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FACULDADE DE MEDICINA

Dissecting the Cellular and Molecular Mechanisms of IL-7-mediated Leukemia T- cell Survival, Proliferation and Cell Growth

Daniel Filipe Silva Ribeiro

Orientador: Prof. Doutor João Pedro Taborda Barata

Tese especialmente elaborada para a obtenção do grau de Doutor em Ciências Biomédicas - Especialidade em Biologia Celular e Molecular

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Preface

This thesis presents data obtained during the research work developed at the *Instituto de Medicina Molecular* and at the *University Medical Center Utrecht* in the period between January 2011 and May 2016 in the scope of my PhD project and under the supervision of João T. Barata, PhD.

This thesis is organized in 6 chapters, which are preceded by a summary written in Portuguese and by an abstract. Before the description of the results obtained, an introductory review of the subject is provided in chapter 1 and the aims of the work are also detailed at the end of chapter 1. In chapters 2, 3, 4 and 5 the original data obtained during this research project is presented and discussed. A general discussion, which integrates and puts into perspective all the results, is presented in chapter 6.

The data presented in this dissertation is purely the result of my own work and it is clearly acknowledged in the text whenever data or reagents produced by others were utilized. I was financially supported by a scholarship from Programa SFRH, Fundação para a Ciência e Tecnologia, Portugal. This work has not been submitted for any degree at this or any university.

The opinions expressed in this publication are from the exclusive responsibility of the author.

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Já estou há tanto tempo no lab e já por cá passou tanta gente que que é impossível nomeá-los a todos (UBCA forever :P). Mas alguns são inesquecíveis. Quero agradecer ao grupo que tão bem me recebeu quando cheguei. As noites de jogos, jantares, saídas, Andanças, concertos e o maravilhoso tempo passado com vocês no lab. Já agora também o apoio científico :) Obrigado Nádia Correia (e André), Leila Martins, Ana Gírio, Ana Silva, Bruno Cardoso e Catarina Henriques.

Como nada do que é bom dura para sempre, saem uns e entram outros. Aos que vieram depois (mais velhos ou mais novos) foi sempre espectacular trabalhar, e divertir-me, convosco. Sinto-me em casa. Obrigado Rita Fragoso, Alice Melão, Joana Silva (a nossa), Inês (da Rita), Mariana, Margarida, Vanda, Leonor, Padma Akkapedi P, Cláudia Faria (as corridas), Carlos, Teresa Serafim, Joana Matos, Isabel Alcobia, Rita Silva (da Isabel) e Inês Antunes. Não posso esquecer as minhas orientandas, a Maui, Inês Lopes e Marta Abreu. Ainda há mais… Não posso esquecer a Marta 'Martinez', a Joana Silva (da Marta) e Inês Martins. Que bons almoços nos Advogados :P

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Por fim, senão isto nunca mais acaba, agradeço a todos os escritores de ficção cientifica e fantasia, pelo escape mental da realidade que me proporcionam. Sim, estou particularmente a pensar em Game of Thrones de George R.R. Martin.

Sumário

A leucemia linfoblástica aguda de células T (LLA-T) constitui um subtipo agressivo de LLA, o cancro pediátrico mais comum. Apesar do grande sucesso obtido com regimes quimio-terapêuticos ajustados ao risco, a sua eficácia está frequentemente associada a efeitos secundários substanciais e os casos que não respondem a terapia ou que recidivam têm muito mau prognóstico. Portanto, são necessárias melhores terapias focadas na eficiência e especificidade contra as células leucémicas. Compreender a biologia e patogénese molecular que contribuem para o desenvolvimento de LLA-T é fundamental para atingir este objectivo.

A interleucina-7 (IL-7) e o seu receptor (IL-7R; heterodímero constituído pelas subunidades IL-7Rα/*IL7R* e γc/*IL2RG*) são essenciais para o desenvolvimento de células T normais, existindo igualmente evidência de que a sinalização mediada por IL-7 promove leucemia. Ratinhos que sobre-expressam IL-7 desenvolvem linfomas de células B e T, e a expressão aumentada de IL-7Rα, presente em ratinhos AKR/J, promove o desenvolvimento de tumores de células T. Adicionalmente, a IL-7 promove a expansão de LLA-T *in vivo* e sobrevivência e proliferação celular *in vitro*. Nós estudámos a existência de mutações activadoras do IL-7R em LLA-T e descobrimos que 9% dos pacientes ao diagnóstico são portadores de mutações somáticas activadoras de *IL7R*. A maioria das mutações introduz uma cisteína não-emparelhada no exão 6 que promove homodimerização de cadeias IL-7Rα, resultando em sinalização constitutiva exclusivamente dependente de Jak1. Também revelámos que as mutações em *IL7R* promovem transformação celular e formação de tumores. É importante salientar que a sinalização do IL-7R mutante, e consequente aumento da viabilidade e proliferação celulares, são significativamente limitadas por inibidores da via Jak/STAT5 (Capítulo 2).

No passado, demonstrámos que a IL-7 promove sobrevivência e proliferação de células leucémicas pela activação da via de sinalização PI3K/Akt/mTOR. No entanto, a observação de que a formação de linfomas murinos mediada por IL-7 requer STAT5 e o facto de células LLA-T com mutação no IL-7R serem sensíveis a inibidores da via Jak/STAT5, levou-nos a investigar o papel desta última via em LLA-T. Neste trabalho nós demonstrámos que STAT5 é essencial para o papel da IL-7 na viabilidade, crescimento e proliferação de células de LLA-T. Contudo, verificámos também que o efeito da IL-7 via STAT5 na sobrevivência das células leucémicas é independente da expressão de Bcl-2. Para tentar identificar o mecanismo envolvido, efectuámos análise de sequenciação de nova geração (NGS) que revelou que a cinase PIM1 é um alvo directo de STAT5 no contexto de

IL-7 e é necessário para os efeitos funcionais do eixo de sinalização IL-7-Jak/STAT5. Adicionalmente, os nossos estudos sugerem que a IL-7 diminui a expressão de *BCL6* e promove a transcrição de um transcrito alternativo (Capítulo 3).

A autofagia pode mitigar o stresse em células cancerígenas resultante, por exemplo, de proliferação mediada por oncogenes ou de quimioterapia. No entanto, quando persistente, o seu papel protector pode alterar-se para o que é designado de morte mediada por autofagia. Dado que a IL-7 promove activação de mTOR, o principal regulador negativo da autofagia, decidimos estudar se a IL-7 poderia regular autofagia em LLA-T. Os nossos estudos demonstram que a IL-7 regula autofagia em LLA-T de uma forma complexa, que envolve a activação de vias pro- (MEK/Erk) e anti- (PI3K/Akt/mTOR) autofágicas. Dependendo do contexto microambiental, a IL-7 usa uma 'estratégia flexível' para alterar a via de sinalização requerida para a sobrevivência. Num microambiente rico em nutrientes (baixa autofagia) a IL-7 inibe autofagia e a sobrevivência celular depende da activação da via PI3K/Akt/mTOR. No entanto, num microambiente pobre em nutrientes a IL-7 passa a aumentar a autofagia e a sobrevivência depende da via MEK/Erk (Capítulo 4).

A IL-7 mantém o tamanho celular e activação metabólica em células T normais. A IL-7 também promove a expressão de Glut1 e hexocinase II (HK2), ambos envolvidos em glicólise. Em LLA-T, demonstrámos previamente que a IL-7 regula o crescimento celular, uso de glucose e expressão de Glut1. Usando dados de NGS obtidos no Capítulo 2, nós aprofundámos o conhecimento relativo à regulação do metabolismo celular em LLA-T mediado por IL-7. Os nossos resultados sugerem que a IL-7 tem um impacto bastante mais generalizado na regulação de glicólise em células de LLA-T do que antecipado. A análise da expressão génica mostrou que a IL-7 promove a expressão precoce de vários genes da glicólise, incluindo os envolvidos em pontos-chave de regulação glicolítica (Capítulo 5).

Tomados em conjunto, os estudos apresentados nesta tese expandem consideravelmente o nosso conhecimento do papel do eixo de sinalização IL-7/IL-7R em LLA-T. A descoberta de mutações oncogénicas no IL-7R poderá ter importantes implicações terapêuticas em LLA-T. Adicionalmente, nós fornecemos evidências claras de que as vias Jak/STAT5/PIM1 e MEK/Erk poderão constituir novos alvos terapêuticos. Finalmente, desvendámos papéis que a IL-7 tem em importantes processos fisiológicos como autofagia e glicólise, o que não apenas aumenta o entendimento corrente da biologia da IL-7 e da leucemia T mas poderá também contribuir para a criação de novas estratégias terapêuticas em LLA-T.

Palavras-chave (5): IL-7; LLA-T; microambiente; vias de sinalização; alvos terapêuticos

Abstract

T-cell acute lymphoblastic leukemia (T-ALL) constitutes an aggressive subset of ALL, the most frequent childhood malignancy. Although risk-adjusted chemotherapeutic regimens are currently extremely effective, they frequently associate with significant long-term side effects. Moreover, cases that do not respond to therapy or that relapse have dismal prognosis. Thus, better therapies focused on efficacy and specificity against T-ALL cells are necessary. Understanding the biology and molecular pathogenesis of T-cell leukemogenesis is critical to carry out this goal.

Interleukin-7 (IL-7) and its receptor (IL-7R; heterodimer constituted by IL-7Rα/*IL7R* and γc/*IL2RG* subunits) are essential for normal T-cell development and there is considerable evidence that IL-7-mediated signaling may promote leukemogenesis. Mice overexpressing IL-7 develop B- and T-cell lymphomas and increased expression of IL-7Rα, present in AKR/J mice, promotes development of T-cell tumors. Furthermore, IL-7 promotes T-ALL expansion *in vivo* and leukemia cell survival and proliferation *in vitro*. We assessed whether activating IL-7R mutations could occur in T-ALL. We found that 9% of T-ALL patients harbor somatic gain-of-function *IL7R* mutations. The majority introduced an unpaired cysteine in exon 6 and promoted IL-7Rα homodimerization, which led to constitutive signaling that relied exclusively on Jak1. We found that *IL7R* mutations promote cell transformation and tumor formation. Importantly, mutant IL-7R signaling (and consequent increase in viability and proliferation) was targetable with Jak/STAT5 pathway inhibitors (Chapter 2).

Previously, we have shown that IL-7 promotes leukemia cell survival and proliferation *in vitro* by activating PI3K/Akt/mTOR signaling pathway. The observation that IL-7-driven murine lymphomagenesis requires STAT5 and the fact that IL-7R-mutated T-ALL are sensitive to Jak/STAT5 pathway inhibitors, led us to investigate the role of this pathway in T-ALL. Here, we showed that inhibition of STAT5 in T-ALL completely abrogates IL-7-mediated T-ALL cell viability, growth and proliferation. Importantly, we demonstrated that survival mediated by IL-7 via STAT5 was independent from expression of Bcl-2 family members. Next-generation sequencing analysis (NGS) revealed that PIM1 kinase is a direct STAT5 target in the context of IL-7 signaling and that PIM1 is required for IL-7/Jak/STAT5-mediated functional effects. In addition, we provide evidence that IL-7 downregulates the expression of *BCL6* and promotes transcription of an alternate transcript (Chapter 3).

Autophagy may mitigate stress, such as that induced by oncogene-driven proliferation or chemotherapy, in cancer cells. However, when persistent, its protective role may shift to what is called autophagic cell death. Since IL-7 promotes activation of mTOR, a master negative regulator of autophagy, we decided to explore whether IL-7 may also regulate T-ALL cell autophagy. We demonstrated that IL-7 modulates autophagy in T-ALL cells in a complex manner that involves triggering both pro- (MEK/Erk) and anti- (PI3K/Akt/mTOR) autophagic signaling pathways. Our data suggest that depending on the microenvironmental cues, IL-7 uses a 'flexible strategy' to shift the signaling pathway required for survival. In a nutrient-rich microenvironment (low autophagy) IL-7 inhibits autophagy and survival relies on PI3K/Akt/mTOR, while in nutrient-poor conditions (high autophagy) IL-7 promotes autophagy and survival relies on MEK/Erk pathway activation (Chapter 4).

IL-7 maintains cell size and metabolic activity in normal T-cells. Also, IL-7 promotes expression of Glut1 and hexokinase II (HK2), both involved in glycolysis. In T-ALL, we previously showed that IL-7 mediated cell growth, promoted glucose use and Glut1 expression. Using NGS data obtained in Chapter 2, we extended the knowledge on IL-7-mediated T-ALL cell metabolism. We provide significant evidence that IL-7 is broadly involved in upregulation of glycolysis in T-ALL. Gene expression analysis showed that IL-7 promotes very early expression of several glycolytic genes, including those involved in key stages of glycolysis regulation (Chapter 5).

Taken together, the studies presented in this work significantly expand our understanding of the role of the IL-7/IL-7R signaling axis in T-ALL. The discovery of oncogenic *IL7R* mutations may have important therapeutic implications in T-ALL. In addition, we provide clear evidence that targeting Jak/STAT5/PIM1 and MEK/Erk pathways in IL-7 signaling constitute new promising therapeutic targets. We also unravel new roles for IL-7 in important physiological processes, such as autophagy and glycolysis, which may help devise new therapeutic strategies in T-cell leukemia.

Keywords (5): IL-7, T-ALL, microenvironment, signaling pathways, therapeutic targets

Abbreviations

1,3BPG	1,3-bisphosphoglycerate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
ABL1	Abelson murine leukemia viral oncogene homolog 1
ALDO	Aldolase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	5' AMP-activated protein kinase
AQP	Aquaporin
Atg	Autophagy-related
ATP	Adenosine triphosphate
bHLH	basic Helix-loop-helix
BM	Bone marrow
CA	Carbonic anhydrase
CCL	C-C motif ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDK	Cyclin-dependent kinases
CDKN	Cyclin-dependent kinase inhibitor
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
CRLF2	Cytokine receptor-like factor 2
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
DC	Dendritic cells
DEG	Delayed early gene
Deptor	DEP domain-containing mTOR-interacting protein
DHAP	Dihydroxyacetone phosphate
DLBCL	Diffuse large B-cell lymphoma
Dll	Delta-like ligand
DN	Double negative
DP	Double positive
ECM	Extracellular matrix
EGIL	European Group for the Immunological Characterization of Leukemias
eIF	Eukaryotic translation initiation factor
EMSA	Electrophoretic mobility shift assay
ENO	Enolase
ER	Endoplasmic reticulum
Erk	Extracellular signal–regulated kinase
ETP	Early-thymic precursors

ETV	ETS-related
EZH	Enhancer of zeste homolog
F1,6BP	Fructose-1,6-bisphosphate
F2,6BP	Fructose-2,6-bisphosphate
F6P	Fructose-6-phosphate
FBXW	F-box/WD repeat-containing protein
FoxO	Forkhead-box O
FSC	Forward scatter
G6P	Glucose-6-phosphate
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GADP	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GPCR	G-protein coupled receptors
GPI	Glucose-6-phosphate isomerase
GSEA	Geneset enrichment analysis
HCQ	Hydroxychloroquine
HK	Hexokinase
HOX	Homeobox
HSC	Hematopoietic stem cell
ICAM	Intercellular adhesion molecule
ICN	Intracellular Notch
IEG	Immediate-early genes
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IkB	Inhibitor of NF-κB
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IL-2RG	Interlukin-2 receptor gamma
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
IL-7Rα	Interleukin-7 receptor alpha cahin
Jak	Janus kinase
JH2	Jak homology 2
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDH	Lactade dehydrogenase
LFA	Lymphocyte function-associated antigen
LIC	Leukemia initiatin cell
LMO	LIM-domain only
LMPP	Lymphoid-primed multipotent progenitor
LRG	Late response genes
LYL	Lymphoblastic leukemia derived sequence
MAP1LC3 (LC3)	Microtubule-associated protein 1 light chain 3

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEF2C	Myocyte-specific enhancer factor 2C
MEK	See MAPKK
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mSIN	mammalian stress-activated protein kinase interacting protein
mTOR	mammalian(mechanistic) target of rapamycin
mTORC	mTOR complex
NAD(H)	Nicotinamide adenine dinucleotide
NF1	Neurofibromin 1
NF-κB	Nuclear factor kappa B
NGS	Next-generation sequencing
OSM	Oncostatin M
OXPHOS	Oxidative phosphorylation
PAS	Periodic-acid Schiff
PDK	3-phosphoinositide dependent protein kinase
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PFK2-F2,6BPase (PFKFB)	Fhosphofructokinase-2-fructose-2,6-bisphosphatase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PH	Pleckstrin homology
PHF	PHD finger
PHLPP	PH domain and leucine rich repeat protein phosphatase
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PI3P	Phosphatidylinositol-3-phosphate
PIAS	Protein inhibitors of activated stats
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PK	Pyruvate kinase
PKB (Akt)	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2a
PRAS40	Proline-rich AKT substrate 40 kDa
PTEN	Phosphatase and tensin homolog
RAG	Recombination-activating genes
Raptor	Regulatory-associated protein of mTOR
RBC	Red blood cell

Rictor	Rapamycin-insensitive companion of mTOR
RTE	Recent thymic emigrants
RTK	Receptor tyrosine kinase
RUNX	Runt-related transcription factor
S6K	p70 ribosomal S6 kinase
SAPK	Stress activated protein kinase
SCF	Stem cell factor
SGK	Serum- and glucocorticoid-induced protein kinase
SHIP	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase
SOCS	Suppressor of cytokine signaling
SP	Single positive
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCA	Tricarboxylic acid
TCR	T-cell receptor
TGF	Transforming growth factor
TM	Transmembrane
TPI	Triose-phosphate isomerase
Treg	Regulatory T-cell
TSC	Tuberous sclerosis complex
TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
TSS	Transcription start site
ULK	unc-51-like kinase
VCAM	Vascular cell adhesion protein
VDAC	Voltage-dependent anion channels
VLA	Very late antigen
Vps34	Vesicle protein sorting
WBC	White blood cells
Wnt	Wingless-related integration site
γC	Gamma common chain

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CHAPTER 1

Introduction

1.1 Hematopoiesis and T-cell development: a brief overview

1.1.1 Hematopoiesis

Hematopoiesis is the process of formation and maturation of blood cellular elements. These include red blood cells (RBCs), white blood cells (WBCs) and platelets. Postnatally and throughout life, hematopoiesis is restricted to the bone marrow (BM) [1]. However, under extreme stress it may occur extramedullary [1-4]. The hematopoietic system is very dynamic, allowing for a fine-tuned and controlled output of newly generated cells under different circumstances. For instance, increased erythropoiesis often occurs in response to hypoxia [5, 6] or malignancy [4].

The challenges posed to the hematopoietic system are met by a hierarchy of stem, progenitor and mature cells, each with a defined role. At the top of the hierarchy sits the hematopoietic stem cell (HSC), a multipotent cell type, capable of self-renewing and giving rise to all hematopoietic cell types [7-9]. During differentiation, two major branches in the hierarchy are established, the myeloid branch and the lymphoid branch [10, 11]. Whereas the later stages of cell maturation are relatively well characterized for each of the various cell types, the early stages of maturation and lineage establishment are less clear. Recent data from murine models suggest the existence of a branching point where the common myeloid progenitor (CMP) gives rise only to cells of the myeloid lineage and the lymphoid-primed multipotent progenitor (LMPP) is capable of giving rise to cells of both the myeloid and lymphoid lineage [11-15].

1.1.2 T-cell development

The preferred model organism to study the hematopoietic development and, in particular, T-cell development is the mouse. To date an extensive list of knock-outs, knock-ins and humanized mouse models have been generated [11] that allow studying both human and murine T-cell development, with their many similarities and important differences.

The thymus is the organ where functional T-cells develop and mature. The thymus is seeded by different populations of precursors coming from the bone marrow which present lymphoid potential. The most studied of these precursors, in mouse and humans, are the common-lymphoid progenitor (CLP) and the LMPP [15-19]. Thymic development is a multi-step process (Figure 1). Under the influence of the thymic microenvironment, the different populations seeding the thymus undergo progressive T-cell lineage restriction,

becoming the most immature thymic cells, early-thymic precursors (ETPs), and culminating with the generation of CD4+ and CD8+ mature T-cells. As the cells progress through the developmental pathway, they acquire T-cell identity and lose the potential to generate other lineages.

The traditional classification of thymic cell populations, or subsets, is based on the expression of the co-receptors CD4 and CD8 [20]. Briefly, thymocytes begin their development as $CD4^-$ $CD8^-$ (double negative; DN) cells, progress through a $CD4^+$ $CD8^+$ (double positive; DP) stage and then become either mature $CD4^+$ $CD8^-$ (single positive CD4; SP4) or mature $CD4^-$ $CD8^+$ (single positive CD8; SP8) cells. Additionally, commitment to αβ and γδ T-cell receptor (TCR)-expressing cells occurs at the DN stage. Moreover, particularly for αβ T-cells, key events such as β-selection and positive and negative selection take place. Cells that have not yet rearranged the TCR β-chain may be referred to as pro-T cells while cells that pass β-selection until the DP stage may be referred to as pre-T cells [21, 22]. More detailed subsets have been identified for both mouse and human thymocytes, though they differ in the expression of cell surface markers between the two species (see sub-sections below) (Figure 1).

Although the main focus of this introduction is the classification of developing thymic subsets based on cell surface markers, it is unavoidable to refer that a number of growth factors and signaling pathways play key roles in thymocyte development in mouse and human. The Stem cell factor (SCF) / Kit (CD117) signaling pathway and the interleukin(IL)-7 (IL-7) / IL-7 receptor (IL-7R) mostly sustain proliferation and viability at the early stages of pro-T cells [23-27]. The Wingless-related integration site (Wnt) pathway was also found to be important in sustaining proliferation of DN cells [28]. Importantly, the Notch signaling pathway is the chief element that is mandatory to establish T-cell lineage commitment and identity in both mouse and humans [29, 30].

1.1.2.1 Mouse

In the mouse, the DN1 stage ($CD44^+$ $CD25^-$) constitutes a broad population of cells which contains the earliest ETPs (Lineage/$Lin^{-/low}$ $CD117^{hi}$ $CD44^+$ $CD25^-$) capable of efficiently originating T-lineage progeny, high proliferative potential and B-/myeloid-lineage potential [16, 31, 32]. The DN2 stage ($CD117^{hi}$ IL-7Rα/$CD127^{hi}$ $CD44^+$ $CD25^+$) further restricts fate towards the T-cells, by loss of some myeloid- and total B-lineage potential, and still retain high proliferative potential [16, 33, 34]. In the DN3 stage ($CD117^-$ $CD44^-$ $CD25^+$) several important events take place. T-cell lineage commitment is completed

[20, 35]. Cells reduce proliferation and either TCR β-chain rearrangement occurs, committing to αβ-lineage, or TCR γ- and δ-chain rearrangements, committing to γδ-lineage [36]. Within the αβ-lineage, a successful β-chain rearrangement coupled with expression of pre-TCR α-chain (pTα) at the cell surface (pre-TCR) that is signaling productive, allows transition to the next stages [37]. Cells that fail to productively rearrange the β-chain die (β-selection). The pre-TCR signal strength is powerful enough for these cells to enter a pre-TCR-dependent proliferative burst and transition to the DN4 stage ($CD117^-$ $CD27^+$ $CD44^-$ $CD25^-$) and then to the DP stage where both CD4 and CD8 co-receptors are expressed [38]. The DP stage comprises ~85% of total thymocytes. In this stage cell undergo a proliferative block and rearrange the TCR α-chain [39]. Thymocytes will continue to mature if they express TCRs with the appropriate characteristics. DP cells interact with antigen presenting cells in the thymus displaying Major Histocompatibility Complex (MHC) molecules to determine their fate. If TCR signals are too weak the developing T cells do not receive enough survival signals and die by neglect. Otherwise, cells will undergo the process of positive selection [40]. Effective interaction with MHC class I will promote development of CD8 SP T-cells and with MHC class II will promote development of CD4 SP T-cells [41]. However, when TCR signals are too strong and self-reactivity may develop, cells are actively killed by negative selection [42], or under particular circumstances, $CD4^+$ cells may develop into regulatory T-cells (T_{reg}) [43].

1.1.2.2 Human

Human thymopoiesis shares many similarities with the murine counterpart. Key events such as T-cell lineage commitment and β-selection during the DN stage, negative and positive selection and CD4/CD8 SP lineage commitment at the DP stage are largely similar. However, DN subset classification based in the murine CD44 *vs* CD25 expression does not have the same representation in human thymocytes [44]. The early T-cell precursors that seed the human thymus are $CD34^+$ $CD38^-$ $CD1a^-$ (DN1). Cells then upregulate CD38 ($CD34^+$ $CD38^+$ $CD1a^-$; DN2), followed by CD1a ($CD34^+$ $CD38^+$ $CD1a^+$; DN3) [45, 46]. Analysis of gene expression profiling and TCR gene rearrangements suggests that an overlap between mouse and human DN stages of development can be established. $CD34^+$ $CD38^-$ $CD1a^-$ resemble mouse DN1/ETP, $CD34^+$ $CD38^+$ $CD1a^-$ the mouse late DN1/DN2 and $CD34^+$ $CD38^+$ $CD1a^+$ mouse DN3 [46]. CD1a upregulation is strongly correlated with T-cell lineage commitment [47] and β-selection can occur as early as this stage [46]. Acquisition of CD7 and cytoplasmic CD3 (cCD3) occurs at the $CD34^+$ $CD38^-$ $CD1a^-$ stage, followed by increase

of CD2 and CD5 expression at the $CD34^+$ $CD38^+$ $CD1a^-$ stage [48]. Cells then lose expression of stem cell marker CD34, progressively gain CD4, CD8 and surface CD3 to become DP thymocytes (CD3+ CD4+ CD8+), which, after TCR α-chain rearrangement, undergo positive and negative selection, to become either mature CD4 or mature CD8 SP T-cells [44]. Acquisition of maturity is accompanied by loss of CD38 and CD1a expression [49, 50].

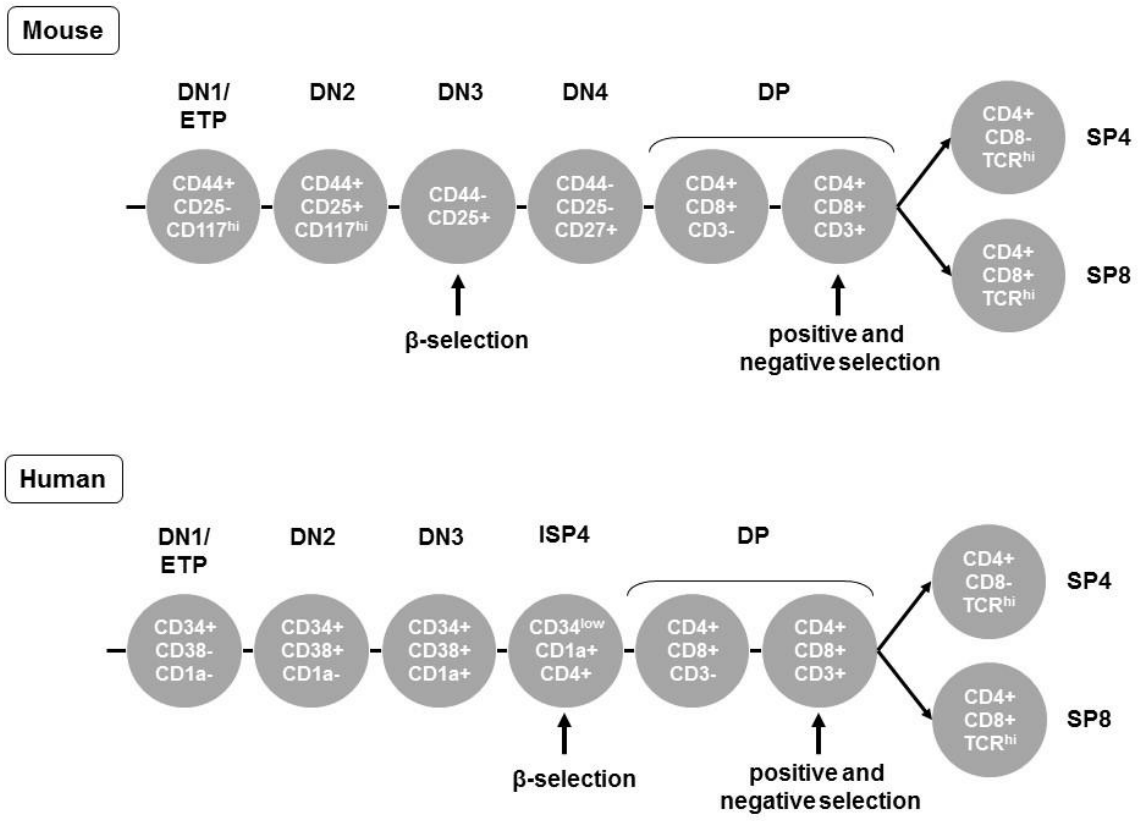

Figure 1. Model overview of human and mouse T-cell development. Precursors migrate from the bone marrow to the thymus. Thymic T-cell development starts at the double negative (DN) stage. It progresses to the double positive (DP) and later to the single positive stage (SP). Important surface markers are represented for each stage. The β-selection and positive and negative selection events are indicated. Further details in the text.

1.2 Acute Lymphoblastic Leukemia (ALL)

Hematopoiesis is regulated by numerous factors. For instance, T-cell development is under the control of survival, proliferative and differentiation signals, such as those elicited by IL-7, Notch or TCR, and determined by recombination-activating gene (RAG)-mediated DNA double-strand break activity during TCR maturation [44, 51]. Although the process is tightly monitored, developing precursors are at risk of transformation. Malignant transformation of cells of lymphoid origin will result in leukemia or lymphoma.

1.2.1 Epidemiology and causes

ALL is the most common childhood cancer, accounting for 26% of the cases. It is more common in males than in females and more prevalent in white than black children [52, 53]. There is evidence that ALL may develop in utero. Studies in identical twins show leukemias with identical genetic rearrangements [54-56]. Additionally, analysis of neonatal blood spots showed the presence of leukemic genetic lesions before the diagnosis of ALL [57, 58]. Despite this, the peak of incidence is characteristically at ages 2 to 4 [52].

The exact causes for ALL are not clearly known, although some genetic conditions associate with predisposition for leukemia development. ALL has increased risk in genetic disorders such as Down syndrome [59], Fanconi anemia [60], Bloom syndrome [61], neurofibromatosis [62], and ataxia-telangiectasia [63].

There is also evidence that non-genetic factors may increase the risk of ALL development. For example, ionizing radiation exposure (e.g. during medical treatment or atomic disasters) was shown as an important physical factor contributing to increased ALL risk [64-66]. Infectious agents (or abnormal responses against them) have also been postulated to contribute to ALL [67, 68]. Other factors include exposure to environmental pesticides, parental smoking and diet of the mother. Secondary leukemia as consequence of cancer therapy is more associated with the development of acute myeloid leukemia (AML) than ALL [67, 69].

1.2.2 Biological characteristics

ALL is characterized by an abnormal accumulation of immature lymphoid cells, or blasts, arrested in their development and bone marrow involvement superior to 20%. Presence of masses in other organs and peripheral blood involvement may vary [70]. ALL originates from malignant clones of B- or T-cell lineage, and the origin is believed to be in

the BM or thymus [71]. ALL is therefore a heterogeneous cancer with combined morphologic, immunologic, cytogenetic and molecular genetic characteristics [72].

Morphological and cytochemical characteristics *per se* have limited ALL sub-classification value and usage is mostly applied to distinguish ALL from AML [73]. ALL blast population cells tend to be small, homogeneous, with a central large nucleus, fine chromatin and scant cytoplasm. Cytochemical analysis of myeloperoxidase, acid phosphatase and periodic-acid Schiff (PAS) stainings, help complementing the diagnostics [74].

Immunophenotyping by flow cytometry and cytogenetic analysis of DNA lesions constitute the gold standard of ALL classification and sub-typing. In childhood, around 85% of cases present with a B phenotype and 13-15% present with a T phenotype. In adults, around 75% have a B phenotype and 25% are of T-cell origin [75]. In our studies, we adopted the criteria of the European Group for Immunological Characterization of Leukemias (EGIL) [76], which correlate the immunophenotype at which the leukemia cells are arrested with that of normal developing lymphocyte precursors. In the case of T-cell leukemia, 4 groups are recognized: pro-T or T-I (cytoplasmic CD3+, CD7+), pre-T or T-II (cCD3+ CD7+ CD2+ and/or CD5+), cortical-T or T-III (cCD3+, Cd1a+) and mature-T or T-IV (CD3+, $CD1a^-$). Recently, a novel sub-type was identified, early T-cell precursor ALL (ETP-ALL), which is defined as $CD1a^-$, $CD8^-$, $CD5^{low/-}$ and expressing at least a myeloid or stem cell surface marker [77].

ALL often presents cytogenetic abnormalities involving numeric and structural chromosomal changes. A comprehensive study of cytogenetics showed that cytogenetic features have biological and prognostic significance [78]. ALL can be classified under 5 major modal groups: diploid (46 chromosomes, no evident structural abnormalities; 31-40%), high hyperdiploid (>50 chromosomes; 23-26%), low hyperdiploid (47-50 chromosomes; 10-11%), pseudodiploid (46 chromosomes with structural abnormalities; 18-26%), hypodiploid (<45 chromosomes; 6%). Regarding translocations, ALL may be divided according to the Lund Chromosomal Group as: t(9;22)(q34;q11.2) or a Philadelphia chromosome (Ph^+); t(4;11)(q21;q23); t(8;14)(q24;q32) or del(8q); other 14q+ abnormalities; del(6q). The classification recognizes 10 groups, being the remaining related to modal chromosome number [78, 79]. Although these findings are useful for predicting clinical outcome and response to treatment, they are not totally accurate. For example, up to 20% of children with favorable genetic features (TEL-AML1 fusion and hyperdiploidy >50 chromosomes) will eventually relapse, although a third of those with high-risk abnormalities

(the Philadelphia chromosome with BCR-ABL fusion and the t(4;11) with MLL-AF4 fusion) can be cured with chemotherapy alone [80]. This fraction is currently even higher due to the introduction of tyrosine kinase inhibitors such as Imatinib and Dasatinib [81-83]. Additionally, genetic factors intrinsic to the individual (e.g. drug-metabolizing enzyme polymorphisms), rather than those acquired by the leukemic cell, may have an important impact on treatment outcome [84].

1.2.3 Symptoms and treatment

Most clinical symptoms of ALL relate to the collapse of normal hematopoiesis. The common clinical signs include fatigue and lethargy due to developing anemia. Bleeding and excessive bruising occurs due to thrombocytopenia. Neutropenia may lead to predisposition to infections and fever. Thymic masses may lead to shortness of breath and superior vena cava syndrome. Tumor spread to the meninges may result in headaches and central nervous system (CNS) involvement [85].

Survival rates of children with ALL has improved dramatically across the decades. In the 1960s, 5-year survival rate was 10%, whereas currently it reaches up to 90% (85% of event-free survival) [86]. These great improvements were built on top of significant advances such as those observed in the biological characterization of ALL, development of more effective drugs and risk-adjusted multi-agent therapy [86, 87]. In adults, however, treatments have been less successful, classically only achieving ~40% of 5-year event-free survival [75]. Lately, however, the application of intensive chemotherapy pediatric protocols on adult T-ALL patients was able to improve 7-year event free survival to 63% [88]

The current treatment approach for ALL includes 3 main phases. A remission induction phase, an intensification (consolidation) phase and continuation long-term treatment [87]. During the remission-induction phase the main objective is to reduce leukemia burden and restore normal hematopoiesis. Therapy includes administration of glucocorticoids (prednisone or dexamethasone), vincristine and at least another agent (asparaginase or anthracycline). Exceptionally, for high risk ALL, regimens include 4 or more drugs [87]. The intensification (consolidation) treatment happens after restoration of hematopoiesis. This phase will deal with possible drug-resistant leukemic cells and decrease the chance of relapse [86]. Treatment regimens may vary, and have different degrees of success, but often include reinduction therapy, high doses of methotrexate and mercaptopurine, and pulses of vincristine and corticosteroid plus high-dose asparaginase [89-92]. The most extreme form of intensification treatment is allogeneic stem cell

transplantation, which is especially beneficial to very-high risk patients [93, 94]. During continuation treatment, regimens are adjusted for long-term tolerance. Mercaptopurine and methotrexate are regularly used in this phase [87]. Increased risk of relapse to the CNS associate with factors such as T-cell immunophenotype, hyperleucocytosis, high-risk genetic abnormalities, and presence of leukemic cells in cerebrospinal fluid. These cases require particular attention to CNS-directed treatment [87, 95-97].

Although the success of the treatments is evident, relapses still occur and aggressive treatments frequently impose severe long-term side effects. Side effects include osteoporosis [98], osteonecrosis [99], thrombocytic complications [100], secondary tumors [101], cardiac dysfunction, along with others [102, 103]. Therefore, a continuous effort is required to develop new, less toxic drugs and therapeutic strategies with improved efficacy against leukemic cells and less side effects. To achieve this, it is indispensable to investigate and improve the knowledge of the etiology and biology of leukemia.

1.3 T-cell Acute Lymphoblastic Leukemia (T-ALL)

T-ALL is a subtype of ALL, characterized by the emergence of malignant immature blasts of T-cell origin arrested during development. T-ALL often presents with higher risk factors such as high white blood cell counts (WBC; >50000/μL), older age, mediastinal mass and enlargement of the spleen, liver, and lymph nodes [72]. Historically, T-ALL cases had higher risk and poorer prognosis than B-ALL cases [104-106]. However, improved protocols, based on risk-adjusted chemotherapy, improved the outcome of T-ALL patients to such an extent that currently ALL patients with a T-cell phenotype benefit from better 5-year disease-free survival for children (up to 78%) [86] and adults (65%; 7-year survival) [88, 107]. Relapses still occur in approximately 25% of the cases, and T-cell phenotype is associated with poorer prognosis after relapse [86, 108, 109].

1.4 Genetic abnormalities in T-ALL

The malignant transformation of healthy thymocytes into T-cell leukemia is believed to be a progressive, multi-step, process where several cell-autonomous mechanisms accumulate to promote a proliferative and survival advantage and a differentiation block to pre-malignant cells, which associates with abnormal signaling and eventually results in leukemia. Those factors range from point and small mutations, to epigenetic changes and to

large chromosomal alterations. Moreover, the microenvironment often impinges on those alterations by promoting and complementing an already signaling-aberrant cell [110-112].

1.4.1 TCR loci-associated chromosomal translocations

In T-ALL, non-random chromosomal translocations involving the juxtaposition of promoter and enhancer elements of TCR gene loci (*TCA@* 14q11, *TRB@* 7q34-35, *TRG@* 7p15, *TRD@* 14q11) and a developmentally important transcription factor are common, found in ~35% of the cases [113] (Figure 2). This leads to unregulated over-expression of the translocated gene and subsequent differentiation blockade [114]. Different families and groups of transcription factors are involved in these abnormalities. Importantly, several groups have classified T-ALL into distinct oncogenetic subgroups that are characterized by rearrangements and aberrant expression of transcription factors and share a similar gene expression profile [115-118]. Major groups include the basic helix-loop-helix (bHLH) family members (SCL/TAL1, TAL2, LYL1 and bHLHB2), the LIM-domain only (LMO) family (LMO1, LMO2, LMO3), several homeobox family members (HOX11/TLX1, HOX11L2/TLX3, NKX2.1, NKX2.2, NKX2.5, HOXA@ cluster) and proto-oncogenes such as TAN1 (truncated from of NOTCH1), MYC, MYB and MEF2C (reviewed in [119]).

1.4.2 Cell Cycle regulators

Cell cycle deregulation is a hallmark of cancer. Deregulation may occur by increased activity/expression of cell cycle promoters, inactivation of cell cycle blockers, or both (**Figure 2**). The most common occurrence in T-ALL is the deletion of the *CDKN2A*/*CDKN2B* locus on chromosome 9p21, present in more than 70% of cases [120, 121]. These genes code for the inhibitors of cyclin-dependent kinase (CDK) 4 proteins ($p16^{INK4a}$ and $p15^{INK4b}$, respectively), thus disrupting the cyclin-CDK complexes [122, 123]. Deletion of these genes will lead to phosphorylation and inactivation of the retinoblastoma protein (Rb), thus promoting cell cycle progression [124]. Additionally, the *CDKN2A* locus, via an alternative reading frame, also codes for $p14^{ARF}$ protein, a negative regulator of HDM2 [125]. Loss of $p14^{ARF}$ will lead to p53 downregulation by allowing HDM2 to promote p53 degradation. Consequently, a decrease in p53 activity, decreases $p21^{Cip1}$ expression, which does not allow proper DNA repair during cell cycle, leading to accumulation of DNA damage [125, 126].

1.4.3 NOTCH1

NOTCH1 is a gene that has a major role in hematopoiesis. It regulates maintenance of stem cells [127] and is required for T-cell lineage specification [30, 128].

NOTCH1 is also one of the most frequently altered genes in T-ALL (Figure 2). Its role in leukemia was first described with the involvement in the translocation t(7;9)(q34;q34.3), but this is a uncommon event (~3% of T-ALL cases) [129]. However, the magnitude of *NOTCH1* importance in cancer was only revealed later on, when it was found that >50% of T-ALL cases had activating mutations in the gene [130]. Although the mechanisms vary, *NOTCH1* translocations or mutations will result in the accumulation of the intracellular, activated form of NOTCH (ICN) [131]. In addition, mutations in the F-box/WD repeat-containing protein 7 (*FBXW7*) gene, found in ~15% of T-ALL patients [132, 133], lead to the stabilization, and consequent increase in activity, of the ICN protein.

1.4.4 Signal transduction elements

During thymopoiesis, both pre-TCR and TCR engagement is required for normal T-cell development. The TCR complex activates a cascade of multiple signaling pathways including the rat sarcoma/ mitogen associated protein kinase (Ras/MAPK), the phosphatidylinositol-3-kinase/ protein kinase B (PI3K/PKB(Akt)) and the phospholipase C γ/ calcineurin (PLCγ/calcineurin) pathways [134, 135]. These pathways are also recruited by pre-TCR signaling transduction.

In T-ALL, multiple signaling components are targets of mutations or translocations (Figure 2). The Src family protein tyrosine kinase lymphocyte-specific protein tyrosine kinase (Lck) is central in TCR signaling [136]. Although rare, ectopic expression of LCK can occur in T-ALL due to the t(1;7)(p34;q34) translocation [137].

The Abelson murine leukemia viral oncogene homolog 1 (ABL1) is a downstream target of Lck [138]. Various ABL1 rearrangements occur in T-ALL. The famous *BCR-ABL1* fusion gene, though present, is uncommon [139]. The most common rearrangement is the NUP214-ABL1 (~6%) [140].

The Ras pathway also suffers from mutations. Activating NRAS mutations occur in 4-10% [141]. Additionally, deletion or inactivating mutations in the Neurofibromin 1 (*NF1*) gene, a negative regulator of the Ras pathway, are found in 3% of the cases [142].

1.4.4.1 Alterations in IL-7/IL7R-related signaling mediators

Altogether, a major fraction of T-ALL cases presents alterations in two major signaling pathways, the PI3K/Akt and the Janus kinase/ signaling transducer and activator of transcription (Jak/STAT) pathways. Both are critical for IL-7R-mediated function in normal thymocytes and T-ALL (discussed later on) [143-145].

The PI3K/Akt pathway is the target of mutations in T-cell leukemia. The most common are in the phosphatase and tensin homolog (PTEN). PTEN is a tumor suppressor and the major negative regulator of the PI3K/Akt pathway [146]. It is mutated in T-ALL (5-30% of the cases) due to non-sense and frameshift mutations and gene deletion occurs in 10% of the cases [147]. Mutations in PI3K family members are uncommon in T-ALL, although gain-of-function mutations in *PIK3CA* (p110α) and inactivating mutations in *PIK3R1* (p85α) have been reported, each in 5% of the T-ALL cases analyzed. *AKT* mutations are even less frequent (around 2% of T-ALLs) [147].

The Jak/STAT pathway is also a target for mutation in T-ALL. The oldest known alteration is the ETV6(TEL)-JAK2 fusion due to the translocation t(9;12)(p24;p13) [148]. Somatic *JAK1* gain-of-function occur mostly in adult T-ALL (18% of the cases) and more rarely in pediatric T-ALL (2% of the cases) [149, 150]. *JAK3* mutations are found in both adult (12% of the cases) [151] and pediatric (7%-25% of the cases) T-ALL [150, 152]. More recently, *STAT5B* gain-of-function mutations have been mutations have been reported in 8% of the patients [153, 154].

1.4.5 Other important alterations

Alterations on the *MYB* locus found in T-ALL, which lead to protein overexpression, include the chromosomal translocation t(6;7)(q23;q32), associated with high expression of proliferation and mitotic genes [155], and duplication of the *MYB* locus [156].

Mutations in chromatin remodeling genes that ultimately benefit T-cell leukemia progression have been reported to occur in *EZH2*, *SUZ12* [157] and *PHF6* [158].

Lastly, inactivating mutations in phosphatases other than *PTEN* were found recently in *PTPN2* [159] and *PTPRC* (CD45) [160] genes.

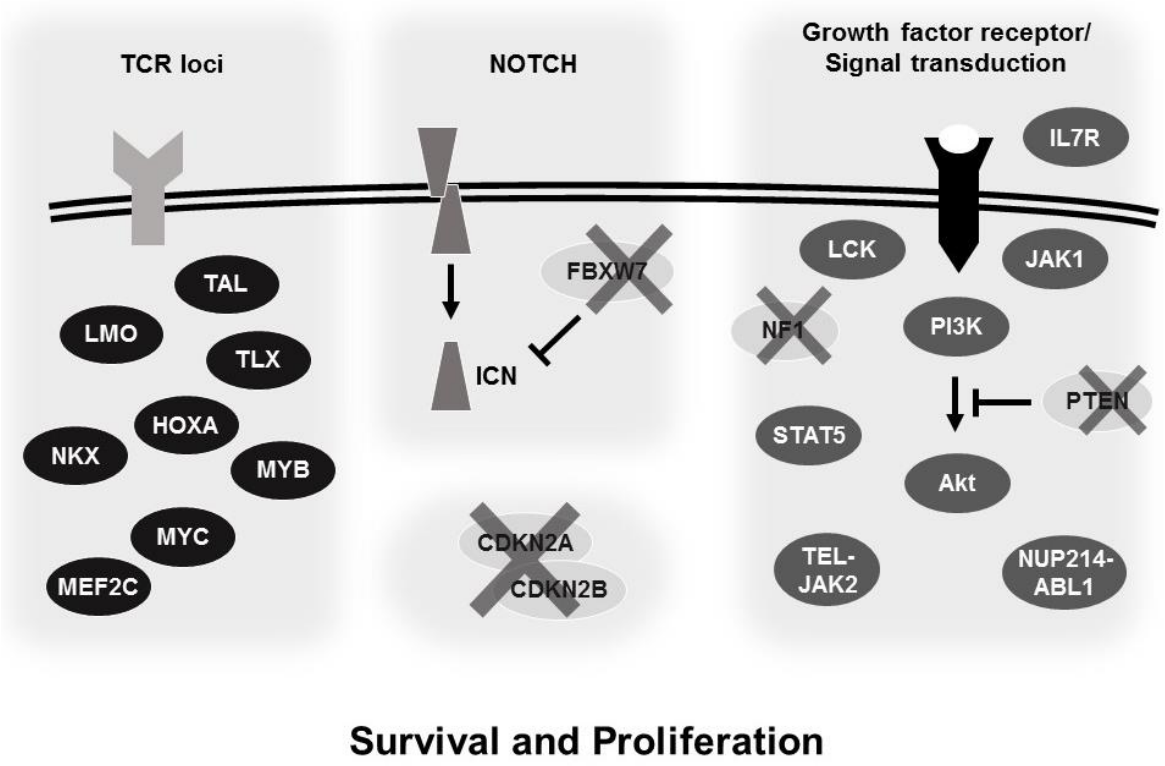

Figure 2. Schematic representation of major genetic alterations in T-ALL driving survival and proliferation. Genetic abnormalities are grouped according to their nature. Represented are common translocations involving TCR loci; NOTCH activating mutations and FBXW7 inactivation; deletion of cell cycle regulators; and mutations in signaling transduction elements and growth factor receptors. Ovals represent either gene families (e.g. NKX) or individual genes (e.g. CDKN2A). The cross over a gene indicates inactivation of the gene or protein. Detailed information is in the text. TCR, T-cell receptor; TAL, T-cell acute lymphoblastic leukemia gene family; LMO, LIM-domain only gene family; TLX, T-cell leukemia homeobox gene family; HOXA, Homeobox A gene cluster; NKX, NK2 homeobox gene family; MEF2C, Myocyte specific enhancer factor 2C; ICN, Intracellular Notch FBXW7, F-box/WD repeat-containing protein 7; CDKN2A/2B, Cyclin-dependent kinase inhibitor 2A/2B; IL7R, Interleukin-7 receptor; JAK, Janus kinase; LCK, Lymphocyte-specific protein tyrosine kinase; PI3K, Phosphatidylinositol-3-kinase family; PTEN, Phosphatase and tensin homolog AKT, also known as protein kinase B (PKB); STAT, Signal transducer and activator of transcription; TEL, also known as ETS variant 6 (ETV6); NUP214, Nucleoporin 214; ABL1, Abelson tyrosine-protein kinase.

1.5 Microenvironment in T-ALL

A tumor is not a homogeneous entity consisting purely of malignant cells entirely self-sufficient. In contrast, it is highly heterogeneous containing both malignant and non-malignant cells of several origins, as well as components such as extracellular matrix and secreted factors. Together, all elements that constitute the tumor microenvironment interact, where the final consequence is to the benefit of the cancer cells [161, 162]. The microenvironment provides cancer cell survival, protects from chemotherapy and may support metastization [112, 162-164]. The studies on the involvement of the

microenvironment in T-ALL have focused mostly in the bone marrow niche, since this is a major site of leukemia burden.

1.5.1 Cell-to-cell contact

Cell adhesion molecules are expressed in T-ALL cells, such as very late antigen 4 / vascular cell adhesion protein 1 (VLA-4/VCAM-1) and lymphocyte function-associated antigen 1 / intercellular adhesion molecule 1 (LFA-1/ICAM-1) [165]. T-ALL cells cultured in BM stroma have increased survival dependent on LFA/ICAM-1 interactions [166]. Interestingly, and in contrast with B-ALL, *in vitro* survival of T-ALL cells on BM stromal cultures appears to correlate with better patient outcome [167]. As mentioned above, the Notch1 receptor is commonly mutated in T-ALL, originating ligand-independent activation of the pathway. However, there is still room for the canonical ligand-dependent Notch signaling to play a role in T-ALL pathogenesis. Blocking of delta-like ligand 4 (Dll4), a Notch ligand, or of the Notch1/2/3 receptors themselves impairs T-ALL growth *in vivo* [168] and T-ALL cell escape from dormancy is associated with Notch3-Dll4 interaction in the microenvironment [169]. It is also noteworthy that PTEN deficient T-ALLs are sensitive to disruption of Notch1-Dll4-dependent signaling [170].

1.5.2 Secreted factors: chemokines

Other than cell-to-cell interactions, cytokines, growth factors and chemokines provide extra means of intercellular communication and behavior conditioning. The whole immune system, including T lymphocytes, is orchestrated by a network of cytokines and chemokines [171]. The stromal cell-derived factor 1 (SDF-1/CXCL12) interaction with its receptor C-X-C chemokine receptor type 4 (CXCR4) was shown to be important for B-ALL homing to bone marrow [172]. More recently, two studies implicated the CXCL12/CXCR4 axis in migration, maintenance and leukemia initiating cell (LIC) activity in human T-ALL xenograft models [173, 174].

Other chemokine signaling elements have also been involved in T-ALL pathogenesis. Particularly, Buonamici and colleagues [175] found that Notch-regulated expression of C-C chemokine receptor 7 (CCR7) in T-ALL and consequent C-C motif ligand 19 (CCL19)/CCR7 signaling was a major regulator of T-ALL infiltration to the CNS. In addition, signaling of the C-C motif ligand 25/ C-C chemokine receptor 9 (CCL25/CCR9) or C-X-C motif ligand 13/ 5 (CXCL13/CXCR5) were shown to contribute to T-ALL cell survival, proliferation and organ infiltration [176-179].

1.5.3 Secreted factors: cytokines and growth factors

Multiple cytokine and growth factors have been implicated in supporting T-cell leukemia. For instance, secretion of IL-18 by the stromal cells upon treatment with mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitors, led to increased T-ALL cell proliferation, suggesting that stroma-leukemic cell cross-talk may provide a protective niche against drug therapy [180].

Also, activation of the insulin-like growth factor 1 receptor (IGF1R) in T-ALL cells is associated with increased LIC activity [181] and growth support from tumor-associated dendritic cells (DCs) [182].

Transforming growth factor β (TGF-β) is a multifunctional cytokine involved in a variety of processes such as cell growth inhibition, cellular senescence, differentiation and apoptosis [183]. The major effectors of TGF-β signaling are Smad2 and Smad3, which directly regulate gene expression [184]. In normal hematopoiesis TGF-β acts as a negative regulator [185]. In T-ALL its role is less explored. Of note, a fraction of primary T-ALL cells do not express Smad3 protein, although they still display non-mutated and normal levels of the Smad3 gene (*MADH3*) mRNA [186]. Additionally, studies in mice suggest that loss of Smad3 can synergize with other oncogenic events, such as the loss of $p27^{kip1}$, to promote T-cell leukemogenesis [187].

1.5.3.1 The γ-common chain (γ_C) family of cytokines

The members of the γ_C family of cytokines all bind to receptors that share the γ_C subunit (IL-2Rγ/CD132), along with one or more specific subunits, to transduce signals. The cytokine family includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [188], all of which have some role in T-cell development, function or homeostasis (reviewed in [188] and [189]). In T-ALL, it was demonstrated that IL-2, IL-4, IL-7, IL-9 and IL-15 are able to promote proliferation of primary samples in vitro, with IL-7 having the most potent effect [190]. Interestingly, synergistic roles in proliferation were observed upon incubation with specific combinations of two γ_C cytokines [190]. IL-21, a more recently discovered γ_C cytokine, has not been tested to date in T-ALL. Nonetheless, given that it supports cell proliferation in other T-cell malignancies [191, 192] and the consistency of the other γ_C cytokines in promoting T-ALL proliferation, it is tempting to speculate that the effect of IL-21 in T-ALL should be similar.

1.6 The IL-7/IL-7R complex

The IL-7R is a heterodimer consisting of the IL-2Rγ/γ_C, shared between the γ_C family of cytokines, and the IL-7Rα (CD127), shared between IL-7 and thymic stromal lymphopoietin (TSLP) [145, 193]. The heterodimerization of γ_C and IL-7Rα upon IL-7 binding gives the specificity to IL-7 [194, 195] and is required for receptor activation (Figure 3A) [196].

1.6.1 The γ_C subunit

As mentioned above the IL-2Rγ (γ_C/CD132) subunit is required for signaling of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [188]. The γ_C gene (*IL2RG*) is located in the X chromosome (Xq13.1) [197, 198], and inactivating mutations often result in X-linked severe combined immunodeficiency (X-SCID). In humans the X-SCID results in a T^- B^+ NK^- phenotype, though B-cells are non-functional [194, 198]. In mice, the phenotype is more severe resulting in T^- B^- NK^- phenotype [199, 200]. It is important to mention that Jak3-deficient mice have a phenotype that closely resembles that of γ_C loss [201-203].

The γ_C receptor belongs to the type I cytokine receptor family and the mature human protein has 374 aminoacid residues (Figure 3B). The extracellular domain possesses two tandem fibronectin type III domains that include two pairs of the conserved cysteine residues, characteristic of the family. A tryptophan-serine-X-tryptophan-serine (WSXWS) motif exists close to the transmembrane domain [204, 205]. The γ_C, as all type I cytokine receptors, does not possess endogenous tyrosine kinase activity, instead it relies on Jak family tyrosine kinases for signal transduction. The intracellular portion possesses, proximal to the transmembrane domain, a Box motif required for Jak3 binding and activation. The short cytoplasmic tail is apparently not directly involved in transducing downstream signaling [204, 206-208].

1.6.2 The IL-7Rα subunit

The IL-7Rα gene (*IL7R*) is located on the chromosome 5p13.2 and is composed of 8 exons. Exon 6 codes for the integral transmembrane domain. The canonical transcript is 4619 nucleotides long. Alternative splicing generates a soluble isoform lacking exon 6 and introducing a premature stop codon [209, 210]. Inactivating mutations on the *IL7R* gene result in a type of SCID. In humans, the SCID results from a T^- B^+ NK^+ phenotype [26, 211, 212]. Mouse deficient in *Il7r* have impaired T- and B-cell development [24]. Importantly,

the presence of B-cell in human individuals and not in mice advocates for important differences in lymphopoiesis in both species. Notably, the phenotype of murine IL-7Rα deficiency is more severe than IL-7 deficiency. This has been attributed to be a consequence of TSLP signaling [213].

The IL-7Rα subunit is a type I cytokine receptor (Figure 3B). As such, it shares many structural similarities to the γ_C subunit described above. The mature form is 439 amino acid residues long. In the extracellular portion, it has two fibronectin type III domains, two paired cysteine residues and a WSXWS motif. The cytoplasmic tail contains a Box1 motif required for Jak1 binding and activity [205]. Additionally, the cytoplasmic tail contains at least 2 tyrosine residues (Y401, Y449) that have been shown to play a role in the activation of downstream signaling pathways [214-217].

As already mentioned, the IL-7Rα chain is also shared with the thymic stromal lymphopoietin/ cytokine receptor-like factor 2 (TSLPR/CRLF2) receptor for TSLP signaling [218, 219].

1.6.3 Interleukin-7

The IL-7 gene (*IL7*) is encoded in chromosome 8q12-13 [220] and requires glycosylation to be fully active [221]. Human and murine IL-7 share 65% aminoacid identity [222]. Human IL-7 can stimulate murine cells [223] and, conversely, murine IL-7 can stimulate human cells [224], although possibly with less potency *in vivo* [225]. IL-7 is produced by the stromal cells of the BM and thymus and by lymphatic endothelial cells [226-230]. IL-7 is a soluble factor, nevertheless it has been observed that it can bind to extracellular matrix (ECM)-associated glycosaminoglycan, heparan sulfate, and to fibronectin [231-233]. Similar to its receptor, IL-7 is essential for normal B- and T-cell development in mice [25].

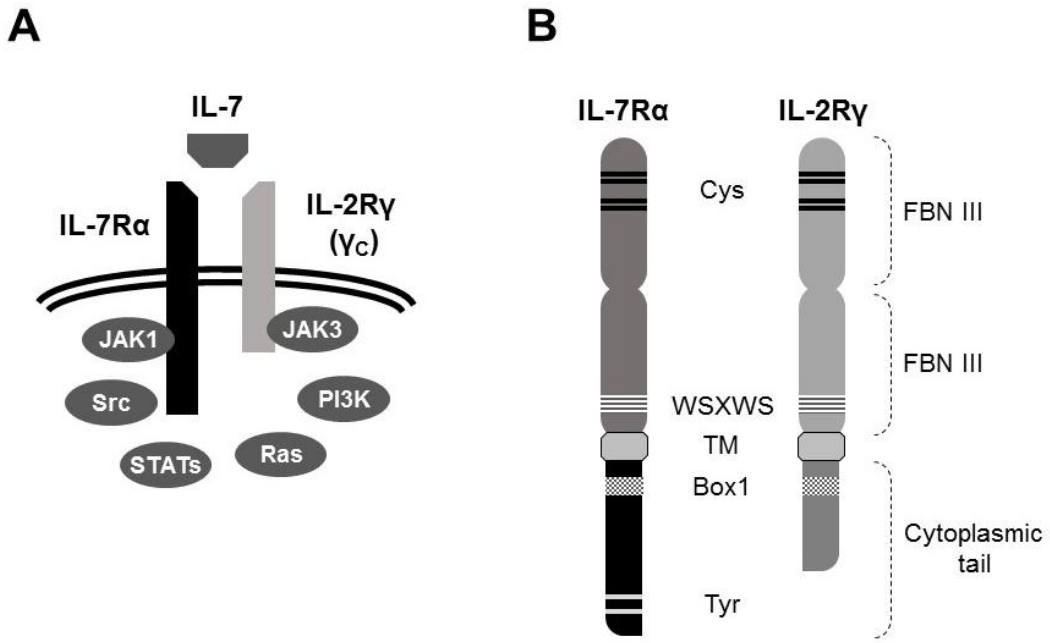

Figure 3. The IL-7/IL-7R signaling complex. (A) IL-7R heterodimer consisting of IL-2Rγ common chain and IL-7Rα, which provides IL-7 specificity. Upon IL-7 binding, downstream signaling is triggered. Janus kinase (JAK) 1 is associated with IL-7Rα and JAK3 with IL-2Rγ. When IL-7R is activated, JAK inter-phosphorylation occurs, IL-7Rα is phosphorylated in the cytoplasmic tail with subsequent recruitment and activation of downstream signaling pathways. In T-ALL signaling pathways include JAK/STAT, PI3K/Akt and Ras/MEK/Erk. Src-family kinases are activated by IL-7, but their role remains undefined. (B) Structural detail of IL-7Rα and IL-2Rγ. The extracellular portion of both receptors possess two tandem fibronectin type-III (FBN III) domains. Within those are found two pairs of conserved cysteine residues (Cys) and a tryptophan-serine-X-tryptophan-serine (WSXWS) motif close to the transmembrane domain (TM). The cytoplasmic tail of both receptors has a Box1 motif required for JAK binding. The IL-2Rγ cytoplasmic tail does not possess downstream signaling transduction capacity. The IL-7Rα has, at least, two phosphorylated tyrosine residues (Tyr; Y401, Y449) involved in the recruitment of downstream signaling elements.

1.6.4 IL-7/IL-7R signaling

IL-7 stimulates both human [27, 234, 235] and murine [236, 237] thymocyte proliferation.

The earliest murine thymocyte population that responds to IL-7 are the DN cells, where IL-7 contributes to survival and proliferation of early T-cell progenitors [238] and is involved in promoting TCRγ rearrangements [239]. More recently, it was found that IL-7 plays a role during β-selection, by promoting DN4 cells self-renewal and preventing premature TCRα rearrangements [240]. Although after β-selection and positive and negative selection (essentially the DP stage) IL-7Rα is downregulated, the role of IL-7 is controversial. It is accepted that during this stage proliferative and survival signals are TCR-dependent [51, 238]. However, there is evidence that IL-7 in this stage is involved in SP8 lineage specification [241-243]. IL-7R is re-expressed in the SP stage and in the periphery,

although heterogeneously. In the periphery, IL-7 signaling promotes homeostatic proliferation of T-cells [145, 244-248].

In humans, similarly to the mouse, the early DN population proliferates in response to IL-7 [27]. Also, the SP populations rely on IL-7 for proliferation [249, 250]. The DP population also appears to be largely IL-7 insensitive, but in contrast to mouse DP cells, this is due to low γ_C expression rather than absence of IL-7Rα expression [250]. In the periphery IL-7 has a homeostatic proliferative effect [251-253].

The severe effects that absence of IL-7-associated signaling has on normal lymphopoiesis, mentioned above, not only delineate its importance in normal T-cells but further incites to investigate its impact on malignant T-cells.

There is increasing evidence in the literature that IL-7/IL-7R-mediated signaling has a role in supporting T-cell malignancies. IL-7 transgenic mice develop T- and B-cell lymphomas [254-257]. Moreover, AKR/J mice, which have a naturally high expression of IL-7Rα, tend to develop spontaneous T-cell lymphomas [258]. Regarding IL-7/IL-7R-associated signaling in T-ALL, IL-7R expression is increased in T-ALL versus other leukemias [259]. Concordantly, T-ALL samples express IL-7R [260-262]. In addition, blocking IL-7R or IL-7 in *in vitro* cultures decreases T-ALL cell viability and proliferation [263, 264]. Several studies demonstrated that IL-7 promotes *in vitro* T-ALL cell proliferation [262, 265-268] and accelerates leukemia *in vivo* [269]. Of note, IL-7Rα is transcriptionally upregulated by NOTCH1, one of the most commonly mutated genes in T-ALL [130] and appears to be involved in Notch-mediated leukemia cell maintenance [270]. Mechanistically, IL-7 signaling in T-ALL was shown to depend on PI3K/Akt and mammalian target of rapamycin (mTOR) signaling, which together promote viability, proliferation and cell growth. Molecularly, IL-7 leads to the down-regulation of the cell cycle inhibitor $p27^{kip1}$, and upregulation of the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) and the glucose transporter GLUT1 – with consequent increase in glucose uptake and in reactive oxygen species which partake in leukemia cell survival [268, 271, 272]. The pathways involved in IL-7-mediated signaling appear to extend to JAK/STAT. IL-7Rα mutant T-ALL cells are sensitive to Jak/STAT pharmacological inhibitors [273] and STAT5 is required for mouse IL-7-dependent T-cell lymphomagenesis [254], indicating a role for this pathway in T-ALL cell survival.

1.7 IL-7-triggered downstream signaling pathways

IL-7R activation triggers multiple intracellular signaling pathways. Several kinases associate directly with the IL-7Rα, including Jak1 [217], PI3K [216] and Src-family kinases $p56^{lck}$ and $p59^{fyn}$ [274, 275]. Jak3 is associated with the γ_C [206, 276]. The canonical IL-7R mechanism of activation requires IL-7 binding, that induces heterodimerization of the IL-7Rα and IL-2Rγ chains, leading to activation by trans-phosphorylation of Jak1/3, respectively, and consequent tyrosine phosphorylation of IL-7Rα cytoplasmic tail (Y401, Y449 particularly). Recruitment of signaling mediators activates downstream signaling pathways that include Jak/STAT, PI3K/Akt/mTOR and Src pathways [277, 278]. STAT5 is the main STAT recruited, but STAT1 and STAT3 may also be recruited by IL-7 signaling [214, 217] (Figure 3A). The role of Src kinases in IL-7 signaling is unclear, since Src kinases in T-cells do not appear to regulate the essential signals delivered by IL-7, as observed by the mild phenotype of the $p59^{fyn\ -/-}$ mice compared to deficiency in IL-7, IL-7Rα or γ_C [279]. Thus, the canonical IL-7 downstream signaling for T-cells considers essentially Jak1/3-STAT5/1/3 and PI3K/Akt/mTOR pathways [188, 189].

Below, the major signaling elements of the pathways described to be activated by IL-7 are introduced.

1.7.1 PI3K/Akt pathway

The PI3K/Akt pathway is a central cellular signaling pathway involved in the regulation of cell growth, survival, metabolism, proliferation, glucose homeostasis and vesicle trafficking [280].

1.7.1.1 Classification of PI3Ks

Although PI3Ks are mostly known as lipid kinases, they can also perform serine/threonine protein kinase activities [281, 282]. The PI3K family is grouped into 3 classes (I-III).

Class I PI3Ks are further divided in 2 sub-classes. The class IA is activated by receptor tyrosine kinases (RTKs) and class IB by G-protein coupled receptors (GPCRs). Class IA forms heterodimers with a p85 regulatory subunit (p85α/55α/50α, p85β and p55γ) and a p110 catalytic subunit (p110α, p110β and p110δ). Class IB forms heterodimers with a p101, p84 and p87PIKAP regulatory subunit and a p110γ catalytic subunit [280].

Class II consists only of a p110-like catalytic subunit. Three isoforms are described: PIK3C2α, PIK3C2β and PIK3C2γ. The functions of this class are not yet well understood, but appear related with membrane trafficking and receptor internalization when activated in response to RTKs, integrins and cytokine receptors [280].

Class III contains the vesicle protein sorting 34 (Vps34) catalytic subunit and the Vps15 regulatory/catalytic subunit [283]. Given its central role in autophagy, this class will be discussed in more detail in the autophagy section (1.8) below.

In vivo, class I PI3Ks primarily catalyze the reaction phosphatidylinositol-4,5-bisphosphate → phosphatidylinositol-3,4,5-trisphosphate (PIP_2 → PIP_3). Class III performs the reaction phosphatidylinositol → phosphatidylinositol-3-phosphate (PI → PI3P). Class II are believed to generate PI3P, PI-3,4-P_2 and possibly PIP_3 [280, 284].

By far, the knowledge of the function of PI3K proteins has relied mostly on the study of the class I and its interaction with Akt(PKB) [285-288], and more recently on class III due to its role in autophagy [283, 289].

PI3K class I has been clearly implicated in cancer. *PIK3CA* (p110α) gene is frequently mutated in multiple tumors such as breast (26%), colon (26%) and hepatocellular (36%) cancers [290, 291]. As discussed before, both PI3K and Akt somatic mutations occur in T-ALL [147].

1.7.1.2 Activation and inactivation of PI3K/Akt pathway

Akt, a serine/threonine kinase, has 3 isoforms: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ [292]. *AKT* gene amplifications are relatively frequent in cancers such as head and neck (30%) [293], pancreatic (20%) [294], gastric (20%) [295], ovarian (12%) and breast (3%) [296] cancers. Activating mutations were first described in breast (8%), colorectal (6%) and ovarian (2%) cancers [297]. In T-ALL, *AKT* activating mutations are rare (2%) [147].

Upon activation, PI3K generates PIP_3 at the plasma membrane [287]. PIP_3 acts as a second messenger and allows the recruitment of proteins containing a pleckstrin homology (PH) domain to the vicinity of the membrane [280]. Those proteins include Akt and the 3-phosphoinositide dependent protein kinase-1 (PDK1) [298]. PDK1 phosphorylates Akt at the activating residue threonine 308 (T308) [299]. The mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Akt at another activation residue, serine 473 (S473) [300].

The dephosphorylation, and consequent inactivation of the pathway, is mediated by the phosphatases PTEN and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase (SHIP) [301, 302]. Akt is also inactivated by protein phosphatase 2a (PP2A) and PH domain and leucine rich repeat protein phosphatase (PHLPP) [303, 304].

1.7.1.3 Downstream targets of Akt activation

Akt phosphorylates multiple substrates with diverse cellular functions, including cell cycle, survival, metabolism and transcription (Figure 4).

A major Akt target is the glycogen synthase kinase-3α/β (GSK3α/β), which is inhibited by Akt [305]. Importantly, GSK3 is involved in the degradation of cyclin D1, thus preventing cell cycle progression [306, 307] and it is also involved in promoting degradation of myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic protein [308]. Thus, by inactivation GSK3, Akt contributes to both proliferation and cell viability.

Other important Akt substrates are the forkhead box O family of transcription factors (FoxOs). Phosphorylation of FoxOs by Akt, lead to their inactivation by cytoplasmic retention and binding to 14-3-3 chaperones [309]. FoxOs promote the transcription of pro-apoptotic genes (e.g. *BCL2L11*/Bim) and cell cycle inhibitors (e.g. *CDKN1B*/$p27^{kip1}$) [310]. Additionally, FoxO1 is a direct transcription factor of *IL7R* [311, 312]. This regulation may provide a negative feedback loop on IL-7 signaling via Akt-mediated FoxO1 inactivation. The NF-κB pathway may be activated by Akt through phosphorylation and activation of inhibitor of NF-κB kinases (IKKs), leading to the degradation of the inhibitor of NF-κB (IκB) [313].

Increased expression of GLUT transporters and glucose uptake is a common event in tumors, which may provide extra energy and metabolic intermediates for cell growth [314, 315]. Akt promotes the expression and translocation to the membrane of GLUT1, GLUT3 and GLUT4 [316, 317]. Notably, IL-7 was shown to increase GLUT1 expression and glucose use in both normal and malignant T-cells in a PI3K/Akt-dependent manner [271, 318].

Importantly, Akt activates mTOR, a central module in the regulation of overall cell growth and metabolism in the cell (Figure 4). Akt phosphorylates the tuberous sclerosis complex 2 (TSC2), consequently destabilizing the heterodimer TSC1/2 [319, 320]. Destabilization of the TSC complex activates the small GTPase Rheb and as a result mTORC1 becomes active [319].

1.7.2 mTOR pathway

mTOR pathway integrates environmental cues from within and without the cell to transition between anabolic and catabolic states, controlling cell metabolism, growth, proliferation and survival (Figure 4) [321].

mTOR is a serine/threonine kinase that forms two complexes: mTOR complex 1 (mTORC1) and mTORC2. mTORC1 has 5 components: mTOR, the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8/GbL); proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor). The mTORC2 complex has 6 components: mTOR; rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (Protor-1); mLST8; and Deptor [321]. mTORC1 is sensitive to rapamycin, whereas mTORC2 is not [322-324].

The regulation and function of mTORC1 is better understood than that of mTORC2. Most signals impinge on regulation of TSC1/TSC2 complex and sometimes directly on mTORC1 (Figure 4). For instance, the canonical insulin and insulin-like growth factor 1 (IGF1) activate PI3K/Akt and Ras/MAPK pathways. Effector kinases of each pathway, Akt, Erk1/2 and $p90^{RSK}$, directly phosphorylate TSC1/2 [320, 325-327]. Akt may also directly stabilize mTORC1 by PRAS40 phosphorylation [328]. Stresses such as low energy, oxygen or DNA damage can input signals to mTORC1. The adenosine monophosphate-activated protein kinase (AMPK), in response to low energy or oxygen, can phosphorylate and promote TSC1/2 activity [329]. AMPK may also phosphorylate Raptor and inhibit mTORC1 [330]. DNA damage signals to mTORC1, in a p53-dependent manner, by promoting the transcription of TSC2, PTEN [331, 332] and Sestrin1/2-dependent activation of AMPK [333]. Upstream signals appear to require the presence of aminoacids to be able to activate mTORC1 [334, 335]. Downstream mTORC1-regulated processes include protein synthesis and inhibition of the catabolic process of autophagy. mTORC1 phosphorylates and inactivates the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), promoting cap-dependent protein translation [336]. In addition, mTORC1 activates the p70 ribosomal S6 kinase 1 (S6K1), increasing mRNA biogenesis and cap-dependent translation [337]. It has been known for some years that rapamycin promotes autophagy in mammalian cells [334]. The precise mechanism was discovered more recently. mTORC1 phosphorylates and inhibits the unc-51-like kinase (ULK) protein complex [338-340], which is required for

autophagy initiation. mTORC1 prevents AMPK, a powerful activator of autophagy, from activating the ULK complex [341, 342].

mTORC2 regulation is less understood. mTORC2 signaling does not seem dependent on nutrients but it is dependent on growth factors. Studies indicate that activation by growth factors is ribosome and PI3K-dependent [343]. Importantly, mTORC2 controls several kinases including Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase Cα (PKCα). mTORC2 directly activates Akt by phosphorylating the Ser473 site required for its maximal activation. This discovery established mTORC2 as the elusive PDK2 known to be responsible for Ser473 phosphorylation [300, 321].

Notably, rapamycin promotes apoptosis in T-ALL cells [268, 344], particularly in the context of IL-7-induced T-ALL cell survival [268]. Moreover, oncogenic Notch activation was shown to promote mTOR activity in a c-Myc-dependent manner [345].

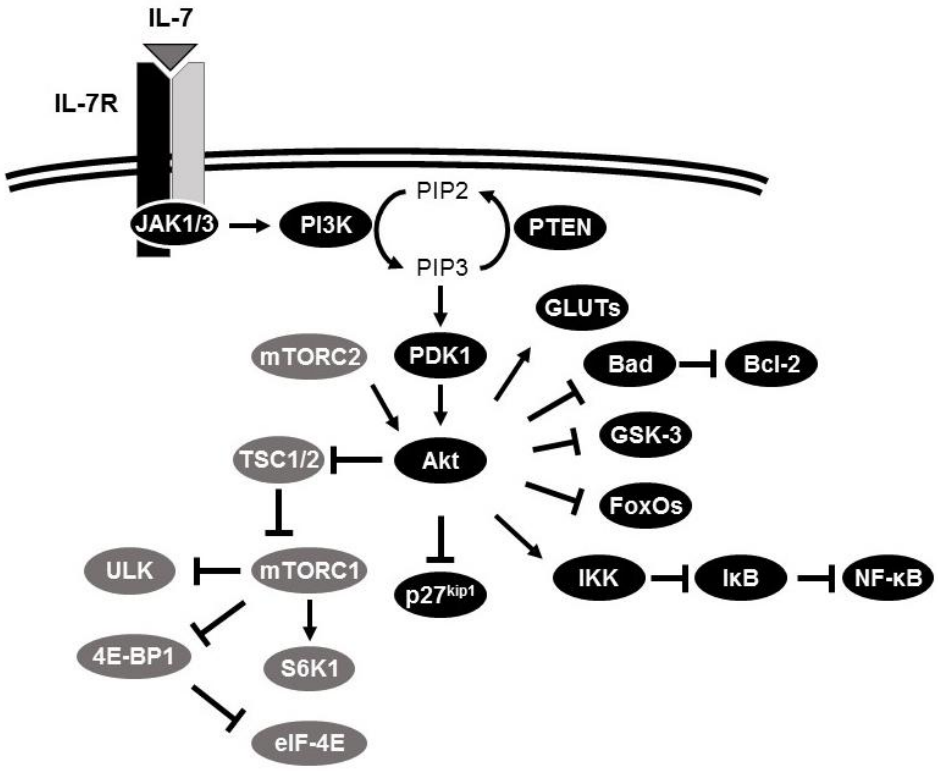

Figure 4. IL-7-mediated activation of PI3K/Akt/mTOR pathway and potential downstream effectors. Activated PI3K phosphorylates PIP2 into PIP3. PTEN antagonizes PI3K by dephosphorylation of PIP3 into PIP2. Generation of the second messenger PIP3 promotes activation of Akt (PKB) by PDK1. mTORC2 acts as PDK2 also activating Akt. Activated Akt has numerous intracellular targets. Direct targets include Bad, GSK-3, FoxO family, IKK and TSC1/2 complex. Akt activation also promotes surface expression of glucose transporters (GLUTs). Phosphorylation and consequent inactivation of the TSC1/2 complex leads to stabilization and activation of the mTORC1 complex, which in turn is involved in downregulation of autophagy via inactivation of the ULK complex. mTORC1 promotes mRNA biogenesis and translation via activation of S6K1 and eIF-4E. Further details and consequences of PI3K/Akt/mTOR pathway activation are described in the main text.

1.7.3 Jak/STAT pathway

The Jak/STAT pathway is the canonical pathway for growth factor and cytokine response. This pathway, due to its simplicity and sophistication, provides very quick signal transduction from the membrane to the nucleus. Upon receptor engagement, activation of Jak tyrosine kinases trans-phosphorylate each other and the cytoplasmic tail of the receptor. This action recruits STAT proteins, which are phosphorylated by Jaks. Activated STATs homo- or heterodimerize translocate to the nucleus. There they bind DNA as dimers or tetramers and regulate gene transcription (Figure 5) [346].

In mammals, there are four Jak proteins: Jak1. Jak2, Jak3 and Tyk2. Jaks selectively bind different receptor chains. There are seven STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. Although different cytokines preferentially activate a

particular STAT, there is often lesser activation of other STATs. Thus, there is a promiscuity in the generation of homo/heterodimers or tetramers, which in turn may have qualitative and quantitative implications in gene transcription [346]. Important genes regulated by STATs include Bcl-2 family members, cyclin D1, $p21^{cip1}$, IL-2Rα, and c-Myc [347-349]. The negative regulators of the pathway belong to three classes: suppressor of cytokine signaling (SOCS), protein inhibitors of activated stats (PIAS) and protein tyrosine phosphatases (PTP) [350, 351].

STAT5a/b are the main IL-7-actived STATs [214, 217]. Mice deficient in either STAT5a or STAT5b do not have major consequences on T or B cell development [352-355]. Interestingly, the initial studies on Stat5a/b double knockout mice also reached a similar conclusion [356, 357]. Thus, the large differences in phenotypes between the double Stat5 knockout and mice lacking IL-7Rα, Jak3 or γ_C, led to the conclusion that IL-7 may regulate lymphocyte development in a STAT5-independent manner. Importantly, STAT gene targeting originated a partially functional protein be expressed, which may have accounted for the mild phenotype observed [358]. Subsequent studies in full Stat5a/b knockout, established that absence of Stat5 led to SCID and was largely similar to deficiencies in IL-7Rα, γ_c, and Jak3 [359].

1.7.4 MAPK pathway

The MAPK pathway has three major signaling modules: the classical extracellular signal-regulated kinase (Erk), the c-Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK) and the p38 MAPK pathways. Each family cascade is activated in a series typically containing three levels: a MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK) that in turn phosphorylates a MAP kinase. Examples of MAPKKK members include Raf-1, B-Raf and c-MOS; of MAPKK members include MEK1 and MEK2; and of MAPK members include Erk-1, Erk-2, p38 and JNK1. The Erk pathway is activated mostly by mitogenic growth factors and cytokines, whereas the p38 MAPK and JNK pathways tend to be activated by stress factors and cytokines (particularly pro-inflammatory cytokines) [326, 360].

1.7.4.1 MEK/Erk pathway

Erk-1/2 (p44/42) are activated by the dual-specificity kinases (serine/threonine-tyrosine) MEK1/2 (Figure 5). Erk phosphorylates and activates several targets, including the kinases $p90^{RSK}$ [361], Mnk [362] and Msk [363], and important transcription factors such as

c-Myc, c-Fos, c-Jun and C/EBPβ [364], and promote cell cycle progression by regulating the expression of cyclin D1, $p27^{kip1}$, $p21^{cip1}$ [365].

The role of MEK/Erk activation by IL-7 in normal T-cells is ill defined. Although, IL-7 activates Erk-1/2 during stages of mouse B-cell development [366] it does not seem to do so in T-cell lines [367, 368]. In humans, IL-7 also does not seem activate the pathway in thymocytes [224] or peripheral blood T-cells [251, 369]. Interestingly, in T-cells from rheumatoid arthritis patients, IL-7 appears to potentiate TCR-mediated Erk-1/2 signaling [370]. Thus, more in-depth studies on the role of IL-7 in normal T-cells is required. However, in T-ALL blasts IL-7 is capable of activating the MEK/Erk pathway [371, 372]. In addition, combination of MEK and PI3K/Akt pathway inhibitors, showed a synergistic effect in several human T-ALL samples, including mutants in *IL7R* or downstream signaling components [372].

1.7.4.2 $p38^{MAPK}$ and JNK/SAPK

$p38^{MAPK}$ has several isoforms (α, β, γ and δ), which are activated by MEK3/6 and to some extent by MEK4 [326]. Targets of $p38^{MAPK}$ include PLA2, Tau, and the transcription factors such as ATF, MEF2A, Elk-1, NF-κB, Ets-1 and p53 [373]. The protein kinase JNK also has multiple isoforms (JNK1/SAPKγ, JNK2/SAPKα, JNK3/SAPKβ), which are activated by MEK4 and MEK7 [326].

Crawley and colleagues [367] showed that both $p38^{MAPK}$ and JNK pathways may be activated by IL-7 in human mature T-cells and murine T-cell lines, where at least $p38^{MAPK}$ mediates IL-7-dependent proliferation. Moreover, $p38^{MAPK}$ is involved in IL-15- and IL-7-dependent proliferation of memory T-cells [374]. Notably, there are no studies performed in developing thymocytes. However, constitutive activation of $p38^{MAPK}$ induces cell cycle arrest and blocks differentiation in DN thymocytes [375], a role that conflicts with IL-7 activity in this stage. Moreover, in a murine IL-7-dependent thymocyte line, IL-7 withdrawal induced a transient stress response that contributed to cell death [376]. Thus, the role of stress-induced MAPK signaling pathways in IL-7-mediated functions in either normal or malignant T-cells remains to be fully explored.

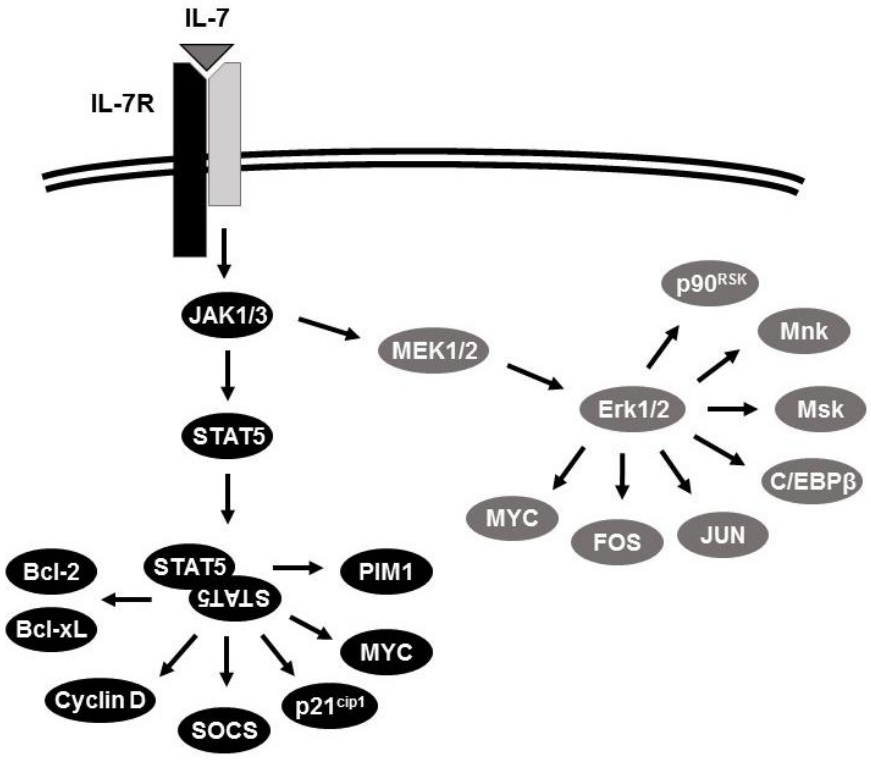

Figure 5. IL-7-mediated activation of JAK/STAT5 and MEK/Erk pathways and potential downstream effectors. IL-7R in T-ALL activates JAK/STAT5 and MEK/Erk signaling pathways. STAT5 is recruited to the IL-7Rα and phosphorylated, leading to dimerization and nuclear translocation. In the nucleus, STAT5 dimers or tetramers bind DNA and may promote transcription of several genes, such as Bcl-2-family members, D-type cyclins, *PIM1*, *MYC*, *CDKN1A* ($p21^{cip1}$) and the *SOCS* family of negative signaling regulators. IL-7 does not appear to activate MEK/Erk pathway in normal T-cells, but it does so in T-ALL. Erk1/2 activation may lead to phosphorylation and activation of Mnk, Msk, Rsk (involved in mRNA translation). In the nucleus, Erk1/2 phosphorylates and potentiates the action of transcription factors such as, MYC, FOS, JUN, C/EBPβ.

1.8 Autophagy and cell metabolism

1.8.1 Autophagy

Autophagy (i.e. self-eating), is a cellular process associated with the degradation of proteins or organelles in a cell, particularly during starvation. Important substrates include long-lived proteins, endoplasmic reticulum (ER), mitochondria, peroxisomes, nucleus and ribosomes. Degradation by autophagy promotes the recycling of nutrients and consequent prolonged cell survival [377]. Autophagy has a homeostatic, housekeeping, function, since autophagy-deficient mice accumulate misfolded and damaged proteins [378-380]. Three major types of autophagy exist: macroautophagy, microautophagy and chaperone-mediated autophagy., In this work we will focus on macroautophagy, which will be referred henceforth simply as autophagy.

Functionally, autophagy can be described as a multi-step process (Figure 6). During the initiation step, the phagophore assembly site (PAS) forms. In the nucleation step, there is assembly of the molecular machinery required for the formation of the double membrane characteristic of autophagy, the phagophore. During the expansion step, the autophagic membrane completely engulfs the autophagic cargo, forming the autophagosome. Subsequently, the autophagosome fuses with the lysosome, forming the autolysosome, then cargo is degraded and nutrients recycled [377].

The core of the autophagic machinery includes four major components: the ULK complex, the Vps34 complex, the autophagy related 12 (Atg12)-Atg5-Atg16 complex and the microtubule-associated protein 1 light chain 3 (LC3/Atg8). The ULK complex contains the serine/threonine kinases ULK1/2 (Atg1), Atg13, FAK family kinase interacting protein of 200 kDa (FIP200/Atg17) and Atg101 [381]. This complex is negatively regulated by mTOR and positively regulated by AMPK [382]. The autophagy-specific Vps34 complex contains the class III PI3Ks Vps34 and Vps15, Beclin 1 (Atg6) and Atg14 [383]. ULK1 and Vps34 complexes drive the nucleation of the isolation membrane and the recruitment of additional ATG proteins, by phosphorylation (ULK) or production of a PI3P pool [383]. During the expansion phase, the Atg12-5-16 complex (an E3-like ligase) assists in the lipidation of LC3 and the family members GATE16 and GABA receptor-associated protein (GABARAP) [383]. The process continues until the completion of the autophagosome. LC3 associated with autophagosome and facing the inner side is degraded in the autolysosome [383].

The cleavage and lipidation of pro-LC3 (LC3-I), a pool of non-autophagosome associated LC3, into lipidated phosphatidylethanolamine (PE)-conjugated LC3 (LC3-II) is a hallmark of autophagy activation and used in autophagy research. Only LC3-II is able to associate with autophagosomes [384]. There are a number of assays to measure and quantify the autophagic flux, each with its own advantages and pitfalls [384]. In this work we chose to use a ratio of LC3-II/LC3-I measured under incubation with the lysosomal inhibitor hydroxychloroquine (HCQ). This assay blocks the final steps of autophagy, promoting the accumulation of autophagosomes/autolysosomes in proportion to the autophagic flux [384].

Deletion of *Atg5* in mouse T-cells decreases total thymocyte and lymphocyte numbers, associates with increased spontaneous apoptosis *in vivo* of mature CD8 T-cells and defective activation-induced proliferation *in vitro* of both CD4 and CD8 T-cells [385]. Deletion of different autophagy genes in early T-cell progenitors suggests that autophagy is important for, at least, DN thymocyte survival or proliferation [386-389]. Additionally, mature Atg3-

deficient T-cells long-term cultured *in vitro* with IL-7 exhibited a higher death rate than autophagy-proficient T cells cultured in the same conditions [390].

In T-ALL, the role of autophagy is poorly understood. For instance, it was reported on several T-ALL cell lines that Akt inhibition was cytotoxic and led to upregulated autophagy [391, 392]. In both reports autophagy was cytoprotective. On the other hand, other studies suggested that autophagy may be cytotoxic. In *in vitro* models of T-ALL glucocorticoid resistance, autophagy-dependent necroptosis was required to overcome glucocorticoid resistance in T-ALL [393]. In another study, autophagy upregulation enhanced cell apoptosis in an *in vitro* model of ER stress induction [394]. Of note, the Jurkat cell line was used in two of the studies which found opposing roles for autophagy [391, 394]. It is likely that the intracellular mechanisms, targeted by the pharmacological inhibitors, either affected or required autophagy in a different manner. This should be taken into account when considering autophagy as a therapeutic target.

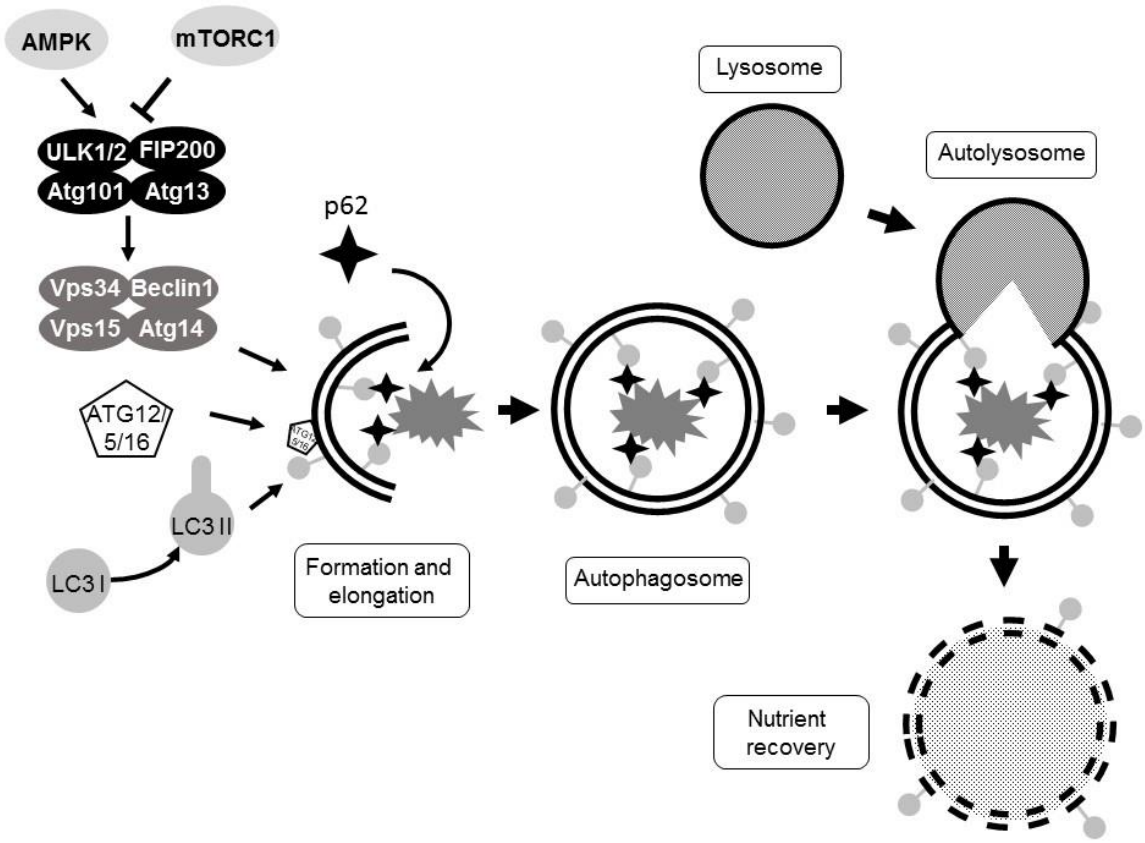

Figure 6. Overview of autophagy. The ULK complex is negatively regulated by mTORC1 and positively regulated by AMPK. Both the ULK and Vps34 complexes are required for autophagy initiation. ULK1 and Vps34 complexes drive the nucleation of the isolation membrane and the recruitment of additional ATG proteins, by phosphorylation (ULK) or production of a PI3P pool (Vps34). To participate in autophagy, LC3-I is cleaved and lipidated into LC3-II. During the formation and elongation phase the Atg12-5-16 assists in the lipidation and anchorage of LC3 to the autophagosome membrane. p62 acts an anchor between the cargo to be degraded and membrane-bound LC3. The process continues until the autophagosome is complete. Subsequently, the autophagosome fuses with the lysosome, forming the autolysosome, then the cargo is degraded and nutrients recycled.

1.8.2 Cell metabolism: a brief overview of aerobic glycolysis

Deregulated cellular energetics and metabolic pathways have emerged as a new hallmarks of cancer [395].

Aerobic glycolysis (Warburg effect), is the conversion of glycose to lactate in the presence of oxygen when one would expect glucose to be metabolized via the tricarboxylic acid (TCA) cycle with oxidative phosphorylation (OXPHOS) [396]. This phenomenon was observed by Otto Warburg in cancer cells many decades ago. However, it is currently accepted that both cancer cells and normal cells may use aerobic glycolysis under specific circumstances, such as in periods of high proliferation. Aerobic glycolysis serves mainly to

replenish metabolic intermediates [396]. A schematic view of glycolysis is presented in Figure 7.

Mature T-cells when transitioning from a resting to an activated state, perform a metabolic switch from OXPHOS to aerobic glycolysis [397, 398].

There is evidence that IL-7 may play a role in the regulation of glycolysis in cells. IL-7 was shown to increase GLUT1 expression and glucose use in both normal [318] and malignant T-cells [271]. Also in a murine IL-7-dependent T-cell line, IL-7 upregulated hexokinase II (HK2), the enzyme that catalyzes the rate-limiting and first obligatory step of glucose metabolism [399].

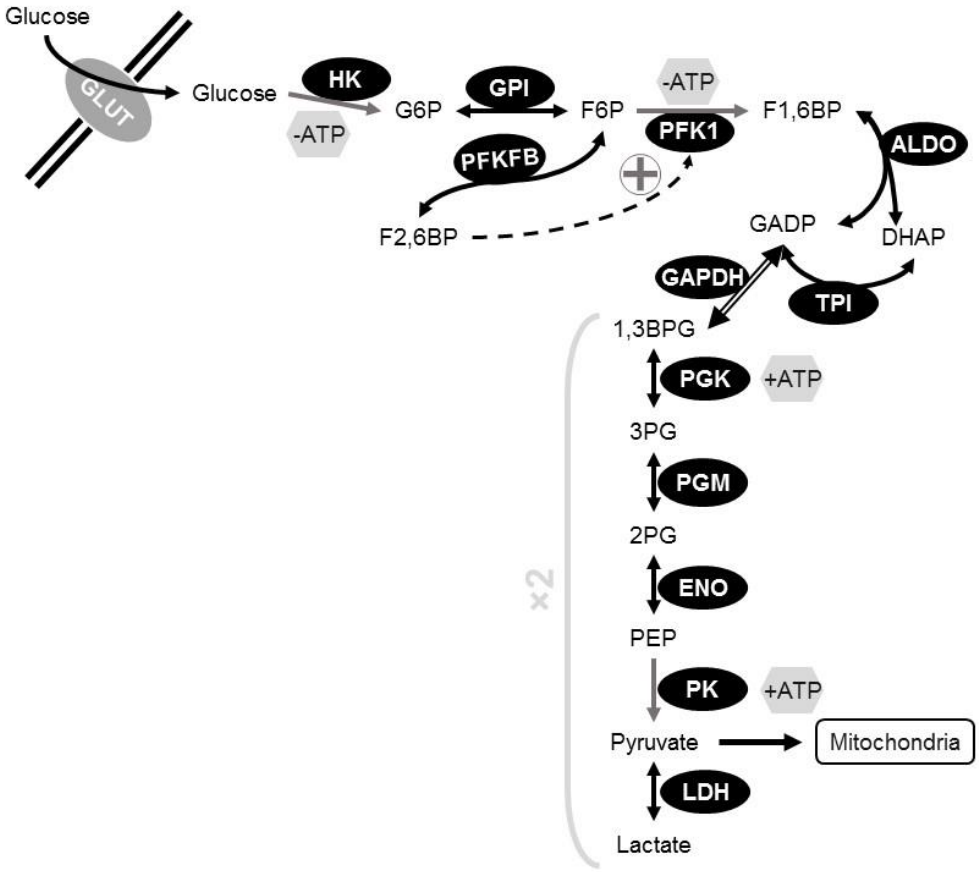

Figure 7. Schematic view of glycolysis. Grey arrows indicate irreversible reactions; the opposite reaction requires a different enzyme. Two-headed arrows indicate reversible reactions. Reaction where ATP is produced or consumed are indicated with +ATP or –ATP, respectively. Glycolysis produces, at most, 2 molecules of pyruvate per molecule of glucose and a gain of 2 NADH and 2 ATP. The glycolytic pathway proceeds as depicted in the scheme. The reverse reactions are associated with gluconeogenesis. Important steps are described next. Glucose is imported into the cell by glucose transporters (GLUT) residing in the plasma membrane, where it is rapidly phosphorylated into G6P by HK enzymes, trapping glucose in the cell. Phosphorylation of F6P into F1,6BP by PFK1 is a rate-limiting step and marks the first committed step of glycolysis. The generation of F2,6BP by the bifunctional enzyme PFK2-F2,6BPase (PFKFB) constitutes an important regulatory step since F2,6BP is the most potent activator of PFK1. NADH is synthesized in the glycolysis and consumed in gluconeogenesis by GAPDH. Pyruvate is the last metabolite of glycolysis. Pyruvate may by imported into the mitochondria to be incorporated into the TCA cycle or converted to lactate

and exported from the cell. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; F2,6BP, fructose-2,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GADP, glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; GLUT, glucose transporter; HK, hexokinase; GPI, glucose-6-phosphate isomerase; PFK1, phosphofructokinase-1; PFK2-F2,6BPase, phosphofructokinase-2-fructose-2,6-bisphosphatase; ALDO, aldolase; TPI, triose-phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase;

1.9 Aims

The global aim of this work is to expand the knowledge of T-ALL biology and pathophysiology, particularly concerning the cell-autonomous mechanisms leading to leukemogenesis and the cross-talk between the microenvironment and the leukemic cell. We put particular emphasis in IL-7/IL-7R-related events and signaling pathways. To achieve these goals, we used primary T-cell leukemia samples coupled with *in vitro* and *in vivo* analysis of relevant cell lines.

In Chapter 2, we sought to evaluate whether IL-7Rα activating mutations exist in T-ALL and if so, provide mechanistic insights and find whether they contribute to the leukemogenic process. First, we screened the coding sequence of the *IL7R* gene in three different cohorts of T-ALL samples and found that roughly 9% of the samples display *IL7R* mutations. Next, we studied the biological and clinical features associated with *IL7R* mutations by cytogenetic and gene expression analysis. We characterized the molecular features of IL-7R signaling by reconstitution of the IL-7R signaling machinery in cell lines and by assessing signaling pathway activation. In parallel, we tested the functional consequences of IL-7Rα signaling. We used Ba/F3 and D1 cell lines for *in vitro* studies. For *in vivo* studies, we used D1 cell line or bone marrow cells of animals with different deletions of IL-7R component genes. Finally, we tested *in vitro* the therapeutic potential of our findings using Jak/STAT pathway inhibitors.

In Chapter 3, we sought to evaluate whether activation of the Jak/STAT5 pathway by IL-7 is necessary and sufficient for IL-7-mediated pro-survival and proliferative effects in T-cell leukemia. We used both IL-7 dependent and responsive cell lines, as well as primary T-ALL samples. First, we demonstrated that IL-7 activated the Jak/STAT5 signaling in T-ALL by western blot and STAT5 DNA binding. We evaluated the functional consequences and molecular mechanisms of STAT5 inhibition in T-ALL upon IL-7 stimulation. For this, we used flow cytometry, western blot, qPCR and radioactive based assays. The results

obtained prompted us to evaluate the STAT5 transcriptional network elicited by IL-7 signaling using next generation sequencing techniques, which in turn drove us to investigate the role of PIM1 in IL-7-mediated signaling in T-ALL. We used flow cytometry, western blot and radioactive based assays to test this.

In Chapter 4, we aimed at discovering if IL-7 could regulate autophagy in T-ALL and exploring the functional consequences of that putative regulation. First, we evaluated whether IL-7 regulated autophagy in T-ALL cells, using IL-7-dependent TAIL7 T-ALL cells. Next, we used pharmacological inhibitors to characterize the molecular actors involved in IL-7-mediated autophagy. To do so, we used flow cytometry, western blot, and confocal and electron microscopy. With the data we obtained, we sought to understand the regulation of IL-7-mediated autophagy regulation in different nutrient (serum) conditions. In this part, we used flow cytometry and western blot analysis of TAIL7 and primary T-ALL cells.

In Chapter 5, building upon data generated in Chapter 2, we aimed at studying cellular pathways affected by IL-7 signaling. We used bioinformatics tools to do so. The results obtained, prompted us to analyze the effect of IL-7 on glycolysis. We determined the glycolytic rate of TAIL7 cells stimulated with IL-7 by determining glucose consumption and lactate production. We used qPCR to assess the expression of genes in the glycolytic pathway.

1.10 References

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CHAPTER 2

Oncogenic *IL7R* gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia

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2.1 Abstract

Interleukin 7 (IL-7) and its receptor, formed by IL-7Rα (*IL7R*) and γc, are essential for normal T-cell development and homeostasis. Here, we show that *IL7R* is a *bona fide* oncogene mutated in T-cell acute lymphoblastic leukemia (T-ALL). Nine percent of T-ALL patients display somatic gain-of-function *IL7R* exon 6 mutations. In most cases *IL7R* mutations introduce an unpaired cysteine in the extracellular juxtamembrane-transmembrane region and promote *de novo* formation of intermolecular disulfide bonds between mutant IL-7Rα subunits, thereby driving constitutive signaling via JAK1 and independently of IL-7, γc or JAK3. *IL7R* mutations induce a gene expression profile partially resembling that provoked by IL-7 and are enriched in the T-ALL subgroup comprising *TLX3* rearranged and *HOXA* deregulated cases. Notably, *IL7R* mutations promote cell transformation and tumor formation. Overall, our findings indicate that *IL7R* mutational activation is involved in human T-cell leukemogenesis, paving the way for therapeutic targeting of IL-7R-mediated signaling in T-ALL.

2.2 Introduction

Signaling mediated by IL-7/IL-7R is essential for normal T-cell development and homeostasis [1, 2]. Mice with IL-7 or IL-7R deficiency display an early block in thymocyte development and reduced numbers of non-functional peripheral T-cells [3, 4]. In humans, *IL7R* inactivating mutations result in the development of SCID [5, 6], whereas *IL7R* polymorphisms have been shown to confer susceptibility to multiple sclerosis [7, 8]. There is circumstantial evidence that IL-7 and IL-7R may also partake in T-cell leukemia progression. IL-7 transgenic mice develop lymphomas [9, 10] and AKR/J mice, which develop spontaneous thymic lymphomas, display high IL-7R levels [11]. In addition, T-cell acute lymphoblastic leukemia (T-ALL) cells respond to IL-7 in vitro in a majority of patients [12-14]. Notably, IL-7Rα is transcriptionally upregulated by Notch [15], one of the most commonly mutated genes in T-ALL [16], and appears to be involved in Notch-mediated leukemia cell maintenance [15]. The possibility that IL-7/IL-7R-mediated signaling may play a role in T-cell leukemia is further supported by the observation that 18% of adult and 2% of pediatric T-ALL patients display activating mutations in JAK1, a tyrosine kinase that directly binds IL-7Rα [17] amongst other receptors. Despite these observations, no direct confirmation exists that IL-7R-mediated signaling plays an active part in the T-cell leukemogenic process in humans.

2.3 Methods

Cells. Primary leukemia cells were obtained from the bone marrow and/or peripheral blood of diagnostic pediatric T-ALL and pre-B ALL patients. Samples were enriched by density centrifugation over Ficoll-Paque (GE Healthcare), washed twice in culture medium (RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin), subjected to immunophenotypic analysis by flow cytometry, and classified according to their maturation stage (Table 1). Informed consent and institutional review board approval were obtained for all primary leukemia collections from Centro Infantil Boldrini, Campinas, SP, Brazil (Boldrini); Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia, Germany (COALL); and Dutch Childhood Oncology Group, The Hague, The Netherlands (DCOG). Primary leukemia cells from patient P1 were cultured in culture medium as $2x10^6$ cells/mL. Growth factor-dependent D1 and Ba/F3 cells were maintained in culture medium plus 50 ng/mL rmIL-7 (PeproTech) or 1% (v/v) WEHI-3B-conditioned medium as source of mIL-3, respectively. Phoenix-Eco packaging cell line and 293T cells were maintained in DMEM (Mediatech or Gibco), supplemented with 10% FBS and penicillin/streptomycin.

***IL7R* sequencing and mutational analysis.** Total RNA was extracted and RNA integrity was confirmed by agarose gel electrophoresis. One microgram of total RNA was reverse transcribed to cDNA using the ImProm II Reverse Transcriptase (Promega). The complete coding sequence of *IL7R* and the JH2 domain of *JAK1* and *JAK3* were amplified by RT-PCR and sequenced on both strands for a total of 68, 52, and 52 T-ALL samples, respectively, from Centro Infantil Boldrini. The same primers were used for amplification and sequencing (see Supplementary Table 2). Mutations found in the *IL7R* were confirmed in the corresponding genomic DNA by PCR amplification of exon 6 coding and flanking intronic sequences followed by homo-heteroduplex formation analysis [32] and/or sequencing. Mutations in exon 6 coding and flanking intronic sequences were further investigated in 119 T-ALL cases from DCOG and COALL patient series, by sequencing, and in 50 precursor B-cell ALL cases from Centro Infantil Boldrini by homo-heteroduplex formation analysis.

Geneset enrichment analysis (GSEA). GSEA was performed on our Affymetrix U133 plus 2.0 microarray expression dataset for 117 T-ALL cases [18] using 100 random

permutations. The microarray expression set is available at http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE26713. Enrichment score and nominal p-value were obtained for genes that are upregulated in human lymphocytes following exposure to IL-7 as described before [19], for which probesets were present on the U133 plus 2.0 expression array (*SOCS2*, *CCL4*, *CCL3*, *TNF*, *PMAIP1*, *LRP1*, *PIM1*, *AHR*, *UPP1*, *GARS*, *CCND2*, *DUSP5*, *FLT3LG*, *IL2RA*, *LIF*, *CEACAM1*, *MX1*, *TNFSF10*, *CSF2*, *CD69*, *CXCR4*, *CSF1*, *SOCS1*, *IL18R1*, *DPP4*, *CASP3*, *XBP1* and *BCL2*).

Gene expression microarray analysis and unsupervised cluster analysis. RNA isolation for 117 pediatric T-ALL patient samples, integrity analyses of RNA, copy-DNA and cRNA syntheses and hybridizations to Human Genome U133 plus2.0 oligonucleotide microarrays have been described before [18]. Differentially expressed genes associated with *IL7R* mutations were obtained by regression analysis using the LIMMA package. Unsupervised cluster analyses were performed in dChip as described previously [18].

Construction of *IL7R* expression vectors. The coding sequence of the *IL7R* was PCR amplified from cDNA of blood mononuclear cells of a healthy donor, using primers *IL7R* 3U32, and *IL7R* 1434L39 (see Supplementary Table 2 for primer information). The reverse primer did not incorporate the stop codon. The undigested PCR product was cloned into pGEM T-Easy (Promega) and verified by sequencing. The cloned fragment was subsequently digested with XmaI, treated with the Klenow fragment of DNA polymerase I, then digested with KpnI and cloned into the XbaI (blunted with Klenow) / KpnI sites of the pUC19 vector, resulting in the clone pUC19/IL7R. By doing so, a stop codon was re-inserted, but the last C-terminal amino acids QNQ of the normal IL7Rα were changed to QNPG. A lentiviral expression vector of *IL7R*, #304/IL7R, was obtained by subcloning the *IL7R* EcoRI(Klenow)-SalI fragment of pUC19/IL7R in place of the ΔLNGFR SmaI-SalI fragment of a pCCL.sin.cPPT.minCMV.eGFP.PGK.ΔNGFR.WPRE lentivirus vector [20] (kindly provided by Dr Luigi Naldini). To obtain a retroviral expression vector, the *IL7R* fragment was amplified from pUC19/IL7R using primers hIL7R5'BglII and hIL7R3'EcoRI. The PCR product was digested with BglII and EcoRI and cloned into pMIG (Addgene 9044, contributed by William Hahn). Equal procedures were used to obtain the expression vectors for the mutants *IL7Rs*. Site-directed mutagenesis of the novel cysteine was obtained by PCR amplification of a BamHI-BbsI fragment spanning positions 803 to 934 of the *IL7R* sequence

(NM_002185.2), using the pUC19/IL7R clone as a template, one of the following forward primers: hIL7R_cP1s**, hIL7R_cP2s, hIL7R_cP2a, and the** reverse primer hIL7R_BbsI. The amplified fragments were digested with BamHI and BbsI and inserted into pUC19/IL7R, thus replacing the *IL7R* fragment containing the cysteine codon. Subsequently, the mutants of the mutant *IL7R* coding sequences were cloned into the lentiviral and retroviral vectors, as described above. All of the above clones were verified by sequencing.

Retroviral infection of D1, Ba/F3 and mouse bone marrow cells. Wild type or mutant full-length human *IL7R* was cloned into pCCL.sin.cPPT.minCMV.eGFP.PGK.ΔNGFR.WPRE lentiviral [20] or pMIG retroviral vectors, both of which also drive the expression of eGFP. Where indicated, C>A or C> S mutations were introduced into the mutant *IL7R* using PCR strategies. All subcloned genes and constructs were verified by DNA sequencing. D1 cells were infected in Retro-Nectin (Takara, Santa Ana, CA)–coated plates with pMIG supernatant produced using the phoenix-Eco packaging cell line. Ba/F3 cells were infected with either pMIG or lentiviral supernatants produced in 293T cells. Equivalent levels of expression of GFP and IL-7Rα were confirmed for all established D1 and Ba/F3 cell lines. BM cells were harvested from tibia and femur of *Il7r*–/– or *Il2rg*–/– mice and progenitors were enriched by lineage cell depletion kit (Miltenyi Biotec), and cultured in X-vivo 10 medium (Bio Whittaker) supplemented with 5% FBS, murine SCF (100 ng/ml), murine IL6 (50 ng/ml) and flt-3 ligand (100 ng/ml) (Peprotech). After 48 h, cells were infected on RetroNectin (TaKaRa)-coated plates overnight with different retroviral supernatant from the packaging line and the infection was repeated after 72 h. On the 4th day, cells were harvested, washed and cultured with or without IL-7.

Transfection of 293T cells. pCDNA3.1 vectors (Invitrogen) bearing human *JAK3*, human γ_C and mouse *Stat5a*, and pMIG-*IL7R* constructs were used, in the indicated combinations, to transfect 293T cells by calcium phosphate precipitation. Transfected cells were stimulated or not with IL-7 (100 ng/mL) for 15 minutes at 37ºC. Where indicated, cells were pretreated with 1mM 2β-Mercaptoethanol or vehicle (PBS) for 2h at 37ºC. Reactions were stopped by placing samples on ice.

siRNA transfection of 293T and Ba/F3 cells. For 293T cells, 50 pmol of ON-TARGETplus Non-Targeting pool or ON-TARGETplus SMARTpool JAK1 siRNA (Dharmacon) were cotransfected with the indicated plasmid DNA constructs (600ng) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were harvested 36h post-transfection and whole cell lysates were resolved by SDS-PAGE. Ba/F3 cells were electroporated (300 V, 1500 microfarads) in a Gene Pulser II (Bio-Rad) with 200 pmol of ON-TARGETplus Non-Targeting pool, ON-TARGETplus SMARTpool *Jak1* or *Jak3* (Dharmacon) or Silencer siRNA *Il2rg* (Ambion) siRNAs. At the indicated time points cells were harvested for viability assay and cell counts.

shRNA transduction of D1 cells. The retroviral vector containing mouse *Jak1* specific 29mer shRNA expressed under U6 promoter and the puromycin selection marker was bought from OriGene. Retroviral supernatant from the packaging line was used to infect mutant IL-7Rα-expressing D1 cells on RetroNectin-coated plates overnight. At 24 hours post-infection, cells were put in fresh culture medium containing 50 ng/ml of mIL-7 and 5 μg/ml of puromycin (Invitrogen) for another 48h. mIL-7 and puromycin were washed away and cells were placed in culture without mIL-7 for 48h. Cell viability and proliferation were measured by MTT assay.

Treatment with pharmacological inhibitors. Ba/F3 cells stably expressing mutant *IL7R* or primary T-ALL cells bearing *IL7R* mutations were cultured in medium alone or with the indicated concentrations of Pyridone 6 (JAK Inhibitor I), STAT5 inhibitor N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (both purchased from Calbiochem), Ruxolitinib (INCB 018424) or Tasocitinib (CP-690550) (both purchased from Axon Medchem) and viability determined at the indicated time by flow cytometry analysis. D1 cells stably expressing mutant *IL7R* were plated in 96-well plate at a density of 1×10^5 cells/well in IL-7 free medium and incubated for 48h with or without JAK inhibitors at the indicated concentrations. Cell viability and proliferation were determined by MTT assay.

Immunoblotting. Cell lysates were resolved by 10% or 12% SDS-PAGE and equal amounts of protein were transferred onto nitrocellulose membranes, and immunoblotted with antibodies against: p-JAK3 (Y980), JAK3, JAK1, STAT5, γ_C, actin, (Santa Cruz Biotechnology), p-STAT5a/b (Y694/Y699) (Upstate Biotechnology), p-TYK2

(Y1054/1055), p-JAK1 (Y1022/1023), p-JAK2 (Y1007/1008), JAK1, p-STAT5 (Y694), p-STAT3 (Y705), p-STAT1 (Y701), p-Akt (S473), Akt, p-Bad (S112), Bad (Cell Signaling Technology), and IL-7Rα (R&D). Immunodetection was performed by incubation with horseradish peroxidase-conjugated appropriate secondary antibodies and developed by chemiluminescence. For the analysis of IL-7Rα dimer formation, whole cell lysates were resolved in denaturing, non-reducing SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted. When indicated, lysates were incubated with 100mM DTT (Sigma-Aldrich) for 5 minutes at room temperature, prior to non-reducing SDS-PAGE.

Cell cycle analysis. Cells were either permeabilized in 0.1% BSA, 0.01 M HEPES, 0.1% saponin in PBS at a concentration of $1x10^6$ cells/ml and an equal volume of detergent buffer containing 50 µg/ml of propidium iodide (Sigma) and 50 µg/ml of RNase (Puregene), or treated as described [13], and analyzed by flow cytometry. Cell cycle distribution was determined using ModFit LT software (Verity).

Cell viability assay. Quantitative determination of cell viability was performed using Annexin V-based apoptosis detection kits and the manufacturers' instructions (R&D Systems or eBioscience). Briefly, cells were resuspended in the appropriate binding buffer, stained with APC-conjugated Annexin V and propidium iodide or 7-AAD at room temperature for 15 minutes, and subsequently analyzed by flow cytometry.

Cell counts. Ba/F3 cells were cultured as $2x10^5$/mL in medium deprived of growth factors or in the presence of IL-3 conditioned medium (1%; v/v) or IL-7 (10 ng/mL). Total cell counts were calculated by trypan blue exclusion using a hemocytometer at the indicated time points.

MTT assay. 8 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml; Sigma) was added to each well, and cells were kept at 37°C for 4h, after which 100 µl of solubilization solution (Promega) was added, and cells were incubated overnight at 37°C. Absorbance was measured by spectrophotometry at wavelengths 590 and 630 nm.

Mice. *Rag1*$^{-/-}$ were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and *Il7*$^{-/-}$ *Rag2*$^{-/-}$mice were obtained from R. Murray (DNAX Research Institute, Palo

Alto, CA). Mice were maintained by homozygous breeding at NCI-Frederick, Maryland. Animal care was provided in accordance with NIH Animal Use and Care guidelines. Experiments were performed following protocols approved by NCI-Frederick Animal Care and Use Committee. All mice used were 8 to 12 weeks old.

Tumor Model. Mice were treated with 0.64 mg/ml of Sulfamethoxazole (SMZ) in drinking water 2 days before the injection, and went up to a week after the injection. Mice received 3 Gy of whole body γ-irradiation 4 hours prior to the injection. D1 cells harboring the empty vector or human *IL7R* (2×10^6 cells in 100 μl of PBS) were injected subcutaneously into the right flank. On day 20, mice were euthanized and tumor size was measured by caliper. Tumor volume was calculated by the modified ellipsoidal formula [21]: Tumor volume = ½ (length x width^2).

Statistical analysis. Fisher's exact test with Bonferroni correction was used to compare the frequency of *IL7R* mutations between T-ALL subgroups. Differences between populations were calculated using unpaired 2-tailed Student's t-test. Differences were considered significant for $p<0.05$.

2.4 Results

2.4.1 Somatic *IL7R* mutations in diagnostic pediatric T-ALL patient samples

Based on the evidence that IL-7/IL-7R-mediated signaling contributes to T-cell leukemia survival and proliferation *in vitro* and *in vivo*, and the existence of JAK1 activating mutations in some T-ALL cases, we hypothesized that gain-of-function mutations in IL-7Rα could be present in some T-ALL. Analysis of *IL7R* complete coding sequence in 68 pediatric diagnostic T-ALL patient samples treated in Centro Infantil Boldrini, Campinas, Brazil revealed that 5 (7%) of the cases had mutations in *IL7R* that affected exclusively exon 6. All mutants displayed in-frame insertions or insertions/deletions (Table 1, Fig. 1a and Supplementary Fig. 1), in the juxtamembrane-transmembrane domain at the interface with the extracellular region (Fig. 1a and Supplementary Fig. 2). The mutations were somatic, since they were detected at diagnosis but not in samples from the same patients in remission (n=5) (Fig. 1b and Supplementary Fig. 1). Subsequent analysis of *IL7R* exon 6 in DCOG and COALL patient series confirmed these results and showed the presence of mutations in 12 out of 133 cases, with the majority of mutations targeting the same hot spot (Table 1, Fig. 1a). In total, 17 of 201 (9%) T-ALL samples from 3 independent cohorts had *IL7R* exon 6 mutations (Fig. 1c). This frequency was confirmed by a parallel study describing *IL7R* mutations in 10.5% of T-ALL cases [22].

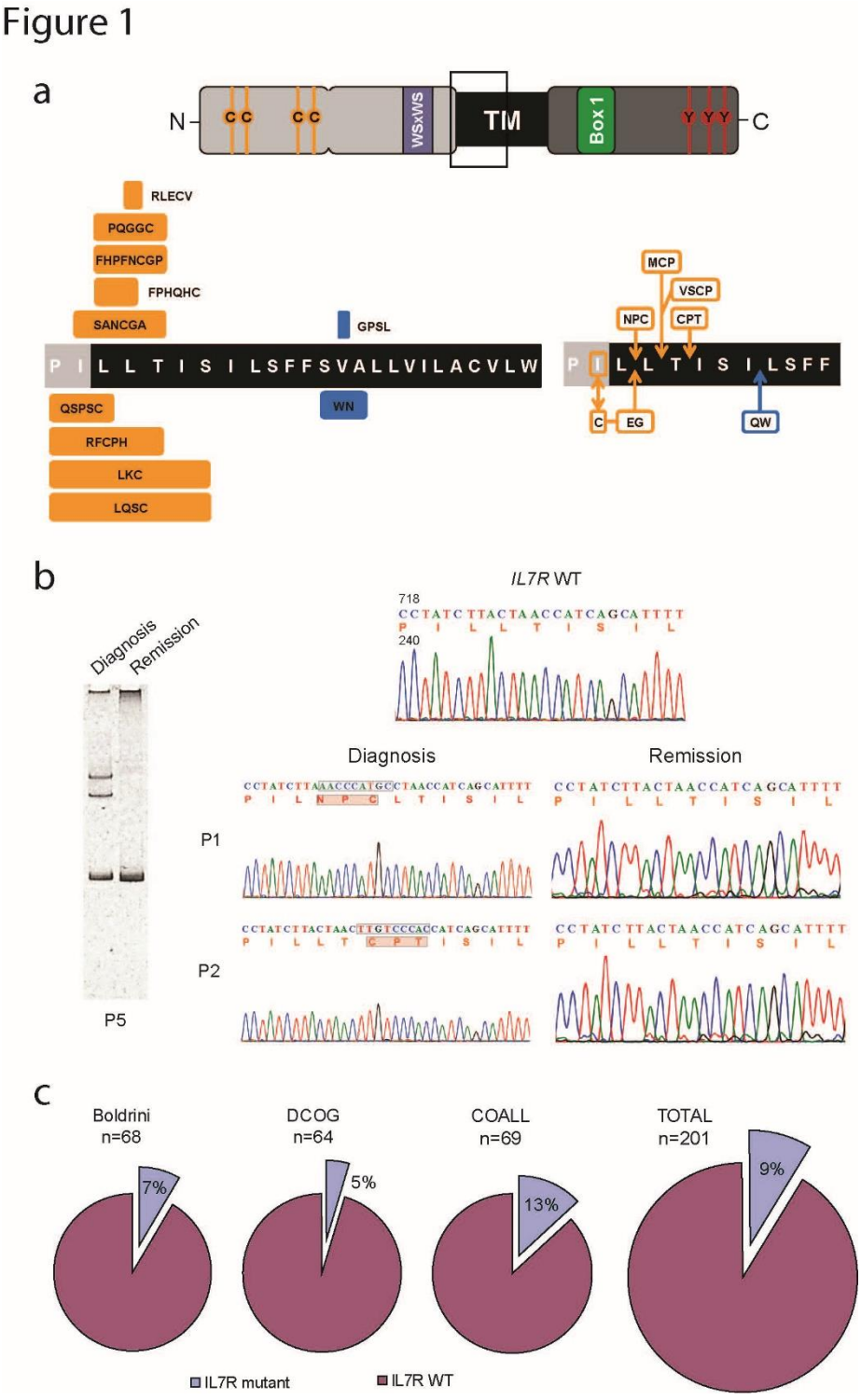

Figure 1. *IL7R* exon 6 somatic mutations in pediatric T-ALL. (a) Scheme of IL-7Rα protein (top) and predicted amino acid alterations (bottom). Indicated are the two extracellular fibronectin type III-like domains, containing 4 paired cysteines and a WSxWS motif, the transmembrane domain, and the cytoplamic tail with the Box 1 motif and the tyrosine residues involved in signal transduction. The region where the mutations occur is denoted by an empty box. Amino acid changes involving introduction *de novo* of a cysteine are indicated in yellow; filled boxes denote deletions-insertions and are aligned with the respective deleted amino acid sequence; arrows point to where simple insertions occur. **(b)** Representative homo/heteroduplex analysis of PCR products (left) and sequencing chromatograms (right) of paired diagnosis and remission samples indicating the somatic, tumor-associated origin of exon 6 mutations. **(c)** Frequency of T-ALL mutations in the three different patient cohorts analyzed.

Table 1. Mutational and immunophenotypical characteristics of *IL7R* mutant T-ALL patients.

Patient #	Cohort	IL7R gene mutation	IL-7Rα predicted aminoacid alterations	NOTCH/ FBXW7 mutational status	PTEN mutational status	Oncogenetic group	EGIL maturation stage	CD3, CD4, CD8 stage
P1	Boldrini	c.[726_727insAACCCATGC] + [=]	p.[L242_L243insNPC] + [=]	WT*	WT	Unknown	cortical	TP
P2	Boldrini	c.[731_732insTTGTCCCAC] + [=]	p.[T244_I245insCPT] + [=]	Unknown	WT	Unknown	pre-T	DP
P3	Boldrini	c.[722_730delTCTTACTAAinsGCGCAAACTGTGGGG] +[=]	p.[I241_T244delinsSANCGA] + [=]	HD*	WT	Unknown	cortical	TP
P4	Boldrini	c.[728_729insGGTATCTTGTCC] + [=]	p.[L243_T244insVSCP] + [=]	WT*	WT	Unknown	cortical	TP
P5	Boldrini	c.[731C>T; 741delTinsCCAATGG] +[=]	p.[T244I; I247_L248insQW] +[=]	WT*	Exon 7 mutation	Unknown	pre-T	ISP4
P6	DCOG	c.[717_727delTCCTATCTTACinsCCAGTCCCCCTCCTGCT] + [=]	p.[P240_L242delinsQSPSC] + [=]	HD	WT	Unknown	pre-T	ISP4
P7	DCOG	c.[721_722delATinsTG; 726_727insGAAGGC] + [=]	p.[I241C; L242_L243insEG] + [=]	HD/PEST	WT	TLX3	n.d.	DN
P8	DCOG	c.[755_761delCTGTCGCinsGGAA] + [=]	p.[S252_254delinsWN] + [=]	WT	WT	HOXA/MLL	pre-T	DP
P9	COALL	c.[719_731delCTATCTTACTAACinsGGTTTTGTCCCCA] + [=]	p.[P240_T244delinsRFCPH] + [=]	HD	WT	TLX3	pre-T	ISP4
P10	COALL	c.[719_736delCTATCTTACTAACCATCAinsTTAAGT] + [=]	p.[P240_S246delinsLKC] + [=]	WT	WT	TLX3	pre-T	DN
P11	COALL	c.[726_730delACTAAinsTCACCCTTTTAACTGTGGAC] + [=]	p.[L242_T244delinsFHPFNCGP] + [=]	HD	WT	TLX3	mature	ISP4
P12	COALL	c.[730_731insTGTGCCCAA] + [=]	p.[L243_T244isnMCP] + [=]	JM	WT	HOXA	mature	DP
P13	COALL	c.[757_758insGCCCATCCC] + [=]	p.[V253delinsGPSL] + [=]	PEST	WT	HOXA	pre-T	DN
P14	COALL	c.[727_728insGACTTGAGTGCG] + [=]	p.[L243delinsRLECV] + [=]	PEST	WT	HOXA/inv-7	mature	DP
P15	COALL	c.[724_736delTTACTAACCATCAinsCCCCAGGGCGGGT] + [=]	p.[L242_S246delinsPQGGC] + [=]	HD/FBXW7	WT	HOXA/SET-NUP214	mature	DP
P16	COALL	c.[719_736delCTATCTTACTAACCATCAinsTCCAATCAT] +[=]	p.[P240_S246delinsLQSC] +[=]	WT	WT	TAL1/LMO2-like	cortical	DP
P17	COALL	c.[726_729delACTAinsTCCCCATCAGCATTGT] + [=]	p.[L242_L243delinsFPHQHC] + [=]	FBXW7	WT	Unknown	mature	ISP4

* *FBXW7* mutational status not analyzed; n.d. - not determined/inconclusive.

2.4.2 Biological and clinical features associated with *IL7R* mutations

To identify possible transcriptional patterns associated with *IL7R* mutations in T-ALL, we analyzed microarray data from 8 *IL7R* mutated and 109 non-mutated diagnostic patient samples. Differential gene expression was tested by regression analysis using the LIMMA package. *IL7R* mutations were associated with upregulation of 39 probesets and downregulation of 41 (FDR p-value <0.05) (Fig. 2a and Supplementary Table 1). Importantly, gene set enrichment analysis (GSEA) of these T-ALL samples showed significant enrichment of a set of genes activated upon IL-7 stimulation in normal lymphocytes (Enrichment score= 0.67, p=0.045) [19]. These genes include *SOCS1*, *SOCS2*, *PIM1*, *BCL2*, *DPP4/CD26* and *CCND2/Cyclin D2* (Fig. 2b), all of which have been reported as transcriptional targets of the JAK/STAT pathway.

T-ALL patients are categorized into several oncogenetic subgroups that are characterized by rearrangements and aberrant expression of transcription factors such as *TAL1* and *LMO1/2*, *TLX1/HOX11*, *TLX3/HOX11L2*, *HOXA*, *NKX2-1* or *MEF2C* [18]. *IL7R* mutations were predominantly found in cases belonging to the *HOXA* subgroup (Table 2). Recently, we identified unsupervised T-ALL gene expression clusters that closely recapitulate oncogenetic T-ALL subgroups, namely the TAL/LMO subgroup (enriched for *TAL1*/*2* and/or *LMO1*/*2*/*3* rearranged cases), the proliferative subgroup (enriched for *TLX1* or *NKX2-1*/*NKX2-2* rearranged cases), the TLX subgroup (enriched for *TLX3* rearranged and *HOXA* deregulated cases) and the immature/ETP-ALL cases (enriched for *MEF2C* deregulated cases) [18]. Our current analyses showed that *IL7R* mutations were especially associated with the TLX subgroup (Fig. 2a and Table 2), in agreement with the fact that this unsupervised gene expression T-ALL subset is enriched in *HOXA* deregulated cases.

As some oncogenic rearrangements in T-ALL are associated with specific immunophenotypic development stages [23, 24], we evaluated whether *IL7R* mutations predominated in particular immunophenotypes. *IL7R* gene alterations did not associate with any specific T-ALL maturation stage based on EGIL criteria [25]. Although *IL7R* mutations were negatively and positively associated with CD2 and CD10 expression, respectively (Supplementary Fig. 3), they did not associate with CD34, CD33, CD5, CD1, CD4, CD8, cytoplasmic CD3, surface CD3, TCR$\alpha\beta$ or TCR$\gamma\delta$ expression.

JAK1 and JAK3 are essential for physiologic IL-7-mediated signaling [1]. None of the *IL7R* mutants analyzed (n=5) displayed gene alterations in the JH2 pseudokinase domain of JAK1 or JAK3, reported to be mutated in pediatric T-ALL [17], and in breast cancer [26] and acute megakaryoblastic leukemia [27], respectively. PI3K/Akt signaling pathway is

activated by IL-7 in T-ALL cells [28], and PTEN, the major negative regulator of PI3K/Akt signaling pathway, is mutated in up to 20% of T-ALL cases [29-33]. Only one of the seventeen *IL7R* mutant samples showed *PTEN* gene alterations (Table 1). *NOTCH1* is a major oncogene in T-ALL, with more than 60% of the cases presenting gene alterations in *NOTCH1* or *FBXW7*, the E3 ubiquitin ligase that targets NOTCH for degradation [16, 34, 35],[36]. No significant difference was observed in the distribution of *IL7R* mutations in *NOTCH1/FBXW7* mutated versus non-mutated patients (Table 2).

We also evaluated whether *IL7R* mutations could predict treatment response and clinical outcome. We did not find any association to initial in vivo prednisone response. Moreover, there was no difference in survival between wild-type and mutant *IL7R* patients. Disease-free (p=0.82, Log-Rank test), event-free (p=0.84) and overall survival (p=0.51; Supplementary Fig. 4) were similar for both groups.

Figure 2

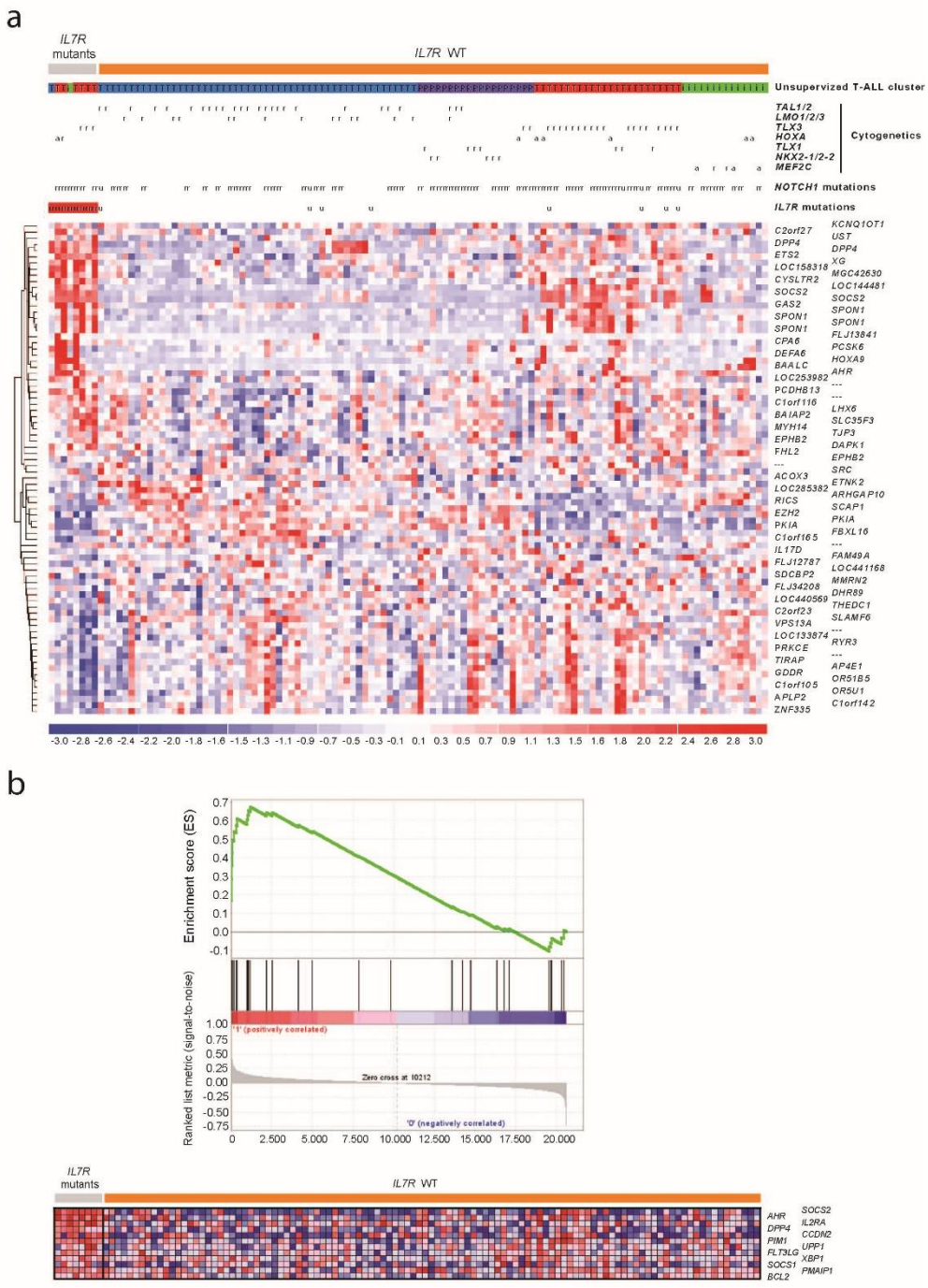

Figure 2. Molecular signatures associated with *IL7R* mutation in T-ALL. (a) Heat-map diagram of the 80 top ranking differentially expressed genes (Supplementary Table 1) in *IL7R* mutants (n=8) compared to wild type (n=109) T-ALLs, as determined by empirical-Bayes linear models (LIMMA package; cut-off FDR p-value=0.05). Genes are shown in rows; each individual sample is shown in one column. The scale bar shows color-coded differential expression from the mean in s.d. (σ) units, with red indicating higher expression and blue lower expression. Unsupervised gene expression T-ALL clusters were defined as previously described [18] and are indicated as: T (blue), TAL/LMO; T (red), TLX; i (green), immature; P (violet), proliferative. Cytogenetic defects are denoted as: r, rearranged/mutated; a, aberrant expression, u, unavailable data. **(b)** Gene set enrichment analysis (GSEA) plot (top) showing that genes over-expressed in human normal lymphocytes following IL-7 exposure [19] were significantly enriched in *IL7R* mutant T-ALL cases (Enrichment score=0.67, p=0.045). Heat-map diagram (bottom) of the 12 top ranking genes in the leading edge.

Table 2. Association of *IL7R* mutations with genetic features of T-ALL patients.

		IL7R		***p*-value**
		mutant	**wild type**	
Gene expression clusters *		**8 (7)**	**101 (93)**	
TAL/LMO	*n*=49	1 (2)	48 (98)	*p*=0.284
Proliferative	*n*=19	0 (0)	19 (100)	*p*=1.0
TLX	*n*=26	6 (23)	20 (77)	***p*=0.008**
Immature	*n*=15	1 (7)	14 (93)	*p*=1.0
Genetics (Oncogenetic subgroups)		**12 (9)**	**123 (91)**	
TAL1/2‡	*n*=28	0 (0)	28 (100)	*p*=0.568
LMO1/2/3‡	*n*=19	0 (0)	19 (100)	*p*=1.0
TLX3	*n*=25	4 (16)	21 (84)	*p*=1.0
TLX1	*n*=8	0 (0)	8 (100)	*p*=1.0
HOXA	*n*=13	5 (38)	8 (62)	***p*=0.016**
NKX2-1/2-2	*n*=6	0 (0)	6 (100)	*p*=1.0
MEF2C	*n*=6	0 (0)	6 (100)	*p*=1.0
unknown	*n*=32	3 (9)	29 (91)	*p*=1.0
NOTCH1/FBXW7		**12 (9)**	**122 (91)**	
mutant	*n*=86	9 (10)	77 (90)	*p*=1.0
wild type	*n*=48	3 (6)	45 (94)	

* Unsupervised gene expression cluster analysis (109 T-ALL cases had known *IL7R* mutational status). Subgroups defined as in Homminga et al. [18]; ‡Two T-ALL cases have both TAL1/2 and LMO1/2 aberrations.

2.4.3 *IL7R* mutations induce constitutive signaling, independently of IL-7, γc and JAK3

The high-affinity IL-7R complex is formed by IL-7Rα and γc. Triggering of IL-7R by IL-7 involves recruitment of both subunits and consequent activation of the tyrosine kinases JAK1 (associated with IL-7Rα) and JAK3 (associated with γc), leading to the downstream activation of different pathways, most prominently PI3K/Akt and STAT5 [1, 2]. We hypothesized that T-ALL-associated *IL7R* mutations should promote either constitutive signaling or increased responsiveness to IL-7. We first compared two primary leukemia samples collected at diagnosis that differed in their *IL7R* mutational status. In contrast to the wild type (WT) T-ALL case, the patient sample harboring an *IL7R* mutation (P1, L242-L243insNPC; Table 1) displayed constitutive JAK1 and STAT5 phosphorylation (Fig. 3a). To exclude the possibility that this difference resulted from lesions other than *IL7R* mutation, we transduced the IL-7-dependent thymocyte cell line D1 [37] with retroviral vectors driving the expression of the human IL-7Rα WT chain or two of the mutants (P1; and P2, T244-I245insCPT). Analysis of JAK/STAT and PI3K/Akt pathways showed that the *IL7R* mutations are gain-of-function, inducing ligand-independent constitutive hyperactivation of IL-7R-mediated signal transduction. *IL7R* mutations induced phosphorylation of JAK1 and STAT5 (Fig. 3b), STAT1 and STAT3 (Supplementary Fig 5), as well as Akt and its direct target Bad (Fig. 3c). Surprisingly, the mutants did not promote JAK3 phosphorylation, which is a hallmark of physiological IL-7-mediated signaling (Supplementary Fig 5). Similar results were obtained with Ba/F3 cells (Supplementary Fig. 6). Strikingly, reconstitution of the IL-7R machinery in 293T cells (which express endogenously only JAK1 and lack IL-7Rα, γc and JAK3) further revealed that the IL-7Rα mutant proteins signal constitutively in a manner that is independent of γc (Fig. 3d,e) and JAK3 (Fig. 3e). In contrast, knock down of JAK1 resulted in abrogation of mutant *IL7R*-dependent constitutive STAT5 phosphorylation (Fig. 3f and Supplementary Fig. 7). Since, similar to JAK3, JAK2 and TYK2 are not activated by the *IL7R* mutants (Supplementary Fig 5), our results indicate that JAK1 is the only Janus kinase mandatory for signaling triggered by mutated IL-7Rα.

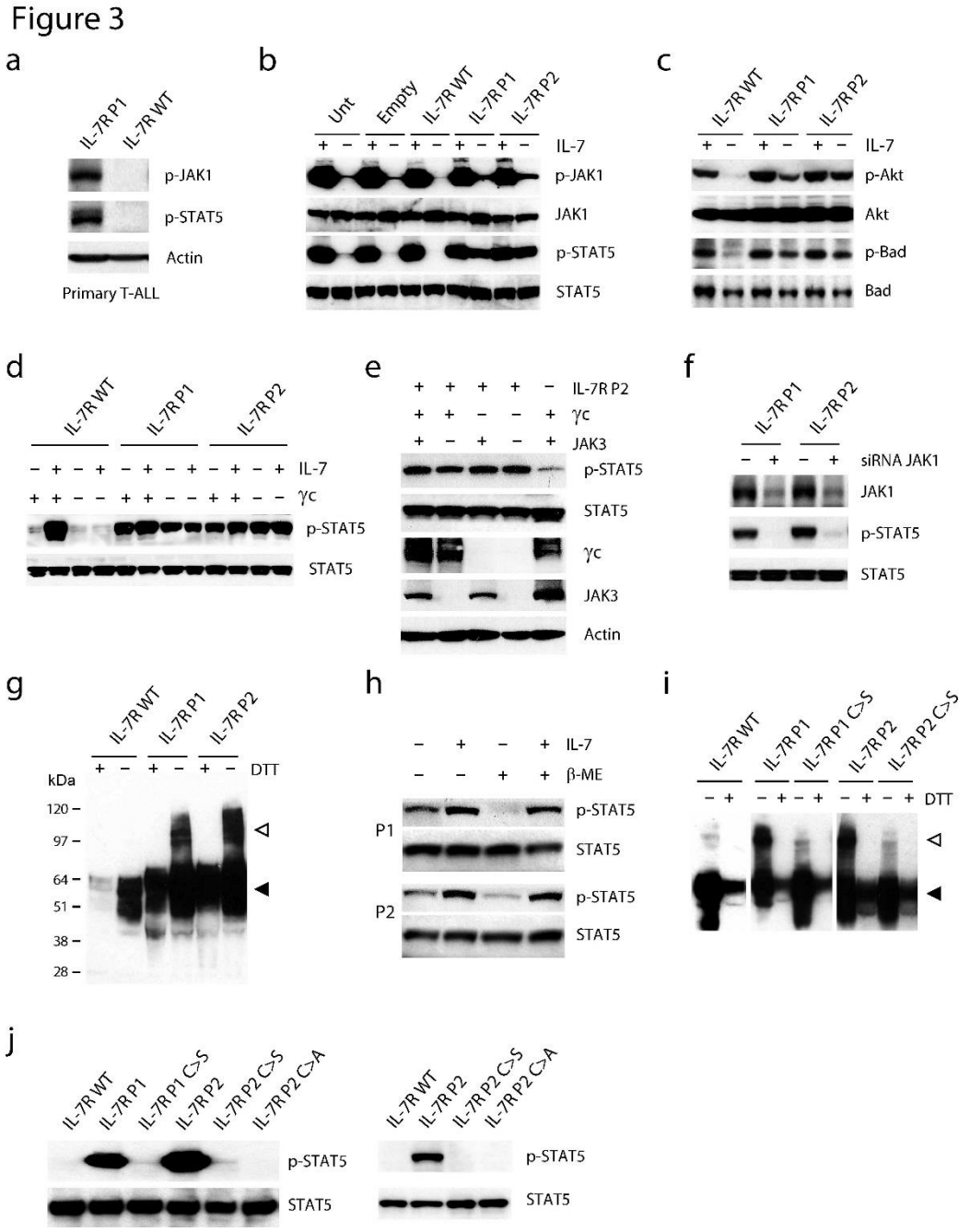

Figure 3. *IL7R* mutations induce constitutive signaling in a manner that is independent of IL-7, γc and JAK3 and relies on disulfide bond promotion of homodimer formation. (a) Primary T-ALL cells collected at diagnostic from *IL7R* mutant (P1) and WT patients were analyzed by immunoblot for JAK1 and STAT5 phosphorylation. D1 cells expressing human WT or mutated (P1 and P2) IL-7Rα were cultured without IL-7 for 4 hr, stimulated or not with IL-7 for 20 min and evaluated for activation of JAK-STAT **(b)** and PI3K/Akt **(c)** pathway activation by immunoblot. **(d)** 293T cells reconstituted with JAK3, STAT5, and WT or mutated IL-7Rα, and expressing or not γc, were analyzed for constitutive and IL-7-induced (15 min. stimulation) STAT5 phosphorylation. **(e)** 293T cells were transfected with IL-7Rα P2 and the remaining components of the IL-7R signaling machinery as indicated, and evaluated for STAT5 phosphorylation. **(f)** 293T cells were transfected with IL-7Rα P1 or P2 and siRNA against JAK1 (+) or control non-targeting siRNA (-) and evaluated after 36 hr for JAK1 expression and STAT5 phosphorylation. **(g)** Lysates from D1 cells expressing WT or mutant IL-7Rα were treated or not with the reducing agent DTT and analyzed for IL-7Rα expression by immunoblot. The monomeric and dimeric forms of the receptor are denoted by black and white arrows, respectively. **(h)** 293T cells expressing IL-7Rα P1 and P2 and the remaining components of the IL-7R signaling machinery were pretreated with β-mercaptoethanol (β-ME) and stimulated or not with IL-7 for 15 min. and subsequently evaluated for STAT5 phosphorylation by immunoblot. **(i)** D1 cells expressing each of

the indicated IL-7R constructs were analyzed for IL-7Rα expression by immunoblot. **(j)** Signaling elicited by each indicated mutant form expressed in D1 (left) or 293T (right) cells was assessed by detection of STAT5 phosphorylation.

2.4.4 Constitutive signaling from *IL7R* mutants is associated with homo-dimerization/oligomerization via disulfide bond formation

Most *IL7R* mutations (14/17; 82%) created an unpaired cysteine residue in the extracellular juxtamembrane/transmembrane interface region (Fig. 1a and Supplementary Fig. 2). Mutations that introduce cysteines in this region in receptors such as EpoR [38], RET [39] and Her2/Neu [40], have been implicated in intermolecular disulfide bond formation, with consequent homodimerization and signaling activation. A similar mechanism was suggested to account for the oncogenic activity of Phe232Cys mutation in the TSLP receptor (CRLF2), recently found in B-ALL [41]. Expression of human IL-7Rα in γc-expressing D1 cells or in 293T cells, which do not express γc, followed by immunoblot analysis under non-reducing conditions showed that the mutants are detected mostly as dimers/oligomers whereas WT IL-7Rα is found mainly in a monomeric form. In contrast, both WT and mutant IL-7Rs were detected essentially in the monomeric form when the protein lysates were resolved under reducing conditions (Fig. 3g and Supplementary Fig. 8). Similar results were obtained by transducing *Il7r*$^{-/-}$ BM cells (Supplementary Fig. 9). Accordingly, constitutive, ligand-independent, phosphorylation of STAT5 was significantly downregulated by pretreatment of mutant IL-7Rα-expressing cells with β-mercaptoethanol (Fig. 3h). Furthermore, receptor dimerization and constitutive signaling were abrogated upon substitution of the mutated cysteine to alanine or serine (Fig. 3i,j). These data indicate that constitutive hyperactivation of IL-7R-mediated signaling in T-ALL cells results, in the majority of the cases, from intermolecular disulfide bond formation arising from the introduction of an unpaired cysteine in the extracellular juxtamembrane/transmembrane region of IL-7Rα that leads to homotypic dimerization/oligomerization.

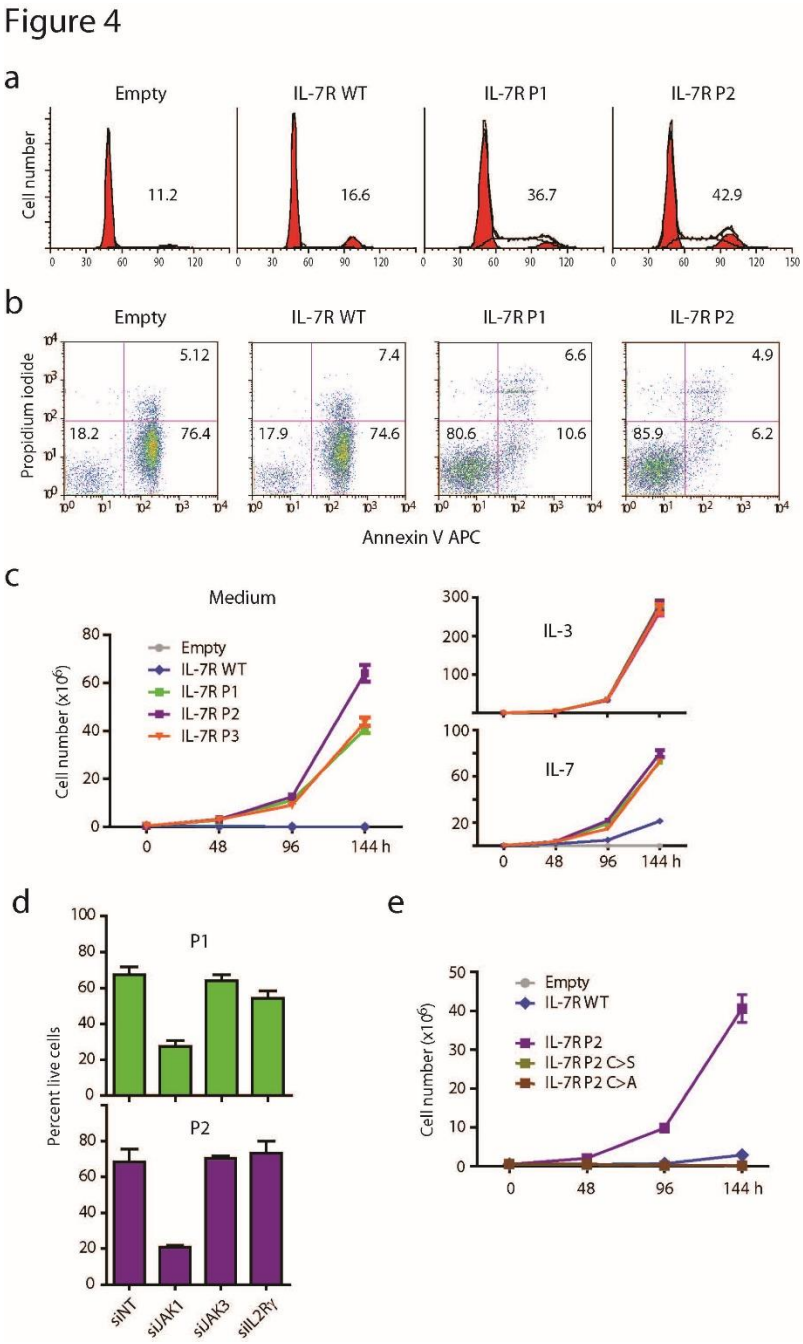

Figure 4. *IL7R* mutations induce cell cycle progression, increase cell viability, and promote growth factor independence. Ba/F3 cells stably expressing WT or mutated IL-7Rα were cultured for 96 hr in medium and analyzed for **(a)** cell cycle distribution (percentage of cells in cycle, S+G2/M, is indicated for each condition), and **(b)** viability (percentage of viable, early apoptotic and late apoptotic/necrotic cells is indicated in the respective quadrant). **(c)** Ba/F3 cells stably expressing IL-7Rα were cultured in the absence of growth factors or with IL-3 or IL-7 and expansion was measured at the indicated time points. **(d)** Ba/F3 cells stably expressing P1 or P2 mutated IL-7Rα were transfected with siRNA against JAK1, JAK3, γc (IL-2Rγ) or with non-targeting (NT) control and evaluated for cell viability after 48 hr. **(e)** Ba/F3 cells transduced with IL-7Rα P2 or with the indicated introduced mutations were cultured in the absence of growth factors and expansion was measured at the indicated time points. Results in panels c-e represent average of triplicates ± sem.

2.4.5 *IL7R* mutations induce cellular transformation *in vitro* and promote tumor formation *in vivo*

We then investigated the cellular consequences of constitutive signaling emanating from IL-7Rα mutants. Expression of mutant, but not wild type, IL-7Rα into IL-7-dependent D1 cells and IL-3-dependent Ba/F3 cells promoted both cell cycle progression (Fig. 4a, Supplementary Fig. 10 and 11) and viability (Fig. 4b, Supplementary Fig. 10 and 11) independently of IL-7. Accordingly, mutation of IL-7Rα conferred growth factor independency to Ba/F3 cells (Fig. 4c), indicating that the *IL7R* mutants have transforming capacity. In agreement with the signaling data (Fig. 3d-f), the functional effect of the mutants was also independent of γc and JAK3, as shown by increased survival of BM cells from *Il2rg*$^{-/-}$ (Supplementary Fig. 12) and *Jak3*$^{-/-}$ (Supplementary Fig. 13) mice transduced with two of the mutants, and reliant on JAK1, as determined by inhibition of mutant *IL7R*-mediated survival in Ba/F3 and D1 cells upon JAK1, but not γc or JAK3, knockdown (Fig 4d and Supplementary Fig. 14). Furthermore, substitution of the *de novo* inserted cysteine residue to serine/alanine resulted in reversal of the transforming capacity of the IL-7Rα mutants (Fig. 4e and Supplementary Fig. 15), suggesting that intermolecular disulfide bond-dependent homodimerization is mandatory not only for signaling but also for the functional effects of IL-7Rα mutants.

Although *IL7R* mutations induced cell transformation, growth factor independence or immortalization *in vitro* does not necessarily implicate the acquisition of a malignant phenotype *in vivo*. Therefore, we next evaluated the *in vivo* tumorigenic potential of *IL7R* mutations. In contrast to D1 cells transduced with empty vector or the WT IL-7Rα, subcutaneous injection of mutant IL-7Rα-expressing D1 cells in *Rag1*$^{-/-}$ mice resulted in tumor formation (Fig. 5a). Notably, ill mice displayed a phenotype typical of T-ALL with substantial homing of mutant IL-7Rα-expressing cells into the bone marrow and infiltration into various organs that are normally affected in advanced stage disease, such as lymph nodes, liver and spleen (Fig. 5b-e, Supplementary Fig. 16 and data not shown). The tumors were transplantable into secondary recipient animals (not shown) and were not dependent on the presence of IL-7, since injection of mutant IL-7Rα-expressing cells led to tumor development in IL-7 deficient mice (Fig. 5f). Taken together, our results indicate that *IL7R* mutational activation is an oncogenic event involved in T-ALL.

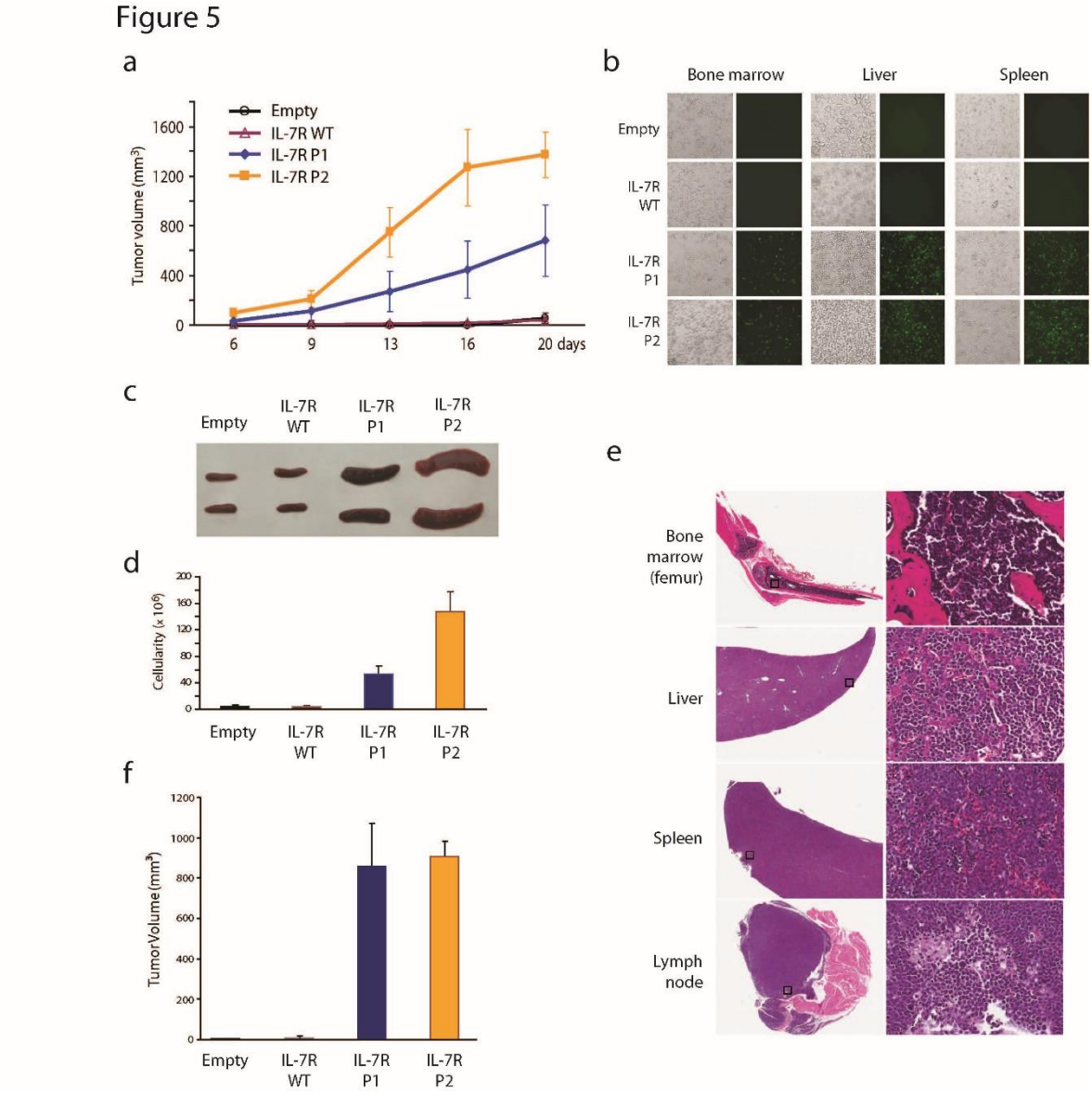

Figure 5. ***In vivo*** **tumorigenic effect of** ***IL7R*** **mutations.** D1 cells expressing WT or mutated IL-7Rα were subcutaneously injected into *Rag1*$^{-/-}$ mice and evaluated for tumor progression and organ infiltration. **(a)** Subcutaneous tumor volume growth curves. **(b)** Phase contrast and fluorescence imaging of D1 cells (GFP-positive) infiltrated into liver, spleen and bone marrow. **(c)** Representative images of spleens from mice culled at day 20 and **(d)** respective spleen cellularity. **(e)** Histological analysis (hematoxylin/eosin staining) of indicated organs from representative mouse transplanted with cells expressing mutant IL-7Rα P2; right panel: 20x magnification of the area denoted by a square on the left panel. **(f)** D1 cells expressing WT or mutated IL-7Rα were subcutaneously injected into *Il7*$^{-/-}$ *Rag2*$^{-/-}$ mice and evaluated for tumor size at day 20. Results in panels a, d and f represent average of triplicates ± sem.

2.4.6 Targeting IL7R mutant cells with JAK/STAT pathway pharmacological inhibitors

To test the potential therapeutic application of our findings, we reasoned that mutant IL-7Rα-expressing cells should rely on constitutive signaling downstream from the receptor. We first evaluated the efficacy of several JAK inhibitors, including Pyridone 6 (JAK inhibitor I), CP-690550 and INCB018424. The latter two are of particular relevance since they are in clinical trials for other rheumatoid arthritis and several cancers, including hematological malignancies. Importantly, all three drugs significantly downregulated JAK1 phosphorylation and consequent downstream activation of STAT5 and Akt (Fig. 6a), and induced cell death in a dose- and time dependent manner (Fig. 6b,c and Supplementary Fig. 17) in Ba/F3 cells expressing mutant IL-7Rα. Likewise, CP-690550, INCB018424 and another clinically-relevant JAK inhibitor, CYT387, inhibited the proliferation of mutant IL-7Rα-expressing D1 cells (Supplementary Fig. 18). Furthermore, a STAT5-specific small molecule inhibitor [42] promoted significant killing of Ba/F3 cells expressing mutant IL-7Rα (Fig. 6d and Supplementary Fig. 19). Finally, we found that primary T-ALL cells harboring *IL7R* mutation are also sensitive to JAK/STAT pathway inhibition. With the exception of CP-690550, the remaining drugs had differential but always significant cytotoxic effects on diagnostic leukemia cells (Fig. 6e). These results illustrate the potential therapeutic value of JAK/STAT pathway small molecule inhibitors in the context of *IL7R* mutant T-ALL.

Figure 6

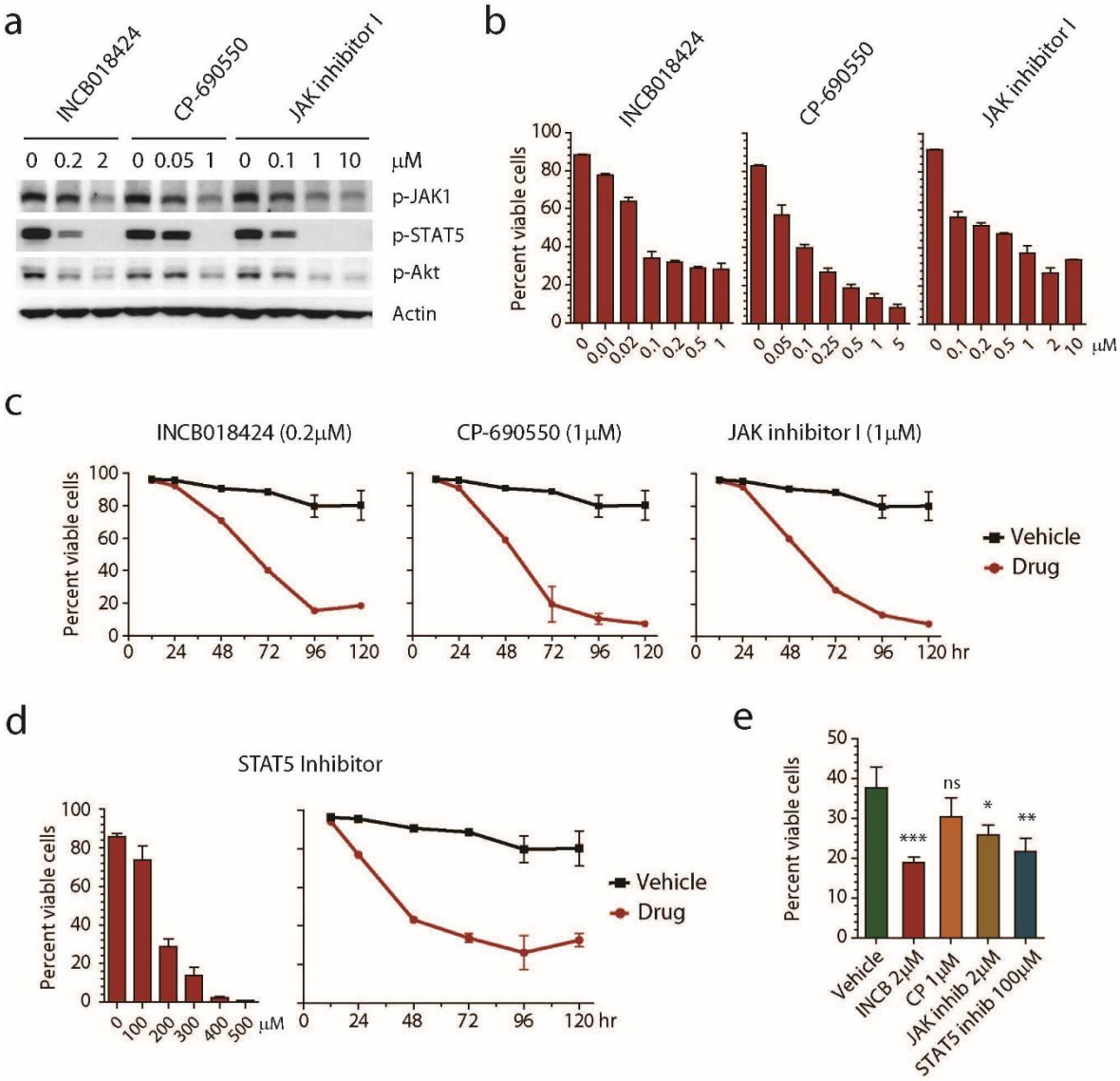

Figure 6. Targeting *IL7R* mutants using JAK/STAT pathway inhibitors. Ba/F3 cells expressing mutated IL-7Rα P1 were cultured in medium alone in the presence or absence of the indicated doses of different JAK and STAT5 pharmacological inhibitors. **(a)** Cells were analyzed at 48 hr for effective JAK/STAT pathway inhibition by immunoblot. Cell viability was analyzed **(b)** at 48 hr (INCB018424) and 72 hr (CP690550 and Pan-JAK inhibitor) after increasing doses of each drug and **(c)** at different time points with a single dose of each inhibitor. **(d)** Cell viability was analyzed at 72 hr with increasing doses or at different culture time points with 200 μM of STAT5-specific inhibitor. **(e)** Primary T-ALL cells from patient P1 were cultured in the presence of the indicated JAK/STAT pathway inhibitors and evaluated for cell viability at 24 hr. ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Viability results panels b-e represent average of triplicates ± sem.

2.5 Discussion

T-ALL is an aggressive hematological cancer resulting from leukemic transformation of thymocytes. Although there has been a remarkable increase in our knowledge of T-ALL molecular pathogenesis, the identification and characterization of the players and mechanisms driving proliferation and survival of leukemia T-cells remains relatively poor. IL-7 and its receptor are essential for normal T-cell development and have been suggested to play a role in T-ALL. In the present study we showed that nine percent of pediatric T-ALL cases display *IL7R* exon 6 mutations that are gain-of-function and have oncogenic ability. Thus, our findings expand the spectrum of disease-associated *IL7R* genetic alterations to cancer. Moreover, this is the first example of an oncogene in the γc family of cytokine receptors, which is critically involved in numerous lymphoid cell functions [43].

Surprisingly, *IL7R* mutations do not occur in the cytoplasmic tail, which recruits signaling effectors, but at the extracellular juxtamembrane/transmembrane interface. The vast majority of *IL7R* mutations identified create an unpaired cysteine residue, which is necessary for disulfide bond-dependent IL-7Rα homodimerization and bypasses the requirement for ligand biding and γc heterodimerization to trigger downstream signaling. Moreover, all *IL7R* mutations insert additional amino acids rather than involving a single amino acid change to a cysteine. This may indicate that these additional amino acids are required for the optimal conformation leading to maximal signaling, perhaps by allowing for the most adequate alignment/exposure of the unpaired cysteine and/or by maximizing the interactions between downstream effectors at the cytoplasmic tail of the receptor. The three remaining cases originated the inclusion of either a tryptophan or an SxxxG motif in the transmembrane domain. Although we did not analyze the mechanisms by which these mutations may contribute to T-cell leukemia, tryptophan residues and SxxxG motifs have both been reported to promote association of transmembrane helices [44, 45] that could result in homo- or heterodimer formation with possibly similar outcomes to cysteine mutations. However, preliminary analyses of mutant P5, which has the insertion of a tryptophan in the transmembrane domain (Table 1), suggest that it does not form dimers (data not shown) and suggest that the pro-survival effect of this mutation is relatively minor: P5 expression in D1 cells deprived of IL-7 for 48h resulted in a 2.8-fold increase in viability relative to IL-7R WT *versus* 7.4-, 9.1-, and 6.0-fold for P1, P2 and P4, respectively. In accordance, P5 appears to be relatively inefficient in inducing constitutive signaling as compared to the other *IL7R* mutations (Supplementary Fig. 7). These results suggest that

IL7R mutations not involving cysteine insertion are not as potent, probably requiring additional cooperating oncogenic events, compared to those that result in the introduction of an unpaired cysteine, which constitute the vast majority of the cases identified in childhood T-ALL and characterized by our study.

IL7R gene alterations appear to be highly predominant in T-cell as compared to B-cell leukemia. We did not detect exon 6 mutations in any of the 50 childhood pre-B ALL cases we analyzed and a recent report indicated that *IL7R* mutations occur in only 0.6% pre-B-ALL cases. In contrast to T-ALL, half of the B-cell-associated mutations affect exon 5, rather than exclusively exon 6, and require cooperation with TSLPR/CRLF2 overexpression [22]. TSLPR expression is rare in T-ALL and not necessary for signaling driven by the *IL7R* mutations, as we showed here in 293T cells – which do not express TSLPR and yet display constitutive signaling after expression of mutated *IL7R*. Interestingly, the fact that IL-7Rα is apparently expressed in various carcinoma cell lines and breast cancer tissue [46], raises the intriguing question of whether mutations in *IL7R* may also occur in solid tumors.

IL7R mutations were found in different T-ALL oncogenetic subgroups, but they tend to associate predominantly with *HOXA* aberrant expression. Although the exact biological significance of this link remains to be fully understood, it is noteworthy that *Hoxa9*$^{-/-}$ mice display impaired early T-cell development, with reduced Bcl-2 and IL-7Rα expression [47]. Curiously, *IL7R* gene alterations did not associate with T-ALL maturation stage or with most T-cell differentiation markers. These observations are reminiscent of the fact that primary T-ALL cells, in contrast to normal developing thymocytes, respond to IL-7 independently of their maturation stage [14].

We demonstrated that pharmacological inhibition of JAK/STAT pathway induces cell death of mutant IL-7Rα-expressing cells. The preliminary data on the effect of these inhibitors in one primary T-ALL patient sample was significant but not as striking as on cell lines. This may relate to the early time point at which viability was assessed (which may have prevented the inhibitors to have the maximal effect), to the importance of other alternative downstream signaling pathways in the regulation of cell survival in primary leukemia, and/or to higher dependence on other oncogenic defects in the leukemia cells of the patient analyzed. Irrespectively of these considerations, our results suggest that JAK/STAT pathway inhibitors are cytotoxic to mutated IL-7Rα-expressing T-ALL cells. Whether inhibitors of other signaling components activated by gain-of-function *IL7R* mutations, such as Akt, can be exploited, *per se* or in combination with JAK/STAT antagonists, to target *IL7R* mutant T-ALL cells requires further investigation.

The extraordinary improvement in T-ALL treatment outcome in recent years is mitigated by the long-term side-effects associated with current regimens and by the dismal prognosis of relapsed patients. Further improvement requires an in-depth understanding of T-ALL molecular genetics and leukemogenic pathways, which will ultimately lead to the identification of novel molecular players and to the development of effective targeted therapies. This line of reasoning has led, for instance, to the identification of *CREBBP*/*CBP* mutations that are associated with ALL relapse [48], or *TSLPR*/*CRLF2* rearrangements, which are particularly frequent in Down syndrome ALL [49]. *PTPN2* and *PHF6* mutational loss [50, 51] are among the most recently characterized genetic lesions involved in T-ALL. Our present work indicates that *IL7R* mutational activation takes part in human T-cell leukemogenesis, thereby expanding the spectrum of genetic alterations in T-ALL to a long recognized major regulator of lymphoid biology. Importantly, our findings provide a strong rationale for specific targeting of IL-7R-mediated signaling as a treatment option for T-ALL.

2.6 Acknowledgements

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2.7 Authorship contributions

JTB and JAY conceived and supervised the study; JTB, JAY, SKD, JPM designed the experiments; JTB wrote the paper and coordinated the different contributions; JAY, SKD, JPM, AF, WL, DR and PZ contributed to the writing of portions of the paper; PPZ, DR, WL, LZ, MCS, MP, JT, JAH, ABS, BAC, LMS and NC performed experiments; JTB, JAY, SKD, JPM, AF, PZ, DR, WL, MCS, LMS analyzed data; MLT, JK, RP, SRB contributed reagents or clinical information.

The authors have no competing financial interests to declare.

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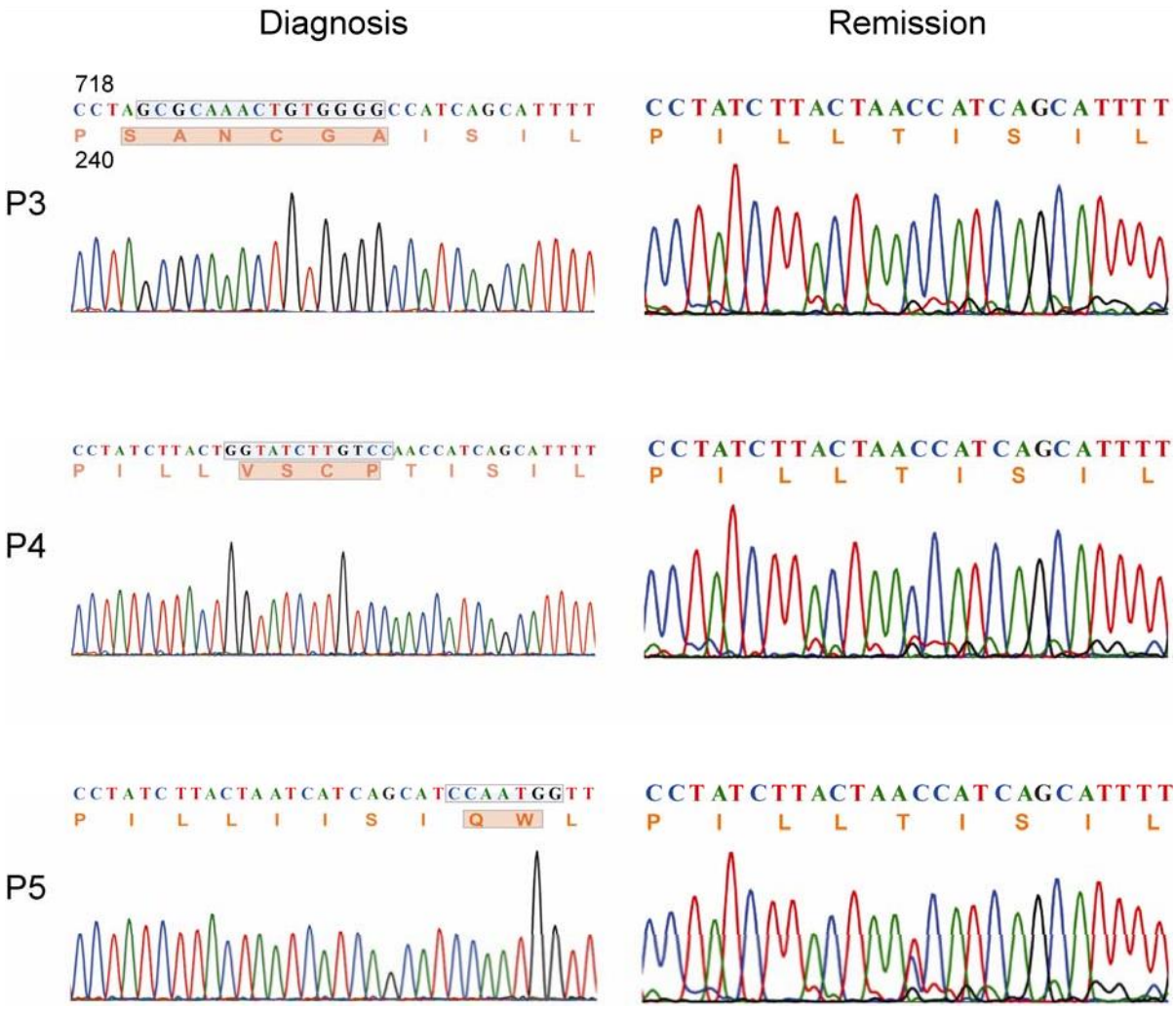
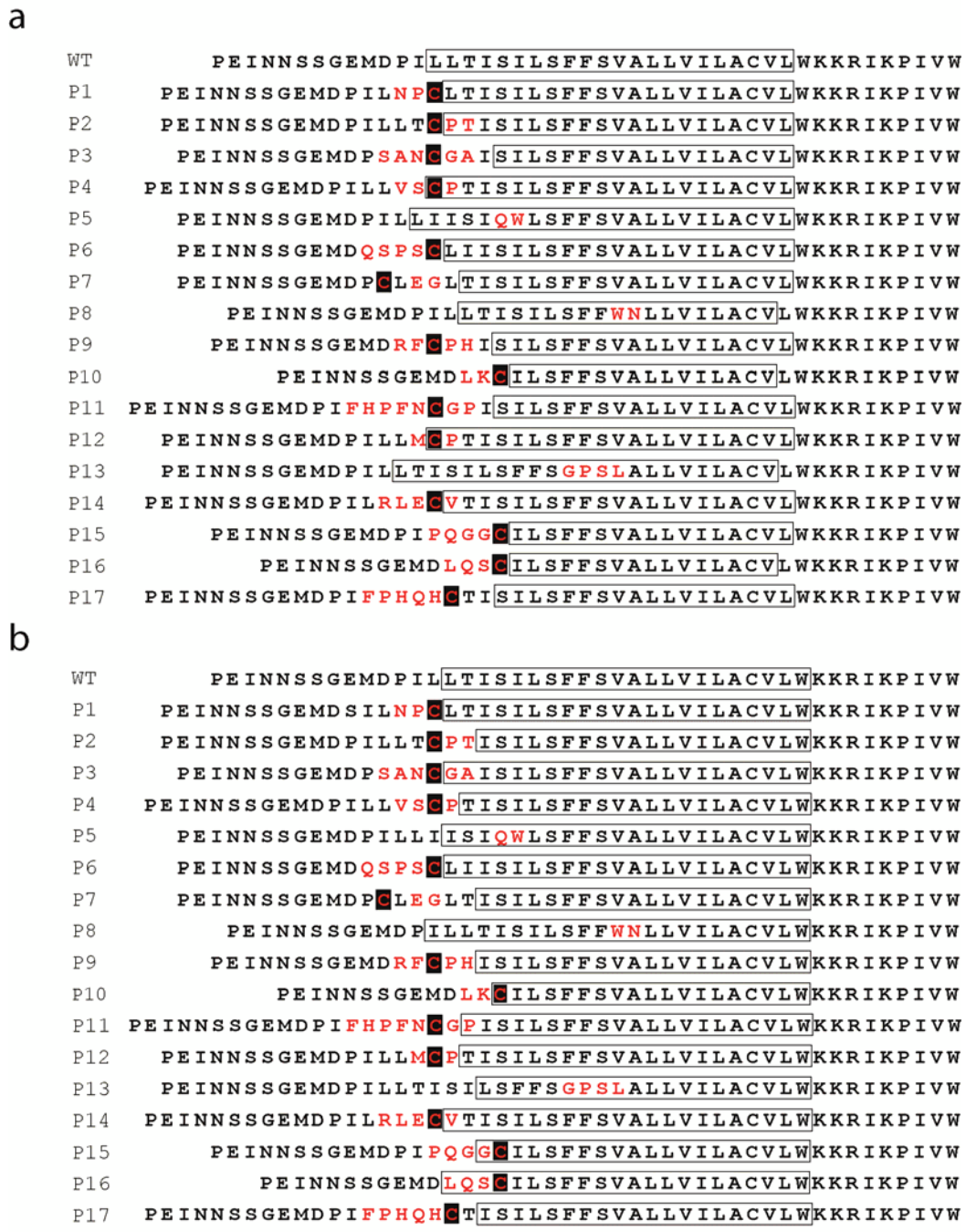
2.9 Supplementary Data

Supplementary Figures 1-19, Tables 1-2 and References

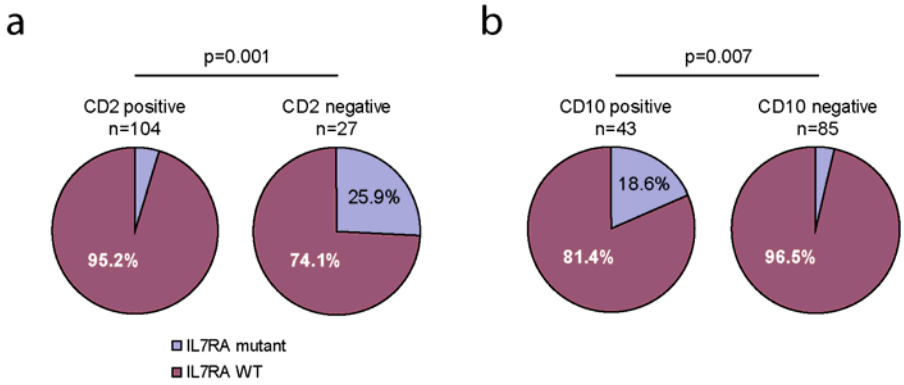
2.9.1 Supplementary Figures



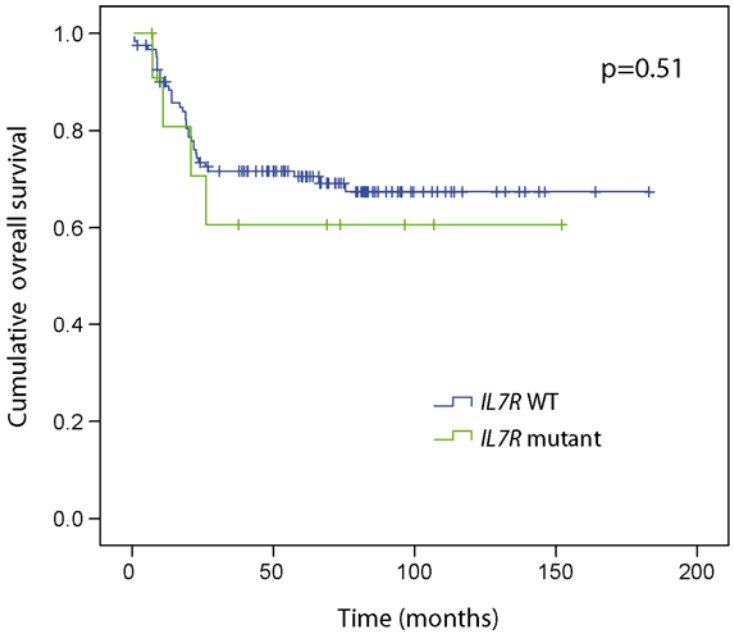
Supplementary Figure 1. *IL7R* exon 6 somatic mutations in pediatric T-ALL. DNA chromatograms of 3 patients from the Boldrini cohort showing the mutated sequences at diagnosis and lack of mutation at remission. The corresponding chromatograms of the remaining 2 patients from the same cohort are shown in Figure 1. Highlighted are the DNA and respective amino acid sequence alterations in each case. Patient P5 has a SNP (c.731C>T; p.T244I) in addition to the indicated QW mutation.



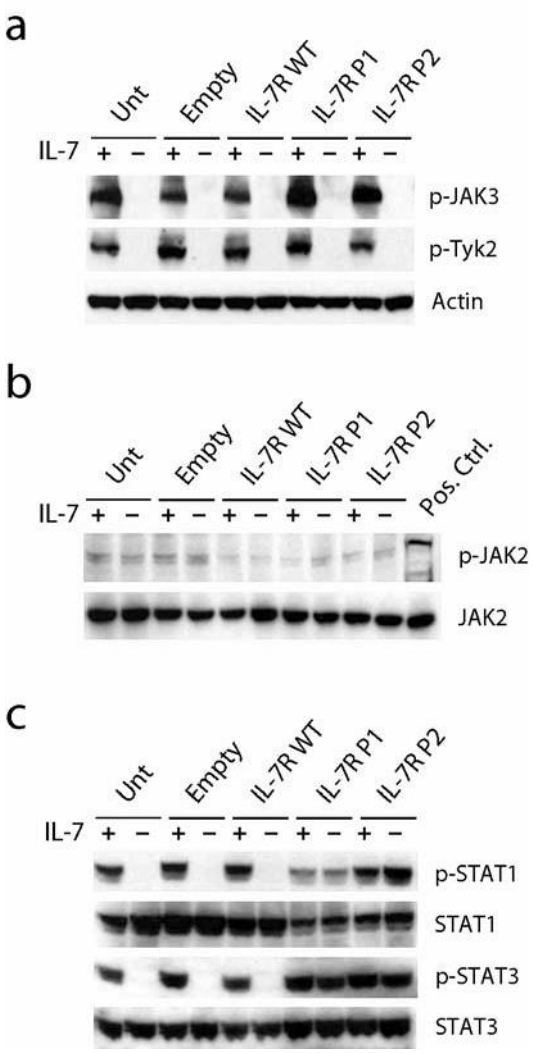
Supplementary Figure 2. The majority of unpaired cysteines created by *IL7R* mutations are predicted to localize extracellularly at the juxtamembrane-transmembrane domain interface. Prediction of core transmembrane helices (TMH, boxed) and localization of mutated amino acids (red), using two distinct bioinformatics tools. **(a)** According to DAS [1] using TM- Library size of 32, the cysteine (cyan) is located extracellularly in 12 patients (6 at the TMH border) and at the extracellular border within the TMH in 2 patients. **(b)** According to TMPRED [2] the cysteine is located extracellularly in 11 patients (4 at the TMD border) and within the TMD in 3 patients (with 1 at the extracellular border).



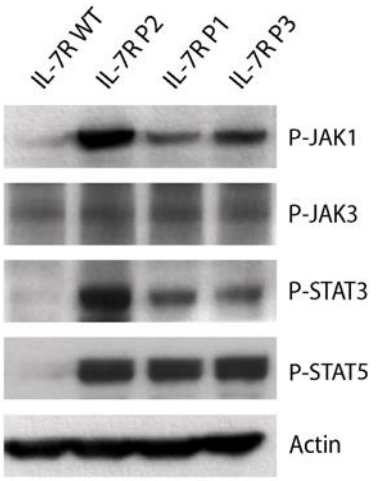
Supplementary Figure 3. Association of *IL7R* mutations with CD2 and CD10. Distribution of *IL7R* mutations among **(a)** CD2 and **(b)** CD10 positive *versus* negative patient samples. P values were calculated using Fisher's exact test.



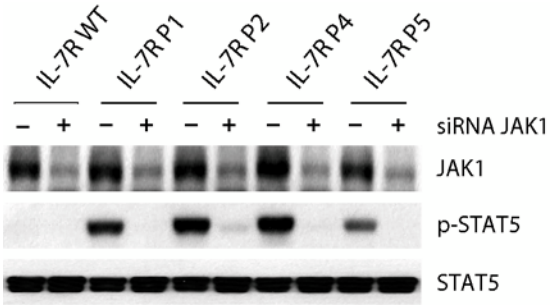
Supplementary Figure 4. Overall survival in pediatric T-ALL cases with and without *IL7R* mutation. Kaplan-Meier survival curve in pediatric T-ALL cases with (*IL7R* mutant, n=12) and without (*IL7R* WT, n=123) mutations in *IL7R* treated on DCOG ALL-7/-8 and -9 (n=66) and COALL-97 protocols (n=69). P value was calculated using Log-Rank test.



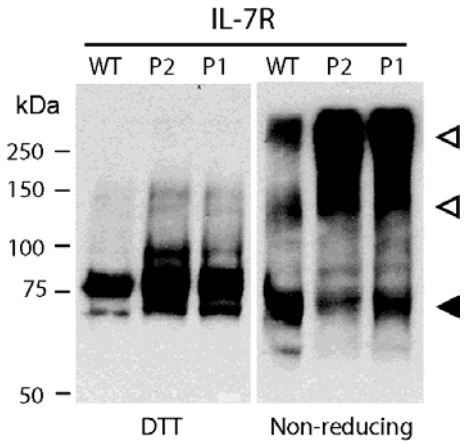
Supplementary Figure 5. Further characterization of JAK/STAT pathway activation in IL-7- versus mutant IL7R-dependent signaling in D1 cells. D1 cells expressing human WT or mutated (P1 and P2) IL-7Rα were cultured without IL-7 for 4 hr, stimulated or not with IL-7 for 20 min and evaluated by immunoblot for phosphorylation of **(a)** JAK3 and TYK2, **(b)** JAK2, and **(c)** STAT1 and STAT3. Whereas *IL7R* mutations induce constitutive phosphorylation of STAT1 and STAT3, they do not drive TYK2, JAK2 or JAK3 phosphorylation.



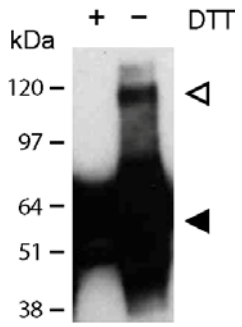
Supplementary Figure 6. *IL7R* mutations induce JAK1, STAT3 and STAT5, but not JAK3, phosphorylation in Ba/F3 cells. Ba/F3 cells expressing human WT or mutated (P1 and P2) IL-7Rα were cultured without IL-3 for 4 hr and evaluated by immunoblot for phosphorylation of the indicated proteins.



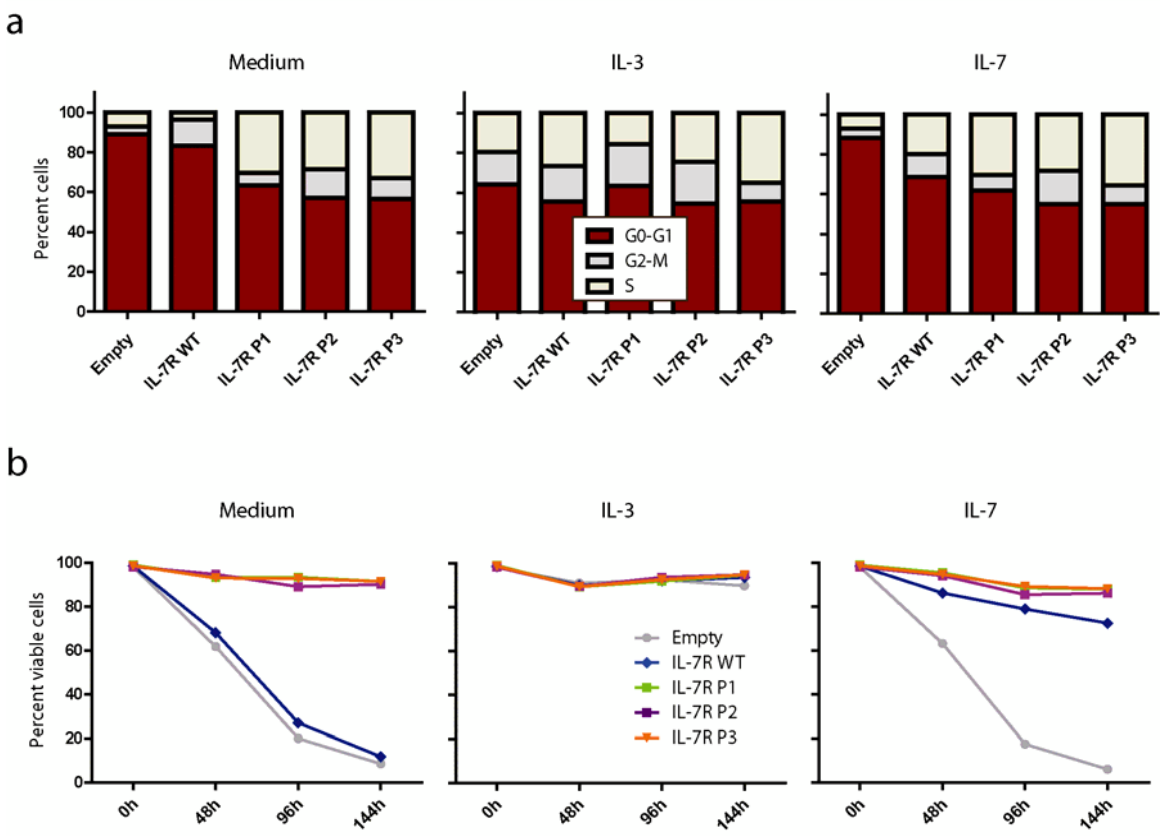
Supplementary Figure 7. JAK1 knockdown abrogates mutant IL*7R*-mediated signaling. 293T cells were transfected with WT or the indicated mutant IL-7Rα together with siRNA against JAK1 (+) or control non-targeting siRNA (-) and evaluated after 36 hr for JAK1 expression and STAT5 phosphorylation. This blot further illustrates that P5, which displays a mutation not introducing a *de novo* cysteine (T244I, I247_L248insQW; Table 1) is able to promote some degree of constitutive STAT5 phosphorylation, which is also sensitive to JAK1 knockdown.



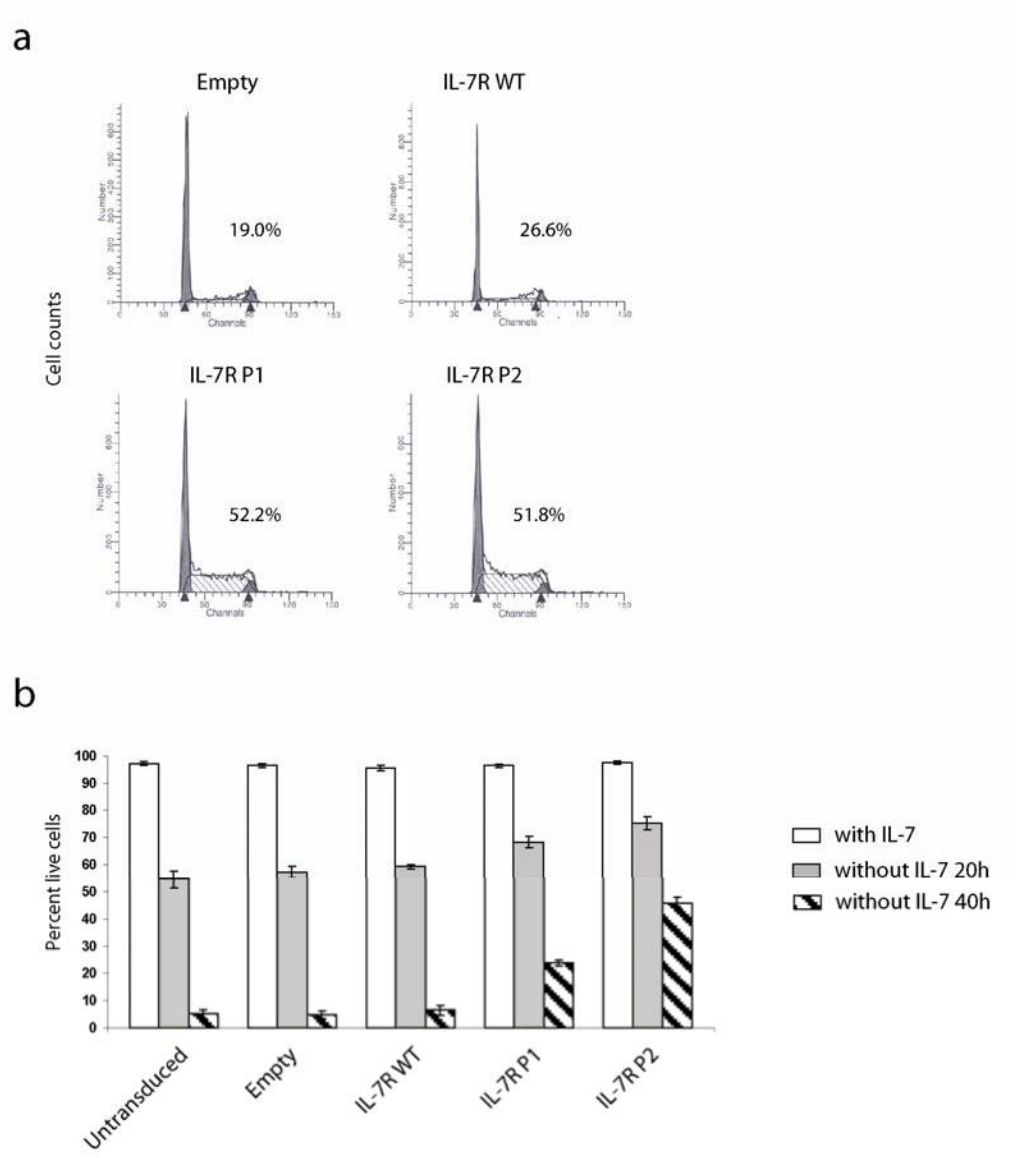
Supplementary Figure 8. IL-7Rα mutant, but not wild type, proteins constitutively form redox-sensitive dimers/oligomers in 293T cells. Lysates from 293T cells expressing wild type (WT) or mutant (P1 and P2) IL-7Rα were treated or not with the reducing agent DTT and analyzed for IL-7Rα expression by immunoblot. The monomeric and dimeric/oligomeric forms of the receptor are denoted by black and white arrows, respectively.



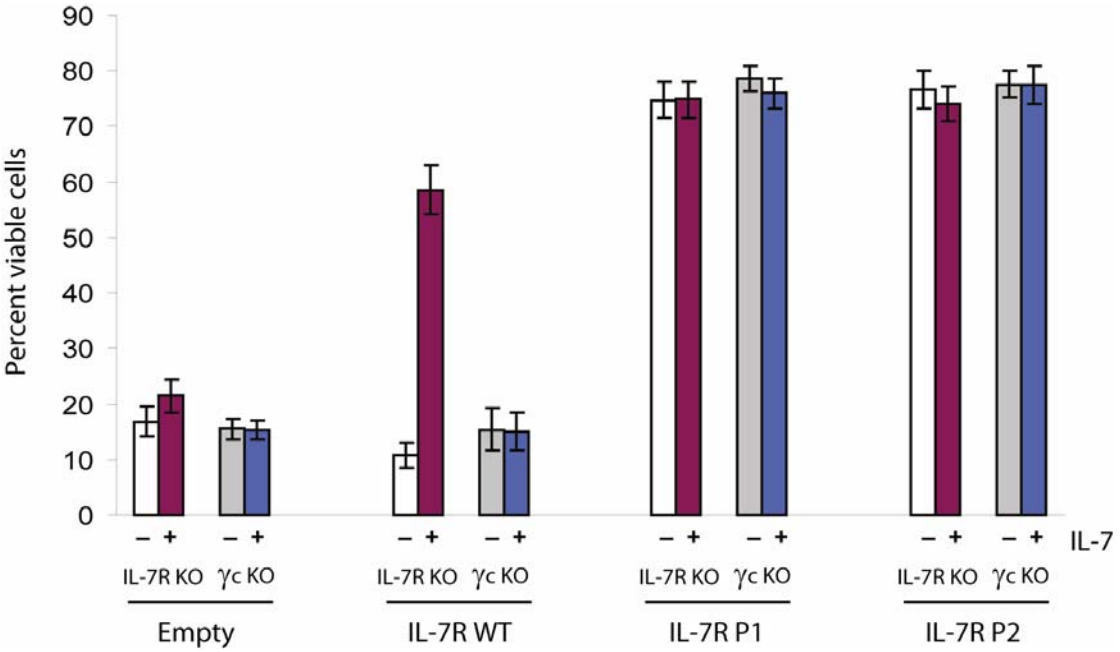
Supplementary Figure 9. IL-7Rα mutant proteins constitutively form redox-sensitive dimers/oligomers in *Il7r* -/- BM cells. Lysates from BM cells from *Il7r* -/- mice transduced with P1 mutant *IL7R* were treated or not with the reducing agent DTT and analyzed for IL-7Rα expression by immunoblot. The monomeric and dimeric forms of the receptor are denoted by black and white arrows, respectively.



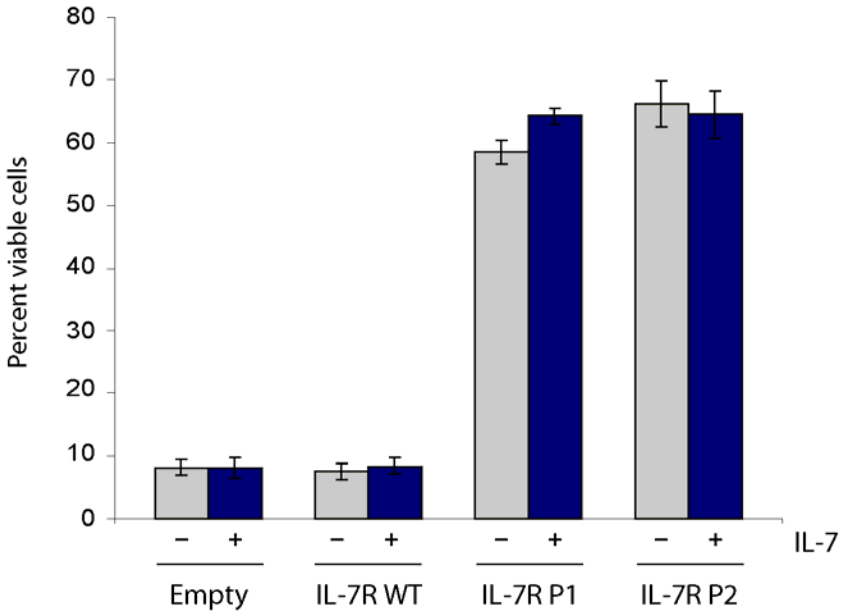
Supplementary Figure 10. *IL7R* mutations induce cell cycle progression and viability in Ba/F3 cells independently of IL-3 or IL-7. Ba/F3 cells stably expressing WT or mutated IL-7Rα were cultured in the absence of growth factors or with IL-3 or IL-7. **(a)** Cell cycle was determined at 96h. **(b)** Viability was evaluated by Annexin V/ 7-AAD staining at the indicated time points. Data represent average of triplicates ± sem.



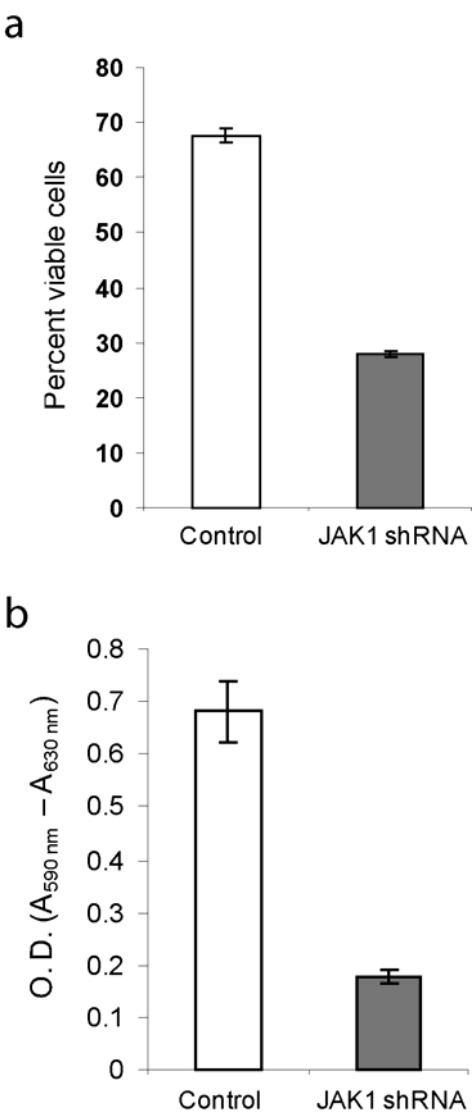
Supplementary Figure 11. *IL7R* mutations induce cell cycle progression and viability in D1 cells independently of IL-7. (a) D1 cells transduced with empty pMIG vector (Empty), IL-7Rα wild type (WT), IL-7Rα P1 or IL-7Rα P2 were analyzed for cell cycle distribution after 24h of IL-7 deprivation. Percentage of cells in cycle (S+G2/M) is indicated for each condition. **(b)** Viability of D1 cells transduced with empty pMIG vector (Empty), IL-7Rα WT, IL-7Rα P1 or IL-7Rα P2 was assessed after 20h and 40h of IL-7 deprivation. D1 cells cultured in the presence of IL-7 are shown as positive controls. Data represent average of triplicates ± sem.



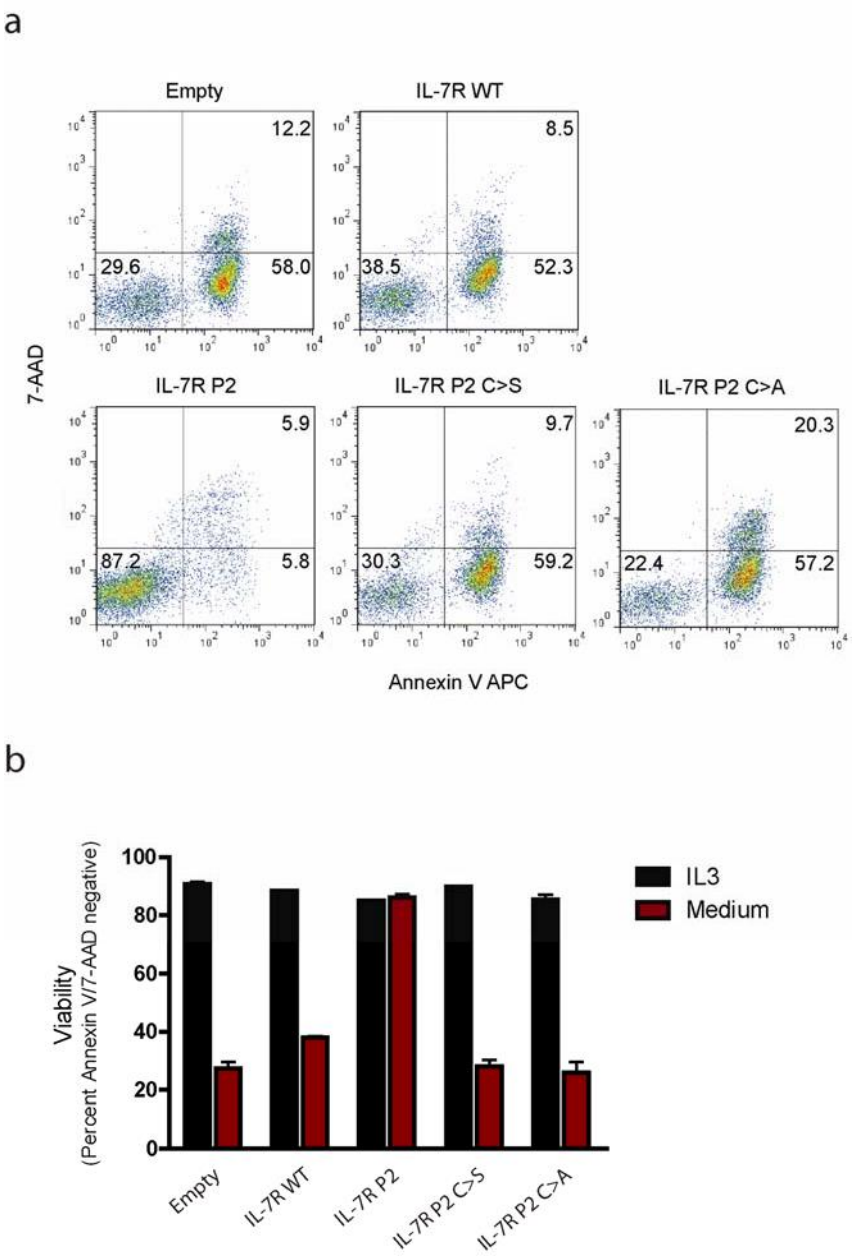
Supplementary Figure 12. *IL7R* mutations induce cell viability independently of γc expression.
Bone marrow cells from *Il7r* -/- (IL-7R KO) or *Il2rg* -/- (γc KO) mice were transduced with the empty pMIG vector (Empty), *IL7R* (IL-7R) WT, *IL7R* P1 or *IL7R* P2 and cultured for 96h with or without IL-7. Viability was evaluated by Annexin V/ 7-AAD staining. Data represent average of triplicates ± sem.



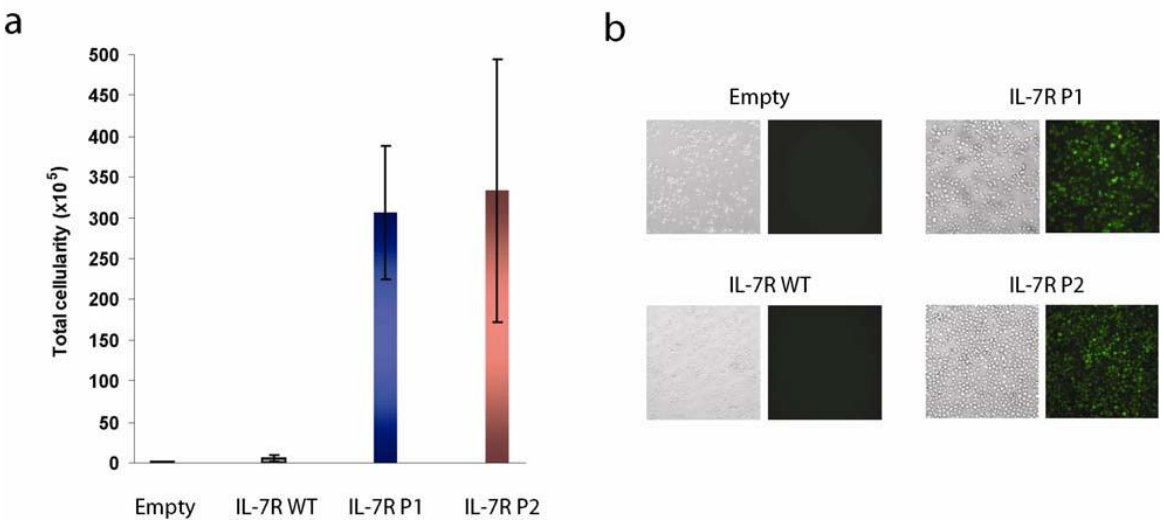
Supplementary Figure 13. *IL7R* mutations induce cell viability independently of JAK3 expression. Bone marrow cells from *Jak3* -/- mice were transduced with the empty pMIG vector (Empty), *IL7R* (IL-7R) WT, *IL7R* P1 or *IL7R* P2 and cultured for 96h with or without IL-7. Viability was evaluated by Annexin V/ 7-AAD staining. Mean ± sem is indicated for triplicates of each condition.



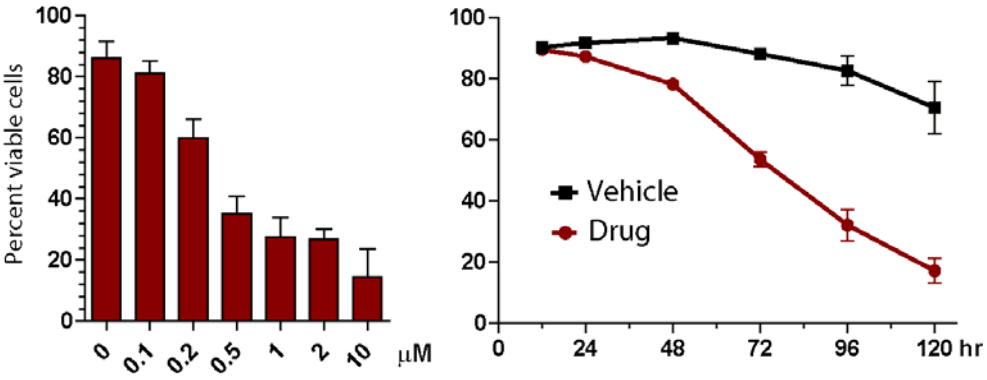
Supplementary Figure 14. *IL7R* mutations induce cell viability and proliferation in a JAK1-dependent manner. D1 cells expressing P1 mutant IL-7Rα were transduced with *Jak1* or control shRNAs. After 24 hr of infection, cells were cultured with IL-7 (50 ng/ml) and puromycin (5μg/ml) for 48 hr, and then cultured in medium alone for 48 hr. Viability/Proliferation was analyzed by Annexin V/7-AAD staining **(a)** and by an MTT assay **(b)**. Mean ± sem is indicated for triplicates of each condition.



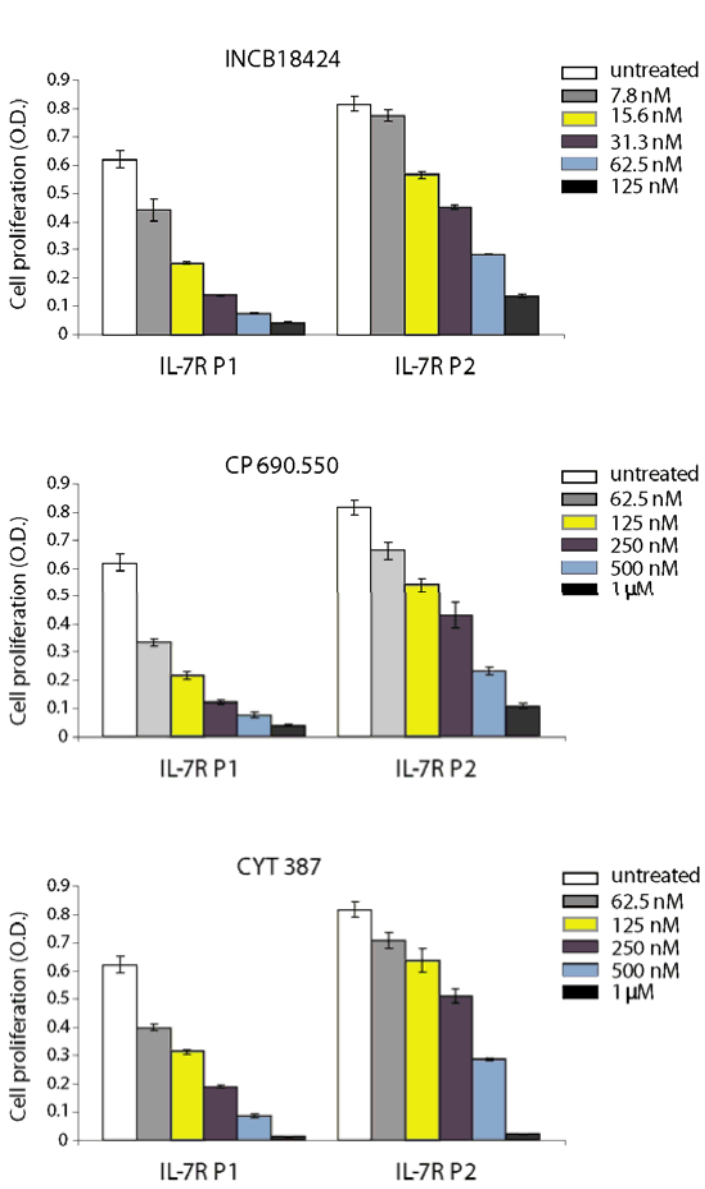
Supplementary Figure 15. Cell viability promoted by *IL7R* mutations depends on the presence of the cysteine introduced *de novo*. Ba/F3 cells stably transduced with pMIG vector (Empty), IL-7Rα WT, or IL-7Rα P2 or the indicated P2 cysteine mutants were cultured in cytokine-deprived medium or in the presence of IL-3, and analyzed for cell viability at 48h. Viability was evaluated by Annexin V/ 7-AAD staining. **(a)** Representative dot plots of cells cultured in the absence of cytokines. **(b)** Mean ± sem of duplicates of each condition.



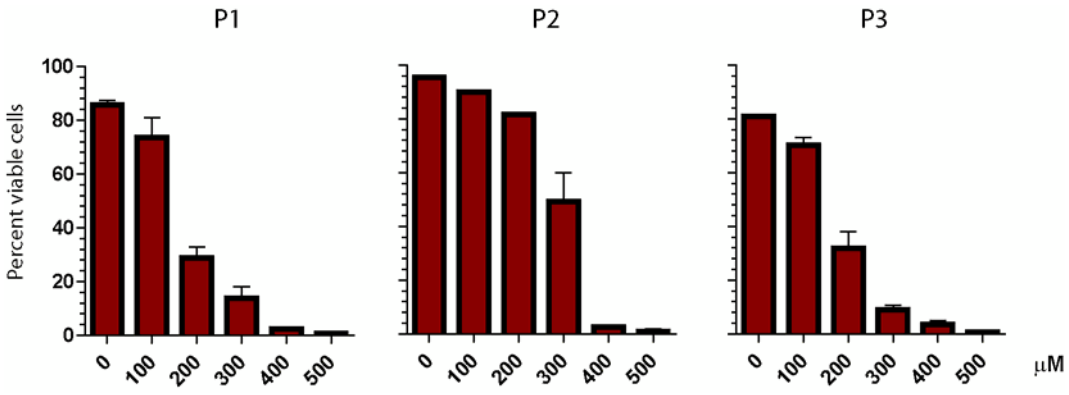
Supplementary Figure 16. Lymph node and liver infiltration in *Rag1* -/- mice subcutaneously injected with D1 cells. (a) Lymph node cellularity and **(b)** representative phase contrast and fluorescence images of lymph nodes from *Rag1*-/- mice 20 days post subcutaneous transplantation of D1 cells. Mean ± sem is indicated for triplicates of each condition.



Supplementary Figure 17. JAK inhibition reduces cell viability of P2 mutant IL-7Rα-expressing Ba/F3 cells. Ba/F3 cells stably expressing mutant IL-7Rα P2 were cultured in medium alone with or without JAK inhibitor I at the indicated doses for 72 hr (left) or for the indicated culture periods at 1μM (right) and analyzed for cell viability by flow cytometry. Mean ± sem is indicated for triplicates of each condition.



Supplementary Figure 18. JAK pharmacological inhibitors in clinical use reduce cell viability/proliferation of mutant IL-7Rα-expressing D1 cells. D1 cells stably expressing mutant IL-7Rα were cultured in medium deprived of IL-7 with or without the indicated clinically-relevant JAK inhibitors and viability/proliferation (shown as O.D.) was determined at 48 hr using an MTT assay. Mean ± sem is indicated for triplicates of each condition.



Supplementary Figure 19. Dose-dependent cell death induced by STAT5 inhibition in P1, P2 and P3 mutant IL-7Rα-expressing Ba/F3 cells. Ba/F3 cells stably expressing the indicated mutant IL-7Rα were cultured in medium alone with or without STAT5 inhibitor at the indicated concentrations for 72 hr and analyzed for cell viability by flow cytometry. Mean ± sem is indicated for triplicates of each condition.

Supplementary Table1.Differentially expressedgenesinIL7RmutantversusIL7RwildtypepediatricT-ALLs(LIMMAanalysis;cut-offFDRp-value=0.05).

Gene Title	Gene Symbol	ID	P.Value	adj.P.Val	logFC
similar to hypothetical protein MGC42630 /// hypothetical protein MGC42630 /// hypothetical LOC504188	**LOC158318 /// MGC42630 /// LOC504188**	**1553590_at**	**3,77E-09**	**0,000103**	**1,284223**
proprotein convertase subtilisin/kexin type 6	PCSK6	207414_s_at	3,54E-09	0,000103	**1,435284**
---	---	220701_at	2,07E-08	0,000378	**-0,86895**
carboxypeptidase A6	CPA6	1552511_a_at	1,04E-	0,001417	**1,247438**
protein kinase (cAMP-dependent, catalytic) inhibitor alpha	PKIA	204612_at	1,62E-07	0,001478	**-1,70916**
interleukin 17D	IL17D	227401_at	1,38E-07	0,001478	**-1,51526**
dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	DPP4	203716_s_at	2,35E-07	0,001839	**2,149853**
BAI1-associated protein 2	BAIAP2	1556145_a_at	3,57E-07	0,002443	**0,85672**
Aryl hydrocarbon receptor	AHR	1559035_a_at	4,21E-07	0,002557	**1,263743**
hypothetical protein LOC253982	LOC253982	214993_at	6,09E-07	0,003328	**0,657823**
chromosome 2 open reading frame 23	C2orf23	204365_s_at	7,23E-07	0,003592	**-0,77833**
toll-interleukin 1 receptor (TIR) domain containing adaptor protein	TIRAP	1554091_a_at	1,34E-06	0,005045	**-1,07611**
tight junction protein 3 (zona occludens 3)	TJP3	213412_at	1,24E-06	0,005045	**1,056165**
hypothetical protein FLJ13841	FLJ13841	219995_s_at	1,38E-06	0,005045	**1,495932**
olfactory receptor, family 51, subfamily B, member 5	OR51B5	234775_at	1,14E-06	0,005045	**-0,86369**
suppressor of cytokine signaling 2	SOCS2	203372_s_at	1,96E-06	0,006691	**2,636933**
CDNA clone IMAGE:5265747	---	1555994_at	3,03E-06	0,009742	**0,923981**
F-box and leucine-rich repeat protein 16	FBXL16	227641_at	3,73E-06	0,011327	**-1,11153**
chromosome 1 open reading frame 142	C1orf142	230810_at	4,01E-06	0,011552	**-0,71443**
dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	DPP4	211478_s_at	4,52E-06	0,012143	**2,123167**
dehydrogenase/reductase (SDR family) member 9	DHRS9	219799_s_at	4,66E-06	0,012143	**-0,69388**
suppressor of cytokine signaling 2	SOCS2	203373_at	5,33E-06	0,01317	**2,454919**
EPH receptor B2	EPHB2	234158_at	5,54E-06	0,01317	**0,806845**
spondin 1, extracellular matrix protein	SPON1	209436_at	8,37E-06	0,018298	**1,19454**
Acyl-Coenzyme A oxidase 3, pristanoyl	ACOX3	243817_at	8,07E-06	0,018298	**-0,77482**
spondin 1, extracellular matrix protein	SPON1	213994_s_at	9,16E-06	0,019257	**1,870146**
Xg blood group (pseudoautosomal boundary-divided on the X chromosome)	XG	1563420_at	1,23E-05	0,021793	**1,451362**
spondin 1, extracellular matrix protein	SPON1	209437_s_at	1,15E-05	0,021793	**1,246723**
Protein kinase C, epsilon	PRKCE	216753_at	1,24E-05	0,021793	**-0,81188**
hypothetical protein MGC42630	MGC42630	227563_at	1,23E-05	0,021793	**0,952581**
zinc finger protein 335	ZNF335	78330_at	1,18E-05	0,021793	**-0,95327**
hypothetical gene supported by AK091454	LOC285382	242447_at	1,37E-05	0,023389	**-0,8158**
Ryanodine receptor 3	RYR3	241901_at	1,52E-05	0,025124	**-0,944**
v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	ETS2	201329_s_at	1,57E-05	0,025297	**1,003391**
LIM homeobox 6	LHX6	224556_s_at	1,62E-05	0,025355	**0,707453**
growth arrest-specific 2	GAS2	205848_at	1,7E-05	0,02579	**2,442036**
SH3-domain GRB2-like 3	---	230959_at	1,84E-05	0,027152	**1,09072**
Death-associated protein kinase 1	DAPK1	237409_at	2,1E-05	0,030229	**1,255728**
family with sequence similarity 49, member A /// family with sequence similarity 49, member A	FAM49A	208092_s_at	2,22E-05	0,03109	**-0,89989**
hypothetical gene supported by BC028053	LOC440569	1569386_at	2,34E-05	0,03183	**0,901727**
cysteinyl leukotriene receptor 2	CYSLTR2	220813_at	2,5E-05	0,03183	**1,224922**
protocadherin beta 13	PCDHB13	221450_x_at	2,41E-05	0,03183	**0,676771**
Adaptor-related protein complex 4, epsilon 1 subunit	AP4E1	241174_at	2,46E-05	0,03183	**-0,90015**
syndecan binding protein (syntenin) 2	SDCBP2	233565_s_at	2,62E-05	0,032525	**-0,83116**
src family associated phosphoprotein 1	SCAP1	205790_at	2,74E-05	0,032776	**-1,33791**
homeo box A9	HOXA9	209905_at	2,82E-05	0,032776	**2,043246**
Rho GTPase activating protein 10	ARHGAP10	239567_at	2,78E-05	0,032776	**-1,09894**
Rho GTPase-activating protein	RICS	203431_s_at	2,9E-05	0,033081	**-1,34478**
enhancer of zeste homolog 2 (Drosophila)	EZH2	203358_s_at	3,19E-05	0,034046	**-0,65741**
defensin, alpha 6, Paneth cell-specific	DEFA6	207814_at	3,3E-05	0,034046	**1,288429**
chromosome 1 open reading frame 105	C1orf105	214357_at	3,17E-05	0,034046	**-1,08944**
brain and acute leukemia, cytoplasmic	BAALC	218899_s_at	3,24E-05	0,034046	**1,652154**
chromosome 1 open reading frame 116	C1orf116	219856_at	3,17E-05	0,034046	**0,519867**
Potassium voltage-gated channel, KQT-like subfamily, member 1	KCNQ1OT1	237249_at	3,36E-05	0,034049	**0,77784**
olfactory receptor, family 5, subfamily U, member 1	OR5U1	234545_at	3,57E-05	0,035492	**-0,88816**
hypothetical gene LOC133874	LOC133874	1554115_at	3,76E-05	0,036728	**-0,59776**
spondin 1, extracellular matrix protein	SPON1	213993_at	3,88E-05	0,036764	**1,5679**
chromosome 1 open reading frame 165	C1orf165	219670_at	3,97E-05	0,036764	**-0,95235**
Hypothetical protein LOC441168	LOC441168	228362_s_at	4,02E-05	0,036764	**0,930994**
Solute carrier family 35, member F3	SLC35F3	231520_at	4,03E-05	0,036764	**0,83601**
Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	PKIA	1563217_at	4,17E-05	0,037264	**-1,61645**
myosin, heavy polypeptide 14	MYH14	217660_at	4,23E-05	0,037264	**0,906789**
CDNA clone IMAGE:4828909	---	1563283_at	4,32E-05	0,037511	**-1,00588**
down-regulated in gastric cancer GDDR	GDDR	238222_at	4,48E-05	0,038288	**-0,75619**
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	1558211_s_at	4,58E-05	0,038522	**-0,7583**
EPH receptor B2	EPHB2	210651_s_at	4,67E-05	0,038692	**-0,68255**
CDNA clone IMAGE:4694535	---	1564760_at	4,79E-05	0,039111	**-0,8968**
Chromosome 2 open reading frame 27	C2orf27	230336_at	4,9E-05	0,039372	**1,039966**
Vacuolar protein sorting 13A (yeast)	VPS13A	1570295_at	4,99E-05	0,039466	**-0,87781**
ethanolamine kinase 2	ETNK2	219268_at	5,05E-05	0,039466	**-0,76813**
uronyl-2-sulfotransferase	UST	205138_s_at	5,16E-05	0,039748	**0,855043**
hypothetical protein LOC144481	LOC144481	1559315_s_at	5,42E-05	0,040594	**1,169696**
thioesterase domain containing 1	THEDC1	222945_x_at	5,36E-05	0,040594	**-1,00074**
UTP15, U3 small nucleolar ribonucleoprotein, homolog (yeast)	FLJ12787	221038_at	5,56E-05	0,041075	**-0,9038**
Similar to ZNF43 protein	---	1565748_at	5,74E-05	0,041867	**-0,69271**
SLAM family member 6	SLAMF6	1552497_a_at	6,07E-05	0,043684	**-0,6851**
Four and a half LIM domains 2	FHL2	1557274_at	6,62E-05	0,046982	**-0,70083**
multimerin 2	MMRN2	219091_s_at	6,8E-05	0,047662	**-0,67255**
hypothetical gene supported by AK091527	FLJ34208	1566761_a_at	7,11E-05	0,048614	**-0,64129**
Amyloid beta (A4) precursor-like protein 2	**APLP2**	**208701_at**	**7,05E-05**	**0,048614**	**-0,91316**

Supplementary Table 2. Primers used for *IL7R*, *JAK1* (JH2), and *JAK3* (JH2) RT-PCR, sequencing and cloning.

Primer	Sequence (5' 3')	5'-3' position (size)	Fragment size
IL7R_exF	CCCTCCCTTCCTCTTACTCTCA	13 – 34 (22) [a]	589 bp
IL7R_exR	TGGCGGTAAGCTACATCGTG	601 – 582 (20) [a]	
IL7R_trF	AGCCAATGACTTTGTGGTGAC	515 – 535 (21) [a]	606 bp
IL7R_trR	ACATCCCCTCCAAGCCTCT	1120 – 1102 (19) [a]	
IL7R_inF	CAGAGGCTTGGAGGGGATGT	1101 – 1120 (20) [a]	416 bp
IL7R_inR	AATCATCTTTGTCGCTCACGGT	1516 – 1495 (22) [a]	
IL7R_hapF	CACTCACTGACCTGTGCTTTT	246 – 266 (21) [a]	673bp
IL7R_hapR	GGAGACTGGGCCATACGATA	918 – 899 (20) [a]	
IL7R_ex8F	TCCTATCTTACTAACCATCAGCATTT	806 – 831 (26) [a]	788bp
IL7R_ex8R	GACTGTGTAGTGGGGTTTTGCT	1593 – 1572 (22) [a]	
Jak1JH2_aF	AGGAGTGGCAGCCCGTCTA	1934 – 1952 (19) [a]	485 bp
Jak1JH2_aR	GGCCAGGAGGAGGTTTTTAGT	2418 – 2398 (21) [a]	
Jak1JH2_bF	AATTCAAAGTTGCCAAACAGCT	2321 – 2342 (22) [a]	527 bp
Jak1JH2_bR	GTCCACTTCAGTTGCTGGTTTT	2847 – 2826 (22) [a]	
Jak3JH2_aF	CGTAGATGGGGTGGCAGTG	1489 – 1507 (19) [a]	526 bp
Jak3JH2_aR	CAGATAGTTGAGGGCGTAGGC	2014 – 1994 (21) [a]	
Jak3JH2_bF	GCCTACGCCCTCAACTATCTG	1994 – 2014 (21) [a]	523 bp
Jak3JH2_bR	CACCATTCCACAGCCCATC	2516 – 2498 (19) [a]	
IL7R_exon6F	CAAAGCACCCTGAGACCCTAC	17400 – 17420 (21) b	278bp
IL7R_exon6R	TTCGTGAAATGCCTTAATCCC	17667 – 17657 (21) b	
IL7R 3U32	GTGGTACCCTCCCTCCCTTCCTCTTACTCTCA	(32) [c]	1470bp
IL7R 1434L39	GGGCCCGGGGTTTTGGTAGAAGCTGGACATGGTGACATA	(39) [c]	
hIL7R5'BglII	CGTACAGATCTCCCTTCCTCTTACTCTCA	(29) [c]	1476bp
hIL7R3'EcoRI	TACGGAATTCTAGCCGGGGTTTTGGTA	(27) [c]	
hIL7R_cP1s	AGATGGATCCTATCTTAAACCCAaGCCTAACCATCAGCAT	799 – 829 (40) [c]	151bp
hIL7R_cP2s	AGATGGATCCTATCTTACTAACTTcTCCCACCATCAGCATTTT	799 - 832 (43) [c]	
hIL7R_cP2a	AGATGGATCCTATCTTACTAACTgcTCCCACCATCAGCATTT	799 – 831 (42)	
hIL7R_BbsI	CAAAGATGTTCCAGAGTCTTCTTATGATCGGGGAGACTG	949 – 911 (39)	

[a] Positions according to NCBI reference of coding sequences NM_002185.2 (*IL7R*), NM_002227.2 (*JAK1*), NM_000215.3 (*JAK3*).

[b] Positions refer to flanking intronic regions of exon 6 according to NCBI reference of genomic sequence NT_006576.16

[c] The cloning restriction sites are underlined. Nucleotide changes for site-directed mutagenesis are noted in small caps. Positions refer to hybridization in the coding sequences of NM_002185.2 (*IL7R)*

2.9.2 Supplementary References

1. Cserzo, M., et al., (2002) On filtering false positive transmembrane protein predictions. *Protein Eng* **15**(9): p. 745-52.

2. Hofmann, K. and Stoffel, W. (1993) TMbase: A database of membrane spanning proteins segments. *Biol Chem Hoppe-Seyler* **374**, 166.

CHAPTER 3

The Jak/STAT5/PIM1 axis activation is required for IL-7-mediated survival and growth of T-cell acute lymphoblastic leukemia cells

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Adapted from manuscript in preparation

3.1 Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive subset of ALL, the most frequent childhood malignancy. Although risk-adjusted chemotherapeutic regimens are currently extremely effective, their efficacy is associated with significant long-term side effects and those cases that relapse have dismal prognosis. Interleukin 7 (IL-7) is produced in the bone marrow and thymus. While IL-7 is essential for normal T-cell development, there is also considerable evidence that it can partake in leukemia expansion. Previously, we have shown that IL-7 promotes T-ALL expansion *in vivo* and leukemia cell survival and proliferation *in vitro* by activating PI3K/Akt/mTOR signaling pathway, consequently downregulating $p27^{kip1}$ and upregulating Bcl-2. However, it is also known that T-cell lymphomas arising spontaneously in IL-7 transgenic mice depend on STAT5 activity and *IL7R* gain-of-function mutations, found in around 10% of T-ALL patients, drive Jak/STAT5 pathway activation. In the present study, we investigated whether the Jak/STAT5 pathway may be involved in the IL-7/IL-7R pro-leukemia effects in human T-ALL. We show that IL-7 induces Jak1/3-STAT5 pathway activation, STAT5 DNA binding and transcriptional activity. Importantly, we show that inhibition of STAT5 in both TAIL7 cell line or primary T-ALL samples abrogates IL-7-mediated T-ALL cell viability, growth and proliferation. Molecularly, STAT5 inhibition results in a complete abrogation of IL-7-induced downmodulation of $p27^{kip1}$, upregulation of cyclin A and increase in transferrin receptor (CD71) surface expression. Interestingly, IL-7-dependent Bcl-2 upregulation at the mRNA or protein level is not affected by STAT5 inhibition. Cross-analysis of STAT5 ChIP-seq and RNA-seq data revealed that IL-7 drives the transcription of the serine/threonine kinase PIM1 and inhibits BCL6 via STAT5. Notably, inhibition of BCL6 mRNA and protein expression appear associate with transcription of an alternate variant that includes the processing of intron 1. Importantly, inhibition of PIM1 kinase activity abrogates IL-7-mediated T-ALL cell growth, viability and proliferation. Overall, our studies indicate that a JAK/STAT5/PIM1 axis is mandatory for IL-7/IL-7R-mediated T-ALL cell survival. Furthermore, these results indicate that JAK/STAT5 pathway inhibitors can eliminate T-ALL cells and that STAT5 plays a major role in mediating IL-7/IL-7R signaling effects in T-ALL cells, therefore constituting a promising target for therapeutic intervention in this malignancy.

3.2 Introduction

Interleukin-7 (IL-7) is a cytokine required for normal T-cell development [1, 2]. In thymocytes, IL-7 activates both the phosphatidylinositol-3-kinase/ Akt (PKB) (PI3K/Akt) and the Janus kinase/ signal transducer and activator of transcription (Jak/STAT) pathways [3, 4]. Importantly, the transcription factor STAT5 is an essential element of IL-7 signaling during normal T-cell development and mature T-cell function [5, 6].

However, IL-7 may also partake in the development of T-cell leukemia. IL-7 transgenic mice develop B- and T-cell lymphomas [7] and overexpression of IL-7R in mouse thymocytes ultimately leads to leukemogenesis [8]. Moreover, IL-7 is present in the microenvironments where the malignant T cells develop [9]. Accordingly, most primary T-ALL samples proliferate *in vitro* in response to IL-7 [10, 11], which furthermore accelerates human T-ALL development *in vivo* [12]. We have previously shown that IL-7 promotes T-ALL cell proliferation and viability via activation of PI3K/Akt(PKB) signaling pathway [13] and consequent downregulation of $p27^{kip1}$ and upregulation of Bcl-2 [14]. Importantly, STAT5 appears to be fundamental to IL-7-dependent murine lymphomagenesis [15], and *IL7R*-mutated T-ALL patient samples are sensitive to both JAK and STAT5 inhibitors [16-18]. However, no studies have yet evaluated the relevance of STAT5 within the context of IL-7 stimulation of human T-ALL cells.

In this study, we show that IL-7 activates the Jak/STAT5 pathway in T-ALL cells and this event is required for IL-7-mediated functional impact on leukemic cells. We observed that inhibition of JAK/STAT5 signaling led to a decrease in cell viability, growth and cell cycle progression induced by IL-7. Notably, we found that IL-7-mediated regulation of Bcl-2 was not dependent on STAT5 activity. On the other hand, STAT5 directly downregulated BCL6 and promoted the expression of PIM1 kinase in an IL-7-dependent manner. Furthermore, we observed that PIM1 plays a major role in mediating IL-7 effects on T-ALL cells.

3.3 Methods

Cell lines, primary T-ALL and cell culture. Primary T-ALL cells of pediatric patients at diagnosis were isolated as described in [12]. In all cases informed consent was obtained in accordance with the Declaration of Helsinki and under institutional ethical review board approval. The TAIL7 cell line, an IL-7-dependent cell line that was established from the peripheral blood of a pediatric T-ALL patient [19], was cultured in RPMI-1640 medium (Life Technologies) supplemented with 5% FBS (Biowest), 2mM glutamine, penicillin/streptomycin (Life Technologies) and 10ng/mL of rhIL-7 (Peprotech). HPB-ALL cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2mM glutamine, and 100U/mL penicillin/streptomycin. Primary T-ALL samples were cultured in conditions similar to those of TAIL7.

Experimental conditions and inhibitors. For all cells, in long term experiments (>24h) IL-7 was used at 20 ng/mL and for short term experiments (0 - 120min) at 50ng/mL, except where indicated otherwise. Before each experiment, TAIL7 cells were deprived of IL-7 for 24h; HPB-ALL were serum-starved (1% FBS) for 24h. We used the STAT5 small-molecule inhibitor N′-((4-Oxo-4H-chromen-3 yl)methylene)nicotinohydrazide (100 μM-TAIL7 cells; 150 μM-primary T-ALL cells) [20] and the PIM1 inhibitor Smi-4a (90μM; Merck/Calbiochem) [21].

Immunoblotting and antibodies. Whole cell lysates prepared as described [13], resolved by 12% SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted with antibodies against p-JAK1/3 (Y1022-Y1023/Y980) (Sigma), p-Akt (S473), Akt, p-STAT5a/b (Y694/Y699) (Cell Signaling Technology), STAT5, BCL6, PIM1, ZAP-70, and Actin (Santa Cruz Biotechnology). Immunodetection was performed by incubation with horseradish-peroxidase–conjugated appropriate secondary antibodies and developed by chemiluminescence.

STAT5 transcriptional activity and DNA binding. To assess STAT5 transcriptional activity, TAIL7 cells were transfected (nucleofected) in a Nucleofector 2b using Solution V (Lonza) with the pGL3-β-casein-Firefly luciferase and pGL4-SV40-Renilla luciferase. Briefly, upon nucleofection, cells were left to recover in RPMI 1%FBS for 12h. Cells were

then stimulated or not with IL-7 (20ng/mL) for 24h and harvested. Luciferase activity was determined using measured luminescence in an Infinite F500 luminometer (Tecan). The Firefly luciferase values in non-nucleofected cells were subtracted from the Firefly luciferase in nucleofected cells. Similar procedure was applied for Renilla luciferase. The ratio between Firefly luciferase and Renilla luciferase was determined for the stimulated condition and normalized to the control (medium) condition. In addition, nuclear extracts of unstimulated or stimulated TAIL7 cells with IL-7 (50ng/mL) were prepared and analyzed by electrophoretic mobility shift assay (EMSA) using DNA oligonucleotides containing a consensus STAT5a/b motif.

STAT5 knockdown. Plasmids encoding lentiviruses expressing shRNAs for *STAT5A* were obtained from the RNAi Consortium [22]. Specific hairpin or scramble hairpin lentiviruses were produced. HPB-ALL cells were transduced by spin infection with polybrene plus lentivirus and viability was monitored daily thereafter.

Proliferation assays. Cells were cultured in triplicates in flat-bottom 96-well plates in the appropriate experimental conditions. Cells were incubated with ^{3}H-thymidine (1 μCi/well) for the last 16h of culture before harvest. DNA synthesis, measured by ^{3}H-thymidine incorporation, was assessed using a liquid scintillation counter. Results were expressed as average and standard deviation of triplicates.

Flow cytometry analysis of viability, cell size, surface and intracellular staining. Viability was determined using Annexin V–based apoptosis detection kits and the manufacturer's instructions (R&D Systems or eBioscience). Briefly, cells were ressuspended in the appropriate binding buffer, stained with APC-conjugated Annexin V and 7-AAD at room temperature for 15 min and subsequently analyzed by flow cytometry. Cell size was assessed by quantitative analysis of forward scatter (FSC) versus side scatter (SSC) cytometry plots gated on the live cell population. Surface analysis of CD71 was done using PE-conjugated CD71 antibodies (eBioscience). Intracellular staining of Bcl-2 was performed using FITC-conjugated Bcl2 antibody (Dako). Briefly, cells were fixed using formaldehyde-based fixation buffer and the manufacturer's instructions (eBioscience), washed in PBS, ressuspended in 1× Perm/Wash Solution (BD Biosciences), stained with Bcl-2 antibody, followed by cytometry analysis. All flow cytometry sample acquisition was performed in a FACS Calibur or an LSR Fortessa (BD Biosciences). Flow cytometry data

analysis was done using FlowJo software (TreeStar). Results are expressed as percentage of positive cells or as mean fluorescence intensity (MFI).

Cell cycle analysis. Cellular DNA content was assessed by staining with propidium iodide followed by flow cytometry analysis. Briefly, 1-2 × 10^6 cells ressuspended in PBS were fixed and permeabilized with an equal volume of ice-cold 80% ethanol. Ribonuclease A was added at 50 μg/ml, and samples were incubated for 30 min at 37 ºC. Propidium iodide was added at a final concentration of 2.5 μg/mL, and samples were analyzed by flow cytometry. Cell cycle distribution was determined using ModFit LT software (Verity).

RT-PCR and qPCR. Total RNA was extracted from 0.5-1 × 10^6 cells using trizol and the manufacturer's instructions (Life Technologies). Total RNA (400ng) was reverse transcribed using the Superscript II reverse transcriptase and random hexamers, according to the manufacturer's instructions (Invitrogen). For qPCR, cDNA (4ng/well) and the relevant primers were mixed in SYBR green master mix (Applied Biosystems) according to the manufacturer's protocol. Reactions were performed in triplicates in a 7500 Fast or Viia7 System instruments (Applied Biosystems). Relative expression of the mRNAs was normalized to 18S expression using the ddCt method. Primer pairs used were (5'-3'):
BCL2 ATGTGTGTGGAGAGCGTCAACC and TGAGCAGAGTCTTCAGAGACAGCC;
BCL2L1 GGAACAATGCAGCAGCCGAG and GTAGAGTGGATGGTCAGTGT;
BCL6 GTTGTGGACACTTGCCGGAA and CTCTTCACGAGGAGGCTTGAT;
CISH AAAACTGGTGCAGCCCTTTGTA and GCCACCAGACGGTTGATGAC;
HRH2 TGGGAGCAGAGAAGAAGCAACC and GATGAGGATGAGGACCGCAAGG;
IL10 CCAGTCTGAGAACAGCTGCAC and GCTGAAGGCATCTCGGAGAT;
OSM CACAGACTGGCCGACTTAGAG and AGTCCTCGATGTTCAGCCCA;
PIM1 CGAGCATGACGAAGAGATCAT and TCGAAGGTTGGCCTATCTGA;
18S GGAGAGGGAGCCTGAGAAACG and CGCGGCTGCTGGCACCAGACTT.

Chromatin immunoprecipitation (ChIP)-sequencing and RNA-sequencing. For either ChIP- or RNA-seq, starved TAIL7 cells were stimulated or not with 50ng/mL of IL-7 in RPMI 5% FBS for 24h. 50-100× 10^6 cells were used in each condition. A ChIP-grade antibody against STAT5 was used for ChIP (Santa Cruz Biotechnology). The RNA-seq library preparation was done to enrich for mRNAs. The protocol for ChIP-seq and RNA-seq

and data analysis was performed as described previously [23], using the human genome assembly hg19.

3.4 Results

3.4.1 IL-7 activates the Jak/STAT5 pathway in T-ALL

To study the hypothesis that STAT5 is an important IL-7 signaling effector in leukemia, we began our studies by assessing JAK/STAT5 activation in response to IL-7 in T-ALL cells. We stimulated the IL-7-dependent human T-ALL cell line TAIL7 with IL-7 and observed a time-dependent (Figure 1A; top) and a dose-dependent (Figure 1A; bottom) increase in the phosphorylation of the Jak1, Jak3 and STAT5. Similar IL-7-dependend STAT5 activation was observed in primary cells collected from pediatric T-ALL patients at diagnosis (Figure 1B). Furthermore, STAT5 phosphorylation associated with increased STAT5 DNA binding (Figure 1C) and transcriptional activity (Figure 1D).

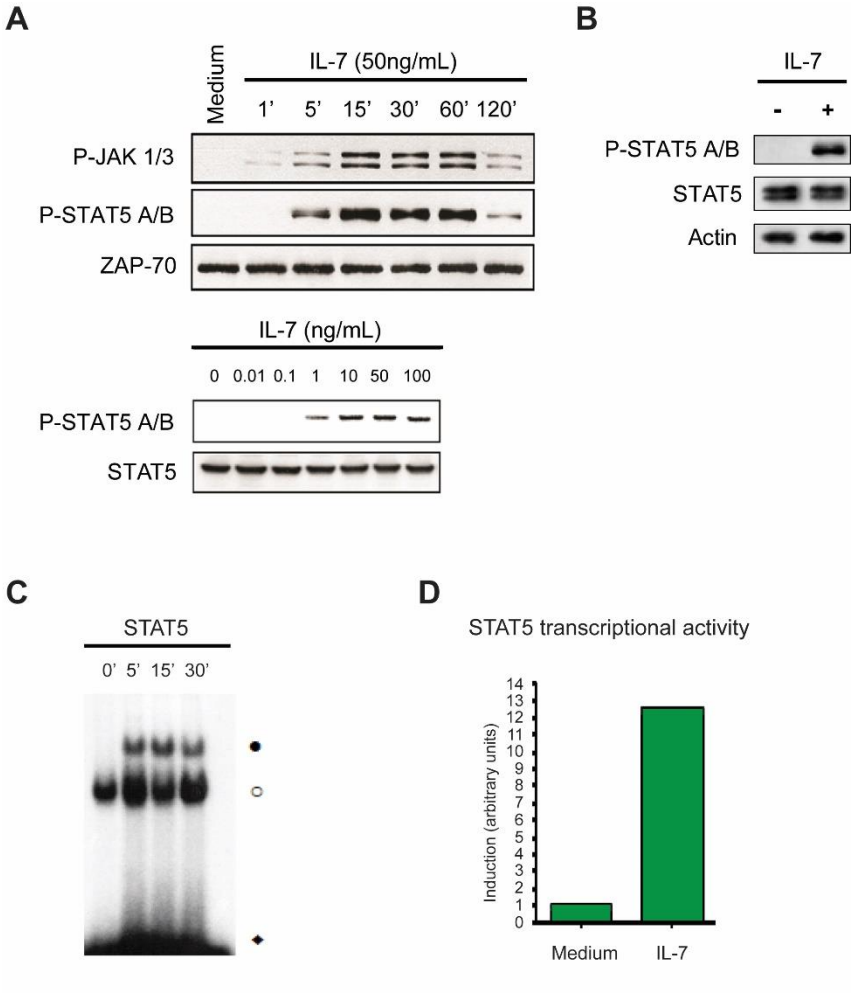

Figure 1. IL-7 induces Jak/STAT5 pathway activation in T-ALL cells. TAIL7 and primary T-ALL were evaluated for Jak/STAT5 pathway activation. (A) IL-7-starved TAIL7 cells were incubated with or without IL-7 (upper panel) for the indicated periods of time or (bottom panel) with the indicated range of IL-7 concentrations for 15 min, followed by immunoblot analysis of JAK-STAT5 pathway activation. In the P-JAK1/3 panel, the upper bands denote P-JAK1 and the lower bands denote P-JAK3. Results are representative

of at least 2 independent experiments. (B) Primary leukemia cells from one T-ALL patient were stimulated with IL-7 for 15 min, followed by immunoblot analysis of STAT5 activation. Data representative of 2 patients analyzed (C) Starved TAIL7 cells were stimulated or not with IL-7 for the indicated time points, and subsequently extracted the nuclear fraction for EMSA analysis using DNA oligonucleotides specific for the STAT5A/B consensus sequence. Indicated by (●) are the STAT5A/B-DNA oligonucleotide specific complex, by (○) unspecific oligonucleotide binding and by (◆) free oligonucleotides. Results are representative of 2 independent experiments. (D) TAIL7 cells were nucleofected with pGL3-β-casein-Firefly Luciferase vector and pGL4-SV40-Renilla Luciferase, followed by IL-7 stimulation for 24h. Luciferase activity from cell extracts was measured in a luminometer. STAT5 transcriptional activity was calculated as described in the 'Methods'. Results are representative of 3 independent experiments or 2 patients.

3.4.2 STAT5 is mandatory to mediate IL-7 pro-survival, growth and proliferation effects in T-ALL cells

Next, to establish the role of STAT5 in the context of IL-7-mediated T-ALL cell stimulation, we investigated the functional consequences of STAT5 downregulation. We used the IL-7-responsive cell line HPB-ALL to stably transduce with lentiviral vectors driving the expression of *STAT5A* shRNA or scramble control. We confirmed the efficiency of *STAT5A* knockdown at the protein level (Figure 2A). Flow cytometry analysis showed that STAT5 downregulation abrogated the IL-7-mediated increase in viability and cell growth in HPB-ALL cells, when compared to the control (Figure 2B,C). These results suggest that STAT5 is required for the survival and growth effects of IL-7 in T-ALL cells.

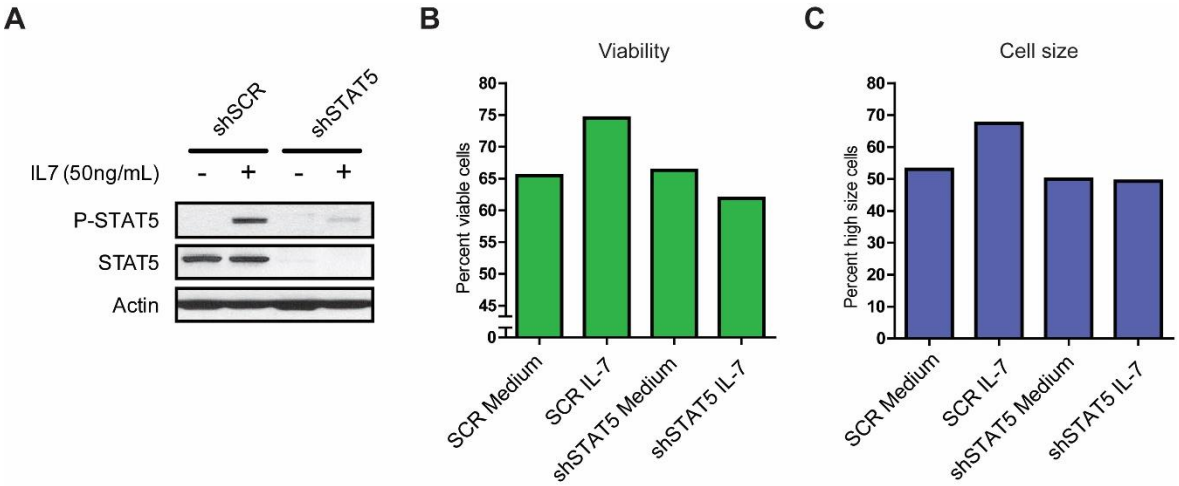

Figure 2. STAT5 knockdown abrogates IL-7-mediated T-ALL cell viability and cell growth. HPB-ALL cells were stably transduced with lentiviral vectors driving the expression of *STAT5A* shRNA (shSTAT5) or scramble control (shSCR). (A) Starved transduced HPB-ALL cells were stimulated or not with IL-7 for 15min and evaluated for knockdown efficiency (total STAT5) and STAT5 activation (P-STAT5) by immunoblot. (B,C) Stably transduced HPB-ALL were stimulated or remained IL-7 free for 72h and assessed for (B) viability and (C) cell growth. Results are representative of 3 independent experiments. Results in panels B,C represent average of triplicates ± sem.

To further dissect the functional and molecular mechanisms associated with STAT5 inhibition and to test the potential clinical applicability of these observations, we treated TAIL7 and primary leukemia T-ALL cells with a specific STAT5 inhibitor (N-((4-Oxo-4H-chromen-3-yl) methylene) nicotinohydrazide; S5i). At the functional level, treatment with the S5i completely abrogated IL-7-induced viability (Figure 3A) and cell growth (Figure 3B) in both TAIL7 cells and primary T-ALL cells. We also observed a decrease in IL-7-mediated cell cycle progression and proliferation in TAIL7 (Figure 3C and 3D, respectively) and primary T-ALL cells (Figure 3E and 3F, respectively).

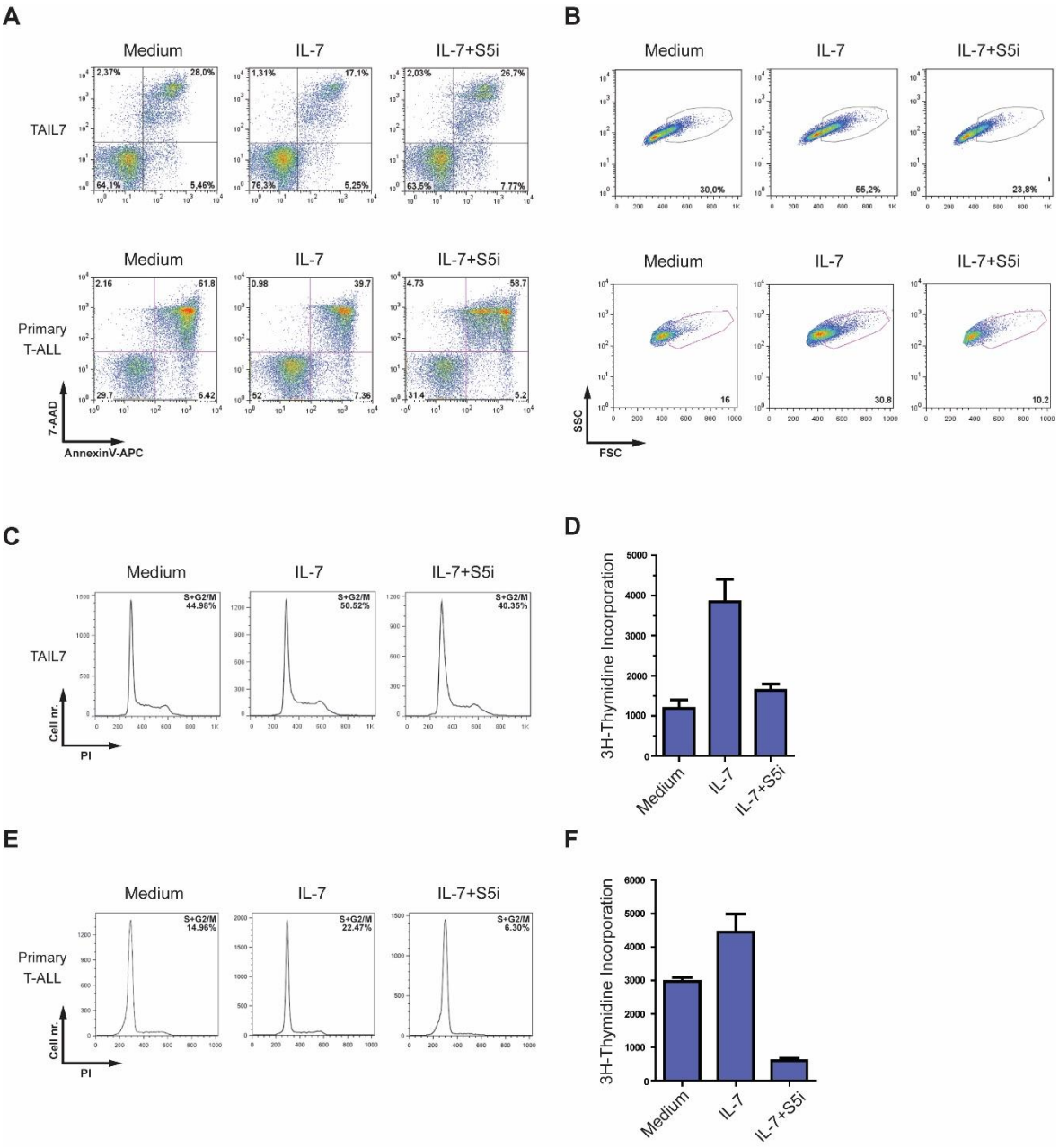

Figure 3. STAT5 inhibition abrogates IL-7-mediated T-ALL cell viability, cell growth, cell cycle progression and proliferation. IL-7-starved TAIL7 or primary T-ALL cells were incubated with IL-7 alone, simultaneously with the S5i (100uM-TAIL7; 150uM-primary), or left untreated. At 72h cells were analyzed by flow cytometry for (A) viability and (B) cell size. (C,E) Flow cytometry analysis of cell cycle at 72h. (D,F) Proliferation assay by ^{3}H-thymidine incorporation at 72h of culture in (D) TAIL7 cells or (F) primary T-ALL. Results are representative of 3 independent experiments or 4 patients. Results in panels D,F represent average of triplicates ± sem.

Next, we decided to dissect the molecular mechanisms associated with decreased viability, cell growth and proliferation. We investigated the surface expression of CD71 (transferrin receptor), a marker associated with cell growth and proliferation [24]; the S-phase cell cycle protein cyclin A; the cell cycle inhibitor $p27^{kip1}$; and the pro-survival Bcl-2 protein, all of which were previously shown to be regulated by IL-7 in T-ALL cells [13]. We found that inhibition of STAT5 prevented the IL-7-induced upregulation of CD71 in TAIL7 and primary T-ALL cells (Figure 4A). Also, we observed complete inhibition of IL-7-induced downmodulation of $p27^{kip1}$ and upregulation of cyclin A (Figure 4B). Remarkably, STAT5 inhibition did not block IL-7-mediated induction of Bcl-2 expression in either TAIL7 or primary T-ALL cells (Figure 4C). Being STAT5 a transcription factor, we also checked *BCL2* and *BCL2L2* (Bcl-xL) mRNA expression, but it was similarly unaffected by STAT5 inhibition (Figure 4D). These results were surprising to some extent, because Bcl-2 family members are known STAT5 target genes and are implicated in IL-7-mediated STAT5 function in developing T lymphocytes and mature T-cells [25-27]. However, in leukemic T-cells IL-7 was shown to upregulate Bcl-2 protein via PI3K/Akt pathway [13], which suggests that STAT5 may regulate leukemia T-cell survival by an alternative, Bcl-2-independent mechanism.

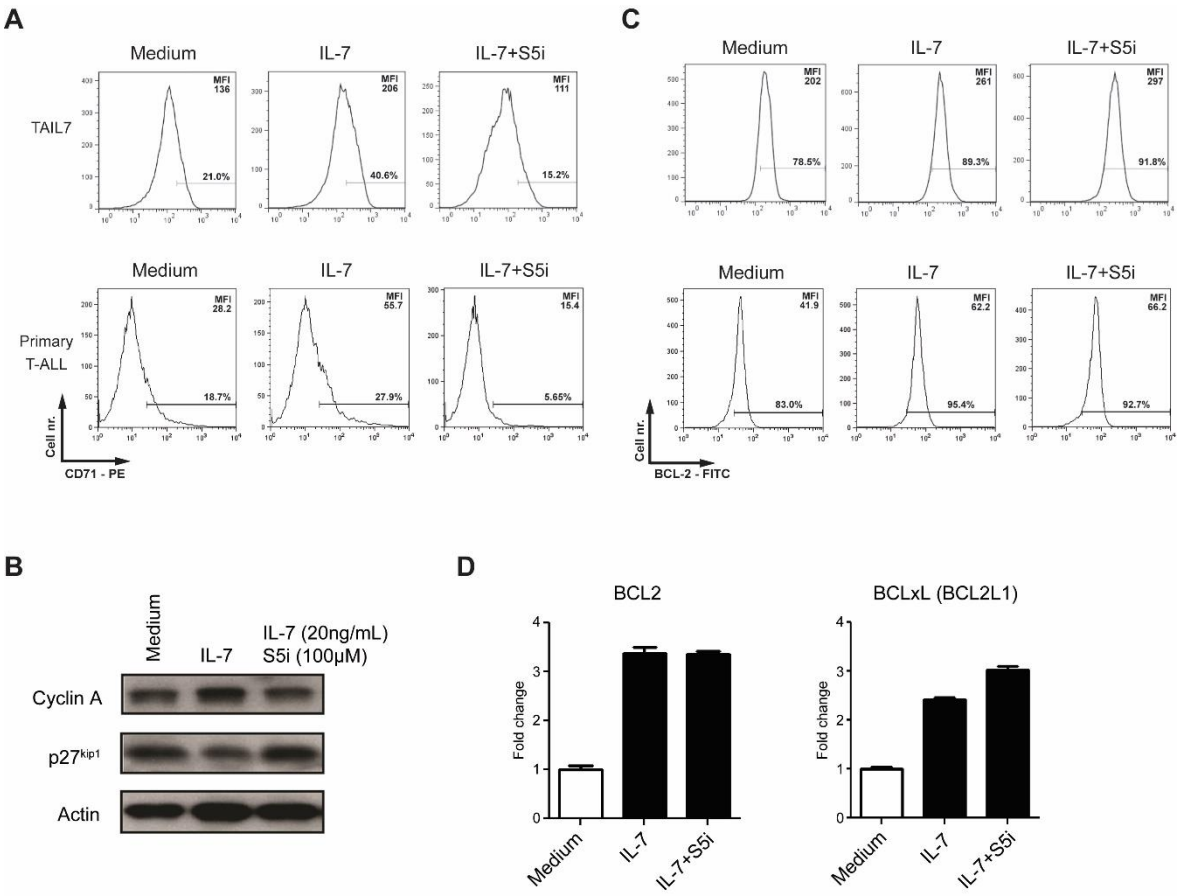

Figure 4. STAT5 inhibition abrogates IL-7-mediated T-ALL upregulation of CD71, and modulation of p27^{kip1} and Cyclin A expression, but not Bcl-2 upregulation in T-ALL cells. IL-7-starved TAIL7 or primary T-ALL cells were incubated with IL-7 alone, simultaneously with the S5i (100uM-TAIL7; 150uM-primary), or left untreated. Cells were collected for flow cytometry or immunoblot analysis at 72h. (A) Flow cytometry of CD71 surface expression. (B) Immunoblot analysis of expression of cell cycle modulators Cyclin A and p27^{kip1}. (C) Flow cytometry analysis of intracellular Bcl-2 expression. (D) Under the same experimental conditions, TAIL7 cells were collected at 24h for mRNA extraction followed by qPCR analysis of *BCL2* and *BCL2L1* gene expression. Fold induction is normalized to medium condition. Results are representative of at least 3 independent experiments or 4 patients. Results in panel D represent average of triplicates ± sem.

3.4.3 STAT5-dependent transcriptional network analysis of IL-7-stimulated T-ALL

To gain insight into the STAT5-dependent transcriptional events associated with IL-7 stimulation and try to unravel the mechanisms by which STAT5 regulates T-ALL cell viability, we performed STAT5 ChIP-seq and RNA-seq in the presence or absence of IL-7 on TAIL7 cells. De novo motif analysis on the ChIP-seq data showed, as expected, a preferential enrichment for STAT DNA binding motifs upon IL-7 stimulation, followed by Runt-related transcription factor (RUNX) binding motifs (Figure 5A). No peaks were found enriched in the unstimulated condition. Activated STAT5 may bind DNA in a dimeric (requires 1 binding site) or tetrameric form (requires 2 binding sites) [28]. Notably, these

peaks usually contain an average of 2.3 STAT motifs per peak, suggesting that STAT5 may favor DNA binding as a tetrameric complex, a feature that has been associated with leukemia [29]. Interestingly, STAT and both RUNX motifs are present in >50% of the peaks. A more detailed analysis revealed that a RUNX motif typically occurs close to a STAT motif (Figure 5B) and is particularly enriched for 1bp distance, indicating potential transcription factor interaction/competition.

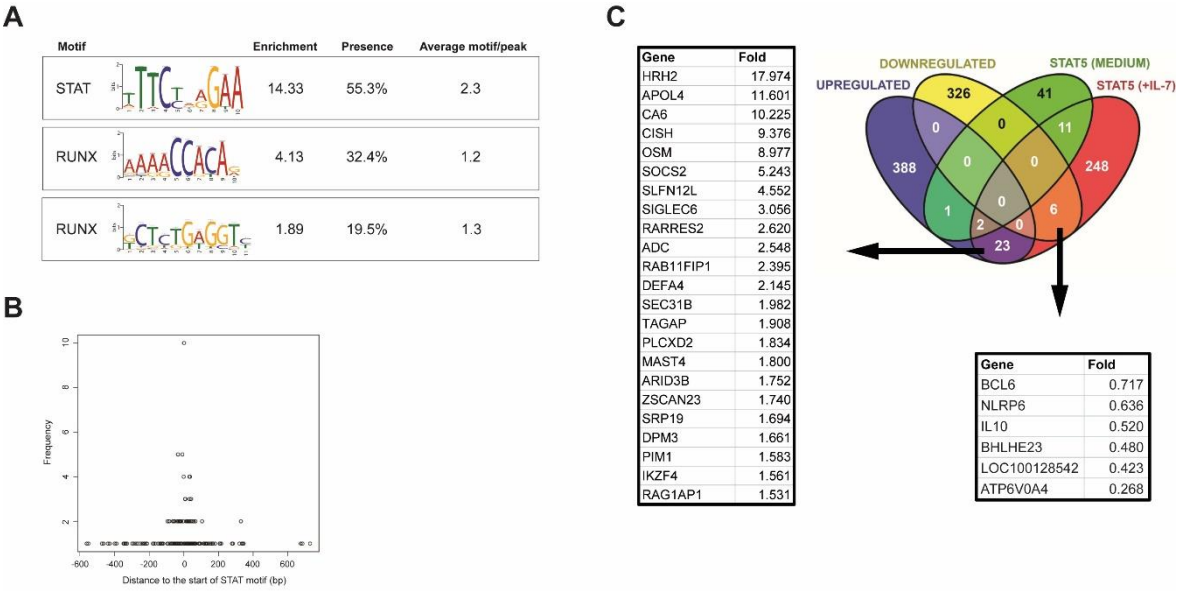

Figure 5. Cross-analysis of STAT5 ChIP-seq and RNA-seq data on IL-7-stimulated TAIL7 cells. IL-7-starved TAIL7 cells were stimulated with IL-7 or left untreated. At 24h, cells were collected for STAT5 ChIP-seq or RNA-seq enriched for mRNA. (A) De novo motif discovery and identification on STAT5 ChiP-seq peaks from IL-7 stimulated cells. Enrichment cut-off at 1.5. Presence denotes the relative presence of the motif on all peaks. Average motif/peak denotes the number of times a motif appears on the peak. (B) Graph showing the distance of RUNX motifs to the STAT motif in base-pair (bp) found in (A) on the horizontal axis, plotted against the frequency of each occurrence. (C) Venn diagram showing overlap of genes found in the RNA-seq analysis (purple and yellow sets) and ChIP-seq analysis (green and red sets). Analysis was performed with genes with a STAT5a peak within 20 kb from the transcription start site (TSS). The gene name and fold induction found in the RNA-seq are listed in the tables. Table on the left lists genes that were upregulated upon IL-7 stimulation and contained a STAT5 peak; the table on the right lists genes that were downregulated upon IL-7 stimulation and contained a STAT5 peak.

Next, we made a cross-analysis of the genes identified in the ChIP-seq with a STAT5 peak within 20kb from the transcription start site (TSS) and their differential expression upon IL-7 stimulation analyzed by RNA-seq (Figure 5C). We then validated identified up- and downregulated genes by performing qPCR analysis in IL-7-treated TAIL7 cells in the presence or absence of S5i (Figure 6). Gene expression measured by qPCR confirmed the RNA-seq results (Figure 5C and 6). In addition, pharmacological inhibition of STAT5

activity consistently diminished the IL-7-mediated increase in expression of genes upregulated by IL-7 (*HRH2*, *CISH*, *OSM*, *PIM1*) and conversely, restored or even potentiated the expression of genes downregulated by IL-7 (*BCL6*, *IL10*) (Figure 6).

To understand if regulation of gene expression would translate into functional impact, we focused our analysis on *PIM1* and *BCL6*, genes that were respectively up- and down-regulated by IL-7/STAT5 and that were likely interesting IL-7/STAT5 effectors based on their known functions [30-34].

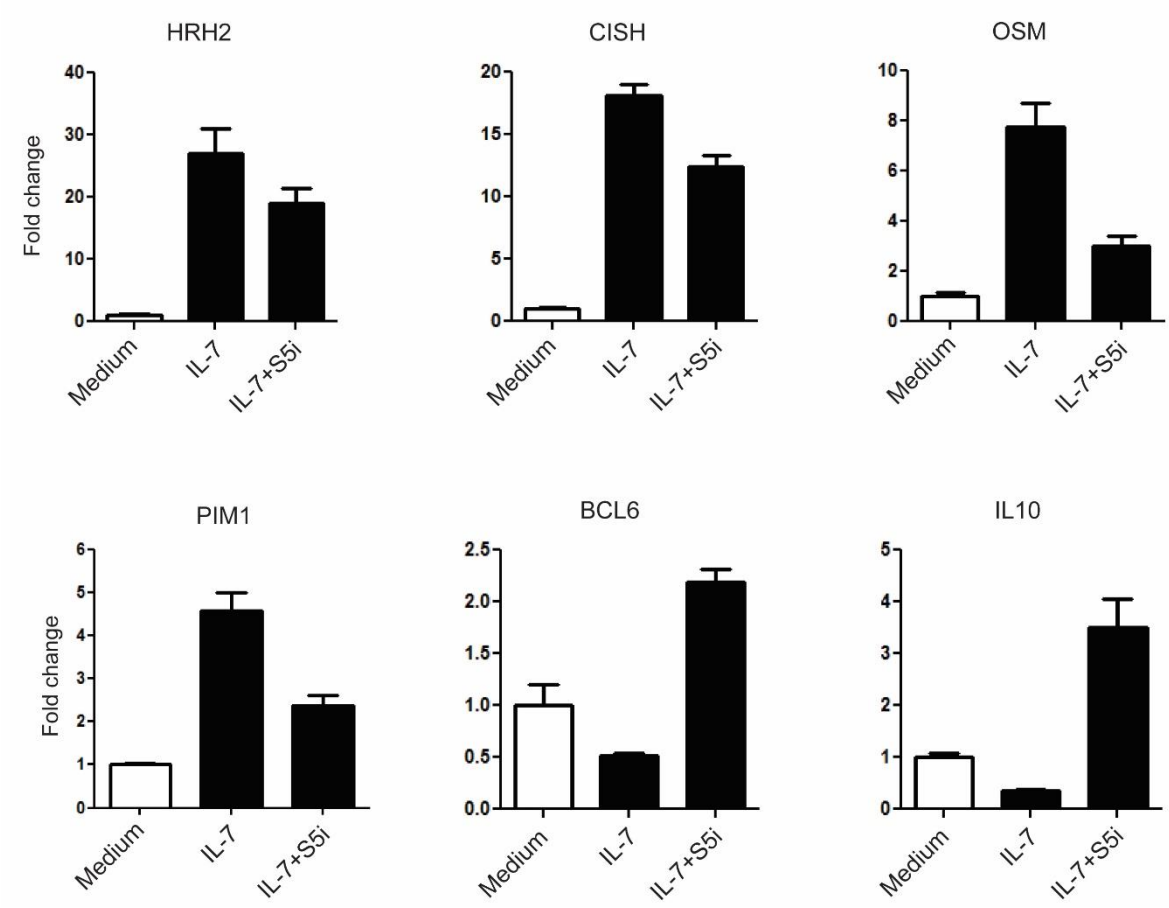

Figure 6. Quantitative PCR validation of ChIP-seq and RNA-seq data using the S5i in TAIL7 cells. IL-7-starved TAIL7 cells were incubated with IL-7 alone, simultaneously with the S5i or left untreated. Cells were collected at 24h and mRNA was extracted for qPCR analysis. Analysis of *BCL6* and *IL10* were done with cells collected at 48h. Fold change is normalized for medium condition. Results are representative of at least 3 independent experiments. Results represent average of triplicates ± sem.

3.4.4 IL-7 downregulates BCL6 expression in T-ALL in a STAT5-dependent manner

BCL6 encodes for B-cell lymphoma 6 protein (BCL6), a transcriptional repressor and an important oncogene in diffuse large B-cell lymphoma (DLBCL) [30]. Moreover, BCL6 was shown to be an effector of resistance to chemotherapy in adult BCR-ABL-positive ALL [31], again indicating its oncogenic role. However, BCL6 can also act as tumor suppressor in certain cancers [35]. In immature T-cells, IL-7 was shown to repress BCL6 [36]. In addition, an IL-7/STAT5/BCL6 link was demonstrated in follicular helper T cell differentiation [37] and B-cell development [38]. However, IL-7-mediated regulation of BCL6 in T-ALL has not been reported. We observed that while IL-7 culture of TAIL7 cells sustained high STAT5 activation and low BCL6 protein expression, IL-7 withdrawal led to loss of STAT5 phosphorylation and increased BCL6 protein levels (Figure 7A). Interestingly, when we inspected the ChIP- and RNA-seq data in the *BCL6* locus, we noticed that binding of STAT5 in the promoter region associated not only with shut-down of expression but also to the transcription of an alternate longer variant that included the processing of intron 1 into the mRNA (Figure 7B).

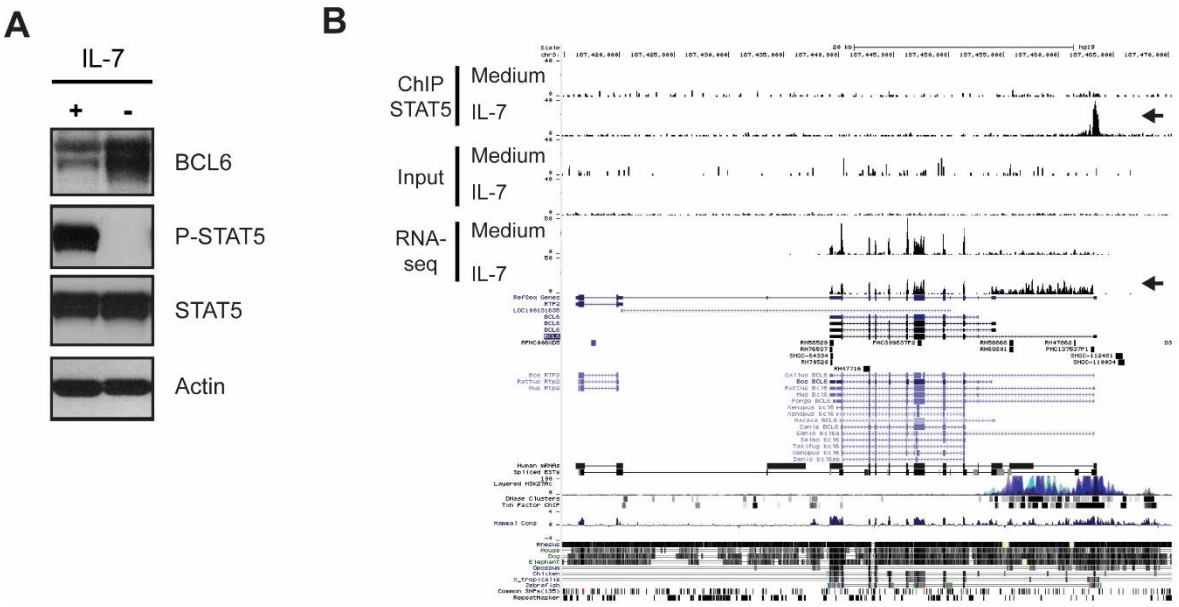

Figure 7. BCL6 protein is downregulated by IL-7 and is a direct target of STAT5-mediated mRNA downregulation and alternative transcription. (A) TAIL7 cells were withdrawn or not from IL-7 for 96h and collected for immunoblot analysis of BCL6, P-STAT5, STAT5 protein expression. (B) Data form ChIP-seq and RNA-seq was uploaded to UCSC genome browser visualization tool (top 6 tracks). The browser is located in the human *BCL6* gene locus (hg19). Custom tracks are paired as control (Medium) and IL-7. ChIP STAT5 track pair represents peaks found upon STAT5 IP, the arrow indicates STAT5 binding. Input track represents control input for ChIP. RNA-seq track represents mRNA expression. Peak height is proportional to the expression. The arrow indicates a decrease in overall *BCL6* gene expression and processing of intron 1 into the mRNA.

3.4.5 IL-7-dependent activation of PIM1 is required for increased survival and proliferation of T-ALL cells

PIM1 kinase, encoded by *PIM1*, is frequently overexpressed in cancer, including hematological malignancies [32]. Moreover, PIM1 is involved in cell cycle regulation [33] and apoptosis [34, 39], thereby being a possible alternative to Bcl-2-dependent prevention of apoptosis. Although shown to be a transcriptional target of either IL-7 or STAT5 in other contexts [32], its role on STAT5-dependent IL-7-mediated effects on T-ALL was never evaluated. Upon treatment of T-ALL cells with S5i, we confirmed that IL-7 upregulates protein expression in a STAT5-dependent manner (Figure 8A). In addition, knockdown of *STAT5A* prevented IL-7-dependent increase in PIM1 expression (Figure 8B). To evaluate the functional consequences of IL-7-dependent PIM1 upregulation, we treated TAIL7 and primary T-ALL cells with a specific PIM1 inhibitor (Smi4a). Treatment with Smi4a completely abrogated IL-7-induced viability (Figure 9A; upper), growth (Figure 9B; upper) and proliferation (Figure 9C) of TAIL7 and primary T-ALL cells (Figure 9A bottom, 9B bottom; and 9D, respectively). Molecularly, inhibition of PIM1 decreased IL-7-mediated CD71 upregulation in TAIL7 (Figure 9E) and primary T-ALL cells (Figure 9F). Surprisingly, and in contrast to STAT5, PIM1 inhibition partially prevented IL-7-dependent Bcl-2 upregulation on TAIL7 (Figure 10A) and primary T-ALL (Figure 10B) cells.

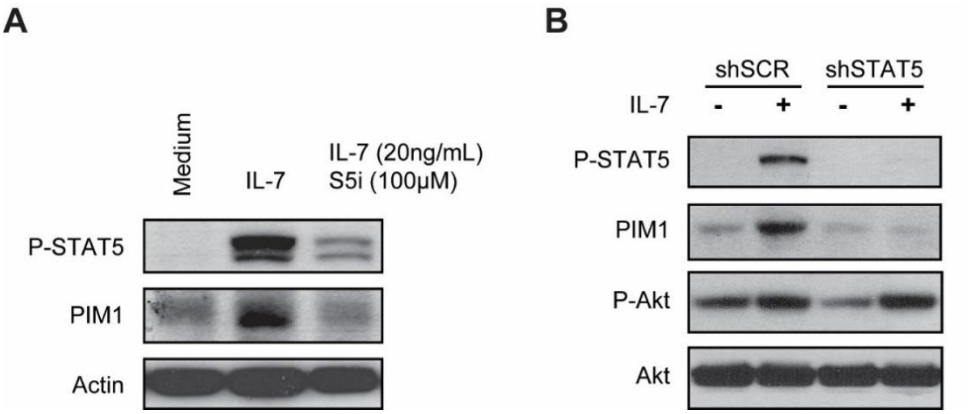

Figure 8. IL-7 upregulates PIM1 via STAT5. (A) IL-7-starved TAIL7 cells were incubated with IL-7 alone, simultaneously with the S5i, or left untreated. At 72h cells were collected for immunoblot analysis STAT5 activation (P-STAT5) and PIM1 expression. (B) Serum-starved, stably transduced HPB-ALL cells were incubated or not with IL-7 for 24h. Cell were collected for immunoblot analysis of STAT5 activation (P-STAT5), PIM1 expression and PI3k/Akt pathway activation (P-Akt).

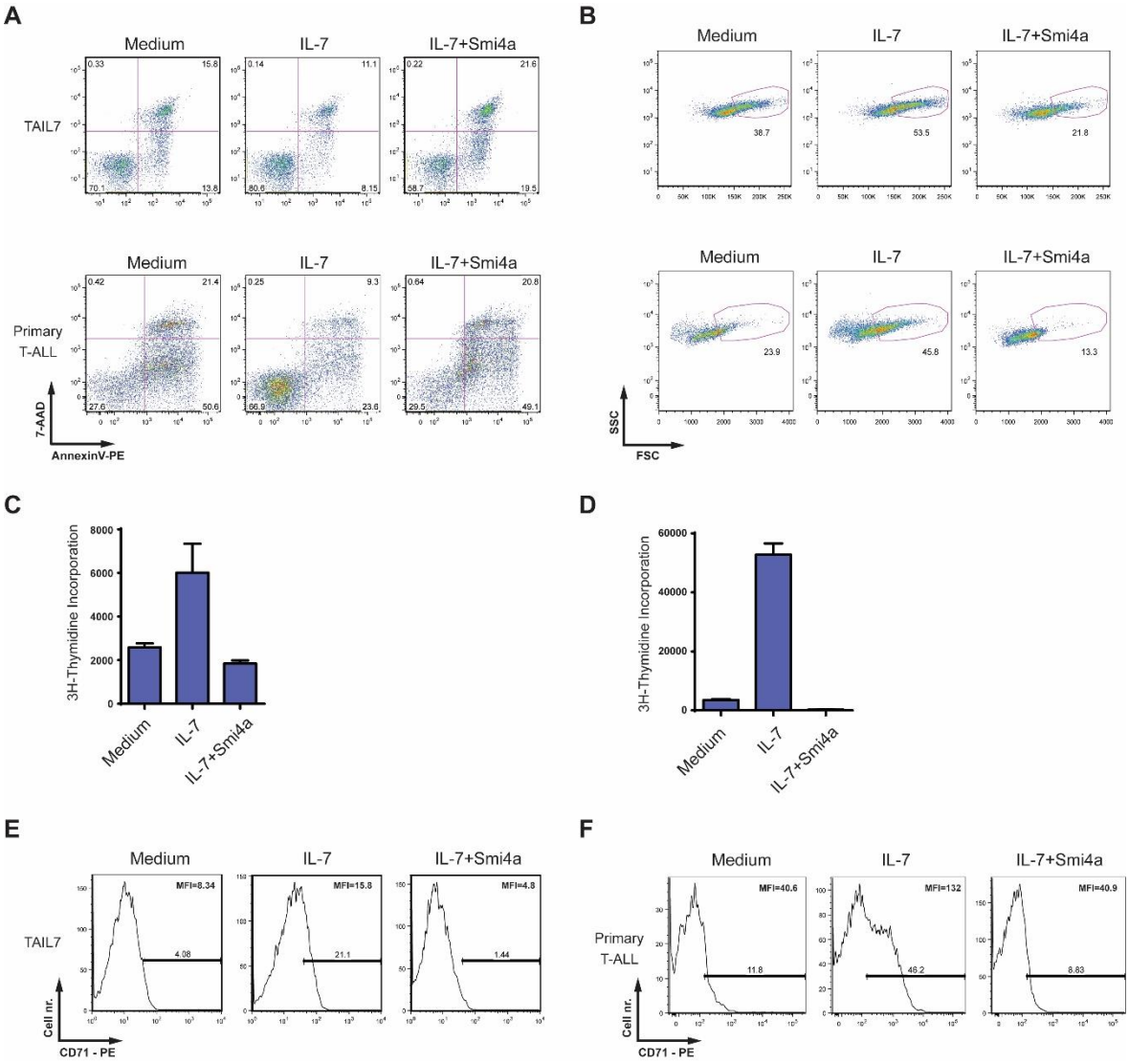

Figure 9. PIM1 inhibition abrogates IL-7-mediated T-ALL cell viability and proliferation. IL-7-starved TAIL7 or primary T-ALL cells were incubated with IL-7 alone, simultaneously with the PIM1 inhibitor Smi4a (90uM), or left untreated. At 72h cells were collected for analysis by flow cytometry of (A) viability and (B) cell size. (E,F) Flow cytometry of CD71 surface expression. (C,D) Proliferation assay by 3H-thymidine incorporation at 72h of culture in (C) TAIL7 cells or (D) primary T-ALL. Results are representative of 3 independent experiments or 4 patients. Results in panels C,D represent average of triplicates ± sem.

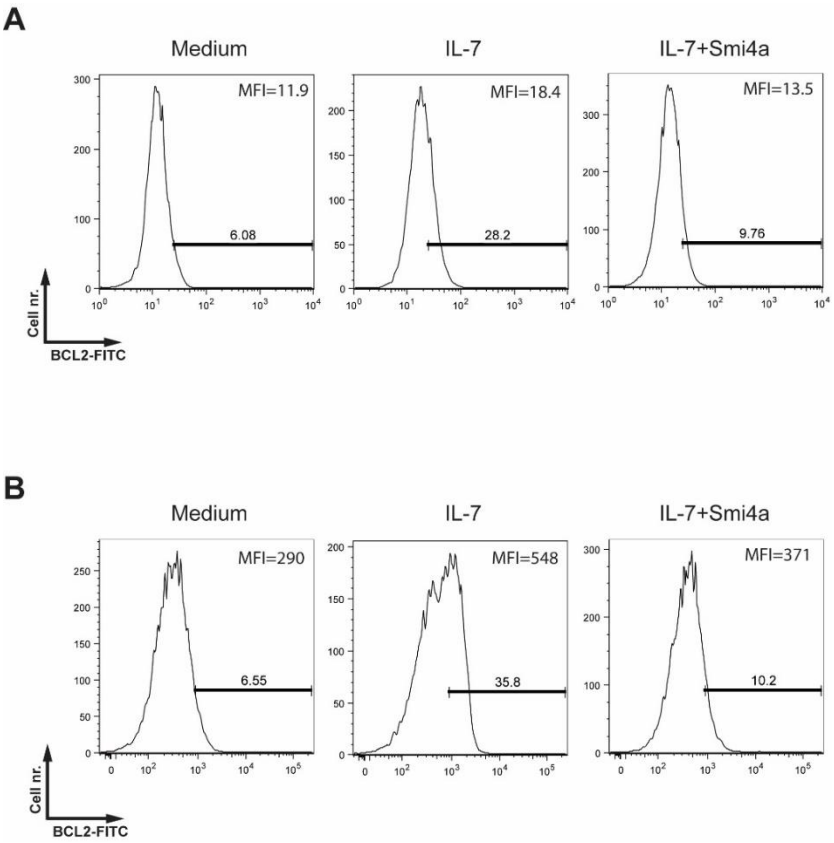

Figure 10. PIM1 inhibition partially abrogates IL-7-mediated Bcl-2 upregulation in T-ALL cells. IL-7-starved TAIL7 or primary T-ALL cells were incubated with IL-7 alone, simultaneously with the PIM1 inhibitor Smi4a, or left untreated. At 72h, cells were collected for analysis by flow cytometry of intracellular Bcl-2 expression. Results are representative of 3 independent experiments or 4 patients.

3.5 Discussion

Despite the improvements in our understanding of T-cell leukemia molecular biology, detailed identification and characterization of etiological mechanisms and potential therapeutic targets remains relatively poor. IL-7/IL-7R signaling is essential for normal T-cell development and has been demonstrated to play a role in T-ALL [40]. While the PI3K/Akt/mTOR pathway plays a critical role in mediating IL-7 effects in leukemia [13, 14], it is essentially unknown whether the Jak/STAT5 pathway, another major signaling pathway activated by IL-7 in T-ALL [41, 42], may also be determinant in IL-7/IL-7R signaling in T-cell leukemia. Here, we demonstrate for the first time that, similar to PI3K/Akt/mTOR, the Jak/STAT5/PIM1 signaling axis is absolutely required for IL-7-mediated survival, proliferation and growth of T-ALL cell lines and primary cells.

STAT5 has been shown to induce the expression of Bcl-2 or Bcl-xL in different circumstances [43-45], including IL-7-dependent signaling [26, 46]. In addition, enforced expression of Bcl-2 in *Il7r* deficient mice could restore normal thymopoiesis in stages where IL-7 has a major pro-survival role [47]. In recent thymic emigrants (RTEs), IL-7-mediated activation of STAT5 is associated with increased survival, whereas activation of PI3K/Akt pathways is associated with increased proliferation [48]. However, in an IL-7-dependent mouse thymocyte cell line where IL-7R was inactivated, STAT5 could prolong cell survival and Bcl-2 expression, but had a more limited, temporary effect compared with full IL-7 stimulation [27]. These observations suggest that in normal T lymphocytes STAT5-mediated regulation of Bcl-2/Bcl-xL, and consequent cell viability, exists elicited by IL-7 signaling, although the physiological regulation of Bcl-2 and survival by IL-7 may require additional signaling components. Thus, our observation that IL-7-mediated increase of *BCL2* and *BCL2L1* was not affected by STAT5 inhibition, was unexpected. However, our previous work demonstrated that, in leukemic T-cells, IL-7 regulates Bcl-2 via a PI3K/Akt-dependent mechanism [13]. Therefore, the evidence suggests that IL-7 regulates Bcl-2-mediated survival in T-ALL cells by activation of PI3K/Akt pathway and not via the Jak/STAT5 pathway, whereas in normal T-cells appear to rely on the latter pathway for Bcl-2 upregulation by IL-7. These subtle differences may have important therapeutic implications in drug design and targeted therapy.

Interestingly, although STAT5-mediated survival was independent of modulation of Bcl-2 expression and that PIM1 expression was STAT5-dependent, we found that PIM1 inhibition negatively regulated Bcl-2 protein expression. One possible explanation for this

conundrum is that STAT5 may trigger contradictory downstream effects that counterbalance each other. For instance, STAT5 may activate both positive (PIM1) and negative regulators of Bcl-2 expression, such that under normal circumstances the final output is neutral (no effect on overall Bcl-2 levels). However, when the PIM1 effector arm of STAT5 signaling is inhibited, the remaining, unaffected STAT5-dependent transcription may create an unbalance leading to downregulation of Bcl-2 expression. In this context it is should be highlighted that genes activated by STAT5 are not restricted to positive regulators of cell cycle and viability. For instance, STAT5 was shown to promote the expression of the cell cycle inhibitor $p21^{WAF/Cip1}$ in various cells [49-51], leading to cell cycle arrest and differentiation. Importantly, PIM1, although being activated transcriptionally by STAT5, can have the opposite effect by phosphorylating and inactivating p21 [52, 53]. In a study using the IL-3-dependent Ba/F3 cell line, a constitutively active mutant of STAT5 could render Ba/F3 cells growth factor-independent [51]. However, IL-3-induced prolonged hyper-phosphorylation of mutant STAT5 led to overexpression of SOCS1 and p21, resulting in apoptosis and differentiation of Ba/F3 cells that could be rescued by PIM1 overexpression [51]. These experiments suggest that a balance in STAT5 signaling is required to consistently promote cell survival. This scenario was not tested in our work, but it is an attractive possibility that PIM1 activity dependent on STAT5 is required to counter-balance other STAT5-mediated effects that alone would be deleterious to leukemia cells, including, for instance, Bcl-2 downregulation.

An interesting observation arising from the de novo motif analysis of our ChIP-seq data is the identification of RUNX DNA-binding motifs close to STAT motifs, with a great proportion being 1bp of distance (Figure 5A,B). Interaction between STAT5 and RUNX proteins has been shown previously and characterized as mutually inhibitory [54]. In addition, *Runx1*$^{-/-}$ mice have a high propensity to develop chemically induced T-cell lymphomas, suggesting a tumor suppressor role for *Runx1* [55] that was confirmed by more recent reports [56, 57]. However, other studies indicate that in TAL1-overexpressing T-ALL, TAL1, GATA3 and RUNX1, form a positive autoregulatory loop and promote expression of *MYB* and *TRIB2* genes, required for the survival of leukemia cells [58], suggesting that RUNX1 may be involved in promoting leukemogenesis in some cases. Notably, expression of IL-7Rα in thymocytes and mature CD4 T-cells was shown to be positively regulated by RUNX1 [59]. The heterogeneous evidence present in the literature on the role of RUNX1 in T-ALL and our own observations suggesting that STAT5 and RUNX1 may interact and thereby modulate IL-7-mediated effects in T-ALL, warrant more detailed studies, namely

regarding whether RUNX1 and STAT5 compete or cooperate in the context of IL-7-dependent signaling in leukemia.

The 2-dimensional approach we took combining STAT5 ChIP-seq with RNA-seq during IL-7 stimulation of T-ALL cells opens new avenues of research into the role of IL-7 signaling in T-ALL. For example, we found the transcriptional repressor BCL6 was directly downregulated by STAT5. However, it was interesting to observe that the repression was not a simple shut-down of transcription but was accompanied by changes in an alternate transcript variant expression (Figure 7). We did not evaluate whether the new mRNA transcript produced the same or a different protein or an unstable/ untranslatable mRNA. Either could lead to down-regulate BCL6 protein. The mechanism by which IL-7 and STAT5 regulate expression of transcript variants was not investigated, but it could involve alternative splicing. The effects of IL-7 in alternative splicing are, to our knowledge, restricted to a recent report showing that in T-cells, IL-7 can regulate alternative splicing of CD95 (Fas) to promote memory CD4 T-cell survival [60]. Thus, assessing the importance of IL-7-regulated changes in transcript variants expression could bring new insights into the complexity of IL-7 signaling in both normal and leukemic T-cells.

Although we focused our functional studies on PIM1, other STAT5-regulated genes may be of relevance as well. For instance, it is interesting that two of the top STAT5-dependent upregulated genes in the context of IL-7 stimulation (*CA6* and *OSM*) have potential to impact on the microenvironment. *CA6* codes for the isozyme carbonic anhydrase (CA) 6, the only secreted isozyme of CA, involved in interconversion of carbon dioxide and bicarbonate to maintain pH balance. Expression of CA isozymes has been associated with tumor growth and metastasis by intra- and extra-cellular pH modulation [61-65]. *OSM* codes for the cytokine oncostatin M (OSM), a member of the IL-6 family of pro-inflammatory cytokines. OSM has been involved in tumor growth [66], tumor invasion and angiogenesis [67], hepatocyte metabolic reprogramming [68] and paracrine pro-tumoral effects in breast cancer [69]. The possibility that IL-7, a primary growth and survival factor for normal T-cells and for T-ALL, may induce a cascade of secondary changes in the leukemic microenvironment driven by the leukemia cells themselves is intriguing. In fact, IL-7 could be promoting leukemia progression not only by directly affecting leukemia cells themselves but also by indirectly modulating the microenvironment. Further studies are required to assess the role of IL-7 as a possible microenvironmental modulator in T-cell leukemia and its therapeutic ‘targetability’.

Overall, this work unveiled the Jak/STAT5/PIM1 axis as mandatory for IL-7/IL-7R-dependent T-ALL cell survival. Furthermore, our results indicate that STAT5 and PIM1 small molecule inhibitors can eliminate IL-7-mediated pro-leukemia cell effects and therefore may constitute promising tools for therapeutic intervention in T-ALL.

3.6 References

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CHAPTER 4

IL-7 increases glucose metabolism and early expression of glucose metabolism-related genes in T-cell acute lymphoblastic leukemia cells: a preliminary study

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4.1 Abstract

Interleukin-7 (IL-7) is essential for normal T-cell development and mature T-cell metabolism. However, there is also considerable evidence that it can partake in leukemia expansion. Previously, we have shown that IL-7 promotes T-cell acute lymphoblastic leukemia (T-ALL) cell survival, growth and proliferation *in vitro* by activating PI3K/Akt/mTOR and JAK/STAT5/PIM1 signaling pathways. Moreover, IL-7 upregulates GLUT1 expression and glucose uptake in T-ALL cells and GLUT activity is required for IL-7-mediated viability of leukemic T-cells. In normal T lymphocytes, IL-7 was shown to upregulate glucose use, trafficking of Glut1 to the cell surface and hexokinase II (HK2) gene transcription. In the present study, we aimed to more broadly characterize the impact of IL-7 on the regulation of metabolism-related genes in leukemia cells. We show, by functional annotation analysis of transcriptome data from IL-7-stimulated TAIL7 T-ALL cells, that IL-7 generally regulates metabolic gene expression, in particular regarding sugar metabolism pathways (namely glycolysis) and oxidative phosphorylation. Functionally, IL-7 increases glucose use and lactate production in T-ALL cells. Additionally, we provide evidence that IL-7 drives very early expression of several glycolysis-related genes in T-ALL. Overall, our preliminary studies indicate that IL-7 has a direct impact on T-ALL cell metabolism.

4.2 Introduction

Cytokines and growth factors influence cell growth and survival [1]. Lymphocytes require extrinsic signals to maintain their cell size and viability [2]. Importantly, interleukin-7 (IL-7) was shown to be able to maintain cell size, metabolic activity and survival of naïve T-cells [3] and required to sustain basal glucose metabolism *in vivo* on resting T-cells [4]. While apoptosis induced by growth factor withdrawal, including IL-7, can be rescued by overexpression of pro-survival Bcl-2-family members, these cannot rescue cell growth or metabolic activity [1-3]. In addition, IL-7 was shown to regulate glucose use, trafficking of Glut1 and increase in hexokinase II (HK2) gene transcription in T lymphocytes [5, 6]. In fact, the knowledge on the impact of IL-7 on T-cell metabolic activity was recently expanded by the finding that IL-7 promotes the expression of the glycerol channel aquaporin 9 (AQP9) in memory CD8 T-cells leading to glycerol transport and triglyceride synthesis, which is essential for IL-7-mediated survival of memory CD8 T lymphocytes [7].

While IL-7 is essential for normal T-cell development [8, 9], there is considerable evidence that IL-7/IL-7R-mediated signaling can promote leukemogenesis [10, 11]. IL-7 is produced in the thymus and bone marrow, microenvironments where the malignant T cells arise and develop [12]. Previously, we showed that IL-7 contributes to T-cell acute lymphoblastic leukemia (T-ALL) cell survival, growth and proliferation by activating PI3K/Akt/mTOR signaling [13, 14] and Jak/STAT5 signaling (Chapter 3). Furthermore, *IL7R* gain-of-function mutations are found in around 9-10% of childhood T-ALL cases [15-17]. In T-ALL, IL-7 positively modulates mTOR activity and promotes transferrin receptor (CD71) expression [13](Chapter 3), both proteins associated with increased T-cell metabolism [18, 19]. Notably, IL-7 also promotes GLUT1 expression and glucose use in T-ALL cells [13], and GLUT activity appears to be required for IL-7-mediated upregulation of reactive oxygen species (which also relies on mitochondrial respiration) and T-ALL cell viability [20].

Although, accumulating evidence suggests that IL-7 may have a non-redundant role in metabolic regulation of both normal and leukemic T-cells, the intervening mechanisms are still poorly understood. In this preliminary study, we provide evidence indicating that IL-7 has a broad impact on T-ALL cell metabolism, as judged by its ability to modulate the expression of genes involved in different metabolic pathways, and particularly in glycolysis and oxidative phosphorylation. Analysis of glucose consumption and lactate production suggest promotion of aerobic glycolysis by IL-7. In addition, we observed very early

induction of key glycolytic pathway genes in response to IL-7. Understanding the metabolic network elicited by IL-7 on T-ALL cells holds the promise of discovering new targets for therapeutic intervention.

4.3 Methods

TAIL7 cell culture. The TAIL7 cell line, an IL-7 dependent cell line that was established from the peripheral blood of a pediatric T-ALL patient [21], was cultured in RPMI-1640 medium (Life Technologies) supplemented with 5% FBS (Biowest), 2mM glutamine, 100U/mL penicillin/streptomycin (Life Technologies) and 10ng/mL of rhIL-7 (Peprotech).

Experimental conditions. TAIL7 cells were deprived of IL-7 for 24h, followed by incubation in pre-warmed culture medium (37ºC) and stimulated, where indicated, with IL-7 (50ng/mL) for the indicated time and collected for the different assays. Cells were cultured as 2×10^6 cells/mL.

RT-PCR and qPCR. Total RNA was extracted from $0.5\text{-}1 \times 10^6$ cells using trizol and the manufacturer's instructions (Life Technologies). Total RNA (400ng) was reverse transcribed using the Superscript II reverse transcriptase according to the manufacturer's instructions and random hexamers (Invitrogen). For qPCR, cDNA (4ng/well) and the relevant primers were mixed in SYBR green master mix (Applied Biosystems) according to the manufacturer's protocol. Reactions were performed in triplicates in a Viia7 System instrument (Applied Biosystems). Relative expression of the mRNAs was normalized to 18S expression using the ddCt method. IL-7-dependent fold change was calculated by dividing the expression of the gene in IL-7 stimulated by IL-7 non-stimulated condition collected at each timepoint. Primer pairs used were (5'-3'):
GLUT1 TCTGGCATCAACGCTGTCTTC and CGATACCGGAGCCAATGGT;
HK2 GAGCCACCACTCACCCTACT and CCAGGCATTCGGCAATGTG;
PFKM AGCGTTTCGATGATGCTTCAG and GGAGTCGTCCTTCTCGTTCC;
PFKL GTACCTGGCGCTGGTATCTG and CCTCTCACACATGAAGTTCTCC;
PFKFB3 ATTGCGGTTTTCGATGCCAC and GCCACAACTGTAGGGTCGT;
ENO1 TGGTGTCTATCGAAGATCCCTT and CCTTGGCGATCCTCTTTGG;
PKM ATAACGCCTACATGGAAAAGTGT and TAAGCCCATCATCCACGTAGA
LDHA TTGACCTACGTGGCTTGGAAG and GGTAACGGAATCGGGCTGAAT;
BCL2 ATGTGTGTGGAGAGCGTCAACC and TGAGCAGAGTCTTCAGAGACAGCC;
CISH AAAACTGGTGCAGCCCTTTGTA and GCCACCAGACGGTTGATGAC;

PIM1 CGAGCATGACGAAGAGATCAT and TCGAAGGTTGGCCTATCTGA;
MYC GCCACGTCTCCACACATCAG and TGGTGCATTTTCGGTTGTTG;
18S GGAGAGGGAGCCTGAGAAACG and CGCGGCTGCTGGCACCAGACTT.

DAVID bioinformatics analysis and KEGG pathway mapping. The RNA-seq data collection and analysis was previously described (Chapter 3). Functional annotation analysis of enriched pathways was done using DAVID Bioinformatics Resources [22, 23]. For the analysis of the top 75% genes with higher mean normalized read counts were selected as background. For the query list, genes with fold difference in expression >1.5x and a p-value <0.05 were selected. DAVID functional annotation analysis was done with default settings. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database [24, 25] was used for global metabolism pathway mapping. The annotation data for KEGG pathway category was retrieved from DAVID and sorted by lowest p-value. Pathways with a Benjamini corrected p-value >0.001 were excluded. Gene names were collected from enriched pathways, converted to KEGG protein identifiers and mapped in the KEGG website in the *Homo sapiens* global metabolism atlas.

Metabolite analysis. Culture cellularity was determined at the beginning of the experiment. Supernatants form experimental cultures were collected at 0h and 24h, centrifuged and frozen at -20ºC. Upon thawing, the concentrations of glucose and lactate were determined using an automatic analyzer YSI 7100MBS (Yellow Springs Instruments). The consumption/production rates of specific metabolites were determined. The variation of nutrient/metabolite amount during the time interval (24h) was calculated and divided by the time interval and the culture cellularity. Results are expressed as $pmol.h^{-1}$ per cell.

4.4 Results

4.4.1 IL-7 regulates the expression of key metabolic pathway genes in T-ALL cells

We have previously produced and validated RNA sequencing (RNA-seq) data to explore the IL-7-mediated transcriptional network in T-ALL (Chapter 3), from stimulated human IL-7-dependent T-ALL cell line TAIL7 with or without IL-7. To digest the data, we performed functional annotation analysis of enriched pathways using DAVID Bioinformatics Resources and KEGG pathway mapping. The top 20 enriched pathways are shown in Figure 1A. Interestingly, we found that many of the enriched pathways were metabolism-related. Those include oxidative phosphorylation, glycolysis/ gluconeogenesis, pentose phosphate and other sugar-related pathways. KEGG Atlas representation overview of IL-7-modulated gene expression, pathway relationship and the potentially affected metabolic enzyme is shown in Figure 1B. Overall, these results indicate that IL-7 stimulates general cell metabolism with emphasis on particular sugar-related pathways and oxidative phosphorylation in T-cell leukemia.

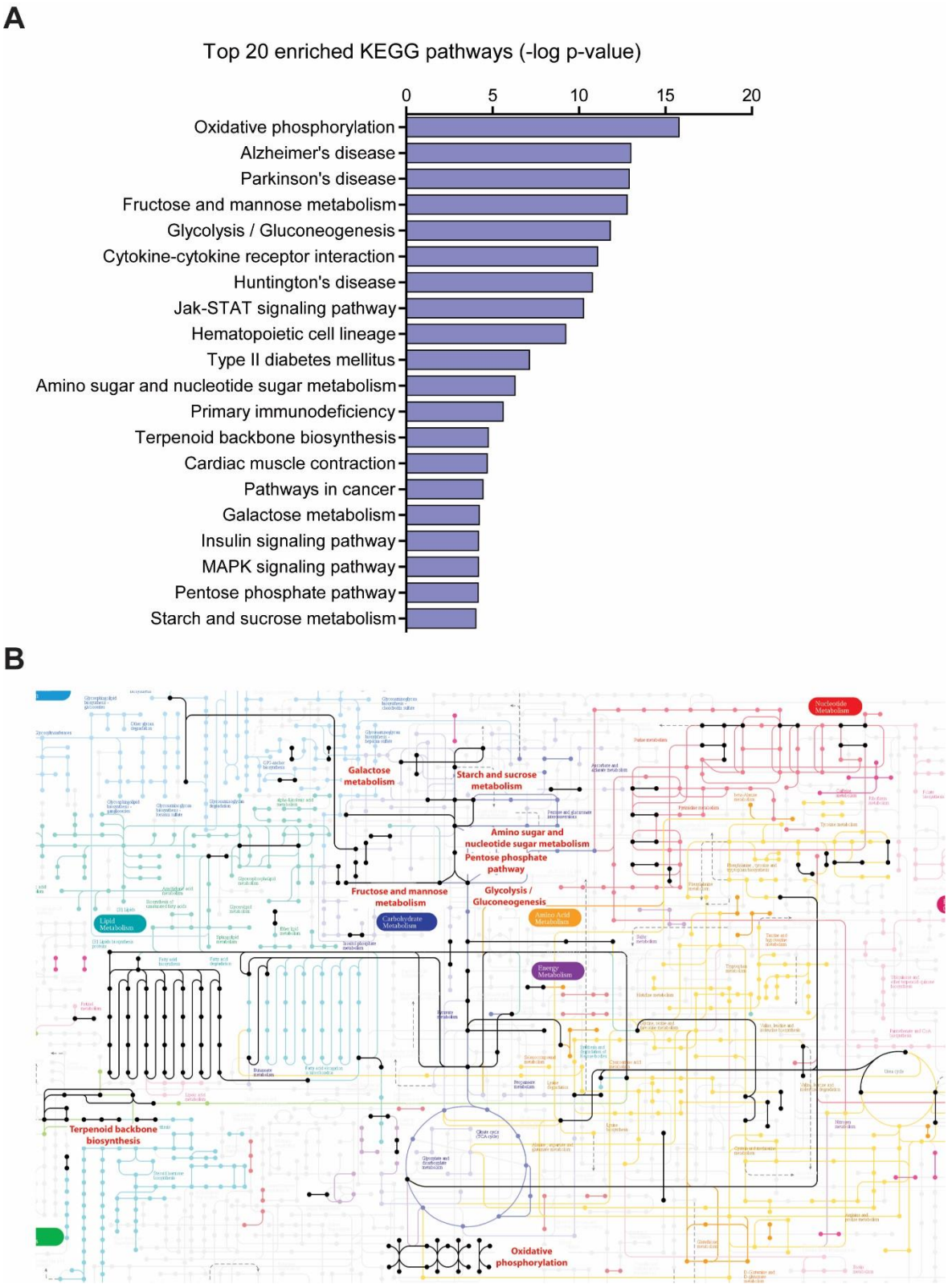

Figure 1. IL-7 promotes gene expression of multiple functional pathways, with emphasis on metabolic and sugar-related pathways in T-ALL. (A) Differential gene expression data from RNA-seq of TAIL7 cells stimulated with IL-7 described in Chapter 3, was subjected to functional annotation analysis using DAVID tools as described in methods. Graphic represents the enriched pathways sorted by -log(p-value). Pathways with Benjamini-corrected p-value >0.001 were excluded. (B) Gene names from pathways found enriched in (A), were converted to KEGG protein identifiers and mapped in human global metabolism atlas. Image was trimmed to display central pathways.

4.4.2 IL-7 promotes glycolytic flux and early expression of glucose metabolism-related genes in T-ALL

Metabolic dysregulation, in particular increased glycolysis, is a common event that supports tumor growth [26]. In T-ALL, metabolic reprogramming has also been associated with resistance to therapy [27]. We evaluated whether alterations in IL-7-mediated metabolic pathway gene expression would functionally affect glycolytic rate in T-ALL cells. We found that IL-7 promoted glucose consumption from and lactate production into the culture medium (Figure 2A), indicating that IL-7 increased the glycolytic rate in T-ALL cells. However, both expression and metabolite data were acquired after 24h of IL-7 stimulation, where it is possible (although unlikely given the very slow doubling time of TAIL7 cells) that the metabolic increase could be related to IL-7-mediated increase in proliferation rather than a primary effect of IL-7 on metabolism. To exclude possible confounding factors, we assessed glycolysis-associated gene expression upon IL-7 stimulation at early time points. We chose genes that either were found upregulated by RNA-seq or were important glycolytic control points. Strikingly, we found that IL-7 induced very early expression (peak expression <1h) of key genes of the glucose and glycolysis metabolism (Figure 2B). We observed increased expression of the glucose transporter *GLUT1*; hexokinase II (*HK2*) that catalyzes the first irreversible step in glucose metabolism; phosphofructokinase-1 isoforms (*PFKL, PFKM*) that catalyze the first irreversible step in glycolysis; Phosphofructokinase 2-fructose bisphosphatase 2 isoform 3 (*PFKFB3*), a central glycolysis regulator; enolase-1 (*ENO1*); pyruvate kinase M (*PKM*) which irreversibly produces pyruvate and is an important regulatory point in glycolysis; and lactate dehydrogenase A (*LDHA*) that produces lactate. We used well-established IL-7 target genes (*BCL2, CISH, PIM1*) as positive controls. Interestingly these control genes did not show the same pattern of expression, and were upregulated at later time points (peak expression >1h), with the exception of Bcl-2, which presented both early and late upregulation (Figure 2B). The first genes to be expressed upon stimulation are expressed very early on and are thus termed immediate-early genes (IEGs). Their downstream targets are termed delayed-early genes (DEGs), whose families contain important proto-oncogenes and tumor suppressors [28]. The proto-oncogene *MYC* may be regulated by IL-7 [29, 30] and is a well-studied IEG [28, 31], thus we also included it on our analysis. However, *MYC* did not present an IEG-like pattern in IL-7-stimulated TAIL7 cells, although it was consistently upregulated throughout time (Figure 2B).

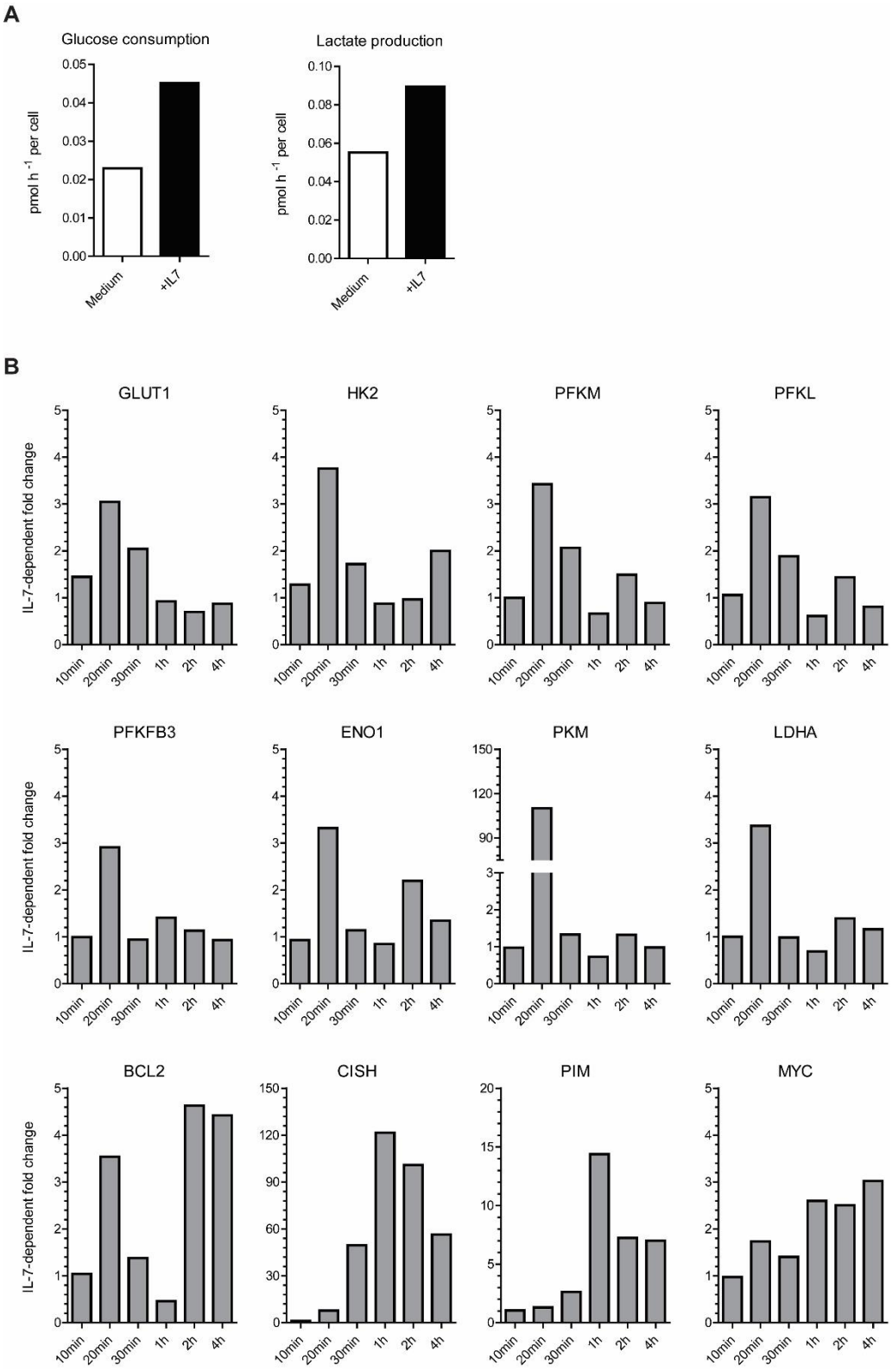

Figure 2. IL-7 increases glucose use and lactate production flux and promotes expression of key glycolysis-related metabolic genes in T-ALL. (A) TAIL7 cells were cultured with or without IL-7 for 24h and the supernatant was collected for metabolite analysis as described in the 'Methods'. Graphics express the specific metabolite production/consumption as pmol.h^{-1} per cell. (B) TAIL7 cells stimulated or not with IL-7, as described in the 'Methods', were collected at the indicated timepoints for mRNA extraction followed by qPCR analysis. Fold induction is normalized to medium condition. Results are representative of 2 (A) and 3 (B) independent experiments.

4.5 Discussion

Metabolic reprogramming is a hallmark of cancer used by cells to sustain cell growth and proliferation [32]. A classical manifestation of metabolic reprogramming in tumors is the Warburg effect or aerobic glycolysis. Although aerobic glycolysis has been associated with tumors, it is in fact used more generally by actively proliferating cells [33]. Importantly, metabolic reprogramming has been incorporated as a requirement into the function of some cell types, such as T-cells [19, 34, 35]. IL-7/IL-7R signaling is essential in normal T-cell development and homeostasis, and plays a critical role in T-ALL cell survival and proliferation [36]. Studies demonstrated that IL-7 modulates lipid synthesis, glycolytic flux and gene expression in T-cells [4-7]. However, little is known about the function of IL-7 in metabolic modulation of T-ALL cells. So far, it was demonstrated that IL-7 promotes Glut1 expression and glucose use in T-ALL cells in a manner that is dependent on PI3K/Akt pathway [13]. Here, we provide evidence that IL-7 promotes a considerably vaster increase in the expression of genes related to sugar metabolism and oxidative phosphorylation, amongst other metabolic pathways. Also, we confirmed our previous studies showing that IL-7 promotes an increase in glucose use [13] and revealed that IL-7 upregulates lactate production, consistent with an apparent increase in glycolytic flux. Furthermore, IL-7 very rapidly increased the expression of glucose metabolism-related genes.

The functional annotation analysis of enriched pathways from RNA-seq data, together with the increase of glucose use on IL-7-stimulated cells, strongly indicates that IL-7 stimulates metabolism in T-ALL cells. Our findings on the use of glucose and lactate production, indicate that their consumption/production ratio is approximately 1:2, respectively. This simple analysis [37] indicates that TAIL7 cells probably use aerobic glycolysis for ATP production mostly. Interestingly, IL-7 stimulation did not seem to alter the ratio but increase the use/production of the molecules. Theoretically, 1 molecule of glucose during glycolysis could generate at most 2 molecules of lactate. If glycolysis intermediates are diverted to other pathways or pyruvate is oxidized in the mitochondria, less lactate is generated from glycolysis [26]. It is possible that glycolysis is used by T-ALL cells to synthesize ATP and support NAD+/NADPH redox balance and other metabolic pathways, such as glutaminolysis, would support biomass production. However, glutaminolysis may also indirectly contribute to lactate production [26], thus complicating the interpretation of the data. Advanced studies of the metabolome using, for instance stable-isotope labeling and analysis of extra parameters (e.g. the ratio of oxygen consumption rate

by extracellular acidification rate), could help discriminate the origin and fate of cell metabolites in the context of IL-7 stimulation [37, 38].

Our early gene expression studies revealed that IL-7 promotes the expression of key glucose-related metabolism genes. Importantly, metabolic genes had an expression peak at <1h. Some genes (*HK2*, *ENO1*) also showed a clear second induction wave at >2h, consistent with previous reports [6]. Although, we did not analyze gene expression between 4h-24h of IL-7 stimulation it is possible that the other glycolytic-related genes also peak their expression within this period. The time of induction and repression of the glycolytic-related genes fits a IEG expression pattern (20-40min). Upon expression, IEGs will then initiate a second wave of transcription of the DEGs (peak 1-2h). Downstream transcription continues with late-response genes (LRGs) [28]. IEGs are the first genes to be transcribed upon extracellular stimuli, thus they do not require previous protein synthesis and their expression is not inhibited by protein synthesis inhibitors such as cycloheximide [28]. Additionally, IEGs have unique features in their mRNA and chromatin that allows their rapid induction [28, 39]. Time of expression *per se* suggests but does not demonstrate that a gene is an IEG. It would be important to complete our preliminary study with cycloheximide experiments to distinguish whether the gene expression is either IEG or DEG. By far, the most well studied IEGs are *FOS*, *JUN*, *MYC* and *EGR1*, all transcription factors and activated by MAPK pathways [28, 39]. However, there is evidence that glycolysis-related genes may be IEGs. *PFKFB3* contains an AUUUA instability element in its mRNA. This motif confers instability and enhanced translational activity on mRNAs and is also found in the IEG family genes [40, 41]. Novellasdemunt and colleagues [42] have recently identified *PKFB3* as an IEG activated by the p38 MAPK pathway in response to stress stimuli. Also, effector-memory CD8 T-cells undergo an immediate-early glycolytic switch upon activation, mediated by PI3K/Akt/mTOR pathway [35].

The pattern of *BCL2* gene expression, in particular the early phase, similar to the glycolytic genes, is intriguing. One possibility is that *BCL2* may have a role in IL-7-dependent metabolic stimulation. Bcl-2 may sequester and inhibit the BH3-domain only Bcl-2 family members of pro-apoptotic factors (e.g. Bad, Bim), thus inhibiting mitochondrial apoptotic pathway [43]. This may allow the cell to quickly allow the oxidative phosphorylation pathway to receive input from glycolysis. In a study using a model of cardiac ischemia [44], it was also shown that Bcl-2 could bind to voltage-dependent anion channels (VDAC) and prevent glycolytic ATP import into the mitochondria and subsequent hydrolysis. This decreased ischemic injury by preventing cytosolic acidification and non-

productive hydrolysis of glycolytic ATP [44]. A role in metabolism for Bad has also been reported [45]. It was found that phosphorylated Bad associated with glucokinase (also known as hexokinase IV) in the mitochondria, promoting its glucokinase activity and increasing mitochondrial respiration in hepatocytes. Furthermore, dephosphorylated Bad dissociated from glucokinase, decreased mitochondrial respiration and promoted cell death [45]. These studies suggest that Bcl-2 family members may have direct and important roles on the regulation of cell metabolism and are in line with our expression data on rapid Bcl-2 upregulation by IL-7 in T-ALL cells. Importantly, we also found that two PFK-1 (*PFKM*, *PFKL*) genes and PFK-2-FBPase2 (PFKFB) isoform 3 (*PFKFB3*) were upregulated by IL-7. The regulation of PFK-1 activity is a major control point in glycolysis. PFK-1 catalyzes the production of the glycolytic intermediate fructose-1,6-bisphosphate (F1,6BP) at the expense of ATP, the first committed step of glycolysis and a rate-limiting step [46]. When ATP levels are high, PFK-1 is allosterically inhibited, thus reducing glycolytic flux [47]. However, the most potent activator of PFK-1, even in the presence of high ATP levels, is fructose-2,6-bisphosphate (F2,6BP) generated by the PFKFB family of enzymes [46]. All PFKFB isoforms are bifunctional kinases and phosphatases, but the highest kinase:phosphatase ratio is found in PFKFB3 (~700:1), making it essentially a kinase that promotes glycolytic flux [48]. PFKFB3 has been highly implicated in cancer, being overexpressed in different cancers and supporting tumor growth [40]. Also, PFKFB3 was shown to promote cell cycle progression via Cdk-1 activation [49]. Importantly, Akt directly phosphorylates PFKFB3 and decreases the affinity to phosphoenolpyruvate (PEP), an allosteric inhibitor [50]. Overall, IL-7-regulated signaling in T-ALL promotes the expression of genes that are central regulators of glycolysis and that may bridge between metabolism and proliferation. It The impact of inhibiting metabolic pathways on IL-7-mediated effects in T-ALL surely warrants investigation.

In summary, our work supports and extends the evidence that IL-7 signaling directly modulates T-ALL cell metabolism. Further studies are required to advance this notion, but it is tempting to speculate that leukemic T-cells exposed to IL-7, very quickly upregulate genes required for glycolysis and other metabolic pathways, which are activated likely to support cell survival and, more importantly, to generate biomass for proliferation and consequent leukemia growth. Dissecting the molecular mechanisms and functional impact of IL-7 modulation of T-ALL cell metabolism may provide multiple, valuable and novel therapeutic targets for this disease.

4.6 References

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CHAPTER 5

IL-7 Flexibly Regulates Autophagy-dependent Viability of T-Cell Acute Lymphoblastic Leukemia Cells

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Adapted from manuscript in preparation

5.1 Abstract

T-cell acute lymphoblastic leukemia (T-ALL) constitutes an aggressive subset of ALL, the most frequent childhood malignancy. Interleukin-7 (IL-7) is essential for normal T-cell development and there is considerable evidence that IL-7-mediated signaling can promote leukemogenesis. Previously, we showed that IL-7 promotes T-ALL cell proliferation, survival and metabolic activation via PI3K/Akt/mTOR pathway. Autophagy is upregulated in rapidly dividing cells, such as cancer cells. However, when persistent, its protective role may shift to what is called autophagic cell death. mTOR is recognized as the master negative regulator of this process, whereas MEK/Erk pathway has been associated with promotion of autophagy. Since IL-7 activates both mTOR and MEK/Erk we decided to explore whether IL-7 may regulate autophagy in T-ALL cells and elucidate its molecular mechanisms and functional consequences. Using the human IL-7-dependent T-ALL cell line TAIL7 and primary leukemia samples, we found that in optimal culture conditions (medium with serum) IL-7 inhibits autophagy in T-ALL, albeit in a complex manner that involves triggering both pro- (via MEK/Erk) and anti- (via PI3K/Akt/mTOR) autophagic signaling pathways. In this scenario, IL-7-mediated viability relies on the latter pathway, as previously described. In contrast, under serum starvation IL-7-mediated survival partially relies on autophagy activation and strictly requires MEK/Erk activation. Our results suggest that IL-7 makes use of a ‘flexible strategy’ to promote T-ALL cell viability by recruiting both pro- and anti-autophagic pathways, which contribute to preventing tumor cell death in different microenvironmental conditions.

5.2 Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. Approximately 15% of all cases present a T-cell origin (T-ALL), and are associated with higher risk and poorer prognosis at presentation [1]. T-ALL arises from transformed T-cell precursors that have undergone a block in development and carry oncogenic lesions that promote self-renewal, proliferation and survival [2].

Interleukin-7 (IL-7) and its receptor (IL-7R) play a crucial role on normal thymocyte development and homeostasis [3-5]. However, IL-7 also promotes T-ALL cell proliferation *in vitro* [6, 7] and accelerates human leukemia expansion *in vivo* [8]. These effects are mediated by IL-7-mediated non-redundant activation of the Phosphatidylinosiol-3-kinase/ Akt/ mammalian Target of rapamycin (PI3K/Akt/mTOR) signaling pathway, which promotes T-ALL cell viability, metabolic activation and proliferation [9-11]. The importance of IL-7/IL-7R signaling is further illustrated by the presence of *IL7R* gain-of-function mutations in around 9% of T-ALL cases [12-14]. In addition, IL-7 can activate the canonical cytokine signaling pathway Janus kinase/ Signal transducer and activator of transcription (JAK/STAT), in particular STAT5, and Mitogen-activated protein kinase kinase/ Extracellular-signal regulated (MEK/Erk) pathway [15].

Macroautophagy (hereafter referred to as autophagy) is an evolutionary-conserved homeostatic intracellular process occurring at basal levels in normal cells and characterized by the sequestration of cytoplasmic compartments through double-membrane vesicles (autophagosomes) to promote their degradation [16]. Autophagy is upregulated during starvation, growth factor withdrawal, cellular stress or in rapidly dividing cells as a compensatory mechanism to provide nutrients and stress relief. Under these situations autophagy may serve as a pro-tumoral mechanism promoting stress mitigation and chemotherapy resistance [17, 18]. On the other hand, organisms with disrupted autophagy are more prone to develop tumors possibly by increased stress from misfolded proteins and non-functioning organelles [19, 20]. Furthermore, when persistent, autophagy can lead to what is termed autophagic cell death, overall suggesting that autophagy may also partake in tumor suppression [17]. This apparent paradox of autophagy function in cancer may be resolved if autophagy initially prevents tumor initiation by reducing intracellular pro-oncogenic stresses, but once a tumor is established it helps the tumor cope with cellular and microenvironmental stresses leading to its development [21].

The mammalian target of rapamycin (mTOR) is a serine/theronine kinase and the master negative regulator of autophagy [22]. The mTOR complex (mTORC) integrates nutritional, energetical (ATP) and growth factor cues from both within the cell and the microenvironment, essential for proper cell growth and proliferation [23]. In the absence these, mTOR is inactivated, which leads to activation of the UNC-51-like kinase 1 / Autophagy-related 13 / 200 kDa FAK family kinase-interacting protein (ULK1/Atg13/FIP200) complex, a required step for autophagy initiation [24]. Whereas class I PI3Ks are involved in down-regulating autophagy indirectly by activating mTOR, the class III PI3K, Vacuolar protein sorting 34 (Vps34), complexes with Beclin 1 to directly mediate autophagosome formation [25, 26]. A hallmark of autophagy is the cleavage and lipidation (with phosphatidylethanolamine) of Microtubule-associated protein 1 light chain 3 (LC3/Atg8), a protein required for the elongation step of the autophagosome and the most reliable and well studied autophagy marker to date [27].

Similar to other cell types, autophagy is an important process in the biology of T-cells. It has been shown that the autophagic process regulates normal T-cell development and function through its role in self-antigen presentation, intracellular organelle homeostasis and energy production [28]. In turn, it would not be surprising if mechanisms controlling autophagy could be deregulated in T-cell leukemogenesis.

To date, no studies have specifically addressed the role of IL-7 in autophagy modulation. Although in a study on the role of Bim isoforms in the context of IL-7 stimulation in lymphocytes it was found that individual Bim isoforms could affect autophagy differently [29]. We hypothesized that since IL-7 activates mTOR it may inhibit autophagy in T-ALL cells, thereby preventing its tumor suppression function and contributing to tumor cell expansion. Consistent with our hypothesis, we found that IL-7 regulates autophagy in a T-ALL cell line model to consistently promote leukemia cell survival, albeit in a complex manner involving the modulation of both pro- and anti-autophagic pathways.

5.3 Methods

Cell culture and experimental conditions. Primary T-ALL cells isolated from pediatric patients at diagnosis and normal thymocytes were isolated as described in [8]. In all cases informed consent was obtained in accordance with the Declaration of Helsinki and under institutional ethical review board approval. The TAIL7 cell line, an IL-7 dependent cell line that was established from the peripheral blood of a pediatric T-ALL patient [15], was cultured in RPMI-1640 medium (Life Technologies) supplemented with 5% FBS, 2mM glutamine, 100U/mL penicillin/streptomycin and 10ng/mL of rhIL-7 (Peprotech). For experiments, TAIL7 cells were deprived of IL-7 and cultured in RPMI supplemented with or without serum for 24h, followed by set-up of experimental conditions. The culture of primary T-ALL samples was done in RPMI-1640 supplemented with 10% FBS, 2mM glutamine, 100U/mL penicillin/streptomycin and 10ng/mL of rhIL-7. For short-term incubations, cells were pre-treated for 1h30 with the indicated inhibitors (or DMSO), followed by 2h stimulation with IL-7 (50ng/mL), and collected for immunoblot analysis, electron or confocal microscopy, where appropriate. For long-term incubations, treatment with inhibitors and IL-7 was done concomitantly for the indicated time, and cells were collected for immunoblot analysis or flow cytometry, where appropriate.

Inhibitors. We used the PI3K inhibitor LY294002 (10/20 μM; long/short-term incubations), the mTOR inhibitor rapamycin (100 nM), the small-molecule inhibitor of STAT5 N′-((4-Oxo-4H-chromen-3 yl)methylene)nicotinohydrazide (100 μM) and the MEK1/2 inhibitor UO126 (10/20 uM) (Merck/Calbiochem). To inhibit autophagy, we used the Vps34 specific inhibitor SAR405 (10μM) [30]. We used hydroxychloroquine (HCQ; 30 μM) (Merck/Calbiochem), an autophagosome/lysosomal inhibitor, as tool to take a "snapshot" of the autophagic flux in the cell at a given moment [27].

Immunoblotting and antibodies. Whole cell lysates were resolved by a 12% or 14% SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted, as described [9], with antibodies against p-STAT5a/b (Y694/Y699), p-Akt (S473), p-S6 (S235/236), p-Erk1/2 (T202/Y204), LC3B (Cell Signaling Technology), p62/SQSTM1, Actin (Santa Cruz Biotechnology) and Tubulin (Roche).

Immunofluorescence and confocal microscopy. Cells were adhered to poly-L-lysine-coated coverslips and fixed with -20ºC cooled methanol for 10 minutes, followed by intracellular incubation with anti-LC3B primary antibody (1:200) in PBS-Tween 20 (0.05%; PBSt) for 1 hour at room temperature. Secondary staining was performed with an anti-rabbit Alexa-488 conjugated antibody (1:400) in PBSt for 30min at room temperature. Coverslips were mounted with Vectashield-DAPI (Vector Labs) and acquired in a confocal microscope (Zeiss LSM 710). DAPI fluorescence was detected with a violet 405 nm diode laser (30 mW nominal output) and a BP 420-480 filter. Both EGFP and Alexa Fluor 488 fluorescence were detected using the 488 nm laser line of an Ar laser (45 mW nominal output) and a BP 505-550 filter.

Flow cytometry. Samples were methanol-fixed and stained as described in the immunofluorescence section. Briefly, cells were methanol fixed, and incubated with anti-LC3B antibody (1:100) in PBSt, followed by secondary staining with an anti-rabbit Alexa-488 conjugated antibody (1:200) in PBSt. Acquisition of samples was performed in an LSR Fortessa or FACS Calibur (BD). Flow cytometry data analysis was done using FlowJo software (TreeStar). Viability was determined by forward scatter (FSC) and side scatter (SSC) parameters and the mean fluorescence intensity (MFI) analysis of LC3 intracellular staining was done within the live cell population.

Electron microscopy. Cells were collected by low-speed centrifugation (2000rpm), 10min, at 4ºC in a bench-top centrifuge. The pellets were immediately carried for electron microscopy fixation using a previously described protocol to improve autophagosome detection [31].

Autophagy and LC3 quantification. The autophagic flux was quantified by LC3 turnover assay, by densitometry analysis, where the ratio between LC3-II and LC3-I. Densitometry analysis was performed on immunoblots using ImageJ software.

5.4 Results

5.4.1 IL-7 inhibits autophagy in T-ALL in nutrient-rich conditions

To find whether IL-7 may regulate the autophagic process in T-ALL, we stimulated TAIL7 cells with IL-7 and performed immunoblot analysis of LC3 cleavage and lipidation (active form; LC3-II) and degradation of the early autophagic substrate p62/SQSTM1, hallmarks of the autophagic process. We observed that IL-7 inhibited processing of LC3 from the inactive form (LC3-I) to its active from (LC3-II), and prevented degradation of p62 (Figure 1A). By electron microscopy, the gold-standard for autophagy assessment, we observed that stimulation with IL-7 decreased the formation of autophagosomes/autolysosomes in the cells (Figure 1B,C). Overall, these data suggest that IL-7 inhibits autophagy in T-ALL cells.

5.4.2 IL-7-dependent activation of PI3K/Akt/mTOR pathway inhibits, whereas MEK/Erk promotes, autophagy in T-ALL cells

To dissect which signaling pathways may be responsible for IL-7-dependent autophagy regulation, we treated TAIL7 cells for 2h with a PI3K inhibitor (LY294002), an mTOR inhibitor (rapamycin), or a MEK1/2 inhibitor (UO126) and evaluated their effects on LC3 by immunoblot analysis. Treatment with either LY294002 or rapamycin reversed IL-7-dependent prevention of LC3 cleavage (Figure 2A), indicating that PI3K/Akt/mTOR mediates IL-7-dependent inhibition of autophagy in T-ALL cells. Interestingly, treatment with UO126 synergized with IL-7 in preventing LC3 conversion (Figure 2A). This indicates that MEK/Erk pathway promotes autophagy in T-ALL. Confocal microscopy analysis of cells cultured for 48h under the same conditions confirmed these data, showing a pattern of LC3 puncta formation and intensity at 48h that was in agreement with the results from the immunoblot analysis at 2h (Figure 2B).

Next, we decided to analyze intracellular LC3 expression by flow cytometry. Although one cannot distinguish directly LC3-I from LC3-II by flow cytometry, we expected that cells with higher autophagic flux would have higher LC3-II expression located on autophagosomes which would in turn increase the mean fluorescence intensity (MFI) of the detected protein. We observed that the data collected by flow cytometry (Figure 3) correlated altogether with LC3 cleavage (Figure 2A) and LC3 puncta formation (Figure 2B). These data further suggest that flow cytometry may be reliably used to measure autophagy in T-

ALL cells at the single cell level. Furthermore, we found that STAT5 does not appear to have a key role in IL-7-mediated regulation of autophagy in T-ALL cells, as STAT5 pharmacological inhibition does not have a major impact on the LC3 MFI (Figure 3).

Overall, these data suggest that IL-7 downregulates autophagy in T-ALL cells via activation of PI3K/Akt/mTOR pathway and promotes autophagy via activation of MEK/Erk pathway. Nonetheless, the inhibitory effect of mTOR on autophagy prevails over that of MEK/Erk signaling in cells maintained in normal (serum-rich) culture conditions (Figure 1).

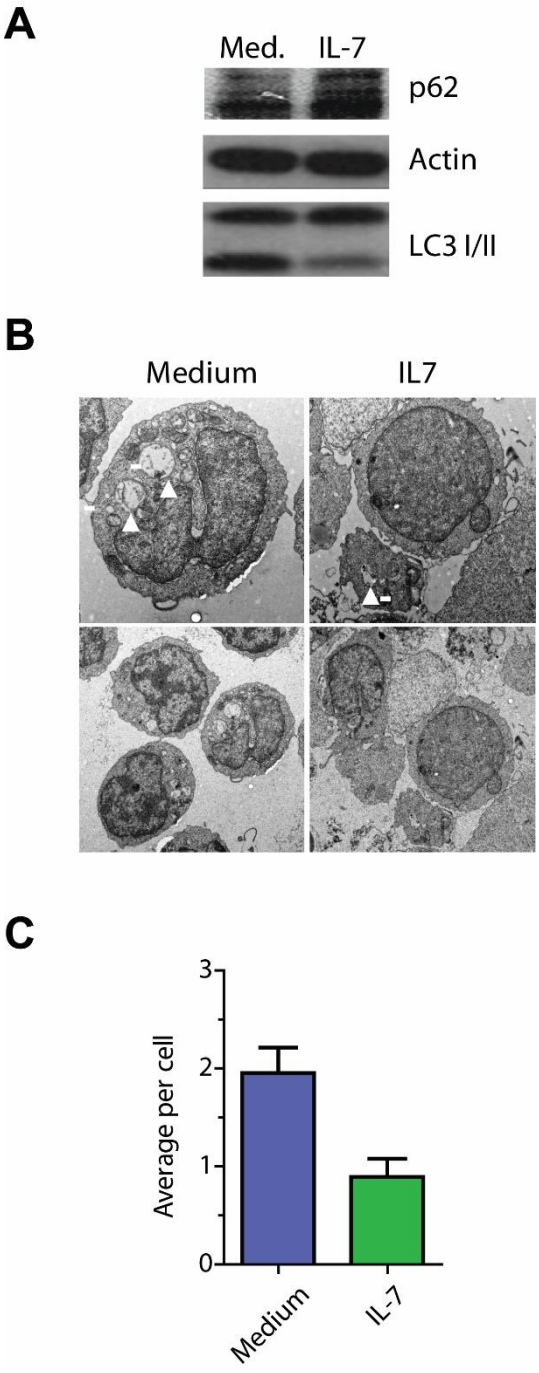

Figure 1. **IL-7 inhibits autophagy in T-ALL cells**. IL-7-deprived TAIL7 cells were incubated with IL-7 (50ng/mL) or left untreated for 2h in the presence of HCQ (30µM). Cells were collected for either (A) immunoblot analysis of LC3 and p62 expression or (B) electron microscopy. (C) Quantification of number of autophagosomes/autolysosomes per cell from analysis of micrographs of (B). Data representative of at least 2 independent experiments. Results in panel C represent average of triplicates ± sem.

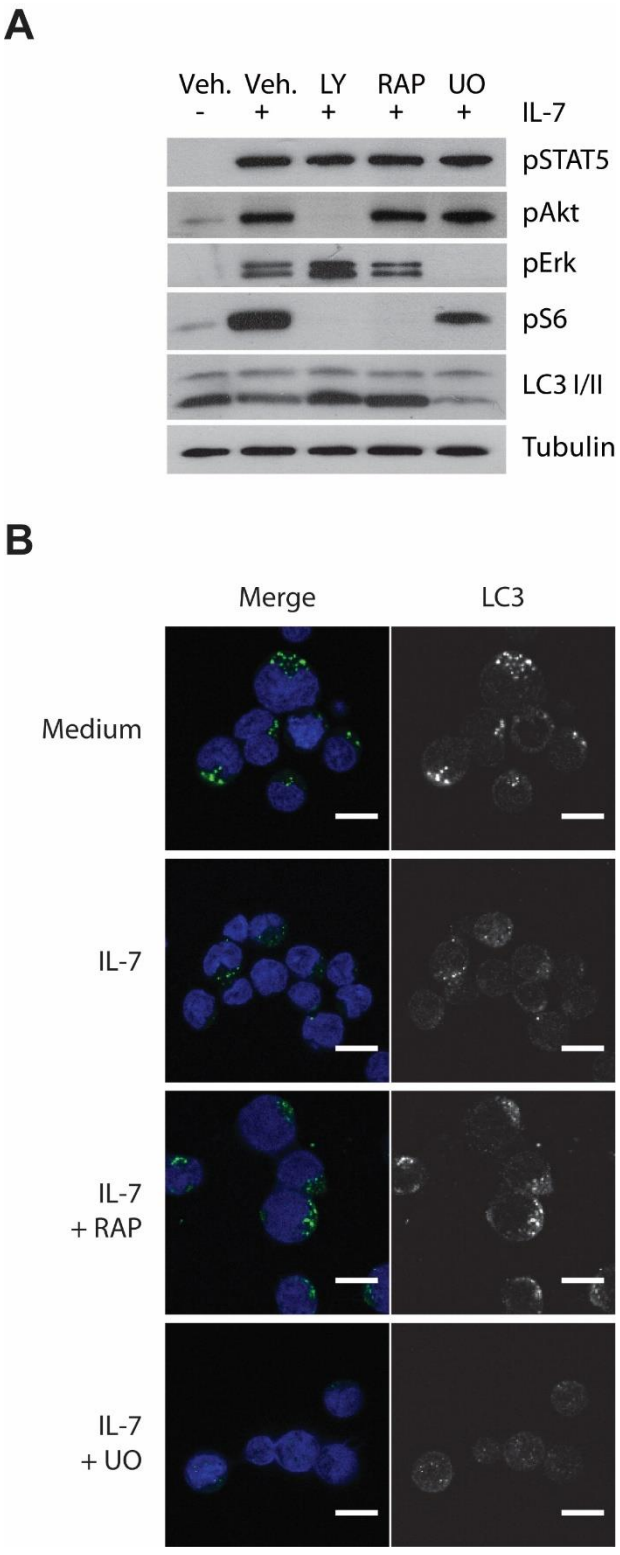

Figure 2. **IL-7 dependent activation of PI3K/Akt/mTOR pathway inhibits, whereas MEK/Erk pathway promotes, autophagy in T-ALL cells.** IL-7-deprived TAIL7 cells were treated for short-term (2h) experiments with LY294002 (LY), rapamycin (RAP), UO126 (UO), followed by an IL-7 stimulus (2h) in the presence of HCQ and collected for analysis. (A) Immunoblot analysis of PI3K/Akt/mTOR, MEK/Erk, JAK/STAT pathway activation and LC3 cleavage. (B) Confocal microscopy analysis of LC3 puncta. Left panels show merge of LC3 puncta (green) and DNA stain with DAPI (blue), right panels show LC3-488 alone. Scale bare represents 10µm. Data representative of at least 3 independent experiments.

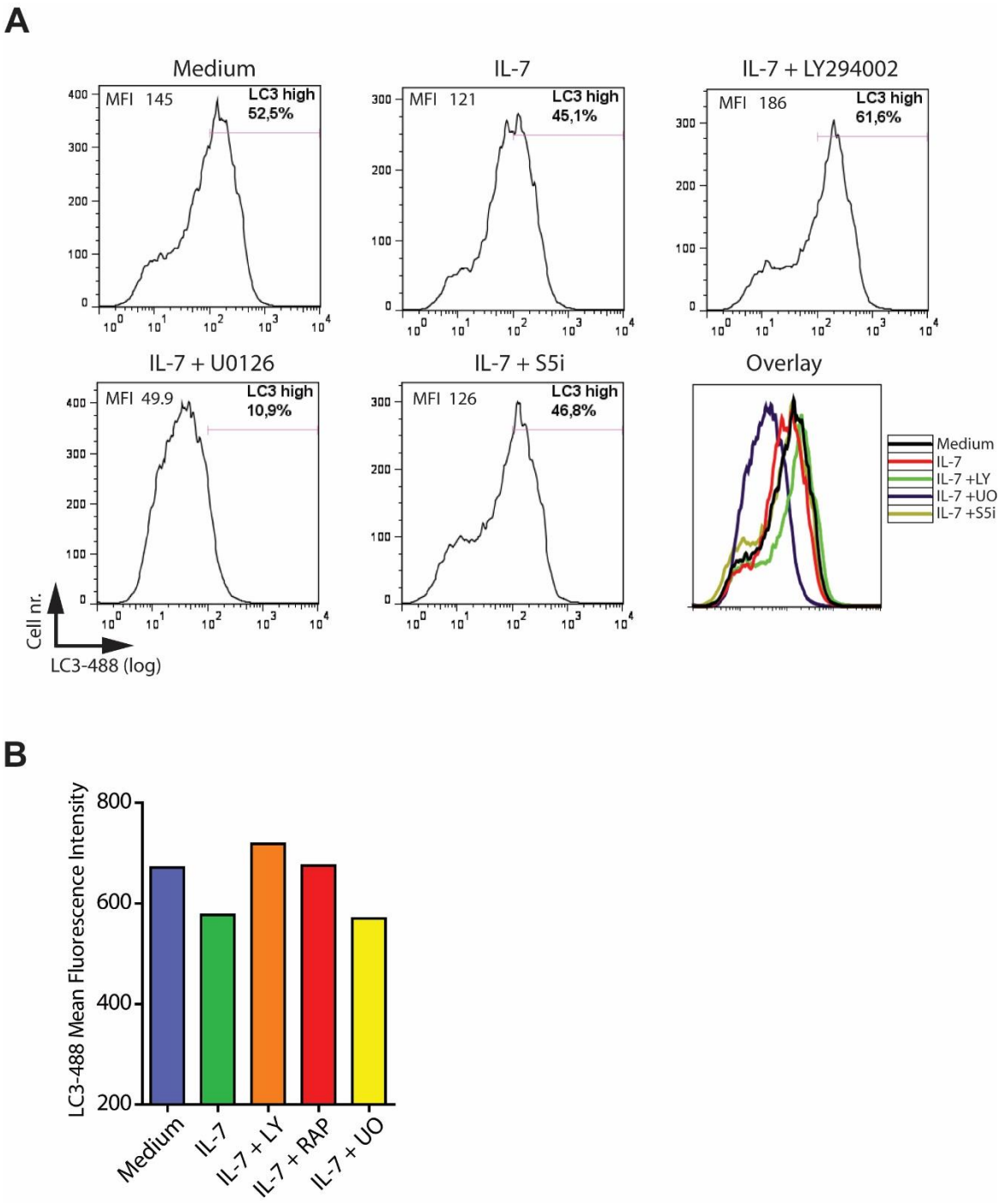

Figure 3. Flow cytometric analysis of LC3 shows IL-7-dependent modulation of LC3 turnover by PI3K/Akt/mTOR and MEK/Erk pathways. IL-7-deprived TAIL7 cells were treated for (A) long-term (48h) or (B) short-term (2h) experiments with LY294002, rapamycin, UO126, STAT5 inhibitor and/or IL-7, as indicated. Following fixation and permeabilization, cells were incubated with an anti-LC3 primary antibody and stained with Alexa-488-conjugated secondary antibody. Samples were analyzed by flow cytometry for LC3-488 intracellular staining. Data representative of at least 2 independent experiments. Results in panel B represent average of triplicates ± sem.

5.4.3 IL-7 relies on MEK/Erk activity and autophagy to promote survival in nutrient-poor conditions

The ability of IL-7 to activate concomitantly pro- and anti-autophagy signaling pathways in T-ALL cells led us to hypothesize that IL-7 may have the ability to protect leukemia cells from stress by positively regulating autophagy. To test this hypothesis, we cultured TAIL7 cells with IL-7 in normal serum conditions or under nutrient stress (no serum). Strikingly, we found that in the absence of serum IL-7 promoted autophagy as observed by LC3 turnover (Figure 4A). In accordance, preliminary densitometry analyses suggest that in serum-poor culture IL-7 appears to induce a shift towards higher MEK/Erk (pro-autophagy) pathway activation than in serum-rich culture, whereas PI3K/Akt/mTOR (anti-autophagy) pathway appears unaffected (Figure 4B).

Next, we reasoned that if in serum-poor medium autophagy constituted an important survival mechanism, then inhibition of the pro-autophagic MEK/Erk pathway should have a negative impact on cell survival. We cultured TAIL7 cells with IL-7 in normal serum conditions or without serum. IL-7 promoted T-ALL survival in both conditions, However, in the presence of serum IL-7-dependent survival required PI3K/Akt/mTOR activation, whereas in the absence of serum MEK/Erk activation was essential for IL-7-dependent survival (Figure 5c; upper-panels). Interestingly, in serum-poor conditions inhibition of PI3K/Akt/mTOR pathway by LY294002, a condition favoring autophagy, promoted cell viability beyond the effect of IL-7. Similar results were found for a primary T-ALL sample (Figure 4C; lower-panels).

To more directly characterize the relevance of autophagy for IL-7-mediated T-ALL cell survival, we cultured TAIL7 cells in the presence of the autophagy inhibitor SAR405. Prevention of autophagy had no significant effect on IL-7-dependent survival in the presence of serum (Figure 5A). In contrast, SAR partially abrogated viability of T-ALL cells cultured with IL-7 in the absence of serum (Figure 5B). These results indicate that autophagy is required, at least in part, for IL-7 to promote T-ALL cell survival under nutrient-poor conditions.

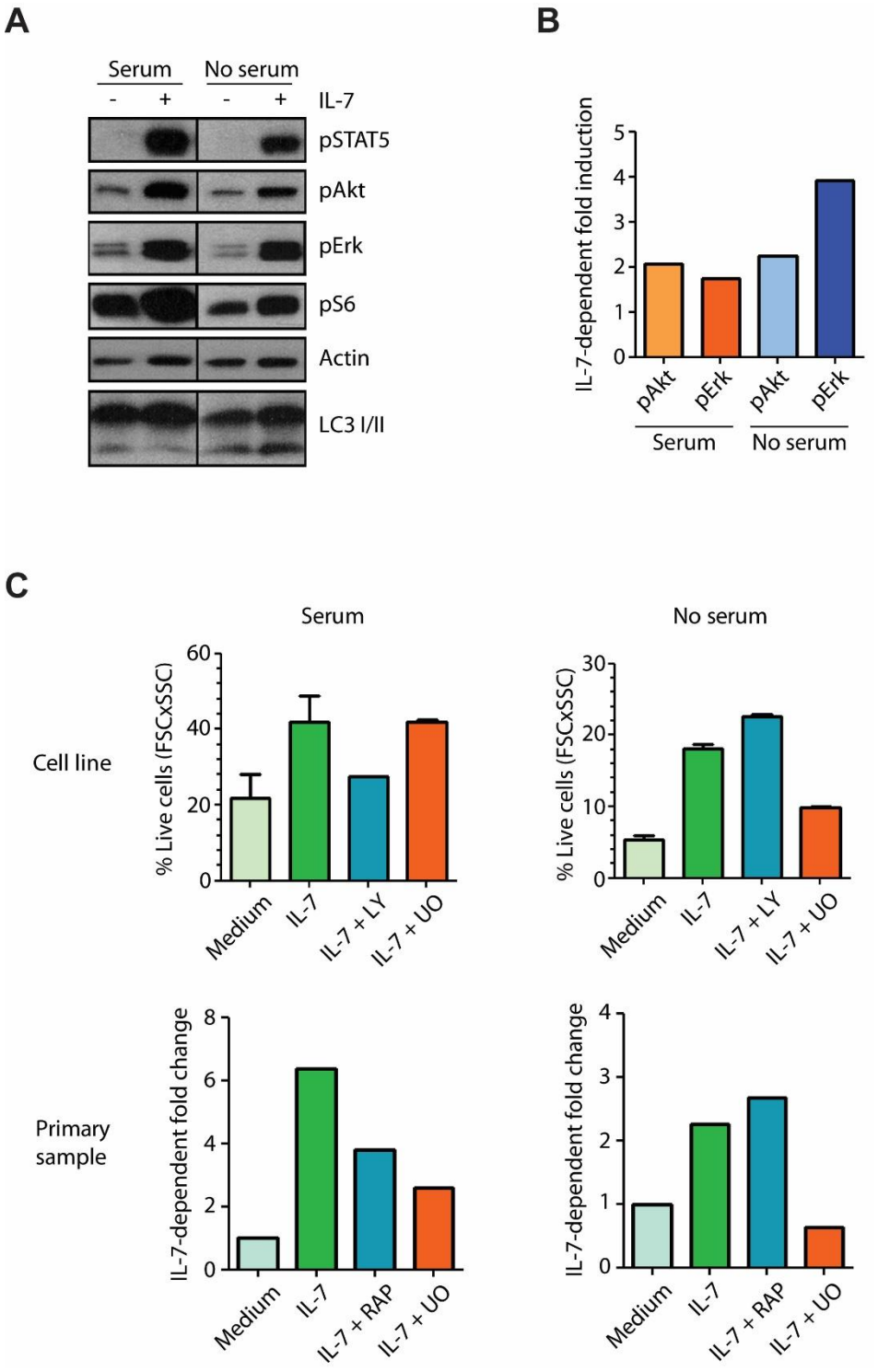

Figure 4. In serum-poor culture IL-7 promotes T-ALL cell viability by MEK/Erk-dependent promotion of autophagy. IL-7-deprived TAIL7 cells or primary leukemia cells were cultured in serum-rich (5% FBS - TAIL7; 10%FBS-primary sample) or in serum-poor (no FBS) conditions, as indicated. (A) Immunoblot analysis of PI3K/Akt/mTOR, MEK/Erk, JAK/STAT pathway activation and LC3 cleavage in TAIL7 cells. (B) Densitometry analysis of IL-7-dependent fold induction of pAkt and pErk observed in **a**. (C) TAIL7 or primary leukemia cells were cultured in serum-rich (5% FBS - TAIL7; 10% FBS - primary T-ALL) or in serum-poor (no FBS) conditions for 96h (TAIL7) or 48h (primary T-ALL) in the absence or presence with LY294002, rapamycin, UO126 and/or IL-7, as indicated. Cells were collected for flow cytometry analysis of viability by FSCxSSC discrimination. Results are representative of 3 independent experiments or 2 patients. Results in panel C (top) represent average of triplicates ± sem.

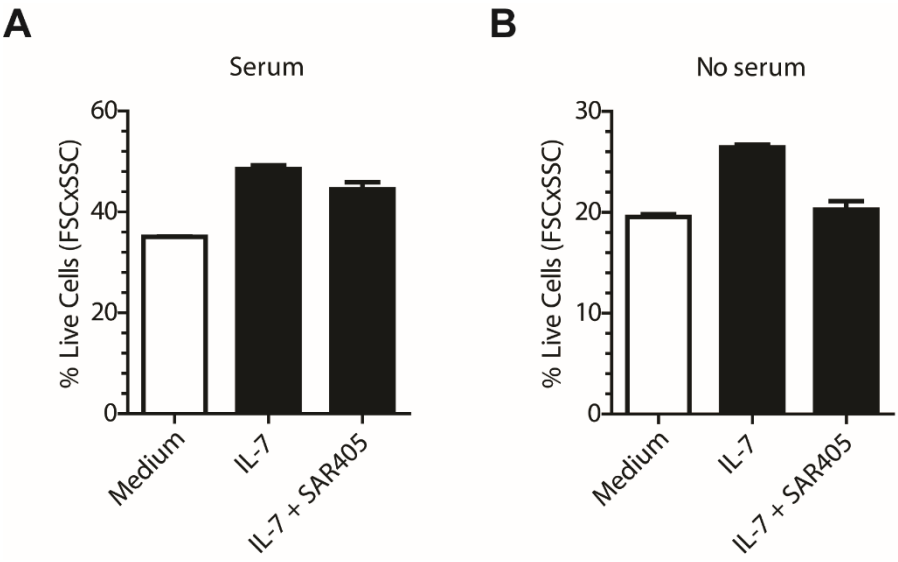

Figure 5. In serum-poor culture inhibition of autophagy abrogates IL-7-mediated T-ALL cell viability. IL-7-deprived TAIL7 cells were cultured in (A) serum-rich (5% FBS) or (B) in serum-poor (no FBS) conditions for 96h in the absence or presence or SAR405 (10μM) and/or IL-7 (50ng/mL), as indicated. Cells were collected for flow cytometry analysis of viability by FSCxSSC discrimination. Data representative of 3 independent experiments. Results represent average of triplicates ± sem.

5.5 Discussion

IL-7 is a major growth factor for both normal and leukemic T-cells, consistently promoting cell proliferation, metabolic activation and cell survival via inhibition of apoptosis [32, 33]. Autophagy is a major cellular process through which long-live proteins and organelles are degraded and functions as a key process for cell survival, tissue remodeling and stress relief. However, alterations in the autophagic process have been described as being involved in many pathologies [17]. In cancer, these alterations can be seen as a double-edged sword. On one hand, by mitigating multiple sources of cellular stress, autophagy may prevent normal cell transformation by avoiding excessive DNA damage and expression of aberrant, potentially oncogenic, proteins. On the other hand, once tumor initiation has occurred, tumors may exploit autophagy to resist stress and increase tumor fitness [18].

Our initial hypothesis was that IL-7 could down-regulate autophagy through the activation of PI3K/Akt/mTOR axis. Interestingly, here we demonstrated that IL-7 modulates autophagy in a manner that takes into account other factors in the microenvironment. We found that in serum-rich conditions IL-7 down-regulates autophagy, while in serum-poor conditions IL-7 promotes autophagy. This may relate to the fact that IL-7 activates two signaling pathways with opposing roles in autophagy: PI3K/Akt/mTOR pathway inhibits autophagy and MEK/Erk pathway promotes autophagy. Indeed, we showed a strong correlation between IL-7-mediated survival of leukemia cells in optimal vs. stress culture conditions (serum-rich vs. serum-poor) and the requirement for active PI3K/Akt/mTOR vs. MEK/Erk signaling, respectively. We postulate that the signaling pathway that IL-7 uses to promote cell viability shifts according to the requirement the cell has on autophagy in a manner that is determined by other cell-autonomous and microenvironmental cues.

Previous studies established that IL-7-mediated increase in T-ALL cell viability requires activation of PI3K/Akt/mTOR pathway to block apoptosis. Whereas the role of IL-7-mediated MEK/Erk activation in T-ALL remained elusive [9, 11, 33]. However, most studies to date investigating the role of IL-7 in T-ALL were performed in optimal culture conditions. Here, for the first time we demonstrated that MEK/Erk plays a role in IL-7-mediated leukemia cell survival and it is correlated with promotion of autophagy to prevent cell death upon serum withdrawal.

The role of mTOR as a master negative regulator of autophagy is well established by its direct control over the ULK1/Atg13/FIP200 complex [22, 26]. The role of MEK/Erk

pathway is often associated with autophagy promotion, but the mechanisms are less well-defined and may involve different layers of regulation [26]. For instance, studies suggest that MEK/Erk pathway may regulate the Vps34/Beclin1 complex assembly [34] or autophagosome vesicle maturation [35]. Conversely, Raf/MEK/Erk complexes were found associated with the autophagosomal membrane and autophagic activity promoted Erk activation [36]. Our results agree with the notion that MEK/Erk activity positively controls autophagy.

The present study opens up futures avenues of research. Autophagy contributes to chemotherapy resistance and several clinical trials are in course testing whether autophagy inhibitors could complement standard chemotherapy [18]. Understanding whether IL-7 contributes to T-ALL chemotherapy resistance and if so, what role autophagy plays in that context, is of particular interest. How (and which) other cues mechanistically determine the ability of IL-7 to promote or inhibit autophagy constitutes and exciting research track. Finally, the role of MEK/Erk signaling in IL-7-dependent T-ALL cell survival, growth and proliferation warrants further investigation.

In summary, our data suggest that IL-7 shifts the balance of intracellular pathway activation to consistently promote T-cell leukemia survival according to the microenvironment.

5.6 Acknowledgments

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5.7 Authorship Contributions

DR designed research, performed experiments, analyzed and interpreted data, and wrote the manuscript; CC, JS, IL, MA performed experiments, analyzed and interpreted data; JTB designed research, analyzed and interpreted data, wrote the manuscript and supervised the study.

5.8 Conflict of Interest Disclosures

The authors have no conflict of interest to declare.

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CHAPTER 6

Discussion

In the present work we found and characterized new aspects of IL-7/IL-7R-mediated signaling in T-cell ALL. Those include the finding of oncogenic mutations in the *IL7R* gene driving constitutive signaling, the characterization of new functions for Jak/STAT5, PI3K/Akt and MEK/Erk signaling pathways and the involvement of IL-7 in modulating the cellular physiological processes of autophagy and metabolism. An overview of our findings is shown in Figure 1.

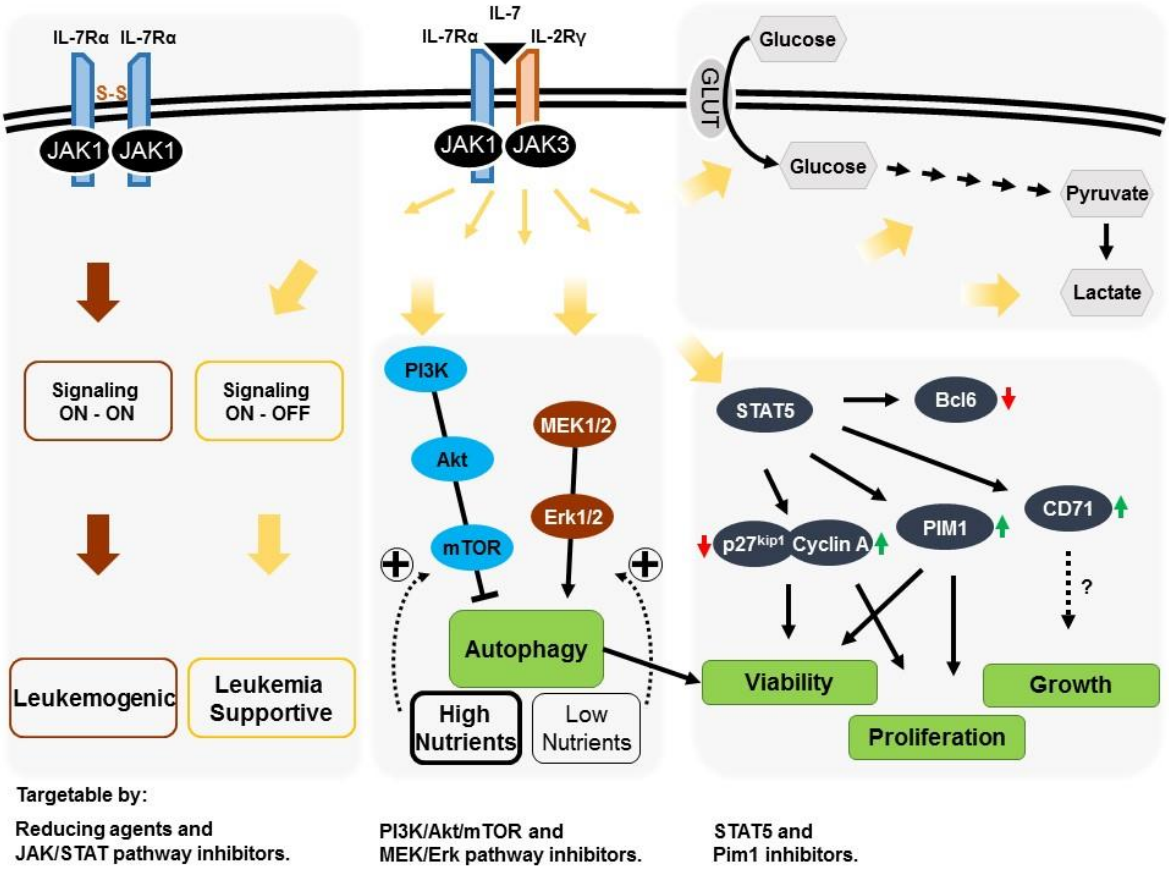

Figure 1. Novel aspects of IL-7 signaling in T-ALL. In Chapter 2, we reported our discovery of gain-of-function mutations in the *IL7R* gene. Most mutations led to homodimerization via insertion of an unpaired cysteine and drove constitutive signaling, transformation and tumor formation. In Chapter 3, we demonstrated that STAT5 was required for IL-7-mediated T-ALL cell viability, proliferation and growth. We also found that PIM1 was a major effector of STAT5 signaling. In addition, evidence suggests that STAT5 downregulated Bcl6 via alternative splicing. In Chapter 4, we discovered that IL-7 downregulated autophagy via PI3k/Akt/mTOR activation while promoting autophagy via MEK/Erk activation. We observed that depending on the nutritional status of T-ALL cells, IL-7 inhibited (high nutrients) or promoted (low nutrients) autophagy. In Chapter 5, we described our preliminary data associating IL-7-dependent increase in glucose consumption and lactate production with the rapid upregulation of several genes involved in glycolysis. Possible therapeutic strategies studied in Chapters 2, 3 and 4 are included bellow each scheme. In-depth discussion of our discoveries is found in the text.

6.1 IL-7R signaling and leukemogenesis: a new oncogene revealed

As mentioned extensively throughout this thesis, while IL-7 [1] and IL-7R [2, 3] signaling are essential for normal T-cell development, there is considerable evidence that they may also contribute to T-ALL development. IL-7 supports T-ALL cell proliferation *in vitro* [4-7] and accelerates human leukemia *in vivo* [8]. In our studies (Chapter 2) we found that 9% of pediatric T-ALL cases have gain-of-function mutations in the exon 6 of *IL7R*. Most mutations inserted an unpaired cysteine in the extracellular juxtamembrane-transmembrane interface domain leading to homodimerization of IL-7Rα chains. This created a ligand-free and γ_C/JAK3-independent triggering of constitutive intracellular signaling that was capable of cell transformation and tumor formation. Parallel independent studies have also demonstrated the presence of *IL7R* mutations [9, 10], validated by numerous subsequent studies in ALL [11-16], including *in vivo* models [17, 18].

Interestingly, the cysteine mutation did not occur alone but required additional aminoacids. Model predictions suggested the additional aminoacids would confer structural conformation changes that allow the associated Jak1 proteins to trans-phosphorylate, mimicking the wild-type IL-7Rα conformational changes elicited by IL-7 and γ_C interaction [19]. The presence of cysteine disulphide bonds provides a rational for targeting mutant signaling with reducing agents. We showed that mutant homodimerization and signaling was affected by treatment with β-mercaptoethanol (β-ME) [20]. These findings were subsequently extended by Mansour and colleagues, who showed that administration of N-acetyl cysteine, another reducing agent, delayed leukemia development in mice engrafted with IL-7Rα-mutant cell lines [21].

The non-cysteine mutations were less prevalent and our data indicated that they are less potent. However, more recently it was shown that some non-cysteine mutations could elicit constitutive signaling and be leukemogenic [13]. *IL7R* mutations also occurred in B-cell ALL (B-ALL), but were rare (<1%), however they tended to cluster with cytokine receptor-like factor 2 (*CRLF2*)-altered cases and some *IL7R* mutation required *CRLF2* co-operation [9]. Nonetheless their biological importance in B-ALL has been highlighted by the fact that they can be found in high risk cases [15].

It is possible that other γ_C family of receptors could harbor similar alterations, a possibility that warrants further studies. IL-2, IL-4, IL-9 and IL-15 (IL-21 has not been studied to date) were all shown to induce proliferation of T-ALL cells to some extent [22], which raises the possibility that such mutations may exist, perhaps at lower frequencies than

for the *IL7R*. Curiously, our analyses have failed to find mutations in the γ_C (*IL2RG*) in the Brazilian patient cohort used in Chapter 2 (data not shown). This is in line with the fact that none of the next generation sequencing studies published to date on B- or T-ALL patients has described mutations in this subunit [10-12, 15]. This is perhaps not completely surprising knowing that γ_C is not expected to provide intracellular signals by itself and merely assists the other subunits (such as IL-7Rα) in their signaling tasks. In the least, this appears to suggest that *IL2RG* mutations, should at the most a rare event in T-ALL.

Curiously, some breast cancer tissue and cell lines express the IL-7R machinery [23], which raises the possibility that other cancer types may benefit from IL-7R signaling, aberrant or not [24].

We also found that mutant IL7Rα-expressing cells were sensitive to Jak/STAT pathway inhibitors. However, it would be important to further investigate whether differences between the mutant signaling (constitutive, elevated, Jak1-dependent) and physiological signaling (regulated, Jak1/3-dependent) may exist from a molecular and therapeutic perspective.

6.2 The Jak/STAT5 pathway: novel mediators of IL-7/IL-7R effector signaling in T-cell ALL

The transcription factor STAT5 is an essential element of IL-7-mediated signaling during normal T-cell development and mature T-cell function [25, 26], but IL-7-dependent murine lymphomagenesis also requires STAT5 [27]. Moreover, mutant IL-7Rα-expressing cells are sensitive to Jak/STAT5 pathway inhibitors (Chapter 2) [17, 28]. We demonstrated in Chapter 3, that a Jak/STAT5/PIM1 signaling axis activated by IL-7 exists in T-ALL, is a required effector of IL-7-mediated signaling and constitutes a drugable target. At the functional level, inhibition of IL-7-dependent Jak/STAT5 or PI3K/Akt pathway activation has similar effects on T-ALL cell viability, proliferation and growth (Chapter 3) [29], indicating that both pathways are indispensable for proper IL-7 signaling and have non-redundant effects in T-ALL. However, dissection of the molecular mechanisms used by each pathway revealed that, at least, the survival mechanism is notably different. Whereas PI3K/Akt/mTOR signaling mediates the expression of the pro-survival protein Bcl-2 downstream from IL-7 stimulation [7, 29], STAT5 does not regulate *BCL2* or *BCL2L1* expression (Chapter3). Thus, in T-cell leukemia IL-7 mediates the expression of Bcl-2 survival protein apparently only via PI3K/Akt signaling, although survival is mediated by

both pathways. This sharply contrasts with IL-7-mediated signaling in normal T-cells, where IL-7-induced viability does not require PI3K or mTOR activation [30, 31]. In this context, STAT5 clearly mediates viability of some T-cell subsets (mature CD8) [32, 33], where it is involved in IL-7-mediated Bcl-2 expression [32]. Overall, evidence suggests that a subtle mechanistic difference, yet with great therapeutic potential, exists between IL-7-mediated signaling in normal *versus* leukemic T-cells. More studies exploring these differences may lead to novel therapeutic strategies.

We found that PIM1 kinase was a direct downstream target of IL-7/STAT5 signaling and could account for STAT5-dependent survival and proliferative effects (Chapter 3). To our surprise, PIM1 inhibition partially abrogated Bcl-2 expression. We postulated this could be due to the existence of opposing arms (both promoting and inhibiting *BCL2* activation) downstream of STAT5 with the final output being neutral effects on *BCL2* transcript levels. Thus, upon inhibition of the PIM1 effector arm (positive regulator of Bcl-2), the remaining STAT5-dependent transcription was unaffected and created an unbalance leading to downregulation of Bcl-2 expression. Excessive STAT5 signaling has been reported to be deleterious [34]. Akt and PIM kinases have a high overlap in function and molecular targets [35, 36]. In addition, inhibition of PI3K pathway has a stronger effect on Bcl-2 expression [29] than inhibition of PIM1 (Chapter 3). It is possible that, in T-ALL, Akt is absolutely required to maintain Bcl-2 expression whereas PIM1 is an adjuvant, or that inhibition of PIM1 while maintaining its protein expression may have a partial dominant-negative effect over Akt. Both possibilities are worth considering.

The analysis of IL-7/STAT5-dependent transcriptome data revealed potential interaction with RUNX transcription factors, which have been implicated in leukemia [37, 38]. In the context of IL-7 signaling, the implications of the interactions with RUNX factors in T-ALL are unknown and particularly intriguing given the contradictory effects of RUNX family members on T-cell leukemogenesis. For instance, the reported RUNX1 tumor suppressor role [39] would be compatible with competition with STAT5 and consequent opposition against IL-7-mediated STAT5-dependent positive functional effects on T-ALL cells.

Interestingly, our studies also showed IL-7-triggered STAT5-dependent downregulation of *BCL6*, apparently mediated by transcription of a possibly unstable alternative transcript. The exact impact of IL-7-mediated BCL6 downregulation in T-ALL cells requires investigation. Notably, a recent report demonstrated that BCL6 downregulation was involved in IL-7-mediated self-renewal capacity of DN4 mouse

thymocytes [40]. This suggests that the leukemogenic impact of aberrant IL-7-mediated signaling on developing T-cells could be achieved in part via downmodulation of BCL6, consequent developmental blockade and self-renewal of immature thymocytes, which would create a pre-leukemogenic context favoring subsequent leukemogenic hits.

6.3 IL-7 and T-ALL cell autophagy: a balance between PI3K/Akt/mTOR and MEK/Erk signaling

Autophagy in cancer has been described as a double-edged sword. On one hand, by mitigating multiple sources of cellular stresses, autophagy may prevent normal cell transformation by, for instance, promoting metabolic homeostasis, preventing excessive DNA damage and expression of aberrant, potentially oncogenic, proteins. On the other hand, once tumor initiation has occurred, tumors may exploit autophagy to resist said stresses, or others, such as chemotherapy, and increase tumor fitness [41]. Our results in Chapter 4 characterize, for the first time, the role of IL-7 signaling in T-ALL cell autophagy. We found that IL-7 balanced autophagy according to microenvironmental conditions and that PI3K/Akt/mTOR activation decreased autophagy, while MEK/Erk activation promoted autophagy. The regulation of autophagy by each pathway is in accordance to previously published data [42]. Interestingly, we demonstrated that in nutrient-rich conditions IL-7 blocked autophagy, and in contrast, in nutrient-poor conditions IL-7 promoted autophagy (Chapter 4). Functionally, we found that in nutrient-rich culture IL-7 mediated survival via PI3K/Akt/mTOR activation, whereas in nutrient-poor culture IL-7 promoted survival via MEK/Erk activation. We postulated that the shift from PI3K/Akt-dependent to MEK/Erk-dependent survival, under IL-7-stimulation, was associated with the differential capacity that each pathway has to regulate autophagy. For instance, in nutrient-poor conditions, autophagy would be beneficial to leukemic cells. In this context, a pathway that promoted autophagy (IL-7-mediated MEK/Erk stimulation) would play a major role in survival. Molecularly, our preliminary data suggest that the shift in IL-7-mediated pro- or anti-autophagic effects is associated with an increase in MEK/Erk pathway activation. How IL-7 increases MEK/Erk activity is unknown and warrants further investigation. Wang and colleagues [43] proposed a model where autophagic stimuli activated AMPK, which in turn activated MEK/Erk signaling. Elevated MEK/Erk activity destabilized mTORC, resulting in high Beclin1 levels which promoted autophagy. It would be interesting to test this model in our studies. These considerations apart, in Chapter 4, we characterized a novel function for MEK/Erk activation

in IL-7-mediated signaling in T-ALL, identifying for the first time a clear role for IL-7-mediated MEK/Erk activation in leukemia T-cells.

6.4 Cell metabolism in T-ALL: does IL-7/IL-7R signaling play a role?

IL-7 promotes cell growth and metabolic activation at several levels in T-cells [30, 44-47]. Less studies have been performed regarding T-ALL, but it has been demonstrated that IL-7 promotes cell growth and CD71 expression dependent on both Jak/STAT5 and PI3K/Akt/mTOR activation and additionally, glucose use dependent on PI3K/Akt/mTOR pathway (Chapter3) [29, 48]. Our subsequent transcriptome analysis of T-ALL cells demonstrated that IL-7 modulates the expression of multiple genes involved in key metabolic pathways, with emphasis on sugar metabolism (Chapter 5).

Our investigation of glucose metabolism-related gene expression revealed that IL-7 very quickly upregulates the expression of several genes involved in the glycolytic process. Some of the investigated genes had two waves of induction by IL-7 (e.g. *HK2*, *ENO1*, *BCL2*), indicating two separate expression mechanisms. Although the quick rise and fall in expression of glycolytic genes suggests an immediate-early gene program [49], whether they are true immediate-early (IEG) or instead delayed-early (DEG) genes still remains to be tested. Most well-known and studied IEGs are transcription factors (e.g. *FOS*, *JUN*, *MYC*) and their expression has been related to the activation of MAPK pathways [49, 50]. Notably, *PFKFB3* expression was associated with IEG response driven by p38 MAPK pathway [51]. The role of IL-7-mediated MEK/Erk activation in T-ALL has only recently started to become apparent (Chapter 4), thus it is also worth considering a role of this pathway in early glycolytic gene expression.

Akt and mTOR pathways as well as PIM kinases (all subject of IL-7 activation) have been associated with metabolic regulation, including glycolysis and oxidative phosphorylation, in T-cells [35]. In addition, the BCL6 transcriptional repressor, downregulated by IL-7/STAT5 signaling (Chapter 3), was found to be a direct repressor of the glycolytic pathway in helper T-cells [52]. Taken together, the available data strongly indicate that IL-7 signaling has a major role in metabolic regulation of T-ALL cells. Dissecting the molecular mechanisms associated with metabolism-related IL-7 effects in T-ALL is of utmost importance.

6.5 Novel molecular targets with therapeutic potential against leukemia

Advances in therapy for T-ALL throughout the years led to great improvements in survival. Still, relapses occur in a significant number of cases and treatments are associated with long-term side effects [53]. Therefore, new, more effective and specific therapies are required.

In Chapter 2, we identified oncogenic gain-of-function mutations in IL-7Rα, driving constitutive signaling via disulphide bond-dependent homodimerization. Antibody-based therapy in cancer has become very successful [54]. Targeting IL-7Rα homodimers with low affinity antibodies, potentially allows specific homodimer recognition due to increased avidity, thereby allowing the selective targeting of mutant IL-7R expressing cells that should spare wild-type IL-7R-expressing cells. On the other hand, the use of antibodies recognizing both mutant and wild-type IL-7Rα, although displaying the potential caveat of targeting normal IL-7R-expressing cells, would have the advantage of targeting all IL-7R-dependent leukemia cells, therefore having probably broader application.

In another approach, the aberrant constitutive signaling may be targeted. We have shown that IL-7R mutants are sensitive to Jak/STAT5 pathway inhibitors (Chapter 2). Mutant signaling relies on Jak1 activation. Ruxolitinib (INCB-018424), a Jak1/2 inhibitor, had the greatest effect on primary mutant T-ALL cells and is already approved for clinical use in myelofibrosis [55]. A small molecule inhibitor of STAT5 also showed promising effects [56]. These data are in accordance with Chapter 3, where we characterized Jak/STAT5 signaling as an important effector of IL-7 signaling and demonstrated that STAT5 small molecule inhibition abrogates viability and proliferation of T-ALL cells. Moreover, we demonstrated that PIM1 kinase is an important downstream effector of STAT5 signaling, using the PIM1 inhibitor Smi-4a [57]. The PIM1 inhibitor PIM447 [58] is currently in phase I trials and may be of interest in the context of T-ALL, including IL-7/IL-7R-dependent cases.

In Chapter 4 we provided evidence that IL-7 modulates autophagy according to microenvironmental conditions, associated with consistent promotion of viability. In stress conditions (absence of serum) IL-7 promoted autophagy in T-ALL cells. The relevance of autophagy in the context of cancer treatment becomes obvious in the website *clinicaltrials.gov*, whose records show multiple registered and ongoing clinical trials using the autophagy inhibitor HCQ as single agent or in combination with other drugs. Also, in our study we found that IL-7-mediated increase in autophagy relied in MEK/Erk pathway

activation. Furthermore, in adverse conditions IL-7 promoted viability via MEK/Erk rather than canonical PI3K/Akt/mTOR. Combination of both pathway inhibitors may have good therapeutic value in targeting leukemia cells living in different microenvironments. MEK inhibitors, such as GDC-0973 or GSK1120212 in phase III trials [59, 60], could be used for this purpose.

The current use of cytarabine, an anti-metabolite, and asparaginase, which catalyzes hydrolysis of asparagine, in therapeutic strategies in ALL [53, 61], highlights the importance of targeting cancer metabolism. Our preliminary metabolism studies in T-ALL suggest that IL-7 increases the activity of the glycolytic pathway. Targeting glycolysis may be a new therapeutic strategy in T-ALL. Some glycolysis pathway inhibitors have entered clinical trials. For example, lonidamine and 2-deoxyglucose (2-DG) are hexokinase inhibitors that target the initial steps of glycolysis; whereas TLN-232 is a PKM2 inhibitor that has the potential to reverse the Warburg effect [62, 63].

In summary, our studies have contributed to improving the knowledge on T-ALL biology, particularly regarding the involvement of IL-7 and its receptor, and in doing so have permitted the identification of several molecular targets for potential therapeutic intervention in this hematological cancer.

6.6 References

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