The Expanding World of N-MYC-Driven Tumors

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ABSTRACT

Enhanced and deregulated expression of N-MYC, a member of the MYC family of transcription factors, drives the development of multiple tumors, including tumors of the nervous and hematologic systems and neuroendocrine tumors in other organs. This review summarizes the cell-of-origin, biological features, associated signaling pathways, and current treatment strategies for N-MYC-driven tumors. We also highlight biological differences within specific tumor types that are driven by the different MYC proteins.

Significance: N-MYC is a driver of multiple tumor types that are derived through a mechanism that involves direct differentiation within the same lineage (e.g., in the case of neuroblastoma, medulloblastoma, and acute myeloid leukemia) and is often associated with a poor prognosis. Emerging data suggest that N-MYC also drives other tumor types through a mechanism that promotes a lineage switch and that this switch may be exploited for therapeutic purposes. Cancer Discov; 8(2); 1–14. ©2018 AACR.

INTRODUCTION

The human MYC gene (encoding the C-MYC protein) was first identified as the cellular homolog of an avian retroviral transforming gene, ν-myc (1). It is the founding member of a small gene family, including two closely related genes, MYCN (encoding N-MYC) and MYCL (encoding L-MYC), identified in neuroblastoma and in small cell lung cancer (SCLC), respectively (2–4). All three genes encode nuclear proteins of slightly different sizes: 439 amino acids (C-MYC), 464 amino acids (N-MYC), and 364 amino acids (L-MYC). The sequence homology among MYC proteins is highest in five relatively short stretches of amino acids (up to 95% amino acid homology; refs. 5–8), which are termed MYCBoxes (numbered I, II, IIIa, IIIb, and IV), and in a carboxy-terminal basic-helix-loop-helix-leucine zipper (bHLH-LZ) domain, that comprises approximately 100 amino acids and enables MYC proteins to form heterodimers with another bHLH-LZ protein termed MAX. MYC/MAX heterodimers bind DNA in a sequence-specific manner (9). Each of the MYCBoxes I–IV interacts with different effector proteins (see below). MYC proteins bind very broadly to active promoters and enhancers and can alter transcription mediated by all three RNA polymerases. Notably, MYC proteins activate and repress transcription of a large number of genes transcribed by RNA polymerase II (RNAPII) that encode proteins involved in fundamental cellular processes, including proliferation, growth, ribosome biogenesis, metabolism, apoptosis, and differentiation (10).

Early studies assessing steady-state mRNA levels in the developing mouse showed that the different Myc family genes have distinct expression patterns that differ in terms of developmental stage and tissue specificity. Both murine Mycn and Myel mRNAs are expressed at high levels during early stages of development, and their expression is restricted to specific tissues. In contrast, murine Myc is expressed throughout the mouse and at each of the developmental stages analyzed (fetal day 15 through postnatal day 21; ref. 11). Early studies also showed that Myc and Mycn (but not Myel; ref. 12) are required for normal development at the onset of organogenesis, as targeted deletions in mice showed that the elimination of either gene was embryonic lethal at midgestation (13–16). Although it was shown that the level of MYC proteins is essential in maintaining development (17, 18), data from epiblast-restricted deletion of Myc showed that the early lethality attributed to the loss of Myc in embryonic cells is instead due to placental insufficiency (19). Tissue-specific conditional deletions demonstrated that C-MYC is necessary for the development and growth of specific hematopoietic cell lineages, crypt progenitor cells in the intestine (20), skin keratinocytes, and other cells where it is expressed. N-MYC and C-MYC are needed for maintaining hematopoietic stem cells, in which both proteins are expressed (19, 21, 22).
addition, N-MYC, but not C-MYC, is essential for the rapid proliferation of progenitor cells during the development of the central nervous system (23). Collectively, these data suggest that the differences in phenotypes observed in knockout animals are mostly due to different expression patterns rather than to differences in protein function. This hypothesis is supported by the observation that a knock-in allele in which endogenous Myc coding sequences have been replaced with Mycn coding sequences rescues growth and development (24).

**N-MYC AND TUMOR BIOLOGY**

Deregulated expression of MYC family members is associated with the development of a large fraction, and possibly the majority, of all human tumors (10). N-MYC is overexpressed both in tumors of the nervous system (e.g., neuroblastoma, medulloblastoma, refs. 25–27; retinoblastoma, ref. 28; astrocytoma, ref. 29; and glioblastoma multiforme, refs. 30–34) and in nonneuronal tumors (e.g., castration-resistant prostate cancer (CRPC) and neuroendocrine prostate cancer (NEPC), ref. 35; hematologic malignancies, refs. 36, 37; rhadomyosarcoma, refs. 38, 39; Wilms tumors, ref. 40; SCLC, ref. 41; and pancreatic tumors, ref. 42). In many cases, N-MYC- or C-MYC–driven tumors appear to arise from cell lineages that express N-MYC or C-MYC during normal development. But the role of N-MYC in tumor biology, in some instances, differs from the role of C-MYC within a specific lineage. For example, in the hematopoietic system, N-MYC is expressed in self-renewing, quiescent stem cells, but this expression switches to C-MYC upon differentiation to transit-amplifying progenitors (43). This suggests that N-MYC plays a role in the activation of stem cell–like properties (e.g., self-renewal). Consistently, enforced expression of N-MYC, but not C-MYC, in mouse bone marrow cells stimulates the proliferation and self-renewal of myeloid cells in vitro and causes rapid development of acute myeloid leukemia (AML) in vivo (Fig. 1; ref. 44). Furthermore, in some solid tumors (e.g., prostate), enhanced N-MYC expression drives a neuroendocrine tumor type that differs from C-MYC–driven tumors in histology and response to androgen receptor (AR) signaling–directed therapies (45–48). In the case of prostate cancer, evidence suggests that N-MYC may play a role in lineage switching from an epithelial origin to a more neuroendocrine one. A shift in expression among MYC proteins during tumor progression can be associated with a shift in cell lineage, tumor progression, and treatment resistance (e.g., in prostate cancer; refs. 35, 49–51).

**Neuroblastoma**

Neuroblastoma is an embryonal tumor that originates from sympatho-adrenergic progenitor cells of the neural crest. It is the most common extracranial solid tumor in childhood and accounts for 15% of all childhood cancer deaths (52–54). The clinical course of neuroblastomas is heterogeneous. Although low-risk neuroblastomas can regress spontaneously or differentiate into benign ganglioneuroma, 50% of high-risk neuroblastomas show an almost-always fatal relapse despite multimodal therapy. Amplification of the MYCN oncogene is present in about 50% of high-risk neuroblastomas and is an adverse prognostic factor (54–56). In addition to MYCN amplification, a somatic variant of MYCN that encodes a proline 44 to leucine (P44L) mutation is present in 1.7% of cases without MYCN amplification (57). The P44L N-MYC variant has also been identified in glioma (58), medulloblastoma (59), Wilms tumor (47), neoplastic cysts of the pancreas (60), and other tumor types (61). The P44L mutant N-MYC protein remains to be biochemically characterized.

Both clinical observations and modeling data show that MYCN amplification is an initiating event that drives the development of high-risk neuroblastomas. In clinical specimens, MYCN amplification is most often present at diagnosis and is seldom subclonal or acquired during progression, and relapsed, low-risk non–MYCN-amplified neuroblastomas never progress to high-risk neuroblastomas (4, 54, 55, 62, 63). Furthermore, transgenic expression of MYCN in migrating neuroectodermal cells of the neural crest using a rat tyrosine hydroxylase (TH) promoter results in the development of neuroblastoma in mice (Fig. 1; ref. 64). More recently, a genetically engineered mouse model (GEMM) designed to inducibly overexpress MYCN in different regions of the sympathetic chain ganglia was shown to develop clinically relevant neuroblastomas (65). A further recent study overexpressed MYCN in wild-type, primary neural crest cells derived from an embryonic neurutube explant (66). Subcutaneous introduction of these transduced cells back into mice led to phenotypically and molecularly accurate tumors that closely model human MYCN-amplified neuroblastoma (66). Ectopic expression of MYCN in the neural crest of zebrafish also induces neuroblastoma, demonstrating that the potential of MYCN to induce neuroblastoma is conserved among species (67). Altogether, these studies unequivocally establish that enhanced expression of N-MYC is a driver of neuroblastoma.

Non–MYCN-amplified neuroblastoma often expresses C-MYC, and the expression of C-MYC and N-MYC appears to be mutually exclusive (68). Tumors with amplified MYCN have a distinct gene expression profile, which comprises multiple genes involved in the development of neuroblastoma and is itself predictive of poor survival, arguing that altering gene expression is a major oncogenic function of N-MYC (68). For example, N-MYC binds to and activates the TERT promoter, resulting in high levels of TERT, the catalytic subunit of telomerase, in MYCN-amplified tumors (69). Intriguingly, chromosomal rearrangements that induce TERT expression and ATRX mutations that activate the alternative lengthening of telomeres pathway are also common in high-risk neuroblastomas (57, 70, 71). Moreover, MYCN amplification, TERT rearrangement, and ATRX mutations are mutually exclusive in neuroblastoma, suggesting that N-MYC-dependent enhancement of TERT expression is a critical function of amplified MYCN in neuroblastoma (57).

New evidence suggests that the MYCN mRNA may also have an oncogenic role in neuroblastoma that is independent of N-MYC protein as a competing endogenous RNA (ceRNA). This notion emerges from studies of the miRNA let-7, which targets MYCN mRNA for degradation (72). Expression of LIN28B, an RNA-binding protein and inhibitor of let-7 (73), maintains high levels of MYCN mRNA in MYCN-amplified neuroblastoma cells. Transgenic LIN28B expression in the mouse sympathetic adrenergic lineage using the Dhh promoter (65, 74) induces the development of neuroblastomas that are marked by low let-7 miRNA levels and high N-MYC protein expression (75). Intriguingly, MYCN-amplified cells...
maintain high MYCN mRNA levels despite robust derepression of let-7 upon LIN28B loss due to sponging of let-7 by MYCN mRNA (72). Enforced expression of LIN28B also induces tumors in mouse models of liver tumors (76), colon cancer (77), and Wilms tumor (78).

**Retinoblastoma**

MYCN overexpression also drives tumorigenesis of retinoblastoma, a rare embryonic neoplasm of retinal origin, in the context of retinoblastoma 1 (RBI) loss (28, 79, 80). Two models of retinoblastoma development that differ in the cell of origin have been proposed (for a review, see ref. 81). In both models, RBI loss leads to ectopic division of progenitor cells, and these proliferating cells acquire other molecular lesions (e.g., epigenetic changes) that result in a transformed cell that drives tumor growth. In early mouse models, retinoblastomas occurred in mice in which Rb loss was coupled with the loss of another retinoblastoma family member (e.g., Rbl, refs. 82, 83; Rbl2, ref. 84; or the CDK inhibitor Cdkn1b, ref. 85). However, alterations in Rbl, Rbl2, or Cdkn1b do not occur in human retinoblastoma. Most recently, a GEMM engineered to express an inducible human MYCN gene in the context of Rb/Rbl loss specifically in retinal progenitor cells showed that MYCN overexpression accelerates formation of retinoblastoma (86). However, although MYCN was required for initial tumor emergence, tumors evolved to a stage that was MYCN...
independent, limiting the potential of targeting N-MYC therapeutically for this subgroup of retinoblastoma.

**N-MYC-Driven Tumors of the Central Nervous System**

Medulloblastoma is the most frequent malignant childhood brain tumor, with a 5-year survival rate for children with average-risk disease of approximately 70% to 80%. For children with high-risk disease, the 5-year survival rate is about 60% to 65% (25–27). Medulloblastoma arises from stem cells or granule neuron progenitor cells (GNP) of the cerebellum. Large-scale genomic and gene expression analyses have shown that medulloblastomas fall into at least 4 distinct subgroups with different clinical and biological features (87–89). The first two subgroups are defined by activation of the WNT and sonic hedgehog (SHH) pathway, respectively. In contrast, subgroups 3 and 4 are not defined by the activation of a single pathway. MYC is amplified and overexpressed in subgroup 3 medulloblastomas, and MYCN is overexpressed and/or amplified in SHH medulloblastomas and a fraction of group 4 medulloblastomas. Although MYCN is a known downstream target of the SHH pathway (via GLI family transcription factors; ref. 90), mechanisms leading to MYCN deregulation in group 4 medulloblastomas are less clear. Genetic activation of the SHH pathway induces medulloblastomas in mice, and one study concluded that deletion of MYCN suppresses tumor formation (90). However, in that model, deletion of MYCN led to mice without a cerebellum, so one cannot conclude solely based on this study that N-MYC is an essential downstream effector of the SHH pathway in medulloblastoma. Deregression of endogenous MYCN by knockout of miR-34a accelerated formation of SHH-driven medulloblastoma (91). Furthermore, inducible expression of MYCN in the cerebellum using the glutamate transporter promoter led to the development of metastatic medulloblastoma and large-cell anaplastic tumors that were independent of SHH signaling (92). Intriguingly, the different subtypes of medulloblastoma appear to be at least in part due to functional differences between N-MYC and C-MYC proteins. Specifically, when GPNs from cerebella of Tprp53-null and Cdcan2c-null mice were transfed with MYC or MYCN ex vivo (93–95), reimplantation of MYCN-transfected GPNs gave rise to SHH-type medulloblastomas, whereas reimplantation of MYC-transfected GPNs resulted in group 3 medulloblastomas. The different biological properties of N-MYC and C-MYC in medulloblastoma are due to a difference in interaction with MIZ1. C-MYC (in contrast to N-MYC) interacts with MIZ1 to suppress genes involved in cell growth, differentiation, and survival of cerebellar neuronal cells, such as the transcription factors ATOH1 and FOXG1 (94).

MYCN is also amplified or overexpressed in a fraction of malignant glioma, which typically localizes to the forebrain (cerebrum) rather than hindbrain (cerebellum; refs. 31–34). To understand whether the difference between both sites of tumor formation is due to a difference in the cell of origin, an allele of MYCN that expresses a more stable protein (N-MYC T58A) was introduced into neural stem cells derived from forebrain, cerebellum, or brain-stem tissues in vitro (34). Upon retransplantation, medulloblastomas/primitive neuroectodermal tumors arose from N-MYC–expressing stem cells from the cerebellum and brain stem, whereas diffuse gliomas arose from N-MYC–expressing neural stem cells derived from forebrain (Fig. 1; ref. 34). These data showed that N-MYC-transduced neural stem cells generate different types of brain tumors depending upon the regional origin of the perinatal neural stem cells. Molecular and clinical data suggest that specific mutations in the H3F3A gene, which encodes histone H3.3, also dictate the type and clinical course of pediatric glioblastomas (30). MYCN amplification and overexpression is associated with H3F3A wild-type glioblastomas. In non-MYCN-amplified tumors, the presence of a specific H3F3A mutation is associated with MYCN transcription induction. In the cerebral hemispheres, a glycine 34 to arginine mutation in H3F3A (H3F3A G34R) results in the induction of MYCN transcription (30, 96). In contrast, other H3F3A-mutant glioblastomas (e.g., K27M) are restricted to thepons and thalamus and are not associated with MYCN induction or amplification (96). H3F3A K27M–mutant tumors arise in younger children and have a worse clinical outcome compared with H3F3A G34R tumors (peak age, 14 years) and H3F3A wild-type tumors (30). These data suggest that there are most likely distinct developmental origins of these tumor subgroups.

**Hematologic Malignancies**

Amplification of MYCN is frequently found in hematologic malignancies such as lymphoma and AML (36, 97), and the MYCN locus is a common target of retroviral integration in mouse T-cell lymphoma (37). Hematopoietic stem cells (HSC) are dormant in the bone marrow but can be activated in response to diverse stresses to replenish all blood cell types. Although it has been shown that N-MYC is not essential for steady-state hematopoiesis (98), data from combined deficiency of C-MYC and N-MYC in adult bone marrow cells have shown that C-MYC and N-MYC are both required for HSC proliferation, metabolic growth, differentiation, long-term self-renewal activity, and survival of stem cells (98, 99). This suggests that both MYC proteins are coexpressed in the most immature populations of HSCs. Most recently, data from an elegant study involving fluorescent fusion alleles of Mycn and Myc in mouse models showed that N-MYC is expressed in the most primitive stem and progenitor compartments and is mutually exclusive to C-MYC expression (43). This study further showed that N-MYC is expressed in self-renewing, quiescent stem cells that switch to higher C-MYC expression in transit-amplifying progenitors that further differentiate. Data from this study suggest that N-MYC’s role in HSCs appears to be in the activation of stem-like properties and is dependent on the ubiquitin ligase HUWE1 (43). Consistent with this idea, transduction of mouse bone marrow cells with N-MYC but not C-MYC stimulated the proliferation and self-renewal of myeloid cells in vitro and rapidly caused AML in vivo (44). Therefore, in hematopoietic malignancies, MYCN amplification and/or overexpression is unequivocally a “driver” mutation that results in tumor formation and depends on the cell of origin, in contrast to other tumors (e.g., prostate, lung) in which N-MYC overexpression arises late during tumor evolution.

**Prostate Tumors**

Data from several solid tumors, such as lung (100–106), pancreas (42, 107, 108), and prostate (35, 46–50, 109–111),
suggest that C-MYC- and N-MYC-driven tumors differ in terms of tumor biology and capacity for cell lineage switching.

Prostate cancer depends on AR signaling, which is why AR-targeted therapy is given to men whose disease is progressing (112). Transformation of prostate cancer toward independence from androgen signaling (i.e., CRPC) has emerged as a resistance mechanism in a subset of metastatic prostate cancer following exposure to AR-targeted therapies, such as abiraterone or enzalutamide. Recent evidence shows that NEPC can arise in later stages of prostate cancer progression from a preexisting adenocarcinoma during the development of treatment resistance to AR-directed therapies (51). NEPC is characterized by low to no AR signaling, loss of TP53 and RB1, and high levels of neuroendocrine markers such as synaptophysin and chromogranin A (35, 49). Both N-MYC and C-MYC are activated and drive the development and progression of prostate cancer, but the resulting tumors are different in terms of histology and dependence on AR signaling, arguing that C-MYC and N-MYC have different biological functions in the context of prostate cancer. MYC overexpression is an early event that occurs in lesions of high-grade prostatic intraepithelial neoplasia and localized prostatic adenocarcinomas, suggesting that C-MYC contributes to the initiation and progression of the disease (50). MYCN, on the other hand, is amplified and overexpressed in late-stage prostate cancer (35, 46, 49, 111) and, based on data from human and mouse model systems, can drive the progression from CRPC toward poorly differentiated NEPC (Fig. 1; refs. 35, 46, 48, 49, 111). N-MYC is overexpressed in the majority of NEPC cases, but there is a spectrum of N-MYC expression in CRPC samples, with 20% of CRPC tumors demonstrating transcript levels in the range of NEPC (46, 49). CRPC tumors with high N-MYC levels also display features of NEPC (e.g., low AR signaling and expression of chromogranin A; refs. 46, 49).

NEPC has a transcriptional signature that is distinct from prostate adenocarcinoma (35, 49). Ectopic MYCN induction in human prostate adenocarcinoma (LNCaP) cells that have high intrinsic C-MYC expression resulted in a dramatic downregulation of MYC mRNA and a transformation to a NEPC-like phenotype (46). The NEPC transcriptional program includes an abrogation of AR signaling, enhanced AKT and epithelial–mesenchymal transition signaling, increased EZH2 levels, and activity and expression of stem cell–related gene signatures and neuroendocrine markers. Consistently, ectopic expression of N-MYC and activated AKT in benign human prostate basal cells results in metastatic castration-resistant tumors with both NEPC and adenocarcinoma foci within the same lesion (48). Data from that study further showed that both histologic subtypes derive from a common epithelial precursor and that N-MYC expression is essential for the propagation of tumor cells enriched for the NEPC component in castrated mice (48).

Conversely, a GEMM engineered to express high C-MYC levels in mouse prostate epithelial cells was shown to develop invasive prostate adenocarcinomas (47), but, unlike N-MYC mouse models, did not exhibit the morphologic, immunohistochemical, or gene expression features of NEPC (47, 109, 110). Molecular assessment of the C-MYC signature in mouse models showed a resemblance to clinical MYC-amplified adenocarcinoma (47). Differences between C-MYC and N-MYC in prostate cancer biology also extend to coregulators such as EZH2. Like N-MYC, C-MYC overexpression has been associated with increased EZH2 activity; however, the evidence to date points to different roles of EZH2 in C-MYC–driven relative to N-MYC–driven prostate cancer. In CRPC and in the absence of N-MYC, EZH2 enhances AR signaling by acting as an AR coactivator (113). In contrast, N-MYC directly interacts with EZH2 to silence AR signaling in NEPC models (46).

Altogether, the mouse and human modeling data show that N-MYC induction results in a shift of cell lineage state (epithelial to neuroendocrine), suggesting that N-MYC drives lineage plasticity in prostate cancer progression. Consistent with this notion, neuroendocrine prostate tumors can coexist with other histologies, such as squamous cell carcinoma or adenocarcinoma (114, 115), suggesting divergent differentiation from a single precursor cell-of-origin. In some cases, these mixed tumors evolve with time toward a predominantly poorly differentiated neuroendocrine phenotype during the course of disease progression and treatment resistance (e.g., AR-directed therapies) with loss of epithelial cell identity and acquisition of an alternative lineage phenotype (49, 116, 117). This plasticity model is supported by the retention of shared common genomic alterations with their preexisting adenocarcinomas (i.e., TPMPRSS2–ERG gene rearrangement and TP53 mutations in prostate cancer; refs. 49, 118, 119). The induction of lineage plasticity by N-MYC is also consistent with data from prostate epithelial cells engineered with a loss of RB1 or TP53 (120–122). A lineage switch driven by N-MYC induction appears to afford the tumor cells the ability to escape cell death induced by anti-AR therapy. As suggested in ref. 120, such lineage plasticity may also facilitate the adaptation of tumor cells to changing environments during metastasis.

Lung Tumors

Other tumor entities (lung and pancreatic) suggest differences between C-MYC and N-MYC but, to date, there is not enough data to convincingly show that they drive different biologies. Twenty percent of neuroendocrine SCLCs harbor genomic amplification of MYCN, MYC, and MYCL, and 9% have a recurrent fusion involving MYCL1 (100, 103–106, 123, 124). In vivo overexpression studies using mouse models, including a recent study by MacPherson and colleagues, demonstrated that L-MYC can promote SCLC (100, 101). Molecular analyses of these models showed that L-MYC activates eIF2 and mTOR signaling pathways, which are both involved in ribosomal biogenesis and protein production and which is consistent with a comparison to N-MYC target genes (101). Although there are no studies that have addressed the ability of N-MYC to act as a driver of SCLC, recent data from a GEMM study suggests that, as in prostate cancer, C-MYC overexpression does not drive a neuroendocrine phenotype in lung cancer (125). In this study, mice were engineered to overexpress C-MYC in the context of Rb1/Trp53 double knockout (RPM) in lung neuroendocrine cells (using the neuroendocrine calcitonin gene-related peptide promoter). As a comparison, similar mice engineered with Rb1/Trp53/Pten triple knockout (RPP) developed classic SCLC. Interestingly, tumors from RPM mice recapitulate
a molecular program consistent with C-MYC–high human SCLC and harbored two populations of cells with distinct morphologies, one that resembled typical “classic” SCLC with small cells and a second characterized by larger cells that are similar to a variant of SCLC in which individual tumors consist of one of these cell types or a mixture of both. Data from this study suggest that SCLC cells maintain a level of lineage plasticity and that C-MYC expression promotes a neuroendocrine-low phenotype based on specific marker expression (e.g., high levels of NEUROD1 and low levels of the neuroendocrine associated master regulator ASCL1 and other neuroendocrine markers).

**Pancreatic Tumors**

The role of MYC proteins in pancreatic cancer is largely unknown, although C-MYC has been suggested to act as an oncogene in a fraction of acinar cell carcinomas (107) and ductal adenocarcinomas (108). A recent study addressed this question through the generation of a GEMM engineered to overexpress MYCN in neural progenitor cells using the human GFAP promoter to drive MYCN expression (42). GFAP is expressed in the developing nervous system as well as in neuroendocrine cells in adulthood. In this system, MYCN induction led to malignancies in the abdomen or head with an incidence of 59%. Abdominal tumors were found in the pancreas, whereas the tumors found in the head were in the pituitary gland. The pancreatic tumors appeared to arise from the islet cells, were found to produce chromogranin A and glucagon, and expressed a molecular program that resembles a program associated with murine models of N-MYC–driven neuroblastoma (42, 65).

**N-MYC–DEPENDENT TRANSCRIPTION**

As described above, N-MYC is a bHLH-LZ protein that binds DNA in a sequence-specific manner together with a heterodimeric partner protein, MAX (Fig. 2; ref. 126). In vitro, the complex recognizes E-box sequences with a core CACA/GTG sequence (“consensus E-box”). Chromatin immunoprecipitation sequencing studies show that N-MYC binds to thousands of active promoters transcribed by RNAPII and to enhancers with an open chromatin structure in embryonic stem cells and in N-MYC–driven neuroblastoma, medulloblastoma, and AML cells (43, 94). This pattern is essentially identical to that of C-MYC. C-MYC is also present at promoters of genes transcribed by RNA polymerases I and III (127), but no studies have been published yet that directly tested for the presence of N-MYC at these promoters.

It is likely that less than half of N-MYC binding sites on chromatin have consensus E-boxes, and many do not even show variant E-boxes. This has been formally been shown for C-MYC in two studies (128, 129). Both studies conclude that the presence of an E-box enhances chromatin association, but is not a prerequisite, and E-boxes are present at less than 50% of all sites. Most likely, therefore, protein–protein interactions play a large role in targeting N-MYC to sites on chromatin. This notion is supported by the observation that the ability of MYC proteins to discriminate between specific and nonspecific...
DNA-binding sequences is lower than observed for other transcription factors (128). Intriguingly, one of the conserved MYCBox (IIIb) interacts with WDR5, a component of a histone methylase complex that marks active promoters, and the interaction enables MYC to preferentially bind to E-boxes that are close to active promoters (130). N-MYC/MAX complexes activate transcription of many of their target genes. As discussed above, high levels of N-MYC expression in MYCN-amplified neuroblastoma, N-MYC-driven medulloblastoma, and NEPC are associated with a specific gene expression profile. These tumor-specific expression profiles comprise only a subset of the genes with N-MYC bound promoters, and it is not completely obvious why this is the case. One model suggests that promoters differ in affinity for MYC proteins such that high-affinity promoters are bound in normal proliferating cells and low-affinity promoters are “invaded” in response to high concentrations of C-MYC or N-MYC found in tumors (129, 131). In this model, tumor-specific gene expression profiles arise as a function of MYC protein concentration, but differences in gene expression among tumors driven by C-MYC and N-MYC are not explained.

One interpretation of these findings is that MYC proteins do not instruct cells to adopt a new gene expression profile, but rather modulate a gene expression pattern that is predefined by the epigenetic landscape. Genes that differ in affinity for C-MYC encode functionally distinct proteins (129). Although genes with high-affinity promoters encode proteins involved in normal cell growth (e.g., proteins that control ribosome biogenesis and protein translation), genes with low-affinity promoters encode proteins involved in cell adhesion, nutrient transport, and responses to hypoxia, which have been linked to tumor-specific functions of MYC. The observations suggest a model in which increases in MYC levels are oncogenic, as they alter expression of low-affinity genes and thereby establish tumor cell-specific physiologic properties. As an alternative model, we speculate that MYC proteins may carry out effector functions at most or even all active promoters and enhancers that are not shared with other transcription factors. More data are needed to properly validate this and to determine whether this extends beyond C-MYC. Importantly, although MYC acts at multiple stages of transcription on promoters transcribed by RNAPII, a hallmark of its activity is the global release of RNAPII from a promoter-proximal pausing site into productive elongation (132, 133). Whether N-MYC has a similar role in promoting pause release by RNAPII and how the known protein–protein interactions of MYC family proteins mediate their global effects on RNAPII are not well understood. Pause release of RNAPII requires phosphorylation of serine 2 in the carboxy-terminal repeat domain by the CDK9 kinase, which is part of the p-TEFB (positive transcription elongation factor B), and MYC proteins may directly recruit p-TEFB to core promoters. Alternatively, recruitment may be more indirect; for example, one of the best validated effector functions of C-MYC is the interaction of MYCBoxI with the TRRAP protein, a scaffold of the NuA4 histone acetylase complex, which has multiple roles in gene expression and in maintaining genome stability (134). C-MYC–induced histone acetylation in turn can recruit p-TEFB via the BRD4 protein (135). Intriguingly, NuA4 has recently been implicated in elongation by RNAPII (136), suggesting that recruitment of NuA4 to core promoters may contribute to MYC-dependent pause release.

N-MYC can also repress transcription of target genes, and N-MYC–dependent repression contributes significantly to the characteristic expression profiles of N-MYC–driven tumors. Interestingly, C-MYC and N-MYC appear to have evolved different mechanisms of repression. C-MYC–dependent transcriptional repression depends on association with a partner protein, MIZ1 (133, 137, 138), and is largely abolished by a point mutant that is unable to bind to MIZ1. As MIZ1 associates with TOPBP1, a protein best known for its role in signaling at stalling replication forks, association of MIZ1 with C-MYC is likely to restrict C-MYC–dependent pause release in the vicinity of stalled replication forks (139). In contrast, N-MYC associates only weakly with MIZ1, and whether an N-MYC/MIZ1 complex has relevant readouts remains unclear. As a result, the difference in expression profiles between C-MYC– and N-MYC–driven medulloblastoma is due to the more efficient complex formation of C-MYC with MIZ1 (94). In addition, N-MYC associates with the EZH2 subunit of the PRC2 histone methylase complex to repress transcription of some of its target genes, and this mechanism may be unique to N-MYC. As mentioned above, one striking example is prostate carcinoma, in which C-MYC enhances expression by AR and promotes androgen-dependent tumor growth (46). In contrast, N-MYC recruits EZH2 to joint target sites with AR and represses androgen-dependent gene expression. As a consequence, deregulated N-MYC expression favors the development of androgen-independent tumors.

REGULATION OF N-MYC STABILITY

MYC proteins are unstable proteins that are rapidly turned over by the ubiquitin–proteasome system (Fig. 2). Three ligases, FBXL7, HUWE1, and TRUSS, ubiquitinate N-MYC and restrict N-MYC function in different biological settings. Although the degrons recognized by TRUSS and HUWE1 on N-MYC are unknown, FBXL7 recognizes a phosphodegron that is part of MYCBox I upon phosphorylation of T58 (140). T58 is phosphorylated by GSK3, which needs a priming phosphorylation at S62 (141). In primary neuroblasts, S62 is phosphorylated by CDK1, and therefore N-MYC degradation is initiated in mitosis (142). Because expression of MYCN mRNA is activated by the E2F transcription factor and therefore begins at the G1–S boundary, the regulated synthesis and degradation results in a saw tooth–like pattern of N-MYC expression throughout the cell cycle, with low N-MYC levels in early G1. MYCBoxI also contains a third highly conserved phosphoresidue (S64). Although the role of this phosphorylation has not been analyzed, the analysis of model substrates shows that the presence of a negative charge at the +2 site inhibits CDK1-dependent phosphorylation (143).

Ubiquitination of N-MYC can be reverted by the deubiquitinating enzymes USP7 and USP28, which deubiquitinate N-MYC and thus enhance N-MYC levels in neuroblastoma cells (144, 145). USP28 binds to MYC proteins via the same phosphodegron that is recognized by FBXL7 and may indeed piggyback on FBXL7. N-MYC is stabilized in human tumor cells and in mouse models of neuroblastoma,
medulloblastoma, and NEPC by association with the Aurora-A kinase. Aurora-A binds N-MYC at residues 28–89 in N-MYC via two interaction surfaces that flank MYCBox1, which contains the phosphodegron recognized by FBXW7 (146). FBXW7, in addition to binding to the phosphodegron, interacts with residues 62–89 of N-MYC. Aurora-A competes with FBXW7 for binding, thereby effectively lowering the affinity of N-MYC for FBXW7 and providing a possible mechanism for how Aurora-A stabilizes N-MYC. Additional kinases that stabilize N-MYC are Polo-like kinase 1 (PLK1), which phosphorylates and thereby inhibits FBXW7, and PI3K, which phosphorylates and inactivates GSK3 and thereby stabilizes N-MYC (147).

**TARGETING N-MYC-DRIVEN CANCER**

Targeting MYC proteins directly using small molecules remains challenging, although compounds that interact with the leucine zipper and antagonize DNA binding show efficacy in N-MYC–driven murine neuroblastoma models (148). The advent of technologies that redirect ubiquitin ligases to new substrates using small molecules [degronomids and heterobifunctional molecules known as proteolysis targeting chimeras (PROTAC); refs. 149, 150] is likely to rapidly enable new strategies to directly target N-MYC. At present, multiple indirect strategies (Fig. 3) to target MYCN-driven tumors are being explored, such as targeting the transcription of MYCN with inhibitors of bromodomain and extraterminal (BET) proteins (e.g., JQ1), targeting proteins that enhance N-MYC stability (e.g., allosteric Aurora-A inhibitors), targeting proteins required for N-MYC–driven transcription (e.g., CDK7 inhibitors), and exploiting synthetic lethality interactions with deregulated N-MYC (e.g., CHK1 inhibitors; ref. 151). Because the specific gene expression patterns of N-MYC–driven tumors include genes encoding targetable proteins involved in tumorigenesis, some of the downstream proteins are also being explored for the therapy of N-MYC–driven tumors (e.g., glutaminase inhibitors).

**Targeting N-MYC Expression and Stability**

BET inhibitors displace the BRD4 oncoprotein from chromatin (152) and from the MYC (153) and MYCN (154) promoter regions. Independent reports have shown that JQ1 targets N-MYC and N-MYC target gene expression to inhibit tumor growth in preclinical models of neuroblastoma (65, 154), pancreatic neuroendocrine tumors (42), and medulloblastoma (155–157). OTX015 (Oncoethix) is a stable small molecule that, like JQ1, disrupts the BRD4–chromatin interaction, repressing MYCN and N-MYC target gene expression in neuroblastoma cell lines and killing neuroblastoma cells in vitro and in vivo (158). OTX015 has also been shown to be...
effective in preclinical models of acute leukemia (159) and in clinical phase I/II trials in adults (160). TEN-010 (Tensha Therapeutics) is a small-molecule BET inhibitor that is currently in two phase Ib clinical trials and is the only compound with a formulation applicable in children.

As discussed above, Aurora-A and N-MYC form a complex that results in N-MYC stabilization and is independent of Aurora-A catalytic activity (161). Capitalizing on this dependence, allosteric Aurora-A inhibitors (e.g., alisertib or CD532) have been used to destabilize N-MYC and induce rapid cell death in N-MYC-addicted neuroblastoma (162–164). These allosteric inhibitors target the ATP-binding site of Aurora-A and alter the conformation of Aurora-A in ways that disrupt the complex and degradation of N-MYC, whereas inhibitors that compete with ATP without causing a conformational change leave the complex intact. This synthetic lethality has been applied successfully in other tumor types. In two recent studies involving NEPC models, knockdown of Aurora-A using siRNA or treatment with the allosteric Aurora-A inhibitors alisertib or CD532 resulted in decreased steady-state levels of N-MYC protein, N-MYC target gene expression, and cell viability (46, 48). This approach also extends beyond N-MYC (e.g., C-MYC-driven SCLC models; refs. 105, 125, 165). On the basis of the structural data (146), it may now be possible to design new strategies that would avoid the toxicity problems of the current Aurora-A inhibitors to inhibit the protein–protein interaction of the Aurora-A/N-MYC complex directly by targeting the interface without disrupting the cell-cycle function of Aurora-A. PLK1 expression also enhances N-MYC protein stability by binding to and phosphorylating FBXW7, leading to its self-polyubiquitination and degradation, which antagonizes FBXW7-mediated N-MYC degradation (147). PLK1 inhibition leads to reduced levels of N-MYC and decreased neuroblastoma and SCLC cell viability (147). Finally, specific inhibitors of several ubiquitin-specific proteases, including USP28, are currently being developed, and inhibitors of USP7 are already available. Therefore, destabilizing N-MYC represents an emerging therapeutic strategy.

**Targeting N-MYC Transcriptional Function**

The efficacy of targeting BRD4 in targeting N-MYC-driven tumors may also be due to a role of BRD4 in MYC-driven transcription, as BRD4 can be recruited by MYC proteins to core promoters (135). Although BRD4 has kinase activity itself, it is best known for its role in recruiting p-TEFB and CDK9 to core promoters. Several inhibitors of transcription-related cyclin-dependent kinases with different specificity profiles are currently being explored for therapy of N-MYC-driven tumors (166, 167). Of note, as the MYCN gene is a target of the E2F transcription factors (168), CDK activity is also required for transcription of the MYCN gene, and CDK inhibitors may block both MYCN transcription and transcriptional activation by the N-MYC protein. As noted above, EZH2 has been shown to cooperate with N-MYC as a transcriptional coregulator to drive CRPC toward an NEPC phenotype (46). Treatment with EZH2 inhibitors reverses N-MYC-driven target gene expression and abrogates tumor cell growth driven either by N-MYC (46) or other drivers of the NEPC phenotype (120, 121). Given the availability of EZH2 inhibitors that are now in phase I clinical trials for advanced solid tumors (e.g., NCT02082977), this characterization will potentially provide a rationale for further clinical development for patients with N-MYC-overexpressed NEPC and CRPC. Finally, the recruitment of MYC proteins to core promoters is enhanced by interaction with WDR5, and the interacting domain may be amenable to inhibition using small-molecule mimetics (130).

**Synthetic Lethal Interactions of Deregulated N-MYC**

A conceptually different approach could be aimed at identifying targetable proteins that are essential for the viability of tumor cells with deregulated N-MYC. For example, a recent study found that MYCN-amplified neuroblastoma cell lines were exclusively sensitive to the BCL2/BCLXL inhibitor ABT-263 (navitoclax) and ABT-199 (venetoclax), a next-generation BH3 mimic (169). This enhanced sensitivity was accounted for by the overexpression of NOXA, an inhibitor of the antiapoptotic protein MCL1. Moreover, inhibition of Aurora-A using the allosteric inhibitor alisertib led to decreased levels of MCL1 through mechanisms associated with mitotic arrest and a loss of p4EBP1-mediated cap-dependent protein translation. As a result, the combination of alisertib and venetoclax was more effective in killing MYCN-amplified tumor cells in vitro and in vivo than either compound alone (169). Similarly, MYCN-amplified neuroblastoma cells were sensitive to disruption of the CHK1 checkpoint kinase that monitors DNA replication (170), and inhibitors of both CHK1 and its upstream kinase ATR are being explored for tumor therapy.

**CONCLUSION AND PERSPECTIVES**

Despite remarkable progress in understanding the role of N-MYC in tumorigenesis, the function of the N-MYC protein, how it is regulated, and strategies for how to target it, there still remain formidable challenges:

1. The almost global presence of MYC proteins at open promoters and enhancers remains a conundrum. Furthermore, despite numerous efforts, no conserved set of target genes that mediate N-MYC’s oncogenic functions in different tumor entities has been identified. Whether C-MYC and N-MYC proteins are oncogenic because they globally enhance transcription of all expressed genes, because they shift gene expression patterns toward an “oncogenic” gene expression spectrum that is specific for each tumor entity, or because they have a mechanistically unique and oncogenic role at all promoters and enhancers remains an open question.

2. To date, virtually all targeting strategies for N-MYC-driven tumors are indirect. It is possible that advances in effectively delivering siRNAs or peptides and peptide mimetics that block N-MYC function in a dominant-negative manner (171) to tumors will enable direct targeting of N-MYC. One of the reasons for the failure to find small molecules that target the N-MYC protein directly is that—most likely—it does not stably fold on its own and is inherently unstructured. However, recent elucidation of crystal structures demonstrates that MYC and N-MYC fold on the surface of stably folded partner proteins and the structures of these complexes reveal docking
sites for small molecules not seen for individual proteins. This raises the expectation that the advent of PROTACs and degronomids will radically enhance the possibilities for direct targeting of N-MYC using small molecules in the near future.

3. At the same time, the dependence of many tumors on continuing N-MYC function, a prerequisite for using it as a drug target, has not been rigorously established. Even in entities where this is likely to be the case (such as MYCN-amplified neuroblastoma), there is no clear view of what cellular phenotype is elicited if N-MYC were to be inhibited in an established tumor and how this might contribute to a favorable therapeutic outcome. Recent work on a transgenic model of C-MYC–driven T-cell lymphoma suggests that an unexpected and critical function of C-MYC in this model is to enable escape of lymphoma cells from T cell–mediated eradication (172). Similarly, an influx of T cells is observed when neuroblastomas are treated with Aurora-A inhibitors (162).

If this scenario is generalizable to all N-MYC–driven tumors, it would suggest rational strategies for the development of combined therapies with immunomodulatory agents such as checkpoint inhibitors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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