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Neuroblastoma and MYCN

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Neuroblastoma, the most common extracranial solid tumor of childhood, is thought to originate from undifferentiated neural crest cells. Amplification of the MYC family member, *MYCN*, is found in ~25% of cases and correlates with high-risk disease and poor prognosis. Currently, amplification of *MYCN* remains the best-characterized genetic marker of risk in neuroblastoma. This article reviews roles for MYCN in neuroblastoma and highlights recent identification of other driver mutations. Strategies to target MYCN at the level of protein stability and transcription are also reviewed.

Neuroblastoma, described by James Wright in 1910, was named because cells were associated with fibrils in arrangements similar to neuroblasts. Nine of 12 cases were in children, suggesting the disease manifests early in life from primitive undifferentiated cells (Wright 1910). Today, neuroblastoma ranks as the most common cancer in infants (<1 year old), with 90% of cases diagnosed by age 5. The primary tumor is frequently located in tissues originating from the sympathetic nervous system, adrenal medulla, or paraspinal ganglia, and metastases are found in a majority of cases at diagnosis, consistent with an origin from multipotent migratory neural crest cells.

RISK IN NEUROBLASTOMA

Risk in neuroblastoma is classified as low, intermediate, or high. Although low- and intermediate-risk patients generally have a favorable outcome (~80%–95% event-free survival rate), high-risk patients have <50% event-free survival rate, and there is also a subset of "ultra-high" risk patients who do not respond to therapy (Maris et al. 2007; Matthay et al. 2012). Current treatment for high-risk patients includes intensive and toxic chemotherapy, followed by surgical resection, myeloablation and autologous stem cell rescue, radiation, and intensive biologic/immunotherapy. Although most high-risk patients initially respond to chemotherapy, the majority relapse and succumb to therapy-resistant disease. Established characteristics for high-risk neuroblastoma patients include age, unfavorable histopathology, loss of heterozygosity for chromosome 1p or 11q, and amplification of *MYCN* (Mueller and Matthay 2009).

MYCN VERSUS MYC

MYCN was identified in 1983 as an amplified gene homologous to v-*myc* but distinct from

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Additional Perspectives on *MYC* and the Pathway to Cancer available at www.perspectivesinmedicine.org

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MYC in human neuroblastoma (Kohl et al. 1983; Schwab et al. 1983). Structurally, the coding regions of both *MYC* and *MYCN* are highly homologous (Fig. 1A), with long 5′ and 3′ untranslated regions (UTRs) and gene products at similar sizes (~50–55 kDa) (Kohl et al. 1986; Stanton et al. 1986). MYC and MYCN proteins both heterodimerize with MAX at consensus E-box sequences (CANNTG), and both proteins have conserved regions for DNA-protein and protein-protein interactions (reviewed in Meyer and Penn 2008). Although a role for MYC in *trans*-repression (through heterodimerization with MIZ1) is well established (see Wiese et al. 2013), comparatively less is known about *trans*-repression by MYCN (Akter et al. 2011; Iraci et al. 2011).

Biologically, MYCN, like MYC, was found to promote transformation in rat embryo fibroblasts and induced proliferation and cell cycle progression in quiescent fibroblasts (Fig. 1B) (Schwab et al. 1985; Yancopoulos et al. 1985; Cavalieri and Goldfarb 1988). Mouse embryonic stem cells (ESCs) homozygous for deletion of either *MYC* or *MYCN* showed normal morphology, and did not show aberrant proliferation or differentiation compared to wild-type ESCs, presumably because MYC and MYCN can compensate for each other (Charron et al. 1992; Davis et al. 1993; Sawai et al. 1993). Further supporting this idea of redundancy is the ability of *MYCN* knocked-in at the *MYC* locus to rescue embryonic lethality and to restore immune functions in *MYC* knockout mice (although MYCN animals were smaller, developed dystrophy of skeletal muscles, and showed differences in growth responses in some cell types; Malynn et al. 2000). Taken together, these findings suggest that MYC and MYCN show prominent, albeit incomplete, redundancy.

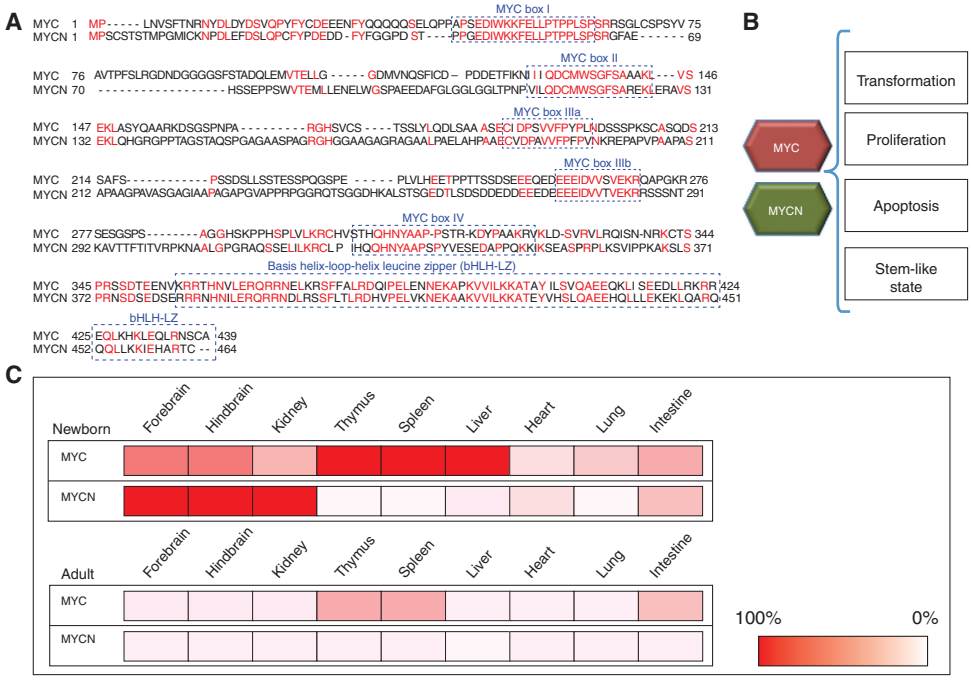

Figure 1. Similarities and differences between MYC and MYCN. MYC and MYCN are similar both in (*A*) structure (homologous sequences in red), and (*B*) biological functions. However, MYC and MYCN differ in (*C*) the spatiotemporal expression levels. In particular, MYCN is preferentially expressed in neural tissue, whereas MYC is more ubiquitously expressed. Expression of MYC and MYCN at each tissue is based on a relative percentage of the highest expressing tissue (newborn forebrain for MYCN and newborn thymus for MYC), which was arbitrarily set to 100%. (Panel *A* based on Hartl et al. 2010. Panel *C* based on data from Zimmerman et al. 1986.)

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What evidence suggests that MYCN is truly distinct from MYC? In *MYCN* null embryos, the regional morphology of the central nervous system (hindbrain prominently) differed from wild-type embryos, despite up-regulation of *MYC* (Stanton et al. 1992). In addition to a lack of compensation, this observation suggests that *MYCN* and *MYC* may regulate each other's expression levels, as observed in other studies (Breit and Schwab 1989; Rosenbaum et al. 1989; Westermann et al. 2008; Helland et al. 2011). Although *MYCN* knockin can compensate for knockout of *MYC,* knockout of either *MYC* or *MYCN* results in embryonic lethality at approximately E10.5–E11.5 (Charron et al. 1992; Davis et al. 1993; Sawai et al. 1993). The inability of endogenous *MYCN* and *MYC* to compensate for these knockout phenotypes may be because of the distinct spatiotemporal expression patterns displayed by MYC family proteins (Fig. 1C).

Expression of *MYCN* is tissue specific, and is found during early developmental stages, whereas expression of *MYC* is more generalized (Fig. 1C). For example, expression of *MYCN* is highest in forebrain, kidney, and hindbrain of newborn mice, and is virtually absent in all tissues of adult mice. In contrast, expression of *MYC* was detected in a broad spectrum of tissues in newborn mice (highest in thymus, spleen, and liver), and subsided substantially in many, but not all, tissues of adult mice (adrenal and thymus maintained high levels; Zimmerman et al. 1986). The differential expression of *MYCN* and *MYC* is particularly striking in the kidneys and B-cell development, in which both *MYCN* and *MYC* are expressed before maturation, with *MYC* alone remaining detectable in kidneys and B cells in adult organisms (Zimmerman et al. 1986). Thus, double knockout of *MYCN* and *MYC* in hematopoietic cells has a significantly more disabling phenotype than either single knockout, suggesting cooperation in the biology of hematopoietic stem cells (Laurenti et al. 2008). Another structure demonstrating differential requirements for MYCN and MYC is the cerebellum. Conditional deletion of *MYCN* in neural stem and progenitor cells markedly reduced proliferation of cerebellar granule neural precursors (GNPs); however, this effect was not seen with deletion of *MYC* (Hatton et al. 2006). This differential requirement for MYCN over MYC is further highlighted by the fact that sonic hedgehog (SHH) signaling drives the expansion of cerebellar GNPs and associates with transcription of *MYCN*, but not *MYC* (Kenney et al. 2003).

Expression of *MYCN* is generally highest in immature cells in newborn mice, with reduced expression in differentiated adult tissues. Consistent with this paradigm is the finding that differentiation of neuroblastoma cells is associated with reduced expression of *MYCN* (Matsumoto et al. 1989; Cinatl et al. 1993; Han et al. 2001; Reddy et al. 2006). Interestingly, *MYCN* is down-regulated during retinoic acid-induced differentiation of neuroblastoma lines before cell cycle and morphological changes (Thiele et al. 1985). Conversely, MYCN has a direct role in blocking differentiation pathways and maintaining pluripotency (Wakamatsu et al. 1997; Kang et al. 2006; Nara et al. 2007; Cotterman and Knoepfler 2009; Lovén et al. 2010; Henriksen et al. 2011). In particular, mice with conditional deletion of *MYCN* in neural progenitor cells showed decreased brain size (especially in the cerebellum where its size was reduced sixfold) and showed a substantial increase in neuronal differentiation compared to control mice (Knoepfler et al. 2002). The enhanced differentiation in *MYCN* null neural progenitor cells may be a result of increased levels of the cyclin-dependent kinase inhibitor p27Kip1, which plays a role in differentiation and is normally degraded by the E3 ubiquitin ligase S-phase kinase associated protein (SKP2), a MYCN transcriptional target (Casaccia-Bonnefil et al. 1997; Gómez-Casares et al. 2013). While MYCN and MYC were both found to have roles in maintaining pluripotency and self-renewal of stem cells, and both proteins can reprogram fibroblasts into induced pluripotent stem (iPS) cells (Nakagawa et al. 2010; Varlakhanova et al. 2010), evidence for differential functions of MYC and MYCN in regulating pluripotency comes from studies in medulloblastoma (see Roussel and Robinson 2013). When misexpressed in GNPs dependent on SHH signaling for survival,

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CSH PERSPECTIVES

MYCN promotes an expected SHH-driven malignancy, whereas MYC drives malignancy down a distinct SHH-independent lineage. These data suggest that MYCN can transform cells in a committed lineage. In contrast, MYC can similarly transform cells, and can also drive cancer stem cell functionality, the latter enabling reprogramming down a distinct lineage (Kawauchi et al. 2012; Pei et al. 2012). It is likely that MYCN can also direct cancer stem cell functionality, through interactions with cooperating transcription factors. Support for this view comes from experiments in which co-expression of SOX9 with MYCN in cerebellar stem and progenitor cells could drive self-renewal, whereas neither SOX9 nor MYCN could drive self-renewal individually (Swartling et al. 2012).

MYCN IN NEUROBLASTOMA

Within two years of *MYCN*'s discovery in neuroblastoma, amplification of *MYCN* was shown to correlate with poor prognosis in patients (Brodeur et al. 1984; Seeger et al. 1985), a biomarker that is still used today to stratify risk. Mice with targeted misexpression of *MYCN* to the peripheral neural crest via the rat tyrosine hydroxylase (TH)-promoter developed neuroblastoma, establishing that misexpression of *MYCN* in migrating neural crest cells can initiate this disease (Weiss et al. 1997). Tumors in these mice had a prolonged latency and showed recurrent chromosomal copy number abnormalities, suggesting that genetic mutations in addition to misexpressed *MYCN* were required to promote neuroblast transformation. This requirement for additional mutations was supported by the fact that loss of tumor suppressors neurofibromin1 or retinoblastoma1 (Rb), when combined with misexpression of *MYCN*, resulted in reduced latency and increased penetrance for tumors. The sections below illustrate some of the many roles that MYCN subserves in neuroblastoma tumorigenesis (Fig. 2).

Metastasis

Metastasis occurs in ~50% of neuroblastoma patients at diagnosis (Maris et al. 2007) with frequent spread to bone marrow (70%), bone (55%), lymph nodes (30%), liver (30%), and brain (18%) (DuBois et al. 1999). Not surprisingly, levels of *MYCN* correlate with invasive and metastatic behavior (Zaizen et al. 1993; Bénard 1995; Goodman et al. 1997). MYCN contributes to all facets of metastasis: adhesion, motility, invasion, and degradation of surrounding matrices. Specifically, MYCN-directed downregulation of integrins α1 and β1 promotes detachment from the extracellular matrix and allow cells to migrate and invade (van Golen et al. 2003; Tanaka and Fukuzawa 2008). MYCN promotes transcription of focal adhesion kinase (FAK), a critical regulator of integrin signaling, and generally promotes increased migration and metastasis in tumor cells (Beierle et al. 2007; Megison et al. 2012). Notably, FAK can also be repressed transcriptionally by p53, indicating a possible competition between MYCN and p53 in regulating FAK levels (Golubovskaya et al. 2008). MYCN also increases activity of matrix metalloproteinases (MMPs). In SHEP neuroblastoma cells, expression of BCL2 led to an increase in expression and secretion of MMP2, whereas co-expression of MYCN and BCL2 suppressed the MMP2 antagonist, TIMP-2 (Noujaim et al. 2002). miR-9, a microRNA activated by MYCN, targets and suppresses E-cadherin, contributing to an epithelial to mesenchymal transition (EMT) (Ma et al. 2010).

Although caspase-8 has been associated mostly with promoting apoptosis, caspase-8 also has a paradoxical role in both promoting and inhibiting metastasis in neuroblastoma, depending on the cellular context. A significantly higher incidence of spontaneous metastasis was observed in primary neuroblastoma tumors without caspase-8, as compared to tumors expressing caspase-8, although the primary tumor sizes were similar. NB7 neuroblastoma lines with ectopic expression of caspase-8 eventually lost expression of caspase-8 at sites of dissemination (Stupack et al. 2006). The lack of caspase-8 triggered integrin-mediated death, a mechanism of apoptosis in cells that have detached from the extracellular matrix and unligated their integrins (Stupack et al. 2001). In the TH-*MYCN* mouse model of neuroblastoma, deletion of ca-

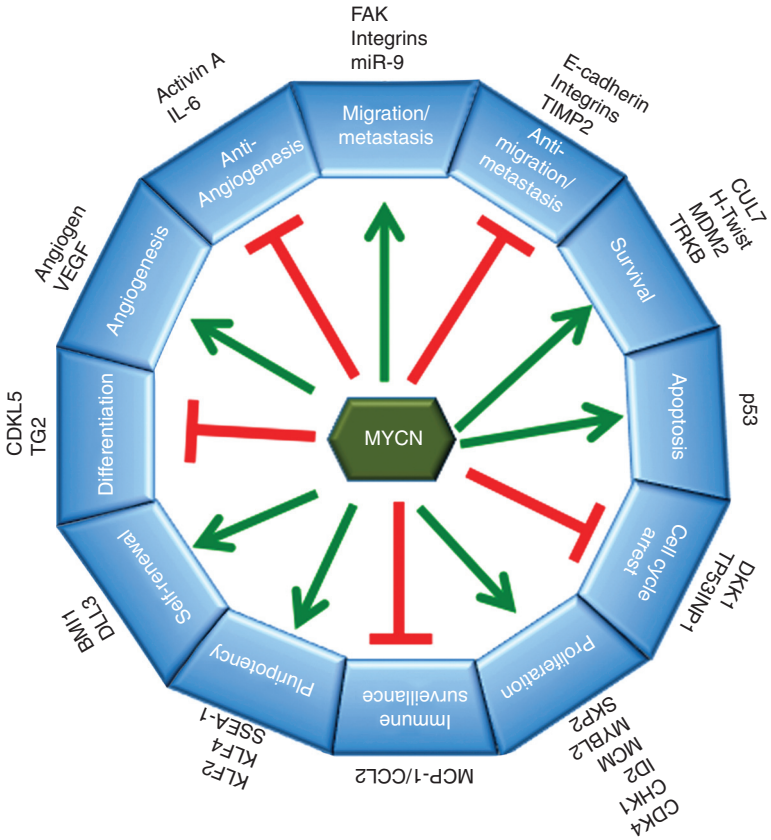

Figure 2. *MYCN* plays multiple roles in malignancy and maintenance of stem-like state. MYCN can activate transcription of genes involved in metastasis, survival, proliferation, pluripotency, self-renewal, and angiogenesis. Additionally, MYCN can suppress expression of genes that promote differentiation, cell cycle arrest, immune surveillance, and genes that antagonize metastasis and angiogenesis. Because MYCN also activates apoptotic pathways involving p53, MYCN is not normally sufficient for transformation.

pase-8 did not affect apoptosis, but instead, increased significantly the incidence of metastasis specifically to the bone marrow, possibly caused by up-regulation of genes involved in EMT, decreased cell adhesion, and increased fibrosis (Teitz et al. 2013). The prometastatic capabilities of caspase-8 were dependent on the tumor being resistant to apoptosis, which occurs when caspase-3 is lost. In this setting, caspase-8 associated with focal adhesion complex proteins, such as SRC, integrins, calpain-2 (CPN2), and FAK. Within this complex, caspase-8, independent of its proteolytic activity, permitted CPN2 to become activated and cleave focal adhesion substrates to promote cell migration (Barbero et al. 2009).

Immune Surveillance

MYCN influences immune surveillance by modulating antigens expressed on tumor cells. One antigen repressed by MYCN is monocyte chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2), required for chemoattraction of natural killer T (NKT) cells (Song et al. 2007). Knockdown of *MYCN* in *MYCN*-amplified neuroblastoma lines rescued MCP-1 production and NKT cell chemoattraction. In contrast, overexpression of *MYCN* in neuroblastoma xenografts inhibited the ability to attract NKT cells. Presumably, MYCN binds to the E-box element of the *MCP-1* promoter to block expression of the chemokine. Support for this model was ob-

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served in patients with *MYCN*-amplified neuroblastoma metastasizing to the bone marrow, which had fourfold fewer bone marrow NKT cells compared to their nonamplified counterparts (Song et al. 2007). Although NKT cells have not been shown directly to be effective against neuroblastoma cells, NKT cells can initially secrete a set of proinflammatory cytokines to recruit immune cells, stimulate the maturation of dendritic cells, and generate antigen-specific T cells to target the tumor.

Angiogenesis

High vascularity in neuroblastoma correlates with poor survival, increased dissemination, and amplification of *MYCN* (Meitar et al. 1996; Ribatti et al. 2002; Ozer et al. 2007). The association of vascularity with amplification of *MYCN* suggests antiangiogenic therapy as a viable approach towards *MYCN*-amplified neuroblastoma. Does MYCN facilitate the secretion of factors to promote growth of endothelial cells and/or block the release of inhibitors of angiogenesis? Conditioned media from neuroblastoma cell lines with induced expression of *MYCN* revealed the loss of inhibitors of endothelial growth (Fotsis et al. 1999). These inhibitors of angiogenesis include Activin A (Hatzi et al. 2000), leukemia inhibitory factor (LIF; Hatzi et al. 2002a), and interleukin 6 (IL-6; Hatzi et al. 2002b). Misexpression of *MYCN* also had a positive effect on proangiogenic factors including angiogenin (Dungwa et al. 2012) and vascular endothelial growth factor (VEGF) via the phosphoinositide 3′-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway (Kang et al. 2008). Indeed, the PI3K/mTOR inhibitor, NVP-BEZ235, suppressed growth of *MYCN*-amplified tumors and disrupted angiogenesis (Chanthery et al. 2012). Here, blockade of PI3K/mTOR signaling destabilized MYCN, which subsequently suppressed transcription and secretion of VEGF.

Self-Renewal and Pluripotency

As described above, neuroblastoma likely arises from neural crest cells, which possess the characteristics of self-renewal and multipotency. MYCN is likely involved in the regulation of both traits, as MYCN can substitute for MYC in reprogramming fibroblasts into iPS cells (Nakagawa et al. 2010). Thus, both MYC and MYCN promote a stem-like state, likely because of blockade of differentiation pathways and expression of self-renewal and pluripotency factors. Indeed, MYCN up-regulates the pluripotency genes *KLF2*, *KLF4*, and *LIN28B* (Cotterman and Knoepfler 2009). Although MYCN also increased *LIF* expression in this study, another report found that MYCN reduced levels of LIF in neuroblastoma, suggesting that MYCN may differentially regulate LIF based on cellular context (Hatzi et al. 2002a). Mouse ESCs knocked out for both *MYC* and *MYCN* showed up-regulation of endodermal and mesodermal markers of differentiation (BMP4, GATA6), as well as activators of lymphocytic differentiation (STAT1, EGR1, ELK3), and sensory organ development (DLL1, BMP4, GBX2, FGFR1), suggesting that MYC proteins repress differentiation pathways (Varlakhanova et al. 2010). Other differentiation proteins suppressed by MYCN include cyclin-dependent kinase-like 5 (CDKL5) and tissue transglutaminase (TG2) (Liu et al. 2007; Valli et al. 2012). *MYC* and *MYCN* deleted mESC also lost expression of the pluripotency marker SSEA-1, although other pluripotency markers NANOG, OCT4, and REX-1 were not affected significantly (Varlakhanova et al. 2010). Nevertheless, the loss of *MYC* and *MYCN* in mouse ESCs resulted in loss of self-renewal, in part, because of an inability to progress efficiently through S-phase and the G_2/M checkpoint. Importantly, MYCN up-regulates expression of the polycomb and self-renewal protein BMI1 in neuroblastoma by directly binding to the E-Box sites within the promoter of *BMI1* (Ochiai et al. 2010; Huang et al. 2011). Additionally, MYCN-mediated expression of DLL3, a Notch1 ligand, has been described as a mechanism to maintain neural stem cell fate (Zhao et al. 2009). Further support for MYCN promoting self-renewal is the finding that *MYCN*-amplified neuroblastoma cell lines tend to undergo symmetric cell division, whereas nonamplified lines preferentially divide asymmetrically (Izumi and

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Kaneko 2012). Thus, MYCN plays a critical role in maintaining a stem-like state by blocking differentiation pathways and activating self-renewal and pluripotency genes (see Chappell and Dalton 2013).

Apoptosis

Like MYC, MYCN activates both proliferation and apoptosis (Evan et al. 1992; Fulda et al. 2000). Whether MYCN promotes a net proliferative response is therefore dependent on the status of cooperating apoptotic factors, such as the antiapoptotic protein, BCL2 (Strasser et al. 1990) or p53 (Elson et al. 1995; Chesler et al. 2008). Interestingly, *TP53* mutations are rare in neuroblastoma at diagnosis, suggesting that MYCN likely cooperates with suppressers of p53 signaling, including miRNA-380-5p, the oncogene CUL7, BMI1, and transcription factor H-Twist, and MDM2 (Valsesia-Wittmann et al. 2004; Slack et al. 2005; Kim et al. 2007; Swarbrick et al. 2010; Huang et al. 2011). In contrast, mutations in *TP53* and p53 pathway members occur commonly in neuroblastoma at relapse, consistent with the idea that such mutations, which drive therapy-resistant disease, may arise in response to cytotoxic chemotherapy. The impor-

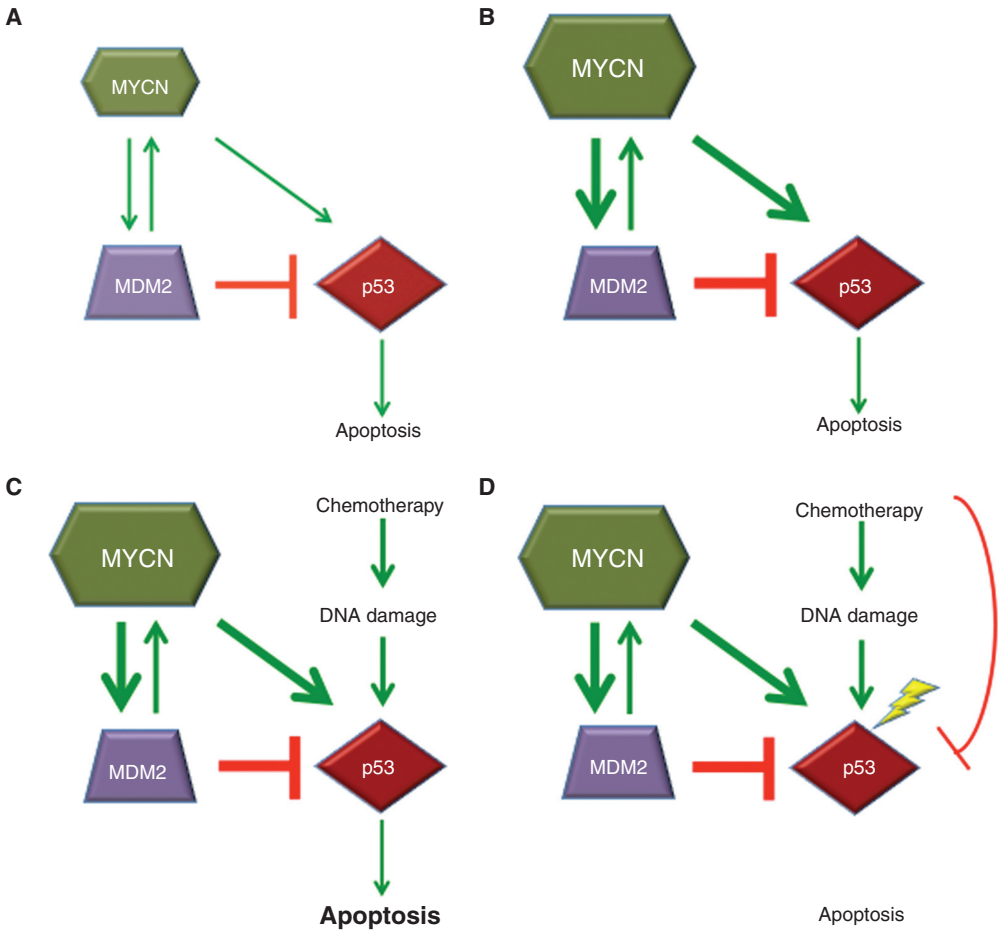

Figure 3. Amplified *MYCN* marks chemoresistance in patients with neuroblastoma. (*A*,*B*) Amplified *MYCN* results in elevated transcription of *MDM2* and *TP53*. High levels of p53 sensitize tumors to apoptosis. (*C*) Patients initially respond to chemotherapy as p53 triggers apoptosis. (*D*) At relapse, tumors show mutation in p53 or p53 pathways, resulting in therapy resistance.

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tance of p53 signaling is further highlighted by the role of MYCN in the MDM2-p53 pathway (Fig. 3). MDM2 is an E3 ubiquitin ligase that promotes survival by ubiquitinating and driving degradation of p53, and has also been implicated in promoting the stability of *MYCN* mRNA by binding to the AU-rich elements of the 3′UTR of *MYCN* (Gu et al. 2012). Interestingly, *MDM2* is a target for p53-mediated transcription, and MYCN can promote transcription of both *MDM2* and *TP53* (Slack et al. 2005; Chen et al. 2010). The role of MYCN in activating both *MDM2* and *TP53* explains conflicting sensitivities to chemotherapy in *MYCN*-amplified neuroblastoma. *MYCN*-amplified neuroblastoma initially respond to chemotherapy, perhaps due, in part, to MYCN-mediated activation of p53. However, these tumors eventually relapse and become resistant, possibly through chemotherapy-induced inactivating mutations of p53, resulting in a feed-forward loop between MDM2 and MYCN to promote survival (Fig. 3).

Loss of caspase-8 function in neuroblastoma has been speculated to be a mechanism of apoptosis evasion in *MYCN*-amplified neuroblastoma (Teitz et al. 2000; Gonzalez-Gomez et al. 2003; Iolascon et al. 2003). A later study did not find correlation between caspase-8 expression and risk in neuroblastoma (*MYCN* status, chromosome 1p36 deletion, disease stage, etc.) (Fulda et al. 2006); however, multiple groups have reported methylation of the promoter for caspase-8 (Casciano et al. 2004; Banelli et al. 2005; Lázcoz et al. 2006). Loss of caspase-8 promotes resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (Eggert et al. 2001).

In addition to associating with suppression of proapoptotic pathways, *MYCN*-amplified neuroblastoma could survive via constitutive activation of prosurvival signaling cascades, such as tropomyosin receptor kinase B (TRKB). Interestingly, levels of TRKB expression are low in neuroblastoma not amplified for *MYCN*. In contrast, TRKB is frequently activated in *MYCN*-amplified neuroblastoma, signaling through both autocrine and paracrine survival pathways (Nakagawara et al. 1994). Activation of TRKB is implicated in resistance to chemotherapy and can up-regulate *MYCN* mRNA, which may provide insights into the association of TRKB with *MYCN*-amplified neuroblastoma (Ho et al. 2002; Dewitt et al. 2013). Conversely, MYCN appears to down-regulate tropomyosin receptor kinase A (TRKA), which is normally found in low-risk neuroblastoma, recruiting HDAC1 to the *TRKA* promoter to induce a repressed chromatin state (Iraci et al. 2011).

Proliferation and Cell Cycle

The best characterized tumorigenic effect of MYCN is to promote proliferation and cell cycle progression. Specifically, *MYCN*-amplified neuroblastomas show an inability to arrest in G_1-phase in response to irradiation and DNA damage, possibly via down-regulation of TP53 inducible nuclear protein 1 (TP53INP1) and up-regulation of both CDK4 and SKP2, allowing CDK2 to escape p21 inhibition (Tweddle et al. 2001; Bell et al. 2007; Muth et al. 2010; Gogolin et al. 2013). MYCN up-regulation of checkpoint kinase 1 (CHK1), an important regulator of S-phase and G_2/M checkpoints, has been suggested as a mechanism through which *MYCN*-amplified neuroblastoma becomes refractory to standard chemotherapy (Cole et al. 2011). Indeed, inhibition of CHK1 promotes chemosensitization in various types of tumor cells (Blasina et al. 2008; Zhang et al. 2009). MYCN also directly represses expression of anti-proliferative proteins such as Dickkopf-1, which disrupts the WNT/β-catenin signaling pathway, and CDKL5, which arrests cells between G_0/G_1 phase (Koppen et al. 2007a; Valli et al. 2012). ID2, a helix-loop-helix transcription factor, is also a target of MYCN. MYCN directly binds to the promoter of *ID2* to stimulate its expression, leading to inactivation of Rb to permit progression through the cell cycle (Iavarone et al. 1994; Lasorella et al. 1996). Consistent with the idea that MYCN activates transcription of *ID2*, *MYCN*-amplified neuroblastoma lines show elevated levels of *ID2*, and *MYCN* and *ID2* expression levels correlate during development (Lasorella et al. 2000; 2002).

Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org
CSH PERSPECTIVES

MYC has also been implicated in progression through the G_1-S phase of the cell cycle by cooperating with RAS to induce cyclin D/CDK4 and/or cyclin E/CDK2 complexes that phosphorylate and inactivate Rb proteins, activating E2F transcription factors to induce S-phase (reviewed in Farrell and Sears 2014). This cycle is further amplified because RAS signaling stabilizes MYC proteins by activating AKT, which phosphorylates and blocks glycogen synthase kinase 3β (GSK-3β)-mediated degradation of MYC proteins. Thus, blockade of RAS signaling can induce growth arrest in *MYCN*-amplified neuroblastoma cells (Yaari et al. 2005). mTOR signals downstream from the RAS/PI3K/AKT pathway and has essential functions in translational control, impacting metabolism, proliferation, and tumorigenesis. Allosteric blockade of mTOR with rapamycin or CCI-779 displayed a more pronounced antiproliferative effect in *MYCN*-amplified than in *MYCN*-nonamplified neuroblastoma tumors. Additionally, allosteric mTOR inhibitors also suppressed expression of VEGF-A and cyclin D1 (Johnsen et al. 2008). Other targets of MYCN that appear to drive proliferation are neuronal leucine-rich repeat protein-1, which also enhances expression of *MYCN*, the transcription factor MYBL2, a downstream target of MYCN and a factor in drug resistance, and minichromosome maintenance (MCM) genes that are responsible for DNA elongation and unwinding during the replication process (Raschella et al. 1999; Koppen et al. 2007b; Hossain et al. 2008; 2012; Gualdrini et al. 2010) .

EPIGENETIC ROLES OF MYCN

Because neuroblastoma typically occurs in early childhood (as opposed to adult cancers that have more time to accumulate mutations that promote transformation), the profound tumorigenic influence of MYCN likely extends beyond its ability to directly regulate expression of any individual gene. MYCN can also indirectly mediate the expression of multiple genes simultaneously via activation of noncoding RNAs, such as miRNA and long noncoding RNA (reviewed in Buechner and Einvik 2012). Additionally, MYCN impacts global histone methylation and acetylation, markers of transcriptional repression and activation, respectively (Knoepfler et al. 2006). Recently, MYCN was found to associate with EZH2, a methyltransferase and member of the polycomb repressor complex 2 (Corvetta et al. 2013). EZH2 has been implicated in the trimethylation of Histone 3 K27, a transcriptional silencing mark (Kotake et al. 2007; Au et al. 2012). Interestingly, the interaction between MYCN and EZH2 requires the MYC box domain III, which is necessary for MYC to promote transformation (Herbst et al. 2005). In support of a transcriptionally repressive function for a MYCN/EZH2 complex is the finding that the interaction between MYC and another DNA methyltransferase, DNMT3A, silences the transcription of p21CIP1, an inhibitor of cyclin-dependent kinases (Brenner et al. 2005). Furthermore, MYCN up-regulates the expression of histone deacetylases (HDACs) in neuroblastoma, including HDAC1, HDAC2, and SIRT1. HDAC1 represses expression of the differentiation protein TG2. HDAC2 silences cyclin G2, a suppressor of cell cycle progression, whereas SIRT1 represses mitogen-activated protein kinase phosphatase 3, activating ERK (Marshall et al. 2010; 2011; Iraci et al. 2011). Notably, SIRT1 stabilizes MYCN, promoting a feed-forward loop between the two proteins (Marshall et al. 2011). Thus, MYCN may enhance silencing of tumor suppressors in neuroblastoma by increasing DNA methylation and deacetylation.

ROLE OF MYCL AND MYC IN NEUROBLASTOMA

Amplification of other MYC family members (i.e., MYCL, MYC) is uncommon in neuroblastoma (Slavc et al. 1990). As compared with levels of *MYCL* and *MYC*, expression of *MYCN* is higher in developing tissues that normally give rise to neuroblastoma (Kohl et al. 1986; Stanton et al. 1992). In fact, loss of heterozygosity for chromosome 1p (a marker of risk) suggests that deletion of *MYCL* (on chromosome 1p34) may indicate unfavorable prognosis (Hiyama et al. 2001), with the caveat that other tumor suppressors also reside in this subchromosomal

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region. *MYC* is infrequently amplified in neuroblastoma, and expression of *MYC* correlates inversely with *MYCN* (Breit and Schwab 1989). Specifically, *MYCN*-nonamplified Stage 4 and 4S neuroblastoma tumors may show high levels of *MYC* mRNA (Westermann et al. 2008), suggesting that amplified *MYC* may compensate in *MYCN*-nonamplified tumors.

TARGETING *MYCN*-AMPLIFIED NEUROBLASTOMA

Knockdown of *MYCN* expression via RNA interference and antisense oligonucleotides in *MYCN*-amplified neuroblastoma has shown an increase in apoptosis and differentiation, and suppression of cell growth, suggesting that blockade of MYCN may be a therapeutic option in *MYCN*-driven neuroblastoma (Burkhart et al. 2003; Kang et al. 2006). However, the development of inhibitors targeting MYC proteins has been challenging, as MYC/MYCN proteins are composed of two extended alpha-helices with no obvious surfaces for small molecule binding. Strategies to circumvent blocking MYCN directly include: (1) targeting epigenetic reader proteins, such as acetyl-lysine binding modules (bromodomains) that link chromatin marks to activation of MYC/MYCN, (2) targeting regulators of *MYCN* mRNA and protein stability, (3) activating p53-induced apoptosis, and (4) triggering differentiation (Fig. 4).

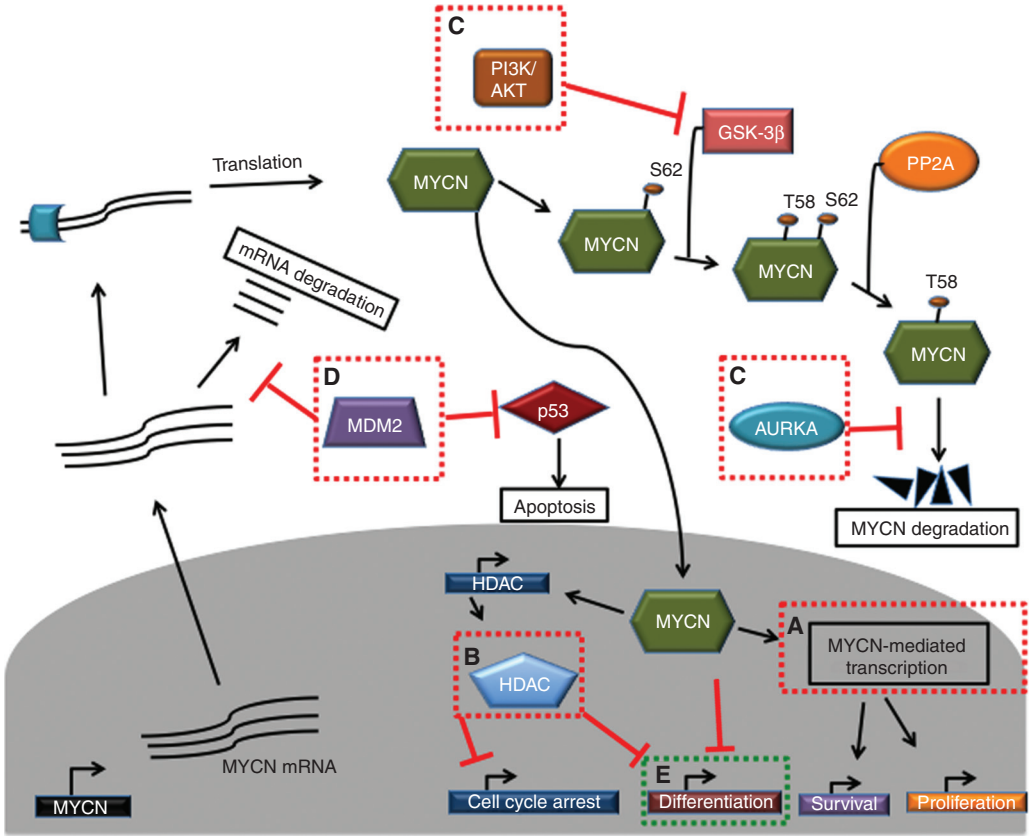

Figure 4. Therapeutic strategies to target MYCN in neuroblastoma. Possible strategies to treat *MYCN*-amplified neuroblastoma patients include (*A*) blocking MYCN-dependent transcription with BET-bromodomain inhibitors, (*B*) inhibiting HDACs, (*C*) antagonizing proteins involved in stabilizing MYCN protein, (*D*) suppressing MDM2 (which stabilizes *MYCN* mRNA and disrupts p53-mediated apoptosis), and (*E*) inducing differentiation.

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BET Bromodomain Inhibitors

Gene expression from enhancer and promoter-bound oncogenic transcription factors is mediated by multiprotein assemblies, such as the mediator complex, the positive transcriptional elongation factor complex (PTEFB), and chromatin-associated protein complexes and chromatin-remodeling complexes (e.g., SWI/SNF). In addition, memory of the cancer-cell state is maintained through cell division by covalent modification of the unstructured amino-terminal tails of histone proteins (see Bradner 2013; Sabó and Amati 2014). Lysine side-chain acetylation figures prominently in activation of MYCN. Acetylation marks are placed in regions of active transcription by histone acetyltransferases. Their interpretation is mediated by acetyl-lysine reader domains or bromodomains, a family of 47 transcriptional coactivators. Members of the bromodomain and extraterminal (BET) subfamily, BRD2-4, are compelling targets owing to characterized binding interactions with SWI/SNF and their interaction with PTEFB. MYC also recruits PTEFB to release RNA polymerase II, promoting transcription. The tool compound and BRD2-4 inhibitor JQ1 was shown to block MYC targets as well as MYC itself in multiple myeloma, a MYC-dependent malignancy (Delmore et al. 2011). JQ1 also suppresses growth of *MYCN*-amplified neuroblastoma in multiple in vivo models including orthotopic transplantation of patient derived xenografts and the TH-*MYCN* mouse model. Mice treated with JQ1 showed a significant increase in overall survival, and tumors showed increased apoptosis and decreased proliferation as well as decreased expression of MYCN itself (Puissant et al. 2013). Thus, BET bromodomain inhibitors may be a therapeutic option for patients with *MYCN*-amplified neuroblastoma (Fig. 4A).

HDAC

As described above, MYCN silences tumor suppressor genes by recruitment of DNA methyltransferases and elevated expression of HDAC, suggesting a possible therapeutic role for HDAC inhibitors. Several preclinical studies have found promising results using HDAC inhibitors in the TH-*MYCN* model of neuroblastoma. For instance, treatment of TH-*MYCN* mice with the SIRT1 inhibitor, Cambinol, reduced tumorigenesis (Marshall et al. 2011). Liu and colleagues found that Trichostatin A (class I and II HDAC inhibitor) restored expression of the differentiation protein TG2, resulting in reduced tumor weight and volume in the same mouse model (Liu et al. 2007). Therefore, HDAC inhibitors may be a viable route to target *MYCN*-amplified neuroblastoma (Fig. 4B).

Regulators of MYC Protein Stability

MYC and MYCN proteins are proteolyzed through a sequential stepwise set of phosphorylation events. Initially, MYC and MYCN are phosphorylated at S62 (via kinases in the RAS signaling pathway, such as MAPK [Seth et al. 1991; Lutterbach and Hann 1994] and CDK1 [Sjostrom et al. 2005]), stabilizing MYC and MYCN and priming these proteins for phosphorylation at T58 via GSK-3β (Pulverer et al. 1994). Dephosphorylation of S62 via protein phosphatase 2A (PP2A) sensitizes MYC and MYCN phosphorylated at T58 to bind F-box and WD repeat domain-containing 7 (FBW7) or other E3 ligases, leading to the ubiquitination and degradation in the proteasome (Fig. 4) (Sears et al. 2000; Welcker et al. 2004; Yada et al. 2004).

PI3K inhibition can decrease MYCN protein levels without impacting the mRNA by blocking a PI3K-driven inhibitory phosphorylation on GSK-3β and promoting MYCN phosphorylation (Fig. 4C) (reviewed in Gustafson and Weiss 2010). Neuroblastoma lines expressing $MYCN^{T58A}$ (lacking a GSK-3β phosphorylation site) were resistant to antiproliferative effects from treatment with the PI3K/mTOR inhibitor, LY294002 (Chesler et al. 2006). These data, and subsequent studies using the clinical inhibitor BEZ235 (Chanthery et al. 2012) suggest that PI3K/mTOR inhibitors represent a viable strategy to target *MYCN*-amplified neuroblastoma tumors.

Proteins that prevent dephosphorylation at T58 stabilize MYCN, which may be the case

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with aurora kinase A (AURKA) in neuroblastoma (Fig. 4C) (Otto et al. 2009). Interestingly, increased expression of AURKA is found in *MYCN*-amplified neuroblastoma, mediated potentially by MYCN itself (Shang et al. 2009). Thus, elevated levels of either MYCN or AURKA promote a potential feed-forward loop that stabilizes both proteins. Because AURKA has possible ligand binding sites for small molecule inhibitors, targeting AURKA could potentially represent a strategy to treat *MYCN*-amplified tumors (Faisal et al. 2011); however, the ability of AURKA to stabilize MYCN has been shown to be independent of kinase activity (Otto et al. 2009). A similar mechanism of tumor suppression has also been proposed for protein tyrosine phosphatase receptor type D (PTPRD). Ectopic expression of PTPRD dephosphorylates tyrosine residues on AURKA, which destabilizes AURKA, and subsequently lowers levels of MYCN and proliferation (Meehan et al. 2012).

MDM2

Down-regulation of MDM2 would presumably have two significant effects in *MYCN*-amplified neuroblastoma. Because MDM2 can bind to the 3′UTR of *MYCN*, loss of MDM2 would destabilize *MYCN* mRNA and subsequently block expression of MYCN protein (Gu et al. 2012). In parallel, decreased MDM2 levels should stabilize p53 and increase the likelihood of p53-dependent apoptosis (Fig. 4D). Indeed, *MYCN*-amplified neuroblastoma cell lines have been found to be more sensitive than *MYCN*-nonamplified lines to antagonists of MDM2: Nutlin-3 and MI-63 (Gamble et al. 2012).

Inducers of Differentiation

Because neuroblastoma is believed to originate from immature neural crest cells, triggering differentiation in these cells should result in reduced proliferation and increased cell death. Indeed, retinoic acid, nitric oxide, and phenylacetate have all been shown to induce differentiation, and inhibit both anchorage independence and cell growth in neuroblastoma (Sidell 1982; Han et al. 2001; Ciani et al. 2004). Although MYCN is known to inhibit differentiation pathways, each of these molecules have been found to reduce MYCN levels as well, indicating that *MYCN*-amplified tumors can still be induced to differentiate as a means to suppress proliferation and promote apoptosis (Fig. 4E).

OTHER GENETIC DRIVER MUTATIONS OF NEUROBLASTOMA

To identify novel driver mutations of neuroblastoma, intense efforts have focused on genome-wide association studies (GWAS), as well as whole exome and whole genome sequencing studies of tumor samples (Table 1). Although

Table 1. Non-MYCN drivers of neuroblastoma

Gene	GOF or LOF	Frequency	Number of tumors
ALK	GOF	6%,[1,2] 8%,[3] 9%,[4] 12.4%,[5] 14%,[6]	87,[1] 215,[2] 93,[3] 240,[4] 194,[5] 130[6]
ATRX	LOF	9.6%,[4] 25%[7,a]	240,[4] 40[7]
ARID1A	LOF	1%–2%,[4] 6%[6]	240,[4] 71[6]
ARID1B	LOF	<1%,[4] 7%[6]	240,[4] 71[6]
LIN28B	GOF	1.1%,[8] 1.4%[6]	263,[8] 71[6]
PTPN11	GOF	2.9%,[4] 3.4%[9]	240,[4] 89[9]
TIAM1	LOF	0%,[4] 3%[1]	240,[4] 87[1]

Tumor susceptibility SNPs were identified in *BARD1* (Capasso et al. 2009; Nguyen et al. 2011; Diskin et al. 2012), *HACE1* (Diskin et al. 2012), *LMO1* (Wang et al. 2011; Diskin et al. 2012; Nguyen et al. 2011), and *LIN28B* (Diskin et al. 2012). Pugh et al. (2013) identified other genetic mutations that had frequencies <1%.

References: [1]Molenaar et al. 2012b, [2]Chen et al. 2008, [3]George et al. 2008, [4]Pugh et al. 2013, [5]Mossé et al. 2008, [6]Sausen et al. 2013. [7]Cheung et al. 2012, [8]Molenaar et al. 2012a, [9]Bentires-Alj et al. 2004.

[a]Half of tumors with *ATRX* mutations were from older patients, which account for a small percentage of neuroblastoma patients.

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subchromosomal aberrations occur robustly in this disease, genetic mutations were quite rare (some could not be verified in independent studies). Two of the more commonly altered genes that have been observed in multiple studies include anaplastic lymphoma kinase (*ALK*) and Alpha thalassemia/mental retardation syndrome X-linked (*ATRX*).

ALK

Familial neuroblastoma occurs rarely compared to sporadic cases. Gain of function mutations in the receptor tyrosine kinase *ALK* (chromosome 2p23) occur in ~50% of familial neuroblastoma cases, and ~7% of sporadic neuroblastoma cases (Chen et al. 2008; George et al. 2008; Janoueix-Lerosey et al. 2008; Mossé et al. 2008; Molenaar et al. 2012b). ALK can drive neuroblastoma in genetically engineered mice misexpressing the most potent mutation of ALK, F1174L (Heukamp et al. 2012; Schulte et al. 2012). Additionally, ALK cooperates with MYCN to drive malignancy, as activation of ALK results in increased expression of MYCN by elevating activity of the *MYCN* promoter, and stabilizing MYCN protein likely via activation of AKT and ERK (Chesler et al. 2006; Berry et al. 2012; Schönherr et al. 2012; Zhu et al. 2012). *ALK* and *MYCN* are also physically linked on chromosome 2p, which may explain why amplification of *MYCN* and *ALK* frequently co-occur (De Brouwer et al. 2010).

ATRX

Whole genome sequencing of 40 neuroblastoma patient samples revealed loss of function mutations in *ATRX* (chromosome Xq21) in 10 samples, five of which belong to older patients, which constitutes a small percentage of overall neuroblastoma cases. Eight of the 10 samples with *ATRX* mutations showed longer telomeres (via alternative lengthening of telomeres [ALT]), and lacked ATRX in the nucleus (Cheung et al. 2012). Patients with *ATRX* mutant neuroblastoma were typically adolescents with a chronic progressive form of this disease. Other than telomere lengthening, ATRX is thought to function epigenetically by recruitment of Histone 3.3 at telomeric regions to maintain chromosome stability (reviewed in De La Fuente et al. 2011). Intriguingly, *ATRX* mutations were mutually exclusive from amplification of *MYCN* in neuroblastoma, which was verified in a separate study (Pugh et al. 2013). In contrast, elevated expression of *MYCN* and mutation of *ATRX* are both associated with mutation of G34 site in Histone 3.3 in pediatric glioblastoma (Schwartzentruber et al. 2012; Wu et al. 2012; Bjerke et al. 2013).

In a separate study, a subset of neuroblastoma tumors (five of 87) with *ATRX* mutations also had defects in *PTPRD* and odd Oz 3 (ODZ3) (Molenaar et al. 2012b). The investigators reasoned that these three genes were likely co-selected, as the probability of finding defects in three concurrent genes in at least five tumors is $<10^{-4}$. The connection among these genes may relate to a role in neuritogenesis, which is critical for differentiation of neuroblasts. PTPRD and ODZ3 are transmembrane receptors that localize to axons and axonal growth cones and are known to enhance neuritogenesis (Arregui et al. 2000). Transgenic mice with *ATRX* mutations showed aberrant dendritic spine morphology and altered signaling through the GTPase, Rac1, which also promotes neuritogenesis (Shioda et al. 2011). Importantly, activators of Rac1 (guanine exchange factors [GEF]), but not inhibitors (GTPase activating proteins [GAP]) are mutated in neuroblastoma, albeit rarely (e.g., *TIAM1* was found mutated in three out of 87 tumors) (Molenaar et al. 2012b). In contrast, Rho signaling opposes neuritogenesis and more mutations in Rho GAPs than GEFs were identified in the same study (Molenaar et al. 2012b). Taken together, this evidence implicates neuritogenesis as a process that is antagonized in neuroblastoma tumorigenesis. Interestingly, a recent study by Pugh et al. (2013) did not detect mutations in GEFs and GAPs involved in Rac/Rho signaling to a statistically significant degree, with TIAM1, the Rac1 GEF most frequently mutated in the Molenaar study, not altered in the larger study by Pugh et al. (2013). The discrepancy between these studies may be a result of the rarity of neuritogenesis mutations and/or biological differences between the two data sets.

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Recently, a correlation was described among different types of tumors with respect to the frequency of mutations within the telomerase reverse transcriptase (*TERT*) promoter, with neural tumors (e.g., glioblastoma, oligodendroglioma, and medulloblastoma), particularly in older patients, showing the highest occurrences (Killela et al. 2013). These mutations are linked to maintaining telomere length, analogous to the effect of ALT, a commonly occurring phenomenon due to *ATRX* mutations. Based on these data, the Yan group hypothesizes that tumor types showing high frequencies of ALT might also show high frequencies of *TERT* mutations, and these mutations, as exemplified by glioblastoma, would be distributed in a mutually exclusive fashion. Older children with neuroblastoma have been shown to have *ATRX* mutations. To our knowledge, however, *TERT* mutations were not examined in these sequencing studies, an intriguing area for future investigation.

Remarks

The search for driver mutations in neuroblastoma has led to the identification of several other candidate genes, although the frequencies of mutations among these candidates are much lower than *ALK* and *ATRX*. These include gain of function in protein tyrosine phosphatase 11 (*PTPN11*) and *LIN28B*, structural mutations in neural growth cone genes (*ODZ3*, *PTPRD*, and *CSMD1*), and heterozygous loss of AT-rich interactive domain 1A and 1B (*ARID1A* and *ARID1B*; Molenaar et al. 2012a,b; Pugh et al. 2013; Sausen et al. 2013). Tumor susceptibility SNPs were associated with loci located at or near BRCA1-associated RING domain-1 (*BARD1*), HECT domain- and ankyrin repeat-containing E3 ubiquitin protein ligase 1 (*HACE1*), *LIN28B*, and LIM domain only 1 (*LMO1*) (Capasso et al. 2009, 2013; Wang et al. 2011; Diskin et al. 2012). In addition to the low mutation frequencies, several of these genes were not validated in multiple studies (e.g., *PTPRD*, *ODZ3*, *CSMD1*, *TIAM1*). In fact, while one study found PTPRD acts as a tumor suppressor in neuroblastoma (Meehan et al. 2012), a separate study found overexpression of PTPRD to have minimal effect on colony formation in neuroblastoma cells, whereas levels of PTPRD were similar between embryonic adrenal cells and neuroblastoma lines, calling into question the role of PTPRD in neuroblastoma (Clark et al. 2012). One of the largest studies to date (240 samples) identified additional mutations (including a recurrent point mutation in *MYCN* (P44L) at a frequency of ~2%, leading to a twofold increased level of expression); however, the vast majority of mutations occurred at frequencies under 1% (Pugh et al. 2013).

The low mutation rate in neuroblastoma resembles other pediatric tumors, including medulloblastoma (Pugh et al. 2012), and stands in contrast to changes in chromosomal copy number, which are quite frequent and robust. Further support for the idea that chromosome aberrations contribute to neuroblastoma is the finding that chromothripsis (shredding of subchromosomal regions and random reassembly of fragments) may be associated with high-risk and late-stage neuroblastoma, as this phenomenon was observed in one of the recent papers reporting sequencing results (Molenaar et al. 2012b). Might neuroblastoma be driven by these subchromosomal gains and losses? For example, deletion of chromosome 1p or 11q occurs in 25%–35% of patients, whereas gain of chromosome 17q occurs in >70% of tumors (Plantaz et al. 1997; Attiyeh et al. 2005; Mossé et al. 2007). Interestingly, multiple studies have found amplification of *MYCN* to be directly correlated with chromosome 1p loss, while inversely related to chromosome 11q deletion (Cheng et al. 1995; Komuro et al. 1998; Caron et al. 2001; Attiyeh et al. 2005; Carén et al. 2010). In addition, gains of chromosome 17q have also been detected in tumors with amplified *MYCN*, although this is not unexpected because of the high frequency of chromosome 17q gains (Plantaz et al. 1997; Valentijn et al. 2005). Potentially, these somatic copy number variations of subchromosomal regions could equate to multiple genetic mutations occurring simultaneously, contributing to transformation. Modeling these chromosomal abnormalities, and identifying minimal regions of gains and losses may lead to the identification of new and potent drivers of neuroblastoma.

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SUMMARY

Although neuroblastoma was first described more than 100 years ago, progress in identifying genetic causes and therapeutic targets has taken time. In 1983, *MYCN* was identified as the second member of the MYC family and two years later, amplification of *MYCN* was identified as among the first genetic biomarkers of any cancer, specifically marking high-risk neuroblastoma (Brodeur et al. 1984; Seeger et al. 1985). A genetically engineered mouse model of neuroblastoma (TH-*MYCN*) was established in 1997 and along with other studies, implicated MYCN in all aspects of tumorigenesis and malignancy (Nakagawara et al. 1994; Weiss et al. 1997; Song et al. 2007; Kang et al. 2008; Cotterman and Knoepfler 2009; Ma et al. 2010). Unfortunately, because MYCN is considered "undruggable," standard care for *MYCN*-amplified neuroblastoma patients does not include targeting MYCN itself. GWAS, whole exome, and whole genome sequencing studies over the past few years have identified few recurrently mutated genes. ALK represents one of the most druggable and promising targets and was validated as a driver of neuroblastoma in both fish and mouse models (Heukamp et al. 2012; Schulte et al. 2012; Zhu et al. 2012). However, ALK mutations occur in $<$10% of neuroblastoma cases and initial clinical studies using targeted therapy against ALK in these patients have been disappointing (Carpenter and Mossé 2012). Preclinical studies using bromodomain inhibitors and targeting molecules that destabilize MYCN show promise preclinically, and should be tested in children with aggressive forms of neuroblastoma. In addition, because chromosomal aberrations appear to be robust in pediatric tumors, clues for uncovering novel drivers and therapeutic targets may depend on developing models of these chromosomal abnormalities.

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