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Myc proteins as therapeutic targets

WC Gustafson[1,2] and **WA Weiss**[1,3,4]

[1] Department of Pediatrics, University of California, San Francisco, CA, USA

[2] Division of Pediatric Hematology/Oncology, University of California, San Francisco, CA, USA

[3] Department of Neurology, University of California, San Francisco, CA, USA

[4] Department of Neurosurgery, University of California, San Francisco, CA, USA

Abstract

Myc proteins (c-myc, Mycn and Mycl) target proliferative and apoptotic pathways vital for progression in cancer. Amplification of the *MYCN* gene has emerged as one of the clearest indicators of aggressive and chemotherapy-refractory disease in children with neuroblastoma, the most common extracranial solid tumor of childhood. Phosphorylation and ubiquitin-mediated modulation of Myc protein influence stability and represent potential targets for therapeutic intervention. Phosphorylation of Myc proteins is controlled in-part by the receptor tyrosine kinase/phosphatidylinositol 3-kinase/Akt/mTOR signaling, with additional contributions from Aurora A kinase. Myc proteins regulate apoptosis in part through interactions with the p53/Mdm2/Arf signaling pathway. Mutation in *p53* is commonly observed in patients with relapsed neuroblastoma, contributing to both biology and therapeutic resistance. This review examines Myc function and regulation in neuroblastoma, and discusses emerging therapies that target Mycn.

Keywords

myc; mycn; neuroblastoma; N-myc; mTor; PI3K

Introduction

Neuroblastoma, a neoplasm of peripheral neural crest origin, is the most common malignant extracranial solid tumor of childhood and accounts for 15% of cancer deaths in children (Park *et al.*, 2008). Approximately 650 new cases are diagnosed in the United States annually with peak incidence in early childhood (ages 0–4 years). The most common site of origin is in the adrenal medulla; however, tumors can occur anywhere along the sympathetic chain.

Patients with new diagnoses are typically stratified into risk groups based on age, stage, histopathology, DNA index and genetic/genomic factors. Amplification of the proto-oncogene *MYCN* occurs in ~25% of tumors and is the best characterized genetic-risk factor for high-risk chemotherapy-refractory disease (Brodeur *et al.*, 1984; Seeger *et al.*, 1985; Riley *et al.*, 2004). Deletion or suppression of caspase 8, loss of chromosomes 1p and 11q, and gain of 17q also correlate with aggressive disease (Bown *et al.*, 1999; Guo *et al.*, 1999; Riley *et al.*, 2004; Attiyeh *et al.*, 2005; Stupack *et al.*, 2006; Maris *et al.*, 2008b).

Correspondence: Dr WC Gustafson, Pediatric Hematology/Oncology, University of California, Helen Diller Family Cancer Research Building, 1450 3rd Street, San Francisco, CA 94158-9001, USA. GustafsonC@peds.ucsf.edu.

Conflict of interest

The authors declare no conflict of interest.

In contrast with most other treatment-refractory cancers, neuroblastomas, irrespective of risk group, generally respond to initial therapy, which typically includes high doses of chemotherapy (Park *et al.*, 2008). Low- and intermediate-risk patients are subsequently cured and rarely progress. Patients with high-risk disease typically relapse with treatment-refractory tumors. It is conceivable that low- and high-risk neuroblastoma are entirely separate disease entities. Overall, prognosis in the high-risk group is quite poor, with long-term survival of 30–40%.

Familial neuroblastoma

Neuroblastoma may be associated with Hirschsprung disease and neurofibromatosis type 1 (Clausen *et al.*, 1989; Rohrer *et al.*, 2002). Mutations in *PHOX2B*, a homeodomain-containing transcription factor important for the development in the autonomic nervous system, underlie the congenital central hypoventilation syndrome and confer a heritable predisposition to neuroblastoma; however, these mutations are not found in spontaneous tumors (Mosse *et al.*, 2004; Trochet *et al.*, 2005; Raabe *et al.*, 2008). In fact, familial neuroblastoma is quite rare, and is most often because of dominant gain-of-function mutations in the orphan receptor tyrosine kinase (RTK) anaplastic lymphoma kinase (*ALK*). *ALK*, which is linked to *MYCN* on chromosome 2p23, also shows sporadic gain-of-function mutation in 8% of spontaneous tumors (Chen *et al.*, 2008; George *et al.*, 2008; Janoueix-Lerosey *et al.*, 2008; Mossé *et al.*, 2008). Although direct connections between *MYCN* and *ALK* have yet to be elucidated, activation of Alk and other RTKs may contribute to stabilization of Mycn protein (detailed below).

Myc family of proto-oncogenes

Broadly implicated in oncogenesis, the human *MYC* family of proto-oncogenes is among the most studied genes in cancer (Meyer and Penn, 2008). Early insertional mutagenesis studies in mouse identified *c-MYC* (homologous to the *v-myc* gene that drives avian myelocytosis) as capable of transformation by retroviral promoter insertion (Payne *et al.*, 1982). *MYCN* was subsequently identified as an *MYC* homolog amplified in neuroblastoma tumors (reviewed in Meyer and Penn, 2008). Amplification of *MYCN* has emerged as among the clearest genetic indicators of high-risk, aggressive disease (Brodeur *et al.*, 1984; Seeger *et al.*, 1985). MYC family members (c-MYC, MYC and MYCL) show differential expression in normal tissues. Expression of murine N-myc in particular is elevated in normal mammalian developing retina, forebrain, hindbrain, intestine, kidney and has functions in neuronal progenitor cells, developing lung tissues, hematopoetic stem cells and programmed cell death in the developing limb (Zimmerman *et al.*, 1986; Mugrauer *et al.*, 1988; Downs *et al.*, 1989; Hirvonen *et al.*, 1990; Hirning *et al.*, 1991; Knoepfler *et al.*, 2002; Bettess *et al.*, 2005; Okubo *et al.*, 2005; Ota *et al.*, 2007; Martins *et al.*, 2008; Xu *et al.*, 2009).

Myc proteins are basic helix-loop-helix leucine zipper transcription factors. Mycn and c-Myc proteins share several regions of homology and share similar cellular functions. Myc proteins localize to the nucleus and form heterodimers with the basic helix-loop-helix molecule, Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Berberich and Cole, 1992; Blackwood *et al.*, 1992; Kato *et al.*, 1992). Myc/Max heterodimers bind to DNA at specific CAC(G/A)TG ‘E-box’ sequences to drive transcription of targets important for proliferation, apoptosis and differentiation (Blackwell *et al.*, 1990, 1993; Amati *et al.*, 1992; Kretzner *et al.*, 1992). Max also heterodimerizes with Mxd or Mnt proteins to influence the transcription of other downstream genes and often to antagonize the proliferative effects of Myc proteins (Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1995, 1997; Walkley *et al.*, 2005). In many systems, Mxd/Max or Mnt/Max heterodimers oppose the actions of Myc/Max to repress transcription; however, very little is known about these interactions in neuroblastoma

(Grandori *et al.*, 2000; Patel *et al.*, 2004). The complex competition among Myc, Mxd and Mnt proteins for binding to Max further modulates the effects of both c-Myc, and probably Mycn, on gene expression.

Downstream of Mycn

E-boxes are common (~25% of known promoters) with >10 000 sites per cell (Fernandez *et al.*, 2003; Li *et al.*, 2003; Zeller *et al.*, 2006). That there are more E-box sequences than Myc molecules in cells represents a conundrum apparently common to many transcription factors (Farnham, 2009). Adding further complexity to this system, Myc proteins also regulate downstream targets through cap-dependent methylation, altering both global translation as well as the translation of specific proteins (Barna *et al.*, 2008; Cole and Cowling, 2008).

Chromatin immunoprecipitation experiments show that c-Myc and Mycn proteins bind to promoters with variable specificity determined by DNA ultrastructure and cellular context (Fernandez *et al.*, 2003; Li *et al.*, 2003; Mao *et al.*, 2003; Chen *et al.*, 2004; Guccione *et al.*, 2006; Zeller *et al.*, 2006; Kim *et al.*, 2008; Martinato *et al.*, 2008; Westermann *et al.*, 2008). Myc/Max heterodimers bind to E-boxes and interact with a variety of histone modifiers, increasing histone acetylation (Bouchard *et al.*, 2001). These alterations modify chromatin at promoters, affecting gene expression (McMahon *et al.*, 2000; Frank *et al.*, 2001; Vervoorts *et al.*, 2003; Guccione *et al.*, 2006; Martinato *et al.*, 2008; Liu *et al.*, 2009). In fact, histone modification by Mycn effectors is being exploited pharmacologically using histone deacetylase inhibitors. These drugs may alter acetylation in neuroblastomas and other *MYC* dependent processes thereby modulating transcription (reviewed in Witt *et al.*, 2009).

Several studies have attempted to elucidate specific transcriptional targets for Mycn in neuroblastoma (Alaminos *et al.*, 2003). A large number of targets important for cell cycle control and differentiation have been characterized, including: downregulation of SKP2 and TP53INP1 with resultant decrease in $p21^{WAF1}$ (Bell *et al.*, 2007), downregulation of DKK1 upstream of the wnt/β-catenin pathway (Koppen *et al.*, 2007), upregulation of NLRR1 both important in neural cell proliferation (Hossain *et al.*, 2008), downregulation of Fyn kinase important in differentiation (Berwanger *et al.*, 2002), regulation of multiple genes responsible for pluripotency (Cotterman and Knoepfler, 2009) and modulation of apoptosis by upregulation of p53 and Mdm2 (Slack *et al.*, 2005b). The multidrug resistance gene MRP1 is regulated by Mycn, driving chemotherapy resistance (Manohar *et al.*, 2004). Importantly, Mycn upregulates oncogenic microRNAs, which have wide ranging effects on cancer (reviewed in Schulte *et al.*, 2009). Mycn also controls several proteins important in ribosome biogenesis (Boon *et al.*, 2001) affecting protein synthesis (reviewed in Ruggero, 2009).

Different groups seeking to stratify neuroblastoma risk using gene expression micoarrays have generated gene lists that are largely non-overlapping (Berwanger *et al.*, 2002; Ohira *et al.*, 2005; Schramm *et al.*, 2005, 2009; Oberthuer *et al.*, 2006). Similar strategies using real-time PCR transcript analysis, are also being performed (Vermeulen *et al.*, 2009). Direct comparison of targets from chromatin immunoprecipitation shows that Mycn and c-Myc have many overlapping targets (Westermann *et al.*, 2008). Further refinement of microarray and chromatin immunoprecipitation techniques and identification of critical transcriptional targets specific to Mycn may provide insights into both biology and therapy for neuroblastoma.

Mycn in cell cycle control

In normal cells, levels of Myc proteins are tightly regulated, with increased levels driven in part through activation of phosphatidylinositol 3-kinase (PI3K), which stabilizes Mycn and c-Myc proteins (Figure 1), enabling entry into the cell cycle (Marqués *et al.*, 2008). In addition to the clearly defined function of Myc family members in control of the cell cycle, c-Myc

contributes non-transcriptionally to the initation of DNA replication (Dominguez-Sola *et al.*, 2007). Myc/Max dimers also bind and inhibit Miz-1, a helix-loop-helix transcription factor (reviewed in Herold *et al.*, 2009). Free Miz-1 promotes transcription of p15INK4b and p21Cip1 proteins associated with cell cycle arrest. Inhibition of Miz-1 in response to Myc/Max binding contributes to immortalization, transformation and oncogenesis (Seoane *et al.*, 2001;Staller *et al.*, 2001;Herold *et al.*, 2008). Interactions between Mycn and Miz-1 are incompletely characterized. Expression of Miz-1 has been associated with favorable outcome in neuroblastoma, however, consistent with an interaction among Miz-1, Mycn and Max (Ikegaki *et al.*, 2007).

Transcription of *MYCN* is downregulated by the neuroblastoma differentiating agent retinoic acid and upregulated by several known transcription factors including E2F and Sp1/Sp3 (Thiele *et al.*, 1985; Inge *et al.*, 2002; Kramps *et al.*, 2004; Kanemaru *et al.*, 2008). Sonic hedgehog indirectly regulates transcription of MYCN in developing neurons (Kenney *et al.*, 2003, 2004; Oliver *et al.*, 2003; Mill *et al.*, 2005). Levels of Myc mRNA are also by alternate internal ribosomal entry sites (Barna *et al.*, 2008; Cobbold *et al.*, 2008). Although natural antisense transcripts are frequently co-amplified with *MYCN* in neuroblastoma, the importance of these co-amplified sequences remains unclear (Krystal *et al.*, 1990; Armstrong and Krystal, 1992; Jacobs *et al.*, 2009).

Mycn and apoptosis

In addition to promoting proliferation, the expression of c-Myc and Mycn actually drives apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992; Fulda *et al.*, 1999; Paffhausen *et al.*, 2007; Ushmorov *et al.*, 2008). Transformation by Myc proteins, therefore, requires concomitant inhibition of apoptosis. Tissue-specific expression of a switchable allele of c-Myc also induced apoptosis *in vivo*. In contrast, proliferation and tumorigenesis required lower level, continuous and deregulated expression of c-Myc (Murphy *et al.*, 2008).

Although the association between *MYCN* amplification and poor outcome has been reproduced in numerous studies over decades, a similar association between expression of Mycn and outcome remains controversial (Chan *et al.*, 1997; Cohn *et al.*, 2000; Tang *et al.*, 2006). Data from preclinical models of switchable myc above suggest that low levels of myc proteins may drive proliferation, with higher levels required to induce apoptosis. If it is true in the context of Mycn and neuroblastoma, then *MYCN* amplification may serve primarily to dysregulate Mycn during the cell cycle, rather than simply driving high-level expression. This hypothesis is consistent with the claims that amplification and not overexpression is predictive of aggressive disease, although inconsistent with observations that *MYCN* is often amplified and overexpressed to extreme levels in neuroblastoma.

Mechanisms through which apoptosis is inhibited as a contributer to Mycn-driven transformation are complex and not yet fully elucidated. Silencing of the apoptotic initiator Casp8 is observed frequently in neuroblastoma (Stupack *et al.*, 2006). Crosstalk with the *p53* pathway has been implicated in apoptosis mediated by both Mycn and c-Myc (reviewed in Hoffman and Liebermann, 2008; Van Maerken *et al.*, 2009b). Mutations in *p53* are rare in primary neuroblastoma (<2%) irrespective of *MYCN* amplification (Vogan *et al.*, 1993). However, inactivating mutations in *p53* and in *p53* pathway members are common at relapse (Keshelava *et al.*, 1997, 2000; Tweddle *et al.*, 2001; Carr *et al.*, 2006). Myc proteins indirectly regulate the p53 pathway and *p53*-dependent apoptosis through the $p14^{ARF}$-MDM2-p53 axis (reviewed in Van Maerken *et al.*, 2009b).

At baseline, p53 is tightly regulated by Mdm2, which binds to and inhibits the transactivation domain of p53 (Oliner *et al.*, 1993; Thut *et al.*, 1997). Mdm2 also ubiquitinates and targets p53 protein for degradation (reviewed in Coutts *et al.*, 2009). Further regulation is conferred by the

tumor suppressor protein $p14^{ARF}$, which binds to and inhibits Mdm2, allowing activation and stabilization of p53 (Kamijo *et al.*, 1998; Zindy *et al.*, 1998; Weber *et al.*, 1999; Midgley *et al.*, 2000; Lin and Lowe, 2001). The balance between apoptosis and survival remains in equilibrium through multiple feedback and feed-forward loops affected by other signaling pathways including external apoptotic stimuli.

In neuroblastoma cells, Mycn directly stimulates transcription of MDM2 (Slack *et al.*, 2005a; Barbieri *et al.*, 2006; Chen *et al.*, 2009). The resulting inhibition of p53 may in part allow cells to escape Mycn-primed apoptosis. Myc and Mycn also indirectly inhibit $p14^{ARF}$, through directly driving transcription factors including TWIST1, resulting in activation of MDM2 and escape from apoptosis (Maestro *et al.*, 1999; Valsesia-Wittmann *et al.*, 2004). The $p14^{ARF}$ protein can in-turn feed back and bind to Myc and Mycn proteins, abrogating their ability to activate downstream targets (Qi *et al.*, 2004; Amente *et al.*, 2007). The complex interactions among Myc/Mycn, Mdm2 and $p14^{ARF}$ provide mechanisms through which Mycn both indirectly activates or inactivates p53, altering the sensitivity of cells to apoptotic stimuli (reviewed in Li and Hann, 2009; Van Maerken *et al.*, 2009b).

Neuroblastoma tumors at diagnosis are generally wild type for p53 and respond to chemotherapy. Children with high-risk tumors generally relapse, whereas those with low and intermediate disease are typically cured. As stated above, Mycn-induced downregulation of the p53 axis could potentially underlie the minimal residual disease that drives subsequent relapse in newly diagnosed *MYCN*-amplified neuroblastomas. This hypothesis is supported by the identification of Mdm2 as an Mycn target, and in studies showing that Mdm2 haploinsufficiency inhibits tumorigenesis in *MYCN*-driven models for neuroblastoma (Slack *et al.*, 2005a; Chen *et al.*, 2009). In fact, small molecule inhibitors of the p53/Mdm2 interaction ('Nutlins') are currently in development and have shown some promise for the preclinical treatment of cancers including neuroblastoma (Barbieri *et al.*, 2006; Chen *et al.*, 2009; Van Maerken *et al.*, 2009a).

Modeling MYCN-amplified neuroblastoma

Mice carrying an *MYCN* transgene under control of the rat tyrosine hydroxylase promoter develop neuroblastoma tumors several months after birth (Norris *et al.*, 2000; Weiss *et al.*, 2000). Tumors from mice transgenic for TH-*MYCN* develop in adrenal and mesenteric ganglia and in paraspinous locations. Histolology and genetics show similarities with high-risk neuroblastoma (Weiss *et al.*, 2000; Hackett *et al.*, 2003; Moore *et al.*, 2008). In relatively resistant strains or subspecies (for example C57BL/6, BALBc or *Mus musculus castaneus*), a range of differentiation was observed in murine tumors. However, when crossed into highly penetrant strains, such as 129 SvJ, the histology was uniformly undifferentiated small round blue cells.

Cell lines derived from TH-*MYCN* tumors retain the ability to form tumors in nude mice (Cheng *et al.*, 2007). Unlike xenograft models of neuroblastoma, TH-*MYCN* tumors show native host interactions with the tumor microenvironment including vascular cells (Chesler *et al.*, 2007). When treated with chemotherapy, tumors in this model, similar to their human *p53* wild-type counterparts, undergo apoptosis in a *p53*-dependent manner. Furthermore, tumors from TH-*MYCN*;*p53*−/+ mice were refractory to cytoxic chemotherapy (Chesler *et al.*, 2008). These data and corresponding data from human tumors (Keshelava *et al.*, 1998, 2000; Xue *et al.*, 2007) collectively suggest a model in which newly diagnosed neuroblastoma tumors impair the $p14^{ARF}$-MDM2-p53 axis in the absence of *p53* mutation, enabling tumors to arise. Subsequent chemotherapy subsequently provides strong selective pressure for inactivating mutations in *p53* or in components of the *p53* pathway, resulting in chemotherapy-refractory tumors seen clinically in relapsed patients. Thus, although mutation at *p53* rarely

contributes to the biology of primary neuroblastoma, therapy-selected mutations in *p53* drive a genetically distinct tumor at relapse. This therapy-associated alteration in the biology of neuroblastoma presents a challenge in identifying effective treatments for relapsed tumors.

Mycn as a target for therapy

In light of both the frequency and importance of *MYCN* amplification in pathogenesis of high-risk neuroblastoma, blockade of Mycn signaling represents an important approach for the developmental therapeutics. The resistance of high-risk relapsed neuroblastoma to conventional chemotherapy and the high morbidity and mortality in these patients present a formidable challenge for clinicians. The prominence of *MYCN* amplification in the pathogenesis of this disease points to Mycn as a potential therapeutic target.

Neuroblastoma presents both common and unique challenges for therapy. The initial response to chemotherapy even in high-risk disease is somewhat unusual. However, the acquisition of *p53* mutant, therapy-refractory disease is common to many cancers (McDermott *et al.*, 2008). Deregulated expression of Mycn may contribute to genomic instability and, in combination with the strong selective pressures of chemotherapy and radiation, select for mutation at *p53* through interactions with Mdm2 and p14Arf detailed above, and by driving the cell cycle and activating *p53*-dependent check points. Alleviation of check-point activation by blocking Mycn itself could conceivably impair the acquisition of *p53* mutant, refractory disease.

Transcription factors have long been considered as targets for cancer therapy; however, clinical approaches to block this class of molecules remain elusive. Clearly, the most direct means of silencing these molecules would be by disrupting Myc synthesis directly through RNAi methods and there are numerous examples of siRNA used successfully on cultured cells. However, although these approaches are extremely useful tools in the laboratory, they have yet to achieve regular use in the clinic largely because of difficulty with delivery (Whitehead *et al.*, 2009).

It is difficult to develop drugs with activities sufficient to block protein–protein interactions or binding of such factors to target sequences. Molecules developed against c-Myc have been primarily directed against the Myc/Max interaction domain and so far have fairly low potency. Progress is being made in this area, however, and the development of inhibitors, which dissociate Myc/Max heterodimers is important (Yin *et al.*, 2003; Wang *et al.*, 2006; Berg, 2008; Brooks and Hurley, 2009). Chemists are good at developing kinase inhibitors, however, and the stability of Myc molecules is regulated at a number of levels by kinases and critical phospho-residues.

Post-transcriptional modification and stabilization of Myc proteins

In neuroblastoma and in neural progenitor cells, Mycn protein stability is regulated by a complex signaling network involving both feedback and feed-forward loops (Figure 1) (Kenney *et al.*, 2004;Sjostrom *et al.*, 2005;Chesler *et al.*, 2006;Kang *et al.*, 2008). Although both c-Myc and Mycn are widely phosphorylated, sites critical to stabilization of Myc proteins are located in the N-terminal Myc Box I, which is commonly mutated in c-Myc in Burkitt lymphoma (Bhatia *et al.*, 1993;Smith-Sørensen *et al.*, 1996;Bahram *et al.*, 2000). Comparable mutations in c-Myc and Mycn are rare in solid tumors including neuroblastoma. The important residues in the Myc Box I region of c-Myc and Mycn are threonine 58 and serine 62 (Henriksson *et al.*, 1993;Pulverer *et al.*, 1994;Lutterbach and Hann, 1999;Sjostrom *et al.*, 2005).

Phosphorylation at S62 stabilizes Mycn protein and primes it for phosphorylation at T58. Candidate kinases proposed to phosphorylate c-Myc at S62 include extracellular signal-regulated kinase, the canonical downstream effector of the Ras/Raf/MAPK; c-Jun N-terminal

kinase and cyclin-dependent kinase 1 pathway (Henriksson *et al.*, 1993; Lutterbach and Hann, 1999; Sears *et al.*, 2000; Benassi *et al.*, 2006). As shown in cerebellar neural progenitor cells and synchronized mitotic SJ8 neuroblastoma cells, cyclin-dependent kinase 1 is thus far the only candidate priming kinase for Mycn at S62 (Sjostrom *et al.*, 2005).

Myc and Mycn proteins monophosphorylated at S62 are substrates for a second phosphorylation at T58, controlled by glycogen synthase kinase 3β (Gsk3β). Gsk3β signals in the Wnt pathway (reviewed in MacDonald *et al.*, 2009), and also downstream of the PI3K/Akt/mTOR pathway (reviewed in Engelman, 2009; Ma and Blenis, 2009; Memmott and Dennis, 2009). The S62 priming phosphorylation site in Myc/Mycn promotes interaction with a complex containing Gsk3β, Pin1, PP2A and Axin. This complex induces the phosphorylation at T58 by Gsk3β in both Myc and Mycn. For c-Myc, Pin1 has been shown to regulate dephosphorylation of S62 by PP2A, a phosphatase and tumor suppressor that itself is regulated by mTOR (Peterson *et al.*, 1999; Gingras *et al.*, 2001; Hartley and Cooper, 2002; Yeh *et al.*, 2004; Arnold and Sears, 2006; Arnold *et al.*, 2009). Regulation of Mycn dephosphorylation by Pin1 and PP2A has not been fully characterized.

Myc proteins monophosphorylated at T58, then bind to the E3 ligase Fbxw7 and are targeted for ubiquitination and degradation (Saksela *et al.*, 1992; Lutterbach and Hann, 1994; Sears *et al.*, 1999; Gregory and Hann, 2000; Oliver *et al.*, 2003; Herbst *et al.*, 2004; Kenney *et al.*, 2004; Welcker *et al.*, 2004; Yada *et al.*, 2004; Otto *et al.*, 2009). Aurora kinase A, which has critical functions in cell cycle regulation and spindle assembly, contributes at this step to the stabilization of phosphorylated and ubiquitinated Mycn, but not of equivalently modified c-myc (Otto *et al.*, 2009). Consistent with these findings, expression of Aurora A kinase is a negative prognostic factor in neuroblastoma (Shang *et al.*, 2009). Although the emerging function of inhibitors of Aurora kinase A in cancer therapy suggested that inhibitors of Aurora kinase A might have unique and multifaceted functions in the therapy of neuroblastoma, stabilization of Mycn apparently requires a scaffold function of Aurora kinase and was independent of Aurora A kinase activity (Maris, 2009; Otto *et al.*, 2009).

Myc proteins as downstream targets of PI3K

The phosphorylation of c-Myc and Mycn is thus regulated directly by Gsk3β, and indirectly by upstream signaling through RTKs, PI3K, Akt and mTOR. RTKs are important to PI3K signaling in neuroblastoma. As mentioned above, activating mutations in the orphan RTK ALK are found in ~9% of neuroblastoma. Other RTKs that could potentially regulate stabilization of Mycn include the insulin-like growth factor receptor and the Trk family of neurotropin receptors (TrkA, TrkB and TrkC). Although mutation in Trk genes is not typically observed in neuroblastoma, expression of TrkA correlates with favorable prognosis, whereas expression of TrkB correlates with amplification of *MYCN* and poor outcome (reviewed in Brodeur *et al.*, 2009). Although neither Trk nor Alk proteins have been directly linked to stabilization of Mycn, these kinases are all upstream activators of PI3K, implying a possible connection (Bai *et al.*, 2000; Norris *et al.*, 2000; Ho *et al.*, 2002; Marzec *et al.*, 2007).

RTKs activate PI3K, which catalyzes the conversion of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) (reviewed in Ma and Blenis, 2009; Memmott and Dennis, 2009). PIP3 binds to Akt and localizes it to the membrane, enabling phosphorylation at T308 by the kinase PDK1. Activation of Akt leads to phosphorylation and inactivation of Gsk3β, stabilizing Mycn by blocking phosphorylation at T58. Activated Akt also phosphorylates and inhibits the tuberous sclerosis 2 (Tsc2) tumor suppressor protein. Tsc2 binds to Tsc1, enabling the Ras-related GTPase Rheb to stimulate mTOR bound in the mTORC1 complex of proteins. As mentioned above, mTORC1 downregulates PP2A, which normally dephosphorylates Myc/Mycn at S62, targeting it for ubiquitination, and promoting

stabilization of Myc and Mycn. Thus, activation of RTKs and PI3K converge on Akt to phosphorylate and inhibit Gsk3β, blocking phosphorylation at T58 and promoting stabilization. In addition, Akt activates mTORC1, inhibiting dephosphorylation of T58/S62-phosphorylated Myc/Mycn at S62, leading to further stabilization of Myc/Mycn.

The critical importance of Mycn phosphorylation and stability as a downstream target of PI3K/Akt/mTOR in neuroblastoma cells is apparent when neuroblastoma cells are treated with a broad spectrum PI3K inhibitor. Activation of Akt predicts poor outcome in neuroblastoma patients (Opel *et al.*, 2007). Treated cells show a decreased proliferation, which is largely rescued when they are engineered to express T58/S62 phosphorylation site-deficient Mycn mutants (Sjostrom *et al.*, 2005; Chesler *et al.*, 2006). These data show that degradation of Mycn is a critical downstream factor in the efficacy of PI3K/mTOR pathway and suggest that clinical inhibitors of PI3K should show activity in Mycn-driven neuroblastoma (detailed review in Fulda, 2009).

Mycn stability as a therapeutic target in neuroblastoma

The post-translational modification and stabilization of Myc and Mycn proteins represents an area with promise for currently available and emerging-targeted therapies. Inhibitors of RTKs (Alk and Trk), PI3K and Akt should activate Gsk3β (which is negatively regulated by phosphorylation), whereas inhibitors of mTOR kinase activity should inhibit the de-activation of PP2A by mTORC1, enabling PP2A to dephosphorylate S62, collectively driving degradation of Mycn in neuroblastoma (Figure 1). Specific Alk and Trk inhibitors are currently in development and have shown promise preclinically and in phase I trials (reviewed in Chiarle *et al.*, 2008;Li and Morris, 2008;Brodeur *et al.*, 2009;Mossé *et al.*, 2009).

Downstream of RTKs, inhibitors of PI3K, mTOR, dual inhibitors of PI3K/mTOR and inhibitors of Akt are all in clinical trials (reviewed in Garcia-Echeverria and Sellers, 2008; Engelman, 2009). Allosteric inhibitors of mTORC1 (so-called rapalogs) block mTOR independently of ATP binding, are currently being tested in neuroblastoma and have shown mixed results preclincally (Houghton *et al.*, 2008; Johnsen *et al.*, 2008; Maris *et al.*, 2008a; Wagner and Danks, 2009). These agents only affect some outputs of mTORC1. In comparison, ATP-competitive inhibitors of mTOR more completely block mTORC1 and also block mTORC2 (Feldman *et al.*, 2009; Thoreen *et al.*, 2009; Yu *et al.*, 2009; Zask *et al.*, 2009). The availability of inhibitors targeting RTKs, PI3K, Akt and mTOR, and the use of these to destabilize Mycn protein, represent important areas of investigation. Further, because of the complex interrelation of pathway members, inhibition at one point often induces feedback activation in other signaling pathways, justifying the need to test these agents in combination using preclinical models.

The regulation of Mycn phosphorylation also involves a priming phosphorylation at S62. As efficient destabilization of Mycn would require activators of kinases responsible for these phosphorylation steps, the ability to finesse phosphorylation at S62 presents a therapeutic challenge. Aurora kinase A represents an additional therapeutic target, as Aurora kinase A stabilizes Mycn at later steps. Inhibitors of Aurora A kinase are currently in clinical trials in neuroblastoma (Shang *et al.*, 2009). However, asMycn is stabilized by a kinase-independent activity of Aurora A, these inhibitors are unlikely to affect Mycn protein (Gautschi *et al.*, 2008; Maris, 2009). An allosteric and ATP-competitive small molecule inhibitor of Aurora A–Mycn interactions, if it could be developed, should retain the ability to block kinase-dependent functions, whereas also twisting Aurora A kinase, thereby disrupting a scaffolding function and degrading Mycn protein. The interplay among RTKs, PI3K, Akt, mTORC1/2, Aurora A and Mycn is quite complex. However, the broad functions for these kinases in cancer biology, and the specific function in regulating the stabilization of Mycn proteins suggests functions in

both Mycn-driven and Mycn-independent cancers including neuroblastoma. The current availability of clinical Alk, Trk, PI3K, mTOR and Aurora inhibitors presents an important translational opportunity to test these agents in children with high-risk neuroblastoma.

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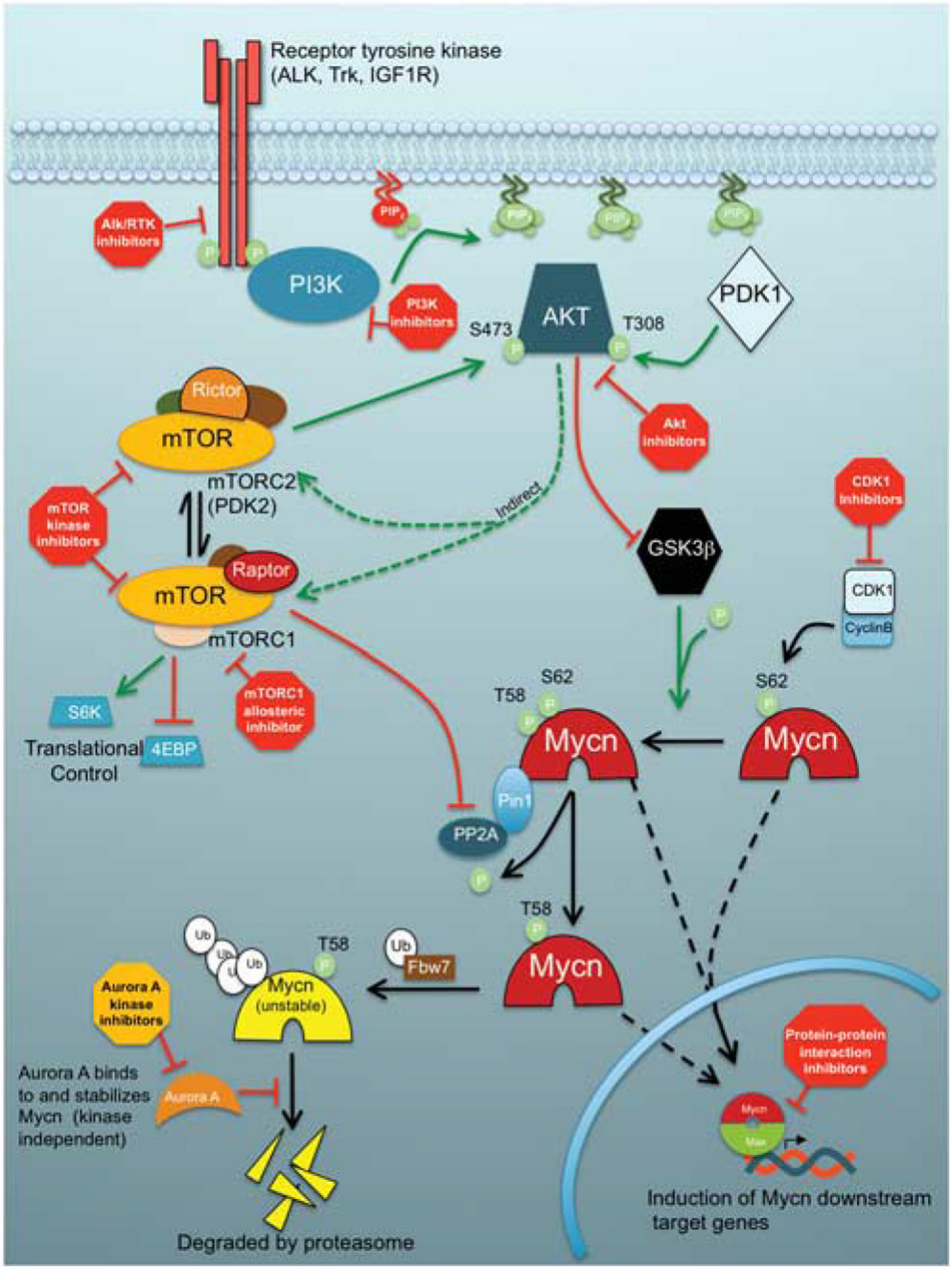

Figure 1.

Schematic model of regulatory pathways involved in Mycn stabilization. In proliferating cells, Mycn is initially phosphorylated at serine 62 by cyclin-dependent kinase 1/CyclinB. The S62-phosphorylated protein is stabilized and competent to enter the nucleus. Nuclear Mycn binds to its partner, the helix-loop-helix transcription factor Max and stimulates the transcription of a target genes important in cell cycle, proliferation, differentiation and apoptosis. This priming phosphorylation at S62 allows the binding of Gsk3β, as well as Pin1 and PP2A in a complex also containing Axin. Active Gsk3β phosphorylates Mycn at threonine 58, producing doubly phosphorylated, stabilized and transcriptionally active Mycn. In a Pin1-mediated process, PP2A dephosphorylates the S62 phosphate, enabling binding of ubiquitin ligase Fbw7. Fbw7

subsequently drives poly-ubiquitination and proteasomal degradation of Mycn. Aurora A kinase can bind to and stabilize polyubiquitinated/phosphorylated Mycn, potentially leaving it competent to bind to Max and activate transcription. In this model, upstream signaling starts at the membrane with receptor tyrosine kinases (RTKs), which are either activated by ligand or by constitutively activating mutations (in Alk, for example). RTKs activate phosphatidylinositol 3-kinase (PI3K), which catalyzes the conversion of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then binds to Akt, localizing it to the membrane and allowing the phosphorylation and activation of Akt by membrane bound Pdk1 at threonine 308. Active Akt then phosphorylates Glycogen synthase kinase 3β (Gsk3β) and inactivates it, blocking Gsk3β-mediated phosphorylation of Mycn T58 and thereby stabilizing Mycn. Akt also activates mammalian target of rapamycin (mTOR) through several indirect signaling mechanisms including through Tsc2/Tsc1 and Rheb. mTOR exists in two distinct complexes. mTORC1 complex, the rapamycin sensitive form, consists of mTOR bound to multiple effector proteins, including Raptor and Lst8, and is important for the promotion of global translation through S6K and 4EBP. mTORC1 also directly phosphorylates and inhibits PP2A, enabling the accumulation of doubly phosphorylated, active Mycn and contributing to the general proliferation-promoting downstream effects of the PI3K/Akt/Mycn pathway (Cheng *et al.*, 2007). The mTORC2 complex, also known as Pdk2, contains mTOR, rictor, Lst8 and mSin1. mTORC2 phosphorylates Akt at serine 473 further activating this kinase. Inhibitors of this pathway could potentially destabilize Mycn. These inhibitors are currently in clinical trials or in clinical development and are denoted by red octagons. The Aurora A Kinase inhibitor is denoted by an orange octagon, which represents targeted kinase inhibition, but no anticipated activity against Mycn itself. Relevant references are contained within the corresponding text.