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Myb proteins: angels and demons in normal and transformed cells

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Abstract

A key regulator of proliferation, differentiation and cell fate, the c-Myb transcription factor regulates the expression of hundreds of genes and is in turn regulated by numerous pathways and protein interactions. However, the most unique feature of c-Myb is that it can be converted into an oncogenic transforming protein through a few mutations that completely change its activity and specificity. The c-Myb protein is a myriad of interactions and activities rolled up in a protein that controls proliferation and differentiation in many different cell types. Here we discuss the background and recent progress that have led to a better understanding of this complex protein, and outline the questions that have yet to be answered.

Keywords

Myb; Oncogene; Stem Cells; Hematopoiesis; Transformation; Transcription; Post-Translational Modifications; Protein-Protein Interactions; SND1; cell cycle; SANT domain; Review

2. INTRODUCTION

2.1. Significance of c-Myb in human disease

The c-*myb* proto-oncogene (MYB) encodes the c-Myb transcription factor, and was originally identified as the cellular counterpart to the v-*myb* oncogenes found in the chicken leukemia viruses Avian Myeloblastosis Virus (AMV) and E26 (1, 2). (We will use c-*myb* and c-Myb to designate the normal gene and protein, respectively, and v-*myb* and v-Myb for the virus-encoded oncogenic forms.) The v-*myb* viruses are highly oncogenic and are capable of transforming immature hematopoietic cells in tissue culture and of inducing acute leukemias in animals. Because of the leukemia-inducing activities of the v-Myb proteins, the c-*myb* gene is often associated mostly with its functions in hematopoietic cells and with hematopoietic tumors. Indeed, in humans, amplification or rearrangement of c-*myb*, caused by chromosomal abnormalities in the region 6q22-24, were originally linked to human acute myelogenous leukemia (3). More recently, deregulated or over-expressed c-*myb* has been detected in a wide range of human cancers and is associated with poorly differentiated tumors in many cell types, including breast cancers (4-6), colon tumors (7-10), pancreatic tumors (11), glioblastomas (12, 13), melanomas (14, 15), head and neck tumors (6) and a variety of leukemias (3, 16-19). 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 and that it is an important human oncogene. However, it has been difficult to obtain direct evidence that c-Myb plays a role in human tumors, in part because the expression of the c-

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myb gene is correlated with proliferation, so it is generally higher in dividing tumor cells than in resting normal cells (8, 15, 20). Despite the many kinds of evidence linking activation of c-*myb* to human tumors, the mechanisms involved in regulating the c-Myb protein and the functions of c-Myb in normal and tumor cells remain the focus of intense research.

Recently, two types of evidence have provided strength to the argument that c-*myb* is a bona fide human oncogene. The first was the finding that the c-*myb* gene is frequently involved in duplications or in genomic duplications in two subsets of pediatric T-cell acute lymphocytic leukemias (T-ALL). In patients harboring the reciprocal translocation t(6;7)(q23;q34) the c-*myb* gene became juxtaposed to the TCRB gene, encoding the T-cell receptor beta chain, leading to c-*myb* over-expression. Another set of patients, identified through comparative genomic hybridization, harbored duplications of the c-*myb* gene. The second type of evidence was the discovery of a recurrent fusion between the c-*myb* and NFIB genes in translocations t(6;9)(q22-23;p23-24), which occurs in adenoid cystic carcinomas of the breast and head and neck. 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 NFIB 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 over-expressed c-Myb in the tumors. These discoveries that the human c-*myb* gene is involved in recurrent translocations in human tumors provide clear evidence that c-*myb* is a bona fide human oncogene.

2.2. Features that make c-Myb unique

Several things make c-*myb* a unique type of oncogene and an unusual transcription factor. Unlike some oncogenes whose normal functions are closely linked to the regulation of the cell cycle or to the mitogenic response (e.g. SRC, MYC, FOS), the normal role of c-*myb* is most closely associated with the regulation of differentiation, especially in immature hematopoietic cells (21, 22). Transformation by the v-*myb* oncogenes is associated with a block in differentiation (23, 24) and disrupting the function of v-*myb* in transformed cells causes them to stop proliferating and to differentiate (25). This raises the question of whether c-Myb regulates both differentiation and proliferation, or whether changes in proliferation occur as a consequence of Myb-regulated changes in differentiation. For example, the more immature transformed cells may respond more vigorously to cytokines or growth factors, resulting in increased proliferation. Alternatively, c-Myb could be involved in the regulation of both proliferation and differentiation. Another unique feature of c-Myb, which may be the key to understanding how it functions as an oncogene, is that its activity is malleable: it regulates different genes in different situations and its activity can be dramatically altered by relatively minor mutations, even single amino acid changes (26, 27). Thus, c-Myb appears to have many different functions and many different roles in various tissue types and situations, and mutations or mechanisms that disrupt these context-specific effects may increase oncogenic activity.

One of the most unique features of c-*myb* is the extremely complex nature of its regulation. The expression of the c-*myb* gene is regulated at several levels, including a sophisticated control of transcriptional elongation that occurs in the first exon and that may be regulated by the transcription factor NFkappaB or by estrogen receptor, depending on the cell type (5, 28, 29). The c-*myb* gene exhibits quite complicated alternative RNA splicing that can produce dozens of different transcripts encoding different versions of c-Myb protein with unique transcriptional activities (19). This makes the c-*myb* gene multifaceted and capable of encoding subtly different transcription factors in different situations or cell types. At the post-transcriptional level the c-*myb* mRNAs are tightly controlled by a system of microRNAs, mostly that bind to the very long 3'-UTR region (30-34). However, the most

sophisticated and least understood regulation occurs after translation. The c-Myb protein is subject to at least 10 and probably many more post-translational modifications, all of which have the ability to change the activity or stability of the protein or to affect which proteins c-Myb is able to interact with and therefore which genes it can regulate. Thus, the c-*myb* gene and the c-Myb protein lie at the intersection of many different regulatory pathways feeding into such diverse mechanisms as the control of transcription, alternative RNA splicing, translation and protein activity and stability.

2.3. Questions to be addressed

It has been over 30 years since v-*myb* was first discovered in a virus that caused leukemia in chickens. The diverse studies and approaches undertaken by numerous laboratories have uncovered a myriad of complexities related to the expression and regulation of c-Myb, but several important questions remain unanswered. It is still not clear how c-Myb regulates both differentiation and proliferation, two processes that seem contradictory. Although c-Myb is a DNA-binding transcription factor (26), it is still not clear which Myb-regulated target genes are the most important ones for the oncogenic activity of v-Myb, or whether Myb proteins regulate different genes in tumors than in normal cells. Although the turnover of c-Myb protein is tightly regulated, whether higher intracellular levels of c-Myb protein lead to a different outcome in terms of gene expression patterns, changes in differentiation or increased proliferation remains to be determined. The goal of this review is to address these and related questions in order to shed light on the most important and most relevant discoveries, and to set a framework for future studies on c-Myb and its role in normal cells and tumors.

3. THE TWO FACES OF MYB: A REGULATOR AND AN ONCOGENE

3.1. Myb as a critical regulator in normal cells

Like many transcription factors, c-Myb has a highly conserved DNA binding domain (Figure 1A) (35), recognizes and binds to specific sequences in DNA (26) and activates the expression of specific target genes (26, 36, 37). The unique feature of c-Myb is that a few mutations can convert it into a transforming protein that regulates a completely different spectrum of target genes (36). Trying to understand the normal functions of c-Myb and how they become corrupted in the oncogenic v-Myb has been the motivation behind many of the studies involving the Myb proteins.

Because the viruses expressing v-Myb caused leukemias, much of the work involving c-Myb has focused on its functions in hematopoietic cells. Early studies showed that anti-sense oligonucleotides targeting c-Myb could block *in vitro* hematopoietic cell differentiation (21) and that homozygous disruption of the murine c-*myb* gene causes a dramatic failure of definitive erythro- and myelopoiesis (22). Since then, c-Myb has been intensively investigated in the context of the hematopoietic system where it has been shown to regulate progenitor cell expansion and differentiation of a number of lineages. A variety of mutant alleles and conditional knockout mouse models have facilitated the study of c-Myb in hematopoiesis. For example, inducible c-*myb* knockout systems have shown that precise expression levels of c-Myb are required at distinct differentiation steps of each hematopoietic cell lineage (38). Tissue-specific deletion of c-Myb in lymphoid progenitors has demonstrated the importance of c-Myb expression during B-cell (39) and T-cell development (40, 41). In addition, low levels of c-*myb* expression lead to a myeloproliferative phenotype resembling a stem cell disorder (42), suggesting that c-Myb is required for proper differentiation and proliferation of hematopoietic stem cells.

Presumably, c-Myb is required in these diverse cell types because it regulates specific genes. Indeed, c-Myb appears to regulate some genes, such as c-Kit (43-46) in many cell types

while other genes, such as Bcl-2 (47, 48), may only be targets in specific lineages. Many other c-Myb targets have been identified in hematopoietic cells, including the first identified target *mim-1* (26), the stem cell antigen CD34 (49), the T-cell receptor delta (50), the cell cycle regulatory genes CDC2 (51) and CCNB1 (52) and the MYC oncogene (53). Although many of these target genes play important roles in hematopoiesis or even in oncogenesis, none of the genes that have been identified have provided more than a partial explanation for what the role of c-Myb is in hematopoietic or other cell types, nor have they led to a good explanation for why c-Myb should have oncogenic activity.

Recent studies have also demonstrated an important need for c-Myb expression in the normal development of some epithelial and other tissues. *In situ* hybridization studies have shown that c-*myb* is expressed at high levels in hematopoietic cells in developing embryos, but also in neural retina and in respiratory epithelial cells (54). In the colon, c-Myb is required for colonic crypt homeostasis, including the integrity, normal differentiation, and steady-state proliferation of colon epithelial stem cells and progenitors (48, 55, 56). Interestingly, c-Myb expression has been found to be important for neural progenitor cell proliferation and for maintenance of neural stem cell niche (57), and an involvement of c-Myb in melanocytes links it to the functions of neural crest cells (44). Clearly, the c-*myb* gene is widely expressed, and the c-Myb protein plays a role in many cell types, not just hematopoietic cells. As conditional knockout systems are expanded to other cell lineages, it seems likely that the number of tissues that require c-Myb for normal development will increase.

One complication of determining the role of the c-Myb protein in normal tissues is the presence of two related transcription factors, A-Myb (MYBL1) and B-Myb (MYBL2), which are often co-expressed with c-Myb. The three Myb proteins have nearly identical DNA binding domains (58) and bind the same DNA sequences *in vitro* and in reporter plasmids (59). However, they are biologically distinct, since mice lacking A-Myb or B-Myb have completely different phenotypes than those lacking c-Myb (22, 60, 61). The genes encoding the three Myb proteins are all induced by activating the estrogen receptor in MCF-7 breast cancer cells (62), making this system a good one for studying the activities of all three proteins. Microarray studies have shown that the three Myb proteins induce almost totally non-overlapping sets of target genes when ectopically over-expressed in MCF-7 cells. This suggests that the similar DNA binding domains are not enough to determine the specificities of the Myb proteins. Instead, the large transcriptional trans-activation and protein-protein interaction domains (Figure 1A) must play a role by helping to steer each of the transcription factors to specific target genes. Thus, it seems likely that the products of the three genes have completely different functions, although they likely evolved from a single progenitor Myb gene similar to the one in Drosophila (63), which is involved in chromosome condensation and genome stability (64, 65).

3.2. Myb unveiled becomes an oncogene

The AMV and E26 viruses expressing different versions of v-Myb are both strongly oncogenic, capable of transforming immature avian hematopoietic cells *in vitro* and inducing leukemias in animals (24, 27, 66, 67). In addition, both forms of v-Myb can induce leukemias in mice when expressed from mouse retroviruses (68, 69). However, both v-Myb proteins are truncated and mutated derivatives of wild type c-Myb (Figure 1A), lacking the N-terminus and C-terminus of c-Myb while retaining the highly conserved DNA binding domain and more than half of the C-terminal domain, including the region required for transcriptional transactivation (70). In contrast to v-Myb, full-length c-Myb is only capable of weak transformation *in vitro* (71, 72) and does not induce leukemias in animals, not even in transgenic mice that over-express c-Myb in all tissues (73). However, C-terminal deletion mutants of c-Myb are much more potent oncogenes and do induce leukemias in mice (74,

75). In addition, leukemias induced by retroviral insertions into the c-*myb* gene express either N- or C-terminal truncations of c-Myb (76, 77), suggesting that truncation of one end or the other of c-Myb is necessary for conversion into a potent oncogenic form. These results lead to the conclusion that c-Myb is subject to auto-inhibition or repression, which can be relieved by deletion of one end or the other, or both.

Researchers studying the differences between v-Myb and c-Myb have focused on the idea that the two proteins have quantitatively different activities: that they have essentially the same activities but the v-Myb protein is de-repressed, lacking the negative controls that keep c-Myb in check. However, this viewpoint now appears to be incorrect, since several types of evidence point to a qualitative difference in the activities of c-Myb and v-Myb. The first hint should have come from the study of the first identified Myb target gene, *mim-1*, which is activated by c-Myb and the v-Myb protein from E26 virus, but not the v-Myb encoded by AMV (26, 27). Instead, researchers focused on the fact that all the Myb proteins can bind the same DNA sequences and activate the same reporter gene plasmids as evidence that they must regulate the same genes. It is now clear that the results from reporter genes are misleading and fail to distinguish between the different Myb proteins. However, microarray assays clearly show that the different Myb proteins activate completely different sets of target genes (36, 37, 78), suggesting that they are qualitatively and functionally distinct. These conclusions have important implications for our understanding of how Myb proteins work and what controls their specificities. If the DNA binding domains of the Myb proteins are not sufficient to direct them to target genes, then protein-protein interactions become much more important and explain why the different Myb proteins have such different activities. However, protein-protein interactions are subject to regulation by many mechanisms, including post-translational modifications, suggesting that not only the activities, but also the specificities of Myb proteins are highly regulated.

3.3. N-terminal deletions of c-Myb

The c-*myb* gene is organized into 15 exons, with exons 4-6 encoding the DNA binding domain (Figure 1B). Variants of c-Myb lacking the N-terminus can arise through several mechanisms, including retrovirus insertions or gene rearrangements. N-terminal truncation appears sufficient to unleash at least some of the transformation potential of c-Myb. Retroviral insertional mutagenesis that results in a truncation of amino acids 1-20 of c-Myb has been found in non-bursal B cell lymphomas caused by ALV (avian leukosis virus) in chickens (79). This deletion occurs in recombinant retrovirus-induced rapid-onset tumors, including B-cell lymphomas, sarcomas, and adenocarcinomas in animals (80), suggesting that the N-terminal deletion is important for increasing the oncogenic activity of c-Myb. The precise role of the N-terminus of c-Myb is unclear, but it has been reported that deletion of the N-terminus can abrogate the ability of c-Myb protein to cooperate with other transcription factors, such as Ets-1 (81), which raises the possibility that oncogenic activation of c-Myb is linked to the loss of cooperation between Myb and other cofactors. Truncation of the N-terminus and the first Myb repeat can cause a decreased affinity for DNA binding and affects interactions between c-Myb and Cyclin D1 (82, 83). Moreover, mutant forms of c-Myb with different N-terminal deletions display differences in transcriptional transactivation activities and the ability to interact with C/EBPbeta (84). All the N-terminal deletions lack a conserved casein kinase II (CKII) phosphorylation site that may influence DNA binding activity (85). However, mutagenesis of the CKII site does not influence the oncogenic activity of c-Myb (82), suggesting that its importance may be limited to specific target genes or cell types.

There is provocative evidence suggesting that modifications of the N-terminus of c-Myb protein may occur in vertebrates through unusual transcriptional mechanisms. The first type of evidence is the identification of a second promoter in the c-*myb* gene, located in the

normal intron 1, which would encode an mRNA lacking the normal exon 1 and a protein with a small N-terminal deletion lacking the CKII phosphorylation sites (86). The implication is that alternative use of the second promoter in some cell types or in some circumstances would produce a subset of c-Myb proteins lacking the N-terminus and whatever negative regulatory controls it confers. The second type of evidence suggests that some c-*myb* transcripts arise via a mechanism of trans-splicing, starting at a completely different promoter located on a different chromosome from the rest of the c-*myb* gene, but resulting in the production of a chimeric transcript encoding a protein that lacks the normal N-terminus of c-Myb (87, 88). This line of evidence originated with the cloning and characterization of one of the first cDNAs for c-*myb*, which contained sequences from two different chromosomes (89). Although the evidence for trans-splicing is compelling, it is not clear how important that mechanism is in the biology of c-*myb* or whether proteins encoded by the trans-spliced chimeric mRNAs have any role in controlling cell fate or in regulating specific sets of genes. However, given the complexity of c-*myb* gene regulation, it would be foolish to rule out trans-splicing just because it is unexpected without proof that it is not important.

3.4. C-terminal deletions affect intra- and intermolecular interactions

All the transforming alleles of c-Myb, including both forms of v-Myb, have C-terminal deletions, suggesting that elements at or near the C-terminus play an important negative regulatory role, perhaps by keeping the activity of the full length protein in check. The fact that overexpression of full-length c-Myb cannot efficiently transform cells *in vitro* or induce tumors in animals suggests that negative regulation of c-Myb is inherent in its structure. In support of this idea, the EVES motif (amino acids 513-563) near the C-terminus of c-Myb (Figure 1A) has been shown to interact with the N-terminal DNA binding domain (90, 91). This intramolecular interaction could form the basis for autoinhibition, explaining why c-Myb fails to transform cells, even if grossly over-expressed. The EVES motif overlaps a site of *in vivo* phosphorylation by p42 Mitogen-Activated Protein Kinase (p42MAPK) (92) and is close to several sites of sumoylation and ubiquitinylation. Thus, activation of signal transduction cascades, leading to changes in p42MAPK activity and c-Myb phosphorylation could affect the intramolecular interactions and transcriptional activity and/or stability of c-Myb (93, 94). Although the potential for these regulatory mechanisms has been demonstrated, a direct link between signaling pathways, p42MAPK activity and changes in the transcriptional or oncogenic activity of c-Myb has yet to be demonstrated.

Recognition of the EVES motif in c-Myb led to the identification of a related domain in the transcriptional co-activator p100, also known as Tudor-SN and recently renamed SND1 (95). The p100/SND1 protein appears to have multiple functions: it is a transcriptional co-activator (96, 97), a component of the RISC complex involved in microRNA metabolism (98, 99) and a key component of RNA editing complexes (100, 101). For c-Myb, the implication is that intramolecular interactions within the c-Myb protein compete for the intermolecular interactions with p100/SND1 (90), which could also affect the interactions between c-Myb and Pim-1 (102), an oncogenic kinase that phosphorylates the DNA binding domain of c-Myb (103). C-terminal truncations of c-Myb could disrupt the intramolecular interaction, allowing other domains of c-Myb to recruit co-activators or co-repressors, leading to changes in transcriptional activity. Three co-repressors (Ski, N-CoR, and mSin3A) that bind to the DNA-binding domain of c-Myb form a complex with TIF1beta that binds to the C-terminus (104). Deletion of the C-terminus decreases the interactions with these co-repressor complexes and also weakens the co-repressor-induced negative regulation of Myb activity. Meanwhile, the deletion of the C-terminus could also unmask binding sites for other proteins in the DNA-binding domain or N-terminal domain, such as CBP/p300 (105). These regulatory mechanisms are likely dependent on enzymes that bind c-

Myb and catalyze changes in protein conformation. Examples include the peptidyl proline isomerases Cyp40, which binds c-Myb and induces a conformational change that affects DNA binding activity (106) and Pin1, which binds c-Myb in a phosphorylation-dependent manner (94). All these results paint an increasingly complex picture of the interdependence of each domain of c-Myb and the interplay of multiple cofactors in modulating its activity, and suggest that multiple different regulatory pathways interface with c-Myb to control its activity.

Deletion of the C-terminus of c-Myb plays a major role in changing the stability of the protein. Full-length c-Myb has a short half-life in most situations (107), which is greatly extended in proteins with C-terminal truncations. Some aspects of c-Myb activity are closely tied to the level of its expression (38, 42), so controlling the stability of the protein could play a critical role in regulating c-Myb activity and cell fate in some situations. The regulation of c-Myb degradation is linked to the presence of a PEST sequence (108), which targets the protein for ubiquitinylation and degradation. The PEST sequence overlaps with the EVES motif and the p42MAPK phosphorylation site, suggesting that the mechanisms that target c-Myb to the proteasome are highly regulated by upstream signaling pathways.

3.5. Wnt signaling and regulation of c-Myb stability

The carboxy terminal domain of c-Myb that is deleted in v-Myb plays a major role in negative regulation of c-Myb activity. Retrovirus-induced truncation of the C-terminus of c-Myb leads to a substantial increase in transactivation (70, 109, 110) and transformation activity (109). Several types of post-translational modifications occur in the C-terminal domain of c-Myb, affecting stability, subcellular location (111) or interactions with other proteins. For instance, activation of the Wnt-1 signaling pathway leads to phosphorylation of c-Myb at multiple sites, followed by ubiquitinylation, targeting to the proteasome and degradation (112). The phosphorylation sites in the C-terminal domain are more critical for the Wnt-1-induced degradation since the interaction of c-Myb with E3 ubiquitin ligases, such as Fbxw7 (113) can be enhanced by these modifications. The oncogenic v-Myb protein lacks the phosphorylation and ubiquitinylation sites and is relatively resistant to this degradation pathway (Figure 2), which may partially explain the differential transforming activity of v-Myb vs c-Myb (114).

Although the number of proteins known to interact with c-Myb increases each year, there is still relatively little information about how any of these interactions affect the activity of c-Myb or whether the interactions are significant for oncogenic activities. These limitations are mostly due to problems with the available assays. A major problem is that most investigators assess the activity of c-Myb only through transfection assays using plasmid-based reporter genes. However, it has been clear since the identification of the very first c-Myb target gene, *mim-1*, that reporter genes do not distinguish between the activities of different Myb proteins or between normal and oncogenic alleles of c-Myb (26, 27). Microarray studies have shown that c-Myb and v-Myb activate completely different, almost completely non-overlapping sets of target genes in human cells (36, 78). It will most likely be necessary to study the effects of the Myb-binding proteins in the context of real target genes in order to determine how they affect c-Myb activity. A related problem is that it is not yet clear which Myb target genes are linked to oncogenic activity. Some target genes, such as cyclin genes (CCNA1, CCNE1, CCNB1), other protooncogenes (c-MYC and c-KIT) or survival genes (BCL-2) seem likely to be important for transformation or oncogenesis. However, no target genes have yet been linked directly to the transformation activity of c-Myb or its oncogenic derivatives.

3.6. Structure and activities of the Myb DNA binding (SANT) domain

The 48 KDa v-Myb protein encoded by AMV, the 135 KDa Gag-Myb-Ets protein encoded by the E26 virus, and the 75 KDa c-Myb protein are all nuclear and bind to the same DNA sequence (PyAACG/TG) (115). All three share a highly conserved DNA binding domain composed of two approximately 50 amino acid long “Myb repeats”, each of which includes three tryptophan residues separated by a characteristic spacing (116). This motif is present in three tandem repeats (R1, R2, and R3) in c-Myb, the first of which is deleted in the AMV and E26 oncoproteins (117, 118). The first repeat has been implicated in stabilizing the binding to DNA(119) but it is not required for DNA binding (120) and deletion of the first repeat increases the oncogenic activity of c-Myb (110). Thus, the core DNA binding domain includes only the second and third Myb repeats (residues 90-192) (120). Four amino acid substitutions are present within the DNA-binding domain of AMV v-Myb. These substitutions do not appear to alter the sequence specificity of DNA binding (115), but do affect the ability of the protein to transform different cell types, to regulate specific genes and to be regulated by other cellular proteins (27, 106, 121).

Although it is named for its ability to bind DNA, the most conserved part of c-Myb (58) is also important for a large number of protein-protein interactions. As discussed above, the DNA binding domain is the site of intramolecular interactions with the C-terminal EVES domain (90) and for intermolecular interactions with the structurally-related p100/SND1 co-activator protein (90, 102). The DNA binding domain is also the site of interactions with and phosphorylation by protein kinases including Protein Kinase A (122) and Pim-1 (103). The tetratricopeptide repeat domain of Cyp40 interacts with the DNA binding domain of c-Myb (106), as do the cell cycle regulator Cyclin D1 (83) and the transcriptional corepressors Ski, N-CoR and mSin3A (104). The solution structure of the Myb DNA binding domain (120) shows that both the structure and the outside surface of the domain – which interacts with other proteins – is perfectly conserved amongst vertebrate Myb proteins. Interestingly, several of the mutations in the oncogenic v-Myb protein change surface residues in the DNA binding domain, suggesting that they enhance the oncogenic activity by affecting interactions with regulators (122) or co-activators that change the specificity of the protein.

In addition to recognizing specific promoters and binding to numerous proteins, the DNA-binding domain of c-Myb is also involved in chromatin remodeling and oncogenic mutations can abolish this function (38, 123). This suggests that the DNA binding domain may play somewhat of a catalytic role, initiating changes in chromatin structure or histone modifications at regulated promoters. Recently the Myb repeat found in the Myb DNA binding domain was relabeled as a SANT domain, variants of which are found in several chromatin remodeling enzymes (123-125). SANT domains are the critical DNA-and chromatin recognition domains in regulators such as Swi3p, Ada2p, Rsc8p and TRF2 and are responsible for recognition of histone tails. Thus, the DNA binding/SANT domain of c-Myb may play an important role in epigenetic regulation of target genes or in the initiation of chromatin remodeling in genes that become activated or repressed.

3.7. The related proteins, A-Myb and B-Myb, are not oncogenic

In addition to c-Myb, all vertebrates also express two closely related proteins with similar overall structures and only a few differences to the surface residues of their DNA binding domains (58). They both have been shown to be transcription factors (126, 127), but neither has been shown to be an oncogene or to have transforming activity. B-Myb (MYBL2) is expressed in all proliferating cells and is required for cell proliferation in which it plays a role in cell cycle progression (128). B-Myb and c-Myb have some similar effects in hematopoietic cells (129), but they are regulated differently and are not interchangeable (130). Over-expressed or amplified B-myb has been reported in different types of human

malignancies (131, 132), but since its expression in linked to proliferation, overexpression of B-Myb may just signal that the tumor cells are dividing. It is suspected that B-Myb plays a role in human cancer, but there is still no direct evidence of its causative role. Although B-Myb recognizes the same DNA sequence as c-Myb (PyAACG/T/G), it regulates completely different sets of genes (37). B-Myb has been identified as a component of LINC (LIN complex), a protein complex involved in the regulation of genes that are expressed in the G2/M phase of the cell cycle (133). LINC is related to similar complexes dREAM and DRM from Drosophila and C. elegans (133), suggesting that this function of B-Myb is evolutionarily conserved and primordial. Thus, B-Myb appears to be the vertebrate equivalent of the original Myb protein, only one of which is found in lower organisms. Compared to BMyb, c-Myb appears to be a specialized version that has acquired oncogenic potential.

The other Myb family member, A-Myb (MYBL1), is the least well studied and remains the most enigmatic. In lymphoid cells, A-Myb expression is normally restricted to the proliferating B-cell centroblasts (134) and is not correlated with that of c-Myb and B-Myb (129). Human A-Myb is a strong transcriptional activator (135) and is therefore more similar to the oncogenic v-Myb than to c-Myb. Transgenic mice with over-expressed AMyb develop hyperplasia of the spleen and lymph nodes with over-expanded B lymphocyte populations (136), suggesting that A-Myb may contribute to hyperplasia by increasing the rate of B cell proliferation. A-Myb is over-expressed or deregulated in several subtypes of human B-cell neoplasia (137) and there is evidence that A-Myb regulates important target genes such as MYC (138). However, there is no direct evidence that A-Myb is oncogenic or that it transforms cells *in vitro* or in animals. Although many studies of A-Myb activity have focused on its role in hematopoietic cells, it is widely expressed and is often co-expressed with c-Myb and B-Myb. For example, the expression of all three Myb proteins is stimulated by estrogen in MCF-7 breast cancer cells (62).

Many types of proliferating cells co-express the A-Myb, B-Myb and c-Myb proteins, and since all three proteins can bind the same DNA sequences and activate the same plasmid-based reporter genes, there is a chance that the three proteins regulate the same genes, perhaps in different phases of the cell cycle. Alternatively, they might have different activities, for example one might activate a promoter that the other represses. However, microarray studies showed that the three Myb proteins activated essentially completely different sets of target genes in human cells (37). Furthermore, swapping the DNA binding domains between A-Myb and c-Myb did not alter their specificities, indicating that the other parts of the proteins, not the conserved DNA binding domains, were most important for determining which genes got regulated by the different Myb proteins. The best explanation is that the DNA binding domain is required for finding Myb binding sites, but the protein-protein interactions mediated by other domains are required for cooperative interactions necessary to stabilize the formation of transcription complexes. Thus, the other parts of the Myb protein determine which promoters get favored, and essentially determine the specificities and which genes get regulated. This has important implications for how the Myb proteins are regulated, since post-translational modifications and mutations can affect protein-protein interactions and therefore determine which target genes get activated in different situations.

3.8. Mutations change the transcriptional activities of c-Myb

Many studies have investigated the regions of the c-Myb and v-Myb proteins involved in transcriptional activation, mostly using standard plasmid-based reporter genes that give information about activity, but not specificity. Deletion studies originally identified the transcriptional activation domain (residues 241-325) of c-Myb as a conserved region downstream from the DNA binding domain (117, 118). However, other studies have shown

that this small central domain is not sufficient for transcriptional activation in the context of the native v-Myb protein (67). Rather, a series of adjacent subdomains are required for transcriptional activation by v-Myb, including a heptad leucine repeat or "leucine zipper" (LZ) and the conserved FAETL motif (139, 140). Substitution of specific leucines in the LZ with proline residues can activate the protein for both transcriptional regulation and for oncogenic transformation (141), suggesting that the LZ is involved in negative regulation of c-Myb. In contrast, mutations in this region decrease the activities of v-Myb (67, 140). The results suggest that the central, transactivation domain of c-Myb and v-Myb is a series of conserved motifs responsible for mediating interactions with co-activators such as p300/CBP (142-145) as well as other proteins whose functions are less well defined, such as p160 (146). It is likely that some of these interactions are more generic, affecting the transcriptional activity of c-Myb measured in reporter gene assays, while others are more specific and may only become evident when tested in a more biologically relevant context or with specific sets of target genes.

As described above, the C-terminal domain of c-Myb plays an important role in controlling which target genes are regulated in different situations. Deletions and domain swap experiments have demonstrated this for panels of human target genes (36), and this is the best explanation for the finding that A-Myb and c-Myb regulate such different sets of genes, despite having nearly identical DNA binding domains (37, 59). The C-terminal domain of c-Myb, which includes the transcriptional activation and LZ regions described above, contains numerous sites of post-translational modifications, including sites of acetylation (147, 148), phosphorylation (92, 112, 122, 149-151), sumoylation (93, 111, 152-154) and ubiquitinylation (107, 113, 155) and is the site of action of the proline isomerases Cyp40 and Pin1 that have dramatic effects on c-Myb activity. It seems likely that the activity and specificity of c-Myb is regulated by post-translational modifications, some of which could change in real time, such as during the cell cycle. Thus, c-Myb is likely to regulate different sets of target genes in different phases of the cell cycle. However, such time-dependent changes may not be detectable using the reporter gene assays or even the endogenous gene activation assays that have been used for most studies involving Myb proteins.

Overall, this section has described the activities of c-Myb and v-Myb, and how mutations can convert one into the other. The results from many laboratories and numerous different types of experiments suggest that the activities of c-Myb are context-specific and affected by numerous post-translational modifications. It seems likely that the mutations that distinguish v-Myb mimic a specific set of modifications in c-Myb, for example by freezing v-Myb into a conformation that stimulates proliferation. Alternatively, the v-Myb mutations may completely change the activity of c-Myb, converting it into something more sinister. If the latter is true, it would mean that v-Myb and c-Myb are as different in activities as A-Myb, B-Myb and c-Myb, and that they are, for all intents and purposes, completely different transcription factors. This is in contrast to the notion that v-Myb is merely a constitutively activated or de-repressed version of c-Myb. The possibility that the normal and necessary activities of c-Myb could be corrupted by mutations into something qualitatively different is one of the defining features of the Myb proteins, and sets it apart from other oncogenes and other transcription factors.

4. DOES MYB REGULATE DIFFERENTIATION, PROLIFERATION OR BOTH?

4.1. Reversible regulation of differentiation by v-Myb

In transformation assays, normal hematopoietic cells form small colonies in semi-solid medium, but eventually stop dividing and differentiate into mature myeloid, erythroid or lymphoid cells. When transformed by oncogenic v-Myb, the cells become fixed at an immature stage of differentiation and continue proliferating. As a result, they form large

colonies of immature dividing “blast-like” cells that can be isolated and expanded for weeks in tissue culture. However, transformation by v-Myb does not constitute a block in differentiation, but an acquired phenotype induced by the oncogenic transcription factor. This is evident from studies performed using a mutant v-Myb that is temperature-sensitive for DNA binding and for transformation (25, 156) and which transforms cells reversibly. Thus, the ts (temperature sensitive)-v-Myb transformed cells remain immature until shifted to the non-permissive temperature, which inactivates v-Myb and allows the cells to differentiate and stop proliferating. If shifted back to the lower temperature the cells de-differentiate, become immature again and resume proliferating (156). These results suggest that the active v-Myb induces a change in the transcription profile, which leads to proliferation and the adoption of an immature phenotype. When v-Myb is inactivated the cells resume their pre-programmed differentiation pathway and become mature, non-proliferating cells. Thus, v-Myb affects both differentiation and proliferation simultaneously. In contrast, cells transformed by the v-Myc oncogene differentiate almost completely, but retain their ability to proliferate, forming large colonies of mature monoblast-like cells (157, 158). The v-Myc oncogene stimulates proliferation without blocking or affecting differentiation. If v-Myc is introduced into cells that are transformed by the ts-v-Myb oncogene, the doubly-transformed cells become temperature-dependent for differentiation, since the ts-v-Myb controls their differentiation state, but continue proliferating at both temperatures, due to the activity of v-Myc (158). This highlights the differences between v-Myb and v-Myc, but also shows the unique nature of v-Myb and its complex functions, which affect both differentiation and proliferation.

4.2. Myb target genes and transforming activities are lineage- and differentiation-specific

The c-Myb protein is expressed in many cell types, including all proliferating hematopoietic cells and most types of proliferating epithelial cells (7, 8, 54, 159). However, evidence of transformation by the v-Myb proteins is restricted to hematopoietic (157) and some neural crest cells (44). Since Myb proteins are transcription factors, the oncogenic activity is presumably linked to the activation or repression of specific target genes. However, Myb proteins must cooperate with other transcription factors in order to regulate cell type-specific genes. The best example of this is activation of the *mim-1* gene, the first identified target gene for c-Myb (26), which is activated by the combinatorial effects of c-Myb plus C/EBPbeta (also called NF-M) that binds adjacent to c-Myb on the *mim-1* promoter (160, 161). Thus, c-Myb can only activate the *mim-1* gene in cells that already express C/EBPbeta. However, co-transfection of c-Myb plus C/EBPbeta into other cell types, such as fibroblasts, can lead to activation of the endogenous *mim-1* gene (161).

Interestingly, the two different v-Myb oncogenes encoded by the AMV and E26 leukemia viruses transform related, but quite distinct types of cells. Both types of transformed cells are immature myeloid cells and require the cytokine cMGF for growth and survival (162) although AMV-transformed cells produce some cMGF and are therefore less cytokine-dependent, due to autocrine stimulation (121). Only the E26 transformed cells express the *mim-1* target gene, which is activated by the E26 form of v-Myb and by c-Myb, but not by AMV v-Myb. The latter protein has three point mutations in its DNA binding domain that disrupt a protein interaction surface and prevent it from activating the *mim-1* gene (27). The two v-Myb proteins have quite different structures (Figure 1B). The AMV protein is truncated at both ends compared to c-Myb, and has 11 point mutations that contribute to its oncogenic potential (82, 121) and affect which target genes it regulates (36, 78). The E26 protein is fused to the retroviral Gag protein at the N-terminal end and a portion of another transcription factor, Ets1, at the C-terminal end. The E26 virus transforms more immature Myeloid Erythroid Progenitor (MEP) cells (163). Interestingly, the ability to transform these immature cells *in vitro* requires both the Myb and Ets proteins, but still works when they are

expressed *in trans* as separate proteins (164). However, the induction of leukemias in animals requires that the proteins be fused (165), suggesting that the fusion protein has unique activities, required for leukemia induction, that are not provided by the separate transcription factors. Indeed, there are even specific target genes that are only activated by the Myb-Ets fusion protein, and not by the two proteins expressed separately (166).

If transformation by v-Myb or mutants of c-Myb requires the activation of specific target genes, then combinatorial effects could explain why v-Myb only transforms hematopoietic cells and not other cell types. A number of c-Myb target genes have been identified that may be linked to transformation or leukemogenesis, including c-kit (43, 45, 167, 168), CD34+ (169) (170), GATA-1 (168) and Flt-3 (168, 171). All of these genes are expressed in hematopoietic stem/progenitor cells but not other cell types and could explain the restriction of v-Myb oncogenic activity to hematopoietic cells. The ability of Myb proteins to activate these genes likely depends on combinatorial interactions with other transcription factors, cofactors, or accessory proteins that influence Myb protein specificity and the genes that are regulated in each cell type (172). This again highlights the importance of protein-protein interactions in gene regulation by Myb proteins. Mutations in c-Myb that disrupt interactions with C/EBPbeta affect the ability to activate the *mim-1* gene (27) and other target genes (36), suggesting that protein-protein interactions are important for Myb protein specificity and for transcriptional activity. This opens up new possibilities for identifying possible drugs targeting specific interactions between Myb proteins and other cooperating factors linked to transformation, that might inhibit the activities of “bad” oncogenic Myb proteins in transformed cells, while leaving “good” c-Myb functional in essential normal cells.

4.3. Myb is a key regulator of stem cell fate

Several types of evidence indicate that c-Myb is a key regulator of proliferation and differentiation in stem cells, and that it plays a central role in the determination of cell fate. In the hematopoietic system, the earliest evidence for an important role of c-Myb came from experiments using antisense oligonucleotides that showed the importance of c-Myb in normal hematopoiesis (21, 173) and from the mouse knockouts showing that c-*myb* gene expression is required for definitive hematopoiesis in mammals (22). More recent studies have used conditional knockout systems to demonstrate that c-Myb is also critical for adult hematopoietic stem cells (174). These studies showed that c-Myb affects all the hematopoietic lineages, suggesting either that it functioned at the earliest stem cell stage or was required in all the lineage-specific progenitors, or both. Another type of early evidence came from transformation studies, which showed that even single amino acid changes in v-Myb led to the induction of completely different phenotypes in the transformed cells (27), suggesting that the oncogenic Myb protein was playing a decisive role by shifting or determining the extent and direction of differentiation along different pathways.

The importance of c-Myb in hematopoietic stem cells has been demonstrated in several different systems. The c-*myb* mRNA is highly expressed in pluripotent hematopoietic stem cells (175) and the products of c-Myb target genes c-Kit (176) and CD34 (169, 170, 177) are used as cell surface antigens to isolate hematopoietic stem cells and immature progenitors. In mice, two different mutagenesis studies designed to find genes involved in stem cell regulation identified mutations in c-*myb* (178, 179), suggesting that even minor changes in the c-Myb protein can have dramatic effects on the balance between differentiation and proliferation in stem cells. However, the absolute levels of c-Myb are also important, since mice engineered to express low levels of c-Myb displayed myeloproliferative defects resembling stem cell disorders (42). Very similar studies have been performed using the zebrafish model, where the c-*myb* gene is a key marker of hematopoietic stem cells (180) and imaging studies have been used to follow the development of c-*myb* positive hematopoietic stem cells (181). Thus, the importance of c-Myb expression for hematopoietic

stem cell proliferation and differentiation has been conserved throughout vertebrate evolution.

Although the role of c-Myb in hematopoietic stem cells has been best documented, several studies have shown that c-Myb plays an important role in stem cells from other tissues, including epithelial stem cells in colonic crypts (56) and neural stem cells in adult brain (57). Recent progress in the stem cell field has led to procedures for generating induced Pluripotent Stem (iPS) cells from adult fibroblasts, keratinocytes and other tissues (182, 183). One of the important inducer genes used for the generation of iPS cells, c-Myc, is a known target gene for c-Myb (53) and bioinformatics approaches have identified c-Myb as a likely regulator of pluripotency and "stemness" in iPS cells (184), but so far no direct tests of the role of c-Myb, or the effects of oncogenic variants such as v-Myb, on the formation of iPS cells have been reported.

4.4. Myb target genes may change during differentiation and the cell cycle

Several c-Myb target genes important for stem cells or early progenitors have been identified, including c-Kit (176) and CD34 (169, 170, 177), suggesting that c-Myb is required for the expression of genes that are important in stem cells and the earliest progenitors. However, c-Myb is also required for the expression of *mim-1* (26), myeloperoxidase (185) and other genes that are expressed in mature cells (186). Ironically, the mature cells express little or no c-Myb, which is primarily expressed in the immature proliferating cells. This suggests that c-Myb may be involved in the initial activation of genes that are kept active by other transcription factors in the mature cells. Although the mechanisms remain poorly defined, it is clear that c-Myb regulates different genes in the immature and more mature cells, another example of its context-specific regulation activity. As described in a previous section, c-Myb regulates genes in combinations with other transcription factors, and its targets change as the presence or absence of the other factors change during differentiation.

Recently, c-Myb was shown to regulate the expression of Cyclin B1, a cell cycle regulatory gene involved in G2/M transition (52). These results are important because they suggest a mechanism for c-Myb regulation of the cell cycle and proliferation. The c-Myb protein has also been shown to form complexes with Cyclin D1 (83) and with the cyclin-dependent kinases CDK4 and CDK6 (187), which also affect c-Myb transcriptional activity via a mechanism that involves p27Kip1 (187). These results raise the possibility that c-Myb activity and specificity could be regulated during the cell cycle. For example, c-Myb might regulate different sets of target genes in different cell cycle compartments. Cell cycle regulation could be controlled by phosphorylation or other post-translational modifications, or by the presence of absence of specific co-regulators, such as E2F or Rb. There is ample evidence that the related transcription factor BMyb is regulated by association with Rb and other cell cycle-regulated proteins (133, 188) and that B-Myb transcriptional activity can be regulated by Cyclin-dependent kinase phosphorylation (189, 190). However, direct evidence that c-Myb activity is regulated during the cell cycle is still unavailable.

5. MECHANISMS AFFECTING C-MYB ACTIVITY AND SPECIFICITY

5.1. Activation of the c-*myb* gene in tumors

The control of c-*myb* transcription is complex and can be affected by several different mechanisms. Overexpression or rearrangement of the c-*myb* gene has been reported in patients with head and neck carcinomas, breast cancer, melanoma and leukemias (14-16, 191), implicating activated alleles of c-*myb* in the development of human tumors. However, the c-*myb* gene promoter is GC rich, resembling a constitutively expressed promoter from a housekeeping gene (192), suggesting that expression of the gene is controlled through

unconventional mechanisms. Early studies in murine hematopoietic cells demonstrated a direct correlation between the relative abundance of c-*myb* mRNA and the level of transcriptional arrest in intron 1 (29, 193). Attenuation of transcription was linked to the rapid down-regulation of c-*myb* mRNA that occurs during the induced differentiation of human colorectal cancer (CRC) cell lines (9) and sequence analysis of primary CRC tumor samples revealed frequent mutations in a poly-T tract disrupting a proposed stem-loop motif in intron 1. The mutations reduced transcriptional attenuation and allowed greater gene expression, suggesting that disruption of the attenuation mechanism plays an important role in the elevated levels of c-*myb* mRNA in CRCs (10). In human breast tumors, c-*myb* was found to be over-expressed in estrogen receptor positive samples and run-on transcription assays showed that c-*myb* transcriptional elongation was directly regulated by estrogen/ER signaling (5). These results show that attenuation or control of elongation is an important mechanism for regulating the levels of c-*myb* in normal and tumor cells.

Elevated levels of c-*myb* mRNA can also be caused by genomic alterations, such as reciprocal translocations or genomic amplification. In human T-cell acute leukemia (T-ALL), recurrent chromosomal translocations t(6;7)(q23;q34) involving the TCRB and c-*myb* loci were identified (Figure 1B) (16). These translocations were reciprocal and balanced, and led to the juxtaposition of the c-*myb* proto-oncogene near the TCRB regulatory sequence, which suggested deregulated expression. Chromosomal translocations involving the c-*myb* gene were also reported in other types of human tumors: the t(6;9)(q22-23;p23-24) translocation in adenoid cystic carcinomas (ACC) of the breast and head and neck consistently resulted in fused MYB-NFIB transcripts predominantly consisting of MYB exon 14 linked to the last coding exon of NFIB (Figure 1B) (6). 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 (Figure 1B). Thus, the translocation appears to deregulate c-*myb* by removing the microRNA binding sites.

The human c-*myb* locus was recently found to be flanked by 257-bp Alu repeats that can mediate c-*myb* gene tandem duplication by homologous recombination between related elements on sister chromatids (17). This Alu-mediated c-*myb* tandem duplication could be one of the reasons for genomic duplication of c-*myb* gene frequently reported in human T-ALL (16, 18), MYST3-linked AMLs (194), BRCA1-mutated breast cancer (4) and some other types of cancer. Intriguingly, comparative studies on healthy individuals and patients with T-ALL showed that this tandem duplication also occurs spontaneously during normal thymocyte development and is clonally selected during the molecular pathogenesis of human T-ALL. So rearrangement or duplication of the c-*myb* gene may occur frequently, but is apparently not sufficient to induce leukemogenesis or transformation without other cooperating events.

5.2. Alternative RNA splicing as a novel mechanism for unleashing c-Myb oncogenicity

Alternative RNA splicing provides a mechanism for encoding multiple different transcripts from a single gene and represents an important molecular mechanism of gene regulation in physiological processes such as developmental programming as well as in disease. Recent studies using next-generation sequencing technologies have shown that up to 94% of human genes undergo alternative RNA splicing (195), suggesting that this mechanism is important for adding to the complexity of expressed transcripts. In cancers, splicing is significantly altered. Many cancer-associated splice variants arise from genes with an established role in oncogenesis, and their functions can be oncogenic. The c-*myb* gene contains 15 classical exons and 6 alternative exons (Figure 1B). It undergoes extensive alternative splicing producing dozens of different splice variants that encode many different isoforms of c-Myb protein (19). Alternative splicing in c-*myb* RNAs has been detected in both normal and tumor cells and in several species (196-200). Recent studies revealed an array of c-*myb*

variant transcripts, expressed in highly regulated, lineage-specific patterns, that are formed through the use of alternative exons 8A, 9A, 9B, 10A, 13A and 14A (Figure 3A) (19). All of these identified splice variants encode Myb proteins sharing the same DNA binding domain but having unique transcriptional activation and C-terminal domains. Several lines of evidence have shown that relatively minor changes in the transcriptional activation domain and/or negative regulatory domain of c-Myb can dramatically affect its transcriptional activity, raising the possibility that the variant c-Myb proteins encoded by these splice variants may display unique transcriptional activities. Indeed, the variant c-Myb proteins encoded as a result of alternative RNA splicing have quantitative and qualitative differences in their transcriptional activities (19).

Perhaps the best characterized of the alternatively spliced c-*myb* transcripts includes alternative exon 9B (previously named 9A) that encodes the p89 variant of c-Myb with a 121-amino-acid in-frame insertion in the transcriptional activation domain (Figure 3A) (201-203). Overexpression of this alternatively spliced isoform in avian hematopoietic cells resulted in increased transactivation and transformation activity (204). The murine homologue of this variant enhanced cell survival in hematopoietic cell assays in which wild-type c-Myb accelerated cell death (205). The 9B variant is conserved throughout vertebrates, suggesting that it plays an important role. However, a targeted knockout of the 9B/p89 variant had no apparent phenotype (206), so it is possible that the variant has specialized functions or that the loss of the variant might be compensated for by other forms of c-Myb.

Recent studies showed that the patterns and relative levels of c-*myb* splice variants changed dramatically in a lineage-specific manner during the *in vitro* differentiation of CD34+ cells (19), suggesting that the alternative splicing of c-*myb* transcripts is highly regulated in hematopoietic cell differentiation and that the ratios or the relative levels of alternatively spliced c-Myb variants play a role in lineage-specific functions of hematopoietic cells. Some alternative exons contain stop codons or cause a translation frame-shift, so the encoded variant proteins have C-terminal deletions, similar to the oncogenic v-Myb (Figure 3A) (19). Since the alternative splicing of c-*myb* transcripts produces a family of transcription factors, it is possible that aberrant or increased alternative splicing in tumors could lead to the production of truncated variants of c-Myb with transforming or leukemogenic activities. This would represent a novel mechanism of activation of an oncogene via alternative RNA splicing. Unfortunately, there is very little information about how alternative splicing is regulated, although some splicing factors have been shown to have oncogene like activities when over-expressed (207).

The alternative RNA splicing allows the c-*myb* gene to produce a family of different transcription factors with unique transcriptional activities. The model is that proteins with different C-terminal domains interact with different sets of co-factors or co-regulatory transcription factors, allowing them to regulate different target genes (Figure 3B). Since the different variants recognize different promoters, they may not even compete with each other, so that a variant representing a relatively small fraction of the total c-Myb protein could play an important role by regulating a different set of genes. This has important implications for the effects of alternative splicing, but also for post-translational modifications, either of which could generate a number of co-expressed subpopulations of c-Myb protein with different activities.

5.3 Regulation of c-Myb expression through microRNAs

MicroRNAs (miRs) are a large and growing class of ~22 nucleotide-long non-coding RNAs which function as repressors, negatively regulating the expression of their target genes, in all known animal and plant genomes (208) (209). In many cases, miRNAs bind to the 3'-UTRs of the mRNAs they regulate (209). The c-*myb* gene encodes an mRNA with an extensive 3'-

UTR that contains binding sites for several different microRNAs, and there are several examples of microRNAs that appear to regulate the turnover or translation of c-*myb* mRNA. For example, the microRNA miR-150, which binds the c-*myb* 3'-UTR, was found to be down regulated in granulocytes that over-expressed c-*myb* and that were isolated from patients with primary myelofibrosis (PMF)(210). The implication is that aberrant expression of miR-150 in the PMF patients led to over-expression of c-*myb* mRNA and subsequent defects in granulocyte differentiation. Similar studies have uncovered an important control circuit in which miR-150 plays a critical role in the regulation of c-*myb* during the development of B-cells (33) and other hematopoietic lineages (32, 211). The link between miR-150 and c-*myb* has even been conserved in zebrafish (31). In addition to miR-150, the c-*myb* 3'UTR also contains binding sites for several other microRNAs (30, 34, 212). These results indicate that deregulation of c-Myb expression by aberrant expression of microRNAs could be another mechanism of activating c-Myb in tumors.

Like the c-Myb protein, which is regulated by many different mechanisms, the c-*myb* gene is controlled at all possible levels, from regulation of the promoter, to control of transcriptional elongation, to alternative RNA splicing, to microRNAs that control the fate of the mRNAs. Each of these is a potential regulatory circuit that can respond to various extracellular cues or activated signaling pathways. Thus, the regulation of c-*myb* expression is a complicated and rich area of study, and offers many opportunities for potential intervention in the design of novel therapeutic approaches.

6. PERSPECTIVE

One of the most surprising and provocative results that has come out of studies of c-Myb and v-Myb is the finding that the activities of Myb proteins are variable and context-dependent and controlled by their interactions with other proteins. Thus, c-Myb regulates different target genes in different cell types, in immature vs. mature cells and perhaps even during different parts of the cell cycle. On the one hand, this realization seems obvious, since c-Myb is expressed in so many different cell types. However, there were expectations that c-Myb would be a "master regulator" of hematopoiesis or that it would have one or a few key targets that would explain its ability to become an oncogenic transforming protein. On the contrary, c-Myb has turned out to regulate hundreds of target genes, and to regulate different sets of genes in different cell types (36, 37, 78, 186). The oncogenic activity of v-Myb may be linked to its ability to regulate different genes than c-Myb, or to do so at different times or in different circumstances than c-Myb. And oncogenic activity may be a combined effect of activating dozens or hundreds of genes, rather than one key target. The other big surprise about c-Myb is the large number of proteins it interacts with, and the large number of post-translational modifications that affect it. Dozens of Myb-binding proteins have been identified, including cell cycle regulators, transcription factors, transcriptional co-activators and modifying enzymes. Although some of these interactions have been shown to affect the activity of c-Myb, it is not at all clear how all these potential interactions relate to one another. For example, do these interactions happen simultaneously in the same cell? Do they happen on the same c-Myb protein or do they define subpopulations of c-Myb with distinct protein partners and activities? It seems clear that the study of c-Myb needs to progress from the discovery phase and into a more systematic study of how the protein is regulated in a time- and context-dependent manner.

Despite more than 25 years of research, the question of what makes v-Myb a transforming protein remains unanswered. This is the central question, and the reason that studying c-Myb is significant and unique. We have learned that the v-Myb protein is lacking the C-terminal domains that cause c-Myb to be rapidly degraded and that promote its intramolecular auto-inhibition, that the v-Myb mutations affect protein-protein interactions and that v-Myb and

c-Myb regulate different sets of genes, but we do not understand why v-Myb is an oncogene, what genes it regulates in transformed or leukemic cells or how those genes contribute to oncogenesis. Answering these questions may require using new technologies, like genome-wide chromatin immunoprecipitation assays, in order to identify all the v-Myb targets in transformed cells. A related question is whether mutants or splice variants of c-Myb that are expressed in tumors can mimic the activity of v-Myb, which appears to be so different from that of c-Myb. It will be necessary to understand the transforming activity of v-Myb in order to answer these questions about c-Myb and derivatives of it.

Although the transforming potential of c-Myb is still not understood, it has become clear that the regulation of c-Myb is extremely complex and involves many different pathways and regulators. The regulation of c-Myb activity involves changes in c-*myb* gene expression, changes in alternative splicing, regulation by microRNAs and then a myriad of post-translational modifications and protein interactions. It seems that c-Myb plays the role of integrator, at the intersection of many different signaling and regulatory pathways. With so many variables affecting its expression and activity, it should be no surprise that the activities of c-Myb are also complex. Hopefully the application of new technologies will illuminate the role of Myb proteins in transformation and oncogenesis, provide an understanding of how post-translational modifications and protein-protein interactions control c-Myb and help explain its important roles in determining cell fate and controlling differentiation and proliferation.

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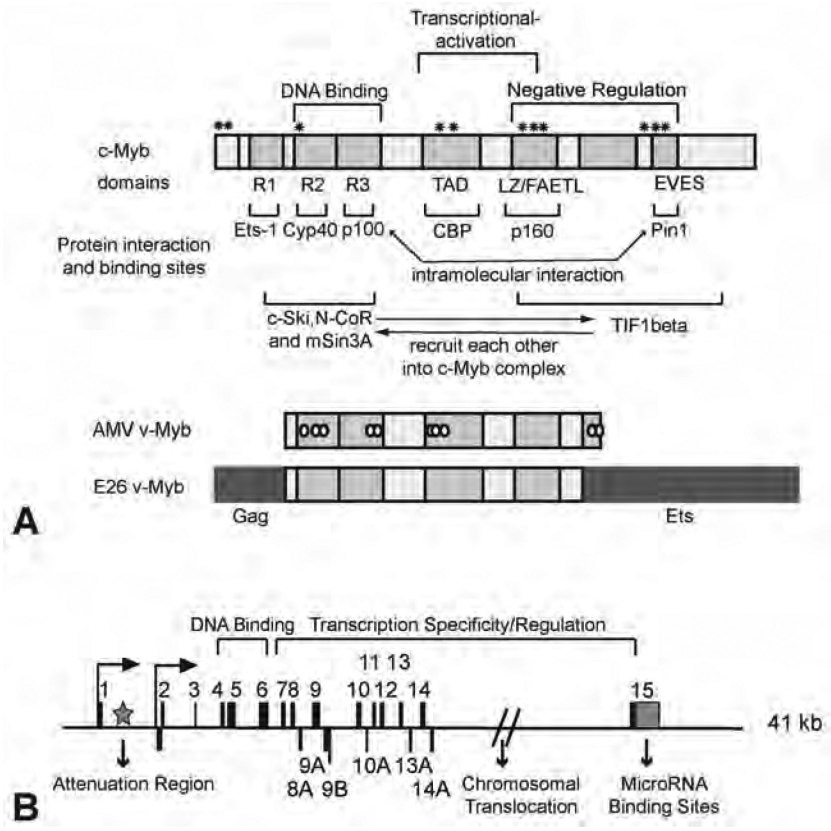

Figure 1.

Myb genomic and protein structure. (A) A diagram of the c-Myb protein structure shows the major functional domains including the highly conserved DNA binding domain (DBD) near the N-terminus, transcriptional trans-activation domain in the middle, and the C-terminal negative regulation domain. Shading indicates the most highly conserved portions of the protein and known sites of post-translational modifications are marked with asterisks. Structural details are labeled below the diagram. The DNA binding domain is comprised of Myb repeats R1, R2 and R3 and is also the binding site for a number of proteins including Ets-1, Cyp40, p100, c-Ski, N-CoR and mSin3A, as indicated. The central transactivation domain (TAD) is the interaction site for p300/CBP. The negative regulatory domain (NRD) extends from the LZ/FAETL region to the EVES motif and includes the binding sites for p160, Pin1 and TIF1beta. Arrows indicate potential intramolecular interactions. The lower section shows the structures of the AMV v-Myb and E26 Gag-Myb-Ets proteins. Compared to c-Myb, both have N- and C-terminal truncations. AMV v-Myb also has 11 point mutations (empty circles) that change its activity. (B) The c-*myb* gene contains 15 core exons (black boxes above the line) and 6 alternative exons (under the line). Important features of the gene are labeled (from left to right): The two promoters (bent arrows) upstream of exons 1 or 2, the translation attenuation site in intron 1 (star), a frequent site of chromosomal translocation in tumors (//) and the 3' untranslated region encoded by exon 15 containing microRNA binding sites.

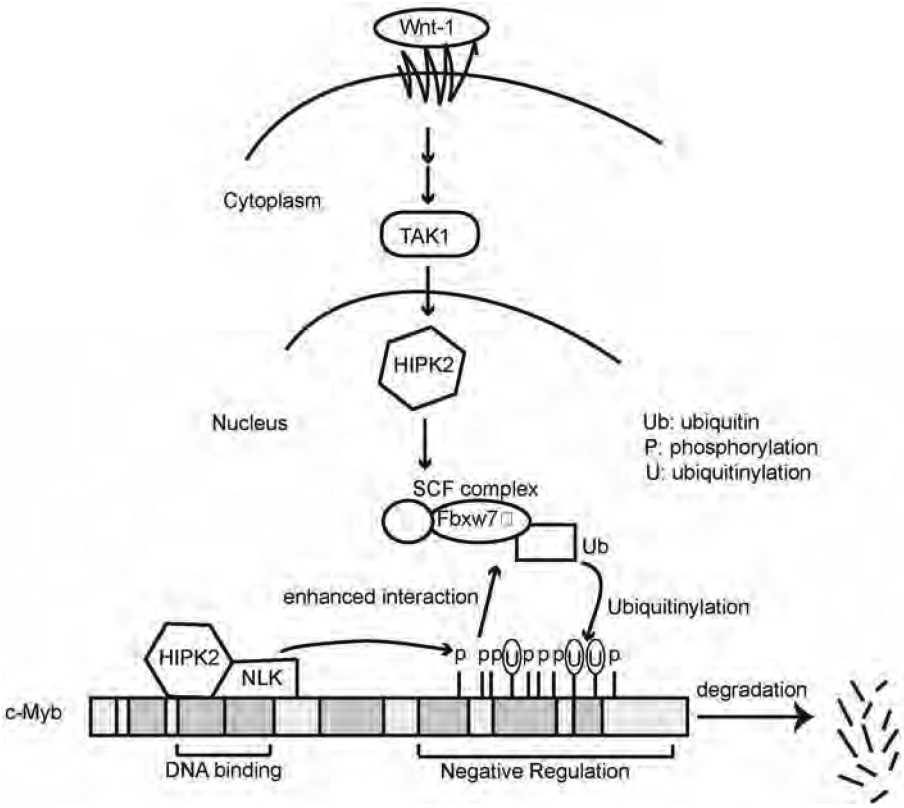

Figure 2.

Wnt signaling affects c-Myb stability and activity. c-Myb protein is phosphorylated and degraded by Wnt-1 signal via the pathway involving TAK1, HIPK2 and NLK. Wnt-1 causes the nuclear entry of TAK1, which then activates HIPK2 and NLK. NLK directly binds to c-Myb DNA binding domain with HIPK2, resulting in the phosphorylation of c-Myb at multiple sites (indicated by P), which enhances the interaction of c-Myb with Fbw7, a ubiquitin ligase, and as well as the interaction with the SCF complex, leading to ubiquitinylation of c-Myb and degradation by the proteasome. Mutations in the DNA binding domain of v-Myb decrease the affinity for HIPK2, and the C-terminal truncation removes NLK phosphorylation and ubiquitinylation sites, helping v-Myb to evade this regulatory mechanism.

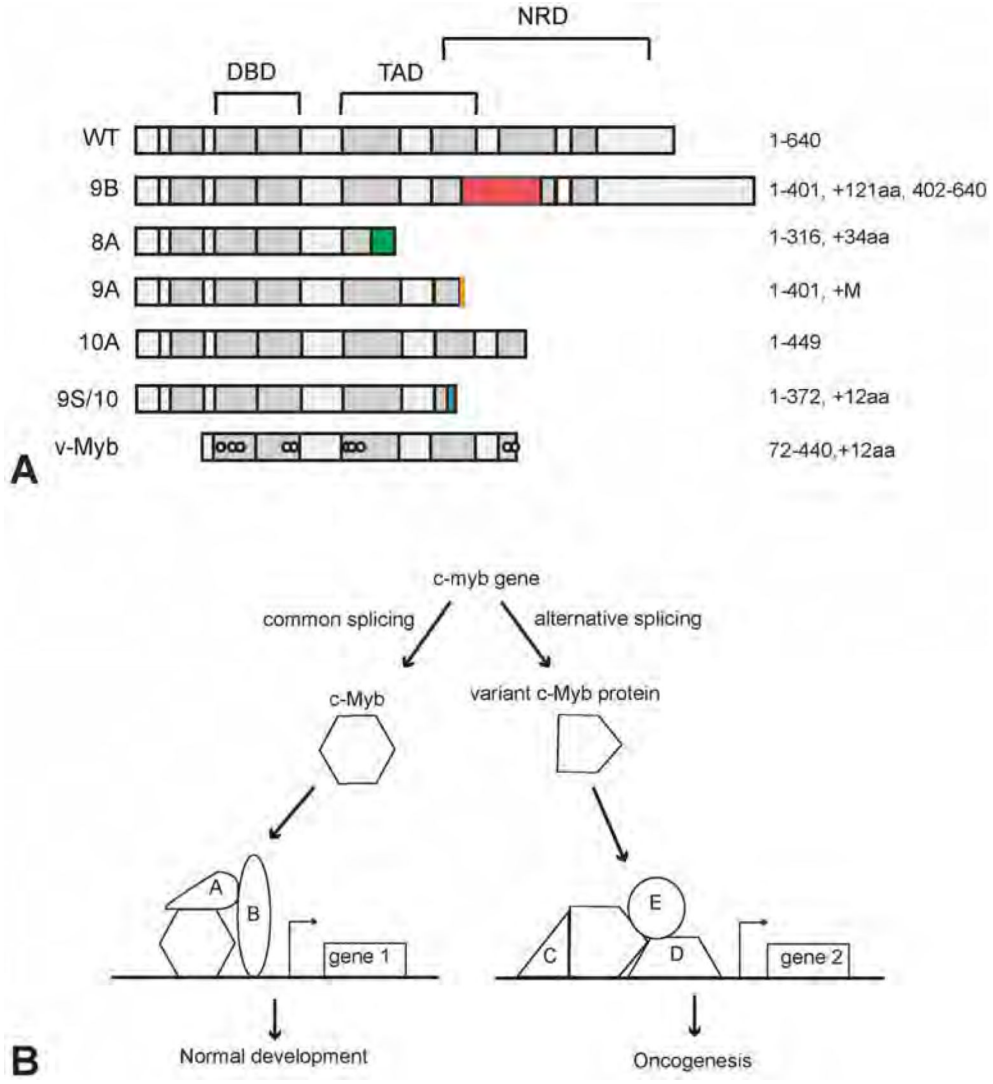

Figure 3.

Alternative splicing as a novel means of activating c-Myb. (A) The diagrams depict the protein structures of wild type c-Myb, alternatively spliced isoforms 9B, 8A, 9A, 10 and 9S/10 and AMV v-Myb. The DNA binding domain (DBD), transactivation domain (TAD) and negative regulatory domain (NRD) are labeled at the top. The numbers on the right indicate amino acid residues from c-Myb plus novel amino acids encoded by the alternative exons. (B) Different versions of Myb protein have different protein structures, permitting them to interact and cooperate with distinctive sets of transcription factors or cofactors to form stable transcription complexes at different promoters.