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Cancer therapeutics: Targeting the dark side of Myc

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Abstract

The potent Myc oncoprotein plays a pivotal role as a regulator of tumorigenesis in numerous human cancers of diverse origin. Experimental evidence shows that inhibiting Myc significantly halts tumour cell growth and proliferation. This review summarises recent progress in understanding the function of Myc as a transcription factor, with emphasis on key protein interactions and target gene regulation. In addition, major advances in drug development aimed at eliminating Myc are described, including antisense and triple helix forming oligonucleotides, porphyrins and siRNA. Future anti-Myc strategies are also discussed that inhibit Myc at the level of expression and/or function. Targeting the dark side of Myc with novel therapeutic agents promises to have a profound impact in combating cancer.

Keywords: Myc; Max; Therapeutics; Deregulation; Transformation; Target genes; Protein-protein interactors; siRNA; Antisense oligonucleotides; ChIP-chip; Cancer

1. Introduction

These are exciting times for clinicians and scientists working at the front line of cancer research. In recent years, our understanding of the genetic etiology and molecular dependence of cancer has advanced to the point where we can begin to exploit this knowledge for the design of novel, effective, anti-cancer therapeutics. Several new classes of anti-cancer agents have been developed to target pathways that are essential for cancer cell growth and survival. To date, the effort has been primarily focused at targeting growth factor receptors which has led to achievements, such as Gleevec (Imatinib, STI-571), a small molecular weight kinase inhibitor, and Trastuzumab (Herceptin), an antibody reagent that targets the HER-2/neu receptor [1–4]. These agents highlight the merits of molecular targeting and provide an important proof-of-concept that targeted therapeutics can be successfully developed and applied to patient care. However, activating mutations can occur at multiple independent points along oncogenic signalling cascades, so targeting cell surface receptors restricts the range of potentially sensitive tumours. Downstream transcription factors that directly control the transformation program provide an alternative target that may be activated in a broader spectrum of cancers. The Myc transcription factor is one of the most potent and frequently deregulated oncoproteins in human cancers [5,6]. Multiple extracellular and intracellular signalling cascades converge to regulate the Myc oncogene (Fig. 1) making it an especially attractive target in the control of transformation.

In this review, we focus on the most recent advances in understanding the molecular mechanisms of Myc function in the etiology of human cancers. Special emphasis is placed on Myc interactions with other proteins and its gene-regulatory mechanisms. As well, we provide an overview of the past, present and potential future strategies used to target Myc.

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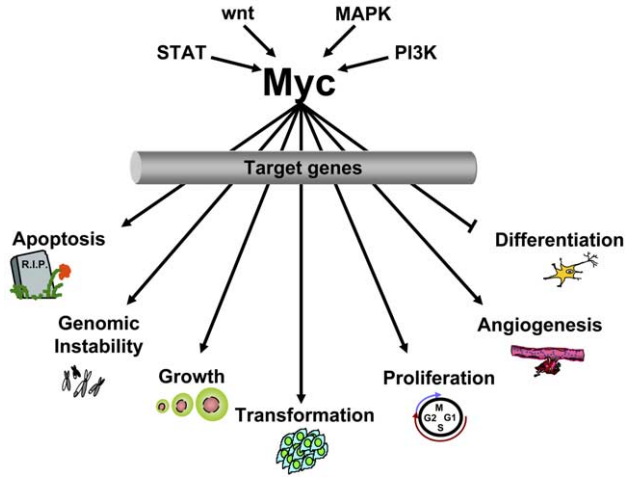

Fig. 1. The regulatory network of Myc. Myc deregulation is a hallmark of many cancers and occurs as a consequence of activation of one or more signalling pathways that induce Myc expression and function as a regulator of gene transcription. These include mitogen activated protein kinases (MAPK) [192–194], phosphatidylinositol-3 kinase (PI3K) [195,196], wnt-TCF/LEF pathway [197], and signal transducer and activator of transcription (STAT) [198] pathways. The target genes regulated by Myc orchestrate the many biological activities attributed to Myc, including apoptosis, genomic instability, growth, transformation, proliferation, angiogenesis and blocking differentiation.

1.1. Myc expression and biological activities

Members of the *myc* family that show oncogenic activity in human cancers include *c-myc*, *MYCN* and *MYCL1*. In a normal physiological state, the protein products of all three genes are expressed during fetal development, whereas only c-Myc protein is expressed in adult tissues. c-Myc protein levels, in non-transformed cells, are induced or suppressed by virtually all signalling cascades bearing proliferative and anti-proliferative cues, respectively. Mitogen stimulation induces c-Myc as an immediate-early response gene, whose expression is essential and sufficient for G1/S progression [7–9]. c-Myc also plays a role in G2/M transition, making it one of the key players in cell cycle regulation [10]. As such, it is important that *c-myc* mRNA and protein have a very short half-life (20–30 min) and are tightly regulated. c-Myc expression is normally rapidly responsive to environmental cues and has been dubbed, "the intracellular sentinel of the extracellular milieu" [11]. By contrast to the highly regulated state of c-Myc and the absence of N-myc and L-myc expression in normal cells, cancer cells often harbour deregulated expression of any one of these three *myc* oncogenes [11].

The founding member of the family, *c-myc*, was first shown to be an oncogene when it was identified as the transduced *v-myc* gene of the transforming avian myelocytomatosis retrovirus [12]. Deregulated c-Myc expression was subsequently shown to be prolific in human cancers. In 100% of Burkitt's lymphoma, c-Myc is translocated with an immunoglobulin enhancer that drives high levels of constitutive *c-myc* mRNA and protein expression, which is instrumental in initiating the disease [13]. Translocations involving the c-Myc locus have also been reported in several additional tumours, including diffuse large cell lymphoma, T-cell acute lymphocytic leukaemia and multiple myeloma [14]. Amplification of c-Myc and/or deregulated expression is evident in many tumours including melanomas and carcinomas of the breast, prostate and colon [5,6,14]. Amplification of *MYCN*, is a hallmark of neuroblastoma [15,16], while *MYCL1* is amplified in ovarian cancer [17]. All three transforming members of the *myc* family can be amplified in non-small cell lung carcinoma [14]. Importantly, in recent years it has become clear that deregulation is not restricted to gross genetic abnormalities of the *myc* gene family, such as translocation or amplification, but can also occur as a consequence of direct or indirect mutations of regulatory molecules controlling *myc* gene expression [11]. Thus, deregulation of Myc expression is evident in numerous human cancers of diverse origin and can result from mutations at one or multiple levels of regulation. Unless otherwise stated, Myc will refer to data described for c-Myc, but these results are often relevant for the highly similar N-Myc and L-Myc oncoproteins.

In response to signals from the cellular environment, Myc can regulate a broad variety of distinct biological activities (Fig. 1). In addition to Myc driving cell proliferation, growth, and transformation, deregulated Myc has also been shown to increase apoptosis, genomic instability, and angiogenesis as well as block differentiation [18–28]. The prevailing model is that Myc controls such a disparate set of activities by regulating distinct cohorts of target genes that then orchestrate each activity. One of the major gaps in the field is linking Myc function, as a transcriptional regulator, to the wide-range of biological activities controlled by Myc [29]. In the following sections we provide an overview of the enormous effort from several labs to identify the target genes regulated by Myc and to uncover the protein–protein interactions that are essential for Myc to function as a transcription factor in transformation.

1.2. Myc structure

Traditionally the Myc protein has been divided into an N-terminal domain (NTD) involved in transactivation and transrepression; and a C-terminal domain (CTD) that is critical for DNA binding. Myc is a transcription factor of the basic helix-loop-helix leucine zipper (b-HLH-LZ) superfamily [30]. The C-terminus harbours both the primary nuclear localisation signal and the basic motif required for binding to the CACGTG E-box DNA recognition sequence (Fig. 2)

[31,32]. The HLH-LZ domain, essential for all known Myc activities, is the heterodimerisation domain that is required for Myc to bind to its primary partner protein, Max (Myc associated protein X).

The N-terminus is a major regulatory region responsible for assembly of the transcriptional machinery [11]. Within the N-terminus there are several highly conserved sequences termed Myc boxes which, together with the C-terminal b-HLH-LZ, define the Myc family of proteins (Fig. 2). Although Myc box I (MBI) is required for gene activation, deletion of this region only partially abolishes the transforming ability of Myc [11,33]. Myc box II (MBII) is essential for the ability of Myc to transform, drive cell proliferation, inhibit differentiation, repress gene transcription, and activate certain target genes [11]. Recently, a third conserved region of Myc has been described, Myc Box III (MBIII), that plays a role in transformation, lymphomagenesis and apoptosis [34]. Interestingly, recent results using circular dichroism indicated that the N-terminal domain shows little to no inherent secondary structure, suggesting that protein–protein interactions are essential for proper folding and function of Myc [35,36]. From a therapeutic perspective this observation offers hope that specific inhibitors can disrupt unique points of interaction between Myc and its binding partners thereby inhibiting transformation. This may offer a novel approach to targeting oncogenic Myc in a tumour-specific manner.

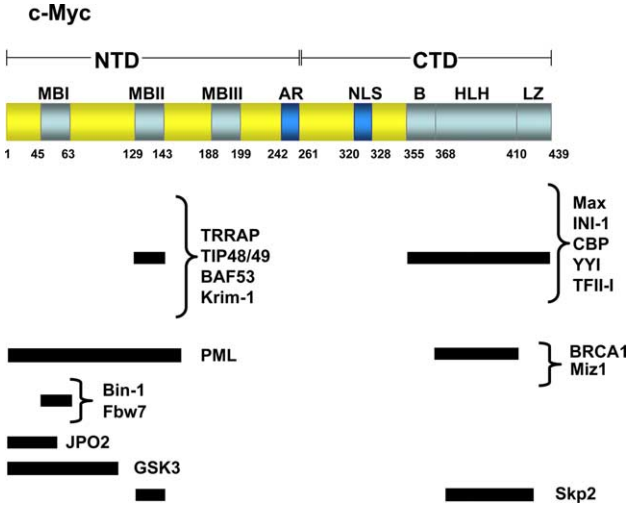

Fig. 2. The structural domains of human c-Myc and their link to protein–protein interactions. c-Myc contains at least six regions which are highly conserved between Myc paralogs and orthologs. The Myc N-terminal domain (NTD) is defined as amino acids 1–262 and contains Myc Box I (MBI), Myc Box II (MBII) and Myc Box III (MBIII) and the acidic region (AR). The Myc C-terminal domain (CTD) is defined as residues 263–439 and contains the primary nuclear localisation signal (NLS) and the basic helix-loop-helix leucine zipper domains (B-HLH-LZ). The regions of c-Myc necessary for the interaction of the specific proteins are shown.

2. Myc interacting proteins

For Myc to hold its extensive role in the control of cellular function, a network of key binding proteins is required (Fig. 2). The identification of this network started with the discovery of Max over a decade ago by screening a human cDNA library with a radiolabeled fusion protein containing the Myc CTD [37]. In recent years, several additional Myc-binding proteins have primarily been identified using biochemical and two-hybrid methodologies. The Myc–Max interaction is already being explored as a therapeutic target, while inhibitors to other key interacting proteins more recently identified, such as TRRAP, have yet to be investigated. A new array of interactors has been identified in recent years, but their role in Myc dependent transformation and the effect of their disruption needs to be further evaluated before they are justifiable targets for therapeutic intervention.

2.1. Myc and Max: from structure to biology

Myc activity is entirely dependent upon dimerisation with Max, an abundant, ubiquitously expressed b-HLH-LZ protein. Recently, the co-crystal structure has been solved and shows that homodimers of Max are held together by polar interactions alone, while Myc–Max heterodimers are stabilised by charged interactions [38]. This results in Myc–Max dimers forming more easily than Max-Max dimers. The crystal structure shows that Myc–Max dimers are stabilised by hydrophobic and polar/charged interactions via helices in the LZ region. Two positively charged residues in Myc form a tetrad with Max, and these two pairs of hydrogen bonds alone control heterodimer specificity with Max. This difference between charged and polar residues also explains the disfavour of Myc–Myc homodimers, caused by electrostatic repulsions between the complementary residues [38,39]. Perhaps one of the most interesting findings from the crystallographic data is the formation of a tetramer with two Myc–Max heterodimers oriented head to tail of the LZ, each binding a DNA E-box. It has been proposed that biologically they may bind widely separated E-box sequences; however, the crystal structure reveals no specific hydrogen bond interactions to stabilise this tetramer [38]. Another function of the heterotetramer might be the formation of a platform for assembly of additional protein factors such as Miz-1, INI1 and BRCA1 which bind to the b-HLH-LZ region of Myc (Fig. 2). The detailed structure of Myc–Max may give rise to novel therapeutic strategies that interfere with either the formation of the tetramer or the association with Myc CTD interactors.

2.2. Myc interaction with chromatin remodeling proteins

In addition to the well-documented association with Max, Myc has been shown to interact with a number of additional transcription factors and co-factors that modulate its activity.

The DNA bound Myc–Max heterodimer interacts through the Myc N-terminal region with a variety of proteins involved in transcription. TRRAP is of particular interest as it forms part of a multiprotein complex with histone acetyl-transferase (HAT) activity. Myc contributes to chromatin remodeling through an MBII-dependent interaction with TRRAP [40,41]. Inhibition of TRRAP synthesis or function blocks Myc-mediated oncogenesis, establishing an essential role for TRRAP in Myc activity [42]. The yeast homologue of TRRAP, tra1 is a component of the SAGA (SPT/ADA/GCN5/Acetyltransferase) complex [43] which also contains the HAT GCN5. The human homologue, hGCN5, has been shown to interact in a complex with Myc through TRRAP [44]. A Myc-Gcn5 fusion protein can partially restore the ability of a Myc MBII deletion mutant to induce transformation, suggesting that recruitment of HAT activity is an important component of Myc function. Myc also interacts with the p400 complex containing TRRAP, surprisingly this complex lacks HAT activity suggesting that Myc-TRRAP interaction may serve additional roles in addition to recruiting HATs. Nevertheless, p400 function in Myc-mediated oncogenesis remains unclear [45]. The recent discovery that CBP (CREB binding protein) binds to the Myc CTD provides an additional link between Myc CTD and activation of transcription. CBP functions partly through its HAT activity and was shown to interact with Myc *in vivo* and to stimulate Myc dependent transactivation. Interestingly, CBP also acetylates Myc *in vitro* and co-expression of the two proteins resulted in stabilisation of Myc [46]. Functionally, the biological significance of this interaction, in particular for Myc-mediated transformation, is yet to be determined.

Myc interacts with several other proteins implicated in chromatin remodeling, namely TIP48 and TIP49 [47]. Interaction of these molecules with Myc requires the Myc NTD and occurs independently of TRRAP binding. TIP48 and TIP49 have ATP hydrolysing activity, as well as suspected helicase activity and have been shown to be required for the foci formation by Myc and Ras in a primary co-transformation assay [47].

Myc may also be involved in a second mechanism of chromatin remodeling: an ATP-dependent process involving the recruitment of the SWI/SNF complex, which regulates transcription through nucleosome repositioning. Myc directly interacts with INI1/hSNF5, a key component of the SWI/SNF complex, and this was suggested to stimulate Myc transcriptional activity [48]. INI1/hSNF5 appears to be a tumour suppressor, and is mutated in the majority of atypical teratoid and malignant rhabdoid tumours [49–51]. More recently, Myc has been shown to interact with BAF53, an actin-related protein which is another integral component of the SWI/SNF chromatin remodeling complex. Targeted mutations in BAF53 inhibit oncogenic transformation by Myc [52]. In that context, it will be important to determine if other proteins of the SWI/SNF complex have the potential to interact with Myc and whether SWI/SNF recruitment may play a role in Myc-mediated transformation. Despite this enormous progress, many questions remain. Which genes are targeted by each of these distinct complexes? Does each complex drive a specific genetic program, such as transformation or apoptosis? Clearly, the precise role of these ATPase/helicase family proteins in Myc biology requires further study and this knowledge will be instructive in determining which complex is of highest priority for therapeutic targeting to block Myc transforming function.

2.3. Interactions with other transcriptional regulators

Whereas TRRAP appears to be a positive regulator of Myc-mediated transformation, Bin-1 (Bridging protein-1) appears to be a negative regulator [53]. The mechanism by which Bin-1 represses transformation is not fully understood, but it includes effects on the cell cycle as well as the promotion of apoptosis in response to Myc over-expression [54]. Since Bin-1 is deleted in a variety of tumours we sought to better define its interaction with Myc. Our group has used nuclear magnetic resonance (NMR) spectroscopy and biochemical assays to define the mechanism of interaction between Bin-1 and the MYC NTD [55]. We showed that a small proline-rich peptide within the conserved MBI interacts with the SH3 domain of Bin1 and that this interaction can be disrupted by phosphorylation of Myc Ser62. Our findings raise the intriguing possibility that the conserved MBI region may bind to other SH3 domain proteins. In addition, the data highlights the significance of post-translational modifications to Myc activity and suggests their modulation as an opportunity for therapeutic intervention.

Due to the evidence that Myc NTD binding proteins are critical to Myc function, we developed a novel high throughput screen termed the repressed transactivator assay (RTA) [56]. This two hybrid approach enables the full Myc NTD to be used as bait in the context of Myc as a transactivator bound to DNA. Novel methods, such as the RTA, will greatly facilitate the identification of Myc-binding proteins and inhibitors to disrupt these interactions. Using the RTA we screened a library derived from medulloblastoma cells with the Myc NTD as bait. A novel Myc interactor, JPO2, was isolated and shown to be a transcription factor containing a putative

LZ and a ring finger domain. JPO2 is closely related to a Myc transcriptional target, JPO1, and was also recently identified as a novel member of this emerging JPO family [57–59]. We show that JPO2 expression affects anchorage independent growth and is associated with metastasis in medulloblastomas [57]. As we have also observed Myc interaction with JPO1 protein, an interesting working model at a molecular level that emerges, sees Myc inducing JPO1 transcription for the formation of a Myc–JPO complex as a positive feedback loop to regulate gene transcription. It will be interesting to determine the validity of this model for the JPO family and to determine if additional Myc induced genes can also form functional transcription regulatory complexes with Myc.

Another novel Myc NTD binding protein is Krim-1, a nuclear zinc finger protein that contains a KRAB domain. Krim-1 was identified using the Ras Recruitment System (RRS) and was shown to associate with MBII [60,61]. In a reporter assay, Krim-1 was able to negatively regulate Myc transactivation and inhibit its oncogenic activity in REFs, a phenotype that is reduced in the presence of a Myc-binding mutant of Krim-1 [61].

Another interactor is the ARF tumour suppressor protein, which binds directly to Myc and inhibits its transcriptional activity in a p53-independent fashion. ARF blocks Myc ability to activate transcription without affecting its ability to repress transcription. ARF prevents Myc-induced transformation whereas Myc-induced apoptosis remains intact even in the absence of p53. ARF was shown to bind both Myc NTD and HLH-LZ domains of Myc [62,63]. These findings suggest a safeguard mechanism for preventing aberrant Myc signalling. The question naturally follows: does ARF differentially bind to a cohort of Myc-target genes related to proliferation and/or transformation? Studies using specific inhibitors of the Myc-ARF interaction will provide a direct link between ARF suppression of tumourigenesis and the control of Myc transcriptional activities. These findings highlight the critical role of MBII in transcriptional regulation and suggest that complexes, such as TRRAP, may either compete or cooperate with other multiprotein complexes for binding with ARF or Krim1.

Recently, several additional Myc-binding proteins have been described, but their role in transformation remains unclear. For example, Myc and PML were shown to co-localise within discrete nuclear structures associated with the nuclear matrix, termed PML bodies [64,65]. These PML bodies associate with regions of high transcriptional activity in the genome and have been implicated in diverse cellular processes, although their specific function remains open [66]. The differential effect that PML appears to have on Myc activation is also interesting. In PML-null mouse embryonic fibroblasts (MEFs) the expression levels of numerous Myc target genes is altered [64,65]. Although PML was demonstrated to physically interact with the Myc NTD and is recruited at Myc regulated promoters, the exact mechanism by which PML influences Myc activity remains unclear. Further insight into the precise contribution of these interactors, as well as others such as PARP-10 [67] or human papillomavirus E6 [68], to the function of oncogenic Myc in the carcinogenic process will determine whether targeting these proteins may be of merit.

2.4. Interactors that regulate Myc stability

Multiple mechanisms ensure proper control of Myc activity in normal cells, including regulation of Myc protein turnover, through the ubiquitin-proteosome pathway [69,70]. Recently, a component of the E3 ubiquitin ligase complex, Skp2, has been reported to mediate Myc turnover *in vivo,* which in turn is linked to Myc transcriptional activity at specific target genes. The regions of Skp2–Myc interaction were mapped to MBII as well as to Myc CTD (Fig. 2) [71,72]. Consistent with its role as a co-factor for Myc, Skp2 induces S-phase entry in a Myc-dependent manner [71]. Since Skp2 is known to have oncogenic properties [73,74] its role in increasing Myc transcriptional activity is not surprising. Although Skp2 is required for the phosphorylation-dependent degradation of various proteins, such as the tumour suppressor FOXO1 [75], there is no evidence suggesting the effect of Skp2 on Myc is regulated at the level of phosphorylation. Phosphorylation-dependent degradation of Myc involves two key residues, T58 and S62 within the MBI. Phosphorylation of S62 is mediated by Ras signalling and is a prerequisite to the phosphorylation of T58 that is performed by glycogen synthase kinase 3 (GSK3) [70,76–78]. Phosphorylation at T58 destabilises Myc protein [70,79]. T58 represents a major hotspot for mutations in Burkitt's and other lymphomas [80]. T58-phosphorylated Myc is specifically bound by the F-box protein, Fbw7, and results in Myc ubiquitination and degradation [80–83]. Fbw7 appears to function as a tumour suppressor gene [84]. Knockdown of Fbw7 increased both the abundance and transactivation activity of endogenous Myc. Surprisingly, one of the Fbw7 isoforms (Fbw7γ) co-localises with Myc in the nucleolus upon proteosome inhibition suggesting that Myc is also regulated in this specialised nuclear compartment for degradation [81]. These data support the idea that Fbw7 and Skp2 have opposing effects on Myc activity by targeting MBI and MBII, respectively. This knowledge can be exploited for therapeutics as targeting MBI might affect Myc stability while targeting MBII would affect Myc transcriptional activity.

3. Myc regulated target genes

To understand the role of Myc in cellular physiology and pathology, it is essential to identify the *bona fide* target genes regulated by Myc. A *bona fide* Myc-target gene is one whose regulatory region is bound, directly or indirectly by Myc, and whose expression is then regulated under an appropriate stimulus. These targets are distinguished from the many regulatory events that occur as a downstream consequence of Myc activity, like cell cycle progression [11]. In the past, many criteria were required to distinguish a gene as a true Myc target gene, but none were absolute. Several cDNA microarray studies have identified many *bona fide* targets and downstream regulated genes, but rarely have the two subsets been clearly distinguished [85–88]. Recently developed techniques have revolutionised this major issue and allowed true target genes to be rapidly identified and profiled *in vivo*, in any given cell or tissue under any given stimulus. One such technique is termed ChIP–chip (or ChIP-on-chip), in which the sensitivity and specificity of chromatin immunoprecipitation (ChIP) is combined with the high throughput capability of microarray (chip) technology.

3.1. Genome-wide Myc binding profile

Knowledge of binding sites found within a genome is essential for understanding the target genes regulated by any transcription factor. With the development of high throughput ChIP technologies, many labs, including our own, have identified the *in vivo* genomic DNA-binding sites of Myc [29]. This is especially important for understanding the mechanisms of Myc role in carcinogenesis. By profiling the many target genes regulated by Myc, the anticipation is that a key subset essential for a particular biological function will be distinguished and the mechanism of co-regulation of this cohort of genes will then be determined. Several novel insights have been forthcoming from these studies on Myc genomic binding in mammals [89–91]. It has been concluded that at sites of transcriptional activation, Myc and Max bind together at high affinity loci containing canonical or non-canonical E-boxes, which are often CpG rich [29,92]. Moreover, these characteristics are evolutionarily conserved from *Drosophila* to mammals [93]. In addition, recent work shows that there is evolutionary conservation of promoter architecture across different species of *Drosophila* containing a single E-box located within the first 100 nucleotides downstream of the transcription start site [94]. These outcomes suggest that a subset of Myc targets share a common and particular mechanism of regulation. By contrast, the repression of the target genes by Myc does not occur at E-boxes, but, rather at proximal promoter regions [95–98]. Interestingly, the Myc–Max interaction is essential for Myc to repress as well as activate gene transcription [91]. A systematic analysis across promoters, employing short oligonucleotide arrays, showed that only 22% of the Myc binding sites are located at the 5′ upstream region of protein-coding genes, while 36% are placed at the 3′ end of well-characterised genes that are associated with non-coding RNAs [99]. In light of this study it is very interesting that two recent papers describe the relationship between Myc and non-coding microRNAs (miRNAs). The first shows that Myc regulates a cluster of miRNAs in chromosome 13 that influences the major cell cycle regulator, E2F1 [100]. The authors proposed that Myc directly binds to this miRNA cluster to negatively regulate E2F1, thereby dampening the runaway effect of Myc inducing E2F1 transcription. It will be interesting to determine the function of this miRNA cluster in tumour cells. The second study shows that another cluster in chromosome 13, which is often overexpressed in lymphomas, strongly cooperates with Myc in lymphomagenesis by inhibiting Myc apoptotic capability [101]. The identification of miRNAs is in its infancy and the identification of transcriptionally active sites by ChIP–chip will greatly aid in delineating oncogenically active miRNAs.

One of the most profound results emanating from the genome-wide localisation studies is that Myc binds to an enormous number of target genes, compared with other transcription factors such as p53 and Sp1 [99]. Moreover, the genomic binding sites are associated with genes whose products are engaged in a wide-range of biological processes. In addition, it is curious that not all Myc bound targets are regulated at the level of expression [89].These observations have been gathered from a limited series of experiments conducted in a variety of labs. Clearly, it will be critical to systematically evaluate the nature of Myc target genes and their regulation under a multiplicity of physiological and pathological conditions. Importantly, these experiments are now feasible thanks to the advances in ChIP–chip technologies. To take advantage of these results, it will be necessary to identify Myc interactors that cooperate in the regulation of genes implicated in Myc related phenotypes. The target genes identified to date are compiled in a well-annotated database http://www.myc-cancer-gene.org/site/mycTargetDB.asp [11,102,103]. Due to space constraints, we will only discuss specific *bona fide* targets in the context of understanding their mechanism of regulation by Myc.

3.2. Mechanisms of Myc dependent transcriptional activation

Activation of target genes by Myc involves at least two regulatory steps; chromatin remodeling and promoter clearance. Mechanistic analyses of Myc-induced genes have clearly shown that Myc participates in chro-

matin remodeling when recruited to promoter regions. For example, the activation of the normally silent telomerase reverse transcriptase gene (TERT) by oncogenic Myc in exponentially growing fibroblasts requires TRRAP recruitment and is accompanied by both H3 and H4 acetylation [104]. Despite the profound role of this target gene in oncogenesis, transformation of primary cells is thought to require the regulation of additional targets by oncogenic Myc, as TERT overexpression alone cannot replace Myc in Rat fibroblast transformation assays [105] and mice lacking telomerase RNA can still be transformed by Myc and H-Ras [106]. Many cell cycle components are also regulated by Myc in a MBII/TRRAP-dependent manner [107]. Myc stimulates expression of the Cyclin D2 and Cdk4 genes, leading to sequestration of the cell cycle inhibitor p27 in CyclinD2/Cdk4 complexes [108–110]. Remarkably, p27 is then degraded by the protein product of two Myc activated genes, Cul1 and Cks [109,111,112]. Evidence suggests that the interaction of Myc-TRRAP to recruit HAT activity to target gene promoters such as TERT and Cyclin D2, is important for Myc transformation and may be dispensable for Myc to drive apoptosis [42,44]. For example, MEFs derived from the Cyclin D1/D2 double knock-out mice showed impaired proliferation in response to ectopic Myc expression, but the ability of Myc to potentiate apoptosis remained intact [113].

In addition to directly regulating chromatin remodeling through the recruitment of HATs, Myc also induces target genes involved in the regulation of chromatin dynamics. Recently, two such target genes have been described. MT-MC1 encodes a nuclear protein with homology to certain DNA helicases, and HMG-I encodes one of the high-mobility group proteins. Interestingly, ectopic expression of these proteins in Myc knock-out rat fibroblasts was shown to reconstitute many Myc phenotypes, such as rescuing the parental cell morphology, correction of the slow growth rate, cell size, genomic instability, clonogenicity, tumourigenicity and the regulation of a subset of Myc target genes [114]. Even though these two genes sometimes differ in the reconstitution of Myc functions, significant overlap exists between them. Remarkably, these chromatin remodeling proteins, either individually or in combination, were not able to complement the ability of Myc to potentiate apoptosis following serum withdrawal. These studies leave the door open to a selective investigation of additional fundamental Myc targets, which can restore, for example, the Myc apoptotic function.

Myc has been shown to participate in the transcription regulatory step associated with promoter clearance of RNA polymerase II (RNAP II) [115]. Myc was previously shown to recruit components of the elongation factor P-TEFb (positive transcription elongation factor b) at the *cad* promoter [115,116]. Inhibition of the P-TEFb complex blocked the effects of Myc in transformed cells [117]. Thus, the rate-limiting step of transcriptional activation of specific target genes may be Myc-mediated recruitment of P-TEFb, which then allows phosphorylation of RNAP II and release of a suspended transcription complex. It will be interesting to determine whether a cohort of genes, like *cad*, is similarly regulated by Myc and involved in the control of a common biological activity.

An attractive new development in understanding Myc function as a regulator of gene transcription is the appreciation that Myc not only regulates RNAP II regulated genes, but also affects genes transcribed by RNAP I and III. In fact, the various components of the ribosomal machinery are synthesised by all three RNA polymerases, RNAP I, II and III (Fig. 3). This is consistent with the regulation of ribosomal targets as a common feature of many expression microarray analyses to identify Myc target genes. Ribosomal biogenesis is a fundamental cellular process that takes place in the nucleolus and is essential for ribosome assembly and protein synthesis. Remarkably, the nucleoli are enlarged in cancer cells and several ribosomal proteins are overexpressed in tumours, suggesting a correlation between the deregulation of protein biosynthesis and cancer, leading to an opportunity for the development of innovative therapeutics targeting the translation machine [26]. Mechanistically, Myc binds to TFIIIB, a component of RNAP III machinery, and stimulates RNAP III transcriptional regulation of the 5S rRNA gene [118,119]. In the nucleoli, Myc directly regulates RNAP I transcription by interacting with SL1 (TIF-IB), an essential complex composed of the TATA binding protein (TBP) and three RNAP I-specific TBP-associated factors (TAFs) [120,121]. Additionally, ChIP experiments show that Myc and Max bind at non-canonical E-box sequences located within ribosomal DNA (rDNA) promoters. This association is followed by recruitment of TRRAP, enhanced histone acetylation, recruitment of RNAP I, and activation of rDNA transcription [122–124]. Taken together, regulation of all three RNA polymerases suggest that Myc plays a key role within the cell to produce molecules implicated in ribosome biogenesis (Fig. 3).

3.3. Mechanisms of Myc dependent transcriptional repression

The molecular mechanism of Myc role in repression of gene transcription remains less well characterised than its role in activation, yet all indications suggest repression is as important as activation for Myc function. For example, structure–function analyses have linked transformation to repression. Moreover, microarray and ChIP–chip analyses show that Myc activates and represses gene expression in similar proportions.

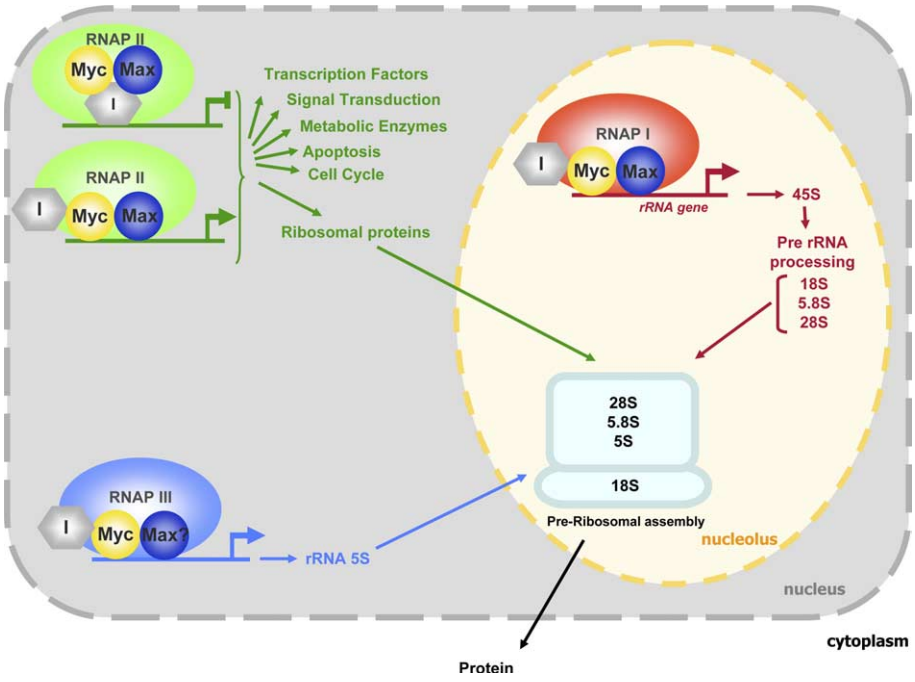

Fig. 3. Myc regulates transcription directed by the three RNA polymerases. Myc, with its partner Max, binds DNA and either activates or represses transcription of numerous target genes of enormous diversity, which are regulated by RNA polymerase II (RNAP II) and implicated in a multiplicity of cellular functions. In the nucleolus, together with Max as a partner, Myc activates the transcription of the rRNA genes, regulated by the RNA polymerase I complex (RNAP I). As well, Myc activates rRNA 5S transcription by interacting with TFIIIB, a component of the RNA polymerase III complex (RNAP III). The collective regulation of these three transcriptional functions by Myc suggests that Myc plays a key role within the cell to produce molecules implicated in ribosome biogenesis. RNAP I, II and III exist as a part of multiprotein complexes: other components are not shown in this simplified figure. The hexagon symbolizes various Myc-protein interactors (I) that contribute to gene regulation. (Figure adapted from Oskarsson and Trumpp [199]).

The target genes repressed by Myc fall into several functional categories. At a molecular level the best characterised targets includes those whose products inhibit cell proliferation (e.g. p15, p21, gadd45). Characterising the mechanism of Myc repression of these and other repressed target genes has shown that Myc does not appear to directly associate with the regulatory regions of repressed targets, but rather is recruited to core promoters through protein–protein interactions. Myc interacts with activating transcription factors, such as TFII-I, NF-Y and Miz-1 [95,125,126]. For example, transcriptional activation by Miz1 is abolished with Myc binding, and the Myc–Miz-1 complex acts as a transcriptional repressor; in part due to competition between p300 and Myc for binding to Miz1 [98]. It was recently shown that Myc represses transcription of p21Cip through recruitment of the DNA methyltransferase corepressor Dnmt3a. Myc and Dnmt3a form a ternary complex with Miz-1 to corepress p21Cip [127]. The precise role of Miz-1, as well as other factors including YY1 [128,129], NF-Y [95,130] and TFII-I [126] in Myc repression remains unclear and will require additional analyses.

Like Myc activation of gene transcription, Myc repression can occur through multiple mechanisms. Myc repression was thought to be dependent upon an initiator (Inr) region within the promoter, however, the presence of an Inr is not essential as genes lacking an Inr, such as gadd45 and PDGFB, are repressed by Myc [95,131]. Myc has been shown to repress gadd45 by a post-RNA polymerase II recruitment mechanism [132]. Interestingly, promoter binding and repression of PDGFBR are separable activities, since mutant Myc proteins that are unable to repress PDGFBR gene expression, still bind to the promoter *in vivo* [130].

Currently a large number of *in vivo* Myc activated or repressed target genes have been compiled from studies in different types of cancer cells. However, many questions remain. For example, which target genes are regulated by Myc and critical for the carcinogenic process? This issue will be essential to further study the subset of Myc regulated genes that directly or indirectly contribute to the tumour formation. Through the characterisation of these target genes, new possibilities will be addressed in the diagnostic field as well as in the development of novel anticancer therapeutics to target the oncogenic activity of Myc.

4. Myc as a target in cancer therapy

Developing therapeutics to inhibit oncogenic Myc would have enormous impact on the treatment of a

wide-range of human cancers. Transgenic mouse models provide a glimpse of how profoundly effective blocking Myc can be as an anti-cancer target *in vivo*. A series of elegant experiments using inducible Myc in hematopoetic cells [133], mammary gland [134], liver [135], skin [135,136] and pancreatic islets [137] have demonstrated that induction of oncogenic Myc leads to full-blown malignancies, while blocking Myc activation in most cases results in tumour regression [138]. This strongly supports the notion that targeting Myc in tumours represents a valid therapeutic approach. Caution should be exercised as not all tumours regressed upon withdrawal of Myc and a small proportion proceeded to hematological malignancies [133], while half of Myc-induced mammary carcinomas acquired mutations in K-Ras or H-Ras, thus rendering them independent of Myc status [134]. It is likely that anti-Myc agents would have to be coupled with therapies targeting Ras or other oncogenic pathways.

Many strategies are under development to target or exploit oncogenic Myc in tumour cells and eradicate the malignant cellular clones (Fig. 4). One approach is to disrupt Myc expression by targeting regulatory steps ranging from transcription to translation. Another strategy is to block Myc function by inhibiting critical protein–protein interactions that are essential for Myc to regulate gene transcription, such as heterodimerisation with Max. The working assumption is that targeting the Myc regulatory network will trigger the lethality of the tumour cell without causing irreversible damage to neighbouring normal cells. Such a tumour-specific effect is evident with other inhibitors that target universally expressed oncogenes. For example, antisense *bcl-2* will sensitise tumour cells to undergo apoptosis in response to low-dose chemotherapy, but non-transformed cells are spared [139–142]. In this case, the tumour cell has become dependent upon the deregulated signalling pathway and even marginal down-regulation of this lifeline renders it susceptible to extinction. Should this assumption be incorrect, Myc inhibitors will have to be targeted by tumour-specific delivery mechanisms. Newer strategies aim to achieve a high therapeutic index by targeting tumour-specific Myc-protein interactions and/or gene regulatory functions. Yet others aim to exploit the presence of oncogenic Myc expression in tumour cells to specifically trigger a suicide response exclusively in these transformed cells. Both ongoing and emerging strategies will be further discussed below.

4.1. Targeting Myc expression

One of the first successful applications of antisense technology targeted Myc expression, and through advancements over the last 15 years, this approach remains at the forefront of anti-Myc therapeutics [8,143]. Antisense oligonucleotides (ASOs) are short single-stranded DNA molecules that specifically target, hybridize and inhibit the mRNA of a selected gene (Fig. 4).

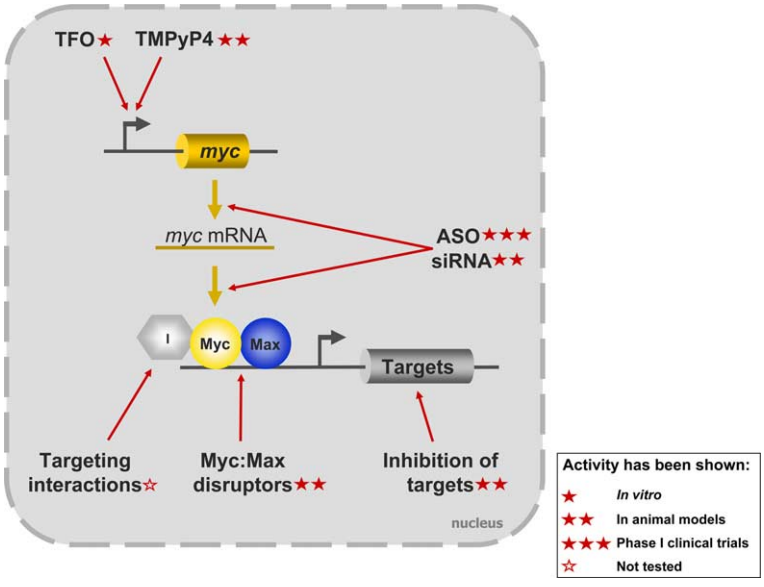

Fig. 4. Strategies for targeting oncogenic Myc in cancer. Therapeutic agents are coded according to their current therapeutic stage of development. Agents that have demonstrated efficacy *in vitro* are indicated with one star. These include inhibitors that block Myc expression, such as triple helix forming oligonucleotides (TFO). Double star agents have been tested in animal tumour models or xenograft models for their efficacy to inhibit Myc. This category includes disruptors of Myc–Max interaction and agents that block Myc target gene function, such as cationic porphyrins (TMPyP4) and small interfering RNAs (siRNA). Some of the antisense oligonucleotides (ASO) that have successfully completed Phase I clinical trials, and are at advanced stage of drug development are indicated by three stars. Finally, a clear star indicates additional potential targets for the therapeutic interference of Myc–protein interactors (I).

The DNA–RNA duplex then recruits RNase H endonuclease to cleave the RNA strand in the duplex [144], leaving the antisense DNA intact to hybridize to additional mRNAs of the target gene [145,146]. Antisense technology has evolved as a viable therapeutic alternative by increasing the functional stability and permeability of the ASOs. This has been largely achieved by replacing the phosphodiester backbone with a nuclease resistant phosphorothioate linkage (PS ASOs) [147,148].

Myc ASOs have advanced successfully through the many stages of preclinical evaluation and have demonstrated anti-cancer potential. Evaluation *in vitro* has shown that downregulating Myc expression by PS ASOs reduces leukaemic cell proliferation, induces differentiation and inhibits G1/S progression [149,150]. Moreover, exposure of MCF-7 breast cancer cells to Myc ASOs induced growth arrest [151]. In mouse models, treatment with Myc PS ASOs resulted in the delay or prevention of Burkitt's lymphoma [152–154] and Myc ASOs enhanced the efficacy of cisplatin to target melanoma both *in vitro* and *in vivo* [155]. Cisplatin resistance can be overcome by inhibiting Myc using AVI-4126, a phosphorodiamidate morpholino oligomer (PMO), in the Lewis lung carcinoma model [156]. AVI-4126 also inhibited growth of a murine prostate cancer xenograft by inducing growth inhibition and apoptosis *in vivo* [157]. These results have led to Phase I clinical trials, which show that intravenous administration of the morpholino oligomer was not accompanied by toxicity or serious adverse events, and importantly, the bioavailability was measurable in malignant tumours. The number of patients studied remains modest, yet this data supports the use of the AVI-4126 PMO as a potential therapeutic for cancer treatment [158]. It will be interesting to monitor the efficacy of this agent as it advances through to Phase II/III clinical trials.

Several additional Myc ASOs show promise for future application as anti-cancer agents. Administration of INX-6295, a 16-mer Myc PS ASO encapsulated in lipid particles, shows antitumour efficacy against a human melanoma xenograft. When administered with cisplatin, INX-6295 produced complete tumour regression in 30% of treated mice [159]. Furthermore, the combined application of *bcl-2* ASO/cisplatin/INX-6295 in mice harbouring human melanoma xenografts overexpressing either *bcl-2* or *c-myc* oncogenes resulted in effective antitumour therapy [160]. Yet another agent, the novel psoralen- or acridine-modified, clamp-forming ASOs, can downregulate Myc expression and synergise with cisplatin to inhibit melanoma cell proliferation and tumour progression [161,162]. The peptide nucleic acid (PNA) ASO also shows promise. PNA is a synthetic DNA in which the sugar-phosphate backbone is replaced with a polyamide-(2-aminoethyl) glycine-skeleton. This modification provides PNAs with a long half-life and enables PNAs to specifically hybridise to DNA and/or RNA in a complementary manner, forming a strong and effective duplex that can inhibit transcription and translation of the target gene [163]. Myc expression was rapidly downregulated in Burkitt's Lymphoma, using a 17-mer anti-*c-myc* PNA covalently linked to a nuclear localisation signal (NLS) (PNA-myc_{wt}-NLS) [164]. However, because this PNA blocked Myc in both transformed and non-transformed cells, further innovative refinements of this strategy are under development to increase tumour specificity [165]. Clearly, ASO technology targeting Myc expression is well advanced and has enormous promise for future application to patient care. Maximal efficacy will depend on achieving synergy with conventional chemotherapies and/or novel molecular anti-cancer agents [166,167].

RNA interference (RNAi) is a modern and popular approach to knockdown gene expression that has potential for drug development. RNAi is a mechanism for silencing gene expression through targeting double-stranded RNA to mRNA resulting in degradation of the targeted mRNA (Fig. 4). In mammalian cells, long double-strand RNAs are cleaved into small interfering RNAs (siRNAs) that, through incorporation into the silencing complex, direct target recognition. RNAi has been touted as the next major tool in targeted cancer therapy, because of its impressive specificity and efficacy. Compared to antisense approaches, siRNAs are 1000-fold more active [168] and a plethora of data shows the efficacy of siRNAs in cell culture. For example, Myc siRNA effectively inhibited Myc protein levels in MCF7 cells [169]. It appears that siRNA can be effective *in vivo* [170], but this has not yet been thoroughly explored. The primary limitations of siRNA *in vivo* remain its stability and delivery. siRNA is quickly degraded in plasma, so the duplex will need to be chemically modified for use as a drug. Perhaps strategies used to modify ASOs to increase stability can be exploited for siRNA. Recently the issue of delivery was addressed by fusing multiple siRNAs, targeting Myc, Hdm2 and VEGF mRNAs, to a positively charged protein that was covalently linked to a specific antibody. This ensured that only melanoma cells ectopically expressing that particular ligand were growth inhibited *in vivo* [171]. With this novel approach it may be possible to deliver such a lethal siRNA cocktail through a Myc-induced cell surface molecule to achieve tumour-specific targeting. Whether siRNAs target mutated oncogenes like Ras [172] and/or deregulated oncogenes like Myc [169], these new tools are likely to make a firm and lasting entrance into the arsenal of therapeutics in the fight against cancer.

Another series of agents have been designed to target *myc* expression at the transcriptional level. One promising agent includes the triple helix forming oligonucleotides (TFOs) which bind to double-stand purine-rich DNA within promoter regions and block transcription factor binding (Fig. 4). Phosphothioate stabilised TFOs

directed against the promoter region of c-*myc* have been shown to successfully inhibit Myc expression in several leukaemia and lymphoma cell lines leading to cellular growth arrest and apoptosis [173,174]. TFOs conjugated to the DNA-intercalating agent daunomycin specifically downregulated endogenous Myc in prostate and breast cancer cell lines [175]. Cationic porphyrin TMPyP4, which inhibits Myc transcription by blocking G quadruplexes, a DNA structure formed from G rich single-strand DNA during active transcription is another approach to target Myc [176] (Fig. 4). TMPyP4 inhibited the *in vitro* transcription of *myc* and decreased tumour growth rates in xenograft models [176]. The original use of cationic porphyrin was to block telomerase by stabilising the telomeric G rich single stand DNA overhangs into G quadruplexes [176]. New Myc-specific porphyrin analogues show promising results, suggesting that this approach has merit for further development [177,178]. Introduction of G quadruplex-forming oligonucleotides into Burkitt's lymphoma cells resulted in growth inhibition by sequestering the factors that normally bind to the native G quadruplexes formed at the Myc promoter [179]. It will be interesting to monitor the development of these various agents that target *myc* transcription for their ultimate use as therapeutics.

4.2. Targeting Myc–Max interaction

Breaking the Myc–Max bond would clearly destroy oncogenic Myc function and several strategies to dissolve this partnership are well underway (Fig. 4). Many of the approaches under development have been advanced because of our knowledge of the detailed structural biology of this protein–protein interaction and the essential residues involved. Molecular modeling and mutagenesis have been used to identify specific amino acids that alter the specificity of dimerisation [180]. Four amino acids within the LZ of Myc were sufficient for homodimerisation, as well as heterodimerisation with Myc and Max. Furthermore, a mutant protein termed Omomyc, which interferes with Myc binding to E-box elements, was able to inhibit colony formation in NIH3T3 cells [181]. Introduction of Omomyc in a Myc-induced skin tumourigenesis model in mice [182] inhibited Myc-induced papillomatosis, as well as restored the normal keratinocyte differentiation program and skin architecture, both of which are otherwise disrupted by Myc activation [183]. Importantly, the positive outcome of Omomyc expression was completely dependent on the presence of an oncogenic Myc and therefore may be of therapeutic value for the specific targeting of Myc-deregulated cells without affecting the surrounding normal cells.

Knowing the structural domains required for the Myc–Max interaction inspired a dominant-negative approach to disrupt this linkage. Myc mutants expressing only the b-HLH-LZ or HLH-LZ domain rapidly induced apoptosis in 3T3-L1 mouse fibroblasts [184]. This study provided the rationale to devise additional strategies to directly exploit Myc heterodimerisation for the design of novel therapeutics. One of the major challenges with this approach lies in achieving efficient delivery of the drug to the nucleus of tumour cells *in vivo*. To this end, a mutant peptide derived from the helix-1 region of Myc was linked to an internalisation sequence [185]. The fusion peptide interfered with the transcriptional activity of Myc leading to the inhibition of MCF-7 cell growth. The stability and activity of this peptidomimetic molecule was increased [186,187] and a variant of the original peptidomimetic has been synthesised and tested in mouse models. Interestingly, analysis of inhibitor interactions of Myc–Max shows the active molecules act through key basic residues at the outer surface of the Myc–Max heterodimer, potentially by binding or interfering with another interactor. The novel peptides were capable of reaching high concentrations in mouse organs and were effective at inhibiting growth of a colon cancer cell line. This peptide is 10-fold larger than traditional small molecules, which may contribute to its highly selective interference with Myc-specific protein–protein interactions [186]. It would be of great interest to test which specific Myc–protein interaction is inhibited *in vivo* and whether knockdown of this co-factor can directly trigger an anti-tumour effect in animals.

Recently, two groups have shown that inhibitors blocking the Myc–Max interaction can be isolated using a high throughput screen. In one study, inhibitors were identified by fluorescence resonance energy transfer in high-throughput screens of peptidomimetic libraries, then confirmed by enzyme-linked immunosorbent assay and electrophoretic mobility shift assay. The antagonists interfered with Myc-induced and Jun-induced oncogenic transformation suggesting the inhibitor may also target the LZ of the Jun oncoprotein [188]. The exact molecular mechanism of inhibition and the utility of these agents in the control of carcinogenesis is the focus of future analysis. In the second study, the yeast two-hybrid assay was used to screen a library of 10 000 compounds to identify those able to disrupt the interaction of the b-HLH-LZ regions of Myc and Max, yet show no general toxicity to the yeast [189]. Several compounds were identified and their ability to inhibit the Myc–Max interaction confirmed by an *in vitro* association assay. Using a reporter assay, the compounds were shown to inhibit Myc transcriptional activity and proved to inhibit growth of Myc transformed rat fibroblasts, but not Myc-null cells. Finally, incubation of transformed cells with the compounds for three days, prior to their injection into nude mice, inhibited tumour growth *in vivo*. Although the compounds used by both groups require relatively high concentrations, these promising studies

provide a platform for future development of more effective small molecule inhibitors of Myc–Max dimerisation.

5. Conclusions and perspectives

Targeting Myc at the level of expression and/or function is an effective approach to eliminate this potent oncoprotein. The agents described in this review successfully block Myc and have passed the first hurdle in targeted drug design. The issues that remain to be resolved include, but are not limited to, evaluating and improving sensitivity, specificity, delivery and efficacy as a single agent and in combination with other anti-neoplastic therapies. The agents that have advanced beyond Phase I clinical trials show enormous promise. It will be fascinating to monitor their progress and learn whether targeting Myc will have the expected impact at the level of patient care.

There are several additional approaches aimed at targeting Myc activity that are in the earliest stages of development. Based on the success of targeting Myc–Max interaction, there is significant interest in targeting other key Myc–protein partnerships, such as Myc-TRRAP. This field of study covers a broad spectrum of experimental analyses, from first identifying the interactor, determining whether it plays an important functional role in Myc transformation, through to mapping the precise points of interaction. Many Myc–protein interactions highlighted here have been described only in recent years and some of them may warrant inhibitor development.

A second major area of fundamental research that has the potential to impact therapeutic design aims at identifying and understanding Myc target genes and the biological pathways they regulate. As a multifunctional master regulator, Myc induces several genes which, in turn, play a critical role in transformation and are now being targeted as potential anti-cancer therapeutics. For example, haplo insufficiency for a Myc target gene, *odc* reduces skin tumourigenesis in mice [190] and a specific inhibitor of ODC, 2-difluoromethylornitive, can block Myc-induced oncogenesis [191]. Clearly, a greater understanding of the Myc transformation program will result in additional opportunities to target Myc function in tumour cells.

Another novel approach does not target Myc directly, but instead aims to exploit the ability of oncogenic Myc to potentiate apoptosis. A cooperating lesion, such as Bcl-2 activation, often inhibits Myc potentiation of apoptosis and collaborates with Myc to drive transformation. Blocking these anti-apoptotic molecules may release the ability of Myc to sensitise tumour cells to undergo apoptosis, elevate the therapeutic index and achieve tumour cell death. There is enormous effort to understand the nature of the genetic abnormalities associated with cancers that can block Myc-potentiation of apoptosis and contribute to transformation. With this knowledge, the innovative approach of exploiting deregulated Myc can be further advanced.

Myc deregulation is often associated with aggressive disease of poor prognosis, which augments the urgency for novel therapeutics targeting this potent oncoprotein. Many such agents are well along the drug development pipeline. Moreover, fundamental research instructs us daily of new opportunities to effectively target Myc expression and function to block malignant transformation. Further growth in the area of anti-Myc therapeutics is warranted and anticipated.

Conflict of interest statement

None declared.

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