TCR Translocations at the Normal-Malignant T cell Interface
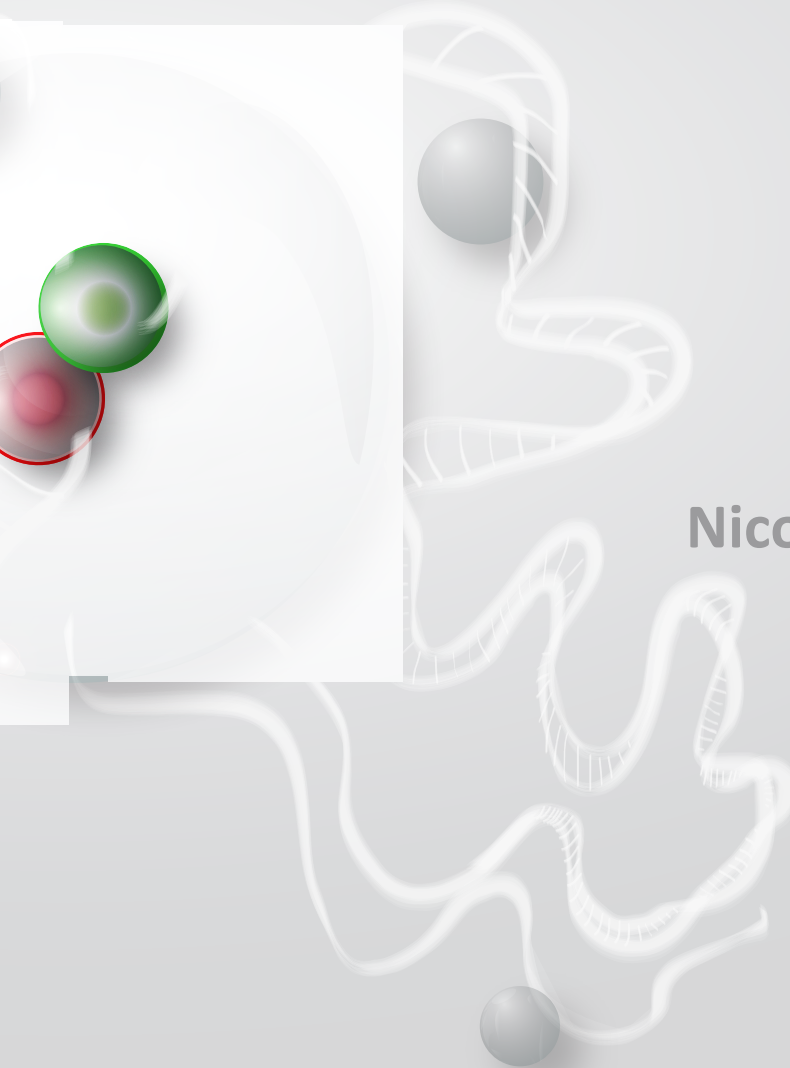
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TCR Translocations at the Normal-Malignant T cell Interface

TCR translocaties aan het normale-maligne T cel raakvlak

Thesis

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Introduction

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INTRODUCTION

Normal and malignant human hematopoiesis

Normal hematopoiesis

Hematopoiesis is the process leading to production and maturation of peripheral blood cells. All blood cells are derived from hematopoietic stem cells (HSCs) which reside in hematopoietic organs [1,2]. In mammals, the site of hematopoiesis changes during development, which is sequentially taking place in different organs starting with primitive erythrocytes in the yolk sac, the aorta-gonad mesonephros (AGM) region, the fetal lever, and finally the bone marrow (BM) during adulthood [1-3]. Blood cells are short-lived, and with a daily demand for more than a billion new hematopoietic cells, a continuous replenishment of progenitor cells committed to specific hematopoietic lineages is required [1,2,4]. HSCs are at the top of the hematopoietic hierarchy, and are the only source of progenitors. HSCs comprise 0.005-0.01% of the bone marrow, and their unique properties, i.e. the ability of self-renewal and multi-lineage differentiation potential in combination with a specific stem cell microenvironment/niche, enable these cells to sustain the hematopoietic system [5,6]. These cells differentiate into progenitor cells, either into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP), which in due course differentiate into mature blood cells, providing cells to the myeloid or lymphoid system respectively [6]. CLPs carry the potential to give rise to B cells, T cells (via the thymus) and NK cells, whereas CMPs have the potential to differentiate into erythrocytes, megakaryocytes, macrophages, and granulocytes. Dendritic cells can arise from both progenitor types [7,8]. The process of hematopoietic lineage determination is tightly regulated by the BM microenvironment's extrinsic factors, such as growth factors and cytokines mediated by cell-cell interactions, which sustain survival and proliferation of committed cells [6,8,9]. Equally important in determining cell fate are the lineage- and cell-type-specific gene expression signatures (intrinsic factors) [10-12]. These signatures are based on the up and down regulation of transcription factors apparently regulated by the epigenetic-micro RNAs regulatory circuit [13]. The strict regulation of both extrinsic and intrinsic signals is of utmost importance, as deregulation of the expression of these factors could result in hematopoietic malignancies such as leukemia or lymphoma. Such deregulation of gene expression is usually caused by irreversible molecular-cytogenetic changes introduced into the genomic DNA sequence. These changes can be caused by mutations, translocations and deletions concerning genes involved in cell cycle, differentiation, proliferation, and self-renewal processes [14,15]. During the last decade it has become evident that, next to genetic aberrations, epigenetic alterations can also contribute to tumorigenesis, for example through gene silencing due to aberrant methylation [16,17].

Malignant hematopoiesis

Hematopoietic malignancies is the generic term for a heterogeneous spectrum of tumors with a specific outcome, depending on the maturation state of the cell type and the cell lineage involved. These malignancies can be either lymphoid in nature, as in the case with acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM), Hodgkin lymphoma (HL), or non-Hodgkin lymphoma (NHL), or they can be of a myeloid nature, as with acute myeloid leukemia (AML), myeloproliferative diseases (MPD), myelodysplastic syndromes (MDS), and chronic myelogenous leukemia (CML). Each of these malignancies is characterized by the presence of specific molecular-cytogenetic alterations, seen at diagnosis in combination with clinical, morphological and immunophenotypic characteristics, which can also give insight into outcome.

From a therapeutic point of view, identification of the different hematopoietic lineage-specific chromosomal aberrations helps to give insight into disease progression and prognosis [18,19]. Most importantly, this knowledge has led to the identification of molecular drug targets and the development of tumor-specific therapies that could result in improved therapy [20-22].

Normal human T cell development

All mammalian T cells originate from BM derived HSCs which can consist of the not T-cell committed lymphoid-primed multipotent progenitors (LMPPs) also termed MPPs, which lack self-renewal capacity or interleukin 7 receptor expressing CLPs with T cell potential [23]. These cells migrate from the BM via the bloodstream and enter the thymus via the cortico-medullary junction (CMJ) [24]. This recruitment into the thymus has been shown to be a highly intermittent regulated process, depending on the availability of intra-thymic niches [24,25]. P-selectin expressed on thymic epithelial cells has been shown to play an important role in regulating the homing of precursor T cells to the thymus [26,27]. The thymus provides these progenitor T cells with the required support and microenvironment to allow these cells to differentiate into functional T cells before re-entering the circulation. In the thymus, thymocytes go through a series of migrations which coincide with their stage of development (Figure 1). The development of thymocytes is a discontinuous process, following continuous key developmental checkpoints, which are marked by T cell receptor (TCR) locus rearrangement and the expression of stage-specific surface markers (Figure 1A). The earliest thymocyte progenitors are referred to as the double negative 1 (DN1) thymocytes (cluster of differentiation ((CD)34+, CD38-, CD1a-) [26,28,] based on the absence of the CD4 or CD8 markers on the cell surface, and these DN1 cells are situated in the direct vicinity of the CMJ (Figure 1A). At this stage, these early thymic progenitors are uncommitted and seem to have retained their myeloid lineage potential [26,29], while they are able to start the TCR delta (TCRD) locus rearrangement, usually by first recombining Dδ-Dδ [28] (Figure 1B and 1C). The subsequent

Figure 1. Schematic representation of T cell development and migration through the thymus. A. The schematic representation of all major consecutive thymocyte subsets, shown with their main markers used for thymic subset immunophenotyping. Bone marrow derived Hematopoietic Stem cells (HSCs; multipotent progenitors (MPPs) and common lymphoid progenitors(CLPs)) enter the thymus at the cortico-medullary junction (CMJ). During the double negative (DN) 1 through DN2 stage, cells migrate from the CMJ through the cortex and progress into the DN3 stage to the sub-capsular zone. DN2 and DN3 thymocytes either differentiate into TCRgd cells (dashed arrows) depending on the TCRgd signal or continue the differentiation process. Cells then progress from the DN3 to the immature single positive stage (ISP), to double positive CD3- (DP3-), DP3+ stage. DP cells migrate into the medulla and differentiate into either CD4+ or CD8+ single positive (SP) cells. SP cells exit the thymus through blood vessels. Curved arrows indicate proliferation. B. During the transition from DN1 to DN2 stage cells undergo rearrangement of the TCRD, TCRG and TCRB loci. At the DN3 stage the pre-TCR is expressed and cells undergo β-selection. Upon survival, cells progress into ISP and DP stages, at which the TCRA locus is rearranged. At the DP3+ stage cells go through positive selection and during the transition into SP cells, cells undergo negative selection. C. Each TCR locus recombines at specific stages of thymocyte development in a particular order. In turn TCR genes will also be recombined in a particular order. Also shown are the percentages of subset composition in the thymus. *Adapted from thesis F. Weerkamp, 2005, p19 and Dik et al* [28].

DN2 thymocytes (CD34+CD38+CD1a-) relocate to a central cortical location. At this stage TCR gamma (TCRG) locus rearrangement takes place and the thymocytes become committed to the T cell lineage [26,28,30,31]. Also at this stage, the first step in TCR beta (TCRB) locus recombination starts (Dβ-Jβ recombination) [28,32] (Figure 1B and 1C).

T cell lineage commitment and development is regulated by the NOTCH1-ligand Delta-like 4 and interleukin-7 (IL-7) signals, derived from stromal cells [26,30,33,34]. Following the DN2 stage, thymocytes progress to a sub-capsular localization as DN3 (CD34+, CD38+, CD1a+) thymocytes [25,28] (Figure 1). During the DN3 stage of thymic development, rearrangement of the TCRB locus is fully ongoing [28]. Cells in which both productive TCRγ and TCRδ chains have been formed before completion of TCRB locus rearrangement further mature as TCRγδ T cells. At this stage the CD3 protein, which is expressed early in thymocyte development, is able to form TCR-CD3 complexes [35,36]. Strong signaling via the TCRγδ receptor drives lineage commitment to TCRγδ T cells [37]. If TCRB locus rearrangement is accomplished on time, DN3 thymocytes undergo beta-selection (β-selection) [28] (Figure 1B). At this stage, DN3 cells are required to signal via a pre-TCR, which comprises the rearranged TCRβ chain, a pre-Tα and CD3 chains [26]. This process allows only the DN3 cells that express a functional TCRβ chain to continue their development, while cells with non-functional TCRβ chains or of which the rearrangement process takes too long to die via apoptosis.

The relocation of the DN thymocytes from the CMJ to the thymic capsule has been shown to be regulated by chemokine receptors such as CXCR4, CCR7 and CCR8, as loss of these receptors leads to accumulation of the DN thymocytes at the CMJ [25,38]. After reaching the capsule, thymocytes migrate back across the cortex towards the medulla, while continuing sequential differentiation (Figure 1A and 1B). When passing through the cortex for the second time, CD34 expression is lost and immature single-positive (ISP) (CD3low, CD4+), doublepositive (DP) CD3- (CD3-, CD8+, CD4+) and DP CD3+ (CD3+, CD8+, CD4+) thymocytes develop sequentially. Also, TCR alpha (TCRA) locus rearrangement is initiated, which results in the expression of the TCRαβ complex (Figure 1B and 1C). At this DP developmental stage, thymocytes undergo the next selection phase. This so-called positive selection depends on the interaction between TCR and peptide-major histocompatibility complex (MHC) which is expressed by cortical thymic epithelium cells [25,26,39]. Thymocytes are only positively selected and rescued from death by neglect upon peptide recognition at low-avidity interactions, and continue survival and migrate in response to CCR7 signaling from the cortex to the medulla [25,39,40]. Simultaneously, these cells undergo negative selection where all thymocytes with a high affinity for tissue-specific self peptide-MHC interaction, therefore being auto-reactive, are deleted, so as to avoid autoimmunity [25,26,39,40]. Eventually, naïve mature T cells leave the thymus via blood or lymphatic vessels and migrate to secondary lymphoid organs [25,26,39].

T-cell acute lymphoblastic leukemia (T-ALL): the malignant counterpart of developing human thymocytes

During human T cell development in the thymus, thymocytes can undergo malignant transformation, which may finally result in the formation of a T-cell malignancy. T cell derived malignancies comprise about 5-10% of the human lymphoproliferative malignancies [41]. T cell acute lymphoblastic leukemia (T-ALL) is derived from progenitor T cells which are blocked at an early stage of thymic differentiation. T-ALL accounts for 10-15% of pediatric and ~25% of adult ALL [14,18]. The different clinico-biological characteristics seen in T-ALL are usually associated with unfavorable clinical features [42]. Differences in morphology, immunophenotype, cytogenetic and molecular characteristics, reveal the heterogeneity of the disorder and show that these malignant cells can originate at different stages of normal precursor T cell development. Hence, these malignant T-ALL cells can be considered as the malignant aberrant counterpart of normal thymocytes.

Molecular-genetic abnormalities in human T-ALL

Molecular and cytogenetic characterization of T-ALL genetic aberrations has resulted in their classification as either 'Type A' or 'Type B' T-ALL [43]. 'Type A' aberrations lead to the over-expression of oncogenes or the formation of fusion genes. The resulting ectopic expression generally leads to a block in the cell differentiation. 'Type A' transformations are thought to be associated with specific T-ALL subgroups [43,44]. 'Type B' aberrations, on the other hand, are not associated with particular T-ALL subgroups, and involve point mutations, insertions or deletions, either leading to loss or gain of function of particular genes [43].

'Type A' genetic aberrations

Malignant transformation in about 30% of T-ALL cases has been attributed to TCR chromosomal translocations [45]. These aberrations are formed by translocations that occur between TCRB (7q34) or TCRA/TCRD (14q11) loci and transcription factor-encoding genes. These genes encode for proteins of for example the helix-loop-helix (bHLH) family, LIM-only domain (LMO) proteins and homeobox proteins (Table 1). Some of these transcription factors are normally expressed in non-malignant thymocytes, and play an important role in regulating normal thymocyte differentiation, while others are not expressed. As a consequence of the translocations, these oncogenes are aberrantly expressed, mostly resulting in an acquired abnormal differentiation and/or cell cycle control and proliferation. As leukemogenesis is a process that requires additional genetic hits, it is generally accepted that these TCR translocations are just one of the genetic events in tumorigenesis. An overview of TCR-associated 'Type A' aberrations and their occurrence in T-ALL is given in Table 1.

Basic helix-loop helix transcription factors (bHLH)

Two classes of bHLH proteins are known. Class A bHLH E proteins, E2A and HEB, bind DNA at specific regulatory elements of T cell-specific genes, and Class B bHLH transcription factors *TAL1*, *LYL1*, *TAL2*, *BHLHB1* and *MYC,* which form heterodimeric DNA binding complexes with Class A bHLH E proteins in T-ALL[46]. Aberrant complex formation has been hypothesized to disturb the normal function of Class A E proteins [47].

TAL1 plays a role in the regulation of hematopoietic stem cell development of hemato-poietic progenitors, mast cells, erythrocytes and megakaryocytes, but is not expressed in thymocytes [48]. In T-ALL, *TAL1* expression is activated either as a consequence of a transloca-tion to the TCRD locus t(1;14)(p32;q11) in ~3% of the cases, or through a deletion leading to the formation of the *SIL-TAL1* fusion gene in about 16-30% of the cases [49,50]. In ~40% of cases, *TAL1* expression is detected in the absence of a detectable *TAL1* rearrangement, which is thought to be caused by biallelic overexpression of *TAL1*, suggesting that other aberrancies may also cause ectopic *TAL1* expression [51]. T-ALL cases positive for *TAL1* mostly have a late DP thymocytic phonotype [52].

LYL1 is closely related to *TAL1*. *LYL1* is only expressed in immature hematopoietic cells and B cells, and in endothelial cells involved in the formation of vascular structures [53,54]. In T-ALL, *LYL1* translocates to TCRB loci, forming the rarely seen t(7;19)(q34;p13) [55] and there T-ALL cells are mostly CD34+ and myeloid express markers, associating *LYL1* expression with an early immature thymocytic phenotype [49].

TAL2 and *OLIG2* (previously referred to as *BHLHB1*) are rarely seen as TCR translocation partners in T-ALL, t(7;9)(q34;q32) involving the TCRB locus and t(14;21)(q11;q22) involving the TCRD locus respectively [56,57]. Expression of *LYL1*, *TAL2* and *BHLHB1* in T-ALL is also thought to cause heterodimer formation Class A with E proteins, thereby disrupting their function [58]. *MYC* is also activated in ~1% of T-ALL cases as the result of t(8;14)(q24;q11) translocations involving the TCRD locus [59].

LIM only domain proteins

The LIM only domain genes, *LMO2* and *LMO1* are involved in ~45% of cases TCR asso-ciated translocations in T-ALL, [52,60]. *LMO1*, has only been observed to translocate to TCRD locus [t(11;14)(p15;q11)]. *LMO1* does not play a role in normal thymocyte development but contributes to leukemogenesis upon ectopic expression in thymocytes [61]. *LMO2* is expressed in the early DN stages of thymocyte development and is important in embryonic stem cell development in mice [62,63]. *LMO2* translocations t(11;14)(p15;q11) and t(7;11)(q34;p13), involve both the TCRD and TCRB locus respectively [61,62,64-66]. Aberrant activation of *LMO2* has also been reported as the result of retroviral integration in a severe combined immunode-ficiency (SCID) gene therapy trail and loss of the negative regulatory element of the *LMO2* promoter [67,68]. *LMO2* over-expression has been shown to lead to the developmental arrest of

Table 1 'Type A' TCR-associated aberrations in T-ALL*

Protein family	Gene	Chromosome band	Chromosomal aberrations	Occurrence in T-ALL	References
Basic helix-loop-helix transcription factors (bHLH)	TAL1	1p32	t(1;14)(p32;q11)/ t(1;7)(p32;q34)	~4%	52,72
	TAL2	9q34	t(7;9)(q34;q34)	~2%	56
	LYL1	19p13	t(7;19)(q34;p13)	~7%	52
	OLIG2	21q22	t(14;21)(q11;q22)	~2%	52
	MYC	8q24	t(8;14)(q24;q11)	~1%	52
Lim only domain (LMO) proteins	LMO2	11p13	t(11;14)(p13;q11)/ t(7;11)(q34;p15)	~6%	62,66
	LMO1	11p15	t(11;14)(p15;q11)/ t(7;11)(q34;p13)	~2%	61
	LMO3	12p12	t(7;12)(q34;p12)	<1	73
Homeobox proteins	TLX1	10q24	t(10;14)(q24;q11)/ t(7;10)(q34;q24)	5-10%[C], ~30%[A]	74,75
	TLX3	5q35	t(5;14)(q35;q11)	20-25%[C],~5%[A]	49,76,77
	HOXA cluster	7p15	t(7;14)(p15;q11)/ inv(7)(p15;q34)	~3%	49,78,79
	NKX2-1	14q13	t(7;14)(q34;q13)/ inv(14)(q13;q32)	<1%	unpub. 80
	NKX2-4	20p11	t(20;14)(p11;q11)	<1%	unpub.
	NKX2-5	5q35	t(5;14)(q35;q32)	<1%	81
Other	MYB	6q23	t(6;7)(q23;q34)	~3%	82
	BCL11B	14q32	inv(14)(q11;q32)	<1%	83
Assumed Type A	TCL1A	14q32	t(7;14)(q35;q32)/ inv(14)(q11;q32)	<1%	84,85
	BMI1	10p12	t(7;10)(q34;p12)	<1%	86

*Classification according to van Vlierberghe *et al.*[43] and Meijerink *et al.*[44] [C] Childhood, [A] Adulthood

thymocytes at the DN, ISP and DP stages [69]. It has been postulated, based on mouse models, that *LMO2* is able to induce self-renewal of committed T cells upon constitutive expression of *LMO2* in thymocytes [70]. *LMO2* and *LMO1* ectopic expression has also been observed in T-ALL cases where *TAL1* or *LYL1* is deregulated. In mouse models, Lmo2 has been shown to physically associate with Tal1 and to form heterocomplexes that are able to regulate different genes [71].

Homeobox transcription factors

Homeobox proteins are involved in the regulation of embryonic development, and play a role in axial patterning, morphogenesis and cellular differentiation. Two homeobox classes are known: Class I homeobox genes comprising the *HOX* genes A, B, and C and Class II homeobox *TLX1* and *TLX3* genes. In particular the *HOXA* cluster genes and the *TLX1* and *TLX3* genes have been associated with T-ALL.

HOXA cluster genes are expressed in the early stages of thymocyte development and are regarded as key players in hematopoietic stem cell self-renewal [87,88]. In T-ALL *HOXA* gene expression is usually deregulated by the recurrent t(7;7)(p15;q34) or inv(7)(p15q34) which is

seen in ~5% of the cases [79]. These aberrations bring *HOXA* cluster genes under control of the TCRB enhancer, causing its ectopic expression [79]. *HOXA* expressing T-ALL is mostly arrested in the late thymocytic development stage [89].

The Class II homeobox genes *TLX1* and *TLX3* are not involved in thymocyte development. *TLX1* (previously referred to as *HOX11*) plays a role in spleen morphogenesis, while *TLX3* (*HOX11L2*) is involved in central nervous system (CNS) development [90]. *TLX1* translocations t(10;14)(q24;q11) and t(7;10)(q34;q24), involving either the TCRD or TCRB locus respectively, and loss of negative regulatory elements upstream of the *TLX1* promoter, have been shown to cause the over-expression of *TLX1* [74,75]. About 30% of T-ALL cases are positive for *TLX1* and show a more early thymocytic phenotype [49,91]. This is in concordance with findings that *TLX1* over-expression in murine fetal lever precursors and human cord blood CD34+ progenitor cells disrupts cell differentiation prior to the DP thymocyte development stage [92].

TLX3 is ectopically expressed in 20-30% of childhood and ~13% of adult T-ALL cases [49,76,77]. TCRD-associated translocation to *TLX3* t(5;14)(q35;q11) do occur but are rare compared to translocations t(5;14)(q35;q32) involving the *BCL11B* gene [49,93,94]. *TLX3* translocations t(5;7)(q35;q21) involving the *CDK6* gene have also been described [95]. *TLX3*-positive T-ALL are heterogeneous and show both an early cortical as seen for *TLX1*-positive cases and a more immature phenotype [43,60].

Genes involved in the more rare 'Type A' mutations

Next to being involved in *TLX3* translocations, the tumor suppressor gene *BCL11B* was shown to be disrupted through inv(14)(q11q32) involving the TCRD locus, resulting in *BCL11B*-TCRD fusion transcripts. Loss of *BCL11B* expression is thought to be involved in tumorigenesis [83]. The TCR translocation involving the proto-oncogene *MYB* has also been described in T-ALL as 'Type A' mutation, while duplications involving this gene have been categorized as 'Type B' mutations [43,82,96]. Other rare but recurrent genetic alterations seen in T-ALL lead to the formation and expression of fusion genes such as *SET-NUP214* and *CALM-AF10* [43]. TCR translocations involving *TCL1A* and *BMI1* (onco) genes have also been described in T-ALL [84-86]. However, these aberrations have not been categorized as yet, mainly due to lack of information regarding the resulting oncogenic activation of these genes upon TCR translocation formation. Nevertheless, it is likely that TCR translocation formation leads to ectopic over-expression of these genes, thus categorizing these aberrations as 'Type A' aberrations (Table 1).

'Type B' genetic aberrations

In addition to TCR translocations, other genetic abnormalities have been associated with T-ALL development. The most common genetic alterations seen in T-ALL are those associated with aberrant NOTCH1 signaling. Normal NOTCH1 signaling plays a role in promoting T-cell

development and has been shown to be essential at different stages of thymocyte development, influencing cell proliferation, TCRB rearrangement, apoptosis and developmental lineage choice [97,98].

More than 50% of T-ALL carry a *NOTCH1*-associated mutation, which results in the constitutive activation of NOTCH signaling [99]. Of these *NOTCH1*-related mutations only ~1% concerns the TCR associated chromosomal aberration (t(7;9)(q34;q34)) [100]. The 9p21 mutations, which lead to the loss of *p16/INK4A* and *p14/ARF* tumor suppressor gene expression, are the most predominant aberrations seen in T-ALL, with about 70% of the cases carrying these mutations [52,101]. Like *p16/INK4A* and *p14/ARF*, other genes; such as *CCND2* and *RB1* and *CDKN1B*, which also play a role in cell cycle regulation, have been implicated in T-ALL, though less frequently. Other rare but recurrent 'Type B' genetic alterations seen in T-ALL lead to mutations that drive cell proliferation, and involve genes such as *LCK*, *JAK1* and *FLT3* or lead to loss of tumor suppressor function of genes such as *PTPN2*, *LEF1, TCF1* and *WT1* [43,44,96,102]. An overview of the few TCR-associated 'Type B' aberrations and their occurrence in T-ALL is given in Table 2.

Molecular mechanism of TCR translocation formation

Normal TCR gene recombination

TCR molecules are heterodimers which consist of either TCRγ and TCRδ chains or TCRα and TCRβ chains. Each TCR chain consists of a variable domain which is involved in antigen recognition and a constant domain which mediates TCR signaling.

The variable domain is encoded by a combination of variable (V), diversity (D) and joining (J) genes. The variability of the TCR allele relies on the coupling of a variety of genes needed for generating a diverse antigen receptor repertoire. These genes are recombined to each other via a tightly regulated and stage-specific process called V(D)J recombination [107,108] (Figure 2).

Table 2 'Type B' TCR-associated aberrations in T-ALL*

Protein family	Gene	Chromosome band	Chromosomal aberrations	Occurrence in T-ALL	References
'Type B'	*NOTCH1*	9q32	t(7;9)(q34;q34)	<1%	[103,104]
	CCND2	12p13	t(7;12)(q34;p13)/ t(12;14)(p13;q11)	<1%	[105]
	LCK	1p34	t(1;7)(p34;q34)	<1%	[106]

*Classification according to van Vlierberghe *et al.*[43] and Meijerink *et al.*[44]
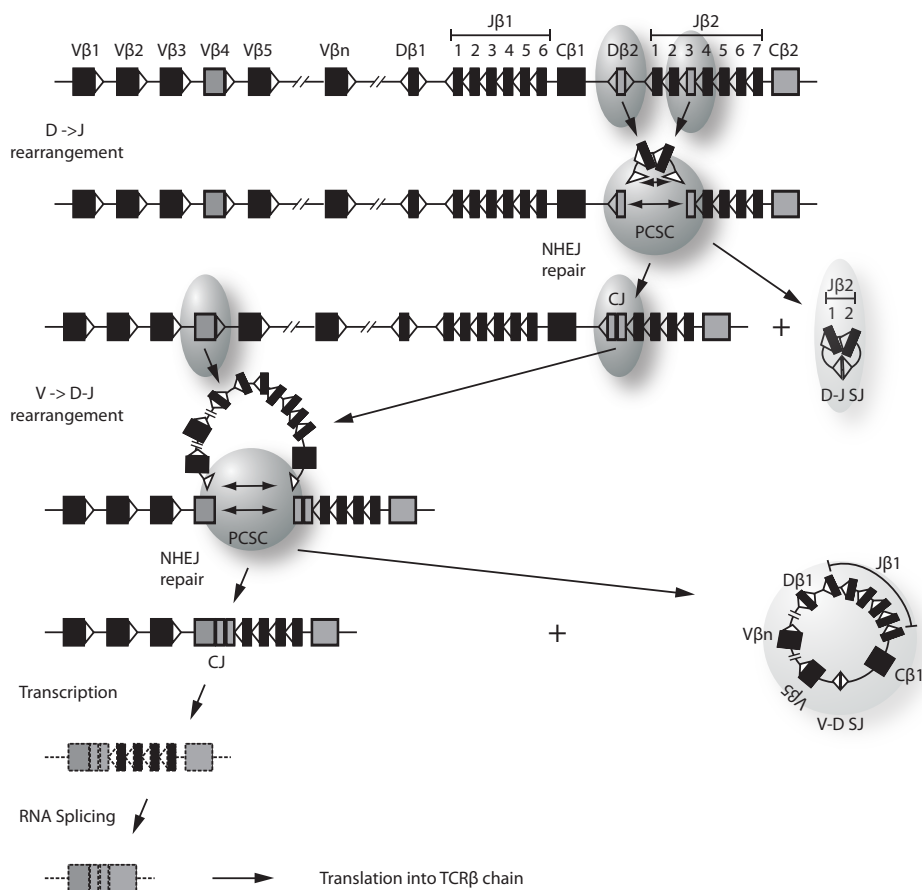
Figure 2. Schematic diagram of sequential rearrangement steps, transcription and translation of the TCRB gene during T cell differentiation. In this example, RAG proteins (gray sphere) recognize and capture the recombination signal sequence (RSS) (white triangle), also called signal end (SE), flanking the Dβ2 gene (coding ends (CE) (rectangle). Here after, RAG captures a second RSS flanking the Jβ2.3 gene. RAG introduces a DSB at the exact border between the CE and the SE. This leads to the formation of a post-cleavage synaptic complex (PCSC) in which the four broken DNA ends are held in close proximity to enable rearrangement. Components of the non-homologous end joining (NHEJ) pathway are recruited to enable repair of the breaks. This forms the Dβ2-Jβ2.3 coding joint (CJ). Following this RAG proteins capture the Vβ4 gene to enable Vβ4-Dβ2-Jβ2.3 rearrangement. The rearranged TCRB gene is transcribed into precursor messenger RNA (mRNA), spliced into mature mRNA, and finally translated into TCRβ protein. The two extrachromosomal TCR excision circles are formed during this recombination process as a byproduct of recombination. Signal joint (SJ). Figure adapted from van Dongen *et al.*

V(D)J recombination of TCR genes occurs in developing T-lymphocytes. The V(D)J recombination process is site-specific, as conserved recombination signal sequences (RSSs) that flank TCR genes limit the recombination process at the TCR locus [28,109]. Each RSS is composed of a palindromic heptamer (consensus sequence: CACAGTG) and an AT-rich nonamer which are separated by non-conserved 12 or 23 bp spacers. Recombination between genes occurs according to the 12/23 rule, which dictates that recombination is restricted to RSSs with dissimilar spacer-length [110], although exceptions to the rule are known. The V(D)J recombination process is mainly driven by the recombination activating gene (RAG) protein complex, composed of RAG1 and RAG2 molecules, which are exclusively expressed in lymphoid cells and at specific stages of cell development. RAG complexes recognize RSSs, and upon proper targeting, capturing and binding of RAG to an RSS, RAG subsequently introduces a nick at the border in between the coding gene and the RSS, after which it captures a second RSS [111]. This forms a pre-cleavage synaptic complex in which two genes are put in close proximity to each other. Next, DNA double-strand breaks (DSBs) are formed, resulting in a hairpin coding end (CE) and a blunt signal end (SE), which are retained in a post-cleavage synaptic complex (PSC) [111,112] (Figure 2). SEs are joined to form a signal joint (SJ) containing the deleted intervening sequences, whereas the CEs form a coding joint (CJ) by recruitment of components of the non-homologous end joining (NHEJ) pathway into the synapse. Since SEs are blunt, they can be directly ligated, while the hair-pinned CEs first need to be opened and processed for ligation. DNA damage is sensed by KU70-KU80 complex, followed by recognition of the hairpin ends in *cis* by the Artemis:DNA-PKcs complex. Binding of DNA-PKcs to the DNA activates kinase and autophosphorylation activity, thus enabling Artemis endonuclease activity to nick hairpins and cause end resection [111,113,114]. During the catalytic process of the DNA ligase 4/XRCC4 complex ligation, terminal deoxynucleotidyl transferase (TdT) adds *de novo* untemplated nucleotides to the junctional region [111,115].

The role of the V(D)J recombination machinery in TCR translocation formation

Although V(D)J recombination for functional TCR gene assembly is restricted to the TCR locus, erroneous TCR recombinations, mainly translocations, do occur. Two types of V(D)J-mediated translocation mechanisms are known: 'Type 1' translocations can occur when RAG proteins mistarget RSS-like sequences located outside the TCR loci, cryptic-RSSs (cRSSs), thereby including these sequences in the TCR recombination process; 'Type 2' translocations can take place when non-RAG-mediated DSBs are erroneously repaired within the PCSC [111,116] (Figure 3). As a result, oncogenes are erroneously joined to TCR genes. This brings oncogenes in the vicinity of TCR enhancers or regulatory elements in *cis,* or they cause the loss of oncogene-negative regulatory elements (NREs), resulting in the aberrant expression of the oncogene [15,111].
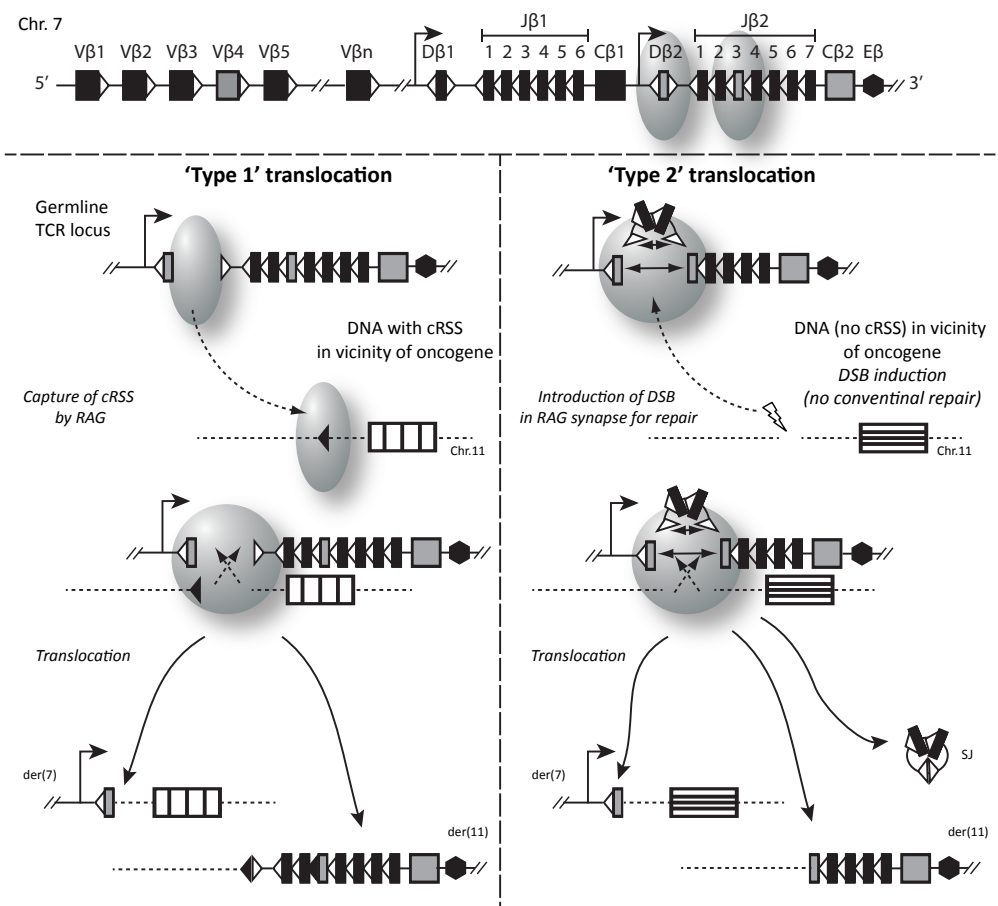
Figure 3. Schematic example of TCR translocation formation via 'Type 1' and 'Type 2' translocations. 'Type 1' translocation: RAG mistakenly recognizes and captures a cRSS adjacent to a oncogene and introduces the sequence into the synaptic complex, where it is recombined with a TCR gene. 'Type 2' translocation: A DNA double strand break (DSB) is introduced in an sequence adjacent to a oncogene and due to a repair mistake, the DSB is repaired in the synaptic complex, where the sequence is erroneously joined to a TCR gene. Due to the translocation, oncogenes are put in the vicinity of regulatory elements such as a TCR promoter (bent arrow) of the TCR genes, causing the aberrant expression of the oncogenes.

Accessibility and nuclear proximity in the context of TCR translocation formation

When considering the high demand for hematopoietic cells from the bone marrow in adults, as well as the many endogenous and exogenous factors that can cause DNA damage in actively proliferating and differentiating cells, it is not unexpected to find a high frequency of chromosomal aberrations in the genome of these cells [4]. Despite the high probability for hematopoietic cells to acquire DNA damage, only a small fraction of the population is ultimately affected by particular genomic aberrations. In terms of TCR translocations, a variety of factors involving the TCR locus involvement, DSB location within the oncogene locus and oncogene function will determine if a translocation will result in a tumorigenic effect [62,117]. Thus a certain oncogenic effect acquired post-translocation is required for a particular genomic aberrations to eventually manifests as leukemogenic aberrations.

The formation of a translocation requires a physical exchange between genomic sequences [4,114,118]. In order to ensure the occurrence of such a physical exchange, two broken ends on distinct chromosomes must be simultaneously present within the same cell, whilst being in close spatial proximity [4]. Therefore pre-translocation conditions must be favorable for the actual occurrence of a physical exchange. Two of these pre-translocation conditions are locus accessibility and nuclear proximity of involved loci.

Locus Accessibility

Eukaryotic genomic DNA is about 2 meters long. In order for it to fit in a nucleus, DNA is hierarchically packaged into chromatin. To achieve this, about 146 bp of DNA is wrapped around a protein octamer composed of four core histones (H3,H4,H2A and H2B) that form the nucleosome core particle [119]. Nucleosomes are about 10 nm in diameter, and form the subunits. These may be regarded as the building blocks of chromatin. The nucleosome array with intervening linker DNA, also referred to by some as "beads on a string", forming the first of three layers of chromatin organization [119,120]. A fifth histone, referred to as linker histone H1 (or its variant H5), associates with the "exit-entry" region of nucleosomal DNA. This binding facilitates the nucleosome array's condensation into a 30 nm fiber, forming the second layer of chromatin organization [121,122]. This second layer of organization has been shown as being assembled in an array of inconsistent, rosette-like loop structures [123-125]. All details regarding chromatin folding into the second and third order of chromatin organization, leading to the final formation of chromosomes, are still not known [119,126]. Nevertheless, nucleosomal organization is considered to be a major determinant to the accessibility of genetic information [126].

Normal cellular processes such as gene transcription, DNA replication, repair and recombination are regulated at the chromatin level and require that proteins have access to DNA sequences for these processes to succeed [119]. However, nucleosome packaging and

positioning restrict transcription factor interaction to the DNA sequence. The regulation of cellular processes depends on the plasticity and dynamics of higher-order chromatin compaction to enable access of protein to the DNA sequences [44,45]. Genome activity is cell-specific, and requires nucleosome modulation to acquire localized accessibility. Nucleosome modulation can be accomplished in two ways. Firstly, through ATP-dependent chromatin remodeling, which causes sliding of the DNA with respect to the histone octamer [119,127]. Secondly, by acetylation, phosphorylation and methylation of N-terminal ends of core histones (histone tails), which are exposed and are able to mediate interaction with adjacent nucleosomes. Modification of the histone tails can stimulate exchange of histone variants of the histone octamer, thereby altering nucleosome stability [119,127,128]. Chromatin remodeling and gene regulation can also be modified directly at the DNA level by DNA methylation. In eukaryotes, DNA can be methylated by the addition of a methyl group at the cytosine of CpG dinucleotides. This process is catalyzed by DNA methyltransferase (DNMT) enzymes. As the result of this methylation, methyl-CpG domain (MBD) proteins recognize and bind methylated CpGs. This impedes the binding of transcription factors, thereby reducing accessibility to the DNA [128-130].

Genome accessibility plays an important role in the process of V(D)J recombination. The process of V(D)J recombination at TCR loci is regulated by the ability of RAG proteins to gain access to RSSs. Many features of open (=active) chromatin such as nuclease sensitivity, germline transcription, activating histone modifications and DNA hypomethylation of TCR genes seem to correlate with the time frame in which the V(D)J recombination process occurs [107,131]. Moreover, there is evidence that promoters, enhancers and transcription control of the TCR genes can regulate RAG1 binding to its substrate [131,132]. The process of TCR recombination requires the TCR locus, more precisely RSSs, to be accessible for targeting by RAG proteins [131] and repair via the NHEJ pathway. Therefore, in the context of TCR translocation formation one can assume that translocations can only succeed if oncogene loci are likewise accessible for V(D)J recombination-associated DNA modulating proteins; either to RAG protein complexes ('Type 1 translocation') or elements of the NHEJ repair pathway ('Type 2' translocations).

Nuclear proximity

It is generally accepted that mammalian genome organization is not random [114]. Based on this non-random organization, great steps have been made towards understanding translocation probability between translocation-prone genes [118,133,134]. Two chromosome translocation models that are applicable to translocation formation in the context of DSB repair and genome organization have been postulated; the 'breakage-first' and the 'contact-first' model [114]. In the 'contact first' model, translocations occur between DSBs that are positioned within translocation distance (inter-locus distances of ≤0.5μm [133]) from each other prior to

the formation of the breaks. In the 'breakage-first' model, translocations occur when DSBs roam the nucleus in search of another DSB and then are erroneously repaired [114].

Within mammalian cells two main DNA repair pathways are known. These are the NHEJ and the homologous recombination (HR) repair pathway [135]. The NHEJ pathway, in addition to being involved in the repair of DSBs that are induced during V(D)J recombination, is involved in the repair of other non-replication-associated breaks and is mainly active at the G1 phase of the cell cycle [135]. Just as during V(D)J recombination, factors such as the Ku80–Ku70 complex, the DNA-PK complex, XRCC4, DNA ligase IV, XLF and Artemis are recruited to the breaks in a hierarchical manner [114,135,136]. Repair via the HR pathway is initiated upon recognition of the DSB by the MRN (MRE11–RAD50–NBS1) complex [114,135]. MRN produces single-stranded DNA by resection upon recognition and binding, whereas replication protein A (RPA) and the recombination factors RAD51 and RAD52 bind to the single-strand ends in order to invade the homologous template and copy it in order to resolve the break. Assembly of particular repair components (either those involved in NHEJ or HR) results in the formation of DNA-repair foci at the site of DNA damage. DNA damage after irradiation in yeast leads to the formation of restricted repair foci [114]. This suggests that in yeast, DNA lesions migrate to localized repair territories. This migration of DSB to repair foci can lead to the formation of translocations in line with the 'breakage-first' translocation model. In mammalian cells, DSBs are mainly immobile in the nuclear space, allowing the different components for repair to diffuse to the location of the lesion. This in contrary to what is observed in yeast [114]. The immobilization of these breaks is thought to protect against formation of translocations. This suggests that translocation in mammalian cells can basically only occur according to the 'contact-first' model [114].

Nuclear proximity and TCR locus folding/ looping also play an important role in the process of V(D)J recombination. Looping allows for long-range interactions between genes for recombination purposes and influences expression of these genes over long genomic distances [125]. TCR genes that have been captured by RAG are kept within close proximity of each other upon DSB induction during the TCR recombination process. This enables joining in the synaptic complex. Nuclear localization of individual loci relative to their translocation partners has been shown to correlate with translocation probability, as well as determine translocation potential [133]. Thus, in the context of the formation of aberrant TCR translocations, one may expect that TCR and oncogene loci are within translocation distance from each other prior to the induction of breaks [3,104,107].

AIM OF THE THESIS AND RESEARCH OBJECTIVES

It is generally accepted that the V(D)J recombination machinery is involved in the for-mation of TCR-associated translocations. Although it is clear that post-translocation positive oncogenic selection ultimately determines if the outcome will be T-ALL, it is still not clear what pre-translocation conditions determine why particular oncogenes, and specific regions within specific oncogene loci, are targeted for recurrent involvement in TCR translocations. We hypothesize that specific regions within a particular oncogene locus are targeted for involvement in TCR translocations due to a combined locus sequence-specific vulnerability, locus accessibility and the required translocation distance of TCR and oncogene loci in the nuclear space. This all at specific stages of thymocyte development, particularly at time of V(D)J recombination. The aim of this project is to determine how sequence-specific features at DSB vulnerability, accessibility of oncogene breakpoint (BP) sites and TCR-oncogene spa-tial proximity all make oncogene loci vulnerable and susceptible to recurrent involvement in TCR translocations using a thymocyte model (Figure 4).
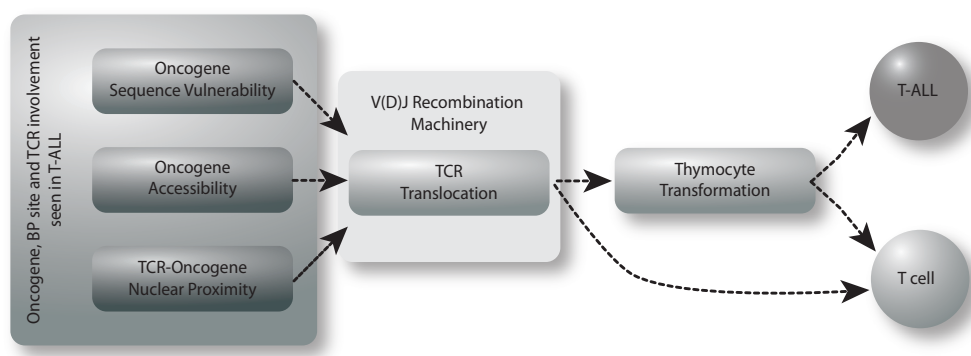
Figure 4. Schematic overview of experimental working model. Based on information on BP site, TCR locus, and oncogene locus involvement as seen in T-ALL we determined 1) if/which/how sequence-specific features could cause sequence vulnerability for DSB induction, 2) if oncogene BP sites are accessible during thymocyte develop-ment which makes these sites vulnerable for DSB inductions, and 3) if recurrence of particular translocations is caused by nuclear proximity between TCR loci and oncogene loci. The role of these factors in the formation of TCR translocations was determined in relation to the involvement and timing of the V(D)J recombination process. Based on our findings we aimed to determine what causes the recurrence of TCR translocations which can lead to T-ALL development.

The specific research objectives are as follows (Figure 4):

1. To determine the basis of localized genetic vulnerability at oncogene BP sites.
2. To determine localized accessibility of oncogene BP sites during normal human thymocyte development and to determine the "window of opportunity" for the formation of TCR translocation.
3. To determine the nuclear position of oncogene and TCR loci during normal human thymocyte development in order to establish translocation probability based on inter-locus distances between the TCR and oncogene loci.

SHORT OUTLINE OF THE THESIS

TCR translocations are formed as the result of mistakes that occur during the V(D)J recombination process. The mechanisms involved in the formation of both 'Type 1' and 'Type 2' translocations are known. However, little is known about the cause for the recurrent occurrence of 'Type 1' and 'Type 2' translocations, the extent of V(D)J recombination machinery involvement, TCR gene and oncogene involvement and BP site vulnerability and reoccurrence in these translocations.

In **Chapter 2** we analyzed a total of 117 breakpoints (BP) identified in TCR translocations and 118 non-TCR chromosomal aberrations to determine the **role of the V(D)J recombination machinery in the formation of TCR translocations.** We evaluated the presence of cRSS at BP sites and established their functionality either *ex vivo* by means of a recombination substrate assay and/or *in silico* with the online recombination information content (RIC) algorithm tool.

Chapter 3 addresses the role of **oncogene accessibility** and **nuclear proximity** between TCR and oncogene loci in the formation of TCR translocation. TCR translocations are formed during the V(D)J recombination process and are mediated by the V(D)J recombination machinery. The process of TCR recombination requires the TCR locus, more precisely RSSs, to be accessible for targeting by RAG [107,131]. Since TCR translocation formations can only succeed if both translocation partners are synchronously accessible to V(D)J associated DNA modulating elements, we determined whether different BP sites within different oncogene loci are accessible during thymocyte development during the time period that V(D)J recombination occurs. Furthermore, whether accessibility of BP sites correlate with oncogene, BP site, and TCR locus frequency of involvement in TCR translocations. In **Chapter 3.1** we assessed whether particular sequence-specific features that can render DNA susceptible to DSB inductions are localized at 'Type 2' translocation BP sites. This was done by determining the free energy (ΔG), GC-content and the presence of transposable elements (TE) at specific T-ALL TCR translocation BP sites found in *LMO2*, *TAL1* and *TLX1* oncogenes. Furthermore, we

determined sequence accessibility of these BP sites. This was done at different thymocyte differentiation stages by determining the methylation status of the BP sites by means of bisulfite sequencing, and by determining nucleosome occupancy by means of formaldehyde assisted isolation of regulatory elements (FAIRE). Nuclear proximity of TCR and oncogene loci is another requirement in enabling the physical exchange of genomic sequences during translocation [114]. The non-random genome organization, the 'contact-first' translocation model and the recurrence of oncogene involvement in TCR translocations suggest that nuclear proximity between oncogene and TCR locus should correlate with oncogene involvement and translocation occurrence in T-ALL [118]. In **Chapter 3.2** we determined whether nuclear inter-locus distances between TCRD or TCRB loci and the oncogenes *TLX1* and *TAL1* are within translocation distance in thymocytes. This to establish whether an decreased inter-loci distance occurs at particular stages of thymocyte development, making these genes more susceptible to translocation involvement at these stages. Three-color three-dimensional fluorescence *in situ* hybridization (3D FISH) analysis was used to determine the inter-chromosomal distances between each TCR and oncogene locus within the nuclear space at different stages of thymocyte development.

Chapter 4 discusses post-translocation associated matters in view of T-ALL development. Oncogene over-expression is a common result of TCR translocations. Specific T-ALL subgroups can be defined based on the presence of oncogene transcripts. However, the notion of oncogenic expression in T-ALL is based on expression differences seen in T-ALL compared to biologically unmatched reference cellular material [51,52,137]. In **Chapter 4.1** we determined the expression levels of *LMO2*, *LYL1*, *TAL1*, *TLX1*, *TLX3* and *NKX2-1* oncogene expression at different stages of normal thymocyte development. In addition to this, these levels are compared to levels found in T-ALL patients to discern between true oncogenic expression and residual expression in normal thymocytes. **Chapter 4.2** describes a novel TCR translocation partner in T-ALL and discuss how this oncogene could be involved in T-ALL development.

Finally, all findings are integrated in a general discussion in **Chapter 5,** and new emerging concepts on TCR translocation formation are discussed.

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Chapter 2

Breakpoint sites disclosure the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell Acute Lymphoblastic Leukemia

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ABSTRACT

Aberrant recombination between T-cell receptor (TCR) genes and onco-genes gives rise to chromosomal translocations that are genetic hallmarks in several subsets of human T-cell acute lymphoblastic leukemias (T-ALL). The V(D)J recombination machinery has been shown to play a role in the formation of these TCR translocations. Other, non-TCR chromosomal aberrations, such as *SIL-TAL1* deletions, have likewise been recognized as V(D)J recombination associated aberrations. Despite the postulated role of V(D)J recombination, the extent of V(D)J recombination involvement in the formation of TCR and non-TCR aberrations in T-ALL is not clear.

We performed a comprehensive *in silico* and *ex vivo* evaluation of 117 breakpoint sites from 22 different TCR translocation partners as well as 118 breakpoint sites from non-TCR chromosomal aberrations. Based on this exten-sive set of breakpoint data, we assessed the role of the V(D)J recombination machinery in the formation of chromosomal aberrations. Collectively, our results indicate that the V(D)J recombination machinery is not the driver, but rather the facilitator in the formation of TCR translocations and other non-TCR chromosomal aberrations in T-ALL. Analysis of pre- and post-translocation con-figurations of the TCR translocations showed that nothing is intrinsically wrong with the V(D)J recombination machinery in the leukemic cells. Based on our findings we propose an updated mechanistic classification on how the V(D)J recombination machinery contributes to the formation of TCR and non-TCR aberrations in human T-ALL.

INTRODUCTION

T cell differentiation is characterized by the tightly regulated process of T cell receptor (TCR) gene rearrangement, also referred to as V(D)J recombination. V(D)J recombination in TCR genes occurs in a precise order: TCRD, TCRG, TCRB, TCRA [1]. The V(D)J recombination process can be divided into two different but equally important phases. In the first phase, recombination activating gene (RAG) protein complexes consisting of heterodimeric RAG1 and RAG2 proteins, recognize, capture and bind recombination signal sequences (RSSs) that flank V, D and J genes [2]. Each RSS comprises a heptamer and a nonamer sequence, which are separated by either a 12 or 23 nucleotide spacer and recombine according to the 12/23 rule [3]. Following capture of an RSS by the RAG complex, DNA double-strand breaks (DSBs) are induced, resulting in coding ends (CEs), that immediately form hairpins, and blunt signal ends (SEs), that are rapidly fused into signal joints (SJs) [2,4,5]. The two hairpinned CEs are kept closely together by the Ku70-Ku80 proteins that associate with the DNA-PKcs, which binds Artemis. In the second phase, the DNA hairpins are nicked by endonuclease activity of Artemis, followed by deletion of nucleotides from the germline sequences and non-templated insertion of *de novo* nucleotides by terminal deoxynucleotidyl transferase (TdT) [5], and finally by joining of the CEs into coding joints (CJs). The entire repair process is orchestrated by components of the non-homologous end joining (NHEJ) pathway within the post cleavage synaptic complex (PCSC) [4,5].

Efficient recombination is restricted to recognition of RSSs by the RAG proteins and the subsequent repair of RAG-induced DSBs is confined to the PCSC. Despite these restrictions, molecular studies on translocation breakpoint (BP) sites have proven the involvement of the V(D)J recombination machinery in the formation of aberrant recombinations [5]. Aberrant recombination between TCR genes and oncogenes gives rise to chromosomal translocations that are common in immature T-lymphoid malignancies, such as T-cell acute lymphoblastic leukemias (T-ALL). These aberrant recombinations result in joining of oncogenes in the vicinity of TCR *cis*-acting regulatory elements such as enhancers, or in removal of negative regulatory elements (NRE) from the oncogene promoter [6]. As a result, expression of the involved oncogene, which mostly encodes for a transcription factor, becomes deregulated. The deregulation of the oncogene is considered as an early key event in T-ALL leukemogenesis [6].

Two TCR translocation mechanisms, designated as 'Type 1' and 'Type 2', have been postulated [4,5]. In 'Type 1' translocations, BP site sequences that are located outside the TCR loci, and resemble RSSs (also referred to as cryptic RSSs; cRSSs), are erroneously targeted by the RAG proteins. As a result, non-TCR sequences are introduced into the V(D)J recombination process and coupled to the TCR locus [5]. 'Type 2' translocations are formed, when DSBs at sites devoid of cRSSs located within the oncogene locus, are repaired and ligated to TCR

genes during the V(D)J recombination process via the NHEJ pathway [5]. Non TCR-associated chromosomal aberrations such as deletional aberrations (*SIL-TAL1*) and insertions (*HPRT1*) have also been appointed as V(D)J recombination-mediated events based on the presence of cRSSs at BP sites [7-10].

Until now, TCR-associated translocation mechanisms have mainly been evaluated for only a few BP sites by means of *ex vivo* experiments [4,6,10,11], basically confirming the concept of RAG mistargeting to cRSSs. These oncogenes and their respective BP sites were usually chosen due to their high frequency in T-ALL as well as for their probability to function as a cRSS based on structural criteria [11].

Despite the postulated role of V(D)J recombination, it is still not clear to what extent the V(D)J recombination machinery is mechanistically involved in the formation of TCR and non-TCR aberrations in T-ALL. Here we examined 117 molecularly defined BP sites and their sequences from 22 different TCR translocation partners as well as 118 BP sites from non-TCR aberrations in our T-ALL cohort and T-ALL cases described in literature. Based on this large and comprehensive *in silico* and *ex vivo* evaluation of BP sites, on analysis of TCR loci and oncogene involvement, and on analysis of pre- and post-translocation configurations of the TCR translocations, we critically re-evaluated the role of the V(D)J recombination machinery in the formation of chromosomal aberrations.

DESIGN AND METHODS

Human material and DNA isolation

Diagnostic bone marrow or peripheral blood samples from T-ALL patients were used. All samples were used after informed consent was obtained in accordance with Institutional Review Board guidelines (IRB; project MEC 2007-394) and the Declaration of Helsinki. The tumor load of the samples was in most cases approximately 90%. DNA isolation was carried out using the Qiagen DNA isolation kit (Qiagen, CA, USA) according to manufacturer's protocol.

Ligation-mediated polymerase chain reaction (LM-PCR)

Translocation junctions were identified by means of ligation-mediated polymerase chain reaction (LM-PCR)[12] In short, 1μg of T-ALL DNA and HeLa DNA were digested with either *DraI, PvuII* (Invitrogen, Grand Island, NY*) HincII,* or *StuI* (New England BioLabs, Ipswich, MA) blunt-end enzymes. After O/N digestion and phenol-chloroform (Sigma, Switzerland) extraction, DNA was precipitated. 50μM of adaptor was ligated to both ends of the DNA fragments. TCR translocation partners were detected via nested PCR with adaptor-specific AP1 and AP2 reverse primers and TCRD- or TCRB-specific primers [6,13]. PCR products of T-ALL samples

that give a dissimilar band size compared to products of the HeLa germline control samples were isolated using the QIAquick gel extraction kit (Qiagen) and sequenced with the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK).

Literature search and definition of TCR translocation BP sites

To create a comprehensive overview of all different TCR-associated translocations in T-ALL including the corresponding translocation partners and to determine the nature of these translocations, a broad survey was conducted on TCR translocation BP sites in human T-ALL. Sequence information on TCR translocations from our T-ALL cohort and T-ALL TCR translocations described in literature were used. First articles which give an overview of TCR and non-TCR associated aberrations seen in T-ALL were enquired [11,14-18]. Based on these results a search for each gene was done in the MEDLINE database through the PubMed search engine (http://www.ncbi.nlm.nih.gov/pubmed/), using a combination of the following search terms: <oncogene name>, and/or T-cell acute lymphoblastic leukemia, T-ALL, and translocation. Furthermore, the GenBank data base was searched. In total, 117 BP sites (from 22 different TCR translocation partners) and 118 BP sites from non-TCR aberrations (involving 3 different genes) were used in this study. In ~40% of the cases both derivate sequences were known. In cases lacking one derivate sequence, a region of 50 bp flanking the known fusion site was considered as potential BP site. The position of every BP site was determined relative to the transcription starting site (TSS) of each particular oncogene involved, using TSS positions as given by the UCSC database (http://genome.ucsc.edu).

In silico determination of cRSS functionality

The online recombination information content (RIC) algorithm tool (http://www.itb.cnr.it/rss/) was used to predict the functionality of regions of translocation junctions identified in our T-ALL patients and those found in the literature. Regions of 100 nucleotides upstream and downstream of the BP sites were analyzed for the presence of functional 12 and 23 nucleotide spacer cRSSs. Pass/fail thresholds given by the RIC tool were used (12 RSS: pass with RIC > -38.81, 23 RSS: pass with RIC >-58.45).

Ex vivo recombination substrate assay

Regions surrounding BP sites involving different oncogenes, or regions surrounding the negative regulatory element (NRE) of the *LMO2* locus were cloned into recombination-constructs as described previously [6]. Primers were designed with restriction enzyme linkers for cloning into the *MluI-SalI* or *SpeI-SacII* cassette (Supplementary Table 1). In total 22 new, different constructs were made. Fourteen inserts were cloned into the *MluI-SalI* cassette as previously described [6] (Supplementary Table 1). The remaining inserts were cloned to produce *LMO2* constructs each of which carried the 12 bp spacer cRSS (391 nt upstream

of the *LMO2* TSS) cloned within the *SpeI-SacII* cassette with one of the three 23 bp cRSSs (at positions 478, 5594 or 9107 upstream *LMO2* TSS) cloned within the *MluI-SalI* cassette (Supplementary Figure 1). *LMO2* 23bp-spacer cRSS (BP position -6,902) with a RIC score of -57.17, which in our previous study was proven to function as cRSS [6], was used as a positive control in the recombination substrate assay. To make sure that we had no bias with respect to the 12/23 rule, recombination vectors carrying an authentic Dδ3 RSS with both a 12 and a 23 bp spacer were used which was also tested in an inverted orientation [6]. The recombination substrate assay was performed as previously described [6].
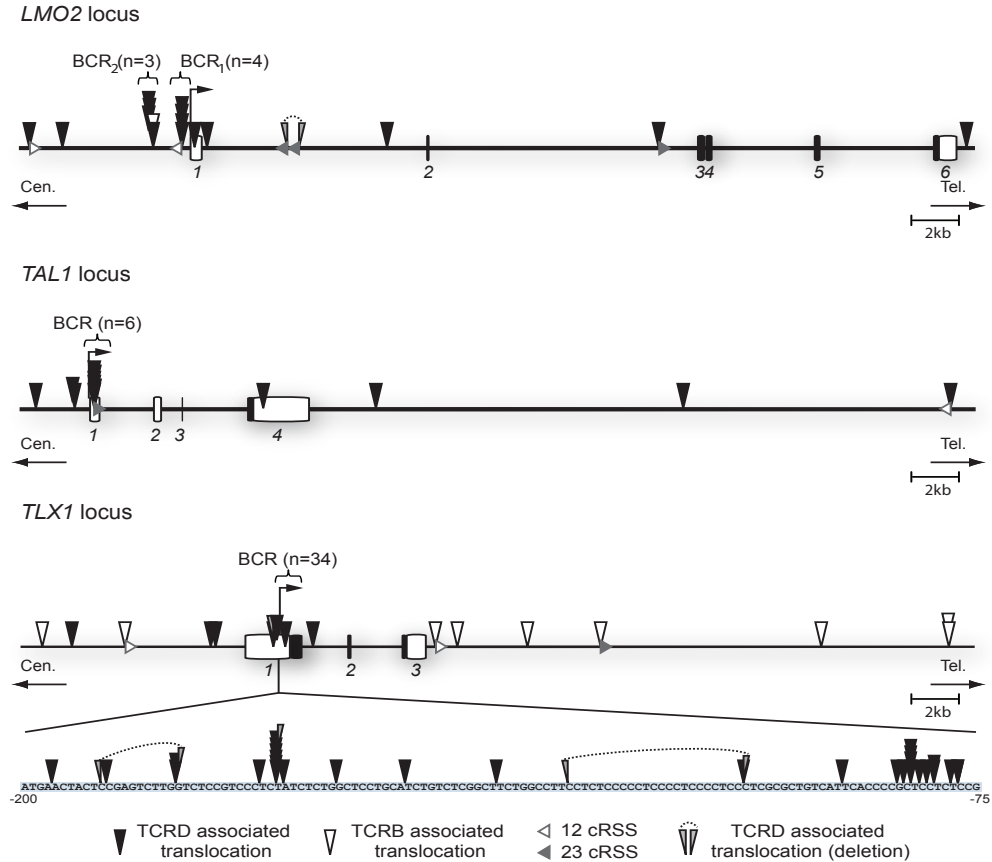
Figure 1. Overview of T-ALL associated BP position and cRSS in *LMO2*, *TAL1* and *TLX1* locus. The position of every BP analyzed in this study is given for *LMO2*, *TAL1* and *TLX1* respectively. For each BP, the associated cRSS is given. Positions of the most upstream and most downstream BP are given relative to the TSS. Breakpoint cluster region (BCR) (a region containing >2 BP sites with ~50bp between adjacent BP sites)

Table 1. Oncogene, TCR locus and translocation type involvement in 117 TCR translocations BP sites

Gene	BP (region)	Distance to TSS (nt)	Evaluated cases (numbers)	TCR involvement (numbers)			Translocation type (numbers)		Reference
				TCRD	TCRA	TCRB	Type 1*	Type 2	
LMO2	5'of BCR$_2$	-6,902/-5,654	2	2	0	0	1	1	6,19
	BCR$_2$	-1,849/-1,846	3	3	0	0	0	3	6 R
	5'of BCR$_1$	-1,659	2	1	0	1	0	2	6,19
	BCR$_1$	-384/-392	4	4	0	0	4	0	6,20,21
	3'of BCR$_1$	+169/+34,160	6	6	0	0	3	3	6,22,23R
			17	16	0	1	8	9	
TAL1	5'of BCR	-3,208/-817	3	3	0	0	0	3	24-26R
	BCR	+427/+431	6	6	0	0	6	0	24,26,27
	3'of BCR	+ 10,780/+53,085	4	3	0	1	1	3	28-31
			13	12	0	1	7	6	
TLX1	5'of BCR	- 10,330/+294	6	4	0	2	1	5	32-34
	BCR	+575/+818	35	35	0	0	0	35	32,35,36
	3'of BCR	+2,112/+3,338	8	1	0	7	2	6	9,32,37
			49	40	0	9	3	46	
BCL11B	n.a	+48,651^i	1	1	0	0	0	1	13
BMI1	n.a.	-277,473	1	0	0	1	0	1	38
CCND2	n.a.	- 61,328	1	0	0	1	1	0	39
MYB	n.a.	+53,891/+53,903	2	0	0	2	0	2	40
		+144,584	1	0	0	1	1	0	40
HOXA6	n.a.	-2,179	1	1	0	0	0	1	41
HOXA9	n.a.	-2,992/-501^i	2	0	0	2	0	2	42,43
LCK	n.a.	+20,253/+25,085	2	0	0	2	1	1	44,45
LMO1	n.a.	-9,644	1	1	0	0	0	1	46
		-5,258	2	2	0	0	2	0	47,48
LMO3	n.a.	+224,944	1	0	0	1	0	1	43
LYL1	n.a.	+8,444	1	0	0	1	0	1	46
		+787	1	0	0	1	0	1	49
MYC	n.a.	+5,96/+10,318	4	0	4	0	1	3	50-53
		+239,565	1	1	0	0	0	1	50,54
NKX2-1	n.a.	+4,286	2	1	0	1	0	2	55
NKX2-4	n.a.	-494	1	1	0	0	0	1	Current Study
NKX2-5	n.a.	+35,271	1	1	0	0	0	1	56
NOTCH1	n.a.	+39,552/+42,969	5	0	0	5	2	3	57-59
OLIG2	n.a.	+84,224	1	0	1	0	0	1	60
TAL2	n.a.	+ 31,795/+31,766	3	0	0	3	3	0	61
TCL1A	n.a.	-145,630/-99,998^i	2	0	1	1	0	2	62,63
TLX3	n.a.	-50,034	1	1	0	0	0	1	64
			38	10	6	22	11	27	-
Total			**117**	**78**	**6**	**33**	**29**	**88**	-

*based on cRSS functionality determined by *in silico* and/or *ex vivo* analysis, ^{i} inversion, n.a.: not applicable
^{R} Sequences found in the GenBank database

RESULTS

LMO2, *TAL1*, and *TLX1* are the predominant TCR translocation partners and show clear translocation BP clusters

In the 117 TCR translocations that we evaluated, a total of 22 different oncogenes were identified as TCR translocation partner. *LMO2* (15%), *TAL1* (11%), and *TLX1* (25%) loci were most frequently involved in these translocations, while other TCR translocation partners were less frequently observed (~32% of total, and each <5%) (Table 1 and Supplementary Table 2). The translocation frequencies reported here are based on the available molecular data, and may therefore not reflect true prevalence of these oncogenic TCR translocations in T-ALL .

Translocation BP sites in the *LMO2*, *TAL1* and *TLX1* loci appeared to be localized in distinct regions and were either densely clustered in breakpoint cluster regions (BCRs; defined as more than two BP sites within a region of 50 bp from each other, and sharing a common translocation DSB type) or localized as single BP sites (Figure 1, Table 1, and Supplementary Table 2).

The *LMO2* locus has two distinct BCRs (hereafter referred to as BCR_1 and BCR_2), with about 40% (7/17) of the BP sites associated to a BCR. About half (6 of 13) of the *TAL1* BP sites and 67% (33/49) of the *TLX1* BP sites formed a BCR (Figure 1).

LMO2, *TAL1* and *TLX1* BCRs localize in the direct vicinity of a transcription starting site (TSS), at the 5'-end of the coding region of the gene, while in contrast the *TAL2* BCR localized at the 3'-end of the oncogene. The localization of BCRs in the vicinity of a TSS suggests a localized accessibility at these sites, presumably as a consequence of transcription activity which in turn is associated with a bias towards mutation formation [65]. The majority (~90%) of the BP sites are found outside of the coding regions of the genes, with ~65% being localized at the 5'-end of the gene and ~25% at the 3'-end, while ~10% are localized within the coding regions (Figure 2A). This indicates that the localization of a translocation BP site, particularly the BCR, is oncogene-specific, which is most likely associated to the selective advantage gained from the translocation, dependent on the function of the transformed gene.

Even though the exact position of the BP site within the coding region of the oncogene locus and the resulting selective advantage ultimately determine if the outcome of a translocation will be the development of a T-ALL, this does not yet elucidate why specific BP sites become recurrently involved in these aberrations while others do not.

TCR translocations in T-ALL mostly involve the TCRD locus

Next, we analyzed whether specific TCR loci show preference for oncogenic translocations in T-ALL. This analysis revealed that the majority (~67%) of all TCR translocations involved the TCRD locus. About 94% of *LMO2*, 92% of *TAL1*, and 82% of *TLX1* translocations
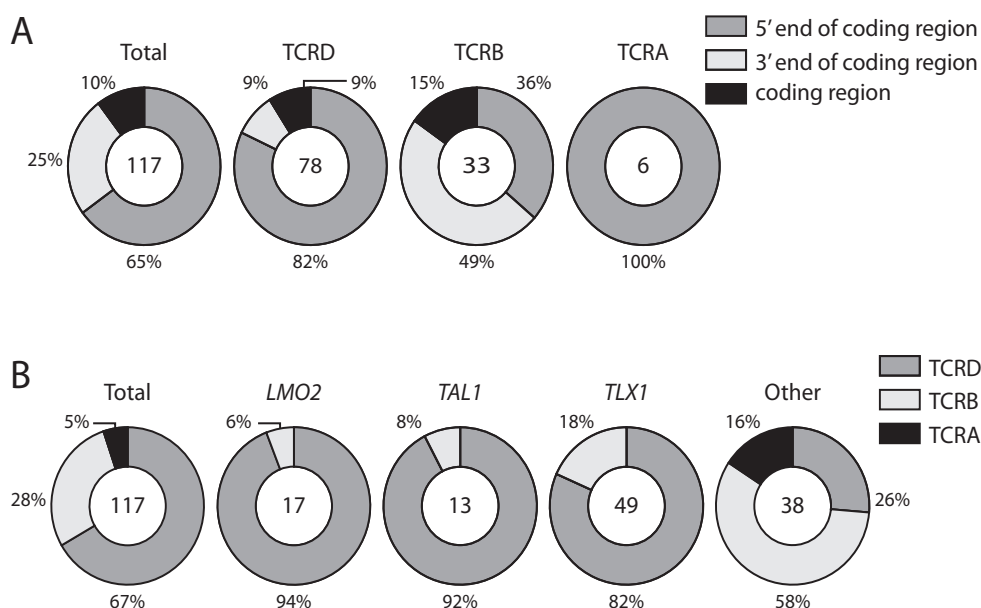
Figure 2. Pie charts illustrating percentages of TCR locus involvement in T-ALL. A. Pie charts illustrating percentages of BP site localization relative to the functional regions of the oncogenes involving all BP sites or involving a particular TCR locus. B. Pie charts illustrating percentages of TCRD, TCRB, and TCRA related translocations involving *LMO2*, *TAL1, TLX1* and the rest of the analyzed translocation partners.

occurred during an attempted TCRD gene rearrangement (Figure 1, 2B and Supplementary Table 2). The BP sites associated to the *TLX1* BCR exclusively concerned TCRD translocations, while all BP sites located at the 3'-end of the *TLX1* exon 3 exclusively involved the TCRB locus (Figure 1). Approximately 58% of BP sites of the less frequent T-ALL TCR translocation partners translocated to the TCRB locus (Figure 2B).

Analysis of the location of the BP site in relation to the TCR locus involved in the translocation, showed that ~82% of all TCRD translocations involved the 5'-end of the oncogenes, while 5'-regions of oncogenes were involved in only ~36% of the TCRB translocations (Figure 2A).

The observed prevalence of TCRD involvement at the 5'-end of oncogenes, together with the high frequency of TCRB translocations (~49%) at the 3'-end of oncogenes, is in concordance with the theory of oncogenic regulation being dependent on the actual TCR locus involved [32,66]. While the majority of translocations (~67%) involves the TCRD locus, TCRA was only involved in ~5% (n=6) of the translocations and exclusively concerned *TCL1A, OLIG2* and

MYC translocations. Involvement of the TCRG locus was not found in any of the analyzed translocations.

Further analysis into TCR gene involvement showed that about ~33%, ~26% and ~26% of the TCRD translocations occurred during attempted Dδ-Dδ, Dδ-Jδ or Vδ-Dδ recombination, respectively. No significant preference for a specific TCRD gene was observed in these translocations. The TCRB translocations predominantly (~76%) occurred during Dβ-Jβ recombinations. Only ~17% of the translocations occurred during Vβ-Dβ recombination. Remarkably in ~4% of the cases, breaks seemed to be induced at two Jβ genes simultaneously. Five translocation events occurred during the attempted Vα-Jα recombinations and in one case the translocation seemed to have occurred during simultaneous induction of DSBs at two Jα genes.
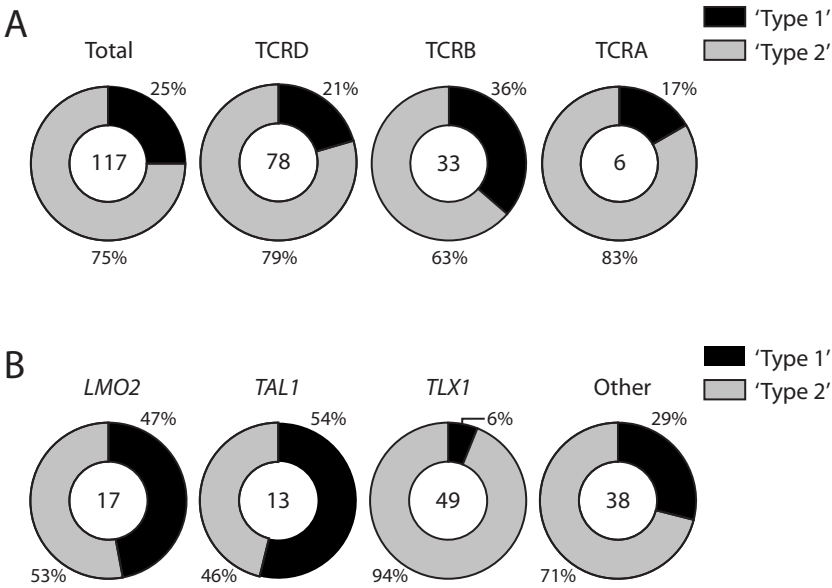
Figure 3. Pie charts illustrating percentages of translocation type occurrence in T-ALL. A. Percentages of the occurrence of 'Type 1' and 'Type 2' translocations involving the TCRD, TCRB, and TCRA genes. B. Percentages of 'Type 1' and 'Type 2' translocations involving either *LMO2*, *TAL1*, *TLX1* and the remaining translocation partners.

TCR translocations in T-ALL are mostly formed via the 'Type 2' translocation pathway

Next we determined which translocation mechanisms ('Type 1' or 'Type 2') were involved in these TCR translocations. The *in silico* testing of the 117 BP sites, identified a cRSS at only 27 BP sites (23%), which were found within the *LMO2*, *TLX1*, *TAL1*, *TAL2*, *MYC*, *NOTCH1*, *LMO1*, *C-MYB* and *LCK* oncogene loci (Table1, Supplementary Table 2). *Ex vivo* studies in our laboratory confirmed that BP sites associated to *TLX1*, *LMO1*, *LMO3,* and *LYL1* that were lacking a cRSS were not induced by RAG (Supplementary Table 3), thus supporting the idea that translocations involving these sites were formed via the 'Type 2' translocation pathway. This further confirmed our previous findings of a high correlation between the RIC score obtained from *in silico* analysis and the translocation efficiency determined by means of *ex vivo* recombination substrate assay [6]. Only about 25% of the whole spectrum of TCR translocations in human T-ALL is driven by RAG mistargeting of cRSSs ('Type 1' translocations). Interestingly, a large fraction (~36%, 16/78) of the TCRB translocations occurred via the 'Type 1' translocation pathway, while this was the case for only ~21% (12/33) of TCRD translocations and ~17% (1/6) of TCRA translocations (Figure 3A). In ~75% of cases the involvement of the V(D)J recombination machinery is mainly apparent due to the involvement of a TCR locus in these formations. Furthermore, this suggests that the V(D)J recombination machinery per se is not the driving force in the induction of DSBs at the majority of BP sites that lead to TCR translocation formation. *TLX1* associated translocations are mostly governed by the 'Type 2' translocation pathway. Contrary to current assumptions, 3 (6%) of the *TLX1* translocations seem to have resulted from the 'Type 1' translocation, as these BP sites are associated with functional cRSSs as defined by *in silico* analysis based on RIC threshold levels. Interestingly, these three cRSS-related *TLX1* translocations are all TCRB-associated translocations (Figure 1). Importantly, the *TLX1* BCR and *LMO2* BCR$_2$ did not associate to a cRSS, while both the *LMO2* BCR$_1$ and the *TAL1* BCR translocations are associated to a cRSS. Other translocation partners mostly (>71% of cases) translocated via the 'Type 2' translocation pathway (Figure 3B). This clearly shows that the majority of TCR translocations are not caused by RAG-cRSS interactions.

cRSSs in oncogene loci are not randomly involved in T-ALL translocations

cRSSs are distributed throughout the entire human genome at an average density of one cRSS per 500 bp [67]. The *LMO2* locus comprised 109 cRSS, of which only 5 (<4%) were associated with a BP site. The 12 bp spacer cRSS with the highest RIC score (-29.21) was identified at the *LMO2* BCR$_1$. Interestingly, the 23 bp spacer cRSS with the highest RIC score (-48.74) was not associated to any of the *LMO2* BP sites in our study, while the position and orientation of this cRSS could have caused the oncogenic activation of *LMO2* upon involvement in a translocation (Supplementary Figure 2). Of the 56 cRSSs identified in *TAL1*, the *TAL1* BCR

did associate to its most efficient 23 bp spacer cRSS (RIC score: -46.34), while no BP sites were associated to the 12 bp spacer cRSS with the highest RIC score (-31.10). Of the 139 *TLX1* cRSSs identified, the single BP site UPN474 was associated to the most efficient 23 bp spacer cRSS (RIC score: -47.754), while no BP sites were associated to the 12 bp spacer cRSS with the highest RIC score (-29.66). Interestingly, the *TLX1* and *LMO2* 12 bp spacer cRSSs as well as the *TLX1* and *TAL1* 23 bp spacer cRSS have comparable RIC scores, implying similar recombination efficiency. However, none of these *TLX1* cRSSs are associated to a *TLX1* BCR, even though they are located within a region (at the 3'end of the *TLX1* locus) known to be involved in translocations (Supplementary Figure 2).

These findings show that the targeting of cRSSs for involvement in a translocation is not random and is independent of the recombination efficiency of a cRSS, implying that other (genetic or epigenetic) factors could be eventually decisive for break susceptibility at these particular sites.

The involvement of the V(D)J recombination mechanism can be extended to non-TCR and non-cRSS associated aberrations.

Involvement of the V(D)J recombination machinery in the formation of TCR transloca-tions in T-ALL is evident, particularly when considering the involvement of the TCR genes in these aberrations. However, V(D)J recombination activity in T-ALL is clearly not limited to rearrangements involving TCR loci, as cross-lineage Ig gene rearrangements (mainly involving IGH) have been observed in low frequencies as well [68,69].

The attribution of the V(D)J recombination machinery in the formation of other, non-TCR related chromosomal aberrations in T-ALL is however less evident. We analyzed 118 BP sites of non-TCR associated aberrations observed in T-ALL by determining the presence of cRSS at the different BP sites and evaluated the potential role of the V(D)J recombination mechanism in the formation of these aberrations (Table 2). *HPRT1* related insertions, though not directly implicated in T-ALL leukemogenesis, do occur in normal peripheral T-lymphocytes [7,9,70]. Even though these insertions do not seem to have direct oncogenic effect, we did analyze them to delineate the mechanism involved.

HPRT1 has frequently been shown to carry a complex deletion/insertion rearrangement, where gross regions of the TCRA locus are inserted into the *HPRT1* locus [7,9,74]. One of the three TCRA insertions identified in this study (the BP site of two other cases could not be identified), showed involvement of a cRSS at the *HPRT1* locus (Table 2). A *BICD* insertion (9q22) into *HPRT1* locus has also been described. In this case both ends of the *HPRT1* BP sites carried a cRSS, while only one end of the inserted *BICD* sequence carried a cRSS. In addition to insertions, deletions are also common in the *HPRT1* locus. Analysis of the different BP sites showed that the BCRs related to the *HPRT1* deletions are associated to a cRSS; however, in about 50% of the deletions no cRSSs could be identified at the deletion BP sites (Table 2).

Table 2. Determination of RAG involvement at BP sites involved in non-TCR chromosomal rearrangements

Mutation	Oncogene Gene	Distance to TSS	in silico determined cRSS present at BP	RIC score		Distance	in silico determined cRSS present at BP	RIC score	Reference*
Deletion									
SIL-TAL1		-3.792/-3.802*	CACAGCC-23-AAGGAAAAG	-46.62		+396/+420*	no	-	8,25,71,72
			CACAGAG-12-GCCAAAACT	-28.12		+405/+420*	no	-	71,72
		+5.292*	CACACAC-12-GATAGAAAC CACACAC-23-ATAGAAACA	-38.63 -50.05	SIL	+407*	no	-	71
		-2.190*	CACAGCC-23-AAGGAAAAG	-46.62		+1.067*	no	-	72
		-6.187*	no	-		+407*	no	-	72
	LMO2	+2,687*	no	-	RAG2	+3,110*	no	-	73
LMO2-RAG2		-31,943*	no	-		-71,278*	no	-	73
Insertion/ Deletion									
		+687*	CAGTGTG-23-GGCAAAACC	-36.92	RSS^Vα24/ RSS^Jα29(ins.)	nd	CACAGTG-23-ACGCAAACC /CACAGTG-23-ACTCAAACC	-27.57 /-27.83	7,9
		nd	nd	nd	RSS^Vα22/ RSS^Jα45(ins.)	nd/nd	CACAGTG-23-ACACAAACC / CACTCTG-12-ACATAAACT	-25.91 /-23.23	9,74
	HPRT1 Deletions/ Insertions	nd/ nd	nd/nd	nd/ nd	RSS^Vα34/ RSS^Jα44(ins.)	nd/nd	CACAGCG-23-CTCCAAACC /CACTGTG-12-ACAGAAACC	-39.11 /-21.54	9,74
		+684 /+20.693(del)*B	CAGTGTG-12-GGCAAAACC/ CACACAC-12-ACAAATACA	-36.92 -28.28	BICD1 (ins.)(del)B	+7,693/ +8,282*	-/ CAAAGTG-23-TGCGCCCGG	-56.46	7,9
		33,400/33,422	no	-		-	-	-	70
		33,359/23,551	no	-		-	-	-	70
		11,405/17,892	no	-		-	-	-	70
		22,664/29,064	no	-		-	-	-	70
		13,588/26,981	no	-		-	-	-	70
		14,949/27,861	no	-		-	-	-	70
	HPRT1 Deletions	1,980/28,526	no	-		-	-	-	70
		23,322/27,499	no	-		-	-	-	70
		13,092/13,447	no	-		-	-	-	70
HPRT1^A		+667/+679 (n=15)	CAGTGTG-12-GGCAAAACC	-36.92		+20,658 /+20,679	CACACAC-12-ACAAATACA/ CACACAC-23-TTTGTGTGT	-28.28 -57.793	75
		+672/+677 (n=2)	CAGTGTG-12-GGCAAAACC	-36.92		+18,581 /+18,590	CACACAC-12-TCTTAATCC CAGAGAG-23-TAAAAAGTG	-36.19 -63.91	75
		+678 (n=1)	CAGTGTG-12-GGCAAAACC	-36.92		+20,998	-	-	75

A HPRT1 associated insertions shown are mutations found in normal human T cells, *The precise BP position is not known due to type of break, No: no functional cRSS found at that BP position according to the RIC algorithm analysis., -: no RIC score, nd: not determined, X/Y(del): Deletion with X as the 5'BP and Y as the 3' BP position, X(ins.):insertion of that particular sequence in to another locus, B The presence of a cRSS tested at both sites.

Role of V(D)J recombination in (non-) TCR aberrations

Analysis of BP sites of *SIL-TAL1* deletions (del(1p)) showed that about 99% (84/85) of all *TAL1* BP sites are associated with a cRSS, whilst in contrast to earlier suggestions [10,11], none of the *SIL* BP sites tested had a cRSS in their vicinity (Table 2). Although *TAL1* also frequently translocates to TCR loci, interestingly none of the *TAL1* BP sites involved in *SIL-TAL1* deletions were in the direct vicinity of the *TAL1* BP sites involved in TCR translocations. The single *SIL-TAL1* case that lacked involvement of a cRSS at either of the BP sites, did have eight untemplated nucleotides at the junctional region [72], suggesting the presence of TdT activity during ligation of these BP sites.

The cryptic deletion del(11)(p12p13) fuses the *LMO2* gene to the *RAG2* gene [73]. No cRSS was identified at the *RAG2* or *LMO2* BP sites. However, a potential untemplated nucleotide is present at the junctional region (Table 2). Despite the high occurrence of *LMO2* in TCR translocations, *LMO2* deletional BP sites do not co-localize with BP sites associated in *LMO2*-TCR translocations. We previously showed that *LMO2* activation was associated with the loss of the *LMO2* negative regulatory element (NRE) rather than due to juxtaposition of *LMO2* to the TCRD enhancer [6]. Furthermore, we identified the presence of one 12 bp spacer cRSS 3' of the *LMO2* NRE and three different 23 bp spacer cRSSs at the 5' side (Supplementary Figure 1) [6]. Since many T-ALL cases have unresolved *LMO2* activation and these cRSS lay in the orientation for RAG-mediated NRE deletion (Supplementary Figure 1A), we determined the mutual recombination potential of these cRSSs. To this end, the capability of these cRSSs to mediate a deletion *ex vivo* was tested by means of a recombination substrate assay. The *ex vivo* analyses showed that none of the 12 bp / 23 bp cRSS combinations tested (Supplementary Table 4, Supplementary Figure 1A) invoked such recombination.

Collectively, these findings suggest that the V(D)J recombination mechanism can also be involved in non-TCR and seemingly in particular cases in non-cRSS associated aberrations.

DISCUSSION

The activity of the V(D)J recombination machinery in thymocytes is primarily limited to the TCR locus. This restriction is determined by an interplay of sequence-specific features and TCR locus accessibility. This recombination process is confined to PCSCs, which in principle ensures the maintenance of genome stability. Nevertheless, it is clear that the V(D)J recombination machinery is involved in the formation of genomic aberrations by mediating DSB induction and repair of breaks in non-TCR loci during the recombination process. Based on our extensive translocation typing analysis on 117 TCR translocation-associated BP sites identified in T-ALL we established that only ~25% of translocations are 'Type 1' translocations. This reinforces previous notions based on smaller series that TCR translocations predominantly occur via the 'Type 2' translocation pathway [5,11]. The data also shows that

the presence or absence of a cRSS at a BP site does not correlate with the frequency of involvement of particular oncogenes in T-ALL.

The observed prevalence (~82%) of TCRD involvement at the 5'-end of oncogenes, and TCRB translocations (~49%) that concern the 3'-end of oncogenes, is in concordance with the theory of oncogenic regulation as a result of the actual TCR locus involved [32,66]. Although the exact position of the BP site within the oncogene locus and the resulting selective advantage ultimately define the occurrence of a translocation in T-ALL, this does not elucidate why specific regions become recurrently involved in aberrations while others do not. The high occurrence of breaks at particular sites, irrespective of the presence or absence of a cRSS, suggests that a certain sequence susceptibility for acquiring DSBs renders translocation BP sites vulnerable for breaks. This theory is substantiated by the fact that particular cRSSs involved in translocations are preferentially targeted over other highly efficient cRSSs found within the same region (as seen for *LMO2* and *TAL1*). This is also underlined by the lack of involvement of *TLX1* cRSSs with RIC scores comparable to *LMO2* and *TAL1* BCR cRSSs, whilst other BP sites in the vicinity of these allegedly highly recombination efficient cRSSs are associated to non-RAG mediated breaks. Thus, other sequence-specific features (transposable elements, association with origin of replication, GC-content or free energy levels [76-78]) could be rendering these sites more susceptible for DSB induction, possibly exceeding the vulnerability level provided by cRSSs in the vicinity.

The majority of translocations (~67%) involve the TCRD locus while TCRG was not found in any of the analyzed translocations and has so far never been reported in TCR translocations in T-ALL. The lack of TCRG translocations is remarkable, as most reported trans-rearrangements do involve the TCRG locus [79,80], suggesting that the locus can be implicated in V(D)J-associated aberrations. The high prevalence of TCRD associated translocations suggests proneness for aberrant TCRD recombinations or a selective advantage upon involvement of the TCRD locus. Most of the TCRD and TCRB translocations involved the Dδ and Dβ genes, respectively. This suggests an increased chance for the occurrence of an erroneous recombination during the earliest phases of TCRD and TCRB recombination, i.e. in the DN1 to DN2 (Dδ-Dδ), the DN3 (Dδ-Jδ) and the ISP to DP CD3- (Vδ-DJδ, Dβ-Jβ) thymocyte subsets [1]. Our findings show that particular oncogenes exclusively translocate with a specific TCR locus. Considering this exclusivity and the fact that TCR loci recombine at specific stages of thymocyte development we can assume that synchronous accessibility of a specific oncogene locus and the TCR locus may play an important role in determining oncogene-TCR locus interactions during V(D)J recombination.

Identification of pre- and post-translocation TCR recombination configurations of derivate chromosomes as seen in T-ALL provides evidence that RAG proteins are able to initially target an authentic RSS prior to mistargeting a cRSS in 'Type 1' translocations. Furthermore, they show that correct joining of CEs within the same cell is possible prior or subsequent to

'Type 2' translocation formation. Ongoing recombination following the translocation event is a phenomenon that has been previously described for *TAL2* translocations which is thought to contribute to the post-translocation oncogenic effect [81,82]. Particular RAG mutations have been shown to cause the formation of unstable RAG post-cleavage synaptic complexes that allow DSBs to participate in the error-prone alternative NHEJ repair pathway [83]. We cannot fully exclude RAG mutations and post-cleavage complex instability as the cause of the T-ALL derived translocations as analyzed in this study. However, the fact that normal recombination can precede or follow a translocation in the very same cell implies that nothing is intrinsically wrong with the V(D)J recombination machinery in these aberrant cells.

Involvement of the V(D)J recombination machinery in the formation of TCR transloca-tions in T-ALL is evident, particularly when considering the involvement of the TCR genes in these aberrations. However, the attribution of the V(D)J recombination machinery in the establishment of other, non-TCR related chromosomal aberrations in T-ALL is less evident. Even though recombination could not be invoked between two *LMO2* NRE flanking cRSSs, the *HPRT1(-BICD1)* deletions/insertions show that interactions between cRSSs can occur [7,9]. Even though such cRSS-cRSS interactions have not yet been identified in T-ALL, involvement of the V(D)J recombination machinery in the formation of these aberrations is evident based on the presence cRSSs at the BP sites and the insertion of untemplated nucleotides at the junctions [7,9].

Our findings indicate that the V(D)J recombination mechanism can also be involved in non-TCR aberrations and in particular cases potentially even in non-cRSS associated aber-rations. Despite the fact that cRSS-cRSS interactions have not been observed in T-ALL so far, they are comparable to 'Type 1' (RSS-cRSS) recombinations. Hence, we propose that these cRSS-cRSS recombinations be denoted 'Type 1B' recombinations in which a second cRSS replaces the authentic TCR RSS (Table 3).
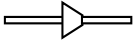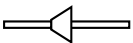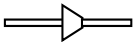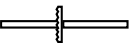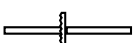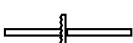
Analogous to *TAL1* translocations, the V(D)J recombination machinery is also involved in *SIL-TAL1* deletions [10], as deduced from the presence of cRSSs at the *TAL1* deletion BP sites only. The addition of random nucleotides at these junctions, a TdT-driven hallmark of the V(D)J recombination process, further confirms this involvement. Notably, it cannot be excluded that DNA polymerase mu (Polμ) can also be involved next to TdT [84]. The lack of cRSSs at the *SIL* BP sites however, suggests that similar to 'Type 2' translocations, non-RAG induced DSBs within *SIL* are repaired via the NHEJ pathway. This could be indicative of a variant of the 'Type 2' recombination pathway. In this proposed 'Type 2B' aberration (Supplementary Figure 3, and Table 3) *TAL1* would act as the carrier of the cRSS instead of the authentic TCR RSS. Although not as obvious, a role for the V(D)J recombination machinery could also be argued in cases such as *SIL-TAL1* and *LMO2-RAG2* deletions [72,73] that despite the lack of a cRSS at the reciprocal BP sites, do show potential non-templated nucleotides at the junctions. Such recombinations could be tentatively referred to as 'Type 3' aberrations (Table 3). Overall

these findings suggest that the involvement of the V(D)J recombination machinery in the formation of chromosomal aberrations is not restricted to TCR loci and/or cRSS participation.

In summary, here we demonstrate that only about 25% of the whole spectrum of TCR translocations in human T-ALL is driven by RAG mistargeting of cRSSs ('Type 1' translocations). In ~75% of cases the involvement of the V(D)J recombination machinery is mainly apparent due to the involvement of a TCR locus in these formations (Type 2 translocations). Non-TCR chromosomal aberrations in T-ALL do not involve RAG mistargeting either, with the exception of *SIL-TAL1* deletions, which can be considered as a 'Type 2B' aberration (Supplementary Figure 3). Thus, V(D)J recombination associated aberrations mostly result from repair mistakes rather than RAG mistargeting of cRSS. Furthermore, our findings argue that nothing is intrinsically wrong with the V(D)J recombination mechanism in these T-ALL, since pre- and post-translocation chromosome configurations indicate normal RAG targeting, repair and recombination of the involved TCR locus in the same cell.

Collectively, our results suggest that the V(D)J recombination machinery is generally not the driving force in the onset of the formation of TCR translocations and other, non-TCR genetic aberrations in human T-ALL. Rather it appears to play a role in facilitating the formation of these aberrations through repair, following any type of DSB breaks in particular

Table 3. Conditions to determine the involvement of the V(D)J recombination mechanism in the formation of chromosomal aberrations and to define the aberration type

V(D)J recombination associated aberration Type	TCR involvement	cRSS involvement	Condition		Frequency*	Typical aberrations
Type 1A	+	+	TCR locus with RSS	locus with cRSS AND	~14%	TCR-*LMO2*
Type 1B**	-	+	locus with cRSS	locus with cRSS AND	-	*HPRT1-BICD***
Type 2A	+	-	TCR locus with RSS	locus without cRSS AND	~40%	TCR-*TLX1*
Type 2B	-	+	locus without cRSS	locus with cRSS AND	~40%	*SIL-TAL1* (frequent)
Type 3***	-	-	locus without cRSS	locus without cRSS AND	~2%	*SIL-TAL1* (rare types)

*percentages based on both TCR translocation and non-TCR molecularly defined aberrations in T-ALL analyzed in this study,
**so far not (yet) identified in T-ALL,
***with untemplated nucleotides at junctional regions, ◀:RSS, ◁:cRSS, ▯: no cRSS.

oncogene regions. Based on these findings we propose an extended and comprehensive mechanistic classification scheme on how the V(D)J recombination machinery contributes to TCR-associated and non-TCR associated aberrations in T-ALL (Table 3).

ACKNOWLEDGEMENTS

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SUPPLEMENTS
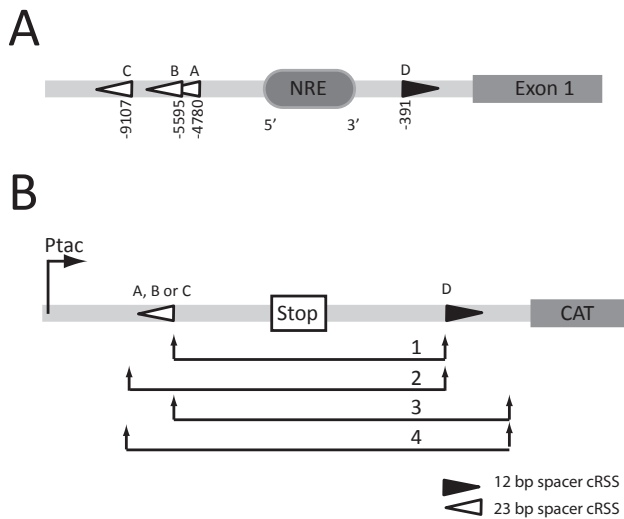
Supplementary Figure 1. A. Overview of position of cRSSs with respect to the NRE in the *LMO2* locus. Black triangle represents the 12 bp spacer cRSS at position 391 upstream of the TSS 3'of the NRE. White triangle represents the three 23 bp spacer cRSSs at positions 4780, 5594 and 9107 upstream of the TSS 5'of the NRE. Shown exon 1 of *LMO2*. B. The *LMO2* NRE 23 bp spacer cRSS were cloned in the upstream *Mlu*I-*Sal*I cassette, and the *LMO2* NRE 12 bp spacer cRSS was cloned in the downstream *Spe*I-*Sac*II cassette. P*tac*: promoter; CAT: chloramphenicol acetyltransferase gene; Stop: transcriptional terminator. Black triangle represents the 12 bp spacer cRSS and the white triangle represents one of the three 23 bp spacer cRSSs. Pathway 1: V(D)J-mediated recombination between 12 bp spacer RSS and 23 bp spacer cRSS; pathway 2: V(D)J-mediated recombination between 12 bp spacer RSS and other BP ; pathway 3: V(D)J-mediated recombination between 23 bp spacer RSS and other BP; pathway 4: break repair mediated recombination (defined as non-V(D)J recombination mediated). At recombination via any of these pathways, the transcription termination sequence is removed enabling the activation of the CAT gene, the selection marker for plasmids having undergone recombination events.

LMO2 locus

TAL1 locus

TLX1 locus

TCRD associated translocation

TCRB associated translocation

TCRD associated translocation (deletion)

12 cRSS

23 cRSS
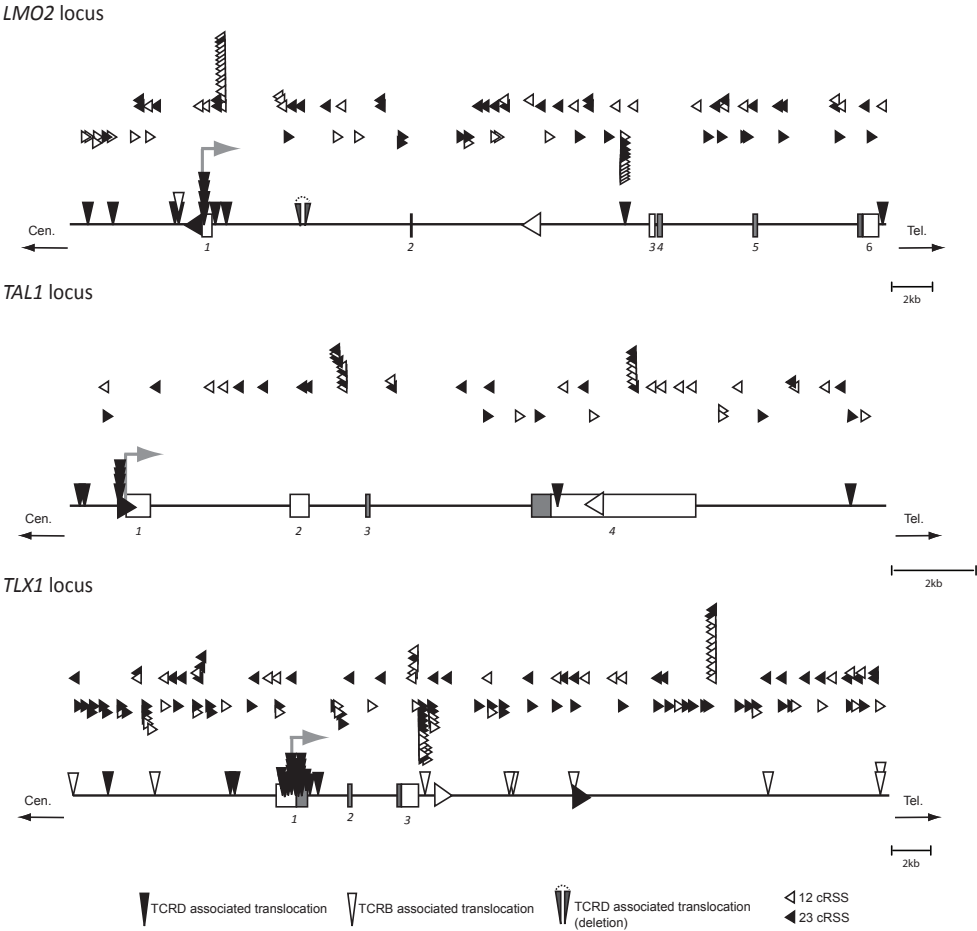
Supplementary Figure 2. Overview of the relative position of all functional cRSSs within the *LMO2*, *TAL1* and *TLX1* locus as determined by recombination information content (RIC) algorithm tool (http://www.itb.cnr.it/rss/). The larger arrows indicate the highest RIC scoring cRSSs.

'Type 2B' Recombination Variant
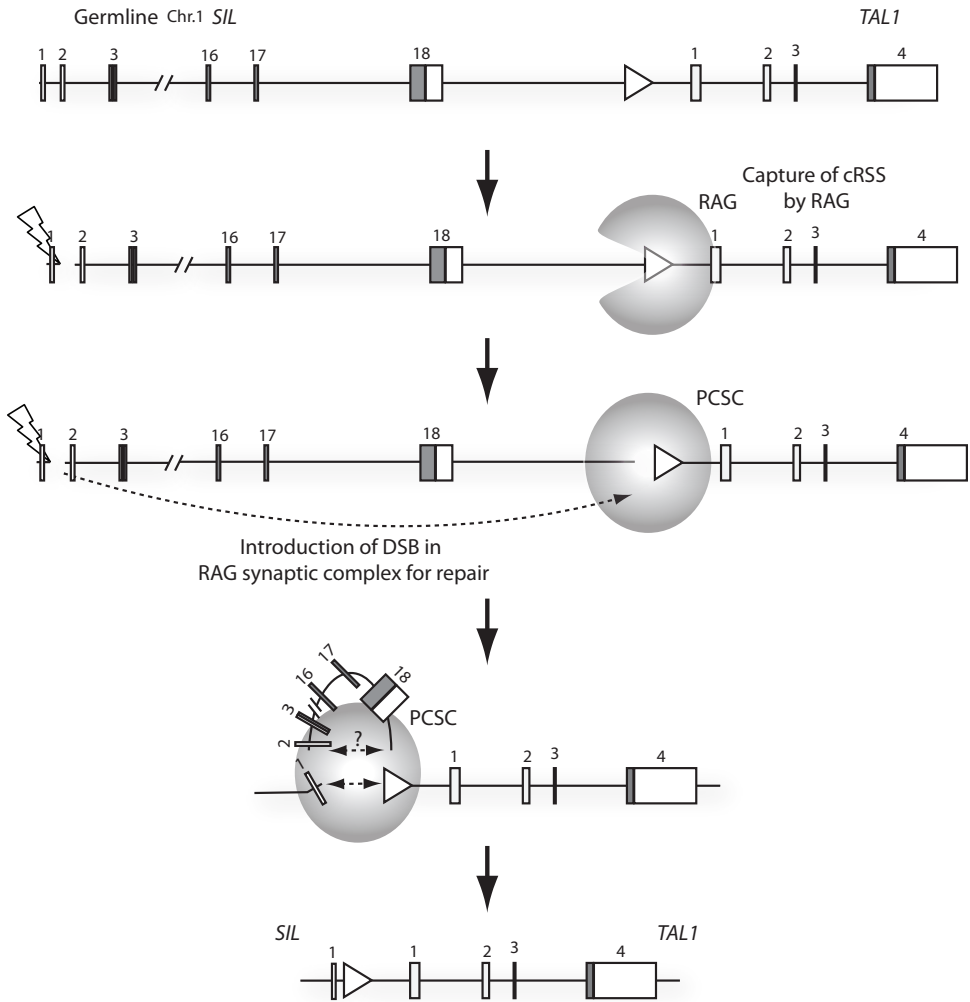
Supplementary Figure 3. Basic representation of the 'Type 2' Recombination Variant involving the *SIL-TAL1* deletion (del(1p)). DSB: double strand break, PCSC; post cleavage synaptic complex.

Supplementary Table 1. BP site specific primers used to produce insert sequence for cloning into the recombination substrate assay cassette

Oncogene	cRSS	BP or cRSS Position to TSS	Forward Primer	Reverse primer
Primers for cRSS functionality test against an authentic RSS				
[A] *LMO2*	23 bp spacer	-6,902	[E] 5' ACGCGT / GGCACTGTTATCTCTTGA 3'	[F] 5' GTCGAC / TGGGGAAAATTGTACTC 3'
[B] *TLX1*	-	-168/-176	[E] 5' ACGCGT / CTTCCCCTCTCTGGCTTCT 3'	[F] 5' GTCGAC / CTCCTTGGGTTTGTCTGTCT 3'
[B] *TLX1*	-	-191/-196	[E] 5' ACGCGT /CCTCCTTGGGTTTGTCTGTCT 3'	[F] 5' GTCGAC / AGAGGAGCGGGGGTGAATGA 3'
[B] *TLX1*	-	+ 11,372	[E] 5' ACGCGT / GGCTGTTACTGACTAGGTT 3'	[F] 5' GTCGAC / GGGCGTGAAACACACAATTAC 3'
[B] *TLX1*	-	+30,526/+30,539	[E] 5' ACGCGT / AAACGAGGGTCCATAGGTGAA 3'	[F] 5' GTCGAC / GGGCGTGAAACACACAATTAC 3'
[B] *LMO1*	-	-9,644	[E] 5' ACGCGT / CACATTTATTTATTTTCTTTTGGGTTTTG 3'	[F] 5' GTCGAC / AGTTTGTTTTATGAGCCAGCATTTT 3'
[B] *LMO3*	-	+224,944	[E] 5' ACGCGT / TTCTTGCCTACATTAGTCTTACTTGGAG 3'	[F] 5' GTCGAC / GAAATCAGAGACCTTACCCAGTGTC 3'
[B] *LYL1*	-	-8,444	[E] 5' ACGCGT / GGAGGGAGAGAATGGGGATG 3'	[F] 5' TGCGAC / CTGGGCTGGGGGATTTTT 3'
Primers for LMO2 12bp spacer-cRSS functionality test against an LMO2 23bp spacer-cRSS				
[B] *LMO2*	12 bp spacer	-391	[C] 5' ACTAGT / GACAGCCGGAGTCCCTTTTATT 3'	[D] 5' CCGCGG / CACCTCACCCCTCATTCATA 3'
[B] *LMO2*	23 bp spacer	-9107	[E] 5' ACGCGT / TACATTGATCCTCCCGCCTC 3'	[F] 5' GTCGAC / AATAACCACAGAGCCCAAC 3'
[B] *LMO2*	23 bp spacer	-5594	[E] 5 'ACGCGT / AGTGATGCTCCCACAGTGA 3'	[F] 5' GTCGAC / GAATAGTGACTGCCCTTTCAACC 3'
[B] *LMO2*	23 bp spacer	-4780	[E] 5' ACGCGT / GCTTGAGCCAGGAGTTGTAGTCT 3'	[F] 5' GTCGAC / GATTTACAGGCCCAGAAATGTT 3'

[A] Dik *et al*[8], [B] This study, [C] *SpeI* restriction site linker, [D] *SacI* restriction site linker, [E] *MluI* restriction site linker, [F] *SalI* restriction site linker.

Supplementary Table 2. An extensive overview of oncogene, TCR locus, BP site and translocation type involvement in TCR translocations of 117 BP sites [5]

Gene	Chr. #	BP Distance to TSS (nt)	1st TCR Derivative (Containing coding oncogene region)	2nd TCR Derivative	Presumed Coupling	Reference*	# of breaks	in silico determined cRSS present	RIC-score (strand)	Functionality [B]	Translocation type [5]
		-6,902**	nd	5'of Dδ2	(Vδ?)-Dδ2Dδ3Jδ1	T064[6]	?	CACTGTG-23-CTTATTCAC	-57.17(-)	yes[C5]	1
		-5,654/-5,644	3'of Dδ2	5'of Vδ3	Vδ3-Dδ2Jδ1	UPN4395[6]	3	no	-	no	2
		-1,849/-1,846**	nd / nd / nd	5'of Jδ1 / 3'of Dδ2 / 3'of Dδ2	(Dδ?)-Jδ1 / Dδ3-(Jδ?) / (Dδ?)-Jδ1	6206[6] / EF455600.1[F], Patient 121 / EF450258.1[F], case 906	?	no	-	yes[C5]	2
LMO2	11	-1,659**	3'of Dδ3 / nd	5'of 3'RSS^Dδ3 / 5'of Jβ2.3	Vδ1Dδ3-Jδ / (Dβ?)-Jβ2.3	T121[6] / 19	2 / ?	no	-	yes[C5]	2
		[D]-390 / -390 / -392 / -384	3'of Dδ2 / 3'of Dδ3 / 3'of Dδ3 / 3'of Dδ2	3'of RSS^Dδ3 / 3'of RSS^Dδ3 / 5'of 3'RSS^Dδ3 / nd	Dδ2-Jδ2 / Dδ1-Jδ2 / Dδ2Dδ3-Jδ2 / Dδ2-(Dδ/Jδ?)	1114[20,21] / 2114[21] / 647[6] / T068[6]	3 / 3 / 2 / ?	CACAGTA-12-GCAATAATT	-29.21(-)	yes[C5]	1
		+169	nd	5'of Dδ1	(Vδ?)-Dδ1Dδ2	LALW-2[23]	?	no	-	nt	2
		+716	5'of Dδ2	3'of 5'RSS^Dδ3	(Vδ?)-Dδ2Dδ3Jδ2	8511(HA)[23,85]	2	CACCGTG-23-TGAATAGAT	-57.56(-)	yes[C11]	1
		[A]+4,284 / [D]+4,778	3'of Dδ2	5'of Jδ1	Dδ2-Jδ1	UPN1589[6]	4	CACAGCA-23-CCCCAACCC / CACAATA-23-CCCATAATC	-55.32(-) / -58.47(-)	nt / nt	1? / 1?
		+8,645	nd	3'of Dδ2	Dδ2-(Jδ?)	EF450768.1[F] Patient 185	?	no	-	nt	2
		+20,600	3'of Dδ3	5'of 3'RSS^Dδ3	Dδ2-Dδ3	T024[6]	2	CACACTA-12-ACAGAAATG	-38.74(+)	yes[C5]	1
		+34,160	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	TALL-104[6]	3	no	-	yes[C5]	2
		-3,208	nd	5'of RSS^Dδ2	(26?/Dδ?)-Dδ2	S65911.1[F]	?	no	-	nt	2
		-932**	nd	3'of Jδ3	Jδ3^(intron)[*]	Patient 1[24]	?	no	-	nt	2
		-817**	nd	5'of Jδ1	(Dδ?)-Jδ1	Patient 5[24]	?	no	-	nt	?
TAL1	1	+431 / [D]+431 / +431	nd / nd / nd	5'of RSS^Dδ2 / 5'of Dδ3 / 5'of Dδ3	(Vδ?/Dδ?)-Dδ2 / (Vδ?)-Dδ3Jδ1 / (Vδ?)-Dδ3Jδ1	S65910.1[F] / 26Patient 4[24] / Patient 2[27]	? / ? / ?	CACACCG-23-CGAAAAGG	-46.33(+)	nt	1?
		+431*	nd	5'of Dδ3	(Vδ?)-Dδ3Jδ1	L23[25]	?	-	-	nt	2
		+420	nd	5'of Dδ2	(Vδ?)-Dδ2Dδ3Jδ1	Patient 0[27]	?	-	-	nt	2
		+427	nd	5'of Dδ2	(Vδ?)-Dδ2Jδ1	S65909.1[F, 26]	?	-	-	nt	2
		+10,786	5'of Dδ3	3'of Dδ3	Dδ2-Dδ3	DU.528[29,30]	3	no	-	nt	2
		+17,719	5'of Jδ3	3'of Dδ2	Dδ2-Jδ3	JU[28]	3	no	-	nt	2
		+36,633	nd	5'of Dδ2	(Vδ?)-Dδ2Jδ1	Patient 6 [86]	?	no	-	nt	2
		+53,085	3'of 5'RSS^Jβ2.1	3'of 5'RSS^Jβ2.7	(Dβ2?)-Jβ2.7	31	?	CACACAC-12-ACACACACA	-32.09(-)	nt	1?

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	Coordinate	3' of	5' of	Structure	Sample	n	CACACAC-23-AGAGAACCC	-49.42(-)	expr	copies
	-10.330**	nd	5'of Dβ2.1	(Vβ?)-Dβ2.1β2.4	PER-255[85]	?			nt	2
	-8,982**	5'of Dδ3	nd	(Dδ/Vδ?)-Dδ3Jδ1	UPN494[32]	?	no	-	nt	2
	-6,525**	nd	3'of 5'RSS[Dβ2]	(Vβ?)-Dβ2	UPN57[32]	?	CACAGGT-12-ACCTAACCA	-34.28(+)	nt	1?
	-2,586**	nd	5'of Dδ3	(Vδ/Dδ?)-Dδ3Jδ1	UPN480[32]	?	no	-	no	2
	-2,359**	nd	5'of Dδ3	?-Jδ1Dδ3[inverted]	[4]	?	no	-	nt	2
	+290/+294	3'of Dδ3	5' of Jδ1	Dδ2Dδ3-Jδ1	UPN200[32]	3	no	-	no	2
	+575/+579	3'of Dδ3	5'of Jδ1	Dδ2Dδ3-Jδ1	UPN528[32]	3	no	-	nt	2
	[D]+608/619	3'of 5'RSS[Dδ3]	5'of Dδ2	Dδ2-Dδ3	T60S046[32]	3	no	-	yes	2
	+602	5'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN503[32]	3				
	+607	nd	nd	TCRD	BA[33]	?				
	[D]+618	3'of 5'RSS[Dδ2]	5'of Dδ3	Dδ2-Dδ3	UPN443[32]	3	no	-	no	2
	+611/+618	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN553[32]	3				
	+621**	3'of Dδ2	nd	Dδ2-(Dδ/Jδ?)	UPN48[32]	?	no	-	nt	2
	[D]+632	3'of Dδ3	5'of Jδ1	Dδ2Dδ3-Jδ1	UPN103[32]	3	no	-	no	2
10	+632	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN242[32]	4				
	+290/+631	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	[A]UPN12[32]	3				
	+632	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN226[32]	3				
	+632	3'of Dδ3	nd	Dδ2Dδ3-(Jδ?)	UPN346[32]	?				
	+633	3'of Dδ2	5' of Dδ3	Dδ2-Dδ3	T6329[32]	3				
	+630/+634	3'of Dδ2	5' of Dδ3	Dδ2-Dδ3	T7001[32]	3				
TLX1	+632	3'of Dδ2	5' of Dδ3	Dδ2-Dδ3	UPN499[32]	3				
		3'of Dδ2	5' of Dδ3	Dδ2-Dδ3	UPN460[32]	3				
	+647**	3'of Dδ2	nd	Dδ2-(Dδ/Jδ?)	UPN461[32]	?	no	-	nt	2
	+660	3'of 5'RSS[Dδ2]	3'of 5'RSS[Dδ3]	Dδ2-Dδ3	UPN259[32]	3	no	-	no	2
	[A]+668/+692	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN496[32]	3	no	-	no	2
	+692**	3'of Dδ2	nd	Dδ2-(Dδ/Jδ?)	UPN501[32]	?	no	-	nt	2
	[D]+720	3'of Dδ3	5'of Jδ1	Dδ2Dδ3-Jδ1	UPN273[32]	3	no	-	yes	2
	+719	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN281[32]	3				
	+714	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN9[32]	3				
	+712	3'of Dδ2	5'of Dδ3	Dδ2-Dδ3	UPN269[32]	3				
	+718	3'of Dδ3	5'of Jδ1	Dδ2Dδ3-Jδ1	UPN362[32]	3				
	+712	3'of Dδ3	5'of Jδ1	Dδ2Dδ3-Jδ1	UPN506[32]	3				
	+718	3'of Dδ2	nd	Dδ2-(Dδ/Jδ?)	UPN452[32]	3				
	+711	nd	5'of Dδ3	Dδ2-Dδ3	BO[36]	3				
	+713	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	JM[35,36]	?				
	+713	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	[A]DW[35,36]	?				
	+716	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	VB[35]	?				
	+713	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	AR[35]	?				
	+719	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	BN[35]	?				
	+732	nd	3'of 5'RSS[Dδ2]	(Vδ/Dδ?)-Dδ2	JS[35]	?				

Gene	Chr	Position			Junction	Reference		RSS	Score	Method	
TLX1	10	+818	nd	5'of Dδ3	(Dδ/Jδ?)-Dδ3Jδ1	UPN430[32]	?	no	-	no	2
		+2,112** +7,738** +11,976	nd	5'of (Dδ3)Jδ1	(Vδ?)-(Dδ3)Jδ1	T-ALL1143[37]	?	no	-	nt	2
		+12,168/+12,171	5'of Jβ2.5 / 5'of Jβ2.1	nd / 3'of Dβ1	(Dβ?)-Jβ2.5 / Dβ1-Jβ2.7	UPN43[32] / UPN537[32]	? / 3	CACACCA-12-GCATACAAT / no	32.87(-) / -	nt / nt	1? / 2
		+15,330	5'of Jβ2.5	3'of Dβ1	Dβ1-Jβ2.5	T178[32]	3	no	-	yes	2
		+25,470**	5'of Jβ1.5	3'of Dβ1	Dβ1-Jβ1.5	UPN474[32]	3	CACAGAG-23-AGGGAAG-CG	-47.75(-)	nt	1?
		D+31,340	nd	3'of Dβ1	Dβ1-(Jβ?)	UPN546[32]	?	no	-	nt	2
		+31,338	5'of Jβ2.1	3'of Dβ2	Dβ1-(Jβ?) / Dβ2-Jβ2.1	TO51[32] / UPN280[32]	? / 3	no	-	yes	2
TAL2	9	D+31,795/+31,766 (us TMEM38B)	5'of Jβ2.3 / 5'of Jβ2.7 / 5'of Jβ2.7	3'of Dβ1.1 / 3'of Dβ1.1 / 3'of Dβ1.1	Dβ1.1-Jβ2.3 / Dβ1.1-Jβ2.7 / Dβ1.1-Jβ2.7	SUP-T3[61] / SUP-T6[61] / BT[61]	2 / 2 / 2	CACTGTG-12-TATAAAAAT / CACACTG-12-GATATAAAA / no	-28.39(-) / -36.38(-) / -	nt / nt	1?
LYL1	19	+8,444	5'of Jβ1.1	3'of Dβ1	Dβ1-Jβ1.1	46	3	no	-	nt	2
		+787**	5'of Jβ1.1	nd	(Dβ?)-Jβ1.1	49	?	no	-	nt	2
CCND2	12	-61,328**	nd	3'of Dβ1	Dβ1-(Jβ?)	46	?	CATAGTC-12-ATCTTCCCC	-38.70(-)	nt	1?
MYC	8	A+10,318 / +1165,291	5'of Jα34	5'of Jα38	Jα38-Jα34	50,52	3	no	-	nt	1?
		+7,906**	5'of Jα31	nd	(Vα?)-Jα31	50,52	?	CACAATC-23-TGAGTATTC	-56.02(+)	nt	1?
		+5,978	5'of 3'RSS^{Vα36Vδ7}	3'of Vα36Vδ7	Vα36Vδ7-(Jα?)	50,51	2	no	-	nt	2
		+239,565**	nd	5'of Dδ3	(Vδ?)-Dδ3Jδ1	50,54	?	no	-	nt	2
		+5,964**	3'of Jα59	nd	Jα59intr.58	53	?	no	-	nt	2
NOTCH1	9	D+39,552**	5'of Dβ2.2(1tm24) / 5'of Jβ2.2(1tm24)	nd / nd	(Vβ?)-Dβ2.2Jβ2.2 / (Dβ?)-Dβ2.2	57,58 / Case 1[57]	? / ?	CACTGAA-23-CTGCTAACC / no	-55.91(-) / -	nt	1?
		D+39,653/+39,649**	5'of Jβ1.1 5'of Jβ1.1	nd / nd	(Dβ?)-Jβ1.1 / (Dβ?)-Jβ1.1	Case 2[57] / Case 3[57]	? / ?	no	-	nt	2
		+42,969**	3'of Jβ2.7^{int}	nd	(Dβ?)-Jβ2.7	59	?	no	-	nt	2
LMO1	11	-9,644	3'of Dδ2	5'of Jδ1	Dδ2-Jδ1	46	3	no	-	no	2
		D-5,258	5'of3'RSS^{Dδ2} / 5'of3'RSS^{Dδ2}	5'of Dδ2/Dδ3 / 5'of Dδ1	Dδ2-Dδ3Jδ1 / Vδ-Dδ1Dδ2Jδ1	47 / 48	3	CACAGTG-12-ACACAGCC / CACGTG-23-ACTCTGGCA	-29.44(+) / -44.10(+)	no / no	1?
LMO3	12	+224,944	5'of Jβ2.1	3'of Vβ2.3	Vβ2.3-Jβ2.1	43	3	no	-	nt	2
NKX2-4	20	-494	3'of Dδ2	3'of Dδ2	Dδ2-Dδ3	Unpub.	3	no	-	no	2
NKX2-1	14	+4,286	5'of Jδ1 / 3 of Dβ1^{int}	nd / nd	(Dδ?)-Jδ1 / Dβ1-(Vβ?)	TO33 unpub. / 9989[55]	2 / 2	no	-	nd	2
NKX2-5	5	+35,271**	nd	3'of Dδ3	Dδ3-(Jδ?)	56	?	no	-	nd	2

Gene		BP position	5' of	3' of	junction	sample/ref	n	cRSS	RIC	tested	n
C-MYB	6	[D]+53,891/+53,903	5'of Jβ1.2 / 5'of Dβ2	3'of 5'RSSDβ1 / 3'of Dβ1	(Dβ1?)-Jβ1.2 / Dβ1-Dβ2	T124[40] / UPN5846[40]	3 / 3	no	-	nt	2
		+144,584 (DS A1H1)	3'of Dβ1	5'of Jβ1.2	Dβ1-Jβ1.2	TL34[40]	3	CACAGTG-12-GTAGGATTT CACAGTG-23-TAAGTGATT	-33.04(-) -53.27(-)	yes	1
BMI1	10	-277,473	5'of Jβ2.3	3'of Dβ2.2	Dβ2.2-Jβ2.3	T003[38]	3	no	-	nt	2
LCK	1	-20,286	5'of Jβ1.2	3'of Dβ1.1	Dβ1.1-Jβ1.2	44	3	CACACAC-12-GCCAAAACA CACAGAC-23-AGC-CAAAAC	-27.16(+) -53.83(+)	yes[C11]	1
		-25,085	5'of Dβ1.1	3'of Vβ11.2	Vβ11.2-Dβ1.1Jβ2.7	45	3	no	-	nt	2
TLX3	5	-50,034** (DS RANBP17)	nd	5'of Dδ2	(Vδ?)-Dδ2D63D6-2Jδ1	64	?	no	-	nt	2
OLIG2 (BHL-HB1)	21	+84,224 (DS OLIG1)	5'of Jα53	3'of Vα29V65	Vα29V65-Jα53	60	3	no	-	nt	2
HOXA6	7	-2,179	3'of Dδ3	5'of Jδ1	Dδ2D63-Jδ1	41	3	no	-	nt	2
HOXA9	7	-2,992	3'of Dβ1	5'of Jβ2.7	Dβ1-Jβ2.7	42	3	no	-	nt	2
		-501[E]	5'of Jβ2.7		(Dβ)-Jβ2.7	43	2	no	-	nt	2
BCL11B	14	+48,651**[E]	5'of Dδ2		(Vδ)-Dδ2	13	2	no	-	nt	2
TCL1A	14	-99,998[E]	5'of Jα42-1		(Vα?)-Jα42-1	63	3	no	-	nt	2
		-145,630	5'of Cβ1	nd	-	62	?	no	-	nt	2

§ Comprehensive overview of the different TCR translocations identified in T-ALL and their corresponding translocation partners. Only those translocations, of which complete sequences and BPs were available, were used in this study. For each BP the distance (nt) relative to its corresponding TSS was determined and the presence of a cRSS was *in silico* determined with the recombination information content (RIC) algorithm tool (http://www.itb.cnr.it/rss/)[12] For a few cases the functionality of possible cRSS was also tested *ex vivo*.

* Reference numbering according to Supplementary Reference list, **The precise BP position is not known due to type of break, A Deletion of > 10nt, B If functionality of the cRSS or BP associated sequence was *ex vivo* tested; yes, no or not tested (nt); C Reference to article where functionality of that particular cRSS was tested, D More than one break was identified to be associated to this BP site in different T-ALL patients, E Inversion, F Accession number GenBank, nd: not determined due to absence of reciprocal translocation sequence, No: no functional cRSS found at that BP position according to the RIC algorithm analysis, -: no RIC score, ?: not known

Supplementary Table 3. *Ex vivo* analysis of functionality BP site associated sequences involved in *LMO1*, *LMO3* and *LYL1* TCR translocations.

Oncogene	Position BP (nt)	[A]cRSS	[B]Vector used	[C]No. Colonies	Recombination Pathway			
					[D]1	[E]2	[F]3	[G]4
LMO2	-6,902	[H]CACAGTG-23-ACGCTCAAC	Dδ3 / Dδ3$_{inverted}$	7 / 10	4 / 0	0 / 1	2 / 2	1 / 6
TLX1	-168/-176	no cRSS	Dδ3 / Dδ3$_{inverted}$	9 / 8	0 / 0	0 / 0	0 / 0	7 / 8
TLX1	-191/-196	no cRSS	[I]Dδ3	1	0	0	1	0
TLX1	+11,372	no cRSS	[I]Dδ3	5	0	0	0	5
TLX1	+30,526/+30,539	no cRSS	Dδ3 / Dδ3$_{inverted}$	1 / 8	0 / 0	0 / 0	0 / 0	1 / 7
LMO1	-9,644	no cRSS	Dδ3 / Dδ3$_{inverted}$	0 / 1	0	0	0	0
LMO3	+224,944	no cRSS	Dδ3 / Dδ3$_{inverted}$	0 / 0	0	0	0	0
LYL1	-8,444	no cRSS	Dδ3 / Dδ3$_{inverted}$	0 / 0	0	0	0	0

[A] Presence of cRSS determined by in silico analysis, result yes or no

[B] Cassette used as described by Dik et al [6]

[C] Total number of clones obtained and analyzed (total number of colonies obtained from one transfections)

[D] Total number of clones which had a V(D)J-mediated recombination between authentic Dδ3 RSS and the oncogene BP sequence

[E] Total number of clones which had a V(D)J-mediated recombination between authentic Dδ3 RSS and other cRSS in the cloned oncogene BP sequence

[F] Total number of clones which had a V(D)J-mediated recombination between authentic Dδ3 RSS and other cRSS in the core vector

[G] Total number of clones which had a break repair mediated recombination (defined as non V(D)J recombination mediated

[H] cRSS sequence from Dik et al [6]

[I] Dδ3$_{inverted}$ not tested

Role of V(D)J recombination in (non-) TCR aberrations

Supplementary Table 5. *Ex vivo* and *in silico* analysis of *LMO2* cRSS functionality at the NRE site.

12 bp spacer cRSS	RIC score	cRSS position to TSS (nt)	23 pb spacer cRSS	RIC score	cRSS position to TSS (nt)	No. Colonies[A]	Recombination Pathway			
							1[B]	2[C]	3[D]	4[E]
CACAGTA-12-GCAATAATT	-29.20	-391	CACACCA-23-GGCAAGACC	-56.93	-4780	0	0	0	0	0
			CACAGTG-23-GGCTGTCAG	-58.68	-5595	0	0	0	0	0
			CAAAGTG-23-AGAGCCTGG	-57.22	-9107	5	0	0	0	5

[A] Total number of clones obtained and analyzed (total number of colonies obtained from two independent transfections)

[B] Total number of clones which had a V(D)J-mediated recombination between 12 bp spacer RSS and 12 bp spacer cRSS

[C] Total number of clones which had a V(D)J-mediated recombination between 12 bp spacer RSS and other BP

[D] Total number of clones which had a V(D)J-mediated recombination between 23 bp spacer RSS and other BP

[E] Total number of clones which had a break repair mediated recombination (defined as non V(D)J recombination mediated)

Chapter 3

Role of oncogene accessibility and nuclear proximity in TCR translocations formation

Chapter 3.1
Genetic and epigenetic determinants mediate proneness of oncogene breakpoint sites for involvement in TCR translocations

Chapter 3.2
Locus-specific nuclear proximity between T cell receptor loci and oncogene loci during human thymocyte development does not impact on TCR translocation formation

Chapter 3.1

Genetic and epigenetic determinants mediate proneness of oncogene breakpoint sites for involvement in TCR translocations

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Chapter 3.2

Locus-specific nuclear proximity between T cell receptor loci and oncogene loci during human thymocyte development does not impact on TCR translocation formation

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Chapter 4

TCR-associated oncogenic events in T-ALL

Chapter 4.1

Correct interpretation of T-ALL oncogene expression relies on
normal human thymocyte subsets as reference material

Chapter 4.2

BMI1 as oncogenic candidate in a novel TCRB-associated
chromosomal aberration in a patient with TCRγδ⁺ T-cell acute
lymphoblastic leukemia

Chapter 4.1

Correct interpretation of T-ALL oncogene expression relies on normal human thymocyte subsets as reference material

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ABSTRACT

Ectopic oncogene expression is common to T-ALL. Presence of these oncogene transcripts is used to define T-ALL subgroups. However, classification of subgroups based on oncogene expression often relies on comparisons made with material other than correct biological reference material.

We have evaluated the expression of T-ALL subgroup-classifying oncogenes *LMO2*, *LYL1*, *TAL1*, *TLX1*, *TLX3* and *NKX2-1* in all major human T-cell developmental stages. Comparison of these transcript levels with levels detected in 39 matched T-ALL counterparts enabled the identification of true oncogenic expression in T-ALL.

These findings redefine the concept of oncogenic expression and will help prevent misclassification of T-ALL subgroups.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) represents the malignant proliferation of T-cell precursors arrested during various stages of T-cell development. Defined molecular-genetic abnormalities, often seen as TCR-associated translocations, are common to this disease. These translocations recurrently implicate specific oncogenes involved in cell cycle, differentiation, proliferation, survival and self-renewal processes [1-4].

Ectopic expression of oncogenes such as *LMO2, LYL1, TLX1*, *TLX3*, *TAL1*, and *NKX2-1* is regarded as a determinant of a T-ALL signature [5,6]. This signature enables molecular subtype classification of T-ALL and identification of the stage of leukemic arrest. This in turn could provide insight into the therapy responses of T-ALL patients [5,7-9]. Although therapies and prognosis of T-ALL patients have improved significantly over the past decades, still a number of T-ALL subgroups do not respond well to treatment.

Ectopic oncogene expression in human T-ALL is often determined by comparison to transcript levels detected in total thymocyte, spleen and peripheral blood lymphocytes, bone marrow or even murine thymic T-cells, which are used as a reference [2,5,10]. Usage of such reference material can result in incorrect interpretation and misclassification of T-ALL subgroups. Although many have speculated and made assumptions regarding the usage of relevant reference material for T-ALL, no real attempt has been made to show that normal human thymocyte subsets is the most optimal biologically relevant reference material for comparison to T-ALL.

Usage of normal thymic counterparts as reference material is essential for correct T-ALL subgroup classification, particularly when such T-ALL classification aims to identify prognostic subgroups with different clinical outcome. Here we redefine the concept of ectopic oncogene expression in T-ALL based on human thymocyte subsets as a reference.

MATERIALS AND METHODS

Patient Material

Diagnostic bone marrow or peripheral blood samples from 39 T-ALL patients (Supplemental Table 1) were immunophenotypically defined using CD1, CD2, surface/cytoplasmic CD3, CD4, CD5, CD7, CD8, CD10, CD34, anti-TdT, anti-TCRαβ and anti-TCRγδ antibodies. TCR-rearrangements were analyzed as described [11]. The T-ALL samples were divided in different categories as described by Asnafi *et al* [4]. Six patients were assigned to the immature (IM) group (either IM0, IMδ or IMγ), 11 patients to a combined IMβ/pre-αβ group (due to lack of cytoplasmic TCRβ staining), and 21 patients to the mature subgroup (8 expressing TCRαβ and 13 expressing TCRγδ).

Normal Thymic subsets

Normal human thymuses, which have been collected from immunologically healthy donors, aged 1 week to 6 years old, were used to isolate all major consecutive thymocyte subsets. Subsets (n=3) were sorted (>90% purity) from pooled thymuses (n=5) as described [12,13].

Real-time quantitative (RQ)-PCR for T-ALL oncogenes.

RNA from T-ALL samples and thymocyte subsets was reverse transcribed into cDNA and RQ-PCR was performed as described [14,15] using an Applied Biosystems 7000 PCR machine (Foster City, CA, USA). Specific primer probe sets (Supplemental Table 2) were used to quantify *LMO2*, *LYL1*, *TLX1*, *TLX3*, and *TAL1* transcripts, following normalization to *ABL* [12].

RESULTS AND DISCUSSION

Expression of T-ALL oncogenes in normal human thymocyte subsets

To evaluate the transcriptional program of T-ALL oncogenes during normal human thymocyte development, *LMO2*, *LYL1*, *TLX1*, *TLX3*, *TAL1* and *NKX2-1* transcript levels were quantified by RQ-PCR and normalized to *ABL* (Supplementary Table 1) [12,14,15].

Our findings showed *LMO2* and *LYL1* expression in the most immature thymic stages. Our research reflected a gradual decline of expression up to the pre-TCRαβ T-cell development stages, whereafter no transcripts were detected (Figure 1). Mean *LMO2* expression levels declined from 150% (DN1) down to 15% (ISP), while mean *LYL1* expression decreased from 165% to 20% between these stages. Of note, murine *Lyl1* transcription is restricted to the DN1/DN2 stage (4), while in human *LYL1* transcripts can be detected up to the ISP stage. Traces of *LMO2* and *LYL1* transcripts were also detected in TCRγδ⁺ thymocytes, at levels comparable to those seen in DN3 subsets (Figure 1). This concurs with the fact that TCRγδ⁺ thymocytes descend from the early DN stages.

In contrast to findings by Ferrando *et al* [2], who showed *tal1* expression in murine thymocytes, no *TAL1* transcripts were detected in any human thymic subset. Furthermore, no transcripts of *TLX1* and *TLX3* were detected in any of the thymocyte subsets (Figure 1). Absence of *TLX3* transcript in all major human thymic subsets conflicts with the findings by Ballerini *et al* [10], who detected low *TLX3* levels in fetal and adult thymus.

No *NKX2-1* transcripts were detectable in any of thymic development stages (Figure 1), which is in concordance with previous findings [6,12].

Oncogenic expression in T-ALL

To establish aberrant expression of the oncogenes in T-ALL, expression levels in 39 T-ALL cases (29 children, 5 adults and 5 cases of unknown age) were compared against the
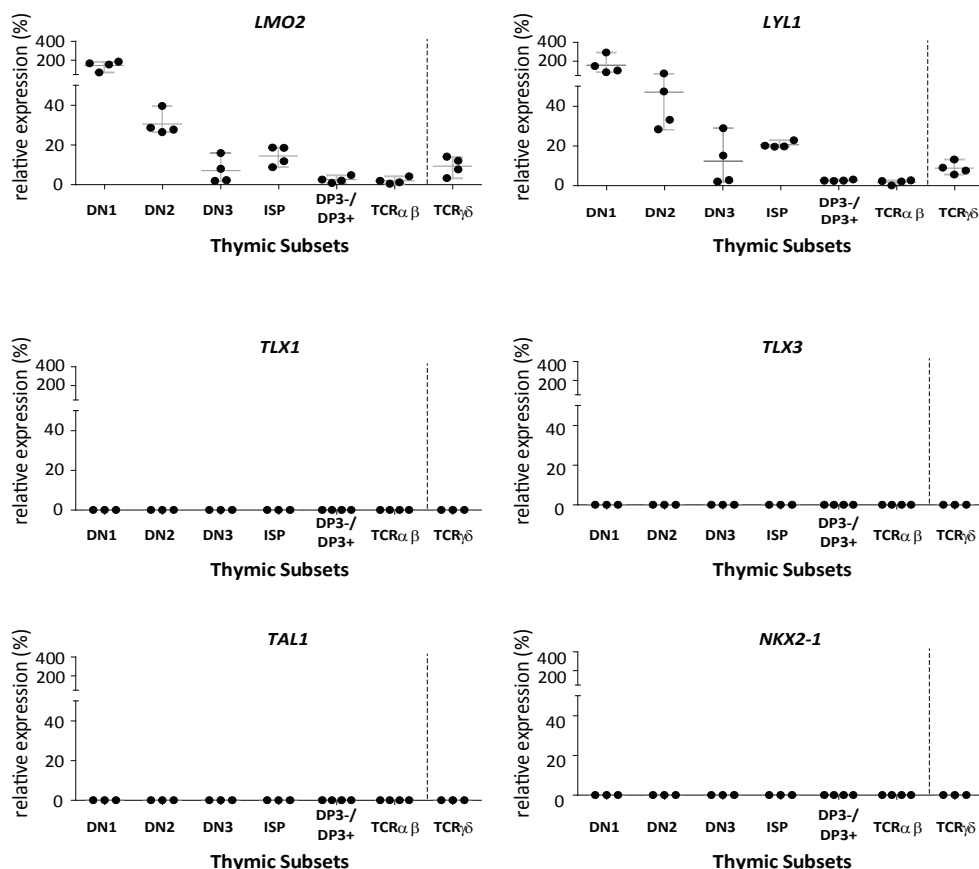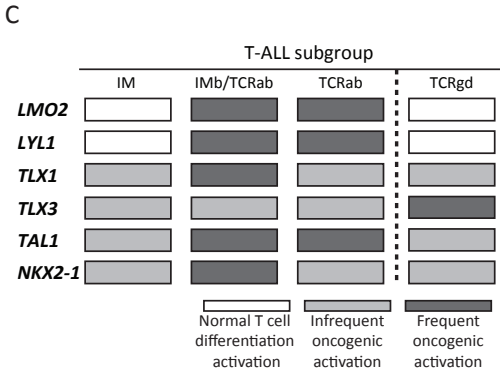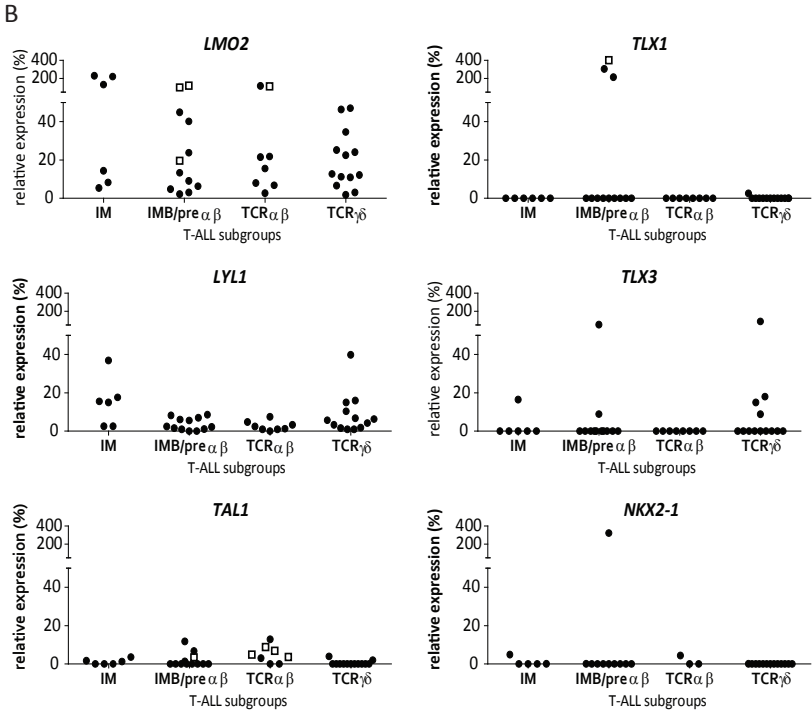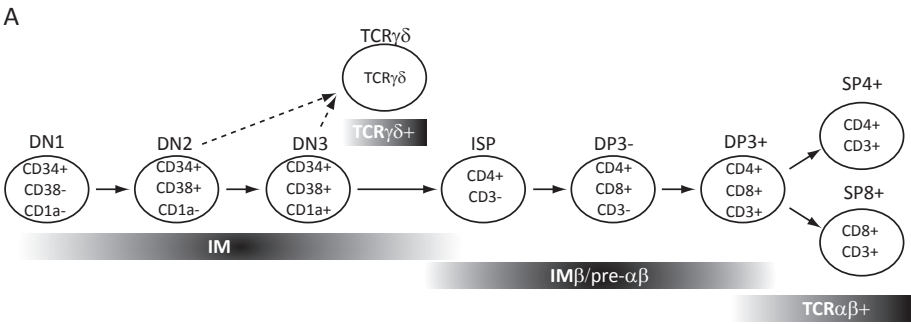
Figure 1. Expression of T-ALL oncogenes in normal human thymocytes. Expression levels of T-ALL oncogenes in consecutive T cell developmental stages. Data is based on 3 to 4 experiments done on independently sorted subsets, each isolated from pooled human thymus samples of five individuals (aged 1 week to 6 years old). Transcript levels of the *LMO2*, *LYL1*, *TLX1*, *TLX3*, *TAL1* and *NKX2*-1 genes were normalized to *ABL* transcript levels and are shown as percentage expression relative to ABL. Mean and range of expression is shown for each subset.

transcriptional program of their normal human thymocyte maturation counterpart (Figures 1, 2A). To this end, T-ALL subgroups were immunophenotypically defined based on their maturation markers and TCR-rearrangements to match them to their thymocyte counterpart [12](Figure 2A).

We observed heterogeneous levels of *LMO2* and *LYL1* transcripts in the different T-ALL cases tested. For *LMO2* this ranged from 2 to 300% relative to *ABL* expression. In most of the T-ALL cases high *LMO2* transcript levels as detected in the immature (IM) (IM0, IMδ, IMγ) and the IMB/preαβ T-ALL groups were comparable to levels detected in their normal human

A

B

C

thymic counterparts (DN1 to ISP)(Figure 1, 2B). High *LMO2* transcript levels in mature T-ALL subgroups (120%-19%, depending on the exact leukemic stage of arrest) are indicative of oncogenic activation and do correlate with an observed *LMO2* activating t(11;14)(p13;q11) translocation in a number of the ectopically *LMO2* expressing cases (Figures 1, 2B).

LYL1 transcripts were mainly detected in the IM and the TCRγδ+ T-ALL subgroups, with levels ranging from 0 to 40% relative to *ABL*. *LYL1* transcript levels detected in T-ALL subgroups were mostly comparable to expression levels detected in their normal thymic counterparts. This implies that *LYL1* activation in immature (double negative) subsets is very often attributed to a normal transcriptional program rather than a mutation involving the *LYL1* locus as previously suggested [5]. Only one case in the TCRγδ+ T-ALL subgroup showed an elevated (ectopic) level of *LYL1* expression. Unfortunately, so far no *LYL1* activating mutation was confirmed for this case.

Only three of the T-ALL cases were positive for *TLX1* transcripts, being exclusively present in the IMβ/pre-αβ subgroup, which corresponds to previous findings [5,6]. A *TLX1* activating translocation, t(7;10)(q34;q24) was confirmed in one case. Seven cases (20%) were positive for *TLX3* transcripts. These were detected in the IM, IMβ/pre-αβ and the TCRγδ+ T-ALL subgroups. *TAL1* transcripts were detected in 13 of the cases analyzed, mostly in the IMβ/pre-αβ and TCRαβ+ T-ALL subgroups. Within this group, five cases contained the *TAL1* activating del(1)p32. As *TAL1*, *TLX1* and *TLX3* are not expressed during any of the thymic developmental stages, the presence of transcripts from these oncogenes in any T-ALL subgroup would be indicative of an abnormal transcriptional program (Figures 2B, 2C).

NKX2-1 transcript levels were determined in 31 of our 39 T-ALL samples, based on availability of material. In this cohort, we found single cases within the IMB/pre-αβ, IM and TCRαβ+ T-ALL subgroups that were positive for *NKX2-1*. We found the highest detectible *NKX2-1* transcript level to be associated to a cortical thymic T-ALL. We also show that *NKX2-1*

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transcripts can be detected, though at lower levels, in other T-ALL subgroups (Figures 2B). As *NKX2-1* is not expressed in thymocytes, we conclude that these cases show an aberrant *NKX2-1* transcriptional program (Figures 2B, 2C).

Usage of normal thymocyte subsets as reference material is a prerequisite for reliable interpretation of T-ALL oncogene expression

Our data show a clear discrepancy between the *LMO2, LYL1* and *TAL1* transcriptional program in human and mice thymocytes. This brings into question whether previous associations suggested between oncogene expression in human T-ALL and transcriptional signature determined in murine thymocytes are accurate enough [2].

Moreover, we show here that *LYL1* and *LMO2* are expressed in the most immature (DN1 to ISP) subsets, which together constitute about 5-10% of the total thymocytic population. Usage of total thymus to determine *LYL1* and *LMO2* expression level [5] would therefore underestimate transcript levels in particular thymocyte subsets. This could result in incorrect interpretation on ectopic *LMO2* and *LYL1* expression in some T-ALL cases.

Ectopic *NKX2-1* expression has been shown to be associated with a cortical thymic arrest in T-ALL [6]. This association was made based on the presence of *NKX2-1* transcripts in T-ALL and the cytogenetic identification of underlying *NKX2-1* rearrangements. Our findings confirm that *NKX2-1* is not expressed in any thymic developmental stage, but also suggests that ectopic *NKX2-1* is not necessarily restricted to T-ALL derived from cortical developmental stages.

CONCLUSION

In conclusion, we provide evidence that usage of correct reference material to determine ectopic oncogene expression in T-ALL is essential for correct interpretation of oncogenic activation to prevent T-ALL subgroup misclassification. Here we show that *TAL1*, *TLX1*, *TLX3 or NKX2-1* expression in T-ALL should be considered aberrant at any maturational stage. *LMO2* and *LYL1* expression in immature and TCRγδ+ expressing T-ALL most likely reflects normal T-cell development rather than oncogenic activation. However, *LMO2* or *LYL1* expression in αβ-lineage T-ALL is truly oncogenic (Figure 2C).

ACKNOWLEDGEMENTS

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SUPPLEMENTS

Supplementary Table 1. Oncogene specific primers probe sets

Gene	Forward primer	Reverse Primer	Probe	Amplicon size
LMO2	FW-LMO2-EMC: CAAACTGGGCCGGAAGC	RV-LMO2-EMC: ACCCGCATTGTCATCTCAT	Tr-LMO2-EMC: CAAAAAGCCTGAGATAGTCTCTCCGGCAG	111 bp
LYL1	FW-LYL1-EMC: CCCCTTCCTCAACAGTGTCTACA	RV-LYL1-EMC: CTCCCGGCTGTTGGTGAA	Tr-LYL1-EMC: CTCACAGTGGCTTGGTCTCCGCTTC	157 bp
TLX1	FW-HOX11-EMC: ACACAAAGGACAGGTTCACAGG	RV-HOX11-EMC: ACGTGCGCGCGGCTTCTTC	T-HOX11-EMC: AGAACCGGACGCCCCCCAAG	72 bp
TLX3	FW-HOX11l2-EMC2: CGCCAAGTCCCTCAAAATGA	RV-HOX11L2-EMC2: TGGAAGGCGTCGTGTTGC	T-HOX11L2-EMC2: CCACTTGGTCCTCCGGTTTTGGA	150 bp
TAL1	FW-TAL1-EMC: CCGGATGCCTTCCCTATGT	RV-TAL1-EMC: TCCCGGCTGTTGGTGAAGA	T-TAL1-EMC: AGACCTTCCCCCTATGAGATGGAGATTACTGAT	125 bp
NKX2-1	FW-NKX2-1-EMC: TCTGGCCCCGGATGGTA	RV-NKX2-1-EMC: CCCATGAAGCGGGAGATG	T-NKX2-1-EMC: CCCAGACCCGCGCTTCCC	65 bp

Supplementary Table 2. Oncogene Expression levels in T-ALL

Patient	T-ALL subclass	age	sex	LMO2	LYL	TLX1	TLX3	TAL1	NKX2-1
								Expression level	
T011	IM0	n.a.	M	220,4	15,5	0	0	1,3	0
T014	IM0	15	F	5,4	2,6	0	0	0	0
T043	IMD	7	F	8,3	2,6	0	0	0	4,9
T135	IMD	4	F	132,9	17,6	0	0	1,7	0
T035	IMG	7	M	14,3	15	0	16,5	0	0
T039	IMG	26	M	229,7	36,9	0	0	3,6	n.d.
T004	TCRGD	17	M	34,6	1	0	0	0	0
T005	TCRGD	13	M	1,8	1	0	0	0	0
T018	TCRGD	11	F	47	4,2	0	0	0	0
T042	TCRGD	n.a.	M	3,1	1,6	0	0	0	0
T062	TCRGD	24	M	12,7	3,3	0	0	0	0
T084	TCRGD	7	M	11,2	16	0	0	2	0
T091	TCRGD	5	M	10,9	5,7	0	0	0	0
T104	TCRGD	2	M	24,1	6,3	0	88,9	4	0

				t(11;14)(p13;q11)		t(7;10)(q34;q24)		del(1)p32	
T106	TCRGD	14	n.a.	12,2	4,8	0	18	0	0
T107	TCRGD	14	M	46,3	39,8	0	8,8	0	0
T108	TCRGD	2	F	25,2	15	2,6	0	0	0
T134	TCRGD	12	M	22,5	6,7	0	0	0	0
T144	TCRGD	4	M	6,6	10,4	0	15,5	0	0
T045	IMB/preAB	3	M	40,1	6,1	0	0	0	0
T051	IMB/preAB	12	M	13,3	8,2	400	0	0	0
T007	IMB/preAB	4	M	4,8	5,6	212,9	0	0	0
T009	IMB/preAB	5	M	9,1	2,2	0	0	0	0
T101	IMB/preAB	3	F	23,8	1,1	305,3	0	0	0
T177	IMB/preAB	22	M	2,2	8,6	0	8,9	0	0
T068	IMB/preAB	6	M	120,6	1,6	0	0	11,8	0
T064	IMB/preAB	8	F	19,6	1	0	0	0	0
6206	IMB/preAB	n.a.	n.a.	101,4	0	0	0	6,7	0
T082	IMB/preAB	n.a.	F	44,8	2,4	0	0	1,4	321,2
T008	IMB/preAB	7	M	6,3	7	0	52,9	0	n.d.
T102	IMB/preAB	11	M	3	0	0	0	3,5	n.d.
T024	TCRAB	5	M	118,9	1,3	0	0	3,1	0
T010	TCRAB	3	M	114	4,8	0	0	12,9	n.d.
T012	TCRAB	34	M	21,5	2,4	0	0	6,9	n.d.
T029	TCRAB	10	F	15,5	7,5	0	0	8,8	n.d.
T087	TCRAB	n.a.	n.a.	6,8	0	0	0	0	4,4
T139	TCRAB	15	M	2,7	1	0	0	4,9	n.d.
T140	TCRAB	29	M	21,8	3,3	0	0	3,7	n.d.
T201	TCRAB	5	n.a.	8	1	0	0	0	0
activating genetic alteration				t(11;14)(p13;q11)		t(7;10)(q34;q24)		del(1)p32	

Expression levels are given relative to ABL. (n.a.: not applicable, n.d.: not determined)

Chapter 4.2

BMI1 as oncogenic candidate in a novel TCRB-associated chromosomal aberration in a patient with TCRγδ⁺ T-cell acute lymphoblastic leukemia

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ABSTRACT

Translocations involving the TCRD locus are more commonly seen in T-cell acute lymphoblastic leukemia (T-ALL) than TCRB translocations. Here we identified a novel TCRB chromosomal aberration in a TCR$\gamma\delta^+$ T-ALL sample, possibly leading to activation of the *BMI1* oncogene. This case illustrates the importance of using new approaches to analyze and uncover cryptic chromosomal rearrangements, which were undecipherable in the past, and adds to the growing list of TCRB-associated chromosomal translocations and our understanding of T-ALL leukemogenesis.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease of progenitor T-cells, which have undergone multiple genetic alterations. Translocations between T-cell receptor (TCR) genes and genes with oncogenic properties are a genetic hallmark of this disease. Most of these oncogenes encode for transcription factors of which their deregulated expression causes a block in the development of the progenitor T-cells.

Until recently, most of these TCR chromosomal rearrangements were attributed to erroneous recombinations involving the alpha/ delta (TCRA/D) locus. However, new approaches uncovering cryptic chromosomal rearrangements revealed a higher incidence of TCR beta (TCRB) locus associated aberrations leading to the identification of new oncogenes involved in T-ALL leukemogenesis [1,2].

Here we present a novel chromosomal aberration, involving the TCRB locus and the 10p12 region, in a previously described TCRγδ+ T-ALL (T003/MA) [3].

CASE REPORT

Cytogenetic analysis on bone marrow material of T003, a 7 year old male patient, revealed a complex karyotype: 46, XY, add(5)(q13), der(7)?inv(7)(p1?q3?), add(10)(q21), del(10)(q25) [26]/45, idem, Y[4], 46, XY[6]. Chromosomal rearrangements involving the q arm of chromosome 7 usually suggest the involvement of *TCRB*-genes in T-ALL cases. Southern blot analysis of the TCRB gene revealed the presence of a 5.35Kb band representing a Dβ1-Jβ2 rearrangement on one allele, while the second allele contained an atypical TCRB gene rearrangement of 4.4Kb within the β2 region (Figure 1) [4].

In order to resolve the nature of this unknown TCRB gene rearrangement, we performed a ligation-mediated PCR (LM-PCR), as previously described[5], using Dβ2 specific nested primers. *S*equencing revealed the presence of a fusion of the 3' end of the Dβ2 gene segment and the p12 region of chromosome 10, representing the derivative chromosome (der)(7) (Figure 2A). Furthermore, a second LM-PCR analysis, performed with nested primer sets directed to the Jβ2.2, Jβ2.5 and Jβ2.7 regions, revealed the reciprocal der(10) containing a fusion between the centromeric side of 10p12 and the 5' site of the Jβ2.3 gene segment (Figure 2A). Additionally, our data showed that the break on chromosome 10 occurred in the absence of cryptic recombination signal sequences (cRSSs) indicative of a 'Type 2' translocation[6].

The breakpoint was localized in-between the p11 and p12 bands on chromosome 10, proximal to the centromere (reference sequence: NT_008705.15) (Figure 2B). This area encompasses the genes *DNAJC1* and *AF10/MLLT10* which are located ~35Kb and ~504Kb
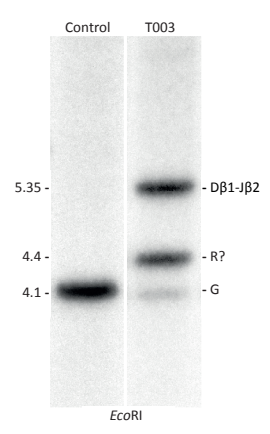
Figure 1. Southern blot analysis of TCRB gene rearrangement in T003. Control and T003 DNA were digested with *Eco*RI preceded by blotting and hybridization with ^{32}P-labelled TCRBJ2 probe. As expected, in the control sample, a germline (G) fragment of 4.1Kb was detected while T003 showed a Dβ1-Jβ2 rearrangement of 5.35KB and an atypical rearrangement (R?) of 4.4 Kb.
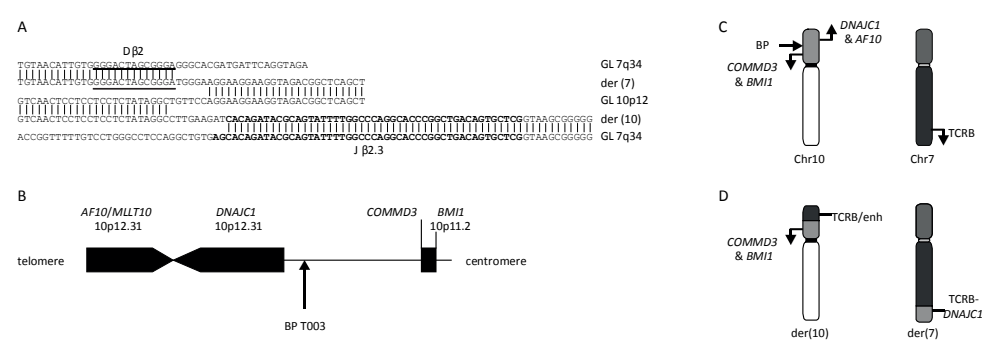

Figure 2. Breakpoint and flanking sequences of both derivative chromosomes in T003. A. GL indicates germline, der; derivative chromosome. Underlined sequence section indicates the Dβ2 gene. Bold sequence section indicated the Jβ2.3 gene. Note the lack of RSS-like sequences in the 10p12 region. B. Area of the breakpoint (brp) is indicated by the arrow (↑) on chromosome 10 (NT_008705.15). C and D. Schematic view of the chromosomal exchange on chromosomes 7 (Chr7) and the reciprocal exchange on Chr10. Bended arrows (⌐) show transcription direction. Arrow (→) shows the breakpoint.

telomeric to the break respectively and *COMMD3* and *BMI1* which are located ~277Kb and ~282Kb centromeric to the break respectively (Figure 2B).

Our data suggest that the der(7) resulted from a possible translocation with the distal part of chromosome 10p, thereby juxtaposing TCRB to *DNAJC1* (Figure 2C,D). The reciprocal chromosomal exchange giving rise to der(10) resulted in positioning of Jβ2.3, Cβ2 segments and TCRB enhancer elements upstream of *COMMD3* and *BMI1,* involving an area of over one hundred kilobases.

The 10p12 region has previously been shown to be involved in other γδ lineage specific t(10;11)(p13;q14-21) translocations resulting in *CALM-AF10* fusions. Breaks within this area have shown to cause the aberrant expression of *BMI1* and *COMMD3,* which is postulated to be induced by transcriptional *cis* activation.

BMI1 plays a critical role in tumorigenesis and its expression is decisive for the proliferative potential and self-renewing ability of stem cells [7]; *BMI1* overexpression is associated with poor prognosis and influences the aggressiveness of tumors in general[7].

As in the *CALM-AF10*^{+} TCRγδ[+] T-ALL cases, *BMI1* stands out as the candidate gene involved in the development of T-ALL in our T003 TCRγδ[+] case. Although we did not have remaining cell material to perform mRNA analysis on T003, we speculate that this translocation might have had a significant effect on the expression of *BMI1* and *COMMD3* either as the result of the presence of strong TCRB enhancer activity upstream of these genes (Figure 2C,D) or due to conformational changes caused by the break [8].

The fact that patient T003 was diagnosed having high risk T-ALL, with a white blood cell count above 50*10[6]/ ml blood, strengthens our speculation that this novel translocation has led to the upregulated expression of *BMI1* causing the aggressiveness of the disease.

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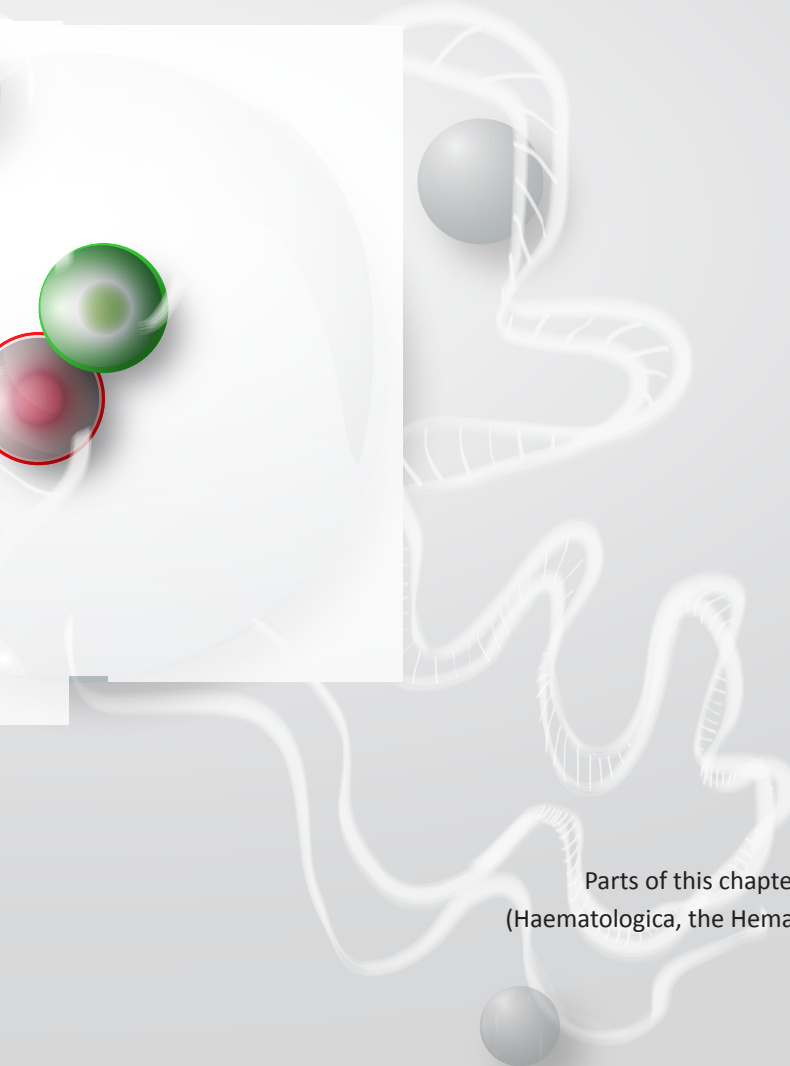
BMI1 as novel candidate oncogene in T-ALL

Chapter 5

GENERAL DISCUSSION

INTRODUCTION

The pathogenesis of T-ALL, like that of other cancers, is the result of a multistep process of mutational events that ultimately causes the uncontrolled expansion of the affected cell [1,2]. TCR translocations are a genetic hallmark seen in about 30% of T-ALL cases [1]. These translocations arise through errors that occur during the V(D)J recombination process, enabling the aberrant coupling of TCR genes to oncogenes [3]. Although the basic mechanism behind TCR translocation formation is generally accepted, it is still not clear why particular oncogenes and specific regions within these oncogene loci are recurrently involved in TCR translocations. We analyzed post-translocation configurations in T-ALL to examine major pre-translocation conditions, which are essential for the formation of a TCR translocation which could lead to the eventual T-ALL leukemogenesis. The whole path to full leukemogenesis is ultimately determined by post-translocation events in the T-ALL cells, but these events are subject to pre-translocation conditions in thymocytes that facilitate actual translocation formation. Here we critically discuss several aspects that are relevant for the occurrence of TCR translocations in precursor T lymphocytes (thymocytes) and their impact on T-ALL leukemogenesis:

1. Involvement of translocation-prone oncogene loci
2. Genetic and epigenetic vulnerability of oncogene loci to DSB induction
3. Spatial organization of TCR and oncogene loci
4. Role of the V(D)J recombination machinery in the formation of (non-) TCR chromosomal aberrations
5. Post-translocation conditions at the normal-malignant T-cell interface

1. Involvement of translocation-prone oncogene loci

At least three factors collectively determine the oncogenic effect of a TCR translocation and its eventual impact on leukemogenesis: 1) the involved TCR gene must provide regulatory elements or the translocation must result in the loss of the oncogene negative regulatory element (NRE), to enhance oncogenic activation [4], 2) the BP site at the oncogene locus must be localized as such that it enables the gain or loss of function effect, depending on the function of the (onco)gene involved [4-6], and 3) the (onco)gene involved must cause a selective/proliferative advantage upon deregulation [5,7].

TCR locus occurrence in TCR translocations

In about 33% of T-ALL cases, TCR-oncogene translocations have been described. TCRD, TCRG, TCRB, and TCRA loci are involved in ~67%, 0%, ~28% and ~5% of translocations, respectively. The majority of translocations (~67%, n=117) involve the TCRD locus. The high prevalence of TCRD associated translocations suggests a proneness for aberrant recombinations of this particular locus or a selective advantage upon involvement of the TCRD locus in these aberrations. TCRB associated translocations are less frequently seen (~28%). This lower frequency might be attributed to the absence of a (functional) pre-TCR in pre-leukemic cells, resulting from incorrect TCRB recombination as a consequence of the translocation. Since the expression of a functional and intact pre-TCR is a prerequisite for cell survival and further differentiation (i.e. β-selection), it is plausible that lack of pre-TCR signaling in these cells will lead to apoptosis before these cells can acquire sequential mutational hits [1]. This might explain why chances for TCRB translocation-mediated leukemogenesis in T-ALL are decreased. Since biallelic gene recombination is common to TCR loci [8], this difference cannot solely be attributed to the aforementioned loss of pre-TCR expression upon TCRB translocation. Until now it is not understood why the TCRA locus is less commonly seen in translocations (in this study ~5% of cases), and why the TCRG locus has never been involved. The TCRG locus is involved in translocus rearrangements (or trans-rearrangements) [9,10], thus suggesting that the locus can be involved in V(D)J recombination-associated aberrations. Regulatory elements drive oncogenic expression; in the case of TCRB translocations the enhancer is involved, while in TCRD translocations oncogenic expression is mostly driven by the TCRD internal promoter [4]. The TCRG locus carries both enhancer and silencer sequences. It has been shown that signaling via the pre-TCR, which has been suggested to be a prerequisite for leukemogenesis [11,12], activates the TCRG silencer [13]. This would suggest that upon TCRG translocation (which would have occurred prior to the TCRB locus rearrangement), pre-TCR expression activates the TCRG silencer, consequently inhibiting oncogenic activation. It might well be that the positive and negative selection phases of TCRαβ thymocytes are implicated in the low occurrence of TCRA locus translocations seen in T-ALL. TCRA translocations are formed at the latest thymocyte developmental stage, which requires less differentiation
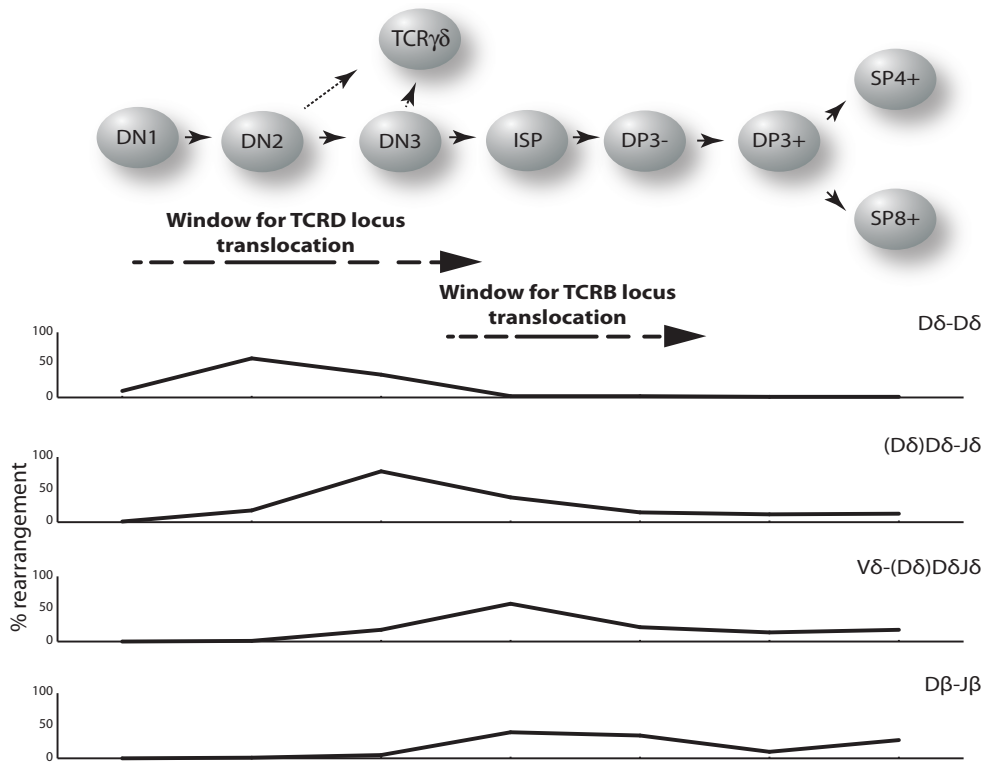
Figure 1. Window of opportunity for formation of TCRD and TCRB locus translocations. Schematic overview of consecutive stages of normal human thymocyte development. Graphs show rearrangement activity (%) during each developmental stage. The arrows indicate the window in which each recombination/ translocation combination can occur. V(D)J recombination graphs are adapted from Dik *et al*. [14] . DN = double negative; ISP = immature single positive; DP = double positive; SP = single positive.

and proliferation steps. It is reasonable to consider that upon translocation, the window for acquiring additional mutational hits is small, thus limiting the chance for tumorigenic transformation. Since the majority (> 90%) of the TCRαβ thymocytes do not survive selection but undergo apoptosis, the majority of the TCRA translocations will therefore be lost.

T cell differentiation is characterized by the tightly regulated process of TCR gene rearrangement, which occurs in a precise order (TCRD, TCRG, TCRB, TCRA) at specific stages of thymocyte development [14]. Identification of the TCR genes involved in TCR aberrations, and understanding of the intended coupling between TCR genes disclosed the developmental stage in which particular aberrations occurred (**Chapter 2**, this thesis). Of note however,

oncogenic transformation might not manifest at the exact stage in which the translocation occurred, but rather at a later developmental stage [15], usually after acquisition of subsequent mutational hits [1,2]. About 42%, 28% and 28% of the TCRD translocations occurred during an attempted Dδ-Dδ, Dδ-Jδ or Vδ-Dδ recombination, respectively, and ~76% and ~17% of TCRB translocations occurred during Dβ-Jβ, Vβ-Dβ recombinations, respectively. Thus the window of opportunity for translocation formation from the perspective of the TCRD locus is mainly during the DN stages of thymic development, whereas the TCRB locus is most likely involved in translocations during the DN3 to DP3- stages of thymocyte development [14]. However, interestingly based on the relative occurrence of TCRD and TCRB translocations and the high frequency of Dδ-Dδ miscouplings in T-ALL, it is evident that the majority of TCR translocations occur during an attempted Dδ-Dδ recombination during the progression from the DN1 to DN2 developmental stages (Figure 1) (**Chapter 2**, this thesis).

Oncogene involvement in TCR translocations

LMO2 (~15%), *TAL1* (~11%) and *TLX1* (~42%) oncogenes are the most commonly involved oncogenes in TCR translocations in T-ALL. Other TCR translocation partners are less frequently observed (~32% of total, and each <5%). Although the translocation frequencies in our study are only based on the available molecular data, and therefore do not reflect true prevalence of TCR-oncogene translocations in T-ALL, they are in line with previously observed relative frequencies [16]. Particular BP sites within the *LMO2*, *TAL1* and *TLX1* loci formed BP cluster regions (BCRs). Of note, other studies refer to a BCR as a region that can vary in size and encompasses areas of up to hundreds of kilobases in which a number of BP sites are localized. Since our interest was in identifying factors involved in causing DSB proneness, BCRs were specifically defined as more than two BP sites within a region of 50 bp sharing a common translocation DSB type (i.e. associated with a cRSS or not). Further analysis on oncogene BP site localization showed that ~82% of all TCRD translocations involved the 5'-end of the genes, while ~36% of the TCRB translocations involved the 3'-end of the genes. This observation fits the theory of effective oncogenic activation as a result of the actual TCR locus involved and its regulatory elements [4,6]. Furthermore, one can assume that the window of opportunity for sequential mutational hits after TCRD translocation formation is larger than for other TCR translocations, as at the TCRD recombination stage cells are more stem cell like and undergo more proliferation and differentiation steps. However, this alone cannot elucidate why TCRD translocations occur more frequently than TCRB translocations. Thus this observed difference could additionally be attributed to: 1) differences in synchronous accessibility between TCR and oncogene loci during the TCR recombination process, 2) differences in nuclear positioning of the TCR loci with respect to particular oncogenes as a prerequisite for translocation formation, and/or 3) better leukemogenic potential of TCRD translocations.

2. Genetic and epigenetic vulnerability of oncogene loci to DSB induction

Occurrence of a particular translocation in T-ALL is determined by the actual position of the BP site within the oncogene locus and by the resulting oncogenic effect of the translocation. However, the selective advantage through oncogenic activation does not elucidate why specific BP sites become recurrently involved in translocations and the cause for the heterogeneity of BP site involvement in TCR translocations. *In silico* and *ex vivo* analyses of 117 molecularly characterized BP sites in T-ALL showed that ~25% of the translocations were formed via the 'Type 1' translocation mechanism, while ~75% of the translocations arise via the 'Type 2' mechanism (**Chapter 2**, this thesis). This clearly shows that the majority of TCR translocations are not caused by RAG-cRSS interactions. A cRSS at a BP site is considered as the most obvious genetic feature known to render DNA susceptible to DSB induction leading to TCR translocation. However, the fact that 'Type 2' BP sites are predominantly seen and even concern BCRs (**Chapter 2**, this thesis), is highly suggestive of the presence and involvement of sequence-specific features that render sequences prone to DSB inductions other than cRSSs at these 'Type 2' translocation BP sites. Furthermore, particular cRSSs with low recombination efficiency and / or non-cRSS sites become preferentially involved in translocations over sites carrying highly efficient cRSSs within the same oncogene locus region. This illustrates that particular sequence-specific features might exceed the vulnerability level provided by cRSSs, but also implies that both 'Type 1' and 'Type 2' BP sites must be accessible to DSB induction and repair during the V(D)J recombination process.

Analysis of accessibility of the BP site sequences in thymocytes, as determined by methylation and nucleosome occupancy, showed that BP sites are differentially accessible (**Chapter 3.1**, this thesis). This difference in accessibility is also seen between cRSSs associated with a BCR (**Chapter 3.1**, this thesis). This implies that a more optimal accessibility could play a role in promoting cRSS involvement in a translocation which in turn could contribute to the differential involvement of cRSSs and oncogenes in T-ALL. cRSS recombination efficiency logically also plays a role in the susceptibility to RAG targeting [5,17]. ChiP analyses of histone marks targeted at cRSSs (mainly H3K4me3) could provide additional information of RAG2 activation at these sites, and thus provide more insight on why particular cRSSs within the same region become involved in translocations while others do not.

BP site localization is often associated with regions of high-GC content [18,19]. Despite an association between oncogene BP site localization and localized / regional high GC-content, no significant correlation was observed between the GC-content and the number of breaks at each BP site or translocation type. Neither did oncogene BP sites localize with the lowest free energy (ΔG) level, as was seen for other types of breaks [20,21]. High GC-content and the lowest ΔG can thus be excluded as the cause of localized and heterogeneity of vulnerability to DSB induction at these sites. All identified BCRs localized within the direct vicinity of the *LMO2*, *TAL1* and *TLX1* oncogenes TSSs. This initially suggested a particular susceptibility of

these sites to DSB induction and/or translocation formation. However, since these onco-genes are not expressed in thymocytes, transcriptional activity cannot be the primary cause of suspected susceptibility and localized vulnerability at these BCR sites [18,22,23] (**Chapter 3.1**, this thesis).

The localization of a *TLX1* BCR with a CpG-rich unmethylated ERV1 transposable element (TE) could be considered as a potential candidate element of genome susceptibility to DSB induction in 'Type 2' translocation breaks. (**Chapter 3.1**, this thesis). Hypomethylated TEs are associated with transcription and retrotransposition which are in turn connected to genetic instability and cancer [24-26]. In our studies, only a single BP site localized within an unmethyl-ated SINE (MIR) TE, but unlike the BCR site associated with ERV1, this single BP site has only been reported only once in T-ALL. Both SINE and ERV sequences have been shown to be associated with particular translocation BP sites [24,25], suggesting that both elements can be the cause of proneness to DSB induction. However, it is not clear what causes the difference in T-ALL BP site frequency between these two TE-associated sites. This could be attributed to the location of the BP site (5' or 3' end oncogene) or the TCR locus involved (TCRD vs. TCRB), which both might impact on the post-translocation oncogenic effect (**Chapter 3.1**, this thesis) [5,6]. Differences in activity and function between the ERV1 and the SINE sequences in thymocytes could likewise disclose a possible differential vulnerability, subsequent insta-bility, and perhaps give insight into the cause of differential frequency in T-ALL. About 75% (6/8) of the 'Type 2' BP sites did not associate with sequences of DSB vulnerability (**Chapter 3.1**, this thesis). Of the six BP sites, only two were localized at the 3'-end of the oncogene. Thus localization within the oncogene locus alone does not explain the low frequency of involvement of these sites in T-ALL.

Other elements like repetitive DNA motifs which have the capacity to adopt non-canon-ical (non-B) DNA structures and are known to induce genetic instability could be involved in vulnerability of these sites to DSB induction [27,28]. These DNA structures have also been reported to be targeted by RAG. It would be of interest to test if indeed a particular non-cRSS motif could be involved in RAG-mediated DSB induction at until now 'Type 2' categorized translocation BP sites. Nevertheless this would only be relevant for BCR sites at which no TEs or cRSSs could be identified, such as the *LMO2* BCR$_2$ (**Chapter 2**, **Chapter 3.1**, this thesis). Since single BP sites do not form BCRs, it is clear that these BP sites are less vulnerable to DSB induction than BCR. This implies that it is unlikely that these lesions resulted from the association with a sequence-specific motif of vulnerability. DSBs at 'Type 2' single BP sites are more likely to have originated by chance or perhaps due to patient–specific sequence vulnerability at these locations.

Finally, as the oncogene chromatin accessibility is stable during all stages of thymocyte development, there is no particular window at which the oncogene is more vulnerable to DSBs thus to increase the opportunity for translocation formation from the perspective of

the oncogene locus. This shows that the accessibility observed at the TCR locus, as seen during the stage-specific recombination process, is the only determinant factor for the time period in which a TCR translocation can occur. As oncogene BP sites (both BCRs and single BP sites) are seemingly continuously accessible, this would suggest an imminent chance for the induction of DSBs at these sites. However, one can assume that other mechanisms could be involved in protecting these sites for DSB induction, as it is unlikely that DSBs are continuously induced at these BP sites.

3. Spatial organization of TCR and oncogene loci

Formation of a translocation can only take place when an erroneous physical exchange occurs between two broken ends on dissimilar chromosomes [7,29,30]. Because DNA broken ends are believed to be immobile in mammalian cells, translocation formation in these cells can only follow the "contact first" translocation model [30,31]. For TCR translocations this means that these are only formed if TCR gene and oncogene broken ends are within translocation distance (≤ 0.5µm [32]) of each other prior to the emergence of the DSBs [30]. Nuclear organization has been considered to be non-random and dependent on cell stage differentiation, gene activity, and GC-content [33-35]. This non-random organization of interphase chromosomes has been postulated to be an important contributor to the translocation frequency. In fact it has been considered to be the determinant of recurrent translocations, since translocation probability has been shown to be increased between genes that are in close nuclear proximity [29,32,33,36,37].

We have shown that oncogene BP sites are accessible during thymocyte development, thus complying with the accessibility condition for DSB induction at these sites. Furthermore we showed that the majority of leukemogenic TCR translocations occur during attempted Dδ-Dδ and Dβ-Jβ recombination, thus indicating that the translocation window is restricted to the DN1 to ISP thymic development stages. The non-random genome organization theory, the contact-first translocation model and the recurrence of particular oncogenes in TCR translocations, collectively suggest that the translocation distance between an oncogene and a TCR locus should correlate with their involvement in translocation formation [29]. However, the nuclear positioning and inter-locus distances observed in 3D nuclear space between TCRD or TCRB loci and *TLX1* or *TAL1* oncogene loci in thymic subsets does not support this notion. No particular nuclear localization of the different loci was observed during any of the analyzed thymocyte development stages. Actually we showed that nuclear organization of gene loci has a strong random characteristic, contrary to previous suggestions [38,39]. Furthermore our findings show that likewise the relative inter-locus distances between translocation-prone genes and non-translocation partners (e.g. the transcriptionally active *GAPDH* gene) is primarily random. This shows that during thymocyte development there is no systematic co-localization of translocation-prone genes that will cause an increase in

translocation probability between these genes. These findings suggest that unlike previously shown [29,36] the inter-locus distance between TCR loci and oncogene loci that is required for chromosomal translocation is acquired by chance and not due to locus-specific nuclear proximity. Furthermore, of all inter-locus distances determined, an average of ~2% of the TCR-*TLX1* and TCR-*TAL1* distances were within the translocation distance of ≤0.5µm. A clear higher occurrence (2.43%) of translocation distances was observed between TCRD and *TLX1* at the DN1 stage compared to the other development stages. This suggests that there is an increased chance for translocation formation at this stage between these genes. TCRD:*TAL1* translocation distances were seen in only ~0.92% of the cases at this stage. This difference in occurrence of translocation distances seen between TCRD and *TLX1* and *TAL1* could elucidate the difference of *TLX1* and *TAL1* involvement in TCRD translocation formations in T-ALL. However, the observation than on average only less than 2% of inter-locus distances are within translocation distance is clearly different from previous findings, since in other cases translocation distances were observed at much higher frequencies. We argue that these observed differences could be attributed to the fact that in other studies experiments were performed in cells that were cultured [29,36], either to drive them into interphase, or to manipulate them through transfection [31]. It is known that culture conditions can change methylation patterns [40,41], and result in loss of epigenetic patterns specific for that cell, thus being non-representative of the situation in primary cells. In other studies nuclear proximity was determined in purified subpopulations of nonmalignant cells of cancer patients [32]. It cannot be fully excluded that these nonmalignant cells already show aberrations or are contaminated with (pre-)malignant cells.
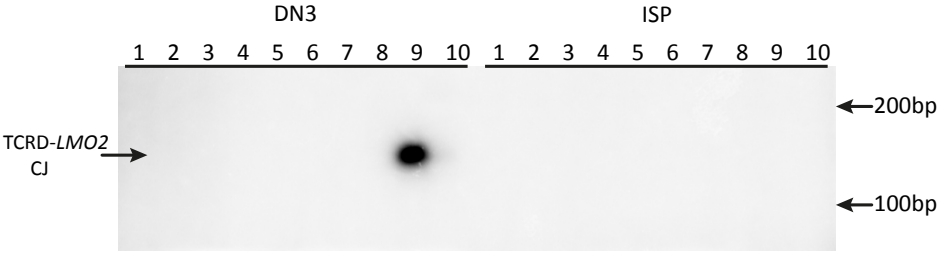
Figure 2. Identification of TCRD-LMO2 translocation in healthy thymic subsets. DNA of sorted thymocyte subsets obtained from five different thymuses from healthy donors was used. 10 nested PCR reactions were done on 500ng DNA followed by Southern Bolt analysis with a probe directed to the Dδ2 TCRD gene. TCRD-*LMO2* coding joint (CJ) represents the presence of a t(11;14)(p13q11) translocation within the normal thymocytes. The presence of the TCRD-*LMO2* CJ was determined in all thymic subsets (DN1-SP stages) (only DN3 and ISP data is shown here) and in total thymic samples. Only in the DN3 stage, the TCRD-*LMO2* CJ was observed in sample 9.

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Based on the presumption that nuclear proximity is required for translocation forma-tion and on the fact a maximum of 2,43% of the TCRD- and 3.37% of the TCRB-oncogene inter-locus distances are within translocation distance, we conclude that TCR translocation formations is a truly exceptional event. To further substantiate this, we have attempted to determine the translocation frequency of TCRD translocations in healthy human thymi, by analyzing these translocation occurrences in each thymic subset. To this end BCRs identified in the *LMO2* and *TLX1* loci were selected for further analysis of translocations in human thymocytes. Nested PCR was performed on thymocyte DNA with Dδ2-*LMO2* BCR [5], and Dδ2-*TLX1* BCR [6] primers. Translocation product was identified by means of Southern Blot analysis using γ-ATP-labeled *Dδ2* and *LMO2* or *TLX1* probes. Unlike previously shown for *LMO2* [5], we were unable to identify TCRD-*LMO2* translocations at the frequency of 10^{-6} -10^{-12}**.** Only one of the ten PCR samples in only the DN3 thymic subset was positive for TCRD-*LMO2* BCR translocation (Figure 2, unpublished data).

TCRD-*TLX1* BCR translocations were neither detected in any of the thymic subsets, nor in total thymus samples (unpublished data). The possibility exists that cells in which trans-locations occur are very quickly cleared, thus reducing the chance for tumorigenic trans-formation. Nevertheless, these findings irrefutably indicate that in normal thymocytes TCR translocations are less common than previously suggested [5,6].

4. Role of the V(D)J recombination machinery in the formation of (non-) TCR chromosomal aberrations

The V(D)J recombination process can be divided into two distinct phases which are of importance in the formation of translocations. Phase one, the induction phase, involves RAG protein complexes that induce DSBs and that are active at particular time points during the V(D)J recombination process [42], whereas in phase two, the repair phase, the non-homologous end joining (NHEJ) repair pathway is active [43]. The translocation mechanism that is leading to the formation of both 'Type 1' (RAG mistargeting) and 'Type 2' (erroneous repair) translo-cation has been described before [3,44,45]. Involvement of the V(D)J recombination machinery in the formation of these TCR translocations can readily be deduced from the involvement of TCR loci in these aberrations. Likewise, particular non-TCR chromosomal aberrations have been argued to be formed by the V(D)J recombination machinery based on the presence of cRSSs at the involved BP sites [46-49]. Despite the postulated role of the V(D)J recombination machinery in the formation of chromosomal aberrations in T-ALL, the precise molecular details of this involvement are still not fully clarified.

In previous studies TCR-associated translocation mechanisms have been evaluated for only a few BP sites by means of *ex vivo* experiments [5,17,49,50]. Unlike these studies, we included known molecularly defined BP sites of both TCR- (117 BP sites) and non-TCR- (118 BP sites)

associated chromosomal aberrations identified in T-ALL, irrespective of the frequency of oncogene involvement in T-ALL and the presence of cRSSs at BP sites (**Chapter 2**, this thesis).

As aforementioned only ~25% of the TCR translocations are formed via the 'Type 1' translocation pathway, while the majority (~75%) of the TCR translocations are 'Type 2' translocations (**Chapter 2**, this thesis). In addition to previous studies, by including non-TCR aberrations we could further subdivide the V(D)J recombination-associated aberration types (Figure 3). RSS-cRSS aberrations involving a cRSS and an authentic TCR RSS (known as 'Type 1' translocations) were categorised as 'Type 1a' aberrations. cRSS-cRSS interactions, as they are seen in non-TCR aberrations such as the *HPRT1(-BICD1)* deletions/insertions –though so far not identified in T-ALL- show clear involvement of the V(D)J recombination machinery (cRSSs at both BP sites and the insertion of untemplated nucleotides at the junctions) [46,48]. As these cRSS-cRSS interactions are 'Type 1a'-like recombinations, we categorized them as 'Type 1B' aberrations. TCR translocations without cRSS involvement at the oncogene site are now referred to as 'Type 2a' aberrations, whereas in analogy to 'Type 1b' aberrations, 'Type 2b' non-TCR aberrations can be identified when an authentic TCR RSS is replaced by a cRSS. One clear example of the latter concerns *SIL-TAL1* deletions/ fusions [49], which show random nucleotides at the junctions, lack a cRSSs at *SIL* BP sites, but do show a cRSS at the *TAL1* BP site. Finally, we have categorized translocations and fusions that do contain random nucleotides in their junctions, but which show neither an authentic TCR RSS nor a cRSSs, as 'Type 3' aberrations. These concerned aberrations of oncogenes that are also known to be involved in TCR translocations, such as *SIL-TAL1* and *LMO2-RAG2* deletion/fusion cases [51,52]. The indication of potential non-templated nucleotides at the junctions is no formal proof of TdT involvement, as DNA polymerase mu (Polμ) could also be involved. Nevertheless, we are convinced that the V(D)J recombination machinery is involved in break-break repair in these aberrations that have originated during early stages of thymocyte development. Overall this shows that the involvement of the V(D)J recombination machinery in the formation of chromosomal aberrations is not restricted to TCR loci and/or cRSS participation. In fact, by combining both TCR and non-TCR chromosomal aberrations a different picture emerges with respect to the V(D)J recombination machinery involvement. When considering the frequency of cRSS associated mutations versus non-cRSS associated mutations we see that ~50% of the chromosomal aberrations analyzed in our study are cRSS-mediated, while ~50% are not. This suggests a considerably higher frequency of cRSS mediated DSB inductions which lead to chromosomal aberrations than previously considered. It may not be unexpected that RAG-cRSS mistargeting would frequently occur, since cRSSs are found throughout the entire human genome, albeit that the recombination efficiency of most of these sequences is lower (i.e. lower RIC score) than that of authentic TCR RSSs (**Chapter 2**, unpublished data). Furthermore, it must also be taken into consideration that even though RAG1 is able to rec-ognize an array of different cRSSs, RAG2 should bind an H3K4me3-modified nucleosome
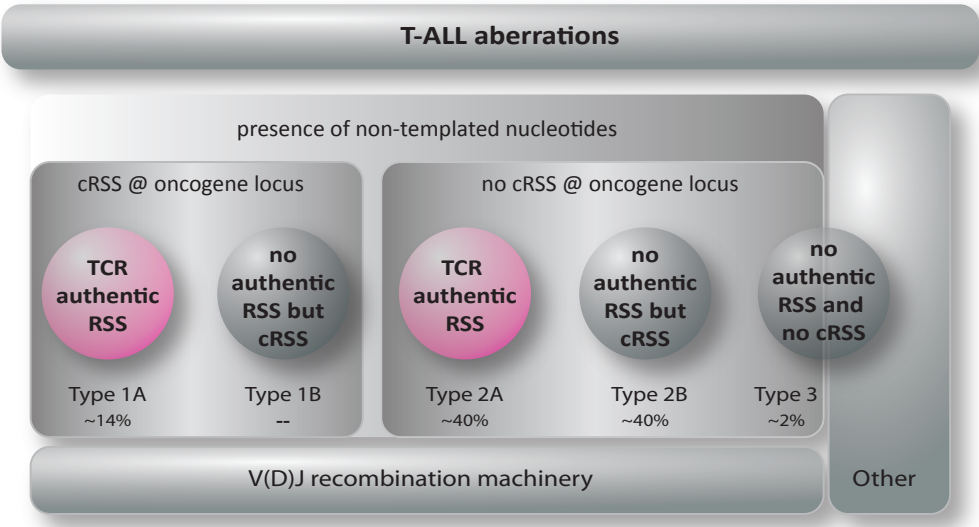
Figure 3. Schematic overview of chromosomal aberration type categorization. Two major chromosomal aberration types can be distinguished based on the presence of a cRSS at the breakpoint (BP) sites involved. Further differentiation between the major subtypes is determined by the involvement of an authentic RSS, a cRSS or no cRSS at the partner loci BP sites. The presence of a (c)RSS at the BP sites suggests the involvement of the V(D)J recombination machinery in the formation of these aberrations. The presence of non-templated nucleotides at the junction regions further substantiates this. At the absence of (c)RSS at the BP sites, the presence of these non-templated nucleotides becomes of more importance to differentiate between the involvement of the V(D)J recombination in the formation of these aberrations, or that they have come about via another mechanism.

adjacent to a cRSS before a complex with RAG1 can be formed, an allosteric change in RAG1 can be induced, and cleavage can occur [53]. Therefore one may assume that RAG recognition of a cRSS does not lead to actual DSB induction in all situations.

Role of RAG in formation of chromosomal aberrations

It has been shown that prior to TCR rearrangement, the involved loci are repositioned through a looping mechanism to facilitate the joining of the involved gene segments [54,55]. If RAG actively targets a cRSS for involvement in the V(D)J recombination process, this would imply that looping of the TCR locus and the high recombination efficiency of an authentic RSS do not restrict recombination to the TCR locus, which seems rather unlikely. In order to describe how RAG targets authentic TCR RSSs two models have been proposed. In the

first model one RAG protein complex recognizes an RSS and afterwards captures a second RSS to form a synaptic complex ("the capture model") [3,54], whereas in the second model two separate RAG protein complexes recognize two separate RSSs and the two RAG-RSS complexes merge to form a synaptic complex ("the merge model") [56,57]. Precise insight in RAG recruitment of an RSS and the synaptic complex formation mechanism should provide better understanding on how RAG is able to bring a cRSS into the RAG-TCR RSS synaptic complex as a first step in translocation formation. In any case, based on pre- and post-translocation TCR recombination configurations of the derivate chromosomes, we showed that normal RAG-mediated recombination can precede or follow a translocation in the very same cell (**Chapter 2**, this thesis). This implies that TCR translocations are not formed through an intrinsic fault of the V(D)J recombination machinery in these aberrant cells. This also virtually excludes RAG mutations as the cause of an unstable post-cleavage synaptic complex, which would allow DSBs to participate in the error-prone alternative NHEJ repair pathway [58]. If one takes into account that RAG protein-complexes are known to function as a shepherd of broken ends into the NHEJ repair pathway [57], thereby hampering repair via the HR pathway, 'Type 1' and 'Type 2' translocations could both be considered as repair mistakes upon coincidental DSB induction. The coincidental erroneous coupling to a TCR locus in which DSBs are actively induced, thereby increases the chance for oncogenic activation.

Role of repair pathway in formation of chromosomal aberration

Based on the abovementioned concept that erroneous repair could be a major cause of TCR translocation formation, we further looked into the role of the repair pathway in the formation of these translocations. The HR pathway requires the MRN complex involving nibrin (NBN) for repair of DSBs [30,59]. In patients with the genetic repair disorder Nijmegen breakage syndrome (NBS), the *NBN* gene is mutated and loss of nibrin jeopardizes genome stability by affecting the cellular response to DNA damage. If HR repair cannot proceed, NHEJ repair is the next option [43,60]. As HR repair is dependent on the presence of a homologue near the DSB, it is likely that upon loss of HR repair the NHEJ pathway, which is less dependent on such proximity, may lead to an increase in erroneous repairs. To determine if a compromised HR repair response influences the frequency of TCR associated translocation formation, we determined the frequency of TCR translocation formation in NBS deficient patients. To this end the frequency of TCRD translocations involving the *TLX1* and *TAL1* oncogenes was compared between a *NBN*-deficient NBS patient, *NBN* heterozygous carriers, and healthy controls (Larmonie and Langerak, unpublished data). Two approaches were followed: 1) split-signal FISH analysis with a TCRA/D probe in combination with *TAL1* or *TLX1* locus probes, and 2) FISH analysis with TCRD locus probes in combination with split-signal FISH probes directed to the *TLX1* and *SIL/TAL1* loci. Interestingly, our findings showed that trans-locations involving the TCRD, *TAL1* and *TLX1* loci are seen in *NBN* heterozygous carriers and
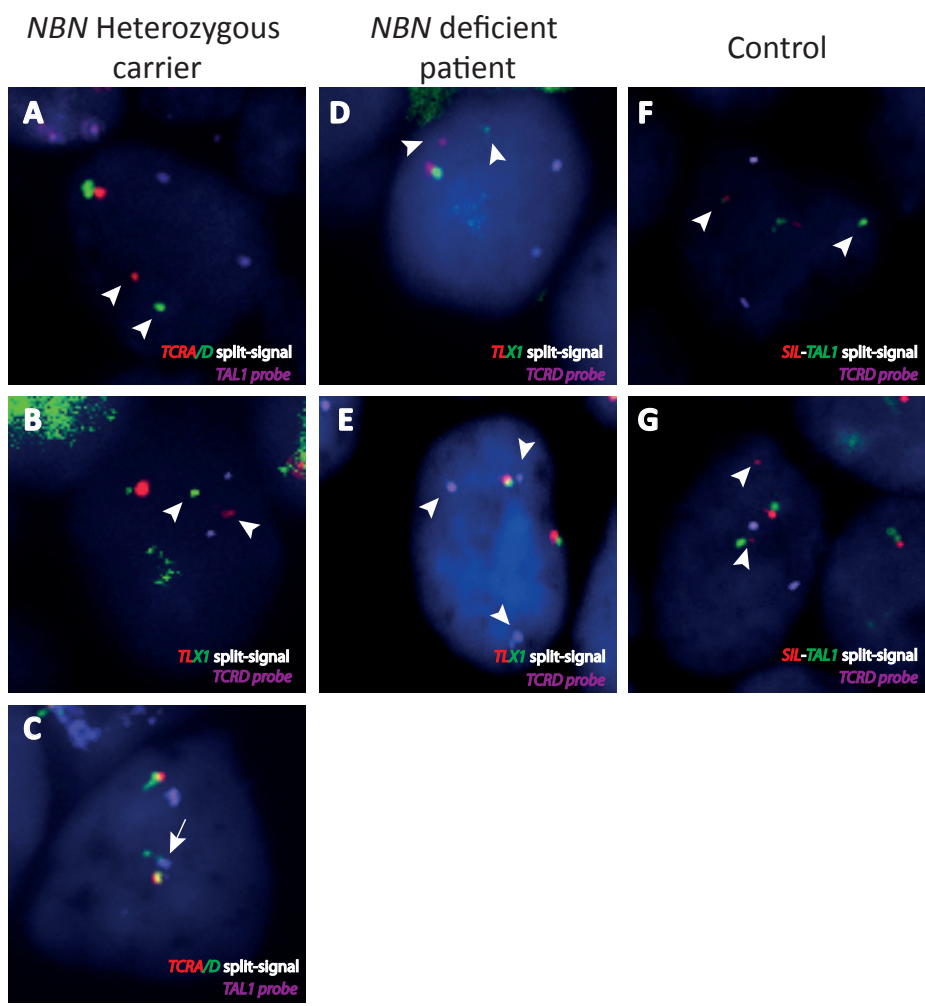
Figure 4. Interphase FISH with split-signal probes and BAC probes on cells obtained from *NBN* heterozygous carriers, an *NBN* deficient patient and control samples. Split-signal FISH analysis with TCRA/D probe (DAKO) in combination with *TAL1* and/or *TLX1* locus BAC probes were used, or FISH analysis was done with TCRD locus BAC probe was done in combination with split-signal FISH probes directed to TLX1 or *SIL/TAL1* loci (DAKO). Nuclear DNA was stained with Dapi. A-C Interphase nuclei of *NBN* Heterozygous carriers with split-signal FISH probes directed to TCRA/D locus combined with *TAL1* probe in A and C, and against split-signal FISH probes against *TLX1* locus and probe against the TCRD locus. In A and B a split in the TCRA/D and *TLX1* probes is seen respectively (arrow heads), indicating the translocation with an unknown partner. In C a possible TCR-*TLX1* translocation is shown (arrow). D and E show the translocation (arrowheads) of both the *TLX1* locus and the TCRD locus respectively in an *NBN* deficient patient. F and G show the presence of *SIL/TAL1* translocations in control cells. (The extended data on the occurrence on translocations in these cells can be found in the Supplementary Table 1).

NBN deficient patients, but also in control samples (Figure 4, Supplementary Table 1). Only in one of the *NBN* heterozygous carrier analysed a potential TCRA/D-*TAL1* translocation was observed (Figure 4C, Supplementary Table 1). These findings suggest that there is no particular increase in translocation formations in *NBN* deficient cells or control cells, although we realize that a larger cohort would be needed to draw firm conclusions.

In contrast, we did find an increase in the occurrence of TCR trans-rearrangements, which involve recombinations between different TCR loci, in these *NBN* deficient patients (Langerak *et al.*, unpublished data). This could suggest that upon loss of *NBN* the TCR loci, in which DSBs are actively induced during V(D)J recombination, are much more susceptible to erroneous rearrangements and subject to genome instability as the result of a faulty repair mechanism.

Furthermore, it is important to note that HR repair mainly takes place in the S/G2 phases of the cell cycle [61,62] and occasionally in the G1 phase. In contrast, NHEJ repair is active throughout the entire cell cycle [63,64], but especially during the G0/G1 phases [61,62], at a stage that the HR repair is least active. The V(D)J recombination relies on the NHEJ repair process and takes place during the G0/G1 cell cycle phase and RAG 2 proteins accumulate during these phases in thymocytes [65]. Lack of NBN during thymocyte development in these patients, therefore does not seem as an obvious cause for increased V(D)J recombination-associated translocation formations through cRSS associated RAG-induced DSBs. Rather, DSBs in the *NBN* deficient condition are probably formed through different mechanisms.

The role of ATM might be different in this respect. ATM is a key sensor in the DNA damage response (DDR) pathway. Defective ATM expression leads to ataxia telangiectasia (AT) and ATM$^{-/-}$ lymphocytes and leukemia/lymphoma cells carry trans-rearrangements at the TCR or IG locus [61,66]. ATM deficiencies in mice have been associated with an increase in TCRD/A translocation occurrence [66]. ATM functions in both the NHEJ and HR pathway [67], which implies that upon loss of ATM both the NHEJ and the HR pathway are compromised. Thus, upon complete loss of the DDR, there is a substantial chance for translocations to occur due to an increase in genome instability.

Overall this suggests that a compromised HR repair pathway by itself does not increase the susceptibility of particular sites to be involved in TCR translocations but that other factors are required to enable TCR translocation formation.

5. Post-translocation conditions at the normal-malignant T-cell interface

Previously, it was assumed that there is a high probability for the occurrence of chromosomal translocations, considering the many endogenous and exogenous factors that can cause DNA damage in actively proliferating and differentiating hematopoietic cells [7]. In spite of that, there is also the assumption that leukemia remains a relatively rare disease, since many factors are required for the eventual formation of a functional leukemogenic

chromosomal translocation. Evaluation of different conditions that are involved in the process of malignant transformation (both pre- and post- translocation conditions) showed that each condition is interrelated in a sequential manner, each being essential for a mutation to manifest itself as tumorigenic. Despite the involvement of endogenous and exogenous factors that increase the chance to DNA damage in proliferating cells, we conclude that, in view of these conditions, the chance that such DNA damage will eventually lead to T-ALL tumorigenesis is much smaller than previously assumed.

As discussed above, at least three specific pre-translocation conditions need to be in place at the time of V(D)J recombination prior to formation of a TCR translocation. These conditions however must exist in different dimensions; consecutively and simultaneously. A translocation can only be formed if at least two DNA breaks exist on two separate chromosomes within the nucleus. Our findings suggest that the formation of breaks in the oncogene locus can be induced by chance, or due to sequence-specific vulnerability to DSB induction. This susceptibility is further amplified by the level of accessibility of the site at that particular time point. The induction of a random break, which seemingly occurs by chance reduces the chance of translocation formation compared to DSBs induced due to an intrinsic susceptibility. However, the presence of a feature of sequence vulnerability to DSB induction does not automatically imply that a break will actually occur at that particular site. However, as BCR sites are recurrently involved in TCR translocations, it can be assumed that the ability of these sites to sustain lesions is higher than expected. The precise cause for the actual formation of a break in particular cells at these vulnerable sites remains to be established.

The second break is actively induced by RAG on the TCR locus, and is constantly present during the V(D)J recombination process. The two broken ends, however, must be located in the nucleus within translocation distance. The observation that less than 2% of inter-locus distances between TCR genes and oncogenes are within translocation distance clearly indicates that translocation-prone genes are not particularly predisposed to translocate with each other as there is no intrinsic established inter-locus distance between these loci. Even though ~2% of cells carry TCR and oncogene loci which are positioned within translocation distance, only a fraction of these loci will be carrying a DSB, at least from the perspective of the oncogene. This reduces the chance for a translocation to occur to less than 2%, if only these two conditions are considered. DSBs originating during the V(D)J recombination process are primarily repaired via the NHEJ repair pathway. As this repair process is error prone, the chance that translocations are formed through this process is increased. Taking into account that genetic and epigenetic determinants collectively mediate susceptibility of oncogene BP sites for DSB induction, and that breaks are primarily repaired via the error-prone NHEJ pathway, we conclude that nuclear proximity is one of the least relevant driving factors of recurrent translocations in T-ALL.

Emergence of oncogenic involvement in T-ALL as the result of a TCR translocation is primarily determined by the oncogenic effect that results from the translocation. If the oncogenic effect does not result in a selective advantage [68,69], such cell will not survive or will not undergo malignant transformation. Oncogenic activation is dependent on the cooperative influence of the level of activation or deactivation of the oncogene as the result of the translocation [5], and the cellular function of the affected (onco)gene. If upon deregulation of oncogene expression, cell cycle, differentiation, proliferation or self-renewal processes are affected [68,69], the involved cell could undergo the next step(s) towards malignant transformation. However, if the oncogene is able to influence one of these cellular processes, but the expression level of the oncogene is not optimal, the tumorigenic pressure diminishes, thus decreasing the chance for malignant transformation [5]. This indicates that the cellular function of an oncogene is more curtail for malignant transformation of the affected cell than the level of oncogene deregulation. In turn, the level of oncogene deregulation is dependent upon BP site localization and TCR locus involvement. The TCR locus provides regulatory elements (enhancers, promoters) to enable or enhance deregulated expression of the oncogene as seen for TCRD translocations, or it could lead to auto-extinction as recently speculated for TCRA-*TLX1* translocation cases [6]. Alternatively, the loss of negative regulatory elements (NRE) influences the activation of the oncogene [5,70-72]. As argued, BP site localization and the occurrence of recurrent involvement of BCRs in these translocations is clearly determined by the vulnerability of these sites to DSB induction as defined by the presence of a sequence-specific feature of vulnerability. The vulnerability to DSB induction and the high frequency of involvement of these sites in T-ALL clearly suggests that this vulnerability, which is dependent on oncogene function for manifestation in T-ALL, influences the frequency of occurrence of these sites, and thus the particular oncogene involved, in T-ALL. We have shown that particular oncogenes (*LMO2*, *TAL1* and *TLX1*) are predominantly seen in T-ALL. This predominance could be primarily explained by the fact that each of these oncogenes carries a sequence of vulnerability to DSB induction. However, other oncogenes, likewise carry a sequence of vulnerability to DSB induction, but are less commonly involved in translocations in T-ALL (**Chapter 2**, this thesis). Thus, the aspect of oncogenic effect based on the function of these particular genes could limit the occurrence of frequency of involvement of these oncogenes in T-ALL. Thus, highly frequent DSBs might not become apparent, if no selective advantage is induced through oncogene deregulation. Surely, the pathogenesis of T-ALL is also dependent upon the multistep process of mutational events that ultimately leads to the uncontrolled expansion of the affected cell. However, without acquiring a primary mutation driving the cell towards subsequent mutations, the cell will not undergo a malignant transformation.

This clearly shows, that the pre-translocation condition of vulnerability to DSB inductions primarily determines the chance of TCR translocation formation, but that the deregulation

and the function of the oncogene involved in the translocation ultimately determine the frequency of oncogene occurrence in T-ALL.

SUMMARY

During T-cell differentiation the process of TCR recombination is the common and defining factor, as its course is decisive for the eventual differentiation of a precursor T cell into a normal or malignant cell. In about 33% of T-ALL cases, TCR-translocations are considered as the first mutational event driving normal T cell development into leukemogenesis. Although the path to leukemogenesis ultimately is determined by post-translocation events, post-translocation events are subject to pre-translocation conditions for the actual translocation formation. Here we showed that sequence-specific features of DSB vulnerability increases the chance for involvement in translocations, and that single BP sites are induced by chance or a patient specific vulnerability. However, nuclear organization of TCR and oncogene loci is random and on average less than 2% of inter-locus distances between the translocation partners is within translocation distance. Thus, we conclude that although oncogene loci are susceptible to DSB induction, the lack of locus nuclear proximity between the translocation partners significantly diminishes the chance for an eventual translocation formation. Furthermore we showed that the actual interchromosomal exchange between TCR and oncogene is not the result of a systemic fault of the V(D)J recombination mechanism, rather they seem coincidental. Since the chance for the occurrence of the physical interchromosomal exchange is small, the reoccurrence of particular TCR-oncogene translocations in T-ALL must primarily be dictated by the post-translocation events, of which the oncogenic effect determined by the oncogene function is most likely to determine the outcome.

ACKNOWLEDGEMENTS

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SUPPLEMENT

Supplementary Table 1. Frequency of translocation aberrations in NBN heterozygous carriers, a NBN patient and healthy controls based on split-signal FISH in combination with FISH probes analysis.

	NBN Heterozygous carriers				*NBN* deficient patient	Control	
Split-signal FISH	*SIL/TAL1*	TCRA/D	*TLX1*	TCRA/D	*TLX1*	*SIL/TAL1*	TCRA/D
FISH Probe	TCRD	*TAL1*	TCRD	*TLX1*	TCRD	TCRD	*TLX1*
Patient #	159	200	8415	159	158	070	209
n	105	105	108	108	104	113	61
Normal cells (%)	93	88	84	72	85	81	87
Aberrant cells (%)	7	12	16	28	15	19	13
TCRD-oncogene translocations (%)	0	1	0	0	0	0	0
Unknown Oncogene Translocation (%)	1	2	4	6	2	2	3
Unknown TCRD translocation (%)	5	2	5	3	4	0	0

Addendum

ABBREVIATIONS

3D FISH	Three-dimensional fluorescence in situ hybridization
ALL	Acute lymphoblastic leukemia
bp	Base pair
BP	Breakpoint
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CE	Coding end
CJ	Coding joint
CMJ	Corticomedullary junction
cRSS	Cryptic recombination signal sequence
D	Diversity gene segment
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DSB	Double strand break
FAIRE	Formaldehyde assisted isolation of regulatory elements
FISH	Fluorescence *in situ* hybridization
HPC	Hematopoietic progenitor cell
HR	Homologous recombination
HSC	Hematopoietic stem cell
IG	Immunoglobulin
ISP	Immature single positive
J	Joining gene segment
LMO1	LIM domain only-1
LMO2	LIM domain only-2
LM-PCR	Ligation mediated polymerase chain reaction
LYL1	Leukemia lymphoid 1
(L)MMPs	(lymphoid-primed) multipotent progenitors
NHEJ	Non-homologous end joining
NKX2-1	NK2 homeobox 1
nt	Nucleotide
PCR	Polymerase chain reaction
RAG	Recombination activating gene
RNA	Ribonucleic acid
RQ-PCR	Real-time quantitative polymerase chain reaction
RSS	Recombination signal sequence
SE	Signal end
SJ	Signal joint
SP	Single positive
TAL1	T-cell acute lymphoblastic leukemia 1
T-ALL	T cell acute lymphoblastic leukemia
TCR	T-cell receptor
TCRA	T-cell receptor alpha
TCRB	T-cell receptor beta
TCRD	T-cell receptor delta
TCRG	T-cell receptor gamma
TLX1	T-cell leukemia homeobox 1
TLX3	T-cell leukemia homeobox 3
TSS	Transcription starting site
V	Variable gene segment

SUMMARY

T-cell receptor (TCR) translocations are a genetic hallmark of T-cell acute lymphoblastic leukemia (T-ALL) that arise due to errors that occur during the V(D)J-recombination process. These translocations result in the juxtaposition of oncogenes, most of which encode for transcription factors, to T-cell receptor (TCR) enhancers and regulatory elements. This results in the aberrant expression of the oncogenes involved. These translocations are considered to be key events in T-ALL leukemogenesis.

Molecular studies on TCR translocation breakpoint (BP) sites that have been identified in T-ALL prove the involvement of the V(D)J recombination machinery in the formation of both 'Type 1' (RAG mistargeting) and 'Type 2' (repair mistake) translocations. These translocations primarily involve the TCRD (delta) and TCRB (beta) loci. TCRD and TCRB loci have been observed to undergo translocations with multiple and particular translocation partners in T-ALL. Although the basic mechanism behind TCR translocation formation is generally accepted, it is still not clear why particular oncogenes and specific regions within these oncogene loci are recurrently involved in TCR translocations. The studies described in this thesis were designed to determine how the localized genomic and nuclear spatial conditions of both TCR locus and oncogene locus enable the occurrence of interchromosomal interactions that lead to the formation of TCR translocations. The research also investigates to what extent the V(D)J recombination mechanism drives these formations.

Chapter 2 presents the results of the detailed *in silico* analysis of 117 BP sites from TCR aberrations and 118 BP sites from non-TCR chromosomal aberrations identified in T-ALL. The role of the V(D)J recombination machinery in the formation of chromosomal aberrations was assessed from these analyses. Only approximately 25% of TCR translocations appeared to be driven by RAG-cRSS mistargeting. This indicates that V(D)J recombination associated translocations, in the context of translocation formation, appear to be more an issues pertaining to the repair process rather than to the mistargeting process. The fact that translocations mainly involve the TCRD locus suggests that the TCRD locus is prone to involvement in translocations, or it enables a more effective oncogenic activation upon translocation formation compared to other TCR loci. The observation of pre- and post-translocation chromosome configurations indicate normal RAG targeting, repair and recombination potential of the involved TCR locus in the same cell. This implies that there is nothing intrinsically wrong with the V(D)J recombination mechanism in T-ALL cells. The chapter furthermore provides an updated mechanistic classification on the contribution of the V(D)J recombination machinery in the formation of both TCR and non-TCR aberrations in human T-ALL. This study also clearly shows that specific oncogenes and regions within a particular oncogene locus are not randomly targeted for involvement in TCR translocations.

Chapter 3 presents the central research question regarding how localized genetic and epigenetic characteristics of oncogene loci (**Chapter 3.1**), as well as the spatial organization of TCR and oncogene loci within the nucleus (**Chapter 3.2**), facilitate the recurring involvement of particular oncogenes in TCR translocations. These factors, known to affect translocation probability, were analyzed using immunologically "healthy" human thymocytes. The usage of thymocytes as primary cells for these studies enabled us to establish these conditions in the "normal" developmental counterpart of T-ALL cells. In addition to this, it allowed us to determine which factors contribute to the emergence of TCR aberrations during the development of precursor T cells. The study also researches the cause of differential involvement of TCRD and TCRB locus in these translocations, as these TCR loci are recombinationally active at specific stage of thymocyte development.

Chapter 3.1 explores the extent to which the accessibility of particular regions within oncogene loci reflects the frequency of oncogene (region) and TCR locus involvement in TCR translocations. The DNA of sorted thymocyte subsets was used to determine the methylation status of BP sites by means of bisulfite sequencing and nucleosome occupancy. This was done through formaldehyde assisted isolation of regulatory elements (FAIRE) in the BPs found in the TCR translocation partners *LMO2*, *TAL1* and *TLX1*. BPs were subsequently analyzed for sequence-specific features of genomic vulnerability to DNA double strand break (DSB) induction. These studies revealed that BP sites tend to cluster in areas where sequence-specific features of DSB vulnerability are present. Furthermore, this proneness is not primarily dependent upon an optimal chromatin accessibility. The observed stability of the oncogene chromatin accessibility during thymocyte development implies that there is no particular opportunity for selective involvement of the TCRD or TCRB loci in these translocations.

Chapter 3.2 examines whether nuclear inter-locus distances between TCRD or TCRB loci on the one hand, and the oncogenes *TLX1* and *TAL1* on the other hand, are within translocation distance and thus involved in promoting translocation formation. We also evaluated whether a decreased inter-locus distance occurs at particular stages of thymocyte development and, which would make these genes more susceptible for translocation involvement at these stages. Three-dimensional fluorescence *in situ* hybridization (3D FISH) analysis showed that the overall inter-locus distances between TCRD and *TLX1* were significantly smaller in particular thymic subsets than TCRB and *TLX1* inter-locus distances. However, on average ≤1% of the TCR-*TLX1* distances and ≤0.8% of the TCR-*TAL1* distances were within the translocation distance (≤ 0.5μm). No significant difference in translocation distances were observed between TCRD and TCRB and the oncogene loci. These findings, in contrast to previous findings regarding other types of translocations, imply that the inter-locus distance required for chromosomal translocation between TCR loci and oncogene loci is primarily based on chance in healthy individuals.

Ectopic oncogene expression is a common result of TCR translocations. The presence of oncogene transcripts allows for specific T-ALL subgroups classification. However, the notion of oncogenic expression in T-ALL is based on expression differences seen in T-ALL compared to biologically unmatched references. **Chapter 4.1** evaluates the expression of the T-ALL subgroup-classifying oncogenes *LMO2*, *LYL1*, *TAL1*, *TLX1*, *TLX3* and *NKX2-1* in all major human T-cell developmental stages. Comparison of these transcript levels with levels detected in 39 matched T-ALL counterparts enabled the identification of true oncogenic expression in T-ALL. Our findings reveal that the usage of the correct reference material to determine ectopic oncogene expression in T-ALL is essential for correct interpretation of oncogenic activation to prevent T-ALL subgroup misclassification. We show that *TAL1*, *TLX1*, *TLX3* and *NKX2-1* are not expressed in thymocytes. Therefore, expression of these oncogenes in T-ALL should be considered aberrant at any maturational stage. *LMO2* and *LYL1* expression in immature and TCRγδ$^+$ T-ALL likely reflect normal T-cell development rather than oncogenic activation. However, their expression in αβ-lineage T-ALL is always indicative of oncogenic activation.

Chapter 4.2 introduces *BMI1* as a new TCRB translocation oncogene in a TCRγδ$^+$ T-ALL. The TCRB-*BMI1* translocation was identified by means of ligation mediated (LM) PCR. The juxtaposition of the TCRB enhancer upstream of *BMI1* suggests that this translocation could have led to the aberrant expression of *BMI1* in this T-ALL. *BMI1* is involved in regulating stem cells proliferation and self-renewing. It is for this reason that we speculate that this novel translocation was the key event leading to tumorigenesis in this T-ALL.

Finally, **Chapter 5** (General Discussion) examines the impact of the studies described in this thesis. In conclusion, BPs tend to cluster at sites that carry sequence-specific features that render DNA prone to breaks. The formations of single BP sites occur either by chance, or due to patient-specific vulnerability of the particular DNA sequence. Increase in vulnerability appears to correlate to increase in accessibility. The induction of DSBs at the oncogene locus is not primarily RAG-cRSS mediated and is not the result of a systemic fault of the V(D)J recombination mechanism. Particular oncogene loci are clearly susceptible to DSB induction. However, the apparently small occurrence on translocation distances between the translocation partners significantly reduces the odds for an eventual successful translocation formation. This suggest that the translocation distance between TCR and oncogene loci is the least driving factor in TCR translocation formation. The likelihood for successful physical inter-chromosomal exchange seems relatively limited. Furthermore, the recurrence of particular TCR-oncogene translocations in T-ALL is primarily dictated by post-translocation events. Of these events, the oncogenic effect is most likely the factor which ultimately determines oncogene occurrence in T-ALL.

SAMENVATTING

T-cel receptor (TCR) translocaties zijn genetische afwijkingen die kenmerkend zijn voor T-cel acute lymfatische leukemie (T-ALL). Deze afwijkingen worden veroorzaakt door fouten die ontstaan gedurende het V(D)J recombinatie proces. Deze translocaties resulteren in koppeling van oncogenen, waarvan de meeste coderen voor transcriptiefactoren, aan regulerende elementen van de TCR genen. De incorrecte koppeling veroorzaakt afwijkende expressie van de betrokken oncogenen. Deze TCR translocaties worden beschouwd als de belangrijkste chromosomale afwijkingen die de T-cel doen ontsporen en die bijdragen aan T-ALL ontwikkeling.

Moleculair onderzoek dat is uitgevoerd op TCR translocatie breukpunten (BP) in T-ALL toont aan dat het V(D)J recombinatiemechanisme betrokken is bij de vorming van zowel 'Type 1' (RAG mistargating) als 'Type 2' (reparatie fout) translocaties. De TCR translocaties betreffen voornamelijk de TCRD (delta) en de TCRB (bèta) genen. De TCRD en TCRB genen kunnen betrokken zijn in translocaties met dezelfde oncogenen maar ook met unieke translocatiepartners. Alhoewel het basismechanisme dat leidt tot de TCR translocaties bekend is, is het nog niet duidelijk waarom bepaalde oncogenen en met name specifieke gebieden in deze oncogenen herhaaldelijk betrokken raken bij TCR translocaties. Doel van de studies zoals die in dit proefschrift worden beschreven, is om te achterhalen hoe lokale (epi)genetische kenmerken en de ruimtelijke positie van de TCR genen en de oncogenen, interacties tussen chromosomen bevorderen die uiteindelijk kunnen leiden tot het ontstaan van TCR translocaties. Het onderzoek heeft zich verder ook gericht op de vraag in welke mate het V(D)J recombinatiemechanisme betrokken is in het ontstaan van deze chromosomale afwijkingen.

Hoofdstuk 2 laat de resultaten zien van een uitgebreide *in silico* analyse zoals die is uitgevoerd op 117 BP van TCR translocaties en 118 BP van niet-TCR afwijkingen in T-ALL. Op basis van deze analyse kon de rol van het V(D)J recombinatiemechanisme in het ontstaan van deze chromosomale afwijkingen worden vastgesteld. Onze bevindingen laten zien dat slechts 25% van de TCR translocaties blijkt te zijn ontstaan door RAG-cRSS 'mistargeting' ('Type 1') fouten. Dit geeft aan dat de vorming van V(D)J recombinatie-geassocieerde TCR translocaties meer een probleem is van het reparatieproces van dubbelstrengs breuken (DSB) dan een 'mistargeting' probleem. Het feit dat de TCRD genen voornamelijk betrokken zijn bij TCR translocaties suggereert een zekere gevoeligheid van de TCRD genen voor translocaties; een alternatieve verklaring is dat de TCRD genen in staat zijn tot effectievere activatie van oncogenen in vergelijking met andere TCR genen. De pre- en post-translocatie chromosomale configuraties suggereren een normaal proces van RAG 'targeting', breukherstel en recombinatie van het betrokken TCR gen in dezelfde cel; dit wijst er op dat er in principe niets mis is met het V(D)J recombinatiemechanisme van T-ALL cellen. Op basis van de uitgebreide translocatie BP analyse wordt in dit hoofdstuk ook een nieuwe indeling

gepresenteerd hoe het V(D)J recombinatiemechanisme bijdraagt aan de vorming van zowel TCR als niet-TCR afwijkingen in humane T-ALL. Deze studie geeft ook aan dat de specifieke oncogenen en regio's binnen een bepaalde oncogene locus niet willekeurig betrokken raken/ worden in TCR translocaties.

In **Hoofdstuk 3** staat de vraag centraal hoe lokale genetische en epigenetische karak-teristieken van de oncogenen (**Hoofdstuk 3.1**) evenals de ruimtelijke organisatie van TCR genen en oncogenen in de kern (**Hoofdstuk 3.2**) de frequente betrokkenheid van bepaalde oncogenen in TCR translocaties bevorderen. Deze factoren, waarvan bekend is dat ze de kans op translocaties kunnen beïnvloeden, werden geanalyseerd in immunologisch "gezonde" humane thymocyten. Het gebruik van primaire thymocyten stelde ons in staat om deze fac-toren te onderzoeken in de "normale" ontwikkelingsstadia waaruit T-ALLs ontstaan en te bepalen welke factoren het meeste bijdragen aan het ontstaan van TCR afwijkingen tijdens de ontwikkeling van voorloper T cellen. Ook konden we op die manier bestuderen of de oorzaak van het verschil in frequentie van voorkomen van TCRD en TCRB translocaties ger-elateerd is aan de specifieke ontwikkelingsstadia van thymocyten waarin de TCR genen actief herschikken.

In **Hoofdstuk 3.1** is onderzocht hoe de toegankelijkheid van bepaalde gebieden binnen het oncogen de frequentie van betrokkenheid van dat gebied in een TCR translocatie beïnv-loedt. Het DNA van gesorteerde thymocytensubsets werd gebruikt om de methylatiestatus van BP gebieden in de TCR translocatiepartners *LMO2*, *TAL1* en *TLX1* te bepalen met behulp van bisulfiet sequentieanalyse. Ook werd de toegankelijkheid van nucleosomen in deze BP gebieden bepaald met behulp van de 'formaldehyde assisted isolation of regulatory ele-ments' (FAIRE) techniek. In de BP gebieden werd vervolgens gekeken naar sequentie-speci-fieke eigenschappen die het genomische DNA kwetsbaar maakt voor DSB breuken. Uit deze studies is gebleken dat BP geclusterd voor lijken te komen in gebieden met sequentie-speci-ifieke factoren die DNA kwetsbaar maken voor DSB en dat dit niet voornamelijk afhangt van een optimale toegankelijkheid van het chromatine. Het feit dat de toegankelijkheid van chromatine stabiel lijkt te zijn tijdens de thymocytenontwikkeling duidt erop dat er geen specifiek moment is tijdens deze ontwikkeling die zorgt voor een selectieve betrokkenheid van een bepaald gebieden in de oncogenen bij TCRD of TCRB translocatievorming.

In **Hoofdstuk 3.2** is onderzocht of de afstanden in de kern tussen enerzijds de TCRD of TCRB genen en anderzijds de *TLX1* of *TAL1* oncogenen (nucleaire inter-locus afstanden), dusdanig klein zijn (binnen zogeheten translocatieafstand liggen) dat de kans op translo-catievorming hierdoor toeneemt. We hebben ook onderzocht of er sprake is van kleinere inter-locus afstanden tussen de TCR genen en oncogenen in bepaalde ontwikkelingsstadia van thymocyten, wat de oncogenen gevoeliger zou kunnen maken voor betrokkenheid bij translocatievorming. Uit de 3D fluorescentie *in situ* hybridisatie (3D-FISH) analyse bleek dat de ruimtelijke positie van de TCR genen en oncogenen in de kern grotendeels willekeurig

is. Verder bleken de inter-locus afstanden tussen TCRD en *TLX1* genen in de thymussubsets gemiddeld gesproken significant kleiner te zijn dan de TCRB-*TLX1* inter-locus afstanden. Gemiddeld hadden ≤ 1% van de TCR-*TLX1* afstanden en ≤ 0.8% van de TCR-*TAL1* afstanden een translocatieafstand van ≤ 0,5 µm. Er werd geen significant verschil in translocatieafstanden waargenomen tussen TCR genen (TCRD of TCRB) en de verschillende oncogenen. Deze bevindingen duiden er op dat bij gezonde personen de benodigde translocatieafstand in geval van chromosomale translocaties tussen TCR genen en oncogenen hoofdzakelijk op toeval berust. Dit is in tegenstelling tot wat eerder is beschreven voor andere typen translocaties.

Een veel voorkomend gevolg van TCR translocaties is de ectopische expressie van het oncogen. Specifieke T-ALL subgroepen kunnen worden gedefinieerd op basis van dergelijke oncogene transcripten. Echter, ectopische oncogene expressie in T-ALL wordt vaak gebaseerd op expressieverschillen tussen T-ALL en biologisch niet-overeenkomend normaal referentiemateriaal. In **Hoofdstuk 4.1** hebben we in alle belangrijke menselijke T-cel ontwikkelingsstadia de expressie bepaald van de oncogenen *LMO2*, *LYL1*, *TAL1*, *TLX1*, *TLX3* en *NKX2*-1 die relevant zijn voor T-ALL subgroepclassificatie. Door de expressieniveaus van deze oncogenen in thymocytensubsets te vergelijken met de expressieniveaus in 39 T-ALL was het mogelijk om vast te stellen wat de daadwerkelijke oncogene expressie was in T-ALL. Op basis van onze bevindingen laten we zien dat het gebruik van het juiste referentiemateriaal essentieel is voor de correcte interpretatie van ectopische oncogene expressie in T-ALL; dit kan een foutieve classificatie van T-ALL subgroepen voorkomen. Aangezien *TAL1*, *TLX1*, *TLX3* en *NKX2-1* niet tot expressie komen in thymocyten moet de aanwezigheid van deze oncogene transcripten als afwijkend worden beschouwd, onafhankelijk van de T-ALL subgroep. Aan de andere kant weerspiegelt *LMO2* en *LYL1* expressie in onrijpe en TCRγδ+ T-ALL subgroepen waarschijnlijk de normale T-cel ontwikkeling in deze cellen in plaats dat er sprake is van oncogene activatie. De aanwezigheid van *LMO2* en *LYL1* oncogene transcripten in de rijpere αβ-T-ALL lijn is daarentegen altijd een indicatie van een ectopische oncogene activatie.

In **Hoofdstuk 4.2** beschrijven we het *BMI1* oncogen als een nieuwe TCRB translocatiepartner in een TCRγδ + T-ALL. Met behulp van de 'ligation mediated' (LM) PCR methode en met Dβ- en Jβ-gen-specifieke 'nested' primers werd de TCRB-*BMI1* translocatie gevonden. De positionering van de TCRB 'enhancer' in de buurt van het *BMI1* gen suggereert dat deze translocatie kan hebben geleid tot de afwijkende expressie van *BMI1* in deze T-ALL. Aangezien *BMI1* betrokken is bij het reguleren van stamcelproliferatie, speculeren we dat de betrokkenheid van dit gen in een TCRB translocatie een cruciale gebeurtenis is die heeft bijgedragen aan de vorming van deze T-ALL.

Tot slot wordt de betekenis van het onderzoeken die beschreven worden in dit proefschrift besproken in de 'General Discussion' **(Hoofdstuk 5)**. Samenvattend, BP lijken geclusterd voor te komen op locaties met sequenties-specifieke kenmerken die het DNA vatbaar maken voor breuken. Plaatsen waar slechts één enkel BP is gevonden zijn vermoedelijk door toeval

ontstaan of als gevolg van een patiënt-specifieke kwetsbaarheid in de DNA sequentie. Deze kwetsbaarheid lijkt te zijn verhoogd op plekken waar de algehele toegankelijkheid van het chromatine in deze gebieden toeneemt. De vorming van DSB in de oncogenen worden niet hoofdzakelijk veroorzaakt door RAG-cRSS interacties en lijken niet het resultaat van een systematische fout van het V(D)J recombinatiemechanisme. De DSB lijken met name bij toeval te ontstaan. Ondanks het feit dat bepaalde oncogenen gevoelig lijken te zijn voor DSB, blijkt de schijnbaar willekeurige positie van TCR genen en oncogenen in de kernen van thymocyten en de geringe kans op de benodigde translocatieafstanden tussen de translocatiepartners de kans op uiteindelijke translocatievorming aanzienlijk te verminderen. Dit suggereert dat de translocatieafstand tussen TCR genen en oncogenen de minst bepalende factor is in TCR translocatievorming; de kans dat er daadwerkelijk een fysieke inter-chromosomale uitwisseling tussen TCR en oncogene loci plaatsvindt lijkt dus relatief klein. Dit alles wijst erop dat de frequentie van bepaalde TCR-oncogen translocaties in T-ALL vooral wordt bepaald door de post-translocatie gebeurtenissen, waarbij selectie door het oncogene effect uiteindelijk de factor is die betrokkenheid van een oncogen in het ontstaan van T-ALL bepaalt.

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Ik wil ook God bedanken voor al Zijn zegeningen.

CURRICULUM VITAE

Nicole Sybelle Dèsirè Larmonie was born on the island of Curaçao on the 16th of January 1978. She attended HAVO secondary school from 1991 to 1996, and VWO from 1996 to 1998 at the Radulphus College on Curaçao. Having decided to further her education, she moved to the Netherlands in 1998 to study Biology en Medical Laboratory Research at Hogeschool van Utrecht where she specialized in Molecular Biotechnology. During this study she conducted research into the function of the carboxyl-terminal tail of connexin46 (Cx46) and its function into the localization of Cx46 to the trans-Golgi network at PENN University, Philadelphia, USA. This research was conducted under the supervision of dr. M. Koval and dr. J. Das Sarma. After obtaining her bachelors degree in 2002, she started her study of Cellular/Molecular Biotechnology at Wageningen University, specializing in Medical Research. She conducted research at the Nederlands Vaccin Instituut (NVI) in Bilthoven during this phase of her studies. Her work was aimed at determining the effect of specific growth rates on the expression of virulence factors and LPS for the production of *Bordetella pertussis* vaccine. The research was conducted under the supervision of dr. A. van Boxel and dr. Z. Soons. Following this, she conducted research at the Department of Immunology at the Erasmus MC, Rotterdam, carrying out functional analysis on T-ALL associated transcription factor under the supervision of dr. W. Dik and dr. A. Langerak. Having obtained her masters degree in 2006, she continued working as a research assistant at the department of Immunology under the supervision of dr. A. Langerak. In 2007 she received the Mosaic NWO Grant (awarded for academic research by the Netherlands Organization for Scientific Research and the Ministry of Education, Culture & Science), which is awarded specifically to promote diversity within the Dutch academic community. This grant enabled her to carry out her PhD research activities at the Departmet of Immunology at the Erasmus MC, Rotterdam. She has succesfully completed her PhD project, and is employed since 2013 as a postdoc at the Department of Pediatric Oncology-Hematology of the Erasmus MC - Sophia Children's Hospital.

PHD PORTFOLIO SUMMARY

Name PhD student:	Nicole Sybelle Dèsirè Larmonie
Erasmus MC Department:	Immunology
Research school:	Molecular Medicine
PhD period:	October 2007- October 2011
Promoter:	Prof. Dr. J.J.M. van Dongen
Copromoter:	Dr. A. W. Langerak

PhD training	Year
In-depth Courses	
Molecular Medicine	2007
Medical Immunology	2007
OIC course "Practical introduction to laser scanning microscopy"	2007
Management for PhD students and Postdocs	2008
Biomedical Research Techniques VII	2008
Molecular Immunology	2009
Basic training didactics	2009
Course Working with Biological Safety Cabinets (Clean air/Telstar)	2010
Scientific English writing	2010
Seminar and Workshops	
Browsing Genes and Genomes with Ensembl	2007
7th Euroconference on Clinical Cell Analysis	2007
2nd Dutch Hematology Conference (NVVH)	2008
3rd Dutch Hematology Conference (NVVH)	2009
Mozaiek Workshop (NWO) Netwerken/onderhandelen	2008
2nd European Congress of Immunology	2009
Access training	2009
Medical Immunology	2010
High resolution & deconvolution workshop	2010
Mozaiek Workshop (NWO) Netwerken	2010
European Hematology Association (EHA-EHA), Scientific workshop on T-cell acute lymphoblastic leukemia (T-ALL) meets normal T-Cell development	2010
16th European Hematology Association (EHA)	2011

Other	
Journal Club	2007-2011
Seminars and minisymposia; Department of Immunology	2007-2011

Teaching	
Supervising Histology Practicum	2007-2011
Internship supervision (4x Bachelor, 2x Master thesis supervision)	2007-2011

National conferences	
11th Molecular Medicine Day, Rotterdam, The Netherlands. Poster presentation.	2007
Dutch Society of Immunology (NVVI) Annual Meeting, Noordwijkerhout, The Netherlands, Poster presentation	2009
12th Molecular Medicine Day, Rotterdam, The Netherlands. Poster presentation.	2010
Dutch Society of Immunology (NVVI) Annual Meeting, Noordwijkerhout, The Netherlands. Poster presentation.	2010

International conferences	
"Epigenetics - Molecular Principles and Mechanisms", Mosbach, Germany	2008
2th European Immunology Meeting, Berlin, Germany. Poster presentation	2009
Scientific workshop on T-cell acute lymphoblastic leukemia (T-ALL) meets normal t-cell development, Mandelieu, France. Poster presentation	2010
16th Congress of the European Hematology Association, Poster presentation London, England	2011

LIST OF PUBLICATIONS

NSD Larmonie, WA Dik, HB Beverloo, ER van Wering, JJM van Dongen and AW Langerak. *BMI1* as oncogenic candidate in a novel TCRB-associated chromosomal aberration in a patient with TCRgamma-delta+ T-cell acute lymphoblastic leukemia. *Leukemia 2008; 22, 1266–1267*

NSD Larmonie, WA Dik, VHJ van der Velden, PG Hoogeveen, HB Beverloo, JPP Meijerink, JJM van Dongen, AW. Langerak. Correct interpretation of T-ALL oncogene expression relies on normal human thymocyte subsets as reference material. *British Journal of Haematology 2012; 157: 125–154*

MD Kraszewska, M Dawidowska, **NSD Larmonie**, M Kosmalska, ŁSędek, M Szczepaniak, W Grzeszczak, AW Langerak, T Szczepański, M Witt and members of the Polish Pediatric Leukemia Lymphoma Study Group (PPLLSG). DNA methylation pattern is altered in child-hood T-cell acute lymphoblastic leukemia patients as compared with normal thymic subsets: insights into CpG island methylator phenotype in T-ALL. *Leukemia 2012; 26: 367-371*

NSD Larmonie, WA Dik, JPP Meijerink, I homminga, JJM van Dongen, AW Langerak. Breakpoint sites disclose the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell acute lymphoblastic leukemia. *Haematologica, the hematology journal 2013: in press.*

NSD Larmonie, A van der Spek, AJJC Bogers, JJM van Dongen, AW Langerak. Genetic and epigenetic determinants mediate proneness of oncogene breakpoint sites for involvement in TCR translocations. *Manuscript in revision.*