Specific regulatory mechanisms constitute c-Myc-mediated global amplification in cancer
by Cherubin Manokaran

Abstract

c-Myc (hereafter Myc) is deregulated in nearly all cancers and directs transcriptional programs that significantly increase global expression. Interestingly, Myc binding sites are not highly conserved across tissue and cell types because deregulated Myc in cancer cells saturates expected binding sites, E-boxes, and enables Myc to bind at low-affinity variable E-box and non-E-box sequences. Increased Myc occupancy then leads to the transcription and, perhaps, the expression of many tissue or lineage-specific, pro-survival genes that contribute to the harmful phenotypes exhibited in different cancer types and subtypes.

Introduction

Myc is a basic helix-loop-helix leucine zipper (bHLHZ) transcription factor that coordinates both somatic and germ cell growth and proliferation by regulating a wide variety of targets. Myc recognizes specific DNA sequences and forms a complex with its binding partner Myc-associated protein X (MAX) to activate and sometimes inactivate the transcription of its targets. Specifically, Myc binds enhancer boxes (E-boxes), or specific DNA response elements with a CANNTG sequence, and has the highest affinity for the canonical CACGTG E-box (26). Although both canonical and non-canonical (variable) E-boxes bind most other transcription factors in the bHLH family, many of them have no effect on cell growth. In fact, many functionally relevant, target genes have binding sites that are specific for Myc. In cancer, frequently deregulated Myc directs dissimilar transcriptional programs between cell types relying on more specific regulatory mechanisms (2).

Endogenous Function

Myc regulates intracellular functions such as cell growth and metabolism as well as the numerous underlying molecular changes. These include increased transcription, decreased growth inhibitory gene expression, microRNA changes, increased mitochondrial function, glycolysis, anapleurosis, and glutaminolysis. Additionally, Myc regulates extracellular processes such as angiogenesis, immune activation and inflammation in response to the microenvironment. Together, the intracellular functions and extracellular processes coordinate cell proliferation. Therefore, Myc is pleiotropic (Figure 1). More importantly, Myc carries out these diverse functions in all somatic cells, from fat to neural cells (26).

Myc has a significant role in organogenesis. Although a related bHLH transcription factor N-myc is responsible for establishing the pluripotent stem cell (progenitor) compartments that constitute organ systems, Myc expression must be induced to complete the process. Myc drives both the self-renewal of progenitors and the proliferative expansion or survival of both lineage-committed cells and differentiated cell types. For the most part, high Myc expression prevents

terminal differentiation; therefore, Myc levels mostly decrease prior to differentiation. To be more precise, following the establishment of progenitor compartments, activated Myc may direct the expansion of specific lineages (Figure 1). For example, WNT/β-catenin signaling likely activates Myc in chondrogenic progenitors to partially mediate the proliferative expansion of chondrocytes within the growth plate and contribute to bone growth (13).

However, the establishment and self-renewal of progenitors, expansion and differentiation of committed cell types and then the proliferation of differentiated cell types are all essential for not only the development but also the maintenance of organs. Therefore, Myc likely provides positive feedback in transcriptional programs that form and maintain cellular or lineage identities (Figure 1). Myc also mediates the reprogramming of somatic cells into induced pluripotent stem cells, which reinforces the role of Myc in assuming a cellular identity (13).

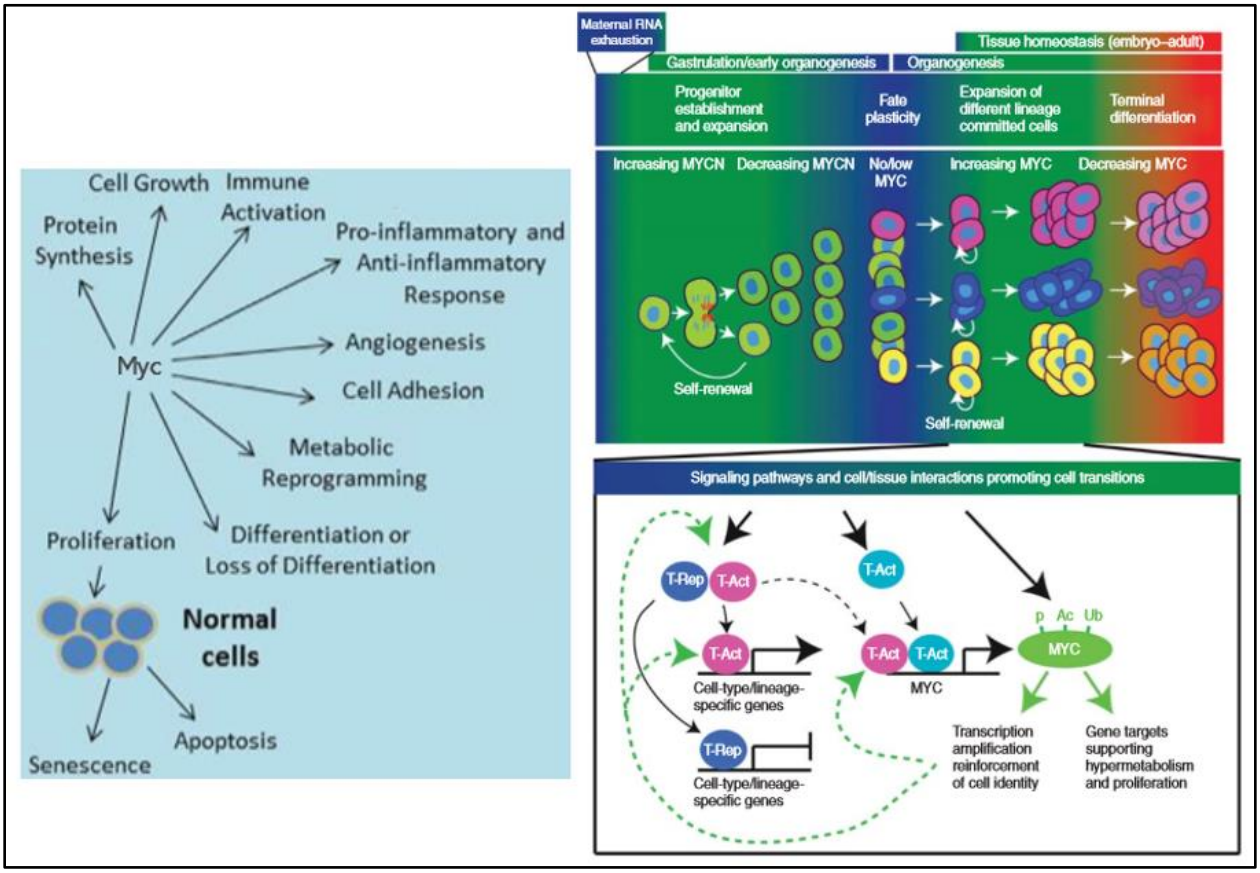

Figure 1. Endogenous Myc function. (Left) Myc is pleiotropic in that it regulates many different cellular processes that may result in as many if not more, different phenotypes (26). (Right). The wide-ranging gene regulation by Myc allows it to direct organogenesis and shown is a potential model for this process (13).

Myc Deregulation in Cancer

Myc is often deregulated in cancer by different alterations that allow it to bypass all regulation and render its negative effects. *Myc* gene amplification is a predominant alteration in many diverse cancer types (Figure 2A and 2C). Amplification alone decreases patient survival ($P<0.05$, Figure 2B). Classification of cancer types based on genetic and epigenetic alterations reveals the two major classes as mutation-based and copy number alteration-based. In the class dominated by copy number alterations, Myc amplification is a hallmark driver of cell proliferation (7, 31).

However, many specific cancer types such as B-cell lymphomas harbor mutations that increase the oncogenicity of Myc. In the MSK study, diffuse large B-cell lymphoma samples harbored 23 missense mutations. Although only four of these are predicted to be mutation hotspots and oncogenic, many of the others have not been characterized (Figure 2A) (31). A similar distribution was observed for B-cell lymphomas in the TCGA study, as well (Figure 2C) (7). Frequently, missense mutations occur in functional domains that stabilize Myc protein preventing degradation and, in turn, increasing its activity. Otherwise, they may also affect Myc interactions and drive entire transcriptional programs such as ones that promote cell apoptosis. In some other cases, hypermutation may give rise to gene fusions; however, co-occurrence was observed in only one sample from the MSK study but likely unrelated even for that one case (30, 31). Unlike *Myc* gene amplification, though, the mutations have no effect on survival ($P>0.05$) (7, 31).

Many cancer types associated with aggressive disease and poor prognosis display Myc amplification. The same cancer types or specific subtypes may involve other important and potentially hallmark Myc alterations. The subsections that follow discuss, in detail, a few of these cancer types and the role of Myc in their pathology.

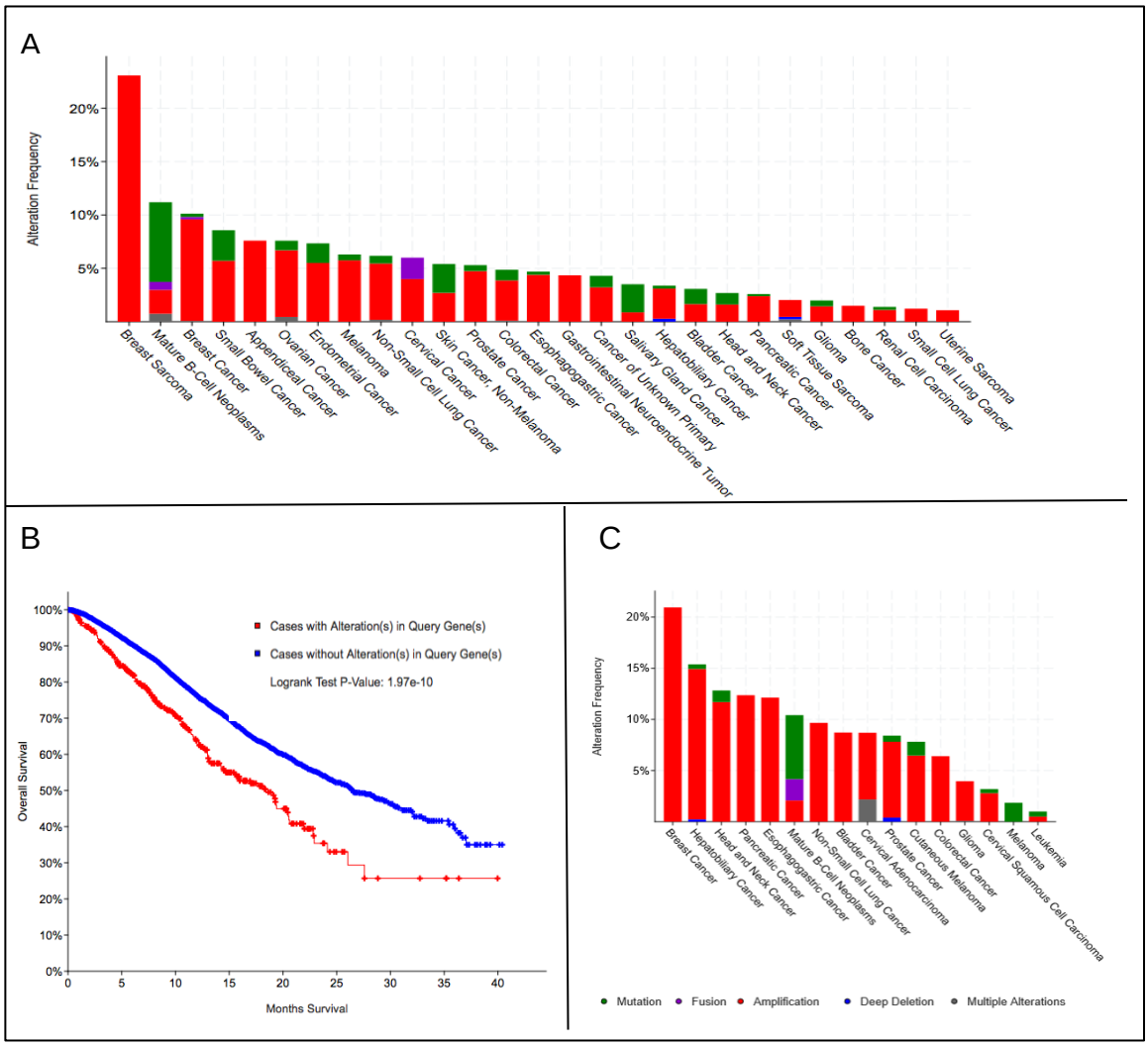

Figure 2. Myc amplification is present in many diverse cancer types and decreases survival. (A) Pan-cancer analyses by the MSK Cancer Center of Myc alterations reveals a significant role for Myc gene amplification in many diverse cancer types. (B) Patient survival decreases as a result of amplifications, alone. Other alterations fail to affect patient survival. (C) In a similar pan-cancer analyses from the TCGA, the largest fraction of patients exhibited gene amplification (7, 31).

Breast Cancer

The pan-cancer studies from MSK and the TCGA identify breast cancer as the type with the highest number of Myc alterations, of which the most frequent is Myc amplification. In the MSK study, Myc-amplified patient samples display histology grade III tumors associated with poor differentiation and, therefore, poor prognosis (Figure 3B). More specifically, Myc amplification is highly represented in the more common but increasingly resistant HR+/HER2- breast cancer subtype as well as triple negative breast cancer, a subtype associated with aggressive disease (Figure 3A) (21). As a challenge to treating HR+/HER2- breast cancer, the subtype is exhibiting increased resistance to hormone therapy. At the same time, triple negative breast cancer is

untreatable with chemotherapy. The phenotype does not allow for the two commonly used strategies for breast cancer treatment, or hormone therapy and HER2-targeted therapy. For this reason, triple negative breast cancer also presents a great challenge for overall breast cancer treatment. In the TCGA study, many patients had an ER- and HER2- status. Patients with ER- status exhibited higher, relative Myc amplification than patients with ER+ status (Figure 3C) (7). For this reason, Myc amplification is not only present in ER-/HER2- breast cancer but likely also a key contributor to the development of this subtype, which includes triple negative breast cancer.

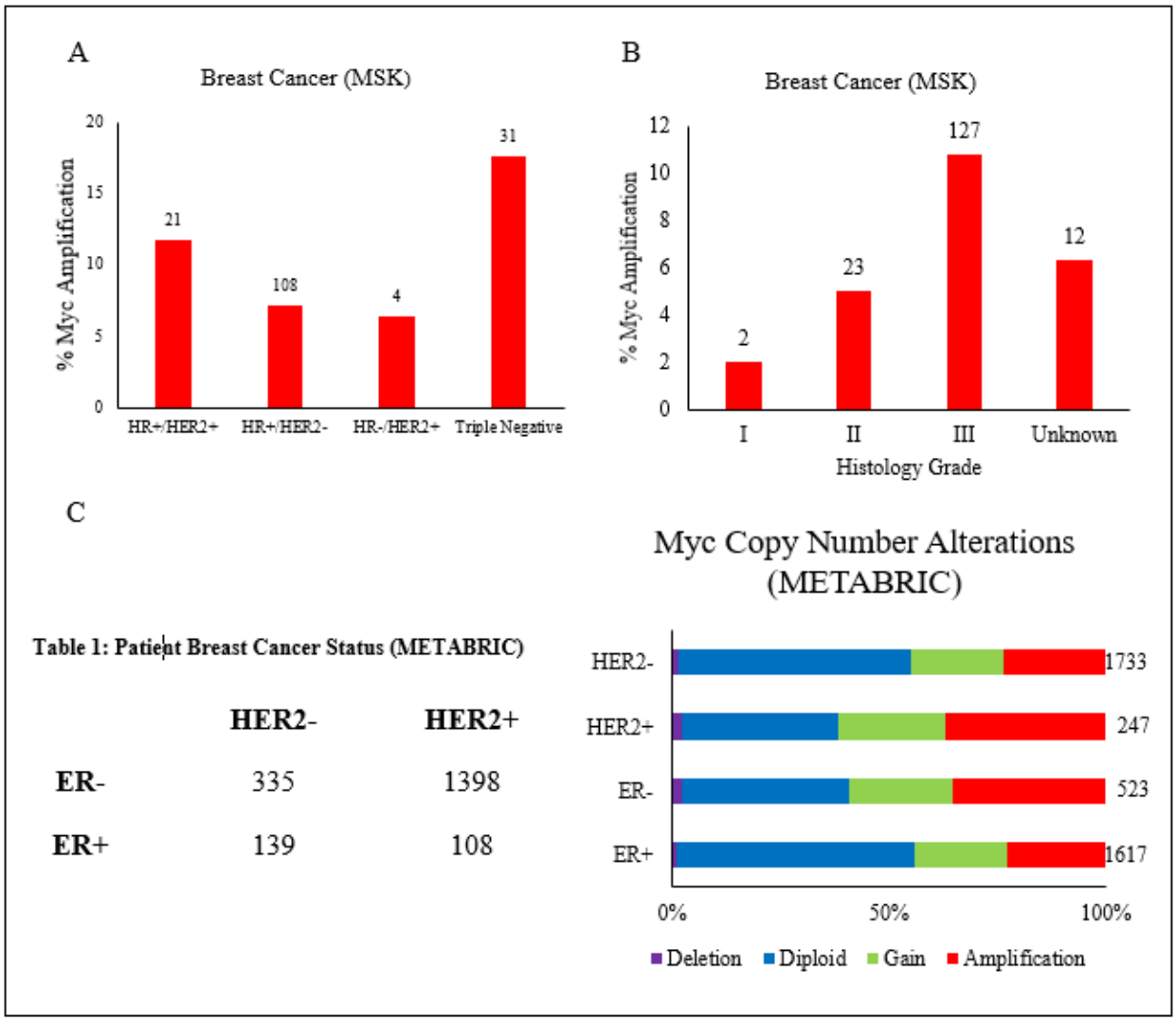

Figure 3. Increased breast cancer severity is related to Myc copy number alterations. (A) Triple negative breast cancer patients from the MSK study showed the largest relative amplification. (B) Myc amplification increased with cancer grade or severity. For example, histology grade III is associated with poor differentiation and prognosis and happens to exhibit the most amplification. (C) ER-/HER2+ breast cancer was the most common subtype in METABRIC followed by ER-/HER2-. In addition, greater relative amplification was observed in the ER- subtype compared to ER+. Taken together, Myc amplification may be a contributor to the aggressive ER-/HER2- and, therefore, triple negative breast cancer subtype (7, 21).

Lung Cancer

A study of 660 NSCLC lung adenocarcinoma and 484 squamous cell carcinoma patients revealed 9% and 8% Myc amplification, respectively. Overall, gain of function and amplification are more highly represented in these samples than deletions, while MAX deletions are more highly represented than gain of function and amplification (Figure 4A). Even more interestingly, Myc copy number gain or amplification and MAX deletions frequently co-occur suggesting that amplified Myc can render its effects on global transcription independently of its canonical binding partner MAX (Figure 4B) (4).

MAX also binds other bHLHZ proteins, some of which decrease Myc activity. For example, MGA is frequently altered and, in turn, inactivated in both NSCLC and SCLC which decreases its effect on Myc activity independently of Myc amplification. In other words, MGA inactivation and Myc amplification are mutually exclusive. Therefore, MGA deletions may be an alternative mechanism of Myc activation. 30 of the 68 MGA missense, nonsense and frameshift mutations in the NSCLC samples cause deletions. 20 of these mutations are predicted to be oncogenic, with the others uncharacterized (5).

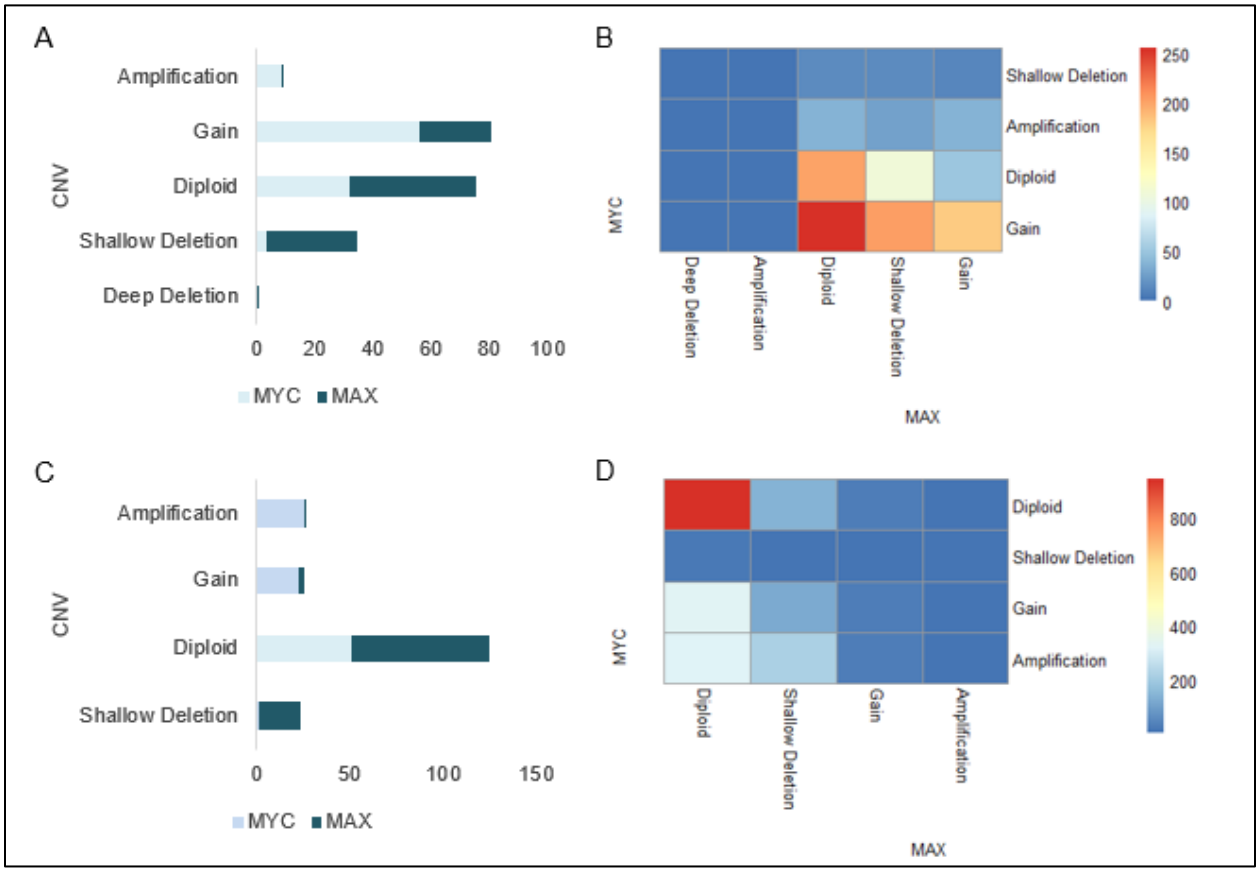

Figure 4. Copy number alterations in lung and breast cancer. (A) Distribution of Myc and Max copy number variations in a pan-lung cancer study (4). (B) Frequency of the co-occurrence of Myc and Max CNVs. (C) and (D) same as (A) and (B) but for breast cancer patient samples from METABRIC (8).

Interestingly, of 642 patients exhibiting gain of Myc function from this pan-lung cancer study, 51% also exhibit a deletion in the *MGA* gene. Of another 100 patients exhibiting Myc amplification, 59% harbor an *MGA* deletion. For both groups, shallow deletions are markedly greater in frequency. Shallow deletions co-occur in MGA and MAX for a total of 162 patients. For these reasons, MGA-dependent Myc activation may also co-occur with Myc amplification, especially when MAX activity decreases (5).

Liver Cancer

Increased Myc activity or expression and increased SIRT1 expression, together, promote liver tumor cell development and therefore decrease the survival of patients with hepatocellular carcinoma (14). Interestingly, Myc deregulation resulted in highly selective transcriptional activation meaning not all Myc-bound genes were deregulated. Conversely, only a fraction of deregulated genes exhibited direct dependence on Myc. Based on similar findings in lymphomas, Myc likely directs highly specific or maybe even tissue-specific transcriptional programs. However, global amplification is a well-established consequence of Myc deregulation; therefore, Myc may indirectly cause the deregulation of many other genes through highly specific direct activation (15).

Glioblastoma

Although not evident from the pan-cancer analyses, Myc may have a large role in gliomas as well. Less than 5% of glioma patients exhibit Myc amplification (7, 31). However, both medulloblastoma and glioma show elevated levels of Myc or Myc amplification (3). Development of high grade glioma induced largely by inactivating mutations to p53 and PTEN is in fact mediated by Myc. Knockdown of Myc restores differentiation of neural stem cells (32). A similar relationship was found in human primary GBM, as well. Additionally, activation of Myc by doxycycline decreases survival in mice treated with GBM-patient-derived neurospheres which can be attributed to more aberrant nuclei from poor differentiation (Figures 5A and 5B). Specifically, Myc inhibition reduces sumoylation and consequently prevents faulty mitosis. Therefore, myc activity or deregulation is likely a key driver of GBM development that may often operate independently of Myc amplification (31).

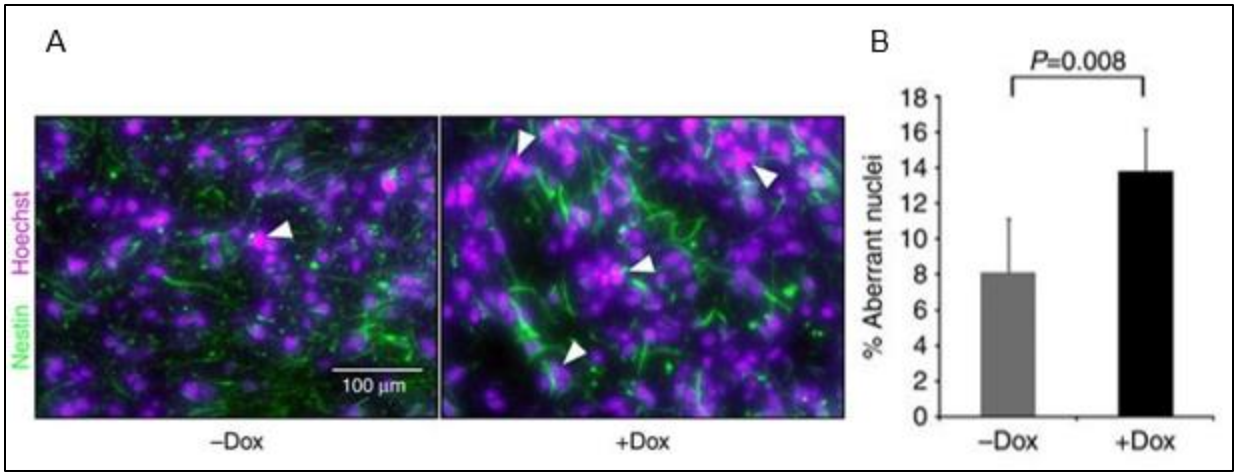

Figure 5. Myc increases growth in glioblastoma growth. (A) Myc (Dox-inducible Omomyc) expression increases the number of aberrant nuclei in patient-derived glioblastoma neurospheres . (B) Quantification reveals a higher frequency of aberrant nuclei upon induction of Myc (3).

Alternative Mechanisms of Myc Activation

Shallow deletions in *MAX* are frequently observed in cancer. Analysis of breast cancer samples from METABRIC suggests Max-independent Myc activity, particularly in the context of Myc amplification. (Figure 4C and 4D). 26% of MGA copy number alterations were deletions in this study, also suggesting MGA-dependent Myc activity, either in the presence or absence of Myc amplification (8). The researchers who first characterized MGA-dependent Myc activity in NSCLC and SCLC began with investigating the role of BRG1 in MAX-independent Myc activity. The known regulator of Myc was also found to act as a switch for MAX expression that drove Myc transcriptional programs in the absence of MAX (22).

Frequently, translocations also drive Myc deregulation. The t(8;14)(q24;q32) translocation was found in nearly 80% of Burkitt's lymphoma patients and activates Myc expression by placing the gene in control of an immunoglobulin (Ig) heavy chain enhancer (Figure 6A). Ig light chain enhancers can also deregulate Myc following translocations to their respective locations on chromosomes 2 and 22. Myc deregulation causes extremely rapid proliferation of B-cells and underlies BL's recognition as the most rapidly growing human tumor (18).

Alternatively, singe nucleotide polymorphisms (SNPs) can activate Myc enhancers. rs6983267 is one that increases the risk of developing either prostate or colorectal cancer. It renders its impact by increasing the binding of the transcription factor TCF4 which augments Myc expression (Figure 6B) (20, 27, 29). In addition, histone modifications, including methylation and acetylation, activate Myc enhancers in a potentially lineage-specific manner (Figure 6C) (1, 16). In both cases, the active enhancers may be lineage, tissue, or even patient-specific and in turn give rise to highly specific Myc expression or activity.

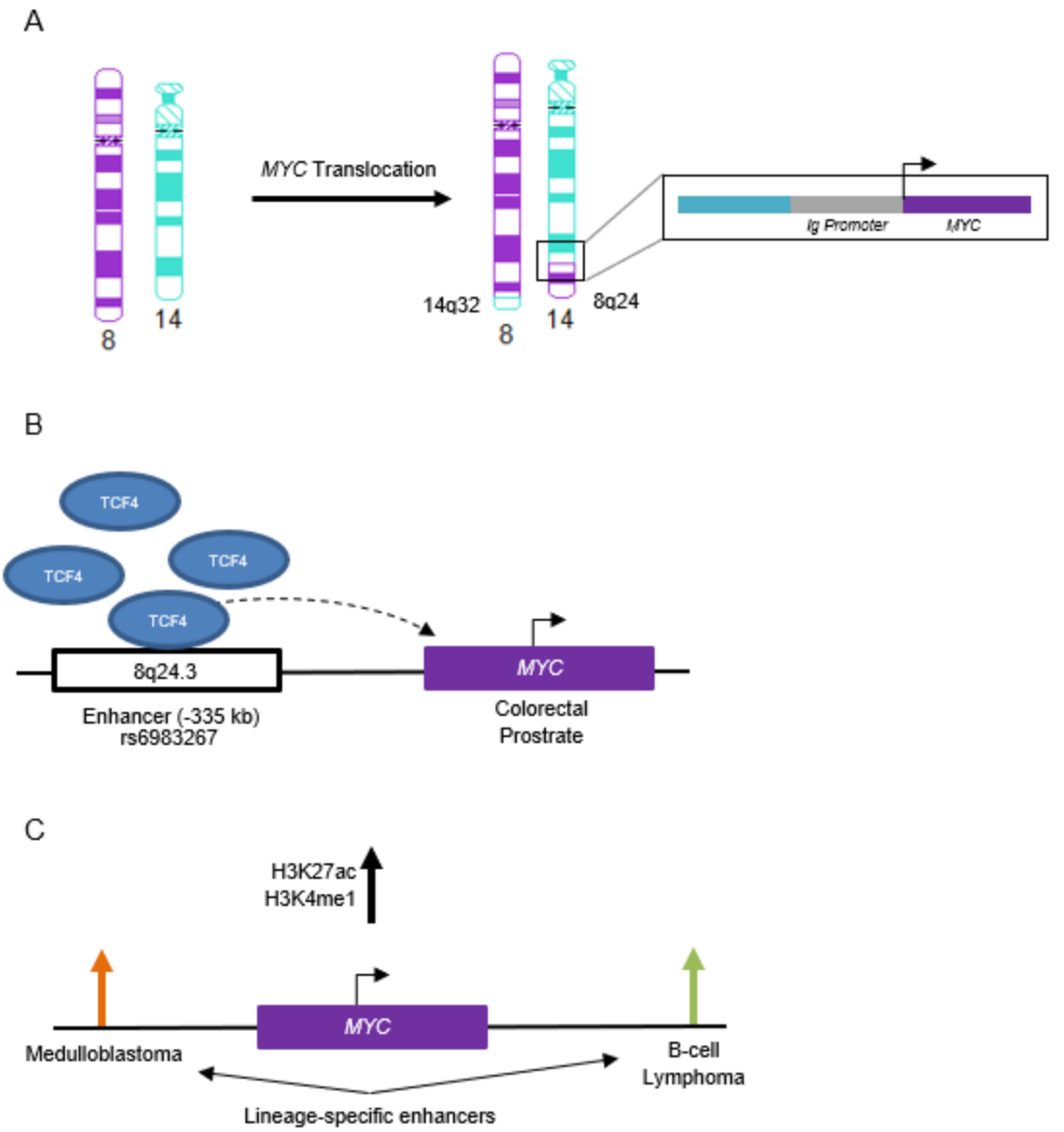

Figure 6. Alternative mechanisms of Myc activation confer lineage-specific changes. (A) Myc translocations place the gene under the control of different enhancers that may switch on Myc expression (18). (B) SNPS and (C) histone modifications activate enhancers and, in turn, Myc expression in its endogenous location. All three mechanisms of activation provide significant specificity (1, 16, 20, 27, 29).

High and low affinity enhancer boxes bind Myc

While c-Myc could be found binding to expected sites, E-boxes, overexpression of c-Myc in the cell saturated these binding sites and led Myc to bind at both variable and non-E-box motifs in a Burkitt's Lymphoma sample (Figure 7B) (17). Although Myc also began to bind long-range enhancers containing the variable E-boxes later in the time course, promoter Myc occupancy correlated more strongly with target gene expression (Figure 7C) (17, 19). Further analysis of Myc binding to various 6-mer motif probes revealed high-affinity, nonspecific binding of the canonical and variable E-boxes. The canonical E-box demonstrates the highest affinity to both

Myc:MAX and MAX:MAX which suggest high Myc affinity but low specificity. Many of the variable E-boxes also display low specificity. In contrary, the AACGTT non-E-box sequence had moderate affinity to Myc:MAX but low affinity to MAX:MAX indicating high Myc specificity (Figure 7D) (2).

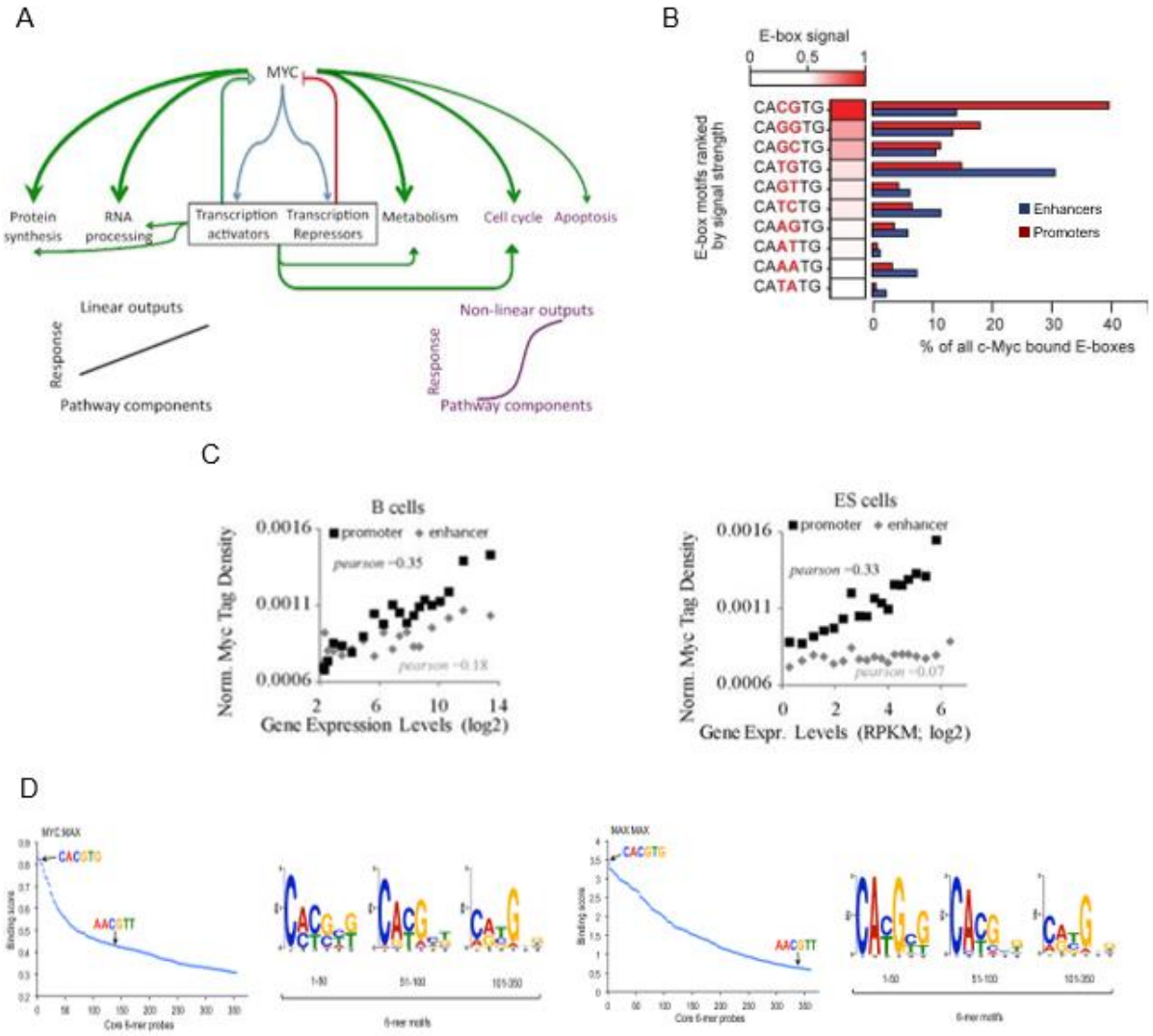

Figure 7. Pleiotropic Myc binds low affinity sites within promoters. (A) Myc activates a variety of transcriptional programs and drive both linear and exponential responses in the cell (19). (B) Canonical E-box and variable E-boxes is distributed between enhancer and promoter regions in B cells (17). (C) Analysis of correlation between Myc promoter or enhancer occupancy and Myc expression in B cells and embryonic stem cell suggests preference for promoter binding (19). (D) Binding affinity of the MYC:MAX and MAX:MAX complexes to a number of 6-mer probes and the motifs associated with different levels of affinity. High binding scores for Myc:MAX implies high Myc affinity, while high scores for MAX:MAX implies low Myc specificity (2).

In general, binding affinity decreased but specificity increased with increasing divergence from the canonical e-box (Figure 7D). Many high specificity, variable and non-E-boxes were also detectable *in vivo* in a Burkitt's lymphoma cell line and promoted the expected target amplification. *In vitro* binding of these non-canonical motifs in the absence of other confounding factors suggests sequence-based recognition and binding (2). For this reason, binding the low-affinity sites likely does not depend on Myc deregulation. However, unlike the canonical, high-affinity sites, they are likely not completely occupied at physiological levels.

Genes activated by low affinity motifs such AACGTT are part of different pathways than the genes activated by canonical motifs. Specifically, the canonical sequences activated genes associated with metabolic processes, whereas the low-affinity sequences activated genes associated with the DNA damage response. Further, increased Myc expression favored binding at the low affinity sequences (2).

Therefore, Myc achieves global deregulation in cancer through specific direct transcriptional activation that mirrors the Myc-activation-based transcriptional programs of normal cell development. The significant role of Myc in the development of cellular identity as well as in the diversity between and within cancer types implies that it influences multiple transcriptional programs in a specific manner. For this reason, Myc binding exclusively to E-boxes would likely be restrictive and potentially evolutionarily disadvantageous for normal and cancer cell development. Ultimately, low-affinity but highly specific Myc binding may be central to the pleiotropic properties of Myc in general and, more so, in the context of overexpression. To support this notion of specific, especially lineage-specific, Myc binding sites have been shown to be significantly non-conserved at promoters in 11 cancer cell types with corresponding differential expression (9).

However, the sequence alone cannot determine occupancy. For example, WDR5 is DNA-bound and part of the H3K4 methyltransferase complex. WDR5 interacts with Myc independently of Myc's binding to DNA. Therefore, this additional interaction also affects Myc binding to DNA, even more so than the highest-affinity, canonical E-box (24). Myc has many known interactors; therefore, protein-protein interactions such as the one between WDR5 and Myc are equally if not more important for promoter binding. Because other proteins bind the same sequences, protein-protein interactions are likely key contributors to Myc binding. Likewise, since Myc can bind to a wide variety of sequences, protein-protein interactions may be essential to foster any true specificity with gene activation. To maintain its effects, Myc must remain bound which may also be indicative of other essential interactions.

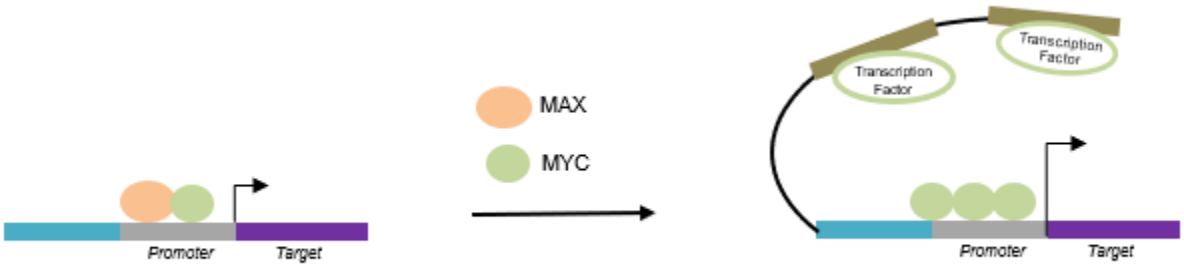

Figure 8. Transcription factors bind enhancers and guide Myc to low-affinity sites.

Taken together, it is possible that transcription factors bind long range-enhancers and, in turn, give rise to protein-protein interactions that directly guide Myc binding (Figure 8). Like WDR5, the enhancer-bound transcription factors may facilitate MAX-independent binding, especially considering the co-occurrence of shallow *MAX* deletions and *Myc* amplification. These enhancers may be rendered active by SNPs and histone modifications, the same mechanisms that drive Myc deregulation (Figure 6B and 6C) (2, 24).

Conclusion

Myc regulates the cell cycle, cell growth, proliferation, and differentiation as well as metabolism and even apoptosis. Myc overactivity causes deregulation of these processes in many types of cancer through a variety of mechanisms and underlies disease development. Interestingly, Myc binds many low-affinity sites to direct tissue or lineage-specific transcriptional programs. Considering the context-specific target activation, elucidating the mechanisms that underly these networks, specifically how Myc successfully occupies low-affinity binding sites, may reveal novel, cancer-specific therapeutic strategies.

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