Cellular MYCro Economics: Balancing MYC Function with MYC Expression

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The expression levels of the MYC oncoprotein have long been recognized to be associated with the outputs of major cellular processes including proliferation, cell growth, apoptosis, differentiation, and metabolism. Therefore, to understand how MYC operates, it is important to define quantitatively the relationship between MYC input and expression output for its targets as well as the higher-order relationships between the expression levels of subnetwork components and the flow of information and materials through those networks. Two different views of MYC are considered, first as a molecular microeconomic manager orchestrating specific positive and negative responses at individual promoters in collaboration with other transcription and chromatin components, and second, as a macroeconomic czar imposing an overarching rule onto all active genes. In either case, *c-myc* promoter output requires multiple inputs and exploits diverse mechanisms to tune expression to the appropriate levels relative to the thresholds of expression that separate health and disease.

MYC was first discovered as the transforming gene of avian retrovirus MC29 (Wasylishen and Penn 2010; Eisenman 2013). It was subsequently shown to be a nuclear DNA-binding protein of cellular origin (Abrams et al. 1982; Moelling et al. 1982; Vennstrom et al. 1982). Further links between MYC and cancer were forged as *c-myc* was shown to be translocated with immunoglobulin genes in Burkitt lymphoma (human) and plasmacytoma (mouse) (Boxer and Dang 2001; Janz 2006), chromosomally amplified or activated by viral insertions such as occurs in cervical carcinoma with human papillomavirus (HPV) (see, e.g., Alitalo et al. 1983; Peter et al. 2006). Chromosomal aberrations of MYC most often leave protein coding sequences

intact. Unlike other proto-oncogenes, MYC requires no changes in protein sequence to become oncogenic; the proto-oncogene becomes the oncogene through abnormal expression (Love et al. 2012). Although coding mutations do occur, they mostly influence the stability and turnover of the protein, thereby increasing its levels rather than modifying fundamental MYC action. MYC oncogenesis would seem to be more a matter of degree than of the quality of MYC function (Liu and Levens 2006; Wierstra and Alves 2008; Levens 2010).

Lots of direct and circumstantial evidence indicates that MYC levels matter. Small changes in MYC levels drive cells from cell-cycle arrest to proliferation or across thresholds that trigger

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apoptosis (Murphy et al. 2008; Shachaf et al. 2008). Whereas normal diploid human fibroblasts are immortalized by retrovirally transduced telomerase, after the knockout of one *c-myc* allele, these cells instead senesce (Guney et al. 2006). Cell size and indeed whole organism size are sensitive to twofold changes in MYC (Iritani and Eisenman 1999; Johnston et al. 1999; Schuhmacher et al. 1999; Trumpp et al. 2001). Using elegant genetic tricks, developmental compartments in *Drosophila* were populated with admixtures of normal cells and cells expressing either twice or half of the normal amounts of MYC. In each case, the low-MYC cells were killed as the high-MYC cells expanded, refilled the compartment, and developed normally (de la Cova et al. 2004; Moreno and Basler 2004; Johnston 2013). If MYC levels are so important, what does it do mechanistically that makes cells so sensitive to its measure, and how are MYC levels controlled?

DNA BINDING BY MYC

MYC encodes a bHLH-ZIP (basic helix-loop-helix-leucine zipper protein) transcription factor (TF) that dimerizes with another member of the same family, MAX. This heterodimer constitutes a DNA-binding domain with maximal selectivity for the sequence CACGTG (E-box) (Blackwood and Eisenman 1991), although interactions with noncanonical E-box sequences also occur. The measured dissociation constant for recombinant MYC-MAX with E-boxes in naked DNA is ~ 100 nM, not especially strong if this number reflects the intracellular K_d , and it is weaker than some other bHLH-ZIP dimers (Park et al. 2004). The ratio of specific to non-specific DNA binding by MYC-MAX is a missing biophysical parameter that is required to model MYC specificity and function.

In crystals, two MYC-MAX heterodimers dimerize, in turn forming a heterotetramer with two diametrically opposed bound E-boxes. Simultaneous engagement of both DNA-binding domains in vivo to the same segment of DNA would create a topologically closed MYC-MAX loop, an arrangement replete with regulatory implications and inviting speculation about

mechanisms to control gene activity (Nair and Burley 2003). However, later experiments failed to detect or directly visualize such loops (Lebel et al. 2007). Moreover, mice heterozygous for fully functional carboxy-terminally tagged MYC-EGFP knock-in and wild-type MYC alleles show no evidence of tetramer formation. If expressed at equal levels, 50% of such tetramers should include one molecule each of MYC-EGFP and MYC, so immunoprecipitation using anti-EGFP would be expected to coimmunoprecipitate MYC; but with the separate formation of MYC-EGFP/MAX or MYC/MAX heterodimers, such coimmunoprecipitation would not occur. Indeed, extracts from serum-stimulated heterozygous fibroblasts showed no MYC-EGFP/MYC coimmunoprecipitation, indicating that tetramerization must require special circumstances if it were to occur in vivo (Nie et al. 2012).

MYC interacts with a large number of proteins and multicomponent complexes. Particular attention has been focused on MYC partners with obvious connections to gene expression, especially those directly participating in the regulation of transcription and chromatin (Amati et al. 2001; Cowling and Cole 2006; Agrawal et al. 2010; Kim et al. 2010). MYC's accomplices include complexes bearing the histone acetylases GCN5 or NuA4, the chromatin remodeler SWI/SNF, general transcription factors (TFs) TFIIF, TBP, and sequence-specific TFs such as YY1 (Shrivastava et al. 1996; Cowling and Cole 2007). However, a functionally unbiased roster of MYC partners includes representatives of diverse sets of regulatory, structural, and catalytic proteins that are involved in a variety of subcellular processes and networks (Agrawal et al. 2010). It is not known whether MYC projects its protean influence as a component of regulatory complexes by modulating the activities of other complex subunits or whether these interactions act as a sink competing against a smaller number of true MYC effectors thereby modulating the levels of active MYC.

WHAT DOES MYC DO?

Extensive literature associates changes in MYC levels, either through pathological activation or

experimental manipulation, with altered outputs of many pathways and regulatory networks, including proliferation; cell-cycle, protein, and RNA biosynthesis; metabolism; and signaling (Eilers and Eisenman 2008; Dang 2010, 2012; Wasylishen and Penn 2010). Careful studies have failed to define a consistent and coherent set of MYC targets that channel MYC's output onto these processes. Most studies attempting to define a pathway that conducts MYC's oncogenic impulse through an explicit series of direct and indirect downstream effectors have been disappointing. However, one recent report indicates that MYC-mediated down-regulation of tristetraprolin (TTP), a protein that destabilizes mRNAs through 3'-UTR (untranslated region) AU-rich elements (AREs), up-regulates hundreds of targets. That TTP is necessary, but not sufficient, for tumorigenesis reveals that the genomic realm of MYC exceeds that of TTP (Ji et al. 2011). So, two views of MYC action have emerged. First, although MYC regulates a large set of targets, camouflaged within this set is a cadre of molecular conspirators that hijack the cell and cause cancer. Second, MYC by itself drives the general activation of all cellular processes reinforcing or exaggerating only those responses already programmed by the cell. In the first scenario, MYC acts digitally, throwing key switches; in the second, it is an analog amplifier and any changes in cellular state arise secondarily from preprogrammed input–output transfer functions.

MYC targets may be operationally defined by genomic DNA-binding differences or expression changes associated with changes in MYC levels. Each of these definitions suffers practical and conceptual limitations. DNA binding, *in vivo*, need not be functionally relevant; all TFs must discriminate between bona fide cognate sites that have evolved to channel TF activity and the vastly greater number of nonspecific sites that have been presumed to be biologically inert. Thus, MYC association with a region of active chromatin might indicate that MYC participates in the genetic processes programmed by these regions, or it might simply reflect the nonspecific association of MYC with the most accessible segments of the genome. If it is the

latter, then many, if not most, MYC-binding sites might be physiologically and pathologically irrelevant. If MYC binds with high selectivity at true target sequences, those sites should saturate at much lower levels of MYC than required to populate the low-affinity or genome-wide nonspecific binding sites to any degree. In fact, the binding distribution of MYC at all of its sites across the genome is distinctly unimodal with no high-affinity tail or peak. Therefore, any threshold used to discriminate specific versus nonspecific binding by MYC is arbitrary (Nie et al. 2012). Binding patterns alone seemed not to spotlight MYC action if gauged by relative, rather than absolute, changes in expression.

The genomic targets of MYC may also be defined by expression, that is, the identification of genes whose expression is increased or decreased in the presence of MYC. Defining MYC targets by expression suffers from issues intrinsic to the measurement of mRNA levels and the comparison of samples. These issues include the choice of thresholds to justify the moniker “MYC-regulated.” Most often, equal amounts of RNA (total or poly[A]) are compared among samples, and the relative changes are noted. In this situation, a threshold demarcates statistical significance and only those targets surmounting the threshold are evaluated further; the more stringent the threshold, the fewer targets recovered. As always, this trade-off between specificity and sensitivity is a problem. This analysis implicitly assumes that the compared cells contain equal amounts of RNA. Sometimes these analyses highlight important biological and mechanistic differences, but in other situations this normalization may obscure global up- or down-regulation of cellular contents among cells with more or less contents. For example, if MYC were a universal activator, normalization to the mean or median of cellular total RNA or mRNA would make genes that are actually up-regulated, but less than the mean/median appear to be down-regulated. In fact, adjusting for total RNA greatly shrinks the set of MYC-repressed mRNAs—so much so that it becomes a contentious issue as to whether any targets are directly repressed by MYC (Lin et al. 2012; Loven

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et al. 2012; Nie et al. 2012). One view holds that MYC is fundamentally an activator, and therefore any apparent direct repression is either a normalization-accounting issue or derives from other gene regulatory mechanisms evolved to override MYC's action. A contrasting view is that MYC activity is context dependent and that MYC itself interacts with transcription and/or chromatin components to direct decreased expression depending on the sequence and chromosomal environment of a particular target (Eilers and Eisenman 2008).

Whether defined by binding or by expression, experiments identifying MYC targets have interrogated a wide range of cell and experimental systems, most often using cancer cell lines expressing pathologically deregulated MYC or experimental systems that express MYC nonphysiologically. Fewer studies have attempted to enumerate the physiological targets of MYC. The diversity of these systems has made it difficult to deconvolute the experimental and biological heterogeneity of this data. To hone in on true MYC biology, informatic approaches have been used to enumerate a "MYC signature"—genes that are unequivocally induced or repressed by MYC and should prove to be reliable markers of MYC expression while providing insight into the biological processes under MYC's purview (Shaffer et al. 2006; Margolin et al. 2009; Wasylshen and Penn 2010; Ji et al. 2011). The signature genes recovered from these attempts incompletely populated diverse pathways, molecular structures, and cellular processes that appeared to be related to the increase in biomass demanded as cells enlarge (Ji et al. 2011).

To distill MYC function, it is important to attempt to separate the direct and indirect effects of its activity. Direct targets serve as MYC's agents to alter the expression of its indirect targets. Determining whether MYC changes a gene's mRNA level by binding to that gene's regulatory sequences and modifying transcription, or indirectly by adjusting the levels or activities of other TFs that bind to the gene's regulatory sequences is a difficult problem (Yap et al. 2011). Moreover, in the case of feed-forward regulation, a target may be simultaneously direct and

indirect (Alon 2007). One way to discriminate direct versus indirect is to use posttranscriptionally activatable MYC-ER_t, a fusion of MYC with the ligand-binding domain of a tamoxifen-specific estrogen receptor (Eilers et al. 1989; Littlewood et al. 1995). Adding tamoxifen and monitoring ligand-induced gene expression in the presence or absence of protein synthesis inhibitors such as cycloheximide may separate direct and indirect targets. Unfortunately because of its short half-life, MYC action cannot be sustained in the absence of new protein synthesis. Furthermore, many mRNAs, especially those bearing AREs, are stabilized by cycloheximide, making it difficult to discriminate changes resulting from MYC versus translation inhibition. Nevertheless, applying rigorous criteria for expression changes in HO15.19 Myc-ER_t cells activated in the presence or absence of cycloheximide identified 21 definitive direct targets, and all 21 were up-regulated (O'Connell et al. 2003). These results are consistent with the notion that MYC is fundamentally an activator and that MYC repression in good measure works through its indirect targets. The identification of the repressive histone methylase Ezh2 as both a direct and indirect MYC target highlights a major pathway for MYC-influenced, but not direct, MYC-driven repression (Nie et al. 2012; Kaur and Cole 2013).

A recent study examining the genomic binding sites of MYC and global gene expression in resting and activated lymphocytes and in embryonic stem cells reevaluated the systems biology of MYC, yielding a model likely to prove at least of heuristic value (Nie et al. 2012). Resting or activated B cells and T cells from mice homozygous for fully functional MYC-EGFP knock-in alleles were compared for MYC binding, RNA polymerase distribution, and mRNA expression across the genome. Using median-mean normalization and fold-change thresholds to analyze mRNA expression, and imposing stringent thresholds for ChIP-Seq peak detection, returned an ontologically and biochemically complex set of targets as typical for MYC genomic binding and expression experiments. Although the expression of most MYC-binding targets seemed unchanged, of those that

changed, two-thirds were up-regulated and one-third were down-regulated.

Reanalysis of the same data normalizing to cell number and removing arbitrary thresholds for expression level or for ChIP-seq peak detection yielded a different perspective. Broad coherence between MYC binding and output was evident with this analysis. Across all samples, the logarithm of mRNA expression level correlated with the magnitude of promoter-bound MYC for all genes, implying a MYC-driven universal increase in RNA synthesis. Consistent with such a role, mRNA levels increased upon activating naïve B cells ~2.5-fold at 12 h post-activation, approaching 15-fold at full activation; this increase was blocked when MYC was inactivated either by conditional knockout or through inhibition of MYC-MAX dimerization. Without MYC, activation proceeds through only the earliest phases until the point when MYC would be induced. Blocking MYC prevented the expression increase across a panel of randomly selected genes not previously recognized as MYC targets when normalized to the median or mean. Genes already switched off and residing in heterochromatin were refractory to MYC amplification. Inducing an exogenous MYC in a lymphoblastoid cell line also supported the concept that it is a universal activator of transcription (Lin et al. 2012; Loven et al. 2012).

Generally after assembling into a preinitiation complex, RNA polymerase initiates transcription, advances a short distance, and pauses. Reinitiation is thwarted until the paused polymerase is released. The amount of MYC at the promoters of active genes, in activated B cells, reflected the amount of RNA polymerase loaded at each transcription start site. The level of RNA polymerase preloaded in the resting state anticipated the magnitude of MYC recruitment during activation. If promoters recruit MYC according to the intensity of transcriptional output and if MYC increases that output, a positive-feedback loop is created, in principle, describing the relationship between MYC binding and expression and operationally making MYC an exponential amplifier. This positive feedback may be passive in part, owing to increased promoter availability following MYC-enhanced pause re-

lease but may indicate that MYC actively facilitates promoter reloading and progression to the pause point (Rahl et al. 2010; Lin et al. 2012; Nie et al. 2012; Rahl and Young 2013).

Thus, we have two fundamentally different views of MYC action (Fig. 1) (Walz and Eilers 2013). In the conventional view, MYC is recruited in a context-dependent manner to particular genes according to the disposition of E-boxes and the spectrum of available TFs and chromatin regulators. In this view, gene-by-gene MYC is helping to set the microeconomic program of the cell, negotiating with its molecular partners at each target whether a gene is on or off and determining the direction (activation vs. repression) and magnitude of promoter output. For each MYC-regulated process, the rate-controlling genes must have evolved the appropriate arrangement of E-boxes and other features to mold the MYC response. In this scheme, the net effect of MYC expression, whether physiological or pathological, is the superimposition of the responses at each promoter; each individual promoter evolves its own MYC transaction more or less apart from the other components of its functional network.

The alternative view is that MYC acts to increase the transcription of genes that are already on, amplifying their output exponentially. In this view, MYC imposes an overarching “macro-economic” rule on the cell, leaving each network and pathway to reach its own set point according to the new levels of expression of its genes and available resources. Exponential amplification, in which highly expressed genes are relatively more up-regulated than genes with lower expression levels, may be indicative of a positive-feedback process in which MYC increases promoter output. This in turn recruits more MYC, etc., until something becomes limiting. Although MYC is an amplifier and not a specifier of cell state, it can contribute indirectly to reprogramming gene expression by driving repressors, TFs, and signaling systems with non-linear dose response curves across key thresholds. A rigorous evaluation of the amplifier model demands determination of the feedback and feed-forward relationships among the amplified active genes.

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Figure 1. Macroeconomic versus microeconomic models of MYC action. (A) In the absence of MYC, target genes are regulated by the integrated inputs of whatever factors are relevant. The logic of the integration is determined locally to set the output. (B) In the macroeconomic model, MYC is recruited both through DNA and protein interactions with the transcription machinery and acts to augment output according to the preexisting level of expression. MYC augments the outputs of more highly expressed genes more than it does of genes expressed at lower levels. MYC does not directly alter the regulatory logic of the promoter. (C) In the microeconomic model, MYC is recruited via E-boxes and then engages in negotiations with the panel of transcription and chromatin factors bound locally. The output of each promoter is situational and determined locally as MYC may activate or repress to different degrees.

WHY CELLS NEED A NONLINEAR AMPLIFIER

How can the preferential amplification of highly expressed genes be explained or at least rationalized? Comparison of resting and activated B cells provokes an attractive speculation. A fully activated B cell contains $\sim 550,000$ total mRNAs, whereas a resting cell contains $\sim 35,000$ transcripts. Under both conditions, the mRNAs are derived from $\sim 12,000$ active genes (Kouzine et al. 2013). Thus, the number of transcripts/gene averages ~ 3 in a resting cell and ~ 45 at full activation. Compared with activated cells, for many if not most genes, expression can be scaled down to the low levels of the naïve lymphocyte. But for those genes already expressed at

low levels (< 10 mRNAs/cell) in activated cells, a proportionate scaling down to resting levels would leave many genes, presumably some essential for cellular viability, present at less than one transcript/cell. To ensure its survival, relative to activated cells, a naïve B cell would need to express relatively more of scarce versus abundant transcripts. Thus, the naïve cell may be thought of as having a logarithmically compressed transcriptome compared with the activated state. An exponential amplifier would expand the transcriptome to functional levels. This hypothesis rationalizes the pulse of MYC expression that occurs almost universally in response to cell activation in metazoan systems as a means to jump-start a quiescent cell. Interestingly, in electrical engineering, logarithmic and exponential

amplifiers are paired to compress and then re-expand signals. Following a pulse of MYC-driven global activation, the panoply of induced TFs and chromatin regulators sustain expression as MYC levels decline. Unremittingly high-level MYC expression is principally a pathologic feature of neoplasia and other proliferative diseases.

Sustained high-level MYC expression in tumors may directly and indirectly overdrive cellular networks past their redlines. In this situation, selection for compensatory changes, for example, antiapoptotic/prosurvival pathway activation, would be expected to countermand the scaled-up output of apoptotic pathways. In addition, although at physiological levels MYC is found at promoters far more than at enhancers (Lin et al. 2012; Nie et al. 2012), at pathological levels, MYC invades enhancers, likely increasing their activity. Besides directly increasing transcriptional output at promoters, relentless MYC action may support epigenetic changes at promoters and enhancers that help to lock cancer cells into their pathologic state via interactions with chromatin-modifying and re-modeling complexes.

The global action of MYC would seem to belie its specific affinity for E-boxes and the consistent enrichment of E-boxes in genome-wide *in vivo* DNA-binding studies. Reconciliation of this E-box paradox has several components. First, the statistical methods used to cull candidate cognate elements from genome-wide studies are very powerful. The sheer magnitude of the data and the abundance of binding sites detected, with or without stringent peak-calling thresholds, impart vast statistical significance onto a biochemically modest enrichment. Overall E-box promoters and non-E-box promoters populate the same unimodal distribution, with the former binding ~15% more MYC on average (Nie et al. 2012). Allowing for degenerate E-boxes, nonspecific binding with DNA and for interactions with the transcription and chromatin machinery, the negative free energy associated with E-box recognition may bias but not license MYC action at its targets. Moreover, the frequency of degenerate E-boxes may be sufficient to assure

MYC recruitment near most transcription start sites.

SETTING MYC LEVELS

If MYC functions as an exponential amplifier of all expressed genes, achieving proper levels of MYC is crucial for the execution, control, and homeostasis of physiological processes (Levens 2010; Liu and Levens 2006; Wierstra and Alves 2008). Inappropriate levels or fluxes in MYC levels may trigger network instability as all downstream direct and indirect targets seek new set points or become dynamically unstable owing to feed-forward and feedback circuitry. Because MYC influences the abundance of all active genes, it would be expected to change the levels of all of its own regulators. In the case of positive feedback, this would create a potentially lethal situation. To avoid this, MYC is regulated at every possible level of macromolecular synthesis and degradation, from chromatin modification and remodeling through transcription to RNA and protein degradation. As the regulation of MYC protein stability is dealt with elsewhere in this collection (Farrell and Sears 2013), the regulation of MYC transcription will be considered here starting with the definition of the MYC locus. The 6-kb coding region situated on the long arm of chromosome 8 resides in a gene desert 50 kb upstream of PVT1 and more than a megabase downstream from the nearest upstream gene (Pomerantz et al. 2009; Tuupainen et al. 2009; Sotelo et al. 2010; Kovalchuk et al. 2012; Sur et al. 2012). MYC mRNA is transcribed principally from the weak P1 promoter and the stronger P2 promoter 150 bp further downstream; additional minor transcription start sites include P0, 600 bp upstream of P2 and P3 in exon 1 as well as others. P1 and P2 reside in constitutive nucleosome-depleted nuclease-hypersensitive sites, as does the CT element (also called nuclease hypersensitive element 1, NHE1) at a sequence with the capacity to assume multiple complex conformations (Liu and Levens 2006). Other hypersensitive sites are associated with induced expression. MYC was among the first genes shown to harbor a transcriptionally engaged RNA polymerase paused

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35–50 nucleotides downstream from the start site, awaiting release into the elongation mode (Spencer and Groudine 1990). Once thought to be rare, it has become apparent that such promoter-paused polymerases may well be the predominant mode of regulation at most active genes (Seila et al. 2009; Levine 2011; Adelman and Lis 2012; Luo et al. 2012). Initial studies of the *MYC* promoter sought to identify the TFs and their binding sites within the vicinity of the transcription start site (TSS) that were necessary or sufficient to confer proper regulation on a reporter (proper meaning the reporter mimics the expression profile of the endogenous gene). However, such efforts proved to be largely unilluminating. Although the minimal promoter itself could suffer a staggering degree of damage by mutagenesis (including to the TATA-box and INR) while still supporting transcription (Krumm et al. 1995), no regulatory domain could be defined that delimited the sequences required for proper regulation.

THE *MYC* LOCUS: FINITE BUT UNBOUNDED?

The current regulatory topography of the *MYC* locus can be charted by superimposing the in vivo/in situ studies of the native *c-myc* gene using methods such as nuclease hypersensitivity assays, genome-wide chromatin immunoprecipitation (ChIP), chromatin conformation capture (3C), and in vivo footprinting. When combined with in vitro binding and transcription assays using manipulated or reconstructed/reconstituted regulatory elements and factors, and with reporter assays (transient and stable transfections) and transgenic animal studies, the complexity of *MYC* regulation is manifest. All of these approaches typically seek to expose the workings of individual pathways among the myriad that converge on the *MYC* promoter. Most signaling systems impact *c-myc* expression, usually by directly modifying the abundance or activity of one or a few among the host of transcription and chromatin regulatory proteins and complexes that bind to the *MYC* gene (Fig. 2). Indeed, because *MYC* is an amplifier, any TF targeting *MYC* magnifies its own effectiveness,

as has been recently shown for the androgen receptor (Ni et al. 2013). Among the regulatory processes influencing *c-myc* transcription are hormones, metabolites, intracellular second messengers, kinase cascades, and protein acetylation. A full catalog of the binding sites of all factors and their elements for *MYC* is beyond the scope of this article, but the reader is referred to reviews by Liu and Levens (2006), Wierstra and Alves (2008), and Levens (2010). Although binding sites for many factors have been mapped, it has been extremely difficult to establish unequivocally the role and significance of many elements. Two issues particularly bedevil these studies. First, many of the factors and pathways impart their influence on *c-myc* through multiple compensatory, if not fully redundant, elements distributed across long distances. Most TFs interact with the *c-myc* chromosomal domain at multiple sites. For example, TCF was first reported to bind ~1.3 kb upstream of the TSS (He et al. 1998); subsequently, TSS sites were reported extending from 30 kb 3' to 400 kb 5' of the TSS (Yochum et al. 2008, 2010; Pomerantz et al. 2009; Tuupanen et al. 2009; Sotelo et al. 2010). The best characterized of these is a TCF7L2-binding site that is 400 kb upstream with a consensus sequence that includes a single-nucleotide polymorphism (SNP) associated with better TCF binding and with colon cancer. This region loops to the promoter, as detected by 3C. The second issue involves the difficulty of assessing the physiological or pathologic function of an element. Deletion of the homologous TCFL2-binding region in mice only slightly lowers *MYC* mRNA levels in the colon while rendering these animals dramatically refractory to anaphase-promoting complex (APC)-mediated polyposis, consistent with the notion that small increments in *MYC* expression are associated with dramatic pathologic consequences (Sur et al. 2012). The marginal effect on expression observed experimentally after deletion of this site would have been discounted, lacking the cancer-associated SNP. Whether this SNP is associated with a larger magnitude effect in a subset of colonic cells or the subtle differences in *MYC* levels observed are sufficient to account for the associated tumori-

Figure 2. Integrating input at the *c-myc* promoter. (A, left) MYC sits in a gene desert removed from other genes and their activating or repressing signals. The MYC region acts as an antenna picking up positive (green lines) and negative (red lines) inputs that flicker on and off. (A, right) The intensity of basal transcriptional output (thin blue line) is too low to generate sufficient mechanical torque to twist open the far upstream element (FUSE). (B, right) When a single signal (thick green line) or the sum of multiple signals (not shown) surmount the torque threshold required to melt FUSE, the FUSE-binding protein (FBP) system kicks in (dark green). (B, left) After melting, FBP or FBP3 (dark green) are recruited to further intensify transcriptional output (thick blue arrow). (C, left) With more intense MYC expression, some MYC targets, including FBP interacting repressor (FIR) (thick red line), directly or indirectly feed back (red lines) to decrease MYC promoter output and restore basal level transcription (thin blue line). (C, right) FIR is recruited to active FUSE by binding with FBP and single-stranded DNA. With declining transcription, FBP is ejected, but FIR lingers at FUSE to enforce low-level expression (thick red line).

genesis remains unknown. The boundaries of the *c-myc* locus have not been enumerated. The numbers and activities of other remote elements remain to be determined.

FUNNY DNA AS A SENSOR OF *c-myc* ACTIVITY

The existence and significance of alternative DNA structures for eukaryotic gene regulation have been largely underexplored and perhaps

underappreciated (Sinden 1994). Typically, computational algorithms validated by biophysical studies in vitro, often using single- or double-stranded oligonucleotides, have been used to infer the potential for alternative DNA structures in vivo. However, these algorithms and indeed, the in vitro studies, have only infrequently been validated by in vivo data limiting their general impact. In vivo variables unaccounted for in computations or biophysical studies have the potential to degrade the accu-

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racy of the calculations. Foremost among these variables is the status of chromatin—the position and density of nucleosomes—as well as consideration of the binding of other TFs and chromatin regulatory complexes and of the magnitude and dynamics of mechanical forces applied to the genome by genetic processes. The *MYC* gene has served as a proving ground for strategies and methods to explore the existence and importance of DNA conformational dynamics in health and disease (Brooks and Hurley 2009).

Several regions of *MYC* have been predicted and shown to adopt non-B DNA conformations in vitro. Included among these alternatives to the conventional right-handed Watson–Crick B-DNA double helix are melted bubbles (single-stranded loops), Z-DNA (a left-handed double helix with a 12 bp repeat), and quadruplex G4 DNA comprised of intrastrand stacks (usually at least three) of planar rings, each composed of four non-Watson–Crick hydrogen-bonded guanines centered on a potassium ion. The backbone stems of the G runs can be oriented parallel or antiparallel to each other and are connected by variable length loops, making G4 DNA highly polymorphous. Other non-B structures include triplex H-DNA and extruded cruciform (hairpins).

All alternative DNA structures must compete for existence against the thermodynamic stability of conventional B DNA. In general, formation of non-B structures must be accompanied by the expenditure of energy via helix-destabilizing enzymes such as helicases, translocases, or DNA and RNA polymerases. In transcription, replication, recombination, and repair, the double helix is actively screwed through the active sites of the engaged enzymes. In situations in which the counter-rotation of the DNA relative to the enzyme is hindered, stress accumulates in the DNA backbone just as in a spring twisted from its relaxed state (Liu and Wang 1987; Kouzine and Levens 2007). The energy of negative supercoiling within the DNA backbone facilitates strand separation and structural reorganization. Supercoiling can be stored stably within a fixed topological domain, or it can be dynamically cranked through an open

domain by ongoing genetic processes, most commonly transcription (Kouzine et al. 2004, 2008, 2013; Kouzine and Levens 2007). Topoisomerases I and II that break one or both strands of the double helix allow rotation of the helix around the nick or passage of one double-helical segment through another, respectively, removing or limiting accumulated topological stress. Because of the coupling between dynamic supercoiling and DNA structure, alternative conformations have the potential to serve as real-time mechanosensors that report the intensity of gene activity. For non-B DNA to serve as a mechanosensor, some effector molecules must, in turn, respond to the mechanosensor, and feedback regulate the source(s) of the dynamic supercoiling. The *c-myc* gene presents several candidate mechanosensing *cis* elements and their associated *trans* factors.

The CT element/nuclease hypersensitive element 1 (NHE1) is a structurally complicated GC-rich region 250 bp upstream of *c-myc* promoter P2. The CT element is constitutively nucleosome depleted (Hay et al. 1987; Liu and Levens 2006; Brooks and Hurley 2009; Levens 2010). The CT element has been reported to bind a variety of TFs such as Sp1 in vitro and has been proposed to form protein-stabilized single-stranded loops, slipped mismatches of its five-repeated segments, G4 and triplex structures. Although in vivo footprinting indicates some sort of conformational disturbance at this element, the patterns are not especially diagnostic of a particular structure (Michelotti et al. 1996). Most likely, the footprints represent the superimposition of several distinct structural populations. Particular states may be enforced preferentially according to the particular transcriptional dynamics and the array of available TFs in a given cell. In B DNA, the CT element presents an array of five Sp1-like binding sites that will dock with Sp1 or other zinc-finger proteins. As a melted loop, the purine-rich strand of the CT element binds with CNBP (also called ZNF9), a single-stranded DNA (ssDNA)- or RNA-binding protein, whereas the pyrimidine-rich strand binds hnRNP K. When folded into quadruplex, the G-rich strand can bind nucleolin (Gonzalez et al. 2009). The transcrip-

tional effector function of nucleolin, if any, has not been elucidated. The complex interplay and competition between alternative DNA conformations and their interactions with CT-binding factors invites the consideration of many hypothetical regulatory modalities that remain largely unproven.

The far upstream element (FUSE) is an AT-rich sequence that in the context of the *MYC* promoter is highly responsive to the unwinding negative supercoiling forces generated by the transcription of downstream sequences. After melting, FUSE binds members of a family of sequence-specific single-strand-binding TFs (the FUSE-binding proteins, FUBPs) as well as the ssDNA-specific FBP interacting repressor (FIR). Both FBP and FIR act through TFIIF downstream from preinitiation complex assembly to control the advancement of the transcription machinery to the point of pause release (Liu et al. 2001, 2006). Once the transcription elicited by any signal surmounts the threshold that melts FUSE, FBP (or FBP3) is recruited to hasten progression to and through pausing. Higher levels of transcription lead to further melting of FUSE, resulting in recruitment of FIR, which in turn drives *MYC* back to basal levels of expression (Fig. 2).

Proteins that bind non-B DNA often do so through protein motifs associated with RNA binding, such as KH domains and RNA recognition motifs (RRMs) (Braddock et al. 2002; Benjamin et al. 2008; Crichlow et al. 2008; Valverde et al. 2008; Mitchell et al. 2010). In the absence of definitive enumeration of their in vivo targets or of quantitative thermodynamic and kinetic data discriminating their RNA- versus DNA-binding characteristics, it would perhaps be prudent to designate these molecules as single-stranded nucleic-acid-binding proteins (or even simply nucleic-acid-binding proteins). Whereas duplex DNA-binding proteins are presumed to conduct the bona fide decoding of regulatory information from the genome, DNA binding by RNA-binding proteins (or other unconventional DNA-binding proteins) is often presumed to be a nonspecific manifestation of RNA binding; however, imagining the transition states between the primordial RNA and

DNA worlds invites speculation that the first regulatory DNA-binding proteins most likely looked like RNA-binding proteins, whereas the evolution of proteins truly specific for double-stranded DNA likely represents a later evolutionary elaboration. Logically, when the first DNAs were templated by RNA, only RNA-binding proteins were available. Subsequently most of the regulatory duties of these early proteins were ceded to canonical B-DNA-binding TFs, although any functions demanding ssDNAs or secondary structures derived from unpaired strands, might be expected to conserve RNA-binding-like protein partners. The intrinsic rigidity of B DNA to bending (persistence length ~ 50 nm, 150 bp) and especially to twisting (torsional persistence length ~ 70 nm, 210 bp) would seem to impose a formidable energetic hurdle opposing the assembly of enhanceosomes and the various loops that have been proposed to juxtapose DNA elements across various scales (Lavelle 2009; Levens and Benham 2011). The strategic placement of sequences adopting non-B conformations, with correspondingly different elastic moduli would seem to be a useful tool coupling *cis*-element function with DNA structure such as at FUSE.

Chromatin must necessarily constrain the role of non-B DNA conformations in the regulation of gene expression. The X-ray crystallographic structures of nucleosomes show complementarity between histone octamer cores and B DNA (Luger et al. 1997; Davey et al. 2002). Alternative DNA structures must be accommodated either by an ill-fitting bulge lifted above the nucleosomal surface or by complete exclusion from nucleosomes, unless the histones undergo a structural transition friendly to other DNA conformations (Mulholland et al. 2012). The CT element is constitutively nucleosome-free, perhaps reflecting an inability to incorporate G4 DNA onto a nucleosome (Michelotti et al. 1996). In contrast, FUSE is nucleosome occluded in *c-myc*-off cells but nucleosome-free in *MYC* cells (Michelotti et al. 1996). Mobilization of the occluding nucleosome must precede the torsional stress that induces melting of FUSE. Application of torque to a nucleo-

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some-embracing FUSE may be accommodated by en bloc nucleosome rotation or shifts in the trajectory of linker regions that protect FUSE from helix-destabilizing stress. The signal and mechanisms mediating mobilization of the FUSE-occluding nucleosome are unknown, although some evidence implicates Swi/Snf chromatin remodeling with the activity of the *MYC* promoter (Nagl et al. 2007). There is also some evidence that Z-DNA may occur in the *MYC* gene, but the regulatory consequences and in vivo significance of Z formation at any of its three proposed sites remain to be explored (Wolff et al. 1995, 1997).

The overview that emerges indicates that the *MYC* promoter is a remarkably robust system comprised of various parallel signaling modules distributed across a vast chromosomal expanse, seemingly isolated from interfering elements from other genes. These modules operate individually and independently when driven by single signals in experimental systems. The ensemble operation of the system in the face of a complex set of physiological and/or pathologic and environmental stimuli remains largely unexplored. The degree to which signals synergize and antagonize and the rules of integration at the *MYC* promoter remain unknown. The existence of FUSE, CT, and Z-DNA elements suggests that the promoter output is coupled not only with chromatin conformation and dynamics, but also with fundamental changes in DNA secondary structure. In the same way that transcription pausing at the *MYC* promoter pre-saged almost global regulation by pause release, *MYC* may also herald the dynamic coupling between DNA conformation and topology with gene activity on a genome-wide scale. Such mechanisms may prove especially important at genes where fast and coarse fluctuations are likely to prove especially deleterious, such as some oncogenes and tumor suppressors.

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David Levens

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