A Genome-Wide Search for Promoters That Respond to Increased MYCN Reveals Both New Oncogenic and Tumor Suppressor MicroRNAs Associated with Aggressive Neuroblastoma

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Abstract

MYCN is a major driver of neuroblastoma tumorigenesis and MYCN amplification is the worst prognostic indicator of aggressive NB. To identify potentially therapeutic tumor suppressor microRNAs for aggressive NB, we utilized a conditional MYCN system to simulate MYCN-amplified and nonamplified tumor types and performed a genome-wide search for MYCN target microRNA promoters differentially repressed under high MYCN conditions. We identified 20 gene promoters hosting 30 microRNAs that were directly bound and differentially regulated by MYCN. Eleven of these genes showed significant clinical correlations for neuroblastoma with 4 genes linked with better survival and 7 genes linked with poor survival. Surprisingly, expression analysis of host genes and microRNAs demonstrated that 8 of 11 pairs were repressed by high levels of MYCN regardless of the clinical correlation of the host gene. We therefore predicted these intronic microRNAs would be tumor suppressors. In fact, detailed gain of function studies for two miRs, miR-591 and miR-558, confirmed potent tumor suppressive effects for miR-591 in orthotopic neuroblastoma xenografts. However, miR-558 markedly increased colony formation, proliferation, and tumor growth in vivo. Our data reveal host-gene independent functions of MYCN-target microRNAs and demonstrate that MYCN represses both tumor suppressive and proproliferative microRNAs.

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Introduction

Neuroblastoma remains a major therapeutic challenge in pediatric oncology, accounting for almost 15% of all childhood cancer deaths (1). To identify potential new therapeutic strategies for this aggressive embryonal malignancy of young children, we investigated the MYCN-dependent regulation and function of microRNAs (miRNAs) in neuroblastoma cell lines and xenograft models. miRNAs are essential regulators of embryogenesis and tissue differentiation, modulate stem cell biology, and act as oncogenes and tumor suppressors (2). These are the same cellular processes that are often perturbed by oncogenes in embryonal malignancies of childhood such as neuroblastoma (3, 4). Furthermore, multiple studies now show that miRNAs collaborate with oncogenic transcription factors to alter cell proliferation and cell cycle control (5-7), thereby contributing to malignant transformation. miRNAs also act as tumor suppressors either by directly suppressing oncogenes such as MYCN (8), activating apoptosis (9), or by indirectly modulating oncogenic transcriptional profiles (10). The tissue-specific functions of miRNAs in multiple types of tumors is thought to be essential for the expression of MYC gene family (c-MYC, MYCN, MYCL) related cellular phenotypes (11, 12) and oncogenic or tumor suppressive roles for individual miRNAs in neuroblastoma continue to be discovered (13).

The MYCN oncogene plays a central role in the biology of neuroblastoma and several other cancers, both as a negative prognostic factor and as a driving oncogene for tumorigenesis (14). In fact, MYCN amplification remains the strongest negative prognostic indicator for neuroblastoma (15) and neural crest targeted expression of MYCN is sufficient to induce neuroblastoma in transgenic animals (16). However, as a bHLH transcription factor, MYCN also directly activates genes which limit proliferation and increase apoptosis and the net
effect on cellular phenotypes is due to a complex balance of pro and anti-oncogenic effects. For example, MYCN directly activates p53 transcription and high levels of wild-type p53 are found in MYCN amplified neuroblastoma (17) and a major downstream target of p53, p21 (CDKN1A), is rapidly upregulated on DNA damage in neuroblastoma. In contrast, MYCN also directly activates transcription of the oncogenic miR-17-92a miRNA cluster, which in turn targets and inhibits p21 translation (18). Thus, the oncogenic consequences of MYCN function is the net integrated result of activation and repression of multiple downstream pathways with contrasting tumor repressive or tumor promoting cellular effects (12).

To identify downstream MYCN-driven genes and miRNAs that are associated with aggressive NB we carried out a genome-wide search for MYCN-binding sites showing differential promoter occupancy at high versus low MYCN condition. Using ChIP-Seq in a tetracycline inducible MYCN cell line (Tet-On) we identified genes and miRNAs that are corepressed under conditions of high MYCN expression with tumor suppressive and, surprisingly, proliferative effects in neuroblastoma tumor models. Detailed in vitro and in vivo characterization of 2 miRNAs strongly repressed by MYCN reveals miR-591 to be a potent tumor suppressor. In contrast miR-558, which was also down-regulated by MYCN, was strongly oncogenic. Our data highlight 2 distinct opposing effects of miRNAs downstream of MYCN. Dissecting the complex interactions of oncogenes such as MYCN and miRNAs which are also potent modulators of gene functions is critical to the rational incorporation of miRNA-mediated therapeutics to current treatment strategies.

Materials and Methods

Chromatin immunoprecipitation

ChIP was performed on MYCN3 cells either untreated or after addition of doxycycline (1 μg/mL) for 24 hours using a previously described method (19) with the following modifications. Cells were fixed in 1% formaldehyde for 10 minutes followed by 0.125M glycine for 15 minutes to prevent over fixation. Following lysis in a 1% SDS buffer, the samples were sonicated in 35 cycles of 30 seconds intervals in a Bioruptor sonicator (Diagenode). The samples were then diluted to 0.1% SDS cleared with protein A-agarose beads blocked with 0.1 mg/mL BSA. The negative control was immunoprecipitated without antibody. Following immunoprecipitation, the samples were washed and the DNA was eluted and purified as described earlier. Input was generated by purifying DNA from the sonicated lysate of treated and untreated cells.

Quantitative PCR (qPCR) was performed using the Qiagen QuantiTect SYBR Green PCR Kit according to the manufacturer’s instructions (Qiagen) using 5 μL of ChIP DNA per 25 μL reaction and 60°C annealing temperature for all primer sets. To control for the difference in DNA concentration between samples, a standard curve of amplification for each primer pair was generated using serial dilution of treated and untreated input. Both anti-MYCN and anti-actin samples were amplified in triplicate from treated and untreated samples.

The qPCR primers used to validate MYCN binding to the E-boxes within the MDM2 and MIRGH1 gene promoters are listed in Table 1.

Parallel sequencing

Chromatin immunoprecipitated DNA was prepared for sequencing following a modified version of Illumina’s Genomic DNA protocol. Sheared DNA fragments undergo end repair by T4 DNA polymerase to fill in 5' overhangs, Klenow polymerase to remove 3' overhangs, and T4PNK to phosphorylate the 5’-OH. For Illumina adapter ligation, a single adenine nucleotide overhang must be added to the polished DNA. A 1:30 dilution of the Adaptor Oligo Mix (20) was used in the ligation step. The resulting adapted constructs are amplified by 18 cycles of PCR enriching the DNA fragments with adapters flanking each end. The amplified product was quantified using a spectrophotometer at 260 nm (Nanodrop) then a portion of final sample is size separated on a 6% TBE (Invitrogen) to verify correct target range amplification. The validated DNA library is submitted at a 10 nmol/L concentration for sequencing. All protocols for Illumina library sequence preparation, sequencing, and quality control are provided by Illumina (20).

Mapping sequencing data

Between 3.7 and 3.9 × 10⁶ sequence tags for each immunoprecipitation condition were mapped onto the human genome reference sequence (HG18) using PASH (21) in an approach similar to Ahn and colleagues (22). Uniquely mapping reads were selected and the read coverage computed over 100bp windows tiling across the entire genome. The read density was then normalized employing a quantile normalization step, ensuring a similar distribution across the 3 data

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**Table 1.** The qPCR primers used to validate MYCN binding to the E-boxes within the MDM2 and MIRGH1 gene promoters

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2 E-box</td>
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<td>MIRGH1 E-box 1</td>
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<td>MIRGH1 E-box 2</td>
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<td>5’-gacggggcagaaaccgcagact-3’</td>
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<tr>
<td>MIRGH1 E-box 3</td>
<td>5’-tctgctagatttctgcacttt-3’</td>
<td>5’-gtgcaagtcagtctacgtagtatga-3’</td>
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<td>MIRGH1 E-boxes 4 and 5</td>
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<td>5’-tgcccctctccaaatgattgacag-3’</td>
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</table>
sets. Next we computed then the difference between each sample (MYCN-induced cell line, a MYCN–non-induced cell line) and the control, and generated P values assuming a normal distribution of the differences. The false discovery rate for each window was computed and an FDR below 0.001 was selected, and used to apply a segmentation algorithm to the remaining windows. Finally, we selected genomic features such as gene promoters, miRNA promoters, CpG islands, etc. and computed enrichment over background to identify promoters that are differentially occupied by MYCN in either the MYCN-induced or MYCN–non-induced sample. The analysis results were stored and visualized using the Genboree Discovery Platform (23).

**PCR assays**

To induce the MYCN expression, MYCN3 cell line was treated with doxycycline (1 μg/mL) for 12, 24, and 48 hours. Snap frozen tumor tissues were processed using RNAlater Ice before the RNA isolation. Total RNA including miR fraction was isolated using Qiagen miRNeasy kit as per the manufacturer’s protocol. For exon/intron/stemloop PCR, total RNA was reverse transcribed using TaqMan Reverse Transcription Reagent and amplified using Taq polymerase in ABI Veriti Thermocycler. Primers (Integrated DNA Technologies) for the respective exons, introns, and stemloops were designed as explained in Figure 3. PCR products were analyzed after 1% to 3% agarose gel electrophoresis.

TaqMan MicroRNA Assay probes for hsa-miR-558 and hsa-miR-591 were used to reverse transcribe and quantify using ABI StepOne RealTime PCR System. StepOne Software, Version 2.1 was used to analyze data. Kruskal–Wallis test (1-way ANOVA) was used for the statistical analysis.

**MTT proliferation assays**

Three thousand cells/well were plated into 96-well microtiter plates. Plates were incubated for 5 days. Proliferation was assessed at baseline and daily in triplicate. To assess proliferation, 15 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added to each well and plates read at 550 nm.

**Colony formation in soft agar**

Colonies were stained with Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich M5655) and visualized and counted with (BioRad Versadoc, Image 1 software).
Animal model

The Institutional Animal Care and Use Committee of Baylor College of Medicine approved the experiments described below. Four- to 6-week-old female NCr nude mice (Taconic Farms) were housed in a barrier facility and acclimated to 12-hour light/dark cycles for at least 48 hours before experimental use.

Tumor implantation

After tumor cells were counted, they were washed and re-suspended in sterile PBS at a concentration of 10^7 per mL. Mice (n = 30) were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylene (5 mg/kg) and 2% inhaled isofluorane. The left flank of mice was surgically prepared in a sterile fashion, and a transverse incision was performed to expose the left kidney. An inoculum of 10^6 tumor cells in 0.1 mL of PBS was injected under the renal capsule using a 27-gauge needle. Tumor cells lines used were CHLA-255 (n = 10), CHLA-255 miR558 (n = 10), and CHLA-255 miR591 (n = 10). The flank musculature was closed with 4-0 Vicryl suture (Ethicon), and the skin incision was closed with a skin staple.

Tissue harvest.

At the time of sacrifice, tumors and contralateral kidneys were resected and weighed. Tumors were visualized under fluorescent microscopy (Olympus SZX16) to assess GFP expression. Portions of tumor were also stored in RNA later (Ambion Inc.) for quantitative miRNA analysis. Portions of tumor were also preserved for histology.

Cell lines.

The neuroblastoma cell lines SH-SY5Y and SHEP were obtained from ATCC. Luciferase positive SH-SY5Y was a kind gift from Dr. Darrell Yamashiro, Columbia University, NY. The MYCN inducible MYCN3 cell line was

Table 2. Host genes and microRNAs with significantly differential MYCN binding

<table>
<thead>
<tr>
<th>Host gene</th>
<th>MicroRNA</th>
<th>Cytogenetic coordinate</th>
<th>Fold change at E-box</th>
<th>Fold change in promoter binding</th>
<th>E-box sequence</th>
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<td>hsa-mir-933</td>
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<td>1.08</td>
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</tbody>
</table>

NOTE: ChIP-Seq identified 20 genes and encoding 29 microRNAs and an intergenic loci encoding a single microRNA with significantly increased binding of MYCN on conditional upregulation (FDR <0.001). Sequence tags within 500bp of promoter E-box sequences or within 3 kb of intergenic microRNA loci were quantified and fold change in occupancy calculated. Fold change for the total promoter region was very similar to E-box binding, suggesting that the majority of increased binding was specific for E-boxes (–, common data for microRNAs expressed as clusters or coexpressed by 1 promoter).
A genome-wide search reveals novel MYCN targets that are repressed under conditions of high MYCN and therefore putative tumor suppressors of aggressive neuroblastoma

To identify gene and miRNA promoters that show increased MYCN binding under high MYCN conditions in the same genetic background, we used a MYCN-conditional cell line (MYCN-3, Tet-on) with rapid and robust inducible expression of functional MYCN for anti-MYCN ChIP (Fig. 1A; ref. 25). Following cross-linking of protein/DNA adducts, we isolated cell lysates under MYCN-induced (24 hours) and non-induced conditions. DNA was isolated, sheared, and MYCN-bound DNA was immunoprecipitated with a MYCN antibody as previously described (19). To confirm the specificity of our immunoprecipitation conditions, the assay was validated using qPCR analysis for the known MYCN target genes MDM2 (19) and MIR17HG (26) and isotype control antibodies (Fig. 1B).

Next, purified ChIP DNA was sequenced on the Illumina GA-I sequencing platform and sequence reads mapped on the human genome reference sequence (hg18, NCBI build 36) using the PASH algorithm (22). For the MYCN-induced cells, of 2,751,696 uniquely mapped sequences, 15,322 tags mapped to E-box DNA sequences. For the noninduced (MYCN-low) cells, we obtained 2,606,250 sequence tags of which 12,986 mapped to E-boxes. After normalization to ensure comparable genomic distribution between data sets, we calculated P values for each enrichment site and then determined false discovery rate (FDR) values for each genomic element sequenced. We next filtered all windows with a stringent FDR (<0.001) to identify MYCN binding to those E-boxes either (a) within 3kb of a known miRNA or (b) E-boxes within promoters of genes hosting intronic miRNAs (see Methods and Supplementary Figs. S1–S4 and Tables ST1–ST3, for details). With this approach we identified a list of 20 genes encoding 29 intronic miRNAs with clear enrichment of E-box–specific MYCN binding (FDR < 0.001) shown in Table 2.

Significant clinical correlations of MYCN-targeted microRNA host genes

To prioritize further functional experiments, we queried the neuroblastoma prognosis data base which presents microarray gene expression data and annotated clinical data for neuroblastoma and other pediatric cancers (http://pob.abcc.ncifcrf.gov/cgi-bin/JK), to obtain gene expression correlations with survival. Here Kaplan–Meier analysis was performed based on target gene expression dichotomized according to overall median gene expression and “good outcome” (survival >3 years from diagnosis) and "poor outcome" (death from disease). Detailed patient cohort descriptions and methods for this statistical analysis can be found in ref. 27. We found 11 of the 20 MYCN target genes identified in our study...
Table 3. Eleven of 20 microRNA hosting genes had increase binding of MYCN and significant clinical correlations with overall survival

| Host gene | MicroRNA   | Worse survival | P
|-----------|------------|----------------|---
| NDUFAF3   | hsa-mir-191| Higher         | <0.01
| SLC25A13  | hsa-mir-591| Higher         | <0.001
| SFRS52    | hsa-mir-636| Higher         | 0.01
| ATAD2     | hsa-mir-548d-1| Higher     | <0.0001
| ABCF1     | hsa-mir-877| Higher         | <0.0003
| EIF4H     | hsa-mir-590| Higher         | 0.004
| LARP7     | hsa-mir-302a-d| Higher     | 0.001
| MIR17HG   | hsa-mir-17-92| Higher       | <0.001
| ATF2      | hsa-mir-933| Lower          | <0.01
| BIRC5     | hsa-mir-558| Lower          | <0.005
| HNRPK     | hsa-mir-7-1| Lower          | <0.001
| DALRD3.2  | hsa-mir-425| NC             |
| RNF130    | hsa-mir-340| NC             |
| FBXL18    | hsa-mir-589| NC             |
| ZNF766    | hsa-mir-643| NC             |
| PANK3     | hsa-mir-103-1| NC        |
| TLE3      | hsa-mir-629| NC             |
| PLEC1     | hsa-mir-661| NC             |
| PANK2     | hsa-mir-103-2| NC        |
| SUP3T3H   | hsa-mir-586| NC             |
| Intergenic| hsa-mir-663| No data       |

NOTE: Correlation of gene expression (by microarray) versus survival was assessed using the public data sets for neuroblastoma gene expression at the Oncogenomics Section, Pediatric Oncology Branch, National Cancer Institute (50). Ten of 20 genes listed had significant correlations with survival. Higher expression correlated with worse survival for 7 genes where 3 genes had lower expression correlate with improved survival. Two microRNA clusters, mir-302a-d and mir-17-92, account for 10 microRNAs. Of note, MIR-17 is a known MYCN target and negative.

demonstrated significant associations with clinical outcome including the well-described oncogenic miRNA cluster miR-17-92 (Fig. 2 and Table 3). As illustrated by representative Kaplan–Meier curves, worse clinical outcomes correlated with both lower gene expression (4 genes) and higher gene expression (7 genes; Fig. 2). We further analyzed the expression of 2 intronic miRNAs, whose host genes had higher expression (SLC25A13, P < 0.001) or lower expression (BIRC5, P < 0.005) correlating with poor outcome (Fig. 2).

The majority of clinically significant microRNA–gene pairs are repressed under conditions of high MYCN

To systematically assess the impact of differential MYCN binding at promoter E-boxes, we designed RT-PCR primer pairs to measure independently the levels of an exon of the host gene, the intron in which the miRNA is located and the stem loop precursor from which the intronic miRNA is processed. We found reduction of expression for the majority of MYCN targeted host gene/miRNA pairs in all of the genomic elements measured (Fig. 3). Since MYCN is a key oncogene driving aggressive NB we would expect that these host gene/miRNA pairs repressed by MYCN should be tumor suppressors whose increased expression is linked with better survival. However, increased expression correlated with better survival in only 3 of the 11 clinically significant host gene/miRNA pairs repressed by MYCN. By contrast, increased expression was correlated with worse survival for the majority of these genes (8 of 11 gene–miRNAs pairs; Table 3). These surprising observations led us to further investigate the function of the associated intronic miRNAs, which in some cases can strongly repress or oppose the function of host genes as part of negative regulator feed-back loops (28).

We focused on 2 representative miRNAs from each group, miR-558 (whose host gene correlates with better survival) and miR-591 (whose host gene correlates with worse survival). First we performed real-time qPCR to validate the MYCN-dependent repression for each miRNA. As shown in Figure 3B, MYCN induction in the MYCN-3 line led to a rapid decline in mature miRNA levels of both miR-558 and miR-591 within 12 hours. This change in the host gene, the stem loop precursor, and the mature miRNA persisted over the time course of the experiments.

These data led us to evaluate whether these miRNAs are specifically repressed in the MYCN-amplified subset of high-risk tumors. Therefore, we analyzed miR-558 and miR-591 expression in an independent cohort of 54 patient samples with qPCR according to a previously established protocol (29). Intriguingly, we found very low to undetectable levels of both these miRNAs in all high-risk samples regardless of MYCN status (P < 0.01). Details on clinical characteristics, stage, and MYCN status can be found in Supplementary Table T4 and in ref. 19. Our results suggest that both these MYCN repressed miRNAs may play a role in blocking MYCN-driven tumor initiation, which occurs prior to MYCN amplification as demonstrated by transgenic animal models (3) and suggested to us that both these miRNAs could be potent tumor suppressors.

It is important to note that a proposed tumor suppressor function for miR-591 contrasts with the antiapoptotic function of the host gene SLC25a13. This gene encodes the mitochondrial solute carrier protein Citrin, which is an aspartate–glutamate exchanger and implicated as the causative gene for both juvenile and adult onset citrullinemia (30). Deficiency or mutations of SLC25a13 interfere with NADH mitochondrial transport and lead to progressive liver disease. ShRNA-mediated knockdown of SLC25a13 activates mitochondrial-mediated apoptosis in hepatocytes (31). Similar proapoptotic cell protective effects are seen BIRC6, the host gene of miR-558 which encodes BRUCE/Apollon a member of the IAP (inhibitor of apoptosis protein) family. Recently, BRUCE has been shown to promote the proliferation of neural progenitor cells (NPCs) and knockdown reduces growth and increases sensitivity of NPCs to glucocorticoids (32). BRUCE has also been shown to protect cardiac myocytes and breast cancer cells from stress-induced apoptosis (33). As shown later, these observations are consistent with the
function of its intronic miR-558 which also strongly promotes proliferation of neuroblastoma cell lines and tumors.

**MYCN-repressed miR-558 and miR-591 show contrasting tumor suppressor and tumorigenic functions in neuroblastoma**

Because our ChIP-seq, qPCR, and profiling of clinical samples suggested that miR-558 and miR-591 may be potent tumor suppressors despite the opposite effects of their host genes on survival, we sought to determine the functional consequences of overexpression of miR-558 and miR-591 in neuroblastoma. Subclones of the SH-SY5Y and ChLA-255 neuroblastoma cell lines were retrovirally transduced with miR-558 or miR-591 stem loops with 140bp of flanking sequence and selected by FACS sorting for GFP expression to avoid artifacts introduced by extended antibiotic selection (pSuper-GFP). Both of these cell lines form highly undifferentiated tumors and metastasize in vivo, reflective of high-risk neuroblastoma with low to moderate MYCN expression.

The impact of these miRNAs on proliferation and apoptosis was evaluated using MTT assays to measure in vitro proliferation, and colony formation using soft agar colony growth as shown in Figure 4. The miR-591 had little effect on cell proliferation relative to the control miRNA-GFP construct with a mutated 5′ seed region or the host cell line without a miRNA construct. This miRNA did not strongly alter colony formation, although a trend towards decreased colony formation relative to the parental or control cell lines was observed. By sharp contrast, miR-558 dramatically and consistently increased both proliferation and colony formation in both cell lines. These in vitro studies clearly demonstrated that miR-558 expression promoted tumor growth consistent with the proliferative function of its host gene BIRC6.

**In vivo orthotopic implantation and tumor formation confirm that miR-591 is a tumor suppressor and miR-558 is an oncomiR**

Because the effects of miRNAs on cell lines growing in culture may be significantly different from their effects on tumors growing in vivo, we generated orthotopic xenografts of our cell lines in nude mice. We implanted tumor cells directly into the left renal capsule of mice and allowed them to grow for 5 weeks. This model recapitulates the growth of human neuroblastomas, which often arise from adrenal or perirenal locations and are highly vascular and invasive (34, 35). At necropsy, tumors were resected, weighed, and analyzed histologically. As shown in Figure 5, miR-591 significantly repressed tumor growth with average weights less than 50% of control tumors ($P < 0.05$). These tumors were small and sclerotic and relatively avascular. In contrast, constitutive expression of miR-558 led to highly vascular tumors that were over twice the average weight of tumors from the nontransduced parental line or the miR-558-mutant line containing a mutated 5′ seed sequence ($P < 0.05$). To confirm expression of the mature miRNAs in our tumors, we performed qRT-PCR for the mature miRNA in multiple tumors. In addition, fluorescent
microscopy of the tumor specimens showed strong expression of the GFP marker in all tumors (Fig. 5).

These in vitro and in vivo functional studies for 2 MYCN-repressed miRNAs reveal contrasting tumor suppressive (miR-591) and oncogenic (miR-558) effects on neuroblastoma growth and tumor formation, which either oppose (SLCA25a13/miR-591) or correlate with (BIRC6/miR-558) functions of their host genes. The therapeutic and biologic implications of these data are discussed later in the context of MYCN-driven neuroblastoma.

Discussion

MYCN amplification is found in about 50% of high-risk neuroblastoma (23% overall) and confers a particularly aggressive phenotype with poor survival (15, 36). By contrast, variations in lower levels of MYCN which are found in nonamplified tumors are clearly not correlated with outcome (37). This suggests that the promoters for MYCN target genes that are differentially regulated at very high levels of MYCN may be particularly important drivers of the highly aggressive MYCN-amplified phenotype.

We simulated MYCN-amplified aggressive neuroblastoma and MYCN-unamplified neuroblastoma by using a conditional cell line that expresses high and low levels of MYCN in the same genetic background and used ChIP-seq to identify gene and miRNA promoters with differential binding of MYCN. Induction of MYCN expression lead to marked changes in promoter occupancy of a subset of genes hosting intronic miRNAs. Interestingly, of the 11 genes identified...
with significant clinical correlations, 8 were strongly repressed by increased MYCN binding independently of the clinical correlation. By contrast, both MIR17HG which encodes the oncogenic miR-17-92a cluster and EIF4H encoding miR-590 showed increased expression on upregulation of MYCN.

Although MYCN and C-Myc are classically defined as oncogenes which activate transcription of proliferative cellular networks, it is increasingly clear that they mediate critical oncogenic networks through repression of target genes including miRNAs. Direct inhibitory interactions between MYC, MIZ-1, and SP1 repress p21 (CDKN1A; ref. 38), and MYC oncogenes can directly regulate promoter methylation (39, 40) and increase HDAC expression (41). It has recently been shown that MYCN binding as a SP1/MIZ1/MYCN complex recruits HDAC1 to local histones as well (42). Our data suggests that some of these repressive effects may be critically regulated by the extent of MYCN promoter occupancy, distinguishing MYCN function in amplified and nonamplified neuroblastoma.

Through this work we identified miR-591 as a potent new tumor suppressor for neuroblastoma, a function not predicted by its expression from an intron in the SLC25a13 host gene. This miRNA is expressed at very low levels in high-risk neuroblastoma and strongly suppresses tumor growth of neuroblastoma xenografts. Exogenous in vivo delivery of this miRNA to neuroblastoma or reactivation of miR-591 target pathways could be a novel therapeutic approach for treating aggressive NB. However, exogenous expression of another MYCN-repressed miRNA, miR-558, produced marked pro-oncogenic effects in line with an antiapoptotic function of its host gene BIRC6.

These contrasting tumor suppressive and effects of MYCN-repressed miRNAs highlight the complexity of interactions of oncogenes and downstream miRNAs in aggressive cancers. MYCN expression shortens the cell cycle and increases contact independent growth and metastasis in vitro and in vivo (43, 44). However, it is also clear from in vitro and in vivo models that MYCN can directly upregulate the p53 protein and sensitize cells to apoptosis from genotoxic stress (44, 45).
Neuroblastoma is uniformly p53 wild type, and p53 signaling can be robustly stimulated in vitro and in vivo in these cancers (25, 46). These multiple effects of MYCN may manifest themselves at distinct time points of tumorigenesis (3, 47). Hence MYCN function may help to prevent differentiation and apoptosis of neuroblast precursors during tumor initiation, and then later restrain proliferation through p53-dependent and -independent mechanisms to match local nutrient and oxygen supplies within the tumor microenvironment.

The role of miRNAs as effectors of the MYCN phenotype is a major focus of interest due to their widespread effects on transcriptional programs and potential therapeutic utility as both biomarkers and direct therapeutic tools (48, 49). Our findings showing that high-level MYCN expression can repress a new oncogenic miRNA, miR-558, and a new tumor suppressor, miR-591, are consistent with the above hypothesis that MYCN must restrain growth as well as promote it. Thus, repression of miR-558 may be critical to the survival of early neuroblasts expanding under high MYCN levels. In contrast, repression of mir-591 may be a prerequisite for the expansion of these tumor precursors after initiation. Finally, these studies reveal an additional and new layer of complexity in the role of MYCN as a driving oncogene for neuroblastoma. This transcription factor suppresses the expression of miRNAs with potent proproliferative effects (e.g., miR-558) which could modify the effectiveness of anti-MYCN–targeted therapies. miRNAs engage in "feed-forward" and "feed-back" regulatory pathways in many cellular processes including reprogramming of induced pluripotent cells and maintenance of embryonic stem cell pluripotency. How oncogenes such as MYCN disrupt and co-opt these highly orchestrated functions of miRNAs that define cellular phenotypes and states of differentiation should be carefully considered in the design of novel therapeutic strategies for neuroblastoma and other MYC-driven malignancies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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