Understanding and Exploiting MYCN Addiction in High-Risk Neuroblastoma Using the Example of CDK13

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Understanding and Exploiting MYCN Addiction in High-Risk Neuroblastoma Using the Example of CDK13

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PD Dr. Frank Westermann
Declaration

The work presented in this thesis was carried out from March 2014 until March 2019 in the group of Neuroblastoma Genomics at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. It was supervised by PD Dr. Frank Westermann.

I declare that this thesis, and the research to which it refers, are the product of my own work that has not yet been submitted for a degree or a diploma at any university. To the best of my knowledge this thesis contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

_____________________
Mona Friedrich
Heidelberg, April 10, 2019
Neuroblastoma (NB) is an extracranial childhood cancer and accounts for 15% of all childhood cancer-related deaths. It is a remarkably heterogeneous disease and cases range from spontaneous regression to aggressive high risk disease. Amplification of the oncogene MYCN occurs in 20-30% of cases and is an indicator of poor prognosis. As a transcription factor, MYCN drives several cellular programs favoring malignant behavior, for example proliferation, angiogenesis and metastasis. In order to gain a better understanding of direct and secondary transcriptional targets of MYCN, gene expression was analyzed in a time course experiment using cell cycle-synchronized cells with regulatable MYCN level. This approach revealed the continued upregulation of a number of cell cycle driver genes as well as genes involved in protein and DNA biosynthesis in MYCN high cells. Differences in the expression of other cell cycle and DNA replication genes occurred only in specific phases of the cell cycle. On the other hand, genes involved in alternative splicing and cell adhesion were constantly downregulated. The expression of snoRNAs strongly increased in the MYCN low condition towards the end of the observation period. An analysis of miRNA expression revealed that many differentially regulated miRNAs targeted genes involved in ribosome biogenesis, cancer processes and signaling pathways. Taken together, this data set indicates that MYCN directly regulates the expression of genes and miRNAs which contribute to accelerated proliferation, metabolism and metastatic growth in NB cells. As a consequence of the induced phenotype, a larger number of secondary targets are deregulated.

As MYCN heavily contributes to tumor malignancy, MYCN-amplified NB cells become addicted to high amounts of the protein. However, MYCN itself is notoriously difficult to target, therefore two siRNA screens were performed to detect synthetic lethal relationships with high MYCN levels. The second part of this thesis deals with the transcriptional kinase CDK13, which was identified as a potential candidate for novel targeted therapies. CDK13 and its highly homologous family member CDK12 were knocked down by several technical approaches. In a cellular MYCN overexpression model, CDK13 repression induced strong cell death only in MYCN high cells. CDK12 repression also elicited a small amount of cell death comparing MYCN high with low cells. Inducible CDK13 knockdown in a MYCN-amplified cell line caused modest reductions in cellular viability and colony formation capacity. CDK13, but not CDK12, knockout induced by the clustered regularly interspaced short palindromic repeats (CRISPR) technique reduced viability and caused a small increase in cells arrested in G1. However, analysis of CDK13 mRNA level revealed significant residual expression, suggesting that the majority of the polyclonal culture might be heterozygous for the induced mutation. A novel small
compound inhibitor against CDK12 and CDK13 (BAY-587) was tested in a panel of eight NB cell lines and the effects were compared to that of a commercially available inhibitor, THZ531. BAY-587 was active at lower concentrations than THZ531. Both compounds strongly reduced viability, colony formation capacity and disrupted cell cycle distributions. BAY-587 treatment reduced the level of CDK12 protein and further induced apoptosis. Gene expression analysis revealed that CDK12/13 inhibition by BAY-587 caused downregulation of genes involved in alternative splicing and DNA damage repair, while transcription regulation genes were upregulated. In summary, CDK13 emerged as a promising new therapeutic candidate for the treatment of high risk NB patients.

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<td>Degrees Celsius</td>
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<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
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<tr>
<td>AAVS1</td>
<td>Adeno-Associated Virus Integration Site 1</td>
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<tr>
<td>act. transl.</td>
<td>Activating translocation</td>
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<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
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<td>Amplified</td>
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<td>Ataxia Telangiectasia Mutated</td>
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<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
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<td>ATR</td>
<td>Ataxia Telangiectasia And Rad3 Related</td>
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<td>Alpha Thalassemia/Mental Retardation Syndrome X-linked</td>
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<td>Bafilomycin A1</td>
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<td>bp</td>
<td>Basepair(s)</td>
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<td>BrdU</td>
<td>Bromdesoxyuridin</td>
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<td>Bovine serum albumin</td>
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<td>Cell Division Cycle 25A</td>
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<td>CDKI</td>
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</tr>
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<td>CDK Inhibitor 1A</td>
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<td>Complementary DNA</td>
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<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>c-MYC</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog</td>
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<tr>
<td>CPM</td>
<td>Count per million</td>
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<td>Clustered regularly interspaced short palindromic repeats</td>
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<td>DAPI</td>
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<tr>
<td>dCas</td>
<td>Deactivated Cas</td>
</tr>
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<td>ddH2O</td>
<td>Double-distilled water</td>
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<tr>
<td>DDR</td>
<td>DNA damage repair</td>
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<td>Differentially expressed genes</td>
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<td>DKFZ</td>
<td>German Cancer Research Center</td>
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<td>DMSO</td>
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<td>Doxy</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Escherichia coli</td>
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</tr>
<tr>
<td>Et al.</td>
<td>Et alii (and others)</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EZH2</td>
<td>Enhancer of Zeste 2</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FCS</td>
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<td>FDR</td>
<td>False discovery rate</td>
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<td>Fer-1</td>
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<td>Forward scatter</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GO</td>
<td>Gene ontology</td>
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<tr>
<td>H</td>
<td>Histone</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>INRG</td>
<td>International neuroblastoma risk group</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KDM</td>
<td>Kinase-dead mutant</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
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<td>KRAB</td>
<td>Krüppel Associated Box</td>
</tr>
<tr>
<td>I</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>M phase</td>
<td>Mitosis</td>
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<td>Methanol</td>
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<td>miRNA</td>
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<td>Minute(s)</td>
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<td>Myc-Interacting Zinc-finger Protein 1</td>
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<td>Milliliter</td>
</tr>
<tr>
<td>MNA</td>
<td>MYCN-amplified/amplification</td>
</tr>
<tr>
<td>MPER</td>
<td>Mammalian protein extraction reagent</td>
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<td>mut</td>
<td>Mutant</td>
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<tr>
<td>MYC</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog</td>
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<td>MYCN</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog</td>
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<td>NB</td>
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<td>not significant</td>
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<tr>
<td>OE</td>
<td>Overexpression</td>
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<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PAA</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gelelectrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCAP</td>
<td>Phospho-CTD Associating Proteins</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PHOX2B</td>
<td>Paired-like Homeobox 2B</td>
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<td>Propidium iodide</td>
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<td>PKMYT1</td>
<td>Protein Kinase Membrane Associated Tyrosine/Threonine 1</td>
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<tr>
<td>PNK</td>
<td>Polynucleotide kinase</td>
</tr>
<tr>
<td>PRC2</td>
<td>Polycomb Repressive Complex 2</td>
</tr>
<tr>
<td>pSer</td>
<td>Phosphorylated serine residue</td>
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<tr>
<td>pTEF-ß</td>
<td>Positive Transcription Elongation Factor ß</td>
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<tr>
<td>R</td>
<td>Arginine</td>
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<td>RB</td>
<td>Retinoblastoma Protein</td>
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<td>RNA polymerase II</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>RT-qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>S / Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single guide RNA</td>
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<td>shRNA</td>
<td>Short hairpin RNA</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>Telomerase Reverse Transcriptase</td>
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<td>Transforming Growth Factor ß</td>
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<td>TMM</td>
<td>Trimmed mean of M values</td>
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<td>Tetracycline repressor</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase -mediated dUTP-biotin nick end labeling</td>
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1 INTRODUCTION

1.1 Basics of Cancer

To this day, cancer represents one of the leading causes of death. According to the World Cancer Report, in 2012 there were 1820 per million people diagnosed with cancer worldwide, which corresponds to a total number of 14.1 million cases (Stewart and Wild, 2014). In the same year, 1020 per million people died from the disease (a total of 8.2 million). The incidence of childhood cancer ranges between 50 and 200 cases per million per year, representing up to 4.5 % of all cancer cases. Growing globalization and human development involve changes in lifestyle that will likely lead to an even higher cancer burden. In 2025, the cancer incidence is predicted to rise above 20 million cases.

Decades of intense cancer research have shed some light on origin and development of this complex disease and given rise to novel concepts of treatment. Despite this, many aspects still remain poorly understood. The following chapter will touch upon the most important concepts in tumor biology.

Cancer is a disease where cells become able to divide uncontrollably and to spread to other tissues (Cooper, 2000). In a landmark article, Hanahan and Weinstein described six distinctive hallmarks that enable previously normal cells to acquire a malignant phenotype (2000). First, the ability to produce mitogenic signals renders cells independent from stimuli by the surrounding tissues. Second, malignant cells override anti-growth signals from the surroundings that in normal tissues maintain quiescence and homeostasis. Third, tumor cells are able to escape programmed cell death, which constitutes a natural barrier to cancer development. Fourth, cells replicate without being limited intrinsically by telomere shortening and ensuing states of senescence or crisis. Fifth, continuous angiogenesis ensures sufficient supply of oxygen and nutrients to the tumor, and sixth, cells invade locally and metastasize to distant tissues.

These traits are acquired stepwise and facilitated by increasing genomic instability (Hanahan and Weinberg, 2011). A series of such genetic changes confers a selective growth advantage to a cell (Nowell, 1976). These changes include somatic mutations as well as chromosome instability associated with losses or gains of chromosome parts or translocations. In addition, epigenetic changes like DNA methylation or histone modifications can impact on gene expression. In tumorigenesis, two classes of genes are of particular importance: proto-oncogenes and tumor suppressor genes. Proto-oncogenes regulate proliferation or inhibit cell death and genetic changes turn them into constitutively active oncogenes (Croce, 2008). Opposed to that, tumor suppressor gene products have a protective function by negatively regulating the cell cycle or promoting cell death. Only a
biallelic inactivation of such a tumor suppressor gene promotes tumorigenesis (Sherr, 2004).

Even though cells need to accumulate several mutations in order to become tumorigenic, several research groups observed that abolishing a single key oncogene may be enough to stop cell growth and induce differentiation or cell death (Jain et al., 2002; Pelengaris et al., 1999). This observation gave rise to the concept of oncogene addiction, which was first described by Weinstein (2002). He postulated that malignant cells depend on the aberrant expression of one or only a few oncogenes to maintain their cancerous behavior. Evidence from clinical studies backs up this concept (Weinstein and Joe, 2006). For instance, breast cancer patients with HER2 overexpression can successfully be treated with antibodies against HER2 (Vogel et al., 2002). Also, reactivation of a key tumor suppressor gene may be sufficient to prevent a cancer cell from proliferating (Weinstein, 2002). This is called tumor suppressor gene hypersensitivity. Both aspects of tumor cell biology bear great potential for the development of new molecularly targeted therapies. In most cases, it will be beneficial to combine targeted drugs or add standard therapy in order to avoid the development of resistances, as observed in targeted therapy studies where tumors relapsed after an initial response. Analysis of the relapsed tumors revealed that they had acquired additional mutations, which blocked the administered antibody from binding (Weinstein and Joe, 2006). Thus, the cells likely depend so heavily on the oncogene in question that selective pressure favors mutations circumventing targeted therapy.

1.2 Neuroblastoma

Neuroblastoma (NB) is a common childhood tumor originating from neural crest precursor cells. Usually, the primary tumor is found in tissues of the nervous system, adrenal medulla or the paraspinal ganglia (Huang and Weiss, 2013). Approximately one in 100,000 children is diagnosed with NB each year and 15% of all childhood cancer deaths are NB cases (Stewart and Wild, 2014). However, neuroblastoma biology and its clinical manifestation are remarkably heterogeneous. Low-risk cases oftentimes regress spontaneously without any treatment. On the other hand, high-risk cases still have survival rates below 50% despite multimodal, intense treatment (Smith and Foster, 2018). It is therefore important to assign the best suitable treatment strategy to each patient and to avoid unnecessary strains through aggressive therapies in patients with a good prognosis. New staging and classification systems were developed by the International Neuroblastoma Risk Group (INRG) in 2009 to reach a worldwide consensus for risk stratification (Cohn et al., 2009; Monclair et al., 2009). Staging is based on image analysis prior to surgery and comprises the stages very low (localized tumors), low, intermediate
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and high (metastatic disease) (L1, L2, M and MS) (Monclair et al., 2009). Classification takes into account stage, age, histology, extent of tumor differentiation, \textit{MYCN (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-Derived Homolog)} status, chromosome 11q status and DNA ploidy (Cohn et al., 2009).

The current standard of care for high-risk patients includes chemotherapy followed by resection (induction phase), high-dose chemotherapy with autologous stem cell rescue and radiation (consolidation phase) and immunotherapy and retinoic acid treatment (maintenance phase) (Smith and Foster, 2018). These therapies come with strong side effects and induce considerable damage. In order to develop less harmful, targeted therapies, a better understanding of the underlying tumor biology of neuroblastoma is indispensable.

1.2.1 Somatic Aberrations in Neuroblastoma

The most common aberration in NB is the amplification of the \textit{MYCN} oncogene, which occurs in 20-30\% of all cases (Brodue et al., 1984; Matthay et al., 2016; Schwab et al., 1983). Additionally, amplifications of \textit{Anaplastic Lymphoma Kinase} (\textit{ALK}) are frequently correlated with that of \textit{MYCN} (Miyake et al., 2002; Trigg and Turner, 2018).

Generally, NBs display relatively few recurrent somatic mutations when compared to adult tumors. Mutations in the \textit{ALK} gene have been observed in up to 10\% of sporadic NB and in half the cases of hereditary NB (2\% of total cases) (Y. Chen et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Moreover, germline or sporadic mutations in the \textit{Paired-like Homeobox 2B} (\textit{PHOX2B}) gene have been identified in a small number of NB tumors (Trochet et al., 2004; van Limpt et al., 2004).

Although mutations of the tumor suppressor gene \textit{TP53} occur in less than 2\% of primary NB tumors, they are found in approximately 15\% of relapsed tumors (Carr-Wilkinson et al., 2010).

1.2.2 \textit{MYCN} in Neuroblastoma and Beyond

\textit{MYCN} is a basic helix-loop-helix leucine zipper transcription factor and belongs to the same protein family as the highly homologous oncogene \textit{MYC} (also known as \textit{c-MYC}). While both \textit{MYCN} and \textit{MYC} deficiency lead to embryonic lethality in mice, it is possible to rescue the effect by introducing \textit{MYCN} at the \textit{MYC} locus (Malynn et al., 2000). However, these mice suffer from growth deficits and muscular dystrophies. Apparently, \textit{MYC} and \textit{MYCN} can assume similar functions, including in proliferation, differentiation and apoptosis, but not completely replace each other. Their exact impact depends on distinct temporal and spatial expression patterns. For example, \textit{MYCN} is expressed rather early
during development and necessary for neurogenesis in healthy mouse embryos (Knoepfler et al., 2002; Zimmerman et al., 1986). 

*MYCN* amplification in NB is highly predictive of a poor prognosis (Brodeur et al., 1984). Moreover, targeted *MYCN* overexpression in neural crest cells of mice is sufficient to cause NB development (Weiss et al., 1997). Apart from NB, amplification and aberrant *MYCN* expression occur in medulloblastoma, retinoblastoma, astrocytoma, non-small cell lung cancer and glioblastoma (Schwab, 2004). High levels of MYC(N) have been correlated with several oncogenic processes, such as malignant transformation and proliferation (Schwab et al., 1985), angiogenesis (Baudino et al., 2002), genome instability (Felsher and Bishop, 1999), metastasis (Megison et al., 2013) or the inhibition of differentiation (Freytag and Geddes, 1992). Somewhat contradictory, tumor inhibiting functions have also been documented, such as sensitization to apoptosis (Fulda et al., 1999).

MYC and MYCN act as transcription factors through different mechanisms. They dimerize with the protein MYC-associated factor X (MAX) to reinforce expression of a large set of target genes by binding to a consensus sequence, the E-Box (Blackwood and Eisenman, 1991). However, at abundant protein levels, the complex is also able to bind to non-canonical sequences (Fernandez et al., 2003). The complex then recruits further proteins to the gene, such as histone acetyltransferase proteins ensuring active chromatin states and positive transcription elongation factor (pTEF-β) for transcript elongation (Frank et al., 2003; Majello et al., 1999). Instead of MYC, MAX can also form a heterodimer with another protein named MAX dimerization protein 1 (MAD), which leads to transcriptional repression of MYC target genes (Ayer et al., 1993).

Beside this, MYC itself also functions as a transcriptional repressor in two scenarios. It forms complexes with MYC-interacting zinc-finger protein 1 (MIZ1), thereby indirectly binding to the DNA (Peukert et al., 1997). MYC-MIZ1 represses the expression of certain cell cycle inhibitory genes, for example *cyclin-dependent kinase inhibitor 1A* (*CDKN1A*, also known as *p21*) or *MAD4* (Kime and Wright, 2003; S. Wu et al., 2003). Furthermore, MYC also interacts with Specific Protein 1 (SP1) (Gartel et al., 2001). SP1 recognizes GC-rich regions and is involved in a range of cellular processes, such as differentiation, growth, cell death and DNA damage response. To achieve this, the MYC(N)-SP1 complex recruits histone deacetylases to its target genes (Iraci et al., 2011). The removal of acetylation marks induces a closed chromatin structure characteristic of transcriptional repression. MYCN also indirectly affects other epigenetic repressors, such as Enhancer of Zeste 2 (EZH2) and other parts of the Polycomb Repressive Complex 2 (PRC2), which is responsible for histone 3 lysine 27 (H3K27) trimethylation (Kaur and Cole, 2013; Neri et al., 2012).
In addition, both MYC and MYCN alter the expression of microRNAs (miRNAs), small non-coding RNAs that repress expression of a whole set of target proteins (Chang et al., 2008; Schulte et al., 2008). Therefore, the impact range of MYC(N) is extensive. For example, several miRNAs from the oncogenic miR-17-92 cluster are regulated by MYC. This cluster controls expression of cell cycle progression protein E2F1, chromatin modifiers and apoptosis regulator BIM (Li et al., 2014; O'Donnell et al., 2005).

Recent publications argued that MYC(N) acts as a transcriptional amplifier of already transcribed genes (Lin et al., 2012; Nie et al., 2012). In contrast, Duffy and colleagues overexpressed MYCN in a non-amplified NB cell line and reported that more genes were downregulated than upregulated in this setting. In addition, a pulse chase experiment revealed that differential gene regulation does not depend on the previous level of expression (Duffy et al., 2015).

Due to its broad effect on gene expression programs, MYCN-amplified (MNA) NB and other cancers are dependent on high levels of the protein. According to the oncogene addiction theory, removal of MYCN should therefore critically hit the tumor. In fact, reducing the MYCN level by short hairpin RNAs (shRNAs) in NB cell lines and mouse models reduces proliferation and tumor incidence (Burkhart et al., 2003). However, targeting MYC or MYCN has proven very difficult in the past (Westermark et al., 2011). Even though some small molecules against MYC-MAX complex formation have been identified recently, they lack in vivo efficiency and are rapidly metabolized (Prochownik and Vogt, 2010). To date, no small molecules targeting MYCN are known. Others tried targeting the bromodomain of putative MYC(N) binding partners by small molecule JQ1, which had anti-proliferative effects and provided a survival advantage in vivo (Delmore et al., 2011; Puissant et al., 2013). While reduction of MYCN expression by RNA interference exhibited promising effects in vitro and in mouse models, there are still technical limitations in the clinic (Westermark et al., 2011). Another strategy aims at identifying genes that are part of the MYCN regulatory network and required for oncogenic activity so that inactivation results in synthetic lethality (Chayka et al., 2015). The inhibition of such a gene will cause tumor cell death but leave normal cells without MYCN amplification (MNA) unaffected.

1.2.3 Chromosomal Instability in Neuroblastoma

Deletions or gains of chromosome parts occur rather frequently in NB, such as gains of 17q (>80% of cases) or deletion of the 11q or 1p chromosome arms (around 30% of cases) (Brodeur et al., 1977; Schleiermacher et al., 2012; Theissen et al., 2014). These alterations are indicators of poorer prognosis. Oftentimes, 17q gain is linked to chromosomal loss at the partner chromosome. For example, 17q may translocate and
attach to chromosome arm 1p (Savelyeva et al., 1994). The recurrence of such events suggests that they might contribute to NB tumorigenesis, for example through the loss of tumor suppressor genes or gain of oncogenes located on the concerned chromosomal arms.

1.2.4 Telomere Lengthening Mechanisms

In recent years, several aberrations affecting telomere maintenance have been identified in NB. In 31% of high-risk cases, the 5p chromosome region is rearranged, which causes very high expression of the neighboring gene for telomerase reverse transcriptase (TERT) (Peifer et al., 2015). High TERT expression also occurs in tumors with MYCN amplification, which is mutually exclusive with TERT rearrangement. Inactivating mutations in the ATRX (alpha thalassemia/mental retardation syndrome X-linked) gene occur predominantly in older patients with chronic disease and adverse outcome (Cheung et al., 2012). They are only found in cases without MNA or TERT rearrangements and are associated with the phenotype of alternative lengthening of telomeres. Taken together, telomere maintenance, which is central to the cancer hallmark unlimited replication, appears to play an essential role in high-risk NB tumorigenesis (Hertwig et al., 2016).

1.3 The Cell Cycle

Cellular proliferation is a tightly regulated process. In order to divide, cells go through a fixed succession of steps and have to pass several checkpoints (Harper and Brooks, 2005). As described above, several of the hallmarks of cancer disrupt aspects of controlled proliferation.
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Progression through the cell cycle phases is driven by specific CDKs and their partner cyclins. Several checkpoints (red bars) prevent cells from proceeding in the event of DNA damage or insufficient growth. R - restriction point. Adapted from Aleem and Arcerci (2015).

Quiescent cells reside in G₀, making up the majority of an organism’s cells. When a quiescent cell receives certain mitogenic growth signals, it re-enters the cell cycle at G₁ phase (Figure 1). In this phase, RNA and proteins are synthesized. Before progressing to S phase, the cell has to pass the restriction point. At this point, cells commit to completing the cell cycle independent of any further growth signals (Pardee, 1974). In the S phase, the cell’s DNA and its centrosomes are duplicated and several checkpoints ensure that replication is complete and potential DNA damage is repaired (Harper and Brooks, 2005). If DNA damage is beyond repair, cell death is initiated as a protective mechanism. In healthy cells, G₂ phase ensues, where the cell prepares for mitosis (M phase) by further protein synthesis and cell growth. During mitosis, nucleus and cytoplasm are divided to form two daughter cells. Progression through the cell cycle is mainly driven by an enzyme family called cyclin-dependent kinases (CDKs).

1.4 Cyclin-Dependent Kinases

1.4.1 Cyclin-Dependent Kinases in the Cell Cycle

CDKs are a family of 20 serine/threonine kinases that share a conserved catalytic core consisting of an ATP-binding pocket (Adenosine triphosphate), a cyclin-binding domain.
and an activating T-loop (Lim and Kaldis, 2013). In their classical function, they need to form a complex with a regulatory subunit from the cyclin family to become activated. The first CDK named Cdc2 was discovered in yeast (Beach 1982, Nurse 1980). Since then, a wealth of research has identified more and more family members, binding partners and functions, as summarized by Malumbres (2014). During the cell cycle, the CDK level remains rather constant, while the different cyclin levels oscillate depending on the cell cycle phase. In the G1 phase, different cyclin D isoforms interact with CDK4 and CDK6. The active complex then phosphorylates Retinoblastoma Proteins (RB), thus liberating transcription factor E2F and allowing expression of genes like cyclin E and A that are necessary for cell cycle progression (Figure 1). CDK2/cyclin E complexes drive transition from G1 into S phase, whereas CDK1/cyclin A complexes are responsible for the initiation of the prophase at G2:M transition. Entry into mitosis is controlled by CDK1/cyclin B complexes, which also regulate centrosome separation (Lindqvist et al., 2007). The activity of cell cycle CDKs is further controlled through activating phosphorylations by CDK7 and through CDK inhibitors (CDKIs) from the INK4 and the CIP/KIP protein family (Kaldis, 1999; Sherr and Roberts, 1999). For example, in the case of DNA damage, p53 binds to the DNA and induces the transcription of p21\textsuperscript{Cip1}. This protein in turn binds to and inhibits CDK2, thereby preventing the transition from G1 to S phase. To abolish G1:S phase arrest, p21\textsuperscript{Cip1} is sequestered by the CDK4,6/cyclin D complex (Sherr and Roberts, 1999).

While the sequence of events in the cell cycle is finely tuned, knockout mouse models have proven that the function of many CDKs can be taken over by other members of the family (Gopinathan et al., 2011).

**1.4.2 Cyclin-dependent Kinases and the Cell Cycle in Cancer**

In many cancer entities, one or more players of the cell cycle are deregulated. Frequently, CDKs 4 or 6 or cyclin D (CCND) or CCNE are overexpressed or the expression of CDKIs or RB is lost due to mutations or chromosomal alterations (Malumbres and Barbacid, 2001). These deregulated components may represent attractive targets in cancer therapy. In the early 2000s, several small molecule CDKIs were developed, such as Flavopiridol and Selicilib. Unfortunately, first clinical studies with CDKIs as single agents showed only very limited effects with suboptimal pharmacokinetics but high toxicity (Diaz-Padilla et al., 2009). All of these CDKIs targeted CDK2 amongst other CDKs, which proved to be replaceable by other family members during cell cycle progression (Ortega et al., 2003). Follow-up studies investigated combination therapies of CDKIs with chemotherapeutics which showed at least some synergies. More potent second-generation CDKIs were designed to be more specific towards single CDKs (Diaz-Padilla et al., 2009). Three
CDK4/6 inhibitors have since demonstrated good activity and have been approved for treatment in advanced breast cancer (Vijayaraghavan et al., 2018). In a range of other solid tumors, ongoing clinical studies are testing the efficiency of CDK4/6 inhibitors. Recent studies demonstrated that up to one third of primary NBs harbor chromosomal aberrations affecting a cell cycle gene (Molenaar et al., 2012). Particularly in high-risk cases, the expression of many cell cycle components is deregulated (Krasnoselsky et al., 2005). Amplifications of CDK4 or CCND1 and loss of CDK inhibitor CDKN2 have been detected in tumors as well as in MNA cell lines (Gogolin et al., 2013; Molenaar et al., 2012). In case of DNA damage, an impaired G1:S arrest has been shown in MNA cell lines despite functional p53 (McKenzie et al., 1999). Most likely, this is due to low levels of p21Cip1 and disturbed downstream signaling (Gogolin et al., 2013; Tweddle et al., 2001). Also, high-risk MNA cases frequently overexpress S-Phase Kinase Associated Protein 2 (SKP2) (Westermann et al., 2007). This protein induces the degradation of cell cycle inhibitors p21Cip1 and p27kip1, thereby promoting G1:S and G2:M phase transition. In p53 wildtype (wt) MNA cell lines, CDK4 suppression via RNA interference (RNAi) or small molecules triggered G1:S arrest and reduced viability (Gogolin et al., 2013). In addition, CDK4 as well as cyclin D inhibition led to neuronal differentiation (Molenaar et al., 2008). Ribociclib (LEE011), a specific CDK4/6 inhibitor, significantly delayed tumor growth in mouse models (Rader et al., 2013). It was further tested in a phase I clinical study, where it achieved stable disease in 47 % of NB patients (Georger et al., 2017).

Molenaar and colleagues also showed that CDK2 is synthetically lethal with high MYCN in NB – a finding that stands in contrast to other entities (2009). A small molecule CDK2 inhibitor was able to reduce tumor size and improve survival in a MYCN transgene mouse model (Dolman et al., 2015). However, these effects cannot be attributed solely to CDK2 inhibition, as it also targets CDKs 1, 4, 5, 6 and 9.

1.4.3 Transcriptional Cyclin-Dependent Kinases

Besides cell cycle regulation, many CDK family members have diverse, often tissue-specific functions, as reviewed by Lim and Kaldis (2013). This includes DNA damage repair (CDK1, 3), epigenetic mechanisms (CDK1, 2), metabolism (CDK5, 8), stem cell renewal (CDK1), spermatogenesis (CDK2, 4, 16) and neuronal function (CDK5, 16). Moreover, a number of CDKs are involved in transcriptional regulation.

Several CDKs sequentially phosphorylate the heptapeptide repeats (YSPTSPS) of the carboxyterminal domain (CTD) of RNA polymerase II (RNA Pol II) during transcription and create a characteristic pattern, the CTD code. Thereby, they impact on RNA Pol II activity, the recruitment of complex members and co-transcriptional processing of pre-mRNA (Sanso and Fisher, 2013). Shortly after transcription is initiated, CDK7/cyclin H within the
Transcription Factor IIA complex phosphorylate serine residues 5 (Ser5) and 7 of the CTD and induce promoter-proximal pausing of RNA Pol II. Next, CDK7 activates CDK9, which together with cyclin T is part of the positive transcription elongation factor b complex (P-TEFb). CDK9 in turn causes pause release and the initiation of elongation by phosphorylating Ser2 (Larochelle et al., 2012). As elongation takes place, Ser5 residues are dephosphorylated, while the amount of phosphorylated Ser2 residues increases (Kohoutek and Blazek, 2012). When transcription terminates, all phosphorylations are removed, which (next to complex interactions with several proteins) primes RNA Pol II for the next round of transcription. During elongation and termination, CDK7, CDK9 and CDK12 activity has been implicated, but the exact mechanisms are still unclear (Fisher, 2017).

CDK8 controls transcriptional repression by preventing the mediator complex from binding RNA Pol II and thereby activating gene expression (Knuesel et al., 2009). In addition, CDK8 and also CDK14 are involved in the WNT/ß-catenin signaling pathway (G. Davidson et al., 2009; Firestein et al., 2008). Even cell cycle CDKs can indirectly impact on gene expression by regulating transcription factors, such as E2F and Forkhead Box Protein M1 (FOXM1) (Lim and Kaldis, 2013). Transcriptional regulation also occurs independently from the kinase function, as is the case for CDK10, which binds to and represses the ETS2 transcription factor (Kasten and Giordano, 2001).

CDK7 inhibition by a covalent inhibitor in NB cells resulted in downregulation of MYCN levels and a decrease in the expression of MYCN target genes (Chipumuro et al., 2014). In a mouse model of high-risk NB, this translated to slower tumor growth.

1.4.4 Cyclin-Dependent Kinases 12 and 13

CDK13 (previously CDC2L5) and the highly homologous CDK12 (previously CrkRS) belong to the group of transcriptional CDKs. Both proteins contain a central kinase domain with 92% shared sequence identity (H. H. Chen et al., 2007). In addition, CDK12 and CDK13 are characterized by serine arginine (SR) and proline-rich motifs in the N-terminus and a low complexity C-terminus with a proline-rich domain, indicating areas of protein-protein interactions (Greenleaf, 2018) (Figure 2). In comparison to other CDKs, CDK12 and 13 are large proteins of around 164 kDa. While early studies claimed cyclin L to be the partner of both CDK12 and CDK13, it turned out that it is in fact cyclin K (Bartkowiak et al., 2010; Blazek et al., 2011). So far, most studies have focused on elucidating CDK12 function and much less is known about CDK13.
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Knockdown of CDK12 and CDK13 by RNA interference (RNAi) in Drosophila and in human cells changes the phosphorylation status of RNA Pol II, arguing for their role as CTD kinases (Bartkowiak et al., 2010; Blazek et al., 2011). Also, chromatin immunoprecipitation experiments demonstrated that CDK12 is found in the middle and at the 3’ end of actively transcribed genes, whereas CDK9 localizes more to the 5’ end. However, the preferred substrate of CDK12/13 is still a matter of debate. Greifenberg and colleagues reported that CDK13 preferentially phosphorylated CTDs with existing phosphorylation marks at Ser7 (2016). They also claimed that CDK13 and CDK12 phosphorylated Ser2 and Ser5 residues, but not Ser7. Davidson et al. suggested that CDK12 might phosphorylate Ser2 and thus contribute to 3 prime end cleavage and polyadenylation (2014). Another study showed that upon CDK12 knockdown, the Ser2 phosphorylation level decreased (Bartkowiak et al., 2010). These conclusions on substrate specificity were based on immunoblotting with pSer antibodies. However, Greenleaf recently pointed out in his review that the extent of antibody binding is influenced by phosphorylation of nearby residues (2018). Consequently, an altered signal only identifies general changes in CTD phosphorylation, but not where exactly these occur. Therefore, conclusions concerning the substrate of CDK12 and CDK13 need to be regarded cautiously. More recently, in vitro phosphorylation studies were able to confirm that CDK12 and CDK13 in complex with cyclin K target predominantly Ser2 and Ser5 (Bosken et al., 2014; Greenleaf, 2018).

Both CDK12 and CDK13 induce alternative splicing when overexpressed in vitro (H. H. Chen et al., 2006; H. H. Chen et al., 2007). This finding is backed up by the observation that the two kinases localize to nuclear speckles (Even et al., 2016; Ko et al., 2001). Also, pull-down assays identified pre-mRNA processing proteins to be associated with CDK12 (Bartkowiak and Greenleaf, 2015). Apart from splicing mechanisms, CDK12 and CDK13 have also been implicated in the regulation of axonal elongation and the maintenance of self-renewal in stem cells (H. R. Chen et al., 2014; Dai et al., 2012).

Several authors performed gene expression analyses to better understand transcriptional targets of CDK12 and CDK13. Blazek et al. found that CDK12/cyclin K particularly

Figure 2: Domain Composition of CDK12 and CDK13
The kinase domains (KD) of CDK12 and CDK13 are flanked by arginine-serine (RS), proline (P), alanine (A) and serine (S) rich domains. Adapted from Kohoutek and Blazek (2012).
regulated long genes with many exons (2011). In their study using human embryonic kidney cells, genes involved in DNA damage repair (DDR) mechanisms were enriched in the set of downregulated genes after knockdown of CDK12 or CCNK, but not CDK13. CDK12 and CCNK knockdown also induced sensitivity towards DNA damaging agents. Greifenberg et al. reported that in colon cancer cells, CDK13 knockdown resulted in the up- and downregulation of a similar number of genes, while CDK12 knockdown induced more downregulation than upregulation (2016). There were relatively few commonly up- or downregulated genes (~25 % of CDK13 target genes). CDK12 knockdown mainly downregulated expression of genes involved in DNA replication and DNA damage, while CDK13 knockdown regulated extracellular and growth signaling pathways and metabolic processes. A different study by Liang and colleagues reported that both CDK12 and CDK13 knockdown by shRNAs almost exclusively led to target gene downregulation (2015). They also reported a much larger overlap of target genes (74 % of CDK13 genes). CDK12 knockdown affected genes involved in RNA processing, DNA repair, chromosome organization and the cell cycle, whereas CDK13 knockdown also affected RNA processing and in addition translation, energy metabolism genes and the expression of small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA). The large discrepancies between these gene expression studies leave open the question in how far CDK12 and CDK13 assume the same or distinct functions in the cell.

Both kinases have been implicated in cancer. CDK13 is amplified in in approximately 30% of hepatocellular carcinomas (Kim et al., 2012). Alterations of the CDK12 gene, such as mutations, rearrangements and amplifications occur in a large number of cancer entities, for example in breast and ovarian cancer (Chila et al., 2016). For instance, a study on ovarian cancer revealed homozygous mutations in about 3% of examined cases, suggesting that CDK12 functions as a tumor suppressor gene. Most CDK12 mutations identified were located in the kinase domain, rendering the protein dysfunctional. As a consequence, the expression of CDK12 target genes was decreased. This was observed for genes involved in DDR like ATM (Ataxia Telangiectasia Mutated), ATR (Ataxia Telangiectasia And Rad3 Related) or RAD51C (Ekumi et al., 2015). In contrast, two other studies in ovarian and prostate cancers with CDK12 inactivation found that DDR genes were not downregulated (Popova et al., 2016; Y. M. Wu et al., 2018). It appears that nonfunctional CDK12 leads to genomic instability in ovarian and prostate cancer in the form of random tandem duplications.
1.5 Aim and Objectives of the Study

Particularly high-risk NB patients with amplification of the MYCN transcription factor face a bad prognosis despite intensive therapy. Therefore, new treatment strategies based on a better understanding of neuroblastoma biology are desperately needed. High abundance of MYCN protein deeply alters the transcriptional signature of affected cells (Oberthuer et al., 2015). Proliferation and especially progression through the cell cycle are accelerated regardless of checkpoints (Bell et al., 2006; Lutz et al., 1996). While it seems obvious that the tumor cells depend on this MYCN signature to maintain this malignant phenotype, the exact transcriptional consequences of high MYCN levels are not completely understood yet.

The first part of this study deals with the gene expression analysis of synchronized NB cells with regulatable MYCN levels as they progress through the cell cycle. It takes into account both RNA and miRNA expression. The use of synchronized cells facilitates the discrimination between cell cycle and MYCN effects at distinct stages of the cell cycle. A promising approach for the identification of new targets is to exploit potential vulnerabilities that arise from high MYCN. A synthetic lethal screen was performed in order to identify vulnerabilities connected to aberrant MYCN and revealed several interesting candidates. The second part of this study deals aims at the validation and ensuing characterization of the candidate CDK13 and the highly homologous CDK12 as potential novel therapeutic targets in MNA tumors.
2 MATERIAL AND METHODS

2.1 Materials

2.1.1 Chemicals

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<td>4,6-diamidino-2-phenylindol (DAPI)</td>
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<td>Formaldehyde</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Giemsa Azure Eosin Methylene Blue</td>
<td>Merck, Darmstadt</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Goat serum</td>
<td>Jackson ImmunoResearch, West Grove, USA</td>
</tr>
<tr>
<td>HEPES KOH</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Lipofectamine RNAiMAX</td>
<td>Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Methanol (MeOH)</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
</tr>
<tr>
<td>Propidium iodide staining solution (PI)</td>
<td>Miltenyi Biotec, Bergisch Gladbach</td>
</tr>
</tbody>
</table>
Polyacrylamide (PAA) 
Potassium acetate 
Potassium chloride 
Ponceau S Solution 
Sodium acetate 
Sodium chloride 
Sodium dodecyl sulfate (SDS) 
Sodium hydroxide 
Sucrose 
TEMED 
Tris base 
Tris-HCl 
Triton X-100 
Trypan Blue 
Tween 20 
Yeast extract

2.1.2 Kits & Ready-Made Reagents

Agencourt AMPure XP
Agilent Kits (High Sensitivity DNA, RNA 6000 Nano, Small RNA)
AP-O-BrdU TUNEL assay kit
Caspase-Glo 3/7 Assay
CellTiter-Blue® Cell Viability Assay
Clarity Western ECL Substrate
cOmplete, Mini Protease Inhibitor Tablets
CytoTox ONE Homogeneous Membrane Integrity Assay
ECL Select Western Blot Detection Reagent
Effectene Transfection Reagent
Expand Long Range dNTPack
GeneArt Site Directional Mutagenesis Plus Kit
LabChip XT DNA 300
Laemmli Sample buffer x4
### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>MATERIAL / METHOD</th>
<th>MANUFACTURER / LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipofectamine RNAiMAX</td>
<td>Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Mammalian Protein Extraction Reagent (M-PER)</td>
<td>Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>NEBNext Ultra RNA Library Prep Kit for Illumina</td>
<td>NEB, Ipswich, USA</td>
</tr>
<tr>
<td>NEBNext Small RNA Library Prep Set for Illumina</td>
<td>NEB, Ipswich, USA</td>
</tr>
<tr>
<td>NuPAGE 3-8% Tris-Acetate Midi Gels</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>NuPAGE Tris-Acetate SDS Running Buffer (20X)</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>PhosSTOP Phosphatase Inhibitor Cocktail Tablets</td>
<td>Roche / Merck, Darmstadt</td>
</tr>
<tr>
<td>NEBNext Small RNA Library Prep Set for Illumina</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Protein Assay Dye Reagent Concentrate</td>
<td>Bio-Rad, Munich</td>
</tr>
<tr>
<td>Qiagen Plasmid Maxi Kit</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QIAprep Spin Miniprep Kit</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>miRNeasy Kit</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>QiazoL Lysis Reagent</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>Qubit Assay Kits (dsDNA BR, dsDNA HS, RNA BR, RNA HS, microRNA)</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>Ribo-Zero Gold rRNA Removal Kit</td>
<td>Illumina, San Diego, USA</td>
</tr>
<tr>
<td>RNase-free DNase Set</td>
<td>Qiagen, Hilden</td>
</tr>
<tr>
<td>SOC medium</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
</tbody>
</table>

### 2.1.3 Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MANUFACTURER / LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accu-Prime Pfx</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>DNA restriction enzymes</td>
<td>NEB, Ipswich, USA</td>
</tr>
<tr>
<td>DNase I</td>
<td>Fermentas, St.Leon-Rot, NEB, Ipswich, USA</td>
</tr>
<tr>
<td>RNase A</td>
<td>Roche / Merck, Darmstadt</td>
</tr>
<tr>
<td>SuperScript IV Reverse Transcriptase</td>
<td>Invitrogen / Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>T4 Polynucleotide Kinase (PNK)</td>
<td>NEB, Ipswich, USA</td>
</tr>
</tbody>
</table>
T7 DNA ligase  
NEB, Ipswich, USA

### 2.1.4 Drugs and Inhibitors

- **Bafilomycin A1**  
  Abcam, Cambridge, England
- **BAY-587**  
  kindly provided by Bayer AG
- **Ferrostatin-1**  
  Sigma Aldrich / Merck, Darmstadt
- **Necrostatin-1**  
  Abcam, Cambridge, England
- **RiboLock RNase Inhibitor**  
  Thermo Fisher Scientific, Waltham, USA
- **RNaseOUT Recombinant Ribonuclease Inhibitor**  
  Invitrogen / Thermo Fisher Scientific, Waltham, USA
- **THZ-531**  
  kindly provided by Bayer AG
- **z-VAD-FMK**  
  Sigma Aldrich / Merck, Darmstadt

### 2.1.5 Buffers and Solutions

**1x PBS**  
137 mM NaCl  
2.7 mM KCl  
10 mM Na$_2$HPO$_4$  
pH 7.4

**LB medium**  
5 g/l yeast extract  
10 g/l bacto tryptone  
5 g/l NaCl

**Versene**  
0.02 % EDTA  
1x PBS

#### 2.1.5.1 Agarose Gel Electrophoresis (DNA Separation)

- **Agarose gel**  
  x% Agarose  
  1x TBE  
  89 mM Tris  
  89 mM Boric acid  
  2 mM EDTA

#### 2.1.5.2 Immunoblotting

- **Lysis buffer**  
  10 ml M-PER  
  40 µl EDTA [2 mM]  
  1x cOmplete Mini  
  1x PhosSTOP  
  10 mM Tris, pH 7.6  
  150 mM NaCl  
  5 % Tween 20
### MATERIAL AND METHODS

**NuPAGE transfer buffer (10x)**  
25 mM Bicine  
25 mM Bis-Tris (free base)  
1 mM EDTA  

pH 7.2  

Prepare 1x solution with 10 % MeOH

**Blocking solution**  
5 % milk powder or BSA in 1x TBS-T

**Complex blocking solution**  
20 % milk powder

20 % FCS

3 % BSA

1 % goat serum

0.2 % Tween 20

in 1x TBS

### 2.1.5.3 Flow Cytometry

**Citric acid buffer**  
2.1 g citric acid  
500 μl Tween 20  
100 ml H2Odd

### 2.1.5.4 Molecular Cloning

**Annealing buffer**  
100 mM potassium acetate  
30 mM HEPES KOH  
2 mM magnesium acetate

### 2.1.6 Neuroblastoma Cell Lines

CHP-134  
HCT116  
IMR-32  
IMR-5-75  
NB-69  
SH-EP TET21N  
SH-EP  
SK-N-BE(2)c  
SK-N-DZ  
TR-14  

Schlesinger *et al.* (1976)

Brattain *et al.* (1981)


Tumilowicz *et al.* (1970)


Lutz *et al.* (1996)

Ross, *et al.* (1983)

Biedler and Spengler (1976)


Cowell and Rupniak (1983)
### Table 2.1: Cell Culture Media Composition

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Additive</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>RPMI 1640 (500 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic medium</td>
<td>FCS</td>
<td>10 %</td>
</tr>
<tr>
<td></td>
<td>P/S</td>
<td>1%</td>
</tr>
<tr>
<td>Freezing medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI 1640</td>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>FCS</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>10%</td>
</tr>
<tr>
<td>IMR-5-75 6TR, IMR-32 6TR</td>
<td>Blasticidin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>IMR-5-75 6TR XYsh, IMR-32 6TR XYsh</td>
<td>Zeocin</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Blasticidin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>IMR-5-75 6TR dCas9</td>
<td>Blasticidin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td></td>
<td>G418</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td>IMR-5-75 6TR dCas9 sgXY</td>
<td>Blasticidin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td></td>
<td>G418</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>IMR-5-75 6TR dCas sg6 pcDNA5/TO</td>
<td>Blasticidin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td></td>
<td>G418</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Hygromycin B</td>
<td>150 µg/ml</td>
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</table>
### 2.1.7 Antibodies

Table 2.2: Primary Antibodies for Immunoblotting

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha Tubulin (DM1A) - HRP</td>
<td>Mouse monoclonal</td>
<td>1:10000</td>
<td>Abcam, Cambridge, England</td>
<td>ab40742</td>
</tr>
<tr>
<td>CDK12 (CrkRS)</td>
<td>Rabbit polyclonal</td>
<td>1:8000</td>
<td>Novus, Minneapolis, USA</td>
<td>NB100-87011</td>
</tr>
<tr>
<td>CDK12 (CrkRS)</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Abcam, Cambridge, England</td>
<td>ab57311</td>
</tr>
<tr>
<td>CDK12 (CrkRS)</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>Cell Signaling Technology, Danvers, USA</td>
<td>#11973</td>
</tr>
<tr>
<td>CDK13 (CDC2L5)</td>
<td>Rabbit polyclonal</td>
<td>1:8000</td>
<td>Novus, Minneapolis, USA</td>
<td>NB100-68268</td>
</tr>
<tr>
<td>CDK13 (CDC2L5)</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Abcam, Cambridge, England</td>
<td>ab58309</td>
</tr>
<tr>
<td>CDK13 (CDC2L5)</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>Sigma Aldrich / Merck, Darmstadt</td>
<td>SAB 2700810</td>
</tr>
<tr>
<td>Vinculin (7F9) - HRP</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Santa Cruz, Dallas, USA</td>
<td>sc-73614</td>
</tr>
</tbody>
</table>
Table 2.3: Secondary Antibodies for Immunoblotting

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Host</th>
<th>Supplier</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-HRP</td>
<td>Goat, polyclonal</td>
<td>Dianova, Hamburg</td>
<td>115-035-003</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>Goat, polyclonal</td>
<td>Dianova, Hamburg</td>
<td>115-035-144</td>
</tr>
<tr>
<td>Anti-rat IgG-HRP</td>
<td>Goat</td>
<td>Santa Cruz, Dallas, USA</td>
<td>sc-2065</td>
</tr>
</tbody>
</table>

2.1.8 *E. coli* Cultivation

- One Shot Top10
  Invitrogen / Thermo Fisher Scientific, Waltham, USA
- One Shot MAX Efficiency DH5α-T1R
  Invitrogen / Thermo Fisher Scientific, Waltham, USA
- One Shot ElectroMAX Stbl4
  Invitrogen / Thermo Fisher Scientific, Waltham, USA

Table 2.4: Antibiotics Used for the Cultivation of *E. coli*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 µg/ml</td>
<td>Serva, Heidelberg</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 µm/ml</td>
<td>Serva, Heidelberg</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>50 µg/ml</td>
<td>Serva, Heidelberg</td>
</tr>
</tbody>
</table>

2.1.9 Nucleic Acids

- GeneRuler 1 kb (Plus) DNA Ladder
  Thermo Fisher Scientific, Waltham, USA
- ERCC RNA Spike-In Control Mix 1
  Thermo Fisher Scientific, Waltham, USA
- NEBNext Multiplex Oligos for Illumina (Index Primers Set 1 + 2)
  NEB, Ipswich, USA
- Adenosine 5′-Triphosphate (ATP)
  NEB, Ipswich, USA
### Material and Methods

#### Table 2.5: Vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Specification</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA6/TR</td>
<td>Tetracycline repressor (TR) protein</td>
<td>Invitrogen, Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>pcDNA5/TO</td>
<td>Tetracycline-regulated expression</td>
<td>Invitrogen, Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>pT-RexTM-DEST30</td>
<td>Tetracycline-regulated expression</td>
<td>Invitrogen, Thermo Fisher Scientific, Waltham, USA</td>
</tr>
<tr>
<td>pLKO.1-puro U6 sgRNA BfuAI stuffer</td>
<td>sgRNA cloning vector</td>
<td>Kearns, et al. (2014), Addgene plasmid # 50920</td>
</tr>
<tr>
<td>AAVS1 TALEN F</td>
<td>Homodimeric TALEN, right pair</td>
<td>Mandegar, et al. (2016)</td>
</tr>
<tr>
<td>AAVS1 TALEN R</td>
<td>Homodimeric TALEN, left pair</td>
<td>Mandegar, et al. (2016)</td>
</tr>
<tr>
<td>pAAVS1-NDi-CRISPRi (Gen1)</td>
<td>Dox-inducible CRISPRi knock-in construct w. mCherry marker</td>
<td>Mandegar, et al. (2016)</td>
</tr>
</tbody>
</table>

#### Table 2.6: Oligonucleotides for Short Hairpin RNA (shRNA) Vectors

<table>
<thead>
<tr>
<th>shRNA oligonucleotides</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK12 sh1 for</td>
<td>GATCCGCCAAGCAATATCGAATTCAAGAGATTCGAT&lt;br&gt;ATTGCTTTCCTCGGTGTTTTTTGGAAA</td>
</tr>
<tr>
<td>CDK12 sh1 rev</td>
<td>CGCCGAAGCAATATCGAATTCAAGAGATTCCGATATTG&lt;br&gt;CTTCTTCGGAATTGTTTTTGGAAAAGCT</td>
</tr>
<tr>
<td>CDK12 sh3 for</td>
<td>GATCCGGACTTGCTCGGCTCTATATTCAAGAGATATAGAG&lt;br&gt;CCGAGCAAGTCTTGTGTTTTGGAAA</td>
</tr>
</tbody>
</table>
## MATERIAL AND METHODS

### shRNA oligonucleotides

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK12 sh3 rev</td>
<td>CGGACTTGCTCGGCTCTATATTCAAGAGATATAGAGCCGA</td>
</tr>
<tr>
<td></td>
<td>GCAAGTCCCTTTTTGGAAAAAGCT</td>
</tr>
<tr>
<td>CDK13 sh1 for</td>
<td>GATCCGGGAACCTTGCAACAACTAGAATTCAAGAGATTCTAGT</td>
</tr>
<tr>
<td></td>
<td>TGTGCAAGTCCCTTTTTGGAAAA</td>
</tr>
<tr>
<td>CDK13 sh1 rev</td>
<td>CGGAACCTTGCAACAACTAGAATTCAAGAGATTCTAGTTGTG</td>
</tr>
<tr>
<td></td>
<td>CAAGTCCCTTTTTGGAAAAAGCT</td>
</tr>
<tr>
<td>CDK13 sh3 for</td>
<td>GATCCGCAGATTGTCTAGATCCAGATTTCAAGAGATCTGGGA</td>
</tr>
<tr>
<td></td>
<td>TCTAGACAATCTGTTTTTTGGAAA</td>
</tr>
<tr>
<td>CDK13 sh3 rev</td>
<td>CGCAGATTGTCTAGATCCAGATTTCAAGAGATCTGGATCTA</td>
</tr>
<tr>
<td></td>
<td>GACAATCTGTTTTTTGGAAAAAGCT</td>
</tr>
<tr>
<td>Scramble for</td>
<td>CTAGCGCAGTCGCGTTTGCAGACTGTTCAAGAGACCAGT</td>
</tr>
<tr>
<td></td>
<td>CGCAAAACCGCACTGTTTTTTGGAAAAG</td>
</tr>
<tr>
<td>Scramble rev</td>
<td>CGCAGTCGCCGTTTGCAGACTGTTCAAGAGACCAGTCGCA</td>
</tr>
<tr>
<td></td>
<td>AACGCGACTGTTTTTTGGAAAGCAGGCGCC</td>
</tr>
</tbody>
</table>

Table 2.7: Silencer Select Small Interfering RNAs (siRNAs) for Transient Gene Knockdown (Ambion)

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence 5'-3'</th>
<th>siRNA ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK12 (CRKRS) si1</td>
<td>CCGAAGAAGCAUAUCGAAAtt</td>
<td>s28621</td>
</tr>
<tr>
<td>CDK12 (CRKRS) si3</td>
<td>GGACUUGUCUGCGCUAUAtt</td>
<td>s28623</td>
</tr>
<tr>
<td>CDK13 (CDC2L5) si1</td>
<td>GGAACUUGACAAACUAGAAtt</td>
<td>s16399</td>
</tr>
<tr>
<td>CDK13 (CDC2L5) si3</td>
<td>CAGAUUGUCAGAUCAGAAtt</td>
<td>s16397</td>
</tr>
<tr>
<td>Silencer Select Neg Ctl 1</td>
<td>Proprietary</td>
<td>4390843</td>
</tr>
<tr>
<td>Silencer Select Neg Ctl 2</td>
<td>Proprietary</td>
<td>4390846</td>
</tr>
</tbody>
</table>
### Table 2.8: Oligonucleotides for Single Guide RNA (sgRNA) Vectors

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA_scr2_rev</td>
<td>AAACCGTGTCAGGGCACGTTTGCCGC</td>
</tr>
<tr>
<td>sgRNA_scr2_fw</td>
<td>ACCGGCGCCAACGTGCCCCTGACGG</td>
</tr>
<tr>
<td>sgRNA_scr3_fw</td>
<td>ACCGGCACTACCAGAGCTAACTCA</td>
</tr>
<tr>
<td>sgRNA_scr3_rev</td>
<td>AAACTGAGTTAGCTCTGTAGTG</td>
</tr>
<tr>
<td>sgRNA10_fw</td>
<td>ACCGTGGTGGCGCGCAGCACCCGC</td>
</tr>
<tr>
<td>sgRNA10_rev</td>
<td>AAACGCCTGTGTGTCGCCGCACCA</td>
</tr>
<tr>
<td>sgRNA6_fw</td>
<td>ACCGTGACAACCAACTCCCGCGCCTAGGG</td>
</tr>
<tr>
<td>sgRNA6_fw neu</td>
<td>ACCGTGACAACCAACTCCCGCGCCTAGGG</td>
</tr>
<tr>
<td>sgRNA6_rev</td>
<td>AAACCCCTAGGCCGGAGTGTGTGTA</td>
</tr>
<tr>
<td>sgRNA6_rev neu</td>
<td>AAACTAGGCCGGAGTGTGTGTA</td>
</tr>
<tr>
<td>sgRNA8_fw</td>
<td>ACCGGGAGGACGACGTCGACGCCGAGG</td>
</tr>
<tr>
<td>sgRNA8_fw neu</td>
<td>ACCGGGAGGACGACGTCGACGCCGAGG</td>
</tr>
<tr>
<td>sgRNA8_rev</td>
<td>AAACCCCTCGGCGTCGACGCCGCTCC</td>
</tr>
<tr>
<td>sgRNA8_rev neu</td>
<td>AAACCGCGTCGACGTCGCTCC</td>
</tr>
<tr>
<td>CDK12 2b_fw</td>
<td>CACCGACTGACCGACTGCCCCTCAGTCG</td>
</tr>
<tr>
<td>CDK12 2b_rev</td>
<td>AAACCGAGAAGGCAGTCGGTGCAGTC</td>
</tr>
<tr>
<td>CDK13 I1_fw</td>
<td>CACCGATCAAAAACAAACCATGTGA</td>
</tr>
<tr>
<td>CDK13 I1_rev</td>
<td>AAACTCACATGGTTTTGGTTGTGATC</td>
</tr>
<tr>
<td>scramble 8.2_fw</td>
<td>CACCGAAAAAGCTTCCCGCCTGATGG</td>
</tr>
<tr>
<td>scramble 8.2_rev</td>
<td>AAACCCATCGCGGAAGCTTTTTC</td>
</tr>
</tbody>
</table>
### Table 2.9: Sequencing Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGH_rev</td>
<td>TAGAAGGCACAGTGCAGG</td>
</tr>
<tr>
<td>CDK12 Amp Zusatz fw</td>
<td>GCAGCAAGATGGATGGAAGG</td>
</tr>
<tr>
<td>CDK12 Amp Zusatz rev</td>
<td>TTCAAGCTTCGTTTTTCTG</td>
</tr>
<tr>
<td>CDK12 Ersatz 1</td>
<td>GATCGCCCAGTCCCTATGGT</td>
</tr>
<tr>
<td>CDK12 Ersatz 2</td>
<td>CTCAGGCAAATTCTCAGCCC</td>
</tr>
<tr>
<td>CDK13 fw Zusatz 1</td>
<td>CGACGTGTCCTAGTCCCC</td>
</tr>
<tr>
<td>CDK13 fw Zusatz 2</td>
<td>CAAAGAAAGATATTGACTGG</td>
</tr>
<tr>
<td>CDK13 Zusatz 3</td>
<td>GTGGAAGCGAGAAAGGA</td>
</tr>
<tr>
<td>CDK13 Zusatz 4</td>
<td>ACCAAAGAAAGCAATTCGGA</td>
</tr>
<tr>
<td>H1 fw</td>
<td>TCGCTATGTGTCTGGGAAA</td>
</tr>
</tbody>
</table>

### Table 2.10: Amplification Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK12 Amp V4 fw</td>
<td>GTCACTCTTAAGGCCACCATGCCAATTTCAGAGAGA</td>
</tr>
<tr>
<td>CDK12 Amp V4 rev</td>
<td>AGTAGCGGATCCCTAGTAAGGAACTCTCTCCCTCTCCT</td>
</tr>
<tr>
<td>CDK13 Amp V4 fw</td>
<td>CTTACACTTAAAGGCCACCATGGCCGAGCAGCT</td>
</tr>
<tr>
<td>CDK13 Amp V4 rev</td>
<td>GTGCATCTCGAGCTAGTATGGTAACCCCTCTGCTCCT</td>
</tr>
</tbody>
</table>
### Table 2.11: Site-Directed Mutagenesis Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK12_3252_FW</td>
<td>GAACTAGTGCTCTGGCAGGAGTGACTAGA</td>
</tr>
<tr>
<td>CDK12_3252_RV</td>
<td>TCTAGTCTCACCTTCGCCCAGGCACTAGTC</td>
</tr>
<tr>
<td>CDK12_3615_FW</td>
<td>CAAATCAAACTAGCAAAATTTTGACTTGCT</td>
</tr>
<tr>
<td>CDK12_3615_RV</td>
<td>GAGCAAGTCAAAAATTTGCTAGTTTGATT</td>
</tr>
<tr>
<td>CDK13 Mut fw1</td>
<td>CTGGAGAAATGGTAGCCTAGCAAAAGTACGT</td>
</tr>
<tr>
<td>CDK13 Mut fw2</td>
<td>GAGGCCAGATAAAAACCTTGGAAAAACTTTTG</td>
</tr>
<tr>
<td>CDK13 Mut rev1</td>
<td>CATATCCAGACGTACTTTTGCTAAGGCTAC</td>
</tr>
<tr>
<td>CDK13 Mut rev2</td>
<td>CAATCGAGCAAAGTTGGCAAGTTTTATCT</td>
</tr>
</tbody>
</table>

### 2.1.10 Laboratory Equipment

- **Analytical Balances PM 4600** Mettler, Gießen
- **ChemiSmart 5100** Vilber Lourmat, Marne-la-Vallée, France
- **CO₂ Incubator Steri-Cult** Thermo Fisher Scientific, Waltham, USA
- **Criterion Vertical Electrophoresis Cell** Bio-Rad, Munich
- **Criterion Blotter** Bio-Rad, Munich
- **Gel documentation system (Geldoc)** Bio-Rad, Munich
- **Horizontal mini-gel systems** GIBCO/BRL Eggenstein Renner, Darmstadt
- **Horizontal mixer RM5** CAT, Staufen
- **iBlot 2 Gel Transfer Device** Thermo Fisher Scientific, Waltham, USA
- **Incubator Function Line** Heraeus, Wehrheim
- **Incubator Shaker, Innova 4300** New Brunswick Scientific, Enfield, USA
- **LightCycler 480** Roche / Merck, Darmstadt
- **Luna Automated Cell Counter** Logos biosystems, Annadale, USA
- **MACSQuant VYB flow cytometer** Miltenyi Biotec, Bergisch Gladbach
- **Magnetic Mixers** Heidolph-Elektro, Kehlheim
Mini Trans-Blot Cell
Mini-PROTEAN 3 electrophoresis system
NanoDrop Spectrophotometer ND-1000
pH-Meter Ph 540 GLP
Platereader FLUOstar OPTIMA
Power supply units, Phero-stab 500
Shaking platform, IKA KS250
Spark Microplate Reader
Spectrophotometer GeneQuant 1300
Sterile bench SAFE 2020
Thermo block mixer compact
Thermo water bath GFL 1083
Thermocycler GeneAmp 9700
Vacuum concentrator, RVC2-18
Vortex Reax top

Centrifuges and Rotors
Avanti-JS-25-I
Allegra X-12
Biofuge fresco
J2-21 M/E
Mini Star
Rotor JA-10
Rotor JA-20
Rotor JS-4.2

Microscopes
Olympus IX81
Olympus CKX41

Bio-Rad, Munich
Bio-Rad, Munich
Peqlab, Erlangen
WTW, Weilheim
BMG Labtech, Ortenberg
Biotec Fischer, Reiskirchen
Janke & Kunkel, Staufen
Tecan, Männedorf, Switzerland
GE Healthcare, Munich
Thermo Fisher Scientific, Waltham, USA
Eppendorf, Hamburg
GFL, Burgwedel
Applied Biosystems, Darmstadt
Christ, Osterode am Harz
Heidolph Instruments, Schwabach
Beckman Coulter, Sinsheim
Beckman Coulter, Sinsheim
Heraeus, Wehrheim
Beckman Coulter, Sinsheim
Neolab, Heidelberg
Beckman Coulter, Sinsheim
Beckman Coulter, Sinsheim
Beckman Coulter, Sinsheim
Olympus, Hamburg
Olympus, Hamburg
2.1.11 Further Materials

- Cell culture dishes
- Cell culture flasks
- Nunc CryoTubes (2 ml)
- Cuvettes Semi-Micro
- FACS tubes with cell-strainer cap
- Filter tips, graduated (10, 100, 200, 1000 µl)
- Luna Cell Counter Slides
- Mr. Frosty Freezing Container
- Nitrocellulose membrane 0.45 µm
- Plastic pipettes (5, 10, 25, 50 ml)
- qPCR 96 well plates, white
- qPCR optical adhesive film
- Reaction tubes (0.5, 1.5, 2.0, 5.0 ml)
- Reaction tubes (15, 50 ml)
- Tissue culturing plates (black, flat bottom 96 wells)
- Tissue culturing plates (transparent, 6, 24, 96 wells)
- Whatman 3MM paper

2.1.12 Software

- Cell B Image Software
- Chromas Lite 2.1
- FlowJo version 10
- FLUOstar Optima
- IGV viewer
- ImageJ version 1.51d
- Microsoft Office package 2010
- R studio
- ScanR acquisition software
Material and Methods

2.1.13 Web Resources

Addgene
DAVID functional annotation tool 6.8
Ensembl genome browser
IC50 Calculator
mirPath v.3 algorithm
National Center for Biotechnology
R2
UCSC Genome Browser

2.2 Methods

2.2.1 Cell Culture Methods

2.2.1.1 Culture and Cryoconservation of Human Neuroblastoma Cells

Neuroblastoma cells were cultured in basic medium with the appropriate selective markers as listed in Table 2.1 in a humidified cell incubator at 37 °C and 5 % CO₂. Every four to seven days the cells were subcultured at ratios from 1:4 to 1:15, according to the individual cell confluence and proliferation rate. Versene or fresh medium were employed to detach the cells from the surface of the culture vessel. Cells were collected for cryoconservation when they reached around 70 % confluence; after centrifugation at 800 rpm for 5 min, they were resuspended in freezing medium and aliquoted into cryovials. The vials were stored in a freezing container and immediately transferred to -80 °C. Cells were stored in liquid nitrogen tanks at -196 °C for the long term. To recultivate cells, they were thawed quickly and dispensed into pre-warmed basic medium. After 48 hours, the medium was replaced with basic medium containing selective markers as applicable.

For experiments, the cell number was determined with a Luna Automated Cell Counter after trypan blue staining (1:1 dilution of 0.1 % trypan blue in PBS). Cells were seeded and, if applicable, doxycycline was directly added to the medium for the induction of
regulatable vectors unless otherwise specified. After completion of the experiment, medium and attached cells were collected for further processing.

### 2.2.1.2 Cell Cycle Synchronization

IMR-5-75 MYCN shRNA cells were synchronized according to the protocol developed by Emma Bell (Ryl *et al.*, 2017). 24 hours after seeding, 1 µg/ml tetracycline or 70 % EtOH was added to the medium. Another 24 hours later, 2 mM thymidine were added. After 18 hours, the medium containing thymidine was removed and cells were washed once before adding fresh medium containing tetracycline. Cells were harvested at regular intervals for the next 22 hours to analyze cell cycle, RNA and miRNA expression.

### 2.2.1.3 Cell Transfection and Selection

DNA vectors were introduced into our cell lines with Effectene Transfection Reagent. Cells of a low passage were plated on a 15 cm dish so that they would reach around 40 % confluence the following day. 3 µg plasmid DNA were diluted in 450 µl EC buffer plus 24 µl Enhancer. After a 2 min incubation step, 90 µl of Effectene Transfection Reagent were added and the DNA mix was vortexed and incubated again for 15 min. Then, 4.5 ml basic medium were added gently to the mix. The medium was removed from the cell culture plate, and 10.5 ml fresh basic medium were added. The DNA transfection mix was added dropwise. After 24 h, the medium was replaced. After another 48 to 72 h, cells were detached from the plate and the full amount was replated, now with the appropriate selection marker. A non-transfected control plate was also exposed to the selection marker in order to determine the time point when all cells not carrying the corresponding resistance gene were killed. After 1-3 weeks, when transfected positive cells had regrown, they were seeded onto 96 well plates at one cell/100 µl for single clone selection. After 6 days, true single clones were identified by visual inspection due to circular, even colony shape and the absence of further colonies in one well. Single clones were raised and analyzed with regard to the expression of the introduced vectors by immunoblotting (chapter 2.2.4).

### 2.2.1.4 Lentiviral Transduction

IMR-5-75 cells were seeded in a 12-well plate (2.5*10^5 cells). The following day, medium was removed and replaced by 500 µl of RPMI + FCS + 8 µg / µl polybrene. Lentivirus containing the lentiCRISPRv.2-706 vector was slowly added to the cells (provided by Friederike Herbst). After 16 hours, the medium was removed, cells were washed once
and fresh medium was added. The polyclonal culture was expanded for validation of the knockout and further use in experiments.

### 2.2.1.5 Gene Knockdown with siRNA

Cells were seeded in 10 cm dishes 24 h before the transfection and doxycycline was directly added where shRNA induction was desired. For transfection, the siRNA stock was diluted in 500 µl serum- and antibiotic-free Opti-MEM medium to a final concentration of 100 nM. Depending on the cell line, 4-5 µl Lipofectamine RNAiMAX were diluted in 500 µl medium in a second tube. The solutions were incubated on the bench for 5 minutes. Then they were combined and incubated for 15 min to allow complex formation. Meanwhile, the medium of the pre-seeded dishes was exchanged with 9 ml RPMI / FCS plus doxycycline, where applicable. The transfection solution was added drop-wise to the dishes. After 72 h, the cells were harvested for further analysis of the achieved knockdown (immunoblotting, cell cycle, cell death).

### 2.2.1.6 CDK12/13 Inhibition

Inhibition of CDK12/13 was achieved using the tool compound BAY-587 or the commercially available THZ531. At first, the IC50 values were determined individually for each cell line. Cells were seeded and after 24 hours treated with a dilution series of the inhibitors. Another 96 hours later, a CellTiter-Blue Assay (CTB) was performed to determine viability. The relative IC50 values were calculated with the four parameter logistic regression model using the online tool “IC50 Calculator” by AAT Bioquest. For experiments, cells were treated with multiples of the IC50 as described above. Cell cycle, cell death and cell confluence were assayed after 96 hours. Apoptosis was assayed after 24 hours, RNA and protein expression after 8 hours.

### 2.2.1.7 Viability Assays

After an experiment, such as treatment with siRNAs or induction of shRNAs, viability of the cells was established by CTB. The reagent was added at a 1:10 ratio to the cells and incubated for 4 hours. Fluorescence resulting from metabolic activity of the cells was recorded using a 540/580 filter with a FLUOstar platereader (0.2 s acquisition time, automatic gain) or Tecan Spark reader (flashes: 30, integration time: 40 µs, gain: 58).

A second measure of viability after treatment was cell confluence. Here, cells were seeded and treated in 96-well plates in a volume of 100 µl. After completion of the experiment, 50 µl of 11 % glutaraldehyde in PBS were added for 30 min to fix the cells.
Subsequently, cells were washed two times with 1x PBS before overnight staining with 10 % Giemsa Azure Eosin Methylene Blue solution in 1x PBS. This was followed by two washes with 1x PBS and a final wash with H2O. After drying, the plates were scanned and confluency was determined with the help of the colony formation plugin of the ImageJ software.

2.2.1.8 Colony Formation Assay

Single cells were seeded at very low density in 6-well plates. They were incubated for 7-10 days, depending on the cell line’s proliferation rate. When colonies could be detected macroscopically, the cells were fixed and stained with Giemsa solution as described above.

2.2.1.9 Rescue from Cell Death

In order to determine the mode of cell death, several inhibitors were used. 30 µM Z-VAD-FMK was employed as an inhibitor of apoptosis, 2.5 – 5 µM ferrostatin-1 as an inhibitor of ferroptosis, 10 – 20 µM necrostatin-1 as an inhibitor of necroptosis and 1.5 nM bafilomycin as an inhibitor of autophagy. For each inhibitor, a cell line-specific maximum tolerable concentration without effect on viability was determined. The rescue experiment was performed by applying the inhibitors at the same time as the treatment and by assessing viability after 96 hours.

2.2.1.10 Cytotox Membrane Integrity Assay

The CytoTox-ONE Homogeneous Membrane Integrity Assay by Promega was used according to the manufacturer’s instructions. Cells were cultured in 5 % FCS to reduce background fluorescence and protein knockdown was induced for 96 hours. Cells were then incubated with the CytoTox-One Reagent for 10 minutes. The fluorescence was recorded with the Tecan Spark reader using the 540/580 filter. Gain was adjusted based on the maximum lactate dehydrogenase release control.

2.2.1.11 Caspase 3/7 Assay

The Caspase-Glo 3/7 Assay by Promega was performed 24 h after treatment of cells in a 96-well format according to the manufacturer’s instructions. Luminescence was recorded with the Tecan Spark reader after 2 h 30 min. The net signal was calculated by subtracting the negative control (basic medium + DMSO) from each sample.
2.2.2 Nucleic Acids Manipulation

2.2.2.1 RNA Extraction for RNA Sequencing

Cells were grown according to the experimental question and harvested. $3 \times 10^6$ cells per condition were pelleted and dissolved in 700 µl QIAzol lysis reagent and stored at -80 °C until further processing. RNA was isolated with the miRNeasy kit by Qiagen according to the manufacturer’s instructions to isolate separate fractions of > 200 nt and small RNA. After ethanol precipitation, the column was further processed for the isolation of total RNA. A DNase I digestion step on the spin column was included into the protocol. RNA was eluted into a volume of 50 µl of RNase-free H$_2$O. The integrity of the isolated RNA was controlled on a bioanalyzer with a RNA Nano 6000 chip and the RNA concentration was determined with the Qubit RNA broad range assay.

2.2.2.2 RNA Library Preparation

Prior to library preparation, the ERCC RNA Spike-In Control Mix 1 was introduced to allow for accurate quantification of the libraries. 1 µl of a 1:10 spike-in dilution was added to a 25 µl volume containing 5 µg total RNA. Next, ribosomal RNA (rRNA) was depleted with the Ribo-Zero Gold rRNA Removal Kit. Afterwards, the sample volume was adjusted to 180 µl and RNA was precipitated by adding 18 µl of 3 M sodium acetate, 2 µl glycogen (10 mg/ml) and 600 µl EtOH (100%) and by incubating it at -20 °C for at least 60 min. This was followed by a centrifugation step at 13000 rpm for 30 min. The supernatant was removed and the pellet was washed with 70% EtOH twice. The pellet was air-dried and dissolved in 11.5 µl ddH$_2$O. The success of rRNA removal was controlled by measuring the new concentration with the Qubit RNA high sensitivity assay and calculating the percentage of mRNA retrieved.

For library preparation, the NEBNext Ultra RNA Library Prep Kit for Illumina was used in combination with Agencourt AMPure XP beads according to the manufacturer’s instructions with the following exceptions. Fragmentation of intact RNA was performed for 20 min at 94 °C. First strand complementary DNA (cDNA) was synthesized during 10 min at 25 °C, 50 min at 42 °C and 15 min at 70 °C. Size selection was done for insert sizes of 300 – 450 bp. For PCR library enrichment, each sample was assigned a unique index primer (NEBNext Multiplex Oligos for Illumina). After library purification, its quality was assessed on the bioanalyzer with a DNA high sensitivity chip. If adapter dimer peaks were visible at <150 bp, the final purification step was repeated. The libraries were then pooled prior to submission to the DKFZ core facility “Genomics & Proteomics” for sequencing. Every sample was adjusted to a concentration of 10 nmol/l in a volume of 10 µl.
The average region size of the library determined by the bioanalyzer was taken as a basis for the calculation of the library concentration. HiSeq 2000 pair-end 100 or 125 bp sequencing was performed on the Illumina platform.

### 2.2.2.3 RNA Extraction for Small RNA Sequencing

Cells were grown according to the experimental question and harvested. 2.5 x 10^6 cells per condition were pelleted and dissolved in 350 µl QIAzol lysis reagent and stored at -80°C until further processing. RNA was isolated with the miRNeasy kit by Qiagen according to the manufacturer’s instructions to isolate total RNA. RNA was eluted in two steps into a total volume of 90 µl RNAse-free H₂O. Subsequently, DNA was digested with the RNase-free DNase set by Qiagen for 30 min at 37 °C:

- 36 µl sample
- 50.5 µl H₂O
- 10 µl 10x buffer RDD
- 2.5 µl RiboLock RNase Inhibitor
- 1 µl DNase I

The RNA was precipitated by adding 10 µl sodium acetate, 2 µl glycogen and 300 µl EtOH and storing it at -20 °C for at least 60 min. It was then centrifuged at 13000 rpm for 30 min. After discarding the supernatant, the pellet was washed with 70% EtOH. The pellet was air-dried and dissolved in 15 µl ddH₂O.

### 2.2.2.4 Small RNA Library preparation

The kit NEBNext Small RNA Library Prep Set for Illumina was used to create microRNA (miRNA) libraries from 1 µg of the isolated total RNA according to the manufacturer’s instructions. The libraries were purified using the QIAquick PCR Purification Kit before the recommended size selection step with AMPure XP Beads. To remove also small fragments, samples were run on a LabChip XT DNA 300. Quality of the libraries was controlled with a bioanalyzer high sensitivity DNA chip. The libraries were pooled and sent for sequencing to the DKFZ core facility “Genomics & Proteomics”. HiSeq 2000 single read 50 bp sequencing was performed on the Illumina platform.
2.2.2.5 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from cells and reversely transcribed to cDNA. Samples were kept constantly on ice. Each reaction contained:

- 11 µl RNA (1 µg)
- 1 µl Oligo (dT)20
- 1 µl dNTP Mix (10 mM)

The mix was incubated for 5 minutes at 65 °C and 1 minute on ice. Then, the following components were added:

- 4 µl 5x Superscript IV Buffer
- 1 µl DTT (0.1 M)
- 1 µl RNaseOUT
- 1 µl Superscript IV Reverse Transcriptase

50 °C 10 min
80 °C 10 min

From each sample of the experiment, 2 µl were pooled and the final volume adjusted to 70 µl. A serial dilution of the cDNA mix by 1:4, 1:16, 1:64 and 1:256 was prepared to generate a standard curve.

RT-qPCR was conducted with the Platinum SYBR Green qPCR SuperMix-UDG by Invitrogen and “QuantiTect” Primers by Qiagen. To measure the expression of the housekeeping genes SDHA and HPRT1, the reaction mix was prepared as follows:

- 13 µl Platinum SYBR Green PCR SuperMix UDG (2x)
- 0.75 µl forward primer (10 µM)
- 0.75 µl reverse primer (10 µM)
- 2.5 µl template cDNA
- 8 µl H₂O

The RT-qPCR reaction mix for genes of interest CDK12 and CDK13 contained:

- 13 µl Platinum SYBR Green PCR SuperMix UDG (2x)
- 2.5 µl 10x QuantiTect Primer
- 2.5 µl template cDNA
- 7 µl H₂O
The RT-qPCR was performed in triplicates in a LightCycler 480 from Roche with cycling conditions as follows:

\[
\begin{align*}
50 \, ^\circ \text{C} & \quad 20 \, \text{s} \\
95 \, ^\circ \text{C} & \quad 2 \, \text{min} \\
95 \, ^\circ \text{C} & \quad 15 \, \text{s} \\
60 \, ^\circ \text{C} & \quad 30 \, \text{s}
\end{align*}
\]

45x

On the basis of the dilution series’ threshold cycles (C<sub>T</sub>), a linear equation was determined and used to calculate the expression levels of genes of interest and housekeeping genes. The expression of genes of interest was normalized with HRPT1 and SDHA housekeeping genes.

### 2.2.2.6 Amplification of CDK12 and CDK13 cDNA

CDK12 and CDK13 cDNA was amplified from purchased plasmids (clone #197093242 from Genomics & Proteomics Core Facility: CDK13 in pENTR223.1; clone GC-E2808 from GeneCopoeia: pShuttleGateway PLUS CDK12 ORF) with the Expand Long Range dNTPack.

\[
\begin{align*}
9.9 \, \mu\text{l} & \quad \text{Expand Long Range buffer with MgCl}_2 \\
2.5 \, \mu\text{l} & \quad \text{dNTPs} \\
1 \, \mu\text{l} & \quad \text{primer fw (1 \, \mu\text{M}) (Table 2.10)} \\
1 \, \mu\text{l} & \quad \text{primer rev (1 \, \mu\text{M})} \\
0.7 \, \mu\text{l} & \quad \text{Expand Long Range enzyme mix} \\
3 \, \mu\text{l} & \quad \text{plasmid DNA (33.3 ng/\mu\text{l})} \\
1 \, \mu\text{l} & \quad \text{DMSO (final concentration 2\%)} \\
30.9 \, \mu\text{l} & \quad \text{H}_2\text{O}
\end{align*}
\]

\[
\begin{align*}
92 \, ^\circ \text{C} & \quad 2 \, \text{min} \\
92 \, ^\circ \text{C} & \quad 10 \, \text{s} \\
64 \, ^\circ \text{C} & \quad 15 \, \text{s} \\
68 \, ^\circ \text{C} & \quad 4 \, \text{min 30} \, \text{s} \\
68 \, ^\circ \text{C} & \quad 7 \, \text{min} \\
\end{align*}
\]

45x

Reaction conditions were optimized for CDK13 amplification (6\% DMSO). The reaction product was separated on a 1.5\% agarose gel, the appropriate band was excised from the gel, purified with the Qiaquick Gel Extraction kit and the cDNA sequence was verified.
2.2.2.7 Introduction of Point Mutations to CDK12 and CDK13 pcDNA5/TO Vector

In order to render the kinase domains of CDK12 and CDK13 inactive, two point mutations were introduced with the Gene Art Site-Directed Mutagenesis Plus kit by Invitrogen according to the manufacturer's instructions. The primers to introduce a K756A and a D877N point mutation in CDK12 were designed using the Thermo Fisher online tool (https://www.thermofisher.com/order/oligoDesigner/mutagenesisplus) and are listed in Table 2.11. The primers to introduce a K734A and a D855N point mutation in CDK13 were designed online on https://www.primergartenesis.com. Primer mixes were prepared as follows:

- **CDK12 mix 1**: CDK12_3615_FW + CDK12_3252_RV
- **CDK12 mix 2**: CDK12_3252_FW + CDK12_3615_RV
- **CDK13 mix 1**: CDK13 Mut fw2 + CDK13 Mut rev1
- **CDK13 mix 2**: CDK13 Mut fw1 + CDK13 Mut rev2

24 ng of CDK12 plasmid and 60 ng of CDK13 plasmid were employed. The PCR was conducted with the following standard parameters:

- **37 °C**: 18 min
- **94 °C**: 2 min
- **94 °C**: 20 s
- **57 °C**: 30 s
- **68 °C**: 4 min 53 s (mix 1) / 11 s (mix 2)
- **68 °C**: 5 min

The CDK13 mutagenesis PCR was further optimized and conducted with 62 °C annealing temperature (PCR step 4) and 10% DMSO.

2.2.3 Molecular Cloning

Standard methods including gel electrophoresis for the separation of DNA fragments, restriction digestions or cryoconservation of *Escherichia coli* (*E. coli*) were performed as described in Sambrook and Russel (2002) and are not explained in full detail here.
2.2.3.1 Cloning of CDK12 and CDK13 shRNAs into the pTER+ Vector

Linearization of the pTER+ vector was carried out at 37 °C for 3 hours:

- 3.5 µg pTER+ plasmid
- 4.5 µg Bgl II restriction enzyme
- 4.5 g Hind III restriction enzyme
- 10 µl Buffer R+
- 77.5 µl H₂O

The enzymes were inactivated at 60 °C for 20 min. The linearized vector was separated on a 1% agarose gel and the corresponding band was excised from the gel. The vector was purified with the QIAquick Gel Extraction Kit.

In an independent reaction, forward and reverse shRNA were annealed (3 min at 95 °C, 1 h at 37 °C):

- 1 µl shRNA fw
- 1 µl shRNA rev
- 5 µl annealing buffer
- 43 µl H₂O

The linearized vector and the annealed oligonucleotides were ligated overnight at 16 °C:

- 1 µl annealed shRNA
- 250 ng linearized pTER+
- 1 µl ligation buffer (10x)
- 4.5 µl H₂O

2.2.3.2 Cloning of sgRNA into Addgene Vector #50920

The vector pLKO.1-puro U6 sgRNA BfuAI stuffer was a gift from Rene Maehr and Scot Wolfe (Addgene plasmid # 50920). The sgRNA oligos were phosphorylated and annealed in the following reaction:

- 1 µl sgRNA fw
- 1 µl sgRNA rev
- 1 µl 10x T4 ligation buffer
- 0.5 µl T4 PNK
- 6.5 µl H₂O
37 °C  30 min
95 °C  5 min, allow to cool down to room temperature.

**Digestion-ligation reaction:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLKO.1-puro U6 sgRNA BfuAI stuffer (#50920)</td>
<td>100 ng</td>
</tr>
<tr>
<td>sgRNA oligo duplex (step 1, 1:250 dilution)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Fast digest buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>DTT</td>
<td>2 µl</td>
</tr>
<tr>
<td>ATP</td>
<td>2 µl</td>
</tr>
<tr>
<td>FastDigest BveI</td>
<td>1 µl</td>
</tr>
<tr>
<td>T7 DNA ligase</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>H₂O to 20 µl</td>
<td></td>
</tr>
</tbody>
</table>

\[
\begin{align*}
37 °C & \quad 5 \text{ min} \\
23 °C & \quad 5 \text{ min} \\
\end{align*}
\]

6x

2 µl of the reaction were used to transform *E.coli* cells.

### 2.2.3.3 Cloning of CDK12/CDK13 into pcDNA5/TO

The receiving vector pcDNA5/TO and the CDK12/CDK13 cDNAs were each digested to introduce matching restriction overlaps. For CDK12, BamHI and BspTI enzymes were employed in Tango buffer. For CDK13, Xho and BspTI were employed in O buffer. The linearized fragments were ligated in a 1:3 vector to insert ratio with T4 ligase and additional ATPs overnight at 16 °C. The products CDK12 pcDNA5/TO and CDK13 pcDNA5/TO were transformed into *E.coli* bacteria.

### 2.2.3.4 Transformation of Competent *E.coli* cells

Chemically competent *E.coli* cells were thawed on ice. 2-5 µl plasmid solution (20 – 50 µg DNA) were added, the solution was carefully mixed and incubated on ice for 30 min. The bacteria were heat shocked at 42 °C for 2 min and cooled down on ice for 3 min. 250 µl of pre-warmed super optimal broth with catabolite repression (SOC) medium were added to the suspension and it was incubated for 30 min at 37 °C. Finally, the transfected bacteria were plated onto Lysogeny broth (LB) dishes with the appropriate antibiotics and incubated at 37 °C for at least 14 hours. Single colonies were picked and used to inoculate 4 ml LB medium. The cultures were incubated with agitation for 14 hours (160 rpm, 37 °C). The next day, glycerol stocks were prepared and the remainder of the culture
was used for plasmid isolation. For larger plasmid yields (using Maxi Kit), 4 ml starter cultures were grown only for 6-8 hours, diluted into 300 ml LB medium (1:500) and grown overnight.

2.2.3.5 Isolation and Purification of Plasmid DNA from *E.coli*

Plasmids were isolated from *E.coli* using the QIAPrep Spin Miniprep or QIAGEN Plasmid Maxi Kit according to the manufacturer’s protocols. The sequence of the plasmids was confirmed by sequencing using the primers listed in Table 2.9.

2.2.4 Immunoblotting

Cells were harvested after an experiment, pelleted and resuspended in an appropriate amount of lysis buffer and again centrifuged to separate the lysate and cellular debris. The protein concentration was determined with the BioRad Protein Assay (Bradford assay). The protein amount was adjusted to 20-30 ng per sample with lysis buffer and Laemmli sample buffer was added. Proteins were then denatured for 2 min at 95 °C.

Separation of proteins was performed with pre-cast NU-PAGE Tris-Acetate Midi gels (3-8% PAA gradient) and matching 1x NuPAGE Tris-Acetate SDS Running Buffer in the Criterion BioRad system.

Protein transfer to a 0.45 µm nitrocellulose membrane was achieved by wet blotting. To this end, gel and membrane were assembled into a stack with two Whatman papers on each side. NU-PAGE gels were blotted in NU-PAGE transfer buffer. The blotting took place at 110 V for 1 – 1.5 hours or at 40 V and 4 °C for 16 hours.

The success of the blotting process was confirmed by staining the membrane with Ponceau S solution. After taking a picture, the staining was washed out. The membrane was then blocked for at least 1 hour on a shaker. Incubation with the primary antibody was done overnight at 4 °C at the indicated dilution in blocking solution (Table 2.2). After three washing steps of 5 minutes with TBS-T, the secondary antibody diluted in TBS-T was added for one hour. Finally, the membrane was again washed three times. The protein bands were detected using the Clarity Western ECL Substrate kit and the ChemiSmart system from Vilber Lourmat.

2.2.5 Flow Cytometry

2.2.5.1 Cell Cycle Analysis

Cells were harvested after an experiment, pelleted and the supernatant removed while retaining ~500 µl. The cells were resuspended and 1 ml of citric acid buffer was added for
fixation. The samples were kept at 4 °C for a maximum of one week until time of analysis. For the staining, cells were pelleted, resuspended in 300 µl DAPI solution (0.5 µg/µl in PBS) and incubated for 30 min at RT in the dark. The samples were analyzed with the MACSQuant VYB flow cytometer. The main cell population was positioned in the lower left quadrant of the dot plot by adjusting the forward scatter (FSC) and side scatter (SSC) voltages. The voltage of laser V1 was adjusted individually for each sample to place the G_1 cell cycle peak at 300. 30,000 events excluding debris and doublets were recorded for each sample. Data analysis was done with the FlowJo cell cycle tool using the Watson pragmatic algorithm. If this was not possible due to strong perturbations of the cell cycle, G_1, S and G_2 phase gates were manually set in the complete experiment.

2.2.5.2 Cell Death Analysis

Cells were harvested after an experiment, pelleted, resuspended in 500 µl PBS and kept on ice. Directly before measurement, 1 µl propidium iodide (PI) / 100µl sample were added to the cell suspension. Samples were acquired as described above. PI was measured in the B2 channel. The gate for the PI-negative population was set in an untreated sample. Where necessary, the gates were adjusted upon visual inspection of the positive and negative population.

2.2.5.3 APO-BrdU TUNEL Assay

To measure apoptosis, the APO-BrdU (Bromdesoxyuridin) TUNEL (Terminal deoxynucleotidyl transferase -mediated dUTP-biotin nick end labeling) kit by Invitrogen was used according to the manufacturer’s instructions. Briefly, cells were harvested after an experiment and fixed in 1% formaldehyde in PBS. After two washing steps in PBS, cells were permeabilized in 70% EtOH overnight at -20 °C. Before staining, cells were pelleted, resuspended in PBS and 1x10^6 cells transferred to a new reaction tube. Incubation in DNA-labeling solution was prolonged to 90 minutes. After staining with the antibody solution and subsequently PI staining buffer, cells were directly analyzed. The BrdU signal was detected in the V2 channel, whereas PI was detected in the B2 channel. Gating of BrdU positive versus negative cells was performed individually for each cell line in untreated samples.
2.2.6 Quantification and Statistical Analyses

2.2.6.1 General Statistics

Differences between mean values were calculated using the Student's t-test for unpaired observations unless specified otherwise. The null hypothesis (differences between tested samples are due to chance) was rejected and differences called significant if the returned p-value was below 0.05. Statistical analysis was performed using Excel (Microsoft).

2.2.6.2 RNA Expression and miRNA Expression Analyses

RNA expression data of the cell cycle synchronization experiments was analyzed by Chunxuan Shao as detailed in Ryl et al. (2017).

For miRNA expression data, the raw reads were trimmed sequentially with cutadapt (Martin, 2011) to remove low quality reads and adapters with the parameters “-q 28 -m 18” and “-O 4 -f fastq -m 18 -a NEBNext=AGATCGGAAGAGCACACGTCT”. Trimmed data were further examined with fqtrim to remove low complexity reads; only reads that were 18 to 25 bps were used in the downstream analysis. Identical reads were collapsed by tally and processed by miRDeep2 for genome alignment, novel miRNA discovery and quantification. The read counts were then normalized with the DESeq2 package in R. Normalization size factors were calculated based on filtered counts (mean counts ≥ 10 reads). Differentially expressed genes were identified with a likelihood ratio test using the DESeq2 with the options “independentFiltering = FALSE, cooksCutoff = FALSE”, while the full model and reduced model were specified as “~ Time + Tet_status + Time:Tet_status” and “~ Time”, respectively. Statistical significant miRNA were selected by FDR <= 0.05.

RNA expression data of CDK12/13 shRNA clones and BAY587-treated cells were analyzed by Umut Toprak. Genes with less than one count per million (CPM) were removed and normalization was performed with the trimmed mean of M values (TMM) method (Robinson and Oshlack, 2010). Next, the log-transformed CPM of the normalized values of control and knockdown/treatment samples were used to calculate the fold-change upon knockdown/treatment (mean and trimean) followed by a log2 transformation. Statistical significance was determined by the rank-based Kruskal-Wallis test with no assumption of the underlying distribution (Kruskal and Wallis, 1952).
3 RESULTS

3.1 The Transcriptional Network of MYCN in Neuroblastoma Cell Lines

As a transcription factor, MYCN regulates large numbers of genes. Effects on proliferation, self-renewal, apoptosis and other cellular processes are well documented (Huang and Weiss, 2013). Yet, many questions remain as to which effects are due to direct regulation of target genes and which to downstream network partners (Lin et al., 2012; Sabo et al., 2014).

3.1.1 MYCN Regulates Transcription of General and Cell Cycle Phase-Specific Drivers of Malignant Phenotype

Previous studies on the regulatory role of MYCN analyzed expression data of bulk cell populations, potentially confounding cell cycle effects with genuine MYCN effects. In order to get a clear picture of the transcriptional effects of MYCN in each cell cycle phase, our group developed a synchronization protocol that allows the observation of cells as they progress through the cell cycle (concept by Emma Bell; Ryl et al., 2017).

Figure 3: Cell Cycle Distribution after Thymidine Synchronization

(A) IMR-5-75 MYCN shRNA cells with high (red) and low (blue) MYCN protein level were arrested in G1/S phase by thymidine addition and released into the cell cycle 18 h later. Samples were taken and analyzed for cell cycle and gene expression. Adapted from Ryl et al. (2017). (B) Cell cycle analysis was performed by DAPI staining and subsequent flow cytometry analysis.
MNA IMR-5-75 cells carrying an shRNA to decrease the endogenous MYCN level were synchronized by thymidine treatment and released into S phase 18 hours later (Figure 3A). Samples were taken after 3, 6 and every two hours for 22 hours, and the synchronization was monitored by cell cycle analysis. Initially, the majority of both MYCN high and MYCN low cells resided in S phase and progressed almost simultaneously through G2 phase, mitosis and G0/1 phase (Figure 3B). MYCN low cells reached G2 and mitosis slightly later than the faster proliferating MYCN high cells. After 16 to 18 hours a major difference became apparent, since MYCN high cells were able to enter a new cell cycle phase, whereas MYCN low cells remained stalled in G0/1 phase.

At every time point, MYCN-induced changes in gene expression were analyzed by RNA sequencing (main experimenter Emma Bell, bioinformatic analysis: Chunxuan Shao). All time points viewed collectively, MYCN significantly upregulated 887 and downregulated 865 genes (Ryl et al., 2017). With few exceptions, the fold changes were moderate and ranged between +/- 2. Unsupervised clustering was performed to detect common patterns of expression changes along the cell cycle. It revealed six distinct clusters (Figure 4). The DAVID functional annotation tool 6.8 was applied separately for each cluster to find significantly enriched gene functions (Huang da et al., 2009a, 2009b). Two of the clusters contained genes that were constitutively up- or downregulated in MYCN high cells throughout the observation period (yellow and green line). In the upregulated cluster, one fifth of gene products were part of the nucleolus and for example involved in ribosomal RNA (rRNA) processing, such as nucleolar protein 56 (NOP56, Figure 5C, Appendix Figure S1). Also, five snoRNAs were in the constantly upregulated cluster. There was also a significant enrichment of genes involved in DNA replication and G1/S transition. This included four major players of cell cycle progression, namely CDK4, cyclin D3, cell division cycle 25A (CDC25A) and protein kinase membrane associated tyrosine/threonine 1 (PKMYT1) (Figure 5A). On the other hand, several inhibitors of cell cycle gene CDK2 were represented in the permanently downregulated cluster (Figure 5A). Genes of this cluster were mostly enriched in the keywords alternative splicing, cell adhesion and synapse (Appendix Figure S2).
RESULTS

Figure 4: Unsupervised Clustering Reveals Cell Cycle-Resolved Gene Expression Patterns after MYCN Knockdown

K-means clustering of differentially expressed genes in RNA expression analysis of synchronized MYCN high and low IMR-5-75 cells. Clusters: “constitutively up” (yellow); “constitutively down” (green); “turbulent” (pink); “G/iS up”; “G/iS down”; “G2/M up”. Adapted from Ryl et al. (2017), experiments were conducted jointly with Emma Bell.

Three clusters showed differential expression in particular cell cycle phases (G/iS up and down, G2/M up). Indeed, cell cycle phase-specific genes were accumulated in these clusters. For example, a remarkable number of keywords related to mitosis were represented in the G2/M cluster gene enrichment (Appendix Figure S 5). In the G/iS up cluster, genes were particularly involved in DNA replication, DNA repair and histones, but also in the cell cycle, more specifically in the G/iS transition (Figure S 4). In the G/iS down cluster, genes were solely enriched for alternative splicing (Figure S 3).

Looking at single cell cycle genes confirmed the phases established by flow cytometry (Figure 3). The expression of CCNE1 was high in both conditions after release from the thymidine block and subsequently dropped strongly (Figure 5B). Only in MYCN high cells, the level rose again, peaking at around 16 hours after release. The expression of CDK2 followed a very similar pattern. These patterns are in line with the established function of cyclin E together with CDK2 in the transition of cells from G1 to S phase (see also Introduction, Figure 1). Going further in the cell cycle, the expression of CCNA2 rose throughout S phase and peaked in G2, again nicely matching its ascribed role. At 18 to 22 hours, the expression rose again in the MYCN high, but not MYCN low condition, in keeping with the stalling in G0/1 that was observed by flow cytometry. CCNB1 and B2 expression peaked at 8 – 12 hours, which corresponds to the time of mitosis, when the protein is known to be active. Its partner CDK1 displayed a very similar expression pattern. Also, the expression peaks of CCNA2 and B1 were shifted by up to two hours between MYCN high and low, confirming the slower cycling of MYCN low cells.
Figure 5: mRNA Expression Profiles of Cell Cycle and Ribosomal Genes
Examples of differentially expressed genes in synchronized MYCN high (red) and low (blue) IMR-5-75 cells. (A) Constitutively MYCN-regulated cell cycle genes; (B) cell cycle phase-dependently regulated cyclins; (C) genes and non-coding RNAs involved in ribosomal processes. Shown are normalized read counts. Affiliation to the clusters “constitutively up” (yellow), “constitutively down” (green), “turbulent” (pink), “G1/S up”; “G1/S down”; “G2/M up” is indicated in the upper left part of the plots. Experiments were conducted jointly with Emma Bell.

The sixth cluster was characterized by a strong increase of transcripts in the MYCN low condition after 14 hours, while they remained constant in MYCN high cells (Figure 4, pink line). Before this time point, read counts in both conditions were typically very low. This small group contained predominantly small nucleolar RNAs (snoRNAs), but also some small nuclear RNAs (snRNAs) and small Cajal body-specific RNAs (scaRNAs). Expression of SNORD15A, for example, reached more than 10,000 reads in MYCN low cells (Figure 5B, middle graph). Eight further snoRNAs were grouped into cluster G1/S down but had a very similar expression pattern to their counterparts in the pink cluster (Figure 5C, right graph).

Taken together, cell cycle-resolved analysis of gene expression in MYCN high and low cells allowed the differentiation between constitutive MYCN target genes, such as CDK4 or NOP56, and more cell cycle phase-dependent genes, such as CCNE1 or several snoRNAs.
3.1.2 MYCN-dependent miRNA Expression Changes Reveal an Additional Layer of Gene Regulation

Since MYCN is known to regulate also the expression of miRNAs, changes in miRNA levels in our cell cycle synchronization experiment were analyzed (data analysis: Chunxuan Shao). 164 miRNAs were significantly differentially expressed, 15 of which had previously not been annotated. 55% of miRNAs were down-regulated, mirroring the balanced distribution of up- and downregulation already observed in the RNA expression data. The list of differentially expressed miRNAs was screened for direct MYCN targets as described by studies of primary NB tumors with our without MYCN amplification and of SH-EP cells with inducible MYCN overexpression (Migliori et al., 2017; Mestdagh et al., 2010; Schulte et al., 2008). There was an intersection of ten miRNAs which were consistently higher expressed in the MYCN high situation (Supplementary Table S 1). Only three miRNAs reported to be induced by MYCN contrarily showed downregulation in the present study (miR-16-2-3p, miR-181-5p, let-7b-5p), while six allegedly downregulated miRNAs could be confirmed. Another five miRNAs were downregulated only in parts of the observation period, while a single miRNA showed inverse expression (miR-1908-5p).

A reverse search with TarBase v7.0 revealed that 29 out of 149 differentially expressed miRNAs regulate cell cycle processes, and 78 miRNAs target genes were involved in ribosome biogenesis (Vlachos, Paraskevopoulou, et al., 2015). Among the cell cycle-regulating subgroup were nine miRNAs that are direct MYCN targets (e.g. members of the miR-17-92 cluster). Finally, there were also miRNAs in this dataset which themselves regulate MYCN expression, miR-let-7e-5p and miR-101-3p (Figure 6B) (Buechner et al., 2011).

Next, an unsupervised k-means clustering was performed, which identified five distinct clusters (Figure 6A). Interestingly, the cluster profiles resembled the ones identified in our RNA expression analysis. There were two clusters which were constitutively up- or downregulated by MYCN (yellow and green lines). In two clusters, the expression level diverged in early G₁ or towards the end of the observation period, when MYCN high cells enter S phase while MYCN low cells remain in G₁ phase (“G₁ up” and “G₁/S up” clusters). A rather small cluster exhibited the most prominent fold changes and peaked in G₂ phase (“turbulent”, pink line). In G₂/M phase, the expression of MYCN high cells clearly exceeded that of MYCN low cells, whereas it was inversed in G₁/S phase. For each cluster, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed with the mirPath v.3 algorithm (Figure 7 and Supplementary Figure S 6 – 9) (Vlachos, Zaggenas, et al., 2015). Despite the comparable patterns of RNA and miRNA clusters, there was no considerable overlap of significantly enriched processes or cellular components. However, many of the top terms appeared in several of the miRNA clusters.
As an example, the pathway “proteoglycans in cancer” was among the top six results in four clusters. In general, there were many KEGG terms related to cancerous processes or particular entities. Also several signaling pathways linked to tumorigenesis were represented, such as the Hippo and the Transforming Growth Factor β (TGF-β) signaling pathways. In addition, several recurrent KEGG terms had to do with synaptic signaling.

Figure 6: Unsupervised Clustering Reveals Cell Cycle-Resolved miRNA Expression Patterns after MYCN Knockdown

(A) K-means clustering of differentially expressed miRNAs in synchronized MYCN high versus low IMR-5-75 cells. Clusters: “constitutively up” (yellow); “constitutively down” (green); “turbulent” (pink); “G1/S up”; “G1 up”.

(B) Example profiles of highly expressed miRNAs. Affiliation to the clusters is indicated in the upper left part of the plots. Shown are normalized read counts. Experiments were conducted jointly with Emma Bell.
Looking at the read counts of the differentially expressed miRNAs, very strong differences became apparent. More than 35% of them had read counts of 200 or below. Opposed to that, seven miRNAs had read counts of 50,000 and higher. Reasoning that high read counts mean that the miRNAs in question have a high impact on gene regulation, they were selected for closer inspection (Figure 6B). Two miRNAs, miR-17-5p and miR-20a-5p, were part of the oncogenic cluster miR-17-92 and their expression was increased in MYCN high cells. Congruously, they and also miR-9-5p were among the MYCN-induced targets established above. Additionally, miR-21-5p and miR-101-3p displayed particularly high expression towards the end of the observation period. Opposed to that, miR-26a-5p and miR-30d-5p read counts dropped in MYCN high compared to MYCN low cells.

**Figure 7: MYCN-Dependent miRNAs Predominantly Regulate Processes Related to Cancer**

KEGG analysis of miRNAs grouped together in the G₁ cluster identified cancer- and synapse-related processes (green and red) and several signaling pathways (yellow). List shows the 30 most significant terms with p ≤ 0.05. Other clusters see Figure S 6 - 9.
In summary, cell cycle-resolved analysis of miRNA expression demonstrated that MYCN regulates the expression level of many miRNAs that play a role in different cancer entities. Cluster-specific gene enrichment analysis showed that different miRNAs fulfill similar functions over the course of the cell cycle.

3.2 CDK13, a Novel Target in MYCN-amplified Neuroblastoma

3.2.1 MYCN Synthetic Lethal Screens Identify CDK13 as One of the Top Candidates

MYCN-amplified tumor cells depend on the transcriptional deregulation caused by aberrant MYCN levels as described in the first part of this study. After all, many oncogenic processes are sustained by MYCN and its downstream interaction partners, for example proliferation, angiogenesis or inhibition of differentiation (Westermann et al., 2008). Since MYC(N) itself is considered to be largely undruggable, new strategies aim at identifying those downstream interaction partners that are indispensable for the survival of MYCN-driven tumors (Kaelin, 2005). In this context, a high-throughput druggable genome synthetic lethal screen in NB cells with regulatable MYCN was performed in a joint project of our group (Sina Gogolin) and Bayer AG. The model system used in the screen was MNA IMR-5-75 cells with an inducible MYCN shRNA that allows reduction of MYCN protein by approximately 65 % (Muth et al., 2010; Ryl et al., 2017).

The screen covered more than 10,000 druggable genes with three siRNAs per gene. Cell numbers were counted as a read-out 96 h after transfection. Candidates were identified based on a strong reduction in viability induced in the MYCN high condition and a strong effect of MYCN (i.e. a large difference between the viability of MYCN low – MYCN high, Figure 8). In addition, these putative MYCN synthetic lethal candidates were subjected to a gene ontology analysis, which revealed an enrichment of CDKs and cyclins among the best-scoring candidates. It pinpointed some well-established players in neuroblastoma biology, such as CDK2, 4 and 7. Also among these candidates was the transcriptional kinase CDK13, which has so far mostly lacked attention of the cancer research community.
IMR-5-75 cells were transfected with 3 siRNAs per gene and incubated for 96 h. Viability was determined by counting Hoechst-stained nuclei. MYCN effect was calculated by subtracting viability of the MYCN high from that of MYCN low cells. Highlighted in yellow are synthetic lethal CDKs and cyclins. Experiments were performed by Sina Gogolin and colleagues at Bayer AG.

A second, independent synthetic lethal screen was performed by collaboration partners in Finland. Here, SH-SY-5Y cells with inducible MYCN overexpression were used as a model system. They were transfected with a pool of four siRNAs per gene, an approach that reduces potential off-target effects. The read-out of this screen was a CellTiter Glo assay measuring metabolic activity of the cells. Cyclin K, the partner cyclin of CDK13, was among the 52 proliferation-reducing hits of this screen, thus fortifying our interest into the kinase as a potential candidate (Figure 9).

CDK13 was therefore picked for further validation next to three other potentially interesting candidates, which formed the intersection of both synthetic lethal screens. The analysis of the additional candidates has been pursued in separate projects and will not be discussed here.
Figure 9: Cyclin K is among the Top Hits of a Synthetic Lethal Screen in MYCN-Overexpressing SH-SY-5Y Cells

Cells were seeded and MYCN expression was induced by tetracycline addition for one day. Cells were transfected with an siRNA pool (four siRNAs per gene) and incubated for 72 h. Cell viability was measured by CellTiter Glo. Shown are normalized, loess fitted viability data points divided by the median of negative controls. Experiments were performed by Saija Haapa-Paanonen and colleagues (VTT Technical Research Centre of Finland).

3.2.2 Evaluation of CDK13 and CDK12 as Potential Therapeutic Candidates

It was decided to include CDK12 next to CDK13 into all further analyses. First of all, it is highly homologous to CDK13 and interacts with the same partner cyclin, namely cyclin K. Differences and similarities in their cellular function have not been fully elucidated until now. Second, CDK12 is mutated or amplified in several cancer entities, including ovarian and breast cancer (Natrajan et al., 2014; Popova et al., 2016). Thus, interest into CDK12 as a potential new target has increased strongly during the last years.

Validation of CDK13 repression in additional neuroblastoma cell lines. As a next step, the effects of knocking down CDK13 or CDK12 were analyzed in a larger panel of cell lines. Viability of the cells after knockdown was analyzed by two methods, first assessment of confluence as a measure of growth and second Cell Titer Blue (CTB) assay as a measure of metabolic activity. Cells treated with negative control siRNAs or with transfection reagent only served as controls. In addition, success of the transfection was controlled by knocking down polo-like kinase 1 (PLK1), which is indispensable in NB cells, leading to strong cell death.

For CDK13, four siRNAs were tested. Of these, in general only siRNA1 and 3 proved effective, while siRNA2 and 4 only had minor effects in one of the cell lines (Figure 10). Of the nine tested neuroblastoma cell lines, SK-N-BE(2)c, NB-69, CHP-134, SK-N-AS and SH-EP cells responded strongly to knockdown of CDK13. In addition, the cell lines
KELLY, LS and IMR-32 responded partly. IMR-5-75 cells responded strongly to siRNA1 and in part to siRNA3. They also showed a slight response to knockdown of CDK12 by siRNA1, but not 3. The same was true for SK-N-BE(2)c. In addition, CDK12 knockdown modestly decreased viability of SH-EP cells. Also two lung and two colon cancer cell lines were tested (shown here: strong responder HCT116). Of these, only one lung cancer cell line did not respond to CDK13 repression. Non-transformed fibroblast cells (NHDF) were not at all affected by knockdown of CDK12 or CDK13.

![Figure 10: CDK13 Repression Reduces Confluence in Most NB Cell Lines](image)

Cells were treated with indicated siRNAs for 96 h, fixed and confluence was evaluated by Giemsa staining. Experiments were performed by Sina Gogolin.

**Expression pattern of CDK12, 13 and CCNK during the cell cycle.** Looking at the RNA levels of our candidates in the cell cycle synchronization experiment revealed that neither CDK13 nor CDK12 or CCNK were differentially expressed in MYCN high and low cells. Expression of CDK12 peaked in late S phase / early G2 phase (Figure 11). Opposed to this, CDK13 expression went up at mitosis and remained constant throughout G1 and early S phase. CCNK levels were constant throughout the observation period and read counts much lower than those of the CDKs.
RESULTS

Figure 11: Expression Profiles of CDK12, 13 and CCNK.
IMR-5-75 cells were synchronized as described in chapter 3.1.1 and RNA sequencing performed at the indicated time points. Shown are read counts of CDK12, 13 and CCNK in MYCN high cells. Experiments were conducted jointly with Emma Bell.

Role of CDK12, 13 and cyclin K in primary tumors. When comparing the proteome of MNA tumors with that of low risk tumors by mass spectrometry, we found that the cyclin K level was strongly decreased ($p < 0.001$, experiments performed by Michal Nadler-Holly, Figure 12A). Also CDK13 and CDK12 were significantly decreased ($p < 0.01$). This finding was confirmed by looking at CDK13 RNA expression in a publicly available cohort of 498 primary NBs, which was significantly lower in MNA tumors ($p = 7.3 \times 10^{-10}$, Figure 12B).

Low expression levels of CDK13 also significantly correlated with poor overall survival ($p = 0.034$, Bonferroni-corrected for multiple testing). While CDK13-high cases had a survival probability of roughly 80 %, it was below 50 % in CDK13-low cases (Figure 12C). For CDK12, the opposite trend was observed, however it was not significant after multiple testing correction (data not shown).

Taken together, data from NB cell lines and primary tumors both indicated an important role of CDK13 in NB biology and thus supported further investigation of CDK13 as a potential therapeutic target.
Figure 12: CDK13 Is Reduced in MYCN-Amplified Neuroblastoma Tumors
(A) Mass spectrometry; proteome of primary neuroblastoma samples with MYCN-amplification compared to single copy status. Experiments were performed by Michal Nadler-Holly (Max Delbrück Center for Molecular Medicine, Berlin). (B) CDK13 RNA expression analysis in primary tumors; sc = single copy, amp = amplified. (C) Kaplan Meier plot showing overall survival for high versus low CDK13 expression. (B), (C): created with R2 Genomics Analysis and Visualization Platform.

3.2.3 Characterization of CDK13 Dependency in MYCN-regulatable Neuroblastoma Cells

Next, cellular models with regulatable MYCN level were employed to learn more about its role regarding sensitivity of neuroblastoma cell lines towards knockdown of CDK13.

**Inducible MYCN knockdown.** First, IMR-32 and IMR-5-75 cells with an inducible MYCN shRNA were used. One day after repression of MYCN, CDK12 or CDK13 were additionally knocked down by siRNA. Knockdown of both kinases induced between 10 - 30 % cell death as determined by uptake of PI due to damaged cell membrane (Figure 13; data for IMR-5-75 MYCN shRNA not shown). CDK13 knockdown tended to induce more cell death than that of CDK12. However, the extent of cell death was irrespective of the MYCN level. With regard to the cell cycle distribution upon CDK13
knockdown, there was a trend towards an accumulation of cells in G₁ phase accompanied by reductions in S and G₂ phase (Figure 13C). Knockdown of CDK12 similarly led to slightly increased cell numbers in G₁ and reduced numbers in S phase in the MYCN low condition (Figure 13B).

**Figure 13: Knockdown of CDK12 or CDK13 in IMR-32 Induces Moderate Cell Death Irrespective of MYCN Level.**

MYCN knockdown was induced in IMR-32 MYCN shRNA cells and after 24 h, cells were transfected with siRNAs against CDK12 or CDK13 or treated with transfection agent only (mock). After 96 h, floating and adherent cells were harvested. (A) Cell death; cells were stained with PI and directly analyzed by flow cytometry. Amount of positive cells was normalized to negative controls 1 and 2; mean ± SEM, n = 2/3. (B), (C) Cell cycle; cells were fixed in citric acid buffer, stained with DAPI and analyzed by flow cytometry; mean ± SEM, n = 2/3.

n.s. = not significant

**Inducible MYCN overexpression.** SH-EP cells have very low levels of endogenous MYCN, but the stable subclone TET21N allows conditional overexpression of MYCN (Lutz et al., 1996). In general, high MYCN led to an increase of S and G₂ phase cells. In this model, knockdown of CDK12 induced moderate cell death in the MYCN overexpression condition (siRNA 1: 20 %, siRNA 3: 10 %, p < 0.05, Figure 14A). In addition, there was a
trend towards more cells residing in G₁ phase compared to one out of two non-targeting controls and mock transfection (Figure 14B). siRNA3 targeting CDK13 induced 76 % cell death in MYCN high cells, but only 18 % in MYCN low (p < 0.001, Figure 14A). Cell cycle analysis revealed less cells in G₁ accompanied by an increase of S and G₂ phase cells in the MYCN low and high condition (Figure 14C). Opposed to that, CDK13 siRNA1 did not induce any cell death at all; instead, cells seemed to be arrested in G₁. Apparently, CDK12 and CDK13 repression elicited arrest in different phases of the cell cycle.

Figure 14: Knockdown of CDK13 Induces Strong Cell Death in MYCN-High SH-EP TET21N Cells
Overexpression of MYCN was induced in SH-EP TET21N cells and after 24 h, cells were transfected with siRNAs against CDK12 or CDK13 or treated with transfection agent only (mock). After 96 h, floating and adherent cells were harvested. (A) Cell death; cells were stained with PI and directly analyzed by flow cytometry. Amount of positive cells was normalized to negative controls 1 and 2; mean ± SEM, n = 2/3. (B), (C) Cell cycle; cells were fixed in citric acid buffer, stained with DAPI and analyzed by flow cytometry; mean ± SEM, n = 2/3.

*** p < 0.001, ** p < 0.01
3.2.4 Conditional CDK12/13 Knockdown in Neuroblastoma Cells

In order to further explore the effects of CDK13 knockdown in neuroblastoma cells, stable cell lines with vectors containing shRNAs against CDK12 or CDK13 were generated. For the creation of the short hairpins, the sequence of the siRNAs was used (CDK12 si1+3, CDK13 si1+3).

**Knockdown efficiency of shRNA clones.** After transfection of IMR-5-75 with the shRNAs, single clone cultures were raised and the extent of the knockdown was determined by immunoblotting. The cell clones with the best knockdown results, which were used in all further analyses, were A8 (CDK13 sh1), L3 (CDK13 sh3), B5 (CDK12 sh1), G1 (CDK12 sh3) and 2C (scramble sh / scr sh). Upon induction of the shRNA, protein was reduced by 70 – 85 % (CDK13 sh1), 45 – 88 % (CDK13 sh3), 35 – 60 % (CDK12 sh1) and 85-98 % (CDK12 sh3) on average (Figure 15). The induction of scramble shRNA did not lead to a reduction in CDK12 or CDK13 level; if anything, the levels were moderately increased.

![Immunoblot images showing knockdown efficiency of shRNA clones](image)

**Figure 15:** Doxycycline-Induced shRNA Targeting CDK13 or CDK12 Results in Decreased Protein Levels.
Expression of CDK12, CDK13 or scramble (scr) shRNA was induced by doxycycline (Dox) addition. After 72 h, floating and adherent cells were harvested and lysed for immunoblotting. Vinculin or α-tubulin were used as loading controls. Representative replicate out of n ≥ 3.
**RESULTS**

**Modest reductions in cell viability and colony formation.** All described experiments were conducted with 72 h and 96 h time windows with concordant results (96 h data not shown). The viability of the cells after knockdown was analyzed by CTB and growth assay. Confluence was reduced by 25% on average in the CDK13 sh1 clone, and only slightly or not at all reduced in the other clones when compared to the scramble control (Figure 16A). The effect on metabolic activity of the cells was even less pronounced (data not shown). A colony formation assay demonstrated decreased ability to form colonies from single cells upon CDK13 knockdown (Figure 16B). The results varied strongly between 10 and 90%, with a mean reduction of 41% for CDK13 sh1. For CDK13 sh3, colony formation was reduced by approximately 70% in four out of five biological replicates (p < 0.001 in each of these replicates). Effects of CDK12 knockdown were less variable with the two CDK12 shRNA clones significantly reducing colony formation by 39% and 98% on average (p < 0.01 in every biological replicate). Also induction of scramble shRNA led to an average reduction of colony formation by 47% (p < 0.01).

![Figure 16: Knockdown of CDK13 in Stable IMR-5-75 Clones Modestly Impacts on Cell Viability and Colony Formation Capacity](image)

Expression of CDK12, CDK13 or scramble (scr) shRNA was induced by doxycycline addition. At the given endpoint, cells were fixed and confluence was evaluated by Giemsa staining. (A) Growth assay; cells were seeded at high density and incubated for 72h; technical replicate’s mean ± SEM. (B) Colony formation assay; cells were seeded at low density and incubated for 7-10 days before fixation and Giemsa staining. Representative biological replicates out of n ≥ 3.

**No effects on cell death or cell cycle distribution observed.** The experiments on viability were complemented by PI stainings to analyze cell death. Neither CDK13 nor CDK12 knockdown did increase the amount of cell death in the cell clones (Figure 17A).
RESULTS

Figure 17: Knockdown of CDK13 in Stable IMR-5-75 Clones Does Not Induce Cell Death or Alter Cell Cycle

Expression of CDK12 or CDK13 shRNA was induced by doxycycline addition for 72 h. (A) Cell death; cells were harvested, stained with PI and directly analyzed by flow cytometry; mean ± SEM, n = 2. (B) Cell cycle; cells were fixed in citric acid buffer, stained with DAPI and analyzed by flow cytometry; mean ± SEM, n = 2. n.s. = not significant

Next, the effect of CDK13 and CDK12 knockdown on the cell cycle tested, but the distribution was not altered (Figure 17B).

RNA expression analysis. In order to better understand the differences between CDK13 and CDK12 function, RNA expression of IMR-5-75 clones 96 h after induction of shRNA was analyzed. Differentially expressed genes (DEGs) were defined as having the same direction of regulation (thus the same sign of the fold change) for both shRNAs and with > 2-fold change in at least one out of two (normalization and bioinformatic analysis: Umut Toprak). Generally speaking, the number of DEGs was very low. Knockdown of CDK13 induced more downregulation than upregulation of genes (66 versus five genes). Knockdown of CDK12 induced upregulation of eight genes, and downregulation of three genes. None of the DEGs overlapped between CDK12 and CDK13. But it was noticeable that all gene lists included a relatively large proportion of transcripts for non-coding RNAs, mainly miRNAs or pseudogenes. Among the most strongly increased transcripts after CDK12 knockdown were five miRNAs. Upon knockdown of CDK13, miRNAs constituted three of the five most upregulated transcripts and eight of the ten most downregulated transcripts. PKIA, a protein kinase inhibitor, was among the most strongly downregulated transcripts after CDK13 knockdown.

CRISPRi-induced knockdown of CDK13. Since some of the shRNA clones only achieved moderate knockdown efficiencies, a different technique promising higher efficiency was employed next. Clustered regularly interspaced short palindromic repeats interference (CRISPRi) works by steric inhibition of Pol II by a deactivated Cas9 (dCas)
enzyme, thus preventing transcription of the targeted gene. In this approach, dCas was integrated into the AAVS1 (Adeno-Associated Virus Integration Site 1) locus by a TALEN (Transcription activator-like effector nuclease)-assisted gene trap. The necessary vectors were a gift of Mandegar and colleagues, who reported consistent knockdown efficiencies of 95% and more in their hands (Mandegar et al., 2016). IMR-5-75 cells were transfected with AAVS1 TALEN F and R and the doxycycline-inducible vector pAAVS1-NDi-CRISPRi with dCas-KRAB (Krüppel associated box) fused to an mCherry construct (“dCas”, step 1). A single clone with even mCherry expression was selected, expanded and again transfected with one of three different sgRNAs against CDK13 and two non-targeting control vectors (sgRNA 6, 8, 10, scr 2 + 3, step 2). Single clones were raised from the polyclonal cultures and the knockdown efficiency was determined by immunoblotting. The protein level of CDK13 was indeed reduced by 50 to 90% in the clones chosen for further analysis, while the scramble controls did not alter CDK13 protein level (Figure 18A). In addition, viability and confluence of the cell clones was reduced by around 40% upon doxycycline addition to induce sgRNA expression (Figure 18C). However, this effect also occurred in dCas9 cell clones of step 1, which did not yet contain the sgRNA vector.

![Diagram](image)

**Figure 18: Induction of dCas9 Vector Negatively Affects Viability in IMR-5-75 Cells**
Expression of sgRNA and OE vector was induced by doxycycline addition. (A) After 96 h, floating and adherent cells were harvested and lysed for immunoblotting. Vinculin or α-tubulin were used as loading controls. (B) Representative light microscopy image of sgRNA 6 clone. Magnification 10x. (C) Viability was assessed by CTB after 96 h. Representative biological replicates out of n = 4. (D) Cell death; cells were harvested, stained with PI and directly analyzed by flow cytometry; mean ± SEM, n = 3.

*** p < 0.001, ** p < 0.01
RESULTS

A live/dead cell exclusion staining to determine the amount of cell death revealed that upon induction by doxycycline, more than 90% cells stained positive for PI in sgRNA 6, dCas9 and sgRNA scr (p < 0.002, Figure 18D). Yet, no change in morphology characteristic of cell death could be observed (Figure 18B). In addition, a cytotox membrane integrity assay did not confirm that induction of the dCas vector should cause a damaged cell membrane (data not shown).

Due to the general loss of viability, further in-depth characterization of these cell clones is not shown here. In order to see whether the effect on viability could be rescued, overexpression (OE) plasmids were introduced into the sgRNA 6 alt clone (CDK12 OE, CDK13 OE and kinase-dead mutants CDK12 KDM and CDK13 KDM). Induction of CDK13 knockdown and simultaneous ectopic overexpression of CDK12 or CDK13 did not improve viability (Figure 18 C and D).

Taken together, partial knockdown of CDK13 was enough to elicit a moderate decrease in NB cell viability and colony formation capacity, while response to CDK12 repression was less pronounced in most cases.

3.2.5 CRISPR-induced CDK12/13 Knockout in Neuroblastoma Cells

A CRISPR knockout of CDK12 and CDK13 was performed in IMR-5-75 cells with the aim of reducing CDK12 or 13 protein to a level close to zero. To this end, the cells were transduced lentivirally with a lentiCRISPRv.2-706 vector containing Cas9, a sgRNA and a GFP reporter (green fluorescent protein). sgRNAs were designed to target CDK12 (#2b), CDK13 (#11) or were a non-targeting control (scr 8.2). The experiments were performed in collaboration with the Friederike Herbst / Hanno Glimm group at the National Center for Tumor Diseases, who kindly provided the necessary viral particles and vectors and had verified the functionality of the sgRNAs in primary colon carcinoma cultures.

Two days after transfection, microscopic survey revealed that the majority of cells had died in the wells transfected with sgRNAs CDK12 and CDK13, while in the scr 8.2 condition cell death was less pronounced. sgRNA CDK12 and CDK13 cells continued to die at day five post transduction despite an additional exchange of culture media (Figure 19). However, a fraction of cells survived and was expanded. The percentage of GFP-positive cells was determined by flow cytometry and ranged between 30-60%. Positive cells were sorted to obtain a more pure polyclonal culture. Re-analysis of sorted cells confirmed that in all three conditions more than 98% of the cells were GFP-positive (Appendix, Figure S 10). These cells were used for all experiments described in the following. By then, the polyclonal cultures had recovered from transduction and grew at a proliferation rate reminiscent of the parental cell line.
Results

Figure 19: High Amount of Cell Death upon Transduction with CRISPR CDK12 and CDK13 Vectors
IMR-5-75 cells were lentivirally transduced with CRISPR vectors containing Cas9, sgRNAs against CDK12, 13 or scrambled and a GFP construct. Microscopic images were acquired five days post transduction and show aggregates of dead, GFP-positive cells predominantly in the CDK12 and 13 knockout condition. Magnification 10x.

Figure 20: Protein and mRNA Expression Analysis of CRISPR Knockout Cells
IMR-5-75 CRISPR polyclonal cells expressing GFP were enriched by fluorescence-activated cell sorting and expanded in culture. 72 h after seeding, floating and adherent cells were harvested and lysed. (A) Immunoblotting; vinculin was used as loading control. (B) RT-qPCR. Representative biological replicates out of n = 3.
The effect of CRISPR on the protein level was examined by immunoblotting, which revealed a reduction of CDK12 by 40 – 60 % (Figure 20A). Due to the lack of a good antibody, immunoblotting of CDK13 was not successful. In addition, mRNA expression of CDK12 and CDK13 was determined by RT-qPCR. CDK12 was not reduced on the mRNA level; while CDK13 was reduced by 35 % on average (Figure 20B).

Next, viability of the CRISPR knockout clones was determined by a CTB assay. It revealed that viability of CDK12 CRISPR clones was not reduced, whereas a trend towards decreased viability was observed in CDK13 CRISPR clones (87 % of scramble control, p = 0.06, Figure 21A).

In addition, the cell cycle distribution was examined by flow cytometry. There were more cells in the G1 phase in CDK13 CRISPR cells compared to scramble controls, even though the increase was not large (Figure 21B).

In conclusion, CRISPR–mediated gene knockout did not lead to the anticipated extensive loss of target protein and mRNA. Therefore, the impact on viability and cell cycle was limited.

3.2.6 CDK12/13 Inhibition by Tool Compound BAY-587

Within the scope of our joint project with Bayer AG, our collaboration partner Gerhard Siemeister provided us with a novel tool compound named BAY-587, an ATP competitive inhibitor of CDK12 and CDK13. In a kinase assay, its half-maximal inhibitory concentration (IC50) was 29 nM for CDK12 and 35 nM for CDK13 (Bayer AG, personal communication). At higher concentrations, BAY-587 also inhibits CDK5 / p25 (86 nM), CDK3 (73 nM) and CDK2 / cyclin E (150 nM).
The CTB assay was used to determine metabolic IC50 values for BAY587 and the commercially available, covalent CDK12/13 inhibitor THZ531 in eight different NB cell lines and one colon carcinoma cell line (Figure S 11). IC50 values for BAY-587 ranged from 61 to 174 nM in NB cell lines and 245 nM in colon carcinoma cells HCT-116 (Table 3.1). Sensitivity to BAY-587 did not correlate with sensitivity to THZ531, which was generally less pronounced.

The response to BAY-587 was subject to considerable biological variation between the biological replicates. Phenotypical analysis was therefore also performed with concentrations above and below the calculated IC50 of each cell line.

Table 3.1: Summary of IC50 Values of CDK12/13 Inhibitors BAY-587 and THZ531 in NB Cell Lines and Colon Carcinoma Cell Line HCT116, as Well as Effects on Cell Death and Colony Formation Capacity upon BAY-587 Treatment

<table>
<thead>
<tr>
<th>MYC/MYCN status</th>
<th>Additional aberrations</th>
<th>IC50 BAY-587 [nM]</th>
<th>IC50 THZ531 [nM]</th>
<th>BAY-587 induced cell death [%]</th>
<th>BAY-587 induced colony formation inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-DZ</td>
<td>amp</td>
<td>61</td>
<td>1956</td>
<td>57</td>
<td>very strong</td>
</tr>
<tr>
<td>IMR-32</td>
<td>amp</td>
<td>62</td>
<td>110</td>
<td>53</td>
<td>complete</td>
</tr>
<tr>
<td>CHP-134</td>
<td>amp</td>
<td>67</td>
<td>1555</td>
<td>62</td>
<td>very strong</td>
</tr>
<tr>
<td>IMR-5-75</td>
<td>amp</td>
<td>108</td>
<td>224</td>
<td>73</td>
<td>very strong</td>
</tr>
<tr>
<td>SK-N-BE(2)c</td>
<td>amp</td>
<td>TP53 mut, NF1 del</td>
<td>113</td>
<td>3709</td>
<td>strong</td>
</tr>
<tr>
<td>NB-69</td>
<td>MYC act transl</td>
<td>143</td>
<td>2607</td>
<td>47</td>
<td>complete</td>
</tr>
<tr>
<td>TR-14</td>
<td>amp</td>
<td>MDM2, CDK4 amp</td>
<td>170</td>
<td>5931</td>
<td>very strong</td>
</tr>
<tr>
<td>SH-EP</td>
<td>MYC act transl</td>
<td>174</td>
<td>584</td>
<td>81</td>
<td>complete</td>
</tr>
<tr>
<td>HCT116</td>
<td>normal / RAS mut</td>
<td>245</td>
<td>205</td>
<td>47</td>
<td>complete</td>
</tr>
</tbody>
</table>

amp = amplified; act. transl = activating translocation; mut = mutant
RESULTS

Figure 22: Treatment with CDK12/13 Inhibitor BAY-587 Reduces the Level of CDK12
Cells were treated with multiples of their individual IC50 value or DMSO as a control. After 8 h, cells were harvested and lysed for immunoblotting. Vinculin was used as loading control. Representative image out of n = 2.

BAY-587 reduced the protein level of CDK12. Immunoblotting of NB-69, SK-N-DZ, SH-EP and IMR-5-75 cells treated with BAY-587 revealed that the CDK12 level was reduced in a dose-dependent manner already 8 h after treatment and remained down until the end of the observation period (96 h, Figure 22). Treatment with a concentration equivalent to the individual IC50 reduced CDK12 level by 70% in NB-69, by 75-85% in SK-N-DZ and by 90-95% in IMR-5-75. The examination of CDK13 levels was hampered by the lack of a good antibody. A single replicate in IMR-5-75 cells indicated that CDK13 protein might increase inversely to CDK12 decline (data not shown).

Colony formation capacity is dramatically reduced. Single cells were seeded at very low density to test their capacity to form colonies upon treatment with either of the two inhibitors. Colony formation was significantly reduced by BAY-587 treatment at IC50 concentrations in all tested cell lines (p < 0.05). In general, BAY-587 had a more profound effect on colony formation capacity than THZ531. In four cell lines, no colonies grew at all after BAY-587 treatment (IMR-32, NB-69, SH-EP, HCT116, Figure 23, left panel). In IMR-5-75, SK-N-DZ, CHP-134, TR-14 and SK-N-BE(2)c, colony formation was also severely compromised (right panel). SK-N-BE(2)c cells were least affected but BAY-587 still led to a 89% reduction of the area covered with colonies when compared to DMSO controls. Treatment with BAY-587 at a concentration of 25% below IC50 was still very effective in reducing colony formation capacity to a minimum (data not shown). When treated with THZ531, colonies were significantly reduced by more than 95% in the three cell lines IMR-5-75, SH-EP and HCT116 (p < 0.05). In the other cell lines, the difference between cells treated with THZ531 and controls was not statistically significant; all the same, colonies were reduced by more than 80% (Figure 23).
**RESULTS**

![Image of colony formation assay](image)

**Figure 23: CDK12/13 Inhibition Impacts on Colony Formation Capacity**
Colony formation assay; cells were seeded at low density, treated with BAY-587 or THZ531 on the following day and incubated for 7 - 10 days before fixation and Giemsa staining. Representative biological replicates out of $n \geq 3$.

*Cell cycle distribution is strongly disturbed.* Next, changes in the cell cycle were analyzed after 96 h. Upon treatment with IC50 + 25% of the inhibitors, the fraction of cells in sub-G1 increased dramatically and the typical cell cycle distribution was frequently lost, making assignment to the phases difficult. At BAY-587 IC50 concentration, the number of cells in G1 phase decreased in cell lines NB-69, SK-N-DZ, CHP-134, TR-14 and HCT116 (Figure 24). This change was accompanied by modest increases in the other cell cycle phases, for example S phase in NB-69 and CHP-134 cells. Opposed to that, in IMR-32, the amount of cells in G1 phase was increased, while it was reduced in S and G2 phase. In IMR-5-75 cells, no changes were apparent. In SH-EP cells, cells from every phase seemed to move towards the sub-G1 fraction with increasing concentrations. Upon treatment with THZ531, G1 phase fractions significantly decreased and S-phase fractions increased in IMR-5-75, SHEP, HCT116 and CHP-134 cells (Figure 24).
**RESULTS**

Figure 24: CDK12/13 Inhibition Induces Cell Cycle Distribution Changes

Cells were treated with inhibitors, grown for 96 h and harvested. For cell cycle analysis, cells were fixed in citric acid buffer, stained with DAPI and analyzed by flow cytometry. Shown are representative histograms comparing DMSO controls with cells treated with IC50 of BAY-587 (upper panel) and average cell cycle distribution (lower panel). Sub-G1 fraction omitted for better comparability; mean - SEM, n ≥ 3.
Figure 24, continued.
**Results**

**Strong induction of cell death.** The amount of cell death induced by both compounds was examined by performing a live/dead cell exclusion staining after 96 h. At IC50 concentrations, BAY-587 induced cell death between 30 – 80 % without any correlation to the cell lines’ sensitivity to the inhibitor (Table 3.1). The amount of induced cell death increased with dose (Figure 25). However, the intensity of PI staining was variable. IMR-5-75, IMR-32 and SK-N-DZ cells stained strongly after inhibitor treatment. Opposed to that, most cells accumulated in an intermediate population with a lower PI signal in SH-EP cells. This population could be separated from live and healthy cells by the forward scatter. Microscopic inspection of the cells did not indicate an increase in floating and thus dead cells. NB-69, TR-14, SK-N-BE(2)c and HCT116 cells had similar PI staining profiles, but in these cases microscopic inspection revealed dead cells floating in the medium. SH-EP cells were treated with BAY-587 and incubated for a prolonged period of seven days. Still, the amount of floating cells did not increase, showing that the onset of cell death was not just delayed in these cells (data not shown).

Four cell lines were selected for further experiments to elucidate the response to BAY-587 treatment. IMR-5-75 and SK-N-DZ represent MNA cell lines with intermediate and sensitive response to BAY-587, respectively. Additionally, NB-69 and SH-EP both harbor a MYC activating translocation and showed an intermediate and weak response to BAY-587, respectively.

To identify the mode of cell death, it was attempted to rescue the selected cell lines from BAY-587-induced cell death. Cells were treated with BAY-587 in combination with inhibitor of ferroptosis Ferrostatin-1, inhibitor of necroptosis Necrostatin-1, inhibitor of apoptosis zVAD or inhibitor of autophagy Bafilomycin A1. For this assay, a new batch of BAY-587 was used and the concentrations were adapted to mitigate its impact and thereby enable potential rescue mechanisms (SK-N-DZ: 47 nM, IMR-5-75: 70 nM, NB-69 and SH-EP: 93 nM). However, no rescue could be achieved with any of the used inhibitors (Figure 26). Next, an Apo-BrdU TUNEL assay was performed 24 h after treatment for the detection of apoptotic cells with DNA strand breaks. In IMR-5-75 and SH-EP cells, an additional 4.7 and 3.4 % of total cells had DNA strand breaks when compared to DMSO controls (Figure 27B). DNA staining by PI revealed that cells with DNA strand breaks stemmed from all phases of the cell cycle (Figure 27A). In SK-N-DZ cells, an extra 14.7 % of cells had DNA strand breaks with a slight prevalence of cells from the S phase. Most DNA strand breaks occurred in NB-69 cells (additional 20.6 % of total cells).

In addition to the TUNEL assay, an assay detecting active Caspase 3/7 was performed also 24 h after treatment (Figure 27C). Active Caspase3/7 was mostly present in NB-69 and SK-N-DZ cells with fivefold and 3.4-fold induction, respectively. A less than twofold induction was detected in IMR-5-75.
Figure 25: CDK12/13 Inhibition Induces Dose-Dependent Cell Death in All Cell Lines Tested

Cell death; cells were treated with BAY-587, THZ531 or DMSO as a control. After 96 h, cells were harvested, stained with PI and directly analyzed by flow cytometry. Shown are representative dot plots with gates indicating live cells (upper panels) and bar charts with the average proportion of PI-positive cells (lower panels; mean ± SEM, n ≥ 3).
**RESULTS**

**Figure 26: BAY-587-Induced Cell Death Cannot Be Rescued**

Cells were treated with BAY-587 and inhibitors of specific cell death types. Viability was assessed by CTB after 96 h. Fer-1 = Ferrostatin-1 (ferroptosis); Nec-1 = Necrostatin-1 (necroptosis); zVAD = zVAD(OMe)-FMK (apoptosis); Baf A1 = Bafilomycin A1 (autophagy). mean ± SEM, n = 3

**Figure 27: Cells from All Phases of the Cell Cycle Enter Apoptosis upon BAY-587 Treatment.**

Cells were treated with BAY-587 for 24 h. (A, B) TUNEL assay; cells were harvested, stained with Apo-BrdU and PI and analyzed by flow cytometry. (A) Representative replicates illustrating gating strategy. (B) Induced DNA strand breaks per cell cycle phase (percentage of BrdU-positive cells in DMSO controls was subtracted from that of treated cells); mean ± SEM, n = 4. (C) Caspase-Glo 3/7 assay; luminescence was measured with a Tecan Spark reader; mean ± SEM, n = 3.


**RESULTS**

**RNA expression analysis.** Since both CDK13 and CDK12 are transcriptional CDKs, their inhibition likely influences gene expression. Therefore RNA expression was examined in IMR-5-75, SK-N-DZ, NB-69 and SH-EP cells after BAY-587 treatment. First, the time window after treatment was determined when no morphological changes indicating cell death had occurred yet (eight hours). Three different concentrations of the inhibitor were used: 0.5 x IC50, 1 x IC50 and 2 x IC50 of BAY-587. Genes with a fold change of larger than two were regarded as differentially expressed (normalization and bioinformatic analysis: Umut Toprak).

The number of significantly DEGs correlated with the concentration of the inhibitor in all cell lines. For example, at 0.5 x IC50, there were 1401 DEGs in SK-N-DZ cells (Figure 28A, sum of down- and upregulated genes). Of these, approximately 90% were also differentially regulated at higher concentrations. At IC50, there were already 3108 DEGs and at 2 x IC50, there were 4442 DEGs. Approximately two to three times more genes were down- than upregulated in all four cell lines. However, there were large differences between the cell lines regarding the number of DEGs. In SH-EP cells, almost no genes were differentially regulated at 0.5 x IC50. The number was slightly higher in IMR-5-75. Differential gene expression was highest in SK-N-DZ and NB-69 cells.

![Figure 28: The Extent of Gene Expression Regulation by BAY-587 Treatment Varies between Cell Lines](image)

RNA expression analysis; cells were treated with BAY-587 and harvested after 8 h for RNA extraction and subsequent library preparation. (A) Number of differentially regulated genes in dependence on cell line and inhibitor concentration. (B) Overlap of DEGs between cell lines at 1 x IC50 of BAY-587.
RESULTS

There was considerable overlap of the genes which were differentially expressed in the four cell lines. For example, there were 448 commonly downregulated genes in the 1 x IC50 condition, corresponding to approximately 20 % of downregulated genes in NB-69 and SK-N-DZ and more than 50 % in SH-EP (Figure 28B). In addition, another 615 downregulated genes were shared in three of the four cell lines. 93 genes were commonly upregulated, making up 10 – 45 % of all DEGs in the different cell lines. A total of 162 upregulated genes were shared between three of four cell lines. When comparing SK-N-DZ and NB-69, the two cell lines with the strongest differential expression, the overlap amounted to roughly 35 % and 50 % of downregulated and upregulated genes, respectively.

The DAVID functional annotation tool 6.8 was employed to search for gene enrichments (Huang da et al., 2009a, 2009b). Regardless of fold-change, the top 500 down- or upregulated genes with significant p-value < 0.05 were included to attain enough genes for this analysis. Only gene ontology (GO) term_direct categories were selected in the interest of clarity. Genes downregulated at concentrations of 0.5 x and 1 x IC50 in SK-N-DZ were significantly enriched in processes related to the cilium and its assembly (Figure 29). Genes downregulated at 1 x and 2 x IC50 concentrations were enriched in the DNA repair mechanisms homologous recombination and nucleotide-excision. Genes with elevated expression at all tested concentrations of the inhibitor were related to the cellular component nucleus. In addition, at IC50, several categories related to transcription were among the results.

Figure 29: CDK12/13 Inhibition Leads to Downregulation of DNA Repair Genes and Upregulation of DNA Transcription Genes

Functional annotation analysis of top 500 DEGs in SK-N-DZ after BAY-587 treatment at IC50 using DAVID; p-values are Benjamini-Hochberg adjusted.
### RESULTS

**Table 3.2:** Intersection of Functional Annotation Terms Enriched in Genes Downregulated upon BAY-587 Treatment

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Functional Annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>all cell lines</td>
<td>alternative splicing</td>
</tr>
<tr>
<td>SK-N-DZ, IMR-5-75,</td>
<td>DNA repair, DNA damage</td>
</tr>
<tr>
<td>SH-EP</td>
<td></td>
</tr>
<tr>
<td>IMR-5-75, SK-N-DZ,</td>
<td>splice variant</td>
</tr>
<tr>
<td>NB-69</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3:** Intersection of Functional Annotation Terms Enriched in Genes Upregulated upon BAY-587 Treatment

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Functional Annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>all cell lines</td>
<td>nucleoplasm, nucleus, isopeptide bond, activator, repressor,</td>
</tr>
<tr>
<td></td>
<td>transcription, cytoplasm, Ubi conjugation, biological rhythms,</td>
</tr>
<tr>
<td></td>
<td>compositionally biased region:Ser-rich, transcription regulation,</td>
</tr>
<tr>
<td></td>
<td>phosphoprotein</td>
</tr>
<tr>
<td>SK-N-DZ, IMR-5-75,</td>
<td>coiled coil, compositionally biased region:Gln-rich,</td>
</tr>
<tr>
<td>SH-EP</td>
<td>compositionally biased region:Poly-Ser, splice variant,</td>
</tr>
<tr>
<td></td>
<td>transferase, chromatin regulator, alternative splicing,</td>
</tr>
<tr>
<td></td>
<td>methylation, SH3 domain, compositionally biased region:Pro-</td>
</tr>
<tr>
<td></td>
<td>rich, chromosomal rearrangement</td>
</tr>
<tr>
<td>IMR-5-75, SH-EP,</td>
<td>protein binding</td>
</tr>
<tr>
<td>NB-69</td>
<td></td>
</tr>
</tbody>
</table>

Many of the above-mentioned categories were also present in the gene enrichment sets of IMR-5-75, NB-69 and SH-EP. In order to determine the intersection of categories between the cell lines, enrichment analyses with all DAVID default annotation categories were performed for DEGs at IC50. Alternative splicing was the only common functional annotation of downregulated gene sets of all cell lines (Table 3.2). Furthermore, DNA repair and DNA damage were common annotations of downregulated genes in SK-N-DZ, IMR-5-75 and SH-EP cells. There were 12 annotations common to upregulated genes of all cell lines, including nucleus, transcription regulation, phosphoproteins and biological rhythms (Table 3.3). SK-N-DZ, IMR-5-75 and SH-EP cells additionally shared annotations related to alternative splicing and chromatin modifications.
In summary, the effects of BAY-587 induced inhibition of CDK12 and CDK13 in NB cell lines included induction of cell death, abolishment of colony formation and profound gene expression changes. The compound was more effective than the commercially available THZ531.
4 Discussion

4.1 The Transcriptional Network of MYCN in Neuroblastoma Cell Lines

Elucidating the transcriptional impact of high MYCN levels in NB cells in a cell cycle-resolved analysis was the central task of the first part of this thesis. A better understanding of direct and secondary transcriptional effects of MYCN can help to devise desperately needed new therapeutic approaches for patients with high-risk tumors with MYCN amplification. It can also advise on the timing of drug administration particularly in combination therapies (Ryl et al., 2017).

4.1.1 MYCN Regulates Transcription of General and Cell Cycle Phase-Specific Drivers of Malignant Phenotype

In order to compare gene regulation in MYCN high versus low cells in a cell cycle-resolved fashion, the cells were synchronized at the $G_1:S$ transition. Gene expression was analyzed every two hours, while the cells went largely synchronously through $S$, $G_2$, $M$ and $G_{0/1}$ phase. At this point, MYCN high cells progressed into $S$ phase, whereas MYCN low cells remained in $G_1$ phase. Overall, equal numbers of genes were up- and downregulated in MYCN high cells. This finding is in contrast to previous reports claiming that MYC acts as a transcriptional amplifier by binding promoters of already actively transcribed genes (Lin et al., 2012; Nie et al., 2012). While this discrepancy might be explained by differences between c-MYC and MYCN function and different entities surveyed (lymphocytes, embryonic stem cells), there are also conflicting studies in the field of NB. Zeid and colleagues found that transcription is globally downregulated upon loss of MYCN (2018). However, mechanistic studies have uncovered multiple ways of MYCN-mediated transcriptional repression (Gherardi et al., 2013). Hence, balanced up- and downregulation of gene expression is likely the consequence of a number of distinct mechanisms.

In this respect, the time course experiment revealed gene sets that were constantly up- and downregulated and can therefore be considered as primary targets of MYCN. Gene enrichment analysis of the constantly upregulated gene set identified the processes $G_1:S$ transition and DNA replication. It seems that high MYCN constitutively induces expression of cell cycle drivers like $CDK4$ or $PKMYT1$. In addition, MYCN constitutively downregulates certain inhibitors of the cell cycle, such as $CDKN1A$. Regulation of these direct MYCN targets entails the fast proliferation characteristic of MYCN high cells.
Other cyclins and CDKs were expressed differentially only towards the end of the observation period, when MYCN high and low cells diverged in their behavior (e.g. \textit{CCNE1}, \textit{CCNA2}, \textit{CCNB}). It is conceivable that these differences arise from MYCN-induced faster progression through the cell cycle.

Next to cell cycle genes, the upregulated gene set was enriched in the interconnected keywords nucleolus, rRNA processing and snoRNAs. Ribosomes translate mRNA into proteins and consist of rRNA and ribosomal proteins. Processing of rRNA predominantly takes place in the nucleolus and involves chemical modifications that are guided by snoRNAs (Pelletier \textit{et al.}, 2018). Increased ribosome biogenesis allowing more protein synthesis is essential for the accelerated proliferation of malignant cells. In particular, it has been linked to MYC-driven cancers (Barna \textit{et al.}, 2008; Wahlstrom and Henriksson, 2015). Also snoRNA expression is often increased in cancer tissue (Gong \textit{et al.}, 2017). Schramm and colleagues reported that snoRNA levels were upregulated by MYCN (Schramm \textit{et al.}, 2013). Contrary to these findings, only five snoRNAs were constantly upregulated in the present study. Interestingly, the transcript level of 36 further snoRNAs rose dramatically in MYCN low cells at the time when they remained stalled in G\textsubscript{0/1} phase, indicating that they must have an important function. Possibly, the cells try to overcome the arrest by fueling protein synthesis. Next to taking part in the processing of rRNA for the genesis of ribosomes, a number of non-canonical functions for snoRNAs have been established recently. These include regulation of splicing and chromatin structure as well as serving as precursors of miRNAs (Dupuis-Sandoval \textit{et al.}, 2015). Thus, it remains to be clarified what the role and consequences of snoRNA upregulation in MYCN low cells are. Among the keywords identified in the constantly downregulated cluster was cell adhesion, which is again concordant with a more aggressive phenotype capable of metastasizing (Seyfried and Huysentruyt, 2013). Genes enriched in this cluster were also associated with alternative splicing and the synapse.

Summed up, MYCN induced and repressed a similar number of genes in this cellular model. This included constitutively regulated genes, many of which are essential for the maintenance of a pro-proliferative phenotype with high biosynthetic activity. In addition, many cell cycle genes were differentially regulated in specific cell cycle phases as a consequence of accelerated cell cycle progression.

\subsection*{4.1.2 MYCN-dependent miRNA Expression Changes Reveal an Additional Layer of Gene Regulation}

Understanding of the transcriptional impact of MYCN in NB is not complete without considering the regulation of miRNAs. Consequentially, the expression of miRNAs was analyzed in our MYCN-regulatable cell cycle model. Of all miRNAs detected, 156 or
roughly 27% were differentially expressed in MYCN high versus low cells. This number is slightly higher than in a study comparing MNA tumors with non-amplified ones (20%) (Megiorni et al., 2017). Approximately the same number of miRNAs was up- and downregulated in MYCN high cells. The direction of MYCN-induced expression changes has previously been debated between different studies. Schulte and colleagues found only upregulated miRNAs by comparing expression in primary MNA tumors with an *in vitro* model of ectopic MYCN overexpression (2008). Opposed to that, Chen and Stallings reported up- and downregulated miRNAs in MNA tumors and cell lines treated with retinoic acid to induce differentiation and downregulation of MYCN (2007). The two studies agreed on the upregulation of four miRNAs by MYCN, let-7b, miR-92, miR-181a, miR-181b. The comparison of such previously reported MYCN targets in NB with results of this study revealed a good degree of overlap. However, four miRNAs did not coincide with the reported direction of regulation.

Among the results were many miRNAs regulating genes belonging to processes identified in the mRNA expression analysis. For instance, the list included 29 miRNAs known to regulate the cell cycle and more than half of the miRNAs were ascribed to be involved in ribosome biogenesis, even though this process did not appear in the functional annotation enrichment analysis. Instead, cluster-wise KEGG analysis showed that the pathways and processes identified for the five clusters strongly resembled each other. Recurrent keywords were different cancer types as well as signaling pathways which are defective in cancer. This suggests that there is a general motif of MYCN-dependent miRNA regulation, which is active throughout all cell cycle phases and serves to drive NB malignancy for example by accelerated cell cycle progression, aberrant signaling pathways and increased biosynthesis of proteins and mRNA. Another recurrent theme was synaptic signaling, which had also been identified in the constantly downregulated genes cluster. This implies that expression of corresponding genes may be repressed by miRNAs.

The notion that miRNAs heavily contribute to the oncogenic effects exerted by MYCN was supported by studying particularly highly expressed miRNAs. Among these were two miRNAs from the miR-17-92 cluster, which were consistently high during the cell cycle in MYCN high cells (miR-20a-5p and miR-17-5p). miRNAs from these clusters have been attributed an oncogenic role in various hematopoietic and solid cancers like leukemia, colon, pancreatic and breast cancer (Mogilyansky and Rigoutsos, 2013). Overexpression of miRNAs from the miR-17-92 cluster was also detected in NB cells overexpressing MYCN and tumors with MYCN amplification (Schulte et al., 2008). What is more, inhibition of this cluster in MNA neuroblastomas increases the expression of CDK2 inhibitor p21 and causes cell cycle arrest and apoptosis (Fontana et al., 2008). However, the regulatory impact of these miRNAs is extremely complex. A target gene study demonstrated that
miR-20a and miR-17 inhibit a whole range of both pro-proliferative and anti-proliferative genes, such as **CCND, E2F1, CDKN1A** or **PTEN**, which in summary mediates a pro-proliferative phenotype (Trompeter et al., 2011). Further, in the present study miR-21-5p expression was increased in MYCN high cells starting from G1 phase. This miRNA has been found to drive bladder, lung and gastric cancer and Hodgkin lymphoma (Gu et al., 2018; Z. Liu et al., 2018; Xu et al., 2014; Yuan et al., 2018). Little is known about the role of miR-21-5p in NB except for a report that inhibition induces apoptosis in NB cell line SK-N-SH (Wang et al., 2017).

Inconsistently, miR-101-3p was also upregulated in MYCN high cells, despite having a tumor suppressive role in endometrial, lung, pancreatic and other cancers and being associated with better survival prognosis (Hu et al., 2017; S. Zhang et al., 2017). Similarly, miR-101-3p was shown to inhibit proliferation and colony formation capacity in a MNA neuroblastoma cell line. Despite these discrepancies, it is quite possible that effects may differ between tumor types or cell lines of the same entity. The role of miR-26a-5p, for example, is still under debate and seems to vary between tumor entities. It is frequently downregulated in hepatocellular carcinoma and prostate cancer tissue and adopts tumor suppressive functions in breast, bladder and gastric cancer (Guo et al., 2016; L. Liang et al., 2017). Opposed to that, it promotes tumor growth in lung cancer and ovarian cancer (B. Liu et al., 2012; Shen et al., 2014). In the present study, miR-26a-5p was downregulated in MYCN high cells, arguing for a rather inhibitory role in MNA neuroblastoma cells. This is in line with a study that found low expression of miR-26a-5p to be correlated with poor outcome and MYCN expression in a mouse model as well as in the same cell model used in this study (Beckers et al., 2015). Finally, in this study miR-30d-5p was also expressed less in the MYCN high condition. In the literature, it is described as a tumor suppressive miRNA by some studies, for example in colon, lung and prostate cancer (Song et al., 2018; Y. Wu et al., 2017; R. Zhang et al., 2017). There is no data available yet regarding the role of miR-30d in neuroblastoma. The expression pattern in the present study suggests that it might be inhibited in more aggressive MNA tumors.

In summary, in this study a number of potentially oncogenic miRNAs were increased in MYCN high cells, while potentially tumor-suppressive miRNAs were decreased. Therefore, it can be concluded that in neuroblastoma, MYCN uses miRNAs as an additional layer of gene regulation promoting hallmarks of malignancy like proliferation, metabolism and metastasis. Each miRNA usually has several target genes, and inversely each gene has multiple binding sites for different miRNAs. This leads to a very complex, delicate system of redundancies, additive effects and feedback loops responsible for the fine-tuning of processes like cell cycle progression (Trompeter et al., 2011).
Also, the study provides first evidence for the activity and effect of miR-30d-5p in neuroblastoma. However, in order to gain an understanding of the role of specific miRNAs, more experiments would need to be conducted, for example phenotypical analysis of knockdowns, bioinformatic target predictions and expression analysis in primary tumors.

4.2 CDK13, a Novel Target in MYCN-amplified Neuroblastoma

4.2.1 MYCN Synthetic Lethal Screens Identify CDK13 as One of the Top Candidates

The aim of the second part of this study was to identify and characterize a novel potential target for the treatment of high-risk neuroblastomas. To this end, synthetic lethal relationships in MYCN-driven tumors were sought. Synthetic lethal genes constitute promising new therapeutic targets, since their inhibition would selectively affect MYCN-driven cancer cells, but not healthy tissue (Cermelli et al., 2014). At the same time, understanding of a synthetic lethal relationship may help to elucidate processes and pathways that are essential for malignant behavior. Several other groups have performed synthetic lethal screens with aberrant MYCN or c-MYC expression in the past. However, work published on this subject so far mostly employed MYCN-normal cells with ectopic overexpression of the protein or compared several cell lines with different genetic background (Chayka et al., 2015; Toyoshima et al., 2012). In this respect, the present approach to screen an isogenic cell line with pre-existing MYCN dependency more closely reflects the actual tumor situation. Not surprisingly, it emerged that CDKs and cyclins were particularly strongly represented among the synthetic lethal candidates identified in the screen.

Work in our own group and by others has established the essential role of CDKs in NB biology. MNA cells depend on high levels of CDK4 to evade drug-induced DNA damage due to a weakened G\(_1\) checkpoint (Gogolin et al., 2013). A dual inhibitor of CDK4 and CDK6 was shown to induce cell cycle arrest and senescence preferentially in MNA neuroblastoma cell lines (Rader et al., 2013). Also treatment with CDK2, CDK7/9 or pan-CDK inhibitors is able to impede NB cell growth (Chipumuro et al., 2014; Dolman et al., 2015; Loschmann et al., 2013).

Toyoshima and colleagues performed a synthetic lethal screen with human foreskin fibroblasts ectopically overexpressing c-MYC. They reported hits related to the transcriptional machinery, DNA repair and mitotic control, among others (Toyoshima et al.,
In particular, CDK2 and CCNK were among the synthetic lethal genes. The relevance of CDK2 as a synthetic lethal candidate in NB has also been demonstrated in a study of Molenaar and colleagues. They reported induction of apoptosis after CDK2 knockdown by different RNA interference techniques exclusively in cell lines with MYCN-amplification (Molenaar et al., 2009).

Chayka and colleagues implemented an shRNA drop out screen in NB cells that ectopically overexpress MYCN (2015). They infected their cells with pools of shRNAs and determined which genes had been depleted after the incubation time of two weeks due to synthetic lethality in high MYCN. This approach identified genes involved in the cell cycle and proliferation, but also in cell death, cellular movement and DNA replication. One of the final candidates was PKMYT1, which is responsible for the inactivation of CDK1 and thus progression of the cell cycle.

Another siRNA screen comparing MNA and MYCN-single copy cells identified the transcriptional kinase CDK9 as a potential candidate (Cermelli et al., 2014).

Taken together, there exists ample evidence to support our interest in CDKs and cyclins as candidates for a synthetic lethal relationship with MYCN. At the same time, the above-mentioned kinases have already been thoroughly studied by us and others. Therefore, this thesis focuses on a less well-known family member, CDK13. Its relevance is confirmed by the fact that its partner cyclin K was among the top candidates in the second synthetic lethal screen performed in SY5Y cells with ectopic MYCN overexpression.

4.2.2 Evaluation of CDK12/13 as Potential Therapeutic Candidates

The evaluation of CDK13 in additional neuroblastoma cell lines revealed that while half of the tested cell lines responded strongly to the knockdown, there were also several intermediate responders, such as IMR-32 and IMR-5-75. However, this does not necessarily have to be a disadvantage. For the validation, siRNA-dependent knockdown was used, which never achieves complete gene inhibition and is applied only transitory. As a consequence, “weak” candidates may be underestimated (Cermelli et al., 2014). A complete inhibition or gene knockout for a prolonged time may lead to more detrimental effects in the cells studied. In addition, inhibition of very strong candidates might induce toxicity also in non-transformed cells.

CDK12 knockdown was effective to some degree in three of nine tested NB cell lines. There was no unifying characteristic in the genetic background of these cell lines. In summary, presence of CDK13 protein is vital in most, if not all NB cell lines, whereas CDK12 seems to be dispensable in many of the tested cell lines. Also the additionally tested lung cancer cells with c-MYC amplification and colon cancer cells with c-MYC overexpression responded to knockdown of CDK13. Findings of the present study
performed in our MNA neuroblastoma cells may therefore be transferrable to other entities, thereby considerably increasing its applicability.

A temporal pattern of transcript abundance characterized the expression of $CDK_{12}$ and $CDK_{13}$ in synchronized MNA IMR-5-75 cells. $CDK_{12}$ mRNA levels were highest before mitosis, which is well in line with its reported function. Greifenberg et al. found that $CDK_{12}$ positively regulates genes involved in DNA metabolic processes, DNA replication and DNA repair (2016). All of these processes are necessary for the cell to prepare for mitosis. $CDK_{13}$, on the other hand, is highly expressed when $CDK_{12}$ levels go down, suggesting that they might have overlapping functions.

Data from primary neuroblastoma showed that protein levels of $CDK_{13}$ together with $CDK_{12}$ and cyclin K are decreased in MNA tumors. This is in contrast to many of the cell cycle CDKs and cyclins, which are elevated in these tumors (Ryl et al., 2017). It is conceivable that MYCN upregulates drivers of the cell cycle to ensure rapid proliferation while neglecting transcriptional CDKs. Consequently, any further reduction of the latter might be difficult to tolerate.

### 4.2.3 Characterization of CDK13 Dependency in MYCN-regulatable Neuroblastoma Cells

We used MYCN-regulatable cell models to confirm the synthetic lethal relationship between CDK13 and high MYCN levels. However, upon siRNA-mediated CDK13 knockdown, a differential induction of cell death was only observed in SH-EP cells with inducible MYCN overexpression, but not in IMR cells with inducible MYCN knockdown. Analysis of the effects of CDK13 knockdown on cell cycle distribution was complicated by the changes already induced by differential MYCN expression. In the MYCN high condition, there are more cells in S and G\textsubscript{2} phase, concordant with their faster proliferation rates (Gogolin et al., 2013). This said, observations diverged between the two models. While CDK13 knockdown led to an increase in G\textsubscript{1} phase in IMR cells, an S phase arrest was observed in SH-EP cells. In addition, CDK12 knockdown induced a G\textsubscript{1} arrest in the SH-EP and, less pronounced, in the IMR-32 model.

The described differences in response to CDK12 and CDK13 knockdown can be attributed to the fact that the three cell lines have a different genetic background. IMR-5-75 and IMR-32 are accustomed and dependent on aberrant MYCN expression. In addition, shRNA-mediated knockdown is not capable of completely eliminate MYCN. Opposed to this, MYCN overexpression is new to SH-EP cells, which have to cope with the concomitant changes in transcription and proliferation, only to name a few.
4.2.4 Conditional CDK12/13 Knockdown in Neuroblastoma Cells

As a next step in the characterization of MNA neuroblastoma vulnerability to CDK12/13 knockdown, IMR-5-75 clones with shRNAs against CDK12 and CDK13 were generated. In comparison to the siRNA technique, shRNAs produce less off-target effects and offer the possibility to select single clones instead of analyzing the bulk population, which includes an unknown proportion of non-transfected cells (Rao et al., 2009).

The selection of suitable single clones with strong knockdown on the protein level was complicated by the lack of good antibodies against CDK12 and CDK13. In the course of this study, antibodies by four different companies were tested. All had high unspecific background staining and weak specific staining. Therefore, accurate protein quantification was very challenging. The protein knockdown was most consistent in CDK13 sh1 and CDK12 sh3 clones. CDK13 sh1 was also the cell clone where knockdown reduced viability most strongly. In line with this, IMR-5-75 cells had also responded most strongly to CDK13 siRNA1, from which the shRNA sequence was adopted. In contrast, knockdown by CDK13sh3 predominantly induced loss of colony formation capacity. Even though knockdown of CDK12 by shRNA3 did not impact on viability, it almost completely abolished colony formation. The lack of a more prominent phenotype upon loss of CDK13 may in part also be explained by leakiness of the shRNA vector. This means that also in the absence of doxycycline, the target gene might be repressed to a certain degree which could already negatively impact on the cells. Given that the knockdown efficiency achieved upon induction of the shRNA was still incomplete, it can be concluded that IMR-5-75 are able to cope reasonably well with minimal residual CDK12 or CDK13 protein. Likely, the two proteins can at least in part compensate the loss of each other due to overlapping functions. However, the extent of overlap is currently still unclear. Two different RNA expression studies claimed that the overlap of CDK13- with CDK12-regulated genes amounted to 25 versus 75 %, respectively. Therefore, RNA expression was studied upon shRNA-induced knockdown of CDK12 and CDK13. However, only a small number of DEGs was identified, and no overlap between genes regulated by CDK12 and CDK13 was detected. miRNAs and pseudogenes were strikingly often represented in the results. Size selection during the library preparation process diminishes smaller RNA species; therefore it is conceivable that the actual number of deregulated miRNAs could be much higher. A specialized small RNA sequencing approach could shed light on this question.
4.2.5 CRISPR-induced CDK12/13 Knockout in Neuroblastoma Cells

Going further, an inducible CRISPR interference model was set up to achieve stronger protein knockdown. The employed vectors were designed by Mandegar and colleagues (2016). They had reported high knockdown efficiencies of up to 99 %, and observed no cell death or changes in proliferation or morphology upon expression of the dCas9-KRAB gene alone. Three different guide RNAs against CDK13 were tested which achieved variable knockdown efficiencies between 50 – 90 %. Moreover, the effect of inducing expression of only the dCas vector without presence of the guide RNA vector was tested. Both alternatives reduced viability and confluence in our neuroblastoma cells. Apparently, the dCas9-KRAB fusion protein itself has a detrimental effect on them, even though most publications using dCas9-KRAB report little to no off-target effects. Many more studies have dealt with the specificity of functional Cas9, but results varied depending on the cell line and species used (X. Wu et al., 2014). Since Mandegar and colleagues worked with human induced pluripotent stem cells, this might explain the differences observed.

Thakore and colleagues specifically compared the effects of dCas9-KRAB to untransduced controls and observed 29 differentially expressed genes (2015). In addition, a ChIP Seq study found more than 2000 peaks where dCas9 without guide RNAs bound to the genome (X. Wu et al., 2014). Conceivably, dCas9 scans the DNA for possible PAM sequences, which are an essential recognition motif for dCas9 binding (Sternberg et al., 2014). Likely, the same happened in the present case, so that dCas-KRAB randomly inhibited parts of the genome.

4.2.6 CDK12/13 Inhibition by Tool Compound BAY-587

Analysis of the IC50 values of CDK12/13 inhibitors BAY-587 and THZ531 in several neuroblastoma cell lines with diverse genetic backgrounds revealed that BAY-587 was active at lower concentrations than THZ531. However, sensitivity of the cell lines to BAY-587 did not predict the extent of sensitivity to THZ531. The same observation was made by our collaboration partners working with primary colon carcinoma cultures (Friederike Herbst, personal communication). This discrepancy probably occurs due to the differing mode of action. MNA cell lines tended to be more sensitive to BAY-587 than those with MYC-activating translocation. However, since only eight cell lines were analyzed in this study, a statistical evaluation was not yet possible.

Treatment with BAY-587 decreased the level of CDK2 protein in a dose-dependent manner. Previous studies demonstrated that cyclin K stabilizes CDK12, but not CDK13 (Blazek et al., 2011). It seems that BAY-587 leads to a degradation of CDK12. Based on
the study by Blazek and colleagues, one explanation might be that binding of cyclin K is prevented.

In general, the effects of CDK12/13 inhibition in NB cells were very promising. BAY-587 treatment at IC50 concentration completely or almost completely abolished colony formation capacity in all studied cell lines. In addition, it compromised cell cycle progression. The sub-G₁ fraction increased dose-dependently, suggesting an increase of cell death. This is in line with results of a study analyzing THZ531 effect in Jurkat cells, a model of acute T cell leukemia (Zhang et al., 2016). They also reported an increasing sub-G₁ fraction, albeit only at concentrations four times the IC50 and above. This indicates that neuroblastoma have a higher vulnerability towards inhibition of CDK12 and CDK13. Interestingly, different cell cycle arrest phenotypes occurred in different cell lines. In the majority of cell lines analyzed, cells tended to accumulate in S phase, only in IMR-32, there was an increase in G₁ phase cells upon BAY-587 treatment. It seems that the cellular response towards CDK12/13 inhibition differs between different NB cell lines. The induction of cell death by CDK12/13 inhibition was confirmed by a live/dead exclusion staining with PI. In SH-EP cells, a population with intermediate PI staining and decreased FSC signal formed with increasing concentrations of BAY-587. This was despite a lack of morphological changes of the cell culture. Since cell death goes along with shrinkage of the cell manifested through low FSC, it could be possible that this population is just beginning to die, but their membrane is not yet completely disrupted preventing heavy uptake of PI (Healy et al., 1998). To explore this possibility, incubation of SH-EP cells treated with BAY-587 was prolonged to seven days and afterwards their morphology was assessed. Still, no increase in cells swimming in the medium could be observed. Possibly, the cells went into senescence, which could be assayed by β-galactosidase staining. Alternatively, it might be conceivable that the cells become smaller without losing viability because CDK12/13 inhibition preferentially leads to downregulation of transcription, eventually leading to a general decrease of protein content. This hypothesis could be tested by live-cell imaging.

The next step was to identify the type of cell death occurring in our cell lines. Neither the addition of apoptosis, necrosis, ferroptosis nor autophagy inhibitors prevented cell death. However, two independent assays detected the presences of DNA strand breaks characteristic of late apoptosis and of active Caspases 3 and 7, which indicate early apoptosis. Therefore, the observed phenotype most likely is a consequence of apoptotic cell death. Gene expression analysis upon BAY-587 treatment revealed that the extent of differential expression mirrored the sensitivity of the cell lines to the inhibitor. The number of up- and downregulated genes was highest in cell lines with strong induction of cell death (NB-69,
Contrarily, SH-EP cells, which had low levels of apoptosis, also lacked large changes in gene expression. Despite this difference, the intersection of DEGs was remarkably large. It accounted for half of the downregulated genes in SH-EP cells and was especially high between NB-69 and SK-N-DZ cells. This suggested that inhibition of CDK12/13 affected similar pathways in our neuroblastoma cells despite their different genetic background and differences in the observed phenotype upon inhibition.

To find out more about the biological implications, a gene enrichment analysis was performed. Downregulated genes were enriched in the cellular component nucleus across increasing concentrations, as was exemplified by SK-N-DZ. As CDK12 and CDK13 are localized in nuclear speckles, this result was in line with expectations (H. H. Chen et al., 2007; Ko et al., 2001). Low concentrations of the inhibitor also led to a decrease in genes belonging to cilia and their formation. The primary cilium is a protuberance from the cell, which is involved in cell signaling, but also the control of cell growth / proliferation. Consequently, loss or decrease of the primary cilium have been attributed to several cancer types (Khan et al., 2016). The downregulation of primary cilium genes seems therefore counterintuitive at first glance and merits further investigation. Higher concentrations of the inhibitor led to reductions in the expression of DNA repair genes.

Next, the functional categories enriched in DEGs of all or three out of four cell lines were determined. There were two recurrent categories of downregulated genes, alternative splicing and DNA damage / repair. Liang and colleagues previously reported the regulation of RNA processing upon both CDK12 and CDK13 knockdown (2015). Earlier functional studies found that overexpression of CDK13 and CDK12 altered splicing (H. H. Chen et al., 2007). Interestingly, alternative splicing was also among the categories downregulated in MYCN high cells in the first part of this thesis. This might be an explanation for the synthetic lethal relationship between MYCN and CDK13. If genes involved in alternative splicing are already downregulated in MYCN high cells, any further reduction mediated by loss of CDK13 might be difficult to tolerate. DNA repair, on the other hand, has previously been linked to CDK12 (K. Liang et al., 2015). In addition, Zhang et al. saw an enrichment of DNA damage repair genes after treatment with THZ531 (2016). In this study, upregulated genes of all cell lines were enriched in terms centered around the nucleus, transcription and its regulation, phosphoprotein and biological rhythms. The first three annotation groups can be explained by the role of CDK12 and 13 in phosphorylating the CTD of RNA Pol II, thereby regulating transcription elongation (Greenleaf, 2018). Further, CDK12/13 seem to preferentially regulate genes with regions rich in particular amino acids (serine, proline). Also, upregulated genes were enriched in alternative splicing mechanisms. Finally, chromatin regulators were upregulated in three of our cell lines. A study performed in C. elegans found that knockdown of CDK12...
reduces the level of histone 3 lysine 36 trimethylations (Bowman et al., 2013). These histone marks serve to recruit DNA damage repair gene RAD51 to the DNA (Aymard et al., 2014). Hence, this could be one mechanism how CDK12 regulates DNA damage repair. Taken together, it can be concluded that the observed effects on gene regulation are fully in agreement with the reported roles of CDK12 and CDK13. This may serve as a confirmation of the specificity of the inhibitor. In addition, novel target gene categories could be identified. However, differences and common functions of the two kinases cannot be elucidated by this approach. The inhibition of CDK12 and CDK13 by BAY-587 induced strong cell death also in those cell lines identified as intermediate responders (IMR-5-75, IMR-32). This can likely be explained by two reasons. First, the inhibitor is active against all CDK12/13 proteins in the cells. Opposed to that, a knockdown induced by siRNA or in the shRNA cell clones is never complete, so that the cells are left with a certain amount of residual active protein. Second, the inhibitor is active against both kinases and therefore redundant functions cannot be fulfilled by the remaining kinase as is the case in the knockdown setting.

4.3 Conclusion and Perspective

This thesis contributes to the understanding of MYCN addiction in NB by examining cell cycle-resolved transcriptional changes in MYCN low and high cells. It distinguished constitutively activated and repressed genes that fuel malignant behavior of MYCN high cells from secondary transcriptional effects arising through this behavior. It showed that MYCN furthermore partly relies on miRNAs to implement and amplify these transcriptional changes. Consequences of high MYCN levels included the acceleration of the cell cycle and increased ribosome biogenesis and protein translation, which is in line with published data. Moreover, a potential role of snoRNAs was discovered, which were heavily upregulated in MYCN low cells towards the end of the observation period. Not much is known about the role of this non-coding RNA species in NB. It would therefore be interesting to functionally investigate the role of selected candidates in NB for example by inducing degradation through antisense oligonucleotides or by overexpression studies.

The second part of this thesis examines CDK13 as a potential new therapeutic target in NB. CDK13 repression using different technical approaches resulted in decreased viability and colony formation capacity as well as altered cell cycle distribution despite residual protein. The highly homologous family member CDK12 seems to be less vital in the NB setting, which is in contrast to other cancer entities. Combined inhibition of CDK13 and
CDK12 by the novel compound BAY-587 elicited impressive effects including a strong induction of cell death, cell cycle arrest mostly in S phase and far reaching changes in gene expression.

Work on CDK13 continues in the context of a joint project with Bayer AG. In order to achieve complete or at least extensive reduction of CDK12 and CDK13, CRISPR knockout single clones will be established and biallelic editing confirmed by sequencing. Should it prove that clones with homozygous knockout of CDK12 or CDK13 are not viable, an inducible system will need to be implemented. In addition to the kinases, the role of their partner cyclin K will also be investigated by corresponding knockout studies. Next to IMR-5-75 cells, which were predominantly used in this thesis, additional cell lines will be selected based on high sensitivity to siRNA-mediated CDK13 knockdown and BAY-587 treatment. Moreover, the analysis of CDK12/13 inhibition suggests that a combined knockout of CDK12 and CDK13 could be more effective than single knockouts. All knockout models will be phenotypically characterized with a particular focus on gene expression and proteome analysis. This will identify common and specific downstream targets of CDK12 and CDK13, thereby adding to the knowledge on general CDK12/13-mediated transcriptional regulation gathered in BAY-587-treated cells.

Going further, ectopical overexpression of CDK12 and CDK13 in the knockout clones will clarify in how far the two proteins may take over each other’s function and thus rescue the observed phenotype. This will be complemented by overexpression of mutant genes with non-functional kinase domains to evaluate the relevance of the enzymatic activity for the knockout phenotype.

Sensitivity to the inhibitor BAY-587 will be examined in a larger panel of NB cell lines to determine the role of MYCN amplification and other genetic aberrations for the response. The most sensitive and least sensitive cell lines will be selected to analyze the effect of BAY-587, hoping to elucidate its mode of action. To verify that BAY-587 induces apoptosis as suggested by the present study, additional experiments should be conducted, for example annexin stainings.

Given that DNA damage repair genes were downregulated upon BAY-587 treatment in all four cell lines examined, it is conceivable that vulnerability towards DNA damage is increased upon inhibition of CDK12/13. It would thus be very interesting to combine the inhibitor with DNA damaging agents, such as doxorubicin, or with irradiation.

In conclusion, this thesis sheds light on the transcriptional consequences of MYCN addiction and establishes CDK13 as a promising drug target in neuroblastoma.
References


Chipumuro, E., Marco, E., Christensen, C. L., Kwiatkowski, N., Zhang, T., Hatheway, C. M., Abraham, B. J., Sharma, B., Yeung, C., Altabe, A., Perez-Atayde, A., Wong,


REFERENCES


methyltransferase". Cancer Res, 73(2), 695-705. doi: 10.1158/0008-5472.CAN-12-2522


amplifier of expressed genes in lymphocytes and embryonic stem cells". Cell, 151(1), 68-79. doi: 10.1016/j.cell.2012.08.033


homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour”. Nature, 305(5931), 245-248.


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<th>Reference</th>
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<th>Journal</th>
<th>Volume, Issue, Pages</th>
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<td>Wu, Y. M., Cieslik, M., Lonigro, R. J., Vats, P., Reimers, M. A., Cao, X.,</td>
<td>&quot;MicroRNA-21 (miR-21) regulates cellular proliferation, invasion, migration, and apoptosis by targeting PTEN, RECK and Bcl-2 in lung squamous carcinoma, Gejiu City, China&quot;.</td>
<td>PLoS One, 9(8), e103698.</td>
<td>10.1371/journal.pone.0103698</td>
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Figure S 1: Gene Enrichment Analysis of mRNAs Grouped Together by Unsupervised Clustering
Differentially expressed mRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific gene enrichment analysis. List shows most significant terms (max. 3) of cluster “constitutively up” (yellow line, p ≤ 0.05).
Figure S2: Gene Enrichment Analysis of mRNAs Grouped Together by Unsupervised Clustering
Differentially expressed mRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific gene enrichment analysis. List shows most significant terms (max. 30) of cluster “constitutively down” (green line, p ≤ 0.05).

Figure S3: Gene Enrichment Analysis of mRNAs Grouped Together by Unsupervised Clustering
Differentially expressed mRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific gene enrichment analysis. List shows significant terms of cluster “G_1/S down” (p ≤ 0.05).
Figure S 4: Gene Enrichment Analysis of mRNAs Grouped Together by Unsupervised Clustering
Differentially expressed mRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific gene enrichment analysis. List shows most significant terms (max. 30) of cluster “G1/S up” (p ≤ 0.05).
Figure S5: Gene Enrichment Analysis of mRNAs Grouped Together by Unsupervised Clustering
Differentially expressed mRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific gene enrichment analysis. List shows most significant terms (max. 30) of cluster “G₂/M up” (p ≤ 0.05).
### Table S 1: Intersection of Differentially MYCN-regulated miRNAs in the Time Course Experiment and the Literature *

<table>
<thead>
<tr>
<th>consistently upregulated</th>
<th>reported as upregulated, downregulated in this study</th>
<th>consistently downregulated</th>
<th>reported as downregulated, not consistently down in this study</th>
</tr>
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<tr>
<td>miR-18a-5p</td>
<td>miR-16-2-3p</td>
<td>miR-30c-2-3p</td>
<td>miR-3065-3p</td>
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<td>miR-181-5p</td>
<td>miR-149-5p</td>
<td>miR-628-5p</td>
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<tr>
<td>miR-20b-5p</td>
<td>let-7b-5p</td>
<td>miR-330-5p</td>
<td>miR-1291</td>
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<td>miR-30a-5p</td>
<td>miR-137</td>
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<td>miR-106a-5p</td>
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<td>miR-1908-5p**</td>
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<td>mir-221-5p</td>
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</tr>
</tbody>
</table>

*(Megiorni et al., 2017; Mestdagh et al., 2010; Schulte et al., 2008)

**consistently upregulated in this study
**Figure S 6: KEGG Analysis of miRNAs Grouped Together by Unsupervised Clustering**
Differentially expressed miRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific KEGG analysis. List shows most significant terms (max. 30) for cluster “constitutively up” (p ≤ 0.05).

**Figure S 7: KEGG Analysis of miRNAs Grouped Together by Unsupervised Clustering**
Differentially expressed miRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific KEGG analysis. List shows most significant terms (max. 30) for cluster “turbulent” (p ≤ 0.05).
Figure S8 KEGG Analysis of miRNAs Grouped Together by Unsupervised Clustering
Differentially expressed miRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific KEGG analysis. List shows most significant terms (max. 30) for cluster “constitutively down” (p ≤ 0.05).
Figure S 9: KEGG Analysis of miRNAs Grouped Together by Unsupervised Clustering
Differentially expressed miRNAs detected comparing synchronized MYCN high and low IMR-5-75 cells were submitted to K-means clustering and subsequent cluster-specific KEGG analysis. List shows most significant terms (max. 30) for cluster “G₁/S up” (p ≤ 0.05).
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Figure S10: Enrichment of GFP-Positive IMR-5-75 CRISPR Clones
IMR-5-75 cells were lentivirally transduced with CRISPR vectors containing Cas9, a specific sgRNA (CDK13, CDK12, scramble) and GFP. Transduced cells were taken into long-term culture and expanded. Fluorescence-activated cell sorting (FACS) was used to determine the percentage of transduced cells with GFP expression of each polyclonal culture (left graphs) and to sort GFP-positive cells, achieving > 98% positive cells in the re-analysis (right graphs).
Figure S 11: Determination of Cell Line-Specific IC50 Values
Cells were seeded and treated with a dilution series of the inhibitors after 24 hours. Another 96 hours later, a CTB assay was performed to determine viability. IC50 values for BAY-587 (blue) and THZ531 (red) were determined with the "IC50 Calculator" by AAT Bioquest. mean ± SEM, n = 3-4.
6.2 Publications

Zunächst bedanke ich mich bei Frank Westermann für die Gelegenheit, in seiner Arbeitsgruppe zu promovieren, für die Betreuung während dieser Zeit, seinen überzeugenden Optimismus, selbst wenn es mal (wieder) nicht so gut lief und die Ermöglichung der Teilnahme an Konferenzen und Summer Schools im Ausland. Mein Dank geht ebenfalls an alle, die sich freundlicherweise als Prüfer oder TAC-Mitglieder in das Projekt Doktorarbeit eingebracht und ihre Zeit geopfert haben: Thomas Höfer, Peter Angel, Matthias Selbach und Jan Lohmann.


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Viel, viel Dank gebührt meiner Familie und meinen Freunden, und dabei ganz besonders meinen Eltern, die mich auf meinem ganzen bisherigen Lebensweg mit allen Kräften unterstützten und immer an mich glaubten.

Zu guter Letzt, aber doch allen voran danke ich dir, Josef, denn du bist mein verlässlicher Rückhalt und sicherer Hafen. Danke, dass du dir Tag für Tag geduldig irgendwelche experimentellen Details oder Sorgen anhörst und dich mit mir über alle noch so kleinen Fortschritte freust!