Acute Leukemias

trends in hematology/oncology

haematologica 2002; 87:1307-1323
http://www.haematologica.org/2002_12/1307.htm

Core binding factor genes and human leukemia

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Background. The core binding factor (CBF) transcription complex, consisting of the interacting proteins RUNX1 and CBFβ, is essential for normal hematopoiesis. Recent studies have shown that mutations and gene rearrangements involving this complex are frequently implicated in leukemogenesis. Understanding the molecular events leading to the disruption of CBF has provided important insights into our understanding of the normal regulatory pathways that control hematopoiesis and has begun to reveal how alterations in these pathways induce leukemia.

Information Sources. Both authors are involved in the identification and characterization of chromosomal abnormalities associated with hematologic malignancy. This has led to contributions to multicenter clinical and laboratory investigations as well as publications in peer-reviewed journals. All of the references cited in this review are published in journals covered by Medline.

State of the Art. The core binding factor (CBF) is a heterodimeric transcription factor composed of the RUNX1 and CBFβ subunits. RUNX1 is the DNA binding element of the complex and its affinity is greatly increased in the presence of CBFβ. Knock-out studies in mice have demonstrated that both RUNX1 and CBFβ are necessary for definitive hematopoiesis. Furthermore, reciprocal chromosomal translocations involving both partners have been directly implicated in leukemogenesis. Evidence is now emerging that at least some of the resulting fusion proteins, namely ETV6-RUNX1, RUNX1-MTG8 and CBFβ-MYH11 dominantly inhibit the function of native CBF by recruiting transcriptional co-repressor complexes. However, knock-in studies have shown that whilst expression of these fusion genes may disrupt normal hematopoiesis, this, by itself, is not sufficient for the subsequent development of leukemia. Mutations of RUNX1 have been identified in familial platelet disorder (FDP), in which there is a congenital predisposition to the development of AML and heterozygous point mutations have been identified in the RUNX1 gene in some leukemias. Moreover, a small number of cases have been reported in which amplification of RUNX1 has been detected in childhood ALL suggesting mechanisms other than loss of function, such as gene dosage may also play a role.

Conclusions. Understanding the role CBF plays in normal hematopoiesis and hematologic malignancies has provided critical reagents for the accurate identification of the broad group of leukemias harboring alterations of CBF. The application of these molecular approaches has already shown an impact on the clinical management of these patients and as more information becomes available, the ability to tailor therapy to improve each patient's chance of a cure becomes feasible.

Key words: core binding factor; Runx1; chromosomal translocation; mutation; hematologic malignancy.

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The realization that gross chromosomal changes such as translocations, deletions, inversions and amplifications could disrupt genes involved in carcinogenesis have led to many of the current molecular approaches to cancer research.[1,2] A major advance of this research has been the identification of consistent chromosomal abnormalities in specific types of tumors. Thus the investigation of leukemia-associated translocations has provided insights into the mechanisms of transformation in human leukemia and the identification and characterization of a series of transcription factors implicated in the regulation of normal hematopoiesis.[3] Among these the core binding factor transcription complex, consisting of the interacting proteins RUNX1 and CBFβ, has been shown to be essential for normal hematopoiesis.[4] Disruptions of the RUNX1/CBFβ complex have frequently been implicated in leukemogenesis with the majority of these events involving a translocation in which part of the RUNX1 or CBFβ protein becomes fused to protein domains encoded by exons from partner chromosomes. More recently, mutations within the *RUNX1* gene have been identified in cases of acute myeloid leukemia (AML) and myelodysplasia and congenital mutations in *RUNX1* have been described in individuals with the rare autosomal dominant disease, familial platelet disorder (FDP), in which there is a congenital predisposition to the development of AML.[5-8] Thus the CBF complex has been shown to be a key target for leukemia-associated mutations in man (Figure 1).

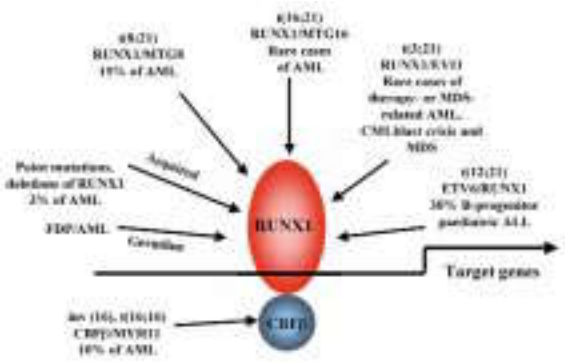


Figure 1. Molecular consequences of genetic mutations that target the CBF transcription factor complex.

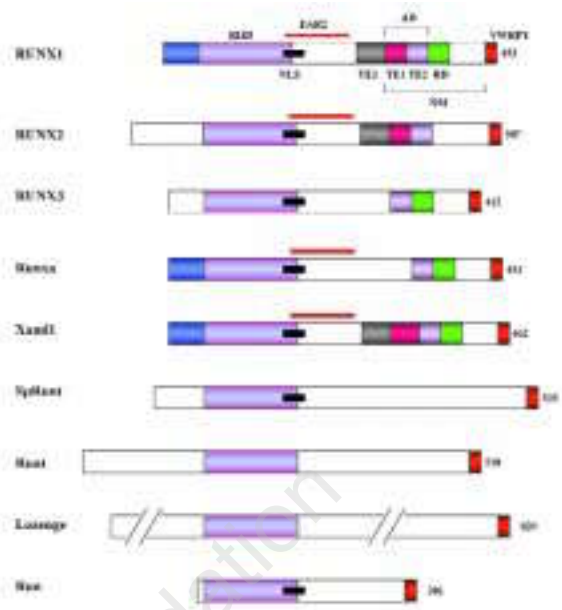


Figure 2. Illustration of the evolutional conservation of the RUNX1 protein. ALL CBFα proteins contain the Runt homology domain (RHD) identified in the mammalian RUNX1, 2 and 3 proteins; zebrafish runxa; *Xenopus* Xaml1; *sea urchin SpRunt-1*; *Drosophila* runt and Lozenge; and *C. elegans* Run. Other, less well conserved functional regions shown are, a nuclear localization sequence (NLS), and three transactivational elements (TE1-3) of which TE1 and 2 make up an activation domain. In addition, the region of *RUNX1* C-terminal to the RHD also contains three repression domains, the more N-terminal domain interacting with Ear-2. A second domain (RD) is located C-terminal to the activation domain. The C-terminal end of *RUNX1* terminates in the amino acid sequence VWRPW (single amino acid code), which binds the Groucho co-repressor. A large area encompassing the AD and ID is required for attachment to the nuclear matrix (NM).

The core binding factor complex

Core binding factors (CBFs) are DNA-binding transcription factor complexes composed of α and β subunits.[9] The α subunit is the DNA binding element of the complex and is capable of binding DNA *in vitro* in the absence of its partner protein, CBFβ. The β subunit stabilizes the binding of CBFα to DNA without direct DNA contact.[10,11] Three mammalian genes encode the α subunit: *RUNX2/AML3/PEBP2αA/Osf2* (herein called (*RUNX2*), *RUNX1/AML1/PEBP2αB* (herein called *RUNX1*) and *RUNX3/AML2/PEBP2αC* (herein called *RUNX3*).[12,13] Only a single β subunit, CBFβ (PEBP2β) is present in mammals.[14] All CBFα subunits contain a highly evolutionarily conserved 128 amino acid domain that is homologous to the *Drosophila* pair-rule protein runt (hence its designation as the runt homology domain or RHD) (Figure 2). The RHD is the DNA-binding domain of the CBFα protein and also contains the heterodimerization domain for the CBFβ subunit.[15] Resolution of the three-dimensional structure of the runt domain of RUNX1 and the heterodimeric complex between the runt domain and the CBFβ subunit have shown that the runt domain is a member of a family of s-type Ig-fold DNA-binding proteins whose other members include p53, NF-κB, NFAT, the T domain, STAT1 and STAT3β.[16-18]

The *Drosophila* gene *runt* participates in several developmental processes, including sex determination, segmentation and neurogenesis.[19-21] A *Drosophila* homolog of the *runt* gene called *lozenge* is involved in the pathway that specifies photoreceptor cell identity during eye development[22] and has recently been shown to be essential for the development of early hematopoietic (crystal) cells during embryonic and larval hematopoiesis.[23] Other runt domain proteins thus far identified include; Run (*Caenorhabditis elegans*),[24] SpRunt-1 (a positive regulator of the aboral ectoderm-specific CyIIIA gene in sea urchin embryos,[25] Xaml1 (involved in the formation of *Xenopus* embryonic blood),[26] runxa and runxb (expressed in separate, specific regions of the developing zebrafish),[27] and RuntB (modulated during chicken chondrocyte differentiation)[28] (Figure 2).

The RUNX1 α subunit can be detected in hematopoietic stem cells, endothelial cells of the aorta, gonad, mesonephros region, chondrogenic centers, olfactory and gustatory mucosa, and neur-

al ganglion cells.[29] After organogenesis, *RUNX1* expression is primarily restricted to cells of the hematopoietic lineage.[30]

RUNX1 recognizes the core DNA sequence TGT/cGGT which is present as a regulatory element in several viral and cellular promoters and enhancers,[31,32] as well as hematopoietic cell-specific genes including those encoding interleukin-3 (IL-3),[33] granulocyte-macrophage colony-stimulating factor (GM-CSF),[34] colony-stimulating factor 1 (CSF1/M-CSF) receptor,[35] myeloperoxidase, neutrophil elastase,[36] granzyme B,[37] and T-cell antigen receptors (TCRs).[38]

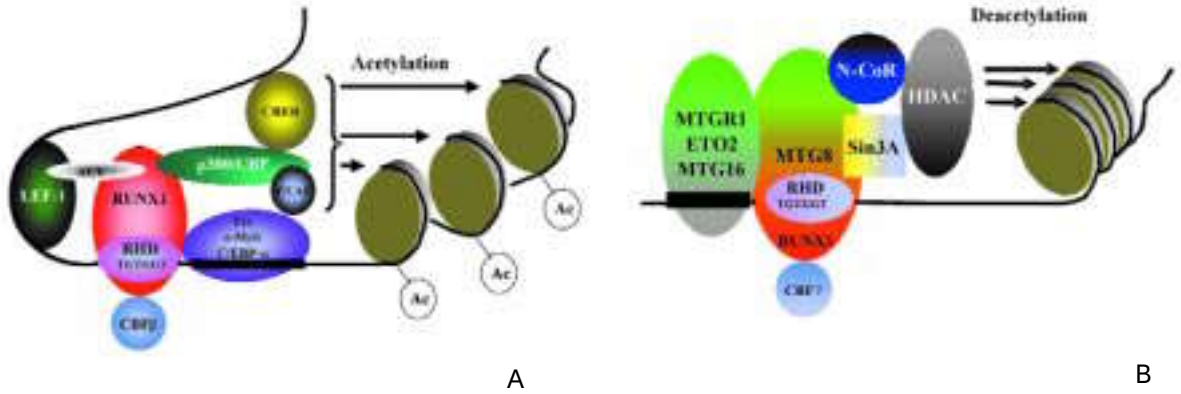
The β-subunit, mapped in humans to 16q22, has a predicted amino acid sequence with no known structural motif.[14] Two *Drosophila* proteins, brother (Bro) and big-brother (Bgb), and a zebrafish homolog (cbfb), structurally and functionally homologous to CBFβ, have been isolated.[39,40] All of these proteins have been shown to increase the DNA-binding affinity of runt, and are also able to increase the DNA-binding affinity of the mammalian CBFα proteins, although to a lesser extent.[39,40] Furthermore, it has recently been shown that dimerization with CBFβ protects RUNX1 from ubiquitin-proteosome-mediated degradation.[41] In contrast to members of the CBFα family, CBFβ appears to be ubiquitously expressed.[14,39] The cellular localization of the CBFβ sub-units also differs in that members of the CBFa family are nuclear proteins whereas CBFβ remains in the cytoplasm and is only recruited to the nucleus upon heterodimerization with the CBFα sub-unit.[42-44]

Although binding of CBF to the core sequence is important for gene expression, adjacent binding sites for lineage-restricted transcription factors, such as c-MYB, C/EBP-α, and ETS family members are also important.[45-47] Thus, CBF may function as a transcriptional organizer that recruits specific factors into a complex that stimulates lineage-specific transcription.[48] This hypothesis is supported by the finding that CBF synergistically activates transcription of the *TCRβ* and *TCRα* enhancers with Ets1,[49,50] the NP-3 promoter with C/EBP-α,[51] and the *CSF-1R* promoter with both C/EPB-α and PU.1.[52] These functions appear to involve a direct physical interaction between RUNX1 and the co-operating transcription factor, resulting in both enhanced DNA binding of each factor and the generation of an activation surface which facilitates interactions with co-activators and the basal transcriptional machinery (Figure 3A).[48]

Although lineage-specific transcription usually involves the recruitment of specific factors which can co-operatively bind DNA, transcriptional synergy between RUNX1 and c-MYB appears to occur without co-operative binding to the *TCRδ* enhancer or the myeloperoxidase promoter.[45] However, this transcriptional synergy again appears to result from interaction of these transcription factors with components of the basal transcriptional machinery. Additional sequence-specific DNA-binding proteins such as LEF-1 also influence the activity of CBF.[49] LEF-1 facilitates interactions between CBF and adjacently bound co-activators by binding to the minor groove of DNA and inducing a bend in the enhancer sequence. The ubiquitous co-activator ALY directly binds to both RUNX1 and LEF-1, thereby stabilizing their juxtaposition[53] (Figure 3A). CBF-mediated transcriptional activation has also been shown to involve binding the transcriptional co-activators p300 and Creb binding protein (CBP) to the transcriptional activation domain of RUNX1.[54] These co-activators may bind other basal transcription factors which have intrinsic histone acetyltransferase (HAT) activity such as CREB, P300/CBP, and P/CAF.[55,56] Together, these HATs induce the acetylation of lysine residues in chromatin-associated histones, resulting in a change in chromatin structure leading to enhanced transcription[57] (Figure 3A). HATs can also directly acetylate transcription factors, thereby altering their transcriptional activity.[58] However, at present it is not known whether CBF is acetylated by the HATs bound to the transcription complex.

In addition to the RHD and transactivation elements (TE), RUNX1 contains several other functional motifs that are important for its biological activity. These include:

- a nuclear matrix targeting area (NM);[59,60]
- a highly conserved nuclear localization signal (NLS);[42]
- two putative transcriptional repression domains;
- the first of the repression domains corresponds to an 80-amino-acid domain immediately C-terminal to the RHD that has been found to bind Ear2 (an orphan member of the nuclear hormone receptor superfamily):[61,62]
- the second repression domain (RD) corresponds to a region in the C-terminal portion of the protein;[63]
- the five C-terminal amino acids, VWRPY, are conserved among all CBFα family members and have been shown to function as a binding site for the transcriptional co-repressor Groucho.[61,64]



A B

Figure 3. The role of CBF in the activation and repression of specific genes. A. The activation of specific gene expression by CBF is dependent on the recruitment of basal transcription factors and chromatin acetylation. The CBF complex binds to the core enhancer sequence and functions as an enhancer-organizing factor. Other proteins in this complex differ according to the particular promoter or enhancer involved. They include the transcription factors C/EBP-α, c-Myb, and Ets family members, the DNA-binding protein LEF-1, which interacts with RUNX1 through an adapter protein called ALY and the transcriptional co-activators p300/CBP. p300/CBP recruits other basal transcription factors such as CREB and also binds to the histone acetyltransferase, P/CAF. B. The RUNX1-MTG8 fusion protein retains the ability to bind to the core enhancer sequence and to heterodimerize with CBFb. However, the fusion protein binds, via MTG8, to co-repressor complexes containing N-CoR, mSin3 and HDAC and to other MTG family members (MTGR1). This co-repressor complex may function to tether these complexes to RUNX1-specific genes resulting in transcriptional repression or to RUNX1-MTG8 specific genes. In addition, MTG8 heterodimer complexes may remove the CBF complex from other transcription factors, thereby altering their transcriptional activity.

By binding Groucho or the related mammalian homologs TLE1-4, RUNX1 changes from a transcriptional activator to a repressor.[65]

Alternative splicing produces at least 3 forms of RUNX1 protein. Two isoforms, RUNX1b and RUNX1c (453 and 480 amino acids, respectively), contain the RHD and the C-terminal transcriptional activation domain, whereas the third isoform, RUNX1a (250 amino acids), contains RHD but lacks the transcriptional activation domain.[66] Although these alternatively spliced forms comprise only a minority of RUNX1 transcripts; changes in the ratio of different isotypes may lead to profound changes in the transcriptional activity of the RUNX1/CBFβ complex. RUNX1 isoforms that lack the transcriptional activation domain have been shown to have a higher DNA-binding affinity, but to be unable to activate transcription.[66,67] Expression of these isoforms would be expected to result in the transcriptional repression of RUNX1 target genes. Consistent with this prediction is the observation that G-CSF-induced differentiation of the myeloid cell line 32Dcl3 can be blocked by RUNX1 isoforms that either lack transcriptional activation sequences C-terminal to the RHD[68] or have N-terminal sequences that lack part of the RHD.[69]

These data suggest that expression of CBF could lead either to transcriptional activation or repression, depending on the specific genes being regulated, the isoform of RUNX1 expressed, and the cellular context in which this occurs. If RUNX1 binds to transcriptional co-activators, then transcriptional activation will result. Alternatively, if RUNX1 were expressed as an isoform that cannot bind co-activators, or in cells that express high levels of co-repressors such as Groucho or Ear2, then RUNX1 would function as a transcriptional repressor. In addition, the interaction of RUNX1 with both transcriptional co-activators and co-repressors may be further regulated by post-translational modifications of each component.

Alterations in the balance of positive and negative signals that are mediated through the RUNX1/CBFβ complex are likely to contribute directly to hematopoietic cell development and transformation. This has been confirmed by using gene-targeting experiments. Both the *Runx1* and *Cbfβ* genes have been inactivated in the germline of mice by homologous recombination and shown to be essential for definitive hematopoiesis of all

lineages. Homozygous null animals, i.e. animals with no functional Runx1 or Cbfβ protein, display normal morphogenesis and yolk sac-derived erythropoiesis, but die between embryonic days 11.5 and 12.5 due to CNS hemorrhage. The defect was shown to be intrinsic to the hematopoietic system by demonstrating that Runx1-null embryonic stem (ES) cells were unable to contribute to any hematopoietic lineage in chimeric mice.[70-72] Furthermore, this hematopoietic defect was rescued by expressing *Runx1* under the control of endogenous *Runx1* regulatory elements through targeted insertion. The targeted *Runx1*$^{-/-}$ ES cell clones contributed to lympho-hematopoiesis within the context of chimeric animals. Rescue was shown to require the transactivation domain of Runx1 but not the C-terminal VWRPY Groucho binding motif.[4]

The *RUNX2* gene, identified as being homologous to the murine *RUNX2* gene, was first cloned in 1994 and mapped to chromosome 6p21.[12] Murine *RUNX2* gene expression is initiated in the mesenchymal condensations of the developing skeleton and is strictly restricted to cells of the osteoblast lineage. Runx2 binds to, and regulates the expression of multiple genes in osteoblasts. The forced expression of Runx2 in non-osteoblastic cells induces the expression of osteoblast-specific genes,[73] whereas mice lacking both copies of the *RUNX2* gene are completely deficient in bone formation, due to maturation arrest of osteoblasts.[74,75]

The *RUNX3* gene has been mapped to chromosome 1p36 and the protein is structurally very similar to the RUNX2 and RUNX1 gene products. RUNX3 is expressed predominantly in cells of hematopoietic origin.[76] Like RUNX1, RUNX3 has been shown to activate transcription of the *TCRβ* gene promoter. RUNX3 forms a complex with Smad3, a receptor-regulated signal transduction protein for members of the transforming growth factor-β (TGF-β) superfamily, and stimulates transcription of the germline Ig Ca promoter.[77] It has recently been shown that, similar to RUNX1, RUNX3 is also capable of interacting with TLE1 and acting as a transcription repressor for T-cell receptor enhancers.[64] Based on these studies it is hypothesized that RUNX3 may play a role in hematopoietic cell differentiation.

Interestingly, mutations within the runt domain of RUNX2 are associated with cleidocranial dysplasia (CCD), an autosomal dominant disorder of skeletal morphogenesis.[78,79] Thus, the conserved runt domain of this family of transcription factors is a key target for disease-associated mutations in man.

Abnormalities involving the core binding factor complex

t(8;21)

By far the most extensively investigated abnormality involving CBFs is the recurrent chromosomal translocation t(8;21) which occurs in ~15% of cases of AML.[80] In this translocation the first five exons of the *RUNX1* gene, containing the RHD are fused to almost the entire *MTG8* gene (*myeloid translocation gene* on chromosome 8, also called *ETO/CDR*) (Figure 4A).[81,82]

Sequence analysis has demonstrated MTG8 as the mammalian homolog of the *Drosophila* gene *nervy*,[83] and recent studies have identified three other mammalian members of this family, *MTGR1*, *MTG16* and *ETO-2*.[84-86] Amino acid sequence comparison between MTG family members and *nervy* reveals four evolutionarily conserved domains (Figure 4A).

MTG8 is expressed as a nuclear phosphoprotein in brain and CD34^{+} hematopoietic progenitor cells, whereas MTGR1 and ETO-2 are ubiquitously expressed.[23,84,86] Although MTG8 is a nuclear zinc-finger-containing protein there is no experimental evidence to suggest that MTG8 can bind directly to DNA. Nevertheless, the structure of MTG8 would suggest that it is likely to function as a transcriptional regulator. This hypothesis is supported by recent experiments demonstrating that MTG8 interact directly with the nuclear receptor co-repressor complex (containing N-CoR, mSin3, histone deacetylase),[87,88] which mediates transcriptional repression by deacetylating histones and creating repressive chromatic structures.[89,90] The co-repressor proteins N-CoR and mSin3 bind to separate regions of MTG8[88,91] suggesting that MTG8 may function as an adapter protein within a nuclear co-repression complex. This function may stabilize the interaction of these co-repressors and tether them to sequence-specific DNA-binding transcription factors or, alternatively, recruit these factors away from other transcription proteins thus inducing a fundamental change in transcriptional activity.

In addition to interacting with transcriptional co-repressors, MTG family members have also been shown to form homo- and heterodimers.[84,86] Dimerization is mediated through the hydrophobic heptad repeat (HHR) region and does not appear to interfere with the ability of these proteins to interact with the N-CoR/mSin3/HDAC co-repressor complex. Thus, the MTG8 family members are likely to form multi-subunit complexes that function in transcriptional regulation. The formation of different het-

erodimers may lead to significant functional differences in the activity of these complexes.

The RUNX1-MTG8 fusion protein contains the RHD of RUNX1 and the MTG8 portions that mediate homo- and heterodimerization with MTG family members as well as interaction with the N-CoR/mSin3/HDAC co-repressor complex,[87,88] but lacks the C-terminal region of RUNX1 that interacts with the p300 and CBP HATs[54] (Figure 4A). Therefore, RUNX1-MTG8 could recruit HDAC and not HAT activity to the promoter of RUNX1-responsive genes, resulting in histone deacetylation and transcriptional repression (Figure 3B). This hypothesis has been confirmed by the finding that RUNX1-MTG8 directly represses RUNX1-mediated transcriptional activation of the TCRβ enhancer,[92] the GM-CSF promoter,[93] TGF-β1,[94] and C/EBPα[95] in transient transcription assays. Moreover, treatment of RUNX1-MTG8 expressing cells with trichostatin A, an HDAC inhibitor, restores cell cycle control.[88] Furthermore, RUNX1-MTG8 has been shown to bind CBFβ more avidly than RUNX1, and therefore accumulates CBFβ more efficiently in the nucleus than does the wild-type protein.[96]

The activity of RUNX1-MTG8 is likely to be modified by the ability of this chimeric protein to homo- and heterodimerize with MTG family members through the HHR domains of MTG8. Recent data demonstrate preferential dimerization with the ubiquitously expressed MTG family member MTGR1, an interaction that augments RUNX1-MTG8-mediated repression of RUNX1-dependent transcription.[84]

To investigate the role of RUNX1-MTG8 in leukemogenesis directly, gene targeting has recently been used to create a *Runx1-Mtg8 knock-in* allele that mimics the t(8;21). Unexpectedly, embryos heterozygous for the fusion gene (*Runx1-Mtg8*$^{+/-}$) died around E13.5 from a complete absence of normal fetal liver-derived definitive hematopoiesis.[97] This phenotype is similar to that seen following homozygous disruption of either *Runx1* or *Cbfβ*[70,71] However, in contrast to *Runx1*- or *Cbfβ*-deficient embryos, fetal livers from *Runx1-MTG8*$^{+/-}$ embryos contained dysplastic multilineage hematopoietic progenitors with abnormally high self-renewal capacity in vitro. When the same group retrovirally transduced the *Runx1-Mtg8* fusion into murine adult bone marrow-derived hematopoietic progenitors *Runx1-Mtg8*-expressing cells were again found to have an increased self-renewal capacity and could be readily established into immortalized cell lines *in vitro*.[97] Taken together, these studies suggest that RUNX1-MTG8 not only neutralizes the normal biological activity of RUNX1 but also has novel gain-of-function activities that alter the expression of genes not normally regulated by RUNX1.

RUNX1-MTG8 has been found to activate transcription of the *BCL-2* promoter through a RUNX1-binding site that resides within a negative regulatory region of the promoter.[98] A further study found that ectopic expression of RUNX1-MTG8 elevates the expression of the G-CSF receptor and that this up-regulation was not dependent on the RUNX1 core binding sequence, but on the binding site of a second transcription factor, C/EBPε.[99] Similarly, RUNX1-MTG8 can synergize with RUNX1 to activate the M-CSF receptor promoter.[100] In a recent study, 24 genes under the downstream control of RUNX1-MTG8 were isolated by using a differential display technique. The regulation of the majority of these genes was found to depend on the integrity of the HHR region through which MTG8 interacts with MTGR1. Among the 24 genes identified, 14 genes were not affected by RUNX1 alone.[101] A further study by the same group analyzed approximately 6,500 genes and identified 32 candidate genes under the downstream control of RUNX1-MTG8. Among the 32 genes, 23 were not known to be regulated by RUNX1-MTG8 suggesting the possibility that RUNX1-MTG8 regulates a number of specific target genes not normally regulated by RUNX1.[102] Importantly, the closely related MTG16 has also been identified as a target of the t(16;21)(q24;q22) translocation,[85] a much rarer but recurrent chromosomal abnormality associated with therapy-related myeloid malignancies.[103] This translocation results in the fusion of *MTG16* to *RUNX1*, producing a RUNX1-MTG16 fusion protein whose structure is similar to RUNX1-MTG8.85 Identification of a second MTG family member involved in a translocation with RUNX1 suggests that MTG sequences are critical for the transforming activity of these fusion oncoproteins.

However, recent studies have shown that expression of a RUNX1-MTG fusion protein is unlikely to be sufficient, by itself, for malignant transformation. Mice in which the expression of Runx1-Mtg8 was under the control of a tetracycline-inducible system did not develop leukemia even though abnormal maturation and proliferation of progenitor cells had been observed.[104] Moreover, transgenic mice in which expression of Runx1-Mtg8 was under the control of the myeloid specific human *MRP8* promoter developed AML only upon treatment with the DNA-alkylating agent *N*-ethyl-*N*-nitrosurea (ENU).[105] RUNX1-MTG8 transcripts have been shown to be present in a fraction of

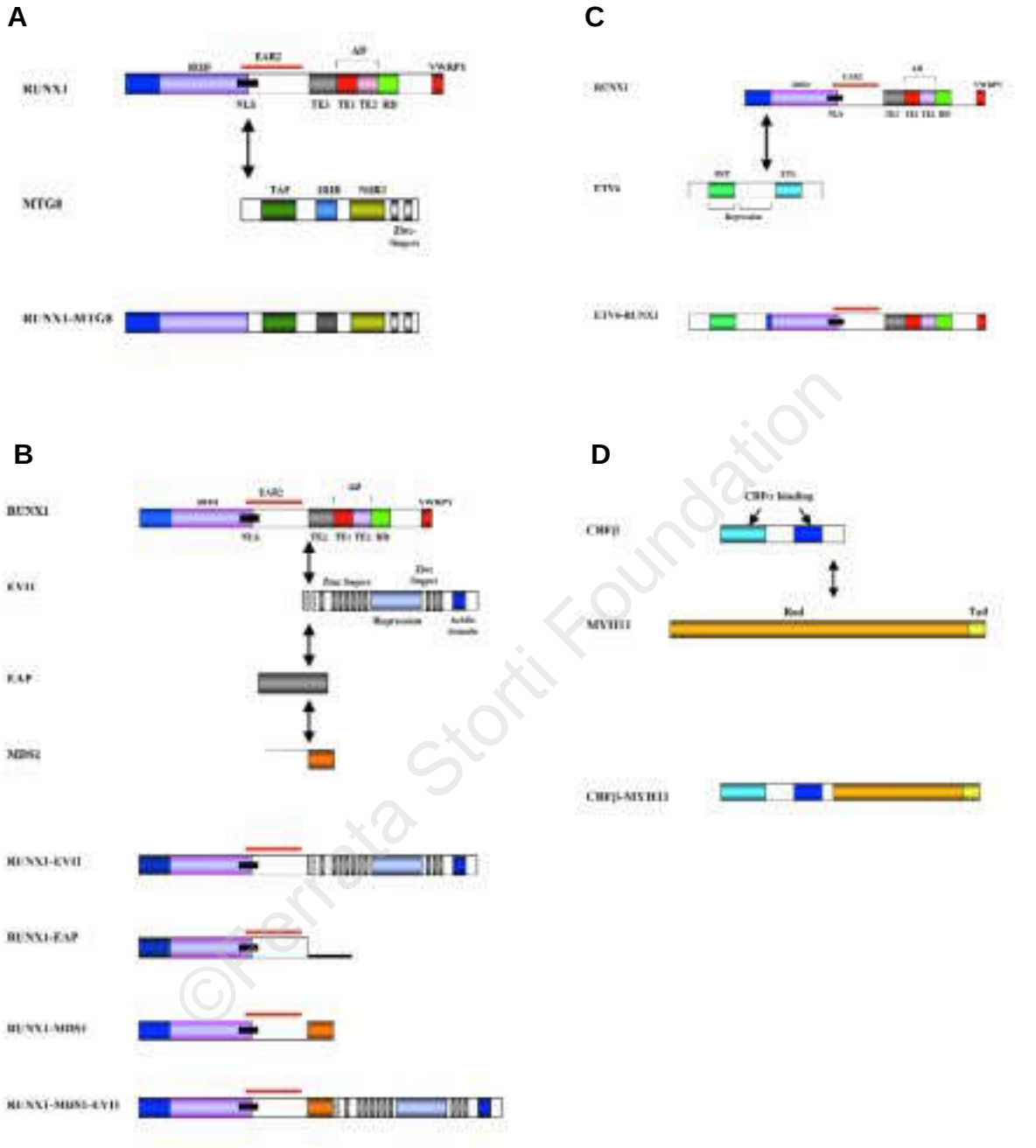


Figure 4. Illustration of the proteins involved in translocations involving CBF. Known protein domains of RUNX1 are as in Figure 2. A. t(8;21) The MTG8 protein contains four regions that have high homology to the *Drosophila* protein nervy and to the MTG family members MTGR1, MTG16 and ETO-2. These regions include an N-terminal domain with homology to transcriptional-activating factors (TAF), a hydrophobic heptad repeat (HHR), a small region with homology to MTG proteins referred to as the nervy homology region 3 (NHR3), and a C-terminal domain that contains two zinc-finger motifs. Vertical arrows indicate breakpoints. B. t(3;12) Known structural motifs are indicated. The fusion of *RUNX1* with *EVI1* includes the second untranslated exon of EVI1, marked as a dashed line with vertical bars. The fusion of RUNX1 with EAP is not in frame and a short line in the RUNX1-EAP fusion product indicates the 17 non-EAP-related amino acids. The unknown amino end of MDS1 is shown as a dashed box. Vertical arrows indicate breakpoints. C. t(12;21) The dimerization (PNT), DNA-binding (ETS), and repression domains of ETV6 are shown. Vertical arrows indicate breakpoints. D. inv(16), t(16;16) The RUNX1 binding domain of CBF and the rod and tail domains of MYH11 are shown. The position of the most commonly identified breakpoint identified in inv(16) and t(16;16) is indicated by vertical arrows.

stem cells, monocytes, and B-cells in remission marrow, and in a fraction of B-cells, but not T-cells, in leukemic marrow. RUNX1-MTG8 transcripts have also been demonstrated in a fraction of colony-forming cells of erythroid, granulocyte-macrophage, and/or megakaryocyte lineages in both leukemic and remission marrow.[106] These data suggest that RUNX1 has a very restricted capacity to transform cells, and that a fraction of RUNX1-MTG8-expressing stem cells undergo additional oncogenic event(s) at a particular stage of hematopoietic differentiation that ultimately leads to AML transformation.

Taken together these data suggest a model for the involvement of RUNX1-MTG8 in hematopoietic cell transformation by:

- actively repressing the normal role of RUNX1 in transcriptional activation;
- repressing transcription by other CBFA family members;
- interfering with the normal function of MTG8 and other MTG family members;
- aberrantly activating the transcription of RUNX1-regulated and novel RUNX1/MTG8-specific target genes.
- predisposing hematopoietic cells to malignant transformation via further oncogenic events.

t(3;21)

The *RUNX1* gene is also involved in another, rarer, recurring translocation, t(3;21)(q26;q22), which occurs mainly in patients with therapy-related AML or MDS who have been previously treated with drugs including topoisomerase II inhibitors, and in patients with CML-BC.[107] This translocation gives rise to the chimeric fusion genes *RUNX1-MDS1 RUNX1-EAP and RUNX1-EVI1*(Figure 4B). *EAP* (Epstein-Barr virus RNA-associated protein) codes for the ribosomal protein L22. However, the *EAP* reading frame is not maintained in the fusion with *RUNX1* and translation of *RUNX1 -EAP* stops after the addition of 17 non-EAP-related amino acid residues to the RHD of RUNX1.[108] This shortened RUNX1 protein may dominantly interfere with normal RUNX1 function during myelopoiesis without a contribution from a partner protein.[109] *MDS1* (myelodysplasia syndrome) is a small gene that is centromeric to *EAP* and encodes a protein of 170 amino acids.[108] *RUNX1-MDS1* contains the same 5' *RUNX1* region as that found in *RUNX1-EAP*, fused in frame to *MDS1*. The function of MDS1 is unknown.

EVI1 encodes a DNA-binding protein with seven zinc-finger motifs at the N-terminus, three zinc-finger motifs in the distal third of the molecule, and an acidic domain at the C-terminus and is not normally expressed in bone marrow or hematopoietic cells.[110] A variant fusion transcript that includes the *MDS1* sequence fused between the *RUNX1* and EVI1 sequences has also been reported in both leukemic and normal cells.[108,111]

The inclusion of both the runt DNA-binding/heterodimerization domain of RUNX1 and the zinc-finger DNA-binding domains of EVI1 in the RUNX1-EVI1 and RUNX1-MDS1-EVI1 fusion proteins afford these proteins striking structural similarities to the RUNX1-MTG8 fusion product (Figure 4A). RUNX1-EVI1 and RUNX1-MDS1-EVI1 can interfere with RUNX1-mediated transactivation, whilst 32D cl3 cells expressing RUNX1-MDS1-EVI1 undergo cell death without differentiation, mimicking the effect of EVI1 alone.[112] Both RUNX1-MDS1-EVI1 and EVI1 interact with Smad3, a downstream effector of TGFβ signaling, thus preventing TGFβ-mediated growth inhibition of 32D cl3 cells and other cell types.[113-115]

Recently, the effect of the *RUNX1-MDS1-EVI1* fusion gene *in vivo* was analyzed by retrovirally transducing the chimeric gene into mouse bone marrow cells. The mice suffered from AML 5-13 months after transplantation with the transduced bone marrow. The disease could be readily transferred into secondary recipients and resulted in a shorter latency of the leukemia.[116]

Thus, not only are the fusion products generated by the t(3;21) translocation strikingly similar to the RUNX1-MTG8 product of the t(8;21) translocation but they may also contribute to leukemogenesis in a similar way by inhibiting normal CBF function and by independent effects of the MDS1/EVI1 domain.

t(12;21)

The *RUNX1* gene was generally considered to be a target for chromosomal translocations in myeloid cells until it was found to be involved in the cytogenetically cryptic t(12;21) translocation detected in approximately 25% of case of childhood B-lineage acute lymphocytic leukemia (ALL), thus making this the most common genetic abnormality in lymphoid leukemias.[117] The partner of t(12;21) translocation on chromosome 12 (p13) was identified in 1994 as the translocated ETS leukemia (TEL) gene involved in the leukemia associated translocation t(5;12).[118] This gene has latterly been renamed ETS-type variant 6 (*ETV6*). The ETS (E26-transformation specific) family of transcription factors is a large group of evolutionarily conserved

transcriptional regulators that play an important role in a variety of cellular processes throughout development and differentiation.[119] All ETS proteins bind DNA via a highly conserved ~85 amino acid region, the ETS domain, which recognizes a purine-rich GGAA/T core motif within promoters and enhancers of various genes.[120] In addition to sequence recognition, DNA binding may also be regulated through phosphorylation of ETS proteins and by protein-protein interactions mediated via other domains within ETS proteins.[121] Although expressed in a variety of tissues, most currently known ETS genes are expressed predominantly in hematopoietic cells and many are key regulators of blood cell development and differentiation.[122]

The ETV6 protein, like one-third of ETS family transcription factors, contains the N-terminal pointed (PNT) dimerization domain which mediates homodimerization,[123,124] and is capable of binding DNA via the C-terminal ETS domain[125] (Figure 4C). The PNT domain of ETV6 is necessary for interaction with the ETS factor, Fli-1, and interferes with the trans-activation of the GPIX promoter by Fli-1.[126] Furthermore, the PNT domain has recently be shown to be required for the interaction with the ubiquitin-conjugating enzyme UBC9.[127] By analogy to other members of the ETS family, it is likely that ETV6 is also a DNA-binding transcription factor. The nature of the genes regulated by ETV6 is not known, but recent studies have shown that ETV6 may act as a transcriptional repressor.[127,128] ETV6 contains two domains that can independently repress transcription of a reporter gene. These are the PNT domain, located at the N-terminus, and a central region of the protein located between the PNT and ETS domains (Figure 4C).[128] The central region represses transcription by interaction with the co-repressors mSin3, SMRT and N-CoR and is sensitive to inhibitors of histone deacetylases. In contrast, the PNT domain represses transcription by a mechanism that does not involve co-repressors sensitive to inhibitors of histone deacetylases. Because the PNT domain is a protein interaction domain, it is likely that other, as yet unidentified, proteins involved in repression, may be recruited.

Unlike the majority of ETS proteins, ETV6 is ubiquitously expressed. The mouse homolog is also widely expressed and mice in which both Etv6 alleles have been deleted die between E10.5-11.5. These mice have normal yolk sac hematopoiesis, but fetal and adult hematopoiesis and lymphopoiesis could not be assessed. However, analysis of chimeric mice showed that *Etv6*$^{-/-}$ cells did not contribute to bone marrow hematopoiesis, although these cells contributed normally to yolk sac and fetal liver myeloid and erythroid progenitors.[129] These studies suggest that the ETV6 protein may be required for hematopoietic cells to home to the bone marrow but not for their differentiation.

Recently, a novel *ETS* gene has been characterized that is highly homologous to *ETV6* and has been called *TEL2*. This gene was discovered via its homology across the ETS domain and it has been localized on chromosome 6p21. Unlike the ubiquitously expressed *ETV6* gene, however, *TEL2* expression appears to be restricted to the hematopoietic tissues. The TEL2 protein associates with itself and with ETV6 in doubly transfected Hela cells and this interaction is mediated through the PNT domain of ETV6.[130]

The t(12;21) translocation results in fusion of the N-terminus of *ETV6* (including the PNT domain) to nearly all of the *RUNX1* gene (Figure 4C).[131,132] ETV6-RUNX1 interferes with RUNX1 DNA-binding and represses activation of the TCRβ and IL-3 promoters by wild-type RUNX1. This repression is dependent upon the integrity of the PNT domain of ETV6, and the RHD and amino acids 216-290 of RUNX1.[133,134] Further studies have shown that the central region of ETV6 which interacts with NcoR is retained in ETV6-RUNX1, and ETV6 lacking this domain is impaired in transcriptional repression.[135] The observation that the PNT domain of ETV6 can mediate heterodimerization between ETV6-RUNX1 and ETV6 suggests that the chimeric molecule may also directly alter the normal function of the wild-type ETV6 protein.[123,136,137]

These data highlight the similarities between ETV6-RUNX1 and RUNX1-MTG8 fusion proteins. In both cases HDAC co-repressor complexes are recruited to genes normally transcribed by RUNX1, whilst dimerization with other transcription factors may involve the expression of fusion protein specific genes. Moreover, transgenic mice expressing ETV6-RUNX1 from the B-cell specific EμPμ promoter failed to develop any hematologic malignancies and, unlike RUNX1-MTG8 transgenic animals, showed no morphologic or phenotypic abnormalities in the bone marrow.[138] Interestingly, the non-translocated *ETV6* allele is frequently deleted in cases of ALL with t(12;21).[139-141] Loss of heterozygosity at the *ETV6* locus is common in childhood ALL,[142-144] and the four ALL cases from which the *ETV6-RUNX1* fusion was first cloned all had deletions of the non-translocated *ETV6* allele.[131,132]

These results indicate that deletion of *ETV6* may be a secondary event in leukemias with t(12;21)

and suggests a consistent association between *ETV6-RUNX1* fusion and deletion of the normal *ETV6* allele.[139] This raises the possibility that the *ETV6* gene could have tumor suppressor activity, although a mitigating argument against this is the failure to detect bi-allelic loss of *ETV6* in the absence of the t(12;21).[131,145,146] In addition, not all cases with loss of *ETV6* contain the *ETV6-RUNX1* fusion.[139,140] One hypothesis for the role of *ETV6* deletions is that the product of the normal *ETV6* allele interferes with the activities of *ETV6-RUNX1* by interaction via the shared PNT dimerization domain. Consistent with this model, several cases of ALL have been identified that carry small deletions within the PNT domain of the *ETV6* locus.[147] An alternative hypothesis is that loss of ETV6 itself provides cells with a proliferative advantage. The defect in marrow homing identified in Etv6$^{-/-}$ mice might also provide ALL blasts containing a similar ETV6$^{-/-}$ phenotype with a proliferative advantage.

Inactivation of normal ETV6 function, both through deletion of the non-translocated allele and disruption of function via fusion to RUNX1 is likely to contribute to the pathogenesis of the ETV6-RUNX1-associated leukemias.

The acquisition of secondary events contributing to the progression of *ETV6-RUNX1* associated ALL is supported by the finding of identical *ETV6-RUNX1* fusion sequences in the lymphoblasts of two sets of identical twins. The first twin of each pair developed ALL at a much earlier age than the second twin (a 1.5- and 9-year interval).[148,149] However, analysis of DNA from archival material from the twin diagnosed at 14 years identified a clone which contained an identical *ETV6-RUNX1* fusion product, when analyzed molecularly, to that of the twin diagnosed at 5 years. Moreover, this clone was present 9 years before clinical diagnosis. These data suggest that the *ETV6-RUNX1* fusion could be generated *in utero*. The long latency period between the generation of the fusion and the development of leukemia suggests that secondary events are required for the development of the leukemic phenotype.

Inv(16), t(16;16)

The importance of CBF in acute leukemias is further demonstrated by chromosomal abnormalities involving the CBFβ subunit. Inv(16)(p13;q22) or the less common t(16;16)(p13;q22) are present in 10% of AML, usually M4eo. The translocation leads to a CBFβ-MYH11 fusion product in which the majority of CBFβ is fused to the tail domain of MYH11 (also known as smooth muscle myosin heavy chain, SMMHC)[150] (Figure 4D). Several variants of CBFβ-MYH11 RNA and protein have been detected in cases of inv(16)[150-152] of which the most common variant includes 165 CBFβ residues fused to 446 MYH11 residues and is detected as a 70 κDa protein. The MYH11 domain is a-helical and consists of multiple, related 28 amino acid regions. One face of the α-helix is hydrophobic, allowing dimerization. The other face is hydrophilic, with alternating positively and negatively charged zones. This face mediates multimerization, which occurs with a 98 amino acid (3.3 repeat) stagger.[153] In addition MYH11 has a non-helical C-terminal tail. Human MYH11 has two isoforms, MYH11204 and MYH11200, which differ in the length of this non-helical C-terminus as a result of alternative splicing.[154] CBFβ-MYH11204 is more highly expressed than the CBFβ-MYH11200 isoform in AML M4eo.[152]

CBFβ-MYH11 can interfere with CBF DNA-binding by sequestering CBFα subunits in complexes formed as a result of multimerization via the MYH11 domain.152;155 In leukemic blasts, CBFβ-MYH11 is detected in small nuclear speckles, and at high concentration CBFβ-MYH11 forms rod-like structures in fibroblastic and hematopoietic cell lines.[152,155,156] The relevance of these structures in leukemogenesis is unknown. CBFβ-MYH11 has been shown to sequester CBFα subunits in the cytoplasm of adherent cell lines.[157,158] This may result from increased affinity of CBFβ-MYH11 for the cytoskeleton, compared with CBFβ, possibly as a result of interaction of its MYH11 segment with cytoskeletal-associated non-muscle myosins.[159] CBFβ-MYH11:CBFα complexes retain the ability to bind DNA allowing the possibility that the fusion gene may also interfere with CBFα trans-activation via local effects on promoter/enhancer transcription complexes.[150,155] Deletion of 11 N-terminal CBFb residues, required for CBFα interaction, as well as 283 C-terminal residues from the MYH11 segment, required for dimerization, prevents CBFβ-MYH11 from interfering with CBFα DNA-binding and trans-activation.[160] Although the mechanism by which the CBFβ-MYH11 fusion oncoprotein contributes to cellular transformation has not been fully elucidated, it may act in a similar manner to the previously described fusion oncoproteins containing RUNX1 by inhibiting normal CBF function and by independent effects of the MYH11 domain. Recently, gene targeting has been used to create a *Cbfβ-Myh11 knock-in* allele that mimics the inv(16). Mouse embryos heterozygous for *Cbfβ-Myh11* lacked definitive hematopoiesis and developed multiple fatal hemorrhages around E12.5.161

This phenotype is very similar to that resulting from homozygous deletions of Runx1 and Cbfβ.[70-72,162,163] Chimeric mice were leukemia-free, but the *knocked-in Cbfβ-Myh11* allele was only identified in erythrocytes, not leukocytes, in the circulating blood.[161,164] These results indicate that hematopoietic stem cells containing the *Cbfβ-Myh11* gene are present in chimera's bone marrow, which have a selective defect in lymphoid and myeloid differentiation. *Cbfβ-Myh11* chimeric mice did not develop tumors in their first year indicating that *CBFβ-MYH11* may contribute to leukemic transformation but additional genetic events are likely to be required. To test this hypothesis, 4-16-week old *Cbfβ-Myh11* chimeric mice were injected with a single sub-lethal dose of ENU, resulting in 84% of the treated chimeric animals developing leukemia 2-6 months after treatment.[164] The tumors in the *Cbfβ-Myh11* chimeras were almost exclusively AML M4, even though ENU causes mutagenesis in cells in many tissues and Cbfβ is broadly expressed,[11] suggesting a strong disease specificity for the *CBFβ-MYH11* oncogene dependent on further, critical, oncogenic events.

Other forms of CBF deregulation

The high incidence of leukemia resulting from deregulation of the CBF complex via chromosomal translocations has led to the hypothesis that other mechanisms of deregulation may be involved in some cases of leukemia. This hypothesis has recently been confirmed by the finding of congenital mutations of *RUNX1* in 6 pedigrees with the rare autosomal dominant disease, familial platelet disorder (FDP), in which there is a congenital predisposition to the development of AML.[8] Furthermore, heterozygous point mutations have been identified in the RHD of the RUNX1 gene in sporadic leukemias, M0 AML, and other myeloid malignancies.[5-7] These mutations interfere with the DNA binding and transactivation properties of *RUNX1* but do not affect dimerization with CBFβ. In co-transfection studies these mutant proteins inhibit wild-type RUNX1 function.[5,6] It has been proposed that haploinsufficiency for *RUNX1* is responsible for FDP.[8] However, hyperactivating, inhibitory, and loss-of-function mutations of RUNX1 have all been described in leukemia.[5] indicating that haploinsufficiency may not be the only mechanism responsible for the predisposition to leukemia seen in these families. Indeed, recent findings suggest that while haploinsufficiency of RUNX1 causes FDP in some families (deletions and frameshifts), mutant RUNX1 proteins (missense and nonsense) may also inhibit wild-type RUNX1, possibly creating a higher propensity to develop leukemia.[165]

A small number of cases have been reported in which amplification of RUNX1 has been detected in cases of childhood ALL.[166-168] However, in a recent study no mutations were detected in any of the amplified copies of the RUNX1 gene[169] suggesting other mechanisms, such as gene dosage may be responsible.

Prognostic implications of core binding factor associated malignancies

Many studies comprising adults with *de novo* AML have demonstrated that the highest complete response rates, and the longest CRD and survival have been associated with t(8:21) and inv(16)/t(16;16).[170] This is also true for children with these abnormalities.[171,172] The molecular basis of a favorable response to treatment in patients with inv(16) or t(8;21) is unknown. However, the improved outcome may be due to an increased sensitivity of the leukemic cells to cytarabine,[171,173] which, together with anthracyclines, constitutes the mainstay of chemotherapy for AML. Furthermore, Tosi *et al*.[174] have demonstrated a significant increase in the incorporation of cytarabine into nuclear DNA *in vitro* and an increase in cytarabine-induced apoptosis in the blast cells from patients with inv(16).

Clinical studies have shown that intensive post-remission therapy with HiDAC in patients with t(8;21), inv(16)/t(16;16) or a normal karyotype, but not in those with other cytogenetic abnormalities, considerably improves outcome.[173] The effect of this treatment is greatest in patients with t(8;21).[175,176]

The presence of the ETV6-RUNX1 fusion protein in childhood ALL has also been associated with a favorable long-term prognosis.[117] However, more recent studies have cast considerable doubt on these findings. In a German co-operative study this lesion was detected in 24% of the relapsed cases studied, a similar frequency to that seen in newly diagnosed cases.[177] In a large retrospective study a Dutch group recently reported that the presence of the *ETV6-RUNX1* fusion did not appear to have independent prognostic significance.[178]

However, three other studies identified the fusion product in 10% or less of relapsed cases of childhood ALL.[179-181] These, seemingly discrepant, results may reflect differences in protocol efficacy. For example, in the studies of Ayigad *et al*.[182] and Takahashi *et al*.,[183] *ETV6-RUNX1* fusion emerged as a favorable prognostic factor in trials that featured intensive

chemotherapy. Recent data have shown that ETV6-RUNX1 patients are nine times more sensitive to L-asparaginase than other C/preB ALL cases[173] and the trials reported by Loh *et al*.[179] were based primarily on intensive L-asparaginase treatment.

The molecular characterization of the events leading to the disruption of CBF has provided important insights into our understanding of the normal regulatory pathways that control hematopoiesis and has begun to reveal how alterations in these pathways induce leukemia. This understanding has also provided critical reagents for the accurate identification of the broad group of leukemias harboring alterations of CBF.

The application of these molecular approaches has already had an impact on the clinical management of these patients and, as more information becomes available, the ability to tailor therapy to improve each patient's chance of a cure.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two inhouse referees and by Professor Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken by Professor Francesco Lo Coco. Manuscript received July 19, 2002; accepted October 15, 2002.

Francesco Lo Coco, Deputy Editor (Rome, Italy)