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Transcriptional regulation of genes by MYCN in PAX3::FOXO1-positive rhabdomyosarcomas and their roles in cell cycle progression

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ABSTRACT

Background: MYCN amplification and high expression is associated with pediatric malignancies including neuroblastoma and alveolar rhabdomyosarcoma. *MYCN* transcription in alveolar rhabdomyosarcomas is driven by a feedback loop with the PAX3::FOXO1 fusion protein. However, the role of MYCN is not well-defined.
Methods: Chromatin ImmunoPrecipitation (ChIP)-sequencing of alveolar rhabdomyosarcoma cell lines was used to identify genome-wide MYCN binding sites. Ontology analyses of genes adjacent to MYCN binding sites corresponding to expression changes after siRNA reduction was performed and confirmed by ChIP-qPCR. Cells from each phase of the cell cycle were isolated by Fluorescence Activated Cell Sorting for assessing protein expression by Western blotting.
Results: Genes encoding transcription factors adjacent to MYCN binding sites and genes with binding sites proximal to their promoters were strongly associated with RNA synthesis and cell cycle pathways, respectively. MYCN binding sites in regions that positively correlated with gene expression changes were linked to cell cycle regulation, consistent with phenotypic absence of cell cycle checkpoint control. Cell cycle regulated genes *CDK4* and *KDM4B* were validated as MYCN-regulated and, in-keeping with unchecked cell cycle progression, expressed throughout the cell cycle, coincident with MYCN.
Conclusions: MYCN binding sites associated with gene expression changes defined the contribution of MYCN to the transcriptional control of key pathways/molecular processes in the development and progression of rhabdomyosarcomas. MYCN aberrantly regulated *CDK4* and *KDM4B* expression throughout the cell cycle in *PAX3::FOXO1* positive rhabdomyosarcoma. The regulatory network defined supports MYCN, CDK4 and KDM4B as therapeutic targets in the treatment of rhabdomyosarcoma patients.

1. Introduction

Rhabdomyosarcoma (RMS) is the most frequent pediatric soft tissue sarcoma and resembles developing skeletal muscle. There are two main histological subtypes, embryonal (ERMS) and alveolar (ARMS). Most ARMS are associated with a characteristic fusion gene, *PAX3::FOXO1* or, less frequently, *PAX7::FOXO1*. Fusion-positive ARMS are generally more aggressive and have a poorer prognosis [1–3]. The fusion genes encode potent transcriptional activators that play a key role in the pathogenesis of these tumors [4–7]. Genes directly bound and regulated by PAX3::FOXO1 have been previously indicated and include *MYCN*, a member of the *MYC* gene family [5,7]. MYC and MYCN regulate many cellular processes including cell proliferation, differentiation, and cell cycle progression, and have been implicated in the progression of many tumor types (reviewed in [8,9]).

MYCN amplification is observed in around 20 % of fusion-positive ARMS through genomic amplification at 2p24 [10]. MYCN has also been shown to auto-regulate as well as regulate PAX3::FOXO1

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expression [11,12]. We have previously shown the dependence of RMS on MYCN through its requirement for tumor cell growth and efficient silencing using peptide nucleic acids (PNAs) resulted in cell death in vitro and reduced rate of tumor growth in vivo [12,13]. Modulation of either MYCN or PAX3::FOXO1 proteins leads to overlapping changes in gene expression including genes involved in myogenesis and cell cycle progression [1,5,7,12].

Here we identified direct binding targets of MYCN associated with transcriptional changes in PAX3::FOXO1 cells and demonstrated that ARMS cell lines were resistant to cell cycle arrest with no cell cycle checkpoint control, as previously indicated [14]. Given our previous demonstration of dependency on MYCN [12], aberrant expression of MYCN and specific downstream proteins were expected to contribute to the RMS phenotype as well as support potential therapeutic opportunities.

2. Materials and methods

2.1. Cell culture

ARMS cell lines RMS-01, RH4, RH41 and RH30 cells were cultured as previously described [15] and detailed in the Supplementary materials and methods. RMS-01 and RH30 have genomic amplification of *MYCN* and *CDK4,* respectively [10,16–19].

2.2. Flow cytometry

Fluorescence Activated Cell Sorting (FACS) isolated cells from each phase of the cell cycle representing G_0/G_1, S and G_2/M (Supplementary materials and methods).

2.3. Western blotting

Total cell lysates were extracted from siRNA experiments in RMS cell lines using Cell Lysis Buffer at 72hrs post-transfection (Cell Signaling Technology) (Supplementary materials and methods).

2.4. Chromatin ImmunoPrecipitation (ChIP), ChIP-sequencing and correlation with gene expression data

ChIP was performed using the MAGnify ChIP kit (Invitrogen) according to manufacturer's instructions. ChIP DNA was sent to Source Bioscience for library preparation and sequencing on an Illumina GAII sequencer (Supplementary materials and methods).

2.5. Bioinformatic analyses

Alignment of reads from ChIP-sequencing was performed using BWA v0.5.8a (http://bio-bwa.sourceforge.net/), samtools v0.1.8 and PICARD v1.5.2 [20]. MACS v1.4.2 was used to identify enrichment peaks between the ChIP and control datasets using default parameters [21]. Enrichment peaks unique to the HA antibody were defined using an FDR threshold of 5 %. Nearest genes were identified using PeakAnnotator [22] (Supplementary materials and methods).

Gene ontology analysis of ChIP-sequencing (ChIP-seq) targets and MYCN-bound DNA motifs was conducted using MetaCore (GeneGo, Thompson Reuters). Statistical-significance threshold levels for all enrichment analyses was set at $p < 0.05$. KEGG pathway analyses used DAVID [23,24].

Analysis of genes correlating with MYCN expression in patient samples used two datasets within R2 (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi); Intergroup Rhabdomyosarcoma Study Group/Pediatric Cooperative Human Tissue Network (IRSG/PCHTN) and Innovative-Therapies for Children with Cancer/Carte d'Identité des Tumeurs (ITCC/CIT). Correlations with *MYCN* where $R > +/-0.339$ and $p < 0.01$.

2.6. Luciferase assays

pGL3 Promoter, pGL3 Control and pRL-CMV (Promega) were used for luciferase assays. DNA containing the putative MYCN binding site within the CDK4 promoter was amplified from RH30 genomic DNA and cloned into pGL3 Promoter. Cells were transfected using Lipofectamine 2000 according to manufacturer's protocol. Dual luciferase assays were performed at 72 h using the Dual-Glo luciferase reporter assay system (Promega) with 3 separate experiments performed for each assay.

3. Results

3.1. Genome-wide ChIP-sequencing identifies binding sites of MYCN in RMS

To identify regions of the genome bound by MYCN we performed ChIP-seq analyses on RH30 cells transfected with a HA-tagged MYCN construct. Using a stringent FDR (< 0.05), we identified peaks at 3278 genes throughout the genome. We analyzed MYCN-bound peaks in the RMS genome, denoted proximal (+/- 5 kb from the transcription start site (TSS) of a gene), distal (+/- 5 kb to +/- 50 kb of TSS) and intergenic (> 50 kb of any TSS). Of the 3278 gene-associated peaks identified, only 20 % were located within the proximal promoter (Fig. 1A). The majority of peaks were located between 5 and 50 kb from any gene transcription start site (TSS), suggesting that MYCN functions primarily via enhancer regions in the RH30 cell line.

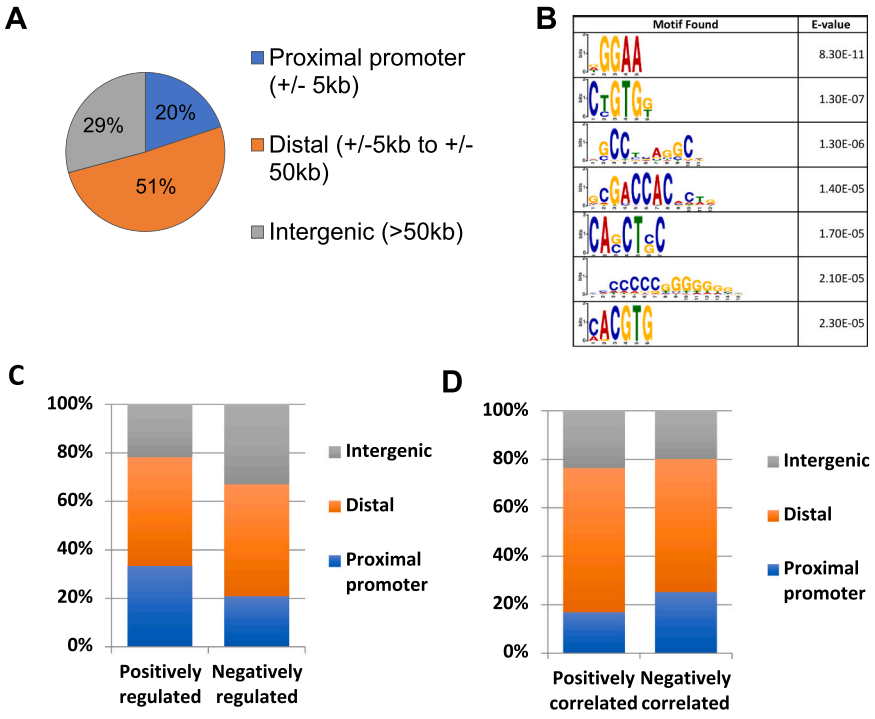
Motif analysis of our MYCN ChIP-seq data using an FDR cut-off of 5 % revealed transcription factor binding motifs associated with MYCN binding peaks, including the common E-box motif CACGTG and a non-canonical E-box CAGCTG (p-value < 0.001, Fig. 1B, Supplementary Table S4). Additionally, motifs for MYOD and MYOG were also found to be associated with MYCN peaks, consistent with the myogenic lineage seen in RMS.

As MYCN and PAX3::FOXO1 regulate the transcription of each other, we sought to determine whether genes might be co-regulated by MYCN and PAX3::FOXO1 in RMS. We compared publicly available ChIP-sequencing data of targets of PAX3::FOXO1 with our MYCN ChIP-sequencing data [5]. We found MYCN binding sites to be predominantly located away from TSS (Fig. 1A), correlating with previously published data for PAX3::FOXO1 that showed binding at distal regions (> 4 kb from TSS). However, of the 3278 regions bound by MYCN, only 7 % of the called genes overlapped with the PAX3::FOXO1 data (1166 sites), suggesting that although MYCN and PAX3::FOXO1 regulate one another, they predominantly regulate different genes in RMS cells.

3.2. Ontology analysis of genes associated with MYCN binding and gene expression changes shows a role for MYCN in cell cycle control in RMS

To identify MYCN binding sites highly likely to correspond to direct transcriptional targets of MYCN, we compared our previously published expression profiling data for MYCN-silenced RH30 cells (PNA (12 h post transfection) and siRNA (24 h post transfection), [12]) with our ChIP-sequencing data. We identified 121 genes that are transcriptionally regulated and are annotated with a binding site for MYCN. Of these genes, 34 have a proximal promoter binding site for MYCN and 58 have a distal binding site. 64 of the 121 genes are positively regulated by MYCN and 57 appear repressed by MYCN, suggesting that MYCN plays a role as both a transcriptional activator and repressor in these cells (Fig. 1C, Supplementary Table S5 and S6).

To identify genes expected to be regulated by MYCN in patient samples, we interrogated our previously derived gene expression profiling data of PAX3::FOXO1-positive RMS patient samples [25]. We compared our MYCN binding sites with genes correlating with *MYCN* expression levels in RMS patient samples (Supplementary Tables S7 and S8). Of the 3521 genes that correlated with *MYCN*, 405 (11.5 %) overlapped with the MYCN ChIP-sequencing data. This was consistent with



E

Go Networks	P-Value
Development_Neurogenesis_Synaptogenesis	2.279E-03
Cell cycle_Meiosis	2.481E-03
Development_Neurogenesis_Axonal guidance	2.906E-03
Transport_Calcium transport	3.397E-03
Cell adhesion_Amyloid proteins	3.735E-03
Cell cycle_G2-M	5.199E-03
DNA damage_Checkpoint	5.390E-03
Signal transduction_Androgen receptor nuclear signaling	5.823E-03
Signal transduction_ESR1-nuclear pathway	6.880E-03
Inflammation_IL-6 signaling	1.913E-02

F

GO Networks	P-Value
Cell cycle_G2-M	1.839E-04
Cytoskeleton_Regulation of cytoskeleton rearrangement	3.573E-04
Cytoskeleton_Cytoplasmic microtubules	4.704E-04
DNA damage_Checkpoint	7.400E-04
Cell cycle_Meiosis	9.224E-03
Development_Hedgehog signaling	1.183E-02
Apoptosis_Apoptotic nucleus	1.267E-02
Inflammation_IL-6 signaling	1.471E-02
Cytoskeleton_Intermediate filaments	1.675E-02
Proliferation_Positive regulation cell proliferation	1.773E-02

Fig. 1. Identification of MYCN binding sites in RMS by ChIP-sequencing. (A) The percentage of peaks in RH30 located within the proximal promoter region (± 5 kb), distal (± 5 kb to ± 50 kb) and intergenic (> 50 kb) to the transcription start site (TSS). (B) Top binding motifs in RH30 identified from analysis of all MYCN peaks with FDR < 5 %, p < 0.0001. (C) Distribution of MYCN peaks related to differentially expressed genes after siRNA or PNA silencing of MYCN. (D) Distribution of MYCN peaks related to genes that are either positively or negatively correlated with MYCN RNA expression levels in RMS patient samples. (E) Ontology analysis of genes located near a MYCN-bound peak and that are positively correlated with MYCN RNA expression levels from our dataset [32]. (F) Ontology analysis of genes located near a MYCN-bound peak and that are positively correlated with MYCN from the IRSG/PCHTN dataset [7].

comparison with another publicly available dataset (IRSG/PCHTN) [26], overlapping genes = 250/2160 (11.6 %). Within the distal region, 233 genes with a binding site as well as 82 genes with a binding site within the proximal promoter region also correlated with *MYCN* expression levels (Fig. 1D). Of these, 185 genes were positively correlated with *MYCN* expression levels and 130 were negatively correlated. Ontology analysis of targets that positively correlated with *MYCN* expression using our dataset [25] showed predominant involvement of MYCN in cell cycle related processes and networks (Fig. 1E), which was also confirmed in the IRSG/PCHTN dataset [26], Fig. 1F).

3.3. *MYCN binds to and regulates CDK4 and KDM4B in RMS*

Of the genes that correlated with *MYCN* expression levels and contained a binding site for MYCN in our dataset, 26 are predicted to play a role in cell cycle regulation (Supplementary Table S10). One of the largest peaks in the MYCN ChIP-sequencing data was in the promoter of *CDK4* (Fold Enrichment = 184.62, FDR = 0 %, p-value < 0.0001, Fig. 2A), a regulator of G1/S phase progression of the cell cycle [27]. To determine whether *MYCN* expression correlates with *CDK4* in RMS we analyzed RMS patient sample expression profiling data [25] using R2 (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) and found a positive correlation between *MYCN* and *CDK4* (Fig. 2B, p-value < 0.05).

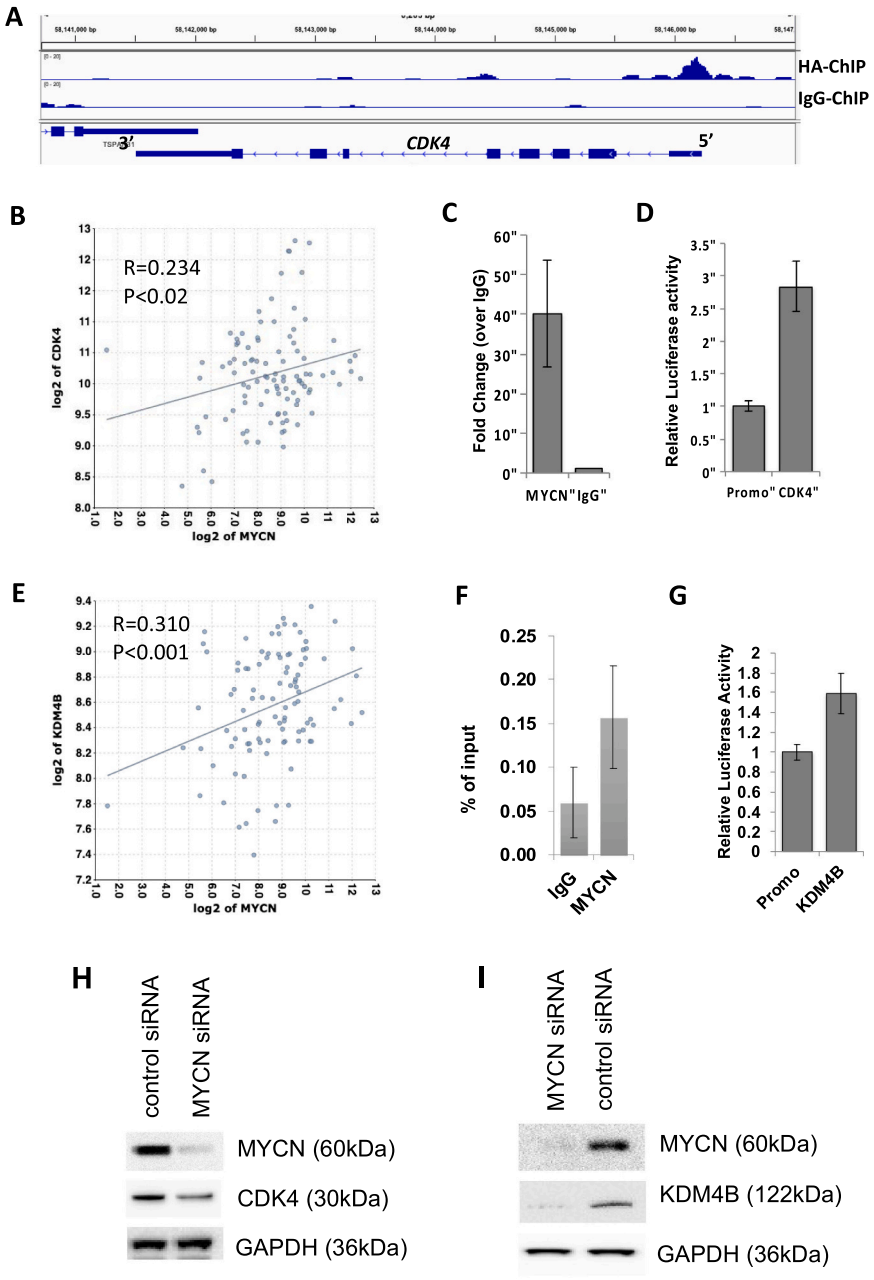


Fig. 2. CDK4 and KDM4B are downstream targets of MYCN. (A) Integrative Genomics Viewer (IGV) screenshot showing MYCN ChIP-sequencing read depth from MACS at the *CDK4* proximal promoter. (B) Correlation between mRNA expression of *CDK4* with *MYCN* in RMS patient samples using the ITCC/CIT dataset [29]. (C) ChIP-qPCR in RH30 HA-MYCN expressing cells showing binding of MYCN at the CDK4 promoter relative to the IgG control. (D) Luciferase assay performed in MYCN amplified RMS cells showing an increase in luciferase activity at the CDK4 promoter relative to the control vector. (E) Correlation between mRNA expression of *KDM4B* and *MYCN* in RMS patient samples using the ITCC/CIT dataset [29]. (F) ChIP-qPCR in RH30 HAMYCN cells showing binding of MYCN at the *KDM4B* promoter relative to the IgG control. (G) Luciferase assay performed in MYCN amplified RMS cells showing an increase in luciferase activity at the *KDM4B* promoter relative to the control vector. Western blot showing siRNA silencing of MYCN at 48hrs post transfection in RH30 cells resulting in a reduction in expression of CDK4 (H) and KDM4B (I) at the protein level.

Although copy number data was not available for analyses of these patient cases, the higher levels of expression of *MYCN* and *CDK4* may be associated with genomic amplification of these regions that has been previously described [10,18]. To confirm that MYCN is binding to the *CDK4* promoter in RMS cell lines we performed a MYCN pull down followed by ChIP-qPCR in RH30 cells and confirmed binding of MYCN to the *CDK4* promoter (Fig. 2C). Luciferase reporter assays surrounding the MYCN binding site within the *CDK4* promoter showed a 3-fold increase in luciferase activity compared to the control vector (Fig. 2D) consistent with MYCN binding to and regulating CDK4.

The cell cycle regulated histone demethylase KDM4B and MYCN have been reported to interact and regulate one another [28,29]. We have recently shown that KDM4B is aberrantly expressed in RMS samples [15,30] and *KDM4B* is reported to be downregulated after MYCN silencing in RMS cells [12]. Analysis of expression profiling data for RMS patient samples [26] identified a positive correlation between *MYCN* and *KDM4B* in RMS patient samples (Fig. 2E, $r = 0.31$, $p < 0.001$). Consistent with this, analysis of our ChIP-sequencing data suggested that there is a binding site for MYCN in the promoter of *KDM4B*. To confirm MYCN binding at this region we performed ChIP-PCR around a region identified as containing an E-box domain and found that MYCN can indeed bind to this region within *KDM4B* (Fig. 2F). Additionally,

luciferase reporter assays surrounding the MYCN binding site within the *KDM4B* promoter showed a fold increase in luciferase activity compared to the control vector (Fig. 2G). To confirm that *CDK4* and *KDM4B* are downstream targets of MYCN we performed siRNA-mediated silencing of MYCN in RH30 cells and demonstrated reduction of CDK4 (Fig. 2G) and KDM4B protein expression (Fig. 2H) upon *MYCN* silencing.

3.4. *MYCN is aberrantly expressed during the cell cycle in ARMS cell lines*

MYCN has previously been shown to be upregulated during G1 with rapid downregulation of the MYCN protein during S and G2 phases of the cell cycle in neuroblastoma [31]. As RMS cells failed to arrest with serum starvation or respond as expected to agents such as thymidine and mimosine that typically synchronize cells in the cell cycle (Supplementary Fig. 1), we assessed MYCN expression in ARMS cells after isolating each phase of the cell cycle using fluorescence activated cell sorting (FACS) (Supplementary Fig. 2). MYCN was expressed in each phase of the cell cycle in ARMS cells (Fig. 3A) indicative of aberrant expression or protein stability. Analysis of CDK4 and MYCN in a non-MYCN or CDK4 amplified ARMS line (RH4), revealed that these were concurrently expressed throughout the cell cycle (Fig. 3B). Similarly, KDM4B was consistently present throughout the cell cycle in RH30 in line with MYCN protein levels (Fig. 3C).

4. Discussion

MYCN is genomically amplified and a key oncogene in a number of pediatric malignancies including neuroblastomas and RMS although little is understood about its role in PAX::FOXO1 positive RMS. Therefore, we aimed to determine genes, and associated processes, that are transcriptionally regulated by MYCN in RMS cells. Analysis of DNA regions bound by MYCN revealed an association with non-canonical E-box motifs in addition to the classic canonical E-box motif which has also seen in neuroblastoma [32]. In addition, we identified a number of other MYCN binding motifs (Supplementary Tables) consistent with the finding that MYC binding is not restricted to E-box sequences (reviewed in [33]). ChIP-seq in combination with gene expression profiling data revealed that MYCN regulates genes involved in other processes including neurogenesis, a feature consistent with the expression of neurological markers in PAX3::FOXO1 positive RMS that may represent a therapy resistant cell population [34,35].

We show that MYCN is a potential transcriptional driver of several cell cycle-related and other genes worthy of further investigation in RMS (Supplementary Tables). These included CDK4 and KDM4B, that links MYCN amplification and/or over-expression to cell cycle progression. Previously we have shown a reciprocal regulatory relationship between PAX3::FOXO1 and MYCN in RMS where *MYCN* contributes to PAX3::FOXO1 expression and vice versa [12]. Also, c-Myc and N-Myc have been shown to regulate the expression of the developmental control gene Pax3 in a cell cycle dependent manner in mouse models [31]. Here we found that MYCN is expressed throughout the cell cycle in ARMS cell lines contrary to the variable pattern of MYC gene expression in normal neuronal [31] and other normal cell types [36].

MYC proteins regulate the expression of cell cycle checkpoint proteins and are involved cell cycle progression (reviewed in [36]). Cell cycle proteins, KDM4B and CDK4 were identified as direct transcriptional targets of MYCN, and their expression upregulated in MYCN-overexpressing cells. CDK4 can be genomically amplified and has been correlated with adverse outcome in fusion-positive RMS [18]. In other cancers, MYCN has been shown to either directly bind to KDM4B [28] or where KDM4B regulates the expression of *c-MYC* [37,38], our data demonstrate that KDM4B is downstream of MYCN in the context of fusion-positive RMS. PAX3::FOXO1 functions as a transcriptional activator in RMS and has been shown to create autoregulatory loops at super enhancers in conjunction with MYCN [5,39]. ChIP-seq data showed that there were no peaks for PAX3::FOXO1 in either the *CDK4* or *KDM4B* promoters and therefore direct regulation of these genes by MYCN is a significant driver [5]. PAX3::FOXO1 is regulated via phosphorylation on serine 430 by CDK4, which in turn leads to enhanced transcriptional activity of the fusion protein. As MYCN drives expression of both *CDK4* and *PAX3::FOXO1* in RMS cells, this is consistent with MYCN directly

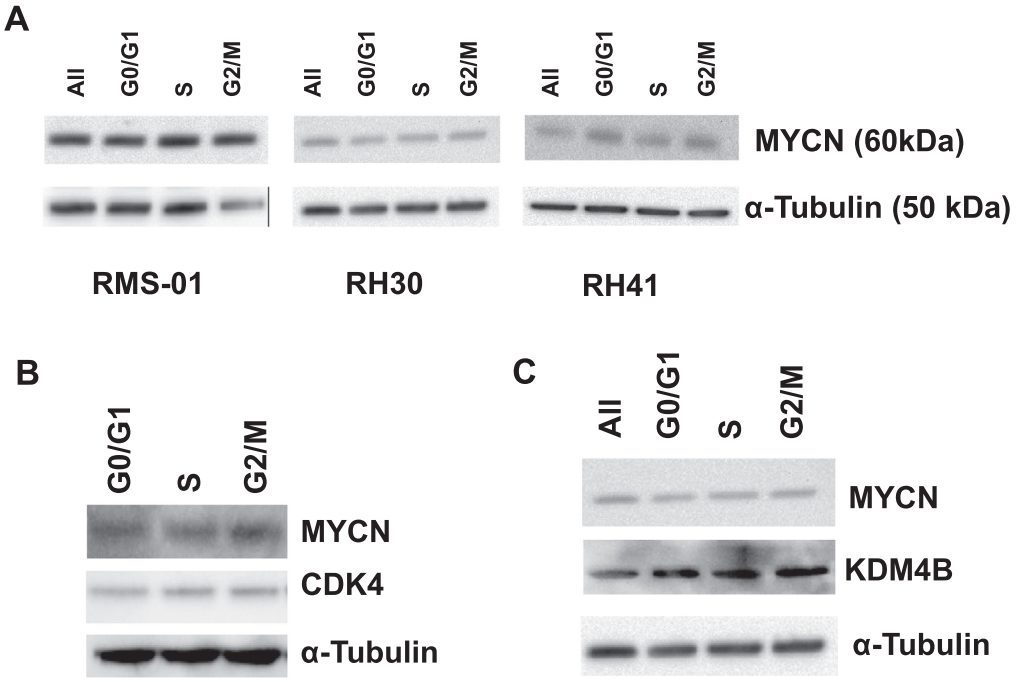


Fig. 3. MYCN is aberrantly expressed throughout the cell cycle in PAX3-FOXO1-positive cell lines. (A) MYCN protein expression analysis by Western blot in 3 ARMS cell lines (RMS-01, RH30, RH41). (B) Expression of MYCN and MYCN-regulated cell cycle protein, CDK4 throughout the cell cycle in the PAX3-FOXO1 positive ARMS cell line (RH4). (C) Expression of MYCN and KDM4B throughout the cell cycle in a PAX3-FOXO1 positive ARMS cell line (RH30).

and indirectly driving the transcriptional output of the fusion protein in these cells thus contributing to the enhanced transcriptional program and aggressive proliferative state of PAX3::FOXO1 RMS.

Here we have shown that MYCN acts as a transcription factor in fusion-positive ARMS to control the expression of selected cell cycle genes throughout the cell cycle thereby maintaining the proliferative state in RMS. Our analyses reveal a complex role for MYCN in regulating genes involved in cell cycle progression, as well as developmental processes including neurogenesis and myogenesis. As such, in addition to recognition of MYCN as a therapeutic target in neuroblastoma, it also represents an attractive target for high-risk fusion-positive RMS [12]. MYCN, together with MYC for other cancer types, are showing increasing promise of being therapeutically targetable [40]. Also, direct downstream targets of MYCN in RMS such as CDK4 and KDM4B demonstrated here highlights potential novel avenues for targeting the downstream activity of MYCN in RMS. Data already support therapeutic potential of inhibiting CDK4 or KDM4B activity in RMS [15,18,30] although selective inhibitors have yet to be developed for KDM4B to enable clinical translation. Future studies may focus on these and other downstream targets of MYCN in new combination strategies, or targeting MYCN itself using, for example, degrader-based strategies, for the treatment of MYCN-positive RMS.

CRediT authorship contribution statement

Shipley Janet: Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Walters Zoë S.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Leongamornlert Daniel:** Writing – review & editing, Methodology, Data curation. **Villarejo-Balcells Barbara:** Writing – review & editing, Validation, Methodology. **Tse Carmen:** Writing – review & editing. **Pengelly Reuben:** Writing – review & editing, Methodology, Data curation. **Ian Titley:** Writing – review & editing, Methodology.

Study ethical approval

No study/ethical approvals or consent was required for this study.

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Conflicts of Interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcped.2025.100230.

Data Availability Statement

Raw data for the ITCC/CIT collection analysed in this study are available in the ArrayExpress database (accession ID E-TABM-1202).

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