RESEARCH PAPER
RNA Biology 12:8, 893–899; August 2015; © 2015 Taylor & Francis Group, LLC

MYCNOS functions as an antisense RNA regulating *MYCN*

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Keywords: Transcriptional regulation, bidirectional promoter, neuroblastoma, RNA, G3BP1, MAP4

Amplification or overexpression of neuronal MYC (MYCN) is associated with poor prognosis of human neuroblastoma. Three isoforms of the MYCN protein have been described as well as a protein encoded by an antisense transcript (*MYCNOS*) that originates from the opposite strand at the MYCN locus. Recent findings suggest that some antisense long non-coding RNAs (lncRNAs) can play a role in epigenetically regulating gene expression. Here we report that *MYCNOS* transcripts function as a modulator of the *MYCN* locus, affecting *MYCN* promoter usage and recruiting various proteins, including the Ras GTPase-activating protein-binding protein G3BP1, to the upstream *MYCN* promoter. Overexpression of *MYCNOS* results in a reduction of upstream *MYCN* promoter usage and increased MYCN expression, suggesting that the protein-coding *MYCNOS* also functions as a regulator of *MYCN* ultimately controlling *MYCN* transcriptional variants. The observations presented here demonstrate that protein-coding transcripts can regulate gene transcription and can tether regulatory proteins to target loci.

Introduction

Neuroblastomas (NBs) are pediatric neuroendocrine tumors and are the most frequent extracranial cancers in young children. An important factor that affects the course of the disease is the expression of the *MYCN* gene. Amplification of *MYCN* in the tumor tissue is linked to aggressiveness of the tumor. *MYCN* encodes a nuclear phosphoprotein that functions as a transcription factor and is a close relative of the MYC oncoprotein, which shows gain of function in most human cancers. Like MYC, the MYCN protein interacts with a partner protein, MAX, as well as with several other proteins including retinoblastoma protein RB1, TBP (TATA box binding protein), YY1 (Ying Yang-1), MIZ1 (Myc-Interacting Zn Finger Protein-1), AP-2 (activating enhancer-binding protein AP-2 α) and NMI (N-Myc interactor) to effect transcriptional changes.[1,2] The amplification of MYCN serves as a prognostic indicator for NB outcome (reviewed in).[3] The *MYCN* locus also generates an antisense transcript *MYCNOS*, (also referred to as *N-cym* (4)) that emanates from intron 1 of MYCN and appears to emanate from a bidirectional promoter at Exon 2 of *MYCN*. *MYCNOS* encodes a protein that is expressed tumor cell lines and several normal tissues (**Fig. 1A**; **Fig. S1**).[4] It inhibits glycogen synthase kinase 3 β (GSK3beta) and thus stabilizes the MYCN protein.[5] High levels of MYCNOS levels relative to MYCN correlate with poor clinical outcome in NB.[6]

An emerging body of evidence suggests that endogenous antisense long non-coding RNAs (lncRNAs) are involved in the epigenetic regulation of gene expression in human cells (reviewed in).[7] Many of these lncRNAs are antisense to their protein-coding counterpart and function in the target-specific recruitment of epigenetic complexes (reviewed in).[8] The observation that *MYCN* is associated with an endogenous antisense transcript, *MYCNOS*, in a discordant manner,[6] suggests that the *MYCN* locus may also be under RNA-directed regulation. To explore this possibility, we examined RNA and protein expression from the *MYCN/MYCNOS* locus in Lan6 neuroblastoma cells. Here we present data suggesting that *MYCNOS* RNA functions as a regulator of the upstream *MYCN* promoter by the targeted recruitment of various proteins including the RAS activating protein G3BP1 to this site. We find that a subset of small regulatory antisense ncRNAs (sasRNAs) is capable of transcriptionally suppressing *MYCNOS* expression by targeting the MYCN intron 1 bidirectional promoter it shares with *MYCN*. We find that the overexpression of MYCNOS results in a decrease of upstream *MYCN* promoter usage and increased MYCN expression, suggesting that the MYCNOS transcript functions both as protein coding mRNA and as regulatory RNA controlling *MYCN* transcriptional variants.

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Submitted: 05/08/2015; Revised: 06/07/2015; Accepted: 06/14/2015
http://dx.doi.org/10.1080/15476286.2015.1063773

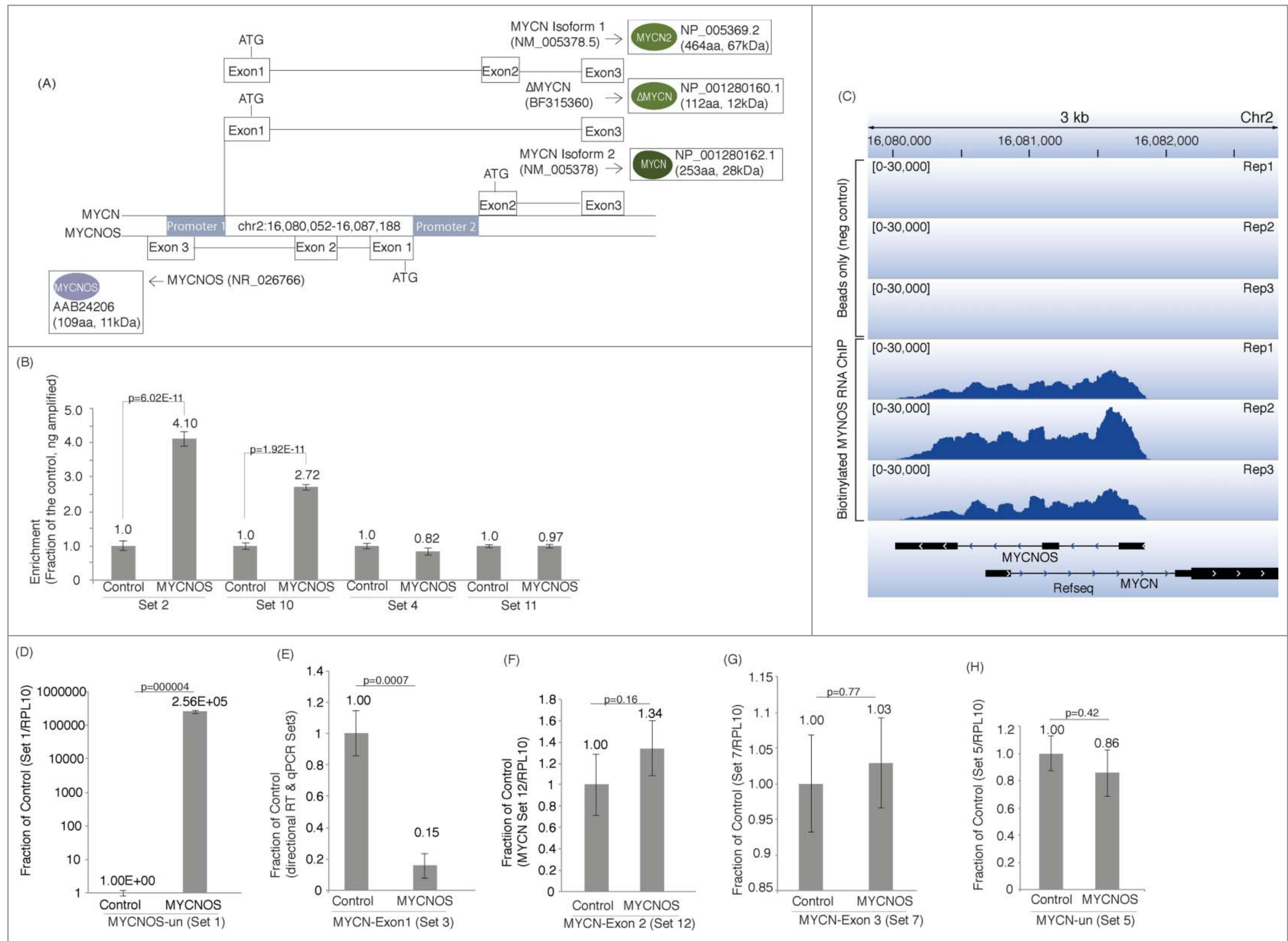


Figure 1. *MYCNOS* directed transcriptional regulation of *MYCN* in Lan6 cells. (**A**) A schematic of the MYCN/*MYCNOS* locus on chromosome 2 (chr2:16,079,790-16,087,217). The *MYCN* isoforms (*ΔMYCN* (accession AAG40001)), *MYCN* (accession AAA36371) and the antisense *MYCNOS* (accession AAB24206) are shown along with their corresponding transcripts (MYCN (NM_005378), and ΔMYCN (BF315360) and MYCNOS (NR_026766)) and the ΔMYCN (Promoter 1) or MYCNOS/MYCN promoters (Promoter 2). (**B and C**) *MYCNOS* biotin transcripts associate at both MYCN promoters. (**B**) CHIP was carried out on *MYCNOS*-biotin treated Lan6 cells. Significant binding was observed at both the upstream MYCN promoter (Set 2) and the internal MYCN promoter/*MYCNOS* exon 1 (Set 10) amplified loci (refer also to Figure S2). The averages of 5 individual samplings of control and treated cultures are shown with the standard deviations. (**C**) *MYCNOS* binds specifically to the MYCN/MYCNOS locus in Lan6 cells. CHIP-sequencing was performed on biotin labeled *MYCNOS*. The triplicate IP-Seq alignments for *MYCNOS* binding and control (beads alone) are shown. (**D–H**) The effects of overexpression of *MYCNOS* on *MYCN* and *MYCNOS* expression. The expression dynamics of the (**D**) *MYCNOS* unspliced at *MYCN* promoter 1, (**E**) upstream *MYCN* exon 1 specific as determined by directional RT and qPCR, (**F**) *MYCN* exon 2, (**G**) *MYCN* exon 3 and (**H**) unspliced variants of *MYCN* in Lan6 cells are shown following treatment with *MYCNOS*. The averages of triplicate treated cultures are shown with the standard error of the means and p values from a paired T-test.

Results

***MYCNOS* RNA localizes to the *MYCN* promoters and modulates *MYCN* expression-**

The *MYCN* locus in humans exhibits characteristics that suggest it may be under antisense RNA-mediated regulation.[9-13] The locus is situated on chromosome 2 (**Fig. 1A**; **Figs. S1–S2**), and encodes 3 previously reported protein isoforms MYCN Isoform 1 (NP_005369.25, 49kDa) which consists of exons 1, 2 and 3, MYCN Isoform 2 which consists of exons 2 and 3 (NP_001280162.1, 28kDa), and ΔMYCN (NP_001280160.1, 12kDa) which incorporates the upstream exon 1 with exon 3 (**Fig. 1A**; **Fig. S1, and Table S2**). The locus also shows an antisense transcript, *MYCNOS*, that codes for a protein (AAB24206, 11kDa), which inhibits GSK3beta to regulate MYCN,[4,5] (**Table S3**). The *MYCNOS* transcript shares a promoter with MYCN (**Fig. 1A**, Promoter 2 in intron 1) and contains 2 exons that are antisense to both exons 1 and 2 of MYCN, one of which overlaps an upstream *MYCN* promoter (**Fig. 1A**; **Figs. S1–S2**). Previous studies have found that antisense transcripts, spanning gene promoters, can exert epigenetically mediated silencing and thus mediate transcriptional control of the particular targeted promoter. This mechanism is inhibited by the action of 5′ aza cytidine (5′Aza-C) and trichostatin A (TSA).[9-11,14] We find that treatment of Lan6 cells with 5′Aza-C and TSA results in a significant increase in the level of *MYCN*

RNA, and the over-expression of MYCNOS resulted in a loss of Histone 3 Lysine 27 and Histone 3 Lysine 36 tri-methylation, suggesting that this locus may also be under antisense RNA-directed regulation (**Figs. S3 and S4**).

Previous studies have found that antisense RNAs interact directly with homology-containing loci to direct epigenetic changes in the target loci, thus affecting transcription.[10] To determine if *MYCNOS* functionally interacts with the *MYCN* locus, *MYCNOS* was generated as a transcript with biotin dUTPs (**Table S3**) and transfected into Lan6 cells. The biotin-linked *MYCNOS* was found to interact directly with both the bidirectional *MYCN/MYCNOS* and the upstream *MYCN* promoter loci (**Fig. 1B**). The only locus found by ChIP-deep sequencing to be enriched with the biotin linked *MYCNOS* was the *MYCN/MYCNOS* locus (**Fig. 1C**), supporting the notion that *MYCNOS* functions specifically at the *MYCN/MYCNOS* locus. When *MYCNOS* was overexpressed in LAN6 cells (**Fig. 1D**), the most dramatic effect was observed for *MYCN*, specifically reduced expression of *MYCN* exon 1 (**Fig. 1E**), with expression of downstream exons not significantly changed (**Fig. 1F–H**). These data suggest that *MYCNOS* directly interacts with the upstream *MYCN* promoter and suppresses transcription of the *MYCN* variant containing exon 1.

Expression of *MYCNOS* is correlated with the expression of *MYCN* Isoform 2-

To further explore the role of *MYCNOS* in regulating *MYCN*, we designed and screened several small antisense RNAs (sasRNAs)[15] targeted to the *MYCNOS* promoter (**Fig. 1A**). This was necessary, as targeting other loci in MYCNOS would result in the targeting of MYCN. The targeting of this bidirectional promoter, which is well defined in the UCSC genome browser, with sasRNAs allows transcriptional gene silencing (TGS) (reviewed in)[8] to be initiated for *MYCNOS*, avoiding dual targeting of the *MYCNOS* and the upstream *MYCN* promoter (**Fig. 1A**; **Fig. S1**). One candidate sasRNA, Dwn2 (**Fig. S2**), was found to reduce expression of unspliced *MYCNOS* (**Fig. 2A**), though not a significant level. The reduction of *MYCNOS* had the inverse effect on unspliced *MYCN* expression, specifically enhancing the

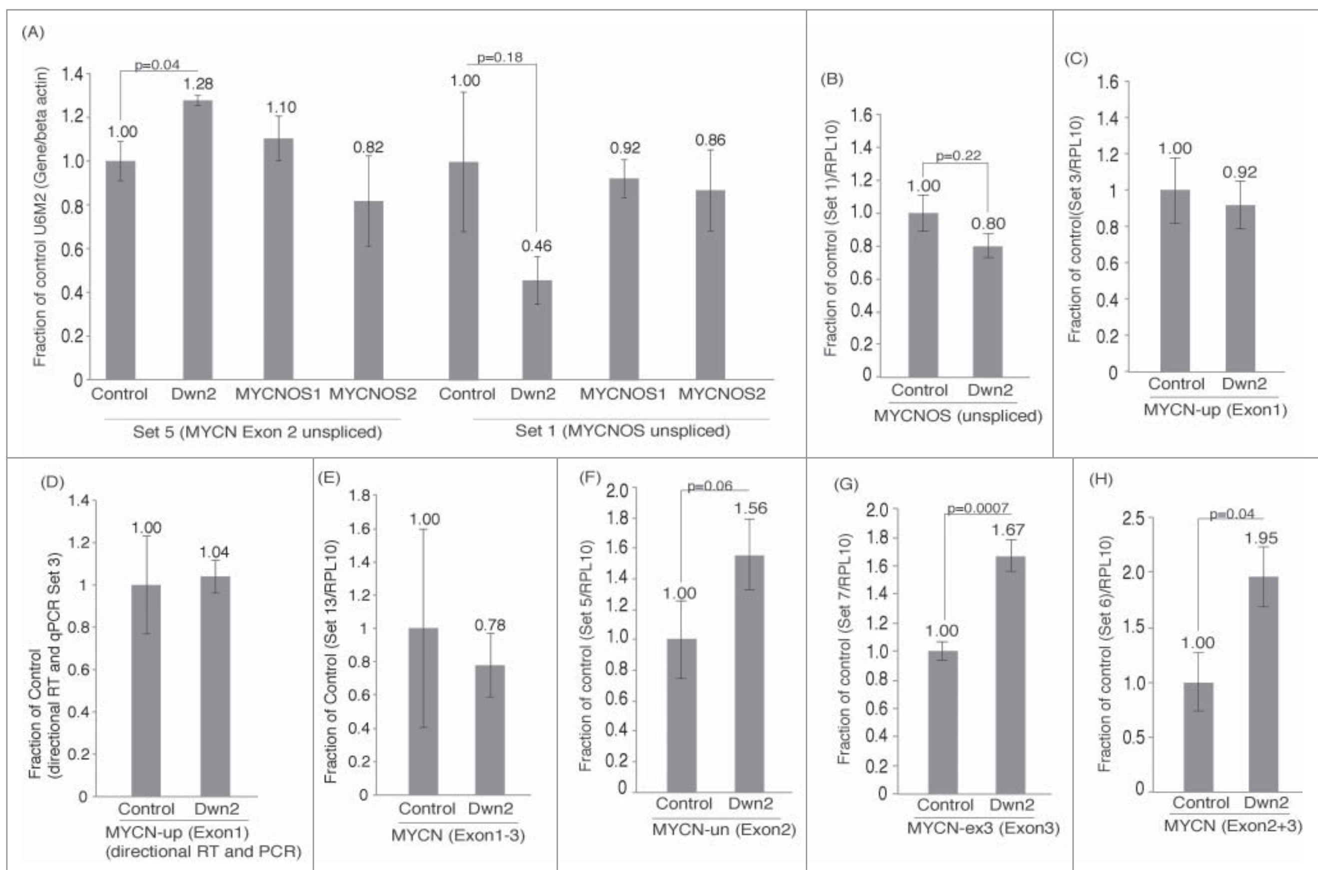
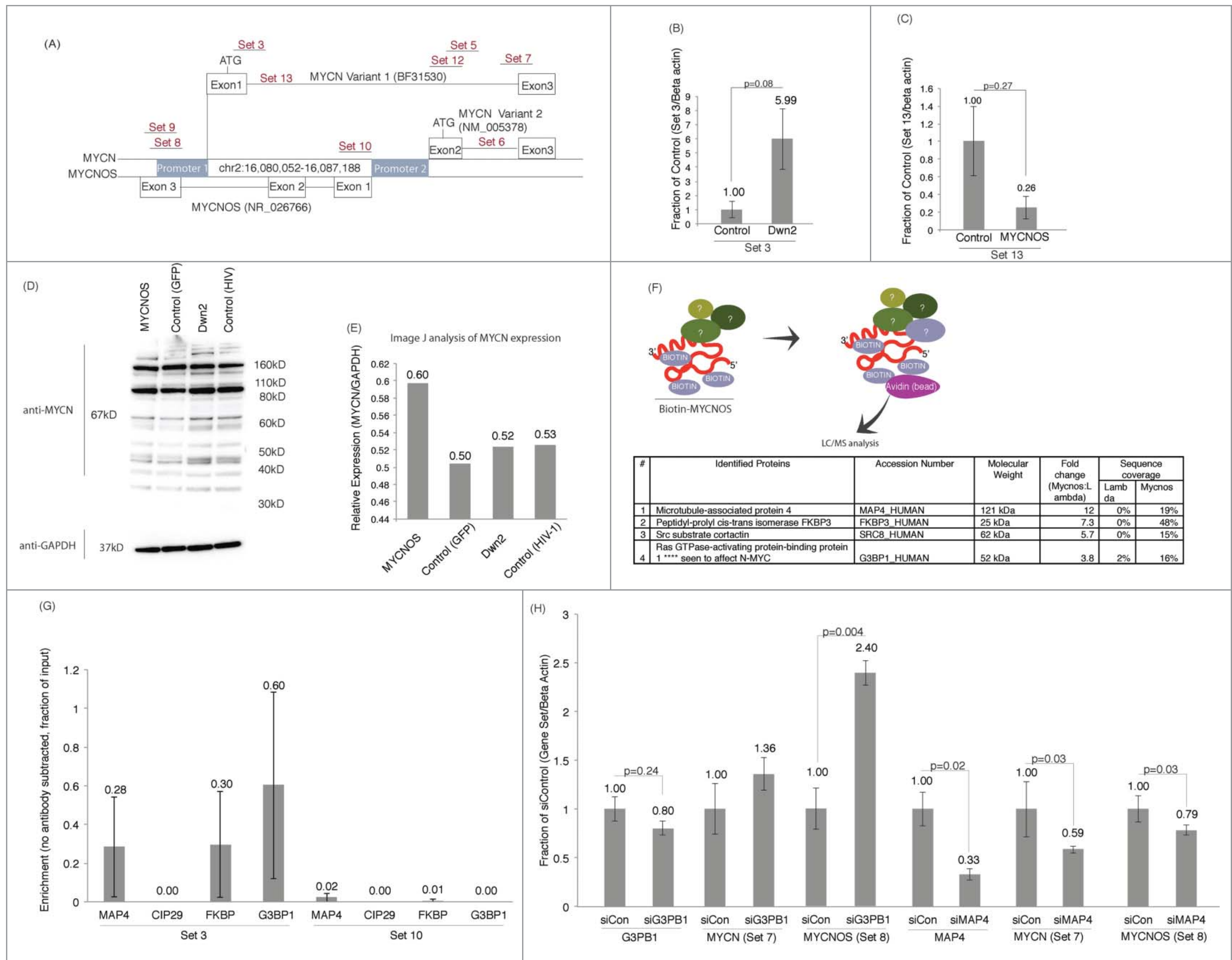


Figure 2. Targeting the *MYCN/MYCNOS* bidirectional promoter. (**A**) The effects of sasRNA targeting of the bidirectional promoter. LAN6 cells were transiently transfected with Dwn2, MYCNOS1 and MYCNOS2 sasRNA expressing plasmids and the expression of *MYCN* and *MYCNOS* unspliced transcript variants determined. (**B–H**) Stable Dwn2 sasRNA expressing and control cell lines were generated and the expression of (**B**) unspliced *MYCNOS* and (**C–H**) MYCN transcripts determined. (**C–H**) Various regions of MYCN transcripts were assessed in the stable Lan6 cell lines including upstream exon 1 by both (**C**) qRT-PCR and (**D**) directional qRT-PCR, (**E**) Exon 1 spliced with exon 3 but lacking exon 2, (**F**) *MYCN* exon 2, (**G**) *MYCN* exon 3, and (**H**) MYCN exon 2 spliced with exon 3. For B-H the averages of triplicate assessed clones are shown with the standard error of the means and p values from a paired T-test.

expression of unspliced variants of *MYCN*, containing exon 2 (**Fig. 2A**). These data suggest that *MYCNOS* might play a role in the transcriptional control of *MYCN*, e.g. the reduction in *MYCNOS* results in increased *MYCN* expression, similar to other genes that are epigenetically regulated by antisense transcripts.[10]

Next, the LAN6 cell line was stably transduced with the sasRNA Dwn2 and compared to vector control-transduced cells. Similar to transient transfections of Dwn2, the stable expression of Dwn2 resulted again in a modest reduction of unspliced variants of *MYCNOS* (**Fig. 2B**). There was no detectable effect on the expression of exon 1 of MYCN (**Fig. 2C–E**), but there was a significant correlation between reduced *MYCNOS* expression and increased expression of *MYCN* Isoform 2 containing exons 2 and 3 (**Fig. 2F–H**). Exons 2–3 encompass the dominant MYCN protein-coding isoform (**Fig. 1A**; **Fig. S1**, and **Table S2**, MYCN Isoform 2 accession AAA36371). These data suggest that (1) sasRNA Dwn2 is capable of reducing *MYCNOS*, (2) that this



#	Identified Proteins	Accession Number	Molecular Weight	Fold change (Mycnos:Lambda)	Sequence coverage	
					Lambda	Mycnos
1	Microtubule-associated protein 4	MAP4_HUMAN	121 kDa	12	0%	19%
2	Peptidyl-prolyl cis-trans isomerase FKBP3	FKBP3_HUMAN	25 kDa	7.3	0%	48%
3	Src substrate cortactin	SRC8_HUMAN	62 kDa	5.7	0%	15%
4	Ras GTPase-activating protein-binding protein 1 **** seen to affect N-MYC	G3BP1_HUMAN	52 kDa	3.8	2%	16%

Figure 3. Transcriptional analysis of the *MYCN/MYCNOS* locus. (**A**) A schematic depicting the primers used in the transcriptional analysis of the *MYCN/MYCNOS* locus and the delineation of MYCN variant 1 (ΔMYCN) and MYCN Variant 2 (MYCN) (**B and C**) Nuclear run-on analysis was carried out on nuclei from the stable lentiviral transduced (Control vs. Dwn2) or transiently transfected (Control vs. *MYCNOS*) Lan6 cells. (**B**) Loss of *MYCNOS* results in increased transcription of *MYCN* exon 1 primer set 3. (**C**) Overexpression of *MYCNOS* leads to transcriptional suppression of *MYCN* exon 1 fused to 3 as determined by primer set 13 (refer also to Figure S2). For B-C the averages of triplicate treated cultures are shown with the standard error of the means and p values from a paired T-test. (**D and E**) Overexpression of *MYCNOS* leads to increased *MYCN* as determined by (**D**) western blot analysis and quantified by (**E**) Image J analysis of *MYCN* expression relative to GAPDH. (**F**) A schematic is shown depicting the avidin IP of Biotin-MYCNOS followed by mass spectrophotometry analysis (LC/MS). A table of the top proteins found associated with biotin labeled *MYCNOS* by LC/MS is also shown. (**G**) IP of the *MYCNOS* associated proteins, MAP4, CIP29, G3BP1 and FKBP were immunoprecipitated and probed by qRTPCR for *MYCNOS* with *MYCNOS* specific primers sets 3 and 10. The averages of triplicate IP's are shown standardized to the input following subtraction of no antibody controls with the standard error of the mean. (**H**) The effects of RNAi of MAP4 and G3BP1 on both MYCN (Set7) and MYCNOS (Set8) expression. The averages of triplicate treated cultures are shown as a fraction of the siRNA control (siCon) with the standard error of the means and p values from a two-sided paired T-test.

reduction in MYCNOS correlates with changes in transcript abundance of *MYCN* Isoform 2 containing exons 2 and 3, (3) that the targeting of the *MYCN/MYCNOS* bidirectional promoter with sasRNAs is a relatively inefficient process relative to the targeting of single directional RNA polymerase 2 expressed genes (reviewed in).[16]

The effects of *MYCNOS* on the *MYCN* transcriptional landscape-

To determine to what extent *MYCNOS* is modulating *MYCN* transcription, nuclear run-on assays were carried out and particular loci in the *MYCN* locus assessed to determine which variant of *MYCN* was affected by *MYCNOS* overexpression (**Fig. 3A**). When *MYCNOS* is constitutively targeted with Dwn2 in stable lentiviral transduced Lan6 cells, the upstream *MYCN* promoter is transcriptionally activated, resulting in increased expression of *MYCN* Isoform 1 (exon 1–3) containing transcripts (**Fig. 3A, B**). Conversely, when *MYCNOS* is overexpressed in Lan6 cells, there is a reduction in the *MYCN* Isoform 1 transcripts incorporating both exons 1 and 3 from the upstream *MYCN* promoter (**Fig. 3C**) and an increase of transcription of *MYCN* Isoform 2 containing exons 2 and 3 (**Fig. S5**). These run-on observations are supported by Western blot analysis, which demonstrates that the overexpression of *MYCNOS* results in increased *MYCN* protein expression of isoform 2 containing exons 2 and 3 (**Fig. 3A, D, E**). These data suggest that *MYCNOS* is involved in transcriptionally modulating *MYCN*, shifting transcription from Isoform 1 (*ΔMYCN*) to the *MYCN* isoform 2 (**Figs. 1A and 3A; Fig. S1, and Table S2**), which is notably concordant with observations from RNA sequencing in the Illumina Bodymap. In the publically available Illumina Bodymap the same discordant regulation is observed in testes, where high MYCNOS correlates with ΔMYCN and MYCN and brain where low MYCNOS correlates with MYCN2 isoforms (**Fig. S6**).

Recent and past observations have documented direct binding of particular lncRNAs to various proteins such as the PRC2,[17,18] hnRNP-K[19] complexes, or DNA methyltransferase 3a (DNMT3a).[10,20,21] To determine whether *MYCNOS* also interacts with proteins at the upstream MYCN locus, an affinity purification of biotin-labeled MYCNOS was carried out on transfected Lan6 cells, and several proteins, including many previously reported to be involved in cancer, were found in association (**Fig. 3F**; **Table S4**). Some of these proteins, microtubule-associated protein 4 (MAP4), peptidyl-prolyl cis-trans isomerase FKBP3 (FKPB), and Ras GTPase-activating protein-binding protein 1 (G3BP), were found to associate directly with MYCNOS, and in particular at regions overlapping the upstream MYCN promoter, as determined by protein immunprecipitation followed by qPCR for *MYCNOS* (**Fig. 3G**). Suppression of MAP4 and G3BP using RNA interference indicated that MAP4 is involved in both MYCN and MYCNOS expression from this locus, while G3BP appeared to be involved in specifically repressing MYCNOS (**Fig. 3H**). These data suggest that *MYCNOS* mediated regulation of the MYCN/MYCNOS locus involves several proteins including MAP4 and the RAS activating protein G3BP1.

Discussion

Studies carried out over the last decade suggest that in human cells, there is an endogenous pathway which utilizes antisense transcripts to regulate the epigenetic and transcriptional state of protein-coding genes (reviewed in).[7] Many of those genes found to date to be under some level of antisense RNA control are involved in tumor suppression,[10,12,22] or transcriptional regulation.[9] The data pre-

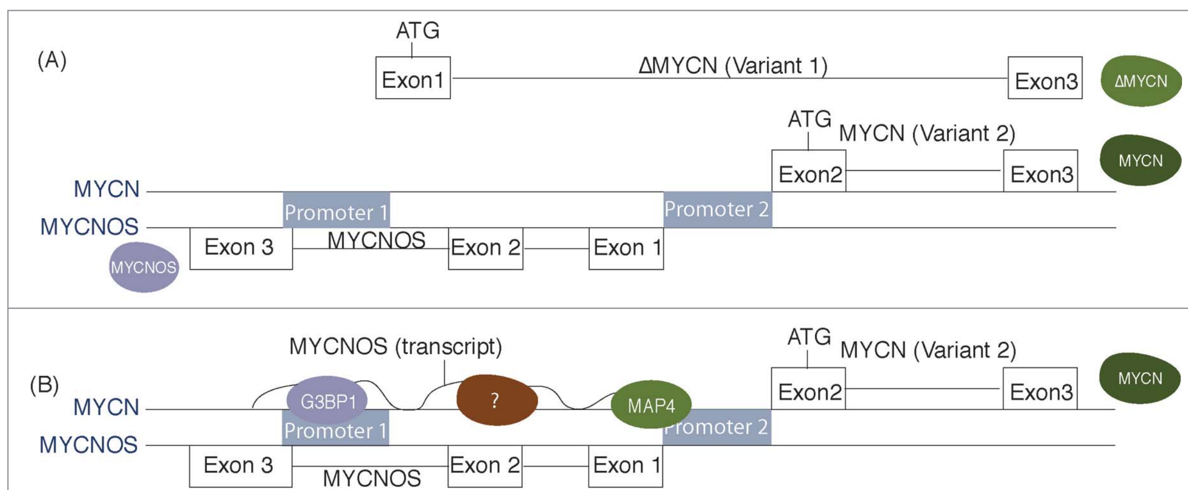


Figure 4. Model for *MYCNOS* mediated regulation of *MYCN*. (**A**) MYCN is expressed from 2 promoters producing two different isoforms; Variant 1, *ΔMYCN* expressed from promoter 1 and variant 2 the dominant protein coding *MYCN* consisting of exons 2 and 3 expressed from promoter 2. (**B**) When MYCNOS is highly-expressed the upstream promoter 1 for *ΔMYCN* is bound with MYCNOS and its associated regulatory protein components consisting of G3BP1, MAP4, and FKBP and possibly several others. This binding results in a reduction of the upstream *MYCN* promoter 1 usage and a transcriptional shift to *MYCN* expression from promoter 2 and *MYCN* exon 2 and 3 containing protein-coding isoforms.

sented here suggest that *MYCNOS*, a bona fide protein-coding gene,[4,5] can also function as an antisense RNA that regulates the transcriptional state of the *MYCN/MYCNOS* locus by recruiting protein complexes to a specific genomic locus.

Previous studies found that *MYCN* expression does not linearly correlate with *MYCNOS*[23] and studies from Jacobs et al.[6] found that the overexpression of *MYCNOS* does not have a noticeable effect on the level of endogenous *MYCN* mRNA or that of upstream exon 1 containing *ΔMYCN* variants and that neither RNA interference nor RNA-editing appear to be mechanisms by which *MYCNOS* regulates *MYCN*. The data presented here are however suggest that the only noticeable effect from either suppressing or overexpressing *MYCNOS* was a shift from *MYCN* Isoform 2 to the other *MYCN* isoform, *ΔMYCN*. Furthermore the observations reported here do not suggest that exon 2 is spliced out due to *MYCNOS* overexpression, as was suggested,[6] but rather that exon 1 incorporation is lost upon MYCNOS overexpression resulting in the *MYCN* Isoform 2 variant containing exons 2 and 3, e.g. there is a relative increase in transcription emanating from the internal *MYCN* promoter and a decrease in transcription from the upstream *MYCN* promoter (**Fig. 4**). This conclusion is in line with observations by Stanton and Bishop who observed alternative processing of *MYCN*[24] and recent findings that observed an upstream lncRNA, lncUSMycN involved in modulating *MYCN* function and oncogenesis.[25] Moreover, RNA sequencing from the Illumina Bodymap shows the same discordant regulation observed here in testes, where high MYCNOS correlates with ΔMYCN and MYCN and brain where low MYCNOS correlates with MYCN2 isoforms (**Fig. S6**).

The observations presented here and previously published studies,[5,6,24] suggest that *MYCNOS* functions as both a protein to inhibit GSK3beta which results in the stabilization of MYCN in human neuroblastomas[5] and as an antisense RNA to directly modulate the usage of the upstream *MYCN* promoter, possibly by either recruiting or acting as a scaffold for various cellular proteins including MAP4 and G3BP1 to tether to the upstream MYCN promoter locus (**Fig. 4**). When the upstream promoter is utilized, exon 1 is incorporated resulting in *MYCN* isoform 1 and an open reading frame contained in this transcript encoding *ΔMYCN* (**Table S2**, accession AAG40001) whereas when the upstream promoter is repressed by MYCNOS the internal MYCN promoter is utilized and the *MYCN* protein-coding isoform 2 containing MYCN exons 2 and 3 is expressed (**Table S2**, accession AAA36371). This mechanism allows *MYCNOS* transcripts to regulate 2 different promoters, one of which is an antisense promoter residing in intron 1 of MYCN that drives *MYCNOS* expression and the other downstream of MYCNOS driving *MYCN* isoform 1 and/or *ΔMYCN* expression (**Fig. 4**). These observations suggest that *MYCNOS* may not only modulate its own expression, but also the transcriptional activity of 2 promoters that can express 3 different protein-coding variants.[5,6] As the binding of *MYCNOS* to the upstream *MYCN* promoter coincides with binding of *MYCNOS* to several proteins, it will be interesting to determine how this *MYCNOS* scaffolding functions. Observations here suggest that MAP4 is required for expression of the entire locus, e.g. both *MYCNOS* and *MYCN*, whereas *G3BP1* appears to be a negative regulator of *MYCNOS* (**Fig. 3H**). It is noteworthy that those proteins found associated with *MYCNOS* include MAP4, a known microtubule promoting, anti-apoptotic protein[26] and mTOR signaling regulator,[27] FKBP3 a protein known to function as a histone deacetylase,[28] and G3BP1, a protein involved in RAS activation. The findings reported here not only implicate *MYCNOS* as an antisense RNA regulator of *MYCN* but also expand on our current understanding of RNA mediated regulatory pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

The project was supported by NIAID R56 AI096861-01, PO1 AI099783-01, Australian Research Council FT1300100572 to KVM and CA151574-01 and RO1 CA153124-01 to PKV.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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