Silencing of the Long Noncoding RNA MYCNOS1 Suppresses Activity of MYCN-Amplified Retinoblastoma Without RB1 Mutation

Duangporn Saengwimol, Pamorn Chittavanich, Natanan Laosillapacharoen, Atthapol Srimongkol, Vijender Chaitankar, Duangnate Rojanaporn, Rangsima Aroonroch, Bhoom Suktitipat, Chonticha Saisawang, Saovaros Svasti, Suradej Hongeng, and Rossukon Kaewkhaw

Correspondence: Rossukon Kaewkhaw, Section of Translational Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand; rossukon.kae@mahidol.edu.

Received: October 9, 2020
Accepted: November 16, 2020
Published: December 3, 2020

PURPOSE. MYCNOS (MYCN opposite strand) is co-amplified with MYCN in pediatric cancers, including retinoblastoma. MYCNOS encodes several RNA variants whose functions have not been elucidated in retinoblastoma. Thus, we attempted to identify MYCNOS variants in retinoblastoma and aimed to decipher the role of MYCNOS variant 1 (MYCNOS1) on the activity of MYCN-amplified retinoblastoma.

METHODS. The profiles of MYCNOS variants and MYCN status were determined in 17 retinoblastoma tissues, cell lines, retinas, and retinal organoids. A functional study of MYCNOS1 expression was conducted in patient-derived tumor cells and in retinoblastoma cell lines via short hairpin RNA-mediated gene silencing. We carried out MYCN expression, cell viability, cell cycle, apoptosis, soft agar colony formation, and transwell assays to examine the role of MYCNOS1 in MYCN and cell behaviors. We analyzed a transcriptome of MYCN-amplified retinoblastoma cells deficient for MYCNOS1 and, finally, tested the responses of these cells to chemotherapeutic agents.

RESULTS. Expression of MYCNOS1 was associated with the expression and copy number of MYCN. Knockdown of MYCNOS1 caused instability of the MYCN protein, leading to cell cycle arrest and impaired proliferation and chemotaxis-directed migration in MYCN-amplified retinoblastoma cells in which RB1 was intact. MYCNOS1 expression was associated with gene signatures of photoreceptor cells and epithelial–mesenchymal transition. MYCNOS1 silencing enhanced the response of retinoblastoma cells to topotecan but not carboplatin.

CONCLUSIONS. MYCNOS1 supports progression of retinoblastoma. Inhibition of MYCNOS1 expression may be necessary to suppress MYCN activity when treating MYCN-amplified cancers without RB1 mutation.

Keywords: retinoblastoma, MYCN, long noncoding RNA MYCNOS1, RB1, photoreceptor signature, drug response

Retinoblastoma is a retinal tumor of infancy and childhood. Although the biallelic loss of RB1 in retina cells has been known for many decades to initiate the disease,1,2 high focal amplification of MYCN has been identified as the primary driver in a novel subtype that is found in the 1% to 2% of patients whose tumors carry the wild-type RB1 gene.3–5 This oncogene-driven retinoblastoma type is a very early-onset unilateral tumor that exhibits more aggression than the classical RB1-deficient retinoblastoma.5 MYCN-amplified retinoblastoma without RB1 mutation appears to

Investigative Ophthalmology & Visual Science

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iovs.arvojournals.org | ISSN: 1552-5783

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have histopathological and genetic characteristics similar to those of other MYCN-amplified embryonic tumors, such as neuroblastoma, even though the tumor features a retinoblastoma-associated gene expression profile. In oncogenic-driven retinoblastoma, MYCN is focally amplified with >28 copies, spanning 1 to 5 Mb and encompassing neighboring genes. MYCN (MYCN opposite strand) is located on the DNA strand opposite to MYCN with extensive head-to-head overlap; it is thus inevitably co-amplified in all cases of MYCN-amplified retinoblastoma. Additionally, the association of high MYCN transcript levels with MYCN amplification and expression has been widely reported in neuroblastoma.

MYCNOS encodes several RNA variants that exert their functions as long noncoding RNA or coding RNA and may functionally characterize human diseases. Most studies have focused on the role of variant 2, or MYCNOS2 (NR_161162.1), in tumorigenesis, where MYCNOS2 is associated with poor clinical outcomes in patients with neuroblastoma. MYCNOS2 transcripts serving as a noncoding RNA facilitate MYCN expression. Moreover, protein-coding MYCNOS2 facilitates the stabilization of MYCN oncprotein, activation of Wnt/beta-catenin signaling, and generation of an anti-apoptotic protein, which supports metastasis, chemoresistance, and survival of cancers.

However, the function of transcript variant 1, or MYCNOSI (NR_110230.2), has not been fully elucidated. One study reported that silencing the long noncoding RNA MYCNOSI results in reduced cell proliferation of MYCN-amplified neuroblastoma and rhabdomyosarcoma. MYCNOS appears to play a key role in cancer progression, but whether it acts as a silent passenger or is a pathogenic consequence of MYCN amplification in retinoblastoma is not known. Here, we characterize the expression profile of all five MYCNOS variants in human retinoblastoma tissues, cell lines, retina, and retinal organoids. Based on these observations, we hypothesize that MYCNOSI promotes oncogenesis and has functional relevance with MYCN in MYCN-amplified retinoblastoma.

**Materials and Methods**

### Human Samples

Retinoblastoma samples were collected from enucleated globes of patients at Ramathibodi Hospital, Mahidol University (Bangkok, Thailand). Fresh surgical specimens were used for genomic DNA and total RNA extraction or directly processed for derivation of cell lines. Blood was drawn from patients, and peripheral blood mononuclear cells were isolated from blood with Ficoll-Paque PLUS reagent (GE Healthcare, Uppsala, Sweden) in accordance with the manufacturer's instructions for genomic DNA extraction. Case characteristics of the 17 patients are listed in the Table. Postmortem eye globes were collected by the Thai Red Cross Eye Bank (Bangkok, Thailand) for cornea donation. The remaining eye globes were used for dissection to extract the neural retina. The lens, iris, and vitreous were discarded, and the choroid/retinal pigment epithelium layers were removed from the retina for sample collection. All experimental protocols were approved by the institutional review board at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University. All methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from a parent of each patient before samples were collected. Retinal organoids were generated from H9 human embryonic stem cells (ESCs) to represent fetal tissues, according to a previous protocol.

### Patient-Derived Cells and Cell Line Cultures

Patient-derived retinoblastoma cells (RB170 cells) were established using a previously reported protocol as suspension and organoid cultures. Briefly, tumor tissue was dissociated, and the resulting cells were cultured in the growth medium developed previously. A final concentration of 1% Matrigel matrix solution (Corning, Inc., Corning, NY, USA) was added in suspension culture, and that of 65% Matrigel matrix (growth factor reduced) was used to embed cells for organoid culture. RB170 was manually dissociated and passaged at a 1:2 or 1:4 ratio once a week for suspension culture or every 3 weeks for organoids. Cold freezing medium (culture medium containing 10% dimethyl sulfoxide) was used to freeze cells at −80°C for 24 hours before long-term storage in liquid nitrogen. A human retinoblastoma cell line (Y79; American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, USA) containing 15% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Medium was changed every 3 days for both RB170 and Y79 cells.

### Short Hairpin RNA-Mediated Gene Silencing

Short hairpins (Supplementary Table S1) targeting MYCNOSI transcripts (two independent target regions) and non-targeting short hairpin controls (sh-NC) were cloned in pZIP-hEF1-alpha-ZsGreen-Puro vectors (Transomic Technologies, Inc., Huntsville, AL, USA). The ZIP lentiviral vector (pZIP) contained a gene cassette in which human elongation factor 1 alpha promoter (hEF-1α) drove the expression of green fluorescent marker (ZsGreen), puromycin-resistant gene, and UltramiR scaffold (Transomic)-loaded short hairpin RNA (shRNA). An element for internal ribosome entry sites was inserted between the fluorescent marker
and puromycin-resistant gene. Lentivirus was produced by transfecting 293T cells with shRNA plasmids and helper plasmids pMDIg/pRRE, pRSV-Rev, and pMD2.G (12251, 12253, and 12259; Addgene, Watertown, MA, USA) using X-tremeGENE HP transfection reagent (Roche, Mannheim, Germany). Viral supernatant was collected 48 and 72 hours after transfection, filtered through a 0.45-μm filter, and concentrated using Lenti-X Concentrator (Takara Bio USA, Inc., Mountain View, CA, USA) in accordance with the manufacturer’s instructions. The multiplicity of infection (MOI) was determined, and an MOI of 3 with 4-μg/mL polybrene was used to transfect 5 × 10^5 cells. Cells were cultured for 72 hours before stable cell lines were selected with 0.4-μg/mL puromycin. The purity of ZsGreen-positive cells was confirmed by flow cytometry after selection.

Methods for genomic analysis, RNA expression analysis, western blotting, histology, immunofluorescence and imaging, live cell imaging, RNA sequencing, soft agar colony formation, cell viability, drug testing, and migration and cell cycle assays are listed in the Supplementary Materials. Data availability is also listed in the Supplementary Materials.

**RESULTS**

**Expression of MYCNOS Variants in Retinoblastoma**

Expression levels of all five MYCNOS variants were examined in retinoblastoma tissues compared with adult retina, retinal organoids, and the Y79 retinoblastoma cell line (Figs. 1A, 1B). We found that MYCNOS1 and MYCNOS2 were expressed in tumors (Figs. 1B, 1C). Although MYCNOS2 was detected in all tumors and normal tissues, MYCNOS1 was rarely expressed in adult retina and retinal organoids (Fig. 1C; Supplementary Figs. S1A–S1C). RB739 and RB941 retinoblastoma tissues showed expression levels of MYCNOS1 and MYCNOS2 similar to those of normal tissues, suggesting possible contamination of normal retina (Fig. 1C). However, MYCNOS1 was highly expressed in RB170 tissue and Y79 (Fig. 1C; Supplementary Figs. S1A–S1C). Expression of MYCNOS1 was downregulated in human ESC-derived retinal organoids representative of fetal retina\textsuperscript{16,19} compared with ESCs, suggesting that its downregulation was required for retinogenesis, whereas high expression levels are implicated in tumorigenesis (Supplementary Figs. S1A, S1B).
Copy Number and Expression of MYCN and Association with MYCNOS1

We then examined MYCN status regarding gene copy number and expression in these tumor tissues. Droplet digital PCR indicated that RB170 tissue carried high copy numbers of MYCN: 90 and 75 based on RPP30 and RLBP reference genes, respectively (Fig. 1D). Ten copies of MYCN were detected in RB775, considered to be low MYCN amplification,5 whereas other tumor tissues carried about two copies compared with blood DNA (Fig. 1D). As expected, MYCN expression levels were relatively high in MYCN-amplified tumors (Fig. 1E; Supplementary Figs. S1D, S1E). The copy number of MYCN was positively correlated with the expression of MYCN and MYCNOS1 (Fig. 1E). Additionally, MYCN expression was correlated with MYCNOS1 expression (Supplementary Fig. S1C). The expression profile of MYCNOS1 implied its oncogenic role, particularly in tumor cells with high MYCN amplification, and suggested functional relevance of MYCN.

MYCN Amplification without RB1 Mutation in RB170 Retinoblastoma

Whole-genome analysis of RB170 tumor tissue and matched germline DNA revealed high MYCN amplification (75 copies) (Supplementary Fig. S1F). MYCN amplicons spanned 1.1 Mb, encompassing MYCNOS, LINC01804, MYCNOT, GACTA3, NBAS, and DDX1, consistent with the amplicion size by whole-genome SNP array (Supplementary Fig. S1F and not shown). Somatic mutations were rarely detected (Supplementary Fig. S1G). No alterations of the RB1 sequence were detected in tumor or blood DNA samples, consistent with the results of Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA; not shown).

Additionally, the sequence of RB1 mRNA from tumors was not changed (Supplementary Fig. S1H). Molecular testing confirmed that RB170 tumor tissue had a wild-type copy of RB1, even though there was evidence of loss of heterozygosity spanning RB1 detected only in the tumor (47 Mb). Histologically, rosettes were absent in tumor tissue, and tumor cells had round nuclei with prominent large nucleoli, which were readily distinguishable from the classical type of retinoblastoma (Supplementary Figs. S1I–S1K). Age at diagnosis was 3 months, and the tumor volume was large. Molecular, histological, and clinical features indicated that RB170 could be classified as a new subtype in which the disease is driven by high MYCN amplification.

Characterization of Tumor Cells Derived from MYCN-Amplified RB170 Tumor Tissue

We generated RB170 cells grown in organoid or cell suspension cultures from MYCN-amplified retinoblastoma without mutations in RB1.17,18 Tumor cells were large and had prominent nucleoli, corresponding to the tumor cells in parental tissue (Fig. 2A; Supplementary Fig. S1I). RB170 tumor cells had cone properties, as shown by CRX and ARX expression (Figs. 2B, 2C). M/L (RXRγ, TRβ2, and M/L opsin) and S (O-opsin) cone-specific proteins were expressed, and glial fibrillary acidic protein (GFAP), indicative of glial cells, was also detected (Figs. 2D–2H). Nevertheless, proteins associated with other retinal cell types, including rod, retinal ganglion, interneuron, and bipolar cells, were not detected in RB170 cells (Supplementary Figs. S2A–S2F). Ki67 staining showed that tumor cultures were comprised of non-proliferative and proliferative cells (Figs. 2B–2H). Interestingly, cells staining positively for retinal markers co-expressed Ki67, suggesting that the proliferative cells had cone (M/L and S) and glial characteristics (Figs. 2B–2H). The proliferative cells of RB170 differed from those of RB1-deficient retinoblastoma, where M/L isopin is primarily expressed.17,20 Additionally, MYCN and MDM2 oncoproteins, which constitute a cone-signal pathway, were detected and co-expressed with Ki67 (Figs. 2I, 2J).

RB170 cells maintained MYCNOS1 expression, MYCN amplification (67 copies), and MYCN expression at high levels while retaining RB1 status, as identified in the tissue by sequencing and MLPA (Fig. 1E). The Y79 cell line, known to be RB1 deficient, carried 50 copies of MYCN and expressed MYCN and MYCNOS1 (Fig. 1E). We then determined the function of MYCNOS1 in RB170 and Y79 tumor cells.

Effect of MYCNOS1 Knockdown on MYCN in MYCN-Amplified Retinoblastoma Cells

Stably expressed shRNA targeting MYCNOS1 (sh-A and sh-B) successfully reduced the levels of MYCNOS1 RNA compared with sh-NC in MYCN-amplified RB170 and Y79 cells (Figs. 3A, 3B). MYCNOS1 expression was not altered following MYCNOS1 depletion, indicating a lack of compensation for the loss of variant 1 (Figs. 3C, 3D). We found that the levels of MYCN mRNA were not affected in RB170 and Y79 deficient for MYCNOS1 (Figs. 3E, 3F). However, levels of MYCN protein were decreased following MYCNOS1 depletion in RB170 (Figs. 3G, 3H). Concurrently, the half-life of MYCN protein was reduced (19.84 ± 4.34 vs. 37.24 ± 2.01 minutes; P = 0.0219), which confirmed that MYCNOS1 modulated MYCN posttranscriptionally (Figs. 3I, 3J). In contrast, MYCN protein levels were not altered in Y79 cells deficient in MYCNOS1; RB1 protein was completely absent in Y79, which might contribute to the levels of MYCN after MYCNOS1 silencing (Figs. 3K, 3L). Regarding MYCN protein levels, the results suggest that knocking down MYCNOS1 mainly affected MYCN-amplified tumor cells without the RB1 mutation.

Activity of MYCN-Amplified Retinoblastoma Cells Following MYCNOS1 Knockdown

The cell viability assay indicated a lower proliferative rate in RB170 deficient for MYCNOS1 (Fig. 4A). This concurred with G1/S cell cycle arrest, which supported the role of MYCN protein when decreased, leading to cell arrest (Fig. 4B). Cell proliferation and cell cycle, however, were not altered following MYCNOS1 knockdown in the Y79 cell line, which appeared to divide more rapidly (2.6 days; 95% confidence interval [CI] 2.2–3.5) than RB170 (3.7 days; 95% CI, 3.0–4.6) (Figs. 4C, 4D). The number of cells staining positive for the apoptotic marker caspase-3 was not altered after MYCNOS1 knockdown in RB170 (Fig. 4E). The soft agar colony formation assay showed that MYCNOS1-deficient tumor cells formed larger colonies (Figs. 4F–4I; Supplementary Fig. S3). Colony areas of 6865.0 ± 153.0 μm² and 5442.0 ± 63.6 μm² were measured for RB170 cells with sh-A and sh-B compared with that of 5171 ± 89.3 μm² for sh-NC-RB170 (mean ± SEM; P < 0.0001 and P = 0.0776,
FIGURE 2. RB170 tumor cells derived from MYCN-amplified retinoblastoma without RB1 mutation maintain cone-specific signaling circuitry. (A) Section of RB170 showing large tumor cells with prominent nucleoli, corresponding to cells in the parental tissue (Supplementary Fig. S1I). (B–J) Immunostaining of RB170 tumor cultures for CRX (B), ARR3 (C), TRβ2 (D), RXRG (E), L/M opsin (F), S opsin (G), GFAP (H), MYCN (I), and MDM2 (J), co-stained with Ki67 indicative of neoplastic cells. See Supplementary Fig. S2 for negative staining for other markers. Scale bar: 50 μm.

respectively) (Figs. 4F, 4G). This was consistent with Y79: 19250.0 ± 1028.0 μm² and 17731.0 ± 1025.0 μm² for sh-A and sh-B vs. 8689.0 ± 336.0 μm² for sh-NC (P < 0.0001 and P = 0.0001, respectively) (Figs. 4H, 4I). The numbers of colonies of both cell lines deficient in MYCNOS1 did not differ statistically from that of cells with sh-NC (Figs. 4F–4I).

Because proliferation of MYCNOS1-deficient RB170 was impaired, a large colony was not solely dependent on cell proliferation (Figs. 4A, 4B, 4F, 4G). Measurement of cell velocity magnitude from live cell imaging indicated that MYCNOS1-deficient RB170 had increased motility, with faster two-dimensional (2D) speed (5.75 ± 0.43 μm/h and 5.13 ± 1.41 μm/h for sh-A and sh-B vs. 4.09 ± 0.55 μm/h for sh-NC; P = 0.0284 and P = 0.2142, respectively) (Supplementary Figs. S4A–S4D). MYCNOS1-depleted RB170 formed dendrites and exhibited increased motility, moving toward or away from others (Supplementary Fig. S4E; Supplementary Video). This increased motility of retinoblastoma cells deficient in MYCNOS1 could contribute to the movement of cells through agarose gel to form aggregates with contacting cells, resulting in large colonies.

We questioned, however, whether this 2D speed was related to chemotaxis-directed migration, which is involved in tumor dissemination, given that this RB170 patient had orbital retinoblastoma and brain metastasis. We then performed a transwell-based, three-dimensional migration assay. Tumor cells were greatly dependent on chemoattractant for migration (16 ± 16 cells without chemoattractant vs.
FIGURE 3. MYCNOS1 silencing reduces stability of MYCN protein in MYCN-amplified retinoblastoma without RB1 mutation. (A, B) MYCNOS1 expression following MYCNOS1 silencing (sh-A and sh-B vs. a non-targeting control, sh-NC) in MYCN-amplified retinoblastoma RB170 (A) and Y79 (B) by quantitative RT-PCR. (C-F) MYCNOS2 (C, D) and MYCN (E, F) transcript levels following MYCNOS1 silencing in RB170 and Y79 by quantitative RT-PCR. (G–L) MYCN protein levels and protein stability assay in RB170 (G–J) and MYCN protein levels in Y79 (K, L) following MYCNOS1 silencing by western blotting of whole lysate and densitometry. Cycloheximide (CHX) was used to inhibit protein synthesis. Means ± SEM are shown, and at least three experiments were conducted independently. One-way ANOVA followed by Tukey’s multiple comparison test and Student’s t-test were used to measure the significance of RNA and protein levels and protein half-lives, respectively. For P < 0.05, the results were concluded to be statistically significant; ns, not significant.

673 ± 44 cells with chemoattractant for sh-NC; P = 0.0002), and MYCNOS1 silencing resulted in decreased transmigration of tumor cells (341 ± 50 cells vs. 673 ± 44 cells for sh-NC; P = 0.0039) (Figs. 4J–4N). Indeed, knocking down MYCNOS1 impaired chemotaxis-directed migrating ability, given that migration of MYCNOS1-deficient RB170 cells was independent of chemoattractant (Figs. 4K, 4M, 4N). This indicated that MYCNOS1 positively controlled migration and that increased 2D speed of MYCNOS1-depleted RB170 was related to cellular processes other than chemotaxis-directed tumor migration.

MYCNOS1-Mediated Regulation of Photoreceptor and Epithelial-Mesenchymal Transition Gene Signatures

A total of 1599 genes were differentially expressed in RB170 deficient for MYCNOS1, with 750 being upregulated and 849 being downregulated (Fig. 5A). BAHCCI1, NAV2, SFRP2, RBP3, TLE4, and ISL1 were the top differentially expressed genes (Fig. 5A). Gene ontology (GO) analysis showed that cell fate commitment and axon development were the most significantly enriched processes in the upregulated gene set, and the associated genes encoded transcription factors directing the differentiation of postsynaptic neurons in retina (ISL1, NEUROG1, NEUROD2, SIX1, PAX6, and PROX1) (Figs. 5A, 5B). Additionally, expression of axon guidance molecules SLIT2, SLIT3, and SEMA6A was upregulated and included in GO terms of axon development and morphogenesis of a branching structure (Fig. 5B). These molecules, which can be repulsive or attractive for growing axons and migrating neurons, are implicated in neural differentiation-related cell motility (Supplementary Video).

In contrast, the expression of photoreceptor-associated genes (RBP3 and ABC4) was greatly downregulated (Fig. 5A), concurrent with the enriched GO term of photoreceptor cell maintenance and several terms relating to the function of photoreceptors (Fig. 5C). We then examined the expression of retinal-associated genes within a set of
**Figure 4.** *MYCNOS1* silencing suppresses cell proliferation and migration. (A–D) Viability assay measuring proliferative rate and cell cycle analysis of RB170 (A, B) and Y79 (C, D). (E) Immunostaining for caspase 3, an apoptotic marker, of RB170. (F–I) Soft agar colony formation assay of RB170 (F, G) and Y79 (H, I). Each point on the scatterplots represents individual colonies (see other initial seeding density in Supplementary Fig. S3). (J–N) Chemotaxis-directed migration of RB170 in transwell assays (J–M) and the number of transmigrating cells (N). Means ± SEM are shown, and three experiments were conducted independently. One-way ANOVA followed by Tukey’s multiple comparison test was used to test the differences between groups. For *P* < 0.05, the results were concluded to be statistically significant; ns, not significant. Scale bar: 200 μm.

differentially expressed genes following *MYCNOS1* silencing and found that expression of cone genes, including *ARR3*, *GNAT*, and *PDE6C*, was downregulated in *MYCNOS1*-depleted tumor cells (Fig. 5D). Several rod photoreceptor-specific genes whose expression is known to be depleted in retinoblastoma were downregulated, but not *NRL* and *GNB1* (Fig. 5D). Interestingly, the expression of genes associated with other retinal cell types, including interneurons, retinal ganglia, and progenitors, was upregulated (Fig. 5D). Because of the absence of cell death, a gene signature change appeared to stem from a cell fate switch after *MYCNOS1* knockdown (Fig. 4E). Together, these results suggest that *MYCNOS1* maintained the cone signature in *MYCN*-amplified retinoblastoma without *RB1* mutation.

Cell–cell adhesion molecules via plasma-membrane adhesion molecules were the next enriched GO terms for upregulated genes (Fig. 5B). *CDH11*, for example, was upregulated and is a candidate tumor suppressor gene whose expression is frequently lost in advanced retinoblastoma.25 In contrast, cell-substrate adhesion, actin filament-based process, and extracellular matrix organization were associated with downregulated genes. These terms suggest a role of *MYCNOS1* in migration and invasiveness (Fig. 5C).

We then examined the expression of genes associated with the epithelial–mesenchymal transition (EMT). Of 1011 genes in the EMT gene database dbEMT2,24 we found 112 genes that were differentially expressed in our dataset, with 49 genes downregulated and 63 genes upregulated in RB170 deficient for *MYCNOS1* (Supplementary Fig. S5A). GO analysis indicated that downregulated genes were associated with the EMT term (Fig. 5E; Supplementary Fig. S5B). Additionally, positive regulation of catenin import into nucleus, SMAD protein complex assembly, and SMAD signal transduction were included in significant GO terms for downregulated genes (Supplementary Fig. S5B). Concordantly, GO terms indicative of EMT inhibition were enriched for upregulated genes, including negative regulations of canonical Wnt signaling pathway and TGF-β1 production (Supplementary Fig. S5C). Furthermore, *SFRP2* and *TLE4* (an inhibitor of Wnt/β-catenin signaling) were among the top five
FIGURE 5. Gene signatures of photoreceptor and epithelial-mesenchymal transition were depleted in MYCN-amplified retinoblastoma cells deficient for MYCNO51. (A) Volcano plot shows differentially expressed genes (blue dots, fold change ≥ 2 and false discovery rate [FDR]-adjusted $P \leq 0.01$) in RB170 following MYCNO51 silencing. (B, C) Gene ontology terms enriched for upregulated (B) and downregulated (C) gene sets shown in the 2D space generated by applying multidimensional scaling to a matrix of the semantic similarities of the GO terms. Similar GO terms are grouped together or are closer in the plots, and cluster representatives are selected based on $P$ values and dispensability.
upregulated genes. In summary, GO analysis indicated that MYCNOS1 controls photoreceptor signature and the EMT process.

**Sensitivity of MYCN-Amplified Retinoblastoma Deficient for MYCNOS1 to Chemotherapeutic Agents**

Topotecan, a cell cycle–specific drug, is frequently used in intravitreal or intra-arterial chemotherapy for refractory intraocular retinoblastoma, whereas carboplatin, a non-specific cell cycle drug, is commonly used in a standard regimen for chemoreduction and is administered intravenously. We found that MYCNOS1 knockdown sensitized RB170 to topotecan for death, indicated by a twofold decrease in the half-maximal effective concentration (EC50, 389.14 ± 16.19 nM vs. 189.7 ± 38.32 nM, P = 0.0409), but did not affect the response of carboplatin-treated cells (Figs. 6A, 6B). This suggests that MYCNOS1 knockdown enhanced the response of MYCN-amplified retinoblastoma without RB1 mutation to a cell cycle–specific drug compared with a non-specific cell cycle drug. However, MYCNOS1 knockdown did not alter the response of Y79 with RB1 null mutation to topotecan or carboplatin (Figs. 6C, 6D). RB1 was significantly upregulated in RB170 deficient for MYCNOS1, suggesting that RB1 status might contribute to the different response against topotecan25,26 in RB170 and Y79 (Supplementary Fig. S5A). In agreement with this, the topotecan EC50 of RB170 was significantly higher than that of Y79 or RB1-deficient retinoblastoma cells without MYCN amplification (RB654),17 suggesting lower sensitivity of MYCN-amplified retinoblastoma with intact RB1 to topotecan (for Y79, 389.14 ± 16.19 nM vs. 114.5 ± 50.9 nM, P = 0.0167; for RB654, 79.81 ± 5.31 nM, P = 0.0100). Nevertheless, MYCNOS1 silencing made RB170 cells responsive to topotecan.

**DISCUSSION**

MYCN amplification initiates an aggressive form of retinoblastoma and co-amplifies MYCNOS1. We showed that MYCNOS1, encoding a long noncoding RNA or variant 1, is an oncogenic driver that promotes cell proliferation and migra-
tion, partly by regulating levels of MYCN protein. Furthermore, MYCNOS1 governs the expression of genes associated with photoreceptor and EMT. The gene signature suggests a role of MYCNOS1 in tumor differentiation and progression, which may contribute to the drug response of this oncogenic-driven retinoblastoma.

Our findings and those of others consistently show that MYCNOS1 transcripts are highly expressed in MYCN-amplified tumor cells compared with non-MYCN-amplified cells. MYCNOS1 transcripts regulate MYCN post-transcriptionally, affecting the levels of MYCN protein, whereas regulation at the transcriptional or posttranscriptional levels has been described for MYCNOS2 in controlling MYCN expression in neuroblastoma. MYCNOS1 promotes cell growth and proliferation by facilitating the G1/S phase transition; G1-phase arrest is indicative of MYCN depletion and occurred in MYCNOS1-deficient RB170. How MYCNOS1 positively controls MYCN protein stability is undetermined and requires further investigation.

MYCNOS1 expression modulated photoreceptor and EMT signatures, which suggests its role in cancer differentiation and progression, adding to the existing body of knowledge about MYCNOS1. Human retinoblastoma is primarily comprised of cone-like cells and has been recognized as being associated with high or low expression of cone genes. MYCN-driven retinoblastoma is in the group characterized by low expression of cone-associated genes, and expression of cone-associated genes was further downregulated following MYCNOS1 depletion. This change in expression of cone genes is not a result of differential death; rather, it could result from a switch in cell fate in tumors deficient for MYCNOS1 (Figs. 4E, 5D). Tumors with reduced cone signature express neuron- or progenitor-associated genes and respond better to chemotherapeutic agents. But, why then do MYCNOS1-deficient cells respond topotocan but not carboplatin? Increased MYCN instability and upregulation of cyclin-dependent kinase inhibitor p27 (CDKN1B) (Supplementary Fig. S5A), an antagonist of MYCN, could induce cell arrest in G1 phase, which would enhance sensitivity to topotecan, a topoisomerase I inhibitor targeting S- and G1-phase cells. Loss of a cone gene signature is suggestive of tumor progression, involving enriched expression of progenitor or neuronal genes. However, it is unlikely that cells deficient for MYCNOS1 represent advanced tumor cells, because they exhibit reduced aggressive behaviors. We hypothesize that MYCNOS1 maintains the features of retinoblastoma/cone signature or “stemness” previously reported for MYCNOS4 and that loss of MYCNOS1 expression induces neuronal differentiation in MYCN-amplified retinoblastoma without RB1 mutation.

EMT allows for migration, invasion, and metastasis of cancer cells and is associated with optic nerve invasion, nodal or distal metastasis, and recurrence of retinoblastoma. Given that this RB170 patient had orbital retinoblastoma and brain metastasis, EMT may contribute to progression of the RB170 tumor. EMT is partly achieved through activation of the SNAI1 transcription factor, which has a major role in repressing E-cadherin transcription. Expression of SNAI1 is induced by SMAD and HMGA2 proteins via the TGF-β/β-catenin signaling pathway, which may uniquely function only in MYCNOS1-promoting Activity of MYCN-Amplified RB.

Our findings indicate that MYCNOS1 coding for MYCNOS1 has a pathogenic consequence of MYCN amplification in MYCN-driven retinoblastoma. In co-amplification with MYCN, MYCNOS1 may uniquely function only in MYCN-amplified tumors for cancer progression. Simultaneous expression of MYCNOS1 with MYCN may be needed to imitate human MYCN-driven retinoblastoma without the loss of RB1 in generating a mouse model. Our findings suggest that MYCNOS1 knockdown is a potential therapeutic strategy for MYCN-amplified retinoblastoma without RB1 mutation. A limitation of this study is the small sample size and that the sample included extremely rare cases of MYCN-amplified retinoblastoma without RB1 mutation. A study with more retinoblastoma samples and additional patient-derived cell lines is necessary to strengthen our findings.

**Acknowledgments**

Supported by the Thailand Research Fund and Commission on Higher Education (MRG6280083); Faculty of Medicine, Ramathibodi Hospital (CF_60002); and Mahidol University.

Disclosure: D. Saengwimol, None; P. Chittavanich, None; N. Laosilipcharoen, None; A. Sirimongkol, None; V. Chatrankar, None; D. Rojanaporn, None; R. Aroonrooch, None; B. Sukittipat, None; C. Saiwasang, None; S. Svasti, None; S. Hongeng, None; R. Kaewkhaw, None

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MYCNOS1-Promoting Activity of MYCN-Amplified RB

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**Supplementary Material**

**Supplementary Video.** Time-lapse video microscopy over the course of 120 hours with images captured every 3 hours.