Prognostic markers in pediatric leukemia and mechanisms of KRAS-induced leukemogenesis

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ABSTRACT

Leukemia results from uncontrolled growth of genetically altered blood cells. Depending on the cell type of origin, the leukemia is defined as T-cell, B-cell, or myeloid and as acute or chronic depending on its characteristics.

However, patients with a certain subtype of leukemia (e.g. acute myeloid leukemia (AML) or acute T-cell leukemia (T-ALL)) still exhibit great heterogeneity in response to treatment and clinical outcome. For optimal survival it is therefore necessary to identify high-risk patients who benefit from more intense treatment and stem cell transplantation as well as patients with lower risk that benefit from less intense treatment. The treatment response is influenced by the genetic alterations that drive the leukemia and mutation status may therefore be used as a prognostic marker for risk stratification. In Paper I and II we focused on genetic markers previously identified as relevant for risk stratification of adult patients with the aim to evaluate them in pediatric patients with acute leukemia. Our results identified presence of *FLT3*-ITD and high *BAALC* expression as independent markers for adverse prognosis in pediatric AML. In addition, we found that high *ERG* expression was predictive for an adverse prognosis in pediatric AML with *MLL*-rearrangement. We also identified that high expression of the NOTCH1 target gene *HES1* was associated with better survival rates in children with T-ALL. This indicates that the level of NOTCH1-activity is predictive for prognosis in pediatric T-ALL.

Paper III focuses on mechanisms of KRAS-induced myeloproliferative neoplasm (MPN) and T-ALL in mice. The mechanistic role of hyperactive RAS is well understood in myeloid malignancies while its role in T-cell leukemogenesis is less clear. We used LSL-*Kras2*^{G12D};Mx1-*Cre* mice that models both MPN and T-ALL induced by hyperactive RAS and found that expression of KRAS^{G12D} had differential effects on the myeloid and T-lymphoid lineages. While increased proliferation of myeloid cells induced MPN by expansion of mature myeloid cells, increased proliferation and partial block in T-cell differentiation led to an expansion of early T-cell progenitors. With time, secondary genetic events resulted in T-ALL transformation and we identified loss of heterozygosity at the *Kras2* locus as a cooperating genetic event in T-ALL induced by KRAS^{G12D}.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

I. **Presence of *FLT3*-ITD and high *BAALC* expression are independent prognostic markers in childhood acute myeloid leukemia**

Staffas A, Kanduri M, Hovland R, Rosenquist R, Ommen HB, Abrahamsson J, Forestier E, Jahnukainen K, Jónsson ÓG, Zeller B, Palle J, Lönnerholm G, Hasle H, Palmqvist L, Ehrencrona H
Blood. 2011, 118:5905-5913

Response letter: High *ERG* gene expression is an unfavorable prognostic marker in pediatric acute myeloid leukemia

Staffas A, Kanduri M, Hovland R, Rosenquist R, Ommen HB, Abrahamsson J, Forestier E, Jahnukainen K, Jónsson ÓG, Zeller B, Palle J, Lönnerholm G, Hasle H, Ehrencrona H, Palmqvist L

Blood, 2012, 119:1087-1088

II. **Prognostic implications of mutations in *NOTCH1* and *FBXW7* in childhood T-ALL treated according to the NOPHO ALL-1992 and ALL-2000 protocols**

Fogelstrand L, Staffas A, Wasslavik C, Sjögren H, Söderhäll S, Frost BM, Forestier E, Degerman S, Behrendtz M, Heldrup J, Karrman K, Johansson B, Heyman M, Abrahamsson J, Palmqvist L

Accepted for publication: Pediatric Blood & Cancer, 2013

III. **$KRAS^{G12D}$-initiated acute T-cell leukemia is accompanied by loss of the wild-type *Kras2* allele**

Staffas A, Karlsson C, Persson M, Palmqvist L, Bergo MO

Manuscript, 2013

CONTENT

ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
B-ALL	Acute B-cell lymphoblastic leukemia
CBF	Core-binding factor
cDNA	Complementary DNA
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CN-AML	Cytogenetically normal AML
DNA	Deoxyribonucleic acid
EFS	Event-free survival
FAB	French-American-British
GAP	GTPase activating protein
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine tri-phosphate
GTPase	Guanosine tri-phosphatase
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem or progenitor cell
ITD	Internal tandem duplication
JMML	Juvenile myelomonocytic leukemia
MPN	Myeloproliferative neoplasm
NOPHO	Nordic society for pediatric hematology and oncology
OS	Overall survival

PCR	Polymerase chain reaction
pI-pC	Polyinosinic-polycytidylic acid
QPCR	Quantitative PCR
T-ALL	Acute T-cell leukemia
TIC	Tumor-initiating cell
TKD	Tyrosine kinase domain
WBC	White blood cell
WHO	World Health Organization
WT	Wild-type

GENES

ABL1	c-abl oncogene 1 [Human], (9q34.1)
BAALC	Brain and acute leukemia, cytoplasmic [Human], (8q22.3)
BCR	Breakpoint cluster region [Human], (22q11.23)
CBFB	Core-binding factor, beta subunit [Human], (16q22.1)
CEBPA	CCAAT/enhancer binding protein α [Human], (19q13.1)
Cre	Cyclization recombinase [Enterobacteria phage P1]
ERG	v-ets avian erythroblastosis virus E26 oncogene homolog [human], (21q22.3)
FBXW7	F-box and WD repeat domain containing 7 [Human], (4q31.3)
FLT3	Fms-related tyrosine kinase 3 [Human], (13q12)
HES1	Hes family bHLH transcription factor 1 [Human], (3q28-q29)
HRAS	Harvey rat sarcoma viral oncogene homolog [Human], (11p15.5)
Hras	Harvey rat sarcoma virus oncogene 1 [Mouse], (7 86.48 cM)
IDH1	Isocitrate dehydrogenase 1, soluble [Human], (2q33.3)
IDH2	Isocitrate dehydrogenase 2, mitochondrial [Human], (15q26.1)
Ikzf1	IKAROS family zinc finger 1 [Mouse], (11 7.02 cM)
JAK2	Janus kinase 2 [Human], (9p24)
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog [Human], (4q11-q12)
KRAS	Kirsten rat sarcoma viral oncogene homolog, [Human], (12p12.1)
Kras2	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, [Mouse], (6 77.37 cM)
MLL	Mixed lineage leukemia [Human], (11q23)
MLLT2	AF4/FMR2 family, member 1 (AFF1) [Human], 4q21
MLLT3	Myeloid/lymphoid or mixed-lineage; translocated to, 3 [Human], (9p22)
MN1	Meningioma 1 [Human], (22q12.1)
MYB	v-myb avian myeloblastosis viral oncogene homolog [Human], (6q22-q23)

MYC	v-myc avian myelocytomatosis viral oncogene homolog [Human], (8q24.21)
MYH11	Myosin, heavy chain 11 [Human], (16p13.11)
NF1	Neurofibromin 1 [Human], (17q11.2)
NOTCH1	Notch1 [Human], (9q34.3)
Notch1	Notch1 [Mouse], (2 18.91 cM)
NPM1	Nucleophosmin [Human], (5q35.1)
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog, [Human], (1p13.2)
Nras	Neuroblastoma ras oncogene [Mouse], (3 45.25 cM)
PML	Promyelocytic leukemia [Human], (15q22)
RARA	Retinoic acid receptor, alpha [Human], (17q21)
RUNX1	Runt-related transcription factor 1 [Human], (21q22.3)
RUNX1T1	Runt-related transcription factor 1; translocated to, 1 [Human], (8q22)
SIL	SCL-interrupting locus [Human], (1p32)
TAL1	T-cell acute leukemia 1 [Human], (1p32)
TET2	Tet methylcytosine dioxygenase 2 [Human], (4q24)
WT1	Wilm's tumor 1 [Human], (11p13)

INTRODUCTION

Cancer

Life of a human, mouse, or any other mammal starts with a single cell formed when a sperm and an egg cell fuses. All later cells of the organism originate from this cell through repeated cell division. Therefore, all cells contain the same genetic material (DNA), which is the code for how the cell appears and behaves. Despite this, all cells in our body do not look the same or function in the same way. Different organs and tissues are formed through the process of differentiation where certain parts of the genome are activated in certain cells and hence their shape and behavior changes. For example, nerve cells create networks that make up our nerves and muscle cells forms contractile fibers.

Throughout life, the cells that make up organs and tissues need to be replaced and cells continue to divide to give rise to new cells. However, the growth and division of cells needs to be tightly regulated. For a cell to divide, external stimuli, for example from growth factors, are needed. In addition, the vast majority of cells are restricted by a limited number of cell divisions that they can go through. Changes in the DNA of a cell (genetic alterations), can make a cell independent of the external growth control and capable of infinitive cell division [1]. This may result in uncontrolled cell-growth that forms the malignant tumor that we call cancer.

Normal hematopoiesis

The production of blood cells is called hematopoiesis and the first hematopoietic cells can be found in the yolk sac during early embryogenesis and later in the aorta-gonad-mesonephros (AGM) region of the dorsal aorta. These early hematopoietic cells later seed the fetal liver, which becomes the main hematopoietic organ during most of life *in utero.* Eventually, hematopoietic cells from the liver settle in the bone marrow, which is the primary site for hematopoiesis during late embryogenesis and throughout life [2]. Our blood cells form the immune system and include a variety of specialized cells. The embryonic yolk sac, AGM, fetal liver, and the adult bone marrow contain rare hematopoietic stem cells (HSCs) with the capacity to divide and differentiate into all kinds of blood cells (Figure 1). In addition, HSCs have the capacity to self-renew, which means they can divide so that one daughter cell inherits the HSC potential [3]. The capacity of HSCs to reconstitute the whole hematopoietic system is the base for hematopoietic stem cell transplantation (HSCT), which is used as treatment of some inherited disorders, autoimmune diseases, and blood cell diseases such as leukemia.

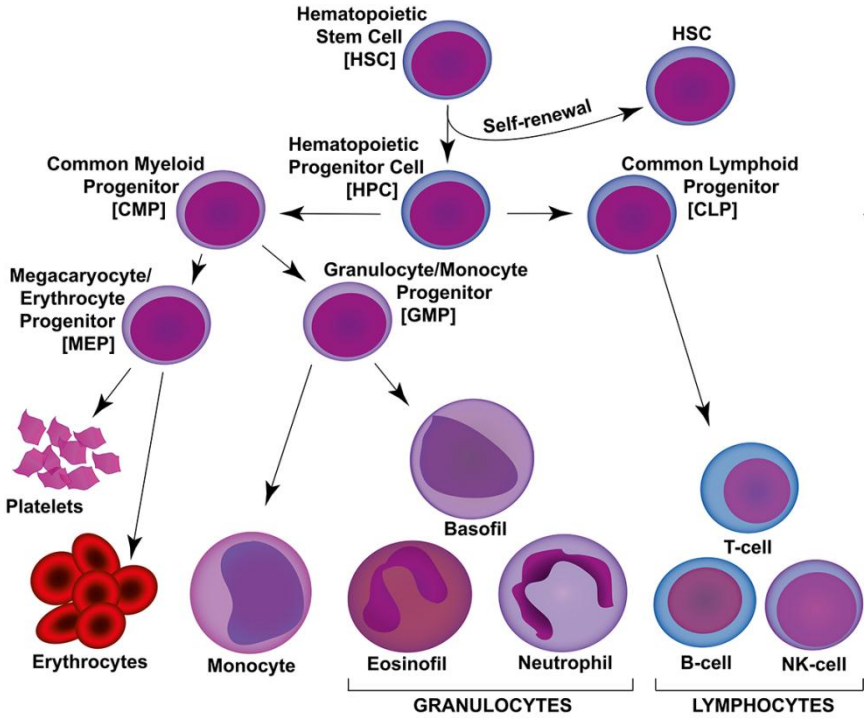

Figure 1. *The hematopoietic hierarchy.*

The specialized hematopoietic cells have different functions in the body. Platelets are responsible for blood coagulation, erythrocytes transport oxygen, and the functions of monocytes and granulocytes are related to our innate immune system. For these processes to function, the production of new cells needs to be balanced to the demand. As outlined in Figure 1, these cells differentiate from a common myeloid progenitor (CMP) and the release of specific growth factors (e.g. thrombopoietin, erythropoietin, and granulocyte-monocyte-colony stimulating factor) stimulates differentiation of the specific lineages. Lymphocytes are part of our adaptive immune system and like the myeloid cells develop from a common progenitor, the common lymphoid progenitor (CLP). Progenitors destined for B-cell differentiation stay within the bone marrow where they develop into mature B-cells but the final steps of B-cell differentiation takes place in the spleen. Early T-cell progenitors leave the bone marrow and settle in the thymus where they develop into mature T-cells. The production of T-cells and B-cells are also dependent on external stimuli for proliferation and differentiation. But instead of responding to the release of growth factors, they are dependent on antigen binding and proper signaling downstream of the T-cell receptor and B-cell receptor, respectively.

Leukemic transformation

The uncontrolled growth of leukemic cells arises from clonal expansion of a transformed hematopoietic stem or progenitor cell (HSPC). The abnormal expansion of cells outcompetes the production of normal hematopoietic cells leading to symptoms as anemia (lack of erythrocytes), bleeding (due to lack of platelets), and sensitivity to infection (due to lack of neutrophils). As mentioned above, normal hematopoietic cells are compromised in terms of cell growth and cell division in several ways; they are dependent on external stimuli for proliferation and they are in general destined for terminal differentiation after a certain number of cell divisions. The transformed cell that gives rise to the leukemic clone has circumvented these control mechanisms by gain of genetic alterations that change the cell's behavior.

Every time a cell divides, all of the over 3 billion DNA base pairs are copied and it is not rare that a mistake is made resulting in a daughter cell with a genetic alteration. Mostly, this alteration does not change the behavior of the cell or it is deleterious leading to cell death. But on rare occasions the genetic alterations make the cell resistant to the normally strict control and transform the cell into a leukemia-initiating cell. Since the number of cell divisions that HSPCs have gone through (and the amount of alterations gained) increases with age [4], so does the risk and incidence of most types of leukemia. Besides age, other factors may also increase the risk for damaging mutations and leukemia. These include environmental factors (e.g. ionizing radiation and chemicals), chemotherapy treatment, and chronic inflammation.

Classification of leukemia

The term leukemia is used to describe all kinds of hematopoietic malignant growth within the bone marrow and blood. The leukemia diagnosis is further defined according to its characteristics and to the cell type of origin.

Acute myeloid leukemia (AML)

The leukemia is of myeloid lineage if the leukemic cells belong to the thrombocytic, erythroid, monocytic, or granulocytic lineage. If also more than 20% of cells within the bone marrow and/or blood are immature cells, called blasts, the leukemia is defined as acute. Since 2004 and revised in 2008, the world health organization (WHO) classification of AML [5] is largely based on identification of somatic genetic alterations (Table 1).

Table 1. *WHO classification of AML and related myeloid neoplasms.*

AML with recurrent genetic abnormalities:
AML with t(8;21)(q22;q22) ;*RUNX1/RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB/MYH11*
Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12);*PML/RARA*
AML with t(9;11)(p22;q23);*MLLT3-MLL*
AML with t(6;9)(p23;q34);*DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21q26.2);*RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13);*RBM15-MKL1*
Provisional entity AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified:
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasms

In the past, AML was instead classified based on morphology of the leukemic cells according to the French-American-British (FAB) system (Table 2). Morphological classification is still in use to classify AML without recurrent genetic abnormalities. This is also displayed by the WHO classification of AML not otherwise specified (Table 1). However, many studies still include FAB classification because patients diagnosed prior to 2004 are included.

Table 2. *FAB classification of AML.*

FAB M0:	AML minimally differentiated
FAB M1:	AML without maturation
FAB M2:	AML with granulocytic maturation
FAB M3:	Acute promyelocytic leukemia (APL)
FAB M4:	Acute myelomonocytic leukemia
FAB M5:	Acute monoblastic/monocytic leukemia
FAB M6:	Acute erythroid leukemia
FAB M7:	Acute megakaryoblastic leukemia

Acute T-cell lymphoblastic leukemia (T-ALL)

Acute lymphoblastic leukemia (ALL) is diagnosed on the basis of the morphological finding of >20% lymphoblasts in peripheral blood and/or bone marrow. Immunophenotyping is used to identify T- or B-cell lineage and expression of terminal deoxynucleotidyl transferase (TdT) and cytoplasmatic CD3 are used as markers for T-cell leukemia. Even though a number of recurrent cytogenetic findings are associated with T-ALL these are not yet included in the classification of T-ALL.

Myeloproliferative neoplasm (MPN)

This disease category describes myeloid neoplasms with increased bone marrow cellularity and increased peripheral blood count but with a blast count below 20%. The most common myeloproliferative neoplasm are chronic myeloid leukemia (CML) which is characterized by the genetic finding t(9;22)(q34;q11);*BCR-ABL*. Other MPNs are polycytemia vera (PV), essential thromocytosis (ET) and primary myelofibrosis (PMF), which typically (PV) or often (ET and PMF) have an activating *JAK2*^{V617F} mutation. Rarer MPNs include chronic myelomonocytic leukemia (CMML), atypical (*BCR-ABL* negative) CML, and juvenile myelomonocytic leukemia (JMML).

The genetic basis of leukemia

The genetic alterations that initiate leukemia can be small (a single altered base within a crucial gene) or they can be large (chunks of DNA moved from one chromosome to another) (Figure 2). The first recurrent alterations that were discovered in leukemia samples were large structural aberrations detected with conventional cytogenetic techniques. In AML, many recurrent cytogenetic findings show great association to clinical outcome and determination of cytogenetic status at diagnosis is largely considered the most powerful genetic prognostic indicator [6-8]. However, cytogenetic aberrations are

found in only about 55% of adult AML and in approximately 75% of pediatric AML [9-11]. The remaining 25-45% of patients have no structural aberration, and this group of patients is termed cytogenetically normal AML (CN-AML). With the emergence of whole genome sequencing, many recurrent alterations affecting only one or a few base pairs have been detected. Many of these are especially common in CN-AML indicating that they may be involved in leukemia initiation. Similarly, many recurrent alterations, both structural alterations and gene mutations, have been discovered in T-ALL [12]. However, the association between genetic findings and prognosis is less clear in T-ALL.

Irrespective of the type of alteration it usually results in inactivation, activation, or altered function of the protein encoded by the affected gene. Genes that encode components normally involved in control of cell division or that promote differentiation are often inactivated in leukemia and are called tumor suppressor genes. Genes encoding proteins

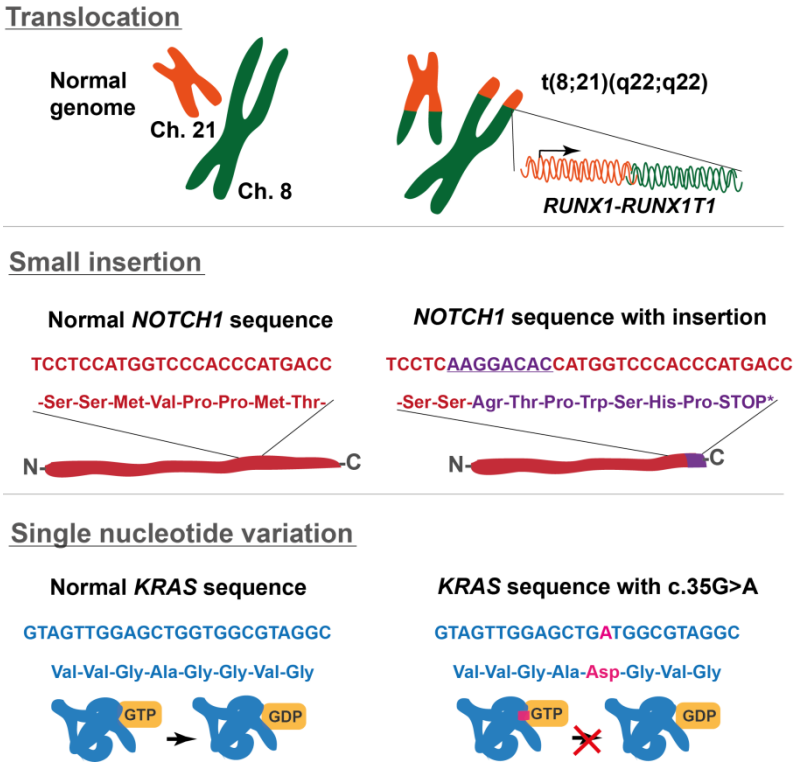

Figure 2. *Translocation between chromosome 8 and chromosome 21 at q22 (t8;21)(q22q22) results in expression of a RUNX1-RUNX1T1 fusion protein. An insertion in the NOTCH1 gene results in a frame-shift in the nucleotide sequence and a premature translation stop. A single nucleotide substitution in the KRAS gene that substitutes glycine for aspartic acid at position 12 renders the KRAS protein constitutively active in the GTP-bound state.*

that promote proliferation or infer capacity of unlimited cell division (i.e. self-renewability) are called oncogenes and are commonly activated in leukemic cells.

In general, the mechanism of how inhibition of tumor suppressor genes and activation of oncogenes cause leukemia falls into two categories; they block differentiation or increase proliferation [13]. It is generally assumed that both these qualities are needed for a cell to transform and to initiate acute leukemia. Therefore, alterations that block differentiation need cooperating mutations that drive proliferation and vice versa. MPN on the other hand, can be initiated by one alteration that mainly increases proliferation. The strict categorization of genetic alterations into these two categories does, however, not always apply. Several recurrent genetic alterations have been shown to infer both proliferative and self-renewing properties. Below follows examples of genes that are commonly altered in AML and T-ALL.

Alterations that block differentiation and/or increase self-renewability

The capacity to divide in a self-renewing manner is highest for the HSCs and gradually decline with lineage commitment and differentiation. An alteration that inhibits differentiation therefore often infers some self-renewability to the cell as well. Mutations that block differentiation often occur in transcription factors or in genes encoding proteins that regulate gene expression by epigenetic modifications (e.g. DNA methylation and histone acetylation or methylation).

CEBPA

The transcription factor CEBPα is crucial for granulocyte differentiation [14] and inactivating mutations in *CEBPA* are found in 4-8% of adult and pediatric AML [15-18]. AML with mutated *CEBPA* is associated with a favorable prognosis [9, 15, 16, 19, 20] and is considered a provisional entity in the WHO classification of AML. The mutations in *CEBPA* are either frame-shift mutations in the N-terminal part resulting in expression of a truncated protein or missense mutations near the C-terminus that cause impaired DNA binding [18].

NPM1

Mutations in *NPM1* are found in approximately 30% of adult and 8% of childhood AML patients and are most common in CN-AML [17, 21-23]. Similar to *CEBPA*, AML with mutated *NPM1* is considered a provisional entity by WHO. The presence of *NPM1* mutation without coexisting *FLT3*-ITD is associated with a favorable prognosis and lower incidence of relapse [9, 21-24]. *NPM1* is usually altered by frame-shift mutations in the region encoding the carboxyl terminus resulting in delocalization of NPM1 from the nucleus to the cytoplasm [21]. AML with mutated *NPM1* is associated with up-regulation of genes normally expressed in HSCs [25] and expression of mutated *NPM1* in mice inhibits megakaryocytic differentiation [26].

inv(16)(p13;q22) or t(16;16)(p13;q22) / CBFB-MYH11

Inversion of chromosome 16 (inv(16)(p13;q22)) or translocation between two copies of chromosome 16 (t(16;16)(p13;q22)) are found in 5-8% of AML patients [5] and are associated with a favorable prognosis [6-8]. The presence of either inv(16)(p13;q22) or t(16;16)(p13;q22) is also diagnostic for AML even if the blast percentage is below 20%. These 16p13q22 alterations result in disruption of the *CBFB* gene and expression of a *CBFB-MYH11* fusion transcript. CBFβ, the protein encoded by CBFB, is part of the core-binding factor (CBF) transcription complex involved in myeloid differentiation. Studies in mice showed that hematopoietic cells with heterozygous expression of *CBFB-MYH11* failed to differentiate into the myeloid lineage, suggesting that CBFB-MYH11 blocks myeloid differentiation through inhibition of CBF-induced transcription [27]. In addition, CBFB-MYH11 has been shown to up-regulate expression of genes implicated in self-renewal [28].

t(8;21)(q22;q22) / RUNX1-RUNX1T1

Translocation between chromosome 8 and chromosome 21 resulting in expression of the fusion transcript *RUNX1-RUNX1T1* (Figure 2) is found in approximately 5% of AML patients [5] and is associated with a favorable prognosis [6-8]. Presence of t(8;21)(q22;q22) is also diagnostic for AML regardless of blast percentage. *RUNX1* encodes the transcription factor CBFα that, like CBFβ, is part of the CBF transcription complex. Expression of *RUNX1-RUNX1T1* in human hematopoietic cells has been shown to inhibit differentiation of lineage-committed myeloid cells [29]. The leukemogenic mechanisms of *RUNX1-RUNX1T1*-expression include both repression of genes normally activated by CBF and activation of other target genes [30].

t(15;17)(q22;q12) / PML-RARA

Translocation between chromosome 15 and 17 (t(15;17)(q22;q12)) is the genetic hallmark of acute promyelocytic leukemia (APL), which comprises 5-8% of AML [5]. With treatment including all-trans retinoic acid (ATRA), this subgroup of AML has a favorable prognosis [6, 7]. The t(15;17)(q22;q12) translocation results in fusion of *RARA* (encoding the transcriptional activator retinoic acid receptor α, RARα) to *PML*. In myeloid cells, binding of retinoic acid to RARα results in expression of genes that are essential for differentiation. The PML-RARα fusion protein has strong affinity for repressor complexes containing co-repressors and histone deacetylases. Since the DNA-binding domain of RARα is intact in the PML-RARα fusion protein, this leads to silencing of RARα target genes. Administration of ATRA gives pharmacological levels of retinoic acid, which induces dissociation of the repressor complex and expression of RARα target genes [31].

MLL

Translocations involving the *MLL* gene are most common in AML but are also frequently found in B-ALL and T-ALL. *MLL*-rearrangement is found in approximately 5% of adult AML

and in 20% of pediatric AML patients [8, 32]. Over 80 different *MLL*-translocations have been identified [33]; t(9;11)(p22;q23);*MLL-MLLT3* and t(4;11)(q21;q23);*MLL-MLLT2* are the most common in AML and ALL, respectively [5]. MLL is a histone methyltransferase that regulates gene expression via chromatin remodeling. MLL-fusions activate transcription of MLL target genes that are involved in regulation of hematopoiesis (including the HOXA-cluster) [34, 35].

TET2 and IDH1/2

TET2 mutations are found in approximately 8% of AML cases [17, 36]. The TET2 enzyme produces 5-hydroxymethylcytosine (5-hmC), which is necessary for DNA demethylation [37]. Inactivating mutations in *TET2* have been associated with increased self-renewal and expansion of HSPCs [36]. Mutations in *IDH1* and *IDH2* are found in 8-17% of adult AML and are most common in CN-AML [17, 36, 38-41]. Altering mutations change the enzymatic activity of IDH1 and IDH2 so that an alternative product, 2-hydroxyglutarate (2HG), is produced [38, 42]. 2HG is similar to the TET2-substrate α-ketoglutarate and high levels of 2HG inhibit TET2 [36, 43]. Thus, mutated IDH1/2 results in inhibition of TET2. *IDH1/2* and *TET2* mutations have also been found to be mutually exclusive in AML, underlining their overlapping oncogenic mechanism [36].

del(1p32) / TAL1

Deletion on chromosome 1 (del(1p32)) is found in 20-25% of all T-ALL cases [44, 45]. This rearrangement results in increased expression of *TAL1* and has been associated with an adverse prognosis in T-ALL [46, 47]. *TAL1* is normally expressed in hematopoietic progenitors and early stages of T-cell development but is down-regulated in mature T-cells [48]. Studies in mice showed that over-expression of *TAL1* can induce T-cell leukemia [49].

NOTCH1 and FBXW7

Approximately 50% of T-ALL cases have an activating mutation in *NOTCH1* that commonly coexist with *FBXW7* mutation [45, 47, 50-56]. The presence of *NOTCH1* mutation was initially associated with an adverse prognosis in T-ALL [55]. However, subsequent studies have either shown that *NOTCH1*/*FBXW7* mutations are associated with better treatment response and a relatively favorable prognosis or have failed to indicate prognostic value [45, 47, 50-53, 56, 57] and the prognostic value of *NOTCH1* and/or FBXW7 mutations in T-ALL is still uncertain. NOTCH1 is a membrane receptor that is cleaved upon ligand binding and the intracellular part locates to the nucleus where it functions as a transcriptional activator. NOTCH1-activation is negatively regulated by phosphorylation of the C-terminal PEST domain and subsequent degradation by the SCF ubiquitin protein E3 ligase complex. FBXW7 is part of this ubiquitin ligase complex and disrupting mutations in *FBXW7* leads to increased levels of active NOTCH1. The activating NOTCH1 mutations either target the C-terminal PEST domain (see Figure 2) resulting in reduced degradation or induce ligand independent activation [54]. NOTCH1 target genes include *MYC* and

HES1 and inactivation of FBXW7 has been shown to infer self-renewability in T-cell leukemia by stabilization of MYC [58]. Coexisting mutations in *NOTCH1* and *FBXW7* is thus likely to create a NOTCH1/MYC/FBXW7 pathway that enhances self-renewability. Consistent with this, *ex vivo* inhibition of NOTCH1 decreased the leukemia initiating potential of T-ALL cells [59]. Also, expression of active NOTCH1 in mouse bone marrow cells induced T-cell leukemia, showing that active NOTCH1 can drive leukemogenesis [60]. However, active NOTCH1 has also been shown to activate pro-proliferative and anti-apoptotic pathways including the PI3K-AKT pathway [61]. Thus, both activation of NOTCH1 and inactivation of FBXW7 (by means of stabilizing NOTCH1 and MYC) mechanistically affect proliferation and self-renewal.

Alterations that increase proliferation and/or survival

As mentioned earlier, HSPCs receive signals from growth factors that induce proliferation and differentiation of certain hematopoietic lineages. Binding of growth factors to membrane bound receptors results in a cascade of intracellular signal transduction that affects proliferation, cell survival, and gene expression. Mutations in growth factor receptors or in proteins involved in downstream signaling induce growth factor-independent proliferation and increased survival and are commonly found in leukemia.

FLT3 and KIT

The FLT3 receptor is expressed mainly on immature myeloid cells and two types of alterations that activate the receptor is identified; internal tandem duplication (ITD) within the juxtamembrane part of the receptor and point mutation within the tyrosine kinase domain (TKD) [62, 63]. *FLT3*-ITD and *FLT3*-TKD mutations are found in approximately 15% and 8% of AML cases, respectively, and are most common in CN-AML [17, 63-68]. The presence of *FLT3*-ITD has repeatedly been associated with an adverse prognosis both in adult and pediatric AML [64, 66, 68-73]. The prognostic value of *FLT3*-TKD mutations are more uncertain; studies have reported association with both adverse [73] and favorable [65, 69] clinical outcome. The stem cell factor (SCF) is a crucial growth factor for myeloid differentiation and signals through the receptor KIT (Figure 3). Mutations in KIT that infer constitutive downstream signaling [74] are found in approximately 4% of AML cases and in up to 30% of patients with CBF AML (i.e. inv(16)/t(16;16)(p13;q22), t(8;21)(q22;q22)) [17, 69, 75, 76]. The mutated KIT and FLT3 receptors are also capable of intracellular signaling from membranes within the golgi and endoplasmatic reticulum adding to the altered signal transduction [77, 78].

t(9;22)(q34;q11) / BCR-ABL1

The presence of a mini-chromosome, termed the ‘Philadelphia chromosome’ relating to the geographic location of its discovery [79], is the hallmark of CML, but is also present in some B-ALL and T-ALL cases. The Philadelphia chromosome is generated by a translocation between chromosome 9 and chromosome 22 and results in expression of a

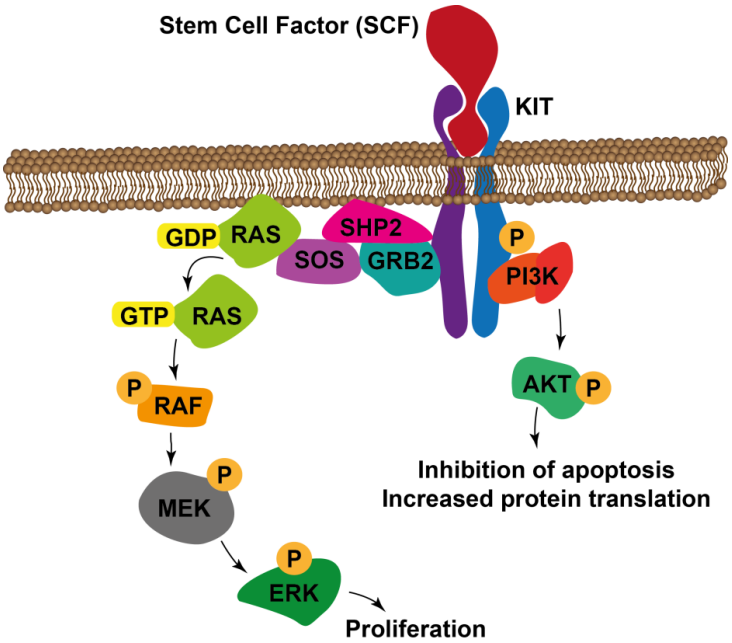

Figure 3. *Binding of the SCF ligand to the KIT receptor induces dimerization and increases the tyrosine kinase activity of the receptor resulting in auto-phosphorylation. The phosphorylated receptor binds and activates PI3K that in turn activates AKT. Activation of AKT affects many processes including apoptosis and protein synthesis. SHP2 and GRB2 also associate with the phosphorylated KIT receptor and recruit SOS. SOS triggers RAS GTP-binding which results in activation of the RAF/MEK/ERK pathway and proliferation.*

BCR-ABL1 fusion protein. ABL1 is a tyrosine kinase that functions downstream of growth factor and adhesion receptors to relay signals for enhanced proliferation, differentiation, and cytoskeletal rearrangement. The BCR-ABL1 fusion has constitutive kinase activity. In addition, motifs in the BCR part create binding sites for SHP2 that bring the fusion protein close to components of the RAS/MEK/ERK and PI3K/AKT signaling pathways; the two most important pathways for BCR-ABL-induced proliferation and survival [80, 81].

WT1

It has been shown that the transcription factor WT1 can bind and stabilize p53, which leads to inactivation of p53-induced apoptosis [82]. On the other hand, over-expression of *WT1* has been reported to inhibit proliferation of cancer cell lines [83], indicating tumor suppressive properties of WT1. Genetic findings in AML mirror these conflicting results; inactivating mutations are observed in 8-14% of patients [84-88] but over-expression of *WT1* is also common [89]. Mutations in *WT1* often co-occur with *FLT3*-ITD [84-86, 88] and have been associated with higher risk for treatment resistance and lower survival rates both in adult and pediatric AML [86-88].

RAS

Mutations in the small GTPase RAS are found in AML, T-ALL, and B-ALL with a frequency of 15-18% [17, 90-92]. Similar to BCR-ABL, activated RAS induce proliferation and inhibit apoptosis through the MEK/ERK and PI3K/AKT pathways. The role of RAS in leukemogenesis is more thoroughly discussed in later chapters of this introduction.

Aberrant gene expression

Approximately 45% of adult AML cases are cytogenetically normal. Of these, around 70% have at least one recurrent gene mutation, but still 10-15% of patients with AML carry no recurrent genetic alteration [9]. Almost all T-ALL tumors on the other hand, show structural alterations, deletions of known tumor suppressor genes or recurrent gene mutations [12]. With the development of micro-array based techniques for gene expression analysis, a lot of effort has been made to profile the gene expression pattern of hematopoietic malignancies in general and of CN-AML in particular. The purpose has been to discover pathways and networks that drive leukemogenesis and to correlate gene expression profiles to prognosis and clinical outcome.

ERG

The importance of the transcription factor ERG in carcinogenesis is indicated by its involvement in chromosomal translocations found in sarcomas, prostate cancer, and leukemia [93-95]. In addition, aberrant expression of *ERG* is common both in T-ALL and in AML [96-98]. Consistent with this, over-expression of *ERG* in mice induces both T-ALL and AML [99, 100]. The mechanisms of ERG-induced leukemia is not fully understood but has been shown to include deregulation of genes involved in myeloid differentiation, activation of the RAS/MAPK-pathway, and activation of the PIM1 kinase [99]. High expression of *ERG* has been associated with high relapse rates and adverse survival in adult CN-AML [96, 98] and in T-ALL [97]. High *ERG* expression was also shown to be associated with adverse survival in *MLL*-rearranged pediatric AML [101].

MN1

The transcriptional co-activator MN1 was identified from its involvement in a t(4;22)(p16;q11) translocation found in a patient with meningioma and in t(12;22)(p13;q11) translocations in AML [102, 103]. Aberrant *MN1* expression is also common in CN-AML [96, 104, 105] and has been associated with poor treatment response and lower survival rates [96, 104, 105]. The leukemogenic mechanism possessed by MN1 is not fully understood but over-expression of *MN1* induced AML in mice [106]. In addition, *MN1* over-expression in mouse bone marrow cells induced gene expression programs reminiscent of early myeloid progenitors [107].

BAALC

The cytoplasmatic protein BAALC was identified in a screen for genes that were over-expressed in AML with sole trisomy 8 [108]. Aberrant expression of *BAALC* was later found to be common also in CN-AML [70, 109] and associated with poor prognosis, high risk for resistant disease, and relapse [70, 96, 108, 109]. BAALC is expressed in hematopoietic progenitor cells and in neuroectodermal tissues but its specific functions remain elusive. Functional studies of *BAALC* over-expression in murine leukemia models showed only minor effect on leukemogenesis and failed to show increase in proliferation or self-renewal [110]. However, the resistance to drug-induced cell-cycle arrest and differentiation was increased in hematopoietic cells over-expressing *BAALC* [110].

Current treatment and risk stratification for pediatric AML and T–ALL

During the last 30 years, the survival rates of pediatric patients with AML and ALL has improved greatly (Figure 4). These improvements are mainly due to risk-adapted therapy with intensification of combined chemotherapy, improvements in stem cell transplant techniques, and better supportive care reducing the number of treatment related deaths [111]. However, a substantial number of children with AML and ALL still relapse and some have resistant disease. The aim of risk stratification is to identify these patients already in the early treatment phase so that further intensification of treatment, often including HSCT, can be given in an attempt to improve outcome. Also, the risk stratification may identify patients with lower risk that benefit from less intense treatment. Some genetic findings are strong prognostic indicators and are also used in risk stratification of pediatric leukemia patients but the most important factor is initial response to treatment.

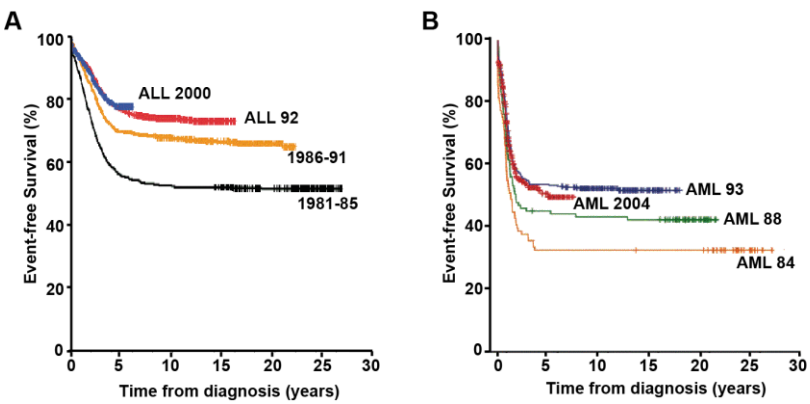

Figure 4. *Event-free survival of children with ALL* ***(A)*** *and AML* ***(B)*** *within Sweden and the Nordic countries and treated according to the NOPHO protocols. Graphs adapted from NOPHO.*

The treatment response is assessed by measuring the amount of remaining leukemic cells, called minimal residual disease (MRD), at certain time points during treatment. MRD is mainly analyzed by flow cytometry or by PCR. For MRD analysis by flow cytometry, the diagnostic sample is used to establish cell surface markers that identify the leukemic blast cells. The presence of leukemic cells in the bone marrow is then analyzed in follow-up samples during treatment. MRD by PCR relies on the identification of a leukemia-specific genetic aberration such as a chromosomal translocation. In ALL, the leukemia-specific rearrangement of the B-cell receptor (BCR) or T-cell receptor (TCR) can be used. The presence of the fusion transcript or the tumor specific BCR/TCR-rearrangement is then analyzed in follow-up samples by quantitative PCR (QPCR). Technical aspects of flow cytometry and PCR are covered in the methods section.

In the pediatric NOPHO-DBH AML2012 protocol, MRD is analyzed by flow cytometry and the risk stratification is as follows:

High-risk patients are identified by any of the following:

- Poor response to the first course of treatment defined as ≥15% leukemic cells in the bone marrow.
- Intermediate response after the second course of treatment defined as 0.1-4.9% leukemic cells in the bone marrow. Patients with ≥ 5% leukemic cells at this time point are classified as having resistant disease.
- The presence of an *FLT3*-ITD mutation without coexisting *NPM1* mutation.

Standard-risk patients are all other patients.

In addition, if the patient fulfils the response criteria for standard-risk and has either inv(16)(p13;q22) or t(16;16)(p13;q22), only two courses of consolidation treatment is given instead of the standard three courses.

In NOPHO ALL2008 [112], MRD is analyzed by QPCR of a leukemia-specific TCR-rearrangement. At diagnosis, all patients with T-ALL are defined as high-risk. If response evaluation after 29 days of therapy shows less than 0.1% leukemic cells, patients are downgraded to the intermediate risk group. If they have a poor response after 29 days of therapy (≥ 5% leukemic cells), HSCT is included in the treatment. High-risk therapy is, however, much more intense than intermediate therapy also without HSCT. In all patients on NOPHO ALL2008, a final response evaluation is performed after 11-12 weeks of therapy and if this shows ≥0.1% leukemic cells, patients are switched to high-risk treatment including HSCT.

The risk stratification for adult AML patients has traditionally been more based on genetic aberrations but recent studies show that treatment response also carries strong predictive power in adult AML [113, 114]. Adults with ALL are increasingly being treated with pediatric protocols with encouraging results. Thus, in the Nordic countries all adults up to 45 years of age are treated on NOPHO ALL2008.

The *RAS* genes

History

In the 1930s, studies showed that a cell- and bacteria-free agent from rat tumors induced neoplasm when injected to animals [115]. Thirty years later, this phenomenon was known to be caused by retroviruses capable of inducing transformation. The identification of the viral genes responsible for the neoplastic transformation led to the identification of viral oncogenes including the Harvey Rat sarcoma virus (v-*HRAS*) and Kirsten Rat sarcoma virus (v-*KRAS*) genes [116, 117]. Studies showed that mammalian cells contained genes (c-*HRAS* and c-*KRAS*) that were highly homologous to these viral genes [118, 119]. Phylogenetic analysis revealed that the mammalian genes were likely to be ancestors to the viral oncogenes, and were accordingly called proto-oncogenes. Further experiments showed that transformation of mammalian cells could be caused by spontaneous mutation within the mammalian homologues [120]. The transformed cells carried a single base pair alteration in *RAS*, resulting in a substitution of glycine for valine at position 12 (RAS^{G12V}) [121]. Subsequent studies in yeast revealed that this alteration increased the activity of RAS [122]. In addition, a third human *RAS* gene was discovered, called neuroblastoma *RAS* (*NRAS*) based on the neuroblastoma cells in which it was characterized [123, 124]. Since these initial discoveries, great efforts and great advances have been made in understanding the normal functions and the oncogenic actions possessed by RAS.

Function and signaling

Human cells express four RAS isoforms, NRAS, HRAS, KRAS4A, and KRAS4B. The two KRAS isoforms are generated by alternative splicing from the *KRAS* locus. Mice express only one variant of KRAS, corresponding to the human KRAS4B. The four RAS isoforms have highly similar sequences but differ in the C-terminal part resulting in slightly different post translational modifications. All RAS isoforms carry a CAAX-box (C - Cysteine, A - Aliphatic amino acid, and X - Any amino acid) at the end of the carboxyl terminus that triggers the attachment of a farnesyl lipid to the cysteine residue, cleavage of the last three amino acids (-AAX), and addition of a methyl group to the farnesylated cysteine. NRAS, HRAS, and KRAS4A are also palmitoylated at cysteine residues upstream of the farnesylated CAAX cysteine whereas KRAS4B contains a hydrophobic lysine-rich stretch in this region [125]. The farnesyl lipid and the palmitoyl groups attach RAS to hydrophobic cellular membranes and anchor RAS in the inner layer of the plasma membrane [126].

NRAS, HRAS, and KRAS4B is ubiquitously expressed in all tissues, although their relative levels vary [125, 127]. The KRAS4A isoform is expressed at lower levels and hereafter KRAS4B will be referred to as KRAS. The function of the different RAS isoforms overlap to some extent but they are also biologically different; their subcellular localization differs [125] and while mice lacking expression of both HRAS and NRAS are viable [128], knockout of KRAS results in embryonic lethality [129]. The RAS proteins are monomeric guanosine tri-phosphatases (GTPases) and bind guanosine di-phosphate (GDP) in their inactive form. Binding of growth factors to membrane bound receptor tyrosine kinases activates guanine nucleotide exchange factors (GEFs, e.g. SOS) that release the bound GDP so that free guanosine tri-phosphate (GTP) can bind instead. Binding of GTP activates RAS by enhancing the affinity for downstream targets. RAS harbors only a weak intrinsic GTPase activity and generally stays GTP-bound until GTPase activating proteins (GAPs, i.e. NF1 and p120RASGAP) catalyze the hydrolysis to GDP. The downstream targets of active RAS are numerous (Figure 5) and some effector pathways are better understood than others, for comprehensive reviews of RAS signaling see [130-133].

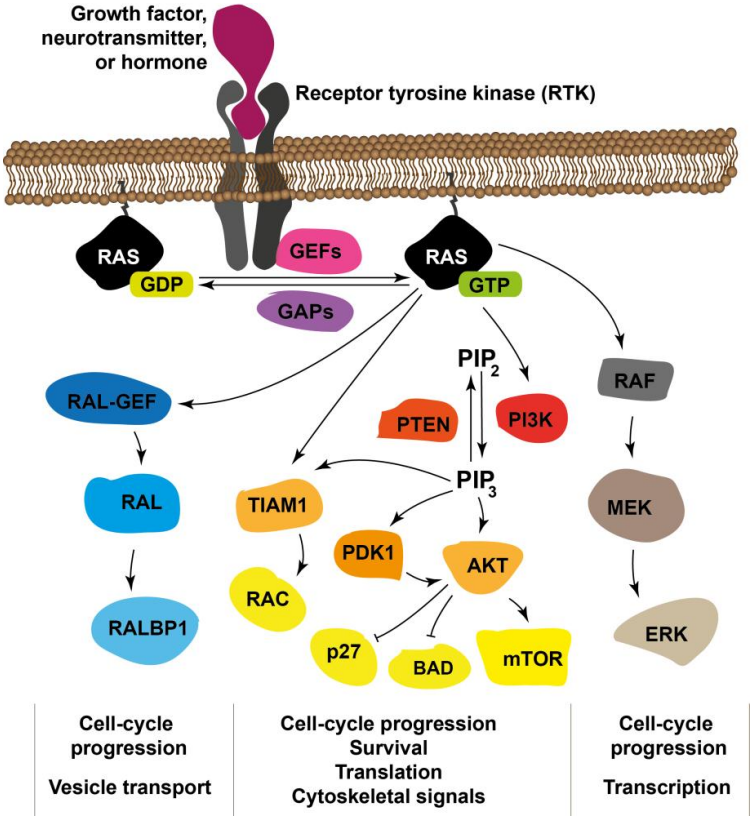

Figure 5. *RAS signaling.*

RAF/MEK/ERK

GTP-bound RAS binds to and activates the three closely related RAF proteins (c-RAF1, BRAF, and ARAF). The active RAF kinases phosphorylate and activate mitogen-activated protein kinase kinase (MAPKK) 1 and 2 (MEK1 and MEK2), that in turn phosphorylate and activate the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2. Active ERK1/2 relocate to the nucleus and regulate transcription through interaction with transcription factors such as ELK1 and c-JUN. Transcriptional activation leads to up-regulation of factors that promote proliferation and cell-cycle entry.

PI3K/AKT/mTOR

Active RAS interacts with and activates type I phosphatidylinositol 3-kinases (PI3Ks) [134]. PI3K phosphorylates phosphatidylinositol-bisphosphate (PIP_2) to produce phosphatidylinositol-trisphosphate (PIP_3). PIP_3 has many downstream targets including phosphatidyl-inositol dependent kinase 1 (PDK1) that phosphorylates and activates AKT. Activated AKT can phosphorylate many different targets resulting in inhibition of apoptosis (phosphorylation of BAD), increased protein translation (phosphorylation of the mTOR-inhibiting complex TSC1/2), and cell-cycle progression (phosphorylation of p27) [135]. In addition, PIP_3 activates the RAC-GEF T-cell lymphoma invasion and metastasis protein 1 (TIAM1), resulting in activation of RAC, which has effects on actin organization and proliferation. RAS has also been shown capable to induce activation of RAC through PI3K-independent mechanisms [136].

RAL-GEF/RAL/RALBP1

Active RAS activates the RAS-like GTPases (RALs) through activation of RAL guanine nucleotide exchange factors (RAL-GEFs). Activated RAL has been implicated in actin organization and cell migration through interaction with Filamin and in vesicle transport through activation of RALBP1. In addition, active RAL affects proliferation through activation of Src [137].

Hyperactivated RAS in leukemia

RAS signaling is crucial for responses induced by cytokines and growth factors in all hematopoietic lineages. It is therefore not surprising that activating mutations in *RAS* are commonly found in AML [17, 90], MPN [138, 139], and in ALL [91, 92]. The most frequent mutations affect codon 12, 13, and 60 and activate RAS by impairment of GTP hydrolysis and impaired binding to RAS-GAPs [140, 141]. In hematological malignancies, mutations in *NRAS* are most frequent while *KRAS* mutations are fairly common and mutations in *HRAS* are rare to nonexistent [90, 92, 142]. In addition to direct *RAS* mutations, RAS can be activated indirectly by inactivation of the RAS-GAP NF1 [143-145] or by activating mutations in receptors upstream of RAS such as FLT3 and KIT [17, 69, 75, 76, 146].

Activation of the RAS pathway in myeloid progenitors results in hypersensitivity to growth factors such as granulocyte-macrophage colony stimulating factor, which is a hallmark of JMML [138]. In CMML, a closely related disease, more than 20% of cases carry a *RAS* mutation, and the presence of mutated *RAS* has been associated with more proliferative phenotypes [139, 147]. In AML, *RAS* mutations are found in approximately 15% of cases and are most frequent in AML with monocytic or granulocytic differentiation [90]. Children with the genetic syndrome neurofibromatosis have inactivating germline mutations in *NF1* and an increased risk of developing JMML, which suggests that hyperactive RAS is capable of initiating MPN [138]. In AML, genetic studies comparing samples at diagnosis to those at relapse have shown that *RAS* mutations are frequently lost or gained as part of the clonal evolution of the leukemic tumor [4, 148], indicating that they are late cooperating events. Consistent with this, hyperactivation of RAS in mouse bone marrow cells induce MPN but not AML [149-151]. The incidence of *RAS* mutations in ALL is approximately 15% [91, 92] and is particularly common in the subgroup of patients with early T-cell progenitor leukemia [145, 152]. Activation of RAS downstream of the T-cell receptor is crucial for normal T-cell proliferation and differentiation [153] but the mechanistic role for hyperactive RAS in T-lymphoid leukemogenesis is not clear.

Mouse models of RAS-induced leukemogenesis

Over-expression of oncogenic RAS

Bone marrow transduction and transplantation using oncogenic viral *HRAS* (MSCV-v-*HRAS*) was shown to induce a mix of B- and T-cell lymphoma in the recipient mice [154]. Studies using a similar model but with retroviral expression of oncogenic *NRAS* showed development of MPN phenotypes in recipient mice [155, 156]. One study investigated and compared the leukemogenic potential of *NRAS*^{G12D}, *HRAS*^{G12V}, and *KRAS4B*^{G12D} when ectopically expressed from retroviral promoters in mouse bone marrow cells [157]. *HRAS*^{G12V} induced leukemia most rapidly but all oncogenes induced disease within 80 days. The phenotypes were also slightly different; expression of *HRAS*^{G12V} and *NRAS*^{G12D} were capable of inducing an AML-like disease with accumulation of immature myeloid cells while expression of *KRAS*^{G12D} induced MPN.

Spontaneous RAS activation

Johnson *et.al.* [158] constructed an altered *Kras2* allele carrying the G12D-mutation but with 2 copies of exon 1. This allele does not express KRAS^{G12D} until homologous recombination has removed one of the duplicated exons. Approximately 50% of mice carrying this allele developed T-cell lymphomas after spontaneous recombination.

Endogenous and tissue specific expression of oncogenic RAS

Development of mouse models with conditional oncogenic *RAS* alleles based on the CRE-*lox*P system (LSL-*Kras2*^{G12D} and LSL-*Nras*^{G12D}, see the methods section for details)

enabled tissue specific expression of hyperactive RAS. These two alleles have extensively been combined with the interferon inducible Mx1-*Cre* allele. Injection of pI-pC in these mice results in expression of the oncogenic *RAS* allele in the bone marrow and in other interferon responsive tissues [159]. pI-pC-injected LSL-*Kras2*^{G12D};Mx1-*Cre* (*KM*) mice rapidly developed MPN resembling human CMML and the mice died after 4–16 weeks with severe anemia, monocytosis, and granulocytosis [150, 151]. LSL-*Nras*^{G12D};Mx1-*Cre* (*NM*) mice also developed MPN with leukocytosis, splenomegaly, and infiltration of mature myeloid cells into non-hematopoietic tissues [160, 161]. The disease latency was much longer in *NM* mice compared to *KM* mice but was shortened by homozygous expression of *Nras*^{G12D} [162]. Interestingly, transplantation of the bone marrow from *KM* mice with MPN to wild-type recipient mice induced T-ALL in several studies [163-169]. Similarly, transplantation of NRAS^{G12D} MPN bone marrow to wild-type recipients induced T-ALL but with a lower penetrance compared to KRAS^{G12D} bone marrow [162]. Transplantation of bone marrow with homozygous NRAS^{G12D} expression did however induce T-ALL with higher penetrance [162].

The mechanism behind the phenotypic switch from myeloid to T-lymphoid after transplantation of bone marrow cells from *KM* and *NM* mice has been investigated but is not clear. Zhang *et.al*. did a limiting dilution transplantation with KRAS^{G12D} bone marrow cells and found that 1.5×10^6 bone marrow cells were needed to induce MPN while T-ALL was induced in the recipient mice by as few cells as 0.3×10^6 [169]. This finding indicates that KRAS^{G12D} T-lymphoid tumor-initiating cells (TICs) are more numerous than KRAS^{G12D} myeloid TICs. However, why the T-lymphoid TICs do not induce T-ALL in the *KM* and *NM* donor mice is not known. The Mx1-*Cre* allele is not hematopoietic specific and pI-pC injected *KM* mice develop lesions also in non-hematopoietic tissues including skin, colon, and liver [170]. Experimental studies in mice have shown that genetic alterations in non-hematopoietic tissues such as the bone marrow stroma and the skin can induce myeloid neoplasms [171, 172]. Thus, the MPN phenotype in *KM* and *NM* mice might result from expression of hyperactive RAS in non-hematopoietic tissues. The low incidence of MPN in the transplanted wild-type mice may then accordingly be explained by lack of this altered tissue microenvironment. Or alternatively, expression of KRAS^{G12D}/NRAS^{G12D} in non-hematopoietic tissues may inhibit T-ALL development in *KM* and *NM* mice.

The TICs are likely immature progenitor cells and studies of the effect of hyperactive RAS-expression on hematopoietic progenitor populations show slightly varied results. Regarding thymic T-lymphoid progenitors, one study reported that pI-pC-injected *KM* mice had a reduced thymic cellularity [164] while another reported increased thymic mass in these mice [166]. Both studies did however report an increased proportion of double negative ($CD4^-CD8^-$) thymocytes compared to control mice, suggesting that expression of KRAS^{G12D} affects T-cell differentiation. Chan *et.al.* reported that the frequency of myeloid progenitors in pI-pC injected *KM* mice were normal in the bone marrow while an increase

was seen in the spleen [151]. Similar studies of *NM* mice showed an increase of myeloid progenitors both in the bone marrow and spleen [161, 162].

The long latency for T-ALL development (2-4 months) after transplantation of $KRAS^{G12D}$ and $NRAS^{G12D}$ bone marrow indicates a need for cooperating secondary mutations. Similar to human T-ALL, these tumors have been shown to carry activating *Notch1* mutations [163-166]. In addition, one study where leukemogenesis was accelerated with viral insertional mutagenesis identified disruption of *Ikzf1* as a cooperating genetic event in $KRAS^{G12D}$-induced T-ALL [163].

AIM

The overall aim of this thesis has been to evaluate genetic alterations as prognostic markers in children with AML and T-ALL and to increase our understanding of RAS-driven leukemogenesis.

Specific aims

- The aim of the study presented in Paper I and the related response letter was to evaluate mutations in *FLT3*, *NPM1*, *CEBPA,* and *WT1* and gene expression levels of *MN1*, *ERG*, *BAALC, FLT3, and WT1* as prognostic markers in children with AML. Since most previous studies had evaluated them separately, the aim was also to determine their relative independence as prognostic markers.

- The study presented in Paper II aimed to evaluate *NOTCH1* and *FBXW7* mutation status and NOTCH1-activity, judged by expression of the target genes *HES1* and *MYC,* as prognostic markers in pediatric T-ALL.

- The aim for the study presented in Paper III was to investigate mechanisms of KRAS-driven leukemogenesis in myeloid and T-lymphoid cells and to determine the cause for the phenotypic switch from MPN to T-ALL after transplantation of bone marrow from LSL-*Kras2*^{G12D};Mx1-*Cre* mice to wild-type recipients.

PATIENTS & METHODS

A detailed description of the patient cohorts and all methods used can be found in the attached papers. This section aims to give an over-view of the methods and to provide a better understanding of their utilities and limitations.

Patient Samples

Pediatric AML patients

Leukemic bone marrow or peripheral blood cells from 185 children newly diagnosed with AML were collected at centers in Denmark, Finland, Iceland, Norway, and Sweden between 1997 and 2007. The age range was 0-18 years with a median age of 6 years. Patients with Down syndrome or APL were not included. The patients were treated according to two consecutive treatment protocols; NOHPO AML93 (1997-2003) [173] and NOPHO AML2004 (2004-2007) [174]. Genomic DNA was obtained from all 185 patients, whereas RNA was available from 149 patients. Cytogenetic data were available for 183 of the patients.

Pediatric T-ALL patients

Leukemic bone marrow cells from 79 children newly diagnosed with T-ALL were collected at the centers for pediatric oncology in Sweden (Gothenburg, Linkoping, Lund, Stockholm, Umea, and Uppsala) between 1992 and 2008. The patients were treated according to the NOPHO ALL92 and the NOPHO ALL2000 protocols [175]. Genomic DNA was obtained from all 79 patients, whereas RNA was available from 30 patients.

The studies presented in Paper I and Paper II were approved by regional ethics committees and informed consent was obtained according to the Declaration of Helsinki.

Methods

Mutation analyses

Gene mutations were analyzed using different polymerase chain reaction (PCR)-based methods. The PCR results in selective amplification of DNA, and is based on the principle of DNA replication. Two oligonucleotides (designated oligos or primers) that are homologous to sequences at the end of the DNA fragment of interest are used as initiation sites for the DNA polymerase. Cycles of repeated denaturation, oligo hybridization, and DNA polymerase elongation creates an exponential increase of the DNA fragment spanned by the two oligos.

Fluorescence based fragment size analysis

To mark the amplified fragment, one of the oligos in the PCR has a fluorophore (e.g. 6-FAM™) attached to its 5'-end which results in incorporation of this fluorophore into the PCR product. The amplified product is then mixed with a number of fragments of known sizes labeled with another fluorophore (e.g. ROX™). The mixed sample is analyzed by capillary electrophoresis where a laser beam excites the fluorophores and their respective emission is detected. The size of the amplified fragment can thus be determined in relation to the known fragments. Deviation in size of the amplified fragment due to insertion or deletion is possible to detect. However, which nucleotide bases within the fragment that is deleted or inserted cannot be revealed. In Paper I, fragment size analysis was used to analyze the presence of *FLT3*-ITD and *NPM1* mutation in the pediatric AML patients.

Restriction fragment length polymorphism (RFLP)

After amplification, the PCR-product is treated with a restriction enzyme that specifically recognizes and cleaves a certain DNA sequence within the fragment. Hence, any alteration in this specific site will impair cleavage. Subsequent analysis of the PCR-product on an agarose gel reveals if cleavage has occurred or not. In Paper I this method was used to detect *FLT3*-TKD mutations. Within the *FLT3* gene, the recognition site for EcoRV ('GATATC') corresponds to the two codons for amino acids Asp835 and Ile836, which is the site for the most common *FLT3*-TKD mutations. However, any alteration in the sequence will not change the encoded amino acids (for example, 'GACATC' still encodes Asp-Ilu, but will impair cleavage by EcoRV. To be certain that the alteration leads to a missense mutation the PCR-product needs to be sequenced.

Sanger sequencing

Fluorescent dye terminator Sanger sequencing with detection by capillary electrophoresis can be used to detect the nucleotide sequence of the amplified DNA or cDNA fragment. The PCR-amplified product is purified from superfluous oligos, deoxynucleotides (dNTPs), and DNA polymerase enzyme and mixed with new primers, dNTPs, fluorescently labeled dideoxynucleotides (ddNTPs), and DNA polymerase. The ddNTPs terminate the polymerase elongation after incorporation, and with the right proportion of dNTPs vs. ddNTPs the reaction results in a distribution of fragments terminated at the different base positions. Subsequent capillary electrophoresis where the fluorescence for each fragment size corresponds to the nucleotide in that position reveals the complete sequence of the fragment. Sequencing can detect single base alterations, deletions, or insertions within the analyzed fragment although the sensitivity is somewhat low and depends on technical quality. Generally, presence of an altered allele below 20% is not possible to detect with Sanger sequencing [176, 177]. In Paper I, sequencing was used to analyze *WT1* mutations in the pediatric AML samples and in Paper II to analyze *NOTCH1* and *FBXW7* mutations in the pediatric T-ALL samples. In Paper III, sequencing was used to analyze *Notch1*

mutations and the absence of wild-type *Kras2* RNA in the mouse KRAS^{G12D} T-ALL tumors.

Gene expression analysis

Quantification of cDNA or genomic DNA can be done using quantitative PCR (QPCR). This method relies on the exponential amplification of genetic material in the PCR resulting in the theoretical formula $N_{Ct}=2^{Ct}\times N_0$ (where N_0 is the amount of starting material and N_{Ct} the amount of material at cycle Ct). The number of cycles it takes to reach a certain amount of template material thus correlates with the amount of starting material. The amount of the analyzed DNA fragment is then normalized to the amount of a fragment from a gene that is thought to be evenly expressed in all cells (called a housekeeping gene) or to the amount of an allele with known copy number if genomic DNA is quantified. There are several ways to quantify the amount of template material during the PCR and two techniques were used in this thesis.

SYBR®green based QPCR

The fluorescence emitted by the fluorophore SYBR®green is enhanced when bound to double stranded DNA. Including SYBR®green in the PCR thus leads to an increase in fluorescence in proportion to the amount of double stranded DNA. However, since SYBR®green binds to any double stranded DNA, any unspecific product will add to the signal. It is therefore crucial that the PCR oligos do not give unspecific amplification. SYBR®green based QPCR on cDNA was used in Paper I to evaluate the gene expression level of *ERG*, *MN1*, *BAALC*, *WT1,* and *FLT3* in the pediatric AML samples. In Paper III, SYBR®green based QPCR on genomic DNA was used to analyze the allelic burden of wild-type and mutated (G12D) *Kras2* allele in the mouse T-ALL cells.

TaqMan® based QPCR

TaqMan® assays include both gene specific oligos and a template specific oligo-probe that is coupled to a fluorophore (e.g. FAM) and a quencher (e.g. TAMRA). The quencher inhibits fluorescence as long as the probe is intact. The probe binds to the single stranded template and in the following cycle, the DNA polymerase degrades the probe resulting in release of the fluorophore. Hence, the amount of fluorescence emitted from the reaction is a measure of the amount of template at that point. The extra specificity gained from the probe means that any unspecific product that the oligos may amplify will not be recorded. In Paper II, TaqMan® assays were used to analyze the gene expression level of *HES1*, *MYC*, *MYB*, and *NOTCH1* in the pediatric T-ALL samples.

The LSL-Kras2^{G12D};Mx1-Cre mouse model

The *Cre-lox*P technique is based on the bacteriophage P1-derived CRE recombinase that recognizes, binds, and recombines a specific sequence designated *lox*P (locus of crossing over P1). The *lox*P site is a 34 base pair (bp) DNA sequence consisting of two 13-bp inverted repeats with an 8-bp spacer in between [178]. CRE recombines the DNA resulting in deletion of the sequence that is flanked by *lox*P sites ('floxed') (Figure 6). By combining a floxed allele with expression of *Cre* recombinase under the control of a certain promoter it is possible to express or delete genes in a tissue- and time-specific manner.

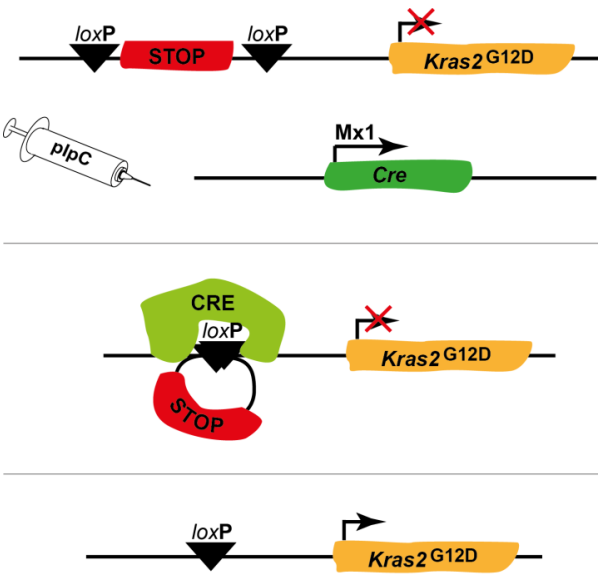

Figure 6. *pI-pC-induced CRE recombination of the LSL-Kras2^{G12D} allele.*

The LSL-*Kras2*^{G12D} allele was engineered to contain two alterations, one point mutation resulting in an aspartic acid (D) instead of a glycine (G) at position 12 (G12D) and a floxed STOP cassette (*lox*P-STOP-*lox*P, LSL) within the promoter region [179]. After CRE recombination, the STOP cassette is removed and transcription from the mutated *Kras2*^{G12D} allele is induced (Figure 6). The LSL-*Kras2*^{G12D} allele equals a *Kras2* knock-out allele before recombination. Since homozygous deletion of *Kras2* is embryonically lethal [129], mice harboring this allele needs to be heterozygous with one copy of wild-type *Kras2*. With the Mx1-*Cre* allele, expression of *Cre* is under control of the interferon-responsive Mx1 promoter [159]. Interferon is produced by the immune system in response to viral infection and cells that express interferon receptors on their cell surface respond by activation of genes with interferon-responsive promoters. To enforce extensive activation of *Cre* expression in Mx1-*Cre* mice, a double stranded RNA, polyinosinic-polycytidylic acid (pI-pC), can be injected intraperitoneally mimicking a viral infection. This triggers interferon production and CRE expression in interferon responsive cells. Cells of the hematopoietic

lineage are highly sensitive to interferon but pI-pC injection induces CRE expression also in cells of some other tissues including liver, intestine, and skin [159, 170]. The combined LSL-*Kras2*^{G12D};Mx1-*Cre* (*KM*) mouse model thus results in expression of oncogenic *Kras2* from the endogenous promoter primarily in the bone marrow but also in some other tissues. Noteworthy, low levels of interferon will be produced even without the administration of pI-pC, which results in expression of *Kras2*^{G12D} in some interferon-responsive cells. In Paper III, *KM* mice were used to model KRAS^{G12D}-induced myeloid and T-lymphoid leukemogenesis. Animal procedures were approved by the animal research ethics committee in Gothenburg, Sweden.

Mouse bone marrow transplantation

Bone marrow transplantation in mice can for example be used to study the tumor-initiating capacity of leukemic cells in secondary recipients or the effect of *ex vivo* manipulation of hematopoietic cells. Whole body irradiation is used to eradicate the bone marrow cells in the recipient mice. Hematopoietic cells from donor mice are injected intravenously, home to the bone marrow, and repopulate the hematopoietic system. In Paper III, mouse bone marrow transplantation was used to distinguish between the effects of KRAS^{G12D} expression in hematopoietic cells and in non-hematopoietic tissues (Figure 7).

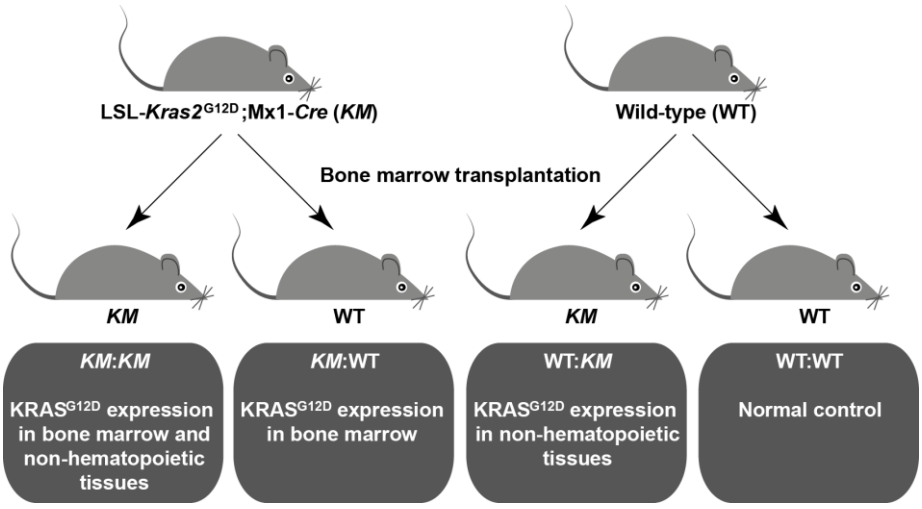

Figure 7. *Experimental strategy for cross bone marrow transplantations to investigate the phenotypic effects of KRAS^{G12D}-expression in hematopoietic cells and in non-hematopoietic tissues.*

BrdU incorporation

Bromodeoxyuridine (BrdU) is an analogue to the thymidine nucleotide. BrdU can be added to cell cultures or injected into animals, which results in incorporation of BrdU into the newly synthesized DNA of proliferating cells. Antibodies that specifically bind to BrdU-containing DNA are then used to detect proliferating cells. Bound antibodies can either be detected with immunohistochemistry or by fluorescent labeling of the antibodies. In Paper III, BrdU incorporation was used to analyze the proportion of proliferating myeloid and T-lymphoid cells in *KM* mice *in vivo*.

Flow cytometry

Flow cytometry, also called fluorescence activated cell sorting (FACS), is used to characterize cells based on their expression of cell surface proteins, intracellular markers, or expression of fluorescent proteins (e.g. GFP). Cells in suspension are incubated with fluorescently labeled antibodies directed to cell surface proteins or alternatively, cells can be treated with agents that permeabilize the cell membrane allowing for binding of anti-bodies to intracellular structures. Presence of cell surface- and intracellular antigens is then analyzed on the single cell level by a flow cytometer that measures the specific fluorescence emitted from the bound antibodies. Defined cell populations may also be sorted and recollected for DNA/RNA preparation or other assays. In Paper III, FACS analysis was used to study subsets of hematopoietic progenitor cells defined by their cell surface antigens, to analyze BrdU incorporation, and to sort T-ALL cells and myeloid hematopoietic cells from mice.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is used to detect specific DNA sequences on chromosomes. DNA corresponding to the sequence of interest is labeled with a fluorophore and after hybridization to the cellular DNA, the presence and location of the sequence can be visualized by fluorescence microscopy. In Paper III, FISH was used to determine the copy number of *Kras2* loci in the mouse T-ALL cells.

Statistical methods

Pearson X^2 test, Fisher's exact test, and Mantel–Haenszel X^2 test

The X^2-test (or Chi-square test) and the Fischer's exact test are used to compare the distribution of a variable within a population to an expected distribution. The X^2-test can be used when the expected frequency is at least five for all outcomes. If that is not the case, the Fisher's exact test should be used instead. The Mantel-Haenszel X^2 test can be used to analyze a linear association of the distribution when the distribution variable has more than two categories and when these can be ordered. In Paper I and Paper II, the Pearson X^2-test and Fisher's exact test were used to compare the distribution of categorical (e.g.

mutation present or not) or dichotomized (e.g. high and low expression level) variables. In Paper II, the Mantel-Haenszel X^2-test was used to test for association between *NOTCH1/FBXW7* mutation status and treatment response. In Paper III, the Pearson X^2-test was used to compare the incidence of T-ALL between experimental groups (e.g. primary pI-pC-injected *KM* mice and mice transplanted with $KRAS^{G12D}$ bone marrow).

Student's t-test

The Student's t-test can be used to compare the means of a numerical variable between two sample populations if the values can be assumed to follow a normal distribution and to have equal variance in both groups. In Paper III, the Student's t-test was used for many pairwise comparisons between experimental groups (e.g. numbers of hematopoietic progenitors).

Mann-Whitney U test and Kruskal-Wallis test

If the sample distribution cannot be assumed to follow a normal distribution the Mann-Whitney *U* test can be used instead of the Student's t-test. This is often the case for gene expression values, which tend to follow a log-normal distribution. To eliminate the impact from the distribution and possible outliers, values are ranked according to their order and then the rank sum is compared between the samples. If the sample distribution is unknown, the Mann-Whitney *U* test can still be used since its efficiency in comparisons of normally distributed samples are nearly as good as the Student's t-test. Similar to Mann-Whitney *U* test, the Kruskal-Wallis test can be used when the sample distribution cannot be assumed to follow the normal distribution or to have equal variance, but is appropriate when comparing more than two groups. In Paper I and Paper II the Mann-Whitney *U* test was used for pairwise comparisons of continuous values and the Kruskal-Wallis test was used in Paper I for comparison of continuous values when more than two groups were compared (e.g. WBC count in patients with wild-type *CEBPA* and with single or double *CEBPA* mutations). In Paper III, the Mann-Whitney *U* test and the Kruskal-Wallis test were used for comparison of relative thymus weight between experimental groups.

Post-hoc tests

When doing multiple independent comparisons (using the Student's t-test, the Mann-Whitney *U* test or the Kruskal-Wallis test) the likelihood for significance in one of the comparisons increases as the number of comparisons increase. To reduce the risk of falsely rejecting the null-hypothesis (type I error) due to this, multiple testing correction sometimes needs to be applied. In Paper I, Bonferroni correction was used to compensate for multiple testing within subgroups (e.g. for multiple comparisons of WBC count between patients with mutation and without). In Paper III, Dunn's multiple comparison test was used for correction in the comparison of thymus weight between several experimental groups.

The Log-rank test and Cox proportional hazard regression

The Log-rank test is used to compare the survival curves generated by Kaplan-Meier estimates for two or more groups and was used in Paper I, Paper II, and Paper III. Cox proportional hazard regression is a method where the contribution of multiple input variables (both time independent and time-dependent) can be analyzed, also called a multivariate analysis. In Paper I, Cox regression was used for analysis of the relative independence of the genetic prognostic markers as well as their independence to other factors such as age and WBC count. In Paper II, Cox regression was used to evaluate the independent prognostic value of increased *HES1* expression in relation to factors such as age and WBC count.

RESULTS & DISCUSSION

FLT3-ITD and high BAALC expression are independent prognostic markers in pediatric AML – Paper I

FLT3-ITD, FLT3-TKD mutations, *WT1* mutations, and high gene expression of *ERG*, *MN1,* and *BAALC* have all been associated with adverse prognosis in AML [64, 66-68, 70, 72, 86-88, 96, 98, 104, 105, 109, 146]. AML with mutated *NPM1* or *CEBPA* on the other hand, has been associated with a relatively favorable prognosis [9, 15, 16, 19, 20, 22-24]. The vast majority of previous studies regarding these genetic markers had focused on adult CN-AML and no previous study included all eight genetic markers. To evaluate the prognostic value of these markers in pediatric AML we analyzed mutation status and gene expression levels in patients treated according to the NOPHO protocols. The frequency of mutations in our material; *FLT3*-ITD 10%, *FLT3*-TKD mutation 4%, *NPM1* mutation 6%, *CEBPA* mutation 6%, and *WT1* mutation 8%, was comparable to previous reports in pediatric AML [15, 22, 66, 68, 72, 85, 86, 180].

In univariate analysis, *FLT3*-ITD, high *ERG,* and high *BAALC* expression were predictive for lower event-free survival (EFS)[1]. Presence of *FLT3*-ITD was also a marker for lower overall survival (OS) of patients that did not receive HSCT in first remission, indicating that this group may benefit from transplantation. In addition, presence of *NPM1* mutation without coexisting *FLT3*-ITD was associated with favorable EFS, which is consistent with previous studies in children and young adults with AML [22, 24]. Factors that reached statistical significance in univariate analysis were combined in a multivariate analysis to determine independence and to correct for WBC count, age, treatment response, cytogenetic risk-group, and HSCT. In line with earlier studies on pediatric AML [66, 68, 72], we found that *FLT3*-ITD was an independent prognostic marker for lower EFS. In addition, high *BAALC* expression was an independent marker for lower EFS. The role of BAALC in AML is however not known and over-expression of *BAALC* in animal models have failed to show an effect on leukemogenesis [110]. This indicates that aberrant *BAALC* expression may be a passive marker for currently unknown events.

In a comment to our study, Pigazzi *et.al*., reported that high *ERG* expression was an independent prognostic marker in pediatric AML with *MLL*-rearrangement [101]. We were invited to respond to this finding and indeed, our data supported the association between high *ERG* expression and adverse EFS in this subgroup. That high *ERG* expression was not an independent prognostic marker in our material including all cytogenetic subgroups largely depended on the correlation between high *ERG* and high *BAALC* expression. However, in the *MLL*-rearranged group, high *ERG* expression was actually a stronger

[1]Defined as time from diagnosis until relapse, resistant disease or death from any cause

predictor for EFS than high *BAALC* expression. The association between high *BAALC*, high *ERG,* and high *MN1* expression has also been observed by others [96, 98, 105], and underlines the importance of investigating the relative independence of genetic prognostic markers.

Prognostic value of NOTCH1-activation in pediatric T-ALL – Paper II

Mutations in *NOTCH1* and/or *FBXW7* are found in approximately 50% of pediatric T-ALL patients [45, 47, 50-52, 54-56]. Several studies have associated *NOTCH1* mutation in T-ALL with a good response to treatment [50-52, 56] and one study also reported an association to better survival [52]. Other studies, including one large meta-study, have not been able to confirm this association [45, 47, 53, 56, 57] and the prognostic value of *NOTCH1*/*FBXW7* mutations in pediatric T-ALL is not clear. To evaluate NOTCH1-activating mutations as prognostic markers we analyzed mutation status as well as gene expression level of NOTCH1-target genes in pediatric T-ALL patients treated according to the NOPHO protocols.

We found *NOTCH1* and/or *FBXW7* mutation in 59% of the patients. No significant difference in EFS or OS could be detected between patients with mutated *NOTCH1*/*FBXW7* and patients with wild-type *NOTCH1*/*FBXW7*. We did however see an association with better early treatment response in patients with *NOTCH1* mutation. Many, but not all, patients with NOTCH1-activating mutations exhibited elevated expression of the NOTCH1 target gene *HES1*. Patients were divided into groups according to *HES1* expression level and patients with high expression of *HES1* had a significantly better EFS and OS. High *HES1* expression was also predictive for better EFS and OS after correction for age, gender, and WBC count. This indicates that NOTCH1-activation, as judged by *HES1* expression, might be a better prognostic marker in pediatric T-ALL than *NOTCH1*/*FBXW7* mutation status.

KRAS-induced T-ALL in mice is accompanied by loss of the wild-type Kras2 allele – Paper III

The LSL-*Kras2*^{G12D};Mx1-*Cre* (*KM*) mouse model displays an intriguing phenotypic shift from MPN to T-ALL after transplantation of the bone marrow to wild-type recipient mice [163-169]. The latency for T-ALL development in the transplanted mice was long (12-30 weeks) compared to the MPN development (9-14 weeks) in the *KM* mice. By transplanting WT bone marrow to *KM* mice we showed that the anemia and death of these mice could not be rescued, suggesting that the rapid death of pI-pC-injected *KM* mice is unrelated to their MPN. We argued that the low incidence of T-ALL in the *KM* mice might be due to that they die before T-ALL has had time to develop. To test this hypothesis we analyzed *KM* mice that were not injected with pI-pC; these mice still exhibit KRAS^{G12D} expression due to endogenous interferon production but survive significantly longer than pI-pC-injected *KM*

mice. We found that the incidence of T-ALL was increased in non-injected *KM* mice compared to pI-pC-injected *KM* mice. This indicates that tumor-life span is a major factor for the malignant phenotype (myeloid vs. T-lymphoid) in *KM* mice.

We also analyzed the effects of $KRAS^{G12D}$-expression on myeloid and T-lymphoid proliferation and differentiation. The results showed that expression of $KRAS^{G12D}$ increased proliferation of both myeloid and T-lymphoid cells. In the myeloid lineage this led to an expansion of mature myeloid cells in peripheral blood, which resulted in the MPN phenotype. In parallel, increased proliferation and a partial block in T-cell differentiation led to expansion of early T-cell progenitors. Transformation to T-ALL occurs after acquisition of secondary mutations [163-166] that likely targets lineage-restricted progenitors [165]. The altered pool of T-cell progenitors induced by $KRAS^{G12D}$-expression may serve as pre-leukemic cells that are targeted by the secondary transforming events.

Interestingly, we found that all T-ALL tumors showed loss of the wild-type *Kras2* allele with gain of a second *Kras2*^{G12D} allele. The gain of a second *Kras2*^{G12D} allele suggests that increased $KRAS^{G12D}$ expression promotes T-ALL, however, loss of the wild-type *Kras2* has been shown to support KRAS-induced transformation pointing to a tumor suppressive role of wild-type KRAS [181-184].

CONCLUSIONS & FUTURE PERSPECTIVES

In Paper I, we showed that *FLT3*-ITD and high *BAALC* expression were independent predictors for an adverse outcome in pediatric AML treated according to the NOPHO AML93 and NOPHO AML2004 protocols. We also found that high *ERG* expression predicts for lower EFS in patients with *MLL*-rearranged AML. In Paper II, we showed that mutation status of *NOTCH1* and *FBXW7* did not add prognostic value for pediatric T-ALL patients treated on the NOPHO ALL92 and NOPHO ALL2000 protocols. However, we did find that high *HES1* expression, an indicator of NOTCH1-activation, correlated with a favorable prognosis.

As a consequence of these results, presence of *FLT3*-ITD (without coexisting *NPM1* mutation) is included in the NOPHO-DBH AML2012 protocol as a high-risk marker and indicator for treatment with HSCT. High *BAALC*, *ERG,* and *HES1* gene expression is currently more difficult to utilize as prognostic markers due to the lack of standardized methods and verified cut-off levels. In addition, there are only a few other reports on the prognostic value of *ERG* and *BAALC* expression in pediatric AML and of *HES1* expression in pediatric T-ALL and the results are not uniformly in concordance with ours [53, 101, 180, 185]. However, once solid data on the prognostic value of expression level of these genes are present, development of assays with better reproducibility may make it possible to use gene expression levels in clinical risk stratification.

In Paper III, we found that $KRAS^{G12D}$-induced T-ALL in mice is accompanied by loss of heterozygosity at the *Kras2* locus. It will be interesting to investigate whether it is the homozygous expression of $KRAS^{G12D}$ or the loss of the wild-type allele that is driving T-ALL. To distinguish between these two possibilities we will perform two experiments. In the first we will transplant $KRAS^{G12D}$ bone marrow with expression of wild-type KRAS from a retroviral promoter. If the wild-type allele possesses tumor suppressive properties, the T-ALL development should be delayed or reduced. In the second experiment we will transplant fetal liver cells with homozygous expression of $KRAS^{G12D}$. Homozygous Mx1-*Cre*;LSL-*Kras2*^{G12D}/LSL-*Kras2*^{G12D} mice die around embryonic day 14.5 [129]. However, fetal liver cells can be harvested before that and transplanted to recipient mice. If a copy number gain of the *Kras2*^{G12D} is inducing T-ALL, these transplanted mice should develop T-ALL more rapidly than mice transplanted with heterozygous fetal liver cells.

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