EUROPEAN JOURNAL OF CANCER 43 (2007) 2413–2422

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Direct regulation of the minichromosome maintenance complex by MYCN in neuroblastoma

Arjen Koppen[a], *Rachida Ait-Aissa*[a], *Jan Koster*[a], *Peter G. van Sluis*[a], *Ingrid Øra*[a,c], *Huib N. Caron*[a,b], *Richard Volckmann*[a], *Rogier Versteeg*[a], *Linda J. Valentijn*[a,*]

[a]*Department of Human Genetics, Academic Medical Center, University of Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands*
[b]*Department of Paediatric Oncology and Haematology, Emma Kinder Ziekenhuis, Academic Medical Center, Amsterdam, The Netherlands*
[c]*Department of Paediatric Oncology and Haematology, Lund University Hospital, 22185 Lund, Sweden*

ARTICLE INFO

Article history:
Received 21 May 2007
Accepted 18 July 2007
Available online 10 September 2007

Keywords:
DNA replication
MCM
Neuroblastoma
MYCN

ABSTRACT

The c-Myc and MYCN oncogenes strongly induce cell proliferation. Although a limited series of cell cycle genes were found to be induced by the myc transcription factors, it is still unclear how they mediate the proliferative phenotype. We therefore analysed a neuroblastoma cell line with inducible *MYCN* expression. We found that all members of the minichromosome maintenance complex (MCM2–7) and MCM8 and MCM10 were up-regulated by MYCN. Expression profiling of 110 neuroblastoma tumours revealed that these genes strongly correlated with *MYCN* expression *in vivo*. Extensive chromatin immunoprecipitation experiments were performed to investigate whether the MCM genes were primary MYCN targets. MYCN was bound to the proximal promoters of the MCM2 to -8 genes. These data suggest that MYCN stimulates the expression of not only MCM7, which is a well defined MYCN target gene, but also of the complete minichromosome maintenance complex.

1. Introduction

Members of the Myc oncogene family are activated in many tumour types and can induce strong changes in the cellular phenotype. They stimulate cell growth, proliferation and invasiveness upon ectopic expression. The Myc oncogene family members, MYC, MYCN and MYCL, are basic-helix–loop–helix-leucine zipper domain containing transcription factors, which activate, together with their dimerization partner Max, gene expression of a number of genes in an E-box (CAC(A/G)TG) dependent manner.[1,2] MYCN and c-myc probably have very similar molecular functions. Mice in which the coding sequence of *MYC* was replaced by that of *MYCN* developed normally. This indicates that MYCN and c-myc can functionally replace each other and control the same cellular processes.[3]

High throughput mRNA profiling studies have identified many genes which are induced upon myc activation, but direct regulation by the myc transcription factors was established for only a limited number of genes. These myc-induced changes in gene expression start to explain the phenotypic changes known to result from myc activation. The Drosophila *MYC* gene promotes cell growth and indeed mammalian c-myc stimulates expression of several genes involved in protein synthesis.[4,5] We observed that MYCN and c-myc stimulate expression of proteins involved in ribosomal RNA processing and ribosome biogenesis.[6] The role of myc oncogenes in cell adhesion and tumour invasion[7–9] is in line with our recent observation that MYCN down-regulates the expression of many genes involved in cell–matrix interactions and in cytoskeleton architecture.[10] Most research has focused on the effect of myc oncogenes on cell proliferation. Indeed a

* *Corresponding author:* Tel.: +31 205666592; fax: +31 206918626.
E-mail address: l.j.valentijn@amc.uva.nl (L.J. Valentijn).
0959-8049/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2007.07.024

number of c-myc target genes are regulators of the cell cycle. c-Myc directly induces the expression of *cyclin-D2* and *CDK4*, eventually leading to increased cyclin-E-CDK2 activity and G1- to S-phase transition.[11,12] Also other cyclins are regulated by c-myc, including *Cyclin-A2*, *-B1*, *-D3* and *-E*.[13–17] In addition, a number of cell cycle genes are transcriptionally suppressed by myc. The cell-cycle inhibitory genes $p15^{INK4B}$ and $p21^{CIP1}$ are repressed by c-myc through interaction with the transcription factor Miz-1 at the Inr sequence in the core promoter.[18,19]

The *MYCN* gene is amplified in 20% of neuroblastoma tumours.[20] Neuroblastoma is a malignant childhood tumour of the sympathetic nervous system with a highly variable prognosis. Amplification of *MYCN* confers a poor prognosis[21] and ectopic expression of *MYCN* in neuroblastoma cell lines was found to stimulate cell cycle progression.[22,23] In this study, we generated gene expression profiles of a neuroblastoma cell line in which ectopic *MYCN* expression can be regulated. Strikingly, all minichromosome maintenance (MCM) genes were induced by MYCN. Chromatin immunoprecipitation (ChIP) experiments identified MCM2 to -8 as direct targets of MYCN. Moreover, microarray analyses of neuroblastoma tumours showed a strong correlation between expression levels of *MYCN* and all MCM genes. These results suggest that MYCN might stimulate the initiation of DNA replication and the accompanying G_1- to S-phase transition by induction of the whole minichromosome maintenance complex.

2. Materials and methods

2.1. Cell lines

The SHEP-21N cell line was grown in RPMI 1640 medium (GIBCO), supplemented with 10% foetal calf serum, 4 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.[22] MYCN expression was switched off by the addition of 50 ng/ml tetracycline. For serum starvation, SHEP-21N cells were incubated for 36 h with the above indicated medium, but without foetal calf serum. Cells were serum stimulated by the addition of fetal calf serum to a final concentration of 10%.

2.2. Oligonucleotide microarray hybridization and analysis

Total RNA of cell lines was isolated by the LiCl-ureum method.[24] Total RNA of neuroblastoma tumours was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol.

RNA concentration and quality were determined using the ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). RNA purification was performed using the RNeasy mini kit (Qiagen, Germantown, USA). Fragmentation of cRNA, hybridization to HG-U133 Plus 2.0 microarrays and scanning were carried out according to the manufacturer's protocol (Affymetrix Inc. Santa Barbara, USA) at the Microarray Department of the Swammerdam Institute of Life Science of the University of Amsterdam. Intensity values and the corresponding detection *p*-values were assigned to each probe set using the MAS5.0 algorithm of GCOS software (Affymetrix Inc. Santa Barbara, USA). The ratios of expression between two time points and their corresponding *p*-values were calculated present-call was <0.01 and the *p*-value of the ratio between the expression at $t = 0$ h and $t = 8$ h was <0.0005. Furthermore, the fold induction had to be >2 or <−2. If more than one probe-set belonged to the same gene, we assigned the expression data of the probe-set with the best *p*-value of the ratio between the two time points to that particular gene. Additional analyses of the microarray data were performed using the RMA and GCRMA normalisation algorithms. With RMA we found 105 of the 109 cell cycle genes described in this report, while with GCRMA we found 108 genes.

2.3. Northern blot analysis

Twenty micrograms of RNA was separated on a 0.8% agarose gel with 6.7% formaldehyde and blotted on Hybond N membranes in the presence of 10× SCC. Sequence-verified DNA probes were hybridized to filters overnight in 0.5 M $NaHPO_4$, pH 7.0, 7% SDS and 1 mM EDTA (incubation buffer) at 65 °C. *MCM* probes were hybridized in the presence of 100 μg human placenta DNA. Filters were washed two times in 40 mM $NaHPO_4$, 1% SDS and one time in 40 mM $NaHPO_4$ at 65 °C. Primers used for probe PCR are listed in the supplementary data.

2.4. Quantitative PCR

For first-strand cDNA synthesis 1 μg of total RNA was reverse transcribed using 125 pM oligodT_{12} primers, 0.5 mM dNTPs, 2 mM $MgCl_2$, RT-buffer (Invitrogen) and 100 U superscript II (Invitrogen) in a total volume of 25 μl.[25] cDNA was diluted two times to a total volume of 50 μl. A fluorescence-based kinetic real-time PCR was performed using the real-time iCycler PCR platform (Biorad, Hercules, USA) in combination with the intercalating fluorescent dye SYBR Green I. Twenty nanograms of cDNA was used for each quantitative PCR reaction. The IQ SYBR Green I Supermix (BioRad, Hercules, USA) was used in accordance with the manufacturer's instructions. A complete list of all the primers used for quantitative PCR is listed in the supplementary data. Expression values were normalised according to Vandesompele et al.[26] by geometric averaging of the genes β-actin and *PSMB4*. The expression of both β-actin and *PSMB4* genes was not regulated in the SHEP-21N cell line according to the microarray data.

2.5. Chromatin immunoprecipitation

SHEP-21N cells were grown in RPMI 1640 supplemented with 50 ng/ml tetracycline for two weeks. Tetracycline was removed by washing the cells three times with HANKS' balanced salts solution (HBSS) and cells were grown on RPMI 1640 for three more days. Cells (3×10^7) were used for four ChIPs (including two negative ChIPs with control antibody). We used a modified protocol of the Upstate ChIP kit (Upstate, Charlottesville, USA). The most important modification was that, after cross-linking and prior to the initial lysis step, nuclei were collected. Nuclei were obtained by harvesting cells in 1 ml Swelling Buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet-P40), incubation on ice for 30 min, homogenation using a syringe and needle (23G) and centrifugation

(4500*g*) at 4 °C. Nuclear lysates were sonicated five times on ice for 30 s with an Ultrasonic Processor (Sonics, Newton, USA) at an amplitude of 30%. Chromatin pre-clearing was performed using protein-A agarose (Roche, Basel, Switzerland) and 25 μg herring sperm DNA for 30 min. at a temperature of 4 °C. Chromatin immunoprecipitation was performed with either 2 μg of monoclonal anti-MYCN antibody (Pharmingen, San Diego, USA) or 2 μg of anti-flag-antibody (Stratagene, La Jolla, USA) and protein-A agarose. For each ChIP sample precipitated chromatin was finally dissolved in 30 μl water. ChIP samples were analysed with SYBR Green based quantitative PCR. Primer pairs were checked for specificity by melting curve analysis and gel electrophoresis. Primer pair efficiencies were determined by the slope of PCR curves, which were comparable at different template concentrations, and by performing PCR reactions with different dilutions of a known template. Precipitated DNA (3 μl) was used as input for each PCR reaction. Each ChIP and following PCR were performed in fourfold. A complete list of all the primers used for ChIP analysis is listed in the supplementary data.

2.6. Flow cytometry

Cells were fixed and stained with propidium iodide using a modified protocol.[27] Histograms of DNA content were generated with a FACS DiVa Flow Cytometer (Becton Dickinson, Franklin Lakes, USA).

2.7. Western analysis

Cell lysates were separated with SDS–polyacrylamide gel electrophoresis and blotted onto Immobilon-P membrane (Millipore, Billerica, USA). Immunostaining of Western blots was performed according to standard procedures and proteins were visualised using the ECL detection system (Amersham, Piscataway, USA). Used antibodies were anti-MCM7 (mouse monoclonal, Santa Cruz, USA), anti-MYCN (mouse monoclonal, Pharmingen, San Diego, USA), anti-β-Actin (mouse monoclonal, Abcam, Cambridge, UK).

3. Results

3.1. MYCN induces expression of genes involved in replication and cell cycle control

In search for molecular pathways involved in the myc-induced proliferation phenotype, we analysed gene regulation by MYCN. We used the neuroblastoma cell line SHEP-21N, in which the ectopic *MYCN* expression can be silenced by tetracycline.[22] *MYCN* expression was switched off for 15 days, after which tetracycline was washed away ($t = 0$) and *MYCN* was re-expressed. RNA was isolated from cells at $t = 0$ and 8, 24 and 120 h after re-expression of *MYCN*. Gene expression profiles of the four time points were made using Affymetrix HG-U133 Plus 2.0 microarrays. One of the most striking observations was that all the minichromosome maintenance complex (MCM complex) genes were significantly ($p < 0.00005$) up-regulated (Fig. 1a). The MCM complex exists of 6 subunits (encoded by *MCM2-7*), is loaded on origins of replication and licenses cells for replication during the G1-phase.[28–30] In addition, MCM8 and MCM10 were induced by MYCN. In a previous study, only the MCM7 gene was found to be a direct target of MYCN.[31] Our data suggest up-regulation of the expression of the whole MCM complex after induction of MYCN.

3.2. Validation of microarray results by qPCR and Northern blot analyses

We also investigated the induction of MCM genes by MYCN in SHEP-21N by quantitative PCR (qPCR). Expression values were normalised by geometric averaging of the control genes β-actin and *PSMB4* (proteasome subunit, beta type, 4). qPCR of *MCM-2* to -8 and *MCM10* showed an up-regulation varying 2–16 fold (Fig. 2a). Up-regulation of these genes was highest at $t = 24$ and *MCM4* and *MCM10* showed the strongest induction. As a control, we included *TK2* (thymidin kinase 2), which was 3-fold down-regulated according to the microarrays. qPCR also showed a 3-fold down-regulation of *TK2*. The regulation of four *MCM* genes by MYCN was also confirmed by Northern blot (Fig. 2b). This analysis showed the expression of *MCM2*, *MCM3*, *MCM4* and *MCM6* to increase with a maximal intensity 24 h after MYCN expression.

3.3. MYCN directly binds to the promoters of MCM genes

The microarray analysis of the time series in SHEP21N indicated that up-regulation of *MCM* gene expression already occurred 8 hours after *MYCN* induction (Fig. 1). Since MYCN activated the promoter activity of one of the *MCM* genes, we analysed whether this effect may result from a direct binding of the MYCN transcription factor to the promoter regions of all the *MCM-2* to -8 and -10 genes. Chromatin immunoprecipitation (ChIP) assays with an MYCN antibody were used to analyze SHEP-21N cells expressing MYCN. The Promoter region of each of the *MCM* genes was determined based on the RefSeq sequences aligned to the genome and primer pairs were chosen at different positions within the promoters (Fig. 3a). As a positive control, we used a primer pair near the E-box within the promoter of *NME1*. *NME1* is a direct target of c-myc[32] and is strongly induced by MYCN in SHEP-21N.[33] A primer pair in the promoter of β-actin functioned as negative control. qPCR was performed on DNA precipitated with either the anti-MYCN antibody or a control antibody and the difference in threshold cycle (ΔC_t) was determined for each primer pair. qPCR with *NME1* specific primers resulted in a ΔC_t of 5.4 cycles, implying a specific ~40-fold enrichment for the E-box containing promoter region by the anti-MYCN antibody (Fig. 3b). For *MCM2*, *MCM7* and *MCM8* the primer pair nearest to the E-boxes showed the largest ΔC_t, varying from 2.6 (*MCM7*, ~6-fold enrichment) to 4.8 (*MCM8*, ~28-fold enrichment). These results indicate that MYCN directly binds to the promoters of these genes. Primer pairs further away from the E-boxes showed a lower ΔC_t, suggesting that MYCN binds in the region of the E-boxes. Also for *MCM3*, *MCM4*, *MCM5* and *MCM6*, which do not contain canonical E-boxes in their proximal promoter, direct binding of MYCN to the promoter was shown. For these *MCM* genes the primer pairs that gave the largest ΔC_t were located near the predicted starts of transcription. MYCN can also bind to non-canonical

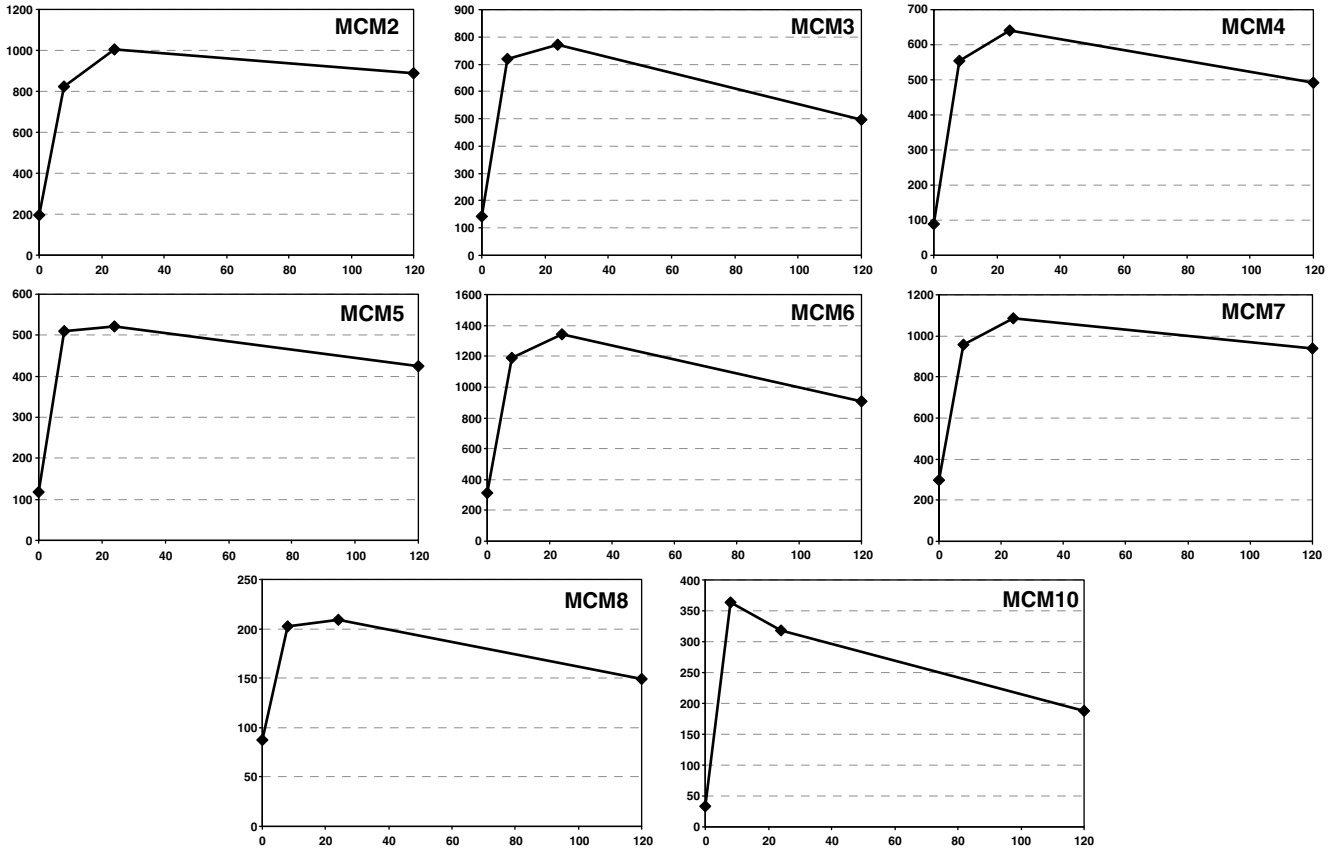

Fig. 1 – MYCN induces the expression of MCM genes. Gene expression was analysed on microarrays at 0, 8, 24 and 120 h after MYCN induction. The Y-axis represents gene expression, the X-axis represents time after MYCN induction (hours).

E-boxes,[34,35] which we could identify for the promoter of *MCM3* (CACGCG), *MCM4* (CACGGG) and *MCM5* (CATGCC). The ChIP analysis did not show a direct interaction of MYCN with the promoter of *MCM10*. Therefore, MYCN does not directly bind to the MCM10 promoter, or MYCN binds outside the analysed promoter region.

In conclusion, we showed that MYCN binds to the promoters of MCM2-8 and thus induces the expression of these genes directly.

3.4. *Increased MCM2, -4 and -7 protein levels correlate with increased S-phase entry by MYCN*

The MCM genes are involved in licensing, initiation of DNA replication and nascent strand elongation during G1- and S-phase.[36] Therefore, we analysed the effect of MYCN on cells entering the S-phase. SHEP-21N cells with or without *MYCN* expression were synchronised by serum starvation for 36 h. After serum stimulation cell cycle distribution was analysed by flow cytometry. In line with previous experiments,[22] more cells entered S-phase when *MYCN* was expressed, showing that *MYCN* expression facilitated S-phase entry (Fig. 4a). This implies that *MYCN* expression results in a higher fraction of cells that are licensed to start DNA replication. In parallel, we analysed whether the MYCN expressing cells in this experiment showed a higher expression of MCM proteins. We monitored the MCM2, MCM4 and MCM7 protein levels during the experiment (Fig. 4b). At t = 0 cells expressing MYCN showed higher MCM2, -4 and -7 protein expression than cells without MYCN expression. This induction of MCM protein levels by MYCN was observed during all subsequent time points of the experiment. Also, in cells which were not synchronised, MCM2, -4 and -7 protein levels were higher when *MYCN* was expressed (Fig. 4b). These results show that the increased MCM mRNA levels induced by MYCN result in increased protein levels, at least for MCM2, -4 and -7. The increased rate of cells entering the S-phase suggests that the activation of the MCM genes by MYCN contributes to enhanced activity of the DNA replication machinery.

3.5. *Expression levels of MYCN and DNA replication genes correlate in neuroblastoma tumours*

The microarray, qPCR and ChIP analyses in the SHEP21N cell line indicate that MYCN regulates the expression of all MCM genes. To investigate whether these *in vitro* data might be relevant *in vivo*, we analysed the expression of *MYCN* and MCM genes in neuroblastoma tumours. mRNA expression profiles were made for 110 neuroblastoma tumours using Affymetrix microarrays. The tumour panel included all histological and clinical stages (INSS classification,[37]) We calculated the Pearson's correlation coefficients of the ^{2}log expression values of *MYCN* and the MCM genes (Table 1). All *MCM* genes showed high correlations with *MYCN* (>0.5), with *MCM2* and *MCM10*

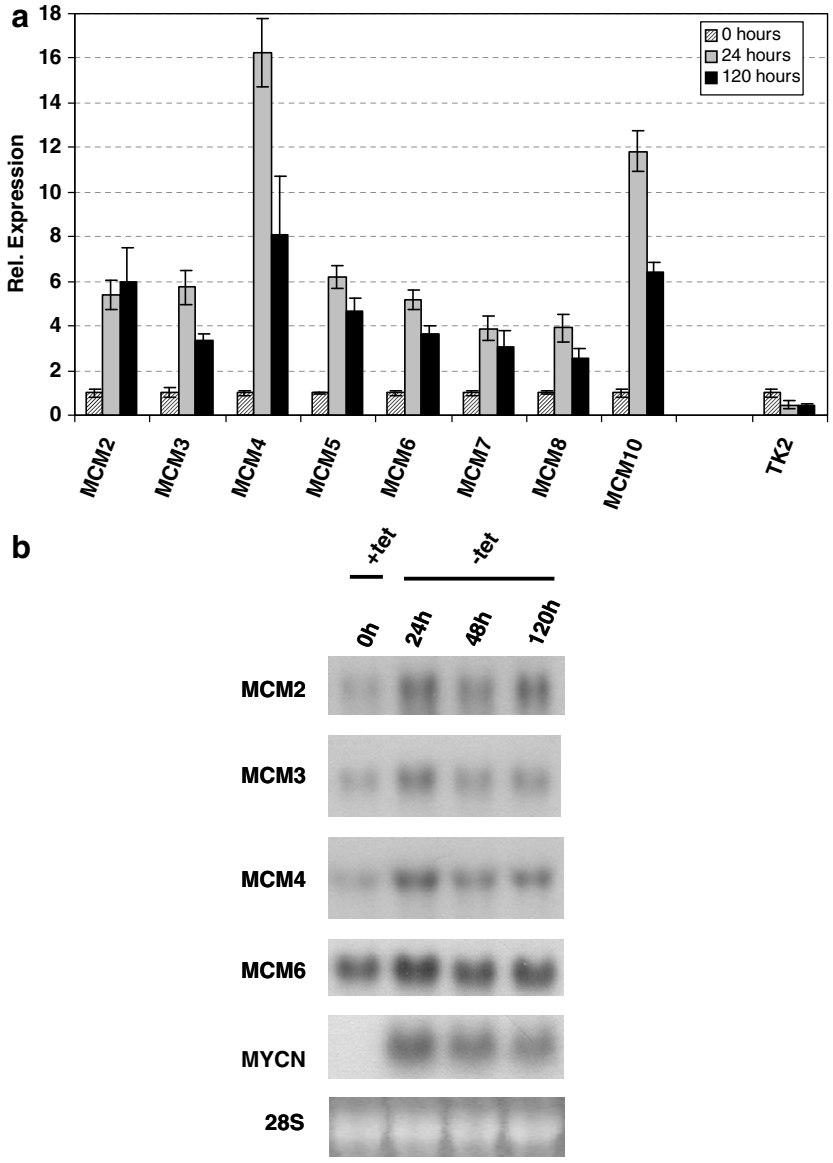

Fig. 2 – qPCR and Northern blot validation of the induction of MCM genes by MYCN. (a) The induction of MCM genes was determined with qPCR at 0, 24 and 120 h after *MYCN* induction. The expression of each gene at time point t = 0 is arbitrarily set on 1. Gene expression was normalised to the geometric averaged expression of control genes β-actin and *PSMB4*. Error bars represent the standard deviation. As control the down-regulation of *TK2* (right panel) is shown. (b) Northern blot analysis of expression of *MCM2*, *MCM3*, *MCM4*, *MCM6* and *MYCN* at the indicated time points. Ethidium bromide staining of 28S ribosomal RNA was used as loading control.

displaying the highest correlation coefficients of $r = 0.72$ and $r = 0.69$ ($p < 0.001$, Fig. 5), respectively. These results are in accordance with our experiments in the SHEP-21N cell line and suggest that MYCN might play a role in the regulation of expression of MCM genes both *in vitro* and *in vivo*.

4. Discussion

Myc oncogenes are known for the induction of a wide array of phenotypes associated with cell growth, proliferation and invasiveness. Many high throughput studies have identified targets of the myc protein family, and these results start to explain the molecular pathways that mediate the myc-induced phenotypes. Myc family members induce ribosomal genes and genes involved in ribosome biogenesis, translational initiation and elongation, which may explain the increased cell growth induced by myc genes.[5,38] Cell proliferation is induced by myc oncogenes by regulation of cell cycle regulatory genes, including cyclins and cyclin dependent kinases, and cell cycle inhibitory genes.[39–41] Also DNA replication is regulated by MYCN since MCM7 has been found as direct transcriptional target. In this paper we describe that in addition to MCM7 also all other MCM members are regulated by MYCN and that at least MCM2 to -6 and MCM8 are direct target genes. Moreover, microarray analysis of a series of 110 neuroblastoma tumours showed that all

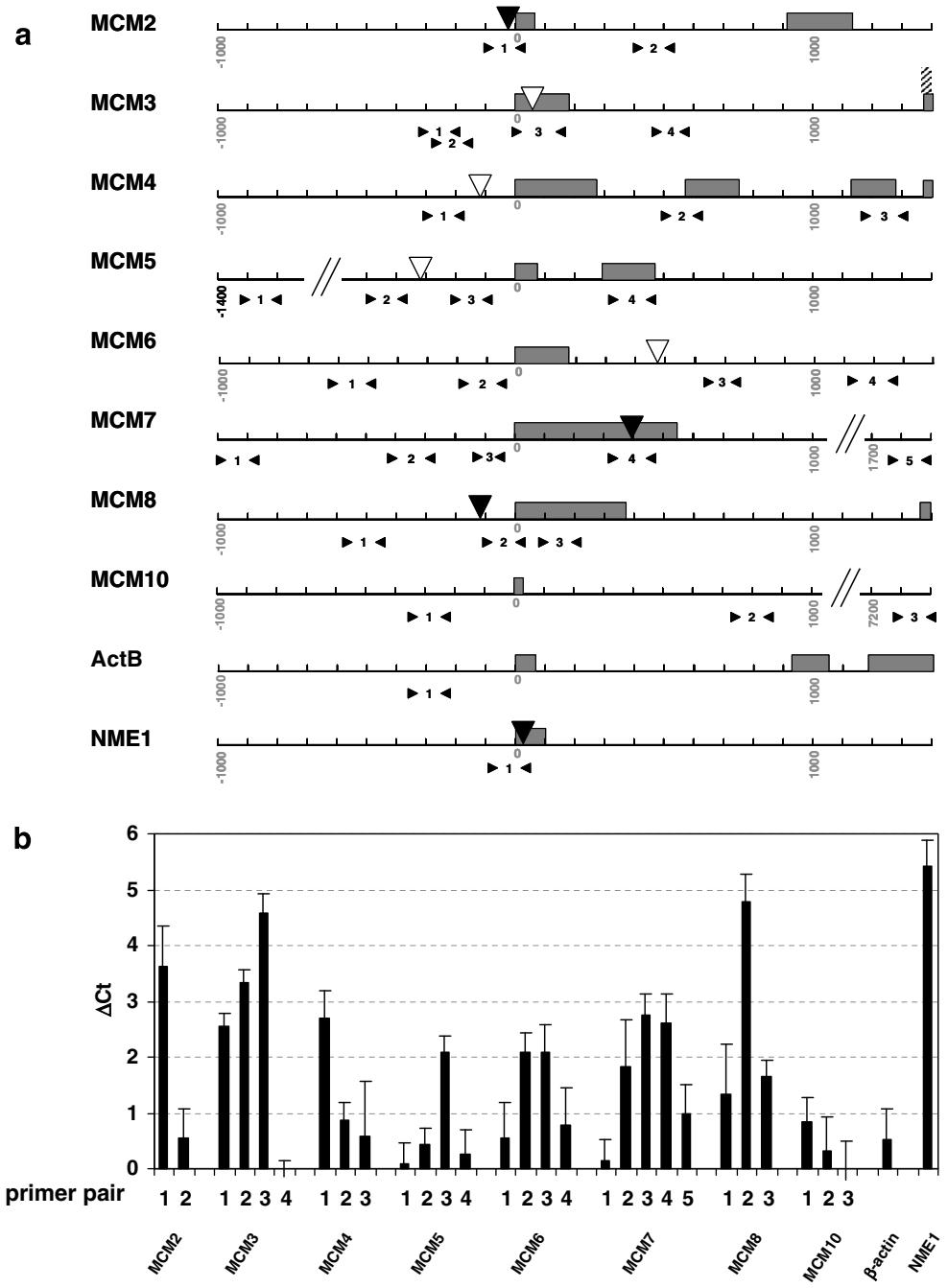

Fig. 3 – ChIP analysis shows a direct binding of MYCN to promoters of minichromosome maintenance genes. (a) Overview of the primer pairs used to analyse binding of MYCN to the *MCM* promoters. Grey squares represent exons. The first exon starts at position 0 and canonical (▼) and non-canonical (▽) E-boxes are indicated. The forward (►) and reversed (◄) primers of each primer pair are shown. (b) ChIP analysis of MYCN binding to the *MCM* genes. Precipitated chromatin was analysed by qPCR with the primer pairs indicated in A. For each primer pair the difference in C_t value between chromatin precipitated with anti-MYCN and an a-specific antibody (ΔC_t) was calculated. β-Actin and *NME1* were used as negative and positive control. Data presented were obtained from four independent ChIP experiments. Error bars represent the standard deviation.

MCM genes highly correlated ($r \geqslant 0.5$, $p < 1 \times 10^{-7}$) with *MYCN* expression, strongly suggesting that the *in vitro* observed regulation by MYCN is important *in vivo*.

During each cell cycle DNA replication is committed to a strict regulatory regime, which ensures that daughter cells end up with the same chromosomal content as the parental cells. The MCM complex, consisting of MCM2 to -7, loads during M-phase on origins of replication after binding of the origin recognition complex (ORC). Subsequently, the CDC7/DBF4 complex follows and the process of origin firing starts.[42,43] MCM8 and MCM10 do not associate with the MCM2 to -7 complex. MCM8 binds chromatin at the onset of the S-phase[44] and

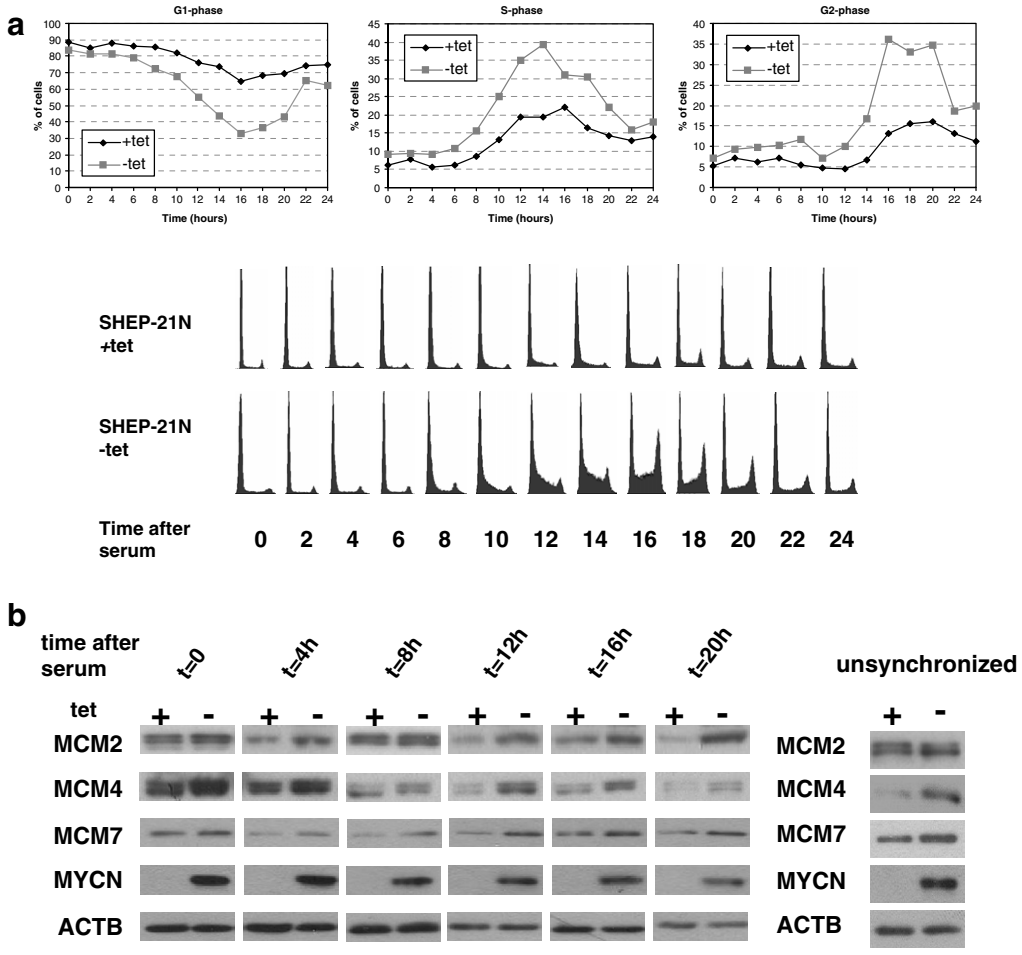

Fig. 4 – *MYCN* expression facilitates S-phase entry and induces MCM7 protein expression. SHEP-21N cells were serum starved for 36 h and serum induced in the presence or absence of tetracycline. (a) Cells were analysed by flow cytometry after serum induction. Percentages of cells (upper panel) in either G1-, S- or G2-phase were determined at regular intervals during 24 h. Histograms of DNA content are represented in the lower panel. (b) Cell lysates were analysed by Western blot with the antibodies directed against the indicated proteins. The last two lanes show the lysates of non-synchronised cells with or without MYCN expression.

Table 1 – All MCM genes correlate *in vivo* with the expression of MYCN

	Correlation (*r*)	Significance (*p*)
MCM2	0.724	4.0e−19
MCM3	0.526	3.5e−09
MCM4	0.506	1.7e−08
MCM5	0.641	4.8e−14
MCM6	0.684	1.7e−16
MCM7	0.683	2.0e−16
MCM8	0.668	1.7e−15
MCM10	0.687	1.3e−16

Pearson's correlation coefficients with corresponding *p*-values (t-test) are shown in the table.

functions as a helicase during elongation of DNA replication.[45] MCM10 is involved in the loading of CDC45[46] and it targets DNA polymerase-a-primase to chromatin.[47] Phosphorylation of MCM proteins by CDC7/DBF4 leads to an allosteric change in the MCM complex that allows binding of CDC45.[48] The CDC45 loading results in the unwinding of DNA and recruitment of replication protein A, which binds single-stranded DNA and is required for DNA polymerase binding and activation.[49] The ATPase activity of the MCM2–7 complex is required for origin unwinding.[50] MCM proteins are released from chromatin during the S/G_2-phase and lose their helicase activity due to phosphorylation by the cyclin dependent kinases CDC2 and cdk2.[51–53] Geminin (GMNN) inhibits re-loading of the MCM complex during S-phase.[54] This system underlies the necessity for the cell to duplicate its DNA only once per cell cycle.

One of the intriguing questions in MCM research concerns the 'MCM-paradox'. The number of MCM2-7 complexes available for each replication origin is 10–40, while only two complexes are required for bidirectional replication.[55] Therefore, it has been hypothesised that the MCM proteins serve other functions in the cell as well, like supporting checkpoint signalling pathways.[56] If so, a considerable fraction of the MCM complex would be occupied with such a function and the

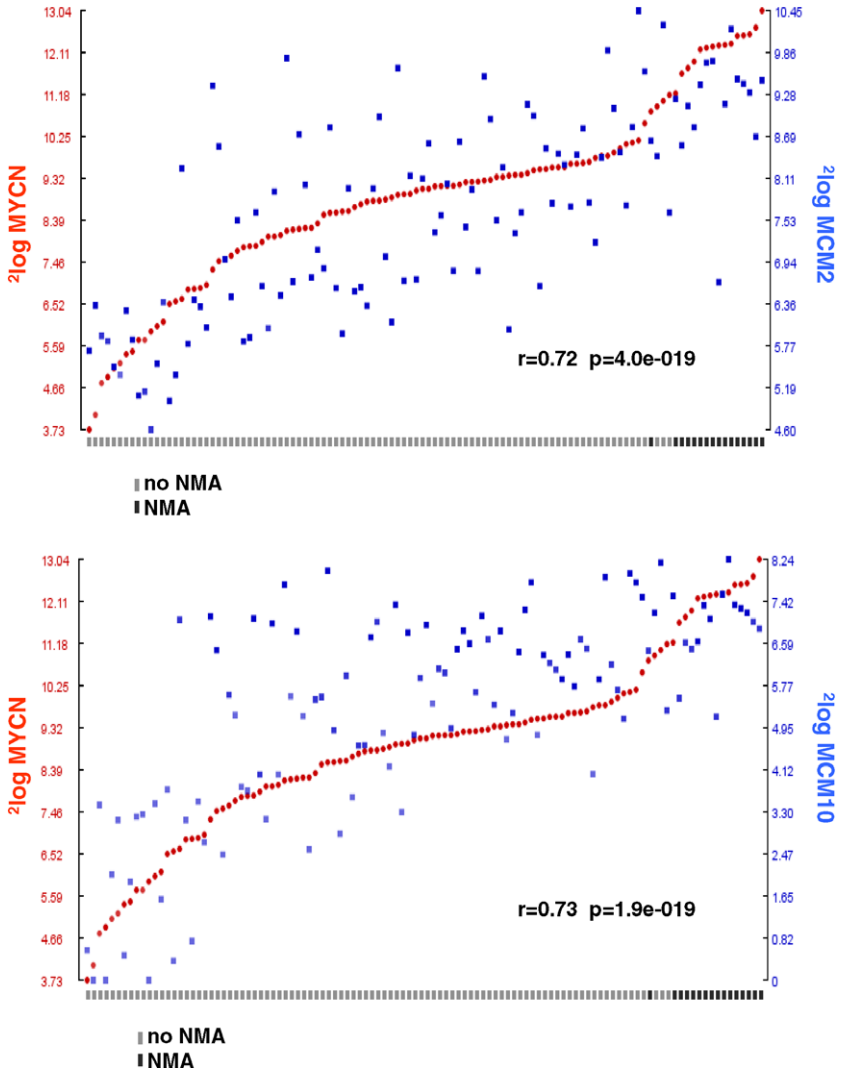

Fig. 5 – The correlation of *MYCN* expression correlates with the expression of *MCM2* and *MCM10* in a series of 110 neuroblastomas. The red *Y*-axis gives the ^{2}log values of the expression of *MYCN*, the blue *Y*-axis gives the ^{2}log MCM2 or MCM10 expression values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase of the MCM gene expression by MYCN could have a profound effect on the availability of a 'free' MCM fraction to engage the pre-RC complex. A further elucidation of all MCM functions is required to solve this question.

Remarkably, it has previously been found that MCM genes are also induced by the E2F/pRB pathway.[57,58] c-Myc can induce E2F1 and E2F2 expressions.[59,60] Therefore, MYCN could stimulate the expression of the MCM genes both directly and indirectly via E2F. Exactly the same was reported for the cell cycle gene MAD2L1. MAD2L1 is directly up-regulated by MYCN as well as by E2F1.[61,62] This network is even more complex, as E2F1 can induce MYCN expression. Furthermore, c-myc can down-tune E2F1 expression by the induction of miR-17-5p and miR-20A, which targets E2F1 mRNA.[63,64] Remarkably, the array data for the MCM genes and the validation by qPCR and Northern blot show a peak at 24 h after MYCN induction, while expression is less increased at 120 h (Figs. 1, 2a and b). The induction is not transient, however, as prolonged MYCN expression keeps the MCM expression at an increased level (2b). This pattern may indicate a cell cycle dependent induction. E2F activity is cell cycle-dependently regulated after Rb phosphorylation by the CyclinD-Cdk4/6 complex during G_1-phase. The pattern of MCM induction might therefore result from direct activation by MYCN combined with a cyclic activation by E2F. Neuroblastomas have low-frequency cyclin-D1 amplifications, but about 70% of neuroblastomas have very high cyclin-D1 expression, reaching levels of 0.4% of all mRNAs present in the cell.[65] As MYCN is amplified in 20% of neuroblastomas, two genes amplified and/or over-expressed in neuroblastoma seem to cooperate in the pathway leading to induction of the MCM genes.

Conflict of interest statement

Not declared.

Acknowledgements

We thank B. Hooibrink and C. van Bree for assistance with the FACS experiments. We thank T. van der Hoeven and J. Verkooijen of the Microarray Department of the Swammerdam Institute of Life Science of the University of Amsterdam for the microarray analyses. This research was supported by grants from the Dutch Cancer Society, Stichting Kindergeneeskundig Kankeronderzoek (SKK), the KIKA foundation and the BioRange programme of the Netherlands Bioinformatics Centre (NBIC).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2007.07.024.

REFERENCES

1. Blackwood EM, Eisenman RN. Max; a helix–loop–helix zipper protein that forms a sequence-specific DNA binding complex with Myc. *Science* 1991;**251**:1211–7.
2. Prendergast GC, Lawa D, Ziff EB. Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and Ras cotransformation. *Cell* 1991;**65**:395–407.
3. Malynn BA, de Alboran IM, O'Hagan RC, et al. N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. *Genes Dev* 2000;**14**(11):1390–9.
4. Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. Drosophila myc regulates cellular growth during development. *Cell* 1999;**98**(6):779–90.
5. Schmidt EV. The role of c-myc in regulation of translation initiation. *Oncogene* 2004;**23**(18):3217–21.
6. Boon K, Caron HN, van Asperen R, et al. N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *Embo J* 2001;**20**(6):1383–93.
7. Coller HA, Grandori C, Tamayo P, et al. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci USA* 2000;**97**(7):3260–5.
8. Goodman LA, Liu BC, Thiele CJ, et al. Modulation of N-myc expression alters the invasiveness of neuroblastoma. *Clin Exp Metastasis* 1997;**15**(2):130–9.
9. Zhang XY, DeSalle LM, Patel JH, et al. Metastasis-associated protein 1 (MTA1) is an essential downstream effector of the c-MYC oncoprotein. *Proc Natl Acad Sci USA* 2005;**102**(39):13968–73.
10. Valentijn LJ, Koppen A, van Asperen R, Root HA, Haneveld F, Versteeg R. Inhibition of a new differentiation pathway in neuroblastoma by copy number defects of N-myc, Cdc42, and nm23 genes. *Cancer Res* 2005;**65**(8):3136–45.
11. Steiner P, Philipp A, Lukas J, et al. Identification of a Myc-dependent step during the formation of active G(1) cyclin-Cdk complexes. *EMBO J* 1995;**14**(19):4814–26.
12. Hermeking H, Rago C, Schuhmacher M, et al. Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci USA* 2000;**97**(5):2229–34.
13. Bouchard C, Marquardt J, Bras A, Medema RH, Eilers M. Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. *Embo J* 2004;**23**(14):2830–40.
14. Jansendurr P, Meichle A, Steiner P, et al. Differential modulation of cyclin gene-expression by Myc. *Proc Natl Acad Sci USA* 1993;**90**(8):3685–9.
15. PerezRoger I, Solomon DLC, Sewing A, Land H. Myc activation of cyclin E Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. *Oncogene* 1997;**14**(20):2373–81.
16. Yin XY, Grove L, Datta NS, Katula K, Long MW, Prochownik EV. Inverse regulation of cyclin B1 by c-Myc and p53 and induction of tetraploidy by cyclin B1 overexpression. *Cancer Res* 2001;**61**(17):6487–93.
17. Schuhmacher M, Kohlhuber F, Holzel M, et al. The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res* 2001;**29**(2):397–406.
18. Staller P, Peukert K, Kiermaier A, et al. Repression of p15(INK4b) expression by Myc through association with Miz-1. *Nat Cell Biol* 2001;**3**(4):392–9.
19. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002;**419**(6908):729–34.
20. Schwab M, Alitalo K, Klempnauer KH, et al. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 1983;**305**(5931):245–8.
21. Rubie H, Hartmann O, Michon J, et al. N-Myc gene amplification is a major prognostic factor in localized neuroblastoma: Results of the French NBL 90 study. *J Clin Oncol* 1997;**15**(3):1171–82.
22. Lutz W, Stohr M, Schurmann J, Wenzel A, Lohr A, Schwab M. Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. *Oncogene* 1996;**13**(4):803–12.
23. Bernards R, Dessain SK, Weinberg RA. N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. *Cell* 1986;**47**:667–74.
24. Auffray C, Rougeon F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumour RNA. *Eur J Biochem* 1980;**107**:303–14.
25. Deprez RHL, Fijnvandraat AC, Ruijter JM, Moorman AFM. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal Biochem* 2002;**307**(1):63–9.
26. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**(7).
27. You JS, Bird RC. Selective induction of cell-cycle regulatory genes Cdk1 (P34(Cdc2)), cyclins A/B, and the tumor-suppressor gene Rb in transformed-cells by okadaic acid. *J Cell Physiol* 1995;**164**(2):424–33.
28. Takisawa H, Mimura S, Kubota Y. Eukaryotic DNA replication: from pre-replication complex to initiation complex. *Curr Opin Cell Biol* 2000;**12**(6):690–6.
29. Tye BK. MCM proteins in DNA replication. *Ann Rev Biochem* 1999;**68**:649–86.
30. Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol* 2005;**6**(6):476–86.
31. Shohet JM, Hicks MJ, Plon SE, et al. Minichromosom maintenance protein MCM7 is a direct target of MYCN transcription factor in neuroblastoma. *Cancer Res* 2002;**62**:1123–8.
32. Li ZR, Van Calcar S, Qu CX, Cavenee WK, Zhang MQ, Ren B. A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci USA* 2003;**100**(14):8164–9.

33. Godfried MB, Veenstra M, Von Sluis P, et al. The N-myc and c-mjc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. *Oncogene* 2002;**21**(13):2097–101.
34. Coulson JM, Edgson JL, Marshal-Jones ZV, Mulgrew R, Quinn PJ, Woll PJ. Upstream stimulatory factor activates the vasopressin promoter via multiple motifs, including a non-canonical E-box. *Biochem J* 2003;**369**:549–61.
35. Blackwell TK, Huang J, Ma A, et al. Binding of Myc proteins to canonical and noncanonical DNA-sequences. *Mol Cell Biol* 1993;**13**(9):5216–24.
36. Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol* 2005;**6**(6):476–86.
37. Brodeur GM, Pritchard J, Berthold F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993;**11**(8): 1466–77.
38. Boon K, Caron HN, van Asperen R, et al. N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *Embo J* 2001;**20**(6):1383–93.
39. Amati B, Alevizopuolos K, Vlach J. Myc and the cell cycle. *Front Biosci* 1998;**3**:250–68.
40. Staller P, Peukert K, Kiermaier A, et al. Repression of p15(INK4b) expression by Myc through association with Miz-1. *Nat Cell Biol* 2001;**3**(4):392–9.
41. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002;**419**(6908):729–34.
42. Weinreich M, Stillman B. Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. *EMBO J* 1999;**18**(19):5334–46.
43. Roberts BT, Ying CY, Gautier J, Maller JL. DNA replication in vertebrates requires a homolog of the Cdc7 protein kinase. *Proc Natl Acad Sci USA* 1999;**96**(6):2800–4.
44. Gozuacik DCM, Lagorce D, et al. Identification and functional characterization of a new member of the human Mcm protein family: hMcm8. *Nucleic Acids Res* 2003;**31**(2):570–9.
45. Maiorano D, Cuvier O, Danis E, Mechali M. MCM8 is an MCM2-7-related protein that functions as a DNA helicase during replication elongation and not initiation. *Cell* 2005;**120**(3):315–28.
46. Sawyer SL, Cheng IH, Chai W, Tye BK. Mcm10 and Cdc45 cooperate in origin activation in *Saccharomyces cerevisiae*. *J Mol Biol* 2004;**340**(2):195–202.
47. Ricke RM, Bielinsky AK. Mcm10 regulates the stability and chromatin association of DNA polymerase-a. *Mol Cell* 2004;**16**(173):185.
48. Jares P, Blow JJ. Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. *Genes Dev* 2000;**14**(12): 1528–40.
49. Walter J, Newport J. Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol Cell* 2000;**5**(4):617–27.
50. Ying CY, Gautier J. The ATPase activity of MCM2-7 is dispensable for pre-RC assembly but is required for DNA unwinding. *EMBO J* 2005;**24**(24):4334–44.
51. Fujita M, Yamada C, Tsurumi T, Hanaoka F, Matsuzawa K, Inagaki M. Cell cycle- and chromatin binding state-dependent phosphorylation of human MCM heterohexameric complexes. A role for cdc2 kinase. *J Biol Chem* 1998;**273**(27):17095–101.
52. Hendrickson M, Madine M, Dalton S, Gautier J. Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. *Proc Natl Acad Sci USA* 1996;**93**(22):12223–8.
53. Ishimi Y, Komamura-Kohno Y, You ZY, Omori A, Kitagawa M. Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2. *J Biol Chem* 2000;**275**(21):16235–41.
54. Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* 2000;**290**(5500):2309–12.
55. Hyrien O, Marheineke K, Goldar A. Paradoxes of eukaryotic DNA replication: MCM proteins and the random completion problem. *Bioessays* 2003;**25**(2):116–25.
56. Cortez D, Glick G, Elledge SJ. Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci USA* 2004;**101**(27):10078–83.
57. Ohtani K, Iwanaga R, Nakamura M, et al. Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene* 1999;**18**(14): 2299–309.
58. Yoshida K, Inoue I. Regulation of Geminin and Cdt1 expression by E2F transcription factors. *Oncogene* 2004;**23**(21):3802–12.
59. Schuldiner O, Benvenisty N. A DNA microarray screen for genes involved in c-MYC and N-MYC oncogenesis in human tumours. *Oncogene* 2001;**20**(36):4984–94.
60. Sears R, Ohtani K, Nevins JR. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. *Mol Cell Biol* 1997;**17**(9):5227–35.
61. Berwanger B, Hartmann O, Bergmann E, et al. Loss of a FYN-regulated differentiation and growth arrest pathway in advanced stage neuroblastoma. *Cancer Cell* 2002;**2**(5):377–86.
62. Hernando E, Nahle Z, Juan G, et al. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature* 2004;**430**(7001):797–802.
63. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005;**435**(7043):839–43.
64. Sears R, Ohtani K, Nevins JR. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. *Mol Cell Biol* 1997;**17**(9):5227–35.
65. Molenaar JJ, van Sluis P, Boon K, Versteeg R, Caron HN. Rearrangements and increased expression of cyclin DI (CCNDI) in neuroblastoma. *Genes Chromosomes Cancer* 2003;**36**(3):242–9.