bacterial culture. Maxiprep was done according to the manufacturer´s protocol (Invitrogen, by Life technologies). With the help of maxiprep high copy number plasmid DNA was isolated and stored at -20ºC.

9.1.4 Transfection of eukaryotic cells

9.1.4.1 Transfection by Electroporation

For transfection by electroporation, 5×10^6 healthy cells were taken from a culture of 70-80% confluent NIT-1 cells. They were firstly centrifuged and resuspended in 400µl of DMEM with no additives. The specific circular DNA (20µg) to be transfected was added to

the medium containing the cells in a 0.4cm gene pulsar cuvette (Biorad). Immediately the cells were electroporated at 250V and 960 microFarads. The cuvette was then kept in the standing position for 10 minutes at RT to recover from the shock. Then, the cells were plated in multiple 12 well culture plate. 3 days after transfection, selection antibiotics were added to select NIT-1 transfectants (final concentration of: Puromycin-1μg/ml and Geniticin G418-400μg/ml. see Table 15).

9.1.4.2 Transfection by METAFECTENE® PRO

Metafectene® Pro is a transfection reagent.

At DAY 0: In a 12 well culture plate 10^6 cells were plated with 2 ml of suitable fresh complete medium. The cells were incubated in the incubator at 37ºC until they reach 90-100% confluency.

At DAY 1: The stock solutions of the circular DNA to be transfected and the transfection reagent were at room temperature. The stock solutions were agitated gently before use. The following solutions were prepared using a cell culture grade 96-well plate or other tubes made of polypropylene, glass or polystyrene (preferentially polypropylene). Medium was pipetted first.

Solution A: 0.5 – 2 μg of circular DNA in 100 μl of PBS.

Solution B: 6.0 μl of METAFECTENE® PRO in 100 μl of PBS.

The DNA solution was added into the transfection reagent solution and was mixed gently carefully by pipetting one time. The solution was incubated at room temperature for 15-20 minutes. After the incubation time the DNA-lipid complexes were added as soon as possible dropwise to the cells and mix the plate with extreme care. Then the cells were incubated at 37°C for 48 hours in a CO_2 incubator.

At DAY 3: The media with the liposomes were removed. Fresh, supplemented media (without antibiotics like Geniticin and puromycin) were added.

At DAY 4, Selecting antibiotics: Geniticin and Puromycin were added (Doses for NIT-1 cells: Puromycin-1μg/ml for the pPUR selection gene and Geniticin G418-400μg/ml for the Neo selection gene).

Prior to transfection, the selective drug concentration required to kill untransfected cells were determined (kill curve; *Ausubel et al. 1995* [341]). This is determined by seeding the cells and then determining at what concentration of the selecting antibiotic 100% cells die. Seventy-two hours after transfection, adherent cells were trypsinized and replated in medium containing the appropriate selection drug. For effective selection, cells should be subconfluent since confluent, nongrowing cells are very resistant to the effects of antibiotics like G-418. For the next 14 days, the drug-containing medium was replaced every 3 to 4 days. During the second week, cells were monitored for distinct clusters of surviving cells. Drug-resistant clones appeared in 2–5 weeks, depending on the cell type and the constructs. Cell death normally occurred after 3–9 days in cultures transfected with the mock control plasmid.

The resistant clones were selected by the selection procedure. Once the resistant clones were selected (two weeks) with the antibiotic G418 and Puromycin (see Table 15), the different experimental groups were exposed to the combination of proinflammatory cytokines and the levels of early apoptosis were measured by Annexin V staining.

Table 15. The concentrations and the dilutions of the antibiotics used to select the NIT-1 cells.

ANTIBIOTIC	WORKING CONCENTRATION	STOCK SOLUTION	COMPANY
G418	1mg/ml(single constructs) 400μg/ml (double constructs)	50mg/ml Geniticin is supplied as 50mg/ml solution in water	PAA (Pasching, Austria)
Puromycin	1μg/ml (double constructs)	10mg/ml Puromycin supplied at 10 mg/mL in 20 mM HEPES buffer (pH 7.2–7.5)	Invitrogen (Scotland)

9.1.5 Measuring of Apoptosis by Annexin-V staining

Day 1:	Plating of cells: Cells were plated in 96 well plates having a density of about 10^5 cells per well without any added antibiotic. Triplicates were made in 5 different experiments.
Day 2:	Treatment of cells with cytokines: Transfected NIT-1 cells were treated with IL-1β (50 Units/ml) (Prospec, Passaic County, NJ, USA) and IFNγ (200 Units/ml) (Prospect, Passaic County, NJ, USA). Control cells were not exposed to cytokine.
Day 3:	Determination of early apoptotic events by annexin V staining by flow cytometry at 24hours after cytokine exposure: Following treatment, approximately 10^5 cells were washed in PBS. The cells were harvested and then stained with Annexin V (an early apoptosis marker) Propidium Iodide (PI) using the Annexin V Detection kit (BD Biosciences). PI fluorescence emission was measured with a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

9.1.6 Retrotranscription of the Rip CDK11p58 transgene mRNA (rTPCR) and Polymerase chain reaction (PCR) to measure CDK11 overexpression in transfected cells

The rTPCR (polymerase chain reaction) is a rapid procedure for *in vitro* enzymatic amplification of a specific mRNA. In the present study, this technique was employed.

9.1.6.1 Isolation of RNA and single strand cDNA synthesis

Total RNA was prepared from each NIT-1 cell transfectant line culture using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The final concentration of RNA in the sample was determined by a Nanodrop (ND-1000) spectrophotometer. Messenger RNA was reverse-transcribed (rT) to cDNA (65ºC for 5mins, in ice for ≥5mins, 55ºC for 1 h and 70ºC for 15 mins) using oligodT and Superscript III reverse transcriptase (Invitrogen). Negative control rT-minus contained the same reagents with the exception of Superscript III were carried out to establish that the target RNA was not contaminated with DNA.

9.1.6.2 PCR

The cDNA product was used as a template for subsequent PCR amplification. Where indicated, rT-PCR primer sequences were obtained from the available scientific literature. The housekeeping gene Hypoxanthine phosphoribosyl transferase (HPRT1/HPRT2) was used as a housekeeping control for proper cDNA synthesis. Table 11 contains primer sequences, product sizes and PCR conditions. For each set of primers, rT-PCR was repeated a minimum 3 times in independent samples. 25µL of reaction mixture contained 10 x reaction buffer (2.5µL), 1.25µL MgCl2 (50mM), 0.5µL dNTPs (10mM), sterile water (14.75µL) and Taq polymerase (0.5µL). The PCR reaction was run in a thermocycler (Gene Amp® PCR System 2700 from Applied Biosystems and PTC-200). 30 cycles of PCR amplification include initialization at 94ºC for 0 minute, denaturation at 94ºC for 1 minute, annealing 55ºC for 1 minute (it depends on the size of the PCR prduct) and elongation at 72ºC2 for 6 minutes. Equal volumes of PCR products (25µL) were loaded and electrophoresed in a SYBR Safe (Life Technologies)-stained 1% agarose gels. The images of agarose gel illuminated by UV light with the help of Alpha Innotech Transilluminator were taken using Kodak EDAS 290 imaging system with Kodak 1D 3.6 software. Experiments were performed in triplicates and representative images are presented.

9.1.7 NIT-1 cell transfectants staining by flow cytometry to measure Cyclin D3 over expression in transfected cells

NIT-1 cell transfectants underwent trypsinization to obtain single cell suspension, and then, they were fixed in cold methanol at -20ºC overnight prior to cellular staining. The cell suspension was centrifugated at 900xg 5 minutes at 4ºC, the supernatant was discarded and the cells were washed with 1mL of blocking solution and stained with the primary antibody of interest in Blocking solution containing 0.01% of triton X-100 which we called permeabilization solution (See Annex I, Section E. Flow cytometry, E.1 Flow cytometry staining buffer). The cells were washed twice with blocking solution with 0.01% of triton X-100, secondary antibody, washing and then the samples were acquired using the FACS-CantoII Flow Cell Cytometer (BD).

Table 16. List of the antibodies used for Cyclin D3 and Ki-67 staining in the NIT-1 cells

Antbodies	Dilutions	Company
Primary Antibodies		
anti-mouse-GLUT-2 biotinilated	1:100	R&D (Germany)
Monoclonal Rat anti-mouse Ki-67	1:100	Dako (California, USA)
Purified Mouse anti-CyclinD3	1:100	BD, Pharmingen (California, USA)
PE-conjugated anti-mouse CD45	1:100	BD, Pharmingen (California, USA)
Secondary antibodies		
anti-mouse CFS405	1:200	Sigma Aldrich (Barcelona ,Spain)
Streptavidin APC	1:300	Immunotools (Friesoythe; Germany)

9.1.8 NIT-1 cell lysis procedure for Western Blot

Cells were trypsinized, washed with PBS and the pellet was collected. 1ml of ice cold RIPA buffer (sc-24948) with freshly added Protease inhibitors was added to the cells and incubated on ice for 30 minutes. Further disruption of the cells were done by hydrodynamic shearing (21-gauge needle) and then incubated in ice for 30 minutes. Then they were transferred in micro centrifuge tube and centrifuged at 10,000g for 10 minutes at 4ºC. The supernatant fluid is the cell lysate which was subjected to SDS-PAGE (10% gel). See 9.1.9.2.

9.1.9 Western blot

9.1.9.1 Protein quantification by Nanodrop:

We quantified the protein concentration of cell lysates according to the Nanodrop (ND-1000) spectrophotometer. Quantification of protein concentration in samples before migrating SDS-PAGE gels is required. If the different samples of the same experiment for the immunodetection of proteins are not equally loaded, no conclusions can be made as we do not know whether the differences are due only to the loading or due to the effects of different treatments.

9.1.9.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Equal amounts of proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE). 10% Resolving gel and 5% stacking gel was used [See Annex 1 Section F. Western Blot, F.2 Composition of (10%) Resolving Gel (1 gel) and F.3 Composition of (5%) Stacking Gel (1 gel]. Electrophoresis was run at constant current of 20mA and voltage kept under 120 volts for 1 hr or until the front bromophenol blue leaked out of the gel. Levels of β-actin signal were used to verify equal protein loading in each lane.

9.1.9.3 Transfer: A Bio RAD wet system was used to transfer the resolved proteins from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). PVDF membrane was activated by methanol for 2 minutes. The membrane can never be let out dried and it has to be kept activated before its use to develop with another antibody.The uniformity of transfer of protein from the gel to the membrane was checked by staining the gel with Coomassie Brilliant Blue. (Composition of Coomassie Brilliant Blue See Annex I, F10, Page no.232)

9.1.9.4 Membrane Blocking: Once the transfer finished, the non-specific binding of proteins to the membrane was blocked by incubation with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) plus 5% nonfat milk for 1hour at RT with mild agitation.

9.1.9.5 Incubation with antibodies: Immunodetection was performed using the appropriate primary antibodies (See Materials, Table 12, Western Blot Primary antibodies) during overnight incubation at 4ºC temperature with agitation. The appropriate secondary Peroxidase-conjugated antibody was diluted in blocking solution and was incubated with gentle agitation for 1hour at RT (See Materials, Table 12, Western Blot Secondary antibodies).

9.1.9.6 Development of peroxidase activity linked to the secondary antibody: Immunoblots were developed and the bound antibody was visualized using the EZ-ECL (Thermo Scientific, Rockford, U.S.A.). With antibodies that produce a faint signal with ECL, chemiluminescent substrate Super Signal (Thermo Scientific, Rockford, U.S.A.) that has a stronger and prolonged signal was used. They were prepared by taking 1:1 ratio each

of the (Detection Reagents 1 and 2 in case of ECL and Stable Peroxide Solution and the Luminol/Enhancer Solution in case of chemiluminescent substrate Super Signal). The membrane was exposed to the substrate for 1 minute and then further developed with the help of an X-ray film (G.E. healthcare, Buckinghamshire,UK) for an appropriate time period usually for 10 seconds, 1 minute, 5 minutes, 10 minutes, 20 minutes and 1 hour. The membrane was developed with the help of a developing machine (Optimax X-ray film processor, Germany).

9.1.9.7 Removing of antibody complexes from the membrane (membrane stripping): Membrane stripping was done using the stripping buffer (See Annex I, Secton F: Western Blot, F.12 Composition of Stripping Solution) for 30minutes at 50°C with constant mild shaking. The membrane was kept washing after stripping for 6-7 times 10 minutes with TBS-T each till the smell of the β-mercaptoethanol completely goes away. The second immunodetection process was initiated with milk blocking.

Notes:

* Gel staining with coomassie: The dye Coomassie blue (Sigma) binds to almost all the proteins, both in denaturalized and not denaturalized, which can be used after electrophoresis. It is used to dye the gel once transfer finishes, marking proteins remnant of the polyacrylamide gels as loading controls of the western blot. The gel was kept in the staining solution overnight (0.1% coomassie, 45% methanol, 10% acid acetic), leaving all the gel below the solution. A destaining solution (5% methanol, 7.5% acid acetic) was used to decolor the gel, which made the gel transparent with protein bands remaining blue.

*Preserving the membrane: The membrane can be used maximum 3 times after stripping. It is preserved for long at -20ºC in the freezer.

9.2 *IN VIVO*

9.2.1 TYPE 1 DIABETES MOUSE MODEL

-All mice used in the experiments performed in this thesis were kept under Specific Pathogen Free (SPF) conditions.

- Only female mice were used in the experiments described in this thesis, unless otherwise stated.

- All animal experimentation procedures performed in this work have been overseen and approved by the Institutional Ethical Committee for Animal Experimentation of the University of Lleida (CEEA) in accordance with the European and U.S. Regulations on Animal Experimentation.

9.2.1.1 The NOD Colony

Our Non-Obese Diabetic (NOD) mouse colony, a model for Type 1 diabetes, originally derives from that held by Chales A. Janeway, Jr.'s laboratory at the Yale University Mouse Facility.

Mice were housed with access to food using standard autoclavable rodent chow (Harlan Iberica) and water ad libitum in SPF conditions in a microisolator cage with positive pressure of HEPA air at 21ºC and at 40-60% humidity, under a 12:12 hours light/dark cycle. SPF is a term used for those housing conditions used for laboratory animals that guarantee the absence of specific pathogens. Use of SPF conditions ensures that specific pathogens do not interfere with our experiments. Upon birth, litters containing distinct genotypes were being housed together until 3 weeks. Ear notches or holes were produced by a sterilized ear punch device to number all the mice at around 2 weeks age of mice (as in Figure 28).

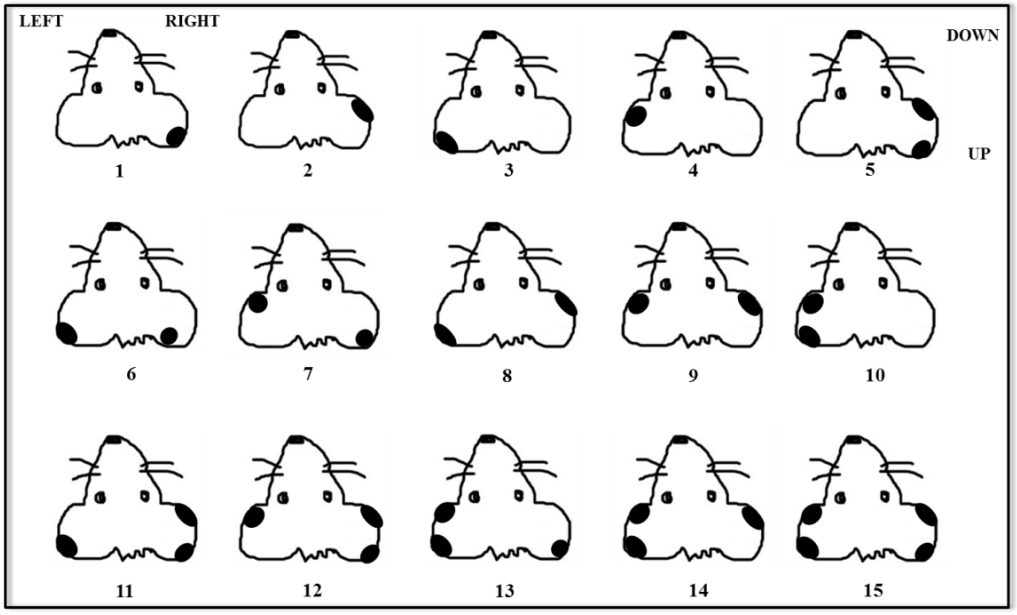


Figure 26. Ear notches made to number all the mice *(belly is kept down).*

The personnel in charge of the food and water supply or carrying out any procedure that involved any contact with the mice always were wearing sterilized lab-overall clothes. The SPF was positively pressurized and air administered to it was HEPA filtered. The manipulations of the animals were done in a laminar flow hood inside the SPF room. Animal food, water and all the material that was in contact with the mice was sterilized by either autoclaving or chemically (peroxide SASS).

Mice were sacrificed by cervical dislocation. For adoptive transfer experiments, inhaled anesthetic was used (Isoflurane; Abbot Laboratories, Madrid; Spain).

9.2.1.2 NODSCID Colony

The SCID mutation was transferred onto a non-obese diabetic background. Animals homozygous for the SCID mutation have impaired T and B cell lymphocyte development. Mice carrying the SCID mutation were kept under SPF conditions on Septrin antibiotic mixture (Sulfamethoxazole 1.2 g/l and trimethoprim 0.24g/l) on a week on-week off basis.

9.2.1.3 Generation of NOD/CcnD3 KO mice

Mice homozygous for the deficiency in Cyclin D3 were originally obtained in the mixed 129/Sv C57BL/6 genetic background [300]. Intensive backcrossing onto the NOD genetic

background and checking for the Idd markers as described was performed [342]. Genotyping for the Idd loci was accomplished by using PCR primers specific for the different loci that are polymorphic for the 129/Sv, C57BL/6, and NOD strains. The primer sets used to ascertain Idd alleles by PCR were: Idd1 (H-2g7; for Idd1 screening a set of PCR primers was used, a, D17 Mit34; 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. The PCR products were run in 4% agarose gels. We reached the 12th generation of backcrosses into NOD (N13), which was used for the experimental procedures. Genotyping of cyclin D3 gene targeting mutation was performed by PCR according to the literature [300].

9.2.1.4 Generation of the NOD/RIPCcnD3 transgenic mice

We generated transgenic NOD mice by direct 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 a generous gift from Martine Roussel [343], 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 alphad 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.

9.2.1.5 Generation of NODCDK11KO mice

Because microarray technology used in our previous work is based on differential expression of RNAs, it does not allow to distinguish which of the two CDK11 gene products (p58 and p130) or both, are downregulated as a result of inflammation in the β-cells. Dr. Lahti has provided both, the cDNA encoding the entire sequence of p130, and that of p58. To determine the degree of involvement of Cdk11p58 in β cell death process, we subcloned the CDK11 p58 into a vector containing the Rat Insulin Promoter (RIP) in a pUC19 backbone. CDK11p58 cDNA obtained initially from Dr. Jill Lahti, was modified to eliminate the endogenous polyadenylation signal since the expression cassette used (RIP-Eα) already contained the signal. Such construction was tested in MIN6 cells, where the messenger was correctly expressed, and thus was ready to be microinjected into fertilized oocytes of NOD females for transgenic lines. MIN6 cells are the insulinoma cell line which is derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic β cells in the C57BL/6 genetic background [344].

9.2.1.6 Generation of NODCDK11 CcnD3KO mice

The CDK11 Δ/+ mice in the C57BL/6 background were provided by Dr. Jill Lahti from St. Jude Children's hospital, Memphis, TN, USA. CDK11 null/null mice were not possible to generate as these mice are embryonically lethal. The blastocyst cells do not proliferate normally and are arrested during mitosis leading to apoptosis [276].

NOD mice deficient in cyclin D3 (NOD Cyclin D3 null/null) were crossed with either NOD mice individuals hemideficient in CDK11 (NOD/CDK11 Δ/+ (HTZ) and NOD mice wild type (WT) for CDK11. All these experimental groups had in common the deficiency in cyclin D3 but they differed in the genotype related to CDK11.
NOD Cyclin D3 (-/-) CDK11 (Δ/+) (Cyclin D3 KO, CDK11 HTZ)

NOD Cyclin D3 (-/-) CDK11 (+/+) (Cyclin D3 KO, CDK11 WT)

9.2.1.7 Generation of NODCDK11 CcnD3 Rip Tg mice

NOD mice hemideficient in CDK11 (NOD/CDK11 Δ/+) were crossed with NOD / RIP-Cyclin D3 6896 line to obtain individuals from the 2 genotypes listed below. All these

experimental groups have in common the CDK11 gene deficiency, and vary according to the RIP-cyclin D3 transgene.

NOD/CDK11 (Δ/+) RIP-CcnD3 Tg+ (CDK11HTZ Cyclin D3Tg+)

NOD/CDK11 (Δ/+) RIP-CcnD3 Tg- (CDK11HTZ Cyclin D3Tg-)

9.2.1.8 Genotyping of the different strains of mice

All strains of mice were genotyped by PCR using specific oligonucleotides of the genomic sequence that was intended to be amplified, in between 15-21 days of age. Genomic DNA was obtained from a fragment of mouse tail length of 0.4 cm from its distal end. Positive and negative controls of amplification were used to ensure the reliability of results.

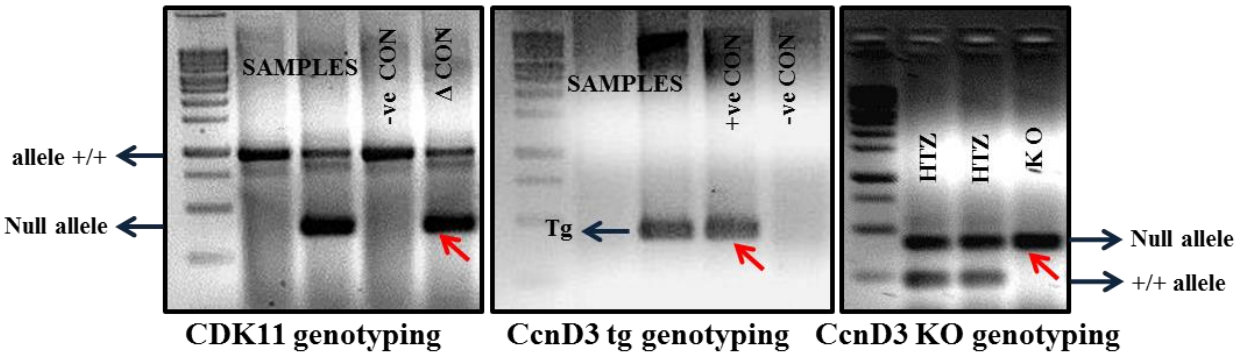


Figure 27. Picture of the gel for the genotyping of the strains of mice

→ *Positive control, Δ= Delta, Tg= Transgenic, KO= Knock out, HTZ= Heterozygous*

9.2.1.9 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) initially, and we built our own colony. 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 conditions are in Annex III. The PCR product was digested with the AluI restriction enzyme at 37ºC.

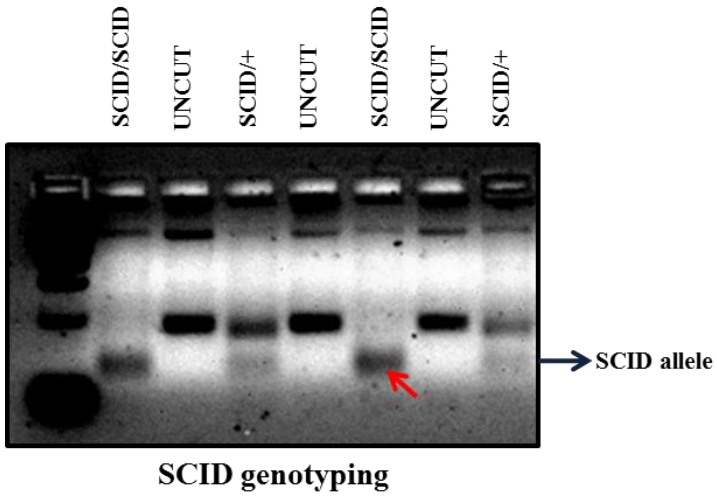


Figure 28. Genotyping of the Scid Mutation. → ***Positive control /+ve allele***

The expected size of fragments obtained after the PCR product was digested with AluI was Mutant=38bp, 28bp and 11bp; WT= 68bp and 11bp.

35 cycles of PCR amplification include initialization at 94ºC for 1.5 minutes, denaturation at 94ºC for 30 seconds, annealing 53ºC for 30 seconds , 72ºC for 30 seconds and elongation at 72ºC for 2 minutes.

The PCR reactions for genotyping were run in a thermocycler (Gene Amp® PCR System 2700 from Applied Biosystems and PTC-200).

(All the PCR Programmes used for the genotyping of mice: see ANNEX II)

9.2.2 Assessment of diabetes

9.2.2.1 Detection of glycosuria (presence of excess glucose in the urine)

Weekly Review of the individuals under observation were done by urine test strips with Medi-Test Glucose 3 (Macherey-Nagel, Düren, Germany) from 3 week to 30 weeks. When an individual tested positive for glycosuria then glycemia was measured. In case of adoptive transfer, recipient female mice were monitored once a week for glycosuria which continued until 17 weeks after adoptive transfer was performed.

9.2.2.2 Glycemia (blood glucose levels)

The glucose was measured after positive detection of glucosuria making a tiny cut at the distal caudal vein, pressing slightly to obtain a drop of about 25 microliters of blood, which was deposited onto Glucocard G sensor detection strips (A. Menarini Diagnostics) to measure blood glucose in the corresponding glucometer (Glucocard Gsensor, Arkray Inc., Shiga. Japan). Glucose levels above 200 mg/dL correspond to a hyperglycemic state, and therefore, the animal was diagnosed as diabetic. To check the autoimmune etiology of diabetes mellitus, the hyperglycemic mouse was killed immediately in order to examine immunohistochemically islet infiltration by leukocytes.

9.2.3 Adoptive transfer experiments

Immunocompromised NOD/SCID female mice (>4 weeks) were used as recipients of total splenocytes from NODCDK11HTZCcnD3Tg+ and NODCDK11HTZ CcnD3Tg- littermate donors of 8 weeks of age in 1 experiment and 5 weeks of age in another set of experiment. Total splenocytes were isolated under sterile conditions by physical disruption of the spleen using frosted glass slides and in RPMI media (Lonza) supplemented with FBS, Gentamycin and β-Mercaptoethanol (see Table 17). Red blood cells were lysed by hypotonic shock with 900µL of pure water and 100 µL of 10x PBS and 10ml of supplemented RPMI was added at the end. Cells were further washed with physiological saline (0.9% NaCl).

In other sets of experiments NODCDK11HTZCcnD3Tg+ and NODCDK11HTZ CcnD3Tg- female mice of >4 weeks of age were used as recipients where the NOD female mice of 8 weeks of age were used as the donor.

Cells were transferred in 220 µL of physiological saline (0.9% NaCl) by i.v. injection.

Table 17. The composition of the RPMI Medium used for the disruption of the splenocytes.

	Volume	Final Concentration
RPMI Medium (1640 with L-Glutamine)	500ml	
Fetal bovine serum	50ml	10%
Gentamycin	580µl	1.21mM (0.058mg/mL)
β-mercaptoethanol	400µl	0.2mM

9.2.4 Intaperitoneal Glucose Tolerance Test (IGTT)

Mice were fasted 16h prior to the Glucose Tolerance Test. Mice were anaesthetized using sodium pentobarbital (60 mg/kg). A single intraperitoneal injection of 2gm glucose per kg of body weight was given. Glucose and insulin were measured at 0, 15, 30, 60 and 120 min after injection of glucose. Glycemia was determined as previously described.

(Pentobarbital dilution and volume of Glucose solution injected per mice- See Annex III)

9.2.5 Immunohistochemical analysis of pancreatic infiltration and insulin content in different strains of mice

Mice were euthanized, pancreas and spleen were extracted and were immersed in 4% paraformaldehyde in PBS for 3 hours in RT. The tissue was dehydrated following this procedure:1 hour in 70%; and then two times, 1h each, in 96% ethanol; three times, 1hr each, in 100% ethanol; and finally, 45 mins in xylene. The tissues were embedded in paraffin for 3hrs at 60ºC, sections were cut 10µm thick, and the sections were placed on adhesion slides (KLeinipath Good(s) in pathology, Netherlands.). After the tissue sections were placed on the slides, these were dried at least overnight at 37ºC on a hot plate.

9.2.5.1 Deparaffinization of the slides followed by Hematoxylin and Eosin staining

This procedure was performed by placing slides three times, 10 min each, in xylene; two times 5 min each, in 100% ethanol; 2 times, 5min each, in 60% ethanol, and in distilled water for at least 10 min. Paraffin sections were counterstained with Hematoxylin & Eosin

(Sigma-Aldrich): 1 bath of 23min of Harris hematoxylin, washed once with distilled water to remove the excess of hematoxylin during 20 min, 10 sec in Acid alcohol, one wash of distilled water for 10 min and a bath of Eosine solution 1-2min were performed. For infiltration studies images were captured with Leica DM R microscope (Leica Microsystems Gmbh, Wetzler, Germany). To assess pancreatic infiltration, a minimum of 4 mice used 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.

9.2.5.2 Immunofluorescence study for insulin content in the strains of mice

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 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. Epifluorescence images were captured with the Confocal microscope (Olympus FV1000, America). (The Buffer preparation: See Annex I, Section D. TISSUE (pancreatic tissue) Staining buffers, D.2 Immunofluorescence Staining).

Table 18. List of the antibodies used for checking insulin in the islets of the strains of mice

Antbodies	Dilutions	Company
Primary antibody		
Polyclonal Guineapig anti-Insulin	1:1000	Dako (California, USA)
Hoeschst 333421mg/mL	1:200	SIGMA Aldrich (St Louis, USA)
Secondary antibody		
antiguineapig-IgG-Cy2	1:500	Jackson Immunoresearch

		laboratories (West Grove, PA, USA)

9.2.6 Morphometric studies

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 alkaline phosphatase substrate. Hematoxylin was used for islet counterstaining. The insulin area was quantified in a blinded fashion using optic microscopy (Leica, Wetzler, Germany) and ImageJ software (NIH, Bethesda, Maryland; USA) using a microscopic scale provided by the software and calibrating the MO with a slide containing a 2mm bar . A minimum of 7 females per experimental group were use ($\geq$ 70 islets counted/mouse).

Table 19. List of the antibodies used for morphometric studies

Antbodies	Dilutions	Company
Primary antibody		
Polyclonal Guineapig anti-Insulin	1:1000	Dako (California, USA)
Secondary antibody		
Biotinylated secondary Ab	1 drop ready to use	Biogenex (California, USA)
Alkaline Phosphatase Substrate	1 drop ready to use	Biogenex (California, USA)
Substrate		
FastRed chromogen	1 tablet makes 1ml	SIGMA Aldrich (St Louis, USA)
Tris Buffer	1 tablet makes 1ml	SIGMA Aldrich (St Louis, USA)

9.2.7 Pancreatic islet isolation

Mice were euthanized by cervical dislocation. Islets were isolated by collagenase P (Roche, Germany) digestion. Each pancreas was injected with 4 mL of collagenase P digestion solution (5 mg collagenase P/ mL Hanks' Balance Salt Solution (HBSS) + 0.1% BSA through the pancreatic duct. After injection, infiltrated pancreas were extracted and

incubated with 3mL of collagenase P digestion solution 7 minutes at 37ºC, tissues were disaggregated mechanically with a Pasteur pipet and for digestion was stopped by adding 45mL of HBSS+0.1%BSA . After digestion, islet preparation was kept in ice during the whole procedure.

The tubes were kept standing for 3 minutes on ice, the lipid layer that remained on top was discarded. Each islet was isolated putting the disaggregated tissue in Petri dishes, and collecting the islets by pipetting with the help of amplifying loop. Islets were found using, a black contrast because of the characteristic islet white glare. Islets were handpicked with micropipette twice under the binocular loop.

9.2.7.1 Islet cell staining by flow cytometry

The isolated islets underwent trypsinization with 500 µL of trypsin/EDTA (Lonza, Verviers, Belgium) for 5 min at 37ºC to obtain single cell suspension, and then, they were fixed in cold methanol at -20ºC overnight prior to cellular staining . The cell suspension was centrifugated at 900xg for 5 minutes, the supernatant was discarded and the cells were washed twice with 1mL of blocking solution containing Hanks Balanced Salt Solution(HBSS) (Lonza, Verviers, Belgium) + 1% FBS (Lonza ,Verviers, Belgium) which reduces non-specific binding of antibodies to the cells. The cells underwent permeabilization with HBSS + 0.01% Trition-X 100 (Sigma Aldrich, St. Louis, USA) by keeping the cells in ice for 5 minutes. Then the cells were stained with the primary antibodies in Blocking solution with 0.01% of triton X-100. The cells were washed twice with blocking solution with 0.01% of triton X-100 after primary antibody incubation. Secondary antibody incubation when required was done in blocking solution with 0.01% of triton X-100 and washed again in the same solution. The samples were acquired using the FACS-CantoII Flow Cell Cytometer (BD; California, USA).

Table 20. List of the antibodies used for cyclin D3 and Ki-67 staining

Antbodies	Dilutions	Company
Primary antibodies		
antimouse-GLUT-2 biotinilated	1:100	R&D (Germany)
Monoclonal Rat antimouse- Ki-67	1:100	Dako (California, USA)
Purified Mouse anti-CyclinD3	1:100	BD, Pharmingen (California, USA)
PE-conjugated antimouse CD45	1:100	BD, Pharmingen (California, USA)
Secondary antibodies		
antimouse CFS405	1:200	Sigma Aldrich (Barcelona ,Spain)
Streptavidin APC	1:300	Immunotools (Friesoythe; Germany)

9.2.8 Statistical Analysis

For statistical descriptives, results are presented as Mean + SEM. To assses differences, Mann-Whitney and ANOVA test were used to compare means and Log-rank tests to compare survival curves. Bonferroni correction was applied to Mann-Whitney pvalues to adjust for multiple testing in multiple comparisons. Chi-square test was applied to analyse the statistical significance in case of iset infiltration. Statistical analyses were performed using SPSS, and images and westen blots were analyzed by Image J software and Scion Image respectively. Threshold for significance was set at $p<0.05$. In the figures, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.005$.

Results

Chapter 1

In vitro Experiments

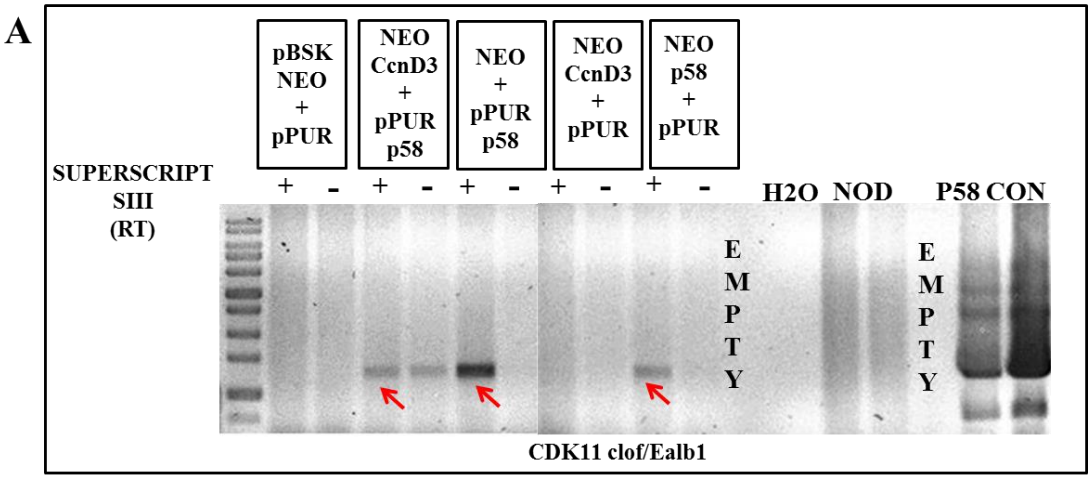
Effects of overexpression of cyclin D3 and/or CDK11 in NIT-1 NOD (Non-obese diabetic) insulinoma cell line

10.1 Assesment of cyclin D3 and/or CDK11 expression in the transfected NIT-1 NOD (Non-obese diabetic) insulinoma cell lines

10.1.1 Assessment of the expression of CDK11p58 mRNA in NIT-1 transfected cells

The NIT-1 transfected cells were cultured in a 75 cm^2 culture flask in the complemented DMEM medium with the antibiotics G418 Sulphate (400µg/ml) and Puromycin (1µg/ml) until the cells reached 60% confluency. RNA extraction was done by TRIZOL extraction procedure and first strand cDNA was obtained from mRNA using OligodT priming.

CDK11p58 transcripts were detected by PCR using the primers CDK11 Clof and Ealb1, (**Figure 29A**) the expression of CDK11p58 transgene is observed in transfected NIT-1 cells (i.e. in the pBSKNEO+pPURp58, pBSKNEOp58+pPUR and in the cells which were co-transfected with pBSKNEOCcnD3+pPURp58). In the lanes with no RT added to the reaction no band was found confirming the results. Only in the cells co-transfected with both pBSKNEOCcnD3+pPURp58 a band was found suggesting it to be genomic DNA contamination for this particular cell. RT- was used as a negative control which contained the same reagents with the exception of Superscript III which helped to establish that the target RNA was not contaminated with genomic DNA. The housekeeping genes HPRT1/HPRT2 were used as a loading control. (**Figure 29B**)



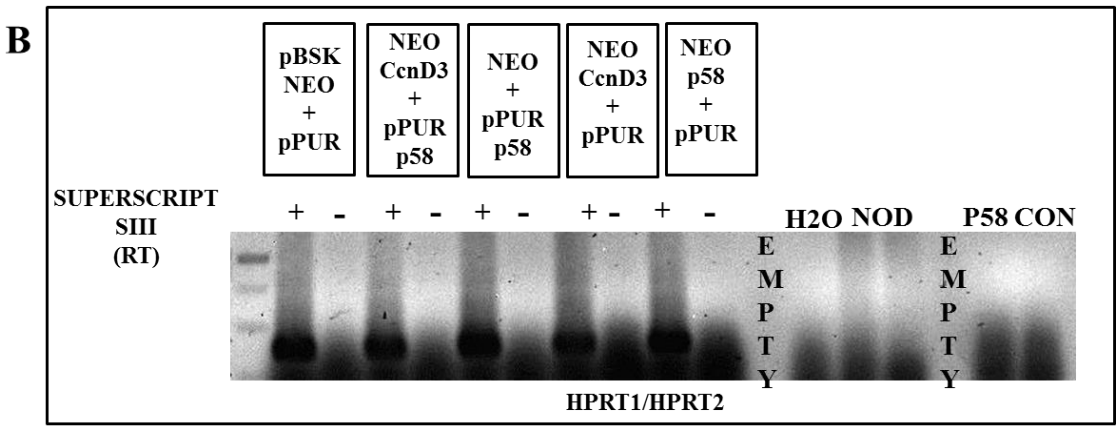


Figure 29. RNA expression levels by RT-PCR in NIT-1 transfected cells. (A)*Upregulation of RNA expression levels in pBSKNEOCcnD3+pPURp58, pBSKNEO+pPURp58 and pBSKNEOCcnD3+pPURp58 in RT positive cells checked by CDK11clof and Ealb1.* ***(B)*** *Control for quality of RNA/cDNA was checked by HPRT-1 and HPRT-2. In each of the cases at least 3 independent experiments were done.*

10.1.2 Assessment of the protein expression of CDK11p58 in NIT-1 transfected cells

The NIT-1 transfected cells were cultured in a 75 cm^2 culture flask in the complemented DMEM medium with the antibiotics G418 Sulphate (400µg/ml) and Puromycin (1µg/ml) until the cells reached 60-70% confluency. Cells were trypsinized, washed with PBS and the pellet was collected. Protein extraction was done and the expression was measured by western blot analysis.

CDK11p58 protein expression was detected in the double transfected NIT-1 cells but in later passages. In **Figure 30,** the overexpression of CDK11p58 transgene is observed more in the empty vectors pBSKNEO+pPUR than the other double transfected cells (pBSKNEOCcnD3+pPURp58, pBSKNEOCcnD3+pPUR, pBSKNEOp58+pPUR and pBSKNEO+pPURp58), which is very unusual. The NIT-1 cells before transfection already has endogenous p58. May be due to transfection and due to many passages the cells transfected with p58 has a conflict with this endogenous p58 which lead the cells to die quickly. Thus, the cells which therefore got selected further by the antibiotic had the selection marker but not the p58 gene. This may lead to the downregulation of p58 in all the transfected cells than the cells transfected with the empty vectors.

β actin was used as the housekeeping gene.

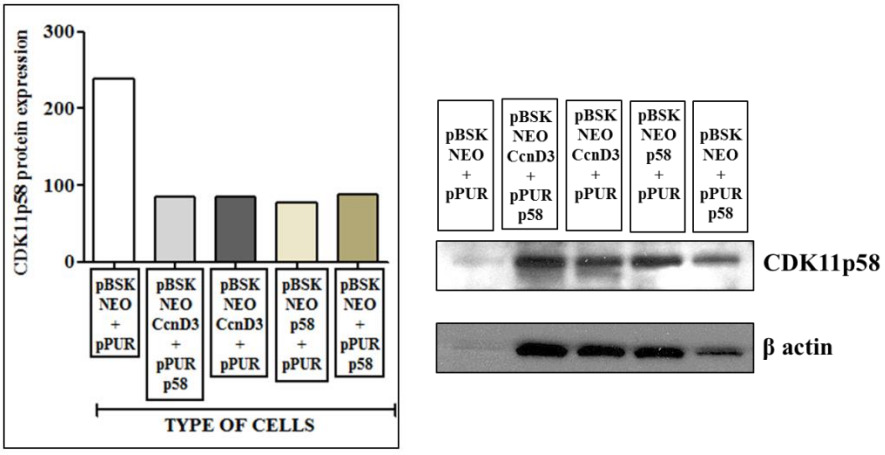


Figure 30. Protein expression levels by western blot analysis in NIT-1 transfected cells. (A)*Upregulation of protein expression levels in pBSKNEO+pPUR than in the double transfected cells with pBSKNEOCcnD3+pPURp58, pBSKNEOCcnD3+pPUR, pBSKNEO p58+pPUR and pBSKNEO+pPURp58. β actin was used as a loading control and the bands were quantified and equalized. They were processed with scion image software. In this case an independent experiment was done in late passages.*

10.1.3 Quantification of the expression levels of cyclin D3 in NIT-1 cells transfected with cyclin D3: Single transfected and double transfected cells

The NIT-1 transfected cells were cultured in a 25cm^2 culture flask in the same complemented DMEM medium with the antibiotics G418 Sulphate (400μg/ml) and Puromycin (1μg/ml) until the cells reached 60%-70% confluency. Cells from the flask were harvested with 1.5 ml of trypsin/EDTA. For cyclin D3 expression analysis, around 100000-200000 cells were used for each experiment.

In **Figure 31A**, we show the parental population gated as NIT-1 according to size and complexity parameters. Glut-2 (β cell marker) was used to assess β cell proliferation of NIT-1 cells. From there, the cyclin D3 positive population was chosen which gave the actual population of cells expressing cyclin D3 and the intensity of median fluorescence (**Figure 31 B and C**).

At first, the cyclin D3 expression level were checked in the single transfected cells where it was confirmed that cyclin D3 mean expression levels were 2 times higher in NIT-1 pBSKNEOCcnD3 transfectants compared to the empty vector NIT-1 pBSKNEO transfectants (means 12191 and 6080.25 respectively, SEM 244.06 and 231.6 respectively with p-value=0.0286). (**Figure 31D**).

Figure 31E showed that no significant difference in the cyclin D3 protein expression profile is shown among all the double transfected cells. Here at first, One-way Anova test was used to compare differences among all groups and, subsequently all paired comparisons were assessed using a Mann Whitney test. Bonferroni method was applied to correct for multiple testing. The values that we got for assessing the cyclin D3 expression were statistically significant when comparing all groups (ANOVA p-value=0.023). Significance was not achieved when comparing individual groups. Hence, the cyclin D3 expression level did not reach significant difference in the double transfected cells.

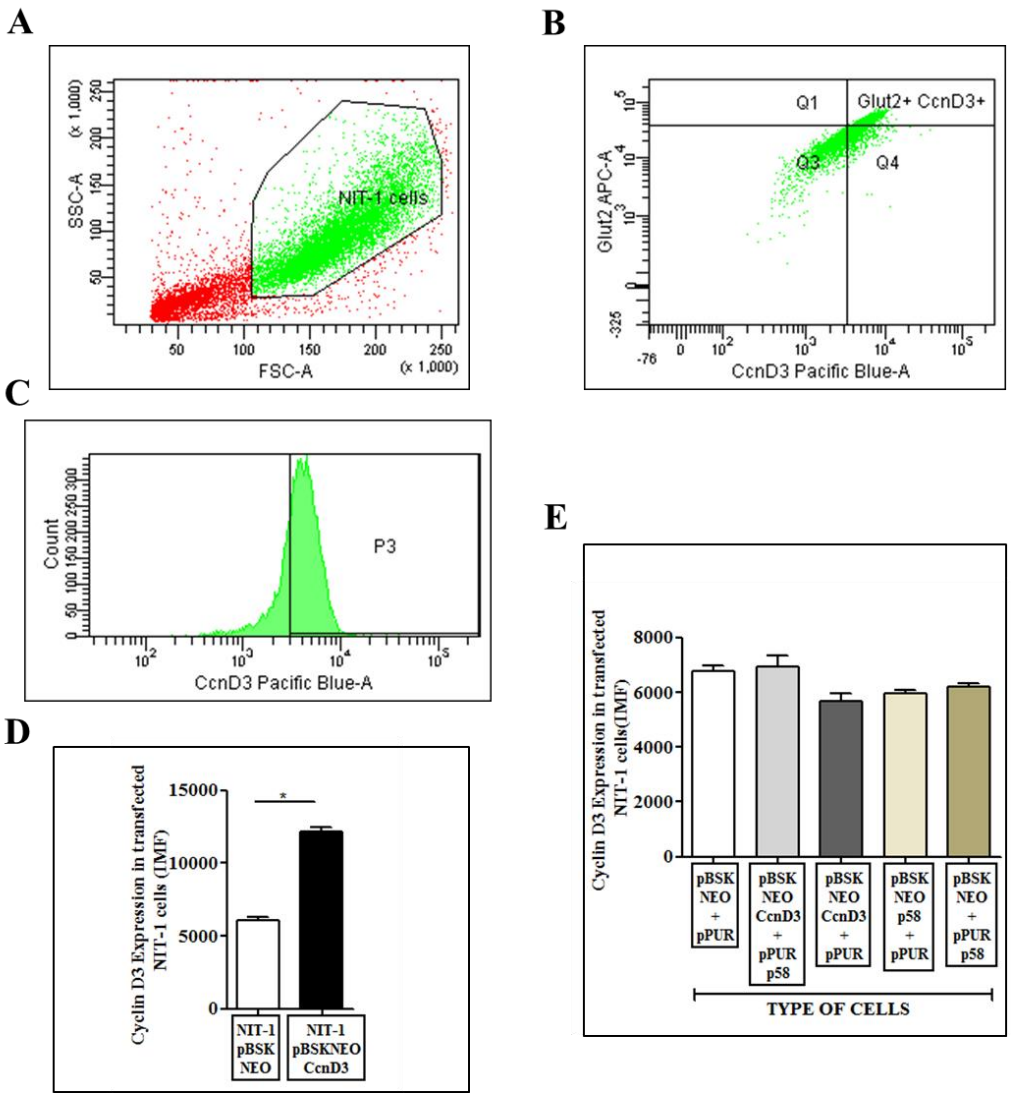


Figure 31. Cyclin D3 expression in NIT-1 cells (single and double transfected cells) *Nit-1 cells were cultured, trypsinized, stained and passed through the flow cytometer.* ***(A)*** *The population of the NIT-1 cells was selected by the SSC (Side Scatter) characteristic and FSC (Forward Scatter) pattern.* ***(B) and (C)*** *The Glut-2^{+} expression was detected with APC (β-cell marker) and cyclin D3^{+} was detected with Pacific Blue.* ***(C)*** *The histogram represents the Intensity of Median Fluorescence of cyclin D3 Pacific Blue.* ***(D)*** *Cyclin D3 expression IMF (Intensity of Median Fluorescence) was measured by flow cytometry on Glut2^{+}CcnD3^{+} cells. Cyclin D3 expression in pBSKNEOCcnD3 was 2.005 times higher than that in pBSKNEO in single transfectants.* ***(E)*** *Cyclin D3 expression IMF was measured by flow cytometry on Glut2^{+}CcnD3^{+} cells. There lies no significant difference between the cells (according to Mann.Whitney test). There lies significant difference among*

the whole group according to ANOVA test (p=0.023). Triplicates were used in the experiment performed.

10.2 NIT-1 cells overexpressing cyclin D3: Effect of cyclin D3 overexpression in proinflammatory cytokine-induced apoptosis and NOD leucocyte-induced necrosis

10.2.1 NIT-1 cells overexpressing cyclin D3 are protected from spontaneous and IL-1β-induced apoptosis

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 mock control insert (**Figure 32A**.) Stable transfectants were selected in DMEM complete media (Methods section Table 13. Composition of DMEM medium). For cytokine-induced cell apoptosis, 100000 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 and 200U/mLfor IFN-γ; on day 2, cells were harvested mechanically (with cell scrappers) and assayed for either annexin V staning, or for proliferation Ki-67. NIT-1 cells stably overexpressing cyclin D3 rendered 3.4 fold more protection against spontaneous and 10.2 fold more protection against IL-1β-induced apoptosis than the mock transfected controls (**Figure 32B**). However, neither residual IFN-γ nor IFN-γ + IL-1β-induced NIT-1 cell apoptosis was affected by cyclin D3 overexpression. No changes in necrosis were observed (**Figure 32D**) amongst the different cell types studied.

NIT-1 cells did not show either enhanced proliferative activity when overexpressing cyclin D3 or impaired necrosis compared with mock-transfected cells (**Figure 32C**) Only IFN-γ either alone (2.76 fold increase) or on association with IL-1β (2.32 fold increase) in pBSKNEO mock transfectants and, in pBSKNEOCcnD3 IFN-γ (4.39 fold increase) or in association with IL-1β (5.25 fold increase) promotes NIT-1 cell replication. Hence, cyclin D3 overexpression only increased NIT-1 cell proliferation in the presence of IFN-γ however, at basal condition or in the presence of IL-1β there was no effect.

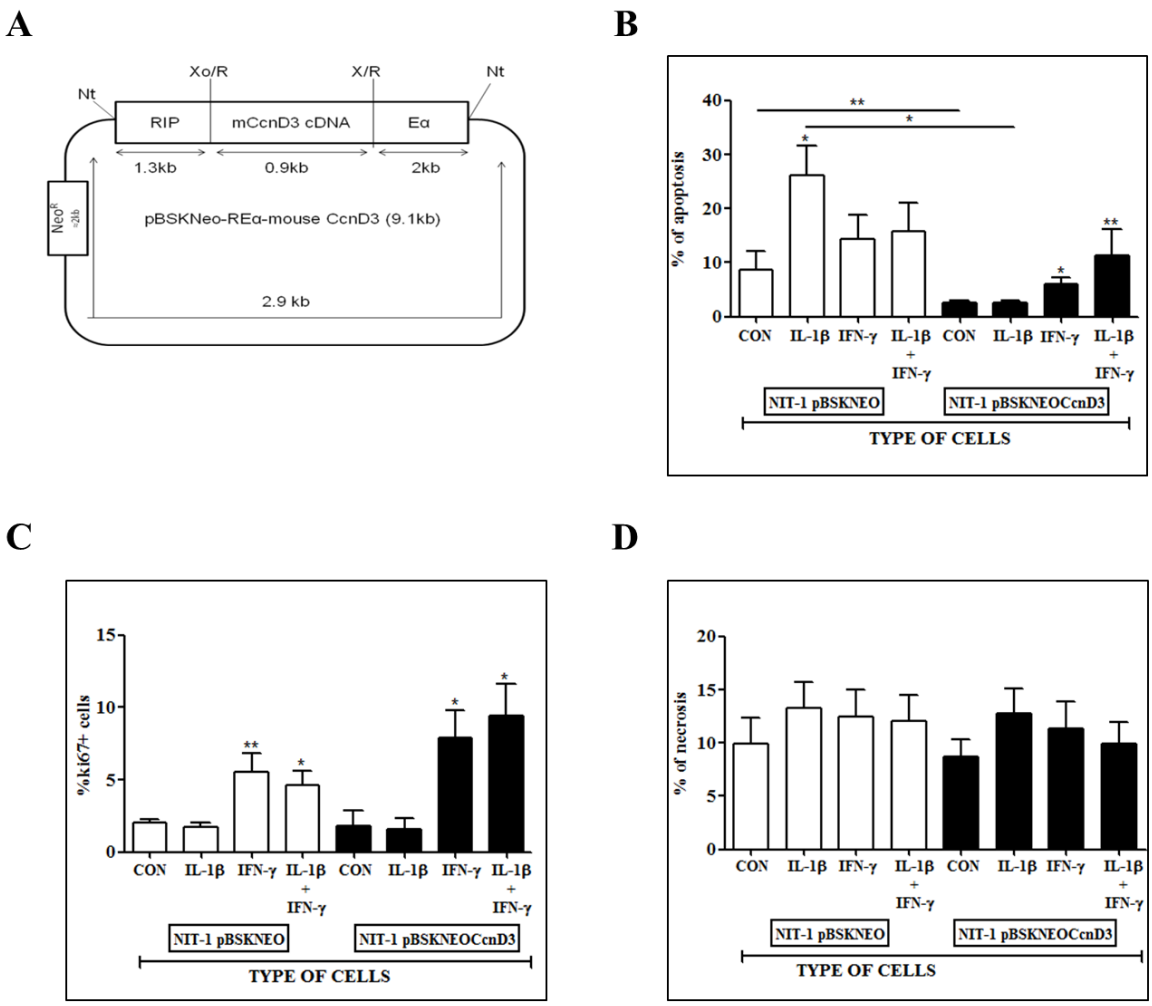


Figure 32. NIT-1 cells overexpressing cyclin D3 are less susceptible to basal and IL-1β induced apoptosis. ***(A)*** *Map of the plasmid pBSKNeo containing the RIP-CcnD3 cassette used to stably transfect NIT-1 cells; H, HindIII; N, NruI; Nh, NheI; Nt, NotI; P, PmeI; R, EcoRI; Xo, XhoI restriction sites* ***(B)*** *NIT-1 Neo cell line with either empty pBSKNEOCcnD3 vector or with pBSKNEO containing the RIP-CcnD3 construct were cultured for 24h with DMEM medium in prescence or absence of IL-1β, IFN-γ or both; % of early apoptosis was measured as AnnexinV^{+} PI^{-} NIT-1cells in each experimental group* ***(C)*** *Proliferation levels were measured as % of Ki67^{+} NIT-1 cells in each group and* ***(D)*** *Necrosis was plotted as % of PI^{+} cells each group. All the graphs were plotted according to the Mean+ SEM. Six experiments were performed. * show differences with respective internal controls; $^{-}$* shows differences between same stimuli but different cell clones NIT-1 pBSKNEO and NIT-1 pBSKNEOCcnD3 respectively; * p≤0.05, ** p≤0.01, ***p≤0.001.*

10.2.2 NIT-1 cells overexpressing cyclin D3 are protected against NOD leucocyte-induced necrosis

Total spleen cells from NOD female mice older than 8 weeks were co-cultured with NIT-1 cells for 24 hours in the absence of G418 sulphate. Stable transfectants were selected in DMEM complete media (Methods section Table 13. Composition of DMEM medium). 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 Ki-67 proliferation. The harvested cells were assayed for apoptosis (**Figure 33A**), necrosis (**Figure 33B**) and proliferative activity (**Figure 33C**).

The results show that at a low splenocyte ratio (1 NIT: 0.1 splenocyte) NIT-1 cells overexpressing cyclin D3 was protected from spleen cell induced necrosis compared to the NIT-1 pBSKNEO transfectants.

However, no protection against spleen cell induced apoptosis was observed in NIT-1 pBSKNEOCcnD3 compared with the NIT-1 pBSKNEO cells, suggesting that IFN-γ + IL-1β (**Figure 33A**) is responsible for spleen cell induced NIT-1 cell apoptosis, and not IL-1β alone.

Cyclin D3 overexpression does not alter the proliferative capacity of these cells in presence of the spleen cells. (**Figure 33C**)

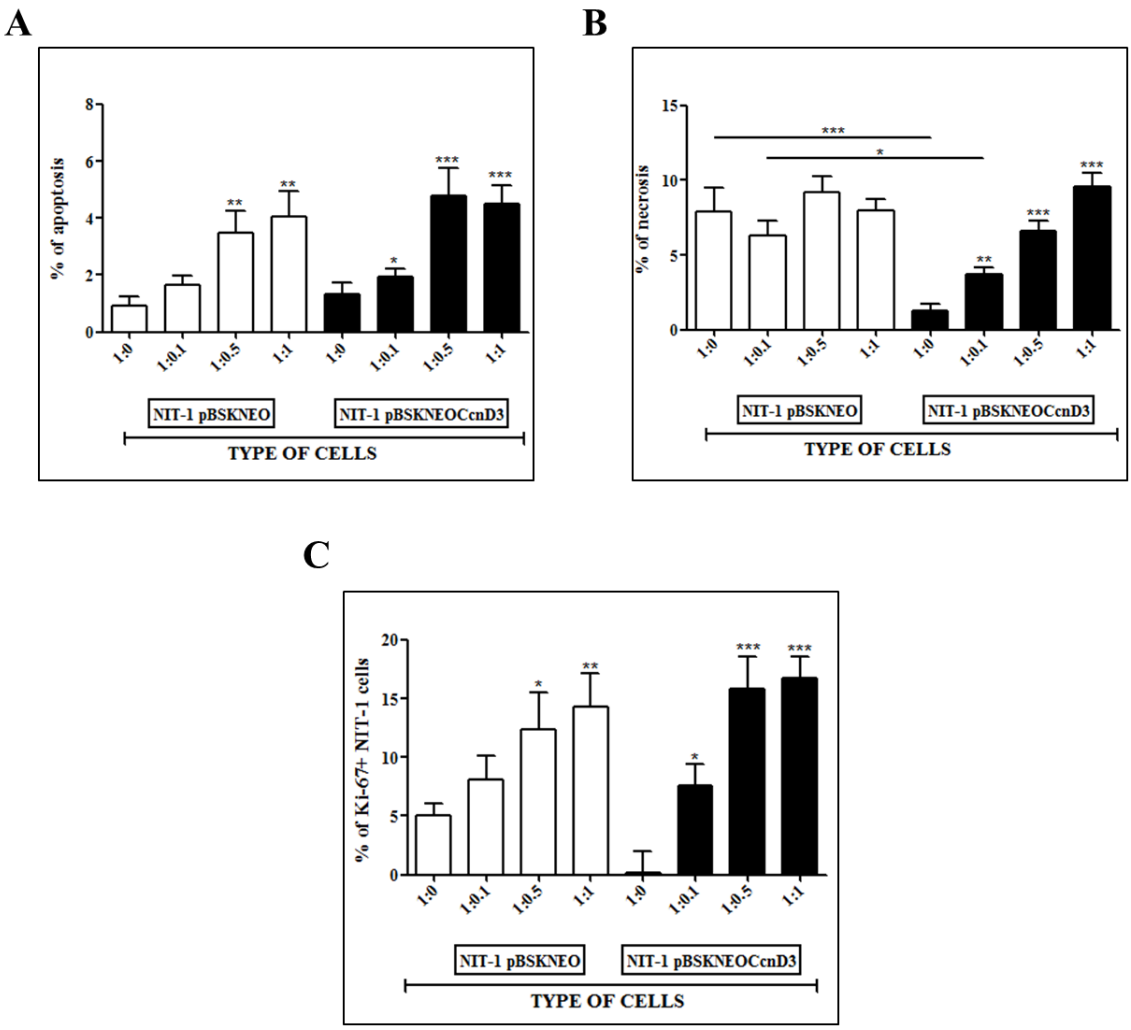


Figure 33. NIT-1 cells overexpressing cyclin D3 cells are protected against necrosis. *NIT-1 cells stably transfected with either the PBSKNeo plasmid or the PBSKNeo plasmid containing the RIP-CcnD3-Eα construct were cultured for24h in DMEM with different ratios of total spleen cells from NOD female mice older than 8 week old. The ratios shown mean the number of spleen cells per one NIT-1 cells (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^{m} AnnexinV^{+}* ***(B)*** *% of Necrotic NIT-1 cells is plotted as % of CD45^{-} Glut2^{+} PI^{+} cells; and* ***(C)*** *Percentage of proliferation NIT-1 cells plotted as of CD45^{-} Glut-2^{+} ki67^{+} cells . All graphs were plotted according to the Mean+ SEM. Four experiments with a minimum of 4 replicates were performed in each of the cases. * show differences with controls; $^{-}$* show differences between same stimuli but different hybridomes NIT-1 pBSKNEO and NIT-1 pBSKNEOCcnD3respectively. * p≤0.05, ** p≤0.01, ***p≤0.001.*

10.3 NIT-1 cells overexpressing cyclin D3 and / or CDK11p58: Effect of cyclin D3 and/or CDK11p58 overexpression in proinflammatory cytokine-induced apoptosis both in early and in late passages

10.3.1 In Early Passages:

10.3.1.1 NIT-1 cells overexpressing both cyclin D3 and CDK11p58 are less susceptible to basal apoptosis

The cDNA of cyclin D3 and/or CDK11p58 was subcloned under the rat insulin promoter (RIP) in the pBSK-NEO plasmid, which confers resistance to Neomycin and, the cDNA of cyclin D3 or CDK11p58 was subcloned under the rat insulin promoter (RIP) in pPUR (See Methods section: **Figure 25A** Map of pPUR vector) which confers resistance to puromycin. NOD insulinoma cell line (NIT-1) was stably transfected by either electroporation or by Metafectene® Pro (Biontex, USA) with the pBSKNEO and pPUR plasmid containing the rat insulin promoter driving the mouse CcnD3 cassette (RIP-CcnD3) transgene and /or the p58 transgene and the empty plasmids as mock control insert (**Figure 32A, Figure 34A** and **34B**).

For checking the basal apoptosis level in the cells overexpressing both cyclin D3 and CDK11p58 10^5 cells per well were seeded in 96-well plates on day 0 without any antibiotics; on day 1, cells were harvested mechanically (with cell scrapers) and assayed for annexin V and propidium iodide staining.

NIT-1 cells stably overexpressing cyclin D3 in pBSKNEOCcnD3+pPURp58 and pBSK NEOCcnD3+pPUR rendered 3.7 fold more protection and 3.1 fold more protection against spontaneous apoptosis respectively than the mock transfected controls (pBSKNEO+pPUR). (**Figure 34C**)

NIT-1 cells stably overexpressing CDK11p58 in pBSKNEOp58+pPUR and pBSKNEO + pPURp58 also rendered 1.79 fold protection and 1.6 fold more protection respectively against spontaneous apoptosis than the mock transfected controls (pBSKNEO+pPUR). (**Figure 34C**)

Moreover these double transfectants overexpressing both cyclin D3 and CDK11p58 or

cyclin D3 alone protects from spontaneous apoptosis when compared to the cells overexpressing only CDK11p58 suggesting that p58 is related to apoptosis. NIT-1 cells stably overexpressing cyclin D3 in pBSKNEOCcnD3+pPURp58 and pBSKNEOCcnD3 +pPUR rendered 2.09 fold more and 1.7 fold more protection when compared to pBSKNEOp58+pPUR respectively and 2.3 fold more and 1.9 more protection when compared to pBSKNEO+pPURp58 respectively (**Figure 33C**).

The statistical difference was done according to Mann-Whitney Test. According to the one way ANOVA test ($p < 0.0001$) and is significant.

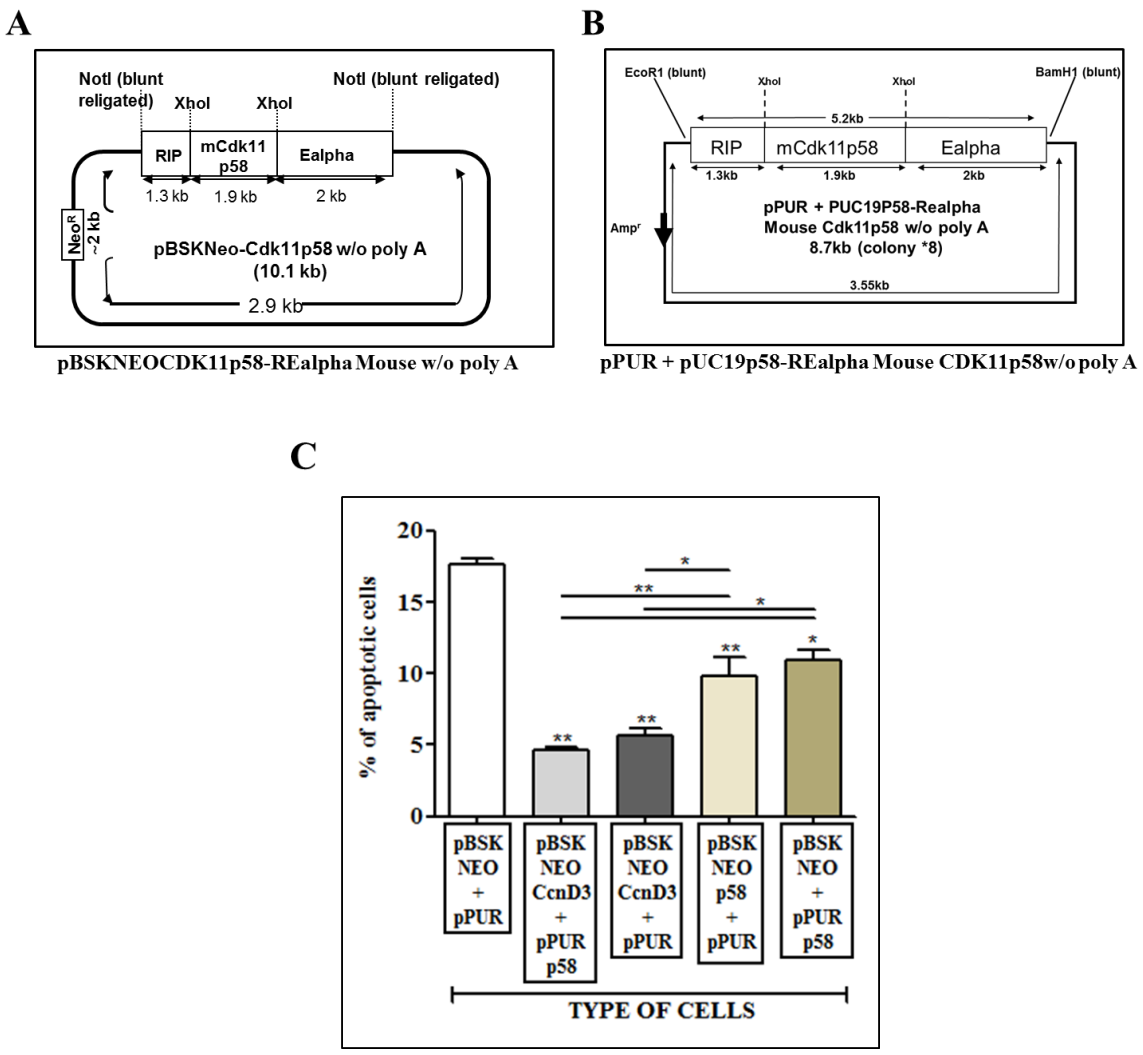


Figure 34. NIT-1 cells overexpressing both cyclin D3 and CDK11p58 are less susceptible to basal apoptosis. (A) *Map of the plasmid pBSKNEO containing the RIP-p58 cassette and*

*pPUR containing pUC19p58 were used to stably transfect NIT-1 cells; BamH1, NotI, EcoR1 and XhoI restriction sites. (**B**) The double transfected NIT-1 cell line with empty vectors and with the RIP-CcnD3 and /or p58 construct were cultured for 24h with DMEM in absence of any cytokine treatmet; % of early apoptosis was measured as AnnexinV^{+} PI^{-} cells in each experimental group.One experiment with 5 replicates were performed. They were expressed as Mean+ SEM. *shows differences between pBSKNEO+pPUR with the double transfected cells, _ * shows differences between different cell clones; * p≤0.05, ** p≤0.01, ***p≤0.001.*

10.3.1.2 Morphological Evidences of the NIT-1 cells in the early passages: Presence of the formation of blebs in the cells overexpressing CDK11p58

The NIT-1 transfected cells were plated in a p60 plate in the absence of cytokines till it reached 60% confluency. The cells were maintained in DMEM complete supplemented medium with the antibiotics G418 Sulphate 6 mM (400µg/ml) and Puromycin (1µg/ml). Photographs were taken with the microscope.

Figure 35, shows morphological features exhibited by the NIT-1 transfected cells. At first in **Figure 35A,** i.e. in pBSKNEO+pPUR, the cells appear to be healthy. In **Figure 35B,** i.e. in the double transfected cells with both cyclin D3 and CDK11p58 there appears a mixture of both healthy and unhealthy blebbing cells suggesting that p58 is actually enhancing apoptosis and so we found the unhealthy blebbing cells mixed with some healthy ones. In **Figure 35C and 35D,** i.e. the cells which are transfected with CDK11p58 either in pBSKNEO or pPUR (pBSKNEOp58+pPUR and pBSKNEO+pPURp58) appear to have the formation of blebs and look unhealthy as compared to **Figure 35A** and **Figure 35E**. Those blebs could be apoptotic bodies. These blebs are visible in **Figure 35C** and **Figure 35D** where CDK11p58 is overexpressed, which evidences the role of CDK11p58 in apoptosis. **Figure 35E,** i.e. the NIT-1 cells which are only transfected with cyclin D3 (pBSKNEO CcnD3+pPUR) had healthy appearance compared to the others showing that cyclin D3 itself might be protecting from apoptosis.

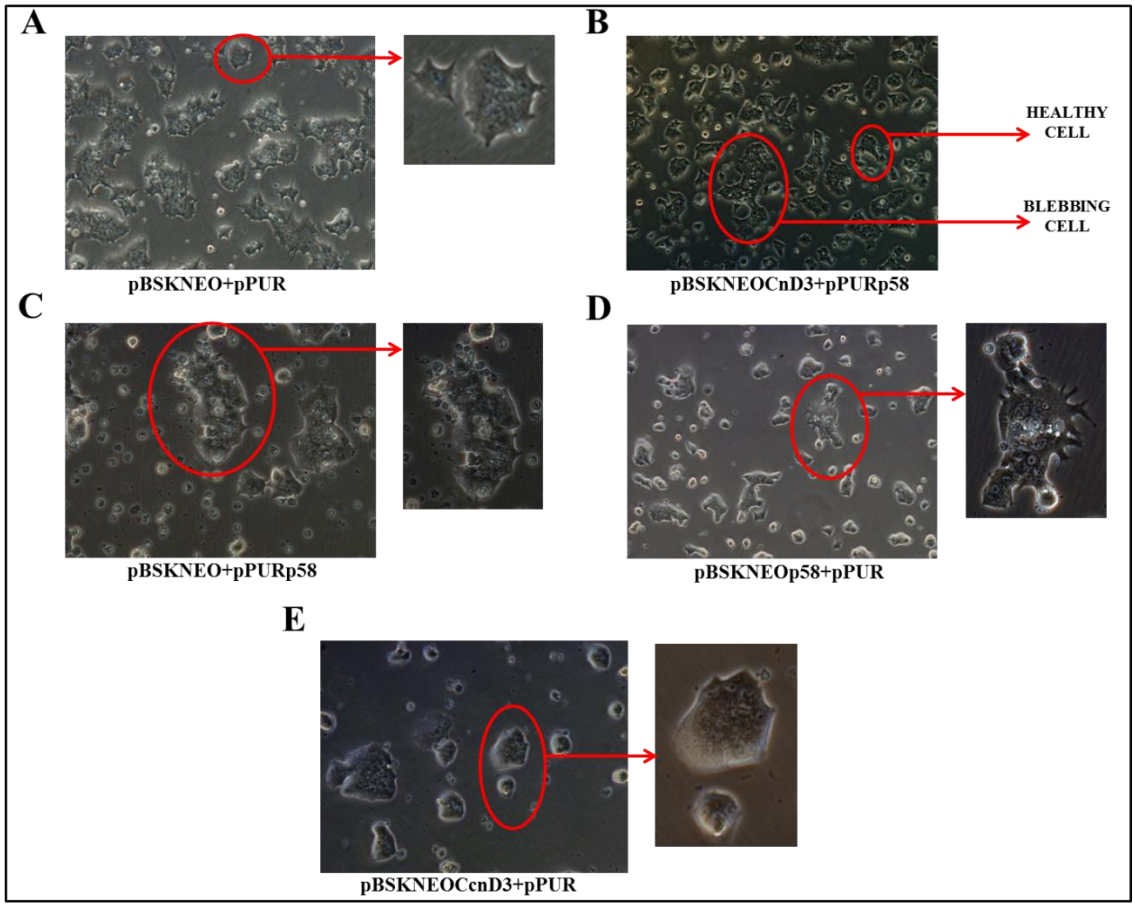


Figure 35. Morphological Evidences from the Nit-1 cells. ***(A)*** *pBSKNEO+pPUR transfected NIT-1 cells have non-apoptotic cells compared to others.* ***(B)*** *NIT-1 cells transfected with pBSKNEOCcnD3+pPURp58 have both type of cells healthy and blebbing cells.* ***(C)*** *&* ***(D)*** *The pBSKNEO+pPURp58 and pBSKNEOp58+pPUR transfected NIT-1 cells have unhealthy cells. The greater magnification shows the formation of blebs or apoptotic body like structures.* ***(E)*** *NIT-1 cells transfected with pBSKNEOCcnD3+pPUR show healthier cells as compared to the others. The pictures were taken by the Olympus IX71 microscope with 10X magnification. The red round lines show the specific cells in a greater magnification. 3 different experiments were performed to check the morphological evidences in these cells.*

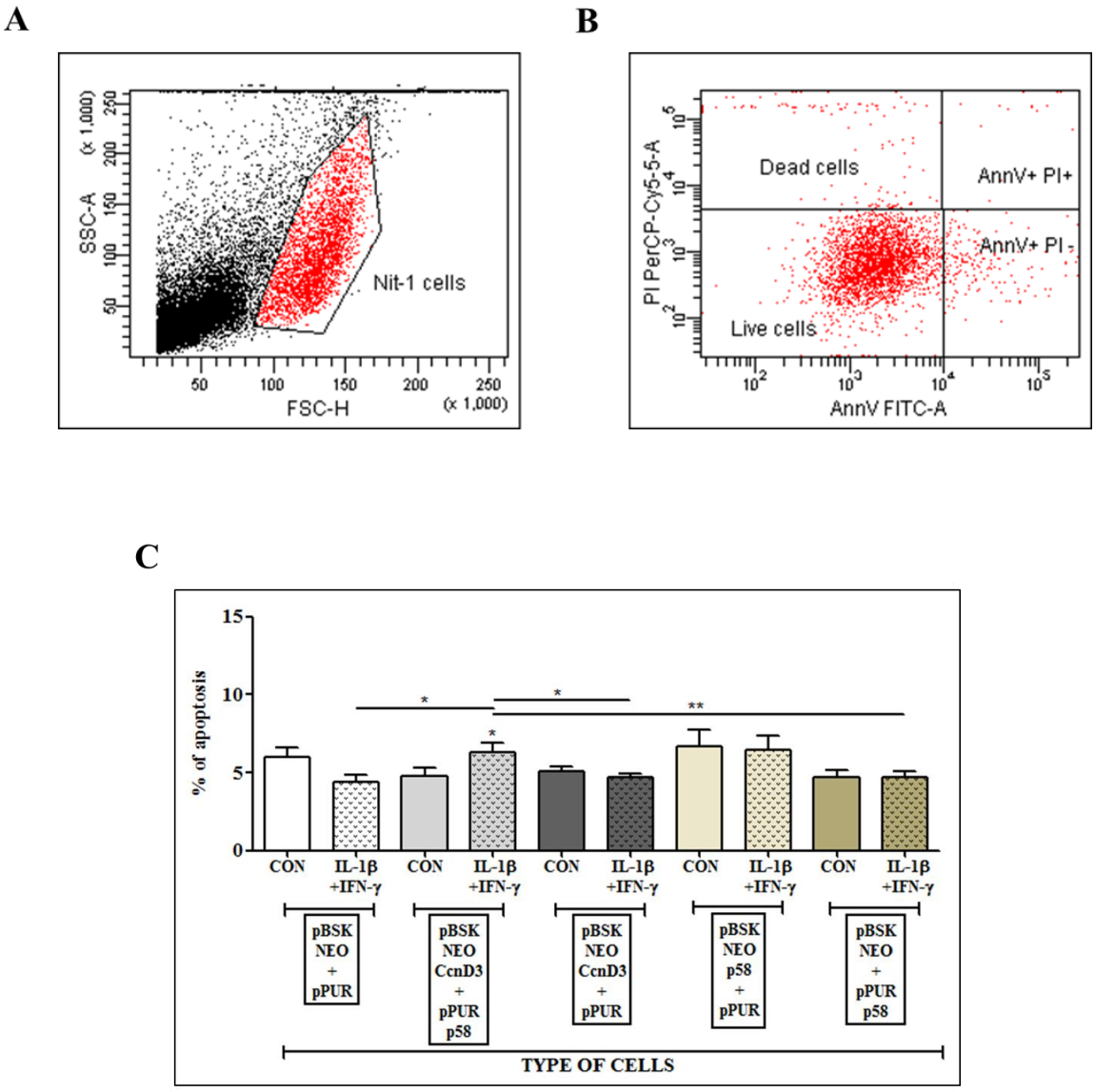
10.3.2 In Late Passages:

10.3.2.1 NIT-1 cells overexpressing both cyclin D3 and CDK11p58 are not protected from spontaneous and IL-1β-induced apoptosis

For cytokine-induced cell apoptosis, 10^5 cells per well were seeded in 96-well plates on day 0; on day 1 cytokines were added at a final concentration of 50U/mL for IL-1β and 200U/mLfor IFN-γ ; on day 2, cells were harvested mechanically and assayed for annexin V and Propidium Iodide staining.

In the later passages of the cells, NIT-1 cells stably overexpressiong cyclin D3 and/or CDK11p58 are not protected against both spontaneous and IL-1β+ IFN-γ induced apoptosis (**Figure 36C**). Only in the presence of overexpressed p58, even in the presence of cyclin D3 i.e. in pBSKNEOCcnD3+pPURp58, apoptosis is induced by cytokines IL-1β and IFN-γ. In case of apoptosis, differences among groups were statistically significant (ANOVA p-value=0.03). When analysing pairs of groups, the most significant difference was found between pBSKNEOCcnD3+pPURp58 and pBSKNEO+pPUR (p-value=0.005), but also other pairs of groups showed significant differences: pBSKNEO+pPURp58vs pBSKNEOCcnD3 +pPURp58, pBSKNEOCcnD3+pPUR vs pBSKNEOCcnD3+pPURp58 and pBSKNEO CcnD3+pPURp58 vs pBSKNEO+pPURp58 in presence of cytokines, but with a lower significance level ($p<0.01$).

Both in the absence and in the presence of cytokines the mock double transfectants have the maximum necrotic behavior (**Figure 36D**). CDK11p58 overexpression causes the NIT-1 cells to reach higher necrotic values, though lower from the mock controls but higher than the cells overexpressing cyclin D3.


A
SSC-A (x 1,000)
FSC-H (x 1,000)
Nit-1 cells
B
PI PerCP-Cy5-5-A
AnnV FITC-A
Dead cells
AnnV+ PI+
AnnV+ PI-
Live cells
C
% of apoptosis
CON
IL-1β +IFN-γ
pBSK NEO + pPUR
pBSK NEO CcnD3 + pPUR p58
pBSK NEO CcnD3 + pPUR
pBSK NEO p58 + pPUR
pBSK NEO + pPUR p58
TYPE OF CELLS

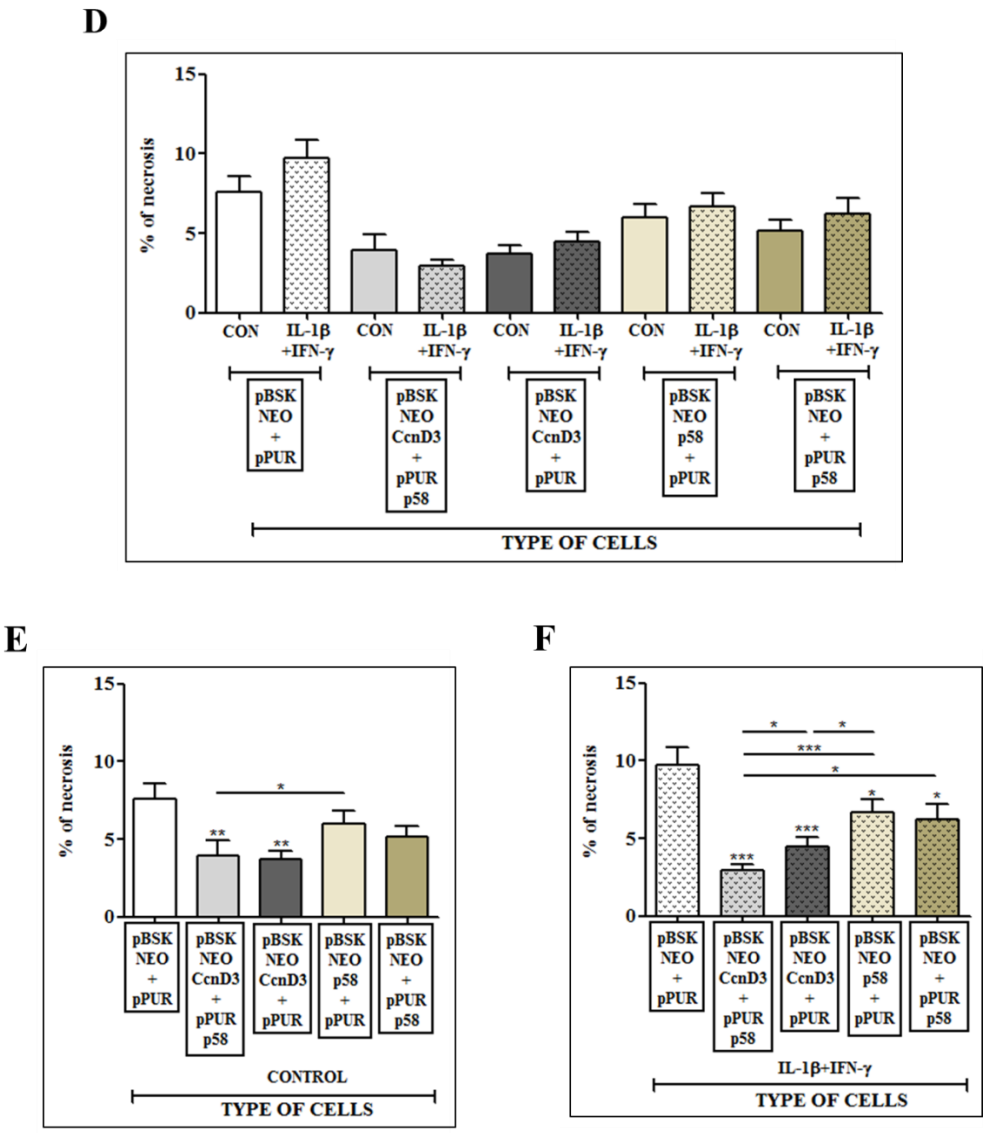


Figure 36. NIT-1 cells overexpressing cyclin D3 and/or CDK11p58 in the double transfected cells. (A) *The population of the NIT-1 cells were selected by the SSC (Side Scatter) and FSC (Forward Scatter).* ***(B)*** *The Annexin-V$^+$ and PI$^-$ subpopulation represent the early apoptotic cells. The propidium iodide$^+$ cells represent the necrotic cells and the dead cells.* ***(C)*** *The double transfected NIT-1 cell line with empty vectors and with the RIP-CcnD3 and /or p58 construct were cultured for 24h with DMEM in prescence or absence of IL-1β+IFN-γ; % of early apoptosis was measured as AnnexinV$^+$ PI$^-$ cells in each experimental group. The controls of the different transfectants are not statistically different from each other. They were expressed as Mean+ SEM.* ***(D)*** *Necrosis was plotted as % of PI$^+$ cells each group and was expressed as Mean+ SEM. The statistical differences are not*

pointed in this graph which gains clarity. ***(E)*** *Percentage of necrotic cells in absence of cytokines.* ***(F)*** *Percentage of necrotic cells in presence of cytokines. All the graphs were plotted according to the Mean+ SEM. Five experiments with a minimum of 4 replicates was performed in each of the cases. * show differences with respective internal controls; *shows differences between pBSKNEO+pPUR with the double transfected cells, _ * shows differences between same stimuli but different cell clones pBSKNEO+pPUR, pBSK NEOCcnD3+pPURp58,pBSKNEOCcnD3+pPUR, pBSKNEOp58+pPUR, pBSKNEO+ pPURp58; * p≤0.05, ** p≤0.01, ***p≤0.001.*

10.4 Assessing the proliferation levels in double transfectants NIT-1 cells

The NIT-1 transfected cells were cultured in a 75cm^2 culture flask in the same complemented DMEM medium with the antibiotics G418 Sulphate (400µg/ml) and Puromycin (1µg/ml) until the cells reached 60% confluency. They were trypsinized and then the proliferation of these transfected cells was measured using the Ki-67 proliferation marker. The cells were plated in DMEM complete medium without antibiotics to perform the experiments.

NIT-1 cells did not show either enhanced proliferative activity when overexpressing cyclin D3 and /or CDK11p58 (**Figure 37D**). This demonstrated the fact that all the double transfected NIT-1 cells had the same proliferative capacity.

The values that we got for assessing the Ki-67 expression were statistically significant when comparing all groups (ANOVA p-value=0.015). When analyzing particular pair of groups statistical significance was not achieved. This is just n=1 experiment with triplicates and these are just preliminary results for the proliferation test.

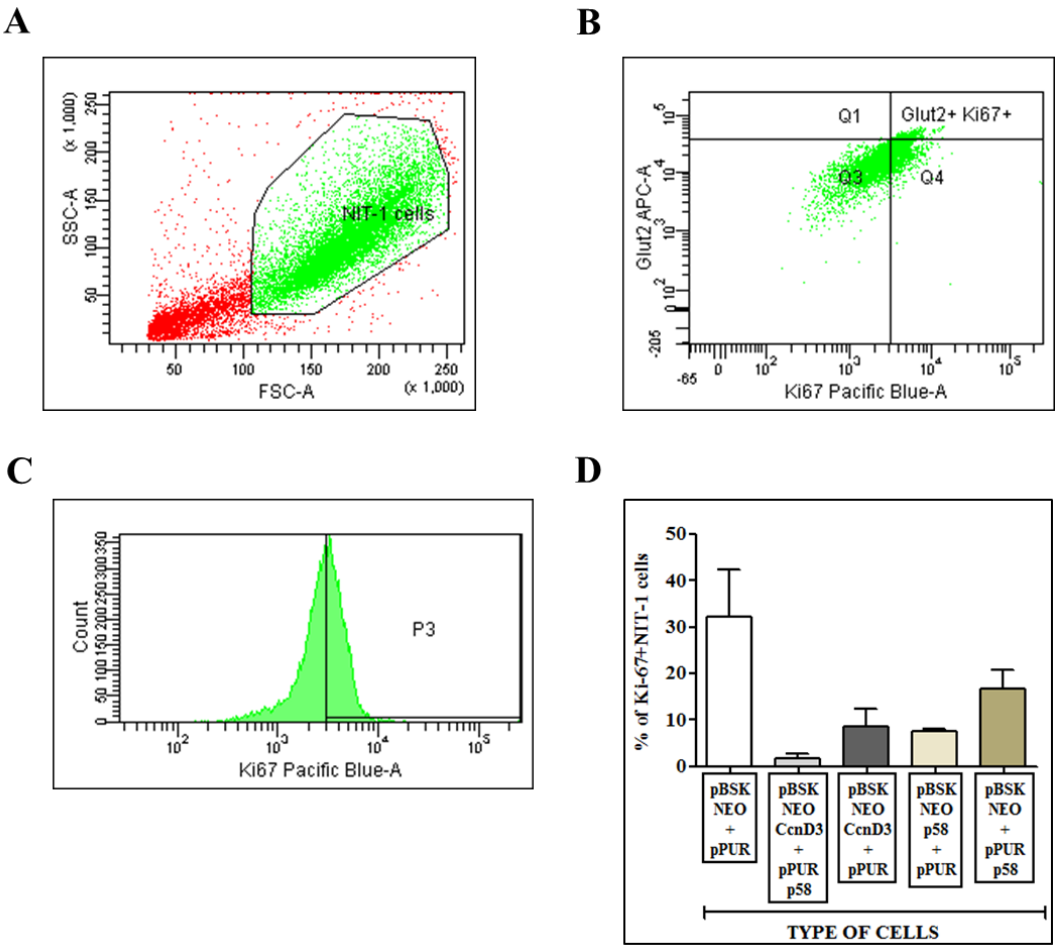


Figure 37. Proliferation levels of the transfected NIT-1 cells. *Nit-1 cells were trypsinized and stained by Flow cytometry.(A) The population of the NIT-1 cells were selected by the SSC (Side Scatter) and FSC (Forward Scatter). (B) & (C) The Glut-2^{+} conjugated with APC (ß-cell marker) and Cyclin D3^{+} conjugated with Pacific Blue were measured. (C) The Intensity of Median Fluorescence of Cyclin D3 Pacific Blue. (D) Proliferation levels were measured as the percentage of Ki-67^{+} cells in each group of transfected cells. Ki-67^{+} cells (% of positive cells) were measured by flow cytometry on Glut2^{+}Ki-67^{+} cells. There lies no significant difference between the cells (according to Mann Whitney test). There lies significant difference among the whole group according to one-way ANOVA test (0.015). The graph was plotted according to the Mean+ SEM. Triplicates were used in the experiment performed. _ * shows differences between different cell clones NIT-1 Neo+pPUR, NIT-1 NeoCcnD3+pPURp58, NIT-1 NeoCcnD3+pPUR, NIT-1 Neop58+pPUR and NIT-1 Neo+pPURp58 , * p≤0.05, ** p≤0.01, ***p≤0.001.*

Chapter 2

In vivo Experiments

Role of Cyclin D3 and CDK11 in the NOD mouse pancreatic beta cells (KO and Tg)

10.5 Generation of NODCDK11CcnD3Tg and NODCDK11CcnD3KO mice: Phenotyping the mice strains

Transgenic NOD mouse overexpressing cyclin D3 in β cells was generated by microinjection of the RIP-CcnD3-Eα construct into fertilized NOD oocytes as in **Figure 38A**. Six different founder lines were generated and one of them was been lost during the SPF rederivation process. Expression of cyclin D3 was verified in 5 different founder lines and only one strain overexpressed cyclin D3 (line 6896) in β cells. We conducted all further experiments with this transgenic line which was crossed with CDK11WT and CDK11HTZ (hemideficient in CDK11 founder, as complete deletion of CDK11 is not possible because it is lethal at the embryonic stage [276]). Thus, NODCDK11CcnD3Tgpos and NODCDK11 CcnD3Tgneg mice were generated and used for further experiments.

Mice homozygous for the deficiency in cyclin D3 were originally obtained in the mixed 129/Sv C57BL/6 genetic background [300]. After intensive backcrossing onto the NOD genetic background and checking the Idd T1D susceptibility loci, we reached the 12th generation (N13) stage. NOD female mice deficient in cyclin D3 (NODCcnD3KO) were further crossed with the CDK11HTZ to generate NODCDK11HTZCcnD3KO mice and NODCDK11WTCcnD3KO mice.

NODCDK11CcnD3Tg pos/neg and NODCDK11CcnD3KO mice were used for the experimental procedures in this thesis.

We observed variations in body size, weight and the number of pups delivered per litter. Among the different NOD strains generated in our study NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+)CcnD3Tg- mice have the same size (data not shown). NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice show no difference in body weight (**Figure 38C**). The number of pups delivered per litter of mice has no significant difference between these two groups (**Figure 38D**).

CDK11(+/+) CcnD3(-/-) mice have a significant small size as compared to the plain wild type NOD mice (**Figure 38B**). CDK11(+/+) CcnD3(-/-) and CDK11(Δ/+) CcnD3(-/-)mice have the same body size (data not shown) . The number of pups delivered per litter of mice

has no significant difference between these two experimental groups NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) (**Figure 38D**).

When comparing the weights of all the groups of mice together we see that there was significant difference between the NODCDK11(+/+) CcnD3(-/-) mice with the other four groups NODCDK11(+/+) CcnD3Tg- and NODCDK11(+/+) CcnD3Tg+, NODCDK11 (Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice (**Figure 38C**). There lies significant difference among the whole group according to one-way ANOVA test ($p < 0.0001$) and after Bonferroni correction. The number of pups delivered per litter in NODCDK11(+/+) CcnD3(-/-) mice was significantly lower than in than both of NODCDK11 (Δ/+) CcnD3 Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice (**Figure 38D**).

Moreover, as we see in **Figure 38A** NODCcnD3(-/-) regardless of CDK11 genotype, exhibit exacerbated diabetes and the litter size is smaller, which decreased exceedingly the possibility of having large experimental groups of these mice.

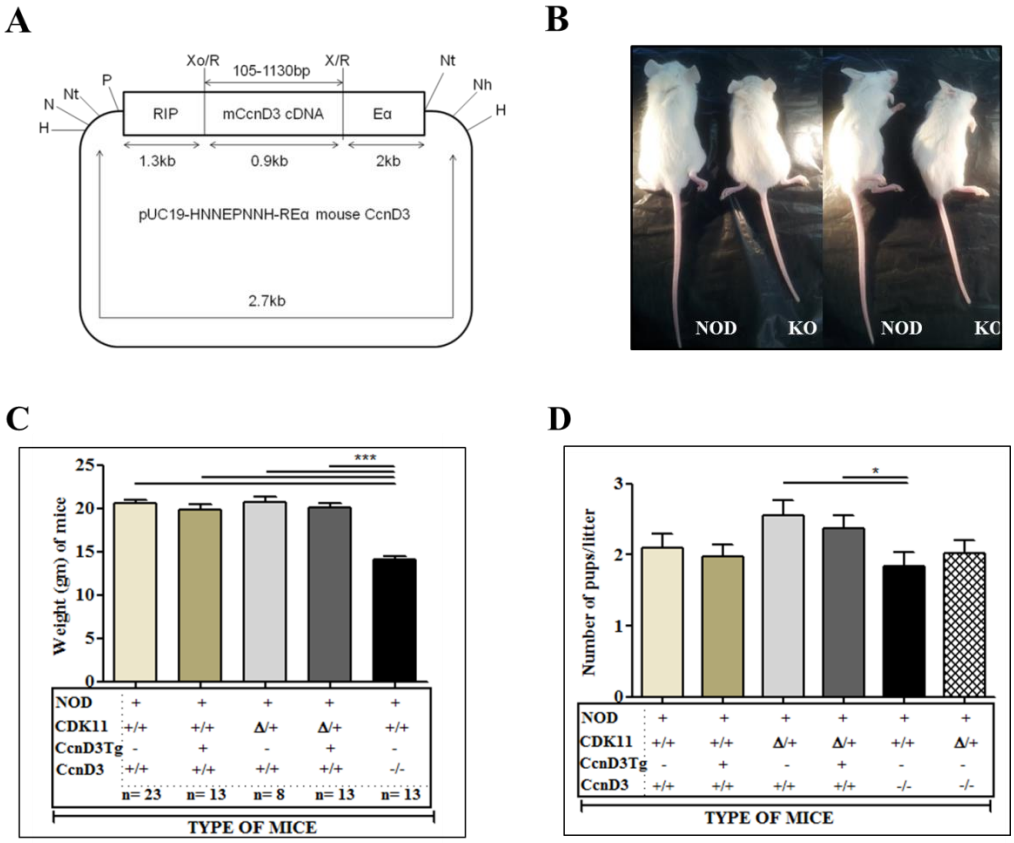


Figure 38. Generation of general features of NODCDK11CcnD3Tg and NODCDK11

CcnD3KO mice. (A) *β-cell specific cyclin D3 construct NOD 6896 Tg strain construct: RIP-CcnD3-Eα, RIP, Rat insulin promoter; Eα, E alpha gene (H-2); H, HindIII; N, NruI; Nh, NheI; Nt, NotI; P, PmeI; R, EcoRI; Xo, XhoI restriction sites.* ***(B)*** *Pictures of NODCDK11(+/+) CcnD3(-/-) mice as compared to the plain wild type NOD mice at 5 weeks of age.* ***(C)*** *Body weight of NODCDK11(+/+) CcnD3(-/-) mice was significantly less compared to the 4 strains of mice i.e. NODCDK11(+/+) CcnD3Tg-, NODCDK11(+/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg- and NODCDK11(Δ/+) CcnD3Tg+ at 12 weeks of age . The graph was plotted according to Mean+ SEM. n= number of pups.* ***(D)*** *The figure represents the number of pups per litter in the 6 different strains of mice namely NODCDK11(+/+)CcnD3Tg-, NODCDK11(+/+) CcnD3Tg+ and NODCDK11(Δ/+)CcnD3 Tg-, NODCDK11(Δ/+)CcnD3Tg+, NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-). NODCDK11 (+/+) CcnD3(-/-) (n=48) mice have significant lower number of pups than NODCDK11(Δ/+) CcnD3Tg- (n=63) and NODCDK11(Δ/+) CcnD3Tg+ (n=63) (according to Mann-Whitney test).The graph was plotted according to the Mean+ SEM. n= number of litters.* p≤0.05, ** p≤0.01, ***p≤0.005.*

10.6 CDK11WT CcnD3KO and CDK11HTZ CcnD3KO mice show the same incidence of diabetes

To demonstrate the causal link between cyclin D3 and CDK11 downregulation and β-cell death *in vivo*, we studied spontaneous diabetes onset in NOD female mice deficient in cyclin D3 and hemi deficient in CDK11 compared to NOD female mice deficient in cyclin D3 but WT for CDK11 littermates. **Figure 39A** shows that both NODCDK11+/+ CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) developed the same diabetic phenotype .

Figure 39B shows that NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) exhibited the same kinetic of the disease, which is faster than that shown by NODCcnD3WT(+/+).

The cumulative incidence of diabetes is higher in the NODCDK11(+/+)CcnD3(-/-) {81.81%} and NOD CDK11(Δ/+) CcnD3(-/-) {78.94%} mice strains compared to wild type littermates {62.85%}. This was due to the deficiency in cyclin D3 in β cells.

This means that cyclin D3 deficiency on its own is enough to accelerate the disease. CDK11 hemideficiency does not further exacerbate the cumulative incidence of diabetes in NOD mice deficient in cyclin D3.

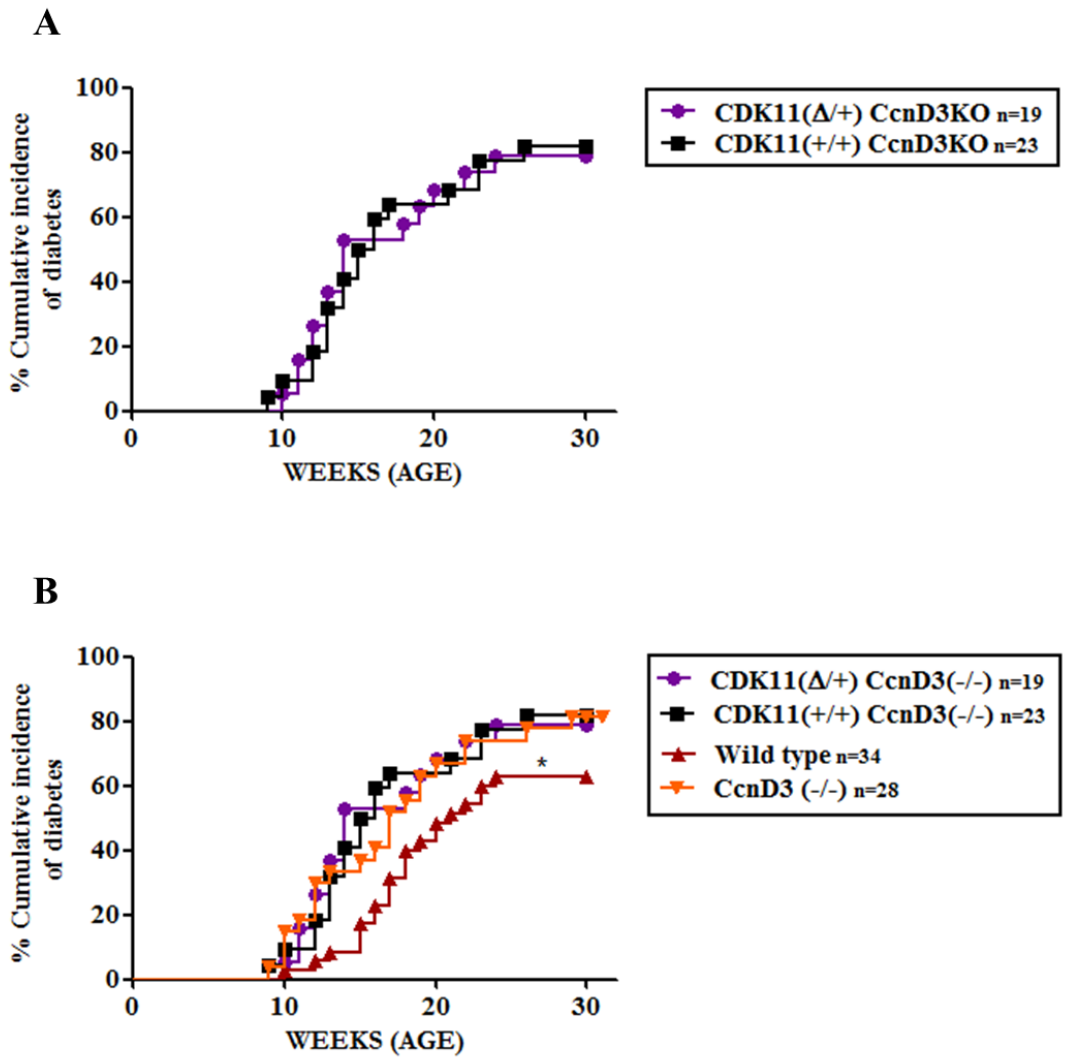


Figure 39. Downregulation of cyclin D3 and CDK11 respective contributions to the development of T1D. (A)*Cumulative incidence of diabetes of NODCDK11(+/+)CcnD3(-/-)(n=19) and NOD CDK11(Δ/+)CcnD3(-/-) (n=23). There is no statistical difference between the two groups.* ***(B)*** *Cumulative incidence of diabetes of NODCDK11 (+/+)CcnD3(-/-) (n=19), NOD CDK11(Δ/+)CcnD3(-/-) (n=23), NODCcnD3(-/-) (n=28) and NODCcnD3WT (n=34). Statistical analysis performed was Logrank test. * $p \leq 0.005$.*

10.7 CDK11HTZ CcnD3Tgpos and CDK11HTZ CcnD3Tgneg mice show the same incidence of diabetes which is lower than that exhibited by NODWT mice

To demonstrate the causal link between cyclin D3 and/or CDK11 downregulation and β-cell death *in vivo,* we studied spontaneous diabetes onset in NOD female mice in two groups where hemideficiency of CDK11 is shared by both groups and cyclin D3 is only overexpressed in β cells in one of them. **Figure 40A** shows that both NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice start developing T1D at 13 and 17 weeks of age respectively.

The cumulative incidence of diabetes in both strains, 56.66% for NODCDK11(Δ/+) CcnD3Tg+ and 38.46% for NODCDK11(Δ/+) CcnD3Tg- is not statistically different.

When compared to T1D onset in NOD mice overexpressing cyclin D3 in β cells (CcnD3Tg) and their transgene negative littermates in NODCcnD3Tg+ mice and the CcnD3Tg- mice, **Figure 40B** shows that NODCDK11(Δ/+) CcnD3Tg+{56.66%}, NODCDK11(Δ/+) CcnD3Tg-{38.46%}and NODCcnD3Tgpos{67.85%} mice exhibit slow T1D kinetics while NODCcnD3Tg- {64.3%} (equivalent to NODWT mice) exhibit faster and more severe diabetes.

Hence, this finding is important because we aimed to see whether the overexpression of cyclin D3 in the absence of half a dose of CDK11 would affect the development of T1D. The overexpression of cyclin D3 in β cells protects, reagardless of CDK11 hemideficiency or not, which would imply that cyclin D3 has an independent role of CDK11. This can be explained by the fact that the kinase activity of CDK11 is regulated by Cyclin D3 [322] and it seems they could interact in the pancreatic β cells.

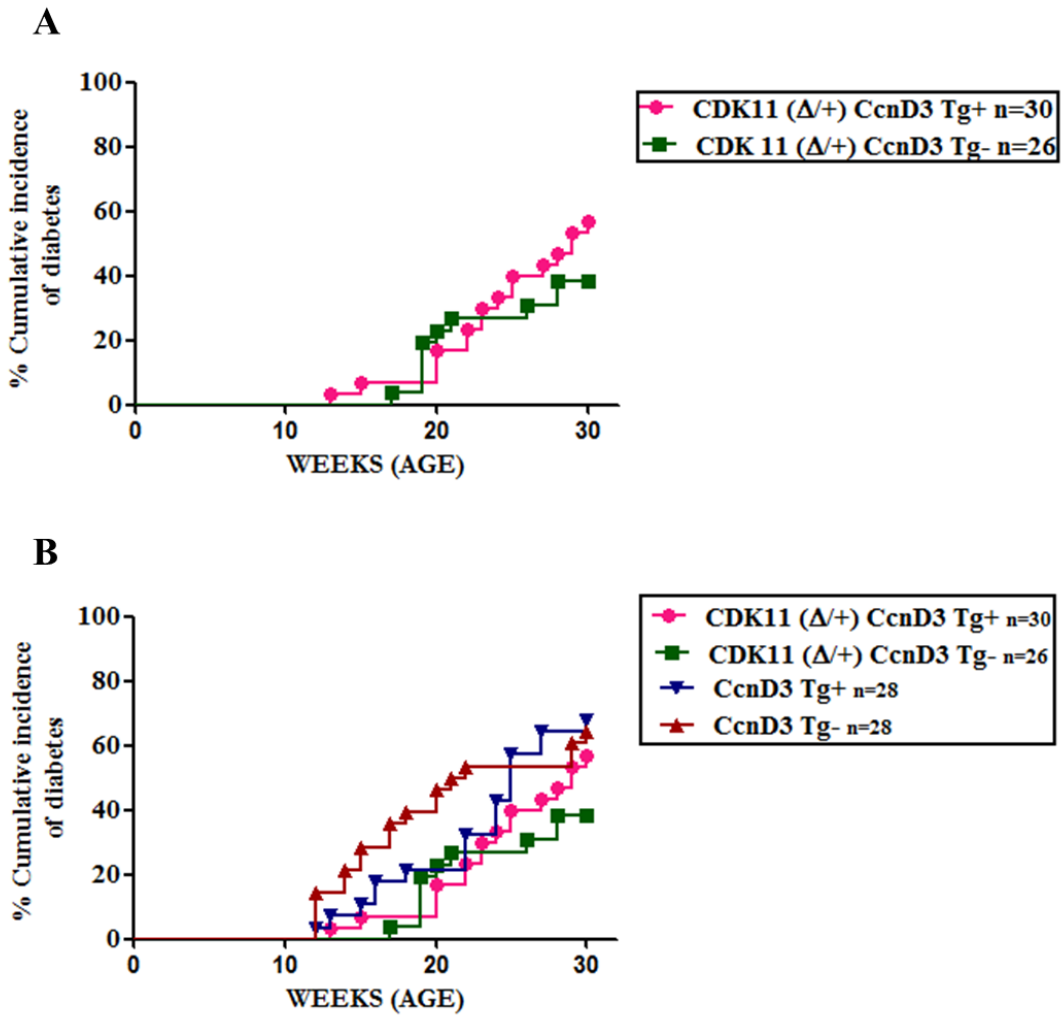


Figure 40.Overexpression of Cyclin D3 and half deletion of CDK11 contributes in the development of T1D. ***(A)*** *Cumulative incidence of diabetes of NODCDK11(Δ/+) CcnD3Tg+(n=30) and NODCDK11(Δ/+) CcnD3Tg- (n=26). There is no statistical difference between the two groups.* ***(B)*** *Cumulative incidence of diabetes of NOD CDK11(Δ/+)CcnD3Tg+ (n=30), NODCDK11(Δ/+) CcnD3Tg- (n=26), NODCcnD3Tg+ (n=28) and NODCcnD3Tg- (n=28). Statistical analysis performed in A and B was Logrank test. * p≤0.05, ** p≤0.01, ***p≤0.005.*

10.8 Susceptibility to adoptively transferred diabetes

10.8.1 Adoptive transfer of diabetes into NOD/SCIDCDK11(Δ/+) CcnD3Tg+ and NOD/SCIDCDK11(Δ/+) CcnD3Tg- recipients

Adoptive transfer experiments were performed with 10 million of total spleen cells from 8 week old NOD female donor mice into either NOD/SCIDCDK11(Δ/+) CcnD3Tg+ or NOD/SCIDCDK11(Δ/+) CcnD3Tg- 3-5 weeks old recipients. NODSCID mice lack lymphocytes, so by adoptive transfer we aimed to check cyclin D3 overexpression could circumvent CDK11 hemideficiency in terms of susceptibility to adoptively transferred diabetes. The diabetes incidence were measured weekly during 17 weeks post transfer.

In **Figure 41,** NOD/SCIDCDK11(Δ/+) CcnD3Tg+ recipient mice show the same susceptibility to develop the disease as that of NOD/SCIDCDK11(Δ/+) CcnD3Tg-. The cumulative incidence of diabetes in both experimental groups is similar: 57.14% in NOD/ SCIDCDK11(Δ/+) CcnD3Tg+ and 66.66% in NOD/SCIDCDK11(Δ/+) CcnD3Tg-. Hence, the result from this adoptive transfer goes hand in hand with the results from the survival graphs showing no statistical difference in the occurrence of the disease. It was confirmed the autoimmune cause of the disease as the pancreas from diabetic mice were extracted and analysed. From the analysis, the pancreatic islets were found to be infiltrated confirming that diabetes was caused by an autoimmune process.

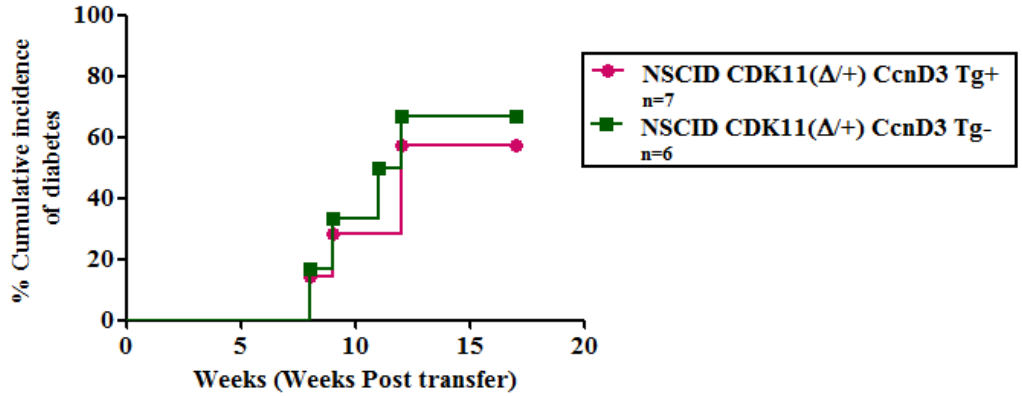


Figure 41. NOD/SCID CDK11(Δ/+) CcnD3Tg+ and NOD/SCIDCDK11(Δ/+) CcnD3Tg-

exhibit the same susceptibility to adoptively transferred diabetes. *Female NOD/SCID CDK11(Δ/+) CcnD3Tg+ (n=7) and NOD/SCIDCDK11(Δ/+) CcnD3Tg- (n=6) of 3-5 weeks of age were adoptively transferred with 10 million of total spleen cells from 8 week old NOD females. There is no statistical difference between the two groups. Statistical analysis performed was Logrank Test.*

10.8.2 Diabetogenecity splenocytes of 8 week old NODCDK11Δ/+CcnD3Tg+ or NOD CDK11Δ/+CcnD3Tg- mice

Adoptive transfer experiments used 10 million of total spleen cells from both types of donors: 8 week old NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- females were transferred into 3-5 weeks old NOD/SCID recipients. By adoptive transfer experiments we wanted to corroborate whether the lymphocytes from both genotypes had the same ability to induce the disease into NOD/SCID recipients. The diabetes incidence was measured weekly during 17 weeks post transfer.

Results show that the immune repertoire from both mouse genotypes had the ability to confer diabetes to the same extent. In **Figure 42,** it is clearly visible that both NODSCID recipients of the NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11(Δ/+)CcnD3Tg splenocytes show the same incidence of diabetes post transfer. The cumulative incidence of diabetes in both genotypes is similar: 35.29% in NODCDK11(Δ/+) CcnD3Tg+ and 64.7% in NODCDK11(Δ/+) CcnD3Tg- mice. This is not statistically different, since they show the same disease kinetics. It was confirmed that mice had autoimmune diabetes. From the analysis, the pancreatic islets were found to be infiltrated confirming it is caused due to autoimmunity.

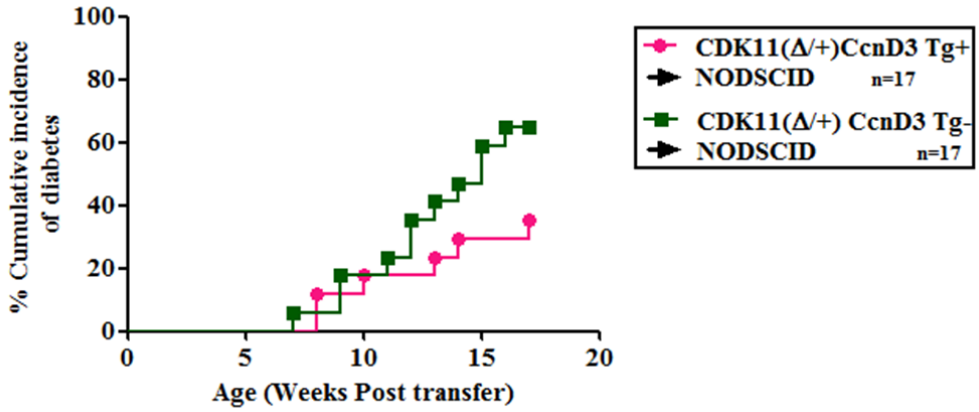


Figure 42. Splenocytes transferred from NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11 (Δ/+)CcnD3Tg- mice to the NOD/SCID causes similar incidence in Type 1 diabetes. *Female NOD/SCID mice of 3-5 weeks were adoptively transferred with 10 million of total spleen cells from different donors NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- (n=17 and n=17 respectively). There is no statistical difference between the two groups. Statistical analysis performed was Logrank Test.*

10.8.3 Diabetogenecity of 5 week old NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11 (Δ/+)CcnD3Tg- splenocytes

Adoptive transfer experiments were done with 10 million total spleen cells from 5 week old NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- female into NOD/ SCID of 3 weeks of age as recipients. By this adoptive transfer we wanted to confirm whether spleen cells from young donors of either of both genotypes analyzed NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- with non-infiltrated islets had the ability to transfer. The diabetes incidence was measured weekly during 17 weeks post transfer.

Results show that the spleen cells of all the genotypes used as donors had the ability to induce diabetes. In **Figure 43,** it is clearly visible that both the NOD/SCID recipients from the NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice of 5 weeks old show the same incidence of diabetes post transfer. This result also follows the same way as the adoptive transfer result in **Figure 42** and **Figure 40A** (as in the survival graph). It shows that since the donors were so young hence the incidence of diabetes in both groups

was very low as compared to that shown in adoptive transfers using 8 weeks old donor mice.

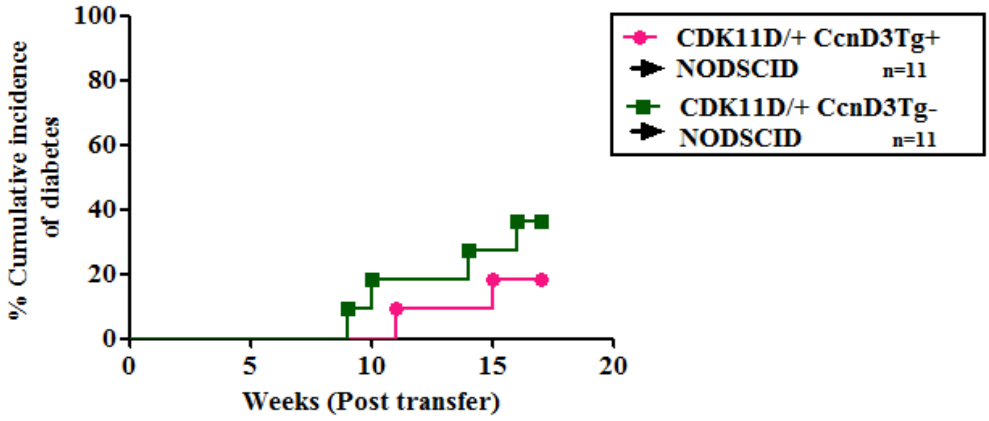


Figure 43. Splenocytes transferred from 5 week old NODCDK11Δ/+CcnD3Tg+ and NODCDK11Δ/+CcnD3Tg- mice to the NOD/SCID causes similar incidence in Type 1 diabetes. *Female NOD/SCID mice of 3-4 weeks were adoptively transferred with 10 million of total spleen cells from 5 week old donors NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- (n=11 and n=11 respectively). There is no statistical difference between the two groups. Statistical analysis performed was Logrank Test.*

10.9 Pancreatic islet infiltration assessment in 5 weeks old NODCDK11(Δ/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg-, NODCDK11(+/+) CcnD3Tg+ and NOD CDK11(+/+) CcnD3Tg- mice

To determine whether there was any alteration in the insulitis score of NODCDK11(Δ/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg-, NODCDK11(+/+) CcnD3Tg+ and NOD CDK11(+/+) CcnD3Tg- mice, we examined histological sections of pancreata from NOD female mice at 5 weeks age of all the genotypes mentioned. The criteria used to select the ages for scoring the infiltration is based on the age of the NOD mice used as donors for transfer and also for checking cyclin D3 expression at these ages. Reflecting the disease exacerbation caused by the CDK11 and cyclin D3 mutation in NOD females, infiltration scores were similar in the 4 genotypes of mice namely: NODCDK11(Δ/+) CcnD3Tg+,

NODCDK11(Δ/+) CcnD3Tg-, NODCDK11(+/+) CcnD3Tg+ and NODCDK11(+/+) CcnD3Tg-.

The pancreatic infiltration score was done in the following way as it is shown in **Figure 44A**: (i) 0= no infiltration, (ii) 1= infiltration surrounding the islet or peri-insulitis, (iii) 2= infiltration covering almost half of the area of the islet and (iv)3= infiltration covering more than half of the islet area (Figure 43A).

Figure 44B shows the percentage of islet infiltration in CDK11(+/+) CcnD3Tg- has the highest infiltration compared to the other three groups. The differences between the proportion of islets among groups were assessed using a Chi-square test. The result indicated that differences were statistically significant (p-value<0.001)

Because NODCDK11(+/+) CcnD3(-/-) mice develop diabetes at an early age, pancreatic islet infiltration was measured in 5 weeks old female mice. **Fig 44C** shows that pancreatic infiltration seemed to be highest in NODCDK11(+/+) CcnD3(-/-) compared to the other 4 groups .

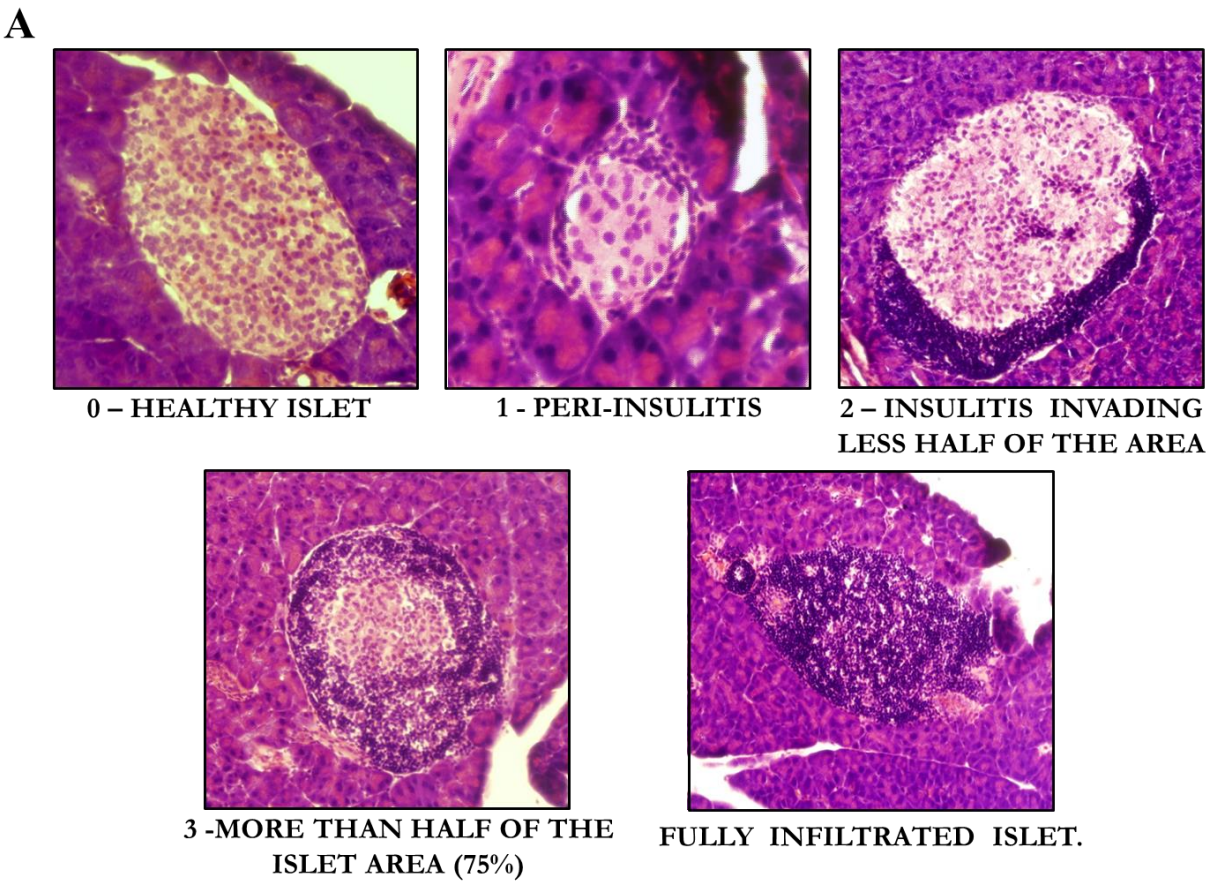


Figure 44. Assessment of the role of the combination of CDK11 hemideficiency and cyclin D3 overexpression in β cells in islet infiltration. (A) *Pancreas of 5 week old NOD female mice were embedded in paraffin and the sections were counterstained with Hematoxilin-Eosin staining. The infiltration score was: 0, no infiltration; 1, pancreatic*

infiltration or peri-insulitis; insulitis invading less; 2, or more; 3 than half of the islet area. ***(B)*** *Percentage of infiltration was measured. According to Chi-square test, the result indicated that differences were statistically significant (p-value<0.001) when comparing the 3 groups NODCDK11(Δ/+)CcnD3Tg+ (n=6),NODCDK11(Δ/+)CcnD3Tg- (n=6), NODCDK11(+/+)CcnD3Tg+(n=6) with that of NODCDK11(+/+)CcnD3Tg- (n=4) but they don't bear any difference among each other.* ***(C)*** *Percentage of infiltration was measured in 5 groups of mice. The 4 groups were compared with CDK11(+/+)CcnD3(-/-) (n=2). Statistical analysis was performed by the Chi-square test.*

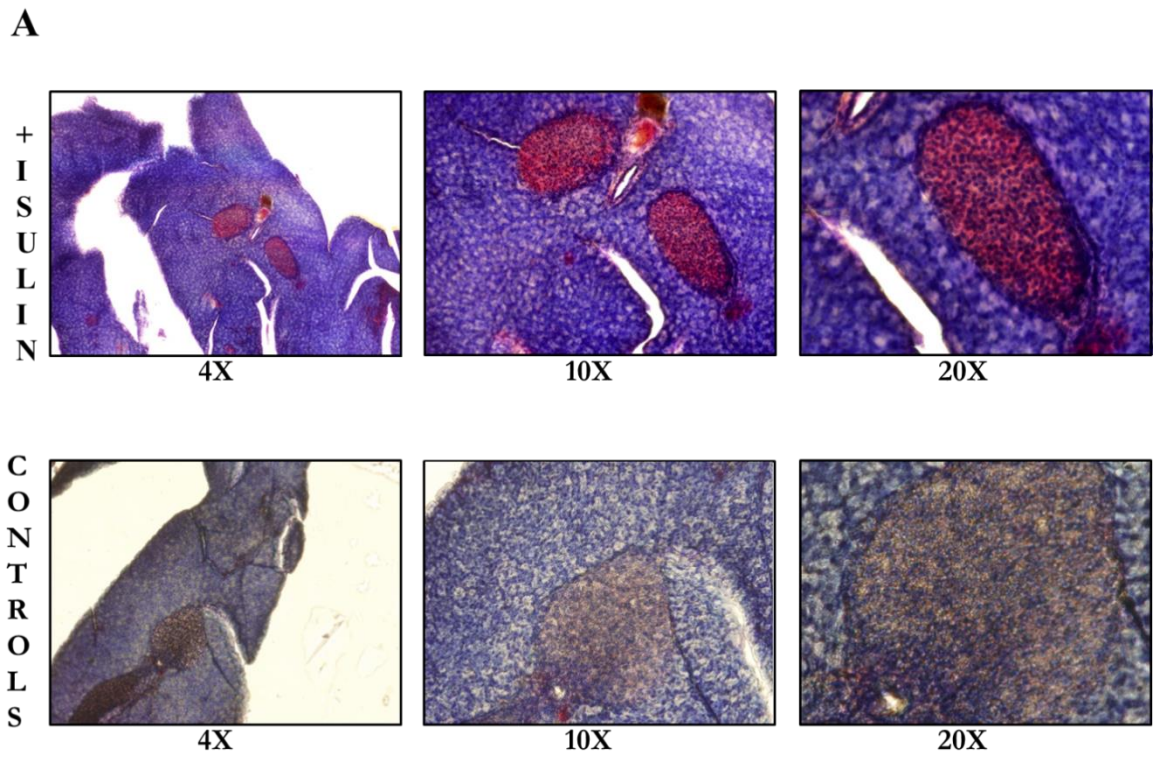
10.10 Assessment of β cell area of different strains of mice and islet count

Pancreata were extracted from 5 weeks old NOD female mice from the strains NOD CDK11(Δ/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg-, NODCDK11(+/+) CcnD3 Tg+ and NODCDK11(+/+) CcnD3Tg- . The pancreatic tissues were dehydrated, and embedded in paraffin to obtain sections, which were deparaffinized, and stained for insulin. **Figure 45A** shows the insulin staining of the islets using Alkaline phosphatase and Fast red as substrate was used as a substrate of it. The tissues were counterstained with Hematoxylin.

Figure 45B shows the β-cell area (in μm^2/ islet) of the pancreatic islets as analysed by ImageJ software. And as we see here, there is no significant difference between the four groups of mice according to ANOVA test. According to Mann Whitney test NODCDK11(+/+)CcnD3Tg+ vs NODCDK11(+/+) CcnD3Tg- and NODCDK11(+/+) CcnD3Tg+ vs NODCDK11(Δ/+) CcnD3Tg+ are significantly different to each other. There was no significant difference among the 2 groups i.e. between NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice. NODCDK11(+/+) CcnD3(-/-) mice has the smallest β-cell area as compared to the other genotypes. Hence, here only overexpression of cyclin D3 in β cells is enough to increase β cell area/islet, but not when the mice are hemideficient in CDK11.

Figure 45C determines the number of islets present per strain of mice.There is no significant difference between the groups and between pairs (according to one-way ANOVA test and Mann Whitney test).

The immunofluorescence study of the islets in 4 different groups along with NODCDK11(+/+) CcnD3(-/-) are shown in **Figure 45D.** It is quite visible from the picture and also we have evidences from our previous experiments of the group that the islets from NODCDK11(+/+) CcnD3(-/-) mice are much smaller than the other four groups and insulin staining was much less intense.



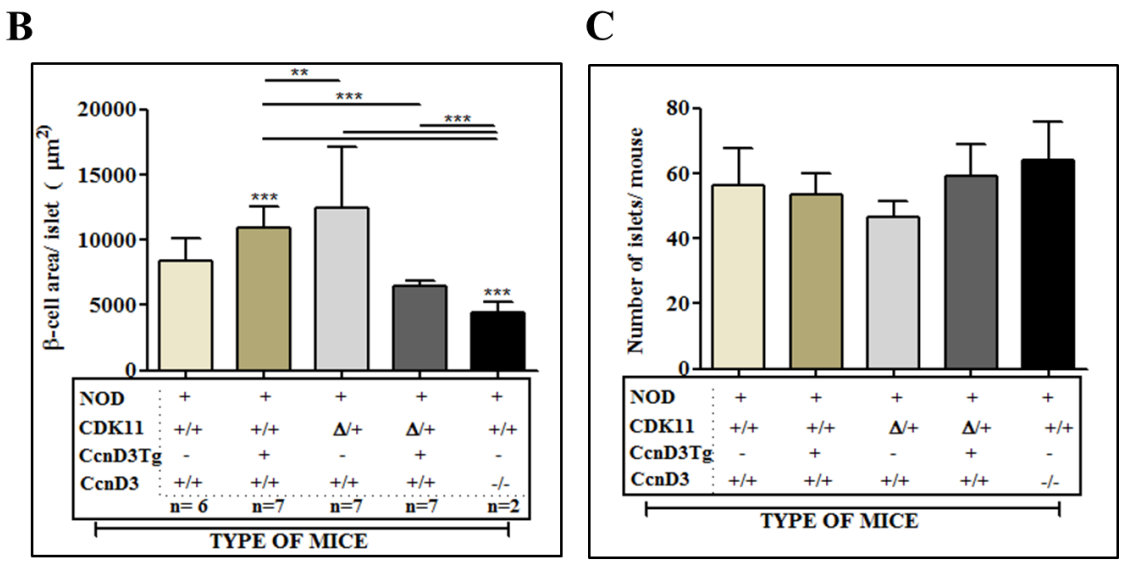


Figure 45. Islet count and β cell area in the different genotypes studied. (A) *Insulin staining was performed in the tissue sections in 4X, 10X and 40 X magnifications. The tissues were stained with insulin staining (+INSULIN) and the other set was stained only with secondary antibody as negative control of staining.* ***(B)*** *Size of β cell area was measured by staining for insulin, and the size of the pancreatic β cell area was measured using ImageJ software. The measurement was done by taking the mean of the β cell/islet area of the mice per group. The mice used were NODCDK11(+/+)CcnD3Tg-(n=6), NODCDK11(+/+)CcnD3Tg+(n=7), NODCDK11(Δ/+)CcnD3Tg-(n=7), NODCDK11(Δ/+) CcnD3Tg+(n=7) and NODCDK11(+/+)CcnD3KO (n=2). The graph was plotted according to Mean+ SEM.* ***(C)*** *Number of islets was measured per group of mice. The graph was plotted according to Mean+ SEM. There was no significant difference between the 4 groups of mice (according to Mann-Whitney). There was no significant difference among the whole group according to one-way ANOVA test. * show differences with respect to the control mice NODCDK11(+/+)CcnD3Tg-; _ * shows differences between the other groups of mice * p≤0.05, ** p≤0.01, ***p≤0.005. n=number of mice.*

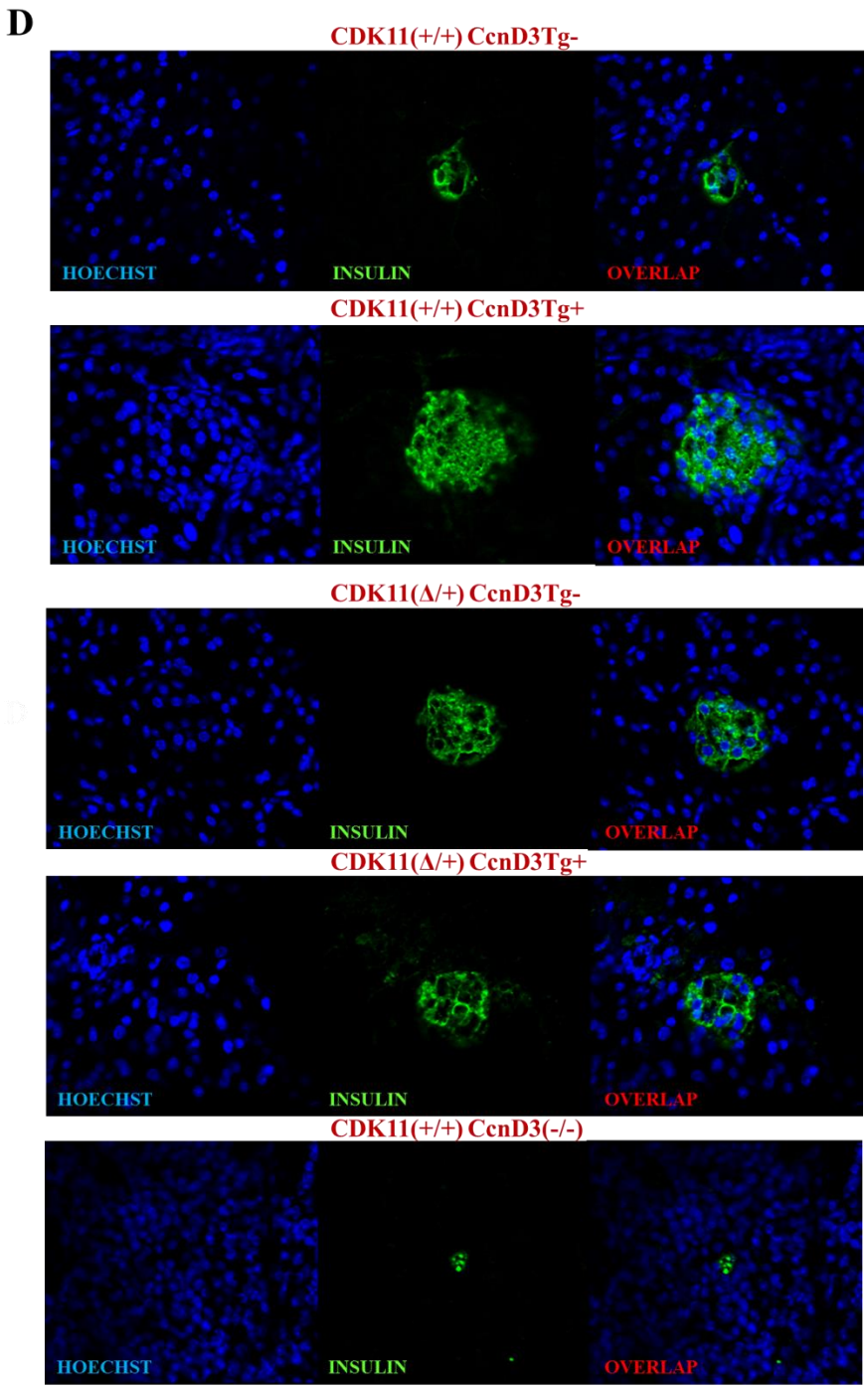


Figure 45. (D) Insulin expression in the 5 genotypes studied. *Pancreata sections from 5 week old female mice from the genotypes studied were stained for insulin (green) and nuclei (blue). The nuclear staining was done with Hoechst staining. Cy-2 was used as the secondary antibody for the insulin staining. The photographs were taken by the confocal microscope Olympus FV1000, America (40 X magnification).*

10.11 Expression profile of cyclin D3 and proliferation rates of the β cells in the different genotypes of mice

To examine the expression pattern of cyclin D3 in the different strains studied, flow cytometric analysis was performed on pancreatic islets for 5 weeks old mice in NOD/SCID background. The 5 week old NOD/SCID mice will have the least infiltrated islets as they lack both T and B lymphocytes. The criteria used to select the age of the mice is based on the age where islet infiltration is minimal (see **Figure 44B**). The pancreatic islets were isolated one by one using a binocular loop, stained (as in Methods: 1.7.1 Islet cell staining by flow cytometry) and passed through the flow cytometer. The plain NOD and the NOD/SCID mice were taken as control mice.

The accurate population of the pancreatic islet cell population was chosen as in **Figure 46A**. Then, hematopoietic cells were excluded using the CD45 marker, and only the pancreatic β cells were chosen as Glut-2^{+} cells. Cyclin D3 positive population was analysed in $CD45^{-}$ $Glut2^{+}$ cell fraction (β cells) which gave the actual population for cells expressing cyclin D3. Again, from the $CD45^{-}$ Glut-2^{+} cell fraction the population of cells positive for Ki-67 (the proliferation marker) was measured.

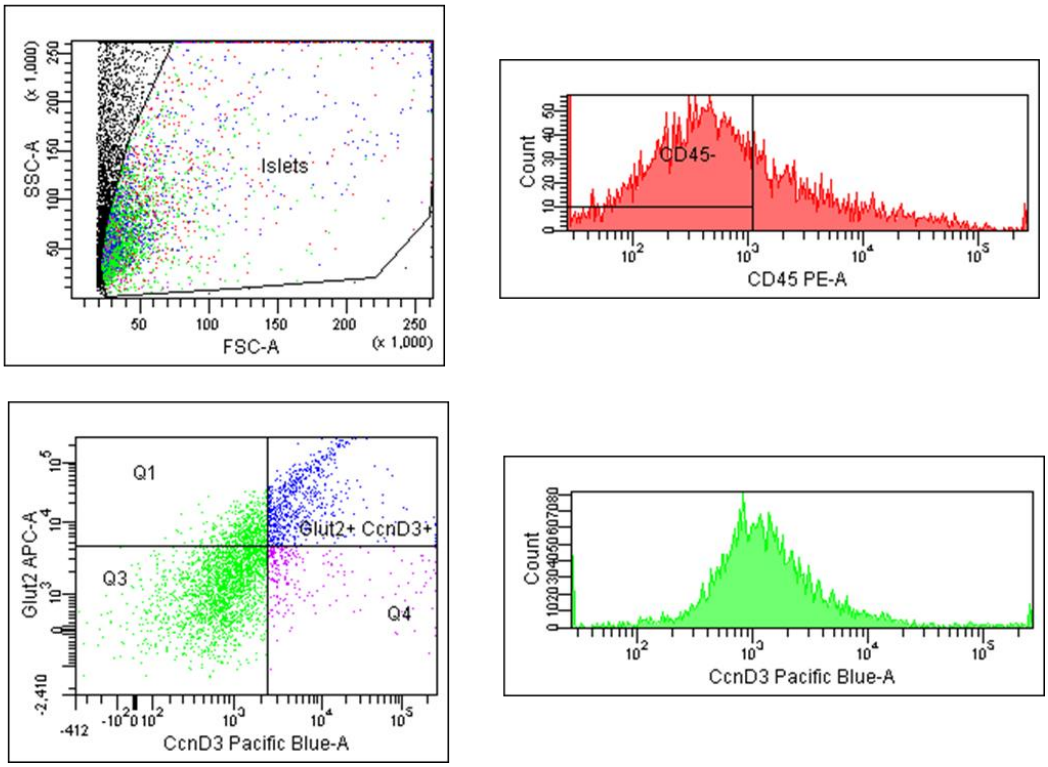
From the results shown in the **Figure 46B,** it is clear that there no significant difference exists between the cyclin D3 protein expression levels observed in the NOD/SCID CDK11(Δ/+) CcnD3Tg+ and NOD/SCIDCDK11(Δ/+)CcnD3Tg- β cells. But as expected negligible cyclin D3 expression was observed in NOD/SCIDCDK11(+/+) CcnD3(-/-) mice compared to the controls and also to the experimental strains. NOD/SCIDCDK11(+/+) CcnD3Tg+ mice was used as positive control which showed the maximum cyclin D3 expression level as compared to the NOD/SCIDCDK11(+/+) CcnD3Tg- and the control NOD and NODSCID mice.

From the results shown in the **Figure 46C and D**, it is clear that there was no significant difference between the Ki-67 expression levels in the NOD/SCIDCDK11(Δ/+) CcnD3Tg+ and NOD/SCIDCDK11(Δ/+)CcnD3Tg- as compared to the control (NOD and NOD/SCID). Hence, the proliferation rates of the cells were similar in these strains. Only the

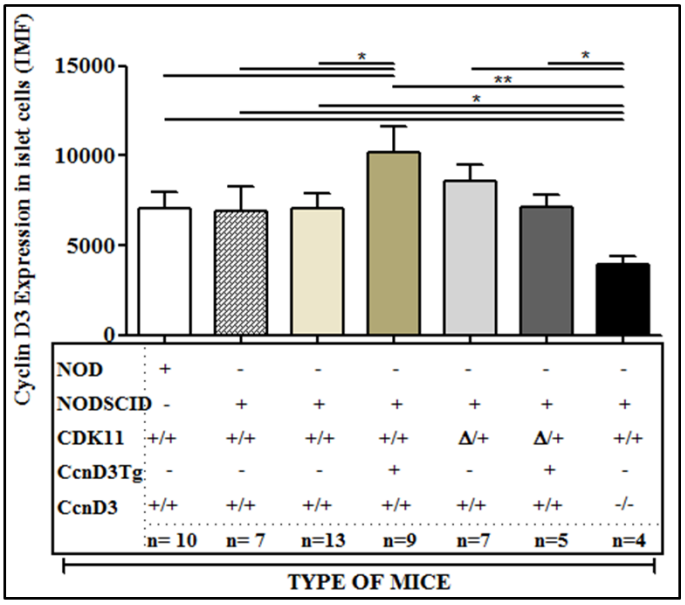
proliferation rate was increased in NOD/SCID CDK11(+/+) CcnD3Tg+ when compared to control NOD mice.

CYCLIN D3 EXPRESSION

A

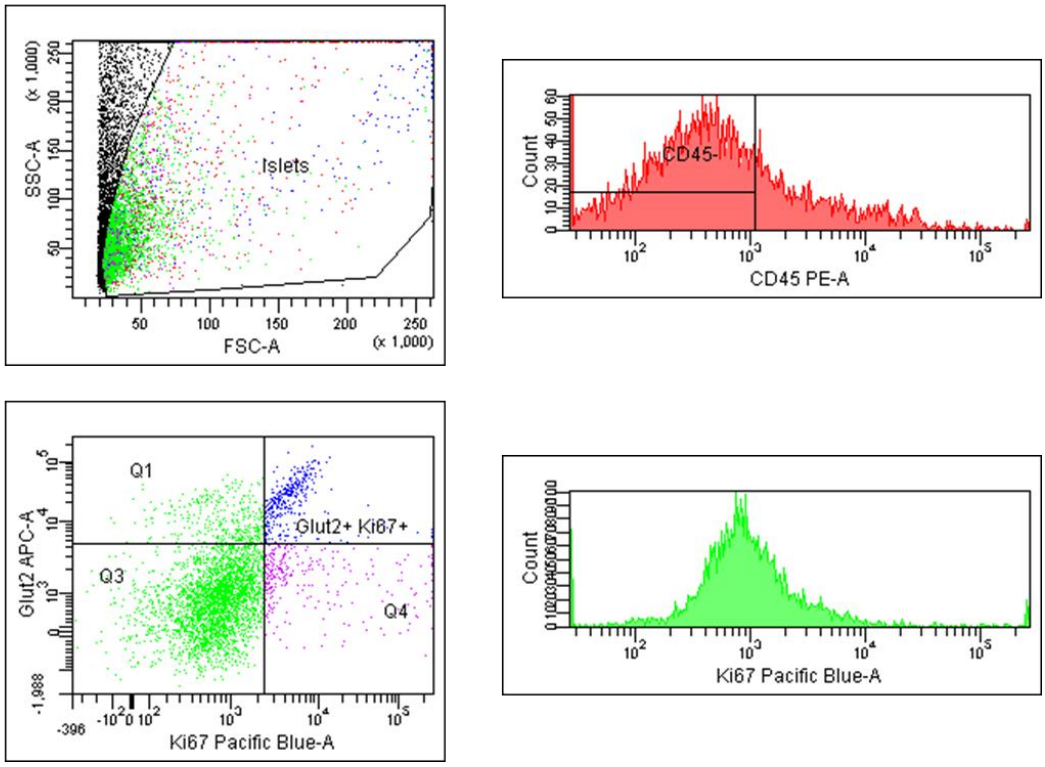


B



PROLIFERATION RATE

C



D

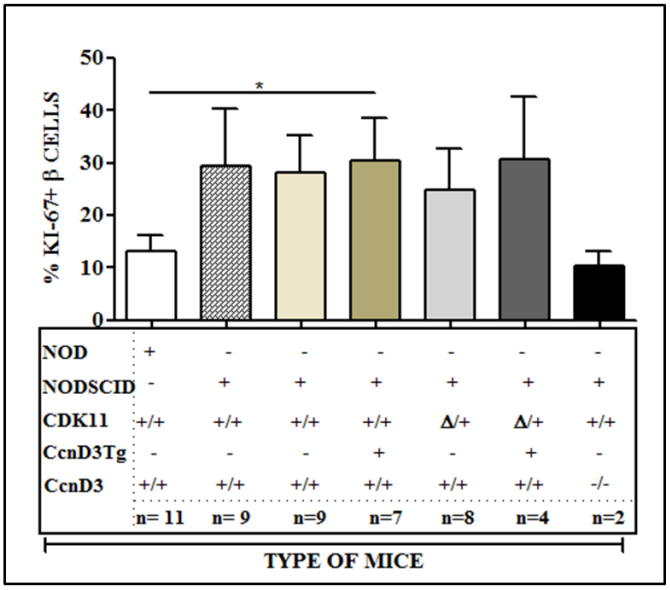


Figure 46. Cyclin D3 expression levels and proliferation rates of β cells in the different

genotypes of mice analysed. (A) and (C) *The population of the islet cells were selected by the SSC (Side Scatter) and FSC (Forward Scatter). The CD45$^-$ (hematopoietic cell marker), Glut-2$^+$ (β-cell marker) and cyclin D3$^+$ and Ki-67$^+$ cells, respectively were measured.* ***(B)*** *Pancreatic islets from 5 week old mice were isolated and stained by Flow cytometry. Cyclin D3 staining IMF (Intensity of Median Fluorescence) was measured by flow cytometry on CD45$^-$Glut2$^+$CcnD3$^+$ cells. NOD (n=10), NODSCID (n=7), NODSCIDCDK11(+/+) CcnD3Tg- (n=13), NODSCIDCDK11(+/+)CcnD3Tg+ (n=9), NODSCID CDK11(Δ/+) CcnD3Tg- (n=7) , NODCDK11(Δ/+)CcnD3Tg+ (n=5), NODSIDCDK11 (+/+)CcnD3(-/-) (n=4).* ***(D)*** *Pancreatic islets from 5 week old mice were isolated and stained by Flow cytometry. Ki-67 staining for cell proliferation was measured by flow cytometry on CD45-Glut2$^+$ Ki-67$^+$cells. There were no significant difference among the groups- NOD (n=11), NODSCID (n=9), NODSCIDCDK11(+/+)CcnD3Tg- (n=9), NODSCIDCDK11(+/+) CcnD3Tg+ (n=7), NODSCIDCDK11(Δ/+)CcnD3Tg- (n=8) , NODCDK11(Δ/+)CcnD3Tg+ (n=4), NODSIDCDK11(+/+)CcnD3(-/-) (n=2). All graphs were plotted according to Mean+ SEM. n=Number of mice. Statistical Analysis was performed according to Mann-Whitney Test and one-way ANOVA test.*

10.12 Cyclin D3 overexpression in the NODCDK11HTZ (Δ/+) mice doesnot alter responses to glucose challenges

Intraperitoneal Glucose Tolerance test (IGTT) was performed in both NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+)CcnD3Tg- mice. This experiment was performed to determine whether cyclin D3 overexpression in β cells from NODCDK11(Δ/+) mice would improve glycemia values upon intraperitoneal challenge with glucose.

Results in **Figure 47** show that both the groups have similar responses to glucose i.e. both groups reach the glycemic peak at 30 mins and then, slowly recover euglycemic condition after 120 mins post challenge. There is no significant difference between them according to Mann-Whitney Test. The diabetic mice were kept as negative controls of glycemic control in the test. The diabetic individuals showed high rise in glycemia after glucose stimulation, and didnot revert back to euglycemia.

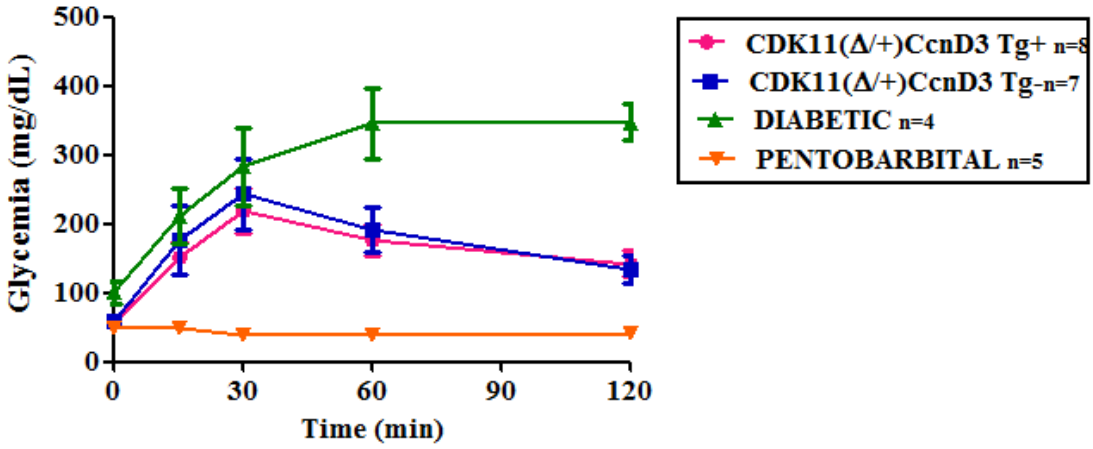


Figure 47. NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11(Δ/+)Tg- mice have similar responses to blood glucose. *Female NOD mice were tested for basal gycemia values at 12-weeks of age, and mice with blood glucose below 150 mg/dL were used (normoglycemic). Mice were administered intraperitoneally with 2g of D-glucose per kg of body weight, and their blood glucose measurements were measured at 15, 30, 60, and 90 minutes post injection. Statistical measurements were done using Mann-Whitney Test. n = number of mice.*

10.13 The experimental groups of mice generated in this work

NOD
NODCDK11(+/+)
NODCDK11(Δ/+)
NODCcnD3 Tg+
NODCcnD3 Tg-
NODCcnD3(-/-)
NODCDK11(+/+) CcnD3Tg+
NODCDK11(+/+) CcnD3Tg-
NODCDK11(Δ/+) CcnD3Tg+
NODCDK11(Δ/+) CcnD3Tg-
NODCDK11(+/+) CcnD3(-/-)
NODCDK11(Δ/+) CcnD3(-/-)
NOD/SCID
NOD/SCIDCDK11(+/+)
NOD/SCIDCDK11(Δ/+)
NOD/SCIDCcnD3 Tg+
NOD/SCIDCcnD3 Tg-
NOD/SCIDCDK11(+/+) CcnD3Tg+
NOD/SCIDCDK11(+/+) CcnD3Tg-
NOD/SCIDCDK11(Δ/+) CcnD3Tg+
NOD/SCIDCDK11(Δ/+) CcnD3Tg-

Discussion

Cyclin D3 and CDK11 are expressed in the β pancreatic cells and the role they play in T1D is a lively field of research. Previously our group found by using the Microarray technology that, cyclin D3 and CDK11 are downregulated in pancreatic islet endocrine cells during the autoimmune attack progression in autoimmune-prone NOD mouse strain. In the present study we have performed a comprehensive analysis of the partnership of cyclin D3 and CDK11 in NOD β cells and in the NOD insulinoma NIT-1 cell line. We have found that cyclin D3 seems to have a role independent of CDK11 to maintain β cell mass homeostasis. This was justified in different models:

(i) In the single transfected and the double transfected cells with cyclin D3 and/or CDK11p58

(ii) In the NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) mice

(iii) In the NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg- mice

11.1 IN VITRO

11.1.1 The single transfected and the double transfected cells with cyclin D3 and/or CDK11p58

Using the glucose-responsive NIT-1 insulinoma cell line as a model for β-pancreatic cells, we sought to understand better the role of cytokine-induced β-cell apoptosis by investigating the effects of cytokines IL-1β and/or IFN-γ in the single and the double transfected cells and as a result induction of apoptosis [337].

The NIT-1 cells stably overexpressing cyclin D3 were protected against both spontaneous and IL-1β-induced apoptosis but not against IFN-γ-induced apoptosis or IL-1β+IFN-γ induced apoptosis. Thus 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. This has given us an 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. Hence, it is obeserved from the results that cyclin D3 is protecting from apoptosis in a proinflammatory niche.

But, in the double transfected cells where the NIT-1 cells were transfected with the pBSKNEO plasmid containing the rat insulin promoter driving the mouse cyclin D3 or CDK11p58 donot protect from apoptosis in a proinflammatory niche in the later passages of the cells. Both the single and the double transfectants are plated and the experiments are done in the same way. Only the difference lies in selecting the cells with antibiotic. The single transfectants were selected by Geneticin (G418) whereas the double transfectants were selected by both geneticin and puromycin. Literatures suggested that among the two antibiotics puromycin is the stronger one because geneticin require much higher concentrations (100-1500mg/ml) than puromycin (0-15µg/ml) for selecting the transfected cells [345, 346]. Previously we had 7 double transfected cell lines:

TYPE OF CONSTRUCTS	NAMES
DOUBLE TRANSFECTED CELLS	pBSKNEO + pPUR (empty vectors)
	pBSKNEOCcnD3 + pPURp58
	pBSKNEOp58 + pPURCcnD3
	pBSKNEOCcnD3 + pPUR
	pBSKNEO + pPURCcnD3
	pBSKNEOp58 + pPUR
	pBSKNEO + pPURp58

The cells transfected with pBSKNEO p58 + pPUR CcnD3 and pBSKNEO + pPUR CcnD3 (highlighted in grey), i.e. both has pPURCcnD3 in common showed puzzling results in presence of proinflammatory cytokines (**Figure 48A**) as because the pBSKNEO +pPURCcnD3 showed significantly higher apoptosis than pBSKNEOCcnD3 +pPUR. We analysed the expression of cyclin D3 in these cells. Surprisingly, it was found that percentage of cells expressing cyclin D3 and also the Intensity Median of fluorescence was not only lower than the pBSKNEOCcnD3 but also from the empty vectors (**Figure 48B and C**). For that reason the cells containing the pPURCcnD3 plasmid were discarded.

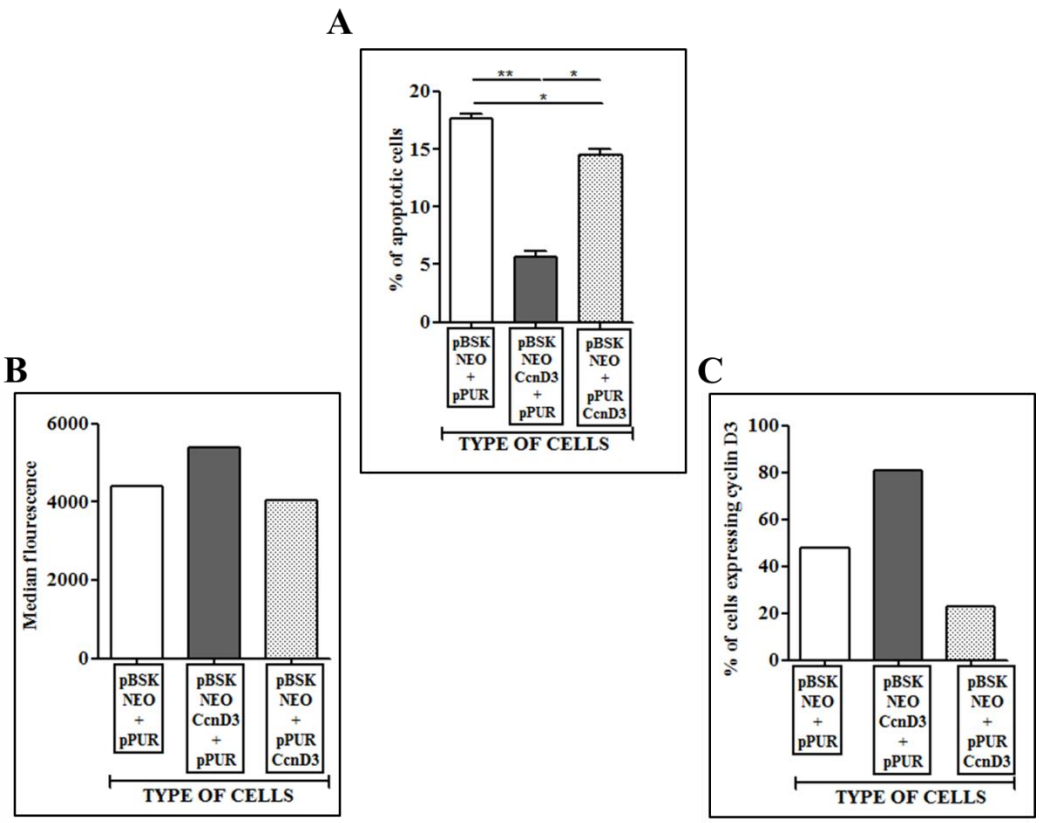


Figure 48. The apoptotic behavior and the cyclin D3 expression of pBSKNEO+ pPUR CcnD3. (A) *The double transfected NIT-1 cell line with empty vectors and with the RIP-CcnD3 construct were cultured for 24h with DMEM; % of early apoptosis was measured as AnnexinV^{+} PI^{-} cells in each experimental group. The pBSKNEO +pPURCcnD3 showed significant higher level of apoptosis than pBSKNEOCcnD3+pPUR. They were expressed as Mean+ SEM.* ***(B)*** *IMF (Intensity of Median Fluorescence) of pBSKNEO+pPUR, pBSKNEO CcnD3+ pPUR and pBSKNEO+pPURCcnD3.* ***(C)*** *Percentage of cells expressing CcnD3 in pBSKNEO+pPUR, pBSKNEOCcnD3+pPUR and pBSKNEO +pPURCcnD3.*

Moreover, we find that cyclin D3 is protecting from apoptosis in a proinflammatory niche in the single transfectants. They behave in the same way also in the double transfectant but only during the early passages of the cells. The double transfectants transfected with cyclin D3 showed much less spontaneous apoptosis compared to the mock controls. The cells transfected with the CDK11p58 showed higher apoptosis compared to the cells transfected with cyclin D3 which explains the reason that p58 is related to apoptosis. This was also evidenced from the morphological analysis of the cells. But in the later passages we donot

see any protection from both spontaneous and cytokine induced apoptosis in the cells overexpressing cyclin D3. The cyclin D3 expression also remains the same in all of them while p58 expression in RNA levels is pronounced. So, it can be concluded that maybe the antibiotic Puromycin is masking the effect of both cyclin D3 and CDK11p58 to express more and by time the cells are loosing their capacity to express the genes due to many passages. This is evidenced by the fact that puromycin inhibits growth of animal cells and is related to that causes premature chain termination during translation in the ribosome [347] and also acts as a reversible inhibitor of dipeptidyl-peptidase II (serine peptidase) [348,349].

Hence, it can be concluded that both in single and in double (early passages) transfected NIT-1 cells overexpressing cyclin D3 protects from apoptosis in a proinflammatory niche as compared to the cells having empty vectors. But in the late passages inspite of expressing CDK11p58 in the cells, the double transfected cells are unable to protect or enhance apoptosis in a proinflammatory niche as compared to the cells having empty vectors.

11.2 IN VIVO

11.2.1 The NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) mice

Experimental evidence shows that the survivability of NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) bears no significant difference with each other. But as we analysed in Figure 39B, the cumulative incidence of diabetes in both the strains and in NODCcnD3KO mice is more than 78% whereas in the NODCcnD3WT is only 62.85%. This is already established in our previous studies that absence of cyclin D3 is enhancing the diabetes onset in mice and hence cyclin D3 is very much important in maintaining the pancreatic beta cell metabolic fitness and viability in a cell. Whereas, the survival graph (Figure 39A) of NODCDK11(+/+) CcnD3(-/-) and NODCDK11(Δ/+) CcnD3(-/-) doesnot show any significant difference which means that presence or absence of CDK11 is incapable to cause any change in the survivability of these mice strain. This explains that cyclin D3 deficiency on its own is enough to accelerate the disease. According to our hypothesis the incidence of diabetes should have been in this order:

NODCDK11(+/+)CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+)CcnD3 (+/+).

But, it is not the case. On the contrary, the survival incidence is –

NODCDK11(+/+)CcnD3(-/-) = NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+)CcnD3 (+/+).

Thus, cyclin D3 is masking the effect of CDK11 and cyclin D3 thus plays a more relevant role than CDK11 when present together in maintaining β cell fitness.

The size of the pups in these KO mice is much smaller than the normal WT mice. Moreover, the number of pups per litter produced is also much less compared to the NODCDK11(+/+)CcnD3Tg- or WT mice.

Some troubles which we faced during maintaining these mice are:-

(i) The number of pups per litter was very less,

(ii) Out of that limited number of pups we used only the females in our experiment, with which mostly the survival graph was done,

(iii) And moreover, these mice became diabetic from the 8^{th} or 9^{th} week onwards. Hence, it was very difficult to maintain their crosses for long.

11.2.2 The NODCDK11(Δ/+)CcnD3Tg+ and NODCDK11(Δ/+)CcnD3Tg- mice

NOD mouse models show that overexpression and normal expression of cyclin D3 on CDK11(Δ/+) doesnot change the survival strategy on spontaneous and adoptive transferred T1D. But the incidence of diabetes in the NODCcnD3Tg- is significantly different from the other strains. If they would have followed our hypothesis, then the survival strategy would have been-

NODCDK11(+/+)CcnD3Tg- > NODCDK11(Δ/+) CcnD3Tg-> NODCDK11(Δ/+)CcnD3 Tg+. Hence, according to our hypothesis, NODCDK11(Δ/+) CcnD3Tg+ mice should have given the maximum protection from T1D. But it is not the case. Instead, all of the three NODCDK11(Δ/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg- and NODCDK11(+/+) CcnD3Tg+are following the same pattern.

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 cyclin D3 over CDK11hemideficient mice, the response to blood glucose and the sensitivity of glucose by the β pancreatic islets are the same and bears no significant difference.

Hence, the overexpression of cyclin D3 in β cells protects, reagardless of CDK11 hemideficiency or not, which would imply that cyclin D3 has an independent role of CDK11.

β cells are responsible for secreting insulin, a key hormone for regulating the glucose metabolism, and changes in expression of the cell-cycle proteins, lead to phenotype changes of pancreatic islets as hyperplasia. We were then interested in assessing whether cyclin D3 and CDK11 are crucial for β-cell physiology. In 5 week old NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+)CcnD3Tg- mice, there is no hyperplasia of pancreatic islets and they have no difference in the β cell area. However, loss of expression of cyclin D3, causes a reduction in the size of the islets. These together with the results from the pancreatic infiltration studies and islet count showed that there lies absolutely no significant difference between the two different strains i.e. NODCDK11(Δ/+) CcnD3Tg+ and NODCDK11(Δ/+) CcnD3Tg-. To prove further we have also checked the cyclin D3 expression in these mice, and the expression also has no significant difference. From previous literatures we know, that the kinase activity of CDK11 is regulated by cyclin D3 [322]. But from the results, it seems that among both CDK11 and cyclin D3, cyclin D3 is the one which have more pronounced function than that of CDK11 when resent together in maintaining the pancreatic β cell function.

11.2.3 Interaction between cyclin D3 and CDK11

This study assessed the relative expression levels between cyclin D3 and Cdk11 required to keep β cell mass homeostasis.

Evidence suggested from previous articles that CDK11p58 donot interact with the other D type cyclins, cyclin D1 and cyclin D2. This was proved in direct two-hybrid assay and GST pull down experiments [322]. But it was found that the G1 cyclin, cyclin D3 not only

functioned as a regulatory subunit of CDK4 and 6 but also acts as a binding partner with G2M/CDK p58 in the G2/M phase during cell cycle progression[322]. Another article also suggested that cyclin D3/CDK11p58 complex (interaction in the nuclear region) is involved in proliferation and apoptosis in Schwann cells induced by lipopolysaccharide [325]. It was also reported in an article the crucial role played by cyclin D3 in cell cycle progression through G1 phase [350] and also its regulation of apoptosis induced by the activation of the T cell receptor in leukemic T cell lines[351]. Moreover as mentioned earlier the kinase activity of CDK11p58 is reduced without binding with cyclin D3 [322].

Hence, keeping these above points in our mind we see that:

(i) NOD mouse strains overexpressing cyclin D3 are protected from T1D.

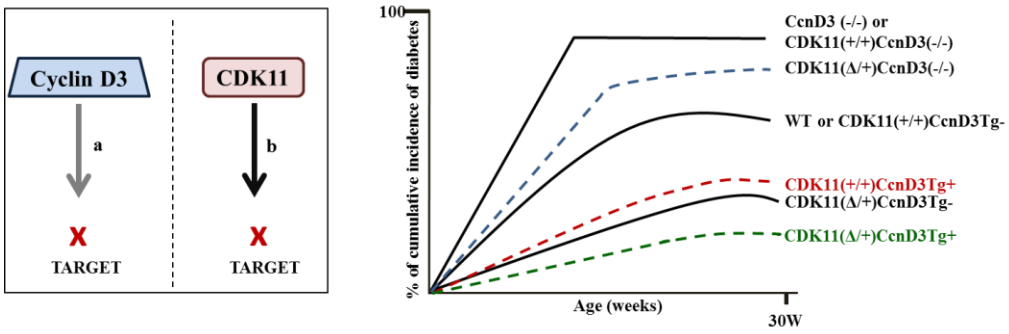
(ii) NOD mouse strains deficient in cyclin D3 show exacerbated T1D.

(iii) NOD mouse strains hemideficient in CDK11 are protected from T1D.

(iv) The addition of the hemideficiency in CDK11 to the deficiency of cyclin D3 does not ameliorate the diabetic phenotype of NOD mice deficient in cyclin D3.

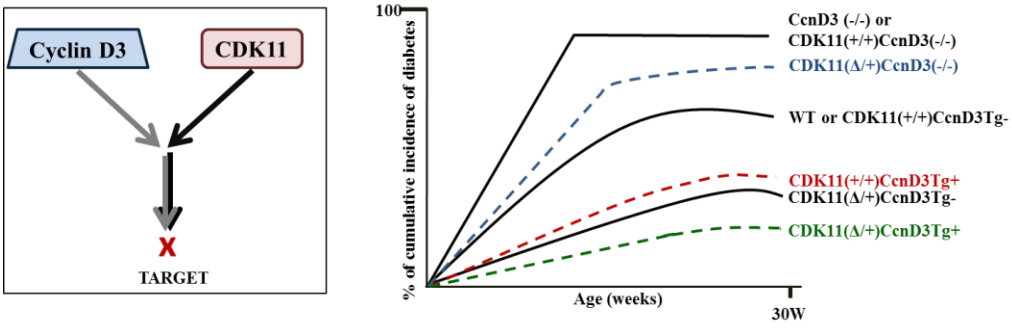
This can be explained by six possible models:

MODEL I: Both cyclin D3 and CDK11 signal through two different pathways. If both of them are following two different pathways then, the diabetes incidence for each genotype should follow this pattern-NODCDK11(+/+) CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) and NODCDK11(+/+) CcnD3Tg- > NOD CDK11(Δ/+) CcnD3Tg- > NODCDK11(Δ/+) CcnD3Tg+. Though they are following different pathways but still they will be affecting each other as they are both associated with the cell cycle progression. Here, they affect as two separate entities.



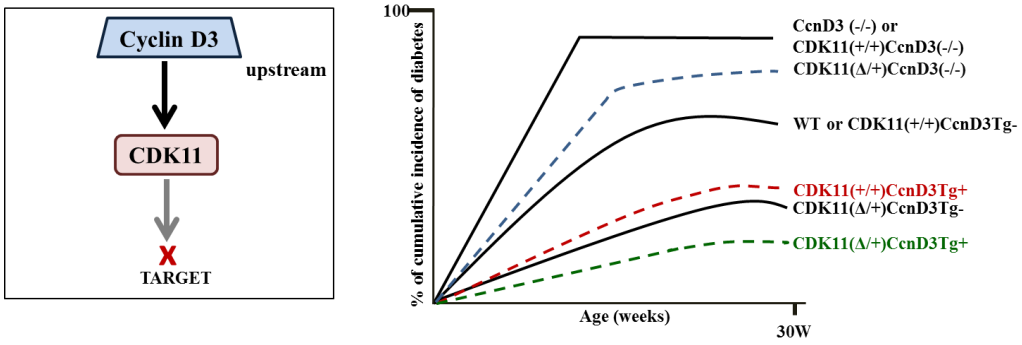
Model I. Cyclin D3 and CDK11 signal through two different pathways.

MODEL II: Both, cyclin D3 and CDK11 signal through the same pathway, and both are at the same level. If both of them are following the same pathway then, the diabetes incidence for each genotype should follow this pattern-NODCDK11(+/+) CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) and NODCDK11(+/+) CcnD3Tg- > NODCDK11(Δ/+) CcnD3Tg-> NODCDK11(Δ/+) CcnD3Tg+. Here, it seems like this model is behaving like MODEL I. But it is not the case. Since, they are following the same pathway both of them affect together as single molecule i.e. as 1 whole entity.



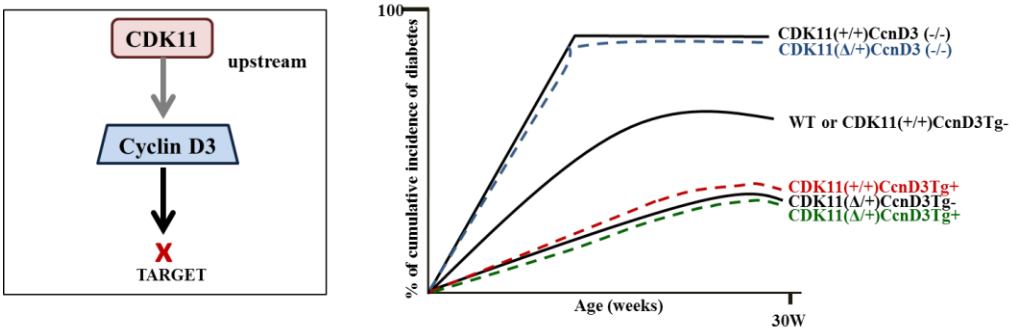
Model II. Cyclin D3 and CDK11 signal through the same pathway and both are in the same level.

MODEL III: Both, cyclin D3 and CDK11 signal through the same pathway, but cyclin D3 is upstream of CDK11. In this case cyclin D3 is upstream of CDK11 which means that CDK11 is closer to the end of the pathway, and its partial removal will hinder cyclin D3 action . CDK11p58 removal, which is related to apoptosis, in the absence of cyclin D3, should not have any additive effect to that observed in the plain removal of CDK11 on its own. And the incidence of diabetes should follow this pattern-NODCDK11(+/+)CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) and NODCDK11 (+/+) CcnD3Tg- = NODCDK11(Δ/+) CcnD3Tg-> NODCDK11(Δ/+) CcnD3 Tg+.



Model III. Cyclin D3 and CDK11 signal through the same pathway, but cyclin D3 is upstream of CDK11.

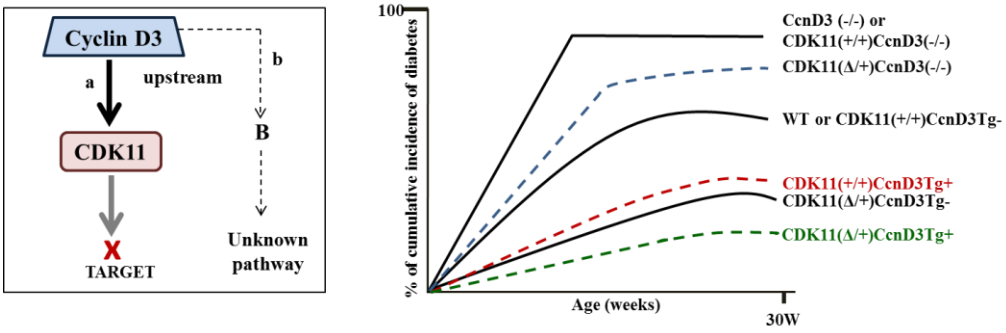
MODEL IV: Both, cyclin D3 and CDK11 signal through the same pathway, but CDK11 is upstream of cyclin D3. In this case cyclin D3 comes after CDK11 in the pathway. Cyclin D3 which is related to maintain the β cell fitness is more prominent here than CDK11 as it is closer to the target. Cyclin D3 irrespective of CDK11 will act more prominently. Thus, the diabetes incidence will be NODCDK11(+/+) CcnD3(-/-) = NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) or WT and NODCDK11 (Δ/+) CcnD3Tg+, NODCDK11(Δ/+) CcnD3Tg- and NODCDK11(+/+) CcnD3Tg+ will follow the same pattern. This model seems to reflect our findings regarding diabetes onset.



Model IV. Cyclin D3 and CDK11 signal through the same pathway, but CDK11 is upstream of cyclin D3.

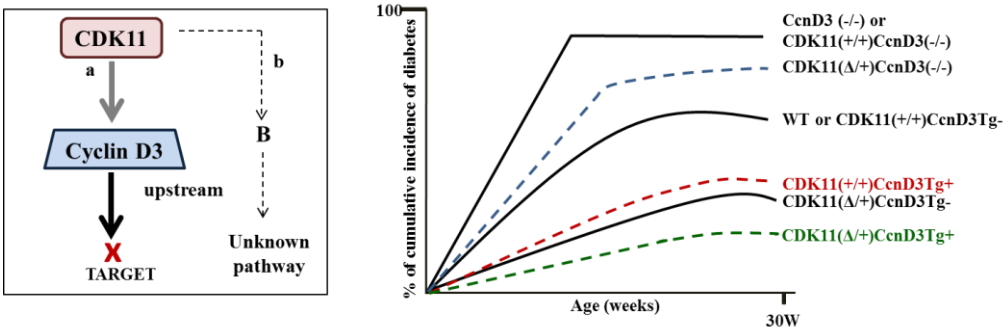
MODEL V: Both, cyclin D3 and CDK11 signal through the same pathway, but cyclin D3 is upstream of CDK11 and also participates in another independent pathway. If cyclin D3 takes part in two different pathways, and one of them includes CDK11, then removal of CDK11 will have less impact on diabetes incidence that just removal of cyclin

D3. Hence here, CDK11 which is related to apoptosis is closer to the target. CDK11 here will have more relevant role than cyclin D3. Thus the survivability will be NODCDK11(+/+) CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) and NODCDK11 (+/+)CcnD3Tg- = NODCDK11(Δ/+) CcnD3Tg-> NODCDK11(Δ/+) CcnD3 Tg+.



Model V. Cyclin D3 and CDK11 signal through the same pathway, but cyclin D3 is upstream of CDK11 and also participates in another independent pathway.

MODEL VI: Both, cyclin D3 and CDK11 signal through the same pathway, but CDK11 is upstream of cyclin D3 and also participates in another independent pathway. Here, CDK11 is upstream of cyclin D3 and it is mainly related to apoptosis as p58 and p46 are both related to apoptosis. Considering this fact it can be suggested that CDK11 will follow another unknown pathway which is related directly to apoptosis. If this is the case then the incidence of T1D should be- NODCDK11(+/+)CcnD3(-/-) > NODCDK11(Δ/+) CcnD3(-/-) > NODCDK11(+/+) CcnD3(+/+) and NODCDK11(+/+) CcnD3Tg-=NODCDK11(Δ/+)CcnD3Tg->NODCDK11(Δ/+)CcnD3Tg+.



Model VI. Cyclin D3 and CDK11 signal through the same pathway, but CDK11 is

upstream of cyclin D3 and also participates in another independent pathway.

The models that better fits our results is **MODEL IV**, since the cumulative of incidence NOD mice deficient in cyclin D3 and either hemideficient or WT for CDK11 is the same in both cases, implying that both, cyclin D3 and CDK11 share the signaling pathway and cyclin D3 is downstream CDK11.

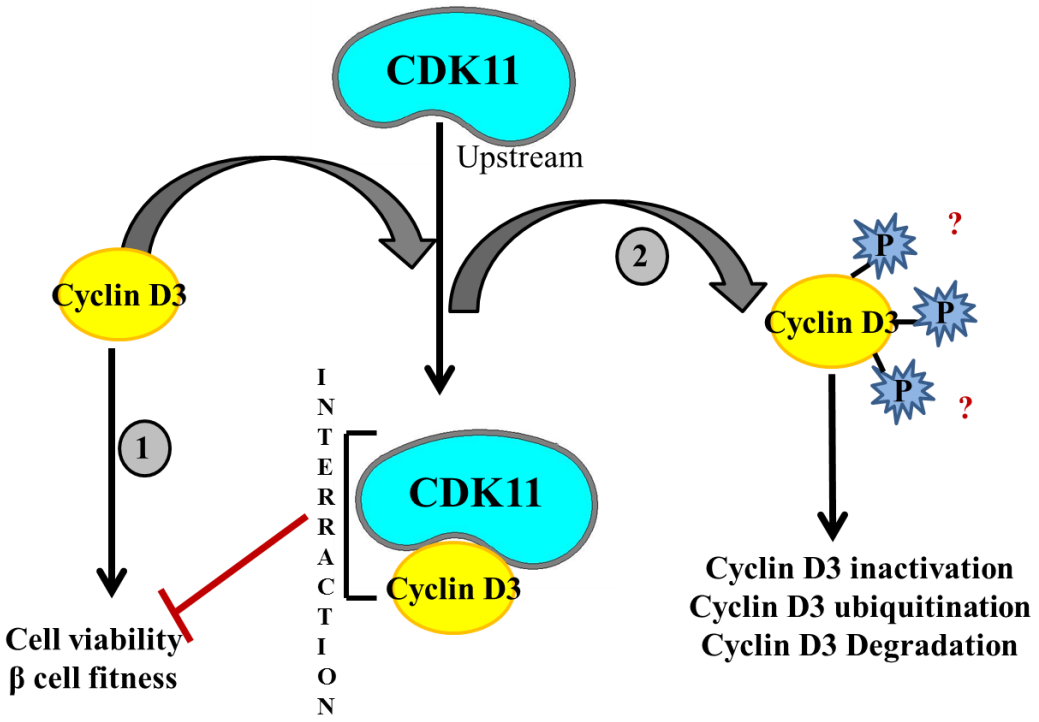


Figure 49: Proposed Model of the role of cyclin D3 and CDK11 on β-cell fitness and viability.

(1) and (2) Pathways

This model thus explains that CDK11 is upstream of cyclin D3. Cyclin D3 when present alone helps in cell viability and β cell fitness. But whenever CDK11 is in association with cyclin D3, cyclin D3 gets inactivated and/or cyclin D3 is sequestered by CDK11, preventing thus the cyclin D3 action.

Summary

12.1 IN VITRO

1. The single transfected NIT-1 cells overexpressing cyclin D3 are protected from spontaneous and IL-1β-induced apoptosis. Not only that, NIT-1 cells over expressing cyclin D3 protected against NOD leucocyte-induced necrosis. This means, that cyclin D3 is protecting the NIT-1 cells in a proinflammatory niche.

2. The double transfected NIT-1 cells overexpressing both cyclin D3 at early passages protected from spontaneous apoptosis. These double transfectants overexpressing CDK11p58 showed higher apoptosis compared to the cells transfected with cyclin D3. This was also proved in the morphological studies in the NIT-1 transfected cells where the cells showed clearly that the cells having an overexpression of CDK11p58 had the formation of blebs. And the other transfected cells with cyclin D3 or the empty vectors were healthier.

3. The double transfected NIT-1 cells overexpressing both cyclin D3 and CDK11p58 at later passages donot protect from spontaneous and cytokine-induced apoptosis.

12.2 IN VIVO

1. The size of NOD mice in both CDK11(+/+)CcnD3(-/-) and CDK11(Δ/+)CcnD3(-/-) were much less than that of the other strains. The size of the pups in both CDK11(Δ/+)CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- mice were of the same and of normal size. The number of pups per litter were also much less in the CDK11(+/+) CcnD3(-/-) and CDK11(Δ/+)CcnD3(-/-) mice compared to the other two strains.

2. CDK11(+/+)CcnD3(-/-), CDK11(Δ/+)CcnD3(-/-) and CcnD3(-/-) mice showed the same incidence of diabetes but they showed exacerbated diabetes in compared to wild type mice. This means that when cyclin D3 is absent alone T1D is exacerbated. When CDK11 is absent then the incidence of T1D is reduced in compared to the wild type mice. But, in case of both deletion of CDK11 (half deletion / HTZ) and cyclin D3 T1D is exacerbated as CDK11(+/+)CcnD3(-/-). This means both these genes are important in the onset of T1D and both their deletion is affecting in the same way. Here the effect of cylcin D3 implies that it has an independent role of CDK11.

3. CcnD3Tg+, CDK11(Δ/+)CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- mice showed the same incidence of diabetes but showed much lesser incidence in diabetes in compared to wild type mice. This suggests that when CDK11 is half deleted, irrespective of cyclin D3 overexpression is protecting from T1D. This means both these genes are important in the onset of T1D and when CDK11 is half deleted it is

giving the same level of protection in presence of normal or overexpression of cyclin D3.

4. Adoptive transfer experiments also showed that both the genotypes CDK11(Δ/+) CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- exhibit the same susceptibility to adoptively transferred diabetes.

5. Both cyclin D3 and CDK11 are important in T1D. From various experiments like the percentage of islet infiltration count and alkaline phosphatase staining where the number of islet count and β cell area were found similar in both the groups CDK11(Δ/+)CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- mice.

6. The cyclin D3 expression assayed in these strains of mice also suggested that both CDK11(Δ/+)CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- groups have the same expression level. The CDK11(+/+)CcnD3(-/-) mice have very less cyclin D3 expression level as expected.

7. In CDK11(+/+)CcnD3(-/-) mice the islet size is the smallest compared to the other groups of mice. The insulin staining in them was much less intense.

8. Both the groups CDK11(Δ/+)CcnD3Tg+ and CDK11(Δ/+)CcnD3Tg- mice also showed the same glycemic value upon intraperitoneal challenge with glucose.

Conclusion

13. CONCLUSION

Cyclin D3 is important for keeping β-cell mass homeostasis and is required for β cell fitness. CDK11 is related to apoptosis and causes exacerbation of T1D. Thus, it seems like that cyclin D3 acts downstream of CDK11 and thus is closer to the target molecules involved in β cell viability.

CONCLUSIÓN

La ciclina D3 es importante para mantener la homeostasis de la masa de células β y es necesaria para la funcionalidad de las células β-pancreáticas. CDK11 está relacionada con la apoptosis y su expresión exacerba la diabetes tipo 1. Por lo tanto, todo apunta a que la ciclina D3 está jerárquicamente por debajo de la CDK11 y por lo tanto está más cerca de las moléculas-diana implicadas en la viabilidad de las células β.

CONCLUSIÓ

La ciclina D3 és important per mantenir l'homeòstasi de la massa de cèl·lules β i és necessari per la funcionalidad de cèl·lules β. CDK11 està relacionada amb l'apoptosi i la seva expressió provoca l'exacerbació de la diabetis tipus 1. Per tant, sembla que la ciclina D3 actua a nivel jeràrquic per sota de CDK11 i per tant està més a prop de les molècules diana implicades en la viabilitat de les cè·lules β.

14. PUBLICATION

Noemí Alejandra Saavedra-Ávila, **Upasana SenGupta**, Begoña Sánchez, Ester Sala , Laura Haba,Thomas Stratmann, Joan Verdaguer, Dídac Mauricio, Belén Mezquita, Ana Bélen Ropero, Angel Nadal, Conchi Mora. Cyclin D3: A new target in autoimmune diabetes. A novel cell cycle-independent role for cyclin D3 in the promotion of pancreatic β cell fitness and viability. (Submitted in Dec 2013).

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Annex I

Buffers & Mediums

A. CELL CULTURE MEDIUMS:

A. 2 RPMI Supplemented Medium

Reagents	Amount	Final Concentration
RPMI	500ml	-
FBS	50ml	10%
Gentamycin Sulfate	580µl	0.058mg/ml
β-mercaptoethanol	400µl	0.2mM

A. 2 DMEM Supplemented Medium

Reagents	Amount	Final Concentration
DMEM w/L-glutamin High glucose	500ml	-
FBS	50ml	10%
Penicillin (100X)	5ml	-
Glutamine	5ml	2.0mM
β-Mercaptoethanol	50µl	1.75mM

B. FOR GENOTYPING OF MICE:

B.1 Mice tail digestion buffer

Reagents	Amount	Final Concentration
TRIS pH8.00	6.005 gm	50mM
KCl	3.72 gm	50mM
EDTA	0.93 mg	2mM
NP-40	4.5 ml	0.45%
Tween-20	4.5 ml	0.45%

C. PCR :

C.1 DNA Loading Buffer (6X)

Reagents	Amount
Ficoll-400	7.5 gm
Bromophenol blue	15 gm
Xylene cyanol	15 mg

0.5M EDTA (pH8.0)	5 ml
100mM TRIS pH 7.5	5 ml

C.2 Preparation of TAE

Reagents	**Amount**
Glacial acetic acid	57.1ml
Tris base	252 gm
Glacial acetic acid	57.1ml
Na2EDTA.2H2O	37.2gm
Water to 1 litre	Final pH 8.5

D. TISSUE (pancreatic tissue) STAINING BUFFERS:

D.1 Hematoxylin and eosin staining

Preparation of Eosin	
Reagents (Eosin stock)	**Amount**
Eosin	2gram
95%EtOH	160ml
Distilled water	40ml
Reagents (Eosin staining solution)	
Eosin stock solution	25ml
80% EtOH	75ml
Acetic Acid	3-4 drops
Preparation of Acid Alcohol	
80% Ethanol	250ml
37% HCl	3ml

D.2 Immunofluorescence Staining

Reagents	**Concentrations**
TRIS Solution	
TRIS $C_4H_{11}NO_3$	12.1g/L
Adjust to pH 7.4	
Permeabilization solution	

Tris Solution	50ml
Triton X-100	1%
Blocking Solution	
Permeabilization solution	50ml
BSA	3%

D.3 Alkaline phosphatase staining

Reagents	**Concentrations**
Blocking Solution	
BSA	3%
Triton-X100	0.01%
Tris Buffer pH7.4	0.1M
Primary Antibody Buffer	
BSA	1%
Triton-X100	0.01%
Tris Buffer pH7.4	0.1M
Washing Buffer	
Tris Buffer pH7.4	0.1M

E. FLOW CYTOMETRY:

E.1 Flow cytometry staining buffer

Reagents	**Concentration**
Before permeabilization	
PBS	1X
Fetal Bovine Serum	1%
Permeabilization	
PBS	1X
Triton-X100	0.01%
After permeabilization	
PBS	1X
Fetal Bovine Serum	1%
Triton-X100	0.01%

F. WESTERN BLOT:

F.1 LYSIS BUFFER:

FORMULATION	VIAL 1	1x Lysis Buffer: 1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide
	VIAL 2	PMSF in DMSO
	VIAL 3	Protease inhibitor cocktail in DMSO
	VIAL 4	Sodim orthovanadate in water
USAGE		Combine 10µl PMSF solution, 10µl sodim orthovanadate solution and 10-20µl protease inhibitor cocktail solution per ml of 1x RIPA Lysis buffer to prepare complete RIPA. 1ml complete RIPA per 2.0×10^7 µl cells in suspension.

F.2 Composition of (10%) Resolving Gel (1 gel)

Reagents	**Amount**
Water	4 ml
30% Acrylamide mix	3.3 ml
1.5M TRIS (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% Ammonium persulphate	0.1 ml
TEMED	0.004ml

F.3 Composition of (5%) Stacking Gel (1 gel)

Reagents	**Amount**
Water	2.1 ml
30% Acrylamide mix	0.5 ml
1M TRIS (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% Ammonium persulphate	0.03 ml
TEMED	0.003 ml

F.4 Composition of Running Buffer

Reagents	Amount 10X (1litre)
Tris	30.2 gm
Glycine	188 gm
SDS 10%	10 gm

F.5 Composition of Transfer Buffer

Reagents	Amount (1litre)
Tris	30.2 gm
Glycine	14.4 gm
Methanol	100 ml

F.6 Composition of TBS (10X)

Reagents	Amount (1litre)	Concentration
Tris pH:7.5	121.1 gm	1M
NaCl	90 gm	9%
Autoclaved		

F.7 Composition of Washing Buffer (TBS-T)

Reagents	Amount (1litre)
1X TBS	2 litre
100% Tween 20	1 ml

F.8 Composition of Blocking Buffer

Reagents	Amount (1litre)
TBST	50 ml
Non-fat dry milk	2.5 gm

F.9 Composition of 2X DNA Loading Buffer

Reagents	Amount (1litre)	Concentration
Water	7ml	
TRIS		1M

Glycerol	8ml	
SDS	16ml	10%
β-Mercaptoethanol	4ml	
Bromophenol Blue	8mg	

F.10 Composition of Coomasie Blue Staining solution

Reagents	Amount (1litre)	Concentration
Methanol	450ml	45%
Coomasie Brilliant Blue	0.5gm	
Acetic acid	100ml	10%
Water	450ml	45%

F.11 Composition of Destaining Solution

Reagents	Amount (1litre)	Concentration
Water	500 ml	-
Methanol	50 ml	5%
Acetic Acid	75 ml	7.5%
Water is added upto 1 litre.		

F.12 Composition of Stripping Solution

Reagents	Concentration
β-Mercaptoethanol	100mM
SDS	2%
Tris HCl pH 6.8	62.5mM

G. MOLECULAR BIOLOGY:

G.1 Bacterial Culture Mediums

Reagents	Amount
LB MEDIUM	
Tryptone	10 gm
Yeast Extract	5 gm
Sodium Chloride	5 gm

1N Sodium Hydroxide	1 ml
LB PLATES	
Tryptone	10 gm
Yeast Extract	5 gm
Sodium Chloride	5 gm
Agar	15 gm

G.2 Reagents for competent cell preparation

SOB		
		FINAL CONCENTRATION
	5 g of bactotriptona	2%
	1.25 g yeast extract	0.5%
	2.5 ml de NaCl 1M	10mM
	0.625 ml de KCl 1M	2.5mM

TfBI		
		FOR 100ml
Potassium Acetate	30mM	0.29 gm
$CaCl_2.2H_2O$	10mM	0.15 gm
RbCl	100mM	1.21 gm
$MnCl2.4H_2O$	50mM	0.99 gm
Glycerol	15%	15 ml
Adjusted to pH 5.8 with acetic acid. Sterilized by filtration. Store at 4 °C.		

TfBII		
		FOR 100ml
MOPS	10mM	10ml from stock of 100mM
$CaCl_2.2H_2O$	75mM	1.1 gm
RbCl	10mM	0.12 gm
Glycerol	15%	15 ml
Sterilize by filtration. Store at 4 ° C.		

Annex II

PCR Programmes

PCR PROGRAMES

PCR Programme for CcnD3 Transgenic

94ºC – 1 minute
60ºC – 1 minute
72°C – 2 minutes
30 Cycles

72°C – 6 minutes

4°C – ∞

PCR Programme for CcnD3 Knock out

94ºC – 3 minutes

94ºC – 1 minute
60ºC – 1 minute
72°C – 1 minute
36 Cycles

72°C – 7 minutes

4°C – ∞

PCR Programme for CDK11 Knock out

94ºC – 2.5 minutes

94ºC – 40 seconds
60ºC – 50 seconds
72°C – 40 seconds
38 Cycles

72°C – 10 minutes

4°C – ∞

PCR Programme for SCID mutation

94ºC – 1.5 minutes

94ºC – 30 seconds
53ºC – 30 seconds
72°C – 30 seconds
35 Cycles

72°C – 2 minutes

4°C – ∞

PCR Programme for CDK11clof/Ealb1 and CDK11clof/Eα

94ºC – 1 minute
62ºC – 1 minute
72°C – 2 minutes
30 Cycles

72°C – 6 minutes

4°C – ∞

PCR Programme for HPRT1/HPRT2

94ºC – 40 minute
62ºC – 20 minute
72°C – 40 minutes
34 Cycles

4°C – ∞

Annex III

Supplementary Informations

Monitoring Type 1 Diabetes

MONITORING DIABETES

OBSERVATION CAGE ID # _____ **STRAIN:** _____

RACK POSITION _____

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

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
Mouse ID ↓																											

Monitoring Adoptive Transfers

MONITORING DIABETES

OBSERVATION CAGE ID # _____ **STRAIN:** _____

TRANSFERRED WITH _____

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

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

Glucose Tolerance Test

Pentobarbital concentration - 60mg/kg

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

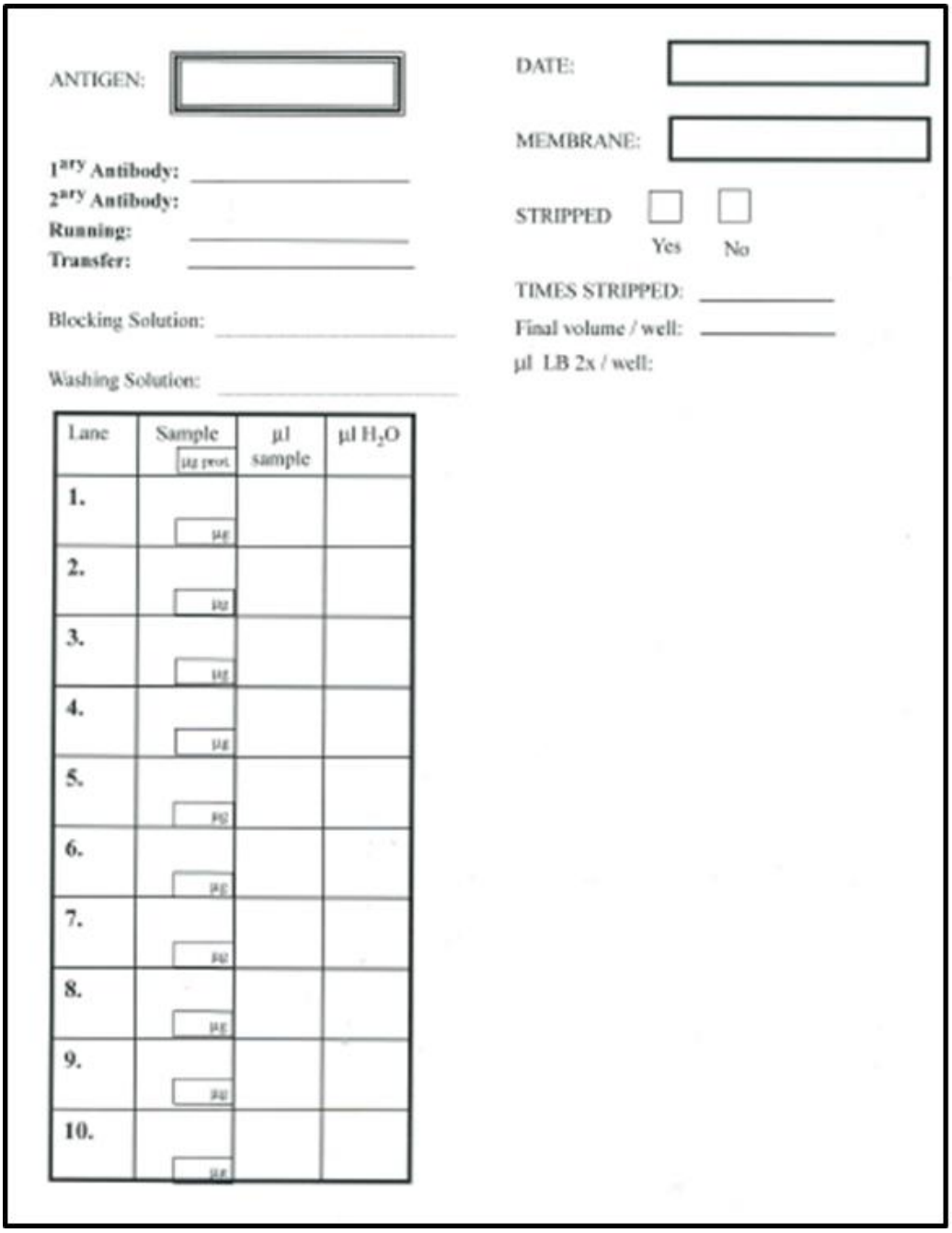
Dilution of Dolethal in physiological saline solution- 0.03mL Dolethal/mL

Glucose solution-5%

Weight (g)	Pentobarbital dilution (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	.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	092
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	Weight (g)	Genotype	Glycemia (mg/dL) t=0	15min	30min	60min	120min

Western Blot Details



ANTIGEN:

DATE:

MEMBRANE:

1^{ary} Antibody: _____
2^{ary} Antibody:
Running: _____
Transfer: _____

STRIPPED ☐ Yes ☐ No

TIMES STRIPPED: _____

Final volume / well: _____

µl LB 2x / well:

Blocking Solution: _____

Washing Solution: _____

Lane	Sample µg prot.	µl sample	µl H_2O
1.	µg		
2.	µg		
3.	µg		
4.	µg		
5.	µg		
6.	µg		
7.	µg		
8.	µg		
9.	µg		
10.	µg		