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Regulation of \textit{MYCN} expression in human neuroblastoma cells

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Abstract

\textbf{Background:} Amplification of the \textit{MYCN} gene in neuroblastoma (NB) is associated with a poor prognosis. However, \textit{MYCN}-amplification does not automatically result in higher expression of \textit{MYCN} in children with NB. We hypothesized that the discrepancy between \textit{MYCN} gene expression and prognosis in these children might be explained by the expression of either \textit{MYCN}-opposite strand (\textit{MYCNOS}) or the shortened \textit{MYCN}-isoform (\textit{\textDelta MYCN}) that was recently identified in fetal tissues. Both \textit{MYCNOS} and \textit{\textDelta MYCN} are potential inhibitors of \textit{MYCN} either at the mRNA or at the protein level.

\textbf{Methods:} Expression of \textit{MYCN}, \textit{MYCNOS} and \textit{\textDelta MYCN} was measured in human NB tissues of different stages. Transcript levels were quantified using a real-time reverse transcriptase polymerase chain reaction assay (QPCR). In addition, relative expression of these three transcripts was compared to the number of \textit{MYCN} copies, which was determined by genomic real-time PCR (gQPCR).

\textbf{Results:} Both \textit{\textDelta MYCN} and \textit{MYCNOS} are expressed in all NBs examined. In NBs with \textit{MYCN}-amplification, these transcripts are significantly higher expressed. The ratio of \textit{MYCN}:\textit{\textDelta MYCN} expression was identical in all tested NBs. This indicates that \textit{\textDelta MYCN} and \textit{MYCN} are co-regulated, which suggests that \textit{\textDelta MYCN} is not a regulator of \textit{MYCN} in NB. However, the ratio of \textit{MYCNOS}:\textit{MYCN} expression is directly correlated with NB disease stage ($p = 0.007$). In the more advanced NB stages and NBs with \textit{MYCN}-amplification, relatively more \textit{MYCNOS} is present as compared to \textit{MYCN}. Expression of the antisense gene \textit{MYCNOS} might be relevant to the progression of NB, potentially by directly inhibiting \textit{MYCN} transcription by transcriptional interference at the DNA level.

\textbf{Conclusion:} The \textit{MYCNOS}:\textit{MYCN}-ratio in NBs is significantly correlated with both \textit{MYCN}-amplification and NB-stage. Our data indicate that in NB, \textit{MYCN} expression levels might be influenced by \textit{MYCNOS} but not by \textit{\textDelta MYCN}. 

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Background
Neuroblastoma cells (NBs) that carry an amplified MYCN gene are extremely malignant. However, MYCN-amplification does not automatically result in higher expression of MYCN in children with NB [1-3]. Thus, it has been suggested that the aggressive phenotype of MYCN amplified NBs may be explained by higher expression levels of other genes co-amplified with MYCN, since the amplified unit of DNA can be up to 1 Mb. To date, three genes have been identified that are frequently co-amplified with MYCN in NBs: DDX1 in 50% of the cases, NAG in 20% of the tumours, and MYCNOS in all cases [4,5]. All three genes demonstrate increased transcript expression when co-amplified in NB cell lines, indicating that they may contribute to tumour phenotype. However, survival analyses in a large study using 75 MYCN-amplified tumours indicate that neither amplified DDX1 nor NAG have an additional adverse effect on the prognosis of the patients [6].

Natural antisense transcripts are abundant in eukaryotic genomes [7-9]. In human, more than 1600 natural antisense transcript are predicted to be present [10]. They can influence gene expression on the DNA level by transcriptional interference, on the transcript level by RNA interference and RNA editing, or direct splicing by RNA masking [11,12]. MYCNOS is the antisense transcript of MYCN [13] and shows overlap with the first exon of MYCN. This antisense transcript could be involved in modulating the expression of MYCN by any of the mechanisms mentioned above. Antisense transcripts are considered to be relevant to the development and progression of tumours [14-16], but until now, only antisense HIF-1α RNA has been shown to be a marker for prognosis in human breast cancer [17].

Recently, we reported a fetal MYCN splice variant (ΔMYCN) lacking exon 2 [18]. The ΔMYCN transcript is expressed in several fetal tissues and contains the acidic region, nuclear localization signal, the basic helix-loop-helix and leucine-zipper domains but lacks the transactivation domain. It has been suggested that the ΔMYCN protein may serve as an obligate dimerization partner for MYCN to convey transcriptional activation or repression.

In this report we analysed whether expression of ΔMYCN and MYCNOS influence MYCN expression levels in NBs of different disease stages.

Methods
Tumour material
Sixteen fresh-frozen NBs were obtained from at the Department of Pathology at the Radboud University Nijmegen Medical Centre. All NBs were derived from pediatric patients (0 to 6-years-old) diagnosed at the Department of Pediatric Hemato-Oncology. Sections of the frozen samples were stained with hematoxylin-eosin and reviewed by the pathologist to verify tumour histology and to evaluate the percentage of tumour cells. Samples were only considered for study if the contents of tumour cells was ≥ 75%. Six out of 16 NBs had MYCN-amplification as shown by southern blot and/or fluorescent in situ hybridization (FISH). All samples were anonymized prior to this study, and the research program was approved by the local ethics committee (Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen).

DNA isolation, RNA isolation and cDNA synthesis
Tumour samples were aliquoted in two parts to isolate both DNA and RNA. Total DNA was isolated with the QIAamp isolation-kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. All samples were RNase treated. Total RNA was isolated with TriZol reagent (Invitrogen, Carlsbad, CA, USA) and treated with Deoxyribonuclease I (Dnase I; Invitrogen). DNase-treated RNA was reverse-transcribed using oligo(dT) primers with the SuperScript First-Strand Synthesis System (Invitrogen).

PCR
MYCN and splice variants were amplified from cDNA by using the GC-RICH PCR System (Roche Applied Science, Almere, The Netherlands). Primers were developed by the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi[19]). Primer sequences are depicted in figure 1. PCR products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing V2.0 Ready Reaction Kit and analysed with the ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA).

QPCR and gQPCR
QPCR and gQPCR was performed by SYBR Green-based quantification (Bio-Rad, Veenendaal, The Netherlands). PCRs were performed on an iCycler (MyiQ single color Real-Time detection System, Bio-Rad, Veenendaal, The Netherlands). Sequences of the primers used to quantify cDNA transcript levels and genomic DNA are shown in table 1 and the position of the primers in MYCN are depicted in figure 1. PCR products were between 80- and 100-bp. Validation of the primer pairs and (g)QPCR experiments were performed as described previously [20,21]. Differences in expression of a gene of interest or in genomic DNA copy number between two samples were calculated by the comparative Ct method [22,23]. Hoebeeck et al. described and validated a similar assay for the determination of MYCN copy numbers in tumor samples [24].

Antibody coupling and immunoprecipitation
NB-samples were homogenized in RIPA-buffer (50 mM Tris-HCl pH 7.4, 0.2% sodium dodecyl sulfate (SDS),
0.2% sodium deoxycholate, 1% triton X-100, 1 mM EDTA) supplemented with 1 mM DTT, 1 mM PMSF, apro- 

tinin 2 μg/ml and leupeptin 2 μg/ml. Total protein con-
centration was determined according to the Bradford 

method (Bio-Rad Laboratories, Hercules, CA, USA).

For immunoprecipitation, 5 μg C-19 mAb (Santa Cruz 

Biotechnology, Heidelberg, Germany) was coupled to 

Prot A sepharose CL-4B beads (Pharmacia Biotechnolo-

gies, Uppsala, Sweden) for 1 hour at 4°C to). NB-lysates 

were precleared O/N with 50 μl packed Prot A sepharose 

CL-4B beads. To the precleared lysates, 20 μl C-19-cou-

pled beads was added and incubated for 24 h at 4°C. Sub-

sequently, the beads were washed with PBS and 

resuspended in SDS sample buffer and stored at -80°C 

until SDS-polyacrylamide gel electrophoresis (PAGE).

**SDS-PAGE and Western blotting**

Samples (30 μg homogenized NB-sample or 20 μl precip- 

itation beads) were separated a 10% polyacrylamide gels 

and transferred to nitrocellulose (Hi-bond, Amersham 

Biosciences, Little Chalfont, UK). Ponceau S staining was 

used to confirm that equal amounts of protein were 

loaded in each lane (additional file 1). The nitrocellulose 

blot was blocked with 1% BSA in 20 mM Tris-HCl pH 7.4 

and 0.1% Tween (Tris-buffered saline/Tween 20; TBS-T) 

for 1 h. The blot was washed for 5 min with TBS-T fol-

lowed by incubation with the C-19 anti-MYC antibody 

1:200 diluted in TBS-T for 1 h at RT. After washing 3× with 

TBS-T, the blot was incubated with HRP-conjugated 

swine-anti-rabbit antibody (1:5000 diluted in TBS-T) for 

1 h at RT. Subsequently, the blot was washed and incu-

bated for 1 min with ECL substrate (Amersham Bio-

sciences, Little Chalfont, UK) and exposed to film (Kodak, 

Rochester, NY, USA).

**Overexpression of MYCNOS in the NB cell line IMR-32**

Primers for the amplification of MYCNOS were developed 

by using the primer3 program (Table 1). MYCNOS was 

amplified from DNA isolated from a healthy control using 

the GC RICH PCR System (Roche, Woerden, The Nether-

lands). Subsequently, MYCNOS was cloned into the Gate-

way donor vector pDONR-201 (Invitrogen). Using the 

Gateway cloning system, MYCNOS was subsequently sub-

cloned into the pcDNA3 expression vector and integrity of 

the construct was validated by sequence analysis. IMR-32 

NB cells at 50% confluence in a 25 cm² flask were co-

transfected with 8 μg pcDNA3-MYCNOS and C1-GFP 

using 120 μl lipofectamine (Invitrogen) in 3 ml Opti-

MEM-I for 20 min at RT.
Table 1: Primer-sequences of primers used in this report.

| Gene of interest | Genbank ID | Forward primer | Reverse primer |
|------------------|------------|----------------|----------------|
| **QPCR**         |            |                |                |
| MYCN             | NM_005378.4| 5'-cacaaggccccctcagataccc-3' | 5'-cacaagtctgatccccctc-3' |
| ΔMYCN            | Not present| 5'-cacaaggccccctcagataccc-3' | 5'-cacaagtctgatccccctc-3' |
| MYCNOS           | S49953.1   | 5'-ttgctctcccttacaaacaacag-3' | 5'-aaactgcctcgaacagcag-3' |
| MYCN total       | NM_005378.4| 5'-cataggaggggctctgctgggctg-3' | 5'-ccctgccctgtctgccctg-3' |
| GUSB             | NM_000181.1| 5'-gggtcttgataaatggcttcc-3' | 5'-tctggcttgcaaaacacaag-3' |
| TRIC             | NM_003234.1| 5'-gttcttctgccctgctgggctg-3' | 5'-ccctgccctgtctgccctg-3' |
| RNF11            | NM_017610.6| 5'-gcagaatgcagcagaagttg-3' | 5'-ccattcttgcagaagttg-3' |
| **gQPCR**        |            |                |                |
| MYCN (exon 1)    | NM_005378.4| 5'-ccgggtgtgtcagatttttc-3' | 5'-tccaacacagttcccaggag-3' |
| MYCN (exon 2)    | NM_005378.4| 5'-gttctctcccttacaaacaacag-3' | 5'-aaactgcctcgaacagcag-3' |
| MYCN (exon 3)    | NM_005378.4| 5'-gttctctcccttacaaacaacag-3' | 5'-aaactgcctcgaacagcag-3' |
| MYCN (3'-UTR)    | NM_005378.4| 5'-taccaggtgcaggagagacc-3' | 5'-agcccaagtagccaagacac-3' |
| MYCNOS           | S49953.1   | 5'-agggggtggtggcgaggc-3' | 5'-gtagctcgcacttatttatttat-3' |
| CFTR             | NM_000492.3| 5'-gggtcttgataaatggcttcc-3' | 5'-tctggcttgcaaaacacaag-3' |
| TBX22            | NM_016954.2| 5'-tttccctggctcctgctgggctg-3' | 5'-ccctgccctgtctgccctg-3' |
| SLC16A2          | NM_006517.2| 5'-tcattcttgcagaagttg-3' | 5'-ccctgccctgtctgccctg-3' |

Table 2: Patient characteristics.

| Pt    | Age¹   | Sex | Diagnosis                              | Localization                      | Histology² | MYCN³ | Treatment and follow-up | Status  |
|-------|--------|-----|----------------------------------------|-----------------------------------|------------|-------|-------------------------|---------|
| 1     | 3 mnts | F   | NB IV S Adnex and liver metastasis     | UD                                | No         | 05/01 surgery + chemo   | Alive   |
| 2     | 3 yrs  | M   | NB IV Supraclavicular and BM metastases| PD                                | No         | 02/03 surgery + chemo   | Alive   |
| 3     | 4 mnts | M   | NB II Pos. lymphnodes with unknown primary tumor | PD                                | No         | 01/03 surgery + chemo   | Alive   |
| 4     | 5 mnts | M   | NB IV S Spine and bone/liver metastases| PD                                | No         | 01/03 surgery + chemo   | Alive   |
| 5     | 14 mnts| F   | NB II Spine                            | PD                                | No         | 02/04 surgery + chemo   | Alive   |
| 6     | 4 mnts | F   | NB II Adnex                            | PD                                | No         | 07/01 surgery + chemo   | Alive   |
| 7     | 2 yrs  | F   | NB III Adnex                           | D                                 | No         | 01/02 surgery + sugery  | Alive   |
| 8     | 3 mnts | M   | NB I Adnex                             | D                                 | No         | 02/02 Surgery           | Alive   |
| 9     | 2 yrs  | M   | NB IV Adnex with bone/BM metastases    | D⁴                                | No         | 12/03 chemo, SCR + RT   | d.o.d.  |
| 10    | 8 mnts | F   | NB III Spine                           | PD                                | No         | 10/04 relapse treatment | d.o.d.  |
| 11    | 18 mnts| F   | NB III Adnex                           | UD⁴                               | 20×        | 12/02 Surgery spinal relapse | d.o.d.  | 09/98 MIBG, chemo + surgery |
| 12    | 2 yrs  | M   | NB IV Adnex and bone metastases       | PD⁴                               | 37×        | 07/98 surgery, chemo + SCR | d.o.d.  |
| 13    | 6 yrs  | M   | NB III Adnex                           | UD                                 | 27×        | 01/00 surgery, chemo + SCR | d.o.d.  |
| 14    | 19 mnts| M   | NB IV Adnex and spine metastasis      | UD                                 | 49×        | 03/98 MIBG, chemo + surgery | d.o.d.  |
| 15    | 18 mnts| M   | NB IV Adnex and multiple distal metastases | n.d.                              | 139×       | 06/99 chemo + RT       | d.o.d.  |
| 16    | 16 mnts| M   | NB IV Adnex and multiple distal metastases | UD                                 | 74×        | 01/97 chemo + surgery | d.o.d.  |

¹Age at diagnosis
²Neuroblastoma differentiation as assessed by pathologist. D = differentiated; PD = poorly differentiated; UD = undifferentiated
³MYCN genomic amplification, as determined with qPCR
⁴Patient has been treated before surgery
BM = bone marrow; d.o.d. = death of disease; MIBG = meta-iodobenzylguanidine; SCR = stem cell rescue; RT = radiotherapy.
Statistical analysis
Statistically significant differences in expression of MYCN-transcripts between NBs with or without MYCN-amplification were calculated with Students’ T-test. Correlation of MYCN-transcript expression with disease stage was calculated using the Spearman rank correlation and correlation of MYCN-transcripts with the MYCN-amplification numbers was calculated with the Pearson correlation test. All statistical tests were two-sided, significance was determined as p < 0.05.

Results
Patient characteristics
We analysed fresh-frozen NBs from 16 pediatric patients (age range: 0–6 years old). The NBs are classified according to the Children’s Cancer Group Neuroblastoma Staging System [25] and treated according to Pediatric Oncology Group-protocols (Table 2). Six out of 16 NBs carried a MYCN-amplification initially detected by Southern blot and/or FISH.

ΔMYCN expression in the neuroblastoma cell line IMR-32
Two splice variants have been described for the proto-oncogene MYCN, the classical transcript that consists of three exons and a shortened ΔMYCN transcript that lacks exon 2. ΔMYCN is expressed in several fetal tissues, but its expression has not been reported in NBs. We used primers spanning exon 2 (Figure 1A and Table 1) to visualize by reverse transcriptase PCR whether or not both transcripts are present in MYCN-amplified IMR-32 neuroblastoma cells. Two fragments were identified that corresponded with the expected product lengths of MYCN and ΔMYCN of respectively 1007 and 100 bp (Figure 1D). Sequence analyses on the excised products confirmed that these fragments were MYCN and ΔMYCN. Absence of additional fragments in the IMR-32 NB cell line suggests that there are no other major MYCN splice variants. To determine whether the ΔMYCN protein is expressed in IMR-32 NB cells, MYCN proteins were visualized with the C-19 antibody that recognizes the c-terminal epitope of both MYCN and ΔMYCN proteins. In the lysate of IMR-32 cells, two protein bands were recognized at approximately 65 and 45 kD, which are the predicted molecular weights of MYCN and ΔMYCN respectively [18] (Figure 2A). For comparison, in a lysate of the melanoma cell line (BLM), which does not carry MYCN-amplification, no reactivity could be observed. We conclude from these experiments that the fetal MYCN isoform ΔMYCN is co-expressed with MYCN in IMR-32 cells.

Quantitative analyses of MYCN, ΔMYCN and MYCNOS expression levels in neuroblastomas
mRNA expression levels of MYCN, ΔMYCN and MYCNOS were measured in 16 human neuroblastoma samples (Table 2) by QPCR relative to three reference genes: GLUSB, TFRC and RNPE [21]. Both MYCN and MYCNOS were found to be expressed in all 16 NBs. In addition, ΔMYCN expression was detected in all NB samples, except for patient 9, who did not carry an amplification of the MYCN region. For MYCN, it has been demonstrated that the relative expression-levels are significantly higher in NBs with MYCN-amplification as compared to non-amplified tumours [26]. Here, we show that besides MYCN, the relative mRNA expression levels of ΔMYCN and MYCNOS are also significantly increased in NBs with MYCN-amplification (p < 0.01; Figure 3A).

Correlation of NB stage with MYCN:ΔMYCN-ratio showed that the MYCN:ΔMYCN-ratio remains constant and does not change with either MYCN-amplification

Figure 2
Detection of MYCN and ΔMYCN in IMR-32 cells. (A) Western blot, visualizing two proteins in the IMR-32 (NB cell line with MYCN-amplification) lysate with the C-19 antibody that recognizes the C-terminal epitope of both MYCN and ΔMYCN. In the BLM (melanoma cell line without MYCN-amplification) lysate, these proteins were not present. (B) Western blot of IMR-32 whole lysate (1), whole lysate minus precipitate (2) and the precipitate (3) using the C-19 antibody. Arrows indicate the positions of MYCN (65 kDa) and ΔMYCN (45 kDa). The additional band in lane 3 is caused by deposition of Ig-heavy chains (50 kDa). Exposure times are indicated below the blots.
**Figure 3**

**MYCN, ΔMYCN, and MYCNOS mRNA expression levels in NBs.** (A) Expression levels of MYCN, ΔMYCN and MYCNOS in MYCN-amplified (closed-triangles) compared to non-amplified (open triangles) tumours. (B) Relative expression-levels of MYCN (closed triangles), ΔMYCN (closed circles) and MYCNOS (open circles) correlated to MYCN-amplification in NBs. (C) Difference of the MYCN:MYCNOS-ratio between NBs with MYCN-amplification (closed circles) and NBs without MYCN-amplification (open circles). (D) Correlation of the number of MYCN copies with MYCN:MYCNOS mRNA ratios (closed circles and non-interrupted line) and with MYCN:MYCN mRNA ratios (open triangles and dotted line). (E) Correlation between NB stage and MYCN:MYCNOS-ratio. (F) Correlation between NB stage and MYCN: ΔMYCN-ratio. NB IV-s is a special type of NB characterized by metastatic disease with spontaneous regression and good survival [31].

NB IV-s is a special type of NB characterized by metastatic disease with spontaneous regression and good survival [31].
Students’ T-test) or NB stage (Figure 3F; two-tailed p-value = 0.025) and NB-stage (Figure 3E; two-tailed p-value = 0.007). The MYCN:MYCNOS-ratio did not significantly change with either MYCN-amplification or NB-stage (two-tailed p-values = 0.58 and 0.24, respectively; data not shown). These data show that in more advanced NB tumours, mRNA expression of MYCNOS increases relative to MYCN.

**ΔMYCN and MYCNOS expression relative to level of MYCN-amplification**

To more exactly determine MYCN copy number in the tumours that were studied, we performed a genomic quantitative PCR (qQPCR) using genomic primers recognizing five different locations within the MYCN-gene (Figure 1A; Table 1). Amplification of these DNA fragments was calculated relative to three reference genes elsewhere on the genome, *CFTR*, *TBX22*, and *SLC16A2*. Among these three reference genes, there were no copy number differences noted in any of the NB samples. All 10 samples with a normal MYCN-copy number based on Southern blotting and/or FISH, carried two to four MYCN copies as determined by qQPCR. The presence of a MYCN duplication in NB cells that lack an overt amplification of MYCN is more often found, although the implications for the progression of the NB are still unclear [5,27]. All 6 samples with multiple copies of MYCN, as determined by Southern blotting and/or FISH, had in between 20 and 139 MYCN gene amplifications (Table 2), which is within the normal range of gene copy numbers observed in NBs with MYCN-amplification [28].

We observed that MYCN mRNA-expression does not linearly correlate with MYCN-amplification, consistent with earlier reports [26]. In addition, also ΔMYCN and MYCNOS do not correlate linearly with the number of MYCN gene copies (Figure 3B). As shown in figure 3D, the relative mRNA expression ratio of MYCN:MYCNOS decreases with an increasing number of MYCN gene copies although this is not significant (non-interrupted line, slope = -0.4; two-tailed p-value = 0.18, calculated with the Pearson correlation test). The MYCN:ΔMYCN ratio does not change with higher MYCN copy numbers (Figure 3D; dotted line, slope = -0.1; two-tailed p-value = 0.88).

**Overexpression of MYCNOS in the NB cell line IMR-32**

The pre-mRNA of MYCNOS, which represents the MYCN antisense transcript, shows overlap with the first exon of MYCN. Therefore MYCNOS may potentially modulate MYCN mRNA expression levels at the mRNA level via RNA-interference or RNA-editing, or direct MYCN splicing by RNA masking [11]. To test this premise, we transfected IMR-32 NB cells with CI-GFP and either the pcDNA3-vector containing MYCNOS or an empty vector. IMR-32 cells have relatively high endogenous expression levels of MYCN, MYCNOS and ΔMYCN, which enables quantification of all three mRNA levels. Flow cytometric analyses showed that there was a transfection efficiency of 74% after 72 hours (Figure 4A). Although there was a 50-fold increase of MYCNOS gene expression in the MYCNOS-transfected cell line relative to the empty vector control cell line (Figure 4B), expression of endogenous MYCN and ΔMYCN was not affected either at the mRNA level or at the protein level (Figure 4B, C). We conclude that although increased expression of MYCNOS relative to MYCN is correlated with an advanced disease state, RNA-interference or RNA-editing are not the mechanisms by which MYCNOS downregulates MYCN expression. In addition, the unchanged MYCN:ΔMYCN ratio in cells with MYCNOS overexpression shows that MYCNOS does not affect splicing by RNA-masking.

**Discussion**

In this report, we have analysed the expression levels of MYCN, ΔMYCN and MYCNOS in NBs. We find that these three mRNA transcripts are expressed in NBs of all stages, but more highly in NBs with MYCN-amplification. The MYCN:MYCNOS expression level ratio is significantly decreased in high grade NBs, whereas the MYCN:ΔMYCN remains constant in NBs of all stages, which indicates that MYCN and ΔMYCN are co-regulated. These results suggest that MYCNOS might be involved in the regulation of MYCN expression levels as has been shown for numerous other antisense transcripts regulating expression of their sense counterparts [11,12]. However, it is important to note that the number of NB samples we investigated is relatively small. Future studies in larger cohorts of patients are needed to further establish a role for MYCNOS in the regulation of MYCN expression in patients with low-, intermediate- and high-risk NB.

Natural antisense RNA can inhibit gene expression at the DNA level by transcriptional interference or at the mRNA level by RNA-interference or RNA-editing, or regulate splicing by RNA-masking [11,12]. In RNA-masking, MYCN-MYCNOS duplex formation modulates RNA processing by preserving a MYCN population that retains intron 1, hence resulting in decreased ΔMYCN expression. Krystal et al. [29] showed that RNA-masking can occur, but they found that only approximately 5% of MYCN RNA interacts with MYCNOS RNA. Our results show that it is unlikely that MYCNOS expression has an effect on splicing, since 50-fold overexpression of MYCNOS in IMR-32 cells did not change the MYCN/ΔMYCN expression ratio at the mRNA level. Therefore, inhibition of (Δ)MYCN expression seems to be the most likely role for
MYCNOS. There are three mechanisms by which this may be accomplished: transcriptional interference, RNA interference and RNA-editing. Since our results show that overexpression of MYCNOS pre-mRNA in NB cell line IMR-32 does not suppress MYCN expression, RNA-interference and RNA-editing do not seem to be the primary inhibitory mechanisms, leaving the possibility that regulation occurs at the DNA level by steric hindrance of the voluminous RNA-polymerase complexes on opposite DNA strands.

It is not clear how increased expression of MYCNOS contributes to the development of NB. Although the increase of MYCNOS expression levels is higher than that of MYCN in NB with amplification, this difference does not appear to influence the prognosis of patients. In patients with NB but without MYCN-amplification, it would be interesting to investigate whether the MYCN:MYCNOS ratio is a good prognostic marker. Differences in MYCNOS expression levels might explain some of the controversies about MYCN expression and prognosis of these patients [1-3].

Besides MYCN and MYCNOS, ΔMYCN, which was previously identified as a fetal transcript [18], is also expressed in NBs. No other MYCN isoforms were detected. This suggests that the alternative splice variant that previously has been described by Stanton et al. [30] and consists of an alternatively spliced exon 1, has little relevance in the progression of NBs. In one tumour, the ΔMYCN transcript could not be identified, but this is probably because ΔMYCN is low expressed in general and in this tumour MYCN was not amplified. The ΔMYCN protein contains a nuclear localization signal, a basic helix-loop-helix, and a

**Figure 4**

*Overexpression of MYCNOS in the NB cell line IMR-32. (A) Transfection-efficiency was measured by GFP expression as analysed by flow cytometry, 74% of the NB cells expressed GFP 72 hours after transfection. (B) In the MYCNOS transfected IMR-32 cells, MYCNOS was 50× upregulated compared to not transfected and mock-transfected IMR-32 cells. Endogenous MYCN expression was not significantly affected. (C) Western blot showing that MYCN protein expression in MYCNOS transfected IMR-32 cells was unaffected. Lane 1 is loaded with lysate from untransfected cells, lane 2 with lysate from cells transfected with the empty vector, and lane 3 with lysate from MYCNOS transfected cells.*
leucine-zipper domain, which may serve to dimerize with MYCN or bind to its DNA binding site. ΔMYCN lacks the transactivation domain including the highly conserved Myc 1 and 2 boxes, from which it was speculated that it competes with MYCN and therefore inhibits the active MYCN protein [18]. However, in all neuroblastoma samples analyzed, the ratio between MYCN and ΔMYCN expression remains constant and does not correlate with MYCN amplification or disease stage, indicating that ΔMYCN induced inhibition of MYCN at the protein level is not of relevance in NB.

Conclusion
In conclusion, our results suggest that the expression of the antisense gene MYCNOS might be relevant to the progression of NB, potentially by directly inhibiting MYCN transcription by transcriptional interference at the DNA level. Analysis of MYCN:MYCNOS expression ratios in patients with NB without MYCN-amplification and clinical follow-up are necessary to establish the relevance of MYCNOS expression to the prognosis of these patients.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JFMJ and APMB designed, performed and analyzed the research and drafted the manuscript. HB and PH conceived of the study. FNL supervised the transfection experiments and helped to draft the manuscript. CAHK performed all pathological characterizations. HB, PMH, GJA and IJMV conducted the study as the principal investigators and contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Supplemental figure. Ponseau S stainings of the immunoblot shown in (A) figure 2a and (B) 4c. Click here for file.
[http://www.biomedcentral.com/content-supplementary/1471-2407-9-239-S1.pdf]

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