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Case Report

MYC and Chromatin

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Abstract. MYC proteins are a family of oncogene-encoded transcriptional regulators that feature prominently in cancer. They are aberrantly expressed in a majority of human malignancies, and derive their extraordinary oncogenic potential from the ability to control expression of genes linked to cell growth, proliferation, metabolism, and genomic instability. MYC proteins are also highly-validated targets for anti-cancer therapies. Over 30 years of research into MYC has revealed the importance of chromatin in regulating both the production of MYC proteins and their ability to recognize target genes and to function as modulators of transcription. Here, we review contemporary understanding of the MYC–chromatin connection, focusing on how the encasement of DNA into chromatin impacts expression of MYC genes, and how MYC responds to and modulates chromatin to exert its transcriptional effects. We also describe ways in which chromatin structure and function are being manipulated by drug-like molecules to inhibit MYC-driven cancers.

Keywords: cancer; MYC; chromatin; histones; cancer therapy

1. Introduction

MYC proteins are a family of transcription factors that lie at the nexus of chromatin, gene regulation, and cancer. It is estimated that more than 50% of all human malignancies display overexpression of one MYC family member [1], and that MYC proteins participate in the cancer-related deaths of up to 100,000 Americans every year—and millions worldwide. The pervasive involvement of MYC proteins in tumorigenesis highlights the importance of studying their actions and regulation, and offers the real hope that intellectual conquest of MYC will lead to the development of broadly-effective anti-cancer therapies.

As regulators of transcription, MYC proteins are dominated by events in the nucleus, specifically those that occur within the context of chromatin. Not only are chromatin-connected processes pivotal in controlling MYC expression, but they profoundly influence MYC activity and, in turn, are influenced by MYC to regulate gene expression. The multifaceted ways that MYC and chromatin interact provides powerful insight into the inner workings of a set of redoubtable human oncoproteins, and have emerged as key entry-points to target MYC in the clinic. Here, we discuss current understanding of the impact of chromatin on MYC, the impact of MYC on chromatin, and how knowledge of the MYC–chromatin equation is being used to gain traction in the fight against cancer.

2. The MYC Family of Proteins

The MYC family of proteins is conserved across metazoan life [2] and consists of three distinct family members, c-MYC, L-MYC, and N-MYC, which arose from gene duplication and are practically distinguished by the spectrum of cancers in which they are implicated [3]. c-MYC is the defining member of the family and is broadly overexpressed in hematologic malignancies, as well as a wide spectrum of solid tumors. L-MYC is most frequently overexpressed in small cell lung carcinoma. And N-MYC is typically overexpressed in tumors of neural origin, such as neuroblastoma. Across the MYC family in any one species, these three proteins typically share between 35 and 50% sequence homology, and are likely to be functionally very similar, as they share critical patches of high sequence homology and display similar architectures and interaction partners. Although some operative differences between MYC family members have been noted [4, 5], it is generally assumed that MYC proteins function through similar mechanisms, and throughout this review we will use the generic term "MYC" unless referring to specific observations regarding a particular family member.

A large number of reviews have been written on MYC over the last 30 years (*e.g.,* [3, 6–9]) describing how it is expressed, regulated, deregulated in cancer, detailing the phenotypic consequences of ectopic MYC expression, and discussing the myriad of ways in which MYC propels cells towards the tumorigenic state. We refer the reader to these sources for a more detailed and expansive view of MYC proteins. Instead, our introduction to MYC will focus on three key concepts that are most important to understanding the MYC–chromatin relationship and its connection to developing anticancer strategies.

2.1. MYC controls cell growth and division. In the normal adult, most cells express very low levels of MYC protein [10], and tightly regulate its expression through a battery of processes that restrict MYC synthesis, protein stability, and activity [9]. Maintaining tight control over MYC—and preserving the signaling pathways that tie MYC production to the proliferative status of the cell—is paramount for the control of normal cellular homeostasis. And the reason is clear. MYC is one of just a handful of proteins that, when forcibly expressed in a growth-factor-deprived cell, can drive that cell from quiescence into S-phase [11, 12], with additional growth-promoting effects on cellular metabolism and protein synthesis [13]. The unique ability of MYC to drive cell growth and division absent of proper signaling processes is arguably key to its potent tumorigenicity—and is something that cancer frequently exploits to its advantage, as levels of MYC in malignant cells can be as much as a hundred-fold higher than their normal counterparts [14]. The pervasive overexpression of MYC in cancer has generated much interest in understanding how MYC levels are established in normal and cancer cells, and as we shall discuss chromatin has surfaced both a major player in the control of MYC expression and as a new route for tempering MYC in cancer.

2.2. MYC functions as a transcription factor. The general architecture of MYC resembles that of a classic sequence-specific transcriptional regulator (Figure 1). The amino-terminus of MYC constitutes a transcriptional activation domain (TAD), which is required for MYC activity [15] and is the primary point of contact of MYC with proteins that influence transcription. The carboxy-terminus of MYC carries a 100 amino acid residue basic helix-loop-helix-leucine-zipper motif (B-HLH-LZ) that dimerizes with the B-HLH-LZ protein MAX [16] to form a sequence-specific DNA-binding domain (DBD) that recognizes the consensus sequence "CACGTG", known as the "E-box" [17]. In the simplest terms, MYC–MAX heterodimers directly bind E-boxes (and variants thereof) in regulatory elements of MYC-target genes via the DBD, while the TAD makes contact with factors that stimulate their productive transcription. Additionally, and like many transcription factors, MYC can also act as a transcriptional repressor [18], a function that depends on association of MYC with DNA, but is mediated via recruitment of a distinct set of gene-inhibitory proteins [18]. Estimates of the number of MYC target genes vary [9], from a few thousand to the entire collection of active genes in any given cell type. Regardless of the precise number of target genes, however, it is generally believed that the function of MYC as a transcriptional regulator, and its ability to initiate widespread transcriptional reprogramming, lies at the heart of its growth-promoting and tumorigenic properties. Importantly, because the DNA template to which MYC binds—and on which it acts—is encased in chromatin, the interactions of MYC with the universe of chromatin modifications and chromatin regulators are vital to its functions as a transcription factor and oncoprotein.

2.3. MYC is a validated target in cancer. Perhaps one of the most surprising concepts to emerge in recent years in our understanding of cancer is that of 'oncogene addiction' [19]—the notion that tumor cells are not irreversibly shunted down the path to tumorigenesis by the actions of proteins such as MYC, but remain dependent on (addicted to) activated oncogenes to sustain the malignant state. Conceptually, oncogene addiction means that strategies to inhibit MYC expression or activity could be tremendously valuable in the clinic; a notion that is supported from results of multiple mouse model systems, where inactivation of MYC in established cancers results in pronounced tumor regression [20–24], including in cases where MYC is not the primary oncogenic driver [25]. Given the pivotal involvement of chromatin in MYC expression and activity, and the accelerating pace with which proteins such as chromatin modifiers are targeted for drug development

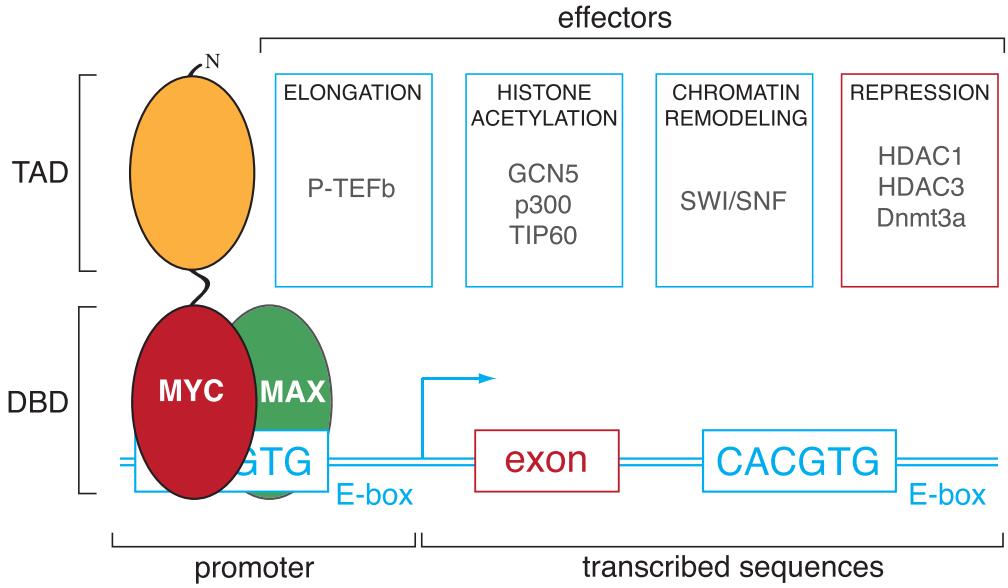

Figure 1: MYC proteins function as sequence-specific transcriptional regulators. The image shows a cartoon of the MYC protein, which carries an amino-terminal (N) transcriptional activation domain (TAD) and forms a functional DNA-binding domain (DBD) via association with MAX. MYC/MAX heterodimers bind variants of the E-box motif, which can be found in promoters as well as transcribed portions of MYC target genes. MAX does not carry a TAD. The image also shows some of the transcriptional effector molecules and complexes that have been shown to mediate various actions of MYC on gene expression.

[26, 27], it is no surprise that the chromatin arena has emerged as fertile territory for developing anti-MYC therapies.

3. Chromatin

The term 'chromatin' was first coined by Walther Flemming in 1882 [28], after observing threadlike structures in the nucleus that take on color after staining with aniline dyes. We now understand chromatin to be the complex of DNA and proteins that condenses chromosomal DNA into the nucleus through hierarchical layers of packaging—between DNA and histones to form the nucleosome, between nucleosomes to form the canonical 30 nm fiber, and between 30 nm fibers to form the final structure of the chromosome. Parceling of DNA into chromatin not only compacts and protects the genetic information, but acts as a physical barrier to processes such as transcription, and subjects DNA to considerable topological restraint. As a result of its unique ability to impact the configuration and availability of DNA, chromatin plays a major role in regulating eukaryotic gene transcription [29]. Below we discuss a few ways in which alterations to chromatin can influence transcription, absent of any changes in the primary DNA sequence. Note that although these examples are discussed individually, they do not occur in isolation, and the functional consequence of any one alteration will be determined by the sum of all regulatory events that descend on a particular piece of chromatin.

3.1. Regulating chromatin via histone modifications. The packaging of DNA into chromatin, and the hierarchical way in which it is assembled, creates a number of interesting routes through which transcription can be regulated. Gene activity can be modulated by processes ranging from incorporation of specialized histone variants [30] or the precise position of nucleosomes on a segment of DNA [31], through to alterations in higher-order chromatin structure [32], interaction of disparate chromatin domains [33], or even the location of a particular piece of chromatin within the nucleus [33]. In terms of MYC, however, perhaps the most salient mechanism of chromatin regulation is post-translational modification (PTM) of histones.

All four core histones are subject to a suite of PTMs that include phosphorylation, acetylation, methylation, ubiquitylation, and SUMOylation [34]. These modifications occur principally (but not exclusively) on the unstructured tails of histones, and establish an intricate system that can regulate gene activity, integrate combinatorial signaling processes, and message the status of a particular segment of chromatin to the cell. The last decade has witnessed an explosion in our understanding of these modifications and how they act, and a few general principals have emerged that are worth considering (Figure 2). First, histone PTMs influence transcriptional processes in multiple ways, such as altering the physical properties of chromatin or signaling recruitment of specific proteins (or protein complexes) known as 'chromatin readers'. As an example, histone acetylation at some sites creates a permissive chromatin environment by 'loosening' the association of DNA with nucleosomes and

by disrupting higher-order compaction processes [35]. At other sites, however, acetylation can promote transcription by selectively recruiting proteins that read the modified histone and, in turn, enlist additional transcription-promoting proteins [36]. Second, histone PTMs are usually in a constant state of flux, and it seems that for every factor that can deposit a specific histone mark—known collectively as 'chromatin writers'—there is a 'chromatin eraser' that can reverse the process. Third, histone PTMs can influence each other and can function combinatorially, constituting a kind of "histone code" that sets—or reflects—the transcriptional state of a particular piece of chromatin [37]. And finally, histone PTMs play a vital role in coordinating transcriptional processes [38], signaling to and from chromatin in response to events such as DNA damage [39], and in mediating transcriptional effects of RNAi-based gene silencing [40]. In this way, histone modifications are functional hubs that tie chromatin to just about every other important cellular process, and create continuous opportunities for cells to adjust their transcriptional output.

Importantly, and as discussed later in this review, targeting the factors that read, write, and erase histone PTMs has become a top priority in the development of drugs to treat cancers, including those driven by MYC.

3.2. Chromatin control through changes in DNA. Unlike protein, the DNA component of chromatin is not subject to an extensive set of regulatory modifications or other changes that influence gene expression. But that does not mean that DNA is invariable, and there are at least two important ways in which DNA can be altered (absent of changes to its sequence) to control gene expression (Figure 3).

In mammalian cells, the most common covalent regulatory modification to DNA is methylation of the fifth carbon of the cytosine base, forming 5-methylcytosine (5meC; [41]). This modification occurs within the context of CpG dinucleotides, is mediated by enzymes known collectively as DNA methyltransferases (DNMTs), and is generally considered a transcriptionally repressive mark, disabling DNA recognition by sequence-specific transcriptional activators [42] or recruiting methylated DNA readers [43] that lead to deposition of further inhibitory marks on chromatin. In normal cells, the distribution of methylated CpG dinucleotides is highly asymmetric, occurring principally at isolated CpG sequences, but not within the high-density CpG islands typically found in gene promoters [44]. The lack of 5meC within promoter-associated CpG islands keeps these elements accessible, and thus permissive for regulation by sequence specific transcription factors. Importantly, cytosine methylation patterns can be changed to alter the transcriptional profile of a cell. *De novo* CpG methylation within promoter DNAs is a mechanism of transcriptional repression [45], and cancer cells frequently exhibit pronounced changes in cytosine methylation, with some regions demethylated and others hypermethylated [46]. The contribution of these changes to cancer pathophysiology are profound, as inhibitors of DNA methylation modulate tumorigenicity in model systems of cancer and indeed are FDA-approved for treatment of malignancies such as acute myeloid leukemia (AML; [47]).

Besides covalent modification, DNA structure can be altered in a number of ways to modulate its biological potential, one of which is the stable formation of triplex or quadruplex configurations that differ dramatically from canonical B-form DNA [48]. For example, triple helical structures (often called "H-DNA" due to their stabilization via hydrogen bonds [49]) form at homopurine-homopyrimidine palindromes, when the DNA duplex at one half of the palindrome denatures and one of the strands pairs with the non-denatured palindrome half. Alternatively, if a DNA segment contains specific configurations of residues rich in blocks of guanine, it can form stable four-stranded structures known as "G-quadruplexes" (G_4-DNA). Depending on the length and nature of the guanine blocks, G4 DNA can involve either one, two, or four separate DNA strands. And if conditions are right, the displaced C-rich strand can fashion a structure known as the 'i-motif', which is a four-stranded structure composed of two intercalated, hemiprotonated, cytosine-cytosine base pairs [50]. Formation of H- and G_4-DNA and i-motif structures could influence transcription in a number of ways, including preventing recognition by sequence-specific DNA-binding proteins, altering the distance or stereospecific alignment of promoter elements, or recruiting new factors that specifically recognize altered DNA configurations. Although the *in vivo* significance of these non-canonical DNA structures has been the subject of much debate [48, 51], accumulating evidence supports the notion that they play a role in gene expression and integrity [52]: For example, triplex DNA formation is mutagenic and can trigger the DNA damage response [53], and sequences capable of forming H-DNA are overrepresented in gene promoters [54], where they have been found to modulate gene activity [52]. Interestingly, much of what is reported on the influence of non-B-form DNA on transcriptional processes centers on control of MYC expression, and we will return to this topic—and its therapeutic potential—later in the review.

4. The Impact of Chromatin on MYC Expression

By the time the archetypal *c-MYC* gene was sequenced in 1982 [55], researchers already knew that control of its transcription made the difference between the normal and the malignant state. Indeed, one of the key discoveries pinpointing *c-MYC* as a cellular oncogene was the finding that avian leukosis virus (ALV) induces tumors by retroviral promoter insertion at the *MYC* locus, stimulating expression of the downstream cellular gene [56–58]. Not surprisingly, therefore, early effort was placed on understanding how *MYC*

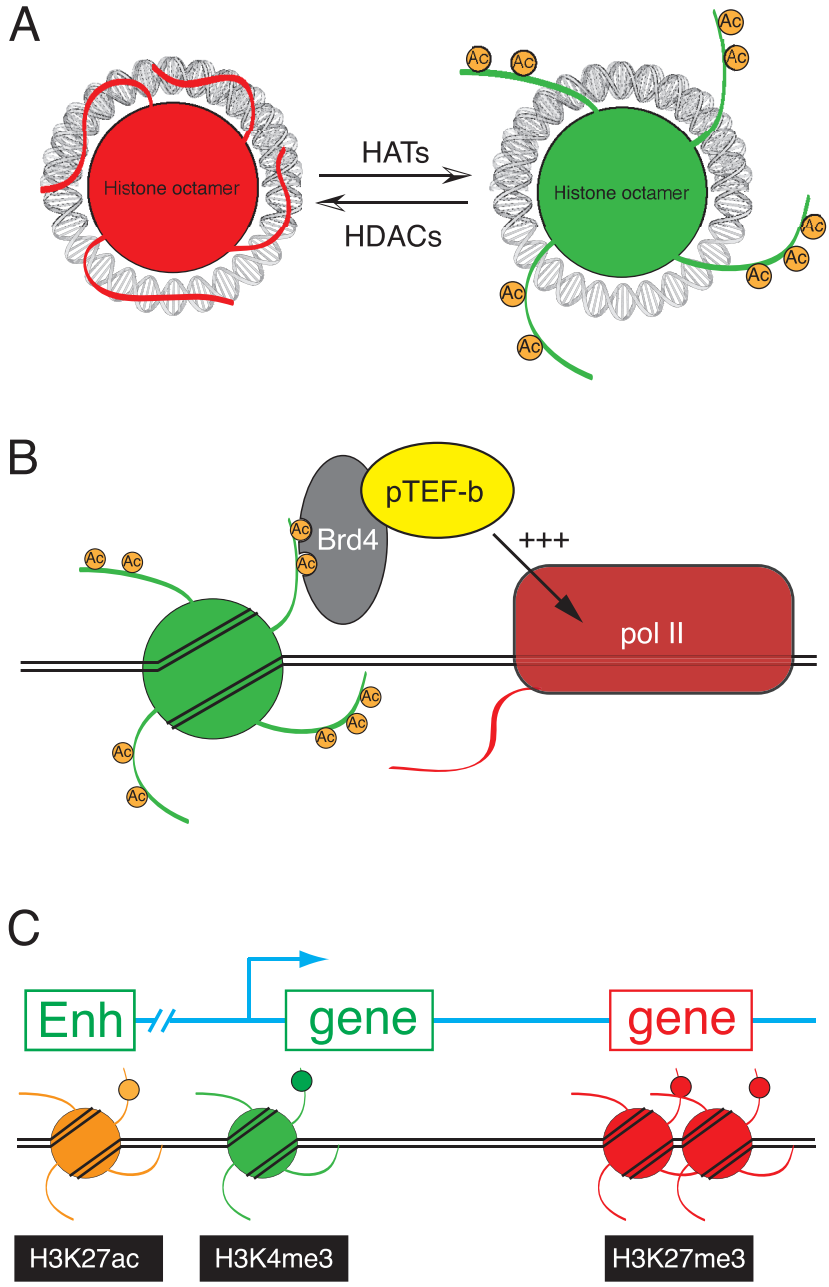

Figure 2: Impact of histone modifications on transcriptional processes. The figure presents some examples of how post-translational modifications to histones influence transcription. (A) Acetylation of histone tails promotes an open chromatin configuration by neutralizing their positive charge and repelling interactions with the negatively-charged DNA backbone. The process is catalyzed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). (B) Histone modifications recruit chromatin "readers". In this example, the dual bromodomain protein Brd4 binds directly to acetylated histones and recruits the elongation factor pTEF-b to stimulate (+++) release of paused polymerases (pol II). (C) Histone modifications as indicators of the transcriptional status of chromatin. Enhancers (Enh), gene-proximal promoters, and repressed genes (red) are indicated by distinct patterns of histone modifications. In this case, just one example of each type of modification is given. "H3K27ac" refers to acetylation of lysine 27 of histone H3. "H3K4me3" refers to trimethylation of lysine 4 of histone H3. "H3K27me3" refers to trimethylation of lysine 27 of histone H3.

gene transcription is controlled. Subsequently, it became clear that control of MYC transcription is a phenomenally complex process (for a thorough review of the *c-MYC* promoter, see [59]) that involves four distinct promoters and more than a dozen transcriptional regulators, many of which integrate signaling events directly relevant to cancer (*e.g.*, *β*-catenin; [60]). It also became apparent that, in addition to an ensemble cast of sequence-specific transcriptional regulators, chromatin plays a leading role in governing MYC transcription [61–66]. Here, we discuss two general and

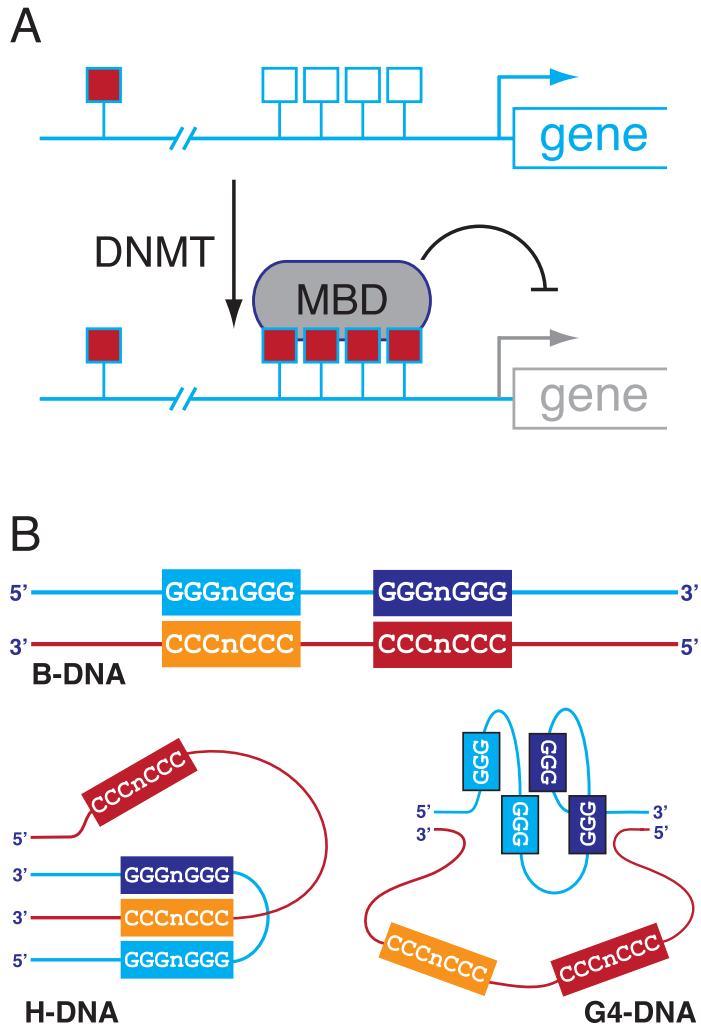

Figure 3: Transcriptionally-relevant changes to DNA that do not affect DNA sequence. (A) Cytosine methylation. CpG doublets are presented as square boxes (open is unmethylated; filled red is methylated cytosine). CpG islands, which are located proximal to ~60% of mammalian promoters, are typically unmethylated. In the cartoon, a DNA methyltransferase (DNMT) catalyzes *de novo* methylation at the CpG island, recruiting a CpG "methyl-binding domain" (MBD) which in turn recruits other factors to repress transcription. CpG methylation can also directly prevent recognition by DNA-binding proteins (*not shown*). (B) Alternative DNA configurations that can form at select repeating sequences, in this case mirror-symmetric homopurine-homopyrimidine stretches. Such elements can form triplex H-DNA via interactions with each repeat, or G-quartets (G4-DNA) via interactions with G residues in each repeat-half. Both structures result in the formation of stretches of single-stranded DNA that confer enhanced sensitivity to S1 nuclease, a common probe for their formation *in vivo*. Modified from [182].

therapeutically tractable ways that MYC transcription is controlled at the level of chromatin: via alternative DNA structures and through long range control by the action of enhancers.

4.1. Alternative DNA structures that regulate MYC transcription. One of the most powerful probes for frank alterations in the configuration of DNA within nucleosomes are nucleases (*e.g.,* DNase 1 and S1 nuclease), which cleave chromatin preferentially at sites of relaxed DNA-nucleosome contact or of single-stranded DNA formation (as occurs upon formation of H- or G_4-DNA; Figure 3). Combined with indirect end-labeling procedures, these enzymes can pinpoint the location of contextual DNA changes, which in turn can then be correlated with specific transcriptional outputs to infer a functional role in gene expression. Such approaches have been instrumental in defining regulatory elements and processes controlling *c-MYC* transcription [61–66], some of which are presented in Figure 4.

As mentioned, *c-MYC* transcription is driven by four distinct promoters, P_0–P_3, with greater than three quarters of *MYC* transcripts originating from the P_2 promoter [59]. Upstream of P_2 lie two elements that can form non-B-DNA structures, likely in response to torsional stresses that are produced as a result of transcription—FUSE and NHE

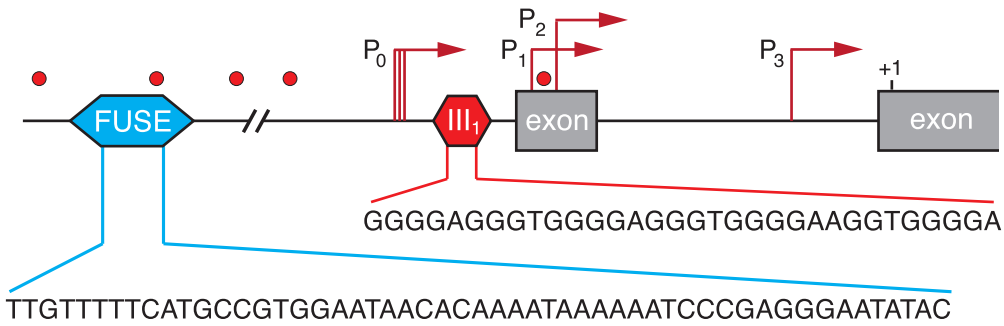

Figure 4: The *c-MYC* promoter. The figure represents an approximate 3 kilobase segment surrounding the 5' end of the human *c-MYC* gene. Red arrows indicate the four MYC promoters (P_0 to P_3), as defined by transcriptional start sites (the P_0 promoter has multiple transcriptional start sites). MYC exons one and two are represented as gray boxes; "+1" indicates the translation start site for the canonical 'p64' MYC protein. Nuclease sensitive regions are indicated as red circles. The relative location of the FUSE and NHE III_1 elements are presented, below which appears the nucleotide sequence of each element.

III_1—that control MYC gene transcription in fascinating and different ways.

FUSE. Located approximately 1.7 kb upstream of the P_2 promoter is the far upstream sequence element, or FUSE [67]. First identified by its nuclease sensitivity, FUSE is a 90 base pair A/T rich *cis*-acting sequence that, in the absence of *c-MYC* gene transcription, is complexed with nucleosomes and adopts a typical double-stranded B-DNA form (Figure 5) [68, 69]. Upon *c-MYC* transcription, however, passage of RNA polymerase II along the DNA creates negative supercoiling stresses at the promoter that destabilize FUSE, morphing the element into a nucleosome-free and single-stranded state that recruits two structure-sensitive regulatory proteins, FBP and FIR [69]. FBP (FUSE-binding protein) is the first to engage the partially unwound FUSE, interacting with single-stranded DNA via a DNA-binding module similar to that found in the RNA-binding protein hnRNP K [70]. Once bound, FBP potently stimulates MYC transcription by making direct contact with the general transcription factor and DNA helicase TFIIH [71]. The physical association between FUSE-bound FBP and P_2-bound TFIIH, together with the increased transcriptional output of the promoter, then conspire to create a topologically constrained loop in the intervening DNA that drives FUSE into the fully-denatured state [72]. Upon transition to the open single-stranded configuration, FUSE is then able to recruit a second single-stranded DNA-binding protein, FIR (FBP-interacting repressor protein [71]), which initiates a new set of events that *inhibit* MYC promoter activity. Specifically, FIR inhibits the helicase activity of TFIIH, causing a reduction in activated transcription, a decrease in torsional stress across the FUSE, loss of FBP binding, and escape of engaged RNA polymerase II molecules into the elongation-competent form. The net effect of these events is to stymie *MYC* promoter function, dissipate the superhelical forces, drive FUSE back to the canonical B-DNA form, and restore MYC transcription to basal levels.

What is the point of such a seemingly counter-productive mechanism? In its simplest terms, the FUSE–FBP–FIR–TFIIH system acts akin to centrifugal governors that maintain the operating speed on rotative engines, tying the actual revolutions per minute of the engine to a device that feeds back to either decrease or increase engine speed. The effectiveness of such devices stems from their ability to directly measure the mechanical output of the engine, and to continue the analogy this is precisely how the FUSE–FBP–FIR–TFIIH system acts. By directly sensing a consequence of transcription—rather than, say, the presence of particular proteins that may be involved in transcription but not always indicative of ongoing transcriptional events—the FUSE–FBP–FIR–TFIIH axis constantly measures the transcriptional output from the MYC gene and feeds back to either inhibit (FIR) or activate (FBP) MYC transcription, thereby keeping MYC expression within the appropriate limits of tolerance.

Three points are worth making regarding the FUSE–FBP–FIR–TFIIH system. First, although there has been considerable debate regarding the *in vivo* significance of alternative DNA structures and the role of torsional stress on transcriptional processes, the Levens group in particular has made a compelling case that *c-MYC* transcription generates sufficient supercoiling to induce unwinding of the FUSE in cells, and that this correlates with both the recruitment of FBP and FIR to FUSE and with the clear functional roles of both proteins in controlling MYC transcription [73]. Second, genome-wide approaches have now shown that dynamic supercoiling is a characteristic of virtually every transcribed gene in human cells [74], meaning that the detailed mechanisms established for MYC are very likely to serve as a paradigm for how all transcription within chromatin can be regulated. And finally, the FUSE–FBP interface has particular structural characteristics (discussed later in Section 7) that may very well make it possible to develop pharmacological inhibitors to attenuate MYC transcription in cancer cells.

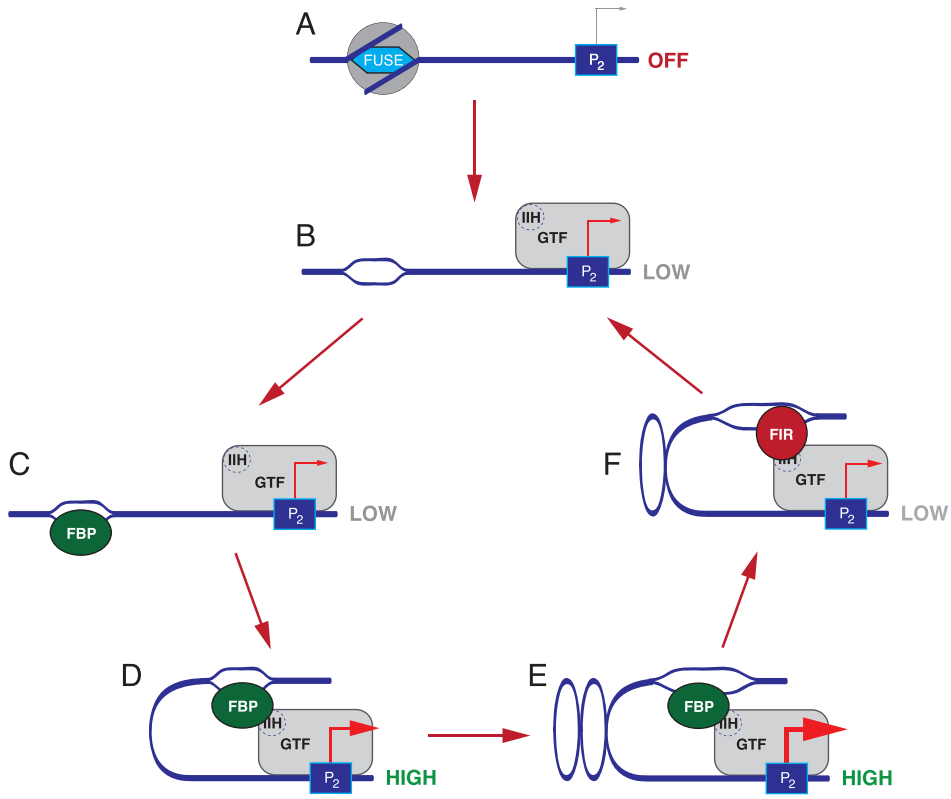

Figure 5: The FUSE–FBP–FIR–TFIIH Governor for *c-MYC* transcription. (A) In the absence of appropriate growth signals, *c-MYC* is not transcribed, and the FUSE element is double-stranded and nucleosome-bound. (B) Upon induction, MYC is transcribed at a low level and as a result the FUSE element transitions to a partially single-stranded state. (C) Single-stranded FUSE is bound by FBP, which then contacts TFIIH (IIH), forming a localized loop in the promoter (D). (E) FBP stimulates transcription, the effect of which is to induce supercoiling in the loop, which in turn fully melts the FUSE element. (F). Melting of FUSE leads to loss of FBP and recruitment of the FIR repressor, which inhibits transcription, leading to a reduction in localized torsional stress, returning the promoter to the basal state (B). Only the P2 promoter is shown for clarity. "GTF" refers to the general transcription factors (including RNA polymerase II).

NHE III$_1$. Downstream of FUSE (Figure 4), and ~100 bp upstream of the P_1 promoter, is nuclease hypersensitive element III_1(NHE III_1). This segment was originally identified via its DNase I hypersensitivity [62], and is noted for its importance in *c-MYC* transcription (particularly the P_1 promoter; [75]), its unusual G-rich sequence composition (Figure 4), and its ability to form triplex DNA structures *in vitro* [76]. Although it has been difficult to establish with certainty that non-B-DNA structures form at NHE III_1 in living cells, the unusual propensity of this DNA segment to adopt alternative configurations is well established *in vitro* [77], and dozens of publications have built a strong and consistent case for their role regulating MYC expression (reviewed in [78]). Additionally, structure-specific antibodies have revealed the presence of G4-DNA in living cells [79], and chemical scaffolds shown to stabilize G_4-DNA configurations *in vitro* have the predicted effects on MYC transcription *in vivo* [80], making it likely that NHE III_1 controls MYC expression, at least in part, via non-canonical DNA structures.

The currently-accepted model for how NHE III_1 functions [81] is depicted in Figure 6 and involves a multi-state mechanism that can either enhance or repress *c-MYC* transcription, depending on protein factors and DNA topology. In the basal state, NHE III_1 is nucleosome-free and in its native B-DNA arrangement. Upon induction (*e.g.,* in response to serum growth factors), the housekeeping transcription factor Sp1 binds the double-stranded G-rich repeats within NHE III_1 (also known as the 'CT elements') and functions in a stereotypical manner to initiate MYC mRNA synthesis [82]. In turn, and as discussed with FUSE, the resulting transcription leads to the induction of negative supercoiling in the wake of RNA polymerase II, which promotes strand separation at NHE III_1. At this point, one of two outcomes are possible. In the presence of additional appropriate signals (*e.g.,* growth factors [83]), MYC transcription can be 'turbocharged' by recruitment of two single-stranded DNA-binding proteins: hnRNP protein K, which binds to the pyrimidine-rich strand [84], and cellular nucleic acid binding protein CNBP, binds to the purine-rich strand [81]. Both factors stabilize single-stranded DNA at NHE III_1 and

accelerate transcription from the *c-MYC* gene. Alternatively, if such signals are not present (or if others are received to shut down MYC expression), each strand of NHE III_1 adopts a unique and different non-B configuration, with the G-rich strand assuming a G_4-DNA structure [85] and the C-rich strand forming an i-motif [86]. These structures act to repress MYC transcription, in large part by preventing binding of Sp1, hnRNP K, and CNBP to their cognate elements in the P_1 promoter [77, 78].

In contrast to the balanced level of transcriptional output afforded by the FUSE–FBP–FIR–TFIIH governor, the topological maneuvers of NHE III_1 appear to provide a binary means of safely turning on and off c-*MYC* transcription. During activation of MYC mRNA synthesis, the action of this element provides a way to first modestly induce the *c-MYC* gene (via Sp1), and then to sample the status of the cell (via hRNP K and CNBP) to determine whether MYC transcription should be increased or shut down. This "toe in the water" approach provides yet another failsafe mechanism to ensure that MYC is fully transcribed only when conditions are right [9]. Additionally, the unique functional characteristics of G_4-DNA formation at NHE III_1 can also integrate signals that acutely shut down MYC transcription and keep it off. For example, the abundant nucleolar protein nucleolin binds directly to NHE III_1 and promotes the formation and stability of the G_4-DNA structure [87], suppressing MYC transcription. Because nucleolin moves from the nucleolus to the nucleoplasm in response to p53 activation [88], this G_4-DNA-mediated mechanism could be a part of the tumor-suppressive program that p53 initiates in times of genomic menace to block cell proliferation. Moreover, because G-quadruplex DNA has a higher melting temperature than the duplex form, this "off" state is likely to be more stable than the permissive B-DNA configuration, and may very well require enzyme-mediated processes to be resolved [89]. If G_4-DNA structures at the MYC promoter have to be actively dismantled to restore P_1 promoter activity, this would provide cells with an additional layer of regulation to prevent *c-MYC* transcription at the wrong time.

Note that although our discussion above deals with FUSE and NHE III_1 separately, their physical proximity, and their functionally thematic similarities, makes it highly likely that topological changes at one element influence actions at the other [77]. Also note that just as the unique spatial rearrangements at FUSE have attracted the attention of those interested in pharmacological inhibition of MYC synthesis, so too have those occurring at NHE III_1.

4.2. The role of enhancers in regulation of MYC transcription. Transcriptional enhancers were first observed in 1981 [90] and defined by their ability to stimulate transcription *in cis* from promoters located many kilobases away. Enhancers are typically several hundred base-pairs in length and recruit collections of *trans*-acting regulatory factors to enhance particular patterns of promoter activity. The ability of enhancers to drive gene expression from a distance can make it difficult to assign each enhancer to a specific target gene (especially as there can be intervening genes between a promoter and its enhancer), and raises the interesting question of how enhancers are able to control gene transcription from such a distance. The unlikely prospect that such expanses are spanned by linear alterations in DNA structure, or assembly of vast protein bridges, led early to the notion that enhancers must function by looping out intervening DNA and engaging in short-range protein-protein contacts with promoter-bound factors. And for the most part this notion appears correct [91, 92]. As with all things connected to MYC, control of its transcription by the action of enhancers is a complex topic, with no unifying model to explain the regulation or deregulation of MYC in all relevant contexts [93]. To highlight some of the ways *c-MYC* gene expression can be controlled by the action of distal enhancers, and the relevance of such mechanisms to cancer, we shall briefly discuss two illustrative examples here—the "gene-desert" enhancers and the 'super-enhancers".

4.3. The Gene Desert Enhancers. As mentioned, the remote action of enhancers can make them difficult to identify by traditional "promoter bashing" analyses, meaning that more global approaches are often required to pinpoint such elements. For example, genome-wide association analyses recently identified a set of single nucleotide polymorphisms (SNPs) on chromosome 8q24 that are associated with markedly increased risk to specific types of epithelial cancers [94–97]. These SNPs cluster in three discrete regions (Figure 7) within a 1.5 Mb "gene dessert" [98] that is hundreds of kilobases away from the nearest gene, *c-MYC*. Despite their desolation, each of these three regions display chromatin marks that are characteristic of enhancers—such as mono-methylation at lysine 4 of histone H3 (H3K4me1) and binding of the chromatin regulator p300 [99]—prompting investigators to examine whether the elements defined by these SNPs are long-range MYC enhancers. Supporting this notion, chromosome conformation capture (3C) assays have revealed that each region is in physical contact with the *c-MYC* gene [99–102], with the intervening DNA looped out, and that these segments can function as enhancers of the MYC promoter in traditional reporter-gene assays. Moreover, the long-range looping that is seen for each of these particular elements closely mirrors the cancer-association of the SNP that defined them, with colon-cancer SNP regions interacting with the MYC promoter in colon, but not breast or prostate, cancer cell lines, and so on [99]. Thus it appears that each enhancer is capable of driving MYC expression in specific tissue-types, and that minor alleles of each SNP are contributory to MYC deregulation in select cancers. But how?

The best understood of the gene desert enhancers is that in prostate/colon-specific risk region 3, defined by SNP rs6983267. Located ~330 kb from the *c-MYC* promoter,

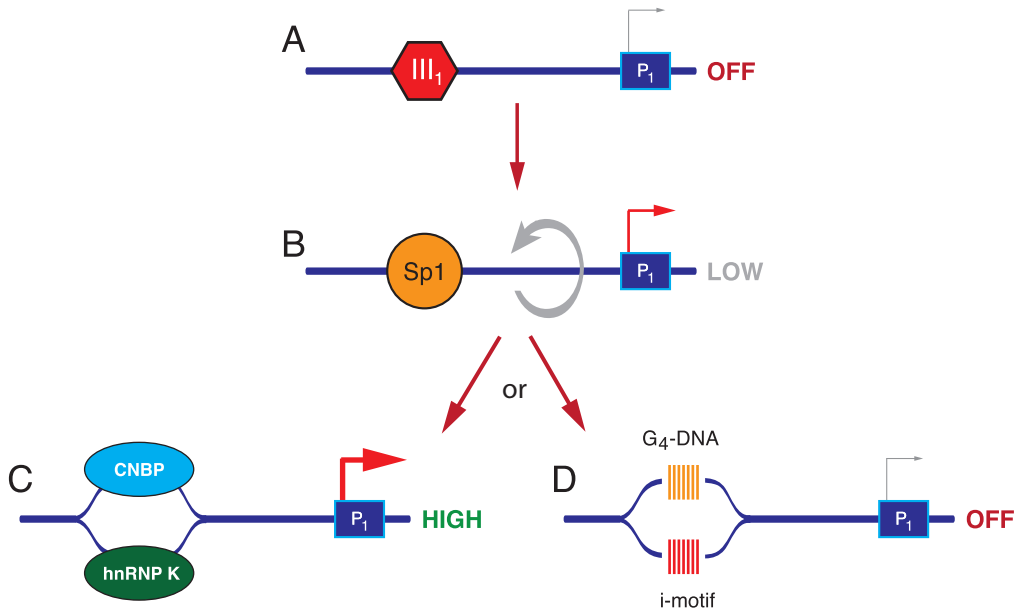

Figure 6: Regulation of MYC transcription through NHE III_1. (A) In the absence of growth factor signals, NHE III_1 is double-stranded and nucleosome free, and the *c-MYC* gene is not transcribed. (B) Signals to transcribe MYC result in the recruitment of transcription factor Sp1 to NHE III_1, and the *MYC* gene is transcribed at a low level. Negative supercoiling occurs as a result of ongoing transcription, causing the NHE III_1 element to denature. At this point, one of two outcomes can occur. (C) If conditions are appropriate for full MYC expression, CNBP and hnRNP K bind separately to each strand of the NHE III_1, and drive high level MYC transcription. (D) If conditions are not appropriate, each single stranded segment of DNA will adopt a G_4 or i-motif configuration, as indicated, which prevents binding of single- and double-stranded regulatory proteins and shuts down the P_1 MYC promoter.

rs6983267 lies at the 3' end of one of two inverted binding sites for the transcription factor TCF/LEF1 [100, 101]—a particularly meaningful occurrence, as TCF/LEF1 is a critical effector of the Wnt/APC/β-catenin pathway that is deregulated in practically all colorectal cancers [103]. The common T variant at this position creates an imperfect consensus site for TCF/LEF1 binding, whereas the tumor-associated G-variant generates a near-optimal TCF/LEF1 site, and is associated with increased TCF/LEF1 binding and two-fold higher levels of *c-MYC* transcription [100]. Interestingly, although the loop that forms between the region 3 enhancer and the MYC promoter is dependent on TCF/LEF1 [101], looping itself is not overtly affected by the G-variation [100]. Given that looping is the most likely mechanism of enhancer-promoter communication, one possibility is that interactions between TCF/LEF1 proteins bound to the MYC enhancer and promoter create a loop that primes the MYC gene for activation, and that tumor-associated perturbations of this system—either by creation of a consensus TCF/LEF1 site at the enhancer or ectopic activation of Wnt signaling—drive the poised ensemble to the active configuration.

A two-fold increase in MYC transcription, as observed with the rs6983267 SNP, may not seem very significant in the context of cancer, where changes in MYC expression levels can be over two orders of magnitude [9]. But one recurring feature with MYC is that it is not simply the overexpression of the protein that is important in tumorigenesis, but that it is the disconnect between MYC and its normal entourage of regulatory mechanisms that leads to malignancy. The fact that the "normal" region 3 enhancer has a highly conserved yet imperfect TCF/LEF1 binding site [100] implies that the ability of cells to regulate this site is an important evolutionary constraint. By extension, conversion of this site to a perfect consensus favors TCF/LEF1 binding and robs cells of the opportunity to appropriately restrain MYC expression. Consistent with this view, mice lacking the region 3 enhancer have only modestly reduced MYC levels and develop normally, but are strikingly resistant to intestinal cancers driven by an APC mutation [104]. Results such as these provide a frank demonstration of the contribution of subtle, long-distance, effects on MYC deregulation in the setting of cancer, and lead to the realization that drug-like molecules capable of inducing even small changes in MYC gene transcription could have tremendous therapeutic utility in certain cancers.

4.4. Super-Enhancers. Very recently, comparative genomic approaches allowed identification of a class of enhancer elements in multiple myeloma cells that can very much be considered the "mothers of all enhancers" [105, 106]. Like typical enhancers, these "super-enhancers" lie distal to transcriptional start sites and can be defined by specific patterns of histone modifications and by binding of positively-acting transcriptional (co)regulators. What sets these elements apart, however, is their scale. Super-enhancers

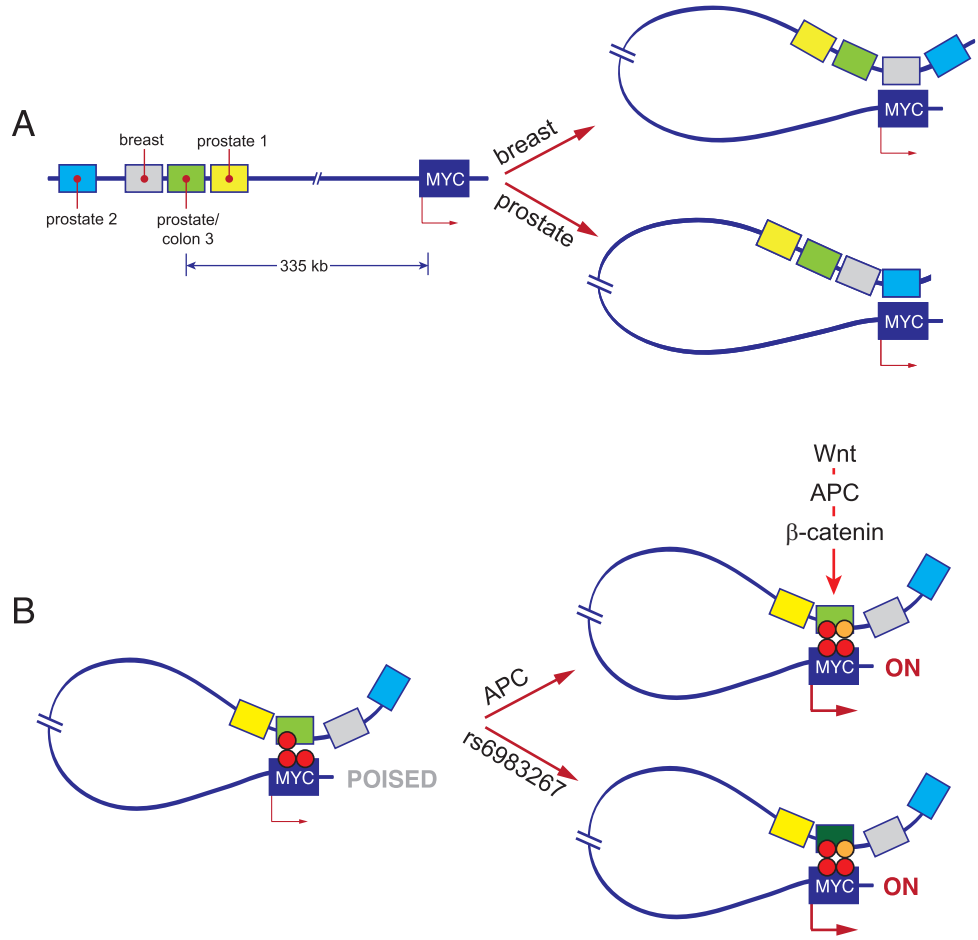

Figure 7: The 8q24 gene desert enhancers. (A) Cartoon of distal *c-MYC* enhancers, including positions where SNPs that are associated with the specific cancer types have been identified. Consistent with the disease-specific association of the various SNP regions, each enhancer makes contact with the *MYC* promoter in a tissue-type specific manner. The breast and prostate 2 enhancers are shown for illustration. (B). The prostate/colon-specific enhancer 3 makes loop-mediated contacts with the *c-MYC* promoter in prostate/colon cells, poising the promoter for activation. Subsequent activation of *MYC* transcription can be achieved by activation of the Wnt/APC/*β*-catenin pathway (APC), which causes *β*-catenin/TCF/LEF1 to occupy a remaining site on the enhancer (orange circle), stimulating MYC expression. Alternatively (or more likely additionally in colorectal carcinomas) *β*-catenin/TCF/LEF1 can be directly stimulated by mutations such as the minor SNP allele of rs6983267, which creates a consensus binding site for *β*-catenin/TCF/LEF1.

are an order of magnitude larger than typical enhancers, bind disproportionally higher levels of transcriptional regulators, and are typically associated with the most actively transcribed genes in the cell. Given their mammoth scale, it is not surprising that super-enhancers tend to associate with genes that most acutely define the identity of a cell [105, 106]. The discovery of super-enhancers reveals that cells take a hierarchical approach to transcriptional regulation, expending some resources to maintain expression of the many genes they need to survive, but marshaling huge conglomerates of transcriptional proteins at a small percentage of sites to regulate those genes most important for establishing who they are and what they do.

In their analysis of super-enhancers in the multiple myeloma cell line MM1.S, which carry a *c-MYC* translocation that places MYC under the control of the *IgH* enhancer, Young and colleagues defined 308 super-enhancers (3% of total enhancers), all of which are associated with genes important for multiple myeloma biology, including *c-MYC*. In this case, super-enhancers are distinguished by unusually high binding of the Mediator co-activator complex, the chromatin reader Brd4, and the histone mark of acetylation of H3 at lysine 27 (H3K27Ac). The MYC super-enhancer in MM1.S cells lies within 50 kb of the translocated *c-MYC* gene and, not surprisingly, is centered on the *IgH* enhancer. Importantly, this element appears to play a major role in controlling MYC expression in this context, as genetic or chemical inhibition of Brd4 (see Section 7) results in a striking decrease in *c-MYC* transcription—and in the tumorigenicity of multiple myeloma cells *in vivo* [105, 107].

Close inspection of super-enhancer architecture reveals that they are actually composed of sets of smaller enhancers that form into a monolithic structure via the action of cooperative protein-protein interactions (Figure 8). The involvement

of cooperativity in super-enhancer assembly allows relatively small increases in transcription factor concentrations to translate to large increases in transcriptional output, and is paramount in establishing the transcriptional dominance of these elements across the genome. Conversely, because such assemblies are built via cooperativity, small decreases in transcription factor concentration or functionality could cause super-enhancers to collapse, leaving typical enhancers largely unscathed. The notion that super-enhancers preside over the control of a set of mission-critical genes for cancer cells, yet are built on an inherently unstable platform, has led to the prospect that they may be a viable point of attack against cancer cells. As discussed later in the review, recent development of a set of Brd4 inhibitors—and their efficacy in pre-clinical models of MYC-driven cancer—has fueled much excitement over this possibility.

4.5. Enhancers: A final thought. The recency with which the *c-MYC* gene-desert and super-enhancers were identified illustrates graphically how difficult it can be to tie the action of a distal enhancer to its target gene(s), but also points to an important opportunity for our understanding of MYC gene transcription. It has been proposed that the typical mammalian genome houses hundreds of thousands of enhancers [108], the vast majority of which have not been systematically studied. If so, it appears likely that additional MYC enhancers will surface in the future, and that their characterization will lead to better understanding of the mechanisms of tumorigenesis. We suggest that characterization of MYC enhancers could be particularly informative with respect to deconvoluting the role of MYC in specific cancer types. Enhancers often play a pivotal role in determining cell-type specific patterns of gene expression, and it is conceivable that deregulation of cell-specific MYC enhancers—either at the level of factors that work through them or phenomena such as focal amplifications [109]—could result in tumorigenesis in one cell type, but not another. If so, and if chromatin factors continue their course as attractive drug targets, understanding which enhancers and super-enhancers control MYC in each tumor type could hold the key for successful implementation of precision medicine therapies.

5. The Impact of Chromatin on MYC Activity

Despite intriguing evidence that MYC proteins preserve some of their functions in the absence of DNA-binding [110], the received wisdom is that the physiological and pathophysiological functions of MYC result from its actions as a canonical transcriptional regulator—binding directly to regulatory elements in target genes and controlling their expression by recruiting factors that modulate the access or activity of RNA polymerase at those sites. In this view, recognition of target genes by MYC underscores all of its activities, and as a result much effort has been placed on understanding how MYC selects its target genes. The presence of an E-box—or variant—has long been recognized as a key determinant for sequence-specific DNA binding by MYC/MAX dimers. But as our understanding of MYC has blossomed, so to has our understanding of the importance of chromatin context in genome recognition by MYC [111].

On average, E-boxes occur every 4 kb within the human genome [6], yet it is clear that not all of these E-boxes are equivalently able to capture MYC. Genome-wide studies have shown that MYC binds preferentially to E-boxes located in regions that can be defined as "active chromatin", characterized by methylation-free CpG islands [112, 113] and specific sets of histone modifications including histone H3 di- and tri-methylation at lysine residues 4 and 79, and acetylation at lysine 27 [14, 114]. Indeed, Guccione *et al*., concluded that histone H3K4/K79 methylation is a "strict pre-requisite for recognition of any target site by MYC" [114]. As H3K4 methylation is also likely to play a major part in keeping CpG islands free of DNA methylation [115], these observations reveal that active histone modifications such as these are every bit as important as primary DNA sequence in determining where MYC will engage an E-box in the genome (Figure 9).

Despite its conceptual simplicity, the notion that MYC favors E-boxes located within chromatin marked by H3K4 and K79 methylation has profound ramifications. First, it gives important insight into MYC's *modus operandi*. Unlike acetylation, which is thought to weaken nucleosome-DNA interaction by neutralizing the positive charge of the lysine side chain (Figure 3), methyl groups are cationic at physiological pH [116], meaning that such modifications are unlikely to simply control whether or not a particular E-box is accessible to MYC/MAX heterodimers. Rather, it appears that H3K4 and 79 methylation function as beacons of active chromatin, signaling to the cell that a particular locus is transcribed or at least poised for transcription. By extension, this realization implies that the function of MYC is not to initiate a novel and defined gene expression program, but instead to supercharge pre-existing transcriptional curricula. This concept lies at the heart of the recently described "amplifier" model [14, 117], which proposes that MYC increases the transcriptional output from all active genes in a given cell, driving tumorigenesis by creating a chaotic state of flux through all extant cellular processes. Although the generality of this model, and its physiological relevance, have yet to be tested [9], MYC's profound appetite for active chromatin marks is very much aligned with the idea that MYC acts by increasing the volume on global transcriptional operations.

Second (and related to the first point), because histone modifications such as H3K4 methylation are heritable, as well as cell- and tissue-type specific [118], their role in governing MYC occupancy leads to the concept that MYC may act in intrinsically different ways in one tumor type versus another (Figure 9A). Efforts to define "smoking gun" transcriptional targets for MYC, searching for the handful

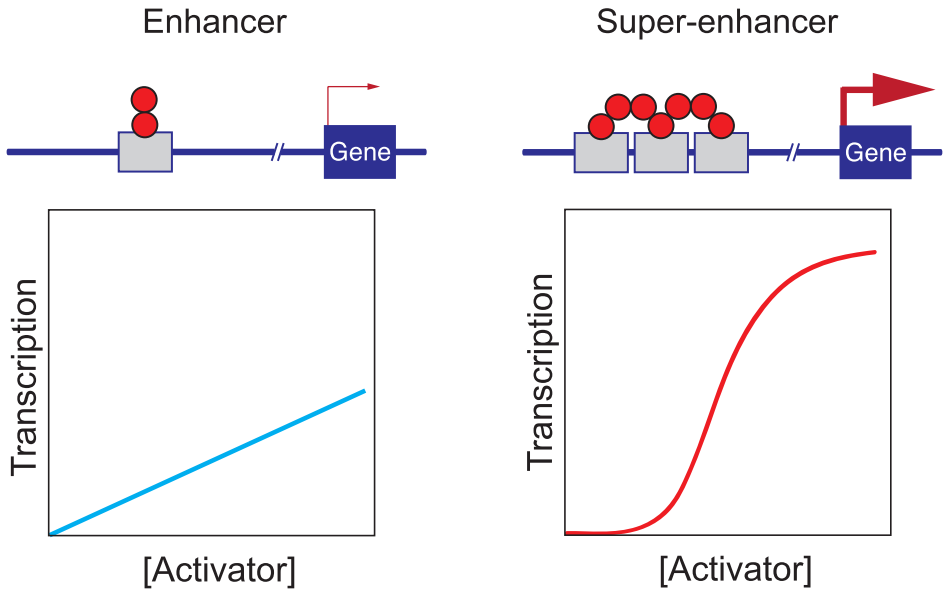

Figure 8: Enhancers versus super-enhancers have different dose-dependent properties. Theoretical dose-response curve for a typical enhancer (left) or a super-enhancer (right). As the concentration of a positively acting factor (activator) increases, the enhancer without cooperative protein-protein interactions responds linearly. The super-enhancer, in contrast, is built via cooperative interactions among enhancers, and thus displays a sigmoidal response. In this case, a small change in the concentration of the activator results in a proportionately larger response in enhancer function and transcriptional output. Adapted from [105].

of select genes responsible for its tumorigenic functions, have generally failed, as have efforts to delineate a global MYC "signature" present in all cancers [119]. Cell type-specific differences in epigenetic histone modifications, such as H3K4/K79 methylation, can readily account for the lack of success in these endeavors, because any differences in these modifications will control which target genes access MYC in any cell- or tumor-type. Going further, it is conceivable that relevant histone modifications may even differ between cells in the same tumor mass, setting vastly different functional states for MYC across the tumor as a whole, and creating a malleable environment that favors tumor evolution to metastasis or therapy resistance. Unlike the relative stability of genetic determinants (*i.e.,* E-boxes), therefore, the inherent plasticity and diversity of epigenetic modifications—and their links to MYC—has the potential to create a constantly changing set of rules that promotes the adaption of MYC-overexpressing cells to any particular challenge in the tumorigenic process.

Finally, it is worth noting that precisely how MYC recognizes genomic targets in the context of select histone modifications is completely unknown (Figure 9B–C). It is formally possible that H3K4 and K79 methylation create a particular chromatin structure that somehow makes E-boxes more accessible to MYC/MAX dimers. As mentioned above, however, it is not clear that methylation can induce these kind of changes in nucleosome configuration. Instead, it seems more likely that these histone methylation events work by recruiting one or more (as yet unidentified) chromatin readers that bind to both the specific histone modifications and to MYC. In this way, MYC would be recruited to its target genes through a bivalent set of interactions, recognizing both DNA (E-box) and specific protein determinants (chromatin reader bound to a methylated histone tail). A growing number of methyllysine binding proteins have been identified [120] that encompass a structurally diverse set of protein domains and binding mechanisms, making it difficult to predict which if any methyllysine readers may conspire with MYC to direct its binding specificity *in vivo*. But if such proteins can be found, targeting either their histone binding pockets, or the surfaces through which they interact with MYC, could provide fertile territory for development of anti-MYC therapies in the future.

6. The Impact of MYC on Chromatin

Once bound to its target genes, MYC elicits changes in the recruitment and activity of transcriptional proteins that stimulate—or in some cases repress [121]—the ability of RNA polymerase to productively transcribe that gene. Multiple mechanisms have been proposed for how chromatin-bound MYC regulates gene activity [6, 7], one of the most important of which appears to be recruitment of the transcription elongation factor pTEF-b and release of pre-engaged, paused, RNA polymerase II molecules across the genome [122]. Additionally, and like many transcriptional regulators, MYC also recruits proteins to modify the local chromatin environment. In this section, we discuss three ways that MYC proteins act upon chromatin to impact transcriptional processes.

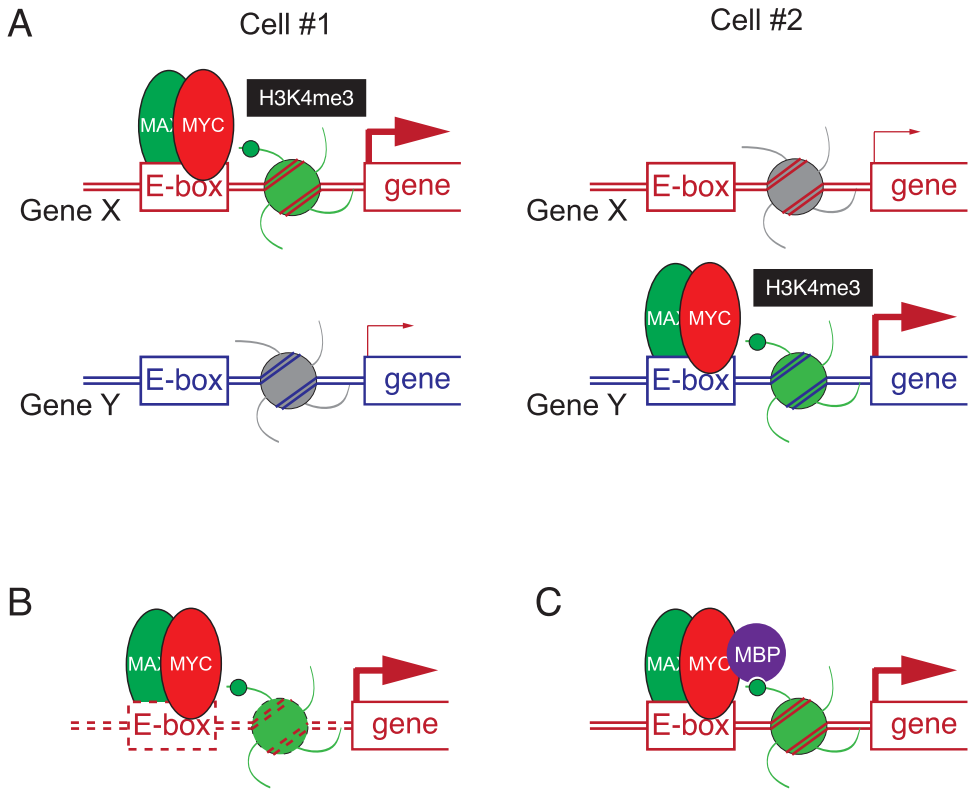

Figure 9: The impact of histone modification on genome recognition by MYC proteins. (A) Two genes (X and Y) are presented in two cell types (1 and 2), both of which have identical E-box elements. In Cell #1, Gene X carries the permissive H3K4me3 mark on an adjacent nucleosome, binds MYC/MAX dimers, and is induced by MYC. In Cell #2, which is *genetically identical*, the pattern of H3K4me3 modification is reversed, and Gene Y binds MYC/MAX. (B–C) Two models for how H3K4me3 promotes MYC binding to chromatin. In (B), the H3K4me3 modification induces structural changes in how the E-box is presented (dotted lines), allowing MYC/MAX heterodimers to bind. In (C) the H3K4me3 modification recruits a methyllysine binding protein (MBP) that recognizes both the modified histone and MYC, actively recruiting MYC/MAX heterodimers to the site.

6.1. The Yin and Yang of MYC and histone acetylation. As described earlier, histone acetylation can regulate transcription through at least two distinct mechanisms: By promoting an open chromatin structure, and by signaling recruitment of specific acetylation-dependent chromatin readers such as Brd4. Conceptually, these two modes of action confer very different functional advantages. Recruitment of chromatin readers in response to histone acetylation is driven by discrete protein interfaces and intramolecular interactions, and as a result can be a very specific and nuanced process, the outcome of which depends on the precise site of modification, as well as the presence or levels of the specific chromatin reader. Acetylation-induced changes in nucleosome-DNA contacts, in contrast, do not require specific effector proteins, are less dependent on the specific sites of modification on histone tails, and can act cumulatively to determine the biological availability of a particular section of chromatin [123]. As a result, changes in the total load of histone acetylation at any given gene act as a molecular "rheostat" that can fine tune transcriptional levels across a broad spectrum of states, from transcriptionally inert to fully active. In line with the notion that MYC induces widespread, and perhaps absolute [14, 117], changes in transcriptional programs, and with its function as both an activator and repressor [18], most evidence indicates that the effect of MYC on histone acetylation is tied to its influence over the acetylation rheostat (Figure 10).

Tied to transcriptional activation, MYC has been shown to induce a plethora of acetylation events at target loci, including at lysine 5 of H2A, lysines 9, 14, and 18 of H3, and lysines 5, 8, 12, and 91 of histone H4 [112, 124]. These marks often occur in combinations and their levels correlate with gene induction, consistent with the notion that MYC is exploiting the cumulative nature of acetylation effects to enhance transcription. Because no single histone acetyltransferase (HAT) is capable of catalyzing all of these events, the scope of histone acetylation induced by MYC implies that it can interact with and recruit multiple HATs to chromatin. Indeed, MYC is known to interact with an assortment of HATs and HAT-containing complexes including GCN5/PCAF [125], Tip60 [126], and p300/CBP [127] as well as the adaptor protein TRRAP which is a component of many HAT complexes [128]. Precisely how MYC manages to coordinate all of these interactions, and the

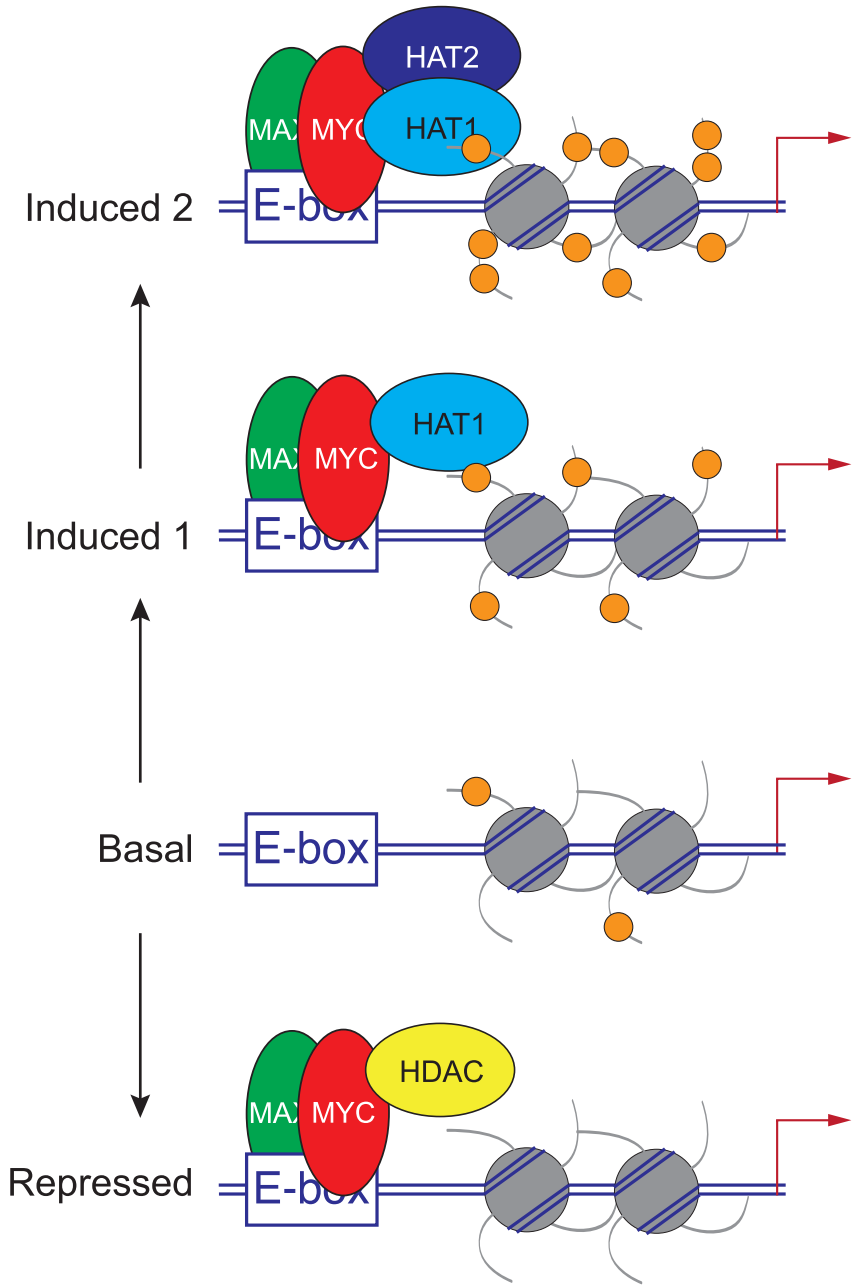

Figure 10: The histone acetylation rheostat. The cartoon shows how the cumulative effects of histone acetylation, and the ability of MYC to recruit HATs and HDACs, can be used to fine-tune the levels of target gene expression, positively or negatively. In the basal state, the gene is moderately active and bears a certain levels of histone acetylation (orange circles). Recruitment of one or two HATs leads to progressive gene induction via increased histone acetylation, leading to induced states 1 and 2. Alternatively, MYC can recruit HDACs, which promote a closed chromatin configuration by removing histone acetyl marks. Note that it is formally possible that MYC simultaneously recruits both HATs and HDACs to a particular locus, with the ultimate effect determined by the balance of these contradictory activities.

specific contribution of individual HATs to MYC function, remains unknown. One possibility is that certain HATs are recruited under specific circumstances or in response to distinct stimuli, providing an additional level of signal integration to control the transcriptional output of MYC. Alternatively, if different interaction surfaces are involved, multiple HATs could be recruited simultaneously by MYC, inducing even greater changes in histone acetylation than could be achieved by recruitment of a single enzyme. Finally, and not beyond the realm of possibility, MYC may take a "whatever's handy" approach, recruiting HATs in relatively non-specific fashion, depending on which enzymes happen to be in the local vicinity of a particular MYC molecule.

Apart from gene-specific changes in histone acetylation, MYC has also been found to influence global patterns of

these modifications [129]. Specifically, Eisenman found that disruption of the *N-MYC* allele in a variety of cell types leads to dramatic, across the board, decreases in histone acetylation, particularly those events catalyzed by the HAT GCN5. Such findings illustrate the high profile connection between MYC and histone acetylation, but also illustrate one of the key issues that limits our ability to understand the direct ways through which MYC functions. Eisenman and colleagues were careful to point out that GCN5 is, in fact, a MYC target gene, and that a fair portion of the global effects they observed upon depletion of GCN5 could be a result of simply reducing intracellular GCN5 levels. But viewed from the more recent perspective that MYC is capable of binding to *every* active gene in a cell [14, 117], it is now impossible to exclude the idea that MYC's control over global acetylation patterns reflects its direct and totalitarian influence over all active loci. Careful analysis of how MYC interacts with its suite of HATs, and generation of precise mutants capable of disrupting these interactions, will be needed to resolve this issue.

Although less studied, control of histone acetylation has also been implicated in transcriptional repression by MYC. Associations have been reported between MYC and two histone deacetylases (HDACs), HDAC1 [130–133] and HDAC3 [134], and in both cases MYC has been shown to recruit HDAC-containing co-repressor complexes to target loci, correlating with a reduction in histone acetylation and repression of gene activity. The relevance of this mode of transcriptional repression to the pro-tumorigenic functions of MYC is not well understood, both in terms of how histone deacetylation compares with other mechanisms of repression [135] and how repression in general contributes to the oncogenic functions of MYC [9, 14, 117]. But it is intriguing, for example, that MYC recruits HDAC3 to repress the expression of the tumor-suppressive microRNAs [136, 137], and that inhibition of their expression is required for the tumorigenic effects of MYC to manifest *in vitro*. It is also intriguing that a mutation that impairs the ability of MYC to recruit HDAC3 to chromatin is compromised in the ability to drive lymphomagensis *in vivo* [138]. Given that HDAC inhibitors are already used in the clinic for treatment of certain hematologic malignancies [139] the issue of how HDAC-mediated transcriptional repression features generally in MYC activity, and more specifically in the context of particular types of MYC-driven cancers, is an area that clearly warrants further investigation.

6.2. Control of histone methylation by MYC proteins. In contrast to histone acetylation, the role that histone methylation plays in regulation of gene expression by MYC is unclear. Although forced expression of MYC can induce both widespread [140] and localized [124] increases in modifications such as H3K4 trimethylation, many of these changes are likely to be indirect, and it remains to be determined whether direct interaction of MYC with methyltransferase components is a bonafide part of its mechanism of action. That said, there are a handful of reports that shed some light on how MYC can directly regulate histone methylation—intriguingly at the level of histone *demethylation*.

In their analysis of *Drosophila* MYC (dMYC), which is functionally interchangeable with mammalian c-MYC in many assays, Eisenman and colleagues found that the Trithorax group protein "Little imaginal discs" (Lid) is required for the ability of dMYC to promote cell growth in the *Drosophila* system [141]. Lid belongs to the JARID1 family of H3K4 demethylases, which preferentially remove the trimethylated H3K4 mark [142] and accordingly are usually associated with transcriptional repression. In this context, however, Lid is required for transcriptional activation by dMYC, raising the paradox of how a repressor can be linked to gene induction. Although precisely how the interaction of dMYC with Lid (and of mammalian MYC with Lid orthologs [141]) promotes transcription is unclear, it is interesting to note that, in addition to binding Lid, dMYC also inhibits its demethylase activity. One possibility is that MYC binds to and inactivates Lid to preserve the H3K4 methylation status of its target genes, insuring that an epigenetic mark that MYC needs to bind to chromatin is preserved in the presence of MYC. Alternatively, Lid could be acting as an adapter protein to tether MYC to select sites on chromatin. In this regard, Eisenman initially proposed that Lid may function as the intermediate between H3K4 tri-methylation and MYC binding to its target genes (Section 5) [141], although subsequent studies failed to detect direct binding of Lid to H3K4 tri-methylated histone tails [142], suggesting that the interaction of Lid with its substrate may not be stable enough to tether MYC to E-boxes *in vivo*. Regardless of the mechanism, the interaction of MYC with JARID1 proteins and the robust connection to MYC biology point to the need for further understanding of the underlying molecular mechanisms at work.

In addition to JARID1 proteins, MYC has also been found to recruit the H3K4 demethylase LSD1 to target genes, again in a manner that correlates with gene induction [143]. In this case, however, the enzymatic activity of LSD1 is not compromised by MYC, but instead LSD1 appears to be fully active and to trigger a transient demethylation of H3K4me2 at MYC target genes. Interestingly, Majello and colleagues [143] argue that it is not the demethylation of H3K4 *per se* that is important to gene activation, but rather a byproduct of the reaction, H_2O_2, which induces localized oxidative DNA damage that, in turn, recruits DNA damage repair factors OGG1 and Ape1 to stimulate transcription. This model provides a very different way of thinking about how MYC regulates transcription, in essence by altering the chemical microenvironment of particular regions of chromatin. The potential of MYC to generate oxidative DNA damage is aligned with its ability to induce formation of reactive oxygen species [144], and there is certainly precedent for factors labeled as "DNA repair proteins" to play mechanistically

important roles in transcription [145]. Thus, although not all aspects of this model have been experimentally challenged, and the possibility remains that LSD1 could stimulate MYC function simply by removing inhibitory methylation marks (such as H3K9 di- and tri-methylation [146]), the mechanisms and significance of the MYC–LSD1 interaction clearly warrant further exploration.

One aspect of the MYC–LSD1 interaction that is particularly instructive—and one that could well inform other studies on the influence of MYC on chromatin—is the transient nature of the effect of MYC on H3K4 dimethylation [143]. Following ectopic induction of MYC, H3K4me2 levels at target genes drop quickly, but return to the basal state within four hours. The fleeting nature of these changes suggest that MYC promotes a highly dynamic and ordered set of events on chromatin, and that studying early events induced by MYC may be more mechanistically informative than static pictures taken at steady-state or after longer periods of MYC activation. Comparatively few studies have looked at the influence of MYC on chromatin with such a degree of temporal resolution, and most models are built from the fairly simple perspective of stable recruitment and long term effects. But if dynamic and ordered processes are at work, early changes on chromatin could be important in setting the functional output of downstream events, and could very likely have been missed in all but a few analyses to date.

6.3. DNA methylation as a mechanism of MYC-mediated repression. The impact of MYC on chromatin extends beyond histone modification to a direct effect on DNA methylation, which has been shown to be important for repression of select MYC target genes [147]. Understanding of this mechanism of repression can be traced back to Eiler's identification of the large multi-zinc finger protein MIZ-1 as a MYC interaction partner [135, 148, 149]. In the absence of MYC, MIZ-1 functions as a transcriptional activator, binding to the initiator element of proliferation-inhibitory genes such as *p15Ink4b* and *p21Cip*, stimulating their expression and inducing a potent growth arrest. When MIZ-1 is complexed with MYC (and MAX), however, the tides are turned (Figure 11). MYC blocks the activation capacity of MIZ-1 by preventing the latter's association with the p300 HAT [149], and converts the ternary complex of proteins into an active repressor by recruiting the *de novo* CpG methyltransferase [150] Dnmt3a [147]. Recruitment of Dnmt3a, in turn, methylates CpG islands within promoters such as *p21Cip*, silencing their expression. The ability of MYC to corrupt the growth-inhibitory functions of MIZ-1 in this way appears important for tumorigenesis, as a single amino-acid substitution in MYC that disrupts interaction with MIZ-1 compromises MYC's oncogenic ability *in vivo* [151].

At the moment, there is no clear indication of the extent of MYC/MIZ-1 target genes that are repressed by this mechanism. Nor is it mechanistically clear how the interaction of MYC with Dnmt3a—which occurs via the MYC TAD [147]—is regulated, so that Dnmt3a is not recruited to the broad set of genes transcriptionally induced by MYC. But it is interesting to note that the maintenance CpG methylase Dnmt1 is required for the development and continuance of MYC-driven T-cell lymphomas [152], suggesting that the involvement of CpG methyltransferases in the tumorigenic actions of MYC may be widespread and worth closer examination.

7. Therapeutic Opportunities

MYC is arguably one of the best-studied proteins in human history and one of the most high-value targets in the war on cancer [9]. It is not surprising, therefore, that significant energy and resources are being placed on development of molecules that either inhibit MYC or take advantage of some unique property conferred on cells by ectopic MYC expression (*e.g.,* glucose addiction [13]) to kill cancer cells [153–155]. Fueled by the realization that the druggable universe is no longer confined to enzymes with small, well-defined, active sites, and by our increasingly sophisticated understanding of transcriptional processes, the realm of MYC and chromatin is proving fertile territory for development of MYC inhibitors.

One of the most striking aspects of how the MYC–chromatin connection is being exploited to develop anti-cancer therapies is the profound concentration of efforts on strategies to inhibit MYC synthesis, rather than to block the downstream actions of MYC on chromatin. HDAC inhibitors, which are promising anti-cancer agents [156], have been shown to attenuate the transforming potential of MYC *in vitro* and in mice [137], presumably via their ability to prevent MYC from repressing transcription of tumor-suppressive microRNAs (Section 6). But examples of downstream blockades of MYC function using such approaches are few. Part of this asymmetry is obviously due to the availability of inhibitors against specific chromatin factors, and the fact that mechanisms controlling MYC gene expression have been studied for longer and are better resolved than those mechanisms through which MYC broadly activates or represses transcription. Reflecting this bias, our discussion here will focus on a few high profile ways that chromatin is being targeted to inhibit MYC gene expression in cancer.

7.1. Targeting DNA topology to inhibit MYC synthesis. Understanding the intricate topological writhing that controls *c-MYC* gene expression—via the FUSE–FBP–FIR–TFIIH governor and by non-B-DNA switches—has led to the development of a number of strategies to curtail MYC transcription by interfering with the formation or stability of "alternative" DNA topologies at the MYC promoter. Theoretically, either the DNA or protein components of these structures could be targeted to reduce MYC synthesis, and the inevitable success or failure of potential therapeutics will be determined

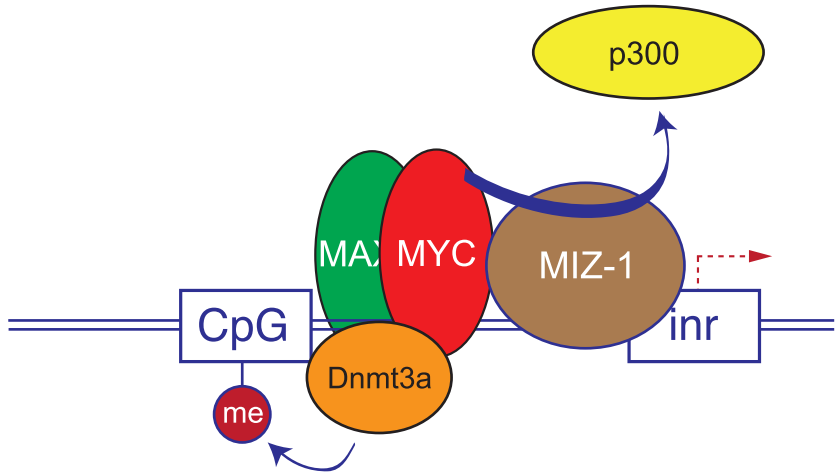

Figure 11: MYC converts MIZ-1 to a transcriptional repressor. MIZ-1 binds the initiator (inr) element in target genes such as *p21Cip* and stimulates their expression, in part, by recruiting the p300 HAT. In the presence of MYC, p300 is no longer recruited to the promoter, preventing activation, and Dnmt3a is recruited, methylating a CpG island and actively inhibiting promoter function.

by how potently these topologies can be targeted, whether their actions outside of MYC are critical for cell survival, and if a therapeutic window can be established to interfere with the tumorigenic MYC expression but leave other cellular events relatively intact. The prospect that tumors (even those driven by other oncogenes [25]) are addicted to MYC [20–24] gives researchers hope that MYC inhibitors will broadly and preferentially kill cancer cells; the real issues are which processes to target, where to attack, and how to build drug-like molecules that get the job done.

The FUSE–FBP–FIR–TFIIH system is one of the best-described mechanisms regulating *c-MYC* expression, and as previously discussed is supported by a wealth of *in vitro* and *in vivo* evidence confirming its importance in MYC transcription. In terms of inhibiting MYC synthesis, the most obvious target within this system is the FUSE–FBP interaction, which is required to accelerate MYC transcription after initial gene activation (Figure 5C–E), and which has a number of distinct attributes, including a relatively shallow hydrophobic DNA-binding interface that is an attractive target for small molecule inhibition, and solid genetic evidence that attenuating the function of FBP stifles MYC transcription and causes cancer cells to stop growing [157]. But surprisingly this area has not been widely pursued. One study used a combination of screening strategies to identify small ligands—benzoylthranilic acids—that bind directly in the hydrophobic pocket of FBP [158] and disrupt the FUSE–FBP interaction *in vitro*. Although the insolubility of these ligands prevented their testing in cells and further development [77], this work showed that it is possible to target the DNA-binding surface of FBP with a small molecule, and if interest in this approach can be expanded, it may very well be possible to develop drug-like molecules that jam the FUSE–FBP–FIR–TFIIH governor.

Outside the realm of targeting protein-DNA interactions is the notion that *c-MYC* transcription can be tempered by developing small molecules that stabilize non-B-DNA structures, such as G4-DNA or i-motifs [77, 159], in the P_1 promoter (Figure 6). This area has been subject to intense interest in recent years [160], encouraged not just by understanding of MYC, but by the realization that G4-structures are involved in regulating the expression of multiple tumor-relevant genes, as well as in telomere maintenance/activation [159, 160]. The general strategy in this area is to find or derive small molecules that stack onto, or intercalate in, G4-DNA, driving them into a biologically inert configuration. From our earlier discussion of G4-DNA, it is clear that stabilizing quartet structures in the MYC promoter has the potential to permanently lock the *c-MYC* gene in the off state (by preventing the binding of CNBP), and this potential has been realized by development of a number of G4-targeting ligands that shut down MYC transcription in cancer cell lines [161–164]. These reagents not only have therapeutic potential, but provide one of the most compelling pieces of evidence that quartet structures form *in vivo* and are directly relevant to *c-MYC* gene activity.

One of the challenges in developing G4-DNA stabilizers as drugs is the issue of specificity. It is clear that G4- and other non-B-DNA configurations are broadly employed in genome events, so how can ligands be developed that are specific to one particular segment of quartet DNA, but not another? Not all quartet DNA is created equal, so it may very well be possible to exploit differences in the properties of different G4-DNA segments to derive fairly-specific inhibitors. But the good news is that selectivity may not actually be required for G4-DNA stabilizers to be effective anti-cancer compounds. One of the best characterized G4 stabilizers, for example, TMPyP4, was originally developed to stabilize telomeric G4 structures

(thus preventing telomere elongation), but has since proven to be particularly effective in achieving the same task at the *c-MYC* promoter [159], attenuating MYC expression. In the context of tumorigenesis, this provides a "one-two" punch to cancer cells, simultaneously choking two critical mechanisms that malignant cells need to survive. Moreover, because of the network of common proteins that regulate G4-DNA formation and stability, it may simply be that disrupting the equilibrium of how quartets and their entourage are distributed is sufficient to push cancer cells over the edge. The G4-binding drug Quarfloxin, for example, was developed to target an interaction between nucleolin and quartet DNA that is important for ribosomal DNA (rDNA) transcription in the nucleolus [165]. Quarfloxin does an admirable job at inhibiting rDNA transcription, induces apoptosis in cancer cells, and even made it to Phase II clinical trials for the treatment of neuroendocrine tumors [160]. What is interesting, however, is that by dislodging nucleolin from the rDNA loci, Quarfloxin forces nucleolin to relocate to the nucleoplasm [165] where, as discussed above, it is free to stabilize G4-DNA in the MYC promoter, silencing MYC expression [160]. Thus the value of these types of inhibitors—much like HDAC inhibitors [166]—may spring from the totality of effects (direct and indirect) they induce, rather than by specific inhibition of a particular molecular event. Unfortunately, Quarfloxin was not pursued beyond Phase II trials because of bioavailability issues, but it did show low toxicity and some measure of therapeutic response, giving hope that future efforts in this direction will lead to effective ways to modulate aberrant MYC expression in cancer patients.

7.2. BET bromodomain inhibitors. Perhaps the most exciting recent developments in targeted anti-MYC therapies center around a class of molecules known as BET bromodomain inhibitors. These compounds have been extensively reviewed elsewhere [167–169], so we will just touch on the highlights here. Collectively, bromodomain-containing proteins are noted for their ability to bind acetylated lysine residues, with BET subfamily members using dual bromodomains to recognize a suite of acetylated proteins, including histones H3 and H4 [170]. Distinct from other chromatin readers, bromodomain proteins have a number of structural characteristics that make them attractive drug targets, including a generally weak interaction with acetylated proteins that is mediated by deep hydrophobic pockets capable of blockage by small molecules. The best-studied member of the BET subfamily, and the one in the crosshairs for inhibition of MYC transcription, is Brd4 [105, 107], which is a global chromatin regulator that binds to acetylated histones to promote transcriptional elongation by RNA polymerase II through the recruitment of PTEFb.

A number of small molecules have been developed that selectively block the interaction of Brd4 with acetylated substrates, including i-BET [171], MMS417 [172], and JQ1 [173]. These cell permeable compounds bind with nanomolar affinity to the two bromodomains in Brd4, preventing association with a number of acetylated proteins, including transcription factors and acetylated histone tails. As expected from the range of proteins bound by Brd4, these inhibitors disrupt a number of critical processes, including inflammation [171, 172] male fertility [174], and viral latency [175], but what is particularly interesting is the impact Brd4 inhibitors have on the expression of MYC. In a host of cancer cell lines and pre-clinical mouse model systems (*e.g.,* [107, 176–181], these molecules result in a frank decrease in *c-MYC* gene transcription and dramatically reduced tumor burdens. Although Brd4 inhibitors may not be highly specific in a molecular sense (*i.e.,* they are not specific inhibitors of MYC expression), they can selectively halt many MYC-driven cancer cells, a phenomenon that can be traced back to the action of super enhancers, which as discussed earlier are acutely sensitive to disturbances in the relevant transcriptional machinery [105]. By displacing Brd4 from active chromatin, i-BET, MMS417, and JQ1 preferentially collapse the molecular house of cards that sustains the MYC super enhancer, leaving many transcriptional processes relatively unaffected. The development of potent and bioavailable Brd4 inhibitors—which will almost certainly impact how cancers are treated within the next decade—not only shows that it is possible to develop drug-like molecules against chromatin readers and to attenuate MYC expression in this way, but it also illustrates one very important point; that small-molecule inhibitors do not need absolute specificity in order to function effectively.

Theoretically, any of the processes discussed here that regulate how MYC is expressed, how it binds to chromatin, or how it influences chromatin structure and dynamics could form the basis of the next wave of MYC inhibitors. And it is clear that new strategies need to be found. BET bromodomain inhibitors are showing great promise, as discussed, but it is apparent that these molecules only work in a limited number of settings where Brd4 and its related machinery dominate MYC expression [176]. This is not necessarily a problem for precision medicine therapies, but it does raise the need for identification of other means to limit MYC in different cancer types. If the phenomenon of super-enhancers proves to be general, and if cancer cells use this hierarchical mechanism to maintain their tumorigenic identity, it is possible that chromatin readers other than Brd4 that sustain MYC super enhancer function would be high value targets for drug development.

8. Future Perspectives

This is an exciting time in our understanding of MYC, and in efforts to exploit the MYC–chromatin connection to intelligently kill cancer cells. Fueled by more than 30 years of basic research into the function and regulation of MYC, richly informative genomic approaches, and a sea-change

in what is considered "druggable", the biomedical research community is poised to make major inroads in development of chromatin-based game-plans to inhibit MYC. Strategies to inhibit MYC synthesis have clearly taken the lead in this regard, but the complexity of *MYC* transcriptional regulation may very well limit the broad utility of these approaches, meaning that additional tactics are needed, ideally ones that target fundamental aspects of how MYC proteins function to control gene expression. Further exploration of HDAC inhibitors seems warranted, as do approaches based on inhibition of HATs, DNA methyltransferases, and of the molecular machinery that directs MYC to active sites of transcription in the genome. Given the pace with which chromatin-centric inhibitors are being developed, and the zealous way in which MYC proteins continue to be studied, it seems that the next few years will bring a critical point of inflection in how chromatin-based events are exploited to treat and cure MYC-driven cancers.

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