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Changes in c-Myb activity during proliferation

Anita Quintana

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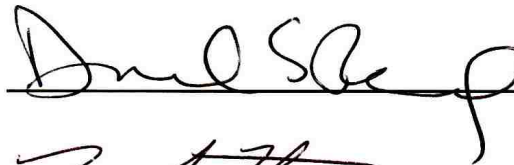
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Changes in c-Myb Activity During Proliferation

BY

Anita M. Quintana

B.S., Microbiology, New Mexico State University, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

August, 2010

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ABSTRACT OF DISSERTATION

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B.S. Microbiology
Ph.D Biomedical Sciences

Abstract

c-myb encodes a transcription factor and is the cellular parent of the v-*myb* oncogene, which causes acute myeloid leukemia in chickens and mice. Truncation or deletion can unmask the oncogenic potential of the human *c-myb* gene and recent reports have established that the human *c-myb* locus is subject to translocation and duplication in human pediatric T-ALL. c-Myb is expressed in all proliferating hematopoietic progenitor cells and has been implicated in some solid tumors, such as breast and colon. Evidence suggests that c-Myb activity is cell type specific and cell cycle dependent. For example, c-Myb interacts with cyclin D1 and CDK6 in Jurkat T-cells and this interaction inhibits c-Myb activity. Furthermore, there are numerous versions of Myb within a cell, each of which has a conserved DNA binding domain, but regulates different sets of endogenous genes. So it seems activity and specificity are regulated by domains outside of the DNA binding domain and possibly in a cell cycle-dependent manner. We hypothesized that protein interactions and post-translational modifications affect c-Myb activity in a proliferation dependent manner. We combined two model cell lines, MCF-7 cells and Jurkat T-cells, to characterize c-Myb specificity in different populations of proliferating cells. We found that c-Myb

protein dynamically repositions from one gene to the next in different phases of the cell cycle. Furthermore, mitotic signals such as estrogen stimulation, affect the detection of conserved domains in the c-Myb proteins suggesting that protein interactions change while c-Myb is bound to target genes. We conclude that c-Myb activity and specificity are regulated by dynamic oscillations in binding and in the event that c-Myb itself does not reposition its binding, partners come and go to its affect activity.

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Chapter I: Introduction

Summary

c-Myb has been implicated in the control of proliferation and differentiation in a number of different cell types. Although several mechanisms for regulating the expression levels and the activity of c-Myb have been identified, several types of evidence suggest that c-Myb has different activities in different cell types, and even at stages of the cell cycle. For example, c-Myb binds to and regulates multiple different cell cycle regulated promoters such as CDC2, CCNB1, and CCNA1, each of which is essential for a different stage of the cell cycle. Furthermore, c-Myb and its truncated-mutated oncogenic derivative regulate distinct endogenous sets of genes. Thus, protein domains outside the DNA binding domain that are targets for post-translational modifications and protein-protein interactions control c-Myb specificity. This suggests that c-Myb activity is highly regulated and modified by a variety of different signaling pathways and interactions, even in a time dependent manner. In this chapter the mechanisms that regulate c-Myb activity during the cell cycle will be highlighted and how changes in these mechanisms could lead to transformation and leukemogenesis will be addressed.

I. The importance of c-Myb in differentiation, proliferation and oncogenesis

The c-Myb protein is a transcription factor with a highly conserved DNA binding domain near its N-terminus (Figure 1) and several additional conserved motifs comprising a large C-terminal domain involved in negative regulation, control of its degradation and half-life, transcriptional activation, and the selection of specific target genes [1, 2]. The interest in c-Myb stems less from its role as a transcriptional regulator, since it activates genes in a manner similar to many other transcription factors, and more from the dualities of its functions. For example, different versions of c-Myb can be either a critical regulator of normal cells or a potent oncoprotein, and the protein is required for the opposing processes of cell proliferation as well as for terminal differentiation. Although the *c-myb* gene was amongst the first cellular proto-oncogenes to be identified and studied, the activities of the c-Myb protein seem paradoxical and its functions remain enigmatic. Because of the changes in activity and specificity, nomenclature becomes important. For the purposes of this chapter we will distinguish between c-Myb the protein and *c-myb* the gene (MYB) in order to differentiate between these cellular forms and the viral forms, v-Myb and *v-myb*.

The origin of c-myb and v-myb

The *c-myb* gene is the cellular progenitor of the viral oncogene, *v-myb*, which transforms immature hematopoietic cells in culture and induces leukemia in animal models [3-5]. The viral derivative of *c-myb* originated in two chicken retroviruses, Avian Myeloblastosis Virus (AMV) and the E26 virus, each of which encodes a truncated and mutated version of the c-Myb protein [6, 7]. Both

viruses are potent inducers of acute leukemia in chickens and rapidly transform immature cells by blocking their differentiation and promoting growth factor-dependent proliferation *in vitro* [8-10]. When the viral oncogenes are expressed from murine retroviruses, they are also capable of inducing leukemia in mice [11, 12], and the *c-myb* gene is frequently activated by retrovirus insertions in mouse models of leukemia [13-17]. Even though the v-Myb proteins are potent oncoproteins, the cellular parent, c-Myb does not induce tumors or leukemia in animals, even when constitutively over-expressed [18]; c-Myb can only transform cells in tissue culture in specialized situations [19]. The viral oncoproteins harbor deletions and point mutations and these modifications to the structure of c-Myb greatly enhance transforming activity [20]. The mutations are now known to affect specificity and target gene selection, since c-Myb and AMV v-Myb regulate distinct sets of endogenous genes [21]. This is one of the dualities of c-Myb: that relatively minor changes in its structure can completely change the spectrum of genes that it regulates. The unexpected plasticity in c-Myb activity may be its signature trait, and could explain its relatively underappreciated role as an oncoprotein in human malignancies.

Expression of c-Myb in human tumors

Because the *v-myb* oncogenes induce leukemia in animals, most of the attention on *c-myb* as a human oncogene has focused on its role in leukemia and other hematopoietic malignancies. In general, the expression of *c-myb* RNA and c-Myb protein in normal hematopoietic cells correlates with proliferation and the level of expression decreases as cells terminally differentiate [22]. Thus, the

highest levels of c-Myb are found in immature, proliferating cells, and little or no c-Myb is found in mature, differentiated, non-dividing cells [23]. In malignancies, the expression of c-Myb is relatively high in most if not all human leukemia such as T-ALL, B-ALL, AML and CML, often at much higher levels than in normal cells [24-27]. Elevated levels are also detected in several types of solid tumors, most notably colon and breast [28-31]. However, the association between *c-myb* expression and proliferation has led to the question of whether *c-myb* is a causal factor in human tumorigenesis, or merely a marker of higher proliferation in tumors compared to normal cells. Recently, a number of studies suggest a causal role by identifying *c-myb* gene amplifications and/or recurrent rearrangements in a variety of human tumors including leukemia and lymphomas, medulloblastomas, squamous cell carcinomas, small cell lung carcinomas and head and neck tumors [27, 32-37]. This evidence strongly suggests that amplification and/or mutation of the *c-myb* gene plays an important and causal role in the development of a wide variety of human hematopoietic, epithelial and neural tumors. Therefore, understanding the functions and activities of the *c-myb* gene and c-Myb protein are critical for understanding a variety of normal processes and for devising treatment strategies targeting this important human oncogene.

Regulation of hematopoietic cell proliferation and differentiation

The primary role of the c-Myb protein in normal development was established by constructing and analyzing *c-myb*^{-/-} knockout mice, which have normal yolk sac hematopoiesis but succumb due to a defect in definitive

erythropoiesis in the fetal liver [38]. Reduction in the level of c-Myb by conditional CreLox alleles rather than complete eradication has demonstrated that lower levels of c-Myb can promote a healthy generation of progenitor cell populations, but decrease their ability to commit to different lineages [39]. For example, reduced levels (5-10% of wildtype mice) of c-Myb lead to increases in monocytes, macrophages, and megakaryocytes. While reduced levels can favor the formation of specific myeloid cells, other lineages such as erythroid cells are decreased. Reduced levels of c-Myb increase immature erythroid progenitor populations, but decrease the level of mature erythroid cells relative to wildtype littermates. These results have demonstrated that thresholds of c-Myb expression and activity are important for cell fate decisions and can impact key differentiation steps. These data suggest that low levels of expression can lead to functional proliferating populations, but higher degrees of expression may be needed for accurate differentiation.

The c-Myb protein is expressed in all proliferating progenitor cell populations and during differentiation the degree of c-Myb expression declines [40-42]. Constitutive c-Myb expression blocks differentiation [43], and anti-sense oligonucleotides inhibit proliferation both *in vitro* and *in vivo* [44-48] demonstrating that c-Myb is an important regulator of proliferation, at the expense of differentiation. However, the identification of many target genes [1] that are expressed in terminally-differentiated cells suggests that c-Myb also plays a role in stimulating differentiation, which seems contradictory. Understanding this paradox: that c-Myb regulates both differentiation and

proliferation, may be the key to understanding the importance and uniqueness of c-Myb as an oncoprotein.

The published results from various laboratories establish that c-Myb has clear biological phenotypes, but do not explain how one protein can have different and apparently contradictory functions. One explanation could be that c-Myb has different functions in different cell types. Most of the studies linking c-Myb to the regulation of proliferation have been performed with hematopoietic cells, usually with leukemia-derived cell lines. For example, c-Myb binds to the CCNA1 (Cyclin A1) and CCNB1 (Cyclin B1) promoters and activates their expression in myeloid leukemia-derived cell lines [49, 50]. In these cells, where c-Myb is highly expressed, it could play an important role in promoting progression through the cell cycle, which could in turn be regulated by c-Myb interactions with Cyclin D1 [51] and Cyclin Dependent Kinases 4 and 6 [52]. The interactions with Cyclin D1 are different for c-Myb and v-Myb, raising the intriguing possibility that regulation of the cell cycle is an integral part of the oncogenic potential of c-Myb [51]. It is not yet clear whether c-Myb also regulates the expression of the Cyclin genes in normal cells, or in only a subset of normal cells such as hematopoietic cell progenitors, although there is some evidence that c-Myb may regulate the Cyclin E1 gene in some proliferating epithelial cells [53] and that c-Myb plays an important role in the estrogen-induced proliferation of mammary cells [54], suggesting that the link between c-Myb and proliferation may be more general.

There is also ample evidence that c-Myb plays an important role in hematopoietic cell differentiation. The original c-Myb target gene, *mim-1*, is only expressed in differentiating neutrophils [55], and this specificity is linked to the requirement for combinatorial cooperation with NF-M, the chicken equivalent of C/EBPbeta [56]. This is a model for the lineage specificity of other c-Myb target genes, which likely require that c-Myb cooperates with other regulators. Such targets include lineage specific genes like neutrophil elastase [57, 58] and myeloperoxidase [59], as well as target genes that are expressed only in immature cells such as CD34 [60] and c-KIT [61, 62].

Alternatively, c-Myb activity could be qualitatively different in different cell types because of lineage-specific post-translational modifications, such as serine or threonine phosphorylation, arginine methylation, lysine acetylation or others [2]. The post-translational modifications could affect the specificity and selection of target genes by c-Myb, analogous to the way point mutations in v-Myb change its activity. These possibilities and the effects of post-translational modifications on c-Myb activity will be discussed below.

Different levels of c-Myb may lead to different outcomes

In addition to qualitative changes in c-Myb activity, or differences in which cooperating factors are available in different lineages, there is also evidence that quantitative changes in c-Myb levels lead to the activation of different pathways. As discussed previously, in mouse models low levels of expression favor the formation of macrophages and monocytes, whereas higher levels favor erythropoiesis [39]. In general, the level of c-Myb activity in different cell types is

difficult to assess, especially since *c-myb* RNA levels decrease during differentiation [41-43, 63]. How is it possible that ablation of *c-myb* is lethal, but small changes in expression dictate lineage selection? While it is true that c-Myb is essential, a threshold of expression may exist that modulates proper hematopoietic cell differentiation. For example, despite the fact that *c-myb* RNA levels decrease substantially during lymphoid cell differentiation, c-Myb protein binds to and activates the promoters of genes that are expressed in fully differentiated cells such as the VDJ recombinase RAG2 [64, 65]. RAG2 is only expressed in fully differentiated T and B-cells, which express little if any *c-myb* mRNA, yet c-Myb protein can cause activation of this gene during an immune response. Results such as these suggest that the level of *c-myb* RNA makes a poor predictor of c-Myb protein activity. Therefore, it seems likely a major part of c-Myb protein regulation occurs through post-translational changes such as stability, modifications, protein interactions that can be reversed quickly, depending on cell context, and using residual c-Myb protein, even in cells that express little or no *c-myb* mRNA.

II. Context-specific changes in c-Myb activity

Structural and conserved domains

The dominant conserved feature in the c-Myb protein is the DNA binding domain near the N-terminus, which is identical in c-Myb proteins from species as diverse as humans, mice and chickens (Figure 1). The c-Myb DNA binding domain has been used as a signature to identify two additional transcription factors, A-Myb (MYBL1) and B-Myb (MYBL2), which have nearly identical DNA

binding domains and similar overall structures, but very different functions. Both crystal and solution structures of the DNA binding domain, either free in solution or bound to DNA, have been determined (reviewed in [1]). Interestingly, even the parts of the DNA binding domain that do not contact DNA have been perfectly conserved amongst vertebrate c-Myb proteins, suggesting that the DNA binding domain also serves as an important protein binding or docking surface [1]. Indeed, several proteins have been identified that interact with the c-Myb or v-Myb DNA binding domains [51, 66-68]. More importantly, three of the mutations in the AMV v-Myb protein change amino acids mapping to protein-interaction surfaces in the DNA binding domain [1]. These amino acid changes alter the activity of the protein [56], change the phenotypes of transformed cells [69] and shift the activity on some target genes [70], showing that relatively subtle changes in the structure of c-Myb, even affecting just a single amino acid residue, can change which target genes are regulated.

There are numerous regions within the c-Myb protein that are highly conserved and have been shown to have very important functions. The conserved trans-activation domain, amino acids 275-327, is essential for the transcriptional activity of c-Myb and v-Myb as measured in reporter gene assays [71, 72]. The extreme C-terminal domains negatively regulate the protein and truncation or deletion of this region causes increased c-Myb activity in some assays [66, 73]. The EVES motif, near the C-terminus and centered at amino acid 530, is involved in negative regulation by inducing an intramolecular fold-back mechanism and interacting with the DNA binding domain [66]. As discussed

further below, the c-Myb protein has many activities that contribute to its ability to regulate genes and control proliferation and differentiation.

Myb proteins have chromatin remodeling activities

Although the Myb DNA binding domain defines a family of related proteins, it recently acquired the additional classifier as a SANT domain protein, defined by a conserved motif found in multiple chromatin remodeling enzymes with homology to the Myb DNA binding domain [74]. The SANT domain is named for 'switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR) and transcription factor (TF)IIIB'[75]. The structural link between c-Myb and these well-known chromatin-remodeling enzymes suggests that c-Myb could also play a role in chromatin remodeling. Although c-Myb does not have enzymatic chromatin remodeling activity, it can modulate chromatin remodeling through interactions with histone tails or through interactions with proteins that modify histone residues. The DNA binding domain of c-Myb has been shown to bind and facilitate the acetylation of the N-terminal tails of histones H3 and H3.3, and this interaction is disrupted by the v-Myb mutations in the DNA binding domain [76], suggesting that changes in the chromatin remodeling activities of c-Myb are important for unleashing its oncogenic activity. Fine mapping of the conserved residues within the c-Myb DNA binding domain also led to the identification of an acidic patch required for interactions with the N-terminal tails of histone H4 [77], suggesting that the c-Myb DNA binding/SANT domain may have multiple histone binding activities that are part of its chromatin remodeling function.

In addition to direct interactions with histone tails, c-Myb can also direct chromatin remodeling by interacting with enzymes that modify histones. For example, c-Myb is found in a complex with the Mixed-Lineage Leukemia protein (MLL), a SET domain-containing histone methyltransferase that is frequently a component of fusion proteins produced by chromosome translocations in human acute leukemia. MLL methylates lysine 4 of histone H3 (H3K4), which is an important step in gene activation. A third protein, Menin, an MLL interacting partner [78], mediates the interaction between MLL and c-Myb. Thus, the gene activation activity of MLL may be directed to specific target genes through MLL-Menin-Myb complex formation. Since MLL is a known human oncogene, this association suggests a possible mechanism of c-Myb in leukemogenesis.

The Myb DNA binding domain recognizes sites in DNA matching a loose consensus sequence: (C/T)AAC(G/T)G [79]. Because this sequence is degenerate, it is expected to occur on average about once per kilobase, or more than 3 million times in the haploid human genome. Clearly, other domains in the c-Myb protein, which likely facilitate specific protein-protein interactions, must also play an important role in determining which binding sites are preferred and therefore which genes are regulated. This is illustrated best by microarray experiments that identified hundreds of target genes that were activated or repressed when different Myb proteins were ectopically expressed either in breast cancer cell lines or in primary human monocytes. To a first approximation, despite having nearly identical DNA binding domains, c-Myb activated totally different sets of genes than either A-Myb or B-Myb [80] and completely different

genes than were activated by its oncogenic derivative, v-Myb [21]. Furthermore, domain swap experiments showed that the biggest effects on target gene selection were caused by the differences outside the DNA binding domains, which were essentially interchangeable [70]. Thus, the regions of c-Myb outside the DNA binding domain, especially the large C-terminal domains that are often referred to as the transcriptional activation and/or negative regulatory domains, are in fact deeply involved in the selection of target genes and in determining the specificities of the c-Myb protein in different situations.

Myb-like domains in other proteins

In addition to chromatin remodeling proteins, Myb-like DNA binding domains are found in the important regulator DMP1, a Cyclin D1-binding transcriptional regulator that has tumor suppressor activity [81, 82], as well as in a group of important regulators called the Telebox proteins [83]. The Telebox domain is found in the human TRF1 and TRF2 proteins and is responsible for the ability of these proteins to bind telomere repeats in DNA after forming homodimers [84]. This mechanism sets the Telebox proteins apart from Myb proteins, since the latter do not form homodimers. Although the Myb-like domains are important for telomere binding, the Telebox proteins can bind to non-telomeric DNA as well, suggesting that these domains are specialized and may also have other functions within the cell [85]. Interestingly, TRF1 and c-Myb also share the ability to interact with the Pin1 proline isomerase, which regulates protein conformation in a phosphorylation-dependent manner [86, 87]. The c-Myb protein also interacts with the proline isomerase Cyp40, which induces

conformational changes in c-Myb but not v-Myb [67], suggesting that interactions with enzymes like Cyp40 and Pin1 could play an important role in suppressing the oncogenic activity of c-Myb.

Post-translational modifications of c-Myb

The c-Myb protein is subject to numerous post-translational modifications that can affect its activity (Figure 2A). SUMOylation is the reversible process of covalent modification of proteins with small ubiquitin like modifiers (SUMO). There are four SUMO proteins and c-Myb is SUMOylated at several different residues in response to different cellular signals. SUMO modification of c-Myb has been divided into distinct groups. The first group is SUMOylation by SUMO protein 1 [88, 89] and occurs in the negative regulatory domain through an interaction with the SUMO modifying enzyme UBC9 [88]. This modification is highly dependent upon the presence of lysine 523 and results in decreased c-Myb transactivation activity. Although SUMOylation of c-Myb by SUMO 2/3 occurs at the same sites, it is a response to stress and requires a separate E3 ubiquitin ligase [90]. SUMO 2/3 modification causes a reduction in the activation of c-Myb target genes [90]. SUMO modification is dependent on the ability of c-Myb to interact with either UBC9 or PIASy and the type and extent of SUMO modification changes based upon these protein interactions. These results suggest that changes in protein interactions and post-translational modifications, even those that occur outside of the DNA binding domain, can have a dramatic effect on c-Myb activity.

Turnover of the c-Myb protein is mediated by ubiquitin and the 26S proteasome. In malignancies c-Myb has a more pronounced half-life compared with normal cells and mutation and deletion of specific regions/residues leads to increased stability [91]. As described, the *c-myb* locus is subject to retroviral insertions that lead to leukemia in mouse models. In many cases these insertions cause truncation of the protein at the N and C-termini and results have confirmed that these truncations, especially in the C-terminus cause increased stability [92]. Therefore, truncation is a simple mechanism by which activity can be regulated. For example, truncated versions may bind to the same target genes as full-length c-Myb except truncated versions are more readily available therefore enhancing c-Myb activity at a specific set of target genes. Since truncation leads to changes in the C-terminal domain, a domain that is highly modified, it is likely that during different stages of proliferation, S-phase versus M-phase, c-Myb is differentially ubiquitinated and has different stability. The stability of c-Myb would allow the protein to interact with specific proteins in a time dependent manner and activate different cyclin genes in different phases of the cell cycle. Results demonstrating c-Myb is constitutively ubiquitinated and does not have altered stability in synchronized cells at different stages of the cell cycle [93] suggests that the stability of full length c-Myb is not a primary means of regulating activity in a cell cycle dependent manner. These data suggest that c-Myb protein does not exhibit increased turnover during the cell cycle and a substantial amount is available at each stage of the cell cycle in cancer cells. Other mechanisms must be readily

available to govern c-Myb activity because a substantial amount of c-Myb is expressed during independent stages of the cell cycle.

Ubiquitin is most commonly associated with degradation, but it is possible that combinations of modifications such as ubiquitin and phosphorylation are needed to properly degrade c-Myb. Coincidentally, results have demonstrated that phosphorylation of the C-terminus induces conformational changes that facilitate proper degradation [94]. Interestingly, hyper-phosphorylated c-Myb and hypo-phosphorylated c-Myb have the same affinity for DNA suggesting that under some conditions the turnover of c-Myb can regulate DNA binding. However, the ability to bind DNA with the same affinity does not translate into activity. These data reinforce the concept that modifications are connected to one another and function as a unit to determine c-Myb activity and binding capacity.

One of the problems with many of these experiments is that they have only used relatively crude measures of c-Myb activity to judge the effects of the modifications. Since the cloning of the original Myb-regulated target gene, *mim-1*, it has been clear that the ability of c-Myb and v-Myb to activate endogenous, chromatin-embedded target genes does not correlate with the ability to activate plasmid-based reporter gene assays [55, 56, 69]. With the advent of microarray assays, the complexities of target gene selection by different versions of c-Myb have become evident [21, 80], and it seems likely that SUMOylation and other post-translational modifications may be responsible for shifting or determining the selection of target genes by c-Myb. It will be necessary to test different versions

of c-Myb in more sensitive, unbiased gene activation assays to determine how these modifications affect c-Myb in the context of the cell.

Myb interacts with proteins during induced acetylation

c-Myb interacts with numerous co-activators and in some cases, these interactions affect Myb activity. Mutation of c-Myb can affect these interactions. One such mutation is M303V, which disrupts an interaction with the transcriptional coactivator and histone acetyltransferase, p300. In a mouse model system, this disruption leads to defects in hematopoiesis and promotes cellular proliferation [95] suggesting that the interaction between c-Myb and p300 affects cell fate decisions. p300 acetylates c-Myb at three lysines within the C-terminal domain and mutation of these residues to alanine leads to an increased DNA binding ability and increased transactivation activity [96]. c-Myb can also interact with the p300-related CREB Binding Protein (CBP) near the c-Myb transactivation domain, resulting in increased transactivation activity [97, 98]. CBP and p300 can acetylate the same residues in c-Myb, suggesting that these two proteins can have redundant functions, but it is unclear which interacting partner is preferred during different developmental stages or in different growth conditions. For example, CBP can interact with the transactivation domain or the negative regulatory domain [98], this difference could account for preferences in which residue gets modified, which in turn might reflect differences in the chronological sequence of protein modifications that dictate c-Myb specificity. If there is a context specific code [2] mediating c-Myb activity then the sequence of modifications could lead to changes in c-Myb specificity.

Varied effects of phosphorylation on Myb activity

Phosphorylation of c-Myb has been reported to have several different effects, including increased or decreased DNA binding activity, decreased stability, defects in negative regulation, and phosphorylation-dependent protein interactions with a number of cofactors [87, 94, 99-105]. Direct phosphorylation has been demonstrated by kinases including Pim1, Casein Kinase II, and p42 Mitogen-Activated Protein Kinase [68, 106, 107]. Phosphorylation can impact specific protein interactions, which could change the activity and/or specificity of c-Myb and determine which target genes become activated. For example, phosphorylation affects protein interactions with HIPK1 and Fbw7 [105, 108], both of which have unique effects on c-Myb activity. Characterization of the interaction between HIPK1 and c-Myb at the *mim-1* promoter established that this interaction inhibits activation of *mim-1*. On the other hand, when c-Myb interacts with Fbw7, c-Myb is subject to ubiquitination and degradation. Thus, modifications can change c-Myb activity directly or in conjunction with other proteins. These studies were performed using plasmid-based reporter gene assays, or a single target gene such as *mim-1*, so the effects of phosphorylation on target gene selection is not known. Therefore, global differences in Myb activities may be dictated by phosphorylation status [80].

Regulation is time dependent: A Mechanism of c-Myb regulation

c-Myb interacts with a wide array of proteins (Figure 2B). Some protein interactions may be dependent on or lead to new post-translational modifications. In addition, post-translational modifications may occur in a chronological order,

analogous to the way modifications are added sequentially to histones during transcriptional activation. These changes in post-translational modifications could account for the ability of c-Myb to bind to and regulate different genes in different biological processes. As discussed, c-Myb regulates the CCNA1, CCNB1, and CCNE1 genes [50, 53, 109] during the cell cycle. In the time-dependent cell cycle, c-Myb could be bound to these genes all the time but only participate in regulating the genes in specific stages of the cell cycle. Alternatively, c-Myb binding to specific promoters could change in a time-dependent manner, oscillating between different promoters as the cells progress through the cell cycle. To distinguish between these possibilities, it will be necessary to measure the association of c-Myb with different target genes as cells progress through the cell cycle.

The idea that c-Myb activity and target gene selection could change in a time-dependent manner during the cell cycle runs counter to expectations of transcription factor activities based solely on plasmid-based reporter gene assays. This may be the reason that the ability of c-Myb to activate endogenous genes does not correlate with the ability to activate reporter genes. If post-translational modifications affect target gene selection by c-Myb, it is likely that the cell contains multiple pools of c-Myb with different, perhaps overlapping specificities. For example, phosphorylation of c-Myb is likely to be sub-stoichiometric, defining a subset or pool of protein with a unique post-translational modification and perhaps a unique specificity for select target genes. However, modification of c-Myb is not limited to phosphorylation since the

protein can also be acetylated [96, 98], SUMOylated [88-90], and ubiquitinated [93], and is likely also to be modified in other ways. Potentially, each modification could distinguish a specific sub-population of c-Myb proteins with a unique spectrum of activities, directed toward a specialized subset of target genes by unique protein-protein interactions that are controlled by the modifications.

Regulating transcription by post-translational modifications or protein interactions offers the ability of regulating activity in a dynamic, time-dependent manner since these types of modifications can occur rapidly in response to mitogenic signals. There is very little known about how c-Myb activity might be affected by upstream signaling pathways, such as cytokine receptor pathways or oncogenic tyrosine kinases. Now that assays are available to follow subtle changes in c-Myb target gene selection, it will be interesting to see how such signaling pathways might affect c-Myb activity and its choice of target genes in specific situations in normal cells and in malignancies.

Mechanisms regulating c-Myb in T-cell and B-cell Development

Although the original *c-myb* knock out animals demonstrated the requirement for c-Myb expression in erythropoiesis, more recent experiments using conditional knock out strains have shown that c-Myb is also necessary for the development of the lymphoid lineages. Conditional knockdown of c-Myb in mice suggests that c-Myb is critical for three points of thymocyte development: double negative, double positive, and single positive [110]. Knockdown of c-Myb has different effects on each stage of development. For example, increases in

double negative thymocytes (DN) upon c-Myb knockdown is not due to defects in the cell cycle or cell survival. In contrast defects in the double positive (DP) T-cells to single positive (SP) cells is related to defects in cell survival [110]. DN cells go through stages of DN (1-4) development and it was demonstrated that at the DN3 stage, levels of TCR β , a c-Myb target gene were decreased, resulting in defects in differentiation [110]. These data suggest that as specific cell types (i.e. different T-cell stages) progress through differentiation the same protein, c-Myb, can facilitate multiple processes (Figure 3). It is likely that c-Myb, or more specifically a unique subset of c-Myb, binds to and regulates the TCR during DN3 development, but a different subset of c-Myb, DP specific c-Myb, regulates other genes to affect cell survival. In theory, this mechanism would affect proliferation by regulating target genes such as cdc2 [111] in a cell cycle dependent manner. c-Myb is essential for proper proliferation and regulates genes such as cdc2, but c-Myb can also regulate genes such as Bcl-2, an anti-apoptotic gene [112, 113]. These results suggest that different sub-populations of c-Myb can regulate different genes in different cell types, or more specifically the same types of cells undergoing different fates. The c-Myb proteins in these sub-populations of cells are likely defined by different post-translational modifications and protein interactions that affect the target gene selection by c-Myb. Thus, knockdown of c-Myb in T-cells has identified different c-Myb subpopulations, each of which is essential for a key stage in cellular development and progression. Recently other groups have expanded on the idea that c-Myb affects different subsets of thymocytes and have demonstrated that during

progression from DP to SP c-Myb regulates Bcl-xL, an anti-apoptotic gene and during c-Myb knockdown, DP thymocytes undergo apoptosis leading to a decrease in specific subsets of SP cells [114]. This concept of sub-populations of c-Myb regulating discrete stages of differentiation can be expanded to other cell types, in particular B-cells, where c-Myb regulates the survival of a specific subset of B-cells [115].

c-Myb's role in T-cell and B-cell development is complex and it is clear that c-Myb target genes vary in different subpopulations of cells. More specifically, the activity of c-Myb varies in different stages of differentiation. This raises an interesting question: If only a subset of c-Myb molecules is involved in regulating specific genes, what is the rest of the c-Myb doing? Is the cell cycle specific fraction of c-Myb expressed, but not active during the cellular response to differentiation cues? Where does it go if its not bound to genes? Some c-Myb interacting partners have variable localization and could sequester c-Myb protein. For example, the interaction between c-Myb and FLASH leads to colocalization into nuclear speckles, however only a fraction of c-Myb is found in these speckles [116]. Unfortunately, it is not clear how many subpopulations of c-Myb exist within a given cell type, or where those subpopulations are localized.

III. Mechanisms that control c-Myb activity and specificity

Alternative RNA splicing produces multiple forms of c-Myb

In addition to post-translational modifications, subpopulations of c-Myb can also be produced through alternative RNA splicing. The *c-myb* gene contains 15 exons and undergoes alternative splicing in both cancer cells and normal cells

[117-120]. The vast majority of alternative splicing occurs in exons 6-15 and excludes the exons that encode the DNA binding domain. Thus, almost all of the variant c-Myb proteins encoded by alternatively spliced mRNAs contain the normal DNA binding domain, but have changes in the large C-terminal domains controlling transcriptional activation, negative regulation and target gene specificity [118]. For example, the addition of alternative exons such as exon 6A, which results in a 400 bp insertion between exon 6 and 7, have been described in normal cells and cancer cells [119]. Exon 9 can form up to four different variants in both chickens and humans and at least two of them are found in hematopoietic cells [118, 121, 122]. With the identification of additional c-Myb target genes and the availability of assays that can detect differences in target gene selection by different versions of c-Myb, it has become clear that many of the proteins encoded by splice variants have distinct transcriptional activities and activate different sets of genes than the “wild type” c-Myb [118]. These results greatly increase the amount of complexity that is possible in the targeting of genes by c-Myb, since different subpopulations of the protein, encoded by different versions of the mRNA, could regulate entirely different sets of target genes.

Comparisons of different types of cells have shown that different patterns of *c-myb* mRNA splicing are found in different cell lineages [118], suggesting that alternative mRNA splicing is as carefully regulated as any other aspect of differentiation. In addition, much higher levels of *c-myb* alternative splicing are found in leukemia than in normal bone marrow cells [118], suggesting that the

mechanisms controlling alternative splicing are defective or modified in malignancies. Thus, amplified alternative splicing may be a novel mechanism for activating oncogenes like *c-myb*, which are capable of producing variant proteins with different, perhaps transforming, activities. This idea is strengthened by recent findings showing that regulators of alternative RNA splicing are themselves oncogenes [123, 124], presumably because they cause the production of alternatively-spliced mRNAs that encode proteins with altered/oncogenic activities, similar to the altered activities displayed by v-Myb compared to c-Myb.

One of the major questions that remains is, do different versions of c-Myb, encoded by different splice variants or produced by sub-stoichiometric post-translational modifications, compete for the same target gene promoters. For example, phosphorylation of specific residues in the N-terminus of c-Myb may only occur on a fraction (1%) of the total molecules of c-Myb. These phosphorylated molecules may have different activities and different binding specificities, due to different cooperative interactions with other proteins that bind some target promoters. If subpopulations of c-Myb are able to bind specific subsets of target promoters, without really competing with the vast bulk of the protein that has a different specificity, then even relatively rare forms of the protein could make important contributions to the patterns of genes that get expressed, and could contribute to transformation. Additional experiments will be required to determine whether different versions of c-Myb, which appear to have

distinct target gene specificities, are able to regulate their target genes in the presence of other, more abundant versions of the protein.

Regulation of c-myb by microRNAs

MicroRNAs are small non-coding RNAs approximately 22 nucleotides in length and were first discovered in plants as regulatory RNAs that can regulate the stability of RNA transcripts [125, 126]. These non-coding RNAs are derived from primordial RNAs that are cleaved into one or more microRNAs complementary to regions within the 3' untranslated region of specific target mRNAs. Genes that encode microRNAs such as *lin-4* and *let-7* were discovered first in *C. elegans* and play important roles in the regulation of larval development [126]. In mammals, deregulated expression of microRNAs can influence a large number of genes and can contribute to many different types of cancer including breast and oral cancer [127-130].

The *c-myb* 3' untranslated region (UTR) is extensive and contains many potential binding sites for microRNAs. MiR150 is expressed in resting T and B-cells, but not proliferating progenitors [131] and deletion of miR150 can cause an increase in *c-myb* expression and expansion of the B1 subset of B-cells. These observations were more pronounced in B-cells than T-cells presumably because the level of miR150 in T-cells is reduced relative to B-cells [132]. The regulatory relationship between miR150 and *c-myb* is conserved in zebrafish suggesting that this ancient regulatory mechanism has been conserved throughout vertebrate evolution [133]. The miR150 mechanism also regulates *c-myb* expression in other cell types. For example, expression of miR150 in

megakaryocyte-erythrocyte progenitors leads to a profound bias of differentiation towards megakaryocytes, and if *c-myb* is expressed without the 3'UTR there is a decrease in the formation of megakaryocytes [134]. This is consistent with results showing that a knockdown of c-Myb expression results in increased megakaryocyte production [135]. These data suggest that miR150 is a potent regulator of *c-myb* and differentiation, however other microRNAs have been demonstrated to be of equal importance in regulating *c-myb*.

MiR15a and miR16 are encoded by the *dleu2* tumor suppressor locus, which is regulated by the Pax5 transcription factor, a c-Myb interacting partner [136]. During levels of high Pax5 activity miR15a/16 are down regulated resulting in increased *c-myb* expression. Likewise, high levels of each microRNA decreases c-myb and A-Myb expression suggesting that some microRNAs regulate multiple Myb family members [136]. Further analysis has established that the miR15a/16 promoter is bound by the c-Myb protein and that functional miR15a can inhibit erythroid and myeloid colony formation [137]. These results suggest that c-Myb protein plays a critical role in regulating its own expression, which is important for proliferation, differentiation, and apoptosis. Other microRNAs such as miR34a are complementary to the *c-myb* 3' UTR and recent studies have identified that miR34a regulates *c-myb* expression during megakaryocyte development [138], suggesting that different microRNAs can promote different cellular processes.

Abnormal regulation of microRNAs has been implicated in numerous forms of cancer. For example, miR15a is deleted in many tumors. Since many

miR15a targets are important for cell cycle regulation [139], these results suggest that loss of miR15a leads to changes in the cell cycle. In contrast, other microRNAs such as miR30 are up-regulated in a subset of cancers [140]. Thus, the regulatory pathways affecting microRNAs are complex and deregulation can occur in the form of deletion, amplification, mutation, and silencing of the genes via epigenetic marks [141].

Attenuation as a regulator of c-myb transcription

There is differential expression of *c-myb* in a variety of different cells during differentiation and changes in *c-myb* expression can be attributed to attenuation of transcriptional elongation in B-cells [142]. This attenuation is mediated by sequences within the first intron of the *c-myb* gene, which includes a DNase I hypersensitive region [143]. During differentiation there is a reduction of protein binding to intron I, correlating with decreased *c-myb* expression [144]. The intron I region contains binding sites for a number of transcription factors including AP-1, SP1, and the estrogen regulated P2 protein [144], and in breast cells the attenuation is relieved in the presence of estrogen [54]. This suggests that intron I attenuation is regulated by mitotic signals in a cell cycle dependent manner. Mutation of the intron I region occurs in some colon cancers, which could lead to the over-expression of *c-myb* and contribute to transformation [145]. Thus, a number of transcriptional and post-transcriptional mechanisms are involved in controlling *c-myb* expression, and these mechanisms play important roles in controlling differentiation and proliferation.

IV. Implications for the regulation of c-Myb during the cell cycle

Myb genes are cell cycle regulated

Stimulation of primary peripheral blood lymphocytes (PBLs) has demonstrated that *c-myb* is expressed in a cell cycle dependent manner. *c-myb* RNA levels peak in expression during the G1/S transition [146, 147] and protein expression can be detected slightly later [148]. These changes in expression level suggest that cell cycle specific mechanisms participate in the induction of c-Myb during different phases of the cell cycle. The *c-myb* gene promoter contains binding sites for the cell cycle transcription factor E2F [149], which could be one mechanism for explaining the induction of the *c-myb* gene during the cell cycle.

The two related proteins, A-Myb and B-Myb, have distinct patterns of expression during the cell cycle. In hematopoietic cells, expression of B-Myb mRNA peaks during the G1/S transition and mutation of the E2F binding site in the B-Myb promoter leads to defective B-Myb gene induction [150]. Both *c-myb* and *myb12* (B-Myb) appear to peak in expression during G1/S, but expression of the A-Myb gene peaks in G0/quiescent hematopoietic cells [22], showing that each of the *myb* genes is regulated differently. Interestingly, some cell type specificity exists, since in vascular smooth muscle cells A-Myb transcription peaks at the G1/S transition [151]. These data establish that all three MYB genes are cell cycle regulated.

Regulation of the cell cycle by Myb proteins

Several types of evidence have linked the Myb transcription factors to cell cycle regulation. For example, other evolutionarily related proteins such as

Drosophila Myb (DMYB), A-Myb, and B-Myb all have documented cell cycle regulatory roles. Mutated alleles of DMYB have detrimental effects on the cell cycle [152] and lead to genomic instability [153]. Recent studies have shown that DMYB is part of the dREAM complex that coordinates specific cell cycle regulation of target genes, particularly in G2/M [154, 155]. B-Myb is the most closely related to the Drosophila DMYB gene [156] and B-Myb, but not c-Myb, is an active component of the Mip/Lin-9 complex, the mammalian ortholog to the dREAM complex. As part of the Mip/Lin-9 complex, B-Myb regulates genes in a G2/M specific manner. B-Myb is also required for proper regulation of the mitotic spindle [157-160] and for inner cell mass formation [161]. These activities appear to be specific for B-Myb, highlighting the importance of B-Myb in both cell cycle regulation and differentiation, and demonstrating that different Myb proteins have completely different functions.

The A-Myb protein is also associated with proper cell cycle progression. Knockdown of A-Myb leads to an arrest of spermatogenesis and defects in breast cell development [162, 163]. These tissues undergo a high level of proliferation suggesting that A-Myb is essential for proliferation in these tissues. Neither A-Myb nor B-Myb is an established oncogene, although expression of each has been detected in B-cell lymphoma or breast cancer, respectively [164, 165]. The relative over-expression of A-Myb and B-Myb may reflect the higher rates of proliferation of the tumor cells compared to the normal tissues.

The importance of c-Myb to cell cycle regulation is less well characterized, but it may be regulated in a way that is analogous to that of B-Myb. The genes

encoding several important cell cycle regulators, including Cyclins A1, B1 and E1, are regulated by c-Myb, at least in some cell types [49, 50, 53]. However, these genes are induced in different phases of the cell cycle, so if c-Myb is involved in their regulation, then c-Myb activity or specificity must also be regulated by the cell cycle. Since c-Myb interacts with Cyclin D1 [51] and with cyclin dependent kinases CDK4 and CDK6 [52], it is possible that c-Myb specificity is regulated through a mechanism of cell cycle stage-specific phosphorylation, similar to the regulation of the Retinoblastoma protein and the E2F transcription factors [166, 167]. However, cell cycle specific phosphorylation of c-Myb has not yet been documented. Alternatively, c-Myb may only be an accessory transcription factor that cooperates with other proteins that are more specifically regulated. For example, c-Myb could form cooperative complexes with E2F at specific promoters, in the way it does with C/EBPbeta. The direct regulation of E2F could recruit c-Myb to specific promoters at different stages of the cell cycle, making c-Myb more of a passive participant, rather than an active component of cell cycle regulation.

Conclusions

c-Myb is not an unlikely candidate to alter the proliferation of cells and the behavior of c-Myb is not completely unique amongst cell cycle controlled transcription factors. For example, for many years studies with *c-myb* were performed in conjunction with *c-myc*. Many studies demonstrated increased expression of *c-myc* was cell cycle dependent and correlated with leukemia [168, 169]. In fact, *c-myc* displays a similar pattern of expression during erythroid

differentiation and although *c-myc* is expressed upstream of *c-myb*, both transcription factors are invaluable for proliferation [170]. *c-Myc* binds to thousands of different genes, which has left a puzzling scenario as to why *c-Myc* binds to a huge portion of the genome, but recent technology has generated data suggesting that *Myc* can have an effect upon chromatin structure [171]. The genome wide targets of *c-Myb* have yet to be described and there are two primary hypotheses to describe *c-Myb* specificity and function. First, *c-Myb* binds to a small fraction of genes and those genes are highly dependent upon *c-Myb* for activation or secondly, *c-Myb* binds to a large fraction of genes and plays a small, but critically important role in activation.

It is likely that *c-Myb* is not regulated in a similar manner as *c-Myc* and is regulated more like cell cycle proteins such as p53 or E2F. The mechanism of p53 cell cycle control is mediated mostly by MDM2, which facilitates proper degradation of p53 [172]. A protein counterpart such as MDM2 has not been discovered, but protein interactions can affect the activity of the protein. On the other hand, E2F is regulated in a similar manner as p53, but there is an entire family of these proteins and E2F proteins can affect both proliferation and apoptosis, which at times appears contradictory [173]. This type of contradiction is similar to *c-Myb*'s role during proliferation and differentiation except there are fewer *Myb* family members. Although there are fewer *c-Myb* family members, alternatively spliced isoforms have distinct activities and different post-translationally modified subsets of *c-Myb* have the potential to mediate the proliferation of cells. Regulation of other proteins could add some insight about *c-*

Myb activity during proliferation and differentiation because c-Myb activity could be mediated through changes in post-translational modifications or protein interactions. Therefore, it is critical to consider how other transcription factors are generally regulated during the cell cycle of other cell cycle because similar factors could regulate c-Myb.

The most highly related transcription factors to compare c-Myb with are A-Myb and B-Myb. Evidence suggests that these Myb proteins are cell cycle regulated. It is established that c-Myb is extensively modified and results with A-Myb and B-Myb extend these results by demonstrating that cyclin/CDK complexes phosphorylate Myb proteins. For instance, A-Myb and B-Myb interact with cyclin A: CDK2, which leads to phosphorylation of each protein [109, 174]. Phosphorylation of A-Myb leads to a relief of transcriptional inhibition and phosphorylation of B-Myb increases transactivation. These results with Myb related partners demonstrate two important results. First, the same complex interacts with and phosphorylates each Myb protein, which has distinct effects on Myb activity. Second, cell cycle machinery such as cyclin A/CDK2 can interact with multiple Myb proteins. This suggests that Myb proteins, including c-Myb can shuffle from one complex to another throughout the cell cycle (Figure 4). This could affect specificity and explain how c-Myb can activate both the CCNB1 and CCNA1 genes. In addition, it is plausible that this shuffling is coordinated amongst Myb proteins. It was described that B-Myb activates G2/M specific genes as a component of the dREAM complex, but c-Myb can bind to some of the same promoters as B-Myb such as CCNB1. In addition c-Myb interacts with

CCND1/CDK6 suggesting that it may participate in complexes similar to the dREAM complex to regulate different sets of genes. These results provide evidence for c-Myb and B-Myb to have some redundant target genes, but different interacting partners at different phases of the cell cycle (Figure 5).

This chapter has described the hypothesis that c-Myb post-translational modifications and protein interactions account for changes in c-Myb activity. As many have shown, there is an extensive regulation of *c-myb* gene expression and RNA turnover that is important for our future understanding of c-myb in cancer formation. A disparity between c-Myb activity and *c-myb* expression exists and results suggest that the differential activities of c-Myb, which may appear at first glance to be antagonizing, actually are due to different sub-populations of the same protein. Hypothetically these populations could be expressed all of the time, but only a minute fraction is active or alternatively, and most likely, is that these populations are formed in response to signaling changes outside of the cell and post-translational modifications and interacting partners define each sub-population. Therefore, future experiments that define these sub-populations will enhance our knowledge and understanding of how c-Myb transforms into an oncoprotein.

Figure Legends

Figure 1: Conserved domains in Myb proteins.

All Myb proteins have a conserved DNA binding domain (shaded dark grey) with a transactivation domain (TAD). The extreme C-termini or negative regulatory domain (NRD) are known to regulate activity, specificity, and negative regulation. v-Myb is truncated, but contains regions of conservation (shaded light grey) and mutated within the remaining portion of the protein (open circles).

Figure 2: c-Myb is actively modified after translation.

A. c-Myb is phosphorylated throughout the protein by a multitude of different kinases and the C-terminus is SUMOylated and acetylated. These modifications affect protein interactions and activity. B. c-Myb interacts with different types of proteins and many of them bind to the DNA binding domain. A select few such as Pim1 and p300 result in post-translational modifications that affect activity.

Figure 3: c-Myb sub-populations regulate T-cell development.

c-Myb is essential for proper T-cell development and defects in c-Myb expression cause different phenotypes in different stages of development. c-Myb has a wide array of target genes and it is likely that different versions of c-Myb have different activity and specificity in different stages of development and different cell types.

Figure 4: Myb proteins are cell cycle regulated.

Components of the cell cycle such as cyclin/CDK complexes can interact with all Myb proteins. A-Myb and B-Myb are phosphorylated by different pairs of cyclin/CDK complexes during the cell cycle. These post-translational modifications alter Myb activity in a cell cycle dependent manner.

Figure 5: Myb proteins have the potential to compensate for each other.

B-Myb and c-Myb have some redundant functions in myeloid cells and can both bind to the CCNB1 gene. It is likely that during proliferation, at different stages of the cell cycle, B-Myb and c-Myb shuffle on and off of the same promoters in a cell cycle dependent manner.

Figure 1

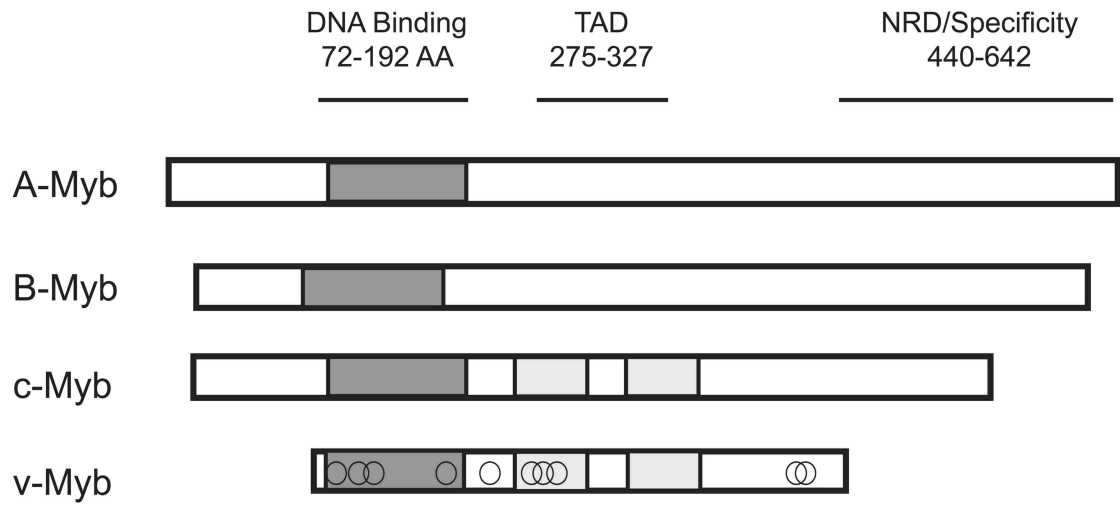
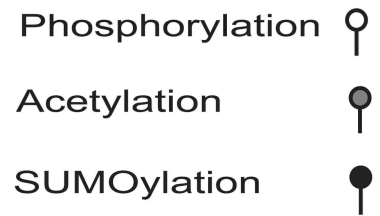
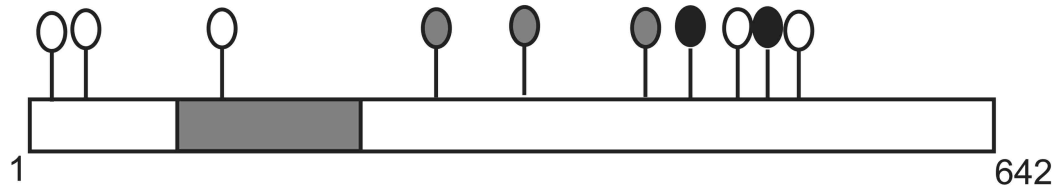


Figure 2

A



B

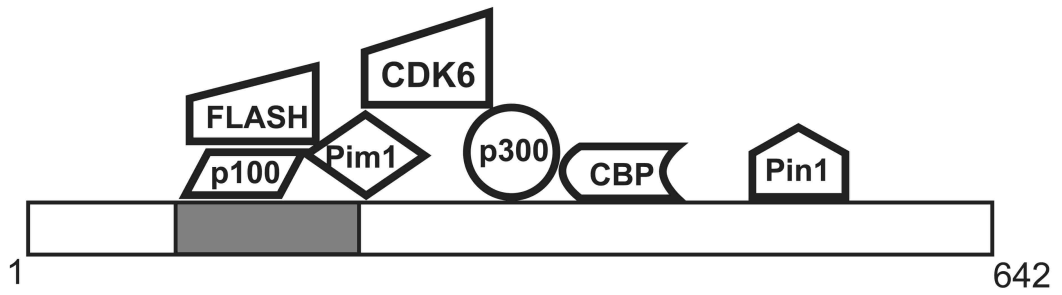


Figure 3

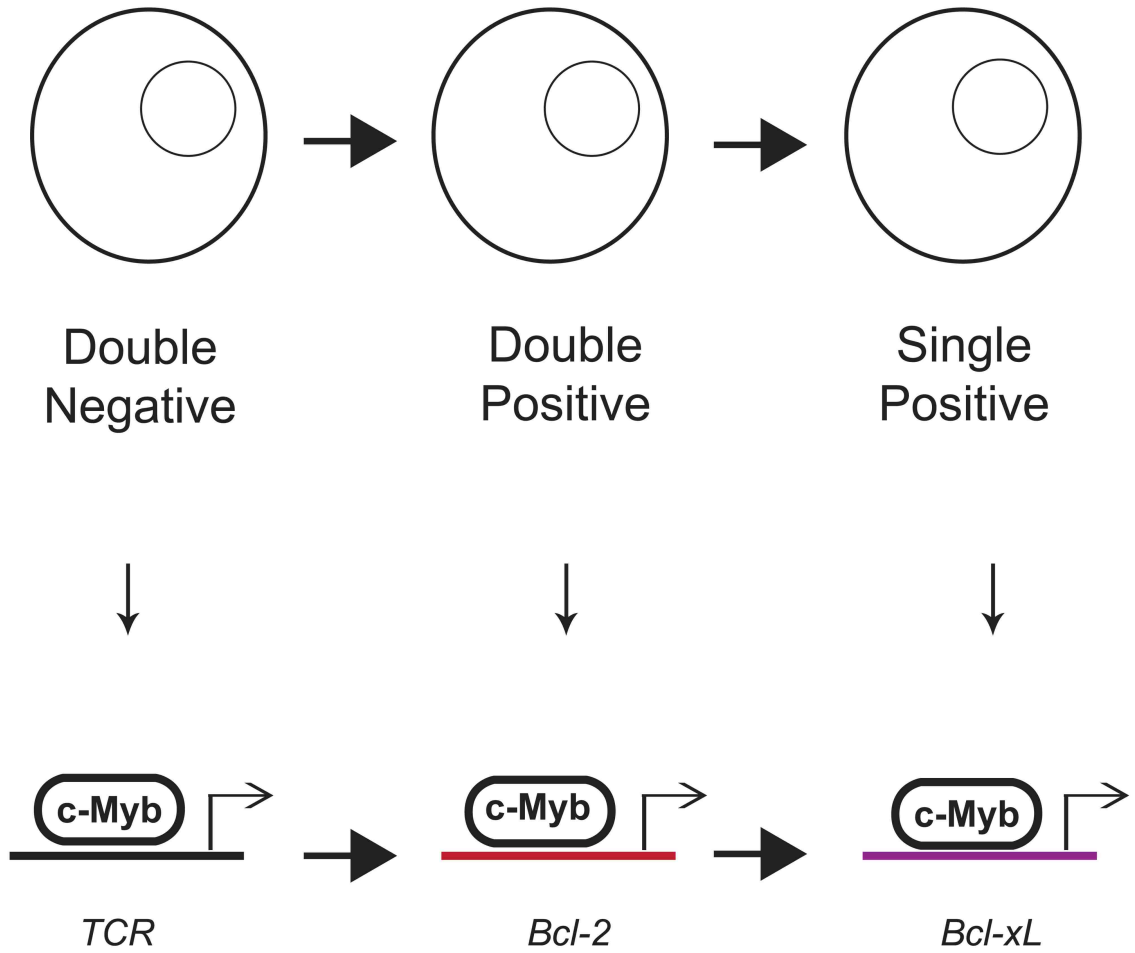


Figure 4

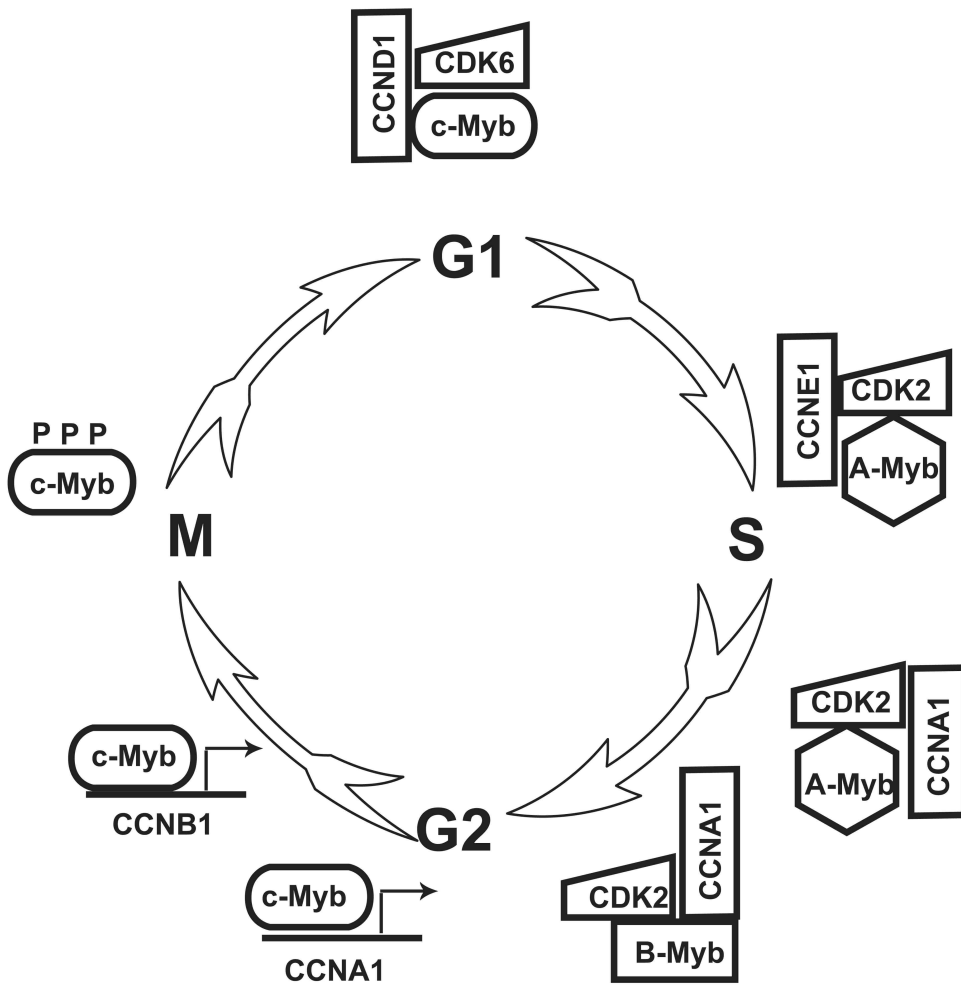
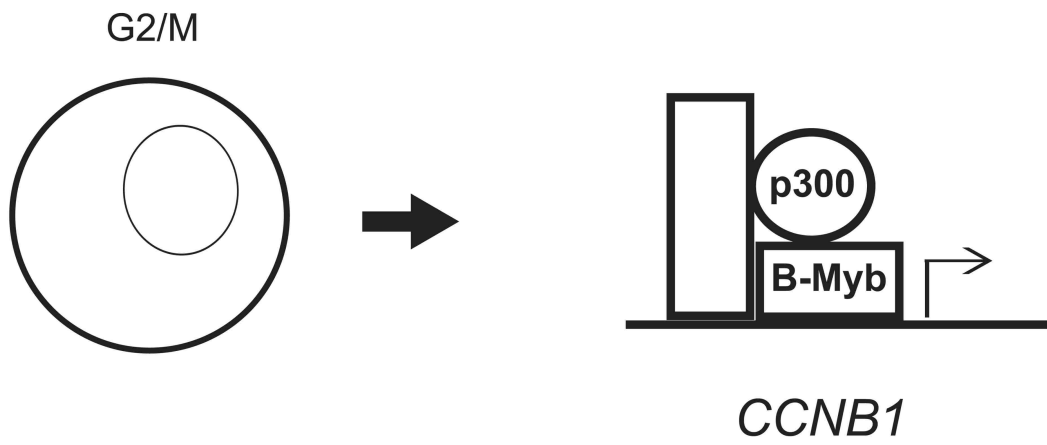
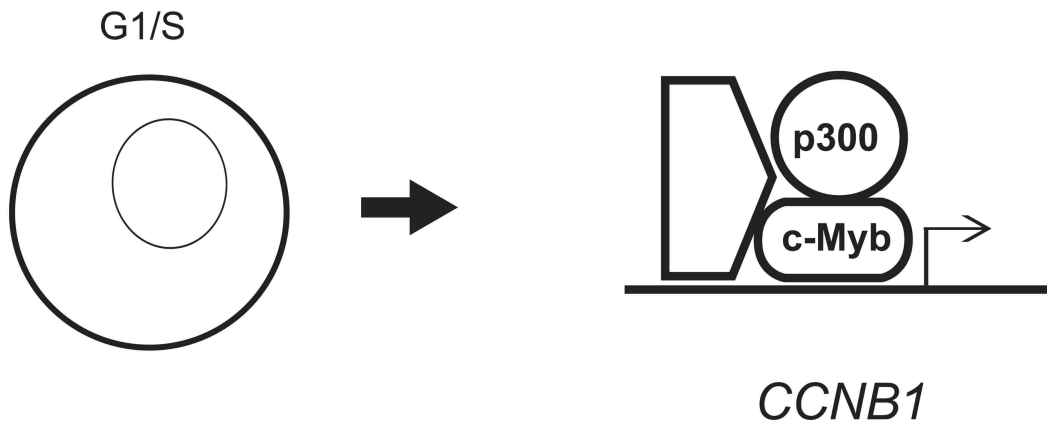


Figure 5



Chapter Two: Specificity in the Cell Cycle

The disparity between the level of *c-myb* expression and c-Myb activity is a difficult to explain, but characterizing this disconnect is essential to describing the effect of c-Myb on cancer formation. As discussed, c-Myb activity can change in single cells types, such as T-cells, depending on the nature of the cell (i.e. double negative, double positive or single positive). It is plausible that different sub-populations of c-Myb exist in different subsets of cells and this hypothesis can be applied to different biological processes. For example, if c-Myb is constitutively expressed during the cell cycle, then different versions of the c-Myb protein could account for the differences in c-Myb activity. c-Myb regulates three cyclin promoters, *CCNB1* [50], *CCNE1* [53], and *CCNA1* [49], and each of these genes is responsible for regulating a different part of the cell cycle; *CCNB1* and *CCNA1* regulate G2/M and *CCNE1* regulates S-phase. The function of these genes and the cyclic regulation of each suggest that if c-Myb regulates their expression, c-Myb must be constitutively expressed in asynchronous cell cultures.

We hypothesize that c-Myb protein expression is relatively constant and although c-Myb half-life is short there is sufficient amounts of protein during all phases of the cell cycle. We developed a model system to characterize c-Myb specificity during the cell cycle. We employed flow cytometry to sort cells into distinct subsets based on DNA content and characterized c-Myb occupancy with Chromatin Immunoprecipitation (ChIP). These data demonstrate that c-Myb protein is constantly present during the cell cycle and that specificity/activity are

dynamic because c-Myb associates with different genes in different sub-populations of cells.

Author Contributions

The following paper discusses our observations in Jurkat T-cells with three independent authors Anita M. Quintana (AMQ), John P. O'Rourke (JPO), and Scott A. Ness (SAN). AMQ performed all of the cell cycle analysis including optimization of staining protocols, flow cytometry, identification of target genes, expression analysis, Western blot, isolation and immunoprecipitation of proteins. JPO kindly made lentiviral particles expressing FLAG-tagged versions of the c-Myb protein and selected for stable expression. SAN mentored AMQ, provided funding and necessary tools to perform the research. SAN also approved the manuscript for publication.

Dynamic Retargeting and Changes in c-Myb Specificity During the Cell Cycle

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Summary

The c-Myb transcription factor is a critical regulator of proliferation and stem cell differentiation, and mutated alleles of c-myb are oncogenic, but little is known about the activities of c-Myb during the cell cycle. We used fluorescence activated cell sorting to divide asynchronously growing populations of human cells into different cell cycle fractions, and then applied chromatin immunoprecipitation assays to determine the occupancy of c-Myb on the promoters of known target genes. We found that the specificity of c-Myb is dramatically different in small subpopulations of cells, for example cells in the G2/M phase of the cell cycle, than in the bulk population. Our results show that c-Myb undergoes dynamic, time-dependent repositioning, regulating different target genes in specific phases of the cell cycle, and that the specificity of c-Myb is actively regulated in real time in human cell lines, primary cells and leukemia.

Highlights

- c-Myb occupancy at cyclin gene promoters is cell cycle dependent
- c-Myb specificity varies dramatically during different phases of the cell cycle
- c-Myb regulates the *CXCR4* gene in leukemia cells

Introduction

The c-Myb transcription factor is responsible for the proper regulation of hematopoiesis and proliferation [175]. Inhibition of the *c-myb* gene blocks hematopoietic cell differentiation *in vitro* [46] and leads to a loss of hematopoietic cells in animals [38]. v-Myb is a truncated mutated derivative of the human *c-myb* gene and causes acute myeloid leukemia in chickens and mice [7]. *c-myb* is amplified in T-ALL [32] and has been implicated in solid tumors such as breast [28] and colon [29] suggesting that c-Myb is important for cancer progression.

Treatment of cancer cell lines with antisense oligonucleotides specific for *c-myb* decreases proliferation demonstrating that *c-myb* is essential for proper proliferation [47, 176]. *c-myb* is expressed in all proliferating progenitor cells [23] and has been implicated in the cell cycle regulation of multiple cell types such as T-lymphocytes and breast cells. For example, *c-myb* expression peaks at the G1/S transition of stimulated peripheral blood lymphocytes [146]. These results suggest that *c-myb* expression is regulated in a cell cycle dependent manner.

c-Myb protein is expressed during stimulation of peripheral blood lymphocytes [148] and the activity of c-Myb is linked to the cell cycle. c-Myb regulates a number of different genes that are essential for cell cycle progression such as CDC2 [111], CCNA1 [49], and CCNB1 [50] but the mechanism controlling c-Myb activity at these promoters is unknown.

At least two mechanisms have been described for transcription factor regulation during the cell cycle. One example is E2F transcription factors, which

are inhibited during the G1 phase of the cell cycle by bound Retinoblastoma tumor suppressor protein. In S phase, Cyclin D1/CDK4 phosphorylation of Retinoblastoma triggers its removal and leads to the activation of E2F target genes [177]. In contrast, receptor-activated signaling cascades lead to activation of kinases like Akt, which phosphorylate FOXO transcription factors, leading to their migration to and sequestering in the cytoplasm and preventing them from regulating the expression of genes encoding cell cycle regulators [178]. Both of these examples are ways in which transcription factors change activity or localization, but not specificity, during the cell cycle.

We set out to monitor the activity of c-Myb and determine whether it undergoes changes in specificity during the cell cycle. We wanted to study the regulation of c-Myb under conditions of asynchronous cell growth without cell cycle arrest agents so we used chromatin immunoprecipitation to follow the association of c-Myb with different gene promoters in cultures of human cells that were progressing normally through the cell cycle. This approach allowed us to fix the proteins to DNA with formaldehyde while the cells were still in the culture dish, locking in the results before the cells were manipulated in any way. Then the cells were harvested, sorted into cell cycle stages and used for chromatin immunoprecipitation experiments. The results show that c-Myb undergoes dramatic and dynamic repositioning onto different gene promoters during the cell cycle and suggest that c-Myb regulates the cell cycle by changing specificity in response to cellular cues.

Results

Fractionation of Jurkat Cells by Hoechst 33342

The development of Chromatin ImmunoPrecipitation (ChIP) assays for the detection of c-Myb target genes [179] provided a means to follow the activity and specificity of c-Myb during the cell cycle. We chose Jurkat T cells, a human cell line to characterize c-Myb specificity during the cell cycle in which we showed previously that c-Myb protein interacts with the cell cycle regulatory proteins CDK4, CDK6 and Cyclin D1 [52], suggesting that c-Myb activity might be regulated during the cell cycle in these cells. We started by developing a system to fractionate asynchronously cultured cell populations into different cell cycle phases based on DNA content. Briefly, asynchronously growing populations of Jurkat cells were stained with the DNA content dye Hoechst 33342 and subjected to flow cytometry. Cellular fractions were then subjected to RNA analysis, protein expression, and ChIP assays. As shown in Figure 1A, Hoechst 33342 is cell permeable and cells stained with this dye show a typical cell cycle distribution of 60.5% G1, 15.1% S-phase, and 15.5% G2/M. As an initial confirmation of our sorting procedure we performed expression analysis of the human CCND1 gene. Figure 1B demonstrates that CCND1 expression is detected in asynchronous cell populations and the sorted G1 fraction of cells, but not the S/G2/M fraction. Our endogenous control gene PPIA was detected in all sorted fractions suggesting that our data with CCND1 accurately reflected cell cycle distribution. We wanted to verify that c-Myb protein is expressed in each phase of the cell cycle. To detect c-Myb protein accurately in sorted cells we first

fixed the cells with formaldehyde as per a ChIP assay then stained and sorted the cells as described. We used the fixed cells to purify c-Myb protein on a hydroxyapatite column to select for c-Myb bound to DNA. Briefly, equal cell numbers of the fixed asynchronously growing cells, the G1 population, or a mixture of the S + G2/M populations were incubated with pre-equilibrated hydroxyapatite, washed, decrosslinked, and analyzed using a Western blot with anti-c-Myb antibodies. c-Myb protein were detected in all the samples, suggesting that c-Myb protein was both expressed and bound to DNA during the cell cycle in these cells (Figure 1C).

c-Myb binding oscillates during the cell cycle

We verified that c-Myb is expressed at each phase of the cell cycle so we performed our sorting procedure followed by ChIP assay to characterize c-Myb specificity at c-Myb target promoters. We wanted to determine how c-Myb regulates the cell cycle so we concentrated on CCNB1 and CCNE1, both of which are documented potential c-Myb target genes [50, 53]. Significant differences in c-Myb activity/specificity were detected when we performed ChIP at these target genes with two independent anti-c-Myb antibodies. The 1493 antibody specifically recognizes an epitope upstream of the FAETL domain and the Ab 1-1 antibody is specific to an epitope in the C-terminal domain (Refer to Chapter 3 Figure 1). Figure 2A shows the results of ChIP assays performed at CCNB1, which demonstrated that c-Myb was bound to the CCNB1 promoter in asynchronous cells, G1 fractionated cells, and G2/M fractionated cells. We used two different c-Myb-specific antibodies for these experiments, and both

antibodies detected significant enrichment of the Cyclin B1 promoter in the same populations. We performed RNA isolation in fractionated cells and detected slightly higher levels of *CCNB1* transcripts in the S/G2/M sample (Figure 2B). In addition, our RNA expression analysis verified that *c-myb* transcript was detectable in G1 and S/G2/M supporting our protein expression data in Figure 1.

We also assessed the association of c-Myb with a binding site that we identified upstream of the Cyclin E1 promoter (Data shown in chapter 3 Supporting Data). Strikingly, c-Myb was not detectable at this enhancer in the asynchronous, G1 or S phase fractions, but c-Myb immunoprecipitation strongly enriched for the Cyclin E1 promoter in the G2/M phase cells (Figure 3A). Expression experiments showed that *CCNE1* mRNA levels correlated with the presence of c-Myb at the enhancer, since the RNA was only detectable in the S/G2/M phase sample (Figure 3B), suggesting that c-Myb binding led to activation of the *CCNE1* gene in the S/G2/M phase cells. We presume that the inability to detect c-Myb at *CCNE1* in asynchronously growing cells was due to the relatively small fraction of G2/M phase cells in the total population. Thus, these experiments show two important results. First, that c-Myb activity and specificity appears to be cell cycle regulated, leading c-Myb to bind to and activate different promoters in different phases of the cell cycle. And second, that c-Myb activity can vary considerably in different subsets of cells, such as the G2/M population of Jurkat cells, and that these activities can be masked or go undetected if only total populations of asynchronously growing cells are analyzed.

Ectopically expressed c-Myb is also cell cycle-regulated

The regulation of c-Myb protein expression is quite complicated, involving changes in *c-myb* gene regulation [40, 54, 149], including mechanisms that affect mRNA elongation [142, 144] as well as alternative splicing [118, 120, 180] and microRNAs that bind in the long 3'-untranslated region to control *c-myb* mRNA stability and translation [132, 138]. The c-Myb protein is also known to be post-translationally modified at numerous positions [88, 96, 99, 103], so there was a possibility that these modifications could affect antibody binding, artificially affecting the outcome of the ChIP assays. To test whether these mechanisms contributed to our cell cycle results, we used lentiviral vectors to generate a stably transduced derivative of Jurkat cells expressing a FLAG epitope-tagged version of c-Myb. In these cells, the FLAG-tagged version of c-Myb is independently expressed from a different promoter, is engineered to react with a different (anti-FLAG) antibody, and the encoded mRNA lacks the normal 3'UTR region containing the regulatory microRNA binding sites [37]. Control experiments showed that the anti-FLAG antibody efficiently immunoprecipitated the epitope-tagged protein from these cells (Figure 4). We performed the same fixation-sorting-ChIP assay with the cells expressing the FLAG-tagged c-Myb, and obtained nearly identical results as with the other antibodies. As shown in Figure 5A, the FLAG-tagged c-Myb was associated with the Cyclin B1 gene promoter in the asynchronous, G1 and G2/M phase cells, but was not detectable in the S phase cells. Similarly, as shown in Figure 5B, the FLAG-tagged c-Myb only associated significantly with the Cyclin E1 promoter in the G2/M phase cells, we obtained the same result using two different c-Myb-specific antibodies (Figure

2 and Figure 3). These results suggest that our ChIP assay results are not due to differences in antibody efficacy and rule out the possibility that our ChIP assays were detecting other transcription factors, e.g. the related B-Myb protein, which has also been shown to regulate the *CCNB1* promoter in the G2/M phase of the cell cycle [157, 159, 160]. Based on all of these results, we conclude that c-Myb activity and/or specificity change dynamically during the cell cycle, and that despite being expressed in all cell cycle stages, even when ectopically expressed from a different promoter, c-Myb associates with different target gene promoters in different phases of the cell cycle.

Cell cycle mechanisms governing c-Myb specificity are not limited to cyclin gene promoters

The results described above suggest that c-Myb associates with the promoters of certain genes, such as *CCNB1* and *CCNE1*, in a cell cycle-dependent manner. However, it was possible that genes important for cell cycle regulation were unusual or different than other Myb-regulated genes. To extend our results we also analyzed the association of c-Myb with the promoters of genes that, unlike cyclins, are not expected to be cell cycle regulated. We previously identified the *CXCR4* gene as a potential c-Myb target, based on its activation in microarray assays [21]. *CXCR4* is the receptor for the chemokine Stromal Cell-Derived Factor-1 and its expression correlates with poor prognosis in several types of leukemia [181, 182]. Over-expression of *CXCR4* has also been demonstrated to increase the proliferation of CD34+ cells and the stem cell potential of CD34+ cells in re-population assays [183]. In Jurkat cells, we did not detect c-Myb at the *CXCR4* promoter in G1 phase cells, but both of our anti-c-

Myb antibodies detected c-Myb at the *CXCR4* promoter in G2/M phase cells (Figure 6A). However, only one of the antibodies, our rabbit antiserum 1493, successfully enriched for the *CXCR4* promoter in the S phase cells, suggesting that the two antibodies detected different forms of c-Myb, or perhaps that the epitope recognized by monoclonal Ab 1-1 was modified or masked in the S phase cells. To confirm these results, we again utilized the Jurkat cells stably expressing FLAG epitope-tagged c-Myb. Using the anti-FLAG antibodies, c-Myb was detected at the *CXCR4* promoter in both the S and G2/M phase cells, but not in G1 phase cells (Figure 6B). As an independent measure, we used a double thymidine block to synchronize the Jurkat cells at the G1/S boundary, then performed ChIP assays at various times after releasing the block. As shown in Figure 6C, we did not detect c-Myb at the *CXCR4* gene promoter in the blocked cells (time 0), but saw a peak in c-Myb binding at 3 hr, which corresponded to 50% of the cell in early S-phase, followed by less binding at later times during a synchronized cell cycle. Thus, c-Myb binding to the *CXCR4* promoter changed in a time-dependent manner as the cells progressed through the cell cycle. Since c-Myb associated with the *CXCR4* promoter in S phase, these results rule out the possibility that c-Myb was in some way inactivated during S phase, which could have explained the lack of S phase binding to the *CCNB1* or *CCNE1* promoters, described above. These results confirm that c-Myb associates with different genes in different phases of the cell cycle in Jurkat T cells, and demonstrate how ChIP results can vary when different antibodies are used. The results also show, quite unexpectedly, that c-Myb only associates with

the *CXCR4* gene promoter in cells that are actively progressing through the cell cycle, and not in the G1 cells that make up more than 60% of the cells in the culture. Thus, the asynchronous population of Jurkat cells contains several subpopulations of cells that can be distinguished both by stage in the cell cycle and by the genes that c-Myb is associated with.

Discussion

There is ample evidence that c-Myb is involved in the regulation of proliferation and differentiation, and that mutated versions of c-Myb affect the cell cycle [175]. Since c-Myb had been implicated in the regulation of cyclin genes *CCNB1* [50] and *CCNE1* [53], which are regulated during the cell cycle, we set out to determine whether c-Myb activity was also cell cycle-regulated. We found that c-Myb binds to different promoters in distinct stages of the cell cycle, suggesting that its specificity was dynamically regulated, allowing it to activate or repress different target genes in different phases of the cell cycle. This is a different mechanism than has been described for the E2F proteins, whose stimulatory and inhibitory family members are regulated through cell cycle-mediated changes in interactions with Retinoblastoma protein and the related pocket proteins p107 and p130 [177, 184]. It is also different than the FOXO proteins, which become inactivated and sequestered in the cytoplasm after phosphorylation by kinases like Akt [178]. Unlike E2F and FOXO proteins, c-Myb remains active throughout the cell cycle, but it interacts with different promoters at different times. For example, we found that c-Myb is associated with the *CCNB1* promoter in G1, the *CXCR4* promoter in S phase, and the *CCNB1*,

CCNE1 and *CXCR4* promoters in G2/M. Thus, c-Myb does not appear to be activated or inactivated during the cell cycle. Instead, as the cell cycle progresses, c-Myb becomes retargeted to different promoters in a time- and stage-dependent manner. The retargeting of c-Myb also distinguishes it from the related transcription factor B-Myb (MYBL2), whose ability to activate transcription is regulated by G2-specific Cyclin A/CDK2 phosphorylation [109, 185] and which is a component of the LINC complex involved in activation of G2/M specific genes [157, 159, 160].

The cell cycle regulation of c-Myb is reminiscent of NF-Y, the ubiquitously expressed transcription factor that binds CCAAT-box promoter elements [186, 187]. NF-Y, which has histone-like subunits, displaces nucleosomes from multimeric CCAAT-boxes at the promoters it regulates and stimulates histone acetylation and gene activation [188, 189]. The c-Myb transcription factor is also involved in chromatin remodeling, via its Myb/SANT domain, which is related to components of chromatin remodeling complexes [74, 75]. The c-Myb Myb/SANT domain binds to histone tails and promotes their acetylation, stimulating the remodeling of chromatin and gene activation [76]. Thus, NF-Y and c-Myb may share the ability to initiate the remodeling of chromatin and the activation of cell cycle regulated genes.

A major unanswered question concerns how c-Myb and NF-Y become targeted to the appropriate promoters at the correct times in the cell cycle. NF-Y regulates different classes of promoters at different times in the cell cycle, but it is thought to activate constitutively: the cell cycle-dependent regulation occurs

through other factors, like E2F, that repress or activate at specific times in the cell cycle [190]. In contrast, the regulation and specificity of c-Myb appears to be regulated during the cell cycle and may be linked to its interactions with Cyclin D1/CDK complexes, since phosphorylation at specific residues could change the affinity of c-Myb for specific promoters by altering its ability to interact in a combinatorial fashion with other proteins [56]. Several types of evidence have shown that the specificity of c-Myb can be affected by mutations [21, 52, 69] and that c-Myb regulates completely different sets of target genes in different cell types [21, 80], suggesting that its specificity is controlled by interactions with other proteins. Our new results suggest that these interactions and the activities of c-Myb are not only tissue-specific, but also change dynamically during the cell cycle.

A final question is whether the changing specificity of c-Myb is linked to its potential oncogenic activity. We have shown that the mutations that render c-Myb oncogenic also completely change its specificity, leading to the activation of different sets of target genes [21, 70]. Our discovery of cell cycle specific changes in c-Myb activity raise the possibility that oncogenic versions of c-Myb may transform cells by regulating some target genes in the incorrect stage of the cell cycle, which could stimulate cell cycle progression or inactivate cell cycle checkpoints. There is evidence that oncogenic versions of c-Myb push transformed cells through the cell cycle, even in the absence of mitogenic growth factor signals [191]. There is also a difference in the interactions between Cyclin D1 and normal or oncogenic versions of c-Myb [51]. Thus, oncogenic versions of

c-Myb may have altered cell cycle-dependent activities, which could be the key to their transforming activities.

Experimental Procedures

Cells and Culture Conditions

Human Jurkat T-cells (ATCC TIB152 Manassas, VA) were cultured at 37°/5% CO₂ in RPMI + Glutamax medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA).

Cell Cycle Analysis

For thymidine block, cells (5×10^5 /ml) were incubated in 2 mM thymidine for 16 hr, released in fresh medium for 9 hr then blocked again with a second round of 2 mM thymidine for 16 hr. After release, cells were collected at different time points and CHIP was performed with anti-Myb 1493 antibodies. For RNA expression analysis, asynchronous cell cultures were stained with Hoechst DNA content dye (Invitrogen, Carlsbad CA) and sorted based on DNA content as described (G1 or S/G2/M populations) [192]. RNA was isolated from equal cell numbers with RNeasy Kit (Qiagen, Valencia CA) according to manufacturer's protocol and reverse transcribed (3 ug) into cDNA template with Reverse Transcriptase III (Invitrogen, Carlsbad CA). Taqman (Applied Biosystems, Foster City, CA) real time PCR was used (except for *CCNE1*, SYBR green was used) to analyze the expression of *c-myb*, *CCNB1*, *CCND1*, and *PPIA*. For protein expression, formaldehyde-fixed proteins were extracted and purified by hydroxyapatite chromatography as described [193, 194]. Briefly, cells were lysed in lysis buffer (5M Urea, 2M guanidine hydrochloride, 200mM potassium

phosphate buffer, and 2M NaCl supplemented with 1 μ M chymostatin, leupeptin, antipain, pepstatin-A, 1mM each phenylmethylsulfonyl fluoride and benzamidine), incubated on pre-equilibrated hydroxyapatite beads (Biorad, Hercules, CA), decrosslinked at 65° for 5 hours, and precipitated with chloroform: methanol (1:4) before analysis by Western blot.

Immunoprecipitation

Cultured cells were harvested by centrifugation and washed with phosphate buffered saline. Cells (1X10⁷ per IP) were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.4, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 0.1% SDS, 150 mM NaCl with 1 μ M each of chymostatin, leupeptin, antipain, pepstatin-A; 1 mM each of phenylmethylsulfonyl fluoride and benzamidine) for 1 hr on a rocker at 4° and centrifuged at maximum speed (13000 g) for 10 min in a microcentrifuge to remove debris. The supernatant (10% of volume) was taken as input control and the remainder was used for subsequent immunoprecipitation (IP) with anti-c-Myb (Millipore, Billerica, MA) or anti-FLAG antibodies (Sigma, St. Louis, MO). Following overnight antibody incubation, protein A/G agarose (30 μ l) (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated for 30 minutes at 4°C. The agarose: antibody complexes were harvested by centrifugation and washed in triplicate with RIPA buffer (50 mM Tris-Cl, pH 7.4, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 0.1% SDS, 150 mM NaCl with 1 μ M each chymostatin, leupeptin, antipain, pepstatin-A; 1 mM each phenylmethylsulfonyl fluoride and benzamidine). Samples were resuspended in 5X SDS-PAGE sample buffer and analyzed by Western blot analysis with anti-c-Myb [66].

Chromatin Immunoprecipitation

Standard methods were used [195, 196] except chromatin was sheared in a 200 μ l volume of 50 mM Tris pH 8.0 (Sigma, St. Louis, MO) by adding 40 units of Micrococcal nuclease (USB, Cleveland, Ohio) for 10 min at 37°C. Adding EDTA to 10 mM stopped the reaction and cells were lysed in 1% v/v SDS. Equal aliquots were subjected to immunoprecipitation with anti-Myb 1493 immune serum (10 μ l) or anti-Myb monoclonal 1-1 antibodies (Millipore, Billerica MA) or control non-immune serum (10 μ l) for 24 hr. Anti-Myb 1493 is a rabbit polyclonal developed against a peptide HQTILDNVKNLLEFAE. Primers used for QPCR reactions using SYBR green (Biorad, Hercules CA) are listed in Supplemental Table 1 (Chapter 2).

Myb Lentivirus Vector

The cDNA encoding an N-terminal FLAG-tagged human c-Myb [21] was cloned into the unique PacI site of the pHR IRES GFP lentiviral vector (kindly provided with packaging vectors by Dr. Bruce Bunnell, Tulane University) directly downstream of the human elongation factor 1 alpha promoter. Viral particles were produced by calcium phosphate transient transfection of 293 FT cells (Invitrogen, Carlsbad CA) along with the lentiviral packaging plasmid delta 8.9 and the pMD.G plasmid expressing the vesicular stomatitis virus glycoprotein. Cell culture supernatant was collected twice in 24 hr intervals post transfection and viral supernatant was concentrated by ultrafiltration using an Ambion Ultracell 100 kDa NMWL filter unit (Millipore, Billerica MA). Jurkat cells were

transduced in the presence of 8 ug/ml of polybrene (Sigma, St. Louis MO) and GFP+ cells were recovered by cell sorting.

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Figure Legends

Figure 1. Accurate fractionation of asynchronous cultures

(A) Jurkat cell cycle analysis. Cell cycle histogram of a representative culture of Jurkat T cells progressing through the cell cycle. The cells were fixed with formaldehyde, stained with Hoechst 33342, analyzed by flow cytometry and sorted into G1, S, or G2/M populations. The fraction of cells in each cell cycle phase is indicated at the top. (B) Expression of CCND1. cDNA was prepared from Jurkat cells sorted into G1 or S/G2/M cell cycle fractions. The samples from asynchronous (Asyn), G1 or S/G2/M fractions were analyzed by semi-

quantitative PCR with CCND1 and PPIA specific primers. (C) Expression of c-Myb protein. Formaldehyde fixed Jurkat cells were sorted into G1 or S/G2/M fractions. Equal cell numbers were used to isolate protein and select for protein bound to DNA through a hydroxyapatite column. Western blot with anti-c-Myb antibodies detected c-Myb in each fraction.

Figure 2. Cell cycle regulation of the CCNB1 promoter.

(A) ChIP analysis with sorted fractions. ChIP assays with anti-c-Myb 1493 antibodies or anti-c-Myb ab-1-1 were performed with chromatin from formaldehyde fixed and stained Jurkat cells. QPCR was used to measure enrichment of the *CCNB1* promoter. Error bars show standard deviation of triplicate QPCR reactions. (B) Levels of *CCNB1* mRNA in sorted G1 or S/G2/M fractions of cells. cDNA was prepared from sorted Jurkat T-cells and the normalized RNA level of *CCNB1* and *c-myb* were measured by Taqman real time PCR. Error bars show standard deviation of triplicate QPCR reactions.

Figure 3. Regulation of CCNE1 is cell cycle dependent.

(A) ChIP analysis at *CCNE1*. ChIP assays with anti-c-Myb 1493 antibodies or anti-c-Myb ab-1-1 were performed with chromatin from formaldehyde fixed and stained Jurkat cells. QPCR was used to measure enrichment of the *CCNE1* promoter. Error bars show standard deviation of triplicate QPCR reactions. (B) Levels of *CCNE1* mRNA in sorted G1 or S/G2/M fractions of cells. cDNA was prepared from sorted Jurkat T-cells and the normalized RNA level of *CCNE1* was measured by Sybr green based real time PCR. Error bars show standard deviation of triplicate QPCR reactions.

Figure 4. Expression of Flag-tagged c-Myb in Jurkat cells.

Whole cell extracts from Jurkat cells stably expressing FLAG epitope-tagged c-Myb were immunoprecipitated with anti-FLAG or anti-Myb 1-1 antibodies. Proteins (input or immunoprecipitated) samples were analyzed by Western blot.

Figure 5. Cell cycle regulation of ectopically expressed, epitope-tagged c-Myb

Stably-transduced Jurkat cells expressing FLAG epitope-tagged c-Myb were fixed and sorted and equal numbers of cells from the asynchronous (Asyn) population or the G1, S or G2/M cell cycle fractions were subjected to ChIP assays as described in Figure 1, using anti-FLAG or IgG (control) antibodies. QPCR assays were used to measure the enrichment of the (A) *CCNB1* and (B) *CCNE1* promoters. Error bars show standard deviation from triplicate QPCR reactions.

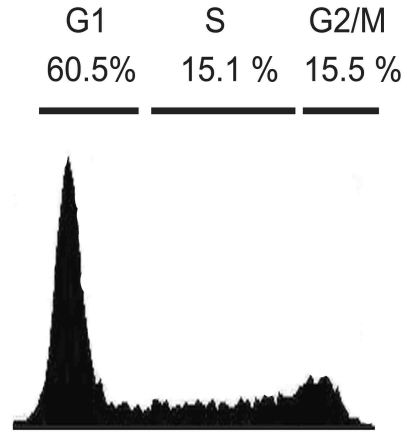
Figure 6. c-Myb cell cycle specificity is not limited to cyclin genes

Jurkat cells (A) or Jurkat cells stably expressing FLAG epitope-tagged c-Myb (B) were fixed, sorted into cell cycle fractions and chromatin was prepared from equal numbers of cells from the G1, S or G2/M fractions and subjected to ChIP assays as described in Figures 2 and 3. QPCR assays were used to measure enrichment of known c-Myb binding sites in the *CXCR4* promoter. The results in panels (A) and (B) were obtained using anti-Myb antibodies 1493 and 1-1, or anti-FLAG antibodies, respectively. (C) Jurkat cells were synchronized with a double thymidine block then samples were collected for ChIP using anti-Myb 1493 antibodies at various times after release. QPCR assays were used to

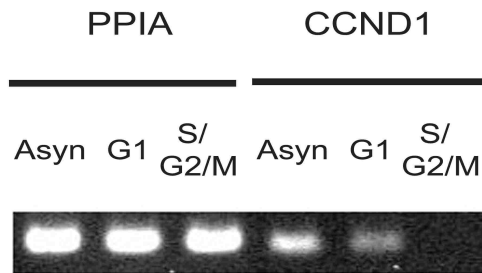
measure enrichment of the *CXCR4* promoter at time points from 0 to 24 hours. Error bars show standard deviation of triplicate QPCR assays. Each sample is normalized to IgG control.

Figure 1:

A



B



C

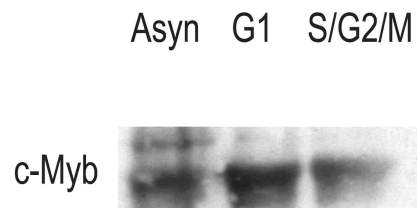
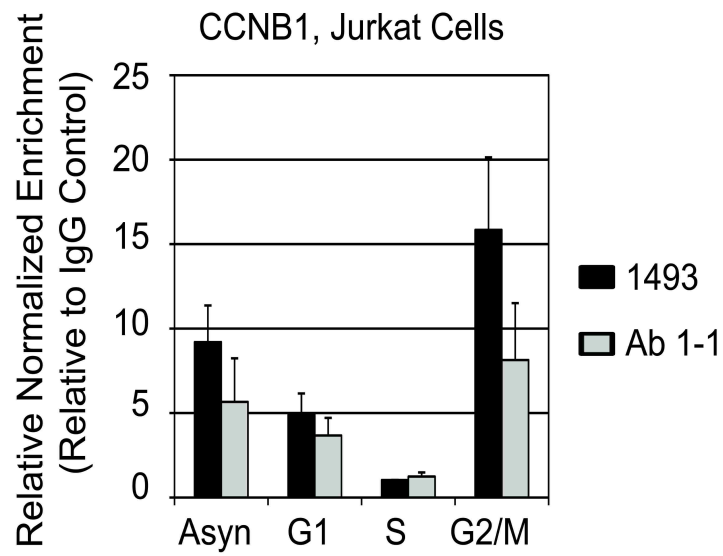


Figure 2

A



B

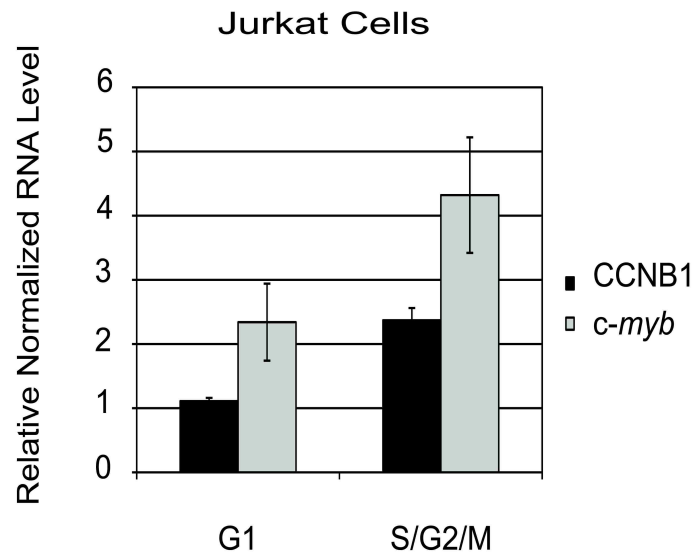
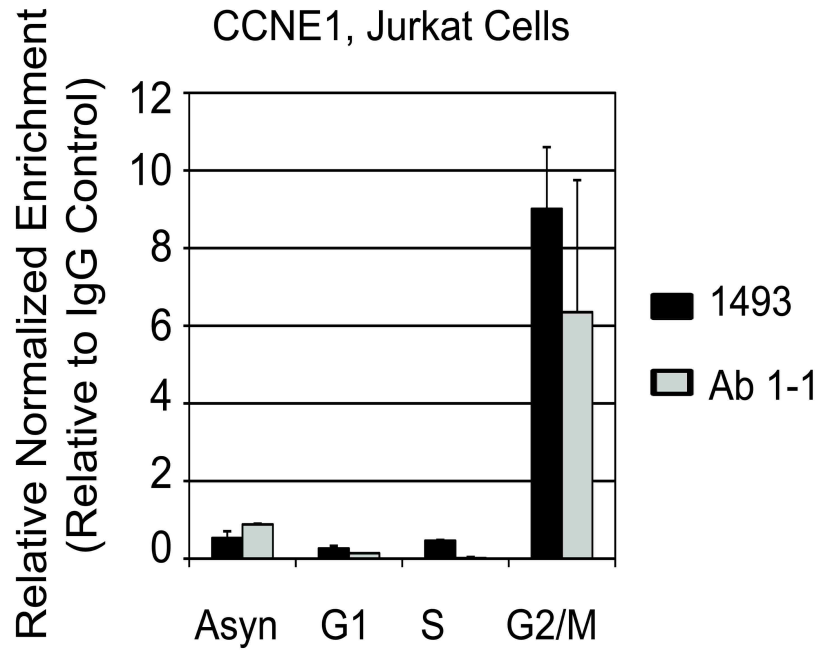


Figure 3

A



B

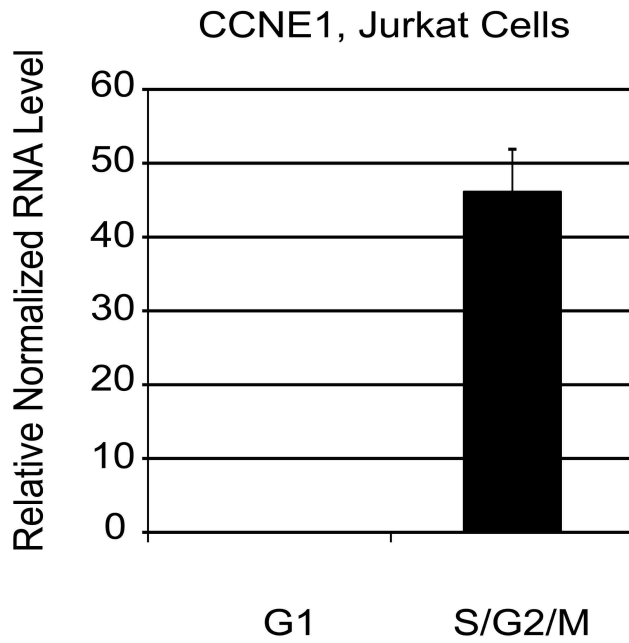
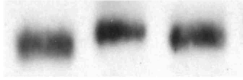


Figure 4

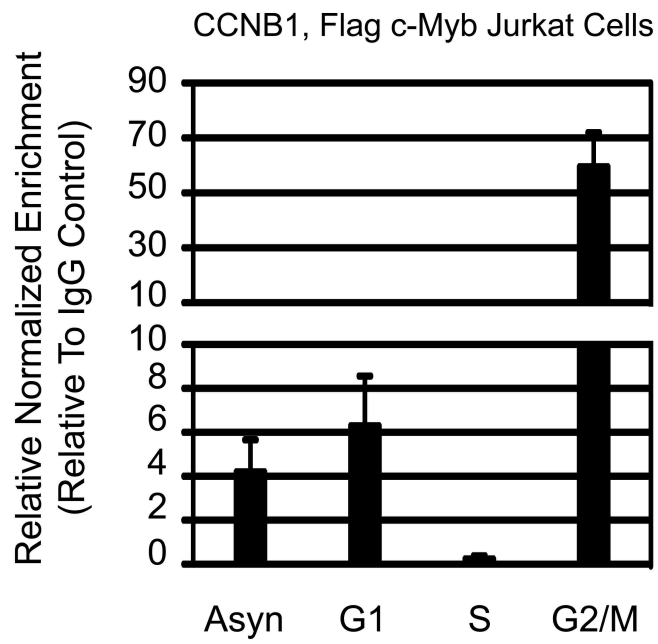
Input Flag Myb



WB: Anti-c-Myb

Figure 5

A



B

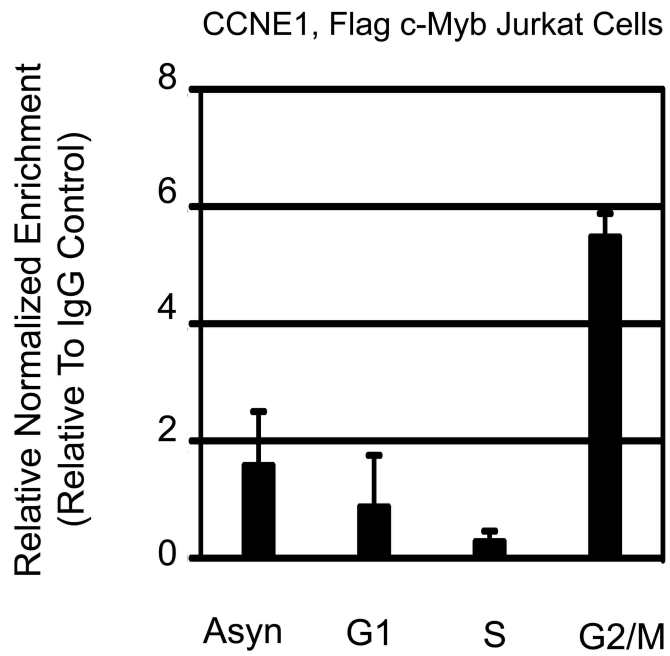
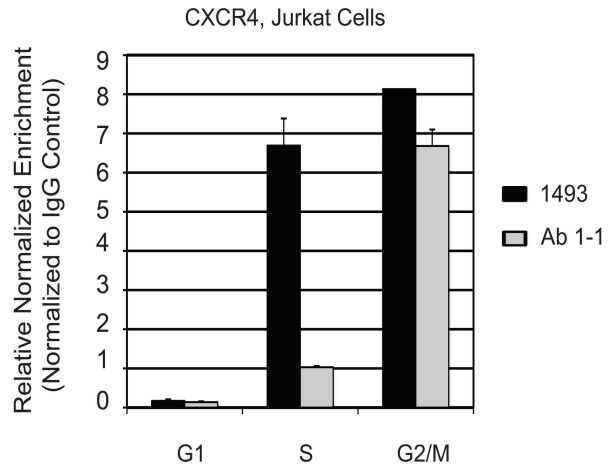
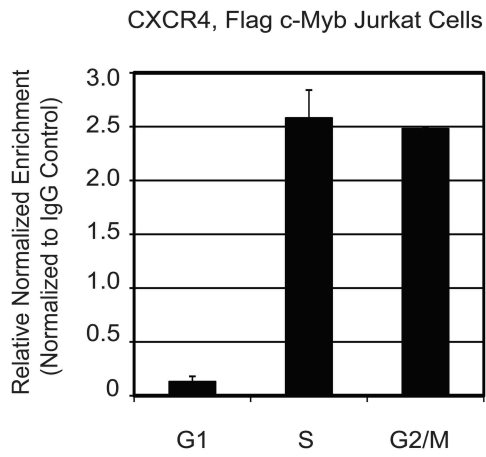


Figure 6

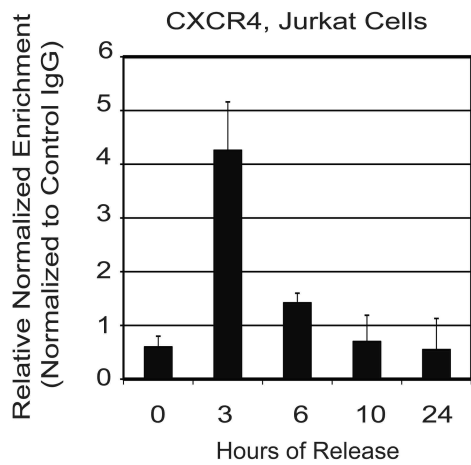
A



B



C



Supplemental Table 1: Real time PCR primer pairs

| Name | Forward Primer | Reverse Primer | Location |
|--------------|---------------------------------|--------------------------------|-----------------|
| CCNB1 | TTGTGCCCCACCTTAAT | CATGTTGATCTTCGCCTTATT | +717 to +837 |
| CCNE1 | GGCCCCCTCCTCTTAGTA | CAGGTCCTCAAAGGCCTTA | -4524 to -4327 |
| CXCR4 | GCACCTGACCCTAGTGATGCT | TTCAGGAGGTAAAAGGAGTAAAGA | -442 to -525 |
| c-Kit | GCGGTGCCAGGAGCTCCTAA | CTCCCCAGACAATAAAGGTCGACT | -444 to -772 |
| GAPDH | GTGCAGGAGTTCATGACCAAC | CACCTGGGCTAGAGTAC | -245 to -425 |
| CCNE1-R | CTGGAGGTGGCTGGTGTACT | CCATCCTTCTCCACCAAAGA | N/A |
| PCR Clone | AGTTAATTAACCACTGCTTACTGGCTTATCG | AGTTAATTAACAACAGATGGCTGGCAACTA | N/A |

Supplemental Table 2: Taqman PCR probes

| Gene Name | Catalog Number |
|------------------|-----------------------|
| c-myb | Hs00193527_m1 |
| PPIA (20X) | 4333763T |
| CCNB1 | Hs00259126_m1 |
| CCND1 | Hs00277039 |

Dynamic changes in c-Myb specificity: Potential Mechanism for Oncogenesis

Until recently it has been difficult to characterize c-Myb specificity in different cell populations, but we have utilized well established techniques such as staining by DNA content, ChIP, and flow cytometry to describe the repositioning of c-Myb from one promoter to the another in a cell cycle dependent manner. These data demonstrate that c-Myb specificity and activity are dynamic and different sub-populations of cells exist in a single culture condition. These results imply that some activity is hidden or undetectable if fractionation of cells is not performed and it is plausible that this hypothesis can be expanded to the differentiation process, for instance, differentiation is characterized by cell surface markers, it is unclear if cells consistently expressing one surface marker are all in the same cell cycle phase. Therefore, in order to characterize all sub-populations of c-Myb we must continue to refine techniques such as ChIP and flow cytometry based sorting.

Identification of target genes in normal primary bone marrow is essential. We have demonstrated that c-Myb repositions in a cancer cell line, but this mechanism may be different in primary cells. If repositioning becomes deregulated and G1 specific genes are activated by c-Myb during G2/M, the cell could push through cell cycle checkpoints and become tumorigenic. It is likely that changes to the regulatory domain of c-Myb (C-terminal domain) such as truncation or deletion could affect specificity in a cell cycle dependent manner because structural changes affect proliferation. However, we have not uncovered

the mechanism that determines where c-Myb binds and how long it binds there. We can only suggest that the wide array of post-translational modifications and protein interactions c-Myb undergoes provide a means of regulating specificity in a very time dependent manner.

Chapter 3: Changes in c-Myb activity during proliferation

c-myb is expressed in epithelial cells and is deregulated in colon cancer, breast cancer, and lung cancer [28, 29, 35], which suggests that c-Myb regulates proliferation or differentiation of these cells. Epithelial cells, such as estrogen positive breast cancer cell lines, offer a unique approach to study the role of c-Myb in proliferation because c-Myb is highly associated with estrogen receptor status [31] and treatment of cells with estrogen relieves a block in *c-myb* transcription elongation [54]. The level of *c-myb* RNA can be manipulated by withholding estrogen thereby synchronizing cells and initiating cell cycle progression with exogenous estrogen treatment. This system allows for the characterization of c-Myb activity during proliferation and since these cells are highly synchronized and therefore, represent a specific subset of cells it is possible to identify c-Myb sub-populations.

These data in MCF-7 cells, an estrogen receptor positive cell line support the hypothesis that protein interactions change while c-Myb is bound to target genes. We have used two independent antibodies to identify genome wide target genes by ChIP on chip (ChIP on a promoter tiling array) and characterized c-Myb activity during different stages of epithelial cell proliferation after estrogen stimulation. We found that antibodies specific to different regions of the c-Myb protein have different detection capabilities in different growth conditions even though a substantial amount of c-Myb is expressed and bound to specific target genes. Our data suggest that protein interactions mask c-Myb epitopes in a

growth dependent manner and furthermore, c-Myb does not bind to the same genes in estrogen deprived cells when c-Myb protein is highly expressed.

Author Contributions

The following paper describes our observations in MCF-7 breast cancer cells. It includes data from four authors Anita M. Quintana (AMQ), Fan Liu (FL), John P. O'Rourke (JPO) and Scott A. Ness (SAN). AMQ performed all ChIP assays, whole genome amplification, ChIP on chip with two independent Myb and H3K4me2 antibodies, data analysis, time course experiments, RNA expression of time course and knockdown and helped write the manuscript. FL developed the CXCR4 reporter construct and performed luciferase assays in Figure 1 at the CXCR4 promoter. JPO made the lentiviral particles for FLAG-tagged c-Myb, FLAG tagged v-Myb, and shRNA constructs. JPO verified knockdown of c-Myb by Western blot. SAN developed the idea, provided funding and mentorship, and helped write and approve the manuscript.

Epithelial cell proliferation triggers changes in c-Myb activities

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Changing activities of c-Myb during proliferation

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5F31HL090024.

Summary

c-Myb is a transcription factor that regulates proper hematopoiesis. While truncated derivatives are oncogenic, the characterization of existing endogenous c-Myb sub-populations with distinct activities is yet to be explored. Several studies have demonstrated that c-Myb interacts with a wealth of proteins such as p300/CBP, PIM1 kinase, and Pin 1 isomerase and many of these interactions are associated with post-translational modifications. These modifications occur in conserved regions and presumably account for differences in the activity of c-Myb and its viral counterpart v-Myb. We performed Chromatin Immunoprecipitation (ChIP) assays with antibodies specific for two highly conserved domains in the C-terminus to determine if these domains are associated with increased c-Myb activity. These domains were masked during estrogen stimulation in an opposing fashion suggesting that c-Myb activity is dependent on the availability of specific conserved domains.

Keywords

c-Myb activity, ChIP, estrogen, MCF-7 cells

Introduction

v-myb is a viral transforming oncogene derived from the cellular proto-oncogene, *c-myb*. *v-Myb* is encoded by a retrovirus that induces acute leukemia in chickens and transforms immature hematopoietic cells in tissue culture [7, 197]. Both *c-myb* and *v-myb* are transcription factors that regulate gene expression and *c-myb* is expressed in a variety of different tissues including breast, colon, endothelial, and hematopoietic cells. The role of *c-myb* in hematopoiesis has been well characterized and deletion of the *c-myb* gene results in an embryonic lethal phenotype due to defects in erythropoiesis [38]. More recently *c-myb* has been associated with duplication or translocation in pediatric T-cell acute leukemia (T-ALL) [32].

Ectopic over-expression of *c-Myb* or *v-Myb* by adenovirus has demonstrated that these two *Myb* proteins regulate distinct sets of endogenous genes [21]. Similar experiments with two highly related proteins, *A-Myb* and *B-Myb*, have demonstrated that this phenomenon is not restricted to truncated and mutated proteins [80]. These data suggest that regions outside of the highly conserved DNA binding domain affect activity of *Myb* proteins.

The *Myb* family of proteins has a highly conserved DNA binding domain, but their C-termini differ suggesting that while these proteins bind to similar DNA sequences, they are regulated by their own unique C-terminus. For example, *v-Myb* is truncated at the N and C-termini and contains eleven point mutations [102, 104, 198]. These deleted and mutated regions in the C-terminus have been

shown to affect the activity of v-Myb. In a similar fashion, modifications to the c-Myb protein can affect binding affinity and activity. The c-Myb protein is extensively modified by phosphorylation of the N-terminus, acetylation of the C-terminus and SUMOylation during stress [90, 96, 99]. In each case of post-translational modification, changes in DNA binding, protein stability, and protein interactions have been confirmed [68, 98, 106]. For example, c-Myb and v-Myb have similar DNA binding domains, but only c-Myb activates the chicken *mim-1* gene. *In vitro* transfection assays have demonstrated that both c-Myb and v-Myb are able to bind to the *mim-1* promoter [1, 55], but activation of the *mim-1* gene is dependent on the interaction of c-Myb with the chicken NF-M protein. v-Myb is unable to interact with and therefore lacks the ability to activate endogenous *mim-1* [56, 199].

These results suggest that post-translational modifications and protein interactions exists in regions not previously characterized and can affect the interpretation of certain types of experiments. Recently, Chromatin Immunoprecipitation (ChIP) has provided the transcription factor field with technology to determine endogenous binding sites. One potential limitation of this technique is that changes in epitopes due to formaldehyde fixation can affect the outcome of the experiment because formaldehyde fixation will fix protein interactions as well as DNA: protein interactions. For example, protein interactions with Pin 1 isomerase and p100 are dependent upon post-translational modifications such as phosphorylation. During formaldehyde fixation, Pin1 may be fixed to the c-Myb C-terminus masking specific antibody

epitopes [87, 198]. Other interactions with proteins such as p100 occur in the c-Myb DNA binding domain. These interactions along with many others would mask the Myb DNA binding domain in a ChIP assay. To overcome this potential limitation we utilized two independent antibodies, which recognized two independent domains to characterize the affect of protein domains, epitope changes, and c-Myb sub-populations on c-Myb activity in MCF-7 breast cancer cells. Our results suggest that c-Myb domains are masked in an estrogen dependent manner and the masking of these domains is associated with increased c-Myb activity.

Results

CXCR4 is a c-Myb target gene

Gene expression profiles of MCF-7 cells expressing c-Myb or the oncogenic variant v-Myb originally identified *CXCR4*, which encodes the receptor for the chemokine SDF-1, as a potential Myb target gene [21]. Both *c-myb* and *CXCR4* have been previously described as estrogen-inducible genes [54, 200]. Therefore, it seemed plausible that estrogen regulation of c-Myb could be responsible for subsequent activation of the *CXCR4* gene. The promoter region of the human *CXCR4* gene contains multiple potential c-Myb binding sites (Figure 1B), and when the promoter was introduced upstream of a luciferase reporter gene it was activated modestly by co-expression of c-Myb and more dramatically by co-expression of v-Myb (Figure 1C), suggesting that both c-Myb and v-Myb can bind and activate the *CXCR4* promoter. These results mimic

those obtained with the endogenous gene, which was more strongly activated by v-Myb than c-Myb [21], suggesting that the *CXCR4* gene is regulated by c-Myb.

CXCR4 and c-Myb are regulated by growth stage

To establish the link between estrogen regulation of c-Myb expression and the subsequent regulation of *CXCR4*, we turned to MCF-7 cells, a widely-used, estrogen-dependent mammary tumor cell line that can be growth-arrested in estrogen-depleted medium, then stimulated by adding back estrogen [54, 200]. In a time-course experiment, we found that estrogen stimulation caused both *c-myb* and *CXCR4* RNAs to increase about 3-4 fold relative to the estrogen-deprived cells by 6 hr (Figure 2A). The stimulation increased to 6-8 fold by 24 hr, but remained well above the basal level even in cells that were allowed to reach high cell density (Confl.). Thus, *c-myb* and *CXCR4* genes were induced with similar kinetics, consistent with the hypothesis that they were regulated by similar mechanisms.

Interestingly, a Western blot showed that c-Myb protein levels were fairly constant in all the samples, although there was some accumulation of a faster-migrating form of c-Myb, especially in the cells that had been stimulated by estrogen for 24 hr (Figure 2B). This suggests that c-Myb protein is fairly stable and remains expressed at similar levels in MCF-7 cells, despite estrogen-induced changes in *c-myb* mRNA levels.

Next, we used a Chromatin Immuno-Precipitation (ChIP) assay to determine whether c-Myb was directly binding the *CXCR4* gene promoter.

Briefly, MCF-7 cells that were deprived of estrogen, stimulated for 24 hr or allowed to reach high cell density (Figure 2C) were fixed with formaldehyde and the chromatin complexes were immunoprecipitated using control non-immune IgG or two different anti-c-Myb antibodies, 1-1 or 1493, which recognize different epitopes in the c-Myb protein (see Figure 1A). Both of these antibodies have been highly characterized and are specific for c-Myb (data not shown, demonstrated by Dr. Fan Liu). The immunoprecipitated chromatin complexes were then assayed for the enrichment of the *CXCR4* promoter, or control promoters, using quantitative real-time PCR (QPCR) assays.

Unexpectedly, the two different anti-Myb antibodies gave completely different results in the ChIP assays. Neither antibody detected c-Myb at the *CXCR4* promoter in the estrogen-deprived cells (Figure 2D), suggesting that the ability of c-Myb to associate with the *CXCR4* promoter is somehow inhibited by estrogen-deprivation, despite the constant levels of c-Myb protein in the cells (Figure 2B). The monoclonal 1-1 antibodies, which recognize an epitope from exon 11 in c-Myb, enriched for the *CXCR4* promoter about 8-fold (Figure 2D, gray bars), but only in the estrogen-stimulated cells and not in the confluent cells. In contrast, the 1493 antiserum (Figure 2D, black bars), which was raised against a peptide from exon 9 in c-Myb, strongly enriched for the *CXCR4* promoter in the confluent cells, but not in the estrogen-stimulated cells. These results differ markedly from our experience using the two antibodies in other cell types, such as hematopoietic cells, where the two antibodies give nearly identical results in ChIP assays (AMQ, JPO and SAN, submitted, Chapter 2). The results suggest

that post-translational modifications, conformational changes or protein-protein interactions were producing two different populations of c-Myb protein at the *CXCR4* promoter, one of which was recognized only by the 1-1 antibody in estrogen-stimulated cells, and a different fraction that was recognized only by the 1493 antiserum in the confluent cells. Thus, there appeared to be a switch in the c-Myb protein associated with the *CXCR4* promoter, triggered by the different growth conditions, which resulted in the ability to be detected by only one or the other antibody.

Growth conditions also affect FLAG-c-Myb binding the CXCR4 promoter

The results described above suggested that growth conditions affected the association of c-Myb with the *CXCR4* promoter and/or the ability of individual anti-Myb antibodies to recognize c-Myb associated with the promoter. In order to have an independent means of measuring whether c-Myb was bound to the *CXCR4* promoter in the confluent and estrogen-stimulated cells, we generated a stably transfected variant of MCF-7 cells expressing a FLAG epitope-tagged version of full-length c-Myb. As shown in Figure 3A, anti-FLAG antibodies efficiently immunoprecipitated the FLAG-tagged c-Myb protein, which ran slightly higher than the endogenous c-Myb protein in an SDS PAGE Western blot. To test specificity, we performed a ChIP assay on the FLAG-Myb MCF-7 cells and compared the ability of the anti-FLAG antibodies to enrich for the *CXCR4* promoter compared to two non-specific regions of the genome that did not contain Myb binding sites. As shown in Figure 3B, the anti-FLAG antibodies efficiently enriched for the *CXCR4* promoter that contains multiple c-Myb binding

sites, but did not enrich other regions of the *CXCR4* gene (NS-1) or a non-specific control region from the *MAT2A* gene promoter (NS-2) [201]. Thus, the ectopically expressed, FLAG-tagged c-Myb protein associated with the same Myb binding site in the *CXCR4* promoter as the endogenous c-Myb protein, and was efficiently immunoprecipitated by the anti-FLAG antibodies.

Next, we repeated the experiment described in Figure 2D, this time using anti-FLAG antibodies to see if FLAG-tagged c-Myb was associated with the *CXCR4* promoter in the three different growth conditions. As shown in Figure 3C, immunoprecipitation with anti-FLAG antibodies gave little to no enrichment of the *CXCR4* promoter in the estrogen-deprived cells, suggesting that c-Myb was not bound to the promoter under those growth conditions. In contrast, anti-FLAG resulted in a modest, 5-fold enrichment of the *CXCR4* promoter in the stimulated cells, and over 90-fold enrichment in the confluent cells. Thus, the results using FLAG antibodies mimicked the results obtained with the 1-1 antibodies in the estrogen-stimulated cells, and mimicked the results obtained with the 1493 antibodies in the confluent cells. Overall, there appeared to be quantitative differences in the amount of c-Myb bound to the *CXCR4* promoter in the different conditions. In addition, there appeared to be a switch in c-Myb which affected the accessibility or availability of the epitopes recognized by the two different antibodies in the stimulated vs. confluent cells.

Global assays detect additional c-Myb target genes in MCF-7 cells

The results we observed with the *CXCR4* gene were intriguing, but we needed to identify additional Myb-regulated genes in MCF-7 cells in order to

determine whether the growth condition-induced switch in the c-Myb epitopes was a general or gene-specific phenomenon. To accomplish this, we performed whole genome chromatin immunoprecipitation coupled with analysis on promoter tiling arrays (ChIP on chip) to identify additional c-Myb target genes. Briefly, DNA purified from chromatin complexes immunoprecipitated by the two different anti-Myb antibodies (1493 or Ab1.1) or a control IgG antibody were amplified, labeled and hybridized to Affymetrix Promoter Tiling arrays with probes spaced at 35 nt intervals around the promoters of approximately 25,000 human genes. This approach identified several thousand statistically significant ($p < 1 \times 10^{-5}$) c-Myb binding sites enriched by one or more of the antibodies in confluent or estrogen-stimulated cells (see Supporting Data), including previously described c-Myb target genes *CCNB1*, *CCNE1*, *CXCR4*, *KIT*, *MYB* and *MYC* and other genes that have not been previously identified as Myb-regulated genes, including *ELK4*, *EPB41*, *JUN*, *KLF4*, *NANOG* and *SND1*. Although we will not present a detailed analysis of the ChIP on chip results here (See Supporting Data), the complete data set is available from the NCBI GEO database (accession GSE18706).

We performed two types of experiments to confirm that these putative target genes were regulated by c-Myb. First, we measured the expression levels of the target gene RNAs following estrogen deprivation, stimulation by estrogen for 6, 12 or 24 hr, or in cells that were allowed to become confluent. As shown in Figure 4A, estrogen led to strong activation of the *CCNB1*, *JUN* and *KLF4* genes, but not the *EPB41* gene, although the kinetics of activation differed somewhat for different genes. Second, we tested whether knocking down the expression of c-

Myb would also lead to knockdown of the target genes. We transduced MCF-7 cells with a previously published retrovirus encoding a doxycycline inducible short hairpin RNA directed against *c-myb* [54]. Briefly, transduced cells (scrambled or *MYB* shRNA) were treated with doxycycline for 24 hr, RNA was harvested and real time PCR was used to measure the relative expression of *c-myb*, *CCNB1*, *CXCR4* and *JUN*. Western blots verified that after 24 hr of doxycycline induction c-Myb protein was significantly reduced (Data not shown, John O'Rourke). As shown in Figure 4B, *c-myb* RNA levels decreased to less than 50% of normal and gene expression for *JUN*, *CCNB1*, and *CXCR4* decreased to 14%, 57%, and 27% of normal, respectively. The scrambled control shRNA did not cause a significant reduction in *c-myb* expression and had no effect on the levels of c-Myb target gene RNAs (Figure 5B). These results suggest that c-Myb is likely to bind and activate the promoters of these genes after estrogen stimulation.

Estrogen stimulation induces a switch in c-Myb

Our ChIP assays for the *CXCR4* promoter (Figure 2) suggested that estrogen stimulation induced a switch in c-Myb, such as conformational changes or post-translational modifications, which affected interactions with the two different anti-Myb antibodies. We next tested whether the switch could also be detected on the additional promoters identified through ChIP on chip assays (See Supporting Material). Briefly, we performed ChIP assays with chromatin purified from cells that had been estrogen deprived, stimulated for 24 hr or allowed to reach confluence. We performed parallel assays with both anti-Myb antibodies:

1-1 and 1493. As shown in Figure 5A, the 1-1 antibodies, which only detected c-Myb at the *CXCR4* promoter in estrogen-stimulated cells, also only detected c-Myb at the *EPB41*, *CCNB1* and *KLF4* promoters in the stimulated cells, but not in deprived or confluent cells. However, the 1-1 antibodies did detect c-Myb at the *JUN* promoter in confluent cells, but at about 10-fold lower levels than the very high enrichment it produced in the stimulated cells. As described above for the *CXCR4* promoter, the 1493 antibodies gave the opposite result (Figure 5B), enriching for the *JUN*, *CCNB1* and *KLF4* promoters only in the confluent cells, but not in the estrogen deprived or stimulated cells. The exception was the *EPB41* gene, since 1493 antibodies did detect c-Myb at that promoter in both the stimulated and confluent cells. As a further control, we repeated this experiment using the MCF-7 cells stably transduced with a FLAG-tagged version of c-Myb. As shown in Figure 5C, FLAG-c-Myb was detected at levels 4- to 10-fold above background at the *JUN*, *EPB41*, *CCNB1*, and *KLF4* gene promoters in both stimulated and confluent cells, but not in the estrogen deprived cells. Taken together, these results suggest that c-Myb is present at all of these promoters in both the stimulated and confluent cells. However, a switch of some sort occurs in c-Myb, making it accessible to the 1-1 antibodies only in the stimulated cells and to the 1493 antibodies only in the confluent cells.

The c-Myb switch is associated with global epigenetic changes

The results described above suggested that our use of two different anti-Myb antibodies had exposed a switch in c-Myb that was triggered in MCF-7 cells grown in different conditions, and that the switch affected c-Myb on multiple,

perhaps many target genes. We turned to a genome wide epigenetics assay to determine how global these changes were, and how many genes might be affected by the switch that we had detected. We performed ChIP on chip with antibodies specific for Histone H3 lysine 4 dimethyl (H3K4me2), which marks genes that are either primed for activation or that are actively being transcribed, and compared chromatin from cells that had been estrogen deprived then stimulated for 24 hr, or had been allowed to become confluent. We found dramatic changes in the number and locations of the H3K4me2 marks in the stimulated vs. the confluent cells, and the differences were gene specific. Examples of four types of genes that were found to harbor these marks are shown in Figure 6A. The *CCNB1* gene had the H3K4me2 mark in both stimulated and confluent cells. In contrast, the *CCNE1* gene, which is usually only expressed during S phase, only had this histone mark in the stimulated cells, and not in the confluent cells. In contrast, the *SND1* gene, which encodes a Myb-binding co-activator protein, was only marked in the confluent cells. The estrogen receptor-regulated *GREB1* gene [202] is representative of many genes that accumulated H3K4me2 marks in the confluent cells. There were also many genes that did not have the histone mark in either cell type.

A striking result comes from the Venn Diagram analysis, shown in Figure 6B. Of the nearly 2700 statistically significant H3K4me2 enriched sites detected in the stimulated cells, less than half (1114) were also detected in the confluent cells. In addition, more than 10,000 new H3K4me2 sites were detected in the confluent cells. The number of H3K4me2 sites is very large in the confluent cells

because many genes, like GREB1, had two or more detectable sites in those cells. Nevertheless, it is clear that there are major differences in the epigenetic patterns in the stimulated and confluent MCF-7 cells.

We also analyzed the overlap between promoters that had the H3K4me2 mark and also were enriched by the two different anti-Myb antibodies in the two different growth conditions. As shown in Figure 6C, more than 80% of the promoters detected by the 1-1 antibodies in the stimulated cells also had the H3K4me2 mark, indicating that they were primed for activation or already actively transcribed, but less than 20% of the cells detected by the 1-1 antibody carried the histone mark in the confluent cells. The 1493 antibodies again gave the opposite result. Only about 20% of the promoters detected by the 1493 antibodies were also positive for the H3K4me2 mark in the stimulated cells, but more than 60% of the c-Myb target genes detected by the 1493 antibodies in the confluent cells also had the H3K4me2 activation mark. Thus, the 1-1 antibody preferentially detects the c-Myb target gene promoters carrying the H3K4me2 mark in the stimulated cells, and the 1493 antibodies preferentially detect those genes in the confluent cells. Taken together, these results suggest that the two different c-Myb antibodies detect different sub-populations of c-Myb protein that are bound to different populations of target gene promoters in the two different growth conditions. We conclude that MCF-7 cells contain two populations of c-Myb protein: one population, recognized by the 1-1 antibodies, which is associated with active genes or genes that are primed for activation in estrogen-stimulated cells, and a second population, recognized by the 1493 antibodies,

which is associated primarily with genes that are primed for activation in confluent cells.

Discussion

These data demonstrated that two highly conserved domains (1493 and Ab1-1 domain) are masked during different phases of estrogen stimulation. Furthermore, the loss of accessibility of the 1493 domain can be associated with increased expression of a number of genes including *CXCR4*, a gene linked to distant metastasis in breast cancer [203]. This is the first report establishing that c-Myb activity rather *c-myb* expression is affected by the presence and absence of estrogen. Previous work has analyzed the relative expression of *c-myb* during estrogen and tamoxifen stimulation, but has not characterized the c-Myb protein [54, 200]. Importantly, we have demonstrated that although the expression level of the *c-myb* gene is dependent upon the presence of estrogen, the amount of c-Myb protein is not affected by estrogen treatment suggesting that there is a discrepancy between *c-myb* expression and c-Myb protein availability. However we cannot determine the function of the c-Myb protein in deprived cells given the lack of target genes identified in this condition.

It is possible that we uncovered this switch because c-Myb binding is dynamic and is displaced as cells are stimulated by estrogen, but our data using FLAG tagged c-Myb suggests that c-Myb is bound to genes in both conditions and that the domains associated with 1493 immune serum and Ab 1-1 are masked in an estrogen dependent manner. This masking is not solely reliant on

changes to the cell cycle because deprived cells do not demonstrate enrichment with the FLAG antibody. The observation that FLAG antibodies bind to each promoter during both cellular confluence and estrogen stimulation, but not estrogen deprivation suggests that the domains recognized by each antibody undergo some degree of epitope masking. This epitope masking might occur through post-translational modifications and protein interactions (Figure 7) since c-Myb interacts with a number of different cofactors such as CBP/p300 [96]. Furthermore, the switch in epitopes from 1493 to Ab 1-1 is critical for activation of a number of genes, which suggests that the Ab 1-1 region is associated with active genes during estrogen stimulation. However, the availability of the 1493 region is associated with H3K4me2 demonstrating that this domain is associated with open chromatin, but not associated with an increased rate of transcription as is the Ab 1-1 population was.

Transcription factors such as c-myc bind to thousands of genes and play critical roles in chromatin remodeling [171]. It is highly likely that c-Myb is important for chromatin remodeling since it binds to nearly 1/3 of the genome and has been associated with chromatin in previous work [204]. This role in chromatin-remodeling maybe highly conserved since *Drosophila* Myb also binds to a large portion of the fly genome [205].

Estrogen treatment altered c-Myb epitopes during ChIP assays. This result corresponds with previously published data suggesting that c-Myb binds to the Stat5a promoter in a progesterone dependent manner [206]. These data suggest that c-Myb is subject to modifications in surface accessible domains that

influence protein interactions. Moreover, domains such as the FAETL domain have been demonstrated to affect Myb transactivation [207]. The epitope recognized by the 1493 antibody is slightly upstream of this region. Although these data correspond well with previously published studies, the 1493 domain has not previously been shown to be modified and does not correspond to known sites of interaction with modifying enzymes [96, 208]. However, this region is surface accessible during cellular confluence and is highly conserved between chickens, mice, and humans. These data identify a novel domain, whose availability is directly affected by estrogen and is important for c-Myb activity in MCF-7 cells.

Finally, our ChIP on chip results revealed a number of target binding regions and multiple different types of target genes (RNA binding, kinases, etc.). We confirmed these targets with primary ChIP and FLAG tagged versions of the c-Myb protein. In addition most of the targets we analyzed have identified Myb response elements (MRE) within the binding region, but we have also shown that c-Myb binds to approximately 1/3 of the genome. This is consistent with what has been documented for other transcription factors such as c-Myc, Sall4, and NFkB [209-211]. Genome wide analysis of these transcription factors has identified approximately 8 000 target genes of c-Myc, 3 200 of Sall4, and 15 000 of NFkB. These data suggest that many transcription factors bind to a multitude of targets. We extended our results by characterizing c-Myb binding during estrogen stimulation. Coincidentally, ER alpha binds to over 10 000 genes [202] suggesting that ER alpha target genes are as wide spread as c-Myb. In

conclusion, our data demonstrate that c-Myb target genes are diverse and that c-Myb can bind to a large fraction of the genome in a similar fashion as other well characterized transcription factors.

Materials and Methods

Cells and Culture Conditions

MCF-7 cells (ATCC Manassas, VA) were cultured at 37°/5% CO₂ in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA). During estrogen starvation, cells (5X10⁵ per/ml) were cultured in phenol red free DMEM (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal stripped serum (Invitrogen, Carlsbad, CA) for 48 hr; following starvation 10nM β -estradiol (Sigma, St. Louis MO) was added to the medium for 24 hr.

Expression analysis, transcription assays, and immunoprecipitation

Expression analysis, quantitative real time PCR (QPCR), transcriptional assays, immunoprecipitation, and Western blots were performed as described [21, 52, 118]. The CXCR4 reporter gene was generated by cloning the 1 kb region upstream of the CXCR4 gene into the pGL2 basic vector (Invitrogen, Carlsbad, CA).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation was performed as previously described [196], but shearing was performed in a 200 μ l volume of 50 mM Tris pH 8.0 (Sigma, St. Louis, MO) by adding 40 units of Micrococcal nuclease (USB,

Cleveland, Ohio) for 10 min at 37°C. EDTA (10 mM) was used to stop the reaction and nuclei were lysed in 1% v/v SDS. CHIP was performed with anti-Myb 1493 immune serum (10 ul) or anti-Myb monoclonal 1-1 antibodies (Millipore, Billerica MA) or control non-immune serum (10 ul) or anti-FLAG (Sigma, St. Louis MO) for 24 hr. Anti-Myb 1493 is a rabbit polyclonal developed against a peptide HQGTILDNVKNLLEFAE. Primers used for QPCR reactions using SYBR green (Biorad, Hercules CA) are listed in Supplemental Table 1. CHIP assay results were normalized for control genes (GAPDH) and control non-immune antibodies. For CHIP on chip assays, DNA (10ng) was amplified according to manufacturer's protocol (www.affymetrix.com). Amplified DNA (6ug) was hybridized to Affymetrix promoter tiling array 1.0R and data analysis was performed with Model-based Analysis for Tiling arrays software [212]. Statistical analysis was performed with PASW 17 (SPSS Inc., Chicago Ill) statistical analysis software. Data were visualized in Integrated Genome Browser (www.affymetrix.com).

Myb Knockdown

Knockdown of *c-myb* was performed as previously described, using an shRNA expression vector kindly provided by Dr. Tom Gonda [54]. Briefly, cells were transduced with a retrovirus expressing shRNA specific to *c-myb* or a scrambled control. Expression of each shRNA was induced for 24 hr with doxycycline and expression of target genes was analyzed by QPCR as described above.

Myb Lentiviral production

N-terminal FLAG-tagged human c-Myb [21] was cloned into the unique PacI site of the pHR IRES GFP lentiviral vector (kindly provided with packaging vectors by Dr. Bruce Bunnell, Tulane University) downstream of an EF1 alpha promoter. Plasmids were transfected into 293 FT cells (Invitrogen, Carlsbad CA) by calcium phosphate transfection along with the lentiviral packaging plasmid delta 8.9 and the pMD.G plasmid expressing the vesicular stomatitis virus glycoprotein. Supernatant was collected at 24 hr intervals for 48 hr. Ultrafiltration using an Ambion Ultracell 100 kDa NMWL filter unit (Millipore, Billerica MA) was used to concentrate viral supernatants. Cells were transduced in the presence of 8 ug/ml of polybrene (Sigma, St. Louis MO) and sorted to enrich for GFP+ transduced cells.

Conflict of Interest

The authors have no competing financial interests in relation to the work described.

Acknowledgments

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by a grant from the W. M. Keck Foundation with additional funding from the State of New Mexico and the University of New Mexico Cancer Center. Some of these experiments used the Shared Flow Cytometry Resource in the UNM Cancer Center. The authors thank Julie Torres and John-Michael Thomas for expert technical assistance and Dr. Tom Gonda for providing the shRNA vector. The authors report no conflicts of interest. Affymetrix tiling array ChIP on chip data has been deposited in the NCBI GEO database with accession number GSE18706.

Figure Legends:

Figure 1. The CXCR4 promoter is regulated by Myb proteins

(A) Structure of c-Myb and v-Myb proteins. The diagrams depict the structure of the c-Myb and v-Myb proteins, which share conserved domains (shaded) involved in DNA binding and regulation. The oncogenic v-Myb protein is truncated at both ends and has a number of point mutations represented by white dots. The locations of the epitopes for antibodies (Abs) 1493 and 1-1 are indicated. (B) Structure of the *CXCR4* gene promoter. The region upstream of the human *CXCR4* gene is diagrammed, with putative Myb binding sites indicated by gray boxes. The arrow indicates the start site and direction of transcription. (C) Activation of a *CXCR4* reporter gene. A reporter construct containing the *CXCR4* promoter upstream of the luciferase reporter gene was co-transfected into HEK293 cells along with control plasmid (vector) or plasmids

expressing c-Myb or v-Myb, as indicated. The figure shows reporter gene activity. Error bars show standard deviation of triplicate assays.

Figure 2. CXCR4 is a c-Myb target gene in MCF-7 cells

(A) MCF-7 cells were deprived of estrogen for 48 hr, then 10nM 17-beta-estradiol was added back for 6, 12 or 24 hr, as indicated. Alternatively, cells were allowed to reach high density (Confl.). The levels of *c-myb* or *CXCR4* were measured by QPCR. Error bars show standard deviation in triplicate PCR reactions, and results are relative to the estrogen-deprived cells. (B) Western blot with anti-c-Myb and anti-beta-actin antibodies of cells grown to high density (Confl.) or deprived of estrogen for 48 hr followed by adding 10nM 17-beta-estradiol for 0, 6, 12 or 24 hr, as indicated. (C) Experimental diagram showing how cells are deprived of estrogen for 48 hr, stimulated for 24 hr or allowed to reach high density (Confluent). (D) ChIP was performed on MCF-7 cells deprived of estrogen for 48 hr, stimulated with 10nM 17-beta-estradiol for 24 hr or allowed to grow to high density (Confl.). Chromatin complexes were immunoprecipitated with anti-c-Myb antibodies 1-1 (gray) or 1493 (black). Enrichment for the *CXCR4* promoter was measured by QPCR. Error Bars represent standard deviation of triplicate PCR reactions.

Figure 3. Flag-c-Myb binds the CXCR4 promoter

(A) Lysate from lentiviral transduced MCF-7 cells was immunoprecipitated with anti-Flag agarose (Sigma) or control IgG agarose (Santa Cruz). Expression of FLAG-tagged c-Myb was visualized with anti-c-Myb antibody 1-1 (Millipore) by Western blot. (B) ChIP assay using anti-FLAG antibodies and chromatin from

FLAG-c-Myb expressing MCF-7 cells cultured to confluence. QPCR was performed with primers specific to the *CXCR4* promoter and primers designed to recognize non-specific binding sites within the *CXCR4* (NS1), *MAT2A* (NS2), and *GAPDH* promoters. Error bars represent standard deviation from triplicate QPCR reactions. (C) FLAG-c-Myb MCF-7 cells were deprived of estrogen for 48 hr, stimulated (Stim.) for 24 hr or grown to high density (Confl.) and subjected to ChIP assay with anti-FLAG or control IgG. QPCR was performed with primers specific to *CXCR4* promoter and *GAPDH*. Data are normalized to the IgG control and *GAPDH*. Error bars represent standard deviation from triplicate PCR reactions.

Figure 4. Additional c-Myb target genes

(A) MCF-7 cells were deprived of estrogen for 48 hr then stimulated with 10nM 17-beta -estradiol for 6, 12 or 24 hr, or grown to high density (Confl.). QPCR was used to measure the levels of *CCNB1*, *EPB41*, *JUN*, and *KLF4* RNAs. Error bars show standard deviation in triplicate PCR reactions, and results are relative to the estrogen-deprived cells. (B) MCF-7 cells were transduced with retroviral vectors expressing doxycycline inducible shRNAs (scrambled or *c-myb* specific). Each cell line was induced for 24 hr with doxycycline and the relative expression of *c-myb*, *CCNB1*, *CXCR4*, and *JUN* RNAs was measured by QPCR. Data are normalized to the non-induced controls.

Figure 5. A switch in c-Myb activity on additional target genes

MCF-7 cells (A,B) or MCF-7 cells expressing FLAG-c-Myb (C) were deprived of estrogen for two days (open bars), stimulated for 24 hr (gray bars) or

grown to high density (black bars), then they were crosslinked and chromatin was isolated and used for ChIP assays using (A) 1-1 (B) 1493 or (C) FLAG antibodies, as described in Figures 2 and 3. QPCR was performed with primers specific for the *JUN*, *EPB41*, *CCNB1* and *KLF4* promoters. Data are relative to control IgG and *GAPDH*. Error bars represent standard deviation of triplicate QPCR reactions.

Figure 6. Changing growth conditions trigger massive epigenetic changes

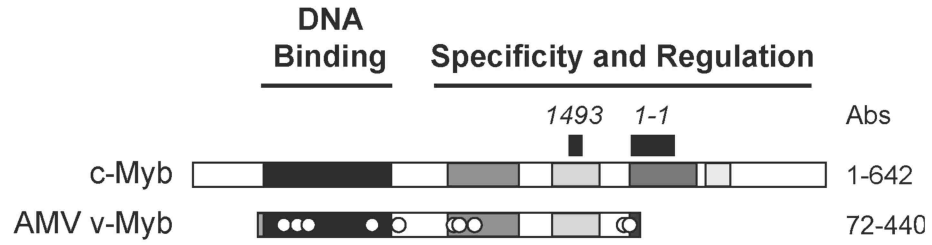
MCF-7 cells were estrogen-deprived for 48 hr then stimulated for 24 hr, or allowed to grow to high density (Confluent) and samples were used in ChIP on chip assays to measure genome-wide changes in the H3K4me2 epigenetic mark, using Affymetrix promoter tiling arrays. (A) Comparison of H3K4me2 marks in Stimulated (red) and Confluent (blue) cells for representative genes *CCNB1*, *CCNE1*, *SND1* and *GREB1*. Histograms show relative signals and the colored bars identify statistically significant ($p < 1 \times 10^{-5}$) regions. Gene diagrams are shown above chromosome number lines, with units in nucleotides from the March 2006 (NCBI 36/hg18) build of the human genome sequence. (B) Venn diagram comparing the number of statistically significant ($p < 1 \times 10^{-5}$) H3K4me2 sites detected in the two growth conditions. (C) Statistically significant regions (.bed files) were compared to find overlaps, and the fraction of genome sites identified by both H3K4me2 and 1-1 (gray) or H3K4me2 and 1493 (black) antibodies in the two growth conditions is shown.

Figure 7. Model for a switch in c-Myb activity

A model for changes in c-Myb activity at the CXCR4 promoter is shown. (A) c-Myb is bound to the promoter and both antibodies, 1493 and 1-1 are able to bind in ChIP assays. (B) Antibody 1493 is unable to bind c-Myb at the CXCR4 promoter in estrogen-stimulated cells and (C) 1-1 antibody is unable to bind in confluent cells. The epitopes may be masked by other proteins as shown here, or affected by conformational changes or post-translational modifications.

Figure 1

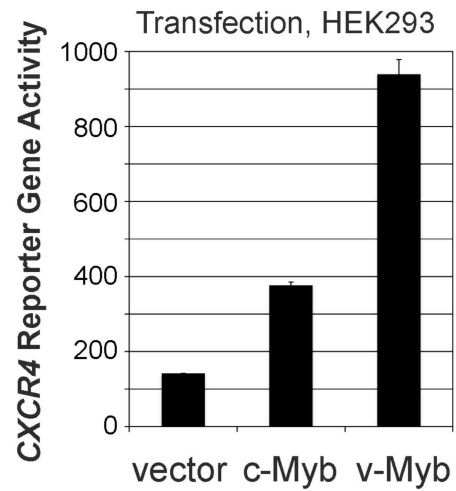
A



B



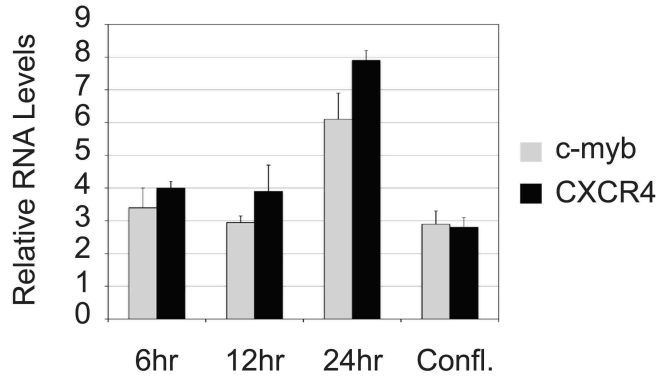
C



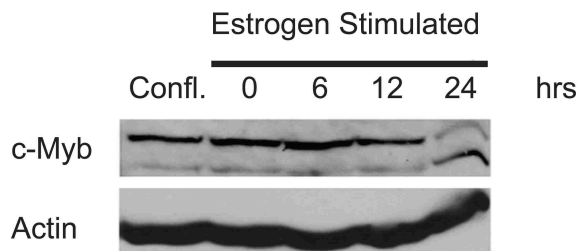
Dr. Fan Liu*

Figure 2

A



B



C

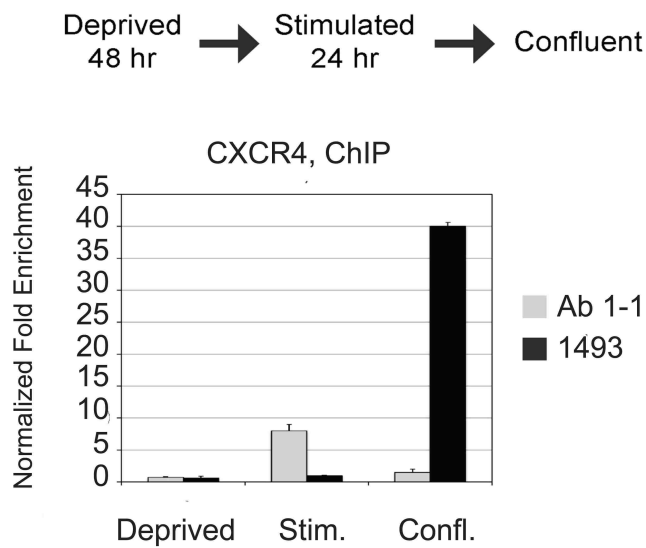
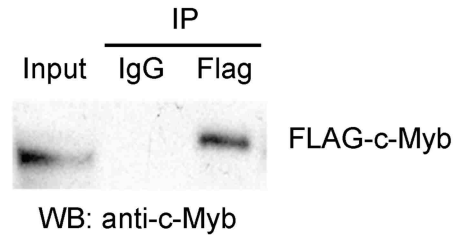
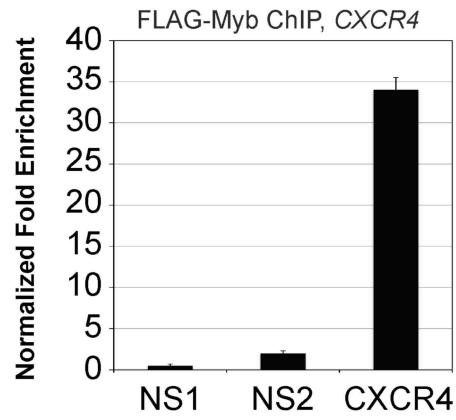


Figure 3

A



B



C

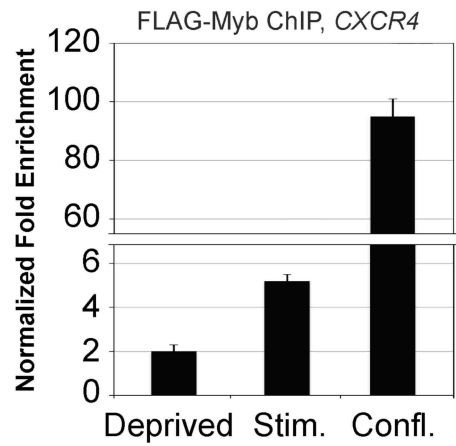
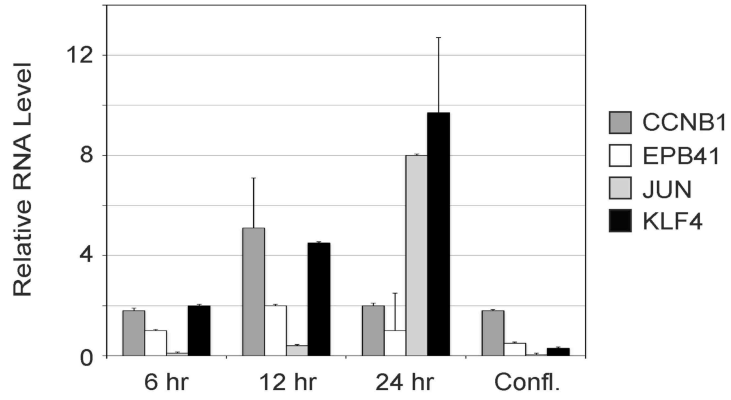


Figure 4

A



B

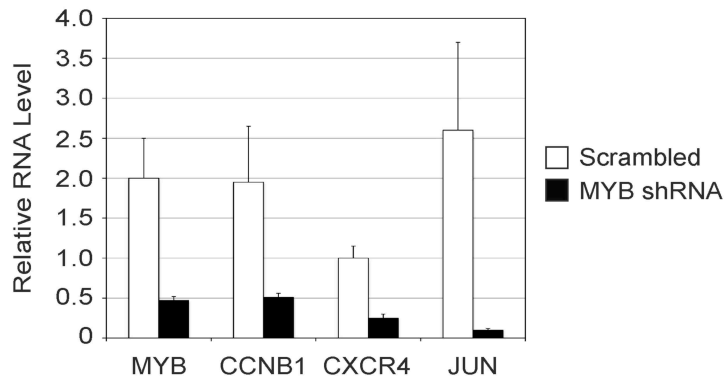
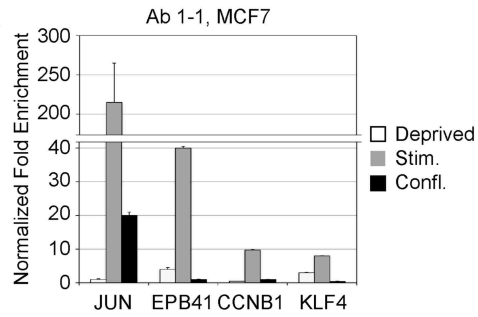
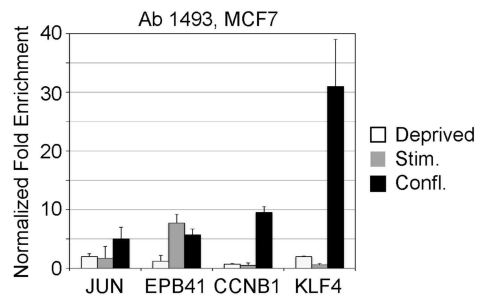


Figure 5

A



B



C

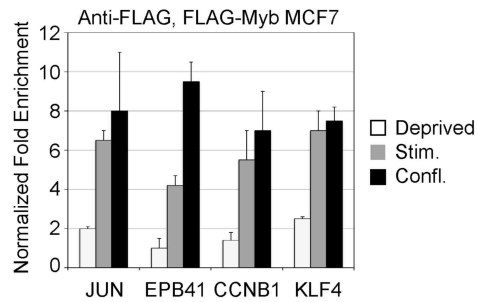
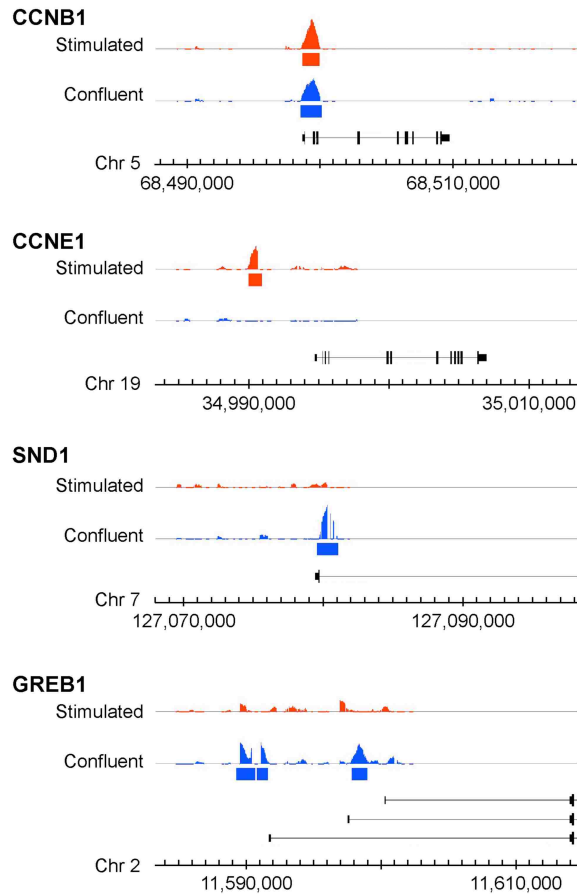
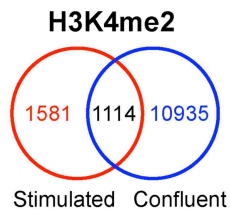


Figure 6

A



B



C

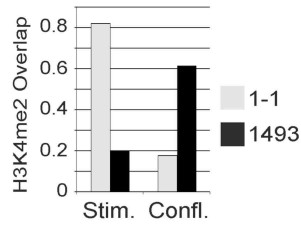
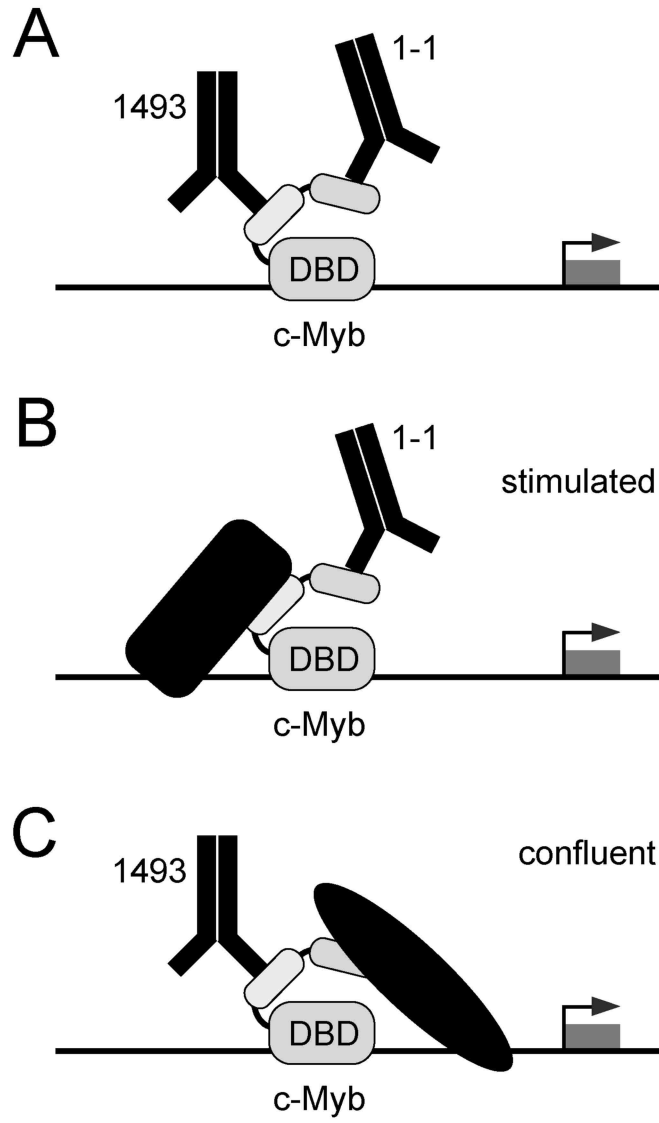


Figure 7



Supplementary Table 1: ChIP primer pairs

| Primer | Forward | Reverse |
|---------------|---------------------------|---------------------------|
| NS-1 | ACCCACCAAACGGGACTCCATACTC | TCTCTTGACGGCGTGGGAATGG |
| NS-2 | TGACATGGACTGCCTGCATA | ACTGAGAAGCATGACGGACAAGTA |
| CXCR4 | CTGCACCTGACCCTAGTGATGCT | CCAGACCTGGGAATGCTACAGTAGA |
| GAPDH | GTGCAGGAGTTCATGACCAAC | CACCTGGGCTAGAGTACA |
| KLF4 | CGTGGGGGCTAAAGCTTGT | CAGAGGTGACTATTCAACCGCATA |
| EPB41 | CAGCGAGCAGAGGGTTAAATCTAT | ATGGCCTGCTGGTCAATCT |
| JUN | GGGGTCGTCTGGCTGCTCGTAGAA | CCAATTGCGCCCACTATAAAA |
| CCNB1 | TGGTAACAAAGTCAGTGAAC | TATCAATGACTTTTCCAGTAG |

Supplementary Table 2: Expression Primers

| Primer | Forward | Reverse |
|---------------|----------------------|---------------------------|
| KLF4 | ACGATCGTCTCCCTCTTT | ACCCTGGGTCTTGAGGAAGT |
| EPB41 | TGGCCCAAAGTGCTGAAGAT | AGTCCGGCCACTGTATCGAA |
| JUN | CCAATTCGCCCACTATAAAA | CTGGGAAACTCCTGAATTTGTAACT |

Supplementary Table 3: Taqman Expression Probes

| Primer | Catalog Number |
|---------------|-----------------------|
| CXCR4 | HS00607978_s1 |
| c-myb | Hs00193527_m1 |
| CCNB1 | Hs00259126_m1 |
| PPIA | 4333763T |

Supporting Data

Introduction

Our data has demonstrated that c-Myb activity and specificity are altered during epithelial cell proliferation. We identified different sub-populations of c-Myb, some of which are more prevalent in highly confluent cell cultures and others are more prominent in estrogen stimulated lower density cultures. Our data is supported by evidence from multiple different target genes, which we initially discovered by ChIP on chip (ChIP on a promoter tiling array). We performed ChIP on chip with cells grown in different culture conditions because characterization of the CXCR4 promoter suggested that our antibodies recognized different sub-populations of c-Myb in a proliferation dependent manner. Genome wide analysis of c-Myb target genes supported our observations at the CXCR4 promoter. We performed a lengthy analysis with our ChIP on chip data, which provided a means to characterize c-Myb activity during estrogen- stimulated proliferation.

Genomics Suggests that c-Myb Sub-populations exist

As stated above, ChIP on chip analysis identified over 10 000 statistically significant c-Myb binding sites, which was consistent with previous work with other transcription factors. However, we extended our genome wide analysis to identify target genes in two different growth conditions. Our work at the CXCR4 promoter in different growth conditions suggested that different sub-populations of c-Myb existed (See above Figures). We hypothesized that the 1493 antibodies and the Ab 1-1 antibodies recognized different fractions of the c-Myb protein in

different populations of cells. We used a filter script written in R (Dr. Scott Ness) to analyze overlapping statistically significant binding sites of our ChIP on chip from both antibodies in each growth condition to determine if these antibodies were specific for different sub-populations of c-Myb on a genome wide scale.

Supporting Results

1493 Specific Sub-population during different growth conditions

Our QPCR data overwhelming suggests that cells cultured to confluence express c-Myb and that our 1493 antibodies detect c-Myb at a number of different genes during cellular confluence even though Ab 1-1 does not detect c-Myb at the same promoters. This striking difference prompted us to perform ChIP on chip in confluent cells and compare the binding sites of 1493 and Ab 1-1 antibodies. Our analysis demonstrated that the 1493 antibody detected over 8,000 target genes in confluent cultures, but only 439 of those binding sites were bound by Ab 1-1 antibodies (Supporting Figure 1A). QPCR at multiple different target genes demonstrated that 1493 consistently enriched for target genes during cellular confluence, but Ab 1-1 did not. Our ChIP on chip data demonstrated a similar trend on a genome wide scale (Supporting Figure 1A), but some genes such as ELK4, were bound by both antibodies according to ChIP on chip. We performed ChIP-QPCR at the ELK4 promoter and demonstrated that in confluent cultures both antibodies detected c-Myb at the ELK4 promoter (Supporting Figure 1B). Our results with the 1493 antibody in stimulated cells demonstrated that these antibodies do not detect the same c-Myb subpopulations in confluent and stimulated cells because as shown in Supporting

Figure 1C, only 382 genes are overlapping in confluent and stimulated cells. These data are supported by our QPCR data demonstrating that 1493 specific c-Myb does not bind to CXCR4, KLF4, CCNB1, JUN, or EPB41 in stimulated cells (See Figure 5 this chapter).

Ab 1-1 c-Myb is distinct from 1493 c-Myb

Our QPCR at other targets such as CXCR4 (See Figure 2 this chapter) suggest that Ab 1-1 did not detect c-Myb at these targets in confluent cells, but did detect c-Myb during estrogen stimulation. We compared the binding of each antibody in estrogen stimulated cells and these genome wide comparisons demonstrated that Ab 1-1 c-Myb recognized a different set of target genes than 1493 in stimulated cells (Supporting Figure 2A). Some overlap was detected and in our QPCR, we demonstrated that c-Myb was bound to JUN under both conditions, but the binding increased 10-fold in estrogen stimulated cells (See Figure 5 this chapter). A comparison of the Ab 1-1 c-Myb in confluent and stimulated cells demonstrated that this population of c-Myb bound to distinct targets in each condition (Supporting Figure 2B). These differences in c-Myb sub-populations led to the characterization of multiple genes during estrogen stimulation as described above.

Identification of new target genes and confirmation of known targets

As mentioned previously, we were able to identify new target genes by ChIP on chip, but our ChIP on chip results confirmed that c-Myb binds to a number of previously reported target genes. These genes have a variety of functions and many of which play important cell cycle regulatory roles. In our

ChIP on chip screen we identified CCNE1 as a direct target gene, which was speculated to be a target gene, but not confirmed [53]. Furthermore, we confirmed that CCNB1 [50], KIT [213], MYC [214], and CXCR4 [21] are all c-Myb target genes. In addition we identified ELK4, EPB41, JUN, KLF4, NANOG, and SND1 as c-Myb target genes. As shown in Supporting Figure 3, the binding site for c-Myb was found in intronic regions, promoter regions, and enhancer regions (CCNE1 ~ 4KB upstream). Interestingly, c-Myb can bind to its own promoter suggesting that c-Myb can operate a feedback loop to control its own expression. These data demonstrate c-Myb binding is versatile and encompasses many different target genes.

Cell Adhesion Governs changes in c-Myb specificity and activity

We observed two different behaviors from our antibodies depending upon the cell type we utilized. In Jurkat T-cells that grow in suspension, our antibodies (1493 and Ab 1-1) gave nearly identical results under all conditions examined (Chapter 2). In contrast, the antibodies displayed opposing behaviors in MCF-7 cells (Chapter 3), which we hypothesized was due to the abundance of cell adhesion and cell contact because confluent cells gave a strikingly different result compared with more asynchronous stimulated cells. To test this hypothesis we characterized antibody efficacy in sub-confluent cultures (asynchronous) and confluent cultures treated with EDTA compared with confluent cultures.

Lack of cell contacts disrupts epitope masking

MCF-7 cells were plated at medium density ensuring that cells would not become excessively confluent or plated at high density so they would grow to

cellular confluence. After a 16-hour incubation, one set of confluent cells was treated with 5mM EDTA for 2 hours and a second set was allowed to maintain cell contacts and cell adhesion. After incubation asynchronous (sub-confluent), confluent, or confluent cells treated with EDTA were subjected to ChIP with both c-Myb antibodies. As shown in Supporting Figure 4, 1493 antibodies detected c-Myb at the CXCR4 promoter during cell confluence, but not in asynchronous, cultures, which have less cell adhesion. Ab 1-1 showed completely opposite results detecting c-Myb during asynchronous growth, but not during cell confluence (Supporting Figure 4). Treatment with EDTA abolished cell: cell adhesion and cell adhesion to the culture plate, similar to suspension cell growth. In EDTA treated cells we detected c-Myb at the CXCR4 promoter with both antibodies mimicking what was observed in Jurkat cells. Therefore, these data demonstrate that epitope masking in MCF-7 cells is directly related to the signaling pathways that are regulated by cell adhesion.

v-Myb binds directly to endogenous c-Myb target genes

We have demonstrated that dynamic repositioning regulates c-Myb specificity and that activity is associated with epitope masking and potential changes in protein interactions. Since c-Myb does not transform cells even though it is associated with various types of cancer [29, 32, 35, 215] so we wanted to determine if the oncogenic derivative of c-Myb, v-Myb binds to different genes thus ensuring its oncogenic potential. v-Myb and c-Myb activate distinct sets of endogenous genes [21], but it is unclear if the differences are due to changes in specificity or activity. Our data thus far suggests two different

hypothesizes: first, that changes to the structure of c-Myb lead to changes in specificity and activity at different genes, or second, that changes in structure do not affect specificity and v-Myb binds to the same target genes, but has different activity at different times. We developed MCF-7 cells that express v-Myb or 3MutC, a protein mutated in the DNA binding domain, which activates c-Myb target genes and not v-Myb target genes and performed ChIP assays to determine occupancy at target genes we identified by ChIP on chip (Supporting Figure 3).

Transduced MCF-7 cells, which express FLAG tagged versions of v-Myb or 3MutC [21] were selected for blasticidin resistance by Dr. Fan Liu and stored for a substantial period time, so we verified that FLAG tagged versions of v-Myb and 3MutC were expressed. Each mutant protein was immunoprecipitated with anti-FLAG and Western blot with anti-c-Myb demonstrated that both proteins were expressed in transduced cells (Supporting Figure 5A). We cultured the cells to confluence and compared the binding of FLAG c-Myb (Chapter 3) with the binding of both mutated proteins at CCNB1, KLF4, JUN, and CXCR4. CCNB1 is essential for the G2/M transition; KLF4 is a reprogramming transcription factor important for induced pluripotent stem cells; JUN is a known oncogene; and CXCR4 is the sole receptor for SDF-1. Therefore regulation of these genes is critically important for cancer and normal cell function. As shown in Supporting Figure 5B, v-Myb bound to CCNB1, JUN, and CXCR4 promoters; all of these genes are activated by c-Myb in MCF-7 in response to estrogen (See Chapter 3). 3MutC occupied the JUN and CXCR4 promoters, but not KLF4 or CCNB1

(Supporting Figure 5B) suggesting that mutation of the DNA binding domain can affect binding at some targets, but a combination of mutation and truncation affects binding in a very unique manner. Furthermore, recent work by Ye Zhou and Dr. John O'Rourke has demonstrated that v-Myb activates CXCR4 and KLF4, but not CCNB1. Together these data suggest that v-Myb binds to some of the same genes that c-Myb does, but can have some unique activities in the absence of direct binding. Furthermore, v-Myb can bind to some genes such as CCNB1 and have a different activity than c-Myb.

Supporting Discussion

We have shown that changes in c-Myb activity and specificity are induced by epithelial cell proliferation. Our ChIP on chip results demonstrated that the changes we have characterized in a small panel of target genes hold true on a genome wide scale. These results are highly significant ($p\text{-value} < 1 \times 10^{-5}$) and have been validated by QPCR. QPCR is very sensitive and yields better results than ChIP on chip. ChIP on chip is also subject to multiple variables that QPCR is not. For example normalization to input control or to control IgG can yield some varying data. In addition, we performed whole genome amplification with random priming. The linearity of this method is controlled for, but it is possible that specific regions of the genome are amplified better than other regions (GC rich or AT rich). Therefore, we performed detailed authentication and validation by identifying Myb response elements in each of the genes we chose to study from our ChIP on chip results. Taken together, the ChIP on chip results provide a

foundation for future characterization of c-Myb activity and specificity by QPCR and other traditional techniques.

Our CHIP on chip results and QPCR data demonstrate that in MCF-7 cells cell adhesion and cell:cell contacts affect c-Myb activity and specificity. This phenomenon is cell type specific and is directly related to the cell adhesion because suspension cells such as Jurkat cells do not display changes in specificity. In addition treatment of cells with EDTA, which abolishes cell contacts alters c-Myb binding. Coincidentally, other groups have demonstrated that the WNT pathway, in particular the TCF family of transcription factors coordinate with c-Myb at specific target genes in colon epithelial cells (Communicated by Rob Ramsay) and in our hands TCF3/4 binds to similar Myb targets during both stimulation and confluence. This suggests that the WNT pathway and c-Myb may coordinate in MCF-7 cells and the activation or suppression (affected by cell adhesion) could affect c-Myb activity. It is important to study this coordination further and determine the role WNT has in c-Myb regulation.

We proposed two different mechanisms of v-Myb regulation. To investigate these proposals, we performed CHIP with FLAG versions of different Myb proteins. We discovered that although c-Myb and v-Myb activate entirely different sets of endogenous genes [21], v-Myb maintains some level of c-Myb specificity. That is, both proteins can bind to overlapping genes, which is consistent with reporter gene assays that suggest v-Myb and c-Myb bind to the same promoters [55, 199]. However, some genes such as KLF4 are not bound by v-Myb, but are induced upon v-Myb over expression demonstrating that v-Myb

can have unique indirect activities. On the other hand, in some cases such as with CCNB1, binding by v-Myb does not translate to activation suggesting that other forms of regulation are absent or v-Myb functions as a repressor. c-Myb and v-Myb have been demonstrated to inhibit specific forms of transformation [216] so it is not unlikely that specific genes can be repressed by v-Myb or c-Myb. Our results support both suggested hypotheses. First both c-Myb and v-Myb can bind to the same genes and v-Myb has a different activity; and second, v-Myb and c-Myb bind to different genes and have unique direct and indirect activities. Our data suggests that these two mechanisms can be differentiated in a gene specific manner.

Furthermore, our studies have identified one gene, KLF4, that is bound by c-Myb and not v-Myb. However, v-Myb can efficiently activate this gene. These data suggest that unique pathways regulate c-Myb and v-Myb and although some aspects of these pathways may converge, each protein has a unique function. KLF4 is important for reprogramming of differentiated cells into transformed pluripotent stem cells, suggesting that c-Myb may indirectly regulate oncogenesis through KLF4. Future work dissecting the mechanisms that Myb proteins utilize to regulate KLF4 is essential to characterize c-Myb induced oncogenesis.

Supporting Data Materials and Methods

ChIP on chip, Immunoprecipitation, and Western Blot

ChIP was performed as described in Chapter 3 Materials and Methods and whole genome amplification was performed according to the manufacturer's

protocol (Affymetrix). A detailed protocol can be found in Appendix II.

Immunoprecipitation was performed as described in Chapter 2 and Chapter 3.

For sub-confluent cultures cells were seeded 5×10^5 per 15 cm dish and cultured for 16 hours. During EDTA treatment, cells were seeded at 1×10^6 per/ml and cultured for 16 hours. Then 5 mM EDTA was added for 2 hours under normal growth conditions.

Lentiviral transduction

Flag tagged versions of v-Myb and 3MutC were cloned into lentiviral vectors with blasticidin resistant marker. Viral particles were produced as described in Chapter 2 and Chapter 3 except no flow cytometry was performed. Cells were selected in $5 \mu\text{g/ml}$ blasticidin and maintained in media with blasticidin. Western blot was performed as described in Chapter 2 and Chapter 3.

Supporting Figure Legends

Supporting Figure 1: ChIP on chip identifies 1493 Specific Sub-population

(A) Comparison of 1493 binding sites in confluent cells. ChIP on chip was performed on confluent MCF-7 cells. A comparison of the binding sites between 1493 immune serum and Ab-1-1 monoclonal antibodies was performed with a script written in R. The Venn diagram demonstrates overlapping genes. (B) ChIP assay in confluent MCF-7 cells. ChIP was performed with anti-c-Myb 1493 (black) or Ab 1-1 (grey). QPCR at the ELK4 promoter demonstrates that both antibodies enrich for this gene in confluent cells. (C) ChIP on chip with 1493. ChIP on chip with confluent MCF-7 cells or estrogen stimulated MCF-7 cells. Venn diagram represents the overlapping statistically significant binding sites.

Supporting Figure 2. Ab 1-1 binds to distinct target genes.

(A) Comparison of Ab 1-1 binding sites. ChIP on chip was performed on stimulated MCF-7 cells with 1493 or Ab 1-1 antibodies. Venn diagram demonstrates each antibody binds to different subsets of genes. (B) Comparison of Ab 1-1 in different growth conditions. Venn diagram demonstrates Ab 1-1 binding sites are unique in confluent and estrogen stimulated cells.

Supporting Figure 3. ChIP on chip target genes

ChIP on chip was performed with 1-1 and 1493 antibodies using chromatin from MCF-7 cells that were deprived of estrogen for 48 hr followed by 24 hr of estrogen stimulation or grown to high density. Histograms show the normalized (relative to input) signal and red bars mark the statistically significant ($p < 1 \times 10^{-5}$) binding sites (histograms) at the indicated promoters. Genes diagrammed above the number line are on the positive strand, with transcription from left to right, and genes below the number line are in the opposite orientation. The images come from Integrated Genome Browser (www.affymetrix.com) and the number lines show nucleotide numbers in the March 2006 build (NCBI 36/hg18) of the human genome.

Supporting Figure 4. Epitope masking is cell adhesion specific.

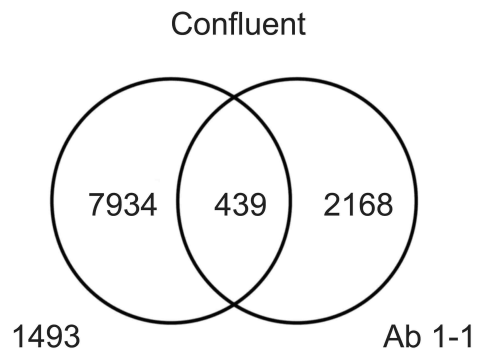
ChIP was performed with MCF-7 cells cultured in different growth conditions. Real time PCR was performed with primers specific for the *CXCR4* promoter. Error bars represent standard deviation of triplicate PCR reactions.

Supporting Figure 5. Oncogenic versions of c-Myb bind to ChIP on chip targets

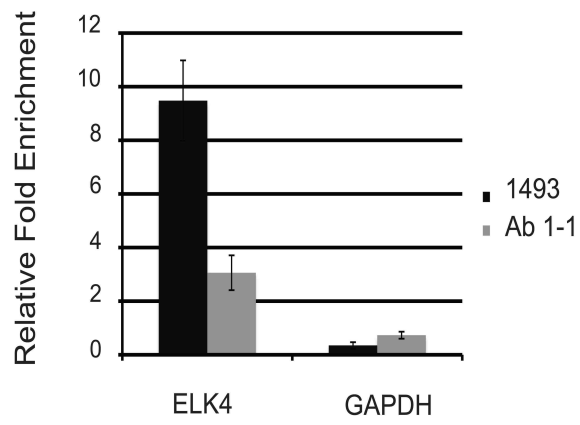
(A) Western blot of lentiviral transduced MCF-7 cells. Cells were subjected to IP with anti-FLAG antibodies or control IgG and Western blot was performed with anti-c-Myb. Each cell line expressed exogenous protein. (B) ChIP of lentiviral transduced MCF-7 cells. ChIP was performed with anti-FLAG antibodies or control IgG. Real time PCR was performed with primers specific to *CCNB1*, *KLF4*, *JUN*, or *CXCR4*. Error bars represent standard deviation of triplicate PCR.

Supporting Figure 1

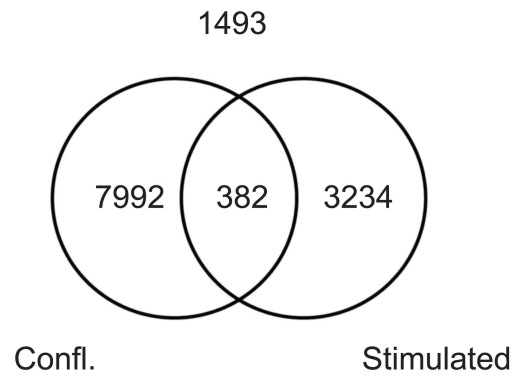
A



B

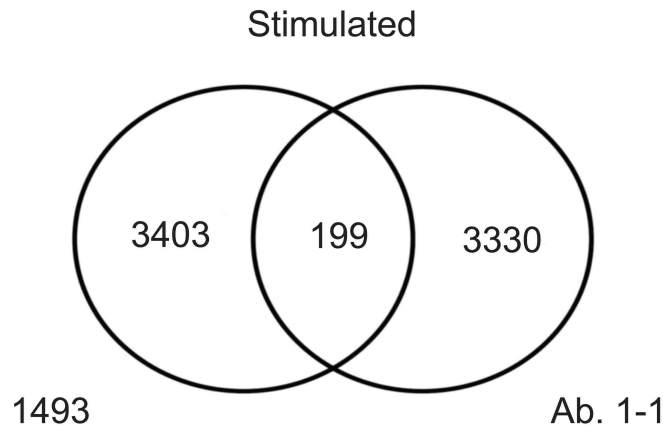


C

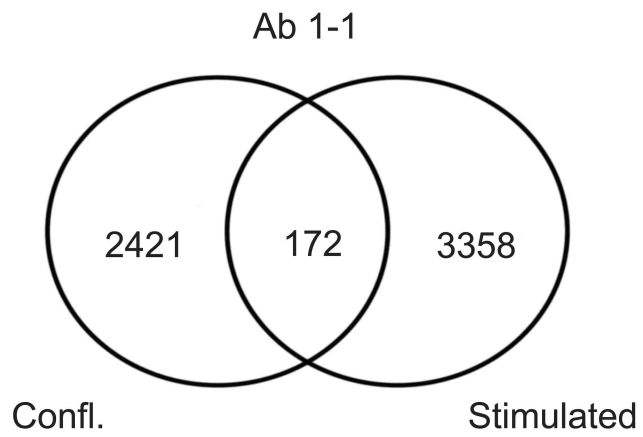


Supporting Figure 2

A

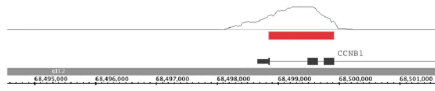


B

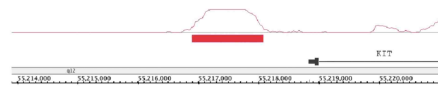


Supporting Figure 3

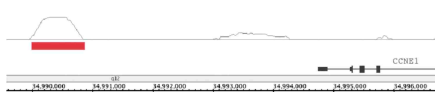
CCNB1



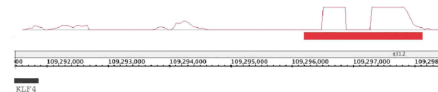
KIT



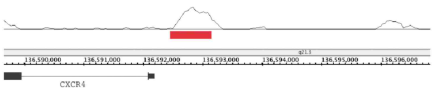
CCNE1



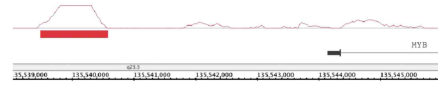
KLF4



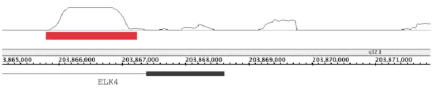
CXCR4



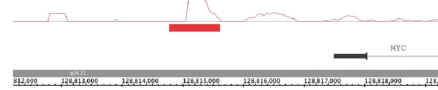
MYB



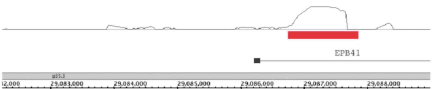
ELK4



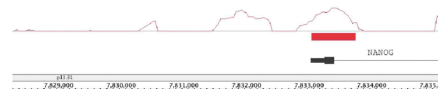
MYC



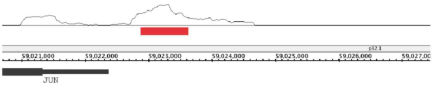
EPB41



NANOG



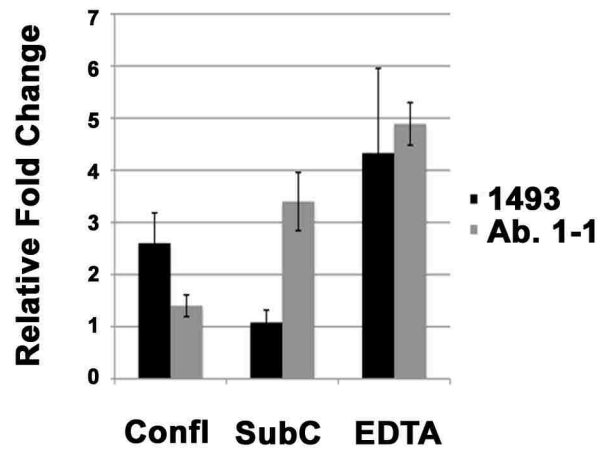
JUN



SND1

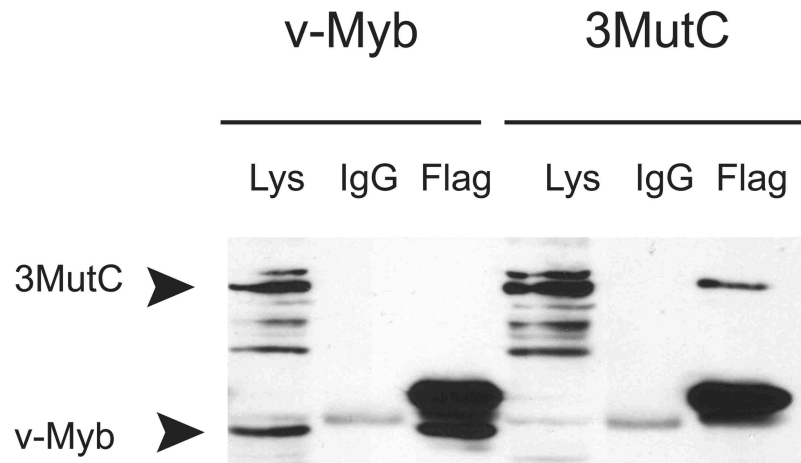


Supporting Figure 4

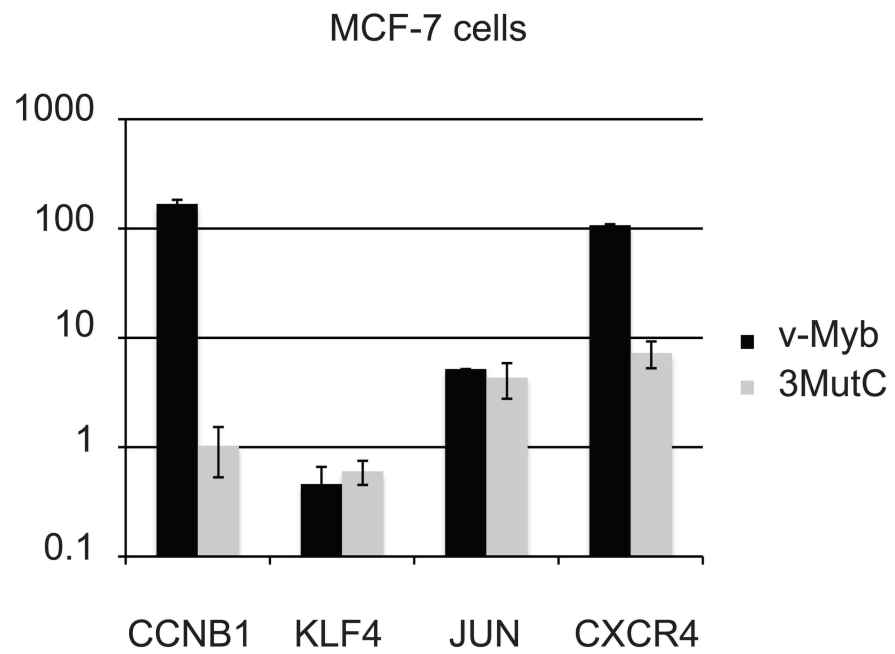


Supporting Figure 5

A



B



Final Words and Implications of Epitope Masking

We identified two sub-populations of c-Myb that are defined by the recognition or lack thereof by two independent antibodies, ab 1-1 or 1493. Our experiments were designed in such a manner that all of our ChIP assays were performed under strikingly different culture conditions. Initially, our hypothesis was designed to determine if c-Myb specificity was estrogen dependent similar to what has been observed when cells are treated with prolactin [206]. Consistent with prior observations, the ab 1-1 specificity was estrogen dependent at most genes analyzed. In addition, c-Myb recognized by ab 1-1 has increased activity, which caused an increase in expression of a number of different genes. However, the inclusion of the 1493 antibody demonstrated that the change observed was not necessarily a change in specificity because 1493 could detect c-Myb when ab 1-1 could not. These data combined with FLAG tagged c-Myb data established that epitope masking was a much more probable explanation for our observations than changes in binding.

Understanding the mechanism of epitope masking is critical for future understanding of c-Myb activity during mitogenic stimulation. Single antibody analysis would suggest that c-Myb specificity is estrogen dependent, but instead our data demonstrated that it is not solely a change in specificity because c-Myb can bind to the same genes in both confluent and stimulated conditions. On the other hand, the changes we see are due to differences in the activity of two unique forms of c-Myb. These forms of c-Myb are defined by protein interactions and protein complexes at the promoter of specific genes, which appear to

change during different stages of estrogen treatment. These data presented do not uncover the proteins interacting with c-Myb. Furthermore, these data have demonstrated that regions outside of the DNA binding domain affect activity, which has been hypothesized based on work that shows Myb proteins activate distinct sets of genes and was extensively described in Chapter 1 [21, 80]. It is likely that the regions characterized (conserved domains) here have pivotal roles in defining what genes are active and at what time.

Our data connects c-Myb activity to changes in cell adhesion and cell contacts because cellular confluence and estrogen stimulation represent cell populations that are highly unique. It is known that cell adhesion and signaling through the WNT pathway is important for cancer progression. Our system suggests that cell adhesion is important for c-Myb activity and others have demonstrated that c-Myb is important for epithelial cell cancers suggesting that the WNT pathway may overlap with changes in c-Myb activity. Results have demonstrated that estrogen treatment with β -estradiol alters cellular motility and cell to cell contacts [217-219]. In addition studies have confirmed that estrogen stimulation (E2) lowers the expression of E-cadherin, which would increase free β -catenin and lead to changes at the molecular level [217, 218, 220, 221]. It seems likely that during cellular confluence β -catenin is sequestered leading to a decrease in WNT signaling, but during estrogen treatment the WNT pathway is activated. c-Myb may interact with components of the WNT pathway in an estrogen dependent manner and some of these components could account for epitope masking. Therefore, the work we have presented in this chapter

establishes a foundation for the study of how c-Myb interacts with the WNT pathway in cancer and normal cells and more specifically, which sub-population, 1493 or Ab 1-1, interacts with these components.

Chapter 4: Discussion

Together these data demonstrate two important points. First, c-Myb undergoes dynamic repositioning in a cell cycle dependent manner and this repositioning is gene specific. Second, protein interactions change while c-Myb is bound to target genes in response to mitogenic stimuli such as estrogen. We posed the hypothesis that c-Myb has dual functionalities because different sub-populations of cells regulate c-Myb in a time dependent manner. These data support the hypothesis that c-Myb sub-populations exist (based on the recognition by different antibodies) and have different activities, which likely explains how c-Myb regulates different processes. Furthermore, we have performed ChIP on chip experiments that identified over 10,000 statistically significant c-Myb target genes demonstrating that c-Myb binds to approximately 1/3 of the human genome. These dual functions of c-Myb are not solely reliant on changes in protein complexes because c-Myb is partially regulated by changes in specificity during the cell cycle. Therefore, there is not one single mechanism governing c-Myb activity and alternatively, multiple mechanisms work together in a time dependent fashion to mediate specificity and activity. c-Myb may be part of an extensive complex of proteins that regulates gene expression in much the same manner as the drosophila dREAM complex in which B-Myb is an important participant.

Are post-translational modifications cell cycle dependent?

Our laboratory has suggested that c-Myb function is regulated by a context specific code or sequential post-translational modifications that work in

concert to regulate c-Myb activity. These modifications can impact protein interactions [67, 87, 94, 108, 141, 198, 222], but these changes have not been associated with cell cycle dependent regulation of c-Myb. Our data confirms that c-Myb is expressed constitutively and repositions onto different promoters and is expressed constitutively. These data provide a foundation to characterize the mechanisms controlling c-Myb specificity. If c-Myb is regulated temporally through post-translational modifications then certain populations of c-Myb (phosphorylated versus SUMOylated) would be in greater abundance at different phases of the cell cycle. Serines 11 and 12 are phosphorylated [107] and c-Myb is hyper-phosphorylated during mitosis [223], which suggests that these residues could be phosphorylated in mitosis and not in other phases defining a large fraction of c-Myb molecules. Alternatively, these residues may be modified constitutively, but only present on a small fraction of c-Myb molecules with unique activities. Therefore, it is essential to develop antibodies that specifically recognize specific modifications in order to study these modifications in cells fractionated according to DNA content. ChIP analysis with these individual antibodies would allow us to determine if different sub-populations of post-translationally modified c-Myb bind to different genes under different growth conditions. Genome wide ChIP correlated with changes in post-translational modifications would determine what sub-populations of c-Myb are bound to clinically relevant genes. A comparison of the expression of these target genes will offer critical insight into the function of c-Myb at specific target genes during different stages of the cell cycle.

Are modifications added in sequence?

A chronological ordering of post-translational modifications associated with activation or repression has been observed with histone modifications [224, 225] and it is entirely likely that c-Myb is regulated in a such a manner. Therefore, some *in vitro* analysis with modified versions of c-Myb could demonstrate if some modifications recruit or signal for a secondary modification. For example, c-Myb is acetylated by p300 and CBP at the same lysine residues in the C-termini [96, 98]. While these residues are the primary sites of acetylation our laboratory has demonstrated that other lysines such as lysine K388 can be acetylated by p300 (Dr. Fan Liu, unpublished data). It is possible that in the event of truncation, such as that observed with v-Myb or splice variant 10A where the C-terminal lysines are deleted, K388 is hyper-acetylated affecting c-Myb activity. Therefore, if we abolish particular modifications through mutation and analyze for the presence of other types of modification such as SUMOylation, we may be able to tease out what modifications occur first and which are associated with an oncogenic phenotype.

A second approach that can be utilized to characterize post-translational modifications is to study the protein interactions that result in modifications. c-Myb interacts with p300, CBP, CDK6, pim1 kinase, and isomerases. Each of these proteins has the ability to change c-Myb activity through post-translational modifications. Acetylation increases c-Myb affinity for CBP, which suggests that some interactions occur in sequential manner. It is possible that p300 acetylates c-Myb before other modifiers such as E3 ubiquitin ligases can cause

SUMOylation or ubiquitylation. Characterizing the time dependent nature of different protein interactions could offer insight about what modifications change c-Myb activity.

c-Myb is displaced from promoters during estrogen deprivation

We demonstrated that in Jurkat T-cells c-Myb repositioned and changed specificity during the cell cycle, but in MCF-7 breast cells, c-Myb did not reposition and epitopes were masked suggesting that protein interactions changed at the promoter. Therefore, repositioning is not the only mechanism c-Myb uses to control gene expression. Our data in MCF-7 cells overwhelmingly suggests that epitope masking occurs. However in estrogen deprived cells c-Myb did not significantly bind to the target genes we identified. c-Myb was expressed in deprived cells so the lack of binding is not simply due to changes in c-Myb expression. The lack of binding to targets during estrogen stimulation in spite of stable expression suggests that in estrogen deprived cells c-Myb underwent repositioning or was simply displaced from promoters. These data coincide with our data in Jurkat cells where c-Myb was expressed and bound to DNA, but oscillated from one promoter to the other. It is likely that in deprived cells c-Myb is shuttled to a different set of promoters, which are yet to be identified.

c-Myb partners change when c-Myb does not

Epitope masking implies that c-Myb is part of a protein complex, which affects c-Myb activity. Potential masking occurred at two very highly conserved regions in the c-Myb protein suggesting that these regions are important for

protein interactions. During cell confluence and estrogen deprivation, c-Myb activity was regulated by the availability of epitopes and not by the oscillation of c-Myb from one promoter to another. This suggests that a second mechanism regulates c-Myb activity, a mechanism that exploits c-Myb protein interactions to change activity. It is likely that c-Myb can bind to the same genes during both cellular confluence and estrogen stimulation, however the factors that cooperate with c-Myb during each growth condition presumably change and alter activity. Therefore, it is important to identify what proteins interact with c-Myb in each condition. For example, other transcription factors such as beta catenin, a WNT signaling protein affected by cell confluence and cell junctions, could interact with c-Myb in stimulated cells, but not confluent cells. Experiments that would define these mechanisms include those detailed below.

In order to identify proteins that interact with each domain (1493 or Ab 1-1) a yeast two-hybrid genetic screen could be performed with isolated fragments of each domain. This type of screening would identify potential interacting partners and in many cases these partners maybe expressed in a tissue specific manner. Alternatively, cells that express-tagged versions of c-Myb could be used to perform proteomics in multiple conditions such as estrogen stimulated cells or those cultured to confluence. These experiments would identify which interactions are the most critical for c-Myb activity at different stages of proliferation or differentiation. There are many molecules of c-Myb within one cell and it is clear that the protein exists in sub-stoichiometric fractions with different activities. It has not been demonstrated that c-Myb is sequestered during

different stages of proliferation, but c-Myb colocalizes with FLASH in distinct nuclear foci [116], which suggests that some protein interactions can sequester c-Myb into different compartments. Therefore, the identification of c-Myb interacting partners could determine if c-Myb has different localization patterns when it is inactive. Estrogen deprived provide good model to study changes in both DNA binding and protein interactions. Since c-Myb in estrogen-deprived cells does not bind to a number of different targets, it suggests that in these cells c-Myb might be sequestered. As a whole, these experiments will characterized c-Myb protein interactions in different growth conditions and determine what mechanisms regulate c-Myb activity.

Do oncogenic versions reposition differently?

It is clear that truncated and mutated versions of c-Myb, like v-Myb, are oncogenic. How these versions transform cells is unknown, but it is known that these proteins regulate distinct sets of endogenous genes [21]. These proteins can bind to the same target genes in reporter gene assays [55], suggesting that the oncogenic version maintains some level of specificity. Our new data suggests that oncogenic versions bind to some of the same genes as wild-type versions of c-Myb. However, this binding might be deregulated in different stages of proliferation or differentiation. To extend our data, cells expressing different variants of c-Myb can be separated into different stages of the cell cycle to characterize if these versions bind to target genes when c-Myb does not. We discovered that c-Myb bound to the CCNB1 promoter in a cell cycle dependent manner. v-Myb can bind to CCNB1promoter in MCF-7 cells so it is plausible that

v-Myb regulation of CCNB1 is deregulated during the cell cycle. Alternatively, it is likely that binding by v-Myb does not always translate into activation. However, at some genes such as CXCR4, v-Myb transactivates the gene to a greater extent than c-Myb [21] suggesting that just the ability to bind could increase activity. Therefore to differentiate between these two scenarios we must identify whether v-Myb repositioning occurs in a manner similar to c-Myb and if v-Myb interacting partners change in response to external stimuli.

Application of fractionation to characterize stem cells

c-Myb is not only necessary for proper proliferation, it is essential for hematopoiesis [38]. In addition, it is expressed in proliferating HSCs [23] and regulates genes such as CD34 [60] that are expressed on primitive HSCs. Furthermore, loss of c-Myb results in defects in self renewal due to impaired proliferation [226] suggesting that the activity of c-Myb during differentiation is critical. Moreover, different subsets of adult stem cells have been with Hoescht dye by changes in side population sorting. These sub-populations are predisposed to differentiate into specific lineages [227]. This suggests that the activity of some transcription factors, such as c-Myb, could be responsible for the differences in “stemness.”

We have expanded a technique to analyze the occupancy of transcription factors during the cell cycle. We can apply our cell cycle analysis with stem cells presorted according to side population scatter. Live side populations can be sorted into three different stem cell compartments and the cell cycle of each

compartment could be characterized with our technique. Therefore, c-Myb activity could be analyzed in different populations of stem cells, where c-Myb is highly expressed and regulates proliferation. To extend these results, the level of c-Myb could be manipulated in mouse models to determine how decreases in c-Myb expression affect the cell cycle of primitive stem cells. These experiments are not limited to using Hoechst dye to identify side populations. Populations of cells could be defined by the expression of different cell surface markers and characterized by cell cycle fractionation. These experiments would characterize the activity and specificity of c-Myb during different stages of hematopoiesis. These experiments would enhance our knowledge of c-Myb activity in cell subsets during differentiation and if applied to some types of cancer cells, could be used to characterize changes in c-Myb activity in cancer.

Implications for the future

It is clear that c-Myb regulation of gene expression is multifaceted and the mechanisms regulating c-Myb activity are complex. If we continue to explore the hypothesis that protein interactions and post-translational modifications dictate c-Myb activity, it is possible to make tremendous strides in the Myb field. We now have the ability to study small fractions of cells. Fractionation of different sub-populations of cells may identify highly active forms of one protein. Future experiments will determine whether protein complexes, modifications, or both, define specific sub-populations of c-Myb and will enhance our understanding of how c-Myb becomes oncogenic.

Appendices

Appendix I: Specificity in primary cells

Characterization of c-Myb in primary cells

Identification of c-Myb target genes is essential for the characterization of c-Myb activity. c-Myb target genes have a variety of functions including functions in proliferation, differentiation, and apoptosis. However, the identification of target genes has been limited to pre-established cell lines. For example, c-Myb binds to the CCNB1 promoter, but the binding has been shown only in Jurkat cells, COS7 cells, and K562 cells [50]. These cells are all established cell lines that have documented expression of c-Myb. Jurkat cells dramatically over-express c-Myb. It is likely that c-Myb activity is deregulated in these cells. In addition, many pathways such as the p53 pathway are amplified or suppressed in some cell lines, suggesting that some target genes may be the product of multiple factors. For example, p53 and c-Myb interact with each other in certain cell types. Cell lines with deleted p53 may exhibit changes in c-Myb activity. Since it is difficult to determine the true activity of c-Myb with cell lines, we used mobilized CD34⁺ in order to characterize c-Myb activity in primary cells. c-Myb is expressed in CD34⁺ immature hematopoietic cells and these cells can be induced to differentiate or proliferate, which allows for the characterization of c-Myb activity at different stages of hematopoiesis.

As discussed, c-Myb plays a complex role during proliferation and differentiation. Given the puzzling fact that c-Myb binds to and activates genes

such as MPO, a myeloid specific differentiation marker expressed in terminally differentiated cells, we wanted to characterize c-Myb specificity and activity during different stages of differentiation. To this end we used cytokine treatment to both expand and differentiate cells into myeloid and erythroid specific progenitors. CD34⁺ cells were expanded for 4 days in media that contained IL-3, IL-6, SCF, and Flt-3 ligand. Cells were then shifted into medium, which contained either IL-6 with GM-CSF or IL-3, SCF with EPO for 10 days. We proceeded to differentiate these two lineages further with a treatment of EPO or G-CSF for 5 more days. We confirmed that *c-myb* RNA was relatively constant during erythroid development and decreased approximately 3 fold or 9 fold in D10 or D15 myeloid cells, respectively [118]. We performed ChIP analysis at different time points to determine c-Myb specificity at both proliferative targets such as CCNB1 or differentiation markers such as MPO and EPB41, a myeloid specific or erythroid specific marker, respectively. These data demonstrate that c-Myb specificity is cell type specific and different populations of cells have varied sub-populations of c-Myb with different binding activity.

Results

c-Myb specificity in Day 4 proliferating progenitors

Stimulated CD34 cells exhibit a typical cell cycle distribution according to Hoechst 33342 DNA content dye. Prior to stimulation, 95% of cells are in G0/G1, however upon cytokine stimulation cells enter the cell cycle and expand approximately 4-fold over 4 days (Figure 1A). These actively proliferating cells have increased *c-myb* expression relative to non-proliferating cells [118], but

ChIP analysis demonstrated that c-Myb was not bound to the proliferative markers, CCNB1 or CCNE1 (Figure 1B). However, c-Myb was bound to c-Kit and EPB41, a gene normally associated with red blood cell membrane structure. Some reports suggest that variants of EPB41 are essential for proper proliferation [228]. Despite expression of CXCR4 in these cells, c-Myb did not occupy the promoter. Consistent with the function of MPO, c-Myb did not occupy the MPO promoter in this population. These results demonstrate that c-Myb specificity is tightly regulated in stimulated CD34+ cells and that c-Myb does not bind to differentiation specific markers when they are not highly expressed.

c-Myb specificity is lineage specific in committed progenitors

As described we differentiated our proliferating day four progenitors and harvested them at day 10. During myeloid specific differentiation, the level of c-Myb decreases approximately 3-fold so we expected the level of c-Myb specificity and activity to decrease, but strikingly, we observed the opposite trend. c-Myb remained bound to the c-Kit promoter, but at least one of two antibodies (1493 or Ab 1-1 refer to chapter 3) detected c-Myb at the CCNB1 or CCNE1 promoter in these myeloid progenitors (Figure 2). c-Myb was detected at the MPO promoter consistent with the hypothesis that c-Myb binds to this promoter in myeloid progenitor cells populations. In contrast, c-Myb did not occupy the MPO promoter in erythroid progenitors, but did occupy CXCR4, c-Kit, CCNB1, and CCNE1 as detected by at least one c-Myb antibody (1493 or Ab 1-1), We presume that the antibodies differ in detection due to the documented epitope masking we have demonstrated in Chapter 3. Taken together these data

demonstrate that c-Myb specificity is lineage specific and that low levels of c-myb mRNA do not correspond to the level of c-Myb binding in primary differentiated cells.

More committed progenitors display less c-Myb activity

c-myb expression decreased as cells differentiated, but our QPCR data demonstrated that c-myb transcript was still detectable after 15 days of differentiation with Epo or G-CSF [118] so we performed ChIP after 15 days of differentiation. Our results with both myeloid and erythroid lineages was more consistent with the level of RNA transcript. We did not detect c-Myb at the MPO, EPB41, CCNB1, or c-Kit promoters in either cell type, but we did detect c-Myb at the CCNE1 gene in myeloid cells with two anti-c-Myb antibodies, but not in erythroid lineage cells (Figure 3). These data demonstrate that more committed progenitors have decreased c-myb RNA transcript levels relative to day 4 or day 10 progenitors and have c-Myb protein that binds to a different subset of genes or a smaller subset of genes since we only detected c-Myb at CCNE1 in these cells.

c-Myb activity is altered in the most immature progenitors.

We have demonstrated that c-Myb protein in progenitor cell populations has different specificity. However, CD34+ cells represent a crude mixture of different cell types. CD34+ is not an accurate measure of stem cell capabilities because other cell surface markers are necessary to accurately define stem cell populations. As a means of purifying immature stem cells from a heterogenous CD34 population we isolated cells based on the expression aldehyde

dehydrogenase expression. Aldehyde dehydrogenase is expressed in more immature stem cells that express the cell surface markers CD34, CD133, and Kit. This population is more accurately defined and therefore represents a more homogenous population of cells. Cells were stained with an aldefluor selection kit (Stem Cell Technology, Vancouver, Canada) and fractionated by fluorescence activated cells sorting. Aldefluor positive cells express aldehyde dehydrogenase and only live cells with intact membranes can retain the fluorescent signal so this technique enriched for only live stem cell populations. We performed ChIP assays with aldefluor positive cells and analyzed c-Myb occupancy at the KIT, CCNE1, CCNB1, and CXCR4 promoters. We detected c-Myb at the KIT promoter in positive cells, which was consistent with what we observed in day 4 CD34+ cells (Figure 4). However, we observed a completely different result at the CCNB1, CCNE1, and CXCR4 promoters. We detected a significant enrichment at both the CCNB1 and CCNE1 promoters in positive cells and a more modest enrichment at the CXCR4 promoter (Figure 4). These data suggest that c-Myb binds to CCNB1, CCNE1, KIT, and CXCR4 in primitive progenitor cell populations, but during stimulated proliferation and differentiation, c-Myb specificity is dramatically different demonstrating that c-Myb specificity and activity are tightly regulated during both proliferation and differentiation.

Materials and Methods

Cells and Culture Conditions

Cytokine-mobilized CD34+ cells (Fred Hutchison Cancer Research Center Large-Scale Cell Processing Core) were cultured in IMDM media (Invitrogen,

Carlsbad, CA) supplemented with BITS serum substitute, IL-3 (20 ng/ml), IL-6 (20 ng/ml), Stem Cell Factor (100 ng/ml), and FLT-3 ligand (100 ng/ml) (all from Stem Cell Technology, Vancouver, Canada) for four days. Cells were then differentiated as previously described [118].

Aldefluor selection and ChIP assays

CD34+ cells were harvested directly from the storage vial and stained for aldehyde dehydrogenase expression according to manufacturer's protocol (Stem Cell Technology, Vancouver, Canada). Cells were by FACS and were fixed as per ChIP assay. A modified micro-ChIP protocol was performed as described [195] and detailed in Chapter 2 and 3. All primer pairs are listed in Chapter 2 and 3 Supplementary Tables except for those listed in Appendix I Table 1.

Implications

This short analysis of c-Myb specificity in primary human CD34+ cells has not been previously reported. Extensive work has been performed in mice, however identification and characterization of c-Myb specificity is unclear, especially in human stem cells. Here we can demonstrate that genes identified in cell lines such as CCNB1, CCNE1, and c-Kit are c-Myb target genes in primary human cells. However, c-Myb occupancy at these targets is restricted cells of a particular cell type, such as day 10 differentiated relative to day 15 differentiated. These results suggest that c-Myb specificity is not redundant in every cell type and the external cues a cell receives (myeloid versus erythroid) impact the target genes c-Myb activates. This was clearly demonstrated by c-Myb specificity at the MPO and EPB41 targets. MPO occupancy was restricted to day 10 myeloid

progenitors. Furthermore, the occupancy of c-Myb at proliferative genes such as CCNB1 and CCNE1 was found in a variety of cell types, especially the most immature stem cells. It is clear from these results that future research and newer technology is necessary to clearly define c-Myb function in hematopoiesis. Defining the mechanisms that differentiate c-Myb specificity in each cell type is a critical stepping-stone to characterize c-Myb oncogenesis.

Figure Legends and Tables

Figure 1. c-Myb specificity in stimulated CD34+ cells.

(A) Cell cycle analysis of CD34+ cells. Cells were fixed in ethanol directly from the storage vial without any stimulation with proliferative cytokines (Left panel) or after 4 days of stimulation with a cytokine cocktail (Right panel). The percentage of cells in each phase is shown. (B) ChIP analysis with CD34+ cells. ChIP was performed with anti-c-Myb 1493 antibodies on CD34+ cells stimulated in culture for 4 days. Data is normalized to control antibody.

Figure 2. c-Myb specificity is lineage specific.

ChIP with differentiated CD34+ cells. Stimulated CD34+ cells (Day 4) were harvested and placed in cytokine specific medium to initiate myeloid or erythroid specific differentiation. Day10 progenitors were subjected to ChIP with anti-c-Myb 1493 and Ab 1-1 antibodies. Erythroid cells are represented as black and gold bars and myeloid cells are white and grey bars.

Figure 3. Successive differentiation changes c-Myb specificity

ChIP with Day 15 differentiated cells. Day10 progenitors were harvested and induced to further differentiate with either erythropoietin or G-CSF for five more days. ChIP was performed with anti-c-Myb 1493 or Ab 1-1 antibodies. Erythroid cells are represented as black and gold bars and myeloid cells are white and grey bars.

Figure 4. c-Myb binds to genes in immature stem cells.

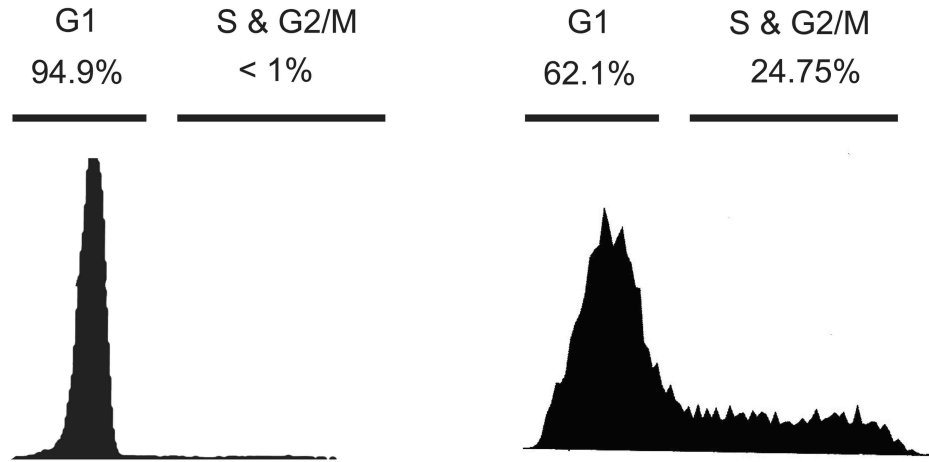
ChIP with aldefluor positive CD34+ cells. Cells were stained for aldehyde dehydrogenase expression and sorted by FACS. ChIP was performed with anti-c-Myb 1493 or Ab 1-1 antibodies. Error bars represent triplicate PCR reactions.

Appendix I Table 1: ChIP primer pairs

| Gene | Forward | Reverse |
|-------------|--------------------------|--------------------------|
| KIT | GCGGTGCCAGGAGCTCCTAA | CTCCCCAGACAATAAAGGTCGACT |
| MPO | CCCTAACTGCATCACTAACCACAA | GCGCAGGACCAAGATAACTT |

Figure 1

A



B

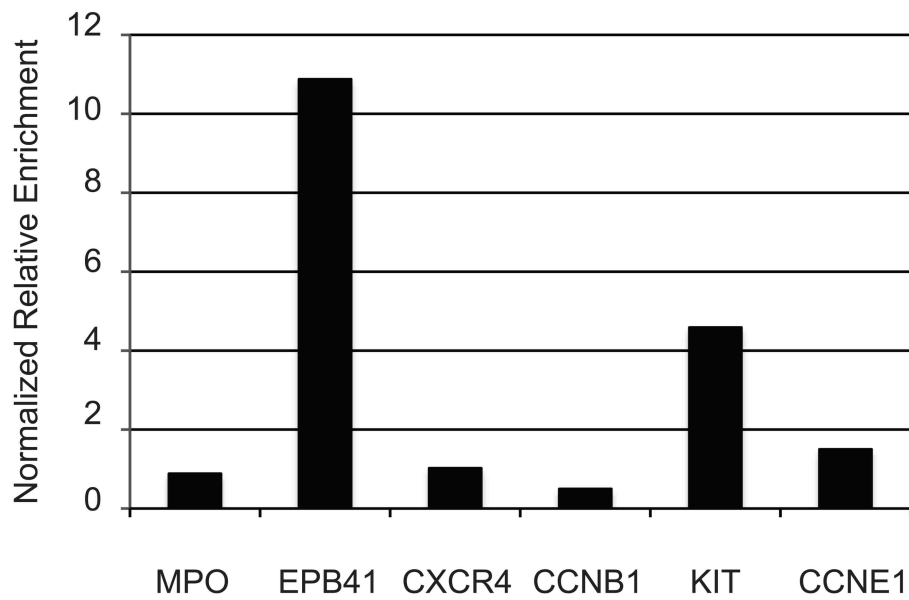


Figure 2:

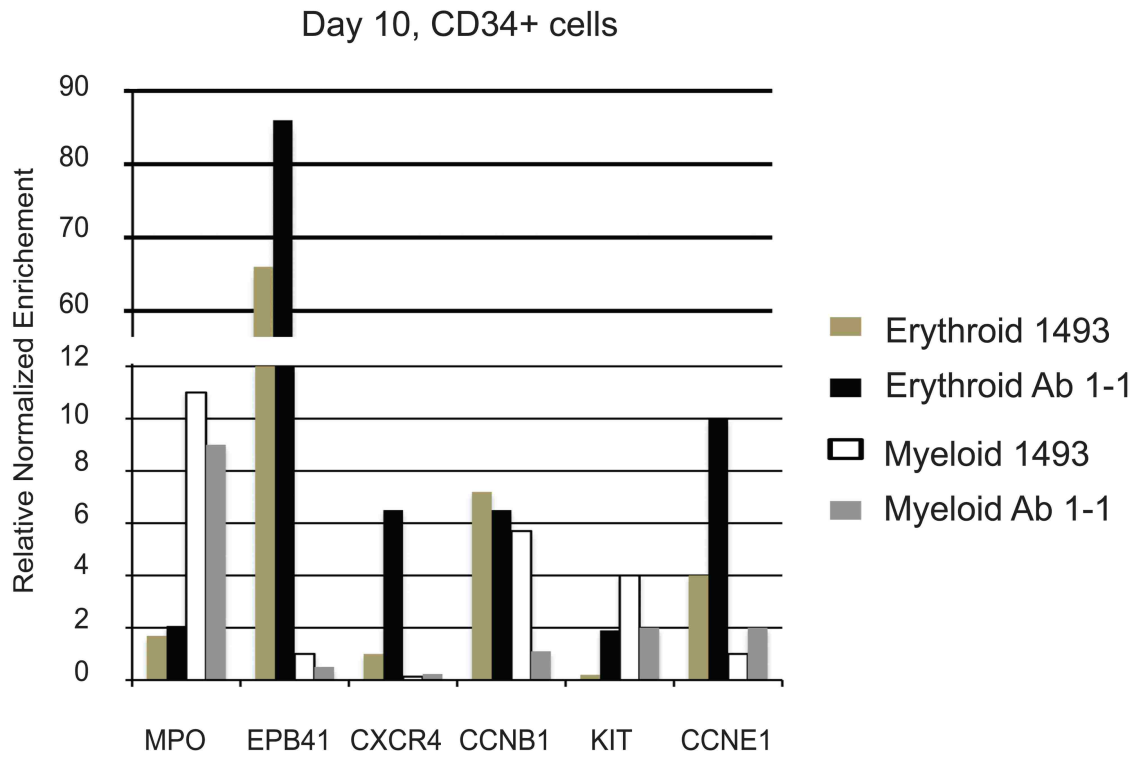


Figure 3:

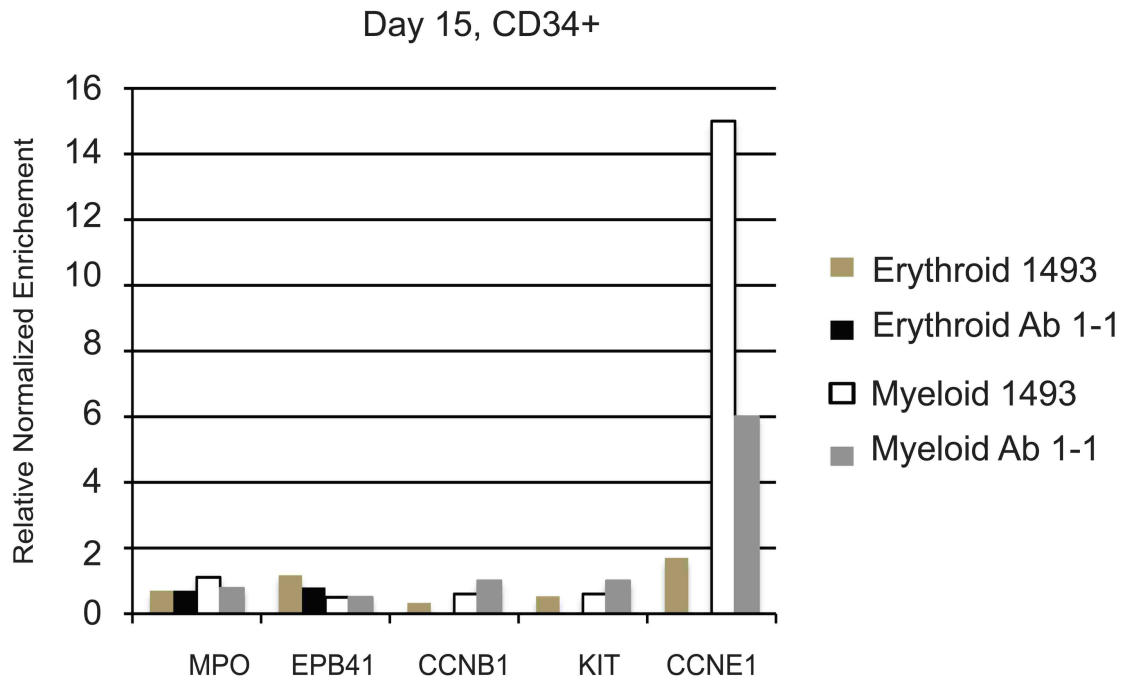
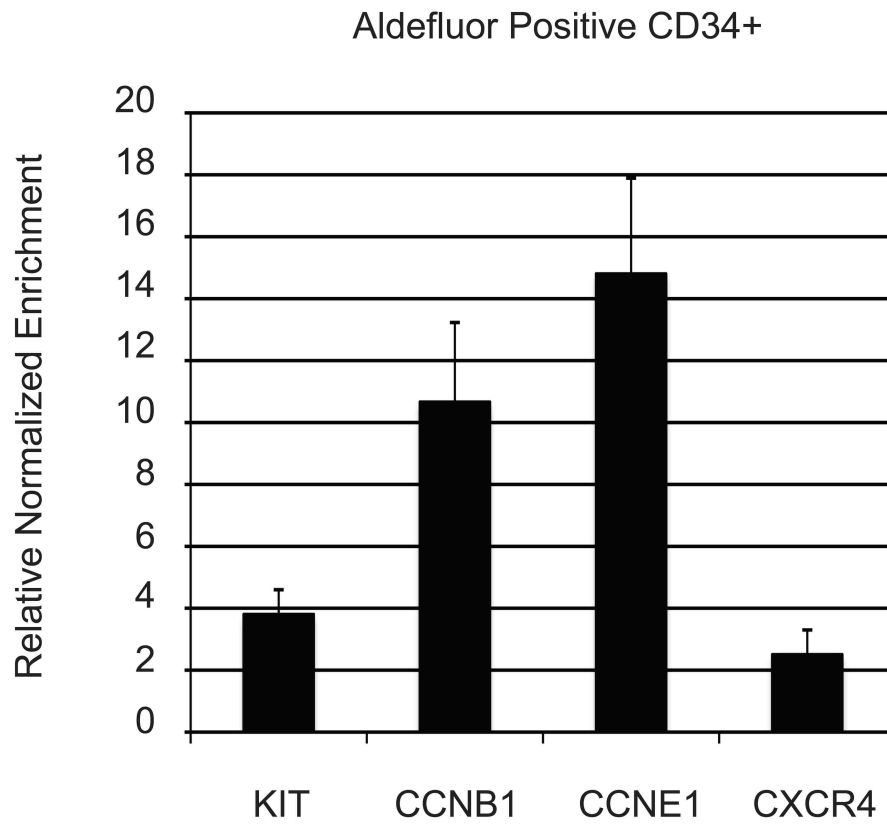


Figure 4



Appendix II: Detailed Whole Genome Amplification Protocol

Whole Genome Amplification (WGA) Protocol

Supplies:

Native Taq polymerase (Invitrogen, 18038-042 5U/ul)

10X Taq Buffer without MgCl (Comes with Taq)

50mM MgCl₂ (comes with Taq)

100uM stock of separate dNTPs (Promega Catalog number U1330)

20uM stock of dUTP (Applied Biosystems Catalog number N808-0095)

Primer A (GTTTCCCAGTCACGGTCNNNNNNNNN) HPLC PURIFIED

Primer B (GTTTCCCAGTCACGGTC) Normal purification

S-300 columns from Amersham (27-5130-01)

Always use Native Taq polymerase and its buffer as it is the most efficient and it must come from Invitrogen.

Step 1:

Quantitate ChIP DNA by Nano drop (For smaller amounts of DNA use a picogram detection kit. There are some available.) Use 10 nanograms of DNA for the concentration of primer used below

Reaction Set Up

2 ul of 10X buffer

2ul of 50mM MgCl₂

1 ul of 5mM dNTPs, mixed from 25mM stock.

1 ul of 1mg/ml BSA

2 ul of Native Taq (5U/ul Invitrogen)

1.16 ul of 40uM primer A

Up to 20 ul of water

PCR Conditions.

94 degrees for 2 min

4-5 cycles:

94 for 60 sec.

28 for 1 min

Ramp from 28-55 at 0.1 degrees/sec

55 for 2 min

68 for 30 sec

Final Extension:

68 for 8 min.

Purify DNA

Use two columns for each sample as follows:

Centrifuge 1500 RPM to remove buffer

Add 200ul of 10mM Tris pH 7.4 to wash

Centrifuge 1500 RPM

Dilute sample to 50 ul

Add samples to the column

Centrifuge 1500 RPM

Collect flow through

Repeat without a second dilution step.

Reaction 2 Setup

10 ul of Sample from Reaction 1

5ul of 10x buffer

1.875ul of dNTPs (10mM of dATP, dGTP, dCTP, and 8mM dTTP with 2mM dUTP)

1.5ul of MgCl₂ 50 mM

2-4ul of 100uM Primer B

1ul Native Taq (5U/ul) Invitrogen

Up to 50 ul of water

(Alternatively use Taq MasterMix with a spike of dUTP)

PCR conditions

94 for 2 min

15 cycles of:

94 for 30 seconds

45 for 30 seconds

55 for 30 seconds

72 for 1 min

Then 15 cycles of:

94 for 30 seconds

45 for 30 seconds

55 for 30 seconds

72 for 1 min but add 5 seconds every cycle

The first cycle will extend for 1 min and subsequent cycles will extend for 1:05, then 1:10 for each consecutive sample.

Run PCR reaction (5ul) on a 2% agarose gel. A smear should be visible in the range of the original chip DNA.

Verification

1st.- Take input DNA and perform PCR with 3-5 gene specific primers.

All of the primers should amplify a known product by regular PCR ensuring there is complete coverage of the genome.

2nd.- Take all of the samples and check them by real time PCR with primers that have displayed significant enrichment. Amplified samples should maintain the same level of enrichment as demonstrated prior to amplification. If they do not then further optimization will be necessary to maintain linear amplification. For example, try reducing the number of cycles. This can be different with different samples. If the ChIP assays have very little background it is possible to perform a higher number of cycles. If everything looks correct than sample can be pooled purified with mini-elute columns (Qiagen, Catalog number 28004), which will give the most product back with minimal losses.

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