Volume 16, Issue 1 2011

CRITICAL REVIEWS™ IN

Oncogenesis

BENJAMIN BONAVIDA
EDITOR-IN-CHIEF

begell house, inc.
publishers

AIMS AND SCOPE

The primary objective of "Critical Reviews™ in Oncogenesis" is the advancement and dissemination of scientific and clinical knowledge relating to the molecular basis of *Oncogenesis* for both its originality and scientific merit. CRO is dedicated to publishing timely critical reviews on topics of current interests with emphasis in basic, molecular and translational applications in the field of *Oncogenesis*. Further emphasis will also be placed on the role *Oncogenesis* in the development of cancer and malignancies as well as the role of cancer stem cells and their oncogenic potential. Reviews will also consider the role of oncogenes as diagnostics and therapeutic targets. This journal will publish special thematic issues on selected topics such as described above and employing many multidisciplinary approaches. Articles are by invitation only.

In contrast to reviews in several other publications, CRO will be focused on its specific and unique thematic approach for each issue that is contributed by various experts in and, often, an invited Guest Editor will be appointed for a particular issue. This multidisciplinary approach for a specific topic in *Oncogenesis* will distinguish CRO from many other journals whereby individual reviews are published. We believe that the new CRO objective will lead to a wide readership and will be used as an essential reference for research scientists, clinicians, students, and health professionals. The new Aims and Scope of CRO are designed to interest a wide readership and make it a reference standard in *Oncogenesis*.

Critical Reviews™ in Oncogenesis (ISSN 0893-9675) is published quarterly and owned by Begell House, Inc., 50 Cross Highway, Redding, Connecticut 06896

Subscriptions: United States rates for 2011 is $745.00. For orders outside the United State and Canada please add an additional $10.00 per issue for foreign airmail shipping and handling fees. All subscriptions are payable in advance. Subscriptions are entered on an annual basis, i.e., January to December. For immediate service and charge card sales, call Begell House at (203) 938-1300. Monday through Friday, 9 A.M.-5 P.M. EST. To order by fax: (203) 938-1304. Send written orders to Begell House, Inc., Subscriptions Department, 50 Cross Highway, Redding, Connecticut 06896. This journal contains information from authentic and highly regarded sources. Reprinted material is quoted with permission, and sources are indicated. A wide variety of references are listed. Reasonable efforts have been made to publish reliable data and information, but the editor and the publisher assume no responsibility for any statements of fact or opinion expressed in the published papers or in the advertisements.

Printed December 23, 2011

Critical Reviews™ in Oncogenesis

Critical Reviews™ in Oncogenesis

Volume 16 / Issue 1-2 **2011**

CONTENTS

Special Issue: Critical Reviews in Leukemogenesis

Guest Editor: Kathleen Sakamoto

Preface: Special Issue on Critical Reviews in Leukemogenesis

Significant progress has been made toward understanding the molecular basis of acute lymphoid and myeloid leukemia. In this issue of *Critical Reviews in Oncogenesis*, we have selected articles that highlight some of the new discoveries over the past decade. Each of the contributing authors is an expert in his or her field and area of research. The topics range from basic mechanisms of leukemogenesis to potential approaches to treating leukemia.

The reviews in this issue include a variety of molecules that are important for the pathogenesis of lymphoid or myeloid leukemias. The role of Gadd45 in hematopoietic stress response and leukemia is discussed. Among the fundamental themes of normal and aberrant hematopoiesis is the role of critical transcription factors involved in leukemogenesis. Abnormal regulation of these proteins can contribute to myeloid transformation. To address this, discussions on C/EBPalpha, Hox proteins, Runx1, GATA-1, and CREB in myeloid leukemogenesis are included. Furthermore, the zinc finger transcription factor, Ikaros, has been shown to be mutated in a significant number of cases of high-risk ALL, and its important role in leukemia is discussed. Studies on a recently described transcription factor, Sall4, and its function in regulating stem cell self-renewal are also found in this issue. The TAM family of receptor tyrosine kinases has recently been shown to activate signaling pathways in leukemia and to act as a target for ALL therapy. Another recent advance in the field of leukemia is the discovery that overexpression of *CRLF2* (*cytokine receptor-like factor 2*) is associated with activating mutations in *IKZF1*, *JAK1* and *JAK2*, and/or *IL-7Rα* and a worse prognosis. Additional approaches to treating leukemia, including targeting Notch1 for T-cell lymphoblastic leukemia, are presented. In summary, the reviews in this issue provide a summary of recently identified molecules and pathways that are involved in leukemogenesis and will advance the field in years to come.

Guest Editor: Kathleen Sakamoto, M.D., Ph.D.
Professor and Chief
Division of Hematology/Oncology
Department of Pediatrics
David Geffen School of Medicine
University of California, Los Angeles

0893-9675/11/$35.00

Critical Reviews™ in Oncogenesis, 16(1–2), 3–12 (2011)

Ikaros and Tumor Suppression in Acute Lymphoblastic Leukemia

Kimberly J. Payne[1] & Sinisa Dovat[2,*]

[1]Loma Linda University, Loma Linda, California; [2]Pennsylvania State University College of Medicine, Hershey, Pennsylvania

*Address all correspondence to: Sinisa Dovat, Department of Pediatrics, Division of Pediatric Hematology/Oncology, Pennsylvania State University College of Medicine, Hershey, PA 17033; sdovat@hmc.psu.edu

ABSTRACT: The *Ikzf1* gene encodes Ikaros—a DNA-binding zinc finger protein. Ikaros functions as a regulator of gene expression and chromatin remodeling. The biological roles of Ikaros include regulating the development and function of the immune system and acting as a master regulator of hematopoietic differentiation. Genomic profiling studies identified *Ikzf1* as an important tumor suppressor in acute lymphoblastic leukemia (ALL), particularly in ALL that is associated with poor prognosis. This review summarizes currently available data regarding the structure and function of Ikaros, the clinical relevance of genetic inactivation of *Ikzf1*, and signal transduction pathways that regulate Ikaros function.

KEY WORDS: Ikaros, leukemia, ALL, CK2, microarray, high-risk, deletion, phosphorylation, casein kinase, tumor suppression

ABBREVIATIONS

ALL, acute lymphoblastic leukemia; HDAC, histone deacetylase; CML, chronic myeloid leukemia; pre-BCR, pre-B cell receptor; *rag*, recombinase activating genes; TdT, terminal deoxynucleotide transferase

I. INTRODUCTION

The *Ikzf1* gene encodes Ikaros protein. Since its discovery, independently, by Georgopoulos et al. and the Smale group, *Ikzf1* has attracted tremendous attention from the scientific community. This interest is due to the biological roles of Ikaros in hematopoiesis, immune function, and tumor suppression, as well as its complex role in the regulation of transcription and chromatin remodeling.[1–3] During the past several years, *Ikzf1* has been established as one of the most clinically relevant tumor suppressors in high-risk acute lymphoblastic leukemia (ALL). This review summarizes our current understanding of the structure and function of Ikaros protein and the clinical relevance of its inactivation, as well as insights into the signal transduction pathways that regulate Ikaros activity.

II. IKAROS MOLECULAR STRUCTURE

The Ikaros proteins contain several functional domains discussed in the following sections.

A. DNA-Binding Domain

The N-terminal end of Ikaros contains a DNA-binding domain that consists of three zinc finger motifs with a typical C2H2 structure, and one CCHC-type zinc finger. A point mutation in the fourth zinc finger has been associated with primary immunodeficiency and pancytopenia in man.[4]

B. Dimerization Domain

The C-terminal end of Ikaros contains two zinc finger motifs that are essential for protein–protein interaction with other Ikaros isoforms or Ikaros family members.[5] This allows for the formation of very diverse protein complexes among different Ikaros family members and/or isoforms.

C. Bipartite Activation Domain

This bipartite activation domain lies adjacent to C-terminal zinc finger region. The presence of this

0893-9675/11/$35.00

domain stimulates basal levels of transcriptional activation of Ikaros target genes.[5,6]

D. Human Ikaros Activation Domain

This 20-amino-acid domain adjacent to the N-terminal zinc fingers regulates DNA-binding specificity and function in transcriptional activation and chromatin remodeling of Ikaros target genes in humans.[7,8]

III. IKAROS IN GENE REGULATION AND CHROMATIN REMODELING

Ikaros has been shown to bind DNA and to directly regulate expression of its target genes.[2,3,9,10] Subsequent experiments have established that Ikaros regulates transcription of its target genes primarily via chromatin remodeling. Ikaros is abundantly localized in pericentromeric heterochromatin in the nucleus.[11] Experiments by several groups have shown that Ikaros regulates expression of its target genes by recruiting them to pericentromeric heterochromatin, resulting in their activation or repression.[8,11,12]

Ikaros associates with histone deacetylase (HDAC)-containing complexes by direct interaction with the NuRD complex ATPase, Mi-2β, and with Sin3A and Sin3B.[13,14] It has been suggested that Ikaros recruits histone deacetylase complex to the upstream regulatory elements of its target genes, which results in chromatin remodeling and repression of the Ikaros target gene.[11,15]

Ikaros has been demonstrated to function as transcriptional repressor in a HDAC-independent way. Ikaros interacts with the corepressor, CtBP [16] and the Ikaros-CtBP complex acts to repress transcription without HDAC involvement, thus Ikaros can function as a transcriptional repressor of its target genes through both HDAC-dependent and HDAC-independent mechanisms.[16]

Ikaros interacts with Brg-1, a catalytic subunit of the SWI/SNF chromatin remodeling complex that functions as an activator of gene expression.[14,17] It has been suggested that Ikaros functions as transcriptional activator by recruiting the SWI/SNF nucleosome remodeling complex to the upstream regions of its target genes, resulting in chromatin remodeling and activation of the gene. Thus, Ikaros can both activate or repress transcription of its target genes via chromatin remodeling, depending on whether it associates with the NuRD, the CtBP or the SWI/SNF complex.

IV. IKAROS IN B-CELL ALL

Since the discovery that Ikaros functions as a master regulator of lymphocyte differentiation and a tumor suppressor in the mouse, human studies have focused on determining whether Ikaros acts as a tumor suppressor in human leukemia. Initial studies focused on the expression of small dominant negative *Ikzf1* isoforms. These studies found that expression of DNA isoforms was associated with adult B cell ALL,[18] as well as with myelodysplastic syndrome,[19] AML,[20] and adult and juvenile CML.[21] However, due to an absence of functional data and the small numbers of patients as well as the lack of genetic evidence for alteration of *Ikzf1*, these studies did not have a profound effect on clinical practice.

During the last several years, multiple microarray-based analyses of genetic changes and alterations in gene expression have been conducted by several groups. These genomic profiling studies have produced strong evidence that that Ikaros plays a key role in tumor suppression in pediatric B-cell ALL and particularly in high-risk B-cell ALL. These findings are summarized as follows.

1. Deletion of a single *Ikzf1* allele or mutation of a single copy of *Ikzf1* were detected in 15% of all cases of pediatric B-cell ALL.[22] It should be noted that all of the described mutations were either nonsense, or frameshift mutations, or mutations that functionally inactivated a particular *Ikzf1* allele. Thus, each of these defects resulted in haploinsufficiency of the *Ikzf1* gene, along with expression of a functionally inactive form of Ikaros which could potentially act as a dominant-negative form.
2. Deletion or mutation of a single copy of the *Ikzf1* allele was detected in over 80% of BCR-ABL1 ALL, a subtype of ALL that are associ-

ated with a poor outcome. Deletion or mutation of an *Ikzf1* allele was also identified in 66% of chronic myeloid leukemia (CML) patients during lymphoid blast crisis.[23–25]

3. Deletion or mutation of *Ikzf1* was identified in one-third of cases of BCR-ABL1 negative ALL. Haploinsufficiency of *Ikzf1* was associated with a three-fold increase in relapse of ALL following treatment.[26-28]
4. Expression profiles of BCR-ABLL1 negative cases with haploinsufficiency of *Ikzf1* and poor prognosis were noted to have similar expression profiles to BCR-ABL1 positive ALL.[26] This led to the definition of the BCR-ABL1-like subtype of B-cell ALL with haploinsufficiency of *Ikzf1* or other transcriptional regulators.[28]
5. Inherited genetic variations of *Ikzf1* are associated with the risk of childhood ALL and poor outcome of the disease.[29,30] Genetic variations have been shown to affect the expression level of Ikaros, suggesting a potential mechanism for leukemogenesis.[30]
6. In 14% of pediatric high-risk leukemia with a poor outcome,[31] the CRLF2 gene is overexpressed due to rearrangement. This CRLF2 defect is significantly associated with JAK mutations and with deletions or mutations of *Ikzf1*.[31,32]
7. The functional, leukemogenic significance of *Ikzf1* haploinsufficiency and/or expression of dominant-negative Ikaros isoforms has been confirmed by several animal models. These models demonstrated that the expression of the dominant negative *Ikzf1* allele in CD34+ cells results in impaired lymphoid differentiation.[33] These models also demonstrate that the haploinsufficiency of *Ikzf1* accelerates the development of leukemia in both retrovirally transduced bone marrow transplants and in a transgenic model of BCR-ABL1 ALL.[34,35]

Overall, the above data established that: a) Ikaros acts as a highly clinically-relevant tumor suppressor in B-cell ALL and particularly in high-risk B-cell ALL, b) The modest decrease in Ikaros activity (e.g., haploinsufficiency) is sufficient to contribute to leukemogenesis, and c) Genetic alterations of *Ikzf1* might serve as a prognostic marker for B-cell ALL outcome. Based on these results, testing for genetic alteration of *Ikzf1* is currently being performed in prospective clinical trials.

V. IKAROS IN T-CELL ALL

Initial studies of Ikaros in T-cell ALL produced somewhat conflicting data: All 18 T-ALL patients in the first study were reported to express dominant negative Ikaros isoforms (assessed by western blot analysis and real-time polymerase chain reaction [RT-PCR]),[36] suggesting a strong correlation of loss of Ikaros function with the development of T-cell ALL. However, in subsequent studies on a total of 14 T-ALL patients (both adult and pediatric) dominant-negative isoforms were not detected by western blot analysis and RT-PCR.[18,37] However, the expression of a dominant-negative isoform of the Ikaros-family member, Helios, was associated with T-cell ALL in one study.[38]

Deletion of one copy of Ikaros was detected in 5% of T-cell ALL patients in more comprehensive studies that utilized high-resolution CGH-arrays on a total of 81 patients.[23,39,40] The most recent study combined western blot, CGH-array analysis, and sequencing of Ikaros cDNA following RT-PCR to provide a more complete view of the relation of Ikaros and T-cell ALL evaluate. That study of 25 cases of human T-cell ALL detected one patient (4%) in which one Ikaros allele had been deleted. The Ikaros protein that was produced by the other intact allele exhibited association with an abnormal cytoplasmic structure and a loss of nuclear localization.[41] This study provided the first definitive functional evidence to link the complete loss of Ikaros function with human T-cell ALL.[42]

In summary, these studies of human T-cell ALL demonstrate that inactivation of the *Ikzf1* gene by deletion occurs in human T-cell ALL in at least 5% of cases. Although Ikaros deletion is less frequent in T-ALL, when compared to B-cell ALL (15%) or BCR-ABL1 ALL (80%), its occurrence in T-Cell All is a notable cause of T-cell ALL, and testing for genetic alteration of *Ikzf1* in newly diagnosed

patients with this disease is warranted. It remains to be determined whether Ikaros deletion will have prognostic significance in T-cell ALL.

VI. MECHANISMS OF IKAROS TUMOR SUPPRESSOR ACTIVITY

The mechanism by which Ikaros suppresses malignant transformation and the development of ALL is largely unknown. The discovery of several Ikaros target genes provided potential mechanisms of the tumor suppressor action of Ikaros in ALL. These are summarized in the following sections.

A. Positive Regulation of B Cell Differentiation

Expression of several genes that are essential for normal B cell differentiation are directly regulated by Ikaros. Ikaros has been shown to bind the *Igll1* promoter and to regulate expression of this gene in early B lineage cells.[43,44] The Ikaros binding site at the *Igll1* promoter overlaps the binding site of the EBF transcriptional activator. Thus, Ikaros regulates *Igll1* transcription by competing with EBF for binding to the *Igll1* promoter and subsequently regulating *Igll1* expression. The *Igll1* gene encodes Lambda5, a component of the pre-B cell receptor (pre-BCR). Pre-BCR expression is essential for progression beyond the pre-B cell stage of differentiation. Thus, Ikaros controls this critical step in early stages of B cell differentiation.

Ikaros binds to the promoter region of the recombinase activating genes (*rag*) and positively regulates transcription of both *rag1* and *rag2*.[45] Upregulation of expression of RAG1 and RAG2, along with Ikaros-mediated control of the compaction of the immunoglobulin heavy-chain locus (*IGH@*) , as well as accessibility of the variable gene segments, promotes *IGH@* gene rearrangement.[45] Thus Ikaros controls another essential step in normal B cell differentiation.

B. Positive Regulation of T Cell Differentiation

Ikaros has been shown to regulate expression of multiple genes that are essential for T cell differentiation. The regulation of *dntt* (terminal deoxynucleotide transferase -TdT) gene expression during thymocyte differentiation by Ikaros has been studied by several groups.[9,46–48] Ikaros binds to the D′ upstream regulatory element of the *dntt* gene promoter. This region contains a consensus binding site that is bound, *in vivo,* by the Elf-1 activator (a member of the Ets family of transcription factors). Ikaros and Elf-1 have been shown to compete for the occupancy of the D′ upstream regulatory element of the *dntt* gene during thymocyte development. Ikaros binding to the *dntt* upstream regulatory element results in repression of TdT transcription, which is associated with repositioning of the *dntt* gene to pericentromeric heterochromatin.[46] During induction of thymocyte differentiation, Ikaros displaces Elf-1 from the D′ upstream regulatory element of *dntt,* which results in downregulation of TdT expression. Phosphorylation of Ikaros has been shown to regulate Ikaros' affinity toward the *dntt* upstream regulatory region.[49]

During thymocyte development, Ikaros binds to the regulatory element of the CD8α gene. It has been suggested that Ikaros positively regulates transcription of the CD8α gene during T cell development[50] and thus plays an important role in CD4 versus CD8 lineage commitment. This hypothesis has been supported by decreased numbers of CD8+ T cells in Ikaros-deficient mice.[50]

Studies by Georgopoulos' group demonstrated that Ikaros binds to the upstream regulatory region of the CD4 gene. Ikaros binding at this site, in complex with the Mi-2β chromatin remodeler, results in expression of CD4, suggesting that Ikaros positively regulates transcription of CD4 via chromatin remodeling.[51]

C. Downregulation of the Notch Pathway

The Notch pathway is essential for T cell development. Activation of the Notch-1 gene has been found in greater than 50% of T-cell ALL.[52] In addition, T-cell ALL cells have high expression of the Notch target genes Hes-1 and pT.[53] In T-cell leukemia derived from Ikaros-deficient mice, the Notch pathway is activated.[54]

The synergism between Notch activation and the loss of Ikaros function in T cell leukemogenesis has been demonstrated by Beverly and Capobianco.[55] Because the consensus binding sequences for the Notch-associated transcriptional activator, CSL, and Ikaros were highly similar, Ikaros was hypothesized to interfere with CSL binding and Notch signaling.[55] Ikaros directly binds to the upstream regulatory element of a Notch target gene Hes-1, and downregulates its expression. Ikaros competes with the transcriptional activator CSL for binding to the upstream regulator element of Hes-1 in a manner similar to that demonstrated for EBF1 and Elf1 (described above).[56] It has been suggested that transcriptional repression of Hes-1 by Ikaros involves chromatin remodeling, as Ikaros binding to the upstream regulatory region of Hes-1 leads to decreased histone H3 acetylation at the Hes-1 locus.[57]

Ikaros competes with CSL for the binding to the upstream regulatory region of Deltex1, another target gene for the Notch signaling pathway.[57] Ikaros represses transcription of Deltex1 by chromatin remodeling as evidenced by decreased histone H3 acetylation at the Deltex1 locus following Ikaros binding to the upstream region of Deltex1.[57]

D. Negative Regulation of Cellular Proliferation

The negative regulation of pre-B cell proliferation by Ikaros has been demonstrated by Ma et al. The mechanism of inhibition of cellular proliferation involves direct binding of Ikaros to the promoter of the c-Myc gene, which results in direct suppression of c-Myc expression in pre-B cells.[58] Repression of c-Myc by Ikaros in pre-B cells also leads to induction of expression of p27, as well as downregulation of cyclin D3.[58] These data provided a potential mechanism by which Ikaros suppresses proliferation of pre-B cells *in vivo*.

It has also been shown that Ikaros can negatively regulate cell cycle progression at the G1/S transition,[59] suggesting that Ikaros has a role in the regulation of the G1/S check point of the cell cycle.

E. Regulation of Apoptosis

The loss of Ikaros function is associated with increased Bcl-xL expression, which suggests that Ikaros downregulates Bcl-xL expression.[20,60,61] These data led to the hypothesis that Ikaros regulates apoptosis, and that decreased Ikaros activity in leukemia cells would increase resistance to chemotherapy. This hypothesis remains speculative due to a lack of mechanistic data to support this assertion.

F. Post-Translational Modifications Regulate Ikaros Tumor Suppressor Function

Post-translational modifications have been shown to regulate Ikaros' activity. Sumoylation regulates Ikaros repressor function.[62] The cell-cycle–specific phosphorylation of Ikaros regulates its DNA-binding ability and nuclear localization during mitosis.[63] In cycling cells Ikaros is a direct substrate for pro-oncogenic kinase CK2. Phosphorylation of Ikaros by CK2 regulates the subcellular localization of Ikaros to pericentromeric heterochromatin, and its DNA-binding affinity toward the upstream regulatory element of the Ikaros' target gene, TdT,[49] as well as its ability to control G1/S cell cycle progression.[59] More recent data showed that Ikaros is a substrate for PP1 phosphatase, and that CK2 and PP1 exert opposite effects on Ikaros function in DNA binding, pericentromeric localization, and chromatin remodeling.[64] Overexpression of CK2 has been shown to increase degradation of Ikaros protein via the ubiquitin pathway, while PP1 counteracts this process (Fig. 1).[64] These data led to the development of a model whereby the loss of Ikaros activity in leukemia can result from genetic defects (deletion, mutation) or functional inactivation of Ikaros due to hyperphosphorylation by CK2.[65] More studies are needed to test this model.

VII. CONCLUSION

Genomic profiling of ALL identified Ikaros as a major tumor suppressor in ALL. Functional studies revealed possible mechanisms of tumor suppression by Ikaros, as well as the regulatory pathways that

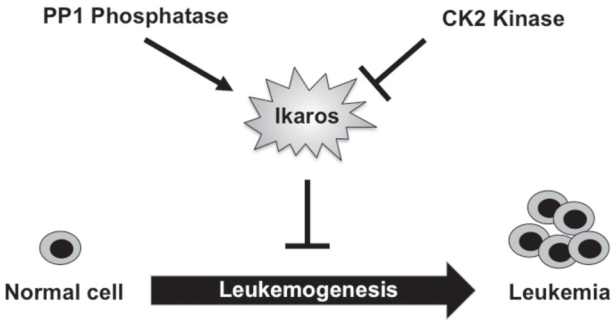


FIGURE 1. Phosphorylation regulates the tumor suppressor function of Ikaros. CK2 kinase directly phosphorylates and functionally inactivates Ikaros, while PP1 phosphatase counteracts this process. Functional inactivation of Ikaros by CK2 kinase promotes leukemogenesis.

control the tumor suppressor function of Ikaros. Future studies will be directed toward evaluating genetic changes in Ikaros as a prognostic marker for ALL, as well as a factor in the decision-making process to design appropriate therapy. Regulatory pathways that control the tumor suppressor function of Ikaros are a potential target for a novel chemotherapy for ALL.

ACKNOWLEDGMENTS

The work was supported in whole or in part by an R01 HL095120 grant, a St. Baldrick's Foundation Career Development Award, the Four Diamonds Fund of the Pennsylvania State University, College of Medicine, and the John Wawrynovic Leukemia Research Scholar Endowment (to S.D.) This work was also supported by the Department of Pathology and Human Anatomy and the Center for Health Disparities and Molecular Medicine at Loma Linda University School of Medicine (to K.J.P). This work was also supported by a Grants for Research and School Partnerships from Loma Linda University (to K.J.P).

REFERENCES

1. Lo K, Landau NR, Smale ST. LyF-1, a transcriptional regulator that interactswith a novel class of promoters for lymphocyte-specific genes. Mol Cell Biol. 1991;11:5229–43.
2. 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.
3. Hahm K, Ernst P, Lo K, Kim GS, Turck C, Smale ST. The lymphoid transcription factor LyF-1 is encoded by specific, alternatively spliced mRNAs derived from the Ikaros gene. Mol Cell Biol. 1994;14(11):7111–23.
4. Goldman FD, Gurel Z, Al-Zubeidi D, Fried AJ, Icardi M, Song C, Dovat S. Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the ikaros gene. Pediatr Blood Cancer. 2011. doi: 10.1002/pbc.23160. [Epub ahead of print]
5. Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. Embo J. 1996;15(19):5358–69.
6. Georgopoulos K, Winandy S, Avitahl N. The role of the Ikaros gene in lymphocyte development and homeostasis. Annu Rev Immunol. 1997;15:155–76.
7. Ronni T, Payne KJ, Ho S, Bradley MN, Dorsam G, Dovat S. Human Ikaros function in activated T cells is regulated by coordinated expression of its largest isoforms. J Biol Chem. 2007;282(4):2538–47.
8. Kim JH, Ebersole T, Kouprina N, Noskov VN, Ohzeki J, Masumoto H, Mravinac B, Sullivan BA, Pavlicek A, Dovat S, Pack SD, Kwon YW, Flanagan PT, Loukinov D, Lobanenkov V, Larionov V. Human gamma-satellite DNA maintains open chromatin structure and protects a transgene from epigenetic silencing. Genome Res. 2009;19(4):533–44.
9. Ernst P, Hahm K, Smale ST. Both LyF-1 and an Ets protein interact with a critical promoter element in the murine terminal transferase gene. Mol Cell Biol. 1993;13(5):2982–92.
10. Molnár A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. Mol Cell Biol. 1994;14(12):8292–303.
11. Brown KE, Guest SS, Smale ST, Hahm K, Merkenschlager M, Fisher AG. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. Cell. 1997;91(6): 845-854.

12. Koipally J, Heller EJ, Seavitt JR, Georgopoulos K. Unconventional potentiation of gene expression by ikaros. J Biol Chem. 2002;277(15):13007–15.

13. Koipally J, Renold A, Kim J, Georgopoulos K. Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. Embo J. 1999;18(11):3090–3100.

14. Kim J, Sif S, Jones B, Jackson A, Koipally J, Heller E, Winandy S, Viel A, Sawyer A, Ikeda T, Kingston R, Georgopoulos K. Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. Immunity. 1999;10(3):345–355.

15. Liberg D, Smale ST, Merkenschlager M. Upstream of Ikaros. Trends Immunol. 2003;24(11):567–570.

16. Koipally J, Georgopoulos K. Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity. J Biol Chem. 2000;275(26):19594–602.

17. O'Neill DW, Schoetz SS, Lopez RA, Castle M, Rabinowitz L, Shor E, Krawchuk D, Goll MG, Renz M, Seelig HP, Han S, Seong RH, Park SD, Agalioti T, Munshi N, Thanos D, Erdjument-Bromage H, Tempst P, Bank A. An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. Mol Cell Biol. 2000;20(20):7572–82.

18. Nakase K, Ishimaru F, Avitahl N, Dansako H, Matsuo K, Fujii K, Sezaki N, Nakayama H, Yano T, Fukuda S, Imajoh K, Takeuchi M, Miyata A, Hara M, Yasukawa M, Takahashi I, Taguchi H, Matsue K, Nakao S, Niho Y, Takenaka K, Shinagawa K, Ikeda K, Niiya K, Harada M. Dominant negative isoform of the Ikaros gene in patients with adult B- cell acute lymphoblastic leukemia. Cancer Res. 2000;60(15):4062–5.

19. Crescenzi B, La Starza R, Romoli S, Beacci D, Matteucci C, Barba G, Aventin A, Marynen P, Ciolli S, Nozzoli C, Martelli MF, Mecucci C. Submicroscopic deletions in 5q- associated malignancies. Haematologica. 2004;89(3):281–5.

20. Yagi T, Hibi S, Takanashi M, Kano G, Tabata Y, Imamura T, Inaba T, Morimoto A, Todo S, Imashuku S. High frequency of Ikaros isoform 6 expression in acute myelomonocytic and monocytic leukemias: implications for up-regulation of the antiapoptotic protein Bcl-XL in leukemogenesis. Blood. 2002;99(4):1350–5.

21. Nakayama H, Ishimaru F, Avitahl N, Sezaki N, Fujii N, Nakase K, Ninomiya Y, Harashima A, Minowada J, Tsuchiyama J, Imajoh K, Tsubota T, Fukuda S, Sezaki T, Kojima K, Hara M, Takimoto H, Yorimitsu S, Takahashi I, Miyata A, Taniguchi S, Tokunaga Y, Gondo H, Niho Y, Nakao S, Kyo T, Dohy H, Kamada N, Harada M. Decreases in Ikaros activity correlate with blast crisis in patients with chronic myelogenous leukemia. Cancer Res. 1999;59(16):3931–4.

22. 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):758–764.

23. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, White D, Hughes TP, Le Beau MM, Pui CH, Relling MV, Shurtleff SA, Downing JR. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature. 2008;453(7191):110–4.

24. Martinelli G, Iacobucci I, Storlazzi CT, Vignetti M, Paoloni F, Cilloni D, Soverini S, Vitale A, Chiaretti S, Cimino G, Papayannidis C, Paolini S, Elia L, Fazi P, Meloni G, Amadori S, Saglio G, Pane F, Baccarani M, Foa R. IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. J Clin Oncol. 2009;27(31):5202–7.

25. Iacobucci I, Storlazzi CT, Cilloni D, Lonetti A, Ottaviani E, Soverini S, Astolfi A, Chiaretti S, Vitale A, Messa F, Impera L, Baldazzi C, D'Addabbo P, Papayannidis C, Lonoce A, Colarossi S, Vignetti M, Piccaluga PP, Paolini S, Russo D, Pane F, Saglio G, Baccarani M, Foa R, Martinelli G. Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood. 2009;114(10):2159–67.

26. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP,

Willman CL, Downing JR. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009;360(5):470–80.

27. Kuiper RP, Waanders E, van der Velden VH, van Reijmersdal SV, Venkatachalam R, Scheijen B, Sonneveld E, van Dongen JJ, Veerman AJ, van Leeuwen FN, van Kessel AG, Hoogerbrugge PM. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. Leukemia. 2010;24(7):1258–64.
28. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, Van Zutven LJ, Beverloo HB, Van der Spek PJ, Escherich G, Horstmann MA, Janka-Schaub GE, Kamps WA, Evans WE, Pieters R. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol. 2009;10(2):125–34.
29. Trevino LR, Yang W, French D, Hunger SP, Carroll WL, Devidas M, Willman C, Neale G, Downing J, Raimondi SC, Pui CH, Evans WE, Relling MV. Germline genomic variants associated with childhood acute lymphoblastic leukemia. Nat Genet. 2009;41(9):1001–5.
30. Papaemmanuil E, Hosking FJ, Vijayakrishnan J, Price A, Olver B, Sheridan E, Kinsey SE, Lightfoot T, Roman E, Irving JA, Allan JM, Tomlinson IP, Taylor M, Greaves M, Houlston RS. Loci on 7p12.2, 10q21.2 and 14q11.2 are associated with risk of childhood acute lymphoblastic leukemia. Nat Genet. 2009;41(9):1006–10.
31. Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, Kang H, Liu W, Dobbin KK, Smith MA, Carroll WL, Devidas M, Bowman WP, Camitta BM, Reaman GH, Hunger SP, Downing JR, Willman CL. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010;115(26):5312–21.
32. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, Schrauder A, Moericke A, Izraeli S, Akasaka T, Dyer MJ, Siebert R, Schrappe M, Stanulla M. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010;115(26):5393–97.
33. Tonnelle C, Bardin F, Maroc C, Imbert AM, Campa F, Dalloul A, Schmitt C, Chabannon C. Forced expression of the Ikaros 6 isoform in human placental blood CD34(+) cells impairs their ability to differentiate toward the B- lymphoid lineage. Blood. 2001;98(9):2673–80.
34. Collins-Underwood JR, Miller CB, Downing JR, Mullighan CG. Ikzf1 Haploinsufficiency Contributes to the Pathogenesis of BCR-ABL1 Positive Acute Lymphoblastic Leukemia. Blood. 2009;114:(abstract 678).
35. Virely C, Moulin S, Cobaleda C, Lasgi C, Alberdi A, Soulier J, Sigaux F, Chan S, Kastner P, Ghysdael J. Haploinsufficiency of the IKZF1 (IKAROS) tumor suppressor gene cooperates with BCR-ABL in a transgenic model of acute lymphoblastic leukemia. Leukemia. 2010;24(6):1200–4.
36. 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.
37. Ruiz A, Jiang J, Kempski H, Brady HJ. Overexpression of the Ikaros 6 isoform is restricted to t(4;11) acute lymphoblastic leukaemia in children and infants and has a role in B-cell survival. Br J Haematol. 2004;125(1):317.
38. 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.
39. Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A, O'Neil J, Gutierrez A, Ivanova E, Perna I, Lin E, Mani V, Jiang S, McNamara K, Zaghlul S, Edkins S, Stevens C, Brennan C, Martin ES, Wiedemeyer R, Kabbarah O, Nogueira C, Histen G, Aster J, Mansour M, Duke V, Foroni L, Fielding AK, Goldstone AH, Rowe JM, Wang YA, Look AT, Stratton MR, Chin L, Futreal PA, DePinho RA. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature. 2007;447(7147):966–71.
40. Kuiper RP, Schoenmakers EF, van Reijmersdal SV, Hehir-Kwa JY, van Kessel AG, van Leeuwen FN, Hoogerbrugge PM. High-resolution genomic profiling of childhood ALL reveals novel recurrent genetic

lesions affecting pathways involved in lymphocyte differentiation and cell cycle progression. Leukemia. 2007;21(6):1258–66.

41. Marcais A, Jeannet R, Hernandez L, Soulier J, Sigaux F, Chan S, Kastner P. Genetic inactivation of Ikaros is a rare event in human T-ALL. Leuk Res. 2010;34(4):426–9.
42. Dovat S, Payne KJ. Tumor suppression in T cell leukemia--the role of Ikaros. Leuk Res. 2010;34(4):416–17.
43. Thompson EC, Cobb BS, Sabbattini P, Meixlsperger S, Parelho V, Liberg D, Taylor B, Dillon N, Georgopoulos K, Jumaa H, Smale ST, Fisher AG, Merkenschlager M. Ikaros DNA-binding proteins as integral components of B cell developmental-stage-specific regulatory circuits. Immunity. 2007;26(3): 335–44.
44. Sabbattini P, Lundgren M, Georgiou A, Chow C, Warnes G, Dillon N. Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. Embo J. 2001;20(11):2812–22.
45. Reynaud D, I AD, K LR, Schjerven H, Bertolino E, Chen Z, Smale ST, Winandy S, Singh H. Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. Nat Immunol. 2008; 9(8):927–936.
46. Trinh LA, Ferrini R, Cobb BS, Weinmann AS, Hahm K, Ernst P, Garraway IP, Merkenschlager M, Smale ST. Down-regulation of TdT transcription in CD4+CD8+ thymocytes by Ikaros proteins in direct competition with an Ets activator. Genes and Development. 2001;15:1817–32.
47. Ernst P, Hahm K, Trinh L, Davis JN, Roussel MF, Turck CW, Smale ST. A potential role for Elf-1 in terminal transferase gene regulation. Mol Cell Biol. 1996;16(11):6121–31.
48. Su RC, Sridharan R, Smale ST. Assembly of silent chromatin during thymocyte development. Semin Immunol. 2005;17(2):129–40.
49. Gurel Z, Ronni T, Ho S, Kuchar J, Payne KJ, Turk CW, Dovat S. Recruitment of ikaros to pericentromeric heterochromatin is regulated by phosphorylation. J Biol Chem. 2008;283(13): 8291–300.
50. Harker N, Naito T, Cortes M, Hostert A, Hirschberg S, Tolaini M, Roderick K, Georgopoulos K, Kioussis D. The CD8alpha gene locus is regulated by the Ikaros family of proteins. Mol Cell. 2002;10(6):1403–15.
51. Naito T, Gomez-Del Arco P, Williams CJ, Georgopoulos K. Antagonistic interactions between Ikaros and the chromatin remodeler Mi-2beta determine silencer activity and Cd4 gene expression. Immunity. 2007;27(5):723–34.
52. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269–71.
53. Chiaramonte R, Basile A, Tassi E, Calzavara E, Cecchinato V, Rossi V, Biondi A, Comi P. A wide role for NOTCH1 signaling in acute leukemia. Cancer Lett. 2005;219(1):113–20.
54. Dumortier A, Jeannet R, Kirstetter P, Kleinmann E, Sellars M, dos Santos NR, Thibault C, Barths J, Ghysdael J, Punt JA, Kastner P, Chan S. Notch activation is an early and critical event during T-Cell leukemogenesis in Ikaros-deficient mice. Mol Cell Biol. 2006;26(1):209–20.
55. Beverly LJ, Capobianco AJ. Perturbation of Ikaros isoform selection by MLV integration is a cooperative event in Notch(IC)-induced T cell leukemogenesis. Cancer Cell. 2003;3(6):551–64.
56. Kleinmann E, Geimer Le Lay AS, Sellars M, Kastner P, Chan S. Ikaros represses the transcriptional response to Notch signaling in T-cell development. Mol Cell Biol. 2008;28(24):7465–75.
57. Kathrein KL, Chari S, Winandy S. Ikaros directly represses the notch target gene Hes1 in a leukemia T cell line: implications for CD4 regulation. J Biol Chem. 2008;283(16):10476–84.
58. Ma S, Pathak S, Mandal M, Trinh L, Clark MR, Lu R. Ikaros and Aiolos inhibit pre-B-cell proliferation by directly suppressing c-Myc expression. Mol Cell Biol;30(17):4149–58.
59. Gomez-del Arco P, Maki K, Georgopoulos K. Phosphorylation controls Ikaros's ability to negatively regulate the G(1)-S transition. Mol Cell Biol. 2004;24(7):2797–807.
60. Ezzat S, Zhu X, Loeper S, Fischer S, Asa SL. Tumor-derived Ikaros 6 acetylates the Bcl-XL promoter to up-regulate a survival signal in pituitary cells. Mol Endocrinol. 2006;20(11):2976–86.

61. Kano G, Morimoto A, Takanashi M, Hibi S, Sugimoto T, Inaba T, Yagi T, Imashuku S. Ikaros dominant negative isoform (Ik6) induces IL-3-independent survival of murine pro-B lymphocytes by activating JAK-STAT and up-regulating Bcl-xl levels. Leuk Lymphoma. 2008;49(5):965–73.
62. Gomez-del Arco P, Koipally J, Georgopoulos K. Ikaros SUMOylation: switching out of repression. Mol Cell Biol. 2005;25(7):2688–97.
63. Dovat S, Ronni T, Russell D, Ferrini R, Cobb BS, Smale ST. A common mechanism for mitotic inactivation of C2H2 zinc finger DNA-binding domains. Genes Dev. 2002;16(23): 2985–90.
64. Popescu M, Gurel Z, Ronni T, Song C, Hung KY, Payne KJ, Dovat S. Ikaros stability and pericentromeric localization are regulated by protein phosphatase 1. J Biol Chem. 2009;284(20): 13869–80.
65. Li Z, Perez-Casellas LA, Savic A, Song C, Dovat S. Ikaros isoforms: The saga continues. World J Biol Chem. 2011;2(6):140–5.

Critical Reviews™ in Oncogenesis, 16(1–2), 13–24 (2011)

Understanding the Biology of *CRLF2*-Overexpressing Acute Lymphoblastic Leukemia

Sarah K. Tasian & Mignon L. Loh*

Department of Pediatrics, Division of Pediatric Hematology-Oncology, University of California San Francisco, San Francisco, California

*Address all correspondence to: Mignon L. Loh, MD, Department of Pediatrics, Division of Pediatric Hematology-Oncology, Helen Diller Comprehensive Cancer Center, University of California-San Francisco, 513 Parnassus Avenue, Health Science East 302, Box 0519, San Francisco, CA 94143; lohm@peds.ucsf.edu

ABSTRACT: Recent genomic analyses of childhood and adult B-precursor acute lymphoblastic leukemia (ALL) samples have identified novel genetic alterations in essential lymphoid development and signal transduction pathways, providing insight into the pathogenesis of high-risk ALL associated with treatment failure. Particular advances have been made in unraveling the genetics of ALL associated with overexpression of the cytokine receptor-like factor 2 gene (*CRLF2*), which is frequently accompanied by simultaneous activating mutations in genes encoding Ikaros *(IKZF1)*, Janus kinase 1 (*JAK1*) and Janus kinase 2 (*JAK2)*, and/or the IL-7 receptor alpha chain (*IL7RA*). Children and adults with high-risk *CRLF2*-overexpressing ALL have high rates of relapse and dismal overall survival. Various groups have thus attempted to characterize the biochemical consequences of these genetic lesions via preclinical models with the goal of identifying targets for new therapies. These studies provide early data suggesting the promise of signal transduction inhibitors (STIs) of the JAK/STAT and PI3K pathways for *CRLF2*-overexpressing ALL. Additional research efforts continue to elucidate these aberrant signaling networks to provide rationale for bringing STIs into the clinic for these high-risk patients. This review highlights the current knowledge of the incidence, prognostic significance, and biology of *CRLF2*-overexpressing ALL and future directions for development of targeted therapies.

KEY WORDS: acute lymphoblastic leukemia, *CRLF2*, *JAK2*, signal transduction, TSLP, TSLPR

ABBREVIATIONS

ALL, acute lymphoblastic leukemia; BFM, Berlin-Frankfurt-Münster; CNA, copy number alteration; COG, Children's Oncology Group; CRLF2, cytokine receptor-like factor 2; DS-ALL, Down Syndrome-associated acute lymphoblastic leukemia; EFS, event-free survival; GEP, gene expression profiling; HR, high risk; IGH@, immunoglobulin heavy locus; IKZF1, Ikaros; IL-7R, IL-7 receptor; IL-7Rα, IL-7 receptor alpha chain protein; IL7Rα, IL-7 receptor alpha gene; JAK, Janus kinase; JAK1, Janus kinase 1; JAK2, Janus kinase 2; MPNs, myeloproliferative neoplasms; MRC, Medical Research Council; NCI, National Cancer Institute; OHS, Oxford Hazard Score; peIF4E, phospho-eIF4E; pERK, phospho-ERK 1/2; pJAK2, phospho-JAK2; pSTAT5, phospho-STAT5; pS6, phospho-S6; p4EBP1, phospho-4EBP1; PI3K, phosphatidylinositol 3-kinase; RFS, relapse-free survival; SR, standard risk; STAT, signal transduction activator of transcription; STIs, signal transduction inhibitors; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor

I. IDENTIFICATION OF NEW GENETIC LESIONS IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

B-precursor acute lymphoblastic leukemia is the most common malignancy of childhood; approximately 3000 new cases are diagnosed annually in the United States in children younger than 21 years of age. Significant improvements in relapse-free survival (RFS) and overall survival have occurred during the past 50 years, largely secondary to the introduction of central nervous system prophylaxes, multi-agent chemotherapy regimens, and the recognition that certain subsets of patients require higher doses of

0893-9675/11/$35.00

these agents to achieve cure. In addition, the recognition of the prognostic significance of leukemia cell-specific cytogenetic and chromosomal abnormalities, as well as studies that have shown the value of early response to therapy, have together improved our ability to refine therapy.[1–3] Approximately 80–85% of children with ALL are cured using these strategies with current multi-agent therapy. However, up to 20% of children fail our current therapies, and most of these patients die from their disease. In addition, adults with ALL have generally worse prognoses, with an overall survival of 35–50%. One-quarter to one-third of pediatric ALL patients have no identifiable leukemia-associated genetic alterations that are predictive of outcome,[4] and that statistic is even higher in adult ALL.[5] Identification of patients who remain at high risk of treatment failure despite contemporary therapies thus remains a priority for basic scientists and clinical investigators with the ultimate hope that these discoveries will lead to new therapies that can improve outcomes. Indeed, a paradigm for such a therapeutic benefit has occurred for children with *BCR/ABL1*-positive ALL; these patients previously experienced a 5-year event-free survival (EFS) of less than 40% when treated with intensive chemotherapy alone, but they now experience an EFS greater than 80% with the addition of the tyrosine kinase inhibitor imatinib in combination with chemotherapy.[6]

With the advent of sophisticated genome-wide analytic technologies, it is now possible to profile the biology of primary human leukemias in a sensitive and high-throughput manner. Recently, several groups have utilized modern microarray-based analyses of genetic alterations and gene expression to identify mutations in key lymphoid development and signaling genes in high-risk childhood ALL. In particular, high frequencies of mutations have been described in genes involved in B lymphocyte development (e.g., *EBF1*, *IKZF1*, *PAX5*) and signaling (e.g., *BTLA*, *CD200, RAS*) and in cell cycle regulation (e.g., *CDKN2A/B, PTEN, RB, TP53*). Additionally, genomic analyses of paired diagnosis and relapse ALL samples have also shed light upon the early origin and clonal nature of such leukemia-associated mutations.[7–9] A comprehensive discussion of these genetic alterations is beyond the scope of this review and has been described elsewhere in greater detail.[8–10]

A. Discovery of *CRLF2, JAK1* and *JAK2,* and *IL7RA* Alterations in Acute Lymphoblastic Leukemia (ALL)

In 2008 and 2009, research groups in Israel and Europe and in the United States first identified somatic mutations in the Janus family of kinases (JAK) in pediatric B-precursor ALL cases via various strategies employing candidate gene sequencing, gene expression profiling (GEP), and single-nucleotide polymorphism arrays.[11–16] Missense and insertional mutations were detected in highly conserved regions of the *JAK1* pseudokinase domain, *JAK2* pseudokinase and kinase domains, and *JAK3* (later determined to be a non-driver mutation), but not in *TYK2*.[12,17] Point mutations within the *JAK2* pseudokinase domain were by far the most common, particularly *JAK2* R683G. Notably, these mutations were distinct from the *JAK2* V617F commonly associated with myeloproliferative neoplasms, which is also located in the pseudokinase domain. In particular, *JAK2* mutations occur frequently in children with Down syndrome-associated ALL (DS-ALL) with an incidence of approximately 20%.[11,13,14,18] Screening of pediatric non-DS-ALL cases demonstrated a 3–10% rate of *JAK* mutations, which was highest in patients classified as "high risk" by either the Oxford Hazard Score (OHS) or the National Cancer Institute (NCI)-Rome risk criteria (age > 10 years or white blood cell count > 50,000/uL at diagnosis). In one study, *JAK* mutations were also noted to be highly associated with *IKZF1* mutations, *CDKN2A/*B deletions, *BCR-ABL1*-like gene expression profiles, and conferred a poor clinical prognosis.[12]

Shortly thereafter, additional genome-wide analyses of DNA copy number alterations (CNAs), transcriptional profiling, and sequencing of these and other DS-ALL and non-DS-ALL cases detected alterations in the *cytokine receptor-like factor 1* gene (*CRLF2*), which encodes the thymic stromal lymphopoietin receptor (TSLPR). Russell *et al*. first described deregulation of various cytokine receptors, such as the erythropoietin receptor, mediated

via juxtaposition to immunoglobulin heavy chain (*IGH@*) transcriptional enhancers in approximately 3% of B-precursor ALL cases.[19,20] Subsequently, the group used serial fluorescent *in situ* hybridization and long-distance inverse polymerase chain reaction to identify recurrent *CRLF2* abnormalities in B-precursor ALL.[21] They discovered that *CRLF2* alterations resulted either from chromosomal breakages and translocation of *CRLF2* (located on the pseudoautosomal region of the sex chromosomes; Xp22.23 or Yp11.32) with *IGH@* (14q32.3) or from interstitial deletion of Xp22.3/Yp11.3 resulting in fusion of *CRLF2* with the G-protein-coupled purinergic receptor P2Y8 gene (*P2RY8*).[21] Both alterations place *CRLF2* under alternate transcriptional control, resulting in *CRLF2* overexpression.[21–23] GEP of these leukemias revealed a *BCR-ABL1*-like kinase signature, although the *BCR-ABL1* translocation was not detected in any *CRLF2*-overexpressing ALL samples.[22,23] *CRLF2* alterations have been detected in 5–15% of pediatric and adult non-DS-ALL, depending upon the risk status of the population studied,[21,23,24] and, strikingly, in 50–60% of children with DS-ALL.[18,22] In general, the *P2RY8-CRLF2* fusion occurs more frequently in younger pediatric patients, while the *IGH@-CRLF2* translocation is more common in adolescents and adults with ALL.[21–24] More recently, Yoda *et al*. described an activating somatic point mutation in *CRLF2* itself resulting in a phenylalanine-to-cysteine change at amino acid 232 (F232C) in a small number of adult ALL patients,[24] which also has been detected infrequently in pediatric ALL patients.[18,25] In general, *CRLF2*-overexpressing ALL samples tend to possess either the *P2RY8-CRLF2* fusion or the *IGH@-CRLF2* translocation, though there are cases that appear to lack these alterations. Although usually mutually exclusive, the fusion and translocation or one alteration in conjunction with *CRLF2* F232C have been detected in a minority of patient samples. It is not yet known whether both mutations occur in a single leukemia cell or in two distinct clonal populations.[23]

CRLF2 alterations are further highly associated with *JAK* mutations in both DS-ALL and non-DS-ALL. Approximately half of patients whose leukemias have *CRLF2* alterations also harbor *JAK* mutations, particularly at the *JAK2* R683 residue. Conversely, virtually all *JAK*-mutated ALL samples reported in the literature overexpress *CRLF2* via the *P2RY8-CRLF2* fusion or *IGH@-CRLF2* translocation, suggesting the cooperative nature of these genetic events in leukemogenesis.[21,22]

Most recently, gain-of-function somatic mutations in the interleukin-7 receptor alpha chain (IL-7Rα; *IL7RA*) have also been described by Shochat *et al*. in a small number of patients with *CRLF2*-overexpressing ALL and in T cell ALL.[26] These mutations occur as S185C point mutations, located in the extracellular domain of the IL-7Rα, or as in-frame insertion and deletion mutations within the transmembrane domain. Three of the eight *CRLF2*-overexpressing B-precursor ALL samples with *IL7RA* mutations also had *JAK2* mutations, which further emphasizes the complex association of these genetic events.[26]

Importantly, it should be noted that ALL-specific alterations in *CRLF2, JAK1* and *JAK2*, and *IL7RA* have not been detected in leukemia cells with known prognostic cytogenetic abnormalities, such as high hyperdiploidy, *ETV6-RUNX1*, *TCF-ECF1*, *MLL* rearrangements, or *BCR-ABL1*,[4,21,27] with the exception of intrachromosomal amplification of chromosome 21 in some non-DS-ALL patients.[21,28,29] *CRLF2* and associated alterations thus seem to comprise a new cytogenetic subtype of B-lineage ALL (Table 1).

B. NCI High Risk Patients With *CRLF2*-Overexpressing ALL Have Poor Outcomes

Following the initial discovery of *IGH@-CRLF2* and *P2RY8-CRLF2* alterations,[21] other groups also identified *CRLF2* alterations via high-resolution genomic profiling of cohorts of pediatric ALL patients who had experienced extremely poor clinical outcomes.[22] Subsequent GEP and CNA microarray analysis by Harvey *et al.* of 207 high-risk patients treated uniformly with an augmented Berlin-Frankfurt-Münster (BFM) regimen on the legacy Children's Oncology Group study P9906 protocol revealed a 14% incidence of *CRLF2* alterations (29 patients), only two of whom had Down Syndrome. *CRLF2* alterations

TABLE 1. Details of Studies Reporting Alterations of *JAK1*, *JAK2*, *CRLF2*, and *IL7RA* in Acute Lymphoblastic Leukemia

Study	Cohort	Findings
JAK mutations		
Malinge 2007[15]	ALL and AML (*N*=90)	Identification of a transforming *JAK2* pseudokinase domain mutation: *JAK2* p.I682_D686del in a single DS-ALL case
Flex 2008[46]	Adult B-ALL (*N*=88) and T-ALL (*N*=38), pediatric B-ALL (*N*=85) and T-ALL (*N*=49)	*JAK1* FERM, SH2, JH2, and JH1 mutations in 18% adult T-ALL; mutations transforming *in vitro*, associated with distinct GEP, older age, poor outcome
Bercovich 2008[11]	DS-ALL (*N*=88), non-DS B-ALL (*N*=109), DS-AMKL (*N*=11), ET (*N*=96)	Missense mutations at/near R683 in the *JAK2* pseudokinase domain in 18% DS-ALL; mutations transforming in Ba/F3-EpoR cells
Kearney 2009[14]	DS-ALL (*N*=42)	JAK2 R683 mutations in 28% cases
Mullighan 2009[12]	High-risk B-progenitor ALL (*N*=187)	*JAK1* (pseudokinase), *JAK2* (pseudokinase and kinase) and *JAK3* mutations in 20 (10.7%) cases; 18 of 20 cases non-DS B-ALL; mutations associated with *IKZF1* deletion, deletions adjacent to *CRLF2*, *BCR-ABL1*-like gene expression profile, and very poor outcome; mutations transform Ba/F3-EpoR cells *in vitro*, and transformation abrogated by pharmacologic JAK inhibitors
Gaikwad 2009[13]	Pediatric DS-ALL (*N*=53)	*JAK2* pseudokinase (R683) mutations in 18.9% DS-ALL cases
CRLF2 alterations		
Russell 2009[21]	Pediatric ALL (*N*>1000)	97 B-ALL cases with *CRLF2* rearrangement; 33 t(X;14)(p22;q32) or t(Y;14)(p11;q32) and 64 with PAR1 deletion (del(X)(p22.33p22.33) or del(Y)(p11.32p11.32)); translocation 0.8% and deletion 4.2% BCP-ALL. 52% of 68 DS-ALL cases have PAR1 deletion
Mullighan 2009[22]	Pediatric B-ALL (*N*=272) and T-ALL (*N*=57)	*IGH@-CRLF2* translocation or Xp/Yp PAR1 deletion in 7% B-ALL and 53% DS-ALL; PAR1 deletion mapped and definitively shown to result in *P2RY8-CRLF2* fusion; rearrangements result in overexpression of *CRLF2;* strong association between *CRLF2* rearrangement and *JAK* mutations; lesions co-transforming in Ba/F3 cells
Hertzberg 2010[18]	Pediatric DS-ALL (*N*=53) and non-DS-ALL	Confirmatory study identifying *CRLF2* rearrangement in 53% DS-ALL samples
Yoda 2010[24]	Adult and pediatric ALL	Overexpression of *CRLF2* in 15% adult and pediatric ALL; confirmation of *CRLF2* rearrangement and association with JAK mutations; identification of *CRLF2* F232C mutation
Harvey 2010[47]	Pediatric high-risk ALL (*N*=207)	Overexpression of *CRLF2* in 14% cases due to *IGH@* translocation (62%) and/or *P2RY8-CRLF2* (34%); *CRLF2* rearrangement associated with JAK mutation, Hispanic/Latino ethnicity, *IKZF1* alteration, and poor outcome
Cario 2010[30]	Pediatric ALL (ALL-BFM-2000; *N*=555)	*CRLF2* overexpression due to rearrangement and associated with poor outcome
IL7RA mutations		
Shochat 2011[26]	Pediatric B-ALL (BFM AIEOP 2000; *N*=286, of which 83 DS-ALL) and T-ALL (BFM ; *N*=285)	Identification of *IL7RA* S185C and insertional/deletional mutations in 3.1% cases of B-ALL (8 of 9 cases also *CRLF2*-overexpressing) and in 10.5 % T-ALL;. mutations transforming in Ba/F3-*CRLF2* cells

Abbreviations: AIEOP, Associazione Italiana Ematologia Oncologia Pediatrica; ALL, acute lymphoblastic leukemia; AMKL, acute megakaryoblastic leukemia; BFM, Berlin-Frankfurt-Münster; DS, Down syndrome; ET, essential thrombocytosis.

Modified from JR Collins-Underwood and CG Mullighan, Genomic profiling of high-risk acute lymphoblastic leukemia. Leukemia. 2010;24:1676-85.[16] Used with permission.

were highly associated with *JAK* and *IKZF1* mutations (69% and 80%, respectively) and with Hispanic/Latino ethnicity. Patients with *CRLF2*-overexpressing ALL responded slowly to chemotherapy and had high rates of minimal residual disease (MRD) at the end of induction. Furthermore, nearly 70% of these patients relapsed. Relapse-free survival (RFS) was 35.3% for *CRLF2* overexpressors vs. 71.3% for non-overexpressors treated on this trial at 4 years. Interestingly, there was no significant effect of the presence of simultaneous *JAK* or *IKZF1* mutations upon MRD or RFS, although the number of patients with *CRLF2*-overexpressing *JAK* wild-type *IKZF1* wild-type ALL in this study was small.[23]

To validate these results and to evaluate *CRLF2* overexpression in standard risk patients, further analyses were performed of 896 ALL samples from children enrolled on the COG 9905 study, comprised of both NCI high-risk (HR) and standard risk (SR) patients. One hundred seventeen patients demonstrated high levels of *CRLF2* expression, which was confirmed by specific mutational analysis. Of the *CRLF2* overexpressors, only those patients classified as NCI HR had a significantly poorer RFS (<40%), which was in sharp contrast to those initially classified as NCI SR (RFS >80%).[25]

Additional analyses of the prognostic significance of *CRLF2* expression have also been performed by Cario *et al*. in 555 leukemia samples from children treated on the ALL-BFM 2000 protocol.[31] Specimens were classified as *CRLF2*-high (49 patients) vs. *CRLF2*–low (506 patients) based upon prior published gene expression data[21, 22] and were further stratified by HR and non-high risk (NHR) clinical groups (classified by *in vivo* response to prednisone). Predictably, DS-ALL patients were enriched in the *CRLF2*-high group. In contrast to the COG studies, these analyses demonstrated no prognostic effect of *CRLF2* overexpression in the HR cohort, whereas *CRLF2*-high NHR patients had worse event-free survival (EFS) than *CRLF2*-low NHR patients (61% and 83%, respectively), particularly those *CRLF2*-high patients harboring the *P2RY8-CRLF2* fusion (29% EFS).[30]

The Medical Research Council (MRC) has also published data regarding the prognostic significance of *CRLF2* overexpression in OHS SR children.[27] Fifty-two of 865 studied patients treated on the MRC ALL97 protocol showed evidence of deregulated *CRLF2* in their leukemia cells (6%), although this rate was markedly higher in DS-ALL patients (54%; 14 of 26 DS patients).[27] Ensor *et al*. noted that *CRLF2*-overexpressing ALL patients were more likely to have hepatosplenomegaly at the time of leukemia diagnosis, although there was no association with age, gender, or initial white blood cell count. *CRLF2* alterations were also associated with *IKZF1*, *CDKN2A/2B*, *PAX5*, and *JAK2* mutations, but to a lesser degree than previously observed in the COG cohorts.[22,23] While univariate analysis demonstrated inferior EFS (but not inferior RFS) of patients with *CRLF2*-overexpressing ALL, this phenomenon did not retain significance in a multivariate analysis. This study also confirmed the association of *IGH@-CRLF2* translocation with older age observed by others[21–24] and noted a non–statistically significant trend of worse EFS for patients with *IGH@-CRLF2* versus those with *P2RY8-CRLF2*. Ultimately, however, no significant difference in RFS or overall survival (OS) was detected between *CRLF2* overexpressors and non-expressors in this SR cohort, as has now been observed in the COG P9905 analysis.[25] The authors concluded that *CRLF2* overexpression in OHS SR ALL patients thus conferred an intermediate-risk, not high-risk, prognosis.[27]

It is important to note the differences among the P9905, P9906, ALL-BFM 2000, and MRC ALL-97 protocols, which may account for the observed variability in outcomes. In particular, striking differences exist between the characteristics of patients treated on the COG 9906 protocol and on the MRC ALL-97 protocol. The P9906 patients were significantly older (median age = 12.8 years), had a higher incidence of *IGH@-CRLF2* translocations and *IKZF1* mutations, and were more likely to be Latino/Hispanic. MRC ALL-97 enrolled patients who were younger, had a five-fold higher rate of *P2RY8-CRLF2* fusions than *IGH@-CRLF2*, and had a higher percentage of DS-ALL patients. In addition, the optimal threshold to define *CRLF2* overexpression by QT-PCR has not yet

been standardized across international consortia, but these efforts are ongoing. Despite these differences, comprehensive data from these analyses do suggest that *CRLF2* overexpression likely portends a poor prognosis for HR, but not SR, ALL patients treated with current therapies. The reason for the discrepant clinical outcomes of HR versus SR *CRLF2*-overexpressing non-DS-ALL patients remains unknown. Additionally, Hertzberg *et al*. noted a trend toward worse OS in DS-ALL *CRLF2* overexpressors,[13,18] while Gaikwad *et al*. observed no difference in EFS between *CRLF2*-overexpressing and non-overexpressing DS-ALL patients.[13]

C. Adults with *CRLF2*-Overexpressing ALL Have the Worst Rates of Survival

While various groups have begun to characterize the outcomes of pediatric ALL patients with *CRLF2* alterations, less is currently known about the prognoses of adults with *CRLF2*-overexpressing ALL. In one study of adult B-precursor ALL patients whose leukemias lacked prognostic cytogenetic abnormalities, 15 of 90 (16.7%) samples demonstrated *CRLF2* overexpression.[24] *CRLF2* overexpressors and non-overexpressors did not differ significantly in age, gender, or presenting white blood cell count, but disease-free and overall survival were markedly worse in the *CRLF2* overexpressors (17.8 months vs. 37.8 months and 25.5 months vs. >100 months, respectively).[24] Although adult patients with ALL have notably poorer outcomes than children, generally ascribed to the higher incidence of *BCR/ABL1*-positive disease and to their inability to tolerate intensive multi-agent chemotherapy, *CRLF2* overexpression also appears to confer a worse prognosis in this population. Larger analyses of RFS and OS in adults with *CRLF2*-overexpressing ALL are indicated and will likely provide important rationale for the identification and development of targeted therapies with potentially fewer toxicities, especially for patients who are unable to tolerate traditional therapy. Various cooperative research groups have thus sought to understand and characterize the biochemical consequences of these genetic lesions with the goal of identifying new therapeutic targets.

II. ELUCIDATING THE BIOCHEMICAL SEQUELAE OF *CRLF2* ALTERATIONS IN ALL

A. The Role of the Thymic Stromal Lymphopoietin Receptor (TSLPR) in ALL

CRLF2 encodes the TSLPR subunit, which heterodimerizes with the IL-7Rα subunit to form the functional TSLPR. Upon binding of its ligand, thymic stromal lymphopoietin (TSLP), the TSLPR is known to induce phosphorylation of the signal transducer and activation of transcription factor 5 (STAT5) in normal dendritic and T cells in response to allergic or inflammatory stimuli, which is likely mediated by antecedent phosphorylation of *JAK2* (pJAK2).[31–36] TSLP has also been shown to promote early development of B cells *in vitro*.[33]

Little is currently known about the role of the TSLPR and TSLP-mediated signal transduction in leukemia, however. Initial studies by Brown *et al*. of transgenic murine non–*CRLF2*-overexpressing B-precursor ALL cell lines demonstrated TSLP- and IL-7–induced phosphorylation of STAT5 (pSTAT5), as well as dose-dependent cellular proliferation in culture.[37] TSLP and IL-7 also induced phosphorylation of S6 (pS6) and 4EBP1 (p4EBP1), which could be inhibited by rapamycin, a mammalian target of rapamycin (mTOR) inhibitor. TSLP further rescued rapamycin-treated primary human ALL cells from apoptosis in short-term culture.[38]

B. *CRLF2* and *JAK* Mutations Confer Gain-of-Function and Result in Aberrant Signal Transduction

Several groups have created preclinical models of human *CRLF2* and *JAK* mutations using retrovirally transduced Ba/F3 cells, an IL-3-dependent murine lymphoma cell line, to evaluate the biochemical effects of these genetic lesions. In an initial study by Mullighan *et al*.,[12] Ba/F3 cells transduced with various patient-derived *JAK1* and *JAK2* mutations, and the murine erythropoietin (EPO) receptor demonstrated moderate cytokine independent growth, as well as preferential growth inhibition when co-cultured

with the chemical JAK inhibitor I. These cell lines also had constitutive levels of pJAK2 and pSTAT5, which were further induced by EPO stimulation.[12] Subsequent work by this group using Ba/F3 cells transduced with the murine IL-7R (Ba/F3-IL-7R), the *P2RY8-CRLF2* fusion, and *JAK2* pseudokinase and kinase domain mutations demonstrated cytokine independence only of the compound *CRLF2*/*JAK2* mutants, but not of cells transduced with either mutation alone or with wild-type constructs.[22] IL-3 independent growth was partially attenuated by short hairpin-RNA knockdown of *CRLF2*. *CRLF2*/*JAK2* mutant Ba/F3 cells also demonstrated constitutive activation of JAK/STAT signaling and preferential growth inhibition by the pharmacologic JAK inhibitor I.[22] Similar observations of constitutive JAK2/STAT5 activation and inhibition with JAK inhibitor I were also made by Hertzberg *et al*. using Ba/F3 cells transduced with wild-type *CRLF2* and *JAK2* R683S, as well as IL-3 independent growth even in the absence of transduced IL-7R.[18]

Another study of *CRLF2* overexpression in adult ALL patients used Ba/F3-IL-7R cells transduced with wild-type *CRLF2* or *CRLF2* F232C to explore the specific effects of this newly discovered point mutation.[24] Using reducing and non-reducing conditions, Yoda *et al*. demonstrated that mutant, but not wild-type, *CRLF2* likely constitutively homodimerizes via intermolecular disulfide bonds of the cysteine residues. TSLP also stimulated modest proliferation of Ba/F3-IL-7R *CRLF2* F232C cells *in vitro*. Immunoblotting of lysates from Ba/F3 *CRLF2* F232 cells demonstrated some constitutive phosphorylation of ERK 1/2 (pERK) and pSTAT5. Additionally, Ba/F3 cells transduced with wild-type *CRLF2* and *JAK2* R683G or R683S had markedly increased basal levels of pJAK2, pSTAT5, and pERK compared to *CRLF2* wild-type/*JAK2* wild-type cells,[24] which was concordant with prior observations.[12,22] Growth of mutant cell lines was also preferentially inhibited by the JAK inhibitor I.[24]

Most recently, studies of primary human *CRLF2*-overexpressing ALL samples with and without simultaneous *JAK* mutations have demonstrated activation of JAK/STAT and phosphatidylinositol 3-kinase (PI3K) pathway signal transduction with TSLP stimulation *in vitro*. JAK2 inhibition with XL019 (Exelixis) abrogated TSLP-induced pSTAT5 and pS6. Minor activation of TSLP-induced MAPK signaling has also been observed in human *CRLF2*-overexpressing ALL cell lines, but less so in primary samples. Studies are ongoing to define more precisely the aberrant signal transduction networks in human *CRLF2*-overexpressing ALL and to test relevant signal transduction inhibitors *in vitro* and *in vivo* in preclinical murine xenograft models.[39]

C. IL-7Rα Mutations Are Also Transforming and Activate JAK/STAT and PI3K Signal Transduction

Given the heterodimeric structure of the TSLPR, research groups have recently begun to evaluate *CRLF2*-overexpressing ALL samples for the presence of *IL7RA* mutations. The group that first identified these somatic missense mutations in *CRLF2*-overexpressing ALL (and in T cell ALL) also tested whether or not these lesions conferred cytokine independent growth using Ba/F3 models.[26] Cells transduced with the transmembrane domain in-frame insertional *IL7RA* mutation InsPPCL demonstrated IL-3 independent growth, while simultaneous *CRLF2* wild-type and *IL7RA* S185C constructs were necessary to confer cytokine independence, suggesting biologic differences between the subtypes of *IL7RA* mutations. TSLP induced preferential proliferation of *CRLF2* wild-type/ *IL7RA* mutant cells in comparison to *CRLF2* wild-type/ *IL7RA* wild-type cells. *CRLF2* wild-type/ *IL7RA* mutant cells demonstrated constitutive pSTAT5 and pS6, which was further inducible with TSLP stimulation. Substitution of the mutant amino acid 185 cysteine residue with a glycine residue in the *IL7RA* constructs markedly diminished cytokine independent growth and constitutive pSTAT5. In addition, Shochat *et al*. observed marked homodimerization of Ba/F3-IL-7Rα InsPPCL cells under non-reducing versus reducing conditions.[26] These data suggest that, as observed with *CRLF2* F232C,[24] introduced cysteine residues likely facilitate IL-7Rα receptor homodimerization and are necessary for the gain-of-function phenotype.

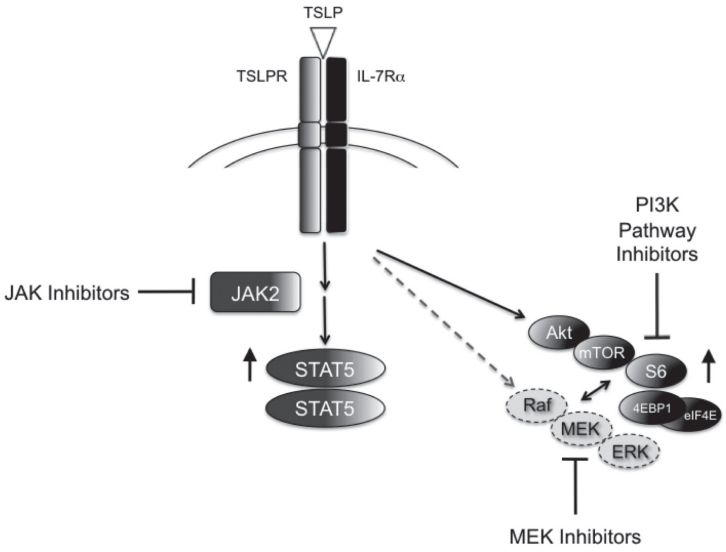


FIGURE 1. Proposed schema of aberrant signal transduction mediated by the TSLPR heterodimer in *CRLF2*-overexpressing ALL. JAK/STAT, PI3K, and possibly MAPK signaling networks are hyperactive. Targeting hyperactive signaling nodes with STIs (such as JAK, PI3K pathway, and MEK inhibitors) is a rational therapeutic strategy for preclinical and clinical testing.

III. ON THE HORIZON: DEVELOPMENT OF TARGETED THERAPIES FOR PATIENTS WITH *CRLF2*-OVEREXPRESSING ALL

Significant efforts have been made by cooperative research groups in a relatively short period of time to improve the biologic understanding of *CRLF2*-overexpressing ALL. *CRLF2* and other associated alterations occur in approximately 5–15% of B-precursor ALL, and children and adults with *CRLF2*-overexpressing ALL suffer unacceptably high rates of relapse with current therapies. These patients comprise an important, newly defined genetic subgroup of HR ALL for whom new treatments are vital to improve RFS and OS.

Extensive preclinical data to date demonstrate aberrant activation of TSLPR-mediated signal transduction in these leukemias, particularly of the JAK/STAT and PI3K pathways. Additional preclinical and translational studies are needed to characterize the biochemical sequelae of *CRLF2* alterations and associated genetic lesions more fully, however. Early *in vitro* work with JAK inhibitors does suggest the promise of targeting aberrant signal transduction with small-molecule inhibitors. Identification of other associated mutations, further delineation of hyperactive signaling networks and specific targets, and evaluation of relevant targeted signal transduction inhibitors (STIs) in *CRLF2*-overexpressing ALL will greatly facilitate these goals (Figure 1).

Precedent exists for the addition of STIs for treatment of pediatric leukemias, as evidenced by the dramatic improvement in long-term survival for children with *BCR-ABL*-positive ALL when imatinib is added to systemic chemotherapy.[6] Currently, STIs targeting hyperactive signaling networks are also being tested in phase II and III clinical trials for adults with *JAK2* V617F-associated MPNs,[40–45] and the clinical, but not molecular, efficacy of the JAK inhibitor INCB018424 (Incyte) in adults with myelofibrosis was recently reported.[41] Determination of the maximum tolerated dose and dose-limiting toxicities of INCB018424 in pediatric patients with relapsed/refractory malignancies is further underway in a Children's Oncology Group phase I trial. Simultaneous preclinical studies of JAK inhibitors and other relevant STIs in combination with cytotoxic chemotherapy are essential, however, and will provide additional rationale for development of such therapeutic approaches in subsequent clinical trials.

Modern genomic profiling techniques have provided important insights regarding the molecular pathogenesis of high-risk ALL that will hopefully identify potential new therapeutic targets. Current data suggest that *CRLF2*-overexpressing ALL is a disorder of hyperactive JAK/STAT and PI3K signaling. Future development of STI-based therapies for adult and pediatric patients with these high-risk leukemias will depend upon the successful identification of specific agents capable of abrogating hyperactive signaling networks shown to be perturbed in these leukemias, as well as our ability to test these agents in accurate preclinical systems, such as genetically engineered mice or xenograft models. Important questions remain, however, regarding why HR, but not SR or DS-ALL, patients fare poorly with current therapeutic strategies. While the first steps toward understanding the biological underpinnings of this subtype of leukemia have been made, much additional work is necessary to unravel fully the mechanisms that contribute to the pathogenesis of *CRLF2*-overexpressing ALL.

ACKNOWLEDGMENT

This work was supported by NIH/NCI 1T32CA12858 and NIH/NICHD 5T32HD044331 (SKT), NIH/NCI K12CA076931 (SKT), a Conquer Cancer Foundation Young Investigator award (SKT), the TeamConnor Childhood Cancer Research Foundation (SKT and MLL), the St. Baldrick's Foundation (MLL), and NIH/NCI RC2CA148529-01 (MLL). MLL is a Clinical Scholar of the Leukemia and Lymphoma Society.

REFERENCES

1. Pui C, Robison L, Look A. Acute lymphoblastic leukaemia. Lancet. 2008;371(9617):1030–43.
2. Hunger SP, Raetz EA, Loh ML, Mullighan CG. Improving outcomes for high-risk ALL: translating new discoveries into clinical care. Pediatr Blood Cancer. 2011 Jun;56(6):984–93.
3. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, Linda S, Martin PL, Pullen DJ, Viswanatha D, Willman CL, Winick N, Camitta BM. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. Blood. 2008 Jun 15;111(12):5477–85.
4. Mullighan CG. New strategies in acute lymphoblastic leukemia: translating advances in genomics into clinical practice. Clin Cancer Res. 2011 Feb 1;17(3):396–400.
5. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. J Clin Oncol. 2011 Feb 10;29(5):532–43.
6. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, Wang C, Davies SM, Gaynon PS, Trigg M, Rutledge R, Burden L, Jorstad D, Carroll A, Heerema NA, Winick N, Borowitz MJ, Hunger SP, Carroll WL, Camitta B. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. J Clin Oncol. 2009 Nov 1;27(31):5175–81.
7. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL, Downing JR. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009 Jan 29;360(5):470–80.
8. Zhang J, Mullighan CG, Harvey RC, Wu G, Chen X, Edmonson M, Buetow KH, Carroll WL, Chen IM, Devidas M, Gerhard DS, Loh ML, Reaman GH, Relling MV, Camitta BM, Bowman WP, Smith MA, Willman CL, Downing JR, Hunger SP. Key pathways are frequently mutated in high risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2011;118(11):3080–7.
9. Hogan LE, Meyer JA, Yang J, Wang J, Wong N, Yang W, Condos G, Hunger SP, Raetz E, Saffery R, Relling MV, Bhojwani D, Morrison DJ, Carroll WL. Integrated genomic analysis of relapsed childhood acute lymphoblastic leukemia reveals therapeutic strategies. Blood. 2011;118(19):5218–26.
10. Collins-Underwood JR, Mullighan CG. Genetic alterations targeting lymphoid development in acute lymphoblastic leukemia. Curr Top Dev Biol. 2011;94:171–96.

11. Bercovich D, Ganmore I, Scott LM, Wainreb G, Birger Y, Elimelech A, Shochat C, Cazzaniga G, Biondi A, Basso G, Cario G, Schrappe M, Stanulla M, Strehl S, Haas OA, Mann G, Binder V, Borkhardt A, Kempski H, Trka J, Bielorei B, Avigad S, Stark B, Smith O, Dastugue N, Bourquin JP, Tal NB, Green AR, Izraeli S. Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome. Lancet. 2008 Oct 25;372(9648):1484–92.
12. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, Tasian SK, Loh ML, Su X, Liu W, Devidas M, Atlas SR, Chen IM, Clifford RJ, Gerhard DS, Carroll WL, Reaman GH, Smith M, Downing JR, Hunger SP, Willman CL. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2009 Jun 9;106(23):9414–8.
13. Gaikwad A, Rye CL, Devidas M, Heerema NA, Carroll AJ, Izraeli S, Plon SE, Basso G, Pession A, Rabin KR. Prevalence and clinical correlates of JAK2 mutations in Down syndrome acute lymphoblastic leukaemia. Br J Haematol. 2009 Mar;144(6):930–2.
14. Kearney L, Gonzalez De Castro D, Yeung J, Procter J, Horsley SW, Eguchi-Ishimae M, Bateman CM, Anderson K, Chaplin T, Young BD, Harrison CJ, Kempski H, So CW, Ford AM, Greaves M. Specific JAK2 mutation (JAK2R683) and multiple gene deletions in Down syndrome acute lymphoblastic leukemia. Blood. 2009 Jan 15;113(3):646–8.
15. Malinge S, Ben-Abdelali R, Settegrana C, Radford-Weiss I, Debre M, Beldjord K, Macintyre EA, Villeval JL, Vainchenker W, Berger R, Bernard OA, Delabesse E, Penard-Lacronique V. Novel activating JAK2 mutation in a patient with Down syndrome and B-cell precursor acute lymphoblastic leukemia. Blood. 2007 Mar 1;109(5):2202–4.
16. Collins-Underwood JR, Mullighan CG. Genomic profiling of high-risk acute lymphoblastic leukemia. Leukemia. 2010 Oct;24(10):1676–85.
17. Mullighan CG. JAK2—a new player in acute lymphoblastic leukaemia. Lancet. 2008 Oct 25; 372(9648):1448–50.
18. Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J, Shiloh R, Iacobucci I, Shochat C, Zeligson S, Cario G, Stanulla M, Strehl S, Russell LJ, Harrison CJ, Bornhauser B, Yoda A, Rechavi G, Bercovich D, Borkhardt A, Kempski H, Te Kronnie G, Bourquin JP, Domany E, Izraeli S. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. Blood. 2010 Feb 4;115(5):1006–17.
19. Akasaka T, Balasas T, Russell LJ, Sugimoto KJ, Majid A, Walewska R, Karran EL, Brown DG, Cain K, Harder L, Gesk S, Martin-Subero JI, Atherton MG, Bruggemann M, Calasanz MJ, Davies T, Haas OA, Hagemeijer A, Kempski H, Lessard M, Lillington DM, Moore S, Nguyen-Khac F, Radford-Weiss I, Schoch C, Struski S, Talley P, Welham MJ, Worley H, Strefford JC, Harrison CJ, Siebert R, Dyer MJ. Five members of the CEBP transcription factor family are targeted by recurrent IGH translocations in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Blood. 2007 Apr 15;109(8):3451–61.
20. Russell LJ, De Castro DG, Griffiths M, Telford N, Bernard O, Panzer-Grumayer R, Heidenreich O, Moorman AV, Harrison CJ. A novel translocation, t(14;19)(q32;p13), involving IGH@ and the cytokine receptor for erythropoietin. Leukemia. 2009 Mar;23(3):614–7.
21. Russell LJ, Capasso M, Vater I, Akasaka T, Bernard OA, Calasanz MJ, Chandrasekaran T, Chapiro E, Gesk S, Griffiths M, Guttery DS, Haferlach C, Harder L, Heidenreich O, Irving J, Kearney L, Nguyen-Khac F, Machado L, Minto L, Majid A, Moorman AV, Morrison H, Rand V, Strefford JC, Schwab C, Tonnies H, Dyer MJ, Siebert R, Harrison CJ. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood. 2009 Sep 24;114(13):2688–98.
22. Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, Ma J, Coustan-Smith E, Harvey RC, Willman CL, Mikhail FM, Meyer J, Carroll AJ, Williams RT, Cheng J, Heerema NA, Basso G, Pession A, Pui CH, Raimondi SC, Hunger SP, Downing JR, Carroll WL, Rabin KR. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009 Nov;41(11):1243–6.
23. Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, Kang H, Liu W, Dobbin KK, Smith MA, Carroll WL, Devidas M, Bowman WP, Camitta BM, Reaman GH, Hunger SP, Downing JR, Willman CL. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1,

Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010 Feb 4;115(26):5312–21.

24. Yoda A, Yoda Y, Chiaretti S, Bar-Natan M, Mani K, Rodig SJ, West N, Xiao Y, Brown JR, Mitsiades C, Sattler M, Kutok JL, DeAngelo DJ, Wadleigh M, Piciocchi A, Dal Cin P, Bradner JE, Griffin JD, Anderson KC, Stone RM, Ritz J, Foa R, Aster JC, Frank DA, Weinstock DM. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 2010 Jan 5;107(1):252–7.
25. Chen I, Harvey R, Mullighan CG, Loh ML, Devidas M, Borowitz MJ, Tasian SK, Payne-Turner D, Wharton W, Pullen J, Carroll AJ, Carroll WL, Camitta BM, Hunger S, Winick NJ, Willman CL. Relationship of CRLF2 expression and outcome in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL): A report from the Children's Oncology Group. J Clin Oncol. 2011;29(Supplemental):9505.
26. Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, Te Kronnie G, Cario G, Cazzaniga G, Kulozik AE, Stanulla M, Schrappe M, Biondi A, Basso G, Bercovich D, Muckenthaler MU, Izraeli S. Gain-of-function mutations in interleukin-7 receptor-α (IL7R) in childhood acute lymphoblastic leukemias. J Exp Med. 2011 May 2;208(5):901–8.
27. Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, Jones L, Kinsey SE, Vora AJ, Mitchell CD, Harrison CJ, Moorman AV. Demographic, clinical and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. Blood. 2010 Nov 29;129(6):734–45.
28. Harewood L, Robinson H, Harris R, Al-Obaidi MJ, Jalali GR, Martineau M, Moorman AV, Sumption N, Richards S, Mitchell C, Harrison CJ. Amplification of AML1 on a duplicated chromosome 21 in acute lymphoblastic leukemia: a study of 20 cases. Leukemia. 2003 Mar;17(3):547–53.
29. Moorman AV, Richards SM, Robinson HM, Strefford JC, Gibson BE, Kinsey SE, Eden TO, Vora AJ, Mitchell CD, Harrison CJ. Prognosis of children with acute lymphoblastic leukemia (ALL) and intrachromosomal amplification of chromosome 21 (iAMP21). Blood. 2007 Mar 15;109(6):2327–30.
30. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, Schrauder A, Moericke A, Izraeli S, Akasaka T, Dyer MJ, Siebert R, Schrappe M, Stanulla M. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010 Jul 1;115(26):5393–7.
31. Isaksen DE, Baumann H, Trobridge PA, Farr AG, Levin SD, Ziegler SF. Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction. J Immunol. 1999 Dec 1;163(11):5971–7.
32. Isaksen DE, Baumann H, Zhou B, Nivollet S, Farr AG, Levin SD, Ziegler SF. Uncoupling of proliferation and Stat5 activation in thymic stromal lymphopoietin-mediated signal transduction. J Immunol. 2002 Apr 1;168(7):3288–94.
33. Levin SD, Koelling RM, Friend SL, Isaksen DE, Ziegler SF, Perlmutter RM, Farr AG. Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. J Immunol. 1999 Jan 15;162(2):677–83.
34. Quentmeier H, Drexler HG, Fleckenstein D, Zaborski M, Armstrong A, Sims JE, Lyman SD. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. Leukemia. 2001 Aug;15(8):1286–92.
35. Wohlmann A, Sebastian K, Borowski A, Krause S, Friedrich K. Signal transduction by the atopy-associated human thymic stromal lymphopoietin (TSLP) receptor depends on Janus kinase function. Biol Chem. 2010 Feb–Mar;391(2–3):181–6.
36. Ziegler SF. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. Curr Opin Immunol. 2010 Dec;22(6):795–9.
37. Brown VI, Fang J, Alcorn K, Barr R, Kim JM, Wasserman R, Grupp SA. Rapamycin is active against B-precursor leukemia in vitro and in vivo, an effect that is modulated by IL-7–mediated signaling. Proc Natl Acad Sci U S A. 2003 Dec 9;100(25):15113–8.
38. Brown VI, Hulitt J, Fish J, Sheen C, Bruno M, Xu Q, Carroll M, Fang J, Teachey D, Grupp SA. Thymic stromal-derived lymphopoietin induces proliferation of pre-B leukemia and antagonizes mTOR inhibitors, suggesting a role for interleukin-7Ralpha signaling. Cancer Res. 2007 Oct 15;67(20):9963–70.

39. Teachey DT, Vincent T, Willman CL, Loh ML, Mullighan CG, Hunger S, Grupp SA. Targeting mTOR signaling is an effective treatment strategy for IKAROS and JAK kinase mutated acute lymphoblastic leukemia. Blood. 2010;116(21): 3251.
40. Verstovsek S. Therapeutic potential of JAK2 inhibitors. Hematology Am Soc Hematol Educ Program. 2009:636–42.
41. Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA, Estrov Z, Bradley EC, Erickson-Viitanen S, Vaddi K, Levy R, Tefferi A. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. N Engl J Med. 2010;363:1117–27.
42. Verstovsek S, Manshouri T, Quintas-Cardama A, Harris D, Cortes J, Giles FJ, Kantarjian H, Priebe W, Estrov Z. WP1066, a novel JAK2 inhibitor, suppresses proliferation and induces apoptosis in erythroid human cells carrying the JAK2 V617F mutation. Clin Cancer Res. 2008 Feb 1;14(3):788–96.
43. Levine RL, Pardanani A, Tefferi A, Gilliland DG. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nat Rev Cancer. 2007;7:673–83.
44. Manshouri T, Quintas-Cardama A, Nussenzveig RH, Gaikwad A, Estrov Z, Prchal J, Cortes JE, Kantarjian HM, Verstovsek S. The JAK kinase inhibitor CP-690,550 suppresses the growth of human polycythemia vera cells carrying the JAK2V617F mutation. Cancer Sci. 2008 Jun;99(6):1265–73.
45. Quintas-Cardama A, Vaddi K, Liu P, Manshouri T, Li J, Scherle PA, Caulder E, Wen X, Li Y, Waeltz P, Rupar M, Burn T, Lo Y, Kelley J, Covington M, Shepard S, Rodgers JD, Haley P, Kantarjian H, Fridman JS, Verstovsek S. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. Blood. 2010 Apr 15;115(15):3109–17.
46. 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.
47. Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, Kang H, Liu W, Dobbin KK, Smith MA, Carroll WL, Devidas M, Bowman WP, Camitta BM, Reaman GH, Hunger SP, Downing JR, Willman CL. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010 Jul 1;115(26):5312-21.

Critical Reviews™ in Oncogenesis, 16(1–2), 25–36 (2011)

Myeloid Leukemia in Down Syndrome

Irum Khan, Sébastien Malinge, & John D. Crispino*

Division of Hematology/Oncology, Northwestern University, Chicago Illinois

*Address all correspondence to: John D. Crispino, Northwestern University, 303 East Superior Street, Lurie 5-113, Chicago, IL 60611; j-crispino@northwestern.edu

ABSTRACT: Although adults with Down syndrome (DS) show a decreased incidence of cancer compared to individuals without DS, children with DS are at an increased risk of leukemia. Nearly half of these childhood leukemias are classified as acute megakaryoblastic leukemia (AMKL), a relatively rare subtype of acute myeloid leukemia (AML). Here, we summarize the clinical features of myeloid leukemia in DS, review recent research on the mechanisms of leukemogenesis, including the roles of *GATA1* mutations and trisomy 21, and discuss treatment strategies. Given that trisomy 21 is a relatively common event in hematologic malignancies, greater knowledge of how the genes on chromosome 21 contribute to DS-AMKL will increase our understanding of a broader class of patients with leukemia.

KEY WORDS: acute megakaryoblastic leukemia (AMKL), GATA-1, transient myeloproliferative disorder (TMD), trisomy 21

ABBREVIATIONS

AMKL, acute megakaryoblastic leukemia; AML, acute myeloid leukemia; Ara-C, cytarabine; ara-U, arabinoside; BFU-E, burst-forming unit erythroid; BST2, bone marrow stromal-cell antigen 2; CDA, cytidine deaminase; CBS, cystathionine beta synthase; CFU-GM, colony-forming unit, granulocyte macrophage; DMR, differentially methylated region; DS, Down syndrome; EFS, event-free survival; ESC, embryonic stem cells; FL, fetal liver; Hsa21, human chromosome 21; HSC, hematopoietic stem cell; IGF-2, insulin-like growth factor 2; ML-DS, myeloid leukemia Down syndrome; MPD, myeloproliferative disorder; MRD, minimal residual disease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOD/SCID, non-obese diabetic/severe combined immunodeficient; OS, overall survival; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; SOD, superoxide dismutase; TL, transient leukemia; TMD, transient myeloproliferative disorder

I. INTRODUCTION

Individuals with Down syndrome (DS) display various developmental abnormalities, including craniofacial dysmorphy, cardiovascular defects and learning disabilities. Paradoxically, individuals with DS have a decreased frequency of solid tumors (epidemiological studies in Denmark, Finland, and Australia indicated an incidence ratio respectively of 0.50, 0.57, and 0.44[1–3]), but a higher incidence of leukemia (10–20-fold).[4] Even more strikingly, young children (<4 years old) with DS have a 500-fold increased incidence of acute megakaryoblastic leukemia (AMKL, also known as ML-DS).[5] The natural history of leukemia in children with DS suggests that trisomy 21 directly contributes to the malignant transformation of hematopoietic cells. In addition, somatic mutations of the *GATA1* gene have been detected in nearly all DS AMKL cases and are notably absent in non-DS AMKL.[6] In this review, we will highlight the clinical manifestations, outcomes, and new observations related to signaling pathways aberrantly controlled by trisomy 21 or *GATA1* mutations during DS-AMKL leukemogenesis.

II. CLINICAL FEATURES

A well-recognized preceding transient myeloproliferative disorder (TMD), aka transient leukemia (TL), occurs in the neonatal period in 4–5% of infants with DS.[7–9] TMD is a clonal pre-leukemia characterized by an accumulation of immature megakaryoblasts in the fetal liver and peripheral blood.[5] The incidence of TMD may be underestimated, as not all cases come to medical attention. The median age of presentation of TMD, based on pooled data from >200 neonates,

0893-9675/11/$35.00

is 3–7 days.[10–12] The clinical presentation of neonates with TMD ranges from a healthy appearance to bruising, respiratory distress, fulminant hepatic failure, *hydrops fetalis,* or even death in 15–20% of cases that have been diagnosed. Overall, though, the majority of cases resolve spontaneously with normal blood counts at a mean of 84 days.[13] After a latency period of 1–4 years, a subset of these children (20–30%), develop acute megakaryoblastic leukemia.[14] In a series of 112 patients with AMKL, the median age of DS patients was 1.8 years old versus approximately 8 years old in non-DS cases.[15,16] Patients with AMKL develop anemia, thrombocytopenia, myelofibrosis, organomegaly, extensive skeletal lesions,[17,18] and leukocytosis, although white blood counts are lower in DS-AMKL than in non-DS-AMKL.[19,20] CNS involvement is unusual.[16]

A. Diagnosis

Histological examination of the bone marrow in AMKL shows replacement with megakaryoblasts and reticulin deposition. Megakaryoblasts are identified by a positive platelet peroxidase reaction,[21] and by immunophenotyping for glycoprotein IIb/IIIa or the von Willebrand factor protein.[22] These blasts are non-reactive for myeloperoxidase and express stem/progenitor markers CD33, CD34, CD117, erythroid markers CD36 and glycophorin A, the lymphoid antigen CD7 and the megakaryocytic markers CD41 and CD42b.[23–25] Of note, cytogenetic differences between DS and non-DS AMKL include the absence of the translocation t(1;22), and instead, the presence of trisomies involving chromosomes 8 and 1,[7] as well as monosomy 7.[26,27] Because Down syndrome is the most common cytogenetic abnormality seen at birth (1/700), improved noninvasive prenatal diagnosis is an area of active research. Strategies are emerging based on screening differentially methylated regions (DMRs) of fetal DNA for chromosome 21 dosage assessment.[28] Moreover, murine models of DS have helped identify differentially expressed genes in DS-fetal livers, some of which may represent potential chromosome 21 specific biomarkers.[29]

B. Prognosis

Prospective, multi-institutional studies in the United States, Germany, and Japan have examined the natural history of TMD in 264 infants.[10–12] Early death occurred in up to 20% of infants and was significantly correlated with higher white blood cell count at diagnosis, increased bilirubin and liver enzymes, and a failure to normalize the blood count. Later development of leukemia occurred in 19% of infants at a mean of 20 months of age and was significantly correlated with karyotypic abnormalities in addition to trisomy 21, including trisomy 11, del 16q, der(14;21), t(5;13), and tetrasomy 21.[10] In DS-AML, age at diagnosis had independent prognostic significance, primarily as a result of poor remission induction in older patients.[30] Cytogenetic abnormalities such as monosomy 7 confer an adverse prognosis in non-DS and DS-AMKL in some studies.[26]

III. MECHANISMS

A. Pathogenesis

1. From Trisomy 21 to TMD toward AMKL: An Incremental Process of Leukemogenesis

If trisomy 21 is considered the first genetic event in DS-AMKL leukemogenesis, the second hit is a mutation of the X-linked gene *GATA1*, encoding a blood-specific transcription factor essential for development of the erythroid and megakaryocytic lineages. *GATA1* mutations are present in nearly all TMD patient samples as early as 21 weeks gestation.[31–34] Using the variable length of nucleotide insertions and deletions as a marker of individual TMD clones, sequential samples collected from the same patient during TMD, remission, and AMKL showed identical *GATA1* mutations that disappeared during remission.[33] This confirms the clonal nature of AMKL and its evolution from TMD.

TMD is a critical model to understand the natural history of AMKL, as 20% of TMD cases evolve into AMKL either overtly, or following an apparent remission. AMKL and TMD blasts express erythroid markers such as gamma globulin and delta aminole-

vulinate synthase as well as *GATA-1* and *GATA-2*, suggesting origin from the megakaryocyte-erythroid progenitor cells.[35] Myeloid and erythroid dysplasia are common as is the presence of karyotypic abnormalities in metaphases from CFU-GM and BFU-E mimicking those seen in megakaryoblasts.[36]

2. Fetal Liver Origin of Leukemia-Initiating Cells

GATA1 mutations are most likely occur *in utero*, based on neonatal blood spot testing, and may precede disease development.[37,38] Mice expressing a *GATA1* mutant ortholog (GATA-1s) of the one seen in human DS specimens display sustained proliferation of a yolk sac/early fetal liver megakaryocyte progenitor, implicating this as the target cell for leukemic transformation in DS-AMKL and TMD.[39,40] Moreover, *GATA1* mutations were detected in 2 of 9 liver samples from terminated fetuses with DS (as early as 21 to 23 weeks of gestation), supporting the fetal liver origin of TMD.[31]

3. Role of Trisomy 21

Second-trimester DS fetal livers (FLs) show increased megakaryocyte-erythroid progenitor frequency and increased clonogenicity.[41] Enhanced erythroid and megakaryocytic differentiation was seen in NOD/SCID mice transplanted with DS FL mononuclear cells.[42] Those observations were obtained from 13- to 23-week-old trisomic FL, preceding the acquisition of any *GATA1* mutation.

Through a high-resolution map of DS generated using a panel of 30 individuals with rare segmental trisomies 21, Korbel *et al*. identified a critical region of 8.35 Mb (35–43.35) that likely contributes to the risk increase for both TMD and AMKL. This region includes previously known oncogenes, such as *RUNX1*, *ERG,* and *ETS2.* [43] Using mouse ES cells (ESCs) bearing an extra copy of human chromosome 21 (Hsa21), disturbances in early hematopoietic differentiation were observed and related to increased expression of *GATA-2*, *Tie-2* and c-*kit*. An siRNA silencing study implicated increased level of *RUNX1* in abnormal Tie-2 and c-kit expression. Using a panel of partially trisomic ESCs mapped with tiling arrays, two non-overlapping regions of Hsa21 were correlated to abnormal hematopoiesis.[44] The distal region contains *RUNX1*, *DYRK1A*,[45] *ETS2,* and *ERG,* while the pericentromeric region frequently harbors chromosome rearrangements and increased disomic homozygosity of DNA markers in DS-TMD and DS-AMKL.[46]

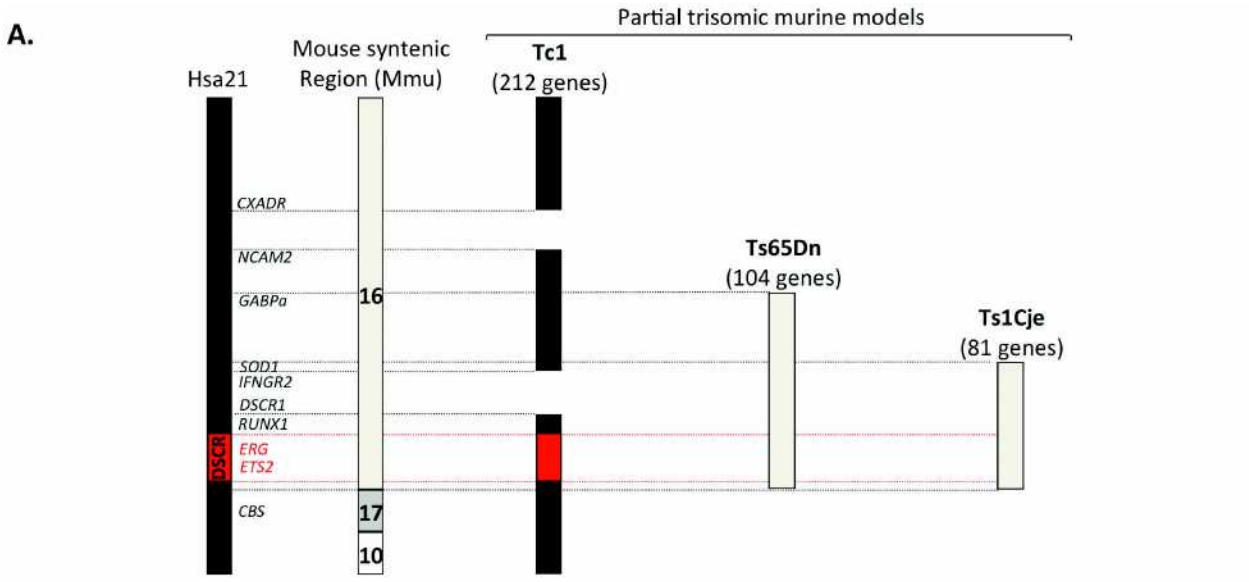
Both ERG and ETS2 bind the hematopoietic enhancer of SCL/TAL1, a key regulator of hematopoietic stem cell and megakaryocytic development.[47] Overexpression of *ETS2* and *ERG* increase the megakaryocytic differentiation of *GATA-1s* progenitors, and immortalize *Gata1s* fetal liver progenitors in replating assays.[48] Coexpression of *ERG* and GATA-1s *in vivo* results in leukemia with an immature megakaryocytic phenotype.[49]

In parallel, several murine models of DS have been developed to identify dosage-sensitive genes that contribute to specific hematopoietic phenotypes (see Figure 1). The Ts65Dn mouse, trisomic for 104 orthologous genes of human chromosome 21, develops a macrocytic anemia and a myeloproliferative disorder (MPD) associated with thrombocytosis.[50] Interestingly, unlike trisomy of *Runx1* in the Ts65Dn murine model of DS, reduction to functional disomy of *Erg* using a loss-of-function allele, corrects the pathologic and hematologic features of myeloproliferation.[51] Other segmental trisomy murine models include the Tc1 murine model (212 genes)[52] and the Ts1Cje mouse (81 genes).[53] Notably, none of these mouse strains develops TMD or AMKL alone or in cooperation with GATA-1s expression, suggesting undiscovered cooperating mutations.

There are also 5 micro-RNAs encoded on chromosome 21, of which miR-125b2 is overexpressed in TMD and AMKL. In both fetal liver and human CD34+ cells, overexpression of *miR-125b-2* led to hyperproliferation and enhanced self-renewal of megakaryocytic progenitors attributed to repression of *DICER1* and the tumor suppressor *ST18.*[54]

4. Role of GATA1

The first insight into the mechanism of DS-AMKL was the discovery of acquired mutations in the *GATA1* gene. These mutations were restricted to the leuke-



B.

	Tc1	Ts65Dn	Ts1Cje
Hematologic phenotype			
	Macrocytic anemia, extramedullary hematopoiesis	MPD with thrombocytosis, megakaryocyte hyperplasia and dysplasia, myelofibrosis, extramedullary hematopoiesis. Altered HSC and myeloid progenitor compartments	Macrocytosis, anemia and abnormal erythroid differentiation. Alterations in FL hematoposisis and reduced LSK cells in BM
Co-operation with GATA-1s			
	Synergistic increase in CFU-Mk number (from 16.5d fetal cells)	Not known	Did not significantly alter the disease spectrum

FIGURE 1. Diagram of Hsa21 and the regions of trisomy in the various murine models of DS. (A) Human chromosome 21 and specific genes in the DS critical region (DSCR) that may contribute to the development of leukemia are shown. The syntenic murine Mmu16 with varying degrees of trisomic representation in the different murine models is depicted on the right. (B) Summary of the hematopoietic phenotype of the murine models and the effect of coexpression of GATA-1s.

mic clones and were not found in normal remission samples.[6] The mutation is not detectable in non-DS leukemia or other sub-types of DS leukemia,[55] emphasizing the specific cooperation of *GATA1* mutation with trisomy 21 in megakaryocytic leukemia. DS and non-DS-AMKL samples exhibit distinct gene expression profiles and a specific signature for DS-AMKL was identified with relatively increased expression of GATA-1 transcripts (as GATA-1s) and failure to down-regulate proliferation-promoting genes that are normally repressed by GATA-1.[56,57] In almost all DS-AMKL and TMD samples, mutations in *GATA1* are detectable in exon 2 producing a premature stop codon within the N-terminal activation domain.[55,58] These mutations prevent the generation of full-length GATA-1, but preserve the translation of GATA-1s, a truncated form of GATA-1 lacking the N-terminal activation domain. Distinct regions in the GATA-1 N terminus are required for terminal megakaryocyte differentiation and controlling growth of immature precursors.[59,60] Analysis of the mutational spectrum at *GATA1* in DS TMD and AMKL blasts shows predominance of insertions/deletions, duplications (74%) and base substitutions (26%).[61] A recent study concluded that the different classes of *GATA1* mutations result in variable translation efficiency of GATA-1s, and further, that the level of GATA-1s protein correlates with risk

of progression to leukemia.[62] However, a subsequent study showed that the *GATA1* mutational spectrum did not differ between TMD or AMKL, and that the type of *GATA1* mutation was unable to predict evolution from TMD to AMKL.[63]

Mice with lineage-specific mutations of the *GATA1* promoter show impaired maturation and dysregulated proliferation of megakaryocytes.[64] Expression profiles of GATA-1s and full-length GATA-1 expressing murine fetal megakaryocytes have been contrasted and showed that GATA-1s fails to repress a number of transcription factor genes (including *Gata2*, *Ikaros*, *Myb,* and *Myc*) that have "pro-proliferative" effect on hematopoietic cell growth.[39,60] Of note, in 2006, a family was discovered with a germline *GATA1* mutation in which affected males generated only the GATA-1s isoform and exhibited anemia and trilineage dysplasia but failed to develop leukemia.[65] This observation established that trisomy 21 is necessary for leukemogenesis in the presence of mutated *GATA1*.

5. Cooperating Mutations

Mutations in the p53 tumor suppressor gene have been demonstrated in a proportion of patients after transformation from TMD to AMKL suggesting a role in disease evolution. To date, only a single case of a p53 mutation in TMD has been reported.[66,67] Several activating mutations of the *JAK3* gene have been identified in TMD, DS AMKL, and non-DS AMKL patients as well as in DS-AMKL cell lines (CMK and CMY). These mutations result in constitutive JAK signaling[13,68,69] and confer responsiveness to treatment with JAK3 inhibitors *in vitro*.[70] Both JAK3 A572V and the recently identified JAK3 P132A[68,71] mutants appear to be oncogenic in murine models. However, recent data have shown that the purported activating *JAK3* mutations are present in DNA samples from normal blood donors, at a frequency similar to that observed in patients with AML, suggesting that they may represent SNPs.[71] Further study in this field is required to clarify the leukemogenic role of *JAK3* mutations in DS-AMKL. In addition, activating mutations affecting *FLT3*, *JAK2*, and *MPL* genes were also identified within DS-AMKL.[72,73] A summary of the stepwise acquisition of mutations is shown in Figure 2A.

6. Abberant Signaling Pathways in DS-AMKL

Fetal liver hepatic stromal cells support hematopoietic stem cell (HSC) expansion by secreting insulin-like growth factor 2 (IGF-2).[74] Constitutive activation of IGF signaling was demonstrated in DS-AMKL and TMD blast cells, as well as in DS-AMKL murine model.[75] Klusmann et al. showed that mutated GATA-1 fails to restrict IGF-mediated activation of the E2F transcription network. This aberrant response converges with overactive IGF signaling to promote enhanced proliferation and increased survival of DS fetal liver progenitors, revealing a fetal stage–specific regulatory network (Figure 2B).

More than 20 genes involved in oxidative metabolism are localized to chromosome 21, including superoxide dismutase (*SOD*)[76] and cystathionine beta synthase (*CBS*). CBS overexpression in DS directs homocysteine to cystathionine synthesis and away from methionine remethylation, creating a folate trap and thymidylate imbalance. Perturbed folate metabolism in turn results in the accumuation of uracil and its misincorporation into DNA. This altered metabolism, when paired to oxidative stress caused by increased *SOD1* activity seen in DS, has been implicated in a model linking chromosome 21 genes (*CBS* and *SOD1*) to the generation of mutations in the *GATA1* gene.[61] Additionally AMKL blasts, unlike TMD cells, have demonstrable telomerase activity, implicating telomerase with the malignant character of a leukemic proliferation.[77]

IV. THERAPY

A. Treatment Options

One of the first clinical trials for this malignancy studied 12 children with DS-AML. These patients (POG8498) showed heightened sensitivity to high-dose cytarabine- and anthracycline-based therapy, with a significantly superior event-free survival (EFS) compared to non-DS AML (3-year EFS 100% in DS-AML vs. 33% in non-DS AML).[78] In subsequent trials, intensive induction showed unacceptable toxicity

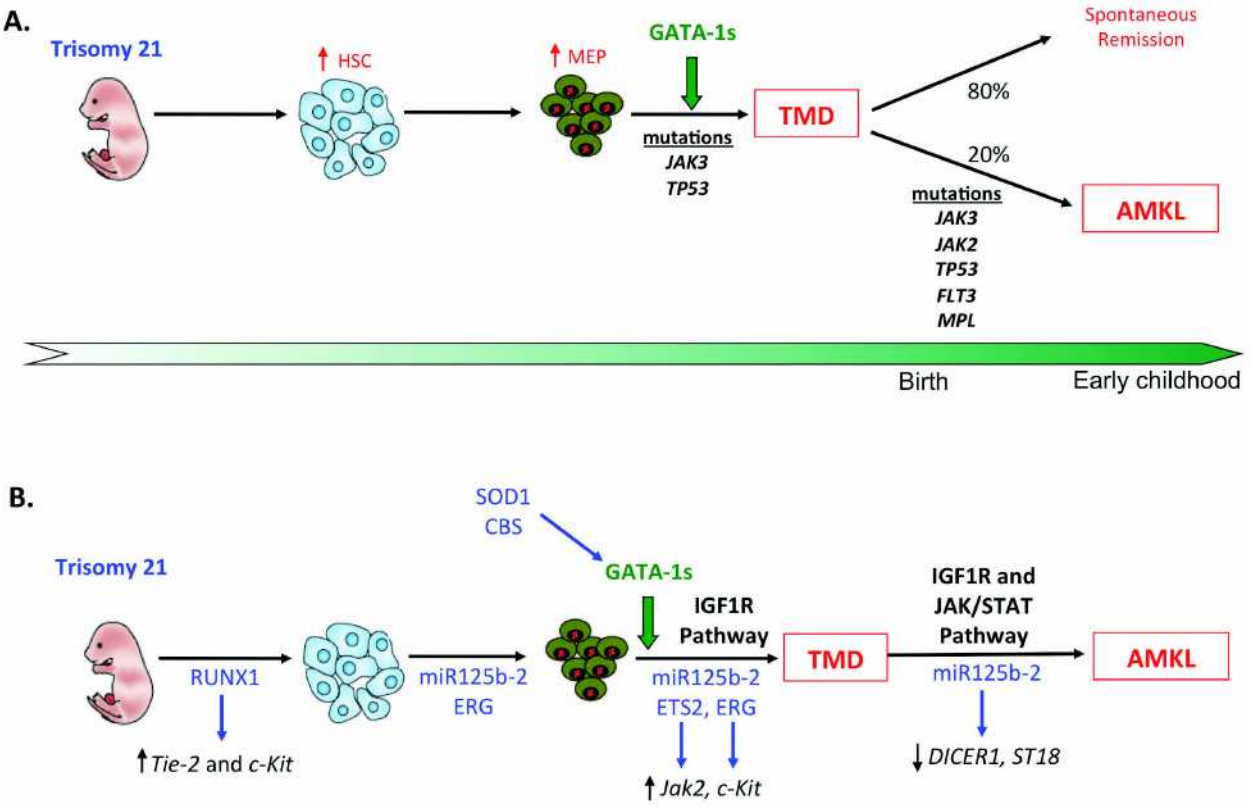


FIGURE 2. Multi-step model of leukemogenesis in Down syndrome. A) Sequential acquisition of known genetic abnormalities and their role in the evolution of DS-AMKL. B) Aberrant signaling pathways implicated in the pathogenesis of DS-AMKL. The chromosome 21 specific genes that appear to have a functional impact in these pathways are highlighted in blue.

and increased mortality in DS-AML as did autologous and allogeneic transplant.[15] AMKL has been treated on protocols involving either conventional (100–300 mg/m^2)[26] or high-dose cytosine arabinoside (3 g/m^2) with reported 3-year overall survival (OS) >80%. However, significant toxicity has been reported with the high-dose Ara-C.[16,19,30,79] Low-dose subcutaneous Ara-C induced remission in almost all cases of AMKL and complicated TMD [80,81] with 5-year EFS and OS comparable to standard chemotherapy.[82]

There was a significant improvement in clinical trials survival outcomes in DS between 1993 and 1998 mainly due to reduction in treatment-related mortality. This resulted from reduced anthracycline and cytarabine dosing and longer intervals of recovery between therapy.[83] Due to the limitations of toxic deaths, infections, and cardiac toxicity in treating DS-AMKL, new, less-intensive protocols have been conducted in the United States, Japan, and Europe.[16,84] In a single prospective study, treatment of TMD with low-dose cytarabine (0.5-1.5 mg/kg) improved 5-year EFS from 28% to 52% in children with risk factors for early death. Treatment of TMD did not alter risk of developing subsequent AMKL.[11] The ML-DS prevention trial (EudraCT no. 2006-002962-20) is ongoing and aims to assess whether the progression from TL to ML-DS may be blocked by eradication of the GATA-1s clone using low-dose cytarabine treatment and monitoring for minimal residual disease (MRD).

B. Chemosensitivity in DS-AMKL

The enhanced sensitivity of DS myeloblasts to Ara-C is due to greater extent of Ara-C incorporation into

DNA and increased relative numbers of double strand DNA strand breaks,[85] attributed to dosage effect of genes localized to chromosome 21, including *CBS*. *In vitro*, DS myeloblasts generate higher concentrations of Ara-CTP, the active cytarabine metabolite. This is thought to be due to increased CBS expression and an elevated ratio of deoxycytidine kinase (*CdK*) to cytidine deaminase (*CDA*). CDA metabolizes Ara-C to the inactive metabolites uridine arabinoside (ara-U), and its levels are lower in DS-myeloblasts than in non-DS myeloblasts. GATA-1 binding sites in the CDAsf promoter suggest the potential role of GATA-1 in regulating CDA transcription.[86]

Blast cells from DS patients are also significantly more sensitive to daunorubicin, melphalan, mitoxantrone, 4-hydroperoxy-cyclophosphamide, vincristine, etoposide, bleomycin, and pirarubicin than those from non-DS patients in MTT assays.[87] Low levels of bone marrow stromal-cell antigen 2 (BST2) in DS megakaryoblasts may lead to decreased interaction of leukemia cells with bone marrow stroma, a mechanism of protection from chemotherapy-induced apoptosis. This may be explained by decreased stimulation of BST2 promoter activity by GATA-1s compared with the full-length protein.[56] DS-AMKL and good prognosis non-DS AMKL blasts demonstrate high expression of CD36, the thrombospondin receptor. CD36 plays a role in fatty acid transport and may exacerbate drug-triggered apoptosis by intracellular lipid accumulation in AMKL.[88] *RUNX1* expression is lower in DS megakaryoblasts compared with non-DS megakaryoblasts.[57] This suggests that RUNX1 may play a role in chemotherapy resistance and contribute to the poor outcomes in non DS-AMKL. Inhibition of RUNX1 may further chemosensitize leukemia cells by inhibition of the PI3 kinase survival pathway.[89]

V. CONCLUSIONS

It is clear that myeloid/megakaryocytic leukemia in DS is the result of a series of genetic events and therefore represents a useful model to understand the role of chromosome 21 in leukemia in general. A trisomic background results in oxidative stress and altered folate metabolism, predisposing the acquisition of *GATA1* mutations, which then allows for the development of TMD. The discovery that mutated GATA-1 is unable to suppress E2F transcription in fetal liver cells may explain the cellular origin of TMD. Research to identify dosage-sensitive genes (or regulators) on chromosome 21 that contribute to megakaryocyte proliferation, implicate the ETS proteins ERG and ETS2. Recently, overactive IGF signaling and overexpression of miR-125b-2, which allow for dis-inhibition of tumor suppressor genes, have also been highlighted. Subsequent clonal selection and evolution to AMKL requires additional insults, including putative cooperating mutations in *JAK3*, *FLT3*, *MPL,* or *TP53*. The multi-step progression to AMKL provides insight into the steps by which normal HSC and/or progenitors are transformed into leukemic cells. Moreover, this is an excellent disease model to understand cell type–specific signaling pathways and their intersection with oncogenes during malignant transformation.

ACKNOWLEDGMENTS

This review was funded in part by grants from the National Cancer Institute (R01 CA101774) and the Leukemia and Lymphoma Society. IK is supported by the Clinical Oncology Training Grant No. (T32 CA079447) from the NCI. SM is a fellow of the Leukemia and Lymphoma Society.

REFERENCES

1. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000 Jan 15;355(9199):165–9.
2. Patja K, Pukkala E, Sund R, Iivanainen M, Kaski M. Cancer incidence of persons with Down syndrome in Finland: a population-based study. Int J Cancer. 2006 Apr 1;118(7):1769–72.
3. Sullivan SG, Hussain R, Glasson EJ, Bittles AH. The profile and incidence of cancer in Down syndrome. J Intellect Disabil Res. 2007 Mar;51(Pt 3):228–31.
4. Mitelman F, Heim S, Mandahl N. Trisomy 21 in neoplastic cells. Am J Med Genet Suppl. 1990;7:262–6.

5. Lange B. The management of neoplastic disorders of haematopoiesis in children with Down's syndrome. Br J Haematol. 2000 Sep;110(3):512–24.
6. Wechsler J, Greene M, McDevitt MA, Anastasi J, Karp JE, Le Beau MM, Crispino JD. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. Nat Genet. 2002 Sep;32(1):148–52.
7. Creutzig U, Ritter J, Vormoor J, Ludwig WD, Niemeyer C, Reinisch I, Stollmann-Gibbels B, Zimmermann M, Harbott J. Myelodysplasia and acute myelogenous leukemia in Down syndrome. A report of 40 children of the AML-BFM Study Group. Leukemia. 1996 Nov;10(11):1677–86.
8. Okada H, Liu PI, Hoshino T, Yamamoto T, Yamaoka H, Murakami M. Down syndrome associated with a myeloproliferative disorder. Am J Dis Child. 1972 Jul;124(1):107–10.
9. Pine SR, Guo Q, Yin C, Jayabose S, Druschel CM, Sandoval C. Incidence and clinical implications of GATA1 mutations in newborns with Down syndrome. Blood. 2007 Sep 15;110(6):2128–31.
10. Massey GV, Zipursky A, Chang MN, Doyle JJ, Nasim S, Taub JW, Ravindranath Y, Dahl G, Weinstein HJ. A prospective study of the natural history of transient leukemia (TL) in neonates with Down syndrome (DS): Children's Oncology Group (COG) study POG-9481. Blood. 2006 Jun 15;107(12):4606–13.
11. Klusmann JH, Creutzig U, Zimmermann M, Dworzak M, Jorch N, Langebrake C, Pekrun A, Macakova-Reinhardt K, Reinhardt D. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood. 2008 Mar 15;111(6):2991–8.
12. Muramatsu H, Kato K, Watanabe N, Matsumoto K, Nakamura T, Horikoshi Y, Mimaya J, Suzuki C, Hayakawa M, Kojima S. Risk factors for early death in neonates with Down syndrome and transient leukaemia. Br J Haematol. 2008 Aug;142(4):610–5.
13. Massey GV. Transient leukemia in newborns with Down syndrome. Pediatr Blood Cancer. 2005 Jan;44(1):29–32.
14. Zipursky A, Poon A, Doyle J. Leukemia in Down syndrome: a review. Pediatr Hematol Oncol. 1992 Apr–Jun;9(2):139–49.
15. Lange BJ, Kobrinsky N, Barnard DR, Arthur DC, Buckley JD, Howells WB, Gold S, Sanders J, Neudorf S, Smith FO, Woods WG. Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. Blood. 1998 Jan 15;91(2):608–15.
16. Creutzig U, Reinhardt D, Diekamp S, Dworzak M, Stary J, Zimmermann M. AML patients with Down syndrome have a high cure rate with AML-BFM therapy with reduced dose intensity. Leukemia. 2005 Aug;19(8):1355–60.
17. Moody A, Simpson E, Shaw D. Florid radiological appearance of megakaryoblastic leukaemia—an aid to earlier diagnosis. Pediatr Radiol. 1989;19(6–7):486–8.
18. Kozlowski K, Halimun E. Generalised bone disease with abundant periosteal reaction in megakaryocytic leukaemia. Eur J Pediatr. 1997 Nov;156(11):845–7.
19. Rao A, Hills RK, Stiller C, Gibson BE, de Graaf SS, Hann IM, O'Marcaigh A, Wheatley K, Webb DK. Treatment for myeloid leukaemia of Down syndrome: population-based experience in the UK and results from the Medical Research Council AML 10 and AML 12 trials. Br J Haematol. 2006 Mar;132(5):576–83.
20. Zeller B, Gustafsson G, Forestier E, Abrahamsson J, Clausen N, Heldrup J, Hovi L, Jonmundsson G, Lie SO, Glomstein A, Hasle H. Acute leukaemia in children with Down syndrome: a population-based Nordic study. Br J Haematol. 2005 Mar;128(6):797–804.
21. Bevan D RM, Greaves M. Leukaemia of platelet precursors: diverse features in four cases. Br J Haematol. 1982 May;51(1):147–64.
22. Windebank KP, Tefferi A, Smithson WA, Li CY, Solberg LA, Jr., Priest JR, Elliott SC, de Alarcon PA, Weinblatt ME, Burgert EO, Jr. Acute megakaryocytic leukemia (M7) in children. Mayo Clin Proc. 1989 Nov;64(11):1339–51.
23. Yumura-Yagi K, Hara J, Kurahashi H, Nishiura T, Kaneyama Y, Osugi Y, Sakata N, Inoue M, Tawa A, Okada S. Mixed phenotype of blasts in acute megakaryocytic leukaemia and transient abnormal myelopoiesis in Down's syndrome. Br J Haematol. 1992 Aug;81(4):520–5.

24. Langebrake C, Creutzig U, Reinhardt D. Immunophenotype of Down syndrome acute myeloid leukemia and transient myeloproliferative disease differs significantly from other diseases with morphologically identical or similar blasts. Klin Padiatr. 2005 May–Jun;217(3):126–34.

25. Litz CE, Davies S, Brunning RD, Kueck B, Parkin JL, Gajl Peczalska K, Arthur DC. Acute leukemia and the transient myeloproliferative disorder associated with Down syndrome: morphologic, immunophenotypic and cytogenetic manifestations. Leukemia. 1995 Sep;9(9):1432–9.

26. Kudo K, Kojima S, Tabuchi K, Yabe H, Tawa A, Imaizumi M, Hanada R, Hamamoto K, Kobayashi R, Morimoto A, Nakayama H, Tsuchida M, Horibe K, Kigasawa H, Tsukimoto I. Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: the Japanese Childhood AML Cooperative Study Group. J Clin Oncol. 2007 Dec 1;25(34): 5442–7.

27. Forestier E, Izraeli S, Beverloo B, Haas O, Pession A, Michalova K, Stark B, Harrison CJ, Teigler-Schlegel A, Johansson B. Cytogenetic features of acute lymphoblastic and myeloid leukemias in pediatric patients with Down syndrome: an iBFM-SG study. Blood. 2008 Feb 1;111(3):1575–83.

28. Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC. Fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21. Nat Med. 2011 Apr;17(4):510–3.

29. Pennings JL, Rodenburg W, Imholz S, Koster MP, van Oostrom CT, Breit TM, Schielen PC, de Vries A. Gene expression profiling in a mouse model identifies fetal liver- and placenta-derived potential biomarkers for Down syndrome screening. PLoS One. 2011;6(4):e18866.

30. Gamis AS, Woods WG, Alonzo TA, Buxton A, Lange B, Barnard DR, Gold S, Smith FO. Increased age at diagnosis has a significantly negative effect on outcome in children with Down syndrome and acute myeloid leukemia: a report from the Children's Cancer Group Study 2891. J Clin Oncol. 2003 Sep 15;21(18):3415–22.

31. Taub JW, Mundschau G, Ge Y, Poulik JM, Qureshi F, Jensen T, James SJ, Matherly LH, Wechsler J, Crispino JD. Prenatal origin of GATA1 mutations may be an initiating step in the development of megakaryocytic leukemia in Down syndrome. Blood. 2004 Sep 1;104(5):1588–9.

32. Mundschau G, Gurbuxani S, Gamis AS, Greene ME, Arceci RJ, Crispino JD. Mutagenesis of GATA1 is an initiating event in Down syndrome leukemogenesis. Blood. 2003 Jun 1;101(11):4298–300.

33. Hitzler JK, Cheung J, Li Y, Scherer SW, Zipursky A. GATA1 mutations in transient leukemia and acute megakaryoblastic leukemia of Down syndrome. Blood. 2003 Jun 1;101(11):4301–4.

34. Groet J, McElwaine S, Spinelli M, Rinaldi A, Burtscher I, Mulligan C, Mensah A, Cavani S, Dagna-Bricarelli F, Basso G, Cotter FE, Nizetic D. Acquired mutations in GATA1 in neonates with Down's syndrome with transient myeloid disorder. Lancet. 2003 May 10;361(9369):1617–20.

35. Ito E, Kasai M, Hayashi Y, Toki T, Arai K, Yokoyama S, Kato K, Tachibana N, Yamamoto M, Yokoyama M. Expression of erythroid-specific genes in acute megakaryoblastic leukaemia and transient myeloproliferative disorder in Down's syndrome. Br J Haematol. 1995 Jul;90(3):607–14.

36. Koike T, Urushiyama M, Narita M, Saitoh H, Ishida F, Imashuku S, Morioka Y, Utsumi J, Ishizuka T, Tsuruta T. Target cell of leukemic transformation in acute megakaryoblastic leukemia. Am J Hematol. 1990 Aug;34(4):252–8.

37. Shimada A, Xu G, Toki T, Kimura H, Hayashi Y, Ito E. Fetal origin of the GATA1 mutation in identical twins with transient myeloproliferative disorder and acute megakaryoblastic leukemia accompanying Down syndrome. Blood. 2004 Jan 1;103(1):366.

38. Ahmed M, Sternberg A, Hall G, Thomas A, Smith O, O'Marcaigh A, Wynn R, Stevens R, Addison M, King D, Stewart B, Gibson B, Roberts I, Vyas P. Natural history of GATA1 mutations in Down syndrome. Blood. 2004 Apr 1;103(7):2480–9.

39. Li Z, Godinho FJ, Klusmann JH, Garriga-Canut M, Yu C, Orkin SH. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. Nat Genet. 2005 Jun;37(6):613–9.

40. Shimizu R, Kobayashi E, Engel JD, Yamamoto M. Induction of hyperproliferative fetal megakaryopoiesis by an N-terminally truncated GATA1 mutant. Genes Cells. 2009 Sep;14(9):1119–31.

41. Tunstall-Pedoe O, Roy A, Karadimitris A, de la Fuente J, Fisk NM, Bennett P, Norton A, Vyas P, Roberts I. Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations. Blood. 2008 Dec 1;112(12):4507–11.

42. Chou ST, Opalinska JB, Yao Y, Fernandes MA, Kalota A, Brooks JS, Choi JK, Gewirtz AM, Danet-Desnoyers GA, Nemiroff RL, Weiss MJ. Trisomy 21 enhances human fetal erythro-megakaryocytic development. Blood. 2008 Dec 1;112(12):4503–6.

43. Korbel JO, Tirosh-Wagner T, Urban AE, Chen XN, Kasowski M, Dai L, Grubert F, Erdman C, Gao MC, Lange K, Sobel EM, Barlow GM, Aylsworth AS, Carpenter NJ, Clark RD, Cohen MY, Doran E, Falik-Zaccai T, Lewin SO, Lott IT, McGillivray BC, Moeschler JB, Pettenati MJ, Pueschel SM, Rao KW, Shaffer LG, Shohat M, Van Riper AJ, Warburton D, Weissman S, Gerstein MB, Snyder M, Korenberg JR. The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies. Proc Natl Acad Sci U S A. 2009 Jul 21;106(29): 12031–6.

44. De Vita S, Canzonetta C, Mulligan C, Delom F, Groet J, Baldo C, Vanes L, Dagna-Bricarelli F, Hoischen A, Veltman J, Fisher EM, Tybulewicz VL, Nizetic D. Trisomic dose of several chromosome 21 genes perturbs haematopoietic stem and progenitor cell differentiation in Down's syndrome. Oncogene. 2010 Nov 18;29(46):6102–14.

45. Canzonetta C, Mulligan C, Deutsch S, Ruf S, O'Doherty A, Lyle R, Borel C, Lin-Marq N, Delom F, Groet J, Schnappauf F, De Vita S, Averill S, Priestley JV, Martin JE, Shipley J, Denyer G, Epstein CJ, Fillat C, Estivill X, Tybulewicz VL, Fisher EM, Antonarakis SE, Nizetic D. DYRK1A-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome. Am J Hum Genet. 2008 Sep;83(3):388–400.

46. Shen JJ, Williams BJ, Zipursky A, Doyle J, Sherman SL, Jacobs PA, Shugar AL, Soukup SW, Hassold TJ. Cytogenetic and molecular studies of Down syndrome individuals with leukemia. Am J Hum Genet. 1995 Apr;56(4):915–25.

47. Rainis L, Toki T, Pimanda JE, Rosenthal E, Machol K, Strehl S, Gottgens B, Ito E, Izraeli S. The proto-oncogene ERG in megakaryoblastic leukemias. Cancer Res. 2005 Sep 1;65(17):7596–602.

48. Stankiewicz MJ, Crispino JD. ETS2 and ERG promote megakaryopoiesis and synergize with alterations in GATA-1 to immortalize hematopoietic progenitor cells. Blood. 2009 Apr 2;113(14):3337–47.

49. Salek-Ardakani S, Smooha G, de Boer J, Sebire NJ, Morrow M, Rainis L, Lee S, Williams O, Izraeli S, Brady HJ. ERG is a megakaryocytic oncogene. Cancer Res. 2009 Jun 1;69(11):4665–73.

50. Kirsammer G, Jilani S, Liu H, Davis E, Gurbuxani S, Le Beau MM, Crispino JD. Highly penetrant myeloproliferative disease in the Ts65Dn mouse model of Down syndrome. Blood. 2008 Jan 15;111(2):767–75.

51. Ng AP, Hyland CD, Metcalf D, Carmichael CL, Loughran SJ, Di Rago L, Kile BT, Alexander WS. Trisomy of Erg is required for myeloproliferation in a mouse model of Down syndrome. Blood. 2010 May 13;115(19):3966–9.

52. Alford KA, Slender A, Vanes L, Li Z, Fisher EM, Nizetic D, Orkin SH, Roberts I, Tybulewicz VL. Perturbed hematopoiesis in the Tc1 mouse model of Down syndrome. Blood. 2010 Apr 8;115(14):2928–37.

53. Carmichael CL, Majewski IJ, Alexander WS, Metcalf D, Hilton DJ, Hewitt CA, Scott HS. Hematopoietic defects in the Ts1Cje mouse model of Down syndrome. Blood. 2009 Feb 26;113(9):1929–37.

54. Klusmann JH, Li Z, Bohmer K, Maroz A, Koch ML, Emmrich S, Godinho FJ, Orkin SH, Reinhardt D. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. Genes Dev. 2010 Mar 1;24(5):478–90.

55. Greene ME, Mundschau G, Wechsler J, McDevitt M, Gamis A, Karp J, Gurbuxani S, Arceci R, Crispino JD. Mutations in GATA1 in both transient myeloproliferative disorder and acute megakaryoblastic leukemia of Down syndrome. Blood Cells Mol Dis. 2003 Nov–Dec;31(3):351–6.

56. Ge Y, Dombkowski AA, LaFiura KM, Tatman D, Yedidi RS, Stout ML, Buck SA, Massey G, Becton DL, Weinstein HJ, Ravindranath Y, Matherly LH, Taub JW. Differential gene expression, GATA1 target genes, and the chemotherapy sensitivity of Down syndrome megakaryocytic leukemia. Blood. 2006 Feb 15;107(4):1570–81.

57. Bourquin JP, Subramanian A, Langebrake C, Reinhardt D, Bernard O, Ballerini P, Baruchel A, Cave H, Dastugue N, Hasle H, Kaspers GL, Lessard M, Michaux L, Vyas P, van Wering E, Zwaan CM, Golub TR, Orkin SH. Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling. Proc Natl Acad Sci U S A. 2006 Feb 28;103(9): 3339–44.

58. Hirose Y, Kudo K, Kiyoi H, Hayashi Y, Naoe T, Kojima S. Comprehensive analysis of gene alterations in acute megakaryoblastic leukemia of Down's syndrome. Leukemia. 2003 Nov;17(11):2250–2.

59. Kuhl C, Atzberger A, Iborra F, Nieswandt B, Porcher C, Vyas P. GATA1-mediated megakaryocyte differentiation and growth control can be uncoupled and mapped to different domains in GATA1. Mol Cell Biol. 2005 Oct;25(19):8592–606.

60. Muntean AG, Crispino JD. Differential requirements for the activation domain and FOG-interaction surface of GATA-1 in megakaryocyte gene expression and development. Blood. 2005 Aug 15;106(4):1223–31.

61. Cabelof DC, Patel HV, Chen Q, van Remmen H, Matherly LH, Ge Y, Taub JW. Mutational spectrum at GATA1 provides insights into mutagenesis and leukemogenesis in Down syndrome. Blood. 2009 Sep 24;114(13):2753–63.

62. Kanezaki R, Toki T, Terui K, Xu G, Wang R, Shimada A, Hama A, Kanegane H, Kawakami K, Endo M, Hasegawa D, Kogawa K, Adachi S, Ikeda Y, Iwamoto S, Taga T, Kosaka Y, Kojima S, Hayashi Y, Ito E. Down syndrome and GATA1 mutations in transient abnormal myeloproliferative disorder: mutation classes correlate with progression to myeloid leukemia. Blood. 2010 Nov 25;116(22): 4631–8.

63. Alford KA, Reinhardt K, Garnett C, Norton A, Bohmer K, von Neuhoff C, Kolenova A, Marchi E, Klusmann JH, Roberts I, Hasle H, Reinhardt D, Vyas P. Analysis of GATA1 mutations in Down syndrome transient myeloproliferative disorder and myeloid leukemia. Blood. 2011 Jun 29.

64. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J. 1997 Jul 1;16(13):3965–73.

65. Hollanda LM, Lima CS, Cunha AF, Albuquerque DM, Vassallo J, Ozelo MC, Joazeiro PP, Saad ST, Costa FF. An inherited mutation leading to production of only the short isoform of GATA-1 is associated with impaired erythropoiesis. Nat Genet. 2006 Jul;38(7):807–12.

66. Malkin D, Brown EJ, Zipursky A. The role of p53 in megakaryocyte differentiation and the megakaryocytic leukemias of Down syndrome. Cancer Genet Cytogenet. 2000 Jan 1;116(1):1–5.

67. Kanezaki R, Toki T, Xu G, Narayanan R, Ito E. Cloning and characterization of the novel chimeric gene p53/FXR2 in the acute megakaryoblastic leukemia cell line CMK11-5. Tohoku J Exp Med. 2006 Jul;209(3):169–80.

68. Walters DK, Mercher T, Gu TL, O'Hare T, Tyner JW, Loriaux M, Goss VL, Lee KA, Eide CA, Wong MJ, Stoffregen EP, McGreevey L, Nardone J, Moore SA, Crispino J, Boggon TJ, Heinrich MC, Deininger MW, Polakiewicz RD, Gilliland DG, Druker BJ. Activating alleles of JAK3 in acute megakaryoblastic leukemia. Cancer Cell. 2006 Jul;10(1):65–75.

69. Kiyoi H, Yamaji S, Kojima S, Naoe T. JAK3 mutations occur in acute megakaryoblastic leukemia both in Down syndrome children and non-Down syndrome adults. Leukemia. 2007 Mar;21(3):574–6.

70. Sato T, Toki T, Kanezaki R, Xu G, Terui K, Kanegane H, Miura M, Adachi S, Migita M, Morinaga S, Nakano T, Endo M, Kojima S, Kiyoi H, Mano H, Ito E. Functional analysis of JAK3 mutations in transient myeloproliferative disorder and acute megakaryoblastic leukaemia accompanying Down syndrome. Br J Haematol. 2008 May;141(5):681–8.

71. Riera L, Lasorsa E, Bonello L, Sismondi F, Tondat F, Di Bello C, Di Celle PF, Chiarle R, Godio L, Pich A, Facchetti F, Ponzoni M, Marmont F, Zanon C, Bardelli A, Inghirami G. Description of a novel Janus kinase 3 P132A mutation in acute megakaryoblastic leukemia and demonstration of previously reported Janus kinase 3 mutations in normal subjects. Leuk Lymphoma. 2011 May 23.

72. Malinge S, Ragu C, Della-Valle V, Pisani D, Constantinescu SN, Perez C, Villeval JL, Reinhardt D, Landman-Parker J, Michaux L, Dastugue N, Baruchel A, Vainchenker W, Bourquin JP, Penard-Lacronique V, Bernard OA. Activating mutations in human acute megakaryoblastic leukemia. Blood. 2008 Nov 15;112(10):4220–6.

73. Hussein K, Bock O, Theophile K, Schulz-Bischof K, Porwit A, Schlue J, Jonigk D, Kreipe H. MPLW515L mutation in acute megakaryoblastic leukaemia. Leukemia. 2009 May;23(5):852–5.

74. Chou S, Lodish HF. Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. Proc Natl Acad Sci U S A. 2010 Apr 27;107(17):7799–804.

75. Klusmann JH, Godinho FJ, Heitmann K, Maroz A, Koch ML, Reinhardt D, Orkin SH, Li Z. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. Genes Dev. 2010 Aug 1;24(15):1659–72.

76. Busciglio J, Yankner BA. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature. 1995 Dec 21–28;378(6559):776–9.

77. Holt SE, Brown EJ, Zipursky A. Telomerase and the benign and malignant megakaryoblastic leukemias of Down syndrome. J Pediatr Hematol Oncol. 2002 Jan;24(1):14–7.

78. Ravindranath Y, Abella E, Krischer JP, Wiley J, Inoue S, Harris M, Chauvenet A, Alvarado CS, Dubowy R, and Ritchey AK. Acute myeloid leukemia (AML) in Down's syndrome is highly responsive to chemotherapy: experience on Pediatric Oncology Group AML Study 8498. Blood. 1992 Nov 1;80(9):2210–4.

79. Ravindranath Y AE, Krischer S. AML in Down syndrome; repsonse and toxicity with high-dose Ara-C. Proc ASCO 1993;12:323a.

80. Tchernia G, Lejeune F, Boccara JF, Denavit MF, Dommergues JP, Bernaudin F. Erythroblastic and/or megakaryoblastic leukemia in Down syndrome: treatment with low-dose arabinosyl cytosine. J Pediatr Hematol Oncol. 1996 Feb;18(1): 59–62.

81. Al-Kasim F, Doyle JJ, Massey GV, Weinstein HJ, Zipursky A. Incidence and treatment of potentially lethal diseases in transient leukemia of Down syndrome: Pediatric Oncology Group Study. J Pediatr Hematol Oncol. 2002 Jan;24(1):9–13.

82. Al-Ahmari A, Shah N, Sung L, Zipursky A, Hitzler J. Long-term results of an ultra low-dose cytarabine-based regimen for the treatment of acute megakaryoblastic leukaemia in children with Down syndrome. Br J Haematol. 2006 Jun;133(6):646–8.

83. Creutzig U, Zimmermann M, Ritter J, Reinhardt D, Hermann J, Henze G, Jurgens H, Kabisch H, Reiter A, Riehm H, Gadner H, Schellong G. Treatment strategies and long-term results in paediatric patients treated in four consecutive AML-BFM trials. Leukemia. 2005 Dec;19(12):2030–42.

84. Taga T, Shimomura Y, Horikoshi Y, Ogawa A, Itoh M, Okada M, Ueyama J, Higa T, Watanabe A, Kanegane H, Iwai A, Saiwakawa Y, Kogawa K, Yamanaka J, Tsurusawa M. Continuous and high-dose cytarabine combined chemotherapy in children with down syndrome and acute myeloid leukemia: Report from the Japanese children's cancer and leukemia study group (JCCLSG) AML 9805 down study. Pediatr Blood Cancer. 2011 Jul 15;57(1):36–40.

85. Taub JW, Matherly LH, Stout ML, Buck SA, Gurney JG, Ravindranath Y. Enhanced metabolism of 1-beta-D-arabinofuranosylcytosine in Down syndrome cells: a contributing factor to the superior event free survival of Down syndrome children with acute myeloid leukemia. Blood. 1996 Apr 15;87(8):3395–403.

86. Ge Y, Jensen TL, Stout ML, Flatley RM, Grohar PJ, Ravindranath Y, Matherly LH, Taub JW. The role of cytidine deaminase and GATA1 mutations in the increased cytosine arabinoside sensitivity of Down syndrome myeloblasts and leukemia cell lines. Cancer Res. 2004 Jan 15;64(2):728–35.

87. Yamada S, Hongo T, Okada S, Watanabe C, Fujii Y, Hori H, Yazaki M, Hanada R, Horikoshi Y. Distinctive multidrug sensitivity and outcome of acute erythroblastic and megakaryoblastic leukemia in children with Down syndrome. Int J Hematol. 2001 Dec;74(4):428–36.

88. Savasan S, Buck S, Raimondi SC, Becton DL, Weinstein H, Chang M, Ravindranath Y. CD36 (thrombospondin receptor) expression in childhood acute megakaryoblastic leukemia: in vitro drug sensitivity and outcome. Leuk Lymphoma. 2006 Oct;47(10): 2076–83.

89. Edwards H, Xie C, LaFiura KM, Dombkowski AA, Buck SA, Boerner JL, Taub JW, Matherly LH, Ge Y. RUNX1 regulates phosphoinositide 3-kinase/AKT pathway: role in chemotherapy sensitivity in acute megakaryocytic leukemia. Blood. 2009 Sep 24;114(13):2744–52.

Critical Reviews™ in Oncogenesis, 16(1–2), 37–46 (2011)

CREB and Leukemogenesis

Er-Chieh Cho, Bryan Mitton, & Kathleen M. Sakamoto*

Division of Hematology/Oncology, Department of Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles California

*Address all correspondence to: Kathleen Sakamoto, Professor and Chief, Division of Hematology/Oncology, Department of Pediatrics, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095-1752; ksakamoto@mednet.ucla.edu

ABSTRACT: Acute myeloid leukemia (AML) is one of the most common leukemias with a 20% 5-year event-free survival in adults and 50% overall survival in children, despite aggressive chemotherapy treatment and bone marrow transplantation. The incidence and mortality rates for acute leukemia have only slightly decreased over the last 20 years, and therefore greater understanding of the molecular mechanisms associated with leukemic progression is needed. To this end, a number of transcription factors that appear to play a central role in leukemogenesis are being investigated; among them is the cAMP response element binding protein (CREB). CREB is a transcription factor that can regulate downstream targets involving in various cellular functions including cell proliferation, survival, and differentiation. In several studies, the majority of bone marrow samples from patients with acute lymphoid and myeloid leukemia demonstrate CREB overexpression. Moreover, CREB overexpression is associated with a poor outcome in AML patients. This review summarizes the role of CREB in leukemogenesis.

KEY WORDS: CREB, leukemia, oncogenesis, transcription factors

ABBREVIATIONS

14-3-3, assigned name based on fractionation on DEAE cellulose and electrophoretic mobility upon starch gel electrophoresis when purifying brain proteins; AKT, a serine-threonine protein kinase called protein kinase B; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATF, activating transcription factor; Bcl-2, B-cell lymphocytic-leukemia proto-oncogene; bZIP, basic leucine zipper; cAMP, cyclic adenosine monophosphate; CBP, CREB-binding protein; CEBPA, CCAAT/enhancer binding protein alpha; c-fos, proto-oncogene whose viral homologue v-fos was identified from FBJ-murine osteosarcoma virus; c-kit, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; CRE, cAMP response element; CREB, cAMP response element binding protein; cyclin, cell cycle regulator; egr-1, early growth response-1 gene; ERK, extracellular signal-regulated kinase; FLT3, *fms*-like *tyrosine kinase 3;* GM-CSF, granulocyte-macrophage colony-stimulating factor; HSCs, hematopoietic stem cells; IL, interleukin; K562, a human leukemia cell line; KG-501, 2-napthol-AS-E-phosphate compound; KID, kinase-inducible domain; KIX:, helical CREB-binding domain of CBP; MEIS1, a homeobox gene found to be activated in myeloid leukemia by retroviral insertion; MEK, mitogen-activated protein kinase; MiR, microRNA; mRNA, messenger RNA; MSK, mitogen- and stress-activated protein kinase; NGF, nerve growth factor; NPM, nucleophosmin; p300, adenovirus E1A-associated cellular p300 transcriptional co-activator protein; PBX1, Pre-B-cell leukemia transcription factor 1; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; pp90RSK, ribosomal protein S6 kinase; Ras, RAt Sarcoma, an oncogene; RNAi, RNA interference; Ser-133, serine 133 amino acid; shRNA, small hairpin RNA; ska, Spindle and KT Associated; TF-1, a human myeloid cell line; TORC, transducer of regulated CREB activity coactivator; WT, Wilms' tumor

I. ACUTE LEUKEMIA

Leukemia develops when a malfunction in the normal regulatory mechanisms of mitosis occurs and allows bone marrow progenitor blood cells to expand in an uncontrolled fashion. The immature blasts proliferate more than normal cells and fail to differentiate normally. Although leukemia affects approximately 10 times more adults than children, it is the most common cancer among children. The most common type of leukemia in adults is acute myeloid leukemia (AML), while acute lymphoblastic leukemia (ALL) accounts for nearly 70% of childhood leukemia.[1,2]

0893-9675/11/$35.00

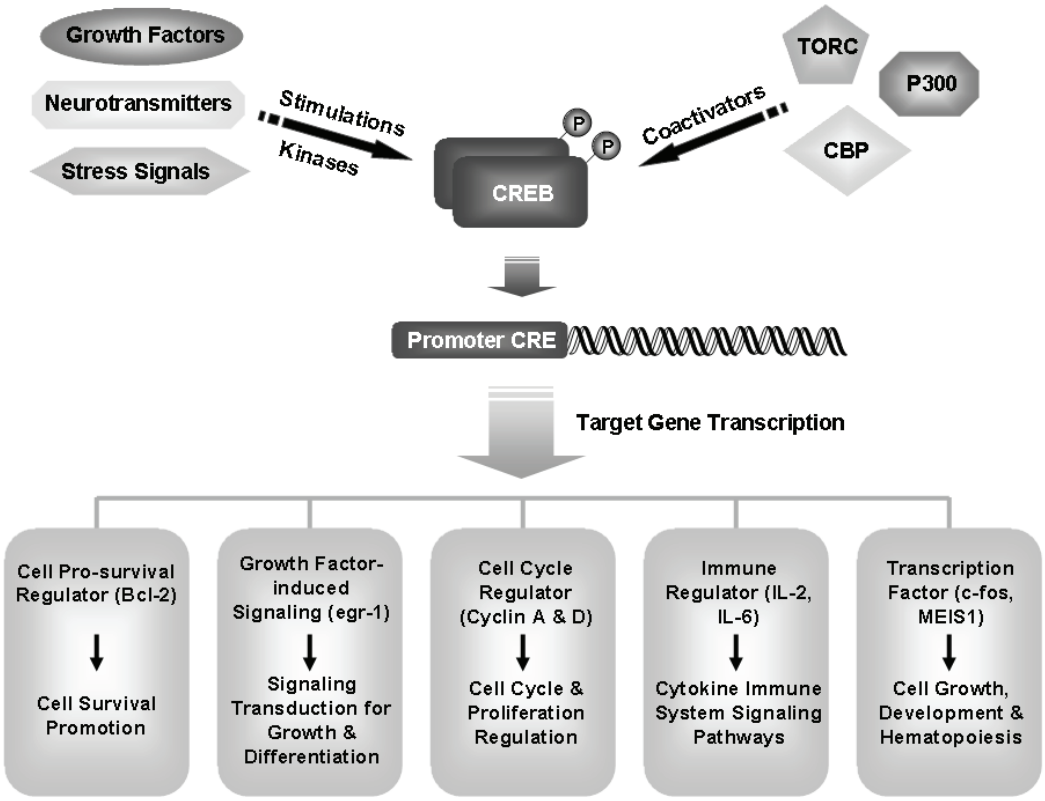


FIGURE 1. The regulation of CREB activation. A variety of extracellular stimuli can promote CREB phosphorylation and activation through different kinases. CREB can then interact with coactivators to promote the transcription of CREB responsive genes. CREB target genes have been shown to mediate effects including cellular proliferation, survival, differentiation, immune response, and hematopoiesis.

Leukemia is the sixth most common cause of cancer deaths in men and seventh most common cause of death among women in the United States. The treatments for ALL and AML have improved with the use of chemotherapy based on stratified risk, molecular markers for prognosis prediction, and supportive care. Generally, the event-free survival rate is lower and the relapse rate is higher in adults than children. The response to treatment for leukemia is variable and associated with the age of the patient, as well as a number of other factors on presentation. The 5-year event-free survival is 70–85% in some of the successful clinical trials in children with ALL and 30–60% in children diagnosed with AML.[1–3] According to the *SEER Cancer Statistics Review*, for ALL patients, the 5-year relative survival rate for adults less than 45 years old is approximately 75%, and for patients more than 45 years old it is less than 20%; for AML patients, the 5-year relative survival rate is over 50% for adults less than 45 years old, and it is less than 40% for patients more than 45 years old.

II. MOLECULAR MECHANISMS OF LEUKEMIA

Acute leukemia has previously been classified by morphology, cytogenetics, and cell surface markers; more recently, it has become clear that molecular characterization of genetic mutations in ALL and AML may relate more strongly to clinical prognosis and can provide information for potential targeted therapies. The advantage of characterizing the phenotype of human leukemia stems from the observation that it

is a heterogeneous disease consisting of a variety of accumulated DNA alterations in progenitor blood cells. Primary genetic defects have been detected by molecular analyses, and these somatic mutations often alter crucial functions of the progenitor cells such as self-renewal and differentiation.

Similar to other cancers, it appears that leukemia arises from the accumulation and synergy of more than one genetic alteration. Many genetic alterations in AML are loss-of-function mutations in transcription factors critical for normal hematopoiesis.[1,4] Data suggest that mutations that alter proliferation and survival functions of the progenitor cells cooperate with the mutations of the transcription factors and result in acute leukemia.[4,5] Examples of genes that are found mutated in AML include biallelic mutations in CCAAT/enhancer binding protein alpha gene (CEBPA), inactivating mutations in Wilms' tumor gene (WT1), activating mutations in *fms*-like *tyrosine kinase 3 gene* (FLT3), and mutation of nucleophosmin (NPM1), which produces mislocalized protein product in cells.[5–9] In recent studies, apart from alterations of genes, differential microRNA expression has also been shown to be involved in leukemia progression.[10]

Acute leukemias are hypothesized to be the consequence of cooperation between mutations that alter proliferation and survival functions of hematopoietic cells and mutations that result from defective differentiation and loss of apoptosis in cells. These mutations may be found at many levels of cellular processes, such as growth-factor receptors, kinase phosphorylation cascades, or cellular transcription programs. Central to all processes, however, are transcription factors, which integrate extranuclear signals and directly influence DNA transcription. Thus, understanding the function of transcription factors in blood malignancies can provide a wealth of information for treatment modalities. CREB, the cAMP-response element binding protein, has become of particular interest in leukemias, as it is known to play a broad range of roles in many critical cellular processes, and the majority of tissue samples from patients with ALL and AML overexpress the CREB in the bone marrow. CREB overexpression is associated with poor outcome in AML patients and increased survival and growth of myeloid cells.[11,12] Transgenic mice expressing CREB in myeloid cells develop aberrant monocytosis and, after a prolonged latency, myeloproliferative disease. Thus, there are both clinical and laboratory data that implicate CREB as a potential critical regulator of leukemogenesis.

III. CREB

CREB is a 43-kDa leucine zipper transcription factor that belongs to the CREB/ATF family and regulates proliferation, differentiation, and survival in a variety of cell types, including neuronal and hematopoietic cells.[13,14]

CREB is a modular protein that contains a kinase-inducible domain (KID), two glutamine-rich domains, and a basic leucine zipper (bZIP) domain. The KID and glutamine-rich domains are critical for transactivation and phosphorylation of CREB.[13,14] A serine 133 (Ser-133) residue within the KID domain is phosphorylated by various kinases, and this phosphorylation promotes the interaction of CREB with a number of transcription coactivators, especially the histone acetyltransferases CREB-binding protein (CBP) or p300.[15,16] CREB can be phosphorylated and thus activated in response to various stimuli such as growth factors, neurotransmitters, stress signals that increase intracellular cAMP, or calcium levels. CREB is also activated by phosphorylation at Ser-133 through nuclear translocation of transducer of regulated CREB activity (TORC) coactivators, which occurs through a Ser-133 phosphorylation–independent mechanism.[17,18]

CREB family member proteins, when activated, bind to the cAMP response elements and promote the recruitment of coactivators such as CBP/p300, thereby initiating the transcriptional machinery and inducing CREB target genes.[19]

IV. PHOSPHORYLATION AND ACTIVATION OF CREB

Phosphorylation is one of the most important post-translational modifications that can modulate the charge, activity, stability, cellular localization, and even

downstream signal transduction of its target proteins, or that can have impact on proteins through crosstalk with other posttranslational modifications.[20] CREB was one of the first transcription factors shown to be regulated by phosphorylation and act as an intracellular signaling second messenger in cells.[21–23] In the late 1980s, CREB was found to be phosphorylated by the cAMP-dependent protein kinase (PKA) *in vitro*, and then phosphorylated by forskolin in cells.[23] CREB is phosphorylated at Ser-133, and various kinases, including ribosomal protein S6 kinase (pp90RSK), protein kinase C (PKC), protein kinase B/AKT, and mitogen- and stress-activated protein kinase (MSK-1), can all phosphorylate CREB at Ser-133.[13,24] Numerous stimuli, including stress signals that increase intracellular cAMP or calcium levels such as neurotransmitters and growth factors, were found to activate CREB in cells. Different growth factors such as mast/stem cell growth factor, basic fibroblast growth factor, and granulocyte-macrophage colony-stimulating factor (GM-CSF), can all induce phosphorylation of CREB.[25,26] CREB, when activated, dimerizes and binds to the promoter regions of its target gene that contains cAMP response element (CRE site), TGACGTCA, or CRE half sites CGTCA/TGACG, and promotes the recruitment of its transcriptional coactivators, CBP/p300, for CREB-mediated transcription. Therefore, CREB can regulate various cellular mechanisms through modulating its target genes (Figure 1).

V. CREB TARGET GENES

Genome-wide analysis revealed that CREB can occupy approximately 4000 promoter sites *in vivo*, emphasizing the broad array of functions CREB may exert; it is important in controlling well-known cell cycle regulators such as Ras, 14-3-3, cyclins, and heat-shock proteins.[27,28] Consistent with that, CREB is involved in a variety of cellular functions, including cell proliferation, survival, apoptosis, differentiation, metabolism, glucose homeostasis, hematopoiesis, immune response, and neuronal activities such as memory and learning.[29,30]

A. Transcription Factors, Metabolic, and Immune Response Regulators

Phosphorylation of CREB at Ser-133 is linked to regulation of transcription factors including c-fos and MEIS1, which contain CREB binding motif on their promoters and can be modulated by CREB.[31–33] CRE binding sites are critical for c-fos transcription, and it was suggested that CREB is a general mediator of stimulus-dependent transcription of c-fos.[31] MEIS1 was upregulated in a microarray analysis in CREB-overexpressing cells, and CREB can induce MEIS1 expression in normal and malignant hematopoietic cells.[32] The importance of CREB in metabolism was also suggested, as numerous CRE-containing genes were found to function in metabolic regulation.[34] Moreover, genes regulating immune response including IL-2 and IL-6, also possess consensus sites for CREB binding and can be modulated by CREB.[13,35,36]

B. Cell Cycle and Proliferation Regulators

CREB is capable of binding to and regulating the promoter regions of cell cycle genes such as cyclin A, D1, and D2, and thus impacts cell proliferation.[37–39] For example, both PI3K and CREB can regulate cyclin D2 promoter activity.[38] Phosphorylation of CREB at Ser-133 is critical for IL-2 induced cyclin D2 transactivation, and the CREB-binding site on cyclin D2 is also important for cyclin D2 promoter activity.[38] PKA inhibitors reduce lymphocyte proliferation and CREB phosphorylation, and thereby CREB and PKA regulate lymphocyte proliferation.[38] Cyclin A1 is also upregulated in leukemia cells that overexpress CREB, while mRNA levels of both cyclin A and D were decreased in CREB shRNA–transduced leukemia cells, suggesting that CREB can promote proliferation of leukemic cells through its downstream targets.[11,12]

C. Growth Factors and Signaling Modulators

Both GM-CSF and interleukin 3 (IL-3) stimulate the proliferation and maturation of myeloid progenitor cells, and each of them can activate signaling pathways

involving a CREB-binding site of the early growth response-1 gene (egr-1) promoter.[40] Also, CREB is phosphorylated on Ser-133 in response to GM-CSF or IL-3 stimulation, and that phosphorylation of CREB on Ser-133 substantially contributes to egr-1 transcriptional activation in response to GM-CSF. In addition, GM-CSF induces pp90RSK activation and phosphorylation of CREB in the human myeloid cell line, TF-1.[11] In TF-1 cells, GM-CSF induces CREB phosphorylation and egr-1 transcription by activating pp90RSK through an MEK-dependent signaling pathway.[11] These studies suggest that phosphorylation of CREB impacts on signal transduction in myeloid cells.

D. Cell Survival Regulation

The role of CREB in cell survival has also been described in a number of tissues. Neurotrophins such as nerve growth factor (NGF) induces phosphorylation of CREB at Ser-133, and it was proposed that Ser-133 phosphorylated CREB induces genes that confer specificity to neurotrophin signals and promote the survival and differentiation of neurons.[41] In addition, CREB-mediated gene expression is necessary for NGF-dependent survival and crucial to promote survival of sympathetic neurons.[42] Moreover, Bcl-2 is activated by NGF and other neurotrophins in a CREB-dependent fashion, and overexpression of Bcl-2 reduces the death-promoting effects of CREB inhibition.[42] Therefore, it appears that activation of CREB promotes survival of neuron cells through activating downstream transcriptional target genes that encode pro-survival factors.

VI. CREB IN HEMATOPOIESIS AND LEUKEMOGENESIS

A. GM-CSF Signaling and CREB Activation

Genetic alterations are involved in leukemogenesis, and they can lead to dysregulated cytokine/growth factor–dependent signal-transduction pathways in leukemic cells.[43–45] Growth factors are produced by myeloid leukemic cells as well as stromal cells, and they bind their own receptors in an autocrine fashion to activate signaling pathways that promote cell growth and survival. Both GM-CSF and IL-3 stimulation result in the proliferation and maturation of early bone marrow progenitor cells. CREB is phosphorylated at Ser-133 in response to GM-CSF or IL-3 stimulation although with different kinetics, and this phosphorylation substantially contributes to transcriptional activation of egr-1 in response to GM-CSF but not IL-3.[40,46] Moreover, egr-1–induced expression by GM-CSF is a PKA-independent event.[47] In TF-1 cells, GM-CSF can induce CREB phosphorylation and egr-1 transcription by activating pp90RSK through an MEK-dependent mechanism.[26] Furthermore, CREB-binding sites have been identified in the promoter of genes regulating proliferation and survival such as Bcl-2 and egr-1, which suggests multiple layers of CREB regulation in leukemic cells. Overall, the role of CREB activation in regulating hematopoietic growth factor signaling in myeloid cells is clearly demonstrated.

B. CREB Is a Proto-Oncogene in Hematopoiesis and AML

Our laboratory showed that the majority of bone marrow samples from patients with acute lymphoid and myeloid leukemia overexpress CREB protein and mRNA.[48] In addition, CREB overexpression is associated with poor outcome of clinical disease in AML patients.[11,48] To understand the role of CREB in leukemogenesis and the biological consequences of CREB overexpression in primary human leukemia cells, leukemia cell lines and transgenic mice were investigated.[11] Overexpression of CREB promotes growth and survival in leukemia cells, while its downregulation leads to suppression of myeloid cell proliferation and survival. Furthermore, CREB transgenic mice developed myeloproliferative disease after 1 year, but not leukemia, suggesting that CREB contributes to leukemic phenotype, but is not sufficient for complete transformation to leukemia.[11] CREB promotes abnormal proliferation and survival of myeloid cells *in vitro* and *in vivo* through upregulation of specific downstream target genes such as cyclin A1.[11,49] It appears that CREB acts as a proto-oncogene to regulate hematopoiesis and that

it contributes to the leukemia phenotype; therefore these results also suggest that CREB-dependent pathways may be targets for directed therapies for leukemia in the future.

C. CREB as a Critical Regulator of Normal Hematopoiesis and Leukemogenesis

CREB appears to be most highly expressed in lineage negative hematopoietic stem cells (HSCs). CREB RNA interference (RNAi) and shRNA techniques were used to knockdown CREB to elucidate its role in hematopoietic progenitors and leukemia cells. Transduction of primary HSCs or myeloid leukemia cells with lentiviral CREB shRNAs resulted in decreased proliferation of stem cells, cell cycle abnormalities, and inhibition of CREB transcription.[12] Transplantation of bone marrow transduced with CREB shRNA in irradiated mice had decreased committed progenitors compared to scrambled control shRNA. However, there was no effect on long-term engraftment, suggesting that CREB insufficiency is not required for HSC activity. Therefore CREB is critical for normal myelopoiesis and leukemia cell proliferation, but not essential for normal function of HSCs.[12]

Compared to patients with leukemia remission or without leukemia, CREB was expressed more highly in bone marrow cells from patients with acute lymphoid or myeloid leukemia.[48] Therefore CREB expression is a potential marker of malignant disease. In an effort to define the target genes of CREB in leukemias, genome-wide analyses were performed and CREB target genes were described; numerous candidate genes have been identified such as transcription regulators and histones, though these await *in vivo* validation.[27,28,50] To identify potential downstream target genes, a microarray analysis with RNA from leukemia K562 cells overexpressing CREB was performed.[51] Approximately 896 genes were differentially expressed in the CREB overexpressing cells compared to control parental cells. Among these, 702 genes were upregulated, and they included members from the MEIS1 and the PBX1 family, which have both been reported to be critical for hematopoietic stem cell self-renewal and leukemogenesis.[51–53]

VII. MICRORNAs AND ONCOGENESIS

Although CREB is overexpressed in leukemia cells, the underlying mechanisms of how CREB regulates leukemogenesis remain largely unknown. Small regulatory non-coding RNA molecules, known as microRNAs, are single-stranded, 20–24 nucleotide-length, RNA molecules that can regulate gene expression in many cellular mechanisms. These microRNAs can modulate gene function at the post-transcriptional level, as they typically reduce the stability of mRNAs that mediate various cellular processes including cell cycle regulation, proliferation, differentiation, and apoptosis and thus have an impact on oncogenesis.[54,55]

Specifically, differential expression of microRNAs in AML appears to have functional relevance in leukemogenesis.[10] MiR-193a, which binds to c-kit proto-oncogene mRNA, was repressed by promoter hypermethylation in AML cell lines and primary AML blasts, but not in normal bone marrow cells.[56] MiR-193a levels were inversely correlated with c-kit levels. Moreover, restoring miR-193a expression in AML cells containing mutated or overexpressed c-kit resulted in reduction in c-kit expression as well as inhibition of cell growth. The growth inhibition activity of miR-193a was suggested to be associated with apoptosis and granulocytic differentiation.[56]

CREB pathways are regulated by micro-RNAs in different cellular backgrounds.[57–59] In myeloid cells that have higher CREB expression levels, miR-34b was expressed less, while overexpression of miR-34b resulted in a reduction of the CREB protein levels.[57] Moreover, miR-34b expression caused abnormal cell cycle progression, reduced cell growth, and altered expression of CREB targets such as Bcl-2, cyclins, protein kinases, and cell survival signaling pathways.[57] The miR-34b promoter is also methylated, which then regulates miR-34b expression level in the leukemia cell lines. The study therefore provides a possible mechanism for CREB overexpression. In another study, miR-301 was found to indirectly regulate ERK/CREB pathway, thereby controlling the transcription and function of its host gene, ska2, a CREB target, in lung cancer cells.[59] Furthermore, inhibition of miR-301 or ska2 leads to an increase of the mitotic

index and a decrease in colony formation, which could contribute to lung cancer transformation.[59]

VIII. CREB AS A POTENTIAL TARGET FOR THERAPY

As described, several lines of evidence support the notion that elevated CREB expression is associated with pathologic growth and survival of hematopoietic cells in primary human leukemic cells, human leukemia cell lines, and transgenic mice, and that CREB and pathways downstream of CREB may represent novel therapeutic targets. CREB levels were found to be elevated at diagnosis, and intriguingly, they were also high in patients with relapsed AML. Patients in remission had similar CREB levels to unaffected controls. Recent evidence also implicates CBP as another important determinant in ALL disease relapse and prognosis. In pediatric patients with relapsed ALL, some 18% demonstrated a focal deletion or gene-sequence alteration in the CBP gene.[60] These alterations were rare in children with ALL who did not relapse, suggesting that the presence of CBP mutations may influence treatment responsiveness. These data demonstrate that CREB and its binding partners influence treatment responsiveness, and they suggest that CREB signaling pathways may represent a novel therapeutic target.

To this end, small molecules that inhibit binding of CREB and CBP have already been identified; this interaction is critical in CREB signaling, and interruption at this step is postulated to reduce CREB activity. Studies of the compound 2-napthol-AS-E-phosphate (KG-501) showed that this molecule specifically inhibits the interaction between the KID of CREB and the helical 'KIX' domain of CBP in a dose-dependent and reversible manner.[61] This molecule does not inhibit forskolin-stimulated phosphorylation of CREB at Ser-133. Furthermore, cAMP-dependent CREB-targeted gene expression was inhibited in the presence of micromolar amounts of this drug, without off-target inhibition of transcriptional machinery. Thus, CREB appears to be a druggable target, and small molecules that inhibit CREB signaling may useful in the clinical setting.[30]

IX. CONCLUSION

In summary, CREB is an important target of growth factor signaling in myeloid cells and promotes the proliferation and differentiation of myeloid progenitor cells. CREB overexpression is observed in the majority of AML and ALL bone marrow cells from patients with leukemia. Ectopic expression of CREB in mice results in myeloproliferative disease but not leukemia, suggesting that additional cooperating oncogenes are required for full transformation. Knockdown of CREB appears to affect myeloid differentiation and myeloid leukemia cell proliferation but does not interfere with long-term engraftment. These results support the possibility of CREB being a potential target for drug development to treat AML. Future directions will focus on understanding how CREB specifically regulates leukemogenesis and targeting this critical protein to treat acute leukemia.

ACKNOWLEDGMENTS

Kathleen M. Sakamoto is supported by National Institutes of Health Grant Nos. R01 HL83077, R01 HL75826, R13 HL103078, R13 CA 159800; the ASH Alternative Training Pathway Award; and the UCLA Stein-Oppenheimer Award. Bryan Mitton is a recipient of an ASH Trainee Research award.

REFERENCES

1. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol. 2011 Feb 10;29(5):551–65.
2. Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. J Clin Oncol. 2011 Feb 10;29(5):487–94.
3. Ofran Y, Rowe JM. Induction and postremission strategies in acute myeloid leukemia: what is new? Curr Opin Hematol. 2011 Mar;18(2):83–8.
4. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol. 2011 Feb 10;29(5):475–86.

5. Scholl S, Fricke HJ, Sayer HG, Hoffken K. Clinical implications of molecular genetic aberrations in acute myeloid leukemia. J Cancer Res Clin Oncol. 2009 Apr;135(4):491–505.
6. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, Meijer J, van Oosterhoud S, van Putten WL, Valk PJ, Berna Beverloo H, Tenen DG, Löwenberg B, Delwel R. Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. Hematol J. 2003;4(1):31–40.
7. Chillon MC, Fernandez C, Garcia-Sanz R, Balanzategui A, Ramos F, Fernandez-Calvo J, González M, Miguel JF. FLT3-activating mutations are associated with poor prognostic features in AML at diagnosis but they are not an independent prognostic factor. Hematol J. 2004;5(3):239–46.
8. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, Döhner H, Döhner K; AML Study Group Ulm. Acute myeloid leukemia. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood. 2002 Dec 15;100(13):4372–80.
9. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF; GIMEMA Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005 Jan 20;352(3):254–66.
10. Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. Blood. 2011 Jan 27;117(4):1121–9.
11. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, Rao NP, Landaw EM, Sakamoto KM. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell. 2005 Apr;7(4):351–62.
12. Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I, Shankar DB, Kasahara N, Stripecke R, Bhatia R, Landaw EM, Sakamoto KM. CREB is a critical regulator of normal hematopoiesis and leukemogenesis. Blood. 2008 Feb 1;111(3):1182–92.
13. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. [Review]. 2001 Aug;2(8):599–609.
14. Sandoval S, Pigazzi M, Sakamoto KM. CREB: A Key Regulator of Normal and Neoplastic Hematopoiesis. Adv Hematol. 2009;2009:634292.
15. Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature. 1993 Oct 28;365(6449):855–9.
16. Kundu TK, Palhan VB, Wang Z, An W, Cole PA, Roeder RG. Activator-dependent transcription from chromatin in vitro involving targeted histone acetylation by p300. Mol Cell. 2000 Sep;6(3): 551–61.
17. Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem. 1999;68:821–61.
18. Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M. TORCs: transducers of regulated CREB activity. Mol Cell. 2003 Aug;12(2):413–23.
19. Johannessen M, Delghandi MP, Moens U. What turns CREB on? Cell Signal. 2004 Nov;16(11):1211–27.
20. Hunter T. The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol Cell. 2007 Dec 14;28(5):730–8.
21. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. 2001 Aug;2(8):599–609.
22. Yamamoto KK, Gonzalez GA, Biggs WH, 3rd, Montminy MR. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature. 1988 Aug 11;334(6182):494–8.
23. Montminy MR, Bilezikjian LM. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature. 1987 Jul 9–15;328(6126):175–8.
24. Bohm M, Moellmann G, Cheng E, Alvarez-Franco M, Wagner S, Sassone-Corsi P, Halaban R. Identification of p90RSK as the probable CREB-Ser133 kinase in human melanocytes. Cell Growth Differ. 1995 Mar;6(3):291–302.
25. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2,

a growth factor-regulated CREB kinase. Science. 1996 Aug 16;273(5277):959–63.

26. Kwon EM, Raines MA, Blenis J, Sakamoto KM. Granulocyte-macrophage colony-stimulating factor stimulation results in phosphorylation of cAMP response element-binding protein through activation of pp90RSK. Blood. 2000 Apr 15;95(8):2552–8.
27. Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell. 2004 Dec 29;119(7):1041–54.
28. Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A. 2005 Mar 22;102(12):4459–64.
29. Siu YT, Jin DY. CREB—a real culprit in oncogenesis. FEBS J. 2007 Jul;274(13):3224–32.
30. Xiao X, Li BX, Mitton B, Ikeda A, Sakamoto KM. Targeting CREB for cancer therapy: friend or foe. Curr Cancer Drug Targets. 2010 Jun;10(4):384–91.
31. Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol. 1998 Feb;18(2):967–77.
32. Esparza SD, Chang J, Shankar DB, Zhang B, Nelson SF, Sakamoto KM. CREB regulates Meis1 expression in normal and malignant hematopoietic cells. Leukemia. 2008 Mar;22(3):665–7.
33. Wang Z, Iwasaki M, Ficara F, Lin C, Matheny C, Wong SH, Smith KS, Cleary ML. GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. Cancer Cell. 2010 Jun 15;17(6):597–608.
34. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol. 2011 Mar;12(3):141–51.
35. Powell JD, Lerner CG, Ewoldt GR, Schwartz RH. The -180 site of the IL-2 promoter is the target of CREB/CREM binding in T cell anergy. J Immunol. 1999 Dec 15;163(12):6631–9.
36. Wen AY, Sakamoto KM, Miller LS. The role of the transcription factor CREB in immune function. J Immunol. 2010 Dec 1;185(11):6413–9.
37. Desdouets C, Matesic G, Molina CA, Foulkes NS, Sassone-Corsi P, Brechot C, Sobczak-Thepot J. Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. Mol Cell Biol. 1995 Jun;15(6):3301–9.
38. White PC, Shore AM, Clement M, McLaren J, Soeiro I, Lam EW, Brennan P. Regulation of cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes. Oncogene. 2006 Apr 6;25(15):2170–80.
39. Fox KE, Colton LA, Erickson PF, Friedman JE, Cha HC, Keller P, MacDougald OA, Klemm DJ. Regulation of cyclin D1 and Wnt10b gene expression by cAMP-responsive element-binding protein during early adipogenesis involves differential promoter methylation. J Biol Chem. 2008 Dec 12;283(50):35096–105.
40. Sakamoto KM, Fraser JK, Lee HJ, Lehman E, Gasson JC. Granulocyte-macrophage colony-stimulating factor and interleukin-3 signaling pathways converge on the CREB-binding site in the human egr-1 promoter. Mol Cell Biol. 1994 Sep;14(9):5975–85.
41. Bonni A, Ginty DD, Dudek H, Greenberg ME. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. Mol Cell Neurosci. 1995 Apr;6(2):168–83.
42. Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science. 1999 Dec 17;286(5448):2358–61.
43. Dai CH, Krantz SB, Dessypris EN, Means RT, Jr., Horn ST, Gilbert HS. Polycythemia vera. II. Hypersensitivity of bone marrow erythroid, granulocyte-macrophage, and megakaryocyte progenitor cells to interleukin-3 and granulocyte-macrophage colony-stimulating factor. Blood. 1992 Aug 15;80(4):891–9.

44. Marvin J, Swaminathan S, Kraker G, Chadburn A, Jacobberger J, Goolsby C. Normal bone marrow signal-transduction profiles: a requisite for enhanced detection of signaling dysregulations in AML. Blood. 2011 Apr 14;117(15):e120–30.

45. Inukai T, Sugita K, Mitsui K, Iijima K, Goi K, Tezuka T, Kojika S, Kagami K, Mori T, Kinoshita A, Suzuki T, Okazaki-Koyama T, Nakazawa S. Participation of granulocyte colony-stimulating factor in the growth regulation of leukemia cells from Philadelphia chromosome-positive acute leukemia and blast crisis of chronic myeloid leukemia. Leukemia. 2000 Aug;14(8):1386–95.

46. Lee HJ, Mignacca RC, Sakamoto KM. Transcriptional activation of egr-1 by granulocyte-macrophage colony-stimulating factor but not interleukin 3 requires phosphorylation of cAMP response element-binding protein (CREB) on serine 133. J Biol Chem. 1995 Jul 7;270(27):15979–83.

47. Wong A, Sakamoto KM. Granulocyte-macrophage colony-stimulating factor induces the transcriptional activation of egr-1 through a protein kinase A-independent signaling pathway. J Biol Chem. 1995 Dec 22;270(51):30271–3.

48. Crans-Vargas HN, Landaw EM, Bhatia S, Sandusky G, Moore TB, Sakamoto KM. Expression of cyclic adenosine monophosphate response-element binding protein in acute leukemia. Blood. 2002 Apr 1;99(7):2617–9.

49. Kinjo K, Sandoval S, Sakamoto KM, Shankar DB. The role of CREB as a proto-oncogene in hematopoiesis. Cell Cycle. [Review]. 2005 Sep;4(9):1134–5.

50. Pellegrini M, Cheng JC, Voutila J, Judelson D, Taylor J, Nelson SF, Sakamoto KM. Expression profile of CREB knockdown in myeloid leukemia cells. BMC Cancer. 2008;8:264.

51. Esparza SD, Chang J, Shankar DB, Zhang B, Nelson SF, Sakamoto KM. CREB regulates Meis1 expression in normal and malignant hematopoietic cells. Leukemia. 2008 Mar;22(3):665–7.

52. Wong P, Iwasaki M, Somervaille TC, So CW, Cleary ML. Meis1 is an essential and rate-limiting regulator of MLL leukemia stem cell potential. Genes Dev. 2007 Nov 1;21(21):2762–74.

53. Ficara F, Murphy MJ, Lin M, Cleary ML. Pbx1 regulates self-renewal of long-term hematopoietic stem cells by maintaining their quiescence. Cell Stem Cell. 2008 May 8;2(5):484–96.

54. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. J Pathol. 2011 Jan;223(2):102–15.

55. Bueno MJ, Malumbres M. MicroRNAs and the cell cycle. Biochim Biophys Acta. 2011 May;1812(5):592–601.

56. Gao XN, Lin J, Li YH, Gao L, Wang XR, Wang W, Kang HY, Yan GT, Wang LL, Yu L. MicroRNA-193a represses c-kit expression and functions as a methylation-silenced tumor suppressor in acute myeloid leukemia. Oncogene. 2011 Aug 4;30(31):3416–28.

57. Pigazzi M, Manara E, Baron E, Basso G. miR-34b targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. Cancer Res. 2009 Mar 15;69(6):2471–8.

58. Gao J, Wang WY, Mao YW, Graff J, Guan JS, Pan L, Mak G, Kim D, Su SC, Tsai LH. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature. 2010 Aug 26;466(7310):1105–9.

59. Cao G, Huang B, Liu Z, Zhang J, Xu H, Xia W, Li J, Li S, Chen L, Ding H, Zhao Q, Fan M, Shen B, Shao N. Intronic miR-301 feedback regulates its host gene, ska2, in A549 cells by targeting MEOX2 to affect ERK/CREB pathways. Biochem Biophys Res Commun. 2010 Jun 11;396(4):978–82.

60. Mullighan CG, Zhang J, Kasper LH, Lerach S, Payne-Turner D, Phillips LA, Heatley SL, Holmfeldt L, Collins-Underwood JR, Ma J, Buetow KH, Pui CH, Baker SD, Brindle PK, Downing JR. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011 Mar 10;471(7337):235–9.

61. Best JL, Amezcua CA, Mayr B, Flechner L, Murawsky CM, Emerson B, Zor T, Gardner KH, Montminy M. Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. Proc Natl Acad Sci U S A. 2004 Dec 21;101(51):17622–7.

Critical Reviews™ in Oncogenesis, 16(1–2), 47–63 (2011)

TAM Receptors in Leukemia: Expression, Signaling, and Therapeutic Implications

Luis Brandão, Justine Migdall-Wilson, Kristen Eisenman, & Douglas K. Graham*

Department of Pediatrics, University of Colorado Denver School of Medicine, Aurora, Colorado

*Address all correspondence to: Douglas K. Graham, University of Colorado Denver School of Medicine, Pediatrics Mail Stop 8302, PO Box 6511, Aurora, CO 80045; Doug.Graham@ucdenver.edu

ABSTRACT: In the past 30 years there has been remarkable progress in the treatment of leukemia and lymphoma. However, current treatments are largely ineffective against relapsed leukemia and, in the case of pediatric patients, are often associated with severe long-term toxicities. Thus, there continues to be a critical need for the development of effective biologically targeted therapies. The TAM family of receptor tyrosine kinases—Tyro3, Axl, and Mer—plays an important role in normal hematopoiesis, including natural killer cell maturation, macrophage function, and platelet activation and signaling. Furthermore, TAM receptor activation leads to upregulation of pro-survival and proliferation signaling pathways, and aberrant TAM receptor expression contributes to cancer development, including myeloid and lymphoid leukemia. This review summarizes the role of TAM receptors in leukemia. We outline TAM receptor expression patterns in different forms of leukemia, describe potential mechanisms leading to their overexpression, and delineate the signaling pathways downstream of receptor activation that have been implicated in leukemogenesis. Finally, we discuss the current research focused on inhibitors against these receptors in an effort to develop new therapeutic strategies for leukemia.

KEY WORDS: leukemia, Axl, Mer, Tyro-3, signal transduction, gene expression, targeted, therapeutics.

ABBREVIATIONS

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CSF1: colony stimulating factor 1; HDAC, histone deacetylase; IFN, type I interferon; LXR, liver X receptor; NK, natural killer; NKT, natural killer T cell; RTK, receptor tyrosine kinase; TAM, Tyro3, Axl, and Mer

I. INTRODUCTION

Cancer is the second leading cause of death in the United States, resulting in an estimated 23.2% of all deaths in 2007. While leukemia and lymphoma only account for approximately 7.6% of these deaths (43,370 in 2010), leukemia is the primary cause of cancer-related death in males under 40 and females under 20 years old. Furthermore, cancer is the leading cause of disease-related deaths in children (1–14 years old), and leukemia is the most common pediatric malignancy.[1–3]

Between 1975 and 2003, the 5-year relative survival for all leukemia patients has increased significantly from 33.4% to 55.6%. This trend is even more dramatic for children with leukemia, where relative survival rates have increased from 43% to 94.6% in cases of acute lymphoblastic leukemia (ALL) and from 18.7% to 56.1% in cases of acute myeloid leukemia (AML).[3] Although much of this improvement stems from changes in treatment regimens and dosing during induction and subsequent courses of therapy, the current success is associated with a 2- to 4-fold increased rate of severe therapy-associated health conditions, including organ damage, infertility, growth decline, reduced mental function, and secondary malignancy.[4,5] Furthermore, current treatment protocols are largely ineffective against relapsed leukemia,[6] highlighting the need for new antileukemic therapeutic agents.

The TAM family of receptor tyrosine kinases (RTKs) includes Tyro-3, Axl, and Mer. In normal hematopoiesis, TAM receptors inhibit inflammation in dendritic cells and macrophages, promote phagocytosis of apoptotic cells and membranous organelles, and are essential for natural killer (NK)

0893-9675/11/$35.00

cell maturation.[7] The TAM family is also vital for platelet activation, platelet signaling during thrombus stabilization,[8,9] and may have an important role in erythropoiesis.[10] In addition to their role in normal hematopoiesis, TAM receptors can activate proliferation- and survival-promoting signaling pathways such as those driven by AKT and ERK1/2, which contribute to oncogenesis in multiple cancers, including myeloid and lymphoid leukemia.[11]

The goal of this review is to summarize the role of the TAM family in leukemia. Because little published data exist on Tyro-3 in hematopoietic malignancy, we have largely focused on Mer and Axl receptor tyrosine kinases. Throughout the discussion, we address leukemia-associated expression patterns observed for TAM receptors, explore potential mechanisms underlying their aberrant expression, and outline the downstream signaling pathways implicated in leukemogenesis. In the final part of our review, we detail the ongoing efforts to develop therapeutic agents against this family of receptors and discuss how specifically targeting TAM receptors can potentially enhance leukemia therapy.

II. TAM RECEPTOR EXPRESSION PATTERNS IN LEUKEMIA

While macrophages, dendritic cells, NK cells, NKT cells, megakaryocytes, and platelets normally express TAM receptors,[7,8,10,12,13] they are not expressed in thymocytes, mature T- or B-lymphocytes, or granulocytes.[14] However, TAM receptors display altered expression patterns in leukemia. Below and in Table 1, we compare TAM receptor expression in different subsets of leukemia.

A. Tyro-3

Tyro-3 (Dtk/Sky/Rse/Brt/Tif) RNA was identified in blasts in 6 of 11 AML patients by RNase protection analysis.[15] More recently, a gene expression microarray found Tyro-3 overexpression in multiple myeloma samples relative to autologous B-lymphoblastoid cell lines, and Tyro-3 mRNA transcript was also detected in primary malignant plasma cells from patients with plasma cell leukemia or multiple myeloma.[16]

B. Axl

Axl (Ufo/Jtk11) was first detected in 1988 as an unidentified gene promoting the transition from chronic phase to blast crisis in two patients with chronic myelogenous leukemia (CML).[17] Three years later, two independent groups cloned the human gene from patients with CML and chronic myeloproliferative disorder.[18,19] Axl mRNA expression was identified in a large number of cell lines derived from myeloid and erythromegakaryocytic leukemias and was notably absent in lymphocytic cell lines.[20] Patient sample analysis confirmed these cell line results, revealing Axl transcript in 56/99 (56.6%) patients with myeloproliferative disorders (AML, CML in chronic phase and in blast crisis, and myelodysplasia), but in only 2.2% of lymphoid leukemias: out of 45 samples, only one patient with chronic lymphocytic leukemia (CLL) had detectable Axl mRNA.[21,22] Similarly, Rochlitz et al. identified Axl transcript in 19 of 54 (35%) AML patient samples, observing that patients with increased CD34 expression also displayed higher levels of Axl, and they determined that Axl expression was associated with worse progression-free and overall survival when adjusted for age, Auer rods, and leukocyte counts.[23] Lastly, a recent study found increased Axl transcript in myeloid leukemia samples from chemotherapy-resistant patients; furthermore, chemotherapy induced Axl expression in an AML cell line, and addition of Gas6—an Axl ligand—enhanced drug resistance in cells during chemotherapeutic treatment.[24]

C. Mer

The human Mer gene (MerTK/RP38/Nyk/Tyro12) was initially cloned from a B-lymphoblastoid expression library.[14] While normal T- and B-lymphocytes do not express Mer mRNA transcript or protein at any developmental stage,[25,26] our lab has detected ectopic Mer mRNA expression in 19/34 (55.8%) patient samples, and Mer protein expression in 8/16 (50%) pediatric T-ALL patient samples.[25] Furthermore, a large-scale microarray analysis demonstrated that significantly high levels of Mer transcript exist in the cytogenetic subset of B-ALL patients expressing

TABLE 1. Expression of TAM Receptors in Leukemia and in the Hematopoietic System

Leukemia	Tyro-3	Axl	Mer	Gas6	Protein S	References
Myeloid						
CML		Pos	Pos	Pos		17, 18, 21, 102
AML	Pos	Pos	Pos	Pos		15, 20, 21, 23, 28, 102
Myeloblastic leukemia		Pos	Pos	Pos		20, 28, 102
Monoblastic leukemia		Pos	Pos	Pos		20, 28, 102
Erythroid leukemia		Pos	Pos	Pos		20, 28, 102
Megakaryoblastic leukemia		Pos	Pos	Pos		20, 28, 102
Lymphoblastic						
ALL		Neg	Pos	Pos	Pos	14, 20, 21, 25–27
CLL		Pos		Pos		21, 95, 102
Plasma cell leukemia	Pos					16
Normal hematopoiesis						
Lymphocytes		Neg	Neg			14, 20, 21
Granulocytes		Neg				21
Basophil/Mast cells		Pos				7
Macrophages	Pos	Pos	Pos	Pos	Pos	7, 13, 14, 21, 103
Megakaryocytes/platelets	Pos	Pos	Pos	Pos		7, 14, 104–106
NK cells, NKT cells	Pos	Pos	Pos	Neg	Pos	7, 13
Dendritic cells	Pos	Pos	Pos	Pos	Pos	7, 13
Bone marrow	Neg	Pos	Pos	Pos		14, 22, 106, 107
Erythroid cells	Neg	Pos	Pos	Pos		10, 108

the *E2A-PBX1*+ fusion protein.[27] Consistently, our lab has identified aberrant Mer protein expression in 16/16 *E2A-PBX1*+ B-ALL patient samples, whereas 11/12 B-ALL samples without *E2A-PBX1* did not express Mer.[26] To date, there are no published reports on the role of Mer in myeloid leukemia, but we have detected increased Mer expression in 11/16 AML cell lines and in 17/26 primary patient samples by western blot and flow cytometry.[28]

The role of Mer in leukemogenesis is further supported by two animal models. Abnormal activation of Eyk, the chicken homologue of Mer, via the naturally occurring RPL30 avian retrovirus, leads to the development of a spectrum of cancers, including lymphomas, in chickens.[29] Additionally, ectopic Mer expression in lymphocytes in the Mer transgenic mouse increases the incidence of leukemia/lymphoma.[30]

III. UPSTREAM REGULATION OF TAM RECEPTOR EXPRESSION

TAM receptor overexpression occurs in many cancers of myeloid lineage, and ectopic expression of Mer, which normal lymphocytes do not express, is found in mantle cell lymphoma, the majority of T cell leukemias, and particular subsets of B cell leukemia.[25,27]

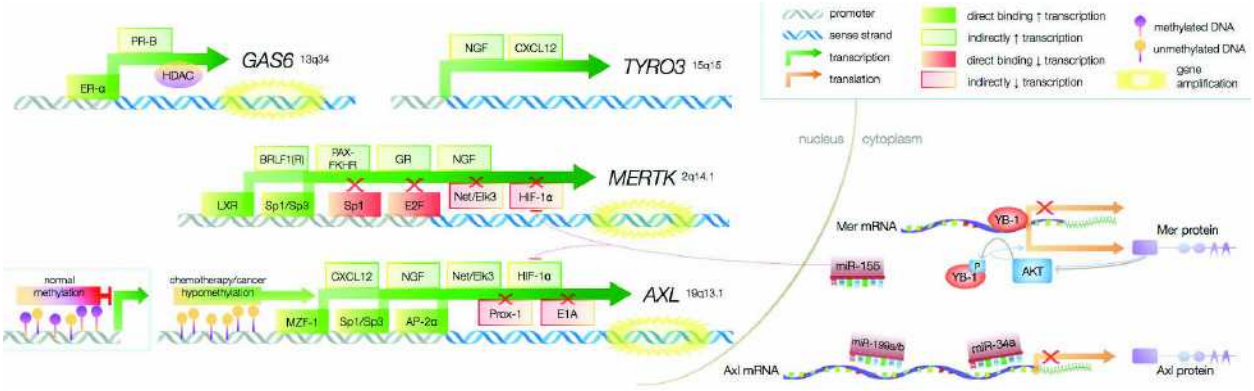


FIGURE 1. Experimentally determined regulators of TAM receptor and ligand gene expression. Nuclear modulators include transcription factors, histone acetylation, promoter methylation, and gene amplification. Outside of the nucleus, several post-transcriptional processes influence protein formation: miRNAs repress translation of Axl and HIF-1a, potentially altering both *MER* and *AXL* transcription; YB-1, an RNA-binding protein, inhibits Mer translation unless it is phosphorylated by AKT, a downstream target of Mer activation.

Although aberrant TAM receptor levels clearly enhance oncogenic potential, much remains unknown about the mechanisms underlying their overexpression. Several studies have begun to explore epigenetic and post-transcriptional regulation of TAM receptor expression, providing us with further insight into the tangled circuitry of cancer progression. Although the studies presented here have been conducted in a variety of systems, their findings may also apply to processes within hematopoietic development and leukemogenesis, which are depicted in Figure 1.

A. Genetic Variation

To date, no activating mutations in the TAM receptor genes have been implicated in malignant transformation, but recent studies have highlighted the potential role of copy number variation in TAM receptor expression. *AXL* gene amplification and corresponding overexpression of its transcript were found in a CGH-based microarray profiling study of glioblastoma samples,[31] and gastric cancer samples displayed increased *AXL* and *MER* copy numbers relative to normal controls.[32] Additionally, DNA copy number analysis identified *AXL* gene amplification in 4/4 lapatinib-resistant breast cancer cell lines,[33] and *GAS6* amplification has also been detected in aggressive mouse mammary tumors.[34]

Analysis of the Axl transcript has also shown that two alternatively spliced isoforms are expressed in tumor and normal samples at different ratios. However, both isoforms have the same transforming capability, suggesting that receptor overexpression—rather than a structural difference in the transcript or protein—drives the oncogenicity of this receptor.[18]

B. Transcriptional Regulation

While several putative transcription factors for the TAM receptor genes have been identified based on promoter binding site specificity, gene expression modulation has been most extensively studied in Axl, as it is the only human TAM receptor for which the gene promoter has been fully characterized. Multiple studies have found that AP-2α, Sp1/Sp3 and MZF-1 directly regulate Axl transcription, with MZF-1 levels directly correlating with Axl expression and metastasis in colorectal and cervical cancers.[18,35–37] More recently, CXCR4/SDF-1 (CXCL12) has been shown to increase transcription of both *AXL* and *TYRO3* in thyroid carcinoma cell lines; although the transcriptional interaction was not further characterized, treatment with a CXCR4 inhibitor did not reduce constitutive Axl expression, suggesting that its overexpression requires additional regulatory mechanisms.[38] Another study found that Gas6, the common ligand for both Axl and Mer,

was transcriptionally upregulated following progesterone receptor activation in breast cancer cells.[39] A complete list of transcription factors and their interactions with the TAM receptor genes has been compiled in Table 2.

Although the human Mer promoter remains completely uncharacterized, Wong et al. identified Sp1, Sp3, and E2F as transcriptional regulators of the mouse *Mertk* gene,[40] which shares considerable homology with its human counterpart. Liver X receptor (LXR) was recently shown to directly bind the *Mertk* promoter and induce its transcription in mouse macrophages (without noticeable effects on Axl or Tyro-3 expression), which promoted phagocytosis of apoptotic cells and maintained immunity.[41] Another study found that CLL cells display increased LXR expression relative to normal lymphocytes, but the potential relationship with Mer expression was not investigated.[42] However, disruption of normal LXR expression in lymphocytes causes age-dependent lymphoid hyperplasia in mouse models,[43] findings similar to those observed upon ectopic Mer expression in transgenic mouse lymphocytes.[30]

Mer has also been identified as a glucocorticoid-responsive gene in mouse mesenchymal stem-like cells: dexamethasone treatment led to a 4.6-fold increase in Mer expression relative to vehicle-treated cells, and ChIP-chip analyses revealed several glucocorticoid binding sequences upstream of the *Mertk* transcription start site.[44] Another study found a similar trend in dexamethasone-induced Mer protein expression on the surface of cultured human macrophages,[45] indicating that Mer upregulation may decrease responsiveness to ALL induction therapy, which involves glucocorticoid treatment. Consistently, our laboratory has found increased Mer expression in patients with relapsed leukemia (Eisenman K and Graham DK, unpublished data), suggesting that Mer may serve as a survival or resistance mechanism in leukemic cells following treatment.

Although Mer is upregulated in the *E2A-PBX1*+ cytogenetic subset of B-ALL patients,[26,27,46] expression of the fusion protein does not appear to induce Mer transcription (Sawczyn K and Graham DK, unpublished data) despite the presence of a PBX1-binding site near the Mer promoter.[47] However, the PBX domain of the fusion protein is directly responsible for inducing apoptosis in hematopoietic precursor B cell lines,[48] consistent with the idea that Mer expression arises as a survival response rather than directly through E2A-PBX1-driven changes in gene transcription.

C. Epigenetic Regulation

Upon identifying Axl as the only consistently upregulated protein tyrosine kinase in drug-resistant AML patient samples, Hong et al. found that treatment of an AML cell line with a chemotherapeutic agent—doxorubicin, cisplatin, or VP16—increased Axl expression, but only when its promoter remained unmethylated.[24] Furthermore, a separate study correlated promoter hypomethylation with the degree of Axl overexpression in Kaposi sarcoma: cell lines expressing high levels of Axl had fewer methylated sites, whereas other lines expressing less Axl—including some derived from other cancer types—displayed more promoter methylation.[49] Lastly, it was shown that SAHA, a histone deacetylase (HDAC) inhibitor, suppresses Gas6 expression in multiple myeloma cells,[50] highlighting another potential mechanism regulating transcription of TAM receptor-related genes.

D. Post-Transcriptional Regulation

Currently, miR-335 is the only microRNA reported to target the Mer 3′UTR.[51] However, the study, which compared expression differences between metastatic and non-metastatic breast cancer lines, only used indirect target-validation methods and never assessed Mer protein expression following manipulation of miR-335 levels. In functional studies of miRNAs in leukemic cells, restoration of miR-335 expression levels with a synthetic mimic did not decrease Mer protein expression (Migdall-Wilson J and Graham DK, unpublished data).

Both miR-155 and miR-34a decrease Axl expression in human monocytes.[52] miR-155, which promotes tumor growth in numerous leukemias and lymphomas when overexpressed,[53] represses translation of several transcription factors that potentially

TABLE 2. Transcriptional Regulators of TAM Receptor Expression

Gene	Interaction	Transcription Factor	Effect	Cell Type	Reference
Tyro-3	Indirect	NGF	↑	Rat neuronal cells	109
Tyro-3	Indirect	CXCR4/SDF-1 (CXCL12)	↑	Thyroid carcinoma	38
Axl	Direct	AP-2a	↑	HeLa, 293T, NIH3T3, NSC-34	37
Axl	Direct	Sp1/Sp3	↑	Rko, HCT116, HeLa	36
Axl	Direct	MZF-1	↑	Colorectal, cervical cancer	110
Axl	Indirect	CXCR4/SDF-1 (CXCL12)	↑	Thyroid carcinoma	38
Axl	Indirect	HIF-1a	↑	Endothelial cells	111
Axl	Indirect	Net/Elk3	↑	Endothelial cells	112
Axl	Indirect	NGF	↑	Rat neuronal cells	109
Axl	Indirect	Prox-1	↓	Blood endothelial cells	113
Axl	Indirect	E1A	↓	Breast cancer	35
Axl	Predicted	AP-1			24
Axl	Predicted	C/EBPb			24
Axl	Predicted	p300			24
Axl	Predicted	CREB			24
Mer	Direct	Sp1	↑,↓	Mouse Sertoli cells	40
Mer	Direct	LXR	↑	Macrophages	41
Mer	Direct	Sp3	↑	Mouse Sertoli cells	40
Mer	Direct	E2F	↓	Mouse Sertoli cells	40
Mer	Indirect	BRLF1 (R)	↑	Several	114
Mer	Indirect	PAX-FKHR	↑	Mouse MSCs	115
Mer	Indirect	GR	↑	Mouse MSCs	44
Mer	Indirect	Net/Elk3	↓	Endothelial cells	112
Mer	Indirect	HIF-1a	↓	Endothelial cells	112
Mer	Predicted	GATA			40
Mer	Predicted	MZF-1			40
Gas6	Direct	ER-a	↑	Breast cancer, normal mammary	116
Gas6	Indirect	PR-B	↑	Breast cancer	39

regulate Axl and Mer expression, including PU.1, CEBPβ, CSF1R, and HIF-1α. In addition to showing that miR-34a and miR-199a/b target the Axl 3′UTR, a recent study also determined that both miRNA genes and *AXL* are regulated by promoter methylation.[54]

Evdokimova et al. found that YB-1, a regulatory RNA-binding protein, normally inhibits translation of Mer mRNA; however, in conditions requiring increased expression of stress- and growth-related proteins, Mer translation is de-repressed upon AKT-mediated phosphorylation.[55] While this mechanism has not been explored in hematopoietic cells, the fact that AKT is a well-known downstream target of Mer raises the possibility of a positive-feedback loop occurring through this post-transcriptional mechanism.

IV. MER AND AXL RECEPTOR SIGNALING IN LEUKEMIA

Mer and Axl activate many different signaling pathways depending on cell type and function, a topic extensively discussed in a review previously published by our lab.[11] In this section, we specifically focus on the Mer- and Axl-activated pathways known to play a role in leukemogenesis (Figure 2).

A. Ligands

Gas6 and Protein S are two structurally similar, vitamin K-dependent proteins that activate the TAM receptors.[56,57] Both ligands are produced in a wide range of tissues, including the bone marrow, thymus, spleen, and plasma,[56,58,59] suggesting that leukemia cells expressing Mer or Axl are constitutively activated through a paracrine mechanism. Consistent with this idea, Gas6 expression has been correlated with the ability of bone marrow stromal cell lines to support hematopoiesis,[60] and primary human osteoblasts have been shown to secrete Gas6 in response to Mer-expressing leukemic cells.[61] Furthermore, overexpression of Gas6 and Protein S has been reported in several cancers and often correlates with poor prognostic markers.[62]

Tubby and Tubby-like protein 1 (Tulp1), which also bind Axl and Tyro-3, were recently described as two new Mer ligands important in phagocytosis.[63] It is unclear whether these two ligands play a role in leukemogenesis.

B. MAPK (ERK) Pathway

Before the TAM receptor ligands had been identified, initial studies on Mer and Axl downstream signaling implemented chimeric receptors[64–66] composed of the Axl or Mer intracellular kinase domain fused to an extracellular domain of a receptor with a known ligand. In a study using a colony stimulating factor 1 (CSF1) receptor/Mer chimeric receptor, stimulation with CSF1 led to phosphorylation of Shc, recruitment of Grb2 and downstream phosphorylation of MEK and ERK1/2 kinases, indicating MAPK pathway activation.[66] These results were later confirmed with a CD8/Mer chimeric receptor in BaF3 cells,[65] and eventually in experiments using the full-length Mer receptor and recombinant Gas6 for activation of the receptor in 293 cells.[67] More recently, our lab demonstrated that Mer expression mediates chemotherapy-induced activation of ERK1/2 in the 697 (human B-ALL) cell line,[26] and related studies showed that Axl stimulation activates the ERK1/2 kinases through Shc, Grb2, and Ras.[64,68] Interestingly, results of other investigations have suggested that Axl-mediated ERK1/2 activation is specifically required to induce proliferation and depends on PI3K/AKT and Src activity.[69,70]

C. PI3K/AKT/mTOR

Many studies exploring downstream activation of the MAPK pathway have also identified the PI3K/AKT kinase cascade as an important component of Axl/Mer signaling.[64,66,67,69] Several groups showed that the p85 subunit of PI3K binds to both Mer and Axl, and determined that this interaction requires both ligand binding and kinase activity.[64,68,71]

In response to Axl stimulation, PI3K/AKT pathway activation is important for both cell proliferation—via ERK1/2 activation—as well as survival, which occurs through phosphorylation of Bad, a Bcl-2 family member;[69,70] furthermore, this antiapoptotic effect appears to be independent of

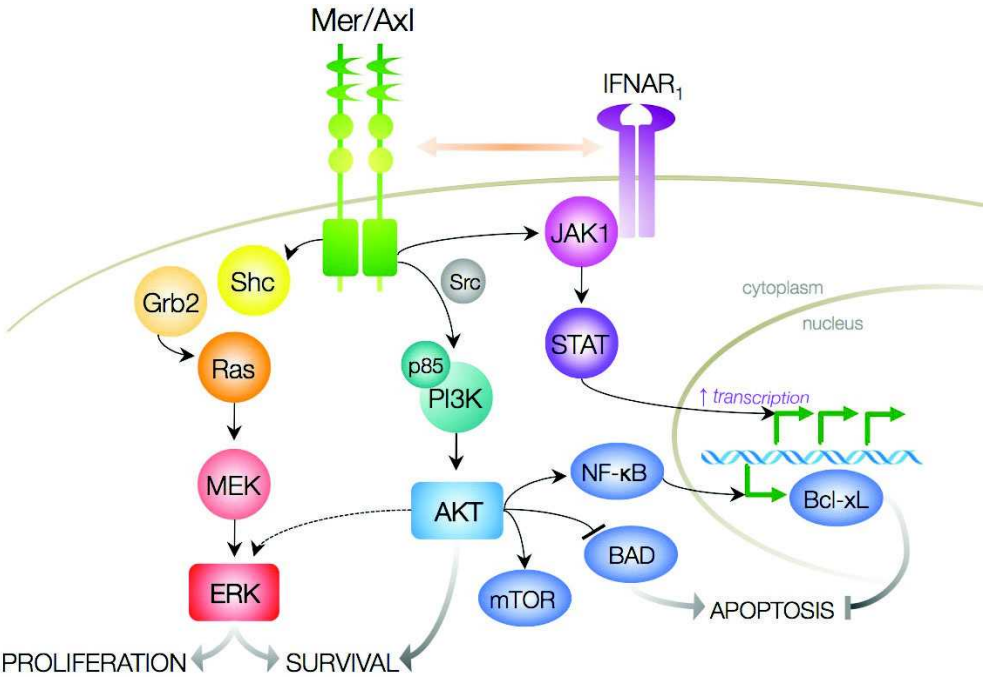


FIGURE 2. Mer/Axl signaling in leukemia. Receptor activation by Gas6 or Protein S leads to activation of MAPK, PI3K/AKT and Jak/STAT pathways, resulting in increased proliferation and survival. Jak/STAT activation has been observed both via direct interaction between Jak and Mer, as well as through interaction between Axl and the type 1 interferon receptor ($IFNAR_1$).

MAPK activity.[69] Interestingly, studies investigating how Mer stimulation affects the PI3K/AKT pathway were less conclusive, partly because they were performed in different cell types: in the Ba/F3 lymphoid cell line, Mer-mediated PI3K activation increased cell survival upon IL-3 withdrawal and minimally impacted cell proliferation.[65] However, in the 32D monocytic cell line, Mer-mediated PI3K activation alone did not sufficiently prevent apoptosis; instead, investigators found that the MAPK and PI3K pathways play parallel roles: inhibition of both cascades was necessary to elicit growth factor withdrawal–induced cell death.[71]

mTOR kinase, a key regulator of protein synthesis and cell growth, is one of the downstream effectors of the PI3K pathway. Goruppi et al. showed that rapamycin, an mTOR inhibitor, blocked Axl-mediated proliferation and survival in NIH 3T3 cells.[69] Furthermore, S6 kinase (S6K)—a downstream target of mTOR—is activated upon stimulation of a Mer chimeric receptor in NIH/3T3 cells.[66]

D. NF-kB

The NF-kB family of transcription factors is a key regulator of cell growth, development, and survival. Aberrant NF-kB activation has been observed in various forms of leukemia/lymphoma[72] and has also been implicated downstream of Mer and Axl/Gas6 signaling:[65,73,74] luciferase reporter assays revealed a 10-fold increase in NF-kB transcriptional activity in Ba/F3 cells expressing a constitutively active chimeric Mer receptor, along with more robust proliferation than vector-only control cells.[65] In NIH/3T3 cells, Gas6 induced a transient decrease in the level of IkB, which binds to and inhibits NF-kB, resulting in enhanced NF-kB DNA-binding activity and an NF-kB–dependent increase in expression of Bcl-xL, an anti-apoptotic protein.[73] Likewise, expression of a dominant negative IkB—which keeps NF-kB in a permanently bound, inactive state—inhibited Gas6-mediated survival, further implying that the anti-apoptotic effects downstream of TAM receptor

activation require NF-kB activity.[73] Lastly, NF-kB activation depends upon PI3K/AKT activation, suggesting that extensive cross-talk exists between the various pathways downstream of Mer or Axl.[65,73]

E. Jak/STAT Pathway

Constitutive activation of STAT proteins has been observed both in AML and ALL, as well as in a variety of other tumors (reviewed in Benekli et al.[75]). Jak/STAT activation arises through various mechanisms, including cytokine overexpression and autocrine/paracrine stimulation,[76] as well as expression of a TEL-JAK2 fusion protein.[77]

Both constitutive Jak activation and STAT phosphorylation have been associated with Mer and Axl activity. COS cells expressing a constitutively active chicken Mer receptor (CD8-Eyk chimera) displayed constitutive activation of STAT1, STAT3, and Jak1; the CD8-Eyk chimera also co-immunoprecipitated with Jak1.[78] More recently, Gas6-induced Axl stimulation led to STAT1 activation in mouse bone marrow dendritic cells, an effect dependent upon type I interferon (IFN) receptor expression.[79] Interestingly, Axl co-immunoprecipitated with the IFN receptor R1 chain, suggesting a possible mechanism for TAM receptor activation of the Jak/STAT pathway.

V. THERAPEUTIC TARGETING

Several novel biologically targeted therapies have shown great promise in improving therapeutic outcomes in leukemia. The most striking examples are imatinib, dasatinib, and nilotinib, tyrosine kinase inhibitors used to treat CML and Philadelphia chromosome-positive (Ph+) ALL.[80] Imatinib therapy resulted in a 73.8% rate of complete cytogenetic response after 19 months, compared with 8.5% for patients on earlier standards of care.[81] Other examples include lestaurtinib, a FLT3 tyrosine kinase inhibitor currently in clinical trials for treatment of MLL-rearranged ALL, and rituximab, a monoclonal antibody against CD20 currently used in therapy for CD20+ leukemia/lymphoma.[82,83]

Mer and Axl represent two novel targets for the development of new anti-leukemia therapies, and multiple studies of receptor inhibition highlight their potential within this realm. Mer inhibition significantly delays leukemia progression in a human ALL cell line xenograft (Brandão and Graham, unpublished data), and Axl inhibition reduces growth of lung and breast cancer xenograft tumors in immuno compromised mice.[84] Furthermore, inhibition of Mer dampens pro-survival pathway activation in a human B-ALL cell line and renders it more sensitive to a spectrum of chemotherapeutic agents currently used in the clinic.[26] Similarly, Mer and Axl inhibition also increases apoptosis and chemosensitivity in astrocytoma cell lines.[85]

Three different strategies are available to hinder TAM receptor signaling: ligand sinks, antibody-mediated downregulation/blocking, and small-molecule kinase inhibitors. Our lab has shown that treatment with a Mer-Fc fusion construct—one example of a ligand sink—inhibits Gas6 signaling, apoptotic cell engulfment by macrophages, and platelet aggregation.[86] Such a construct could be used as adjuvant therapy to transiently inhibit TAM signaling in leukemia cells at the time of chemotherapy administration.

Two separate groups have reported that Axl-specific antibodies inhibit signaling and enhance the effect of standard chemotherapies on tumor cells.[84,87,88] Zhang et al. showed that treatment with a polyclonal anti-Axl antibody significantly diminishes the motility and invasive properties of human breast cancer cells.[88] Furthermore, Ye et al. demonstrated that an anti-Axl monoclonal antibody blocks Gas6 signaling, reduces Axl surface expression, and synergizes with standard chemotherapy agents to inhibit tumor growth in xenograft models of lung and breast cancer.[87] While there are no published studies regarding the use of Mer-specific antibodies as a therapeutic agent, our lab has characterized monoclonal anti-Mer antibodies that decrease surface expression of Mer in human ALL cell lines.

Although there are few reports of TAM receptor-specific small-molecule inhibitors, several inhibitors developed against closely related receptor tyrosine kinases,[89] such as c-Kit and Met, are also active against

Axl and/or Mer. Foretinib (GSK1363089, GlaxoSmithKline), a Met inhibitor currently in several clinical trials for use against solid tumors, inhibits *in vitro* Axl phosphorylation in the low nanomolar range (IC_{50}=11 nM).[33] MP470 (Amuvatinib, SuperGen), originally designed as a c-Kit inhibitor, inhibits Axl activation[90,91] and is currently in a Phase II clinical trial to test it as combination therapy with Platinum-Etoposide against small cell lung carcinoma. Crizotinib (PF-2341066, Pfizer), a Met inhibitor also active against ALK kinase, inhibits Axl with an IC_{50}=322 nM,[92] and is currently being evaluated in a variety of clinical trials. Lastly, BMS777607 (Bristol-Meyers Squibb)—also developed against Met—has low nanomolar activity *in vitro* against Axl, Mer and Tyro-3 (IC_{50}= 1.1, 14, and 4.3 nM, respectively) and has been evaluated in a Phase I dose-escalation clinical trial.[93]

Unlike the inhibitors mentioned above, R428 (Rigel) was developed specifically against Axl.[94] Treatment with R428, which inhibits Axl with an IC_{50} = 14 nM but shows low activity for Mer and Tyro3, blocked both *in vitro* invasion and *in vivo* metastatic potential of a human breast cancer cell line, and also prolonged survival in a mouse model of post-mastectomy metastasis.[94] In a separate study, R428 was also shown to induce apoptosis in CLL B cells.[95]

In contrast with Axl, there is no information on specific small molecule inhibitors targeting Mer or Tyro-3. In a crystal structural study of Mer, a library of kinase inhibitors was screened *in vitro* against the kinase domain of the receptor[96]: several compounds were identified, but no further reports of biological activity or toxicity are available.

VI. CONCLUSION

In this review, we have described the role of the TAM receptor family in leukemia to spotlight the aberrant expression patterns within different subsets and illustrate how receptor activation upregulates particular proliferative and antiapoptotic signaling pathways known to contribute to leukemogenesis. Although the mechanisms underlying abnormal TAM receptor expression are still unclear, the existing data—insight gained from studies conducted on various cell types—underscore how multiple, and perhaps context-dependent, processes facilitate overexpression of TAM receptors in leukemic cells.

The preclinical data presented here strongly support the development of specific inhibitors of TAM receptors: Mer and/or Axl inhibition can enhance leukemia cell sensitivity to chemotherapeutic agents currently used in the clinic, thus requiring lower doses to achieve equal or better efficiency and decreasing the severity of side effects observed with current therapies. Given that patients with inactivating Mer mutations and TAM receptor-knockout mice experience late-onset health defects,[97–101] transient TAM inhibition during chemotherapy will not likely bear the same long-term consequences. Furthermore, current immunophenotyping assays can easily be adapted to characterize TAM expression in leukemia patient samples,[25,28] highlighting the diagnostic value of TAM family receptors. For all of these reasons, we believe that incorporating TAM receptor-targeted therapies into treatment regimens for leukemia, as well as for other cancers, is an exciting strategy in the ongoing fight against this devastating disease.

ACKNOWLEDGMENTS

This article was supported in part by grants from the National Institutes of Health (RO1CA137078, DKG); the National Heart, Lung, and Blood Institute (F32HL096416, LB); and from the American Cancer Society (RSG-08-291-01-LIB, DKG).

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009 Jul–Aug;59(4):225–49.
2. Kaatsch P. Epidemiology of childhood cancer. Cancer Treat Rev. 2010 Jun;36(4):277–85.
3. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK. SEER Cancer Statistics Review, 1975–2008. 2011.
4. Landier W, Bhatia S. Cancer survivorship: a pediatric perspective. Oncologist. 2008 Nov;13(11):1181–92.

5. Oeffinger KC, Mertens AC, Sklar CA, Kawashima T, Hudson MM, Meadows AT, Friedman DL, Marina N, Hobbie W, Kadan-Lottick NS, Schwartz CL, Leisenring W, Robison LL. Chronic health conditions in adult survivors of childhood cancer. N Engl J Med. 2006 Oct 12;355(15):1572–82.
6. Nguyen K, Devidas M, Cheng SC, La M, Raetz EA, Carroll WL, Winick NJ, Hunger SP, Gaynon PS, Loh ML. Factors influencing survival after relapse from acute lymphoblastic leukemia: a Children's Oncology Group study. Leukemia. 2008 Dec;22(12):2142–50.
7. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nat Rev Immunol. 2008 May;8(5):327–36.
8. Angelillo-Scherrer A, Burnier L, Flores N, Savi P, DeMol M, Schaeffer P, Herbert JM, Lemke G, Goff SP, Matsushima GK, Earp HS, Vesin C, Hoylaerts MF, Plaisance S, Collen D, Conway EM, Wehrle-Haller B, Carmeliet P. Role of Gas6 receptors in platelet signaling during thrombus stabilization and implications for antithrombotic therapy. J Clin Invest. 2005 Feb;115(2):237–46.
9. Gould WR, Baxi SM, Schroeder R, Peng YW, Leadley RJ, Peterson JT, Perrin LA. Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic responses. J Thromb Haemost. 2005 Apr;3(4):733–41.
10. Angelillo-Scherrer A, Burnier L, Lambrechts D, Fish RJ, Tjwa M, Plaisance S, Sugamele R, DeMol M, Martinez-Soria E, Maxwell PH, Lemke G, Goff SP, Matsushima GK, Earp HS, Chanson M, Collen D, Izui S, Schapira M, Conway EM, Carmeliet P. Role of Gas6 in erythropoiesis and anemia in mice. J Clin Invest. 2008 Feb;118(2):583–96.
11. Linger RM, Keating AK, Earp HS, Graham DK. TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res. 2008;100: 35–83.
12. Caraux A, Lu Q, Fernandez N, Riou S, Di Santo JP, Raulet DH, Lemke G, Roth C. Natural killer cell differentiation driven by Tyro3 receptor tyrosine kinases. Nat Immunol. 2006 Jul;7(7):747–54.
13. Behrens EM, Gadue P, Gong SY, Garrett S, Stein PL, Cohen PL. The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. Eur J Immunol. 2003 Aug;33(8):2160–7.
14. Graham DK, Dawson TL, Mullaney DL, Snodgrass HR, Earp HS. Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. Cell Growth Differ. 1994 Jun;5(6):647–57.
15. Crosier PS, Hall LR, Vitas MR, Lewis PM, Crosier KE. Identification of a novel receptor tyrosine kinase expressed in acute myeloid leukemic blasts. Leuk Lymphoma. 1995 Aug;18(5–6):443–9.
16. De Vos J, Couderc G, Tarte K, Jourdan M, Requirand G, Delteil MC, Rossi JF, Mechti N, Klein B. Identifying intercellular signaling genes expressed in malignant plasma cells by using complementary DNA arrays. Blood. 2001 Aug 1;98(3):771–80.
17. Liu E, Hjelle B, Bishop JM. Transforming genes in chronic myelogenous leukemia. Proc Natl Acad Sci U S A. 1988 Mar;85(6):1952–6.
18. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, Espinosa R, 3rd, Le Beau MM, Earp HS, Liu ET. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol. 1991 Oct;11(10):5016–31.
19. Janssen JW, Schulz AS, Steenvoorden AC, Schmidberger M, Strehl S, Ambros PF, Bartram CR. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene. 1991 Nov;6(11):2113–20.
20. Challier C, Uphoff CC, Janssen JW, Drexler HG. Differential expression of the ufo/axl oncogene in human leukemia-lymphoma cell lines. Leukemia. 1996 May;10(5):781–7.
21. Neubauer A, Fiebeler A, Graham DK, O'Bryan JP, Schmidt CA, Barckow P, Serke S, Siegert W, Snodgrass HR, Huhn D. Expression of axl, a transforming receptor tyrosine kinase, in normal and malignant hematopoiesis. Blood. 1994 Sep 15;84(6):1931–41.
22. Neubauer A, Burchert A, Maiwald C, Gruss HJ, Serke S, Huhn D, Wittig B, Liu E. Recent progress on the role of Axl, a receptor tyrosine kinase, in malignant transformation of myeloid leukemias. Leuk Lymphoma. 1997 Mar;25(1–2):91–6.
23. Rochlitz C, Lohri A, Bacchi M, Schmidt M, Nagel S, Fopp M, Fey MF, Herrmann R, Neubauer A. Axl expression is associated with adverse prognosis and with expression of Bcl-2 and CD34 in de novo acute myeloid leukemia (AML): results from a multicenter trial of the Swiss Group for Clinical Cancer Research (SAKK). Leukemia. 1999 Sep;13(9):1352–8.

24. Hong CC, Lay JD, Huang JS, Cheng AL, Tang JL, Lin MT, Lai GM, Chuang SE. Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. Cancer Lett. 2008 Sep 18;268(2):314–24.

25. Graham DK, Salzberg DB, Kurtzberg J, Sather S, Matsushima GK, Keating AK, Liang X, Lovell MA, Williams SA, Dawson TL, Schell MJ, Anwar AA, Snodgrass HR, Earp HS. Ectopic expression of the proto-oncogene Mer in pediatric T-cell acute lymphoblastic leukemia. Clin Cancer Res. 2006 May 1;12(9):2662–9.

26. Linger RM, DeRyckere D, Brandao L, Sawczyn KK, Jacobsen KM, Liang X, Keating AK, Graham DK. Mer receptor tyrosine kinase is a novel therapeutic target in pediatric B-cell acute lymphoblastic leukemia. Blood. 2009 Sep 24;114(13):2678–87.

27. 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 Mar;1(2):133–43.

28. Eisenman K, Sather S, McGranahan A, Liang X, Desch A, Cannon C, Martinson H, Keating AK, Graham DK. Mer as a Potential Novel Target in Acute Myeloid Leukemia. Pediatr Blood Cancer. 2010 June;54(6):790.

29. Jia R, Mayer BJ, Hanafusa T, Hanafusa H. A novel oncogene, v-ryk, encoding a truncated receptor tyrosine kinase is transduced into the RPL30 virus without loss of viral sequences. J Virol. 1992 Oct;66(10):5975–87.

30. Keating AK, Salzberg DB, Sather S, Liang X, Nickoloff S, Anwar A, Deryckere D, Hill K, Joung D, Sawczyn KK, Park J, Curran-Everett D, McGavran L, Meltesen L, Gore L, Johnson GL, Graham DK. Lymphoblastic leukemia/lymphoma in mice overexpressing the Mer (MerTK) receptor tyrosine kinase. Oncogene. 2006 Oct 5;25(45):6092–100.

31. Margareto J, Leis O, Larrarte E, Pomposo IC, Garibi JM, Lafuente JV. DNA copy number variation and gene expression analyses reveal the implication of specific oncogenes and genes in GBM. Cancer Invest. 2009 Jun;27(5):541–8.

32. Kubo T, Kuroda Y, Shimizu H, Kokubu A, Okada N, Hosoda F, Arai Y, Nakamura Y, Taniguchi H, Yanagihara K, Imoto I, Inazawa J, Hirohashi S, Shibata T. Resequencing and copy number analysis of the human tyrosine kinase gene family in poorly differentiated gastric cancer. Carcinogenesis. 2009 Nov;30(11):1857–64.

33. Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, Halsey W, Sathe GM, Martin AM, Gilmer TM. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. 2009 Sep 1;69(17):6871–8.

34. Abba MC, Fabris VT, Hu Y, Kittrell FS, Cai WW, Donehower LA, Sahin A, Medina D, Aldaz CM. Identification of novel amplification gene targets in mouse and human breast cancer at a syntenic cluster mapping to mouse ch8A1 and human ch13q34. Cancer Res. 2007 May 1;67(9):4104–12.

35. Liao Y, Yu D, Hung MC. Novel approaches for chemosensitization of breast cancer cells: the E1A story. Adv Exp Med Biol. 2007;608: 144–69.

36. Mudduluru G, Allgayer H. The human receptor tyrosine kinase Axl gene—promoter characterization and regulation of constitutive expression by Sp1, Sp3 and CpG methylation. Biosci Rep. 2008 Jun;28(3):161–76.

37. Orso F, Jager R, Calogero RA, Schorle H, Sismondi P, De Bortoli M, Taverna D. AP-2alpha regulates migration of GN-11 neurons via a specific genetic programme involving the Axl receptor tyrosine kinase. BMC Biol. 2009;7:25.

38. Avilla E, Guarino V, Visciano C, Liotti F, Svelto M, Krishnamoorthy G, Franco R, Melillo RM. Activation of TYRO3/AXL tyrosine kinase receptors in thyroid cancer. Cancer Res. 2011 Mar 1;71(5):1792–804.

39. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem. 2002 Feb 15;277(7):5209–18.

40. Wong CC, Lee WM. The proximal cis-acting elements Sp1, Sp3 and E2F regulate mouse mer gene transcription in Sertoli cells. Eur J Biochem. 2002 Aug;269(15):3789–800.

41. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, Deniz J, Ramirez C, Diaz

M, Gallardo G, de Galarreta CR, Salazar J, Lopez F, Edwards P, Parks J, Andujar M, Tontonoz P, Castrillo A. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. Immunity. 2009 Aug 21;31(2):245–58.

42. Geyeregger R, Shehata M, Zeyda M, Kiefer FW, Stuhlmeier KM, Porpaczy E, Zlabinger GJ, Jager U, Stulnig TM. Liver X receptors interfere with cytokine-induced proliferation and cell survival in normal and leukemic lymphocytes. J Leukoc Biol. 2009 Nov;86(5):1039–48.
43. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell. 2008 Jul 11;134(1): 97–111.
44. So AY, Cooper SB, Feldman BJ, Manuchehri M, Yamamoto KR. Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at glucocorticoid-induced genes. Proc Natl Acad Sci U S A. 2008 Apr 15;105(15):5745–9.
45. McColl A, Bournazos S, Franz S, Perretti M, Morgan BP, Haslett C, Dransfield I. Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages. J Immunol. 2009 Aug 1;183(3):2167–75.
46. Chiaretti S, Li X, Gentleman R, Vitale A, Wang KS, Mandelli F, Foa R, Ritz J. Gene expression profiles of B-lineage adult acute lymphocytic leukemia reveal genetic patterns that identify lineage derivation and distinct mechanisms of transformation. Clin Cancer Res. 2005 Oct 15;11(20):7209–19.
47. Fujita PA, Rhead B, Zweig AS, Hinrichs AS, Karolchik D, Cline MS, Goldman M, Barber GP, Clawson H, Coelho A, Diekhans M, Dreszer TR, Giardine BM, Harte RA, Hillman-Jackson J, Hsu F, Kirkup V, Kuhn RM, Learned K, Li CH, Meyer LR, Pohl A, Raney BJ, Rosenbloom KR, Smith KE, Haussler D, Kent WJ. The UCSC Genome Browser database: update 2011. Nucleic Acids Res. 2011 Jan;39:D876–82.
48. Smith KS, Jacobs Y, Chang CP, Cleary ML. Chimeric oncoprotein E2a-Pbx1 induces apoptosis of hematopoietic cells by a p53-independent mechanism that is suppressed by Bcl-2. Oncogene. 1997 Jun 19;14(24):2917–26.
49. Liu R, Gong M, Li X, Zhou Y, Gao W, Tulpule A, Chaudhary PM, Jung J, Gill PS. Induction, regulation, and biologic function of Axl receptor tyrosine kinase in Kaposi sarcoma. Blood. 2010 Jul 15;116(2):297–305.
50. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Hideshima T, Akiyama M, Chauhan D, Munshi N, Gu X, Bailey C, Joseph M, Libermann TA, Richon VM, Marks PA, Anderson KC. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A. 2004 Jan 13;101(2):540–5.
51. Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J. Endogenous human microRNAs that suppress breast cancer metastasis. Nature. 2008 Jan 10;451(7175):147–52.
52. Kurowska-Stolarska M, Ballantine L, Stolarski B, Hunter J, Hueber A, Gracie JA, Liew FY, McInnes IB. miR-155 and miR-34a regulate proinflammatory cytokine production by human monocytes. Annals of the Rheumatic Diseases. 2010 March 1, 2010;69(Suppl 2):A30.
53. Bryant A, Lutherborrow M, Ma D. The clinicopathological relevance of microRNA in normal and malignant haematopoiesis. Pathology. 2009;41(3):204–13.
54. Mudduluru G, Ceppi P, Kumarswamy R, Scagliotti GV, Papotti M, Allgayer H. Regulation of Axl receptor tyrosine kinase expression by miR-34a and miR-199a/b in solid cancer. Oncogene. 2011 Jun 23;30(25):2888–99.
55. Evdokimova V, Ruzanov P, Anglesio MS, Sorokin AV, Ovchinnikov LP, Buckley J, Triche TJ, Sonenberg N, Sorensen PH. Akt-mediated YB-1 phosphorylation activates translation of silent mRNA species. Mol Cell Biol. 2006 Jan;26(1): 277–92.
56. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, Masiakowski P, Ryan TE, Tobkes NJ, Chen DH, DiStefano PS, Long GL, Basilico C, Goldfarb MP, Lemke G, Glass DJ, Yancopoulos GD. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell. 1995 Feb 24;80(4):661–70.

57. Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, Hunt RW, Trail G, Clogston C, Toso RJ, Yanagihara D, Bennett L, Sylber M, Merewether LA, Tseng A, Escobar E, Edison TL, Yamane HK. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature. 1995 Feb 16;373(6515):623–6.

58. Balogh I, Hafizi S, Stenhoff J, Hansson K, Dahlback B. Analysis of Gas6 in human platelets and plasma. Arterioscler Thromb Vasc Biol. 2005 Jun;25(6):1280–6.

59. Manfioletti G, Brancolini C, Avanzi G, Schneider C. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol. 1993 Aug;13(8):4976–85.

60. Dormady SP, Zhang XM, Basch RS. Hematopoietic progenitor cells grow on 3T3 fibroblast monolayers that overexpress growth arrest-specific gene-6 (GAS6). Proc Natl Acad Sci U S A. 2000 Oct 24;97(22):12260–5.

61. Shiozawa Y, Pedersen EA, Taichman RS. GAS6/Mer axis regulates the homing and survival of the E2A/PBX1-positive B-cell precursor acute lymphoblastic leukemia in the bone marrow niche. Exp Hematol. 2010 Feb;38(2):132–40.

62. Linger RM, Keating AK, Earp HS, Graham DK. Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. Expert Opin Ther Targets. 2010 Oct;14(10):1073–90.

63. Caberoy NB, Zhou Y, Li W. Tubby and tubby-like protein 1 are new MerTK ligands for phagocytosis. EMBO J. 2010 Dec 1;29(23):3898–910.

64. Fridell YW, Jin Y, Quilliam LA, Burchert A, McCloskey P, Spizz G, Varnum B, Der C, Liu ET. Differential activation of the Ras/extracellular-signal-regulated protein kinase pathway is responsible for the biological consequences induced by the Axl receptor tyrosine kinase. Mol Cell Biol. 1996 Jan;16(1):135–45.

65. Georgescu MM, Kirsch KH, Shishido T, Zong C, Hanafusa H. Biological effects of c-Mer receptor tyrosine kinase in hematopoietic cells depend on the Grb2 binding site in the receptor and activation of NF-kappaB. Mol Cell Biol. 1999 Feb;19(2):1171–81.

66. Ling L, Kung HJ. Mitogenic signals and transforming potential of Nyk, a newly identified neural cell adhesion molecule-related receptor tyrosine kinase. Mol Cell Biol. 1995 Dec;15(12):6582–92.

67. Chen J, Carey K, Godowski PJ. Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. Oncogene. 1997 May 1;14(17):2033–9.

68. Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, Bartram CR, Janssen JW. Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. Oncogene. 1997 Jun 5;14(22):2619–31.

69. Goruppi S, Ruaro E, Varnum B, Schneider C. Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts. Mol Cell Biol. 1997 Aug;17(8):4442–53.

70. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. J Cell Physiol. 2005 Jul;204(1):36–44.

71. Guttridge KL, Luft JC, Dawson TL, Kozlowska E, Mahajan NP, Varnum B, Earp HS. Mer receptor tyrosine kinase signaling: prevention of apoptosis and alteration of cytoskeletal architecture without stimulation or proliferation. J Biol Chem. 2002 Jul 5;277(27):24057–66.

72. Lopez-Guerra M, Colomer D. NF-kappaB as a therapeutic target in chronic lymphocytic leukemia. Expert Opin Ther Targets. 2010 Mar;14(3):275–88.

73. Demarchi F, Verardo R, Varnum B, Brancolini C, Schneider C. Gas6 anti-apoptotic signaling requires NF-kappa B activation. J Biol Chem. 2001 Aug 24;276(34):31738–44.

74. Tibrewal N, Wu Y, D'Mello V, Akakura R, George TC, Varnum B, Birge RB. Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of lipopolysaccharide-inducible NF-kappaB transcriptional activation. J Biol Chem. 2008 Feb 8;283(6):3618–27.

75. Benekli M, Baer MR, Baumann H, Wetzler M. Signal transducer and activator of transcrip-

tion proteins in leukemias. Blood. 2003 Apr 15;101(8):2940–54.

76. Qazi S, Uckun FM. Gene expression profiles of infant acute lymphoblastic leukaemia and its prognostically distinct subsets. Br J Haematol. 2010 Jun;149(6):865–73.
77. Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, Berthou C, Lessard M, Berger R, Ghysdael J, Bernard OA. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science. 1997 Nov 14;278(5341):1309–12.
78. Zong C, Yan R, August A, Darnell JE Jr, Hanafusa H. Unique signal transduction of Eyk: constitutive stimulation of the JAK-STAT pathway by an oncogenic receptor-type tyrosine kinase. EMBO J. 1996 Sep 2;15(17):4515–25.
79. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell. 2007 Dec 14;131(6):1124–36.
80. O'Hare T, Deininger MW. Toward a cure for chronic myeloid leukemia. Clin Cancer Res. 2008 Dec 15;14(24):7971–4.
81. O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R, Druker BJ. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003 Mar 13;348(11):994–1004.
82. Small D. Targeting FLT3 for the treatment of leukemia. Semin Hematol. 2008 Jul;45(3 Suppl 2):S17–21.
83. Griffin TC, Weitzman S, Weinstein H, Chang M, Cairo M, Hutchison R, Shiramizu B, Wiley J, Woods D, Barnich M, Gross TG. A study of rituximab and ifosfamide, carboplatin, and etoposide chemotherapy in children with recurrent/refractory B-cell (CD20+) non-Hodgkin lymphoma and mature B-cell acute lymphoblastic leukemia: a report from the Children's Oncology Group. Pediatr Blood Cancer. 2009 Feb;52(2):177–81.
84. Li Y, Ye X, Tan C, Hongo JA, Zha J, Liu J, Kallop D, Ludlam MJ, Pei L. Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. Oncogene. 2009 Oct 1;28(39):3442–55.
85. Keating AK, Kim GK, Jones AE, Donson AM, Ware K, Mulcahy JM, Salzberg DB, Foreman NK, Liang X, Thorburn A, Graham DK. Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. Mol Cancer Ther. 2010 May;9(5):1298–307.
86. Sather S, Kenyon KD, Lefkowitz JB, Liang X, Varnum BC, Henson PM, Graham DK. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood. 2007 Feb 1;109(3):1026–33.
87. Ye X, Li Y, Stawicki S, Couto S, Eastham-Anderson J, Kallop D, Weimer R, Wu Y, Pei L. An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. Oncogene. 2010 Sep 23;29(38):5254–64.
88. Zhang YX, Knyazev PG, Cheburkin YV, Sharma K, Knyazev YP, Orfi L, Szabadkai I, Daub H, Keri G, Ullrich A. AXL is a potential target for therapeutic intervention in breast cancer progression. Cancer Res. 2008 Mar 15;68(6):1905–15.
89. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002 Dec 6;298(5600):1912–34.
90. Mahadevan D, Cooke L, Riley C, Swart R, Simons B, Della Croce K, Wisner L, Iorio M, Shakalya K, Garewal H, Nagle R, Bearss D. A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal stromal tumors. Oncogene. 2007 Jun 7;26(27):3909–19.
91. Qi W, Cooke LS, Stejskal A, Riley C, Croce KD, Saldanha JW, Bearss D, Mahadevan D. MP470, a novel receptor tyrosine kinase inhibitor, in combination with Erlotinib inhibits the HER family/PI3K/Akt pathway and tumor growth in prostate cancer. BMC Cancer. 2009;9:142.
92. Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S, Koudriakova TB, Alton G, Cui JJ, Kung PP, Nambu MD, Los G, Bender SL, Mroczkowski B, Christensen JG. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through

antiproliferative and antiangiogenic mechanisms. Cancer Res. 2007 May 1;67(9):4408–17.

93. Schroeder GM, An Y, Cai ZW, Chen XT, Clark C, Cornelius LA, Dai J, Gullo-Brown J, Gupta A, Henley B, Hunt JT, Jeyaseelan R, Kamath A, Kim K, Lippy J, Lombardo LJ, Manne V, Oppenheimer S, Sack JS, Schmidt RJ, Shen G, Stefanski K, Tokarski JS, Trainor GL, Wautlet BS, Wei D, Williams DK, Zhang Y, Fargnoli J, Borzilleri RM. Discovery of N-(4-(2-amino-3-chloropyridin-4-yloxy)-3-fluorophenyl)-4-ethoxy-1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (BMS-777607), a selective and orally efficacious inhibitor of the Met kinase superfamily. J Med Chem. 2009 Mar 12;52(5):1251–4.

94. Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, Duan M, Torneros A, Yu J, Heckrodt TJ, Zhang J, Ding P, Apatira A, Chua J, Brandt R, Pine P, Goff D, Singh R, Payan DG, Hitoshi Y. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res. 2010 Feb 15;70(4):1544–54.

95. Ghosh AK, Secreto C, Boysen J, Sassoon T, Shanafelt TD, Mukhopadhyay D, Kay NE. The novel receptor tyrosine kinase Axl is constitutively active in B-cell chronic lymphocytic leukemia and acts as a docking site of nonreceptor kinases: implications for therapy. Blood. 2011 Feb 10;117(6):1928–37.

96. Huang X, Finerty P, Jr., Walker JR, Butler-Cole C, Vedadi M, Schapira M, Parker SA, Turk BE, Thompson DA, Dhe-Paganon S. Structural insights into the inhibited states of the Mer receptor tyrosine kinase. J Struct Biol. 2009 Feb;165(2):88–96.

97. McHenry CL, Liu Y, Feng W, Nair AR, Feathers KL, Ding X, Gal A, Vollrath D, Sieving PA, Thompson DA. MERTK arginine-844-cysteine in a patient with severe rod-cone dystrophy: loss of mutant protein function in transfected cells. Invest Ophthalmol Vis Sci. 2004 May;45(5):1456–63.

98. Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, Apfelstedt-Sylla E, Vollrath D. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat Genet. 2000 Nov;26(3):270–1.

99. Tschernutter M, Jenkins SA, Waseem NH, Saihan Z, Holder GE, Bird AC, Bhattacharya SS, Ali RR, Webster AR. Clinical characterisation of a family with retinal dystrophy caused by mutation in the Mertk gene. Br J Ophthalmol. 2006 Jun;90(6):718–23.

100. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, Lai C, Skinner MK, Klein R, Matsushima GK, Earp HS, Goff SP, Lemke G. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature. 1999 Apr 22;398(6729):723–8.

101. Camenisch TD, Koller BH, Earp HS, Matsushima GK. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. J Immunol. 1999 Mar 15;162(6):3498–503.

102. Dirks W, Rome D, Ringel F, Jager K, MacLeod RA, Drexler HG. Expression of the growth arrest-specific gene 6 (GAS6) in leukemia and lymphoma cell lines. Leuk Res. 1999 Jul;23(7):643–51.

103. Graham DK, Bowman GW, Dawson TL, Stanford WL, Earp HS, Snodgrass HR. Cloning and developmental expression analysis of the murine c-mer tyrosine kinase. Oncogene. 1995 Jun 15;10(12):2349–59.

104. Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, Arnout J, Dewerchin M, Hoylaerts M, Herbert J, Collen D, Dahlback B, Carmeliet P. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med. 2001 Feb;7(2):215–21.

105. Cosemans JM, Van Kruchten R, Olieslagers S, Schurgers LJ, Verheyen FK, Munnix IC, Waltenberger J, Angelillo-Scherrer A, Hoylaerts MF, Carmeliet P, Heemskerk JW. Potentiating role of Gas6 and Tyro3, Axl and Mer (TAM) receptors in human and murine platelet activation and thrombus stabilization. J Thromb Haemost. 2010 Aug;8(8):1797–808.

106. Avanzi GC, Gallicchio M, Cavalloni G, Gammaitoni L, Leone F, Rosina A, Boldorini R, Monga G, Pegoraro L, Varnum B, Aglietta M. GAS6, the ligand of Axl and Rse receptors, is expressed in hematopoietic tissue but lacks mitogenic activity. Exp Hematol. 1997 Nov;25(12):1219–26.

107. Crosier PS, Freeman SA, Orlic D, Bodine DM, Crosier KE. The Dtk receptor tyrosine kinase, which binds protein S, is expressed during hematopoiesis. Exp Hematol. 1996 Feb;24(2):318–23.

108. Tang H, Chen S, Wang H, Wu H, Lu Q, Han D. TAM receptors and the regulation of erythropoiesis in mice. Haematologica. 2009 Mar;94(3):326–34.

109. Zheng Y, Zhang L, Lu Q, Wang X, Yu F. NGF-induced Tyro3 and Axl function as survival factors for differentiating PC12 cells. Biochem Biophys Res Commun. 2009 Jan 16;378(3):371–5.

110. Mudduluru G, Vajkoczy P, Allgayer H. Myeloid zinc finger 1 induces migration, invasion, and in vivo metastasis through Axl gene expression in solid cancer. Mol Cancer Res. 2010 Feb;8(2):159–69.

111. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood. 2005 Jan 15;105(2):659–69.

112. Gross C, Dubois-Pot H, Wasylyk B. The ternary complex factor Net/Elk-3 participates in the transcriptional response to hypoxia and regulates HIF-1 alpha. Oncogene. 2008 Feb 21;27(9):1333–41.

113. Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J. 2002 Sep 2;21(17):4593–9.

114. Li Y, Mahajan NP, Webster-Cyriaque J, Bhende P, Hong GK, Earp HS, Kenney S. The C-mer gene is induced by Epstein-Barr virus immediate-early protein BRLF1. J Virol. 2004 Nov;78(21): 11778–85.

115. Khan J, Bittner ML, Saal LH, Teichmann U, Azorsa DO, Gooden GC, Pavan WJ, Trent JM, Meltzer PS. cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proc Natl Acad Sci U S A. 1999 Nov 9;96(23):13264–9.

116. Mo R, Tony Zhu Y, Zhang Z, Rao SM, Zhu YJ. GAS6 is an estrogen-inducible gene in mammary epithelial cells. Biochem Biophys Res Commun. 2007 Feb 2;353(1):189–94.

Critical Reviews™ in Oncogenesis, 16(1–2), 65–76 (2011)

The Role of Hox Proteins in Leukemogenesis: Insights Into Key Regulatory Events in Hematopoiesis

E.A. Eklund

The Feinberg School at Northwestern University and Jesse Brown VA Medical Center, 303 E. Superior St., Lurie 5-105, Chicago, IL 60611; e-eklund@northwestern.edu

ABSTRACT: Acute myeloid leukemia (AML) is a heterogeneous disease with highly variable prognoses. Identification of recurring chromosomal translocations provides some prognostic information for individual AML subjects. Population based gene-expression profiling studies also identified abnormalities relevant to prognosis. Such studies associate increased expression of a set of homeodomain transcription factors with poor prognosis in AML. This set includes HoxB3, B4, A7-11 and Meis1, which are dysregulated as a group in the bone marrow in poor prognosis AML. Aberrant expression of these homeodomain transcription factors is found in AML with chromosomal translocations involving the *MLL*, *MYST3* and *CREBBP* genes, and in a poor prognosis subset with normal cytogenetics. Studies in murine models suggest that Hox protein overexpression is functionally significant for myeloid malignancies. Overexpression of individual Hox proteins expanded various bone marrow populations *in vitro*, leading to myeloproliferation and in some cases differentiation block and AML *in vivo*. Therefore, dysregulated expression of key Hox target genes may contribute to adverse prognosis in AML. Identification of these genes will provide insights into the pathobiology of prognosis in AML. Studies are beginning to identify Hox target genes which may be rational targets for therapeutic approaches to this poor prognosis leukemia subset.

KEY WORDS: leukemogenesis, gene regulation, transcription factor, Hox, myelopoiesis.

ABBREVIATIONS

AML, acute myeloid leukemia; CBP, CREB-binding protein; DNMT, DNA-methyl transferase; GMP, granulocyte/monocyte progenitors; HD, homeodomain; Jak2, Janus kinase 2; MLL, mixed lineage leukemia; MPN, myeloproliferative neoplasm; PCG, polycomb group proteins

I. HOX PROTEINS ARE HOMEODOMAIN TRANSCRIPTION FACTORS

A. Characteristics of Hox Proteins

Gene expression profiling studies in human AML correlated increased expression of a subset of Hox proteins with poor prognosis.[1–5] These studies led to increased interest in understanding this family of homeodomain transcription factors and their roles in normal and leukemic myelopoiesis.

1. Highly Conserved Homeodomain Transcription Factors

HOX genes encode homeodomain transcription factors which are highly conserved from *Drosophila* to man. *HOX* genes were initially identified due to homology to *Drosophila HOMC* genes, and naming of mammalian *HOX* genes follows this homology (Figure 1) (reviewed by Eklund[6,7]). Human and murine *HOX* genes are arranged in four groups (referred to as paralog groups A–D) which are found on four different chromosomes. Numbering of individual *HOX* genes follows homology between groups, with greatest similarity between Hox proteins of different groups with the same number. During embryogenesis, Hox1–4 are most highly expressed in the head, Hox5–7 in the thorax, and Hox8–11 in the abdomen and pelvis.[6,7] This tight spacial regulation is hypothesized to result in regulation of organ specific genes by groups of Hox proteins, although few such genes have been identified.

Similarly, *HOX* gene transcription during definitive hematopoiesis is tightly regulated, but in a temporal manner. Maximal expression of Hox1-4 occurs in hematopoietic stem cells ($CD34^+CD38^-$

0893-9675/11/$35.00

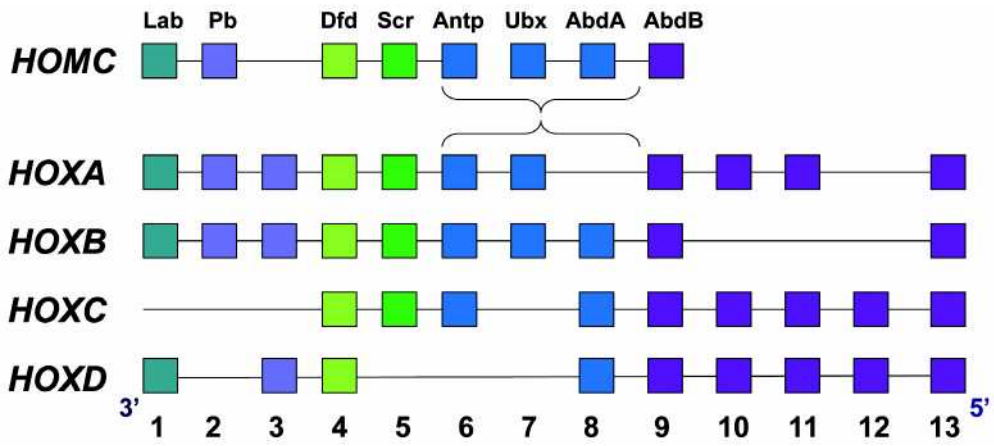


FIGURE 1. Homology of mammalian *HOX* genes to *Drosophila HOMC* genes. Mammalian *HOX* genes are found on four different chromosomes which are arranged similarly to homologous *Drosophila HOMC* genes.

in humans), with down regulation of these genes during $CD38^+$ differentiation. Hox7-11 expression is maximal in lineage committee progenitors ($CD34^{+/-}CD38^+$) with down regulation as differentiation proceeds.[2,8] In AML, increased expression of HoxB3, B4, A7-11 is found in the most primitive progenitors with expression of A7-11 aberrantly sustained in differentiating progenitors.[1,2] These observations suggest that identification of Hox target genes would provide useful insights into stem cell biology, myelopoiesis, and myeloid leukemogenesis. Recent studies have identified Hox target genes which begin to explain the crucial role these proteins play in hematopoiesis.

2. Mechanisms of Gene Regulation by Hox Proteins

a. Conserved Domains in Hox Proteins

Hox proteins with the same number but from different groups are well conserved in comparison to adjacent Hox proteins in the same group (Figure 2). Hox proteins bind to DNA through a homeodomain (HD) which is found in the C-terminus of the protein. The HD is highly conserved between Hox proteins, and Hox-HD are highly conserved between species. The HD includes conserved tyrosine residues which may regulate Hox activity.[9–11] For example, HD-tyrosine phosphorylation of HoxA10 decreases the binding affinity for cis elements in phagocyte effector genes, but increases the binding affinity of HoxA9 to the same genes.[9,10] Tyrosine phosphorylation of HoxA9 and HoxA10 occurs in a Jak2 dependent manner in myeloid progenitor cells in response to differentiating cytokines.[12] HD-tyrosine phosphorylation alters both protein-DNA and protein–protein interactions which may change the profile of interacting target genes.[13]

Hox proteins also include a conserved hexapeptide domain which is N-terminal to the HD (Figure 2). This domain interacts with proteins of the Pbx family of transcription factors. Pbx proteins are frequent DNA-binding partners for Hox proteins, and they participate in binding site selection, as is discussed below.[14] Meis proteins are also frequent Hox partners, but the domains involved in Hox/Meis interactions have not been well defined.

Hox proteins can either activate or repress transcription, depending on the sequence of the binding site, the partner proteins, and the cellular context. An activation domain was identified for HoxA10 which is conserved with other Hox10 proteins. This domain facilitates interaction with the Creb-binding protein (CBP) and is homologous to PQ domains in E1a interacting proteins.[15] This domain does not exist in HoxA9, suggesting that another mechanism is used for transcriptional activation by this protein.

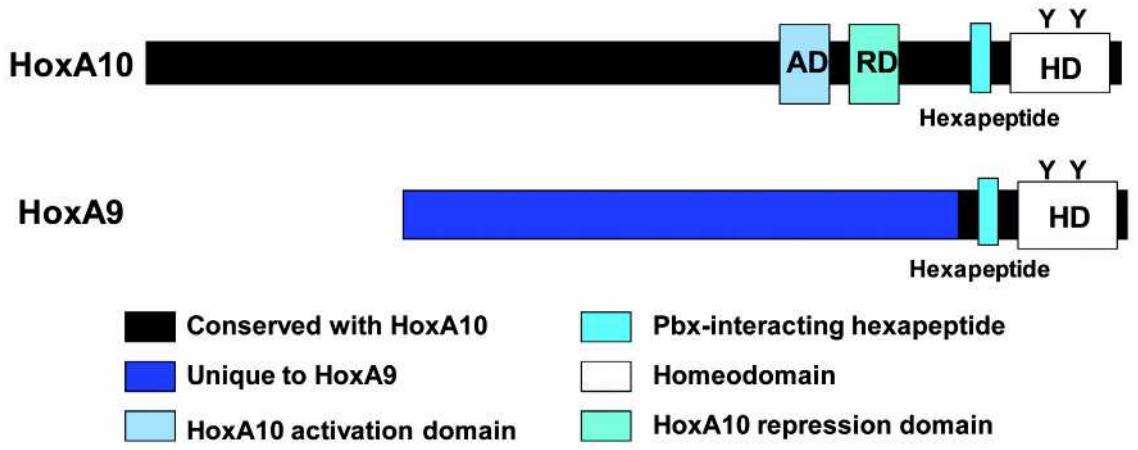


FIGURE 2. Hox proteins share conserved domains. HoxA9 and HoxA10 share conserved domains, including the DNA-binding homeodomain and the hexapeptide domain which mediates interaction with Pbx proteins. The remainder of the HoxA9 and HoxA10 proteins are divergent.

A HoxA10 repression domain was described between the activation domain and the hexapeptide (Figure 2). This domain facilitates interaction between a HoxA10/Pbx1 heterodimer and histone deacetylase 2.[16]

b. Binding Site Consensus Sequences

Even before identification of the first genuine Hox target gene, studies were performed to identify the Hox-DNA binding site consensus sequences. These studies used iterative binding site selection techniques to identify DNA sequences preferentially selected by various Hox proteins. A relatively loose consensus (5′-TNATNN-3′) was identified by these studies.[17] These studies also determined a binding site preference across each Hox locus from 5′-TTAT-3′ on the Hox1 end of each locus to 5′-TGAT-3′ on the Hox13 end of the locus.[17] Given the extensive homology between Hox-HDs, this suggests that other, less conserved domains are also involved in binding site selection. The short length and relative degeneracy of the Hox-DNA binding consensus make genome-wide sequence analysis of limited use for identifying candidate Hox target genes. Other approaches, including gene expression studies and chromatin immuno-precipitation based assays have been employed for this purpose, as discussed below.

c. DNA Binding Partners

Hox proteins often bind DNA as heterodimers with Pbx proteins.[14] DNA-binding consensus sequences for Hox-Pbx heterodimers were also identified by binding-site selection studies.[17] In these studies, 5′-ATGATTNATNN-3′ was identified as the composite consensus sequence, with Pbx proteins recognizing the 5′-ATGAT-3′ end of the sequence.[17] Identified target genes for Hox-Pbx dimers include the *ITGB3* and *DUSP4* (activated by HoxA10+Pbx2), and *CYBB* and *NCF2* (repressed by HoxA10+Pbx1).[15,16,18,19] Pbx proteins have not been found to directly influence transcriptional repression or activation of any target gene, but they are hypothesized to assist the Hox protein in selecting DNA-binding sites and/or increasing affinity of Hox-DNA binding.

AbdB-Hox proteins (i.e., A9-13) may also bind DNA as a heterodimer with Meis proteins, or as a trimer with both Pbx and Meis. Binding-site selection studies determined that the consensus for the Meis half of the DNA binding site is 5′-TGACAG-3′; quite different than the Pbx recognition sequence.[20] However, activation of the *CYBB* gene by HoxA9 involves interaction with Meis1 at a binding site that overlaps the HoxA10-Pbx1 repressor element on this gene.[11]

B. Mechanisms of *HOX* Gene Regulation

Differentiation stage–specific transcription of various *HOX* genes is an important mechanism that regulates Hox protein activity. Although expression proceeds 5′ to 3′ through each *HOX* locus during hematopoiesis, mechanisms that regulate this process have not been completely defined. No control regions for *HOX* loci have been identified, but several transcription factors have been identified that regulate the promoter regions of a number of *HOX* genes.

1. Regulation by the Mixed Lineage Leukemia (Mll) Protein

Increased and sustained Hox expression is found in a subset of leukemia with chromosomal translocations involving the *MLL* gene.[1–3] This observation suggested that the *MLL* gene product (Mixed Lineage Leukemia or Mll protein) might be involved in *HOX* regulation. Consistent with this hypothesis, Mll binds to the promoter region of multiple *HOX* genes of the AbdA and AbdB groups.[21] Mll is a complex proteins with AT hooks (involved in DNA binding), DNA methyltransferase domain (DNMT), a PHD domain, and a SET domain (reviewed by Marschalek[22]). Functional studies suggest that Mll binding to *HOX* promoters maintains differentiation stage specific transcription but does not initiate transcription.[21] Leukemia associated Mll-fusion proteins are hypothesized to induce aberrant *HOX* transcription because the Mll domains involved in maintaining transcription are present in the fusion protein, but domains essential for stage-specific down-regulation are not. The SET domain has been implicated in this latter function, as it is reproducibly deleted in leukemia associated Mll-fusion proteins.

Because "anterior" *HOX* genes (Hox1-5) also exhibit aberrant transcription in AML with *MLL* translocations, Mll-fusion proteins must influence events which indirectly dysregulate transcription of these genes. Conditional knockout of the *MLL* gene is an embryonic lethal at day 16.5 in mice. These mice have a reduced number of hematopoietic stem cells (HSC) that are unable to compete in repopulation assays.[23] These results also suggest that Mll influences 5′*HOX* genes.

2. Regulation by Cdx Proteins

Another family of transcription factors that influence *HOX* gene transcription and/or Hox protein expression are Cdx proteins. Cdx are HD transcription factors and increased expression of Cdx1, 2 and 4 have been variously documented in human AML. Gene disruption studies identified roles for Cdx proteins in *HOX* gene regulation and hematopoiesis. Knockout of individual *CDX* genes impairs hematopoiesis during embryogenesis in murine models, and impairs transcription of multiple *HOX* genes, including HoxB2-5 and HoxA7-13.[24,25] Conversely, overexpression of Cdx4 in murine bone marrow induces a myeloproliferative disorder and AML in murine transplantation experiments.[26] Interestingly, overexpression of Cdx4 rescues the hematopoietic defect in murine Mll–/- bone marrow.[27]

Mechanisms for cross regulation by Cdx and Hox proteins have been recently defined. *HOXA10* has been identified as a direct Cdx4 target gene in myeloid progenitor cells.[28] In these studies, Cdx4 overexpression activated the *HOXA10* promoter and increased HoxA10 expression. Additionally, these studies identified *CDX4* as a HoxA10 activation target gene, establishing a positive feedback relationship between Cdx and Hox proteins.[28] This has implications for understanding myeloid leukemogenesis with Hox overexpression.

3. Regulation by Polycomb Proteins

Studies in *Drosophila* determined that *HOMC* gene transcription is regulated by Polycomb Group (PcG) proteins (reviewed by Takihara[29]). Subsequent studies in mammalian models determined that PcG proteins bind to *HOX* promoter regions where they are thought to generally repress transcription. This has been described for YY1 binding to the *HOXB4* promoter and Bmi1 binding to the *HOXA9* promoter.[30,31] PcG knockout in murine models impairs HSC function in various assays, which is hypothesized to be due to dysregulated *HOX* gene transcription.[29] Molecular mechanisms for these effects remain to be clarified.

C. Function of Hox Proteins During Hematopoiesis

Investigation of specific activities for various Hox proteins is complicated by functional redundancy between groups and between adjacent members of the same group. This makes gene-disruption studies difficult to interpret unless multiple members of a group are knocked out, which produces additional complications for expression of other members of the locus. More information has been obtained from overexpression studies, as discussed below.

1. HoxB3 and B4

a. Studies in Knockout Mice

Homologous recombination was used to generate murine models with disruption of *HOXB4* or double knockout of *HOXB3/B4*.[32] These mice have a minor hemato-phenotype and mild abnormalities in hematopoiesis. These abnormalities include impairment of HSC function as indicted by decreased competitive repopulating activity. The results of studies with either *HOXB4* or *HOXB3/B4* knockout are similar, suggesting functional redundancy between these two Hox proteins. The mild nature of the phenotype suggests there is redundancy with other HoxB proteins, or anterior Hox proteins of other groups.

b. Overexpression Studies

More information was obtained with gain of function studies for HoxB3 and HoxB4. These studies determined that overexpression of either HoxB3 or HoxB4 in murine or human bone marrow expanded the most immature cell populations *in vitro*.[33–35] Consistent with these results, mice that were transplanted with HoxB3 or HoxB4 overexpressing bone marrow developed a myeloproliferative neoplasm (MPN) with expansion of the long-term repopulating HSC population in the bone marrow.[33–35] However, the MPN did not progress to AML in these mice.

2. HoxA9 and A10

a. Studies in Knockout Mice

Constitutive knockout of the *HOXA9* or *HOXA10* gene in mice results in abnormal development of the genitourinary system, impaired fertility in heterozygous knockout animals, and a very mild hemato-phenotype. Mice with knockout of either gene are reported to have either mild pancytopenia, or unremarkable blood counts, depending upon the report. Studies of bone marrow function are similarly unremarkable. There is some decrease in competitive repopulating ability in *HOXA9*–/– bone marrow compared to normal, but the difference is not profound.[36] Similar studies have not been performed with *HOXA10*–/– bone marrow, but serial plating assays are only slightly less efficient in comparison to wild-type bone marrow. Double knockout of *HOXA9* and *HOXA10* has not been studied nor has conditional knockout in bone marrow cells only.

b. Overexpression Studies

Investigations of HoxA9 and HoxA10 by *in vitro* and *in vivo* overexpression have been quite informative. *In vitro*, overexpression of either protein in murine or human bone marrow preferentially expands the granulocyte/monocyte progenitor (GMP) population and immortalizes the cells.[37–40] Mice transplanted with HoxA10-overexpressing bone marrow develop a MPN with mature neutrophils that progresses to differentiation block and AML over 6–8 months.[40] Mice transplanted with HoxA9-overexpressing bone marrow also develop a MPN, which only progresses to AML after an extremely long lag time, or if the bone marrow is co-overexpressing Meis1.[36] These results suggest some redundancy and some unique functions for HoxA9 and HoxA10. Additional studies of murine models suggest the hypothesis that HoxA10 induces differentiation block, while HoxA9 is involved in selection of myeloid versus lymphoid lineage commitment.

II. HOX PROTEINS ARE ABERRANTLY EXPRESSED IN AML

A. Association Between Hox Expression and *MLL*-Translocation

The *MLL* gene is involved in leukemia associated translocations with more than 70 different partner genes (the most common of which are listed in Table 1) (reviewed by Marschalek[22]). These leukemias are characterized by expression of a fusion protein, which include the same set of domains from the Mll N terminus and from the C-terminal domains from the fusion partner. Gene expression profiles and clinical characteristics are fairly similar for all leukemias with translocations involving the *MLL* gene, suggesting that Mll domains are the dominant factor driving pathogenesis. Because *MLL* is located on the q23 region of chromosome 11, these are referred to as 11q23 leukemias. In adults, 11q23 leukemia is often associated with prior exposure to topoisomerase II inhibitors and has extremely poor prognosis, even among therapy-related leukemias. In pediatric patients, *MLL* translocations are found predominantly in a poor prognosis subset of infant leukemias with both lymphoid and myeloid blasts (hence the term mixed lineage leukemia).[3] As discussed above, 11q23 leukemias are associated with increased expression of a set of HD transcription factors in CD34+ bone marrow cells and CD34+CD38+ circulating blasts; therefore expression of these genes is both increased and aberrantly sustained in differentiating myeloid cells. This set includes HoxB3, B4, A7-11 and Meis1. A similar gene-expression profile is found in AML with normal cytogenetics, but with tandem duplication of the *MLL* gene.[41]

B. Other Forms of Leukemia with Increased Hox Expression

Studies of the pathogenesis of 11q23 leukemias suggest the hypothesis that aberrant HD-transcription–factor expression is prognosis driving in AML. Additional studies in forms of leukemia without *MLL* translocations confirmed this hypothesis. Increased expression of similar sets of Hox proteins and Meis1 were also described in AML with translocations involving *MYST3* and *CREBBP* genes.[5] Statistical analysis also revealed a poor prognosis subset of AML subjects with normal cytogenetics (and without tandem duplications of *MLL*) with increased Hox and Meis1 expression.[4] These studies suggest that Hox proteins may regulate a network of genes which influence molecular events especially associated with adverse outcomes in AML.

TABLE 1. MLL Fusion Proteins

MLL-ELL	MLL-SEPTIN6
MLL-GAS	MLL-AF4
MLL-CBP	MLL-AF6
MLL-EEN	MLL-AF9
MLL-LTG9	MLL-AF17

C. Murine Models to of Leukemogenesis

The functional contribution of various Hox proteins to myeloid leukemogenesis has been studied in murine models. These murine models have been crucial to develop the causal links between increased Hox expression and the pathogenesis of myeloid malignancy.

1. HoxA10 and Shp2-PTP

As discussed above, overexpression of HoxA10 or HoxA9 + Meis1in murine bone marrow expands the GMP population *in vitro* and results in a myeloproliferative neoplasm in murine transplantation experiments. However, AML does not occur immediately in mice transplanted with HoxA10 or HoxA9 overexpressing bone marrow but develops over the course of months.[38,40] These results suggest that Hox protein overexpression is sufficient to induce myeloproliferation and that it also predisposes to acquisition of additional mutations, which are necessary for differentiation block and AML. Posttranslational modification of HoxA10 is involved in modulation of both transcriptional repression and activation functions.[9–13] *In vitro* studies suggest that posttranslational modification of HoxA10 prevents the overexpressed

protein from participating in gene regulatory activities which might lead to differentiation block. Therefore, mutations that impair cytokine induced tyrosine phosphorylation of HoxA10 might lead to AML in HoxA10 overexpressing bone marrow.

HoxA10 is a substrate for Shp2 protein tyrosine phosphatase.[10] Downregulation of Shp2-PTP activity during myelopoiesis is one factor that contributes to HoxA10 tyrosine phosphorylation in response to differentiating cytokines. Leukemia-associated mutations in the gene encoding Shp2 have been described that result in constitutive activation of the PTP[42]. These mutations are found in 10% of AML and perhaps a higher percentage of 11q23-AML. Consistent with this hypothesis, mice transplanted with bone marrow overexpressing HoxA10 plus a constitutively active form of Shp2 develop leukemia immediately.[43]

2. Mll-Fusion Proteins

Various leukemia-associated Mll-fusion proteins have been overexpressed in murine bone marrow cells and analyzed *in vitro* and *in vivo*. Similar to overexpression studies with individual Hox proteins, Mll-fusion protein expressing murine bone marrow has increased serial plating capacity and is immortalized *in vitro*. Mice transplanted with such bone marrow develop a MPN with predominance of mature neutrophils. This MPN progresses to differentiation block and AML over 6–8 months.[44,45] This *in vivo* process is delayed in mice transplanted with Mll-fusion protein expressing, Cdx4–/– bone marrow.[27] Various *MLL*-fusion partners have been tested in such experiments, and aberrant Hox expression has been identified which follows a very similar pattern to that observed in human 11q23-AML.[46] Results from studies of Mll-fusion protein expression in murine bone marrow with knockout of various Hox proteins has been inconsistent, but the majority indicate development of MPN and AML in mice transplanted with HoxA7–/– or HoxA9–/– bone marrow expressing various Mll-fusion proteins.[47,48]

TABLE 2. Target Genes

HOXB3	HOXB4
*DNMT3B^*	*FLASH*
*OTX2^*	*HEMGN**
	IGFBP1^
	MYC#
	RAP1#

^Increased in 11q23.
#Decreased in 11q23.
*No difference.

III. HOX TARGET GENES AND DOWNSTREAM REGULATORY EVENTS

A. HoxB3 and B4

Despite the documented importance of HoxB3 and HoxB4 in HSC maintenance and function, relatively few relevant target genes have been identified that explain these activities (Table 2). HoxB3 interacts with the promoter region of *OTX2,* which encodes a homeodomain protein involved in regulating development of eye structures, but without a documented role in hematopoiesis.[49] The gene-encoding DNA methyltransferase 3B is activated by HoxB3 and may be involved in epigenetic regulation of HSC relevant genes.[50] A number of HoxB4 target genes have been identified which impact cell proliferation and survival (Figure 2), including the genes encoding myc, IGF binding protein 1, FLASH, and Rap1.[51–54] Interestingly, expression of myc and Rap1 are relatively decreased in 11q23-AML. HoxB4 also activates the *HEMGN* gene encoding a nuclear protein of unknown significance, which is expressed in bone marrow progenitor cells.[55]

B. HoxA9

A number of target genes for HoxA9 have been identified, generally using expression microarray screening for altered gene expression in HoxA9 overexpressing cells (Table 3). HoxA9 activates several genes of potential significance for the pathogenesis of AML, including genes encoding Flt3, Pim1 and

TABLE 3. HOXA9 Target Genes

Cytokines/Receptors	Proliferation	Inflammation
EPHB4#	*miR155*	*CYBB**
*FLT3^*	*PIM1^*	*NCF2**
OPN		SELE*

^Increased in 11q23.
#Decreased in 11q23.
*No difference.

TABLE 4. HOXA10 Target Genes

Cytokines/ Receptors	Proliferation	Inflammation
*ITGB3**	*MAPK6PS1**	*ATPGV1H*
*SELL**	*NUDT6^*	*CYBB**
*TGFB2**	*PLCB1#*	*IL11^*
*TGFBR3**	*PRKAR2A^*	*NCF2**
*TMEM8^*		*TBXAS1**
Ubiquitination	**DNA-Binding Proteins**	**Apoptosis**
*ARIH2^*	*CDX4^*	*DUSP4**
*UBE2S^*	*CUX1**	*BRSK1#*
	*MEIS1^*	PDCD5^
	PBX2^	

^Increased in 11q23.
#Decreased in 11q23.
*No difference.

miR155.[56–60] Flt3 is the receptor for Flt3L and is expressed on HSC and myeloid progenitor cells. Aberrant activation of this receptor due to *FLT3* gene mutation is associated with poor prognosis in AML. Gene expression studies indicate a statistically significant association between the presence of *FLT3* mutation and HoxA9 and HoxA10 overexpression in AML.[61] Pim1 is a kinase, which is frequently activated by the Moloney murine leukemia virus and participates in leukemic transformation of such cells. Expression of miR155 has been described in AML, and a number of putative target mRNAs have been identified that may mediate a functional contribution to leukemogenesis. HoxA9 also activates several genes that confer the mature phagocyte phenotype, including genes encoding the NADPH oxidase component gp91phox, and E selectin.[11,62] This is consistent with the hypothesis that HoxA9 is involved in selection of myeloid lineage commitment.

C. HoxA10

A slightly larger number of HoxA10 target genes have been identified (Table 4). Similar to HoxA9, HoxA10 activates a number of genes involved in cell proliferation and survival.[63] HoxA10 activates transcription of several genes involved in proliferation of HSC and myeloid progenitors, including the genes encoding Tgfb2 and one a cognate receptor, and the gene encoding b3 integrin.[11,64] HoxA10 also activates the *CDX4* gene in myeloid progenitor cells, which suggests an important role for HoxA10 in regulation of the Hox-code.[63] HoxA10 also activates the gene encoding Mkp2 (the *DUSP4* gene) in myeloid progenitors.[65] Mkp2 inactivates Jnk and p36 MapKkinases, resulting in HoxA10-dependent apoptosis resistance of progenitor cells. Hoxa10 also regulates genes that confer the mature phagocyte phenotype (Table 4). However, in contrast to HoxA9, HoxA10 represses transcription of these genes in myeloid progenitor cells. This is consistent with descriptive observations suggesting that HoxA10 induces differentiation block and identifies potentially antagonistic functions for HoxA9 and HoxA10 for regulating this aspect of myelopoiesis.

IV. CONCLUSIONS

Dysregulated Hox expression is found in a subset of poor prognosis AML, including leukemias with *MLL* gene translocations. Studies in murine models and human primary cells suggest that increased and sustained expression of Hox proteins plays a functional role in leukemogenesis through dysregulation of HSC and GMP populations. Studies have identified some Hox target genes that may be relevant to this process. The products of such genes, and cognate pathways, may be rational therapeutic targets for personalized therapeutic approaches to AML with dysregulated Hox expression.

ACKNOWLEDGMENT

This article was supported by funding from the National Institutes of Health, Grant No. Ro1 HL87717 for Dr. Eklund's project, "The Role of HoxA10 in Myelopoiesis."

REFERENCES

1. Drabkin HA, Parsy C, Ferguson K, Guilhot F, Lacotte L, Roy L, Zeng C, Baron A, Hunger SP, Varella-Garcia M, Gemmill R, Brizard F, Brizard A, Roche J. Quantitative HOX expression in chromosomally defined subsets of acute myelogenous leukemia. Leukemia. 2002;16:186–5.
2. Kawagoe H, Humphries RK, Blair A, Sutherland HJ, Hogge DE. Expression of HOX genes, HOX cofactors and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells. Leukemia. 1999;13:687–98.
3. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, Minden MD, Sallan SE, Lander ES, Golub TR, Korsmeyer SJ. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet. 2002;30:41–7.
4. Riche J, Zeng C, Baron A, Gadgil S, Gemmill RM, Tigaud I, Thomas X, Drabkin HA. Hox expression in AML identified a distinct subset of patients with intermediate cytogenetics. Leukemia. 2004;18:1059–63.
5. Camos M, Esteve J, Jares P, Colomer D, Rozman M, Villamor N, Costa D, Carrio A, Nomdedue J, Montserrat E, Campo E. Gene expression profiling of acute myeloid leukemia with translocation t(8;16)(p11;q13) and *MYST3-CREBBP* rearrangement reveals a distinctive signature with a specific pattern of HOX gene expression. Cancer Res. 2006;66:6947–54.
6. Eklund EA. The role of Hox proteins in myeloid leukemia. Curr Opin Hematol. 2006;13:67–73.
7. Eklund EA. The role of *HOX* genes in malignant myeloid disease. Curr Opin Hematol. 2007;14: 85–9.
8. Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc Natl Acad Sci USA. 1994;91:12223–7.
9. Eklund EA, Jalava A, Kakar R. Tyrosine phosphorylation decreases HoxA10 DNA-binding and transcriptional repression during IFNg induced differentiation in myeloid cell lines. J Biol Chem. 2000;275:20117–26.
10. Lindsay S, Huang W, Wang H, Horvath E, Zhu C, Eklund EA. Activation of SHP2 protein-tyrosine phosphatase increases HoxA10-induced repression of the genes encoding $gp91^{PHOX}$ and $p67^{PHOX}$. J Biol Chem. 2007;282:2237–49.
11. Bei L, Lu YF, Eklund EA. HoxA9 activates transcription of the gene encoding $gp91^{PHOX}$ during myeloid differentiation. J Biol Chem. 2005;280:12359–70.
12. Kakar R, Kautz B, Eklund EA. JAK2 is necessary and sufficient for interferon gamma-induced transcription of the gene encoding $gp91^{PHOX}$. J Leukocyte Biol. 2005;77:120–7.
13. Eklund EA, Goldenberg I, Lu Y, Andrejic J, Kakar R. SHP1 protein tyrosine phosphatase regulates HoxA10 DNA-binding and transcriptional repression activity in undifferentiated myeloid cells. J Biol Chem. 2002;39:36878–88.
14. Chang CP, Shen WF, Rozenfeld S, Lawrence HJ, Largman C, Cleary ML. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev. 1995;9:663–74.
15. Bei L, Lu YF, Bellis SL, Zhou W, Horvath E, Eklund EA. Identification of a HoxA10 activation domain necessary for transcription of the gene encoding Beta 3 integrin during myeloid differentiation. J Biol Chem. 2007;282:16846–59.
16. Lu Y, Goldenberg I, Bei L, Andrejic J, Eklund EA. HoxA10 represses gene transcription in un-differentiated myeloid cells by interaction with Histone deacetylase 2. J Biol Chem. 2003;278:47792–802.
17. Chang CP, Brocchieri L, Shen WF, Largman C, Cleary ML. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox Locus. Mol Cell Biol. 1996;16:1734–45.
18. Wang H, Lu YF, Huang W, Papoutsakis ET, Fuhrken P, Eklund EA. HoxA10 activates tran-

scription of the gene encoding Mkp2 in myeloid cells. J Biol Chem. 2007;282:16164–76.

19. Lindsey S, Zhu C, Lu YF, Eklund EA. HoxA10 represses transcription of the gene encoding p67phox in phagocytic cells. J Immunol. 2005;175: 5269–5279.
20. Shen WF, Montgomery JC, Rozenfeld S, Moscow JJ, Lawrence HJ, Buchberg AM, Largman C. AbdB-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. Mol Cell Biol. 1997;17:6448–58.
21. Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, Hess JL. MLL targets SET domain methyltransferase activity to Hox gene promoters. Molecular Cell. 2002;10:1107–17
22. Marschalek R. Mechanisms of leukemogenesis by MLL fusion proteins. Br J Haem. 2010;152: 141–54.
23. McMahon KA, Hiew SY, Hadjur S, Veiga-Fernandes G, Menzel U, Price AJ, Kioussis D, Williams O, Brady HJM. Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell. 2007;1:338–45.
24. Davidson AJ, Ernst P, Wang Y, Dekens MPS, Kingsley PD, Palis J, Korsmeyer SJ, Daley GQ, Zon LI. Cdx4 mutants fail to specify blood progenitors and can be rescued by multiple Hox genes. Nature. 2003;425:300–6.
25. Bansal D, Scholl C, Frohling S, McDowell E, Lee BH, Dohner K. Ernst P, Davidson AJ, Daley GQ, Zon LI, Gilliland DG, Huntly BJP. Cdx4 dysregulates Hox gene expression and generates acute myeloid leukemia alone and in cooperation with Meis1a in a murine model. Proc Natl Acad Sci USA. 2006;103:16924–16929.
26. Wang Y, Yabuuchi A, McKinney-Freeman S, Ducharme DMK, Ray MK, Chawengsaksophak K, Archer TK, Daley GQ. Cdx gene deficiency compromises embryonic hematopoiesis in the mouse Proc Natl Acad Sci USA. 2008;105:7756–61.
27. Koo S, Huntly BJ, Wang Y, Chen J, Brumme K, Ball B, McKinney-Freeman SL, Yabuuchi A, Scholl C, Bansal D, Zon LI, Fröhling S, Daley GQ, Gilliland DG, Mercher T. Cdx4 is dispensable for murine adult hematopoietic stem cells but promotes MLL-AF9-mediated leukemogenesis. Haematologica. 2010;95:1642–50.
28. Bei L, Huang W, Wang H, Shah C, Horvath E, Eklund EA. HoxA10 activates *CDX4* transcription and Cdx4 activates *HOXA10* transcription in myeloid cells. J Biol Chem. 2011;286:19047–64.
29. Takihara Y. Role of Polycomb-group genes in sustaining activities of normal and malignant stem cells. Int J Hematol. 2008;87:25–34.
30. Gilthorpe J, Vandromme M, Brend T, Gutman A, Summerbell1 D, Totty N, Rigby PWJ. Spatially specific expression of Hoxb4 is dependent on the ubiquitous transcription factor NFY. Development. 2002;129:3887–99.
31. Smith LL, Yeung J, Zeisig BB, Popov N, Huijbers I, Barnes J, Wilson AJ, Taskesen E, Delwel R, Gil, J, Van Lohuizen M, So CWE. Functional crosstalk between Bmi1and MLL/Hoxa9 Axis in establishment of normal hematopoietic and leukemic stem cells. Cell Stem Cell. 2011;8:649–62.
32. Bjornsson JM, Larsson N, Brun ACM, Magnusson M, Andersson E, Lundstrom P, Larsson J, Repetowska E, Ehinger M, Humphries RK, Karlsson S. Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4. Mol Cell Biol. 2003;23:3872–83.
33. Sauvageau G, Thorsteinsdottir U, Hough MR, Hugo P, Lawrence HJ, Largman C, Humphries RK. Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. Immunity. 1997;6:13–22.
34. Antonchuka J, Sauvageau, G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Expl Hem. 2001;29:1125–34.
35. Schmittwolf C, Porsch, M, Greiner, A, Avots, A, Muller AM. HOXB4 confers a constant rate of in vitro proliferation to transduced bone marrow cells. Oncogene. 2005;24:561–72.
36. Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK, Largman C. Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood. 1997;89:,1922–30.
37. Thorsteinsdottir, U, Mamo, A, Kroon, E, Jerome, L, Bijl, J, Lawrence HJ, Humphries, K, Sauvageau, G. Overexpression of the myeloid leukemia-associated HoxA9 gene in bone marrow cells induces stem cell expansion. Blood. 2002;99:121–9.

38. 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;17:3714–25.

39. Bjornsson JM, Andersson E, Lundstrom P, Larsson N, Xu X, Repetowska E, Humpthries KR, Karlsson S. Proliferation of primitive myeloid progenitors can be reversibly induced by HOXA10. Blood. 2001;98:3301–8.

40. Thorsteinsdottir U, Sauvageau G. Hough MR, Dragowska W, Lansdorp PM, Lawrence HJ, Largman C, Humphries RK. Overexpression of HoxA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. Mol Cell Biol. 1995;17:495–505.

41. Dorrance AM, Liu S, Yuan W, Becknell B, Arnoczky KJ, Guimond M, Strout MP, Feng L, Nakamura T, Yu L, Rush LJ, Weinstein M, Leone G, Wu L, Ferketich A, Whitman SP, Marcucci G, Caligiuri MA. Mll partial tandem duplication induces aberrant Hox expression *in vivo* via specific epigenetic alterations. J Clin Invest. 2006;116:2707–16.

42. Tartaglia M, Niemeyer CM, Fragale A, Song X, Beuchner J, Jung A, Hahlen K, Hasle H, Licht JD, Gleb BD. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nature Genet. 2003;34:148–50.

43. Wang H, Lindsey S, Konieczna I, Bei L, Horvath E, Huang W, Saberwal G, Eklund EA. Constitutively active SHP2 cooperates with HoxA10-overexpression to induce acute myeloid leukemia. J Biol Chem. 2009;284:2549–67.

44. Horton SJ, Grier DG, McGonigle GJ, Thompson A, Morrow M, De Silva I, Moulding DA, Kioussis D, Lappin TR, Brady HJ, Williams O. Continuous MLL-ENL expression is necessary to establish a Hox code and maintain immortalization of hematopoietic progenitor cells. Cancer Res. 2005;65:9245–52.

45. Lavau C, Luo RT, Du C, Thirman MJ. Retrovirus mediated gene transfer of MLL-ELL transforms primary myeloid progenitors and causes acute myeloid leukemia in mice. Proc Natl Acad Sci USA. 2000;60:10984–9.

46. Li Z, Luo RT, Mi S, Sun M, Chen P, Bao J, Neilly MB, Jayathilaka N, Johnson DS, Wang L, Lavau C, Zhang Y, Tseng C, Zhang X, Wang J, Yu J, Yang H, Wang SM, Rowley JD, Chen J, Thirman MJ. Consistent deregulation of gene expression between human and murine MLL rearrangement leukemias. Cancer Res. 2009;69:1109–16.

47. So CH, Karsunky H, Wong P, Weissman IL, Cleary ML. Leukemic transformation of hematopoietic progenitors by MLL-GAS7 in the absence of HoxA7 or HoxA9. Blood. 2004;103:3192–9.

48. Kumar AR, Hudson WA, Chen W, Nishiuchi R, Yao Q, Kersey JH. HoxA9 influences the phenotype but not the incidence of MLL-AF9 fusion gene leukemia. Blood. 2004;103:1823–8.

49. Guazzi S, Pintonello ML, Vigano A, Boncinelli E. Regulatory interactions between the human HOXB1, HOXB2, and HOXB3 proteins and the upstream sequence of the Otx2 gene in embryonal carcinoma cells. J Biol Chem. 1998;273:11092–9.

50. Palakurthy RK. Epigenetic silencing of the RASSF1A tumor suppressor gene through HOXB3-mediated induction of DNMT3B expression. Molecular Cell. 2009;36:219–30.

51. Pan Q, Simpson RU. c-myc intron element-binding proteins are required for 1, 25-dihydroxyvitamin D3 regulation of c-myc during HL-60 cell differentiation and the involvement of HOXB4. J Biol Chem. 1999;274:8437–44.

52. Gao J, Mazella J, Tseng L. Hox proteins activate the IGFBP-1 promoter and suppress the function of hPR in human endometrial cells. DNA Cell Biol. 2002;21:819–25.

53. Morgan R, Nalliah A, Morsi El-Kadi AS. FLASH, a component of the FAS-CAPSASE8 apoptotic pathway, is directly regulated by Hoxb4 in the notochord. Dev Biol. 2004;265:105–12.

54. Morsi El-Kadi AS, in der Reiden P, Durston A, Morgan R. The small GTPase Rap1 is an immediate downstream target for Hoxb4 transcriptional regulation. Mech Dev. 2002;113:131–9.

55. Jiang J, Yu H, Shou Y, Neale G, Zhou S, Lu T, Sorrentino BP. Hemgn is a direct transcriptional target of HOXB4 and induces expansion of murine myeloid progenitor cells. Blood. 2010;116:711–9.

56. Bruhl T. Homeobox A9 transcriptionally regulates EphB4 to modulate endothelial cell migration and tube formation. Circ Res. 2004;94:743–51.
57. Gwin K, Frank E, Bossou A, Medina KL Hoxa9 regulates Flt3 in lymphohematopoietic progenitors. J Immunol. 2010;185:6572–83.
58. Shi X, Bai S, Li L, Cao X. Hoxa-9 represses transforming growth factor-beta-induced osteopontin gene transcription. J Biol Chem. 2001;276:850–5.
59. Hu YL, Fong S, Largman C, Shen WF. HOXA9 regulates miR-155 in hematopoietic cells. Nucleic Acids Res. 2010;38:5472–8.
60. Hu YL, Passegué E, Fong S, Largman C, Lawrence HJ. Evidence that the Pim1 kinase gene is a direct target of HOXA9. Blood. 2007;1094732–8.
61. Oncomine 4.4 Research Edition, Compendia Biosciences. Available from: www.oncomine.org/resource/.
62. Bandyopadhyay S, Ashraf MZ, Daher P, Howe PH, DiCorleto PE. HOXA9 participates in the transcriptional activation of E-selectin in endothelial cells. Mol Cell Biol. 2007;27:4207–16.
63. Bei L, Huang W, Wang H, Shah C, Horvath E, Eklund EA. HoxA10 activates CDX4 transcription and Cdx4 activates HOXA10 transcription in myeloid cells. J Biol Chem. 2011;286:19047–64.
64. Shah CA, Wang H, Bei L, Platanias LC, Eklund EA. HoxA10 regulates transcription of the gene encoding transforming growth factor beta 2 (TGFB2) in myeloid cells. J Biol Chem. 2011;286:3161–76.
65. Wang H, Lu YF, Huang W, Papoutsakis ET, Fuhrken P, Eklund EA. HoxA10 activates transcription of the gene encoding Mkp2 in myeloid cells. J Biol Chem. 2007;282:16164–76.

Critical Reviews™ in Oncogenesis, 16(1–2), 77–91 (2011)

RUNX1 Mutations in Clonal Myeloid Disorders: From Conventional Cytogenetics to Next Generation Sequencing, A Story 40 Years in the Making

James K. Mangan[1] & Nancy A. Speck[2,*]

[1]Department of Internal Medicine, Division of Hematology-Oncology and the Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania; [2]Abramson Family Cancer Research Institute and Department of Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

[*]Address all correspondence to: Nancy A. Speck, Abramson Family Cancer Research Institute and Department of Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, nancyas@exchange.upenn.edu

ABSTRACT: Translocations and mutations in the core binding factor genes, *RUNX1* or *CBFB*, are found in acute myeloid and lymphocytic leukemia, therapy-related myeloid leukemia, myelodysplastic syndrome, chronic myelomonocytic leukemia, and in familial platelet disorder with predisposition to acute myeloid leukemia. Here we review the biochemical and biological properties of the normal Runx1 protein, discuss the nature of *RUNX1* mutations in myeloid leukemia, their prognostic significance, and the mutations that cooperate or co-exist with them in these various diseases.

KEY WORDS: Runx1, AML, MDS, hematopoietic stem cells.

ABBREVIATIONS

ALL, acute lymphocytic leukemia, AML, acute myeloid leukemia, AML1, acute myeloid leukemia 1, CALGB, Cancer and Leukemia Group B, CBFB, Core Binding Factor Beta, CMML, chronic myelomonocytic leukemia, FAB, French-American-British, FLT3-ITD, Fms-like tyrosine kinase 3 internal tandem duplication, FPD-AML, familial platelet disorder with predisposition to acute myeloid leukemia, GM, granulocyte-monocyte, HHR, hydrophobic heptad repeat, HSCs, hematopoietic stem cells, MDS, myelodysplastic syndrome, MYND, myeloid-nervy-DEAF-1, N-CoR, nuclear receptor co-repressor, NHR1, nervy homology region 1, NHR2, nervy homology region 2, NHR4, nervy homology region 4, s-AML, secondary AML, SMRT, silencing mediator of retinoid and thyroid hormone receptor

I. INTRODUCTION

In 1973 Janet Rowley, using new chromosome banding techniques, identified a reciprocal translocation between chromosomes 8 and 21 in two female patients with acute myeloid leukemia (AML).[1] That same month, Rowley published another paper demonstrating that the end of the long arm of chromosome 22 thought to be missing in the Philadelphia chromosome (a cytogenetic abnormality frequently associated with chronic myeloid leukemia) had in fact not been lost but was instead relocated to the end of chromosome 9.[2] These contemporaneous papers established that consistent chromosomal translocations could be correlated with specific leukemia subtypes. Eighteen years later the acute myeloid leukemia 1 (AML1) residing at the breakpoint in t(8;21)(q22;q22) was cloned[3] and later renamed *RUNX1*.[4] Hence the discovery of *RUNX1* mutations was an important milestone in the history of cancer genetics. Janet Rowley's keen powers of observation and intuition that translocations were causative in leukemia, and not simply correlative, begat an era of intensive research in cancer genetics, which may have reached its zenith with the application of next generation sequencing technology.

Fast forward nearly 40 years, and we now know much about the *RUNX1* gene and its encoded protein. Runx1 is a sequence-specific DNA binding protein and has an obligate non-DNA binding partner called core binding factor β (CBFβ), the gene for which is also targeted by translocations important in

0893-9675/11/$35.00

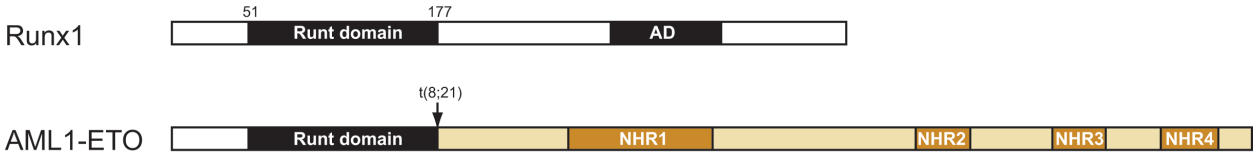


FIGURE 1. Schematic diagram of Runx1 and AML1-ETO. White/black represent sequences from Runx1, and gold from ETO. TAD, transactivation domain; NHR1–4, nervy homology domains 1–4.

AML, the inv(16)(p13;q22) and t(16;16)(p13;q22).[5] AML with any of these three translocations is often referred to as "core binding factor leukemia." Runx1 has essential functions in normal hematopoiesis in the embryo and the adult. In addition to the t(8;21), translocations, mutations in *RUNX1* have been found in *de novo* and therapy-related AML, myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML), acute lymphocytic leukemia (ALL), and in the autosomal dominant pre-leukemia syndrome familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML).

II. THE Runx1 PROTEIN: DOMAIN STRUCTURE AND FUNCTION, AND INTERACTING PROTEINS

Runx1 is a sequence-specific DNA binding protein, and like most proteins of its ilk, it contains effecter domains linked by less structured sequences. By far the most well-characterized domain of Runx1 is its DNA binding "Runt" domain, named after the first member of the family to be cloned, the *Drosophila* runt protein.[6,7] (Figure 1) Multiple structures of the Runt domain have been solved.[8–11] The DNA and CBFβ interacting interfaces are on opposite sides of the Runt domain and do not overlap, and CBFβ does not touch the DNA. The primary role of CBFβ is to increase binding of Runx1 to DNA by stabilizing a particular conformation of the Runt domain.[11,12] As will be described later, many missense mutations in the Runt domain have been identified in AML, MDS, CMML, and FPD/AML, the vast majority of which involve residues at the DNA binding interface.

The second most well-characterized domain in Runx1 is the transactivation domain, which is located midway between the Runt domain and the C-terminus; it is essential for Runx1's *in vivo* functions.[13–16] No structures of this domain have been solved, although computational analysis[17] predicts that parts of the transactivation domain and an adjacent inhibitory domain are likely to be structured. Multiple proteins have been identified that interact with sequences that are C-terminal to the Runt domain that presumably mediate its activities.[18–28] Mutations that are C-terminal to the Runt domain are also found in leukemia and are primarily nonsense or frame-shift mutations that result in the production of proteins lacking all or part of the transactivation domain. A few missense mutations have also been found, but their functional significance has not been established.[29,30]

Less well-characterized sequences in the C-terminus of Runx1 affect Runx1's DNA binding potential. Specifically, deletion of C-terminal sequences causes Runx1 to bind DNA with an affinity approximately 40-fold greater than that of the full-length protein.[14,31] Therefore Runx1 proteins lacking the inhibitory sequences can presumably out-compete binding of the functional full-length protein to DNA, and dominantly inhibit its activity. For simplicity's sake we use the term "Runx1 mutations" to refer to all mutations other than translocations, including loss-of-function (amorphic) mutations, hypomorphic mutations, and antimorphic mutations that create dominant negative *RUNX1* alleles.

III. Runx1 FUNCTION IN NORMAL HEMATOPOIESIS

Runx1's earliest role in development is for the differentiation of hematopoietic progenitors and stem cells (HSCs) from a small population of endothelial

cells in the conceptus.[32–34] Because mutations in the germline caused mid-gestation lethality, conditional deletion strategies were necessary to ascertain its role in adult hematopoiesis. Deletion of Runx1 in adult HSCs caused multi-lineage blocks in B and T lymphoid development and megakaryocyte maturation, and thus the mice are lymphopenic and thrombocytopenic.[35–37] Notably, Runx1 loss in HSCs does not cause AML on its own but establishes a pre-leukemic state that predisposes to AML following the acquisition of secondary mutations.[38,39] The effects of Runx1 loss on HSCs and progenitors are not entirely understood. One outcome is an increase in a population of cells in mouse bone marrow that lacks lineage markers and expresses the HSC markers Sca-1 and c-Kit (LSK cells).[32,35,36,38,39] Runx1 loss also increases the number of granulocyte and megakaryocyte progenitors in the bone marrow and causes what has been alternatively described as a myeloproliferative disease or myelodysplasia,[35,37] and in one study a lower penetrance lymphoma was noted.[37] Runx1 loss does not, however, cause a notable decline in functional long-term repopulating HSCs,[39,40] which is probably a critical property contributing to the pre-leukemic state, as a mutant HSC that is rapidly lost from the bone marrow cannot provide a target population for secondary mutations. Runx1 loss enhances the ability of mouse hematopoietic progenitors to undergo serial replating in culture,[36] which is regarded as a measure of self-renewal activity and may also contribute to the maintenance of a dysfunctional progenitor population.

IV. *RUNX1* MUTATIONS IN AML

A. The t(8;21) Subgroup

AML can be subdivided into several subtypes, including AML with recurrent cytogenetic abnormalities, AML with multi-lineage dysplasia (this includes patients with an antecedent MDS or myeloproliferative disease), AML and MDS therapy related (following chemotherapy or radiation exposure), and AML not otherwise categorized.[41] Translocations or loss of function *RUNX1* mutations have been found in all of these subtypes.

Characteristic genetic abnormalities include the t(8;21) and inv(16) in *RUNX1* and *CBFB*, respectively, each of which defines subgroups within the category of recurrent cytogenetic abnormalities, and confer a favorable prognosis. The t(8;21) breaks the *RUNX1* gene in intron 5, and results in fusion of the N-terminal portion of Runx1 (including the Runt domain, but minus the transactivation domain) to a protein most commonly known as ETO (encoded by *RUNX1T1*) (Figure 1).[3,42–45] ETO contains four domains conserved with its *Drosophila* homologue nervy, the structures for all of which, along with their interacting proteins, have been solved.[46–51] Mutations that specifically disrupt the interaction between individual domains in ETO and their associated proteins revealed that one domain in particular, nervy homology region 2 (NHR2, also known as hydrophobic heptad repeat or HHR) is critical for AML1-ETO's leukemogenic activity in retroviral transduction based assays.[52,53] NHR2 forms a four-helix bundle (a dimer of dimers), and mutations that reduced the tetramer to dimer abrogated AML1-ETO's leukemogenic activity.[53] That oligomerization of AML1-ETO *per se* was important was demonstrated by the ability of an oligomerization domain from the forkhead binding protein to substitute for NHR2 and enable AML1-ETO to confer serial replating activity to primary bone marrow cells.[54]

On the other hand, the most C-terminal domain, NHR4, also known as the myeloid-Nervy-DEAF-1 (MYND), appears to restrain AML1-ETO's leukemogenic activity, as mutations that severely disrupt the NHR4 fold promote AML1-ETO's activity.[55] In fact, the full-length unaltered form of AML1-ETO is not by itself leukemogenic, and it can only cause AML in mice when combined with another oncogene such as Fms-like tyrosine kinase 3 internal tandem duplication (*FLT3-ITD), TEL-PDGFBR*, or activated *KIT*.[56–58] But either the deletion or mutation of NHR4 will allow AML1-ETO to induce leukemia in the absence of a co-transduced oncogene.[55] NHR4 binds the silencing mediator of retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) complexes, as well as a protein called SON, and presumably one or more of these complexes is responsible for dampening AML1-

ETO's activity.[55,59–61] Mutations involving NHR4 have not been found in t(8;21) leukemia, thus this does not appear to represent a common mechanism for augmenting AML1-ETO's activity.[62]

Deletions of the NHR1 domain have yielded conflicting results, with one group reporting an effect on AML1-ETO's activity[63] and others not.[52,54] The NHR1 deletion in the former study[63] was larger than in the latter two studies[52,54] and included sequences C-terminal to the conserved NHR1 domain that were previously shown to bind N-CoR and to contribute to leukemogenesis.[52,64] The larger deletion prevented association with p300 and acetylation of two lysine residues within the Runx1 portion of the AML1-ETO protein.[63] Substituting one of those lysines with an arginine impaired AML1-ETO's leukemogenic activity, indicating that the ability to recruit p300 and become acetylated is important for AML1-ETO function.[63]

The two other interactions mediated by AML1-ETO that are essential for its activity are DNA and CBFβ binding by the Runt domain, although there is some disagreement about the importance of the latter.[52,54,65–67] Thus AML1-ETO has several surfaces amenable to targeting with small-molecule or peptide inhibitors: the Runt domain:DNA interface, potentially the Runt domain:CBFβ interface, and NHR2 oligomerization. Inhibition of p300 or other histone acetylases may also provide a therapeutic option.[63]

Multiple lines of evidence indicate that the acquisition of AML1-ETO confers a different phenotype than Runx1 mutations. Genetic experiments in *Drosophila* showed that AML1-ETO behaves as a constitutive repressor, blunting the transcription of genes the Runx1 homologue lozenge activates, but additionally those that lozenge would normally repress.[68] The phenotype of conditional knock-in mice in which AML1-ETO expression is activated in the adult bone marrow resembles a somewhat milder version of Runx1 loss, as the mice had no evidence of lymphopenia or thrombocytopenia.[69] AML1-ETO conditional knock-in mice did, however, display some of the aberrations in progenitors seen upon Runx1 loss, including increased numbers of granulocyte-monocyte (GM) progenitors and enhanced serial replating activity. AML1-ETO in the conditional knock-in mice cooperated with the *HIP1-PDGFBR* oncogene to induce a very rapid myeloproliferative disease (MPD) that was not observed with either mutation alone.[70] As hematopoietic differentiation was not impeded in this AML1-ETO knock-in mouse model, this suggests that defects in the stem/progenitor pool caused by AML1-ETO are fundamental to the leukemic process. The phenotypes in mice correlate with the leukemic phenotypes, as AML1-ETO is found in the French-American-British (FAB) M2 subtype, also known as acute myeloblastic leukemia with maturation, whereas biallelic *RUNX1* mutations have been found in minimally differentiated acute myeloblastic leukemia, AML M0. Finally, as discussed in more detail below, *RUNX1* mutations confer a considerably worse prognosis than the t(8;21).

The t(8;21) is the most common translocation in pediatric AML patients (10–20%).[71–74] One study documented a prenatal origin of the t(8;21) from the Guthrie cards of half of the t(8;21) pediatric patients analyzed.[75] Two of the positive patients were between 10 and 12 years of age at the time of diagnosis, therefore harbored a pre-leukemic clone for more than a decade before developing AML. Patients in long-term remission can also harbor residual t(8;21)-containing cells in their bone marrow for many years.[45,76] Eighteen percent of healthy individuals have t(8;21) containing cells detectable by polymerase chain reaction, and AML1-ETO transcripts were detected in 40% of cord blood samples.[77,78] Thus the t(8;21) results in the acquisition of a long-lived pre-leukemic HSC that has no overt clinical manifestations.

The mutations that cause AML are often divided into two classes: class I mutations which activate signaling pathways, hence proliferation and survival, and class II mutations that generally involve transcription factors and cause impaired differentiation, decreased apoptosis, and growth arrest. Another class of frequently mutated genes encodes epigenetic regulators. The class I mutations most frequently found in t(8;21) AML include *KIT, NRAS,* and *KRAS*. Approximately 20% of t(8;21) patients have activating mutations in *KIT*, and exon 17 *KIT* mutations have been found to confer an unfavorable prognosis in multiple studies.[79–81] *FLT3* is the most commonly mutated gene in AML,

but *FLT3* mutations occur at a relatively low rate in t(8;21) leukemia.

B. Amorphic and Antimorphic *RUNX1* Mutations in AML

RUNX1 mutations were first described in AML M0 and FPD/AML,[82,83] followed shortly thereafter in MDS,[84] and more recently in CMML.[85,86] More recent larger-scale sequencing efforts are providing a more comprehensive picture of the frequency and scope of *RUNX1* mutations, their prognostic significance, and the co-existing mutations.

Two groups recently analyzed large numbers of AML patients for the presence of *RUNX1* mutations.[29,30] A report from the German-Austrian AML study group, which evaluated 18- to 60-year-old AML patients (primarily *de novo* AML, but including a smaller number of secondary and therapy-related AML patients) found *RUNX1* mutations in 53 of 945 (5.6%) cases.[29] An earlier study of an older 15- to 90-year-old Taiwanese patient population with *de novo* AML reported a higher incidence of *RUNX1* mutations (13.2%, 62 of 470 patients).[30] It was suggested by authors of the former study that the higher frequency of *RUNX1* mutations in the Taiwanese study could be caused by their inclusion of older patients, as the mutation frequency increases with age. In both studies most *RUNX1* mutations were frame-shift mutations, the remainder included missense, nonsense, in-frame, or silent mutations, and the vast majority were mono-allelic. The mutations were primarily located within the Runt domain and C-terminal to the Runt domain. Both groups reported *RUNX1* mutations were mainly found in the cytogenetic intermediate-risk group and were closely associated with trisomy 8. No *RUNX1* mutations were found in the favorable risk group with characteristic genetic abnormalities that include t(8;21) and inv(16). In univariate analyses *RUNX1* mutations were found to be associated with refractory disease and inferior event-free, relapse-free, and overall survival. Allogeneic hematopoietic stem cell transplant improved the outcome of patients with *RUNX1* mutations,[29,30] while patients who instead received repetitive cycles of high-dose cytarabine or autologous hematopoietic stem cell transplant relapsed or died.[29] *RUNX1* mutations were associated with the presence of *MLL- PTD* mutations in both studies and *IDH1/IDH2* in one study[29] but were inversely correlated with *CEBPA* and *NPM1* mutations.[29,30] No significant correlation was found with *FLT3, NRAS*, *KRAS, KIT, PTPN11, or WT1* mutations, despite the fact that several of these, and in particular *KIT* mutations, are frequent in t(8;21) AML. Both groups found *RUNX1* mutations highly associated with AML M0; one group also reported association with M1[30] and the other with M2 morphologies.[29]

An analysis of 111 pediatric AML cases identified 5 cases with *RUNX1* mutations, in addition to 20 t(8;21) cases and 16 inv(16) cases, bringing the total of core binding factor mutations in this pediatric AML cohort to 36.9%.[87] If one combines the frequencies of *RUNX1* mutations in adult AML (13% in the unselected Taiwanese study), with the t(8;21) (7% in a Cancer and Leukemia Group B (CALGB) study with a median age of 52); and inv(16) (8% in the same CALGB study),[88] this results in an overall frequency of core binding factor mutations in adult AML of approximately 28%. *RUNX1* mutations, t(8;21), and inversion 16 are mutually exclusive.

V. *RUNX1* MUTATIONS IN MDS

MDS is a clonal stem cell disorder characterized by ineffective production of myeloid lineage cells with associated dysplasia that can involve one or more myeloid lineages. There are multiple subcategories of MDS, including refractory cytopenia with unilineage dysplasia, refractory anemia with ringed sideroblasts with associated thrombocytosis, refractory cytopenia with multilineage dysplasia, refractory anemia with excess blasts I and II, 5q-syndrome, myelodysplasia (unclassifiable), and refractory cytopenia of childhood. Approximately one-third of MDS patients progress to AML over time. Fewer than half of MDS patients have chromosomal abnormalities, and balanced translocations are rare.

Nevertheless, the first report of a *RUNX1* mutation in MDS was a balanced translocation, the t(3;21)(q26.2;q22).[89] However loss-of-function

RUNX1 mutations are far more common in MDS, and numerous reports have documented them.[84,90,91] At the time of writing the most recent report was a mutational screen in 439 MDS patients for a broad array of cancer-associated genes, in which mutations in *RUNX1* along with 17 other genes were identified.[92] *RUNX1* mutations were the third most frequent (8.7%), surpassed only by mutations in the epigenetic regulators *TET2* (20.5%) and *ASXL1* (14.4%). A multivariate analysis that included risk stratification using the International Prognostic Scoring System[93] showed that mutations in *RUNX1, ASXL1*, *TP53*, *EZH2*, and *ETV6* were independent predictors of poor overall survival in all but the highest risk category. Mutations in *RUNX1, TP53*, and *NRAS* correlated with severe thrombocytopenia and elevated blast counts, but not with neutropenia or anemia. Loss of function *Runx1* mutations in mice affect megakaryocyte but not granulocyte or erythroid differentiation, consistent with the MDS phenotype seen in human patients with *RUNX1* mutations. A 13.8% frequency of *RUNX1* mutations was reported in an earlier study of 188 MDS + CMML patients.[94] Samples from MDS patients who progressed to secondary AML (s-AML) were analyzed for mutations at both stages.[94,95] In most cases *RUNX1* mutations were present in both the MDS and s-AML samples, and in a smaller number of cases *RUNX1* mutations were found in the s-AML but not in the antecedent MDS. Thus *RUNX1* mutations are likely to be early events in many cases, but can also be later events in disease progression. Conversion from mono- to biallelic *RUNX1* mutations was also observed in several s-AML samples, either through acquisition of an independent mutation or uniparental disomy.[95]

In the fourteen samples in the Bejar et al.[92] study that had mutations in addition to *RUNX1*, they were most often in *TET2* (12), *ASXL1* (12), *EZH2* (8), and *NRAS* (6), and there was no overlap with mutations in *TP53, JAK2, ETV6, IDH1/2, NPM1, GNAS, BRAF, PTEN,* or *CDKN2A*. Thus although the types of mutations in *RUNX1* found in AML and MDS were similar, the cooperating mutations were distinct. In general, very few activated tyrosine kinases were identified in MDS, confirming the previous hypotheses that MDS is generally associated with class II mutations and mutations in epigenetic regulators and MPD is generally associated with class I mutations.

An intriguing observation is that loss of function *RUNX1* mutations in MDS are highly correlated with previous exposure to radiation, both therapeutic and accidental, the latter in atomic bomb survivors and individuals who lived in close proximity to the Semipalatinsk nuclear test site in what today is Kazakhstan.[90,96] The close association of *RUNX1* mutations with radiation suggest either that the *RUNX1* gene is particularly sensitive to DNA damage following radiation or that preexisting *RUNX1* mutations may predispose patients to MDS following DNA damage.

RUNX1 mutations were also recently described in Fanconi anemia (FA) patients, who have a 30–40% probability of developing MDS and AML by age 40.[97] A screen of 57 FA bone marrows for chromosome copy-number changes and mutations in common MDS/AML genes (*TET2, CBL, NRAS, TP53, RUNX1, CEBPA, NPM1, FLT3,* and *MLL)* found that the somatic acquisition of only three abnormalities correlated with MDS/AML in FA patients: 3q+, 7/7q-, and *RUNX1* translocations, deletions, and mutations.[98]

VI. *RUNX1* MUTATIONS IN CMML

CMML has overlapping features of MDS and myeloproliferative neoplasms, including peripheral monocytosis > 1×10^9/L, <20% blood or bone marrow blasts, and bone marrow dysplasia in one or more myeloid lineage. CMML progresses to AML in 15–20% of patients. CMML is a relatively rare disease, thus sequencing studies of the scale described above for AML and MDS have not been performed. In a smaller-scale analysis by the Munich Leukemia Group, mutations in *TET, CBL, NRAS, KRAS, JAK2, RUNX1,* and *MPL* were interrogated in 81 CMML samples.[99] The majority (72.8%) of CMML samples had a mutation in *TET, CBL, NRAS, KRAS, JAK2,* or *RUNX1,* with *RUNX1* mutations in 8.6% of patients. *RUNX1* mutations in this study were not found to be of prognostic relevance. Another analysis was performed in a Taiwanese population, and *RUNX1*

mutations were found in 30/81 patients (37%). Both were unselected groups with a preponderance of elderly patients. In the Taiwanese cohort, there was a trend toward faster progression to AML in the *RUNX1* mutated group, which was especially pronounced when *RUNX1* mutations occurred in the C-terminus.[86]

VII. *RUNX1* MUTATIONS IN FPD/AML

FPD/AML is an autosomal dominant disorder caused by mutations in *RUNX1*. Many but not all FPD/AML patients have low platelet counts or platelet activation defects.[100,101] The penetrance of MDS/AML in FPD/AML patients is >40%, with a median age of incidence of 33 years.[102] Large intragenic deletions in *RUNX1* in FPD/AML established haploinsufficiency is one mechanism for the disease,[83] but mutations are also frequently found in the Runt domain. FPD/AML is clearly an intriguing syndrome and an improved understanding of the pathogenesis of MDS and AML in this disorder would seem to be key for unraveling the mechanisms underlying *RUNX1* mutant AML and MDS in general. However, the ability to gain more insight into the pathogenesis of MDS/AML in FPD/AML has been hampered by small patient numbers and the heterogeneity of the disease presentation.

VIII. BIOCHEMICAL AND FUNCTIONAL ANALYSES OF *RUNX1* MUTATIONS

The majority of *RUNX1* mutations can be categorized based on their potential impact on the protein (Table 1). These include 1) large deletions; 2) mutations resulting in truncation within the Runt domain; 3) missense mutations in the Runt domain at the DNA interface that affect DNA but not CBFβ binding; 4) missense mutations in the Runt domain at the CBFβ interface that affect CBFβ but not DNA binding; 5) missense mutations in the Runt domain that affect both DNA and CBFβ binding through destabilizing the Runt domain fold; 6) mutations that truncate Runx1 C-terminal to the Runt domain and remove all or part of the transactivation domain; and 7) missense mutations that are C-terminal to the Runt domain (rare). It has been hypothesized that these various mutations would have different biological effects, with some behaving as loss of function (amorphic) mutations, others as hypomorphic mutations, and some as antimorphic mutations that could create dominant interfering Runx1 proteins.

Matheny et al.[103] compared different categories of missense mutations in the Runt domain using both biophysical and genetic approaches, and they could confirm that mutations that perturbed CBFβ binding or the Runt domain fold resulted in hypomorphic *Runx1* alleles in mice, while a mutation that severely impaired DNA but not CBFβ binding generated a weakly antimorphic allele. The mechanism for the antimorphic activity was not clear, but it could involve sequestering a limiting protein with a Runx1:CBFβ heterodimer that cannot bind DNA. The majority of leukemia mutations in the Runt domain are at the DNA interface, indicating that severe disruption of Runx1 activity is more likely to be pathogenic.

Watanabe et al.[104] compared mutations in the Runt domain that affected DNA binding to a truncation that was C-terminal to the Runt domain that removed the transactivation domain by overexpressing the mutant proteins in a bone marrow transplant model. Both induced MDS, but with different properties, in that the DNA binding mutant caused leukocytosis while the C-terminal truncation mutant caused leukopenia. Thus, different mutations indeed contribute different biological properties to the Runx1 protein, and presumably to disease phenotype.

Most *RUNX1* mutations are mono-allelic, and unfortunately a disease caused by mono-allelic mutations has been very difficult to model in the mouse. Although moderately (15%) decreased platelet counts were reported in mice haploinsufficient for *Runx1,*[105] this was not reproduced in another lab,[103] potentially due to differences in genetic background of the mice. More pronounced thrombocytopenia was observed in mice homozygous for a hypomorphic *Runx1* allele,[103] suggesting that reducing the effective dosage by more than 50% may provide a strategy for more faithfully modeling at least some aspects of *RUNX1* haploinsufficiency in human disease. Hence one of the more interesting unresolved questions is

TABLE 1. *RUNX1* Mutations in AML, MDS, CMML, and FPD/AML

Mutation	Affects				Type
	DNA binding	CBFβ binding	Runt domain fold	Transactivation	
Large deletion	Yes	Yes	NA	Yes	Amorphic
Truncation before or within Runt domain	Yes	Yes	NA	Yes	Amorphic
Missense mutation in Runt domain at DNA interface[a]	Yes	Yes or no	Yes or no	Yes	Antimorphic or amorphic
Missense mutation in Runt domain at CBFβ interface[b]	No	Yes	No	Yes	Hypomorphic
Missense mutation in Runt domain, not at DNA or CBFβ interface[b]	Yes	Yes	Yes	Yes	Hypomorphic
Truncation C-terminal to Runt domain	No	No	No	Yes	Antimorphic
Missense mutation C-terminal to Runt domain[b]	No	No	No	Yes	Antimorphic[c]

[a]Common in leukemia. Mutations that affect DNA but not CBFβ binding result in antimorphic alleles, and are more common than those that affect both DNA and CBFβ binding.
[b]Rare in leukemia.
[c]Presumed, not tested. NA, not applicable.

why mono-allelic *RUNX1* mutations would confer a more adverse phenotype than t(8;21)? One possible explanation is that mono-allelic *RUNX1* mutations tend to occur in older AML patients, who may have accumulated more cooperating mutations than younger patients with t(8;21).

IX. CONCLUSION

Patients with t(8;21) and inv(16) for the most part do well with standard induction and high-dose cytarabine consolidation alone without the need for allogeneic stem cell transplantation. However even for these so-called favorable prognosis core binding factor leukemias, long-term leukemia-free survival is only 50%.[106] Prognosis for patients with *RUNX1* mutations, which typically fall into the intermediate risk cytogenetic categories (normal and non-complex), is even worse. Our increased knowledge of core binding factor mutations and function has not yet resulted in novel therapeutic approaches. This may be due, in part, to *RUNX1's* role as a class II mutation that may be involved in altering the expression of a multitude of target genes. This is illustrated by the recognition that t(8;21) AML is associated with its own unique gene methylation profile that is predictive of outcome.[107] Similarly, the presence or absence of a *RUNX1* mutation in AML M0 can be ascertained through the use of gene expression profiling, because *RUNX1* mutations result in altered expression of key target genes in a reproducible manner.[108] Given the multitude of potential Runx1 targets, one approach would be to focus on therapies that alter expression of many target genes simultaneously, as is the case for hypomethylating agents in MDS. A second approach would be to focus on combinatorial therapies, simultaneously targeting the class II effects of *RUNX1* and the class I mutations in tyrosine kinases that frequently accompany *RUNX1*, such as the *FLT3-ITD* or activated *KIT*. One such phase I trial is currently underway at MD Anderson Cancer Center, in which the hypomethylating agent 5-azacitadine is being tested in combination with the FLT3 inhibitor sorafenib in relapsed and refractory

AML. A third approach would be to incorporate knowledge about specific targets of Runx1 in AML: a so-called "target the target" approach. For example, a recent study showed that *AKT3* and *RARA* are upregulated in AML M0,[108] providing a rationale for further investigation into the efficacy of small molecules targeting these pathways. Given the difficulties in modeling leukemias with mono-allelic *Runx1* deficiency in the mouse (described earlier), more robust *Runx1* deficient leukemia models are needed, and could potentially be created by crossing *Runx1* haploinsufficient mice with mice carrying tyrosine kinase mutations that are known to cooperate with *Runx1*. Such improved models may ultimately allow us to better "target the target."

The incentive to engage in research with the goal of unlocking the secrets of the pathogenesis of AML with *RUNX1* mutations would also be increased if we had an improved understanding of the prognostic relevance of *RUNX1* mutations in specific therapeutic situations. How does *RUNX1* mutation status affect response to hypomethylating agents? FLT3 inhibitors? HDAC inhibitors? Therapy with high-dose anthracyclines or high-dose cytarabine? In MDS? In AML? These questions remain unanswered, as the prognostic relevance of *RUNX1* mutations have really only been examined on a global level. Our knowledge about the role of *RUNX1* in AML has clearly made great strides since Dr. Rowley identified the reciprocal translocation between chromosomes 8 and 21 nearly 40 years ago, but much remains undiscovered.

ACKNOWLEDGMENTS

We gratefully acknowledge support from the National Institutes of Health (Award Nos. R01 CA149976 and T32 CA009615) and the Abramson Family Cancer Research Institute.

REFERENCES

1. Rowley JD. Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet. 1973;16:109–112.
2. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290–3.
3. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y and Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, *AML1*. Proc Natl Acad Sci USA. 1991;88:10431–4.
4. van Wijnen A, Stein G, Lian J, Stein J, Hiebert S, Ito Y, Speck N, Komori T, Neil J and Quarles D. Systematic nomenclature for the runt homology transcription factors, CBFA/PEBP2a/AML, involved in hematopoiesis and skeletal development. J Cell Biochem. 2004;23:4209–10.
5. Liu P, Tarle SA, Hajra A, Claxton DF, Marlton P, Freedman M, Siciliano MJ and Collins FS. Fusion between transcription factor CBFb/PEBP2b and a myosin heavy chain in acute myeloid leukemia. Science. 1993;261:1041–4.
6. Kania MA, Bonner AS, Duffy JB and Gergen JP. The Drosophila segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev. 1990;4:1701–3.
7. Kagoshima H, Shigesada K, Satake M, Ito Y, Miyoshi H, Ohki M, Pepling M and Gergen JP. The Runt-domain identifies a new family of heteromeric DNA-binding transcriptional regulatory proteins. Trends Genet. 1993;9:338–41.
8. Berardi M, Sun C, Zehr M, Abildgaard F, Peng J, Speck NA and Bushweller JH. The Ig fold of the core binding factor a Runt domain is a member of a family of structurally and functionally related Ig fold DNA binding domains. Structure Fold Des. 1999;7:1247–56.
9. Bravo J, Li Z, Speck NA and Warren AJ. The leukaemia-associated AML1 (Runx1)-CBFb complex functions as a DNA-induced molecular clamp. Nat Struct Biol. 2001;8:371–7.
10. Nagata T, Gupta V, Sorce D, Kim W-Y, Sali A, Chait BT, Shigesada K, Ito Y and Werner MH. Immunoglobulin motif DNA-binding and heterodimerization for the PEBP2/CBF Runt-domain. Nat Struct Biol. 1999;6:615–9.
11. Tahirov TH, Inoue-Bungo T, Morii H, Fujikawa A, Sasaki M, Kimura K, Shiina M, Sato K, Kumasaka T, Yamamoto M, Ishii S and Ogata K. Structural

analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFb. Cell. 2001;104:755–67.

12. Yan J, Liu Y, Lukasik SM, Speck NA and Bushweller JH. CBFbeta allosterically regulates the Runx1 Runt domain via a dynamic conformational equilibrium. Nat Struct Mol Biol. 2004;11:901–6.
13. Bae S-C, Ogawa E, Maruyama M, Oka H, Satake M, Shigesada K, Jenkins NA, Gilbert DJ, Copeland NG and Ito Y. PEBP2aB/Mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. Mol Cell Biol. 1994;14:3242–52.
14. Kanno T, Kanno Y, Chen L-F, Ogawa E, Kim W-Y and Ito Y. Intrinsic transcriptional activation-inhibition domains of the polyomavirus enhancer binding protein 2/core binding factor a subunit revealed in the presence of the b subunit. Mol Cell Biol. 1998;18:2444–54.
15. Okuda T, Takeda K, Fujita Y, Nishimura M, Yagyu S, Yoshida M, Akira S, Downing JR and Abe T. Biological characteristics of the leukemia-associated transcriptional factor AML1 disclosed by hematopoietic rescue of AML1-deficient embryonic stem cells by using a knock-in strategy. Mol Cell Biol. 2000;20:319–28.
16. Dowdy CR, Xie R, Frederick D, Hussain S, Zaidi SK, Vradii D, Javed A, Li X, Jones SN, Lian JB, van Wijnen AJ, Stein JL and Stein GS. Definitive hematopoiesis requires Runx1 C-terminal-mediated subnuclear targeting and transactivation. Hum Mol Genet. 2010;19:1048–57.
17. Linding R, Jensen LJ, Diella F, Bork P, Gibson TJ and Russell RB. Protein disorder prediction: implications for structural proteomics. Structure. 2003;11:1453–9.
18. Kitabayashi I, Yokoyama A, Shimizu K and Ohki M. Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. EMBO J. 1998;17:2294–3004.
19. Kitabayashi I, Aikawa Y, Nguyen LA, Yokoyama A and Ohki M. Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. Embo J. 2001;20:7184–96.
20. Aikawa Y, Nguyen LA, Isono K, Takakura N, Tagata Y, Schmitz ML, Koseki H and Kitabayashi I. Roles of HIPK1 and HIPK2 in AML1- and p300-dependent transcription, hematopoiesis and blood vessel formation. Embo J. 2006;25:3955–65.
21. Bruhn L, Munnerlyn A and Grosschedl R. ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRa enhancer function. Genes Dev. 1997;11:640–53.
22. Yagi R, Chen L-F, Shigesda K, Murakami Y and Ito Y. A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator. EMBO J. 1999;18:2551–62.
23. Lutterbach B, Westendorf JJ, Linggi B, Issac S, Seto E and Hiebert SW. A mechanism of repression by Acute Myeloid Leukemia-1, the target of multiple chromosomal translocations in acute leukemia. J Biol Chem. 2000;275:651–6.
24. McLarren KW, Lo R, Grbavec D, Thirunavukkarasu K, Karsenty G and Stifani S. The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. J Biol Chem. 2000;275:530–8.
25. Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ and Stein GS. Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci. 2000;113 (Pt 12):2221–31.
26. Schroeder TM, Kahler RA, Li X and Westendorf JJ. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J Biol Chem. 2004;279:41998–2007.
27. Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G and Olson EN. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell. 2004;119:555–66.
28. Chakraborty S, Sinha KK, Senyuk V and Nucifora G. SUV39H1 interacts with AML1 and abrogates AML1 transactivity. AML1 is methylated in vivo. Oncogene. 2003;22:5229–37.
29. Gaidzik VI, Bullinger L, Schlenk RF, Zimmermann AS, Rock J, Paschka P, Corbacioglu A, Krauter J, Schlegelberger B, Ganser A, Spath D, Kundgen A, Schmidt-Wolf IG, Gotze K, Nachbaur D, Pfreundschuh M, Horst HA, Dohner H and Dohner K. RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML study group. J Clin Oncol. 2011;29:1364–72.

30. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, Huang CF, Lee FY, Liu MC, Yao M, Huang SY, Ko BS, Hsu SC, Wu SJ, Tsay W, Chen YC, Lin LI and Tien HF. AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood. 2009;114:5352–61.
31. Gu T-L, Goetz TL, Graves BJ and Speck NA. Autoinhibition and partner proteins, CBFb and Ets-1, modulate DNA binding by CBFa2(AML1). Mol Cell Biol. 2000;20:91–103.
32. Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E and Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature. 2009;457:889–91.
33. North TE, Gu T-L, Stacy T, Wang Q, Howard L, Binder M, Marín-Padilla M and Speck NA. *Cbfa2* is required for the formation of intra-aortic hematopoietic clusters. Development. 1999;126:2563–75.
34. Yokomizo T, Ogawa M, Osato M, Kanno T, Yoshida H, Fujimoto T, Fraser S, Nishikawa S, Okada H, Satake M, Noda T, Nishikawa SI and Ito Y. Requirement of Runx1/AML1/PEBP2aB for the generation of haematopoietic cells from endothelial cells. Genes Cells. 2001;6:13–23.
35. Growney JD, Shigematsu H, Li Z, Lee BH, Adelsperger J, Rowan R, Curley DP, Kutok JL, Akashi K, Williams IR, Speck NA and Gilliland DG. Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood. 2005;106:494–504.
36. Ichikawa M, Asai T, Saito T, Yamamoto G, Seo S, Yamazaki I, Yamagata T, Mitani K, Chiba S, Ogawa S, Kurokawa M and Hirai H. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med. 2004;10:299–304.
37. Putz G, Rosner A, Nuesslein I, Schmitz N and Buchholz F. AML1 deletion in adult mice causes splenomegaly and lymphomas. Oncogene. 2006;25:929–39.
38. Motoda L, Osato M, Yamashita N, Jacob B, Chen LQ, Yanagida M, Ida H, Wee HJ, Sun AX, Taniuchi I, Littman D and Ito Y. Runx1 protects hematopoietic stem/progenitor cells from oncogenic insult. Stem Cells. 2007;25:2976–86.
39. Jacob B, Osato M, Yamashita N, Wang CQ, Taniuchi I, Littman DR, Asou N and Ito Y. Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis. Blood. 2009;1610–20.
40. Ichikawa M, Goyama S, Asai T, Kawazu M, Nakagawa M, Takeshita M, Chiba S, Ogawa S and Kurokawa M. AML1/Runx1 negatively regulates quiescent hematopoietic stem cells in adult hematopoiesis. J Immunol. 2008;180:4402–8.
41. Vardiman JW, Harris NL and Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292–302.
42. Erickson P, Gau J, Chang K-S, Look T, Whisenant E, Raimondi S, Lasher R, Trujillo J, Rowley J and Drabkin H. Identification of breakpoints in t(8;21) AML and isolation of a fusion transcript with similarity to Drosophila segmentation gene *runt*. Blood. 1992;80:1825–31.
43. Miyoshi H, Kozu T, Shimizu K, Enomoto K, Maseki N, Kaneko Y, Kamada N and Ohki M. The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J. 1993;12:2715–21.
44. Nucifora G, Birn DJ, Erickson P, Gao J, LeBeau MM, Drabkin HA and Rowley JD. Detection of DNA rearrangement in the AML1 and ETO loci and of an AML1/ETO fusion mRNA in patients with t(8;21). Blood. 1993;81:883.
45. Chang KS, Fan YH, Stass SA, Estey EH, Wang G, Trujillo JM, Erickson P and Drabkin H. Expression of AML1-ETO fusion transcripts and detection of minimal residual disease in t(8;21)-positive acute myeloid leukemia. Oncogene. 1993;8:983–8.
46. Liu Y, Chen W, Gaudet J, Cheney MD, Roudaia L, Cierpicki T, Klet RC, Hartman K, Laue TM, Speck NA and Bushweller JH. Structural basis for recognition of SMRT/N-CoR by the MYND domain and its contribution to AML1/ETO's activity. Cancer Cell. 2007;11:483–97.
47. Liu Y, Cheney MD, Gaudet JJ, Chruszcz M, Lukasik SM, Sugiyama D, Lary J, Cole J, Dauter Z, Minor W, Speck NA and Bushweller JH. The tetramer structure of the Nervy homology two domain, NHR2, is critical for AML1/ETO's activity. Cancer Cell. 2006;9:249–60.

48. Park S, Chen W, Cierpicki T, Tonelli M, Cai X, Speck NA and Bushweller JH. Structure of the AML1-ETO eTAFH domain-HEB peptide complex and its contribution to AML1-ETO activity. Blood. 2009;113:3558–67.

49. Corpora T, Roudaia L, Oo ZM, Chen W, Manuylova E, Cai X, Chen MJ, Cierpicki T, Speck NA and Bushweller JH. Structure of the AML1-ETO NHR3-PKA(RIIalpha) complex and its contribution to AML1-ETO activity. J Mol Biol. 2010;402: 560–77.

50. Plevin MJ, Zhang J, Guo C, Roeder RG and Ikura M. The acute myeloid leukemia fusion protein AML1-ETO targets E proteins via a paired amphipathic helix-like TBP-associated factor homology domain. Proc Natl Acad Sci U S A. 2006;103:10242–7.

51. Wei Y, Liu S, Lausen J, Woodrell C, Cho S, Biris N, Kobayashi N, Wei Y, Yokoyama S and Werner MH. A TAF4-homology domain from the corepressor ETO is a docking platform for positive and negative regulators of transcription. Nat Struct Mol Biol. 2007;14:653–61.

52. Yan M, Ahn EY, Hiebert SW and Zhang DE. RUNX1/AML1 DNA binding domain and ETO/MTG8 NHR2 dimerization domain are critical to AML1-ETO9a leukemogenesis. Blood. 2008:883–6.

53. Wichmann C, Becker Y, Chen-Wichmann L, Vogel V, Vojtkova A, Herglotz J, Moore S, Koch J, Lausen J, Mantele W, Gohlke H and Grez M. Dimer-tetramer transition controls RUNX1/ETO leukemogenic activity. Blood. 2010;116:603–13.

54. Kwok C, Zeisig BB, Qiu J, Dong S and So CW. Transforming activity of AML1-ETO is independent of CBFbeta and ETO interaction but requires formation of homo-oligomeric complexes. Proc Natl Acad Sci U S A. 2009;106:2853–8.

55. Ahn EY, Yan M, Malakhova OA, Lo MC, Boyapati A, Ommen HB, Hines R, Hokland P and Zhang DE. Disruption of the NHR4 domain structure in AML1-ETO abrogates SON binding and promotes leukemogenesis. Proc Natl Acad Sci U S A. 2008;105:17103–8.

56. Grisolano JL, O'Neal J, Cain J and Tomasson MH. An activated receptor tyrosine kinase, TEL/PDGFßR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci USA. 2003;100:9506–11.

57. Wang YY, Zhao LJ, Wu CF, Liu P, Shi L, Liang Y, Xiong SM, Mi JQ, Chen Z, Ren R and Chen SJ. C-KIT mutation cooperates with full-length AML1-ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci U S A. 2011;108:2450–5.

58. Schessl C, Rawat VP, Cusan M, Deshpande A, Kohl TM, Rosten PM, Spiekermann K, Humphries RK, Schnittger S, Kern W, Hiddemann W, Quintanilla-Martinez L, Bohlander SK, Feuring-Buske M and Buske C. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. J Clin Invest. 2005;115:2159–68.

59. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG and Lazar MA. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol. 1998;18:7185–91.

60. Lutterbach B, Westendorf JJ, Linggi B, Patten A, Moniwa M, Davie JR, K.D. H, Bardwell VJ, Lavinsky RM, Rosenfeld MG, Glass C, Seto E and Hiebert SW. ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. Mol Cell Biol. 1998;18:7176–84.

61. Wang J, Hoshino T, Redner RL, Kajigaya S and Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC complex. Proc Natl Acad Sci USA. 1998;95:10860–65.

62. Hackanson B, Abdelkarim M, Jansen JH and Lubbert M. NHR4 domain mutations of ETO are probably very infrequent in AML1-ETO positive myeloid leukemia cells. Leukemia. 2010;24:860–1.

63. Wang L, Gural A, Sun XJ, Zhao X, Perna F, Huang G, Hatlen MA, Vu L, Liu F, Xu H, Asai T, Deblasio T, Menendez S, Voza F, Jiang Y, Cole PA, Zhang J, Melnick A, Roeder RG and Nimer SD. The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. Science. 2011;333:765–9.

64. Amann JM, Nip J, Strom DK, Lutterbach B, Harada H, Lenny N, Downing JR, Meyers S and Hiebert SW. ETO, a target of t(8;21) in acute leukemia, makes distinct contacts with multiple histone deacetylases and binds mSin3A through its oligomerization domain. Mol Cell Biol. 2001;21:6470–83.

65. Park S, Speck NA and Bushweller JH. The role of CBFbeta in AML1-ETO's activity. Blood. 2009;114:2849–50.

66. Roudaia L, Cheney MD, Manuylova E, Chen W, Morrow M, Park S, Lee CT, Kaur P, Williams O, Bushweller JH and Speck NA. CBFbeta is critical for AML1-ETO and TEL-AML1 activity. Blood. 2009;113:3070–9.
67. Kwok C, Zeisig BB, Dong S and So CW. The role of CBFbeta in AML1-ETO's activity. Blood. 2010;115:3176–7.
68. Wildonger J and Mann RS. The t(8;21) translocation converts AML1 into a constitutive transcriptional repressor. Development. 2005;132:2263–72.
69. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EH and Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell. 2002;1:63–74.
70. Oravecz-Wilson KI, Philips ST, Yilmaz OH, Ames HM, Li L, Crawford BD, Gauvin AM, Lucas PC, Sitwala K, Downing JR, Morrison SJ and Ross TS. Persistence of leukemia-initiating cells in a conditional knockin model of an imatinib-responsive myeloproliferative disorder. Cancer Cell. 2009;16:137–48.
71. Rubnitz JE, Raimondi SC, Halbert AR, Tong X, Srivastava DK, Razzouk BI, Pui CH, Downing JR, Ribeiro RC and Behm FG. Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: a single institution's experience. Leukemia. 2002;16:2072–7.
72. Raimondi SC, Chang MN, Ravindranath Y, Behm FG, Gresik MV, Steuber CP, Weinstein HJ and Carroll AJ. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a cooperative pediatric oncology group study-POG 8821. Blood. 1999;94:3707–16.
73. Pession A, Rondelli R, Basso G, Rizzari C, Testi AM, Fagioli F, De Stefano P and Locatelli F. Treatment and long-term results in children with acute myeloid leukaemia treated according to the AIEOP AML protocols. Leukemia. 2005;19:2043–53.
74. Perel Y, Auvrignon A, Leblanc T, Michel G, Reguerre Y, Vannier JP, Dalle JH, Gandemer V, Schmitt C, Mechinaud F, Lejars O, Piguet C, Couillaud G, Pautard B, Landman-Parker J, Thuret I, Aladjidi N, Baruchel A and Leverger G. Treatment of childhood acute myeloblastic leukemia: dose intensification improves outcome and maintenance therapy is of no benefit—multicenter studies of the French LAME (Leucemie Aigue Myeloblastique Enfant) Cooperative Group. Leukemia. 2005;19:2082–9.
75. Wiemels JL, Xiao Z, Buffler PA, Maia AT, Ma X, Dicks BM, Smith MT, Zhang L, Feusner J, Wiencke J, Pritchard-Jones K, Kempski H and Greaves M. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002;99:3801–5.
76. Nucifora G, Larson RA and Rowley JD. Persistence of the 8;21 translocation in patients with acute myeloid leukemia type M2 in long-term remission. Blood. 1993;82:712–5.
77. Song J, Mercer D, Hu X, Liu H and Li MM. Common leukemia- and lymphoma-associated genetic aberrations in healthy individuals. J Mol Diagn. 2011;13:213–19.
78. Basecke J, Cepek L, Mannhalter C, Krauter J, Hildenhagen S, Brittinger G, Trumper L and Griesinger F. Transcription of AML1/ETO in bone marrow and cord blood of individuals without acute myelogenous leukemia. Blood. 2002;100:2267–8.
79. Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, Vukosavljevic T, Perrotti D, Vardiman JW, Carroll AJ, Kolitz JE, Larson RA and Bloomfield CD. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol. 2006;24:3904–11.
80. Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB, Colapietro P, Nichelatti M, Pezzetti L, Lunghi M, Cuneo A, Viola A, Ferrara F, Lazzarino M, Rodeghiero F, Pizzolo G, Larizza L and Morra E. Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. Blood. 2006;107:3463–68.
81. Schnittger S, Kohl TM, Haferlach T, Kern W, Hiddemann W, Spiekermann K and Schoch C. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. Blood. 2006;107:1791–99.
82. Osato M, Asou N, Abdalla E, Hoshino K, Yamasaki H, Okubo T, Suzushima H, Takatsuki K, Kanno T, Shigesada K and Ito Y. Biallelic and heterozygous point mutations in the Runt domain of the *AML1/PEBP2aB* gene associated with myeloblastic leukemias. Blood. 1999;93:1817–24.

83. Song W-J, Sullivan MG, Legare RD, Hutchings S, Tan X, Kufrin D, Ratajczak J, Resende IC, Haworth C, Hock R, Loh M, Felix C, Roy D-C, Busque L, Kurnit D, Willman C, Gewirtz AM, Speck NA, Bushweller JH, Li FP, Gardiner K, Poncz M, Maris JM and Gilliland DG. Haploinsufficiency of *CBFA2* (*AML1*) causes familial thrombocytopenia with propensity to develop acute myelogenous leukamia (FPD/AML). Nature Genet. 1999;23:166–75.

84. Imai Y, Kurokawa M, Izutsu K, Hangaishi A, Takeuchi K, Maki K, Ogawa S, Chiba S, Mitani K and Hirai H. Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis. Blood. 2000;96:3154–60.

85. Gelsi-Boyer V, Trouplin V, Adelaide J, Aceto N, Remy V, Pinson S, Houdayer C, Arnoulet C, Sainty D, Bentires-Alj M, Olschwang S, Vey N, Mozziconacci MJ, Birnbaum D and Chaffanet M. Genome profiling of chronic myelomonocytic leukemia: frequent alterations of RAS and RUNX1 genes. BMC Cancer. 2008;8:299.

86. Kuo MC, Liang DC, Huang CF, Shih YS, Wu JH, Lin TL and Shih LY. RUNX1 mutations are frequent in chronic myelomonocytic leukemia and mutations at the C-terminal region might predict acute myeloid leukemia transformation. Leukemia. 2009;23:1426–31.

87. Radtke I, Mullighan CG, Ishii M, Su X, Cheng J, Ma J, Ganti R, Cai Z, Goorha S, Pounds SB, Cao X, Obert C, Armstrong J, Zhang J, Song G, Ribeiro RC, Rubnitz JE, Raimondi SC, Shurtleff SA and Downing JR. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc Natl Acad Sci U S A. 2009;106:12944–9.

88. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, Pettenati MJ, Patil SR, Rao KW, Watson MS, Koduru PR, Moore JO, Stone RM, Mayer RJ, Feldman EJ, Davey FR, Schiffer CA, Larson RA and Bloomfield CD. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100:4325–36.

89. Nucifora G, Birn DJ, Espinosa III R, Erickson P, LeBeau MM, Roulston D, McKeithan TW, Drabkin H and Rowley JD. Involvement of the AML1 gene in the t(3;21) in therapy-related leukemia and in chronic myeloid leukemia in blast crisis. Blood. 1993;81:2728–34.

90. Harada H, Harada Y, Tanaka H, Kimura A and Inaba T. Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. Blood. 2003;101:673–80.

91. Steensma DP, Gibbons RJ, Mesa RA, Tefferi A and Higgs DR. Somatic point mutations in RUNX1/CBFA2/AML1 are common in high-risk myelodysplastic syndrome, but not in myelofibrosis with myeloid metaplasia. Eur J Haematol. 2005;74:47–53.

92. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, Kantarjian H, Raza A, Levine RL, Neuberg D and Ebert BL. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364:2496–506.

93. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, Sanz M, Vallespi T, Hamblin T, Oscier D, Ohyashiki K, Toyama K, Aul C, Mufti G and Bennett J. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89:2079–88.

94. Dicker F, Haferlach C, Sundermann J, Wendland N, Weiss T, Kern W, Haferlach T and Schnittger S. Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. Leukemia. 2010;24:1528–32.

95. Flach J, Dicker F, Schnittger S, Schindela S, Kohlmann A, Haferlach T, Kern W and Haferlach C. An accumulation of cytogenetic and molecular genetic events characterizes the progression from MDS to secondary AML: an analysis of 38 paired samples analyzed by cytogenetics, molecular mutation analysis and SNP microarray profiling. Leukemia. 2011;25:713–8.

96. Zharlyganova D, Harada H, Harada Y, Shinkarev S, Zhumadilov Z, Zhunusova A, Tchaizhunusova NJ, Apsalikov KN, Kemaikin V, Zhumadilov K, Kawano N, Kimura A and Hoshi M. High frequency of AML1/RUNX1 point mutations in radiation-associated myelodysplastic syndrome around Semipalatinsk nuclear test site. J Radiat Res (Tokyo). 2008;49:549–55.

97. Alter BP. Cancer in Fanconi anemia, 1927–2001. Cancer. 2003;97:425–40.

98. Quentin S, Cuccuini W, Ceccaldi R, Nibourel O, Pondarre C, Pages MP, Vasquez N, Dubois d'Enghien C, Larghero J, Peffault de Latour R, Rocha V, Dalle JH, Schneider P, Michallet M, Michel G, Baruchel A, Sigaux F, Gluckman E, Leblanc T, Stoppa-Lyonnet D, Preudhomme C, Socie G and Soulier J. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. Blood. 2011;117:e161–170.

99. Kohlmann A, Grossmann V, Klein HU, Schindela S, Weiss T, Kazak B, Dicker F, Schnittger S, Dugas M, Kern W, Haferlach C and Haferlach T. Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. J Clin Oncol. 2010;28:3858–65.

100. Owen C, Barnett M and Fitzgibbon J. Familial myelodysplasia and acute myeloid leukaemia—a review. Br J Haematol. 2008;140:123–32.

101. Owen C. Insights into familial platelet disorder with propensity to myeloid malignancy (FPD/AML). Leuk Res. 2010;34:141–142.

102. Liew E and Owen CJ. Familial myelodysplastic syndromes—a review of the literature. Haematologica. 2011;96:1536–42.

103. Matheny CJ, Speck ME, Cushing PR, Zhou Y, Corpora T, Regan M, Newman M, Roudaia L, Speck CL, Gu TL, Griffey SM, Bushweller JH and Speck NA. Disease mutations in RUNX1 and RUNX2 create nonfunctional, dominant-negative, or hypomorphic alleles. Embo J. 2007;26:1163–75.

104. Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y, Nakajima H, Nosaka T, Inaba T and Kitamura T. AML1 mutations induced MDS and MDS/AML in a mouse BMT model. Blood. 2008;111:4297–308.

105. Sun W and Downing JR. Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs while simultaneously inducing an increase in more mature progenitors. Blood. 2004;104:3565–72.

106. Marcucci G, Mrozek K, Ruppert AS, Maharry K, Kolitz JE, Moore JO, Mayer RJ, Pettenati MJ, Powell BL, Edwards CG, Sterling LJ, Vardiman JW, Schiffer CA, Carroll AJ, Larson RA and Bloomfield CD. Prognostic factors and outcome of core binding factor acute myeloid leukemia patients with t(8;21) differ from those of patients with inv(16): a Cancer and Leukemia Group B study. J Clin Oncol. 2005;23:5705–17.

107. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, Schifano E, Booth J, van Putten W, Skrabanek L, Campagne F, Mazumdar M, Greally JM, Valk PJ, Lowenberg B, Delwel R and Melnick A. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell. 2010;17:13–27.

108. Silva FP, Swagemakers SM, Erpelinck-Verschueren C, Wouters BJ, Delwel R, Vrieling H, van der Spek P, Valk PJ and Giphart-Gassler M. Gene expression profiling of minimally differentiated acute myeloid leukemia: M0 is a distinct entity subdivided by RUNX1 mutation status. Blood. 2009:3001–7.

Critical Reviews™ in Oncogenesis, 16(1–2), 93–102 (2011)

C/EBPα Dysregulation in AML and ALL

Ido Paz-Priel & Alan D. Friedman*

Division of Pediatric Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland

*Address all correspondence to: Dr. Alan D. Friedman. Division of Pediatric Oncology, Johns Hopkins University School of Medicine, CRB I, Rm. 253, 1650 Orleans St., Baltimore, MD 21231, afriedm2@jhmi.edu

ABSTRACT: The transcription factor CCAAT/enhancer binding protein α (C/EBPα) is a critical regulator of myeloid development, directing granulocyte, and monocyte differentiation. As such, it is dysregulated in more than half of patients with acute myeloid leukemia (AML). C/EBPα expression is suppressed as result of common leukemia-associated genetic and epigenetic alterations such as AML1-ETO, BCR-ABL, FLT3-ITD, or *CEBPA* promoter methylation. In addition, 10–15% of patients with AML with intermediate risk cytogenetics are characterized by mutations of the *CEBPA* gene. Two classes of mutations are described. N-terminal changes result in expression of a truncated dominant negative C/EBPαp30 isoform. C-terminal mutations are in-frame insertions or deletions resulting in alteration of the leucine zipper preventing dimerization and DNA binding. Often, patients carry both N- and C-terminal mutations each affecting a different allele, and a mouse model recapitulates the human phenotype. Patients with mutated *CEBPA* AML comprise a clinically distinct group with favorable outcome consistently seen in patients with biallelic mutations. In addition, C/EBP family members are aberrantly expressing from the immunoglobulin heavy chain locus in 2% of pre-B ALLs. This review summarizes the normal hematopoietic developmental pathways regulated by C/EBPα and discusses the molecular pathways involved in mutated *CEBPA* AML and ALL.

KEY WORDS: leukemia, myeloid, differentiation, hematopoiesis

ABBREVIATIONS

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BR-LZ or bZIP, basic region-leucine zipper; C/EBPα, CCAAT/enhancer binding protein α; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; HSC, hematopoietic stem cell; miRNA, microRNA; TAD, trans-activation domain

I. REGULATION OF NORMAL MYELOID DEVELOPMENT BY C/EBPα

A. The Biochemistry of C/EBPα

CCAAT/enhancer binding protein α (C/EBPα) contains 358 amino acid residues, with the intron-less human *CEBPA* gene located on chromosome 19q13.1. C/EBPα has an α-helical, 86 residue, C-terminal basic region-leucine zipper (BR-LZ or bZIP) DNA-binding domain.[1] The LZ contains a hydrophobic surface that allows homo-dimerization or hetero-dimerization with other bZIP proteins as a coiled-coil structure, thereby positioning the more N-terminal BR to enter the major groove and contact DNA (Figure 1).[2] Additional members of the C/EBP family of bZIP transcription factors include C/EBPβ, C/EBPδ, and C/EBPε. C/EBP as obligatory homo- or hetero-dimers bind the DNA motif 5′-T(T/G)NNGNAA(T/G). C/EBP proteins also heterodimerize with members of the CREB or AP-1 families of bZIP proteins to bind hybrid DNA elements.[3,4] Once bound to DNA, C/EBPα activates transcription via its two N-terminal trans-activation domains.[5]

Full-length C/EBPα is 42 kd in molecular weight. Initiation of translation from an internal ATG located at amino acid 120 of the human protein leads to co-expression of a shorter, 30 kd isoform in a subset of normal tissues, though typically the p30 isoform is less abundant (Figure 1).[6] C/EBPαp30 retains the ability to dimerize and bind DNA but lacks a potent trans-activation domain (TAD), allowing the p30 isoform to dominantly inhibit trans-activation by C/EBPα42, at least for a subset of C/EBP target genes.

0893-9675/11/$35.00

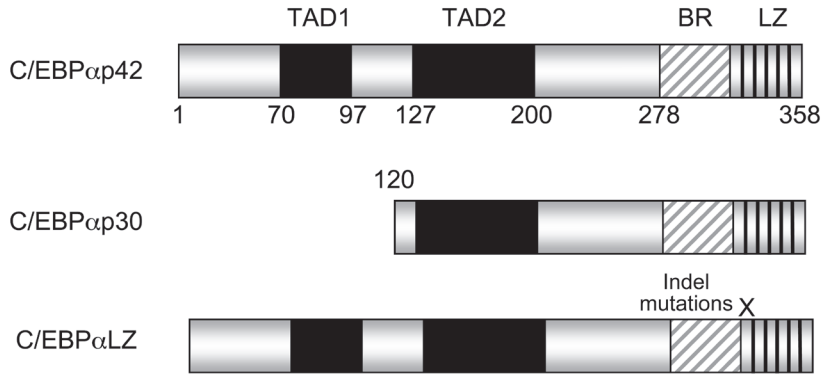


FIGURE 1. Diagram depicting C/EBPαp42, truncated C/EBPαp30, and the location of C/EBPαLZ in-frame insertions and deletions. BR, basic region; LZ, leucine zipper; TAD, trans-activation domain.

B. The Biology of C/EBPα During Normal Myelopoiesis

Within hematopoiesis, C/EBPα is specifically expressed in granulocytes, monocytes, and eosinophils,[7] though it is also found in hepatocytes, adipocytes, and type II pneumocytes.[8] Low-level C/EBPα expression is detectable in the hematopoietic stem cell (HSC) population, and expression increases as these cells develop into the common myeloid progenitor (CMP) and subsequently into the granulocyte-monocyte progenitor (GMP); C/EBPα levels finally diminish as immature myeloid cells mature to neutrophils or monocytes.[7,9] Deletion of the C/EBPα gene leads to arrest at the CMP to GMP transition, with reduced formation of both granulocytes and monocytes.[9] When expressed in 32Dcl3 cells, representative of granulocytic progenitors, exogenous C/EBPα directs granulopoiesis.[10] However, transduction of marrow cells with C/EBPα leads to increased monopoiesis at the expense of granulopoiesis;[11] this may reflect formation of C/EBP:AP-1 heterodimers, as C/EBPα proteins containing artificial acid and basic LZs that force their homodimerization do not induce increased monopoiesis, whereas forced heterodimerization of C/EBPα with c-Jun or c-Fos strongly favors monocytic development.[12] Induction of AP-1 proteins in myeloid cell lines using phorbol ester or IL-6 allows formation of endogenous C/EBP:AP-1 complexes during monopoiesis, whereas C/EBPα homodimers are more abundant in cells undergoing granulopoiesis in response to G-CSF.[3] Reduced levels or activity of C/EBPα may be sufficient for monopoiesis via interaction with AP-1 proteins but not for granulopoiesis, as suggested by the finding that absence of RUNX1 or NF-κB p50 leads to approximately 25% of normal C/EBPα protein expression in marrow and diminished granulopoiesis.[11,13]

In addition to interacting with AP-1 proteins, C/EBPα may stimulate monopoiesis by inducing transcription of the *PU.1* gene via interaction with its promoter and -14 kb distal enhancer.[14,15] Notably, in contrast to C/EBPα reduced PU.1 protein levels due to gene or enhancer deletion favors granulopoiesis over monopoiesis,[16–18] and exogenous C/EBPα induces granulopoiesis rather than monopoiesis in marrow cells lacking the *PU.1* distal enhancer.[15] A summary of the transcriptional control of myeloid development by RUNX1, C/EBPα, PU.1 and cooperating factors is shown (Figure 2).

As a major regulator of differentiation C/EBPα has a strong anti-proliferative effect,[19,20] and in myeloid cells it inhibits the progression from G1 to S phase.[10] This effect is independent of DNA binding[21] and occurs at least in part through protein:protein interaction with E2F mediated by the basic region.[22] As a result, C/EBPα down regulates pro-proliferative transcription factors such as c-Myc. [23] Inhibition of cell cycle is part of the differentiation program

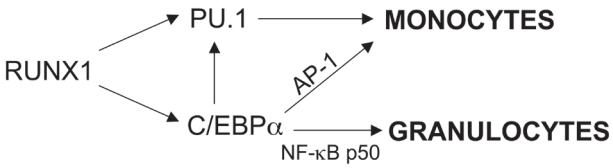


FIGURE 2. Model for the transcriptional control of myeloid development by RUNX1, C/EBPα, and PU.1. RUNX1 stimulates transcription of the genes encoding C/EBPα and PU.1, and C/EBPα also activates the PU.1 gene. C/EBPα then hetero-dimerizes with AP-1 proteins and cooperates with PU.1 to favor monopoiesis, whereas C/EBPα homo-dimers cooperate with NF-κB p50 to favor granulopoiesis.

induced by C/EBPα but is not sufficient by itself to direct terminal differentiation.[21]

Cytokine signals cooperate with C/EBPα to regulate myeloid development. Study of individual GMP exposed to G-CSF or M-CSF demonstrates that these cytokines not only provide survival and proliferative signals but also contribute to lineage specification.[24] Comparison of M-CSF with G-CSF signaling in lineage-negative marrow cells demonstrates that M-CSF more potently activates ERK via PLCγ, whereas G-CSF more potently activates STAT3 and specifically induces SHP2 tyrosine phosphorylation.[25] Moreover, ERK inhibitors reduce formation of monocytic progenitor colonies whereas a SHP2 inhibitor reduces formation of granulocytic colonies. shRNA-mediated knockdown of SHP2 similarly reduces granulopoiesis via inhibition of *CEBPA* gene transcription.[26] G-CSF activation of SHP2 may increase *CEBPA* mRNA expression due to the ability of SHP2 to increase the activity of RUNX1,[27] with RUNX1 then directly inducing *CEBPA* gene transcription.[13] ERK phosphorylates C/EBPα serine 21, reducing the activity of C/EBPα, perhaps by weakening its N-terminal TAD.[28] Partial inactivation of C/EBPα by ERK downstream of M-CSF receptor would be expected to favor monopoiesis; in particular, weakened C/EBPα might be incapable of directing granulopoiesis but remain able to hetero-dimerize with AP-1 proteins to direct monopoiesis.

II. C/EBPα DYSREGULATION DURING MYELOID TRANSFORMATION

Two mechanisms affect C/EBPα function in AML: reduced expression as a downstream result of other AML-related mutation or mutation of the *CEBPA* gene.

A. Inhibition of C/EBPα in AML

Inhibition of C/EBPα expression or activity occurs via several mechanisms in different subsets of AML. As discussed above, deletion of the RUNX1 gene reduces *CEBPA* expression, and RUNX1 might directly activate *CEBPA* transcription. Related to these findings, mutation of RUNX1 leading to reduced RUNX1 levels or expression or fusion proteins that dominantly inhibit RUNX1 activity occurs in at least 30% of AML cases.[29] In particular, t(8;21) leads to expression of RUNX1-ETO, which binds DNA via RUNX1 *cis* elements to repress target expression; inv(16) leads to expression of CBFβ-SMMHC, which interacts with RUNX1 to either sequester RUNX1 off chromatin or inhibit its activity on chromatin; t(3;21) expresses RUNX1-MDS1/EVI1, which also inhibits RUNX1 activity; and point mutations that inactivate RUNX1, the majority heterozygous, are present in a subset of patients.[29,30] RUNX1-ETO directly represses the *CEBPA* promoter, and blasts from patients with t(8;21)-associated AML indeed have reduced C/EBPα protein levels.[31] Abnormal expression of EVI1 through translocations involving chromosome 3, is associated with high risk for AML or MDS. EVI1 fusion protein expression is associated with translational suppression of C/EBPα expression. [32]

Signaling pathways activated in AML can inhibit C/EBPα expression or activity. The activated receptor tyrosine kinase receptor mutant, FLT3-ITD, found in 30% of AML cases, reduces *CEBPA* transcription and leads to ERK modification of C/EBPα S21 to reduce the activity of C/EBPα.[33,34] BCR-ABL, an intracellular, constitutively active tyrosine kinase, inhibits translation of the *CEBPA* mRNA.[35] Trib2 induces C/EBPα proteosomal degradation, dependent upon interaction with COP1.[36]

CEBPA promoter methylation is found in half of AML cases, often as the indirect consequence of several of the above mechanisms that down-modulate *CEBPA* transcription, and it is most commonly associated with inv(16) and t(15;17).[37] In addition, a subgroup of AML whose gene expression profile aggregates with mutated *CEBPA* AML is characterized by *CEBPA* silencing through promoter hypermethylation and expression of T cell markers. The majority of these patients harbor activating *NOTCH1* mutations and have poor outcome.[38,39]

Finally, the *CEBPA* gene open-reading frame itself is subject to mutation in approximately 10% of AML cases. Biologically and clinically this is a distinct subtype of AML as recognized in the 2008 World Health Organization classification of myeloid neoplasms[40] and elaborated on the next section.

B. *CEBPA* Gene Mutation in AML

As a key regulator of myeloid differentiation *CEBPA* is mutated in approximately 5–15% of patients with AML.[41–45] Two categories *CEBPA* mutations are found in AML cases (Figure 1).[42,44] Interestingly, C/EBPα null mutations are rare, and the mutated proteins are expressed by the leukemic blasts, suggesting a selective pressure and an active role in leukemogenesis for the mutated C/EBPα proteins. N-terminal mutations typically lead to premature termination and translational reinitiation at methionine 120, leading to expression of the N-terminally-truncated C/EBPαp30, lacking a major TAD. C/EBPαp30 retains the capacity to bind DNA and to hetero-dimerize with C/EBPαp42, thereby dominantly inhibiting C/EBPαp42-mediated trans-activation. In addition, C/EBPαp30 induces expression of Ubc9, an E2 conjugating enzyme, which in turn sumoylates C/EBPαp42 on lysine 161. Sumoylated C/EBPαp42 has reduced capacity to activate transcription or slow proliferation.[46,47] C-terminal mutations typically occur in the vicinity of the first α-helix of the LZ, preventing dimerization and therefore preclude DNA-binding. Strikingly, these C/EBPαLZ variants are in-frame insertions or deletions, indicating that the resulting proteins contribute to leukemic transformation. Indeed, although they themselves cannot bind DNA, several C/EBPαLZ oncoproteins inhibit apoptosis via induction of bcl-2 or Flice inhibitory protein, dependent upon interaction of their BR with NF-κB p50 bound to DNA in the promoter regions of these target genes.[48,49] Interaction of C/EBPαLZ oncoproteins with NF-κB p50 displaces HDACs, inducing transcriptional derepression, with the C/EBPα TAD then directing transcriptional activation.[50]

In two-thirds of AML cases harboring *CEBPA* gene mutations, one allele harbors an N-terminal variant and the other allele a C-terminal variant. Of note, in a mouse model, C/EBPαp30 and a C/EBPαLZ oncoprotein synergistically contribute to AML formation.[51]

Patients with mutated *CEBPA* AML typically present with myeloblastic French-American-British types M1 or M2 morphology and associated with the following immunophenotype: HLA-DR(+), CD7(+), CD13(+), CD14(-), CD15(+), CD33(+), CD34(+).[41–44,52] *CEBPA* mutations occur almost exclusively in patients with intermediate-risk cytogenetics and predominantly in those with normal karyotype. Moreover, other class II mutations such as core binding factor (CBF) leukemia, mutated NPM1, or MLL-PTD only rarely overlap with mutated *CEBPA*.[41,43,45,53]

Multiple cooperative groups reported that patients with mut*CEBPA* have significantly improved outcome compared to wt*CEBPA*, in par with patients with favorable risk cytogenetics, such as CBF leukemia. Further analysis of several,[53–57] but not all,[43,57] studies suggests that this benefit is restricted to patients with biallelic mutations. Further, patients with biallelic *CEBPA* mutations have a specific gene expression pattern, while monoallelic mutations do not aggregate in a specific pattern. [56,57] Of particular importance to risk stratification of normal karyotype AML is the interplay of several prognostic markers. For example, mutated *NPM1*/wt-FLT3 cases have a favorable outcome while wt-*NPM1*/FLT-ITD is associated with a dismal prognosis. *NPM1* is rarely mutated in patients with biallelic mut*CEBPA*, and these patients have a 2–4-fold lower incidence of FLT3-ITD.[41,45,53,57] Interpretation of the combinatorial effect of FLT3-ITD and mut*CEBPA* is compli-

cated by inconsistent analysis of mono vs. biallelic mutations and FLT3-ITD allelic ratio. However, in several reports mutCEBPA predicts favorable outcome independent of FLT3 status.[41,43,57–59] The incidence of FLT3-ITD or mutated *NPM1* is similar in wt*CEBPA* and monoallelic mut*CEBPA*.[53,57]

Germline *CEBPA* N-terminal mutation were found in pedigrees with familial AML, and progression to AML is typically associated with acquisition of a somatic C-terminal mutation.[57,60,61] Importantly, approximately 10% of patients with mut*CEBPA* AML harbor a germline mutation.[57,61]

MicroRNAs (miRNA) are small (20–22 bp) noncoding RNAs that play a key role in transcriptional regulation. miRNAs silence target genes by binding untranslated regions of mRNA, resulting in translation inhibition or cleavage of coding mRNA. Several miRNAs are induced by C/EBPα in the course of normal myeloid differentiation.[62–64] Dysregulation of miRNA expression is increasingly appreciated as a common feature of cancer. Global miRNA expression patterns accurately classify molecular subtypes of AML, including mut*CEBPA* leukemia that is characterized by down-regulation of multiple miRNAs including the 181 family and miR-34a.[59,65] C/EBPα induces expression of miR-34a to silence E2F3 and suppress proliferation during normal granulopoiesis. Restoration of miR-34a expression in mut*CEBPA* AML blasts slows proliferation and induces differentiation.[64]

In contrast to *RUNX1* and *CEBPA*, mutation of the PU.1 gene is rare in AML cases, despite the finding that deletion of the PU.1–14 kb distal enhancer in mice leads to 20% of control PU.1 expression and highly penetrant AML.[18] Nevertheless, as both RUNX1 and C/EBPα activate the PU.1 distal enhancer,[15,66] their diminished expression or activity would be expected to lead to reduced PU.1 levels, contributing to AML formation.

In addition to stimulating myeloid differentiation and inhibiting apoptosis in cooperation with NF-κB p50, C/EBPα inhibits G1 to S cell cycle transition via direct interaction with E2F in myeloid progenitors, and this effect requires integrity of the C/EBPα N-terminus via an unclear mechanism.[20–22] Thus, reduced C/EBPα expression or its N-terminal mutation might contribute to the myeloid transformation in part via removal of this cell cycle inhibitory effect.

III. C/EBPα OVEREXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Within hematopoiesis, C/EBPα is restricted to the myeloid lineages and it is not expressed in lymphocytes or their progenitors or in the erythroid/megakaryocytic lineages.[7] Moreover, ectopic expression of C/EBPα leads to supression of Pax5, a central transcription factor in B-cell development, and redirects the fate of B cells into macrophages.[67] Overexpression of wild-type C/EBPα occurs in B precursor ALL carrying the t(14;19)(q32;q13) translocation which juxtaposes *CEBPA* and the immunoglobulin heavy chain enhancer locus.[68] The leukemic blasts from patients with pre-B ALL carrying this translocation express high levels of wild-type C/EBPα mRNA and protein.[68] Importantly, no mutations were found in *CEBPA*, and the AML-associated p30 isoform[44] was not excessively expressed. Akasaka et al. extended these findings and demonstrated involvement of other C/EBP family members in translocations with the IgH locus in pre-B ALL patients, and consequently overexpression of wild-type C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, or C/EBPγ in approximately 2% of patients with pre B ALL.[69] The similar phenotype resulting from translocations of the various C/EBPs suggests an important role for the bZIP domain that is highly conserved among the different family members. Of note, this region mediates protein:protein interaction with NF-κB p50 and consequent induction of bcl-2 and FLIP and protection from apoptosis.[22,48,49]

IV. SUMMARY

C/EBPα is a key mediator of normal myeloid differentiation, contributing to both granulopoiesis and monopoiesis. C/EBPα also inhibits cell cycle progression and stimulates cell survival in cooperation with NF-κB p50. Alterations in the *CEBPA* gene or in pathways that down-modulate C/EBPα expression at the transcriptional, translational, or posttranslational

levels likely contribute to myeloid transformation by inhibiting myeloid differentiation while favoring myeloid progenitor cell cycle progression. In addition, C/EBPαp30, C/EBPαLZ variants, or residual wild-type C/EBPα may also contribute to myeloid transformation by inhibiting apoptosis. Restoration of C/EBPα expression in AML might provide a means to induce cell differentiation and slow cell proliferation to contribute to AML therapy.

ACKNOWLEDGMENTS

I.P.P. is a St. Baldrick's Foundation Scholar and the recipient of a Hyundai Hope on Wheels Award. A.D.F. is supported by National Institutes of Health (Grant Nos. R01 HL089176 and U01 HL099775/HL100397) and the Maryland Stem Cell Research Foundation.

REFERENCES

1. Landschulz WH, Johnson PF, McKnight SL. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science. 1989;243:1681–8.
2. Miller M, Shuman JD, Sebastian T, Dauter Z, Johnson PF. Structural basis for DNA recognition by the basic region leucine zipper transcription factor CCAAT/enhancer-binding protein α. J Biol Chem. 2003;278:15178–84.
3. Hong S, Skaist AM, Wheelan SJ, Friedman AD. AP-1 protein induction during monopoiesis favors C/EBP:AP-1 heterodimers over C/EBP homodimerization and stimulates FosB transcription. J Leukoc Biol. 2011:90:643–51.
4. Shuman JD, Cheong J, Coligan JE. ATF–2 and C/EBPα can form a heterodimeric DNA binding complex *in vitro*. Functional implications for transcriptional regulation. J Biol Chem. 1997;272:12793–800.
5. Friedman AD, McKnight SL. Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. Genes Dev. 1990;4:1416–26.
6. Ossipow V, Descombes P, Schibler U. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci U S A. 1993;90:8219–23.
7. Scott LM, Civin CI, Rorth P, Friedman AD. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood. 1992;80:1725–35.
8. Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, McKnight SL. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. 1989;3:1146–56.
9. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, Shigematsu H, Levantini E, Huettner CS, Lekstrom-Himes JA, Akashi K, Tenen DG. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBPα. Immunity. 2004;21:853–63.
10. Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPα bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. Blood. 1999;94:560–71.
11. Wang D, D'Costa J, Civin CI, Friedman AD. C/EBPα directs monocytic commitment of primary myeloid progenitors. Blood. 2006;108:1223–9.
12. Cai DH, Wang D, Keefer J, Yeamans C, Hensley K, Friedman AD. C/EBPα:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBPα homodimers or AP-1. Oncogene. 2007;27:2772–9.
13. Guo H, Friedman AD. Myeloid expansion in the absence of RUNX1 is associated with increased monopoiesis, reduced granulopoiesis, and diminished *CEBPA* gene expression, effects of potential relevance to myeloid transformation Blood. 2010;116:1296a.
14. Kummalue T, Friedman AD. Cross-talk between regulators of myeloid development: C/EBPα binds and activates the promoter of the PU.1 gene. J Leukoc Biol. 2003;74:464–70.
15. Yeamans C, Wang D, Paz-Priel I, Torbett BE, Tenen DG, Friedman AD. C/EBPα binds and activates the PU.1 distal enhancer to induce monocyte lineage commitment. Blood. 2007;110:3136–42.

16. Dahl R, Walsh JC, Lancki D, Laslo P, Iyer SR, Singh H, Simon MC. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPα ratio and granulocyte colony-stimulating factor. Nat Immunol. 2003;4:1029–36.

17. Dakic A, Metcalf D, Di Rago L, Mifsud S, Wu L, Nutt SL. PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. J Exp Med. 2005;201:1487–502.

18. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, Akashi K, Fiering S, Tenen DG. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. Nat Genet. 2004;36:624–30.

19. Friedman AD, Landschulz WH, McKnight SL. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. 1989;3: 1314–22.

20. Umek RM, Friedman AD, McKnight SL. CCAAT-enhancer binding protein: a component of a differentiation switch. Science. 1991;251:288–92.

21. Wang QF, Cleaves R, Kummalue T, Nerlov C, Friedman AD. Cell cycle inhibition mediated by the outer surface of the C/EBPα basic region is required but not sufficient for granulopoiesis. Oncogene. 2003;22:2548–57.

22. Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L, Nerlov C. E2F repression by C/EBPα is required for adipogenesis and granulopoiesis in vivo. Cell. 2001;107:247–58.

23. Johansen LM, Iwama A, Lodie TA, Sasaki K, Felsher DW, Golub TR, Tenen DG. c-Myc is a critical target for C/EBPα in granulopoiesis. Mol Cell Biol. 2001;21:3789–806.

24. Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC, Schroeder T. Hematopoietic cytokines can instruct lineage choice. Science. 2009;325:217–8.

25. Jack GD, Zhang L, Friedman AD. M-CSF elevates c-Fos and phospho-C/EBPα(S21) via ERK whereas G-CSF stimulates SHP2 phosphorylation in marrow progenitors to contribute to myeloid lineage specification. Blood. 2009;114:2172–80.

26. Zhang L, Friedman AD. SHP2 tyrosine phosphatase enhances *CEBPA* gene expression to mediate cytokine-dependent granulopoiesis. Blood. 2011;118:2266–74.

27. Huang H, Waldon Z, Chan G, Zhu H, Steen H, Feng GS, Neel B, Cantor A. Tyrosine phosphorylation of Runx1 in megakaryocytes by Src family kinases. Blood. 2010;116:327a.

28. Ross SE, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L, Wright WS, Schaufele F, Tenen DG, MacDougald OA. Phosphorylation of C/EBPα inhibits granulopoiesis. Mol Cell Biol. 2004;24:675–86.

29. Friedman AD. Leukemogenesis by CBF oncoproteins. Leukemia. 1999;13:1932–42.

30. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, Huang CF, Lee FY, Liu MC, Yao M, Huang SY, Ko BS, Hsu SC, Wu SJ, Tsay W, Chen YC, Lin LI, Tien HF. AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood. 2009;114:5352–61.

31. Pabst T, Mueller BU, Harakawa N, Schoch C, Haferlach T, Behre G, Hiddemann W, Zhang DE, Tenen DG. AML1-ETO downregulates the granulocytic differentiation factor C/EBPα in t(8;21) myeloid leukemia. Nat Med. 2001;7:444–51.

32. Helbling D, Mueller BU, Timchenko NA, Hagemeijer A, Jotterand M, Meyer-Monard S, Lister A, Rowley JD, Huegli B, Fey MF, Pabst T. The leukemic fusion gene AML1-MDS1-EVI1 suppresses *CEBPA* in acute myeloid leukemia by activation of calreticulin. Proc Natl Acad Sci U S A. 2004;101:13312–7.

33. Radomska HS, Basseres DS, Zheng R, Zhang P, Dayaram T, Yamamoto Y, Sternberg DW, Lokker N, Giese NA, Bohlander SK, Schnittger S, Delmotte MH, Davis RJ, Small D, Hiddemann W, Gilliland DG, Tenen DG. Block of C/EBPα function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. J Exp Med. 2006;203:371–81.

34. Zheng R, Friedman AD, Levis M, Li L, Weir EG, Small D. Internal tandem duplication mutation of FLT3 blocks myeloid differentiation through suppression of C/EBPα expression. Blood. 2004;103:1883–90.

35. Perrotti D, Cesi V, Trotta R, Guerzoni C, Santilli G, Campbell K, Iervolino A, Condorelli F, Gambacorti-Passerini C, Caligiuri MA, Calabretta B. BCR-ABL suppresses C/EBPα expression through inhibitory action of hnRNP E2. Nat Genet. 2002;30:48–58.

36. Keeshan K, Bailis W, Dedhia PH, Vega ME, Shestova O, Xu L, Toscano K, Uljon SN, Blacklow SC, Pear WS. Transformation by Tribbles homolog 2 (Trib2) requires both the Trib2 kinase domain and COP1 binding. Blood. 2010;116:4948–57.

37. Hackanson B, Bennett KL, Brena RM, Jiang J, Claus R, Chen SS, Blagitko-Dorfs N, Maharry K, Whitman SP, Schmittgen TD, Lübbert M, Marcucci G, Bloomfield CD, Plass C. Epigenetic modification of CCAAT/enhancer binding protein α expression in acute myeloid leukemia. Cancer Res. 2008;68:3142–51.

38. Figueroa ME, Wouters BJ, Skrabanek L, Glass J, Li Y, Erpelinck-Verschueren CA, Langerak AW, Löwenberg B, Fazzari M, Greally JM, Valk PJ, Melnick A, Delwel R. Genome-wide epigenetic analysis delineates a biologically distinct immature acute leukemia with myeloid/T-lymphoid features. Blood. 2009;113(12):2795–804.

39. Wouters BJ, Jorda MA, Keeshan K, Louwers I, Erpelinck-Verschueren CA, Tielemans D, Langerak AW, He Y, Yashiro-Ohtani Y, Zhang P, Hetherington CJ, Verhaak RG, Valk PJ, Löwenberg B, Tenen DG, Pear WS, Delwel R. Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced *CEBPA* and mutations in NOTCH1. Blood. 2007;110:3706–14.

40. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937–51.

41. Frohling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Döhner H, Döhner K. *CEBPA* mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol. 2004;22:624–33.

42. Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, Larsen RJ, Asou H, Miller CW, Hoelzer D, Koeffler HP. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein α in myelodysplastic syndromes and acute myeloid leukemias. Blood. 2002;99:1332–40.

43. Ho PA, Alonzo TA, Gerbing RB, Pollard J, Stirewalt DL, Hurwitz C, Heerema NA, Hirsch B, Raimondi SC, Lange B, Franklin JL, Radich JP, Meshinchi S. Prevalence and prognostic implications of *CEBPA* mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2009;113:6558–66.

44. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG. Dominant-negative mutations of *CEBPA*, encoding CCAAT/enhancer binding protein-α (C/EBPα), in acute myeloid leukemia. Nat Genet. 2001;27:263–70.

45. Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L, Habdank M, Späth D, Morgan M, Benner A, Schlegelberger B, Heil G, Ganser A, Döhner H; German-Austrian Acute Myeloid Leukemia Study Group. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358:1909–18.

46. Geletu M, Balkhi MY, Peer Zada AA, Christopeit M, Pulikkan JA, Trivedi AK, Tenen DG, Behre G. Target proteins of C/EBPαp30 in AML: C/EBPαp30 enhances sumoylation of C/EBPαp42 via up-regulation of Ubc9. Blood. 2007;110:3301–9.

47. Sato Y, Miyake K, Kaneoka H, Iijima S. Sumoylation of CCAAT/enhancer-binding protein α and its functional roles in hepatocyte differentiation. J Biol Chem. 2006;281:21629–39.

48. Paz-Priel I, Cai DH, Wang D, Kowalski J, Blackford A, Liu H, Heckman CA, Gombart AF, Koeffler HP, Boxer LM, Friedman AD. CCAAT/enhancer binding protein α (C/EBPα) and C/EBPα myeloid oncoproteins induce bcl-2 via interaction of their basic regions with nuclear factor-κB p50. Mol Cancer Res. 2005;3:585–96.

49. Paz-Priel I, Ghosal AK, Kowalski J, Friedman AD. C/EBPα or C/EBPα oncoproteins regulate the intrinsic and extrinsic apoptotic pathways by direct interaction with NF-κB p50 bound to the bcl-2 and FLIP gene promoters. Leukemia. 2009;23:365–74.

50. Paz-Priel I, Houng S, Dooher J, Friedman AD. C/EBPα and C/EBPα oncoproteins regulate nfkb1 and displace histone deacetylases from NF-κB p50 homodimers to induce NF-κB target genes. Blood. 2011;117:4085–94.

51. Kirstetter P, Schuster MB, Bereshchenko O, Moore S, Dvinge H, Kurz E, Theilgaard-Mönch K, Månsson R, Pedersen TA, Pabst T, Schrock E, Porse BT, Jacobsen SE, Bertone P, Tenen DG, Nerlov C. Modeling of C/EBPα mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. Cancer Cell. 2008;13:299–310.

52. Lin LI, Chen CY, Lin DT, Tsay W, Tang JL, Yeh YC, Shen HL, Su FH, Yao M, Huang SY, Tien HF. Characterization of *CEBPA* mutations in acute myeloid leukemia: most patients with *CEBPA* mutations have biallelic mutations and show a distinct immunophenotype of the leukemic cells. Clin Cancer Res. 2005;11:1372–9.

53. Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of *CEBPA* mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double *CEBPA* mutations and the interaction with *FLT3* and *NPM1* mutations. J Clin Oncol. 2010;28:2739–47.

54. Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E, Benthaus T, Sauerland MC, Berdel WE, Büchner T, Wörmann B, Braess J, Hiddemann W, Bohlander SK, Spiekermann K. Acute myeloid leukemia with biallelic *CEBPA* gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. J Clin Oncol. 2010;28:570–7.

55. Pabst T, Eyholzer M, Fos J, Mueller BU. Heterogeneity within AML with *CEBPA* mutations; only *CEBPA* double mutations, but not single *CEBPA* mutations are associated with favourable prognosis. Br J Cancer. 2009;100:1343–6.

56. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double *CEBPA* mutations, but not single *CEBPA* mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113:3088–91.

57. Taskesen E, Bullinger L, Corbacioglu A, Sanders MA, Erpelinck CA, Wouters BJ, van der Poel-van de Luytgaarde SC, Damm F, Krauter J, Ganser A, Schlenk RF, Löwenberg B, Delwel R, Döhner H, Valk PJ, Döhner K. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with *CEBPA* mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for *CEBPA* double mutant AML as a distinctive disease entity. Blood. 2011;117:2469–75.

58. Damm F, Heuser M, Morgan M, Wagner K, Gorlich K, Grosshennig A, Hamwi I, Thol F, Surdziel E, Fiedler W, Lübbert M, Kanz L, Reuter C, Heil G, Delwel R, Löwenberg B, Valk PJ, Krauter J, Ganser A. Integrative prognostic risk score in acute myeloid leukemia with normal karyotype. Blood. 2011;117:4561–8.

59. Marcucci G, Maharry K, Radmacher MD, Mrozek K, Vukosavljevic T, Paschka P, Whitman SP, Langer C, Baldus CD, Liu CG, Ruppert AS, Powell BL, Carroll AJ, Caligiuri MA, Kolitz JE, Larson RA, Bloomfield CD. Prognostic significance of, and gene and microRNA expression signatures associated with, *CEBPA* mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. J Clin Oncol. 2008;26:5078–87.

60. Smith ML, Cavenagh JD, Lister TA, Fitzgibbon J. Mutation of *CEBPA* in familial acute myeloid leukemia. N Engl J Med. 2004;351:2403–7.

61. Pabst T, Eyholzer M, Haefliger S, Schardt J, Mueller BU. Somatic *CEBPA* mutations are a frequent second event in families with germline *CEBPA* mutations and familial acute myeloid leukemia. J Clin Oncol. 2008;26:5088–93.

62. Eyholzer M, Schmid S, Wilkens L, Mueller BU, Pabst T. The tumour-suppressive miR-29a/b1 cluster is regulated by *CEBPA* and blocked in human AML. Br J Cancer. 2010;103:275–84.

63. Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. Blood. 2010;115:1768–78.

64. Pulikkan JA, Peramangalam PS, Dengler V, Ho PA, Preudhomme C, Meshinchi S, Christopeit M, Nibourel O, Müller-Tidow C, Bohlander SK, Tenen DG, Behre G. C/EBPα regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with *CEBPA* mutations. Blood. 2010;116:5638–49.

65. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling

in relation to the genetic heterogeneity of acute myeloid leukemia. Blood. 2008;111:5078–85.

66. Huang G, Zhang P, Hirai H, Elf S, Yan X, Chen Z, Koschmieder S, Okuno Y, Dayaram T, Growney JD, Shivdasani RA, Gilliland DG, Speck NA, Nimer SD, Tenen DG. PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis. Nat Genet. 2008;40:51–60.
67. Xie H, Ye M, Feng R, Graf T. Stepwise reprogramming of B cells into macrophages. Cell. 2004;117:663–76.
68. Chapiro E, Russell L, Radford-Weiss I, Bastard C, Lessard M, Struski S, Cave H, Fert-Ferrer S, Barin C, Maarek O, Della-Valle V, Strefford JC, Berger R, Harrison CJ, Bernard OA, Nguyen-Khac F. Overexpression of *CEBPA* resulting from the translocation t(14;19)(q32;q13) of human precursor B acute lymphoblastic leukemia. Blood. 2006;108:3560–3.
69. Akasaka T, Balasas T, Russell LJ, Sugimoto KJ, Majid A, Walewska R, Karran EL, Brown DG, Cain K, Harder L, Gesk S, Martin-Subero JI, Atherton MG, Brüggemann M, Calasanz MJ, Davies T, Haas OA, Hagemeijer A, Kempski H, Lessard M, Lillington DM, Moore S, Nguyen-Khac F, Radford-Weiss I, Schoch C, Struski S, Talley P, Welham MJ, Worley H, Strefford JC, Harrison CJ, Siebert R, Dyer MJ. Five members of the CEBP transcription factor family are targeted by recurrent IGH translocations in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Blood. 2007;109:3451–61.

Critical Reviews™ in Oncogenesis, 16(1–2), 103–115 (2011)

Targeting NOTCH1 in Hematopoietic Malignancy

Giovanni Roti[1] & Kimberly Stegmaier[1,2,*]

[1]Department of Pediatric Oncology, Dana-Farber Cancer Institute and Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts; [2]The Broad Institute of Harvard University and Massachusetts Institute of Technology, Cambridge, Massachusetts

*Address all correspondence to: Kimberly Stegmaier, Department of Pediatric Oncology, Dana-Farber Cancer Institute and Children's Hospital Boston, 450 Brookline Ave, Boston, MA 02215; kimberly_stegmaier@dfci.harvard.edu

ABSTRACT: NOTCH1 is a well-validated target in hematopoietic malignancy, with *NOTCH1* activating mutations identified in more than 50% of T-cell acute lymphoblastic leukemias. Moreover, a recent report has identified *NOTCH1* activating mutations in 12% of chronic lymphocytic leukemias. While the frequency of *NOTCH1* mutations and the well-documented role of this protein in the pathogenesis and maintenance of T-ALL support targeting NOTCH1 as a therapeutic strategy, the critical role of this protein in normal cell-fate specification and differentiation lead to complexities in its successful targeting. In this review, we will discuss potential approaches to targeting NOTCH1 in hematopoietic malignancies, including inhibition of the enzymes involved in its activation, antibodies directed against either the receptor or its ligands, and direct interference with the NOTCH1 transcriptional complex. Moreover, we will discuss the challenges to each of these approaches as well as potential solutions to overcoming these difficulties.

KEY WORDS: NOTCH1, T-ALL, targeted therapy, T-cell acute lymphoblastic leukemia, γ-secretase inhibitors.

ABBREVIATIONS

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ANK, ankyrin; CSL, CBF1/RBP-Jκ/suppressor of hairless/LAG-1; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CCND2, cyclin D2; DLL4, delta-like ligand 4; EGFR, epidermal growth factor receptor; EGF-like, epidermal growth factor-like motifs; GE-HTS, gene expression-based high-throughput screening; GSI, γ-secretase inhibitor; HD, heterodimerization domain; IDENTITY, interrupting Alzheimer's dementia by evaluating treatment of amyloid pathology); KLF4, Kruppel-like factor 4; LBD, ligand-binding domain; LMA, ligation-mediated amplification; LNR, LIN12/NOTCH repeats; MAML, mastermind-like; NSCLC, non-small cell lung cancer; ICN, NOTCH intracellular domain; NRR, NOTCH negative regulatory region; N^{EC}, NOTCH non-covalently associated extracellular domain; N^{TM}, NOTCH transmembrane domain; PEST domain, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich sequence; RAG, recombination activating gene; RAM, RBP-Jκ-associated module; SAHM1, stapled α-helical peptides derived from MAML1; T-ALL, T-cell acute lymphoblastic leukemia; TCRB, T-cell receptor β; TAD, transactivation domain; VEGF, vascular endothelial growth factor

I. INTRODUCTION

Great strides have been made in treating cancer with multiagent cytotoxic chemotherapy, such as the treatment of pediatric acute lymphoblastic leukemia (ALL). However, we remain without curative therapy for many malignancies, and even in the case of pediatric ALL we have reached a plateau in survival rates. Moreover, for those who are cured, therapy-related toxicity can be life-long. New approaches to cancer treatment are needed. The success of imatinib in the treatment of *BCR-ABL*-rearranged chronic myelogenous leukemia (CML) ushered in a new era in the treatment of cancer with so-called "targeted" therapies.[1,2] While the activity of imatinib in CML has been dramatic, response to other targeted agents has been both tempered and complex, such as response to FLT-3 inhibitors in acute myeloid leukemia (AML)[3] and response to epidermal growth factor receptor (EGFR) inhibitors in non-small cell lung cancer (NSCLC).[4,5] In this review, we focus on an emerging target in both hematological malignancies and solid tumors: NOTCH1. We discuss the role of NOTCH1 in normal hematopoiesis, the data

0893-9675/11/$35.00

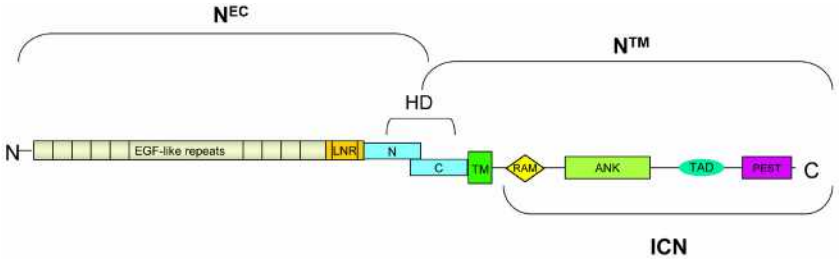


FIGURE 1. Structure of NOTCH1 receptor. The functional domains of NOTCH1 are annotated. During maturation, NOTCH1 is cleaved by furin producing a heterodimeric complex consisting of two subunits: the non-covalently associated extracellular (N^{EC}) and the transmembrane (N^{TM}) subunits. The N^{EC} contains EGF-like repeats that bind to NOTCH ligands, three NOTCH/LIN-12 repeats (LNR), and the heterodimerization domain (HD-N). The HD-N interfaces with the heterodimerization domain (HD-C) of the N^{EC} to maintain a stable association of N^{EC} and N^{TM} subunits. N^{TM} subunit also contains the transmembrane domain (TM), the RAM domain, a series of ankyrin (ANK) repeats, a transactivation domain (TAD), and a C-terminal PEST domain (proline (P), glutamic acid (E), serine (S), and threonine (T) rich) sequence.

supporting a driver role for NOTCH1 in hematological malignancies, and potential approaches and challenges to targeting NOTCH1.

II. NOTCH STRUCTURE AND ACTIVATION

NOTCH receptors mediate a conserved signaling pathway critical in controlling cell fate specification, stem cell maintenance, and differentiation.[6] In mammals, the NOTCH family includes four transmembrane class I receptors (NOTCH 1–4). NOTCH proteins are synthesized as single precursors (proNOTCH1) and are cleaved by a furin-convertase activity (S1) in the trans-Golgi network, resulting in a heterodimer receptor consisting of a non-covalently associated extracellular domain (N^{EC}) and a transmembrane subunit (N^{TM}) interacting via a heterodimerization domain (HD) (Figure 1).[7] The N^{EC} domain contains repeated epidermal growth factor-like motifs (EGF-like), critical for the ligand binding, followed by cysteine rich LIN12/NOTCH (LNR) repeats. Along with the HD, these LNR repeats prevent spontaneous activation of the receptor in the absence of a ligand. The N^{TM} subunit contains a RAM domain, nuclear localization signals, seven tandem ankyrin repeats, a transcriptional activation domain and a C-terminal PEST sequence that mediates NOTCH stability and turnover.

In mammals, canonical NOTCH signaling requires activation by ligands of the Delta (Delta-like 1, 3, and 4) and Jagged/Serrate (Jagged 1 and 2) families expressed on the surface of neighboring cells. Ligand binding initiates two consecutive proteolytic cleavages and endocytosis of the receptor. The first cleavage is mediated by ADAM10 (and possibly ADAM17 in some contexts) and occurs just external to the transmembrane domain site of N^{TM} at site S2. The second cleavage (S3) occurs within the transmembrane domain and is mediated by γ-secretase, a complex composed of four proteins: presenilin, nicastrin, APH-1, and PEN-2. This highly regulated process culminates with the release of the NOTCH intracellular domain (ICN). After translocation to the nucleus, ICN binds the DNA-binding factor CSL and recruits co-activators of the mastermind-like (MAML) family, forming a complex that activates the transcription of target genes such as *MYC*, *DTX1*, and *HES1*.

A. NOTCH1 in T-Cell Development

In hematopoiesis, the NOTCH signaling pathway is central to cell-fate commitment and differentiation. The best characterized function of NOTCH1 in hematopoiesis is its essential role in T-cell fate specification.[8] Early lymphocyte progenitors require NOTCH1 activation for T-cell commitment as demonstrated by the absence of T cells and increased

intrathymic B cells with deletion of *NOTCH1* prior to T-cell commitment.[9] Later stages of T-cell maturation are also NOTCH1 dependent. NOTCH1 signals support pro-T cell to pre-T-cell maturation and progression through the DN1, DN2, and DN3 phases.[10] NOTCH1 signaling also regulates *TCRB* gene rearrangements and lineage specification between αβ versus γδ lineages.[11–13]

B. Activating *NOTCH* Mutations in Hematological Malignancies

The oncogenic role of NOTCH1 was first identified through its involvement in a rare, recurrent chromosomal translocation t(7;9)(q34;q34.3) in T-cell acute lymphoblastic leukemia (T-ALL).[14,15] This translocation fuses the 3′ end of *NOTCH1* with the *TCRb* locus, resulting in the synthesis of truncated and constitutively activated NOTCH1 polypeptides. In 2004 Weng et al. discovered activating *NOTCH1* mutations in 55–60% of human T-ALLs, making *NOTCH1* mutations the most common genetic alteration reported in this disease.[16] The majority of *NOTCH1* mutations are located in either the HD or PEST domains. Class 1 HD mutations are single amino acid substitution or in frame insertions or deletions generating a constitutively active form of NOTCH1 that does not require ligand activation.[16,17] A second group of HD mutations (class 2) are longer tandem duplications located in exon 27 that result in high levels of ligand-independent activation of NOTCH1 by creating an unprotected S2 cleavage site.[17] An additional class of mutations, class 3, was described more recently by Sulis et al.[18] These mutations consist of internal tandem duplications of exon 28 and adjacent intronic sequences in the *NOTCH1* gene, which result in expansion of the extracellular juxtamembrane region of the NOTCH1 receptor.[18] In contrast, mutations leading to the loss of the PEST domain render ICN1 less susceptible to proteosomal degradation.[16] Similarly, more rare mutations in *FBXW7*, a gene that encodes an ubiquitin ligase, result in increased stability of ICN1.[19,20]

Additional lines of evidence indicate that aberrant activation of NOTCH1 is a critical event in T-ALL leukemogenesis (reviewed in Grabher et al.[22] and Aster et al.[23,24]). For example, it has been demonstrated that activated intracellular NOTCH1 is important for cellular transformation and tumor progression and that the tumor initiating activity of T-ALL primary blasts is dependent on NOTCH1 activation.[16,25–28] Furthermore, Demarest et al. demonstrated that in inducible mouse models MYC cannot substitute for activated NOTCH1, indicating that multiple targets downstream of NOTCH1 contribute to the maintenance of leukemia.[29]

Further evidence that NOTCH activation plays a key role in the pathogenesis of T-ALL is demonstrated by the frequent finding of *NOTCH1* mutations in murine models of T-ALL. *NOTCH1* is a common site of proviral integration in murine T-ALLs induced by retroviral mutagenesis,[30–32] and spontaneous mutations in *NOTCH1*, many RAG-mediated[33] are common in T-ALLs arising in *SCL/LMO1*, *OLIG2/LMO1*, *OLIG2*, *LMO1*, and *NUP98/HOXD13* murine models.[34] In addition, mice transplanted with hematopoietic progenitors transduced with vectors driving the expression of ICN1 or the NOTCH ligand Delta-like 4 develop T-ALL.[35,36] Moreover, O'Neil et al. described that in a mouse model of *TAL1*-induced leukemia or in thymic lymphomas in mice defective for *H2AX*, *TP53*, and *RAG2* genes, the frequency of *NOTCH1* mutations ranged from 31% to 74%.[37]

While activating *NOTCH1* mutations have been most commonly reported in T-ALL, *NOTCH1* PEST domain mutations have also been reported in 5.3 to 12.2% of chronic lymphocytic leukemia (CLL).[38,39] In both publications, *NOTCH1* mutations were associated with a low number of somatic hypermutations in the variable region of immunoglobulin genes and with a poor prognosis. Sporadic cases of activating *NOTCH1* mutations in acute myeloid leukemia (AML)[40] and *NOTCH2* mutations in lymphomas have also been reported.[41]

C. NOTCH Activation in Solid Tumors

Given the critical importance of NOTCH signaling in a wide range of cell specifications, it is not surprising that alterations of this pathway are also reported in non-hematological malignancies. In non–small-cell

lung carcinoma, for example, Westhoff et al. reported two major alterations in the NOTCH pathway: loss of the expression of *NUMB*, a negative regulator of NOTCH signaling, and gain-of-function *NOTCH1* mutations in 6 of 49 samples evaluated.[42] A role for NOTCH signaling in human breast cancer has been suggested by both the development of adenocarcinomas in the murine mammary gland upon NOTCH1 activation[43] and loss of NUMB-mediated negative regulation of NOTCH signaling in 50% of human mammary carcinoma.[44] Furthermore, activation of NOTCH pathway components has been reported in numerous other solid tumors.[45]

III. APPROACHES TO INHIBITING NOTCH1

The studies summarized above strongly support the development of NOTCH1 inhibitors for targeted cancer therapy, particularly for T-ALL where recurrent *NOTCH1* mutations are common and cancer dependency has been established. Several approaches to targeting NOTCH1 have been explored, including the inhibition of the enzymes involved in its activation, the application of inhibitory antibodies, and the direct inhibition of the NOTCH transcription factor complex (Figure 2). Below, we discuss these strategies, as well as the potential challenges in targeting NOTCH1 by each of these approaches.

A. γ-Secretase Inhibitors

Over the past decade, inhibitors of γ-secretase (GSI), the enzyme critical in the final activation cleavage of NOTCH1, have been investigated for cancer therapy. GSIs were initially identified and developed for their ability to block the generation of Aβ polypeptides, a pathogenic feature of Alzheimer's disease. With the discovery of activating *NOTCH1* mutations in T-ALL, GSIs have been repurposed to prevent NOTCH1 receptor activation. Several studies have confirmed the initial observation that GSI treatment in T-ALL cells induces G0/G1 arrest along with a rapid clearance of intracellular NOTCH1.[15,16,37,46] Based on these findings, a small phase I clinical trial testing the Merck GSI MK-0752 was conducted.[47] Six adult and two pediatric patients with leukemia (seven with T-ALL and one with AML) were enrolled in this study. *NOTCH1* mutations were identified in four of the seven T-ALLs. Treatment duration ranged from 2 to 56 days before patients discontinued the drug for disease progression or drug-related toxicity. One patient with T-ALL and a *NOTCH1* activating mutation achieved a 45% reduction in a mediastinal mass but subsequently progressed by 56 days. Dose-limiting gastrointestinal toxicity, primarily diarrhea, was observed at drug doses of 300 mg/m^2. It was hypothesized that gastrointestinal toxicity was due to blockade of NOTCH1 and NOTCH2 in the gut leading to intestinal secretory metaplasia, an increased number of goblet cells and arrested proliferation in the crypts of the small intestine.[48]

Because of the limited anti-leukemic activity in this trial and the severe gastrointestinal toxicity, studies were conducted to identify combination therapies with GSIs. Real et al. demonstrated that glucocorticoid therapy in combination with NOTCH1 inhibition by GSIs improved the antileukemic effect of GSIs as well as reduced their gut toxicity *in vivo*.[49,50] Moreover, this combination abrogated glucocorticoid resistance in T-ALL cell lines and primary patient blasts and induced an apoptotic cell death through induction of *BCL2L11*. Mice treated with glucocorticoids and a GSI showed decreased gastrointestinal toxicity mediated by the induction of cyclin D2 (CCND2), a cyclin associated with cell cycle progression, and by the downregulation of Kruppel-like factor 4 (KLF4), a negative regulator of the cell cycle that is required for goblet cell differentiation.[49] Similar results were observed using PF-03084014, a noncompetitive, reversible GSI developed by Pfizer.[51] This molecule was reported to induce an anti-leukemic effect *in vitro* and *in vivo* in T-ALL cell lines expressing mutant *NOTCH1*. An intermittent dosing schedule of PF-03084014 attenuated gut toxicity, and the addition of glucocorticoids to PF-03084014 therapy also abrogated PF-03084014-induced gastrointestinal toxicity in mice. A dose escalating study to determine the safety profile and maximum tolerated dose of PF-03084014 in patients with advanced cancer and leukemia is ongoing (NCT00878189).

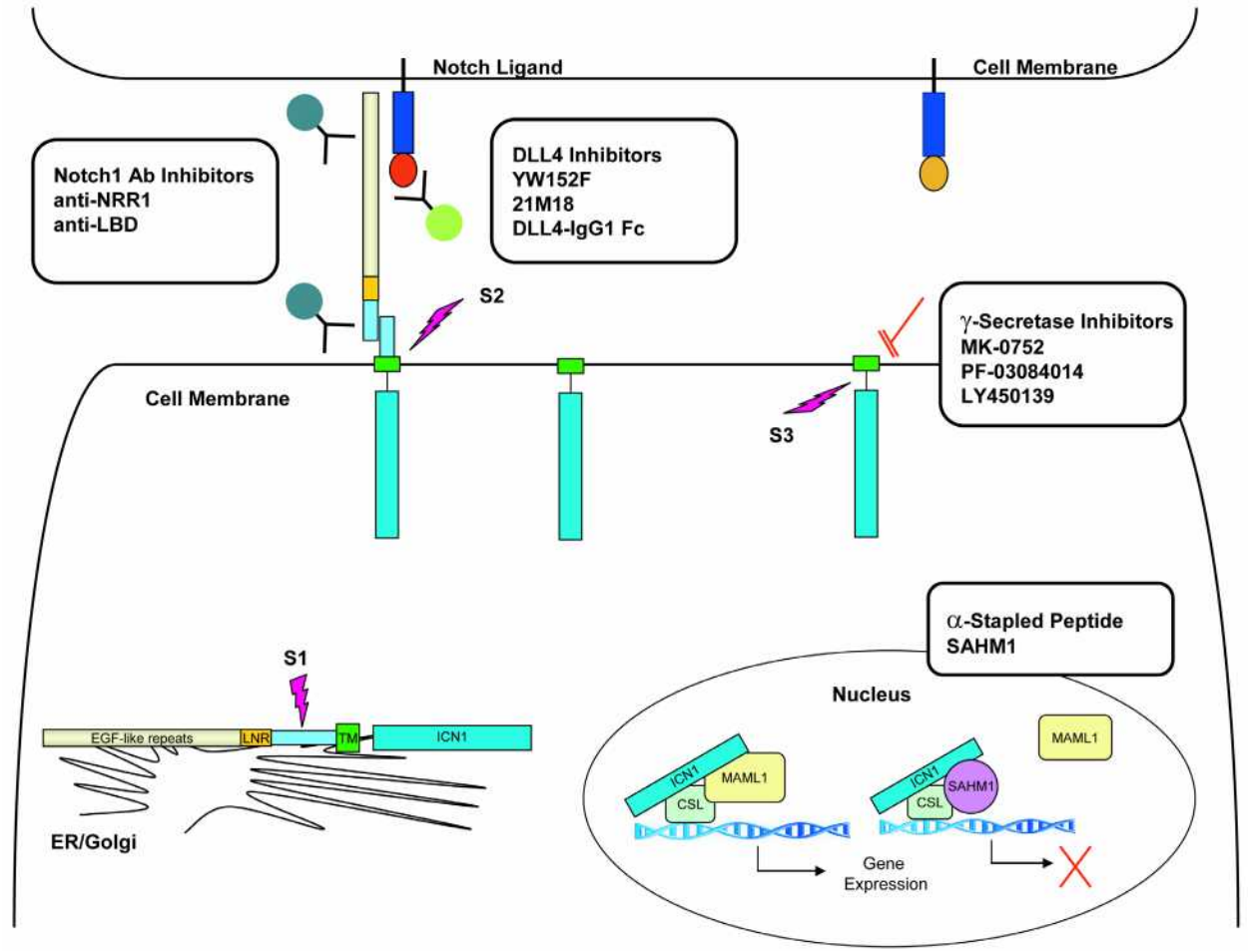


FIGURE 2. Approaches to targeting NOTCH1. NOTCH1 is normally activated by a series of proteolytic cleavage events beginning with the S1 cleavage by a furin-like protease resulting in the mature heterodimeric receptor. NOTCH1 is then activated by ligand on an adjacent cell leading to a metalloprotease cleavage at S2 followed by cleavage at S3 by the γ-secretase complex and the release of the activated form of NOTCH1, ICN1. ICN1 then translocates to the nucleus where it associates with other transcription factors in a complex to alter gene transcription. The activation of NOTCH1 enables several approaches to its inhibition, such as antibody-based targeting of the ligand or receptor, inhibition of the enzymes involved in its maturation, and direct inhibition of the transcription factor complex.

Although GSIs represent a rational approach to target the NOTCH1 pathway, additional safety concerns have emerged. In two phase III trials, the IDENTITY and IDENTITY-2 studies (interrupting Alzheimer's dementia by evaluating treatment of amyloid pathology), LY450139 (semagacestat), a GSI developed by Lilly, was compared with placebo in more than 2,600 patients with mild-to-moderate Alzheimer's disease. An interim analysis reported in a press release by Lilly on August 17, 2010, revealed that cognition and the ability to complete activities of daily living actually worsened in patients treated with semagacestat compared to those treated with placebo. Moreover, data showed that semagacestat is associated with an increased risk of skin cancer compared with those who received placebo, likely due to inhibition of NOTCH in the skin by chronic GSI administration. Whether this risk will be ameliorated

by intermittent, pulsed therapy with GSI, as would be the schedule in cancer-directed therapy, is still to be determined.

A tumor suppressor role for NOTCH1 in the skin has been supported by several prior studies.[52,53] In a tissue-specific inducible gene-targeting approach to study the role of the NOTCH1 receptor in the adult mouse epidermis and the corneal epithelium, loss of *NOTCH1* resulted in epidermal and corneal hyperplasia, the development of skin tumors, and enhanced chemical-induced skin carcinogenesis.[54] The role of impaired NOTCH1 signaling in promoting squamous cell carcinoma development was supported by several additional studies,[55,56] including recent work suggesting that promotion of skin tumorigenesis by *NOTCH1* loss is through an impact on the stromal microenvironment.[57]

B. Antibody-Based Targeting of the NOTCH1 Receptor

Because of the therapeutic challenges of GSI therapy (inhibition of other signaling pathways, inhibition of multiple NOTCH receptors, and gastrointestinal toxicity) the development of NOTCH1-directed antibodies has been explored. In one study, investigators identified two classes of antibodies with selectivity for NOTCH1 from cell-based and solid-phase screening of a phage display library: one directed against the EGF repeat region of the ligand-binding domain (LBD) and the second directed against the NOTCH negative regulatory region (NRR).[58] The antibodies were selective for NOTCH1, were bound to NOTCH1 on human tumor cell lines, and inhibited the expression of NOTCH1 target genes. While the NRR antibodies inhibited ligand-independent signaling in heterologous cells expressing the more common class 1 HD point mutations, they did not antagonize rare class 2 or class 3 mutant receptors. Moreover, the NRR antibodies were incomplete antagonists of NOTCH1 signaling and their effects were weaker than GSI in T-ALL cell lines expressing class 1 mutations.

A second group used a similar phage display strategy to generate antibodies which specifically antagonize NOTCH1 versus NOTCH2. The antibodies were confirmed to stabilize the auto-inhibited NRR and selectively block NOTCH1 in T-ALL cells inhibiting growth in vitro and in a xenograft model of class 1 mutant T-ALL.[59] Moreover, the anti-NRR1 antibody deregulated angiogenesis. Interestingly, the anti-NRR2 antibody alone did not affect intestinal morphology while the anti-NRR1 antibody induced modest goblet cell metaplasia. Combined therapies resulted in weight loss and severe goblet cell metaplasia. These studies confirm that selective inhibition of NOTCH receptors with an antibody-based approach is feasible, leads to decreased gut toxicity, and has anti-tumor activity in *NOTCH1* mutant models of disease.

C. Targeting NOTCH Ligands

An alternative strategy to ablate NOTCH activation is to inhibit NOTCH ligands. This approach has been evaluated in solid tumor models with the inhibition of delta-like ligand 4 (DLL4). DLL4 is essential for normal embryonic vasculature development,[60,61] and its expression is increased with tumor angiogenesis.[61–63] One study by Noguera-Troise et al. reported that the vascular endothelial growth factor (VEGF) sustains high expression of DLL4 in the tumor endothelial cells.[64] The authors then blocked DLL4/NOTCH signaling using a retroviral approach to express forms of DLL4 predicted to activate (full-length membrane-bound DLL4) or inhibit (the extracellular region of DLL4 fused to the human IgG1 Fc constant region) DLL4. In a C6 rat glioma model, blockade of DLL4 by expressing DLL4-IgG1 Fc compared to a full-length DLL4 resulted in decreased tumor growth. Paradoxically DLL4 blockade resulted in more highly branched and finely interconnected vessels with increased tumor vascular density leading to the hypothesis that the DLL4/NOTCH pathway normally negatively regulates sprouting and branching during tumor angiogenesis and that the increased vascularity with NOTCH inhibition is "nonproductive." Indeed, there was increased tumor hypoxia with blockade of DLL4 and decreased vessel perfusion. These results were recapitulated using adenovirus expressing DLL4-Fc injected intravenously into a C6 glioma mouse xenograft model.

Similar results were described using YW152F, a Genentech antibody which inhibits the interaction of DLL4 and NOTCH but not an interaction with other NOTCH ligands.[65] YW152F had activity in multiple solid tumor xenograft models and also induced a paradoxical increase in tumor vascular density. Moreover, because DLL4/NOTCH signaling is largely restricted to the vascular system, short-term treatment was well tolerated in mice with no evidence of gastrointestinal toxicity observed. Additional studies using another antibody confirmed to inhibit DLL4-induced NOTCH pathway activation, 21M18, demonstrated anti-tumor activity in colon tumor xenografts.[66] Furthermore, treatment with 21M18 alone, or in combination with irinotecan, reduced tumor-initiating cells and delayed tumor recurrence.

Although targeting DLL4 was well-tolerated and efficacious in the treatment of solid tumors in short-term treatment studies, recent reports raise concern about chronic inhibition of DLL4.[67,68] In particular, Yan et al. evaluated the effect of chronic inhibition of DLL4 in adult mice, rats, and cynomolgus monkeys either using a DLL4-specific antibody or using the antagonist soluble protein (DLL4-IgG1 Fc). The authors observed changes in liver histopathology, such as sinusoidal dilatation and centrilobular hepatocyte atrophy after 3 to 8 weeks of treatment in mice and monkeys. The development of subcutaneous vascular tumors was also observed in adult male rats.[68] Similarly, in a model of sporadic *NOTCH1* loss of heterozygosity, Liu and colleagues observed widespread vascular tumors and lethal hemorrhaging.[67] Again, as in the case of the chronic administration of GSI, these studies raise safety concerns regarding the chronic administration of inhibitors of NOTCH signaling. Moreover, the targeting of ligand will not be an effective strategy in the context of *NOTCH1* mutations in T-ALL rendering NOTCH1 activation ligand independent.

D. Direct Inhibition of the NOTCH Transcriptional Complex

Another approach to inhibiting NOTCH is to directly modulate the transcription factor complex. Historically, transcription factors have been considered among the most chemically intractable of protein targets because of their lack of hydrophobic pockets and the challenge of designing high-throughput screening assays to measure protein–protein or protein–DNA interactions. New chemistry strategies, however, may enable more effective targeting of this challenging protein class. One alternative approach to targeting pharmacologically intractable proteins employs hydrocarbon stapling to generate peptides with improved pharmacological properties compared to unstapled peptides, including increased metabolic stability, binding affinity and serum half-life.[69] A hydrocarbon stapling approach was used to target the NOTCH1 transcriptional complex.[70] It was previously shown that a dominant negative fragment of MAML1 antagonizes NOTCH signaling and cell proliferation in T-ALL cell lines [71,72] and forms an α-helix engaging an elongated groove resulting from the interaction of ICN1 and CSL.[73] In light of these findings, Moellering et al. hypothesized that the NOTCH transcriptional complex might be amenable to targeting with hydrocarbon-stapled, α-helical peptides whereby a stapled dnMAML1 fragment might impair binding of MAML1 to the ICN1-CSL complex. In a proof-of-concept study, they demonstrated that SAHM1 (stapled α-helical peptides derived from MAML1) prevents assembly of the NOTCH1 transcriptional complex and inhibits the expression of NOTCH1 target genes. Moreover, SAHM1 treatment resulted in anti-leukemia activity in vitro and in vivo in *NOTCH1* mutant T-ALL. While this peptide approach has not yet been tested in human clinical trials, Aileron Therapeutics has advanced an α-helical peptide, designed to activate the apoptosis pathway, into late-stage preclinical studies.

E. Emerging Approaches to NOTCH1 Inhibition

As our understanding of NOTCH signaling and regulation improves, our opportunity for therapeutic intervention also increases. For example, it was recently discovered that the NAD^+-dependent deacetylase SIRT1 serves as a negative modulator of NOTCH signaling in endothelial cells.[74] Acetyla-

tion of ICN1 appears to control the amplitude and duration of NOTCH responses by promoting ICN1 stability. SIRT1 functions as an ICN1 deacetylase, promoting ICN1 protein turnover. Thus, the alteration of NOTCH acetylation may be yet another approach to its inhibition.

Discovery efforts to identify NOTCH1 inhibitors with unbiased screening are also in progress. For example, our laboratory developed a gene expression-based signature approach (gene expression-based high-throughput screening (GE-HTS)) for cell-based, small-molecule discovery as an alternative to traditional target- and phenotype-based screening.[75] We applied GE-HTS to the modulation of mutant *NOTCH1* in T-ALL.[76] We developed a gene expression signature for the NOTCH1 *on* versus *off* states using microarray expression profiling of seven different *NOTCH1* mutant T-ALL cell lines treated in duplicate with vehicle versus the GSI compound E.[77] From a set of ~500 genes with differences of $p < 0.01$ by two-sided Student's *t*-test, 28 genes were selected to define the NOTCH1 *off* signature based on mean fold changes >1.5 between the NOTCH1 *on* versus *off* states. We next adapted this signature to our GE-HTS assay, which uses ligation-mediated amplification (LMA) and a Luminex bead-based detection system.[78] To facilitate future clinical translation, we intentionally selected a library of approximately 4,500 compounds enriched for FDA-approved drugs and known bioactives to inform studies of compound mechanism of action and to enable more rapid advancement to the clinic. Interestingly, molecules that alter ion fluxes scored highly in the screen, and the characterization of their phenotypic effects in T-ALL and mechanism of altering NOTCH1 is underway.

IV. CONCLUSION

While the genetic and functional data to support targeting NOTCH1 in hematological malignancy are compelling, the road ahead has evident challenges. One difficulty is the on-target side effects of inhibiting NOTCH. As illustrated in the early trials of GSIs, gut toxicity poses a significant challenge due to inhibition of both NOTCH1 and NOTCH2 in the gut. Potential solutions to this challenge include intermittent dosing, the addition of dexamethasone, and the development of agents which specifically target NOTCH1 without effecting NOTCH2, such as antibody-directed therapies. Another concerning toxicity, reported in a recent trial performed in patients with Alzheimer's disease, is the development of secondary malignancies, particularly skin cancer. Moreover, as the list of malignancies in which NOTCH may have a tumor suppressor role grows (e.g., chronic myelomonocytic leukemia[79]), so, too, does the concern that chronic NOTCH suppression will lead to secondary malignancies. The hope is that intermittent dosing of the NOTCH inhibitor will mitigate this risk. Another possibility is "targeted" delivery of a "targeted" therapy to tumor cells, such as a caged derivative or a molecule-antibody conjugate directed specifically to T-ALL blasts.

A second concern in targeting NOTCH1 has been the largely cytostatic effect of NOTCH1 inhibition with GSIs and antibody-based therapy in preclinical testing. As in the case of cytotoxic therapy for cancer, targeted therapies will likely need to be used in combination with other drugs to clinical efficacy. Preclinical studies have already demonstrated the success of combining NOTCH1 inhibitors with glucocorticoids, both in terms of reducing gut toxicity but also in terms of enhancing anti-tumor activity[49–51]. Moreover, several studies support the combination of NOTCH1 inhibitors with inhibitors of the PI3K-AKT-mTOR pathway.[80,81]

The road ahead will not be easy, but with a compelling target and a strong clinical need for new approaches to treating these diseases, NOTCH is an important target to pursue. In looking toward a future hope of targeted treatments, we should not forget the lessons of the past: combination therapy is critical and some toxicity has been acceptable when therapy is curative.

ACKNOWLEDGMENTS

This work was supported by Leukemia and Lymphoma Society Special Fellow award and Fondazione Umberto Veronesi (G.R.) and by the Leukemia and Lymphoma Society, William Lawrence and Blanche

Hughes Foundation, SynCure Cancer Research Foundation, and the National Cancer Institute (K.S.). We thank Annie Carlton for assistance with the figures and Jon Aster for critical reading of the manuscript.

REFERENCES

1. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001 Apr 5;344(14):1031–7.
2. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med. 2002 Feb 28;346(9):645–52.
3. Wiernik PH. FLT3 inhibitors for the treatment of acute myeloid leukemia. Clin Adv Hematol Oncol. 2010 Jun;8(6):429–36, 44.
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004 May 20;350(21):2129–39.
5. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004 Jun 4;304(5676):1497–500.
6. Artavanis-Tsakonas S, Matsuno K, Fortini ME. Notch signaling. Science. 1995 Apr 14;268(5208): 225–32.
7. Logeat F, Bessia C, Brou C, LeBail O, Jarriault S, Seidah NG, Israel A. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci U S A. 1998 Jul 7;95(14):8108–12.
8. Pear WS, Radtke F. Notch signaling in lymphopoiesis. Semin Immunol. 2003 Apr;15(2):69–79.
9. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC, Pear WS. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity. 1999 Sep;11(3):299–308.
10. Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. Nat Immunol. 2004 Mar;5(3):247–53.
11. 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 Jun;16(6):869–79.
12. Washburn T, Schweighoffer E, Gridley T, Chang D, Fowlkes BJ, Cado D, Robey E. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. Cell. 1997 Mar 21;88(6):833–43.
13. Deftos ML, Bevan MJ. Notch signaling in T cell development. Curr Opin Immunol. 2000 Apr;12(2):166–72.
14. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell. 1991 Aug 23;66(4):649–61.
15. Palomero T, Barnes KC, Real PJ, Glade Bender JL, Sulis ML, Murty VV, Colovai AI, Balbin M, Ferrando AA. CUTLL1, a novel human T-cell lymphoma cell line with t(7;9) rearrangement, aberrant NOTCH1 activation and high sensitivity to gamma-secretase inhibitors. Leukemia. 2006 Jul;20(7):1279–87.
16. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC. Activating mutations of NOTCH1 in human T-cell acute lymphoblastic leukemia. Science. 2004 Oct 8;306(5694):269–71.
17. Malecki MJ, Sanchez-Irizarry C, Mitchell JL, Histen G, Xu ML, Aster JC, Blacklow SC. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two

distinct mechanistic classes. Mol Cell Biol. 2006 Jun;26(12):4642–51.

18. 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 Aug 1;112(3):733–40.
19. 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 Aug 6;204(8):1825–35.
20. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, Hardwick J, Welcker M, Meijerink JP, Pieters R, Draetta G, Sears R, Clurman BE, Look AT. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. J Exp Med. 2007 Aug 6;204(8):1813–24.
21. Demarest RM, Ratti F, Capobianco AJ. It's T-ALL about Notch. Oncogene. 2008 Sep 1;27(38):5082–91.
22. Grabher C, von Boehmer H, Look AT. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. Nat Rev Cancer. 2006 May;6(5):347–59.
23. Aster JC, Blacklow SC, Pear WS. Notch signalling in T-cell lymphoblastic leukaemia/lymphoma and other haematological malignancies. J Pathol. 2011 Jan;223(2):262–73.
24. Aster JC, Pear WS, Blacklow SC. Notch signaling in leukemia. Annu Rev Pathol. 2008;3:587–613.
25. Aster JC, Xu L, Karnell FG, Patriub V, Pui JC, Pear WS. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by Notch1. Mol Cell Biol. 2000 Oct;20(20):7505–15.
26. Capobianco AJ, Zagouras P, Blaumueller CM, Artavanis-Tsakonas S, Bishop JM. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. Mol Cell Biol. 1997 Nov;17(11):6265–73.
27. Beverly LJ, Felsher DW, Capobianco AJ. Suppression of p53 by Notch in lymphomagenesis: implications for initiation and regression. Cancer Res. 2005 Aug 15;65(16):7159–68.
28. Armstrong F, Brunet de la Grange P, Gerby B, Rouyez MC, Calvo J, Fontenay M, Boissel N, Dombret H, Baruchel A, Landman-Parker J, Romeo PH, Ballerini P, Pflumio F. NOTCH is a key regulator of human T-cell acute leukemia initiating cell activity. Blood. 2009 Feb 19;113(8):1730–40.
29. Demarest RM, Dahmane N, Capobianco AJ. Notch is oncogenic dominant in T-cell acute lymphoblastic leukemia. Blood. 2011 Mar 10;117(10):2901–9.
30. Dail M, Li Q, McDaniel A, Wong J, Akagi K, Huang B, Kang HC, Kogan SC, Shokat K, Wolff L, Braun BS, Shannon K. Mutant Ikzf1, KrasG12D, and Notch1 cooperate in T lineage leukemogenesis and modulate responses to targeted agents. Proc Natl Acad Sci U S A. 2010 Mar 16;107(11):5106–11.
31. Girard L, Hanna Z, Beaulieu N, Hoemann CD, Simard C, Kozak CA, Jolicoeur P. Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev. 1996 Aug 1;10(15):1930–44.
32. Yanagawa S, Lee JS, Kakimi K, Matsuda Y, Honjo T, Ishimoto A. Identification of Notch1 as a frequent target for provirus insertional mutagenesis in T-cell lymphomas induced by leukemogenic mutants of mouse mammary tumor virus. J Virol. 2000 Oct;74(20):9786–91.
33. Ashworth TD, Pear WS, Chiang MY, Blacklow SC, Mastio J, Xu L, Kelliher M, Kastner P, Chan S, Aster JC. Deletion-based mechanisms of Notch1 activation in T-ALL: key roles for RAG recombinase and a conserved internal translational start site in Notch1. Blood. 2010 Dec 16;116(25):5455–64.
34. Lin YW, Nichols RA, Letterio JJ, Aplan PD. Notch1 mutations are important for leukemic transformation in murine models of precursor-T leukemia/lymphoma. Blood. 2006 Mar 15;107(6):2540–3.
35. Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J, Baltimore D. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med. 1996 May 1;183(5):2283–91.
36. Yan XQ, Sarmiento U, Sun Y, Huang G, Guo J, Juan T, Van G, Qi MY, Scully S, Senaldi G, Fletcher FA. A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. Blood. 2001 Dec 15;98(13):3793–9.

37. O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH, Alt FW, Kelliher M, Look AT. Activating Notch1 mutations in mouse models of T-ALL. Blood. 2006 Jan 15;107(2):781–5.

38. Di Ianni M, Baldoni S, Rosati E, Ciurnelli R, Cavalli L, Martelli MF, Marconi P, Screpanti I, Falzetti F. A new genetic lesion in B-CLL: a NOTCH1 PEST domain mutation. Br J Haematol. 2009 Sep;146(6):689–91.

39. Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, Escaramis G, Jares P, Bea S, Gonzalez-Diaz M, Bassaganyas L, Baumann T, Juan M, Lopez-Guerra M, Colomer D, Tubio JM, Lopez C, Navarro A, Tornador C, Aymerich M, Rozman M, Hernandez JM, Puente DA, Freije JM, Velasco G, Gutierrez-Fernandez A, Costa D, Carrio A, Guijarro S, Enjuanes A, Hernandez L, Yague J, Nicolas P, Romeo-Casabona CM, Himmelbauer H, Castillo E, Dohm JC, de Sanjose S, Piris MA, de Alava E, Miguel JS, Royo R, Gelpi JL, Torrents D, Orozco M, Pisano DG, Valencia A, Guigo R, Bayes M, Heath S, Gut M, Klatt P, Marshall J, Raine K, Stebbings LA, Futreal PA, Stratton MR, Campbell PJ, Gut I, Lopez-Guillermo A, Estivill X, Montserrat E, Lopez-Otin C, Campo E. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature. 2011 Jun 5.

40. Palomero T, McKenna K, J ON, Galinsky I, Stone R, Suzukawa K, Stiakaki E, Kalmanti M, Fox EA, Caligiuri MA, Aster JC, Look AT, Ferrando AA. Activating mutations in NOTCH1 in acute myeloid leukemia and lineage switch leukemias. Leukemia. 2006 Nov;20(11):1963–6.

41. Lee SY, Kumano K, Nakazaki K, Sanada M, Matsumoto A, Yamamoto G, Nannya Y, Suzuki R, Ota S, Ota Y, Izutsu K, Sakata-Yanagimoto M, Hangaishi A, Yagita H, Fukayama M, Seto M, Kurokawa M, Ogawa S, Chiba S. Gain-of-function mutations and copy number increases of Notch2 in diffuse large B-cell lymphoma. Cancer Sci. 2009 May;100(5):920–6.

42. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, Pece S, Di Fiore PP. Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci U S A. 2009 Dec 29;106(52):22293–8.

43. Smith GH, Gallahan D, Diella F, Jhappan C, Merlino G, Callahan R. Constitutive expression of a truncated INT3 gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth Differ. 1995 May;6(5):563–77.

44. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, Zurrida S, Maisonneuve P, Viale G, Di Fiore PP. Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol. 2004 Oct 25;167(2):215–21.

45. Koch U, Radtke F. Notch signaling in solid tumors. Curr Top Dev Biol. 2010;92:411–55.

46. De Keersmaecker K, Lahortiga I, Mentens N, Folens C, Van Neste L, Bekaert S, Vandenberghe P, Odero MD, Marynen P, Cools J. In vitro validation of gamma-secretase inhibitors alone or in combination with other anti-cancer drugs for the treatment of T-cell acute lymphoblastic leukemia. Haematologica. 2008 Apr;93(4):533–42.

47. Deangelo DJ, Stone RM, Silverman LB, Stock W, Attar EC, Fearen I, Dallob A, Matthews C, Stone J, Freedman SJ, Aster J. A phase I clinical trial of the notch inhibitor MK-0752 in patients with T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and other leukemias. J Clin Onc. 2006;24(18S):6585.

48. Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadient R, Jacobs RT, Zacco A, Greenberg B, Ciaccio PJ. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci. 2004 Nov;82(1):341–58.

49. Real PJ, Tosello V, Palomero T, Castillo M, Hernando E, de Stanchina E, Sulis ML, Barnes K, Sawai C, Homminga I, Meijerink J, Aifantis I, Basso G, Cordon-Cardo C, Ai W, Ferrando A. Gamma-secretase inhibitors reverse glucocorticoid resistance in T-cell acute lymphoblastic leukemia. Nat Med. 2009 Jan;15(1):50–8.

50. Real PJ, Ferrando AA. NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia. Leukemia. 2009 Aug;23(8):1374–7.

51. Wei P, Walls M, Qiu M, Ding R, Denlinger RH, Wong A, Tsaparikos K, Jani JP, Hosea N, Sands M, Randolph S, Smeal T. Evaluation of selective gamma-secretase inhibitor PF-03084014 for its antitumor efficacy and gastrointestinal safety to guide optimal clinical trial design. Mol Cancer Ther. 2010 Jun;9(6):1618–28.

52. Dotto GP. Notch tumor suppressor function. Oncogene. 2008 Sep 1;27(38):5115–23.

53. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer. 2003 Oct;3(10):756–67.

54. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet. 2003 Mar;33(3):416–21.

55. Proweller A, Tu L, Lepore JJ, Cheng L, Lu MM, Seykora J, Millar SE, Pear WS, Parmacek MS. Impaired notch signaling promotes de novo squamous cell carcinoma formation. Cancer Res. 2006 Aug 1;66(15):7438–44.

56. Lefort K, Mandinova A, Ostano P, Kolev V, Calpini V, Kolfschoten I, Devgan V, Lieb J, Raffoul W, Hohl D, Neel V, Garlick J, Chiorino G, Dotto GP. Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. Genes Dev. 2007 Mar 1;21(5):562–77.

57. Demehri S, Turkoz A, Kopan R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. Cancer Cell. 2009 Jul 7;16(1):55–66.

58. Aste-Amezaga M, Zhang N, Lineberger JE, Arnold BA, Toner TJ, Gu M, Huang L, Vitelli S, Vo KT, Haytko P, Zhao JZ, Baleydier F, L'Heureux S, Wang H, Gordon WR, Thoryk E, Andrawes MB, Tiyanont K, Stegmaier K, Roti G, Ross KN, Franlin LL, Wang F, Chastain M, Bett AJ, Audoly LP, Aster JC, Blacklow SC, Huber HE. Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS One. 2009;5(2):e9094.

59. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, Finkle D, Venook R, Wu X, Ridgway J, Schahin-Reed D, Dow GJ, Shelton A, Stawicki S, Watts RJ, Zhang J, Choy R, Howard P, Kadyk L, Yan M, Zha J, Callahan CA, Hymowitz SG, Siebel CW. Therapeutic antibody targeting of individual Notch receptors. Nature. 2010 Apr 15;464(7291):1052–7.

60. Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J. Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 2004 Oct 15;18(20):2474–8.

61. Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A. 2004 Nov 9;101(45):15949–54.

62. Patel NS, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. Cancer Res. 2005 Oct 1;65(19):8690–7.

63. Mailhos C, Modlich U, Lewis J, Harris A, Bicknell R, Ish-Horowicz D. Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. Differentiation. 2001 Dec;69(2–3):135–44.

64. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature. 2006 Dec 21;444(7122):1032–7.

65. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo JA, de Sauvage F, Plowman G, Yan M. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature. 2006 Dec 21;444(7122):1083–7.

66. Hoey T, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, Fitch-Bruhns M, Lazetic S, Park IK, Sato A, Satyal S, Wang X, Clarke MF, Lewicki J, Gurney A. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. Cell Stem Cell. 2009 Aug 7;5(2):168–77.

67. Liu Z, Turkoz A, Jackson EN, Corbo JC, Engelbach JA, Garbow JR, Piwnica-Worms DR, Kopan R. Notch1 loss of heterozygosity causes vascular tumors and lethal hemorrhage in mice. J Clin Invest. 2010 Feb 1;121(2):800–8.

68. Yan M, Callahan CA, Beyer JC, Allamneni KP, Zhang G, Ridgway JB, Niessen K, Plowman GD. Chronic DLL4 blockade induces vascular neoplasms. Nature. 2010 Feb 11;463(7282):E6–7.

69. Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. Activation of apoptosis in vivo by a

hydrocarbon-stapled BH3 helix. Science. 2004 Sep 3;305(5689):1466–70.

70. Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, Kung AL, Gilliland DG, Verdine GL, Bradner JE. Direct inhibition of the NOTCH transcription factor complex. Nature. 2009 Nov 12;462(7270):182–8.
71. Maillard I, Weng AP, Carpenter AC, Rodriguez CG, Sai H, Xu L, Allman D, Aster JC, Pear WS. Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. Blood. 2004 Sep 15;104(6):1696–702.
72. Weng AP, Nam Y, Wolfe MS, Pear WS, Griffin JD, Blacklow SC, Aster JC. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. Mol Cell Biol. 2003 Jan;23(2):655–64.
73. Nam Y, Sliz P, Song L, Aster JC, Blacklow SC. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Cell. 2006 Mar 10;124(5):973–83.
74. Guarani V, Deflorian G, Franco CA, Kruger M, Phng LK, Bentley K, Toussaint L, Dequiedt F, Mostoslavsky R, Schmidt MH, Zimmermann B, Brandes RP, Mione M, Westphal CH, Braun T, Zeiher AM, Gerhardt H, Dimmeler S, Potente M. Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. Nature. 2011 May 12;473(7346):234–8.
75. Stegmaier K, Ross KN, Colavito SA, O'Malley S, Stockwell BR, Golub TR. Gene expression-based high-throughput screening(GE-HTS) and application to leukemia differentiation. Nat Genet. 2004 Mar;36(3):257–63.
76. Roti G, Ross KN, Mecucci C, Ferrando AA, Aster JC, Stegmaier K. Chemical genomic screen identifies Ionophores as modulators of Notch1 in T-ALL. Blood. 2008;12(11):81.
77. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, Barnes KC, O'Neil J, Neuberg D, Weng AP, Aster JC, Sigaux F, Soulier J, Look AT, Young RA, Califano A, Ferrando AA. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A. 2006 Nov 28;103(48):18261–6.
78. Peck D, Crawford ED, Ross KN, Stegmaier K, Golub TR, Lamb J. A method for high-throughput gene expression signature analysis. Genome Biol. 2006;7(7):R61.
79. Klinakis A, Lobry C, Abdel-Wahab O, Oh P, Haeno H, Buonamici S, van De Walle I, Cathelin S, Trimarchi T, Araldi E, Liu C, Ibrahim S, Beran M, Zavadil J, Efstratiadis A, Taghon T, Michor F, Levine RL, Aifantis I. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature. 2011 May 12;473(7346):230–3.
80. Cullion K, Draheim KM, Hermance N, Tammam J, Sharma VM, Ware C, Nikov G, Krishnamoorthy V, Majumder PK, Kelliher MA. Targeting the Notch1 and mTOR pathways in a mouse T-ALL model. Blood. 2009 Jun 11;113(24):6172–81.
81. Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. Blood. 2007 Jul 1;110(1): 278–86.

Critical Reviews™ in Oncogenesis, 16(1–2), 117–127 (2011)

The Role of Stem Cell Factor SALL4 in Leukemogenesis

Chong Gao, Nikki R. Kong, & Li Chai*

Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

*Address all correspondence to: L. Chai, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115; lchai@partners.org

ABSTRACT: SALL4, a member of the SALL gene family, is one of the most important transcriptional regulators of stem cells. It is of particular interest to stem cell biologists because it is linked to the self-renewal of both embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs), and it is involved in human leukemia. In ESCs, the Sall4/Oct4/Nanog core transcriptional network governs the self-renewal and pluripotent properties of human and murine ESCs. In normal HSCs and leukemic stem cells (LSCs), SALL4 is linked to three known pathways that are involved in self-renewal: Wnt/β-catenin, Bmi-1, and Pten. Despite the important shared role of SALL4 in self-renewal of HSCs and LSCs, our recent studies obtained through correlating global downstream target genes and unique functional studies in normal versus leukemic cells have demonstrated that SALL4 has differential effects on both pro- and anti-apoptotic pathways in normal and leukemic cells. Targeting SALL4, particularly when combined with the use of ABT-737, a BCL2 antagonist, could lead to leukemic cell-specific apoptosis. This review summarizes our current knowledge on the SALL gene family development, particularly on the role of SALL4 in stem cells, as well as tumorigenesis, especially leukemogenesis.

KEY WORDS: transcription factor, acute myeloid leukemia, survival factor

ABBREVIATIONS

AML, acute myeloid leukemia; ChIP, chromatin-immunoprecipitation; DRRS, Duane-radial ray syndrome; ESCs, embryonic stem cells; HDAC, histone deacetylase; HSCs, hematopoietic stem cells; IVIC, Instituto Venezolano de Investigaciones Cientìficas syndrome; LSCx, leukemic stem cells; MDS, myelodysplastic syndrome; NuRD, Mi-2/nucleosome remodeling and deacetylase; PRCs, polycomb-repressive complexes; SP, side population; TBS, Townes-Brockes syndrome

I. THE SALL GENE FAMILY

SALL4, a member of the SALL (spalt-like/sall) gene family (SALL1 to SALL4), was originally cloned based on DNA sequence homology to *Drosophila* gene spalt (sal) and has two major isoforms: SALL4A and SALL4B.[1] Sal is a nonclustered, region-specific homeobox gene and is essential for the development of the posterior head and anterior tail segments of the fly.[2,3] Sal proteins belong to a group of C2H2 zinc finger transcription factors characterized by multiple zinc finger domains distributed over the entire protein.[1] Structural characteristics of human SALL include a single C2HC zinc finger near the N-terminus and several C2H2 zinc fingers in the middle portion of and at the C-terminus of the protein (Figure 1A).[4] C2H2 Zinc finger domains can bind to DNA, and in some cases, to RNA and proteins.

II. THE ROLE OF SALL4 IN ESCs

Over the past few years, remarkable progress has been made in identifying stem cell factors that are essential in the maintenance of self-renewal and pluripotent capacities of embryonic stem cells (ESCs). Several research groups, including ours, have demonstrated that Sall4 plays an essential role in this process[5–10] by interacting with two other key regulators in ESCs: Nanog and Oct4. Sall4 regulates Oct4 expression by directly binding to the highly conserved regulatory region of Oct4, as demonstrated both *in vivo* and *in vitro*. Sall4$^{+/-}$ ESCs have significantly reduced levels of pluripotency markers (Oct4, Sox2, and Nanog) compared to wild-type (WT) ESCs. These findings were consistent with the results obtained from RNAi-mediated knockdown of Sall4 using an RNAi lentiviral vector.[5] Similar to Oct4, reduction of Sall4

0893-9675/11/$35.00

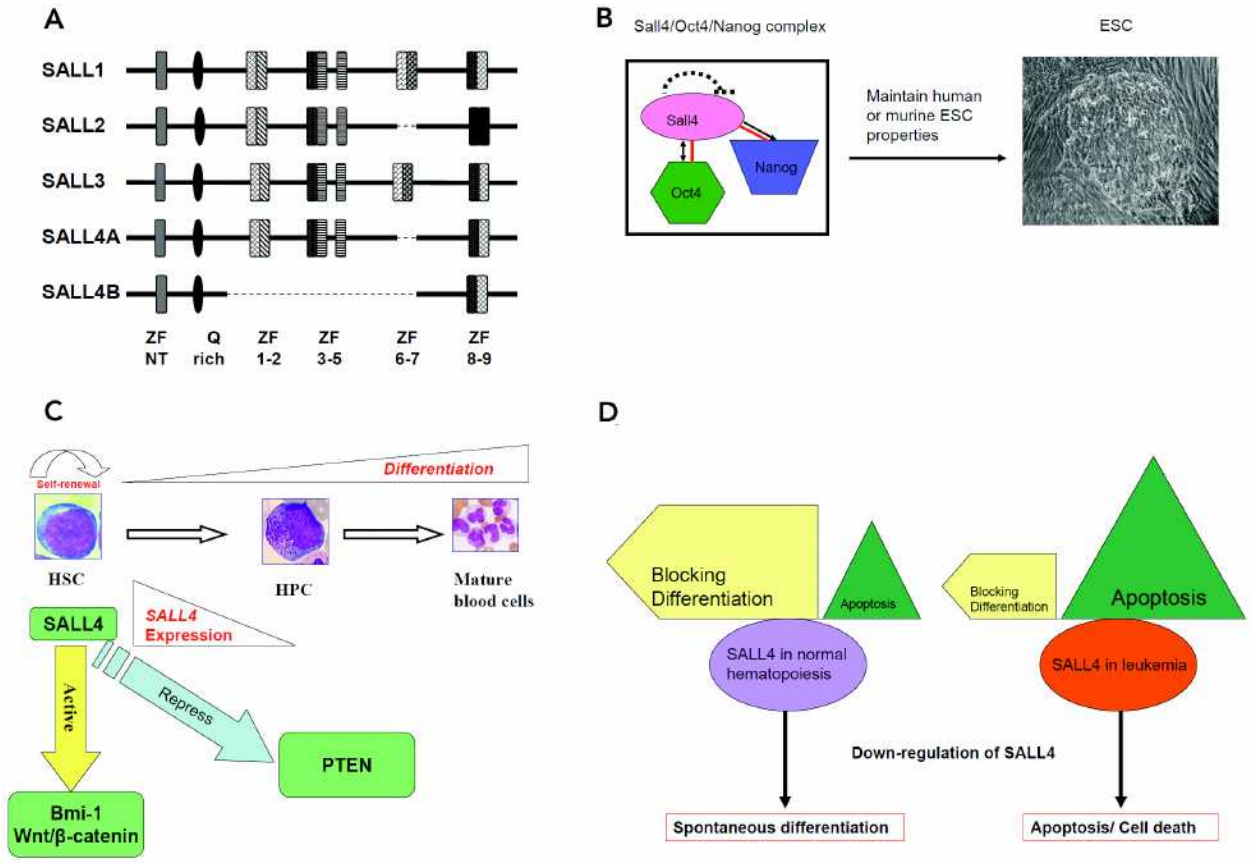


FIGURE 1. The role of SALL4 in ESC, HSC and LSC. (A) Schematic representation of the main domains present in human SALL proteins. Rectangles represent the N-terminal C2HC type zinc finger (NT ZF) and C2H2 type zinc fingers (ZF 1 to 9). Ovals represent the polyQ regions. The black rectangles indicate the C-terminal zinc fingers in SALL2 which are not homologous to those in other SALL proteins. The break lines represent the regions that are absent in these proteins. (B) Sall4/Oct4/Nanog core transcriptional network in ESC. Sall44 contributes to the maineince of murine and human ESC stemness by regulating and interacting with Oct4 and Nanog. In addtion, while we have observed that a positive feedback relationship is present between human SALL4 and OCT4, the strong self-repression of SALL4 seems to be the "break" for this loop. Black arrow: activation, black dash arrow: inhibition. Red solid line: interaction. (C) The SALL4/Wnt/Bmi-1/PTEN network in normal and leukemic hematopoiesis. SALL4 contributes to hematopoietic differentiation at least, in part, through repression of PTEN, activation of Bmi-1 and interacts with Wnt /β-catenin. During normal hematopoiesis, SALL4 is preferentially expressed in HSCs, down-regulated as HSCs differentiate into HPCs and is absent in mature myeloid populations such as neutrophils. Down-regulation of SALL4 leads to differentiation. Dys-regulateion of SALL4/Wnt/Bmi-1/PTEN network can lead to leukemic development. (D) Proposed model on differential effects of SALL4 in normal and leukemic hematopoiesis. While the main effect of SALL4 in leukemic cells is to promote cell survival; the major functional role of SALL4 in normal hematopoiesis is to maintain cells at the stem/progenitor state with expression of CD34 and to block differentiation. Upon down-regulation of SALL4 expression, leukemic cells undergo apoptosis and cell death, while normal hematopoietic stem/progenitor cells are induced to differentiate spontaneously.

in murine ESCs results in respecification of ESCs to the trophoblast lineage.[5]

In addition, we and others have mapped the global gene targets of Sall4 using chromatin-immunoprecipitation followed by microarray hybridization (ChIP-chip).[11,12] Over thousands of Sall4 target genes have been identified, and most have been classified as genes related to stem cell maintenance and development. Co-immunoprecipitation showed that Sall4, Oct4, and Nanog formed a complex; this

is consistent with the finding that Sall4 co-occupied the promoters of Oct4 and Nanog target genes.[11] Sall4 regulation of pluripotency appears to be tightly linked to transcriptional regulation as well as the chromatin-based epigenetic events mediated by polycomb-repressive complexes (PRCs) and bivalent domains.[11] This evidence suggests that Sall4 plays a central but diverse role during early embryonic development.

Most recently, we and others have reported a similar role of SALL4 in human ESCs and have shown for the first time that SALL4 is required for the maintenance of human ESC characteristics.[13] Furthermore, we found a novel SALL4/OCT4 transcription regulatory loop in balancing the proper expression dosage of SALL4 and OCT4 for the maintenance of ESC stemness. While we have observed that a positive feedback relationship is present between SALL4 and OCT4, the strong self-repression of SALL4 seems to be the "break" in this loop. In addition, we have shown that SALL4 can repress the promoters of other SALL family members, such as SALL1 and SALL3, and competes with the activation of these two genes by OCT4. Overall these studies suggest that SALL4 is a master regulator that controls its own expression and the expression of OCT4. Moreover, SALL4 and OCT4 work antagonistically to balance the expressions of other SALL gene family members.[13]

III. THE FUNCTION OF THE SALL GENE FAMILY IN DEVELOPMENT

Sal plays an important role in the embryonic development of the larval tracheal system and the adult wing. Sal-related genes have been identified in *C. elegans*,[14] fish,[15] frogs (*Xenopus*),[16,17] mice,[18] and humans.[1] Sall1 and Sall3 are normally expressed in mouse from embryonic day 7 (E7). The expression of Sall2 in early murine development has not been well studied yet. Sall1$^{-/-}$ mice die in the perinatal period and have kidney abnormalities,[19] whereas Sall2-deficient mice show no apparent abnormal phenotypes.[20] Sall3$^{-/-}$ mice survive until birth but fail to feed and eventually die on the first postnatal day.[21] In contrast, Sall4 is visible in the 8- to 16-cell stage of the early embryo and becomes enriched in the inner cell mass,[10] from which the ESCs are derived. Sall4 null mice die at E6.5,[9] suggesting that among the Sall family, Sall4 is most critical for early embryonic development.

In humans, SALL1 is mutated in patients with Townes-Brockes Syndrome (TBS), a disorder characterized by urogenital, limb, anal and cardiac malformations.[22–24] Defects in hematopoiesis have not been reported to date in patients with TBS. Similar to SALL1, SALL2 is expressed in the developing neuroectoderm of the brain, inner ear, and urogenital ridge-derived structures (e.g., testes, ovaries, and kidneys), though Sall2-deficient mice show no apparent abnormal phenotypes and no evidence shows the association between SALL2 and developmental disorder in human. SALL3 is expressed in the developing central nervous system: heart, limb buds, kidneys, ears, and palate. SALL3 is mapped to human chromosome 18q23.[25] Because the Sall3-null mice have multiple developmental defects, it has been suggested that the SALL3 gene product may be involved in the phenotype of patients with 18q deletion syndrome characterized by developmental delay, hypotonia, growth retardation, midface hypoplasia, hearing loss, and tapered fingers.[25] The latest SALL gene member, SALL4, is mutated in human Duane-radial ray syndrome (DRRS) and Instituto Venezolano de Investigaciones Cientìficas syndrome (IVIC).[26–29] Both syndromes are autosomal-dominant developmental disorders involving radial-sided hand anomalies and congenital strabismus. IVIC is also characterized by leukocytosis and thrombocytopenia, suggesting that SALL4 may be involved in normal hematopoiesis.[30]

IV. SALL GENE FAMILY IN CANCER

In addition to genetic diseases, the SALL gene family has been reported in several types of human tumors (Table 1). Consistently high expression of SALL1 was detected in Wilms tumor, a pediatric renal cancer.[31] Also, overexpression of SALL1 mRNA was identified in the carcinoma *in situ* cells of testicular germ cell tumor.[32] However, hypermethylation of multiple CpG islands of the SALL1 gene was reported in

TABLE 1. SALL Gene Expression in Cancers

Gene	Tumor	Status of Gene	Reference
	Wilms tumor and trophoblast tumors	Upregulated	54, 55
	Sex hormone–producing tumors	Upregulated	55
SALL1	Testicular carcinoma	Upregulated	56
	Acute lymphocytic leukemia	Methylation	57
	Breast cancer	Methylation	33
SALL2	Prostate and breast cancer	Downregulated	58
	Lung carcinoma	Downregulated	59
	Colon and prostate adenocarcinoma	Downregulated	59
	Ovarian carcinoma	Downregulated	60
SALL3	Hepatocellular carcinoma	Methylation	38
	Bladder cancer	Methylation	61
	Acute myeloid leukemia	Upregulated	42
	Acute lymphoblastic leukemia	Upregulated	62
	Ovarian primitive germ cell tumors	Upregulated	39
SALL4	Metastatic germ cell tumors	Upregulated	63
	Testicular primitive germ cell tumors	Upregulated	40, 64
	Extragonadal yolk sac tumors	Upregulated	65
	Gastric cancers	Upregulated	41
	Kidney cancer	Upregulated	66
	Breast cancer	Up-regulated	44, 67, 68

acute lymphocytic leukemia and breast cancer,[33] and all patients with methylation of multiple CpG islands of SALL1 had a worse overall survival rate. Therefore, the role of SALL1 in cancer still needs to be defined.

The SALL2 gene is mapped to chromosome 14q12.1–13, a region that is associated with loss of heterozygosity in 49% of ovarian cancers[34] and 25% of bladder cancers. SALL2 is expressed in a number of mouse and human tissues. Our previous study demonstrated loss of SALL2 expression in some solid tumors[35] and suggested that SALL2 may function as a tumor suppressor. Murine Sall2 was able to bind to the large T antigen of polyoma virus, a DNA virus that induces a broad variety of neoplasms in its natural host.[36] Overexpression of the SALL2 gene by transfection inhibits the growth and DNA synthesis of the ovarian cancer cell line SKVO3 and tumor formation in nude mice, as a result of the induction of $p21^{Cip1/Waf1}$.[37] These observations further indicate that SALL2 may have roles in suppressing tumorigenesis.

To date, only one study on the role of SALL3 in cancer has been published. SALL3 can interact with DNMT3A, a DNA methytransferase, in CpG island methylation *in vitro*. In addition, downregulation of SALL3 results in acceleration of DNA methylation in hepatocellular carcinoma.[38]

The most well-studied SALL gene in cancer is SALL4. Recent studies have shown that SALL4 is a novel sensitive and specific diagnostic marker of ovarian primitive germ cell tumors and testicular germ cell tumors.[39,40] SALL4 has also been identified as a sensitive marker for AFP-producing gastric carcinoma that is especially useful in distinguishing hepatoid gastric carcinoma from hepatocellular carcinoma.[41]

V. THE ROLE OF SALL4 IN LEUKEMOGENESIS

A. SALL4 as a Diagnostic Marker of Leukemia

During normal hematopoiesis, SALL4 is preferentially expressed in human CD34+ hematopoietic stem/progenitor cells (HSCs) and down-regulated in CD34 cells during hematopoietic differentiation.[42] Using immunohistological staining and quantitative RT-PCR analysis, we found that SALL4 was aberrantly expressed in many leukemia cell lines and primary leukemia cells of acute myeloid leukemia (AML) and precursor B-cell lymphoblastic leukemia/lymphomas.[42] Recent publications from our group have shown that SALL4 expression correlates with disease progression in human chronic myeloid leukemia,[43] and its expression in AML patients correlated with treatment status. We further explored the role of SALL4 in drug resistance and found that SALL4 was involved in the maintenance of side-population (SP) cells by regulating ATP-binding cassette drug transport genes.[44] Therefore, SALL4 may be used as a marker for diagnosis and prognosis for AML.

B. Functional Studies of SALL4 in Leukemia

Our research group has demonstrated that constitutive expression of SALL4 contributes to leukemogenesis in adult mice.[42] Mice transgenic for SALL4B, one of the SALL4 isoforms that we identified, developed pre-leukemic myelodysplastic syndrome (MDS)-like features and subsequent acute myeloid leukemia (AML), suggesting that SALL4 contributes to the initiation of leukemia.[42] In addition, loss-of-function studies have demonstrated that SALL4 is a key regulator in leukemic cell survival and that down-regulation of SALL4 leads to significant apoptosis of leukemic cells,[45] suggesting that SALL4 is essential for the maintenance of leukemia.

C. SALL4 Protein Partners

In searching for the mechanism of SALL4-meidated leukemogenesis, we found that SALL4 could bind to β-catenin and synergistically enhance the Wnt/β-catenin signaling pathway. The expression of cyclin-D1 and c-Myc, the two known targets of the Wnt/β-catenin pathway, were also increased in the SALL4B transgenic bone marrow cells.[42]

We also sought to identify SALL4-associated proteins by tandem mass spectrometry. Components of a transcriptional repressor Mi-2/nucleosome remodeling and deacetylase (NuRD) complex were found in the SALL4-immunocomplexes with histone deacetylase (HDAC) activity both in ESCs with endogenously high SALL4 expression and 293T cells overexpressing SALL4.[46]

D. Important Downstream Target Genes of SALL4 in Self-Renewal in HSCs and LSCs

The SALL4-mediated transcriptional repression was tested on PTEN, one potential SALL4 target gene. PTEN was confirmed to be a SALL4 downstream target by chromatin-immunoprecipitation (ChIP). Moreover, the SALL4 binding site in the promoter region of PTEN was co-occupied by NuRD components, suggesting that SALL4 repressed the transcription of PTEN through its interactions with the Mi-2/NuRD complex. The *in vivo* repressive effect(s) of SALL4 were evaluated in SALL4B transgenic mice, where decreased expression of Pten was associated with myeloid leukemia.[46]

In addition to Pten,[46] we and others have previously demonstrated that Bmi-1[45,47] is also a SALL4 target gene. We have shown that transcription from the Bmi-1 promoter is activated by SALL4 in a dose-dependent manner when using a luciferase reporter gene assay. Both promoter deletion construct studies and ChIP from a myeloid stem cell line, 32D, demonstrating that SALL4 binds to a specific region of the Bmi-1 promoter. Down-regulation of SALL4 by siRNA in the HL-60 leukemia cell line results in decreased Bmi-1 expression. Furthermore, Bmi-1 expression is up-regulated in SALL4B transgenic mice, and the levels of Bmi-1 in these mice increase as they progress from normal to preleukemic (MDS) and leukemic (AML) stages.

In summary, both PTEN and Bmi-1, the two key regulators of self-renewal of normal HSCs and leukemic stem cells (LSCs),[48–50] are direct targets of

SALL4. Bmi-1 is up-regulated and Pten is down-regulated in SALL4B murine leukemic model.[46,47]

E. Differential Effects on Pro-/Anti-Apoptotic Pathways Upon Down-regulation of SALL4 in Normal and Leukemic Cells

Recently, SALL4 was found to be a robust stimulator in the expansion of hematopoietic stem cells.[51] To determine the transcriptional network that may be responsible for the function of SALL4, we performed a genome-wide analysis of SALL4 target genes in human CD34+ and myeloid leukemic cells. Chromatin-immunoprecipitated (ChIP) DNA was obtained from immunomagnetically selected CD34+ cells, leukemic blasts, and leukemic cell line NB4 cells using an affinity-purified polyclonal anti-SALL4 antibody. This SALL4 antibody has been previously used in immunohistochemistry, flow cytometry, and western blot analysis, and it has been used to identify SALL4 targets in ChIP experiments in NB4 cells and murine ES cells.[11,45]

When SALL4 targets from leukemia and CD34+ cells were compared, many genes that were regulated by SALL4 in normal CD34+ cells could not be found in the data sets from leukemia cells. Comparison between the two groups of targets in CD34+ and leukemia cells yielded some common, but mostly unique, SALL4 targets, particularly the pro-/anti-apoptotic pathways.

We next sought to test whether SALL4 has differential effects on the pro- and anti-apoptotic pathways in these two cell types. While down-regulation of SALL4 in leukemia triggered significant cell death, knocking down of SALL4 in normal CD34+ cells did not affect cell viability. We noticed that three genes were differentially affected by SALL4 in leukemic versus normal CD34+ cells. In normal CD34+ cells, while no obvious apoptotic phenotypes were observed, the expression of apoptosis induction genes TRO and ABL1 was decreased, and the expression of apoptosis inhibition gene, BCL2, was consistently increased in SALL4-reduced CD34+ cells. In contrast, when SALL4 was down-regulated in the leukemic cell lines KG1a, NB4 and KBM5, and in primary AML cells, along with the apoptotic phenotypes, the expression of TRO and ABL1 increased while the expression of BCL2 decreased.

Next, we tested whether the SALL4/BCL2 pathway is essential for maintaining the survival of the leukemic cells by using the small-molecule drug, ABT-737. ABT-737 is a BH3 domain mimetic that can interrupt the interaction of BCL2 and its family members and results in the inhibition of the anti-apoptotic function of BCL2. It can trigger leukemic cell apoptosis but has no effects on normal hematopoiesis.[52,53] Because SALL4 can regulate BCL2 expression and ABT-737 can affect its function, we hypothesized that we may observe an enhanced effect in promoting leukemic cell apoptosis if we combined the use of ABT-737 with down-regulation of SALL4. To test our theory, 2.5×10^5 primary human AML cells infected with SALL4 shRNA lentivirus, control shRNA, or no virus control were treated with either DMSO or 10nM ABT-737 for 12 hours. The cells were tested for apoptosis and cell death by Annexin V/PI staining. As reported previously by others,[53] ABT-737 treatment alone can lead to apoptosis and cell death in primary AML samples. Interestingly, by knocking down SALL4 in these cells, in combination with ABT-737 treatment, we observed an additional two- to three-fold increase in apoptosis compared to cells treated with ABT-737 alone or ABT-737 with control shRNA. Cells treated with solvent control DMSO did not show significant cell death. These results show that the combination of ABT-737 and down-regulation of SALL4 could be a novel therapeutic strategy in treating AML patients.

VI. CONCLUSION

In conclusion, SALL4 is one of few genes that bridge the self-renewal properties of ESCs, normal HSCs, and LSCs**.** In ESCs, the Sall/Oct4/Nanog core transcriptional network governs the self-renewal and pluripotent properties of human and mouse ESCs (Figure 1B). In normal HSCs and LSCs, SALL4 is involved in three known self-renewal pathways: Wnt/β-catenin, Bmi-1, and Pten pathways (Figure 1C).

Our more recent unpublished studies by correlating global downstream target genes and unique

function(s) in normal versus leukemic cells have demonstrate that SALL4 has differential effects on both pro- and anti-apoptotic pathways in normal and leukemic cells. It has been proposed that cancer stem cells arise from dysregulated normal stem or progenitor cells; however, the key regulator(s) responsible for the dysregulated cellular programs directing proliferation versus differentiation have not been well defined. There are very few reports of a single gene inducing differential effects on normal stem/progenitor cells and their malignant counterparts. While the main effect of SALL4 in leukemic cells is to maintain cell survival, the major functional role of SALL4 in normal hematopoiesis is to retain stem and progenitor cells in an undifferentiated stage and to antagonize myeloid differentiation (Figure 1D). The differential biological effects of SALL4 in normal versus leukemic hematopoiesis correlate with the limited number of overlapping SALL4 direct target genes and its differential regulations of these target genes in the two cell types. Therefore, SALL4-mediated stem cell function is cell-context–dependent. Based on our observation that SALL4 is an essential factor for survival of the leukemic cells, it is reasonable to propose future anti-cancer applications that combine the strategies targeting SALL4. In addition, ABT-737 can be used to exploit the unique property that reduced SALL4 expression or function leads to apoptosis of leukemic but not normal hematopoietic stem cells.

Finally, many questions regarding the role of SALL4 in leukemogenesis, such as the mechanism(s) of SALL4 gene expression regulation in normal hematopoiesis and dysregulation in leukemia, still remain unanswered and require future investigation.

ACKNOWLEDGMENT

This work was supported in part through National Iinstitutes of Health (Grant Nos.sRO1HL092437, RO1HL092437-A1S1, and PO1HL095489 and PO1DK080665) (to L.C). We also want to thank Kol Jia Yong, Xi Tian and Youyang Yang for assisting with the preparation of the manuscript.

REFERENCES

1. Kohlhase J, Schuh R, Dowe G, Kuhnlein R, Jackle H, Schroeder B, Schulz-Schaeffer W, Kretzchmar H, Kohler A, Muller U, Raab-Vetter M, Burkhardt E, Engel W, Stick R. Isolation, characterization and organ-specific expression of two novel human zinc finger genes related to the *Drosophila* gene Spalt. Genomics. 1996;38(3):291–8.
2. Kuhnlein RP, Frommer G, Friedrich M, Gonsalez-Gaitan M, Weber A, Wagner-Bernholz UF, Gehring WJ, Jackle H, Schuh R. Spalt encodes an evolutionarily conservative zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of *Drosophila* embryo. EMBO J. 1994;13(1):168–79.
3. Jurgens G. Head and tail development of the *Drosophila* embryo involves Spalt, a novel homeotic gene. EMBO J. 1988;7:189–96.
4. de Celis JF, Barrio R. Regulation and function of Spalt proteins during animal development. The Int J Dev Biol. 2009;53(8–10):1385–98.
5. Zhang J, Tam WL, Tong GQ, Wu Q, Chan HY, Soh BS, Lou Y, Yang J, Ma Y, Chai L, Ng HH, Lufkin T, Robson P, Lim B. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol. 2006 Oct;8(10):1114–23.
6. Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. A protein interaction network for pluripotency of embryonic stem cells. Nature. 2006 Nov 16;444(7117):364–8.
7. Wu Q, Chen X, Zhang J, Loh YH, Low TY, Zhang W, Sze SK, Lim B, Ng HH. Sall4 interacts with Nanog and co-occupies Nanog genomic sites in embryonic stem cells. J Biol Chem. 2006 Aug 25;281(34):24090–4.
8. Warren M, Wang W, Spiden S, Chen-Murchie D, Tannahill D, Steel KP, Bradley A. A Sall4 mutant mouse model useful for studying the role of Sall4 in early embryonic development and organogenesis. Genesis. 2007 Jan;45(1):51–8.
9. Sakaki-Yumoto M, Kobayashi C, Sato A, Fujimura S, Matsumoto Y, Takasato M, Kodama T, Aburatani H, Asashima M, Yoshida N, Nishinakamura R. The murine homolog of Sall4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with sall1 in anorectal,

heart, brain and kidney development. Development. 2006 Aug;133(15):3005–13.

10. Elling U, Klasen C, Eisenberger T, Anlag K, Treier M. Murine inner cell mass-derived lineages depend on Sall4 function. Proc Natl Acad Sci U S A. 2006 Oct 31;103(44):16319–24.
11. Yang J, Chai L, Fowles TC, Alipio Z, Xu D, Fink LM, Ward DC, Ma Y. Genome-wide analysis reveals Sall4 to be a major regulator of pluripotency in murine-embryonic stem cells. Proc Natl Acad Sci U S A. 2008 Dec 16;105(50):19756–61.
12. Lim CY, Tam WL, Zhang J, Ang HS, Jia H, Lipovich L, Ng HH, Wei CL, Sung WK, Robson P, Yang H, Lim B. Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages. Cell Stem Cell. 2008 Nov 6;3(5):543–54.
13. Yang J, Gao C, Chai L, Ma Y. A novel Sall4/Oct4 transcriptional feedback network for pluripotency of embryonic stem cells. PLoS One. 2010;5(5):e10766.
14. Basson M, Horvitz HR. The *Caenorhabditis Elegans* gene Sem-4 controls neuronal and mesodermal cell development and encodes a zinc finger protein. Genes Dev. 1996;10(15):1953–65.
15. Koster R, Stick R, Loosli F, Wittbrodt J. Medaka Spalt acts as a target gene of Hedgehog signaling. Development. 1997;124(16):3147–56.
16. Onuma Y, Nishinakamura R, Takahashi S, Yokota T, Asashima M. Molecular cloning of a novel *Xenopus* Spalt gene (Xsal-3). Biochem Biophys Res Commun. 1999;264(1):151–6.
17. Hollemann T, Schuh R, Pieler T, Stick R. *Xenopus* Xsal-1, a vertebrate homolog of the region specific homeotic gene Spalt of *Drosophila*. Mech Dev. 1996;55(1):19–32.
18. Ott T, Kaestner KH, Monaghan AP, Schutz G. The mouse homolog of the region specific homeotic gene Spalt of *Drosophila* is expressed in the developing nervous system and in mesoderm-derived structures. Mech Dev. 1996;56(1–2):117–28.
19. Nishinakamura R, Matsumoto Y, Nakao K, Nakamura K, Sato A, Copeland NG, Gilbert DJ, Jenkins NA, Scully S, Lacey DL, Katsuki M, Asashima M, Yokota T. Murine homolog of Sall1 is essential for ureteric bud invasion in kidney development. Development. 2001 Aug;128(16):3105–15.
20. Sato A, Matsumoto Y, Koide U, Kataoka Y, Yoshida N, Yokota T, Asashima M, Nishinakamura R. Zinc finger protein Sall2 is not essential for embryonic and kidney development. Mol Cell Biol. 2003 Jan;23(1):62–9.
21. Parrish M, Ott T, Lance-Jones C, Schuetz G, Schwaeger-Nickolenko A, Monaghan AP. Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. Mol Cell Biol. 2004 Aug;24(16):7102–12.
22. de Vries-Van der Weerd MA, Willems PJ, Mandema HM, ten Kate LP. A new family with the Townes-Brocks syndrome. Clin Genet. 1988;34(3):195–200.
23. Marlin S, Toublanc JE, Petit C. Two cases of Townes-Brocks syndrome with previously undescribed anomalies. Clin Dysmorphol. 1998;7(4):295–8.
24. Kohlhase J, Taschner P, Burfeind P, Pasche B, Newman B, Blanck C, Breuning M, ten Kate L, Maaswinkel-Mooy P, Mitulla B, Seidel J, Kirkpatrick S, Pauli R, Wargowski D, Devriendt K, Proesmans W, Gabrielli O, Coppa G, Wesby-van Sway E, Trembath R, Schnizel A, Reardon W, Seemanova E, Engel W. Molecular analysis of Sall1 mutations in Townes-Brocks syndrome. Am J Hum Genet. 1999;64(2):435–45.
25. Kohlhase J, Hausmann S, Stojmenovic G, Dixkens C, Bink K, Schulz-Schaeffer W, Altmann M, Engel W. Sall3, a new member of the human Spalt-like gene family, maps to 18q23. Genomics. 1999;62(2):216–22.
26. Al-Baradie R, Yamada K, St Hilaire C, Chan WM, Andrews C, McIntosh N, Nakano M, Martonyi EJ, Raymond WR, Okumura S, Okihiro MM, Engle EC. Duane-radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in Sall4, a new member of the Sal family. Am J Hum Genet. 2002 Nov;71(5):1195–9.
27. Borozdin W, Wright MJ, Hennekam RC, Hannibal MC, Crow YJ, Neumann TE, Kohlhase J. Novel Mutations in the Gene Sall4 provide further evidence for acro-renal-ocular and Okihiro syndromes being allelic entities, and extend the phenotypic spectrum. J Med Genet. 2004 Aug;41(8):e102.
28. Kohlhase J, Schubert L, Liebers M, Rauch A, Becker K, Mohammed SN, Newbury-Ecob R, Reardon W. Mutations at the Sall4 locus on chromosome

20 result in a range of clinically overlapping phenotypes, including Okihiro syndrome, Holt-Oram syndrome, acro-renal-ocular syndrome, and patients previously reported to represent thalidomide embryopathy. J Med Genet. 2003 Jul;40(7):473–8.

29. Kohlhase J, Heinrich M, Schubert L, Liebers M, Kispert A, Laccone F, Turnpenny P, Winter RM, Reardon W. Okihiro syndrome is caused by Sall4 mutations. Hum Mol Genet. 2002 Nov 1;11(23):2979–87.
30. Paradisi I, Arias S. Ivic syndrome is caused by a C.2607dela mutation in the Sall4 locus. Am J Med Genet A. 2007 Feb 15;143(4):326–32.
31. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG. Dominant-negative mutations of Cebpa, encoding Ccaat/enhancer binding protein-alpha (C/Ebpalpha), in acute myeloid leukemia. Nat Genet. 2001 Mar;27(3):263–70.
32. Hoei-Hansen CE, Nielsen JE, Almstrup K, Hansen MA, Skakkebaek NE, Rajpert-DeMeyts E, Leffers H. Identification of genes differentially expressed in testes containing carcinoma in situ. Mol Hum Reprod. 2004 Jun;10(6):423–31.
33. Hill VK, Hesson LB, Dansranjavin T, Dallol A, Bieche I, Vacher S, Tommasi S, Dobbins T, Gentle D, Euhus D, Lewis C, Dammann R, Ward RL, Minna J, Maher ER, Pfeifer GP, Latif F. Identification of 5 novel genes methylated in breast and other epithelial cancers. Mol Cancer. 2010; 9:51. doi:10.1186/1476-4598-9-51.
34. Bandera CA, Takahashi H, Behbakht K, Liu PC, LiVolsi VA, Benjamin I, Morgan MA, King SA, Rubin SC, Boyd J. Deletion mapping of two potential chromosome 14 tumor suppressor gene loci in ovarian carcinoma. Cancer Res. 1997;57(3):513–5.
35. Ma Y, Li D, Chai L, Luciani AM, Ford D, Morgan J, Maizel AL. Cloning and characterization of two promoters for the human Hsal2 gene and their transcriptional repression by the Wilms tumor suppressor gene product. J Biol Chem. 2001;276(51): 48223–30.
36. Li D, Dower K, Ma Y, Tian Y, Benjamin TL. A tumor host range selection procedure identifies P150(Sal2) as a target of polyoma virus large T antigen. Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14619–24.
37. Li D, Tian Y, Ma Y, Benjamin T. P150sal2 is a P53-independent regulator of P21waf1/Cip. Mol Cell Biol. 2004 May 1, 24(9):3885–93.
38. Shikauchi Y, Saiura A, Kubo T, Niwa Y, Yamamoto J, Murase Y, Yoshikawa H. Sall3 interacts with Dnmt3a and shows the ability to inhibit Cpg island methylation in hepatocellular carcinoma. Mol Cell Biol. 2009 Apr;29(7):1944–58.
39. Cao D, Guo S, Allan RW, Molberg KH, Peng Y. Sall4 is a novel sensitive and specific marker of ovarian primitive germ cell tumors and is particularly useful in distinguishing yolk sac tumor from clear cell carcinoma. Am J Surg Pathol. 2009 Jun;33(6):894–904.
40. Cao D, Li J, Guo CC, Allan RW, Humphrey PA. Sall4 is a novel diagnostic marker for testicular germ cell tumors. Am JSurg Pathol. 2009 Jul;33(7):1065–77.
41. Ushiku T, Shinozaki A, Shibahara J, Iwasaki Y, Tateishi Y, Funata N, Fukayama M. Sall4 represents fetal gut differentiation of gastric cancer, and is diagnostically useful in distinguishing hepatoid gastric carcinoma from hepatocellular carcinoma. Am J Surg Pathol. 2010; Apr;34(4):533–40.
42. Ma Y, Cui W, Yang J, Qu J, Di C, Amin HM, Lai R, Ritz J, Krause DS, Chai L. Sall4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. Blood. 2006 Oct 15;108(8):2726–35.
43. Lu J, Ma Y, Kong N, Alipio Z, Gao C, Krause DS, Silberstein LE, Chai L. Dissecting the role of sall4, a newly identified stem cell factor, in chronic myelogenous leukemia. Leukemia **25**, 1211–1213 (July 2011) | doi:10.1038/leu.2011.65
44. Jeong HW, Cui W, Yang Y, Lu J, He J, Li A, Song D, Guo Y, Liu BH, Chai L. Sall4, a stem cell factor, affects the side population by regulation of the ATP-binding cassette drug transport genes. PLoS One. 2011 Apr 19;6(4):e18372.
45. Yang J, Chai L, Gao C, Fowles TC, Alipio Z, Dang H, Xu D, Fink LM, Ward DC, Ma Y. Sall4 Is a key regulator of survival and apoptosis in human leukemic cells. Blood. 2008 Aug 1;112(3):805–13.
46. Lu J, Jeong HW, Kong N, Yang Y, Carroll J, Luo HR, Silberstein LE, Yupoma, Chai L. Stem cell factor Sall4 represses the transcriptions of Pten and Sall1 through an epigenetic repressor complex. PloS ONE. 2009;4(5):e5577.
47. Yang J, Chai L, Liu F, Fink LM, Lin P, Silberstein LE, Amin HM, Ward DC, Ma Y. Bmi-1 is a target gene for Sall4 in hematopoietic and leukemic cells. Proc Natl Acad Sci U S A. 2007 Jun 19;104(25):10494–9.

48. Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L. Pten maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature. 2006 May 25;441(7092):518–22.

49. Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, Ema H, Kamijo T, Katoh-Fukui Y, Koseki H, van Lohuizen M, Nakauchi H. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity. 2004 Dec;21(6):843–51.

50. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature. 2003 May 15;423(6937):302–5.

51. Aguila JR, Liao W, Yang J, Avila C, Hagag N, Senzel L, Ma Y. Sall4 is a robust stimulator for the expansion of hematopoietic stem cells. Blood. May 20. 2011 doi:10.1182/blood-2011-01-333641

52. Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S, Deng X, Zhai D, Shi YX, Sneed T, Verhaegen M, Soengas M, Ruvolo VR, McQueen T, Schober WD, Watt JC, Jiffar T, Ling X, Marini FC, Harris D, Dietrich M, Estrov Z, McCubrey J, May WS, Reed JC, Andreeff M. Mechanisms of apoptosis sensitivity and resistance to the Bh3 mimetic Abt-737 in acute myeloid leukemia. Cancer Cell. 2006 Nov;10(5):375–88.

53. Will B, Siddiqi T, Jorda MA, Shimamura T, Luptakova K, Staber PB, Costa DB, Steidl U, Tenen DG, Kobayashi S. Apoptosis induced by Jak2 inhibition is mediated by bim and enhanced by the Bh3 mimetic Abt-737 in Jak2 mutant human erythroid cells. Blood. 2010 Apr 8;115(14):2901–9.

54. Ma Y, Chai L, Cortez SC, Stopa EG, Steinhoff MM, Ford D, Morgan J, Maizel AL. Sall1 expression in the human pituitary-adrenal/gonadal axis. J Endocrinol. 2002 Jun;173(3):437–48.

55. Ma Y, Singer DB, Gozman A, Ford D, Chai L, Steinhoff MM, Hansen K, Maizel AL. Hsal 1 is related to kidney and gonad development and is expressed in Wilms tumor. Pediatr Nephrol. 2001 Sep;16(9):701–9.

56. Hoeflich A, Wu M, Mohan S, Foll J, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb HJ, Wolf E. Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. Endocrinology. 1999 Dec;140(12):5488–96.

57. Kuang SQ, Tong WG, Yang H, Lin W, Lee MK, Fang ZH, Wei Y, Jelinek J, Issa JP, Garcia-Manero G. Genome-wide identification of aberrantly methylated promoter associated Cpg islands in acute lymphocytic leukemia. Leukemia. 2008 Aug;22(8):1529–38.

58. Ma Y, Li D, Chai L, Luciani AM, Ford D, Morgan J, Maizel AL. Cloning and characterization of two promoters for the human Hsal2 gene and their transcriptional repression by the Wilms tumor suppressor gene product. J Biol Chem. 2001 Dec 21;276(51):48223–30.

59. Li D, Tian Y, Ma Y, Benjamin T. P150(Sal2) Is a P53-independent regulator of P21(Waf1/Cip). Mol Cell Biol. 2004 May;24(9):3885–93.

60. Liu H, Adler AS, Segal E, Chang HY. A transcriptional program mediating entry into cellular quiescence. PLoS Genetics. 2007 Jun;3(6):e91.

61. Yu J, Zhu T, Wang Z, Zhang H, Qian Z, Xu H, Gao B, Wang W, Gu L, Meng J, Wang J, Feng X, Li Y, Yao X, Zhu J. A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer. Clin Cancer Res. 2007 Dec 15;13(24):7296–304.

62. Cui W, Kong NR, Ma Y, Amin HM, Lai R, Chai L. Differential expression of the novel oncogene, Sall4, in lymphoma, plasma cell myeloma, and acute lymphoblastic leukemia. Mod Pathol. 2006 Dec;19(12):1585–92.

63. Cao D, Humphrey PA, Allan RW. Sall4 is a novel sensitive and specific marker for metastatic germ cell tumors, with particular utility in detection of metastatic yolk sac tumors. Cancer. 2009 Jun 15;115(12):2640–51.

64. Mei K, Liu A, Allan RW, Wang P, Lane Z, Abel TW, Wei L, Cheng H, Guo S, Peng Y, Rakheja D, Wang M, Ma J, Rodriguez MM, Li J, Cao D. Diagnostic utility of Sall4 in primary germ cell tumors of the central nervous system: a study of 77 cases. Mod Pathol. 2009 Dec;22(12):1628–36.

65. Wang F, Liu A, Peng Y, Rakheja D, Wei L, Xue D, Allan RW, Molberg KH, Li J, Cao D. Diagnostic utility of Sall4 in extragonadal yolk sac tumors: An immunohistochemical study of 59 cases with comparison to placental-like alkaline phosphatase,

alpha-fetoprotein, and glypican-3. Am J Surg Pathol. 2009 Oct;33(10):1529–39.

66. Deisch J, Raisanen J, Rakheja D. Immunoexpression of Sall4 in Wilms tumors and developing kidney. Pathol Oncol Res. 2011 Sep;17(3):639–44.

67. Kobayashi D, Kuribayshi K, Tanaka M, Watanabe N. Sall4 is essential for cancer cell proliferation and is overexpressed at early clinical stages in breast cancer. Int J Oncol. 2011 Apr;38(4):933–9.

68. Bard JD, Gelebart P, Amin HM, Young LC, Ma Y, Lai R. Signal transducer and activator of transcription 3 is a transcriptional factor regulating the gene expression of Sall4. FASEB J. 2009 May;23(5):1405–14.

Critical Reviews™ in Oncogenesis, 16(1–2), 129–140 (2011)

Gadd45 Stress Sensors in Malignancy and Leukemia

Dan A. Liebermann,[1,2,*] Jennifer S. Tront,[1] Xiogen Sha,[2] Ksushiki Mukherjee,[2] Alisha-Mohamed-Hadley,[1] & Barbara Hoffman[1,2]

[1]Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, Pennsylvania; [2]Department of Biochemistry, Temple University, Philadelphia, Pennsylvania

*Address all correspondence to: Dan A. Liebermann, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3400 N. Broad Street, Room 150 Allied Health Building, Philadelphia, PA 19140; lieberma@temple.edu

ABSTRACT: Gadd45 proteins, including Gadd45a, Gadd45b, and Gadd45g, have been implicated in stress signaling in response to physiological and environmental stress, including oncogenic stress, which can result in cell cycle arrest, DNA repair, cell survival, senescence, and apoptosis. The function of Gadd45 as a stress sensor is mediated via a complex interplay of physical interactions with other cellular proteins implicated in cell cycle regulation and the response of cells to stress, notably PCNA, p21, cdc2/cyclinB1, and the p38 and JNK stress response kinases. Altered expression of Gadd45 has been observed in multiple types of solid tumors as well as in hematopoietic malignancies. Using genetically engineered mouse models and bone-marrow transplantation, evidence has been obtained indicating that Gadd45 proteins can function to either promote or suppress tumor development and leukemia; this is dependent on the molecular nature of the activated oncogene and the cell type, via engagement of different signaling pathways.

KEY WORDS: Gadd45, cancer, leukemia

ABBREVIATIONS

BM, bone marrow; DNR, daunorubicin; HCV, hepatitis C virus; IR, g-irradiation; MMS, methylmethanesulfonate; UV, ultraviolet radiation

I. Gadd45 IN CELLULAR STRESS RESPONSES

The gadd45 family of genes includes three genes, gadd45a, gadd45b, and gadd45g, whose cognate proteins are key players in cellular stress responses. Gadd45 genes encode for small (18 kDa), evolutionarily conserved proteins that are highly homologous (55–57% overall identity at the amino acid level; Figure 1), are highly acidic, and are localized within both the cell cytoplasm and nucleus.[1–4]

Gadd45a, originally termed gadd45, was cloned as one of many growth arrest and DNA damage-inducible (gadd) genes that are rapidly induced by UV radiation in Chinese hamster ovary (CHO) cells.[2] Gadd45b, originally termed MyD118, was cloned in this laboratory, as one of many myeloid differentiation primary response (MyD) genes, induced in the absence of protein synthesis following treatment of M1 myeloblastic leukemia cells with differentiation inducers.[5] Gadd45g, was cloned in this laboratory using an MyD118 (gadd45b) cDNA probe. It was subsequently found to encode for the murine homologue of human CR6[4], originally cloned as an immediate early response gene in T cells stimulated by interleukin-2.[6]

Each of the gadd45 genes is expressed in multiple murine tissues (including heart, brain, spleen, lung, liver, skeletal muscle, kidney and testes) but at different levels,[4] yet mice null for each of these genes are viable.[7,8] Furthermore, expression of gadd45a, gadd45b, and gadd45g is induced in response to multiple environmental and physiological stresses, including methylmethanesulfonate (MMS), g-irradiation (IR), ultraviolet radiation (UV), VP-16, daunorubicin (DNR), and inflammatory cytokines.[3,8,9] In all cases, the pattern of expression for each gadd45 gene is unique, consistent with each gadd45 family member playing distinct roles in response to each source of stress. During myeloid differentiation, using either normal bone marrow (BM) stimulated with different hematopoietic cytokines or various hematopoietic cell lines induced to undergo terminal myeloid differen-

0893-9675/11/$35.00

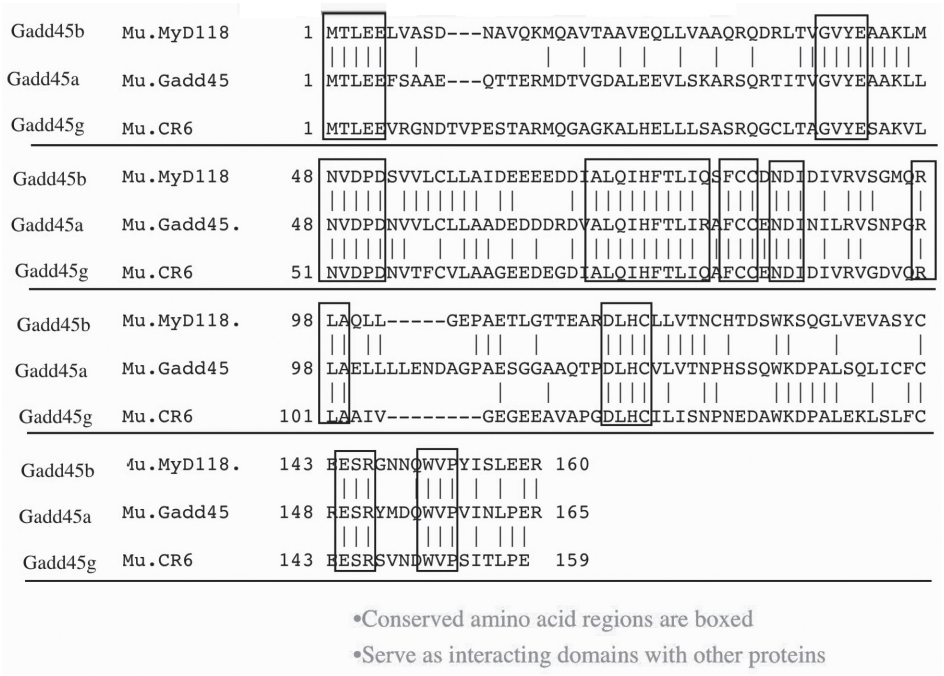


FIGURE 1. Comparison of Gadd45 protein sequences.

tiation, each gadd45 gene has a distinctive pattern of expression. Consistent with the distinctive expression patterns, regulation of expression for each gadd45 gene is unique. For instance, gadd45a is a p53 target gene, although its induction can also be p53 independent, whereas gadd45b is a primary response gene to both IL-6 and TGF-b, and gadd45g is induced as a primary response to IL-2 and IL-6.[1,4,6,10–11]

Gadd45 proteins have been shown to participate in cell cycle arrest, DNA repair, cell survival, and apoptosis in response to environmental and physiological stress, as well as having a role in development and carcinogenesis. Gadd45 function by interacting with and modulating the structure/function of partner proteins that are implicated in cell cycle regulation and the response of cells to stress (Figure 2). The ultimate biological outcome is highly dependent on the magnitude and type of stress stimulus as well as cell type.[12] Proteins that interact with Gadd45 include PCNA and histones, cdk1, p21, MEKK4, MKK7, and p38. There is evidence that interaction of the Gadd45 proteins with PCNA promotes nucleotide excision repair (NER) of DNA.[13–16] Interaction with PCNA and/or histones may also play a role in epigenetic gene activation by repair-mediated DNA demethylation.[17–21] Interaction with the cdc2/cyclinB1 complex inhibits the kinase activity of the complex, thereby resulting in G2/M cell cycle arrest.[22,23] It has been suggested that when Gadd45a interacts with p21, it serves to augment its CDKI activity, promoting G1 arrest.[16,24] Association of Gadd45 proteins with MEKK4 and p38 results in their activation.[12] It was recently reported that the MEKK4 N-terminus binds to its C-terminal segment, thereby inhibiting the C-terminal kinase domain, and that binding of Gadd45 to the MEKK4 N-terminal Gadd45-binding site disrupts this N–C binding, resulting in kinase activation.[25] Gadd45 binding can also induce dimerization of MEKK4.[25] Gadd45 proteins have also been implicated in activation or repression of JNK activity via interaction with and modulation of the structure/function of upstream kinase modulators,[9,26–32] thereby promoting apoptosis or cell survival depending on the stimulus and/or cell type. In this context, Gadd45b is a major player in the endogenous

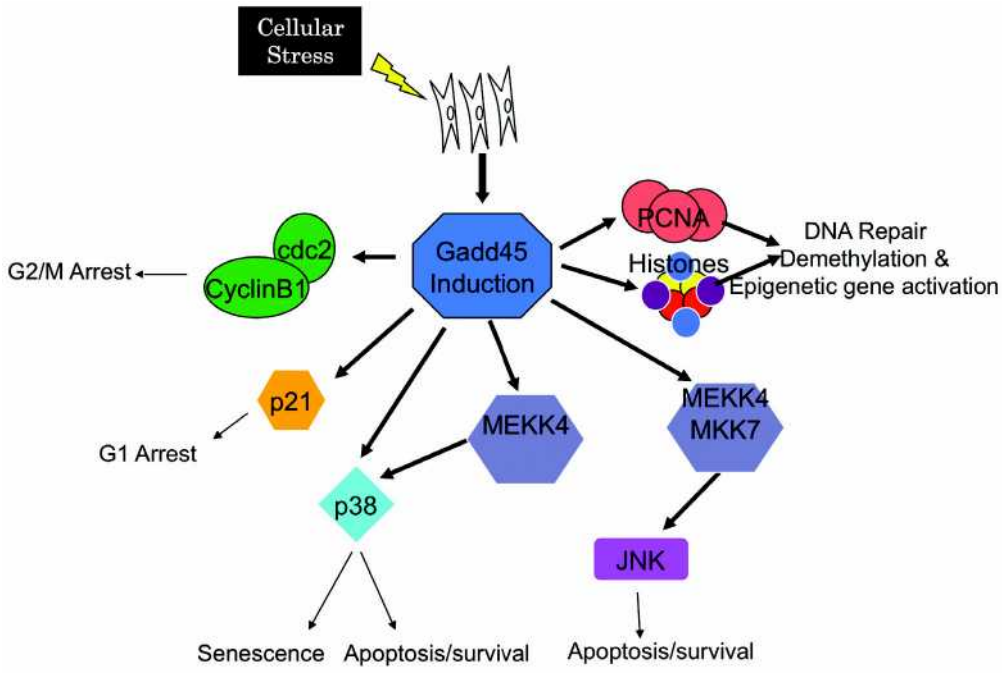


FIGURE 2. Gadd45 function in stress signaling. Summary of the various protein–protein interactions of Gadd45 proteins that affect cellular processes such as cell cycle arrest, DNA repair, survival, apoptosis, senescence as well as epigenetic gene activation.

NF-kB-mediated resistance to apoptosis in a variety of cell lines. In fibroblasts this mechanism involves activation of MKK7,[29] upstream of JNK, by directly binding with the kinase ATP pocket. Evidence supports the existence of a large complex containing an MKK7-Gadd45b:Gadd45b-MKK7 tetrameric unit, whose complexity could be further increased by the dimeric nature of the isolated MKK7.[31] Using the Technical University of Denmark web site (linked to Entrez), Gadd45 proteins were observed to harbor several threonine and tyrosine phosphorylation sites, as well as a sites that are amenable for acetylation. How post-translational modifications of Gadd45 proteins might alter their function has not been documented, and this issue is currently under investigation. Finally, it is important to note that Gadd45 proteins can form homo- and hetero-oligomers with different family members; however, their role in mediating Gadd45 functions has not been established.[12,33]

If protein–protein interactions govern the many functions of the Gadd45 family of proteins, what determines these interactions is the next question of interest. It is predicted that the interaction of Gadd45 with its partner protein is regulated by level of expression, cellular localization, and posttranslational modifications of both the Gadd45 proteins and their interacting partners, which in turn may be determined by the type and magnitude of the stress stimulus and the cell type.

II. Gadd45 AS SENSORS OF ONCOGENIC STRESS THAT MODULATE TUMOR DEVELOPMENT

The complex role of stress sensors in monitoring oncogenic stress and in impacting on tumor development is not fully understood. The best and most studied example of oncogenic stress sensors in tumorigenesis is p53 and its varied cellular functions. Recent observations have implicated Gadd45 proteins as important sensors of oncogenic stress, both *in vitro* and *in vivo*.

It is known that, whereas primary mouse cells require introduction of two activated oncogenes for transformation, disruption of certain key growth control genes allows single oncogene transformation.[34,35] For MEFs obtained from Gadd45a$^{-/-}$mice, H-ras has been shown to be sufficient for transformation.[7,36] The role Gadd45b and/or Gadd45g play in susceptibility of MEFs to single oncogene transformation remains to be assessed.

Evidence was obtained that Gadd45 proteins also play a role in modulation of tumor development *in vivo*. Gadd45a$^{-/-}$ and Gadd45b$^{-/-}$ mice were observed to display increased mutation frequency, susceptibility to ionizing radiation (IR), and chemical carcinogenesis.[7] (unpublished data, Liebermann et al., 2011) NF-κB–mediated repression of Gadd45a and Gadd45g has also been documented to be essential for cancer cell survival.[37]

The exact frequency of mutations in Gadd45 family members in different types of cancer remains to be established. Nevertheless, reduced expression of the three Gadd45 family members due to promoter methylation has been frequently observed in several types of human cancer. The Gadd45a promoter is methylated in the majority of breast cancers, resulting in reduced expression when compared with normal breast epithelium.[38] In pituitary adenomas, silencing of the Gadd45g gene is seen in 67% of patients. This down-regulation is primarily associated with methylation of the Gadd45g gene, and reversal of this epigenetic change results in re-expression of the protein.[39] Gadd45g is also down- regulated in anaplastic thyroid cancer and in 65% of hepatocellular carcinomas due to hypermethylation of the Gadd45g promoter.[40] In another study, all three Gadd45 genes were observed to be methylated and silenced in hepatocellular carcinoma, indicating a strong linkage between Gadd45 gene expression and liver cancer.[41] Furthermore, down-regulation of Gadd45b expression by hepatitis C virus was observed to lead to defective cell cycle arrest. Hypermethylation of the Gadd45b promoter in the presence of HCV was shown to be responsible for this defect, and has been correlated to HCV-associated hepatocellular carcinomas.[42] Ying et al. analyzed the methylation status of two regions in the Gadd45g promoter in a total of 75 cell lines as well as primary tissues and tumors.[43] They show that promoter hypermethylation is frequently detected in tumors cell lines, including 85% of non-Hodgkin, 50% of Hodgkin lymphoma, 73% of naso-pharyngeal carcinoma, 50% of cervical carcinoma, 29% of esophageal carcinoma, and 40% of lung carcinoma, but not in immortalized normal epithelial cell lines, normal tissues, or peripheral blood mononuclear cells. To gain more insight into the Gadd45g methylation, they also performed high-resolution bisulfite genomic sequencing. They found that densely methylated CpG sites were detected in all silenced cell lines, indicating that epigenetic silencing of Gadd45g could be involved in the pathogenesis of tumors. A methylation-mediated repression of Gadd45a was observed also in prostate cancer.[44] The role of Gadd45a as a potential therapeutic target has been highlighted by the fact that it is up-regulated on docetaxel treatment and may contribute to docetaxel-mediated cytotoxicity of prostate cancer cells.[44] Other observations have shown that activated NF-κB leads to repression of GADD45a and GADD45g in various types of cancer.[37] Thus, constitutive activation of NF-κB in cancers and/or promoter methylation may co-operate to suppress Gadd45 genes in cancer.

Abnormal Gadd45a expression has been documented in pancreatic cancer. One study has shown that gadd45a expression is elevated in several pancreatic ductal adenocarcinoma cell lines, and loss of Gadd45a expression limits growth and survival of one cell line in culture.[45] In another study, it was observed that ectopic expression of Gadd45a in the PANC1 pancreatic cancer cell line resulted in apoptosis and cell cycle arrest.[46] These two studies suggest contradictory roles of gadd45a in pancreatic tumor cell growth and survival. Nevertheless, a study in Japan attempted to correlate expression of Gadd45a and p53 inactivation in human pancreatic cancer.[47] This is important because gadd45a is a p53 target gene, though it has been also shown to be expressed also independently of p53. Interestingly, elevated Gadd45a expression levels were reported in 54% of human pancreatic ductal carcinomas, and the frequency of point mutations was found to be almost 14%.[47] Moreover, overexpression of Gadd45a protein, along

with possible p53 loss of function, significantly contributed to poor prognosis, compared with patients with undetectable Gadd45a expression levels.[47] It was observed that in resectable invasive pancreatic ductal carcinomas, Gadd45a is frequently mutated, and this mutation combined with the p53 status affects the survival of these patients.[47] We have observed that inhibition of endogenous gadd45a expression in the PANC1 cell line by shRNA limits cell number, due to cell cycle arrest and/or apoptosis.[48] As such, further investigation is needed to better define a role for Gadd45a and other family members in pancreatic cancer development.

Recent work, conducted in this laboratory, has highlighted the role of Gadd45 proteins, notably Gadd45a, as sensors of oncogenic stress in breast carcinogenesis.[49,50] Generation and side-by-side analysis of MMTV-Myc versus MMTV-Ras mice strains, either wild-type or null for gadd45a, have highlighted a unique role for Gadd45a as either a suppressor or promoter of breast cancer development, via employment of distinct signaling pathways in response to distinct oncogene stressors (Figure 3). Our data indicate that the Gadd45a tumor suppressor function, mediated via activation of JNK and p38 stress kinases, contributes to Ras-induced apoptosis and senescence, respectively, and is a unique response to Ras oncogenic stress. In contrast, the tumor promoter function of Gadd45a, mediated through negative regulation of MMP10 expression via the GSK3b/b-catenin signaling cascade, results in increased tumor vascularization and is a unique response to oncogenic Myc. These novel findings indicate that Gadd45a can function to either promote or suppress breast tumor development through engagement of different signaling pathways, depending on the molecular nature of the activated oncogene.

III. Gadd45 IN LEUKEMIA

Expression of gadd45 is rapidly induced by different stressors in hematopoietic cells; this includes differentiation-inducing cytokines and genotoxic agents. Induction of gadd45 genes at the onset of myeloid differentiation suggests that Gadd45 protein(s) play a role in hematopoiesis, yet no apparent abnormalities were observed in either the bone marrow (BM) or peripheral blood compartments of mice deficient for either gadd45a or gadd45b. However, under conditions of hematological stress, including acute stimulation with cytokines, myeloablation, inflammation, and genotoxic stress, both gadd45a-deficient and gadd45b-deficient mice exhibit abnormal responses.[8,9,32,51]

The role Gadd45 proteins play as sensors of oncogenic stress, and their regulated expression during development of hematopoietic cells and upon response to stress, suggest that altered expression may play a role in leukemogenesis. Recent documented observations, as well as unpublished work conducted in this laboratory, support this notion.

Activating mutations in FLT3 (Fms-like tyrosine kinase) are among the most common genetic lesions in AML, occurring in approximately 30–35% of AML cases.[52] The most common mutation is an internal tandem duplication (ITD) in the intracellular, juxtamembrane domain present in approximately 20–25% of patients. In a recent study GADD45A mRNA levels were measured in a panel of AML samples to determine the association with the FLT3-ITD mutation.[53] It was observed that Gadd45a expression levels were significantly lower in FLT3-ITD$^+$ AML than in FLT3-ITD$^-$ AML, which is consistent with FLT3-ITD–induced down-regulation of Gadd45a in human AML. Furthermore, Gadd45a expression data for primary AML samples, extracted from a published microarray study,[54] indicate significant lower expression of Gadd45a relative to normal controls, in a number of AML clusters defined by their gene expression signature, including a cluster consisting only of patients with FLT3-ITD mutations. Down-regulation in a cluster characterized by a t(11q23) rearrangement (predominantly MLL-AF9), may be via a related mechanism, as 11q23 events have been associated with increased FLT3 expression in several studies.[55,56] Lower expression of Gadd45a was observed also in a cluster characterized by the presence of t(8;21) translocations involving the AML-ETO fusion oncoprotein.

Based on observations made with breast cancer mouse models, where Gadd45a was found to behave as a tumor suppressor in response to H-RAS and as

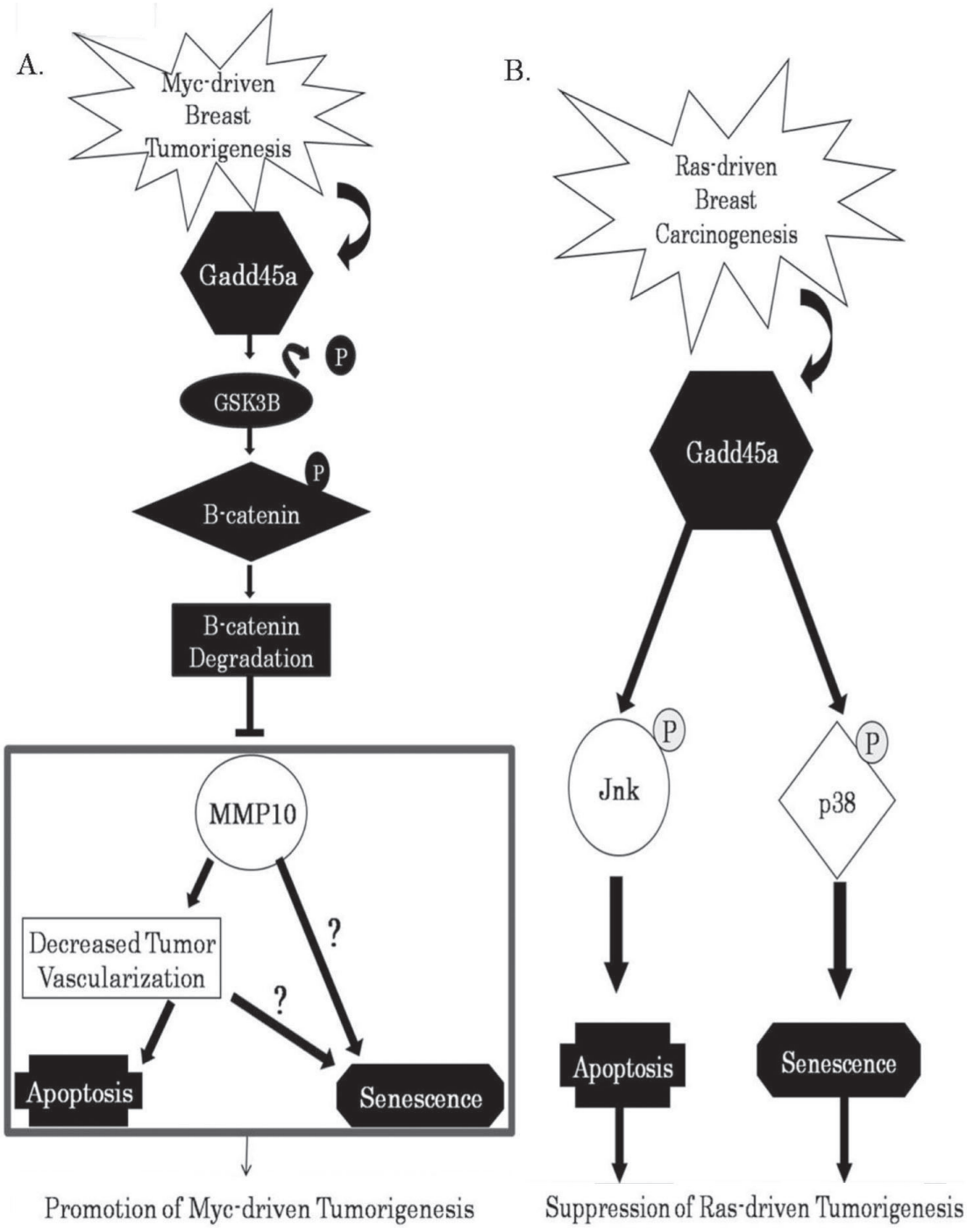


FIGURE 3. Schematic diagram demonstrating how Gadd45a modulates mammary tumor development in RAS-driven compared to Myc-driven tumors. Gadd45a suppresses RAS-driven tumorigenesis via Jnk-mediated apoptosis and p38-mediated senescence. In contrast, Gadd45a promotes Myc-driven tumorigenesis via GSK3B/B-catenin signaling which suppresses MMP10 expression, resulting in increased tumor vascularization, decreased apoptosis and senescence, ultimately accelerating tumor growth.

an oncogene in response to Myc[49,50] (Figure 2), this laboratory embarked on assessing if and how Gadd45 proteins may modulate leukemogenesis involving constitutive ras signaling, BCR/ABL signaling or deregulated myc expression.

Ras mutations occur at a frequency of 25% in AML, 30% in myeloma and 6–20% in ALL. The highest incidence, however, is in MDS, where the frequency is approximately 30–40%, with highest frequency (50–70%) in the CMML subset.[57] The most common mutations are found in N-RAS (~30%), less frequently in K-RAS (~15%), and rarely in H-RAS.[57–59] Recently, evidence was obtained showing that oncogenic N-RAS, K-RAS, and H-RAS exhibit different leukemogenic potentials in mice,[60] with N-RAS inducing either an AML- or CMML-like disease and H-RAS always an AML-like disease, whereas K-RAS invariably induces a CMML-like disease. Using bone marrow transplantation (BMT) in mice, data obtained in this laboratory (unpublished data, Liebermann et al., 2011**)**, indicate that loss of Gadd45a impeded N-RAS–driven leukemia. This is in contrast to the tumor suppressor function of Gadd45a in H-RAS–driven breast cancer.[49,50] These observations are in line with the hypothesis that the nature of the Gadd45 oncogenic stress sensor function depends upon the biological setting, including cell type, developmental stage, and stress and/or stimulus. Thus, further understanding the role of Gadd45 proteins as mediators of oncogenic Ras signaling in the context of Ras-driven leukemogenesis and extending the work to human patients is a highly critical pursuit.

BCR-ABL is known as the most common translocation in the myeloproliferative (MPD) disorder chronic myelogenous leukemia (CML); it is the first leukemia to be described[61,62] that is associated with a consistent cytogenetic abnormality, termed the Philadelphia chromosome (Ph1).[62] The Philadelphia translocation is an acquired somatic mutation in the hematopoietic stem cell[63] that results in fusion of the ABL gene (225 kb) from chromosome 9 to the BCR gene (135 kb) on chromosome 22.[64–68] The chromosome 9 breakpoint involves a large (200 kb) region within the ABL alternative first exons (1a and 1b) that results in fusion genes that incorporate ABL exon 2.[64] The breakpoints on chromosome 22 are clustered within three much smaller regions of the BCR gene.[65] A chimeric mRNA (8.5 kb) is translated to an activated BCR-ABL oncoprotein most commonly 210 kD in size.[66,67] BCR-ABL is known to localize to the cytoskeleton and to display constitutively active tyrosine kinase activity that leads to the recruitment of downstream effectors of cell proliferation and survival, via several adapter proteins (e.g., GRB2, GAB2, and CRKL) and signaling pathways (e.g., RAS, PI3K, JAK-STAT, and PDk2-NFkB), which are all thought to contribute to the pathogenesis of CML.[69] Cross-stalk signaling between Bcr-Abl/Ras/Gadd45 is depicted in Figure 4. The complex nature by which these signaling pathways contribute to the initiation and progression of CML is only partially understood. Data obtained in this laboratory indicates that loss of either Gadd45a or Gadd4b accelerates the development of BCR-ABL-driven leukemia in mouse BM transplantation studies, and Gadd45 expression was observed to be altered in human CML samples, correlating with disease progression, thereby identifying Gadd45 as a tumor suppressor in the context of BCR-ABL driven-leukemia (unpublished data, Liebermann et al., 2011). Clearly elucidating the role Gadd45 plays as a tumor suppressor in the context of BCR-ABL driven leukemia, and what signaling pathways and downstream effectors are modulated by Gadd45 to suppress leukemogenesis is important to better understand the molecular pathology of CML.

Finally, elevated Myc expression is associated with many leukemias, as well as solid tumors including breast (45%), colon (67%), gastric (47%), ovarian (44%), prostate (70%) and medulloblastoma (35%).[70] Multiple extracellular and intracellular signaling cascades converge to regulate the Myc oncogene.[71] Deregulation of Myc is not restricted to gross genetic abnormalities of the Myc gene, but it can also occur as a consequence of direct or indirect mutations of regulatory molecules controlling myc gene expression. This is exemplified by the observations that Myc deregulation is associated with its amplification as well as by activation by other oncogenes associated with myeloid leukemia, including AML1-ETO, PML-RARA, and PLZF-RAR, activating mutations

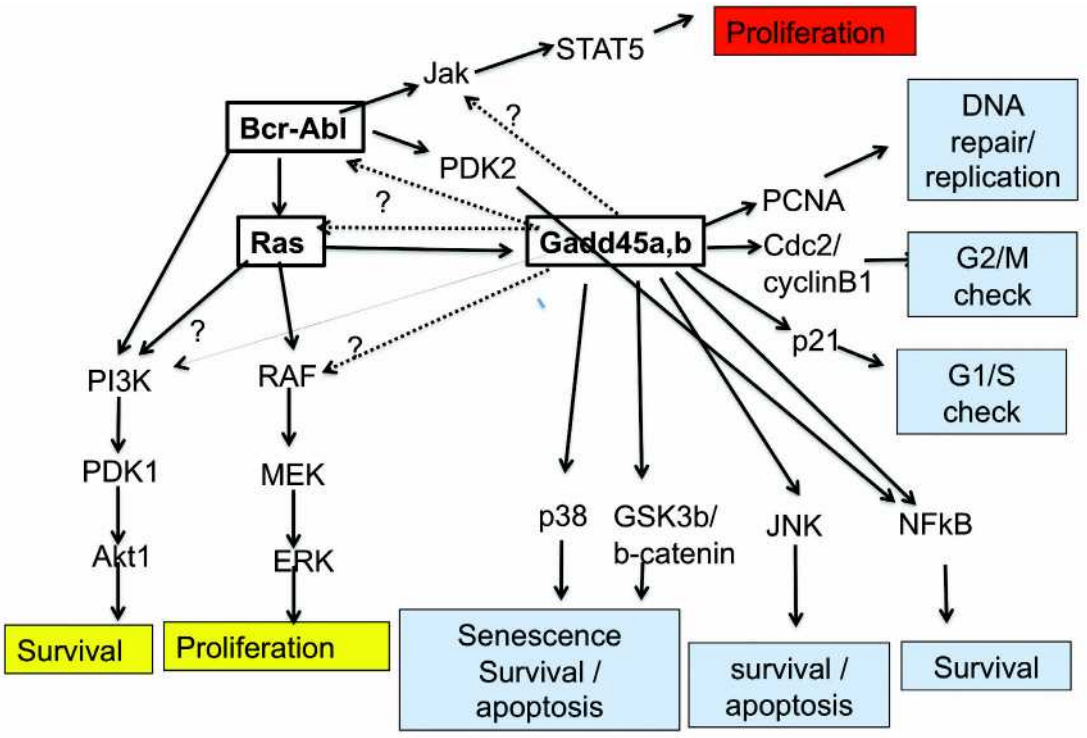


FIGURE 4. Schematic diagram of Bcr-Abl/Ras/Gadd45 signaling.

in the FLT3 receptor tyrosine kinase (associated with one-third of AML patients), and BCR-ABL.[72–77] In mouse models, myc was observed to rapidly induce acute myeloid leukemia without evidence of lymphoma-associated antiapoptotic mutations, and Mcl1 haploinsufficiency was found to protect the mice from myc-induced acute myeloid leukemia.[78,79] Data obtained in this laboratory, has shown that loss of gadd45a in myc over-expressing myeloid cells reduced apoptosis, thereby increasing proliferation (unpublished data, Liebermann et al., 2011). This data is consistent with Gadd45a playing a tumor suppressor role in modulating leukemia associated with elevated Myc (unpublished data, Liebermann et al., 2011). Thus understanding how Gadd45 proteins modulate myc-driven leukemogenesis, and extending these observations to human AML is important.

IV. CONCLUSION

Altered expression of Gadd45 has been observed in multiple types of solid tumors as well as in hematopoietic malignancies. Using genetically engineered mouse models, accumulated evidence indicates that the function of Gadd45 proteins to either promote or suppress tumor development and leukemia depends on the molecular nature of the activated oncogene and the cell type, engaging different signaling pathways. Further research is aimed at better understanding how Gadd45 proteins interface with different signaling cascades to either suppress or promote tumor development and leukemia in response to distinct oncogenic stressors. Thus Gadd45 proteins, and their signaling targets, may represent a novel class of molecules for therapeutic intervention in cancer and leukemia.

ACKNOWLEDGMENTS

Supported by National Institutes of Health Grant Nos. 1R01HL084114-01 (DAL), 1R01CA122376-01 (DAL), and RO1 CA081168-06 (BH).

REFERENCES

1. Carrier F, Smith ML, Bae I, Kilpatrick KE, Lansing TJ, Chen CY, Engelstein M, Friend SH, Henner WD, Gilmer TM, Kastan MB, Fornace AJ Jr. Characterization of human Gadd45, a p53-regulated protein. J Biol Chem. 1994:269:32672–7.
2. Zhan Q, Lord KA, Alamo I Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ Jr. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol Cell Biol. 1994;14:2361–71.
3. Liebermann DA, Hoffman B. MyD genes in negative growth control. Oncogene. 1998;17: 3319–30.
4. Zhang W, Bae I, Krishnaraju K, Azam N, Fan W, Smith K, Hoffman B, Liebermann DA. CR6:A third member in the MyD118 & Gadd45 gene family which functions in negative growth control. Oncogene. 1999;18:4899–907.
5. Abdollahi A, Lord KA, Hoffman-Liebermann B, Liebermann DA. Sequence and expression of a cDNA encoding MyD118: A novel myeloid differentiation primary response gene induced by multiple cytokines. Oncogene. 1991;6:165–7.
6. Beadling C, Johnson KW, Smith KA. Isolation of interleukin 2-induced immediate-early genes. Proc Natl Acad Sci USA. 1993;90:2719–23.
7. Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-SalgueroPM, Morgan WF, Deng CX, Fornace AJ Jr. Genomic instability in Gadd45a-deficientmice. Nat Genet. 1999;23:176–84.
8. Gupta M, Gupta S, Balliet AG, Hollander MC, Fornace AJ, Hoffman B, Liebernmann DA. Hematopoietic cells from Gadd45a and Gadd45b deficient mice are sensitized to genotoxic-stress Induced apoptosis. Oncogene. 2005;24:7170–9.
9. Gupta S, Gupta M, Hoffman B, Liebermann DA. Hematopoietic cells from gadd45a deficient and gadd45b deficient mice exhibit impaired stress responses to acute stimulation with cytokines, myeloablation, and inflammation. Oncogene. 2006;25:5537–46.
10. Selvakumaran M, Lin HK, Tjin Tham Sjin R, Reed J, Liebermann DA, Hoffman B. The novel primary response gene MyD118 and the proto-oncogenes myb, myc and bcl-2modulate transforming growth factor b1-induced apoptosis of myeloid leukemia cells. MolCell Biol. 1994;14:2352–60.
11. Yoo J, Ghiassi M, Jirmanova L, Balliet AG, Hoffman B, Fornace AJ Jr, Liebermann DA, Bottinger EP, Roberts AB. TGF-beta-induced apoptosis is mediated by Smaddependentexpression of GADD45B through p38 activation. J Biol Chem. 2003;278:43001–7.
12. Liebermann DA, Hoffman B. Gadd45 in stress signaling. J Mol Signal. 2008;3:15.
13. Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM, Fornace AJ Jr. AntisenseGADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. Oncogene. 1996;13:2255–63.
14. Vairapandi M, Azam N, Balliet AG, Hoffman B, Liebermann DA. Characterization of MyD118, Gadd45, and PCNA interacting domains: PCNA impedes MyD/Gadd mediatednegative growth control. J Biol Chem. 2000; 275:16810–9.
15. Azam N, Vairapandi M, Zhang W, Hoffman B, Liebermann DA, Azam N, Vairapandi M, Zhang W, Hoffman B, Liebermann DA. Interaction of CR6 (GADD45gamma) with proliferating cell nuclear antigen impedes negative growth control. J Biol Chem. 2001;276:2766–74.
16. Liebermann DA, Hoffman B. Gadd45 in the response of hematopoietic cells togenotoxic stress. Blood Cells Mol Dis. 2007;39:329–35.
17. Barreto G, Schäfer A, Marhold J, Stach D, Swaminathan SK, Handa V, Döderlein G, Maltry N, Wu W, Lyko F, Niehrs C. Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature. 2007;445, 671–5.
18. Schmitz KM, Schmitt N, Hoffmann-Rohrer U, Schäfer A, Grummt I, Mayer C. TAF12 recruits Gadd45a and the nucleotide excision repair complex to the promoter of rRNA genes leading to active DNA demethylation. Mol Cell. 2009;33:344–53.
19. Engel N, Tront JS, Erinle T, Nguyen N, Latham KE, Sapienza C, Hoffman B, Liebermann DA. Conserved DNA methylation in Gadd45a(-/-) mice. Epigenetics 2009;4:98–9.
20. Rai K, Huggins IJ, James SR, Karpf AR, Jones DA, Cairns BR. DNA demethylation in zebrafish

involves the coupling of a deaminase, a glycosylase, Gadd45. Cell. 2008;135:1201–12.

21. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H. Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science. 2009;323:1074–7.
22. Wang XW, ZhanQ, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ Jr, Harris CC. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci USA. 1999;96:3706–11.
23. Vairapandi M, Balliet AG, Hoffman B, Liebermann DA. GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J Cell Physiol. 2002;192:327–38.
24. Fan W, Richter G, Cereseto A, Beadling C, Smith KA. Cytokine response gene 6induces p21 and regulates both cell growth and arrest. Oncogene. 1999;18:6573–82.
25. Miyake Z, Takekawa M, Ge Q, Saito H. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans autophosphorylation of the MTK1 kinase domain. Mol Cell Biol. 2007;27:2765–76.
26. Papa S, Zazzeroni F, Bubici C, Jayawardena S, Alvarez K, Matsuda S, Nguyen DU, Pham CG, Nelsbach AH, Melis T, De Smaele E, Tang WJ,D'Adamio L, Franzoso G. Gadd45 betamediates the NF-kappa B suppression of JNK signalling by targeting MKK7/JNKK2. Nat Cell Biol. 2004;6:146–53.
27. Takekawa M, Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4/MAPKKK. Cell. 1998;95:521–30.
28. Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ Jr. Gadd45a protects against UV irradiation-induced skin tumors, and promotesapoptosis and stress signaling via MAPK and p53. Cancer Res. 2002;62:7305–15.
29. Zhu N, Shao Y, Xu L, Yu L, Sun L. Gadd45-alpha and Gadd45-gamma utilize p38 and JNK signaling pathways to induce cell cycle G2/M arrest in Hep-G2 hepatoma cells. Mol Biol Rep. 2009 36:2075–85.
30. Amanullah A, Azam N, Balliet A, Hollander C, Hoffman B, Fornace A, Liebermann D. Cell signalling: Cell survival and a Gadd45-factor deficiency. Nature. 2003;424:741–2.
31. Tornatore L, Marasco D, Dathan N, Vitale RM, Benedetti E, Papa S, Franzoso G, Ruvo M, Monti SM. Gadd45 beta forms a homodimeric complex that binds tightly to MKK7. J Mol Biol. 2008;378:97–111.
32. Gupta M, Gupta S, Hoffman B, Liebermann DA. Gadd45a and gadd45b protect hematopoietic cells from ultraviolet radiation induced apoptosis via distinct signaling pathways. J Biol Chem. 2006;281:17552–8.
33. Kovalsky O, Lung FD, Roller PP, Fornace AJ Jr. Oligomerization of human Gadd45a protein. J Biol Chem. 2001;276:39330–9.
34. Lundberg AS, Hahn WC, Gupta P, Weinberg RA. Genes involved in senescence and immortalization. Curr Opin Cell Biol. 2000;12:705–9.
35. Drayton S, Peters G. Immortalisation and transformation revisited. Curr Opin Genet Dev. 2002;12: 98–104.
36. Bulavin DV, Kovalsky O, Hollander MC, Fornace AJ Jr. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of Gadd45a. Mol Cell Biol. 2003;23:3859–71.
37. Zerbini LF, Wang Y, Czibere A, Correa RG, Cho JY, Ijiri K, Wei W, Joseph M, Gu X, Grall F, Goldring MB, Zhou JR, Libermann TA. NF-kappa B-mediated repression of growth arrest- and DNA-damage-inducible proteins 45alpha and gamma is essential for cancer cell survival. Proc Natl Acad Sci USA. 2004;101:13618–23.
38. Wang W, Huper G, Guo Y, Murphy SK, Olson JA Jr, Marks JR. Analysis of methylation-sensitive transcriptome identifies GADD45a as a frequently methylated gene in breast cancer. Oncogene. 2005;24:2705–14.
39. Bahar A, Bicknell JE, Simpson DJ, Clayton RN, Farrell WE. Loss of expression of the growth inhibitory gene GADD45gamma, in human pituitary adenomas, is associated with CpG island methylation. Oncogene. 2004;23:936–44.
40. Sun L, Gong R, Wan B, Huang X, Wu C, Zhang X, Zhao S, Yu L. GADD45g, down-regulated in

65 % hepatocellular carcinoma (HCC) from 23 Chinese patients, inhibits cell growth and induces cell cycle G2/M arrest for hepatoma Hep-G2 cell lines. Mol Biol Rep. 2003;30:249–53.

41. Na YK, Lee SM, Hong HS, Kim JB, Park JY, Kim DS. Hypermethylation of growth arrest DNA-damage-inducible gene 45 in non-small cell lung cancer and its relationship with clinicopathologic features. Mol Cells. 2010;30:89–92.
42. Higgs MR, Lerat H, Pawlotsky JM. Downregulation of Gadd45beta expression by hepatitis C virus leads to defective cell cycle arrest. Cancer Res. 2010;70:4901–11.
43. Ying J, Srivastava G, Hsieh WS, Gao Z, Murray P, Liao SK, Ambinder R, Tao Q. The stress-responsive gene GADD45G is a functional tumor suppressor, with its response to environmental stresses frequently disrupted epigenetically in multiple tumors. Clin Cancer Res. 2005;11:6442–9.
44. Ramachandran K, Gopisetty G, Gordian E, Navarro L, Hader C, Reis IM, Schulz WA, Singal R. Methylation-mediated repression of GADD45α in prostate cancer and its role as a potential therapeutic target. Cancer Res. 2009;69:1527–35.
45. Schneider G, Weber A, Zechner U, Oswald F, Friess HM, Schmid RM, Liptay S. Gadd45a is highly expressed in pancreatic ductal adenocarcinoma cells and required for tumor cell viability. Int J Cancer. 2006;11:2405–11.
46. Li Y, Qian H, Li X, Wang H, Yu J, Liu Y, Zhang X, Liang X, Fu M, Zhan Q, Lin C. Adenoviral-mediated gene transfer of Gadd45a results in suppression by inducing apoptosis and cell cycle arrest in pancreatic cancer cell. J Gene Med. 2009;11:3–13.
47. Yamasawa K, NioY, Dong M, Yamaguchi K, Itakura M. Clinicopathological significance of abnormalities in Gadd45 expression and its relationship to p53 in human human pancreatic cancer. Clin Cancer Res. 2002;8:2563–9.
48. Cretu A, Sha X, Tront J, Hoffman B, Liebermann DA. Stress sensor Gadd45 genes as therapeutic targets in cancer. Cancer Therapy. 2009;7(A):268–76.
49. Tront JS, Hoffman B, Liebermann DA. Gadd45a suppresses Ras-driven mammary tumorigenesis by activation of JNK and p38 stress signaling resulting in apoptosis and senescence. Cancer Research. 2006;66:8448–54.
50. Tront JS, Huang Y, Fornace A, Hoffman B, Liebermann DA. Gadd45a promotes or suppresses breast cancer dependent on the oncogenic stress. Cancer Research. 2010;70:9671–81.
51. Hoffman B, Liebermann DA. Gadd45 modulation of intrinsic and extrinsic stress responses in myeloid cells. J Cell Physiol. 2009;218:26–31.
52. Parcells BW, Ikeda AK, Simms-Waldrip T, Moore TB, Sakamoto KM. FMS-like tyrosine kinase 3 in normal hematopoiesis and acute myeloid leukemia. Stem Cells. 2006;24:1174–84.
53. Perugini M, Kok CH, Brown AL, Wilkinson CR, Salerno DG, Young SM, Diakiw SM, Lewis ID, Gonda TJ, D'Andrea RJ. Repression of Gadd45a by activated FLT3 and GM-CSF receptor mutants contributes to growth, survival and blocked differentiation. Leukemia. 2009;23:729–38.
54. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, Beverloo HB, Moorhouse MJ, van der Spek PJ, Löwenberg B, Delwel R. Prognostically useful gene-expression profiles in acutemyeloid leukemia. N Engl J Med. 2004;350:1617–28.
55. Kuchenbauer F, Kern W, Schoch C, Kohlmann A, Hiddemann W, Haferlach T, Schnittger S. Detailed analysis of FLT3 expression levels in acute myeloid leukemia. Haematologica. 2005;90:1617–25.
56. Ono R, Nakajima H, Ozaki K, Kumagai H, Kawashima T, Taki T, Kitamura T, Hayashi Y, Nosaka T. Dimerization of MLL fusion proteins and FLT3 activationsynergize to induce multiple-lineage leukemogenesis. J Clin Invest. 2005;115:919–29.
57. Ikeda A, Shankar DB, Watanabe M, Tamanoi F, Moore TB, Sakamoto KM. Molecular targets and the treatment of myeloid leukemia. Mol Genet Metabol. 2006;88;216–24.
58. Reimann C, Arola M, Bierings M, Karow A, van den Heuvel-Eibrink MM, Hasle H, Niemeyer CM, Kratz CP. A novel somatic K-Ras mutation in juvenile myelomonocytic leukemia. Leukemia. 2006;20:1637–8.
59. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. Blood. 1994;83:2248–54.
60. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS, KRAS, and HRAS exhibit different

leukemogenic potentials in mice. Cancer Res. 2007;67:7139–46.

61. Virchow R. Weisses Blut. Froiep's Notzien. 1845; 36:151.
62. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. J Nat Cancer Inst. 1960;25:85–109.
63. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. Proc Natl Acad Sci USA. 1967;58:1468–71.
64. Bernards A, Rubin CM, Westbrook CA, Paskind M, Baltimore D. The first intron in the human c-abl gene is at least 200 kilobases long and is a target for translocations in chronic myelogenous leukemia. Mol Cell Biol. 1987;7:3231–6.
65. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell. 1984;36:93–9.
66. 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;300:765–7.
67. Heisterkamp N, Stam K, Groffen J, de Klein A, Grosveld G. Structural organization of the bcr gene and its role in the Ph' translocation. Nature. 1985;315:758–61.
68. Melo JV. BCR-ABL gene variants. Baillieres Clin Haematol 1997;10:203–22.
69. Tefferi AD, Gilliland DG. Oncogenes in myeloproliferative disorders. Cell Cycle. 2007;6: 550–66.
70. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol. 2006;16:318–30.
71. Ponzielli R, Katz S, Barsyte-Lovejoy D, Penn LZ. Cancer therapeutics: targeting the dark side of Myc. Eur J of Cancer. 2005;41:2485–501.
72. Afar DE, Goga A, McLaughlin J, Witte ON, Sawyers CL. Differential complementation of Bcr-Abl point mutants with c-Myc. Science. 1994;264:424–6.
73. Alitalo K, Saksela K, Winqvist R, Alitalo R, Keski-Oja J, Laiho M, Ilvonen M, Knuutila S, de la Chapelle A. Acute myelogenous leukaemia with c-myc amplification and double minute chromosomes. Lancet. 1985;2:1035–9.
74. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100: 1532–42.
75. Sawyers CL, Callahan W, Witte ON. Dominant negative MYC blocks transformation by ABL oncogenes. Cell. 1992;70:901–910.
76. Müller-Tidow C, Steffen B, Cauvet T, Tickenbrock L, Ji P, Diederichs S, Sargin B, Köhler G, Stelljes M, Puccetti E, Ruthardt M, deVos S, Hiebert SW, Koeffler HP, Berdel WE, Serve H. Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. Mol Cel Biol. 2004;24:2890–904.
77. Slovak ML, Ho JP, Pettenati MJ, Khan A, Douer D, Lal S, Traweek ST. Localization of amplified MYC gene sequences to double minute chromosomes in acute myelogenous leukemia. Genes Chromosomes Cancer. 1994;9:62–7.
78. Luo H, Li Q, O'Neal J, Kreisel F, Le Beau MM, Tomasson MH. c-Myc rapidly induces acute myeloid leukemia in mice without evidence of lymphoma-associated antiapoptotic mutations. Blood. 2005;106:2452–61.
79. Xiang Z, Luo H, Payton JE, Cain J, Ley TJ, Opferman JT, Tomasson MH. Mcl1 haploinsufficiency protects mice from myc-induced acute myeloid leukemia. J Clin Invest. 2010;120: 2109–18.