The cell cycle in neuroblastoma: from genomic aberrations to targeted intervention
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Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells

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Abstract
Two genes have a synthetically lethal relationship, when silencing or inhibition of one gene is only lethal in the context of a mutation or activation of the second gene. This situation offers an attractive therapeutic strategy, as inhibition of such a gene will only trigger cell death in tumor cells with an activated second oncogene but spare normal cells without activation of the second oncogene. Here we present evidence that CDK2 is synthetically lethal to neuroblastoma cells with MYCN amplification and over-expression. Neuroblastomas are childhood tumors with an often lethal outcome. Twenty percent of the tumors have MYCN amplification and these tumors are ultimately refractory to any therapy. Targeted silencing of CDK2 by three RNA interference techniques induced apoptosis in MYCN-amplified neuroblastoma cell lines, but not in MYCN single copy cells. Silencing of MYCN abrogated this apoptotic response in MYCN-amplified cells. Inversely, silencing of CDK2 in MYCN single copy cells did not trigger apoptosis, unless a MYCN transgene was activated. The MYCN induced apoptosis after CDK2 silencing was accompanied by nuclear stabilization of P53 and mRNA profiling showed up-regulation of P53 target genes. Silencing of P53 rescued the cells from MYCN-driven apoptosis. The synthetic lethality of CDK2 silencing in MYCN activated neuroblastoma cells can also be triggered by inhibition of CDK2 with a small molecule drug. Treatment of neuroblastoma cells with Roscovitine, a CDK inhibitor, at clinically achievable concentrations induced MYCN-dependent apoptosis. The synthetically lethal relation between CDK2 and MYCN indicates CDK2 inhibitors as potential MYCN-selective cancer therapeutics.

Introduction
Two genes are considered to be ‘synthetically lethal’ if mutation of either gene alone is compatible with viability but simultaneous mutation of both genes causes death. This mechanism is best described for loss of function genes but also exists for gain of function genes. For example gene A can become essential for survival if gene B is over-expressed. This mechanism is potentially very attractive for the development of targeted anti cancer compounds that could specifically kill tumor cells while leaving normal cells alive(1-3). However, examples of synthetic lethal oncogenes in human tumors are hardly documented. Neuroblastomas are embryonal tumors that originate from the developing sympathetic nervous system and though neuroblastomas have a low incidence, they are the second cause of cancer related deaths in children(4;5). The MYCN gene is amplified in 20-30% of neuroblastoma tumors and amplification strongly correlates with a bad prognosis. MYC genes are potent oncogenes that drive unrestrained cell growth and proliferation. They function as transcription factors that cause upregulation or repression of genes involved in a variety of oncogenetic pathways. Recently MYC genes were also shown to control protein expression through mRNA translation and to directly regulate DNA replication(6-9). Apart from the function in oncogenesis, MYC genes have also been described to induce apoptosis if over-expressed in non MYC-amplified cells(10). This could indicate that the amplification of the MYC oncogenes requires a specific genetic
background and that there could be synthetic lethal relations with other (onco)genes. Cell cycle aberrations occur in all tumors and many targeted compounds inhibiting specific cell cycle kinases have been developed\(^\text{(11;12).}\) These efforts were based on the idea that the targeting of aberrant cell cycle checkpoints in cancer cells could lead to tumor growth inhibition and cell death\(^\text{(13).}\) Several studies have recently shown that most cell cycle kinases are not essential for cell survival in vitro and in vivo\(^\text{(14).}\) Cyclin Dependent Kinase 2 (CDK2) was thought to be a crucial regulator of S phase progression and was therefore evaluated as an anticancer drug target. Tetsu et al. however showed that CDK2 inhibition in several cancer cell types did not result in cell death and Santamaria et al. showed that genetic ablation of CDK2 in mice could be compensated for by CDK1\(^\text{(15;16).}\) These findings severely reduced optimism about CDK2 as therapeutic target.

Several cell cycle aberrations involving G1-regulating genes have been identified in neuroblastomas. Cyclin D1 and CDK4 gene amplifications occur at a low frequency and a CDK6 mutation which inactivates p16-binding has been found\(^\text{(17-19).}\) Cyclin D1 was found to be extremely over-expressed in about 75\% of neuroblastoma tumors. Inhibition of the G1 regulating genes CDK4 or Cyclin D1 in neuroblastoma cell lines resulted in restoration of the G1 checkpoint and subsequent neuronal differentiation\(^\text{(20).}\) The absence of an apoptotic response made these G1 regulating genes less attractive as a therapeutic target.

In this paper we show that inhibition of the next step of the cell cycle which is controlled by CDK2 results in massive cell death. This is in clear contrast with the previous findings of redundancy of CDK2. Inhibiting CDK2 using various siRNA based techniques showed an apoptotic response in MYCN-amplified neuroblastoma cell lines and not in MYCN single copy cells. We could show a synthetic lethal relation in which CDK2 silencing only results in cell death in the presence of MYCN. This lethal response is depending on functional p53. We show that this MYCN dependent P53 mediated apoptosis can also be induced by a CDK2 inhibiting small molecule.

**Results**

*Silencing of CDK2 is lethal to MYCN amplified Neuroblastoma cells.*

To identify new drug targets in the cell cycle, we analyzed the mRNA expression pattern of cell cycle regulating genes in 88 neuroblastoma tumors. High CDK2 expression was strongly correlated with a bad prognosis (Fig. S1A). To evaluate CDK2 as a potential drug target, we silenced CDK2 expression in human neuroblastoma cell lines by transient siRNA. Surprisingly, CDK2 knock-down resulted in a strong induction of apoptosis in three MYCN-amplified neuroblastoma cell lines, while leaving one MYCN single copy neuroblastoma cell line and exponentially growing fibroblasts unaffected (Fig. 1A). To further analyze the effects of CDK2 knock-down, we introduced an inducible shRNA construct into the neuroblastoma cell line IMR32 (targeting another sequence in the CDK2 coding region) and performed time course analysis. Induction of CDK2 shRNA resulted in a decrease in CDK2-specific pRb phosphorylation, followed by PARP and Caspase 3 cleavage (Fig. 1B). An E2F reporter construct revealed a strong reduction
Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells

Fig. 1. CDK2 inhibition causes apoptosis in MYCN-amplified neuroblastoma cells
(A) Three MYCN amplified neuroblastoma cell lines, one MYCN single copy cell line (s.c.) and exponentially growing fibroblasts were transfected with 21 bp double strand siRNA against CDK2 or GFP as control. Samples were harvested at 48 hours and immunoblotted for CDK2, PARP and β-Actin. The PARP antibody recognizes total and cleaved PARP (lower band). Cleavage of PARP indicates activation of the apoptotic pathway. Light microscopy pictures were taken just before harvesting the cells.
(B) The neuroblastoma cell line IMR32 was transfected with a pcDNA6/TR vector and a vector containing CDK2 shRNA under a Tet Operator controlled CMV promoter generating the inducible cell line IMR32-pcDNA6-CDK2sh. Doxycycline was added at time-point 0 to induce CDK2 shRNA expression and silence CDK2. Cells were harvested at various time-points and immunoblotted for CDK2, Threonine 821-phosphorylated pRb, PARP, Cleaved Caspase 3 and β-Actin. Cleavage of PARP and Caspase 3 indicates activation of the apoptotic pathway.
(C) A firefly luciferase vector containing 6 E2F-binding sites was transfected together with a renilla luciferase vector in IMR32-pcDNA6-CDK2sh. After transfection, doxycycline was added. Control samples were not treated. Dual-luciferase assays were performed after 48 and 72 hours to measure the relative E2F activity.
(D) IMR32-pcDNA6-CDK2sh and the IMR32-pcDNA6 from which this cell line was derived were grown in the presence or absence of doxycycline. Cells were harvested at various time points and counted using a Coulter counter.

of E2F activity (Fig. 1C). FACS analysis showed a G1 arrest after 48 hours followed by an increase in sub-G1 fraction, indicative of apoptosis (Fig. S1B). Cell counting after induction of CDK2 shRNA confirmed progressive cell death at 48, 72 and 96 hours after CDK2 silencing (Fig. 1D), eventually leading to 100% cell death after 6 days. We used a lentiviral-mediated shRNA targeting a third CDK2 mRNA sequence, but now located in the non-coding 3’UTR. Again CDK2 silencing in IMR32 resulted in PARP and Caspase 3
The Cell Cycle in Neuroblastoma

Fig. 1. CDK2 inhibition causes apoptosis in MYCN-amplified neuroblastoma cells
(E) IMR32 cells were infected with the lentiviral vector encoding either CDK2 shRNA or the control shRNA and harvested at various time-points after infection. Samples were immunoblotted for CDK2, PARP, Cleaved Caspase 3 and β-Actin.
(F) A CDK2 cDNA expression vector and an empty vector were transfected in IMR32. Clones were isolated using Neomycin selection and one empty vector control clone and two clones expressing ectopic CDK2 were selected for further analysis. Cells were treated with increasing concentrations of lentiviral CDK2 shRNA. Non treated (NT) samples were also included. Samples were immunoblotted for CDK2, PARP and β-Actin.

cleavage indicating apoptosis (Fig. 1E). To rescue this phenotype, we ectopically over-expressed the coding region of CDK2 in the neuroblastoma cell line IMR32. CDK2 silencing in this cell line using the lentiviral encoded shRNA targeting the 3’UTR, silenced the endogenous CDK2 but not the ectopic CDK2 (Fig. S1C and S1D). These cells did not show an apoptotic response, thus excluding off-target effects of the CDK2 shRNA (Fig. 1F and S1E). Our findings are in clear contrast with previous reports on the lack of apoptosis after CDK2 inhibition(16). We hypothesized that the apoptotic response after CDK2 silencing depends on the genetic background of the cell system as we observed apoptosis only in MYCN-amplified neuroblastoma cells and not in a MYCN single copy neuroblastoma cell line and exponentially growing fibroblasts (Fig. 1A).

Apoptosis after CDK2 inhibition is depending on MYCN over expression.
To test whether apoptosis induced by CDK2 inhibition is MYCN dependent, we used SHEP-21N, a MYCN single copy neuroblastoma cell line with a MYCN transgene that can be repressed by the addition of tetracyclin (Tet-off system). CDK2 silencing by transient CDK2 siRNA induced apoptosis when MYCN was expressed but not when MYCN was silent (Fig. 2A and 2B). To further validate the MYCN-dependency, we tested this in MYCN-amplified neuroblastoma cells (IMR32). Silencing of CDK2 using transient siRNA in IMR32 resulted in massive apoptosis which could be rescued by silencing of MYCN with transient siRNA (Fig. 2C and 2D). These findings show that apoptosis after CDK2 silencing is indeed depending on MYCN over-expression.

The apoptotic induction after CDK2 silencing is mediated by the P53 pathway.
To identify genes involved in this apoptotic response we generated Affymetrix mRNA expression profiles of IMR32 at various time-points after inducible silencing of CDK2. In addition, we generated Affymetrix profiles of IMR32 after treatment with transient CDK2 siRNA alone, MYCN siRNA alone or the combination of CDK2 and MYCN siRNA. We selected for genes that were up-regulated after CDK2 silencing but not after com-
Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells. Combined CDK2 and MYCN silencing (see materials and methods for selection procedure). These stringent criteria identified genes involved in Rac GTPase signaling (CYFIP2), peroxide signaling (SESN2), autophagy (DRAM), apoptotic signaling (TRAIL-R2, FDXR) and one a member of the histone H2B family (HIST1H2B). Strikingly, five out of six of the most strongly regulated genes are established direct transcriptional target genes of P53 (Fig. 3A). This suggests an involvement of P53 in the apoptotic response(21-25). P53

Fig. 2. Apoptosis after CDK2 inhibition in neuroblastoma is depending on MYCN over-expression.
(A) SHEP-21N, a neuroblastoma cell line containing MYCN under a Tet-repressor, was cultured with and without tetracycline for 3 days and then re-plated on 6 well plates at a density of 6x10^6 cells per well and treated with CDK2 siRNA or a control siRNA. Cells were harvested 48 hours after siRNA treatment and immunoblotted for CDK2, MYCN, PARP, cleaved caspase3 and β-Actin.
(B) Light microscopy pictures were taken just before harvesting the cells. Samples with MYCN on and CDK2 off settings showed a >94% reduction in cell density compared to all control samples.
(C) IMR32, a neuroblastoma cell line with MYCN amplification, was seeded on 6 cm plates at a density of 2.5X10^5 cells per plate and treated with control siRNA, CDK2 siRNA, MYCN siRNA or combination of MYCN and CDK2 siRNA. Cells were harvested after 48 hours and immunoblotted for CDK2, MYCN, PARP, cleaved caspase3 and β-Actin.
(D) Light microscopy pictures were taken just before harvesting the cells. Samples with MYCN on and CDK2 off settings sowed a >85% reduction in cell density compared to all control samples.
Fig. 3. Apoptosis after CDK2 silencing in MYCN-amplified cell is P53 mediated. 
(A) IMR32-pcDNA6-CDK2sh with doxycycline and IMR32-pcDNA6 were harvested for RNA isolation at various time-points after the addition of doxycycline. Also, transient siRNA experiments were performed in IMR32 using GFP siRNA, CDK2 siRNA, MYCN siRNA or CDK2 and MYCN siRNA. RNA was isolated at 24 and 48 hours after transfection. Affymetrix microarray profiling was performed for both the inducible shRNA time-course experiments and the transient siRNA experiments. Affymetrix expression levels are shown for the six most strongly regulated genes. (See materials and methods for the selection procedure.)
Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells

Fig. 3. Apoptosis after CDK2 silencing in MYCN-amplified cell is P53 mediated.
(B) IMR32-pcDNA6-CDK2sh was seeded on 6cm plates at a density of 2.5x10^5 cells per plate and treated with doxycycline to induce CDK2 shRNA. Cells were harvested at various time points. Samples were immunoblotted for CDK2, P53 and β-Actin.
(C) IMR32 was seeded on 6cm plates at a density of 2.5x10^5 cells per plate and treated with control siRNA, CDK2 siRNA, MYCN siRNA or combination of MYCN and CDK2 siRNA. Cells were harvested after 48 hours and immunoblotted for CDK2, MYCN, P53 and β-Actin.
(D) IMR32 was seeded as described under fig 3c and treated with CDK2 siRNA or GFP siRNA and harvested 48 hours after transfection. Total lysates and nuclear lysates were isolated. Total lysates were stained with CDK2 and P53 and the nuclear lysates with P53 and Histone H3 for loading control.
(E) IMR32 was seeded on 6 well plates at a density of 1x10^5 cells per well and infected with a lentiviral vector encoding either CDK2 shRNA or a P53 shRNA or with both shRNAs. Non coding SHC002 control shRNA was added to experiments with CDK2 or P53 shRNA only and in the control samples to equal the amount of shRNA in each sample. Also a non-transfected control was included (o). Protein was harvested after 48 hours and immunoblotted for CDK2, P53, PARP and β-Actin.
mRNA levels are constant (Affymetrix expression data not shown) but the P53 protein levels increase after inducible and transient CDK2 silencing in MYCN over-expressing cells (Fig. 3B and 3C). This indicates that P53 protein levels are up-regulated by stabilization. In addition, P53 showed nuclear translocation after CDK2 silencing (Fig. 3D). Finally, to analyze the P53-dependency in the apoptotic response, we used lentiviral silencing of P53 and CDK2. Silencing of P53 indeed prevents PARP cleavage in cells with CDK2 silencing (Fig. 3E).

**The CDK2-targeting drug roscovitine induces P53 dependent apoptosis in MYCN amplified cells.**

To extend these findings to clinically applicable compounds, we used a CDK2-inhibiting small molecule. Roscovitine is a CDK inhibitor with a relatively high affinity for the CDK2 ATP-binding pocket and IC50 levels in micro-molar range(26;27). In the MYCN-amplified neuroblastoma cell line IMR32, Roscovitine causes an inhibition of CDK2-specific pRb phosphorylation and a strong apoptotic response followed by P53 stabilization and PARP and Caspase 3 cleavage (Fig. 4A). The fast response (6 hours after exposure to the compounds) probably reflects the direct effect of the small molecules on the CDK2 kinase activity compared to the slower decrease of CDK2 protein levels after siRNA mediated silencing. We determined Roscovitine LC50 levels for IMR32 and SHEP-21N (respectively 3.0 μM and 7.5 μM) and used those concentrations to compare the sensitivity in MYCN on and off setting. In both cell lines Roscovitine induced cell death when MYCN was expressed but not when MYCN was silenced (Fig. 4D, 4C, S4A and S4B).
Discussion

Despite previous reports of redundancy of CDK2 in various tumor and non-malignant cell systems, we show a strong dependency on CDK2 in the subset of MYCN over-expressing neuroblastoma. Off-target RNA interference effects can not explain these findings as we have used three different RNAi techniques with different target sequences that gave the same results and we could rescue the phenotype by over-expressing CDK2. Moreover, Affymetrix profiling of the time course of IMR32 cells with inducible CDK2 shRNA showed no up-regulation of genes involved in the interferon response (28). Finally, a CDK2-inhibiting small molecule gives similar phenotypic effects. We show that these findings are the result of a synthetic lethal relation between CDK2 and over-expressed MYCN. A search for the etiology of the apoptotic response revealed a strong induction of P53 at various levels but we do not exclude involvement of other signal transduction routes. It seems unlikely that this apoptotic response involves E2F, as down-regulation of E2F transcriptional activity after CDK4 or Cyclin D1 silencing in neuroblastoma cell line does not result in apoptosis but leads to neuronal differentiation (20). We did not test whether cells with over-expression of the MYC (c-Myc) oncogene show comparable sensitivity for CDK2 inhibition, but Goga et al. showed that MYC over-expressing cells are more sensitive to CDK1/2 inhibitors (29). This suggests that the synthetic lethal relation between CDK2 and MYCN described here could also exist between CDK2 and MYC.

Our results suggest that CDK2 inhibitors might be potential MYCN-selective cancer therapeutics in a p53 wild type background. Most neuroblastoma primary tumors have intact and functional P53 (30;31). The MYCN gene is amplified in 20-25% of the tumors and relates to an extremely poor prognosis. These two characteristics make neuroblastoma tumors good candidates for in vivo validation and subsequent clinical trials with small molecule CDK2 inhibitors.

Materials and methods

Patient material and cell lines.

The neuroblastoma tumor panel used for Affymetrix Microarray analysis contains 88 neuroblastoma samples. All samples were derived from primary tumors of untreated patients. Material was obtained during surgery and immediately frozen in liquid nitrogen. Cell lines were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 20 mM L-glutamine, 10 U/ml penicillin and 10 µg/ml streptomycin. Cells were maintained at 37°C under 5% CO2. For primary references of these cell lines, see Cheng et al (32). The IMR32 sub-clones with over-expression of CDK2 and the empty vector controls were generated by transfecting 4µg of pCMV-neo-Bam or pCMV-neo-Bam CDK2 expression vector (Addgene)(33) using Lipofectamin 2000® according to manufacturers’ protocol. Clones were selected using 300µg/ml Neomycine.
RNA interference.

The synthetic siRNA oligonucleotides were synthesized by Eurogentec. siRNAs were targeting CDK2 on nucleotide 401-419 in NM_001798.2 and MYCN on nucleotide 348-366 at NM_005378 (NCBI Genebank) Previously designed siRNA directed against GFP was used as negative control (sense sequence: GACCCGGCGAGUGAGAATT). Neuroblastoma cell lines were cultured for 24 hours in 6 cm plates and transfected with 5.5μg siRNA using lipofectamin® according to manufacturers’ protocol. The lentiviral shRNAs were obtained from Sigma (MISSION® shRNA Lentiviral Transduction Particles). IMR32 cells were counted and 100,000 cells were plated in 6cm wells in 2ml of culture medium. The culture medium was changed after 16 hours and the cells were infected with either the shCDK2-lentivirus (MISSION® shRNA TRCN0000000587) the P53 shRNA (MISSION® shRNA TRCN0000003755) or the control lentivirus (MISSION® Non-Target shRNA Control SHC002 ). Virus concentrations were determined using a p24 ELISA. The IMR32 clone capable of inducible CDK2 shRNA expression was generated in a step-wise process. First, IMR32 cells were transfected with the pcDNA6/TR vector (Invitrogen) encoding the tetracycline/doxycycline repressor behind a constitutive CMV promoter, using Lipofectamine 2000®. Clones were selected using 5μg/ml Blasticidin-S (Invitrogen), resulting in the isolation of IMR32-pcDNA6. The CDK2 shRNA expression constructs were prepared as follows: Oligonucleotides targeting CDK2 on nucleotide 546-566 in NM_001798.2 (genebank) were annealed and ligated into pTER restricted with BglII and HindIII as described (34;35), creating pTER/CDK2. This construct was used for transfection of IMR32-pcDNA6 using Lipofectamin 2000®. Clones were again selected using 5μg/ml Blasticidin-S and 10μg/ml Zeocine resulting in the isolation of IMR32-pcDNA6-CDK2sh. For induction of the shRNA expression vector, doxycycline was added to the culture medium to a concentration of 100ng/ml.

Compounds.

Roscovitine was obtained from Sigma and diluted to a concentration of 30mM in DMSO. Cells were treated with various concentrations of Roscovitine with a constant DMSO concentration of 0.5%.

Western Blotting.

The neuroblastoma cell lines were harvested on ice and washed twice with PBS. Cells were lysed in a 20% glycerol, 4% SDS, 100 mM TrisHCl pH6.8 buffer. Protein was quantified with RC-DC protein assay (Bio-Rad). Loading was controlled by Bio-Rad Coomassie staining of a reference SDS-PAGE gel. Lysates were separated on a 10% SDS-PAGE gel and electro-blotted on a transfer membrane (Millipore). Blocking and incubation were performed using standard procedures. The following antibodies were used as primary antibodies: CDK2 clone 55 mouse monoclonal (BD Bioscience), PARP clone 4C10-5 mouse monoclonal (BD Pharmingen), pRb (pT821) (Biosource), P53 clone DO-7 mouse monoclonal (Labvision), Cleaved caspase 3 (Asp175) rabbit polyclonal (Cell Signaling Technology), MYCN Mouse monoclonal (Pharmingen), Histone H3 mouse monoclonal (Upstate) and Actin C2 mouse monoclonal (Santa Cruz). After incubation with a secondary sheep anti-mouse or anti-rabbit horseradish peroxidase linked antibody (Amersham), proteins were visualized using an ECL detection kit (Amersham). Antibod-
Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells.

**Cell counting and FACS analysis.**

All cell counting experiments were performed twice. Cells were collected by trypsinization and diluted in 2ml of medium. Two samples of 100μl of this medium were diluted in 100μl TritonX-100/saponine and 10ml isotope II was added. Duplicate counting of each sample was performed using a coulter counter (Beckman Coulter™). For FACS (fluorescence-activated cell sorter) analysis cells were grown for 24 hours in 6 well plates and then transfected with siRNA as described before. At the indicated time-point after transfection, cells were lysed with a 3.4mM Tri-sodiumcitrate, 0.1% Triton X-100 solution containing 50μg/μl of propidium iodide. After 1 hour incubation, the DNA content of the nuclei was analyzed using a FACS. A total of 30,000 nuclei per sample were counted. The cell cycle distribution and apoptotic sub G1 fraction was determined using WinMDI version 2.8.

**Transactivation assays.**

The following luciferase constructs were used in the transactivation assays: pGL3 TATA basic-6xE2F (pGL3 containing a TATA box and 6 E2F-binding sites was previously tested for E2F selectivity and was a kind gift of Prof. R. Bernards, The Dutch Cancer Institute(36)) and pRL-CMV (Renilla luciferase vector under CMV promoter). Cells were cultured for 24 hours in 6 well plates and transfections were conducted using lipofectamin® according to manufacturers’ protocol. 1μg of the pGL3 TATA basic-6xE2F vector was transfected together with 0.8μg pRL-CMV vector. Dual luciferase assays were performed after 48 and 72 hours using the Promega dual-luciferase reporter assay system. For each assay three separate experiments were performed.

**RNA isolation and Affymetrix Microarray analysis.**

Total RNA of neuroblastic cell lines and tumors was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration was determined using the NanoDrop ND-1000 and quality was determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). For Affymetrix Microarray analysis, fragmentation of RNA, labeling, hybridization to HG-U133 Plus 2.0 microarrays and scanning was carried out according to the manufacturer’s protocol (Affymetrix Inc.). The expression data were normalized with the MAS5.0 algorithm within the GCOS program of Affymetrix. Target intensity was set to 100 (α1=0.04 and α2 0.06). If more than one probe set was available for one gene the probe set with the highest expression was selected, considered that the probe set was correctly located on the gene of interest. For the selection of genes involved in apoptosis after CDK2 silencing, we used the following procedure. First, genes were selected that showed a minimal expression of 100 (MAS 5.0) in at least one time-point in all time-courses and in the transient experiments. Next, genes were selected that were up-regulated at least 1 2log fold at time-point 48 compared to time-point 0 in the biological triplicate of the inducible CDK2 shRNA experiments. Genes that were regulated more than 0.5 2log fold in the control time courses were excluded. These genes were further analyzed using
the transient siRNA experiments. Genes were only included if they were up-regulated more than 1 2log fold at time-point 24 in the CDK2 siRNA samples compared to the GFP control sample. Finally, genes were excluded if they were up-regulated more than 0.5 2log fold in the CDK2 and MYCN siRNA experiment.

**Q-PCR.**

1μg of Trizol isolated RNA was used for cDNA synthesis with 125pM oligoT12 primers, 0.5mM dNTPs, 2mM MgCl2, RT-buffer (Invitrogen) and 100U Superscript II (Invitrogen) in a total volume of 25μl. 1ul of this cDNA was used for Q-PCR. A fluorescence-based kinetic real-time PCR was performed using the real-time iCycler PCR platform (Biorad) in combination with the intercalating fluorescent dye SYBR Green I. The IQ SYBR Green I Supermix (BioRad) was used in accordance with the manufacturer’s instructions. The following primers were used: β-actin forward 5’-CCCAGCACAATGAAGATCAA-3’ and reverse 5’-ACATCTGCTGGAAGTGAC-3’; CDK2 Coding sequence forward 5’-ACACAGCTCTTCGGGATCTCTTT-3’ and reverse 5’-CATCCTGGAAGAAAGGTGA-3’; CDK2 3’UTR forward 5’-CCCTTTCTTCCAGGATGTGA-3’ and reverse 5’-TGAGTCAAATAGCCTAAAGG-3’.

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Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells

Reference List

The Cell Cycle in Neuroblastoma


Supplementary figures

Supplementary Figure 1.
(A) Kaplan-Meier analysis of 88 patients with neuroblastoma with high versus low CDK2 expression. The cutoff was put at a CDK2 expression level of 130 (MASS 5 determined) giving an optimal significance of 9.7 $10^{-5}$, as determined by a LogRank test.

Supplementary Figure 1.
(B) FACS analysis of IMR32-pcDNA6-CDK2sh at various time-points after addition of doxycycline, resulting in CDK2 shRNA activation and subsequent CDK2 silencing (for the corresponding Western blot see fig 1b).
Supplementary Figure 1. 
(C) Q-PCR on cDNA of the IMR32-EV sub clone harvested 72 hours after lentiviral transfection with CDK2 shRNA or control shRNA (SHC002). RT-PCR using primers against the coding sequence and the 3’ UTR of CDK2 mRNA and against β-actin mRNA as a control, was performed. The figures show signal intensity on a 10 log scale for three separate experiments.

Supplementary Figure 1. 
(D) Q-PCR on cDNA of the IMR32-CDK2-C1 with over-expression of CDK2. Analyses are identical to those performed with IMR32-EV (see supplementary figure1c)
Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells

Supplementary Figure 1.
(E) Light microscopy pictures of IMR32-EV and IMR32-CDK2-C1 72 hours after infection with control shRNA (SHC002) and CDK shRNA.

Supplementary Figure 2.
(A) The IMR32 cell line was treated with MYCN siRNA and 24 hours after transfection Roscovitine was added in an end concentration of 3µM. Samples were harvested 24 hours after start of treatment and immunoblotted for MYCN, PARP and β-Actin.

Supplementary Figure 2.
(B) Light microscopy pictures were taken just before harvesting the cells.