Chapter 18

The MYCN Oncogene

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1. Introduction

MYCN is a member of the *MYC* family of oncogenes, which also includes *c-MYC* and *MYCL*. Despite knowing about the existence of MYCN for nearly thirty years, the majority of functional studies involving MYC family members have focused on c-MYC due to the limited expression profile of MYCN in human cancers, and also in part due to the existence of highly conserved functional domains between c-MYC and MYCN [1]. MYCN is normally expressed during embryonal development and orchestrates cell proliferation and differentiation in the developing peripheral neural crest [2]. However, the deregulated expression of MYCN has been shown to contribute to tumorigenesis and neuronal transformation [3]. Thus, MYCN represents a highly desirable therapeutic target. Previous studies have shown that downregulating *MYCN* expression, via antisense oligonucleotides, resulted in lower tumour incidence and decreased tumour mass in a murine neuroblastoma tumour model [4]. However, to date, no molecularly targeted therapies have been developed that are able to mimic this response in the clinic, and further studies are required to help elucidate the mechanisms that drive *MYCN* tumour formation and progression.

2. The MYC family and the discovery of MYCN

The eventual discovery of *MYC* oncogenes arose from early pioneering work on the Rous sarcoma virus (RSV), a transforming retrovirus able to cause sarcomas in infected chicken cells. Using the information provided by RSV, hybridisation studies were performed on a specific group of avian tumours involving a retrovirus responsible for inducing myeloid leukaemia. This led to the identification of a sequence that was named *v-gag-myc*, or *v-myc* for myelocytomatosis (the leukaemia that is induced following the transduction of avian cells with this virus) and supported the idea that viral integration into a host genome could activate a nearby host oncogene [5, 6]. As it transpired, the human homologue of *v-myc*, termed *c-MYC* (cellular-*MYC*) was the first cellular oncogene whose overexpression was

shown to be activated through retroviral insertional mutagenesis [7]. Deregulated expression of *c-MYC* has since been implicated in a range of cancers, and allowed the discovery of other important *MYC* family members including *MYCN* and *MYCL*.

Neuroblastoma is the most common extracranial solid tumour of early childhood and accounts for approximately 15% of all cancer related deaths in children. Aggressive drug refractory neuroblastoma cells have been frequently observed to contain genomic aberrations referred to as double-minute chromatin bodies and homogeneously staining regions. Both of these types of aberrations were found to contain multiple copies or amplification of specific genes, and in particular, the critical gene within these regions was later identified to be the *c-MYC*-related oncogene, *MYCN*, so-called because of its identification in neuroblastoma cells [8]. Amplification of the *MYCN* oncogene has also been demonstrated in retinoblastoma, glioblastoma, medulloblastoma, astrocytoma and small cell lung cancer cells [9]. In addition, another member of the *MYC*-oncogene family, *MYCL*, was identified in small cell lung cancer (SCLC), and demonstrated homology to a small region of both *c-MYC* and *MYCN*. Gene mapping studies assigned *MYCL* to human chromosome region 1p32, a location that is distinct from that of either *c-MYC* or *MYCN* (regions 8q24 and 2p24 respectively) but is also associated with cytogenetic abnormalities in certain human tumours such as thyroid cancer and lung cancer [10, 11]. *MYCL* was found to be amplified in some SCLC cells [12]. In mammals, a fourth member of the *MYC* family, *s-Myc* has been identified, however only *c-MYC*, *MYCN* and *MYCL* have been implicated in the tumorigenesis of specific human cancers [13].

All three tumour-associated *MYC* genes have the same characteristic three-exon structure with the major polypeptide open reading frame residing in the second and third exons. The first exon is not conserved between the genes, but rather possesses regulatory functions, whereas the two coding exons produce highly homologous sections of amino acids interspersed with areas of diminished conservation, leading to the suggestion that individual MYC polypeptides have discrete, independent, functional domains [14]. In tumour biology, many cancers have been shown to exhibit increased levels of MYC protein in tumour tissue relative to the surrounding normal tissues, and this has been shown to contribute to the aggressiveness of the tumour [15]. Importantly, the MYC family of proteins share functionally similar roles, acting as transcription factors to drive cellular proliferation and vasculogenesis, promote metastasis and genomic instability, as well as inhibit cell differentiation and reduce cell adhesion [13, 16]. However, recent findings have also raised the possibility of transcriptionally-independent functions of the MYC proteins [17].

3. The functional activity of MYCN

MYC proteins are well established as nuclear phosphoproteins that act as regulators of transcription, and can both activate and repress the expression of its target genes [16]. *MYCN* encodes a 60kDa protein that has affinity for and binds to DNA, and is phosphorylated by casein kinase II [18, 19]. Phosphorylation is important for the transforming abilities of MYC family members and also for the regulation of MYC protein stability and activity [20]. The

affinity of MYCN protein for DNA relies on the presence of certain motifs, comprising a basic DNA binding region, an α-helical protein-protein interaction domain or helix-loop-helix (HLH), and a leucine zipper motif (Zip) encompassing the bHLH-Zip domain at the carboxy or C-terminus of the protein [9]. The mechanism that mediates the DNA-binding capacity of MYC proteins was confirmed via the identification of MAX, also a bHLH-Zip protein [21]. MYCN and MAX (Figure 1) interact to form a complex that binds to DNA in a sequence specific manner [22]. MYCN binds to MAX protein via its bHLH-LZ region. Several other proteins have also been shown to interact with the C-terminus of MYCN, including YY-1, AP-2, TFII-I and BRCA1 [23], or with the central region of MYCN such as NMi [24], all of which are associated with MYCN's function as a transcriptional regulator.

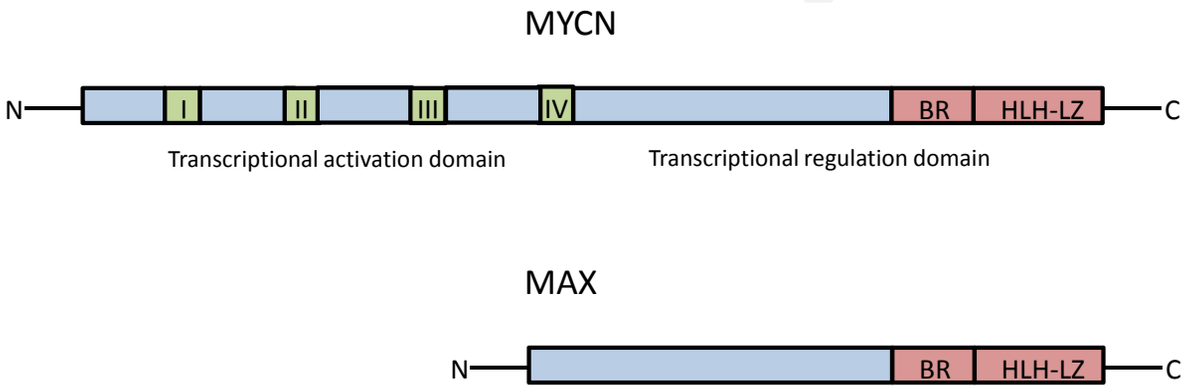

Figure 1. Domains of the MYCN and MAX proteins. The N-terminus of MYCN has three elements, known as MYC homology boxes I-III, which are highly conserved in MYC proteins. The C-terminus contains the basic-region/helix-loop-helix/leucine-zipper that is responsible for interaction with the MAX protein.

The amino or N-terminus of MYCN acts as a transactivation domain that contains two highly conserved regions called Myc Homology Boxes I and II (MBI and MBII) [1]. This region has been shown to bind to nuclear cofactors, including TRRAP, p107, BIN1, MM-1, AMY-1, PAM, α-Tubulin, TIP48 and TIP49, to assist the targeting of protein to specific gene promoters [23, 25]. Another protein YAF2, has been demonstrated to bind to the central region of MYCN to further stimulate transcription upon MYCN-MAX transactivation [26]. All of these interacting proteins are a part of a transcription factor complex by which target genes are activated. Myc Homology Box III (MBIII) is conserved only within c-MYC and MYCN, but not MYCL, and is necessary for cellular transformation [27]. A fourth Myc Homology Box (MBIV) is also necessary for MYC transforming activity [28].

Recent studies have provided evidence of a function of MYCN that is independent from its role as a classical transcription factor. MYCN was shown to remodel large domains of euchromatin, regions of lightly packaged chromatin that contain active, functioning genes, by regulating histone acetylation [29, 30]. Two possibilities have been suggested for this role. The first is that MYCN maintains the activity of euchromatin, whilst the second is that MYCN maintains euchromatin at remote sites to act as an enhancer and regulator of genes at a distance. Novel functions of other MYC proteins have been identified through mutational analyses that have uncoupled the transforming ability of c-MYC from its role as

a transcription factor [28, 31]. c-MYC was found to increase the translation of specific mRNAs by promoting the methylation of the 5′ mRNA guanine "cap", including mRNAs encoding cyclin T1 and CDK9 [31]. A role for c-MYC has also been described in the initiation of DNA replication by binding to various components of the pre-replicative complex and localising to early sites of DNA replication [32]. These observations suggest that c-MYC may play a role in controlling initiation of the S phase of the cell cycle and contribute to replicative stress and genomic instability, to further accelerate tumorigenesis [17]. Even though the evidence has yet to be provided, given the high level of homology between c-MYC and MYCN, the described transcription-independent roles of c-MYC suggest similar roles will be identified for MYCN in contributing to tumour cell biology.

4. MYCN as a transcriptional activator

As indicated above, MYCN heterodimerises with MAX and binds with high affinity to a CACA/GTG E-box sequence found upstream of promoter target sequences [13]. The MYCN-MAX heterodimer activates transcription via several mechanisms. TRRAP (or TRansactivation/tRansformation Associated Protein) binds to the N-terminal region of MYCN and is essential for MYCN transformation. Through TRRAP, MYCN recruits histone acetylation (HAT) complexes to chromatin, including the 1.8 megaDalton SAGA complex (SPT/ADA/GCN5/Acetyltransferase) [33]. Histone acetylation is associated with gene activation by chromatin modification influencing histone-DNA and histone-histone contact [34]. TRRAP is involved with another HAT complex, TIP60, an H2A/H4 acetylase [35]. Interestingly, *in vivo* acetylation of histone H4 is highly associated with MYC target gene activation [36]. Two other proteins, TIP48 and TIP49 that are found in the TIP60 complex also bind to the N-terminus of MYCN [25]. Both proteins are highly conserved hexameric ATPases that are involved in chromatin remodelling involving the movement or displacement of nucleosomes, as opposed to chromatin modification [37].

The MYC family represents a particularly unusual set of transcription factors in that they can bind to and regulate approximately 10-15% of the entire genome [14]. Some MYCN target genes have been shown to be activated independently of TRRAP and HAT complexes. Investigation into HAT independent activation has revealed the involvement of RNA polymerase II at the promoter regions of target genes. c-MYC protein binding has been shown to stimulate the clearance of RNA polymerase II from the promoter region to allow for efficient transcription elongation by the RNA pol II kinases, TFIIH and positive transcription elongation factor b (PTEFb) [38]. c-MYC also regulates RNA pol II promoter clearance by controlling the expression of RNA pol II kinases via mRNA cap methylation, polysome loading, and the rate of translation [31].

5. MYCN as a transcriptional repressor

Most studies have focused on the role of MYC proteins as transcriptional activators. However, cells transformed by constitutive expression of c-MYC are characterised by the loss of expression of numerous genes such as those involved in cell adhesion and cell cycle regulation, and even loss of *c-MYC* itself [39-41]. An early indicator of the transcriptional

repressor role of MYC proteins was the involvement of c-MYC in a negative feedback loop, where the introduction of ectopic c-MYC or MYCN was able to downregulate endogenous expression of c-MYC in mouse fibroblast cells [42]. Structure and function analyses found that the regions of c-MYC that are required for transformation are also required for negative autoregulation [43] and led to the idea that the repression of target genes by MYC proteins could also contribute to transformation.

The understanding of transcriptional repression by MYC proteins was greatly advanced via the identification of repressed target genes such as *transglutaminase-2* (*TG2*) and *interleukin-6* (*IL-6*) [44]. Genomic studies have now revealed that MYC proteins repress as many targets as they activate, emphasising the role of gene repression by these oncoproteins during cellular transformation [14]. One recent example is the identification of *TG2* repression by MYCN in neuroblastoma, which occurs via the interaction between MYCN with Specificity Protein I (SP1) [45]. *TG2* is a multifunctional enzyme that catalyses the transamidation and multimerisation of proteins, but also promotes programmed cell death and induces neuritic differentiation in neuroblastoma cells [46]. Hence downregulation of *TG2* by MYCN would allow neuroblastoma cells to overcome apoptosis and continue to proliferate. Similarly, MYCN has been shown to interact with SP1 to downregulate the expression of *MRP3* (also known as *ABCC3*), the gene encoding an intermembrane transporter which is involved in the transport of organic anions, prostaglandins, leukotrienes and selected chemotherapeutics [47-49]. Another important gene that is downregulated by MYCN is *IL-6*, which has been shown to play an important anti-angiogenic role by inhibiting vascular endothelial cell proliferation [50]. The transcriptional repression by MYCN is also supported by the interaction between MYC proteins and another transcription factor MIZ-1 (Myc-interacting zinc finger protein-1) [51]. MIZ-1 is a POZ/BTB (poxvirus and zinc finger/bric-a-brac, tramtrack and broad complex) domain protein that transactivates genes involved in cell cycle regulation as well as tumour suppressor genes via the recruitment of the p300 histone acetyltransferase [52]. Interestingly, high-level MIZ-1 expression is associated with a favourable disease outcome of neuroblastoma [53]. MIZ-1 interacts with the carboxy-terminal HLH region of c-MYC and MYCN, where the binding of the MYC-MAX heterodimer to MIZ-1 disrupts the interaction between MIZ-1 and p300, causing the transcriptional repression of tumour suppressor genes [54]. MYC has also been shown to recruit a DNA methyltransferase, DNMT3a to the MYC-MIZ-1 complex, suggesting that repression can be mediated by the methylation of target gene promoters [55].

6. Mechanisms of regulating MYCN expression

Due to the gross transforming ability of deregulated expression of MYC proteins, the expression of these protooncogenes is tightly regulated in normal cells at both the transcriptional and protein level. For example, MYC mRNA transcripts and proteins have very short half-lives and are expressed at constant levels as cells enter the cell cycle [56, 57]. Furthermore, anti-proliferative signals trigger rapid down-regulation in expression, and the phosphorylation patterns of MYC proteins are known to influence their stability. In addition to these mechanisms, expression of MYCN is particularly tightly regulated with regards to

timing and tissue specificity. Thus, MYCN is normally expressed during embryonal development of the peripheral nervous system in neural crest cells [2]. Neural crest cells migrate during mid-gestation to populate the entire peripheral nervous system, including autonomic and peripheral ganglia and the adrenal gland. These migrating progenitor cells represent a highly proliferative population, and during normal development exit the cell-cycle and undergo differentiation following the colonisation to the ganglia and spinal cord area. This event is orchestrated by extracellular signalling molecules such as mitogens and cytokines and coincides with decreased expression of MYCN [56, 58]. Without this strict control, dysregulated MYCN expression impairs the ability of progenitor cells to undergo differentiation. Studies which sustained MYCN expression in murine neural crest cells under the control of a tyrosine hydroxylase promoter, demonstrated the capacity to cause neuroblastoma in transgenic mice [3]. Despite this transforming ability, MYCN is vital for normal embryonic development, and murine embryos lacking MYCN exhibit profound hypoplasia, particularly in the central and peripheral nervous system, disorganized architecture of the brain, defective heart development and defects in the lung, genitourinary system, stomach, intestines and limb buds [59].

In order to understand how extracellular stimuli controlled MYC expression in cells, gene mapping studies in association with *MYC* transcription studies were undertaken, and these identified response elements within the *MYC* transcript as well as their regulators. In neuronal cells, *MYCN* has been shown to be regulated in its promoter region as well as in an enhancer region upstream of the coding region. The elongation transcription factor, E2F binds to the promoter region of *MYCN* in response to different mitogenic signals [60]. The promoter region also contains positive transcription factor binding sites for SP1, SP3 and TGFβ [61]. However, the presence of a retinoic acid response element (RARE) within this region allows for negative regulation of *MYCN* by retinoic acid [62].

A key finding was made in 1986 which identified *c-MYC* as the first eukaryotic gene to be negatively regulated by transcriptional elongation control, where a block in the elongation of mRNA during transcription occurred during cellular differentiation [63]. This finding was later confirmed in *MYCN* studies where transcription elongation pausing sites were identified in exon 1 and intron 1 of human *MYCN* [64, 65]. Furthermore, there is *in vivo* evidence that the downregulation of *MYCN* during mouse embryogenesis is partly regulated by the control of transcriptional elongation [66].

Transcription alone cannot account for the large difference in mRNA levels following the introduction of proliferative or anti-proliferative stimuli. The rapid turnover of mRNA was also associated with the discovery of two distinct mechanisms of *MYC* mRNA decay. The first involves a translation-independent mechanism involving poly(A) tail shortening of the untranslated region of the transcript, while the second represents a translation-dependent mechanism that is regulated by a region of mRNA which corresponds to the C-terminus of the protein, called the coding region determinant [67-69]. This region is bound to a 75kDa protein that protects the region of mRNA from endonuclease attack, in response to growth signals that induce c-MYC stabilisation. In the case of *MYCN*, RNA stability factors have also been identified which bind to the untranslated region of *MYCN* mRNA. In addition, an

internal ribosomal entry segment (IRES) in the transcript acts to enhance neuronal specific translation [70, 71].

7. Regulation of MYCN protein expression

The regulation of MYCN protein levels has also been investigated and phosphopeptide analysis has revealed that specific serine and threonine residues of MYCN are phosphorylated *in vivo*. Two residues in particular, Threonine 58 (Thr58) and Serine 62 (Ser62) have been demonstrated as important determinants of transformation and MYCN protein stability and activity [20]. Proliferative stimuli activate phosphorylation of Ser62 by cyclin B and Cdk1 during prophase to increase MYCN protein stability [72]. Phospho-ser62 via a feedback mechanism, then serves as a platform for the phosphorylation of Thr58 by glycogen synthase kinase 3 (GSK3), allowing the tumour suppressor FBW7 to bind and recruit a ubiquitylation complex, directing MYCN protein for degradation. Mitotic degradation of MYCN in the absence of growth factor-dependent signals allows cell cycle exit and the commencement of differentiation [73]. Another kinase, Aurora A, has recently been identified and shown to inhibit degradation of ubiquitinated MYCN by supporting the synthesis of non-degradable ubiquitin chains [74].

8. MYCN downstream target genes

The first transcriptional target for a MYC protein was discovered ten years after the identification of human *c-MYC*. The development of a conditionally expressed *c-MYC* construct, via the fusion of human *c-MYC* to the hormone-binding domain of the oestrogen receptor, led to the identification of a downstream target involved in cell cycle progression, α-prothymosin [75]. This approach was then used to identify additional targets including ornithine decarboxylase 1 (ODC1), the rate-limiting enzyme involved in polyamine synthesis [76]. A different method of identifying MYC targets utilised *MYC*-null models to determine whether the regulation of expression of genes was dependent on the presence of a *MYC* oncogene. Such examples of labour-intensive techniques were invaluable in determining single *bona fide* MYC targets, however recent advances in technology have allowed for large-scale analyses of MYC-regulated genes [77, 78].

Expression microarrays and chromatin immunoprecipitation assays (ChIP) have helped researchers identify MYC-regulated targets as well as link MYC-target expression to functional cellular pathways which are associated with transformation [79, 80]. MYC and MYCN-regulated targets have since been linked to a number of transforming activities involving the cell cycle (eg. cyclin D2, CDK4, p21), cell proliferation (e.g. MDM2), growth, metabolism (e.g. ribosomal proteins, proteins involved in nucleotide biosynthesis such as thymidylate synthase and ODC1), cell adhesion and migration (e.g. integrins) and angiogenesis (e.g. thrombospondin) [81-86]. Indeed, the activation and repression of MYC target genes is a well-coordinated event. Time course studies using microarray have identified differences between early and delayed gene expression responses, following MYC activation in a MYC-inducible cell system [87]. Early-response MYC target genes are

primarily involved in MAPK signalling, RNA metabolism and transcription factors, which suggests a program that prepares cells for entry into the S phase. On the other hand, delayed-response MYC target genes are involved in ribosomal biogenesis, nucleotide metabolism and energy metabolism, suggesting subsequent maintenance of cells during the S phase. Finally, late steady-state MYC-mediated transcription involved genes that regulate the cell cycle, nucleotide metabolism and DNA replication. Most genes that were activated in the early response were then repressed during this late steady-state phase. Furthermore, sustained MYC activation led to the silencing of differentiation-related genes and upregulation of genes that are involved cell proliferation.

During tumorigenesis, MYCN promotes cell cycle progression by the activation of cyclins (such as cyclin D1 and D2) as well as cyclin-dependent kinase 4 (CDK4), and represses the expression of mediators of cell cycle arrest such as p21 [73]. One important MYCN-regulated metabolic pathway involves the synthesis of polyamines, which are organic cations that enhance transcription, translation and replication [88]. *MYCN* expression is strongly correlated with *ODC1* expression in neuroblastoma, and the high levels of *ODC1* expression that are driven by *MYCN*-amplification and over-expression are strongly associated with poor clinical outcome of this disease [89].

Another gene whose expression is strongly correlated with *MYCN* expression in neuroblastoma is that encoding the multidrug resistance-associated protein, MRP1, a glycoprotein that belongs to the superfamily of ATP-binding cassette (ABC) transmembrane transporters [90-92]. MRP1, also known as ABCC1, is able to confer resistance to a broad range of structurally unrelated chemotherapeutic drugs [93]. MRP1 has since been shown to be a downstream transcriptional target of MYCN in neuroblastoma, whose expression is highly predictive of outcome in this disease [91, 94, 95]. The expression of another gene that is also a member of the ABC family of transporters, *MRP4* (or *ABCC4*), has also been demonstrated to be positively correlated to *MYCN* expression in neuroblastoma and like *MRP1*, its over-expression is a prognostic indicator of neuroblastoma outcome [95, 96]. In fact, it has recently been shown that MYCN can coordinate the transcription of a large set of *ABC* genes, and the expression profiles of these genes correlate with MYCN function [48].

9. MYCN tumorigenesis

The evidence for a clinical role of MYCN in the tumorigenesis of neuroblastoma was first recognised when the amplification of the *MYCN* oncogene was identified in 24 out of 63 primary untreated neuroblastoma tumour samples and appeared to correlate with more advanced stage of disease [97]. *MYCN*-amplification was subsequently associated with rapid disease progression as well as poor patient outcome in this disease [98]. Importantly, the progression-free survival of neuroblastoma patients was then shown to be dose-dependent on *MYCN* where higher copy number resulted in lower survival. This association was independent of patient age and disease stage. *MYCN*-amplification was later confirmed in numerous studies to be a powerful prognostic marker for predicting neuroblastoma patient outcome, independent of other clinical variables [99-102]. Determination of *MYCN*

amplification status is now routinely determined in primary neuroblastomas and is one of the most powerful prognostic markers yet identified for this disease.

The *MYCN* oncogene is normally located on the distal short arm of chromosome 2 (2p24). This region was found to be amplified across a panel of neuroblastoma cell lines [8], and although the exact mechanism by which this occurs is unknown, the process of amplification usually results in 50 to 400 copies of the gene per cell, leading to the production of abnormally high levels of *MYCN* RNA and protein, presumably conferring a selective advantage to the tumour cell [103].

The potent transforming ability of MYCN has been demonstrated by several studies, while *MYCN* transfection studies have demonstrated that the oncoprotein plays a crucial role in neuroblastoma progression [104, 105]. Conditional overexpression of *MYCN* in neuroblastoma cell lines was shown to dramatically increase the growth rates and metastatic ability of these tumour cells, increase DNA synthesis, and inhibit exit from the cell cycle and neuronal differentiation [106, 107]. Furthermore, targeted expression of the *MYCN* oncogene in neuroectodermal cells of transgenic mice resulted in the development of neuroblastoma [3]. In these animals, human *MYCN* (*hMYCN*) oncogene expression was targeted to neural crest cells via an upstream rat tyrosine hydroxylase promoter. Tyrosine hydroxylase is the first and rate-limiting step in catecholamine synthesis. In contrast, reduction in the *MYCN* RNA levels via introduction of *MYCN* antisense oligonucleotides *in vitro* as well as *in vivo* led to reduced rates of growth and of tumorigenicity [4, 108, 109].

Whilst *MYCN*-amplification has been shown to be associated with a highly malignant neuroblastoma phenotype, the precise role of this oncogene in non-amplified tumours remains controversial. Approximately 40% of those neuroblastomas that lack *MYCN*-amplification are nevertheless still clinically aggressive, and the clinical significance of *MYCN* expression in the absence of *MYCN*-amplification, remains elusive with evidence both for and against an association with adverse outcome [110, 111]. One study that analysed both *MYCN* mRNA and protein levels in a cohort of non-amplified tumours, found no prognostic significance attributable to expression of this oncogene [110]. Rather, since the survival rates for older children with or without high MYCN expression were poor, the results suggested that additional factors contribute to tumour aggressiveness in this subgroup. Furthermore, in a more recent study involving 91 neuroblastoma patients, high *MYCN* expression was found to be associated with a favourable outcome in neuroblastomas lacking *MYCN*-amplification [111]. Interestingly, in this study, the forced expression of *MYCN* significantly suppressed growth of non-amplified neuroblastoma cells by inducing apoptosis. It is possible that the prognostic value of *MYCN* gene expression in neuroblastoma may be an artefact of the different biology of neuroblastoma in infants compared to older children, and further well-controlled, large cohort studies will be needed in order to clarify the precise role of MYCN in non-amplified neuroblastoma.

Although the majority of the literature investigating *MYCN* in cancer comes from studies on neuroblastoma, this oncogene has also been shown to play a role in the tumorigenesis of other cancers, both adult and paediatric. For example, *MYCN* amplification and/or over-

expression has been observed in high grade C5 serous ovarian tumours, small cell lung cancer, rhabdomyosarcoma and neuroendocrine prostate cancer [112-115], while gain of 2p (and *MYCN*) plays a role in chronic lymphocytic leukaemia [116]. In childhood medulloblastoma, MYCN, c-MYC, and to a lesser extent MYCL, appear to be involved in the biology of this disease [117]. *MYCN* amplification occurs in up to 10% of medulloblastoma patients and is associated with poor clinical outcome, and like neuroblastoma, the risk of death increases with increasing copy number [117]. Furthermore, *MYCN* expression was found to be high in foetal cerebella, with the levels decreasing to almost absent in adult cerebella, suggesting that MYCN is essential to normal foetal development [118]. Interestingly, in this study, *MYCN* expression was absent from the medulloblastoma cell lines tested, which differed from the expression pattern observed in the primary tumours [118]. Finally, as with neuroblastoma, the association of *MYCN* mRNA levels with clinical outcome remains unclear [119] and it has been postulated that mRNA levels of both *c-MYC* and *MYCN* may only be clinically relevant in subgroups of medulloblastoma [117].

The most compelling evidence for a role of MYCN in the biology of medulloblastoma comes from two mouse models of this disease. Firstly, targeted expression of MYCN to the cerebellum in transgenic mice has demonstrated the importance of MYCN in contributing to the initiation and progression of medulloblastoma and also in the metastatic spread of disease to the spinal and paraspinal tissues via cerebral spinal fluid. Furthermore, the MYCN downstream targets Odc1, MDM2 and Fb1 were upregulated and correlated with *MYCN* mRNA levels [118]. The second model used targeted Smoothened (SmoA1) to the cerebella of transgenic mice, which were then crossed with mice harbouring conditional knock-out of MYCN, to demonstrate that MYCN was essential for medulloblastoma tumorigenesis [120]. These two models thus serve to demonstrate the importance of MYCN in the initiation and progression of this disease.

10. Molecular targeting of MYCN for therapeutic benefit

Molecular targeted therapy involves targeting malignant cell growth by directly inhibiting the function of specific molecules within a cell, namely those that are responsible for driving cancer progression. Such agents aim to block or exploit various aspects of cancer biology, such as genetic instability, proliferative signal transduction, aberrant cell cycle control, deregulated survival, angiogenesis and metastasis [121]. Numerous methods of molecular targeted therapy have been investigated, including antisense oligonucleotides (ASOs) that hybridise to and inhibit the mRNA of a specific gene; peptide nucleic acids (PNAs), which are DNA analogues that specifically hybridise to DNA and/or RNA in a complementary manner to inhibit transcription/translation of a target gene; and small interfering RNA (siRNA), which silences gene expression by inducing the sequence specific degradation of complementary mRNA or by inhibiting translation [122]. However, such technologies although useful in the laboratory, have had limited success in the clinic due to problems associated with their delivery.

Immunotherapy has also generated interest, and utilises the body's immune system to target and remove cancer cells by the recognition of certain molecular markers, or block specific

cell receptor pathways. Another approach to molecular targeting, involves the development of synthetic small molecule inhibitors which potentially have the ability to interfere with a molecular target at multiple levels [122]. These small molecules may diffuse into cells to act directly on intracellular targets, such as inhibiting the expression of a target gene at the transcriptional or translational level, or inhibiting the function of a protein by directly binding to the protein and inducing conformational changes that prevent its interaction with other factors [123]. Synthetic small molecules are generally defined by a molecular weight cut-off of <500Da. They are favoured by the pharmaceutical industry because of their attractive pharmacokinetic properties, especially tumour cell penetration, and their relative ease of development and pharmaceutical production [123]. At present, strategies to develop novel small molecule inhibitors as viable therapies are aimed at using these technologies in combination with other cytotoxic drugs, with the hope of reducing drug dosages, and thus overcoming drug resistance associated with intensive chemotherapy, and reducing drug-related toxicity and side effects.

A number of molecular mechanisms have been identified as possible targets for the treatment of neuroblastoma. However, the prominent deregulated expression and amplification of *MYCN* suggests that this oncogene represents an ideal target for therapeutic inhibition [124]. In addition, normal MYCN expression is restricted to the early stages of embryonic development and is virtually undetectable in normal post-natal tissues, therefore weighing in its favour as a target for inhibition. Inhibition of *MYCN* expression by antisense treatment against *MYCN* mRNA or by retinoic acid has been demonstrated to decrease proliferation and induce neuronal differentiation in neuroblastoma cells [125-127]. Furthermore, the introduction of *MYCN* antisense oligonucleotides in the human *MYCN* (*hMYCN*) trangenic mouse model led to reduced rates of tumour growth in these animals [4, 108, 109].

Inhibition of MYCN protein through its protein-protein interactions and protein-DNA interactions was previously seen as too difficult to target by small molecules [128]. However, it has been reported that small-molecule antagonists of MYC/MAX dimerisation interfered with c-MYC-induced oncogenic transformation of chicken embryo fibroblasts *in vitro* [129]. In addition, a number of endogenous MYCN/MAX antagonists such as MAX/MAX have been found to compete for binding to E-box sequences and repress transcription [130], causing cell cycle arrest, terminal differentiation or apoptosis. More recently, inhibition of c-MYC transcription via a Bromodomain and Extra Terminal Domain (BET) inhibitor, JQ1, has been described [131]. This inhibitor has been shown to disrupt c-MYC mRNA synthesis by preventing the recruitment of coactivator proteins required for c-MYC transcriptional initiation and mRNA elongation [131]. Furthermore, this molecule was able to decrease the tumour burden in an orthotopic mouse model of multiple myeloma. Treatment of several MYCN-amplified neuroblastoma cell lines with JQ1, resulted in a decrease in MYCN expression, although this effect was far less dramatic that that observed in a c-MYC driven cell line [132]. Despite promising evidence for targeting MYCN as a therapeutic strategy, no MYC or MYCN inhibitors have yet entered clinical trial, and further studies are required to develop effective MYCN inhibitors.

11. Future perspectives

The validity for targeting MYCN for therapeutic benefit relies on the gross transforming ability of this transcription factor. MYCN represents a particularly attractive target due to its lack of expression in adult and normal paediatric tissues. Although MYCN, and MYC proteins in general are commonly viewed as "undruggable" due to the nature of these proteins, MYCN offers potential advantages at a number of levels for therapeutic inhibition, either upstream, or downstream along the MYCN transcriptional pathway. If clinically useful MYCN inhibitors can be successfully developed, they are likely to find application in combination therapies involving conventional chemotherapeutic drugs and be used as an improved approach to target aggressive cancers that are driven by this oncoprotein.

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