Chemosensitivity of testicular germ cell tumors is based on high constitutive Noxa protein levels and a functional p53 response

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und unter ausschließlicher Verwendung der angegebenen Hilfsmittel und der Ratschläge von jeweils namentlich aufgeführten Personen angefertigt habe.

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Matthias Gutekunst

Contents

List of abbreviations

3'-UTR	3' untranslated region
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
approx.	approximately
BH3	Bcl-2 homology domain 3
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CIS	carcinoma in situ
CLL	chronic lymphoid leukemia
CML	chronic myelogenous leukemia
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSB	double strand break
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
e.g.	*"exempli gratia"*, for example
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gravitational acceleration
GCT	germ cell tumor

GWAS	genome-wide association study
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	human papillomavirus
HRP	horse radish peroxidase
ICP MS	inductively-coupled-plasma mass-spectrometry
i.e.	*"id est"*, that is
IGCNU	intratubular germ cell neoplasia unclassified
kDa	kilo Dalton
µg	microgram
µl	microliter
µM	micromolar
M	molar
mA	milliampere
MCL	mantle cell lymphoma
MEF	mouse embryonic fibroblast
mg	milligram
min	minute
miRNA	micro ribonucleic acid
ml	milliliter
MOMP	mitochondrial outer membrane permeabilization
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mW	milliwatt
NER	nucleotide excision repair
ng	nanogram
nm	nanometer
nM	nanomolar

OMM	outer mitochondrial membrane
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cells
PI	propidium iodide
PIKK	PI3K-like protein kinase
Pt	Platinum
qPCR	quantitative real time polymerase chain reaction
RA	retinoic acid
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
ROX	X-rhodamine
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
siRNA	small interfering ribonucleic acid
SRB	sulforhodamine B
TBST	TRIS-buffered saline with Tween 20
TEMED	tetramethylethylenediamine
TGCT	testicular germ cell tumor
TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volt
v/v	volume/volume
w/v	weight/volume
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Summary

In contrast to the majority of tumors, testicular germ cell tumors (TGCTs) can be cured by chemotherapy even in advanced metastatic stages. Thus, these neoplasms are considered a paradigm of chemosensitive tumors. It is well accepted that their intrinsic susceptibility to apoptosis determines the unique responsiveness to Cisplatin-based chemotherapy. Although p53 has been implicated in the chemotherapeutic response of TGCTs, the role of this tumor suppressor remains controversial to date. In this study, RNAi-mediated silencing of p53 is shown to completely abrogate Cisplatin hypersensitivity of TGCTs. The central role of p53 is further demonstrated by the finding that the amount of p53 protein induced by Cisplatin treatment is tightly correlated with apoptosis induction. In this context, a reduced capacity to repair damaged DNA is rather unlikely to account for hypersensitivity, a conjecture that is strengthened by the finding that TGCT cells are capable of removing Cisplatin adducts from DNA if apoptosis induction is blocked by caspase inhibition. On the contrary, the hypersensitive phenotype is a result of the unique responsiveness of TGCTs to p53 activation in general, since treatment with the non-genotoxic p53 activators Nutlin-3 or Bortezomib leads to p53-dependent apoptosis to a similar extent as does Cisplatin treatment.

Although functional p53 is a mandatory requirement for Cisplatin hypersensitivity in TGCTs, the proapoptotic character of the p53 response is dependent on the pluripotent cellular context of TGCTs mediated by Oct-4. Differentiation or silencing of Oct-4 expression abrogates Cisplatin sensitivity and reduces apoptosis induction by Nutlin-3 treatment. This is not due to differential activation of p53 since no substantial alterations in Cisplatin-induced target gene activation are observed. Furthermore, p53 accumulation is unaffected by differentiation or Oct-4 depletion. Rather, examination of the Bcl-2 family profile in pluripotent vs. differentiated or Oct-4-depleted cells revealed that high constitutive Noxa protein levels dictate an extremely low apoptotic threshold in TGCT cells. Oct-4 status and Noxa protein levels are correlated to Cisplatin sensitivity in a panel of TGCT cell lines as well as in TGCT patient samples. Importantly, this correlation is also seen with other genotoxic agents such as Etoposide or Doxorubicin and the non-genotoxic p53 activator Nutlin-3. Since Oct-4 depletion leads to a concomitant loss of Noxa protein

and *NOXA/PMAIP1* transcript, an Oct-4-dependent transcriptional mechanism might account for the high Noxa protein levels.

RNAi experiments show that Puma is another important determinant of TGCT hypersensitivity. In contrast to Noxa, Puma is not present at high levels in TGCT cells but exerts its proapoptotic function upon activation by p53.

In conclusion, this study demonstrates that pluripotent TGCTs are primed for apoptosis by Oct-4-mediated high constitutive Noxa protein levels. Thus, in response to genotoxic or other stresses p53 activation results in an efficient induction of apoptosis mediated by transcriptional target genes such as *PUMA/BBC3*.

Zusammenfassung

Testikuläre Keimzelltumore können im Gegensatz zu vielen anderen Tumorerkrankungen selbst im fortgeschrittenen Stadium nach Metastasierung erfolgreich behandelt werden und werden daher als Modellbeispiel chemosensitiver Tumore betrachtet. Es ist allgemein anerkannt, dass die intrinsische Suszeptibilität dieser Tumore für apoptotischen Zelltod deren einzigartiges Ansprechen auf Cisplatin basierte Chemotherapie bestimmt. Obwohl eine prominente Rolle des Tumorsuppressors p53 in der Chemosensitivität testikulärer Keimzelltumore bereits vermutet wurde, konnte dessen Bedeutung bisher nicht zweifelsfrei geklärt werden. In der vorliegenden Arbeit gelang es mittels RNAi basiertem Gen-Silencing zu zeigen, dass p53 für die Cisplatin Sensitivität dieser Tumore unabdingbar ist. Die zentrale Rolle von p53 wird weiterhin durch die Beobachtung gestärkt, dass die durch Cisplatin induzierte Menge an p53 Protein direkt mit dem Ausmaß an Apoptose korreliert. Diese Beobachtungen deuten darauf hin, dass eine verminderte DNA Reparatur Kapazität keine Rolle für die exklusive Cisplatin Sensitivität testikulärer Keimzelltumore spielt. Dies wird dadurch bestätigt, dass eine Reparatur der durch Cisplatin Behandlung entstandenen DNA Addukte festgestellt werden konnte, wenn die Induktion von Zelltod durch einen Caspase Inhibitor blockiert wurde. Darüber hinaus konnte nachgewiesen werden, dass der hypersensitive Phänotyp auf einer allgemeinen Sensitivität testikulärer Keimzelltumore gegenüber der Aktivierung von p53 basiert. Eine Behandlung mit den nicht genotoxischen p53 Aktivatoren Nutlin-3 und Bortezomib induzierte Zelltod in ähnlichem Maße wie eine Cisplatin Behandlung.

Obwohl p53 obligatorisch für die Cisplatin Sensitivität testikulärer Keimzelltumore ist, ist der vorwiegend proapoptotische Charakter der zellulären p53 Antwort durch den pluripotenten zellulären Kontext bedingt, welcher u.a. durch Oct-4 bestimmt wird. Differenzierung bzw. Silencing von Oct-4 führt zu einer substanziellen Reduktion sowohl der Cisplatin Sensitivität als auch der Nutlin-3 Sensitivität. Dies ist nicht auf eine differentielle Aktivierung von p53 zurückzuführen, da keine signifikanten Unterschiede in der Aktivierung von p53 Zielgenen in Oct-4 positiven und Oct-4 negativen Zellen zu beobachten war. Darüber hinaus konnte kein Einfluss auf die Akkumulation von p53 festgestellt werden. Stattdessen ergab eine Untersuchung von Proteinen der Bcl-2 Familie, dass hohe konstitutive Noxa Protein Level zu einer erheblichen Verringerung der

apoptotischen Schwelle führen. Sowohl in Zelllinien testikulärer Keimzelltumore als auch in primärem Tumormaterial wurde eine sehr enge Korrelation zwischen Oct-4 Status, Noxa Protein Level und Cisplatin Sensitivität beobachtet. Darüber hinaus ist dieser Zusammenhang sowohl für andere genotoxische Substanzen wie Etoposid und Doxorubicin, als auch für nicht genotoxische Substanzen wie Nutlin-3 gegeben. Die Tatsache, dass der Verlust von Oct-4 außer zu einer Reduktion von Noxa Protein auch zur Verringerung des entsprechenden Transkripts führt weist darauf hin, dass es sich um eine transkriptionelle Art der Regulation handeln könnte.

Außerdem wurde Puma als wichtiger Faktor in der Cisplatin Sensitivität testikulärer Keimzelltumore identifiziert. Diese Tumore weisen jedoch keine konstitutiv hohen Puma Protein Level auf. Es muss daher davon ausgegangen werden, dass Puma seine proapoptotische Funktion nach Aktivierung durch p53 ausübt.

In der vorliegenden Arbeit konnte nachgewiesen werden, dass die Suszeptibilität pluripotenter testikulärer Keimzelltumore gegenüber Apoptose auf konstitutiv hohe Noxa Protein Level zurückzuführen ist. Aus diesem Grund führt eine Aktivierung von p53 durch zellulären Stress genotoxischer oder nicht genotoxischer Art zu einer effizienten Induktion von Zelltod, welche durch p53 Zielgene wie z.B. *PUMA/BBC3* vermittelt wird.

1 Introduction

1.1 Testicular germ cell tumors

1.1.1 Epidemiology

Testicular germ cell tumors (TGCTs) represent a small percentage of all tumor incidences (approx. 1 %) with a still increasing frequency worldwide. However, these neoplasms are the most prevalent solid tumors of young adult males between 15 and 40 years of age (Adami et al., 1994; Bosl and Motzer, 1997; Mannuel et al., 2012). Moreover, in this age group, TGCTs are the leading cause of cancer-related mortality and morbidity (Gilbert et al., 2011; Winter and Albers, 2011).

Incidences vary among populations, however, an increased risk is associated with a family history of TGCTs. Compared to other cancers, a high proportion of TGCT susceptibility is considered to depend on genetic effects (approx. 25 %) (Czene et al., 2002). Besides other genomic abnormalities, amplification of chromosome 12p either as an isochromosome or tandem duplications was reported to be involved in tumorigenesis (Houldsworth et al., 2006). These amplified regions include candidate genes for involvement in the pathogenesis of TGCTs such as *KRAS* or *CCND2* (Gilbert et al., 2011). In addition, recent GWAS studies identified *KITLG*, *SPRY4*, *BAK1*, *DMRT1*, *TERT* and *ATF7IP* as genes predisposing to TGCTs (Gilbert et al., 2011).

In contrast to other tumor types TGCTs mostly harbor wildtype p53 (Heimdal et al., 1993; Peng et al., 1993) suggesting a minor role of p53 in transformation or indicating a selective pressure to retain wildtype p53. miR-372 and miR-373 were found to interfere with the p53 pathway implicating a compensatory mechanism for the loss of p53 in tumorigenesis (Voorhoeve et al., 2006).

Non-genetic risk factors for TGCTs include cryptochordism, inguinal hernia, hydrocele and spermatic and testicular dysgenesis. Moreover, syndrome-associated abnormal testicular development such as Klinefelter's syndrome, XY dysgenesis and Down's syndrome are associated with an elevated risk of TGCTs (Houldsworth et al., 2006).

1.1.2   Pathogenesis

Primordial germ cells (PGCs) are induced from the pluripotent stem cell population during early embryogenesis. These pluripotent germ cell precursors then migrate to the gonadal ridge, the location of the developing gonads. There, PGCs differentiate into spermatogonia which give rise to mature germ cells capable of producing gametes (Oosterhuis and Looijenga, 2005). TGCTs arise from PGCs with a defect in normal development that consequently causes malignant transformation. These precursor lesions are termed carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (IGCNU) (Dieckmann and Skakkebaek, 1999). The majority of CIS are localized in the gonads, however, extragonadal germ cell tumors (GCTs) can occur due to misplacement of PGCs during embryogenesis (Bosl and Motzer, 1997).

1.1.3   Classification

The majority (95 %) of testicular cancers are GCTs, whereas the remaining 5 % consist of non-germinal neoplasms such as Leydig cell tumors, Sertoli cell tumors and lymphomas (Winter and Albers, 2011). TGCTs are classified into seminoma and non-seminoma based on histological and biochemical characteristics (Ulbright, 2005).

Seminoma consist of uniform, undifferentiated cells similar to PGCs and do not retain pluripotency. They show low metastatic potential and can generally be cured by orchiectomy and radiation therapy (Houldsworth et al., 2006; Winter and Albers, 2011).

Non-seminoma are characterized by different histological subtypes representing variable differentiation statuses and stages of embryonic development. These tumors contain undifferentiated embryonal carcinoma (EC) cells which are the precursors for the more differentiated embryonic tumors such as teratoma or teratocarcinoma and extra-embryonic tumors such as yolk sac tumors or choriocarcinoma (Chaganti and Houldsworth, 2000; Winter and Albers, 2011). Besides seminoma, most TGCTs occur as mixtures of non-seminomatous histological subtypes or a combination of seminomatous and non-seminomatous components. In most cases, non-seminomatous TGCTs can be cured by a combination of orchiectomy and Cisplatin-based chemotherapy with teratomas representing a TGCT component found to be relatively resistant to chemotherapy (Houldsworth et al., 2006). EC cells represent the malignant counterpart of embryonic

stem cells (ESCs). However, in contrast to ESCs, non-reprogrammable genetic modifications confine their developmental potential and drive tumorigenesis (Blelloch et al., 2004).

Due to their rapid progression, TGCTs cause early lymph node or distant metastases. Therefore, a high percentage of patients already suffer from metastatic disease at the time of diagnosis (Di Pietro et al., 2005).

1.1.4 The pluripotency marker Oct-4

Along with Nanog and Sox2, the transcription factor Oct-4 is regarded as one of the key regulators of pluripotency (De Jong and Looijenga, 2006). *OCT4/POU5F1* is exclusively expressed in cells of non-malignant pluripotent nature, i.e. ESCs and PGCs, as well as their malignant counterparts EC and seminoma (De Jong and Looijenga, 2006; Mueller et al., 2009). Oct-4 belongs to a family of octamer binding proteins that specifically bind to the conserved ATTTGCAT motive in transcriptional control elements of genes via their POU domain (De Jong and Looijenga, 2006).

Oct-4 must be tightly regulated since even a slight decrease can initiate differentiation (Niwa et al., 2000). A plethora of factors bind upstream of the *OCT4/POU5F1* gene, interact physically with the Oct-4 protein or are transcriptionally targeted by Oct-4. Furthermore, expression of *OCT4/POU5F1* is regulated by epigenetic modifications (De Jong and Looijenga, 2006).

OCT4/POU5F1 expression was initially associated with TGCTs by a study showing expression of *OCT4/POU5F1* mRNA in EC and seminoma but not in differentiated components of teratoma and yolk sac tumors (Palumbo et al., 2002). Immunohistological analysis revealed exclusive expression in CIS, EC and seminoma but not in the differentiated types of non-seminoma (Looijenga et al., 2003). Following studies conclusively established Oct-4 as a marker for TGCTs and their precursor cells (De Jong and Looijenga, 2006).

1.1.5   Cisplatin-based chemotherapy

Cisplatin-based chemotherapy is widely used in cancer therapy. Testicular, ovarian, cervical, head and neck, and non-small-cell lung cancers are commonly treated with platinum-based therapies (Jamieson and Lippard, 1999). Upon cellular uptake, Cisplatin becomes aquated, losing chloride ions and gaining water molecules. The resulting positively charged molecule is then capable of binding to nucleophilic molecules such as DNA, RNA or proteins (Rabik and Dolan, 2007). Monoadducts as well as intrastrand and interstrand lesions occur in DNA, with 1,2-d(GpG) intrastrand crosslinks accounting for the majority of DNA lesions (Rabik and Dolan, 2007). These crosslinks are primarily eliminated by nucleotide excision repair (NER) (Zamble et al., 1996).

Problems of platinum-based chemotherapy include side effects such as ototoxicity, peripheral neuropathy, nephrotoxicity and myelosuppression (Hartmann and Lipp, 2003) and intrinsic or acquired resistance. Second generation agents such as Carboplatin, Oxaliplatin or Satraplatin have been developed to counter these adverse effects (Rabik and Dolan, 2007).

In contrast to most other solid tumors, TGCTs can be cured by Cisplatin-based chemotherapy at high rates even in advanced metastatic stages (Bosl and Motzer, 1997; Einhorn, 2002).

1.1.6   Cisplatin hypersensitivity

A better understanding of the unique Cisplatin sensitivity of TGCTs might help to improve the therapeutic index of chemoresistant tumors. Various attempts have been made to explain the underlying molecular mechanisms. Initially, classical determinants of drug resistance such as drug uptake and efflux, drug metabolism and drug binding to DNA were studied. However, no considerable differences were found in TGCTs when compared to other tumor types (Masters and Köberle, 2003). Since apoptosis is a constant feature of normal spermatogenesis in a variety of mammalian species (Sinha Hikim and Swerdloff, 1999) and most types of spermatogonia are sensitive to genotoxic agents such as Cisplatin (Zhang et al., 2001), TGCTs derive from an intrinsically apoptosis-prone tissue. Therefore, the unique responsiveness to chemotherapy was proposed to be due to inherited biological characteristics (Blagosklonny, 2011).

In contrast to other tumor types most TGCTs are characterized by the expression of wildtype *TP53* (Heimdal et al., 1993; Peng et al., 1993; Riou et al., 1995; Lutzker, 1998). Several groups reported a correlation of *TP53* status with susceptibility to chemotherapy (Eid et al., 1997; Heidenreich et al., 1998; Houldsworth et al., 1998) whereas other reports failed to confirm such a correlation (Burger et al., 1997, 1998b). Similarly, there is some dispute as to whether p53 is overexpressed in TGCTs (Riou et al., 1995; Guillou et al., 1996; Lutzker and Levine, 1996; Kersemaekers et al., 2002). Functional analysis confirmed an important role for p53 in TGCT hypersensitivity (Lutzker et al., 2001; Kerley-Hamilton et al., 2005), however, other studies revealed conflicting data (Burger et al., 1999; Kersemaekers et al., 2002). In some cases, transactivation of target genes by p53 appeared to be impaired (Guillou et al., 1996; Lutzker and Levine, 1996). However, Mdm2, a transcriptional target of p53, was found to be highly expressed in p53 overexpressing TGCTs supporting the notion that p53 is transcriptionally competent (Riou et al., 1995; Kersemaekers et al., 2002). Indeed, Kerley-Hamilton et al. proposed a transcriptional response to Cisplatin that is dominated by the activation of p53 target genes (Kerley-Hamilton et al., 2005). A recent study proposes that transactivation specificity of p53 is cell context dependent and varies between sensitive and resistant TGCT cells (Di Pietro et al., 2012).

The above mentioned transactivation deficiency was thought to account for the lack of *P21/CDKN1A* expression in TGCTs (Bartkova et al., 2000; Datta et al., 2001). Also, this CDK inhibitor was found to be induced only slightly upon chemotherapy (Chresta et al., 1996). Therefore, low p21 protein levels were suggested to sensitize TGCTs to Cisplatin via a non-efficient inhibition of cell cycle progression (Spierings et al., 2004). In addition, several factors associated with G_1/S-phase transition were described to be deregulated in TGCTs, further compromising proper cell cycle progression (Houldsworth et al., 1997; Bartkova et al., 1999, 2000, 2003; Schmidt et al., 2001). An impaired capacity of cytoplasmic p21 to prevent apoptosis induction was also considered to explain TGCT hypersensitivity (Koster et al., 2010). In this context, low levels of p21 were ascribed to Oct-4, which was previously shown to mediate TGCT hypersensitivity (Mueller et al., 2006). Differentiation, a process in which Oct-4 expression is turned off (Houldsworth et al., 2002), also led to reduced Cisplatin hypersensitivity (Timmer-Bosscha et al., 1998).

Several DNA repair mechanisms, including DNA polymerases that are capable of bypassing Cisplatin-induced DNA lesions, have been linked to the cellular Cisplatin

response (Masters and Köberle, 2003). Moreover, initial experiments in TGCTs indicated that these tumors are characterized by a reduced NER capacity (Bedford et al., 1988; Hill et al., 1994; Köberle et al., 1997) that was attributed to low levels of the NER proteins XPA and ERCC1-XPF (Köberle et al., 1999). Whereas addition of XPA to cellular extracts of TGCTs conferred full NER capacity *in vitro* (Köberle et al., 1999), overexpression of XPA did not lead to an elevated Cisplatin resistance *in vivo* (Köberle et al., 2008). Likewise, functional analysis suggested a role for ERCC1-XPF in Cisplatin sensitivity (Usanova et al., 2010). In addition, a reduced proficiency in homologous recombination could indicate a general defect in DNA repair and may also account for Cisplatin sensitivity of TGCTs (Cavallo et al., 2012).

Several attempts have been made to link expression of proapoptotic and antiapoptotic Bcl-2 proteins to Cisplatin sensitivity since members of this protein family regulate cell fate through the control of mitochondrial outer membrane integrity and apoptosis (Chipuk et al., 2010). Low Bcl-2 and Bcl-xL levels were suggested to favor apoptosis induction upon chemotherapeutic agents (Chresta et al., 1996; Mayer et al., 2003), however, exogenous expression of Bcl-2 led to an increased Cisplatin susceptibility due to concomitant downregulation of Bcl-xL (Arriola et al., 1999). Furthermore, levels of Bcl-2, Bcl-xL, Bax and Bak could not explain Cisplatin resistance in TGCT cell lines (Burger et al., 1997, 1998b; Mueller et al., 2003). Rather, failure to activate the caspase-9 pathway was suggested to result in a higher apoptotic threshold (Mueller et al., 2003). Besides Noxa which was proposed as a prognostic marker for clinical outcome in EC patients (Grande et al., 2012), proapoptotic BH3-only proteins have not been implicated in TGCT Cisplatin hypersensitivity to date.

1.2   p53 stress response

Cells are continuously exposed to a wide range of extrinsic and intrinsic stress stimuli that, if not dealt with adequately, can cause severe cellular damage and confer oncogenic potential to the cell. The decision to eliminate such cells often depends on p53 (Aylon and Oren, 2007). Stress stimuli cause p53 responses leading to induction of apoptosis, cell cycle arrest or senescence which mediate its tumor suppressor capacity (Vousden and Prives, 2009) or determine the cellular responsiveness to genotoxic chemotherapy (Appella

and Anderson, 2001). Most tumors harbor *TP53* mutations that perturb p53 function or disrupt the regulatory network controlled by p53 (Kruse and Gu, 2009). Although the suitability of *TP53* status as a predictive marker in chemotherapy remains controversial, it is indicative for the clinical outcome in certain tumors (Robles and Harris, 2010). Moreover, as described above, wildtype *TP53* expression might be of importance in the unique hypersensitivity of TGCTs to genotoxic agents (Lutzker et al., 2001; Kerley-Hamilton et al., 2005).

1.2.1   Stabilization of p53 upon DNA damage

Genotoxic agents such as Cisplatin cause major cytotoxic lesions, including various forms of DNA crosslinks (Rabik and Dolan, 2007). Cells deficient in NER and DNA double-strand break (DSB) repair by homologous recombination were shown to be sensitive to such agents (Frankenberg-Schwager et al., 2005). DNA lesions are recognized principally by three kinases belonging to the PI3K-like protein kinase (PIKK) family, ATM, ATR and DNA-PK. ATM and DNA-PK respond primarily to DSBs whereas ATR responds to UV-light induced lesions as well as DSBs and stalled replication forks (Shiloh, 2003). Upon DNA damage, ATM is activated by autophosphorylation (Bakkenist and Kastan, 2003), whereas ATR stability requires ATRIP, an ATR-binding protein (Cortez et al., 2001). The mechanism of DNA-PK activation still remains unclear (Neal and Meek, 2011). The DNA damage signal is transduced to p53 via phosphorylation of p53 itself or other ATM, ATR and DNA-PK targets (Shiloh, 2003). ATM phosphorylates p53 at the N-terminal serine residues 15 and 20 (Kruse and Gu, 2009) resulting in disruption of its binding to Mdm2. Mdm2 is one of several E3 ubiquitin ligases that target p53 for proteasomal degradation (Michael and Oren, 2003; Murray-Zmijewski et al., 2008) and is phosphorylated by ATM leading to a reduction of its capacity to foster p53 degradation (Cheng and Chen, 2010). Besides phosphorylation by ATM, ATR and DNA-PK, p53 is also phosphorylated by CHK1 and CHK2 which are targets of ATM and ATR, respectively (Bartek and Lukas, 2003). Histone acetyltransferases (HATs) such as CBP, p300 or PCAF mediate acetylation of p53 at different C-terminal lysine residues upon DNA damage (Liu et al., 1999). Interdependent with N-terminal phosphorylation events, these covalent modifications stabilize p53 and enable its function as a transcription factor by altering its DNA binding capacity (Sakaguchi et al., 1998). Beyond the described modifications, p53 stability and

function are regulated by various other posttranslational modifications, its cellular localization and a wide variety of cofactors (Murray-Zmijewski et al., 2008). It is therefore likely that other factors can contribute to stabilization of p53 upon DNA damage.

1.2.2   Apoptosis mediated by p53

One of the major mechanisms by which p53 exerts its tumor suppressor function is initiation of the apoptotic program (Aylon and Oren, 2007). According to the type of cellular stress and tissue, differential activation of distinct subsets of target genes was suspected to determine the cellular response to p53 accumulation (Murray-Zmijewski et al., 2008). The amount of p53 protein, posttranslational modifications, cofactors and promoter affinity dictate which subset of genes is activated or repressed (Aylon and Oren, 2007; Vousden and Prives, 2009), e.g. phosphorylation at serine 46 as well as acetylation at the lysine residues 120 and 382 direct p53 to promoters of proapoptotic target genes (Puca et al., 2009; Gu and Zhu, 2012). Cofactors such as p63, p73, p300, Brn3b and ASPP1/2 also enhance p53's proapoptotic capabilities (Aylon and Oren, 2007). As a consequence, induction of proapoptotic target genes such as *BAX*, *FAS/CD95*, *DR5/TNFRSF10B*, *TP53AIP1*, *TP53INP1*, *PUMA/BBC3* and *NOXA/PMAIP1* triggers apoptosis (Vousden and Lu, 2002; Murray-Zmijewski et al., 2008).

Recent work demonstrated that p53 also has transcription-independent functions leading to the induction of apoptosis (Green and Kroemer, 2009). Upon export to the cytoplasm, p53 is capable of binding to the antiapoptotic proteins Bcl-2 and Bcl-xL resulting in a reduced cellular antiapoptotic capacity. Moreover, p53 can directly activate Bax and Bak to trigger apoptosis (Moll et al., 2006). Thus, in principle p53 is acting as a BH3-only protein. Interestingly, binding to Bcl-2 proteins happens through the p53 DNA binding domain (Pietsch et al., 2008). Therefore, mutations in this domain can disrupt both nuclear and cytoplasmic activity. Induction of apoptosis by cytoplasmic p53 can occur independently of its nuclear function. However, transactivation of BH3-only genes such as *PUMA/BBC3* or *NOXA/PMAIP1* might cooperate with cytoplasmic p53 in apoptosis induction, e.g. Puma can release p53 from Bcl-2 or Bcl-xL allowing activation of Bax or Bak (Green and Kroemer, 2009).

1.3   Bcl-2 protein family

The mitochondrial pathway of apoptosis is the most common form of programmed cell death in metazoan organisms. It has a major role in development, tissue homeostasis and immunity. Moreover, it provides an important block against oncogenesis and the development of other diseases (Strasser et al., 2011). The apoptotic program can be triggered by the extrinsic pathway through death receptor activation or by the intrinsic pathway of apoptosis that is controlled by proteins of the Bcl-2 family. Complex interactions between these proteins determine the integrity of the outer mitochondrial membrane (OMM) (Chipuk et al., 2010). Once antiapoptotic Bcl-2 family proteins are kept in check by their proapoptotic counterparts, Bax and Bak can be activated to induce mitochondrial outer membrane permeabilization (MOMP) leading to the release of proteins such as cytochrome c or Smac/Diablo to the cytoplasm. Subsequently, caspases are activated to execute the apoptotic program (Strasser et al., 2011).

1.3.1   Bcl-2 family members and their function

The Bcl-2 family contains antiapoptotic and proapoptotic proteins. Complex interactions of these proteins dictate cell fate and determine if MOMP occurs. Antiapoptotic Bcl-2 proteins include Bcl-2 itself, Bcl-xL, Bcl-w, Mcl-1 and A1 that bind to and directly inhibit proapoptotic proteins. The proapoptotic effector proteins Bax and Bak homooligomerize upon activation and form pores in the OMM which allow cytochrome c and Smac/Diablo release from the intermembrane space (Chipuk et al., 2010). Among BH3-only proteins, activator proteins such as Bim, Bid and potentially Puma can directly bind to Bax and Bak to trigger pore formation. Proteins such as Bad and Noxa bind to antiapoptotic Bcl-2 family proteins resulting in a low antiapoptotic capacity which allows stress-induced activator proteins to activate Bax and/or Bak ("sensitization") (Chipuk et al., 2008). Another hypothesis suggests that sensitizer proteins compete with activator proteins for binding sites of antiapoptotic Bcl-2 family proteins thereby releasing activator proteins which then bind to Bax and Bak ("de-repression") (Lovell et al., 2008). A third model ("neutralization") proposes that occupation of Bax and Bak by antiapoptotic Bcl-2 family proteins inhibits MOMP and therefore sequestering antiapoptotic Bcl-2 family proteins is sufficient to induce cytochrome c release (Willis et al., 2007). Recent studies show that

apoptosis induction potentially occurs by features of all models *in vivo* (Mérino et al., 2009).

BH3-only proteins engage antiapoptotic Bcl-2 family proteins via their BH3 domain. Studies using peptides that mimic different BH3 domains revealed a defined interaction pattern between pro and antiapoptotic Bcl-2 family proteins. Bim and Puma were shown to bind all antiapoptotic proteins with high affinity whereas Bad can engage Bcl-2 and Bcl-xl but not Mcl-1 and A1. In contrast, Noxa is capable of binding to Mcl-1 and A1 but shows very low affinity to other antiapoptotic proteins (Chen et al., 2005b; Certo et al., 2006).

1.3.2   Bcl-2 family and chemotherapeutic response

Deregulation of the expression of Bcl-2 family proteins substantially contributes to tumor development, maintenance and therapeutic response (Kelly and Strasser, 2011), since blockade of apoptosis induction by overexpression of antiapoptotic Bcl-2 family proteins is a common feature of human cancers and cooperates with oncogenes that promote proliferation to drive tumorigenesis (Adams and Cory, 2007). In addition, increased antiapoptotic Bcl-2 family proteins can mediate therapeutic resistance, e.g. Bcl-xL conferred resistance of Bcr/Abl$^+$ chronic myelogenous leukemia (CML) cells to Imatinib (Horita et al., 2000) and high Mcl-1 protein levels were shown to render HeLa cells resistant to the BH3 mimetic ABT-737 (Van Delft et al., 2006). In contrast, anticancer agents rely on the proapoptotic activity of distinct BH3-only proteins, for instance genotoxic agents trigger a p53 response that activates Puma and/or Noxa (Villunger et al., 2003), Bortezomib induces Noxa protein (Qin et al., 2005; Fernández et al., 2006) and Paclitaxel relies on the proapoptotic activity of Bim (Tan et al., 2005). Letai and colleagues recently developed a method termed "BH3 profiling" which allows the identification of cellular dependence on specific antiapoptotic Bcl-2 family proteins for survival (Certo et al., 2006). Incubation of purified mouse mitochondria with peptides corresponding to BH3 domains of BH3-only sensitizer proteins in the presence of an activator protein (tBid) and one antiapoptotic Bcl-2 family protein at a time revealed that mitochondrial integrity relied on specific antiapoptotic proteins depending on BH3 peptide treatment (Certo et al., 2006). Importantly, mitochondria from murine leukemia cells were shown to be depolarized by treatment with BH3 sensitizer peptides alone implying that cancer cells may chronically express activator BH3-only proteins that necessitates the antagonizing function of

antiapoptotic Bcl-2 family proteins (Certo et al., 2006). A cancer cell's reliance on a balance of these proteins was termed "primed for death" and several successive studies have shown that BH3 profiling can be used to predict chemotherapeutic response (Deng et al., 2007; Brunelle et al., 2009). Moreover, heightened mitochondrial priming was shown to account for hypersensitivity of $CD4^+$ $CD8^+$ thymocytes (Ryan et al., 2010) and acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and ovarian cancer patients showed a superior clinical response when cancer cells were highly primed (Ni Chonghaile et al., 2011; Vo et al., 2012).

1.3.3   Noxa

The *NOXA/PMAIP1* gene was first described to be induced by phorbol 12-myristate 13-acetate (PMA) (Hijikata et al., 1990) and was later re-discovered as a p53-dependent proapoptotic gene induced upon DNA damage (Oda et al., 2000). It codes for a 54 amino acid protein characterized by a BH3 motif classifying it as a BH3-only protein in the family of Bcl-2 proteins. Besides p53, other transcription factors have been described to transactivate *NOXA/PMAIP1*, e.g. HIF-1α (Kim et al., 2004), E2F1 (Hershko and Ginsberg, 2004) FoxO1 (Valis et al., 2011), ATF3 and ATF4 (Wang et al., 2009), c-myc (Nikiforov et al., 2007) and p73 (Rocco et al., 2006) whereas Bmi1 was shown to repress *NOXA/PMAIP1* transcription (Yamashita et al., 2008). In addition to transcriptional regulation, recent reports suggested various posttranscriptional regulatory mechanisms to control Noxa protein stability. KLF6-SV1 was reported to target Noxa for HDM2-dependent degradation (Difeo et al., 2009) and ubiquitinated forms of Noxa were found to accumulate upon Bortezomib treatment (Baou et al., 2010) indicating a regulation of Noxa by the ubiquitin-proteasome system. Craxton et al. demonstrated that proteasomal degradation of Noxa protein occurs independently of ubiquitin as well (Craxton et al., 2012). Also, a phosphorylation site at serine residue 13 was identified to determine cellular localization of Noxa (Lowman et al., 2010). Another mechanism of posttranscriptional regulation involves miR-200c which targets the 3'-UTR of Noxa therefore repressing its expression (Lerner et al., 2012).

Noxa exerts its proapoptotic functions by binding to antiapoptotic Bcl-2 family proteins dependent on its BH3 domain (Oda et al., 2000). As described above, Noxa binds to Mcl-1 and A1 with high affinity antagonizing their antiapoptotic functions but exhibits low

binding affinity for other antiapoptotic Bcl-2 family proteins (Chen et al., 2005b; Certo et al., 2006). Binding of Noxa to Mcl-1 not only reduces Mcl-1's capacity to bind proapoptotic activator proteins but also promotes its degradation by the E3 Ligase Mule and decreases its interaction with the deubiquitinase USP9X (Gomez-Bougie et al., 2011) exemplifying complex regulatory processes among Bcl-2 family proteins. Furthermore, recent studies reported binding of Noxa to Bcl-xL (Lopez et al., 2010) and direct activation of Bax and Bak by Noxa (Du et al., 2011) indicating that its binding capacity might not be limited to Mcl-1 and A1.

The apoptotic potential of overexpressed Noxa was suggested to vary depending on the cellular context due to its moderate capacity to antagonize Bcl-2 and Bcl-xL (Ploner et al., 2008). However, several studies demonstrate an important role for Noxa in cell death upon a variety of stresses engaging multiple apoptotic pathways (Shibue et al., 2003; Zhang et al., 2011) and, in contrast to Puma, propose an important role for Noxa in tumor-selective apoptosis induction (Suzuki et al., 2009).

1.4   Aims

As described in 1.1.6, various attempts have been made to understand the molecular mechanisms underlying the exclusive sensitivity of TGCTs to Cisplatin. However, no explanation has generally been accepted to date. An early hypothesis that was supported by several studies suggested that TGCTs are sensitive to DNA damage due to retention of wildtype p53. Contrarily, other studies came to the conclusion that p53 is not a determinant of Cisplatin sensitivity. Thus, the first part of this study sought to clarify the role of p53 in TGCT's susceptibility to chemotherapeutics.

For this purpose, cell lines resembling the hypersensitive phenotype of EC cells were used

(I)   to clarify if the presence of functional p53 is a requirement for the hypersensitive phenotype of TGCT cells.

(II)   to examine pathways that lead to the activation of p53 upon DNA damage in these cells.

(III)   to discriminate effector pathways triggered by p53 in the cellular context of TGCT cells.

Evidence that the pluripotent cellular context of EC cells confers hypersensitivity to genotoxic agents came from studies showing that Cisplatin sensitivity is reduced upon differentiation. Moreover, loss of the stem cell factor Oct-4 was proposed to account for acquired Cisplatin resistance in refractory tumors. A general determinant of chemosensitivity includes Bcl-2 family proteins. Therefore, further experiments were performed to examine

(IV)   the p53 stress response in the context of pluripotent, Oct-4-positive TGCT cells
(V)   further possible determinants of the chemosensitive phenotype such as Bcl-2 family proteins

In summary, this study was aimed towards better understanding the role of p53 and the pluripotent cellular context mediated by Oct-4 in TGCT hypersensitivity. Moreover, factors that are associated with either p53 or Oct-4 and contribute to TGCT hypersensitivity were sought.

2   Material and Methods

2.1   Cell culture

2.1.1   Cell lines

Cell lines specified in Table 1 were used in this study.

Table 1: Cell lines used.

cell line	source	obtained from
NTERA-2D1	embryonal carcinoma	ATCC
2102EP	embryonal carcinoma	kindly provided by F. Honecker (Hamburg University,
H12.1	embryonal carcinoma	frozen pellets of these cell lines were kindly provided by T. Mueller (Martin Luther University of Halle-Wittenberg, Halle, Germany)
H12.1ODM	in vitro differentiated derivative of H12.1	
H12.1RA	in vitro differentiated derivative of H12.1	
H12.5	embryonal carcinoma	
1777NRpmet	differentiated state, isolated from mixed GCT	
1411HP	yolk sac tumor	
833K	embryonal carcinoma	
GCT-72	yolk sac tumor	
MCF7	breast cancer	ATCC
H460	non-small cell lung cancer	ATCC
A549	lung adenocarcinoma	ATCC
A2780	ovarian carcinoma	ATCC

2.1.2   Cultivation procedures

Cell lines were cultured in supplemented RPMI-1640 medium as follows.

RPMI-1640 medium	Biochrom
500 ml were supplemented with	
10 % (v/v) FBS	Gibco
0.1 g/l Penicillin/Streptomycin	Gibco
10 mM HEPES, pH 7.4	Merck
2 mM L-glutamine	Biochrom
0.13 mM L-asparagine	Serva
0.05 mM 2-mercaptoethanol	Merck
1 mM sodium pyruvate	Gibco
3 ml 100x non-essential amino acids	Biochrom

Cells were maintained in cell culture flasks at 37 °C with 5 % CO_2 and passaged every 2-3 days using Trypsin/EDTA.

Cell culture flasks	Sarstedt
Serological pipettes	Corning
6-, 96-well-plates	Greiner Bio-One
15 ml tubes	Greiner Bio-One
50 ml tubes	Becton Dickinson
Trypsin/EDTA	Gibco
Laminar flow hood	Heraeus
Incubator	Heraeus

Cell count and viability was assessed by trypan blue staining using a hemocytometer. Trypan blue is a vital stain selectively coloring dead tissues or cells blue.

Trypan blue 0.5 % (w/v)	Biochrom
Neubauer hemocytometer	Roth
Microscope	Zeiss

2.1.3   Cryopreservation of cell lines

Cells maintained in culture were replaced every 3 months to prevent genetic changes by selection processes during cell culture. To cryopreserve cells, 1-2 Mio. cells were collected in ice-cold DMSO:FBS (1:10) and transferred to a cryo-tube. Cells were then gradually cooled down to -80 °C in a special freezing container before they were stored at -196 °C in liquid nitrogen. Cell lines were thawed at 37 °C, transferred to RPMI-1640 medium and centrifuged (5 min, 1400 rpm) to get rid of DMSO. Subsequently, cells were cultivated as described above.

DMSO	Sigma
1.8 ml CryoTube™	Nalgene Nunc International
5100 Cryo 1 °C Freezing container "Mr. Frosty"	Nalgene Nunc International

2.2   Reagents

2.2.1   Genotoxic chemotherapeutics

Cisplatin (cis-diamminedichloroplatinum(II)) was used as reference chemotherapeutic since it is a standard treatment for TGCTs. This platinum complex binds to DNA and causes crosslinks which ultimately trigger induction of cell death. Cisplatin was used at a concentration of 10 µM. Other genotoxic agents used in this study include Etoposide (used at 5 µM) and Doxorubicin (used at 100 ng/ml).

Cisplatin (1 mg/ml)	Robert-Bosch-Hospital, Stuttgart
Etoposide (10 mg/ml)	Robert-Bosch-Hospital, Stuttgart
Doxorubicin (2 mg/ml)	Robert-Bosch-Hospital, Stuttgart

2.2.2   Activation of p53 by small molecule inhibitors

In order to investigate DNA damage-independent p53 activation, the Mdm-2 antagonist Nutlin-3 (Vassilev et al., 2004) was used to achieve p53 accumulation. Nutlin-3 binds to Mdm2 and thereby disrupts its interaction with p53 preventing it from proteasomal degradation via the ubiquitin ligase activity of Mdm2 (Thompson et al., 2004). Furthermore, the proteasome inhibitor Bortezomib was used to accumulate p53 by shutting off proteasome function (Williams and McConkey, 2003). Nutlin-3 was used at a concentration of 10 µM, Bortezomib at 10 nM.

Nutlin-3	Sigma
Bortezomib	Toronto Research Chemicals

2.2.3   Kinase inhibitor library

An influence of protein kinases on Cisplatin sensitivity was investigated using a protein kinase inhibitor library containing 160 well-characterized, cell permeable protein kinase inhibitors. Cells were pretreated with inhibitors for 2 h followed by Cisplatin treatment and subsequent determination of cytotoxicity by MTT assay. Inhibitors were used at 10 µM.

Cytotoxicity values were normalized to an untreated control. Subsequently, the difference in cytotoxicity of the combination of Cisplatin and inhibitor and inhibitor alone was

determined. Inhibitors that had minor effects on Cisplatin-induced cytotoxicity (within standard deviation of values obtained for Cisplatin treatment alone) were considered not to change Cisplatin cytotoxicity.

InhibitorSelect 96-Well Protein Kinase Inhibitor Library I	Calbiochem
InhibitorSelect 96-Well Protein Kinase Inhibitor Library II	Calbiochem

2.2.4 Histone deacetylase (HDAC) inhibition

A possible influence of acetylation of lysine 382 on p53 stability and activity was investigated using the HDAC inhibitor SAHA which induces accumulation of acetylated histones as well as non-histone proteins. SAHA was used at a concentration of 1, 2, 4, 8, 16 μM.

SAHA	Biomol

2.2.5 Caspase inhibition

To exclude apoptosis induction as a possible reason for the incapability to repair damaged DNA, apoptosis was inhibited by carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK), a cell-permeant pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases. Z-VAD-FMK was used at a concentration of 50 μM.

Caspase Inhibitor Z-VAD-FMK	Bachem

2.3   Cell viability assays

2.3.1 Flow cytometry-based apoptosis assay

Flow cytometry was used to distinguish between vital and apoptotic cells. Cells were harvested by trypsinization, washed once in ice-cold PBS followed by a washing step in ice-cold Annexin V binding buffer (10 mM HEPES, 140 mM sodium chloride, 25 mM

calcium chloride, pH 7.4). The cell pellet was then resuspended in 100 µl binding buffer containing 5 µl Annexin V-FITC and 2 µl propidium iodide (PI; 50 µg/ml stock solution in PBS). The suspension was incubated at room temperature for 10 min. Additional 300 µl binding buffer were added to the cells before analysis using FACScan and CELLQuest software.

Fluorescence Activated Cell Analyzer "FACScan"	Becton Dickinson
Laser:	
15 mW, 488 nm, argon-ion laser	
Detectors/Filters:	
530 nm (FITC)	
585 nm (propidium iodide)	
650 nm (dark red fluorescence)	
CELLQuest software	Becton Dickinson

In early apoptotic stages, the cellular membrane already undergoes changes. Under these conditions, the phospholipid phosphatidylserine is no longer restricted to the inner membrane leaflet and can be bound by Annexin V, a phospholipid binding protein. Labeling of Annexin V with fluorescein isothiocyanate (FITC), a fluorophor with an excitation wavelength of 488 nm, allows to distinguish between cells in early and late stages of apoptosis. In combination with FITC-labeled Annexin V, PI (excitation wavelength 536 nm) was used to detect cells in late stages of apoptosis. This fluorescent dye penetrates cells whose membranes are already damaged and can therefore be used as an indicator of necrosis or late stages of apoptosis.

Annexin V-FITC	BD Pharmingen
Propidium iodide stock solution (1 mg/ml)	Sigma
Annexin V binding buffer (10x)	BD Pharmingen
PBS	Biochrom

2.3.2 MTT assay

Cytotoxicity was measured by MTT assay (Mosmann, 1983). In living cell, the tetrazole MTT is reduced by a mitochondrial dehydrogenase to purple formazan, which can be quantified by a spectrophotometer. Therefore, formazan staining indicates proliferating cells. 7,500 cells were seeded in 96 well plates and grown for 48 h after treatment. Subsequently, 10 µl MTT solution (10 mg/ml MTT in PBS) were added per well before the plate was incubated for 2 h at 37 °C and lysed with 90 µl MTT lysis buffer (15 % SDS

in DMF-water (1:1); pH 4.5). Upon incubation over night at room temperature with light shaking, formazan staining was quantified by a spectrophotometer at 570 nm.

MTT	Sigma
ELISA-Reader "Wallac"	SLT Labinstruments

2.3.3   SRB microculture colorimetric assay

IC_{50} values were determined by Dr. Thomas Mueller (Martin-Luther-University of Halle-Wittenberg, Halle, Germany) as described (Skehan et al., 1990).

2.4   Subcellular fractionation

Cytoplasm and nuclei were separated using a modified protocol of Mitochondria Isolation Kit for Cultured Cells (Pierce). Cells were treated as desired, harvested and pelleted. Subsequently, the cell pellet was resuspended in 800 µl Mitochondria Isolation Reagent A and vortexed for 5 sec followed by 20 min incubation on ice, vortexing at maximum speed every minute. Cellular disruption was verified using trypan blue staining. To pellet the nuclear fraction, lysates were centrifuged (10 min, 300 g, 4 °C). Nuclear pellet was washed three times with a mixture of Mitochondria Isolation Reagent A and Mitochondria Isolation Reagent C and flash frozen in liquid nitrogen. The supernatant was collected and centrifuged three times (15 min, 12,000 g, 4 °C) to obtain the cytosolic fraction. Subcellular fractionation was verified by Western blot analysis of tubulin which is located in the cytoplasmic compartment and TBP which is located the nuclear compartment.

Mitochondria Isolation Kit for Cultured Cells	Pierce Biotechnology

2.5   Protein analysis

2.5.1   Western Blot

2.5.1.1 Protein extraction from cell lines

Cells were grown to desired confluence and harvested by trypsinization. Subsequently cells were pelleted by centrifugation (5 min, 1400 rpm, 4 °C) and washed once in ice-cold PBS followed by flash freezing the pellets in liquid nitrogen. A Triton-X based cell lysis buffer (50 mM TRIS; 250 mM sodium chloride; 0.1 % Triton X-100; 5 mM EDTA pH 7.6; protease and phosphatase inhibitor cocktails added freshly) was used to lyse cells. To support cell lysis, cells were sonicated for 20 sec, cell debris was pelleted by centrifugation (15 min, 13,000 rpm, 4 °C) and discarded. Protein concentration of purified lysates was determined by Bradford assay. To separate lysates by SDS-PAGE, identical amounts of cellular protein were boiled with 1x Laemmli buffer (62.5 mM TRIS; 20 % (v/v) glycerol; 5 % (v/v) β-mercaptoethanol; 2 % (w/v) SDS; 1 % bromophenol blue, pH 6.8) for 5 min at 95 °C.

Ultrasound homogenizator *Sonopuls HD200*; *MS72* titan microtip	Bandelin Elektronik

2.5.1.2 Separation of proteins by SDS-PAGE

Proteins were separated according to their molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels consist of acrylamide, bisacrylamide, SDS and a pH-adjusted buffer. Variable concentrations of acrylamide can be used to resolve proteins with different molecular weights. Lower concentrations were used to resolve proteins with high molecular weights whereas high concentrations were used to resolve proteins with lower molecular weights. Furthermore, SDS gradient gels (5-20 % acrylamid) were used to resolve very small proteins (<10 kDa) or to detect proteins of differing molecular weights on the same gel. Protein weights were detected using a molecular weight marker. Separation of proteins was done at 6 mA over night in electrophoresis buffer (25 mM TRIS; 0.2 M glycine; 1 % SDS) using a vertical electrophoresis system.

Ammonium persulfate	Bio-Rad
TEMED	Roth
Vertical electrophoresis system, Protean II xi Cell	Bio-Rad
30 % acrylamid/Bis-solution (37.5:1)	Bio-Rad
Molecular weight marker	Cell Signaling
Thermomixer Comfort	Eppendorf

2.5.1.3 Western Blot

Separated proteins were blotted electrophoretically on nitrocellulose membrane using a Trans-Blot semi-dry transfer cell. For this, the SDS gel was applied to the membrane avoiding air bubbles. Gel and membrane were fitted between three layers of Whatman filter paper, respectively, which was drenched in transfer buffer (0.025 M TRIS; 0.192 M glycine; 20 % (v/v) methanol; 1 % (w/v) SDS). Protein transfer was done at 15 V for 1.5 h. Afterwards, the nitrocellulose membrane was washed in TBS (137 mM sodium chloride; 2.7 mM potassium chloride; 25 mM TRIS; pH 7.4) to eliminate residual methanol. Subsequently, the membrane was blocked with 5 % (w/v) skim milk in TBST (TBS with 0.1 % Tween-20) for 1 h at room temperature. Membranes were incubated with primary antibodies in 5 % (w/v) skim milk in TBST, 5 % BSA in TBST or 1 % Roche blocking solution in TBS according to Table 2.

Following primary antibody incubation, the membrane was washed 4 x 15 min in TBST. Dependent on the primary antibody host, the membrane was then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Table 3). Upon 4 washing steps á 15 min, the protein of interest was detected by chemoluminescence using SuperSignal West Dura Extended Duration Substrate and x-ray films according to manufacturer's instructions. Intensities of protein bands were quantified using AIDA (advanced image data analyzer; version 2.31) software.

Table 2: Primary antibodies used.

antibody	host	company	dilution
β-actin	mouse	Sigma	1:5000 in 5 % (w/v) skim milk in TBST
ATM	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
ATR	goat	Santa Cruz	1:200 in 5 % (w/v) skim milk in TBST
Bak	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
Bax	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bcl-2	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bcl-w	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bcl-xL	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bim	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
CHK2	mouse	Santa Cruz	1:500 in 5 % (w/v) skim milk in TBST
DNA-PK	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
GAPDH	rabbit	Cell Signaling	1:5000 in 5 % BSA in TBST
Mcl-1	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
Noxa	mouse	Calbiochem	1:500 1 % Roche blocking solution in TBS
Oct-4	mouse	Cell Signaling	1:2000 in 5 % BSA in TBST
p21	mouse	BD Pharmingen	1:1000 in 5 % (w/v) skim milk in TBST
p53 (Pab 240)	mouse	Santa Cruz	1:2000 in 5 % (w/v) skim milk in TBST
p53 (DO-1)	mouse	Santa Cruz	1:2000 in 5 % (w/v) skim milk in TBST
lys382-p53	rabbit	Cell Signaling	1:1000 in 5 % (w/v) skim milk in TBST
ser15-p53	mouse	Cell Signaling	1:1000 in 5 % (w/v) skim milk in TBST
ser20-p53	rabbit	Cell Signaling	1:250 in 5 % (w/v) skim milk in TBST
ser46-p53	rabbit	Abcam	1:500 in 5 % (w/v) skim milk in TBST
Puma	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
TBP	mouse	Novus	1:1000 in 5 % (w/v) skim milk in TBST
Tubulin	mouse	Abcam	1:2000 in 5 % (w/v) skim milk in TBST

Table 3: Secondary antibodies used.

antibody	host	company	dilution
anti-rabbit-IgG-HRP	goat	Cell Signaling	1:2000 in 5 % (w/v) skim milk in TBST
anti-mouse-IgG-HRP	goat	Cell Signaling	1:2000 in 5 % (w/v) skim milk in TBST
anti-goat-IgG-HRP	rabbit	BioVision	1:5000 in 5 % (w/v) skim milk in TBST

Nitrocellulose membrane	Boehringer
Trans-Blot semi-dry transfer cell	Bio-Rad
Filter paper, Gel-blotting paper	Schleicher & Schuell
Western blocking reagent	Roche
Autoradiography cassette	Amersham Biosciences
Lumi-Film, Chemoluminescence detection film	Roche
SuperSignal West Dura Extended Duration Substrate	Pierce Biotechnology
Advanced image data analyzer (AIDA 2.31)	Raytest

To reanalyze nitrocellulose membranes e.g. for the analysis of housekeeping proteins such as β-actin and GAPDH, membranes were incubated in SDS-β-mercaptoethanol stripping solution (62.5 mM TRIS; 2 % (w/v) SDS; 100 mM β-mercaptoethanol; pH 6.7) for 20 min at 52 °C in order to get rid of bound antibodies.

2.5.2   Flow cytometry

Cells were grown to desired confluence and harvested by trypsinization. Subsequently, cells were pelleted by centrifugation (5 min, 1400 rpm, 4 °C). A FITC-conjugated antibody was used to detect Fas/CD95 protein on the cellular surface. In addition, a negative control containing an isotypic control antibody was used to exclude false positive results. For each test an antibody solution (100 µl) was prepared containing 5 µl Fas/CD95 antibody or 20 µl isotype control, respectively. Pelleted cells were resuspended in antibody solution and incubated for 15 min at 4 °C. 1 ml of PBS-BSA (1 % BSA) was added followed by a centrifugation step (5 min, 1200 rpm, 4 °C). Supernatant was discarded, cells were resuspended in 1 ml PBS-BSA and subjected to FACS analysis using FACScan and CELLQuest software (see 2.3.1).

Anti-Human CD95 (APO-1/Fas) FITC	NatuTec
FITC Mouse IgG$_1$ κ Isotype Control	BD Pharmingen

2.6   RNA interference

RNA interference (RNAi) was used to study the cellular pathways involved in Cisplatin hypersensitivity. Cells were transfected with a mixture of four siRNAs directed against one target gene to obtain an adequate knockdown efficiency. For this, cells were seeded at low confluence in cell culture flasks. Dharmacon siRNAs were diluted 1:166 (to obtain a final

siRNA concentration of 15 nM) in antibiotics-free transfection medium with reduced serum, vortexed and incubated for 5 min at room temperature. At the same time transfection reagent was diluted 1:25 in transfection medium and incubated for 5 min at room temperature as well. Subsequently, diluted siRNA and transfection reagent were mixed 1:1, vortexed and incubated for 20 min at room temperature to allow liposome formation.

Table 4: Sequences of SMARTpool siGenome siRNAs.

target gene	SMARTpool siGenome sequences	
ATM	GCAAAGCCCUAGUAACAUA	GGGCAUUACGGGUGUUGAA
	UCGCUUAGCAGGAGGUGUA	UGAUGAAGAGAGACGGAAU
ATR	GAACAACACUGCUGGUUUG	GCAACUCGCCUAACAGAUA
	UCUCAGAAGUCAACCGAUU	GAAUUGUGUUGCAGAGCUU
CHEK2	GAAAUUGCACUGUCACUAA	CUCAGGAACUCUAUUCUAU
	AAACGCCGUCCUUUGAAUA	GCUAAAUCAUCCUUGCAUC
DNAPK/PRKDC	GCAAAGAGGUGGCAGUUAA	GAGCAUCACUUGCCUUUAA
	GAUGAGAAGUCCUUAGGUA	GCAGGACCGUGCAAGGUUA
FAS/CD95	UAGAUGAGAUCAAGAAUGA	GAAAGAAGCGUAUGACACA
	GCUGGAGUCAUGACACUAA	GUUCAACUGCUUCGUAAUU
NOXA/PMAIP1	AAACUGAACUUCCGGCAGA	AAUCUGAUAUCCAAACUCU
	CUGGAAGUCGAGUGUGCUA	GCAAGAACGCUCAACCGAG
OCT4/POU5F1	UCCCCAUGCAUUCAAACUGA	GCGAUCAAGCAGCGACUAU
	GAUAUACACAGGCCGAUGU	CAUCAAAGCUCUGCAGAAA
TP53	GAGGUUGGCUCUGACUGUA	GCACAGAGGAAGAGAAUCU
	GAAGAAACCACUGGAUGGA	GCUUCGAGAUGUUCCGAGA
PUMA/BBC3	CGGACGACCUCAACGCACA	CCGAGAUGGAGCCCAAUUA
	CCUGGAGGGUCCUGUACAA	GGCGGAGACAAGAGGAGCA
BIM/BCL2L11	sequence unknown	

Opti-MEM I Reduced Serum Media	Gibco
SMARTpool siGenome siRNA	Dharmacon
DharmaFECT 3	Dharmacon

Afterwards, transfection solution was diluted 1:10 in cell culture medium and cells were incubated with transfection solution. After 6 h, medium was changed to cell culture medium. Experiments were done 48 h later to ensure optimal silencing of the relevant protein. siRNA sequences are provided in Table 4. Bim was silenced using Bim siRNA from Santa Cruz (30 nM) according to the transfection procedures described above.

2.7   RNA quantification

2.7.1   RNA isolation

Isolation of cellular RNA was done using the RNeasy kit according to manufacturer's instructions.

RNeasy kit	Quiagen

2.7.2   cDNA synthesis

RNA was transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit. 500 ng RNA were transcribed per reaction.

RevertAid First Strand cDNA Synthesis Kit	Fermentas
Mastercycler Gradient	Eppendorf

2.7.3   SYBR green-based qPCR assays

qPCR was used to quantify gene expression in unstressed and Cisplatin-treated cells. For this, a mixture of 2.5 µl Platinum SYBR Green qPCR-SuperMix-UDG, 0.0125 µl forward- and reverse-Primer (from 10 µM stock solution), 0.15 µl H_2O, 0.1 µl X-rhodamine (ROX) and 2 µl cDNA was prepared in 384 well plates. cDNA was diluted 1:10 before use. Established primers listed in Table 5 were used to amplify indicated RNAs. *GAPDH* primers were kindly provided by Dr. Efrat Lidor Nili (Weizmann Institute of Science, Rehovot, Israel), *NOXA/PMAIP1* primers by Dr. Nadya Rakovitsky (Weizmann Institute of Science, Rehovot, Israel), *P21/CDKN1A* primers by Dr. Yael Aylon (Weizmann Institute of Science, Rehovot, Israel), *BAX* primers by Dr. Maike Sonnenberg (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart) and *ACTB* primers by Dr. Claudia Kalla (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart).

Table 5: Primer sequences for SYBR green-based qPCR assays.

mRNA	forward primer	reverse primer
FAS/CD95	TGGACCCTCCTACCTCTGGTTCT	GCAGGGCACGCAGTCTGGTT
GAPDH	AGCCTCAAGATCATCAGCAATG	CACGATACCAAAGTTGTCATGG
NOXA/PMAIP1	GCAGAGCTGGAAGTCGAGTGT	AAGTTTCTGCCGGAAGTTCAG
P21/CDKN1A	GGCAGACCAGCATGACAGATT	GCGGATTAGGGCTTCCTCTT
PUMA/BBC3	ACGACCTCAACGCACAGTACG	TCCCATGATGAGATTGTACAGG
BAX	ACCAAGAAGCTGAGCGAGTGT	ACAAACATGGTCACGGTCTGC
mature *NOXA/PMAIP1*	GAGATGCCTGGGAAGAAGG	CCTGAGTTGAGTAGCACACTCG
pre *NOXA/PMAIP1*	TTGCTTTCCTTCTCAGAGCTG	TTCTGCCGGAAGTTCAGTTT
ACTB	GCGATATCATCATCCATGG	AGCCTCGCCTTTGCCGA **

* (Gomes et al., 2006)

** (Kreuzer et al., 1999)

7900HT Fast Real-Time PCR System was used to run SYBR green-based qPCR assays applying the following cycling program:

50 °C for 2 min

95 °C for 2 min

40 cycles of:

      95 °C for 15 sec

      60 °C for 60 sec

7900HT Fast Real-Time PCR System	Applied Biosystems
Platinum SYBR® Green qPCR-SuperMix-UDG	Invitrogen
MicroAmp® Optical 384-well Reaction Plate	Applied Biosystems
Optical Adhesive Covers	Applied Biosystems

2.7.4   TaqMan-based qPCR assays

Gene expression of a panel of 46 *bona fide* p53 target genes was analyzed using a TaqMan-based qPCR system.

Table 6: TaqMan assays (Applied Biosystems) used for quantification of p53 target genes.

	gene name	assay #			gene name	assay #
1	*APAF1*	Hs00559441_m1		25	*TNFRSF10C*	Hs00182570_m1
2	*ZMAT3*	Hs00536976_m1		26	*BCL2*	Hs00608023_m1
3	*TAP63*	Hs00978348_m1		27	*CDKN1A*	Hs00355782_m1
4	*MYC*	Hs00905030_m1		28	*ABCB1*	Hs01067802_m1
5	*IGFBP3*	Hs00426287_m1		29	*CCNK*	Hs00171095_m1
6	*TERT*	Hs00972656_m1		30	*IER3*	Hs00174674_m1
7	*BAX*	Hs00180269_m1		31	*TNFRSF10D*	Hs00388742_m1
8	*NOXA/PMAIP1*	Hs00560402_m1		32	*BCL2L1*	Hs00236329_m1
9	*TP63*	Hs00978340_m1		33	*EGFR*	Hs01076078_m1
10	*AIFM2*	Hs01097300_m1		34	*CCNA2*	Hs00996788_m1
11	*MDM2*	Hs00234753_m1		35	*MDM4*	Hs00159092_m1
12	*ATF3*	Hs00231069_m1		36	*PIDD*	Hs00388035_m1
13	*PUMA/BBC3*	Hs00248075_m1		37	*TP53I3*	Hs00153280_m1
14	*TNFRSF10A*	Hs00269492_m1		38	*FASLG*	Hs00181225_m1
15	*TAP73*	Hs01056228_m1		39	*GADD45A*	Hs00169255_m1
16	*CCNG1*	Hs00171112_m1		40	*CCNB1*	Hs01030097_m1
17	*MET*	Hs01565584_m1		41	*RB1*	Hs01078066_m1
18	*BID*	Hs00609632_m1		42	*P53AIP1*	Hs00986095_m1
19	*TNFRSF10B*	Hs00366278_m1		43	*TP53INP1*	Hs01003820_m1
20	*TP73*	Hs01056230_m1		44	*FOS*	Hs00170630_m1
21	*CDC25C*	Hs00156411_m1		45	*HGF*	Hs00300159_m1
22	*PCNA*	Hs00696862_m1		46	*CDK1*	Hs00938777_m1
23	*BTG2*	Hs00198887_m1		47	*TBP*	Hs00427621_m1
24	*FAS/CD95*	Hs00531110_m1				

TaqMan assays (Table 6)	Applied Biosystems
BioMark HD System	Fluidigm

2.8   Microarray analysis

RNA was isolated according to 2.7.1. Microarray analysis was performed by Microarray Facility Tuebingen, Germany. To assess global gene expression, Human Gene 1.1 ST Array Plate (Affymetrix) was used. In order to examine gene expression prior to induction

of apoptosis, cells were treated with Cisplatin for 6 h before they were harvested. Microarray data are MIAME compliant and were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-410.

2.9   miRNA analysis

2.9.1   RNA isolation

Isolation of cellular RNA was done using mirVana miRNA Isolation Kit according to manufacturer's instructions.

mirVana miRNA Isolation Kit	Ambion

2.9.2   TaqMan miRNA low density arrays

miRNA low density arrays were used to investigate a possible Oct-4-dependent regulation of *NOXA/PMAIP1* expression by miRNAs. These arrays provide the possibility to assess the expression of 365 miRNAs. Cells were treated as desired and total RNA was isolated. TaqMan Arrays were performed according to manufacturer's instructions. First, 10 ng total RNA was reverse transcribed using predefined primers that are specific for mature miRNA species. Second, templates were pre-amplified by qPCR. Third, cDNA templates were mixed together with TaqMan Universal PCR Master Mix and loaded onto the TaqMan Array. The array was briefly centrifuged to distribute samples to multiple wells on the array and sealed. The qPCR was run using the Applied Biosystems 7900HT Fast Real-Time PCR system.

TaqMan Human MicroRNA Array A	Applied Biosystems
TaqMan MicroRNA RT Kit	Applied Biosystems
Megaplex RT Primers Human Pool A	Applied Biosystems
TaqMan PreAmp Mastermix (2x)	Applied Biosystems
Megaplex PreAmp Primers Human Pool A	Applied Biosystems
TaqMan Universal PCR Mastermix noUNG	Applied Biosystems
7900HT Fast Real-Time PCR system	Applied Biosystems

2.9.3   Transfection with miRNA mimics

miRNA mimics are double-stranded RNA oligonucleotides used to characterize miRNA function. First, miRNAs identified to be upregulated upon Oct-4 depletion by low density arrays were screened for *NOXA/PMAIP1* mRNA binding potential (microrna.org; (Betel et al., 2008)). mirSVR scores (Betel et al., 2010) were used to estimate the capacity of the identified miRNAs to target *NOXA/PMAIP1* mRNA. Predicted miRNAs upregulated at least 2.0fold upon Oct-4 depletion were considered as candidate miRNAs (Table 7). Subsequently, cells were transfected with RNAs mimicking the identified miRNAs (Table 7) in order to investigate possible effects on *NOXA/PMAIP1* expression. A non-targeting RNA was used as a negative control. Transfection was done according to the protocol for siRNAs (see 2.6) using 25 nM miRNA mimics per transfection. Transfection efficiency was monitored by qPCR (see Table 7 for TaqMan assays).

Table 7: miRIDIAN mimics (Dharmacon) and corresponding TaqMan assays (Applied Biosystems).

miRNA	miRNA mimic	assay	mature sequence
U6 snRNA		001973	
hsa-miR-145	C-300613-05-0005	002278	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-181a	C-300553-05-0005	000480	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-193b	C-300764-05-0005	002367	AACUGGCCCUCAAAGUCCCGCU

2.10   Cancer patient samples

Tissue samples from EC and seminoma patients were provided by the Department of Urology, Eberhard-Karls-University of Tuebingen, Tuebingen, Germany. The local ethics committee approved the collection of patient samples (315/2012BO2 and 396/2005V) and informed consent was obtained from the patients. Specimen were frozen in liquid nitrogen immediately after surgery and stored at -80°C for further use. To obtain protein lysates for Western Blot, frozen tissue was crashed and subsequently lysed using a FastPrep-24 tissue homogenizer. For this, tissue samples were collected in cell lysis buffer (50 mM TRIS; 250 mM sodium chloride; 0.1 % Triton X-100; 5 mM EDTA pH 7.6; protease and phosphatase inhibitor cocktails added freshly) and lysed with lysing matrix D followed by a sonication step to obtain optimal cell disruption (all steps at 4 °C). Tissue debris was

discarded after a centrifugation step (30 min, 1,400 rpm, 4 °C) and cell lysate subjected to Western Blot analysis (see 2.5.1).

Lysing Matrix D	MP Biomedicals
FastPrep-24 Instrument	MP Biomedicals

2.11   Statistics

Data are expressed as standard deviation of the mean (SD). Changes in paired samples were analyzed using two-sided paired t-Test. p-values were designated as follows: *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001. Correlations are expressed as Spearman rank correlation coefficient.

3   Results

3.1   p53 determines the responsiveness of TGCTs to Cisplatin

TGCTs are highly apoptosis-prone cancers which can be cured by Cisplatin-based chemotherapy alone even in advanced metastatic stages (Bosl and Motzer, 1997; Einhorn, 2002). In spite of the considerable effort made to understand the underlying molecular mechanisms, the reasons for the exclusive Cisplatin sensitivity of these tumors are not completely understood yet. Since more than 97 % of TGCTs express wildtype *TP53* (Lutzker, 1998), this tumor suppressor was proposed to be an important player in Cisplatin hypersensitivity and several studies confirmed such a role (Lutzker et al., 2001; Kerley-Hamilton et al., 2005). However, others reported controversial data showing that TGCTs responded to Cisplatin in a p53-independent manner (Burger et al., 1999; Kersemaekers et al., 2002). Consequently, the first part of this study aimed at a better understanding of p53's role in Cisplatin hypersensitivity of TGCTs.

3.1.1   Cisplatin hypersensitivity

In this study, the mechanisms underlying Cisplatin hypersensitivity of TGCTs were investigated in EC cell lines. In most experiments, NTERA-2D1 and 2102EP cells were used, both of which are derived from primary human testicular teratocarcinoma (Wang et al., 1980; Andrews et al., 1984) and exhibit high Cisplatin sensitivity (Mueller et al., 2003; Kerley-Hamilton et al., 2005). Figure 1A illustrates their increased sensitivity to Cisplatin as determined by MTT assay. NTERA-2D1 and 2102EP cells showed an IC_{50} of Cisplatin about 10fold lower compared to other cell types. Notably, even when apoptosis induction in NTERA-2D1 cells was compared to H460 cells that have a similar IC_{50}, the EC cell line showed an earlier onset of apoptosis (Figure 1B). Thus, EC cell lines are characterized by a massive and rapid induction of apoptosis upon Cisplatin treatment and resemble the exceptional sensitivity of TGCTs to genotoxic agents.
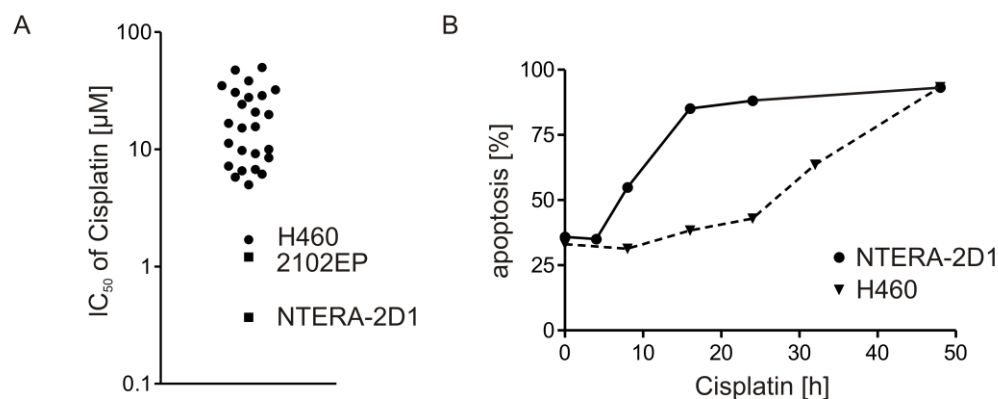
Figure 1: Cisplatin IC$_{50}$ determined by MTT assay in 28 cell lines.
(A) A panel of 28 cell lines including NTERA-2D1 and 2102EP cell lines was treated with increasing concentrations of Cisplatin to determine IC$_{50}$ as measured by MTT assay (data kindly provided by Dr. Heiko van der Kuip (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart)). (B) NTERA-2D1 and H460 cells were treated with Cisplatin and apoptosis was determined by Annexin V-FITC and PI staining at different time points.

3.1.2   Cisplatin hypersensitivity depends on p53

To address the question whether p53 is a determinant of Cisplatin hypersensitivity, RNAi-mediated gene silencing was used to diminish accumulation of p53 in response to Cisplatin-induced DNA damage. Knockdown of p53 in the EC cell lines NTERA-2D1 and 2102EP resulted in an almost complete loss of residual constitutive p53 protein and prevented its induction upon Cisplatin treatment (Figure 2A). In order to investigate effects of p53 on cell death induction, p53-depleted cells together with control cells were treated with Cisplatin, stained with Annexin V-FITC and PI and subjected to FACS analysis. Importantly, depletion of p53 completely rescued NTERA-2D1 cells from Cisplatin-induced apoptosis (99.8±5.8 % survival in p53-depleted vs. 28.1±17.0 % in control cells; Figure 2B, left panel). A similar picture was seen with 2102EP cells where 74.7±15.8 % of p53-depleted cells survived Cisplatin treatment compared to 31.8±5.5 % of control cells (Figure 2B, right panel).
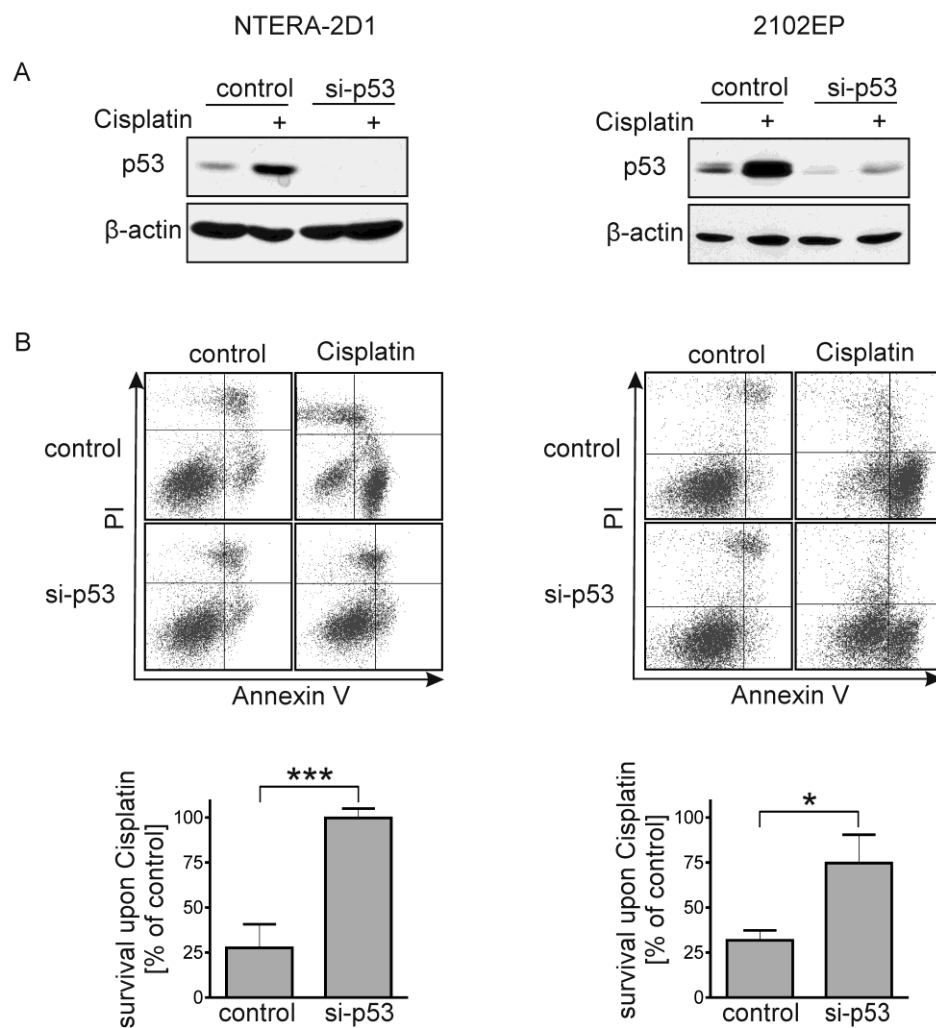
Figure 2: Cisplatin hypersensitivity is dependent on p53.

NTERA-2D1 and 2102EP cells were transfected with a p53-targeting siRNA and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. (A) Verification of p53 knockdown by Western Blot analysis. (B) Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. Graph reflects means ±SD of survival upon Cisplatin treatment (lower left quadrant of FACS analysis) relative to corresponding controls (cells treated with the same siRNA but cultivated in the absence of Cisplatin) from five (***: p≤0.001) or three (*: p≤0.05) independent experiments, respectively.

These results implicate a direct correlation between the presence of p53 protein and the degree of cell death induced by Cisplatin. Importantly, titration of p53 protein with increasing concentrations of p53-targeting siRNA revealed a close dose-response relationship between the amount of p53 protein and the amount of Cisplatin-induced cell death (Figure 3).

Together, these data demonstrate that Cisplatin hypersensitivity of TGCTs is highly
dependent on p53 and positively correlates with the amount of p53 protein.
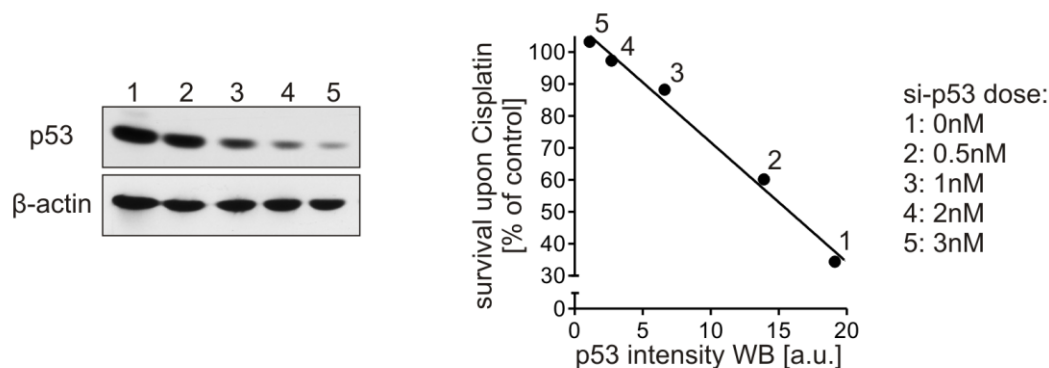
Figure 3: Dose-dependent relationship between the amount of p53 protein and Cisplatin-induced cell death.
NTERA-2D1 cells were transfected with the indicated amounts of p53-targeting siRNA and incubated for
48 h prior to Cisplatin treatment (16 h). p53 protein levels were analyzed by Western Blot. Densitometric
analysis was done to quantify p53 protein. Cells were stained with Annexin V-FITC and PI and analyzed for
apoptosis by flow cytometry. Graph reflects survival upon Cisplatin treatment relative to corresponding
controls vs. p53 protein levels.

3.1.3   TGCTs do not exhibit high p53 protein levels

It was previously proposed that the exceptional sensitivity of TGCTs is dependent on high
p53 protein levels (Guillou et al., 1996; Lutzker and Levine, 1996). To investigate if
differential p53 protein levels may account for the response to Cisplatin, four cancer cell
lines covering a broad spectrum of Cisplatin sensitivity were chosen from a panel of 28
cell lines (Figure 1) and analyzed for p53 protein. Interestingly, NTERA-2D1 cells did not
exhibit particularly high levels of p53 protein when compared to A549, H460 and MCF-7
cells. Neither constitutive nor Cisplatin-induced levels were found to be elevated in these
cells when analyzed by Western Blot (Figure 4). In fact, NTERA-2D1 cells rather showed
slightly lower p53 protein levels compared to other cell lines (Figure 4). Therefore, the
predominantly proapoptotic characteristics of p53 leading to Cisplatin hypersensitivity do
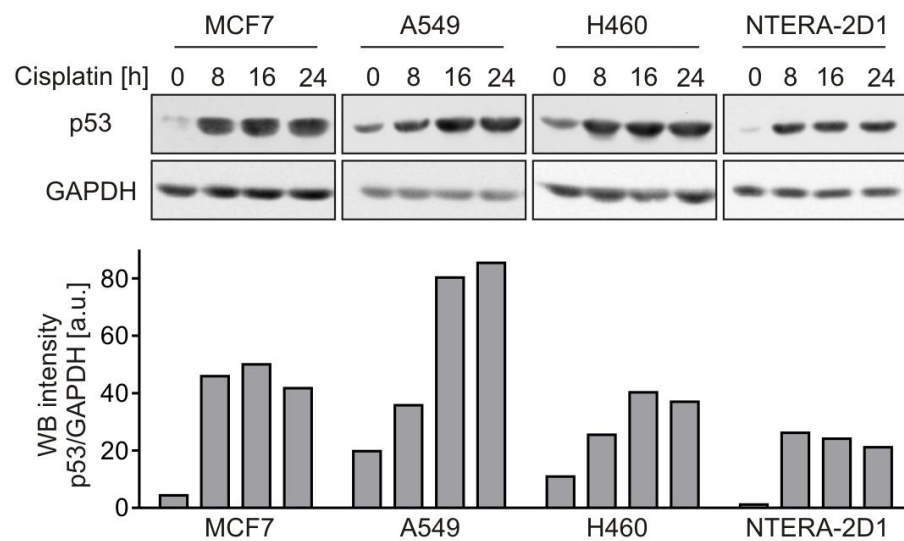not seem to rely on exceptionally high p53 protein levels.

Figure 4: TGCTs do not harbor high levels of p53 protein.

MCF-7, A549, H460 and NTERA-2D1 cell lines were treated with Cisplatin for indicated times and p53 protein levels were compared by Western Blot analysis. Graph reflects intensity of p53 bands determined by densitometry relative to the amount of GAPDH protein.

3.1.4 CHK2 but not ATM, ATR and DNA-PK affect Cisplatin sensitivity

DNA lesions are translated into cellular DNA damage signals by a complex network of protein kinases via phosphorylation (Bensimon et al., 2011). By means of these posttranslational modifications, kinases govern the activity of a multitude of proteins such as p53 to guarantee an effective DNA damage response. Major signal transducers of DNA damage include the PIKKs ATM, ATR and DNA-PK (Shiloh, 2003) as well as the checkpoint kinases CHK1 and CHK2 (Bartek and Lukas, 2003). Phosphorylation events that depend on these kinases can directly or indirectly lead to stabilization of p53 and affect its target gene specificity (Shieh et al., 1997; Chehab et al., 1999; Bartek and Lukas, 2003; Amano et al., 2009). In order to identify signal transducers involved in the proapoptotic response of p53 to Cisplatin-induced DNA damage, the effect of 160 kinase inhibitors on Cisplatin cytotoxicity was examined by MTT assay. Interestingly, out of 160 kinase inhibitors, a CHK2 inhibitor decreased Cisplatin cytotoxicity substantially (Figure 5). Similarly, one ATM inhibitor had an alleviative effect on the cytotoxic effects of Cisplatin treatment, however, this effect was weaker than the effect seen with CHK2 inhibition (Figure 5). Notably, three different DNA-PK inhibitors and an ATM/ATR

inhibitor did not affect the cellular response to Cisplatin (Figure 5). Inhibitors targeting other kinases such as p38, rho kinase, MK2a, ERK, CDK4, casein kinase and GSK-3b were also found to reduce Cisplatin cytotoxicity in NTERA-2D1 cells (Figure 5). These date indicate that CHK2 may have a central role in p53-mediated hypersensitivity of TGCTs. Besides CHK2, ATM and other kinases might also participate in DNA damage signal transduction upon Cisplatin treatment.
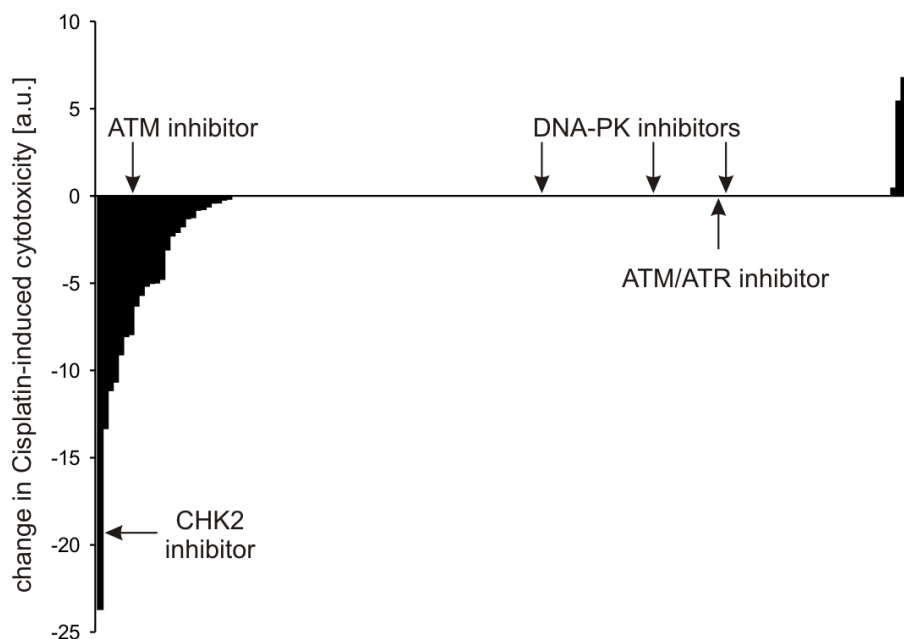

Figure 5: CHK2 inhibition reduces Cisplatin-induced cytotoxicity.

Cells were treated with one of 160 kinase inhibitors at a time for 2 h. Subsequently, Cisplatin was added. Control cells were treated with inhibitor alone. Graph reflects effects of inhibitor pretreatment on Cisplatin-induced cytotoxicity as determined by MTT assay (see 2.2.3).

In order to verify the results obtained from kinase inhibitor experiments, RNAi-mediated gene silencing was used to diminish expression of selected kinases. Single and combinatorial knockdowns of ATM, ATR and DNA-PK were performed in NTERA-2D1 cells. Western Blot analysis demonstrated that all siRNAs efficiently repressed their target genes and blocked protein synthesis (Figure 6A).

A

B
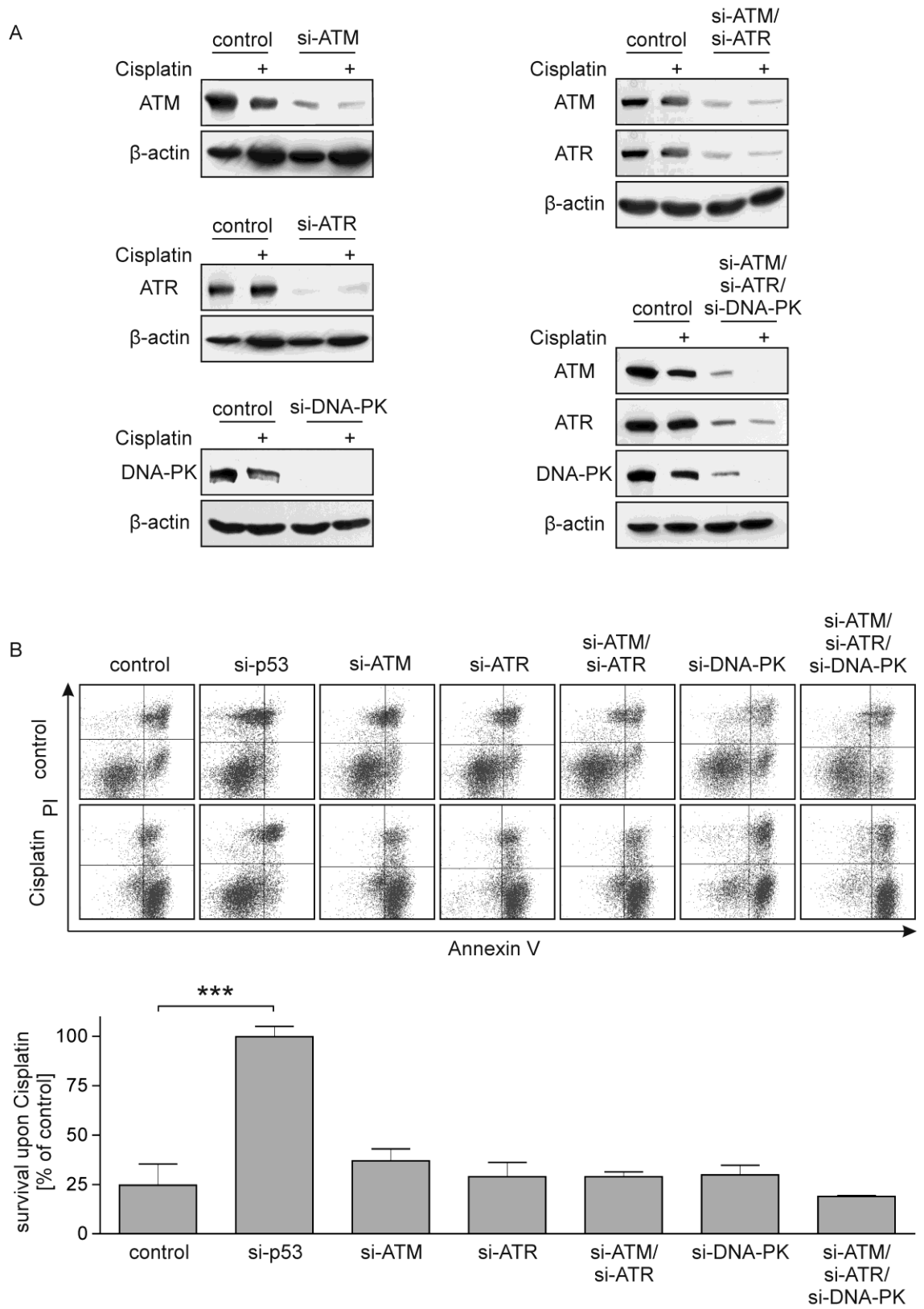
Figure 6: ATM, ATR and DNA-PK are dispensable for p53-dependent apoptosis upon Cisplatin treatment.
NTERA-2D1 cells were transfected with the indicated siRNAs and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. (A) Verification of knockdowns by Western Blot analysis. (B) Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graph reflects means ±SD of survival upon Cisplatin treatment (lower left quadrant of FACS analysis) relative to corresponding controls (cells treated with the same siRNA but cultivated in absence of Cisplatin) from three independent experiments (***: $p \leq 0.001$).

Transfected cells were treated with Cisplatin for 16 h, stained with Annexin V-FITC and PI and survival was examined by FACS analysis. Interestingly, loss ATM, ATR or DNA-PK, respectively, did not influence Cisplatin sensitivity (Figure 6B). Surprisingly, not even combinatorial knockdown of ATM and ATR or simultaneous knockdown of all three kinases did affect Cisplatin sensitivity (Figure 6B). In contrast, simultaneous knockdown of ATM, ATR and DNA-PK even increased Cisplatin-induced apoptosis (Figure 6B).

It was previously suggested that the serine residues 15 and 20 are key phosphorylation sites contributing to p53's capacity to induce apoptosis upon DNA damage (Unger et al., 1999; Amano et al., 2009). Due to the predominantly proapoptotic p53 response of EC cells to Cisplatin, these phosphorylation sites were analyzed in ATM-, ATR- or DNA-PK-depleted NTERA-2D1 cells to clarify if phosphorylation occurs independently of these kinases. For this, serine 15 and serine 20 were detected by Western Blot analysis using phospho-specific antibodies. As expected, single knockdown of ATM or ATR, respectively, reduced phosphorylation at serine 15 (Figure 7). Double knockdown of both kinases almost prevented phosphorylation at this site, whereas phosphorylation of serine 20 was dependent on all three kinases (Figure 7). Interestingly, despite a significant reduction in phosphorylation at both serine residues, p53 accumulation was not compromised (Figure 7). These data indicate that phosphorylation at serine 15, serine 20 or other residues by ATM, ATR and DNA-PK may not be critical for p53's stabilization as well as its capability to induce apoptosis upon DNA damage in the context of EC cells.
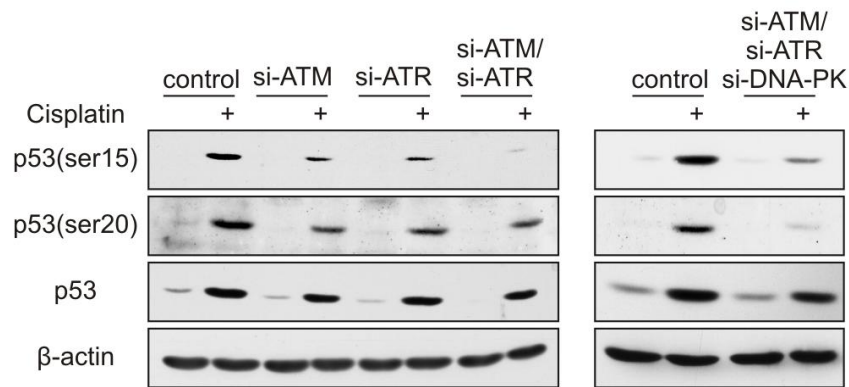
Figure 7: Phosphorylation at serines 15 and 20 is dispensable for p53 stabilization upon Cisplatin treatment.
NTERA-2D1 cells were transfected with the indicated siRNAs and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. p53 protein as well as phosphorylation at serines 15 and 20 was assessed by Western Blot analysis.

Next, NTERA-2D1 cells were depleted of CHK2 by RNAi-mediated silencing to verify the effect of CHK2 inhibition on Cisplatin toxicity (Figure 5). Importantly, loss of CHK2 significantly reduced Cisplatin sensitivity (Figure 8B). In spite of its impact on DNA damage-induced apoptosis induction, CHK2 depletion did not substantially affect p53 protein stability since p53 accumulated to a similar extent in CHK2-depleted cells and control cells (Figure 8A). Nevertheless, these data confirm a role of CHK2 in the sensitivity of TGCT cells to Cisplatin.
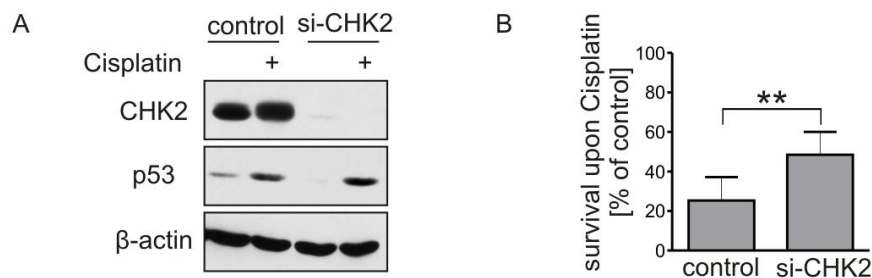
Figure 8: CHK2 is involved in Cisplatin hypersensitivity.
NTERA-2D1 cells were transfected with a CHK2-targeting siRNA and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. (A) Analysis of CHK2 and p53 protein levels by Western Blot. (B) Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graph reflects means ±SD of survival upon Cisplatin treatment relative to corresponding controls (cells treated with the same siRNA but cultivated in absence of Cisplatin) from four independent experiments (**: $p \leq 0.01$).

3.1.5   Noxa and Puma play a major role in Cisplatin hypersensitivity

To further explore the mechanisms underlying the proapoptotic capacity of p53 in TGCTs, a subset of its transcriptional targets involved in apoptosis induction and cell cycle arrest was screened for induction upon Cisplatin treatment.
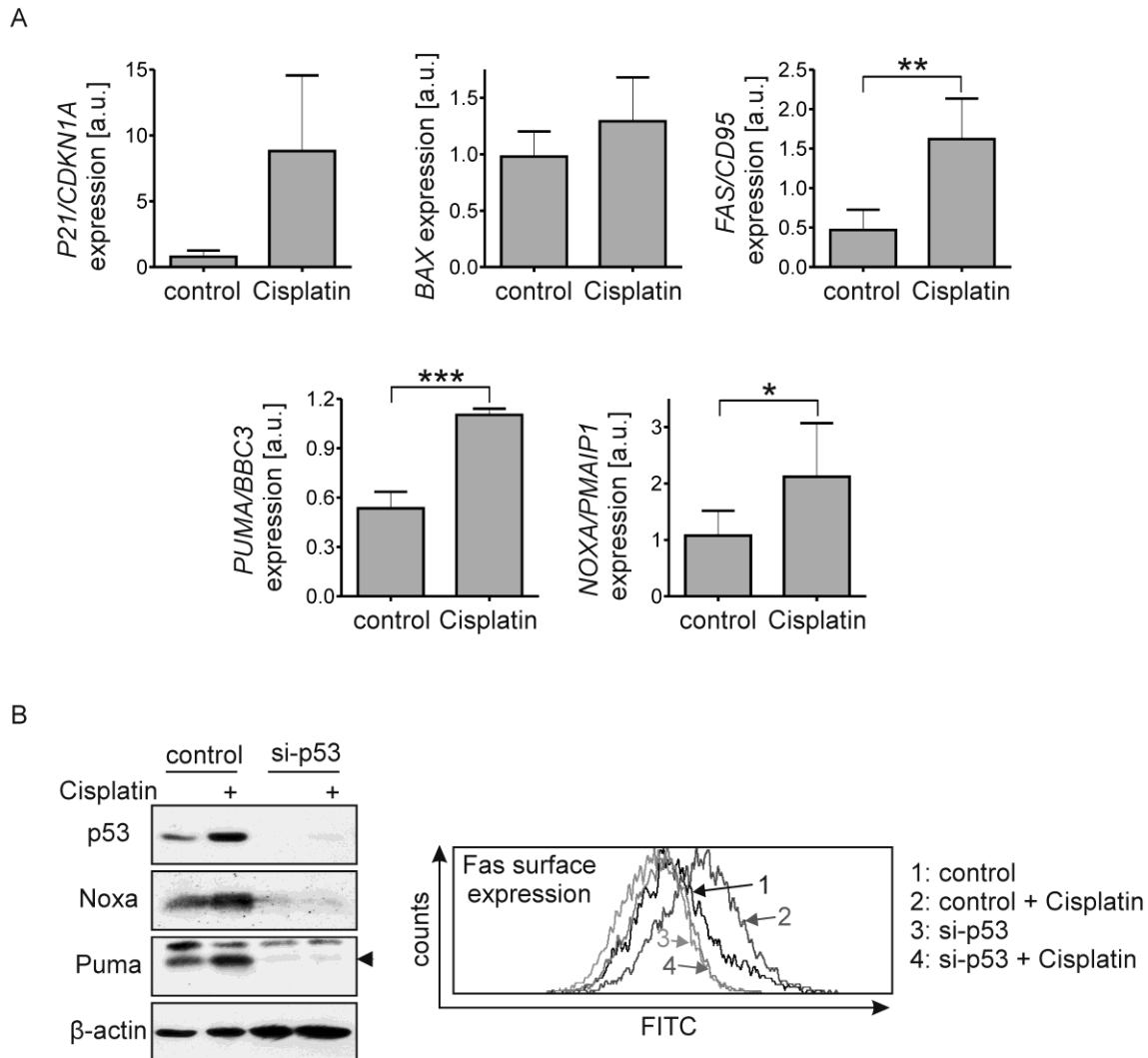
A

B

Figure 9: *NOXA/PMAIP1*, *PUMA/BBC3* and *FAS/CD95* mRNA and protein are induced upon Cisplatin treatment in a p53-dependent manner.

(A) NTERA-2D1 cells were treated with Cisplatin for 8 h. RNA was prepared and transcribed to cDNA. Expression of the p53 target genes *P21/CDKN1A*, *BAX*, *NOXA/PMAIP1*, *PUMA/BBC3* and *FAS/CD95* was determined by SYBR green-based qPCR. Graphs reflect means ±SD of mRNA levels normalized to *GAPDH* from three independent experiments (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$). (B) NTERA-2D1 cells were transfected with a p53-targeting siRNA and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. Efficacy of p53 knockdown as well as protein levels of Noxa, Puma and Fas/CD95 were analyzed by Western Blot or immunostaining followed by FACS analysis.

P21/CDKN1A, *BAX, FAS/CD95, PUMA/BBC3* and *NOXA/PMAIP1* transcripts were quantified by SYBR green-based qPCR. *P21/CDKN1A* was induced upon Cisplatin treatment (Figure 9A), however, transcript levels were found to be extremely low in NTERA-2D1 cells when compared to fibroblasts and did not lead to appreciable amounts of p21 protein (Figure 10). *FAS/CD95, PUMA/BBC3* and *NOXA/PMAIP1* transcripts were significantly increased upon Cisplatin (Figure 9A), paralleled by an induction at the protein level (Figure 9B). In contrast, *BAX* expression was not significantly changed (Figure 9A). Silencing of p53 led to a diminished induction of Fas/CD95, Puma and Noxa protein upon Cisplatin treatment, confirming that the corresponding genes are transactivated in a p53-dependent manner in EC cells (Figure 9B).
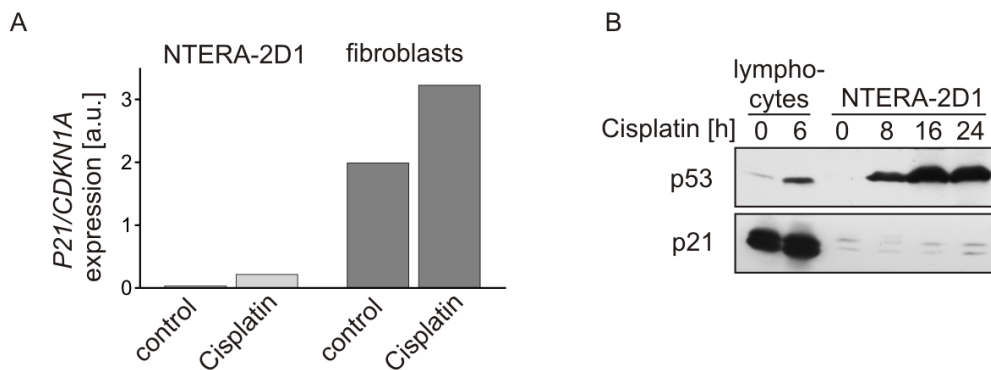
Figure 10: p21 is expressed at low levels in TGCT cells.
(A) NTERA-2D1 cells as well as primary fibroblasts isolated from human lung tissue were treated with Cisplatin for 8 h. RNA was isolated, transcribed to cDNA and analyzed for *P21/CDKN1A* expression by SYBR green-based qPCR. (B) NTERA-2D1 cells and lymphocytes were treated with Cisplatin for indicated times and p53 and p21 protein levels were determined by Western Blot analysis.

A possible role of these proapoptotic proteins in Cisplatin hypersensitivity was examined using RNAi-mediated gene silencing. Efficient repression of Noxa, Puma and Fas/CD95 was verified by Western Blot analysis or immunofluorescence, respectively (Figure 11A, upper panels), followed by FACS-based cell death analysis (Figure 11A, lower panels). Silencing of Puma led to a significant decrease in Cisplatin hypersensitivity in NTERA-2D1 cells (56.8±8.9 % survival in Puma-depleted vs. 24.9±9.5 % in control cells; Figure 11A, lower panels). Importantly, this effect was even more pronounced in Noxa-depleted cells, where survival upon Cisplatin treatment was increased to 68.8±11.3 % compared to 24.9±9.5 % in control cells (Figure 11A, lower panels). Moreover, double knockdown of

both Noxa and Puma almost mimicked the effect seen in p53-depleted cells (79.9±11.0 %
survival in Noxa- and Puma-depleted vs. 24.4±14.4 % in control cells; Figure 11A, lower
panels). In contrast, depletion of Fas/CD95 did not affect Cisplatin sensitivity significantly
(Figure 11A, lower panels). A similar picture was seen with 2102EP cells (Figure 11B),
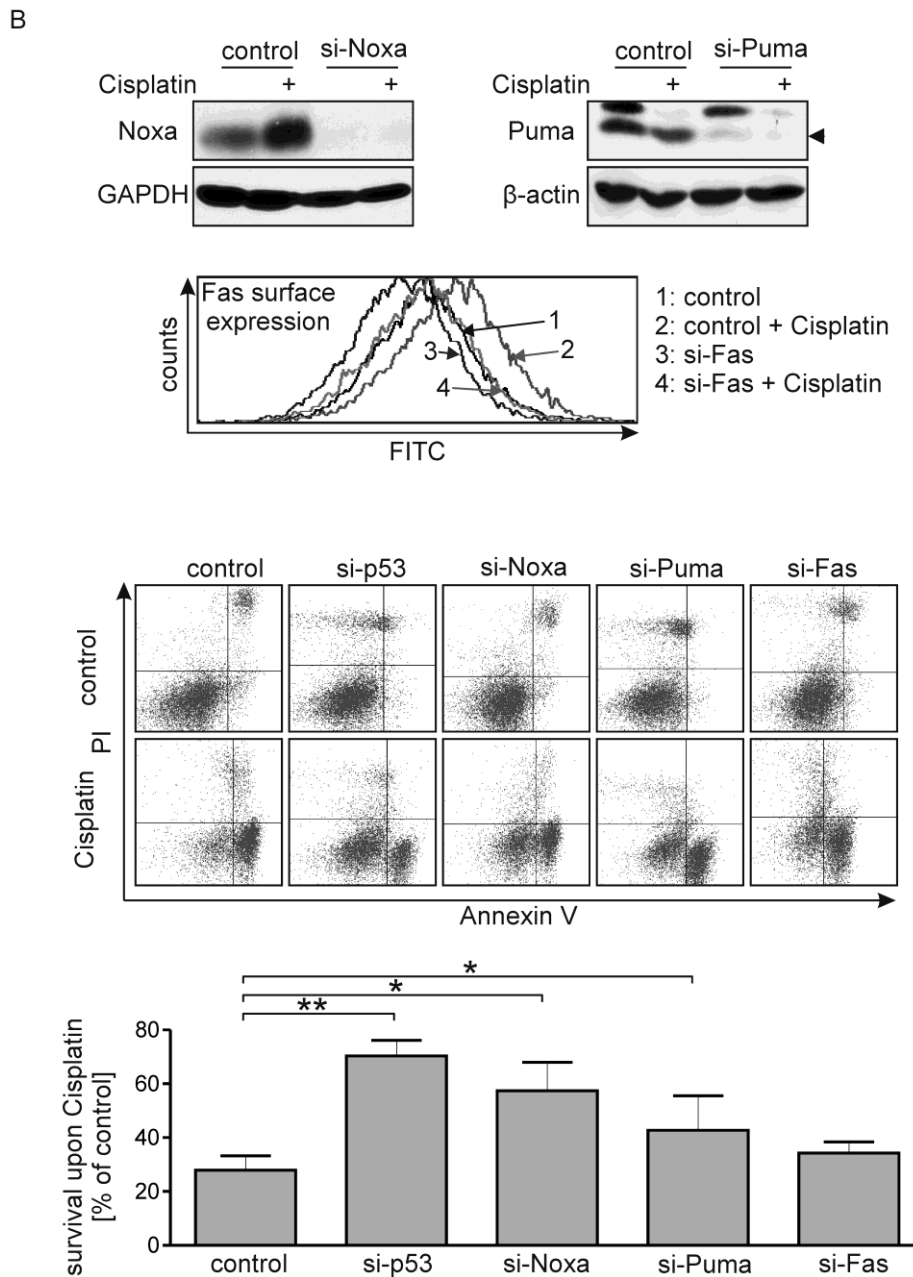confirming an important role of Noxa and Puma, but not Fas/CD95 in Cisplatin sensitivity.

A
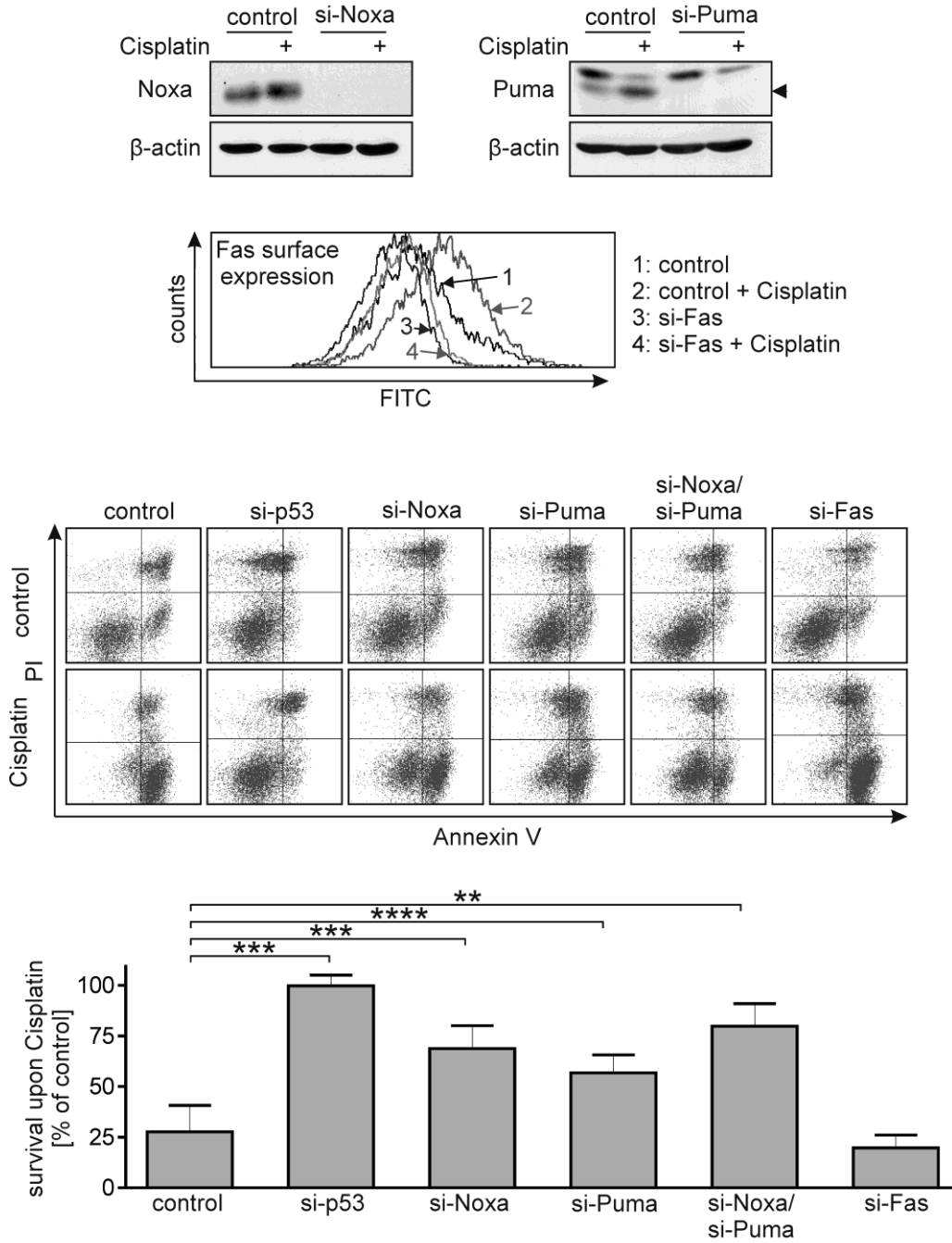
Figure 11: Noxa and Puma are important mediators of p53-dependent apoptosis induction.
NTERA-2D1 (A) or 2102EP (B) cells, respectively, were transfected with the indicated siRNAs and incubated for 48 h prior to Cisplatin treatment (16 h). Controls reflect cells treated with non-targeting siRNA. Validation of knockdown efficiency was done by Western Blot analysis (Noxa and Puma) or by evaluation of surface expression using a FITC-labeled antibody and flow cytometry (Fas/CD95). Survival upon Cisplatin treatment was measured by Annexin V-FITC and PI staining (lower left quadrant of FACS analysis), followed by flow cytometry analysis. Graph reflects means ±SD of survival upon Cisplatin treatment relative to corresponding controls (cells treated with the same siRNA but cultivated in absence of Cisplatin) from three independent experiments (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001).

3.1.6 TGCT hypersensitivity is not restricted to genotoxic agents

The amount of p53 protein was found to be highly correlated with Cisplatin-induced cell death and stabilization of p53 occurred independently of the major DNA damage signal transducers (Figure 3 and Figure 6). In addition, it was previously demonstrated that TGCTs do not only exhibit an exclusive sensitivity to genotoxic agents such as Cisplatin but are also readily eliminated by Nutlin-3, a DNA damage-independent activator of p53 (Bauer et al., 2010; Li et al., 2010). Thus, accumulation of p53 seems to be sufficient to launch the apoptotic program in EC cells. To further test this hypothesis, NTERA-2D1 cells were treated with Nutlin-3 or Bortezomib, a proteasome inhibitor which was shown to cause accumulation of p53 (Williams and McConkey, 2003). Both compounds stabilize p53 in a non-genotoxic manner. As shown by Western Blot analysis (Figure 12A), Nutlin-3 and Bortezomib treatment led to accumulation of p53 similar to Cisplatin treatment in NTERA-2D1 cells. Furthermore, apoptotic cell death upon Nutlin-3 or Bortezomib treatment, respectively, was comparable to cell death seen upon Cisplatin treatment (Figure 12B).
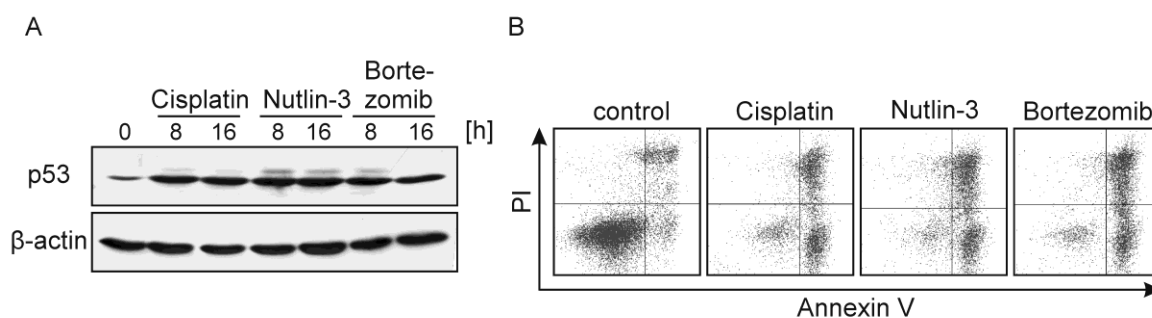

Figure 12: TGCT cells are hypersensitive to non-genotoxic p53 inducers.
(A) NTERA-2D1 cells were treated with Cisplatin, Nutlin-3 or Bortezomib, respectively, for indicated times and p53 protein levels were analyzed by Western Blot analysis. (B) Cells were treated with Cisplatin for 24 h and stained with Annexin V-FITC and PI to analyze apoptosis by flow cytometry.

Importantly, neither Nutlin-3 nor Bortezomib elicited phosphorylation of p53 at serine residues 15 and 20 as seen upon Cisplatin treatment confirming the DNA damage-independent character of p53 accumulation (Figure 13).
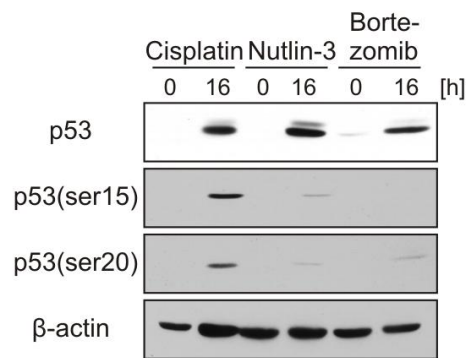
Figure 13: Nutlin-3 and Bortezomib do not induce phosphorylation of p53 at serine residues 15 and 20.
NTERA-2D1 cells were treated with Cisplatin, Nutlin-3 or Bortezomib, respectively (16 h). The amount of p53 protein as well as phosphorylation at serine 15 and serine 20 were assessed by Western Blot analysis.
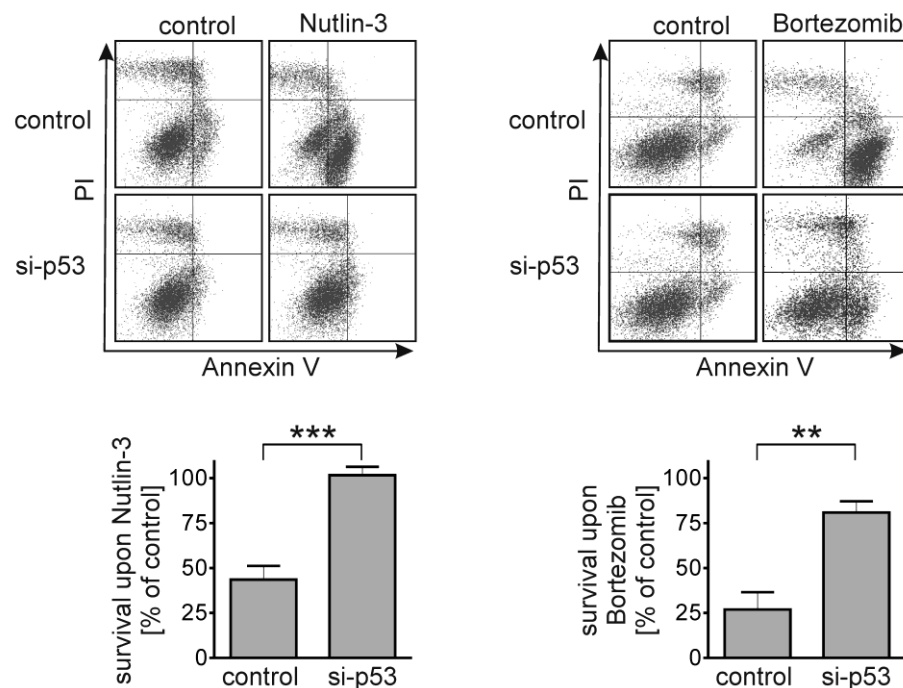

Figure 14: Accumulation of p53 is sufficient to induce apoptosis in TGCTs.
NTERA-2D1 cells were transfected with a p53-targeting siRNA and incubated for 48 h prior to Nutlin-3 or Bortezomib treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graphs reflect means ±SD of survival upon Nutlin-3 or Bortezomib treatment (lower left quadrant of FACS analysis) relative to corresponding controls (cells treated with the same siRNA but cultivated in absence of Cisplatin) from three independent experiments (**: $p \leq 0.01$, ***: $p \leq 0.001$).

These data lead to the notion that EC cells are sensitive to p53 accumulation in general. To strengthen this hypothesis, a similar approach to that described in 3.1.2 was used and survival of NTERA-2D1 cells upon Nutlin-3 or Bortezomib treatment in the absence and presence of p53 was quantified. Importantly, RNAi-mediated knockdown of p53 was sufficient to prevent Nutlin-3-induced apoptosis (101.7±4.6 % survival in p53-depleted vs. 43.6±7.6 % in control cells, Figure 14) and reduced Bortezomib-induced apoptosis significantly (81.0±6.2 % survival in p53-depleted vs. 27.0±9.6 % in control cells, Figure 14).

It is of note that the close dose-response relationship between the amount of p53 protein and cell death observed upon Cisplatin treatment (Figure 3) persists when p53 accumulated independently of DNA damage in cells treated with Nutlin-3 (Figure 15).
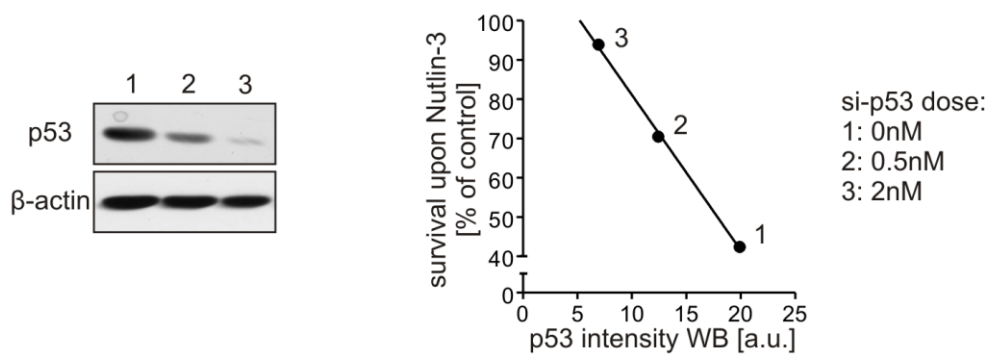

Figure 15: Close dose-response relationship between the amount of p53 protein and Nutlin-3-induced cell death.
NTERA-2D1 cells were transfected with indicated amounts of a p53-targeting siRNA and incubated for 48 h prior to Nutlin-3 treatment (16 h). p53 protein levels were analyzed by Western Blot. Densitometric analysis was performed to measure p53 protein levels. Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graph reflects survival upon Nutlin-3 treatment relative to corresponding controls vs. p53 protein levels.

Since accumulation of p53 upon treatment with Cisplatin as well as its accumulation in a non-genotoxic manner leads to induction of the apoptotic program in TGCTs, a reduced DNA repair capacity might not be a major requirement for hypersensitivity as previously proposed (Köberle et al., 1997). This is supported by the fact that prevention of apoptosis induction by the caspase inhibitor Z-VAD-FMK allowed the removal of DNA adducts formed by Cisplatin (Figure 16), a process that is usually preceded by the onset of apoptosis in EC cells.
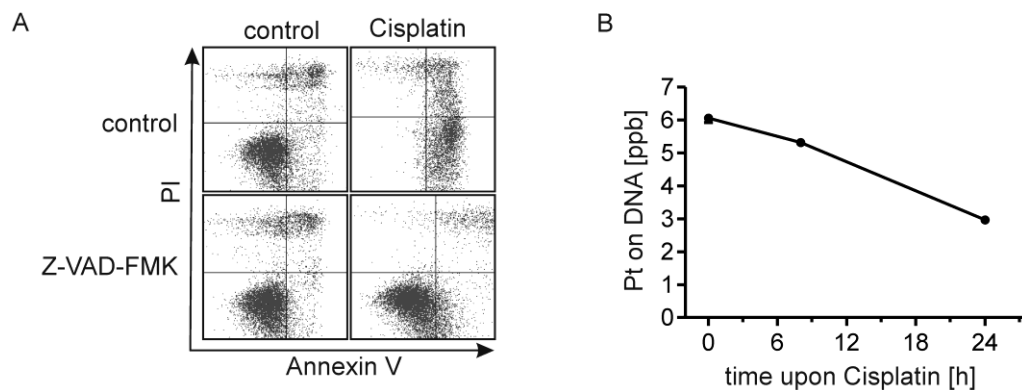
Figure 16: NTERA-2D1 cells are capable of removing Pt adducts from DNA.

(A) NTERA-2D1 cells were treated with Z-VAD-FMK (2 h) prior to Cisplatin exposure. Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry after 24 h. (B) NTERA-2D1 cells were pre-treated with 50 µM Z-VAD-FMK (2 h) and incubated with 30 µM Cisplatin (2 h). Subsequently, cells were washed and incubated in fresh medium containing Z-VAD-FMK for the indicated time period, DNA was isolated and Pt was quantified by inductively-coupled-plasma mass-spectrometry (ICP MS) (data kindly provided by Dr. Christiane Alber (Alber, 2011)).

These data indicate that the cellular Cisplatin response in TGCTs is dictated by a predominantly proapoptotic p53 response involving the downstream effectors Noxa and Puma. Moreover, TGCTs are characterized by a general hypersensitivity to p53 activation.

3.2   The p53 response in the Oct-4-mediated pluripotent context of TGCTs

EC represent the pluripotent entity of TGCTs and are characterized by expression of Oct-4, a key regulator of pluripotency (De Jong and Looijenga, 2006). Differentiation by retinoic acid (RA), a process in which Oct-4 expression is turned off (Houldsworth et al., 2002), was shown to reduce Cisplatin sensitivity in EC cells (Timmer-Bosscha et al., 1998). Similarly, loss of Oct-4 causes a significant reduction of Cisplatin sensitivity and was proposed to account for acquired Cisplatin resistance in refractory tumors (Mueller et al., 2006; Koster et al., 2010). Due to the central role of p53 in TGCT hypersensitivity demonstrated above, subsequently, p53 activity was examined with respect to differentiation and Oct-4 status.

3.2.1   The differentiation status determines the sensitivity to p53 activation

NTERA-2D1 cells can be differentiated along a neuronal lineage by RA (Andrews, 1984) which is associated with a decrease in Cisplatin sensitivity (Timmer-Bosscha et al., 1998). Since TGCTs were shown to be sensitive to p53 activation in general, a possible impact of differentiation on induction of apoptosis by the non-genotoxic p53 activators Nutlin-3 and Bortezomib was investigated. For this, NTERA-2D1 cells were differentiated with RA and treated with Cisplatin as well as the p53 activators Nutlin-3 and Bortezomib. Subsequently, cell death was determined by flow cytometry. Short-term differentiation for 48 h led to a tremendous decrease in hypersensitivity to non-genotoxic p53 activators and Cisplatin (Figure 17).
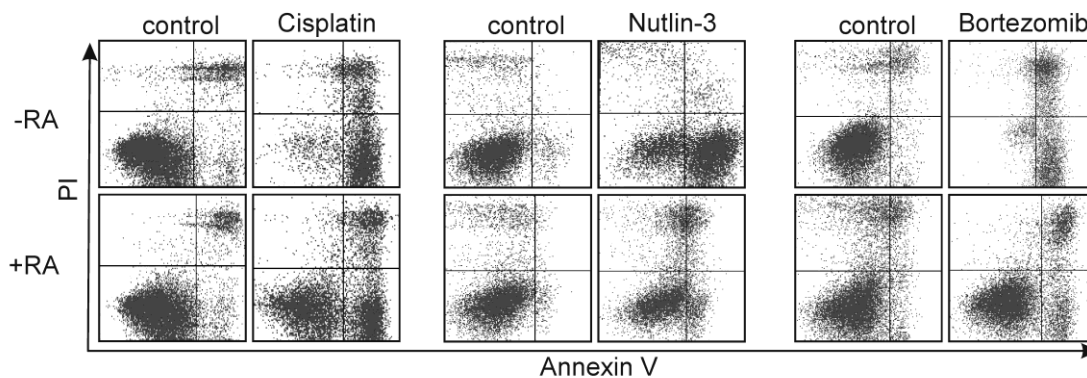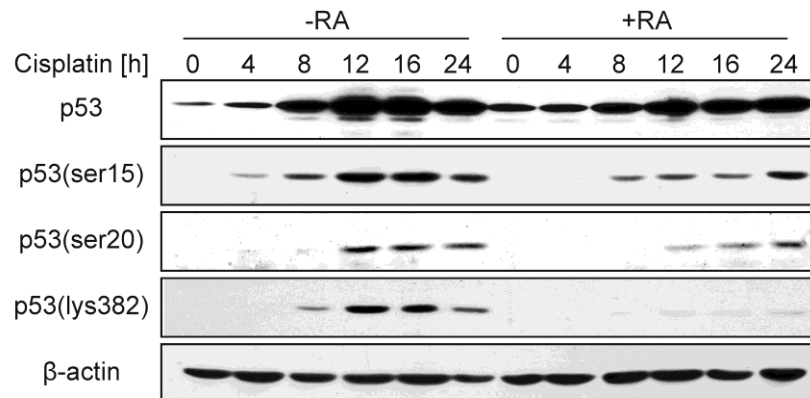

Figure 17: Differentiation decreases TGCT sensitivity to p53 activation.
NTERA-2D1 cells were short-term differentiated with RA for 48 h prior to drug treatment. Subsequently, cells were incubated with Cisplatin, Nutlin-3 or Bortezomib (16 h) and stained with Annexin V-FITC and PI in order to analyze cell death by flow cytometry.

Since differentiation abolishes p53's capacity to induce apoptosis, possible changes in the accumulation of p53 and its posttranslational modifications were compared to undifferentiated NTERA-2D1 cells by Western Blot analysis. p53 accumulation upon Cisplatin treatment was observed to be similar in differentiated vs. undifferentiated cells and no major changes were seen concerning phosphorylation at serine residues 15 and 20 (Figure 18A). However, acetylation at lysine 382, a modification that was previously suggested to direct p53 to promoters of apoptotic genes (Puca et al., 2009) was almost completely abrogated upon short-term differentiation (Figure 18A). To clarify whether the

proapoptotic activity of p53 could be restored when acetylation at lysine 382 is rescued, differentiated NTERA-2D1 cells were treated with the HDAC inhibitor SAHA (Marks and Breslow, 2007). As shown in Figure 18B (left panel), SAHA restored acetylation at lysine 382, however, when treated with Cisplatin, no sensitization of these cells was observed as compared to differentiated control cells (Figure 18B, right panel).
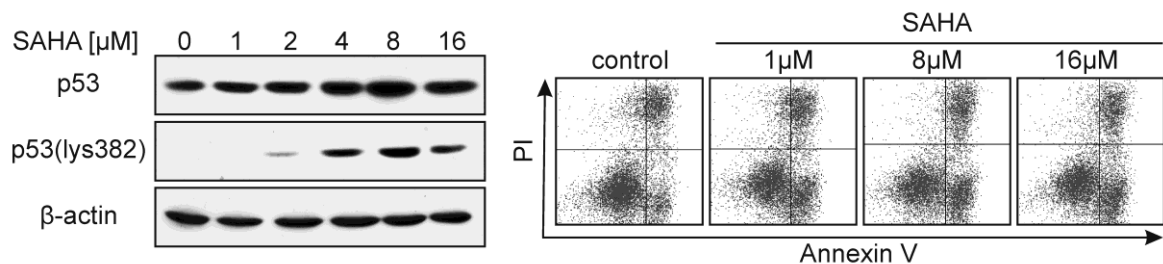
A

| Cisplatin [h] | 0 | 4 | 8 | 12 | 16 | 24 | 0 | 4 | 8 | 12 | 16 | 24 |

(Panels: p53, p53(ser15), p53(ser20), p53(lys382), β-actin; -RA and +RA)

B

SAHA [µM] 0 1 2 4 8 16

(Panels: p53, p53(lys382), β-actin)

SAHA: control, 1µM, 8µM, 16µM (PI vs Annexin V flow cytometry)

Figure 18: Changes in p53 accumulation and posttranslational modifications do not occur upon short-term differentiation.

(A) NTERA-2D1 cells were differentiated with RA, treated with Cisplatin for indicated times and analyzed by Western Blot for accumulation of p53 and posttranslational modifications at serine residues 15 and 20 as well as at lysine 382 in comparison to undifferentiated control cells. (B) Differentiated cells were pre-treated with indicated concentrations of the HDAC inhibitor SAHA for 2 h followed by Cisplatin treatment. Cells were stained with Annexin V-FITC and PI and analyzed for cell death by flow cytometry. Protein levels of p53 and acetylation at lysine 382 were analyzed by Western Blot.

To examine possible changes in p53 target gene expression, microarray analysis was done. 161 *bona fide* p53 target genes (Wei et al., 2006; Riley et al., 2008) were screened for differential regulation by Cisplatin in differentiated and undifferentiated cells. Therefore, untreated NTERA-2D1 cells and cells treated with RA for 48 h were incubated with

Cisplatin for 6 h. From 161 annotated p53 target genes, 51 were regulated at least 1.5fold by Cisplatin treatment. Out of these, 12 were related to apoptosis induction. Interestingly, besides *IER3*, *NOXA/PMAIP1* and *PUMA/BBC3* transcripts which were suggested to play an important role in TGCT hypersensitivity (Figure 11) were found to be expressed at lower levels in differentiated cells treated with Cisplatin compared to control cells (Figure 19). These data imply that the predominantly proapoptotic p53 response in TGCT depends on the pluripotent context of EC cells and suggests a differential expression of *NOXA/PMAIP1* and *PUMA/BBC3* as a characteristic of these cells.
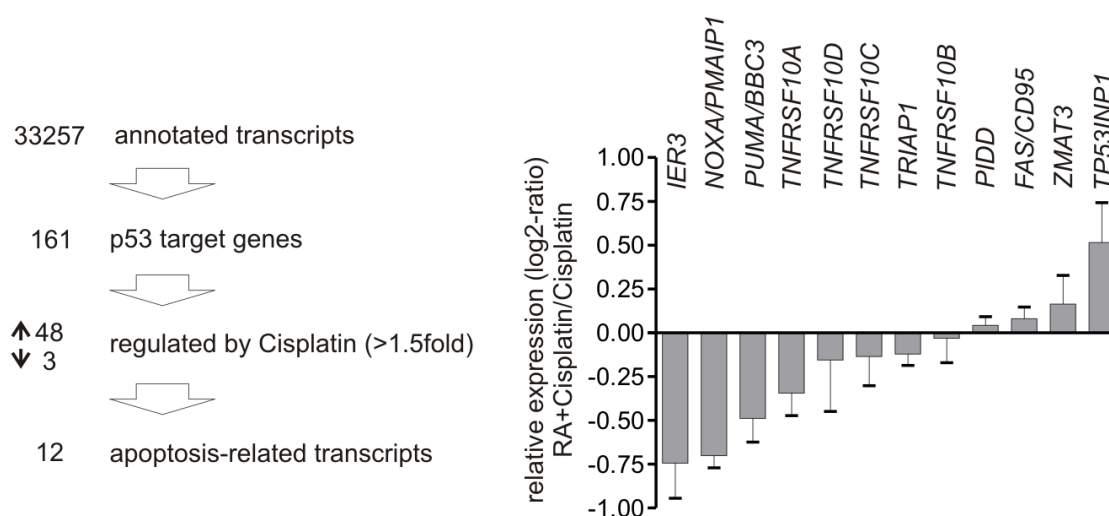

Figure 19: Cisplatin-induced mRNA levels of *NOXA/PMAIP1* and *PUMA/BBC3* are reduced in differentiated cells.

NTERA-2D1 cells were differentiated with RA and, together with undifferentiated control cells, treated with Cisplatin for 6 h. RNA was isolated and applied to microarray analysis to assess global gene expression. Left panel: selection criteria for Cisplatin-induced p53 target genes related to apoptosis. Right panel: expression levels of the 12 apoptosis-related p53 targets upon Cisplatin treatment were compared to cells differentiated prior to Cisplatin treatment. Graph reflects means ±SD from three independent experiments.

3.2.2   TGCT hypersensitivity depends on Oct-4

In addition to changes in p53 activity triggered by differentiation, a possible direct influence of Oct-4 on p53 was investigated. At first, analogous to the experiments described in Figure 17, it was tested whether Oct-4 is required for the general sensitivity of TGCTs to p53 activation. For this, NTERA-2D1 cells were depleted of Oct-4 by RNAi-

mediated gene silencing and treated with Nutlin-3. Efficiency of Oct-4 knockdown was verified by Western Blot analysis (Figure 20A). Oct-4 depletion reduced Nutlin-3-induced apoptosis to a degree comparable to Cisplatin-induced cell death (Figure 20B). This observation is in line with the results observed upon short-term differentiation (Figure 17) and emphasizes a link between the pluripotent cellular context mediated by Oct-4 and efficient induction of apoptosis by p53.
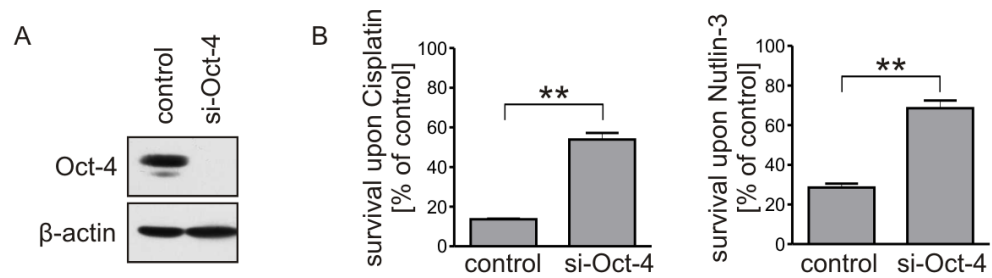

Figure 20: p53-mediated hypersensitivity depends on Oct-4.
NTERA-2D1 cells were transfected with an Oct-4-targeting siRNA and incubated for 48 h prior to Cisplatin or Nutlin-3 treatment (16 h). Controls reflect cells treated with a non-targeting siRNA. (A) Verification of Oct-4 knockdown by Western Blot analysis. (B) Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graphs reflect means ±SD of survival upon Cisplatin or Nutlin-3 treatment (lower left quadrant of FACS analysis) relative to corresponding controls from three independent experiments (**: $p \leq 0.01$).

3.2.3   p53 activity in the absence and presence of Oct-4

In order to clarify if Oct-4 modulates the p53 response in pluripotent EC cells, accumulation and activity of p53 were analyzed in the presence and absence of Oct-4. In addition to NTERA-2D1 cells, experiments were conducted in 2102EP cells, a cell line that harbors a defective differentiation program (Matthaei et al., 1983). Thus, secondary effects of differentiation initiation upon knockdown of Oct-4 could be excluded. First, transcriptional activity was screened using a qPCR system which allows simultaneous acquisition of 46 *bona fide* p53 target genes (Wei et al., 2006; Riley et al., 2008). Oct-4 expression was diminished by RNAi-mediated gene silencing (Figure 21A). Surprisingly, despite its impact on apoptosis induction, Oct-4 depletion did not affect transactivation by p53 upon Cisplatin treatment in either cell line (Figure 21B).
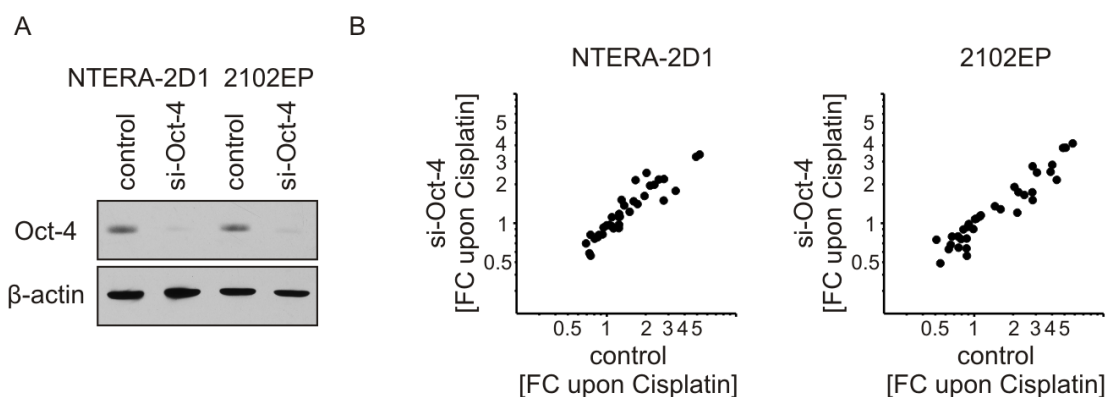
Figure 21: Oct-4 depletion does not influence the transcriptional p53 response to Cisplatin.
NTERA-2D1 and 2102EP cells were transfected with a Oct-4-targeting siRNA and incubated for 48 h prior
to Cisplatin treatment (6 h). Controls reflect cells treated with a non-targeting siRNA. RNA was isolated,
transcribed to cDNA and applied to qPCR analysis using primers for 46 *bona fide* p53 target genes (Table 6).
Graph shows means of fold changes (FC) upon Cisplatin treatment of detected transcripts from three
independent experiments. Dots depict fold change values of each transcript.

To investigate a possible role of cytoplasmic p53 in apoptosis induction, Cisplatin-induced
accumulation and cellular localization of p53 were studied in the presence and absence of
Oct-4. For this, nuclear and cytoplasmic protein lysates of NTERA-2D1 and 2102EP cells
were obtained and analyzed by Western Blot where tubulin (cytoplasm) and TBP (nucleus)
served as controls for efficient subcellular fractionation. In both cell lines, accumulation of
p53 was found to be similar in both compartments upon Cisplatin treatment (Figure 22).
Moreover, neither p53 accumulation nor its localization was significantly influenced by
knockdown of Oct-4 (Figure 22).

Together, these results indicate that rather than directly altering p53 activity, Oct-4
provides an apoptosis-prone cellular context that augments the proapoptotic efficacy of
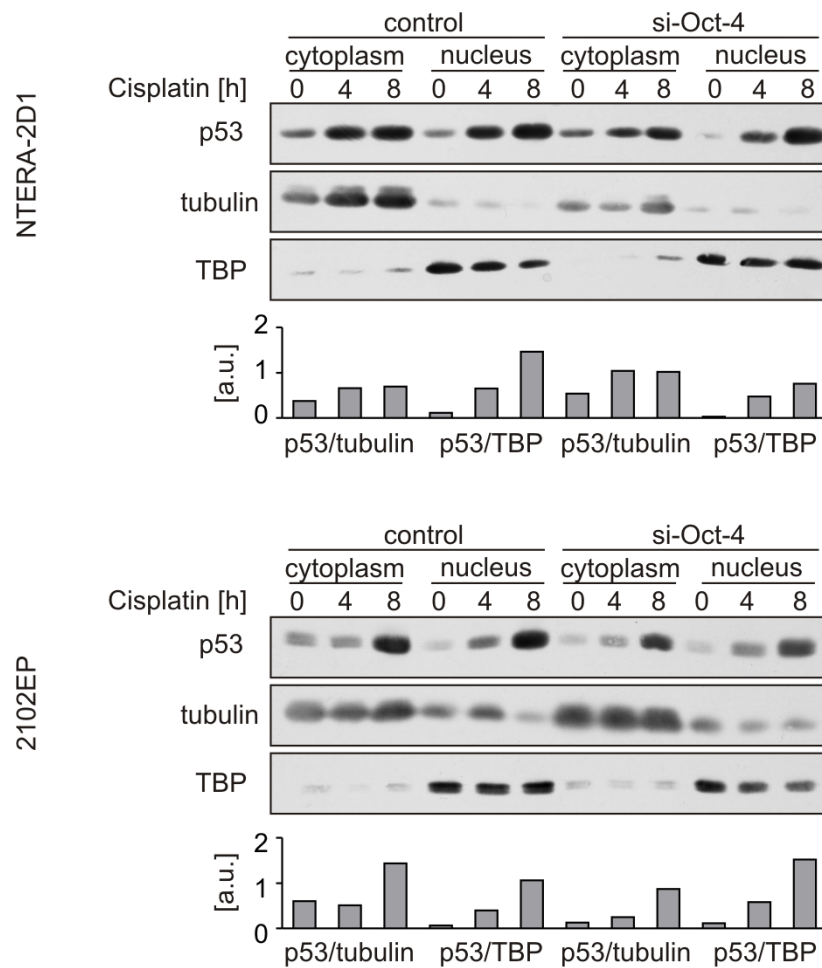p53.

Figure 22: Oct-4 depletion does not affect p53 accumulation and localization.
NTERA-2D1 and 2102EP cells were transfected with Oct-4-targeting siRNA and incubated for 48 h. Controls reflect cells treated with non-targeting siRNA. Cells were then treated with Cisplatin for indicated times and subjected to subcellular fractionation. p53 protein was analyzed by Western Blot along with tubulin, representing the cytoplasmic compartment and TBP representing the nuclear compartment. Graphs reflect densitometric quantification of p53 levels relative to the amount of TBP or tubulin, respectively.

3.3   Oct-4-dependent high constitutive Noxa levels determine Cisplatin sensitivity

The mitochondrial composition of Bcl-2 family proteins has recently been suggested to determine responsiveness to chemotherapy (Ni Chonghaile et al., 2011). Expectedly, Bcl-2 proteins have also been supposed to shape the hypersensitive phenotype of TGCTs. It was

found that antiapoptotic factors such as Bcl-2 and Bcl-x were expressed at low levels in these tumors (Soini and Paakko, 1996; Mayer et al., 2003). Other studies, however, could not confirm a correlation between levels of these proteins and Cisplatin hypersensitivity (Burger et al., 1997; Mueller et al., 2003). Nevertheless, in these studies, proapoptotic BH3-only proteins such as Noxa and Puma were not investigated. However, these proteins mediate Cisplatin hypersensitivity (Figure 11). Hence, an investigation of the Bcl-2 protein composition in the context of pluripotent, Oct-4-positive EC cells, might help uncover what dictates the low apoptotic threshold in TGCT cells.

3.3.1   Bcl-2 family protein levels in Oct-4-positive cells

To investigate the role of the Bcl-2 protein family in TGCT hypersensitivity, constitutive protein levels of the proapoptotic BH3-only proteins Bax, Bak, Noxa, Puma, Bim and Bid as well as the antiapoptotic Bcl-2 family members Bcl-2, Bcl-xL, Bcl-w and Mcl-1 were analyzed in the presence and absence of Oct-4. For this, Oct-4 was silenced by RNAi-mediated knockdown in NTERA-2D1 and 2102EP cells. Lysates were prepared from both Oct-4-depleted and control cells and compared by Western Blot analysis. Notably, Noxa was the only Bcl-2 family protein which could selectively be detected in Oct-4-positive cells (Figure 23). The fact that this was observed in both pluripotent NTERA-2D1 and nullipotent 2102EP cells (Figure 23) suggests that the reduction of Noxa protein is not a secondary effect of differentiation but a direct consequence of Oct-4 depletion. Mcl-1, the major antiapoptotic binding partner of Noxa, was also reduced but still detectable upon Oct-4 knockdown (Figure 23). Moreover, levels of the BH3-only proteins Puma and Bim were found to be decreased especially in 2102EP cells, although the reduction was not as obvious as observed for Noxa (Figure 23). Protein levels of all other Bcl-2 family members analyzed did not exhibit concomitant changes in both cell lines upon Oct-4 depletion (Figure 23).
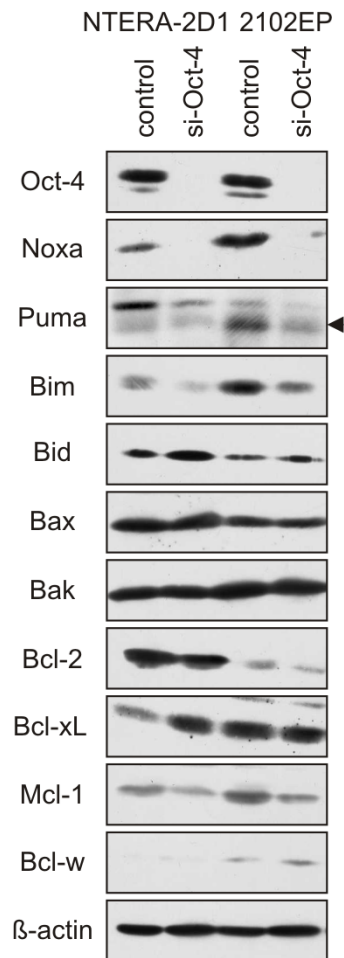
Figure 23: Constitutive protein levels of Noxa, Puma and Bim are dependent on Oct-4.
NTERA-2D1 and 2102EP cells were transfected with an Oct-4-targeting siRNA and incubated for 48 h. Controls reflect cells treated with a non-targeting siRNA. Transfected cells were lysed and subjected to Western Blot analysis to determine protein levels of indicated Bcl-2 family proteins.

3.3.2 Noxa and Puma mediate Cisplatin hypersensitivity

NOXA/PMAIP1 and *PUMA/BBC3* were identified as p53 target genes that play a role in Cisplatin hypersensitivity of TGCTs (Figure 11). The fact that constitutive protein levels of Noxa, Puma and Bim were found to be dependent on Oct-4 suggests that besides transcriptional activation upon Cisplatin, constitutive levels of proapoptotic Bcl-2 family members could play an important role in Oct-4-mediated TGCT hypersensitivity. Therefore, survival upon Cisplatin treatment in Noxa-, Puma- and Bim-depleted cells was compared to survival in cells depleted of Oct-4. All siRNAs reduced the respective protein

levels to a similar degree as monitored by Western Blot analysis (Figure 24A). Interestingly, Noxa knockdown rescued NTERA-2D1 and 2102EP cells from Cisplatin-induced cell death to a similar extend as did Oct-4 knockdown (Figure 24B). A comparable picture was seen with Puma, although in NTERA-2D1 cells, silencing of Puma did not reduce apoptosis as efficiently as did Noxa knockdown (Figure 24B). In contrast, Bim depletion had only minor effects on Cisplatin hypersensitivity (Figure 24B).
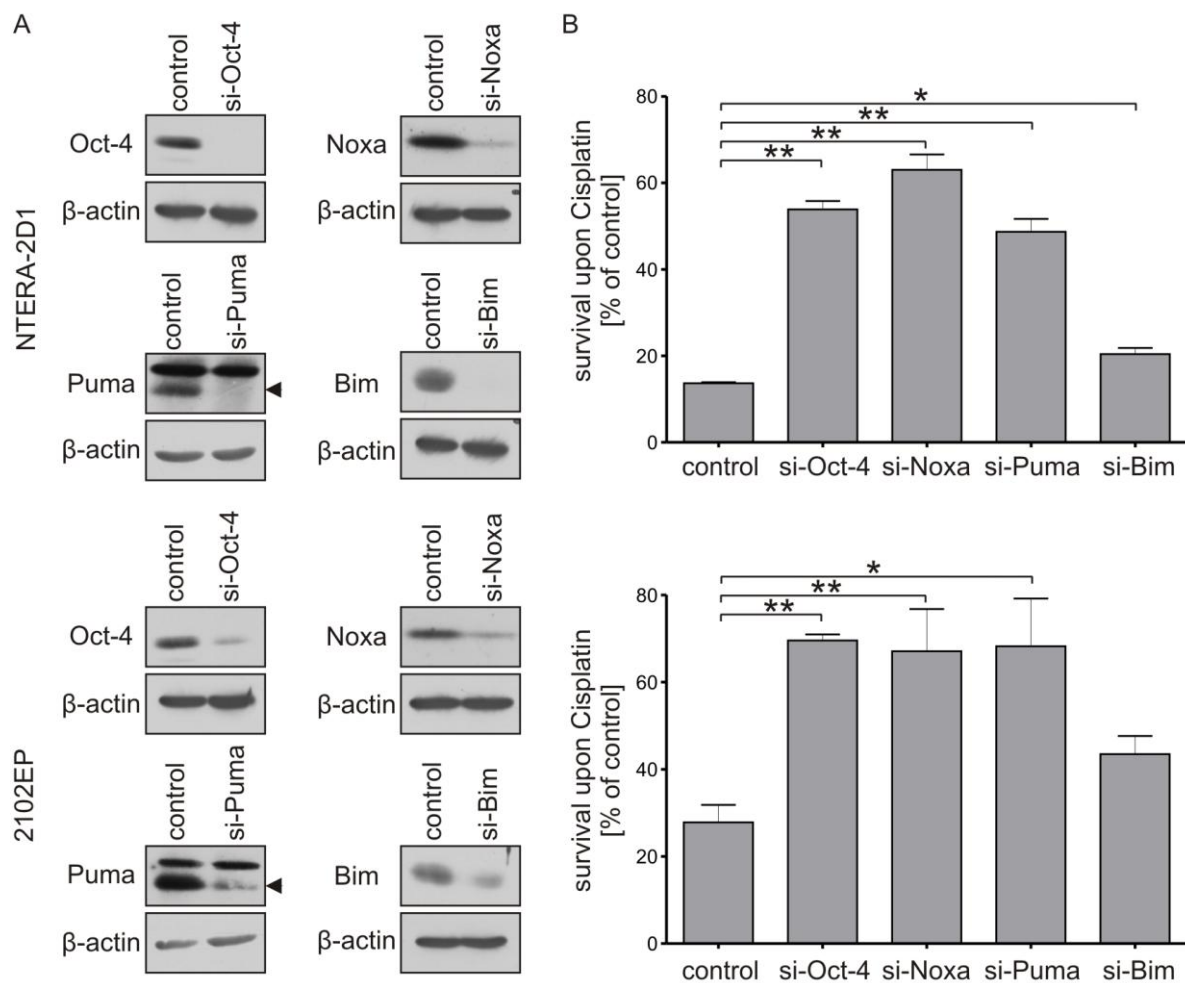

Figure 24: Noxa and Puma depletion reduces Cisplatin hypersensitivity comparable to Oct-4 depletion. NTERA-2D1 and 2102EP cells were transfected with the indicated siRNAs and incubated for 48 h prior to Cisplatin treatment (NTERA-2D1: 16 h; 2102EP: 12 h). Controls reflect cells treated with a non-targeting siRNA. (A) Verification of knockdowns by Western Blot analysis. (B) Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graphs reflect means ±SD of survival upon Cisplatin treatment (lower left quadrant of FACS analysis) relative to corresponding controls from three independent experiments (*: $p \leq 0.05$, **: $p \leq 0.01$).

3.3.3   TGCTs express high constitutive Noxa protein levels

According to the results shown above, differential expression of Noxa and Puma in unstressed, Oct-4-positive TGCT cells might determine their extremely low apoptotic threshold. In order to test this hypothesis, constitutive Noxa and Puma protein levels were compared between Oct-4-positive and Oct-4-depleted TGCT cell lines as well as Oct-4-negative lymphocytes, fibroblasts and cancer cell lines. Interestingly, absolute levels of Noxa protein in Oct-4-positive cells were found to be significantly higher when compared to Oct-4-negative cells (Figure 25). Furthermore, knockdown of Oct-4 in NTERA-2D1 and 2102EP cells reduced Noxa protein to a level comparable to that observed in other cell types (Figure 25). In contrast to Noxa, Puma protein levels were found to be higher only in 2102EP cells (Figure 25).
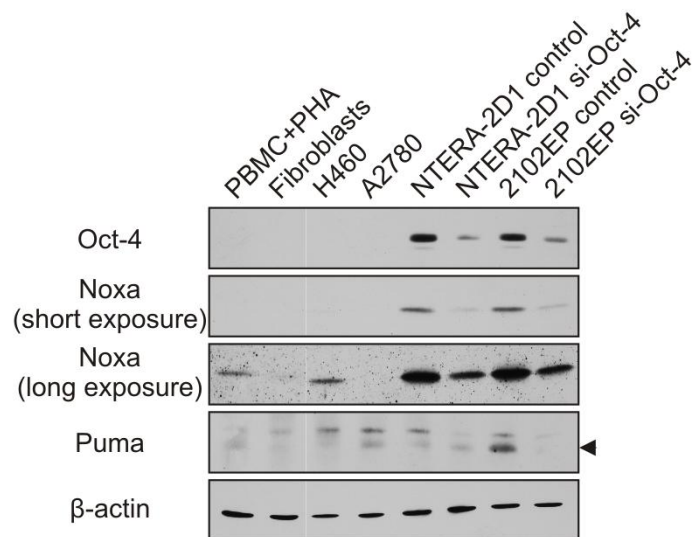

Figure 25: TGCTs are characterized by high constitutive Noxa protein levels.
NTERA-2D1 and 2102EP cells were transfected with an Oct-4-targeting siRNA and incubated for 48 h. Controls reflect cells treated with a non-targeting siRNA. Protein lysates from TGCT cell lines and PBMCs, fibroblasts, H460 and A2780 cells were prepared and Oct-4, Noxa and Puma levels were determined by Western Blot analysis.

To investigate if high constitutive Noxa protein levels are maintained via transcriptional mechanisms in EC cells, *NOXA/PMAIP1* mRNA expression was determined by qPCR. In contrast to *PUMA/BBC3*, *NOXA/PMAIP1* transcript was expressed at significantly higher levels in Oct-4-positive NTERA-2D1 and 2102EP cells when compared to Oct-4-negative

lymphocytes, fibroblasts or other cancer cell lines (Figure 26). Oct-4 knockdown revealed a direct effect of Oct-4 on *NOXA/PMAIP1* mRNA expression (Figure 26), although this effect was less pronounced than at the protein level (Figure 25). Moreover, Oct-4 depletion reduced *NOXA/PMAIP1* transcript in NTERA-2D1 and 2102EP cells to a level comparable to that observed in differentiated cells, further proving that TGCTs maintain high *NOXA/PMAIP1* levels also at the RNA level. *PUMA/BBC3* transcript, on the other hand, was only slightly reduced upon Oct-4 depletion and did not correlate with Oct-4 status (Figure 26).
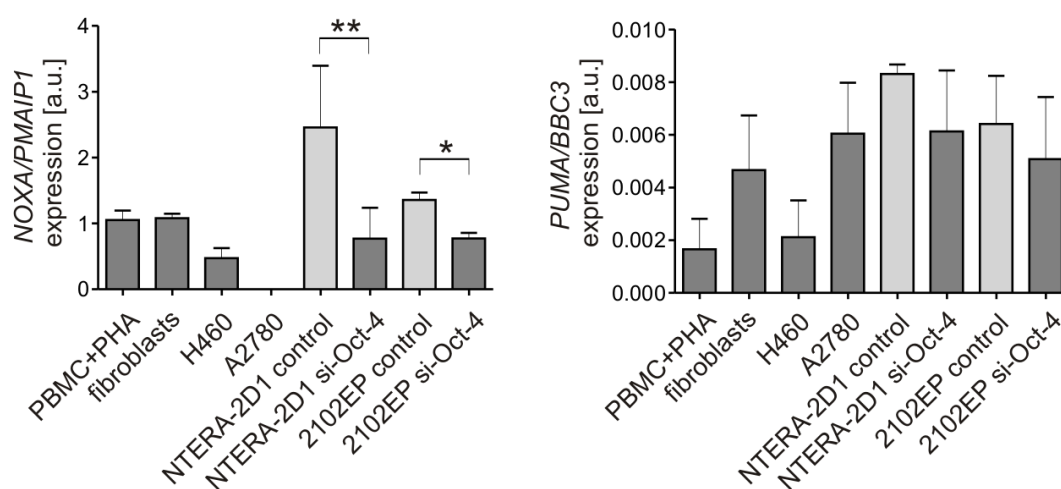

Figure 26: *NOXA/PMAIP1* mRNA is correlated with Oct-4 expression in TGCTs.
NTERA-2D1 and 2102EP cells were transfected with an Oct-4-targeting siRNA and incubated for 48 h prior to analysis. Controls reflect cell treated with a non-targeting siRNA. RNA from TGCT cell lines and PBMCs, fibroblasts, H460 and A2780 cells was obtained, transcribed to cDNA and applied to qPCR analysis in order to determine *NOXA/PMAIP1* and *PUMA/BBC3* transcript levels. Graph reflects means ±SD of *NOXA/PMAIP1* and *PUMA/BBC3* transcript from three independent experiments (*: $p \leq 0.05$, **: $p \leq 0.01$).

Since Oct-4 mainly functions as a transcription factor, it might directly transactivate *NOXA/PMAIP1*. However, the fact that Oct-4 regulates a plethora of target genes (Babaie et al., 2007) and is involved in numerous protein-protein interactions (Pardo et al., 2010), it is also possible that Oct-4 might control *NOXA/PMAIP1* mRNA stability. To address this question, *NOXA/PMAIP1* pre-mRNA expression was examined following knockdown of Oct-4. *NOXA/PMAIP1* mRNA was detected using primers spanning exon-exon junctions to prevent amplification of genomic DNA. Primers for the detection of *NOXA/PMAIP1* pre-mRNA were designed to span intron-exon junctions. As shown in Figure 27,

NOXA/PMAIP1 pre-mRNA was found to be regulated similarly to *NOXA/PMAIP1* mRNA leading to the conjecture that Oct-4 indeed is involved in transactivation of the *NOXA/PMAIP1* gene.
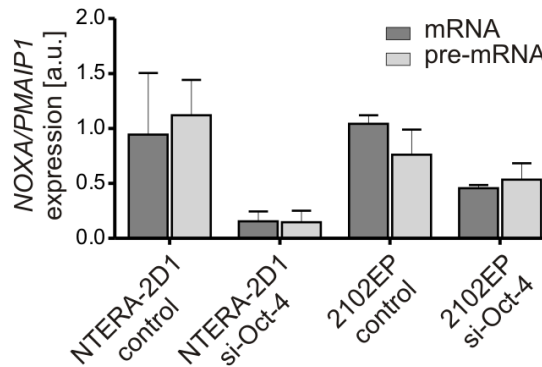

Figure 27: Constitutive *NOXA/PMAIP1* mRNA levels are maintained via Oct-4-dependent transcriptional mechanisms.

Cells were transfected with an Oct-4-targeting siRNA and incubated for 48 h. Controls reflect cells treated with a non-targeting siRNA. RNA was extracted and transcribed to cDNA. Contamination of RNA samples with genomic DNA was excluded by DNase digestion. Expression of *NOXA/PMAIP1* mRNA was detected by qPCR using a primer pair spanning exon-exon junctions. Primers for pre-mRNA detection were designed to span exon-intron junctions. Graphs reflect means ±SD from three experiments. RNA levels were normalized to *ACTB* mRNA.

3.3.4 Noxa protein levels are tightly correlated with Oct-4 status and Cisplatin sensitivity

The preceding experiments suggest a close relationship between Oct-4 status, Cisplatin sensitivity and constitutive Noxa protein levels. In contrast, Puma protein levels were not closely related to Oct-4 despite the important role of Puma in TGCT hypersensitivity (Figure 11 and Figure 24). These results were verified in an extended TGCT cell line panel including 5 undifferentiated, Oct-4-positive cell lines and 5 differentiated, Oct-4-negative cell lines.
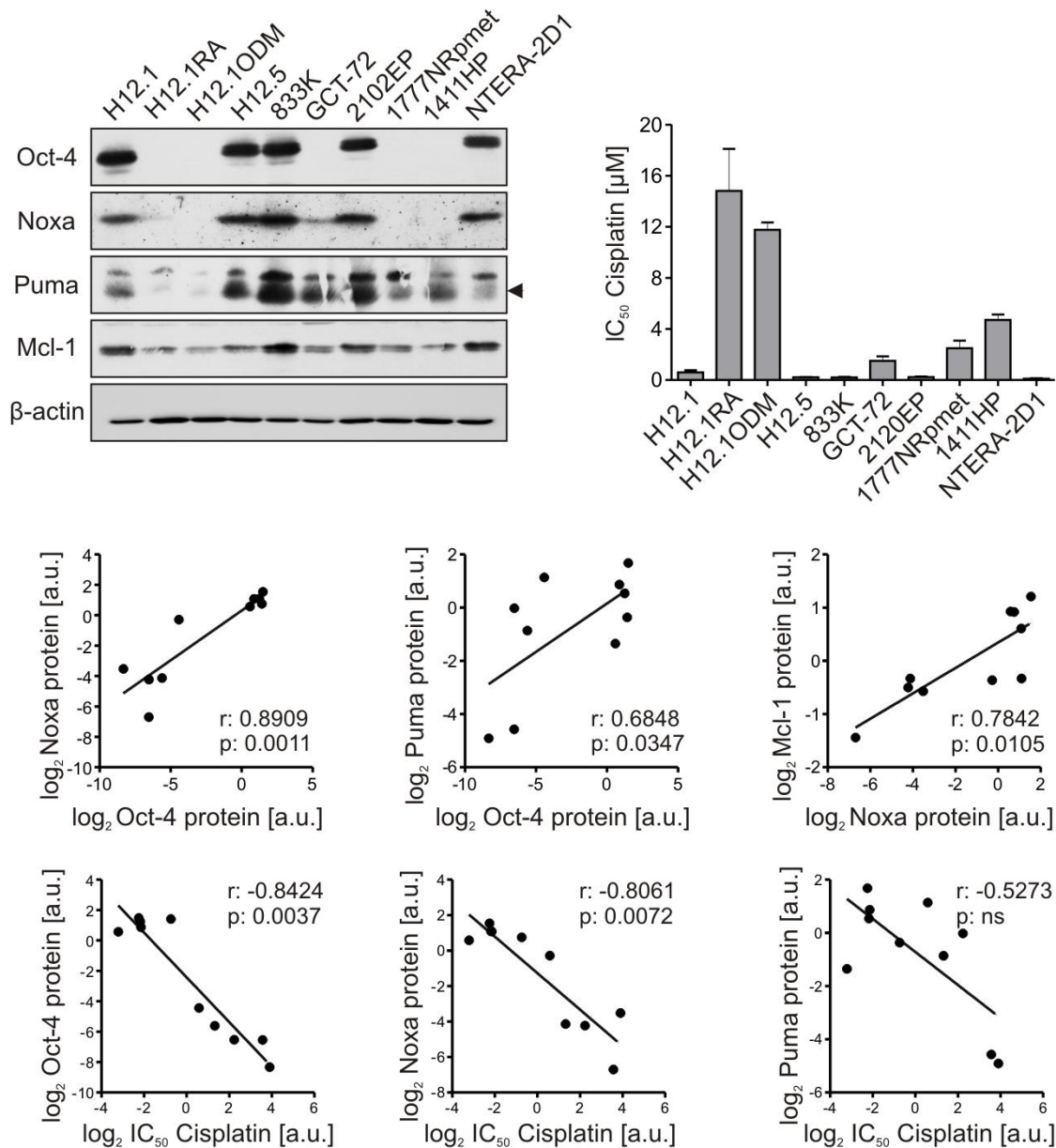
Figure 28: Constitutive Noxa protein levels are highly correlated with Oct-4 status and Cisplatin sensitivity in TGCT cell lines.

Upper left panel: cell lysates from 10 TGCT cell lines were prepared to analyze Oct-4, Noxa, Puma and Mcl-1 protein levels by Western Blot. Upper right panel: IC_{50} of Cisplatin were determined by SRB microculture colorimetric assay. Graph reflects means ±SD from three independent experiments (Data kindly provided by Dr. Thomas Mueller, University of Halle-Wittenberg, Halle). Middle panels: Dot blots reflect correlations of protein levels determined by densitometric analysis. Lower panels: Dot blots show correlation between Oct-4, Noxa and Puma protein levels and IC_{50} of Cisplatin. Correlations are expressed as Spearman rank correlation coefficient (r). p values are given for significant correlations ($p \leq 0.05$).

For this, Cisplatin IC_{50} were determined by SRB microculture colorimetric assay and correlated with the protein levels of Oct-4, Noxa and Puma determined by Western Blot analysis and quantified by densitometry. The previously described correlation between Oct-4 status and Cisplatin sensitivity (Mueller et al., 2006) was confirmed (Spearman r=-0.842, p=0.004; Figure 28). Importantly, Noxa was significantly correlated with Oct-4 expression (Spearman r=0.891, p=0.001; Figure 28) as well as with Cisplatin sensitivity (Spearman r=-0.806, p=0.007; Figure 28). Additionally, Puma protein levels were positively correlated with Oct-4 status, however, this correlation was weaker than for Noxa (Spearman r=0.685, p=0.035; Figure 28) and no significant correlation was seen with Cisplatin sensitivity (Spearman r=-0.527, p>0.05; Figure 28). Interestingly, protein levels of Mcl-1, the antagonistic binding partner of Noxa, were positively correlated with Noxa protein levels (Figure 28) suggesting a simultaneous upregulation of Mcl-1 in cells that contain high constitutive Noxa protein levels in order to prevent spontaneous induction of apoptosis.
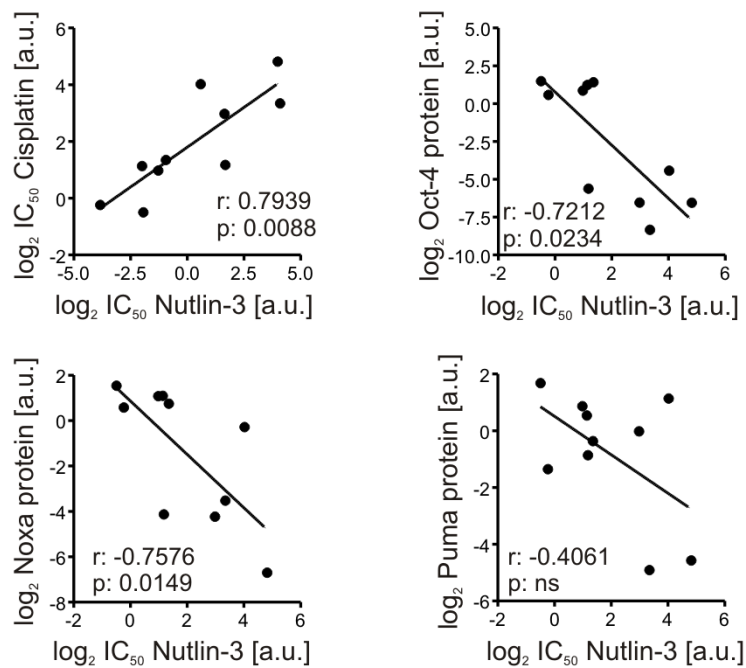
Figure 29: Oct-4 and Noxa protein levels are correlated with Nutlin-3 sensitivity in TGCT cell lines.
Dot blots reflect correlations between Cisplatin and Nutlin-3 sensitivity determined by SRB microculture colorimetric assay as well as between protein levels of Oct-4, Noxa and Puma (analyzed by densitometric analysis; Figure 28) and IC_{50} of Nutlin-3 in 10 TGCT cell lines (Figure 28). Correlations are expressed as Spearman rank correlation coefficient (r). p values are given for significant correlations (p≤0.05).

Notably, similar results were observed with Nutlin-3 as sensitivity to this compound was closely correlated with Cisplatin sensitivity among TGCT cell lines (Spearman r=0.794, p=0.009; Figure 29). Moreover, as seen with Cisplatin sensitivity, Oct-4 and Noxa protein levels were significantly correlated with Nutlin-3 sensitivity (Spearman r=-0.721, p=0.023 and r=-0.758, p=0.015; Figure 29) whereas no significant correlation was detected with Puma protein levels (Spearman r=-0.527, p>0.05; Figure 29). These data further support the conjecture that Oct-4 lowers the apoptotic threshold in TGCTs by maintaining high constitutive Noxa levels. In contrast to that, Puma, although dependent on Oct-4, might function mainly as a mediator of the Cisplatin-induced p53 response.

It is of note that RNAi-mediated knockdown of Oct-4 and Noxa revealed a similar impact of both proteins on the sensitivity of TGCTs to other genotoxic agents such as Etoposide or Doxorubicin as well as on the non-genotoxic p53 activator Nutlin-3 (Figure 30). These data strengthen the concept that Oct-4 and Noxa are major determinants of TGCT hypersensitivity.
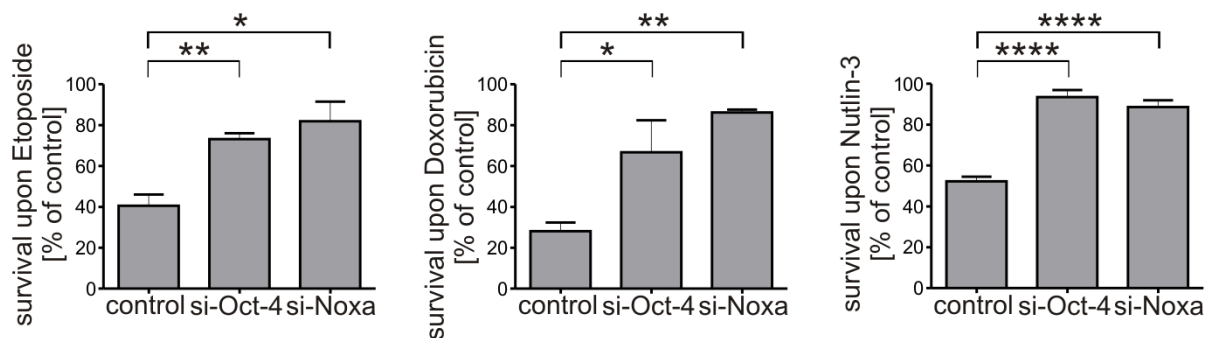
Figure 30: Oct-4 and Noxa determine the sensitivity of TGCTs to genotoxic and non-genotoxic p53 activators.

Cells were transfected with the indicated siRNAs and incubated for 48 h prior to Etoposide, Doxorubicin or Nutlin-3 treatment. Controls reflect cells treated with a non-targeting siRNA. To analyze survival, cells were stained with Annexin-V/FITC and PI upon treatment (16 h) and quantified by flow cytometry. Graph reflects means ±SD of survival upon Etoposide and Doxorubicin treatment from three independent experiments and survival upon Nutlin-3 treatment from six independent experiments (*: p≤0.05, **: p≤0.01, ****: p≤0.0001).

Next, the correlation between Oct-4 and Noxa observed in cancer cell lines was verified in cancer patient samples. Thus, samples from 8 seminoma and 5 EC patients were analyzed for Oct-4 and Noxa protein levels by Western Blot in comparison to a lung tumor sample. NTERA-2D1 cells served as a positive control, 1777NRpmet cells as a negative control.

Importantly, as seen in TGCT cell lines (Figure 28), Oct-4 protein levels were significantly correlated with the amount of Noxa protein (Spearman r=0.832, p<0.0001; Figure 31).
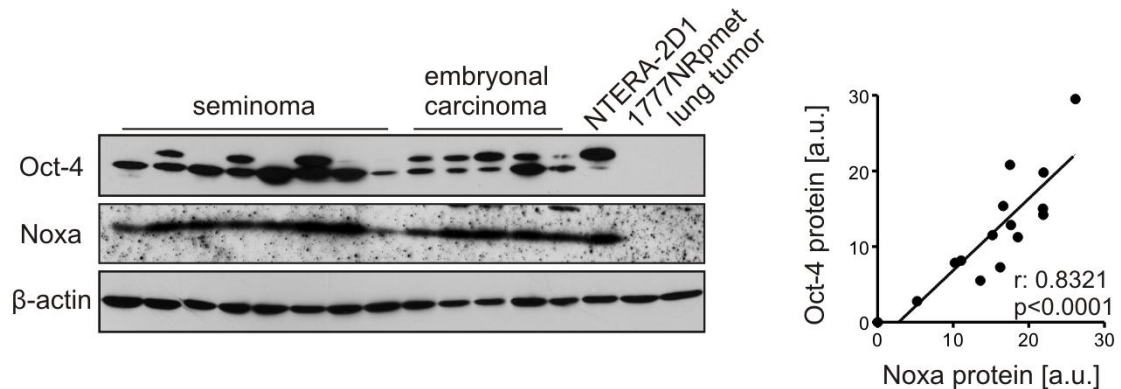

Figure 31: Constitutive Noxa protein levels are highly correlated with Oct-4 status in cancer patient samples.

Whole protein lysates were obtained from frozen patient samples of 5 EC, 8 seminoma and a lung tumor and applied to Western Blot analysis to determine Oct-4 and Noxa protein levels. Lysates from NTERA-2D1 and 1777NRpmet cell lines served as positive or negative control, respectively. Graph reflects correlation of Oct-4 and Noxa protein levels. Correlation is expressed as Spearman rank correlation coefficient (r).

3.3.5   Regulation of Noxa by miRNAs

NOXA/PMAIP1 was commonly seen as a transcriptionally activated stress-responsive gene (Ploner et al., 2008) rather than a protein present at high levels in unstressed cells. Therefore, besides a transcriptional mechanism, another level of regulation might exist. miRNAs are a family of 21-25 nucleotide small non-coding RNAs that negatively regulate gene expression at the posttranscriptional level (He and Hannon, 2004). Low density miRNA arrays were applied to identify miRNAs regulated by Oct-4 which potentially target *NOXA/PMAIP1* mRNA. Out of 365 miRNAs, 22 were found to be upregulated ≥2.0fold upon Oct-4 depletion in both NTERA-2D1 and 2102EP cells (Figure 32A). miRNA target prediction (microrna.org) revealed that of these miRNAs, miR-145, miR-181a and miR-193b potentially target *NOXA/PMAIP1* mRNA. In addition, 23 miRNAs were downregulated ≥2.0fold upon Oct-4 depletion (Figure 32B). These might be possible candidate miRNAs suppressing negative regulators of *NOXA/PMAIP1* expression in Oct-4-positive TGCT cells.
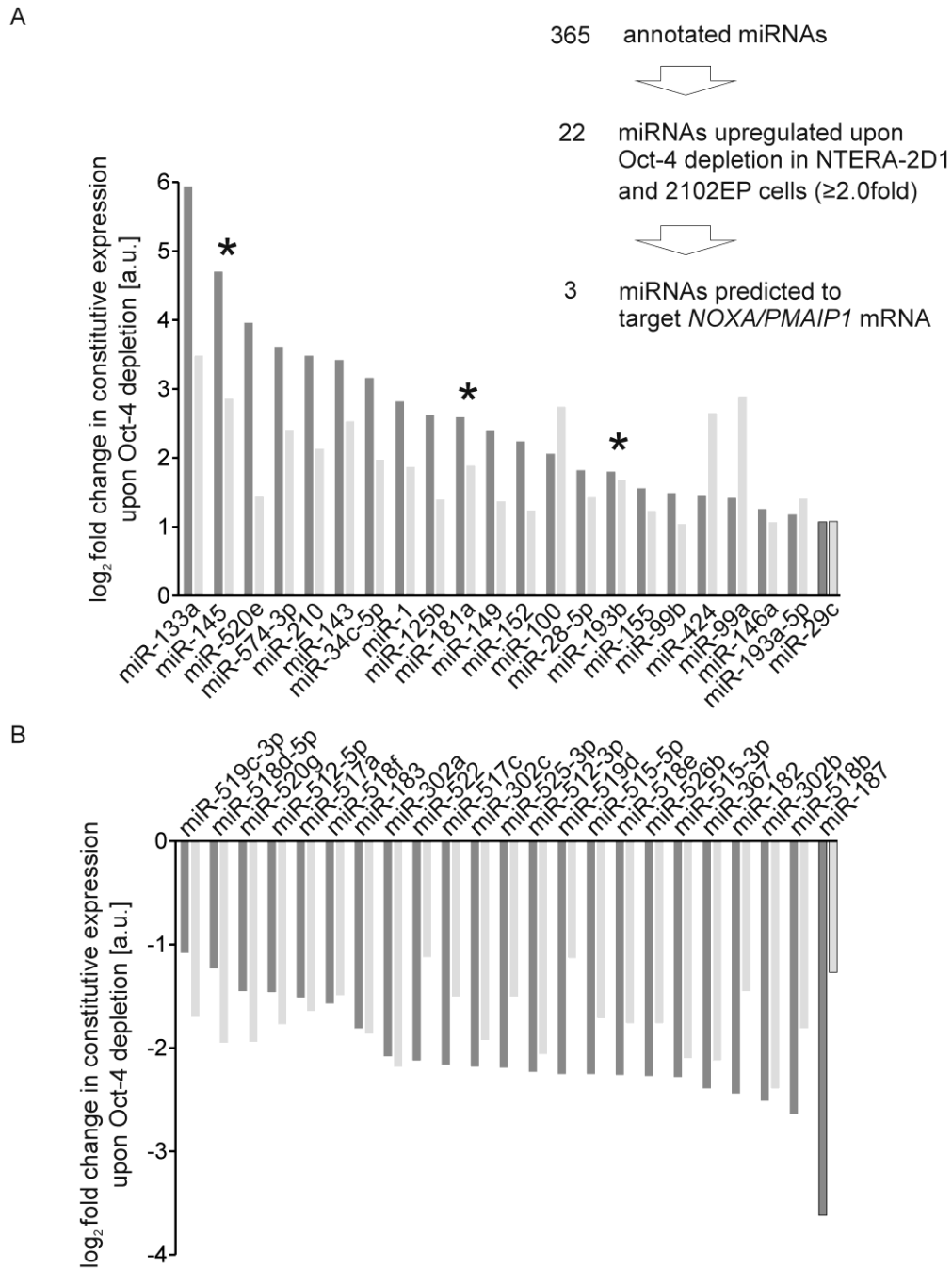
Figure 32: Out of 22 miRNAs upregulated upon Oct-4 depletion, miR-145, miR-181a and miR-193b potentially target _NOXA/PMAIP1_ mRNA.

Low density miRNA arrays were performed to detect miRNAs dependent on Oct-4 in NTERA-2D1 cells (dark grey bars) and 2102EP cell (light grey bars). (A) miRNAs upregulated upon Oct-4 depletion in both cell lines (≥2.0fold). miR-145, miR-181a and miR193b (marked with asterisks) were predicted to target _NOXA/PMAIP1_ mRNA (prediction tool: microrna.org). (B) miRNAs downregulated upon Oct-4 knockdown (≥2.0fold).

Subsequently, both cell lines were transfected with small RNAs mimicking miR-145, miR-181a and miR-193b in order to analyze their impact on Noxa protein levels as well as Cisplatin hypersensitivity. As shown in Figure 33A, cells were efficiently transfected with miRNA mimics. Control cells were transfected with non-targeting RNA. Importantly, all three miRNA mimics did not affect Noxa protein levels (Figure 33B). Consequently, quantification of cell death by FACS analysis did not reveal any changes in Cisplatin hypersensitivity of transfected cells (Figure 33C). Thus, the identified miRNAs do not contribute to Oct-4-mediated Noxa regulation in EC cells.
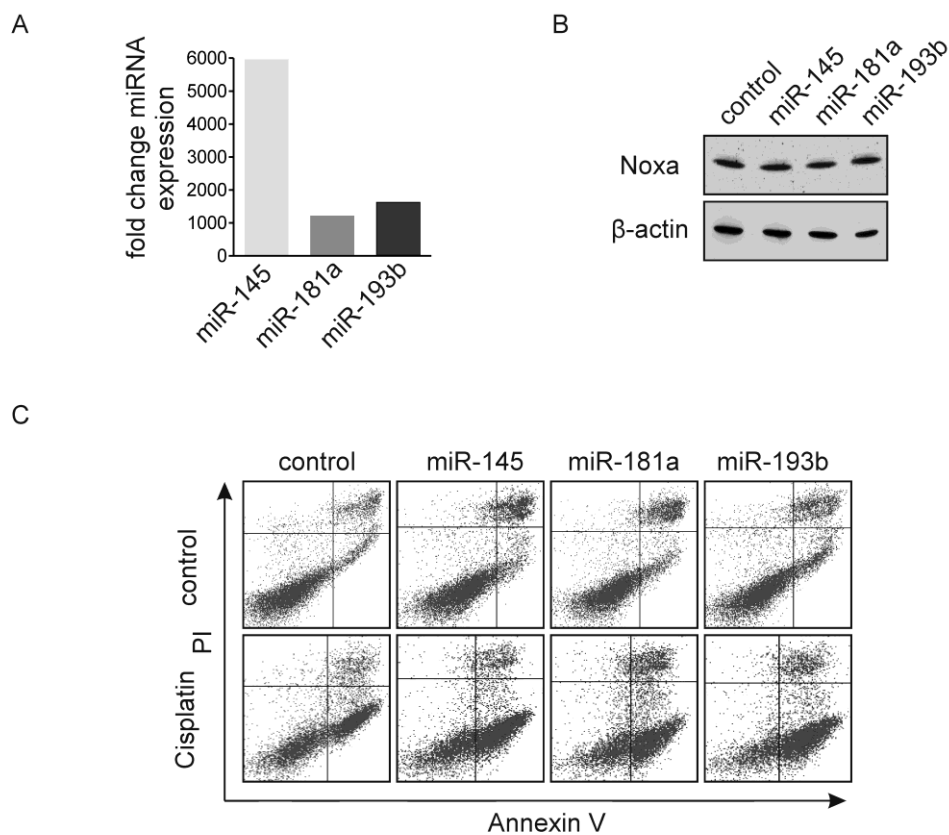
Figure 33: miR-145, miR-181a and miR-193b do not control high constitutive Noxa protein levels and Cisplatin hypersensitivity.
NTERA2-D1 cells were transfected with mimics of miR-145, miR-181a and miR-193b. Controls were transfected with a non-targeting RNA. (A) Verification of overexpression of miRNA mimics. RNA was isolated from transfected cells, transcribed to cDNA and levels of miRNA mimics were determined by qPCR. Graph reflects fold change in expression of transfected vs. control cells. (B) miR-145, miR-181a and miR-193b do not control Noxa protein levels. Whole protein lysates from transfected cells were analyzed by Western Blot analysis to determine Noxa protein levels. (C) miR-145, miR-181a and miR-193b do not prevent Cisplatin-induced apoptosis. Transfected cells were incubated with Cisplatin for 16 h and stained with Annexin V-FITC and PI in order to analyze cell death by flow cytometry.

3.4   Both Noxa and p53 are required to induce cell death in TGCTs

This study established a central role for p53 in TGCT hypersensitivity and proposes that its proapoptotic function in this tumor type is dependent on high constitutive Noxa protein levels. This characteristic is exclusive to the Oct-4-positive cellular context of pluripotent TGCTs and primes these tumors for apoptosis. Therefore, simultaneous induction of p53 and Noxa should cause cell death in Oct-4-depleted cells.
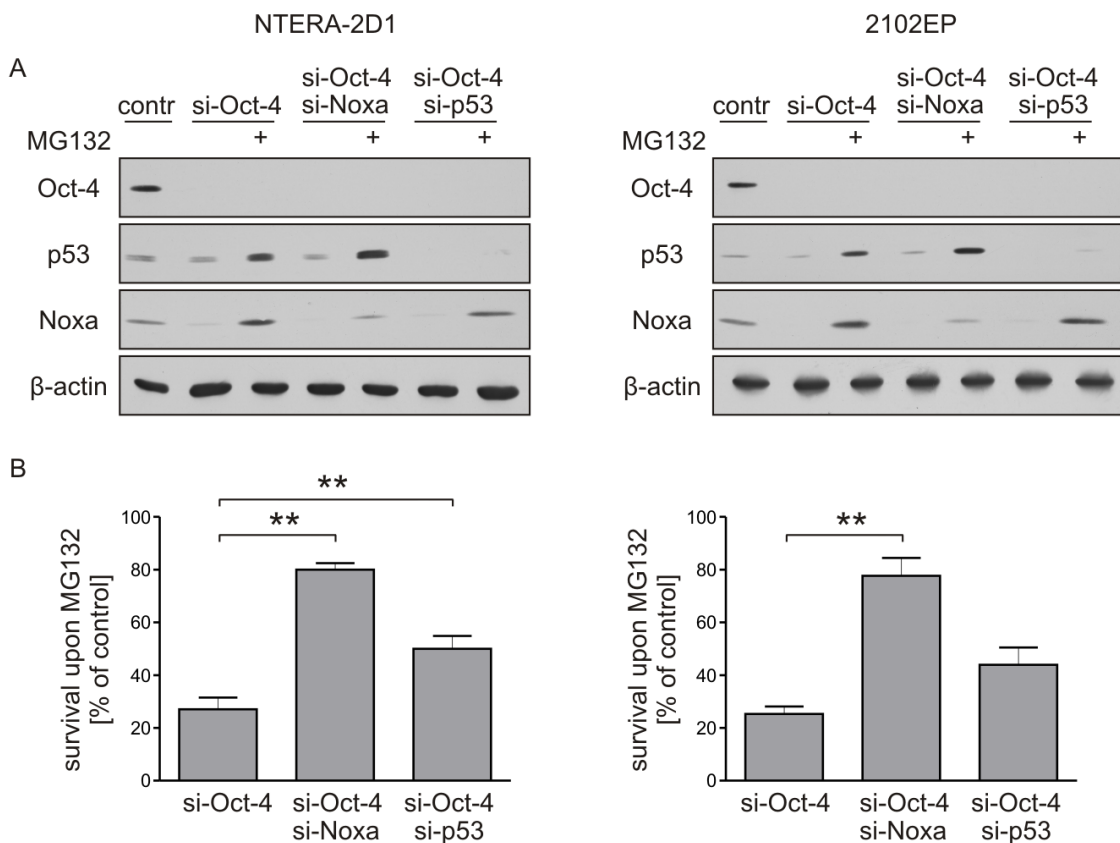
Figure 34: Accumulation of both Noxa and p53 is required to induce cell death in Oct-4-depleted cells. Cells were transfected with the indicated siRNAs and incubated for 48 h before MG132 treatment. Controls reflect cells treated with a non-targeting siRNA. Transfected cells were treated with MG132 for 20 h (NTERA-2D1) or 16 h (2102EP), respectively. (A) Noxa and p53 protein is accumulated upon MG132 treatment. Protein lysates were prepared and subjected to Western Blot analysis to determine protein levels of p53 and Noxa and to verify efficient gene silencing. (B) Both Noxa and p53 rescue Oct-4-depleted cells from MG132-induced apoptosis. Cells were stained with Annexin V-FITC and PI and analyzed for apoptosis by flow cytometry. Graphs reflect means ±SD of survival upon MG132 treatment relative to corresponding controls from three independent experiments (**: $p \leq 0.01$).

To test this hypothesis, NTERA-2D1 and 2102EP cells were depleted of Oct-4 by RNAi-mediated knockdown and treated with the proteasome inhibitor MG132. Bortezomib, another inhibitor of the proteasome has previously been shown to accumulate p53 and Noxa protein (Williams and McConkey, 2003; Fernández et al., 2005; Qin et al., 2005). In contrast to Cisplatin or Nutlin-3 treatment (Figure 20), proteasomal inhibition resulted in cell death in Oct-4-depleted NTERA-2D1 and 2102EP cells (Figure 34B).

Importantly, MG132 treatment led to accumulation of Noxa to levels comparable to those seen in Oct-4-positive control cells (Figure 34A). Concomitant induction of p53 protein was required to eliminate Oct-4-depleted cells as silencing of either Noxa or p53 protein reduced apoptosis induction (Figure 34B). These data further support the conjecture that high Noxa protein levels as well as activation of p53 determine the exclusive hypersensitivity of TGCT cells.

4   Discussion

Tumors that can be cured by chemotherapy are relatively rare. Although patients with tumors derived from lung, ovary, breast or stomach tissue initially respond to chemotherapy, 5-year survival is generally low. In contrast, certain tumors are highly sensitive to chemotherapy including childhood cancers, leukemias and lymphomas (Masters and Köberle, 2003). Also, TGCTs are successfully cured by Cisplatin-based chemotherapy. Considerable effort has been made to understand the underlying molecular mechanisms of this exceptional sensitivity to chemotherapeutic treatment (see 1.1.6) in order to improve the therapeutic index of other tumor types. Despite these efforts, the reasons for the exclusive Cisplatin hypersensitivity of TGCTs have remained obscure. This study gains insight into the molecular mechanisms and requirements that render TGCTs sensitive. Hopefully, it may provide possible starting points for more effective therapies in chemorefractory TGCTs, as well as for tumors derived from other tissues.

In this study, p53 was identified as a central determinant of Cisplatin hypersensitivity in TGCTs. Furthermore, the predominantly proapoptotic response to p53 accumulation was demonstrated to be a general phenomenon of TGCTs rather than a response restricted to genotoxic agents such as Cisplatin. Unexpectedly, p53 activity was found to be unaltered in pluripotent Oct-4-positive TGCT cells when compared to Oct-4-depleted or short-term-differentiated tumor cells even though these cells exhibit a significantly lower susceptibility to genotoxic and non-genotoxic p53 activators. The diminished sensitivity upon loss of Oct-4 was demonstrated to be a consequence of the concomitant loss of the BH3-only protein Noxa, which is present at high levels in unstressed pluripotent TGCT cells harboring Oct-4. Accordingly, a close correlation was established between Oct-4 status, high constitutive Noxa protein levels and Cisplatin sensitivity. These data led to the conjecture that pluripotent Oct-4-positive TGCT cells exist in a state primed for cell death. Thus, accumulation of p53 by Cisplatin, other genotoxic or non-genotoxic agents results in induction of apoptosis presumably mediated by transactivation of the p53 target gene *PUMA/BBC3*.

4.1   TGCTs are hypersensitive to p53 accumulation

In unstressed cells, the tumor suppressor p53 is maintained at low levels by ubiquitin-dependent degradation mediated by the E3 ligase Mdm2 (Michael and Oren, 2003). Upon a variety of genotoxic stresses p53 is transiently stabilized and activated as a transcription factor which results in cell cycle arrest or leads to apoptosis induction (Appella and Anderson, 2001). Alternatively, p53 can initiate apoptosis via transcription-independent mechanisms (Green and Kroemer, 2009). Direct disruption of p53 function by gene mutation occurs in about half of all cancers and most other cancers exhibit defects in pathways that activate p53 or mediate its tumor suppressor activity (Vogelstein et al., 2000). Thus, *TP53* status could be expected to represent an indicative marker for clinical response to chemotherapy. However, intensive research done in this field produced controversial results indicating that the role of p53 in chemotherapeutic response is complex and depends on cell type and drug regimen (Robles and Harris, 2010; Lai et al., 2012).

In this study, the role of p53 in Cisplatin hypersensitivity of TGCTs was investigated taking into account the relevance of functional p53, potential upstream activators and factors facilitating p53 activation. In the course of these experiments, the requirement of wildtype p53 for the hypersensitive phenotype of TGCTs was demonstrated. Furthermore, hypersensitivity of TGCTs is shown to be a general phenomenon not restricted to genotoxic agents, instead it is a result of their unique responsiveness to p53 activation. This property is lost upon differentiation and Oct-4 depletion due to changes in the cellular context rather than a differential p53 response.

4.1.1   p53 is major determinant of Cisplatin hypersensitivity

TGCTs differ considerably from most tumors as they almost exclusively express wildtype *TP53* (Heimdal et al., 1993; Peng et al., 1993). Early on, functional *TP53* was implicated in the sensitivity of TGCTs to genotoxic agents such as Cisplatin or Etoposide (Riou et al., 1995; Chresta et al., 1996). Rare cases of resistant tumors have been attributed to *TP53* gene mutations (Houldsworth et al., 1998). The finding that patient samples of TGCTs express elevated levels of wildtype p53 protein supported the conjecture that p53 was involved in hypersensitivity of these tumors (Riou et al., 1995; Guillou et al., 1996;

Heidenreich et al., 1998). Tolerance of high p53 protein levels has been attributed to a transcriptionally inactive p53 protein that exists in a latent state and is activated upon DNA damage (Guillou et al., 1996; Lutzker and Levine, 1996). Further evidence supporting the importance of p53 for chemosensitivity of TGCTs came from Lutzker et al. who reported a relationship between the amount of constitutive p53 protein levels and sensitivity to DNA damage in isogenic murine teratocarcinoma cells (Lutzker et al., 2001). In the present study, it was demonstrated that RNAi-mediated p53 depletion abrogated Cisplatin sensitivity of TGCT cell lines (Figure 2). These data are in line with the finding of Kerley-Hamilton et al. who reported reduced sensitivity to Cisplatin treatment upon transfection with p53-targeting siRNAs (Kerley-Hamilton et al., 2005) and suggest a central role for p53 in Cisplatin sensitivity of TGCTs. It is of importance that apoptosis induction was closely related to the extent of p53 accumulation upon Cisplatin treatment (Figure 3) further emphasizing the relevance of p53 for the hypersensitive phenotype of TGCTs. Interestingly, in this study, it was found that NTERA-2D1 cells did neither exhibit high constitutive nor Cisplatin-induced p53 protein levels when compared to rather Cisplatin-resistant cell lines derived from other tumor entities such as MCF-7 (Figure 4). This result matches data from a study analyzing p53 levels by immunohistochemistry in samples from TGCT patients before as well as after chemotherapy. Overall, relatively low levels of p53 were detected in tumors from all patient groups (Kersemaekers et al., 2002). Consistent with the findings of the present study, TGCT cell lines were also shown to exhibit lower constitutive p53 levels compared to MCF-7 or other cell lines (Kersemaekers et al., 2002). NTERA-2D1 cells were challenged as a model to study the importance of high p53 protein levels for Cisplatin sensitivity due to their relatively low p53 protein levels compared to other TGCT cell lines (Lutzker et al., 2001). However, NTERA-2D1 cells showed comparably low IC_{50} of Cisplatin as cell lines described to exhibit higher p53 protein levels (Figure 31; Burger et al., 1999). These data further indicate that high constitutive p53 protein levels are not a prerequisite for the exclusive sensitivity of TGCTs to Cisplatin.

Stoter and colleagues observed no significant differences in sensitivity to a variety of genotoxic stresses between NTERA-2D1 cells harboring wildtype p53 and NCCIT cells which express mutant p53 (Burger et al., 1997, 1998a, 1998b, 1999). Therefore, they proposed that the presence of functional, transactivation-competent p53 was not a prerequisite for efficient induction of apoptosis in TGCT cell lines. Burger et al. showed that abrogation of p53 function in NTERA-2D1 cells by expression of the human

papillomavirus (HPV) E6 protein did not affect hypersensitivity to apoptotic stimuli (Burger et al., 1999). However, HPV16-E6 might target proteins other than p53 as this virus protein has been implicated in degradation of prosurvival proteins by the ubiquitin-proteasome system (Xu et al., 1995) as well as in augmentation of DNA damage signaling pathways such as the Wnt pathway (Lichtig et al., 2010). Therefore, RNAi-mediated silencing of p53 used in the present study represents a more specific approach to study the functions of p53 in Cisplatin sensitivity.

In summary, these data demonstrate a central role of p53 in hypersensitivity of TGCTs to Cisplatin.

4.1.2   Stabilization/activation of p53 in TGCTs

The major signal transduction pathways leading to posttranslational modification and subsequent accumulation of p53 upon genotoxic stress include activation of the protein kinases ATM, ATR and DNA-PK (Shiloh, 2003). Unexpectedly, these kinases were found to be dispensable for Cisplatin sensitivity in NTERA-2D1 cells since silencing of either kinase or combinatorial knockdowns did not affect Cisplatin-induced apoptosis (Figure 6). This was not due to a diminished capacity of these kinases to phosphorylate p53 as phosphorylation at serine residues 15 and 20 was detected in response to Cisplatin treatment and dependent on ATM, ATR or DNA-PK (Figure 7). However, absence of phosphorylation at these sites did not impair Cisplatin-induced accumulation of p53 (Figure 7). These data demonstrate that ATM, ATR and DNA-PK as well as phosphorylation at serine residues 15 and 20 are not a requirement for p53 stabilization in TGCTs and accordingly for Cisplatin sensitivity.

In contrast to ATM, ATR and DNA-PK, the checkpoint kinase CHK2 significantly decreased Cisplatin-induced apoptosis in NTERA-2D1 cells (Figure 8) confirming results obtained with a CHK2 inhibitor (Figure 5). Although CHK2 is commonly seen to be activated in an ATM/ATR-dependent manner (Bartek and Lukas, 2003), recent studies suggest that ATM-independent mechanism are also conceivable (Hirao et al., 2002) and that DNA damage is not a prerequisite for CHK2 activation (Chen et al., 2005a). Thus, in TGCT cells, CHK2 might transduce DNA damage signals independently of ATM and ATR. In spite of its impact on Cisplatin sensitivity, CHK2 depletion did not affect p53

stabilization upon Cisplatin treatment indicating that CHK2 does not function upstream of p53. Interestingly, CHK2 was recently suggested to be activated downstream of p53 since p53 overexpression increased and p53 silencing decreased the activating phosphorylation of CHK2 at threonine 68, respectively (Liang et al., 2011). In addition, CHK2 was implicated in apoptosis induction in cancer cells without functional p53 (Chen et al., 2005a), suggesting a p53-independent contribution of CHK2 to Cisplatin hypersensitivity of TGCT cells.

Besides ATM, ATR, DNA-PK and CHK2 a complex network of kinases was described to be related to the DNA damage response (Bensimon et al., 2011). Importantly, about 40% of DSB-induced phosphorylations were found to be ATM-independent (Bensimon et al., 2010). In addition, a multitude of posttranslational modifications regulate p53 activation (Murray-Zmijewski et al., 2008). It therefore seems possible that p53 regulators other than ATM, ATR and DNA-PK sense and transduce DNA damage signals to p53. Besides CHK2, screening of a protein kinase inhibitor library for inhibitors with a negative impact on Cisplatin sensitivity identified kinases which have previously been described to increase p53 protein stability in response to DNA damage via direct phosphorylation including p38 (Bulavin et al., 1999; Thornton and Rincon, 2009), ERK (Persons et al., 2000; She et al., 2000) and casein kinase (Sakaguchi et al., 2000). Interestingly, ERK has previously been implicated in Cisplatin-induced apoptosis in TGCTs (Schweyer et al., 2004; Fung et al., 2006). These data indicate that kinases other than the major DNA damage signaling transducers are involved in p53 stabilization upon DNA damage in TGCTs. Moreover, phosphorylation of p53 is not necessarily required for DNA damage induced p53 stabilization (Blattner et al., 1999). Changes in Mdm2 or Mdm2-regulating factors such as ARF rather than in p53 itself can lead to a reduced p53 turnover resulting in p53 accumulation (Michael and Oren, 2003). Due to the insignificance of major DNA damage signal transducers in the response of TGCT cells to Cisplatin treatment, DNA damage-independent effects of Cisplatin treatment such as cellular ROS formation should also be considered to play a role in apoptosis induction as previously described for other cell types (Berndtsson et al., 2007).

Together, these data indicate that classical DNA damage response mediators such as ATM, ATR and DNA-PK are not involved in DNA damage-induced p53 stabilization upon Cisplatin treatment and therefore do not contribute to Cisplatin sensitivity. Other kinases such as p38, ERK or casein kinase might instead adopt such a role in TGCTs. Furthermore,

DNA damage could lead to the repression of factors promoting p53 turnover such as Mdm2. However, the exact mechanism of p53 stabilization and activation upon Cisplatin treatment in TGCTs remains to be elucidated. Interestingly, CHK2 plays a role in Cisplatin sensitivity that is not related to p53 stabilization.

TGCTs are also sensitive to p53 activation by Nutlin-3 (Bauer et al., 2010; Li et al., 2010; Koster et al., 2011). Cell death assays confirmed that treatment with Nutlin-3 induced apoptosis in NTERA-2D1 cells (Figure 12). In fact, apoptosis was induced to a similar extent as did Cisplatin treatment and was accompanied by a similar accumulation of p53 protein (Figure 12). As expected, Nutlin-3-induced apoptosis was dependent on p53 (Figure 14) confirming results from Koster et al. who demonstrated that suppression of p53 induced resistance in a panel of TGCT cell lines (Koster et al., 2011). Interestingly, Nutlin-3-induced cell death was highly correlated with the amount of accumulated p53 protein (Figure 15) analogous to the results observed upon Cisplatin treatment (Figure 3). It is of importance that Nutlin-3 activated p53 independently of DNA damage signaling pathways as DNA damage-associated p53 phosphorylation did not occur upon Nutlin-3 treatment (Figure 13). Previous studies reported that phosphorylation of p53 on key serines is dispensable for its transcriptional activation and apoptosis induction (Thompson et al., 2004) supporting the conjecture that p53 stabilization leading to cell death can occur independently of DNA damage signaling pathways. This hypothesis is strengthened by the finding that treatment of NTERA-2D1 cells with the proteasome inhibitor Bortezomib resulted in accumulation of p53 and apoptosis induction similar to Cisplatin and Nutlin-3 treatment, respectively (Figure 12). As observed upon Nutlin-3 treatment, proteasomal inhibition did not lead to phosphorylation of p53 at serine residues associated with DNA damage-dependent p53 stabilization (Figure 13). Nevertheless, Bortezomib-induced cell death required p53 activation (Figure 14) as described for other cell types (Vaziri et al., 2009; Chen et al., 2010). Taken together, these data demonstrate that TGCT hypersensitivity is not restricted to genotoxic agents such as Cisplatin but suggest that rather these tumors are highly susceptible to p53 accumulation in general. In this context, a reduced DNA repair capacity seems hardly conceivable as a cause for Cisplatin hypersensitivity as previously proposed (Bedford et al., 1988; Hill et al., 1994; Köberle et al., 1997). Data presented in Figure 16 further support this conjecture showing that NTERA-2D1 cells were capable of removing Pt adducts from DNA if apoptosis induction, which usually precedes DNA repair, is blocked efficiently.

4.1.3   The proapoptotic p53 response

p53 triggers differential cellular responses to genotoxic agents by transactivation of distinct subsets of target genes (Murray-Zmijewski et al., 2008). Keeping in mind the central role of p53 in TGCT hypersensitivity, it is obvious that a robust transcriptional activation of p53-associated target genes is linked to hypersensitivity of TGCTs (Kerley-Hamilton et al., 2005). Therefore, classical mediators of apoptosis as well as cell cycle regulators were analyzed for a role in Cisplatin hypersensitivity.

PUMA/BBC3 and *NOXA/PMAIP1* were previously shown to be induced by p53 in response to DNA damage (Oda et al., 2000; Nakano and Vousden, 2001; Yu et al., 2001). Their integral function in apoptosis in general and in drug-induced cell death in particular has been demonstrated in gene-targeting approaches in mice (Jeffers et al., 2003; Shibue et al., 2003; Villunger et al., 2003). In NTERA-2D1 cells, both *PUMA/BBC3* and *NOXA/PMAIP1* mRNA were found to be transcriptionally upregulated following Cisplatin treatment (Figure 9). RNAi-mediated silencing of either transcript caused a significant reduction of Cisplatin-induced apoptosis in NTERA-2D1 and 2102EP cells (Figure 11), establishing Puma and Noxa as important factors in the sensitivity of TGCTs to Cisplatin. In contrast to Puma, stress-induced Noxa was previously proposed to be involved in the response of TGCT cells to Cisplatin (Grande et al., 2012). However, it was suggested that p73 rather than p53 plays a key role in Noxa induction upon Cisplatin treatment (Grande et al., 2012). Interestingly, both *PUMA/BBC3* and *NOXA/PMAIP1* transcripts were found to be reduced in Cisplatin-resistant differentiated compared to undifferentiated NTERA-2D1 cells (Figure 19) further supporting a role of these genes in Cisplatin sensitivity. It is of importance that simultaneous silencing of Puma and Noxa promoted survival in NTERA-2D1 cells to a comparable extend as did p53 knockdown (Figure 11). Interestingly, HeLa and neural precursor cells also require both Puma and Noxa for efficient apoptosis induction (Akhtar et al., 2006; Nakajima and Tanaka, 2007). In other cell types, either Puma or Noxa are sufficient for apoptosis induction depending on the type of genotoxic stress (Jeffers et al., 2003; Erlacher et al., 2005; Naik et al., 2007). In summary, these data indicate that in TGCT cells, both Puma and Noxa are essential mediators of the p53-dependent proapoptotic response to Cisplatin treatment.

Besides *NOXA/PMAIP1* and *PUMA/BBC3* mRNA, *FAS/CD95* mRNA was found to be upregulated in NTERA-2D1 cells treated with Cisplatin (Figure 9). The Fas/CD95 pathway

of apoptosis has previously been implicated in Cisplatin sensitivity of TGCTs (Spierings et al., 2003). However, silencing of Fas/CD95 did not affect Cisplatin sensitivity of both examined cell lines (Figure 11) suggesting that this cell death pathway has a minor impact on Cisplatin sensitivity of these tumor cells.

A role of the cell cycle inhibitor p21 has previously been implicated in Cisplatin hypersensitivity (Koster and De Jong, 2010). Although *P21/CDKN1A* mRNA was induced upon Cisplatin treatment (Figure 9), NTERA-2D1 cells were observed to exhibit low p21 protein levels when compared to other cell types (Figure 10). These data confirmed several studies reporting that most TGCTs lack p21 protein (Guillou et al., 1996; Bartkova et al., 2000; Datta et al., 2001; Mayer et al., 2003), a characteristic that was proposed to account for the hypersensitive phenotype of TGCT cells (Spierings et al., 2004). Unexpectedly, a deficiency of apoptosis inhibition by cytoplasmic p21 was proposed as the underlying mechanism (Koster et al., 2010). Since p21 is best known for its function as a cell cycle inhibitor (Abbas and Dutta, 2009), low p21 protein levels might in addition contribute to the deregulated G1/S phase transition described in TGCTs (Strohmeyer et al., 1991; Houldsworth et al., 1997; Bartkova et al., 1999, 2000) and thus affect Cisplatin hypersensitivity. Together, these data indicate that low p21 protein levels play a role in Cisplatin hypersensitivity of TGCTs. However, this might not be related to a reduced capacity of p53 to transactivate cell cycle arrest-related genes since induction of *P21/CDKN1A* mRNA was seen upon Cisplatin treatment (Figure 9; Kerley-Hamilton et al., 2005). The finding that p21 was repressed by a miRNA-mediated mechanism in an Oct-4-dependent manner in pluripotent TGCT cells (Koster et al., 2010) further strengthens the conjecture that low p21 protein levels are not a consequence of an inability of p53 to transactivate *P21/CDKN1A*.

Cisplatin hypersensitivity of TGCT cell lines is lost upon differentiation or Oct-4 depletion, respectively (Timmer-Bosscha et al., 1998; Mueller et al., 2006). In addition, this study revealed that the sensitivity of TGCTs to non-genotoxic p53 activators such as Nutlin-3 was abrogated upon short-term differentiation (Figure 17). Analogous, Oct-4-depleted cells showed a decreased sensitivity to Nutlin-3 treatment when compared to Oct-4-positive cells (Figure 20). It is of importance that in spite of the substantial reduction of Cisplatin sensitivity, p53 accumulation was not altered in differentiated NTERA-2D1 cells in comparison to Cisplatin-sensitive undifferentiated cells (Figure 18). A similar picture

was seen upon Oct-4 depletion which did not result in a differential accumulation of p53 upon genotoxic stress (Figure 22). In accordance with these findings, Mueller et al. observed p53 accumulation in TGCT cell lines independent of their Oct-4 status when treated with the respective IC_{90} dose (Mueller et al., 2006). These data indicate that efficient apoptosis induction by p53 seems to be related to the pluripotent context of TGCTs. Moreover, the finding that Cisplatin-sensitive and Cisplatin-resistant TGCTs accumulate p53 to a similar extent upon genotoxic stress supports the conjecture that factors downstream of p53 such as Puma or Noxa must be decisive for the predominantly proapoptotic p53 response of TGCTs.

Pluripotent cells are known to exhibit high global transcriptional activity and undergo large-scale silencing as cells differentiate (Efroni et al., 2008). Therefore, it was speculated that differentiation of TGCT cells, a process in which Oct-4 expression is turned off (Houldsworth et al., 2002), causes changes in the accessibility of p53-responsive promoters and thus affects the transcriptional p53 response (Kerley-Hamilton et al., 2005). However, short-term differentiation or RNAi-mediated Oct-4 depletion, respectively, might not result in extensive genome-remodeling as expression of genes related to these processes was only observed at later time points in mouse P19 EC cells during differentiation with RA (Spin et al., 2010). Nevertheless, both short-term differentiation and Oct-4 depletion were sufficient to induce resistance in TGCT cells (Figure 17 and Figure 20). Therefore, a broader approach was taken to study possible alterations in p53 target gene activation in pluripotent Oct-4-positive TGCT cells. In spite of the profound impact of Oct-4 depletion on hypersensitivity in NTERA-2D1 cells, it did not significantly alter transactivation of 46 bona fide p53 target genes upon Cisplatin treatment (Figure 21). These data indicate that resistance induced by Oct-4 depletion does not depend on changes in the transcriptional capacity of p53. In fact, the central role of p53 in TGCT hypersensitivity implicates that pluripotent, Oct-4-positive TGCT cells are characterized by an apoptosis-prone cellular context that allows efficient cell death induction upon p53 activation. Although no differential target gene activation was detected between Oct-4-positive and Oct-4-negative cells, these data do not contradict the proposition that Cisplatin-induced apoptosis is characterized by an extensive activation of p53 target genes (Kerley-Hamilton et al., 2005).

Besides activation of the transcriptional proapoptotic program, p53 is capable of facilitating apoptosis induction in the cytoplasm via protein-protein interactions with prosurvival Bcl-2 family proteins (Green and Kroemer, 2009). This function of p53

provides an additional opportunity of apoptosis induction in TGCT cells. Indeed, subcellular fractionation revealed that p53 accumulated in the cytoplasm to a similar extend as it did in the nucleus upon Cisplatin treatment suggesting a role for cytoplasmic p53 in Cisplatin hypersensitivity. However, depletion of Oct-4 did not alter cytoplasmic p53 accumulation (Figure 22). Notably, cytoplasmic p53 has been proposed to be involved in apoptosis induction upon DNA damage in ESCs (Grandela et al., 2007; Qin et al., 2007). Together, these data indicate that hypersensitive TGCTs do not exhibit a differential p53 response. Thus, other mechanisms presumably related to the Oct-4-mediated pluripotent properties of TGCTs must dictate a cellular context which allows an efficient apoptosis induction by p53.

4.2   High constitutive Noxa protein levels dictate a low apoptotic threshold

Bcl-2 family proteins control the integrity of the mitochondrial outer membrane and thus govern life/death decisions (Chipuk et al., 2010). Accordingly, Letai and coworkers recently established a critical role of this protein family in the clinical response to chemotherapy (Ni Chonghaile et al., 2011; Vo et al., 2012). Their model suggests that a differential occupation of antiapoptotic Bcl-2 family proteins by their proapoptotic counterparts determines the cell's proximity to the apoptotic threshold, a property called mitochondrial priming (Ni Chonghaile et al., 2011). In this study, analysis of constitutive Bcl-2 family protein levels with reference to Oct-4 status and Cisplatin sensitivity revealed that the hypersensitive phenotype of TGCTs is closely related to the constitutive presence of Noxa protein (Figure 24 and Figure 28). Thus, the Oct-4-mediated cellular context dictates a Bcl-2 family profile that primes pluripotent TGCTs for apoptosis induction via high constitutive Noxa protein levels.

4.2.1   Bcl-2 family proteins in hypersensitivity

A differential composition of Bcl-2 family proteins has previously been implicated in Cisplatin sensitivity of TGCTs. Low Bcl-2 and Bcl-xL levels in combination with a high Bax/Bcl-2 ratio were proposed to account for the low apoptotic threshold and the excellent

chemotherapeutic responsiveness of these tumors (Chresta et al., 1996; Soini and Paakko, 1996; Mayer et al., 2003). However, in spite of the marked decrease in TGCT sensitivity (Figure 20), Bcl-2, Bax and Bak protein levels were not found to be affected by RNAi-mediated silencing of Oct-4 (Figure 23). This is in line with the finding that the amount of Bcl-2, Bcl-xL, Bax or Bak protein was not associated with Cisplatin sensitivity in different TGCT cell lines (Burger et al., 1997; Mueller et al., 2003). Nevertheless, low expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL could enhance the proapoptotic capacity of Noxa due to its inability to antagonize either protein (Chen et al., 2005b; Certo et al., 2006). This might be of particular importance since Bcl-xL was proposed to have a chemoprotective role in TGCTs (Arriola et al., 1999). Recent reports indicate that Noxa is capable of antagonizing Bcl-xL upon DNA damage (Lopez et al., 2010), suggesting an additional mechanism that broadens Noxa's proapoptotic capacity. In contrast to Bcl-2, Bax and Bak, constitutive Puma as well as Bim protein levels were found to be dependent on Oct-4 (Figure 23). However, a correlation with Cisplatin sensitivity was not observed (Figure 24 and Figure 28). Therefore, Puma possibly exerts its prominent proapoptotic function (Figure 11 and Figure 24) only upon induction by p53 as previously described for other cell types (Yu et al., 2006). Then, Puma may sequester further antiapoptotic Bcl-2 family proteins and/or activate Bax or Bak leading to rapid induction of apoptosis. Together, these data indicate that Noxa is the only Bcl-2 family protein that is tightly correlated with both Oct-4 status and Cisplatin sensitivity, suggesting that high constitutive Noxa levels are a key determinant of the exclusive sensitivity of Oct-4-positive TGCT cells to Cisplatin. This conjecture is strengthened by the finding that RNAi-mediated silencing of Noxa reduced Cisplatin sensitivity to a similar extent as did Oct-4 depletion (Figure 24). Consistent with these results, cell death induced by Doxorubicin and Etoposide was found to be dependent to a similar extent on both Oct-4 and Noxa (Figure 30). Moreover, a similar picture was seen with the non-genotoxic p53 activator Nutlin-3 (Figure 30). These data provide further evidence for the dominant role of Noxa in TGCT hypersensitivity and indicate that Noxa is central to their hypersensitive phenotype in general. This hypothesis is supported by Grande et al. who reported a correlation between the amount of Noxa protein and good clinical prognosis in EC patients (Grande et al., 2012).

Noxa preferentially binds to Mcl-1 (Chen et al., 2005b; Certo et al., 2006). This antiapoptotic Bcl-2 family protein was shown to be correlated with Oct-4 status and Noxa

expression in a panel of TGCT cell lines (Figure 28). In line with these findings, Sano et al. found an increased expression of Mcl-1 in patient samples from seminoma and EC (Sano et al., 2005). Moreover, Oct-4 depletion led to reduced Mcl-1 protein levels (Figure 23). However, this reduction was significantly weaker than the decrease in Noxa protein which was barely detectable in Oct-4-depleted cells. Thus, the Noxa/Mcl-1 ratio was shifted considerably in favor of Mcl-1, establishing a higher apoptotic threshold (Figure 23). These data indicate that Oct-4-positive cells may require high Mcl-1 protein levels to prevent spontaneous apoptosis induction due to the low levels of other Bcl-2 family proteins (see above). Similarly, mouse embryonic fibroblasts (MEFs) treated with the BH3 mimetic ABT-737, an antagonist of Bcl-2 and Bcl-xL, were entirely dependent on Mcl-1 for survival (Van Delft et al., 2006). In addition, it was previously described that Noxa facilitates Mcl-1 degradation upon genotoxic stress (Gomez-Bougie et al., 2011), a mechanism that may contribute to the efficient apoptosis induction in TGCTs. Together, these data demonstrate that the Noxa/Mcl-1 balance decisively influences cell fate in TGCT cells. Interestingly, HeLa and pancreatic cancer cells were also reported to be dependent on the Noxa/Mcl-1 balance when treated with Camptothecin (Mei et al., 2007; Naumann et al., 2012). Thus, in contrast to Puma which was reported to kill cells unselectively due to its capacity to sequester all antiapoptotic Bcl-2 family proteins (Suzuki et al., 2009), Noxa together with Mcl-1 provides a fine-tuned system determining a cell's apoptotic threshold. This scenario is particularly evident in EC cells which resemble the malignant counterpart of ESCs and thus must be subject to rigorous control to ensure their genomic integrity.

4.2.2 Mechanisms leading to high Noxa protein levels

This study demonstrates that Oct-4-positive TGCT cells express high constitutive Noxa protein levels (Figure 25 and Figure 28). The fact that Oct-4 mainly functions as a transcription factor suggests a transcriptional mechanism to account for the heightened Noxa protein levels. Indeed, *NOXA/PMAIP1* mRNA expression was found to be dependent on Oct-4 since RNAi-mediated Oct-4 depletion reduced *NOXA/PMAIP1* mRNA to a level detected in other cell types (Figure 26). Moreover, comparison of pre-mRNA and mRNA expression revealed that *NOXA/PMAIP1* pre-mRNA is downregulated upon Oct-4 depletion to a similar extent as is *NOXA/PMAIP1* mRNA, implicating a transcriptional

mechanism of regulation rather than an impact of Oct-4 on *NOXA/PMAIP1* mRNA stability. Notably, Oct-4 is involved in the transcriptional control of a plethora of genes including other transcription factors (Babaie et al., 2007). Therefore, transcriptional control of *NOXA/PMAIP1* by Oct-4 might as well include indirect mechanisms. In addition, the Oct-4 interactome includes regulators of gene expression and other regulatory factors (Pardo et al., 2010) providing a possible second level of Oct-4-mediated regulation of gene expression. Interestingly, *NOXA/PMAIP1* mRNA was shown to be upregulated in ESCs and to cluster with the pluripotency markers *NANOG* and *SOX2* in Bcl-2 family expression profiles in ESCs and non-ESCs (Madden et al., 2011). Moreover, *NOXA/PMAIP1* expression was found to be correlated with Oct-4 in this cell type (Cai et al., 2006; Babaie et al., 2007). These data add to the view that high expression of *NOXA/PMAIP1* mRNA is associated with the pluripotent cellular context of TGCTs. Another study reported an Oct-4 binding site within 8 kb of the *NOXA/PMAIP1* promoter (Boyer et al., 2005) indicating that a direct regulation by Oct-4 must also be considered.

Commonly, *NOXA/PMAIP1* was seen as a transcriptionally activated stress-responsive gene rather than encoding a protein present at high levels in unstressed cells (Ploner et al., 2008). However, in addition to Oct-4-positive TGCTs, high Noxa protein levels were also detected in lymphocytes increasing their susceptibility to apoptosis in response to glucose deprivation (Figure 25; Alves et al., 2006). These data together with the finding that the effect of Oct-4 depletion on Noxa was even more pronounced at the protein than at the mRNA level (Figure 25 and Figure 26) indicates that, in addition to a transcriptional mechanism, modulation of Noxa translation and/or protein stability might account for the high constitutive protein levels observed. Since MG132 treatment led to accumulation of Noxa in Oct-4-depleted cells, a posttranslational regulation of Noxa by the ubiquitin-proteasome system is conceivable (Figure 34). Indeed, proteasomal degradation was previously described to control Noxa protein levels (Difeo et al., 2009; Baou et al., 2010; Craxton et al., 2012). However, other studies report that Noxa is upregulated via a transcriptional mechanism upon proteasomal inhibition (Fernández et al., 2005; Qin et al., 2005; Pérez-Galán et al., 2006). Thus, accumulation of Noxa induced by proteasome inhibition is not necessarily based on changes in protein stability.

Another possibility of posttranscriptional regulation includes miRNAs which were proposed to target more than a third of mammalian protein-coding genes (Bartel, 2004; Lewis et al., 2005). A hint to miRNA-mediated regulation of Noxa came from Lerner et al.

who identified miR-200c as a negative regulator (Lerner et al., 2012). Besides transcriptional activation of protein-coding genes, Oct-4 is also known to control expression of miRNAs (Boyer et al., 2005). Therefore, low density arrays were used to identify miRNAs repressed by Oct-4 which in turn might be upregulated upon loss of Oct-4 and therefore could be involved in Noxa regulation. Indeed, multiple miRNAs were identified to be upregulated upon Oct-4 depletion, among them miR-145, miR-181a and miR-193b which were predicted to target the *NOXA/PMAIP1* 3'-UTR (Figure 32A). However, transfection of NTERA-2D1 cells with RNA molecules mimicking these miRNAs did neither result in decreased Noxa protein levels nor affected Cisplatin sensitivity (Figure 33) indicating that Noxa might not be subject to direct miRNA regulation. It is of note that Oct-4 depletion also reduced expression of a subset of miRNAs (Figure 32B). Therefore, factors that negatively regulate Noxa protein stability such as E3 ligases or kinases which are otherwise repressed by miRNAs might be active under these conditions. These data indicate that although direct regulation of Noxa by miRNAs could not be observed, this mechanism of regulation might as well indirectly influence Noxa protein levels.

In summary, an Oct-4-dependent transcriptional mechanism accounts at least partially for high Noxa protein levels. In addition, posttranscriptional mechanisms might contribute to Noxa stabilization. Further experiments are required to elucidate the exact mechanisms leading to high constitutive Noxa protein levels in Oct-4-positive TGCT cells.

4.3   Conclusion and outlook

In this study two molecular characteristics of TGCTs were found to determine their exclusive Cisplatin hypersensitivity. On the one hand, their functional p53 response is a prerequisite for the intrinsic sensitivity of these tumors. On the other hand, the Oct-4-mediated pluripotent context dictates high constitutive Noxa protein levels which establish a low apoptotic threshold resulting in an exceptional sensitivity to p53 activation. Once constitutive Noxa protein is lost, e.g. during differentiation or loss of Oct-4, the sensitivity to p53 activation is reduced significantly due to a then higher apoptotic threshold. In this study, Oct-4-positive TGCT cells were found to be sensitive to p53 accumulation by the proteasome inhibitor Bortezomib (Figure 14). In contrast to Cisplatin or Nutlin-3

treatment, Oct-4 depletion accompanied by loss of Noxa did not affect their susceptibility to proteasome inhibition (Figure 34). This was due to the concomitant accumulation of both p53 and Noxa protein which does not occur upon Cisplatin or Nutlin-3 treatment. In fact, RNAi-mediated silencing of Noxa as well as p53 reduced apoptosis induction upon MG132 treatment (Figure 34). These data further support the conjecture that hypersensitivity of TGCTs is dependent on both high levels of Noxa protein and a functional p53 response.

In conclusion, this study demonstrates that functional p53 is a mandatory requirement for the hypersensitive phenotype of TGCTs. Genotoxic as well as non-genotoxic p53 accumulation readily triggers apoptosis in these cells. Thus, TGCT cells are hypersensitive to p53 activation in general. Surprisingly, this was not due to differential p53 activation. Instead, the predominantly proapoptotic p53 response in TGCTs is entirely dependent on their Oct-4-shaped pluripotent context characterized by high constitutive Noxa protein levels. Thus, Noxa dictates a low apoptotic threshold which accounts for the highly apoptosis-prone phenotype. Consequently, the induction of additional proapoptotic Bcl-2 family proteins such as Puma by p53 results in immediate initiation of the apoptotic program.

Although the majority of TGCTs can be cured even in advanced metastatic stages, about 15 % of patients with metastatic disease will relapse with a response rate of about 30 % (Horwich et al., 2006). Mechanisms of chemoresistance in refractory tumors include loss of Oct-4 (Mueller et al., 2006). Therefore, it was hypothesized that Oct-4-negative EC cells drive the malignant growth of such tumors (Mueller et al., 2010). Data presented in this study indicate that concomitant loss of high Noxa protein levels could account for this phenotype. Thus, further investigations of the mechanisms that result in the high constitutive Noxa protein levels may provide new possibilities to abrogate Cisplatin resistance by restoration of Noxa protein levels and could further improve the efficacy of Cisplatin-based chemotherapy in TGCTs. Moreover, this study demonstrates that TGCTs are hypersensitive to p53 activation in general. Therefore, non-genotoxic p53 activators such as Nutlin-3 might represent an opportunity to replace cytotoxic Cisplatin-based chemotherapy in order to avoid side effects such as ototoxicity, peripheral neuropathy, nephrotoxicity and myelosuppression (Hartmann and Lipp, 2003).

In contrast to TGCTs, most other tumors exhibit low cure rates when treated with chemotherapy. The results of this study indicate that upregulation of Noxa in these

chemoresistant tumors may lower the apoptotic threshold leading to an increased therapeutic index. Interestingly, in mantle cell lymphoma (MCL), upregulation of Noxa protein by the proteasome inhibitor Bortezomib leads to apoptosis induction (Pérez-Galán et al., 2006). Also, combination of Cisplatin with Fludarabine induced apoptosis due to reactive oxygen species (ROS)-mediated upregulation of Noxa in chemorefractory tumors of chronic lymphoid leukemia (CLL) (Tonino et al., 2011). Recently, Bcl-2 family proteins have emerged as players in cellular pathways other than the mitochondrial pathway of apoptosis, e.g. mitochondrial dynamics, endoplasmic reticulum (ER) calcium stores and autophagy (Chipuk et al., 2010). These data indicate that compounds interfering with a multitude of cellular pathways could ultimately target Noxa protein levels. Thus, a conceivable approach includes screening of drugs for their potential to increase Noxa protein levels. As described above, a deeper understanding of the mechanisms leading to high Noxa levels in TGCTs might provide information that could be utilized in a targeted approach.

Apoptosis is eventually triggered by p53-dependent induction of additional BH3-only proteins such as Puma in TGCTs. In other cell types, however, activation of Puma upon chemotherapeutic treatment does not require functional p53 (Yu and Zhang, 2008), e.g. p73 was shown to induce Puma in p53-deficient cells upon Doxorubicin treatment (Kawahara et al., 2008). Another BH3-only protein that targets all antiapoptotic Bcl-2 family proteins includes Bim which is not known to be a direct transcriptional target of p53 (Wei et al., 2006) and therefore is conceivable to serve as an apoptotic trigger in Noxa-primed p53 deficient cells. These data indicate that pharmacological upregulation of Noxa protein could also sensitize cells harboring mutant p53 or even p53-deficient cells for chemotherapy.

In summary, this study provides further insight into the molecular mechanisms underlying the exceptional Cisplatin sensitivity of TGCTs. It remains to be determined to what extent these results can be exploited to improve the therapeutic index of refractory TGCTs as well as other chemoresistant tumors.

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7   Publications

This work was published in part in the following publications:

Original articles

M Gutekunst, T Mueller, A Weilbacher, MA Dengler, J Bedke, S Kruck, M Oren, WE Aulitzky, H van der Kuip. Cisplatin hypersensitivity of testicular germ cell tumors is determined by high constitutive Noxa levels mediated by Oct-4. Cancer Res. 2013 Mar 1;73(5):1460-9. Epub 2013 Jan 9.

M Gutekunst, M Oren, A Weilbacher, MA Dengler, C Markwardt, J Thomale, WE Aulitzky, H van der Kuip. p53 hypersensitivity is the predominant mechanism of the unique responsiveness of testicular germ cell tumor (TGCT) cells to cisplatin. PLoS One. 2011 Apr 21;6(4):e19198.

Oral presentations

M Gutekunst, T. Müller, A Weilbacher, MA Dengler, J Bedke, S Kruck, H van der Kuip, WE Aulitzky. Oct-4-dependent high constitutive Noxa determines p53-mediated Cisplatin hypersensitivity in testicular germ cell tumors. Onkologie 2012;35(Suppl.6): 183

M Gutekunst, M Oren, A Weilbacher, MA Dengler, WE Aulitzky, H van der Kuip. High expression levels of NOXA are important for p53-mediated hypersensitivity in testicular germ cell tumor (TGCT) cells. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 4693

M Gutekunst, C Markwardt, MA Dengler, H van der Kuip, WE Aulitzky. Cisplatin-hypersensitivity of NTERA cells is mediated through a CHK2/p53 dependent activation of NOXA. Onkologie 2010; 33 (suppl. 6): 87

Poster presentations

M Gutekunst, T Mueller, A Weilbacher, MA Dengler, M Oren, WE Aulitzky, H van der Kuip. Testicular germ cell tumors are hypersensitive to p53 activation based on their Oct-4/Noxa-mediated cellular context rather than on differential p53 activity. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; 2013. Abstract nr 1721.

M Gutekunst, T Mueller, A Weilbacher, MA Dengler, S Kruck, J Bedke, WE Aulitzky, H van der Kuip. OCT-3/4 expression is associated with high levels of the pro-apoptotic BH3 only protein NOXA in testicular germ cell tumors (TGCTs). In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr. 2002.

Other original articles

MA Dengler, AM Staiger, **M Gutekunst**, U Hofmann, M Doszczak, P Scheurich, M Schwab, WE Aulitzky, H van der Kuip. Oncogenic stress induced by acute hyper-activation of Bcr-Abl leads to cell death upon induction of excessive aerobic glycolysis. PLoS One. 2011;6(9):e25139. Epub 2011 Sep 20.

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Other abstracts

MA Dengler, A Weilbacher, **M Gutekunst**, AM Staiger, H Horn, G Ott, H van der Kuip, WE Aulitzky. High NOXA (PMAIP1) transcript levels combined with a short-lived NOXA protein primes mantle cell lymphoma (MCL) cells for death by inhibition of the ubiquitin proteasome system. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; 2013. Abstract nr 1717.

MA Dengler, **M Gutekunst**, AM Staiger, H Horn, G Ott, WE Aulitzky, H van der Kuip. Fatty acid metabolism as a promising target for treatment of mantle cell lymphoma. Onkologie 2012;35(Suppl.6): 42

A Weilbacher, **M Gutekunst**, M Oren, WE Aulitzky, H van der Kuip. Differential effect of the p53 inducers Nutlin-3 and RITA on cell lines with different p53 status. Onkologie 2012;35(Suppl.6): 195

MA Dengler, **M Gutekunst**, AM Staiger, S Kopacz, G Ott, H van der Kuip, WE Aulitzky. Fatty acid metabolism is a possible target for treatment of cyclin D1 over-expressing mantle cell lymphoma. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract no. 4675.

MA Dengler, **M Gutekunst**, S Kopacz, H Horn, U Hofmann, M Schwab, G Ott, H van der Kuip WE Aulitzky. Cyclin D1 Over-Expressing Mantle Cell Lymphoma Cells Are Hypersensitive to Inhibition of Fatty Acid Synthase (FASN). ASH Annual Meeting Abstracts 2011 118:1656. Achievement Award, American Society of Hematology (ASH).

MA Dengler, A Staiger, **M Gutekunst**, U Hofmann, WE Aulitzky, H van der Kuip. Oncogenic stress induced by Bcr-Abl over-activation leads to cell death mediated by a massively enhanced glycolysis. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract no. 4693

MA Dengler, AM Staiger, **M Gutekunst**, WE Aulitzky, H van der Kuip. Oncogenic stress-induced cell death following Imatinib deprivation in Bcr-Abl overexpressing Imatinib-resistant ALL cells. In: Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; 2010 Apr 17-21; Washington, DC. Philadelphia (PA): AACR; 2010. Abstract no. 4522

MA Dengler, A Staiger, **M Gutekunst**, H van der Kuip, WE Aulitzky. Bcr-Abl-mediated oncogenic stress induces ER stress response and programmed necrosis-like cell death in Bcr-Abl overexpressing Imatinib-resistant ALL cells. Onkologie 2010; 33 (suppl. 6): 211

MA Dengler, A Staiger, **M Gutekunst**, Ute Hofmann, H van der Kuip, WE Aulitzky. Glucocorticoids inhibit cell death induced by oncogenic stress after imatinib withdrawal in TKI resistant p190Bcr/Abl overexpressing cells. Blood (ASH Annual Meeting Abstracts) 2010 116: Abstract no. 3150.

C Markwardt, **M Gutekunst**, I Skorta, H van der Kuip, W Aulitzky. Possible role of ASPP1 (Apoptosis Stimulating Protein of p53 1) for the unusual high sensitivity of germ cell tumor cells to Cisplatin. Onkologie 2009; 32(suppl. 4): 218.

8 Curriculum vitae

Personal Details

Name: Matthias Gutekunst

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Education and Training

03/09 - 01/13 **PhD thesis at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart**
Qualification: Dr. rer. nat.

Prof. Dr. Walter E. Aulitzky, Robert Bosch Hospital, Stuttgart

Prof. Dr. Peter Scheurich, Institute of Cell Biology and Immunology, University of Stuttgart

"Chemosensitivity of testicular germ cell tumors is based on high constitutive Noxa protein levels and a functional p53 response"

01/10 - 03/10 **Study visit at the Weizmann Institute of Science, Rehovot, Israel**
Prof. Dr. Moshe Oren, Department of Molecular Cell Biology

10/03 - 02/09 **Degree in Biology at the University of Stuttgart**
Qualification: Diplom

Focus subjects: Immunology / Microbial Biotechnology

Diploma thesis at the Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart

Prof. Dr. Heike Walles, Cell and Tissue Engineering

"Monitoring cell viability by Raman spectroscopy"

02/07 - 07/07 **Visiting student at the University of California, San Francisco, USA**
Prof. Dr. Philip Coffino, Department of Microbiology and Immunology

"Mutations in pore residues of different Rpts affect proteasome function in S. cerevisiae"

09/93 - 06/02 **Abitur at Gymnasium Ebingen, Albstadt**

Stuttgart, August 8, 2013