REARRANGEMENTS/AMPLIFICATION OF THE *ABL1* GENE AS AN EXAMPLE OF KINASE ACTIVATION IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Carlos GRAUX

Promotor: Professor André Bosly (UCL)
Co-promotor: Professor Jan Cools (KUL)

Thesis submitted in fulfillment of the requirements for the degree of
'Doctor in Biomedical and Pharmaceutical Sciences'

Brussels, 18th December 2008

Jury :

Promotor: Professor André Bosly (UCL)

Co-promotor: Professor Jan Cools (KUL)

President: Professor Jean-Luc Balligand (UCL)

Members: Professor Christine Harrison (University of Newcastle)
Professor Frank Speleman (Universiteit Gent)
Professor Anne Hagemeijer (KUL)
Professor Lucienne Michaux (KUL/UCL)
Professor Hélène Poirel (UCL)

The research described in this thesis was conducted at:

- the Center for Human Genetics, University of Leuven, Leuven, Belgium
  - in the laboratory of Genetics of Malignant Disorders
    (Prof. Anne Hagemeijer and Prof. Peter Vandenberghe)
  - in the laboratory of Molecular Pathogenesis of Leukemia
    (Prof. Jan Cools)

- the Human Genetics Center, Catholic University of Louvain, Brussels, Belgium
  - in the Hematologic Section laboratory
    (Prof. Hélène Poirel)

The picture on the cover corresponds to "La Chimère" from Gustave Moreau. It illustrates the principle of a chimeric protein that keeps some of the characteristics of the original proteins, acquires new functionalities (wings) and is able to attract specific ligands. It has been found on the web at the following address: www.lettres.ac-versailles.fr.

A mon Papa,
A la maman de Sarah,
A Aude et à tous les patients qui auraient tant voulu rester encore un peu parmi nous.

Acknowledgements

Mener à bien une thèse représente une quantité de travail incalculable. On y arrive probablement parce que, quand on aime, on ne compte pas. Derrière ce travail, il y a aussi beaucoup d'enjeux personnels, professionnels, familiaux, linguistiques, … Avec le temps j'ai réalisé la richesse de la récolte… Récolte qui dépasse largement l'investissement. Un placement sûr qui n'est pas le fruit de la spéculation mais celui d'un travail acharné dans le cadre de collaborations équitables… car vous l'aurez vite compris, ce travail dépasse largement ce qui peut être réalisé par une seule personne en 5 ans.

Il y a d'ailleurs tellement de personnes à remercier…

Je commencerai par ma famille et en particulier par Sarah, mon épouse, qui m'a soutenu depuis le début dans ce projet et qui a essayé de m'épargner la culpabilisation liée aux innombrables soirées et week-ends passés devant l'ordinateur. Je lui avais promis qu'après l'assistanat ça irait mieux. Finalement, l'échéance a été quelque peu repoussée. Mais je reste convaincu que cette thèse est avant tout source de liberté... Merci aux filles, Célestine et Charlotte, qui donnent un sens supplémentaire à notre vie. Merci à mes parents qui ont guidé positivement ma curiosité. Petit, elle m'a amené à démonter (casser) pas mal de choses, … Aujourd'hui, mon Papa aurait été tellement fier de venir m'écouter. Merci à mon frère et mes sœurs, à toute ma famille, à celle de Sarah et à mes amis pour leur regard valorisant vis-à-vis de mon travail.

Puis, je voudrais remercier la chance… Chance d'avoir rencontré le Professeur Lucienne Michaux qui a souhaité pour moi ce sentiment de liberté d'une thèse accomplie, qui a cru en mes capacités de la mener à bien et m'a aidé concrètement à y parvenir avec une disponibilité unique.
Elle m'a aussi introduit auprès du Professeur Anne Hagemeijer dont elle connaisait la rigueur, l'honnêteté et surtout la bienveillance. En me confiant un sujet porteur, le Professeur Hagemeijer m'a ouvert une porte pour laisser entrer la chance. Elle fût de découvrir la fusion *NUP214-ABL1* dans la leucémie aiguë lymphoblastique T, une nouvelle anomalie avec des implications thérapeutiques à la clef. Une opportunité à saisir et à mériter, qui fût le fil conducteur de ce travail. Toutes deux ont pris à cœur la réussite de ma thèse et ont investi généreusement de leur temps pour m'aider à publier l'avancement de la recherche.
Autre chance, au bout du même couloir, dans le laboratoire du Professeur Peter Marynen, travaillait un certain Professeur Jan Cools, de retour des Etats-Unis. Il venait de publier dans le NEJM une histoire bien semblable: la fusion *FIP1L1-PDGFRa* dans le syndrome hypereosinophilique. La bonne personne, au bon endroit et au bon moment qui pouvait valoriser notre découverte. J'ai rencontré chez Jan Cools un énorme respect, une volonté de valoriser chacun dans son travail et de partager ses connaissances et découvertes. J'ai rencontré également une véritable vision à long terme. Merci également à tous les membres de son équipe de recherche, en particulier à Kim de Keersmaecker qui s'est investie dans l'étude fonctionnelle de la fusion *NUP214-ABL1* avec une qualité exceptionnelle. Sa disponibilité pour relire mes articles et fournir des conseils m'a été très précieuse. Merci à Idoya Lahortiga et à Nicole Mentens qui m'ont appris avec beaucoup de patience les techniques de biologie moléculaire, domaine largement inconnu pour un médecin. Nous

avons partagé pendant deux ans le même enthousiasme pour le projet *IKZF2-ERBB4* qui n'a pas encore révélé tous ses secrets. Merci tout particulièrement à Idoya pour l'aide à la réalisation de certaines figures de ce manuscrit. Merci également aux autres membres du laboratoire de Jan pour l'athmosphère de travail tellement cordiale et leur patience par rapport à mon « néerlanglais » parfois douteux. Merci également aux Professeurs Iwona Wlodarska et Peter Vandenberghe ainsi qu'à leurs équipes respectives pour leur accueil, leurs conseils et leur soutien logistique.

Merci ensuite à toute l'équipe d'hématologie des Cliniques universitaires UCL Saint-Luc (Professeur Augustin Ferrant, Docteur Marie-Christiane Vekemans, Professeur Eric van den Neste, Professeur Cédric Hermans, Professeur André Delannoy, Docteur Marie Marevoet, les infirmières, les aides soignantes, les secrétaires, les data-managers, …) et à l'équipe de génétique (Professeur Hélène Poirel, Khadija Bahloula, Jeanne-Marie Libouton, Geneviève Ameye, Violaine Havelange, Jean-François Duhoux et l'ensemble de l'équipe) qui m'ont permis d'organiser mon temps et m'ont offert un cadre pour poursuivre mon activité de recherche au cours de mes deux années de résidence dans l'institution (2005-2006). En particulier, merci à Khadija Bahloula pour son aide importante et précieuse pour toute une série de manipulations.

Et un immense merci à mes collègues du servive d'hématologie des Cliniques universitaires UCL de Mont-Godinne au sein duquel j'ai le plaisir de travailler depuis 2007. Merci de m'avoir encouragé et soutenu d'une manière cohérente dans mon projet de thèse. Merci au Professeur André Bosly d'avoir accepté d'en être le promoteur et pour son regard bienveillant sur mon parcours professionnel. Merci de m'avoir intégré dans cette équipe d'hématologie de Mont-Godinne dont j'avais tellement apprécié la qualité du travail ainsi que les qualités humaines au cours de mon assistanat. Un merci tout particulier au Docteur Anne Sonet qui a fait preuve d'une grande souplesse pour m'aider à trouver le temps scientifique qu'elle n'a jamais réussi à dégager pour elle-même. Merci à mes autres collègues, les Professeurs Chantal Doyen et Christian Chatelain pour leur considération par rapport mon travail. Merci à toute l'équipe exceptionnelle du nursing, des data managers, des secrétaires, en particulier à Véronique Wergifosse, et à tous les collègues de l'hôpital pour leurs encouragements.
Ce n'est pas toujours facile pour les collègues « cliniciens » submergés au quotidien par la dure réalité du travail de terrain de percevoir toujours positivement les enjeux d'un travail de recherche dont les bénéfices n'apparaissent pas toujours de façon aussi concrète. Je n'ai jamais ressenti de culpabilisation sinon la mienne de devoir transmettre à mes collègues un service en chantier pour partir à Leuven assumer ma semaine de recherche. J'espère sincèrement avoir acquis au cours de cette période de thèse des outils qui me donneront les moyens d'assumer ce que l'équipe de Mont-Godinne attendait de moi en m'engageant. J'espère surtout que tout cet effort servira une qualité toujours croissante de mon travail au service des patients.

Merci à tous les assistants avec qui j'ai eu le plaisir de travailler pendant cette période. J'espère leur avoir communiqué davantage mon enthousisme que mon stress.

Merci à la direction de Mont-Godinne, en particulier au Professeur Patrick De Coster, Médecin Directeur, et à tous ceux qui s'appliquent à promouvoir une dynamique de

recherche au sein de l'institution, entre autre par la valorisation des clinicien-chercheurs. Je remercie également le Recteur de l'UCL, le Professeur Bernard Coulie, ainsi que le Professeur Jean-François Denef, Prorecteur aux Affaires Médicales, pour l'attribution du mandat de clinicien-chercheur. Merci au FNRS, à Salus Sanguinis, à la fondation Franqui-De Roover, au « Cycle for Life » et à la Société Belge d'Hématologie pour le soutien financier.

Merci à tous les membres du jury: au Professeur Jean-Luc Balligand d'avoir accepté d'être le président de ma thèse malgré un emploi du temps très chargé. J'ai apprécié sa rigueur, son efficacité et sa capacité de créer une ambiance constructive et valorisante lors des réunions du comité d'encadrement. Merci aux Professeurs Christine Harrison et Frank Speleman qui ont non seulement accepté le déplacement mais aussi contribué à cette recherche. Merci aussi au Professeur Harrison pour la relecture du manuscrit qui rend le texte plus agréable à lire.

Merci à Marie-Bernadette Jacqmain, dessinatrice à Mont-Godinne, qui est habituée de voir débarquer dans son bureau des médecins affolés par une « deadline » imminante et qui ont besoin d'un schéma pour … tout de suite… enfin, si possible.

Ce travail est aussi le fruit d'une collaboration fructueuse avec de nombreux laboratoires de recherche en Belgique, en France et ailleurs, entre autre dans le cadre du Groupe Francophone de Cytogénétique Hématologique (GFCH) à Paris et du Groupe Belge de Cytogénétique Hématologique et Oncologique (BCGHO), avec l'occasion de tisser de nombreux liens. J'espère que tout le monde y a trouvé ou y trouvera son compte.

« Last but not least », je terminerai en remerciant les patients. Leur évoquer mon travail de recherche a souvent été l'occasion de créer des complicités. Ils ont bien souvent été les premiers à m'encourager, percevant dans leur corps l'importance de rechercher des traitements plus efficaces et surtout moins toxiques. Même si la recherche est à certains moments grisante pour elle-même, c'est la folle envie de les aider davantage qui m'a poussé à donner le meilleur de moi-même dans ce travail de thèse. En regardant le fond des éprouvettes, mon regard et mes pensées se sont souvent évadées vers eux, m'obligeant parfois à répéter l'expérience en cours… Cette double activité de recherche et de clinique m'a permis de garder le contact avec la finalité de la recherche et aussi d'apporter au laboratoire ma sensibilité de clinicien. J'espère dans l'avenir pouvoir valoriser cette thèse en développant une activité de recherche translationnelle au sein du service.

Cette thèse a representé aussi un véritable « challenge » linguistique. La pratique de l'anglais et du néerlandais même imparfaite m'offre une liberté gigantesque que j'apprécie particulièrement dans mes contacts avec les collègues hématologues néerlandophones.

Si vous me demandez donc la clef de la réussite d'une thèse, je vous dirai que le travail, même acharné, ne suffit pas. La rencontre de personnes bienveillantes et constructives qui ont la volonté de vous pousser en avant est primordiale. Si en plus la chance vous sourit dès le début, alors, il n'y a vraiment plus qu'à sauter dans le train quand il passe et ... travailler.

Contents

List of abbreviations

Ag:	antigen
AML:	acute myeloid leukemia
Array-CGH:	array-comparative genomic hybridization
ATP:	adenosine triphosphate
BAC:	bacterial artificial chromosome
B-ALL:	B-cell acute lymphoblastic leukemia
bHLH:	basic helix-loop-helix
CDK :	cyclin dependent kinase
cDNA:	complementary deoxyribonucleic acid
CEL:	chronic eosinophilic leukemia
CLP:	common lymphoid progenitor
CML:	chronic myeloid leukemia
CNS:	central nervous system
Ct:	threshold cycle
D:	diversity
DAG:	diacylglycerol
del:	deletion
der:	derivative chromosome
dmin:	double minute
DNA:	deoxyribonucleic acid
DSL:	Delta-Serrate-Lag2
FAB:	French-American-British
FISH:	fluorescent *in situ* hybridization
FTI:	farnesyl transferase inhibitor
GSI:	γ-secretase inhibitor
HDAC:	histone deacetylase
HOX:	homeobox
HSC:	hematopoietic stem cell
hsr:	homogeneously staining region
ins:	insertion
inv:	inversion
IL-3:	interleukine 3
IL-7:	interleukine 7
IP3:	inositol triphosphate
ITAM:	immunoreceptor tyrosine-based activation motif
J:	joining
Kb:	kilobase (1000 nucleotides)
Mb:	megabase (1000000 nucleotides)
M-bcr:	major breakpoint cluster region
m-bcr:	minor breakpoint cluster region
μ-bcr:	micro breakpoint cluster region
MHC:	major histocompatibility complex
MLL:	mixed lineage leukemia
MRD:	minimal residual disease

mRNA:	messenger ribonucleic acid
MSCV:	murine stem-cell virus
NA:	non available
ND:	not done
NES:	nuclear export signal
NLS:	nuclear localization signal
p:	short arm of a chromosome
PCR:	polymerase chain reaction
PEST:	proline-, glutamate-, serine-, and threonine-rich
PI3K:	phosphatidylinositol 3-kinase
PKC:	enzyme protein kinase C
PLCγ1:	phopholipase Cγ1
PIP2:	phosphatidylinositol biphosphate
PIP3:	phosphatidylinositol triphosphate
pre-TCR:	precursor T-cell receptor
pTα:	TCRα chain 'substitute' for immature T-cells
Puro:	puromycine
q:	long arm of a gene
Q:	quantitative
RACE:	rapid amplification of cDNA ends
RAG:	recombinase activating gene regulator
RNA:	ribonucleic acid
RNAi:	RNA interference
RT-PCR:	reverse transcriptase-polymerase chain reaction
SH2:	SRC homology 2
SH3:	SRC homology 3
siRNA:	small interfering ribonucleic acid
SNP:	single nucleotide polymorphism
T-ALL:	T-cell acute lymphoblastic leukemia
TCR:	T-cell receptor
TK:	tyrosine kinase
TKI:	tyrosine kinase inhibitor
V:	variable
WBC:	white blood-cell count
WHO:	World Health Organization
WT:	wild type

Summary

T-cell acute lymphoblastic leukemia (T-ALL) is a neoplastic disorder that develops from a single hematopoietic T-cell precursor that acquired oncogenic anomalies. T-ALL is a heterogeneous disease comprising several clinico-biological entities characterized by distinct underlying genetic defects.
In the first part of this work, we attempted to correlate those numerous anomalies with the role of the corresponding non mutated genes or pathways in normal T-cell development. Mutations targeting self-renewal, differentiation, proliferation, survival and death signals cooperate in a multistep process leading to a clinically overt leukemia.

Starting from the fortuitous observation by FISH of *ABL1* gene amplification in one T-ALL case, we found a new fusion gene, *NUP214-ABL1*, on amplified episomes in 6% of T-ALL, encoding a constitutively activated chimeric tyrosine kinase. Sensitivity of this oncogenic protein to the tyrosine kinase inhibitor (TKI) imatinib was demonstrated in the T-ALL cell line ALL-SIL carrying the *NUP214-ABL1* transcript.
Using cytogenetic and molecular analyses, we focused mainly our experimental work on kinase alterations. This class of mutations confers a proliferation advantage to the cell and is frequently observed in cancer, but was only rarely described in T-ALL. The recurrent cryptic *NUP214-ABL1* fusion was always amplified, usually extrachromosomally on episomes and/or intrachromosomally in homogeneous staining regions (hsr). In some T-ALL cases both episomes and hsr coexisted. In addition, the *NUP214-ABL1* rearrangement was usually associated with other genetic abnormalities, i.e. increased *TLX1* or *TLX3* expression, deletion of the tumor suppressor gene *p16* and mutations of *NOTCH1*, consistent with a multistep pathogenesis of leukemia. The *NUP214-ABL1* fusion gene was sometimes only present in a fraction of the leukemic cells (i.e. in subclones), suggesting its relatively late occurrence.

We next investigated whether other *ABL1* fusions were involved in T-ALL. We identified *EML1-ABL1* in a single T-ALL case as a fusion gene also encoding a constitutively activated tyrosine kinase sensitive to imatinib. Again, coexistence with *TLX1*, *p16* and *NOTCH1* anomalies was detected. At disease relapse, a transition from *EML1-ABL1* to *NUP214-ABL1* positivity was observed. However, retrospective screening for *TLX1* and *p16* demonstrated that aberrations of these genes persisted at the time of hematological remission. This observation suggested that a latent preleukemic clone with a restricted number of mutations evolved to an aggressive clone with the acquisition of *EML1-ABL1*, defied chemotherapy, and eventually caused relapse after having acquired another kinase mutation, i.e. *NUP214-ABL1*.

Finally, we explored other tyrosine kinase aberrations in T-ALL by performing genome wide array-CGH screening of copy number abnormalities in T-ALL cell lines. We identified and characterized a new transcript, *IKZF2-ERBB4*, in T-ALL cell-lines and T-ALL patients. In two T-ALL cell lines, a small deletion at the 3' end of *IKZF2* was responsible for the generation of an *IKZF2-ERBB4* fusion gene. Although 10 % of T-ALL patients also expressed a similar fusion transcript, no deletions could be detected in these cases, leaving the exact molecular cause for the presence of this fusion transcript currently unknown. The transcript was expressed as a transcription induced chimera or trans-spliced

transcript in normal mice brain and kidney. The existence of a putative IKZF2-ERBB4 chimeric protein could so far not be confirmed and we can only speculate on its biological or pathological consequences. Further investigations are needed to solve this particularly intriguing enigma.

In conclusion, this work demonstrates a pathological role of *ABL1* fusion variants in T-ALL. For *NUP214-ABL1*, episomes represent a new mechanism of formation of a fusion. If we consider targeted therapy and the use of TKI (active in vitro) for the treatment of T-ALL we should take into account some T-ALL specificities. Firstly, episomes, when present, are an intrinsic mechanism of drug resistance by virtue of their amplification potential. Secondly, *ABL1* fusions (*EML1-ABL1* and *NUP214-ABL1*) represent secondary cooperating events, which suggest that therapy targeting these ABL1 fusion proteins should be given in addition to drugs effective against the associated mutations.

Résumé

La leucémie aiguë lymphoblastique T (LAL-T) est une maladie néoplasique qui se développe à partir d'un précurseur hématopoiétique T ayant accumulé des anomalies oncogéniques. C'est une maladie hétérogène comprenant plusieurs entités clinico-biologiques caractérisées par des aberrations génétiques sous-jacentes distinctes.
Dans la première partie de ce travail, nous avons essayé de corréler ces nombreuses anomalies avec le rôle de la forme non mutée des gènes ou des voies de signalisation correspondants au cours du développement thymocytaire normal. Des mutations affectant l'auto-renouvellement, la différentiation, la prolifération, la survie et la mort cellulaire agissent en concert selon un processus multi-étapes qui aboutit au développement d'une leucémie floride.

En partant de l'observation fortuite d'une amplification du gène *ABL1* au cours d'un examen de routine par FISH dans un cas isolé de LAL-T, nous avons découvert un nouveau gène de fusion, *NUP214-ABL1*, supporté par des épisomes amplifiés dans 6% des LAL-T. Ce gène de fusion encode une tyrosine kinase chimérique présentant une activité constitutive. Sa sensibilité à l'inhibiteur de tyrosine kinase imatinib a pu être démontrée dans la lignée cellulaire issue de LAL-T, ALL-SIL, qui exprime le transcrit de fusion *NUP214-ABL1*.
En utilisant les outils de la cytogénétique et de la biologie moléculaire, nous avons ensuite concentré notre travail sur les altérations de tyrosines kinases. Cette classe d'anomalies donne un avantage prolifératif à la cellule et est fréquemment rencontré dans le cancer, mais rarement rapporté dans la LAL-T. La fusion *NUP214-ABL1* est cryptique et est toujours amplifiée, habituellement en extra-chromosomique sur des épisomes ou plus rarement en intra-chromosomique au niveau de zones qui colorent de façon homogène (hsr). Dans certains cas, les deux présentations coexistent. De plus *NUP214-ABL1* est habituellement associé à d'autres anomalies génétiques comme la surexpression des gènes *TLX1* ou *TLX3*, la perte du gène suppresseur de tumeur *p16* et à des mutations activatrices de *NOTCH1*, ce qui cadre bien avec une vision multi-étapes de la leucémogenèse T. La fusion *NUP214-ABL1* est parfois détectée dans une fraction seulement des cellules leucémiques (sous-clone), ce qui suggère qu'il s'agit d'un évènement relativement tardif.

Nous avons ensuite recherché l'implication d'autres fusions avec *ABL1* dans la LAL-T. Nous avons identifié *EML1-ABL1* dans un cas isolé. Comme *NUP214-ABL1*, ce gène de fusion encode une tyrosine kinase chimérique constitutivement activée et sensible à l'action inhibitrice de l'imatinib. Nous avons retrouvé également la coexistence d'anomalies de *TLX1*, *p16* et de *NOTCH1*. Lors de la rechute de la maladie, la fusion *EML1-ABL1* n'était plus détectable mais par contre, le patient avait acquis un transcrit de fusion *NUP214-ABL1*. Retrospectivement, nous avons démontré la persistance des anomalies de *TLX1* et de *p16* dans les échantillons de la rémission. Cette observation suggère l'existence d'un clone pré-leucémique au départ, présentant un nombre plus restreint de mutations, qui a évolué vers un clone agressif suite à l'acquisition de *EML1-ABL1*, a résisté à la chimiothérapie et a entraîné la rechute suite à l'acquisition d'une autre mutation de tyrosine kinase, dans le cas présent, une fusion *NUP214-ABL1*.

Finalement, nous avons recherché d'autres anomalies de tyrosine kinase dans la LAL-T à l'aide d'une technique d'hybridation génomique comparative utilisant des micro-damiers (array-CGH). Cette technique permet une analyse globale du génome à la recherche d'anomalies du nombre de copies des gènes. Nous avons ainsi identifié et caractérisé un nouveau transcrit, *IKZF2-ERBB4*, dans des lignées cellulaires et des échantillons de LAL-T. Dans deux lignées cellulaires, la présence d'une petite délétion à la partie 3' de *IKZF2* est responsable de la génération d'un gène de fusion *IKZF2-ERBB4*. Bien que 10% des patients avec une LAL-T expriment aussi un transcrit de fusion similaire, nous n'avons pas pu mettre en évidence de délétion. Le mécanisme de formation du transcrit de fusion *IKZF2-ERBB4* reste inconnu dans ces cas. Le transcrit de fusion est également exprimé sous forme de chimère induite par la transcription ou de transcrit issu d'un trans-épissage dans le tissu rénal et cérébral normal de la souris. L'existence de la proteine chimérique hypothétique IKZF2-ERBB4 n'a pas pu être confirmée à ce jour. Nous ne pouvons donc, à ce stade, qu'émettre des hypothèses en ce qui concerne ses conséquences biologiques ou pathologiques. Des investigations complémentaires sont requises pour résoudre cette énigme particulièrement intrigante.

En conclusion, ce travail démontre le rôle pathologique de fusions impliquant *ABL1* dans la LAL-T. Dans le cas de *NUP214-ABL1*, les épisomes représentent un nouveau mécanisme de formation d'un gène de fusion. Par rapport à une thérapie ciblée, si l'on considère les inhibiteurs de tyrosine kinase (actifs *in vitro*), il faut tenir compte de certaines caractéristiques des ces LAL-T. Tout d'abord, les épisomes, lorqu'ils sont présents, représentent un mécanisme intrinsèque de résistance aux médicaments suite à leur proriété d'auto-amplification. Deuxièmement, les fusions impliquant *ABL1* (*EML1-ABL1* et *NUP214-ABL1*) sont des événements secondaires qui coopèrent avec d'autres mutations. Cela suggère que les traitements qui ciblent *ABL1* doivent être administrés en plus de drogues qui visent efficacement les mutations associées.

Samenvatting

T-cel acute lymfoblastische leukemie (T-ALL) is een neoplasme dat zich ontwikkelt vanuit één enkele hematopoietische T-cell voorloper die oncogene afwijkingen accumuleerde. Het is een heterogene ziekte die verschillende klinisch-biologische entiteiten omvat en gekenmerkt wordt door talrijke onderliggende genetische defecten.
Het eerste deel van dit werk richt zich op het vergelijken van de rol van deze verschillende afwijkingen met die van de corresponderende niet gemuteerde genen of signaalwegen in de normale T-cell ontwikkeling. Mutaties met invloed op zelfvernieuwingscapaciteit, differentiatie, proliferatie, celoverleving en celdood werken samen in een meerstaps proces dat uiteindelijk resulteert in een klinisch detecteerbare leukemie.

Vertrekkend van de toevallige FISH observatie van *ABL1* amplificatie in één T-ALL patiënt identificeerden we *NUP214-ABL1*, een nieuw fusiegen dat voorkomt op geamplificeerde episomen in 6% van de T-ALL patiënten en dat codeert voor een continu geactiveerd chimeer tyrosine kinase. Gevoeligheid voor de tyrosine kinase inhibitor (TKI) imatinib werd aangetoond in de T-ALL cellijn ALL-SIL die het *NUP214-ABL1* transcript tot expressie brengt.
Het experimenteel werk is voornamelijk gericht op het karakteriseren van afwijkingen in kinasen met behulp van cytogenetische en moleculaire analyses. Kinase mutaties bieden de cel een proliferatie voordeel en komen vaak voor in kanker, doch zelden in T-ALL. De recurrente cryptische *NUP214-ABL1* fusie is steeds geamplificeed, meestal extrachrosmosomaal op episomen of intrachromosomaal op hsr, met beide presentaties tegelijkertijd in sommige patiënten. Bovendien is *NUP214-ABL1* meestal geassocieerd met andere genetische defecten, namelijk verhoogde *TLX1* of *TLX3* expressie, deletie van het *P16* tumor suppressor gen en mutaties in *NOTCH1,* wat overeenstemt met de meerstaps pathogenese van leukemie. *NUP214-ABL1* is soms slechts aanwezig in een fractie van de leukemische cellen (namelijk in subklonen), wat een relatief laat optreden van *NUP214-ABL1* suggereert.

We onderzochten vervolgens of andere *ABL1* fusies betrokken zijn in T-ALL. We identificeerden één patiënt met een *EML1-ABL1* fusiegen dat eveneens codeert voor een imatinib gevoelig continu geactiveerd tyrosine kinase. Weerom werd gelijktijdig voorkomen met *TLX1*, *P16* en *NOTCH1* mutaties gedetecteerd. Overgang van *EML1-ABL1* naar *NUP214-ABL1* werd geobserveerd bij ziekteherval. Echter, retrospectieve studies toonden aan dat de *TLX1* en *P16* afwijkingen ook aanwezig waren gedurende hematologische remissie. Deze observatie suggereert dan een latente preleukemische kloon met een beperkt aantal mutaties evolueerde tot een agressieve kloon met het verwerven van *EML1-ABL1,* ontsnapte aan chemotherapie, en uiteindelijk herval veroorzaakte na het verwerven van een andere kinase mutatie, in dit geval *NUP214-ABL1*.

Tenslotte gingen we op zoek naar andere tyrosine kinase afwijkingen in T-ALL met behulp van genoomwijde array-CGH screening voor afwijkingen in kopij aantallen in T-ALL cellijnen. In T-ALL cellijnen en T-ALL patiënten identificeerden en karakteriseerden we een nieuw transcript, *IKZF2-ERBB4*. In twee cellijnen identificeerden we de onderliggende genetische herrangschikking verantwoordelijk voor de fusie als een kleine deletie tussen *IKZF2* en *ERBB4,* maar totnogtoe kon een gelijkaardig defect niet in andere T-ALL

cellijnen of patiënten gedetecteerd worden. Het transcript werd tot expressie gebracht als een transcriptie geïnduceerd chimeer in muizen. Het bestaan van een *IKZF2-ERBB4* chimeer eiwit kon totnogtoe niet bevestigd worden en we kunnen enkel hypothesen vormen rond zijn biologische of pathologische implicaties. Verder onderzoek is vereist voor het ophelderen van deze intrigerende observatie.

We besluiten dat dit werk een pathologische rol voor *ABL1* fusie varianten in T-ALL aantoont. Voor *NUP214-ABL1* vertegenwoordigen episomen een nieuw mechanisme voor het vormen van een fusie. Wanneer we gerichte therapie en het gebruik van TKI (actief in vitro) overwegen voor de behandeling van T-ALL moeten we een aantal T-ALL bijzonderheden in beschouwing nemen. Ten eerste, episomen, wanneer deze voorkomen, vormen een intrinsiek mechanisme van resistentie omwille van hun selectieve amplificatie. Ten tweede vormen *ABL1* fusies (*EML1-ABL1* en *NUP214-ABL1*) secundaire oncogenen wat suggereert dat therapie gericht tegen *ABL1* zal moeten gecombineerd worden met geneesmiddelen gericht tegen de geassocieerde mutaties.

I Introduction (adapted from « Graux C *et al. Leukemia* 2006 »)

1.1 Definition of leukemia

Leukemia is a neoplastic disorder originating from a single hematopoietic cell that following changes in its genetic making acquires the capacity to proliferate independently of growth signals and to escape apoptosis. It leads to uncontrolled proliferation of hematopoietic cells in the bone marrow, blood and other tissues. The resulting suppression of normal hematopoiesis is responsible for anemia, thrombopenia and leucopenia, and the classical symptoms of fatigue, bleedings and infections.

1.2 Classification of leukemias

The degree of maturity and aggressiveness of the leukemic cell population distinguishes between chronic and acute leukemias. In the context of **chronic leukemias**, enhanced proliferation and/or decreased apoptosis lead to proliferation and accumulation of mature hematopoietic cells. The natural course is usually indolent. In the case of **acute leukemias**, there is additionally a block of differentiation that leads to proliferation of cells arrested at an immature stage. The behavior of those cells is more aggressive and the natural course is rapidly fatal in the absence of appropriate treatment. These cells have usually accumulated several mutations prior to cause full-blown leukemia.
Based on the cell type of origin a distinction is made between **myeloid** and **lymphoid** leukemias. Lymphoid leukemias are further divided into B and T-cell leukemias according to the lymphoid lineage concerned.

Leukemias represent about 2% of all cancers.[1] The incidence is approximately 10 cases/100000/year.[2,3] Acute myeloid leukemias (AML) account for 32% of all cases, acute lymphoblastic leukemias (ALL) for 11%, chronic lymphocytic leukemias (CLL) for 26% and chronic myeloid leukemias (CML) for 15%. The remainder is unclassified.
Leukemia occurs in children and adults but the incidence of a particular subtype differs with age. Chronic leukemias are mainly found in adults, particularly in the elderly for CLL. Acute myeloid leukemias occur at all ages but the incidence exponentially increases after 60 years old. ALL is also found in all age groups but the highest peak of incidence is between ages 2 and 5 years.

Acute leukemias are further subclassified according to the **stage of differentiation** of the leukemic blast cells.
The widely used French-American-British (FAB) classification is based on cellular morphology and histochemical techniques.[4]
More recently, the World Health Organization (WHO) classification of leukemias integrated new techniques including immunohistochemistry, immunophenotyping, cytogenetic, fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR), as well as anamnestic information, such as previous exposition to cytotoxic drugs, to propose a new classification taking into account the **pathophysiology** of leukemias. Some proposed subgroups bear a particular prognosis and/or require specific treatment modalities.[5]

Efforts are made to further classify acute myeloid and lymphoid leukemias into molecular subtypes of **prognostic** relevance in order to adapt the intensity of the treatment to the aggressiveness of the disease. It is already the case in AML, where several molecular prognostic markers have been found which are used to stratify the treatment according to the risk of relapse. The aim is to avoid exposing patients to unnecessary toxicity in the case of good prognosis leukemia and to offer more aggressive therapies, such as allogeneic stem cell transplantation, to patients with a high risk of relapse.

Finally, efforts are also made to isolate leukemias with **specific abnormalities** that can be used as target for treatment. The best example of targeted therapy is the inhibition of the BCR-ABL1 fusion protein with imatinib in CML.

In the next paragraph, we will develop the role of *ABL1* in hematological malignancies to introduce the aim of this thesis to elucidate the involvement of the tyrosine kinase encoding gene *ABL1* in T-ALL.

1.3 Focus on *ABL1* in hematological malignancies

The *ABL1* gene is located on chromosome 9 at 9q34. It encodes a non receptor member of the tyrosine kinase family of proteins that regulates intracellular signal transduction pathways involved in cell proliferation, differentiation and survival. The gene contains eleven exons including two alternative exons 1 that give rise to ABL1a and ABL1b isoforms. The different domains encoded by *ABL1* are the NH2-terminal region that contains a myristate group in the ABL1b variant, the SRC homology 3 (SH3) and SH2 domains, the tyrosine kinase domain, the DNA binding and the F-actin binding domains. The SH3 and SH2 domains contribute to maintaining the conformation of the protein in which the activation loop blocks substrate entry in the kinase domain. Three nuclear localization signals (NLS) are located between the kinase and the DNA binding domains and one nuclear export signal (NES) is located in the actin binding domain. The COOH-terminal region also contains binding sites for various proteins acting downstream of ABL1.[6] The role of wild type ABL1 is not completely understood, in particular in hematopoietic cells. The protein shuttles between the cytoplasm and the nucleus by means of its nuclear localization and nuclear export signals.[7] Nuclear ABL1 plays an important role in cell cycle arrest observed in response to genotoxic stress while cytoplasmic ABL1 associates with the F-actin cytoskeleton in the cytosol.[8,9]

In several hematological malignancies, chromosomal translocations involving 9q34 are responsible for the creation of *ABL1* fusion genes. The most frequent is the *BCR-ABL1* fusion gene that was first described in chronic myeloid leukemia (CML) where it constitutes the hallmark of the disease[10] **(Figure 1)**. The occurrence of breakpoints within the *BCR* gene at 22q11 and the *ABL1* gene at 9q34 lead to the formation of the Philadelphia translocation, t(9;22)(q34;q11), that creates the reciprocal fusion genes: *BCR-ABL1* and *ABL1-BCR*. *BCR-ABL1* gene encodes a chimeric tyrosine kinase with oncogenic properties. *BCR-ABL1* is also found in 3% and 25% of childhood and adults B-ALL[11], respectively, rare cases of AML[12], atypical myeloid proliferative disorders[13] and exceptionally in T-ALL[14].

Dinstinct breakpoint regions within *BCR* are responsible for three different *BCR-ABL1* variants.[15] The major breakpoint cluster region (M-bcr) corresponds to genomic sequences spanning exons 13 and 14 (also termed as b2 and b3). It is responsible for the production of a P210 variant (210 kDa) found in 95% of CML and 20-30% of *BCR-ABL1* positive ALL cases. The minor breakpoint cluster region (m-bcr) covers intronic sequences between the two alternative exons 2. The resulting P190 protein (190 kDa) is found in 70-80% of *BCR-ABL1* positive adult ALL cases and in most pediatric *BCR-ABL1* positive ALL. More rarely, a P230 variant is found in a mild form of myeloproliferative disease recognized as neutrophilic-chronic myeloid leukemia. In this case the *BCR* breakpoint is located in intron 19 (µ-bcr) **(Figure 1)**.

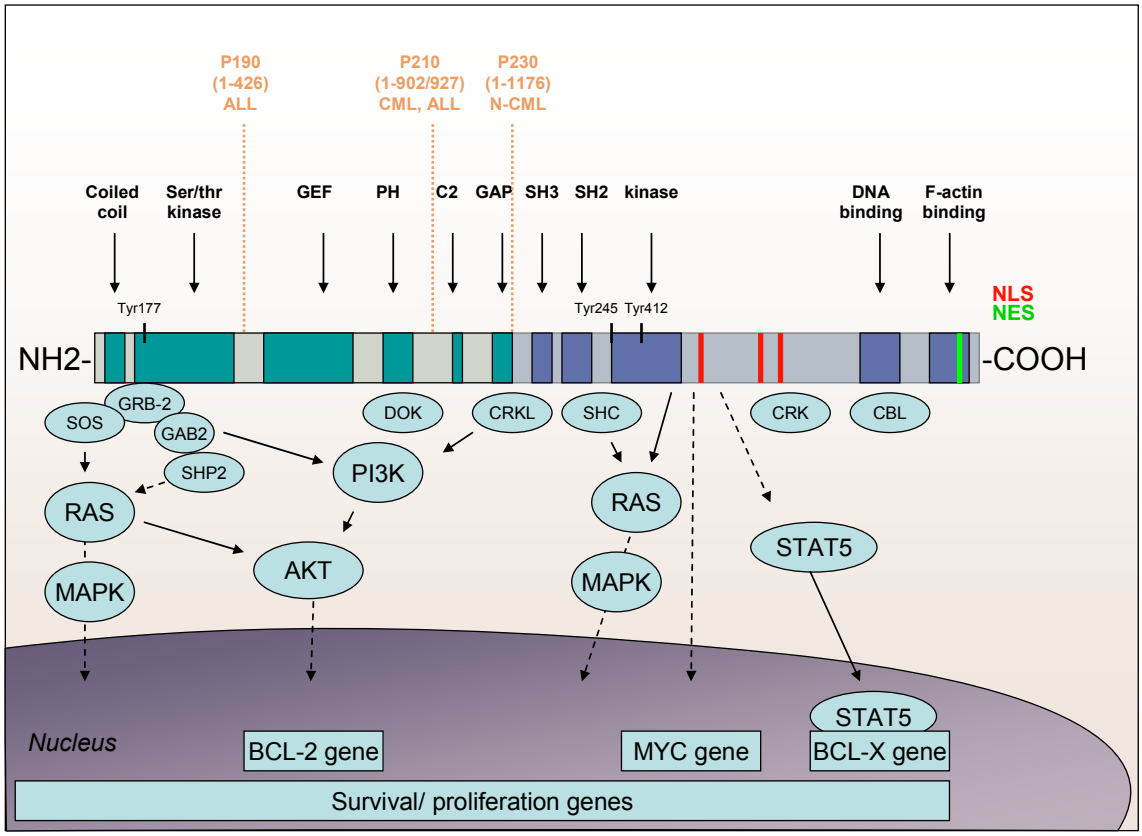


Figure 1 BCR-ABL1 chimeric protein structure and signaling
P190, P210 and P230 indicate the points at which *BCR* most commonly fuses to *ABL1*. GEF: guanine exchange factor; PH: pleckstrin homology domain; C2: calcium dependent lipid binding site; GAP: guanosine triphosphate activating protein domain; SH3/SH2: SRC homology 3/2 domains; NLS: nuclear localization signal; NES: nuclear export signal. BCR-ABL1 interactions result mostly in tyrosine phosphorylation and require binding to adaptor proteins such as GRB-2, CRKL, CBL, CRK, DOK, SHC. The main signaling pathways involved in cellular transformation by BCR-ABL1 are the RAS/MAP kinase, the PI3K/AKT1 and the STAT pathways. Their activation leads to the inhibition of apoptosis through direct or transcriptional activation of anti-apoptotic BCL2 (AKT1) or BCL-X (STAT) and to the transcription of survival and proliferation genes.

The leukemogenic potential of the BCR-ABL1 chimeric protein results from the constitutive activation of the normally fine-regulated kinase domain of ABL1 by the juxtaposed BCR domains.[16] The importance of two particular domains of BCR for transforming properties has been nicely demonstrated in mouse transplant experiments.[17] Firstly, the NH2-terminal coiled-coil domain of BCR acts as oligomerization motif that

promotes tetramerization of BCR-ABL1.[18] It changes the intramolecular structure of ABL1 which probably disrupts the inhibitory role exerted by SH2 and SH3 on the kinase domain and allows the adjacent BCR-ABL1 molecules to phosphorylate each other on tyrosine residues in their kinase-activation loop. Secondly, a tyrosine at position 177 of BCR serves as GRB2 binding site allowing maximal BCR-ABL1 autophosphorylation and linking to the RAS/MAP kinase and the phosphatidylinositol 3-kinase (P13K) signaling pathways.[19] The structure of the BCR-ABL1 protein and signaling are shown in **Figure 1**.

BCR-ABL1 is exclusively located in the cytoplasm, mainly at the cortical actin cytoskeleton, where it interacts with various proteins that transduce the oncogenic signal through different signaling pathways[20,21] **(Figure 1)**. Beside activation of signaling pathways, the BCR-ABL1 protein disturbs normal mechanisms of DNA repair induced by cellular stress.[22] It causes genomic instability that manifests by the accumulation of additional genetic defects characterizing the evolution from the chronic to blast crisis phase of the disease. ABL1 interacts also with integrin functions giving a possible explanation for the decreased adhesion of CML progenitors to the bone marrow stroma.[23]

The mechanisms by which the three BCR-ABL1 protein variants drive particular transforming capacities leading to different hematological diseases remain unclear. The intensity of tyrosine kinase activity of the different fusion proteins may contribute to the differences of transformed phenotypes. It has been shown that P230 exhibited lower intrinsic tyrosine kinase activity than P210 and P190.[24] The hypothesis that their expression might be restricted to different hematopoietic compartments as an explanation for differences in transforming capacities is more controversial.[25,26] Finally, the different domains of BCR retained in the fusion may also play a role in driving the transformation of myeloid rather than lymphoid clones through differential role on the metabolic pathways that regulate normal granulocytic differentiation. This has been suggested for the Dbl/CDC24 guanine-nucleotide exchange factor (GEF) homology domain retained in the P210 and P230 isoforms.[27]

The central role played by the kinase domain of ABL1 in transformation led to the design of **ABL1 kinase inhibitors**.[28] Screening of small compound libraries identified imatinib mesylate (Glivec®) as selectively inhibiting a small group of tyrosine kinases (TK) comprising PDGFRA/B, KIT and ABL1/2, including BCR-ABL1, by competing with ATP for its binding site in the kinase domain. Results of first line treatment of CML with imatinib are quite impressive with only 7% of patients progressing to accelerated- and blast-phase at 5 y.[29] Resistance to imatinib is mainly due to de novo or acquired mutations in the kinase domain.[30] Other mechanisms of resistance such as amplification of the fusion gene have been described. In advanced phase of CML (accelerated phase and blast crisis) and in *BCR-ABL1* positive B-ALL, results are less impressive, with less than 20% of patients showing long lasting remission with a combination of imatinib and chemotherapy. The presence of numerous other genetic events including loss of function of tumor suppressor genes (*PAX5* or *IKAROS*) explain this relative resistance to imatinib, as well as rapid acquisition of kinase domain mutations and frequent expression of the p-glycoprotein. Most of the mutant BCR-ABL1 tyrosine kinase proteins can be inhibited by second generation tyrosine kinase inhibitors (TKI) such as nilotinib (Tasigna®), a structural 10-20 fold more potent variant of imatinib.[31] Dasatinib (Sprycel®), a structurally different dual

ABL/SRK kinase inhibitor, covers also most mutated forms of the fusion by binding ABL1 in both active and inactive form.[32] However, the BCR-ABL1 T315I mutant is resistant to both second generation TK inhibitors. More recently, the non selective ATP competitive aurora kinase inhibitor MK-0457 has shown activity against the T315I variant.[33]

1.4 The normal T-cell development and signaling

1.4.1 Hematopoiesis

Hematopoiesis corresponds to the formation of blood cells from pluripotent hematopoietic stem cells (HSC). This process takes place essentially in the bone marrow where complex signals from the microenvironment drive HSC to start asymmetric division to form another stem cell and an early progenitor that undergoes differentiation and proliferation steps to finally produce the different cells found in the blood. The fate decision of a particular progenitor towards the myeloid or lymphoid lineage commitment depends on a complex array of signals that are poorly understood. Cells engaged in the myeloid lineage differentiate into red blood cells, neutrophils, monocytes/macrophages, eosinophils, basophils, mast cells and megacaryocytes. Cells engaged in the lymphoid lineage differentiate into mature B- or T-lymphocytes. Hematopoiesis implies a finely tuned balance between self-renewal of progenitors and terminal differentiation of committed cells. It requires an adequate coupling between proliferation, differentiation and apoptosis **(Figure 2)**.

1.4.2 T-cell receptor

Mature T-cells are characterized by the membrane expression of TCR/CD3 complex. T-cell receptor (TCR) is a unique transmembrane heterodimer composed of two chains: either αβ or γδ. The α, β, γ and δ proteins all consist of a variable domain and a constant domain. The variable domains are polymorphic and mediate the unique antigen (Ag) binding properties of each individual TCR. Gene loci encoding α- and δ-chains (*TCRA* and *TCRD*) are clustered on chromosome 14q11, those encoding the β-chain (*TCRB*) and the γ-chain (*TCRG*) are located on 7q34 and 7p15, respectively. The genomic locus of the various chains contains several gene clusters corresponding to the Variable (V), the Diversity (D), the Joining (J) and the Constant (C) regions. *TCRA* and *TCRG* genes do not contain the D segment.

During T-lymphocyte development, strictly ordered gene rearrangements take place leading to the formation of a linear coding unit with exclusion of intervening sequences. Through a random choice between the various V, D and J segments and through the deletion or addition of extra nucleotides at the junctions, a broad T-cell repertoire is generated.[34] The recombination activating genes *RAG-1* and *RAG-2* are essential for the rearrangement of variable region genes. The variable region of the αβTCR recognizes antigenic peptides presented on major histocompatibility complex (MHC) molecules by Ag-presenting cells.[35]

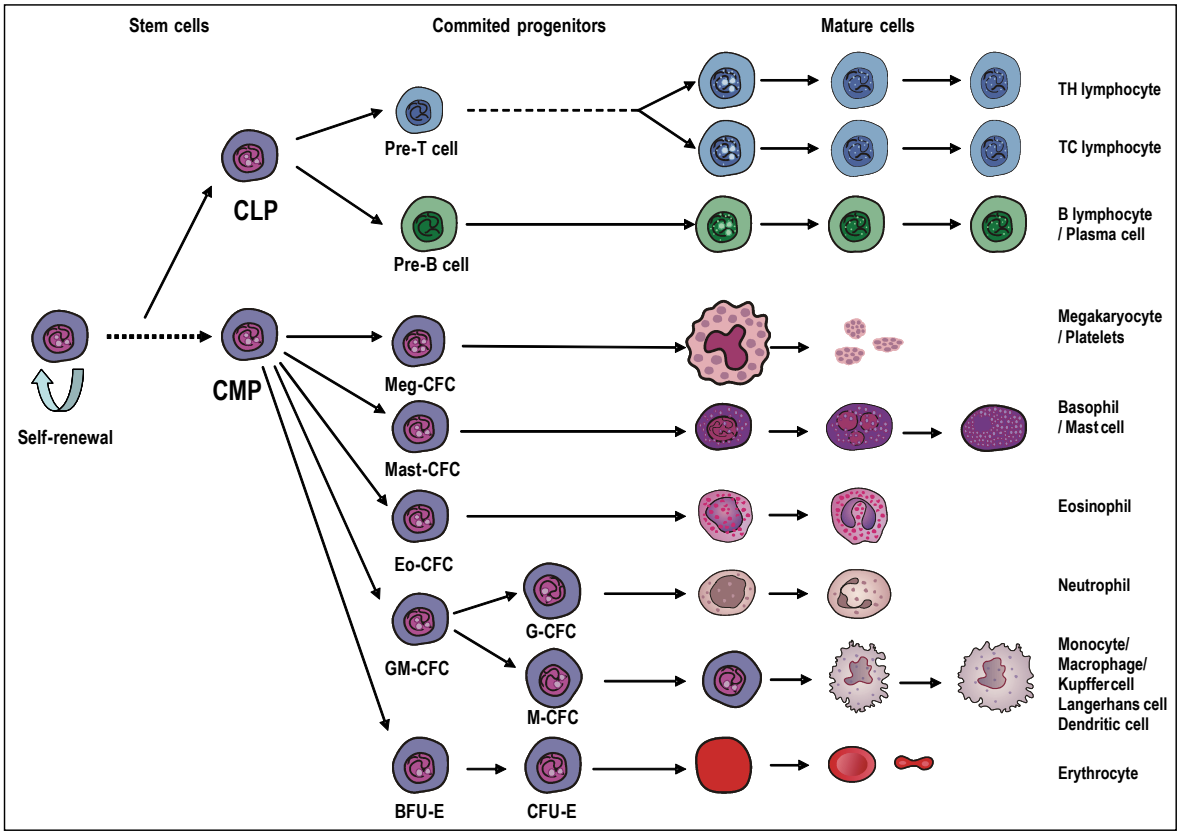


Figure 2 Normal hematopoiesis
CLP: common lymphoid progenitor; CMP: common myeloid progenitor; Meg-CFC: megacaryocyte-colony forming cell; Mast-CFC: mast cell-colony forming cell; Eo-CFC: eosinophil-colony forming cell; GM-CFC: granulocyte macrophage-colony forming cell; G-CFC: granulocyte-colony forming cell; M-CFC: macrophage-colony forming cell; BFU-E: burst forming unit-erythrocyte cell; CFU-E: colony forming unit-erythrocyte cell; TH lymphocyte: T helper lymphocyte; TC lymphocyte: T cytotoxic lymphocyte.

1.4.3 Thymocyte development and selection

Unlike the other bone marrow progenitors, common lymphoid progenitors (CLP) leave adult bone marrow or migrate from the embryonic liver to colonize the thymus, the site of education and maturation of T-lymphocytes **(Figure 3)**. At this stage, CLP are still able to differentiate into T, NK, dendritic and possibly B cells.[36] The commitment to the T-cell lineage depends on signals from the thymic microenvironment, where IL7 is indispensable for survival and proliferation of human T-cell precursors through the induction of the anti-apoptotic MCL-1 protein.[37,38] It first starts with the rearrangement of the genes coding for the TCRδ and TCRγ followed by the rearrangement of those coding for the TCRβ. TCRβ expression allows the formation of the pre-T-cell receptor (pre-TCR). The pre-TCR complex is composed of the TCRβ chain, the TCRα chain 'substitute' for immature T-cells (pTα) and the CD3 chains.[39,40] Subsequently, *TCRA* genes rearrangement leads to the expression of the mature αβTCR.[41] The pre-TCR and the mature αβTCR are sequentially expressed and both act as checkpoints: signals from the pre-TCR allow survival, cellular expansion and further differentiation of T-cells with productive rearrangement of the TCRβ (β selection).[42,43] Thymocytes with αβTCR that recognize self-MHC molecules are rescued from programmed cell death by survival signals from the αβTCR and progress further

towards maturation (αβ selection or positive selection). Others die by neglect. T-cells with strong αβTCR affinity for endogenous MHC complex are eliminated to avoid self-reactivity (clonal deletion or negative selection). Thus, signaling from the mature αβTCR, triggered by its interaction with MHC molecules on thymic epithelial cells, allows the selection of αβTCR non-self-reactive thymocytes that are MHC-restricted and Ag-specific.

Committed T-cells down-regulate the surface antigen CD34 and sequentially up-regulate CD5, CD1a, CD4 and CD8. During positive selection, double positive thymocytes, characterized by CD4+ and CD8+ expression, undergo transition to single positive mature stages: either CD4+ and MHC class II restricted or CD8+ and MHC class I restricted. The γδ lineage diverges from the αβ at the pre-T1 stage and does not use the pre-TCR complex to mature.

Mature immunocompetent lymphocytes migrate to peripheral lymphoid organs where encounters with Ag lead to cell mediated immune reactions driven by either cytotoxic CD8+ T-cells or CD4+ helper T-cells.

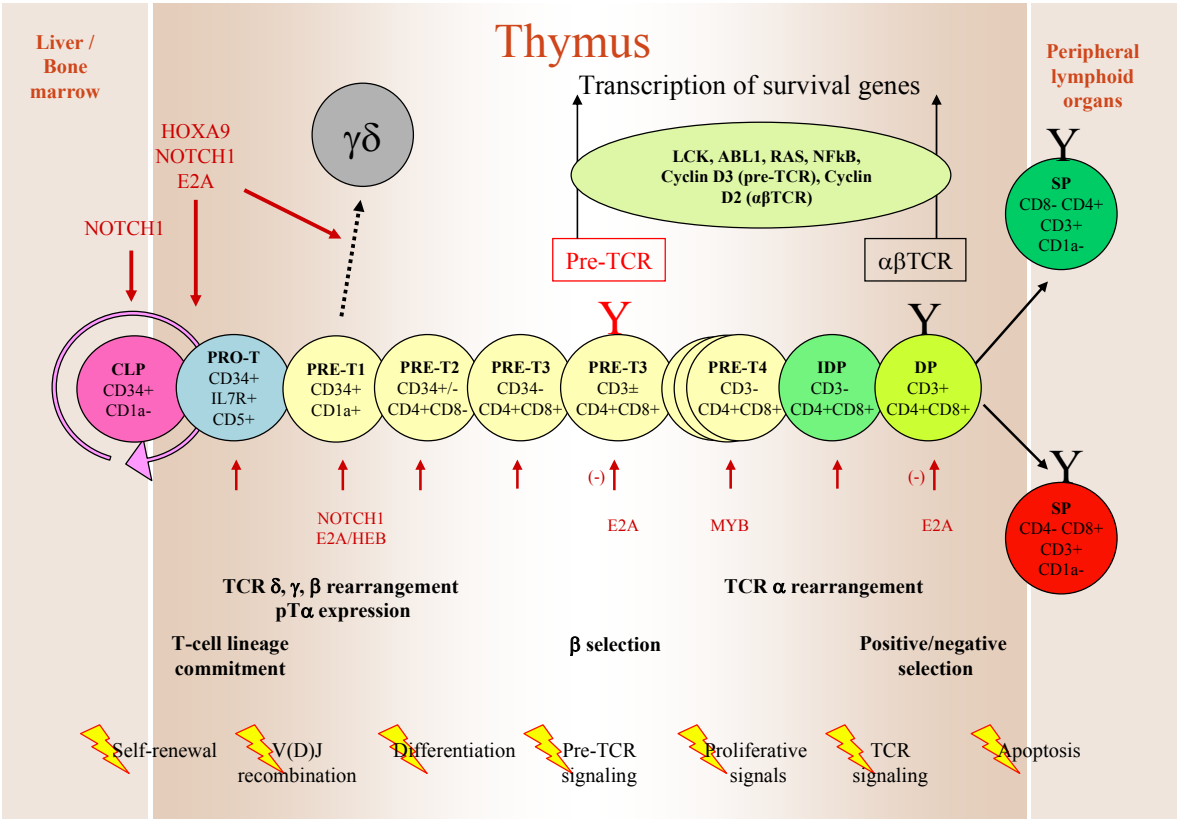


Figure 3 Thymocyte development

CLP: common lymphoid progenitor; IDP: immature double positive thymocyte; DP: CD8+ CD4+ double positive thymocyte; SP: CD4+ or CD8+ single positive thymocyte; pre-Tα: TCRα chain "substitute" for immature T cells; pre-TCR: pre-T-cell receptor. There are many differences between human and mouse thymocyte maturation but they both follow the same main developmental stages.[44] The understanding of the role of the thymopoiesis regulators, E2A/HEB, NOTCH1, Homeobox proteins and MYB in the control of key steps throughout thymocyte development derives mainly from experiments in mice. Also, their site of action as indicated in this figure is an extrapolation to the context of human thymopoiesis.
The lower part of the figure summarizes the critical steps of thymocyte development that can be targeted by oncogenic events.

Thymocyte development requires a fine regulation of proliferation, differentiation, survival and death signals. Key steps are controlled by several transcriptional regulators.[45,46] Among these, the E-proteins E2A and HEB, proteins from the family of NOTCH receptors, Homeobox proteins and MYB are major players in normal T-cell development as well as in T-cell leukemogenesis.[47,48] Their role during the differentiation of lymphocytes progenitors in the thymus will be discussed below together with their role in T-cell leukemogenesis.

1.4.4 Pre-TCR and TCR signaling

Biological consequences of pre-TCR, αβTCR and γδTCR signaling are not the same even though all three receptors use identical signaling pathways and activate similar transcription factors.[49] One difference between pre-TCR and TCR receptors might lie within the cytoplasmic tail of the pTα chain. It has been suggested that differences in the strength of the signal produced by the pre-TCR and the γδTCR might be responsible for the activation of distinct transcription factors and "molecular signatures" that define the commitment of cells towards the αβ or γδ lineage: commitment to the αβ lineage depending on the weak signal from the pre-TCR and commitment to the γδ lineage depending on the strong signal from the γδTCR.[50,51]

Pre-TCR signaling induces rapid cell-cycle entry, several cycles of division and maturation of thymocytes. Pre-TCR expressing thymocytes display several characteristics of cell-cycle entry such as cyclin D3 expression, increased CDK2 activity and down-regulation of p27.[52,53,54] The Ras/Raf pathway has been proposed as the link between the pre-TCR and cell cycle entry by activating the E2A inhibitor Id3, releasing the block exerted by E2A on proliferation and maturation at the β selection check-point **(Figure 3)**.[55,56] The transcription factor Myb could also play an important role in pre-T-cell induced proliferation.[47,57,58] It is now accepted that immature thymocytes are subject to apoptosis until they receive survival signals from functional pre-TCR and TCR receptor. It has been suggested that one of those apoptotic signals might be triggered by DNA breaks induced by recombination events in pre-T cells through action of p53.[59] Very little is known of the signaling pathways that are used for survival of thymocytes having acquired a functional pre-TCR, but data suggest that activation of the NFkB pathway and upregulation of the anti-apoptotic protein BCL2A1 could play a major role.[60,61,62]

Signals from **αβTCR** in response to antigen recognition are important for lymphocyte survival. They are illustrated and commented on **Figure 4**. The different signaling cascades activate NFAT, FOS and NFkB transcription factors that will induce the transcription of various genes including *IL2*, encoding a pivotal growth factor supporting clonal expansion of Ag-reactive T-cells. Recent reports suggest the requirement for the ABL1 kinase in the regulation of T-cell receptor mediated signal transduction leading to IL-2 production and cell proliferation, in particular by indirect activation of ZAP70.[63,64]

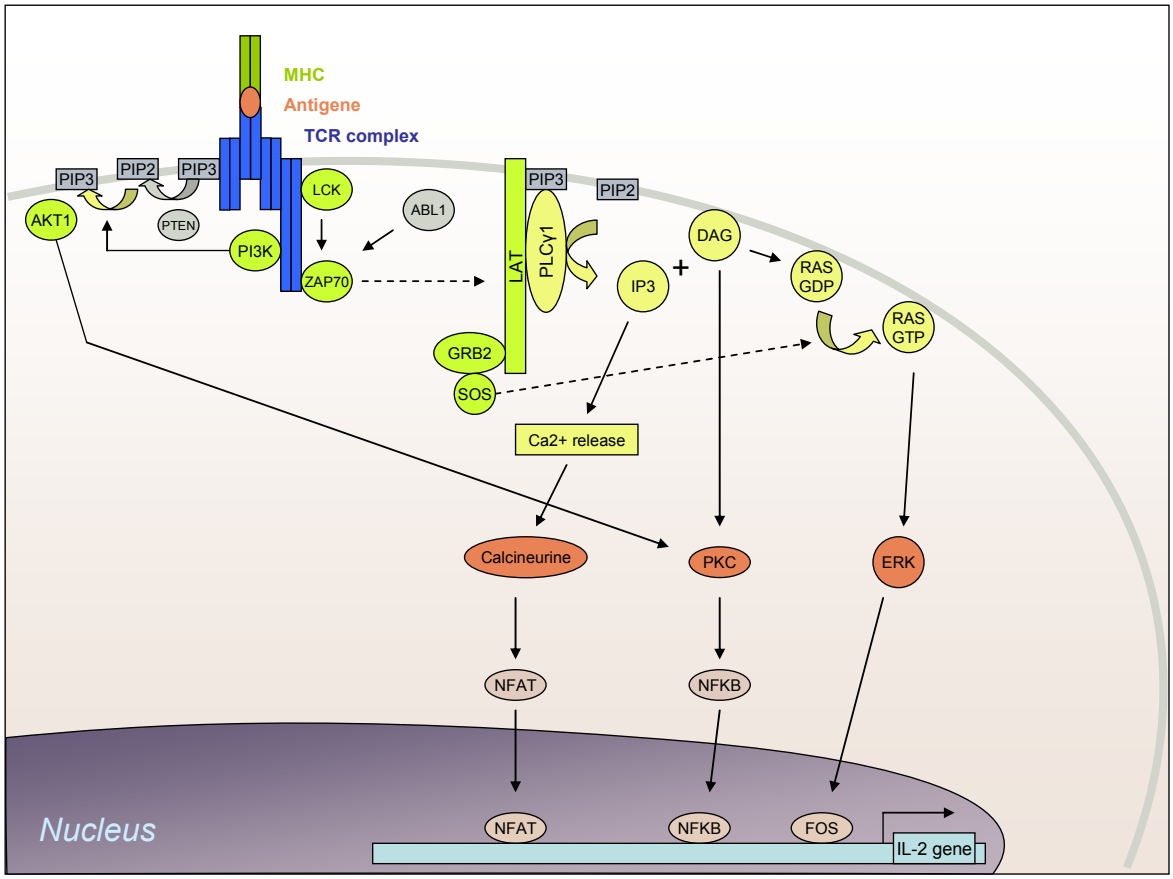


Figure 4 TCR signaling
TCR forms a complex at the cell surface with CD3 and the ζ chains, which will activate intracellular signaling upon Ag binding. SRC protein kinases LCK and FYN brought into proximity of TCR/CD3 through interaction with the co-stimulatory molecules CD4 or CD8 phosphorylate the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) present on the cytoplasmic portion of the ζ chains. This leads to the recruitment and activation of other kinases such as PI3K[65] and ZAP-70.[66] Activated PI3K phosphorylates in turn PIP2 into PIP3.[67] The exact opposite activity is exerted by the phosphatase PTEN.[68] PIP3 recruits pleckstrin homology (PH) domain containing proteins, such as phopholipase Cγ1 (PLCγ1) and the protein tyrosine kinase ITK, to the membrane. Activated ZAP70 phosphorylates transmembrane adaptor proteins including the linker of activation of T cells (LAT). This membrane-anchored adaptor protein brings PLCγ1 and ITK together allowing the latter to phosphorylate and activate PLCγ1, which subsequently hydrolyses PIP2 into the second messengers IP3 and diacylglycerol (DAG). IP3 mobilizes Ca^{2+} which initiates calcineurin phosphatase activity that in turn dephosphorylates NFAT allowing them to translocates to the nucleus. LAT recruits another adaptor protein GRB2 that, like DAG, activates the RAS/ERK pathway resulting in increased MAP kinase signaling and eventually FOS transcription. FOS participates to the AP1 transcription factor complex.[69] DAG also activates the enzyme protein kinase C (PKC) that participates to the generation of active transcription factors like NFkB. Accumulation of PIP3 at the plasma membrane recruits AKT1 and induces its phosphorylation by PDK1 and the rector-mTOR complex leading also to the activation of NFkB.[62,70,71]

1.5 T-cell acute lymphoblastic leukemia

1.5.1 Definition

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplastic disorder that originates in a single lymphoid progenitor committed to the T-cell lineage. Acquisition of genetic anomalies during its thymic development allows clonal expansion of immature

leukemic cells (lymphoblasts) in the bone marrow, blood, nodal and extra-nodal sites. T-ALL represents 15% of childhood and 25% of adult ALL. High white blood cell count, bulky mediastinal mass and central nervous system (CNS) involvement are unfavorable clinical findings more commonly encountered in T-ALL compared to B-ALL. When the patient presents with an important neoplastic mass, usually mediastinal, and 25% or less of lymphoblasts in the bone marrow, the designation lymphoma is preferred. But the distinction between T-cell acute lymphoblastic lymphoma (T-LL) and T-ALL is arbitrary since they correspond to the same biologic unit. Current treatment is still based on the administration of intensive chemotherapy. In children, the disease is curable in 70-80% of cases.[72,73] In adults, cure rate remains low (< 40%).[73] Toxicity of the treatment remains a major concern, particularly in the elderly in whom accumulation of comorbidities does not allow the optimal use of the most efficient chemotherapy drugs. It is a heterogeneous disease comprising several clinico-biological entities. In T-ALL, there are no good molecular prognostic markers that reliably correlate with outcome. Response to induction therapy as evaluated by the measurement of the minimal residual disease (MRD) integrates disease as well as patient characteristics and serves to adapt treatment strategy according to the risk of relapse.

1.5.2 Classification

1.5.2.1 Classification based on immunophenotype

The antigen expression profile of leukemic lymphoblasts parallels those of the normal stages of T-cell differentiation and maturation. The different T-ALL subtypes correspond to differentiation arrest at specific stages of T-cell development and immunophenotype can be used to classify the disease according to the degree of maturation.
Several immunophenotypic classifications of T-ALL have been proposed. Among these, the classification proposed by the European Group for the Immunological Characterization of Leukemias (EGIL) is commonly used in Europe in clinical practice.[74] According to EGIL, the presence of cytoplasmic or membrane expression of CD3 defines T-ALL. Four subgroups are proposed: the immature subgroup or pro-T-ALL (TI) is defined by the expression of only CD7; pre-T-ALL (TII) also expresses CD2 and/or CD5 and/or CD8; cortical T-ALL (TIII) shows CD1a positivity; finally, mature T-ALL (TIV) is characterized by the presence of surface CD3 and CD1a negativity. Depending on the mutually exclusive membrane expression of αβ- or γδTCR, two subgroups, group a (αβ) and group b (γδ), are distinguished.
A classification of T-ALL taking also into account the status of *TCR* genes rearrangement, *pre-Tα* and *RAG1* expression has been proposed but is not used in clinical practice.[75]

1.5.2.2 Classification based on gene expression signatures

Recent investigations using quantitative RT-PCR and gene expression profiling have shown that different T-cell oncogenes (mainly transcription factors) can also be aberrantly expressed in T-ALL, even in the absence of specific chromosomal abnormalities.[76] Large-scale expression profiling of T-ALL has identified several gene expression signatures indicative of leukemic arrest at specific stages of normal thymocyte development.[77,78,79]

For example, T-ALL expressing the oncogene *LYL1* (*LYL1*+ T-ALL) have a signature corresponding to immature thymocytes (pro-T), *TLX1*+ T-ALL to early cortical and *TAL1*+ T-ALL to late cortical thymocytes. T-ALL characterized by *MLL*, *SET-NUP214* and *CALM-AF10* rearrangements cluster together with T-ALL that rearrange the *HOXA* locus following translocations with the *TCRB*. They all present the same signature of upregulation of some *HOXA* genes and form therefore the *HOXA*+ subgroup of immature T-ALL.[80] More recently, rare T-ALL overexpressing the *MYB* oncogene following a translocation with *TCRB* have been shown to represent a new subgroup of which the exact differentiation arrest remains to be determined. It suggests that those different oncogenic transcription factors are probably oncogenic by interfering with the normal transcriptional programs that regulate thymocyte development.

Gene expression profiling of these specific subgroups may identify genes involved in responsiveness to chemotherapy. In addition, these gene expression signatures can clarify specific characteristics of a subgroup related to cell growth, apoptosis and thymocyte development. These insights can assist in the selection of therapeutic agents that are most likely to be efficient in particular subtypes.

Table 2 summarizes the different developmental stages of normal thymocytes matched with corresponding T-ALL counterparts.

1.5.3 (Cyto)genetic changes

In approximately 50% of T-ALL, structural chromosomal aberrations are identified by conventional karyotyping.[81] Numerical changes are rare, except for tetraploidy which is seen in approximately 5% of cases, and are without prognostic significance. A high percentage of cryptic abnormalities is revealed by FISH, mainly cryptic deletions at 9p21 and at 1p32. In addition, translocations with breakpoints in near terminal regions of chromosomes, for example the t(5;14)(q35;q32), rearrangements of *TCRB* at 7q34 and 9q34 breakpoints, are often disclosed only by using FISH with the appropriate probes as illustrated in **Figure 5**. The recurrent cytogenetic changes observed in T-ALL are summarized in **Table 1**.
Structural rearrangements in T-ALL result in oncogene activation either by transcriptional activation, usually the consequence of translocations involving TCR loci, or by gene fusion which leads to the expression of chimeric proteins. Deletions are the hallmark for loss of tumor suppressor genes. Genomic amplification of oncogenes has been also recently identified. Finally, mutational analysis of genes encoding key regulators of T-cell development and signaling proteins has shown activating or inhibiting mutations in a high proportion of T-ALL.

1.5.3.1 Translocations involving *TCR* loci

Breakpoints involving *TCR* loci are recurrent on 14q11 (*TCRA/D*) and 7q34 (*TCRB*). During T-cell development with V(D)J recombination taking place, several other genes transcribed at an early stage of thymocyte development are in ‘open’ chromatin configuration and vulnerable to the action of recombinase enzymes. Therefore, exit from the cell cycle is normally required for pre-T cells undergoing V(D)J recombination in order

to avoid the generation of illegitimate recombinations during mitosis.[59] Loss of cell cycle control at this stage may thus allow the juxtaposition of transcription factor genes to strong promoter and enhancer elements of the TCR genes leading to their aberrant expression in developing thymocytes and give rise to T-ALL with differentiation block at various stages of maturation. Altogether, translocations involving the *TCR* loci are found in about 35% of T-ALL with unidentified partner genes in 5–10% of cases.[82]

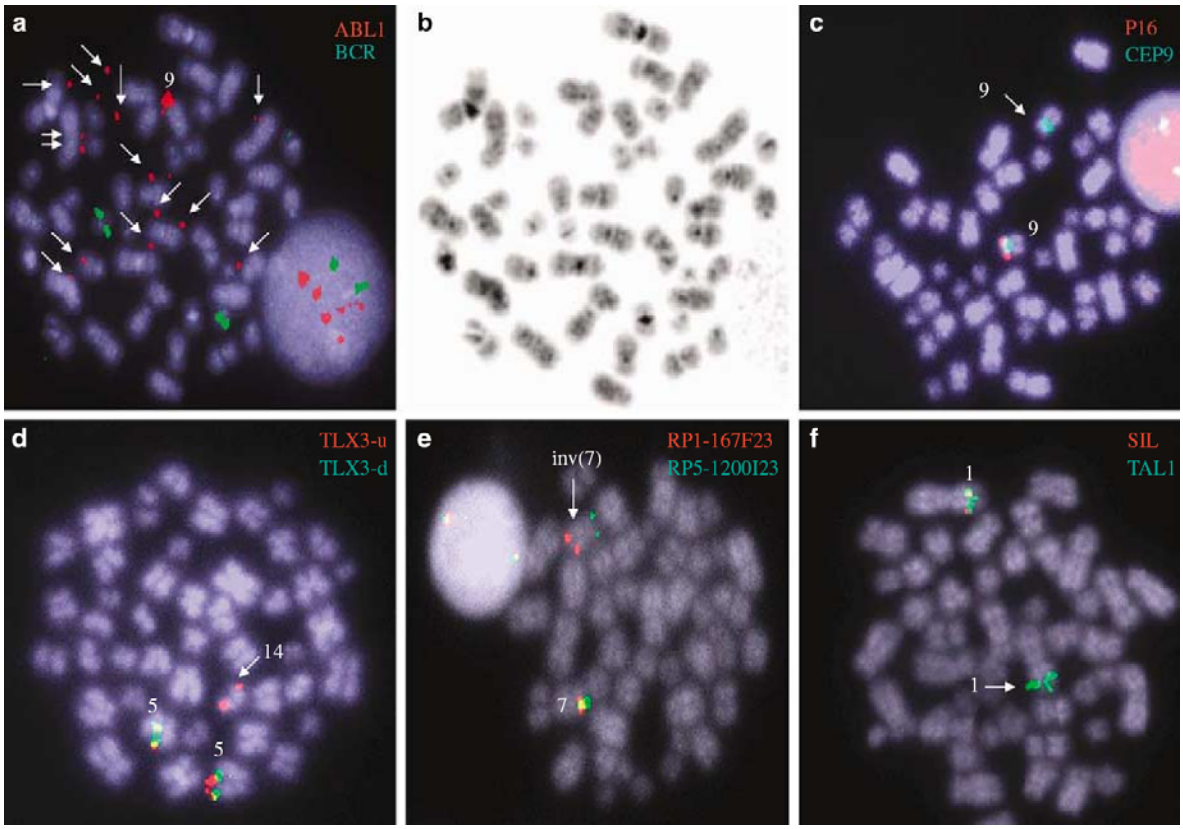


Figure 5 FISH detection of cryptic cytogenetic aberrations in T-ALL
Arrows indicate abnormal hybridization patterns. (a/b) *ABL1* containing episomes visible as small dots in between chromosomes and not visible in G-banded metaphase (b). Probe used: LSI-*BCR/ABL1* ES (Vysis). (c) Hemizygous deletion of *p16* (9p21) detected with LSI-*p16*/CEP9 (Vysis). (d) Detection of t(5;14) with *TLX3* DNA probe/split signal (DAKOcytomation). Sequences labeled in red are translocated to chromosome 14. (e) Detection of inv(7)(p15q34) using BAC probes: RPI-167F23 (in red) and RP5-1200I23 (in green) mapping centromeric and telomeric of the *HOXA* locus (7p15), respectively. (f) Deletion of *SIL* resulting in SIL-TAL1 fusion gene, demonstrated with the *SIL-TAL1* DNA probe (DAKOcytomation). The *SIL* probe (in red) is absent from del(1).

1.5.3.2 Generation of fusion genes

A second type of rearrangement, mostly translocations, results in the formation of 'fusion genes'. Parts of both genes located at the chromosomal breakpoints are fused 'in frame' and encode a new chimeric protein with oncogenic properties. The *SIL-TAL1* fusion gene results from a cryptic interstitial deletion at 1p32 and is found in 9–30% of childhood T-ALL, with decreasing frequency in adults.[83,84] The t(10;11)(p13;q14) encoding *CALM-AF10* is found in about 10% of cases, but is often cryptic.[85] Translocations implicating *MLL* with various partners represent about 8% of cases[86], and translocations of *ABL1* are rare except for *NUP214-ABL1* fusion we recently identified in up to 6% of T-ALL as a

result of episomal formation with amplification.[87] Another recently described fusion involves *SET* and *NUP214* on chromosome 9.[88] Recurrent translocations involving *NUP98*, another protein of the nucleopore complex, are reported very rarely.[89,90]

1.5.3.3 Deletions/amplifications/duplications

Genomic copy number abnormalities are most frequently cryptic but can be now detectable using high-density DNA microarrays.[91] Cryptic **deletions** are frequent and may be concomitant with other changes. The most frequent is loss of the *INK4/ARF* locus at 9p21 that leads to loss of G1 control of the cell cycle.[92] Another recurrent aberration is del(6)(q) of variable size. The common deleted region focuses around 6q16 but the target gene(s) have not yet been formally identified.[93] Recently, a new recurrent and cryptic deletion on chromosome 11, del(11)(p12p13) has been identified in childhood T-ALL. In this case, loss of the regulatory region upstream of *LMO2* causes activation of the proximal *LMO2* promoter.[94] Numerous other sporadic copy number anomalies are now detected by genome wide screening. These findings, even at low frequency, help to identify genes with sometimes a broader role in T-ALL, giving insight in T-cell leukemogenesis.[91] Among others, deletions in which microRNAs might be deregulated are recurrent, highlighting their potential role in T-cell pathogenesis.[91] **Amplification** of the *NUP214-ABL1* fusion gene has been recently described on amplified episomes.[87] **Duplication** of a gene or small chromosome region has been reported for *MYB* and for the 9q34 locus, respectively.[95,96] In the case of 9q34 duplication, the amplified region contains several oncogenes such as *ABL1*, *NUP214*, *VAV2*, *TRAF2* and *NOTCH1*, but their subsequent enhanced expression is difficult to assess since this abnormality seems to be present in minor leukemic clone only.[97]

1.5.3.4 Mutations

Mutations activating the NOTCH1 pathway represent a finding shared by nearly 70% of T-ALL. Recently, mutations affecting *JAK1* have been found to provide proliferation advantage to the lymphoblasts in 18% of adult T-ALL cases. It is likely that numerous other mutations affecting genes involved in cell signaling will be identified in the future with the new high throughput sequencing technologies.

1.5.4. Main deregulated genes and signaling pathways

1.5.4.1 Aberrant expression of oncogenes and deregulation of thymopoiesis regulators

1.5.4.1.1 Deregulation of NOTCH signaling

The NOTCH family proteins are transmembrane receptors involved in regulation of cell fate in a broad spectrum of tissues and species.[98] NOTCH signaling regulates hematopoietic stem cell maintenance (self-renewal)[99] and plays a critical role in T-cell development.[100] NOTCH1 is an important player in T-cell commitment decisions of the CLP[101] and in the

assembly and signaling of pre-TCR in immature thymocytes.[102,103] NOTCH1 could play a role in differentiation by controlling the turnover of E2A proteins.[104] The PI3K/AKT pathway has been shown to be an important effector of NOTCH1 induced differentiation, proliferation and survival of T-cell progenitors after the β selection.[105]

Mature NOTCH1 is a heterodimeric transmembrane receptor consisting of an extracellular (NEC) and a transmembrane (NTM) subunit that are not covalently associated by the heterodimerization domain (HD). Binding of the ligand from the Delta- Serrate-Lag2 (DSL) family to the NEC results in the removal of the NH2-terminus fragment of NOTCH1 triggering a cascade of proteolytic cleavages of the NTM and NOTCH1 activation. The first cleavage is catalyzed by an ADAM protease and the final cleavage by the γ-secretase complex of proteases that allow the release of intracellular NOTCH1 (ICN). ICN translocates to the nucleus where it becomes part of a large transcription activator complex including the CSL transcription factor. ICN has a C-terminal PEST domain, a motif implicated in protein turnover and responsible for the short half-life of the protein.[106] Phosphorylation of the COOH-terminus of PEST by CDK8 results in its FBXW7-CSF-mediated proteasomal degradation.[107]

In T-ALL, *NOTCH1* was identified as a fusion partner of *TCRB* in the rare **translocation**, t(7;9)(q34;q34.3) leading to the formation of N-terminally truncated constitutively active NOTCH1 peptides.[108] Mice transplanted with bone marrow expressing activated NOTCH1 develop T-cell neoplasms.[109] Recently, *NOTCH1* activating **mutations** have been found in the HD domain, the juxtamembrane extracellular region (JME) and the PEST domain in more than 50% of T-ALL of all molecular subtypes.[110,111] Mutations in HD and JME result in ligand independent ICN production; mutations in PEST extend the half-life of ICN transcription activator complex.
The presence of *NOTCH1* mutations in a high percentage of T-ALL of all molecular subtypes suggests that they are relatively early events in T-cell leukemogenesis, occurring in immature progenitors.
Inhibition of NOTCH1 signaling with γ-secretase inhibitors has been proposed as targeted therapy in T-ALL.[112] Activating *NOTCH1* mutations predict favorable early treatment response and long-term outcome in childhood T-ALL.[113]

In addition, heterozygous missense mutations of the ***FBXW7*** gene affecting four essential arginine residues involved in the interaction with PEST have been found in 15% of T-ALL, preventing FBXW7-mediated ubiquitination of oncogenic nuclear NOTCH1.[114,115] Combined *NOTCH1* HD or JME and *NOTCH1* PEST or *FBXW7* mutations were found in 15-25% of T-ALL and were shown to have a synergistic effect on NOTCH1 activation.[116] Of interest, in addition to its role in NOTCH1 turnover, FBXW7 has been shown to target mTOR and c-Myc for degradation.[117,118]

The signaling pathways and target genes responsible for NOTCH1 transforming activity in T-ALL is still unclear and results probably from its role in maintenance of stem cells and from the deregulation of its normal functions during T-cell development.[119]
Recently, analyses of gene expression profiles of T-ALL cell-lines before and after treatment with γ-secretase inhibitors has highlighted the crucial role of **MYC** as a direct NOTCH1 transcriptional target in controlling NOTCH1 induced cell growth.[116] Direct

involvement of the *MYC* gene has been previously reported in rare T-ALL cases harboring a t(8;14)(q24;q11) fusing *MYC* to *TCRA/D* locus.[120,121]
A pathological role of the **PI3K/AKT pathway** in T-ALL, downstream of NOTCH1, has been confirmed by the recent finding of loss of expression of ***PTEN*** in 17% of T-ALL patients following frameshift and non-sense mutations. [116,122,123,124] On one hand, PTEN expression has been shown to be negatively controlled in T-ALL by activated NOTCH1 via HES1[124]; on the other hand, PTEN negatively regulates the PI3K/AKT/mTOR pathway by dephosphorylating PIP3 into PIP2 **(Figure 4)**. Thus, loss of PTEN expression bypasses the requirement for aberrant NOTCH1 signaling through the direct activation of the PI3K/AKT/mTOR pathway. This provides an explanation for the resistance of *PTEN* mutant T-ALL cell lines to γ-secretase inhibitors acting on NOTCH1, and their persistent sensitivity to AKT inhibitors acting downstream of PTEN.[125]
Finally, **NFkB** has been found to be positively regulated by NOTCH1 in the context of T-ALL, representing therefore a potential therapeutic target.[126]

1.5.4.1.2 Deregulation of E-proteins

The E proteins, including **E2A** and **HEB,** are class A bHLH proteins that are ubiquitously expressed as homo- or heterodimers. Members of the basic helix–loop–helix (bHLH) family of transcription factors share the bHLH motif (60 aa) allowing homo- or hetero-dimerization through the HLH domain and DNA binding through the basic part of dimerized proteins.[127] E-proteins bind DNA at specific E-Box sites in the enhancers of many T-cell specific regulatory genes such as *CD4* and *pTa*.[128,129] E2A and HEB are the bHLH transcription factors expressed in the thymus. The E2A gene encodes two distinct E2A bHLH proteins, E12 and E47, by alternative splicing. A portion of E47 protein binds DNA as a complex with HEB. The role of E proteins in thymocyte development is very complex.[44,45] In mice, the absence of the *E2A* gene products leads to accumulation of double-negative thymocytes without *TCRB* gene rearrangement and to the development of aggressive T-cell lymphomas.[130,131] E2A proteins regulate V(D)J recombination, the expression of *RAG* and *pTa* genes required for the formation of pre-TCR.[132] E47/HEB heterodimers show proapoptotic activities.[133] E2A proteins act as negative regulators of cell proliferation in thymic precursors.[134] They behave as gatekeepers at pre-TCR and αβTCR checkpoints, controlling further proliferation and differentiation until inhibitory signals from efficiently rearranged pre-/αβTCR release this block.[45] Specific inhibitors of E proteins, Id proteins, are playing opposing roles in regulating cell proliferation and oncogenic transformation.[135]
As will be discussed below, E proteins are targeted by several oncoproteins but have never been directly involved in genetic rearrangements.

Class B bHLH proteins. Class B bHLH proteins form heterodimers with class A bHLH and are expressed in a tissue-specific manner. *LYL1*, *TAL1* and *TAL2* are not expressed in the normal thymus[136] but can be ectopically expressed in T-ALL.[137] It has been proposed that by binding to E2A and HEB, they interfere with the normal activities of these proteins.[138,139]

TAL1 *(SCL)* maps to chromosome band 1p32. Rearrangements of this locus are frequent in childhood T-ALL, resulting in *TAL1* activation either as a consequence of the translocation,

t(1;14)(p32;q11)[140] or more often due to a submicroscopic interstitial deletion generating the *SIL-TAL1* fusion gene.[83,84] Other rare translocations targeting *TAL1* have also been described.[141] In addition, high expression levels of *TAL1* in the absence of detectable *TAL1* rearrangement are observed in about 40% of T-ALL.[76,142] These show maturation arrest at the late cortical stage with heterogeneous coexpression of the transcription factor encoding genes *LMO1/LMO2* and overexpression of the antiapoptotic gene *BCL2A1*.[77] TAL1 is involved in embryonic and adult hematopoiesis[143,144] and in angiogenesis[145], but is not normally expressed in developing T-cells. However, it has been shown that TAL1 and its partners LMO1/2 are coexpressed in the most primitive thymocytes.[146] The leukemogenic activity of TAL1 is not completely understood. Ectopically expressed TAL1 may bind to its normal interacting proteins, the transcription factors E47 and HEB, and therefore compete with the formation of E47/HEB heterodimers, abolishing their normal transcriptional activity.[146,147,148] Such dominant negative action of ectopically expressed TAL1 on genes normally targeted by E2A is supported by data showing that, in mice, enforced expression of TAL1 lacking the transactivation domain still leads to aggressive T-cell malignancies[149,150]. In addition, enforced TAL1 thymic expression is associated with repression of several genes, some of which are controlled by E47/HEB such as *CD5*, *RAG1/2* and *TCRB*.[147] It has also been suggested that this transcriptional repression might be mediated by recruitment of histone deacetylase to the target genes. Also, in agreement, is the observation that HDAC inhibitors induce apoptosis of TAL1 positive tumors, supporting the hypothesis that TAL1 causes gene silencing.[147,149] More recently, by combining *TAL1* knockdown by RNA interference (RNAi) and gene-expression profiling, it has been demonstrated that TAL1 binding can be associated not only with repression but also with activation of genes whose promoters are occupied by TAL1, E2A, and HEB.[151]

LYL1 is a partner gene of *TCRB* in the rare translocation, t(7;19)(q34;p13).[152] *LYL1* is also constitutively overexpressed in a subset of T-ALL, in the absence of chromosome rearrangements.[77] *LYL1* expression identifies T-ALL with an immature phenotype, expressing CD34 and sometimes myeloid markers.[77] Transgenic mice overexpressing *LYL1* develop long latency T- or B-cell lymphoma.[153] Poor outcome of T-ALL patients overexpressing *LYL1* is possibly due to parallel overexpression of antiapoptotic molecules, which is characteristic of early precursors.

TAL2 and BHLH1 are upregulated in T-ALL as a consequence of recurrent but rare translocations: t(7;9)(q34;q32) which juxtaposes *TAL2* and *TCRB*[154], and t(14;21)(q11;q22) which juxtaposes *BHLH1* and *TCRA*.[155] Their homologies with TAL1 bHLH domain suggest a similar mechanism for leukemogenesis.

LIM domain only genes *LMO1* and *LMO2*. The genes encoding the LIM domain only proteins *LMO1 (RTBN1)* and *LMO2 (RTBN2)* mapping to chromosome bands 11p15 and 11p13, respectively, are frequently rearranged in T-ALL.[156] Most common are the translocations, t(11;14)(p15;q11) and t(11;14)(p13;q11) juxtaposing *LMO1/LMO2* to the *TCRA/D* locus.[157,158] Translocations involving *TCRB* and *LMO1* or *LMO2* loci have also been reported.[82] Abnormal expression of *LMO1/2* has been found in 45% of T-ALL, even in the absence of typical chromosomal changes, but often in association with deregulation of *LYL1* (*LMO2*) or *TAL1* (*LMO1* and *2*).[76,159] Activation of *LMO1/2* in translocated cases occurs by promoter swap and/or by removing tissue specific negative regulatory elements

located 5' of promoter.[160] Recently, *LMO2* activation has been reported as a result of a cryptic recurrent deletion of negative regulatory elements upstream of the first promoter of *LMO2* in 4% of childhood T-ALLs.[94] Aberrant activation of *LMO2* via retroviral integration has been reported in two T-cell lymphoproliferative disorders in children participating in a gene therapy trial of X-linked Severe Combined Immunodeficiency.[161]
LMO2 is essential for erythroid development in mice.[162] In erythroid precursors, LMO2 physically associates with other nuclear factors (GATA-1, TAL1, LDB1 and E2A) to form pentameric complexes involved in the transcription of several genes.[163] Such complexes have been described in one T-ALL cell line.[164] Studies in mice show that *LMO2* or *TAL1* overexpression induces leukemia with a long latency.[165] Of interest, co-expression of *LMO1* in the thymus of transgenic mice overexpressing *TAL1* shortens the latency period before leukemia onset.[141,166] Along these lines, one case with T-cell lymphoproliferative disorder and retroviral activation of *LMO2* mentioned above also displayed an acquired mutation in *TAL1*.[161] The fact that null mice for *TAL1* or for *LMO2* develop the same phenotype and that in some leukemic patients, *TAL1* or *LYL1* and *LMO2* are co-expressed argues in favor of common oncogenic pathways.[77]

1.5.4.1.3 Deregulation of homeobox genes

Homeobox (*HOX*) genes are key regulators of embryonic development, involved in axial patterning, morphogenesis and cellular differentiation. All homeobox genes share a 61 amino acids motif, the homeodomain, a DNA binding domain, regulating transcription of target genes.[167] There are two classes of homeobox genes.

Class I homeobox genes. Class I *HOX* genes comprise 39 genes distributed in four HOX clusters (A–D) located on chromosomes 7p15, 17q21, 12q13 and 2q31, respectively. These regulatory genes share extensive homology with the *HOM-C* genes of Drosophila.[168] In addition to embryonic development, some *HOX* genes of clusters A, B and C were shown to play a role in normal hematopoiesis, influencing stem cell renewal and lineage commitment.[169] *HOXA* genes (*A7, A9, A10, A11*) are expressed during the early stages of human T-cell development.[170] In knockout mice, the absence of *HOXA9* results in an early block of thymocyte differentiation with reduced expression of IL7 receptor.[171] Conversely, overexpression of *HOXA9* also results in defective T-cell development.[172] *HOXB3* is expressed in very immature progenitors and probably interferes with the choice of αβ versus γδ lineage in pro-T-cells.[173] *HOXC4* is expressed at all stages of T-cell development.[170]

In T-ALL, ***HOXA* genes** (especially *HOXA10* and *A11*) can be upregulated as a consequence of an often cryptic inversion of chromosome 7 or translocation, t(7;7), which brings the *TCRB* enhancer within the *HOXA* locus.[80] FISH analysis disclosed these rearrangements in up to 5% of T-ALL showing a rather mature phenotype (EGIL) with characteristic CD2 negative and CD4 single positive lymphoblasts. Expression-array of *HOXA* positive T-ALL indicates an arrest after lineage choice but before β selection.[78]
The exact mechanism of activation of these *HOXA* genes remains to be explored. Beyond the classical activation due to the juxtaposition of *TCRB* regulatory elements in the vicinity of the *HOXA* genes, it has been suggested that the breakpoint in the *HOXA* gene cluster could disrupt the normal schedule of sequential up- and downregulation of these genes

within the *HOXA* locus.[80] Indeed, the expression of each *HOX* gene in blood progenitors follows stage and lineage-specific programs that are tightly regulated and differentiation into mature blood cells is accompanied by a progressive downregulation of *HOX* gene expression.[174]
HOX genes are known targets of MLL and are upregulated in ALL cases with *MLL* translocations.[175] Similarly, the *CALM-AF10* translocation results in *HOXA* upregulation[176] as will be discussed below. Altogether, expression studies (microarrays and/or RQPCR) have identified a subgroup of *HOXA* expressing T-ALL.[78] Included in this group are the T-ALL with *TCRB-HOXA*[80], *MLL*[175] and *CALM-AF10*[176] rearrangements. A few cases without these translocations, but also clustering in the *HOXA* expressing group have been recently identified as having a ***SET-NUP214*** fusion gene following a recurrent cryptic deletion, del(9)(q34.11q34.13). The chimeric SET-NUP214 protein binds in the promoter regions of specific *HOXA* genes and interacts in vivo with the methyltransferase DOTL1, as previously shown for *CALM-AF10* and *MLL-AF10* fusions, leading to their transcriptional activation.[88]

Class II homeobox genes. Class II homeobox genes (also named non-*HOX* genes or divergent homeobox genes) are dispersed throughout the genome.[177] They encode cofactors for HOX proteins and are not involved in homeotic transformations. Their pattern of expression is more restricted. They participate in organogenesis and in differentiation of specific cell types. In T-ALL, the class II orphan homeobox *TLX1 (HOX11)* and *TLX3 (HOX11L2)* have been extensively studied.

TLX1 (HOX11) is not normally expressed in developing T-cells and *HOX11* null mice are asplenic, suggesting a role in spleen development.[178] The *TLX1* homeobox gene has been identified as the gene located at the 10q24 breakpoint region of the translocations, t(10;14)(q24;q11) and t(7;10)(q34;q24).[179] As a result of the juxtaposition with promoter elements of *TCRA* and *B*, respectively, the full-length protein is expressed at a high level. In addition, loss of negative regulatory elements upstream of the promoter could also explain the ectopic transcription of *TLX1*.[180] In addition, *TLX1* is frequently activated in T-ALL in the absence of an overt genetic rearrangement.[76,77,181] In those cases, it has been suggested that aberrant promoter demethylation might disrupt gene silencing mechanisms that normally operate during normal T cell development.[182] *TLX1* is expressed in up to 30% of T-ALL, more often in adults than in children. These leukemias show an early cortical phenotype and a more favorable outcome than all other classes of T-ALL.[183] It has been suggested that downregulation of antiapoptotic genes at this stage of thymocyte development could explain their better prognosis.[77] In addition, *TLX1* positive T-ALL have been associated to elevated expression of the glucocorticoid receptor that may explain the particular sensitivity of this subgroup to steroids.[77] The oncogenic potential of TLX1 is well established but its transforming mechanisms remain unclear and are the subject of intense research. Enforced expression of *TLX1* in murine bone marrow gives rise to T-ALL-like malignancies after long latencies, which suggests that additional mutations are required for full blown leukemia.[184,185] Mechanisms requiring homeodomain-DNA interactions have been proposed.[186] In addition, recent works suggest that TLX1 modulates G1/S transcriptional network of T-ALL by interacting with the catalytic subunits of the protein serine/threonine phosphatases PP2A and PP1.[187,188]

TLX3 (HOX11L2) expression in T-ALL is in most cases caused by the cryptic translocation, t(5;14)(q35;q32) juxtaposing *TLX3* to the distal region of *BCL11B*[189], a gene universally expressed during T-cell differentiation. The t(5;14) is found in approximately 20% of childhood T-ALL and 13% of adult cases. Rare translocation variants have been reported: a t(5;14) that involves *NKX2-5*, another homeobox gene instead of *TLX3*[190], a t(5;7)(q35;q21) involving *TLX3* and *CDK6*[191], a t(5;14)(q35;q11) and a t(5;12)(q35;p13) juxtaposing *TLX3* to *TCRA/D* and *ETV6* genes, respectively.[192,193] *TLX3* is very similar to *TLX1* and microarray studies indicate that *TLX1* and *TLX3* positive T-ALLs cluster together[77,78], suggesting common mechanisms of action. *TLX3* expressing T-ALLs show a less narrow phenotype than *TLX1*, either more immature or slightly more mature[78] and they do not have the favorable outcome reported for *TLX1* positive cases.[77,194,195] More studies, including also analysis of combined involvement of other T-cell oncogenes, are needed to clarify this point.[196]

<u>*MLL* fusion genes.</u> The *MLL* gene at chromosome band 11q23 has structural and functional homologies with the Drosophila Trithorax gene. It acts as a transcriptional regulator and represents a major component of the cellular memory system, which maintains the established transcription patterns. The MLL protein is required to maintain the transcription of specific members of the *HOX* gene family.[197,198,199] *MLL* is known to rearrange with more than 50 partners in several translocations encoding chimeric proteins in which the N-terminal portion of MLL is fused to the C-terminal portion of the partner.[200] Translocations involving *MLL* are seen in both myeloid and lymphoid malignancies, frequently in infant acute leukemias and in secondary leukemias in patients treated with topoisomerase II inhibitors. Gene expression profiling has shown that B-precursor ALL associated with *MLL* translocations form a distinct subtype of acute leukemia, readily distinguished from either AML or ALL.[201,79] *MLL* fusions are found in 4–8% of T-ALL.[86,175,201] The preferential partner is *ENL (MLLT1)*. Translocation, t(11;19) (q23;p13.3), encoding *MLL-ENL* is often found in young adolescents and carries a better prognosis than usually associated with *MLL* rearrangements.[73,202] Other partners *(AF10, AF6, AF4, AFX1)* are occasionally seen. Their prognostic impact is not known. Expression-array analysis demonstrated increased expression of a subset of *HOX* genes *(HOXA9, HOXA10, HOXC6)* and of *MEIS1*, the HOX gene co-regulator, to be a central mechanism of leukemic transformation by MLL oncoprotein in *MLL* positive T- and B-ALL. On the other hand, the upregulation of myeloid genes and expression of FLT3 characteristic of *MLL* positive B-ALL is not found in *MLL* positive T-ALL.[175] T-ALL with *MLL* fusion represent a distinct molecular subtype with a specific expression profile, characterized by differentiation arrest at an early stage of thymocyte development and with commitment to γδ lineage.[77,78] Lymphoid and myeloid leukemogenic potential of *MLL* fusions has been well established in murine models.[203]

<u>*CALM-AF10 (PICALM-MLLT10)* fusion.</u> The translocation, t(10;11)(p13;q14), is recurrent in T-ALL[204], but is also found in leukemias of other lineages like AML. This translocation fuses *CALM* (Clathrin Assembly protein-like Lymphoid-Myeloid Leukemia gene, also called *PICALM*) to *AF10* (also called *MLLT10*).[205] *CALM* and *AF10* are occasional *MLL* fusion partners.[206] Both are ubiquitously expressed and AF10 functions as a transcription factor. In mice, retroviral expression of *CALM-AF10* was reported to induce biphenotypic acute leukemia.[207] *CALM-AF10* fusion is detected in 9–10% of T-ALL and is restricted to mature types expressing γδTCR and their precursor, immature γδ (IM γ/δ) T-ALL.[85] The

translocation is often cryptic and requires molecular detection either by FISH or RT-PCR. Several different fusion transcripts have been described. Within *CALM-AF10* positive T-ALL, *AF10* content differs with the stage of maturation arrest: the more 5' breakpoints are associated to mature TCRγδ cases and the more 3' breakpoints are associated to IM γ/δ T-ALL.[85] These findings suggest a possible role for AF10 protein in TCRγδ lineage commitment at the early stages of hematopoietic differentiation. The mechanism by which the fusion transcript is leukemogenic is not clear. Expression-arrays disclosed upregulation of the *HOXA5*, *HOXA9* and *HOXA10* genes and their cofactor *MEIS1*.[78,176] This strongly indicates common oncogenic pathways in *CALM-AF10* and *MLL* T-ALLs. Unique to *CALM-AF10* is the overexpression of *BMI1*, a gene located at 10p12.3 in close vicinity of *AF10*. BMI1 is essential for self-renewal of normal and leukemic stem cells.[208] BMI1 controls cell proliferation by inhibiting the *CDKN2A (p16)* gene[209] and is thus an alternative to the deletion of the *CDKN2* locus observed in 70% of T-ALL, but not present in *CALM-AF10* T-ALL. Prognosis is globally poor, particularly for the most immature subtype.[206,85]

1.5.4.1.4 Elevated MYB expression

MYB encodes a nuclear transcription factor important for differentiation, proliferation and survival of hematopoietic cells. Myb is essential at several stages during T-cell development in the thymus, notably for the proliferation of immature thymocytes following β selection.[210] Bone marrow enforced expression of *MYB* leads to leukemia in mice.[211-213] Involvement of *MYB* in T-cell leukemogenesis has been first reported in rare cases harboring a translocation, t(6;7)(q23;q24), fusing *MYB* to *TCRB* loci.[214] More recently, duplication of the *MYB* oncogene has been reported in 8% of T-ALL cases.[215,216] The resulting MYB overexpression is responsible for differentiation impairment as assessed by siRNA knockdown experiments in *MYB* duplicated T-ALL cell lines. Of interest, unlike *MYB* translocated cases[96], *MYB* duplicated cases do not cluster in a particular subgroup of T-ALL but are detected in T-ALL expressing other genetic defects associated to differentiation arrest like *TLX3*, *TLX1*, *SIL-TAL1*, *MLL-ENL*, *CALM-AF10*, *HOXA* or *TAL2*. It suggests cooperating activity of MYB in impairing differentiation. Combined inhibition of *MYB* and NOTCH1 shows synergistic effects on proliferation and viability of cell-lines making MYB suitable for targeted therapy.

1.5.4.2 Cell cycle defects

***p16/p14* deletion.** Cell cycle progression is tightly regulated with various checkpoint controls in order to maintain genomic integrity of the cell and to obtain an adequate balance between cell proliferation and differentiation. The *INK4/ARF* locus (or *CDKN2A/B* locus) on chromosome 9p21 contains genes coding for three proteins, p16INK4A, p14ARF and p15INK4B involved in cell cycle regulation.[217,218] *p16INK4A* and *p14ARF* are transcribed from the same gene *CDKN2A*. Both transcripts have identical exons 2 and 3 but differ in their promoters and exon 1 (exons 1a and 1b, respectively). Alternative splicing of exon 1 gives rise to alternative reading frames and therefore to unrelated proteins. p16INK4A and p15INK4B are inhibitors of cyclin-CDK protein complexes and maintain the cells in a quiescent stage. Loss of cyclin-CDK inhibition results in phosphorylation of the retinoblastoma protein (Rb1) abrogating its ability to inhibit cell cycle activity.[219] p14ARF

also controls cell cycle entry through interaction with p53 pathway. Indeed, p14 associates with MDM2 (a negative regulator of p53), resulting in upregulation of p53 and subsequent CDKN1A (p21) activation. p21 is also a cyclin-CDK inhibitor causing cell cycle arrest in G1/G2 in order to allow DNA repair or apoptosis in case of major DNA damage.[220]
As mentioned before, maintenance of immature T-cells in a quiescent stage during V(D)J recombination is particularly important to avoid illegitimate recombination phenomena and to allow p53 induced repair of DNA damage caused by double strand breaks occurring during recombination. p16 expression has been proposed to play an important role at this stage, being subsequently downregulated to allow the proliferation step in response to β selection.[221]

Cryptic deletion (detectable by FISH) of the *INK4/ARF* locus at 9p21 is the most frequent anomaly detected in T-ALL.[92,222] Homozygous/heterozygous deletions are seen in 65/15% of cases, respectively. These deletions target the *CDKN2A* (p16/p14) and in part also *CDKN2B* (p15) genes. In addition, mutation or methylation of promoters of these genes is present in a substantial number of cases. Inactivation at transcriptional and post-transcriptional level has also been reported.[223,224] Globally, functional inactivation occurs in nearly all childhood T-ALL[225] and in the vast majority of adult T-ALL. The majority of *TAL1* positive and *HOX11* positive T-ALL presents with homozygous deletion of *p16*. This indicates that inactivation of these tumor suppressor genes is directly involved in T-cell leukemogenesis. The respective contribution of p16 and p14 inactivation still remains unclear. It also identifies the Rb1 and p53 pathways as potential targets for therapy. Prognostic significance of *INK4/ARF* locus deletion in acute leukemia is inconsistent but seems to be unfavorable in childhood T-ALL, as shown in one study in which *p16* homozygously deleted T-ALL patients had a significantly lower 5-year disease-free survival.[226]

***CCND2*.** This gene encodes cyclin D2. The mature TCR preferentially induces cyclin D2 expression whereas stimulation of the pre-TCR preferentially induces cyclin D3 and down-regulates cyclin D2 expression.[54,227] Recently, three cases of T-ALL have been reported with marked overexpression of *CCND2*, as a consequence of translocation of *CCND2* locus at 12p13 to regulator elements of the *TCRB* or *TCRA/D* locus.[228] Aberrant high *CCND2* expression in these three cases was associated with *TAL1*, *TLX1* and *TLX3* positive molecular subtypes, *NOTCH1* mutation and *CDKN2A* deletion, indicating a role for *CCND2* in multistep leukemogenesis of T-ALL.

1.5.4.3 Deregulation of (pre)-TCR and other signaling components

1.5.4.3.1 Tyrosine kinase activation

Tyrosine kinases play a key role in (pre-)TCR signaling **(Figure 4)**. They are critical in regulation of T-cell survival, proliferation and T-cell immune response.
In T-ALL, a number of these genes are activated either by gene fusion as a consequence of chromosomal rearrangements or by mutations.

ABL1* fusions.** ABL1 is a ubiquitously expressed cytoplasmic tyrosine kinase, encoded by a gene mapping on 9q34. It has recently been shown to play a role in TCR signaling **(Figure 4)**.[63,64] The translocation, t(9;22)(q34;q11), encoding the **BCR-ABL1**[6] fusion kinase, characteristic of chronic myeloid leukemia, is found in 25% of precursor B-ALL and only rarely (1%) in T-ALL.[11,14] Specific for T-ALL is the ***NUP214-ABL1 fusion found in up to 6% of T-ALL.[87] Interestingly, this fusion gene is found on amplified episomes, which are not visible cytogenetically, and exceptionally on homogeneously stained regions (hsr) or double minute chromosomes (dmin).[196,229] Molecular detection by FISH or RT-PCR is required to diagnose such cases **(Figure 5)**. *NUP214-ABL1* positive T-ALL are characterized by ectopic expression of *TLX1* or *TLX3*, deletion of *CDKN2A* locus and activating *NOTCH1* mutations.[87,218,230, 231]
Other *ABL1* fusion variants have rarely been reported. Like *BCR-ABL1*, ***ETV6-ABL1*** resulting from translocation, t(9;12)(q34;p13), is found in CML, precursor B-ALL and exceptionally in T-ALL.[232] A translocation, t(9;14)(q34;q32), encoding an ***EML1-ABL1*** fusion protein has been described in a single T-ALL patient.[233] All these fusions result in constitutive activation of *ABL1* leading to activation of survival and proliferation pathways.[234,235] Recently, T-ALL patients have been described displaying *ABL1* overexpression without evidence of *ABL1* fusions.[236] This suggests that other alterations affecting *ABL1* gene are still to be discovered.
Interestingly, imatinib, a specific inhibitor of ABL1 kinase seems to be effective in controlling the activity of these ABL1 fusion proteins *in vitro*.[87,233,237]
An ***ETV6-ABL2*** fusion transcript in combination with an *ETV6* point mutation has been recently reported in a T-ALL cell line.[238]

JAK*.** Non receptor tyrosine kinases JAK proteins are an essential relay for transmitting signals from cytokine receptors to downstream signaling. In a childhood T-ALL, a translocation, t(9;12)(p24;p13) encoding ***ETV6-JAK2 fusion gene was shown to result in constitutive tyrosine kinase activity.[239] Subsequently, the transforming role of this fusion protein was demonstrated in mice transgenic for *ETV6-JAK2*, which developed leukemia.[240]
Very recently, activating mutations in the ***JAK1*** gene, localized on chromosome 1 at 1p31.3, have been reported in 18% and 3% of adult T-ALL and B-ALL, respectively. *JAK1* mutations were more prevalent in the elderly and associated with poor response to chemotherapy and outcome. The high frequency of this mutation in adult T-ALL makes this anomaly particularly suitable for targeted therapy.[241]

***LCK*.** LCK, a member of the SRC family of tyrosine kinases, is specifically expressed in T-cells and is a key player in proximal (pre)-TCR signaling cascade, upstream to ABL1 **(Figure 4)**. In T-ALL it has been shown to be overexpressed in rare cases with translocation, t(1;7)(p34;q34), joining *LCK* to the *TCRB* locus.[242,243]

***FLT3*.** This gene, localized on chromosome 13 at 13q12.3, encodes a receptor tyrosine kinase playing an important role in the development of hematopoietic stem cells. Activating mutations such as internal tandem duplication (ITD) in the juxtamembrane domain encoding sequences or point mutations in sequences coding for the activation loop of the kinase domain are most common in AML (30%), rare in precursor-B ALL and infrequent in T-ALL.[244] In adult T-ALL, *FLT3* mutations seem to be restricted to cases with a very immature phenotype, expressing LYL1, LMO2 and the KIT receptor.[244] In pediatric T-

ALL, the association with KIT receptor remains to be determined.[245] Occasional expression of myeloid markers supports the contention that FLT3 positive T-ALL are leukemias with expansion of early progenitor cells.[175]

1.5.4.3.2 Other defects in signal transduction

RAS. *RAS* genes encode proteins that play a critical role in transmitting survival signals from the cell membrane receptors to the intracellular transduction pathways. Mutations of *RAS* genes are common and have been described in various malignancies[246] including acute leukemias.[247] They lead to the constitutive activation of the RAS-MAP kinase signaling cascade **(Figure 4)**. Activating mutations of *N-RAS*, localized on chromosome 1 at 1p13.2, have been detected in 10% of a series of childhood T-ALL.[248] Other studies indicate that RAS is highly activated in 50% of T-ALL[249] suggesting a key role for RAS activation in T-ALL pathogenesis. This also supports therapeutic approach using farnesyltransferase inhibitors.[250] Recently, deletion of ***NF1*** on chromosome 17 at 17q11.2, encoding a negative regulator of the RAS pathway, has been proposed as an alternative for RAS activation.[251]

1.5.5 From thymocyte to lymphoblast: a multistep process

From the above description and as represented in **Figure 6**, it emerges clearly that multiple hits are cooperating in the generation of T-ALL, in agreement with the paradigm of multistep oncogenesis first developed for colon tumors by Vogelstein and Kinzler.[252]

1. The very high incidence of *NOTCH1* mutations, detected in all molecular subtypes of T-ALL strongly suggests that mutations occur in very immature progenitors, conferring them **increased self-renewal capacity**[99] and resulting in accumulation of progenitor cells susceptible to undergo additional molecular hits.

2. Aberrant expression of transcription factors is a universal finding in T-ALL. Overexpression of oncogenes is often the consequence of chromosomal translocations, but is also found in the absence of specific cytogenetic alterations and constitutes the basis of the molecular classification established after analysis of microarray gene expression profiles.[77,78] These gene expression signatures correspond to different stages of **differentiation arrest** resulting from deregulated transcription factors activity. bHLH proteins (TAL1, LYL1, TAL2) are oncogenic through inhibition of the E2A proteins regulatory activity. *LMO1* and *LMO2* overexpression is frequently found in *TAL1* or *LYL1* positive T-ALL[77] and contributes to transforming activity of TAL1.[149,166] *TLX1 (HOX11)* and *TLX3 (HOX11L2)* are not normally expressed in thymocytes, but are aberrantly expressed in a substantial number of cases, classically with an early cortical phenotype.[77] Several *HOX* genes play a role in thymocyte development but their expression is transient. Recently, a subgroup of T-ALL has been identified with sustained aberrant expression of members of the *HOXA* family (A5, A10, A11).[78] *HOXA* genes are targets of MLL, SET-NUP214 and of CALM-AF10 fusion proteins and are overexpressed in immature T-ALL carrying these translocations.[175,176] In addition, *HOXA* genes can be upregulated by virtue of translocation in *TCRB* locus, as seen in cases with inv(7) or t(7;7) in more mature cases[80] **(Table 2)**. Finally, T-ALL overexpressing *MYB* following translocation t(6;7)(q23;q24)

might represent a distinct subgroup.[220] Of interest, in T-ALL, E2A proteins are targeted by several oncogenic proteins but have never been directly involved in genetic rearrangements.

3. **Defects in cell cycle control** are a universal phenomenon in T-ALL, mostly due to loss of the *INK4/ARF* locus.[248] Disruption of cell cycle control can also be operated by transcription factor modulation. For example, *CALM-AF10* T-ALL overexpress *BMI1* that control cellular proliferation by suppressing *p16* gene.[176] Also, enforced TAL1 expression negatively regulates *p16* gene through interfering with the E boxes sequences of its promoter.[253] Conversely, E2A proteins positively regulate several CDK inhibitors' promoters and negatively affect cell growth, consistent with their tumor suppressor properties. HOX11 has been shown to act on the cell cycle by interacting with the protein serine/threonine phosphatases PP2A and PP1.[187]

4. Constitutive activation of tyrosine kinases (LCK, FLT3, ABL1, JAK1/2) or other signaling components (RAS) associated to response to growth/survival signals, are found in 30-40% of T-ALL. It provides cells with **proliferative and survival advantage** and, if associated to mutations affecting cell cycle control, can be responsible for the rapid expansion of the leukemic clone. It can be expected that additional events activating signaling proteins or inhibiting their regulators like phosphatases remain to be identified.

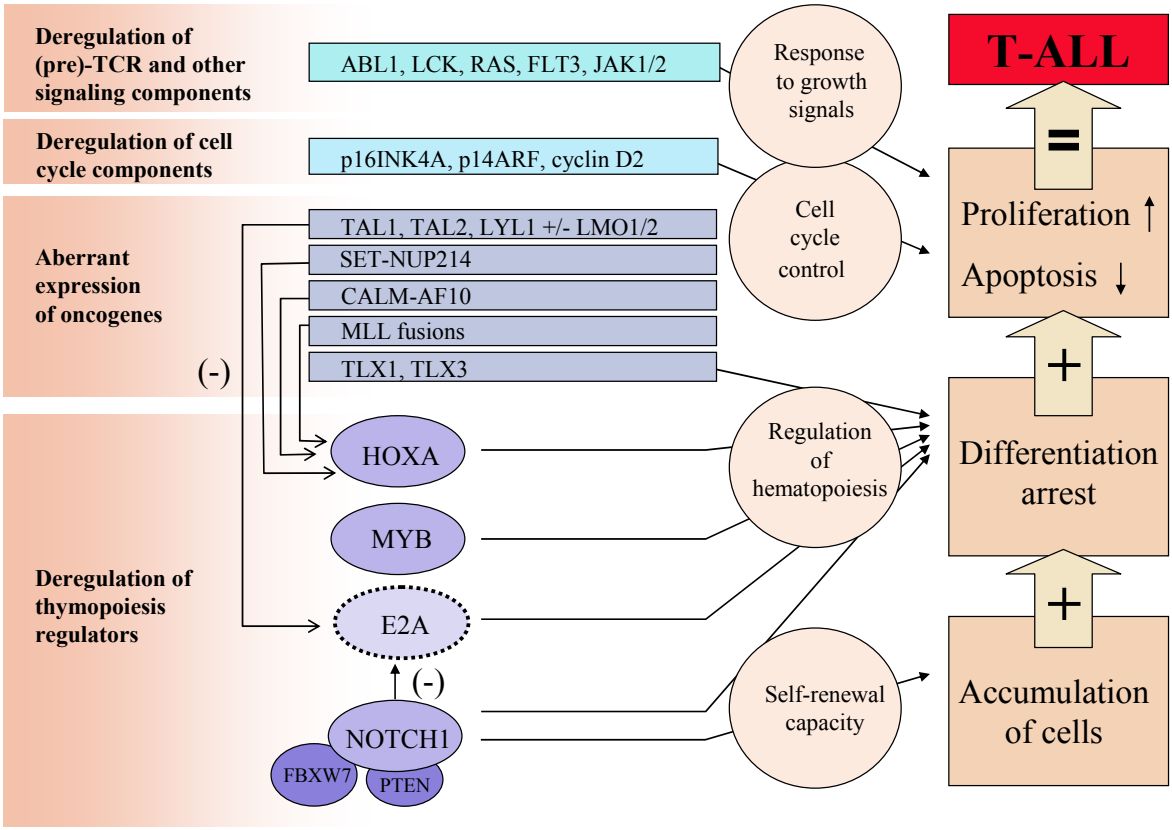


Figure 6 Multistep T-cell leukemogenesis
The multistep process of T-cell leukemogenesis is explained in the text above this figure.

1.5.6 From bench to bedside: translational advances in T-ALL

Accumulated data on the underlying mechanisms responsible for T-cell leukemogenesis[91,254,255], led to consider the use of rationally designed therapies targeting specific elements that are essential for T-lymphoblast survival.

A paradigm of such approach is the use of **γ-secretase inhibitors** in *NOTCH1* mutated T-ALL. Arguments in favor of the use of such therapy are the high prevalence of oncogenic activating *NOTCH1* mutations in T-ALL coupled to the availability of γ-secretase inhibitors (GSI) capable of blocking NOTCH1 signaling. GSI are already tested for the treatment of Alzheimer's disease. Despite a strong rationale, first clinical trials with GSI in *NOTCH1* mutated T-ALL were of reduced effect and too toxic. One explanation for the lack of response in some cases was the discovery of mutational loss of PTEN in γ-secretase insensitive cases, bypassing the requirement for aberrant NOTCH1 signaling for leukemogenic activation of the PI3K/AKT/mTOR pathway since wild type (WT) PTEN normally exerts a negative control on this pathway.[98] Agents directly targeting this pathway such as the **mTOR inhibitor** rapamycine and **AKT inhibitors** could circumvent this lack of efficacy of γ-secretase inhibitors in NOTCH1 activated/PTEN deleted T-ALL. Those agents could also increase the intrinsic activity of GSI in cases without *PTEN* deletion as PTEN has been shown to be negatively regulated downstream of oncogenic NOTCH1 and so participate to NOTCH1 induced activation of the PI3K/AKT/mTOR pathway.[256,257] The gastrointestinal dose limiting toxicity observed with γ-secretase inhibitors reflects the requirement for NOTCH1 in self-renewal of the gastrointestinal mucosa. Modification of dose, timing and combination of schedule are needed to produce a differential effect of the drug in neoplastic and normal tissues.

Activated tyrosine kinases represent another type of defect that can be targeted by therapy. Their major interest lies in the recent development of **specific kinase inhibitors**.[258] These include specific inhibitors of ABL1, SRC, FLT3 and JAK kinases among others. Some of them have already been shown to be effective in other hematological malignancies. Activated tyrosine kinases found in T-ALL are secondary cooperating mutations involved in pathway regulating growth or apoptosis. Therefore, use of tyrosine kinase inhibitors can be only considered in combination with chemotherapy or drugs targeting more specifically the primary genetic defects. **Farnesyl transferase inhibitors** (FTI) could be useful for T-ALL patients with activated RAS.

Specific inhibitors of transcription factors are not yet available but more general approaches should be investigated like the use of **histone deacetylase** (HDAC) **inhibitors**, **proteasome inhibitors** and **short interfering RNA** (siRNA).

Inactivation of the *p16* tumor suppressor gene is encountered in most T-ALL cases, suggesting its direct implication in T-cell leukemogenesis. In a substantial number of cases, *p16* suppression is due to promoter methylation giving a rationale for the use of **demethylating agents** in those cases. Since p16INK4A and p14ARF inhibits Rb1 and p53, respectively, these pathways may also represent interesting targets for therapy.

In conclusion, as T-ALL is a very heterogeneous disease, future targeted therapy will require a precise knowledge of the multiple molecular hits that cooperate in the various subtypes of T-ALL in order to define adequate drug combinations that target the different defects responsible for the persistence of the transformed phenotype. Also, a precise diagnosis of these genetic changes and gene expression signatures will be necessary to improve treatment adequacy and outcome.

II Rationale and aims

With current chemotherapy-based treatments, cure rate for T-ALL is about 70-80% for childhood cases, but less than 40% for adults.[176] Chemotherapy drugs have a narrow therapeutic index and are responsible for serious immediate and late adverse cytotoxic effects. More efficient and less toxic treatments are required to improve outcome of T-ALL patients. The success of targeted therapy with imatinib in chronic myeloid leukemia (CML) encourages searching for tumor specific abnormalities that may serve as new targets.

The aim of our work was to investigate the genetic alterations responsible for the development of T-ALL. Our approach was based on the use of cytogenetic and molecular analyses and focused mainly on kinase alterations. The final aim was the improvement of therapy by identifying possible anomalies suitable for therapy.

To reach this objective, in addition to the experimental work, we first reviewed the literature on normal (T-cell ontogeny) and abnormal T-cell development (T-cell leukemogenesis) in order to identify the critical steps of thymocyte development susceptible to be targeted by the different oncogenic genetic defects in T-ALL.[259]

Many oncogenes involved in T-ALL have been discovered by analyzing the breakpoints of recurrent or unique chromosomal translocations.
By chance, performing routine FISH analysis with an *ABL1* probe on a T-ALL, we observed a novel amplification of the *ABL1* signal. Since targeted therapy for ABL1 was already available, we were particularly interested to provide further insight in this finding that represents the initiating element of this thesis.

The aims of the present thesis were:

(1) Evaluation of the recurrent nature (incidence) of the *ABL1* amplification observed in this isolated T-ALL case by performing FISH screening of additional T-ALL samples. We aimed also to elucidate its significance and potential role in T-cell oncogenesis (functional consequences).

As the *ABL1* amplification led to the identification of a *NUP214-ABL1* fusion in 6% of T-ALL, the additional aims were:

(2) Study of the genomic presentation of the *NUP214-ABL1* fusion and assessment of its place in the multistep process of T-cell leukemogenesis. The underlying aim was to provide insight in therapeutic implications.

(3) Identification of novel *ABL1* fusion genes in T-ALL.

As constitutively active tyrosine kinases play a critical role in proliferation and survival of numerous cancers and are identified in 30-40% of T-ALL only, we aimed to:

(4) Identify new genetic anomalies, other than *ABL1* fusions, responsible for the activation of kinases in T-ALL.

III Results

3.1 *ABL1* aberrations in T-ALL

3.1.1 *ABL1* amplification

3.1.1.1 Fusion of *NUP214* to *ABL1* on amplified episomes in T-cell acute lymphoblastic leukemia (*Nature Genetics* 2004)

Graux C[1,2,13], Cools J[1,3,13], Melotte C[1], Quentmeier H[4], Ferrando A[5], Levine R[5], Vermeesch JR[1], Stul M[1], Dutta B[6], Boeckx N[2], Bosly A[7], Heimann P[8], Uyttebroeck A[9], Mentens N[1,3], Somers R[1,3], MacLeod RA[4], Drexler HG[4], Look AT[5], Gilliland DG[5,10,11], Michaux L[12], Vandenberghe P[1,2], Wlodarska I[1], Marynen P[1,3] & Hagemeijer A[1].

[1]Department of Human Genetics and [2]Department of Hematology, University of Leuven, Leuven, Belgium. [3]Department of Human Genetics, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium. [4]German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. [5]Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA. [6]Microarray Facility, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium. [7]Department of Hematology, Cliniques universitaires UCL de Mont-Godinne, Yvoir, Brussels, Belgium. [8]Department of Medical Genetics, Erasme Hospital, Free University of Brussels, Brussels, Belgium. [9]Department of Pediatric Hemato-oncology, University of Leuven, Leuven, Belgium. [10]Brigham and Women's Hospital and [11]Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts, USA. [12]St. Luc, Brussels, Belgium.

[13]These authors contributed equally to this work.

In T-cell acute lymphoblastic leukemia (T-ALL), transcription factors are known to be deregulated by chromosomal translocations, but mutations in protein tyrosine kinases have only rarely been identified. Here we describe the extrachromosomal (episomal) amplification of ABL1 in 5 of 90 (5.6%) individuals with T-ALL, an aberration that is not detectable by conventional cytogenetics. Molecular analyses delineated the amplicon as a 500-kb region from chromosome band 9q34, containing the oncogenes ABL1 and NUP214 (refs. 5,6). We identified a previously undescribed mechanism for activation of tyrosine kinases in cancer: the formation of episomes resulting in a fusion between NUP214 and ABL1. We detected the NUP214-ABL1 transcript in five individuals with the ABL1 amplification, in 5 of 85 (5.8%) additional individuals with T-ALL and in 3 of 22 T-ALL cell lines. The constitutively phosphorylated tyrosine kinase NUP214-ABL1 is sensitive to the tyrosine kinase inhibitor imatinib. The recurrent cryptic NUP214-ABL1 rearrangement is associated with increased HOX expression and deletion of CDKN2A, consistent with a multistep pathogenesis of T-ALL. NUP214-ABL1 expression defines a new subgroup of individuals with T-ALL who could benefit from treatment with imatinib.

3.1.1.2 Heterogeneous patterns of amplification of the *NUP214-ABL1* fusion gene in T-cell acute lymphoblastic **leukemia (*Leukemia* 2008)**

Graux C[1,11,14,15], Stevens-Kroef M[2], Lafage M[3], Dastugue N[4], Harrison CJ[5], Mugneret F[6], Bahloula K[1], Struski S[7], Grégoire MJ[8], Nadal N[9], Lippert E[10], Taviaux S[12], Simons A[2], Kuiper RP[2], Moorman AV[5], Barber K[13], Bosly A[14], Michaux L[15], Vandenberghe P[15], Lahortiga I[11,15], De Keersmaecker K[11,15], Wlodarska I[15], Cools J[11,15], Hagemeijer A[15,16] and Poirel HA[1,16] on behalf of the GFCH (Groupe Francophone de Cytogénétique Hématologique) and the BCGHO (Belgian Cytogenetic Group for Hematology and Oncology).

[1]Hematologic Section of the Human Genetics Centre, Cliniques universitaires UCL Saint-Luc, Brussels, Belgium; [2]Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [3]Département de Génétique, CHU Timone, Marseille, France; [4]Laboratoire d'Hématologie - Génétique des Hémopathies, Hôpital Purpan, Toulouse, France; [5]Leukemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; [6]Laboratoire de Cytogénétique, CHU Le Bocage, Dijon, France; [7]Laboratoire d'Hématologie, Hôpital Haute Pierre, Strasbourg, France; [8]Laboratoire de Génétique, CHU de Nancy-Brabois, Vandoeuvre-Les-Nancy, France; [9]Laboratoire d'Hématologie - Pavillon de Biologie, CHU Hôpital Nord, St Etienne, France; [10]Laboratoire d'Hématologie, CHU de Bordeaux, Bordeaux, France; [11]Human Genetics - Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium; [12]Laboratoire d'Hématologie – Génétique moléculaire et chromosomique, Hôpital Arnaud de Villeneuve, Montpellier, France; [13]Leukemia Research Cytogenetics Group, Cancer Sciences Division, University of Southampton, Southampton, United Kingdom; [14]Department of Hematology, Cliniques universitaires UCL de Mont-Godinne, Yvoir, Belgium; [15]Centre for Human Genetics, University of Leuven, Leuven, Belgium. [16] Hagemeijer A & Poirel HA share senior authorship.

Episomes with the NUP214-ABL1 fusion gene have been observed in 6% of T-ALL. In this multicentric study we collected 27 cases of NUP214-ABL1-positive T-ALL. Median age was 15 years with male predominance. Outcome was poor in 12 patients. An associated abnormality involving TLX1 or TLX3 was found in all investigated cases. Fluorescent in situ hybridization revealed a heterogeneous pattern of NUP214-ABL1 amplification. Multiple episomes carrying the fusion were detected in 24 patients. Episomes were observed in a significant number of nuclei in 18 cases, but in only 1-5% of nuclei in 6. In addition, intrachromosomal amplification (small hsr) was identified either as the only change or in association with episomes in four cases and two T-ALL cell lines (PEER and ALL-SIL). One case showed insertion of apparently non-amplified NUP214-ABL1 sequences at 14q12. The amplified sequences were analyzed using array-based CGH.These findings confirm that the NUP214-ABL1 gene requires amplification for oncogenicity; it is part of a multistep process of leukemogenesis; and it can be a late event present only in subpopulations. Data also provide in vivo evidence for a model of episome formation, amplification and optional reintegration into the genome. Implications for the use of kinase inhibitors are discussed.

3.1.2 Novel fusion gene involving *ABL1*

3.1.2.1 Overrepresentation of chromosome 9q34 region, including *ABL1*, is recurrent in T-cell acute lymphoblastic leukemia (poster BHS 2004)

Graux C[1], Michaux L[1], Wlodarska I[1], Stul M[1], Ferrant A[2], Bosly A[3], Vandenberghe P[1], Hagemeijer A[1].

[1]Department of Human Genetics, University of Leuven, Leuven, Belgium; [2]Department of Hematology, Cliniques universitaires UCL Saint-Luc, Catholic University of Louvain, Brussels, Belgium; [3] Department of Hematology, Cliniques universitaires UCL de Mont-Godinne, Yvoir, Belgium.

Abstract: Use of a wide panel of FISH probes for the diagnosis of hematological malignancy may lead to the discovery of unsuspected chromosomal abnormalities. By application of a BCR-ABL1 probe (LSI BCR/ABL1 ES Dual Color, Vysis®) in a T-cell ALL we fortuitously found a major amplification of the red signal (> 10 copies) covering the ABL1 gene in more than 80% of cells. We subsequently decided to apply the same probe on a series of T-ALL cases, in order to assess the prevalence of this finding.

We analysed 59 T-ALL and 23 T-LL for which archival fixed cells were available. Two cases showed a major amplification of the red signal (>10) in the majority of the cells. Two additional patients showed such a major amplification in a minority of the cells (<20%). In 6 other cases (patients 6 to 11), a third copy of the region 9q34 was observed in addition to both normal chromosomes 9. In patient 6, the third copy of 9q34 translocated to an extra chromosome 1p. In patient 7, two subclones were observed with the third copy of 9q34 translocated either on chromosome 1q or on a marker. Patient 8 displayed a translocation of the extra region 9q34 on chromosome 7p. Patient 9 showed 2 abnormal tetraploid clones, one with 2 extra copies of the region 9q34 on a der(2) and one with 1 extra copy of the region 9q34 on a marker. Patient 10 presented with an isochromosome 9 leading to observation of 1 extra red signal. Patient 11 displayed 3 red signals in 10% of the nuclei but, due the lack of material, no further investigation could be performed. A twelfth patient presented with a pericentric inversion of chromosome 9 with split of the red signal, suggesting involvement of ABL1 in this structural aberration. The outcome of 2/5 patients with major amplification was poor. One was primary refractory to the treatment and the other relapsed rapidly. Interestinly, the latter did not show 9q34 amplification anymore at relapse. The remaining cases with major amplification are in remission, with follow-up ranging from 6 to 32 months. Three of the 6 cases with 1 extra-copy of 9q34 relapsed. The remaining cases are in remission, with a follow-up ranging from 5 to 28 months. Patient 12 relapsed after 6 months.

Gene amplification, potentially leading to gene overexpression, is a rare finding in hematological malignancy. Involved genes include MLL, AML1, JAK2, E2F1 and ETS1. Amplification of the fusion gene BCR-ABL1 has been described as a recurrent mechanism of resistance to STI. ABL1 amplification has been reported so far only in 2 papers: in one case of lymphoid blast crisis of CML and in 4 secondary leukemia as segmental jumping

translocation. Interestingly, a high percentage of 9q34 amplifications has been described in enteropathy-type T-cell lymphoma.
Further investgations are ongoing in order to confirm genomic amplification of the ABL1 locus, to assess expression of ABL1 and to investigate whether other genes located at 9q34 could be target gene in T-ALL.

In conclusion, aberrations involving the region 9q34 are recurrently observed in T-cell ALL, and mainly consist in both amplifications and overrepresentation of this region. In addition, we observed one case with a breakpoint involving the region covered by the ABL1 probe. This finding could provide information about the mechanisms of oncogenesis in T-ALL and give clues for novel therapeutic approach. Whether 9q34 amplification is of clinical relevance is not yet determined.

3.1.2.2 Fusion of *EML1* to *ABL1* in T-cell acute lymphoblastic leukemia with cryptic t(9;14)(q34;q32) (*Blood* 2005)

De Keersmaecker K[1], Graux C[2], Odero MD[3], Mentens N[1], Somers R[1], Maertens J[2], Wlodarska I[2], Vandenberghe P[2], Hagemeijer A[2], Marynen P[1], Cools J[1].

[1]Department of Human Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), Leuven, Belgium; [2]Departments of Human Genetics and Hematology, University of Leuven, Leuven, Belgium; and [3]Department of Genetics, University of Navarra, Pamplona, Spain.

The BCR-ABL1 fusion kinase is frequently associated with chronic myeloid leukemia and B-cell acute lymphoblastic leukemia but is rare in T-cell acute lymphoblastic leukemia (T-ALL). We recently identified NUP214-ABL1 as a variant ABL1 fusion gene in 6% of T-ALL patients. Here we describe the identification of another ABL1 fusion, EML1-ABL1, in a T-ALL patient with a cryptic t(9;14)(q34;q32) associated with deletion of CDKN2A (p16) and expression of TLX1 (HOX11). Echinoderm microtubule-associated protein-like 1-Abelson 1 (EML1-ABL1) is a constitutively phosphorylated tyrosine kinase that transforms Ba/F3 cells to growth factor-independent growth through activation of survival and proliferation pathways, including extracellular signal-related kinase 1/2 (Erk1/2), signal transducers and activators of transcription 5 (Stat5), and Lyn kinase. Deletion of the coiled-coil domain of EML1 abrogated the transforming properties of the fusion kinase. EML1-ABL1 and breakpoint cluster region (BCR)-ABL1 were equally sensitive to the tyrosine kinase inhibitor imatinib. These data further demonstrate the involvement of ABL1 fusions in the pathogenesis of T-ALL and identify EML1-ABL1 as a novel therapeutic target of imatinib.

Erratum:

Study design

Patients

The 16-year-old male patient with a cryptic t(9;14) presented with very high leukocytosis (455 X 10^9/L), with 99% blasts with the phenotype of cortical thymocytes, and normal karyotype. He is in first complete remission 15 months after diagnosis.

3.1.2.3 Transition from *EML1-ABL1* to *NUP214-ABL1* positivity in a patient with acute T-cell lymphoblastic leukemia (*Leukemia* 2006)

De Keersmaecker K[1], Lahortiga1 I[2], Graux C[3], Marynen P[1], Maertens J[4], Cools J[1] and Vandenberghe P[5].

[1]Department of Molecular and Developmental Genetics – Flanders Interuniversity Institute for Biotechnology (VIB), University of Leuven, Leuven, Belgium; [2]CIMA, University of Pamplona, Pamplona, Spain; [3]Department of Hematology, Cliniques Universitaires StLuc, Catholic University of Louvain, Brussels, Belgium; [4]Department of Hematology, University of Leuven, Leuven, Belgium; [5]Department of Human Genetics, University of Leuven, Leuven, Belgium.

3.2 Other tyrosine kinase aberrations in T-ALL

IKZF2-ERBB4 fusion in T-cell acute lymphoblastic leukemia (unpublished work)

Introduction

T-ALL is a neoplastic disorder that originates in a single lymphoid progenitor cell committed to the T-cell lineage. Proliferation and accumulation of those cells arrested at a very immature stage is responsible for bone marrow failure and enlargement of various tissues. The disease is rapidly fatal if not correctly treated.[1] Current treatment continue to be based on the administration of intensive chemotherapy. Although the disease is curable in 70-80% of childhood cases, in adults, the cure rate remains low (< 40%). It is particularly true in the elderly, where co-morbidities limit the optimal use of toxic drugs that usually have a very narrow therapeutic index. Beside host and drug related factors, the disparity of the response to treatment is largely explained by the heterogeneity of the underlying genetic alterations leading to leukemic transformation.[2,3]
Efforts are being made to identify new genetic defects that may be specifically targeted by therapy in order to decrease toxicity and increase the efficacy of treatment.[4] Genomic amplification of oncogenes and deletion of tumor suppressor genes are recurrent mechanisms contributing to transformation. These genomic copy number abnormalities are most frequently cryptic but are now detectable using high-density DNA microarrays.[5]
In this paper, we report a new recurrent *IKZF2-ERBB4* fusion gene detected in T-ALL cell lines resulting from a microdeletion at the 3' end of the *IKZF2* gene. The fusion transcript was also detected in other cell lines and T-ALL samples in the absence of an underlying genomic rearrangement. Moreover, we showed that the *IKZF2-ERBB4* transcript was present at very low expression level in several normal tissues as transcription induced chimera (TIC). *IKZF2* and *ERBB4* are members of gene families that have been linked to oncogenesis as a tumor suppressor gene or oncogene, respectively. We hypothesize that expression of this fusion *IKZF2-ERBB4* transcript in an inappropriate context might be oncogenic.

Material and methods

Patients and cell lines
Bone marrow and peripheral blood samples from 88 T-ALL patients were selected from the database of the Department of Human Genetics (Leuven) and the Centre for Medical Genetics (Gent). Samples were obtained according to the guidelines of the local ethical committees. Diagnosis of T-ALL was based on morphology, cytochemistry and immunophenotyping according to the WHO and EGIL criteria. The following cell lines, KE37, HPB-ALL, DND-41, SUP-T1, RPMI-8402, MOLT-4, MOLT-14, MOLT-16, JURKAT, LOUCY, P12-ICHIKAWA, CCRF-CEM, KARPAS-45 (T-ALL/T-LL), SKM1 (AML), BV-173 (blast crisis CML) were obtained from DSMZ, Braunschweig, Germany. Total RNA for the normal tissue panel was purchased from BD Biosciences, San Jose, CA. Normal blood samples were obtained from healthy volunteers. The following strains of mice were purchased from Harlan, Oxon, UK: BALB/cOlaHsd, C57BL/6JOlaHsd, 129P2/OlaHsd, FVB/NHsd, NIH/OlaHsd. Animals were 10 weeks of age with 1 male and 1 female of each strain.

Cytogenetics and fluorescence in situ hybridization (FISH)
Cytogenetic analyses were carried out on T-ALL bone marrow or peripheral blood cells using direct or short-term cultures without mitogen and using R banding. Fluorescence *in situ* hybridization (FISH) was performed on stored fixed cell suspension originally used for karyotyping.

RT-PCR
RNA was extracted from cell lines, bone marrow or peripheral blood samples and tissues using the RNAeasy system (Quiagen, Hilden, Germany). RNA was DNase treated and reverse transcribed using Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). The following primers were used for detection of the *IKZF2-ERBB4* fusion: IKZF2-ex2-F: 5'GGAAACAGAGGCTATTGATGG3'; IKZF2-ex3-F: ATGGCAATTGACCTCACCTC; ERBB4 ex4-R: 5'CCGAACAATATCTTGCCAATG3'. Primers used for cloning, SNPs analysis and sequencing are available. Long range amplification of 10 Kb segments for 454 genomic sequencing was performed with the Phire Hot Start DNA polymerase (Finnzymes Oy, Espoo, Finland).

Quantitative (RT)-PCR.
Primers were designed with Primer Express software (Applied Biosystems). The efficiency of amplification of the chosen primer sets was validated to be equal to that of the normalizer. The reaction mixtures consisted of LightCycler 480 SYBR Green I Master (Roche) with 500 nM of each primer and 20 ng DNA or 10 ng cDNA in a total volume of 15 µl. After an initial denaturation step for 10 min at 95ºC, thermal cycling conditions were 15 sec at 95 ºC and 1 min at 60 ºC for 40 cycles. Finally, the dissociation curves for each reaction were determined. All samples were run in duplicate/triplicate on a LightCycler 480 instrument (Roche).
For Q-PCR, a primer set for the *ABL1* gene was used for normalization. For RT-Q-PCR, the expression level of *ERBB4* transcripts was quantified relative to the expression level of the gene *HPRT1*. We used the comparative ddCt method (Sequence Detection System bulletin 2 (Applied Biosystems)) with SYBR-green. The quantification/expression levels relative to the *ABL1*/*HPRT1* housekeeping genes were calculated following the equation: relative target quantification/expression level as percentage of *ABL1/HPRT1* quantification/expression = 2-Ct x 100%, whereby Ct = Ct target–Ct *ABL1*/*HPRT1*

Microarray comparative genomic hybridization (array-CGH)
Array-CGH was performed using Code Linked Slides (AP Biotech) containing the 3,527 BAC clones from the Wellcome Trust Sanger Institute 1 Mb Clone Set, a gift from N. P. Carter (The Wellcome Trust Sanger Institute, UK), as previously described. Additional clones covering all 90 protein tyrosine kinase genes were added to this set. The human genome CGH-microarray 244A (Agilent, Santa Clara, California) containing 244.000 oligonucleotides with 8.9 Kb overall median probe spacing was used for high resolution analysis.

Constructs
Wild type (WT) ERBB4, truncated ERBB4 and the different *IKZF2-ERBB4* constructs were generated by PCR (Phusion: Finnzymes Oy, Espoo, Finland), and cloned in the retroviral vector pMSCV-puro (Clontech, Palo Alto, CA). All constructs were verified by sequencing.

Cell culture
Cell lines were cultured in RPMI-1640 supplemented with 20% fetal calf serum. Human 293T cells and murine Ba/F3 cells were transduced with the different constructs containing plasmids/viral vectors. Stably transduced Ba/F3 cells were selected with puromycin and cultured in presence or absence of IL3 and the ERBB4 ligands Neuregulin1 (Heregulin) and Betacellulin (PeproTech Inc., Rocky Hill, NJ) and in the presence of different concentrations of the pan-ERBB inhibitor CI-1033 (ACCC, San Diego, California). The proliferation/viability was counted by FACS (BD FACS Canto Flow Cytometer).

Western blotting
Total lysates of cell line cultures were analyzed by standard methods using the following antibodies: anti-carboxy-terminal ERBB4, anti-phospho-ERBB4, anti-amino-terminal IKZF2 (Santa Cruz, Santa Cruz , CA).

Results

1. Identification of a deletion at the ERBB4 locus in the KE37 T-ALL cell line

In order to identify the presence of novel unbalanced genomic rearrangements, we performed array-CGH in 12 T-ALL derived cell lines using a BAC array. The BAC array had an overall resolution of 1 Mb, but included additional BAC probes covering the loci of each of the 90 tyrosine kinase genes, increasing the resolution to about 100 Kb at these loci. Among other abnormalities, we detected a small deletion of ~ 400 Kb at chromosome 2q34 in the KE37 cell line. Of interest, the two corresponding BAC probes RP11-641C18 and RP11-694B12 covered the 5' part of *ERBB4* **(Figure 1a)**.
To determine which exons of *ERBB4* were lost by this deletion, we performed Q-PCR analysis on DNA from KE37, confirming loss of exon 1 of *ERBB4* while the remaining exons were present (data not shown).
Since the *IKZF2* gene is located upstream of *ERBB4* and has the same transcriptional orientation, we hypothesized that loss of material between both genes might fuse them, generating an *IKZF2-ERBB4* fusion gene. Indeed, using RT-PCR, with forward primers located to exon 2 and 3 of *IKZF2* (hemi-nested) and reverse primer to exon 4 of *ERBB4*, we amplified an in frame fusion between exon 3 of *IKZF2* and exon 4 of *ERBB4* **(Figure 1b and 1c)**.

2. Detection of an IKZF2-ERBB4 transcript in T-ALL cell lines and patient samples

We tested for the presence of the *IKZF2-ERBB4* transcript in additional cell lines including a selection of T-ALL, B-ALL, and AML cell lines. The fusion was detected in five of the remaining 14 other independent cell lines (HPB-ALL, SUPT1, DND41 (T-ALL/T-LL), BV173 (B-ALL), SKM1 (AML)). Depending on the location of the primers used for RT-PCR, we amplified different fusion transcripts involving variable exons of *IKZF2* and/or of *ERBB4*. Additionally, SUPT1 showed an insertion between *IKZF2* and *ERBB4* sequences of 51 base pairs (bp) derived from the genomic region present between both genes. In DND41, the fusion between *IKZF2* and *ERBB4* was not in frame due to the presence of a 102 bp region derived from intron 1 of *ERBB4* and containing stop codons in all 3 reading frames **(Figure 1c)**.

We further screened 88 diagnostic T-ALL patient samples for the presence of the *IKZF2-ERBB4* transcript and detected 9 positive cases (≈ 10%). Different variants of the *IKZF2-ERBB4* fusion were observed **(Figure 1c)**. One patient expressed two different variants and another presented with an out-of-frame fusion transcript due to the presence of a 484 bp sequence that was included as an extra exon between *IKZF2* and *ERBB4* exons. Of interest, this 484 bp sequence corresponded to a number of 'cryptic' exons (with correct splice donor and splice acceptor sites) located in the genomic region between *IKZF2* and *ERBB4*. One of these cryptic exons corresponded exactly to the additional sequence found in SUPT1 which was already described as an Expressed Sequence Tag (EST).

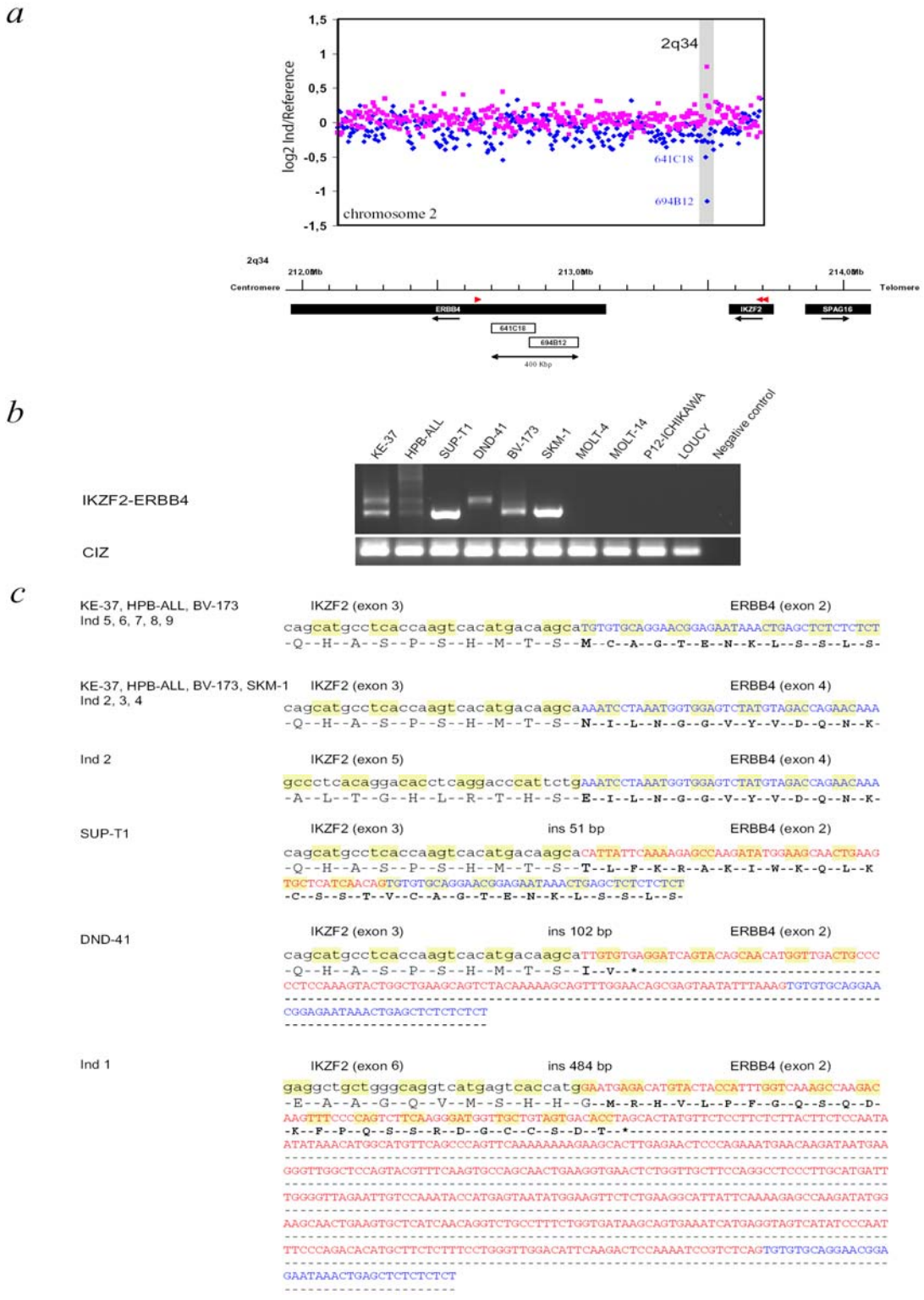


Figure 1 **A.** BAC array based CGH analysis of the KE37 T-ALL cell line. Hybridization signals of chromosome 2 probes are shown. Each dot represents the log2 of the value for KE37 ('Ind') versus a control sample ('Reference') for each probe, ordered based on the probes'chromosomal location. Detection of a deletion of BAC clones RP11-641C18 and RP11-694B12 at the *ERBB4* locus (2q34). **B.** Detection of an *IKZF2-ERBB4* transcript by RT-PCR using forward primers in exon 2 and 3 (hemi-nested) of *IKZF2* combined with a reverse primer in exon 4 of *ERBB4* in different cell lines. **C.** Sequences of the different *IKZF2-ERBB4* transcript variants detected in patients and cell lines.

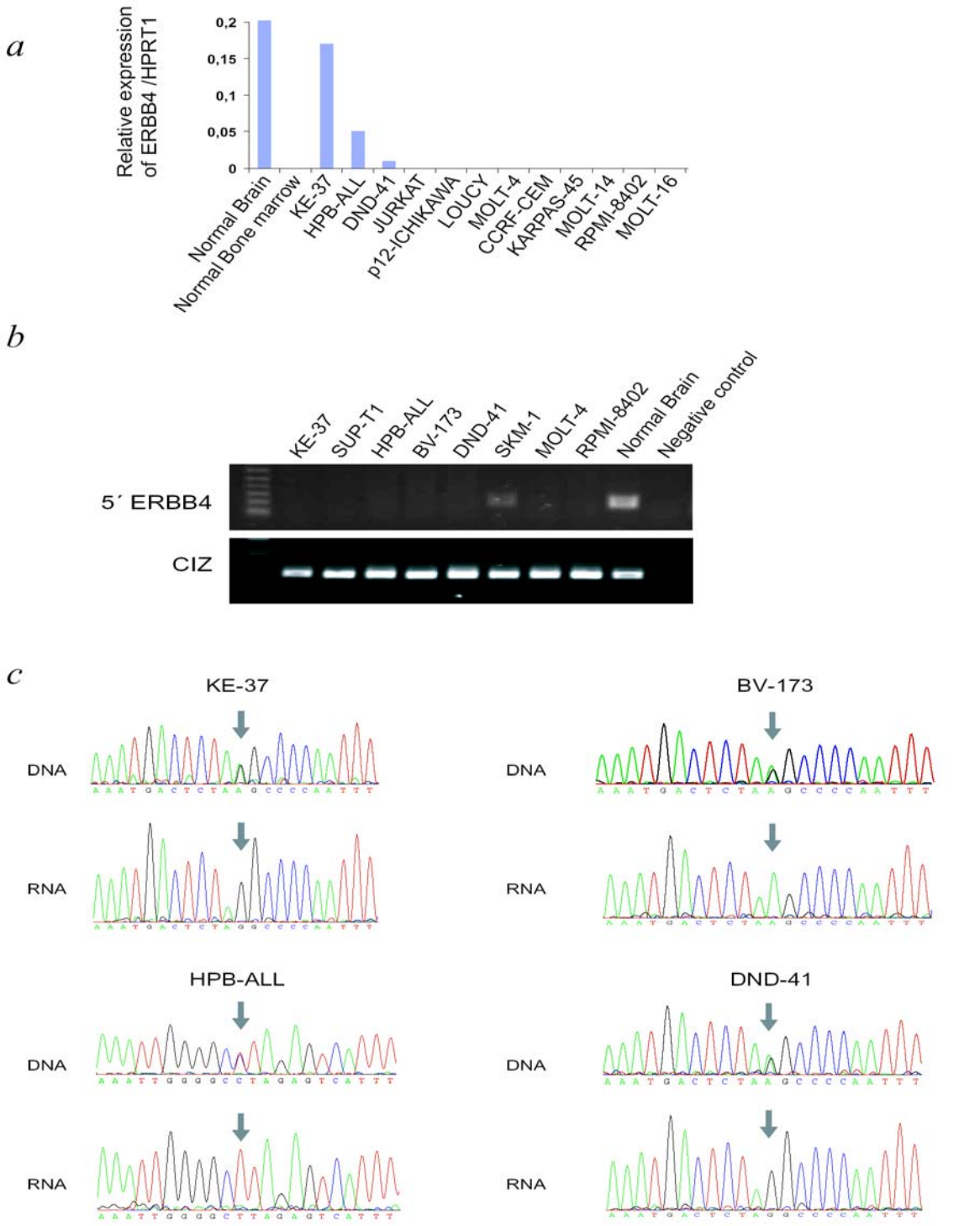


Figure 2 **A.** Expression of *3'ERBB4* by RT-Q-PCR in cell lines positive for the *IKZF2-ERBB4* transcript (KE37, HBP-ALL and DND-41) and absence of expression in control cell lines that do not express the fusion transcript. BV173 expresses also *3'ERBB4* (not shown). Brain is used as positive control and normal bone marrow as negative control. Expression of *3'ERBB4* has been calculated relative to *HPRT1* expression. **B.** Except for the myeloid SKM-1 cell line, absence of detection of *5'ERBB4* (exon 1) by RT-PCR both in cell lines expressing or not expressing the *IKZF2-ERBB4* transcript. **C.** Results of the sequencing of the 3'UTR region of *ERBB4* containing 3 informative SNPs (heterozygosity at genomic DNA level) showing monoallelic expression at cDNA level. Black arrows indicate SNP rs1684599.

Since *ERBB4* is normally not expressed in the bone marrow, detection of expression of the 3' end of *ERBB4* would theoretically be the consequence of the *IKZF2-ERBB4* fusion. Indeed, we demonstrated by RT-Q-PCR that expression of 3'*ERBB4* was restricted to cell lines carrying the *IKZF2-ERBB4* fusion **(Figure 2a)**. To exclude that this might correspond to wild type (WT) expression of *ERBB4*, we also used a primer pair located in exon 1 of *ERBB4 (5'ERBB4)* that is normally lost as a consequence of the fusion. We could not detect expression of *5'ERBB4* in the cell lines except in the myeloid cell line SKM-1 **(Figure 2b)**. Moreover, based on 3 informative SNPs in the 3'UTR region of *ERBB4*, we could demonstrate monoallelic expression of the *IKZF2-ERBB4* fusion in the KE-37, HPB-ALL, BV-173 and DND-41 cell lines, arguing for *IKZF2-ERBB4* being an acquired somatic abnormality rather than the consequence of a defect in splicing events **(Figure 2c)**.

3. Microdeletion supporting the formation of the fusion in KE-37 and HPB-ALL

High resolution array-CGH was carried out using the 244K oligonucleotide array (Agilent) in order to further delineate the deleted sequences in KE37 and to confirm the deletion as the underlying mechanism of the fusion formation in the other cell lines and T-ALL patient samples. This analysis revealed the presence of 2 distinct deletions in the KE37 cell line: one deletion deleting the 3' end of *IKZF2* and the other deleting the 5' end of *ERBB4*. In the HPB-ALL cell line, there was only one deletion comprising the 3' end of *IKZF2* **(Figure 3a)**. Of interest, the common deleted region included the poly(A) signal sequences of *IKZF2*. No genomic defect was detected with the resolution of the 244K array in the other cell lines or T-ALL patients expressing the *IKZF2-ERBB4* fusion transcript.
We confirmed the presence of two separate deletions in KE37 and the unique deletion in HPB-ALL by FISH and Q-PCR (data not shown), and determined their exact breakpoints by sequence analysis **(Figure 3b)**. This showed that the deletions were regular and did not involve inversions or more complex rearrangements in KE37 and HPB-ALL.
In all other cell lines and patient samples, no loss of material was seen by FISH, Q-PCR or array-CGH, strongly suggesting that these samples did not contain deletions similar to those observed in HPB-ALL and KE37, although all expressed the *IKZF2-ERBB4* fusion at the RNA level. In addition, we excluded by FISH the presence of a translocation t(2;2)(q34;q34) as an alternative rearrangement to generate the *IKZF2-ERBB4* fusion (data not shown).
In order to exclude the presence of mutations in the intron-exon boundaries and mutation of the poly(A) signal of *IKZF2* in the samples without obvious deletions, we sequenced these regions, but no mutation were detected. To exclude deep intronic mutations, very small deletions, or any other possible mutations, we sequenced the entire region containing the *IKZF2* and *ERBB4* genes in the BV-173 and SUP-T1 cell lines. We amplified the complete genomic region spanning the *IKZF2* and *ERBB4* genes in these samples by generating overlapping long distance PCR products. We used the 454 sequencing technology to sequence this 600 Kb region. Analysis of this sequence is ongoing.
Finally, we explored the possibility that abnormal expression of transacting splicing factors might be responsible for aberrant splicing between *IKZF2* and *ERBB4* in T-ALL cell lines and patients lacking obvious underlying mechanism for the fusion formation. Careful analysis of 244 array-CGH data (Agilent) revealed, in 3 of four tested *IKZF2-ERBB4* positive patients, decreased hybridization signals at locus 18q12.1 that corresponded to

oligonucleotides covering the *BRUNOL4* gene, encoding an RNA binding splicing factor (data not shown). We could however not confirm expression of *BRUNOL4* in T-ALL cells, and it is described that this region on 18q12 is a copy number variation in the general population.

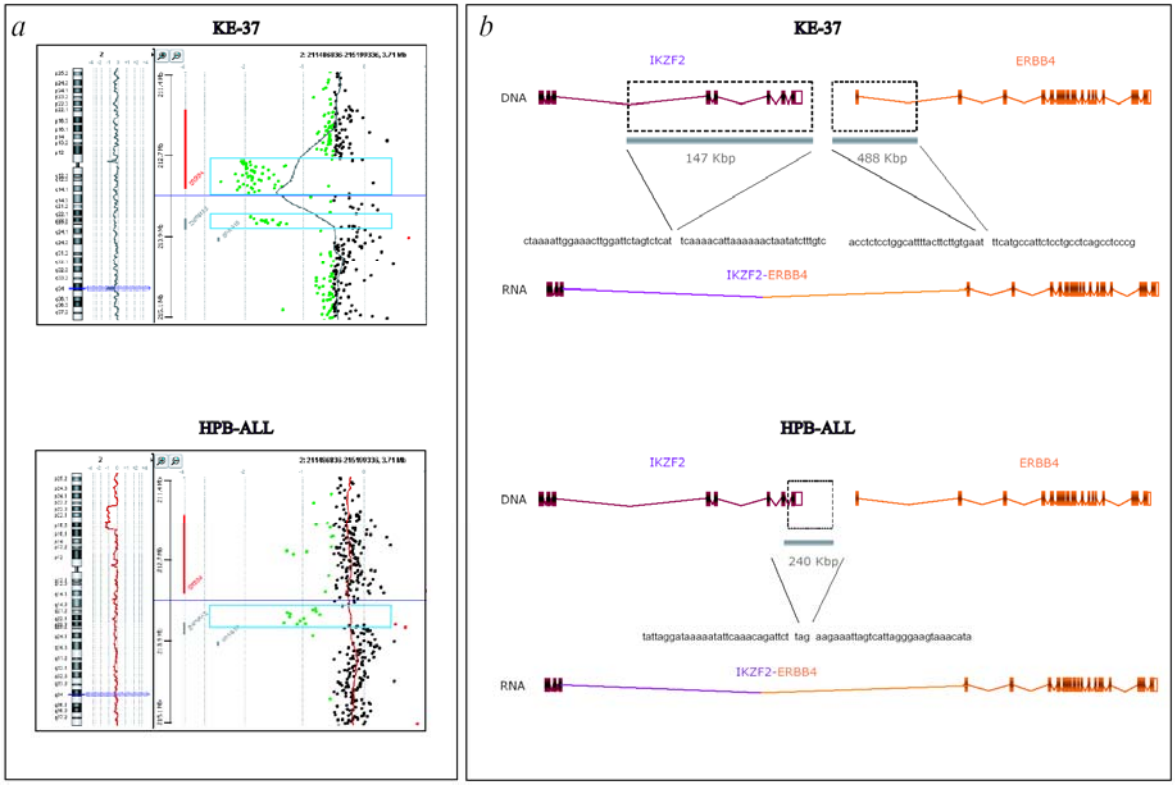
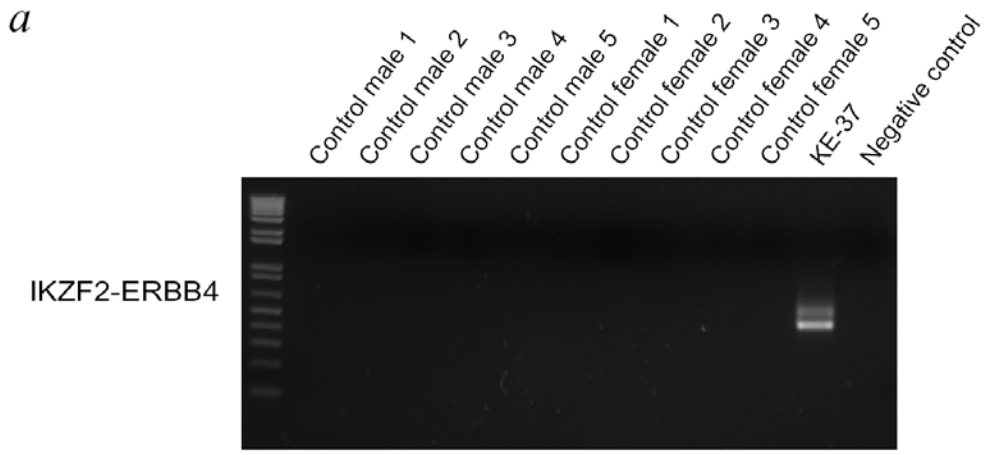


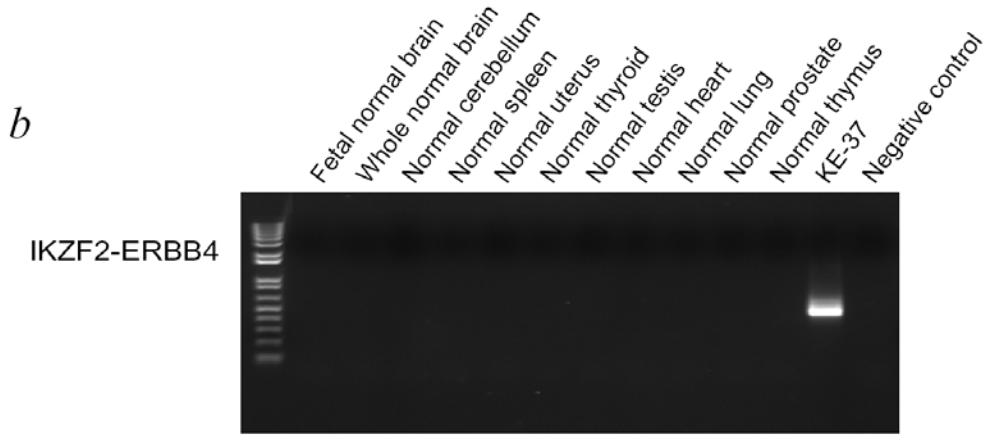
Figure 3 **A.** High resolution 244 array-CGH (Agilent) analyse delineates the deletion found with the BAC array as two separated deleted fragments in KE37 and detects a unique deleted fragment in HPB-ALL. **B.** Sequencing of the intronic breakpoints reveals that, in KE37, the first deletion of 147 Kb of length spans exons 4 to 8 of *IKZF2* and that the second deletion (488 Kb) eliminates the first exon of *IKZF2*. In HPB-ALL the deletion (240 Kb) eliminates exon 8 of IKZF2. The common deleted region includes the poly(A) signal sequences of *IKZF2*.

4. IKZF2-ERBB4 fusion transcript: a transcription induced chimera?

In the absence of an underlying genomic defect that might explain the formation of the *IKZF2-ERBB4* fusion transcript in most cases, we wanted to exclude that the *IKZF2-ERBB4* fusion was a transcription induced chimera normally expressed in some tissues.
We first investigated by RT-PCR a panel of normal human tissues for the presence of the *IKZF2-ERBB4* fusion transcript, but no fusion transcript was detected **(Figure 4a and 4b)**. We also studied human thymic sorted cell subpopulations to determine whether *ERBB4* expression might be present at certain stages of T-cell differentiation. There was no significant expression of *ERBB4* at any stage of thymic cell development (data not shown).
Finally, we examined bone marrow, brain, kidney, spleen and thymus tissues from 5 different strains of mice. We clearly detect at the *Ikzf2-Erbb4* fusion transcript in brain and/or kidney of several mice **(Figure 4c)**. As observed in cell lines and patient samples, different variants of the transcript were detected. In addition, some transcripts showed inserted sequences between *IKZF2* and *ERBB4*. In these normal mouse tissues, *Ikzf2-Erbb4*

was never detected in the absence of wild type Erbb4 expression and *Ikzf2* was found to be expressed in all tissues analyzed. It is important to point out that hemi-nested RT-PCR was needed to reveal amplification of the fusion suggesting that the expression is relatively low, but this also was true for the T-ALL samples and some cell lines.





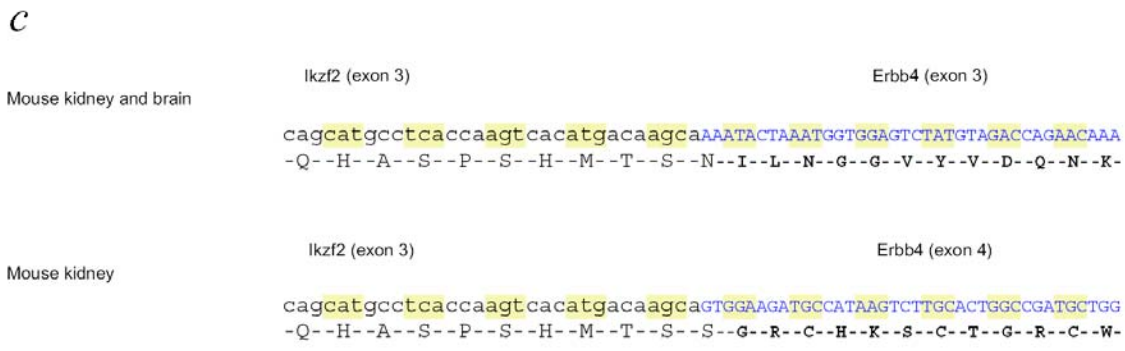


Figure 4 No detection of an *IKZF2-ERBB4* transcript in 10 normal blood samples **(A)** and a panel of normal human tissues **(B)**. **C.** Detection of an *IKZF2-ERBB4* fusion transcript in brain and kidney of mice. Two different transcript variants are shown in kidney and one in brain.

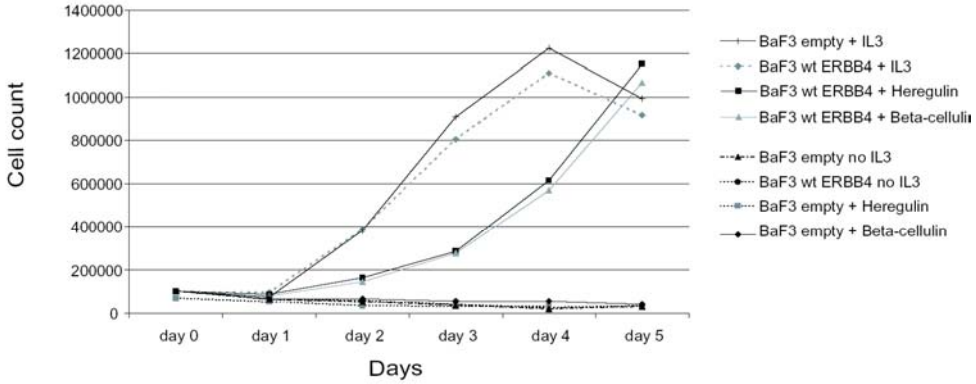
5. No detection of an IKZF2-ERBB4 protein.

In order to detect and further characterise the expected IKZF2-ERBB4 fusion protein (sub-cellular localization, phosphorylation status of the kinase domain of ERBB4, …), we performed western blotting using lysates of cell lines expressing the *IKZF2-ERBB4* fusion transcript. As expected, we could detect wild type IKZF2 with an antibody directed against its N-terminal end, but no signal that might correspond to the IKZF2-ERBB4 fusion protein. Since wild type ERBB4 is normally not expressed in haematological cells, we only expected to detect an IKZF2-ERBB4 fusion protein using an antibody directed against the C-terminal end of ERBB4. In this case also, no fusion protein was detected. Efficiency of the antibody was verified in Ba/F3 cells with enforced expression of wild type ERBB4.

6. Functional consequences of the fusion?

Since no expression of an IKZF2-ERBB4 fusion protein could be demonstrated in cell lines expressing the fusion transcript, we cloned different variants of the *IKZF2-ERBB4* fusion, truncated *ERBB4* and wild type (WT) *ERBB4* transcripts into retroviral vectors for subsequent transduction in Ba/F3 cells to provide detectable expression for functional studies.
We first verified whether WT *ERBB4* alone was able to transform Ba/F3 cells to growth factor independency. We cultured Ba/F3 cells engineered to express WT *ERBB4* in the presence or absence of IL3 and in the presence or absence of the ERBB4 ligands, Neuregulin1 (Heregulin) or Beta-cellulin. Ba/F3 cells expressing WT *ERBB4* were able to grow in the absence of IL3 when the specific ligands were added, suggesting that activation of ERBB4 kinase activity could transform Ba/F3 cells to IL3 independent growth **(Figure 5a)**. We next added the pan-ERBB tyrosine kinase inhibitor CI-1033 to the Ba/F3 cells, and observed an inhibition of the growth of WT *ERBB4* transduced Ba/F3 cells **(Figure 5b)**. These data indicate that the Ba/F3 cell line can be transformed by activated ERBB4 kinase activity.We next cloned different variants of the *IKZF2-ERBB4* fusion into the retroviral MSCV-puro vector. Expression of these *IKZF2-ERBB4* fusions in the Ba/F3 cell line did not result in growth factor independent proliferation. We have to note here that we did not obtain detectable protein expression of the fusions in Ba/F3 cells, while the WT ERBB4 protein was expressed at detectable levels. The lack of clear expression of the fusion proteins prevents us to take definitive conclusions about their oncogenic function in this assay.
Finally, in view of the failure in demonstrating the existence of an IKZF2-ERBB4 protein, we used the pan-ERBB tyrosine kinase inhibitor CI-1033 directly on T-ALL cell lines expressing the *IKZF2-ERBB4* transcript to evaluate the ability of blocking the tyrosine kinase activity of ERBB4 to inhibit their growth. We could not observe a clear inhibitory effect of CI-1033 on the proliferation and survival of the *IKZF2-ERBB4* expressing cell lines (data not shown).

a



b

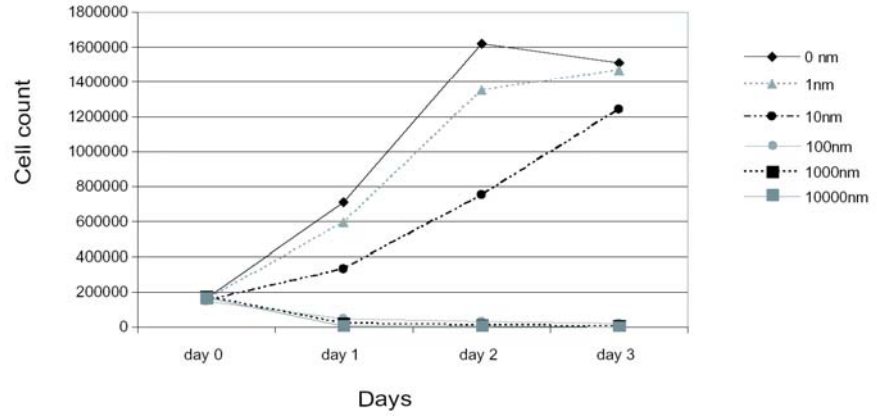


Figure 5 **A.** Growth curves of Ba/F3 cells transfected with wild type *ERBB4* containing retrovirus or with empty retrovirus in presence or absence of IL3 and of the ligands, neuregulin1 or beta-cellulin. Ba/F3 cells transfected with WT *ERBB4* are independent on IL3 for their growth in presence of the specific ligands. **B.** Treatment with the pan-ErbB inhibitor has a dose dependent inhibitory effect on the growth of WT-*ERBB4* transformed Ba/F3 cells cultured in presence of specific ligand but in absence of IL3.

7. Consequences of the fusion on IKZF2 splice variants expression

IKZF2 RNA is normally spliced to produce different mRNA variants. We therefore explored by RT-PCR changes in *IKZF2* splicing patterns in cell lines carrying the *IKZF2-ERBB4* transcript in comparison to patterns in cell lines without the fusion transcript. Using a pair of primers located inside both UTR sequences of *IKZF2*, we could amplify in the KE-37 cell line, a previously unrecognized transcript variant fusing exon 3 to exon 8 **(Figure 6)**. This was confirmed by quantitative RT-PCR (data not shown). There were no significant changes in the splicing pattern of the other cell lines.

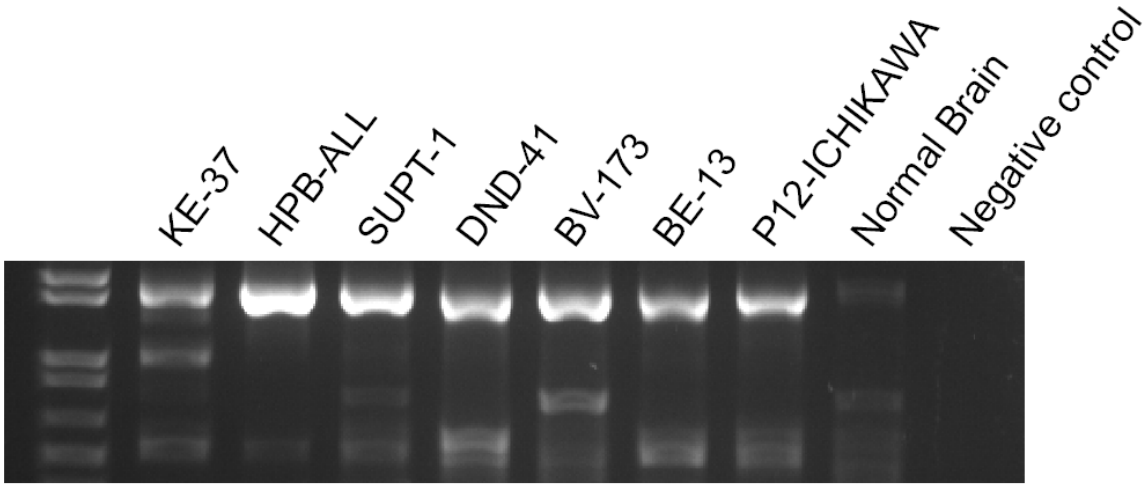


Figure 6 Different transcripts of *IKZF2* in cell lines detected by RT-PCR with primers located in both UTR regions. Sequencing of an additional band in KE-37 (middle band) shows an alternative transcript variant fusing exon 3 to exon 8.

Discussion

We report here a new fusion transcript, *IKZF2-ERBB4*, initially detected in a T-ALL cell line as a consequence of a cryptic deletion of genomic material at the 3' end of *IKZF2*. The *IKZF2-ERBB4* fusion transcript was subsequently also detected in additional T-ALL cell lines, and in about 10 % of T-ALL patients. The mechanism of generation of the fusion gene remains, however, unclear in the majority of cases. Since the fusion gene was also detected in normal tissues, it is possible that the *IKZF2-ERBB4* fusion transcript is a kind of normal splice variant, with currently unknown function. It remains to be answered if its expression in T-ALL plays a role in the pathogenesis of the disease, or its present is a consequence of a general deregulation of splicing factors.
In any case, linking *IKZF2* to *ERBB4* in leukemia may be oncogenic, since both *IKZF2* and *ERBB4* have already been linked to cancer through tumor suppressor and oncogenic properties respectively. This fusion combines loss of *IKZF2* sequences coding for DNA interaction domains of Helios and juxtaposition of the remaining sequences to those of *ERBB4* encoding the kinase domain; two changes that can potentially lead to transformation.

IKZF2 mapped at 2q34 encodes Helios, a member of the Ikaros family of zinc-finger nuclear proteins including also Ikaros and Aiolos encoded by *IKZF1* and *IKZF3*, respectively. Ikaros proteins regulate early developmental decisions and transcription during B- and T-cell development[6,7] and Helios is normally expressed in early double-negative (DN) thymocytes.[8] They all three contain four central zinc fingers for DNA-binding and two carboxy-terminal zinc fingers mediating dimerization between family members **(Figure 7)**. Alternative mRNA splicing leads to the expression of multiple isoforms that are differentially expressed in subsets of hematopoietic cells. There is *in vitro* evidence that isoforms lacking the DNA-binding domain can dimerize with isoforms that retain the ability to bind DNA, inhibiting their transactivation potential. Short Ikaros and Helios isoforms lacking N-terminal zinc fingers have been found to be aberrantly expressed

in B- and T-cell leukemias and to function as a dominant-negative inhibitor of the normal Ikaros transcriptional activity.[9,10,11] More recently, cryptic deletions in IKZF1 associated with the expression of non-DNA binding isoforms of Ikaros have been found in Philadelphia positive ALL and CML blast crisis, demonstrating genomic abnormalities as underlying mechanism.[5] Enforced expression in mice models of non-DNA binding isoforms of Ikaros and Helios leads to the development of leukemia and lymphoma.[12,13]

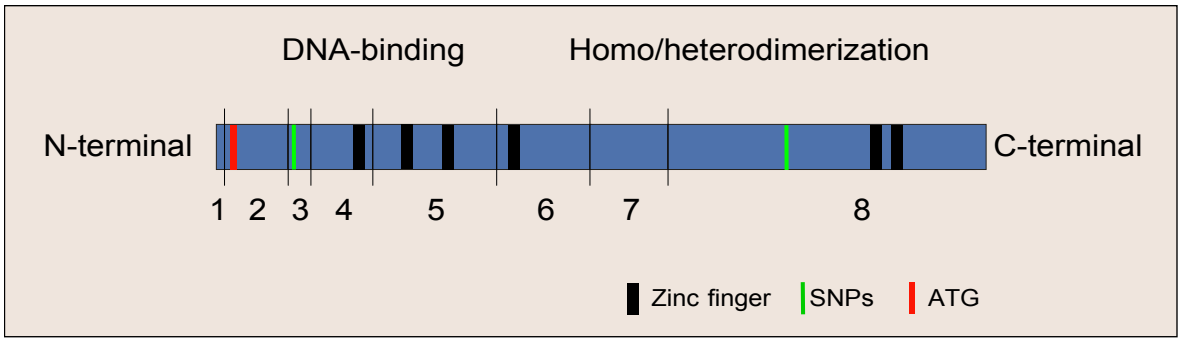


Figure 7 Schematic representation of the IKZF2 protein. The four N-terminal zinc finger domains for DNA interaction and the two carboxy-terminal zinc finger domains for protein dimerization are indicated.

As a consequence of the *IKZF2-ERBB4* fusion, both the DNA binding and the heterodimerization domains encoding sequences of *IKZF2* are disrupted, making it unlikely that the putative IKZF2-ERBB4 fusion protein functions as a dominant negative variant of Ikaros proteins. Moreover, to date, we failed to demonstrate the expression of an IKZF2-ERBB4 protein, suggesting that if protein expression is present, it will be very low. We can however hypothesize that even discrete changes in the balance between the different Helios isoforms may affect its global activity towards loss of tumor suppression function. Of interest, the KE37 cell line express a previously non described splice variant of *IKZF2* fusing exon 3 to exon 8 that therefore lacks sequences coding for DNA-binding domains normally encoded by exons 4 to 6 but keeps intact exon 8 encoding the dimerization domains. The resulting Helios isoform has the potential to act as a dominant negative inhibitor of Ikaros proteins family transcription. Nevertheless, it can not be generated by the *IKZF2-ERBB4* allele as we demonstrated loss of exon 8 sequences with the deletion. It can only be generated by the non fused allele that appears, however, still able to produce the full-length *IKZF2* transcript as observed in the other cell lines.

Another consequence of the *IKZF2-ERBB4* fusion may be the activation of the tyrosine kinase domain of ERBB4 following its juxtaposition to Helios domains. ERBB4 mapped at 2q34 belongs to the ERBB subfamily of receptor tyrosine kinases that consists of four homologous members. Their activation by EGF-like growth factor ligands leads to the formation of homo- or heterodimeric complexes that display different signaling properties regulating cellular responses such as cell proliferation, survival, migration and differentiation.[14] *ERBB1* and *ERBB2* are well described oncogenes targeted by oncogenic somatic mutations is several cancers. *ERBB1 (EGFR, HER1)* is overexpressed in a variety of solid tumors and is a target for therapy with monoclonal antibodies directed against the extracellular domain (cetuximab, Erbitux®) or with ATP competitive tyrosine kinase inhibitors (gefitinib, Iressa® and erlotinib, Tarceva®).[15] *ERBB2 (HER2, c-Neu)* is overexpressed at very high levels in about one fifth of breast cancers and is indicative of

poor prognosis. It is also a target for therapy with monoclonal antibodies (trastuzumab, Herceptin®) and kinase inhibitor (lapatinib, Tykerb®).[16] Involvement of *ERBB3* in cancer is less clear.[26] *ERBB4* plays an important role in the maturation of the mammary gland during lactation but unlike *ERBB2*, its role in breast carcinogenesis is controversial. Some reports suggest that *ERBB4* might promote growth while others favor a role in suppression of growth.[14] Amplification and tyrosine kinase domain mutations of *ERBB4* have been reported in breast cancer without clear assessment of their functional consequences and both overexpression and downregulation of *ERBB4* have been observed.[17] *ERBB4* is transcribed into four transcripts with very different activation mechanisms. The structure of ERBB4 isoforms and downstream signaling are shown and commented on **Figure 8.** This diversity among ERBB4 isoforms and the property to heterodimerize with other members of the ERBB family probably explain the extreme complexity in understanding the biological responses regulated by ERBB4 and the controversy around its oncogenic potential.[18] ERBB4 JM-a CYT-2 isoform has been shown to have more oncogenic potential than the others **(Figure 8)**.[19]
As sequences corresponding to the peptide signal of ERBB4 are lost with the *IKZF2-ERBB4* fusion, surface expression of the putative chimeric protein is not possible and potential oncogenic activity of the chimeric protein has therefore to be independent of ligand binding. We first hypothesized that IKZF2-ERBB4 might carry a constitutive kinase activity even though, unlike other chimeric kinase proteins, no dimerization domain was present in the juxtaposed domains of the fusion partner Helios. We tested whether *IKZF2-ERBB4* expressing cell lines were sensitive to treatment with an pan-ERBB tyrosine kinase inhibitor (CI-1088). Our data indicates that none of the cell lines was inhibited by this drug, demonstrating at least that those cell lines do not depend directly on the tyrosine kinase activity of IKZF2-ERBB4 to proliferate. Failure to reveal expression of an IKZF2-ERBB4 chimeric protein both in cell lines expressing the transcript and in 293T cells or Ba/F3 cells with retroviral enforced expression of the *IKZF2-ERBB4* cDNA, has not allowed further functional analysis of the IKZF2-ERBB4 fusion protein so far. Likewise, its cellular sublocalization could not be determined. An eventual nuclear staining would had been an indication of the role of the fusion protein as a transcription factor as described for the intracellular domain (ICD) of WT ERBB4 **(Figure 8)**.[20]

The mechanism underlying the formation of the *IKZF2-ERBB4* transcript has been clearly identified as the result of a microdeletion in two T-ALL cell lines that moreover showed monoallelic expression of the fusion transcript. For the other cell lines and T-ALL patient samples, the mechanism remains unexplained. Monoallelic expression of *IKZF2-ERBB4* was also demonstrated in DND-41 and BV-173 despite the absence of an obvious underlying denetic defect. Monoallelic expression of *IKZF2-ERBB4* suggests that the fusion transcript could be the result of an undetected acquired somatic abnormality, but does not exclude that the mechanism of transcription induced chimera is at play. Indeed, sporadic reports suggest that chimeric transcripts can be generated from two adjacent genes oriented in the same orientation on the same chromosome.[21,22] Similarly, transplicing between two normal unrelated precursor RNAs (from genes located on different chromosomes) has been shown to give rise to physiological chimeric transcripts. A recent example of such fusion transcript generated by trans-splicing is the *JAZF1-JJAZ1* fusion, which is found in normal cells, but also in tumor cells where it is generated by a chromosomal translocation t(7;17)(p15;q21).[23] In both cases, the splicing and translation of

such “fusion” RNAs can lead to the expression of new proteins with novel functions. It represents an additional mechanism like alternative splicing to generate protein diversity.
While we could not yet detect the *IKZF2-ERBB4* fusion in normal human tissues, we found that the *Ikzf2-Erbb4* fusion was expressed in kidney and brain of healthy mice. In these normal tissues, expression of the *Ikzf2-Erbb4* fusion transcript was always associated with expression of wild type *Erbb4*. This is in sharp contrast with the results of T-ALL samples and cell lines in which wild type *ERBB4* was always absent (except in the myeloid cell line SKM1). Nevertheless, the detection of the *Ikzf2-Erbb4* fusion in normal tissues confirms that this fusion transcript can be generated in normal cells as a kind of alternatively spliced mRNA, either by the mechanism of transcription induced chimera or by trans-splicing. The physiological significance of the fusion transcript remains to be determined. Of interest, *ERBB4* is particularly highly expressed in brain where predominance of some particular isoforms has been linked to susceptibility to schizophrenia.[24]

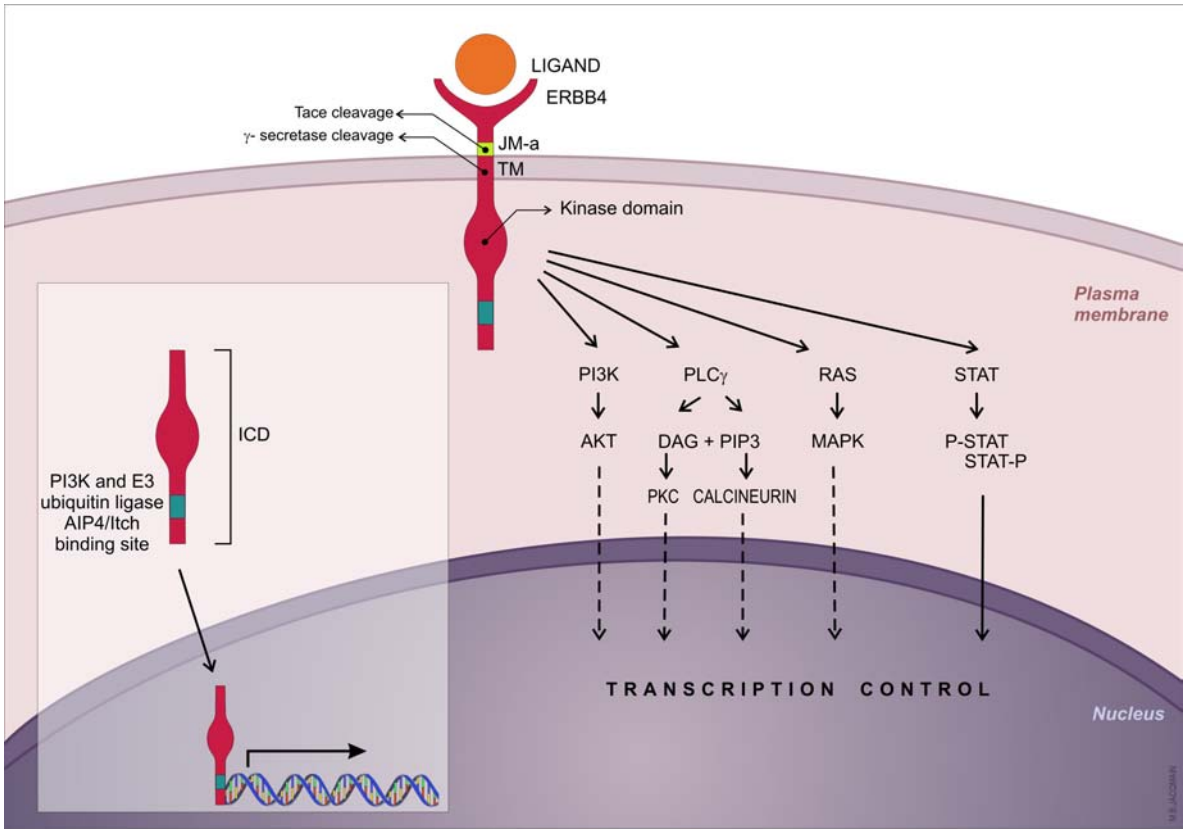


Figure 8 *ERBB4* encodes an extracellular domain for ligand binding, a transmembrane and an intracytoplasmic region containing a kinase domain (encoded by exon 18–23) and a regulatory domain. *ERBB4* pre-mRNA is spliced to produce four structurally, functionally and tissue specific different isoforms. JM-a and JM-b isoforms differ at the juxtamembrane domain by the presence or not of a cleavage site for the tumor necrosis factor a-converting enzyme (TACE); CYT1 and CYT2 isoforms differ at the intracytoplasmic domain by the presence or not of a PI3-K binding site and an interaction motif (PPAY) for the WW domain-containing E3 ubiquitin ligase AIP4/Itch that regulates CYT1 isoform endocytosis, subcellular localization and degradation. Only the JM-a CYT1 isoform is shown. Binding of ERBB4 with various ligands (neuregulin1(heregulin), beta-cellulin, epiregulin and heparin-binding epidermal growth factor) allows sequential proteolytic cleavages of CYT1 isoforms by TACE first, followed by γ-secretase that allows the release of an intracellular domain (ICD) that can move to the nucleus and act as a transcription factor. This mechanism is reminiscent to that used by amyloid precursor and the Notch protein. Beside this direct nuclear pathway of transcription activation depending on a proteolytic processing, a canonical pathway depending on the kinase domain activity links receptor activation to proliferation by the intermediate of the PI3K/AKT, the PLCγ, the RAS/MAP kinase and the STAT pathways.

The presence of cryptic exons in the *IKZF2-ERBB4* fusion transcript in only some cell lines and some patients might indicate that defects in the splicing machinery may allow recognition of those sequences as exons, while they are not recognized as such in other cell lines and patients. We detected copy number variation of *BRUNOL4* in patients based on 244K array-CGH data (Agilent). *BRUNOL4* is a nice candidate of such trans-acting splicing factor of which deletion might influence the splicing pattern of other genes. Indeed, this gene is a member of the CELF family of RNA binding proteins implicated in cell-specific and developmentally regulated alternative splicing. Based on the Database of Genomic Variants, the observed copy number changes of this gene represent copy number variations also observed in the general population.[25,26] Moreover, its expression in the hematopoietic system was particularly weak and the same array profile of deletion has been detected in other samples unrelated to the *IKZF2-ERBB4* deletion.

In conclusion, the *IKZF2-ERBB4* transcript is a new fusion transcript, that can be identified in normal tissues, and is likely to be generated as transcription induced chimera or by the mechanism of trans-splicing. It is also found in a pathologic context as the consequence of a microdeletion occurring at the 3' end of the *IKZF2* gene or following other mechanisms that still remain to be discovered. The expression of the transcript is particularly low as nested RT-PCR was usually required to reveal its expression. The existence of a putative IKZF2-ERBB4 chimeric protein could so far not be confirmed and we can only speculate on the biological or pathological consequences of this fusion gene. We can not rule out that the presence of the fusion transcript in some patients lacking obvious underlying defects might reflect an arrest of differentiation at a stage where the transcript is normally slightly expressed. Further investigations are needed to solve this particularly intriguing enigma.

References

1 Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004; 350: 1535–1548.

2 Harrison CJ, Foroni L. Cytogenetics and molecular genetics of acute lymphoblastic leukemia. *Rev Clin Exp Hematol*. 2002; 6: 91–113.

3 Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC *et al*. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002; 1: 75–87.

4 Pui CH, Jeha S. New therapeutic strategies for the treatment of acute lymphoblastic leukaemia. *Nat Rev Drug Discov*. 2007; 6(2): 149-65.

5 Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, Girtman K, Mathew S, Ma J, Pounds SB, Su X, Pui CH, Relling MV, Evans WE, Shurtleff SA, Downing JR. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007; 446(7137): 758-64.

6 Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science*. 1992; 258(5083): 808-12.

7 Winandy S, Wu L, Wang JH, Georgopoulos K. Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. *J Exp Med*. 1999;190(8): 1039-48.

8 Kelley CM, Ikeda T, Koipally J, Avitahl N, Wu L, Georgopoulos K, Morgan BA. .Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol*. 1998; 8(9): 508-15.

9 Sun L, Crotty ML, Sensel M, Sather H, Navara C, Nachman J, Steinherz PG, Gaynon PS, Seibel N, Mao C, Vassilev A, Reaman GH, Uckun FM. Expression of dominant-negative Ikaros isoforms in T-cell acute lymphoblastic leukemia. *Clin Cancer Res*. 1999; 5(8): 2112-20.

10 Nakase K, Ishimaru F, Fujii K, Tabayashi T, Kozuka T, Sezaki N, Matsuo Y, Harada M. Overexpression of novel short isoforms of Helios in a patient with T-cell acute lymphoblastic leukemia. *Exp Hematol*. 2002; 30(4): 313-7.
11 Tabayashi T, Ishimaru F, Takata M, Kataoka I, Nakase K, Kozuka T, Tanimoto M. Characterization of the short isoform of Helios overexpressed in patients with T-cell malignancies. *Cancer Sci*. 2007; 98(2): 182-8.
12 Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell*. 1995; 83(2): 289-99.
13 Zhang Z, Swindle CS, Bates JT, Ko R, Cotta CV, Klug CA. Expression of a non-DNA-binding isoform of Helios induces T-cell lymphoma in mice. *Blood*. 2007;109(5): 2190-7.
14 Gullick WJ. c-erbB-4/HER4: friend or foe? *J Pathol*. 2003; 200(3): 279-81.
15 Milano G, Spano JP, Leyland-Jones B. EGFR-targeting drugs in combination with cytotoxic agents: from bench to bedside, a contrasted reality. *Br J Cancer*. 2008; 99(1): 1-5.
16 Azim H, Azim HA Jr. Targeting Her-2/neu in breast cancer: as easy as this! *Oncology*. 2008; 74(3-4):150-7.
17 Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi OP, Elenius K. Role of ErbB4 in breast cancer. *J Mammary Gland Biol Neoplasia*. 2008; 13(2): 259-68.
18 Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001; 2(2): 127-37.
19 Määttä JA, Sundvall M, Junttila TT, Peri L, Laine VJ, Isola J, Egeblad M, Elenius K. Proteolytic cleavage and phosphorylation of a tumor-associated ErbB4 isoform promote ligand-independent survival and cancer cell growth. *Mol Biol Cell*. 2006; 17(1): 67-79.
20 Schlessinger J, Lemmon MA. Nuclear signaling by receptor tyrosine kinases: the first robin of spring. *Cell*. 2006; 127(1): 45-8.
21 Parra G, Reymond A, Dabbouseh N, Dermitzakis ET, Castelo R, Thomson TM, Antonarakis SE, Guigó R. Tandem chimerism as a means to increase protein complexity in the human genome. *Genome Res*. 2006; 16(1): 37-44.
22 Akiva P, Toporik A, Edelheit S, Peretz Y, Diber A, Shemesh R, Novik A, Sorek R. Transcription-mediated gene fusion in the human genome. *Genome Res*. 2006; 16(1): 30-6.
23 Li H, Wang J, Mor G, Sklar J. A neoplastic gene fusion mimics trans-splicing of RNAs in normal human cells. *Science*. 2008; 321(5894): 1357-61.
24 Law AJ, Kleinman JE, Weinberger DR, Weickert CS. Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. *Hum Mol Genet*. 2007; 16(2): 129-41.
25 Good PJ, Chen Q, Warner SJ, Herring DC. A family of human RNA-binding proteins related to the Drosophila Bruno translational regulator. *J Biol Chem*. 2000; 275(37): 28583-92.
26 Meins M, Schlickum S, Wilhelm C, Missbach J, Yadav S, Gläser B, Grzmil M, Burfeind P, Laccone F. Identification and characterization of murine Brunol4, a new member of the elav/bruno family. *Cytogenet Genome Res*. 2002; 97(3-4): 254-60.
27 Burgess AW. EGFR family: structure physiology signalling and therapeutic targets. *Growth Factors*. 2008; 26(5): 263-74.

IV Discussion and future perspectives

Many genetic abnormalities involved in cancer have been identified by analyzing the breakpoints of newly discovered translocations or by determining the gene content of deleted/amplified chromosomal regions.
In this work, we started from the observation of an *ABL1* gene amplification discovered during routine FISH examination of a T-ALL sample. The screening of additional samples confirmed the recurrent nature of this abnormality as 6% of the T-ALL cases harbored such an *ABL1* amplification. The same observation has been subsequently reported by the team of Ch. Harrison on an independent series of T-ALL.[260] We demonstrated that this *ABL1* amplification was not restricted to this gene but also involved the two adjacent genes *LAMC3* and *NUP214*. Since only the 3' part of *ABL1* and the 5' part of *NUP214* were amplified, we suspected that both genes might be involved in the formation of a fusion. We hypothesized that the small DNA fragment between *ABL1* and *NUP214* might circularize, forming both an episome and a fusion between the *5'NUP214* and *3'ABL1*, as the simplest way to explain why *3'ABL1*, *LAMC3* and *5'NUP214* were co-amplified as extrachromosomal FISH signals in the absence of cytogenetically visible structure to support this amplification. We confirmed this hypothesis by demonstrating the existence of a *NUP214-ABL1* transcript in those patients initially detected with *ABL1* amplification. The transcript was translated into a chimeric protein with characteristics of an activated tyrosine kinase, as both the NUP214-ABL1 protein and its direct downstream ligand CRKL were constitutively phosphorylated. Finally, we demonstrated that the growth of the *NUP214-ABL1* containing T-ALL cell-lines ALL-SIL can be inhibited by the use of the tyrosine kinase inhibitor (TKI) imatinib that specifically targets the kinase domain of ABL1 conserved in the NUP214-ABL1 chimeric protein.
This work illustrates how a single observation can lead to important insights in the molecular genetics of a disease than can be further translated into potential clinical improvement. These *ABL1* FISH amplification, which might have been considered as background signal before, represents in fact a new fusion gene and a new mechanism of creation of a fusion. This underlines the importance of case report oriented research alongside more objective oriented research.

In the setting of a multicentric collaboration, we collected and characterized 27 additional *NUP214-ABL1* T-ALL cases in order to gain more insight into the contribution of this abnormality in the transformation of leukemic cells and attempt to evaluate the interest in targeting it with kinase inhibitors like imatinib.
Our data revealed an important heterogeneity of the genomic presentation of the *NUP214-ABL1* amplification. Indeed, the amplification was not only supported by extrachromosomal episomes as initially reported but it also presented in some cases as a clustered intrachromosomal amplification forming small homogeneously staining region (hsr). In one patient, hsr became the only form of amplification after several relapses. Hsr was also the only presentation in both *NUP214-ABL1* positive T-ALL cell lines analyzed. Additionally, we observed that when the *NUP214-ABL1* amplification was present in a major clone, cells tended to have accumulated more episomes per nuclei than when present in minor clones. These data allowed us to present a model in which the formation of the episome is the primary event generating the *NUP214-ABL1* fusion gene. In absence of a centromeric structure, episomes segregate unequally during cell division and cells

containing the highest number of *NUP214-ABL1* fusion containing episomes are preferentially selected. After this amplification step, in some cases, reintegration of episomes into the genome (hsr) may occur, conferring more stability to the fusion through the successive rounds of cell division.

Amplification of the *NUP214-ABL1* fusion gene was the hallmark of all cases, confirming the data published by K. De Keersmaecker showing that *NUP214-ABL1* has weaker transforming properties compared to other *ABL1* fusions.[234,235] *NUP214-ABL1* needs therefore to be amplified to reach clinically relevant oncogenic activity. In apparent contradiction with this, one patient presented with an intrachromosomal insertion of one *NUP214-ABL1* containing episome without evidence of amplification. We postulated that in this case reintegration might have placed the fusion in the vicinity of a strong promoter increasing the transcription rate of the fusion as an alternative for fusion amplification.

Another important observation from this FISH study was the presence of the *NUP214-ABL1* fusion, only in a small subset of the blast cells in some cases. The presence of subclones seems to contradict the hypothesis that acquisition of *NUP214-ABL1* might be responsible for the transition from a latent to a proliferative and aggressive leukemic clone. However, we can speculate that in those cases, another kinase mutation might be responsible for the proliferation of the majority of the cells and that further expansion of the *NUP214-ABL1* clone following selection of cells with the highest number of episomes might eventually supplant this, becoming in turn the major clone.

Presence of subclones has several implications. Firstly, very small subclones can escape FISH detection, being only disclosed by RT-PCR techniques. It allows us to recommend the combination of both techniques for the screening of the *NUP214-ABL1* fusion at diagnosis. Secondly, it raises the question of the interest in therapeutic targeting specifically NUP214-ABL1 when present in a very small clone only. As the *NUP214-ABL1* containing episomes have the property to easily self-amplify, such subclones probably represent a population of cells more susceptible to induce relapse. Of interest, in our series, *NUP214-ABL1* was almost always remained present at the time of relapse. Therefore, drugs targeting NUP214-ABL1 may have therapeutic potential in this subgroup of T-ALL patients whatever the importance of the NUP214-ABL1 positive clone. Thirdly, the presence of the *NUP214-ABL1* amplification in subclones strongly argues in favor of the secondary nature of this fusion. This is also suggested by the observation of cases with *TLX1/TLX3* expression and *p16* deletion in which the *NUP214-ABL1* fusion represents only a small subclone.

As *NUP214-ABL1* appears to be a relatively late event in the multistep process of T-ALL leukemogenesis, tyrosine kinase inhibitors would have to be administered in addition to drugs which target the earlier genetic defects to avoid relapse from latent pre-leukemic clones. At the present time, chemotherapy only can achieve this goal but we would expect that, in the future, combinations of tyrosine kinase inhibitors with drugs targeting more specifically the other leukemogenic hits will contribute towards decreasing the toxicity of the treatments. The situation with *NUP214-ABL1* in T-ALL is relatively different to that observed in *BCR-ABL1* positive B-ALL where the fusion represents a primary abnormality allowing treatment of patients with a combination of TKI and less intensive chemotherapy. Moreover, in *NUP214-ABL1* positive T-ALL, selection of cells having accumulated the greatest number of episomes represents an intrinsic mechanism of resistance to TKI. Finally, the schedule of administration of tyrosine kinase inhibitors with respect to the delivery of chemotherapy may be of great importance, as TKI may potentially decrease the

number of cycling cells available to incorporate the cytotoxic drugs and thus decrease their efficacy, as experienced in early trials with FLT3 inhibitors in AML.
A recent encouraging paper reports rapid complete cytogenetic remission after upfront dasatinib tyrosine kinase inhibitor monotherapy in a patient with a *NUP214-ABL1* positive T-ALL. He presented initially with an acute abdomen rending him not suitable for classical induction therapy. After having recovered from surgery and obtained remission with dasatinib, he received the classical chemotherapeutic drugs and eventually underwent bone marrow transplantation.[261]

During our initial screening of T-ALL samples with an *ABL1* FISH probe, we found 2 cases showing a split of one signal corresponding to the presence of a breakpoint in *ABL1*. In one patient, it resulted from an inversion of chromosome nine as detected by conventional cytogenetics. By FISH, we mapped the other breakpoint on 9p slightly more telomeric than *PAX5*, but were unable to amplify an *ABL1* fusion transcript by RACE-PCR (Graux C *et al*., BHS 2004. Poster).
FISH analysis of the second case identified a new cryptic translocation t(9;14)(q34;q32). In this case RACE-PCR revealed an *EML1-ABL1* transcript. Like the *NUP214-ABL1* transcript, *EML1-ABL1* was translated into a chimeric protein with constitutive tyrosine kinase activity sensitive to the ABL1 tyrosine kinase inhibitor imatinib. Of interest, this patient, like the *NUP214-ABL1* cases also exhibited additional mutations such as *p16* deletion, *NOTCH1* mutation and *TLX1* expression supporting the view that T-cell leukemogenesis is a multistep process involving mutations affecting cell cycle control, cell-renewal, differentiation and proliferation. Unfortunately, after having achieved clinical remission, this patient relapsed 27 months after diagnosis. Compliance to treatment had been mediocre. At relapse, the leukemic cells had lost *EML1-ABL1* positivity but had acquired instead *NUP214-ABL1* positivity while the other cooperating mutations (*TLX1*, *p16* and *NOTCH1*) initially found were still present. Retrospective analysis of remission samples revealed that, in fact, *TLX1* positivity was still present in spite of the apparent clinical remission but that neither *EML1-ABL1* nor *NUP214-ABL1* were detectable. This observation suggests that *NUP214-ABL1* and *EML1-ABL1* are secondary and late events that are able to transform a latent preleukemic clone into a highly proliferative clone responsible for a clinically overt leukemia.

Finally, the screening of T-ALL samples for the presence of *ABL1* amplification led us to discover other abnormalities of the 9q34 region. Indeed, 10% of screened patients presented a trisomy of the 9q34 region. In those cases, the whole region was amplified and did not seem to be involved in translocation phenomena. The amplified region comprised not only the entire *ABL1* gene but also other oncogenes like *VAV2*, *TRAF2*, *NOTCH1,* all being involved in normal and pathological T-cell development (Graux C *et al*., BHS 2004. Poster). The same observation has been subsequently reported by the group of Rotterdam that used array-comparative genomic hybridization (array-CGH) as screening method for the detection of new genomic abnormalities in childhood T-ALL.[97] They found that 33% of cases harbored a 9q34 amplification of variable size. FISH characterization revealed 9q34 duplication on one chromosome, similar to what we found in our cases. However, increased expression of *ABL1*, *VAV2*, *TRAF2* or *NOTCH1* as a consequence of gene dosage could not be demonstrated. This could be related to the fact that 9q34 duplication was present in minor clones only and that it was therefore technical almost impossible to prove activation

of specific genes located in the common duplicated region. Three genes, *MRLP41*, *SSNA1*, *PHPT1* not previously related to cancer where however significantly overexpressed. The link between 9q34 duplication and oncogenesis has not been established. It probably corresponds to a marker of progression of the disease more than an initiating leukemogenic event.
Among more than 400 T-ALL patients screened in this work, we did not detect other *EML1-ABL1* fusions or additional *ABL1* fusion variants, except two *BCR-ABL1* cases, making *NUP214-ABL1* the most frequent *ABL1* fusion in T-ALL.

The genetic defect responsible for growth factor independent proliferation remains unknown in about 60-70% of T-ALL cases. *NUP214-ABL1* in T-ALL and *FIP1L1-PDGFRa* in chronic eosinophilic leukemia (CEL) are examples of tyrosine kinase fusion genes that can be revealed following gain or loss of genomic material, respectively. We therefore performed array-CGH screening of T-ALL cell-lines in order to identify novel unbalanced genomic rearrangements that could be responsible for the activation of other tyrosine kinases. Among other abnormalities, we detected in a cell line a small deletion of ~ 400 kb responsible for a fusion between the flanking genes, *IKZF2* and *ERBB4*. We suspected that this fusion might result in the production of a chimeric protein with constitutive activation of the tyrosine kinase domain of ERBB4. Unfortunately, we never could detect the hypothetical IKZF2-ERBB4 protein. In addition, if a deletion was clearly the cause of the expression of the *IKZF2-ERBB4* fusion transcript in 2 T-ALL cell lines, in other positive cell lines and T-ALL patients expressing the transcript, no underlying defect could be observed. Sporadic reports suggest that chimeric transcripts can occur in human as a consequence of the transcription of two adjacent genes oriented in the same orientation into one single RNA.[262,263] Similarly, transplicing between two normal unrelated precursor RNAs has been shown to give rise to physiological chimeric transcripts.[264] The splicing and translation of such RNAs can lead to a new, fused protein having domains from both original proteins, representing an additional mechanism to generate protein diversity. Accordingly, we could demonstrate that the *IKZF2-ERBB4* transcript was present in normal kidney and brain tissues of different strains of mice. In this case, expression of the *IKZF2-ERBB4* fusion was systematically detected together with the wild type *ERBB4* transcript, supporting the hypothesis that *IKZF2-ERBB4* might exist in some tissues as a kind of alternative splicing between both genes. It was not the case in the context of T-ALL samples and cell lines where wild type *ERBB4* was no detectable. It argues for *IKZF2-ERBB4* being an acquired abnormality in those cases. Of interest, *ERBB4* is particularly highly expressed in brain where predominance of some particular isoforms has been linked to susceptibility to schizophrenia.[265]
We hypothesized that aberrant expression of the *IKZF2-ERBB4* transcript in an inappropriate context might be oncogenic. Both *IKZF2* and *ERBB4* have been already linked to cancer through tumor suppressor and oncogenic properties, respectively. The *IKZF2-ERBB4* fusion combines loss of sequences coding for DNA interaction domains of IKZF2 and juxtaposition of the remaining sequences to those encoding the kinase domain of ERBB4, two changes that can potentially lead to transformation. Further investigations are needed to understand the mechanisms of fusion creation in cases expressing the transcript in absence of obvious underlying defect and, if exists, to formally determine the potential oncogenic role of this fusion protein by establishing the contribution of loss and gain of function of *IKZF2* and *ERBB4* respectively.

In conclusion, the understanding of the molecular mechanisms underlying neoplastic transformation of hematopoietic cells has greatly improved during the last decade, and this is particularly true for T-cell leukemogenesis.
In the introduction, we attempted to review in a comprehensive way the numerous genetic defects described in T-cell acute lymphoblastic leukemia by paralleling them with the normal T-cell development. Thymopoieis requires a fine regulation of self-renewal, differentiation, proliferation, survival and death signals and it is now clear that a combination of mutations targeting each of those different cellular processes is required to give rise to a clinically overt leukemia.

In this study, we identified fusion genes involving the ABL1 tyrosine kinase as novel oncogenes in T-ALL. Anomalies leading to tyrosine kinase activation such as *NUP214-ABL1* and *EML1-ABL1* or the recently described *JAK1* mutations provide cells with a proliferation advantage. However, these lesions are detected in only a small proportion of T-ALL cases. Therefore it is important to continue our search for additional similar mutations in the remaining cases since they represent interesting targets for therapy with tyrosine kinase inhibitors.
One potential problem associated with targeting these tyrosine kinases is their relatively late occurrence during the leukemic process and the systematic presence of associated genetic lesions, as demonstrated in this work for *NUP214-ABL1* and *EML1-ABL1*. In addition, in the case of *NUP214-ABL1*, the episomal amplification represents an intrinsic mechanism of resistance to tyrosine kinase inhibitors. These observations suggest that the future probably resides in combining drugs that target the different hits in a tumor-specific way. Currently, most "specific" therapies available or in development and that are potentially useful for the treatment of T-ALL, target secondary cooperating mutations or pathways regulating growth or apoptosis (ABL1/SRC, FLT3, JAK and farnesyl transferase inhibitors, …). The next step will be to determine how those drugs can be combined with chemotherapy or other agents in a way that takes into account the different hits involved in the pathogenesis and maintenance of each T-ALL subtype. The final aim is to improve efficacy and decrease toxicity of the treatments. The success of such strategy will depend on its ability to target the leukemic stem cell. If not targeted by the treatment, the leukemic stem cell will persist as a source of relapse, and disease control will probably require long term intake of a combination of drugs impacting on quality of life and health budgets.

Another major challenge will be the translation of data on sensitivity to new drugs obtained in cell lines into clinical trials. As T-ALL is a rare disease, the randomized evaluation of the benefits of new therapeutic options specific for T-ALL subgroups will be difficult, especially in children in whom remission rate with current chemotherapeutic based treatments is already high. Moreover, numerous therapeutic options will have to be tested. These limitations underline the need for cooperation between cooperative groups in order to establish a global strategy that takes all aspects into account, including the economic ones.

V References

1 Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. *CA Cancer J Clin*. 2001; 51(1): 15-36.

2 Xie Y, Davies SM, Xiang Y, Robison LL, Ross JA. Trends in leukemia incidence and survival in the United States (1973-1998). *Cancer*. 2003; 97(9): 2229-35.

3 Groves FD, Linet MS, Devesa SS. Patterns of occurrence of the leukaemias. *Eur J Cancer.* 1995; 31A: 941–949.

4 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976; 33(4): 451-8.

5 Jaffe E, Harris N, Stein H, Wardiman J. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.* IARC Press: Lyon, 2001, p115-117.

6 Wong S, Witte ON. The BCR-ABL story: bench to bedside and back. *Annu Rev Immunol* 2004; 22: 247–306.

7 Taagepera S, McDonald D, Loeb JE, Whitaker LL, McElroy AK, Wang JY, Hope TJ. Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. *Proc Natl Acad Sci U S A*. 1998; 95(13): 7457-62.

8 Sawyers CL, McLaughlin J, Goga A, Havlik M, Witte O. The nuclear tyrosine kinase c-Abl negatively regulates cell growth. *Cell*. 1994; 77(1): 121-31.

9 Van Etten RA.Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol*. 1999; 9(5): 179-86.

10 de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*. 1982; 300(5894): 765-7.

11 Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004; 350: 1535–1548.

12 Soupir CP, Vergilio JA, Dal Cin P, Muzikansky A, Kantarjian H, Jones D, Hasserjian RP *et al.* Philadelphia chromosome-positive acute myeloid leukemia: a rare aggressive leukemia with clinicopathologic features distinct from chronic myeloid leukemia in myeloid blast crisis. *Am J Clin Pathol* 2007; 127(4): 642-50.

13 Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B. Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction. *Blood*. 1996; 88(7): 2410-4.

14 Quentmeier H, Cools J, MacLeod RA, Marynen P, Uphoff CC, Drexler HG *et al.* e6-a2 BCR-ABL1 fusion in T-cell acute lymphoblastic leukemia. *Leukemia* 2005; 19: 295–296.

15 Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996; 88(7): 2375–84.

16 Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat Rev Cancer*. 2005 Mar;5(3):172-83.

17 McWhirter JR, Galasso DL, Wang JY. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol Cell Biol*. 1993; 13(12): 7587-95.

18 He Y, Wertheim JA, Xu L, Miller JP, Karnell FG, Choi JK, Ren R, Pear WS. The coiled-coil domain and Tyr177 of bcr are required to induce a murine chronic myelogenous leukemia-like disease by bcr/abl. *Blood*. 2002; 99(8): 2957-68.

19 Gishizky ML, Cortez D, Pendergast AM. Mutant forms of growth factor-binding protein-2 reverse BCR-ABL-induced transformation. *Proc Natl Acad Sci U S A*. 1995; 92(24): 10889-93.

20 Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood*. 2000; 96(10): 3343-56.

21 Hantschel O, Wiesner S, Güttler T, Mackereth CD, Rix LL, Mikes Z, Dehne J, Görlich D, Sattler M, Superti-Furga G.Structural basis for the cytoskeletal association of Bcr-Abl/c-Abl. *Mol Cell*. 2005; 19(4): 461-73.

22 Dierov J, Dierova R, Carroll M. BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. *Cancer Cell*. 2004; 5(3): 275-85.

23 Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF. Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature*. 1987; 328(6128): 342-4.

24 Li S, Ilaria RL Jr, Million RP, Daley GQ, Van Etten RA. .The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have different lymphoid leukemogenic activity. *J Exp Med*. 1999; 189(9): 1399-412.

25 Schenk TM, Keyhani A, Bottcher S, Kliche KO, Goodacre A, Guo JQ, Arlinghaus RB, Kantarjian HM, Andreeff M. Multilineage involvement of Philadelphia chromosome positive acute lymphoblastic leukemia. *Leukemia*. 1998; 12(5): 666-74.

26 Haferlach T, Winkemann M, Ramm-Petersen L, Meeder M, Schoch R, Weber-Matthiesen K, Schlegelberger B, Schoch C, Ludwig WD, Hiddemann W, Löffler H. New insights into the biology of Philadelphia-chromosome-positive acute lymphoblastic leukaemia using a combination of May-Grünwald-Giemsa staining and fluorescence in situ hybridization techniques at the single cell level. *Br J Haematol*. 1997; 99(2): 452-9.

27 Martinelli G, Amabile M, Giannini B, Terragna C, Ottaviani E, Soverini S, Saglio G, Rosti G, Baccarani M. Novel types of bcr-abl transcript with breakpoints in BCR exon 8 found in Philadelphia positive patients with typical chronic myeloid leukemia retain the sequence encoding for the DBL- and CDC24 homology domains but not the pleckstrin homology one. *Haematologica*. 2002; 87(7): 688-94.

28 Goldman JM, Melo JV. Chronic myeloid leukemia--advances in biology and new approaches to treatment. *N Engl J Med*. 2003; 349(15): 1451-64.

29 Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell*. 2002; 2(2): 117-25.

30 Druker BJ, Guilhot F, O'Brien SG, *et al*. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *NEngl J Med*. 2006; 355: 2408-2417.

31 Weisberg E, Manley PW, Breitenstein W, Brüggen J, Cowan-Jacob SW, Ray A, Huntly B, Fabbro D, Fendrich G, Hall-Meyers E, Kung AL, Mestan J, Daley GQ, Callahan L, Catley L, Cavazza C, Azam M, Neuberg D, Wright RD, Gilliland DG, Griffin JD. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell*. 2005; 7(2): 129-41.

32 Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science*. 2004; 305(5682): 399-401.

33 Giles FJ, Cortes J, Jones D, Bergstrom D, Kantarjian H, Freedman SJ. MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood*. 2007; 109(2): 500-2.

34 Tonegawa S. Somatic generation of antibody diversity. *Nature.* 1983; 302: 575–581.

35 Garcia KC, Teyton L, Wilson IA. Structural basis of T cell recognition. *Annu Rev Immunol.* 1999; 17: 369–397.

36 Res P, Martinez-Caceres E, Cristina JA, Staal F, Noteboom E, Weijer K *et al*. CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood*. 1996; 87: 5196–5206.

37 Plum J, De Smedt M, Leclercq G, Verhasselt B, Vandekerckhove B. Interleukin-7 is a critical growth factor in early human T-cell development. *Blood*. 1996; 88: 4239–4245.

38 Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003; 426(6967): 671-6.

39 Carrasco YR, Navarro MN, de Yebenes VG, Ramiro AR, Toribio ML. Regulation of surface expression of the human pre-T cell receptor complex. *Semin Immunol.* 2002; 14: 325–334.

40 Blom B, Verschuren MC, Heemskerk MH, Bakker AQ, Gastel-Mol EJ, Wolvers-Tettero IL *et al*. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. *Blood.* 1999; 93: 3033–3043.

41 Spits H. Development of alphabeta T cells in the human thymus. Nat Rev *Immunol*. 2002; 2: 760–772.

42 von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. *Annu Rev Immunol.* 1997; 15: 433–452.

43 Kruisbeek AM, Haks MC, Carleton M, Michie AM, Zuniga-Pflucker JC, Wiest DL. Branching out to gain control: how the pre-TCR is linked to multiple functions. *Immunol Today*. 2000; 21: 637–644.

44 Blom B, Spits H. Development of human lymphoid cells. *Annu Rev Immunol.* 2006; 24: 287–320.

45 Murre C. Intertwining proteins in thymocyte development and cancer. *Nat Immunol.* 2000; 1: 97–98.

46 Engel I, Murre C. E2A proteins enforce a proliferation checkpoint in developing thymocytes. *EMBO J.* 2004; 23: 202–211.

47 Ciofani M, Zúñiga-Pflücker JC. The thymus as an inductive site for T lymphopoiesis. *Annu Rev Cell Dev Biol*. 2007; 23: 463-93.

48 Allen RD 3rd, Bender TP, Siu G. c-Myb is essential for early T cell development. *Genes Dev*. 1999; 13(9): 1073-8.

49 Aifantis I, Mandal M, Sawai K, Ferrando A, Vilimas T. Regulation of T-cell progenitor survival and cell-cycle entry by the pre-T-cell receptor. *Immunol Rev*. 2006; 209:159-69.

50 Haks MC, Lefebvre JM, Lauritsen JP, Carleton M, Rhodes M, Miyazaki T, Kappes DJ, Wiest DL. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity*. 2005; 22(5): 595-606.

51 Hayes SM, Li L, Love PE. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity*. 2005; 22(5): 583-93.

52 Pénit C, Lucas B, Vasseur F. Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *J Immunol*. 1995; 154(10): 5103-13.

53 Hoffman ES, Passoni L, Crompton T, Leu TM, Schatz DG, Koff A, Owen MJ, Hayday AC. Productive T cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev*. 1996; 10(8): 948-62.

54 Sicinska E, Aifantis I, Le Cam L, Swat W, Borowski C, Yu Q, Ferrando AA, Levin SD, Geng Y, von Boehmer H, Sicinski P. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell*. 2003; 4(6): 451-61.

55 Engel I, Murre C. The function of E- and Id proteins in lymphocyte development. *Nat Rev Immunol*. 2001; 1(3): 193-9.

56 Engel I, Murre C. Disruption of pre-TCR expression accelerates lymphomagenesis in E2A-deficient mice. *Proc Natl Acad Sci U S A*. 2002; 99(17): 11322-7.

57 Allen RD 3rd, Kim HK, Sarafova SD, Siu G. Negative regulation of CD4 gene expression by a HES-1-c-Myb complex. *Mol Cell Biol*. 2001; 21(9): 3071-82.

58 Pearson R, Weston K. c-Myb regulates the proliferation of immature thymocytes following beta selection. *EMBO J*. 2000; 19(22): 6112-20.

59 Lin WC, Desiderio S. Cell cycle regulation of V(D)J recombination-activating protein RAG-2. *Proc Natl Acad Sci U S A*. 1994; 91(7): 2733-7.

60 Mandal M, Borowski C, Palomero T, Ferrando AA, Oberdoerffer P, Meng F, Ruiz-Vela A, Ciofani M, Zuniga-Pflucker JC, Screpanti I, Look AT, Korsmeyer SJ, Rajewsky K, von Boehmer H, Aifantis I. The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J Exp Med*. 2005; 201(4): 603-14.

61 Voll RE, Jimi E, Phillips RJ, Barber DF, Rincon M, Hayday AC *et al*. NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity*. 2000; 13: 677–689.

62 Kim D, Xu M, Nie L, Peng XC, Jimi E, Voll RE *et al*. Helix–loop–helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF-kappaB activities. *Immunity*. 2002; 16: 9–21.

63 Zipfel PA, Zhang W, Quiroz M, Pendergast AM. Requirement for Abl kinases in T cell receptor signaling. *Curr Biol.* 2004; 14: 1222–1231.

64 Wange RL. TCR signaling: another Abl-bodied kinase joins the cascade. *Curr Biol.* 2004; 14: 562–564.

65 Carrera AC. T cell receptor-associated alpha-phosphatydyl-inositol-3-kinase becomes activated by t cell receptor cross-linking and requires p56lck. *J Biol Chem*. 1994.

66 Ward SG, Cantrell DA. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr Opin Immunol*. 2001; 13: 332–338.

67 Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. 2003; 3(4): 317-30.

68 Wang X. The tumor suppressor PTEN regulates T cell survival and antigen receptor signaling by acting as a phosphatidylinositol 3-phosphatase. *J Immunol*. 2000

69 Leo A, Wienands J. Adapters in lymphocyte signaling. *J Clin Invest*. 2002; 109: 301-309.

70 Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol*. 2005; 17(6): 596-603.

71 Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307(5712): 1098-101.

72 Goldberg JM, Silverman LB, Levy DE, *et al*. Childhood T-cell acute lymphoblastic leukemia: the Dana–Farber Cancer Institute acute lymphoblastic leukemia consortium experience. *J Clin Oncol*. 2003; 21: 37.

73 Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006; 354: 166–178.

74 Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A *et al*. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia.* 1995; 9: 1783–1786.

75 Asnafi V, Beldjord K, Boulanger E, Comba B, Le Tutour P, Estienne MH *et al*. Analysis of TCR, pT alpha, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment. *Blood* 2003; 101: 2693–2703.

76 Ferrando AA, Herblot S, Palomero T, Hansen M, Hoang T, Fox EA *et al*. Biallelic transcriptional activation of oncogenic transcription factors in T-cell acute lymphoblastic leukemia. *Blood.* 2004; 103: 1909–1911

77 Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC *et al*. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell.* 2002; 1: 75–87.

78 Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H *et al*. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood.* 2005; 106: 274–286.

79 Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Liu H, Pui CH, Evans WE, Naeve C, Wong L, Downing JR. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*. 2002; 1(2): 133-43.

80 Speleman F, Cauwelier B, Dastugue N, Cools J, Verhasselt B, Poppe B *et al*. A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of HOXA10 and HOXA11 in a subset of T-cell acute lymphoblastic leukemias. *Leukemia*. 2005; 19: 358–366.

81 Harrison CJ, Foroni L. Cytogenetics and molecular genetics of acute lymphoblastic leukemia. *Rev Clin Exp Hematol.* 2002; 6: 91–113.

82 Cauwelier B, Dastugue N, Cools J, Poppe B, Herens C, De Paepe A *et al*. Molecular cytogenetic study of 126 unselected T-ALL cases reveals high incidence of TCRbeta locus rearrangements and putative new T-cell oncogenes. *Leukemia* 2007; 21(1): 121-8.

83 Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G *et al*. Site-specific recombination of the tal-1 gene is a common occurrence in human T cell leukemia. *EMBO J.* 1990; 9: 3343–3351.

84 Janssen JW, Ludwig WD, Sterry W, Bartram CR. SIL-TAL1 deletion in T-cell acute lymphoblastic leukemia. *Leukemia.* 1993; 7: 1204–1210.

85 Asnafi V, Radford-Weiss I, Dastugue N, Bayle C, Leboeuf D, Charrin C *et al*. CALM-AF10 is a common fusion transcript in T-ALL and is specific to the TCRgammadelta lineage. *Blood.* 2003; 102: 1000–1006.

86 Hayette S, Tigaud I, Maguer-Satta V, Bartholin L, Thomas X, Charrin C *et al*. Recurrent involvement of the MLL gene in adult T-lineage acute lymphoblastic leukemia. *Blood.* 2002; 99: 4647–4649.

87 Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R *et al*. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. *Nat Genet.* 2004; 36: 1084–1089.

88 Van Vlierberghe P, van Grotel M, Tchinda J, Lee C, Beverloo HB, van der Spek PJ, Stubbs A, Cools J, Nagata K, Fornerod M, Buijs-Gladdines J, Horstmann M, van Wering ER, Soulier J, Pieters R, Meijerink JP. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. *Blood*. 2008; 111(9):4668-80.

89 Hussey DJ, Nicola M, Moore S, Peters GB, Dobrovic A. The (4;11)(q21;p15) translocation fuses the NUP98 and RAP1GDS1 genes and is recurrent in T-cell acute lymphocytic leukemia. *Blood.* 1999; 94: 2072–2079.

90 Lahortiga I, Vizmanos JL, Agirre X, Vazquez I, Cigudosa JC, Larrayoz MJ *et al*. NUP98 is fused to adducin 3 in a patient with T-cell acute lymphoblastic leukemia and myeloid markers, with a new translocation t(10;11)(q25;p15). *Cancer Res*. 2003; 63: 3079–3083.

91 Van Vlierberghe P, Pieters R, Beverloo HB, Meijerink JP. Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia. *Br J Haematol*. 2008; 143(2): 153-68.

92 Hebert J, Cayuela JM, Berkeley J, Sigaux F. Candidate tumorsuppressor genes MTS1 (p16INK4A) and MTS2 (p15INK4B) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. *Blood.* 1994; 84: 4038–4044.

93 Sinclair PB, Sorour A, Martineau M, Harrison CJ, Mitchell WA, O'Neill E *et al*. A fluorescence in situ hybridization map of 6q deletions in acute lymphocytic leukemia: identification and analysis of a candidate tumor suppressor gene. *Cancer Res.* 2004; 64: 4089–4098.

94 Van Vlierberghe P, van Grotel M, Beverloo HB, Lee C, Helgason T, Buijs-Gladdines J, Passier M, van Wering ER, Veerman AJ, Kamps WA, Meijerink JP, Pieters R. The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. *Blood*. 2006; 108(10): 3520-9.

95 Ferrando AA, Look AT. Gene expression profiling in T-cell acute lymphoblastic leukemia. *Semin Hematol.* 2003; 40(4): 274-80.

96 Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, Langerak AW, Montpellier B, Nadel B, Walrafen P, Delattre O, Aurias A, Leblanc T, Dombret H, Gewirtz AM, Baruchel A, Sigaux F, Soulier J.The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood*. 2007; 110(4): 1251-61.

97 van Vlierberghe P, Meijerink JP, Lee C, Ferrando AA, Look AT, van Wering ER, Beverloo HB, Aster JC, Pieters R.A new recurrent 9q34 duplication in pediatric T-cell acute lymphoblastic leukemia. *Leukemia*. 2006; 20(7): 1245-53.

98 Lai EC. Notch signaling: control of cell communication and cell fate. *Development* 2004; 131: 965–973.

99 Duncan AW, Rattis FM, DiMascio LN, Congdon KL, Pazianos G, Zhao C *et al*. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol.* 2005; 6: 314–322.

100 Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu Rev Immunol.* 2005; 23: 945–974.

101 Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR *et al*. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity*. 1999; 10: 547–558.

102 Ciofani M, Schmitt TM, Ciofani A, Michie AM, Cuburu N, Aublin A *et al*. Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. *J Immunol*. 2004; 172: 5230–5239.

103 Wolfer A, Wilson A, Nemir M, MacDonald HR, Radtke F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta lineage thymocytes. *Immunity*. 2002; 16: 869–879.

104 Nie L, Xu M, Vladimirova A, Sun XH. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J*. 2003; 22: 5780–5792.

105 Ciofani M, Zúñiga-Pflücker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol*. 2005; 6(9): 881-8.

106 Schweisguth F. Notch signaling activity. *Curr Biol*. 2004; 14: 129–138.

107 Fujii Y, Yada M, Nishiyama M, Kamura T, Takahashi H, Tsunematsu R, Susaki E, Nakagawa T, Matsumoto A, Nakayama KI. Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation. *Cancer Sci*. 2006; 97(8): 729-36.

108 Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD *et al*. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell.* 1991; 66: 649–661.

109 Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J *et al*. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med*. 1996; 183: 2283–2291.

110 Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C *et al*. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306: 269–271.

111 Sulis ML, Williams O, Palomero T, Tosello V, Pallikuppam S, Real PJ, Barnes K, Zuurbier L, Meijerink JP, Ferrando AA. NOTCH1 extracellular juxtamembrane expansion mutations in T-ALL. *Blood*. 2008; 112(3): 733-40.

112 Weng AP, Nam Y, Wolfe MS, Pear WS, Griffin JD, Blacklow SC *et al*. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol*. 2003; 23: 655–664.

113 Breit S, Stanulla M, Flohr T, Schrappe M, Ludwig WD, Tolle G, Happich M, Muckenthaler MU, Kulozik AE. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. *Blood*. 2006;108: 1151-1157

114 Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, Ferrando A, Aifantis I. The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J Exp Med*. 2007; 204(8): 1825-35.

115. Malyukova A, Dohda T, von der Lehr N, Akhoondi S, Corcoran M, Heyman M, Spruck C, Grandér D, Lendahl U, Sangfelt O. The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res*. 2007; 67(12): 5611-6.

116 Palomero T, Ferrando A. Oncogenic NOTCH1 control of MYC and PI3K: challenges and opportunities for anti-NOTCH1 therapy in T-cell acute lymphoblastic leukemias and lymphomas. *Clin Cancer Res*. 2008; 14(17): 5314-7.

117 Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, Balmain A. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. *Science*. 2008; 321(5895): 1499-502.

118 Welcker M, Orian A, Jin J, Grim JE, Harper JW, Eisenman RN, Clurman BE. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc Natl Acad Sci U S A*. 2004; 101(24): 9085-90.

119 Aster JC. Deregulated NOTCH signaling in acute T-cell lymphoblastic leukemia/lymphoma: new insights, questions, and opportunities. *Int J Hematol.* 2005; 82: 295–301.

120 Shima EA, Le Beau MM, McKeithan TW, Minowada J, Showe LC, Mak TW, Minden MD, Rowley JD, Diaz MO. Gene encoding the alpha chain of the T-cell receptor is moved immediately downstream of c-myc in a chromosomal 8;14 translocation in a cell line from a human T-cell leukemia. *Proc Natl Acad Sci U S A.* 1986; 83(10): 3439-43.

121 Erikson J, Finger L, Sun L, ar-Rushdi A, Nishikura K, Minowada J, Finan J, Emanuel BS, Nowell PC, Croce CM. Deregulation of c-myc by translocation of the alpha-locus of the T-cell receptor in T-cell leukemias. *Science*. 1986; 232(4752): 884-6.

122 Uddin S, Hussain A, Al Hussein K, Platanias LC, Bhatia KG. Inhibition of phosphatidylinositol 3'-kinase induces preferentially killing o PTEN-null T leukemias through AKT pathway. *Biochem Biophys Res Commun*. 2004; 320: 932–938.

123 Seminario MC, Wange RL. Lipid phosphatases in the regulation of T cell activation: living up to their PTEN-tial. *Immunol Rev.* 2003; 192: 80–97.

124 Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, Caparros E, Buteau J, Brown K, Perkins SL, Bhagat G, Agarwal AM, Basso G, Castillo M, Nagase S, Cordon-Cardo C, Parsons R, Zúñiga-Pflücker JC, Dominguez M, Ferrando AA. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med*. 2007; 13(10): 1203-10.

125 Palomero T, Dominguez M, Ferrando AA. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. *Cell Cycle*. 2008; 7(8): 965-70.

126 Vilimas T, Mascarenhas J, Palomero T, Mandal M, Buonamici S, Meng F, Thompson B, Spaulding C, Macaroun S, Alegre ML, Kee BL, Ferrando A, Miele L, Aifantis I. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med*. 2007; 13(1): 70-7.

127 Jones S. An overview of the basic helix–loop–helix proteins. *Genome Biol.* 2004; 5: 226.

128 Tremblay M, Herblot S, Lecuyer E, Hoang T. Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL. *J Biol Chem.* 2003; 278: 12680–12687.

129 Sawada S, Littman DR. A heterodimer of HEB and an E12-related protein interacts with the CD4 enhancer and regulates its activity in T-cell lines. *Mol Cell Biol.* 1993; 13: 5620–5628.

130 Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL *et al*. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol.* 1997; 17: 4782–4791.

131 Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol Cell Biol.* 1997; 17: 7317–7327.

132 Bain G, Romanow WJ, Albers K, Havran WL, Murre C. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med*. 1999; 189: 289–300.

133 Engel I, Murre C. Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc Natl Acad Sci USA*. 1999; 96: 996–1001.

134 Pagliuca A, Gallo P, De Luca P, Lania L. Class A helix–loop–helix proteins are positive regulators of several cyclin-dependent kinase inhibitors' promoter activity and negatively affect cell growth. *Cancer Res.* 2000; 60: 1376–1382.

135 Kim D, Peng XC, Sun XH. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol.* 1999; 19: 8240–8253.

136 Begley CG, Aplan PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. *Proc Natl Acad Sci USA*. 1989; 86: 10128–10132.

137 Baer R. TAL1, TAL2 and LYL1: a family of basic helix–loop–helix proteins implicated in T cell acute leukaemia. *Semin Cancer Biol.* 1993; 4: 341–347.

138 Hsu HL, Cheng JT, Chen Q, Baer R. Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. *Mol Cell Biol*. 1991; 11(6): 3037-42.

139 Hsu HL, Wadman I, Baer R. Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc *Natl Acad Sci U S A*. 1994; 91(8): 3181-5.

140 Carroll AJ, Crist WM, Link MP, Amylon MD, Pullen DJ, Ragab AH *et al*. The t(1;14)(p34;q11) is nonrandom and restricted to T-cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. *Blood.* 1990; 76: 1220–1224.

141 Aplan PD, Raimondi SC, Kirsch IR. Disruption of the SCL gene by a t(1;3) translocation in a patient with T cell acute lymphoblastic leukemia. *J Exp Med.* 1992; 176: 1303–1310.

142 Bash RO, Hall S, Timmons CF, Crist WM, Amylon M, Smith RG *et al*. Does activation of the TAL1 gene occur in a majority of patients with T-cell acute lymphoblastic leukemia? A pediatric oncology group study. *Blood*. 1995; 86: 666–676.

143 Robb L, Lyons I, Li R, Hartley L, Kontgen F, Harvey RP *et al*. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc Natl Acad Sci USA*. 1995; 92: 7075–7079.

144 Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature*. 1995; 373: 432–434.

145 Visvader JE, Fujiwara Y, Orkin SH. Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev*. 1998; 12: 473–479.

146 Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T. SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. *Nat Immunol.* 2000; 1: 138–144.

147 O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB. *Cancer Cell*. 2004; 5: 587–596.

148 Park ST, Sun XH. The Tal1 oncoprotein inhibits E47-mediated transcription. Mechanism of inhibition. J *Biol Chem*. 1998; 273(12): 7030-7.

149 Aplan PD, Jones CA, Chervinsky DS, Zhao X, Ellsworth M, Wu C *et al*. An scl gene product lacking the transactivation domain induces bony abnormalities and cooperates with LMO1 to generate T-cell malignancies in transgenic mice. *EMBO J.* 1997; 16: 2408–2419.

150 O'Neil J, Billa M, Oikemus S, Kelliher M. The DNA binding activity of TAL-1 is not required to induce leukemia/lymphoma in mice. *Oncogene.* 2001; 20: 3897–3905.

151 Palomero T, Odom DT, O'Neil J, Ferrando AA, Margolin A, Neuberg DS, Winter SS, Larson RS, Li W, Liu XS, Young RA, Look AT. Transcriptional regulatory networks downstream of TAL1/SCL in T-cell acute lymphoblastic leukemia. *Blood*. 2006; 108(3) :986-92.

152 Mellentin JD, Smith SD, Cleary ML. lyl-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix–loop–helix DNA binding motif. *Cell.* 1989; 58: 77–83.

153 Zhong Y, Jiang L, Hiai H, Toyokuni S, Yamada Y. Overexpression of a transcription factor LYL1 induces T- and B-cell lymphoma in mice. *Oncogene*. 2007; 26(48): 6937-47.

154 Xia Y, Brown L, Yang CY, Tsan JT, Siciliano MJ, Espinosa III R *et al*. TAL2, a helix-loop-helix gene activated by the (7;9)(q34;q32) translocation in human T-cell leukemia. *Proc Natl Acad Sci USA.* 1991; 88: 11416–11420.

155 Wang J, Jani-Sait SN, Escalon EA, Carroll AJ, de Jong PJ, Kirsch IR *et al*. The t(14;21)(q11.2;q22) chromosomal translocation associated with T-cell acute lymphoblastic leukemia activates the BHLHB1 gene. *Proc Natl Acad Sci USA*. 2000; 97: 3497–3502.

156 Rabbitts TH. LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. *Genes Dev.* 1998; 12: 2651–2657.

157 McGuire EA, Hockett RD, Pollock KM, Bartholdi MF, O'Brien SJ, Korsmeyer SJ. The t(11;14)(p15;q11) in a T-cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. *Mol Cell Biol.* 1989; 9: 2124–2132.

158 Royer-Pokora B, Loos U, Ludwig WD. TTG-2, a new gene encoding a cysteine-rich protein with the LIM motif, is overexpressed in acute T-cell leukaemia with the t(11;14)(p13;q11). *Oncogene*. 1991; 6: 1887–1893.

159 Asnafi V, Beldjord K, Libura M, Villarese P, Millien C, Ballerini P *et al*. Age-related phenotypic and oncogenic differences in T-cell acute lymphoblastic leukemias may reflect thymic atrophy. *Blood.* 2004; 104: 4173–4180.

160 Hammond SM, Crable SC, Anderson KP. Negative regulatory elements are present in the human LMO2 oncogene and may contribute to its expression in leukemia. *Leuk Res*. 2005; 29: 89–97.

161 Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science.* 2003; 302: 415–419.

162 Warren AJ, Colledge WH, Carlton MB, Evans MJ, Smith AJ, Rabbitts TH. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell*. 1994; 78: 45–57.

163 Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A *et al*. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.* 1997; 16: 3145–3157.

164 Ono Y, Fukuhara N, Yoshie O. TAL1 and LIM-only proteins synergistically induce retinaldehyde dehydrogenase 2 expression in T-cell acute lymphoblastic leukemia by acting as cofactors for GATA3. *Mol Cell Biol*. 1998; 18: 6939–6950.

165 Larson RC, Osada H, Larson TA, Lavenir I, Rabbitts TH. The oncogenic LIM protein Rbtn2 causes thymic developmental aberrations that precede malignancy in transgenic mice. *Oncogene*. 1995; 11: 853–862.

166 Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ, Wadman I *et al*. Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. *EMBO J.* 1996; 15: 1021–1027

167 Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D *et al*. Homeodomain-DNA recognition. *Cell.* 1994; 78: 211–223.

168 Lawrence PA, Morata G. Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell*. 1994; 78: 181–189.

169 van Oostveen J, Bijl J, Raaphorst F, Walboomers J, Meijer C. The role of homeobox genes in normal hematopoiesis and hematological malignancies. *Leukemia.* 1999; 13: 1675–1690.

170 Taghon T, Thys K, De Smedt M, Weerkamp F, Staal FJ, Plum J *et al*. Homeobox gene expression profile in human hematopoietic multipotent stem cells and T-cell progenitors: implications for human T-cell development. *Leukemia.* 2003; 17: 1157–1163.

171 Izon DJ, Rozenfeld S, Fong ST, Komuves L, Largman C, Lawrence HJ. Loss of function of the homeobox gene Hoxa-9 perturbs early T-cell development and induces apoptosis in primitive thymocytes. *Blood*. 1998; 92: 383–393.

172 Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J.* 1998; 17: 3714–3725.

173 Sauvageau G, Thorsteinsdottir U, Hough MR, Hugo P, Lawrence HJ, Largman C *et al*. Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. *Immunity*. 1997; 6: 13–22.

174 Look AT. Oncogenic transcription factors in the human acute leukemias. *Science*. 1997; 278: 1059–1064.

175 Ferrando AA, Armstrong SA, Neuberg DS, Sallan SE, Silverman LB, Korsmeyer SJ *et al*. Gene expression signatures in MLL rearranged T-lineage and B-precursor acute leukemias: dominance of HOX dysregulation. *Blood*. 2003; 102: 262–268.

176 Dik WA, Brahim W, Braun C, Asnafi V, Dastugue N, Bernard OA *et al*. CALM-AF10+ T-ALL expression profiles are characterized by overexpression of HOXA and BMI1 oncogenes. *Leukemia*. 2005; 19: 1948–1957.

177 Owens BM, Hawley RG. HOX and non-HOX homeobox genes in leukemic hematopoiesis. *Stem Cells*. 2002; 20: 364–379.

178 Roberts CW, Shutter JR, Korsmeyer SJ. Hox11 controls the genesis of the spleen. *Nature*. 1994; 368: 747–749.

179 Hatano M, Roberts CW, Minden M, Crist WM, Korsmeyer SJ. Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science*. 1991; 253: 79–82.

180 Brake RL, Kees UR, Watt PM. Multiple negative elements contribute to repression of the HOX11 proto-oncogene. *Oncogene*. 1998; 17: 1787–1795.

181 Kees UR, Heerema NA, Kumar R, Watt PM, Baker DL, La MK *et al*. Expression of HOX11 in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG). *Leukemia*. 2003; 17: 887–893.

182 Watt PM, Kumar R, Kees UR. Promoter demethylation accompanies reactivation of the HOX11 proto-oncogene in leukemia. *Genes Chromosomes Cancer*. 2000; 29: 371–377.

183 Ferrando AA, Neuberg DS, Dodge RK, Paietta E, Larson RA, Wiernik PH *et al*. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. *Lancet*. 2004; 363: 535–536.

184 Hawley RG, Fong AZ, Lu M, Hawley TS. The HOX11 homeoboxcontaining gene of human leukemia immortalizes murine hematopoietic precursors. *Oncogene*. 1994; 9: 1–12.

185 Hawley RG, Fong AZ, Reis MD, Zhang N, Lu M, Hawley TS. Transforming function of the HOX11/TCL3 homeobox gene. *Cancer Res.* 1997; 57: 337–345.

186 Owens BM, Zhu YX, Suen TC, Wang PX, Greenblatt JF, Goss PE *et al*. Specific homeodomain–DNA interactions are required for HOX11-mediated transformation. *Blood*. 2003; 101: 4966–4974.

187 Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. *Nature*. 1997; 385: 454–458.

188 Riz I, Hawley RG. G1/S transcriptional networks modulated by the HOX11/TLX1 oncogene of T-cell acute lymphoblastic leukemia. *Oncogene*. 2005; 24: 5561–5575.

189 Bernard OA, Busson-LeConiat M, Ballerini P, Mauchauffe M, Della VV, Monni R *et al*. A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. *Leukemia*. 2001; 15: 1495–1504.

190 Nagel S, Kaufmann M, Drexler HG, MacLeod RA. The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2). *Cancer Res*. 2003; 63: 5329–5334.

191 Su XY, Busson M, Della VV, Ballerini P, Dastugue N, Talmant P *et al*. Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia. *Genes Chromosomes Cancer*. 2004; 41: 243–249.

192 Hansen-Hagge TE, Schafer M, Kiyoi H, Morris SW, Whitlock JA, Koch P *et al*. Disruption of the RanBP17/Hox11L2 region by recombination with the TCRdelta locus in acute lympho- blastic leukemias with t(5;14)(q34;q11). *Leukemia*. 2002; 16: 2205–2212.

193 Jalali GR, An Q, Konn ZJ, Worley H, Wright SL, Harrison CJ, Strefford JC, Martineau M. Disruption of ETV6 in intron 2 results in upregulatory and insertional events in childhood acute lymphoblastic leukaemia. *Leukemia*. 2008; 22(1): 114-23.

194 Ballerini P, Blaise A, Busson-Le Coniat M, Su XY, Zucman-Rossi J, Adam M *et al*. HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. *Blood*. 2002; 100: 991–997.

195 Cave H, Suciu S, Preudhomme C, Poppe B, Robert A, Uyttebroeck A *et al*. Clinical significance of HOX11L2 expression linked to t(5;14)(q35;q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. *Blood*. 2004; 103: 442–450.

196 Ballerini P, Busson M, Fasola S, van den AJ, Lapillonne H, Romana SP *et al*. NUP214-ABL1 amplification in t(5;14)/ HOX11L2-positive ALL present with several forms and may have a prognostic significance. *Leukemia*. 2005; 19: 468–470.

197 Yu BD, Hess JL, Horning SE, Brown GA, Korsmeyer SJ. Altered Hox expression and segmental identity in Mll-mutant mice. *Nature*. 1995; 378: 505–508.

198 Schumacher A, Magnuson T. Murine Polycomb- and trithoraxgroup genes regulate homeotic pathways and beyond. *Trends Genet*. 1997; 13: 167–170.

199 Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD *et al*. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell*. 2002; 10: 1107–1117.

200 Meyer C, Schneider B, Jacob S, Strehl S, Attarbaschi A, Schnittger S *et al*. The MLL recombinome of acute leukemias. *Leukemia*. 2006; 20: 777–784.

201 Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD *et al*. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*. 2002; 30: 41–47.

202 Rubnitz JE, Camitta BM, Mahmoud H, Raimondi SC, Carroll AJ, Borowitz MJ *et al*. Childhood acute lymphoblastic leukemia with the MLL-ENL fusion and t(11;19)(q23;p13.3) translocation. *J Clin Oncol*. 1999; 17: 191–196.

203 Corral J, Lavenir I, Impey H, Warren AJ, Forster A, Larson TA, Bell S, McKenzie AN, King G, Rabbitts TH. An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell*. 1996; 85(6): 853-61.

204 Groupe Francais de Cytogenetique Hematologique (GFCH). t(10;11)(p13–14;q14–21): a new recurrent translocation in T-cell acute lymphoblastic leukemias. Genes Chromosomes Cancer 1991; 3: 411–415.

205 Dreyling MH, Martinez-Climent JA, Zheng M, Mao J, Rowley JD, Bohlander SK. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. Proc Natl Acad Sci USA 1996; 93: 4804–4809.

206 Dreyling MH, Schrader K, Fonatsch C, Schlegelberger B, Haase D, Schoch C *et al*. MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): both rearrangements are associated with a poor prognosis. *Blood*. 1998; 91: 4662–4667.

207. Deshpande AJ, Cusan M, Rawat VP, Reuter H, Krause A, Pott C, Quintanilla-Martinez L, Kakadia P, Kuchenbauer F, Ahmed F, Delabesse E, Hahn M, Lichter P, Kneba M, Hiddemann W, Macintyre E, Mecucci C, Ludwig WD, Humphries RK, Bohlander SK, Feuring-Buske M, Buske C. Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of CALM/AF10-positive leukemia. *Cancer Cell*. 2006; 10(5): 363-74.

208 Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL *et al*. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature*. 2003; 423: 302–305.

209 Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*. 1999; 397: 164–168

210 Bender TP, Kremer CS, Kraus M, Buch T, Rajewsky K. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat Immunol*. 2004; 5: 721-729.

211 Lipsick JS, Wang DM. Transformation by v-Myb. *Oncogene*. 1999; 18: 3047-3055

212. Badiani PA, Kioussis D, Swirsky DM, Lampert IA, Weston K. T-cell lymphomas in v-Myb transgenic mice. *Oncogene*. 1996; 13: 2205-2212.

213. Davies J, Badiani P, Weston K. Cooperation of Myb and Myc proteins in T cell lymphomagenesis. *Oncogene*. 1999; 18: 3643-3647.

214 Sinclair P, Harrison CJ, Jarosová M, Foroni L. Analysis of balanced rearrangements of chromosome 6 in acute leukemia: clustered breakpoints in q22-q23 and possible involvement of c-MYB in a new recurrent translocation, t(6;7)(q23;q32 through 36). *Haematologica*. 2005; 90(5): 602-11.

215 Lahortiga I, De Keersmaecker K, Van Vlierberghe P, Graux C, Cauwelier B, Lambert F, Mentens N, Beverloo HB, Pieters R, Speleman F, Odero MD, Bauters M, Froyen G, Marynen P, Vandenberghe P, Wlodarska I, Meijerink JP, Cools J. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. *Nat Genet*. 2007; 39(5): 593-5.

216 Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, Langerak AW, Montpellier B, Nadel B, Walrafen P, Delattre O, Aurias A, Leblanc T, Dombret H, Gewirtz AM, Baruchel A, Sigaux F, Soulier J. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood*. 2007: 1251-61.

217 Stone S, Jiang P, Dayananth P, Tavtigian SV, Katcher H, Parry D *et al*. Complex structure and regulation of the P16 (MTS1) locus. *Cancer Re*. 1995; 55: 2988–2994.

218 Stone S, Dayananth P, Jiang P, Weaver-Feldhaus JM, Tavtigian SV, Cannon-Albright L *et al*. Genomic structure, expression and mutational analysis of the P15 (MTS2) gene. *Oncogene*. 1995; 11: 987–991.

219 Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev*. 2003; 13: 77–83.

220 Sherr CJ, Weber JD. The ARF/p53 pathway. *Curr Opin Genet Dev*. 2000; 10: 94–99.

221 Lagresle C, Gardie B, Eyquem S, Fasseu M, Vieville JC, Pla M, Sigaux F, Bories JC. Transgenic expression of the p16(INK4a) cyclin-dependent kinase inhibitor leads to enhanced apoptosis and differentiation arrest of CD4-CD8- immature thymocytes. *J Immunol.* 2002; 168(5): 2325-31.

222 Cayuela JM, Madani A, Sanhes L, Stern MH, Sigaux F. Multiple tumor-suppressor gene 1 inactivation is the most frequent genetic alteration in T-cell acute lymphoblastic leukemia. *Blood*. 1996; 87: 2180–2186.

223 Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP *et al*. Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA*. 1994; 91: 11045–11049.

224 Garcia-Manero G, Jeha S, Daniel J, Williamson J, Albitar M, Kantarjian HM *et al*. Aberrant DNA methylation in pediatric patients with acute lymphocytic leukemia. *Cancer*. 2003; 97: 695–702.

225 Omura-Minamisawa M, Diccianni MB, Batova A, Chang RC, Bridgeman LJ, Yu J *et al*. Universal inactivation of both p16 and p15 but not downstream components is an essential event in the pathogenesis of T-cell acute lymphoblastic leukemia. *Clin Cancer Res*. 2000; 6: 1219–1228.

226 Ramakers-van Woerden NL, Pieters R, Slater RM, Loonen AH, Beverloo HB, van Drunen E *et al*. In vitro drug resistance and prognostic impact of p16INK4A/P15INK4B deletions in childhood T-cell acute lymphoblastic leukaemia. *Br J Haematol*. 2001; 112: 680–690.

227 Weng AP, Aster JC. No T without D3: a critical role for cyclin D3 in normal and malignant precursor T cells. *Cancer Cell*. 2003; 4: 417–418.

228 Clappier E, Cuccuini W, Cayuela JM, Vecchione D, Baruchel A, Dombret H *et al*. Cyclin D2 dysregulation by chromosomal translocations to TCR loci in T-cell acute lymphoblastic leukemias. *Leukemia*. 2006; 20: 82–86.

229 Kawamata N, Zhang L, Ogawa S, Nannya Y, Dashti A, Lu D, Lim S, Schreck R, Koeffler HP. Double minute chromosomes containing MYB gene and NUP214-ABL1 fusion gene in T-cell leukemia detected by single nucleotide polymorphism DNA microarray and fluorescence in situ hybridization. *Leuk Res*. 2008 Sep 15.

230 Graux C, Stevens-Kroef M, Lafage M, Dastugue N, Harrison CJ, Mugneret F, Bahloula K, Struski S, Grégoire MJ, Nadal N, Lippert E, Taviaux S, Simons A, Kuiper RP, Moorman AV, Barber K, Bosly A, Michaux L, Vandenberghe P, Lahortiga I, De Keersmaecker K, Wlodarska I, Cools J, Hagemeijer A, Poirel HA. Heterogeneous patterns of amplification of the NUP214-ABL1 fusion gene in T-cell acute lymphoblastic leukemia. *Leukemia*. 2008

231 Bernasconi P, Calatroni S, Giardini I, Inzoli A, Castagnola C, Cavigliano PM *et al*. ABL1 amplification in T-cell acute lymphoblastic leukemia. Cancer Genet Cytogenet 2005; 162: 146-150.

232 Golub TR, Goga A, Barker GF, Afar DE, McLaughlin J, Bohlander SK *et al*. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol*. 1996; 16: 4107–4116.

233 De Keersmaecker K, Graux C, Odero MD, Mentens N, Somers R, Maertens J *et al*. Fusion of EML1 to ABL1 in T-cell acute lymphoblastic leukemia with cryptic t(9;14)(q34;q32). *Blood*. 2005; 105: 4849–4852.

234 De Keersmaecker K, Rocnik JL, Bernad R, Lee BH, Leeman D, Gielen O, Verachtert H, Folens C, Munck S, Marynen P, Fornerod M, Gilliland DG, Cools J. Kinase activation and transformation by NUP214-ABL1 is dependent on the context of the nuclear pore. *Mol Cell*. 2008; 31(1): 134-42.

235 De Keersmaecker K, Versele M, Cools J, Superti-Furga G, Hantschel O. Intrinsic differences between the catalytic properties of the oncogenic NUP214-ABL1 and BCR-ABL1 fusion protein kinases. *Leukemia*. 2008.

236 Chiaretti S, Tavolaro S, Ghia EM, Ariola C, Matteucci C, Elia L, Maggio R, Messina M, Ricciardi MR, Vitale A, Ritz J, Mecucci C, Guarini A, Foà R. Characterization of ABL1 expression in adult T-cell acute lymphoblastic leukemia by oligonucleotide array analysis. *Haematologica*. 2007; 92(5): 619-26.

237 Quintás-Cardama A, Tong W, Manshouri T, Vega F, Lennon PA, Cools J, Gilliland DG, Lee F, Cortes J, Kantarjian H, Garcia-Manero G. Activity of tyrosine kinase inhibitors against human NUP214-ABL1-positive T cell malignancies. *Leukemia*. 2008; 22(6): 1117-24.

238 Griesinger F, Janke A, Podleschny M, Bohlander SK. Identification of an ETV6-ABL2 fusion transcript in combination with an ETV6 point mutation in a T-cell acute lymphoblastic leukaemia cell line. *Br J Haematol*. 2002; 119: 454–458.

239 Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P *et al*. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood*. 1997; 90: 2535–2540.

240 Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M *et al*. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*. 1997; 278: 1309–1312.

241 Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, Knoops L, Ariola C, Fodale V, Clappier E, Paoloni F, Martinelli S, Fragale A, Sanchez M, Tavolaro S, Messina M, Cazzaniga G, Camera A, Pizzolo G, Tornesello A, Vignetti M, Battistini A, Cavé H, Gelb BD, Renauld JC, Biondi A, Constantinescu SN, Foà R, Tartaglia M. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med. 2008; 205(4): 751-8.

242 Burnett RC, Thirman MJ, Rowley JD, Diaz MO. Molecular analysis of the T-cell acute lymphoblastic leukemia-associated t(1;7)(p34;q34) that fuses LCK and TCRB. *Blood*. 1994; 84: 1232–1236.

243 Tycko B, Smith SD, Sklar J. Chromosomal translocations joining LCK and TCRB loci in human T cell leukemia. *J Exp Med*. 1991; 174: 867–873.

244 Paietta E, Ferrando AA, Neuberg D, Bennett JM, Racevskis J, Lazarus H *et al*. Activating FLT3 mutations in CD117/KIT(+) T-cell acute lymphoblastic leukemias. *Blood*. 2004; 104: 558–560.

245 Van Vlierberghe P, Meijerink JP, Stam RW, van der SW, van Wering ER, Beverloo HB *et al*. Activating FLT3 mutations in CD4+/CD8- pediatric T-cell acute lymphoblastic leukemias. *Blood*. 2005; 106: 4414–4415.

246 Bos JL. ras oncogenes in human cancer: a review. *Cancer Res*. 1989; 49: 4682–4689.

247 Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA *et al*. Prognostic importance of mutations in the ras protooncogenes in de novo acute myeloid leukemia. *Blood*. 1994; 83: 1603–1611.

248 Yokota S, Nakao M, Horiike S, Seriu T, Iwai T, Kaneko H *et al*. Mutational analysis of the N-ras gene in acute lymphoblastic leukemia: a study of 125 Japanese pediatric cases. *Int J Hematol*. 1998; 67: 379–387.

249 von Lintig FC, Huvar I, Law P, Diccianni MB, Yu AL, Boss GR. Ras activation in normal white blood cells and childhood acute lymphoblastic leukemia. *Clin Cancer Res*. 2000; 6: 1804–1810.

250 Goemans BF, Zwaan CM, Harlow A, Loonen AH, Gibson BE, Hahlen K *et al*. In vitro profiling of the sensitivity of pediatric leukemia cells to tipifarnib: identification of T-cell ALL and FAB M5 AML as the most sensitive subsets. *Blood*. 2005; 106: 3532–3537.

251 Van Vlierberghe P, Beverloo HB, Buijs-Gladdines J, van Wering ER, Horstmann M, Pieters R, Meijerink JP. Monoallelic or biallelic LMO2 expression in relation to the LMO2 rearrangement status in pediatric T-cell acute lymphoblastic leukemia. *Leukemia*. 2008; 22(7): 1434-7.

252 Vogelstein B, Kinzler KW. The multistep nature of cancer. *Trends Genet*. 1993; 9: 138–141.

253 Hansson A, Manetopoulos C, Jonsson JI, Axelson H. The basic helix–loop–helix transcription factor TAL1/SCL inhibits the expression of the p16INK4A and pTalpha genes. *Biochem Biophys Res Commun*. 2003; 312: 1073–1081.

254 De Keersmaecker K, Marynen P, Cools J. Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia. *Haematologica*. 2005; 90: 1116–1127.

255 Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. Nat Rev Immunol. 2008; 8(5): 380-90.

256 Stephens L, Williams R, Hawkins P. Phosphoinositide 3-kinases as drug targets in cancer. *Curr Opin Pharmacol*. 2005; 5(4): 357-65.

257 Sengupta TK, Leclerc GM, Hsieh-Kinser TT, Leclerc GJ, Singh I, Barredo JC. Cytotoxic effect of 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: implication for targeted therapy. *Mol Cancer*. 2007; 6: 46.

258 Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer*. 2004; 4: 361-70.

259 Graux C, Cools J, Michaux L, Vandenberghe P, Hagemeijer A. Cytogenetics and molecular genetics of T-cell acute lymphoblastic leukemia: from thymocyte to lymphoblast. *Leukemia*. 2006; 20: 1496-1510.

260 Barber KE, Martineau M, Harewood L, Stewart M, Cameron E, Strefford JC *et al.* Amplification of the ABL gene in T cell acute lymphoblastic leukemia. *Leukemia.* 2004; 18: 1153-1156.

261 Deenik W, Beverloo HB, van der Poel-van de Luytgaarde SC, Wattel MM, van Esser JW, Valk PJ, Cornelissen JJ. Rapid complete cytogenetic remission after upfront dasatinib monotherapy in a patient with a NUP214-ABL1-positive T-cell acute lymphoblastic leukemia. *Leukemia*. 2008 Nov 6.

262 Parra G, Reymond A, Dabbouseh N, Dermitzakis ET, Castelo R, Thomson TM, Antonarakis SE, Guigó R. Tandem chimerism as a means to increase protein complexity in the human genome. *Genome Res*. 2006; 16(1): 37-44.

263 Akiva P, Toporik A, Edelheit S, Peretz Y, Diber A, Shemesh R, Novik A, Sorek R. Transcription-mediated gene fusion in the human genome. *Genome Res*. 2006; 16(1): 30-6.

264 Li H, Wang J, Mor G, Sklar J. A neoplastic gene fusion mimics trans-splicing of RNAs in normal human cells. *Science*. 2008; 321(5894): 1357-61.

265. Law AJ, Kleinman JE, Weinberger DR, Weickert CS. Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. *Hum Mol Genet*. 2007; 16(2): 129-41.

Curriculum Vitae

Carlos Graux was born in Namur, Belgium, September 10th 1973. He is married to Sarah Grevisse and they have two children named Célestine and Charlotte.
He attended secondary school (Latin-Greek) at the Saint-Louis Institute, Namur.
He went on to take his degree in medicine at the Facultés Universitaires Notre-Dame de la Paix of Namur (FUNDP) and at the Catholic University of Louvain (UCL) and graduated magna cum laude in June 1998. He took up specialist training in Internal Medicine and in Hematology at the UCL and spent the last year of his training at the Katholieke Universiteit Leuven (KUL).
He next worked during two years (2005-2006) as resident hematologist at the Hematology Department of the University Hospital UCL Saint-Luc, Brussels (Professor Augustin Ferrant).
Since 2007, he is hematologist in the staff of the Hematology Department of the University Hospital UCL of Mont-Godinne, Yvoir (Professor André Bosly).
From October 2003, he combined clinical work and research at the the Center for Human Genetics of Leuven: first, in the laboratory of Genetics of Malignant Disorders under the guidance of Professor Anne Hagemeijer and then, in the laboratory of Molecular Pathogenesis of Leukemia under the supervision of Professor Jan Cools. He also performed some experimental work in the Hematologic Section laboratory of the Human Genetics Center of Louvain (Professor Hélène Poirel).

Publication list

Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R, Vermeesch JR, Stul M, Dutta B, Boeckx N, Bosly A, Heimann P, Uyttebroeck A, Mentens N, Somers R, MacLeod RA, Drexler HG, Look AT, Gilliland DG, Michaux L, Vandenberghe P, Wlodarska I, Marynen P, Hagemeijer A. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. ***Nature Genetics***. 2004; 36(10): 1084-9.

Speleman F, Cauwelier B, Dastugue N, Cools J, Verhasselt B, Poppe B, Van Roy N, Vandesompele J, **Graux C,** Uyttebroeck A, Boogaerts M, De Moerloose B, Benoit Y, Selleslag D, Billiet J, Robert A, Huguet F, Vandenberghe P, De Paepe A, Marynen P, Hagemeijer A. A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of HOXA10 and HOXA11 in a subset of T-cell acute lymphoblastic leukemias. ***Leukemia***. 2005; 19(3): 358-66.

De Keersmaecker K, **Graux C**, Odero MD, Mentens N, Somers R, Maertens J, Wlodarska I, Vandenberghe P, Hagemeijer A, Marynen P, Cools J. Fusion of EML1 to ABL1 in T-cell acute lymphoblastic leukemia with cryptic t(9;14)(q34;q32). ***Blood***. 2005; 105(12): 4849-52.

Graux C, Cools J, Michaux L, Vandenberghe P, Hagemeijer A. Cytogenetics and molecular genetics of T-cell acute lymphoblastic leukemia: from thymocyte to lymphoblast. ***Leukemia***. 2006; 20(9): 1496-510. Review.

De Keersmaecker K, Lahortiga I, **Graux C**, Marynen P, Maertens J, Cools J, Vandenberghe P. Transition from EML1-ABL1 to NUP214-ABL1 positivity in a patient with acute T-lymphoblastic leukemia. ***Leukemia***. 2006; 20(12): 2202-4.

Sonet A, **Graux C**, Nollevaux MC, Krug B, Bosly A, Vander Borght T. Unsuspected FDG-PET findings in the follow-up of patients with lymphoma. ***Ann Hematol***. 2007; 86(1): 9-15.

Cauwelier B, Cave H, Gervais C, Lessard M, Barin C, Perot C, Van den Akker J, Mugneret F, Charrin C, Pages MP, Gregoire MJ, Jonveaux P, Lafage-Pochitaloff M, Mozziconacci MJ, Terre C, Luquet I, Cornillet-Lefebvre P, Laurence B, Plessis G, Lefebvre C, Leroux D, Antoine-Poirel H, **Graux C**, Mauvieux L, Heimann P, Chalas C, Clappier E, Verhasselt B, Benoit Y, Moerloose BD, Poppe B, Van Roy N, Keersmaecker KD, Cools J, Sigaux F, Soulier J, Hagemeijer A, Paepe AD, Dastugue N, Berger R, Speleman F. Clinical, cytogenetic and molecular characteristics of 14 T-ALL patients carrying the TCRbeta-HOXA rearrangement: a study of the Groupe Francophone de Cytogenetique Hematologique. ***Leukemia***. 2007; 21(1): 121-8.

Lahortiga I, De Keersmaecker K, Van Vlierberghe P, **Graux C**, Cauwelier B, Lambert L, Mentens N, Beverloo HB, Pieters R, Speleman S, D Odero M, Bauters M, Froyen G, Marynen P Vandenberghe P, Wlodarska I, Meijerink JPP & Jan Cools J. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. ***Nat Genet***. 2007; 39(5): 593-5.

Delande S, Goffette P, Verbaandert C, Rahier J, **Graux C**, Mazzeo F, Humblet Y, Machiels JP. Bronchobiliary fistula and cholangiocarcinoma: a case report and principles of management. ***Acta Clin Belg***. 2007; 62(6): 438-41.

Haroche J, Amoura Z, Charlotte F, Salvatierra J, Wechsler B, **Graux C**, Brousse N, Piette JC.Imatinib mesylate for platelet-derived growth factor receptor-beta-positive Erdheim-Chester histiocytosis. ***Blood***. 2008; 111(11): 5413-5.

Graux C, Stevens-Kroef M, Lafage M, Dastugue N, Harrison CJ, Mugneret F, Bahloula K, Struski S, Grégoire MJ, Nadal N, Lippert E, Taviaux S, Simons A, Kuiper RP, Moorman AV, Barber K, Bosly A, Michaux L, Vandenberghe P, Lahortiga I, De Keersmaecker K, Wlodarska I, Cools J, Hagemeijer A and Poirel HA on behalf of the GFCH (Groupe Francophone de Cytogénétique Hématologique) and the BCGHO (Belgian Cytogenetic Group for Hematology and Oncology). Heterogeneous patterns of amplification of the *NUP214-ABL1* fusion gene in T-cell acute lymphoblastic leukemia. ***Leukemia***. 2008. Oct 16.