UPPSALA
UNIVERSITET

UPTEC X 15 018
Examensarbete 30 hp
September 2015

Identification of MYCN and SOX9 target genes and a study of drug treatment effects in medulloblastoma

Tiolina Östergren

UPPSALA UNIVERSITET

Degree Project in Molecular Biotechnology

Masters Programme in Molecular Biotechnology Engineering,
Uppsala University School of Engineering

UPTEC X 15 018	**Date of issue 2015-09**

Author

Tiolina Östergren

Title (English)

Identification of MYCN and SOX9 target genes and a study of drug treatment effects in medulloblastoma

Title (Swedish)

Abstract

Medulloblastoma (MB) is the most common malignant brain tumor affecting children. The transcription factors MYCN and SOX9 are associated with initiation, maintenance and recurrence of MB and are also connected to more aggressive tumors. In this study, a ChIP was performed to isolate DNA from genes that are transcriptionally regulated by these proteins. Identification of these target genes will reveal new potential drug targets and help us better understand the functions of MYCN and SOX9. The ChIP was not fully optimized during this project and the target genes were not sent for sequencing and identified. To study the connection between SOX9 and recurrence, cells with different levels of SOX9 were treated with drugs, after which cell viability was measured. No significant difference in resistance could be measured. Change in expression level of MYCN, SOX9 and other relevant genes after drug treatment was also studied. The results show an increase in SOX9 and HES1, suggesting that these genes are involved in tumor recurrence.

Keywords

Medulloblastoma, MYCN, MYC, SOX9, HES1, ChIP, recurrence

Supervisors

Fredrik Swartling, Vasil Savov, Holger Weishaupt
Department of Immunology, Genetics and Pathology, Uppsala University

Scientific reviewer

Tobias Sjöblom
Department of Immunology, Genetics and Pathology, Uppsala University

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **29**

Biology Education Centre Biomedical Center Husargatan 3, Uppsala
Box 592, S-751 24 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Identification of MYCN and SOX9 target genes and a study of drug treatment effects in medulloblastoma

Tiolina Östergren

Populärvetenskaplig sammanfattning

Medulloblastom (MB) är en form av malign hjärncancer som framförallt drabbar barn. De behandlingar som används idag ger en genomsnittlig överlevnadsgrad på 70-80%. Överlevande drabbas ofta av allvarliga bieffekter av behandlingen och återfall av MB får nästan alltid dödlig utgång. Förändringar hos de gener som ger upphov till transkriptionsfaktorerna MYC och MYCN har kopplats till utveckling av MB respektive till mer aggressiva tumörer. Även transkriptionsfaktorn SOX9 har kopplats till mer aggressiv tillväxt av cancern och tros även kunna driva återfall. Identifiering av gener som påverkas av dessa transkriptionsfaktorer kan ge exempel på nya mål för läkemedel och ge oss en bättre bild av hur det kommer sig att de driver MB.

Detta arbete beskriver hur kromatin immunoprecipitation (ChIP) användes, med målet att identifiera de gener som regleras av MYCN eller SOX9 i olika cellinjer. För att undersöka kopplingen mellan SOX9 och återfall, behandlades medulloblastomcellinjer med olika uttryck av SOX9 med läkemedel, efter vilket mängden överlevande celler mättes. Ingen skillnad i överlevnad som kunde koppla SOX9-uttryck till överlevnad kunde mätas. Förändringar i uttrycket av MYCN, SOX9 och de cancerrelaterade generna MYC och HES1 mättes även efter läkemedelsbehandling. Det visades att det genomsnittliga uttrycket av SOX9 och HES1 ökat under behandlingen, vilket kopplar dessa gener till återfall av MB.

Examensarbete 30 hp
Civilingenjörsprogrammet i Molekylär Bioteknik

Uppsala universitet, september 2015

TABLE OF CONTENTS

ABBREVIATIONS

cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
DPBS	dulbecco's phosphate-buffered saline
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transformation
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hCMYC	human MYC
HES1	hairy and enhancer of split-1
hSOX9	human SOX9
MB	medulloblastoma
mSOX9	mouse SOX9
qPCR	quantitative polymerase chain reaction
rtTA	reverse transcriptional transactivator
SHH	sonic hedgehog
TB	transcription factor
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TF	transcription factor
TRE	tetracycline-response element
uni-MYCN	universal MYCN
WNT	wingless

INTRODUCTION

Medulloblastoma

The most common pediatric malignant primary brain tumor is Medulloblastoma (MB), a cancer that originates in the hindbrain (cerebellum) and only rarely affects adults (1,2). Treatment with surgery, radiation and chemotherapy give 5-year survival rates of 70-80% for average risk disease, but survival rates can be much lower for patient groups with certain risk factors and decrease over time (3,4). These treatments can also result in side effects such as hearing loss (5), neuropathy (6) and nephrotoxicity (7), decreasing the quality of life for survivors (8) and limiting the level of treatment for sensitive developing brains in young children. There are 4 transcriptionally characterized subgroups of MB (9,10):

- The WNT subgroup, mainly associated with changes in the Wingless (WNT) signaling, is the rarest type and has a high long term survival rate.
- The SHH subgroup, defined by and often driven by Sonic Hedgehog (SHH) signaling, with an intermediate prognosis.
- Group 3, where the driving signaling pathway is not fully known, but immunohistochemical positivity for NPR3 could be used as a marker. This group has the worst prognosis.
- Group 4, with largely unknown molecular characteristics, and a prognosis similar to SHH.

One of many other differences between the subgroups is the recurrence pattern. Local recurrence is most common for SHH and metastatic recurrence for Group 3 and 4 (11). Recurrence is almost always fatal (12). These differences need to be taken into consideration regarding choice of treatment and the molecular causes behind them further studied in order to develop more specialized and effective drugs.

MYC and MYCN

Mis-expression of the MYCN gene is associated with tumor development in cancers such as Medulloblastoma, Glioblastoma, Neuroblastoma and malignant Glioma (1,13–15). In more than 50% of all MBs there is a mis-expression of the MYCN gene. It has been shown that targeted expression of MYCN can initiate MB independently of SHH and that it is involved in progression, maintenance and metastasis (16).

In 10% of MB there is an amplification of the MYCN gene or the related MYC gene. While high expression levels are indicators of MB, only amplifications are correlated with aggressive tumors and poor survival (17). MYCN is amplified in SHH or Group 4 (18). Expression is high in the SHH and WNT group, while Group 4 has a rather low expression level of both MYC and MYCN. MYC is expressed in Group 3 and WNT, but amplified only in Group 3. These cases are the most lethal. Group 3 tumors without this amplification do not have a worse prognosis than having the same prognosis as the SHH group (1,17).

The MYCN gene codes for the N-myc proto-oncogene protein, a transcription factor (TF) with an important role in regulating spine, forebrain and hindbrain development (1,19). It is not expressed in adult cerebella, but during its development and in MB (16). The related MYC gene codes for the TF c-Myc, which affects cell cycle progression, apoptosis and cellular transformation and is a mediator of Notch signaling (20, 21).

SOX9

Another gene associated with MB and aggressive tumor progression is SOX9 (22). The SOX9 gene codes for the transcription factor SOX9, a downstream target of Notch involved in development, differentiation and lineage commitment during embryogenesis. It can induce proliferation and inhibit senescence and is overexpressed in many cancer types (23, 24). An important function of the

SOX9 gene is regulation of cartilage extracellular matrix (ECM) genes and several genes for enzymes involved in ECM modification (25). It is highly involved in epithelial-mesenchymal transformation (EMT), an important part of tumor cell migration, which is dependent on alterations in the ECM (26). Preliminary experiments show that increased SOX9 levels in MYCN-driven MB cells can drive migration to the forebrain and SOX9 positive cells in SHH-independent MB are able to initiate tumor recurrence. Also, high SOX9 expression is associated with the SHH subgroup, where it regulates SHH-dependence (1,18). It is also known to inhibit WNT signaling (27).

HES1

The HES1 gene codes for the transcriptional repressor Hairy and enhancer of split-1. It is also a downstream target of Notch signaling, involved in embryonic development and cell migration (28-30). Overexpression of HES1 and mutations of other genes in this pathway have been associated with development of several cancer types (31, 32).

Aims of this study

- To identify target genes that are transcriptionally regulated by MYCN or SOX9 in different MB cell lines.
- To study if different levels of SOX9 expression affect drug resistance.
- To evaluate how the expression of genes SOX9, MYCN, MYC and HES1 change during drug treatment in cells with different starting levels of SOX9 expression.

For target gene identification, chromatin immunoprecipitation sequencing (ChIP)-sequencing will be used. DNA fragments to which MYCN and SOX9 bind will be isolated through ChIP, for four different cell lines. These fragments sequences contain binding sites for one of the TFs and represent genes that are being regulated. These sequences can later be compared to the sequencing of RNA from the same cell lines, to see which of those genes that are being expressed and thereby are likely to be activated by TFs.

MB002 and MB002/S9wt cells will be treated with Doxycycline, Vincristine and Cisplatin. Viability will then be measured through alamarBlue® Cell Viability Assay (33). The gene expression of the drug treated cells will also be analyzed through RNA extraction, complementary (cDNA) synthesis and quantitative polymerase chain reaction (qPCR).

MATERIAL AND METHODS

Cell lines

GTS1905

The GTS cells come from recurred MB tumors in mice. These mice have a transgene where the SOX9 promoter drives expression of a reverse transcriptional transactivator (rtTA) (18). When bound by Doxycycline, the rtTA activates a bidirectional tetracycline-response element (TRE) that expresses the MYCN oncogene and Luciferase. This expression will only occur in SOX9 positive cells, where the SOX9 promoter is active, and results in a tumor. Doxycycline treatment then kills the SOX9 negative cells with less MYCN expression. When the Doxycycline treatment stops, the SOX9 positive cells can proliferate again and a tumor can recur. In contrary to what could be expected, all GTS cells are not SOX9 positive.

MB002 and MB002/S9wt

The MB002 cells come from a human MB cell line. The MYC gene is amplified and they belong to Group 3 (34). MB002/S9wt have been transduced with two different lentiviruses. One expresses mouse SOX9. The other one expresses a repressor of that mSOX9 gene. Doxycycline binds and inhibits the repressor, resulting in mSOX9 expression.

SF8368 and SF8638/S9wt

The SF8638 are primary human MB cells that belong to the the SHH subgroup and have a relatively high SOX9 expression. SF8638/S9wt is transduced with the same lentiviruses as MB002/S9wt.

Cell culturing

All cell lines were grown in cell incubators at 37°C, 5 % CO_2 and 100 % humidity. They were split using StemPro Acutase (Gibco) for cell separation and DPBS (Gibco) for washing. GTS1905C1 and GTML3 cells were grown in Complete Neurobasal Culture Media consisting of Neurobasal Media without vitamin A (Gibco) supplemented with 1xB27 without vitamin A (Gibco), Penicillin-Streptomycin (Gibco), L-glutamine (Gibco), 20 ng/ml fibroblast growth factor (FGF) (PeproTech) and epidermal growth factor (EGF) (PeproTech). SF8368 was grown in Complete Neurobasal Culture Media additionally supplemented with 1xN_2 (Gibco). MB002 and MB002/S9wt was grown in Stem Cell Base Media consisting of equal amounts of Neurobasal Media without vitamin A and DMEM/F-12 (Gibco) supplemented with MEM NEAA (Gibco), sodium pyruvate (Gibco), Hepes (Gibco), GlutaMAX (Gibco), leukemia inhibitory factor (Millipore), heparin (Stemcell Technology), Penicillin-Streptomycin, B-27 and 20 ng/ml EGF and FGF.

Isolation of DNA fragments with MYCN or SOX9 binding sites using ChIP

Cross-linking transcription factors to target genes

10 cm cell culture dishes (Falcon) with 8 million cells each were cultured overnight. Transcription factors were then cross-linked to their target genes by adding Complete Cell Fixation Solution prepared according to the Instruction Manual for ChIP-IT® High Sensitivity (Active Motif). Cell fixation was stopped using Stop Solution (Active Motif). The cells were washed twice with PBS Wash Buffer prepared according to the Instruction Manual (Active Motif). This step binds the TFs to the DNA until reversal of cross-linking (Figure 1).

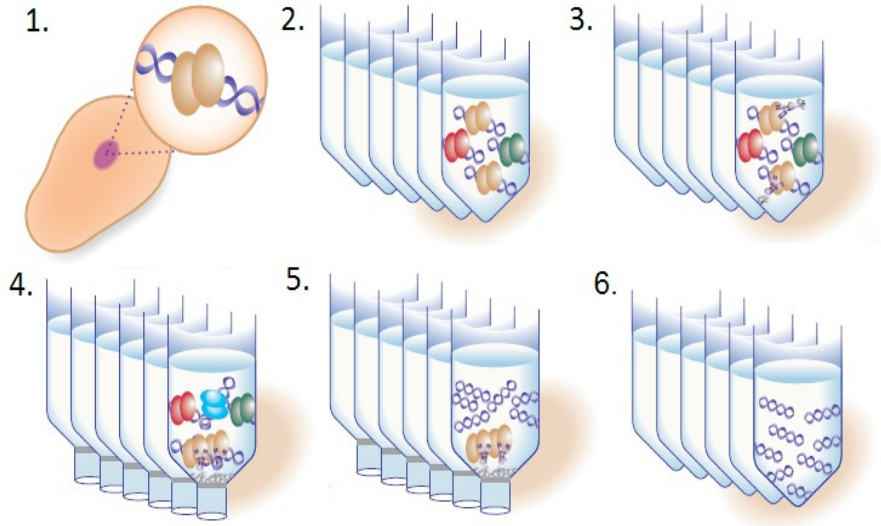

Figure 1. The ChIP process. 1. All transcription factors are cross-linked to their target sequences. 2. The cells are lysed and DNA is sheared to shorter fragments. 3. Antibodies are added that bind desired proteins (MYCN and SOX9). 4. Agarose beads, which bind the antibodies, are added and unbound DNA is washed away. 5. The antibody-bound protein/DNA-complexes are eluted. 6. Cross-links are reversed and the DNA is purifed. Results are controlled with qPCR. The DNA is sent for sequencing. **(**Modified from ChIP-IT® High Sensitivity Intruction Manual (Active Motif).)

Sonication

Lysis, Wash and Shearing buffers were prepared according to the ChIP protocol for Chromatin fragmentation using the Covaris S2 sonicator by Ethan Ford. The cells were lysed in Lysis buffer. After this step the cells were kept on ice and buffers added were cold. The cells were washed using Wash Buffer and centrifuged. The Wash buffer was carefully removed from the pellet and tube using Shearing Buffer and the cells were then resuspended in Shearing Buffer and transferred to a 1 ml sonication tube (Covaris). To get shorter fragments of DNA, the cells were sonicated using a Covaris S2. Some of the fragments contain a binding site for MYCN or SOX9. Different settings (Duty cycle, Intensity, Cycles per burst, Time) were tested on new samples until the length of the fragments were measured to be around 200-500 bp long (Table 1). Sonication was alternating between on and off every 30 seconds for all sonications.

Table 1. Sonication settings. Sonications were performed with different settings to achieve fragments of around 200-500 bp. Some settings were tried more than once. Lengths measured with the bioanalyser might have been incorrect due to problems with measuring the concentration.

No.	**Cell line**	**Parameters** (Duty cycle, Intensity, Cycles per burst)	**Time** (min)
1	GTS1905	5%, 4, 200	12
2	SF8638	10%, 5, 200	12
3	MB002	10%, 5, 200	15-33
4	GTS1905	20%, 10, 1000	10,15
5	SF8638	10%, 7, 500	5, 10, 15
6	MB002	20%, 8, 500	5, 10, 15
7	MB002	10%, 8, 500	10,15
8	GTS1905	10%, 6, 500	15, 20, 25
9	GTS1905	10%, 5, 500	15, 20, 25
10	MB002	5%, 4, 200	15, 20, 25
11	SF8638	5%, 5, 200	15, 20, 25
12	GTS1905	10%, 4, 200	15, 20, 25
13	SF8638	5%, 5, 200	10,12
14	MB002	5%, 5, 200	10,12
15	SF8638	5%, 4, 200	12
16	MB002	5%, 3, 200	12
17	MB002	5%, 4, 200	12
18	MB002	5%, 3, 200	12
19	MB002	2%, 4, 200	12
20	SF8638	5%, 4, 200	12
21	GTS1905	5%, 4, 200	12

DNA purification and measuring the length of the DNA fragments
Chloroform (Life Technologies) was added to a part of the sonicated sample and mixed thoroughly. After centrifugation, the upper phase with the DNA was collected and the step was repeated. The upper phase was then mixed with glycogen (Life Technologies) and 3M sodium acetate (pH 5.2). Cold 100% ethanol was added and the sample was kept in -80ºC for 1 h. After centrifugation, the supernatant was removed and the pellet washed with cold 70% ethanol, dried and resuspended in TE-buffer (Sigma Aldrich). The DNA concentration was measured using the Qubit® dsDNA BR assay (Life Technologies) for sonicated samples 1-14 and using NanoDrop 2000 (Thermo Scientific) for samples 15-21.

Fragment analysis was performed using the Agilent 2100 Bioanalyzer or gel electrophoresis. For measuring with the bioanalyser, the High Sensitivity DNA Assay Protocol (Agilent Technologies) was performed. For measuring with gel electrophoresis, a gel with 1.5% Agarose (Sigma Aldrich) in 0.5x TBE-buffer was used. The samples were mixed with 6x DNA Loading Dye (Thermo Scientific) and Gene Ruler 100 bp (Thermo Scientific) was used as ladder.

Immunoprecipitation
Immunoprecipitation was performed on sonicated samples from GTS1905, MB002 and SF8638 cells (No. 12, 16, 20 and 21, Table 1). MYCN antibody ab16898 (Abcam) or NMYC-1 (Thermo Scientific) was added to sonicated no. 12 and 16. SOX9 antibodies ab3697 (Abcam) or ab5535 (Millipore) was added to no. 20 and 21. All samples were also treated with IgG antibody (Millipore) to produce a negative control for the qPCR ChIP control. The amount of antibody added was based on recommended concentrations for ChIP according to their manuals. Blocker (Active Motif) and Protease Inhibitor Cocktail (Roche) was added and the samples were kept in 4ºC overnight. Protein G agarose beads (Active Motif) were washed in TE-buffer (pH 8.0) (Sigma Aldrich) and added to the samples, which were then incubated rotating in 4ºC for 3 h. The beads bind the antibodies. In this way the DNA fragments that are bound by MYCN, SOX9 or IgG, depending on antibody used, are indirectly bound to the beads. ChIP Buffer (Active Motif) was added and the samples were filtered through a ChIP Filtration Column (Active Motif). Wash Buffer AM1 (Active Motif) was added and filtered through the columns five times to wash away unbound DNA. The bound DNA was eluted in Elution Buffer AM4 (Active Motif). This DNA represents the fragments containing binding sites for MYCN, SOX9 or IgG.

Reversal of cross-links and DNA purification
The DNA samples were treated with 0.2 µg Proteinase K (Active Motif) per µl to remove protein. The samples were diluted 1:5 in DNA Purification Binding Buffer (Active Motif) and 3M Sodium Acetate was added to adjust the pH. The samples were filtered through a DNA Purification column (Active Motif) and washed with DNA Purification Wash Buffer (Active Motif). The DNA was then eluted in DNA Purification Elution Buffer (Active Motif). This DNA should represent fragments containing binding sites for MYCN, SOX9 or IgG.

Primer design
Primers for the ChIP control qPCRs were designed and ordered from Sigma Aldrich. Positive control primer pairs were designed to match within around 500 bp upstream from the first exon of genes that have been identified as probable MYCN or SOX9 targets in previous data (Table 2) (23, 35–39). This is where a TF is likely to bind if activating expression of a gene. Negative control primer pairs were designed to match somewhere at the end of a random gene, where any TF is unlikely to bind. For human and mouse positive control primers, corresponding genes were chosen. Genes for negative control primers were chosen separately.

Table 2. Primers used for ChIP control. Primers 1-11 match mouse genes associated with SOX9 and primers 12-22 to MYCN. Primers 23-25 match randomly chosen mouse genes. Primers 26-35 match corresponding human genes associated with SOX9 and primers 36-46 to those associated with MYCN. Primers 47-49 match randomly chosen human genes.

No.	Gene	Forward primer sequence	Reverse primer sequence
1	Fzd2	CCTTTCTCCTTCACTTCACC	GCACAAAGGAGCCCATTA
2	Lhx2	GAGAATGGAGACTTCCTCAAG	GAACAGTTTCCTGTGTTTCTTC
3	Wwp2	AATGTTCAGTTTACTCCGTAGG	GACAACCTGACACCCTTTC
4	Sulf2	CAGCACTGAGACTGGAATG	CACCTTTGGCTAGTGTAGAG
5	Id4	CGTAGGAACTTCTCTCTCTCTT	GCACTCCATTCCGTCAAC
6	Col9a2	GCTAACAGGATGCCTCAAG	CCTCCACCATCAATTCCATTA
7	Adamtsl4	GCATAGGCAGGCCTTTAATC	GGTTTGTTGTTTGTCTTTGAGG
8	Col11a2	GACCTTTCCATCAGCATCG	GAACGGAGAAAGCTGAGATG
9	Col2a1	TCCATCTGCTCATGCTTTG	GGTTGTTTGCAGAGGCATA
10	Adamts4	GGTCCCATCCTCTCTTCTT	TCATTTATGCTACCCTTTACCC
11	Nfatc1	CCAGCGTTGATTGCTTCA	CCCAACATGGCTTCTCTTAG
12	Cdk4	GCATACCAACTGGAAGGAAG	GCCAGAAATGGTGCTGTAA
13	Hspd1	CCCTGTCCTATTAGTCCCAAA	AGAGTCATGGTGAGTGTCTC
14	Npm1	AGCGATAGGCCTTCATTTG	CCAAAGAGAAACCCAGTCAG
15	Ncl	AGAGGTCACCGACATTACA	AACGGCTTCTTGGCTATG
16	Ahcy	GATAGCTCCGCAGTCATTTC	CTCCCTCTGTTTGCCTTTC
17	Nme2	CTGGAAGGCTCTTAACTACAAA	CAGGAGGATCAGGAAAGGA
18	Cct4	GGGATTATGAGGATTAGTGTGG	TAACCACTGACTCTTAGGGAA
19	Phb	GAGAAGCCATACACATGATCTC	AAGGGAGTCGGTCATTCA
20	Mcm7	GACACAGACTGATCTGGATTC	CCCAGTTCTTCATTCTGTCTT
21	Nras	TTGCTGCTGCTTAGATCAC	AGGATTCATTTCAGGCACAA
22	Trap1	CTTGCTACTAGTGTCTGCTAAC	GACAGGTTCTCGCTTGATTC
23	Oog1	CTTGGATTGCCTGCCCTATT	TCAGGGTATCTCCAACTCTCTC
24	Vmn2r10	AAACCAGGCCACTTGTATCC	GAAAGCCATGAGCACAGTTATTG
25	Prl7a2	GGAATGACAGACAAATGGAGAA	ATCCCAATAGTCCCATCTACTT
26	Fzd2	ATTGGCCGAGAGCACATT	GCCTCTCCGATCCCAATTA
27	Lhx2	GAAGATATCGCAGGAAGCTGTT	CAAGAGGAGCAGAGGAGAGT
28	Wwp2	AACAGTCTGAAGGTCTCGTTTC	ATGGTCCCAGGCAATCAAATA
29	Sulf2	AACAGTCTGAAGGTCTCGTTTC	ATGGTCCCAGGCAATCAAATA
30	Id4	TCCCTTGGGCACACATCAG	AATGACGCTCGGGCCAAT
31	Col9a2	CTGGAGAGGACGTAGGGTA	CTGGAGAGGACGTAGGGTA
32	Adamtsl4	TCGCTGAGTCCCGAGTGTAA	TCAGTCACTCCTGCCTCTCC
33	Col2a1	AAGCGTGACTCCCAGAGA	GCTGGGCTGTAACCTGAAC
34	Adamts4	CTCACTCTCTGCCTCTCCT	CTCTAGCAGCCGAATGGATAAT

35	Nfatc1	GCGGGATGGGAATTTCCTTTCTA	AAAGCCCGGCATGCTGAA
36	Cdk4	CCACATACACACTGGAAGCA	TTTCGCTCCACCATGAGAAA
37	Hspd1	ACTGAGTGGCCACCAATCC	TCTGAGGCGGAGGGAGTAAT
38	Npm1	TAAGCCGCAATTCACTCTCTC	TAAGCCGCAATTCACTCTCTC
39	Ncl	GCTCGCTTTGTGCAAGGT	GCTCGCTTTGTGCAAGGT
40	Ahcy	CCTGGCCTGCAACTTTACT	AACCCAGCGGAACTGAAAT
41	Nme2	ACTCCTCCCGTTCCCTCTT	GCAAGCTGCAAGGGAATCT
42	Cct4	TGGCTGACGCCTGTAATC	TGGCTGACGCCTGTAATC
43	Phb	GTTCCACCTGTCCTCTTCATC	CTAAGACTGCCGCAAGGAG
44	Mcm7	TGTTGGCCAGGCTGATTT	GGTGCATTCCGCCAAATAAAG
45	Nras	CCCTTGGTGTAGCTTCTGTT	TCTTAGACAAAGCCTTCAGTCC
46	Trap1	ATTCACAAAGGTCGCAGACA	TAGCTGTGCGCATTCCTG
47	Hipk4	CCCTCAGAACAGAATCCAAACT	GCTTCTTGAGCTTGGGAGAT
48	Ssbp2	TCTCCAGTGAGGAGCTGAG	GGGAATTAGAAACTGCTCTAGAAGG
49	Spata9	GTGGTATCACTGCAGAATTTGG	GAGAGGAAGAGGAGTGTGTTTG

ChIP control qPCR
All primers (Table 2) were combined with MYCN, SOX9 or IgG immunoprecipitated DNA samples or with input DNA, which had not been immunoprecipitated and should contain all genes. With MYCN and SOX9 treated DNA, positive control primers should result in a high amount of product while negative control primers should not. With IgG treated DNA, no primers should create a product. The input DNA should result in a product in combination with any primer.

All DNA samples were diluted to 6 ng/µl and mixed with SYBR green mix (Applied Biosystems), distilled water and one pair of forward and reverse primers from the primer group matching the expression of the cell line and the antibodies used for immunoprecipitation (Table 3). The following program was set for the StepOnePlus Real Time PCR system (Applied Biosystems):

95ºC 10 min
(95ºC 15 sec
60ºC 60 sec with data acquisition) x45
95ºC 15 sec
60ºC 60 sec
(0.3ºC increase, hold for 15 sec with data acquisition) until reaching 95ºC

Table 3. qPCRs were performed to test the primers. All primers were tested by being combined with different DNA samples, where their target sequence was expected to be present or not.

qPCR no.	Cell line	Primers in qPCR
1 and 2	GTS1905	Mouse MYCN
3 and 4	MB002	Human MYCN
5 and 6	SF8638	Human SOX9
7 and 8	GTS1905	Mouse SOX9

Drug treatment of MB002 and MB002/S9wt

Doxycycline is a tetracycline antibiotic that induces expression of SOX9 in MB002/S9wt transgenic cells. Vincristine and cisplatin are chemotherapeutic drugs that are both being used to treat several cancer types, including MB. Vincristine inhibits mitosis through binding of the tubulin protein (40). Cisplatin induces cytotoxicity by interfering with transcription and replication mechanisms and can also induce apoptosis (41).

MB002 and MB002/S9wt cells were plated 10.000 cells/well in 96-well plates (Falcon) and 300.000 cells/well in 6-well plates (Falcon). After incubating the cells for 24 h, drugs were added. 5 different concentrations of Doxycycline (0, 1, 5, 10, 20 ng/ml), Vincristine (0, 10, 50, 100, 200nM) or Cisplatin (0, 0.08, 0.4, 2, 10 µM) were added to each cell line (Table 4). MB002 cells were not treated with Doxycyline.

Table 4. Setup of drug treatment of cells. Cell lines with different levels of SOX9 expression were treated with different concentrations of drugs for viability assay and expression analysis.

Cell line	Drug: concentrations
MB002	Doxycycline: 0, 1, 5, 10, 20 ng/ml
MB002/S9wt	Doxycycline: 0, 1, 5, 10, 20 ng/ml
MB002	Vincristine: 0, 10, 50, 100, 200nM
MB002/S9wt	Vincristine: 0, 10, 50, 100, 200nM
MB002	Cisplatin: 0, 0.08, 0.4, 2, 10 µM
MB002/S9wt	Cisplatin: 0, 0.08, 0.4, 2, 10 µM

Cell viability assay of drug treated cells

Viability of the drug treated cells in 96-well plates (Table 4) was measured after 24, 48 and 72 h using the alamarBlue® Cell Viability Assay. Resazurin was added to the wells. During a 2 h incubation, it is reduced by metabolically active cells into resorufin. The fluorescence of resorufin was measured, which is proportional to the number of living cells (24).

Expression analysis of drug treated cells

The cells in 6-well plates (Table 4) treated with Doxycycline were collected after 24 and 72 h and cells treated with Vincristine or Cisplatin for 72 h and kept in Trizol (Qiagen Sciences) in -80ºC for at least 24 h. Expression levels of human SOX9 (hSOX9), universal MYCN (uni-MYCN), human MYC (hCMYC), mouse SOX9 (mSOX9), human HES1 and human GAPDH (for normalization) were analyzed for drug treated MB002 and MB002/S9wt cells using qPCR.

RNA extraction

100 µl Chloroform was mixed with the thawed samples. After centrifugation, the top layer containing the RNA was transferred to a new tube and mixed with 70% Ethanol. The mixture was filtered through an RNeasy column (Qiagen Sciences). RW1 (Qiagen kit) was added and spun through as well as RPE (Qiagen Sciences). The RNA was eluted in RNase free water (Qiagen Sciences) and the concentration was measured with a NanoDrop.

cDNA synthesis

5x VILO Reaction Mix (Life Technologies), 10x SuperScript Enzyme Mix (Life Technologies) and 50 ng RNA was mixed. The volume was adjusted to 20 µl with DEPC-treated water. The samples were incubated in 25ºC for 10 min, 42ºC for 60 minutes and 85ºC for 5 min.

qPCR

Each cDNA sample was diluted 10 times and a qPCR was performed following the same instructions as for the ChIP control qPCR. Primers matching genes GAPDH, human SOX9 (Sigma Aldrich), universal MYCN (Eurofins Genomics), human MYC (Sigma Aldrich), mSOX9 (Eurofins Genomics) and HES1 were used. Recieved cDNA samples from MB002/S9wt treated with 0, 1, 10 and 100 ng/ml of Doxycycline, MB002 treated with 0 and 1000 ng/ml Doxycycline and SF8368/S9wt treated with 0 and 100 ng/ml of Doxycycline were analyzed with the same primers.

RESULTS

Target genes for MYCN and SOX9 in MB cell lines

Sonication settings

After sonication, most samples had a majority of fragments shorter than the desired 200-500 bp (Figure 2.A,B). When comparing the concentrations of DNA in the sonicated samples measured with the Qubit and NanoDrop, it was discovered that the Qubit showed much lower concentrations. Some measurements with the bioanalyser gave results where the length of the fragments could not be interpreted (Figure 2.C,D,E). Sonication number 17, MB002 cells sonicated with Duty cycle 5%, Intensity 4 and 200 Cycles per burst for 12 minutes, showed a broad range of lengths of fragments with a peak around 200 bp (Figure 3.A). The same settings were used for sonication of SF8638 (number 20) and GTS1905 (number 21) cells, which resulted in similar length intervals when measured with gel electrophoresis (Figure 3.B).

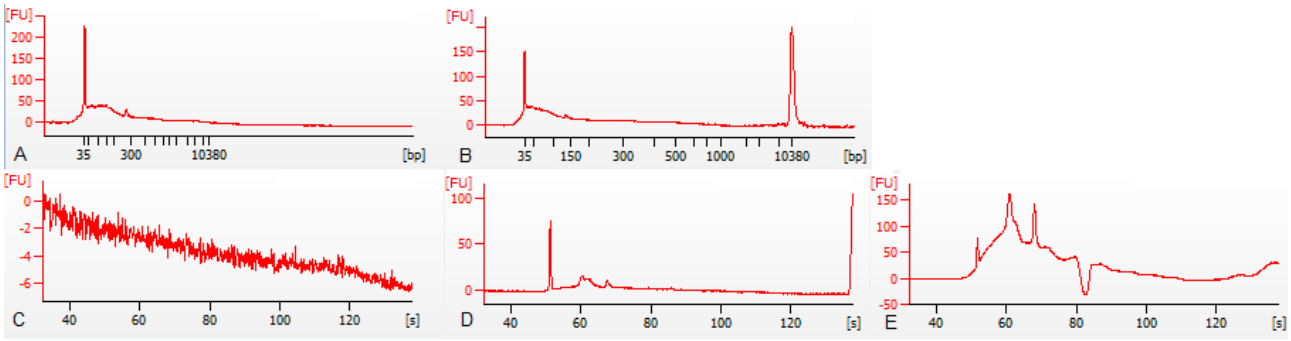

Figure 2. Examples of fragment analysis showing that the fragments are too short or unclear results (using bioanalyser). (A) and (B) show examples of bioanalyser results from sonications which resulted in too short fragments. The sharp peaks represent markers. The round, lower peaks represent DNA fragments, in these cases with lengths mostly shorter than 200 bp. (C), (D) and (E) show exaples of results that were hard to interpret. Too high concentration of DNA in the samples or shaking of the building might have been the reason.

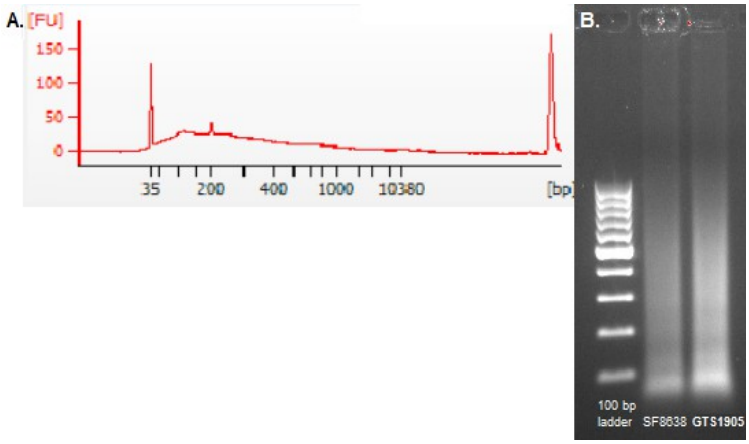

Figure 3. The lengths of fragments from cells sonicated with the most successful settings of parameters. Sonication of (A) MB002 and (B) SF8638 and GTS1905 with Duty cycle 5%, Intensity 4 and 200 Cycles per burst for 12 minutes resulted in a wide range of fragment lengths.

Immunoprecipitation and qPCR

Some primers resulted in melt curves with more than one peak, meaning that they matched more than one location in the DNA and created products of two different lengths (Figure 4). The expected pattern of expression was that all primers should result in a product when combined with input

DNA, which should contain all genes of the cell line. Positive control primers were expected to give a product when combined with DNA immunoprecipitated with MYCN or SOX9 antibodies, while negative control primers were not (Figure 5.A, B). No products should arise from matches with DNA immunoprecipitated with IgG. Almost no positive primers gave any product when combined with DNA that had been immunoprecipitated with one of the MYCN or SOX9 antibodies (Figure 5.C, D).

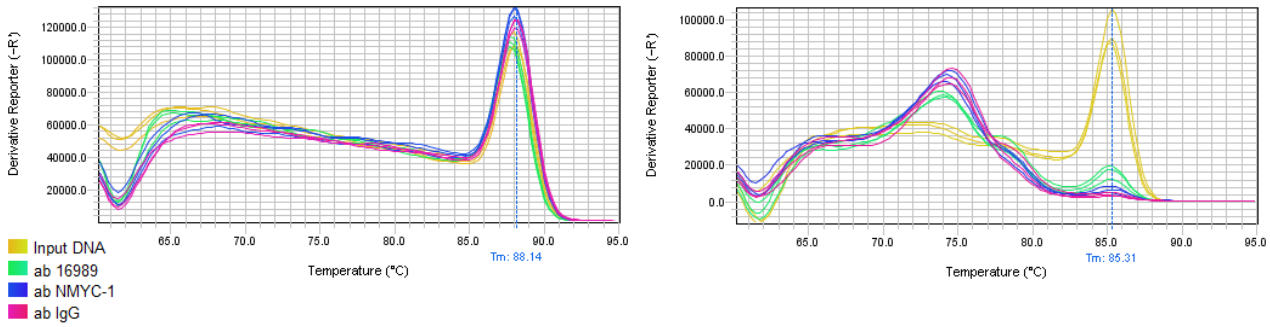

Figure 4. Examples of melt curves for primers giving one or two products in the qPCR. Melt curves show if a primer gives more than one type of product, represented by peaks. (A) The melt curve for the mouse Hspd1 primer has one clear peak and the primer seems to be working as desired. (B) The mouse Npm1 primer results in more than one peak in its melt curve and will not be used for further studies.

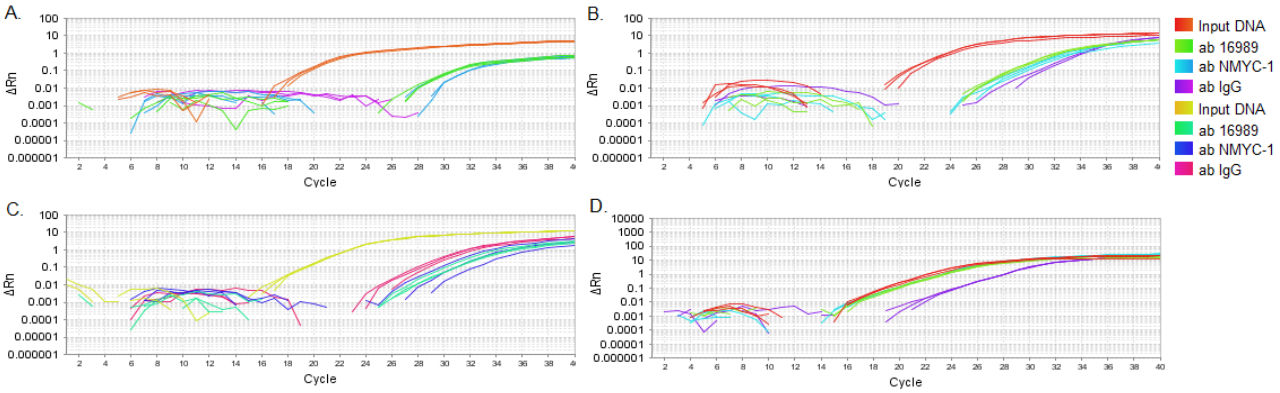

Figure 5. Examples of expected and unexpected results in CHIP control qPCR. Low threshold cycle (C_T) values in these amplification plots represent a high amount of product. Examples of a expected results for (A) a positive control primer (human Cdk4 primer run with MB002) and (B) a negative control primer (human Hipk4 primer run with MB002). Examples of unexpected results for (C) a positive control primer (mouse Achy primer run with MB002) and (D) a negative control primer (mouse Oog1 primer run with GTS1905).

Differences in viability of mSOX9 positive and negative cell lines

Doxycyline concentrations of 10 ng/ml or less did not have a significant effect on the cell viability, while 20 ng/ml left few surviving cells in both cell lines. However, MB002/S9wt did seem to recover from Doxycycline better than MB002 (Figure 6.A). Vincristine concentrations of 10 nM or more greatly reduced the cell viability, especially for MB002/S9wt (Figure 6.B). Cisplatin concentrations between 0.4 and 10 µM killed gradually more cells, with MB002 cells showing a slightly higher relative cell viability after 72 h (Figure 6.C). There is no significant overall difference in relative cell viability between MB002 and MB002/S9wt cells after treatment with the drugs.

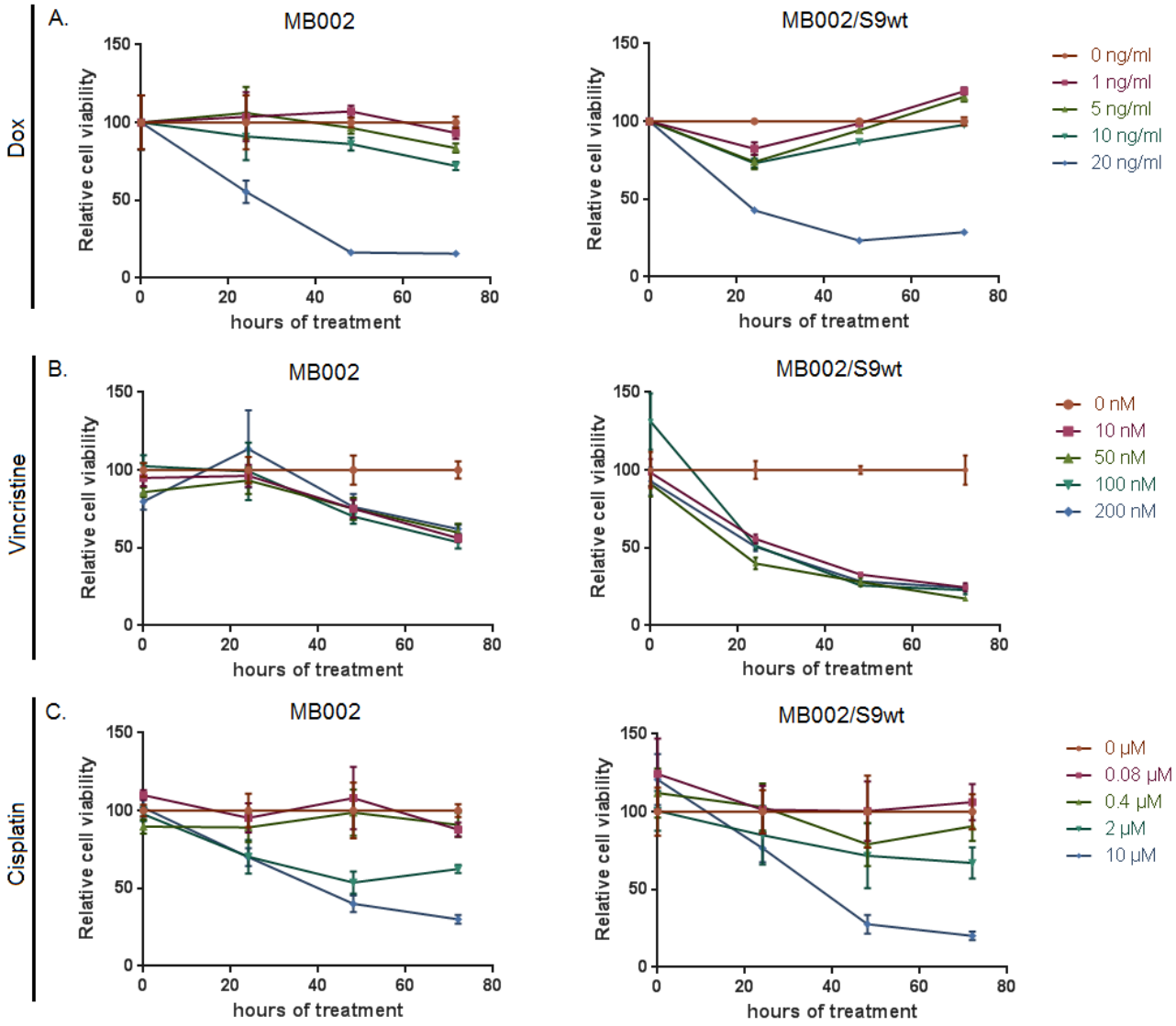

Figure 6. Cell viability of MB002 and MB002/S9wt after drug treatment. MB002 and MB002/S9wt were treated with increasing concentrations of Doxycycline, Vincristine and Cisplatin. Cell viability was measured with the alamarBlue® Cell Viability Assay after 24, 48 and 72 h. No significant overall difference in relative viability could be seen after 72 hours of drug treatment.

Expression changes after drug treatment of mSOX9 positive and negative cell lines

MB002 and MB002/S9wt were treated with drugs and expression of the following five genes was evaluated:

mSOX9 expression

mSOX9 was expressed by the MB002/S9wt cells as expected, but expression levels did not increase significantly when the concentration of Doxycycline was increased (Figure 7). MB002 cells did not express mSOX9 (Figure 8). SF8638/S9wt follows the same pattern, showing an expression of mSOX9 that is not regulated by Doxycycline (Figure 9).

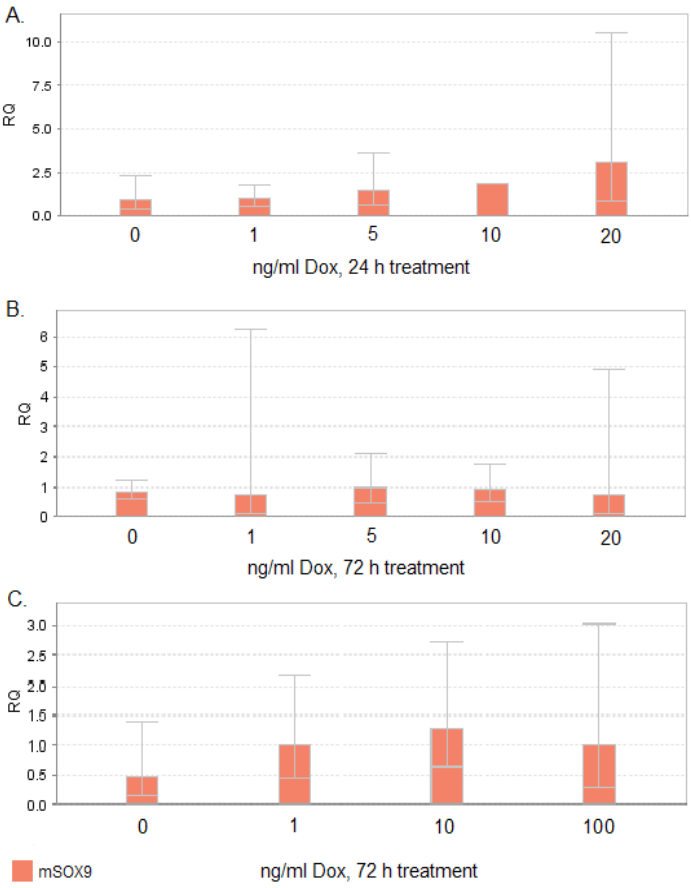

Figure 7. Expression of mSOX9 in MB002/S9wt cells after treatment with Doxycycline. Relative quantity of mSOX9 did not increase significantly when MB002/S9wt cells were treated with Doxycycline for (A) 24 h or (B and C)72 h.

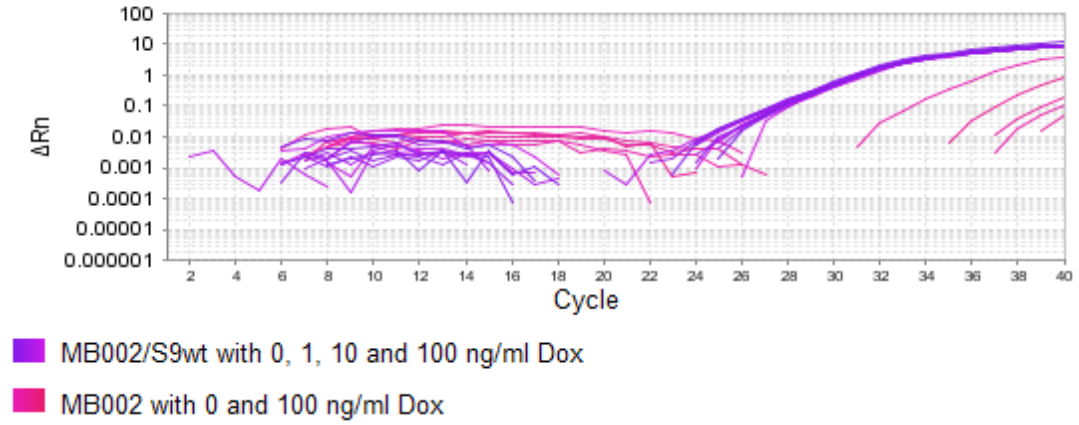

Figure 8. Difference in expression of mSOX9 in MB002 and MB002/S9wt cells after drug treatment. The mSOX9 amplification plot from the qPCR performed with MB002 and MB002/S9wt cells show that mSOX9 is expressed in MB002/S9wt but not in MB002.

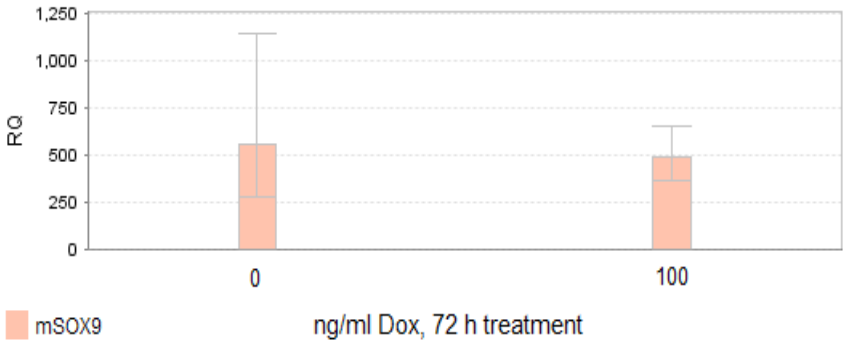

Figure 9. Expression of mSOX9 after drug treatment. Relative quantity of mSOX9 in SF8638/S9wt follows the pattern of being unregulated by Doxycycline.

hSOX9 expression

The average expression of hSOX9, naturally expressed by the cells, was significantly increased in MB002/S9wt cells treated with the highest concentrations of Doxycycline and in both MB002 and MB002/S9wt cells treated with Vincristine. Treatment with Cisplatin did not give this effect (Figure 10).

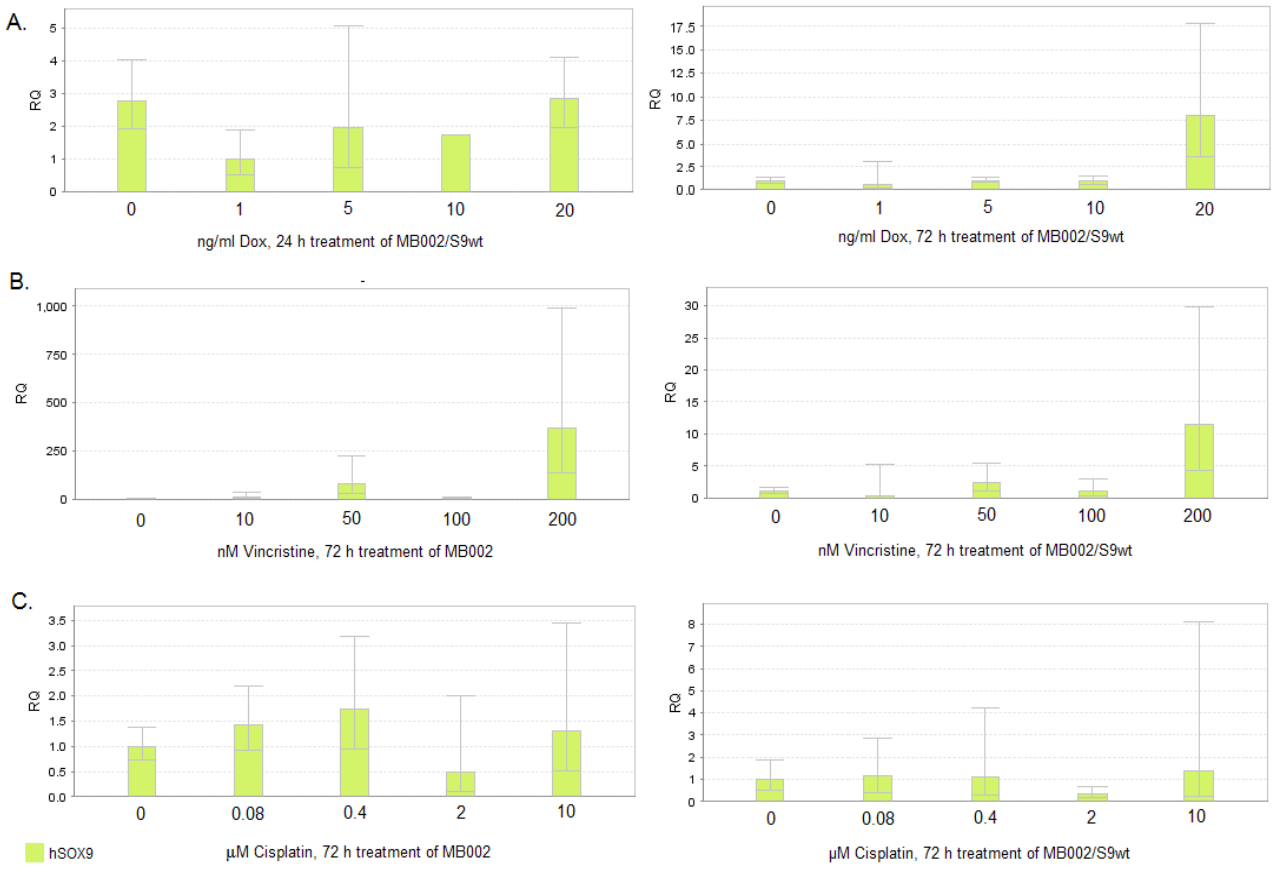

Figure 10. hSOX9 expression after drug treatment. Relative quantity of hSOX9 expression increased with Doxycycline concentration (A) and Vincristine concentration (B), but was not affected by Cisplatin treatment (C).

MYCN expression
There was no clear pattern of change in MYCN expression after drug treatments. Vincristine treated samples showed a rather significant increase, but neither Doxycycline nor Cisplatin gave the same result (Figure 11).

MYC expression
No increase or decrease in expression of MYC was seen in any of the cell lines after drug treatment (Figure 12).

HES1 expression
Expression of HES1 was significantly increased when treated with a high concentration of Doxycycline (Figure 13).

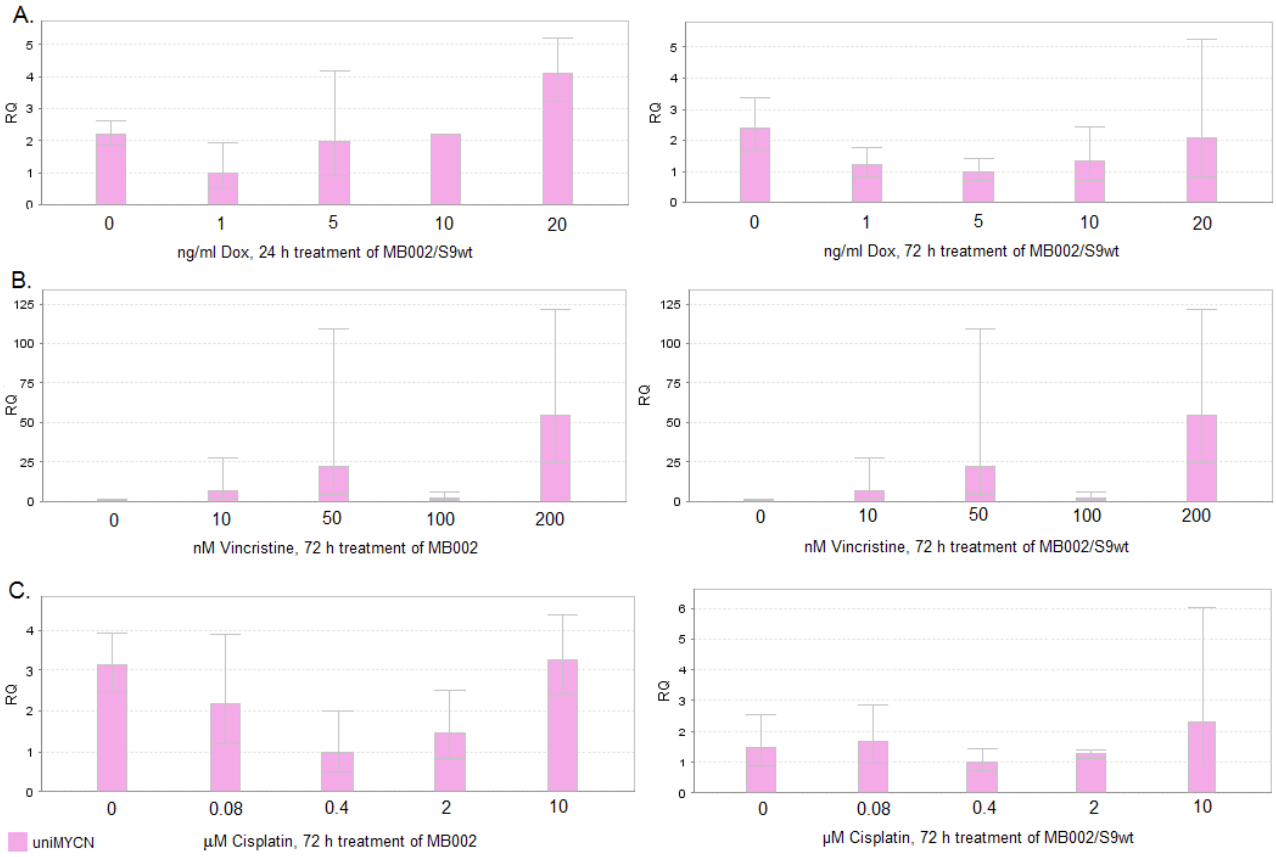

Figure 11. MYCN expression after drug treatment. Relative quantity of MYCN only increases with high concentrations of Vincristine treatment. These were the cells with the lowest viability efter drug treatment, which might explain why the effect is only seen for these samples (A) MB002/S9wt cells were treated with increasing concentrations of Doxycycline for 24 and 72 h. This did not affect MYCN expression. (B) MB002 and MB002/S9wt cells were treated with Vincristine for 72 h. MYCN expression is highly increased with Vincristine concentration. (C) Treatment with increasing concentrations of Cisplatin did not affect MYCN expression.

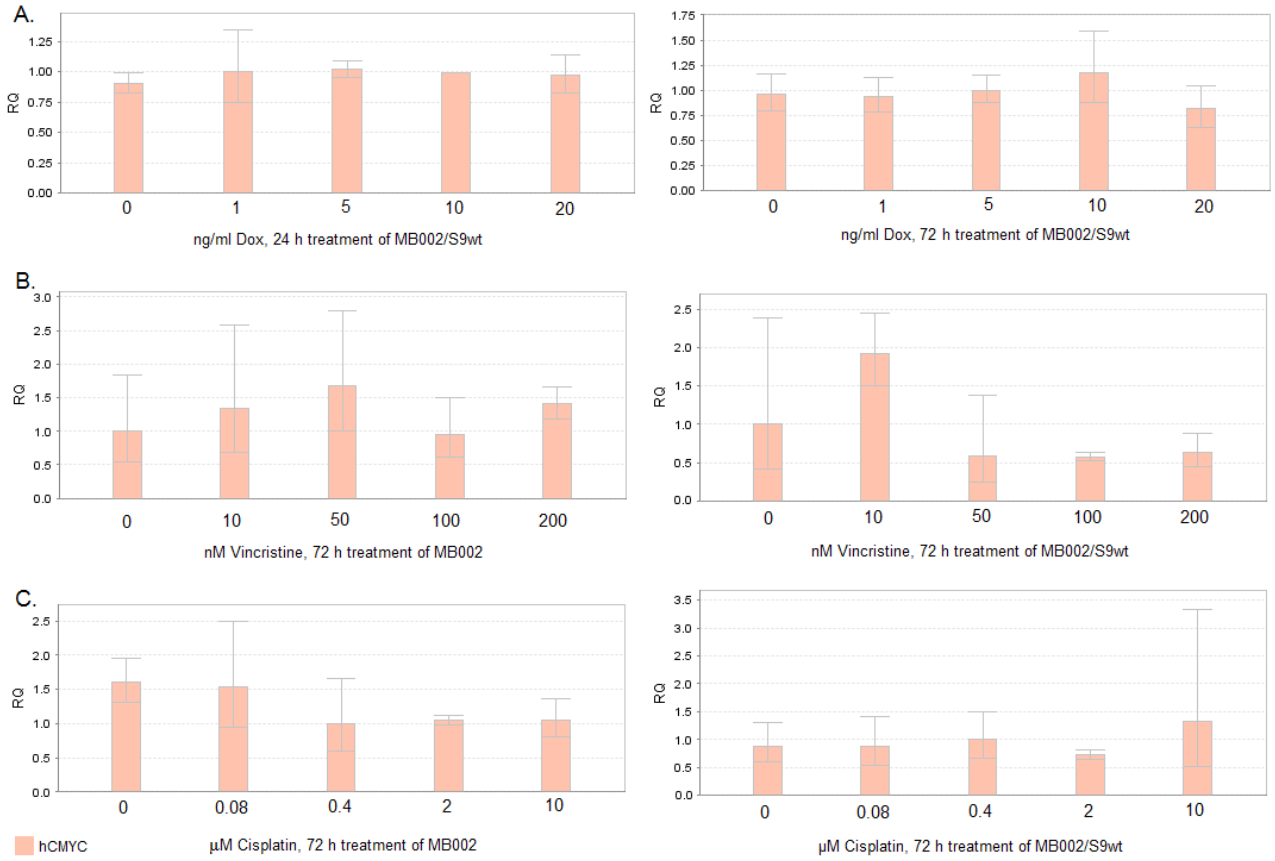

Figure 12. Expression of MYC after drug treatment. No significant variations in quantity of MYC could be seen after treatment with (A) Doxycycline (B) Vincristine or (C) Cisplatin.

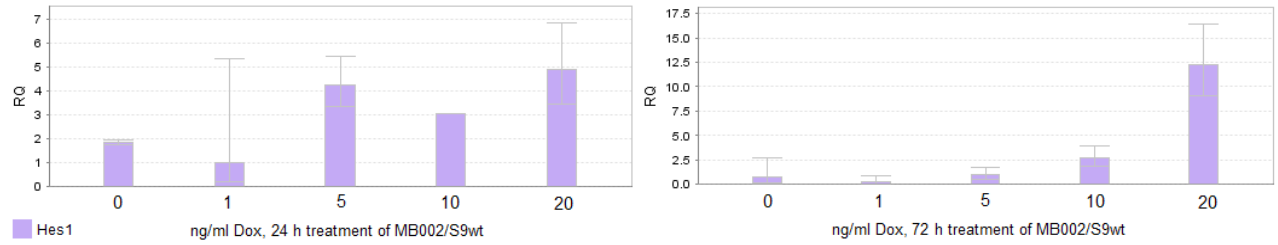

Figure 13. Expression of HES1 after treatment with Doxycycline. There was a significant increase of relative quantity of HES1 after treatment with Doxycycline. The expression is high in cells that survive Doxycycline treatment or is increased as the cells struggle to survive. There could possibly be a connection between this increase and the increase of hSOX9.

DISCUSSION

The faulty measurements of DNA concentration in the sonicated samples might have led to usage of too high concentrations in the bioanalyser. This might be the reason for the sometimes poor quality of the length measurements. After the differences in concentration measurements were discovered, only the NanoDrop was used and for these samples results looked more as expected.

Duty cycle 5%, Intensity 4 and 200 Cycles per burst for 12 minutes were the sonication settings that resulted in fragments closest to the desired lengths. They created a wide variety of lengths with a peak around 200 bp for GTS1905, MB002 and SF8638. Other cell lines might need different settings. The wide range of fragment lengths decreases the specificity of the locations that will be found for binding sites.

There are several possible reasons for the unexpected patterns of the qPCR results. The primer target sequences might be located to far away from the TF binding sites, preventing them from passing through the ChIP process. There is also a small risk that a fragment matching a negative primer also contains a binding site. It is however unlikely that this is the case for all the fragments. A more likely reason is the concentration of the antibodies. Few fragments would pass the ChIP and be able to be detected in the qPCR if the amounts of antibodies were too low, even if they contained both a TF binding site and a primer target sequence. For a more correct qPCR result, the concentrations of all antibodies should be higher and as similar too each other as possible. Provided that the previous steps of the ChIP process are working, there should be a set of concentrations that give the expected qPCR results, without more than a few exceptions.

Primers matching more than one location in the DNA were detected by looking at the melt curves, where each peak represents a primer product. Primers with melt curves with more then one peak will be excluded from further studies.

Expression of mSOX9 was expected to increase in MB002/S9wt and SF8638/S9wt cells when treated with Doxycycline, according to the design of the lentiviruses with which those cells were transduced. No significant increase could be seen. Although not regulated by Doxycycline, mSOX9 is expressed in these cells and not in MB002. An unregulated product created in the qPCR by the mSOX9 primer could be the effect of the primer binding to a similar sequence in the genome, but since there was no product for the MB002 cells, this is unlikely. The conclusion is that the lentivirus expressing mSOX9 has been successfully transduced, while transduction of the repressing lentivirus has not.

hSOX9, naturally expressed by the human cell line, increased significantly in both MB002 and MB002/S9wt cells after treatment with high concentrations of Doxycycline or Vincristine, but not with Cisplatin. This would indicate either that the cells of each cell line with high hSOX9 expression from the start have a higher resistance to drugs, or that hSOX9 expression increases in the cells during drug treatment. This supports the association between SOX9 and recurrence. The viability results do not. The viability assay should be repeated to confirm the results. If the results are consistent, the increase of hSOX9 might not be correlated with drug resistance or might not be high enough to have an effect. It is also possible that the transduced mSOX9 and the hSOX9 do not function the same way.

The expression results from Vincristine treated cells indicate some increase of MYCN expression during the drug treatment. This increase does not occur in Doxycycline and Cisplatin treated cells and overall MYCN levels are low. The lack of change in MYCN expression might be due to the fact

that the MB002 cell line is driven by MYC and not MYCN. There was no significant change in MYC expression either. Both MYCN and MYC are involved in proliferation, which Vincristine and Cisplatin targets. This might be the reason why cells with high expression of those genes do not have an advantage and are not involved in recurrence.

The increased levels of HES1 during Doxycycline treatment indicates that cells with a higher HES1 expression are more likely to survive Doxycycline treatment or that the gene increasingly expressed as the cell is adapting to survive. This supports the idea of HES1 being involved in recurrence. If this is a direct effect of HES1 expression increasing survival, or an indirect effect of increased overall Notch signaling, remains to be evaluated.

Identification of MYCN and SOX9 target genes will increase the understanding of the functions and relationships of the transcription factors. New potential drug treatment targets might be revealed, specific for each cell line. Target genes found to be associated with drug resistance and recurrence should be prioritized.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor Fredrik Swartling for giving me the opportunity to do my Master thesis project at IGP and for guiding me along the way. I am sincerely thankful to my supervisor Vasil Savov for great guidance and support during every day of this project and to my supervisor Holger Weishaupt. To all of my supervisors, thank you for your support, encouragement and scientific expertise. I also want to thank all members of the Fredrik Swartling research group, as well as many members from the Neuro-oncology group, for helping me with daily laboratory work and endless searching for material and especially for making me feel welcome during my time at IGP.

REFERENCES

1. Swartling FJ, Savov V, Persson AI, Chen J, Hackett CS, Northcott PA, m.fl. Distinct Neural Stem Cell Populations Give Rise to Disparate Brain Tumors in Response to N-MYC. Cancer Cell. 25 May 2012;21(5):601–13.

2. Packer RJ, Vezina G. Management of and prognosis with medulloblastoma: Therapy at a crossroads. Arch Neurol. 10 November 2008;65(11):1419–24.

3. Medulloblastoma - Childhood - Statistics [Internet]. Cancer.Net. [cited 27 May 2015]. Retrieved from: http://www.cancer.net/cancer-types/medulloblastoma-childhood/statistics

4. MacDonald TJ, Rood BR, Santi MR, Vezina G, Bingaman K, Cogen PH, m.fl. Advances in the Diagnosis, Molecular Genetics, and Treatment of Pediatric Embryonal CNS Tumors. The Oncologist. 04 January 2003;8(2):174–86.

5. Gupta T, Mohanty S, Sadhana Kannan, Jalali R. Prospective longitudinal assessment of sensorineural hearing loss with hyperfractionated radiation therapy alone in patients with average-risk medulloblastoma. Neuro-Oncol Pract. 09 January 2014;1(3):86–93.

6. Grisold W, Cavaletti G, Windebank AJ. Peripheral neuropathies from chemotherapeutics and targeted agents: diagnosis, treatment, and prevention. Neuro-Oncol. 09 January 2012;14(suppl 4):iv45–54.

7. Taylor RE, Bailey CC, Robinson K, Weston CL, Ellison D, Ironside J, m.fl. Results of a Randomized Study of Preradiation Chemotherapy Versus Radiotherapy Alone for Nonmetastatic Medulloblastoma: The International Society of Paediatric Oncology/United Kingdom Children's Cancer Study Group PNET-3 Study. J Clin Oncol. 15 April 2003;21(8):1581–91.

8. Bull KS, Spoudeas HA, Yadegarfar G, Kennedy CR. Reduction of Health Status 7 Years After Addition of Chemotherapy to Craniospinal Irradiation for Medulloblastoma: A Follow-Up Study in PNET 3 Trial Survivors on Behalf of the CCLG (formerly UKCCSG). J Clin Oncol. 20 September 2007;25(27):4239–45.

9. Kool M, Korshunov A, Remke M, Jones DTW, Schlanstein M, Northcott PA, m.fl. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. Acta Neuropathol (Berl). April 2012;123(4):473–84.

10. Roussel MF, Robinson GW. Role of MYC in Medulloblastoma. Cold Spring Harb Perspect Med. 11 January 2013;3(11):a014308.

11. Ramaswamy V, Remke M, Bouffet E, Faria CC, Perreault S, Cho Y-J, m.fl. Recurrence patterns across medulloblastoma subgroups: an integrated clinical and molecular analysis. Lancet Oncol. November 2013;14(12):1200–7.

12. Pizer B, Donachie PHJ, Robinson K, Taylor RE, Michalski A, Punt J, m.fl. Treatment of recurrent central nervous system primitive neuroectodermal tumours in children and adolescents: Results of a Children's Cancer and Leukaemia Group study. Eur J Cancer. June 2011;47(9):1389–97.

13. Hui AB-Y, Lo K-W, Yin X-L, Poon W-S, Ng H-K. Detection of Multiple Gene Amplifications

in Glioblastoma Multiforme Using Array-Based Comparative Genomic Hybridization. Lab Invest. 05 January 2001;81(5):717–23.

14. Perry A, Miller CR, Gujrati M, Scheithauer BW, Zambrano SC, Jost SC, m.fl. Malignant Gliomas with Primitive Neuroectodermal Tumor-like Components: A Clinicopathologic and Genetic Study of 53 Cases. Brain Pathol. 01 January 2009;19(1):81–90.

15. Oude Luttikhuis MEM, Iyer VK, Dyer S, Ramani P, McConville CM. Detection of MYCN Amplification in Neuroblastoma using Competitive PCR Quantitation. Lab Invest. 02 January 2000;80(2):271–3.

16. Swartling FJ, Grimmer MR, Hackett CS, Northcott PA, Fan Q-W, Goldenberg DD, m.fl. Pleiotropic role for MYCN in medulloblastoma. Genes Dev. 15 May 2010;24(10):1059–72.

17. Korshunov A, Remke M, Kool M, Hielscher T, Northcott PA, Williamson D, m.fl. Biological and clinical heterogeneity of MYCN-amplified medulloblastoma. Acta Neuropathol (Berl). 09 December 2011;123(4):515–27.

18. Swartling FJ, Savov V, Čančer M, Bolin S, Fotaki G, Dubuc A, m.fl. Metastasis and Tumor Recurrence from Rare Sox9-Positive Cells in Mycn-Driven Medulloblastoma. Neuro-Oncol. 07 January 2014;16(suppl 3):iii28–iii28.

19. Knoepfler PS, Cheng PF, Eisenman RN. N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev. 15 October 2002;16(20):2699–712.

20. Hoffman B, Liebermann DA. Apoptotic signaling by c-MYC. Oncogene. 2008;27(50):6462–72.

21. Wong GW, Knowles GC, Mak TW, Ferrando AA, Zúñiga-Pflücker JC. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. Blood. 16 August 2012;120(7):1439–48.

22. CaMay P, Jäckel C, Krebs S, De Toni EN, Blum H, Jauch K-W, m.fl. Hypoxia-independent gene expression mediated by SOX9 promotes aggressive pancreatic tumor biology. Mol Cancer Res MCR. March 2014;12(3):421–32.

23. Matheu A, Collado M, Wise C, Manterola L, Cekaite L, Tye AJ, m.fl. Oncogenicity of the developmental transcription factor Sox9. Cancer Res. 01 March 2012;72(5):1301–15.

24. Zhu M-Y, Gasperowicz M, Chow RL. The expression of NOTCH2, HES1 and SOX9 during mouse retinal development. Gene Expr Patterns GEP. April 2013;13(3-4):78–83.

25. Oh C, Lu Y, Liang S, Mori-Akiyama Y, Chen D, de Crombrugghe B, m.fl. SOX9 regulates multiple genes in chondrocytes, including genes encoding ECM proteins, ECM modification enzymes, receptors, and transporters. PloS One. 2014;9(9):e107577.

26. Akiyama H, Chaboissier M-C, Behringer RR, Rowitch DH, Schedl A, Epstein JA, m.fl. Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. Proc Natl Acad Sci U S A. 27 April 2004;101(17):6502–7.

27. Topol L, Chen W, Song H, Day TF, Yang Y. Sox9 Inhibits Wnt Signaling by Promoting β-Catenin Phosphorylation in the Nucleus. J Biol Chem. 30 January 2009;284(5):3323–33.

28. Iso T, Kedes L, Hamamori Y. HES and HERP families: Multiple effectors of the notch signaling pathway. J Cell Physiol. 01 March 2003;194(3):237–55.

29. Fischer A, Gessler M. Delta–Notch—and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res. July 2007;35(14):4583–96.

30. Aujla PK, Bora A, Monahan P, Sweedler JV, Raetzman LT. The Notch effector gene Hes1 regulates migration of hypothalamic neurons, neuropeptide content and axon targeting to the pituitary. Dev Biol. 01 May 2011;353(1):61–71.

31. Shih I-M, Wang T-L. Notch Signaling, γ-Secretase Inhibitors, and Cancer Therapy. Cancer Res. 03 January 2007;67(5):1879–82.

32. Liu J, Ye F, Chen H, Lü W, Zhou C, Xie X. Expression of differentiation associated protein HES1 and Hes5 in cervical squamous carcinoma and its precursors. Int J Gynecol Cancer Off J Int Gynecol Cancer Soc. December 2007;17(6):1293–9.

33. alamarBlue® Cell Viability Assay Protocol [Internet]. [citerad 29 May 2015]. Retrieved from: https://www.lifetechnologies.com/se/en/home/references/protocols/cell-and-tissue-analysis/cell-profilteration-assay-protocols/cell-viability-with-alamarblue.html

34. Bandopadhayay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y, m.fl. BET-bromodomain inhibition of MYC-amplified medulloblastoma. Clin Cancer Res Off J Am Assoc Cancer Res. 15 February 2014;20(4):912–25.

35. Murphy DM, Buckley PG, Bryan K, Watters KM, Koster J, van Sluis P, m.fl. Dissection of the oncogenic MYCN transcriptional network reveals a large set of clinically relevant cell cycle genes as drivers of neuroblastoma tumorigenesis. Mol Carcinog. 01 June 2011;50(6):403–11.

36. Murphy DM, Buckley PG, Bryan K, Das S, Alcock L, Foley NH, m.fl. Global MYCN Transcription Factor Binding Analysis in Neuroblastoma Reveals Association with Distinct E-Box Motifs and Regions of DNA Hypermethylation. PLoS ONE [Internet]. 04 December 2009 [cited 29 May 2015];4(12). Retrieved from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2781550/

37. Westermann F, Muth D, Benner A, Bauer T, Henrich K-O, Oberthuer A, m.fl. Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas. Genome Biol. 2008;9(10):R150.

38. Kadaja M, Keyes BE, Lin M, Pasolli HA, Genander M, Polak L, m.fl. SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. Genes Dev. 15 February 2014;28(4):328–41.

39. Li Y, Zheng M, Lau Y-FC. The Sex-Determining Factors SRY and SOX9 Regulate Similar Target Genes and Promote Testis Cord Formation during Testicular Differentiation. Cell Rep. 07 August 2014;8(3):723–33.

40. Jordan MA. Mechanism of Action of Antitumor Drugs that Interact with Microtubules and Tubulin. Curr Med Chem -Anti-Cancer Agents. 01 January 2002;2(1):1–17.

41. Tanida S, Mizoshita T, Ozeki K, Tsukamoto H, Kamiya T, Kataoka H, et al. Mechanisms of Cisplatin-Induced Apoptosis and of Cisplatin Sensitivity: Potential of BIN1 to Act as a Potent Predictor of Cisplatin Sensitivity in Gastric Cancer Treatment. Int J Surg Oncol. 12 June 2012;2012:e862879.