

Investigation of BRD9 inhibition as alternative treatment option for testicular germ cell tumors

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
-R	cisplatin-resistant
°C	degree celsius
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
Ac	acetyl group
AFP	α-Fetoprotein
AKT	serine/threonine-protein kinase
ALDH	aldehyde dehydrogenase
AML	acute myeloid leukemia
APS	ammonium persulfate
ASH1L	histone-lysine N-methyltransferase ASH1L
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATP7A	copper-transporting ATPase 1
ATP7B	copper-transporting ATPase 2
AUC	area under the curve
BAF	BRG1/BRM-associated factor
Bax	apoptosis regulator BAX
BCA	bicinchoninic acid assay
Bcl-xL	B-cell lymphoma-extra large
BCP	bromodomain-containing protein
BEP	bleomycin, etoposide and platinum chemotherapy
BER	base excision repair
BET	bromodomain and extra terminal
BMP	bone morphogenetic protein
BRAD1	BRCA1-associated RING domain 1
BRCA1	breast cancer type 1 susceptibility protein
BRCA2	breast cancer type 2 susceptibility protein

Abbreviation	Meaning
BRD	bromodomain
BRD2	bromodomain-containing protein 2
BRD3	bromodomain-containing protein 3
BRD4	bromodomain-containing protein 4
BRD7	bromodomain-containing protein 7
BRD9	bromodomain-containing protein 9
BRDT	bromodomain testis-specific protein
BRIP1	BRCA1 interacting helicase 1
BSA	bovine serum albumin
Casp6	caspase-6
Cc	choriocarcinoma
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CDX2	homeobox protein CDX-2
CH ₃	methyl group
CHEK2	checkpoint kinase 2 protein
CLDN1	claudin-1
CLEC1	C-type lectin domain family 1
cm	centimeter
cm ²	square centimeter
CNS	central nervous system
CO ₂	carbon dioxide
COAD	colon adenocarcinoma
CRISPR	clustered regularly interspaced short palindromic repeats
CTR1	copper transporter 1
DAPI	6-diamidino-2-phenylindole dihydrochloride
DAZL	deleted in azoospermia-like
DLX6	homeobox protein DLX-6
DMEM	dulbecco's modified eagle's medium
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

Abbreviation	Meaning
DND1	dead end protein homolog 1
DNMT	DNA methyltransferase
DNMT33B	DNA (cytosine-5)-methyltransferase 33 B
DNMT3L	DNA (cytosine-5)-methyltransferase 3 L
dNTP	deoxynucleotide triphosphates
DPPA3	developmental pluripotency-associated protein 3
DUSP6	dual specificity phosphatase 6
EC	embryonal carcinoma
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ERCC1	excision repair cross-complementing 1
et al.	et alii, aliae, alia; latin: and others
EtOH	ethanol
ExE	extraembryonic endoderm
EXO1	exonuclease 1
FACS	fluorescence activated cell sorting
FAS	fatty acid synthase
FASL	fatty acid synthase ligand
FBS	fetal bovine serum
FDA	food and drug administration
FDR	false discovery rate
FES	tyrosine-protein kinase Fes/Fps
FGF3	fibroblast growth factor 3
FGF4	fibroblast growth factor 4
FLIP	filamin-A-interacting protein
fwd	forward
FZD5	frizzled-5
FZD6	frizzled-6
FZD10	frizzled-10
g	gram
GADD45	growth arrest and DNA-damage-inducible protein 45
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

Abbreviation	Meaning
GATA4	transcription factor GATA-4
GATA5	transcription factor GATA-5
GBC	gallbladder cancer
GCNIS	germ cell neoplasia in situ
GCT	germ cell tumor
GEO	gene expression omnibus
GFP	green fluorescent protein
GO	gene ontology
GSH	glutathione
h	hour
H ₂ O	water
HAND1	heart- and neural crest derivatives-expressed protein 1
HAT	histone acetyltransferase
HCl	hydrochloric acid
HDAC	histone deacetylase
hESC	human embryonic stem cell
HESX1	homeobox expressed in ES cells 1
HEYL	hairy/enhancer-of-split related with YRPW motif-like protein
HHR	homologous recombination machinery
HMGB1	high-mobility group box protein 1
hPGCLC	human primordial germ cell like cell
HR	homologous recombination
HRAS	GTPase HRas
HRP	horseradish per
HRR	homologous recombination repair
HSF4	heat shock factor protein 4oxidase
IF	immunofluorescence
IGCCCG	international germ cell cancer collaborative group
IHC	immunohistochemistry
ISWI	imitation SWItch
K _{ac}	ε-N-acetylation of lysine residues
kDa	kilo dalton

Abbreviation	Meaning
KIT	mast/stem cell growth factor receptor Kit
KLF2	krueppel-like factor 2
KLF4	krueppel-like factor 4
KLF5	krueppel-like factor 5
KLF15	krueppel-like factor 15
LACC	locally advanced cervical cancer
LDH	lactate dehydrogenase
LEF1	lymphoid enhancer-binding factor 1
M	molar
MDM2	E3 ubiquitin-protein ligase Mdm2
MeOH	methanol
mg	milligram
MgCl ₂	magnesium chloride
min	minute
ml	milliliter
MLH1	DNA mismatch repair protein Mlh1
mm	millimeter
mM	millimolar
MMR	mispaired base repair
mRNA	messenger ribonucleic acid
MRP	multidrug resistance proteins
MRT	malignant rhabdoid tumor
MSH2	DNA mismatch repair protein Msh2
MT	metallothionein
mTOR	mammalian target of rapamycin
NAE1	NEDD8-activating enzyme E1 regulatory subunit
NANOG	homeobox protein NANOG
NCAM	neural cell adhesion molecule 1
NBS1	nibrin
ncBAF	non-canonical BAF
NEAA	non-essential amino acid
NER	nucleotide excision pathway

Abbreviation	Meaning
NH ₃	amine
NHL	non-hodgkin lymphoma
nm	nanometer
nM	nanomolar
N-MYC	N-MYC proto-oncogene protein
NODAL	nodal homolog
NOTCH2	neurogenic locus notch homolog protein 2
ns	not significant
OCT	organic cation transporter
PAGE	polyacrylamid gel electrophoresis
PALB2	partner and localizer of BRCA2
PARP	poly (ADP-ribose) polymerase
PBAF	polybromo-associated BAF
PBRM1	protein polybromo-1
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCR	polymerase chain reaction
PD-1	programmed cell death 1
PDGF	platelet-derived growth factor
PDGFb	platelet-derived growth factor subunit B
PDGFRb	platelet-derived growth factor receptor b
PD-L1	programmed cell death ligand 1
PE	platinum and etoposide chemotherapy
PFA	paraformaldehyde
PGC	primordial germ cell
PI3K	phosphoinositid-3-kinase
PMS	N-methyl dibenzo pyrazine methyl sulfate
PMS2	mismatch repair endonuclease PMS2
POU5F1	POU domain, class 5, transcription factor 1
PRDM1	PR domain zinc finger protein 1
PRDM14	PR domain zinc finger protein 14
PROTAC	proteolysis targeting chimera

Abbreviation	Meaning
p-RPLND	primary retroperitoneal lymph node dissection
PTM	posttranslational modification
PUMA	P53 upregulated modulator of apoptosis
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RAD51	DNA repair protein RAD51
RAD54	DNA repair protein RAD54
rev	reverse
RIN	RNA integrity
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPMI	roswell park memorial institute medium
SALL4	SAL-like protein 4
SAMSN1	SAM domain-containing protein SAMSN-1
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second
SEMA3C	semaphorin-3C
SFRP1	secreted frizzled-related protein 1
SFRP4	secreted frizzled-related protein 4
shRNA	small hairpin RNA
siRNA	small interfering RNA
SMARCA2	probable global transcription activator SNF2L2
SMARCA4	transcription activator BRG1
SOX2	transcription factor SOX-2
SOX15	transcription factor SOX-15
SOX17	transcription factor SOX-17
SOX21	transcription factor SOX-21
SPG	spermatogonia
SqCLC	squamous cell lung cancer

Abbreviation	Meaning
SSC	spermatogonial stem cell
SWI/SNF	SWItch/sucrose non-fermentable
TAE	tris-acetate-EDTA-buffer
TEMED	tetramethyl ethylenediamine
TFAP2C	transcription factor AP-2 gamma
TGCT	testicular germ cell tumor
TKI	tyrosine kinase
TMA	tissue microarray
U	unit
UTF1	undifferentiated embryonic cell transcription factor 1
UV	ultraviolet
V	volt
v/v	volume per volume
VEGFR	vascular endothelial growth factor receptor
VIP	etoposide, ifosfamide and platinum chemotherapy
w/v	weight per volume
WB	western blot
WHO	world health organisation
WNT2B	protein Wnt-2b
xg	relative centrifugal force
XPA	DNA repair protein complementing XP-A cells
XPF	xeroderma pigmentosum complementation group F
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilid
YST	yolk sac tumor
ZMYND8	MYND-type zinc finger-containing chromatin reader ZMYND8
ZSCAN10	zinc finger and SCAN domain-containing protein 10
β-hCG	beta subunit of human chorionic gonadotropin

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SUMMARY

Type II germ cell tumors are the most prevalent tumor in young men between the ages of 15 and 35 years. They arise from a block in primordial germ cell differentiation leading to a precursor lesion called germ cell neoplasia in situ (GCNIS). They are subgrouped into seminomas and non-seminomas. Non-seminomas comprise embryonal carcinomas which are able to differentiate into cells of all three germ layers as well as extraembryonic tissue classified as teratoma, yolk sac tumor and choriocarcinoma.

The standard treatment of testicular germ cell tumors (TGCT) is orchiectomy followed by cisplatin-based chemotherapy leading to high 5-year survival rates of 95 % but unfortunately 15-20 % of patients are still resistant to the treatment. Therefore, we found it very important to investigate alternative treatment options like epigenetic drugs. BET protein inhibitors interfering with the epigenetic landscape have already been shown to be effective in different cancer types like prostate cancer, glioblastoma and breast cancer. The bromodomain-containing protein-9 (BRD9) is part of a chromatin remodeling complex and an epigenetic reader which binds to acetylated lysine residues to activate gene expression by recruitment of other transcription factors. BRD9 shows significantly increased protein levels in cervical cancer and in malignant rhabdoid tumor (MRT) cells. For example, in acute myeloid leukemia (AML) cells the inhibition of BRD9 led to reduction of cell growth. Therefore, we found it very interesting to investigate the effect of the BRD9 inhibitor I-BRD9 in TGCTs.

First, we analyzed the expression of the target BRD9 in TGCT tissues and cell lines. Meta-analysis of microarray data in tissues as well as cell lines showed expression of BRD9. On protein level Western Blot also revealed comparable protein levels in TGCT cell lines while a tissue microarray (TMA) showed heterogenous expression of BRD9 in TGCT tissues. On the other hand, the lowest expression was found in the control cell line MPAF and in normal testis tissue indicating a promising starting point for testing the BRD9 inhibitor in TGCTs. XTT viability assays after I-BRD9 application led to reduced cell growth in all TGCT cells while the control cells were only slightly affected. FACS analysis revealed induction of apoptosis as well as G1-phase cell cycle arrest already after 24 hours of treatment with I-BRD9 in the TGCT cell lines while the control cells remained unaffected. RNAseq analysis displayed downregulation of a prominent network of pluripotency markers including NANOG, NODAL and KLF4 while

genes involved in epithelium development were upregulated. These data suggest loss of the pluripotency state and differentiation towards an epithelial cell fate (Figure 1).

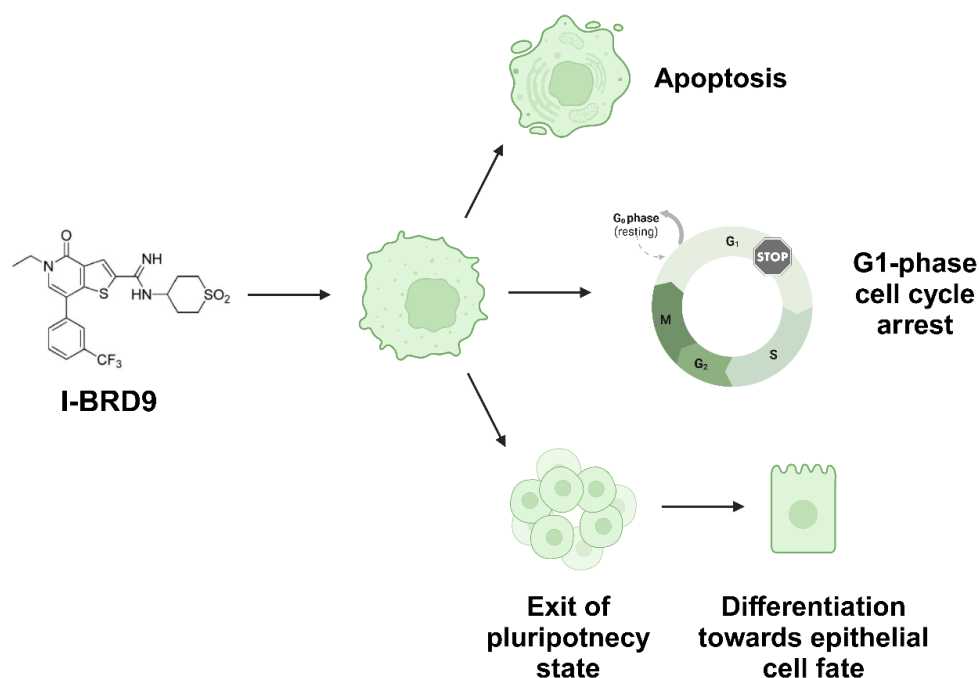


Figure 1: Overview of effects of I-BRD9 in TGCTs. BRD9 inhibition led to induction of apoptosis as well as G1-phase cell cycle arrest. Transcriptome analysis revealed downregulation of pluripotency markers and induction of epithelium development. Created in <https://BioRender.com>.

Taken together, the BRD9 inhibitor I-BRD9 led to severe effects in TGCT cell lines like reduction of viability, induction of apoptosis and G1-phase cell cycle arrest while the control cells were only slightly affected. BRD9 inhibition induces loss of the pluripotency state and differentiation towards an epithelial cell fate. Most importantly, the data suggest I-BRD9 as a potential treatment alternative for TGCTs.

1 INTRODUCTION

1.1 Germ cell development

The start of every mammalian embryo is the fertilization of the oocyte by the sperm resulting in a totipotent zygote. The zygote arises all cell lineages including the germ line (Figure 2) ². Origin of the germ cell lineage are primordial germ cells (PGC) which give rise to spermatogonia or oocytes ^{3,4}.

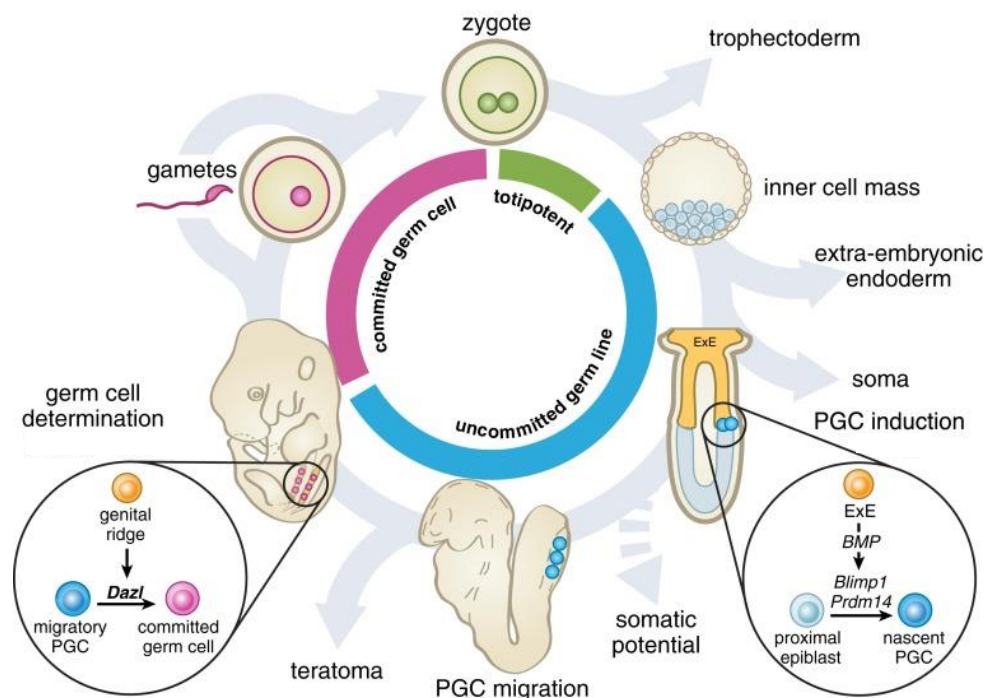


Figure 2: Human germ line development. The fertilized oocyte develops into the zygote which further develops into the blastocyst. The pre-implantation epiblast cells arise all lineages including the germ line. PGCs are found near the yolk sac before they migrate to the genital ridge. Meiosis and gametogenesis give rise to gametes (oocytes and sperm) which are able to restart the circle by fertilization. PGC – primordial germ cell, ExE – Extraembryonic endoderm. Modified from ⁵.

Specification occurs during early embryonic development and is orchestrated by a specific network of genes which is induced by signals from the extra-embryonic tissues ^{2,6}. BMP and WNT signaling result in induction of expression of BLIMP1 which specifies PGCs ^{6,7}. Afterwards, PRDM14 as well as TFAP2C are upregulated ⁸. They represent a regulatory transcription factor network of PGCs ^{2,6}. During migration of the PGCs

along the developing hindgut to the genital ridges global DNA methylation as well as deletion of imprinting occurs ⁷. At the genital ridge PGCs undergo licensing in preparation for gametogenesis which is induced by DAZL ⁹. Furthermore, PGCs differentiate into gonocytes and enter cell cycle arrest in G0-phase and therefore are prevented from mitosis until after birth. Afterwards, gonocytes differentiate into spermatogonia (SPG) which stay dormant for 5-7 years. Mitosis starts and the number of SPGs is increased to enable differentiation into spermatozoa in the process of spermiogenesis ¹⁰.

Upon PGC specification, pluripotency markers including OCT4, LIN28, SOX2, KLF2, KLF5, N-MYC and NANOG are expressed in PGCs. In contrast to induced pluripotency PGC fate is already initiated in the epiblast cells which comprise key factors of pluripotency ⁴. PGCs maintain the expression of SOX2, KLF2, KLF5, N-MYC and NANOG during specification while migratory PGCs express POU5F1, PRDM1, SALL4 and NANOG ^{4,11}. After arrival in the gonadal niche PGCs downregulate pluripotency markers ¹². Of note, the expression of many pluripotency factors in PGCs as well as absence of correct germ cell differentiation signals might lead to the development of germ cell tumors ¹³.

1.2 Germ cell tumors

According to the World Health Organization (WHO) the second leading cause of death worldwide is cancer accounting nearly 10 million deaths in 2018. The prevalent cancers in males are lung, prostate, colorectal, stomach and liver cancer while breast, colorectal, lung, cervical and thyroid cancer are most predominant in females ¹⁴. Testis cancer only accounted 72040 (1.7 %) of new cases in 2022 but germ cell tumors (GCT) are the most prominent cancer in younger males between the ages of 15 and 35 (Figure 3) ^{15,16}.

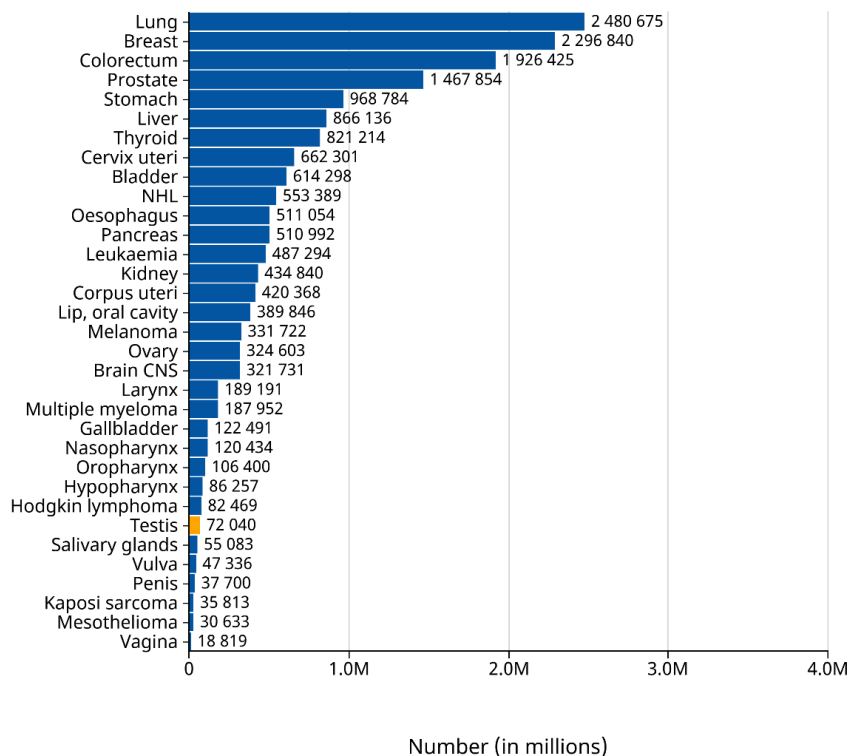


Figure 3: New cases in testis cancer in 2022 worldwide. Absolute numbers of incidences in both sexes in 2022. Testis cancer accounted for 72040 new cases. NHL – Non-Hodgkin lymphoma; CNS – central nervous system. Modified from ¹⁵.

GCTs are a group of rare neoplasms which occur in the gonads (ovary and testis), in extragonadal sites along the body midline and in the midline of the brain ^{12,16}. Incidences are rising steadily highlighting the clinical importance. A high prevalence is visible in the Caucasian populations and in total there is a five times higher incidence rate in industrialized countries compared to less developed regions ^{16,17}. The familial risk factor is higher than in most other cancers and the predominant abnormality is a short arm of the chromosome 12 ^{16,18}. Other risk factors are for example cryptorchidism, infertility, contralateral testicular cancer or Klinefelter's syndrome ¹⁹. Based on the cell of origin, sex and age of the patient and developmental potential 7 types (Type 0-VI) of GCT were classified ¹².

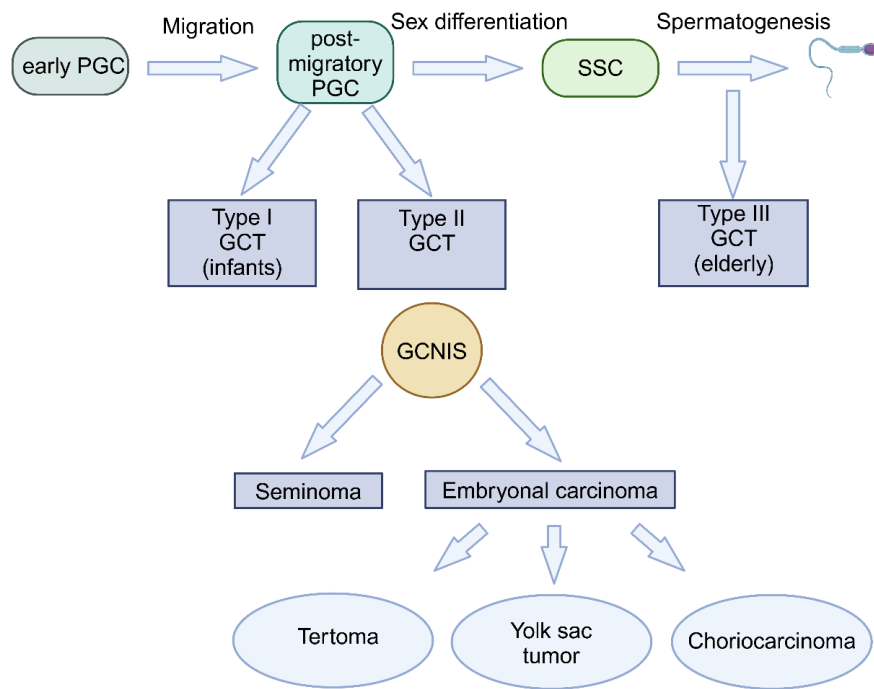


Figure 4: Development of GCTs. Type I GCTs derive directly from post-migratory PGCs. In contrast, type II GCTs arise from a precursor lesion called germ cell neoplasia in situ (GCNIS). They subgroup in seminoma and non-seminoma (embryonal carcinomas) which comprise teratoma, yolk sac tumor and choriocarcinoma. Type III GCTs arise in spermatogenesis. PGC – primordial germ cell; SSC – spermatogonial stem cell; GCT – germ cell tumor; GCNIS – germ cell neoplasia in situ. Modified from ⁷. Created in <https://BioRender.com>.

1.2.1 Type I germ cell tumors

Type I GCTs are yolk sac tumors and teratomas of neonates and children up to 6 years ¹⁷. They occur along the midline of the body corresponding to the PGC migration route. The origin of these tumors are PGCs or gonocytes (Figure 4) ¹². Downregulation of PGC-specific genes like TFAP2C, PRDM1 and PRDM14 is initiated but they fail to also downregulate pluripotency markers ⁷. Type I teratomas usually have no chromosomal abnormalities while type I yolk sac tumors show aneuploidy with chromosomal changes ²⁰.

1.2.2 Type II germ cell tumors

Type II GCTs occur mainly in the testis where they are referred to as testicular germ cell tumors (TGCT). Only in a few cases (5 %) tumors localize extragonadal along the body midline. TGCTs account for 60 % of all malignant tumors in young men (20-40 years). The gain of chromosome 12p was found in 80 % of all TGCTs indicating

importance for development of TGCTs ²⁰. They arise from a precursor lesion called germ cell neoplasia in situ (GCNIS) ⁷. Arrested PGCs with (epi-) genetic aberrations fail to downregulate pluripotency and germ cell markers and give rise to this lesions ^{7,21}. GCNIS stay dormant until puberty accumulating chromosomal abnormalities which activate malignant growth developing into seminoma or embryonal carcinoma (Figure 4) ²¹. Seminomas display restricted abilities of differentiation and therefore are considered to be the default pathway of GCNIS development. They share gene expression patterns of PGC maintaining pluripotency markers including OCT4, NANOG and LIN28 ⁷. Irie et al. found that seminoma derived TCam-2 cells aligned with human PGC-like cells (hPGCLC) and gonadal hPGCs in PCA indicate the progress of the early germline from pre-induced cells over hPGCLCs and TCam-2 cells to gonadal hPGCs (Figure 5). All three share expression of early germ cell markers (BLIMP1, TFAP2C, DND1 and KIT) ²².

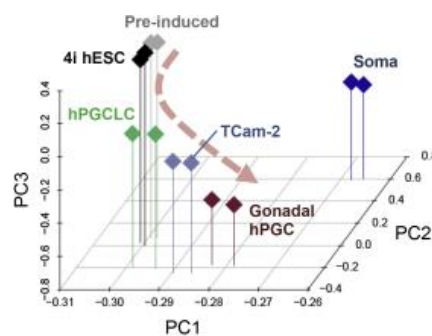


Figure 5: Human PGC-like cells resemble seminoma (TCam-2) cells. Principle component analysis (PCA) of RNA-seq data. Arrowline indicates possible progression of the germline. hESC – human embryonic stem cell; hPGCLC – human primordial germ cell-like cell; hPGC – human primordial germ cell. Modified from ²².

Of note, seminoma cells (TCam-2) also express SOX17 which is the key specifier for PGC fate. SOX17 regulates BLIMP1, PRDM14 and TFAP2C which are necessary for depletion of somatic genes and therefore inducing latent pluripotency ²².

Embryonal carcinomas are totipotent and able to differentiate into all three germ layers (meso-, ecto- and endoderm) as well as extraembryonic tissue and therefore differentiating into teratoma, yolk sac tumor or choriocarcinoma (Figure 4) ⁷. Non-seminomas share the expression of OCT4, NANOG and DPPA3 with seminomas but in addition also express NODAL, DNMT33B, DNMT3L and CD30 ²³. Of note, in

embryonal carcinomas SOX2 is upregulated¹². Therefore, SOX2 and SOX17 are used as diagnostic biomarkers for distinguishing TGCT subtypes²⁴.

1.2.3 Type III germ cell tumors

Type III GCTs are spermatocytic tumors of older males between 40 and 55 years. The origin of these tumors are probably germ cells capable of spermatogonia maturation (Figure 4)⁷. Mutations in FGF3 and HRAS are predisposing for type III GCTs¹². Chromosomal abnormalities are rare, the repetitive change are losses or gains of whole chromosomes¹².

1.3 Treatment of TGCTs

TGCTs are diagnosed as an unilateral palpable mass by the patient or incidentally via scrotal ultrasonography. Patients may also report scrotal pain, flank or back pain and in very few cases gynaecomastia^{25,26}. To support the diagnosis serum tumor marker levels including α -Fetoprotein (AFP), beta subunit of human chorionic gonadotropin (β -hCG) and lactate dehydrogenase (LDH) are determined. These markers correlate with germ cell cancer histology²⁵. Radical orchiectomy allows for histopathologic reports on tumor size, histology and lymphovascular invasion²⁶. Staging is performed based on the International Germ Cell Cancer Collaborative Group (IGCCCG). Seminomas and non-seminomas can be distinguished in clinical stages I-III.

1.3.1 Non-Seminomas

Stage I non-seminomas are diagnosed in 70 % of patients at this stage and are grouped into low-risk or high risk according to absence or presence of vascular invasion and display high survival rates of 98-100 %^{26,27}. For low-risk patients active surveillance is the recommended procedure and as an alternative one cycle of bleomycin, etoposide and platinum (BEP) chemotherapy is used. High risk patients are usually treated with one adjuvant BEP cycle but surveillance can also be an alternative²⁵. Non-seminoma stage II tumors involve also the retroperitoneal lymph nodes and are distinguished according to involved lymph node size in IIA (nodes <2 cm), IIB (nodes 2-5 cm) and IIC (nodes >5 cm)²⁶. For stage IIA with negative tumor markers close follow up of the lymph node is recommended with primary retroperitoneal lymph

node dissection (p-RPLND) when volume of the lymph node increases. Alternatively, tumors are treated with 3 cycles of BEP chemotherapy or 4 cycles of platinum and etoposide (PE) chemotherapy. In case of stage IIA with positive tumor marker as well as stage IIC chemotherapy is recommended ²⁶. For stage IIB and III tumors treatment is based on good, intermediate or poor prognosis. Stage III tumors are defined as tumors that involve lymph nodes or any other organs ^{25,26}. In case of good prognosis tumors are treated by 3 cycles of BEP, 4 cycles of PE or RPLND. For intermediate prognosis 4 cycles of BEP or 4 cycles of etoposide, ifosfamide and platinum (VIP) chemotherapy are recommended. In patients with poor prognosis 4 cycles of either BEP or VIP including dose intensification are used ²⁵.

1.3.2 Seminomas

About 80 % of patients are diagnosed at stage I and the 5-year relapse rates after orchiectomy account 15-20 % ²⁶. After orchiectomy there are different treatment options based on low and high risk. Stage I tumors with low risk undergo active surveillance while high risk tumors are treated by 1-2 cycles of platinum-based chemotherapy at a dose in the area under the curve (AUC) of 7 or also surveillance ²⁵. For Stage IIA tumors radiotherapy or chemotherapy with either 3 BEP cycles or 4 PE cycles are recommended ²⁶. Stage IIB-III tumors are treated by 3-4 cycles of BEP chemotherapy ²⁵.

1.3.3 Cisplatin

Cisplatin is the first generation of platinum-based drugs and was first synthesized in 1844 while the chemical structure was discovered in 1893. In 1965 Dr. Rosenberg unveiled the ability of cell division inhibition and cisplatin was FDA approved for cancer treatment in 1978 ²⁸⁻³². In patients with testis cancer the survival rate was significantly increased by the establishment of cisplatin-based chemotherapy. Before treatment with cisplatin the 5-year survival rate was 72 % while the use of cisplatin increased the survival rate to 95 % ³³. Cisplatin is a metallic coordination molecule with square planar geometry. It is composed of a platinum ion enclosed by two amine (NH₃) ligands and two chloride (Cl) ligands (Figure 6). At room temperature cisplatin is a white or yellow crystalline powder and is soluble in *N,N*-dimethylformamide (DMF) ²⁹.

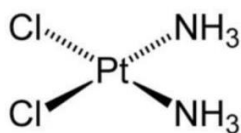


Figure 6: Chemical structure of cisplatin. Pt – platinum; Cl – chloride; NH₃ – amine. Modified from ²⁹.

Cisplatin enters the tumor cells through the copper transporter 1 (CTR1). Afterwards, activation is initiated by significantly lower intracellular chloride ion concentration which leads to the replacement of the chloride ligands by water molecules. Following chemical reactions in the cytoplasm enable cisplatin to bind to DNA and therefore changing the DNA structure leading to DNA damage ³⁰. Oxidative stress is common in cisplatin induced cytotoxic effects. It is defined by formation of reactive oxygen species (ROS) as well as reduced mitochondrial glutathione (GSH) resulting in DNA damage ^{28,29}. Ataxia telangiectasia mutated (ATM) leads to phosphorylation and therefore the activation of p53 followed by GADD45, p21 and MDM2 mediated cell cycle arrest ²⁸. In addition, p53 results in apoptosis by several mechanisms including activation of Bax, PUMA and Casp6 as well as degradation of FLIP or inhibition of the antiapoptotic role of Bcl-xL (Figure 7) ²⁹.

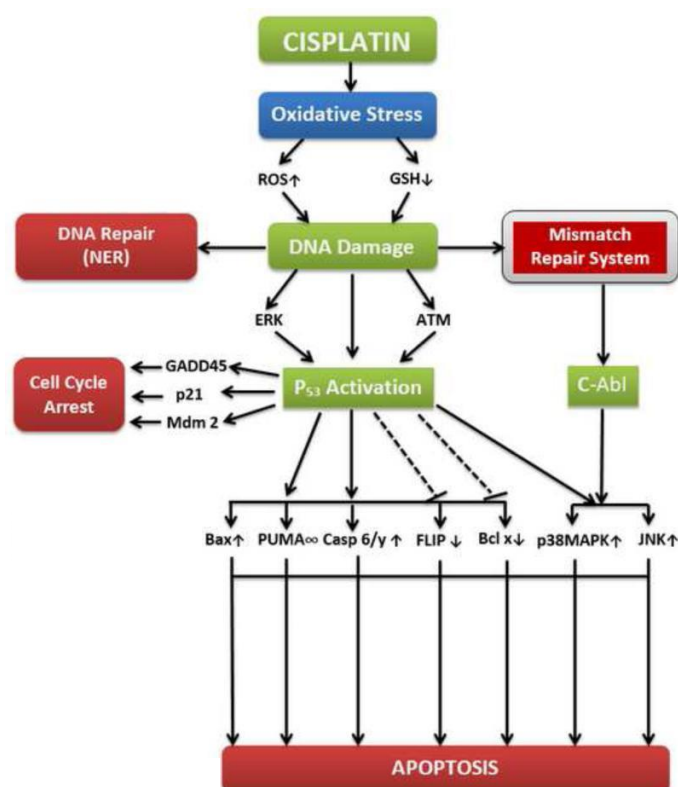


Figure 7: Mode of action of cisplatin in cancer treatment. Modified from ²⁹.

Sensitivity of TGCTs to cisplatin is based on impaired DNA repair induced by DNA damage as well as increased apoptotic response ³⁴. Cisplatin-induced DNA damage is usually repaired by the nucleotide excision pathway (NER) ³⁵. In TGCTs proteins involved in NER including ERCC1, XPA and XPF show low expression levels and therefore sensitizing TGCT cells for cisplatin therapy ³⁴. Furthermore, cisplatin induces increased expression of the FAS receptor which is a target of p53 and results in the activation of the intrinsic apoptosis pathway executed by the interaction of FAS and its ligand FASL ³⁶. Of note, DNA methylation correlates with cisplatin-sensitivity. While seminomas are hypomethylated, embryonal carcinomas show an intermediate methylation profile and the more differentiated tumors including yolk sac tumors, choriocarcinomas and teratomas display hypermethylation. In line, seminomas as well as embryonal carcinomas show high sensitivity towards cisplatin while on the other hand, highly differentiated teratomas are insensitive to cisplatin ³⁷.

1.4 Cisplatin resistance

While survival rates improve there are still 15-20 % of patients resistant to the treatment and 50 % of patients develop intrinsic resistance or establish multidrug resistance after treatment with cisplatin ^{38,39}. Cisplatin resistance is multifactorial and is divided in pre-, on-, post- and off target resistance (Figure 8, Figure 9) ⁴⁰. Pre-target mechanisms play a role prior to binding of cisplatin to the DNA while on-target mechanisms are directly involved in cisplatin-induced DNA damage. Pathways activated by cisplatin-induced DNA damage are grouped in post-target mechanisms. Off-target mechanisms comprise pathways not directly related to cisplatin-induced DNA lesions ⁴¹.

1.4.1 Pre-target

Pre-target mechanisms are based on reduction of cisplatin influx, increase of cisplatin efflux as well as cisplatin inactivation (Figure 8) ³⁹. Cisplatin uptake into the cell is mediated by passive diffusion or facilitated transport by the copper transporter CTR1 or organic cation transporters (OCT) while copper efflux transporters ATP7A and ATP7B as well as multidrug resistance proteins (MRP) enable the export of cisplatin ^{40,42}. Application of copper (substrate of CTR1) resulted in less cytotoxic effects of cisplatin, whereas copper chelators lead to accumulation of cisplatin and escalate cytotoxicity ^{43,44}. In TGCTs cisplatin resistance is not related to drug transporters ³⁸. Detoxification is performed by cytoplasmic scavengers like metallothioneins (MT) or glutathione (GSH) binding to cisplatin and therefore reducing the amount of active cisplatin ^{45,46}. Low levels of scavengers were found in TGCTs suggesting no significant contribution of pre-target mechanisms to cisplatin resistance in TGCTs ^{38,47}.

1.4.2 On-target

Cisplatin induces DNA damage which usually results in apoptotic signals ⁴⁰. However, cisplatin-resistant cells are able to repair adducts by activation of DNA repair mechanisms or cells can tolerate unrepaired DNA lesions ^{39,40}. Nucleotide excision repair (NER) is the most common system to overcome cisplatin-induced DNA damage (Figure 8) ⁴⁸. The bonds formed between cisplatin and DNA are removed by excision repair cross-complementing 1 (ERCC1) and Xeroderma pigmentosum complementation group F (XPF) proteins ³⁹. ERCC1 expression is negatively

correlated with sensitivity to cisplatin in different cancer types including bladder and ovarian cancer ^{49,50}. In TGCTs high-mobility group box protein 1 (HMGB1) which binds to cisplatin DNA crosslinks and interferes with NER is highly expressed leading to cisplatin resistance ³⁸. Tolerance of cisplatin-induced DNA damage is related to impaired mismatch base repair (MMR) ³⁹. Cisplatin-induced DNA lesions are detected but not repaired by MMR-related proteins including MSH2 and MLH1 ^{40,51,52}. In TGCT patients impaired MMR is correlated with treatment failure and relapse. Decreased expression of MHL2 in refractory TGCTs indicate defective MMR as a mechanism of cisplatin resistance in TGCTs ³⁸. The homologous recombination machinery (HRR) usually repairs cisplatin-induced DNA double-strand breaks ³⁹. Two components of the HRR are BRCA1 and BRCA2 which are both mutated in ovarian and breast cancer ⁵³. HRR deficiency is correlated with increased sensitivity to cisplatin ^{54–56}. Poly (ADP-ribose) polymerase (PARP) plays a role in repair of single-strand breaks induced by cisplatin via base excision repair (BER) ³⁸. In TGCTs overexpression of PARP was detected suggesting to be involved in a possible on-target mechanism in TGCTs ^{57,58}.

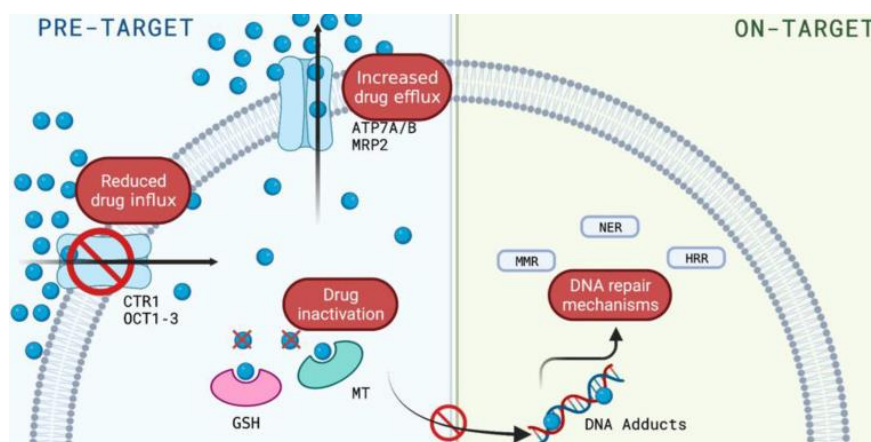


Figure 8: Mechanisms of cisplatin resistance. Mechanisms are subgrouped into pre-, on-, post- and off-target. Pre-target mechanisms comprise reduced drug influx, increased drug efflux as well as drug inactivation. On-target mechanisms are based on DNA repair mechanisms. MMR – mismatch base repair; NER – nucleotide excision repair; HRR – homologous recombination repair. Modified from ³⁹.

1.4.3 Post-target

The inactivation of the TP53 gene encoding for the p53 protein is the predominant post-target mechanism (Figure 9) ⁵⁹. Depletion of p53 results in reduction of apoptosis and induction of resistance in 50 % of cancers ⁶⁰. Of note, TGCTs are among few cancers showing rarely inactivation of TP53 ⁶¹. In fact, hyperactivation of p53 leads to increased sensitivity to cisplatin in TGCTs ^{62–64}. High abundance of MDM2 interacting with p53 is related to cisplatin resistance in TGCTs ³⁶. Upregulation of platelet-derived growth factor receptor b (PDGFRb) and its ligand PDGF-b in TGCT results in activation of PDGFR/ PI3K/ AKT pathway and subsequent phosphorylation of p21 and activation of MDM2. Thus, cells are prevented from apoptosis and induce G1-phase cell cycle arrest ^{65,66}. Another mechanism is the inactivation of caspases like caspase 3, 8 and 9 which are important in apoptosis. Caspase inactivation is related to cisplatin resistance in different types of cancer ^{39,67}.

1.4.4 Off-target

A general pathway in response to stress is autophagy or heat-shock response which are classified as off-target mechanism (Figure 9) ^{40,68,69}. In ovarian and non small cell lung cancer upregulation of proteins of the autophagy pathway is positively correlated with cisplatin resistance while inhibition of autophagy resensitized cells to cisplatin ^{70,71}.

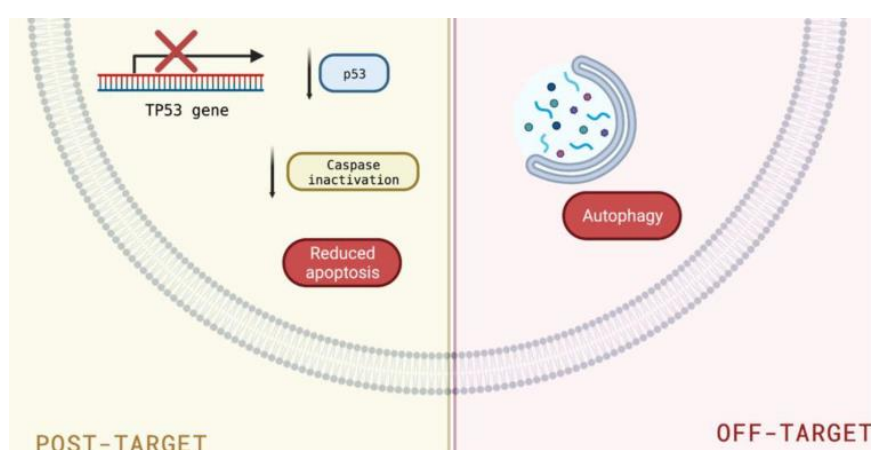


Figure 9: Mechanisms of cisplatin resistance. Mechanisms are subgrouped into pre-, on-, post- and off-target. Post-target mechanisms result in reduced apoptosis. Autophagy is categorized as off-target mechanisms. Modified from ³⁹.

All these pre-, on-, post- and off-target mechanisms contribute to cisplatin resistance emphasizing the necessity of investigating alternative treatments.

1.5 Alternative treatment options

Different preclinical and clinical trials studied various alternative treatment options for refractory GCTs including targeted therapy, immunotherapy epigenetic drugs and other therapeutic agents ⁷².

1.5.1 Targeted therapy

One possibility of targeted therapy are tyrosine kinase inhibitors (TKI) inhibiting receptor tyrosine kinases for example vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor (PDGF) which are overexpressed in GCTs ^{66,72}. Sunitinib, a multikinase inhibitor, re-sensitized cisplatin-resistant GCT cell lines to cisplatin and in a phase II clinical trial sunitinib led to a response rate of 13 % ^{73,74}.

Another target is the mammalian target of rapamycin (mTOR) which upon overactivation is associated with enhanced cell growth, proliferation and survival. The mTOR inhibitor everolimus was tested in phase II clinical trials but showed only limited efficacy ^{75–77}.

Targeted therapy also includes the inhibition of PARP. High PARP expression is correlated with lower overall survival in GCTs ⁵⁷. Olaparib inhibiting PARP was tested in phase II clinical trials with marginal efficacy ⁷⁸.

Possible targets are cyclin-dependent kinases (CDK). Inhibitors of cell cycle-associated CDKs (CDK1/2/4/6) like palbociclib and ribociclib were investigated in phase II clinical studies ^{79,80}. Transcriptional CDK (CDK7/8/9/12/13) inhibitors were studied in vitro and showed cytotoxic effects in TGCT cell lines ⁸¹.

1.5.2 Immunotherapy

The Programmed cell death 1 (PD-1)/ Programmed cell death ligand 1 (PD-L1) interaction is the main target of immune checkpoint inhibitors. PD-L1 is highly expressed in GCTs compared to normal testis tissue ^{82,83}. Unfortunately, phase II

clinical trials of pembrolizumab (PD-1 inhibitor) showed no objective response ^{84,85}. Only one refractory GCT patient responded rapidly to pembrolizumab ⁸⁶. The PD-L1 inhibitor avelumab also showed no efficacy in patients with multiple relapsed non-seminomas ⁸⁷.

1.5.3 Other therapeutic agents

Aldehyde dehydrogenase (ALDH) showed high expression in GCTs compared to normal testis tissue. Inhibition of ALDH by disulfiram in combination with cisplatin led to synergistic effects in cisplatin-resistant embryonal carcinoma cell lines as well as inhibition of growth in resistant xenografts ⁸⁸. A phase II clinical trial is still ongoing ⁷².

Targeting the WNT/ β -catenin signaling pathway which is deregulated in GCT by the inhibitor PRI-724 led to apoptosis in cisplatin-resistant GCTs ⁸⁹. Unfortunately, no clinical trials are initiated ⁷².

Inhibition of the interaction of MDM2 and p53 by the small-molecule MDM2 inhibitor Nutlin-3 led to induction of apoptosis and in combination with cisplatin synergistic effects were observed ^{36,90}. Again, clinical studies are missing ⁷².

1.5.4 Epigenetic drugs

Since DNA hypermethylation as well as histone deacetylation are known to be involved in cisplatin resistance targeting DNA methyltransferases (DNMT) and histone deacetylases (HDAC) was investigated ⁹¹. A hypomethylating agent guadecitabine led to induction of complete regression of cisplatin-resistant embryonal carcinoma xenografts ⁹². A phase I clinical study of combination of guadecitabine and cisplatin also showed complete response in two patients ⁹³.

HDAC inhibitors indicated preclinical efficacy in vitro and in vivo ⁷². Romidepsin led to decreased viability in TGCT cell lines as well as reduced tumor growth in xenografted mice treated with romidepsin ⁹⁴.

BET protein inhibitors like the bromodomain inhibitor JQ1 inhibiting BRD4 induced apoptosis as well as cell cycle arrest in TGCT cell lines and reduced tumor growth in xenografted mice ⁹⁵.

Taken together, in contrast to promising preclinical studies targeted therapy including TKI, mTOR and PARP inhibitors as well as immune checkpoint inhibitors led to questionable results in clinical trials. Only inhibitors interfering with the epigenetic landscape showed the most promising results. Therefore, investigation of alternative treatment options like epigenetic drugs is very important.

1.6 Bromodomain-containing proteins

Bromodomains (BRD) are able to bind specifically to ϵ -N-acetylation of lysine residues (K_{ac}) which are the most frequent posttranslational modification (PTM) in proteins ^{96,97}. BRDs got the name from the *Drosophila* gene *brahma* where the bromodomain was first reported by Tamkun et al. in 1992 ^{98,99}. They are evolutionary conserved and comprise ~110 amino acids. All BRDs share a fold including a left-handed bundle of four α helices (α_Z , α_A , α_B , α_C) which are linked by two interhelical loops (ZA and BC loops) forming a hydrophobic binding pocket including the K_{ac} binding site and therefore influencing the binding specificity (Figure 10) ^{97,100,101}.

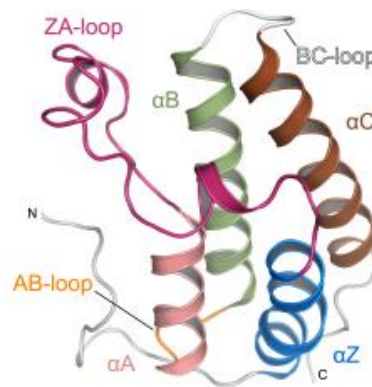


Figure 10: Structure of the bromodomain of human BRD4. Modified from ⁹⁷.

Proteins containing BRDs have various functions like chromatin remodeling, recruitment of transcription factors and histone modifications ¹⁰². In humans 61 bromodomains are known in 46 different bromodomain-containing proteins (BCP). They are subgrouped in bromodomain and extra-terminal (BET) and non-BET families ¹⁰³. BET proteins comprise BRD2, BRD3, BRD4 and testis-specific BRDT while non-BET proteins comprise histone acetyltransferases (HAT), SWI/SNF complexes, AAA ATPase proteins, ISWI complexes and E3 SUMO/ubiquitin ligases ^{102,103}. Since BRDs

play important roles in chromatin-based gene transcription targeting BCPs by small molecules is a promising starting point to overcome therapy resistance and to discover alternative treatment options for TGCTs. The inhibition of BET proteins has already been shown to be effective. Therefore, we focused on BRD9. The effect of BRD9 inhibition is not studied yet in TGCTs but seems to be a possible target for an alternative treatment option.

1.6.1 BRD9

BRD9 comprises 597 amino acids and contains one bromodomain ¹⁰³. It belongs to the non-BET families and is classified as a group IIIa member. Group IIIa comprises chromatin remodeling factors like SMARCA2, SMARCA4, PBRM1, BRD7 and BRD9. They are subunits of the Switch/Sucrose Non-Fermentable (SWI/SNF) complex which remodels chromatin in an ATP-dependent manner. The SWI/SNF complex regulates DNA damage, gene transcription and cell growth as well as differentiation ¹⁰⁴. The ATPase component of the complex contains several functional domains including BRD which mediates histone binding ¹⁰². The SWI/SNF complexes are subgrouped into three classes: canonical ATPase BRG1/BRM-associated factor (BAF), polybromo-associated BAF (PBAF) and non-canonical BAF (ncBAF) complexes ¹⁰⁵. BRD7 is part of the PBAF complex while the smallest ncBAF complex comprises BRD9. The bromodomain and its K_{ac} binding ability mediate the assembly of the complex by propelling ATPase-driven movement along the chromatin ¹⁰². Therefore, BRD9 is an epigenetic reader activating gene expression by recruitment of transcriptions factors (Figure 11) ⁴¹.

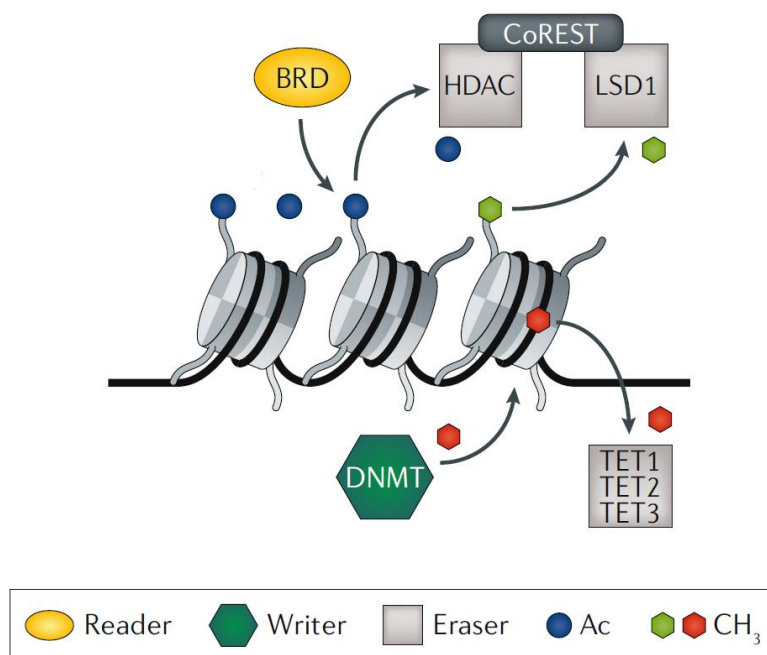


Figure 11: Epigenetic modifications in TGCTs. BRDs are epigenetic readers activating or repressing gene expression by recruitment of transcription factors. BRD – bromodomain proteins; HDAC – histone deacetylase; DNMT – DNA methyltransferase; Ac – acetyl group; CH₃ – methyl group. Modified from ⁴¹.

Furthermore, BRD9 is known to play an oncogenic role, promotes tumor progression and is deregulated in 23 malignancies ¹⁰⁶. For example, BRD9 is upregulated in acute myeloid leukemia (AML) cells and targeting BRD9 led to decreased proliferation of mouse and human AML cells ¹⁰⁷. In squamous cell lung cancer (SqCLC) inhibition of BRD9 by overexpression of miR-140-3p reduced tumorigenesis by downregulation of c-myc ¹⁰⁸. LP99 was the first BRD7/BRD9 inhibitor that revealed anti-inflammatory ability in human monocytic leukemia cells ¹⁰⁹. Two BRD9-specific inhibitors BI-7273 and BI-9564 were designed and showed both antiproliferative effects in AML cells while BI-9564 also showed decreased tumor burden in xenografted mice ¹¹⁰. I-BRD9 is the most selective and potent BRD9 inhibitor ¹⁰². Of note, BRD9 inhibition was not studied in TGCTs yet.

1.6.2 I-BRD9

I-BRD9 is a chemical probe that was described by Theodoulou et al. in 2016 (Figure 12 A). It is a K_{ac} mimetic binding to the bromodomain binding pocket of BRD9 (Figure

12 B) ¹¹¹. I-BRD9 was discovered by structure-based design and showed more than 700-fold selectivity over BET proteins and 200-fold higher over BRD7 ¹⁰³.

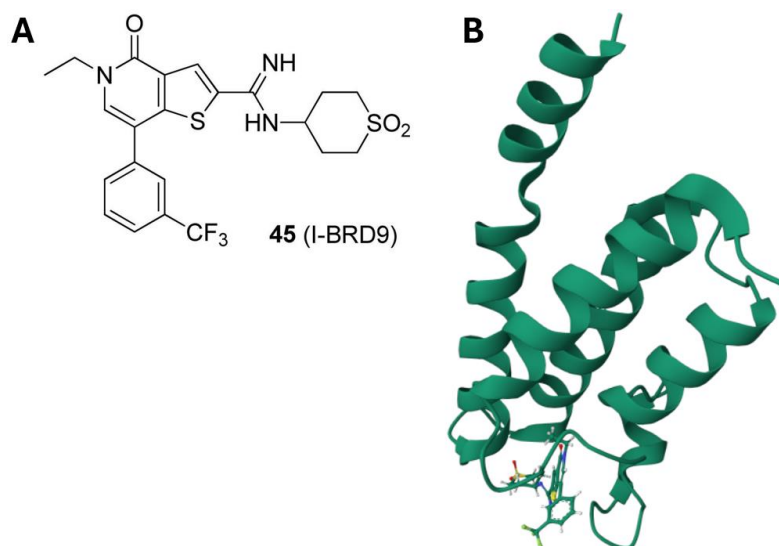


Figure 12: K_{ac} mimetic I-BRD9 binds to the bromodomain of BRD9. (A) Structure of I-BRD9 ¹¹¹. (B) Crystal structure of I-BRD9 binding to BRD9. Image from the RCSB PDB (RCSB.org) of PDB ID 6V1B ¹¹². Modified from ^{111,112}.

The thienopyridine I-BRD9 led to downregulation of immunology and cancer-related genes including CLEC1, FES, SAMSN1 and DUSP6 ^{113–116}. In AML cells I-BRD9 led to reduction of cell growth as well as induction of apoptosis ¹¹⁷. I-BRD9 resulted in decreased cell proliferation in gallbladder cancer (GBC) as well as reduction of tumor growth in a GBC mouse tumor model without significant side-effects ¹¹⁸. In colon adenocarcinoma (COAD) cells application of I-BRD9 suppressed cell growth as well as reduced tumor growth in xenografted mice ¹¹⁹. Treatment of rhabdoid tumors with I-BRD9 resulted in reduction of cell proliferation, induction of apoptosis as well as G1-phase cell cycle arrest ¹²⁰. These data suggest I-BRD9 as an effective compound in different tumors. Of note, I-BRD9 was not studied in TGCTs so far. Therefore, investigation of the effect of I-BRD9 in TGCTs is an interesting approach to elucidate alternative treatment options.

1.6.3 TGCT cell lines

For in vitro studies of TGCTs there are several cell lines described. Embryonal carcinoma cell lines 2102 EP, NCCIT and NT2/D1 were derived from mixed non-seminoma patients ^{121–123}. NCCIT and NT2/D1 cells are pluripotent while 2102 EP cells display a nullipotent character ^{123–125}. Cisplatin-resistant subclones 2102 EP-R, NCCIT-R and NT2/D1-R were established by cisplatin treatment with increasing sublethal concentrations ^{126,127}. JAR cell line resembles choriocarcinoma and was derived from a trophoblastic tumor of the placenta from the male fetus ¹²⁸. The cell line TCam-2 was established from a testicular seminoma ¹²⁹. As control cells FS1 and MPAF were used. Immortalized adult human sertoli cells (FS1) were derived from testis tissue of a Frasier syndrome patient ¹³⁰. MPAF cells are human adult fibroblasts.

1.7 Aim of the project

In this project the aim was to elucidate an alternative treatment option for testicular germ cell tumors to overcome cisplatin therapy resistance. Therefore, the BRD9 inhibitor I-BRD9 which has already been shown to be effective in different cancer types but was not yet tested in TGCTs was studied. First, expression of the target BRD9 was analyzed on RNA level by meta-analysis of microarray data in TGCT tissues as well as cell lines and for protein level Western Blot as well as TMA were performed. In different TGCT cell lines cytotoxicity of the inhibitor was measured by viability assays. The effect of application of I-BRD9 on apoptosis as well as cell cycle distribution were assessed via FACS. To reveal changes on transcriptome level induced by BRD9 inhibition 3'mRNA sequencing was performed.

The findings will elucidate the effect of the BRD9 inhibitor I-BRD9 in TGCTs and the possibility to be used as alternative treatment option with reduced side effects and to overcome therapy resistance.

2 MATERIALS

2.1 Cell lines

Cell line	Description	Standard culture medium	Source
2102 EP	Embryonal carcinoma	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	Prof. Dr. L. Looijenga (Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands)
2102 EP-R	Cisplatin-resistant subline derived from 2102EP	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	PD Dr. F. Honecker (ZeTup Silberturm, St. Gallen, Switzerland)
NCCIT	Embryonal carcinoma	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	Prof. Dr. L. Looijenga (Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands)
NCCIT-R	Cisplatin-resistant subline derived from NCCIT	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	PD Dr. F. Honecker (ZeTup Silberturm, St. Gallen, Switzerland)
NT2/D1	Embryonal carcinoma	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	Prof. Dr. L. Looijenga (Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands)
NT2/D1-R	Cisplatin-resistant subline derived from NT2/D1	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	PD Dr. F. Honecker (ZeTup Silberturm, St. Gallen, Switzerland)

Cell line	Description	Standard culture medium	Source
TCam-2	Seminoma	RPMI, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	Prof. Dr. L. Looijenga (Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands)
JAR	Choriocarcinoma	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	ATCC (Manassas, VA, USA)
FS1	Sertoli cells	DMEM, 20% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM)	Dr. Valerie Schumacher (Nephrology Research Center, Boston, USA)
MPAF	Fibroblasts	DMEM, 10% FBS, 1% penicillin/streptomycin (10,000 U/ml), 1% L-glutamine (200 mM), 1% non-essential amino acids (100x)	PD Dr. M. Peitz (Institute of Reconstructive Neurobiology, Bonn University, Bonn, Germany)

2.2 Cell culture media and reagents

Medium/ reagent	Manufacturer
0.05% Trypsin-EDTA	Thermo Fisher Scientific, Waltham, USA
Dimethyl sulfoxide (DMSO)	AppliChem, Darmstadt, Germany
Dulbecco's Modified Eagle's Medium (DMEM) high glucose	Thermo Fisher Scientific, Waltham, USA
Fetal bovine serum (FBS)	Merck, Darmstadt, Germany
L-Glutamine 200 mM	Thermo Fisher Scientific, Waltham, USA
Non-essential amino acids (NEAA)	Thermo Fisher Scientific, Waltham, USA
Penicillin/ streptomycin	Thermo Fisher Scientific, Waltham, USA

Medium/ reagent	Manufacturer
Phosphate buffered saline (PBS)	Thermo Fisher Scientific, Waltham, USA
Roswell Park Memorial Institute (RPMI) medium	Thermo Fisher Scientific, Waltham, USA
Sodium pyruvate	Merck, Darmstadt, Germany

2.3 Consumables

Consumable	Manufacturer
384-well micro titer plate	4titude, Wotton, UK
Cell Culture Dishes 55 cm ²	VWR, Darmstadt, Germany
Cell Culture Flasks, Filter Cap, CELLSTAR® (T25, T75)	Greiner bio-one, Frickenhausen, Germany
Cell Culture Multiwell Plates (6-, 12-, 24-, 96-well-plate)	TPP, Trasadingen, Austria
Cell scraper 25 cm	SARSTEDT, Nümbrecht, Germany
Cryogenic vials 2 ml, internal thread	Greiner bio-one, Frickenhausen, Germany
Eppendorf tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Extra Thick Blot Filter Paper	BioRad Laboratories, Hercules, USA
FACS tubes	BD Biosciences, Heidelberg, Germany
Filter tips (10 µl, 100 µl, 1000 µl)	Nerbe Plus, Winsen/Luhe, Germany
Microplate, 96 well, PS, F-bottom, clear	Greiner bio-one, Frickenhausen, Germany
Microscope Cover Glasses 14 mm	Marienfeld GmbH, Lauda-Königshofen, Germany
Microscope slides	Marienfeld GmbH, Lauda-Königshofen, Germany
Parafilm M®	Pechiney Plastic Packaging, Menasha, USA
PCR® strip tubes	Axygen Scientific, Union City, USA
Petri dishes	Greiner bio-one, Frickenhausen, Germany

Consumable	Manufacturer
Pipette tips (10 µl, 100 µl, 1000 µl)	Greiner bio-one, Frickenhausen, Germany
Polypropylene tubes CELLSTAR® (15 ml, 50 ml)	Corning, Amsterdam, Netherlands
Roti-PVDF membrane	Carl Roth, Karlsruhe, Germany
Stripettes Costar® (5 ml, 10 ml, 25 ml)	Greiner bio-one, Frickenhausen, Germany

2.4 Chemicals and reagents

Chemical/ Reagent	Manufacturer
2-Mercaptoethanol	Merck, Darmstadt, Germany
6-Diamidino-2-phenylindole dihydrochloride (DAPI)	AppliChem, Darmstadt, Germany
10x reaction buffer + MgCl ₂	Thermo Fisher Scientific, Waltham, USA
Albumin fraction V (BSA)	AppliChem, Darmstadt, Germany
Ammonium persulfate (APS)	Carl Roth, Karlsruhe, Germany
AnnexinV binding buffer	BioLegend, San Diego, USA
BRD9 inhibitor I-BRD9 (SML1534)	Sigma-Aldrich, St. Louis, USA
cOmplete™ ULTRA Tablets, Mini Protease Inhibitor Cocktail	Roche, Basel, Switzerland
Coomassie Brilliant Blue G250	Biomol, Hamburg, Germany
Ethylenediaminetetraacetic acid (EDTA) (50 mM)	Thermo Fisher Scientific, Waltham, USA
Ethanol	VWR, Darmstadt, Germany
Fluoroshield™	Merck, Darmstadt, Germany
Glycine	Carl Roth, Karlsruhe, Germany
Hematoxylin	Merck, Darmstadt, Germany
Hoechst bisBenzimid H 33342	Sigma-Aldrich, St. Louis, USA
Isopropanol	AppliChem, Darmstadt, Germany
Maxima SYBR Green/ROX qPCR Master Mix (2X)	Thermo Fisher Scientific, Waltham, USA

Chemical/ Reagent	Manufacturer
Methanol	VWR, Darmstadt, Germany
Oligo(dT) ₁₈ primer	Thermo Fisher Scientific, Waltham, USA
PageRuler™ Plus Prestained Protein Ladder, 10 bis 250 kDa	Thermo Fischer Scientific, Waltham, USA
PE-AnnexinV	BioLegend, San Diego, CA USA
PBS tablets	AppliChem, Darmstadt, Germany
Phenazine methosulfate (PMS)	Sigma-Aldrich, St. Louis, USA
RiboLock RNase Inhibitor (40 U/μl)	Thermo Fischer Scientific, Waltham, USA
Roti-Load 1, reducing, 4 x concentrated	Carl Roth, Karlsruhe, Germany
Rotiphorese ® Gel 30 (37,5:1)	Carl Roth, Karlsruhe, Germany
Rotiphorese ®10x SDS-PAGE	Carl Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Merck, Darmstadt, Germany
Tetramethyl ethylenediamine (TEMED)	VWR, Darmstadt, Germany
Tris-HCl	Carl Roth, Karlsruhe, Germany
Triton X-100	AppliChem, Darmstadt, Germany
Tween 20	AppliChem, Darmstadt, Germany
UltraPure™ agarose	Invitrogen™, Thermo Fisher Scientific, Waltham, USA
Water, nuclease free	Thermo Fisher Scientific, Waltham, USA
XTT sodium salt	AppliChem, Darmstadt, Germany

2.5 Buffers and solutions

Buffer/ Solution	Recipe/ Supplier
5x RT buffer	Thermo Fisher Scientific, Waltham, USA
Ammonium persulfate (APS) 10%	10 % (w/v) ammonium persulfate in H ₂ O
BSA-Blocking solution	5 % (w/v) BSA in PBST
BSA-Antibody dilution solution	1 % (w/v) BSA in PBS
PBST	200 ml 10x PBS, 1 ml Tween20, ad 2l H ₂ O
RIPA buffer	Cell Signaling, Danvers, USA

Buffer/ Solution	Recipe/ Supplier
SDS Polyacrylamide gel	12 % separation gel: 3.2 ml H ₂ O, 4 ml Rotiphorese® Gel 30 (37,5:1), 2.6 ml 1.5 M Tris-HCl (pH 8.8), 100 µl 10 % SDS, 100 µl 10 % APS, 4 µl TEMED 5 % stacking gel: 3.4 ml H ₂ O, 830 µl Rotiphorese® Gel 30 (37,5:1), 630 µl 1 M Tris-HCl (pH 6.8), 50 µl 10 % SDS, 50 µl 10 % APS, 5 µl TEMED
SDS running buffer	100 ml ROTIPHORESE® 10x SDS-PAGE, ad 1l H ₂ O
Tris-acetate-EDTA-buffer (TAE) (50 x)	2 M Tris base, 50 mM EDTA, 1 M acetic acid
Western Blot transfer buffer (10x)	20 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, 20% (v/v) Methanol
Western Blot transfer buffer (1x)	200 ml Methanol, 100 ml Western Blot transfer buffer (10X), ad 1 l H ₂ O

2.6 Kits

Kit	Manufacturer
Pierce™ BCA protein assay kit	Thermo Fisher, Waltham, USA
RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Fisher, Waltham, USA
DyLight™ 594 Antibody Kit, R.T.U. (DI-2794)	Vector Laboratories, Newark, CA, USA
VectaFluor™ Horse Anti-Rabbit IgG, DyLight™ 594 Antibody Kit, R.T.U. (DI-1794-15)	Vector Laboratories, Newark, CA, USA
WESTAR NOVA 2.0 Western Blot Substrate	Cyanagen, Bologna, Italy

2.7 Enzymes

Enzyme	Manufacturer
DNaseI	Thermo Fisher Scientific, Waltham, USA
Maxima H Minus Reverse Transkriptase (200 U/μl)	Thermo Fisher Scientific, Waltham, USA
RNase A	AppliChem, Darmstadt, Germany

2.8 Equipment

Equipment	Manufacturer
2100 Bioanalyzer Instrument	Agilent Technologies, Santa Clara, USA
Advanced Digital Shaker	VWR, Darmstadt, Germany
Agarose gel chamber	Peqlab, Erlangen, Germany
Autostainer BenchMark Ultra	Roche, Basel, Switzerland
Balance BP211S	Sartorius, Göttingen, Germany
Balance PT 120	Sartorius, Göttingen, Germany
BD FACSCanto II Flow Cytometer	BD Biosciences, New Jersey, USA
Cell culture hood BSB 6A	Gelaire, Sydney, Australia
Cell culture hood Safety cabinet HERAsafe®	Kendro, Langenselbold, Germany
Centrifuge 5417R	Eppendorf, Hamburg, Germany
Centrifuge Heraeus™ Megafuge™ 1.0	Thermo Fisher Scientific, Waltham, USA
Centrifuge Heraeus™ Multifuge™ 3 S-R	Thermo Fisher Scientific, Waltham, USA
ChemiDoc MP Imaging System	BioRad Laboratories, Hercules, USA
Electrophoresis Power Supply Consort EV243	Peqlab, Erlangen, Germany
Gel Documentation System GEL iX20 Imager	Intas Science Imaging Instruments GmbH, Göttingen, Germany
HiSeq 2500 V4	Illumina, San Diego, USA
Incubator Heracell 240i	Thermo Fisher Scientific, Waltham, USA
Incubator UM200	Memmert, Schwabach, Germany
iMark™ Microplate Absorbance Reader	BioRad Laboratories, Hercules, USA
Magnetic stirrer MR 3001	Heidolph, Schwabach, Germany

Equipment	Manufacturer
Mini-PROTEAN® Tetra Cell Casting Module	BioRad Laboratories, Hercules, USA
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	BioRad Laboratories, Hercules, USA
Microscope Labovert FS	Leica Microsystems, Wetzlar, Germany
Microscope Axiovert 40C	Zeiss, Jena, Germany
Multichanel pipette	Eppendorf, Hamburg, Germany
Neubauer improved counting chamber	Brand, Wertheim, Germany
pH-Meter	Schott Instruments, Mainz, Germany
Pipette controller Accu-Jet® Pro	Brand, Wertheim, Germany
Pipettes (10, 20, 100, 1000 µl)	Eppendorf, Hamburg, Germany
Spectrophotometer Nano Drop 1000	Thermo Fisher Scientific, Waltham, USA
Sonicator Bioruptor®	Diagenode, Seraing, Belgium
Thermal cycler 2720	Applied Biosystems® by Thermo Fisher Scientific Inc., Carlsbad, USA
Thermomixer Compact	Eppendorf, Hamburg, Germany
Tilt/roller mixer RS-TR 05	Phoenix Instrument, Garbsen, Germany
Trans-Blot® Turbo™ Transfer System	BioRad Laboratories, Hercules, USA
ViiA 7 Real-Time PCR System	Thermo Fisher Scientific, Waltham, USA
VisiScope CSU-W1	Vistron Systems, Puchheim, Germany
Vortex Genie 2	Scientific Industries Inc., New York, USA
Waterbath TW8	Julabo, Seelbach, Germany
Western Blot Imaging System ChemiDoc MP	Bio-Rad Laboratories, Hercules, USA
Western Blot Transfer System Trans-Blot® Turbo™	Bio-Rad Laboratories, Hercules, USA

2.9 Antibodies

Antibody	Manufacturer	Order number	Species	Application	Dilution
SALL4	Cell Marque, Rocklin, USA	385M	Mouse	IHC	1:50
BRD9	Abcam, Cambridge, UK	ab277488	Rabbit	IHC	1:500
BRD9	Cell Signaling, Danvers, USA	58906S	Rabbit	WB	1:1000
β -actin	Merck, Darmstadt, Germany	A5441	Mouse	WB	1:10000
Anti-Rabbit HRP	Agilent Technologies (Dako), Santa Clara, USA	P0448	Goat	WB	1:2000
Anti-Mouse HRP	Agilent Technologies (Dako), Santa Clara, USA	P0260	Rabbit	WB	1:2000
PRDM14	Cell Signaling, Danvers, USA	83527	Rabbit	IF	1:100
NANOG	Abcam, Cambridge, UK	ab109250	Rabbit	IF	1:250

2.10 Oligonucleotides

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
HSF4	GGACCAGTTTCCTCGTAAGCGA	CTCACCACCTTCCGAAAACCGT
SEMA3C	TTTGCGTGTTGGTTGGAGTAT	TCCTGTAGTCTAAAGGATGGTGG
DLX6	TCGCTTTCAGCAGACACAGT	CGGCTTCTTGCCACACTTAT
LEF1	GGTCCTCCTGGTCCCCACACAA	TCATGCTGAGGCTTCACGTGCA
GATA5	CCTGCGGCCTCTACCACAA	GGCGCGGCGGGACGAGGAC
HEYL	GAGAAACAGGGCTCTTCCAA	CTTCAAGGACCCCCAGGTA

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
NANOG	ATGGAGGAGGGAAGAGGAGA	GATTTGTGGGCCTGAAGAAA
NODAL	ATGCCAGATCCTCTTGTTGG	AGACATCATCCGCAGCCTAC
KLF4	ATCTCAAGGCACACCTGCG	CCTGGTCAGTTCATCTGAGCG
PRDM14	TCCACACAGGGGGTGTACTT	GAGCCTTCAGGTCACAGAGC
FGF4	GGCGAGAGCTCCAGCAG	CGGCTCTACTGCAACGTG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

2.11 Software

Name	Description	Source
BD FACSDiva™ Software	Flow cytometer application setup for data acquisition	BD Biosciences, New Jersey, USA
Bioconductor v3.20	Software packages for R-based analysis of omics data	https://www.bioconductor.org/
BioRender Online Tool	Creation of scientific schematics	https://www.biorender.com/
Enrichr	Pathway analysis tool for transcriptome data	https://maayanlab.cloud/Enrichr/
GraphPad Prism 10	Statistical analysis and visualization of graphs and figures	https://www.graphpad.com/
ImageJ	Image processing and analysis	https://imagej.net/ij/
Image Lab Software	Acquisition and analysis software for Western Blot	BioRad Laboratories, Hercules, USA
Microsoft 365 (Word, Excel, Power Point)	Word and data processing and presentation software	Microsoft, Redmond, USA
Microplate Manager Software 6	Acquisition software for XTT data	BioRad Laboratories, Hercules, USA

Name	Description	Source
NCBI Pubmed	Literature database	https://pubmed.ncbi.nlm.nih.gov/
QuPath	Software for bioimage analysis	https://qupath.github.io/
R v4.4.2	Software environment for statistical computing and graphics	https://www.r-project.org/
R studio v024.09.1+394	Coding environment for R	https://posit.co/
STRING	Protein-protein interaction network and functional enrichment analysis tool	https://string-db.org/
ViiA™ 7 Software	Acquisition of qRT-PCR data	Thermo Fisher Scientific, Waltham, USA
VisiView Software	Imaging software	Vistron Systems GmbH, Puchheim, Germany
Zotero	Reference management software	https://www.zotero.org/

3 METHODS

3.1 Affymetrix expression microarray analysis of GCT tissues

The Affymetrix expression microarray was previously published by Eckert et al. ¹³¹. The data were re-analyzed in context of this study regarding BRD9 expression in normal testis tissue (n=4), GCNIS (n=3), seminoma (n=4), embryonal carcinoma (n=3) and teratoma (n=3).

3.2 Tissue microarray

For tissue microarray (TMA) a cohort of 159 clinically annotated TGCT patients with curative or palliative surgical treatment (2005-2011) at the University Hospital Bonn and diagnosed by the Institute of Pathology was used. Approval of the study was given by the institutional review board of the University of Bonn (#187/16). TMA was performed by Dr. Christine Sanders and Dr. Florian Fronhoffs.

3.3 Immunohistochemistry staining

Immunohistochemical staining of BRD9 or SALL4 in the different tissues was performed using the Autostainer BenchMark Ultra. The antibodies are listed in the material (2.9). The counterstaining of the tissue sections was done with hematoxylin. The images were analyzed and quantified in QuPath and the mean H-score was determined. The analysis was performed by Dr. Christine Sanders.

3.4 Cell culture maintenance

The cell lines 2102 EP ¹²⁵, 2102 EP-R ¹²⁶, NCCIT ¹²², NCCIT-R ¹²⁶, NT2/D1 ¹²³, NT2/D1-R ¹²⁷ (embryonal carcinoma cell lines), JAR ¹³² (choriocarcinoma cell line), FS1 ¹³³ (sertoli cell line) and MPAF ¹³⁴ (human adult fibroblast cell line) were cultured in standard culture medium (2.1) in the incubator at 37 °C and 7.5 % CO₂. Splitting was performed twice a week. Cells were washed with PBS. For detachment 0.05 % Trypsin-EDTA was added and incubated for 5 minutes in the incubator. Cells were resuspended in standard culture medium. In a specific ratio cells were transferred into a cell culture flask for further maintenance. For long-term storage cell suspension was

centrifuged at 400 g for 5 minutes, media was discarded and cell pellet was resuspended in freezing medium (50 % FBS, 40 % standard culture medium and 10 % DMSO). Cells were aliquoted in cryogenic vials and stored at -80 °C. Thawing of cells was performed by incubation of the vial at 37 °C in a water bath and subsequent transfer into a cell culture flask filled with standard culture medium.

3.5 Illumina HT-12v4 expression microarray analysis of TGCT cell lines

The Illumina expression microarray was previously published by Nettersheim et al.⁹⁴. The dataset is publicly available at GEO (ncbi.nlm.nih.gov/geo/, GSE71239). The data were re-analyzed in context of this study regarding BRD9 expression in 2102 EP (n=5), NCCIT (n=4), TCam-2 (n=5), JAR (n=2), FS1 (n=4) and MPAF (n=4) cells.

3.6 Protein extraction, SDS PAGE and Western Blot analysis

For protein extraction all steps need to be performed on ice. Cells were lysed with RIPA buffer supplemented with protease inhibitor cOmplete ULTRA Tablets. Lysate was sonicated for 30 seconds at medium intensity and centrifuged for 15 minutes at 20817 g and 4 °C. Cell debris were pelleted and protein containing supernatant was transferred into a fresh tube. Protein lysates were stored at -20 °C.

For determination of protein concentration BCA assay was performed using the Pierce™ BCA Protein Assay Kit according to the manufacturer's manual. In brief, 9 bovine serum albumin (BSA) standards in different concentrations (25, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml) were prepared. Triplicates of standards as well as diluted (1:10) lysates were pipetted into a microplate and 200 µl working solution (50:1 ratio of BCA reagents A and B) were added. The plate was incubated at 37 °C for 30 minutes. Absorbance was measured at a wavelength of 595 nm in a plate reader. Using the standard curve generated by the measurements of the different BSA standards protein concentrations of lysates were determined.

For sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) protein lysates were diluted (1:10) and mixed with 4x ROTI-Load (reducing) and subsequently denatured for 10 minutes at 95 °C in a heating block. A SDS gel with a 5 % stacking

gel and a 12 % separation gel was used. The gel was prepared with a Mini-PROTEAN® Tetra Cell Casting Module. 30 µg of protein were loaded in the pocket. The PageRuler™ Plus Prestained Protein Ladder (10 bis 250 kDa) was used to determine the size of the proteins. The electrophoresis was performed in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for 10 minutes at 90 V and afterwards at 120 V for approximately 1.5 hours in SDS running buffer.

Proteins were transferred to a methanol-activated PVDF membrane by the Trans-Blot® Turbo™ Transfer System using transfer buffer. Coomassie staining was performed to evaluate equal protein amounts. Membrane was blocked in 5 % BSA in PBST for 1 hour at room temperature. Primary antibodies were diluted in 5 % BSA in PBST and incubated over night at 4 °C. The antibodies are listed in the materials (2.9). Membrane was washed three times in PBST for 10 minutes on a shaking plate. Incubation with secondary antibody coupled to horseradish peroxidase (HRP) diluted in 5 % BSA in PBST was conducted for 1 hour at room temperature. Three washing steps with PBST for 10 minutes were performed. For detection of bands WESTAR NOVA 2.0 enhanced chemiluminescent (ECL) substrate or for more sensitive detection SuperSignal™ West Femto Maximum Sensitivity Substrate was used. Substrate A and B were mixed in a 1:1 ratio and applied to the membrane. Detection was performed using the ChemiDoc MP Imaging System.

3.7 Cell viability assay

For measurement of viability XTT assay was performed. Cells were seeded at a concentration of 3×10^4 cells/ml and 100 µl of cell suspension were transferred in wells of a 96 well plate. The next day treatment with BRD9 inhibitor or corresponding solvent control DMSO was performed in five different concentrations (2, 10, 15, 20 and 25 µM). Viability was assessed after 24, 48 and 72 hours. XTT was dissolved in medium without supplements in a concentration of 0.7 mg/ml. PMS (1.25 mM) was added and 50 µl of XTT/PMS solution was pipetted onto the cells. Incubation was performed in the incubator for 4 hours. Afterwards, absorbance was measured in a plate reader at 450 and 650 nm.

3.8 DAPI/ AnnexinV (Apoptosis) FACS analysis

For fluorescence activated cell sorting (FACS) analysis cells were seeded in a 6 well plate in a concentration of 1.5×10^5 cells/well. The next day treatment with 15 μ M I-BRD9 or DMSO control was performed. After 24 and 48 hours of treatment the supernatant was collected in a FACS tube and harvested cells were added. Three washing steps with PBS and subsequent centrifugation at 400 g for 5 minutes were performed. For staining cells were resuspended in 100 μ l staining solution (100 μ l AnnexinV binding buffer, 0,1 μ l DAPI and 4 μ l PE-AnnexinV) and incubated at 37 °C for 30 minutes in the dark. Afterwards, 200 μ l of PBS were added and measurement was performed at the Flow Cytometry Core Facility (University Bonn, Germany) at the BD FACSCanto II Flow Cytometer and analyzed with the BD FACSDiva software™.

3.9 Hoechst (Cell Cycle) FACS Analysis

Cells were seeded in a 6-well plate (1.5×10^5 cells/well) and the next day treated with 15 μ M I-BRD9 or DMSO control. Measurement was performed after 24 and 48 hours of treatment. Washing with PBS and subsequent centrifugation at 400 g for 5 minutes was applied three times. Cells were fixed with ice-cold methanol for 60 minutes at 4 °C. Washing with PBS and centrifugation was performed and afterwards cells were incubated in staining solution (1.2 μ g/ml Hoechst33258 and 50 μ g/ml RNaseA in PBS) for 30 minutes at 37 °C. Measurement was performed at the Flow Cytometry Core Facility (University Bonn, Germany) at the BD FACSCanto II Flow Cytometer and analyzed with the BD FACSDiva software™. For further analysis FlowJo™v10.8 software was used.

3.10 RNA isolation

For isolation of RNA cell suspension was centrifuged at 10621 g for 5 minutes. Cell pellet was washed with PBS two times with subsequent centrifugation. Afterwards the RNeasy Mini Kit was used according to the manufacturer's manual. Concentration was measured with the Spectrophotometer Nano Drop 1000 and purity was checked by the 260 nm/ 280 nm ratio. High purity was considered at a ratio between 1.8 and 2.1.

3.11 3'mRNA sequencing analysis

For transcriptome analysis 2102 EP, TCam-2 and MPAF cells were seeded in a concentration of 2×10^5 cells/ well in a 6 well plate. Cells were treated with I-BRD9 (15 μ M) or DMSO control the next day for 24 hours. RNA was isolated as described in chapter 3.10. RNA integrity (RIN) was determined by NANO 6000 Assay kit with the Agilent Bioanalyzer 2100 system. RNA samples with RNA integrity > 7 were used for RNA sequencing analysis. The Core Facility Next Generation Sequencing (University of Bonn) performed RNA quality control, library preparation (QuantSeq 3'-mRNA Library Prep) and RNA sequencing using Illumina HiSeq 2500 V4 device at 10M reads per sample. The data are publicly available at Gene Expression Omnibus (GEO) with the accession number GSE282367 ¹³⁵.

3.12 RNAseq data analysis

The Core Facility Bioinformatics (University of Bonn) performed the bioinformatic analysis. For preprocessing and quantification of the reads using default parameters nf-core RNAseq pipeline (version 3.17) was applied ¹³⁶. First quality and adapter trimming were performed with TrimGalore followed by alignment of the trimmed reads against the human genome (GRCh38) with STAR (version 2.7.11b) ¹³⁷. Pseudoalignment of the aligned data to estimate transcript abundances was performed using Salmon (version 1.9.0) ¹³⁸. To obtain gene-level expression estimates the transcript-level quantifications were aggregated. R environment (version 4.2.0) was used to execute statistical analyses ¹³⁹. Only genes with a minimum count of 5 in at least three samples were used for the inference analysis to ensure the robustness of the results. For differential gene expression analysis the Bioconductor package DESeq2 was utilized ^{140,141}. For calculation of multiple testing adjusted p-values (false discovery rate, FDR) for each contrast the Benjamini-Hochberg method was applied. Differential expression data obtained by the Core Facility Bioinformatics were further analyzed using STRING 11.5 database and Enrichr analysis tools ^{142,143}.

3.13 Quantitative RT-PCR

RNA isolation of cells was performed as described in 3.10. For DNase digest 1 µg of RNA were mixed with 1 µl 10x Buffer + MgCl₂, 0.5 µl DNaseI filled up to 10 µl with H₂O. Incubation was performed at 37 °C for 30 minutes. For deactivation of DNaseI 1 µl EDTA was added and incubated for 10 minutes at 75 °C. For cDNA synthesis 1 µl oligo(dT)₁₈ primer as well as 1 µl dNTP mix (10 mM) were added and filled up to 14.5 µl with H₂O. Incubation was performed for 5 minutes at 65 °C. Afterwards, 4 µL 5x RT buffer, 0.5 µl RiboLock RNase inhibitor and 1 µl Maxima H Minus reverse transcriptase were added and incubated for 30 minutes at 65 °C and subsequent at 85 °C for 5 minutes. The cDNA was diluted 1:16 in H₂O and master mix containing 15 µl SYBR Green qPCR Master Mix (2x) as well as 1 µl forward primer (10 mM) and 1 µl reverse primer (10 mM) was prepared. Oligonucleotide sequences are listed in the materials (2.10). 16 µl of diluted cDNA was mixed with 16 µl of master mix and triplicates of 10 µl were pipetted into a 384 well plate. A master mix only (without cDNA) control was used for each primer pair. The housekeeping gene GAPDH was utilized as control. For quantitative real-time polymerase chain reaction (qRT-PCR) the Applied Biosystems ViiA™ 7 Real-Time PCR System was used. The expression fold change $2^{-\Delta\Delta CT}$ was calculated¹⁴⁴.

3.14 Immunofluorescence staining

For immunofluorescence staining cells were seeded in a concentration of 1×10^4 cells/ml in a 24 well plate onto coverslips. Treatment with I-BRD9 (15 µM) or DMSO control was performed the following day for 72 hours. Cells were washed with PBS and fixation was performed with 4 % PFA for 15 minutes. Afterwards cells were washed with PBS for 5 minutes three times. Permeabilization was performed by incubation with 0.3 % Triton-X100 in PBS for 10 minutes. Afterwards three washing steps were applied. For blocking normal horse serum was utilized for 20 minutes followed by three washing steps. Second blocking was performed with 5 % BSA in PBS for 30 minutes. The cells were washed three times. Primary antibody was incubated in 1 % BSA in PBS overnight at 4 °C. All antibodies are listed in the materials (2.9). Next day, three washing steps were performed. Secondary antibody kit (VectaFluor Horse Anti-Rabbit IgG, DyLight™ 594 Antibody Kit, R.T.U. or VectaFluor Horse Anti-Mouse IgG, DyLight 594 Antibody Kit, R.T.U.) was used for 1 hour in the dark. Three washing steps were

performed. Cells were stained with Hoechst Bisbenzimid H 33342 (0.01 mg/ml) diluted 1:1000 in PBS for 10 minutes. The cells were washed three times. Coverslips were mounted with Fluoroshield on slides. For imaging the VisiScope CSU-W1 (Vistron, Puchheim, Germany) of the Core Facility Microscopy (University of Bonn) with the VisiView Software was used.

3.15 Statistical analysis

All independent biological replicates are presented as mean with standard deviation as error bars. Statistical significance was calculated by student's t test. P-values < 0.05 were considered significant and were indicated by asterisk (ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001). Statistical analysis was performed with GraphPad Prism 10.

4 RESULTS

TGCT patients have high 5-year survival rates of up to 95 %. The standard treatment regime is orchiectomy followed by cisplatin-based chemotherapy but unfortunately 15-20 % of the patients are resistant to the standard therapy ^{38,39}. Therefore, investigation of alternative treatment options like epigenetic drugs is very important to address and eventually overcome therapy resistance. Further, new treatment options could offer reduction of side effects.

4.1 BRD9 is expressed in TGCT tissue as well as cell lines

Expression of the target BRD9 in TGCT tissues was analyzed by meta-analysis of previously published Affymetrix expression microarray data in normal testis tissue, GCNIS, seminoma, embryonal carcinoma and teratoma ¹³¹. The analysis revealed the highest expression of BRD9 in embryonal carcinoma while GCNIS displayed the lowest expression. Of note, normal testis tissue also showed high expression of BRD9 (Figure 13A). A tissue microarray comprising GCNIS, seminoma and embryonal carcinoma of 159 patients as well as 5 normal testis tissues was performed and validated by SALL4 staining (Figure S 1). Immunohistochemical staining showed protein level and distribution of BRD9 in the different tissues. BRD9 was upregulated (moderate or high protein level) in 35.2 % of the tumor tissues compared to normal testis tissue. The protein levels of BRD9 showed significant differences between GCNIS, seminoma and embryonal carcinoma ($p=0.001$). The highest BRD9 protein level was found in GCNIS while tumor associated normal testis tissue displayed no or weak protein levels of BRD9 (Figure 13 B,C, Table 1).

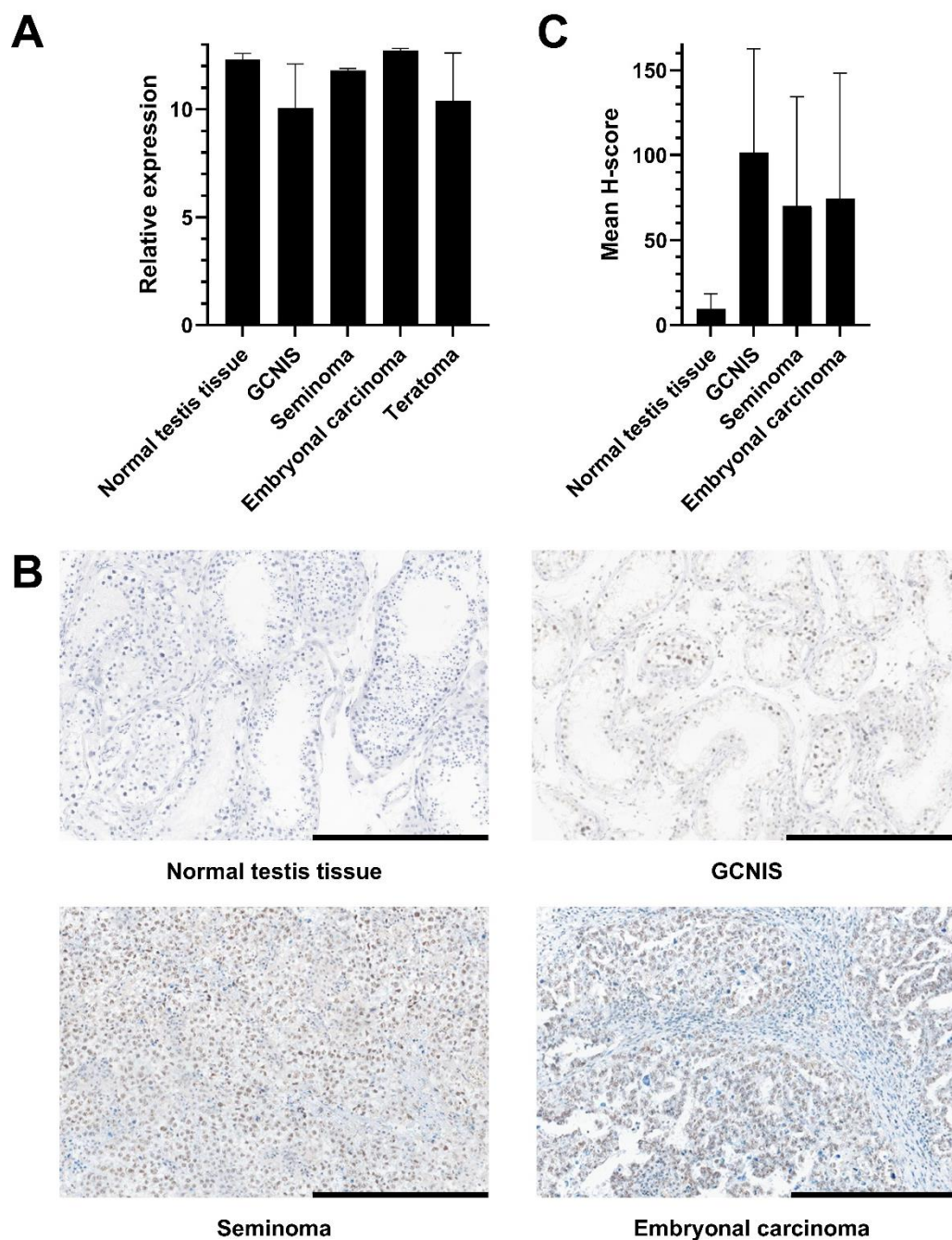


Figure 13: Expression of BRD9 in TGCT tissues. (A) Meta-analysis of Affymetrix expression microarray data in normal testis tissue, GCNIS, seminoma, embryonal carcinoma and teratoma. (B) Representative images of immunohistochemical stainings of BRD9 in normal testis tissue, GCNIS, seminoma and embryonal carcinoma (TMA). Scale bar: 500 μ m. (C) Mean staining intensity (H-score) of BRD9 in normal testis tissue, GCNIS, seminoma and embryonal carcinoma. TMA as well as analysis was performed by Dr. Christine Sanders and Dr. Florian Fronhoffs. GCNIS – germ cell neoplasia in situ. Modified from ¹.

Table 1: Expression of BRD9 in testicular tumor and normal testis tissue. Testing for differences in BRD9 expression between histological subtypes (Pearson-Chi-Square, $p=0.001$). The analysis was performed by Dr. Christine Sanders. SD – standard derivation; GCNIS – germ cell neoplasia in situ. Modified from ¹.

Tissue	Number of patients	Mean H-score [SD]	Visual analysis in categories			
			0 negative	1 weak	2 moderate	3 high
GCNIS	52	101.35 [61.23]	4 (7.7 %)	24 (46.2 %)	20 (38.5 %)	4 (7.7 %)
Seminoma	82	70.08 [64.19]	33 (40.2 %)	28 (34.1 %)	15 (18.3 %)	6 (7.3 %)
Embryonal carcinoma	25	74.27 [74.02]	9 (36 %)	5 (20 %)	10 (40 %)	1 (4 %)
Normal testis tissue	5	9.59 [8.93]	3 (60 %)	2 (40 %)	0	0

For investigation of BRD9 expression in TGCT cell lines meta-analysis of previously published Illumina expression microarray data in embryonal carcinoma (2102 EP and NCCIT), seminoma (TCam-2), choriocarcinoma (JAR) and control cell lines (FS1 and MPAF) was performed ⁹⁴. The TGCT cell lines showed comparable expression levels while the control cell line MPAF (human adult fibroblasts) showed the lowest BRD9 expression (Figure 14 A). For analysis of BRD9 protein level Western Blot was performed. Here, again TGCT cell lines showed similar protein levels while MPAF displayed only a low BRD9 protein level (Figure 14 B).

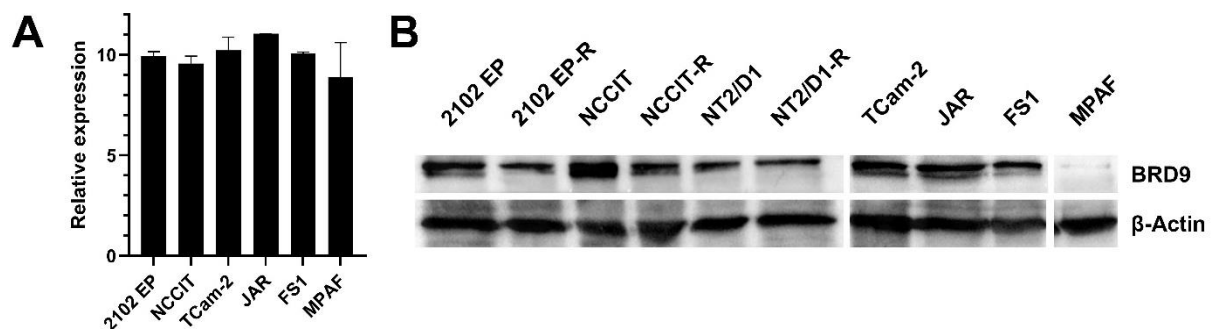


Figure 14: Expression of BRD9 in TGCT cell lines. (A) Meta-analysis of Illumina expression microarray data in 2102 EP, NCCIT, TCam-2, JAR, FS1 and MPAF cells. (B) BRD9 protein level in 2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2, JAR, FS1 and MPAF cells with corresponding load control β -actin detected by Western Blot. Original Western Blot can be found in the appendix (Figure S 2). Modified from ¹.

These data suggest that BRD9 is expressed in TGCT tissues as well as cell lines. In addition, the lowest expression was detected in the control indicating a good starting point for further investigation of targeting BRD9 in TGCT cell lines using the inhibitor I-BRD9.

4.2 Inhibition of BRD9 decreases viability in TGCT cell lines in a dose- and time-dependent manner

The viability of TGCT cell lines (2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2 and JAR) as well as two control cell lines (FS1 and MPAF) was investigated by XTT assay after treatment with I-BRD9. Measurement was performed after 24, 48 and 72 hours of treatment and five different concentrations (2, 10, 15, 20 and 25 μ M) were tested. Initial induction of decreased viability was detected after 24 hours. A clear decrease was visible after 48 hours and even stronger after 72 hours which indicated a time-dependent effect. All TGCT cell lines showed the strongest reduction of viability after treatment with the highest concentration (25 μ M). In NCCIT and NT2/D1 cells also 20 μ M led to a similar decrease in viability as the highest concentration (25 μ M). The cisplatin-resistant cell lines (2102 EP-R, NCCIT-R and NT2/D1-R) showed comparable effects as the parental cell lines (2102 EP, NCCIT and NT2/D1). In total, embryonal carcinoma cell lines and especially NCCIT cells were affected the most (Figure 15). The seminoma cell line TCam-2 displayed the lowest cytotoxic effect compared to the other TGCT cell lines. Choriocarcinoma cells (JAR) also showed a strong decrease of viability in the two highest concentrations (20 and 25 μ M). On the other hand, control cell lines were only slightly affected. Sertoli cells (FS1) showed only a reduction of viability to 70 % in the highest concentration (25 μ M) of I-BRD9 treatment while the fibroblast cell line (MPAF) showed no decrease in viability suggesting only low side-effects on somatic cells (Figure 16).

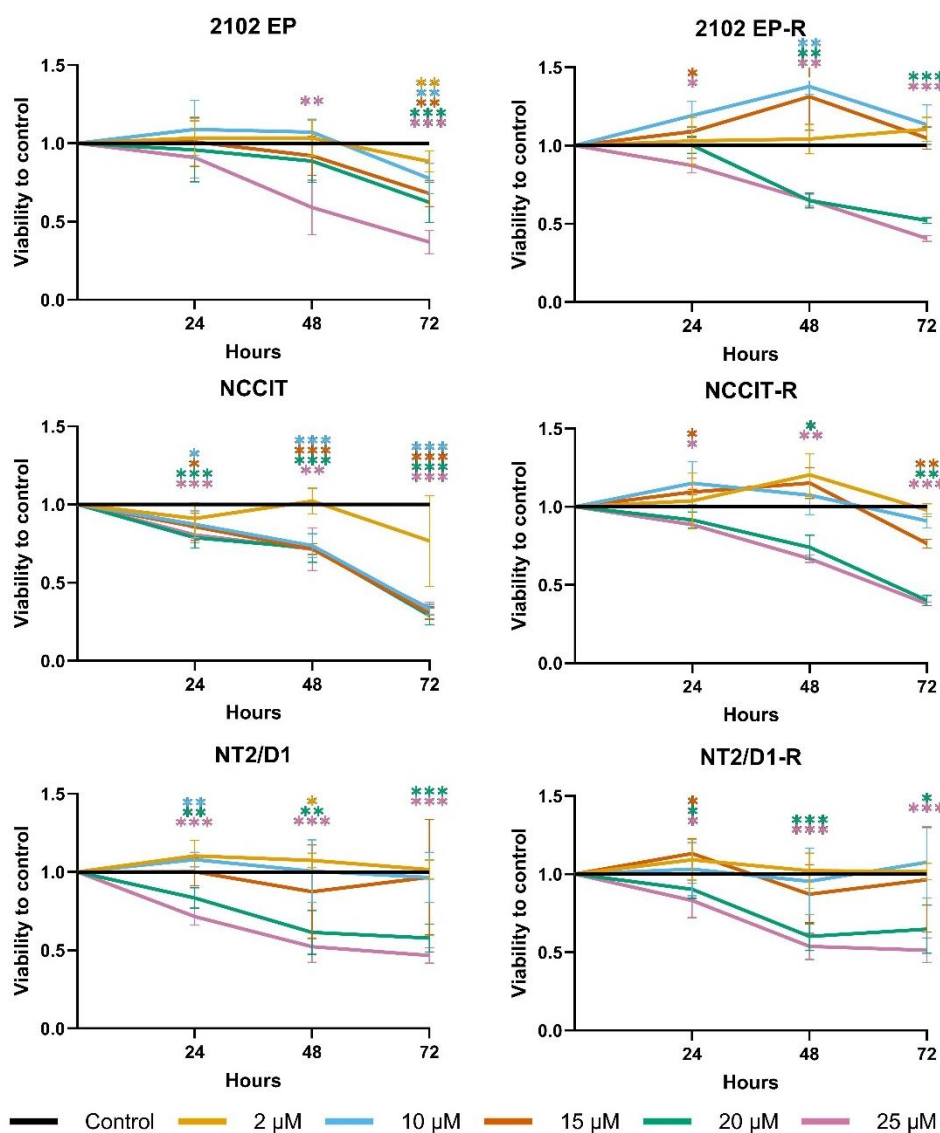


Figure 15: Viability of TGCT cell lines treated with I-BRD9. XTT assay in embryonal carcinoma cell lines as well as cisplatin-resistant subclones (2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1 and NT2/D1-R) was performed at three different time points (24, 48 and 72 hours) after treatment with I-BRD9 at five different concentrations (2, 10, 15, 20 and 25 μ M). The values were normalized to the corresponding DMSO control. Significance between I-BRD9 and DMSO treated control cells is indicated by asterisk (*p < 0.05; **p < 0.01; ***p < 0.001). The colors of asterisks indicate corresponding concentrations. n=3-6. Modified from ¹.

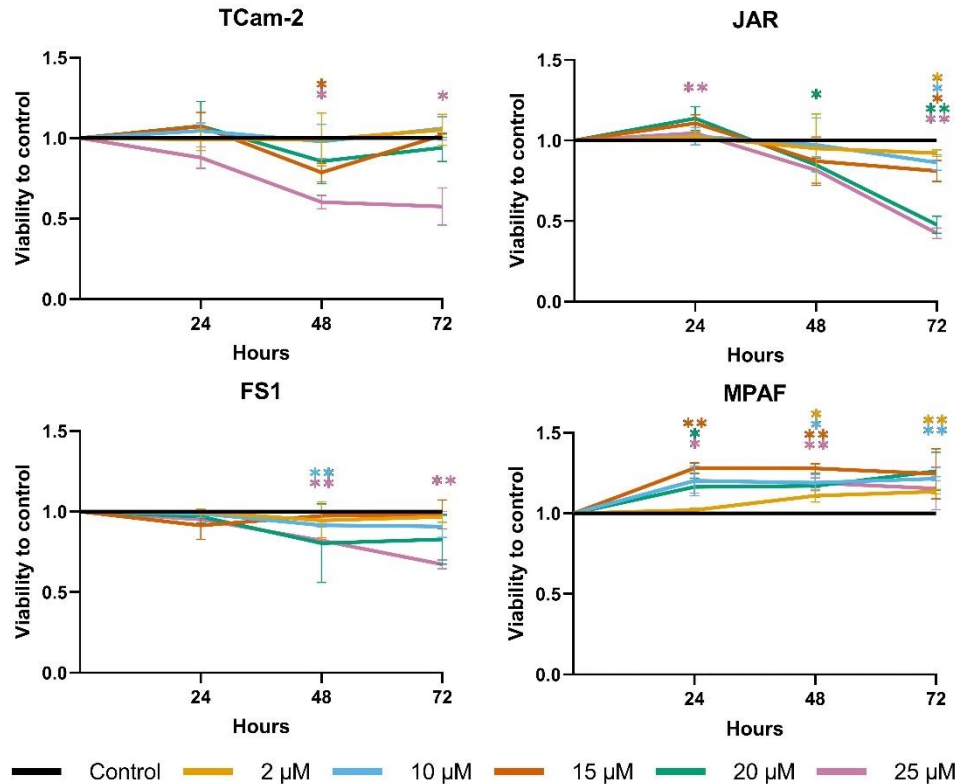


Figure 16: Viability of TGCT cell lines treated with I-BRD9. XTT assay in seminoma (TCam-2), choriocarcinoma (JAR) as well as control cell lines (FS1 and MPAF) was performed at three different time points (24, 48 and 72 hours) after treatment with I-BRD9 at five different concentrations (2, 10, 15, 20 and 25 µM). The values were normalized to the corresponding DMSO control. Significance between I-BRD9 and DMSO treated control cells is indicated by asterisk (*p < 0.05; **p < 0.01; ***p < 0.001). The colors of asterisks indicate corresponding concentrations. n=3-6. Modified from ¹.

For comparison of therapeutic potency IC₅₀ values were calculated using logarithmic regression curves based on XTT assay data (Figure S 3, Figure S 4). The lowest IC₅₀ value and therefore the highest potency of I-BRD9 was determined in NCCIT cells at 6 µM after 72 hours of treatment. The lowest potency of I-BRD9 in TGCT cell lines was found in TCam-2 cells after 72 hours at 29 µM. In total, I-BRD9 showed very high potency in all TGCT cell lines after 72 hours. In 2102 EP, 2102 EP-R, NCCIT-R, NT2/D1 and NT2/D1-R cells the BRD9 inhibitor displayed also very high potency after 48 hours of treatment suggesting a high sensitivity of embryonal carcinomas towards I-BRD9. On the other hand, control cell line FS1 (sertoli cells) revealed low sensitivity after 24 hours (>500 µM) and moderate sensitivity after 48 hours (64 µM). After 72 hours I-BRD9 also showed high potency at 34 µM in FS1 cells but the TGCT cell lines were all already sensitive to I-BRD9 in lower concentrations (6-29 µM). Of note, in

human adult fibroblasts (MPAF) the BRD9 inhibitor showed only low potency at all time points (Table 2).

Table 2: IC₅₀ values of TGCT cell lines and control cell lines. IC₅₀ values [μM] were calculated based on XTT assays in 2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2, JAR, FS1 and MPAF cells after treatment with I-BRD9 at three different time points (24, 48 and 72 hours). The values were grouped according to very high potency (green, 0-30 μM), high potency (orange, 31-50 μM), moderate potency (red, 51-500 μM) and low potency (white, >500 μM). Modified from ¹.

	24 h	48 h	72 h
2102 EP	201	30	21
2102 EP-R	85	28	23
NCCIT	249	80	6
NCCIT-R	60	28	20
NT2/D1	37	25	23
NT2/D1-R	44	27	26
TCam-2	78	32	29
JAR	>500	170	21
FS1	>500	64	34
MPAF	>500	>500	>500

Taken together, viability assays showed a cytotoxic effect in all TGCT cell lines while control cell lines remained only slightly affected. Embryonal carcinoma cells showed the strongest effect after I-BRD9 treatment. The data indicate a time- and dose-dependent effect in all cell lines. IC₅₀ values also displayed very high potency to I-BRD9 after 72 hours and in some TGCT cell lines already after 48 hours. On the other hand, control cell lines showed lower potency compared to TGCT cell lines.

4.3 I-BRD9 led to induction of apoptosis and G1-phase cell cycle arrest in TGCT cell lines

To investigate the effect of I-BRD9 on apoptosis DAPI/ AnnexinV-based fluorescence activated cell sorting (FACS) was performed after 24 and 48 hours of treatment. TGCT cell lines (2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2 and

JAR) and two control cell lines (FS1 and MPAF) were treated with 15 μ M I-BRD9 or the solvent control DMSO. In 2102 EP, 2102 EP-R, NT2/D1-R and TCam-2 cells the highest induction of apoptosis was detectable already after 24 hours of treatment with I-BRD9 compared to the DMSO control. In NCCIT, NCCIT-R, NT2/D1 and JAR cells an increase of apoptotic cells was visible after 24 hours but even stronger after 48 hours of treatment. The highest accumulation of apoptotic cells was detected in embryonal carcinoma cells (NCCIT-R) after 48 hours. In contrast, the control cell lines FS1 and MPAF showed no significant differences between I-BRD9 and DMSO treated cells (Figure 17).

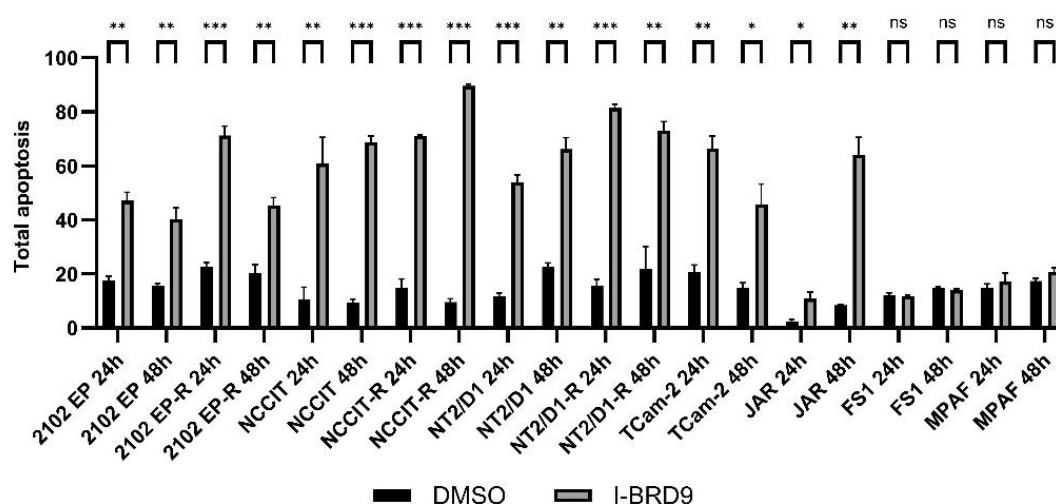


Figure 17: BRD9 inhibition induced apoptosis in TGCT cell lines. DAPI/ AnnexinV-based FACS analysis was performed in 2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2, JAR, FS1 and MPAF cells after 24 and 48 hours of treatment with I-BRD9 (15 μ M) or solvent control DMSO. Asterisks indicate significant differences between treated cells and control group (ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001). n=3. Modified from ¹.

Cell cycle analysis was performed by Hoechst staining in 2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2, JAR and FS1 cells treated with I-BRD9 (15 μ M) or DMSO control. FACS analysis was performed after 24 and 48 hours. Induction of G1-phase cell cycle arrest was visible in all TGCT tumor cell lines after 24 hours of treatment with I-BRD9 in comparison to the DMSO control. In 2102 EP, 2102 EP-R and NT2/D1 cells an even stronger increase was visible after 48 hours. The highest accumulation of cells in G1-phase was detected in embryonal carcinoma cells (NCCIT and NT2/D1-R). The embryonal cell line 2102 EP showed the lowest effect on cell cycle

distribution after treatment with I-BRD9. The seminoma cell line TCam-2 as well as choriocarcinoma cells (JAR) displayed also strong induction after 24 hours of BRD9 inhibition. In contrast, control cell line FS1 (sertoli cells) showed no increase of cells in G1-phase (Figure 18).

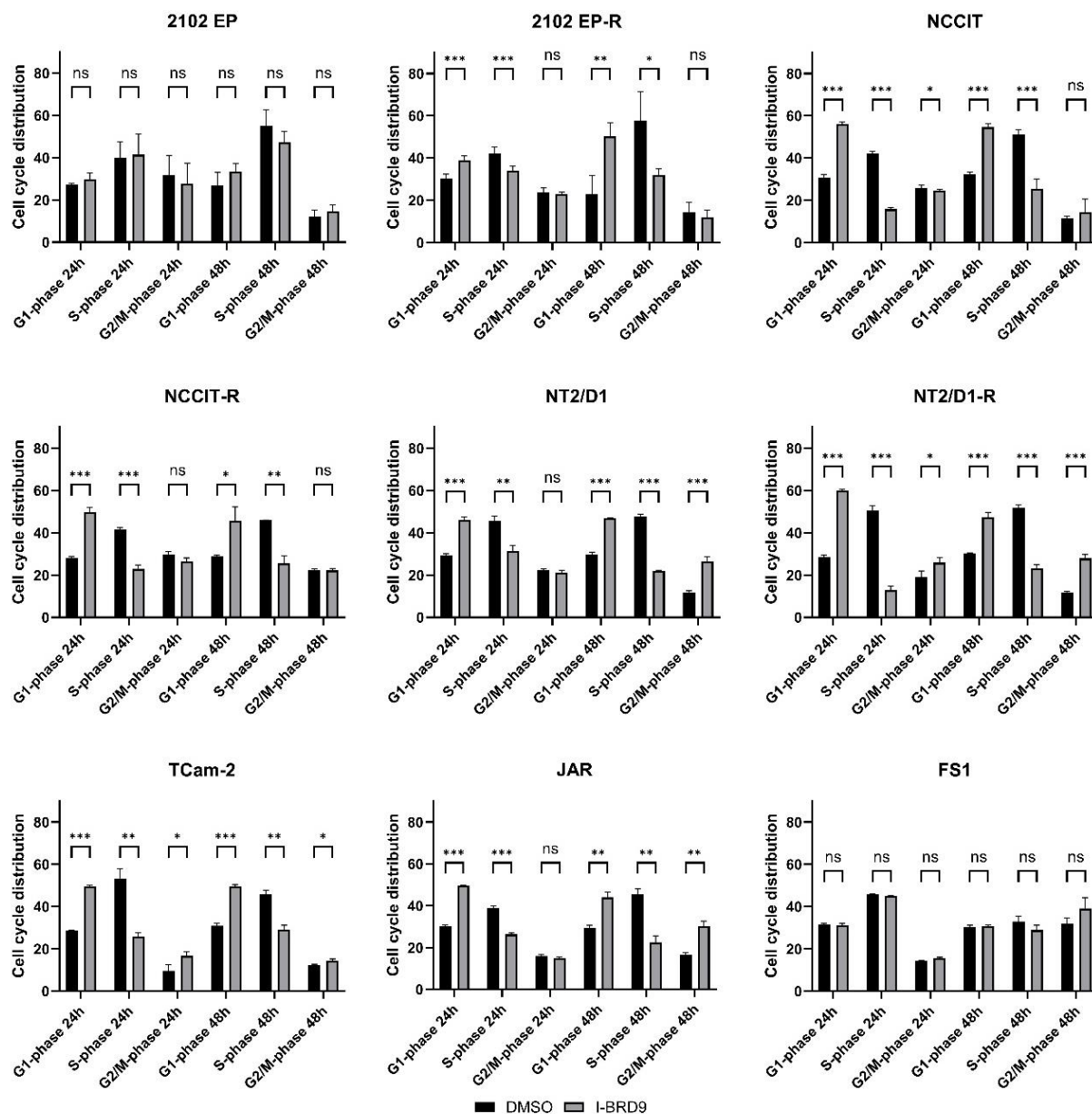


Figure 18: I-BRD9 induced G1-phase cell cycle arrest in TGCT cell lines. Staining of Hoechst in 2102 EP, 2102 EP-R, NCCIT, NCCIT-R, NT2/D1, NT2/D1-R, TCam-2, JAR and FS1 cells was analyzed via FACS after 24 and 48 hours of treatment. Cells were treated with I-BRD9 (15 μ M) or corresponding DMSO control. Asterisks indicate significant change between I-BRD9 treated and control cells (ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001). n=3-9. Modified from ¹.

BRD9 inhibition induced apoptosis and G1-phase cell cycle arrest in TGCT cell lines already after 24 hours of treatment with I-BRD9 in comparison to DMSO treated control cells. Embryonal carcinoma cells showed the strongest effect after BRD9 inhibition. On the other hand, I-BRD9 had no significant effect on control cells in apoptosis or cell cycle distribution analysis.

4.4 BRD9 inhibition in embryonal carcinoma and seminoma induces differentiation to an epithelial cell fate and loss of pluripotency

The impact of I-BRD9 on gene expression was analyzed by 3'mRNA sequencing after 24 hours of BRD9 inhibition in comparison to DMSO treated cells. The time point (24 hours) was chosen to analyze early gene expression changes before effects were observed in viability assays (48 and 72 hours). To get an overview of the different TGCT entities RNAseq was performed in embryonal carcinoma (2102 EP), seminoma (TCam-2) and control cells (MPAF).

In total, 2102 EP cells displayed 5536 significantly deregulated genes. 983 genes with a $\log_2$ foldchange higher than 1 were upregulated and 983 genes with a $\log_2$ foldchange lower than -1 were downregulated (Figure 19). In TCam-2 cells 753 genes with a $\log_2$ foldchange higher than 1 showed upregulation while 898 genes with a $\log_2$ foldchange lower than -1 were downregulated. In sum, 4833 genes were significantly deregulated after 24 hours of I-BRD9 treatment compared to the DMSO control (Figure 20). Control cells MPAF showed the lowest amount of significantly deregulated genes (4781). 1179 genes with a $\log_2$ foldchange lower than -1 displayed downregulation and 695 genes with a $\log_2$ foldchange higher than 1 were upregulated after 24 hours of BRD9 inhibition in comparison to DMSO treated cells (Figure 21).

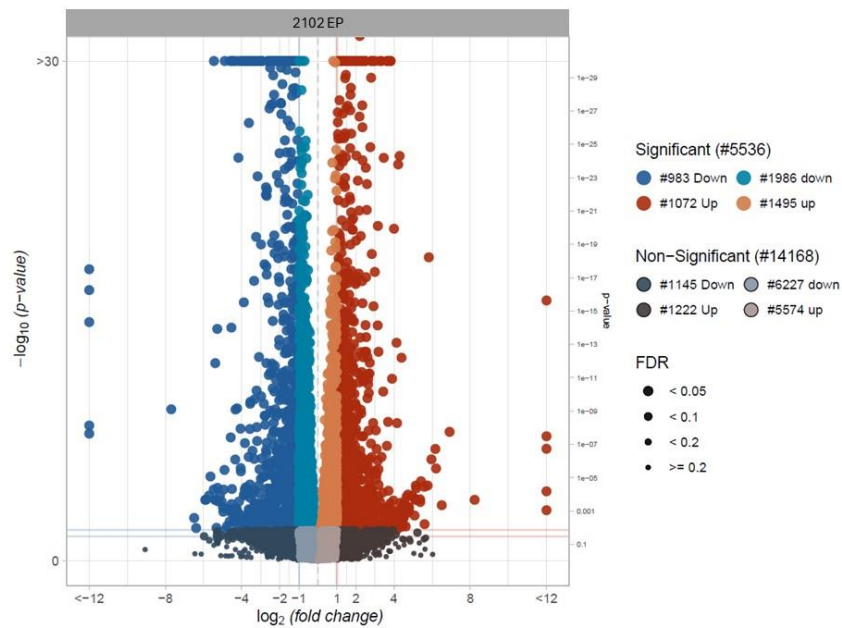


Figure 19: Differentially expressed genes in 2102 EP cells. Volcano plot displaying differentially expressed genes after 24 hours of treatment with I-BRD9 in comparison to DMSO control. Significantly upregulated genes are depicted in red ($\log_2$ foldchange >1) or orange ($\log_2$ foldchange between 0 and 1) and significantly downregulated genes are highlighted in dark ($\log_2$ foldchange <-1) or light blue ($\log_2$ foldchange between 0 and -1) (adjusted p-value < 0.05). FDR – false discovery rate. Modified from ¹.

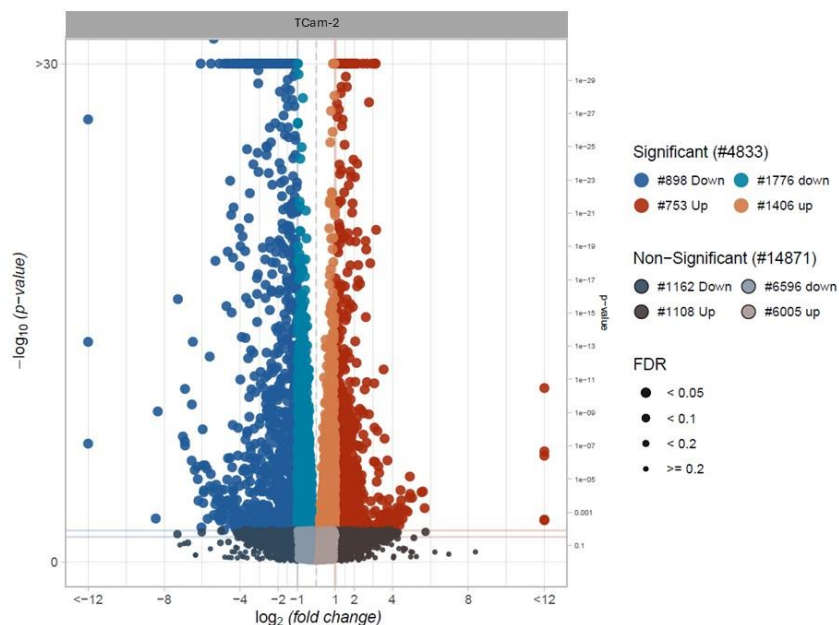


Figure 20: Differentially expressed genes in TCam-2 cells. Volcano plot displaying differentially expressed genes after 24 hours of treatment with I-BRD9 in comparison to DMSO control. Significantly upregulated genes are depicted in red ($\log_2$ foldchange >1) or orange ($\log_2$ foldchange between 0 and 1) and significantly downregulated genes are highlighted in

dark ($\log_2$ foldchange <-1) or light blue ($\log_2$ foldchange between 0 and -1) (adjusted p-value < 0.05). FDR – false discovery rate. Modified from ¹.

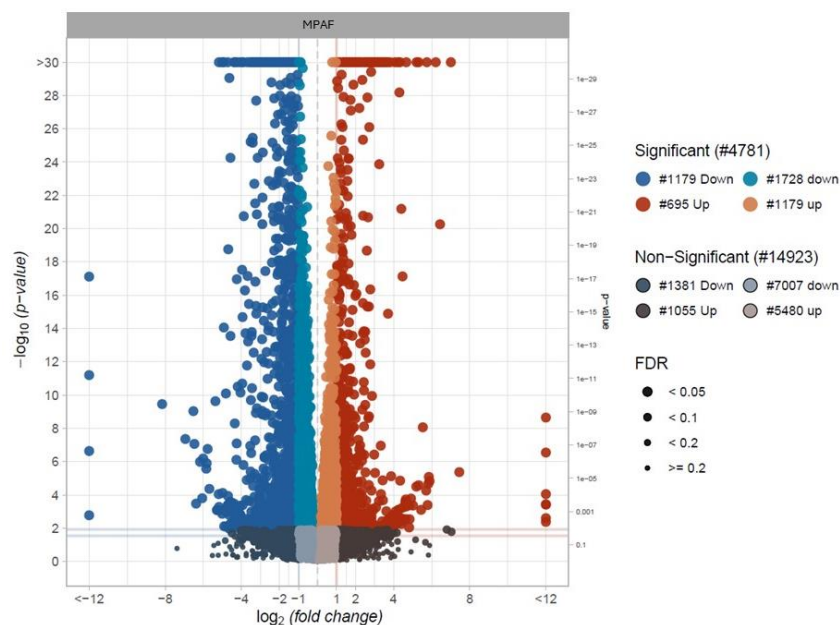


Figure 21: Differentially expressed genes in MPAF cells. Volcano plot displaying differentially expressed genes after 24 hours of treatment with I-BRD9 in comparison to DMSO control. Significantly upregulated genes are depicted in red ($\log_2$ foldchange >1) or orange ($\log_2$ foldchange between 0 and 1) and significantly downregulated genes are highlighted in dark ($\log_2$ foldchange <-1) or light blue ($\log_2$ foldchange between 0 and -1) (adjusted p-value < 0.05). FDR – false discovery rate.

First, enrichment analysis of significantly upregulated genes was performed. In 2102 EP (embryonal carcinoma) as well as TCam-2 (seminoma) cells the GO term “epithelium development” (GO:0060429) was among the TOP3 with deregulation of 197 genes in 2102 EP and 176 genes in TCam-2 cells (Figure 22 A, B, Figure S 5, Figure S 6). In control cells MPAF GO terms associated with metabolic processes like “organic acid metabolic process” (GO:0006082, 148 genes), “oxoacid metabolic process” (GO:0043436, 147 genes) and “cellular lipid metabolic process” (GO:0044255, 145 genes) were enriched (Figure 22 C). Of note, enrichment of epithelium development was not found in MPAF cells after BRD9 inhibition suggesting that this is a unique response of TGCT cell lines.

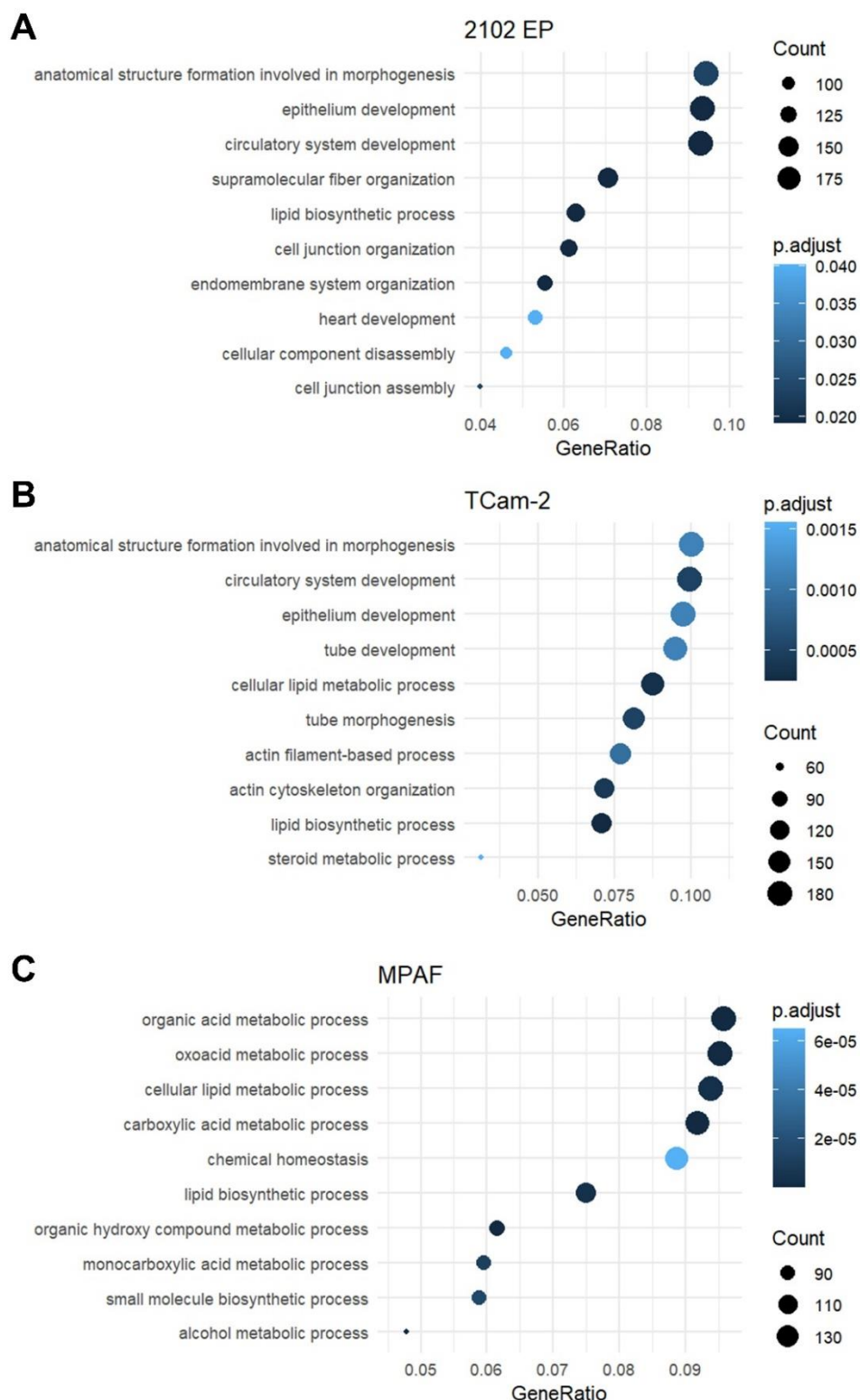


Figure 22: Epithelium development is enriched in 2102 EP and TCam-2 cells while metabolic processes are upregulated in MPAF cells. Dot plot of enrichment analysis of TOP 10 enriched biological processes based on all upregulated genes after 24 hours of I-BRD9 treatment compared to the DMSO control in (A) 2102 EP, (B) TCam-2 and (C) MPAF cells. Dot size displays the gene count associated with the GO term. Modified from ¹.

For validation in 2102 EP and TCam-2 cells different genes involved in epithelium development were selected based on the $\log_2$ foldchange ($\log_2$ foldchange >2). HSF4 was upregulated in both cell lines while SEMA3C, DLX6 and LEF1 were upregulated in 2102 EP cells and GATA5 as well as HEYL were enriched in TCam-2 cells. For 2102 EP cells SEMA3C and for TCam-2 cells GATA5 showed the strongest upregulation while HSF4 for 2102 EP and HEYL for TCam-2 cells showed the lowest $\log_2$ foldchange (Figure 23 A). Validation was performed by qRT-PCR of the selected genes after 24 hours of treatment with I-BRD9 compared to DMSO treated cells. In 2102 EP cells, all four genes (HSF4, SEMA3C, DLX6 and LEF1) showed upregulation compared to the control. In line with the $\log_2$ foldchanges in the RNAseq, SEMA3C showed the strongest upregulation while HSF4 was only slightly upregulated. TCam-2 cells also displayed upregulation of all three genes (HSF4, GATA5 and HEYL). HEYL showed the lowest upregulation while GATA5 was affected the most as already visible in the $\log_2$ foldchanges (Figure 23 B). These data validate the upregulation of genes involved in epithelium development in 2102 EP and TCam-2 cells and suggest that differentiation of the TGCT cells was initiated.

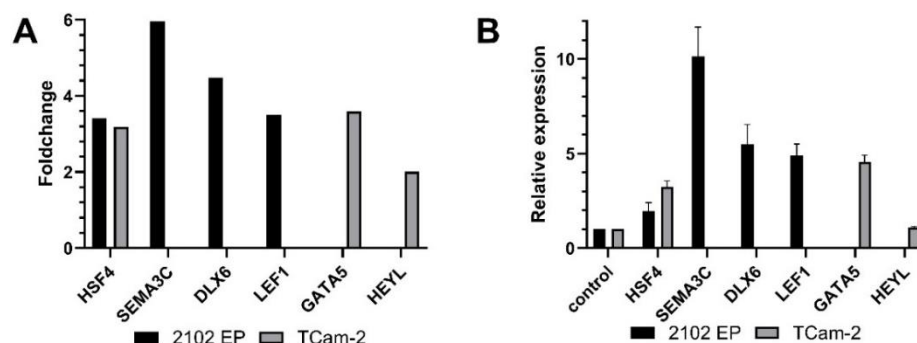


Figure 23: Genes associated with epithelium development were upregulated in 2102 EP and TCam-2 cells. (A) The $\log_2$ foldchange of chosen validation genes in 2102 EP and TCam-2 cells after 24 hours of treatment with I-BRD9 in comparison to the DMSO control. (B) Relative expression of validation genes in 2102 EP and TCam-2 cells after BRD9 inhibition for 24 hours compared to DMSO treated control cells analyzed by qRT-PCR. Values were normalized to the housekeeping gene GAPDH. n=3. Modified from ¹.

Next, pathway analysis was performed for downregulated genes after 24 hours of treatment with I-BRD9 in comparison to DMSO treated control cells. In embryonal carcinoma (2102 EP) and seminoma (TCam-2) cells “signaling pathways regulating pluripotency of stem cells” were mostly affected (Figure 24 A, B). In addition, the

STRING interaction networks of genes associated with the GO term “stem cell population maintenance” (GO:0019827) in 2102 EP and TCam-2 cells were overlapping including pluripotency markers like NANOG, PRDM14 and KLF4 (Figure 24 D, E). In MPAF cells, pathway analysis revealed an enriched “p53 signaling pathway” while “signaling pathways regulating pluripotency of stem cells” were as expected not affected (Figure 24 C).

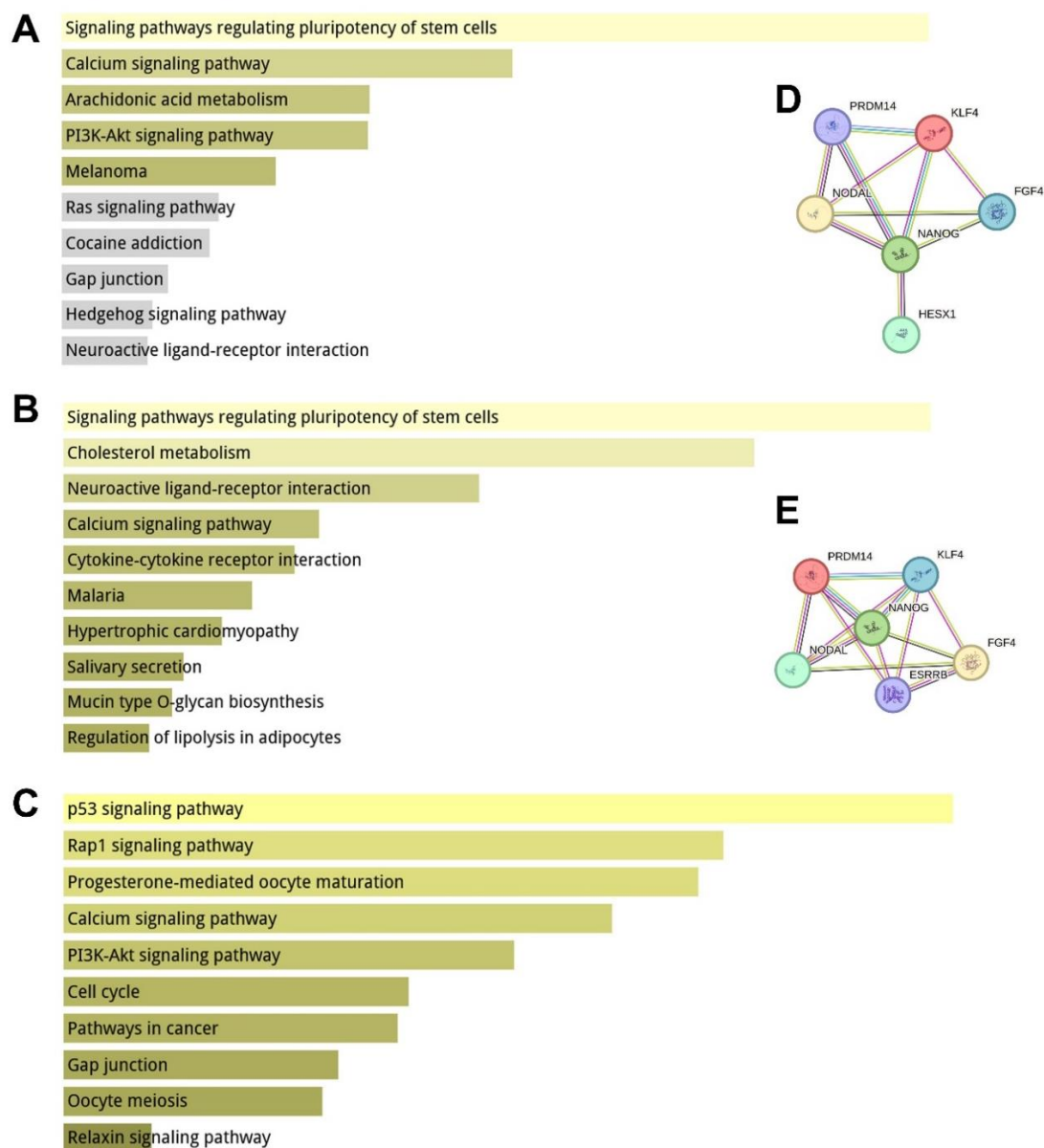


Figure 24: Downregulation of genes associated with stem cell population maintenance in 2102 EP and TCam-2 cells. Pathway analysis of downregulated genes in (A) 2102 EP, (B) TCam-2 and (C) MPAF cells after 24 hours of treatment with I-BRD9 compared to DMSO control analyzed with Enrichr. STRING interaction network of genes associated with stem cell population maintenance in (D) 2102 EP and (E) TCam-2 cells. Modified from ¹.

Downregulation of pluripotency markers was validated by qRT-PCR after 24 hours of BRD9 inhibition compared to the DMSO control. Five genes indicative of pluripotency were selected based on the $\log_2$ foldchange ($\log_2$ foldchange < -2). NANOG, NODAL, KLF4, PRDM14 and FGF4 were all downregulated in both cell lines after 24 hours of I-BRD9 treatment. In 2102 EP cells, FGF4 showed the strongest downregulation while NODAL was the least affected. FGF4 was also the most affected gene in TCam-2 cells and KLF4 displayed the lowest downregulation (Figure 25 A). Relative mRNA levels were detected by qRT-PCR and showed downregulation for all genes in both cell lines. NANOG and FGF4 displayed the strongest downregulation while KLF4 was the least affected (Figure 25 B).

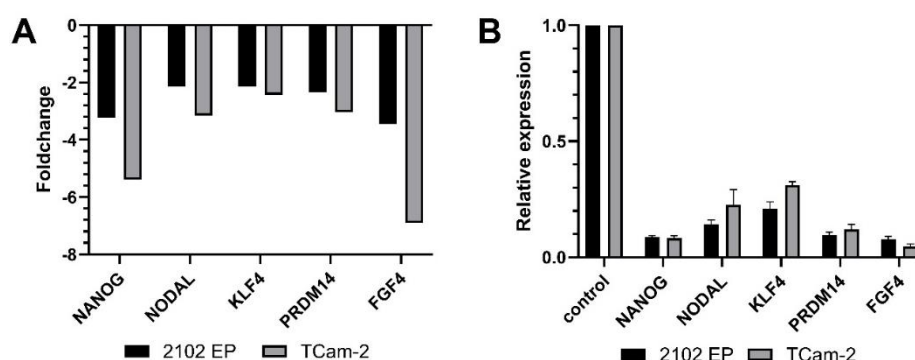


Figure 25: Stem cell population maintenance associated genes were downregulated in 2102 EP and TCam-2 cells. (A) The $\log_2$ foldchange of chosen validation genes in 2102 EP and TCam-2 cells after BRD9 inhibition for 24 hours compared to DMSO treated cells. (B) Relative expression of validation genes in 2102 EP and TCam-2 cells after treatment with I-BRD9 for 24 hours in comparison with DMSO control analyzed by qRT-PCR. Normalization to the housekeeping gene GAPDH was performed. $n=3$. Modified from ¹.

Validation of downregulation of pluripotency markers was also performed on protein level by immunofluorescence staining. 2102 EP and TCam-2 cells were treated for 72 hours with I-BRD9 or DMSO and stainings against NANOG and PRDM14 were performed. For NANOG as well as PRDM14 a strong signal was detected in DMSO treated control cells while signal was absent after BRD9 inhibition in both cell lines (Figure 26, Figure 27).

Validation was successful on RNA and protein level for pluripotency markers in 2102 EP and TCam-2 cells.

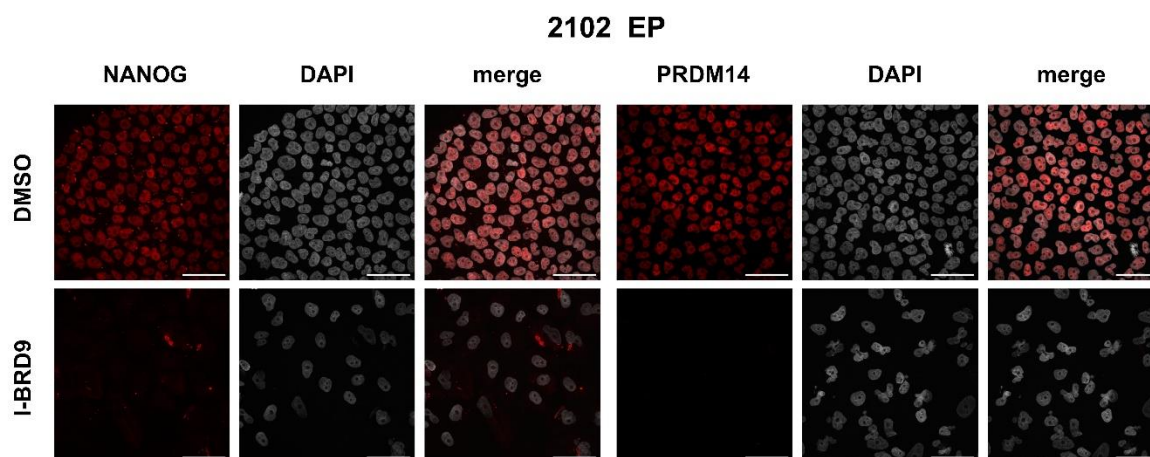


Figure 26: NANOG and PRDM14 were downregulated in 2102 EP cells on protein level. Immunofluorescence staining of NANOG (left panel) and PRDM14 (right panel) after 72 hours of BRD9 inhibition (lower panel) or DMSO control (upper panel) in 2102 EP cells. Target proteins (NANOG and PRDM14) were visualized in red while cell nuclei were depicted in grey. Nuclei were stained with DAPI. Scale bar: 50 μ M. Modified from ¹.

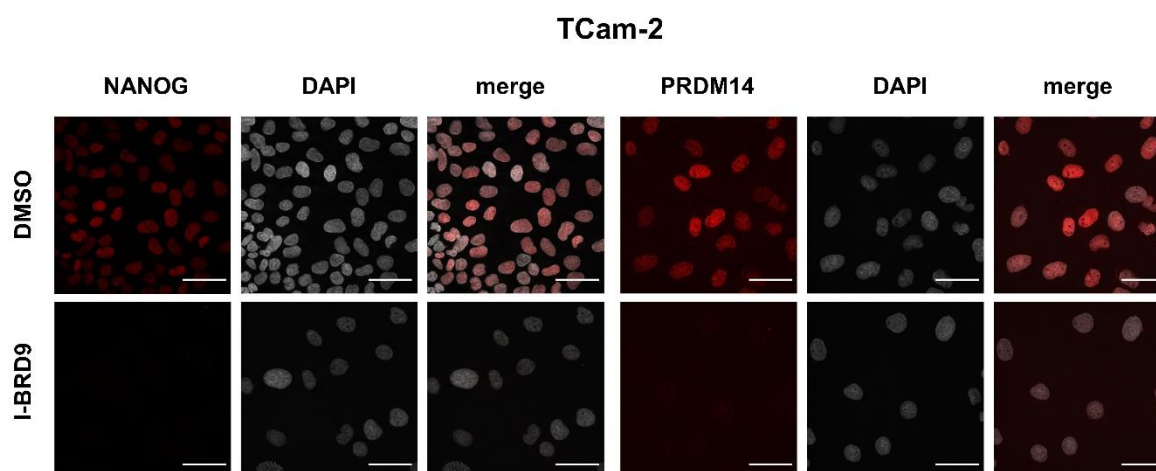


Figure 27: NANOG and PRDM14 were downregulated in TCam-2 cells on protein level. Immunofluorescence staining of NANOG (left panel) and PRDM14 (right panel) after 72 hours of BRD9 inhibition (lower panel) or DMSO control (upper panel) in TCam-2 cells. Target proteins (NANOG and PRDM14) were visualized in red while cell nuclei were depicted in grey. Nuclei were stained with DAPI. Scale bar: 50 μ M. Modified from ¹.

In summary, BRD9 inhibition led to induction of epithelial differentiation while pluripotency markers were downregulated in embryonal (2102 EP) and seminoma (TCam-2) cells. On the other hand, control cells (MPAF) showed upregulation of metabolic processes and downregulation of p53 signaling pathways.

4.5 Confirmation of induction of apoptosis and cell cycle arrest on transcriptome level

FACS analysis showed induction of apoptosis and cell cycle arrest after I-BRD9 treatment in TGCT cell lines compared to DMSO treated control cells (4.3). Here, the effect of BRD9 inhibition on the transcriptome level in context of deregulations in genes important for apoptosis and cell cycle was confirmed.

RNAseq analysis showed upregulation of genes involved in “apoptotic process” (GO term: 0006915) after 24 hours of I-BRD9 treatment. In embryonal carcinoma cells (2102 EP) a total of 38 genes associated with apoptotic processes were upregulated while in seminoma cells (TCam-2) 15 genes showed upregulation confirming induction of apoptosis by I-BRD9 treatment. TNF is the most upregulated gene in both 2102 EP ($\log_2$ foldchange: 4.8) and TCam-2 ($\log_2$ foldchange: 3.6) (Figure 28 A, B). On the other hand, control cells (MPAF) also showed upregulation of 18 genes involved in apoptotic processes (Figure 28 C). This contrasts with the FACS analysis where MPAF cells displayed no significant changes in apoptotic cells after 24 or 48 hours of treatment with I-BRD9 (4.3).

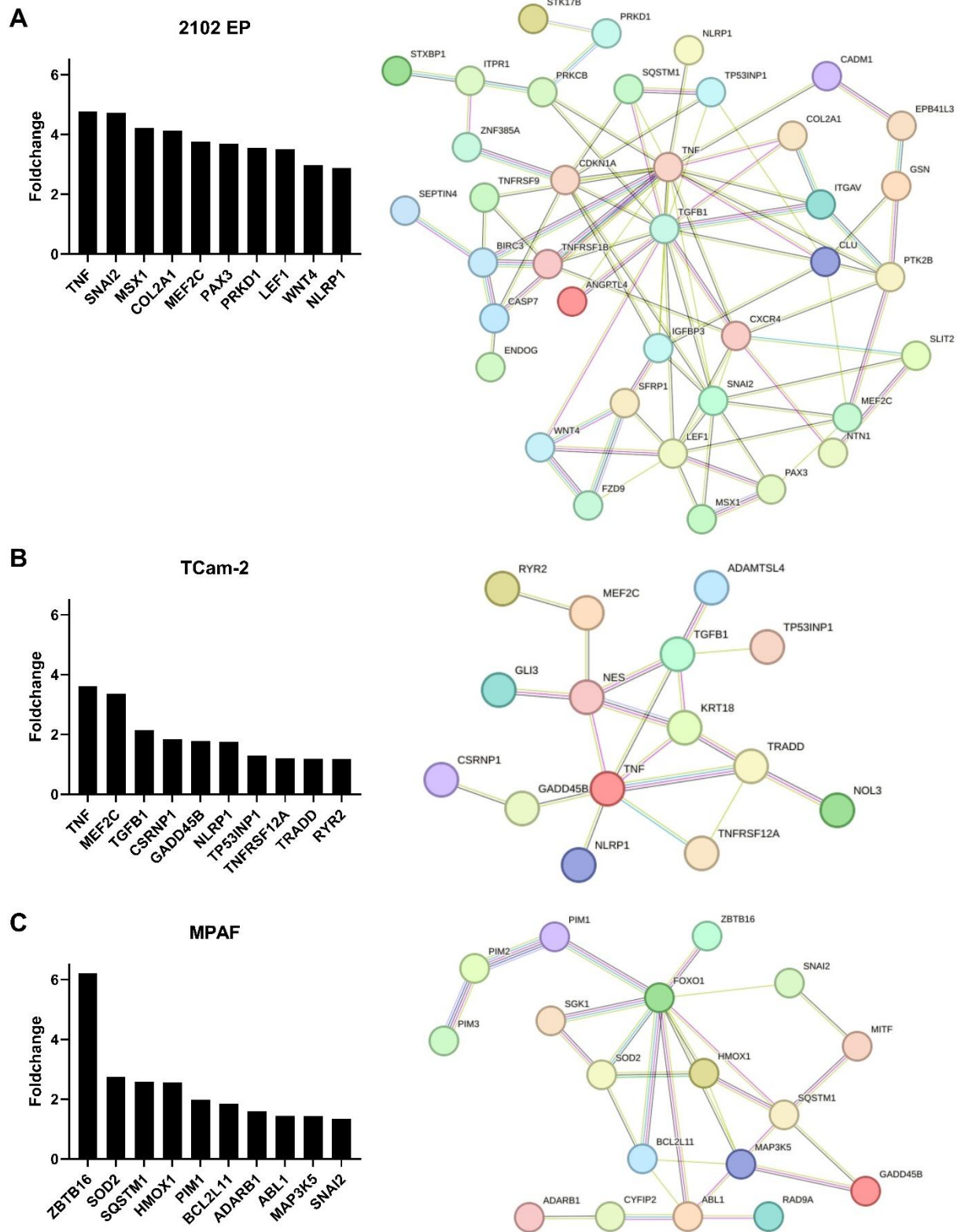


Figure 28: Transcriptome analysis confirmed induction of apoptosis. RNAseq analysis was performed in 2102 EP, TCam-2 and MPAF cells after 24 hours of I-BRD9 treatment. STRING interaction network of genes involved in the GO term “apoptotic process” as well as log₂ foldchanges of TOP10 upregulated genes for (A) 2102 EP, (B) TCam-2 and (C) MPAF cells. n=3.

In embryonal carcinoma cells (2102 EP) 11 genes associated with “cell cycle process” (GO term: 0022402) were downregulated while seminoma cells (TCam-2) displayed downregulation of 23 genes. CHEK2 was downregulated in both 2102 EP ($\log_2$ foldchange: 1.3) and TCam-2 ($\log_2$ foldchange: 1.3) (Figure 29). This is in line with the findings of FACS analysis revealing induction of cell cycle arrest after 24 hours of I-BRD9 treatment (4.3).

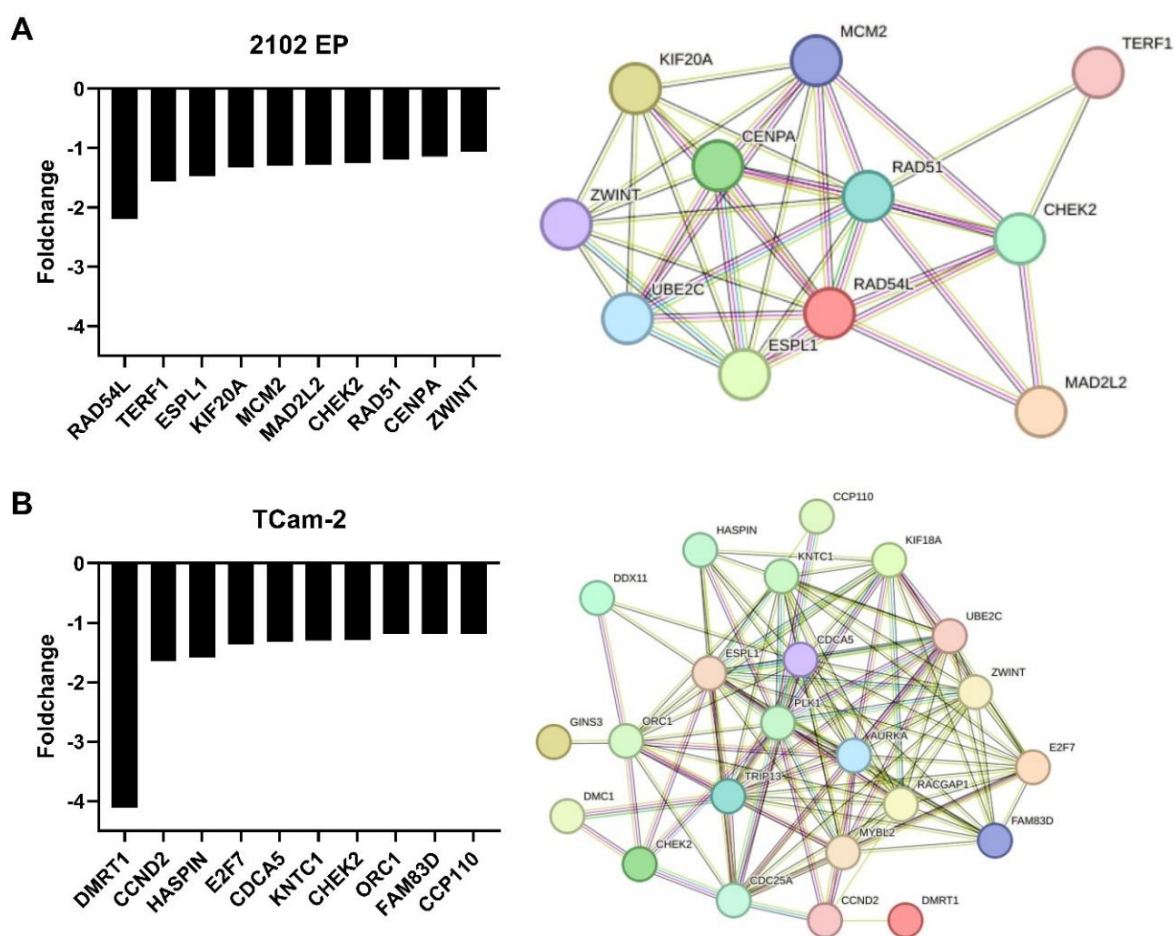


Figure 29: Transcriptome analysis confirmed induction of cell cycle arrest. RNAseq analysis was performed in 2102 EP, TCam-2 and MPAF cells after 24 hours of I-BRD9 treatment. STRING interaction network of downregulated genes involved in cell cycle processes and $\log_2$ foldchanges of TOP10 downregulated genes in (A) 2102 EP and (B) TCam-2 cells. n=3.

Induction of apoptosis in 2102 EP and TCam-2 cells was confirmed on RNA level by the upregulation of genes associated with apoptotic processes. In contrast, also MPAF cells showing no induction of apoptosis in FACS analysis displayed upregulation of

genes involved in apoptotic processes. Cell cycle distributions found in 2102 EP and TCam-2 cell by FACS analysis were validated on RNA level by downregulation of genes involved in cell cycle processes.

5 DISCUSSION

This project showed that the inhibition of BRD9 with the inhibitor I-BRD9 might be an effective treatment alternative for TGCTs. Application of I-BRD9 resulted in severe effects like decrease of viability, induction of apoptosis as well as G1-phase cell cycle arrest in TGCT cell lines while the control cells remained only slightly affected. Transcriptome analysis revealed loss of the pluripotency state along with differentiation towards an epithelial cell fate.

First, the expression of the target BRD9 was analyzed. Meta-analysis of microarray data in TGCT tissues revealed heterogeneous expression with the highest expression in embryonal carcinoma while the lowest expression was found in GCNIS. Normal testis tissue showed also high expression of BRD9 on RNA level. On the other hand, a TMA displayed the highest BRD9 protein levels in GCNIS while normal testis tissue revealed the lowest expression. In TGCT cell lines meta-analysis of microarray data showed expression of BRD9 in all TGCT cell lines while the control cell line MPAF displayed the lowest expression. On protein level a comparable BRD9 abundance was visible compared to RNA level. BRD9 expression in TGCTs on protein level along with low abundance in controls indicated a good starting point for targeting BRD9 in TGCT cell lines. Viability assays after treatment with I-BRD9 displayed cytotoxic effects in a dose- and time-dependent manner in all TGCT cell lines. The strongest cytotoxicity was detected in the embryonal carcinoma cell line NCCIT while the sertoli cells (FS1) were only slightly affected and the fibroblasts (MPAF) showed no effect by the I-BRD9 treatment. In line, the IC_{50} values showed the highest sensitivity for I-BRD9 in NCCIT cells (6 μ M) while MPAF cells revealed IC_{50} values above 500 μ M. FACS analysis showed induction of apoptosis already after 24 hours of treatment with I-BRD9 in all TGCT cell lines and in most cell lines an even stronger induction after 48 hours. The control cells (FS1 and MPAF) displayed no significant increase in apoptosis. Again, an embryonal carcinoma cell line (NCCIT-R) showed the strongest effect. Cell cycle distribution analysis via FACS revealed G1-phase cell cycle arrest already 24 hours after BRD9 inhibition in all TGCT cell lines. While the strongest arrest was visible in embryonal carcinoma cells (NCCIT and NT2/D1-R) the sertoli cells (FS1) showed no induction of cell cycle arrest. Transcriptome analysis in embryonal carcinoma (2102 EP) and seminoma (TCam-2) revealed downregulation of a prominent network of pluripotency markers along with upregulation of genes involved in epithelium

development. Of note, these changes on transcriptome level were not detected in the fibroblasts (MPAF).

In our experiments the embryonal carcinoma cell lines showed the strongest effects after BRD9 inhibition indicating I-BRD9 as a possible alternative treatment option especially for embryonal carcinomas. In line, the BRD4 inhibitor JQ1 also displayed stronger impact on apoptosis in embryonal carcinoma cells compared to seminomas⁹⁵.

I-BRD9 application showed no significant differences between parental and cisplatin-resistant subclones in our analyses. The role of BRD9 in cisplatin resistance is still elusive. In locally advanced cervical cancer (LACC) methylation of the BRD9 promoter region and therefore low expression levels of BRD9 led to higher sensitivity to cisplatin-radiotherapy¹⁴⁵. Furthermore, in BRD9 overexpressing gastric cancer cells BRD9 inhibitors enhanced the sensitivity to cisplatin¹⁴⁶. Zhou et al. found that in ovarian cancer cells I-BRD9 treatment reduced homologous recombination (HR) and re-sensitized the cells to cisplatin. Three BRD-containing proteins (BRD9, ZMYND8 and ASH1L) comprise a high HR signature and positively influence HR. BRD9 especially plays an important role in homologous recombination by orchestrating RAD54-RAD51 interaction. BRD9 is recruited by RAD51 leading to the binding of BRD9 to K515 acetylation on RAD54 which is induced by DNA damage. Therefore, BRD9 enhances the interaction of RAD54 and RAD51. Upon binding, RAD51 forms filaments on single-strand DNA leading to the search for homologous sequences on the intact DNA strand which are used as a model for error-free repair. This leads to the conclusion that BRD9 might be a possible treatment target for HR-proficient tumors¹⁴⁷. Deficiencies in homologous recombination mechanisms (HRR) are known to be involved in cisplatin³⁹. For example, in ovarian and breast cancer BRCA1 and BRCA2 which code for proteins of the HRR machinery comprise mutations. BRCA2 has a key function in HR while BRCA1 is also involved in cell cycle-checkpoint control, chromatin remodeling and DNA repair indicating its role in carcinogenesis. BRCA1 and BRCA2 together with RAD51 induce HR and double-strand break repair. Cells that express mutated BRCA1 and BRCA2 are more sensitive to ionizing radiation and the following repair is error-prone⁵³. Not only BRCA1, BRCA2 and RAD51, but also other genes (BRAD1, BRIP1, ATM, NBS1, CHEK2 and PALB2) of the HR pathway are known to be involved in inherited susceptibility for different cancer types. The first step of HR is the sensing of

double-strand breaks. Here, NBS1 is necessary to recruit ATM to the damaged site leading to induction of checkpoint activation. ATM in combination with CHEK2 initiates phosphorylation of downstream proteins including p53 and BRCA1. BARD1 and BRIP1 assist BRCA1 in the assembly with other proteins involved in the repair. PALB2 is a linker between BRCA1 and BRCA2 and mediates the recruitment of RAD51 initiating the HR by DNA synthesis ¹⁴⁸. CHEK2 and ATM are the most common breast cancer susceptibility genes while BRIP1 is highly associated with susceptibility in ovarian cancer ^{149,150}. BARD1 and PALB2 are also involved in breast cancer susceptibility ^{151,152}. In general, deficiency in HRR is correlated with higher sensitivity to cisplatin. These findings suggest that BRD9 might play a role in cisplatin resistance but in this study the question cannot be answered. The origin of the cisplatin resistance in our cisplatin-resistant cell lines is unclear. Therefore, BRD9 involvement in resistance mechanisms needs to be investigated.

Interestingly, the control cell lines FS1 (sertoli cells) showed only slight sensitivity to I-BRD9 indicating weak side-effects on healthy testis tissue and the control cell line MPAF (fibroblasts) revealed no sensitivity to I-BRD9 application indicative for only slight or no effect on somatic cells. In fact, I-BRD9 was already tested in a mouse xenograft with colon adenocarcinoma cells and no side-effects were reported ¹¹⁹. Nevertheless, side-effects were only studied in control cells implying the need for further investigation of possible side-effects in healthy testis tissue.

Interfering with the epigenetic landscape and targeting bromodomain-containing proteins has already been shown to be effective. In colon cancer cells depletion of BRD8 significantly reduced cell viability and induced G1-phase cell cycle arrest and apoptosis ¹⁵³. The BRD4 inhibitor JQ1 led to induction of apoptosis as well as G1/G0-phase cell cycle arrest in TGCT cell lines ⁹⁵. Similar results were obtained by the epigenetic readers LP99 (targeting BRD7 and BRD9) as well as MZ-1 which is a proteolysis targeting chimera (PROTAC) targeting BRD4. Both reduced cell viability and induced apoptosis and cell cycle arrest in TGCT cell lines ¹⁵⁴. BRD9 inhibition showed already antitumor effects in different tumor entities. BI-7273 and BI-9564 both targeting BRD9 resulted in reduced growth of acute myeloid leukemia cells (AML) ¹¹⁰. The effect of I-BRD9 was also studied in AML cells resulting in reduced proliferation dependent on cell cycle inhibition and apoptosis ¹¹⁷. In rhabdoid tumor cells I-BRD9 led to reduced cell proliferation and induced G1-phase cell cycle arrest ¹²⁰. Application

of I-BRD9 in colon cancer cells decreased cell growth in vitro and arrested tumor growth in xenografted mice after BRD9 inhibition in vivo ¹¹⁹. In gallbladder cancer I-BRD9 significantly affected cell proliferation and reduced tumor growth in mouse tumor models ¹¹⁸. Inhibition of BRD9 in uterine fibroid cells reduced cell proliferation as well as induced apoptosis and G1-phase cell cycle arrest ¹⁵⁵. Taken together, BRD9 inhibition by I-BRD9 led to reduced viability, cell cycle arrest as well as apoptosis in different tumor entities which is in line with our findings in TGCT cell lines.

Despite the inhibition of BRD9 also degradation and depletion of BRD9 revealed severe effects in different tumor entities. The BRD9 degrader QA-68 induces ubiquitination and degradation of BRD9 therefore reducing cell proliferation of AML cells. The authors also investigated BRD9 CRISPR knockout as well as doxycycline-induced knockdown of BRD9 in AML cell lines again resulting in decreased cell growth ¹⁵⁶. Another BRD9 degrader, dBRD9-A, led to reduced viability as well as cell cycle arrest in synovial sarcoma cell lines. Furthermore, an in vivo xenograft model treated with the BRD9 degrader showed decreased tumor progression ¹⁵⁷. In AML cells the shRNA-mediated BRD9 depletion resulted in reduced cell survival as well as induction of G0/G1-phase cell cycle arrest and apoptosis ¹⁵⁸. Depletion of BRD9 by siRNA in squamous cell lung cancer cells resulted in inhibited cell proliferation ¹⁰⁸. In prostate cancer using shRNA for knockdown of BRD9 led to decreased cell viability and furthermore a xenograft mouse model treated with shRNA targeting BRD9 revealed reduced tumor growth. In addition, the BRD9 PROTAC degrader dBRD9 decreased cell proliferation in prostate cancer cell lines ¹⁵⁹. In summary, the studies show that interfering with BRD9 by inhibition, depletion or degradation resulted in reduced cell viability as well as induction of cell cycle arrest and apoptosis in different tumor entities which is in line with the effects of I-BRD9 application in TGCT cell lines and stresses the possibility of targeting BRD9 as potential treatment option.

Treatment with I-BRD9 led to loss of pluripotency as well as differentiation towards an epithelial cell fate in embryonal carcinoma (2102 EP) and seminoma (TCam-2) cells. In fact, retinoic acid induced differentiation was already studied in different testicular germ cell tumor cell lines. Application of retinoic acid in embryonal carcinoma cells led to alterations of expression of WNT2B, FZD5, FZD6, FZD10, SFRP1 and SFRP4 resulting in a reprogrammed WNT signaling pathway and therefore induction of canonical WNT signaling ¹⁶⁰. The embryonal carcinoma cell line NT2/D1 differentiates

towards a neuronal cell fate after retinoic acid treatment along with reduction of cell growth and tumorigenicity ^{123,161}. Retinoic acid application in the embryonal carcinoma cell line NTera2 led to significant downregulation of genes involved in DNA mismatch repair like PMS2, MLH1, MSH2 and EXO1 as well as pluripotency factors including OCT4 and NODAL ¹⁶². In fact, exit of pluripotency along with induction of differentiation seems to be a common response of GCT reacting to drugs. In TGCT cell lines the inhibition of BRD4 by JQ1 led to downregulation of pluripotency markers including LIN28, ZSCAN10 and UTF1 along with upregulation of mesodermal differentiation marker HAND1 leading to the conclusion that the cells exit the pluripotency state and differentiate towards a mesodermal cell fate ⁹⁵. Funke et al. found that neddylation pathway inhibition by the NAE1 inhibitor MLN4924 induced upregulation of cell differentiation markers like HAND1, CLDN1, SOX15 and CDX2 in 2102 EP cells indicating mesoderm/ endoderm differentiation. Again, downregulation of pluripotency markers including SOX21, SOX2, TCFL1, KLF15 and HESX1 was detected indicative for loss of pluripotency and induction of mesodermal/ endodermal differentiation ¹⁶³. In fact, this is in line with our findings showing downregulation of a prominent network of pluripotency markers including NANOG, KLF4, PRDM14 and NODAL while genes involved in epithelium development were upregulated. In conclusion, loss of pluripotency along with differentiation seems to be a common aspect in TGCT cell lines after application of different drugs.

Of note, differentiation therapy is an approach where differentiating agents induce tumor reprogramming and therefore leading to reduce proliferative capacity and induce terminal differentiation ¹⁶⁴. Application of berberin, a plant-alkaloid, reduced cancer stemness markers including N-MYC, β -catenin, CD133, NOTCH2, SOX2 and Nestin along with increase of neuronal differentiation markers like NCAM, MAP2 and β -III tubulin generating viable neurons in neuroblastoma cells ¹⁶⁵. Treatment with differentiating agents salinomycin and thioridazine resulted in differentiation of embryonal carcinoma cells as well as decreased expression of pluripotency markers like OCT4. In xenografted mice thioridazine application increased survival and decreased the amount of pluripotent cells in the tumor and therefore reduced tumorigenicity ¹⁶⁶. This underlines our conclusion, that I-BRD9 treatment in TGCT cells could induce terminal differentiation and therefore reduce the risk of progression of the tumor as well as reduction of cytotoxicity of standard chemotherapies.

The next step of testing the suitability of I-BRD9 as a potential treatment option for TGCTs would be to use other models than cell lines. Cell line-derived and patient-derived xenograft models could be used. The cell line-derived xenografts are easily available and have a rapid growth rate while they lack heterogeneity as well as the tumor microenvironment. Patient-derived xenograft models enable studies retaining the heterogeneity, mutations as well as microenvironment and the model allows for personalized medicine. A limitation is the use of immune suppressed mice ¹⁶⁷. Another possibility is the use of 3D culture methods from embryonic or adult stem cells which comprise self-organizing abilities leading to the formation of organoids allowing for studying human pathologies in vitro. In addition, patient-derived organoids can be used for personalized medicine ¹⁶⁸. Cancer organoids resemble human tumors and enable not only drug screening but additionally basic research ¹⁶⁹. An in vitro testis organoid model was established to study reproductive toxicants providing a promising model for reproductive toxicology investigations ¹⁷⁰. Unfortunately, no TGCT organoids are described yet indicating necessity for further research.

In conclusion, the treatment of TGCT cell lines with I-BRD9 led to severe effects like reduction of cell viability as well as induction of cell cycle arrest in G1-phase and apoptosis while the control cell lines remained only slightly effected. I-BRD9 induced loss of pluripotency in embryonal carcinoma and seminoma cells along with differentiation towards an epithelial cell fate indicating terminal differentiation as a benefit of BRD9 inhibition. Most importantly, the findings suggest I-BRD9 as a potential alternative treatment option for TGCTs.

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7 APPENDIX

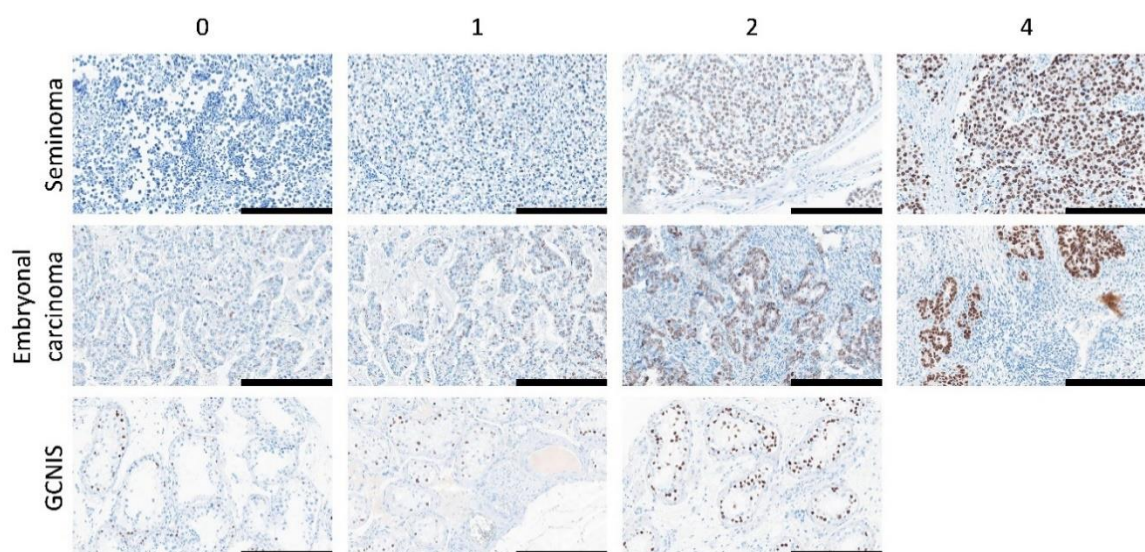


Figure S 1: Example images for the staining intensities of SALL4 in seminomas, embryonal carcinomas and GCNIS. Staining intensity was scored as negative (0), weak (1), moderate (2) and strong (3). The example images for GCNIS show the heterogeneity of SALL4 expression. GCNIS – germ cell neoplasia in situ.

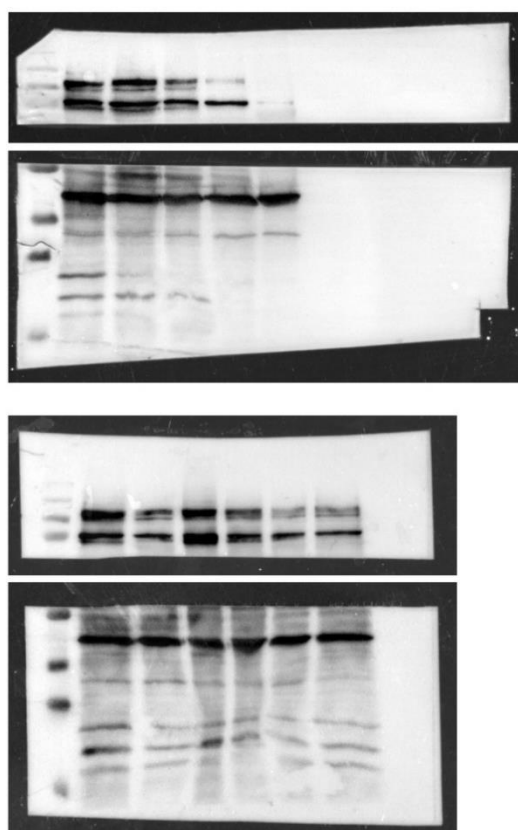


Figure S 2: Original Western Blot images of BRD9 protein level and corresponding load control β -actin in different TGCT cell lines as well as control cell lines. Modified from ¹.

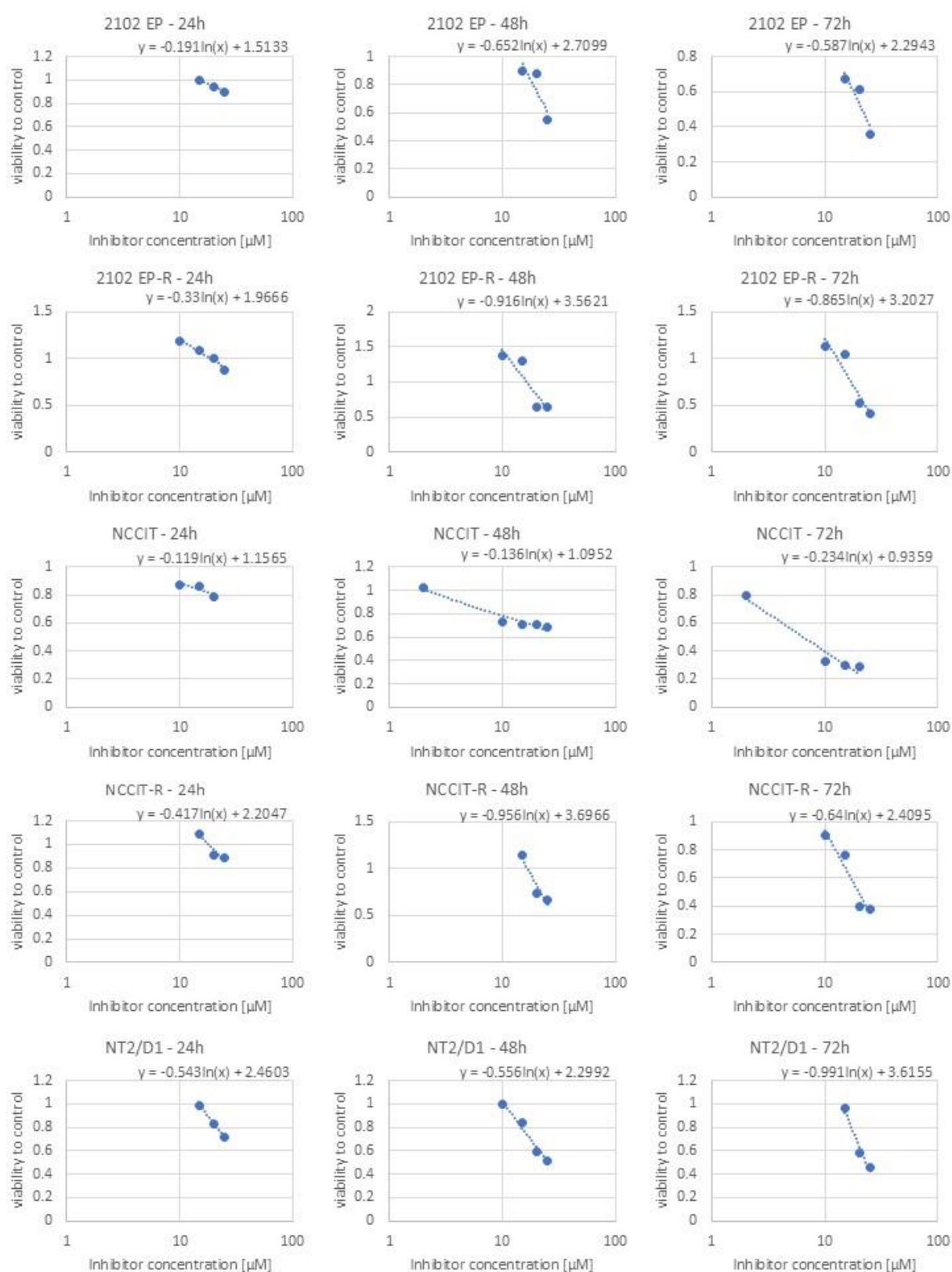


Figure S 3: Utilization of logarithmic regression curve for calculation of IC_{50} values based on XTT assay data (Figure 15).

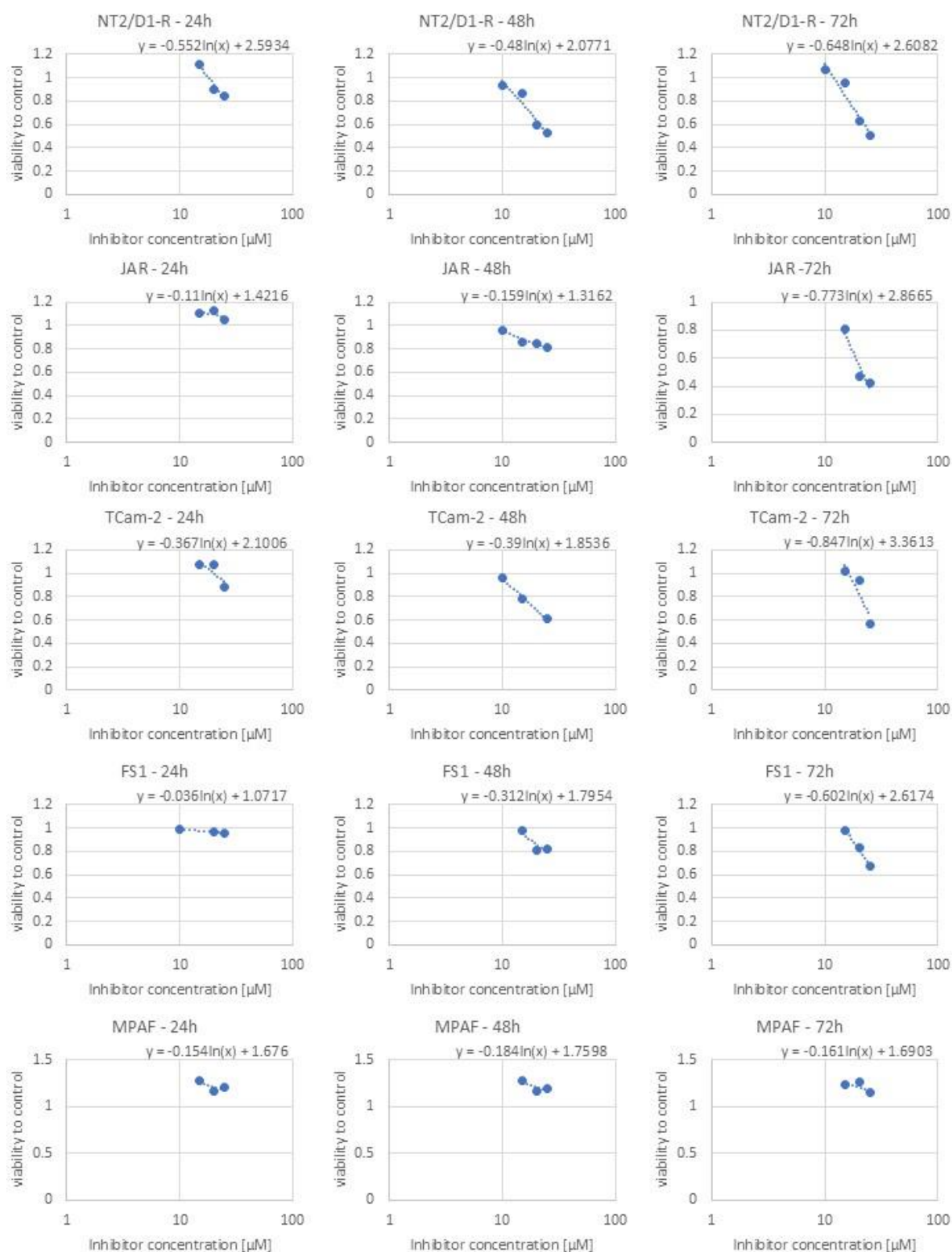


Figure S 4: Utilization of logarithmic regression curve for calculation of IC₅₀ values based on XTT assay data (Figure 15).

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