Exploiting the DNA-damage repair defect of BRCA1-deficient pancreatic cancer cells in drug therapy

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Abstract

Introduction:

Germline mutations of the *BRCA1* gene are a major cause of familial breast cancer and also increase the risk of pancreatic cancer approximately two-fold. To evaluate the role of BRCA1 in suppression of pancreatic tumors, we inactivated Brca1 in an established mouse model of pancreatic cancer. The establish if a targeted therapy of these carcinoma would be possible we determined the sensitivity of primary tumor cells to various drugs routinely used in chemotherapy.

Methods:

To test whether Brca1 suppresses formation of pancreatic tumors, we generated *Pdx-1-Cre* transgenic animals carrying the conditional *K-Ras*$^{G12D/WT}$ allele in combination with conditional-null alleles of *Brca1* and/or *p53*.

Cohort 1 (n=39): *Pdx1-Cre; K-Ras*$^{G12D/WT}$*; p53*$^{cond/cond}$*; Brca1*$^{WT/WT}$

Cohort 2 (n=36): *Pdx1-Cre; K-Ras*$^{G12D/WT}$*; p53*$^{cond/cond}$*; Brca1*$^{cond/cond}$

Tumor-free survival was analysed by Kaplan-Meier survival plots and primary tumor cell lines were established. Histological features and karyotypers were analyzed. To determine the sensitivity of cells to different chemotherapeutic agents, cells were grown for 72 hrs with increasing concentrations of gemcitabine, fluorouracil, paclitaxel, mitomycin C or cisplatin to determine the half inhibitory concentrations (IC50). Results were compared with ANOVA or Student's t-test using GraphPad Prism 5 software.

Results:

Double-mutant mice (cohort 1; n=39) succumbed to pancreatic tumors with an average latency of approximately 10 weeks (T_{50}=68 days). However, tumor latency was dramatically reduced in triple-mutant mice (cohort 2, n=36; T_{50}=40 days; $p<0.0001$), indicating that wildtype Brca1 suppresses pancreatic tumor development.

Pancreatic tumors of all genotypes showed typical features of pancreatic ductal adenocarcinoma; Brca1 deficient mice additionally presented with macrocystic lesions of the pancreas. Spectral karyotype analysis of the Brca1 deficient tumors showed an abundance of structural abnormalities.

In the drug sensitivity assay significant differences in sensitivity between Brca1-proficient and Brca1-deficient pancreatic tumor cells was seen after treatment of cells with the DNA-damaging agents cisplatin ($p<0.01$) and mitomycin C ($p<0.05$), while anti-metabolites (gemcitabine, fluorouracil) or taxanes (paclitaxel) showed no differential effects.

Conclusions:

Brca1-deficient pancreatic tumor cells are hypersensitive to DNA-damaging agents like cisplatin and mitomycin C. Therefore, inclusion of a DNA damaging agent in the treatment protocol may be beneficial to pancreatic cancer patients with *BRCA1* mutations.

Zusammenfassung:

Einleitung:

Keimbahnmutationen des BRCA1-Gens sind eine der Hauptursachen für familiären Brustkrebs und erhöhen auch das Risiko von Pankreaskarzinomen um ca. das Zweifache. Um die Rolle von BRCA1 in der Suppression von Tumoren des Pankreas zu untersuchen, inaktivierten wir BRCA1 in einem etablierten Mausmodell des duktalen Adenokarzinoms des Pankreas.

Um festzustellen, ob eine gezielte Therapie dieser Tumoren möglich wäre, bestimmten wir die Empfindlichkeit primäre Tumorzellkulturen gegenüber verschiedener Medikamente, welche routinemäßig in der Chemotherapie verwendet werden.

Methoden:

Um zu testen, ob BRCA1 die Bildung von Tumoren des Pankreas unterdrückt, generierten wir Pdx1-cre transgene Tiere mit kondtionalem *K-Ras*$^{G12D/WT}$ Allel in Kombination mit konditionalen Null-Allelen von *Brca1* und/oder *p53*.

Kohorte 1 (n=39): *Pdx1-Cre; K-Ras*$^{G12D/WT}$*; p53*$^{cond/cond}$*; Brca1*$^{WT/WT}$

Kohorte 2 (n=36): *Pdx1-Cre; K-Ras*$^{G12D/WT}$*; p53*$^{cond/cond}$*; Brca1*$^{cond/cond}$

Tumor-freies Überleben wurde mit Hilfe einer Kaplan-Meier-Kurve dargestellt. Primäre Tumorzelllinien wurden etabliert und histologische Merkmale und Karyotypen wurden analysiert.

Zur Bestimmung der Empfindlichkeit der Zellen gegenüber verschiedenen Chemotherapeutika wurden die Zellen für 72 Stunden mit steigenden Konzentrationen von Gemcitabin, 5-Fluorouracil, Paclitaxel, Mitomycin C oder Cisplatin inkubiert, um die mittlere inhibitorische Konzentration (IC50) zu bestimmen. Die Ergebnisse wurden mittels ANOVA oder Student-t-Test mit Hilfe der GraphPad Prism 5-Software verglichen.

Ergebnisse:

Doppel-mutante Mäuse (Kohorte 1; n= 39) erlagen invasiven Pankreastumoren mit einer durchschnittlichen Latenz von etwa 10 Wochen (T50 = 68 Tage). Die

Tumorlatenz der dreifach-mutanten Mäuse war dramatisch reduziert (Kohorte 2; n = 36; T50 = 40 Tage, p <0,0001), was darauf hinweist, dass Wildtyp-BRCA1 hilft, die Entwicklung von Pankreas-Tumoren zu unterdrücken.

Pankreastumoren aller Genotypen zeigten typische histologische Merkmale des duktalen Adenokarzinomes des Pankreas. Bei Brca1-defizienten Mäusen wurden zusätzlich macrozystische Läsionen des Pankreas bemerkt.

In der Spektralen Karyotypisierung zeigten Brca1-defiziente Tumoren eine Fülle von strukturellen Anomalien.

Nach Behandlung Brca1-kompetenter und Brca1-defizienter Pankreas-Tumorzellen mit den DNA-schädigenden Medikamenten Cisplatin (p <0,01) und Mitomycin C (p <0,05) konnte ein signifikanter Unterschied in der Sensitivität nachgewiesen werden, während die Behandlung mit Anti-Metaboliten (Gemcitabin, Fluorouracil) oder Taxanen (Paclitaxel) keine unterschiedlichen Auswirkungen auf die verschiedenen Zelllinien zeigte.

1 Introduction

1.1 Motivation

Each year about 12.800 people in Germany are confronted with the diagnosis of pancreatic cancer [1]. Prognosis for these patients is poor, median survival of patients with pancreatic carcinoma is six months; 5-year-survival is between five and seven percent. Pancreatic cancer is the fourth most common malignant cause of death in Germany.

Apart from smoking, chronic pancreatitis and chemical noxa, an important risk factor for pancreatic cancer is a genetic predisposition. Mutations of the oncogene K-Ras and the tumor suppressor gene *TP53* are common in pancreatic cancer cells and it is known that carriers of a deficiency in the breast cancer susceptibility genes BRCA1 and BRCA2 have a higher risk to develop ductal adenocarcinoma of the pancreas. In Germany between two and three percent of pancreatic cancers depend on a genetic predisposition.

Depending on clinical stage patients with pancreatic cancer are currently treated, in curative or palliative intention, with surgery and adjuvant or neoadjuvant radiation and chemotherapy, which is usually administered in clinical therapy trials as a combination therapy of gemcitabine or fluorouracil (5-FU) with various other cytostatics, e.g. erlotinib. Unfortunately only 10-15% of the tumors are still operable at the time of diagnosis and un-operable tumors usually do not respond well to chemotherapy.

Multiple adverse effects of the administered drugs like myelosuppression, mucositis, dermatitis, nausea, diarrhea and cardiac toxicity often limit chemotherapeutic treatment. Therefore, it would be desirable to find a chemotherapy regimen with a strong specificity against tumor cells. A promising way to achieve this is to use the special genetic make-up of hereditary pancreatic cancer as a target for such a specific therapy.

In order to study the role of BRCA1 in the development of pancreatic cancer a mouse model was generated combining conditional loss-of-function mutations of the Brca1 and the p53 genes and an activating mutation of one of the K-Ras alleles into the murine genome, using a pancreas specific Cre-LoxP system (Pdx-1-Cre). For comparison animals carrying only the mutations in the p53 and K-Ras genes were generated.

Primary tumor cell lines derived from these mice can be used as an *in vitro* model system to evaluate the sensitivity of the different genotypes to treatment with various drugs.

Due to the importance of BRCA1 for genomic stability we expect BRCA1-deficient carcinoma cells to be highly sensitive to DNA cross-linking or alkylating agents like cisplatin and mitomycin C, while other drugs used routinely in chemotherapy of pancreatic tumors like 5-FU or gemcitabine should inhibit the cellular proliferation to the same extent independent of the genetic make-up of the tumor cells.

1.2 Ductal adenocarcinoma of the pancreas

1.2.1 Epidemiology

Pancreatic cancer is a rare disease, standardized incidence in Germany is estimated to be 12.6/100 000 in men and 8.7/100 000 in women, resulting in 12.800 cases each year.

Pancreatic adenocarcinoma is a disease of the old age; mean age at diagnosis differs between men and women, being 69 years and 76 years respectively.

In Germany, pancreatic ductal adenocarcinoma is only the ninth most common cause of cancer for men and only the seventh for women, accounting for only about 3% of all cancers. However it is the fourth most common cause of death due to a malignant disease, being responsible for 5.8% in men and 6.7% in women of all cancer casualties. This fact is owned to the high mortality rate of the ductal adenocarcinoma that is almost as high as the incidence, viz. 12.6/100,000 in men and 8.4/100,000 in women [1].

The figures in the USA are similar; incidence for men and women being 13/100,000 and 10.6/100,000 respectively and mortality being 12.2/100,000 and 9.3/100,000 respectively. It has been estimated that 37,680 men and women (18,770 men and 18,910 women) will be diagnosed with and 34,290 men and women will die of cancer of the pancreas in 2008 [2].

There are only few known risk factors for the development of pancreatic ductal adenocarcinoma: Age, smoking, the consumption of red meat and chronic pancreatitis. An elevated risk has also been described for patients with diabetes and obesity. Manifestation of pancreatic cancer in family members can also lead to an increased risk; about 5-10% of pancreatic cancers are estimated to be based on a familial predisposition [3].

Germline mutations that have been associated with elevated pancreatic cancers risk include the tumor suppressors p16INK4a (CDK inhibitor 2A (melanoma, p16, inhibits CDK4)), BRCA1 (Breast cancer susceptibility gene 1), BRCA2 (Breast cancer susceptibility gene 2) and STK11/LKB1 (Serine/threonine kinase 11), the DNA mismatch repair gene MLH1 (MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)) and the protease gene PRSS1 (protease, serine, 1 (trypsin 1)). As the age of diagnosis for patients with one of these germline mutations is not younger than for

patients with sporadic PDAC it seems likely that these genes contribute to carcinogenesis only at a later stage, i.e. during the progression of non-invasive precursor lesions to invasive carcinoma.

1.2.2 Histology

Pancreatic neoplasms encompass a variety of different subtypes, including both benign and malignant tumors. Histological they are separated into the common tumors of the exocrine gland and rare endocrine tumors like insulinomas. Benign neoplasms of the pancreas are rare; the most common is the serous cystadenoma, which accounts for about 1% of all pancreatic tumors. The most important malignant entity is the ductal adenocarcinoma, accounting for up to 90% of all pancreatic neoplasms. There are several other rare types of malignant pancreatic neoplasms including serous or mucinous cystadenocarcinoma.

Table 1**:** Histological classification of pancreatic neoplasms

Pancreatic neoplasm	Histological features	Genetic alterations	Frequency
Ductal adenocarcinoma	Ductal morphology; desmoplasia	K-Ras, $p16^{INK4a}$, *TP53,* SMAD4	80-90%
Variants of ductal adenocarcinoma:			
a. Medullary carcinoma	Poorly differentiated; intratumoral lymphocytes	hMLH1, hMSH2	
b. Colloid (mucinous noncystic) carcinoma	Mucin pools	MUC2 overexpression	
Acinar cell carcinoma	Zymogen granulas	APC/ β-catenin	1%
Pancreatoblastoma	Squamoid nests, multilineage differentiation	APC/ β-catenin	
Solid pseudopapillary neoplasm	“Pseudo” papillae, solid and cystic areas, hyaline globules	APC/ β-catenin, CD10 expression	
Serous cystadenoma	Multilocular cysts; glycogen-rich epithelium	VHL	
Pancreatic endocrine tumors	Hormone production	MEN I	2-3%

Ductal adenocarcinomas are originating from epithelial lining of small pancreatic ductules as it is demonstrated by the occurrence of in-situ precursor lesions termed pancreatic intraepithelial neoplasia (PanIN). Invasive pancreatic adenocarcinomas are located in 70% of the cases in the head, in 20% in the body and in 10% in the tail of the pancreas.

1.2.3 Carcinogenesis in the pancreas

In 1953 Nordling first proposed that cancer was the result of accumulating DNA damage [4]. Based on this model, Knudson later formulated his two-hit-hypothesis showing that retinoblastoma are the result of two independent events (hits) affecting the DNA [5].

Based on the multiple hit hypothesis tumor progression models were developed, in which the combination of loss of tumor-suppressor genes and the activation of oncogenes leads to uncontrolled proliferation of a cell and thus to cancer. The most commonly known of these tumor-progression models is the adenoma-carcinoma sequence of colorectal adenocarcinoma, which is developing from benign adenomatous polyps to invasive carcinoma. Analogically, a progression model for the adenocarcinoma of the pancreas has been introduced.

There are three distinct types of precursor lesions to pancreatic neoplasms: Pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN). The most common of these are the PanIN, the most common precursors of ductal adenocarcinoma, which arise in the smaller pancreatic ducts. Low-grade PanINs are found in up to 30% of pancreatic specimen of elder adults [3].

Based on the degree of architectural and cytonuclear atypia PanINs are classified in three grades [6].

PanIN-1 is the lowest grade lesion, characterized by single layer of cells typically with mucinous metaplasia and absence or minimal nuclear atypia. It is subcategorized in PanIN-1A and PanIN-1B, with presence or absence of micropapillary infoldings of the epithelium, respectively. It is histological difficult to distinguish between PanIN 1A/B and mucinous change of pancreatic ductules due to other mechanisms (e.g. obstruction or inflammation).

PanIN-2 lesions show a moderate degree of nuclear atypia including enlarged nuclei, loss of polarity, nuclear crowding, pseudostratification and hyperchromatism in a pseudostratified epithelium similar to colorectal adenomas.

PanIN-3 lesions, the carcinoma in situ, are characterized by severe degree of nuclear atypia accompanied by proliferating epithelial cells commonly forming a cribriform pattern with luminal necrosis and atypical mitoses. In contrast to the invasive

pancreatic carcinoma PanIN-3 lesions are still contained within the basement membrane of the pancreatic ducts.

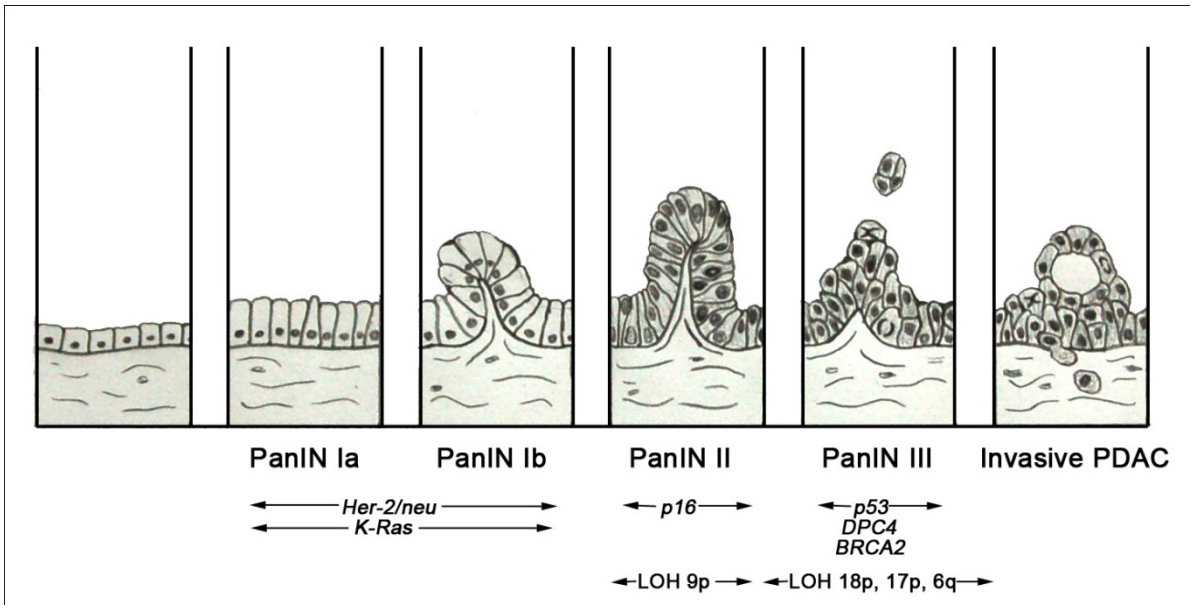

Figure 1: Progression model for pancreatic cancer. The progression from histological normal epithelium to low-grade PanIN to high-grade PanIN (left to right) is associated with the accumulation of specific genetic alterations. (Basd on [7])

Beside PanIN, intraductal papillary mucinous neoplasms (IPMN) can be a precursor of PDAC, but IPMN also are a virtually obligate precursor lesion for the colloid mucin producing subtype of PDAC. IPMN arise from the epithelium of either the main pancreatic duct or its major side-branches. They are made up of dilated ductal segments, often with papillary projections that secrete mucin. Like mucinous cysts, IPMN have a high potential for malignant transformation.

Mucinous cystic neoplasms (MCN) are mucin-producing cysts characteristically surrounded by an ovarian-like stroma, which similar to granulosa-theca cells is positive for estrogen and progesterone receptors and alpha-inhibin. The mucinous lining of the cyst shows a wide variety of cytological and architectural atypia. MCN can develop to mucinous cystadenocarcinoma that can be of tubular or ductal type. It is still controversial whether carcinomas developing from MCN are biologically different from ductal adenocarcinoma or if MCN are a precursor lesion to ductal adenocarcinoma.

The histological progression is inevitably accompanied by genetic alterations [7]. Often the first mutations occurring in pancreatic carcinogenesis are activating

mutations of *K-RAS*, an oncogene of the RAS family. Mutations in the *K-RAS* gene can infrequently already be found in normal pancreatic tissue and they can be detected in 30% of early neoplasms and in up to 95% of advanced PDACs [8].

Another early event is the over-expression of the Her2/neu receptor, which is usually not expressed in pancreatic ductal epithelial cells. Overexpression in precursor lesions seems to correlate with the severity of dysplasia and is most prominent in well-differentiated ductal adenocarcinoma [9].

Mutations in the cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4), also known as *CDKN2A* gene can lead to functional inactivation of the $p16^{INK4a}$ protein. Physiologically $P16^{INK4a}$ is involved in the retinoblastoma protein pathway, which ultimately leads to the entry of the cell into S-phase. $P16^{INK4a}$ binds to CDK4/CDK6, thus preventing the formation of an active cyclin D/CDK4/CDK6 complex and subsequently the phosphorylation of the retinoblastoma protein. Loss of the $P16^{INK4a}$ tumor suppressor through mutation or promoter hypermethylation and the resulting uncontrolled entry into S-phase has been described in early and moderately progressed lesions like PanIN-1b and PanIN2 and have been found in 80-95% of pancreatic cancers [10].

A protein related with $P16^{INK4a}$ is $P19^{ARF}$, both proteins are encoded by the 9p21 locus but differ in their first exon and alternative reading frames of the shared downstream exons. Functional $P19^{ARF}$ inhibits the MDM2 dependant degradation of the P53 tumor suppressor protein (see below); loss of $P19^{ARF}$ accordingly leads to increased degradation of P53. Despite the fact that $P16^{INK4a}$ and $P19^{ARF}$ are encoded by the same gene, there are germline and sporadic mutations targeting $P16^{INK4a}$ but sparing $P19^{ARF}$. Thus $P19^{ARF}$ probably plays a less significant role in pancreatic carcinogenesis. Yet the role of P53 independent mechanisms of action such as inhibition of ribosomal RNA processing still has to be explored.

However, more important than a lowered cellular concentration of P53 through increased degradation is another mechanism, which also leads to an impaired P53 function: Mutations in the *TP53* locus itself. Mutations in the *TP53* locus are common in all kinds of cancer (up to 50% of all tumors) and they can be frequently found in pancreatic cancers (76%). Mutations of *TP53* seem to be a late event in carcinogenesis, they can be found in high grade PanIN with significant features of dysplasia, illustrating their role in malignant progression [11].

Further changes in the genome found in PanIN-3 lesions include inactivity of the tumor suppressor gene *SMAD4* (SMAD family member 4), also known as *MADH4* or *DPC4* and the DNA-maintenance genes *BRCA1* and *BRCA2*.

The *SMAD4* tumor suppressor gene, which is located on chromosome 18q21, is part of the signaling cascade downstream from TGF-β and Activin, thus playing an important role for growth inhibition via pro-apoptotic signaling and regulation of G1/S transition. Loss of SMAD4 accordingly leads to an inadequate cell growth and proliferation of the cell. Inactivation of SMAD4 has been shown to occur in about 55% of pancreatic cancers [12] and more than 90% show loss of heterozygosity at this locus.

Inactivating mutations of DNA repair genes like *BRCA1* or *BRCA2* also are late events in carcinogenesis, as they require an appropriate genomic cell context to be tolerated by a cell. The increasing amount of DNA damage would usually lead the cell to apoptosis or senescence, but for example in absence of tumor suppressors like P53 these mechanisms are suppressed and even extensive genetic damage can be tolerated. 17% of familial pancreatic cancers have been shown to exhibit mutations in the *BRCA2* gene [13]. Unfortunately there is currently no consistent data evaluating the contribution of acquired mutations in the *BRCA* genes to non-familial pancreatic carcinoma.

Beside these changes in distinct genes and proteins reduced telomere length has been described in both early and late PanIN lesions. The resulting unstable chromosome ends can lead to abnormal fusion events and chromosomal rearrangements.

1.2.4 Hereditary pancreatic cancer

As explained above the ductal adenocarcinoma of the pancreas is a genetic disease. Mutations can be the result of spontaneous mutations or the influence of carcinogens, for example found in cigarette smoke, or they can be inherited. It is estimated that about 5 - 10% of pancreatic cancers are hereditary [14]. Many of these hereditary cancers are part of rare medical syndromes that have known underlying genetic defects.

Peutz-Jeghers syndrome is an autosomal-dominantly inherited syndrome characterized by mucocutaneous pigmentation and gastrointestinal polyposis, caused by germ-line mutations in the serine/threonine kinase gene (*STK11/LKB1*) on chromosome 19p13.3 [15]. Patients have a high risk to develop cancer, by the age of 70 85% are estimated to develop cancer, especially gastrointestinal cancer (33% at age 70), including pancreatic cancer (11% at age 70) [16]. Earlier studies even reported a 36 – 42% lifetime risk to suffer from pancreatic ductal adenocarcinoma [17].

The Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome is a rare hereditary syndrome causing affected family members to develop skin moles and melanomas. These patients also have an increased risk of developing pancreatic cancer. Especially patients from pedigrees carrying a *CDKN2A* mutation in context of the FAMMM syndrome are known to have a 13-22 fold increased risk to develop pancreatic adenocarcinoma, so that this constellation has been termed familial atypical multiple mole melanoma-pancreatic carcinoma syndrome (MPCS) [18].

There are further cancer predisposition syndromes like the hereditary non-polyposis colorectal cancer (HNPCC), the familial adenomatous polyposis (AFP) or the Li-Fraumeni syndrome but the risk for these patients to develop cancer of the pancreas is estimated to be below 5%.

It is known that pancreatic cancer itself (not as part of any known syndrome) can appear as a familial disease accordingly called familial pancreatic cancer (FPC). Criteria for FPC are at least two cases of pancreatic carcinoma in first-grade relatives. In Germany 1 – 3% of pancreatic cancers fulfill the criteria for FPC. FPC families show the phenomenon of anticipation; in the younger generation the carcinoma seems to manifest at a younger age (about 10 years) than the previous generation.

With the help of affected families it has been possible to map a candidate gene to 4q31.3. Recently, it was proposed that the responsible gene was Palladin, which is involved in the organization of the actin skeleton, but further studies showed that mutations in this gene could not be detected in other families and that Palladin is mostly overexpressed in the non-neoplastic stroma of infiltrating ductal adenocarcinomas, but is only rarely overexpressed in the cancerous cells [19].

Between 6 and 19% of familial pancreatic cancer families carry a germline mutation of *BRCA2*. Notably, not all of these families have a history of breast or ovarian

cancer [20]. PDAC connected with a mutation in *BRCA2* occurs 8-10 years earlier than the sporadic disease.

It is well known that the hereditary breast-ovarian cancer syndrome caused by mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* involves an increased risk to develop other types of cancers. Especially a deficiency in *BRCA2*, but also of *BRCA1* leads, beside to the name-giving breast and ovary cancer, to a high risk to develop a pancreatic carcinoma. Relative risk for carriers of *BRCA1* or *BRCA2* mutations to develop PDAC is 2.26 and 5.9 respectively [21, 22].

Additionally, there are some hereditary diseases carrying an elevated risk to develop pancreatic cancer, although the gene defects themselves are not carcinogenic.

Hereditary pancreatitis, which is a disease characterized by recurrent pancreatitis, is caused by a defect in either *PRSS1* (7q35) or *SPINK1* (Serine peptidase inhibitor, Kazal type 1) (5q31). Result of these mutations is an altered cationic trypsinogen that is not autolysed in the pancreas cells and thus starts to digest the pancreas from within. The resulting recurrent or chronic pancreatitis is a known risk factor for pancreatic adenocarcinoma as the chronic inflammation is a proliferation stimulus. Relative risk to develop PDAC is estimated to be 69 [23]

Cystic fibrosis iscaused by defects in the cystic fibrosis transmem-brane regulator (*CFTR*) gene. The faulty chloride channel leads to an obstruction of mucinous glandula in the pancreas and to chronic inflammation. Relative risk for PDAC forpatients with cystic fibrosis is estimated to be elevated between 2.26 and 32 fold [24], [25].

Table 2: Genetic syndromes with a gentic predisposition to pancreatic cancer (based on [26])

Syndrome	Mutated gene (locus)	Pancreatic cancer risk
Peutz-Jeghers	*STK11* (19p13.3)	RR = 132 (95% CI, 44–261)
Hereditary pancreatitis	*PRSS1* (7q35)	RR = 69 (95% CI, 56–84)
	SPINK1 (5q31)	
Cystic fibrosis	*CFTR* (7q35)	2.26 – 32-fold
FAMMM	*CDKN2A* (9p21)	13 – 22-fold
Hereditary breast-ovarian cancer	*BRCA1* (17q21)	RR = 2.26 (95% CI, 1.2–4.0)
	BRCA2 (13q12)	RR = 5.9 (95% CI, 3.2 to 10.0
Familial adenomatous polyposis (FAP)	*APC* (5q21)	RR = 4,5 (95% CI, 1.2–11.4)
Lynch syndrome	*MSH2* (2p22-21)	Increased
	MLH1 (3p21)c	
Ataxia teleangiectasia	*ATM* (11q22-23)	Increased
Von Hippel-Lindaud	*VHL* (3p25)	Increased

1.2.5 The UICC TNM Classification and staging of the ductal adeno-carcinoma of the pancreas

The “International Union Against Cancer” (UICC) created a system of classification and staging based on the extension of the primary tumor, the infiltration of local lymph node groups and the presence of distant metastases. This classification, called the TNM-classification and the resulting stage grouping was created to allow an objective assessment of a tumor and thus enable the physician to give a prognosis for the individual patient at the time of diagnosis, plan an adequate treatment and evaluate the results of treatment

The TNM cancer staging system comprises three main dimensions: T describes the local extent of the tumor and a possible infiltration into the surrounding tissue; N illustrates the infiltration of surrounding lymph node areas and M indicates the presence of distant metastases. An additional R dimension is added if the patient has undergone surgery to describe the completeness of the tumor resection (table 3).

Based on the values in the TNM scale, invasive carcinomas are classified in four (I-IV) staging groups (table 4).

Table 3: The stage grouping of the UICC TNM-Classification (based on [27])

	M0		M1
	N0	N1	
Tis	0		IV
T1	IA	IIB	
T2	IB	IIB	
T3	IIA	IIB	
T4	III	III	

Table 4: The UICC TNM-Classification of pancreatic ductal adenocarcinoma (based on [27])

Extent of primary tumor (T category)	
Tis	Carcinoma in situ
T1	Tumor limited to the pancreas, ≤2cm in greatest dimension
T2	Tumor limited to the pancreas, >2cm in greatest dimension
T3	Tumor extends beyond the pancreas, but without involvement of celiac axis or superior mesenteric artery
T4	Tumor involves celiac axis or superior mesenteric artery
Infiltration of regional lymph nodes (N category)	
N1	Along the common hepatic artery, splenic hilum, along the splenic artery, along the inferior margin of the pancreatic body-tail.
N2	Along the left gastric artery, around the celiac artery, along the superior mesenteric artery, along the middle colic artery
N3	Suprapyloric, infrapyloric, in the hepatoduodenal ligament, on the posterior surface of the pancreatic head, around the abdominal aorta, on the anterior surface of the pancreatic head
Presence of distant metastasis (M category)	
M1	Metastasis to distant organs, peritoneum or distant lymph nodes
Resection margins	
R0	Tumor-free resection margins (with 1mm minimum clearance)
R1	Microscopic residual tumor
R2	Macroscopic residual tumor

1.3 Therapy of the ductal adenocarcinoma of the pancreas

1.3.1 Curative therapy

A curative intended therapy is only possible in absence of distant metastases. Long-term survival following R0 resection of the tumor is still below 20%. Both distant metastases and local recrudescence can be causes of tumor relapse.
Following surgical therapy in UICC stages I-III the treatment protocol includes adjuvant chemotherapy. Chemotherapy succeeded to increase disease-free survival, but failed to increase overall survival significantly [28].

1.3.1.1 Surgical therapy

The purpose of surgical therapy of a pancreatic carcinoma in curative intention is the complete resection of the tumor (R0 resection) with tumor free margins of 10mm. Complete resection is indicated independently of localization and infiltration of other organs or blood vessels, although a possibly required resection of arteries (A. mesenterica superior or celiac trunk) usually is a limit to complete resection.
For carcinoma of the pancreas head the procedure of choice is usually the pancreaticoduodenectomy with hemigastrectomy (Classic Whipple procedure) or the pylorus sparing pancreaticoduodenectomy. From the oncological point of view long-term outcome is the same for both procedures. If possible it is avoided to carry out a total pancreatectomy as patients often develop Brittle-Diabetes after this procedure.
For tumors of the body or the tail a total or subtotal distal pancreatectomy, in some cases with splenectomy, is indicated.
Although mortality of the Whipple procedure even in specialized centers still is around 5% and usually pancreatectomy is not indicated in the presence of distant metastasis, the indication to carry out these procedures can be given, if distant metastases are first discovered during the procedure as a palliative measure to reduce tumor pain.

1.3.1.2 Drug therapy

Due to the low incidence of PDAC most clinical trials on drug therapy of PDAC with curative intention only include small numbers of patients. Trials with a large number of treated patients have only been published for adjuvant therapy with gemcitabine. Therefore, patients treated with adjuvant chemotherapy following an R0 resection should be included in clinical treatment studies. The most important protocols include the Mayo-protocol, the AIO protocol (both 5-FU/ folic acid) and single-agent therapy with gemcitabine, which is the best evaluated one [28]. Adjuvant chemotherapy should start within six weeks after resection and should be administered over six months.

There is no evidence that adjuvant radiochemotherapy has any benefit considering clinical outcome or survival when compared to chemotherapy. Due to the lower incidence of dose-limiting adverse reactions chemotherapy is usually chosen over radiochemotherapy.

Following R1 resection patients are treated with additive chemotherapy, consisting of a single agent regimen with gemcitabine. There is not enough data yet to appraise the role of additive radiochemotherapy after R1 resection, again patients eligible for this option should be included in clinical treatment studies.

A neoadjuvant therapy, i.e. a treatment that is given prior to an operation to reduce the tumor in size and allow a R0 resection, is only recommended in clinical trials. There is no data yet supporting the benefit of a neoadjuvant therapy, although it has been shown that 10% of primarily non-resectable, locally advanced tumors can reach a secondary resectibility following neoadjuvant radiochemotherapy [29].

The role of targeted therapies and immunotherapeutic agents in curative therapy of pancreatic cancer has not been evaluated yet and use is restricted to clinical trials.

1.3.2 Palliative therapy

As mentioned above the majority of patients with pancreatic adenocarcinoma present with symptoms only when the tumor has already infiltrated adjacent tissue or metastasized. Currently there is no curative therapy for these patients. In various

studies palliative chemotherapy showed an improvement of clinical performance and survival compared to the best supportive care [30].

Palliative first-line chemotherapy can be administered as a single agent therapy with gemcitabine, which showed a significantly longer progression-free survival than a treatment with 5-FU [31].

Further phase II and III studies evaluating combinations of gemcitabine with various other drugs, like the tyrosine kinase inhibitor erlotinib or the platinum agent cisplatin are currently under way.

The combination of gemcitabine with the small molecule drug erlotinib (targeting specifically the epidermal growth factor receptor (EGFR) tyrosine kinase) showed a promising increase of 1-year-survival to 24% compared to 19% in the gemcitabine-only group. For patients with metastasized disease the one-year survival was raised by 22%, yet for patients with locally advanced and thus inoperable disease a non-significant difference between the two groups has been shown, approving the use of gemcitabine/erlotinib only in metastatic disease [32].

Another promising drug for the use in locally advanced and metastatic disease is Endo-tag®-1. Endo-tag®-1 is a drug composed of the mitotic inhibitor paclitaxel contained in a cationic, i.e. positively charged, liposome. Due to the positive charge these liposomes are able to attach themselves selectively to the newly developing endothelial cells of the tumor, which are negatively charged.

A phase II study showed a one-year-survival of 36% for patients with locally advanced or metastatic pancreatic cancer who were treated with a combination of gemcitabine with Endo-tag®-1, compared to 17% for patients treated with gemcitabine only.

A combination of gemcitabine with platinum derived drugs like oxaliplatin or cisplatin or the antimetabolite prodrug capecitabine showed a benefit, but only for patients with good clinical status (Karnofsky Index >90% or ECOG 0-1) [33, 34].

Other drugs and combination therapies (i.e. mitomycin C, the combination of cisplatin, epirubicin, 5-FU and gemcitabine (PEFG) or gemcitabine plus docetaxel) showed either no clinical benefit or led to an increase in adverse reactions with only marginal effects on survival or performance.

The role of radiochemotherapy for patients with advanced disease still needs to be further investigated. On one hand, radiochemotherapy has been shown to be able to

allow a secondary R0 resection, on the other hand treatment with radiochemotherapy can be accompanied by the early development of distant metastases in some patients. Due to the favorably profile of side effects 5-FU is preferred over gemcitabine as radiosensitizer.

1.4 Oncogenes and tumor suppressor genes

As described above carcinomas develop in various steps involving the activation of oncogenes or the functional loss of tumor suppressor genes.

Proto-oncogenes encode for proteins, e.g. kinases or growth factors, involved in the regulation of cell differentiation, proliferation and cell cycle control. The conversion of proto-oncogenes to oncogenes can occur via two mechanisms, either via an increase in the number of gene products, as a result of increased transcription/translation or reduced degradation, or via changes in the genomic sequence, providing the gene product with new capabilities, e.g. a higher affinity to its substrate. Activating mutations of oncogenes function in a dominant manner, the resulting signaling activity can lead to a premature entry to mitosis or inadequate proliferation.

Tumor suppressor genes on the other hand encode proteins that, if they work properly, counteract the development of carcinomas. They are often involved in processes important for the maintenance of cell integrity such as DNA damage repair or induction of apoptosis. The lack of control or repair mechanisms enables the cell to proliferate even if alterations are present that would typically lead to cell cycle arrest or induction of apoptosis.

In most cases loss of function of a tumor-suppressor gene is recessive; if one allele of the gene is lost the product of the intact allele is usually sufficient to perform the task of the gene. This explains why patients suffering from an inherited deficiency in a tumor-suppressor gene like *BRCA1* do not develop carcinoma at a very early age but only after the remaining wildtype allele is lost.

A third group of genes whose malfunction could possibly lead to the development of a malignant tumor are micro-RNA genes. Micro-RNA genes do not encode proteins; instead they are involved in the regulation of gene expression. Micro-RNAs are small RNA molecules with a length between 20 and 23 nucleotides that are able to anneal to messenger RNA (mRNA). Micro-RNAs can act both as oncogenes and as tumor suppressors by either blocking the translation of the mRNA to protein or hindering its degradation [35].

1.4.1 The tumor suppressor BRCA1

The observation that 10% of breast tumor cases have a familial background led to the idea that a hereditary mutation in a tumor suppressor gene could be responsible for this group of cancers.

The gene encoding the tumor suppressor protein BRCA1 was first identified by linkage analysis in 1990 [36, 37] and then cloned in 1994. It is located on the long arm of chromosome 17 (17q21) (mouse chromosome 11). It contains 24 exons and encodes a protein of 1863 amino acids [38].

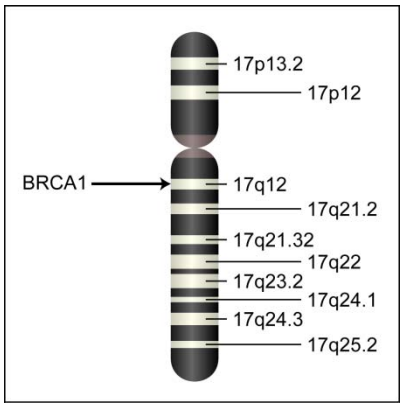

Figure 2: Location of BRCA1 on the long arm of chromosome 17.

BRCA1 is mostly known for the high incidence of breast and ovary carcinoma in context of the hereditary breast ovarian cancer syndrome (HBOC). The risk for patients with a germline mutation of *BRCA1* to develop cancer of the breast or the ovary is estimated between 45% and 87% by the age of 70 for breast cancer and between 36% and 66% by the age of 70 for ovary cancer. Furthermore, carriers of *BRCA1* mutations are at a statistically significantly increased risk to develop carcinomas of several other tissues, including pancreatic cancer (RR = 2.26) and carcinomas of the uterine body (RR = 2.65) and cervix (RR = 3.72). Additionally the relative risk for male carriers to develop prostate cancer before the age of 65 years is elevated (RR = 1.82) [21].

BRCA1 is essential for normal embryonic development. Mice carrying two *Brca1* null alleles (*Brca1*$^{-/-}$) exhibit growth retardation and die during early embryogenesis. This developmental faultiness of the *Brca1* null embryos could be ameliorated or partly rescued in a *p53*-deficient background [39]. While *Brca1*$^{-/-}$ animals die already at day e7.5 of embryonic development, this embryonic lethality can be delayed until e9.5-e10.5 by generating compound homozygous knock-out embryos with *p53* deletion (i.e., *Brca1*$^{-/-}$; *p53*$^{-/-}$).

The BRCA1 protein is usually localized in the nucleus. Expression is regulated cell-cycle dependant, mRNA levels rise at G_1/S remains high during S, G_2 and M phases and decreases again in G_1 phase [40]. Post-translational modifications of BRCA1 include phosphorylation and ubiquitination.

Considering that it is a protein with tumor suppressor function the sequence of *BRCA1* is very weakly conserved in mammalian evolution. The human *BRCA1* sequence is only 56% identical with murine *Brca1*. *TP53* for example is conserved 77% and *RAD51* even 99% [41]. Surprisingly, in spite of this poor conservation, human *BRCA1*, introduced via an artificial chromosome, is able to rescue the embryonic lethality of *Brca1* null mutations in mice [42].

Proper function of the BRCA1 protein requires three different functional domains. Located at the N-terminal region of *BRCA1* is a RING (Really interesting new gene) Zinc finger domain (residue 5-98) and at the C-terminal region there are two tandem BRCT (BRCA1 C-Terminus) motifs (residue 1650-1859). Furthermore, BRCA1 contains a nuclear localization sequence (NLS) (residue 200–300). Unlike the BRCA1 protein as a whole, these functional domains are highly conserved in mammalian evolution.

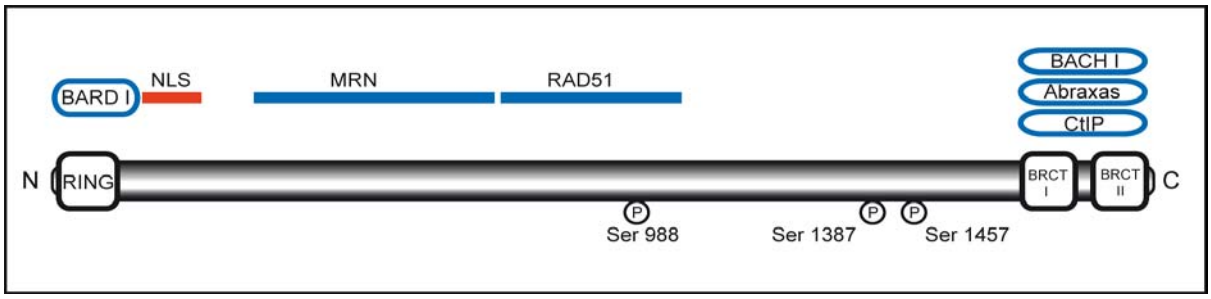

Figure 3: Functional domains of the BRCA1 protein. Schematic representation of the functional domains of BRCA1. Representative proteins are marked above the site of interaction. Important sites of phosphorylation are indicated.

The BRCT domain contains a repeated sequence of approximately 100 amino acids, which has been identified in several proteins involved in DNA repair. The two BRCT repeats form a phosphoprotein-binding pocket, which is required for interaction with other proteins. For example, the activation of the G_2 accumulation checkpoint by BRCA1 requires interaction between the BRCA1-BRCT motifs and a Ser-990-phophorylated BRCA1-Associated Carboxyl-terminal Helicase (BACH1) [43] and the activation of the transient G_2/M checkpoint requires BRCA1-BRCT interaction with a Ser-327-phosphorylated C-terminal binding protein interacting protein (CtIP) [44].

So far the only enzymatic activity of BRCA1 that has been shown both *in vitro* and *in vivo* is its E3 Ubiquitin ligase activity, which is catalyzed by the RING Zinc finger. The RING domain includes a pattern of seven cysteine residues and one histidine

residue, which bind two Zn^{2+} atoms. This pattern was immediately recognized when BRCA1 was cloned, as it was well known from several other proteins interacting with DNA.

Besides the characteristic Cysteine residues the RING domain contains two antiparallel α-helices required for heterodimerization with a structurally related protein called BARD1 (BRCA1 associated Ring domain) [45].

It is important to know that virtually all the BRCA1 in the cell is associated with BARD1 [46]. The importance of this interaction is obvious as $BARD1^{-/-}$ mice show a phenotype indistinguishable from $BRCA1^{-/-}$ or double $BRCA1^{-/-}$; $BARD1^{-/-}$ mice and die during early embryogenesis [47]. Furthermore the *in vitro* E3 ubiquitin ligase activity of BRCA1 is increased dramatically if BARD1 is introduced to the reaction [48].

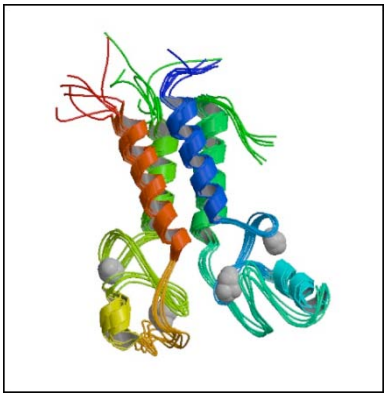

Figure 4: 3D-structure of the BRCA1-BARD1 heterodimeric RING-RING complex [37].

Although much shorter (777 amino acids) than BRCA1 the BARD1 protein also contains a N-terminal RING-domain and two C-terminal BRCT repeats as well as additional Ankyrin repeats, whose functions are still unknown.

More than 300 cancer-disposing missense mutations have been described for *BRCA1*. These include missense mutations located in five of the seven cysteine residues of the RING domain and in the BRCT domains and protein truncating mutations along the whole protein.

BRCA1 has been included in a group of tumor-suppressor proteins known as caretakers of the cell. It has a pivotal role in the repair of DNA double-strand breaks, regulation of centrosome amplification and cell cycle checkpoint control and thus for genomic stability of the cell. Deficiency of the *BRCA1* gene consequently results in the accumulation of gross chromosomal rearrangements like translocations, major deletions and fusions. This fact is impressively illustrated by Spectral Karyotype Imaging (SKY) analysis of BRCA1-deficient tumor cells, as shown below.

The cellular response to DNA damage requires three steps, damage sensoring, initiation and regulation of the cellular response including regulation of the cell cycle and finally the repair of the damaged strand.

Following a double strand break, BRCA1 is involved in cell cycle checkpoint activation, the selection of the repair pathway and the actual repair of the DNA damage.

The eukaryotic cell possesses three distinct pathways for the repair of double-strand breaks: The error-prone single-strand annealing (SSA) pathway, the homologous recombination repair pathway (HR) and non-homologous end joining (NHEJ). BRCA1 contributes to both homologous recombination and non-homologous end joining.

Homologous recombination repair is limited to S- and G_2-phase as it requires a homologous strand of the sister chromatid. HR is not only required to repair double strand breaks but also to repair DNA interstrand crosslinks introduced by DNA damaging drugs like mitomycin C or platinum derivates.

Double-strand breaks are first recognized and bound by the MRE11-RAD50-NBS1 (MRN) complex, which recruits the ataxia-teleangiectasia-mutated (ATM) protein kinase. Then BRCA1 is relocated to the damage site by the histone H2AX that has been phosphorylated by ATR following the double strand break (γ-H2AX). There, BRCA1 forms a complex with RAD51 and BRCA2 (BRCC complex) that activates the pathway for DNA repair via homologous recombination. At the site of the DNA damage BRCA1 also co-localizes with the MRN complex. The re-localization of BRCA1 to nuclear foci in response to DNA damage is the result of enhanced phosphorylation of the BRCA1 protein [49]. BRCA1 can be phosphorylated by various damage sensing kinases. Different damage sensing kinases phosphorylate BRCA1 on different target Serine residues, a fact that could help to explain how BRCA1 is able to fulfill its distinct functions in the cell. While phosphorylation by Checkpoint kinase 2 (CHK2) at serine 988 leads to the arrest of the G_2/M checkpoint [50], both phosphorylation by the damage sensing kinases ATM (Ataxia teleangiectasia mutated) at serine residue 1387 [51] and ATR (ATM and RAD3-related) at serine 1457 [52] lead the cell to DNA damage repair.

Besides co-localization with the MRN complex and the BRCC complex in response to DNA damage [53], the genome-associated BRCA1 has been demonstrated to be part of several other multi protein complexes including the BRCA1 associated surveillance complex (BASC) [54] and the BRCA1 A (containing Abraxas), BRCA1 B (containing BACH1) and BRCA1 C (containing CtIP) foci.

Through collaboration with the other proteins in these complexes BRCA1 seems to be involved in regulation of DNA-processing, namely the regulation of the MRN

complex protein MRE11 and most importantly the control of the length of the single-strand DNA that is processed by CtIP. Processing of single-strand DNA is one of the first steps of DNA damage repair and it seems to be the decisive step in the selection of the repair pathway that is chosen for DNA repair. This process has been shown to depend on the collaboration of BRCA1 and CtIP throughout the cell cycle [55], but unfortunately it is not fully understood yet.

By activation and re-localization of the MRN complex, BRCA1 is also involved in one of the first steps of the NHEJ pathway. One of the main proteins of the NHEJ pathway is a DNA-dependent serine/threonine protein kinase termed DNA-PK, a protein composed of the DNA end-binding complex (Ku) built from the subunits Ku70 and Ku80 and the catalytic subunit DNA-PKcs. The DNA-PK is able to bridge broken DNA strand ends that have been processed by the MRN complex to make them available for ligation by DNA ligase IV and XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4).

BRCA1 is not only involved in the repair of double-strand breaks but also in the nucleotide excision repair (NER) pathway, which is responsible for removing damaged bases. NER especially recognizes bulky distortions in the integrity of the DNA double helix. Damage of bases occurs regularly in daily life, prominent examples are thymidine dimers caused by UV-light. Patients suffering from Xeroderma pigmentosum, a genetic disease with in-born mutations of one of the genes involved in the NER pathway like XPE (Xeroderma pigmentosum E) or XPC (Xeroderma pigmentosum C), are highly photosensitive. BRCA1 and P53 are known to regulate transcription for both XPE and XPC; these proteins are involved in the DNA damage sensoring of the NER pathway and it has been suggested that BRCA1 can do so in an P53-independent manner, although the clinical relevance of this function remains to be shown [56].

This is not the only case where BRCA1 is involved in the regulation of transcription. The C-terminus of BRCA1 is able to bind and activate RNA polymerase II through the RNA helicase A. Also the BRCA1/BARD1 heterodimer is able to bind the transcriptional factor ZNF350 (Zink Finger Protein 350) and promote transcription of P21 and GADD45 (Growth Arrest and DNA Damage 45), two proteins involved in pathways leading to cell cycle arrest.

BRCA1 is involved in cell cycle control in various ways, especially in the G_2/M checkpoint control. As mentioned above G_2/M checkpoint control is dependant on the interaction of BRCA1 with CtIP [44]. Upon DNA damage BRCA1 is essential for the activation of the CHK1 kinase that controls G2/M checkpoint arrest. Furthermore it controls the expression, phosphorylation and cellular localization of both the CDC2/cyclinB kinase and CDC25C, two proteins that are essential for the G_2/M transition [57].

Activation of the S-phase checkpoint via BRCA1 requires ATM-mediated phosphorylation of BRCA1, NBS1 (Nijmegen breakage syndrome 1) and SMC1 (structural maintenance of chromosome protein 1). ATM, NBS1 and BRCA1 are part of the BRCA1-associated genome surveillance complex (BASC). This implicates the BASC complex in the phosphorylation of SMC1A (Structural maintenance of chromosomes 1A) in reaction to DNA damage, yet how S-Phase checkpoint arrest is eventually achieved is not fully understood.

BRCA1 has also been found to regulate transcription of both MAD2 (Mitotic Arrest Deficient 2) and BUBR1 (BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)), two key components of the mitotic spindle checkpoint that control the CDC20/Anaphase Promoting Complex/C (APC/C).

Another function of BRCA1 is the regulation of centrosome amplification. During prophase the centrosomes migrate to opposite cell poles and build the origins of the mitotic spindles. In a healthy cell the centrosome, composed of two centrioles is duplicated during S-phase. BRCA1 ubiquitin ligase activity has been implicated in the inhibition of centrosome-dependent microtubule nucleation by ubiquitination of γ-tubulin and probably a second unknown protein, although *in vitro* ubiquitination of γ-tubulin is not proven. Still, in absence of the E3 ubiquitin ligase activity of BRCA1 or a γ-tubulin eligible for ubiquitination the centrosomes are amplified.

The activity of BRCA1 in centrosome regulation appears to be regulated by Aurora-A kinase and protein phosphatase 1α-mediated phosphoregulation [58]. This effect has so far only been observed in breast tissue, suggesting that other cell types possess other proteins that take charge of this function of BRCA1. This has even been considered a possible explanation for the tissue specificity of BRCA1-associated carcinomas.

Due to the fact that the only known enzymatic activity of BRCA1 is the E3 Ubiquitin ligase activity it appears obvious that the ubiquitination reaction is responsible for

many, if not all cellular functions of BRCA1. Various proteins have been shown to be ubiquitinated by the BRCA1/BARD1 heterodimer *in vitro*, yet until recently only auto-ubiquitination with K6 linked chains, that do not target proteins for degradation, of the BRCA1/BARD1 heterodimer has been demonstrated *in vivo*.

Using a Isoleucine-26 to Alanine point mutation of BRCA1 ($BRCA1^{FH\text{-}I26A/-}$) that is not able to bind the corresponding E2 proteins Reid et al. were able to demonstrate that the E3 ligase activity of BRCA1 is not necessary for the viability of embryonic stem cells, the suppression of spontaneous chromosomal rearrangements, cellular response to genotoxic distress and the response to double-strand breaks like the repair via homologous recombination or the assembly of the RAD51 focus. Yet the reaction to double-strand breaks induced by mitomycin C seems to be impaired partially, though not as extensively as with a functional null-allele and the level of the BRCA1/BARD1 heterodimer is lower in $BRCA1^{FH\text{-}I26A/-}$ cells, implicating the autoubiquitination of the BRCA1/BARD1 heterodimer in stabilization of the complex [59].

There still is an on-going discussion if the tumor-suppressor-function is indeed linked to the DNA damage repair function or what other cellular effect is the mediator of BRCA1 tumor suppression. It has been shown that the BRCT domain [60] and the BRCA1/BARD1 heterodimer [61] are essential for tumor-suppression. Recently it has been demonstrated that the BRCT domains bind to the phosphorylated serine/threonine protein kinase AKT (pAKT) and lead to its ubiquitination and thus toward protein degradation. BRCA1 mutant cells lacking the BRCT repeats or the E3 ubiquitin ligase activity fail to ubiquitinate pAKT and accumulate nuclear pAKT. Subsequently the transcription functions of FOXO3a, a main nuclear target of pAKT are diminished [62]. The AKT pathway regulates growth and metabolism of the cell via various effectors. Activation of the AKT pathway by PI3K (Phosphoinositide 3-kinase) or PTEN (Phosphatase and tensin homolog) has been shown to have oncogenic effects.

An animal model investigating the role of the RING domain and its E3 ubiquitin ligase activity in tumorigenesis has not been published yet but might be helpful to further evaluate the contribution of ubiquitination to BRCA1 tumor suppressor function.

Additionally BRCA1 is also involved in functions not directly related to its role as a caretaker protein.

It has been shown that BRCA1, together with other proteins involved in DNA damage repair, also has a role in meiotic sex chromosome inactivation (MSCI). On entry into pachytene, BRCA1 and ATR coat the entire length of the unsynapsed regions of the X and Y axial elements, followed by the translocation of ATR to the surrounding chromatin, where H2AX phosphorylation through ATR and consequently MSCI takes place [63].

Furthermore it has been shown that BRCA1 co-localized with markers of the inactive X chromosome (Xi) in cells carrying two x chromosomes and co-stained with the non-coding X-inactive specific transcript RNA (XiST RNA). BRCA1-deficient carcinoma cells showed evidence of defects in Xi chromatin structure. Reconstitution of these cells with wild-type BRCA1 showed focal XiST RNA staining without altering XiST abundance. Inhibition of BRCA1 synthesis led to increased expression of an otherwise silenced Xi-located GFP transgene. These findings suggested BRCA1 deficiency might disturb the inactivation of the mitotic sex body [64].

1.4.2 The tumor suppressor P53

Mutations in the locus *TP53* that encodes the tumor suppressor protein P53 are very common in malign cells; about half of these cells acquire a mutation in this gene during malignant progression [65]. For adenocarcinomas of the pancreas mutations in *TP53* are described for up to 76% of examined tumors. [66]

TP53 is positioned on the short arm of chromosome 17 (17p13.1) (the murine analog *p53* is located on mouse chromosome 11), it contains 11 exons encoding for a 393 amino-acid nuclear protein, which plays a central role in the regulation of the cell cycle.

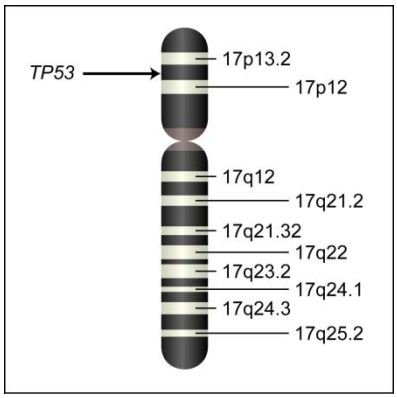

Figure 5: *TP53* on the short arm of chromosome 17.

The encoded protein P53 contains several functional domains, the N-terminal transcription activation domain (TAD) (residue 1-42) that is important for the activation of transcription factors and binding of MDM2, a proline rich domain (residues 80-94) important for apoptotic function, the central sequence-specific DNA-binding domain (DBD) (residues 100-300), a homo-oligomerisation domain (OD) (residues 307-355) and the C-terminal regulatory domain (residues 356-393) that is involved in down-regulation of the DNA-binding activity of the central DNA binding domain.

The homo-oligomerisation domain is needed for tetramerisation of P53. This oligomer of four P53s is the active form of the protein.

The protein P53 is part of a signaling cascade mediating the cells response to stress factors like heat, DNA damage or hypoxia [67]. Therefore, P53 is acting predominantly as transcription factor, regulating the expression of several target genes in both positive and negative direction. [68] Due to its importance for the response to DNA damage it has been given the name guardian of the genome.

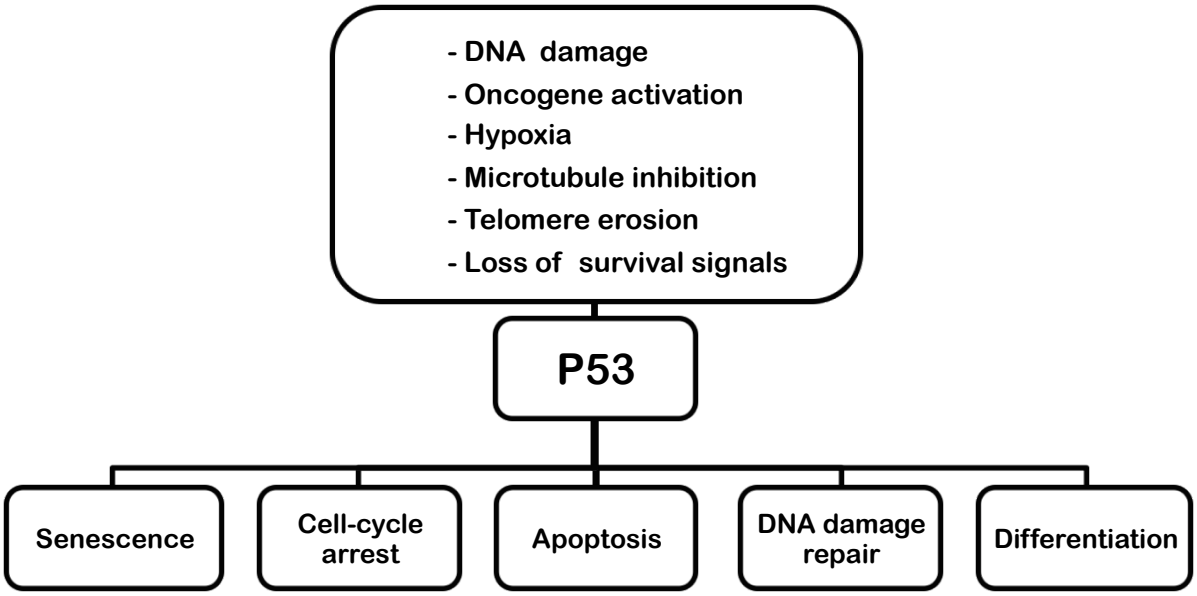

Figure 6: Activation and activities of P53. The importance of P53 results from its position at the intersection of several pathways responding to various kinds of damage to cellular integrity.

P53's activity is controlled mainly by the protein MDM2 (Murine Double Minute) (in humans alternatively called HDM2 [Human double minute]) an ubiquitin ligase which is the transcription product of the murine double minute 2 (*MDM2*) oncogene.

MDM2 uses several mechanisms to inhibit P53. MDM2 binds directly to the P53 transactivation domain, hindering its transcriptional activity. Furthermore MDM2 tags P53 for degradation by ubiquitination, inhibits acetylation and helps transporting P53 from the nucleus to cytoplasm. In the unstressed state of the cell, half-life of P53 is only 5-20min, thus concentration and activity of P53 is kept at a low level.

P53 regulation is based on the interaction of P53 and MDM2 and posttranslational modifications. Various upstream pathways target the P53/MDM2 interaction, to regulate the levels of P53.

In response to oncogene expression the levels of the $P14^{ARF}$ protein can raise by up-regulation of its transcription or stabilization of the protein. $P14^{ARF}$ binds to MDM2, which then is no longer able to control the levels of P53.

Another possibility is the phosphorylation of the P53 protein close to the N-terminal MDM2 binding region, thus hindering the interaction with MDM2 and stabilizing P53. This phosphorylation has been observed in cellular reaction to DNA damage, catalyzed by numerous kinases like ATM (Ataxia teleangiectasia mutated) or CHK1 and CHK2.

Via the interaction with MDM2 P53 possesses a powerful self-control mechanism, as the MDM2 oncogene is a target gene of P53 transcription regulation. [69]
Posttranslational modifications used by upstream proteins to regulate the activity of P53 include phosphorylation, sumoylation, ubiquitination, acetylation, glycosylation, ribosylation or redox regulation, influencing for example its sequence specific DNA binding and transcriptional activity, promoter specificity or oligomerization state. These varying post-translational modifications also allow P53 to react to the activation by different stress factors in a specific way.
Low concentrations of the P53 protein can be detected in all human cells. At these constitutive low levels numerous functions of P53 have been described, including for example regulation of anti-oxidative function, repair of genotoxic damage or arrest of the cell cycle.
Furthermore there is evidence that P53 has a role in regulation of glycolysis [70], autophagy [71], invasion and motility, cellular senescence, angiogenesis, differentiation and bone remodeling.
In a healthy cell, one of P53's main functions in response to minor cellular stress is to arrest the cell cycle to provide the cell with time to cope with minor impairments like DNA damage. The signal to arrest the cell cycle is conducted by various proteins including GADD15 (growth arrest and DNA damage-inducible gene 15) and $P21^{WAF}$/CIP1, which is an inhibitor of various cyclin-dependant checkpoint-kinases (CDK).
After suffering irreparable damage to the genome the cellular concentration of P53 rises to the high levels, similar to those that can be found in tumor cells. The most important effect of such high concentrations of P53 is the induction of apoptosis.
P53 uses two distinct pathways and various messenger proteins to induce apoptosis, the intrinsic and the extrinsic pathway. Several proteins like for example PUMA (p53 up-regulated modulator of apoptosis), BAX or P53AIP1 are part of the intrinsic apoptotic pathway that targets mitochondria by reducing the mitochondria membrane potential. The lower membrane potential leads to the release of Cytochrome C and thus to the induction of the APAF-1/Caspase-9-cascade that finally results in apoptosis.
Another possibility for P53 to induct apoptosis is via the extrinsic apoptotic pathway by enhancement of the expression of TNF-receptor family members like FAS/APO1

and DR5/KILLER death receptors. Activation of these receptors initiates the Caspase cascade via Caspase 8.

Similar to BRCA1 it is not fully understood which of the numerous functions of P53 is responsible for its tumor suppressor function. As described above various messenger proteins reacting on various specific stimuli regulate the expression of P53. For example, P53 induced reaction to the activation of oncogenes is transmitted by the protein ARF (alternative reading frame). On the other hand ARF is not necessary for the activation of P53 following DNA damage. As it has been shown that the ARF protein is responsible for virtually all of P53's tumor suppressor activity it has been proposed that the reaction to oncogene activation and not the response to DNA damage is responsible for the P53 tumor suppressor activity. [72]

A deficiency in one or more of the P53-pathways can be the result of a mutation in one or both of the *TP53* alleles or any other participating protein. The importance of P53 results from its central position on the crossroad of various pathways. The activation of an alternative pathway might be able to surpass a failure of a signaling cascade leading up to or down from P53, yet for P53 itself this possibility does not exist. Cells that are deficient of P53 and thus in induction of apoptosis have a selective advantage compared to those with faultless apoptotic function and tend to tumorous proliferation.

The importance of the tumor suppressor function of the P53 protein can be easily observed in patients suffering from Li-Fraumeni syndrome, the hereditary deficiency of the *TP53* gene, which is a rare, but well described disease. 71% of the Li-Fraumeni syndrome patients have a proven germ-cell mutation in one of the *TP53* alleles. [73] Li-Fraumeni syndrome patients develop tumors at young age, the risk for developing tumors like soft-tissue sarcomas at the age of 30 or earlier has been described to be over 50%. [74].

As described above, tumor suppressor genes are usually recessive, yet *TP53* is a prominent counter-example. 74% of the mutations in the *TP53* gene in malignant cells are missense mutations. Some of the mutant P53 proteins are able to influence P53-dependent apoptosis using a dominant-negative mechanism, which means that wild-type P53 loses its tumor suppressor activity in presence of mutant P53 [75]. This has been explained by the fact that the P53 protein needs to form a tetramer for its tumor suppressor function; the dominant-negative effect of mutant P53 is probably

due to heterodimerization of wild-type P53 with the mutant P53 forming a non-functional heterodimer.

1.4.3 The oncogene K-RAS

The most common genetic alteration in PDAC is an activation of the Kirsten rat sarcoma oncogene (*K-RAS*). *K-RAS* is a member of the RAS subfamily of proto-oncogenes, which also includes *H-RAS* (Harvey-RAS) and *N-RAS* (Neuroblastoma-RAS). Together with other subfamilies (Rho, Rab, Ran, Arf and Rad) the RAS subfamily is member of the RAS superfamily of proto-oncogenes that are small GTPases located at the cell membrane. These small GTPases function as signal transduction proteins passing on extracellular signals to a variety of intracellular response mechanisms.

The *K-RAS* gene (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is located on chromosome 12 (12p12.1) (mouse chromosome 6) containing 4 exons. Alternative splicing leads to variants encoding two isoforms of the K-RAS protein with two alternative fourth coding exons K-RAS 2a and K-RAS 2b with a length of 189 and 188 amino acids, respectively.

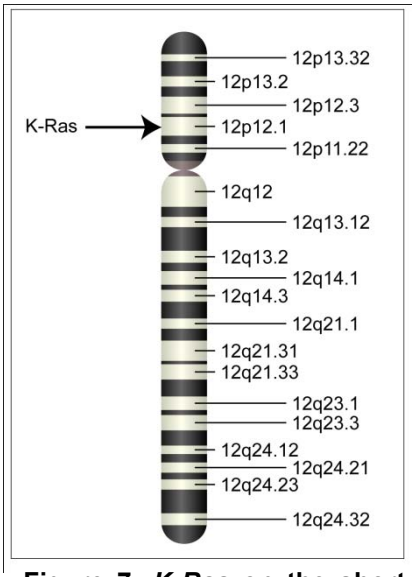

Figure 7: *K-Ras* on the short arm of chromosome 12.

K-RAS contains several functional domains including the core effector domain (res. 32-40), the GTPase domain (res 86-165) and a hypervariable region with a conserved CAAX sequence at the C-Terminus. K-RAS is subject to various posttranslational modifications at the C-Terminal hypervariable region, e.g. by palmitoylation and especially at the CAAX sequence at the C-Terminus, where farnesyltransferase and geranylgeranyltransferase I catalyze the covalent addition of a farnesyl or geranylgeranyl isoprenoid, respectively. This posttranslational processing allows relocation of K-RAS to the plasma membrane, which is required for the signal transduction activity.

Signal transduction via the K-RAS proteins is switched on and off depending on the presence of GTP molecules in the GTPase domain. K-RAS is in its active state as long as GTP is bound to the protein. The structural arrangement of K-RAS, especially the orientation of two regions termed Switch I (residues 32-38) and Switch II (residues 59-67) is influenced by the γ-phosphate of GTP. The Switch I and II regions

are required for the interaction of K-RAS with its regulators and effectors as the GTP bound state possesses high affinity to effector proteins.

Due to the GTPase activity of K-RAS GTP is converted to GDP and thus signal transduction is switched off. Reactivation of K-RAS takes place when the resulting GDP is ejected from the binding site and GTP, which is abundant in the cytosol, recaptures its place.

The activity of K-RAS is modulated by two mechanisms mediated by two groups of regulatory proteins. The first group are GTPase activating proteins (GAP) that can accelerate the intrinsic slow conversion rate by the factor 1000 and thus reduce the signaling activity.

The second group of regulatory proteins are guanine nucleotide exchange factors (GEF). RasGEFs activate K-RAS by stimulating the exchange between GDP and GTP. Prominent members of the GEFs are the proteins RasGEF and Son of the sevenless (SOS).

As mentioned above, the *K-RAS* proto-oncogene is mutated in more than 90% of ductal adenocarcinomas. The most common mutation is a missense mutation in codon 12 with an exchange of the glycine to valine (*K-RAS*^{G12V}) or Aspartic acid (*K-RAS*^{G12D}), less common mutations include codon 13 or 61. The loss of the Glycine as residue 12 leads to a loss of the GTPase function and thus consequently to a constant activity of the K-RAS signal [76].

K-RAS transduces extracellular signals to the nucleus. The signaling cascade leading up to K-RAS starts with growth factors stimulating the receptor tyrosine kinase (RTK) that recruits RAS to the cell membrane. There, the activated RAS engages a multitude of effector molecules and pathways, including the class-1 phosphatidyl inositol 3'-OH kinase catalytic subunits (PI3K) pathway leading to inhibition of apoptosis and the promoting of cell survival and the MAPK/ERK pathway initiated by the RAF1 Ser/Thr kinase that is one of the main cellular pathways for mitogen signals [77].

The ability of oncogenic transformation of a cell through members of the RAS family has been shown to depend on additional changes to the cells genomic integrity. In primary fibroblasts, the effect of oncogenic (activated) RAS was a halt in proliferation and the induction of senescence mediated by the P53-P21^{WAF} or the P16^{INK4a} tumor suppressor pathways. Yet, if these primary cells are immortalized by another

carcinogen before transfection, activation of RAS signaling can lead to transformation, a fact supporting the multiple-hit theory of carcinogenesis [78].

Beside their role in carcinogenic transformation the RAS proteins have been described to play a role in development. The cardio-facio-cutaneus diseases, including neurofibromatosis type-1 and Costello and Noonan syndromes have been associated with the RAS signaling pathways. For example, neurofibromatosis type 1 is a dominantly transmitted disease caused by a defect in the *NF-1* gene. The *NF-1* gene encodes the protein Neurofibromin-1, which is a RasGAP.

1.5 Transgenic mice

In 2007 the Nobel Price in Physiology or Medicine was awarded to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells" [79]. By modifying the genomic sequence of embryonic stem cells using homologous recombination, the techniques developed by the laureates allow investigating the *in vivo* function of targeted genes and creating models of hereditary diseases caused by genomic alterations.
In cancer research animal models provide the opportunity to investigate the functions of tumor suppressor genes and oncogenes by introducing inactivating or activating mutations in these genes. Tumor development can easily be monitored in small animals like mice.

1.5.1 Techniques

A specific genomic sequence can be introduced into the genome by two ways: Either via introduction of a linear DNA construct into a fertilized egg i.e., at single-cell stage (transgenic mice) or via a process termed homologous recombination in embryonic stem cells (gene targeting).
For the first method a construct of DNA containing the gene and a promoter region is generated using plasmid DNA. This plasmid is linearized and injected directly into the pro-nucleus of a fertilized single cell mouse zygote. This zygote can then be implanted into the reproductive tract of a pseudo-pregnant mouse.
A major advantage of transgenic mice is that they can be generated within a short period of time. However his method is not site specific; the transgene can integrate randomly anywhere in the genome with a varying number of copies in tandem arrays i.e., head to tail orientation. The introduced gene is expressed in virtually all cells of the organism, depending completely on the attached promoter sequence. However, if the introduced gene happens to integrate in a genomic locus that is ubiquitously expressed in all tissues of the animal, it will be expressed in all cell types of the animal in spite of being linked to a tissue-specific promoter. As a consequence, a

large number of founder animals might have to be screened for tissue-specific expression of the transgene. This is one of the major shortcomings of this technique of genome manipulation.

Homologous recombination in embryonic stem cells on the other hand provides the means to target a specific region of the genome and is often used to create distinct changes in a gene. Expression of this changed gene will be regulated by its endogenous promoter region in the same way as the wild-type gene was.

To introduce a genetic alteration into an animal a target construct is generated. The targeting construct consists of two homologous sequences flanking the gene of interest (known as 5' and 3' homology arms). The desired change is engineered into the gene of interest through site-directed-mutagenesis. The modified gene is then inserted in between the two homology arms along with a positive selection marker. This positive selection marker placed in between the homology arms adjacent to the modified gene is a bacterial gene that confers resistance to antibiotics such as neomycin (neo^R), hygromycin (hyg^R), puromycin ($puro^R$) etc. Often, the targeting construct also includes a negative selection marker. Frequently, this negative selection marker is the gene that encodes for the thymidine kinase of the herpes simplex virus (tk+) and, unlike the positive selection markers, is added outside the region of homology. The tk+gene confers extreme sensitivity to the nucleoside analog ganciclovir.

The targeting construct is linearized and introduced into embryonic stem cells (ES cells) by electroporation. During homologous recombination two DNA strands align at sites with identical or similar sequences and a crossover between the two strands can lead to an exchange of genetic information. The medium for the transfected ES cells contains neomycin and ganciclovir, thus only cells which have acquired the neo^R-gene by recombination can survive, a process called positive selection. The cells recombining homologous lose the tk+-gene because it is located outside the homologous section, but on occasion the targeting construct may also randomly integrate into the genome. In these instances, the ES cell clones will be resistant to antibiotics, but these cells will be extremely sensitive to ganciclovir in the medium as they still have the tk+ gene. The thus prepared embryonic stem cell can then be introduced into the blastocoel of embryonic day 3.5 embryos (blastocysts). These blastocysts are transferred into the oviducts of pseudo-pregnant female mice to be carried to term.

The resulting pups are chimeric, grown partially out of the original blastocyst cells, partially out of the injected embryonic stem cells. Typically, the blastocysts and the ES cells are of different genetic backgrounds in regards to their fur color, e.g. blastocysts are of C57B6 background which confers a black fur color in contrast to ES cells of 129SV background which confers an agouti fur color. Thus, depending on the extent to which ES cells contribute to the embryogenesis, the resulting chimeric pups will have agouti fur color to varying degree. Typically the chimeric pups with the most agouti fur color are also likely to have their germ cells derived from the ES cells. The animals with germ cells derived from the ES cells will produce pups that are heterozygous for the introduced mutation. Inter-breeding of heterozygous animals will produce animals that are homozygous for the mutation.

1.5.2 Site-specific recombinase systems

Site-specific recombinase (SSR) systems like Cre-LoxP or FLP-FRT revolutionized genetics in mice. These powerful tools enable scientists to express specific genes in specific tissues *in vitro* and *in vivo*.

As many genes play crucial roles during embryogenesis introducing null-mutations (commonly known as “knock-out” mutation) into the mouse genome often results in embryonic lethality. Indeed, mice carrying null-alleles instead of wildtype *Brca1* die during early embryogenesis [80]. Site-specific recombinase technology allows scientists to bypass the early embryonic lethality from knocking-out essential genes such as *Brca1*.

The two most commonly used site-specific recombinase systems are Cre-LoxP and FLP-FRT. The site-specific recombinase Cre (Causes recombination of the bacteriophage P1 genome) and Flp (Flipase) recombine DNA at specific target sites named LoxP (locus of crossover (x) in bacteriophage P1) or FRT (Flipase recognition target) respectively.

LoxP is a 34 base pair (bp) structure containing a 13 bp palindrome or inverted repeats sequence flanking an 8 bp asymmetrical core termed spacer. The 34 bp Flipase recognition target (FRT) contains two 13 bp reverse complement recognition sites also flanking an 8 bp asymmetrical core. Cre and Flipase excise a circular piece of DNA from in between two directly following target sites by recombination of the asymmetrical spacer sequences (see fig. 8). As the circular excision pro-duct is lost, this reaction is irreversible.

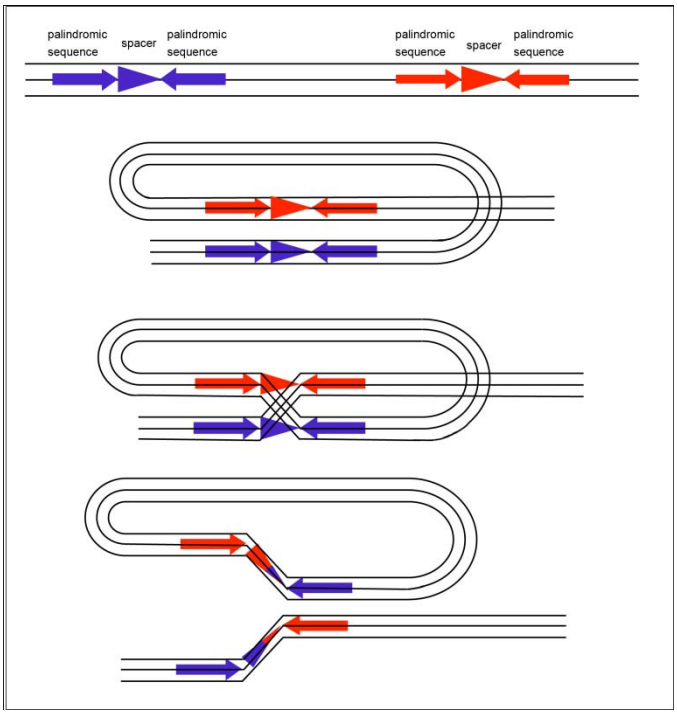

Figure 8: Site-specific recombination by the Cre-LoxP system. Recombination occurs at the spacer (triangular), which is located between two palindromic sequences (arrows) in the LoxP sites.

To express an engineered gene in a specific tissue the Cre-recombinase or the Flipase is linked to a specific promoter region that is only activated in the targeted tissue or at a certain stage of development. The activation of the promoter region leads to expression of the Cre-recombinase, which then excises the LoxP or FRT marked sequence out of the targeted gene.

2 Material and Methods

For a detailed list of solvents and reagents please refer to the appendix.

2.1 Transgenic mice

All animals sacrificed for the experiments were bred in the laboratory of Prof. Thomas Ludwig, PhD at the Institute for Cancer Genetics at Columbia University, New York. Constructs for the conditional *Brca1* and *p53* knock-outs were designed and engineered in the Ludwig Lab and described before [61, 81]. Mice expressing *Pdx-1-Cre* recombinase and an activated *K-Ras* allele were obtained for breeding from Prof. Tyler Jacks' laboratory at the Massachusetts Institute of Technology, Cambridge, MA and have been described before [82]. All mice included in the breeding program were on a mixed background of 129/Sv x C57BL/6J. All experiments involving mice were performed according to Columbia University New York Institutional Animal Care and Use Committee-approved protocols. Mice were sacrificed upon appearing moribund.
For selective expression of the Cre recombinase in the pancreas the promoter of the *Pdx-1* gene (pancreatic and duodenal homeobox gene 1) was used. During early embryogenesis as early as embryonic day 8.5 (e8.5), *Pdx-1* is expressed in the posterior foregut endoderm, a region that will develop into antral stomach, rostral duodenum and pancreas. Later high expression of *Pdx-1* can be found in both endocrine and exocrine cells of the pancreas and low expression in enteroendocrine cells of duodenum and stomach. In adults the expression of *Pdx-1* is restricted to β-cells in the islets of Langerhans and, though in lower levels, acinar cells. [83]
The *Pdx-1-Cre* transgenic animals were generated via injection of a DNA sequence consisting of the Pdx-1 promoter attached to the Cre-recombinase sequence into the pronucleus of single-cell stage embryos as previously described [82]. It has been shown that *Pdx-1-Cre* mediated recombination results in a mosaic expression of the target gene with few recombined exocrine cells in an otherwise normal pancreas [84]. Activation of the *K-Ras* oncogene was achieved using a targeting vector carrying an activating Glycine to aspartic acid mutation at codon 12 ($K\text{-}Ras^{G12D}$). This dominant mutation is compromising both K-Ras' intrinsic and extrinsic GTPase activities and results in constitutive downstream signaling of the Ras effector pathways. The

expression of oncogenic *K-Ras*^{G12D} is controlled by a removable polyA transcriptional termination Stop element. The targeting construct consists of a *K-Ras*^{G12D} gene with an upstream LoxP–Stop–LoxP sequence (construct referred to as LSL-*K-Ras*^{G12D}). Via homologous recombination the LSL-*K-Ras*^{G12D} vector was targeted to the endogenous *K-Ras* locus. Upon Cre-mediated excision-recombination the polyA Stop codon is removed, activating the G12D gain-of-function mutation of *K-Ras*. As *K-Ras* is an essential gene during development [85] and the targeted allele is a functional null allele until Cre-mediated recombination occurs, the mice are maintained in a heterozygous state.

To achieve a pancreas specific knock-out of *p53* and *Brca1* targeting vectors carrying LoxP sites were introduced into the loci of the *Brca1* and *p53* genes in mouse embryonic stem cells by homologous recombination. Upon *Cre*-expression essential parts of the *Brca1* and *p53* genes are deleted, thus both these conditional constructs result in a functional null-allele.

The targeting construct for the conditional knock-out of the *Brca1* gene consisted of a 6.1-kb fragment containing exons 1 and 2. An FRT-flanked PGK-promoter driven neomycin resistant marker gene (PGK-neo cassette) together with a single LoxP site was cloned into intron 1 and a second LoxP site was introduced into intron 2. The PGK-neo cassette contains a stop codon, so that the conditional knock-out allele of *Brca1* is non-functional until the PGK-neo cassette is excised following Flipase-mediated recombination. The conditional knock-out allele is targeted into ES cells by homologous recombination and the correctly targeted ES cells are injected into blastocysts to get germline transmission of the conditional-null *Brca1* allele. The resulting conditional-null *Brca1* heterozygous animals are bred with animals expressing Flipase ubiquitously, including the germ cells, to excise the PGK-neo cassette as well as the stop codon from the conditional-null *Brca1* allele which would otherwise cause premature termination of the Brca1 transcripts. Therefore, the animals born from these matings will be heterozygous for the conditional-null *Brca1* allele while expressing functional Brca1 protein from both the conditional as well as the remaining wild-type *Brca1* alleles. Subsequently, these animals can be inter-bred to generate homozygous conditional-null *Brca1* animals, which are viable and fertile. Only upon further breeding these animals with those expressing Cre, it leads to an excision of *exon 2* within the *Brca1* gene, creating a pre-mRNA, which is not eligible

for correct splicing and is thus rapidly degraded. For our purposes, these conditional-null *Brca1* homozygous animals are bred with *Pdx-1-Cre* transgenic animals to delete *Brca1* specifically in the pancreas while all other organs will express fully functional Brca1 protein.

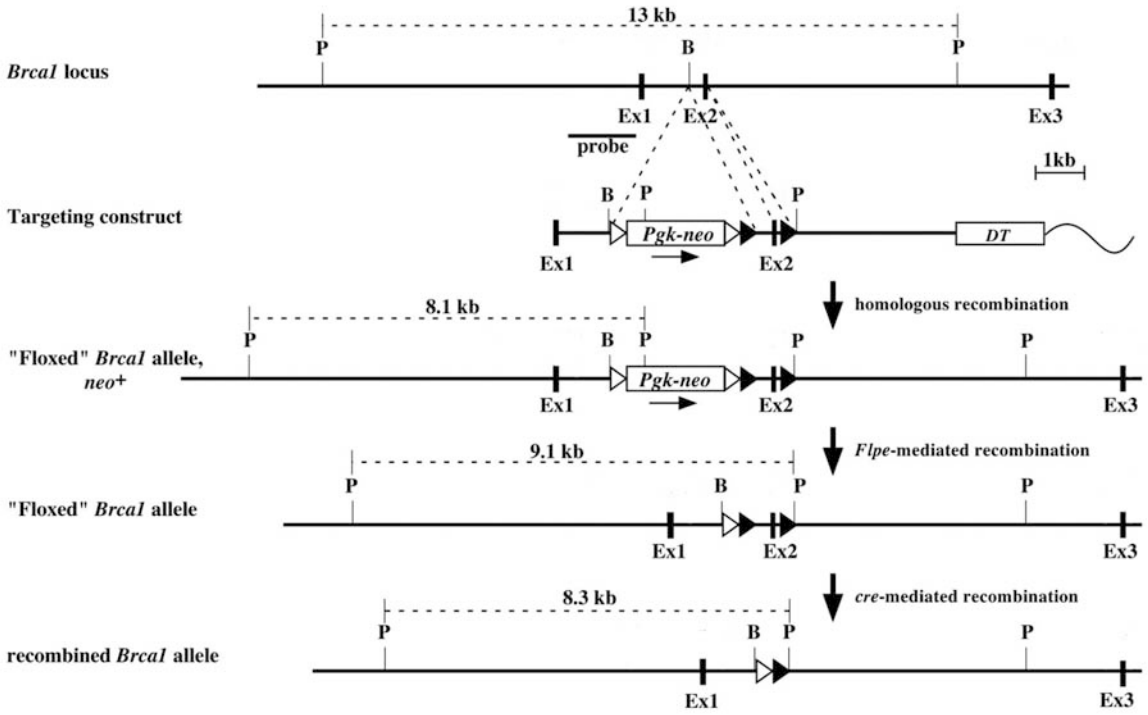

Figure 9: Construct and targeting of the conditional *Brca1* allele. A partial restriction map of the wildtype *Brca1* locus encompassing exons 1 and 2 (Ex1 and Ex2; black boxes) is shown on top, followed by a diagram of the targeting vector used to flank exon 2 with LoxP sites, and maps of the *Brca1* locus following homologous, Flp-mediated, and Cre-mediated recombination. Relevant restriction enzyme sites are BamH I (B) and Pst I (P). The position of the 5' flanking *Brca1* probe used for Southern analysis and the expected sizes of the Pst I fragments recognized by the probe are also indicated.

The targeting vector for the generation of a conditional-null *p53* allele was designed to contain exon 7, which is part of the DNA-binding domain of *p53*. In the construct exon 7 is flanked by LoxP recognition sites, thus the critical DNA-binding domain of *p53* is lost upon recombination. However, prior to breeding these animals with Cre-expressing animals, they were mated with Flipase-expressing animals to remove the PGK-neo cassette and the following stop codon. This will ensure a functionally intact conditional-null *p53* allele until the Cre-recombinase is expressed in the pancreas.

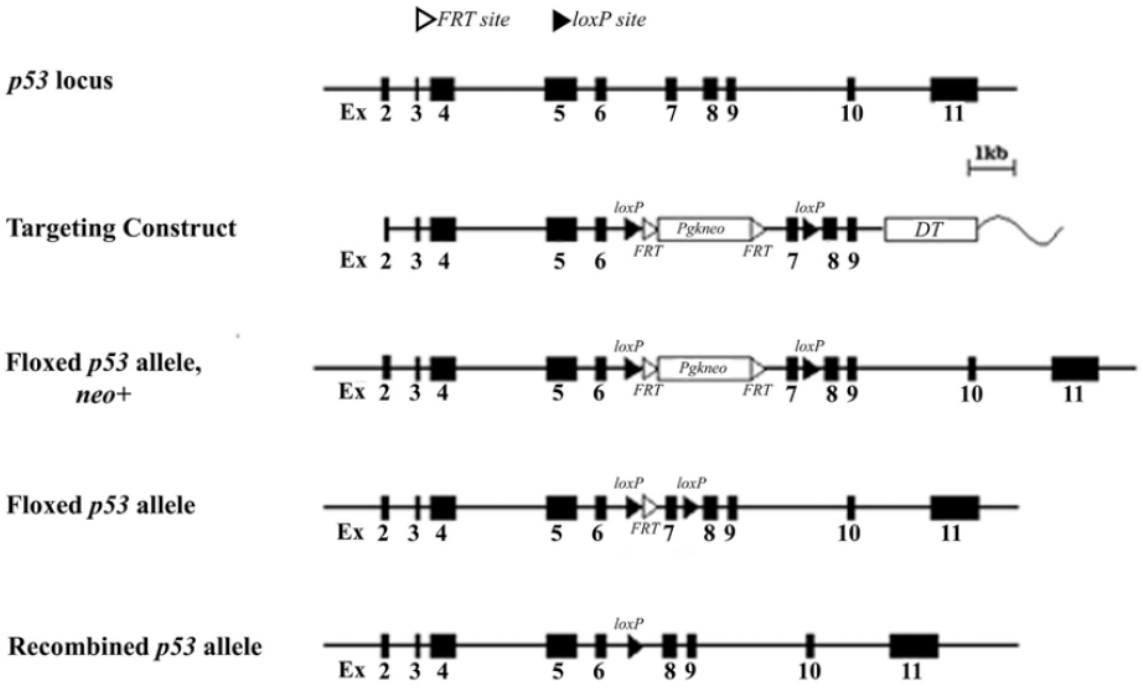

Figure 10: Construct and targeting of conditional *p53* allele. A partial restriction map of the wildtype *p53* locus encompassing exon 7 (Ex7) is shown on top, followed by a diagram of the targeting vector used to flank exon 7 with *LoxP* sites, and maps of the *p53* locus following homologous, Flp-mediated, and Cre-mediated recombination.

To evaluate the participation of the *Brca1* tumor suppressor in pancreatic carcinogenesis and the implications for drug therapy animals with three distinct genotypes were generated by Prof. Ludwig's team. All 95 experimental animals expressed *Pdx-1-Cre* and the activated *K-Ras*^{G12D} allele. The mice were divided into two groups, carrying either the homozygous conditional-null or the homozygous wildtype *Brca1* alleles (*Brca1*$^{cond/cond}$ or *BRCA1*$^{WT/WT}$). All *Brca1* wildtype animals were homozygous for the conditional-null *p53* alleles (*Pdx-1-Cre*; *Brca1*$^{WT/WT}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ (n=35)). The homozygous conditional-null *Brca1* animals were divided into two subgroups with the conditional-null *p53* alleles either in homozygous or heterozygous state (*Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ (n=44) or *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/WT}$; *K-Ras*$^{G12D/WT}$ (n=16)). Additionally six wildtype animals with the same genetic background were raised.
Mice were genotyped using a small biopsy of the tail. The animals were screened for *Brca1* wildtype, conditional or null allele, *p53* wildtype, conditional or null alleles, *Pdx-1-Cre* and expression of activated *K-Ras*.

2.2 Genotyping

2.2.1 DNA extraction

First the material for DNA extraction, e.g. tails or pancreata was incubated in 500 µl Proteinase K buffer for at least 12 hours at 56º C to digest the protein. After shaking for 5 minutes 200 µl of saturated NaCl solution (~5.7M) was added. The solution was shaken again and then spun down for 10 minutes to sediment the salt and complexed proteins. Then the supernatant was removed and mixed with 500 µl of 2-Isopropanol to precipitate the DNA. The solution was centrifuged again but this time the pellet containing the DNA was kept and washed with 500 µl of 70% ethanol. Afterwards the pellet was dried 20 minutes at 40° C in vacuum. For further processing the DNA was resolved in 1x TE-buffer and stored at 4º C.

2.2.2 Southern Blot

To prepare the genomic DNA for Southern Blot analysis, it was digested with a restriction endonuclease at 37º C for at least six hours.

Reaction conditions for digest of genomic DNA with restriction enzymes:

DNA	10 µl
Enzyme (5U/µl)	1 µl
Corresponding Buffer	3 µl
H_20	16 µl

BamH I:

Restriction site: 5'…GGATCC…3'
3'…CCTAGG…5'

Corresponding Buffer: NEBuffer 2

EcoR V:

Restriction site: 5'…GATATC…3'
3'…CTATAG…5'

Corresponding Buffer: NEBuffer 3

Pst I:

Restriction site: 5'…CTGCAG…3'
3'…GACGTC…5'

Corresponding Buffer: NEBuffer 3

Following the restriction digest, the reaction product was separated by fragment size via electrophoresis in a 0.8% agarose gel.

Afterwards the samples were depurinated in 0.25M HCL for 20 minutes and then denatured in 0.5M NaOH 1.5M NaCl for 15 minutes to render the DNA single-stranded.

Then the DNA was allowed to transfer from the gel onto a Biodyne B positively charged nylon membrane through simple diffusion for at least six hours. After successful transfer the membranes were labeled and air-dried for 10 minutes. The membrane was subsequently baked at 80ºC for at least 15 minutes before being hybridized with a radioactively labeled probe.

Afterwards the membranes were immersed in 2X Standard Saline Citrate (SSC) and then pre-hybridized for 30 minutes at 65º C with 10 ml rapid hybridization buffer and 350 µl salmon sperm to reduce background on the blot. After pre-hybridization the probe could be added and the blot then hybridized for 2 hours at 65º C.

Prior to hybridization, P32-radiolabeled probes were synthesized using Random Primer Labeling Kit from the Stratagene Company according to manufacturer's protocol. Briefly, 1.5µl of genomic DNA fragment to be labeled (~25ng), 22.5µl of ddH2O and 10µl of random oligonucleotide primers were mixed and then heated on a 100ºC block for 5 minutes followed by brief centrifugation. To the mix, 10µl of 5xdCTP buffer, 5µl of P32-radioisotope labeled dCTP and 1µl of Klenow-polymerase were added and placed on a 37ºC heat block for 8-10 minutes. Tubes were

centrifuged again and 2µl of stop mix, EDTA, and 250µl of salmon sperm genomic DNA were added and boiled at 100ºC for 10 minutes to denature the probe.

After hybridization for at least 2h the membranes were washed in declining concentrations of SSC and SDS (first wash: 2X SSC, 0.5% SDS, second wash: 1X SSC, 0.5% SDS, third wash: 0.1X SSC, 0.1% SDS) and subsequently dried.

In the next step an X-ray film was exposed to the membrane overnight and developed the next morning.

2.3 Tissue culture

2.3.1 Establishment of primary cancer cell lines

For the drug sensitivity experiments and immunocytochemistry primary tumor cells cultures were derived from the pancreatic tumors of sacrificed animals.
Animals exhibiting a palpable tumor in the abdominal cavity were sacrificed. During dissection the tumor was excised. A small non-necrotic piece of the tumor was used to establish a cell line. Therefore, the tumor was instantly reduced to small pieces mechanically with a scalpel and then incubated with trypsin for several minutes to break the intercellular bonds. After stopping the trypsin digest with the fetal bovine serum contained in D-MEM medium the solution was passed three times through a 10ml syringe using an 18-gauge needle first, followed by three passages with a 21-gauge needle to generate a single-cell suspension of tumor cells. The cell suspension was plated on a 10cm dish and left in the incubator at 37°C undisturbed for a week to allow epithelial cells to settle down and proliferate.
The remaining parts of the tumor were used for histology or genotyping.

2.3.2 Drug sensitivity assay

Exponentially growing cells were seeded in 6-well plates at a density of 10^4 cells/well in 2ml D-MEM medium. After 24 hours the D-MEM medium was exchanged against 2ml D-MEM medium containing the evaluated drug in declining concentrations or, as a negative control, no drug. For every drug concentrations were chosen so that the highest concentration was the lowest possible concentration allowing no proliferation of all evaluated cell lines while the lowest concentration was the highest possible concentration that had no influence on cell proliferation. The remaining concentrations were distributed evenly between those two borders (for actual concentrations refer to chart in appendix).
Subsequently cells were allowed to proliferate for 96 hours. Then cells were washed twice with PBS, trypsinized in 300µl Trypsin-EDTA for 5-7 minutes and centrifuged for 5 minutes at 1400 rpm. The cells were resuspended in D-MEM medium, were stained with Trypan Blue and vital cells counted using a hematocytometer.

Proliferation was calculated as percent of number of cells in the untreated wells. In each experiment 2-4 different cell lines were treated simultaneously using the same drug and concentrations. Each experiment was done in triplicates and repeated for every cell line at least twice.

2.4 Interpretation of data

Statistical analysis of tumor-free survival and of the drug sensitivity assay was done with GraphPad Prism® 5 Software.

Tumor-free survival was illustrated using a Kaplan-Meier survival plot.

To demonstrate the differences in inhibition of proliferation data resulting from each experiment of the drug sensitivity assay was evaluated independently. Determination of the half maximal inhibitory concentration (IC_{50}) was calculated using a non-linear fit curve with variable slope. Lower border was limited to 0%. As mentioned above, all experiments were performed in triplicates. The half maximal inhibitory concentration calculated for each set of experiments thus resulted in three IC_{50} values that were used for further statistical analysis.

Half maximal inhibitory concentrations were compared using Student's t-test for two different cell lines or 1 way ANOVA with Tukey's post test for three or more different cell lines.

To create graphs showing the influence of the genotype on drug sensitivity, for each drug concentration the averages of the survival in percent of each cell line were calculated. Again analysis has been done for each experiment individually.

2.5 Cytostatic drugs

2.5.1 Crosslinking and alkylating agents

Crosslinking and alkylating drugs introduce intrastrand or interstrand links into the DNA double helix. These changes in the tertiary structure of the DNA molecule interfere with various cellular processes like replication and transcription.

2.5.1.1 Cis-diamminedichloridoplatinum(II) (Cisplatin)

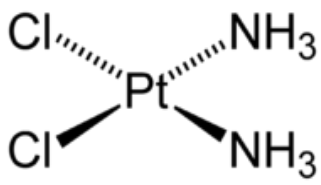

Figure 11: Cisplatin

Cisplatin is a cytostatic drug based on platinum whose inhibitory influence on cell division was first described in 1965 [86]. Following its activation by a process termed aquation, in which one or both of the *cis*-chloride ligands are displaced by water molecules, the complex binds to nucleophilic species like the nucleic acids of DNA. The primary target of cisplatin are the nucleophilic N7-sites of the purine bases in DNA. Cisplatin forms DNA-protein, DNA-DNA interstrand and most important intrastrand crosslinks, especially 1,2-intrastrand ApG and GpG crosslinks, latter being largely responsible for the cytotoxic effect of cisplatin [87].

The inflicted damage leads to conformity changes of the DNA double helix and thus to difficulties with transcription and stalling replication forks. In contrast to the interstrand lesions introduced by other crosslinking agents like Mitomycin C (see below), the intrastrand lesions are repaired primarily by NER without the participation of the homologous recombination pathway [88].

The administration of cisplatin is limited by multiple adverse drug effects including nephrotoxicity (20-41%), peripheral neuropathy (30-86%), ototoxicity (adults 23-50%, children >50%), drug-induced nausea and vomiting, anaphylaxis and electrolyte disturbance [89] [90].

2.5.1.2 Mitomycin C

Mitomycin C is anti-neoplastic agent from the group of antitumor antibiotics. Following a cascade of spontaneous transformations mitomycin C acts as a bifunctional alkylator crosslinking the complementary strands of the DNA double helix. These interstrand crosslinks are so lethal that one single crosslink is sufficient to induce apoptosis in a bacterial cell [91].

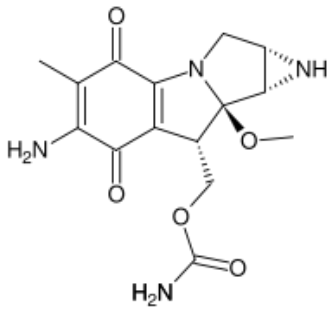

Figure 12: Mitomycin C

Mitomycin C has a strong specifity for 5'-CpG-3' sequences, especially for the second alkylation reaction.

It is not fully understood how the resulting interstrand lesions are repaired in the cell. A proposed model includes the unhooking of the interstrand crosslink by NER on one strand, combined with repair via the homologous recombination pathway [92].

Adverse effects of mitomycin C include anemia (19-24%), congestive heart failure, drug-induced nausea and vomiting (14%), hemolytic uremic syndrome and especially myelosuppression (64%), which is dose-limiting.

2.5.2 Mitotic Inhibitors and antimetabolites

Mitotic inhibitors and antimetabolites are cell-cycle dependent drugs. Especially antimetabolites are only active during S-phase when nucleosides are required as building-blocks for the duplication of the cellular DNA. The main effect of mitotic inhibitors is of course limited to the M-phase although depending on their mechanisms of action they can also have some cellular activity during interphase.

2.5.2.1 Gemcitabine

Gemcitabine (2',2'-Difluordesoxycytidine) is a nucleoside analog based on the nucleoside deoxycytidine. Fluorine atoms replace the hydrogen atoms on the 2’ carbons of deoxycytidine. Being a pro-drug gemcitabine only is active after it was metabolized intracellularly to the active diphosphate (dFdCDP) and triphosphate

(dFdCTP) nucleotides. The cytotoxic effects of gemcitabine are exerted through dFdCDP-assisted incorporation of dFdCTP into DNA, resulting in inhibition of DNA synthesis and induction of apoptosis.

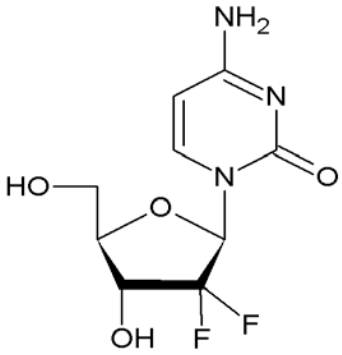

Figure 13: Gemcitabine

A therapy with gemcitabine is less debilitating than with other chemo-therapeutics, yet there are several adverse effects, like myelosuppression, which is dose-limiting. Other commonly reported adverse drug reactions are nausea, abnormal liver function tests, proteinuria, hematuria, dyspnea and skin reactions.

2.5.2.2 Paclitaxel

Paclitaxel is an antimicrotubule agent isolated from the bark of the pacific yew tree, Taxus brevifolia that stabilizes microtubules by preventing depolymerisation. Paclitaxel binds to the β-subunit of tubulin, an essential part of the microtubules that make up the cell skeleton. Usually microtubules are dynamic structures, whose assembling and disassembling is required for vital cellular functions during interphase and mitosis, like assembling of the spindle apparatus during prophase or the development of pseudopodia. Treatment with paclitaxel thus leads to difficulties during mitosis, where the static microtubules do not allow the proper organization of the spindle apparatus. The defective spindle formation subsequently activates the mitotic spindle checkpoint, which prevents mitosis and leads to apoptotic cell death.

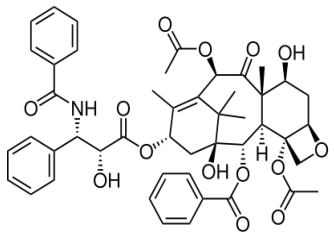

Figure 14: Paclitaxel

Dose-limiting adverse drug effects of paclitaxel include myelosuppression, infections, neurotoxicity (e.g. peripheral neuropathy) and myalgia/arthralgia. Common side effects also include gastrointestinal symptoms like nausea or vomiting, alopecia, skin reactions, abnormal liver function tests, bradycardia or hypotonia.

Recent research showed that *BRCA1*-deficient cells might be resistant to a treatment with taxoids [93]. *BRCA1* is involved in mediating cellular response to taxanes by various mechanisms, including the activation of the mitotic spindle checkpoint, ergo

BRCA1 deficiency allows the cell to enter mitosis even after erroneous assembly of the spindle apparatus.

2.5.2.3 Fluorouracil (5-FU)

Fluorouracil (5-FU) is a pyrimidine analog. After metabolization to the nucleotides fluorouridine 5'-triphophosphate (FUTP), 5'-fluoro-2'-desoxy-uridine 5'-triphosphate (FdUTP) und 5-fluoro-2'-desoxyuridine 5'-monophosphate (FdUMP) it can be incorporated into the nucleic acid instead of the pyrimidine nucleotides cytidine triphosphate (CTP), deoxycytidine triphosphate (dCTP), thymidine triphosphate (dTTP) and uridine triphosphate (UTP) [94].

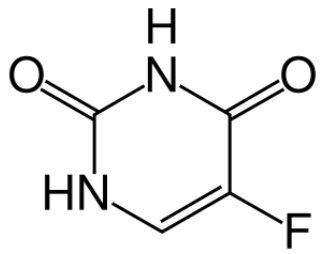

Figure 15: 5-Fluorouracil

Furthermore, fluorouracil acts as a thymidylate synthase inhibitor. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP), which is the only intracellular mechanism for thymidine de-novo synthesis.

Dose-limiting adverse effects of fluorouracil include myelosuppression, mucositis of the digestive tract, especially stomatitis, neurotoxicity and cardiotoxicity. Further common adverse drug reactions are palmar plantar erythrodysesthesia, bronchospasm and alopecia.

Notably, there is a genetic inability to metabolize the pyrimidine-based drugs. Patients suffering from this dihydropyrimidine dehydrogenase deficiency (DPD deficiency) may develop life-threatening toxicity following a treatment with fluorouracil.

2.6 Spectral Karyotyping (SKY)

For spectral karyotyping metaphase preparations were made from logarithmically growing tumor cells. To be able to analyze a large number of metaphases of the same cell clone, the cells were synchronized by serum starving. A total of 10–20 metaphases were analyzed in each tumor line.

Hybridization and detection of SKY probes was performed following the manufacturer's protocol (Applied Spectral Imaging) in the cytogenetics laboratory of Dr. Vundavalli Murty at Columbia University.

After pre-treatment with pepsin the chromosomes were denatured at 72˚C for 1.5 minutes using a 70% formamide; 2X SSC; pH 7.0 denaturation solution and dehydrated using serial washings in increasing concentrations of ethanol.

Then the Spectral karyotyping (mouse) reagent was added to the metaphase spreads and allowed to hybridize for 24-36 hours in a humidified chamber.

Slides were washed with SKY washing solution I (50% formamide; 2X SSC), SKY washing solution II (1X SSC) and SKY washing solution III (4X SSC, 0.1% Tween 20) before the blocking reagent and subsequently the Cy5 Staining Reagent and Cy5.5 Staining reagent have been added. In-between the different Staining Reagents the slides were washed with SKY washing solution III.

After counterstaining with anti-fade DAPI-Reagent metaphases were captured by using the SD300-C SpectraCube and analyzed by using SkyView spectral imaging system software.

3 Results

3.1 Genotyping

Pdx-1-Cre is expressed in cells of the exocrine lineage within the pancreas as early as embryonic day 8.5 (e8.5), and continues to be expressed through adulthood in acinar cells. The bulk of the pancreas is composed of cells of exocrine lineage derived from the *Pdx-1* expressing progenitor cells (e.g., acinar and ductal cells); other minor cell types found include cells of endocrine lineage (e.g., islets), stromal cells, endothelial or immune cells.

Therefore, in the pancreas of an animal expressing *Pdx-1-Cre*, one would expect more or less full recombination of the conditional-Brca1 allele, while one does not expect any recombination in the tail from the same animal.

To assess the pancreas-specific, *Pdx-1-Cre* dependent recombination of the targeted conditional *Brca1* allele, a Southern blot using the 5'-flanking *Brca1* probe ("B1 5' ") was conducted with genomic DNA from the tails and pancreata of four week old *Pdx-1-Cre*; $Brca1^{cond/WT}$ and *Pdx-1-Cre*; $Brca1^{cond/-}$ animals. (figure 16) On Pst I-digested genomic DNA the probe hybridizes to a 13kb fragment for the wild type allele of *Brca1*, a 9.1kb fragment for the conditional allele and a 8.3kb fragment for the recombined allele.

Among the *Pdx-1-Cre*; $Brca1^{cond/WT}$ animals, the conditional *Brca1* allele (9.1kb band) was completely recombined within the pancreata (note the appearance of the 8.3kb recombined lower band); as expected, the tail of the same animals did not show any recombination of the conditional *Brca1* allele as indicated by the intact 9.1kb conditional allele band. Surprisingly, among the *Pdx-1-Cre*; $Brca1^{cond/-}$ animals, the conditional *Brca1* allele did not show any recombination in the pancreas (note the intact 9.1kb conditional allele which is in equimolar ratio to the null-allele (~8.3kb)); again, as expected the tail of the animals did not show any recombination of the conditional *Brca1* allele. Therefore, it appears that recombination of the conditional *Brca1* allele in the pancreas was detectable only in the context of a remaining wildtype *Brca1* allele. In animals carrying both a conditional and a null *Brca1* allele no recombination of the conditional *Brca1* allele is taking place.

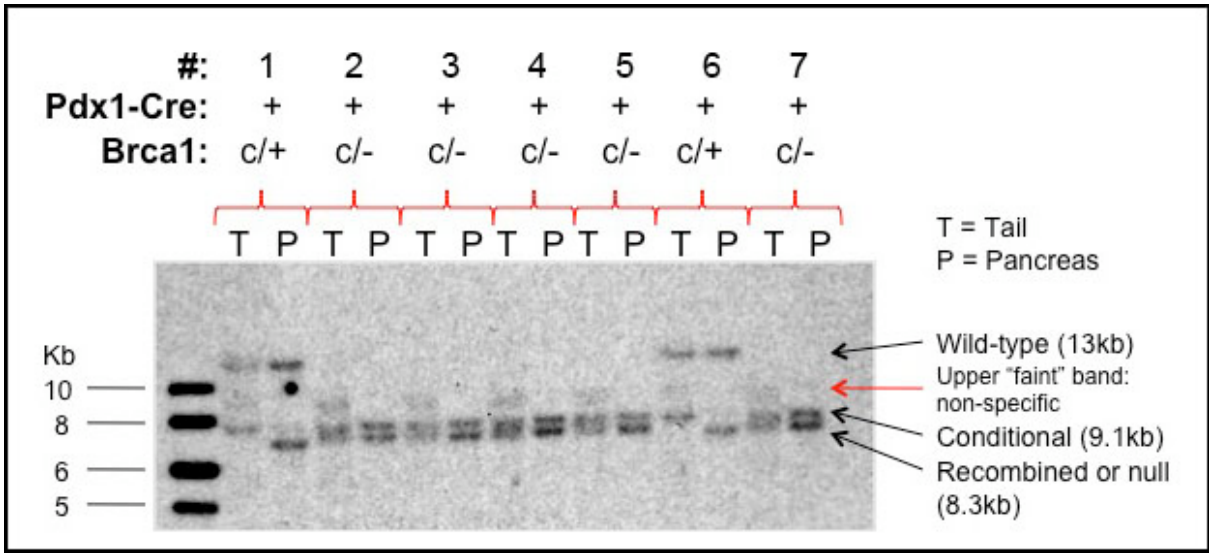

Figure 16: Southern Blot of Pst I-digested tail and pancreatic genomic DNA hybridized with the "B1 5' " probe of experimental animals (Pdx-1-Cre; *Brca1*$^{cond/–}$; # 2, 3, 4, 5, 7) and control animals (Pdx-1-Cre; *Brca1*$^{cond/WT}$; # 1, 6). Recombination of the conditional allele is seen in the pancreata of control animals (Compare tail and pancreas lanes for *Brca1*$^{cond/WT}$ animals # 1, 6). Yet, in absence of a functional wildtype allele, the equimolar ratio of the conditional and null alleles in the pancreata of the *Brca1*$^{cond/–}$ animals clearly indicates that no recombination took place.

Interestingly, the pancreata of animals with conditional and null *Brca1* alleles (*Pdx-1-Cre*; *Brca1*$^{cond/–}$) are smaller than the pancreata of control animals carrying a wildtype and a conditional allele (*Pdx-1-Cre*; *Brca1*$^{cond/WT}$) (figure 17).

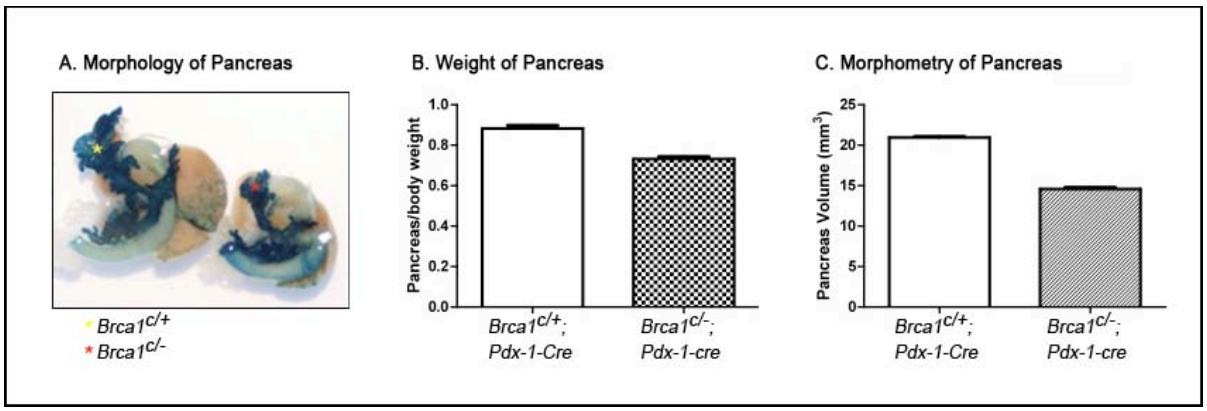

Figure 17: The weight of the pancreata and their 3-D volume is reduced among BRCA1$^{cond/–}$; Pdx-1-Cre (n=3) animals compared to their littermate controls (Brca1$^{cond/WT}$; Pdx-1-Cre, n=3)

If indeed, *Brca1*-deleted cells are not viable, then, are pancreatic tumors among *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ animals indeed the result of Pdx-1-Cre dependent recombination and inactivation of the conditional alleles of *Brca1* and *p53* and activation of *K-Ras*?

To address this question six cell-lines were established from pancreatic tumors. Three of the sacrificed animals were homozygous for the *Brca1* wildtype allele (Pdx-1-Cre; BRCA1$^{WT/WT}$; p53$^{cond/cond}$; Kras$^{G12D/WT}$; three were homozygous for the conditional BRCA1 allele (Pdx-1-Cre; BRCA1$^{cond/cond}$; p53$^{cond/cond}$; Kras$^{G12D/WT}$). All six animals carried the conditional allele at the *p53* and *K-Ras* loci. To determinate the status of these conditional alleles in the tumor cells we did a series of Southern Blots. To determine the status of the *Brca1* locus the DNA obtained of the tumor cell lines was incubated with the restriction enzyme BamH I, creating a ~2.5kb large fragment for the wildtype and conditional allele and a ~1.9kb large fragment for the recombined allele, visible after hybridization with the "B1 3'" probe. The cell lines 43-16, 41-05 and 43-11 show a homozygous ~2.5kb band and thus, as they express the *Pdx-1-Cre*, the wildtype allele. The cell lines B1-10, B1-8 and 8-1 produce a single ~1.9kb band, showing that the introduced conditional alleles were fully recombined.

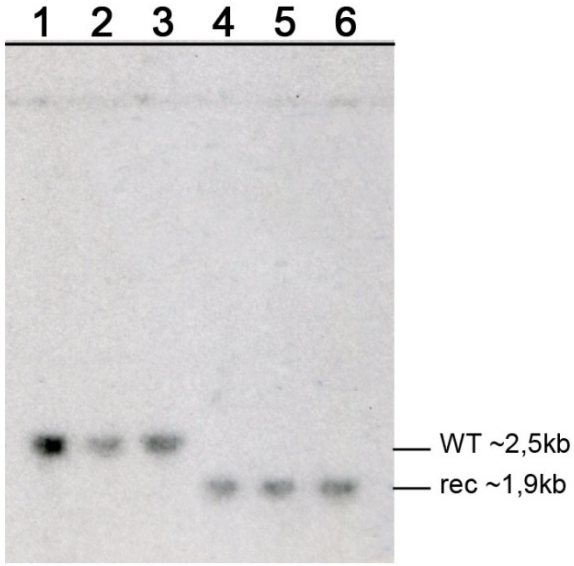

Figure 18: Southern Blot Brca1. Southern Blot with "B1 3'" probe after restriction with BamH I shows the homozygous wildtype allele for cell lines 1 – 3, and the homozygous recombined alleles for cell lines 4 – 6. (Abbr.: 1: 43-16; 2: 41-05; 3: 43-11; 4: B1-10; 5: B1-8; 6: 8-1; WT: Wildtype allele; rec: recombined allele)

To demonstrate the status of the *p53* gene we also did a Southern Blot with the "p53 cond" probe after restriction of the tumor DNA with EcoR V (figure 19). This digest yields a ~4.6kb fragment for the wildtype allele, a ~2.5kb fragment for the conditional allele and a ~4.3kb fragment for the recombined conditional allele. All cell lines showed complete disappearance of the conditional allele and carry the homozygous

recombined allele. Notably the cell line 8-1 shows an additional band at ~4.1kb. This indicates that one of the recombined *p53* alleles must have undergone a chromosomal rearrangement during recombination.

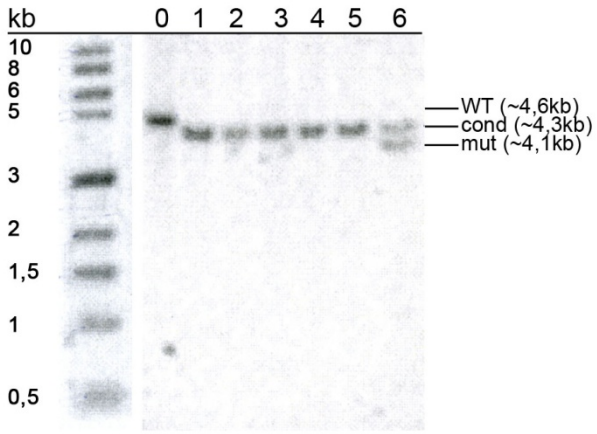

Figure 19: Southern Blot p53. Southern Blot with "p53 cond" probe after restriction with EcoR V shows the homozygous recombined alleles for cell lines 1 – 5. Cell line 6 shows an additional ~4.1kb band. (Abbr.: 0: Wildtype control; 1: 43-16; 2: 41-05; 3: 43-11; 4: B1-10; 5: B1-8; 6: 8-1; WT: Wildtype allele; rec: recombined allele, mut: mutated recombined allele)

The same membrane used for the *p53* Southern Blot was used to show the recombination of the conditional *K-Ras* allele. The membrane was stripped of the "p53 cond" probe and subsequently hybridized with the "SV40 polyA" probe (figure 20). During engineering of the conditional allele a LoxP-flanked neomycin gene containing the SV40 polyadenylation (polyA) sequence is introduced into the K-Ras gene. The neomycin polyA sequence is then lost during cre-mediated recombination leading to expression of the oncogenic K-Ras^{G12D}. The absence of a band in the Southern Blot thus shows the full recombination of the conditional *K-Ras* allele in all cell lines.

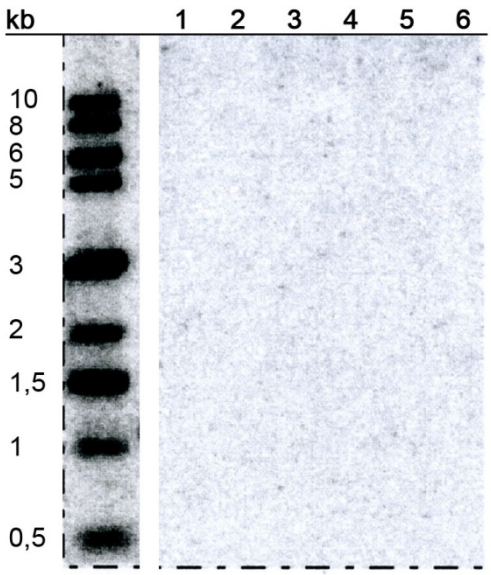

Figure 20: Southern Blot K-Ras. Southern Blot with "SV40 polyA" probe after restriction with EcoR V shows the full recombination of the conditional allele in all cell lines (Abbr.: 1: 43-16; 2: 41-05; 3: 43-11; 4: B1-10; 5: B1-8; 6: 8-1)

The genotypes of the various cell lines determined with these experiments are summarized in table 5.

Table 3**:** Genotypes of the cell lines employed in the drug sensitivity assay

Murine primary pancreatic tumor cell lines			
	Brca1	*p53*	*K-Ras*
43-11	$^{WT}/_{WT}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$
43-16	$^{WT}/_{WT}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$
41-05	$^{WT}/_{WT}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$
B1-8	$^{rec}/_{rec}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$
B1-10	$^{rec}/_{rec}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$
8-1	$^{rec}/_{rec}$	$^{rec}/_{rec}$	$^{G12D}/_{WT}$

3.2 Tumor-free survival

To evaluate the influence of the various genotypes on tumor initiation and progression we observed the tumor-free survival of the various genotypes. Tumor free survival has been defined as the age of sacrifice due to the appearance of a palpable tumor or when animals appeared moribund.

All animals lacking functional Brca1 (*Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$) developed pancreatic tumors at very young age. Median age at development of a palpable tumor was 5 weeks, although microscopic changes were recognizable already at an age of 6d.

The median tumor-free survival was significantly longer for mice with wildtype *Brca1* (*Pdx-1-Cre*; *Brca1*$^{WT/WT}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$). Median tumor-free survival of these animals (n=35) was 10 weeks, compared to the 5 weeks of the animals with the conditional Brca1 allele (n=44; P<0.0001).

Median tumor-free survival of animals heterozygous for the *p53* allele (*Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/WT}$; *K-Ras*$^{G12D/WT}$ (n=16)) was 12 weeks, which is also considerably shorter than survival of Pdx-1-Cre; BRCA1$^{WT/WT}$; p53$^{cond/WT}$ (or p53$^{R172H/+}$); K-Ras$^{G12D/WT}$ animals (~5 months) [95].

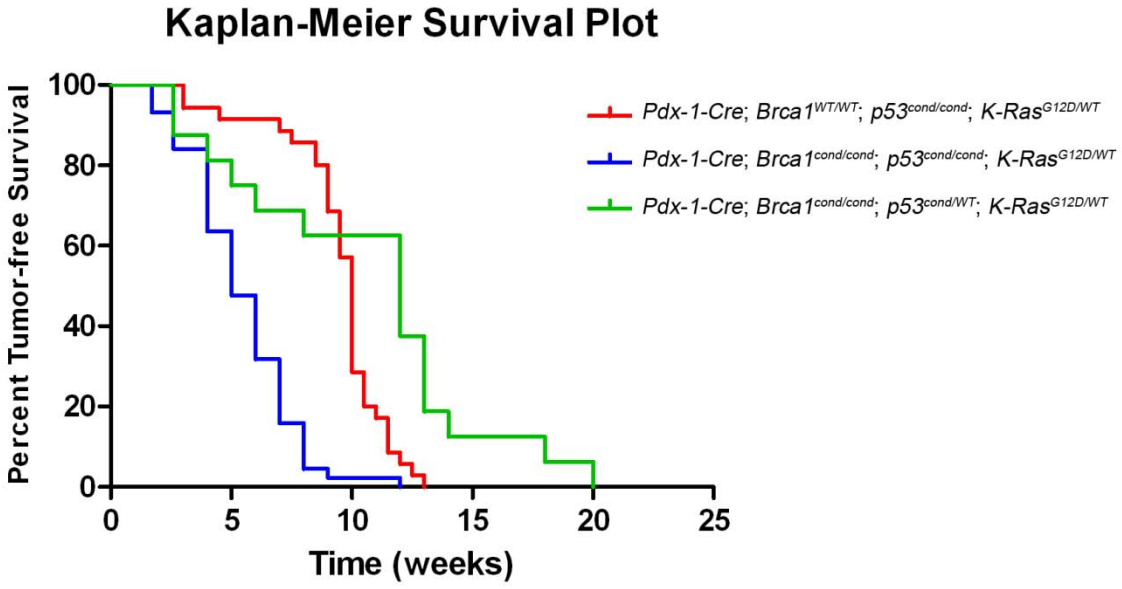

Figure 21: Tumor free survival. The Kaplan-Meier survival plot illustrates the significant difference in tumor free survival of the various genotypes.

3.3 Drug sensitivity assay

As described above primary carcinoma cell lines were treated with various drugs to determine any possible differences in sensitivity to specific drugs between *Brca1*$^{rec/rec}$ and *Brca1*$^{WT/WT}$. Significant differences of the IC_{50} of the two genotypes were demonstrated for mitomycin C and cisplatin, while there were no significant differences between the different genotypes after treatment with 5-FU, gemcitabine or paclitaxel.

After treatment with mitomycin C the half maximal inhibitory concentration was lower in Brca1-deficient cells when compared to Brca1 wildtype cells. In 7 out of 8 independent experiments these differences were statistically significant ($p < 0.01$). Also in experiment #3 the *Brca1*-deficient cells were more sensitive to the treatment than the *Brca1* wildtype cells, yet due to an exceptional low IC_{50} in one of the 41-05 triplicates (IC_{50} I), these differences are not significant.

For detailed results of the drug sensitivity assay and the variance analysis refer to table S2 and S3 in the supplement.

Table 4: Half inhibitory concentrations for treatment with mitomycin C

Mitomycin C						
#	**Cell line**	**IC_{50} I**	**IC_{50} II**	**IC_{50} III**	**IC_{50} Mean**	**95% CI of Mean**
#1	43-11	28.00	13.01	18.42	19.81	0.95 – 38.67
	B1-10	5.63	10.60	4.65	9.96	-0.97 – 14.89
#2	41-05	31.88	34.24	30.53	32.22	27.55 – 36.88
	B1-10	20.56	14.36	20.64	18.52	9.57 – 27.47
#3	41-05	17.09	51.30	28.64	32.34	-10.89 – 75.57
	B1-8	8.27	11.16	10.70	10.04	6.18 – 13.91
	8-1	18.82	8.19	15.46	14.16	0.66 – 27.65
#4	43-11	84.97	93.62	83.23	87.27	73.45 – 101.1
	8-1	25.04	28.04	25.05	26.04	21.75 – 30.34
	B1-10	19.78	17.27	12.39	16.48	7.15 – 25.81
#5	43-16	41.81	40.36	45.55	42.57	35.92 – 49.23
	43-11	34.45	34.48	39.38	36.10	29.05 – 43.15
	B1-8	12.13	13.57	10.60	12.10	8.41 – 15.79
#6	43-16	31.53	35.10	37.19	34.61	27.50 – 41.72
	8-1	14.94	18.74	20.83	18.17	10.75 – 25.59
#7	43-16	44.45	38.18	39.22	40.62	32.27 – 48.96
	41-05	47.42	57.00	54.58	53.00	40.63 – 65.37
	B1-8	16.93	20.81	16.19	17.98	11.81 – 24.14
	8-1	20.63	26.26	15.22	20.70	6.99 – 34.42
#8	43-11	20.71	20.39	19.43	20.18	18.52 – 21.83
	B1-10	15.88	9.74	10.39	12.00	3.63 – 20.38

Treatment with the intercalating agent cisplatin produced similar results. The detected differences between Brca1-deficient and proficient cell-lines have been significant in all experiments. For detailed results and statistical tests refer to table S4 and S5 in the supplement.

Table 5: Half inhibitory concentration for treatment with cisplatin

Cisplatin						
#	**Cell line**	**IC_{50} I**	**IC_{50} II**	**IC_{50} III**	**IC_{50} Mean**	**95% CI of Mean**
#1	41-05	0.78	0.69	0.70	**0.72**	0.60 - 0.85
	8-1	0.28	0.28	0.30	**0.29**	0.26 - 0.32
	B1-10	0.19	0.15	0.16	**0.16**	0.11 - 0.21
#2	43-16	0.41	0.44	0.52	**0.45**	0.30 - 0.60
	B1-8	0.22	0.16	0.20	**0.19**	0.11 - 0.28
	B1-10	0.28	0.22	0.32	**0.27**	0.15 - 0.40
#3	43-11	0.48	0.60	0.46	**0.51**	0.32 - 0.70
	8-1	0.29	0.25	0.27	**0.27**	0.22 - 0.32
	B1-8	0.25	0.25	0.30	**0.27**	0.20 - 0.34
#4	43-11	0.52	0.51	0.55	**0.53**	0.48 - 0.57
	B1-10	0.20	0.17	0.22	**0.20**	0.14 - 0.26
#5	41-05	1.09	1.22	0.93	**1.08**	0.72 - 1.40
	43-16	0.88	0.73	0.84	**0.82**	0.63 - 1.00
	8-1	0.37	0.30	0.33	**0.33**	0.24 - 0.43
	B1-8	0.30	0.24	0.23	**0.26**	0.16 - 0.36

Drugs with other mechanisms of action than the induction of DNA damage, like the drugs used routinely in the therapy of PDAC (gemcitabine, 5-fluorouracil or paclitaxel) showed different results. Here it was not possible to show a consistent significant discrepancy between any of the tested cell-lines. The detailed results and variance analysis for the antimetabolites gemcitabine and 5-fluorouracil can be found in tables S6 to S9 in the supplement.

Table 6: Half inhibitory concentrations for treatment with gemcitabine

Gemcitabine						
#	**Cell line**	**IC_{50} I**	**IC_{50} II**	**IC_{50} III**	**IC_{50} Mean**	**95% CI of Mean**
#1	43-11	0.033	0.027	0.036	0.032	0.021 - 0.043
	B1-10	0.019	0.014	0.022	0.018	0.001 - 0.028
	B1-8	0.032	0.041	0.038	0.037	0.025 - 0.049
#2	43-16	0.028	0.027	0.032	0.029	0.022 - 0.036
	B1-8	0.026	0.029	0.031	0.029	0.021 - 0.036
	8-1	0.053	0.054	0.045	0.051	0.038 - 0.064
#3	41-05	0.062	0.047	0.051	0.053	0.034 - 0.072
	43-16	0.062	0.047	0.051	0.033	0.012 - 0.054
	B1-8	0.022	0.029	0.032	0.027	0.015 - 0.040
	B1-10	0.047	0.045	0.048	0.047	0.042 - 0.051
#4	43-11	0.029	0.027	0.028	0.028	0.024 - 0.032
	41-05	0.037	0.033	0.029	0.033	0.023 - 0.043
	B1-10	0.033	0.030	0.032	0.032	0.027 - 0.036
	8-1	0.027	0.030	0.029	0.029	0.025 - 0.032

Table 7: Half inhibitory concentration for treatment with 5-fluorouracil

5-Fluorouracil						
#	**Cell line**	**IC_{50} I**	**IC_{50} II**	**IC_{50} III**	**IC_{50} Mean**	**95% CI of Mean**
#1	43-16	0.60	0.73	0.71	0.68	0.50 - 0.86
	B1-8	0.49	0.61	0.68	0.59	0.35 - 0.83
	B1-10	0.46	0.39	0.37	0.41	0.29 - 0.53
#2	41-05	0.20	0.25	0.26	0.24	0.15 - 0.32
	43-16	0.28	0.27	0.35	0.30	0.19 - 0.41
	B1-10	0.35	0.52	0.44	0.44	0.23 - 0.64
#3	41-05	0.25	0.26	0.26	0.25	0.24 - 0.27
	43-11	0.25	0.17	0.27	0.23	0.10 - 0.36
	8-1	0.33	0.42	0.30	0.35	0.20 - 0.51
#4	43-11	0.86	0.80	0.68	0.78	0.56 - 1.01
	43-16	0.81	0.50	0.68	0.66	0.27 - 1.05
	B1-10	1.11	1.02	1.33	1.15	0.75 - 1.55
	B1-8	0.67	0.61	0.45	0.51	0.16 - 0.86
#5	43-16	0.68	0.94	0.62	0.75	0.32 - 1.18
	41-05	0.72	0.76	0.65	0.71	0.57 - 0.84
	8-1	0.92	1.03	1.09	1.01	0.81 - 1.22
	B1-8	0.90	1.00	0.98	0.96	0.84 - 1.09

A previously described resistance of *Brca1*-deficient cells to the mitotic inhibitor paclitaxel has been observed but has not been significant.

The results of cell line 43-16 in experiment #4 seem to be the result of a systematic error and have thus not been used for further statistical analysis. Details of the drug sensitivity assay and variance analysis can be found in table S10 and S11 in the supplement.

Table 8: Half inhibitory concentration for treatment with paclitaxel

Paclitaxel						
#	**Cell line**	**IC_{50} I**	**IC_{50} II**	**IC_{50} III**	**IC_{50} Mean**	**95% CI of Mean**
#1	43-11	0.82	0.94	1.47	1.08	0.22 - 1.93
	B1-10	1.19	1.12	0.91	1.07	0.71 - 1.44
	8-1	2.24	1.85	1.88	1.99	1.45 - 2.53
#2	41-05	1.65	1.01	1.09	1.25	0.39 - 2.11
	8-1	1.61	1.47	1.35	1.47	1.15 - 1.80
#3	41-05	1.65	1.61	1.63	1.63	1.58 - 1.68
	B1-10	2.16	2.09	3.25	2.50	0.88 - 4.11
	8-1	3.04	1.68	1.87	2.20	0.38 - 4.02
#4	(43-16)	(3.41)	(2.78)	(2.61)	(2.94)	(1.88 - 3.99)
	43-11	1.37	1.25	1.16	1.26	1.01 - 1.51
	8-1	1.35	1.63	1.77	1.58	1.06 - 2.10
	B1-10	1.36	1.21	1.23	1.26	1.06 - 1.47
#5	43-16	2.03	3.52	4.21	3.25	0.48 - 6.02
	B1-8	5.27	4.73	5.16	5.05	4.34 - 5.76
	B1-10	3.31	4.01	3.82	3.71	2.82 - 4.61
#6	43-11	1.14	0.75	1.00	0.97	0.48 - 1.45
	B1-10	1.39	1.35	1.46	1.40	1.26 - 1.54
	8-1	1.72	2.83	2.11	2.22	0.82 - 3.62

3.3.1 Dose-response curves

To further illustrate the influence of the *Brca1* status on drug response I created dose-response curves, plotting drug dose of the various drugs against cell survival in percent. A higher sensibility to a given drug results in a left shift of the curve, a resistance in a right shift of the curve. According to the results presented above, curves of Brca1-negative cell lines show a left shift for mitomycin C and cisplatin and a slight right shift for paclitaxel.

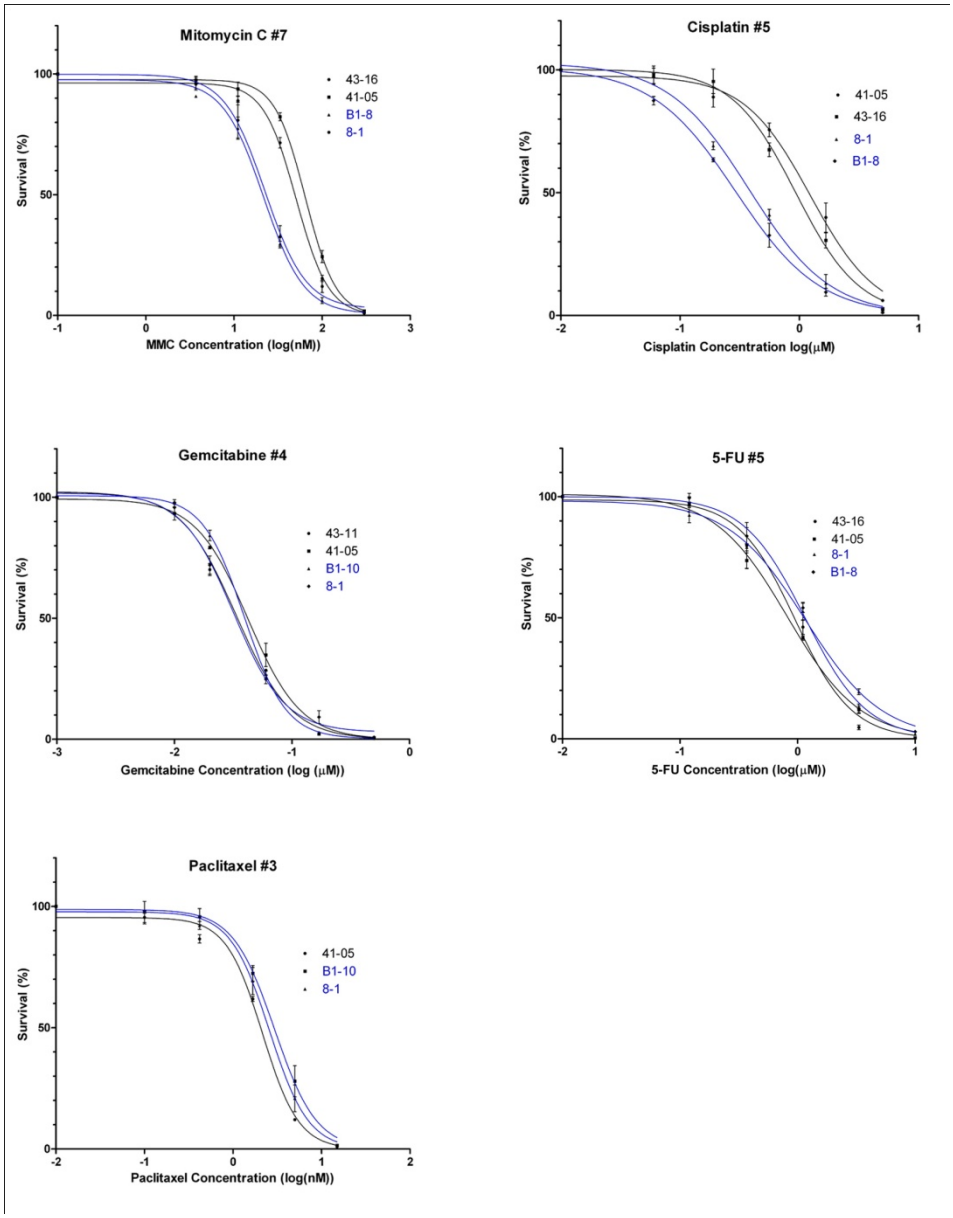

Figure 22: Graphs illustrating the survival assay. The figure shows a collection of graphs calculated from representative experiments. Brca1-deficient cell lines are presented in blue, BRCA1-proficient cell lines in black. The reduction of the IC_{50} is illustrated by a left shift of the curves after treatment with cisplatin or mitomycin C.

3.4 Pathology

To assess the histological properties of PDAC in Brca1-deficient mice a total of 101 mice of various genotypes were maintained in four different cohorts as noted below:

1.) *Pdx-1-Cre*; *Brca1*$^{WT/WT}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$
(n=38; age 6 days to 26 weeks)

2.) *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$
(n=41; age 2 days to 8 weeks)

3.) *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/WT}$; *K-Ras*$^{G12D/WT}$
(n=16; age 18 days to 20 weeks)

4.) Wildtype controls
(n=6; age 2 days to 43 weeks)

Except for the wild-type animals, all others were sacrificed within 48 hours of appearing moribund as indicated by their distended abdomens. Besides the pancreatic tumors and any other lesions, we sampled all internal organs such as lungs, liver, gall bladder, kidney, gut, spleen, thymus, diaphragm, salivary glands and reproductive organs for metastases. Tissues were analyzed by routine histology (H&E staining) and pancreatic lesions found were classified according to Hruban et al. [96] with lesions classified as acinar-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PANIN) or invasive pancreatic carcinoma (PCA).

Unlike control wild-type animals, all others developed ductal adenocarcinoma of the pancreas at an early age. The invasive pancreatic carcinomas (PCA) that developed among these animals were often poorly differentiated adenocarcinomas (figure 23). However, remnants of glandular differentiation were consistently found among these pancreatic ductal adenocarcinomas. Signet-ring cells with large mucin droplets pushing the cell nucleus to the cellular border represent a vestige of glandular differentiation recognizable in these poorly differentiated pancreatic carcinomas (figure 23E). Sarcomatous metaplasia featuring spindle cells arranged in fascicles was commonly seen. Another common growth pattern was the anaplastic carcinoma featuring markedly pleomorphic cells including bizarre giant cells (figure 23D).

It is noteworthy that ductal carcinomas that express wildtype-Brca1 (i.e., pancreatic cancers that developed among *Pdx-1-Cre; Brca1*$^{WT/WT}$*; p53*$^{cond/cond}$*; K-Ras*$^{G12D/WT}$

cohort) tended to have larger glandular elements often accompanied by mucinous metaplasia, whereas Brca1-deficient carcinomas frequently displayed a more prominent anaplastic component.

We sacrificed some of these animals at defined ages to compare the pancreatic cancer initiation and progression among various cohorts of animals. Mice that concomitantly lacked Brca1 and p53, and expressed activated-K-Ras (i.e., animals of *Pdx-1-Cre; Brca1$^{cond/cond}$; p53$^{cond/cond}$; K-RAS^{G12D}* genotype) displayed PANIN-3 lesions already at the age of two days; adenocarcinoma of the pancreas was microscopically detectable as early as at an age of six days. Almost all of the animals sacrificed at an age of six days and older (39/40) presented with adenocarcinoma of the pancreatic ducts with anaplastic features like bizarre giant cells. Although these were mostly poorly differentiated, highly invasive adenocarcinomas, remnants of glandular differentiation such as signet ring cells were almost invariably present in all cases analyzed. Only one of the animals (six weeks) had metastasized to the liver, yet several animals (15 of 41) had primary neoplasms of the gall bladder.

Development of carcinoma had a longer latency in animals heterozygous at the p53 locus (i.e., animals of *Pdx-1-Cre; Brca1$^{cond/cond}$; p53$^{cond/WT}$; K-Ras$^{G12D/WT}$* genotype). PANIN-3 was first discovered at an age of 18 days (one of two animals), first carcinomas were discovered at an age of 5 weeks and all 13 animals older than 5 weeks exhibited malignant neoplasms of the pancreas. In addition more than half of these animals (7 of 13; 4 of 5 with an age of 13 weeks and older) exhibited metastases to lymph nodes, liver, lung and salivary gland.

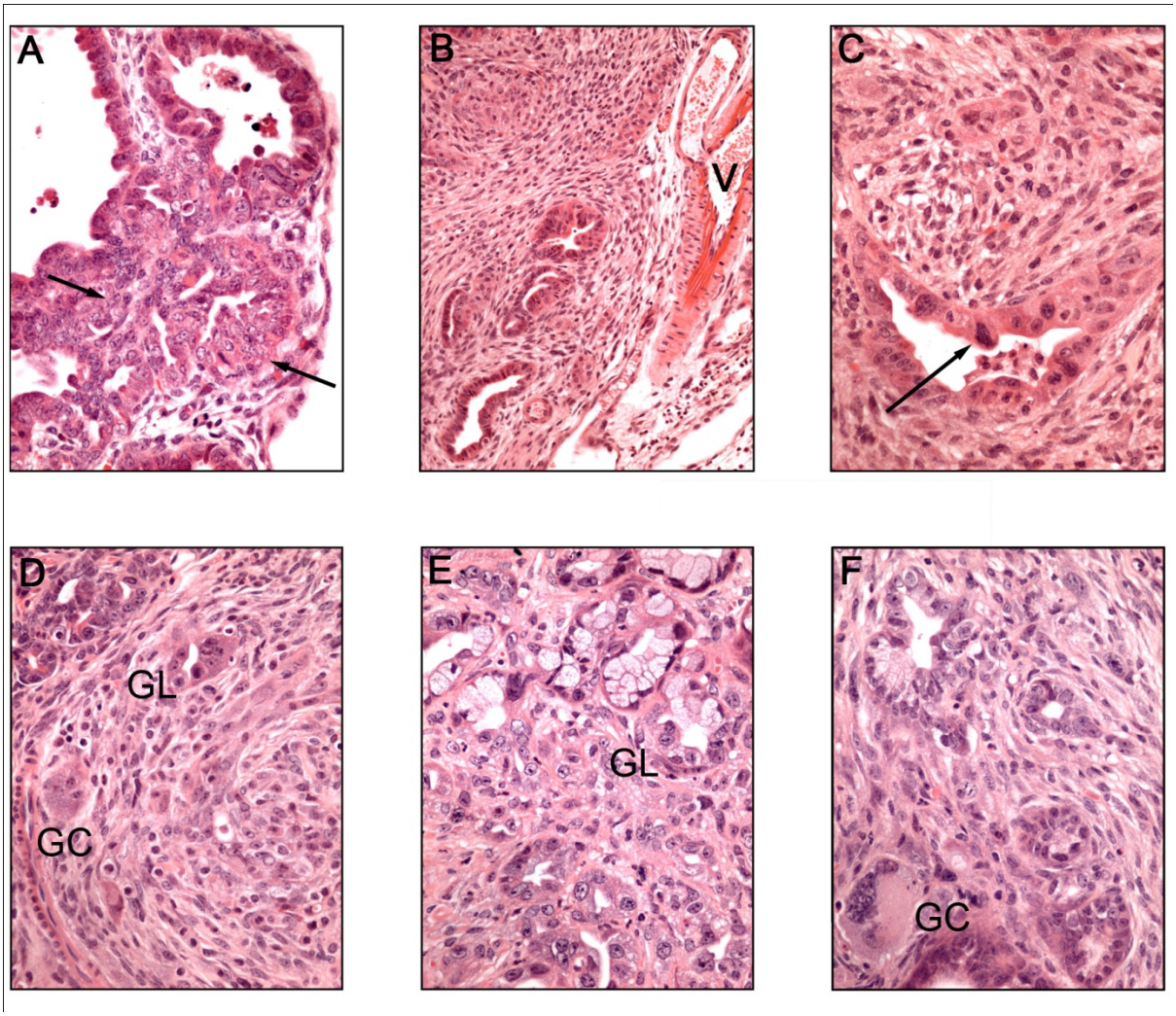

Figure 23: Histological features of ductal adenocarcinoma of the pancreas. A: Pancreas of a 1-week-old *Brca1*$^{cond/cond}$ animal. In-between arrows lies PANIN III lesion. **B** and **C**: Pancreas of a 2-week-old *Brca1*$^{cond/cond}$ animal. **B**: Anaplastic carcinoma with spindle cell metaplasia approaching pancreatic capsule with large blood vessels (V). **C**: Arrow indicates cancerous gland with bizarre giant cell. **D**: Pancreas of a 3-week-old *Brca1*$^{cond/cond}$ animal. Carcinoma is composed of poorly formed glandular elements (GL) and bizarre giant cells (GC). **E**: Pancreas of a 10-week-old *Brca1*$^{WT/WT}$ animal and **F**: Pancreas of a 5-week-old *Brca1*$^{cond/cond}$ animal: The invasive carcinoma of all mice have a glandular, anaplastic (i.e. bizarre giant cells) and spindle cell component. *Brca1*$^{WT/WT}$ cancers tend to have larger glandular elements commonly with mucinous metaplasia (GL), whereas the *Brca1*$^{cond/cond}$ tend to display a more prominent anaplastic component (GC).

A striking phenotype of mice lacking functional *Brca1* is the development of macroscopic visible cystic lesions of the pancreas. These extensive growing macrocystic lesions impress with cysts growing to a size up to 8 mm (figure 24).

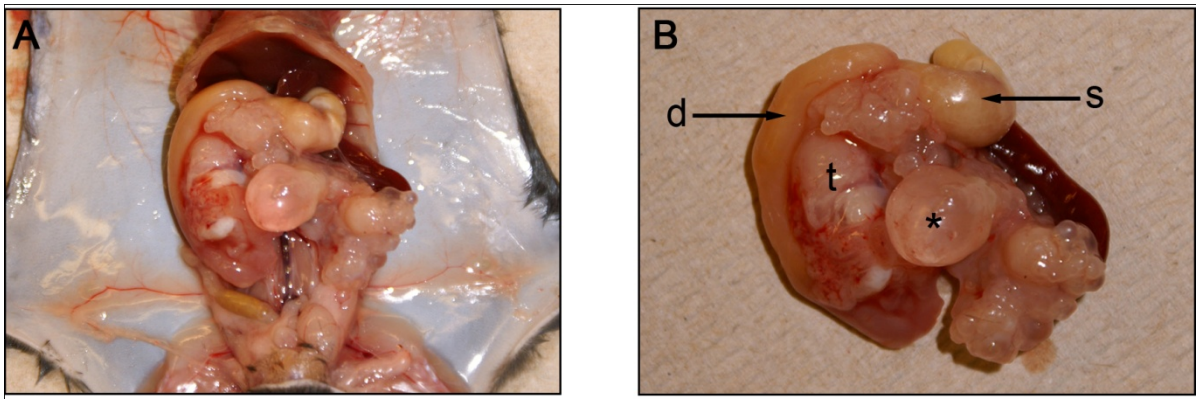

Figure 24: Macroscopic aspect of pancreatic Tumor. A: Brca1-deficient pancreatic tumor in situ. **B:** the same tumor after preparation. Tumor is made up of solid areas (t) and macrocystic areas (*). Arrows indicate duodenum (d) and stomach (s).

The benign cysts of the pancreas are lined by cuboidal to flat inform cells with no obvious atypia. The lining contains numerous apoptotic cells, which then seem to be released into the lumen of the cysts. The cysts develop predominantly in the body and tail of the pancreas (figure 25A-C). While most of these lesions have the features of serous cysts, some present with a more mucinous appearance.

Interestingly, the cystic lesions were often surrounded by an ovarian-like stroma, which is characterized by a stroma with wavy nuclei and expression of estrogen and progesterone receptors (figure 25D to F) and alpha-inhibin.

This benign neoplasm has been observed in virtually all of the *Pdx-1-Cre*; *BRCA1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ mice and can be found as early as at an age of two days. In older animals these cystic lesions are still present but are often overgrown by the aggressively growing anaplastic ductal adenocarcinoma.

Two animals heterozygous at the *p53* locus (*Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/WT}$; *K-Ras*$^{G12D/WT}$) were examined at an age of 18 days. Both already had developed the described cystic lesion. One of the animals with this genotype sacrificed at the age of twelve weeks even demonstrated the rare entity of a papillary serous cystadenocarcinoma (figure 25G).

To fully apprehend the decisive role played by *Brca1* in the development of these cystic lesions it is of note that none of the animals with wildtype *Brca1* displayed any of these macrocystic lesions.

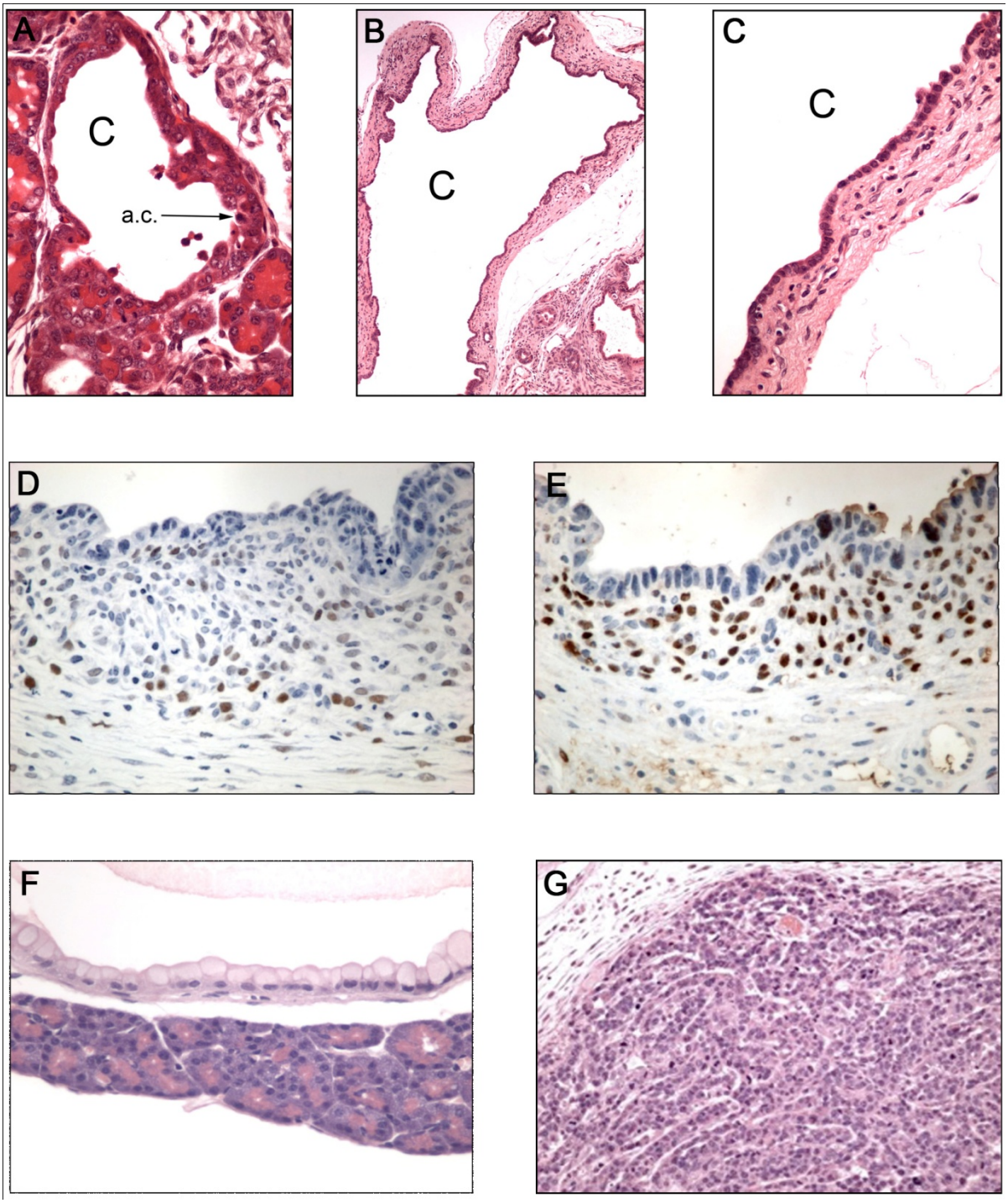

Figure 25: Histological features of macrocystic lesions. A: Cystic lesion in the pancreas of a 2d old animal. Apoptotic cells are released in the lumen of the cyst. B & C: Cyst in the pancreas of a 3 weeks old animal in 10x (B) and 40x (C) magnification. D & E: Immunostaining with anti-PR (D), and anti-ER (E). F: Cyst with single layer of epithelial cells that contain mucin droplets. G: Papillary serous cystadenocarcinoma in the pancreas of a 12-week-old animal heterozygous in the p53 locus. (Abbr.: C: Lumen of the cyst; a.c.: Apoptotic cell).

As mentioned above the animals with functional *Brca1* (*Pdx-1-Cre*; *BRCA1*$^{WT/WT}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$) also developed ductal adenocarcinoma of the pancreas, albeit with a more glandular appearance. At an age of six days none of the examined animals (n=2) showed signs of invasive malignancy or precursor lesions. Precursor lesions like PANIN-3 and first signs of malignancy appeared at an age of three weeks, 34 of 36 animals older than three weeks showed ductal adenocarcinoma. 25 of these carcinomas had a glandular differentiation with signet ring cells. Notably, while only one of the *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ animals showed metastases, 19 oft the 34 *Pdx-1-Cre*; *Brca1*$^{WT/WT}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ animals with invasive carcinoma had metastasized, mainly to the peritoneum, mesentery, lymph nodes and the liver.

3.5 SKY Analysis

Spectral karyotype analysis was employed to illustrate the influence of *Brca1* deficiency on the frequency of chromosomal aberrations like translocations and aneuploidy. The euploid mouse karyotype (2n=40) is composed of 2 x 19 = 38 autosomes plus 2 sex chromosomes.

Cells derived from the pancreatic tumor cell lines 43-11 and 43-16 that have functional *Brca1* alleles, show typical features of malign cells like polyploidy and translocations.

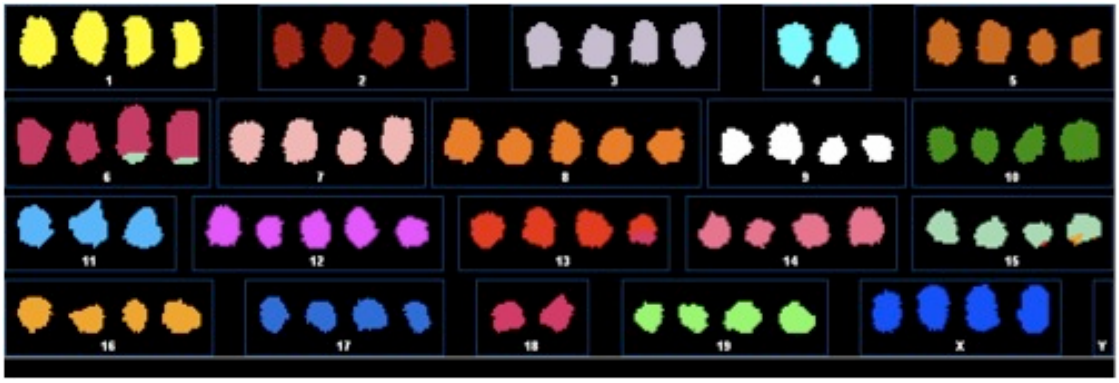

Figure 26: Karyotype of cell line 43-11

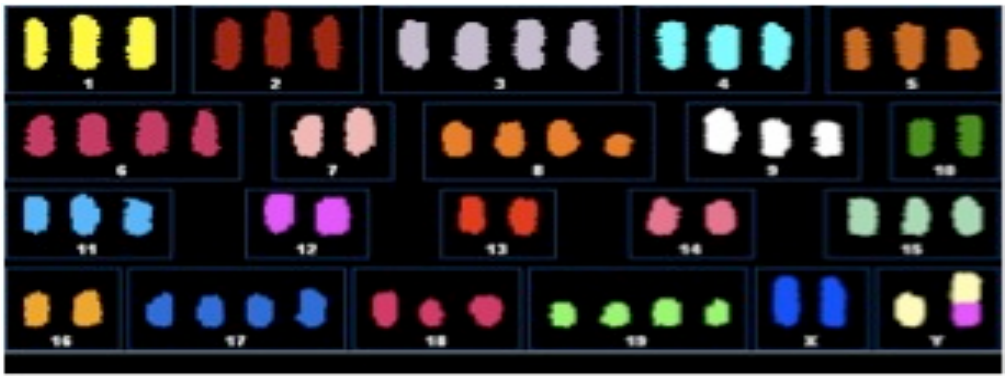

Figure 27: Karyotype of cell line 43-16

The same characteristics of tumor cells have of course been found in the Brca1-deficient tumors. Just like it has been described for Brca1-deficient mouse embryonic fibroblasts or mammary or ovarian epithelial tumors, no Brca1 pancreatic tumor cell was euploid; the cells were usually tetra- to pentaploid.

The tumor cells expressing no functional Brca1 additionally contained an abundance of structural aberrations such as insertions, deletions, translocations, di-, tri- or even tetracentric chromosomes, and chromatid breaks.

By immunofluorescence analysis we could also detect supernumerary functional centrosomes with intact centrioles whose presence results in multipolar mitoses and aneuploidy (data not shown).

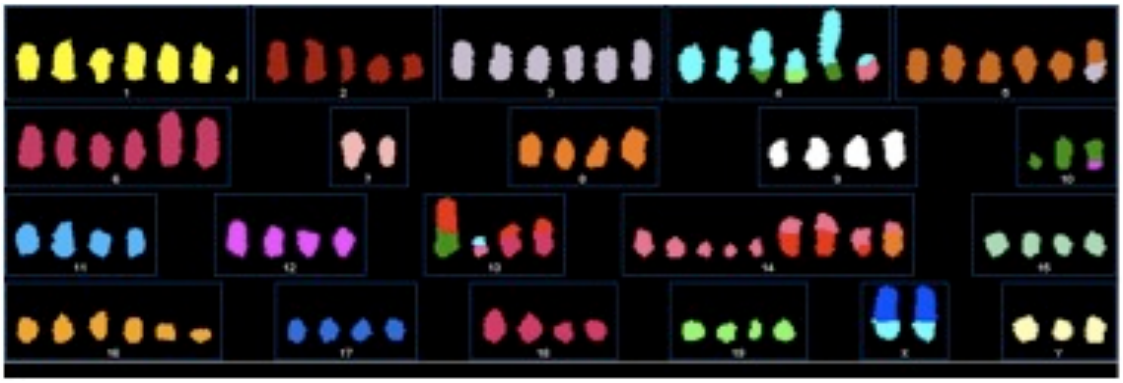

Figure 28: Karyotype of cell line 8-1

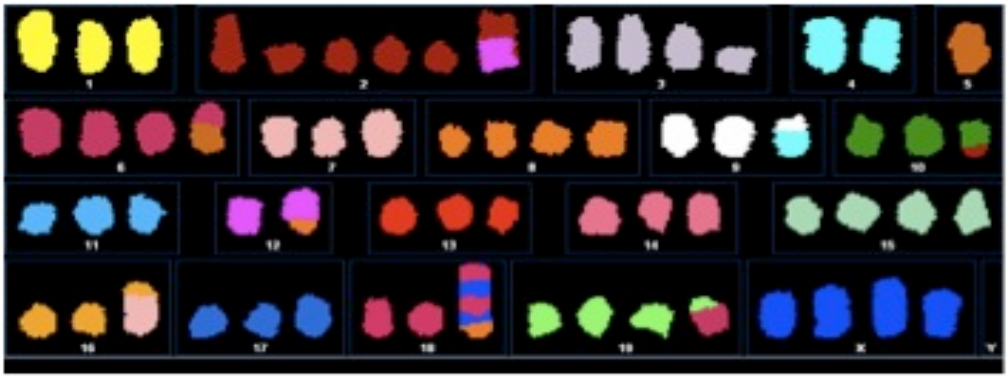

Figure 29: Karyotype of cell line B1-10

For full karyotypes of the four cell lines SKY analysis was done with please refer to the Supplement (S12), where a sample karyotype of each cell line is noted.

4 Discussion

4.1 Histological features of BRCA1-deficient pancreata

The typical histopathology of ductal adenocarcinoma seen in both, the *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ and the Pdx-1-Cre; p53$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ mice has been described earlier in other pancreatic tumor models using mutations of the *p53* and *K-Ras* genes. Thus, this suggests that the development of ductal adenocarcinoma in our model depends mostly on the functionality of the p53 and K-Ras pathways. However the discrepancy of the tumor-free survival between Brca1-deficient and proficient mice and the differences in the microscopic appearance are remarkable and clearly indicate a participation of Brca1 if not in tumorigenesis then in tumor progression or regulation of growth and proliferation.

As described above among the BRCA1 proficient animals PANIN-3 lesions were first detected in animals with the age of 18 days, compared to Brca1/p53-double homozygous animals which showed PANIN-3 lesions as early as 2 days of age. In-situ carcinomas were discovered at the earliest at an age of 5 weeks among BRCA1 wildtype animals – again, compared to Brca1/p53-double homozygous animals which displayed invasive carcinoma among animals as young as 6 days of age.

Brca1-deficient tumors showed a slightly more anaplastic component than the more glandular *Brca1*-wildtype tumors. In most cases of sporadic human PDAC a glandular architecture is evident, while anaplastic carcinoma is a rare and highly aggressive subtype of PDAC. Yet, the histological differences between the two genotypes in our mouse model are not substantial enough to allow a classification of *Brca1* negative carcinomas as anaplastic and *Brca1* wildtype carcinomas as well differentiated PDAC.

The longer tumor latency of the animals heterozygous at the *p53* locus suggests that a fraction of the oncogenic drive results from the lack of p53 function, proposing that loss of the wildtype *p53* allele of the heterozygous animals is required for the Brca1-deficient putative malignant cell to bypass p53-induced apoptosis, survive and proliferate.

This would be a comparable mechanism as it has been demonstrated for the *in-utero* development of mice with a non-conditional knock-out of *Brca1*. As described above, these animals die early during embryogenesis, but can be partially rescued by

introducing an additional knock-out of *p53* in the genome [39]. But, the observation that the embryonic lethality is not rescued completely among *Brca1*$^{-/-}$; *p53*$^{-/-}$ mouse embryos suggests that *Brca1* gene has other functions independent of *p53*, which are critical for cell-survival. Oncogenic signals, such as expression of activated K-Ras (i.e. K-Ras^{G12D}) can help Brca1-deleted cells to overcome these additional survival or proliferative barriers. Interestingly, among *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$ animals, a few developed PDAC with long latency. One of these animals had acquired a spontaneous *K-Ras*^{G12D} mutation, underscoring the importance of oncogenic *K-Ras*^{G12D} expression in PDAC development in these animals (data not shown, private communication, T. Ludwig).

In animals carrying both a conditional and a null *Brca1* allele *(Pdx-1-Cre; Brca1*$^{cond/-}$) no recombination of the conditional *Brca1* allele is taking place in the pancreas. Thus, it is likely that the expression of functional *Brca1* is also required during the development of the pancreas.

We hypothesize that in the *Pdx-1-Cre*; *Brca1*$^{cond/-}$ animals, the pancreatic cells that recombine the conditional *Brca1* allele are either non-viable or undergo proliferative arrest, and hence do not contribute to pancreatic development. However, since *Pdx-1-Cre* transgene is expressed in a stochastic pattern in the pancreas [82], enough cells that do not recombine the conditional *Brca1* allele remain within these pancreata, and thus survive and proliferate to give rise to the entire pancreata. Accordingly, the pancreata of animals with conditional and null *Brca1* alleles (*Pdx-1-Cre*; *Brca1*$^{cond/-}$) are smaller than the pancreata of control animals carrying a wildtype and a conditional allele (*Pdx-1-Cre*; *Brca1*$^{cond/WT}$).

Albeit the pancreata of *Pdx-1-Cre* animals that carry only the *Brca1* conditional allele (*Pdx-1-Cre*; *Brca1*$^{cond/cond}$) contain hardly any cells that express *Pdx-1* and recombine the conditional alleles, all the *Pdx-1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ animals develop PDAC, growing from *Pdx-1-Cre* expressing, fully recombined cells.

Thus, we postulate that, in contrast to the *Pdx1-Cre*; *BRCA1*$^{cond/-}$ pancreata, among the *Pdx1-Cre*; *Brca1*$^{cond/cond}$; *p53*$^{cond/cond}$; *K-Ras*$^{G12D/WT}$ pancreata, the Brca1-deleted pancreatic ductal cells are more likely to survive and proliferate due to the concomitant loss of p53 and expression of activated K-Ras. Moreover, given the role of Brca1 as “the care-taker” of the genome, the loss of Brca1 from these cells likely results in unstable genomes, which, in turn, could lead to rapid accumulation of

mutations, some of which could be favorable for PDAC tumorigenesis. Indeed, this latter hypothesis is supported by the observation that the *Pdx-1-Cre*, *Brca1*$^{cond/cond}$, *p53*$^{cond/cond}$, *K-Ras*$^{G12D/WT}$ cohort of animals developed PDAC with a much shorter latency (5-6 weeks) compared to cohort of animals that were *Pdx-1-Cre, p53*$^{cond/cond}$, *K-Ras*$^{G12D/WT}$ (10-12 weeks).

While the rationale for the mouse model was the evaluation of the contribution of a *Brca1* defect to the development of PDAC and the assessment of the feasibility of a targeted therapy, we were surprised to find a pancreatic neoplasm that, in our model, is proprietary to the Brca1-deficient pancreas.

The expression of activated K-Ras obviously has a specific effect in *Brca1*$^{-/-}$ pancreatic cells, as the development of the described cystic lesions seems to be typical for mice with a combination of non-functional Brca1 and activated K-Ras. In the pancreata of animals that were either *Pdx-1-Cre, Brca1*$^{cond/cond}$ or *Pdx-1-Cre, K-Ras*$^{G12D/WT}$ no cystic lesions were discovered (data not shown, private communication, T. Ludwig).

A possible scenario to explain the initiation of distinct types of neoplasms in the Brca1-deficient pancreas would be, that during the short period the Cre recombinase is expressed during pancreatic development not all conditional alleles do actually recombine. While full recombination of all three conditional alleles initiates PDAC, recombination of only the *Brca1* and *K-Ras* conditional alleles might initiate the development of cysts. Another possible explanation would be, that the cystic lesions originate from a specific subset of cells in the pancreatic ducts or from special kind of precursor cells.

At first glance the cystic lesions containing hardly any atypia in their lining cells appear to be serous cystadenoma. In accordance with other tumor-progression models one would eventually expect malign transformation of the cystadenoma. But transformation of benign cysts to the malign entity papillary serous cystadenocarcinoma is a rare event observed in only one of the animals heterozygous at the *p53* locus. This leads to the assumption that, in accordance with the multiple-hit theory, additional genetic modifications independent of p53, are required to allow malign transformation of this kind of neoplasm.

At second glance the presentation of these cystic lesions with surrounding ovarian-like stroma and the evidence of Estrogen receptor suggests an analogy between these lesions and mucinous cystic neoplasm (MCN), which is a precursor lesion to

pancreatic adenocarcinoma in humans and has the same histological features. Indeed we were even able to detect mucinous elements among the serous cysts. However, MCN usually present with cellular atypia along the cyst lining.

Among older animals the cystic lesions are usually overgrown by the anaplastic adenocarcinoma. Histology did not allow to decide whether these adenocarcinomas are originating from the cystic lesions or from other precursor lesions like PanINs. Again, this re-emphasizes the validity of the multiple-hit theory, as one possible explanation would be that a specific set of “hits” is required for initiation of papillary serous cystadenocarcinoma, while a different set might initiate transformation of the benign cysts to PDAC and a third might not enable the cell to overcome apoptotic signals.

The pathogenesis of these cystic lesions and the role of Brca1 remain unknown. Considering the amount of apoptotic cells in the lumen of the cysts, we propose a model where luminal apoptosis leads to an extraluminal proliferation of ductal cells thus forming cystic structures with a growing lumen and diameter.

Further studies will be required to fully understand these Brca1-dependant neoplasms and to address the question if these cystic lesions might even serve as a mouse model for human mucinous cystic neoplasm.

Up to this point there have been no reports about cystic lesions in *Brca1* knock-out mice or members from families with germline mutations of *BRCA1*.

BRCA1-depending cystic lesions were only described in the context of ovarian carcinoma, where epithelial inclusion cysts are common precursor lesions of epithelial tumors [97]. Still, a study examining the ovaries of healthy women with a germline mutation of BRCA1 did not show any precancerous lesions including epithelial inclusion cysts [98].

On the other hand ovarian carcinomas in women with a germline mutation of *Brca1* are with a higher-than-average rate serous cyst adenocarcinomas, proposing an influence of *Brca1* deficiency in the development of this subtype of ovarian cancer.

The unusual lack of metastasis of the ductal adenocarcinoma in the *Pdx-1-Cre*; $Brca1^{cond/cond}$; $p53^{cond/cond}$; $K\text{-}Ras^{G12D/WT}$ mice can easily be explained by the rapidity of the development of the carcinoma. The animals usually die due to the tumor burden and the ensuing massive peritonitis caused by the rapidly proliferating primary tumor before metastases have time to develop. The presence of metastases

in animals heterozygous for p53 (*Pdx-1-Cre*; *BRCA1*$^{cond/cond}$; *p53*$^{cond/WT}$; *K-Ras*$^{G12D/WT}$), which show slower tumor progression, supports this theory.

The only tissue besides pancreas that developed neoplasms was the gall bladder (n=16). Gall bladder adenomas (n=7) and Carcinoma in-situ (n=6) could be microscopically detected as early as at an age of 4 weeks, indicating that the transformation of the cells has possibly already taken place during embryogenesis. An explanation would be the activation of the *Pdx-1* promoter in progenitor cells of the gall bladder epithelial cells during development, indeed expression of *Pdx-1-Cre* has been described in extra-pancreatic sites such as the gall bladder or gastric epithelia [82].

Another possible explanation for the development of gall bladder neoplasms would be that a furiously growing carcinoma of the pancreas may lead to an obstruction of the bile duct and consequently to a chronic cholecystitis. The chronic inflammation could act as a proliferation stimulus in the gall bladder. Arguing against this is the early appearance of the neoplasms and the missing evidence of chronic inflammation in the bile ducts or the gall bladder.

The fact that no other tumors that are commonly found in mouse models like lymphomas have been detected is probably also based on to the short latency and aggressive growth of the engineered pancreatic tumors and the high specificity of the *Pdx-1* promoter to the pancreas.

4.2 The mouse as a model for human carcinogenesis

Given that our engineered tumors are the result of the combination of three very potent mechanisms to generate malignancies, it raises the question if it might be possible to create carcinomas of any kind of epithelial cells after tissue- specific expression of the Cre-recombinase and in how far these engineered neoplasms have any analogy to human carcinogenesis.

Loss of function mutations of *TP53* and the activation of *K-Ras* are established to be a factor in the malignant transformation of pancreatic cells. We believe that by targeting the Cre-recombinase to the *Pdx-1* locus it was possible to construct a close model of human carcinogenesis in the pancreas. The *Pdx-1* promoter generates a small number of *Cre*-expressing cells in a mostly normal pancreas. The analysis of various promoters expressed in the pancreas using *Cre*-dependant *K-Ras* activation showed that tumors engineered using the *Pdx-1* promoter showed a high resemblance to the PanIN to PDAC progression suggesting that these mouse neoplasms may share a common cellular origin with the human counterpart [3].

The resemblance to tumor genesis and progression of sporadic human PDAC we were able to observe in our model validates the applicability of our mouse model as a model for human carcinogenesis.

Undoubtedly, there is a large contribution to PDAC development by the activation of K-Ras and the loss of p53 to tumorigenesis. PDX-1 cre mediated activation of the oncogenic $Kras^{G12D}$ allele results in PDAC with an average latency of approximately 1 year [82]. Concomitant activation of $Kras^{G12D}$ and inactivation of a conditonal p53 allele or activation of a point mutant $p53^{R172H}$ allele causes PDAC with an average latency of five months (21 weeks) [95, 99]. Finally, activation of the $Kras^{G12D}$ allele and concomitant inactivation of both conditonal p53 alleles results in PDAC with an average latency of ~10 weeks.

As mentioned above, the Pdx-1-Cre; $BRCA1^{cond/cond}$; $p53^{cond/WT}$; $K\text{-}Ras^{G12D/WT}$ animals had a medium tumor-free survival of 12 weeks compared to the average latency of 21 weeks of Pdx-1-Cre; $p53^{cond/WT}$; $K\text{-}Ras^{G12D/WT}$ and Pdx-1-Cre; $p53^{172H/WT}$; $K\text{-}Ras^{G12D/WT}$ mice. Similarly, the Pdx-1-Cre; $BRCA1^{cond/cond}$; $p53^{cond/cond}$; $K\text{-}Ras^{G12D/WT}$ animals developed PDAC in 5 weeks compared to Pdx-1-Cre; $p53^{cond/cond}$; $K\text{-}Ras^{G12D/WT}$ where it takes on average 10 weeks.

These differences are statistically highly significant and demonstrate that the loss of BRCA1 function contributes to the development or progression of PDAC. Additional loss of BRCA1 clearly accelerates tumor initiation and subsequently tumor growth.

In human carcinogenesis most likely only one cell is transformed through accumulation of genetic alterations. The rapid growth of the tumors in the Pdx-1-Cre; $BRCA1^{cond/cond}$; $p53^{cond/cond}$; $K\text{-}Ras^{G12D/WT}$ animals is of course also a result of the fact that there is not only one but multiple transformed cells as origin of the tumor. In fact, tumors in this model develop so fast that metastasis is almost never observed.

A closer reproduction of human carcinogenesis is achieved with animals heterozygous for the targeted genes where tumor initiation depends on an additional loss-of-function mutation of the wildtype allele.

Thus, in Pdx-1-Cre; $BRCA1^{cond/cond}$; $p53^{cond/WT}$; $K\text{-}Ras^{G12D/WT}$ animals a prerequisite for tumor development is the LOH of the remaining p53 wild-type allele. As LOH is a stochastic event tumors in these animals are oligoclonal, develop with longer latency and also metastasize.

Yet for our purposes, which were the evaluation of histological features and the establishment of cell cultures, the fast developing carcinomas of our model fulfill all requirements.

While the importance of K-Ras activation and a defect of *TP53* for the transformation of pancreatic epithelial cells has been published before, the role of *BRCA1* in the development of pancreatic cancer is still controversial.

Although risk estimates for members of families with a *BRCA1* germline mutation demonstrate an elevated relative risk to contract pancreatic cancer, a study investigating the rate of *BRCA1* mutations in familial pancreatic cancer cases failed to show an involvement of *BRCA1*. In 66 cases of familial pancreatic cancer no deleterious mutations of *BRCA1* were detected by sequencing of the *BRCA1* gene. These results were found even in families reporting a family history of breast or ovarian cancer [100]. A different study examining 101 cases of sporadic pancreatic adenocarcinoma was only able to detect one patient with a mutation of *BRCA1* [101].

Still pancreatic cancer is currently the only additional malignancy for which there is unequivocal evidence for increased risk in both *BRCA1* and *BRCA2* carriers, although the absolute risk is small. In *BRCA1* carriers pancreatic cancer risk carriers by age 70 years has been estimated to be 1.16% (95% CI 0.83–1.61%) in men, and

1.26% (95% CI 0.92–1.72%) in women, reflecting the mentioned elevated RR of 2.26 [21].

The inability to detect *BRCA1* mutations in the analyzed familial pancreatic tumors with a history of ovarian or breast cancer could be explained by a phenomenon known as “BRCAness” [102]. This refers to sporadic or familial carcinomas of various tissues, which exhibit a phenotype that resembles the carcinomas of patients with known mutations of the *BRCA1* or *BRCA2* gene. Possible mechanisms to suppress the DNA damage repair via the homologous recombination pathway include epigenetic changes or alterations of upstream or downstream proteins involved in the BRCA1 pathway. A prominent example for epigenetic changes is the hypermethylation of the *BRCA1* gene promoter, which results in suppression of BRCA1 expression and a loss of BRCA1 function that is comparable to that of genomic mutations. In breast cancer *BRCA1* promoter hypermethylation leads to a phenotype comparable to that of *BRCA1*-deficient tumors. These tumors tend to be ER negative and show an association with similar subtypes of breast cancer like familial *BRCA1* cancers. Furthermore expression microarrays show similarities between *BRCA1* promoter methylated and *BRCA1*-deficient tumors [103].

With the help of our mouse model I was able to demonstrate that murine pancreatic tumor cells deficient in Brca1 dependent DNA repair are sensible to a treatment with DNA damaging drugs. These results are not only applicable to carcinoma developing due to a germline mutation of BRCA1 but also to tumors developing due to other defects of BRCA1 pathways. Thus it will be important for individual treatment decisions to find possibilities to discern not only between tumors with germline mutations of BRCA1 but also to identify tumors carrying the features of BRCAness. For example the high grade of genomic instability we were able to demonstrate using spectral karyotype imaging is a feature typically found in BRCA1-negative carcinomas. This should also be observable in tumors with BRCA1 promoter hypermethylation or other causes of BRCAness and could help to identify patients eligible for a targeted therapy.

4.3 Targeted therapies for BRCA1-negative pancreatic ductal adenocarcinoma

4.3.1 Drug sensitivity assay

To evaluate the inhibition of proliferation a drug sensitivity assay was established. The first experimental design included the assessment of drug sensitivity test with an MTT assay. The MTT assay is a colorimetric method using the cleavage of the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan crystals by metabolic active cells. [104]. Yet the MTT assay measures metabolic activity instead of cellular proliferation. As the cell lines we used to investigate drug sensitivity were highly differing in their metabolic activity I decided to use a more direct assay to survey proliferation of the treated cells.

The determination of cell number using a hematocytometer of course bears the risk of information bias. Blinding of the counting person to the genotype of the evaluated cell line and the drug concentration has counteracted this risk. The results obtained with this assay are consistent internally and with comparable results published for BRCA2-deficient pancreatic cancer cell lines.

The results verified the expected sensitivity of *Brca1*-deficient pancreatic adenocarcinoma to a treatment with DNA damaging drugs.

4.3.2 Implications for the therapy of BRCA1-negative pancreatic ductal adenocarcinoma

During the past years individualized and targeted therapies achieved stunning results and the effectiveness of individualized therapies and the importance of the transfer of the acquired knowledge are slowly gaining more and more attention in hospitals and clinics.

While many of these targeted therapies predominantly exist in scientific papers and propaganda leaflets of pharmaceuticals companies other methods and compounds are already well established in the clinical setting.

A prominent example is the treatment of breast cancer. Today it is common sense to examine not only the histological features of breast cancers but also to examinate the receptor status (ER, PR and HER-2/neu) and to use this information to treat patients

accordingly with drugs like tamoxifen and herceptin. Compared to earlier results this proves to create not only better survival but also better performance of patients during treatment.

As there is only a small number of BRCA1 related pancreatic cancer it will not be possible to conduct large clinical studies to determine an effective and specific therapy and other ways will have to be established. But as we know more and more about the biology of cancer and are increasingly able to determine the differences between carcinomas of various tissues and also between individual patients, we use this knowledge to identify new ways of drug treatment.

BRCA1-deficient tumors are associated with a flawed repair of DNA damage, the logical consequence of this knowledge is to try and inflict DNA damage to the carcinomatous cells.

Subject of this research was to show that the important role Brca1 has in the repair of DNA damage via the homologous recombination pathway could indeed be exploited in the therapy of *Brca1* negative pancreatic tumors. The results clearly show that tumor cells that have no functional *Brca1* allele have a significantly higher chemosensitivity to DNA damage inducing drugs. These results are consistent with the results of several other studies investigating the *in vitro* drug sensitivity of Brca1 negative mouse embryonic stem cells [105] and tumor cells of various tissues like breast or ovarian cancer [106].

Platinum compounds and other DNA damage inducing drugs like Mitomycin C are well established in the treatment of cancer. Yet for the treatment of PDAC these drugs have only minor relevance. This is due to the results of several studies investigating the use of Cisplatin or Oxaliplatin in combination with Gemcitabine in the palliative situation. The combination showed a non-significant improve of outcome, unfortunately accompanied by a higher toxicity, a significant improve of survival has only been shown for patients with good clinical performance (i.e. Karnofsky index ≥ 90%). Also the use of Mitomycin C in combination with 5-FU showed no positive effect [107].

It is now important to use the knowledge about the efficiency of these drugs in a BRCA1-negative context that has been acquired in the past years to develop special study protocols also for patients with pancreatic cancer.

As we expect a substantially improved response to the treatment with these drugs in patients with defects in DNA damage repair the increase in adverse effects can be accepted. It might even be possible to treat these patients with lesser drug doses and thus to reduce adverse effects.
First trials could be carried out with platinum salts in combination with the well-established Gemcitabine as this combination already showed promising results in patients with good clinical status.

4.3.3 Novel therapeutic strategies

Beside the development of new treatment protocols using established drugs, new anticancer agents are identified. Often members of this new generation of anticancer drugs are designed to specifically attack pathways that are corrupted in transformed cells.
The identification of the *BRCA1* gene and the effect of its absence naturally led to trials to restore *BRCA1* function via gene therapy. Yet after promising phase I trials with retroviral *BRCA1*, in phase II trials no effect of the *BRCA1* therapy on ovarian tumors has been observed [108]. Due to these disappointing results the concept of *BRCA1* gene therapy has not been pursued further.
As explained above *BRCA1*-deficient cells have a selective advantage if the cell also carries an additional mutation of *TP53* bypassing the apoptotic signal induced by the accumulating DNA damage in *BRCA1*-deficient cells. Cancer cells thus often carry a combination of *BRCA1* and *TP53* mutation. Another new approach to selectively attack carcinomatous cells is to re-establish P53 expression.
This can be achieved by introduction of the *TP53* gene via adenoviral vectors over-expressing external p53 in tumor cells. A serious problem of adenoviral P53 is limited efficiency of gene delivery to tumor cells. Local therapy has proven to be effective in head and neck cancer, yet the intravenous application of adenoviral P53 showed little effect. A possible solution for pancreatic carcinomas might be the intra-operative injection of the drug into the surrounding tissue.
P53 expression can also be reconstituted with the help of small molecules. These compounds can act via inhibition of degradation of P53 by MDM2 thus raising the levels of P53 or reconstitution of the wildtype configuration of mutant P53 proteins.

The re-establishment of P53 expression is thought to be especially effective in a *BRCA1*-deficient background as the DNA damage accumulated through the defect in BRCA1 mediated homologous recombination will induce apoptosis upon adequate P53 expression.

Another new group of anticancer drugs are inhibitors of Poly(ADP-ribose) polymerases (PARP). Poly(ADP-ribose)polymerases are enzymes that are involved in base excision repair, a key pathway in the repair of DNA single-strand breaks. Loss of PARP function leads to accumulating single-strand breaks and subsequently to stalling replication forks and double strand breaks that are usually repaired by homologous recombination. This is illustrated by the formation of RAD51 foci upon inhibition of PARP. Yet, as in *BRCA1*-deficient tumors the homologous recombination pathway is lost, DNA damage is either repaired via error-prone mechanisms such as non-homologous end joining (NHEJ) and single-strand annealing (SSA) leading to complex chromatid re-arrangements and further accumulation of errors in the genetic information This causes cells to arrest at the G2/M checkpoint and permanently arrest or undergo apoptosis [109].

Inhibition of PARP-1 via siRNA or small molecules showed impressive results when used in *BRCA1*- or *BRCA2*-deficient cells *in vitro* and in various phase I trials [110].

Currently PARP inhibitors are major area of research for new therapeutic interventions against tumors that have lost functional BRCA1. Several phase II trials evaluating PARP inhibiting drugs are currently active or recruiting.

During the 2009 Meeting of the American Society of Clinical Oncology (ASCO) preliminary results of two international multi-center Phase II proof-of-concept studies were presented. 54 patients (18 *BRCA1*-deficient and 9 *BRCA2*-deficient) pre-treated breast cancer and 57 patients (39 BRCA1-deficient and 18 BRCA2-deficient) with advanced chemotherapy-refractory ovarian cancer were treated with the orally active PARP inhibitor olaparib (AZD2281). Objective response rates (RECIST criteria) were 38% and 33% at 400 mg bd. respectively [111] [112].

While these short-term results raise high hopes there is still nothing known about long-term toxicity of PARP inhibitors. It is feared that a treatment impairing alternative pathways of DNA damage repair in a *BRCA1* heterozygous background might facilitate the development of secondary neoplasms of other tissues.

The same objections apply to radiochemotherapy of *BRCA1*-deficient tumors.

Breast cancer patients with a germline mutation of *BRCA1* who were treated with breast-conserving surgery and radiotherapy are known to bear a higher risk to develop a secondary carcinoma of the ipsilateral breast than patients with sporadic carcinoma, these secondary carcinoma are considered to be genuine new primaries rather than recurrences of the initial irradiated tumors. If these secondary tumors are caused by the radiation therapy or by the inherited predisposition remains unclear although the rate of ipsilateral secondaries matches the rate of contralateral secondaries.

The rate of acute or chronic radiation associated complications showed no differences between carriers of a germline mutation and patients with sporadic breast cancer [113].

While breast cancer is generally considered to be well-responding to radiotherapy, this therapy is not generally recommended for patients with PDAC, due to a plus in adverse reactions following combined radio-chemotherapy. Yet as the principle of radiotherapy is the induction of DNA damage tumors with a *BRCA1* deficiency are considered to be highly sensitive to irradiation. To further investigate the applicability of radiochemotherapy in tumors with a defect in DNA repair patients considering this therapy should be monitored closely and with a long follow-up for secondary cancers.

4.3.4 Secondary resistance to platinum compounds and PARP inhibitors

Unfortunately there is a setback to a possible therapy of *BRCA1* or *BRCA2* mutated carcinomas with platinum compounds or PARP inhibitors - the development of a secondary resistance. Secondary resistance to these agents has been observed shortly after the discovery of their selective effectivity [114].

A possible explanation how *BRCA1*-deficient tumors acquire drug resistance is frame shift mutations. As explained the sensibility of tumors to platinum or PARP inhibitors depends on the dysfunctional DNA repair. If a tumor that has developed on the background of a frame-shift mutation in a tumor suppressor like *BRCA1* acquires another frame shift mutation in the same allele, the open reading frame can be restored and the mechanisms of DNA repair reconstituted. Other secondary intragenic mutations include the reversion of amino acid deletions to wildtype. Swisher et al. demonstrated that three out of four recurrent ovarian cancers with

acquired platinum resistance showed a back mutation of the 185delAG deletion and restoration of *BRCA1* expression [115]. Interestingly an *in vivo* study with a conditional mouse model for hereditary breast cancer creating an irreparable *BRCA1* null allele only showed the development of resistance to doxorubicin and docetaxel, while the tumors did not develop a secondary resistance to Cisplatin [116].
A secondary resistance to platinum compounds has been observed in both ovarian and mammary carcinomas and is consequently also to be expected in pancreatic carcinomas. Of course tumor cells with a secondary resistance have a secondary selective advantage during therapy. These cells can be the origin of a tumor relapse.

4.3.5 Innovations in the treatment of *BRCA1*-deficient breast cancer

An alternative approach to the identification of efficient therapies is to consider the drug sensibilities of other, more common, tumors associated with BRCA1 or faulty DNA damage repair like breast cancer.
While the number of pancreatic carcinomas, especially of *BRCA1*-deficient pancreatic carcinomas is comparable low, there are large numbers of patients with carcinomas of the breast or the ovary, usually treated in large clinical studies.
Trials comparing the response of BRCA1 patients and non-BRCA1 patients to treatment with DNA damaging drugs do not exist. It would be of great interest to re-evaluate some of the large breast cancer studies for sub-groups like *BRCA1* status or depending on histological subtype and hormone receptor status.
Breast cancer that is associated with a deficiency of *BRCA1* is often associated with the basal-like or triple negative subtype. It has even been suggested that the basal-like subtype is associated with a deficiency in the *BRCA1* DNA damage repair pathway, although there is not enough data yet to confirm this assumption.
The thought that triple-negative breast cancer might be associated with deficiency in DNA repair lead to therapy trials with DNA-damaging drugs like the platinum derivates carboplatin or cisplatin that thus far show promising results. A trial investigating the putative highly active combination of a platinum agent and a PARP inhibitor is currently being planned.

Furthermore there are several studies examining the role of novel therapeutic agents in therapy of advanced triple negative breast cancer [117]. These new compounds include antiangiogenic agents like the monoclonal antibody against vascular endothelial growth factor-A (VEGF-A) bevacizumab and the multikinase vascular endothelial growth factor receptor inhibitor sunitinib.
Further newly developed drugs that are currently in phase II trials are epithelial growth factor receptor (EGFR) inhibitors like the anti-EGFR monoclonal antibody cetuximab, MAP-kinase inhibitors like the oral multikinase inhibitor sorafenib and mammalian target of rapamycin (mTOR) inhibitors like temsirolimus.
The results of these studies might be in some way transferable to other types of BRCA1-related carcinomas like PDAC and give new impulses to the research on therapy of these cancers.

4.3.6 Individualization of drug therapy

A downside of large clinical trials including hundreds of patients is that it is inevitable to stratify patients into subgroups and it is thus impossible to pay tribute to the diversity of tumors. Just like there is not “the” breast cancer but various distinct subtypes there are probably various subtypes of ductal adenocarcinoma of the pancreas defined by the genetic make-up of the tumor. For the successfully treatment of this genetic diversity of tumors it will be important to try and develop a personal therapy for each individual patient.
To plan an individual therapy for each patient it is necessary to gather as much information as possible about the characteristic properties of the tumor. Besides taking a thorough patient history investigating personal risk factors and family history of certain carcinomas and subtypes one of the most important steps is to acquire a tissue sample of the tumor.
A tissue sample can be obtained either via biopsy or, if the patient has undergone surgery, the resected tumor is processed for further investigations. The pathologist can assess the histological subtype of the tumor and gain additional information by specific stains, immunohistochemistry or SKY analysis. Further parts of the sample might be used to assess the genomic properties of the tumor, for example the integrity of tumor suppressor genes like *BRCA1* or *TP53*. Following the same train of

thoughts it could be interesting to investigate the gene expression levels of relevant proteins like P53 or BRCA1.

The use of microarray and proteomic technologies for the molecular characterization of human tumors has made great progress in the past years. As mentioned above it has been possible to demonstrate similarities between familial *BRCA1*-deficient tumors and tumors with *BRCA1* promoter hypermethylation . Tumors with a similar gene expression profile consequently could also show a similar chemosensitivity.

Besides these analyses it will be important to obtain a sample of vital tumor cells for further research. This tissue sample can be used to generate a tissue culture.

Besides providing the laboratory with a sufficient amount of DNA material to thoroughly assess the genomic configuration of the tumor, cultured cells can be employed for *in vitro* drug testing, as I have done in this work or to evaluate the integrity of various pathways like the cell's response to DNA damage.

With the help of a cell culture it is possible to directly test chemosensitivity of the tumor cells to a variety of drugs and drug combinations in considerably short time. These assays would not only enable clinicians to identify the most effective drugs but also to exclude those agents for which the tumor has an inherent or acquired resistance and thus to spare the patient from a futile but harmful chemotherapy.

There are various *in vitro* assays to evaluate the drug sensitivity of tissue cultures including the clonogenicity assay, the MTT assay or the ATP-based tumor chemosensitivity assay (ATP-TCA).

Unfortunately *in vitro* assessment of drug sensibility has limited validity. The treatment of cells *in vitro* does not pay respect to the heterogeneity of *in vivo* tumors, that could even be made up of various progenitor cells, or to the individual tumor micro-environment and the pharmacokinetic and pharmacodynamic makeup of each individual patient, that plays an important role for drug half-life, distribution, metabolization, elimination, activation of pro-drugs or for the delivery of certain drugs to the tumor cell, which is an important factor for targeted therapies like e.g. gene therapy.

On the other hand there is increasing evidence that the results of *in vitro* drug sensibility assays are associated with the clinical outcome of cancer patients. Several trials published in the recent years were able to demonstrate the predictive value of chemosensitivity testing with the MTT assay in malignomas of various origins

including acute leukemia [118], leukemic non-Hodgkins lymphoma [119] and gastric carcinoma [120]. Another trial was able to reliably predict sensitivity to different chemotherapy agents and resistance to platinum-containing chemotherapy with the ATP tumor chemosensitivity assay ovarian cancer [121].

Powell and Kachnic have described a new and promising concept for the evaluation of putative *BRCA1*-deficient cells. They used a core biopsy of human breast cancer for the evaluation of the functionality of the homologous recombination pathway. The biopsy was irradiated immediately after obtaining the sample. After some time to allow repair foci formation in response to the inflicted DNA damage immunohistochemistry was used to identify tumors with defects in the ability to form Rad51 or BRCA1 containing foci. This is a matter of particular interest as they were able to show a lack in foci formation even in tumors from patients with no known genetic predisposition [122].

A serious impediment to the described methods of drug sensibility assessment is the requirement to obtain a tissue sample. Only for a few tissues and assays (those that need only small numbers of cells) these can be easily acquired, e.g. by aspiration of ascites, but for most patients this would mean to undergo invasive procedures like a core biopsy or even surgery.

While an operable tumor allows the acquisition of tumor tissue during surgery before start of an adjuvant therapy, for patients with inoperable adenocarcinoma of the pancreas neither biopsy nor surgery is a feasible method.

In vivo tumors have the capability to develop a drug resistance during the course of treatment. It will thus be necessary to obtain cellular material during the course of treatment to re-evaluate the efficiency of the current therapy. But especially in pancreatic cancer, there is hardly any possibility to obtain a biopsy to test for secondary resistance except during initial surgery.

Trials to establish cell cultures from tumors cells accumulated from the blood stream are still in their infancy and not yet reliable.

A non-invasive method to evaluate the response of malignant tissue to chemotherapy is monitoring of the tumor with Positron emission tomography (PET) or the combination of PET/computed tomography (CT) using the glucose analog ^{18}F-fluorodeoxyglucose (FDG). During a PET/CT the morphologic information of computed tomography and the functional information of the positron emission

tomography are integrated yielding high-resolution images of the tumor and its glucose utilization.

PET/CT scans of course do not allow determining which drug to use for a tumor, but they can help to verify if the individual tumor really is responding to a drug chosen empirically. An advantage of this technology is the possibility to recognize a possible secondary resistance to an administered drug during the course of treatment [123]

A gene test examining the status of the *BRCA1* gene might help to identify patients with a germline mutation of one *BRCA1* allele, whose carcinoma are likely to be result to an additional LOH mutation of the intact allele. Yet the determination of the *BRCA1* status in patients with no history of familial breast or ovary cancer is controversial. While a positive result might influence treatment decisions a negative result will not exclude a BRCA1 dysfunction in the actual tumor. Furthermore direct gene testing does not take into account the idea of BRCAness. Patients with no identified mutation of *BRCA1* might have a false sense of security.

Additionally, there are many ethical objections to *BRCA* genotyping as the genes and thus the test for mutations are patented in the U.S. by a revenue-orientated company (Myriad Genetics, Inc.; Salt Lake City, UT), especially as the patent owning company is targeting advertisement directly to patients. Although Myriad Genetics transferred the patents to the University of Utah (Salt Lake City, UT) the financial interest is still reflected by the fact that the cost for the BRCA1 test in the U.S. is, due to license fees, $3000 compared to a prize of $1500 for the combined test of BRCA1 and BRCA2 in laboratories in Europe, where the patent has been revoked by the European patent office.

The general ethical issue of gene patenting and direct-to-customer marketing in medicine has been discussed thoroughly and will not be commented in greater detail here [124].

4.4 Implementation of screening programs

Even with highly specific drug therapies like DNA cross-linking agents or PARP inhibitors it will probably not be possible to defeat pancreatic cancer by drug therapy alone. Thus, the primary objective will always be to identify pancreatic carcinoma and precursor lesions in early stages, when resection with tumor free margins is still possible. Unfortunately, currently only 10-15% of pancreatic carcinomas fulfill this condition at the time of diagnosis.

Recently, Picozzi and colleagues re-emphasized the importance of an early and correct diagnose of pancreatic tumors. They showed that the medium delay between first symptoms, diagnosis and start of an adequate therapy is 4 months (112 days), and that 25 of 134 patients (19%) experienced a delay of 6 months or longer. Considering the overall survival time of patients with pancreatic cancer this delay could obviously have a negative effect on treatment outcome [125].

As mentioned above, symptoms of pancreatic cancer include unspecific symptoms like abdominal pain or loss of appetite and weight, but patients are often not diagnosed until they develop more severe symptoms like jaundice or diabetes mellitus. These symptoms often only develop when the carcinoma has already grown infiltrative.

Today there is no fast, easy and non-invasive screening method for pancreatic lesions like the prostate specific antigen (PSA) test for prostate cancer or the detection of fecal occult blood for colon carcinoma.

Available screening methods like multi-slice CT or endoscopic ultrasound on the other hand are expensive and given the low incidence of pancreatic cancer a screening of the whole population with these methods is not reasonable.

Thus, it will be important to determine further risk factors. Most of the known risk factors like the consumption of red meat or smoking are abundant in the western society. Ergo a risk assessment employing these will not be useful to identify a population with an elevated risk. Thus far, the only patients eligible for clinical screening programs are members of familial pancreatic cancer kindred.

Also there appears to be a high number of pancreatic cystic neoplasms that are incidentally diagnosed when an examination of the abdomen is conducted for other reasons. The number of these will even rise further, as the number of radiological examinations of the abdominal organs is. How to proceed with these so-called

incidentalomas has been discussed in literature lately. There have been reports about “benign” incidentalomas that were left in situ and later showed malign features. Given the unfortunate prognosis of pancreatic carcinoma patients at risk should at least consider accepting the risks of a surgical excision of these incidentalomas.
This is especially true for patients with further risk factors, in particular for patients with a familial cancer background. If these patients carry a cancer predisposing mutation like a mutation of *BRCA1*, extra caution should be exercised.

5 Conclusions

Pancreatic carcinoma is still associated with an unfavorable prognosis. Although recent trials with Gemcitabine showed a significant improvement in survival and clinical performance, 1-year survival of patients with inoperable tumors is still only 18-20%.

In this work I was able to demonstrate that PDACs lacking the tumor suppressor protein BRCA1 develop with shorter latency, that concomitant loss of BRCA1 and P53 cooperate in onset and progression of PDACs, and that BRCA1-deficient pancreatic tumor cells have an increased susceptibility to treatment with DNA damaging drugs like mitomycin C or cisplatin.

Recent research suggests that these tumors might also have an increased susceptibility to additional new therapeutic agents interfering with the DNA damage repair pathway or the induction of apoptosis like PARP inhibitors or a gene therapy with *TP53*. Ultimately, it might be even possible to knock out BRCA1 function e.g. via tumor specific siRNA agents to sensitize other tumors to treatment with DNA damaging drugs.

These new treatment options are able to raise hopes of many affected patients, not only those suffering from pancreatic cancer. Results from pre-clinical and first clinical trials investigating the feasibility and effectiveness of targeted therapies promise a significant improvement of both performance and survival. It will now be of utmost importance to transfer scientific findings to hospitals and clinics and to incorporate them in treatment decisions. Therefore, patients eligible for a targeted therapy need to be identified. Naturally, the required diagnostics will further increase the costs of the already expensive care of cancer patients.

Especially in economically uncertain times, when public health service is short of funds, it will reveal itself whether patients with pancreatic or other carcinoma are already “given up on” or whether society is willing to bear the additional costs of the individualization of cancer therapy. Hopefully, in the future the patient and his attending doctor will still have the freedom to choose the individual best therapy.

Supplement

Tables

Table S1: Drug concentrations used for the drug sensitivity assay

			#1	#2	#3	#4	#5	#6
Mitomycin C	#1-8	(nM)	0.00	3.70	11.11	33.33	100.00	300.00
Cisplatin	#1-5	(µM)	0.00	0.06	0.19	0.56	1.67	5.00
Gemcitabine	#1	(nM)	0.00	12.35	37.04	111.11	333.33	1000.00
Gemcitabine	#2-4	(nM)	0.00	6.17	18.52	55.56	166.67	500.00
5-Fluorouracil	#1-5	(µM)	0.00	0.12	0.37	1.11	3.33	10.00
Paclitaxel	#1-2	(nM)	0.00	0.07	0.13	0.40	2.00	10.00
Paclitaxel	#3-6	(nM)	0.00	0.10	0.42	1.67	5.00	15.00

Table S2: Results drug sensitivity assay mitomycin C

Mitomycin C

#1	B1-10 #1		B1-10 #2		B1-10 #3		43-11 #1		43-11 #2		43-11 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	2000	0,9	1000	0,5	1000	0,4	5500	4,3	7000	3,5	7000	3,2
100	5500	2,5	3500	1,8	2000	0,8	20500	16,1	13000	6,5	2000	0,9
33,3	26500	12,0	27000	13,5	21500	8,1	62000	48,8	69000	34,3	83500	38,3
11,1	91500	41,6	117000	58,6	73500	27,8	110000	86,6	96000	47,8	157500	72,2
3,7	119000	54,1	170000	85,2	165500	62,7	142500	112,2	187500	93,3	197000	90,4
0	220000	100,0	199500	100,0	264000	100,0	127000	100,0	201000	100,0	218000	100,0

#2	B1-10 #1		B1-10 #2		B1-10 #3		41-05 #1		41-05 #2		41-05 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	3000	1,0	3500	1,0	6000	1,7	2500	1,4	3500	1,6	2000	1,1
100	35000	12,2	20000	5,6	2000	0,6	31500	17,7	45000	20,2	31500	17,7
33,3	117500	41,0	118000	33,1	146500	40,6	93500	52,5	132000	59,2	122000	68,7
11,1	194000	67,7	210500	59,1	292000	80,9	163500	91,9	190000	85,2	131000	73,8
3,7	227000	79,2	278000	78,1	346500	96,0	178000	100,0	232500	104,3	155000	87,3
0	286500	100,0	356000	100,0	361000	100,0	178000	100,0	223000	100,0	177500	100,0

#3	41-05 #1		41-05 #2		41-05 #3		B1-8 #1		B1-8 #2		B1-8 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	11000	0,4	18000	2,2	9000	0,8	500	0,2	1500	0,6	1000	0,3	1500	2,5	5500	6,4	1500	1,8
100	218500	8,9	257000	31,5	169000	15,3	6000	1,9	8000	3,3	6000	1,9	7500	12,3	13000	15,1	10500	12,7
33,3	359000	14,6	578000	70,9	536000	48,5	48500	15,6	45500	19,0	43000	13,4	20000	32,8	31500	36,6	20000	24,1
11,1	2220000	90,1	725000	89,0	975000	88,2	140000	45,2	137500	57,3	170000	53,1	44000	72,1	42000	48,8	56500	68,1
3,7	2350000	95,3	770000	94,5	1075000	97,3	237500	76,6	200000	83,3	327500	102,3	53000	86,9	53000	61,6	72000	86,7
0	2465000	100,0	815000	100,0	1105000	100,0	310000	100,0	240000	100,0	320000	100,0	61000	100,0	86000	100,0	83000	100,0

#4	43-11 #1		43-11 #2		43-11 #3		8-1 #1		8-1 #2		8-1 #3		B1-10 #1		B1-10 #2		B1-10 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	72500	9,4	72500	10,9	35000	4,9	8000	8,5	6500	8,2	7000	6,8	5000	0,6	15000	1,6	10000	0,9
100	395000	51,5	367500	55,1	395000	55,1	22500	23,8	17000	21,4	21500	20,8	90000	11,0	90000	9,4	90000	8,3
33,3	612500	79,8	572500	85,8	520000	72,5	41000	43,4	36000	45,3	46500	44,9	330000	40,2	370000	38,5	295000	27,2
11,1	697500	90,9	577500	86,5	555000	77,4	66000	69,8	58000	73,0	69000	66,7	560000	68,3	585000	60,9	570000	52,5
3,7	772500	100,7	692500	103,7	650000	90,6	87500	92,6	68000	85,5	94000	90,8	795000	97,0	865000	90,1	955000	88,0
0	767500	100,0	667500	100,0	717500	100,0	94500	100,0	79500	100,0	103500	100,0	820000	100,0	960000	100,0	1085000	100,0

#5	43-16 #1		43-16 #2		43-16 #3		43-11 #1		43-11 #2		43-11 #3		B1-8 #1		B1-8 #2		B1-8 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	10000	3,2	5000	1,8	10000	2,7	7500	2,2	7500	2,0	7500	2,0	2500	0,4	5000	1,1	1000	1,0
100	85000	27,4	50000	18,2	102500	27,9	67500	19,9	100000	26,1	80000	21,8	35000	6,1	10000	2,2	4000	3,8
33,3	200000	64,5	195000	70,9	247500	67,3	197500	58,1	207500	54,2	242500	66,0	135000	23,7	120000	27,0	15000	14,3
11,1	255000	82,3	235000	85,5	335000	91,2	287500	84,6	295000	77,1	310000	84,4	315000	55,3	275000	61,8	56000	53,3
3,7	310000	100,0	275000	100,0	365000	99,3	332500	97,8	352500	92,2	367500	100,0	510000	89,5	395000	88,8	100000	95,2
0	310000	100,0	275000	100,0	367500	100,0	340000	100,0	382500	100,0	367500	100,0	570000	100,0	445000	100,0	105000	100,0

#6	43-16 #1		43-16 #2		43-16 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	10000	1,9	40000	6,4	12500	4,3	10000	2,9	17500	4,3	10000	3,3
100	75000	14,4	110000	17,6	57500	20,0	42500	12,1	45000	11,2	37500	12,3
33,3	305000	58,7	365000	58,4	180000	62,6	117500	33,6	155000	38,5	130000	42,6
11,1	425000	81,7	540000	86,4	235000	81,7	190000	54,3	260000	64,6	202500	66,4
3,7	515000	99,0	605000	96,8	277500	96,5	327500	93,6	377500	93,8	275000	90,2
0	520000	100,0	625000	100,0	287500	100,0	350000	100,0	402500	100,0	305000	100,0

#7-1	43-16 #1		43-16 #2		43-16 #3		41-05 #1		41-05 #2		41-05 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	1500	3,0	500	0,9	500	1,0	10000	1,0	17500	1,8	22500	2,3
100	9000	17,8	8500	14,7	7000	13,5	197500	19,6	267500	28,1	255000	25,5
33,3	37500	74,3	39000	67,2	38000	73,1	797500	79,0	802500	84,3	837500	83,8
11,1	43500	86,1	53500	92,2	46000	88,5	922500	91,3	947500	99,5	905000	90,5
3,7	46000	91,1	56000	96,6	52500	101,0	967500	95,8	947500	99,5	972500	97,3
0	50500	100,0	58000	100,0	52000	100,0	1010000	100,0	952500	100,0	1000000	100,0

#7-2	B1-8 #1		B1-8 #2		B1-8 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	10000	1,7	5000	1,0	5000	1,0	1500	0,7	1500	1,1	1500	0,9
100	30000	5,0	45000	8,7	25000	4,8	16500	8,0	22500	16,7	19500	11,3
33,3	170000	28,3	165000	31,7	150000	28,8	64500	31,4	55500	41,1	43500	25,2
11,1	450000	75,0	450000	86,5	370000	71,2	180000	87,6	120000	88,9	114000	66,1
3,7	550000	91,7	470000	90,4	470000	90,4	202500	98,5	130500	96,7	159000	92,2
0	600000	100,0	520000	100,0	520000	100,0	205500	100,0	135000	100,0	172500	100,0

#8	B1-10 #1		B1-10 #2		B1-10 #3		43-11 #1		43-11 #2		43-11 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
300	0	0,0	0	0,0	1500	0,7	500	0,4	500	0,4	0	0,0
100	6000	2,1	9000	3,8	6000	2,7	5000	3,5	6500	4,8	6000	3,8
33,3	87000	30,5	43500	18,6	34500	15,5	51000	36,0	46000	33,7	52500	32,8
11,1	196500	68,9	108000	46,2	118500	53,4	121000	85,5	117000	85,7	133000	83,1
3,7	280500	98,4	210000	89,7	199500	89,9	136000	96,1	125000	91,6	143500	89,7
0	285000	100,0	234000	100,0	222000	100,0	141500	100,0	136500	100,0	160000	100,0

Table S3: Variance analysis of half maximal inhibitory concentration after treatment with mitomycin C

Mitomycin C							
#	**test**	**vs.**	**Mean Δ**	**p**	**q**	**P <0.01**	**99% CI of Δ**
#1	t-test	43-11 vs. B1-10		0.027		yes	
#2	t-test	41-05 vs. B1-10		0.004		yes	
#3	ANOVA	41-05 vs. B1-8	22.30	0.0864	3.66	No	-16.31 to 60.92
		41-05 vs. 8-1	18.19	0.0864	2.98	No	-20.43 to 56.80
		B1-8 vs. 8-1	-4.12	0.0864	0.67	No	-42.73 to 34.50
#4	ANOVA	43-11 vs. 8-1	61.23	< 0.0001	26.49	Yes	46.60 to 75.86
		43-11 vs. B1-10	70.79	< 0.0001	30.63	Yes	56.16 to 85.43
		8-1 vs. B1-10	9.56	< 0.0001	4.14	No	-5.07 to 24.20
#5	ANOVA	43-16 vs. 43-11	6.47	< 0.0001	4.65	No	-2.34 to 15.28
		43-16 vs. B1-8	30.47	< 0.0001	21.90	Yes	21.66 to 39.28
		43-11 vs. B1-8	24.00	< 0.0001	17.25	Yes	15.19 to 32.81
#6	t-test	43-16 vs. 8-1		0.002		yes	
#7	ANOVA	43-16 vs. 41-05	-12.38	< 0.0001	5.03	No	-27.66 to 2.89
		43-16 vs. B1-8	22.64	< 0.0001	9.20	Yes	7.37 to 37.91
		43-16 vs. 8-1	19.91	< 0.0001	8.09	Yes	4.64 to 35.19
		41-05 vs. B1-8	35.02	< 0.0001	14.23	Yes	19.75 to 50.30
		41-05 vs. 8-1	32.30	< 0.0001	13.12	Yes	17.02 to 47.57
		B1-8 vs. 8-1	-2.73	< 0.0001	1.11	No	-18.00 to 12.55
#8	t-test	43-11 vs. B1-10		0.0146		Yes	

Table S4: Results drug sensitivity assay cisplatin

Cisplatin

#1	41-05 #1		41-05 #2		41-05 #3		B1-10 #1		B1-10 #2		B1-10 #3		8-1 #1		8-1 #2		8-1 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	20000	3,8	32500	3,0	17500	1,8	5000	0,5	10000	1,0	10000	1,0	1000	0,5	2000	0,9	1000	0,5
1,7	120000	22,6	142500	13,1	130000	13,6	10000	1,0	20000	2,0	25000	2,4	2000	1,1	3000	1,3	5000	2,5
0,6	325000	61,3	667500	61,4	560000	58,5	235000	23,5	235000	22,9	235000	22,9	31000	16,6	29000	12,4	36000	17,9
0,2	475000	89,6	1007500	92,6	962500	100,5	640000	64,0	715000	69,8	765000	74,6	79000	42,2	86000	36,8	92000	45,8
0,1	565000	106,6	1047500	96,3	897500	93,7	1045000	104,5	955000	93,2	965000	94,1	183000	97,9	203000	86,8	154000	76,6
0,0	530000	100,0	1087500	100,0	957500	100,0	1000000	100,0	1025000	100,0	1025000	100,0	187000	100,0	234000	100,0	201000	100,0

#2	43-16 #1		43-16 #2		43-16 #3		B1-8 #1		B1-8 #2		B1-8 #3		B1-10 #1		B1-10 #2		B1-10 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	5000	0,9	2500	0,9	2500	0,9	5000	1,5	2500	0,7	2500	0,7	1000	0,5	3000	1,6	2000	1,3
1,7	60000	10,3	25000	8,7	15000	5,3	22500	6,6	15000	4,0	12500	3,4	19000	9,4	20000	10,4	9000	5,6
0,6	260000	44,8	135000	47,0	175000	61,4	100000	29,2	82500	22,1	97500	26,9	69000	34,0	64000	33,3	54000	33,8
0,2	495000	85,3	265000	92,2	277500	97,4	210000	61,3	175000	47,0	210000	57,9	137000	67,5	103000	53,6	132000	82,5
0,1	565000	97,4	277500	96,5	292500	102,6	252500	73,7	345000	92,6	350000	96,6	187000	92,1	178000	92,7	146000	91,3
0,0	580000	100,0	287500	100,0	285000	100,0	342500	100,0	372500	100,0	362500	100,0	203000	100,0	192000	100,0	160000	100,0

#3	43-11 #1		43-11 #2		43-11 #3		8-1 #1		8-1 #2		8-1 #3		B1-8 #1		B1-8 #2		B1-8 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	5000	0,6	0	0,0	2500	0,6	2500	0,6	5000	1,3	2500	0,5	1000	0,5	3000	1,3	2000	1,0
1,7	90000	10,9	35000	10,5	42500	10,7	27500	6,3	35000	8,9	20000	4,1	8000	3,8	12000	5,1	14000	7,2
0,6	425000	51,5	215000	64,7	207500	52,2	140000	32,2	115000	29,3	125000	25,9	64000	30,2	57000	24,4	62000	31,8
0,2	755000	91,5	335000	100,8	337500	84,9	322500	74,1	257500	65,6	380000	78,8	146000	68,9	176000	75,2	155000	79,5
0,1	790000	95,8	325000	97,7	385000	96,9	387500	89,1	310000	79,0	440000	91,2	212000	100,0	218000	93,2	183000	93,8
0,0	825000	100,0	332500	100,0	397500	100,0	435000	100,0	392500	100,0	482500	100,0	212000	100,0	234000	100,0	195000	100,0

#4	43-11 #1		43-11 #2		43-11 #3		B1-10 #1		B1-10 #2		B1-10 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	15000	2,2	2500	0,6	2500	0,7	2500	0,5	2500	0,6	2500	0,6
1,7	95000	13,7	70000	16,8	50000	13,6	40000	8,6	30000	7,1	27500	6,4
0,6	390000	56,1	227500	54,5	220000	59,9	130000	28,1	122500	29,2	110000	25,6
0,2	605000	87,1	390000	93,4	332500	90,5	252500	54,6	207500	49,4	280000	65,1
0,1	670000	96,4	455000	109,0	362500	98,6	435000	94,1	297500	70,8	345000	80,2
0,0	695000	100,0	417500	100,0	367500	100,0	462500	100,0	420000	100,0	430000	100,0

#5-1	41-05 #1		41-05 #2		41-05 #3		43-16 #1		43-16 #2		43-16 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	25000	5,7	30000	7,2	22500	5,4	2500	1,3	9000	3,6	6000	2,8
1,7	192500	44,3	197500	47,3	117500	28,1	70000	36,4	64000	25,9	63000	29,4
0,6	325000	74,7	297500	71,3	337500	80,8	122500	63,6	163000	66,0	156000	72,9
0,2	400000	92,0	337500	80,8	392500	94,0	165000	85,7	242000	98,0	219000	102,3
0,1	445000	102,3	380000	91,0	412500	98,8	175000	90,9	249000	100,8	219000	102,3
0,0	435000	100,0	417500	100,0	417500	100,0	192500	100,0	247000	100,0	214000	100,0

#5-2	8-1 #1		8-1 #2		8-1 #3		B1-8 #1		B1-8 #2		B1-8 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
5,0	7500	3,0	5000	1,9	1000	0,4	5000	1,4	5000	1,5	2500	0,6
1,7	50000	20,0	30000	11,2	22000	8,2	23000	6,3	37500	11,4	42500	10,7
0,6	102500	41,0	100000	37,4	120000	44,8	154000	42,4	90000	27,3	112500	28,3
0,2	180000	72,0	177500	66,4	185000	69,0	236000	65,0	207500	62,9	247500	62,3
0,1	247500	99,0	257500	96,3	263000	98,1	310000	85,4	300000	90,9	342500	86,2
0,0	250000	100,0	267500	100,0	268000	100,0	363000	100,0	330000	100,0	397500	100,0

Table S5: Variance analysis of half maximal inhibitory concentration after treatment with cisplatin

Cisplatin							
#	**test**	**vs.**	**Mean Δ**	**p**	**q**	**P < 0.01**	**99% CI of Δ**
#1	ANOVA	41-05 vs. 8-1	0.4344	<0.0001	23.90	Yes	0.319 to 0.550
		41-05 vs. B1-10	0.5602	<0.0001	30.82	Yes	0.445 to 0.675
		8-1 vs. B1-10	0.1258	<0.0001	6.92	Yes	0.011 to 0.241
#2	ANOVA	43-16 vs. B1-8	0.2605	0.0016	9.33	Yes	0.084 to 0.437
		43-16 vs. B1-10	0.1808	0.0016	6.47	Yes	0.004 to 0.358
		B1-8 vs. B1-10	-0.0797	0.0016	2.85	No	-0.257 to 0.097
#3	ANOVA	43-11 vs. 8-1	0.2425	0.0012	8.71	Yes	0.066 to 0.419
		43-11 vs. B1-8	0.2397	0.0012	8.61	Yes	0.063 to 0.416
		8-1 vs. B1-8	-0.0027	0.0012	0.10	No	-0.179 to 0.174
#4	t-test	43-11 vs. B1-10		<0.0001		Yes	
#5	ANOVA	41-05 vs. 8-1	0.7447	<0.0001	14.96	Yes	0.298 to 1.192
		41-05 vs. B1-8	0.8205	<0.0001	16.48	Yes	0.374 to 1.267
		43-16 vs. 8-1	0.4832	<0.0001	9.71	Yes	0.037 to 0.930
		43-16 vs. B1-8	0.5590	<0.0001	11.23	Yes	0.112 to 1.006
		41-05 vs. 43-16	0.2614	<0.0001	5.25	No	-0.186 to 0.708
		8-1 vs. B1-8	0.0758	<0.0001	1.52	No	-0.371 to 0.523

Table S6: Results drug sensitivity assay gemcitabine

Gemcitabine

#1	43-11 #1		43-11 #2		43-11 #3		B1-10 #1		B1-10 #2		B1-10 #3		B1-8 #1		B1-8 #2		B1-8 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
1000	3000	0,8	3000	0,7	3000	0,7	0	0,0	0	0,0	0	0,0	0	0,0	3000	1,4	3000	1,4
333	15000	3,8	9000	2,1	3000	0,7	3000	0,7	3000	0,7	3000	0,9	0	0,0	3000	1,4	9000	4,2
111	54000	13,7	63000	14,4	75000	16,8	9000	2,2	9000	2,2	15000	4,3	27000	10,2	27000	12,5	45000	20,8
37,0	198000	50,4	183000	41,8	252000	56,4	93000	22,3	93000	22,3	111000	31,6	156000	59,1	150000	69,4	123000	56,9
12,3	420000	106,9	417000	95,2	459000	102,7	321000	77,0	321000	77,0	366000	104,3	213000	80,7	219000	101,4	198000	91,7
0,01	393000	100,0	438000	100,0	447000	100,0	417000	100,0	417000	100,0	351000	100,0	264000	100,0	216000	100,0	216000	100,0

#2	43-16 #1		43-16 #2		43-16 #3		B1-8 #1		B1-8 #2		B1-8 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
500	3000	0,9	3000	0,9	0	0,0	0	0,0	6000	2,4	3000	1,1	12000	3,8	18000	6,4	21000	6,0
167	18000	5,3	12000	3,6	12000	4,0	15000	6,2	12000	4,8	18000	6,7	84000	26,4	63000	22,3	69000	19,8
55,6	87000	25,7	72000	21,6	93000	31,0	78000	32,1	57000	22,9	87000	32,6	144000	45,3	135000	47,9	156000	44,8
18,5	243000	71,7	237000	71,2	243000	81,0	135000	55,6	189000	75,9	192000	71,9	267000	84,0	243000	86,2	252000	72,4
6,2	333000	98,2	336000	100,9	285000	95,0	231000	95,1	258000	103,6	261000	97,8	294000	92,5	264000	93,6	315000	90,5
0,1	339000	100,0	333000	100,0	300000	100,0	243000	100,0	249000	100,0	267000	100,0	318000	100,0	282000	100,0	348000	100,0

#3-1	41-05 #1		41-05 #2		41-05 #3		43-16 #1		43-16 #2		43-16 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
500	3000	0,5	6000	0,7	6000	0,6	0	0,0	6000	1,1	3000	0,5
167	135000	23,8	105000	12,3	168000	16,9	27000	5,3	51000	9,3	27000	4,5
55,6	336000	59,3	468000	54,7	513000	51,7	234000	45,6	243000	44,5	237000	39,3
18,5	492000	86,8	642000	75,1	843000	84,9	267000	52,0	468000	85,7	357000	59,2
6,2	585000	103,2	753000	88,1	1008000	101,5	420000	81,9	597000	109,3	522000	86,6
0,1	567000	100,0	855000	100,0	993000	100,0	513000	100,0	546000	100,0	603000	100,0

#3-2	B1-8 #1		B1-8 #2		B1-8 #3		B1-10 #1		B1-10 #2		B1-10 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
500	0	0,0	6000	0,8	3000	0,3	9000	1,6	3000	0,5	3000	0,5
167	3000	0,4	15000	2,1	6000	0,7	57000	10,4	45000	8,2	63000	11,5
55,6	141000	20,7	219000	30,7	312000	34,9	300000	54,9	279000	50,8	288000	52,5
18,5	483000	70,9	522000	73,1	672000	75,2	408000	74,7	456000	83,1	465000	84,7
6,2	462000	67,8	636000	89,1	906000	101,3	498000	91,2	546000	99,5	519000	94,5
0,1	681000	100,0	714000	100,0	894000	100,0	546000	100,0	549000	100,0	549000	100,0

#4-1	43-11 #1		43-11 #2		43-11 #3		41-05 #1		41-05 #2		41-05 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
500	1200	0,4	600	0,2	1200	0,4	3000	0,8	0	0,0	3000	0,9
167	7800	2,5	4800	1,4	10200	3,1	9000	2,5	9000	2,4	6000	1,9
55,6	90600	28,8	99000	28,4	94200	28,4	150000	42,4	138000	36,2	84000	25,9
18,5	235200	74,7	230400	66,2	230400	69,4	285000	80,5	300000	78,7	255000	78,7
6,2	308400	97,9	336000	96,6	324600	97,8	348000	98,3	339000	89,0	300000	92,6
0,1	315000	100,0	348000	100,0	331800	100,0	354000	100,0	381000	100,0	324000	100,0

#4-2	B1-10 #1		B1-10 #2		B1-10 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
500	3000	0,4	3000	0,4	6000	0,8	3000	0,6	6000	1,3	3000	0,6
167	18000	2,4	21000	2,7	27000	3,5	21000	4,2	48000	10,3	69000	13,0
55,6	216000	28,9	186000	24,1	213000	27,4	129000	25,9	99000	21,3	144000	27,1
18,5	660000	88,4	627000	81,3	645000	83,0	345000	69,3	369000	79,4	357000	67,2
6,2	738000	98,8	744000	96,5	771000	99,2	483000	97,0	459000	98,7	486000	91,5
0,1	747000	100,0	771000	100,0	777000	100,0	498000	100,0	465000	100,0	531000	100,0

Table S7: Variance analysis of half maximal inhibitory concentration after treatment with gemcitabine

Gemcitabine							
#	**test**	**vs.**	**Mean Δ**	**p**	**q**	**P < 0.01**	**99% CI of Δ**
#1	ANOVA	43-11 vs. B1-10	0.0136	0.0052	5.37	No	-0.002 to 0.030
		43-11 vs. B1-8	-0.0049	0.0052	1.93	No	-0.021 to 0.011
		B1-10 vs. B1-8	-0.0185	0.0052	7.31	Yes	-0.035 to -0.002
#2	ANOVA	43-16 vs. B1-8	0.0005	0.0006	0.21	No	-0.013 to 0.015
		43-16 vs. 8-1	-0.0217	0.0006	9.73	Yes	-0.036 to -0.008
		B1-8 vs. 8-1	-0.0222	0.0006	9.95	Yes	-0.036 to -0.008
#3	ANOVA	41-05 vs. 43-16	0.0204	0.0034	5.62	No	-0.002 to 0.043
		41-05 vs. B1-8	0.0259	0.0034	7.14	Yes	0.003 to 0.049
		41-05 vs. B1-10	0.0068	0.0034	1.87	No	-0.016 to 0.029
		43-16 vs. B1-8	0.0055	0.0034	1.52	No	-0.017 to 0.028
		43-16 vs. B1-10	-0.0136	0.0034	3.75	No	-0.036 to 0.009
		B1-8 vs. B1-10	-0.0192	0.0034	5.27	No	-0.042 to 0.003
#4	ANOVA	43-11 vs. 41-05	-0.0050	0.1038	3.56	No	-0.014 to 0.004
		43-11 vs. B1-10	-0.0035	0.1038	2.49	No	-0.012 to 0.005
		43-11 vs. 8-1	-0.0006	0.1038	0.41	No	-0.009 to 0.008
		41-05 vs. B1-10	0.0015	0.1038	1.07	No	-0.007 to 0.010
		41-05 vs. 8-1	0.0045	0.1038	3.15	No	-0.004 to 0.013
		B1-10 vs. 8-1	0.0030	0.1038	2.08	No	-0.006 to 0.012

Table S8: Results drug sensitivity assay 5-fluorouracil

5-Fluorouracil

#1	B1-10 #1		B1-10 #2		B1-10 #3		B1-8 #1		B1-8 #2		B1-8 #3		43-16 #1		43-16 #2		43-16 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	2500	0,7	2500	0,7	0	0,0	2500	0,7	2500	0,8	2500	0,8	7500	1,2	2500	0,4	2500	0,4
3,33	7500	2,0	5000	1,3	2500	0,6	10000	3,0	7500	2,5	12500	3,9	20000	3,2	20000	3,4	40000	5,9
1,11	87500	23,8	70000	18,5	85000	21,1	87500	26,1	122500	40,5	122500	38,6	237500	37,8	240000	40,5	250000	36,8
0,37	250000	68,0	242500	64,2	222500	55,3	235000	70,1	212500	70,2	260000	81,9	447500	71,3	517500	87,3	585000	86,0
0,12	365000	99,3	325000	86,1	395000	98,1	282500	84,3	307500	101,7	307500	96,9	550000	87,6	560000	94,5	627500	92,3
0,01	367500	100,0	377500	100,0	402500	100,0	335000	100,0	302500	100,0	317500	100,0	627500	100,0	592500	100,0	680000	100,0

#2	41-05 #1		41-05 #2		41-05 #3		43-16 #1		43-16 #2		43-16 #3		B1-10 #1		B1-10 #2		B1-10 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	0	0,0	6000	0,6	6000	0,6	6000	0,6	6000	0,6	6000	0,6	1800	0,5	3600	1,0	3600	0,9
3,33	12000	1,2	18000	1,8	12000	1,2	12000	1,2	12000	1,2	6000	0,6	28800	7,2	37800	10,7	36000	9,3
1,11	42000	4,1	36000	3,6	42000	4,2	78000	7,9	78000	7,6	156000	15,9	97200	24,3	109800	31,0	111600	28,8
0,37	312000	30,2	480000	47,9	444000	44,3	444000	44,8	492000	48,0	528000	53,7	199800	50,0	225000	63,5	216000	55,8
0,12	912000	88,4	858000	85,6	966000	96,4	978000	98,8	888000	86,5	1020000	103,7	336600	84,2	336600	94,9	369000	95,3
0,01	1032000	100,0	1002000	100,0	1002000	100,0	990000	100,0	1026000	100,0	984000	100,0	399600	100,0	354600	100,0	387000	100,0

#3	41-05 #1		41-05 #2		41-05 #3		43-11 #1		43-11 #2		43-11 #3		8-1 #1		8-1 #2		8-1 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	0	0,0	0	0,0	0	0,0	0	0,0	3000	0,7	3000	0,6	3000	2,2	0	0,0	3000	1,6
3,33	2400	0,7	4800	1,4	2400	1,1	3000	0,5	3000	0,7	6000	1,3	9000	6,7	3000	2,2	15000	7,8
1,11	24000	6,9	21600	6,1	16800	7,8	18000	2,9	30000	6,7	18000	3,8	24000	17,8	30000	21,7	33000	17,2
0,37	139200	40,0	148800	41,9	93600	43,3	294000	47,1	129000	28,7	255000	53,8	72000	53,3	90000	65,2	87000	45,3
0,12	328800	94,5	338400	95,3	196800	91,1	540000	86,5	336000	74,7	390000	82,3	111000	82,2	120000	87,0	165000	85,9
0,01	348000	100,0	355200	100,0	216000	100,0	624000	100,0	450000	100,0	474000	100,0	135000	100,0	138000	100,0	192000	100,0

#4-1	B1-8 #1		B1-8 #2		B1-8 #3		43-16 #1		43-16 #2		43-16 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	3000	1,4	3000	1,3	0	0,0	0	0,0	2400	1,6	1200	1,0
3,33	3000	1,4	3000	1,3	12000	4,8	14400	11,3	7200	4,8	15600	12,5
1,11	108000	49,3	81000	34,6	57000	22,6	63600	50,0	42000	28,2	44400	35,6
0,37	129000	58,9	114000	48,7	162000	64,3	94800	74,5	99600	66,9	96000	76,9
0,12	180000	82,2	189000	80,8	267000	106,0	124800	98,1	152400	102,4	115200	92,3
0,01	219000	100,0	234000	100,0	252000	100,0	127200	100,0	148800	100,0	124800	100,0

#4-2	B1-10 #1		B1-10 #2		B1-10 #3		43-11 #1		43-11 #2		43-11 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	32400	7,5	37800	9,3	21600	6,1	30000	7,8	24000	6,3	18000	4,2
3,33	106200	24,6	88200	21,6	108000	30,5	66000	17,2	36000	9,4	48000	11,1
1,11	226800	52,5	207000	50,7	190800	53,8	174000	45,3	177000	46,1	147000	34,0
0,37	318600	73,8	320400	78,4	271800	76,6	276000	71,9	312000	81,3	351000	81,3
0,12	388800	90,0	412200	100,9	313200	88,3	342000	89,1	402000	104,7	426000	98,6
0,01	432000	100,0	408600	100,0	354600	100,0	384000	100,0	384000	100,0	432000	100,0

#5-1	43-16 #1		43-16 #2		43-16 #3		41-05 #1		41-05 #2		41-05 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	500	0,3	1000	0,5	500	0,3	2500	0,3	2500	0,3	5000	0,5
3,33	5500	3,0	10000	4,9	12000	6,2	77500	9,2	122500	14,5	117500	12,3
1,11	79000	43,5	106000	52,1	82500	42,9	352500	41,8	365000	43,2	380000	39,9
0,37	137500	75,8	199500	98,0	126500	65,7	662500	78,6	630000	74,6	645000	67,7
0,12	177000	97,5	199500	98,0	183500	95,3	865000	102,7	810000	95,9	875000	91,9
0,01	181500	100,0	203500	100,0	192500	100,0	842500	100,0	845000	100,0	952500	100,0

#5-2	8-1 #1		8-1 #2		8-1 #3		B1-8 #1		B1-8 #2		B1-8 #3	
µM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	2000	2,0	1500	1,5	2000	2,4	12500	2,4	15000	3,3	12500	3,1
3,33	21500	21,7	19500	19,0	15000	17,6	85000	16,2	47500	10,5	45000	11,1
1,11	45500	46,0	54500	53,2	50000	58,8	265000	50,5	257500	56,9	222500	54,9
0,37	75500	76,3	82000	80,0	69000	81,2	405000	77,1	400000	88,4	347500	85,8
0,12	86500	87,4	94000	91,7	83500	98,2	512500	97,6	467500	103,3	395000	97,5
0,01	99000	100,0	102500	100,0	85000	100,0	525000	100,0	452500	100,0	405000	100,0

Table S9: Variance analysis of half maximal inhibitory concentration after treatment with 5-fluorouracil

5-Fluorouracil							
#	**test**	**vs.**	**Mean Δ**	**p**	**q**	**P < 0.01**	**99% CI of Δ**
#1	ANOVA	43-16 vs. B1-8	0.0884	0.0130	2.02	No	-0.189 to 0.366
		43-16 vs. B1-10	0.2685	0.0130	6.13	No	-0.008 to 0.546
		B1-8 vs. B1-10	0.1801	0.0130	4.11	No	-0.097 to 0.457
#2	ANOVA	41-05 vs. 43-16	-0.0596	0.0154	1.77	No	-0.273 to 0.153
		41-05 vs. B1-10	-0.1973	0.0154	5.87	No	-0.410 to 0.016
		43-16 vs. B1-10	-0.1377	0.0154	4.09	No	-0.351 to 0.075
#3	ANOVA	41-05 vs. 43-11	0.0243	0.0455	0.88	No	-0.150 to 0.199
		41-05 vs. 8-1	-0.0969	0.0455	3.51	No	-0.272 to 0.078
		43-11 vs. 8-1	-0.1212	0.0455	4.39	No	-0.296 to 0.053
#4	ANOVA	43-11 vs. 43-16	0.1217	0.0028	1.51	No	-0.378 to 0.622
		43-11 vs. B1-10	-0.3671	0.0028	4.55	No	-0.867 to 0.133
		43-11 vs. B1-8	0.2752	0.0028	3.41	No	-0.225 to 0.775
		43-16 vs. B1-10	-0.4888	0.0028	6.06	No	-0.989 to 0.011
		43-16 vs. B1-8	0.1535	0.0028	1.90	No	-0.347 to 0.654
		B1-10 vs. B1-8	0.6423	0.0028	7.97	Yes	0.142 to 1.142
#5	ANOVA	43-16 vs. 41-05	0.0392	0.0149	0.66	No	-0.329 to 0.408
		43-16 vs. 8-1	-0.2662	0.0149	4.48	No	-0.635 to 0.102
		43-16 vs. B1-8	-0.2147	0.0149	3.61	No	-0.583 to 0.154
		41-05 vs. 8-1	-0.3054	0.0149	5.14	No	-0.674 to 0.063
		41-05 vs. B1-8	-0.2539	0.0149	4.27	No	-0.622 to 0.115
		8-1 vs. B1-8	0.0515	0.0149	0.87	No	-0.317 to 0.420

Table S10: Results drug sensitivity assay paclitaxel

Paclitaxel

#1	43-11 #1		43-11 #2		43-11 #3		B1-10 #1		B1-10 #2		B1-10 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	25000	2,7	20000	2,2	40000	8,2	12500	1,4	15000	1,5	25000	2,4	125000	29,5	110000	17,7	180000	32,5
2,00	315000	34,6	365000	41,0	235000	48,5	365000	39,9	400000	39,0	335000	32,2	375000	88,6	545000	56,9	575000	61,6
0,40	575000	63,2	560000	62,9	350000	72,2	745000	81,4	765000	74,6	775000	74,5	575000	87,1	750000	71,8	690000	68,0
0,13	925000	101,6	735000	82,6	500000	103,1	930000	101,6	965000	94,1	1040000	100,0	705000	106,8	915000	87,6	975000	96,1
0,07	925000	101,6	900000	101,1	475000	97,9	902500	98,6	1010000	98,5	1045000	100,5	705000	106,8	1010000	96,7	915000	90,1
0,00	910000	100,0	890000	100,0	485000	100,0	915000	100,0	1025000	100,0	1040000	100,0	660000	100,0	1045000	100,0	1015000	100,0

#2	41-05 #1		41-05 #2		41-05 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
10,00	47500	6,6	40000	2,9	15000	1,9	25000	7,7	10000	2,8	2500	0,8
2,00	415000	57,2	565000	40,6	335000	42,9	157500	48,5	175000	49,3	167500	50,8
0,40	452500	62,4	870000	62,6	495000	63,5	252500	77,7	250000	70,4	227500	68,9
0,13	730000	100,7	1295000	93,2	710000	91,0	325000	100,0	317500	89,4	322500	97,7
0,07	695000	95,9	1200000	86,3	695000	89,1	315000	96,9	347500	97,9	320000	97,0
0,00	725000	100,0	1390000	100,0	780000	100,0	325000	100,0	355000	100,0	330000	100,0

#3	41-05 #1		41-05 #2		41-05 #3		B1-10 #1		B1-10 #1		B1-10 #3		8-1 #1		8-1 #2		8-1 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
15,00	17500	1,9	7500	0,8	2500	0,3	7500	1,4	5000	0,7	10000	2,3	2500	1,3	5000	2,0	1000	0,2
5,00	105000	11,3	117500	12,9	120000	12,0	140000	25,7	127500	18,1	175000	40,0	60000	31,6	32500	13,3	97000	17,9
1,67	582500	62,6	542500	59,8	630000	63,2	375000	68,8	490000	69,5	345000	78,9	152500	80,3	152500	62,2	353000	65,1
0,42	795000	85,5	765000	84,3	897500	90,0	530000	97,2	630000	89,4	440000	100,6	177500	93,4	217500	88,8	510000	94,1
0,10	872500	93,8	832500	91,7	1005000	100,8	575000	105,5	637500	90,4	425000	97,1	185000	97,4	237500	96,9	537000	99,1
0,00	930000	100,0	907500	100,0	997500	100,0	545000	100,0	705000	100,0	437500	100,0	190000	100,0	245000	100,0	542000	100,0

#4-1	43-16 #1		43-16 #2		43-16 #3		43-11 #1		43-11 #2		43-11 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
15,00	57500	10,4	62500	11,1	70000	12,0	12500	1,4	15000	1,5	15000	1,6
5,00	222500	40,3	212500	37,8	210000	36,1	120000	13,8	182500	17,7	150000	15,6
1,67	372500	67,4	360000	64,0	342500	58,8	397500	45,8	415000	40,3	372500	38,7
0,42	497500	90,0	530000	94,2	495000	85,0	875000	100,9	927500	90,0	862500	89,6
0,10	495000	89,6	552500	98,2	532500	91,4	875000	100,9	972500	94,4	930000	96,6
0,00	552500	100,0	562500	100,0	582500	100,0	867500	100,0	1030000	100,0	962500	100,0

#4-2	8-1 #1		8-1 #2		8-1 #3		B1-10 #1		B1-10 #2		B1-10 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
15,00	3000	1,0	2000	0,8	3000	1,1	3000	1,0	1000	0,3	1000	0,3
5,00	39000	13,6	33000	13,1	49000	17,3	24000	7,8	18000	4,5	32000	9,5
1,67	134000	46,9	147000	58,6	162000	57,0	155000	50,2	173000	43,6	138000	40,9
0,42	278000	97,2	243000	96,8	251000	88,4	293000	94,8	365000	91,9	351000	104,2
0,10	286000	100,0	246000	98,0	236000	83,1	288000	93,2	372000	93,7	343000	101,8
0,00	286000	100,0	251000	100,0	284000	100,0	309000	100,0	397000	100,0	337000	100,0

#5	43-16 #1		43-16 #2		43-16 #3		B1-8 #1		B1-8 #2		B1-8 #3		B1-10 #1		B1-10 #2		B1-10 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
15,00	297500	24,6	165000	13,8	175000	13,3	137500	23,3	72500	11,6	135000	21,2	120000	4,2	210000	8,9	157500	10,4
5,00	610000	50,5	562500	47,0	430000	32,6	312500	53,0	347500	55,4	330000	51,8	967500	34,1	1035000	43,7	660000	43,8
1,67	805000	66,7	752500	62,8	760000	57,6	452500	76,7	495000	78,9	477500	74,9	2445000	86,2	1980000	83,5	1245000	82,6
0,42	925000	76,6	982500	82,0	970000	73,5	575000	97,5	592500	94,4	495000	77,6	2805000	98,9	2160000	91,1	1500000	99,5
0,10	1087500	90,1	940000	78,5	1195000	90,5	565000	95,8	605000	96,4	612500	96,1	2737500	96,6	2295000	96,8	1560000	103,5
0,00	1207500	100,0	1197500	100,0	1320000	100,0	590000	100,0	627500	100,0	637500	100,0	2835000	100,0	2370000	100,0	1507500	100,0

#6	43-11 #1		43-11 #2		43-11 #3		41-05 #1		41-05 #2		41-05 #3		B1-8 #1		B1-8 #2		B1-8 #3	
nM	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%	cells/well	%
15,00	5000	1,1	5000	0,9	2500	0,6	7500	0,9	7500	0,9	2500	0,4	70000	10,5	50000	8,5	47500	5,5
5,00	57500	12,6	55000	10,0	47500	11,5	72500	8,4	72500	8,8	65000	9,5	225000	33,7	210000	35,9	287500	33,2
1,67	220000	48,4	177500	32,3	142500	34,5	490000	56,8	420000	51,1	377500	54,9	322500	48,3	380000	65,0	510000	59,0
0,42	340000	74,7	382500	69,5	362500	87,9	705000	81,7	750000	91,2	632500	92,0	560000	83,9	502500	85,9	732500	84,7
0,10	455000	100,0	547500	99,5	410000	99,4	810000	93,9	780000	94,8	645000	93,8	645000	96,6	535000	91,5	887500	102,6
0,00	455000	100,0	550000	100,0	412500	100,0	862500	100,0	822500	100,0	687500	100,0	667500	100,0	585000	100,0	865000	100,0

Table S11: Variance analysis of half maximal inhibitory concentration after treatment with paclitaxel

Paclitaxel							
#	**Test**	**vs.**	**Mean Δ**	**p**	**q**	**P < 0.01**	**99% CI of Δ**
#1	ANOVA	43-11 vs. B1-10	0.0029	0.0062	0.02	No	-0.910 to 0.916
		43-11 vs. 8-1	-0.9109	0.0062	6.32	No	-1.824 to 0.002
		B1-10 vs. 8-1	-0.9139	0.0062	6.34	Yes	-1.827 to -0.001
#2	t-test	41-05 vs. 8-1		0.3512		No	
#3	ANOVA	41-05 vs. B1-10	-0.8680	0.2405	2.66	No	-2.934 to 1.198
		41-05 vs. 8-1	-0.5667	0.2405	1.74	No	-2.633 to 1.499
		B1-10 vs. 8-1	0.3013	0.2405	0.92	No	-1.765 to 2.367
#4	ANOVA	43-11 vs. 8-1	-0.3237	0.0507	3.94	No	-0.843 to 0.196
		43-11 vs. B1-10	-0.0050	0.0507	0.06	No	-0.525 to 0.515
		8-1 vs. B1-10	0.3187	0.0507	3.88	No	-0.201 to 0.838
#5	ANOVA	43-16 vs. B1-8	-1.7990	0.0454	4.48	No	-4.345 to 0.746
		43-16 vs. B1-10	-0.4593	0.0454	1.14	No	-3.005 to 2.086
		B1-8 vs. B1-10	1.3400	0.0454	3.33	No	-1.205 to 3.885
#6	ANOVA	43-11 vs. B1-10	-0.4329	0.0120	2.16	No	-1.701 to 0.836
		43-11 vs. 8-1	-1.2540	0.0120	6.26	No	-2.522 to 0.015
		B1-10 vs. 8-1	-0.8207	0.0120	4.10	No	-2.089 to 0.448

S12: Representative karyotypes of cell lines 43-11, 43-16, 8-1 and B1-10
(Recurrent chromosomal aberrations are shown in italics)

43-11:
68-79, (4N), XXXX, *dic(X;14)*, dic(X), -2, *der(2)t(2;12)*, -4, del(4), *dic(5)*, der(6)dup(6)t(6;15) x2, *dic(9;10), del(9) x2, del(10) x2*, -11, *dic(11)*, -12, del(15), der(15)t(15;17), der(16)t(5;16), -16, *cen(17) x2*, -17, -18 x2, -19 [cp10]

43-16:
60-69 (3n) XXY, *cen(x)*, der(Y)t(Y;12) x2, *der(Y)t(X;Y), del(1)*, +2, del(3), +3, *ace(3), der(4)t(Y;4)*, +5, +6, der(6)t(6;7), *hsr(X;6),* -7, +8, *dup(8), dic(9), dic(9;10)*, -10, -12, -13, *t(13;13)*, -14, *der(14)t(11;14) x2, der(14)t(8;14)*, -16, *der(16)t(16;6;19;6;8)*, +17, +19 [cp5]

8-1:
3n – 5n, 55 – 97, XXYYYYY, der(X)t(X;13), *+der(X)t(X;6), +der(X)t(X;2), t(Y;11), t(Y;3), +del(X),* +cen(1), dup(1), *der(1)t(1;8), +del(1), der(2)t(2;15), t(2;2), t(2;14), +ace(2), -2*, +del(2), +del(3), +cen(3), -3, der(3)t(3;9), *der(3)t(3;4) x2, der(3)t(3;6) x2, +dup(3), der(4)t(1;4), der(4)t(4;19), der(4)t(4;15;10)*, -4, +del(4) x3, +cen(4), der(4)t(4;10), -5 x2, *der(5)t(5;11), dic(5) x2, der(5)t(3;5), +del(5), +dup(6) x2*, der(6)t(6;9) x2, *der(6)t(6;18)*, -7 x2, *der(7)t(3;7), +del(7), der(7)t(7;18), der(7)t(8;7), cen(7), der(7)t(7;14),* -8, +del(8), *der(8)t(2;8), der(8)t(8;16)*, +del(9), -9, *der(9)t(1;9) x2, der(9)t(8;9), der(9)t(9;17), dic(3;9), der(9)t(3;9)*, -10, *del(10), cen(10), der(10)t(10;12),* -11 x2, +del(11), *dup(cen(11))*, +del(12) x2, -12, cen(12), *der(12)t(X;12), der(12)t(12;18) x2, -13*, der(13)t(13;18), der(13)t(13;4;10), *der(13)t(13;18;4;10) x2, der(13)t(3;13) x2, der(13)t(13;18;6), der(13)t(13;1;5)*, der(13)t13;14), *der(13)t(12;13), der(13)t(X;13), der(13)t(13;10), +ace(13)*, der(14)t(13;14), der(14)t(7;14), *der(14)t(8;14), der(14)t(10;14), er(14)t(11;14), der(14)t(9;14) x2, del(14) x2, dup(14), +cen(14), +14*, -15, der(15)t(14;15), +del(15) x2, cen(16), +del(16), -16, *der(16)t(13;16),* -17, del(17), der(17)t(17;18) x2, -18, *der(18)t(16;18), der(18)t(6;18)*, del(18), -19 [cp7]

B1-10:
64-77 (4N), XXXX, +X, cen(X), del(X), -1, *cen(1)*, der (1)t(1;3), der(2)t(2;12), del(2) x3, dic(2), *dic(3)*, del (3), *ace(3)*, -4, del(4), *der(4)t(4;11), dic(4)*, -5 x2, *cen(5), der(6)t(6;13;10)*, der(6)t(6;14), der(6)t(6;13), der(6)t(6;5, *del(6) x2*, +7, *der(8)t(8;13;10), der(8)t(8;7), der(8)t(2;8)*, del(8), -8, *dup(cen(8))*, -9, der(9)t(4;9), *t(9;14)*, del(9), -10, der(10)t(10;14) x2, *der(10)t(10;4;8), der(10)t(X;10)*, -11, del(11), *der(11)t(5;11)*, -12, *der(12)t(8;12)*, *del(12), der(13)t(5;13), dup(13) x2, cen(13)*, -14 x2, del(14) x2, *der(14)t(4;17)*, +15, *del(15)*, dup(cen(16)), -16, dup(16), +17, *cen(17)*, *der(17)t(14;17), dup[cen(17), dic(17;5)]*, -18, dup(18), dup[cen(18)t(18;X;18;8)], *der(18)t(8;18), der(18)t(10;18), der(18)t(18;19)*, +19, der(19)t(6;19), *-19, der(19)t(19;13;19)*

Materials

Solutions and Reagents:

Tris (Tris(hydroxymethyl)-aminomethan)	*Sigma*®
Tris-Acetate	*Sigma*®
EDTA (Ethylenediaminetetraacetic acid)	*Fisher Scientific*®
SDS (Sodium dodecyl sulfate)	*USB*®
NaCl	*Sigma*®
NaCl Solution saturated (~5.8M)	
Agarose	*Denville Scientific*®
Isopropanol (2-N-Propanol)	*Fisher Scientific*®
100% Ethanol	*Fisher Scientific*®
70% Ethanol	*Fisher Scientific*®
Salmon Sperm Testes DNA	*Sigma*®
Tween 20	*Sigma*®
Formamide	*Sigma*®

Buffer:

1X PBS (Phosphate buffered saline)
(137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4; pH 7.4)

1X SSC (Saline-sodium citrate)
(150mM NaCl, 15mM Sodium Citrate pH7.0)

20x SSC (Saline-sodium citrate)
(3M NaCl, 300 mM Sodium Citrate; pH 7.0)

NEBuffer 2 *New England Biolabs*®
(150mM NaCl; 10mM Tris-HCl; 10mM $MgCl_2$;1mM Dithiothreitol (pH7.5 at 25º C)

NEBuffer 3 *New England Biolabs*®
(100mM NaCl; 50mM Tris-HCl; 10mM $MgCl_2$; 1mM Dithiothreitol (pH7.5 at 25º C)

Proteinase K buffer
(50mM Tris-HCl pH8.0, 100mM NaCl, 100mM EDTA, 1% SDS, 200ug/ml proteinase K)

Rapid Hybridization Buffer
(5xSSC, 10% Polyethyleneglycol, 5% Poly(sodium 4-styrenesulfonate), 0.2% Cetylpyridinium chloride monohydrate)

Tail Buffer
(50mM Tris Buffer pH8; 100mM NaCl; 100mM EDTA; 1% SDS)

1X TE Buffer
(10mM Tris Buffer pH8; 1mM EDTA, pH 8)

1X TAE Buffer
(40mM Tris-acetate; 1mM EDTA, pH8 adjusted with glacial acetic acid)

Agarose gels:

0.8% Agarose Gel
(0.8% Agarose; 1x TAE Buffer; ethidium bromide, final concentration: 0.5µg/ml)
2% Agarose Gel
(2% Agarose; 1x TAE Buffer; ethidium bromide, final concentration: 0.5µg/ml)
Nylon Membrane (positively charged) *Biodyne B*®

Enzymes:

Proteinase K (10 mg/ml)	*Isc Bioexpress*®
Klenow Exon9 Fragment	*New England Biolabs*®
BamH I	*New England Biolabs*®
EcoR I	*New England Biolabs*®
PST I	*New England Biolabs*®

Tissue Culture:

Cell culture dish (10cm)	*Fisher Scientific*®
6-well plates	*Fisher Scientific*®
Culture Medium	
440ml Dulbecco's modified Eagle's Medium (DMEM)	*Sigma*®
50ml Fetal bovine serum	*Sigma*®
5ml Penicillin-Streptomycin solution	*Cellgro*®
5ml L-glutamine	*Sigma*®
Trypsin	*M**tech*®
PBS	
Gelatin (0.1%)	*Sigma*®
DMSO (Dimethyl sulfoxide)	*Fisher Scientific*®
Trypan Blue (0.4% solution)	*Invitrogen*®

SKY:

Denaturation solution
(70% formamide; 2X SSC; pH 7.0)

SKY washing solution I
(50% formamide; 2X SSC)

SKY washing solution II
(1X SSC)

SKY washing solution III
(4X SSC, 0.1% Tween 20)

Spectral karyotyping (mouse) Reagent	*Applied Spectral Imaging*®
Blocking Reagent	*Applied Spectral Imaging*®
Cy5 Staining Reagent	*Applied Spectral Imaging*®
Cy5.5 Staining Reagent	*Applied Spectral Imaging*®
Anti-Fade-DAPI Reagent	*Applied Spectral Imaging*®

Drugs:

Mitomycin C from Streptomyces caespitosus (2mg)	*Sigma*®
Cis-diamminedichloridoplatinum (II) (cisplatin) (25mg)	*Sigma*®
Paclitaxel	*Sigma*®
Gemcitabine	*Lilly*®
5-FU (5-Fluorouracil)	*Sigma*®

Abbreviations

aa	Amino acid
ARF	Alternative reading frame
ATM	Ataxia teleangiectasia mutated
ATR	ATM and RAD3-related
bp	Basepair
BACH1	BRCA1-Associated Carboxyl-terminal Helicase
BARD1	BRCA1 associated Ring domain 1
BASC	BRCA1-associated genome surveillance complex
BRCA1	Breast cancer susceptibility gene/protein 1
BRCA2	Breast cancer susceptibility gene/protein 2
BRCC	BRCA1-BRCA2-containing complex
BRCT	BRCA 1 C-Terminus
CDK	Cyclin-dependant checkpoint kinases
CHK2	Checkpoint kinase 2
Cre	Causes recombination of the bacteriophage P1 genome
CtIP	C-terminal binding protein interacting protein
DNA	Deoxyribonucleic acid
GAP	GTPase activating proteins
GEF	Guanine nucleotide exchange factors
GTP	Guanosine-5'-triphosphate
HR	Homologous recombination
HDM	Human double minute
IC_{50}	Half maximal inhibitory concentration
IPMN	Intraductal papillary mucinous neoplasm
kbp	Kilo-basepair
LoxP	Locus of crossover (x) in bacteriophage P1
MCN	Mucinous cystic neoplasm
MDM	Murine double minute
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MRN	MRE11-RAD50-NBS1
MSCI	Meiotic sex chromosome inactivation
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
PanIN	Pancreatic intraepithelial neoplasia
PCR	Polymerase Chain Reaction
Pdx-1	Pancreatic and duodenal homeobox gene 1
PI3K	Phosphoinositide 3-kinase
$P16^{INK4A}$	CDK inhibitor 2A (melanoma, p16, inhibits CDK4)
PRSS1	Protease, serine, 1 (trypsin 1)
PTEN	Phosphatase and tensin homolog
PUMA	p53 up-regulated modulator of apoptosis
RING	Really interesting new gene
SSA	Single-strand annealing
SKY	Spectral Karyotype Imaging
SMAD4	SMAD family member 4
SSC	Standard Saline Citrate
SSR	Site-specific recombinase
STK11/LBK1	Serine/threonine kinase 11
Xi	Inactive X chromosome
XiST RNA	X-inactive specific transcript RNA
XPC	Xeroderma pigmentosum C
XPE	Xeroderma pigmentosum E

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List of publications

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