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c-MYB and DMTF1 in cancer

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Abstract

The *c-Myb* gene encodes a transcription factor that regulates cell proliferation, differentiation, and apoptosis through protein-protein interaction and transcriptional regulation of signaling pathways. The protein is frequently overexpressed in human leukemias, breast cancers, and other solid tumors suggesting that it is a *bona fide* oncogene. c-MYB is often overexpressed by translocation in human tumors with t(6;7)(q23;q34) resulting in *c-MYB-TCRβ* in T cell ALL, t(X;6)(p11;q23) with *c-MYB-GATA1* in acute basophilic leukemia, and t(6;9)(q22–23;p23–24) with *c-MYB-NF1B* in adenoid cystic carcinoma. Antisense oligonucleotides to *c-MYB* were developed to purge bone marrow cells to eliminate tumor cells in leukemias. Recently small molecules that inhibit c-MYB activity have been developed to disrupt its interaction with p300. The *Dmp1* (cyclin D binding myb-like protein 1; *Dmtf1*) gene was isolated through its virtue for binding to cyclin D2. It is a transcription factor that has a Myb-like repeat for DNA binding. The *Dmtf1* protein directly binds to the *Arf* promoter for transactivation and physically interacts with p53 to activate the p53 pathway. The gene is hemizygously deleted in 35–42% of human cancers, and is associated with longer survival. The significances of aberrant expression of c-MYB and DMTF1 proteins in human cancers and their clinical significances are discussed.

Keywords

c-MYB; DMP1 (DMTF1); expression; leukemia; solid tumor; breast cancer; therapy; anti-sense oliodeoxynucleotides; super enhancer; small molecule

Introduction

The c-Myb proto-oncogene encodes the transcription factor that was originally identified as the cellular proto-oncogene for the v-Myb found in two different chicken leukemia viruses: Avian Myeloblastosis Virus (AMV) and E26 (1–3; Fig. 1A). The v-Myb retroviruses are highly oncogenic and are capable of transforming immature hematopoietic cells in culture and induce acute leukemias in animals. The c-*Myb* gene is expressed in hematopoietic cell precursors, colonic crypts, intestines, kidneys, and brain (4, 5). In humans, amplification or rearrangement of *c-MYB*, caused by chromosomal abnormalities in the region 6q22–24, were originally linked to acute myelogenous leukemia (6). More recently, deregulated or

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overexpressed c-MYB has been detected in a wide range of human cancers and is associated with poorly differentiated tumors in many cell types, including hematopoietic malignancies (7–14), breast cancers (BCs, 15–20), colon cancers (21–25), pancreatic cancers (26, 27), glioblastomas (28–30), melanomas (31–33), head and neck cancers (34) and esophageal cancers (35), vulvar cancers (36), and lacrimal gland cancers (37). The widespread association of c-Myb with many types of tumors in a diverse set of tissues suggests that it plays a critical role in tumorigenesis (38 for a review). The mechanisms of aberrant expression of c-MYB in human cancers compatible with its oncogenic role include gene amplification, chromosomal translocation, increased transcription, mRNA stabilization due to loss of microRNA-binding sites (34, 39).

Recently, three types of evidence have provided strength to the argument that c-MYB is a *bona fide* oncogene. The first was the finding that the *c-MYB* gene is frequently involved in genomic duplications in two subsets of pediatric T-cell acute lymphocytic leukemias (T-ALL) (7–9, 14). In patients harboring the reciprocal translocation t(6;7)(q23;q34), the *c-MYB* gene became juxtaposed to the *TCRβ* gene leading to *c-MYB* overexpression (8, 40; Fig. 2A). The second evidence comes with acute basophilic leukemia (ABL) where the *c-MYB* gene is overexpressed as a result of t(X;6)(p11;q23) translocation resulting in *c-MYB-GATA1* expression (41, 42; Fig. 2B). The third type of evidence was the discovery of a recurrent fusion between the *c-Myb* and Nuclear Factor 1B (*NF1B*) genes in translocations t(6;9)(q22–23;p23–24), which occurs in adenoid cystic carcinomas of the breast, salivary glands of the head and neck, and other organs (17, 34, 36, 37, 38, 43; Fig. 3A). The translocation results in the expression of chimeric transcripts in which the normal 3’-UTR of the *c-MYB* transcript is replaced by a portion of the *NF1B* mRNA. The chimeric transcript lacks a number of binding sites for microRNAs that would normally down-regulate the expression of c-MYB protein, resulting in overexpressed c-MYB in human tumors (17, 34, 43). Recently, small molecules to c-MYB have been developed to disrupt its interaction with p300 promising their therapeutic effects in human cancer overexpressing c-MYB (44, 45 for review). The general consensus is that super enhancers (46, 47) to *c-MYB* as a consequence of chromosomal translocation cause overexpression of c-MYB resulting in carcinogenesis. These discoveries that the human *c-MYB* gene is involved in recurrent translocations in tumors provide evidence that *c-MYB* is a *bona fide* human proto-oncogene.

Scientists have been engaged in the development of antisense oligonucleotides that are more effective for eliminating leukemic cells from the bone marrow (48). They have also been used to treat chronic myelogenous leukemia (CML) by systemic infusion with some success (49). Genomic abnormalities that decrease c-MYB expression have been reported in human melanomas suggesting its tumor-suppressive role in this neoplasm (50, 51).

There are two other Myb family members in mammals: *A-Myb* (*MYBL1*), *B-Myb (MYBL2)*, in addition to *c-Myb* (52–56; Fig. 1B). These two genes had been isolated during screening of human cDNA libraries at low stringency with the *v-Myb* probe (51). Orthologues of all these three are present in both mice and humans while two vertebrates, Drosophila melanogaster and sea urchins have a single homolog of *Myb* (56).

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The Myb-like transcription factor DMP1 (cyclin D binding Myb-like Protein 1; DMTF1; Fig. 1C) governs the activity of the ARF-p53 tumor suppressor pathway by binding to the *ARF* promoter in response to oncogenic stresses driven by Myc/Ras/cyclin D1 (57–73) and through physical interaction with p53 in response to DNA damage (74, 75; 76–83 for reviews of DMP1; 84–86 for reviews of ARF). DMP1 also regulates $p16^{INK4a}$ transcription involved in the RB tumor suppressor pathway (87). It encodes transcriptional activator of amphiregulin, thrombospondin-1, JunB, and Egr1 indicating that it is a mediator of a variety of signal transduction pathways (69). One unique feature of the *DMTF1* locus is the fact that the locus generates at least three splice variants: *DMTF1*α, β, and γ with antagonizing activities (88, 89; 80, 90 for review). The *hDMTF1*α gene corresponds to mouse *Dmtf1*α with tumor suppressive activity (89). The hDMTF1-ARF-MDM2-p53 pathway provides cell autonomous tumor surveillance that detects and force early stage cancer cells to undergo senescence and/or apoptosis to prevent the development of cancer (71). The *hDMTF1* expression is suppressed by WT1 in leukemic cells via direct binding to an EGR/SP1 site (91). Since the *WT1* gene is aberrantly overexpressed in human leukemic cells, it can be used as a molecular marker of the minimal residual disease (92–99). These studies delineated a new oncogenic WT1-mediated mechanism of control of cell proliferation in the hematopoietic cells (99).

We have shown that *Eμ-Myc, K-Ras*^{LA}, *HER2/neu,* or *cyclin D1*-driven tumor development was significantly accelerated in both *Dmtf1*$^{+/-}$ and *Dmtf1*$^{-/-}$ mice with no significant differences in the survival between the two cohorts, suggesting that Dmtf1 is haplo-insufficient tumor suppressor (83 for review). In *Eμ-Myc* lymphomas, the combined frequencies of *p53* mutation and *Arf* deletion in mice of *Dmtf1*$^{+/-}$ or *Dmtf1*$^{-/-}$ background were significantly lower than that in *Dmtf1*$^{+/+}$ littermates, indicating that Dmtf1 is a physiological regulator of the Arf-p53 pathway *in vivo* (63). Consistently, Kobayashi and Srour reported that Dmtf1 regulates hematopoietic stem cell function under both steady-state and stress conditions through regulation of Arf and $p21^{Cip1}$ (70).

Loss of heterozygosity (LOH) of the h*DMTF1* locus was found in 42 % of human breast cancer (71) and 35 % of non-small cell lung cancer (67, 100) in mutually exclusive fashion with that of *INK4a/ARF* or *p53* (67, 71). Recent studies suggest the critical roles of oncogenic splice variants from human genomic loci in carcinogenesis (reviewed in 80, 101–104). We found overexpression of the splice variant DMTF1β in human breast cancer primary samples and conducted clinic-pathological and transgenic mouse studies focusing on DMTF1 (88).

In this review, we will summarize the finding on aberrant c-MYB protein expressions in cancer (both overexpression and underexpression) that had been reported in the literatures focusing on c-MYB and DMTF1. We also make comments on overexpression of other MYB proteins in cancer. Understanding the mechanisms for altered expression of c-MYB and DMTF1 proteins is important not only for the understanding of pathophysiology of cancer, but also play significant roles in the development of novel therapies targeting these molecules.

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Aberrant expression of c-Myb in cancer

c-Myb: cloning and expression

In 1979, Beug et al. (1) reported that cells transformed by AMV and E26 viruses resemble myeloblasts in that they weakly express Fc receptors, phagocytic capacity, and macrophage cell surface antigen, but strongly express myeloblast cell surface antigen and ATPase activity. AMV-infected cells contain two viral mRNAs: 7.5 kilobase (kb) genomic mRNA and 2.5 kb subgenomic mRNA, which contains the AMV-specific sequences, v-Myb (1). *In vitro* translation of AMV virion RNA size-fractionated by sucrose density gradient centrifugation yielded 76-, 56-, 48.5-, 47 -, and 32- kilo dalton (kDa) products. Three of these might represent the product of the AMV *Myb* gene (2). In 1982, the oncogene (*v-Myb*) of AMV and its cellular homolog (*c-Myb*: chicken) have been molecularly cloned and sequenced (105). Comparisons between the sequences of *v-Myb* and *c-Myb* indicated that transduction of c-Myb to form v-Myb possibly resulted from an initial DNA rearrangement and the subsequent use of a spliced RNA (105). The data indicate that the *c-Myb* gene contains at least eight exons which span a total of about 16 kbps (106).

Nucleotide sequencing has shown that mouse clones have both 5' and 3' to the sequences homologous to the *v-Myb* oncogenes of AMV and avian leukemia virus E26 (1, 2) with an open reading frame of 1,944 nucleotides encoding a protein highly homologous to the chicken c-Myb protein (106). Examination of the predicted amino acid sequence of the murine c-Myb protein revealed the presence of a 3 tandem repeat of 52 amino acid residues near the N-terminus of the protein (i.e. Myb repeats responsible for DNA-binding; Fig. 1A) having a high α-helix content, a basic region toward the N-terminus of the protein, and an overall globular configuration (Fig. 1A). c-Myb and v-Myb comparison suggested that the v-Myb protein lacks the N-terminal region of c-Myb (106–109; Fig 1A, B).

Global and conditional knockout mouse models for *c-Myb*

Loss of *c-Myb* function in mice results in embryonic lethality due to failure of fetal hepatic hematopoiesis (110). Although embryonic erythropoiesis was not impaired by the *c-Myb* alteration, adult-type erythropoiesis, which first takes place in the fetal liver, was greatly diminished in *c-Myb* mutants (110). Additional hematopoietic lineages were similarly affected. These results are consistent with a role for *c-Myb* in maintaining the proliferative potential of hematopoietic progenitor cells.

Since the global knockout of *c-Myb* was embryonic lethal, Emambokus et al. (111) generated a knockdown allele of *c-Myb* (*c-Myb*$^{LoxP/LoxP}$), expressing low levels of the protein, which enabled them to investigate further the involvement of *c-Myb* in hemopoiesis (111). Low levels of c-Myb were sufficient to allow progenitor expansion but, importantly, the progression of progenitors towards terminal differentiation was significantly impaired (111). Decreased levels of c-Myb expression allowed differentiation of macrophage and megakaryocytes, while higher levels seem to be important in the control of erythro- and lymphopoiesis. The transition from the CFU-E to erythroblasts was critically dependent on c-Myb levels (111). During T cell development, c-Myb regulated immature cell numbers and differentiation prior to expression of CD4 and CD8. Overall, their results pointed to a

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complex involvement of *c-Myb* in the regulation of proliferation and commitment within the hematopoietic hierarchy (111).

Liu and Reddy disrupted the *c-Myb* proto-oncogene specifically in adult bone marrow (BM; i.e. conditional knockout) to demonstrate that this transcription factor was a regulator of proliferation and differentiation of adult hematopoietic stem cells (HSCs, ref. 112). Targeted disruption of the *c-Myb* gene resulted in depletion of the HSC pool. In addition, BM hematopoiesis in adult mice was impaired in *c-Myb*-deficient cells, resulting in profound reductions of nearly all hematopoietic lineages (112). Serial BM transplantation into lethally irradiated recipient mice indicated an essential role for *c-Myb* in self-renewal process. In conclusion, their data indicated a critical role for *c-Myb* self-renewal in adult BM hematopoiesis and multi-lineage differentiation of adult HSCs (112).

The same group later showed that conditional disruption of the *c-Myb* proto-oncogene resulted in dramatic reductions in common myeloid progenitors (CMP; $CD34^{+}CD16/CD32^{-}$), granulocyte macrophage progenitors (GMP; $CD34^{+}CD16/CD32^{+}$), and megakaryocyte erythrocyte progenitors (MEP; $CD34^{-}CD16/CD32^{-}$) in adult mice, leading to a reduction of neutrophils, basophils, monocytes and platelets in peripheral blood (113). In short, c-Myb plays an essential role in the regulation of multiple stages in adult myeloid hemopoiesis (113).

The colonic crypt is a functional unit of the colon mucosa with a central role in ion and water reabsorption (114). The distal colonic crypt has a single stem cell at its base that gives rise to highly proliferative progenitor cells differentiating into columnar, goblet, and endocrine cells. Malaterre et al. (114) studied three genetically distinct hypomorphic *c-Myb*-mutant mouse strains (i.e. $c\text{-}myb^{Plt3/plt3}$, $c\text{-}myb^{Plt4/Plt4}$, and $c\text{-}myb^{M303V/M303V}$); all of which show reduced colonic crypt size. *In vivo* proliferation and cell cycle marker studies suggested that these mice had a progenitor cell proliferation defect mediated in part by reduced cyclin E1 expression. To evaluate the extent to which c-Myb was required for colonic crypt homeostasis, they created a tissue-specific, mouse knockout model for *c-Myb* deletion, where they showed that *c-Myb* was required for crypt integrity, differentiation, and proliferation (114).

Mouse models for aberrant overexpression of c-Myb in thrombopoiesis

Mice null for the thrombopoietin (TPO) receptor *Mpl* are profoundly thrombocytopenic, as are humans with mutations in the *MPL* gene (115). Levin et al. (116) studied whether 5-FU produced thrombocytosis in $c\text{-}Mpl^{-/-}$ mice (116). They found that mice can produce a normal level of platelets after administration of 5-FU by producing large numbers of megakaryocytic, myeloid, and erythroid progenitors through a TPO - independent mechanism (116). To identify mutations for causing thrombocytopenia and to define the molecular pathways regulating platelet production, Carpinelli et al. (117) performed a suppressor screen in *Mpl*-null mice using N-ethyl-N-nitrosourea (ENU). They showed that mutations in the *c-Myb* gene caused a myeloproliferative syndrome and expansion of megakaryocytes and platelets in the absence of TPO signaling, demonstrating the utility of large scale ENU mutagenesis suppressor screens for the discovery and validation of therapeutic targets in mice (117).

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Sandberg et al. (118) created knock-in mice with a homozygous Met303 to Val (M303V) mutation in the *c-Myb* gene where the interaction between c-Myb and the transcriptional coactivator p300 was disrupted (used in study 114 as well). The biologic consequences of the mutation included thrombocytosis, megakaryocytosis, anemia, lymphopenia, and absence of eosinophils. Detailed analysis of hematopoiesis in mutant mice revealed distinct blocks in T-cell, B-cell, and red blood cell development, as well as a 10-fold increase in the number of hematopoietic stem cells (118). Cell cycle analysis showed that twice as many hematopoietic stem cells were actively cycling in mutant mice compared with wild type mice. In conclusion, c-MYB, through its interaction with p300, controls the proliferation and differentiation of hematopoietic stem and progenitor cells (118).

Overexpression of c-MYB in human cancer

c-MYB: human chromosomal mapping and cytogenetics

In 1982, Dalla-Favera et al. (119) assigned the *c-MYB* proto-oncogene to chromosome 6 through study of somatic cell hybrids. Harper et al. (120) then conducted detailed mapping of the c-*MYB* locus to chromosome 6q22–6q24 by *in situ* hybridization, which was confirmed by Winqvist et al. (121) and Janssen et al. (122). These studies indicated that the *c-MYB* gene was localized at the breakpoints of translocations frequently involved in T-cell acute lymphatic leukemia (T-ALL), ovarian cancers, and melanomas.

c-MYB expression in human hematopoietic malignancies – role of translocations

It has been reported that *Alu* repeat clusters act as mediators of recurrent chromosomal alterations in tumors (123). The human *c-MYB* locus was found to be flanked by 257-bp *Alu* repeats and the duplication was mediated somatically by homologous recombination between the flanking *Alu* elements on sister chromatids (124). This *Alu* - mediated *c-MYB* tandem duplication could be one of the mechanisms for genomic duplication for *c-MYB* in human T-ALL (8, 14), MYST3-linked acute myeloid leukemia (AML; ref. 11), and *BRCA1*-mutated BC (15).

Overexpression of the *c-MYB* gene has been reported most frequently in human hematopoietic malignancies. Barletta et al. and others (125, 126) found that deletions of the long arm of chromosome 6 (6q-), was frequently found in acute lymphoblastic leukemias (ALLs), non-Hodgkin lymphomas, and myeloid leukemias with high levels of *c-MYB* expression although the *c-MYB* locus was not allelic. The *c-MYB* gene was retained on 6q22, which was bordered by chromosomal breakpoints in both interstitial and terminal 6q-deletions.

In human T-ALL, recurrent chromosomal translocations t(6;7)(q23;q34) involving the *TCRβ* and *c-MYB* loci have been reported (8, 38; Fig. 2A). This translocation led to the juxtaposition of the *c-MYB* proto-oncogene near the *TCRβ* regulatory sequence suggesting aberrant expression of the protein (Fig. 2A). Moreover, Lahortiga et al. (9) reported that a duplication of the *c-MYB* gene was found in 8.4 % of patients with T-ALL, associated with a 3-fold increase in *c-MYB* expression. Consistently, knockdown of c-MYB initiated T cell differentiation indicating that *c-MYB* depletion is a therapeutic target for T-ALL.

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Acute basophilic leukemia (ABL) is a rare subtype of acute leukemia with clinical features related to hyperhistaminemia because of excessive growth of basophils. Cases of t(X;6)(p11;q23) translocation have been reported in this disease (41). Quelen et al. (42) reported the four cases of ABL with a t(X;6)(p11;q23) translocation occurring in infants. The *in situ* hybridization and rapid amplification of cDNA ends revealed that the translocation generated a *MYB-GATA1* fusion gene (42; Fig. 2B). Expression of *MYB-GATA1* in mouse lineage(−) cells committed them to the granulocyte lineage and blocked them at an early stage of differentiation. These results establish a link between chromosomal translocation and the development of ABL with t(X;6)(p11;q23) translocation (42).

Although human cancers have complex genotypes and genomically unstable, they are often dependent on the continued presence of oncogenic mutation(s) that caused the disease - a phenomenon called “oncogene addiction” (127). Such dependencies have also been demonstrated in mouse models, where conditional expression of oncogenes to initiate cancer is required for tumor maintenance and progression; thus they can be used as pathways for therapeutic targets (e.g. *dox-neuNT*; refs. 128, 129). Zuber et al. (127) performed an integrative approach that combines genetically modified mouse models, transcriptional profilings, and inducible RNAis to characterize cellular programs that underlie addiction to the MLL-AF9 fusion protein found in aggressive AML. They showed that MLL-AF9 strengthened c-Myb-governed programs for aberrant self-renewal associates with leukemic stem cell activity and poor prognosis in AML (127). c-Myb suppression precisely mimicked MLL-AF9 withdrawal and eradicated aggressive AML *in vivo* without affecting normal myelopoiesis, indicating that strategies to inhibit Myb-dependent self-renewal programs are promising in cancer therapeutics (127).

Self-renewal is a characteristic of both hematopoietic stem cells (HSCs) and leukemic stem cells (LSCs); therefore, the identification of mechanisms required their functions could provide therapeutic opportunities that are more effective and less toxic than current ones. Zhu et al. (130) performed an *in vivo* short hairpin RNA (shRNA) screen and identified jumonji domain - containing protein JMJD1C as an important driver of MLL-AF9 leukemia. Using a conditional mouse model, they showed that loss of *Jmjd1c* substantially decreased LSC frequency and caused differentiation of MLL-AF9- and homeobox A9 (HOXA9)-driven leukemias (130). They found that JMJD1C directly interacted with HOXA9 and modulated a HOXA9 - controlled gene expression program. In contrast, loss of *Jmjd1c* led to only minor defects in blood homeostasis and modest effects on HSC self-renewal (130). Together, these data establish JMJD1C as an important mediator of MLL-AF9- and HOXA9- driven LSC function that is largely dispensable for normal HSC function.

Sroczynska et al. (131) used a mouse model of human AML induced by the *MLL-AF9* fusion oncogene; they also performed screening with a shRNA library to find novel drug targets. One of the best candidate drug targets identified in these screens was again Jmjd1c. Depletion of *JMJD1C* impaired the growth and colony formation of mouse MLL-AF9 cells *in vitro* (131). Depletion of *JMJD1C* impaired expansion and colony formation of human leukemic cell lines, with the strongest effect observed in the MLL-rearranged ALL cell line. The growth defect upon *JMJD1C* depletion was caused by increased apoptosis indicating that JMJD1C as a potential therapeutic target for human leukemias (131). Overexpression of

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c-Myb or c-Myc significantly provided a growth advantage over *Jmjd1c*-depleted cells, indicating the functional relationship between c-Myb/c-Myc and Jmjd1c in colony formation of leukemic cells (131).

Antisense oligonucleotides to *c-MYB* in cancer therapy

For the past decades, scientists have been engaged in the development of antisense oligodeoxynucleotide (ODN) drugs that might be more effective for the treatment of leukemias and other human malignancies. c*-MYB, c-MYC*, and *BCR/ABL* genes have been chosen since they are often overexpressed in human malignancies and have short half-life mRNAs/proteins.

Since the *c-Myb* gene encodes proteins that are critical for hematopoietic cell proliferation and development, Gewirtz et al. (132) developed a model for testing the *in vivo* efficacy of phosphorothioate antisense ODNs for disrupting *c-MYB*. Their model was a human leukemia - SCID mouse chimera with K562 cells derived from a patient with chronic myelogenous leukemia (CML). The tumor cells carried the Philadelphia chromosome: Ph1 and the *BCR/ABL* hybrid gene (133, 134) that could be used to track human cells in the mouse host. Animals treated with antisense *c-MYB* survived at least 3.5 times longer than controls. Moreover, animals receiving antisense *c-MYB* DNA had significantly less disease at sites most frequently involved by leukemic cell infiltration (CNS and ovary), promising the future direction of AS-ODN therapy (134).

ODNs to *c-MYB* had been developed to purge marrow autografts administered to allograft BMT-ineligible CML patients. Luger et al. (135) purged CD34(+) marrow cells with ODN to *c-MYB* for either 24 or 72 hours. After treatment, *c-MYB* mRNA levels declined significantly in ~50 % of patients. Analysis of BCR/ABL expression in long-term culture-initiating cells suggested that purging had been successful in more than 50 % of patients. Overall 6 of 14 patients (43 %) had achieved a major cytogenetic response (135). These results lead to the speculation that enhanced delivery of ODNs targeted to critical proteins of short half-life lead to the development of effective therapeutic agents in the future.

In the Phase I systemic infusion study, Myb AS-ODN was delivered by continuous infusion at dose levels ranging between 0.3 to 2 mg/kg/day for 2 months (132). One BC patient survived ~14 months with transient restoration of chronic phase disease. These studies indicate that ODN may be administered safely to leukemic patients through systemic infusion, which may eventually demonstrate therapeutic utility in the treatment of human leukemias (132, 135). The Gewirtz lab also reported the increase efficiency with phosphorothioated 2’-deoxy-2’-fluoro-beta-D-arabinonucleic acid modified antisense *c-MYB* ODNs in leukemic cell killing in comparison to unmodified ODNs (136). The modifications and synergisms with other antisense therapy should be pursued further to increase the therapy of antisense *c-MYB* in leukemia therapy. Although antisense *MYB* therapy have been studied mostly in CML due to the presence of universal breakpoint marker BCR/ABL, the possibility for purging should be pursued in human AMLs using other breakpoint markers such as *PML/RARA* (137), *AML1/ETO* (138), or by using *WT1* as an universal marker for human acute leukemias (92, 94, 96–98), the expression of which increases on leukemic relapse (95).

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Small molecules that antagonize c-MYB activity

Although these antisense approaches to *c-MYB* looked very promising in leukemia therapy, it was not pursued later clinically to remove leukemic cells in patients. Instead, small molecules to inhibit c-MYB activity, e.g. by disrupting interactions between c-MYB and its co-regulator p300, has been was attempted to destabilizing the protein itself (139–142; 143 for review). Uttakar et al. (139) identified the triterpenoid Celastrol as a potent low-molecular-weight inhibitor of the interaction of Myb with its cooperation partner p300. They reported that Celastrol inhibited the proliferative of acute myeloid leukemia (AML) cells while not affecting normal hematopoietic progenitor cells. Moreover, Celastrol prolonged the survival of mice in a model of an aggressive AML. Their work demonstrates the therapeutic potential of a small molecule inhibitor Celastrol that disrupt the Myb-p300 interaction for the treatment of AML through a combination of molecular biological, chemical biological, and *in vivo* experiments with primary samples from AML patients (139). The same group later proved the effectiveness of nathoquinone blumbagin using the same method (140). These outstanding studies provide a starting point for the further development of Myb-inhibitory compounds for the treatment of leukemia and possibly other tumors driven by deregulated Myb.

Coulibaly et al. (141) showed that helenalin acetate inhibited C/EBPβ by binding to the N-terminal part of C/EBPβ, thereby disrupting the cooperation of C/EBPβ with the co-activator p300. They reported that helenalin acetate is the first small-molecule C/EBPβ inhibitor by direct binding (141). The same group extended the study to clarify the mechanism of effectiveness of the c-Myb inhibitor Celastrol in cancer therapy. They became aware that the reporter system used for c-Myb inhibitor screening also responded to inhibition of C/EBPβ, a transcription factor known to cooperate with c-Myb in myeloid cells (142, 143). They found that Celastrol strongly inhibited the activity of C/EBPβ by disrupting its interaction with the Taz2 domain of p300. Helanalin Acetate independently targets c-Myb and C/EBPβ by disrupting the interaction of both transcription factors with p300 (142). c-Myb, C/EBPβ, and p300 cooperate in myeloid-specific gene expression and are associated with ‘super-enhancers’ (46, 47) in AML cells that have been implicated in the maintenance of the leukemia (144, 145). They posit that the ability of Celastrol to disrupt the activity of a transcriptional Myb-C/EBPβ-p300 module might explain its promising anti-leukemic activity (142).

MYB-NF1B translocation and c-MYB overexpression in adenoid cystic carcinomas (ACCs)

Chromosomal translocations involving the *c-MYB* gene have been reported in other types of human tumors than leukemias. The t(6;9)(q22–23;p23–24) translocation was found in adenoid cystic carcinomas (ACCs) of the breast, head and neck, vulva, lacrimal gland and other tissues (17, 36, 37), which consistently result in *MYB-NF1B* fusion transcripts consisting of *c-MYB* exon 14 linked to the last coding exon of nuclear factor 1B (NF1B). This leads to loss of *c-MYB* exon 15, which encodes the 3’-UTR, where several highly conserved target sites for microRNAs (miR15a/16 and miR-150) are located (Fig. 3A). Thus, the translocation appears to deregulate *c-MYB* by removing the microRNA binding sites, and the product is different from that of wild type c-Myb.

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Mitani et al. (34) did an extensive analysis with 123 primary tumors of the salivary gland, including primary and metastatic ACCs, and non-ACC salivary carcinomas. The *c-MYB-NF1B* fusion genes were identified by reverse transcriptase-PCR. The *MYB-NF1B* fusion was detected in 28% primary and 35% metastatic ACCs, but not in any of the non-ACC salivary carcinomas (34). Different exons in both the *c-MYB* [13, 8b, 11, 15, 9b, 8a, 16 (the order of frequency)] and *NF1B* [12, 11, 9, 9'] genes were involved in the fusions, resulting in expression of multiple chimeric variants (34). c-MYB was overexpressed in the vast majority of the ACCs although c-MYB expression was significantly higher in tumors carrying the *c-MYB-NF1B* fusion. They concluded that the c-MYB-NFIB fusion characterizes a subset of ACCs contributing to c-MYB overexpression (34).

In 2016, Mitani et al. (146) reported the genetic alterations in ACC lacking the classical translocation and fusion transcript for t(6;9)(q22–23;p23–24) and identified new abnormalities in translocation (+) tumors (reviewed in 147, 148) (Fig. 3B). They identified a novel *MYBL1-NF1B* (i.e. *A-MYB-NF1B*) gene fusion as a result of t(8;9) translocation and multiple rearrangements in the *MYBL1* (*A-MYB*) gene in 35 % of the t(6;9)(−) ACC (146; Fig. 3B). All *MYBL1* alterations involved deletion of the C-terminal negative regulatory domain showed high MYBL1 expression (146). Reciprocal relationship between c-MYB and MYBL1 (A-MYB) expression was consistently found in ACC. The breakpoints in the four tumors with *MYBL1-NF1B* fusions were located in introns 8 and 14 of the *MYBL1* gene and in intron 10 of the *NF1B* gene. Accordingly, only the last two exons of *NF1B* were part of the gene fusions (146; Fig. 3B). Three of the t(6:9)(+)/*MYB-NF1B* (−) tumors had the 5' end of the *NF1B* gene fused to different gene partners (non-*MYB/MYBL1*) including *XRCC4*, *NKAIN2*, *PTPRD* and *AIG1*, suggesting a biologic importance of the C-terminal part of these fusions (146). The role of *MYBL1* in translocation in ACC was also reported by a different group (149). Brayer et al. (149) analyzed ACC tumors with t(8;9) and t(8;14) translocations and found that the *MYBL1 (A-MYB)* gene was fused to the *NFIB* and *RAD51B* genes, respectively. Interestingly, tumors with c-*MYB* and *MYBL1* translocations showed similar gene expression profiles in RNA-seq, correlated with clinical outcomes, suggesting that the related MYB proteins are interchangeable oncogenic drivers in ACC (149).

Driver of c-MYB expression in human cancer

Accumulating studies show that *c-MYB* overexpression itself caused by translocation rather than creation of chimeric protein by translocation is essential for the pathogenesis of ACC (150, 151). Drier et al. (151) identified the juxtaposition of super-enhancer regions to the *c-MYB* locus as the unifying feature of ACC translocations. Detailed genomic and epigenomic analyses of ACCs revealed alternate rearrangements that translocated super-enhancers in the *NFIB* and *TGFBR3* loci either upstream or downstream of the *c-MYB* gene. c-MYB protein bound these super-enhancers, which loop to the *c-MYB* promoter, thereby establishing a positive feedback loop that sustains expression of this master regulator (151). They also showed that c-MYB bound to a larger repertoire of enhancers genome-wide, which appeared to support alternate ACC expression signatures in the myoepithelial and luminal epithelial compartments of ACC. Consistently, BET bromodomain inhibitors, which disrupt enhancer functions, slowed tumor growth in ACC

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primagraft models *in vivo* (151). In conclusion, the major driver of c-MYB overexpression in cancer is the translocation of enhancer(s), with the protein fusion event playing a minor part. The situation is similar to WT1 overexpression in human leukemias where the gene overexpression itself rather than translocation(s) play a major role in leukemogenesis (92–99).

Expression of c-MYB and MYB-like protein DMTF1 in breast cancer

Breast cancer (BC) is important for c-MYB because 1) ACC is found in BC, 2) c-MYB expression correlates strongly with ER positivity (19, 20). This observation was confirmed by analysis of data from studies of a large number of tumors by using microarray expression profiling (16). In many cell types, *c-MYB* expression appears to be regulated by transcriptional repression in the first intron (152), which involves a region potentially capable of forming a stem-loop structure in the transcript and an adjacent poly(dT) region (153, 154). Intriguingly, this motif is frequently mutated in colon carcinomas, but not in BC (153), suggesting that another mechanism is responsible for overcoming attenuation in BC cells. Elevated levels of *c-MYB* mRNA in human cancers can also be caused by genomic amplification in hereditary BRCA1(+) breast cancer (15, 155).

To determine the frequency and patterns of inactivation of the hDMTF1-ARF-Hdm2-p53 pathway in human BCs, we extracted DNA from 110 pairs of clinical samples and conducted LOH analyses for h*DMTF1*, *INK4a/ARF*, *p53*, and gene copy number assay for *Hdm2* (71). LOH for as found in 27 samples with the 5’ probe (25 %), 30 cases (27 %) with the 3’ probe, and 46 of 110 cases (42 %) with either the 5’ or 3’ probes. None of the 61 samples we studied showed methylation of the h*DMTF1* promoter (71) suggesting that complete inactivation of h*DMTF1* does not happen in tumor cells. Detailed mapping of the genomic fragment deleted in BC showed that gene deletion was limited to the h*DMTF1* locus in 30 of 32 cases of LOH (94 %) (71), suggesting that the gene deletion was selective to the h*DMTF1* locus (71). With I*NK4a/ARF* probes, LOH or homozygous deletion was detectable in 19 cases with the 5’ probe (17 %), 10 cases (9 %) with the 3’ probe, and 22 of 110 (20 %) with either of these. Likewise, LOH for the *TP53* locus was detectable in 22 cases (20 %) with the 5’ probe, 30 with the 3’ probe (27 %), and 37 of 110 (34 %) with either of these (71). Overexpression of the p53 protein by immunohistochemistry was found in 6 of 13 *p53* LOH(+) cases (46 %), but not in any of the p53 LOH(−) BCs, consistent with the previous report that showed association of *p53* mutations with loss of the *p53* allele in BC (156). LOH for h*DMTF1* and *INK4a/ARF* was found to be mutually exclusive in 62 of 65 cases (95 %, $p = 0.0027$, $\chi^2 = 9.0$) (71). Likewise, LOH for h*DMTF1* and *p53* was also mutually exclusive in 63 of 73 cases (86 %, $p = 0.025$, $\chi^2 = 5.0$). The *Hdm2* gene amplification was found in 14 of 110 samples (13 %), which occurred independently of the LOH for h*DMTF1*. Thus, our data demonstrate that 1) LOH for h*DMTF1* is frequently found in BCs with wild-type *INK4a/ARF* and *p53* genomic loci, and 2) LOH for h*DMTF1* and *Hdm2* amplification occur at random. We also found significant correlation between cases with h*DMTF1* LOH (+) BC with low Ki67 expression ($p = 0.027$, $\chi^2 = 4.9$) and diploid content of genomic DNA ($p = 0.046$, $\chi^2 = 4.0$). Conversely, BCs with LOH for *p53* were associated with high Ki67 ($p = 0.015$, $\chi^2 = 5.9$) and aneuploidy ($p = 0.014$, $\chi^2 = 6.0$) suggesting their association with specific BC subtype(s) with poor prognosis. Indeed we

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found that h*DMTF1* LOH (+) BCs were significantly associated with luminal A group of BCs ($p = 0.0085$; $\chi^2 = 6.9$) while *p53* LOH(+) BCs were associated with non-luminal A subtype ($p = 0.023$; $\chi^2 = 5.1$) (71).

Consistent with these findings, BCs with LOH for h*DMTF1* had longer relapse-free survival than those without LOH ($p = 0.0092$, $\chi^2 = 6.8$) (71). Conversely, LOH for *p53* had negative impact on patients' disease-free survival ($p = 0.021$, $\chi^2 = 5.4$) (71) consistent with our observation that ~50 % of *p53* LOH cases showed mutation of the remaining *p53* allele (71). LOH for *INK4a/ARF* had no impact on patients' survival while BC with *Hdm2* amplification showed significantly shorter survival than those without gene amplification ($p = 0.022$, $\chi^2 = 5.3$) (71). Together, our data indicate that the more downstream the molecule is localized in DMTF1-ARF-Hdm2-p53 signaling, the more negative impact the marker shows on BC patients' survival.

Correlation of DMTF1 protein expression with *hDMTF1* LOH and HER2 status in human BC

BC samples without LOH for h*DMTF1* showed more intense nuclear staining for hDMTF1 (mostly grades 2–3) while tumors with LOH showed weaker staining (mostly grades 0–1) ($p = 0.0006$). Normal breast epithelial cells also showed weak (1+) hDMTF1 staining. We found a significant increase in hDMTF1 staining in BCs that showed HER2 overexpression (2+ or 3+) ($p = 0.0038$), regardless of the genomic status for h*DMTF1* (68, 81). Together, our data show that: 1) hDMTF1 protein is downregulated in clinical samples that showed LOH for h*DMTF1* and 2) HER2 and hDMTF1 protein expression levels are positively correlated.

The impact of hemizygous loss of *DMTF1* on breast cancer or mammary tumors

Our study shows that LOH for h*DMTF1* is associated with low Ki67 index and increased frequency of diploid DNA, both of which are indicators for favorable prognoses of BCs (157–159). Nevertheless, in wild type *MMTV-neu* tumors, loss of *Dmtf1* was associated with higher histological grades with increased local invasion (68), and thus with more aggressive disease than wild type tumors without *Dmtf1* involvement. Likewise, $K\text{-}Ras^{LA}$ or *cyclin D1*-driven mouse models of cancer exhibit more aggressive tumors with invasion/ metastasis in *Dmtf1*-deficient mice (67, 72) when p53 is wild type. Moreover our GeneChip Microarray (69) showed that i) Dmtf1α upregulates *Thbs1*, an inhibitor of angiogenesis, ii) it increases the transcription of *Egr1* that inhibits cancer metastasis through regulation of TGFβ1, PTEN, and p53 (160). The differential effects of Dmtf1 for human BC and mouse mammary tumors in tumor aggressiveness can be explained by the fact most of the BC patients without LOH for h*DMTF1* showed the involvement of the *TP53* and/or *INK4a/ARF* locus; the deletion of both of which is associated with poor prognoses of patients (161–164). By contrast, involvement of the *p53* or *Ink4a/Arf* locus is rare in mammary tumors from *MMTV-neuNT* mice (68). Instead, these mouse tumors often overexpressed *Ink4a/Arf* repressors such as Tbx2 or Pokemon (76) to inactivate the Rb and p53 pathways.

The low incidence of *p53* or *Ink4a/Arf* mutation in *MMTV-neuNT* tumors does not mean that p53 or Ink4a/Arf has no role in HER2/neu-induced mammary tumorigenesis in mice. Indeed, it was reported that *MMTV-neu* (wild type); *WAP-p53–172H* double transgenic

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mice exhibited dramatically shortened survival than in *MMTV-neu* single-transgenic mice, with tumors showing anaplastic and aneuploid phenotypes indicating strong cooperativity of ErbB2 and mutant p53 in tumor development (165, 166). It was also reported that *MMTV-neu*; *Ink4a/Arf*$^{+/-}$ mammary tumors showed increased Ki67 expression, higher expression of cyclin D1, and decreased mammary tumor apoptosis as compared with those from *MMTV-neu* mice (167). Thus, tumors with inactivation of any components of the Dmtf1-Arf-p53 pathway lead to more aggressive phenotypes than those without involvement of the pathway in mice. In human BCs, phenotypic comparison of tumors with or without the involvement of the DMTF1-ARF-p53 pathway is very difficult since one of these components is almost always inactivated (51/66, 77 % in our study) in clinical samples (71).

Concluding remarks and future prospects

Among Myb-like proteins, c-Myb is by far the most intensively studied for their roles in signal transduction related to cell proliferation, apoptosis, and differentiation. The *c-MYB* genomic locus has been mapped to the breakpoint that is frequently translocated in human leukemia and solid tumors; hence the gene product is overexpressed in human cancers including hematopoietic malignancies and carcinoma. Since leukemic cells are often dependent on c-MYB for their growth, the gene has been a target for removal of leukemic cells from the bone marrow needed to autologous bone marrow transplantation for CML. Development of antisense ODNs to other leukemias than CML has not even been undertaken, esp. treatment of acute leukemias through continuous intravenous infusion of antisense ODNs. One major obstacle for antisense ODN-mediated cancer therapy is the stability of the ODNs *in vivo*. Recently morpholino-modified ODNs have shown outstanding stability *in vivo*, which should be studied further together with collaboration of several antisense ODNs that have anti leukemic or tumor cell activity.

Although these antisense approaches to *c-MYB* looked very promising in leukemia therapy, it was not pursued clinically to remove leukemic cells in patients. Instead, small molecules to inhibit c-MYB activity, e.g. by disrupting interactions between c-MYB and its co-regulator p300, has been was attempted to destabilizing the protein itself. Since the approaches look very promising for cancer therapy, clinical trials for small inhibitors celastrol and/or blumbagin should begin soon for future cancer therapy.

Recent studies show that *c-MYB* overexpression itself caused by translocation rather than creation of chimeric protein is essential for the pathogenesis of human cancer (150, 151). Indeed, juxtaposition of super-enhancer regions to the *c-MYB* locus has been identified as the unifying feature of ACC translocations. Whatever the mechanism is, c-MYB overexpression itself is the most important event in carcinogenesis than creation of fusion protein(s) by translocation, which can be targeted by immune therapy or small molecule inhibitors.

In contrast to c-MYB, the DMTF1 transcription factor is often (30–40 %) underexpressed in human cancers, due to specific LOH of the locus (67, 71). Both gene knockout and transgenic mouse models (62, 88; 168, 169) have been created for *DMTF1* to demonstrate the tumor suppressive and oncogenic functions of the splice variants. Interestingly LOH of

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h*DMTF1* has been reported to be mutually exclusive of those of the *INK4a/ARF* or the *p53* locus in lung and breast cancers, suggesting that *DMTF1*-loss a new disease category of cancer. Since the disease-free survival is longer in BC with LOH for h*DMTF1*, it is highly possible that tumors that overexpress hDMTF1 have *p53* mutation(s) associated with poor prognosis. Indeed, Dmp1 can bind to both wild type and mutant p53. Future research can thus focus on the role of hDMTF1 in tumors with p53 mutation(s).

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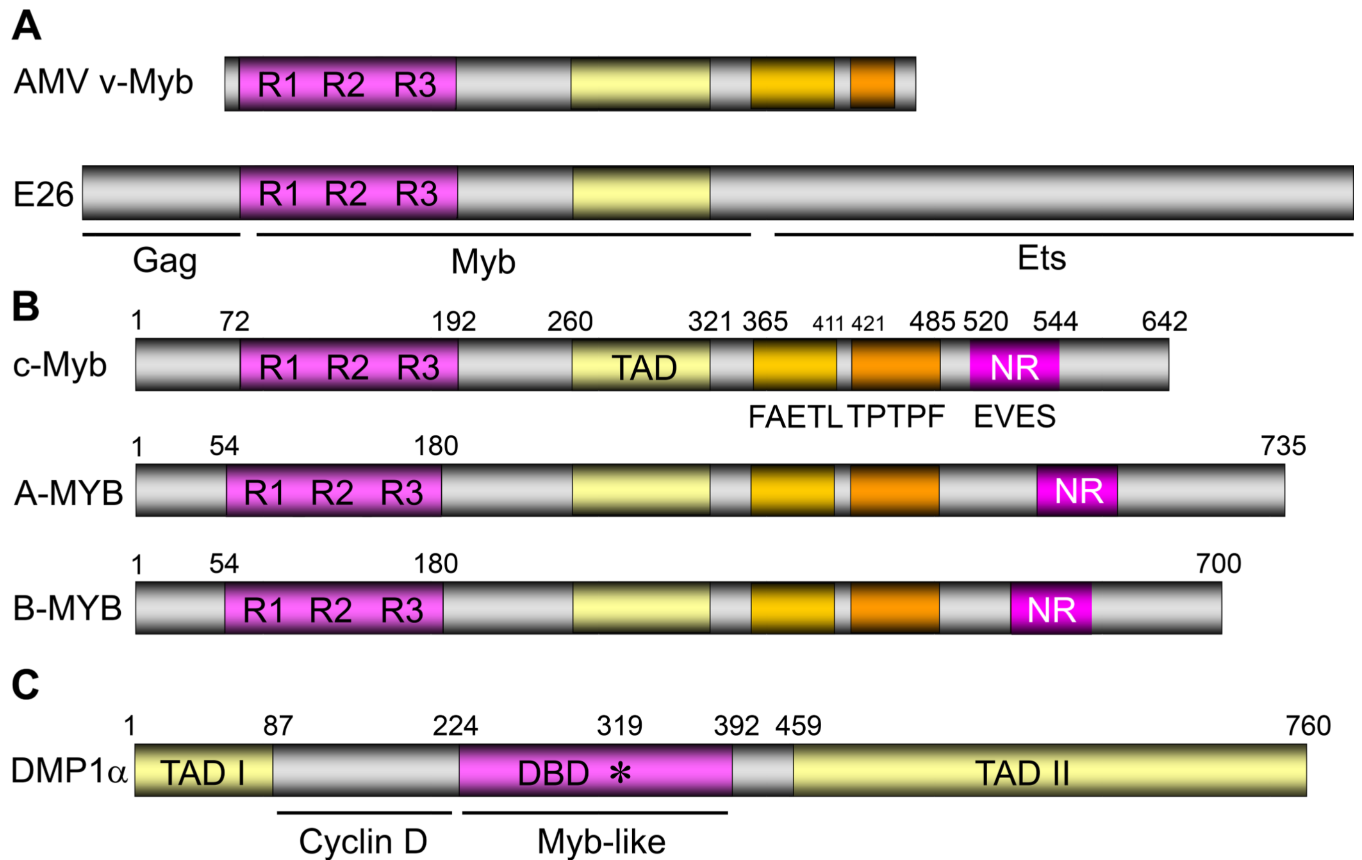

Figure 1. The domain structures of Myb-like proteins.

A. The structure of AMV and E26 proteins that encode v-Myb. R1–3 represents the three repeats within the Myb protein responsible for DNA binding. See refs. 1–3, 105–111.

B. The domain structures of A-Myb, B-Myb, and c-Myb (53, 170). TAD: transactivation domain; NR: negative regulatory domain. The "FAETL" domain (171) is required for oncogenic activity, the "TPTPF" domain conserved in the other Myb proteins, and the "EVES" domain (172) that is involved in intra-molecular interactions and negative regulation.

C. The domain structure of the human DMTF1α protein (58). DBD: DNA-binding domain. This protein has three tandem MYB-like repeats and two transactivation domains.

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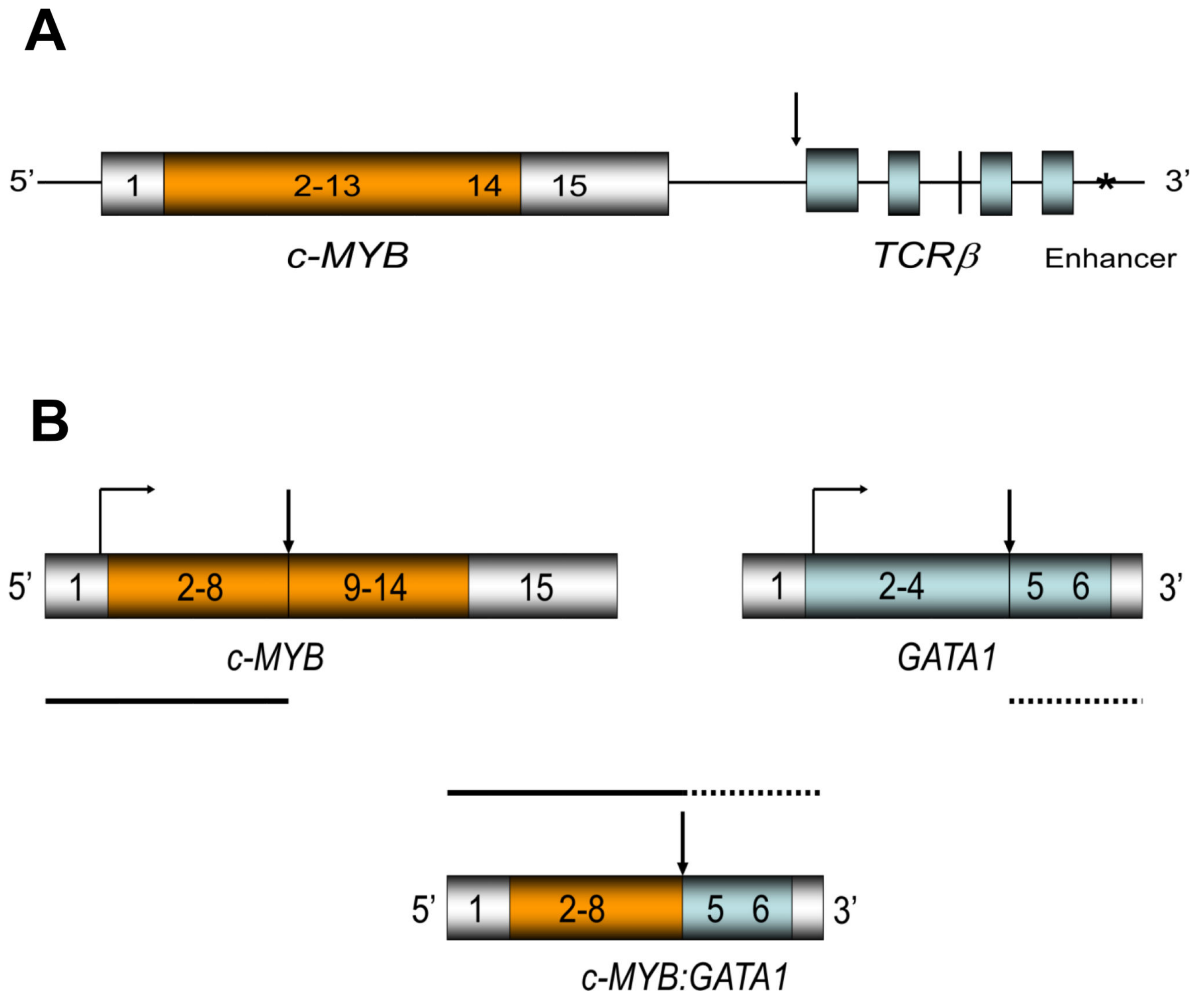

Figure 2. The genomic structures of translocations involving c-MYB.

A: The genomic structure for the chimeric *c-MYB-TCRβ* locus (8, 38, 40). The transcription of the entire *c-MYB* locus is enhanced by the telomeric enhancer for *TCRβ*.

B: The genomic structure for the *c-MYB*, *GATA1*, and *c-MYB:GATA1* chimeric loci (39, 40) implicated in human acute basophilic leukemia. The vertical arrows represent the breakpoint of translocation.

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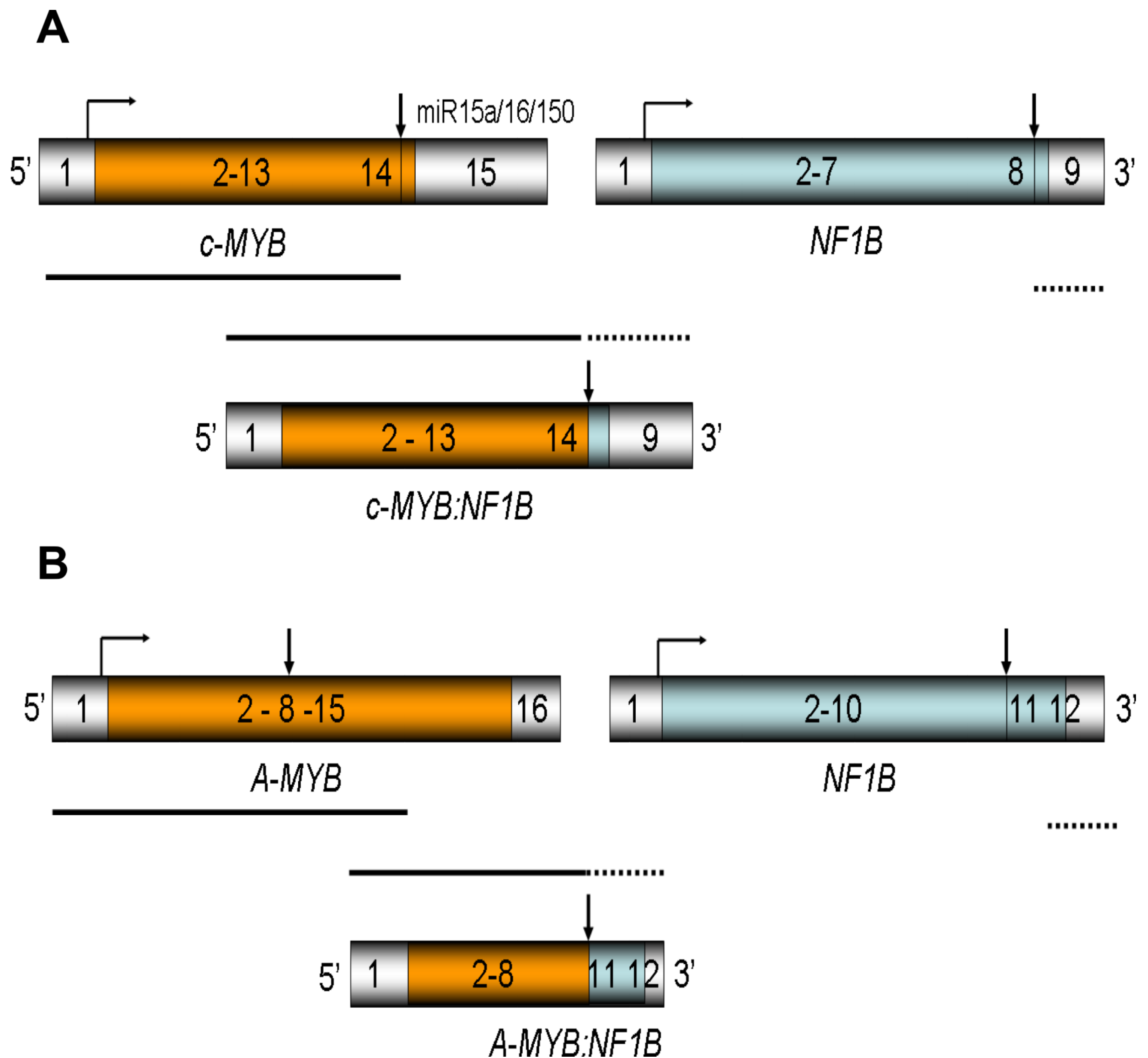

Figure 3. The genomic structures and protein products of translocations involving c-MYB and A-MYB in adenoid cystic carcinomas.

A. The genomic structure for the *c-MYB*, *NF1B*, and chimeric *c-MYB:NF1B* locus with t(6:9) (17, 41). The vertical arrows represent the breakpoint of translocation. This translocation has been found in adenoid cystic carcinomas in the breast, head and neck, vulva, lacrimal gland, and other organs regardless of the site of origin.

B. The genomic structure for the *A-MYB*, *NF1B*, and chimeric *A-MYB:NF1B* locus without t(6:9) (146).