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Taming of the beast: Shaping Myc-dependent amplification

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Abstract

Myc deregulation is a hallmark oncogenic event where overexpression of the transcription factor gives rise to numerous tumorigenic phenotypes. The complex consequences of Myc deregulation have obfuscated clear mechanistic interpretations of its function. A synthesis of recent experimental observations offers a consensus on Myc's direct transcriptional function — when overexpressed, Myc broadly engages the cell's established euchromatic *cis*-regulatory landscape where the factor generally amplifies transcription. The level of Myc binding at target genes and the transcriptional output are differentially modulated by additional regulators, including Miz1. Targeting Myc oncogenic activity will require an understanding of whether amplification promotes tumorigenesis and the consequences of amplification in tumors adapted to oncogenic Myc.

Keywords

Myc; cancer; transcription

Myc transcription in cancer

The intense interest in Myc proteins stems from their pervasive role in the genesis of human tumors. A large body of evidence has established that levels of one out of three Myc proteins (c-Myc, N-Myc, or L-Myc) are enhanced and its expression released from its normally tight dependence on growth factors in a large fraction of all human tumors [1, 2]. The three Myc genes are differentially expressed during development, but the proteins are functionally equivalent in most biological systems, allowing us to focus on c-Myc (called “Myc” from here on) in this review [3]. Multiple experiments in transgenic models of human tumors

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show that this "deregulated" expression of Myc proteins promotes tumorigenesis and that tumors generated by different oncogenes depend on elevated Myc levels [4]. Accordingly, several proof-of-principle studies suggest that targeting Myc proteins may have considerable benefit for tumor therapy [5, 6]. This hope awaits clinical confirmation, since therapies directed against Myc proteins have yet to enter the clinic. Myc proteins are nuclear proteins and as such may have several functions; however, there is consensus in the field that one major activity of Myc proteins is to regulate transcription [7]. This has stimulated a vigorous debate about why Myc-dependent transcription is so critically important for tumorigenesis. Here we review the current state of this debate.

The evolution of Myc

One of the best-understood Myc transcription factor networks is that of *Drosophila*. dMyc, a helix-loop-helix basic leucine zipper protein, binds together with another HLH-bZIP protein, dMax, to target genes via a conserved sequence element termed an E-box (CACGTG) and to activate transcription [8]. The dMyc antagonist dMnt, also pairs with dMax to repress transcription from the same DNA sequence [9]. Deletion of dMnt largely rescues the developmental defects of dMyc deletion, providing strong evidence that transcriptional activation is the critical biological function of dMyc [10]. The majority of target genes of dMyc and dMax encode proteins involved in RNA and protein synthesis [11]. Consistently, dMyc proteins stimulate cell growth, but not cell proliferation, and loss-of-function alleles show a "minute" phenotype [8, 12]. Transferring this model to mammalian tumorigenesis would suggest that the oncogenic activity of Myc is due to the deregulated and constitutive activation of its target genes. According to this model, sustained high levels of Myc in human tumors over-drive RNA biogenesis and protein translation enabling growth-factor independent cell growth. Multiple observations are consistent with this model: for example, Myc-driven lymphomas, in contrast to their normal counterparts, rely on supraphysiological rates of protein translation to support growth [13].

Like *Drosophila* dMyc, mammalian Myc proteins, when dimerized with Max, are sequence-specific DNA binding proteins that bind to E-Boxes [7]. Unlike prokaryotic repressors that discriminate operators from non-operator DNAs by $\sim 10^{5-6}$ -fold, Max homodimers and, most likely, Myc/Max heterodimers have considerable affinity for variant E-boxes and even generic DNA [14]. It is almost certain, therefore, that Myc/Max heterodimers have non-negligible affinity for most promoters. Reflecting its continuum of association to targets, the intra-nuclear dynamics of Myc suggest engagement with intra-nuclear partners, indicating that it is globally distributed, is almost always bound directly or indirectly with chromatin and is available to all active genes [15, 16]. While broadly anticipated, there is currently only limited formal evidence that transcriptional activation is the critical oncogenic function of Myc. One example is the finding that inactivation of Mga, a repressive partner protein of Max, occurs commonly, but mutually exclusively with focal amplification of the Myc gene in lung cancer suggesting that Mga inactivation is pathologically equivalent to Myc overexpression in this entity [17].

Clues to how Myc regulates gene expression are provided in the list of co-activators it recruits to induce transcription: a conserved sequence in Myc — MycBoxII, binds the

adaptor protein TRRAP via TRRAP GCN5-containing histone acetylase complexes [18, 19]. Myc also recruits the p300 histone acetylase and other histone-modifying enzymes to its target sites, although the requisite interaction surfaces on Myc are less well defined [20, 21]. Myc also interacts with TBP [22], the chromatin regulators Tip60 [23], and SWI/SNF [24], and it inhibits the H3K4Me3 demethylase dKDM5/LID[25]. Myc's ability to interact with and recruit a multitude of general chromatin regulators is consistent with its global role in regulating chromatin structure as Myc activity has been broadly linked with large nuclei containing high levels of euchromatic and hyperacetylated chromatin [18, 26, 27].

Myc may also interact directly with the transcription apparatus. Most notably, the extreme amino-terminus of Myc binds cyclin T1 *in vitro* [28]. Cyclin T1 is part of the P-TEFb elongation complex, which phosphorylates the C-terminal domain of RNA polymerase II at serine 2 and thereby promotes the transition from promoter binding to productive elongation. The relevance of this finding stems from observations that Myc can stimulate transcription at a step after initiation of transcription and that RNA polymerase II accumulates in the body of transcribed genes after activation of Myc [29-31]. Furthermore, ectopic expression of Myc globally enhances phosphorylation of RNA polymerase [31, 32]. Together, these observations suggest that the release of RNA polymerase from a paused position may be a key mechanism of transcriptional activation by Myc [31] (Figure 1). There is no doubt that other effectors of Myc remain to be defined. How Myc exploits these partners individually or in combination at any given promoter is not known.

The general amplifier model

The identification of Myc as a transcriptional activator raised the expectation that an enumeration of the direct target genes of Myc would provide a list of critical downstream targets and biological processes that mediate the physiological and oncogenic functions of Myc. This expectation prompted a series of studies to identify the Myc-regulated genes by comparing RNA expression profiles and the genome-wide map of Myc-bound chromatin using microarray or NextGen Sequencing technology. Interrogation of a broad range of cell types surprisingly revealed mammalian Myc proteins at nearly all promoters in open chromatin [33]. This was not only true for genes transcribed by RNA polymerase II, but also for genes transcribed by RNA polymerases I and III (although rRNA promoters tend to bind less Myc than other promoters) [34, 35]. For RNA polymerase II-transcribed genes, the presence of histone marks indicative of open chromatin, in particular K4-trimethylated histone H3, highly correlated with Myc binding [36]. When over-expressed, Myc proteins also bound to many enhancers, again correlating with histone marks indicative of activity. Since many genes encoding proteins involved in translation (e.g. ribosomal protein genes) are highly transcribed from promoters in CpG islands where E-boxes are more frequently found in an open chromatin conformation, these results parallel those obtained in *Drosophila*.

Collectively, these data have led to an exciting model, in which Myc proteins act as general amplifiers that globally enhance transcription; the specificity and degree of amplification at individual genes is determined by the exact pattern of open promoters that pre-exists in a cell even prior to Myc activation [37, 38]. In this model, the unique biological role of Myc

stems from the ability to accelerate transcriptional activation (i.e. releasing a paused RNA polymerase from the promoter) rather than by specifying a particular program of gene expression [31]. Strong support for this model comes from the analysis of primary B-cells [37]. Upon stimulation of resting B-cells, Myc binds to all promoters of transcriptionally active genes in these cells and Myc binding follows the pattern of loading of RNA polymerase II that pre-exists in resting low Myc cells. In response to mitogen stimulation there is a global increase in mRNA levels that depends on Myc — confirmed by examining the mRNA levels of multiple individual genes. There are indications that the "global amplifier" model operates not only in primary cells, but also in tumors. Comparing normal B-cells with Myc-driven B-cell lymphomas reveals a strong increase in total cellular mRNA content in lymphomas [38]. ChIP-sequencing shows that Myc binds to 90% of all open promoters in lymphomas — an easy explanation might be that Myc broadly enhances expression of the genes to which it binds and this increases cellular mRNA content. Similarly, the comparison of human tumor cell lines with different Myc levels suggests that Myc-driven amplification accounts for differences in mRNA content between different tumor cells [39].

According to a strict amplifier model, the direct effect of Myc on promoters is always positive. The effect of Myc is not equally strong for each gene since promoters and relevant enhancers differ in their affinity for Myc: promoter binding dictates differences in response. Moreover, the overall promoter response to Myc is not directly proportional to the amount Myc recruited there. Furthermore, at high and oncogenic levels, Myc "invades" enhancers and promoters, enabling the pathological regulation of genes that bind little Myc at physiological levels [38-40]. The general amplifier model therefore can account for the observation that oncogenic levels of Myc regulate a specific set of genes in particular tumors [41]. The model can also account for apparent Myc-dependent repression. Specifically, if expression of most mRNAs is amplified and if total mRNA content therefore increases in a cell upon Myc stimulation, then the relative amount of mRNAs transcribed from genes that have low affinity promoters and are therefore poorly activated genes will decrease and - depending on the mode of normalization - these genes will appear repressed [42]. Whether the general amplifier model can account for Myc-dependent repression is therefore intimately tied to the question of whether Myc induces a general increase in cellular RNA levels and, by inference, whether cells grow as a result of Myc activation: if cells grow, seemingly repressed genes may actually be low affinity Myc targets that are relatively "left behind" [42]. Myc drives both growth and proliferation of mammalian cells, unlike *Drosophila* [43]. Because cell proliferation and division reduce cell size, some of the apparent Myc-dependent repression may reflect the post-mitotic partition of cellular contents compared with non-dividing cells; therefore, a complete description of amplification will be sensitive to cell cycle parameters. Further, if Myc acts at transcription, then gauging Myc action from mRNA abundance is imprecise because mRNA synthesis becomes conflated with mRNA half-lives, and cell cycle parameters. Yet this too becomes even more complicated as the factors that regulate mRNA stability and the cell cycle may themselves be important direct or indirect Myc targets.

An important aspect of the amplifier model is the postulate that in any given cell type, all transcription initiating genes are potential targets of Myc: individual promoters differ

quantitatively in their affinity for Myc, but no sharp cut-off discriminates Myc-targets from non-targets. Occupancy at each promoter changes as Myc concentrations vary and this change defines the degree of the transcriptional response. We suggest that there is a continuum of activity as Myc flickers on and off of weakly bound, weakly expressed promoters, but stays longer or more frequently at high output promoters (Figure 2). This view of Myc as a general regulator capable of interacting with all active promoters has implications for the way that ChIP-sequencing data need to be interpreted, since it suggests that cut-offs that are introduced by analysis (e.g. peak-calling) programs may generate artificial distinctions between target and non-target genes.

This quantitative view also suggests that in some experimental settings, the response of some genes to Myc may plateau as occupancy by Myc at promoters and enhancers and corresponding transcriptional output is saturated. Whereas Myc abundance is low in most, but not all primary cells (luminal cells in mammary ducts are a notable exception), recent estimates for Myc in tumor cells appear high enough to allow the saturation of some promoters [38]. A recent study provides evidence that high affinity promoters are Myc-saturated in proliferating cells and that further increases in Myc therefore only increase occupancy and gene expression at low affinity promoters [40]. As a result, gene expression patterns that discriminate cells with oncogenic Myc levels from normally proliferating cells are different from the gene expression changes that occur as Myc-levels increase during the physiological transition from quiescence to proliferation.

These considerations allow the conclusion that even within the framework of the general amplifier model, the observed effects of Myc on gene expression depend (i) on the precise Myc levels that are being compared, (ii) on whether cells grow or not upon Myc activation and (iii) on the precise pattern of ongoing transcription that determines which genes are accessible to Myc.

Direct and Indirect transcriptional repression

In many studies almost as many genes are downregulated by Myc as go up evoking the notion that Myc can act as a repressor [39, 44]. Setting aside pseudo-downregulation arising from normalization issues (above), such down-regulation can stem from a direct and context dependent repressive function of Myc that is unmasked by factors bound locally at the regulatory sequences of particular target genes. For example, Myc blunts the induction of the *CDKN2B* and *CDKN1A* genes in response to TGF β signaling by a direct interaction with the Miz1 and Smad3 proteins at both promoters [45, 46]. Alternatively, repression can be indirect; as a universal amplifier, Myc will increase the expression of repressive transcriptional and chromatin components. As repressors are upregulated, downregulation of sets of targets may ensue as the system seeks a new steady state. For example Myc up-regulated PTEN inactivates Akt, which disinhibits Ezh2, promoting widespread PRC2-mediated repression [47]. Furthermore, microRNAs, such as miR17-92 that represses Sin3b, Hbp1, Suv420h1, and Btg1 to regulate chromatin structure as well as the apoptosis facilitator Bim [48] are repressive mediators of Myc action. Direct and indirect mechanisms may become entangled as feedforward and feedback arms of genetic circuits adjust to new conditions. In principle, the dynamics of the temporal profile of the transcriptome could help

to discriminate direct from indirect regulation, as changes requiring newly synthesized protein intermediaries are expected to be delayed relative to the induction of Myc protein. However, few studies have had the temporal resolution to discriminate direct versus indirect targets. Because they are translation independent, microRNAs and other non-coding RNAs are likely to act after only a brief delay. This rapid onset of activity is likely to hinder the discrimination of the direct and indirect effects of Myc.

Shaping Transcriptional Amplification

Transcriptional amplification and cell growth induced by Myc cannot continue indefinitely and unopposed. Mechanisms must therefore exist that adjust transcriptional amplification to available metabolic resources and that provide feedback from the physiological status of a cell to Myc activity. Consequently, supraphysiological levels of Myc would be expected to elicit either compensatory measures or, if they fail, promote cell death. As Myc saturates its strongest and most highly expressed sites (these being most likely to be physiological targets), it may pathologically spill over onto other promoters and enhancers, increasing their activity and overall cellular RNA production, provided that the cell can marshal sufficient resources to augment net transcription.

There is evidence for feedback mechanisms that limit Myc-dependent transactivation operating at both physiological and supraphysiological levels. The apoptosis elicited by over-expression provides an example of an upper-bound limit for Myc levels [49]. As another example, the ribosomal protein L11, encoded by a direct target gene of Myc, inhibits Myc-dependent transactivation and expression [50, 51]. Supraphysiological levels of Myc induce expression of the Arf tumor suppressor protein, which in turn interacts with Myc and inhibits Myc-dependent transactivation [52, 53]. One broadly acting mechanism that limits transcriptome amplification driven by supraphysiological Myc is the recruitment of the zinc finger protein Miz1 at many promoters [54]. In primary cells, and in the absence of high levels of Myc, Miz1 is a highly sequence specific transcription factor that activates a relatively small number of targets that share an extended consensus sequence and that control autophagy [55]. As Myc levels rise to supraphysiological levels, Miz1 joins Myc via a protein-protein interaction at a large fraction of promoters that then decreases output [40]. Miz1 is unstable and continuously degraded by the Huwe1 ubiquitin ligase [56]; upon inhibition of Huwe1, Miz1 broadly accumulates at Myc-bound promoters, blunts activation and enhances repression by Myc [56, 57]. Interestingly, the Arf protein binds to and inhibits Huwe1 and promotes association of Myc with Miz1 [58, 59], strongly suggesting that Arf and Miz1 are part of a common stress-response to oncogenic Myc levels.

A second mechanism that is likely to limit and shape Myc amplification is competition between Myc and other HLH-bZIP dimers for binding at E-boxes and perhaps at other sites (Figure 3). Because Max is more abundant than Myc even in tumors, Myc-Max must operate against an aggregate excess of Max homodimers as well as heterodimers of Max with repressive members of the HLH-bZIP family (Mxd-14, Mnt, etc.) [23]. The degree to which the levels of these other complexes alter the physical distribution and activity of Myc at E-box versus non-E-box promoters remains to be rigorously evaluated. The specialized regulation of these other E-box-binding proteins (such as Mondo) have the capacity to tune

the Myc- response toward or away from cellular processes such as metabolism [60] or circadian rhythms via coupling with proteins such as CLOCK [61].

Almost certainly, steady-state patterns of gene expression observed after Myc expression do not only reflect the direct action of Myc, but are also due to secondary effects of proteins or RNAs encoded by Myc target genes that feed-back on gene expression [39]. In a rapidly growing cell, such as a B-cell becoming activated from the naïve state, increasing synthesis of macromolecules is required to satisfy a growing demand for the transcription machinery and other components required to activate genes. Examples for such indirect effects are the induction of the Gcn5 histone acetylase that mediates a global opening of chromatin upon Myc expression and enhanced expression of phosphoribosyl-pyrophosphate synthetase 2 (PRPS2), which promotes the enhanced nucleotide biosynthesis of Myc-driven tumors [18, 26, 27]. At the same time, rapid growth and enhanced global transcription may induce the redeployment of limiting components of the gene expression machinery to more or less active transcription start sites. Under conditions of limiting gene expression components, increasing Myc at strongly expressed promoters might recruit transcription machinery otherwise assigned to weakly transcribed genes. Such diversion would yield the up-regulation of some genes with the down regulation of others. This repression by redistribution of the transcriptional machinery has been recently observed during NF- κ B activation in endothelial cells [62].

Conceptually, other mechanisms may activate to counterbalance transcriptome amplification. For example in yeast, rates of RNA synthesis and RNA degradation are coupled [18]. Mutations and strategies that alter the levels of one elicit compensatory changes in the other. Additional amplification counteracting pressures may arise from rate limiting levels of translation initiation in tumors [17]. Notably, disruption of the translation initiation factor eIF4F is synthetic lethal in tumors with deregulated Myc suggesting that the high levels of mRNA output need to be supported by high levels of protein expression, perhaps exposing an orthogonal vulnerability arising due to amplification [63]. For RNA levels to rise globally in response to MYC deregulation, these compensatory countermeasures may need to be defeated or overwhelmed.

Concluding Remarks

Deregulated in tumors, Myc acts as a universal regulator of the cell's pre-existing gene expression program. Whether amplification alone provides a sufficient oncogenic impulse or whether Myc-dependent repressive mechanisms are required for Myc-driven tumorigenesis warrants further investigation. Simply, as a universal regulator, mutations in other oncogenes and tumor suppressors must first specify particular genes to be turned on or off, and Myc then develops the new gene expression program. This model is also consistent with data in induced pluripotent stem cell (iPS) reprogramming where Myc overexpression increases the efficiency of an embryonic stem cell specifying Oct4/Sox2/Klf4 transcription factors [64].

Alternatively, the pathways for growth, proliferation, apoptosis and neoplastic metabolism do not scale linearly, i.e. increasing the components of these pathways drives them across

thresholds that gate their activity as a step function. In this situation, malignant behavior would manifest as an emergent property explainable by the control theory (Box 1) and architecture of subsystems and networks whose components are up-regulated by Myc.

Although predominantly an amplifier of transcription, context specific conditions and constraints at individual genes modulate Myc transcriptional output and response. To elucidate the molecular mechanism of Myc-driven transcriptome amplification, a better description of its transfer function — the function describing how the Myc transcriptional effect depends on initial parameters at any gene, i.e. the input-output curve — will be required to account for its physiological and pathological activities. If proportional and linear, for example all Myc targets will be up-regulated equivalently, any changes in the relative expression of particular genes would demand parallel changes in the levels of the gene specific factors that differentially up or down-regulate these targets (Figure 4A). However, if the transfer function is non-linear, as proposed in [65], then different zones across the expression spectrum will be disproportionately regulated. If highly expressed genes are preferentially amplified, then a Myc signature may emerge comprised of genes that are highly expressed across many tissues (Figure 4B). Of course such amplification cannot continue indefinitely and unopposed; supraphysiological levels of Myc are expected to elicit either compensatory measures or cell death (Figure 4C). Increasing amplification of all genes demands that the cell can marshal sufficient resources to augment net transcription. In the presence of limiting materials, the competition between promoters may modify the transfer function from one that supports only universal amplification to one that allows for repression at those promoters deprived of limiting factors that have been deployed to support the expression of other targets. In this scheme repression (e.g. Miz1-dependent repression) reflects feedback control that responds to the unbalanced and excessive Myc-enforced expression of other targets (Figure 4D).

Universal activation by Myc does not preclude the functional emergence of preferred, and hence specific targets. Moreover, the preferential up or down regulation of specific genes that occurs as tumors achieve homeostasis in high Myc conditions may underlie Myc's ability to act as one of the rare universal oncogenes capable of promoting tumorigenesis in a wide variety of tissues. Only when experiments challenge models that make quantitative, precise and testable predictions will a durable consensus on the role of Myc emerge. Beyond having heuristic value as an organizing framework for diverse observations, the universal non-linear amplifier model may yet account for quantitative differences in gene expression between cell states in health and disease.

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Box 1**Control Theory**

A dynamic analysis of network behavior as modeled by the effect of inputs and their consequent feedback on system output. In engineering, this can describe how different components affect the output of a system. For instance, a compressor either boosts or dampens signal depending on initial input signal. A reverb module would apply a delay to input signal before entering them back into the system, creating a recursive feedback loop. An amplifier increases signal by an amount relative to the initial input signal. In each of these examples, the transformations are surmised through the transfer function — a mathematical representation of the relationship between input and output. Thus a control theory analysis of Myc examines the consequence of adding Myc to the transcriptional system. The Myc transfer function describes the transcriptional output of Myc activity as a function of the initial transcription parameters.

Highlights

1. Myc is a general regulator that binds to established euchromatin in the cell
2. Myc acts as a non-linear amplifier of transcription
3. 3. Miz1 and other regulators shape Myc transcriptional amplification

Figure 1. Schematic of MYC's transcription elongation promoting activity
 MYC/MAX bind to E-box (CACGTG) sequences at enhancers and promoters and recruits the elongation factor complex P-TEFb, which promotes elongation in part by phosphorylating the C-terminal domain of RNA Polymerase II.

Figure 2. Schematic of MYC genomic binding at (A) low, (B) medium, and (C) high concentrations of the factor

Cis-regulatory elements (boxes) are shaded by their affinity for Myc. Myc transcriptional activity at genes is depicted as a circle sized by Myc's impact.

Figure 3. Schematic showing the dynamic occupancy of E-box sites by Myc and other transcriptional regulators

Multiple bHLH transcription factor pairings compete for binding at E-box sites. The net positive or negative transcriptional consequence of regulator binding at E-boxes is depicted.

Figure 4. Schematic of Myc transfer functions

Transfer functions are shown providing the relationship between expression changes at genes as a function of various parameters as measured either by absolute or relative change in expression. **(A)** A linear amplifier transfer function where the change in expression in high vs. low Myc conditions is constant across all transcriptionally active genes. **(B)** A non-linear amplifier transfer function where the change in expression in high vs. low Myc conditions is proportional to the exponent of the expression in low Myc conditions. **(C)** A Myc/Miz1 repression transfer function where the change in expression is proportional to the ratio of Myc binding divided by Miz1 binding. **(D)** A combined Miz1 shaped non-linear amplifier model that combines the transfer functions in **(B)** and **(C)**. A two dimensional heatmap is provided showing the relative change in expression as a function of both the ratio of Myc binding divided by Miz1 binding (y-axis) and the initial expression in low Myc conditions (x-axis).