Universidade de Lisboa

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Departamento de Biologia Vegetal

The role and therapeutic potential of regulators and effectors of PI3K/Akt pathway in T-cell leukemia

Daniel Filipe Silva Ribeiro

Mestrado em Biologia Molecular Humana

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ABBREVIATURE INDEX

β-ME: β-mercaptoethanol

$\Delta\Psi_M$: Mitochondrial Transmembrane Potential

γ_C: gamma-common chain of the IL-2 receptor family

^{3}H-TdR: Tritiated Thymidine

7AAD: 7-Aminoactinomycin D

AEBSF: 4-(2-Aminoethyl) Enzenesulfonyl Fluoride Hydrochloride

Akt/PKB: v-akt Murine Thymoma Viral Oncogene Homolog 1 / Protein Kinase B

AnnV: Annexin V

APC: Allophycocyani

APS: Ammonium Persulfate

BAD: BCL2-associated agonist of cell death

Bcl-2: B-cell CLL/Lymphoma 2

Bcl-XL: Bcl-2 like 1

Bim: BCL2-like 11

BSA: Bovine Serum Albumin

CDK: Cyclin Dependent Kinases

cDNA: coding Deoxyribonucleic Acid

CK2: Protein Kinase CK2

CKIP1: CK2-interating protein-1

CREB: cAMP Response Element inding

CSF: Colony Stimulating Factor

DAPT: N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

dNTP: deoxyribonucleotide

DRB: 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole

EDTA: Ethylenediaminetetraacetic Acid

EtOH: Ethanol

FACS: Fluorescence Activated Cell Sorting

FasL: Fas Ligand

FBS: Fetal Bovine Serum

FOXO3a: Forkhead Box O3a

Gadd45: Growth Arrest and DNA-Damage-inducible

GFP: Green Fluorescent Protein

GSK3β: Glycogen Synthase Kinase 3β

HA-tag: Hemagglutinin tag

HBSP: Hanks' Buffered Saline Plus Supplements

HEPES: 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid

Hes: Hairy and Enhancer of Split 1

Hey: Hairy/Enhancer-of-split Related with YRPW Motif 1

hPGK: Human Phosphoglycerate Kinase

HRP: Horseradish Peroxidase

IKK: IκB Kinase

IL-2: Interleukin 2

IL-7: Interleukin 7

IL-7R: IL-7 Receptor

Jak: Janus Kinase

LB medium: Lysogeny broth

LLA-T: Leucemia Linfoblástica Aguda de Células T.

MAPK: Mitogen Activated Protein Kinases

Mcl-1: myeloid cell leukemia sequence 1

MCS: Multiple Cloning Sites

Mdm2: Transformed Mouse 3T3 Cell Double Minute 2

minhCMV: Minimum Human CMV Promoter

MOI: Multiplicity of Infection

mRNA: messenger Ribonucleic Acid

Myc: Myelocytomatosis Viral Oncogene Homolog
NICD: Notch Intracellular Domain
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PEG: Polyethylene Glycol
PH domain: Pleckstrin Homology domain
PI: Propidium iodide
PI3K: Phosphoinositide 3-kinase
PIP_2: Phosphatidylinositol 4,5-bisphosphate
PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate
PML protein: Promyelocytic Leukemia protein
PTEN: Phosphatase and Tensin Homolog
Ras: Rat Sarcoma Viral Oncogene Homolog
SCF: Stem Cell Factor
SDS: Sodium Dodecyl Sulfate
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2 domain: Src Homology 2 domains
SOB: Super Optimal Broth
SOC: SOB medium with catabolic repression
STAT: Signal Transducers and Activators of Transcription
TACE: Tumor Necrosis Factor-α-Converting Enzyme
TAE buffer: Tris-acetate EDTA
T-ALL: T-cell Acute Lymphoblastic Leukemia
TBB: 4,5,6,7-Tetrabromobenzotriazole
TBS: Tris Buffered Saline
TEMED: Tetramethylethylenediamine
TMRE: Tetramethylrhodamine Ethyl Ester
TRAIL: TNF-related Apoptosis-inducing Ligand

ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is a hematological cancer that mainly affects children. Although current treatments are effective, they originate significant long-term side-effects and significant number of relapses occur. Both microenvironmental and cell-autonomous cues contribute to T-ALL progression. IL-7 is an essential cytokine that has been shown to promote survival and proliferation of T-ALL cells by activating the PI3K/Akt pathway. Notch and CK2 have been implicated in transformation and survival of T-ALL cells by downregulating or functionally inactivating PTEN protein, a tumor suppressor and negative regulator of PI3K/Akt pathway.

In the present thesis, two main objectives were defined: to establish the relative importance of two proteins, GSK3β and FOXO3a, which are negatively regulated by the PI3K/Akt pathway, in IL-7-mediated proliferation and viability of T-ALL cells; and to assess the therapeutic potential of combined Notch and CK2 inhibition in the regulation of the tumor suppressor PTEN. Both objectives can contribute to the rational definition of new therapeutic targets and the development of novel treatment strategies.

To accomplish the first task, lentiviral vectors were constructed bearing constitutively active mutants of GSK3β and FOXO3a (GSK3β.S9A and FOXO3a.A3). Preliminary data suggest that downregulation of FOXO3a activity may be mandatory for IL-7 to exert its effects upon leukemia cells. T-ALL cells transduced with this mutant show decreased viability and IL-7 stimulation cannot rescue the viability to the control levels.

To accomplish the second task, PTEN+ cell lines were incubated with the γ-secretase inhibitor (Notch inhibitor) DAPT, the CK2 inhibitors DRB or TBB, or with both. Our results show that the combined use of γ-secretase and CK2 inhibitors can affect the cell size, cell number and proliferation of T-ALL cell lines in a cooperative manner.

RESUMO

Introdução: A leucemia linfoblástica aguda de células T (LLA-T) é um cancro hematológico que afecta especialmente crianças até 5 anos de idade, sendo um dos tipos de cancro mais comum nesta faixa etária. Esta doença caracteriza-se pela expansão clonal descontrolada de linfócitos T imaturos na medula óssea e timo, que extravasam para o sangue periférico e invadem outros órgãos. Os tratamentos actualmente existentes têm uma eficácia considerável: em média, 70% dos doentes apresentam-se livres de doença 5 anos após o tratamento. No entanto, a percentagem de recidivas (cujo prognóstico é extremamente reservado) e as complicações a longo prazo devido à elevada toxicidade dos tratamentos, tornam premente a necessidade de novas terapias, mais específicas e

eficazes. Nesse sentido, as vias de transdução de sinal que promovem a proliferação e sobrevivência das células tumorais constituem promissores alvos terapêuticos.

São conhecidas várias causas para este tipo de leucemia: factores hereditários, exposição excessiva a radiação ou químicos, entre outros. Além de factores intrínsecos à célula maligna, os factores microambientais desempenham um papel fundamental no estabelecimento do tumor. A IL-7 é uma citocina essencial para a linfopoiése, funcionando como factor de crescimento e sobrevivência para células T normais. Contudo, as células T tumorais também beneficiam da IL-7, proliferando significativamente em resposta a esta citocina..

A IL-7 utiliza a via Jak/STAT para transdução de sinal pelo IL-7R. Após activação do receptor, são imediatamente activadas as cinases de tirosina Jak1 e Jak3 e subsequentemente as STATs. As Jak não têm apenas as STAT como substratos mas também outros intervenientes essenciais em vias de sinalização como a PI3K na via PI3K/Akt. A família de cinases PI3K está envolvida na síntese de fosfatidilinositóis trifosfatados (PIP_3) na membrana. A síntese de PIP_3 recruta as cinases PDK e Akt, com a consequente activação de Akt por PDK. A Akt é a cinase de serina/treonina efectora da via PI3K/Akt, tendo numerosos alvos envolvidos na regulação de diferentes processos celulares A fosforilação catalisada por esta cinase pode ser activadora ou repressora, levando a que, de uma forma geral, a proliferação celular e a sobrevivência sejam aumentadas. Exemplos de proteínas inactivadas por Akt incluem membros da família *Forkhead* e GSK3. É frequente esta via de sinalização estar hiper-activada em tumores, incluindo LLA-T.

O factor de transcrição FOXO3a (membro da família *Forkhead*) é inactivado em resultado da fosforilação catalisada por Akt. FOXO3a tem sido implicado como tendo um papel protector em processos como envelhecimento, neurodegeneração e cancro. FOXO3a promove a expressão de Gadd45, TRAIL, Bim, Noxa, FasL e $p27^{Kip1}$, entre outros indutores de apoptose e inibidores do ciclo celular. Após estimulação com IL-7, ocorre a activação da via PI3K/Akt e subsequente fosforilação/inactivação de FOXO3a. GSK3β é uma cinase de serina/treonina que também se possui diversos substratos A fosforilação mediada por GSK3β origina a activação de proteínas pró-apoptóticas (Bax, Bim, p53) e inactivação e/ou degradação de proteínas anti-apoptóticas e promotoras do ciclo celular (Mcl-1, CREB, Ciclina D1, c-Myc). A activação da via PI3K/Akt resultante da estimulação com IL-7 resulta na fosforilação e consequente inactivação de GSK3β. No entanto, não se sabe se qualquer um destes dois processos (inactivação de FOXO3a e inactivação de GSK3β) é necessário para que a IL-7 funcione como citocina indutora de viabilidade e proliferação das células LLA-T.

PTEN, um supressor tumoral, é o principal regulador negativo da via PI3K/Akt. Trata-se de uma fosfatase de lípidos cuja função é directamente antagónica da de PI3K. Como tal, este

gene sofre frequentemente mutações ou deleções em muitos tipos de cancro. Em LLA-T é frequente encontrar-se uma baixa actividade de PTEN devido a mecanismos de regulação transcricional, mediados por Notch, e pós-traducional, mediados por CK2.

A via de sinalização Notch está implicada em eventos como diferenciação, proliferação e apoptose, sendo fundamental durante a embriogénese. Existem diversos receptores Notch e também diversos ligandos. Canonicamente, após a ligação do ligando, Notch é clivado por TACE e pelo complexo γ-secretase. Esta última clivagem liberta dentro das células, a forma activa do receptor Notch (NICD), que funciona como factor de transcrição. São transcritos, directa e indirectamente, genes como Hes1, Hey, c-Myc ou Ciclina D1, todos eles implicados num aumento da sobrevivência e proliferação. A sinalização alterada de Notch tem sido descrita em diferentes tipos de cancro. Em LLA-T, mais de 50% dos casos têm mutações activadoras de Notch1, ou seja, mutações em NOTCH que levam a uma maior processividade do complexo γ-secretase. A activação de Notch conduz a um silenciamento transcricional de PTEN, por intermédio de Hes1, tendo como consequência uma sobre-activação da via PI3K/Akt.

A proteína CK2, é uma cinase de serina/treonina com um enorme leque de substratos na célula. Devido à extensa diversidade de substratos, a sua regulação é complexa e pouco compreendida. No entanto, um aumento de actividade de CK2 tem sido implicado na transformação maligna e aumento de agressividade do tumor. Em leucemia, foi demonstrado que a actividade de CK2 pode levar tanto a uma degradação aumentada de supressores tumorais, mas também a uma estabilização de proteínas em formas inactivas. CK2 está frequentemente sobre-activa em células primárias de doentes com LLA-T, promovendo a estabilização e inactivação de PTEN. Consequentemente, embora as células leucémicas apresentem elevados níveis de expressão de PTEN, a actividade da fosfatase é extremamente baixa, levando a uma hiper-activação da via PI3K/Akt.

Objectivos: Tendo em conta a importância que tanto factores do microambiente como alterações intrínsecas à célula têm na progressão tumoral através da regulação da via PI3K/Akt, pretendeu-se cumprir dois objectivos complementares nesta tese: estabelecer a importância relativa que GSK3β e FOXO3a têm aquando da estimulação de células de LLA-T por IL-7; e definir o potencial terapêutico que a inibição conjunta de Notch e CK2 têm sobre a regulação do supressor tumoral PTEN em LLA-T. No primeiro objectivo, tentou-se perceber se a inactivação de GSK3β e FOXO3a é necessária para o completo efeito da IL-7. Para tal, subclonou-se mutantes constitutivamente activos (GSK3β.S9A e FOXO3a.A3) no vector lentiviral #304 e procedeu-se à transdução de células HPB-ALL e TAIL7. Foram feitas análises de sobrevivência e activação (por citometria de fluxo), proliferação (através da avaliação da incorporação de ^{3}H-timidina) e estudos moleculares de expressão proteica (por *Western Blot*). No segundo objectivo, foram usadas linhas celulares PTEN+ (HPB-ALL,

TALL-1 e TAIL7) e procedeu-se à incubação das células com inibidores da γ-secretase (DAPT), para impedir activação de Notch, e inibidores de CK2 (DRB ou TBB), quer de forma isolada, quer de forma conjunta. Foram feitos estudos de activação celular (por citometria de fluxo), contagem do número total de células (usando o hemocitómetro, com exclusão de azul tripano) e proliferação (avaliação da incorporação de ^{3}H-timidina). Em suma, como objectivo geral da tese pretendeu-se contribuir para a compreensão do papel e potencial terapêutico de proteínas envolvidas na regulação e função da via PI3K/Akt em LLA-T. Em última análise, as conclusões obtidas poderão eventualmente levar à definição de novos alvos terapêuticos e de novas estratégias de tratamento desta doença.

Resultados: Relativamente à subclonagem de GSK3β.S9A, tento u-se inicialmente clonar no vector intermédio pBSKS^{-} para depois se subclonar no vector lentiviral #304. Esta estratégia não funcionou devido ao elevado número de eventos de recombinação que ocorreram nas bactérias transformadas. Procedeu-se então à clonagem deste mutante por PCR. Desenharam-se e testaram-se primers específicos para o gene que continham locais para enzimas de restrição compatíveis com o vector #304. Usando o vector TOPO como vector intermédio, conseguiu-se então construir o vector final #304.GSK3β.S9A. Os resultados da sequenciação da clonagem revelaram existir uma delecção *in frame* de 39 nucleótidos no gene. Por expressão transiente em células, verificou-se por *Western Blot* que a proteína mutante possuía um peso molecular superior ao esperado. Perante estes resultados aparentemente contraditório, reviram-se os dados obtidos e fez-se uma extensa pesquisa na literatura e PubMed. Descobriu-se então que se estava perante uma variante de *splicing* funcional do gene GSK3β, frequentemente expressa, que não inclui o exão 8b (daí os resultados da sequenciação apresentarem uma delecção), mas que inclui o exão 10 (daí a proteína no *Western Blot* ter um peso superior à nativa das células). Portanto, a clonagem e expressão do gene estavam correctas e o mutante pode ser usado em experiências.

Relativamente à clonagem de FOXO3a.A3 no #304, tendo este uma HA-*tag*, procedeu-se à encomenda de primers específicos para clonagem por PCR. Perante o insucesso em amplificar uma banda específica quando se pretendia incluir a HA-*tag*, decidiu-se então descartar a possibilidade de ter a HA-*tag* (não essencial para a execução do trabalho) e procedeu-se à clonagem de apenas FOXO3a.A3. A clonagem TA no vector TOPO não resultou, muito provavelmente devido, a este gene ser rico em GC,. Portanto, procurou-se um vector específico para clonagem GC. Após clonagem no vector intermédio e alguma optimização na ligação do mutante FOXO3a.A3 no #304, conseguiu-se finalmente construir o vector #304.FOXO3a.A3. A sequenciação do plasmídeo e expressão transiente em células 293 confirmaram que o mutante poderia ser usado em experiências.

Procedeu-se então à produção de lentivírus para transdução de da linha celular HPB-ALL. Infelizmente, a eficiência de transdução é baixa e, como tal, as análises funcionais subsequentes padecem de um efeito de diluição das células transduzidas nas não-transduzidas (significativamente mais numerosas). As células foram então cultivadas com ou sem IL-7 e os resultados para o mutante FOXO3a.A3, embora não estatisticamente significativos, são promissores. FOXO3a constitutivamente activo em LLA-T parece baixar consistentemente a viabilidade, sendo que a presença de IL-7 não é suficiente para inverter esse efeito. Estes resultados preliminares sugerem que pelo menos a inactivação de FOXO3a.A3 parece ser necessária para que a IL-7 exerça completamente os seus efeitos anti-apoptóticos.

No que respeita ao segundo objectivo, procedeu-se à incubação de linhas LLA-T positivas para PTEN com inibidores da γ-secretase e de CK2. Os resultados obtidos mostram uma diminuição do tamanho celular, viabilidade e proliferação das células quando incubadas com cada um dos inibidores. Mas este efeito é significativamente aumentado quando as células são incubadas com ambos os inibidores, demonstrando um efeito cooperativo entre os dois tipos de inibidores. Estes resultados apontam para uma possível nova estratégia terapêutica. Os dados aqui descritos estão incluídos num artigo original recentemente aceite para publicação (Silva, Jotta, Silveira, Ribeiro, et al. *Regulation of PTEN by CK2 and Notch1 in primary T-cell acute lymphoblastic leukemia: rationale for combined use of CK2- and gamma-secretase inhibitors*. Haematologica – aceite para publicção)

Conclusões: Os objectivos do Capítulo 1 foram parcialmente atingidos, sugerindo que, pelo menos, a inactivação de FOXO3a é necessária à completa actividade da IL-7. No presente momento estão a pesquisar-se alternativas para contornar o problema da baixa infecciosidade das linhas celulares pelos lentivírus. Os objectivos do capítulo 2 foram completamente atingidos, levando a que se possa ponderar a inclusão do uso combinado de inibidores de Notch e CK2 no desenvolvimento de novas estratégia terapêuticas para tratamento de doentes com LLA-T.

KEYWORDS

T-cell Acute Lymphoblastic Leukemia, GSK3β, FOXO3a, PTEN, CK2, Notch

INTRODUCTION

This thesis contains two major parts. In the first part, the role of two effectors of PI3K/Akt pathway is explored in the context of IL-7-mediated signaling. The second part investigates the therapeutic potential of targeting two known regulators of the PI3K/Akt pathway. To adequately this Introduction includes a brief characterization of: 1) T-ALL and the importance of microenvironmental factors for tumor progression, highlighting the possible role of IL-7 in T-ALL establishment and progression; 2) the Jak/STAT pathway, which is important for normal T cell signal transduction and the signaling pathways that are the focus of this study (PI3K/Akt and Notch); 3) the PI3K/Akt effectors GSK3β and FOXO3a regulated in response to IL-7; 4) the PI3K/Akt negative regulator PTEN and its relation with Notch and CK2. Finally, the role of GSK3β, FOXO3a, PTEN and CK2 in normal cells and malignant cells is discussed.

T cell Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia is a type of leukemia characterized by an abnormal proliferation of immature cells of lymphoid lineage in the bone marrow or thymus that intravasate the peripheral blood and invade other organs. These cells are not immunologically functional and because of their increased proliferation rate they can impair normal hematopoiesis. ALL has a higher incidence in children until 5 years of age and is one of the most common cancers in this age-group. In young adults, the incidence of the disease decreases significantly but rises again in elderly [1, 17]. T-ALL is a subtype of ALL in which the immature cells are of the T lineage. The importance of studying T-ALL resides in part in the fact that the majority of the affected individuals are children and in the fact that, although the existing treatments are effective (more than 70% disease free survival at 5 years in the most successful protocols), a significant number of relapses still occur and have a dismal outcome. Moreover, severe long term complications often arise because of the elevated toxicity of the treatments [4]. There are many known alterations in the cellular genome that may lead to the development of ALLs, this are resultant from the exposure to several factors such as the inheritance of mutated alleles, overexposure to certain radiations and chemicals or late exposure to pathogens [1, 17]. However, genomic alterations are not the only events promoting the transformation of a normal cell into a cancer cell: the microenvironment has a fundamental role in the establishment of a tumor. Cancer cells are in constant cross-talk with the normal surrounding cells which are frequently subverted by the tumorigenic process and can, for instance, synthesize factors that promote tumor progression [4, 7].

Interleukin 7

Normal hematopoiesis is stimulated by various cytokines, including, for example, SCF, CSFs and IL-7. Each one of these factors leads to the differentiation of certain hematopoietic lineages. IL-7 is mainly secreted by the stromal cells in the bone marrow and thymus, being essential to the proliferation of B and T cell precursors. Its receptor (IL-7R) is composed of two chains: IL-7Rα, specific for IL-7, and IL-2Rγ/γc, the gamma-common chain shared by receptors of the IL-2 cytokine family (IL-2, IL-4, IL-9, IL-15 and IL-21) [4, 18, 19].

It has been demonstrated that IL-7 performs the function of growth and survival factor for normal cells as for T-ALL malignant cells, acting mainly through the PI3K/Akt pathway in the latter case. These functions are resultant of the increased expression of Bcl-2 and Cyclin D1 proteins and decreased expression of $p27^{Kip1}$, a CDK inhibitor [3, 4, 12]. Since malignant T-ALL cells originate from normal T cell precursors, which rely on IL-7 for their development, they are easily exposed to the IL-7 present in the BM and thymic microenvironments. Normal T cell precursors stop dividing and differentiate in response to different stimuli, and are, at certain stages, highly prone to apoptosis, In contrast, T-ALL cells are blocked at a certain stage of differentiation, and thus may rely largely on the proliferative and survival signals of IL-7 without going through the apoptosis-prone process of T-cell differentiation. [4].

Jak/STAT pathway

Jak/STAT pathway is the canonical signaling cascada used by cytokines to transduce signals to target cells. Jaks are a family of tyrosine kinases with 4 known members: Jak1, Jak2, Jak3 and TYK2. Jaks are found associated with the intracellular part of the receptors. STAT proteins are a family of transcription factors with 7 members: STAT1, 2, 3, 4, 5a, 5b and 6. STATs are found in the cytoplasm in a monomeric and inactive form. After phosphorylation by Jaks they dimerize and become activated, being translocated to the nucleus, where they function as transcription factors and promote proliferation, differentiation and apoptosis [16, 19].

In the case of IL-7 stimulation, after receptor binding, the dimerization of the receptor is promoted and the associated Jak proteins, Jak3 in γc and Jak1 in IL-7Rα, are interphosphorylated in the tyrosine residues of the Jak Homology 1 domain, becoming activated. After that, Jak 1 and 3 phosphorylate the receptor in target tyrosines, leading to the anchorage of STAT proteins, mainly STAT5a and 5b. In this way, STATs are recruited to the proximity of Jaks and are phosphorylated/activated, dimerizing and translocating to the nucleus. Once in the nucleus, STAT5 functions as a transcription factor for several genes that code for proteins involved in inducing survival, proliferation and cell cycle progression, such as Bcl-2, Bcl-X_L, c-Myc, IL-2Rα e cyclin D1. Importantly, Jak substrates are not limited to STAT proteins, and include proteins which intervene in other signaling pathways, such as Ras/MAPK and PI3K/Akt [4, 16].

PI3K/Akt pathway

PI3K/Akt signaling pathway is a master regulator of cellular homeostasis. The main elements of this pathway are PI3K, PTEN, PDK and Akt/PKB [11].

The PI3K proteins belong to a family divided in 3 classes, being class I the most studied. This class is further divided in sub-classes Ia and Ib. The sub-class Ia contains the regulatory subunits of PI3K: p85α, p55α, p50α, p85β, p55γ, possessing two SH2 domains that allow the recognition of phosphorylated and between them the catalytic subunit (p110α, p110β ou p110δ) interaction domain. The sub-class Ib contains the catalytic and regulatory subunits p110γ and p101, respectively [15]. The various isoforms of PI3K are involved in the synthesis of phosphatidylinositols mono-, bi-, or triphosphate from the membrane phospholipids. The synthesis of PIP_3 near the membrane recruits proteins that possess PH domains, such as PDK and Akt, allowing the activation of Akt. The antagonist of PIP3 synthesis is the phosphatase PTEN, converting PIP_3 into PIP_2, thus inactivating the PI3K/Akt pathway [10].

When inactive, the serine/threonine kinase PDK1 locates in the cytosol, whereas when actiave it is found near the plasma membrane. This kinase is responsible for the phosphorylation of one threonine (T308) in the kinase domain of Akt, while PDK2 is responsible for the phosphorylation of one serine (S473) in C-terminal domain of Akt. The identity of PDK2 is still a matter of debate, although the protein complex mTORC2 is likely to constitute the PDK2 activity in most cells [4, 15].

Akt is a serine/threonine kinase and the effector of the PI3K/Akt pathway. Three isoforms of this kinase are known: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. This enzyme has a great diversity of targets and phosphorylation by this kinase can either activate or repress the activity of the substrates. For example, Akt-mediated phoshorylation leads to inactivation of BAD, Caspase 9, Forkhead family members and GSK3β, while promoting the activation of IKK, Mdm2, and CREB. In this way, Akt influences many features that control the cell behavior, namely it promotes proliferation, inhibition of apoptosis and control of the metabolism [5, 8, 11, 15]. Summarizing, the activation of a receptor by its ligand or the cross-talk with another signaling pathway triggers the activation of PI3K, leading to the synthesis of PIP_3 which favors the activity of PDKs directing the phosphorylation and activation of Akt. Upon activation, Akt will interfere with the activity of many proteins tipping the balance towards survival and proliferation. Interestingly, it has been shown that activation of this pathway is essential for the viability and cell cycle progression of T-ALL cells mediated by IL-7 in vitro, leading to the postulation that IL-7 may have a role in the establishment or maintenance of T-ALL [3, 4].

The tumor suppressor PTEN is a lipid phosphatase that acts as the antagonist of PI3K, and is the most important downregulator of the PI3K/Akt pathway. The *PTEN* gene is frequently

silenced, mutated or deleted in different types of cancers, includin glioblastoma, melanoma, prostate and endometrium cancers, amongst many others. Mice heterozygous for the *PTEN* gene (*PTEN*$^{+/-}$) have increased manifestation of several cancers, including leukemias and lymphomas. In T-ALL, low levels of PTEN protein activity are frequent, resultant mainly form transcriptional down regulation or gene silencing and posttranslational modification. Transcriptional downregulation is discussed in the Notch pathway section below, and the posttranslational modification is discussed in the CK2 section. [29, 30].

Notch pathway

Notch signaling pathway is a highly conserved pathway across the animal kingdom. Notch is implicated in stem cell fate determination and patterning events, controlling differentiation, proliferation and apoptosis. Correct Notch signaling is fundamental during embryogenesis [25].

In mammals, four Notch surface receptors (Notch1-4) and five ligands (Delta-like 1, 3 and 4, and Jagged 1 and 2) are described. Upon ligand-receptor binding, Notch receptor undergoes two proteolytic cleavages: first by TACE, releasing the ligand-bound part of Notch, and second by the γ-secretase complex, releasing the Notch Intracellular Domain (NICD), the active signal-transducing fragment. NICD is then imported to the nucleus promoting the transcription of direct and indirect target genes: Hes1, Hey, c-Myc, Cyclin D1, implicated in augmented survival and proliferation [21].

Alterations in Notch signaling mechanisms have several implications due to abnormal signaling patterns. In cancer, increased Notch signaling has been correlated with the development of melanoma, breast cancer, myeloma and leukemia. [21, 25]. In T-ALL, Notch1 activating mutations have been described for more than 50% of diagnostic T-ALL cases leading to an increased γ-secretase complex activity and production of NICD. Also, increased Notch activity has been associated with a decrease in PTEN transcription leading to an upregulation of the PI3K/Akt pathway. This effect appears to be exerted by upregulation of HES1, a direct transcriptional target of Notch and a transcriptional repressor of PTEN [20, 23].

FOXO3a

The protein Forkhead box O3a (FOXO3a) belongs to an extense family of transcription factors with the Forkhead Box domain organized in groups ranging from FOXA to FOXS. The FOXOs have a wide distribution in all tissues of the body and have been implicated in several processes like: aging, cancer, diabetes, neurodegeneration and infertility [13]. FOXO3a directly induces the expression of Gadd45, TRAIL, Bim, Noxa, FasL, p27Kip1 and other promoters of apoptosis and inhibitors of the cell cycle [9, 12, 13]. Phosphorylation of

FOXO3a by Akt leads to FOXO3a inactivation due to the export of the transcription factor from the nucleus and subsequent retention in the cytoplasm [9, 13]. Another antagonist of FOXO3a is IKK (also activated by Akt) which phosphorylates and marks it for destruction by the proteasome [9]. After stimulation by IL-7, PI3K/Akt pathway is activated and consequently FOXO3a is phosphorylated/inactivated. In this thesis the mutant FOXO3a.A3 will be used. This mutant has the three Akt serines substituted by non-phosphorylatable alanines, rendering it permanently active even in the presence of activated Akt.

GSK3

The enzyme Glycogen Synthase Kinase 3 (GSK3) is a serine/threonine kinase initially discovered as an enzyme capable of phosphorylating glycogen synthase, however today there are more than fifty known substrates. This kinase regulates several processes, namely cell structure, metabolism and apoptosis. Two isoforms of this enzyme exist: GSK3α and GSK3β. GSK3β activates pro-apoptotic proteins – BAX, Bim, p53 – and inactivates anti-apoptotic proteins and promoters of the cell cycle – Mcl-1, CREB, Cyclin D1, c-Myc [14]. Upon activation of the PI3K/Akt pathway, GSK3β is phosphorylated and thereby inactivated by Akt [6, 14]. In this thesis the mutant GSK3β.S9A will be used. This mutant has a substitution of serine to alanine, making it permanently active even in the presence of activated Akt.

CK2

Protein kinase CK2 is a ubiquitous serine/threonine kinase highly conserved in eukaryotes. It is composed of two regulatory α subunits, for which two isoforms exist (α and α') and two catalytic β subunits. These isoforms have the same catalytic activity, although they seem to have specialized functions, since they are differently modified by other proteins (e.g. $p34^{cdc2}$), and have different binding capacities (e.g. towards CKIP1) [31].

CK2 has a broad range of substrates either in the nucleus or cytoplasm. These substrates often include proteins involved in the proliferation and survival of the cell. The failure to produce CK2 knock-out mice, shows that this kinase is essential for survival. Given the extensive amount of CK2 substrates and interactions, its regulation appears to be highly complex and poorly understood [27].

A great number of studies have implicated an increased activity of CK2 in malignant transformation, and tumor aggressiveness. Increased expression of CK2 is found in kidney, prostate, breast, lung cancers as well as in leukemias. CK2 was shown to phosphorylate tumor suppressors in key residues, promoting their faster degradation (PML protein) or their stabilization in its inactive form (PTEN protein). Oncogenes are also targets of CK2, but in their case the phosphorylation by CK2 leads to an altered and increased activity (e.g. c-Myc,

NK-κB or Akt). Also, CK2 can modulate the apoptotic machinery. Inhibition of CK2 in prostate cancer cells, leads to a decrease in the anti-apoptotic Bcl-2 and Bcl-xL proteins, and an increase in the pro-apoptotic Bax protein [29, 31].

In T-ALL, CK2 was shown to be frequently overexpressed and to promote the inactivation and stabilization of the tumor suppressor PTEN, leading to a state in which the cells had high levels of inactive PTEN and consequent hyperactivation of the PI3K/Akt pathway [29].

Figure 1 shows a summarized scheme of the signaling effects of IL-7, Notch and CK2 upon PI3K/Akt pathway, based on what is currently known regarding T-ALL cells. The first part of this project focused on the attempt to assess the role of FOXO3a and GSK3β in IL7-mediated signaling towards a therapeutic approach. The second part of the thesis focused in testing whether combined use of CK2- and γ-secretase (Notch) inhibitors may have therapeutic potential in T-ALL.

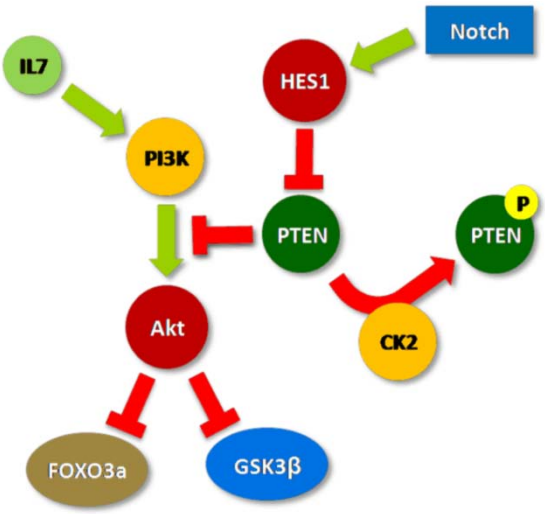

Figure 1 – Schematic representation of the effects on the PI3K/Akt pathway of the external stimulus IL-7 and the cell-intrinsic molecules Notch and CK2 in T-ALL cells.

OBJECTIVES

The major aim of this thesis is to the understand the role and therapeutic potential of proteins involved in the regulation and functional activity of the PI3K/Akt signaling pathway in T-ALL. The conclusions obtained may contribute to the rational definition of new therapeutic targets and development of novel treatment strategies.

Chapter 1 detailed objectives:

The main objective of this part of the thesis was to establish the relative importance of two proteins, GSK3β and FOXO3a, regulated by the PI3K/Akt pathway, upon IL-7 stimulation, in the proliferation and viability of T-ALL cells. In an initial approach, studies were made to investigate if the inactivation of these proteins is necessary for the full functionality of IL-7.

To accomplish the task, GSK3β.S9A and FOXO3a.A3 constitutively active mutants will be subcloned in the #304 lentiviral vector, lentiviruses will be produced. To obtain robust conclusions, transductions of either an IL-7 responsive cell line (HPB-ALL) and IL-7 dependent cell line (TAIL7) should be made. Several techniques were used to analyze the functional effects of the genes: flow cytometry, incorporation of ^{3}H-TdR and Western Blot.

Chapter 2 detailed objectives:

The main objective of this part of the thesis was to establish the therapeutical potential of combined Notch inhibition by using DAPT as a γ-secretase inhibitor, and CK2 inhibition by using DRB or TBB as CK2 inhibitors, in the regulation of the tumor suppressor PTEN. The following PTEN positive cell lines were used: HPB-ALL, TALL-1 and TAIL7.

To accomplish the task, the cell lines were cultured with DAPT, DRB or TBB, or both DAPT and DRB/TBB. To analyze the effects of the inhibitors flow cytometry, cell counts (using a hemocytometer and trypan blue exclusion) and proliferation by ^{3}H-TdR incorporation were used.

MATERIALS AND METHODS:

To simplify the reading, brands and models of equipment and reagents will be indicated only once.

1 – Culture of bacteria cells and DNA cloning:

The manipulation of bacteria cells, which required sterile conditions, was performed under the flame. The culture in Petri dishes was incubated at 37ºC in an incubator. The suspension culture was made at 37ºC in an orbital mixer Agitorb 200 (Aralab). The measurements of DNA concentration were performed in a spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific).

Digestion with restriction enzymes: For a maximum volume of 20µL, the following reagents we added by this order: needed volume of H_2O, 1-10µg of DNA, 2µL of adequate enzyme Buffer 10x (Promega), 2µL BSA 10x (Promega), 1-2µL enzyme(s) (Promega), the reaction was incubated at 37ºC, 1h to 1h30min.

Klenow Fragment reaction: after purification od the DNA with the Wizard® SV Gel and PCR Clean-Up System kit (Promega), reagents were added by this order: for 20uL of volume: needed H_2O, needed amount of DNA, 2µL of Klenow Buffer 10x (Fermentas), 2µL of BSA 10x, 0.5uL of Klenow (Fermentas), 0.5µL of dNTPs at 10mM. Incubate at 25ºC, 15min, followed by heat-inactivation for 20min at 75ºC and then cooled in ice.

Agarose gel electrophoresis in TAE buffer: An agarose gel was prepared using a 1% w/v agarose proportion in TAE buffer 1x supplemented with 0.5µg/mL of Ethidium Bromide. *Orange G sample Buffer* 5x was added to the samples to a final concentration of 1x. The molecular weight marker 1kb Gene Ruler DNA Ladder (Fermentas) was used (Figure 2). The electrophoresis ran at 86V during 45-60min according to the size of the fragments to resolve. The gels were visualized under a UV light (285nm) transillumintor (UVP) and photographed with a *Kodak Edas* 290 camera (Kodak). When the fragment was one of interest, we proceeded to the excision and purification of the DNA from the gel with the Wizard® SV Gel and PCR Clean-Up System kit.

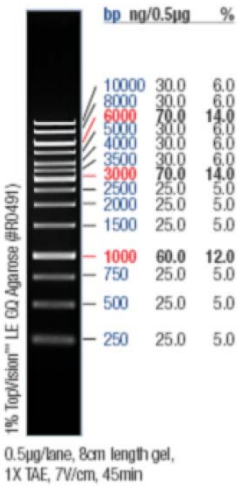

Figure 2 – Range and resolution of the DNA molecular weight marker 1Kb Gene Ruler DNA Ladder, by standard manufacturer's conditions. From the manufacturer's website.

Cloning in commercial TOPO (Invitrogen) and pGC.Blue (Lucigen): the cloning in both these commercial vectors was made according to the manufacturer's instructions. The *E. coli* strain provided with the TOPO vector is DH5α and the one provided with the pGC.Blue kit is 10G.

Cloning in non-commercial vectors: The cloning was performed for a maximum of 200ng of DNA and a maximum volume of 15µL of the reaction, in the proportion of DNA vector to DNA insert**:** 100ng vector ÷ vector size (Kb) = Xng insert ÷ insert size. To increase the odds of ligation X was multiplied by 3. By this order the following, reagents were added: needed volume of H_2O, needed amount of vector DNA and insert DNA, 1.5µL T4 DNA Ligase Buffer 10x (Fermentas), 1µL T4 DNA Ligase (Fermentas). The mix was incubated at 16ºC in a termocycler MyCycler (Bio Rad), *over-night,* followed by transformation of competent bacteria.

Chemical Transformation of competent *E. coli*: *LB medium (1L):* 10g Tryptone, 5g Yeast Extract, 10g NaCl in 1L ddH_2O was autoclaved. *LB Agar (1L):* 15g Agar (IDG) in 1L LB. *SOB medium (1L):* 20g Tryptone (Becton-Dickinson), 5.0g yeast extract (Becton-Dickinson), 0.5g NaCl (Sigma), added H_2O until 1L. After autoclave, added sterile 10mL of $MgCl_2$ 1M (Sigma) and 10mL of $MgSO_4$ 1M (Sigma). *SOC Medium (100mL):* 1mL glucose 2M (Sigma) sterile and 99mL SOB medium.

For the amplification of #304 based vectors the *E. coli* strain JM109 (Stratagene) was used. 1-5µL of DNA was used to transform ice thawed competent bacteria, followed by 30min incubation on ice. Then a thermal shock of 45seg at 42ºC followed by 2min in ice was done. Next, 900µL of SOC medium was added and bacteria were incubated for 1h at 37 ºC in orbital agitation 250rpm. Bacteria were then centrifuged at 13000rpm for 1min and the pellet was ressuspended in LB and plated in LB-Agar dishes pre-warmed at 37ºC. The blue/white screening was performed by spreading in the surface of the Petri dishes 40µL of X-Gal (Promega) 50mg/mL. The antibiotic screening was made by adding 100µg/mL ampicillin to LB-Agar or kanamycin 30µg/mL to LB-Agar.

PCR:

Primers for GSK3.S9A:

GSK3 Fwd (SphI) – 5' CATGCATGCAAGATGTCAGGGCGGCCCAGAA 3'.

GSK3 Ver (SalI) – 5' GCGTCGACTAATCAGGTGGAGTTGGAAGCTG 3'.

Primers for FOXO3a.A3:

HA Fwd (SphI) – 5' CATGCATGCTGGATGTAC CCA TAC GAT GTT CC 3'.

HA New (SphI) – 5' CATGCATGCTGGATGTAC CCA TAC GAC GTC CCA GA 3'.

FOXO Fwd (SphI) – 5' CATGCATGCTGGATGGCAGAGGCACCG 3'.

FOXO Rev (SalI) – 5' GCGTCGACTAATCAGCCTGGCACCCAGCT 3'.

Restriction enzyme recognition site is underlines in the primers.

For the PCR reaction were added in the following order: needed volume of H_2O, Pfu Buffer 10x (Promega) and DMSO (Sigma) to a final concentration of 10% (v/v) each, followed by addition of each Primer (Invitrogen) to a final concentration of 100nM, between 100-500ng of DNA and 2Units/µL of reaction of Pfu DNA Polymerase (Promega) . The PCR program used was the following, in a termocycler MyCycler: 94ºC – 5min (denaturing), [94ºC – 30sec (denaturing), variable annealing temperature for 1min and 30sec, 72ºC for variable extension time]x32, 72ºC – 7min (final extension), 4ºC – 5min.

Bacteria culture for miniprep: Glycerol Stocks were performed in the following way: a 300µL of an over-night culture of bacteria was mixed with 300µL of LB medium supplemented with 30% (v/v) glycerol (Sigma) and supplemented with the respective selection antibiotic.

2mL of LB medium supplemented with the respective selection antibiotic was poured in a Falcon tube (Orange Scientific). A bacterial colony or a part of a glycerol stock was picked to the Falcon tube. The Falcon tube was incubated in an orbital mixer, 250rpm, over-night. Bacteria was precipitated at 13000rpm, 5min, followed by supernatant discard and stored at -20ºC.

Small scale DNA extraction by the 1,2,3 process for: *Solution 1:* 50mM Glucose, 25mM Tris-HCl pH=8, 10mM EDTA (Sigma) and 100µg/ml Rnase (Promega). Stored at 4 C. *Solution 2:* 0,2 N NaOH and 1% SDS (Sigma) w/v. Stored at room temperature. *Solution 3:* 11,5% v/v Glacial Acetic Acid and 3M KOAc. Stored at 4ºC.

From a previously prepared miniprep, the pellet was ressuspended in 250µL of Solution 1 by vortexing. Then 250µL of Solution 2 was added, inverted and rested for 3min at room temperature. Next, added 350µL of Solution 3, inverted and centrifuged at 13000rpm, 10min. The supernatant was transferred to a new microtube and 500µL of isopropanol were added and the tube inverted, followed by a centrifugation at 13000rpm, 30min, 4ºC. Next, 500uL of -20ºC stored EtOH was added to the pellet, followed by centrifugation at 13000rpm, 10min at room temperature. Then, all EtOH was removed and the DNA pellet was air dryed. The DNA was eluted in 30µL of ddH_2O.

Bacteria culture for maxiprep: A previous miniprep culture was added to 150mL of LB supplemented with the respective selection antibiotics and incubated in an orbital mixer, 200rpm, over-night, followed by centrifugation at 4000rpm, 4ºC in Eppendorf 5810R centrifuge. The pellet was stored at -20ºC.

Maxiprep DNA extraction: the extraction was made using the Genopure Plasmid Maxi Kit (Roche) following manufacturer's instructions.

Sequentiation analysis: the analysis was made with the freeware software BioEdit.

2 – Culture of human cells, production and viral transduction, experimental and analytical techniques:

The manipulation of cells, which required sterile conditions was made in a vertical laminar flow chamber Hera safe (Heraeus) and its culture inside a Hera cell (Heraeus) CO_2 incubator at 37ºC, 5% CO_2. Washing/centrifugation of cells, in Falcon tubes, was performed in a Sorvall RT7 plus centrifuge at 10ºC, 1700rpm, 7min. Washing/centrifugation of cells in microtubes was performed in a 4ºC Biofuge fresco (Heraeus) centrifuge at 4ºC, 3200rpm, 7min. Cell counts were performed by Trypan Blue (Sigma-.Aldrich) exclusion in a 0.08% solution. Suspension cells were cultured in RPMI (Invitogen) with 5% or 10% FBS, designated R5 or R10, respectively. Adherent cells were cultured in DMEM (Invitrogen) with 10% FBS, designated D10. All culture mediums were supplemented with Penicillin/Streptomycin 1x (Invitrogen).

293T adherent cell culture: *Thawing:* a vial was thawed into 37ºC pre-warmed D10. Cells were seeded in a T75 flask (Nunc). The medium was changed in the following day. *Culture:* enough volume of pre-warmed D10 was used to cover the bottom of the flask. *Cell passage:* in this step, pre-warmed PBS and D10 were used. The flask bottom was washed and trypsin-EDTA 1x 0.05% (Invitrogen) was added and incubated at 37ºC until the cells detached. Trypsin was then inactivated by adding D10 medium. Cells were then filtered through a 0.70µm cell strainer (BD Falcon™), followed by centrifugation. Cells were then counted and seeded again.

HPB-ALL and TALL1 cell culture: *Thawing:* a vial was thawed into pre-warmed R10. Cells were seeded in a T25 flask. In the following day the cells were washed and counted. When the proportion of dead cells was higher than 20% of total cells, Lympholyte (Cederlane) was used, according to the manufacturer's instructions to separate live from dead cells. *Culture:* every three days the cells were counted and diluted to a concentration of $1*10^6$cells/mL for HPB-ALL or $0.5*10^6$cells/mL for TALL1 in R10.

TAIL7 cell culture: *Thawing:* a vial was thawed into pre-warmed R5. Cells were washed, counted and Lympholyte was used to purify the live cells. *Culture:* every 3 days the cells were counted and diluted at $2*10^6$cells/mL in R5 supplemented with 10ng/mL of IL-7, in 24-well plates (TPP). Once a week, live cells were purified by using Lympholyte.

Cell freezing (per cryovial): Cells were frozen in a suspension of 1.8mL of FBS with 10% (v/v) DMSO. Cryovials of 1.8mL (Nunc) were pre-cooled at 4ºC. After washing cells were ressuspended in 900µL of FBS and stored at 4ºC for 1h. A solution of 900µL FBS with 20% (v/v) DMSO was prepared and refrigerated at 4ºC and then was added dropwise to the cell suspension, stirred gently. Cryovials were stored at –80ºC in a cryobox. The following day the cryovials were moved to liquid N_2.

Mammalian cell lysis for Western Blot: *Lysis buffer:* 50mM Tris-Base, 150mM NaCl, 5mM EDTA, 1mM NaOVa (Sigma), 10mM NaF, 10mM Sodium Pyrophosphate (Sigma), 1% NP-40, 10µg/ml Aprotinin (Sigma), 1µg/ml Pepstatin (Sigma), 10µg/ml Leupeptin (Sigma). Stored at 4ºC.

A minimum of $5*10^6$ cells was pelleted, the supernatant discarded, 1µL of AEBSF 100mM (Sigma) protease inhibitor was added and the pellet was ressuspend in a 70-100µL of Lysis Buffer, according to the number of cells and pellet size. A centrifugation at 13000rpm, 10min, 4ºC was performed and the supernatant (protein extract) was transferred to a new microtube and Stored at -20ºC.

Protein quantification: a sample of the protein extract was added into a 20% solution of Bradford Reagent (Bio Rad) performing a 1:500 (v/v) dilution of sample. The quantification was assessed in a GeneQuant*pro* (Amersham Biosciences) spectrophotometer at 595nm wavelength.

SDS-PAGE electrophoresis: *12% acrylamide resolving gel at (per gel):* 5mL Resolving buffer (375mM Tris base, pH=8.8), 3.85mL of ddH_2O, 6mL Acrylamide (National Diagnostics), 33.5µL APS 20%, 150µL SDS 10%, 15µL TMED (Bio Rad). *Stacking gel (per gel):* 1.3mL Stacking buffer (125mM Tris Base, pH=6.8), 5mL ddH_2O, 0.75mL Acrylamide, 22.5µL APS 20%, 13µL TEMED. *Running buffer (5L, 1x):* 30.25g Tris base, 144g glycine (Sigma), 5g SDS, H_2O to 5L.

Lysis Buffer was added to the volume of protein extract correspondent to 50µg of protein. to to uniformize the volume of the samples. 3x Sample Buffer (Bio Rad) was added to the samples for a final concentration of 1x and incubated at 90ºC,10min, for denaturation. After cooling on ice, the samples were loaded in the gel. To equilibrate the gel the same volume of Sample Buffer was added to empty wells. The gel ran in Running Buffer, at 90V during the Stacking half and at 100V during the Resolving half.

Western Blot transference, probing, development and stripping: *Transfer buffer basic (8L, 1x):* 30g Tris base, 144g glycine, filled with H_2O until 8L. *Transfer buffer for use (1L):* 800mL Transfer Buffer basic, 200mL methanol. *TBS (5L, 10x):* 440g NaCl, 60g Tris base, pH=7.6, filled with H_2O to 5L. *TBSt 1% (1L):* 1L TBS 1x, 1000µL Tween 20. *Blocking solution for Western Blot:* TBSt added with 3% (w/v) of skimmed milk.

Transference: the polyacrylamide gel was assembled next to a nitrocellulose membrane, in Transfer Buffer, so that when exposed to electrical current (100V, 1h30min) the proteins would pass from the gel to the membrane. The Western Blot membranes were always washed in TBSt with permanent agitation. *Primary antibody probing:* depending on the antibody, different dilutions were done in TBSt supplemented with 1:100 (v/v) of NaN_3 2% (w/v) solution, then incubated over-night at 4ºC with slow agitation, followed by three washes 15+5+5min. *Secondary antibody probing:* HRP conjugated antibody, directed against the

species of the primary antibody, was diluted 1:5000 (v/v) in TBSt 3% milk, then incubated at room temperature, 1h with slow agitation. Three washes of 15+5+5min were made. *Development:* the membranes were incubated in a mix of 500µL of two Pierce ECL Western Blotting Substrate (Thermo Scientific) solutions and after 3min were developed, by . *Stripping:* the membranes were incubated in Stripping Buffer (15 mM Tris base, 100 mM 2-β-Mercaptoethanol (Sigma), pH=6.7) and supplemented with 1:1000 (v/v) dilution of 2-β-Mercaptoethanol, followed by Incubation for 45min, at 56ºC with agitation.

Lentivirus production: 293T cells passed frequently (2-3x a week) and of early passage (max. P20) were used. All supernatants were collected and filtered by 0.45µm filters, frozen in liquid N_2 and stored at -80ºC. The DMEM-10 and PBS were always used pre-warmed at 37ºC. *Day 1:* Petri plates were covered with 3mL PDL 10µg/mL. for 20min. Per plate, $2*10^6$ 293T were seeded in 10mL of D10. *Day 2:* 1h before transfection the medium was changed to 10mL of D10 supplemented with 25µM of chloroquine. *Transfection per plate:* 5.1µg #304 vector, 3.1µg pMDLg, 1.4µg pRSV.Rev, 2.0µg pMD2.VSVg were mixed in a 450µL solution of $CaCl_2$ 0.25M . The DNA mix was added dropwise, to 450µL of HBSP 2x (1.5mM Na_2HPO_4 (Sigma),10mM KCl (Sigma), 280mM NaCl, 12mM glucose, 50mM HEPES, adjusted to pH=6.95). After 5min rest at room temperature, 900µL of the DNA precipitate was added dropwise to each plate. *Day 3:* in the morning the dishes were washed with PBS. From this point on, all fluids and tips were discarded to a Virkon solution (Antec International). To each plate, 6mL of D10 supplemented with 20mM HEPES (Sigma) and 10mM Sodium Butyrate was added. 8h after the 1st supernatant and a titration aliquot were collected and D10 + HEPES medium was added. *Day 4:* in the morning the 2nd supernatant and a titration aliquot were collected and D10 + HEPES medium was added. *Day 5*: the 3rd viral aliquot and the titration aliquot were collected. The plated were washed and the cells ressuspended in PBS for acquisition in a Flow Cytometer to determine the transfection efficiency (GFP+ cells).

TAIL7 lentiviral transduction: The infection was done using a MOI between 10 to 25 in 24-well plates. *Day 1* The viruses were concentrated in an Amicon Column (Millipore) following the manufacturers' instructions. Half the volume of concentrated supernatant was frozen in N_2 liquid for the next day. The plates were covered with 250 µL of Retronectin (Takara) at 20µg/mL and left to rest 2h at room temperature. The wells were washed with PBS and 500µL of TAIL7 cells in R5 supplemented with 40ng/mL of IL7 were seeded together with 500µL of viral supernatant, per well. *Day 2:* 700µL of medium were carefully removed from the wells and 500µL of R5 supplemented with 40ng/mL of IL7 was mixed with 500µL of viral supernatant, per well. *Day 3:* The transduction efficiency was assessed in FACS (GFP+ cells) and the cells readied for sorting.

HPB-ALL lentiviral transduction: The infection was done using a MOI=2.5 in 24-well plates (TPP). Per well, a maximum of 500µL of concentrated viral supernatant and 500µL of

cell containing medium were mixed and supplemented with 8µg/mL of polybrene (Sigma). The cells were cultured with viruses for 48h. The viruses were washed out and HPB-ALL were cultured for 24h in R10, for recovery. The transduction efficiency was analyzed in a Flow Cytometer (GFP+ cells).

Flow Cytometry staining: All sample acquisitions were performed in a Flow Cytometer BD FACSCalibur™. The cell sorting was performed in a BD FACSAria™ *cell sorter.* Flow cytometry data analysis was made in a Mac with the FlowJo™ software. *Antibodies:* cells were washed with PBS (maximum of 10^6 cells for described volumes). Supernatant was discarded and cells ressuspended in 100µL of PBS. The antibody was added and incubated in the dark for 30min at 4ºC. Excess antibody was washed by adding 1000µL of PBS, followed by centrifugation. The cells were ressuspended in 200uL of PBS and acquired. *TMRE*: cells were collected and incubated in RPMI-1 with 100nM TMRE (Invitrogen) in the dark at 37ºC for 15min in a final volume of 200µL and then acquired. *AnnV/7AAD*: cells were washed/centrifuged in PBS, and the pellet was ressuspended in 100µL of AnnV Binding Buffer 1x (BD). 1µL of AnnV-APC (BD) and 2µL 7AAD (BD) were added per sample. The samples were incubated in the dark at room temperature, 15min. Before acquisition 100µL of AnnV Binding Buffer 1x was added and samples were acquired.

HPB-ALL survival experiments: after transduction and recovery, the cells were washed and incubated in 96-well plates with RPMI-1% FBS and with or without IL-7, in triplicates. After 48h and 72h post-incubation the cells were harvested and the adequate flow cytometry stainings were performed: TMRE, AnnexinV-APC and 7AAD. When analyzing the data, the gating strategy was the following: gated on the whole cell population to exclude debris; subgated on the live cells; subgated on the GFP+ cells; then either subgated to distinguish TMRE-/+ cells or subgated to distinguish AnnV-7AAD-/AnnV+7AAD-/AnnV+7AAD+.

Cell Sorting: The cell sorting was performed in a BD FACSAria™ Cell Sorter. *Sample preparation:* the cells were passed through a 0.70µm cell strainer and washed with PBS.Cells were ressuspended PBS- EDTA 5mM supplemented with twice the concentration of Penicillin/Streptomycin used in the culture medium and Gentamicin at 100µg/mL. The cells were sorted to R20 medium containing the same concentration of antibiotics as in the pre-sort medium. The sorted cells were the GFP+ cells.

Inhibitors protocol: cells were cultured in 24- or 96-well plates, in conditions already described. The medium was supplemented with DAPT, DRB, TBB or DMSO (vehicle). Inhibitors were stocked at 25mM. Cell size was analyzed by Flow Cytometry, cell counts by trypan blue exclusion in a hemocytometer and proliferation by ^{3}H-TdR incorporation. Minimum tested dose of each inhibitor (1, 5 or 10µM DAPT; 12.5 or 25µM DRB/TBB) that originated at least a 10% inhibitory effect was identified for each cell line, for each functional assay, and used in combination for the assessment of cooperative effects. For primary T-ALL

cells, the TBB dose had been previously determined (4) and DAPT was tested at a single, high concentration (5□M).

^{3}H-TdR protocol: a stock of 250µL of tritiated thymidine in 6mL of RPMI was prepared. 25µL of stock solution was added per well of a 96-well plate to the cells, followed by a 16h culture with the tritiated thymidine. Plates were frozen and then Analyzed in a β liquid scintillation counter.

Statistical analysis: Graphics and statistical analysis were performed in the software GraphPad Prism 5.

RESULTS AND DISCUSSION – CHAPTER 1:

Constructing Lentiviral Vectors to Express Effector Genes Involved in PI3K/Akt Pathway

Assessing Their Role in IL7 Mediated Signaling of T-ALL

In this thesis lentiviruses were chosen, over other kinds of viruses, to transduce HPB-ALL and TAIL7 cells, because of their reported ability to transduce non-dividing or slow dividing cells such as TAIL7. The #304 lentiviral vector (kindly provided by Prof. Luigi Naldini via Prof. João Gonçalves) allows, together with other vectors of the packaging system (pMDLg, pRSV.REV, pMD2.VSVg), the construction of replication-defective lentiviruses. The #304 plasmid has another interesting feature, a bidirectional promoter system (fig S5). This system has the reporter gene GFP transcribed under the minhCMV promoter in one direction and the gene of interest transcribed under the hPGK promoter in the opposite direction, this allows a more stable and stronger expression of the gene of interest.

Cloning GSK3β.S9A into the #304 vector and assessing its expression:

The initial plan was to subclone the GSK3β.S9A gene from the pSG5.GSK3β.S9A (fig. S1) vector to the #304.Ø vector. Due to the lack of adequate restriction sites present in both pSG5 and #304 vectors and because it is known from previous experiments that it is very hard to perform blunt end ligations in the #304 vector, the subcloning strategy passed by cloning the fragment of interest in a intermediate vector, pBSKS$^-$ (fig. S2), which contains identical restriction sites to those found in the #304 vector. The steps undertaken were the following: first, the pSG5.GSK3β.S9A vector was digested with BglII, with subsequent polymerization with the Klenow Fragment in order to create a blunt extremity followed by the digestion with EcoRI. In parallel, the pBSKS$^-$ vector was digested with HindIII, and a subsequent polymerization with the Klenow Fragment was made to create a blunt extremity, followed by a final digestion with EcoRI. Next, the fragment generated from the digestion of pSG5.GSK3β.S9A was ligated into the digested pBSKS$^-$. After cloning the GSK3β.S9A gene in the pBSKS$^-$ vector, a parallel digestion of this vector and the #304.Ø vector with PstI and SalI was done and the GSK3β.S9A gene containing PstI (5')/SalI (3') extremities were ligated in the digested #304.Ø vector, therefore constructing the #304.S9A. All the digestions were performed as described in Methods but with some alterations imposed by the enzyme BglII, which was not heat-inactivated and had to be purified by the Wizard® SV Gel and PCR Clean-Up System kit, and by the use of the Klenow Fragment which was used in a reaction alone and then heat-inactivated.

Digestion of pSG5.GSK3β.S9A and pBSKS$^-$: following the digestion of both vectors, all the products were loaded in a 1% agarose gel and an electrophoresis was performed. The results obtained are displayed in the Figure 3.

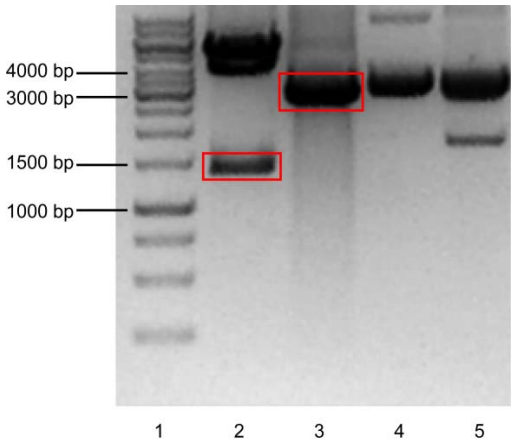

Figure 3 – Parallel digestion of pSG5.GSK3β.S9A and pBSKS$^-$ vectors evidenced in a 1% agarose gel electrophoresis. Lane 1 – Molecular weight marker. Lane 2 – pSG5.GSK3β.S9A after digestion with BglII and EcoRI, with GSK3β.S9A marked within the red rectangle. Lane 3 – pBSKS$^-$ after digeston with HindIII and EcoRI, with the linearized vector marked within the red rectangle. Lane 4 – uncut pSG5.GSK3β.S9A. Lane 5 – uncut pBSKS$^-$.

The pSG5.GSK3β.S9A plasmid had ~5.4Kb of size. After the digestion with both enzymes, two fragments were expected: one containing the GSK3β.S9A (~1.3Kb) and the remaining vector (~4.1Kb). Lane 2 of fig. 3 shows the expected fragments. The pBSKS$^-$ plasmid had ~3.0Kb of size. Two fragments were expected after the digestion: one with 12bp of very fast migration that could not be detected in the gel, and another with ~3.0Kb of size. Lane 3 of fig. 3 shows the linearized vector. Given the positive result obtained, the fragments marked within the red rectangle in fig. 3 (GSK3β.S9A and pBSKS$^-$) were excised from the gel, purified, ligated and transformed in JM109 *E. coli* as described in the Methods. The transformed bacteria were grown and selected in Petri dishes prepared for colorimetric (lacZ based) and ampicillin selection. Using this strategy, the white colonies were supposed to be the positive transformants, because the ligation in the MCS of this vector disrupts the lacZ gene, thereby keeping the colonies white in the presence of X-Gal. In the next day, ten white colonies were grown for minipreps. After DNA extraction, a digestion of all the colonies plus the #304-Ø vector was performed with the enzymes PstI e SalI. The results obtained are shown in Figure 4.

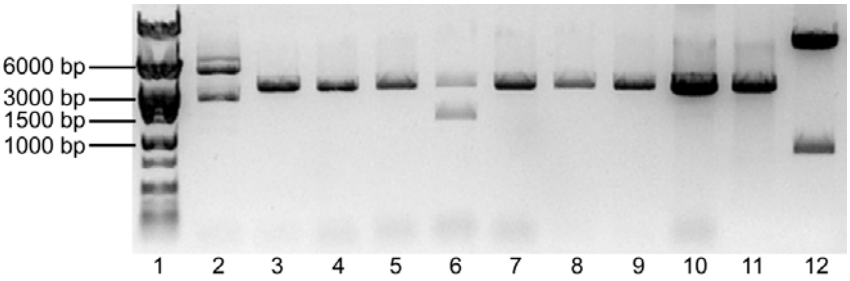

Figure 4 – Digestion of DNA from the selected colonies transformed with the pBSKS$^-$.GSK3β.S9A ligation evidenced in a 1% Agarose gel electrophoresis. Lane 1 – Molecular weight marker. Lanes 2 to 10 – Selected colonies digested with PstI and SalI. Lane 11 – pBSKS$^-$ digested with PstI and SalI. Lane 12 - #304.Ø digested with PstI and SalI.

If no recombination had occurred, the digestion of the colonies should yield 2 fragments: a ~3.0Kb fragment (pBSKS$^-$) and a ~1.3 Kb fragment (GSK3β.S9A). The only colony that seemed to have the expected result was on lane 6. But the GSK3β.S9A fragment did not appear to have ~1.3 Kb when compared to the molecular marker – it seemed bigger. To solve this problem, the colony from lane 6 was digested with BamHI, which is expected to cut once in the MCS of pBSKS- and once inside the GSK3β.S9A sequence, theoretically resulting in two fragments with ~3.7 Kb and 0.6 Kb, if no recombination had occurred. Figure 5 shows this result.

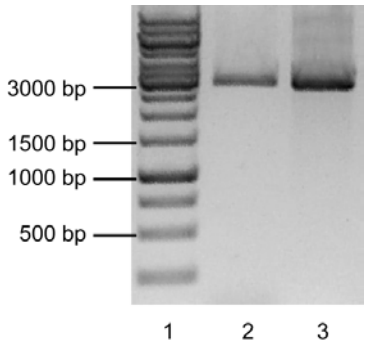

Figure 5 – Digestion of colony 6 with BamHI restriction. Lane 1 – Molecular weight marker. Lane 2 – Colony 6 digested with BamHI. Lane 3 – Uncut colony 6.

The digestion of colony 6 with BamHI did not give the 2 expected bands in the gel (fig. 5), meaning that the ligation did not work as expected. Since none of the 10 selected white colonies had the expected vector, another method had to be planned to perform the subcloning of GSK3β.S9A in the #304.

The alternative method thought was performing a PCR of pSG5.GSK3β.S9A with primers containing restriction sites for SphI (5') and SalI (3'), GSK Fwd and GSK Rev, respectively, to clone in the TOPO vector (fig. S3). This vector was digested with the selected restriction enzymes and ligated in the #304 vector. The PCR reaction of pSG5.GSK3β.S9A was made

as described in the Methods, using 61ºC for annealing temperature and an extension time of 2'30".

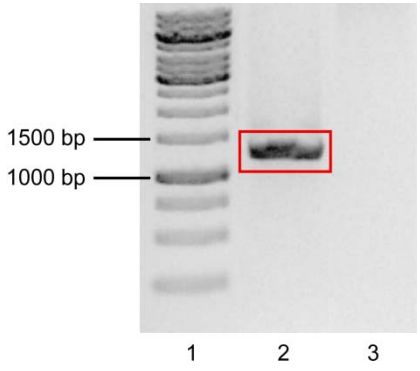

Figure 6 – Agarose gel at 1% showing the PCR reaction of pSG5.GSK3β.S9A. Lane 1 – Molecular weight marker. Lane 2 – PCR reaction of pSG5.GSK3β.S9A with selected primers. The fragment within the red rectangle was purified. Lane 3 – PCR reaction without template.

The Figure 6 shows the PCR of pSG5.GSK3β.S9A, which was highly specific as intended and produced just one band with the expected size of ~1.3Kb. This fragment was excised, purified and ligated in the TOPO vector, following the manufacturer's instructions, and transformed into *E. coli* bacteria grown in Petri dishes prepared for colorimetric (lacZ based) and kanamycin selection. Five white colonies were selected and grown for minipreps, following a digestion with SphI and SalI restriction enzymes. Figure 7 shows the result of the digestion.

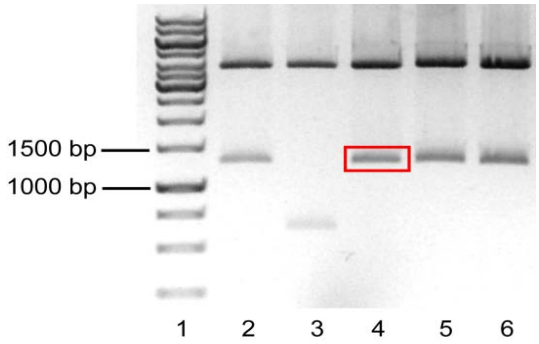

Figure 7 - Digestion of the ligation of the GSK3β.S9A in TOPO vector evidenced in a 1% agarose gel electrophoresis.. Lane 1 – Molecular weight marker. Lanes 2-6 – Selected colonies digested with SphI and SalI. The fragment within the red rectangle was purified.

In Figure 7, lanes 2, 4, 5 and 6 seemed to have the fragments with the expected size. The fragment in lane 4 that contained GSK3β.S9A shown within the red rectangle was chosen, purified and a ligation reaction with the #304.Ø vector previously digested with SphI and SalI was performed. JM109 bacteria were transformed with the ligation reaction and spread over a Petri dish prepared for ampicillin selection. Five colonies were selected from the Petri dish to prepare minipreps. To verify if the GSK3β.S9A gene was correctly inserted in the #304 vector, a digestion with EcoRV and SalI was made. EcoRV is a 'single cutter' in #304 vector and does not cut inside de GSK3β.S9A, being the expected bands of ~2.0Kb and ~7.8Kb. Figure 8 shows the digestion of the colonies with EcoRV and SalI.

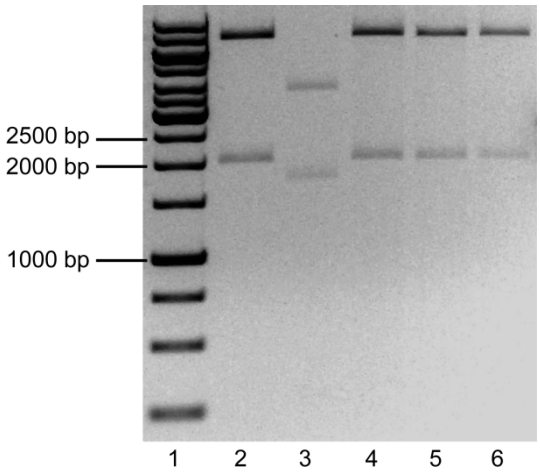

Figure 8 - Digestion of the ligation of the GSK3β.S9A in #304.Ø vector evidenced in a 1% agarose gel electrophoresis.. Lane 1 – Molecular weight marker. Lanes 2-6 – Selected colonies digested with EcoRV and SalI.

All the colonies had the expected bands, except for the colony of lane 3 in Fig 8. This suggests that the #304.GSK3β.S9A vector was constructed. One positive colony was selected to be used for viral production. In addition, the DNA extracted from the miniprep was sequenced, and 293T cells were transfected to determined if the protein was correctly expressed. The 293T cells were transfected using Fugene 6 (Roche) according to the manufacturer's instructions. One day post transfection, cells were lysed for protein extraction, resolved by 12% SDS-PAGE, and analysed for the expression of GSK3β by Western Blot. Figure 9 shows the Western Blot probed for Phospho-GSK3β, GSK3β, Actin and GFP.

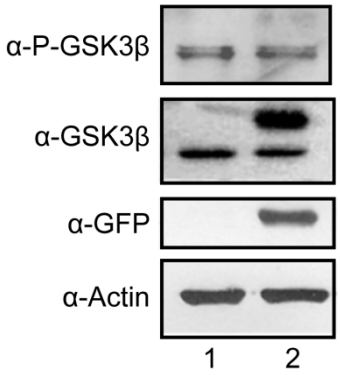

Figure 9 – Expression of GSK3b in 293T cells transfected with #304.GSK3β.S9A. Protein was detected by Western Blot analysis using antibodies against P-GSK3β, GSK3β, Actin and GFP. Lane 1 – Untransfected cells. Lane 2 – Fugene 6 transfected cells.

It was expected from the probing in this Blot that GFP protein was only detected in lane 2, an equal expression of Phospho-GSK3β in both lanes and an increased expression of total GSK3β in lane 2. All was according to the expected, except for the probing of total GSK3β. Lane 1 had only one band, corresponding to the endogenous GSK3β protein. However, lane 2 showed two bands probing for GSK3β, which was puzzling. Could it be that the S9A mutation induced a shift in the Blot? This was unlikely. GSK3β has 46-47 kDa of molecular weight, corresponding to the lower band apparent in both lanes. The upper band was positioned at ~50 kDa, but one aminoacid change was very unlikely to cause such a great

shift in migration. Plus, the polyacrylamide gel was ran under denaturing conditions, so only the molecular weight of the peptide should influence migration, not the nature or structure of its aminoacids. So, the most likely explanation was that a mutation (in-frame insertion) occurred while the vector amplified in the bacteria. However, the sequencing results initially did not shed light on the problem. Sequencing data was checked against the coding sequence retrieved from the PubMed website with the reference NM_002093.2 in September 2008. The analysis showed that the sequence of the GSK3β.S9A in the #304.GSK3β.S9A vector had the expected S9A mutation, but also a supposed in-frame deletion of 39 nucleotides (nt911-nt949) that corresponded to 13 aminoacids. Paradoxically, instead of an insertion of nucleotides that could result in the observed increase of molecular weight of the protein, there seemed to be a deletion.

To try and find a proper explanation for this discrepancy, a more thorough search was conducted in the PubMed website and the NM_002093.2 reference was checked in more detail. It was surprising to see that this reference had meanwhile been updated in March 2009 and it redirected to the new reference NM_002093.3. This new reference indicated that the sequence retrieved belonged to the mRNA transcript variant 1, giving rise to GSK3β1. The other transcript variant (GSK3β2) was described as being 13 aminoacids shorter. After comparing the sequence of GSK3β2 with that of #304.GSK3β.S9A, the problem was partially solved: the cDNA used to construct the GSK3β.S9A mutant was retrieved from cells that expressed the transcript variant 2 (GSK3β2). However, this still did not explain how an isoform with less aminoacids could have a bigger molecular weight. We tried to look for an answer by carefully investigating the literature. Two articles were found describing GSK3β isoforms. The article by Mukai et al. [33] described the finding of the alternatively spliced exon (8b) containing the already mentioned 13 aminoacids. In this article, it was shown that the molecular weight of the 8b- isoform was clearly below 50 kDa. The article by Schaffer and colleagues [32], also reported on exon 8b. Importantly, the authors further described the alternative splicing of exon 10 (nt1135-1234) of GSK3β, which corresponds to 33 aminoacids. Although the work did not state the molecular weight of the GSK3β isoform that contained the exon 10, Western Blot data in the article, showed clearly a higher molecular weight isoform, which contained exon 10. This isoform displayed a shift similar to the upper GSK3β band observed in Fig. 9. The puzzle was solved. It appears that 293T cells display an isoform of GSK3β that does not contain exon 10, whereas the cloned GSK3β.S9A gene includes exon 10 while lacking exon 8b. Isoforms containing exon 10 were found in all tissues analyzed by Schaffer et al (including lung, heart, liver, brain, etc). In conclusion, the GSK3β.S9A mutant cloned in the #304 vector was cloned from a functional GSK3β isoform and can be further used in experiments.

Cloning FOXO3a.A3 into the #304 vector and assessing its expression:

The plan was to subclone the FOXO3a.A3 gene from the pECE.HA-FOXO3a.A3 (fig S4) vector to the #304.Ø vector. Similarly to the GSK3β cloning, the lack of adequate restriction sites in pECE, #304 or other intermediate vector and the difficulty of performing blunt-end ligations in the #304 vector, made us decide to delineate primers for PCR cloning with SphI (5') and SalI (3') restriction sites, HA Fwd and FOXO Rev, respectively, to ligate HA-FOXO3a.A3 in the TOPO vector. From the TOPO vector with the cloned FOXO3a.A3, digestions with SphI and SalI were performed and the FOXO3a.A3 mutant was ligated the #304 vector, thus cloning HA-FOXO3a.A3 into the #304 vector.

The PCR reaction of pECE.HA-FOXO3a.A3 was made as described in the Methods, using 61ºC for annealing temperature and an extension time of 2'30". Figure 10, shows the result of this PCR.

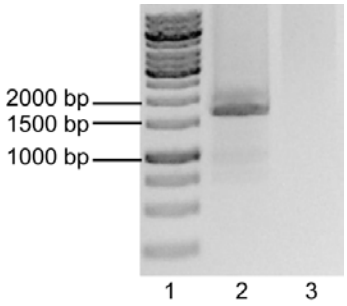

Figure 10 – Agarose gel at 1% showing the PCR reaction of pECE.HA-FOXO3a.A3. Lane 1 – Molecular weight marker. Lane 2 – PCR reaction of pECE.HA-FOXO3a.A3 with selected primers. Lane 3 – PCR reaction without template.

The expected size of the amplified fragment was ~2.1Kb. Lane 2 of Fig. 10 shows the amplification of a band with less than 2.0Kb size, which was not the band expected. This may have happened for two reasons: an annealing temperature too low may have directed the primers for unspecific annealing sites or the extension time may not have been enough. The processivity of the *Pfu* polymerase is ~1000bp.min^{-1}. An extension time of 2'30" was expected to amplify up to 2.5Kb fragments, but maybe optimal conditions were not achieved. We tried to surpass these two possible problems by repeating the PCR with an annealing temperature of 63ºC and an extension time of 3'30". Figure 11 shows the result of this PCR.

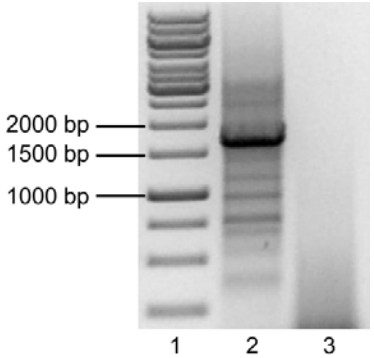

Figure 11 – Agarose gel at 1% showing the PCR reaction of pECE.HA-FOXO3a.A3. Lane 1 – Molecular weight marker. Lane 2 – PCR reaction of pECE.HA-FOXO3a.A3 with increased annealing temperature and extension time. Lane 3 – PCR reaction without template.

However, the increased annealing temperature and extension time did not solve the problem, as shown in Lane 2 of Figure 11. The unspecific band displayed in Fig. 10 continued to be amplified. Another possible solution came from the fact that in the pECE vector there might have been very similar sequences to the template of one of the primers that caused unspecific amplification. Therefore, the new strategy was to cut the vector with a restriction enzyme that excised a fragment containing the HA-FOXO3a.A3 gene, ran the digestion in a gel to have the fragment purified and then made a PCR reaction from this fragment avoiding the unspecific annealing. The selected restriction enzyme was SphI. From its digestion, a fragment with ~2.7Kb of size containing the HA-FOXO3a.A3 gene and a little part of the pECE was obtained and purified. A PCR reaction in the same conditions as the previous one was performed.

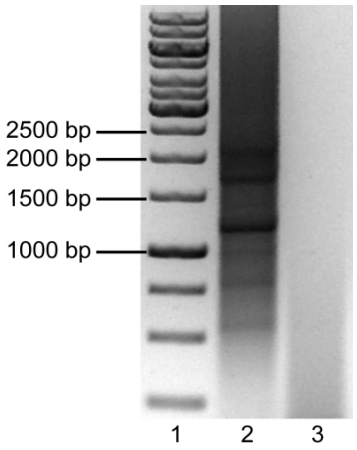

Figure 12 – Agarose gel at 1% showing the PCR reaction of a fragment excised from the pECE vector containing the HA-FOXO3a.A3 gene. Lane 1 – Molecular weight marker. Lane 2 – PCR reaction of the fragment containing HA-FOXO3a.A3. Lane 3 – PCR reaction without template.

Figure 12 shows the result of the PCR reaction. In Lane 2, rather than the expected specific band, a smear with a few stronger bands could be seen. Another problem may have been the primers used, particularly the forward primer which contained the HA-tag sequence. More than one nucleotide sequence could have originated from the same aminoacid sequence. So, when the HA Fwd primer was firstly designed, an Internet search in several companies' websites was made to find the most common nucleotide sequence for HA-tag, since the sequence present in the pECE vector was not available. Possibly, the HA sequence in the HA Fwd primer was not the correct one. Then, a new primer with another very common HA-tag sequence was ordered (HA new). The PCR reaction with the new primer was made with an annealing temperature of 62ºC and an extension time of 3'30". Unfortunately, the PCR continued to amplify a band with the size seen in Fig 11. After this unsuccess, the HA-tag was discarded and a new forward primer was designed to amplify only the FOXO3a.A3 gene.

A new PCR reaction was set with an annealing temperature of 61ºC and an extension time of 3'30". Figure 13 shows the result of this PCR.

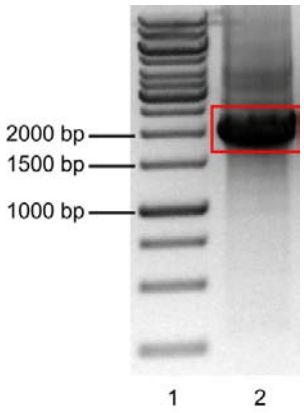

Figure 13 – Agarose gel at 1% showing the PCR reaction of pECE.HA-FOXO3a.A3 vector with FOXO Fwd and Rev primers. Lane 1 – Molecular weight marker. Lane 2 – PCR reaction showing the expected band within the red rectangle.

Lane 2 of Fig 13 shows that finally the PCR amplified the ~2.1Kb expected band, seen within the red rectangle. This band was then excised from the gel, purified, ligated in the TOPO vector and DH5α *E. coli* were transformed and grown in Petri dishes prepared for colorimetric (lacZ based) and kanamycin selection. However, no colonies of bacteria could be grown in the plates, which suggested that the ligation in the TOPO vector was not occurring even after several attempts.

After an extensive search for possible solutions to clone FOXO3a.A3, a new cloning kit was found: the pGC.Blue cloning kit from Lucigen Corporation. This kit works based in CG cloning as opposed to TOPO that works based in TA cloning. The GC cloning was the reason to choose this kit over other kits similar to TOPO, because generally a GC interaction is stronger than a TA interaction and also because FOXO3a.A3 was a GC rich gene (>60% GC content) which could have affected the TA cloning. After the ligation reaction with this new kit was performed, 10G *E. coli* were transformed and grown in Petri dishes prepared for colorimetric (lacZ based) and kanamycin selection. Seven white colonies plus one blue colony (negative control) were picked for minipreps. The minipreps DNA were extracted and a digestion with SphI and SalI was performed. Figure 14 shows the result of the digestion.

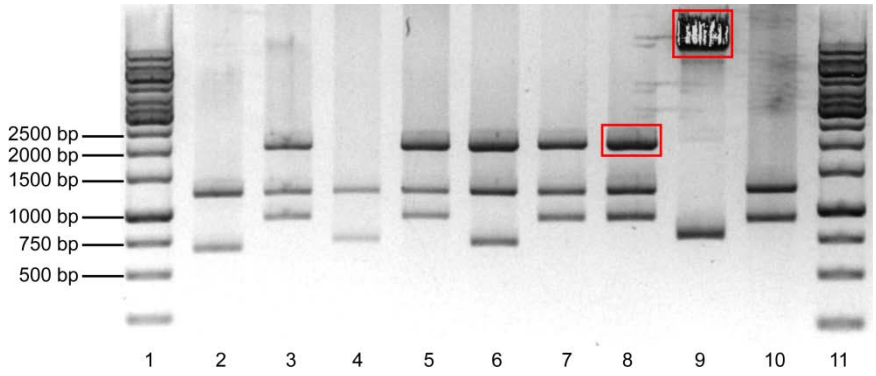

Figure 14 - Agarose gel at 1% showing the digestion of the ligation of the FOXO3a.A3 in pGC.Blue vector. Lanes 1 and 11 – Molecular weight marker. Lanes 2-8 – Selected white colonies digested with SphI and SalI. The fragment within the red rectangle was purified Lane 9 - #304.Ø digested with SphI and SalI. The fragment within

the red rectangle was purified. Lane 10 – Digestion of a blue colony with SphI and SalI as a negative cloning control.

In theory, a digestion of the pGC.Blue vector with the FOXO3a.A3 gene cloned in it with SphI and SalI restriction enzymes should have resulted in 4 fragments: 48bp (not visible), ~0.9Kb and ~1.2Kb that belonged to the vector and ~2.1Kb, the FOXO3a.A3 gene. In fig 14 it can be seen that lanes 3, 5, 7 and 8 had the expected restriction pattern, this showed that FOXO3a.A3 was finally cloned in an intermediate vector. The fragments highlighted by the red rectangles, FOXO3a.A3 and digested #304, were purified and a ligation reaction performed as described in the Methods. JM109 *E. coli* were transformed as described and grown in Petri dishes prepared for ampicillin selection. In this first attempt no colonies grew in the plate. This could have been because the insert-to-vector ratio was not optimized for this vector, although this ligation followed the 3:1 insert-to-vector ratio based on size. Therefore, a new ratio of 1:1 (insert:vector) based on the weight of the DNA was attempted. Again, no colonies formed in the Petri dish.

Looking at the troubleshooting of the datasheet from the reagents (Fermentas) and bacteria (Stratagene) used in the ligation reaction, an experiment for optimization of ligation and transformation was devised (Table 1), where all the other settings were kept as described in Methods:

A	**2H LIGATION**	**+2ML PEG, +0.8ML B-ME**
B		+2µL PEG, - β-ME

Table 1 **-** Testing of two different conditions (A and B) to optimize the ligation of FOXO3a.A3 in #304. The variables changed are time of ligation reaction, addition of PEG and addition of beta-ME to the bacteria prior to transformation.

In sticky-end ligations with the presence of 2µL PEG 4000 at 50%, the maximum recommended duration of the reaction was 2 hours. For the transformation of bacteria 0.8µL of β-ME at 1,4M was added/not added to the thawed aliquot on ice, followed by a resting of 10min and the addition of the DNA.

After performing these two different conditions, bacteria were grown in Petri dishes prepared for ampicillin selection. Colonies were selected for minipreps and DNA extraction, followed by a digestion with SphI and SalI. Figure 15 shows the result of the digestion.

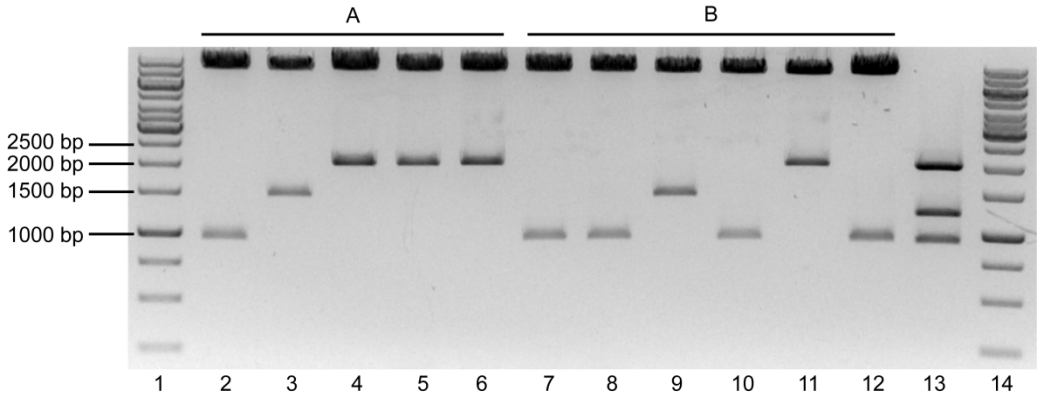

Figure 15 - Agarose gel at 1% showing the digestion of the ligation of the FOXO3a.A3 in #304 vector. Lanes 1 and 14 – Molecular weight marker. Lanes 2-6 – Digestion with SphI and SalI of selected colonies from condition A. Lanes 7-12 - Digestion with SphI and SalI of selected colonies from condition B. Lane 13 – Digestion of pGC.Blue.FOXO3a.A3 with SphI and SalI as a FOXO3a.A3 positive control.

Four colonies had the expected fragments of ~2.1Kb and ~8.6Kb for FOXO3a.A3 and #304.Ø, respectively: Lanes 4, 5, 6 and 11. This suggested that the #304.FOXO3a.A3 vector was finally constructed. The colony corresponding to lane 4 was selected for viral production. The DNA extracted from the maxiprep was sequenced and 293T cells were transfected in order to determine if the protein was correctly expressed.

The 293T cells were transfected using Fugene 6 according to the manufacturer's instructions. One day post transfection, cells were lysed for protein extraction and resolved by 12% SDS-PAGE, and analysed by Western Blot. Figure 16 shows the expression of Phospho-FOXO3a, FOXO3a and GFP.

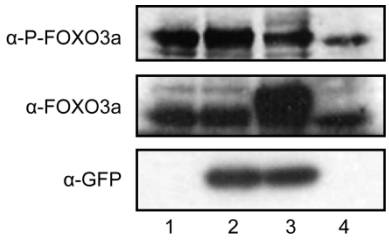

Figure 16 – Western Blot of a 12% polyacrylamide gel showing the expression of P-FOXO3a (Upstate), FOXO3a (Upstate) and GFP. Lane 1 – Untransfected 293T cells. Lane 2 – Fugene 6 transfected 293T cells with #304.Ø. Lane 3 – Fugene 6 transfected 293T cells with #304.FOXO3a.A3. Lane 4 – Wild type HPB cells

In Lane 3 the over expression of FOXO3a.A3 was clearly seen, showing that the FOXO3a.A3 gene was correctly expressed. Moreover, the integrity of the FOXO3a.A3 gene was confirmed by the sequencing results. In conclusion, the #304.FOXO3a.A3 vector can be further used in experiments.

Production of lentiviruses and transduction of HPB-ALL and TAIL7 cells.

In order to start fulfilling the main objective of this chapter, lentiviruses had to be produced in high titers. The cell line 293T was used as a packaging cell line for lentivirus production,

calcium chloride transfection of all the necessary plasmids, as described in the Methods. Viruses were then harvested and stored for later use. After the last harvest, cells were washed and analyzed by Flow Cytometry to assess the efficiency of transfection. The supplementary Figure S6, shows a typical result of the transfection efficiency, higher than 90%. During the traineeship, many viral productions were made. The titers varied usually in the range of $1*10^5$ to $9*10^5$ viral particles/mL. After concentration, the viral titer increased, in average, 1 to 1.5 log. For a transduction experiment, two or three viral productions were used.

Effects of GSK3β.S9A and FOXO3a.A3 in IL7-mediated effects in T-ALL cells:

HPB-ALL transductions were performed as described in the Methods section. The yield of these transductions was disappointing, with percentages of GFP+ cells ranging from 5-12%. After the transduction, HPB-ALL cells were incubated to assess survival. Cells were stained for flow cytometry with AnnexinV-APC and 7AAD, which allow for a sensitive determination of viability of lymphocytes, including T-ALL cells. It should be noted that the data were acquired by first gating in the “live” cell population as determined by FSC x SSC distribution, and subsequent subgating in the GFP+ cells. This implicates that we analyzed the effects on viability of cells that were still expressing GFP (excluding all those that may have lost GFP expression as a consequence of the cell death process). The reason for this approach was that we did not want to bias our analysis by including non-transduced cells. On the other hand, this implicates that we could only compare viable cells versus early apoptotic cells, and that most of the cells included in the gate were *a priori* mostly viable. Nonetheless, we found that the presence of constitutively active FOXO3a resulted in a general lowering in viability, both at 48h (data not shown) and 72h (Figure 17). At 48h IL-7 boosted the viability of empty transduced cells and could partially rescue the viability of the FOXO3a.A3 transduced cells (not shown). However, at 72h IL-7 was no longer able to rescue the viability of FOXO3a.A3 transduced cells, while it still was able to rescue the viability of empty transduced cells (Fig 17).

The strategy we used resulted in a great loss of information about the late apoptotic/necrotic cells. Therefore, the next step taken will be to sort the GFP+ cells and then perform this same experiment using the different constructs (#304-Empty, #304-GSK3β.S9A and #304-FOXO3a.A4).

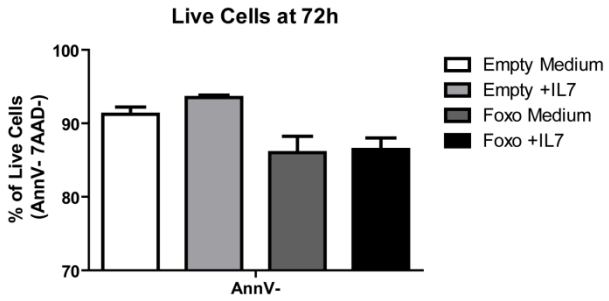

Figure 17 – Viability assay of HPB-ALL transduced with the empty vector or FOXO3a.A3 bearing vector. Cells were cultured with or without IL-7 for 48h (A) and 72h (B). Stainings for TMRE, AnnexinV-APC and 7AAD were made. For each time point graphics are shown with the proportion of live (AnnV-7AAD-), very early apoptotic (TMRE-), early apoptotic (AnnV+7AAD-) and late apoptotic (AnnV+7AAD+) cells. Error bars from 3 replicates are presented.

RESULTS AND DISCUSSION – CHAPTER 2:

Regulation of the Tumor Suppressor PTEN

The Therapeutic Potential of Combined γ-secretase and CK2 Inhibition in T-ALL

As already discussed, in T-ALL PTEN expression and activity can be downregulated by two independent processes, Notch1 and CK2 activation, respectively. Therefore, an interesting hypothesis to test was if the combined inhibition of Notch and CK2 could have an impact on the proliferation and viability of T-ALL cells. Experiments with the PTEN positive cells lines HPB-ALL, TAIL7 and TALL-1 were performed as described and cell size, cell counts and proliferation assays were made.

Incubation with the γ-secretase (i.e., Notch) inhibitor DAPT, or with the CK2 inhibitors DRB/TBB, lowered the total cell counts of cultured HPB and TAIL7 cells when compared to medium alone (Figure 18). This effect was further enhanced by the combined presence of both types of inhibitors, which suggested a cooperative effect of Notch and CK2 inhibition as potentiators of PTEN's activity. Further strengthening this result was the cell size measurement, an indirect indicator of the cells' metabolic activity that is also associated with cell cycle stage [5, 12] . HPB-ALL and TAIL7 cells were consistently smaller (thus, likely non-activated, quiescent) when cultured with one inhibitor, and even less when cultured with both inhibitors (Fig 19). Additionally, proliferation of HPB-ALL, TAIL7 and TALL1 cell lines, assessed by incorporation of ^{3}H-thymidine during DNA synthesis, was greatly diminished in the presence of the inhibitors, particularly when used in combination (fig 20). The reason for the cooperative effects of these inhibitors likely rely on the fact that they converge on the regulation of PTEN by different, and complementary, mechanisms. On one hand, γ-secretase inhibitors, by inhibiting Notch, increase the protein level of PTEN via transcriptional upregulation; on the other hand, inhibition of CK2 prevents the CK2-mediated posttranslational inactivation of PTEN, by making the PTEN less stable but more active. The use of both inhibitors has the potential to more effeciently block the PI3K/Akt pathway (Fig 1). Altogether, our results suggest that the combined administration of γ-secretase inhibitors and CK2 inhibitors might have therapeutic potential in T-ALL.

These data, along with other results, were accepted for publication in Haematologica as a manuscript entitled: "Regulation of PTEN by CK2 and Notch1 in primary T-cell acute lymphoblastic leukemia: rationale for combined use of CK2- and gamma-secretase inhibitors"..

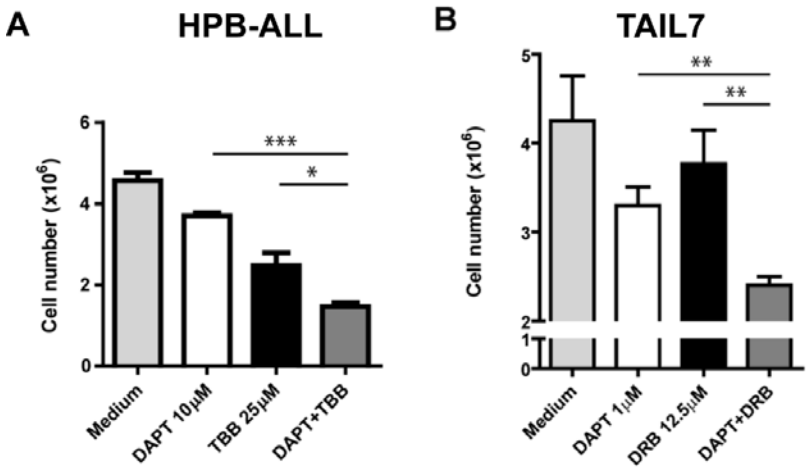

Figure 18 – The γ-secretase inhibitor DAPT and CK2 inhibitors DRB/TBB act cooperatively to decrease the total cell number of T-ALL cells. Cells were counted in triplicates after 4 days (A – HPB-ALL) or 7 days (B – TAIL7) of culture. Viable number of cells was assessed in a hemocytometer by trypan blue exclusion. * p<0.05, ** p <0.01, *** p <0.001.

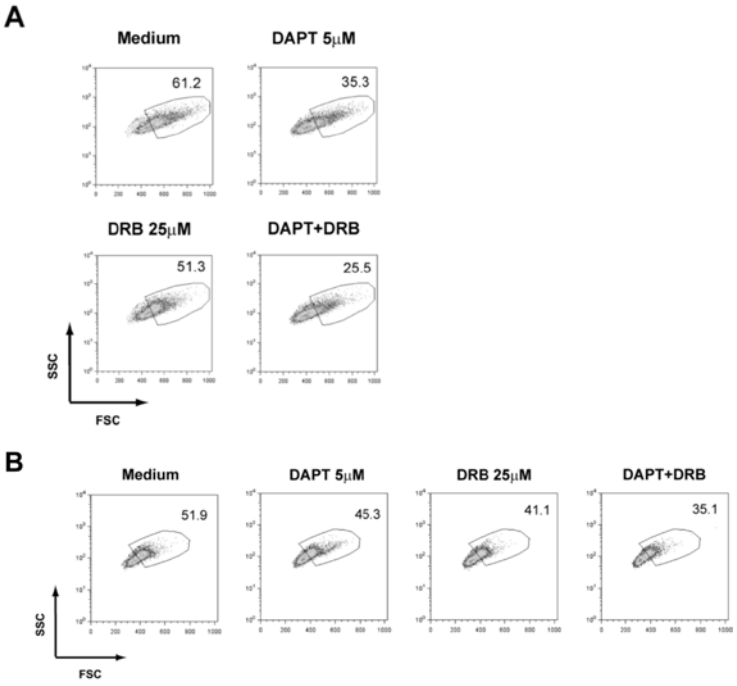

Figure 19 – Upon treatment, the γ-secretase inhibitor DAPT and CK2 inhibitors DRB or TBB act synergistically to decrease the cell size. Cell size was assessed by Flow Cytometry in triplicates after 72h of culture. A – HPB-ALL cells; B – TAIL7 cells.

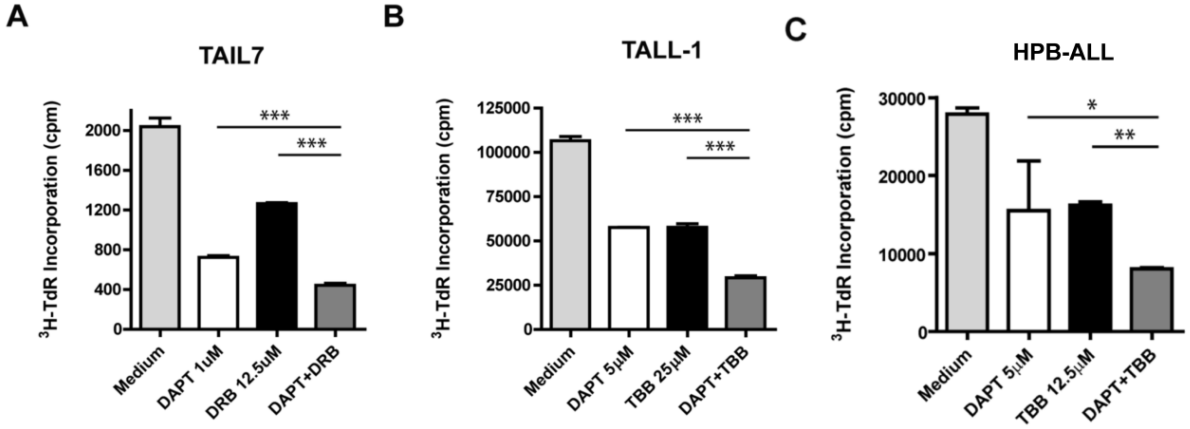

Figure 20 – Proliferation is reduced when cells are treated with the γ-secretase inhibitor DAPT and CK2 inhibitors DRB or TBB. Cells were cultured for 7 days in triplicates. 16h prior to analysis cells are incubated with ^{3}H-Thymidine. A – TAIL7 cells; B – TALL-1; C – HPB-ALL cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

CONCLUSIONS

This thesis aimed to explore two complementary issues important for leukemia progression: the signals from the miroenvironment, here represented by IL-7, and the molecular aberrations occurring within the cancer cell, here represented by CK2 and Notch, both of which converge upon PI3K/Akt pathway. In the first case, we planned to evaluate the role of GSK3 and FOXO3a on IL-7-mediated effects in T-ALL. In the second case, we planned to determine the therapeutic potential of simultaneously inhibiting Notch and CK2 activity in T-ALL cells.

The objectives of the first chapter of the thesis were only partially achieved. Despite the many obstacles, the FOXO3a.A3 and GSK3β.S9A mutants were subcloned into the #304 lentiviral vector. Their expression, assayed in 293T cells, was correct and they were not phosphorylated. The preliminary data obtained in the functional experiments with HPB-ALL was highly suggestive that at least FOXO3a inhibition was mandatory for IL-7 mediated survival and proliferation of T-ALL cells lines.

The objectives of the second chapter of the thesis were totally achieved. We demonstrated that the use of γ-secretase inhibitors combined with CK2 inhibitors, lead to a cooperative negative effect on T-ALL cell number, cell size and proliferation.

In conclusion, the work here developed has in part contributed to the potential opening of new therapeutic strategies and may lead to a better characterization of PI3K/Akt pathway in the context of T-ALL.

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SUPPLEMENTARY INFORMATION

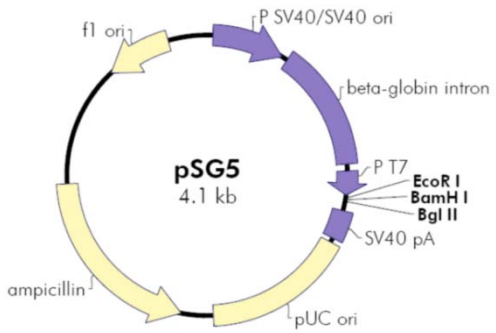

Figure S1 – Circular map of pSG5 and its characteristics. The GSK3β.S9A gene is cloned in the EcoRI/ΔSmaI-ΔMBamHI restiction sites. Adapted from Statagene's pSG5 datasheet.

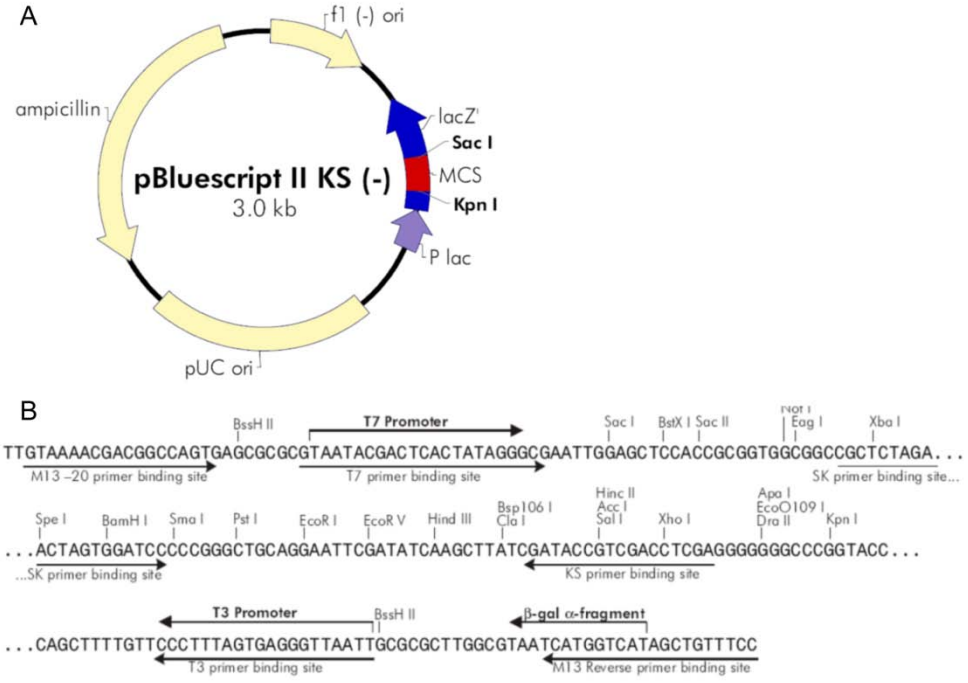

Figure S2 – **A –** pBSKS$^-$ circular map, its characteristics and **B –** MCS detail. Adapted from Stratagene's pBSKS$^-$ datasheet.

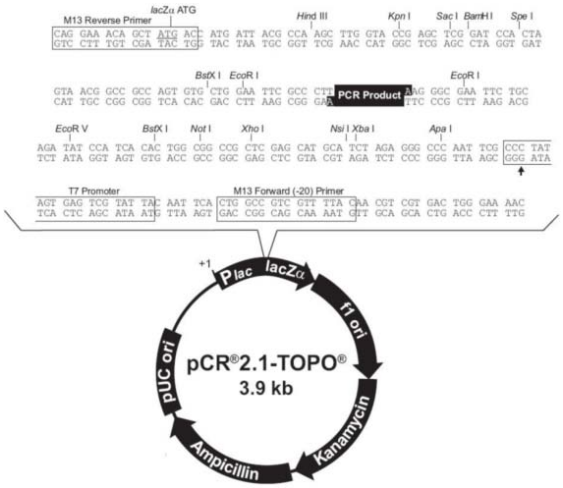

Figure S3 – Circular map of TOPO vector with its characteristics and MCS detail. Adapted from Invitrogen's datasheet.

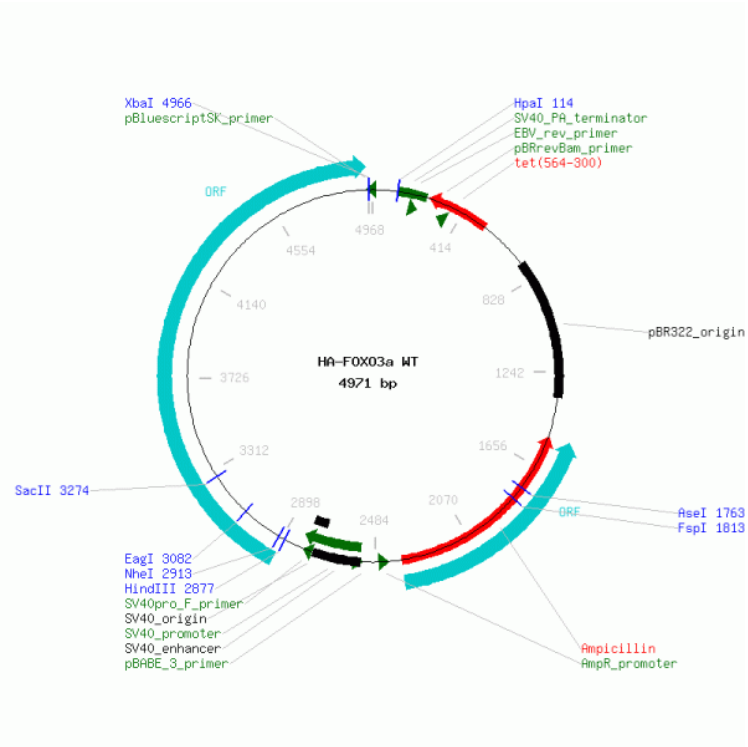

Figure S4 – Circular map of pECE. HA-FOXO3a (not FOXO3a.A3) vector with its characteristics. The bigger blue ORF represents the place where, in a equivalent way, HA-FOXO3a.A3 is cloned. Adapted from Addgene website: http://www.addgene.org.

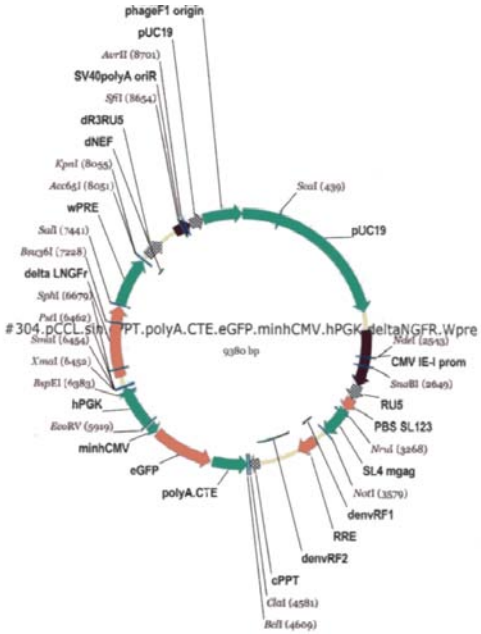

Figure S5 – Circular map of the #304.deltaLNGFr vector ant its characteristics. The #304.Ø vector was reconstructed by the excision of the delta LNGFr fragment

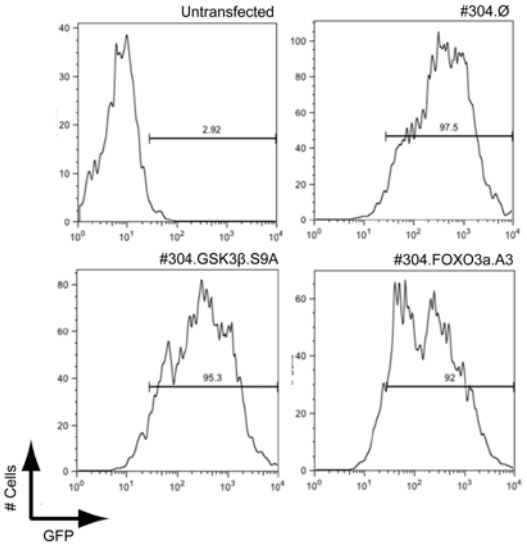

Figure S6 – Representative result of transfection efficiency of 293T cells obtained by the calcium phosphate method. Over 90% of the cells are transfected by the #304 based vectors. Assessed by flow cytometry for the presence of GFP.