Abstract

Background. MYCN has been an attractive therapeutic target in neuroblastoma considering the widespread amplification of the MYCN locus in neuroblastoma, and its established role in neuroblastoma development and progression. Thus, understanding neuroblastoma-specific control of MYCN expression at the transcriptional and post-transcriptional level would lead to identification of novel MYCN-dependent oncogenic pathways and potential therapeutic strategies.

Methods. By performing loss- and gain-of-function experiments of the neuroblastoma hotspot locus 6p22.3 derived IncRNAs CASC15-003 and NBAT1, together with coimmunoprecipitation and immunoblotting of MYCN, we have shown that both IncRNAs post-translationally control the expression of MYCN through regulating a deubiquitinase enzyme USP36. USP36 oncogenic properties were investigated using cancer cell lines and in vivo models. RNA-seq analysis of loss-of-function experiments of CASC15-003/NBAT1/MYCN/USP36 and JQ1-treated neuroblastoma cells uncovered MYCN-dependent oncogenic pathways.

Results. We show that NBAT1/CASC15-003 control the stability of MYCN protein through their common interacting protein partner USP36. USP36 harbors oncogenic properties and its higher expression in neuroblastoma patients correlates with poor prognosis, and its downregulation significantly reduces tumor growth in neuroblastoma cell lines and xenograft models. Unbiased integration of RNA-seq data from CASC15-003, NBAT1, USP36, and MYCN knockdowns and neuroblastoma cells treated with MYCN inhibitor JQ1, identified genes that are jointly regulated by the NBAT1/CASC15-003/USP36/MYCN pathway. Functional experiments on one of the target genes, COL18A1, revealed its role in the NBAT1/CASC15-003-dependent cell adhesion feature in neuroblastoma cells.

Conclusion. Our data show post-translational regulation of MYCN by NBAT1/CASC15-003/USP36, which represents a new regulatory layer in the complex multilayered gene regulatory network that controls MYCN expression.

Key Points

- Post-translational control of MYCN expression by sense-antisense IncRNAs CASC15/NBAT1.
- NBAT1/CASC15/MYCN/USP36/COL18A1 controlled novel oncogenic pathway in neuroblastoma.
Importance of the Study

*MYCN* amplification and its expression have served as reliable biomarkers in the stratification of neuroblastoma risk status. Hence, there is a greater appreciation in the neuroblastoma research community in understanding the mechanisms that control *MYCN* oncogene expression at the transcriptional and post-transcriptional levels, and also, in characterizing the novel MYCN-dependent downstream oncogenic pathways to devise potential therapeutic opportunities for the treatment of high-risk NBs. In this investigation, we show that *NBAT1* and *CASC15-003* IncRNAs regulate *MYCN* expression post-translationally through their interacting protein partner USP36, which is a deubiquitinating enzyme. Our study also characterizes the \( \text{NBAT1/CASC15-003/MYCN/USP36/} \text{COL18A1} \)-controlled oncogenic pathway in neuroblastoma pathogenesis.

Neuroblastoma (NB), a tumor of neural crest origin, is the most common extracranial solid tumor. It typically develops from sympathetic ganglia in the abdomen, the majority of which develop from adrenal medulla.\(^1\,2\) It is the second most common solid tumor after central nervous system tumors affecting children. Being a complex disease, patients are classified into low-, intermediate-, or high-risk groups based on a set of well-defined prognostic indicators such as age of diagnosis, segmental chromosomal aberrations, and stage of the disease.\(^3,4\)

In NB, nonrandom segmental chromosomal aberrations are considered well-defined prognostic markers in the clinical setting, with implications in therapeutic decision strategy. For example, chromosome alterations such as 1p deletion, 11q deletion, 17q gain, and *MYCN* oncogene amplification are routinely used in therapeutic decision strategy.\(^5,6\) Interestingly, about 25% of NB cases show *MYCN* gene amplification, which is a well-characterized chromosomal alteration. *MYCN* gene amplification is associated with aggressive and highly metastatic NB, and confers dismal prognosis and poor survival.\(^7,8\) Aggressive NBs with the *MYCN* gene amplification (MNA) have <50% survival rates with a higher frequency of relapse of the disease, which poses a challenge to rigorous therapeutic procedures. The MYCN protein belongs to the 3-member MYC family of oncoproteins that also includes c-MYC and l-MYC. Unlike the ubiquitously expressed c-MYC, MYCN expression is confined to the embryonic nervous system and mesenchymal tissue. Moreover, MYCN expression is reduced in adult tissues and differentiated neurons.\(^9,10\) MYCN is implicated in a wide range of biological functions associated with cancer development and progression, including pluripotency, self-renewal, proliferation, metastasis, and angiogenesis.\(^9\) Experimental overexpression of MYCN promotes tumor phenotypes in vitro and in vivo,\(^11,12\) further reinforcing its role in oncogenesis. MYCN expression is regulated at the transcriptional\(^13,14\) and post-transcriptional level\(^15–18\) through cooperation between transcriptional factors and long noncoding RNAs (lncRNAs). In addition to the elevated levels of *MYCN* RNA, the stability of the MYCN protein also plays a vital role in NB.\(^19,21\) However, the mechanisms by which lncRNAs control MYCN protein stability, and their role in MYCN-dependent oncogenic pathways, remain poorly investigated.

Materials and Methods

Cell Lines

SH-SY5Y, IMR-32, and Kelly cells were purchased from CLS Cell Lines Service. SK-N-BE2 cells were obtained from DSMZ, German Collection of Microorganisms and Cell Cultures GmbH. Cell lines used in this study were maintained at 37°C with 5% CO\(_2\) and cultured in respective media as per manufacturer’s instructions.

Plasmid Constructs

For details, please refer to Supplementary Materials and Methods.

Transient Transfections

For details, please refer to Supplementary Materials and Methods.
Generation of Stable Knockdown Cells

Lentiviral shRNA particles targeting CASC15-003 and NBAT1, USP36, and non-target shRNA control were purchased from Sigma-Aldrich and stable shRNA cells were generated using a method described previously.22,23

Co-immunoprecipitation

Co-immunoprecipitation was performed as described previously23 except for a few modifications. Cells were lysed in BC100 buffer (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 10% Glycerol, 0.2 mM EDTA, 0.1% Triton X-100, fresh protease inhibitor) and 5 µg of anti-MYCN antibody was added and incubated at 4°C overnight on a rotor. Next day antibody:lysate mixture was incubated with ProteinA, magnetic beads for 1 h at 4°C. Beads were washed thrice in BC100 buffer and the bead bound antibody:protein complexes were analyzed by Western blot.

Immunoprecipitation of Ubiquitinated Proteins

Immunoprecipitation to detect the ubiquitination of endogenous or ectopically expressed MYCN in MG132 (15 µM for 15 h) treated cells was performed as described previously.23 Unlike cultured cells, xenografts were not treated with MG132 and instead MG132 was added to the IP lysis buffer in which the tumors were homogenized using a glass mortar and pestle. Lysates were harvested from the homogenized tissue by centrifugation at 14,000 rpm for 30 min at 4°C. IP was performed with respective antibodies followed by immunoblotting to analyze the ubiquitination status using FK2 antibody (anti-ubiquitin antibody).

Immunoblot

For details, please refer to Supplementary Materials and Methods.

Immunofluorescence

For details, please refer to Supplementary Materials and Methods.

Proliferation Assay

Please refer to Supplementary Materials and Methods for details.

Cell Adhesion Assay

Cell adhesion assay was performed as described previously.24 In brief, cell culture plates were coated with 15 µg/mL of bovine collagen 1 (Cat# A1064401, Thermofisher scientific) for at least 2 h at room temperature. Collagen solution was then removed and the plates were allowed to dry for about 1 h before seeding the cells. Twenty-five thousand cells were seeded and allowed to settle for 30 min in a cell incubator. The unattached cells were removed and the adherent cells were gently washed a couple of times with 1x PBS. The adherent cells were then estimated by performing MTT assay using CellTiter 96 Non-Radioactive Cell Proliferation assay kit from Promega (G4000) as per manufacturer’s instructions.

Colonization Assay

For details, please refer to Supplementary Materials and Methods.

Wound Healing Assay

Please refer to Supplementary Materials and Methods for details.

Xenografts in Mice

Xenografts in 5- to 6-week-old Bagg Albino (inbred research mouse strain) nude mice (n = 4) with control or USP36Sh KD IMR-32 cells were generated as described previously.23 All the mouse experiments were approved by the Animal Ethical Review Board, University of Gothenburg, Gothenburg, Sweden (Ethical permit number-5.8.18-02708/2017).

Chromatin Immunoprecipitation (ChIP)

For details, please refer to Supplementary Materials and Methods.

Differential Gene Expression Analysis

Please refer to Supplementary Materials and Methods for details.

Statistical Analysis

P values were calculated using Student’s t-test or analysis of variance where statistical significance was represented as (*) P-value ≤ .05, (**) P-value ≤ .01, (***) P-value ≤ .001. (*) P-value ≤ .05 is considered significant.

Results

6p22.3 IncRNAs CASC15-003 and NBAT1 Regulate MYCN Protein Levels in NB Via USP36

6p22.3 IncRNAs CASC15-003 and NBAT1 show higher expression in low-risk NBs compared to the high-risk patients, and their higher levels in NB patients correlates with better clinical outcome. MYCN amplification in NB patients, on the other hand, predicts worse prognosis and is often associated with metastasis and relapse. MYCN amplified (MNA) tumors have low CASC15-003 and NBAT1 compared to the
Figure 1. 6p22.3 IncRNAs CASC15-003 and NBAT1 regulate MYCN levels in neuroblastoma (NB) cells. (A) Immunoblot shows the MYCN protein levels in vector control, CASC15-003 and NBAT1 overexpressing SK-N-BE(2), IMR-32, and SH-SY5Y cells (upper panel) and in Ctrl Sh, CASC15-003 and NBAT1 shRNA transduced IMR-32 and SH-SY5Y cells (lower panel). Vector control and Ctrl Sh were used as controls for overexpression and KD, respectively. GAPDH is used as a loading control. (B) Immunoblot of MYCN and USP36 in USP36 KD SK-N-BE(2), IMR-32, and SH-SY5Y cells. KD was carried out using lentiviral shRNA particles in SK-N-BE(2), IMR-32, and SH-SY5Y, whereas transient USP36 KD was performed using siRNA in SK-N-BE(2) (last panel on the right). Ctrl Sh or Ctrl Si was used as a control and GAPDH as a loading control. (C) Immunoblot of MYCN and
MYCN nonamplified (nonMNA) ones.22,23 This intrigued us to check for the abundance of these RNAs and their connection to MYCN protein levels in MNA and non-MNA cell lines. For this, we examined the expression levels of 6p22.3 IncRNAs in MNA cell lines (IMR-32, SK-N-BE(2) and KELLY) and non-MNA cell lines (NB69 and SH-SY5Y). In line with the tumor data, MNA cell lines showed reduced expression of CASC15-003 and NBAT1 as compared to the non-MNA cell lines (Supplementary Figure S1A). This inverse correlation between CASC15-003/NBAT1 expression and MYCN protein levels encouraged us to probe the functional connection between MYCN and CASC15-003/NBAT1 in NB. Toward this end, we analyzed MYCN protein levels following loss- and gain-of-function assays for these 2 IncRNAs. Overexpression of CASC15-003 or NBAT1 in SK-N-BE(2), IMR-32 and SH-SY5Y cell lines resulted in reduced MYCN protein levels (Figure 1A, upper panel), whereas shRNA mediated knockdown (KD) of these 2 IncRNAs in IMR-32 and SH-SY5Y led to an increase in MYCN protein levels (Figure 1A, lower panel). KD of CASC15-003 or NBAT1 could not be achieved in SK-N-BE(2) as the endogenous abundance of these two RNAs is quite low (Supplementary Figure S1A). On the other hand, MYCN mRNA levels were not altered significantly in SK-N-BE(2) cells overexpressing the 2 IncRNAs (Supplementary Figure S1B). These findings suggest that MYCN is not regulated at the transcriptional level by CASC15-003 and NBAT1, but rather at the post-translational level.

LncRNAs are indeed known to regulate the stability and abundance of their target proteins by direct or indirect mechanisms. Previous studies identified ubiquitin-specific protease USP36 as a common interacting protein partner of CASC15-003 and NBAT1, and these IncRNAs control USP36 activity through modulating its spatial organization.23 USP36 was shown to deubiquitinate target proteins to determine their stability,23,25,26 C-MYC, a member of the MYC family of proteins, was shown to be stabilized by the deubiquitinase activity of USP36.26 Considering that MYCN is a paralogue of c-MYC, we sought to investigate if USP36 could regulate MYCN protein levels through its deubiquitinase activity. To test this hypothesis, USP36 was stably downregulated in SK-N-BE(2), IMR-32, and SH-SY5Y cell lines by transduction of 2 independent short hairpin RNAs (shRNAs). In addition, transient KD of USP36 was performed in SK-N-BE(2) cells using siRNA. Both stable and transient USP36 KD led to reduced levels of MYCN protein (Figure 1B). As expected, USP36 overexpression in SK-N-BE(2), IMR-32, and SH-SY5Y cells resulted in an increase in MYCN protein levels (Figure 1C). These results strongly suggest that USP36 positively regulates MYCN protein levels. However, MYCN RNA levels were not affected following KD or overexpression of USP36 (Supplementary Figure S1C), suggesting a post-transcriptional regulation of MYCN by USP36, most probably through regulating MYCN ubiquitination levels. We tested the stability of the MYCN protein in USP36 siRNA KD cells by a cycloheximide chase experiment. A striking reduction in the MYCN protein was observed in USP36 KD cells in comparison to the control cells (Figure 1D), suggesting that USP36 determines the stability of MYCN through post-transcriptional regulation.

**USP36 Interacts With MYCN and Regulates Its Stability by Deubiquitination**

To understand the role of USP36 in regulating MYCN protein stability, we sought to investigate the interaction between these 2 proteins by immunocytocytochemistry. Endogenous MYCN and USP36 co-localize with the nucleolar-specific protein NPM1 in IMR-32 cells (Figure 2A). Also, Halo-USP36 and HA-MYCN showed co-localization when overexpressed in SH-SY5Y (Figure 2B). To further investigate the interaction between USP36 and MYCN, we performed a co-immunoprecipitation (Co-IP) of MYCN to verify its association with USP36 in SK-N-BE(2) and IMR-32 cell lines. We found that endogenous MYCN interacts with USP36 in both of the cells lines (Figure 2C, left and middle panels). Consistent with the endogenous MYCN and USP36 interaction data, ectopically expressed Halo-USP36 was immunoprecipitated with the endogenous MYCN in HeLa cells, further confirming the interaction between MYCN and USP36 (Figure 2C, right panel).

Considering that the ubiquitin-specific protease USP36 enhances the stability of target proteins through the removal of ubiquitin moieties, we thought USP36 interaction with MYCN could be involved in the regulation of MYCN stability via ubiquitin mediated proteasomal degradation. We first confirmed the presence of MYCN in the ubiquitinated protein pool by immunoprecipitation with the ubiquitin targeting FK2 antibody (Figure 2D, upper panel). We next verified the role of USP36 in the maintenance of MYCN ubiquitination status by overexpressing both MYCN and USP36. We found that ubiquitination of ectopic MYCN was lower in cells that overexpress USP36 compared to cells overexpressing MYCN alone (Figure 2D, lower panel), indicating that USP36 is a deubiquitinating enzyme for MYCN. To address this further, we tested the ubiquitination status of the endogenous MYCN protein following USP36 loss-of-function and gain-of-function experiments. USP36 KD using shRNAs revealed a significant increase in ubiquitinated MYCN levels (Figure 2E, upper panel), whereas its overexpression led to decreased accumulation of ubiquitinated MYCN (Figure 2E, lower panel). Similarly, USP30Sh KD xenografts also showed higher MYCN ubiquitination as compared to the control tumors (Supplementary Figure S2A).
Figure 2. USP36 interacts with and regulates MYCN protein. (A) Upper panel: Immunostaining analysis in IMR-32 cells shows the endogenous MYCN in green and nucleolar marker nucleophosmin (NPM1) in red, stained with anti-MYCN and anti-NPM1 antibodies, respectively. DAPI was used as the nuclear stain. Lower panel: Immunostaining of USP36 (green) and NPM1 (red) stained with anti-USP36 and anti-NPM1 antibodies, respectively, in IMR-32 cells. DAPI was the nuclear stain. Scale bar, 5 µm. (B) SH-SY5Y cells transiently transfected with Halo-USP36 and HA-MYCN expressing plasmids followed by immunostaining analysis. Upper panel: Immunostaining of Halo-USP36 and HA-MYCN stained with anti-Halo and anti-HA antibodies, respectively. DAPI was the nuclear stain. Lower panel: Immunostaining for Halo-USP36 and NPM1 stained with anti-Halo or anti-NPM1 antibodies, respectively. DAPI was the nuclear stain. Scale bar, 5 µm. (C) Co-immunoprecipitation (Co-IP) showing the interaction between MYCN and USP36. Left and middle panels show the endogenous MYCN immunoprecipitation using anti-MYCN antibody in SK-N-BE(2) and IMR-32 cells, respectively. Anti-MYCN and anti-USP36 antibodies detected the presence of MYCN and USP36 in the anti-MYCN immunoprecipitation, IP:MYCN (lane3 from left). Left and middle lanes represent 5 or 10% input and negative control IgG, respectively. GAPDH immunoblot rules out the
**Integrated Transcriptome Analyses Identify Common Target Genes Regulated by CASC15-003, NBAT1, USP36, and MYCN**

Our findings indicate that the 6p22.3 locus-derived IncRNAs CASC15-003 and NBAT1 can determine cell proliferation and tumorigenesis in NB by regulating MYCN stability via modulating USP36 function. To further understand how the functional interplay between CASC15-003/NBAT1/USP36 and MYCN control NB pathogenesis, we performed transcriptomic analysis following the individual KD of CASC15-003, NBAT1, USP36, and MYCN. We also performed RNA-seq on NB cells treated with JQ1, a transcriptional inhibitor of MYCN, specifically, since NBAT1/CASC15 are tumor suppressors, and USP36/MYCN oncogenic genes are gained in CASC15/NBAT1 KD cells, we therefore compared upregulated genes from CASC15/NBAT1 KD RNA-seq data with downregulated genes from RNA-seq datasets obtained from USP36 KD/MYCN KD/JQ1 treated cells (Supplementary Tables 1–3). These integrated transcriptomic analyses identified four jointly regulated target genes, COL18A1, EGFL7, ITGB5, and OAF (Figure 4A and B), which show significant deregulation in the transcriptomic data from all 4 KDs as well as the JQ1 treatment (Figure 4B–D). We further validated the expression of the four genes using RT-qPCR following KD of CASC15-003, NBAT1, MYCN, and USP36, as well as in the JQ1 treated cells (Figure 4E–H). COL18A1, EGFL7, ITGB5, and OAF showed significant upregulation after NBAT1 and CASC15-003 KD (Figure 4E), whereas they were downregulated in the MYCN KD, USP36 KD, and JQ1-treated samples (Figure 4F–H, Supplementary Figure S1E). These results suggest that COL18A1, EGFL7, ITGB5, and OAF genes are part of a CASC15-003/NBAT1/MYCN/USP36 controlled regulatory network.

**COL18A1 Contributes to the MYCN-Dependent Tumor Phenotype in NB Cells**

The COL18A1 gene encodes alpha chain of type XVIII collagen, which is an extracellular matrix protein. Hence, we became particularly interested in understanding the functional role of COL18A1 in the context of CASC15-003/NBAT1/USP36/MYCN regulated pathways. We first investigated whether COL18A1 is directly regulated by MYCN. For this, we overexpressed MYCN in USP36 siRNA KD SK-N-BE(2) cells. COL18A1 expression was rescued in MYCN overexpressed cells compared to USP36 KD cells (Figure 5A). To further confirm the direct regulation of COL18A1 by MYCN, chromatin immunoprecipitation (ChiP) with MYCN antibody was performed in CASC15-003 and NBAT1 depleted SH-SY5Y cells. The ChiP assays on CASC15-003 and NBAT1 depleted cells showed increased MYCN occupancy at the COL18A1 promoter compared with the control cells (Figure 5B). Since COL18A1 is an extracellular matrix protein, we investigated its role in wound healing, cell proliferation and extra cellular matrix organization in SK-N-BE(2) cells. siRNA-mediated KD of COL18A1 significantly reduced the wound healing efficiency (Figure 5C) and the cell proliferation rate (Figure 5D) of SK-N-BE(2) cells. COL18A1 KD also affected the cell adhesion efficacy of SK-N-BE(2) cells (Figure 5E, left panel). Interestingly, we found an increase in the cell adhesion non-specific interactions in the Co-IP. Right panel: shows interaction between ectopically expressed MYCN and USP36 in HeLa cells. MYCN and Halo-USP36 were ectopically expressed in HeLa cells and the lysates were subjected to immunoprecipitation with anti-MYC antibody in IP:MYCN. Anti-MYC and anti-Halo antibodies detected the presence of MYCN and USP36 in both IP:MYCN and input. IgG is the negative control for IP. (D) Ubiquitination of MYCN in NB cells. Upper panel: Immunoblot shows the presence of MYCN in lysates (lane 3) immunoprecipitated with anti-FK2 antibody in SK-N-BE(2) cells treated overnight with MG132, IP:FK2. IgG used as a negative control and GAPDH immunoblot served as a control for nonspecific binding. Lower panel: Ectopically expressed Halo-USP36 deubiquitates HA-MYC in HeLa cells. HA-MYC and Halo-USP36 were ectopically expressed in HeLa cells. Two days after transfection, cells were treated with MG132 overnight and subjected to IP with anti-HA antibody. Immunoblot shows the ubiquitinated HA-MYC in IP:HA-MYC detected by FK2 antibody. IP:HA-MYC also shows the levels of immunoprecipitated HA-MYC. Anti-HA, Anti-Halo and Anti-GAPDH detect the levels of ectopic MYCN, USP36 and endogenous GAPDH, respectively. (E) Immunoblot for ubiquitinated MYCN in USP36 stable KD or USP36 overexpressing SK-N-BE(2) cells. Cells were treated with MG132 overnight and MYCN was immunoprecipitated and analyzed by Immunoblot to check the ubiquitination status of MYCN. Upper panel: Lysates from IMR-32 cells, stably transfected with Ctrl or USP36Sh1 and Sh3, were immunoprecipitated with anti-MYC antibody. Immunoblot shows the ubiquitination of immunoprecipitated MYCN in IP:MYCN detected by FK2 antibody. Also, IP:MYCN shows the levels of immunoprecipitated MYCN. Anti-MYC, anti-USP36, and anti-GAPDH antibodies detect the MYCN, USP36, and GAPDH levels, respectively, in IP:MYCN and input. IgG is the negative control for IP. Lower panel: MYCN was immunoprecipitated from HeLa cells, ectopically expressing vector Ctrl or Halo-USP36. Immunoblot shows the ubiquitinated MYCN detected by FK2 antibody and the corresponding levels of MYCN detected by anti-MYC antibody in IP:MYCN. Anti-Halo, anti-MYC and anti-GAPDH antibodies detect the levels of Halo-USP36, MYCN, and GAPDH, respectively, in the input.
efficacy of CASC15-003 and NBAT1 KD cells (Figure 5E, middle panel) and this increased cell adhesion was lost following COL18A1 KD (Figure 5E, right panel). Taken together, our data indicate that COL18A1 plays an important role in CASC15-003/NBAT1/USP36/MYCN controlled extracellular matrix organization in NB.
Figure 4. Transcriptome analysis to identify common targets between CASC15-003, NBAT1, USP36, and MYCN. (A) Venn diagram showing the overlap of downregulated genes from USP36 KD, MYCN KD and JQ1-treated SK-N-BE(2) cells with upregulated genes from CASC15-003 and NBAT1 KD SH-SY5Y cells. (B) Bar graph showing the fold change of common genes (Figure 4A) in NBAT1 and CASC15-003 KD SH-SY5Y cells. (C) Bar graph showing fold change of common genes (Figure 4A) in MYCN KD and JQ1-treated SK-N-BE(2) cells. The significances are indicated by *P < .05, **P < .01, and ***P < .001. (D) Bar graph showing fold change of common genes (Figure 4A) in USP36 KD SK-N-BE(2) cells. (E–H) Expression analysis of target genes COL18A1, EGFL7, OAF, and ITGB5 by RT-qPCR. (E) RT-qPCR analysis of COL18A1, EGFL7, OAF, and ITGB5 in SH-SY5Y cells stably KD for CASC15-003 and NBAT1. (F) SK-N-BE(2) cells were transiently transfected with control siRNA or USP36 siRNA for 48 h followed by RNA extraction and RT-qPCR. Bar graph represents the expression of COL18A1, EGFL7, ITGB5, OAF, USP36, and MYCN. (G) SK-N-BE(2) cells transiently transfected with Control siRNA or MYCN siRNA for 48 h and then expression of COL18A1, EGFL7, OAF, ITGB5, and MYCN genes was analyzed by RT-qPCR. (H) Bar graph represents the relative expression of COL18A1, EGFL7, OAF, ITGB5, and MYCN mRNA in SK-N-BE(2) cells treated with vehicle control DMSO or JQ1 for 14 h. (B–H) The significances are indicated by *P < .05, **P < .01, and ***P < .001.
Figure 5. COL18A1 contributes to extracellular matrix organization in neuroblastoma. (A) qPCR analysis of COL18A1 showing the rescue of COL18A1 expression in SH-SY5Y cells. COL18A1 following USP36 KD/MYCN overexpression compared to the cells transfected with USP36 siRNA or CSI. (B) Schematic showing the exonic structure of the COL18A1 gene with purple and green pointers indicating the MYCN binding sites on the COL18A1 promoters. ChIP-qPCR shows the occupancy of MYCN at the promoters of COL18A1 isoforms in stable CSh or NBAT1Sh and CASC15-003Sh SH-SY5Y cells. (C) Wound healing assay depicting the cell migration potential in COL18A1 KD SK-N-BE(2) cells. Cells were transiently transfected with CSI or COL18A1 siRNA 1 and 2 for 36 h followed by making a scratch on the monolayer. The initial (0 h) and final (24 h) wound lengths were measured, and relative migration was plotted as a bar graph on the right. Scale bar represents 500 µM. (D) Proliferation assay depicts the relative proliferation status of SK-N-BE(2) transfected with CSI or COL18A1 siRNA 1 and 2. MTT assay was performed 48 h post-transfection to determine the relative proliferation, left panel. Bar graph represents the relative expression of COL18A1 in CSI.
Discussion

Nearly half of the high-risk NB patients harbor MYCN amplification and display poor clinical outcome with metastatic dissemination and increased risk of relapse. Since MYCN is a critical oncogenic driver in NB pathogenesis, there is an increasing appreciation for understanding MYCN driven downstream oncogenic pathways, and also upstream pathways that control MYCN expression at the transcriptional, post-transcriptional and post-translational level. Previous studies have demonstrated the role of protein coding RNAs in the regulation of MYCN-dependent oncogenic pathways. However, not much has been learnt on the role of IncRNAs in MYCN dependent oncogenic pathways. Nevertheless, a few recent investigations have shown that IncRNAs such as MYCNOS and LncUSMycN, both of which are located in the vicinity of the MYCN gene, regulate MYCN at the transcriptional and post-transcriptional level. For example, MYCNOS IncRNA regulates MYCN expression either by altering the usage of MYCN promoters or by activating MYCN transcription by directly recruiting CTCF to the MYCN promoter. MYCNOS has also been shown to act as an mRNA, encoding the NCYM protein which is implicated in MYCN stability. On the other hand, LncUSMycN interacts with the spliceosome complex protein NONO and stabilizes MYCN transcript. Although these studies reveal functional connections between IncRNAs and MYCN transcriptional and post-transcriptional regulation, the molecular mechanisms by which IncRNAs control the MYCN dependent oncogenic pathways are lacking.

In this investigation, we demonstrate that the NB hotspot locus derived IncRNAs NBAT1 and CASC15-003 regulate MYCN expression post-translationally through the regulation of the deubiquitinating enzyme USP36. Both NBAT1 and CASC15-003 lack any sequence homology, yet they jointly regulate MYCN expression post-translationally via the ubiquitin-proteasome system. Post-translational regulation of MYCN by NBAT1/CASC15-003/USP36 represents a new regulatory layer in the complex multilayered gene regulatory network that controls MYCN expression. Another interesting aspect of the current investigation is the demonstration of USP36 as an oncogene. Its higher expression in NB patients correlates with poor prognosis and its downregulation significantly reduces tumor growth in cultured cancer cells and xenograft models. These observations coupled with the published literature on NBAT1, CASC15-003 and MYCN indicate that, in the NBAT1/CASC15-003/USP36/MYCN regulatory axis, NBAT1/CASC15-003 serve as tumor suppressors, whereas MYCN and USP36 behave as oncogenes, and that the functional interplay between NBAT1/CASC15-003 and USP36/MYCN may contribute to NB pathogenesis.

This investigation also characterizes novel MYCN-dependent pathways in the NB context. These novel MYCN-dependent pathways were obtained following an unbiased integration of RNA-seq datasets from CASC15-003, NBAT1, USP36, and MYCN knockdowns and NB cells treated with the MYCN inhibitor JQ1. The integrated RNA-seq analysis revealed four genes, COL18A1, EGFL7, ITGB5, and OAF. Among the 4 genes, COL18A1 is an interesting candidate considering its role in the organization of the extracellular matrix, and closure of the neural tube and brain development which are closely connected to NB development and progression.31,32 In vitro cell culture assays revealed that COL18A1 knockdown interferes with cell migration, cell proliferation and cell adhesion. Its ability to rescue the increased cell adhesion property of the NBAT1/CASC15-003 KD cells suggests that COL18A1 may play an important role in MYCN dependent tumor phenotype. COL18A1, which encodes human collagen XVIII protein, is a widely expressed proteoglycan in the basement membrane. It generates three isoforms with varied N terminal regions and a conserved C-terminal region that encodes an angiogenic inhibitor, endostatin.33 Human collagen XVIII protein shows higher expression in many solid tumors.34-37 Its higher expression in stromal cells is associated with metastatic activity.38,39 Lower expression of COL18A1 was also observed in several cancers38,40–42 and this in particular is consistent with endostatin antitumorigenic and antiangiogenic properties.43,44 These observations indicate that COL18A1 possesses both oncogenic and tumor suppressor properties in a context dependent manner. COL18A1 expression dependence on the NBAT1/CASC15-003/USP36/MYCN regulatory axis indicates that it may possess oncogenic properties. NBAT1/CASC15-003 play a crucial role in the regulatory axis through modulating the activity of deubiquitinase USP36, leading to the enhanced stability of MYCN. Interestingly, both MYCN and USP36 are colocaled in the nucleolus and show endogenous interaction. The stabilization of MYCN in turn activates COL18A1, thereby increasing cell migration and cell adhesion properties. These observations indicate that the NBAT1/CASC15-003/USP36/MYCN/ COL18A1 regulatory axis may have crucial role in metastatic activity in high-risk NB patients. However, the higher expression of COL18A1 in certain tumors correlates with increased overall and event-free survival, including for NB.45 If one considers the tumor suppressor properties of COL18A1 in certain tumors together with antiangiogenic and antitumorigenic properties of its cleaved product endostatin, it might give us the impression that higher COL18A1 expression in tumors may help in tumor regression or sensitize tumors to chemotheraphy. The latter contention is consistent with the fact that MYCN expressing tumors are more sensitive to chemotheraphy than nonexpressing tumors.46,47 Furthermore, COL18A1 shows higher expression in stage 4S NB tumors compared to the other INSS stages.
S1F), and 4S stage tumors with higher metastatic activity are prone to tumor regression. Thus, we speculate that the NBAT1/CASC15-003/USP36/MYCN/COL18A1 regulatory axis may help in the tumor regression of high-risk 4S NB tumors. Alternatively, this regulatory axis may take part in higher metastatic activity. Further work is warranted to distinguish the oncogenic and/or tumor suppressor functions of COL18A1 in the context of the NBAT1/CASC15-003/USP36/MYCN regulatory axis.

Supplementary Material
Supplementary material is available at Neuro-Oncology Advances online.

Keywords
long noncoding RNA | neuroblastoma | USP36 | MYCN | COL18A1 | CASC15 | NBAT1

Acknowledgments
The computations for RNA-sequencing datasets were performed on resources provided by the Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) high-performance computing (HPC) which is part of the Swedish National Infrastructure for Computing (SNIC). We thank Jennifer Uhler, University of Gothenburg, for help with the manuscript editing.

Funding
This work was supported by grants from Knut and Alice Wallenberg Foundation (KAW2014.0057); Swedish Cancer Research Foundation [ Cancerfonden: Kontrakt no. CAN2018/591 ]; Swedish Research Council [PR2018-0090]; Ingabritt och Arne Lundbergs forskningsstiftelse (LU2020-0017) and LU/ALF (to C.K.) and Assar Gabrielssons Fond FB-18-10 (to P.K.J.).

Conflicts of interest statement. Authors do not have any conflicts of interest to declare.

Authorship Statement. C.K. and P.K.J. conceptualized the study, wrote the manuscript, P.K.J. performed co-immunoprecipitations, functional experiments and analyzed data, T.M. performed immunostaining, and performed functional experiments, M.D.M. performed functional experiments on COL18A1, S.T.K. performed bioinformatic analysis, and M.K. performed ChIP-qPCR analysis.

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