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The role of LMO4 in T cell acute lymphoblastic leukaemia

by

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A thesis submitted to the University of Birmingham for the degree of MRes in Cancer Sciences.

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ABSTRACT

The combination of chemotherapy regimens has markedly improved the survival rates of patients suffering with T-cell acute lymphoblastic leukaemia (T-ALL). Nevertheless, there are still patients with poor prognoses, particularly for those experiencing a relapse of the disease. Early T-cell precursor ALL (ETP-ALL) is a subtype of T-ALL with such a poor prognosis and has an immature immunophenotype with a gene expression signature similar to ETPs. The LIM-only 4 (LMO4) protein has recently been found to be overexpressed in this subgroup, where it is supposed to play a critical role in early T-cell development. However, overexpression of other LMO family members such as LMO1 and LMO2 was suggested to displace LMO4 from a DNA-binding complex and thus deregulate the normal T-cell maturation process. To address the role of LMO4 in T-cell maturation we first sought to determine its presence in ETP-ALL cell lines and to identify potential interacting partners that could be present in the same transcriptional complex. We also performed *LMO4* gene silencing experiments in order to investigate if it is indeed fundamental for T-cell maturation and survival processes. This research allowed us to characterise two cell lines that can serve as a model for ETP-ALL and provided us with a better insight into the role of the LMO proteins in this type of leukaemia. The presence of LMO4 during ETP maturation process indicates that it could be directly involved during the onset of ETP-ALL.

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AIMS

The purpose of this project was to investigate the role of the LMO4 protein in T-ALL and more specifically in immature T-ALL cases. To achieve this, we first had to examine whether this protein was present in our ETP-ALL cell lines. We also wanted to determine whether it was present in the transcriptional complex comprising Ldb1 and whether it was indeed displaced from the complex by LMO2 overexpression. Finally, silencing of the *LMO4* gene would allow us to assess its involvement in T-cell development and check whether it is a critical factor for the survival of the immature T-cell lineages.

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LIST OF ABBREVIATIONS

AKT1: RAC-alpha serine/threonine-protein kinase

bHLH: basic helix-loop-helix

BMP: Bone morphogenic protein

BRCA1: Breast cancer type 1

C/EBPα: CCAAT-enhancer binding protein α

CBFβ: Core-binding factor beta subunit

CHIP: C terminus of HSC70-Interacting protein

CLP: Common lymphoid progenitor

CtIP: CtBP interacting protein

Cy5: Cyanine 5

DC: Dentritic cell

DLL4: Delta-like ligand 4

DN: Double negative

DP: Double positive

DSB: Double-strand break

ELISA: Enzyme-linked immunosorbent assay

ETP: Early thymic progenitor

ETP-ALL: Early T-cell precursor acute lymphoblastic leukaemia

FACS: Fluorescence-activated cell sorting

GATA: GATA sequence-binding factor

GFI1: Zinc-finger repressor growth factor independence 1

GFP: Green Fluorescent Protein

GM-CSF: Granulocyte-macrophage colony-stimulating factor

H2AFV: H2A histone family member V

HDAC1: Histone deacetylase 1

Hes1: Hairy and enhancer of split 1

Hhex: Haematopoietically-expressed homeobox protein

hnRNP: heterogeneous ribonucleoprotein particles

HNRNPA1: Heterogeneous nuclear ribonucleoprotein A1

HNRNPC: Heterogeneous nuclear ribonucleoprotein C

HSC: Haematopoietic Stem Cell

HSPA9: Heat shock protein 9

Ig: Immunoglobulin

IL-3: Interleukin-3

IL-7: Interleukin-7

KHDRBS1: KH domain containing RNA binding signal transduction associated 1

LCK: Lymphocyte specific protein tyrosine kinase

Ldb1: LIM domain binding protein 1

LGALS1: Lectin galactoside-binding soluble 1

LIM: Linl-1, Isl-1 and Mec-3

LMO: LIM-only

LRF: Leukaemia/lymphoma related factor

Lyl1: Lymphoblastic leukaemia derived sequence 1

MHC: Major histocompatibility complex

MINT: MSX2-interacting protein

MPP: Multipotent progenitor

MYB: Myeloblastosis proto-oncogene

NHEJ: Non-homologous end joining

NK: Natural killer cell

PPP1CA: Protein phosphatase 1 catalytic subunit alpha isoform

PRKDC: Protein kinase DNA-activated catalytic polypeptide

q-PCR: Quantitative polymerase chain reaction

RAG: Recombination activating gene

RUNX-CBFβ: Acute myelogenous leukaemia factor-core binding factor β

Sca-1: Stem cell antigen-1

SCF: Skp, cullin, F-box containing complex

SCL: Stem cell leukaemia

SF3B1: Splicing factor 3b, subunit 1

Shh: Sonic hedgehog

shRNA: small hairpin RNA

SIL: SCL interrupting locus

siRNA: small interfering RNA

Skp2: S-phase kinase-associated enzyme

SP: Single positive

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TAL1: T-cell acute lymphoblastic leukaemia protein 1

T-ALL: T-cell acute lymphoblastic leukaemia

TCF-1: T-cell factor 1

TCR: T-cell receptor

TGF- β: Transforming growth factor beta

VDJ: Variable, Diverse, Joining

YWHAQ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta

INTRODUCTION

Blood cell production, or haematopoiesis, is a process that starts during embryonic development and continues throughout life (1). There are two waves of haematopoiesis in verterbrates, the primitive and definitive wave (2). The primitive wave produces red blood cells, in order to enable tissue oxygenation during the embryo growth and myeloid cells (3), but these cells do not exhibit long term self-renewal. In contrast, definitive haematopoiesis occurs later in development and involves Haematopoietic Stem Cells (HSCs) which are able to give rise to myeloid, erythroid and lymphoid blood lineages of the adult organism (1). In humans, primitive haematopoiesis begins in the yolk sac and subsequently definitive haematopoietic stem cells are generated in the ventral wall of the dorsal aorta. These stem cells then migrate temporarily into the liver, before reaching at their final destination, the bone marrow. Although most cell types are specified within the bone marrow, some early progenitors move from the bone marrow into the thymus, where T-cell development occurs (1).

The main focus of this research was childhood T-cell acute lymphoblastic leukaemia (T-ALL). In order to better define the molecular processes causing blood disorders, such as T-ALL, it is important to have an understanding of the same processes during normal T-cell development.

T-lymphocyte development

The progenitors of T-lymphocytes arise in the bone marrow from the pool of HSCs that progress via multipotent progenitors (MPPs) into common lymphoid progenitors (CLPs). These then migrate into the blood circulation and through a chemotaxis process end up in the thymus (Figure 1.1) (4). The immigrant precursors differentiate into early thymic progenitors (ETPs), also called double negative 1 (DN1) (5). These are double negative for the T-cell markers CD4 and CD8 that contain multilineage

differentiation potential, which means they can still give rise to dendritic cells (DCs), natural killer cells (NKs) and macrophages when given the right stimuli (5).

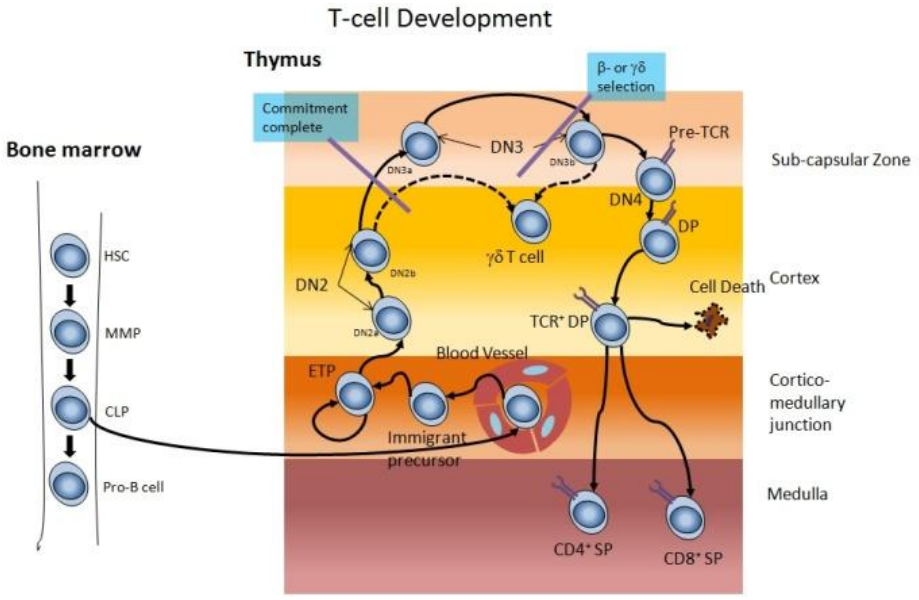


Figure 1.1. T-cell developmental stages. The progression of haematopoietic stem cells (HSCs), multipotent progenitors (MPPs) and common lymphoid progenitors (CLPs) in the bone marrow are shown to the left. Lymphoid progenitors migrate through the blood to the cortico-medullary junction of the thymus where they differentiate into early T-cell progenitors (ETPs). Migration of ETPs to the cortex and then to the subcapsular zone initiates their differentiation to the double negative (DN) stages. The return of DN cells from the sub-capsular zone to the medulla induces their differentiation to double positive (DP) and then single positive (SP) stages. Complete T-cell lineage commitment is shown with a line between the DN2b and DN3a stages. β or γδ selection is indicated between the DN3a and DN3b stages. This figure was taken from Michael Litt *et al.,*(2013) (7).

Many factors contribute to T-cell differentiation, one of which is the thymic microenvironment, which expresses Notch ligands, interleukin-7 (IL-7)and sonic hedgehog (Shh) proteins that control cell fate specification and progressively turn off genes that maintain the ETP/stem cell phenotype (6, 7). The Notch signalling pathway was the first to be demonstrated as critical for the generation of ETPs (8), where the Delta-like 4(DLL4) ligand, which is present in the thymus, interacts with the Notch1 receptor and initiates T-cell development (9, 10).However, Notch signalling is not sufficient to activate T-cell lineage genes directly (11). Blood cells can generally be identified by the presence of specific combinations of surface molecules. During T-cell maturation, T-cells express multiple CD

molecules on their membrane that change in their expression levels during their migration into the different thymic areas and thereby developmental stages. Such markers are the CD4, CD8, CD25, CD44, CD3, CD117 (c-kit), CD24 and the T-cell receptor (TCR) chains (12). The DN thymocytes do not express the cell surface glycoproteins CD4 and CD8 until they become double positive (DP; CD4+ CD8+) and thereafter, single positive (SP; CD4+ CD8- or CD4-CD8+). The differentiation process of the DN subpopulation is divided into four stages according to their expression of CD25 and CD44 and the thymus microenvironment (Figure 1.1) (12). Starting from the ETP/DN1 (CD44+CD25-) heterogeneous population that contains high levels of CD117, stem cell antigen-1 (Sca-1) but not CD3, this group can be further divided to DN2a(CD44+CD25+CD117+) and DN2b (CD44+CD25+CD117^{int}) in the thymic cortex(13, 14). This is followed by T-cell lineage commitment and differentiation into a DN3a (CD44-CD25+CD117-CD27-) and subsequently DN3b (CD44-CD25+CD117-CD27+) population in the sub-capsular thymic zone (7). After gaining T-lineage properties, the DN3 T-cells stop proliferating (12). T-cells pass from a proliferative expansion stage to cell-cycle arrest that enables the TCR gene rearrangement process to be activated (12). Successful V (D) J rearrangement is required to express functional TCRβ, TCRγ and/or TCRδ chains and components of CD3 chains. This is initiated by the recombinases RAG-1,RAG-2 and the Notch pathway which together activate the V(D)J recombination process where the Variable, Diverse, Joining (VDJ) gene segments are randomly combined to form a unique TCR (21). After acquiring a TCRβ chain, the resulting β chain pairs with a surrogate α chain (pre-Tα) and forms a pre-TCR. The expression of the pre-TCR on the cell surface allows cells to pass a check-point, known as β-selection and proceed through the DN4 (CD44-CD25-CD117-CD27+) stage where they lose their dependence on Notch ligand, IL-7 and sonic hedgehog (15, 16). Then, the cells with a correctly assembled αβTCR, recognizing the peptide-major histocompatibility complex (MHC) ligand on thymic epithelial cells, undergo positive selection. Depending on the class of MHC recognition, T-cells become either CD4 SP (CD4+CD8-) if they have previously interacted with an MHC class II or CD8 SP (CD4-CD8+) if they interacted with an MHC class I .Finally, SP cells undergo negative

selection in order to eliminate T-cells which have high affinity to self-antigens and thus reduce chances of generating auto-reactive T-cells (17). Alternatively, DN3 T cells that successfully rearrange TCRγ and TCRδ chains are selected as γδ T-cells and are CD4- CD8-.

The existence of a transcription factor group during T-cell development is essential for the T-cell differentiation process from the ETP/DN1 to the DN4 stage. It is composed of different types of transcription factors or transcription factor families that work with Notch and includes GATA sequence-binding factor 3(GATA3)(18), the myeloblastosis proto-oncogene (MYB), the acute myelogenous leukaemia factor-core binding factor β (RUNX-CBFβ) complexes, the basic helix-loop-helix E-proteins E2A (E47/E12) and HEB(19),the dominant negative helix-loop-helix protein ID3, the transcription factor Bcl11b, the T-cell factor 1(TCF-1),the zinc-finger repressor growth factor independence 1(GFI1) and the tumour suppressor Ikaros family member (20-23). Moreover, T-cell development depends on the decreased expression of some other transcription factors such as the transcriptional activator PU.1, the stem cell leukaemia (SCL/ TAL1) protein, CCAAT-enhancer binding protein α (C/EBPα), GATA-2, LIM-domain-only protein 2 (LMO2) and haematopoietically-expressed homeobox protein (Hhex) between the DN1-DN3 stages (24, 25).

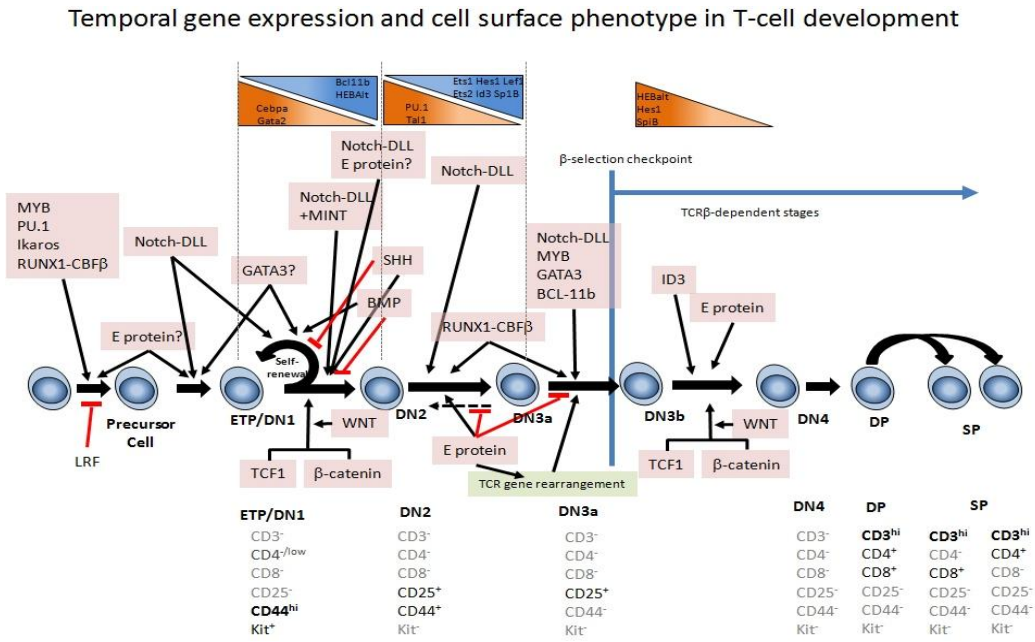


Figure 1.2. Regulatory factors in early T-cell development. The different developmental stages are shown in the center beginning from HSCs which progress to SP cells. Regulatory factors important for the progression from one stage to another are indicated above and below the stages. Red lines indicate the negatively active factors. The triangles indicate regulatory factors which are either downregulated or upregulated at indicated stages. The blue line indicates the β-selection checkpoint starting after the DN3a stage and the long horizontal blue arrow shows the TCRβ dependent stages. This figure was taken from Michael Litt *et al.,* (2013) (7).

Figure 1.2 illustrates known regulatory factors that have a distinct or repeated role in early T-cell development. Beginning from the very early stage of development where HSCs differentiate into T-cell precursors in the bone marrow, expression of MYB, PU.1, Ikaros, RUNX1-CBFβ and E-proteins is fundamental for the initiation of the T-cell developmental programme(7)(24). The transcriptional repressor leukaemia/lymphoma related factor (LRF) keeps T-cell lineage genes silent and makes B-cell development possible by limiting the expression of Notch. LRF absence promotes the activation of the Notch signalling pathway, which is critical for the generation and maintenance of the self-renewal properties of ETPs/DN1 (8, 26).

TCF1 and GFI1 are important for the DN1 to DN2 transition(27, 28) as well as the WNT signalling pathway that is analogous to Notch pathway and converts TCF1 into an activator via its interaction with β-catenin (29, 30). Another factor that affects DN1 to DN2 transition is Notch-MINT interaction.

The MSX2-interacting protein (MINT) is a natural negative regulator of the Notch-dependent transcription (31) and is normally expressed in ETPs, but its levels decline while cells enter the DN3 stage and β-selection (24). Moreover, although GATA-3 is also essential for ETPs to become DN2, its overexpression leads ETPs and DN2 cells to differentiate into mast cells (32). This shows that it is fundamental to have a balance between T-cell activator and repressor genes in order for the immature T-cells to further differentiate and in this case Notch-DLL interaction maintains GATA-3 levels down (32). Finally, Bcl11b which is important to fully commit to the T-lineage, starts being expressed at the DN2 stage (33) and causes, through an unknown mechanism, the downregulation of PU.1, which is a transcription factor known for its importance for the B-cell and myeloid lineage development (34).

The DN2 to DN3 stage transition is depended on RUNX1-CBFβ, MYB, Notch-DLL, GATA-3,Bcl11b and the E-proteins E2A and HEB, which are also needed for TCRβ gene rearrangement and the ability to undergo β-selection (35).Conditional deletion of MYB causes a developmental block at the DN3 stage with decreased V(D)J recombination at the TCRβ locus (36). Deletion of GATA-3 also leads to an accumulation of DN3 cells with a defect in the TCRβ protein expression and RUNX1 is essential for the proliferative expansion of cells after β-selection (22, 23). As during all the previous stages, Notch and E-proteins are very important as they “give a ticket” to pass the DN3 stage and recent studies have shown that E2A and Notch pathways converge (37). Activation of the *Notch1* gene is downstream of E2A and together they regulate the previously called Notch1 targets such as hairy and enhancer of split 1(Hes1) and Ptcra (21), which in turn decrease the MINT expression of Notch (31). Before cells enter the β-selection checkpoint, cell-cycle arrest is crucial for TCRβ gene rearrangement (19) and for the normal β-selection process to occur. This is achieved by the activity of E2A and HEB proteins which also prevent DN3 cells to undergo reverse differentiation and return to a DN2-cell-like phenotype (38). At this point we can conclude that the DN3 stage depends on both

Notch and E-protein activity and it is important to bear in mind that removal of E-protein activity can be enough to restore the progenitor-cell regulatory factors.

Moving through the TCRβ-dependent stages which are the final T-cell developmental stages (DN3b to SP). ID3 protein is upregulated and inhibits the bHLH activity of the E2A protein, as well as Notch1, and is a mechanism that apparently ensures allelic exclusion and allows cell expansion (20, 39). Finally, the existence of non-T-cell factors that promote specific alternative pathways are sufficient to change the cell fate of the immature T-cells into other lineages so it is important to keep their levels low. Such factors are ID2, SCL/TAL1, C/EBPα, PU.1, LMO2, GATA-2 and the bone morphogenic protein (BMP). If C/EBPα and PU.1 are expressed ectopically at the DN2 stage, they can impose a myeloid or dendritic cell developmental programme on T-cell precursors (40). Similarly, ID2 and TAL1 work as E-protein antagonists of the DN2 cells, which is sufficient for ID2 to promote natural killer-cell development (41).

In conclusion, lineage commitment from multipotent HSCs to mature T-cells is a continuous process involving coordinated developmental stages regulated through receptor-ligand pair interactions and an underlying complex transcriptional network. Thus, limiting the potential of progenitor cells to differentiate into other lineages and inducing their survival and proliferation. It is therefore likely that these transcriptional networks are mis-regulated when normal blood development transforms into malignant haematopoiesis.

Genetic rearrangements during lymphopoiesis induce T-ALL

Carcinogenesis is a multistep process that accumulates various molecular, genetic and epigenetic abnormalities such as point mutations, amplifications, deletions, and chromosomal translocations which usually activate oncogenes or inactivate tumour suppressor genes and thus deregulate the normal balance between proliferation and cell death of the cell (42, 43). Lymphoid malignancies have been best described to carry tumour-associated chromosomal rearrangements (17) that lead to the activation of genes. Lymphoid gene activation occurs most commonly by bringing a gene presumed oncogenic into the same chromosomal context as an immunoglobulin (Ig) or a TCR gene (17). This shows that the enzyme systems (RAG-1, RAG-2) responsible for the normal V(D)J recombination get deregulated and create tumour-specific chromosomal rearrangements(44).

T-cell acute lymphoblastic leukaemia (T-ALL) is a good example of a disease often caused by gene activation as a result of genomic translocations as it helped to discover many different novel genes that could lead to tumourigenesis (45). However, there are many T-ALL cases where no obvious genomic translocation is detected and where the underlying mechanism leading to leukaemogenesis is unclear. This needs to be further investigated in order to better understand how these diseases are initiated and develop, as this is vital information that can be utilised to improve leukaemia therapy.

T-ALL is a malignant clonal expansion of immature T-cells that happens in 10-15% of childhood and 25% of adult leukaemic cases (46). Nowadays, intensive use of chemotherapy has improved childhood prognosis and nearly 90% of children can be currently cured (47). Recent studies have observed that the genetic deregulations involving T-ALL development correlated with prognosis but this information is not sufficient for the use of these abnormalities in treatment planning (48). Coustan-Smith *et al.,(*2009) hypothesized that a part of T-ALL cases would arise from oncogenically transformed ETPs and this would be the reason why patients and more specifically children, respond poorly to lymphoid cell-directed chemotherapy (46). The high risk of remission induction failure or

relapse of children treated with ALL intensive chemotherapy, lead scientists to center their research on this genomically unstable subtype of childhood T-ALL. ETPs-ALL have distinct cell surface markers such as CD1a-,CD5 ^{low} ,CD8- and one or more myeloid or stem cell-associated markers which facilitate their identification. Moreover, gene expression profiling studies showed that paediatric T-ALL seems to involve four subgroups of genetic rearrangements including TAL/LMO, TLX3/HOX11L2, TLX1/HOX11 and HOXA (48-50). Recently, an additional immature T-ALL subgroup was identified that was enriched for ETP-ALL cases (49). This subgroup was characterised by a gene expression signature containing a number of genes generally associated with the early T-cell stages, including LMO4 and Hhex (51).In this research we wanted to further investigate the immature subgroup of childhood T-ALL and in particular the role of LMO proteins and their interacting partners have in its development.

Investigating the role of the LMO proteins in T-ALL

During the last two decades many studies have been done on the LIM-only domain (LMO) family of nuclear and cytoplasmic proteins in various types of human cancers but more specifically in T cell acute lymphoblastic leukaemia (T-ALL). To date, four LMO proteins (LMO1-LMO4) have been identified after the first characterization of *LMO1* (previously called *RBTN1* or *TTG1*) near the breakpoint of the chromosomal translocation t(11;14)(p15;q11) involving the TCR- δ gene (52-54).

By using the *LMO1* cDNA as a probe, researchers managed to isolate the two related genes *LMO2* (previously called *RBTN2* or *TTG2*) and *LMO3* (previously called *RBTN3*), of which *LMO2* was found in T-ALL cases located at the junction of the chromosomal translocations t(11;14)(p13;q11) or t(7;11)(q34;p13), involving TCR-α and TCR-δ or TCR-β respectively (55, 56) (Table 1.1).

Recurring Translocations in T-ALL					
TCR Rearrangements			**Non-TCR Rerrangements**		
Gene	**Rearrangement**	**Frequency**	**Gene**	**Rearrangement**	**Frequency**
TAL1	t(1;14)(p32;q11) t(1;7)(p32;q34)	~3 of T-ALL	TAL1	STIL-TAL1 (1p32 deletion)	12-25% T-ALL
TAL2	t(7;9)(q34;q32)	rare	HOXA	PICALM-MLLT10 (t(10;11)(p13;q14)) MLL-MLLT1 (t(11;19)(q23;p13)) SET-NUP214 9q34 deletions	
LMO1	t(11;14)(p15;q11) t(7;11)(q34;p15)	6-8% of T-ALL	ABL1	EML1-ABL1 (t(9:14)(q34;q32)) BCR-ABL1 (t(9;22)(q34;q11)) ETV6-ABl1 (t(9;12)(q34;p13)) NUP214-ABL1	8% T-ALL for ABL1 6% T-ALL for NUP214
LMO2	t(11;14)(p13;q11) t(7;11)(q34;p13) 11p13 deletions		ETV6	ETV6-JAK2 (t(9;12)(p24;p13) ETV6-ARNT (t(1;12)(q21;p13)	Rare
HOX11	t(10;14)(q24;q11) t(7;10)(q34;q24)	30% of T-ALL			
HOX11L2	t(5;14)(q35;q32)	20% Childhood T-ALL 4% Adult T-ALL			
HOXA	Inv(7)(p15q34) t(7;7)(p15;q34)				
LYL1	t(7;19)(q34;p13)	rare			

Table 1.1. Table showing recurring translocations in T-ALL. The rearrangements are divided into those involving TCR and non-TCR loci. This table was taken from Michael Litt *et al.,* (2013) (7).

The LMO proteins are a subclass of LIM (Linl-1, Isl-1 and Mec-3) proteins that are characterized by the presence of two LIM domains, with limited additional amino acids at the N- and C-termini (57). Each LIM domain defines a conserved cysteine-rich structure comprising two tandem-repeated zinc fingers and has a size of 50-60 amino acids(Figure 1.3A)(58). It acts as a DNA recognition scaffolding site for the formation of multiprotein complexes that can regulate gene transcription, cell cycle progress and apoptosis (59-61). Even though the LIM domain is structurally similar to GATA-type zinc fingers (62, 63), it does not bind directly to DNA but functions as a protein-protein linker. Proteins containing LIM domains are found both in the cytoplasm and the nucleus, suggesting that the LMO proteins can bind to a vast number of proteins involved in different biological processes. As mentioned before, the LMO proteins are generally involved in transcriptional regulation, through nucleating the formation of transcription factor complexes. This is the reason why they are important in cell differentiation and cell fate (64).

Characterization of LMO2

The LMO2 protein is the most researched protein within the LMO family as it is involved in haematopoiesis and more particular leukaemogenesis. It has an important role during embryogenesis and angiogenesis as it is required for the production of primitive erythrocytes and the haemogenic endothelium which leads to the generation of the definitive haematopoietic system (65).

During haematopoiesis, LMO2 is found in multiprotein complexes comprising the LIM domain binding protein 1 (Ldb1), TAL1 a bHLH protein, which dimerizes with the bHLH proteins E12 and E47 (66), and GATA-1, GATA-2 or GATA-3 (67). More specifically, LMO2 bridges between the DNA binding GATA factors and E-box factors, thereby recognizing a bipartite DNA-binding motif of an E-box sequence separated about one helix turn from the GATA site (Figure 1.3B) (68). This complex

regulates transcription and it was confirmed by other studies that it is essential for erythropoiesis as Lmo2-null mice did not survive after day 10 of embryogenesis (65).

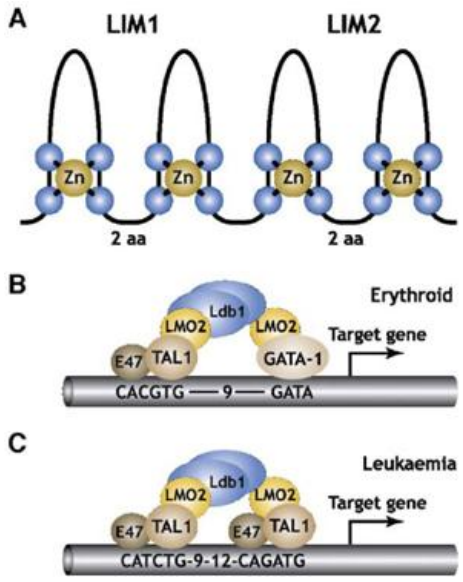


Figure 1.3. Diagrammatic respresentation of LMO2 protein structure and transcription complex. A) Represents the LMO protein structure composed of two LIM domains. Each LIM domain has two zinc-binding LIM fingers. B) Transcription complex where LMO2 binds to TAL1, E47, Ldb1, GATA-1 in normal erythroid cells.C) In leukaemic T-cells, a different complex occurs which comprises LMO2, Ldb1, TAL1 and E47. This figure was taken from Nam CH, Rabbitts TH (2006) (69).

What is more, LMO2 and TAL1 are translocated in T-ALL due to RAG recombination mistakes during T-cell development. This induces an overexpression of LMO2 and TAL1 which bind to two E-box binding sites in the bipartite DNA binding elements. As a result, abnormal regulation of the downstream target genes is observed, a blockade of the T-cell development and activation of HSC-associated genes that cause self-renewal of the DN cells (Figure 1.3C)(69) . A possible explanation of this transcriptional deregulation would be the simple mass action effect, based on the LMO2, TAL1 concentrations which affect the availability of individual proteins (74). LMO2-TAL1 complex could apply its tumourigenic effects by inhibiting the normal functions of E2A and HEB, important in ETP cell differentiation and displace GATA-2 from its normal function (74). It is also important to know that although LMO2 disrupts the normal functioning of T-cell development, T-ALL takes place only

after a long latency period (2-6 years) (70). This is because these mutations are not sufficient on their own to cause leukaemia. Inactivation of tumour suppressor genes, activation of other proto-oncogenes or disruption of the Notch pathway are extra mutations that need to happen in order to cause T-ALL(64).

Characterization of LMO4

Lmo4 was the most recently characterized family member and was identified as an auto-antigen in breast cancer patients (71) were its overexpression induced mammary hyperplasia in transgenic mice (72). The exact pathway by which Lmo4 causes tumour induction is still unknown, but a number of potential roads have been identified in different types of cancers. One of these, is its involvement in the cell cycle progress. Montañez-Wiscovich *et al.,* (2010) demonstrated that Lmo4 knock-down in all murine breast cancer subtypes resulted in G2/M arrest in the cell cycle (73), as well as increased cell death, amplification of centromeres and faults in spindle formation. Lmo4 knock-down also causes exencephaly in mice due to defective neuronal tube closure. Cranial nerve abnormalities and skeletal defects were also observed (74).This suggests that Lmo4 expression levels have to be highly controlled for keeping the normal balance of cells.

Despite its presence in many human tissues such as the brain, skin, pancreas and the oral cavity (75), Lmo4 was also detected in mice thymocytes and more specifically in the DN and DP immature T cells (76, 77). Similar to Lmo2, Lmo4 is also a binding partner of Ldb1 in T-cells (78). Lmo4 exists in a complex comprising the tumour suppressor gene BRCA1 and CtIP in breast epithelial mice cells (77). Lmo4 inactivates the transcriptional activation of BRCA1, promoting cancerous cell growth in breast tissues (79). Moreover, Grutz *et al.,* (1998) hypothesised that the enforced expression of LMO1 and LMO2 would displace the interaction of LMO4 in a complex comprising Ldb1 and thus influence the normal T cell differentiation programme (75).However, the most intriguing finding was that of Matthews *et al.,* (2008) ten years later where the binding affinities of LMO2 and LMO4 for Ldb1 were

measured by developing a competition ELISA strategy (78). The dissociation constants of binding of Ldb1 for the LIM domains of LMO4 and LMO2 demonstrated that LMO4 had a higher affinity for Ldb1 (K_d=10nM) than LMO2 (K_d=20nM) (78). Previous work within the Hoogenkamp lab was performed on mouse ES cell lines, using a shRNA construct, which indicated that the knockdown of *Lmo4* lead to a loss of cells, at least in part through increased apoptosis. Our hypothesis is that LMO4 is present at the very early T-cell developmental stage (ETP) in a complex comprising Ldb1. Under normal expression levels, LMO4 would allow ETP cells to differentiate and keep their cell number constant. However, mutations occurring in the T-cell progenitor stage would disrupt the transcriptional network, upregulating transcription factors associated with stem cell/early progenitor properties, including LMO4, thereby preventing the normal T-cell differentiation process. Finally, accumulation of secondary mutations would induce T-ALL by complementing the increased self-renewal with a proliferative and survival advantage.

To further understand the role of LMO4 in human T-ALL, we performed a number of experiments on four immature T cell lines. We have characterized and measured its relative mRNA expression level by doing quantitative polymerase chain reaction (q-PCR). Western Blot analysis using an anti-LMO4 antibody was also performed on the T-cell protein extracts, in order to confirm that the LMO4 protein is expressed. We also performed immunoprecipitation of Ldb1, followed by mass spectrometry in order to validate and identify novel interacting partners that could be part of the DNA-binding complex. Western blots were performed on already known interacting partners such as Ldb1, TAL1, GATA-2, GATA-3 and on a new LMO2 target, Hhex. Finally, we decided to knock-down *LMO4* in order to assess the viability and growth of the transfected T cells.

This project is one of the first to focus on the role of the LMO4 protein in human T-ALL. Identification of novel interacting partners in the transcriptional complex suggests that there are still many interacting processes that have not yet been characterized. Moreover, knocking-down the LMO4

protein will help us to ascertain if LMO4 is fundamental to the T-cell development and whether it might hopefully be used as a future therapeutic target.

MATERIALS AND METHODS

Cell culture

T-ALL cell lines were grown in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% Fetal bovine serum, 2mM Glutamax (GIBCO), 5 U/ml Penicillin/Streptomycin and 0.075mM MTG. The cell lines were grown in a humidified incubator at 37°C and 5% CO_2. Molt-4 cells were kept at a cell density between 0.4 - 2 million/ml and the other cell lines between 0.2 - 2 million/ml. Human lymphoid progenitor cell lines ARR and DU.528 were kindly provided by Dr. A.W. Langerak, Erasmus Medical Centre, Rotterdam, The Netherlands.

RNA isolation and cDNA synthesis

RNA was isolated from the cells using an RNeasy Mini Kit (QIAGEN) according to the enclosed protocol. RNA concentration and quality was determined on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). For cDNA synthesis, 2µg RNA was denatured at 70°C for 10minutes in the presence of 0.1mM Oligo$(dT)_{20}$ and cDNA was synthesized using 200U SuperScript II Reverse Transcriptase, 1mM dNTPs in 1X first-strand buffer in a total volume of 20ul at 40°C for 30minutes (Life Technologies).

Quantitative PCR

Quantitative PCR (qPCR) was performed using cDNA in order to assess the expression of GAPDH, LMO1, LMO2, LMO4 and Tal1.The forward and reverse primers that were used are listed in Table 2.1. qPCR reactions were composed of 5µl 2X SYBR Green master mix (Life technologies), 0.25µl forward and reverse primers (0.25µM final concentration each), 2.5µl of 10X diluted cDNA in H_2O and 2.25µl H_2O. A standard curve was produced for each set of primers and each time they were used, using serial dilutions of cDNA. Primers for the *GAPDH* gene were used for normalisation. Reactions were loaded on 96 well plates and the

qPCR was run on a ABI 7500 Real-Time PCR System. Calculations were carried out using the standard curve, and normalised to the GAPDH expression level.

Table 2.1. Forward and reverse primers.

Name of primer	Sequence
Forward hLMO2 cDNA	5'-ATGGAAGGGAGCGCGGTGA-3'
Reverse hLMO2 cDNA	5'-CTATATCATCCCATTGATCTTAG-3'
Forward hLMO4 cDNA	5'-ATGGTGAATCCGGGCAGCAG-3'
Reverse hLMO4 cDNA	5'-TTAGCAGACCTTCTGGTCTGG-3'
Forward TAL1 cDNA	5'-GTTCTTTGGGGAGCCGGATG-3'
Reverse TAL1 cDNA	5'-TGAAGATACGCCGCACAACT-3'
Forward Si hCTRL RNA	5'-GUGCAUUGCUAGUACCAAC[dT][dT][Cyanine5]-3'
Reverse Si hCTRL RNA	5'-GUUGGUACUAGCAAUGCAC[dT][dT]-3'
Forward Si hLMO4 RNA	5'-GCAUGAUCCUUUGCAGAAA[dT][dT][Cyanine5]-3'
Reverse Si hLMO4 RNA	5 '-UUUCUGCAAAGGAUCAUGC[dT][dT]-3'

Whole cell and nuclear extract preparation

Crude nuclear extracts were prepared by lysing 10^7 cells in 1ml Hypotonic cell lysis buffer (10mM HEPES pH7.6, 10mM KCl, 1.5mM Mg_2Cl) on ice for 15minutes. Nuclei were pelleted by centrifuging at 16000g for 30 seconds. Thereafter, nuclei were lysed in 50µl Hypertonic buffer (20mM HEPES pH7.6, 420mM NaCl, 1.5mM MgCl, 0.2mM EDTA, 0.5% NP40, 20% Glycerol) for 20minutes on ice and centrifuged at maximum speed for 2minutes. Finally, the supernatants were collected and diluted 1.8-fold with No-Salt buffer (20mM HEPES pH7.6, 1.5mM Mg2Cl, 0.2mM EDTA, 0.5% NP40, 20% Glycerol) and either used immediately or stored at -20°C. In order to prevent protease activity, 1:1000 readymade protease inhibitor cocktail (PIC) (Roche) was added together with Phenylmethylsulfonyl fluoride (PMSF) (Sigma) in a final concentration of 10ng/ml at all isolation steps. Protein extracts were assayed to determine the protein concentration, using a BCA Protein Assay Kit (Pierce Biotechnology).

Western blot analysis

Nuclear extracts (75µg) were separated on 4-12% gradient Bis-Tris Plus Bolt Mini Gels (Novex by Life Technologies) at 165V for 40minutes. The gel was transferred to nitrocellulose, using gel transfer stacks (Life Technologies), for 330 seconds and the membrane was blocked in 5% skimmed milk (Marvel) for 30 minutes. The membrane was incubated overnight with primary antibody, and for 1hour with the appropriate secondary antibody. The membrane was washed in excess PBS between each incubation step and just before the exposure. The antibodies used were: TAL1 (sc-12984X), GATA-2 (sc-9008), GATA-3 (sc-9009) from Santa Cruz Biotechnology; LMO4 (ab131030), Ldb1 (ab96799), Hhex (ab34222) from Abcam; LMO2 (AF2726, R&D systems) and secondary antibodies IRDye 680RD or 800RD Donkey anti-Rabbit IgG, IRDye 680RD or 800RD Donkey anti-Mouse IgG and IRDye 680RD Donkey anti-Goat IgG. Membranes were exposed and fluorescence was detected on a Odyssey CLx. Infrared imaging system (Li-Cor).

Protein pull down assays and mass spectrometry

Immunoprecipitation

60µl Protein G coated Dynabeads (Life Technologies) were first washed twice with PBS-0.03% tween, then with BSA/PBS-0.03% tween and mixed with 4µg anti-Ldb1 or rabbit IgG antibodies and rotated for 30minutes at 4°C. 600µg of the ARR and DU.528 nuclear extracts were incubated with beads for 2hours at 4°C. Beads were washed 3 times between each incubation step in 10 volumes (600µl) of beads wash buffer (20mM HEPES pH7.6, 150mM NaCl, 1.5mM MgCl, 0.2mM EDTA, 0.5% NP40, 20% Glycerol and 1:1000 PIC and PMSF). Pulled-down proteins were resuspended in 40µl 4X LDS sample loading buffer (Novex) and 5µl 10X sample reducing agent (Novex) and boiled at 95°C for 5minutes. Successful immunopreciptiation was confirmed by LMO2 western blot analysis.

Mass spectrometry

For mass spectrometry analysis, the immunoprecipitates were separated on 4-12% Bis-Tris Plus Bolt Mini Gels, on 165V for 40minutes, stained with coomassie blue staining dye for 2 hours and destained with 1% acetic acid overnight. Each lane was cut into approximately 16 small slices. Each slice was placed into low binding-polypropylene tubes and washed with 500µl of 50% acetonitrile, 50mM ammonium bicarbonate for 30minutes, followed by incubation in 250µl of 100mM iodoacetamide in 50% acetonitrile, 50mM ammonium bicarbonate at room temperature and in the dark for 30minutes. Finally, gel slices were washed with 500µl of 10% acetonitrile, 50mM ammonium bicarbonate for 30minutes at room temperature with agitation. Organic solvent was evaporated in a vacuum centrifuge for 2 hours or till dry. Trypsin protein digestion to peptides was achieved with 20µg of trypsin (Promega) which was dissolved in 500µl of 10% acetonitrile, 50mM ammonium bicarbonate and desiccated acrylamide-gel slice were incubated in 20µl trypsin solution over night at room temperature. Peptides were extracted in 3 steps; the first incubation in 100µl of 1% formic acid in 10% acetonitrile for 1 hour, the second incubation in 100µl of 2% formic in 60% acetonitrile, and the third incubation again in 100µl of 1% formic acid in 10% acetonitrile for 1 hour. Extracted volumes were pooled and peptides were lyophilised in a vacuum centrifuge until dry. Finally, the pellets were reconstituted in 20µl 1% formic acid. Samples were sent for analysis at the mass spectrometry facility of the School of Cancer Sciences, University of Birmingham and run on a Bruker Impact ESI-TOF machine with the running software Otof control/hystar.

siRNA electroporation

Cells were spun down at 300g and resuspended at 10^7 cells per 700µl of PBS. 300nM of the control siRNA (siCtrl) or hLMO4 siRNA (siLMO4) were added to cuvettes, followed by 700µl of the cells and kept on ice. Electroporation was performed at 350V for 15ms, unless stated otherwise, on a Pischer EPI 3500 electroporator. Immediately after electroporation cells were transferred into flasks with 12ml culture medium to recover and after an hour cells were gently pelleted and resuspended in fresh medium to remove

free siRNA. The next day the cells were analysed by flow cytometry or single-cell sorted on a MoFlo Fluorescence-activated cell sorting (FACS) machine. siRNAs were tagged with a fluorescent Cyanine(Cy5) group at the 3' end of the upper strand, which gives emission in the far red spectrum and can be detected by flow cytometry as for the detection of APC (650nm).This facilitates the analysis of transfection efficiency and can be used to specifically FACS sort the transfected cells. siRNA sequences are shown in Table 2.1.

Flow cytometry

Cells were analysed by flow cytometry to check their differentiation stage for expression of cell surface receptor with an APC-conjugated antibody specific for human CD3 (17-0036-41, eBioscience). Samples were washed with PBS and then incubated in 100µl of 0.25ng/µl hCD3 antibody in PBS, 0.5% BSA, 0.2mM EDTA buffer for 20-30minutes on ice. Samples were then washed from the antibody with PBS, 0.5% BSA, 0.2mM EDTA and resuspended in 500µl of the same buffer. Analysis on a CyanADP (DakoCytomation-Beckman Coulter) machine was performed afterwards. As a gating strategy, live cells were selected on basis of the forward and side scatter profile, followed by gating on single cells using the pulse width plot.

Analysis of siRNA transfected cells was similarly performed by spinning down a small volume of cells at 1100g for 3 minutes. Cells were resuspended in 1ml of PBS, 0.5% BSA, 0.2mM EDTA buffer and analysed on a CyanADP machine, gating live single cells, detecting Cy5 using the APC laser/filter settings.

Single-cell sorting

Three 96 well plates were prepared for each siCtrl or siLMO4 triplicate transfection. To each well 300µl of culture medium was added and Cy5 positive single ARR cells were sorted into each well by a MoFlo High-Speed Cell Sorter (Beckman Coulter). As a control for sorting efficiency and the effect of cell sorting on cell viability, a plate of untransfected ARR was also prepared.

RESULTS

LMO4 was recently identified as part of an expression signature characterising a subgroup of paediatric T-ALL, namely T-ALL with an immature phenotype (51). As LMO4 is a known oncogene in other tissues, such as breast cancer (76), we wished to gain insight in the involvement of LMO4 in childhood T-ALL with an immature phenotype and identify its interaction partners. To this end we first had to identify cell lines that would best suit to our criteria. As previously mentioned, ETP/DN T-ALL cell lines do not possess TCRs or CD3 chains and have distinct cell surface markers such as CD1a-,CD5 ^{low} ,CD8- and one or more myeloid or stem cell-associated markers. The more differentiated T-ALL cell lines that have entered further into the recombination process start expressing CD1a,CD3 and CD5.

T-ALL cell lines

In 2007, Sandberg *et al.* characterized 30 T-cell lines, amongst which 23 human T-ALL cell lines (Table 3.1), performing immunophenotypical analyses, which was very helpful for the set-up of our experiments (80). At the top of the list in Table 3.1, ARR and DU.528 are the least differentiated T-ALL cell lines. We were kindly provided with these two cell lines by the corresponding author of the publication, Dr. A.W. Langerak, Erasmus Medical Centre, Rotterdam,The Netherlands. We decided to analyse the Molt-4 (DP) and Jurkat (SP) T-ALL cell lines as well, to compare the different genes, markers and proteins of interest between the early and later T-ALL developmental stages.

Cell line	T-cell malignancy of origin	CD7	CD5	CD1[a]	CyCD3	CD3	TCR	CD4[b]	CD8	TdT	CD2
ARR	CD3- T-ALL	+	–	–	ND	–	–	–	–	ND	–
DU.528	CD3- T-ALL	+	ND	–	ND	–	–	–	–	–	–
H-SB2	CD3- T-ALL	+	+	–	+	–	–	–	–	–	–
RPMI 8402	CD3- T-ALL	+	+	–	+	–	–	–	–	+	+
CML-T1	CD3- T-ALL	+	+	–	ND	–	–	+	+	–	–
Karpas 45	CD3- T-ALL	+	+	–	+	–	–	+	+	+	+
KE-37/SKW-3	CD3- T-ALL	+	+	+	+	–	–	+	–	ND	+
SUP-T1	CD3- T-ALL	+	+	+	ND	–	–	+	+	+	–
SUP-T3	CD3- T-ALL	+	+	+	ND	–	–	+	+	+	+
MOLT 3/4	CD3- T-ALL	+	+	+	+	–	–	+	+	+	+
P12-Ichikawa	CD3- T-ALL	+	+	+	+	–	–	+	–	+	+
PF-382	CD3- T-ALL	+	+	+	ND	–	–	+	+	–	+
CCRF-CEM	TCR$\alpha\beta$+ T-ALL	+	+	+	+	+	$\alpha\beta$	+	–	+	–
HPB-ALL	TCR$\alpha\beta$+ T-ALL	+	+	+	+	+	$\alpha\beta$	+	+	+	+
JURKAT	TCR$\alpha\beta$+ T-ALL	+	+	+	+	+	$\alpha\beta$	+	–	+	+
K-T1	TCR$\alpha\beta$+ T-ALL	+	+	+	ND	+	$\alpha\beta$	–	–	+	+
TALL-1	TCR$\alpha\beta$+ T-ALL	+	+	+	+	+	$\alpha\beta$	+	+	+	+
MOLT 16/17	TCR$\alpha\beta$+ T-ALL	+	+	–	+	+	$\alpha\beta$	–	–	+	+
TALL-104	TCR$\alpha\beta$+ T-ALL	+	ND	–	ND	+	$\alpha\beta$	–	+	ND	+
DND-41	TCR$\beta\delta$+ T-ALL	+	+	+	+	+	$\beta\delta$	+	–	+	+
Loucy	TCR$\gamma\delta$+ T-ALL	+	+	–	ND	+	$\gamma\delta$	–	–	–	–
MOLT 13	TCR$\gamma\delta$+ T-ALL	+	+	–	+	+	$\gamma\delta$	–	–	+	–
Peer/Be13	TCR$\gamma\delta$+ T-ALL	+	+	–	+	+	$\gamma\delta$	+	+	+	–
HUT 78/H9	CTCL (SS)	+	+	–	+	+	$\alpha\beta$	+	–	–	–
HUT 102	CTCL (MF)	–	+	–	–	–	–	+	–	–	+
MT-1	ATLL	–	+	–	ND	–	–	–	–	–	–
DEL[c]	ALCL	–	–	–	ND	–	ND	–	–	ND	–
JB6[c]	ALCL	+	–	ND	ND	–	–	ND	ND	ND	+
Karpas 299[c]	ALCL	–	+	ND	ND	–	–	+	–	ND	–
SU-DHL1[c]	ALCL	–	+	–	ND	–	–	–	–	–	–

Abbreviations: ALCL, anaplastic large cell lymphoma; ATLL, adult T-cell lymphoma leukemia; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; SS, Sézary syndrome; T-ALL, T-cell acute lymphoblastic leukemia; ND, not done. Tint is used to emphasize the expression pattern of the tested leukocyte markers.
[a]Clone T6.
[b]Clone Leu 3a.
[c]Cell lines express CD30.

Table 3.1. Shown are 30 human T-cell lines, of which the first 23 represent T-ALL cells. At the top of the list, ARR and DU.528 are the least differentiated T-ALL cell lines, as indicated by their surface marker expression profile. Further down Molt-4 and Jurkat cell lines are indicated. This table was taken from Sandberg *et al.,* (2007).

CD3 expression on T-ALL cells

In order to confirm the developmental hierarchy between the four cell lines, cells were incubated with an APC-conjugated antibody recognising the human CD3ε subunit. This was followed by flow cytometry analysis. Unstained controls were run for every cell line and the unstained Jurkat is shown (Figure 2.1). After gating on the live single cells, the results showed that the ARR and DU.528 cell lines did not express the CD3 marker on their cell surface, as shown by the absence of any APC staining. Molt-4 and Jurkat were both positive for CD3 with Jurkat showing clearly higher fluorescence expression levels.

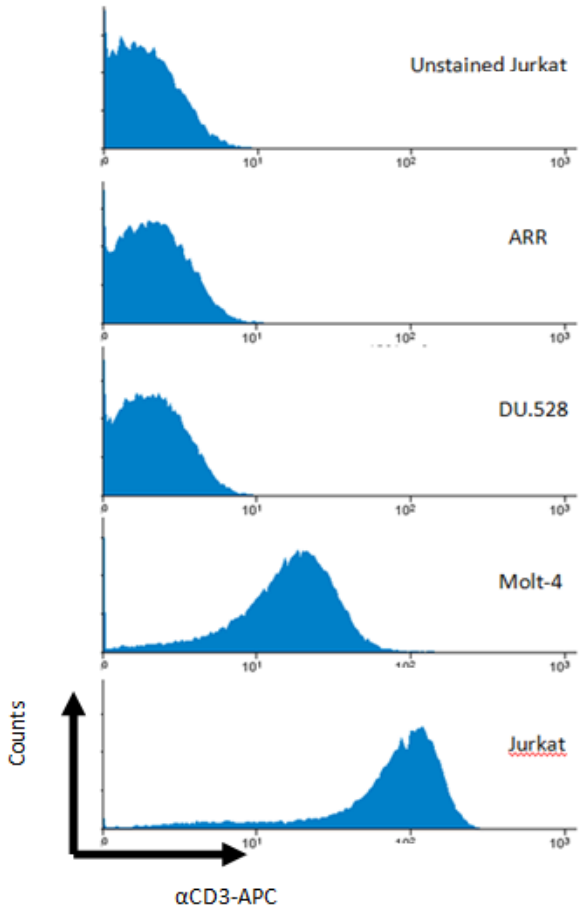


Figure 2.1.Flow cytometry analysis of CD3 expression. Unstained controls and CD3 stained samples were run for the ARR, DU.528, Molt-4 and Jurkat cell lines in duplicate. The unstained Jurkat (control) as well as the CD3 stained cells are shown. After gating on live-single cells, the graphs show the APC fluorescence on the x-axis and the numbers of events (cell counts) on the y-axis. αCD3 stained ARR and DU.528 cell lines graphs are similar to unstained Jurkat, indicating that these cells do not express CD3 on their cell surface. Molt-4 and Jurkat graphs show an increase in fluorescence emission, indicating CD3 surface expression.

Exponential growth of the cell lines

In order to properly culture the different cell lines, we examined the growth characteristics of each of the four cell lines. We aimed to maintain their density relatively low and subculture them at the right time. Therefore the cells were maintained at densities between 0.2 to 2.0 x10^6/ml and subcultured every two or three days. At regular intervals a small amount of culture medium was removed, the cells were counted, and the resulting numbers were plotted to perform a growth curve analysis.

Starting with an initial concentration of 0.4x10^6/ml cells, all cell lines grew exponentially. The ARR cell line was the fastest growing of the four, following a daily growth rate of 2.06 ($y = e^{0.7233x}$), which translates into a doubling time of 23.3 hours. Next was Jurkat, with a daily growth rate of 1.81 ($y = e^{0.5951x}$), doubling in 26.5 hours , followed by DU.528 at a daily growth rate of 1.7 ($y = e^{0.5337x}$), doubling in 28 hours and finally Molt-4, which doubled in 29.8 hours having a daily growth rate of 1.6 ($y = e^{0.4754x}$). Further observations showed that Molt-4 did not grow as well when its density was clearly below 0.4x10^6/ml, whereas the other three cell lines grew equally well at 0.2x10^6/ml.

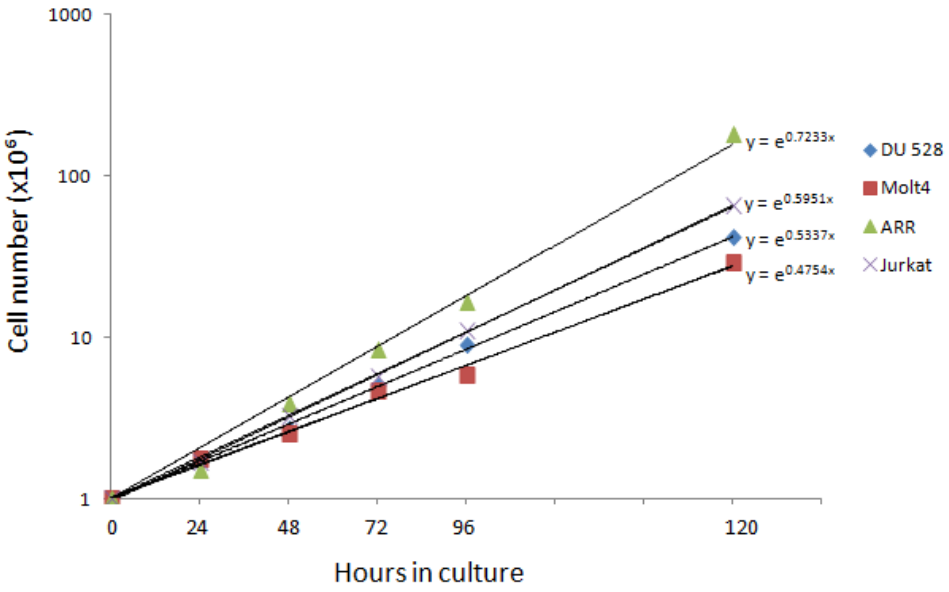


Figure 2.2. Exponential growth of the T-ALL cell lines. Starting from an initial concentration of 0.4x10^6/ml, each cell line was counted every 24hours. The graph shows the cell counts on the y-axis and the hours in culture on the x-axis. An exponential growth curve was fitted through the data points and the formula was used to calculate the doubling times.

Being aware of the doubling times of each cell line enabled us to grow the cells at an appropriate density and at the right time to isolate their RNA, or their protein extracts. Cells were kept growing in their exponential phase and usually harvested for RNA or protein isolation when these were at a density of around 0.8-1.5x10^6/ml.

Relative mRNA and protein expression

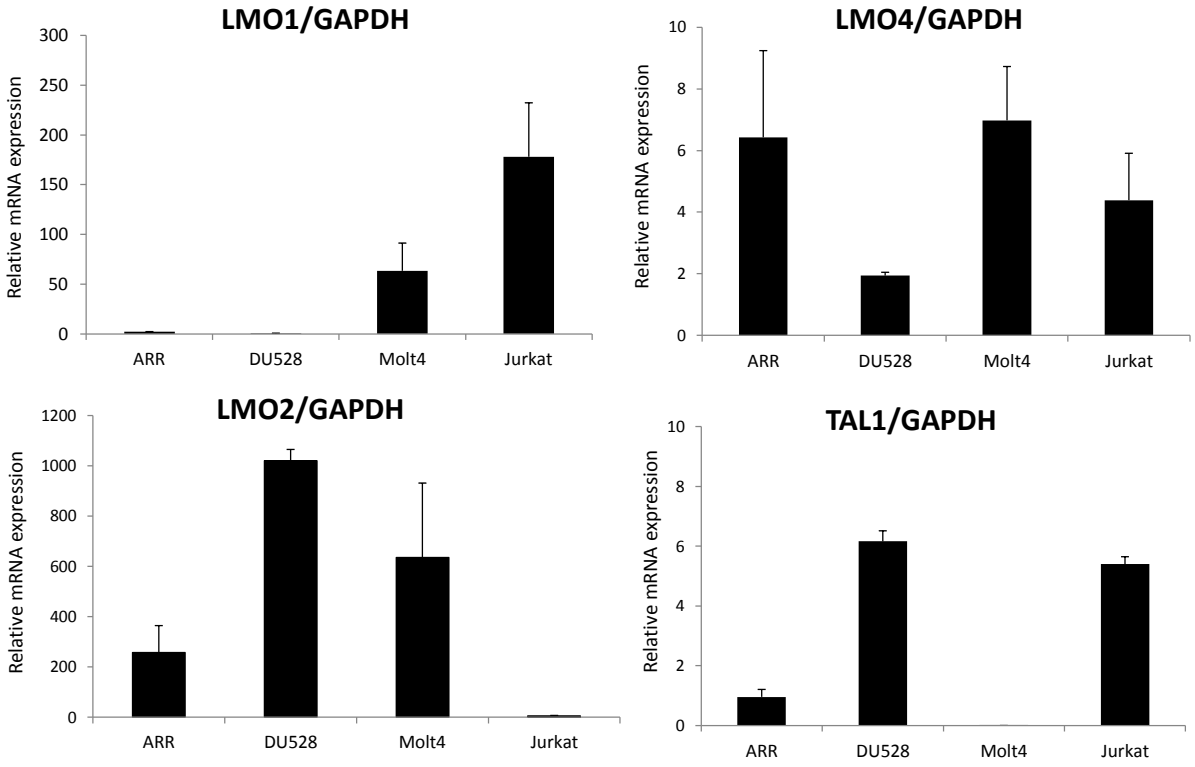
The quantitative PCR data for *LMO1* showed that it was expressed at high levels in Jurkat, to a lesser extent in Molt-4 and was absent in ARR and DU.528 (Figure 2.3A). These data are in line with published data on *LMO1* mRNA expression levels in Molt-4 and Jurkat (81). *LMO2* mRNA and protein expression was undetectable in the Jurkat cell line, which was expected as high LMO1 and LMO2 seem usually mutually exclusive (Figure 2.3A). In the remaining three cell lines, Western blot analysis showed that LMO2 was more abundant in the DU.528 and Molt-4 cell lines than in ARR. A similar protein expression pattern was observed for Ldb1, showing a low, but detectable, level in Jurkat, a higher level in ARR and more in DU.528 and Molt-4 (Figure 2.3B).

LMO4 protein expression levels followed an anti-correlation with the differentiation stages of the cell lines, as it was more abundant in ARR and DU.528 than in Molt-4 and further reduced in Jurkat cells (Figure 2.3B). Interestingly, the protein expression pattern was not fully concurrent with its mRNA levels as it had clearly lower mRNA levels in DU.528 (Figure 2.3A). This indicates that there is a level of post-transcriptional regulation taking place. Similar to LMO4, also the protein expression of Hhex and GATA-2 followed a decrease over the four cell lines representing increasing differentiation stages. This was within expectation, as these factors are known to be associated with a haematopoietic stem cell / early progenitor phenotype and not the committed T-cell phenotype.

An intriguing result was observed for TAL1 expression. Previous studies showed that the DU.528 cell line carried a SIL-TAL1 translocation, which normally causes high TAL1 expression. As expected, *TAL1* mRNA is most highly expressed in DU.528 and Jurkat cell lines, less in ARR and absent in Molt-4 (Figure 2.3A). Surprisingly, TAL1 Western blots show that it is only present at detectable levels in ARR and Jurkat cells. These data show that although the mRNA expression of a gene is high, this not always automatically means that protein levels are high as well.

Finally, GATA-3 shows a similar expression profile to TAL1 as it is present only in ARR and Jurkat cells (Figure 2.3B). This could suggest that both proteins are present within the same complex.

A)



B)

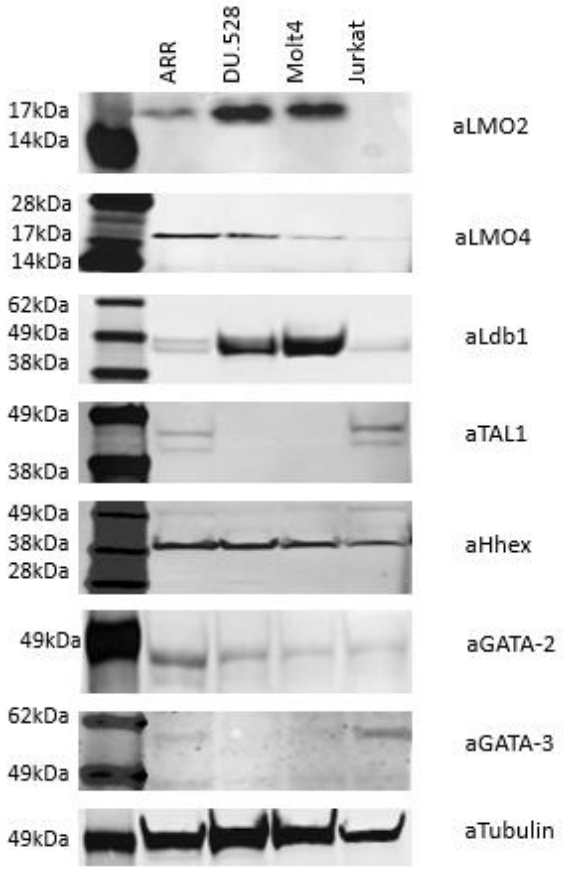


Figure 2.3. Relative mRNA and protein expression in the four T-ALL cell lines. Figure A) shows the relative mRNA expression data of *LMO1, LMO2, LMO4* and *TAL1* as a ratio to *GAPDH* mRNA expression. For each cell line biological triplicates of cDNAs were synthesized, each measured in duplicate. Figure B) shows the protein levels of LMO2 (17kDa) and LMO4 (18kDa), as well as that of some of their potential interaction partners in the four T-ALL cell lines.Ldb1 (49kDa), TAL1(42kDa), Hhex(42kDa),GATA-2(50kDa),GATA-3(50kDa). Equal amounts (75µg) of the four nuclear extracts were loaded on the gel which is confirmed by the tubulin (49kDa) expression. The qPCR and Western Blot experiments were both performed at least twice.

Ldb1 pull-down and mass spectrometry analysis

The mRNA and protein expression level experiments presented in figure 2.3 showed to which extent the three LMO proteins are expressed, as well as the levels of several proteins generally associated with them. However, transcription factor complexes are composed of many proteins and by performing protein pull downs followed by mass spectrometry analysis, we aimed to identify more proteins, including those unknown to be interacting partners. By performing a αLdb1 immunoprecipitation we wanted to see whether this protein indeed interacts in the early human T-

ALL cell complex of the cell lines and whether known and novel interacting proteins could be found. We first sought to control if LMO2 was indeed interacting with Ldb1 and decided to run the pulled down proteins on a 4-12% Bis-Tris Plus Bolt Mini gel. The gel was then transferred to a nitrocellulose membrane, separated into two parts and incubated either with an Ldb1 or LMO2 antibody (Figure 2.4A). We showed that LMO2 was present in the Ldb1 pulled down proteins, thus interacting together.

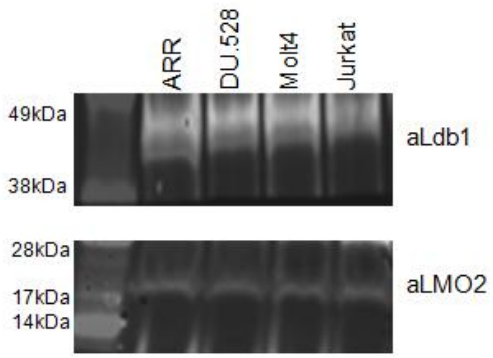
We then performed the same immunoprecipitation using an αLdb1 antibody or IgG as a control on the ARR and DU.528 nuclear extracts and the pulled down proteins were run on a 4-12% Bis-Tris Plus Bolt Mini gel. The gel was stained with coomassie blue dye, staining all the pulled down proteins (Figure 2.4B). This facilitated the cutting of each of the lanes into 16 individual gel slices. These were then processed as described in the Material and Methods section, trypsin digested and analysed on a mass spectrometer.

The results revealed a number of already known proteins involved in the complex and proteins that have still not well defined roles in it (Appendix 1). The generation of Venn Diagrams helped us to better see the number of unique proteins identified in this pull down assay (Figure 2.4C). The ARR Venn diagram shows 71 unique proteins that supposedly interact with Ldb1 and only 20 common proteins between the Ldb1 and IgG immunoprecipitates. The DU.528 Venn diagram identifies 38 unique proteins that could interact with Ldb1 and 93 proteins that are common to Ldb1 and IgG immunoprecipitates.

In order to understand the pathways where all these proteins are involved in, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) networks were generated, using the online database STRING 9.1, and showed all reported interactions between the proteins (Figure 2.4D). Many ribosomal subunits were identified, which show within the network as a dense cluster. These are

likely contaminants from the cytoplasm, as there ribosomal proteins are expressed in abundance. Alternatively, these could be pulled down by Ldb1 during mRNA translation.

A)



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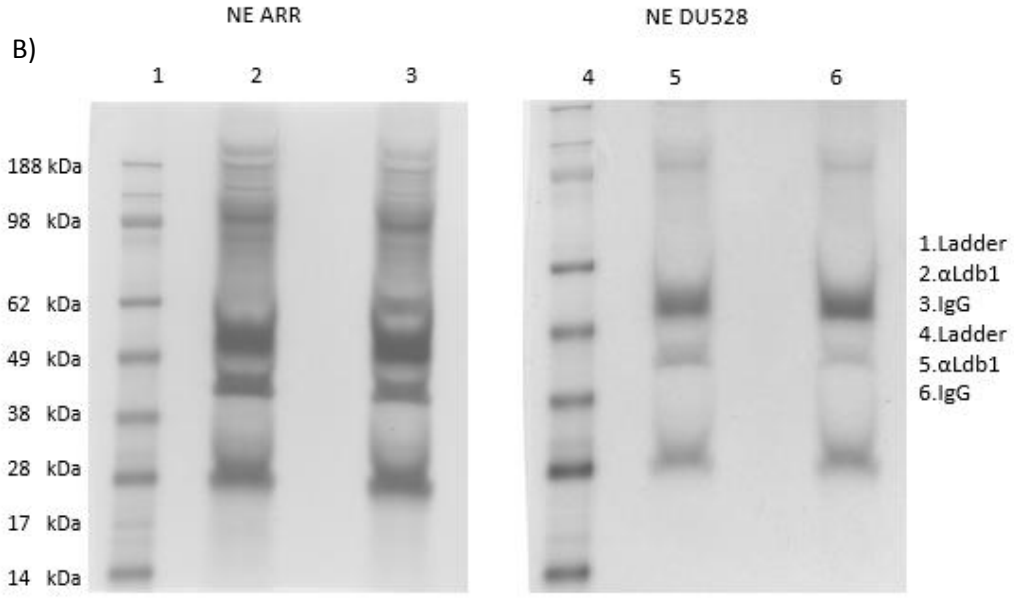
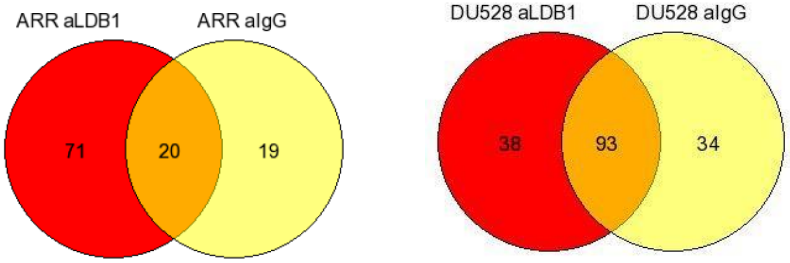
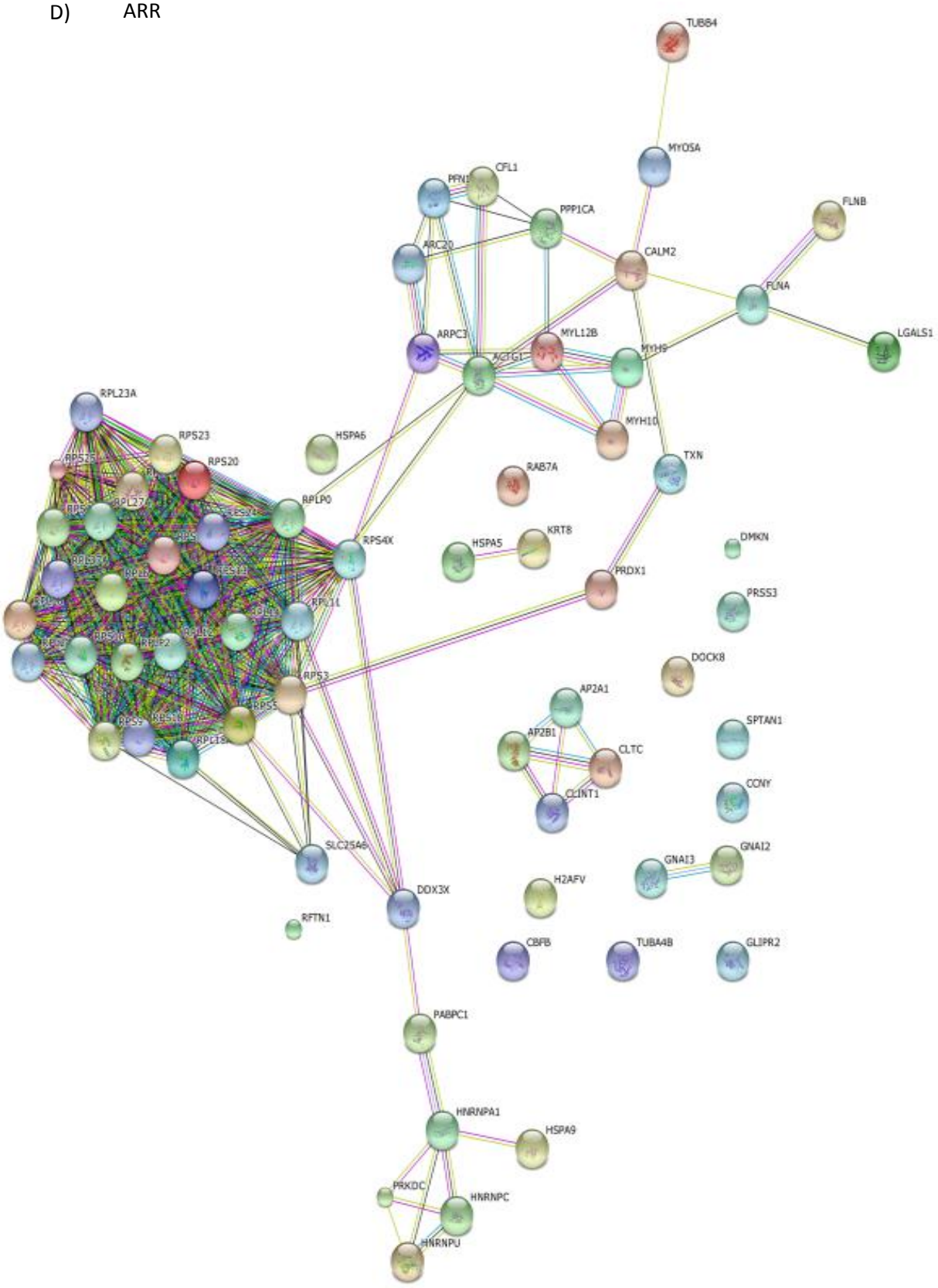


C)



D) ARR


TUBB4
MYO5A
CFL1
PFN1
PPP1CA
FLNB
ARC20
CALM2
FLNA
LGALS1
ARPC3
MYL12B
ACTG1
MYH9
MYH10
RPL23A
RPS23
HSPA6
RPS20
TXN
RAB7A
RPLP0
RPS4X
HSPA5
KRT8
DMKN
PRDX1
PRSS3
RPL11
RPL12
RPLP2
DOCK8
RPS3
AP2A1
SPTAN1
AP2B1
CLTC
CLINT1
CCNY
SLC25A6
GNAI3
GNAI2
H2AFV
DDX3X
RFTN1
CBFB
TUBA4B
GLIPR2
PABPC1
HNRNPA1
HSPA9
PRKDC
HNRNPC
HNRNPU

DU.528

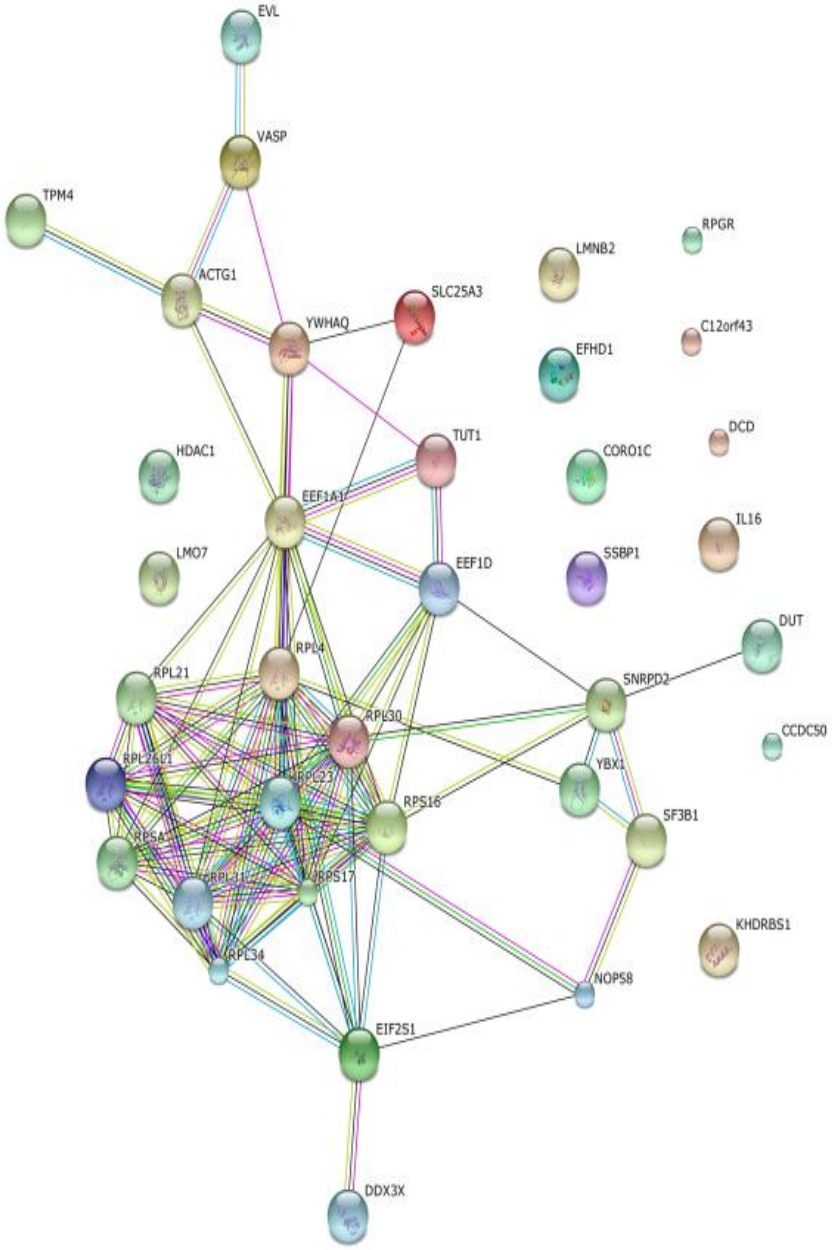


Figure 2.4. A) Immunoprecipitation of Ldb1 shows LMO2-Ldb1 interaction in the four cell lines. After pulling down Ldb1 from the nuclear extracts of the four T-ALL cell lines, the immunoprecipitates were separated on a gel. This was then transferred to a membrane which was afterwards cut and incubated either with Ldb1 or LMO2 antibodies.**Identification of the Ldb1 pulled down proteins.**Figure B) illustrates the Coomassie blue stained Bis-Tris Plus Bolt Mini gels of the αLdb1 pulled down proteins in the ARR and DU.528 cells.Figure C) illustrates the Venn Diagrams generated for ARR and DU.528 which show in orange colour the common proteins between the αLdb1 and IgG immunoprecipitations. The unique pulled down proteins to the cell lines in red and to IgG in yellow. Figure D) shows the String Networks generated for the two cell lines where all reported interactions between the pulled down proteins are identified.

***LMO4* gene silencing in ARR cell line**

To study the role of LMO4 protein in early T-ALL, we decided to silence its gene in the ARR cell line by using double stranded small interfering RNA (siRNA).

Previously, our laboratory silenced *Lmo4* in mouse cells by using a Retrovirus that expressed shRNA sequences from a miR-30 background. The ability and specificity of this shRNA in targeting the *Lmo4* gene was used for the design of the siRNA. In order to target the human *LMO4* gene we first compared the human and mouse LMO4 DNA/RNA sequences. We then identified which nucleotides did not blast the sequence to find out the differences and changed them in order to be an exact match to the human sequence (siLMO4). The oligonucleotides were ordered from Sigma-Aldrich with a Cyanine5 (Cy5) tag at the 3' of the top strand, which is the one that will not bind to the *LMO4* mRNA. This fluorescent label facilitated the determination of transfection efficiency by flow cytometry or FACS. A control siRNA was designed (siCtrl), with no predicted binding to any human mRNA, as a negative control for nonspecific effects of the transfection procedure.

Our first round of transfections was performed by placing 200nM of the siCtrl or siLMO4 into cuvettes with 10^7/ml ARR cells and electroporating at 350V for 10ms. An extra control sample, where the siCtrl was added to cells, but in the absence of electroporation was also generated. After 24h, we analysed their fluorescence signal by flow cytometry. Surprisingly, we observed that all samples were stained with the Cy5 dye, including the non-electroporated sample (data not shown). We concluded from these first results that the siRNA was either binding to the cell membrane or taken up by the cells. Thus, we decided to perform a wash to remove free siRNA from the medium 1h after electroporation, giving the cells time to recover after electroporation.

From further optimisations we concluded that the best voltage and pulse length for transfecting ARR cells was 350v for 15ms. Three hundred nanomolar of the siCtrl or siLMO4 were placed into cuvettes with 10^7 ARR cells and electroporated. The experiment was performed in duplicate and the

electroporated cells were transferred into flasks together with 12ml of medium. Again an extra control sample similar to the siRNA control was also used, which has not been electroporated. One hour later, the medium was replaced and the fluorescence signal was analyzed by flow cytometry.

Analysis of the graphs showed efficient transfection (Fig.2.5A). The shift of the siCtrl and siLMO4 curves to the right according to the population of untransfected ARR cells, indicated that more than half of the cells were having a fluorescence intensity higher than the control. In fact, the appearance that the whole peak had shifted and the absence of a separate negative peak within the samples, indicates that it is likely that all cells were containing the siRNA. Twenty-four hours after transfection, cells lost a high percentage of the Cy5 fluorescence signal; *i.e.* 35% for the control and 25% for the siLMO4 siRNA. We expected this to be due to the cellular degradation of the Cy5 dye. Unfortunately, we did not detect any clear differences between the cells that were transfected with siLMO4 compared to the siCtrl transfected cells. We obtained more cell death than we had expected with the square wave electroporator, which obscured potential effects on cell population scale.

Therefore, we performed another experiment in triplicate, where we sorted the cells by FACS. We decided to sort single cells using a droplet-based microfluid sorter (MoFlo), enabling single-cell sorting into 96-well plates. This technique allowed us to quantify the number of successfully transfected cells that could grow on and see if the *LMO4* gene is important for the survival and proliferation of T-ALL cells.

For each of the triplicates of the siCtrl or siLMO4 transfections we sorted one 96-well plate with single cells. In addition, we sorted one plate of untransfected cells in order to determine the efficiency of single cell sorting and the influence of FACS on cell viability. After approximately one week, all the wells were assessed for the presence of cells. The results, as shown in figure 2.5B, showed that 76 out of 96 wells contained living cells for the untransfected cells. The three plates

containing siCtrl electroporated samples had on average 41 wells with living cells and those with the siLMO4 47 wells with living cells.

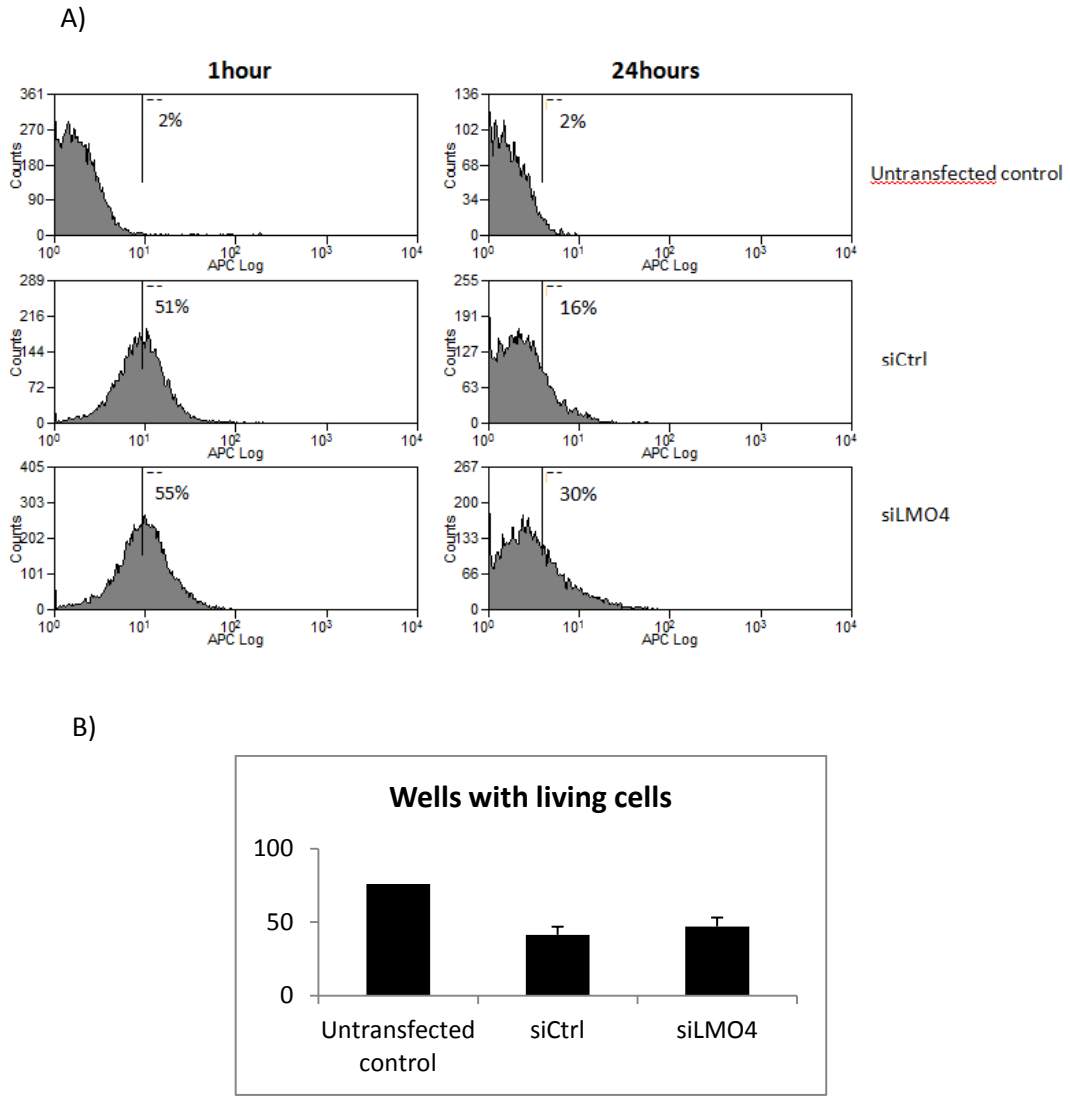


Figure 2.5. *LMO4* gene silencing in the ARR cell line. A) Shows the siRNA mediated knockdown of *LMO4* in the ARR cell line. ARR cells were electroporated either with a siCtrl or a siLMO4 at 350V for 10ms. Untransfected control cells did have siRNA added to the medium, but were not electroporated. One hour after their electroporation, the medium was changed and cells were analyzed on a flow cytometry machine. The same protocol was repeated 24 hours later after electroporation. After gating the live, single cells, graphs were plotted, showing the number of cell counts on the y-axis and the intensity of APC fluorescence on the x-axis. B) Shows the single-cell sorting of ARR cells. siCtrl and siLMO4 were transfected in triplicate and 16 hours later each culture was single cell sorted into 96 well plates. Five days later, the number of wells containing living cells were counted. A single untransfected control was also included in the experiment.

These results indicate that the *LMO4* gene is not easily silenced when siRNAs are introduced into ARR cells. A reason would be that the Cy5 fluorescent dye is degraded by the cells and that the transfection approaches are not as efficient as we would expect. Thus, *LMO4* knock down should be tested by other approaches.

DISCUSSION

Definitive haematopoiesis is a process that involves HSCs which can give rise to all blood lineages of the adult organism (1). T lymphocytes are derived from these HSCs through a series of differentiation stages, becoming functional and protecting the organism from harmful antigens. During differentiation, ETPs are subjected to multiple cellular and molecular changes, switching off non-lymphoid genes and procuring a functional T-cell phenotype. The DN3 stage is fundamental for T-cell fate as it is at this point where immature cells stop proliferating and enter the process of V(D) J recombination. Successful recombination will allow cells to express a functional TCR, important for recognizing multiple cells and antigens. However, when the recombination system gets deregulated, translocations occur which together with other mutations lead to malignant clonal expansion of immature T-cells called T-ALL (46).

Our research mainly focused on a phenotypically immature T-ALL subtype, where it has been hypothesized that ETPs are oncogenically transformed and respond poorly to lymphoid cell-directed chemotherapy (46). Analysis of this ETP-ALL subgroup demonstrated that *LMO4* gene was present at relatively high expression levels in this early T-ALL group (49). As LMO4 has a known role in carcinogenesis in several non-haematopoietic tissues and is closely related to LMO1 and LMO2, which are well known for their role in T-ALL, we wanted to find out more about its involvement in T-ALL. In order to enlighten the role of LMO4 in the transcriptional complex of T-ALL, we aimed to characterize it by performing proteomics approaches together with gene knockdown.

Phenotypical analysis of utilised cell lines

The first objective that we wanted to study was to investigate the presence of the LMO1, LMO2, and LMO4 proteins in immature T-ALL cell lines. Identification of human early T-cell progenitor T-ALL cell lines was of major importance as it would help us to set up all our experiments. Previous studies of Sandberg *et al.,* (2007), characterized 23 human T-ALL cell lines by doing immunophenotypical analyses (80). In their list the ARR and DU.528 cell lines had a distinct cell immunophenotype (CD1a-,CD2-,CD3-,CD4-,CD5- ,CD7+,CD8- and TCR-) which correlated with studies done on ETP-ALL (46). As controls we decided to work on Jurkat cells, a T-ALL cell line blocked at a relatively mature stage, with a TCR expression and was SP (CD4+, CD8-). We also wanted to include Molt-4 cells in our experiments as it is a DP (CD4+, CD8+) cell line that does not express a TCR. Together these cell lines offered the opportunity to put our data in the context of developmental stages.

Before starting the experiments, we first wanted to confirm the developmental hierarchy between the four cell lines. Flow cytometry was performed with an APC-conjugated antibody recognising the human CD3ε subunit. We decided to analyze the CD3 expression as it is a defining feature of cells fully committing to the T-cell lineage, together with the TCR. CD3 is composed of four polypeptide chains (ε , γ ,δ, ζ) which together assemble as three pairs of dimmers (εγ,εδ,ζζ) and act as a T-cell co-receptor (82). Thus, if cells do express the CD3 marker, under normal circumstances they will also express the TCR, which means that they are not very immature anymore.

The flow cytometry results showed the relative fluorescence of the CD3 conjugated antibody to APC on a logarithmic scale. Comparing the CD3 stained ARR and DU.528 cell lines to the control, this showed that they did not express the CD3 marker on their cell surface. Molt-4 and Jurkat did both express CD3 with Jurkat showing clearly higher expression levels on the logarithmic scale. These data were in line with a developmental hierarchy of ARR and DU.528 as CD4, CD8 double negative early T-cells, followed by the CD4, CD8 double positive Molt-4 and CD4 single positive Jurkat having the most

mature T-cell phenotype. These results correlated with the published research of Sandberg *et al.,* (2007) (46).

As part of the characterisation of the four cell lines, we wanted to measure their growth rates. Cells were counted regularly during one week. A growth curve analysis was performed showing the doubling time of each cell line. All four cell lines were fast growing with relatively similar doubling times. Faster growth could be an indication of a more aggressive form of leukaemia. In this case, the early progenitor cell line ARR was the fastest growing of the four, following a doubling time of 23.3 hours. However, DU.528 was third out of the four cell lines with 28 hours, which makes it less likely that the immature T-ALL are characterized by particularly fast proliferation as compared to other T-ALL.

Expression of LMO proteins and functionally related transcription factors

T-ALL is a type of blood cancer that can lead to rapid progression and relapse within the first years following diagnosis. During the last decades, much effort was put in characterizing the molecular and genetic alterations that cause leukaemia. As a result, novel genes and molecular mechanisms have been identified in being involved in T-ALL. Although a number of specific translocations have been well studied, many details on the alterations at the molecular level have yet to be unravelled, especially in those cases where the underlying initiating event is not known. The purpose of our work was to gain a better understanding of the genetic and molecular expression of the LMO proteins and interaction partners in immature ETP or T-ALL.

We started our experiments by quantifying the mRNA expression levels of the *LMO1, LMO2, LMO4* and *TAL1* genes in the four cell lines. The qPCR results showed that the *LMO1* relative mRNA level was expressed at high levels in Jurkat, to a lesser extent in Molt-4 and absent in ARR and DU.528. These results correlated with studies by Oram SH *et al.*, (2013), who used these two cell lines and

looked at the regulation of the *LMO1* gene in T-ALL (81). The absence of *LMO1* in the ETP-ALL (ARR and DU.528) lead us to exclude LMO1 as focus of any further experiments. Following this, we found that *LMO2* mRNA and protein expression was not detectable in Jurkat cells, which was expected, as high expression of LMO1 and LMO2 seem to be mutually exclusive. This is likely to be due to their highly similar role in leukaemogenesis, *i.e.* once high expression of either LMO1 or LMO2 is established, there is no advantage for cells to gain high expression of the other (83). Furthermore, the other three cell lines were found to express LMO2 protein, being at higher levels in DU.528 and Molt-4. This shows that the mRNA abundance of *LMO2* is successfully transcribed into a functional protein with a high stability against proteosomal degradation, *e.g.* due to its interaction with TAL1. TAL1-LMO2 interaction links protein stabilization with higher order protein complex assembly and thus modulates its post-transcriptional degradation (84), which is not always the case as it will be explained below.

For the LMO4 protein expression levels, Western blot analysis showed an anti-correlation with the differentiation stages of the cell lines, as it was more abundant in ARR and DU.528 than in Molt-4 and further reduced in Jurkat cells. Interestingly, the mRNA expression levels were not fully correlating with the protein expression pattern as DU.528 cells had clearly lower *LMO4* mRNA levels. A hypothesis would be that the post-transcriptional regulation renders it more stable in DU.528, producing higher amounts of steady state protein levels per mRNA than in the other cell lines.

These results are of high importance as it allowed us to evaluate the LMO protein expression in different developmental stages of human T-ALL cell lines. To begin with, it seems that LMO4 is expressed during all the developmental stages of T-ALL with higher levels in the most immature stages, declining during differentiation. This may represent normal development and can point in the direction that LMO4 is necessary during the ETP stage and that a failure to downregulate LMO4 leads to aberrant differentiation. On the other hand, LMO2 is highly expressed in DU.528 cell line which

carries a *TAL1* gene alteration, as detailed below, which together with secondary hit mutations is sufficient for the T-cell developmental arrest and clonal expansion at immature stages (85, 86). Furthermore, despite that LMO2 is expressed in immature T-ALL cell lines, it is also present in more differentiated stages. This indicates that the exact developmental stage at which leukaemic cells with LMO2 overexpression are blocked is dependent on more parameters than LMO2 alone.

Having studied the LMO transcriptomic and proteomic expression in the cell lines, we decided to perform the same experimental approaches to the proteins that are present in the same transcription complex as LMOs. Ldb1 is a ubiquitously nuclear LIM domain binding protein, which does not bind to DNA but participates in large transcriptional complexes as a co-factor (87). It dimerizes through its N-terminal domain and binds LMO proteins or other LIM homeodomains through its C-terminal LID domain (88). As mentioned before, there are differences in affinities of LMO proteins for Ldb1 (78). Enforced expression of LMO2 as a result of chromosomal translocations was hypothesized to displace LMO4 from Ldb1 causing T-cell developmental arrest (75). This may indeed be the case in DU.528 and Molt-4, as the protein expression levels of Ldb1 are higher in these cell lines, as is LMO2.This also indicates that different components of the same complex have a similar mode of regulation of their expression, possibly through positive feedback mechanisms. We can also predict that Ldb1 interacts more with LMO4 in the ARR cell lines where LMO2 is less abundant. Finally, Ldb1 interacts with LMO1 in Jurkat cells as LMO2 and LMO4 are nearly absent.

TAL1 is another protein of the transcriptional complex which in normal haematopoiesis gets downregulated. TAL1 is a bHLH protein which binds to other E-box factors, such as E2A (E47/E12). E2A and the bHLH component of TAL1 are able to bind the E-box motif found in transcriptional enhancers regulating transcription during haematopoiesis (89). What is more, TAL1 binds to LMO2 and GATA-2 in normal conditions. However, TAL1 gets frequently deregulated exerting its oncogenic

effect by inhibiting the tumour suppressor characteristics of E2A and thus stopping lymphoid cell differentiation (90).

It is also important to mention that 12-25% of T-ALL cases have a submicroscopic 90 kb deletion that fuses the coding sequence of TAL1 to the first exon of the SCL interrupting locus (SIL) gene and results to a SIL/TAL1 fusion gene (91, 92). Although the latter leads to a fusion transcript, there is no SIL-TAL1 fusion protein formation, but only TAL1 deregulated expression (93). The DU.528 cell line has been shown to contain the SIL-TAL fusion (103).Interestingly, our results indicate that TAL1 protein expression is only detectable in the ARR and Jurkat cell lines. Intriguingly, *TAL1* mRNA expression levels are high in DU.528, as would be expected. We therefore conclude that although the DU.528 cell line possesses the SIL-TAL translocation, which leads to high levels of transcription, this is not reflected in high protein levels. This may be due to high levels of protein degradation, or possibly mutations in the coding region causing a frame shift. Published data have identified two ways of degrading TAL1 through the ubiquitin-proteasome pathway. The first pathway involves Notch signalling which requires Skp2, a substrate-binding subunit of SCF ubiquitin ligase complexes which together with CHIP, a protein with a ubiquitin ligase activity degrade TAL1 (94).The second pathway induces TAL1 degradation through TGF-β which activates AKT1, phosphorylates TAL1 and binds to CHIP (95). Importantly, this data shows that the analysis of mRNA expression levels in leukaemia does not always automatically represent protein levels. This indication should be considered in studies on gene expression in T-ALL, or other cells, as protein expression levels have to be considered in order to confirm gene expression levels.

It would be interesting to perform whole cell extract mass spectrometry analysis on patient samples to see whether this improves the link between expression profile clustering and clinical outcome. Another fascinating fact is that in DU.528 cells, the TAL1 translocation was the reason for the leukaemia to develop, but that at a later stage the TAL1 protein was apparently not needed any more

for an unknown reason. It needs to be kept in mind however, that the time point when this occurred, may have been after isolation and establishment of the cell line, rather than within the patient.

An alternative oncogenic factor would be Lyl1, a bHLH transcription factor which also interacts with LMO1 and LMO2 (96). Lyl1 and Tal1 are both important during haematopoiesis but in different devepmental stages. During early haematopoietic development, LMO2 and Tal1 interact in a DNA binding complex that we previously explained. However, gene expression analyses of LMO2 transgenic thymocytes have demonstrated a gene expression profile that is similar to human immature T-ALL cases where LYL1 is overexpressed instead of TAL1 (97). Researchers showed that LMO2 binding to LYL1 induced its promoter activation, which together with the downstream target gene Hhex caused T-ALL growth (98). Thus, it may be that the DU.528 cell line expresses LYL1 instead of TAL1.

As mentioned above, GATA-2 and GATA-3 are also important for lymphoid differentiation. GATA-2 needs to become downregulated at early stages and GATA-3 is needed for T-cell lineage commitment and regulates TCR gene rearrangement (7). To check the expression levels of GATA-2 and GATA-3, Western blot analysis was performed. The results showed that GATA-2 had a similar expression profile to LMO4 protein, showing that it followed its normal downregulation during differentiation. Whether the presence of LMO4 and GATA-2 in ARR cells means they can co-occupy the same transcriptional complex, as is the case for LMO2 and GATA-2, remains to be determined. GATA-3 was clearly detected in the ARR and Jurkat cell lines. As a true T-cell transcription factor this was expected for the Jurkat cell line, but it is interesting that also the early ARR cells already contain relatively high levels of GATA-3.

Finally, identification of the *Haematopoietically-expressed homeobox (Hhex)* gene in ETP-ALL cell lines (99) has raised our curiosity to analyse its protein expression. Hhex is a divergent orphan homeobox gene essential for the embryonic formation of the foregut, the central nervous system, as

well as the endothelial and haematopoietic cells (100).High levels of *Hhex* expression in haematopoietic lymphoid precursor cells inhibit their maturation process disrupting normal T-cell development. Alex George *et al.,* (2003) demonstrated that lower levels of Hhex expression in the same progenitors induced immature T-cell lymphomas in bone marrow recipient mice (101). They also suggested that *Hhex* can act as an oncogene in lymphoid cells and that it is fundamental for progenitor cell contribution to mature blood cell lineages. Recently, Stephen Smith *et al., (*2014) identified ETP-ALL oncogenes that are upregulated such as *Lyl1, Hhex and Mycn* in LMO2 transgenic T-ALL mice (99). They also showed that by knocking down *LMO2* gene, *Hhex* and *Mycn* mRNAs decreased by 40% in ETP-ALL cells, suggesting that *LMO2* induces the expression of *Hhex* to trigger T-ALL. We concluded from these data that Hhex is a transcriptional target of LMO2 which can be involved in the onset of T-ALL by an as yet unidentified mechanism. We performed Western blot analysis to identify the Hhex protein and found its expression pattern to be similar to that of LMO4 and GATA-2, *i.e.* protein levels decreased as in the normal T-cell maturation stages. Despite the high levels of LMO1 in Jurkat cells, Hhex levels were relatively low in these cells. It is interesting to speculate whether this means that LMO1 cannot activate the *Hhex* gene in the way LMO2 does. This would be interesting to follow up, as it is currently unclear whether LMO1 and LMO2 are having completely identical functions in the onset and maintenance of T-ALL.

Identification of new interacting partners of Ldb1

In our effort to better understand the transcription factor complexes containing LMO2 or LMO4, we decided to start with a focus on Ldb1. We expect Ldb1 to be an obligate partner for both LMO2 and LMO4 and have an antibody that recognises it efficiently. We performed immunoprecipitations of the Ldb1 protein from the nuclear extracts of the two immature cell lines ARR and DU.528. This was followed by several preparation processes in order to prepare the samples for mass spectrometry analysis. The results identified many proteins, some of which were common to the negative control

and some of which were unique and therefore potentially interacting with Ldb1. To analyse this list further, we only kept the unique pulled down proteins from Ldb1 and generated a “String Network”, which reports all known common interactions between proteins. Many ribosomal subunits were identified, which show within the network as a dense cluster, especially in ARR. These are likely contaminants from the cytoplasm, as these ribosomal proteins are expressed in abundance. Alternatively, these could be pulled down during *Ldb1* mRNA translation. A suggestion to improve the results would be to completely purify nuclei by following a longer and more laborious nuclear extraction protocol.

Despite this, we managed to identify proteins such as the core-binding factor beta subunit (CBFβ) which binds to the core site, 5'-PYGPYGGT-3', of a number of enhancers and promoters, including murine leukaemia virus, polyomavirus enhancer, T-cell receptor enhancers, LCK, IL-3 and GM-CSF promoters in the ARR cell line. CBFβ enhances DNA binding by RUNX1 and is important in inducing T-cell differentiation. The protein kinase DNA-activated catalytic polypeptide (PRKDC) is a Serine/threonine-protein kinase that acts as a molecular sensor for DNA damage. It is involved in DNA non-homologous end joining (NHEJ) required for double-strand break (DSB) repair and V (D) J recombination. PRKDC interacts with the heterogeneous nuclear ribonucleoprotein A1 and C (HNRNPA1, HNRNPC) involved in the packaging of pre-mRNA into hnRNP particles and modulate the stability and level of translation of mRNA molecules. The protein phosphatase 1 catalytic subunit alpha isoform (PPP1CA) is essential for cell division, and participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. It also may play an important role in dephosphorylating substrates. The H2A histone family member V (H2AFV) which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. The heat shock 70kDa protein 9 (HSPA9) is related in the control of cell proliferation and

cellular aging. Lectin galactoside-binding soluble 1(LGALS1) may regulate apoptosis, cell proliferation and cell differentiation.

Concerning the pulled down proteins in DU.528 cell line, more intriguing proteins were identified, such as histone deacetylase 1(HDAC1), which is responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Histone deacetylation gives a tag for epigenetic repression and plays an important role in transcriptional regulation, cell cycle progression and developmental events. The splicing factor 3b, subunit 1 (SF3B1) may be involved in the assembly of the E-complex. The LIM domain 7 (LMO7) protein, which may be involved in protein-protein interaction. The KH domain containing RNA binding signal transduction associated 1(KHDRBS1) which has a role in G2-M progression in the cell cycle. It represses CBP-dependent transcriptional activation apparently by competing with other nuclear factors for binding to CBP. The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide (YWHAQ) is an adapter protein implicated in the regulation of a large spectrum of both general and specialized signalling pathway. We conclude that many interacting proteins are important in the regulation of T-cell maturation such as CBP, the E-complex and possibly LMO7. Identifying LMO7 is very interesting as little is known about its functions and mode of action especially in lymphoid cell lines. A suggestion would be that LMO7 could be associated to DU.528 tumourigenesis as a consequence of TAL1 translocation and absence in this cell line.

Identification of these different proteins as potential interactors with Ldb1 show that Ldb1 might be important in T-cell differentiation as its interacting partners are involved in DNA replication, DNA repair, transcription regulation, cell proliferation and differentiation. However, the interactions of these proteins need to be further confirmed by repeating the mass spectrometry analysis and performing pull down experiments followed by Western blotting. Interestingly, the LMO proteins themselves were not identified, despite our ability to detect LMO2 by Western blotting in the Ldb1

pull down samples that were sent for mass spectrometry. After assessing the prediction of the tryptic digestion on the relatively small LMO proteins within the procedure, it is likely that the generated peptides are not ideal for detection by the mass spectrometer. Usage of a different enzyme to digest proteins could improve this, but may have detrimental effects on the detection of other proteins.

***LMO4* knockdown using siRNA transfection**

The purpose of knocking down the *LMO4* gene was to study its role in early T-ALL. siRNA was transferred into the cells by electroporation. The siRNA is subsequently intracellularly separated into two strands, one of which is specific for a region of the target mRNA, in this case *LMO4*. This strand then interacts with the mRNA targeting it for degradation and inhibiting mRNA translation (102).

Two possibilities to perform *LMO4* knockdown in ARR cells were considered. One was by transduction of the cells with a Lentivirus, the other by delivery of siRNA by electroporation. Previous work within the Hoogenkamp laboratory had the *Lmo4* gene successfully silenced in mouse cells by expressing shRNAs from a mir-30 backbone by a Retrovirus. This work lead to the conclusion that there was an apoptotic phenotype upon knockdown of *Lmo4*, which was problematic for the analysis, as cells were dying at the same time or before the Green Fluorescent Protein (GFP) selection marker was becoming expressed. This made, that for the current research, we would need a Lentivirus with a doxycycline inducible siRNA module. Time constraints related to obtaining and cloning the Lentiviral vector, made us choose for the transfection of siRNA. The siRNA sequence was designed based on the successful mouse shRNA knockdown.

We performed several optimizations concerning the voltage, pulse length and siRNAs concentration and the results indicated that the majority of cells were transfected. Initially we found that the addition of siRNA to the medium without electroporation lead to an accumulation of Cy5 in or on the cells after several hours. When we changed to washing the free dye out of the medium one hour

after transfection this problem was solved, however, we noticed that the presence of the dye was quickly reduced at later time points. We related this to the cellular degradation of the Cy5 dye, suggesting that with future experiments we could change the type of fluorescence tag to see whether that would be more long-lived.

The fact that we could not detect any clear differences between the total siLMO4 and siCtrl cultures, lead us to sort single cells into individual wells and quantify the surviving cells several days after transfection. The results showed that the same number of single cell derived colonies were observed for cells transfected with a shLMO4 or siCtrl. This means that either the *LMO4* gene was not sufficiently silenced, which we hoped to avoid by having a siRNA sequence that was proven to work in mouse, or that the siRNA works faster, at the time many cells are already going into apoptosis due to the transfection procedure itself. Because of this it would be wiser to perform silencing of *LMO4* by cloning the sequence of interest (shLMO4) into a doxycycline inducible Lentiviral vector. Cells can then be transduced and the addition of doxycycline to the medium can induce silencing. In this way, cells that are successfully transduced can be selected for using a selectable marker, while the knockdown is not yet occurring keeping the cells in a non-stressed state that is effectively wild type. Upon induction the effect of the knockdown can then be analysed in isolation.

This research allowed us to characterise two cells lines that can serve as a model for ETP-ALL and provided us with a better insight into the role of the LMO proteins in this leukaemia. The presence of LMO4 during ETP maturation process indicates that it could be directly involved during the onset of ETP-ALL, but this has to be further confirmed. The results with TAL1 can also be very significant for future research, as we showed that it is not present at high levels despite the SIL/TAL fusion. An interesting approach would be to perform mass spectrometry analyses on whole cell extracts of T-ALL samples. Additionally, as LYL1 is a protein that can have similar functions within the same complexes as TAL1, this oncogenic factor should be included in future studies within ETP-ALL. Thus,

more investigations still have to be done on this genetic instable ETP-ALL subtype with poor prognosis in order to understand and prevent transformed ETPs from entering proliferation and self-renewal.

APPENDIX 1

Ldb1 immunoprecipitated proteins from the DU.528 cell line
Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4
ATP-dependent RNA helicase DDX3X OS=Homo sapiens GN=DDX3X PE=1 SV=3
Vasodilator-stimulated phosphoprotein OS=Homo sapiens GN=VASP PE=1 SV=3
Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3
Lamin-B2 OS=Homo sapiens GN=LMNB2 PE=1 SV=3
EF-hand domain-containing protein D1 OS=Homo sapiens GN=EFHD1 PE=1 SV=1
Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC PE=1 SV=5
60S ribosomal protein L34 OS=Homo sapiens GN=RPL34 PE=1 SV=3
60S ribosomal protein L21 OS=Homo sapiens GN=RPL21 PE=1 SV=2
60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=5
60S ribosomal protein L31 OS=Homo sapiens GN=RPL31 PE=1 SV=1
Nuclease-sensitive element-binding protein 1 OS=Homo sapiens GN=YBX1 PE=1 SV=3
LIM domain only protein 7 OS=Homo sapiens GN=LMO7 PE=1 SV=2
Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 PE=1 SV=1
60S ribosomal protein L26-like 1 OS=Homo sapiens GN=RPL26L1 PE=1 SV=1
40S ribosomal protein S17 OS=Homo sapiens GN=RPS17 PE=1 SV=2
14-3-3 protein theta OS=Homo sapiens GN=YWHAQ PE=1 SV=1
Splicing factor 3B subunit 1 OS=Homo sapiens GN=SF3B1 PE=1 SV=3
Coiled-coil domain-containing protein 50 OS=Homo sapiens GN=CCDC50 PE=1 SV=1
Eukaryotic translation initiation factor 2 subunit 1 OS=Homo sapiens GN=EIF2S1 PE=1 SV=3
Elongation factor 1-delta OS=Homo sapiens GN=EEF1D PE=1 SV=5
Ena/VASP-like protein OS=Homo sapiens GN=EVL PE=1 SV=2
60S ribosomal protein L30 OS=Homo sapiens GN=RPL30 PE=1 SV=2
Uncharacterized protein C12orf43 OS=Homo sapiens GN=C12orf43 PE=1 SV=2
Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2
40S ribosomal protein SA OS=Homo sapiens GN=RPSA PE=1 SV=4
Coronin-1C OS=Homo sapiens GN=CORO1C PE=1 SV=1
Histone deacetylase 1 OS=Homo sapiens GN=HDAC1 PE=1 SV=1
Pro-interleukin-16 OS=Homo sapiens GN=IL16 PE=1 SV=4
Nucleolar protein 58 OS=Homo sapiens GN=NOP58 PE=1 SV=1
Single-stranded DNA-binding protein, mitochondrial OS=Homo sapiens GN=SSBP1 PE=1 SV=1
40S ribosomal protein S16 OS=Homo sapiens GN=RPS16 PE=1 SV=2
Elongation factor 1-gamma OS=Homo sapiens GN=EEF1G PE=1 SV=3
Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial OS=Homo sapiens GN=DUT PE=1 SV=3
X-linked retinitis pigmentosa GTPase regulator OS=Homo sapiens GN=RPGR PE=1 SV=2
KH domain-containing, RNA-binding, signal transduction-associated protein 1 OS=Homo sapiens GN=KHDRBS1 PE=1 SV=1
Phosphate carrier protein, mitochondrial OS=Homo sapiens GN=SLC25A3 PE=1 SV=2
Small nuclear ribonucleoprotein Sm D2 OS=Homo sapiens GN=SNRPD2 PE=1 SV=1
ATP-dependent DNA helicase 2 subunit 1 OS=Homo sapiens GN=XRCC6 PE=1 SV=2
Moesin OS=Homo sapiens GN=MSN PE=1 SV=3
Actin-related protein 2/3 complex subunit 1B OS=Homo sapiens GN=ARPC1B PE=1 SV=3
Histone H2B type 1-K OS=Homo sapiens GN=HIST1H2BK PE=1 SV=3

Ldb1 immunoprecipitated proteins from the ARR cell line
Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1
Transferrin receptor protein 1 OS=Homo sapiens GN=TFRC PE=1 SV=2
78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2
Myosin-Va OS=Homo sapiens GN=MYO5A PE=1 SV=1
Calmodulin OS=Homo sapiens GN=CALM1 PE=1 SV=2
Myosin-10 OS=Homo sapiens GN=MYH10 PE=1 SV=3
F-actin-capping protein subunit alpha-1 OS=Homo sapiens GN=CAPZA1 PE=1 SV=3
Guanine nucleotide-binding protein Gsubunit alpha-2 OS=Homo sapiens GN=GNAI2 PE=1 SV=3
Dedicator of cytokinesis protein 8 OS=Homo sapiens GN=DOCK8 PE=1 SV=3
Probable ATP-dependent RNA helicase DDX5 OS=Homo sapiens GN=DDX5 PE=1 SV=1
40S ribosomal protein S13 OS=Homo sapiens GN=RPS13 PE=1 SV=2
AP-2 complex subunit beta OS=Homo sapiens GN=AP2B1 PE=1 SV=1
Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2
LIM domain and actin-binding protein 1 OS=Homo sapiens GN=LIMA1 PE=1 SV=1
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Homo sapiens GN=PPP1CA PE=1 SV=1
60S ribosomal protein L24 OS=Homo sapiens GN=RPL24 PE=1 SV=1
40S ribosomal protein S10 OS=Homo sapiens GN=RPS10 PE=1 SV=1
Heat shock 70 kDa protein 6 OS=Homo sapiens GN=HSPA6 PE=1 SV=2
ATP-dependent RNA helicase DDX3X OS=Homo sapiens GN=DDX3X PE=1 SV=3
60S ribosomal protein L27a OS=Homo sapiens GN=RPL27A PE=1 SV=2
Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1
40S ribosomal protein S9 OS=Homo sapiens GN=RPS9 PE=1 SV=3
Guanine nucleotide-binding protein G subunit alpha OS=Homo sapiens GN=GNAI3 PE=1 SV=3
AP-2 complex subunit alpha-1 OS=Homo sapiens GN=AP2A1 PE=1 SV=3
60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1
60S ribosomal protein L30 OS=Homo sapiens GN=RPL30 PE=1 SV=2
Putative tubulin-like protein alpha-4B OS=Homo sapiens GN=TUBA4B PE=5 SV=2
Putative heterogeneous nuclear ribonucleoprotein A1-like 3 OS=Homo sapiens GN=HNRPA1L3 PE=5 SV=1
60S ribosomal protein L11 OS=Homo sapiens GN=RPL11 PE=1 SV=2
Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2
60S acidic ribosomal protein P0 OS=Homo sapiens GN=RPLP0 PE=1 SV=1
Core-binding factor subunit beta OS=Homo sapiens GN=CBFB PE=1 SV=2
Actin-related protein 2/3 complex subunit 4 OS=Homo sapiens GN=ARPC4 PE=1 SV=3
40S ribosomal protein S25 OS=Homo sapiens GN=RPS25 PE=1 SV=1
60S ribosomal protein L6 OS=Homo sapiens GN=RPL6 PE=1 SV=3
DNA-dependent protein kinase catalytic subunit OS=Homo sapiens GN=PRKDC PE=1 SV=3
Ras-related protein Rab-7a OS=Homo sapiens GN=RAB7A PE=1 SV=1
40S ribosomal protein S4, X isoform OS=Homo sapiens GN=RPS4X PE=1 SV=2
60S ribosomal protein L18a OS=Homo sapiens GN=RPL18A PE=1 SV=2
Actin-related protein 2/3 complex subunit 3 OS=Homo sapiens GN=ARPC3 PE=1 SV=3
Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=7
40S ribosomal protein S16 OS=Homo sapiens GN=RPS16 PE=1 SV=2
40S ribosomal protein S20 OS=Homo sapiens GN=RPS20 PE=1 SV=1
Galectin-1 OS=Homo sapiens GN=LGALS1 PE=1 SV=2
Cyclin-Y OS=Homo sapiens GN=CCNY PE=1 SV=2
Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN=HNRNPU PE=1 SV=6
Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2
40S ribosomal protein S23 OS=Homo sapiens GN=RPS23 PE=1 SV=3
Dermokine OS=Homo sapiens GN=DMKN PE=1 SV=3

Golgi-associated plant pathogenesis-related protein 1 OS=Homo sapiens GN=GLIPR2 PE=1 SV=3
60S ribosomal protein L37a OS=Homo sapiens GN=RPL37A PE=1 SV=2
60S ribosomal protein L14 OS=Homo sapiens GN=RPL14 PE=1 SV=4
Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3
Tubulin beta-4 chain OS=Homo sapiens GN=TUBB4 PE=1 SV=2
Heterogeneous nuclear ribonucleoproteins C1/C2 OS=Homo sapiens GN=HNRNPC PE=1 SV=4
40S ribosomal protein S24 OS=Homo sapiens GN=RPS24 PE=1 SV=1
40S ribosomal protein S18 OS=Homo sapiens GN=RPS18 PE=1 SV=3
ADP/ATP translocase 3 OS=Homo sapiens GN=SLC25A6 PE=1 SV=4
Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9 PE=1 SV=2
Clathrin interactor 1 OS=Homo sapiens GN=CLINT1 PE=1 SV=1
Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3
Histone H2A.V OS=Homo sapiens GN=H2AFV PE=1 SV=3
Raftlin OS=Homo sapiens GN=RFTN1 PE=1 SV=4
40S ribosomal protein S15a OS=Homo sapiens GN=RPS15A PE=1 SV=2

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