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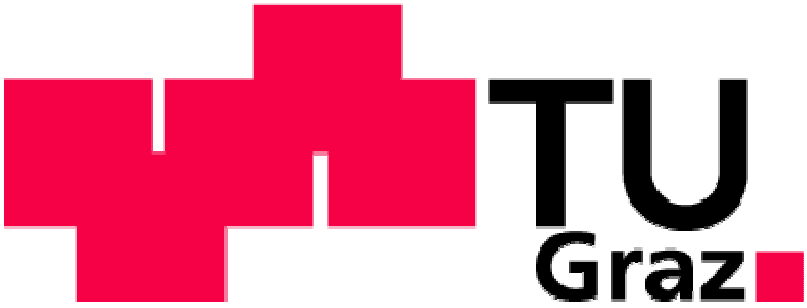
Mitotic origin and subsequent consequences of CNVs

Master Thesis



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Graz, June, 2010

Kurzfassung

Mitotic origin and subsequent consequences of CNVs

Die Mehrheit solider Tumore weist eine chromosomale Instabilität auf, die durch Gewinne oder Verluste einzelner Chromosomen oder Chromosomenfragmente charakterisiert ist. Die Ursache der chromosomalen Instabilität und die Mechanismen, die zu Chromosomenumbauten führen, sind nicht geklärt. Es gibt Hinweise, dass bestimmte Regionen im Genom, die „Fragile Sites“ dabei eine wichtige Rolle spielen. Fragile Sites kommen in jedem Genom vor und können unter bestimmten Bedingungen brechen oder Konstruktionen bilden, die in der Chromosomenanalyse sichtbar sind.

Ziel der vorliegenden Arbeit ist die detaillierte Untersuchung eines speziellen Mechanismus, dem „oncogene induced senescence“ (OIS), speziell in Hinblick auf eine mögliche Rolle für die Tumorentstehung. Vorarbeiten einer italienischen Arbeitsgruppe zeigten, dass OIS zu Stress während der DNA-Replikation führen kann. Dieser Stress kann an den „Fragile Sites“ oder an den Stellen des Replikationsursprungs zu Veränderungen führen, die die Anzahl der betroffenen Regionen verändern kann. Die Kopienzahlveränderungen innerhalb eines Genoms können mittels FISH (Fluoreszenz In Situ Hybridisierung) oder mit der Array-CGH (Array-Comparative Genomic Hybridization) erfasst werden. Um numerische Veränderungen mit hoher Auflösung zu untersuchen, wurden verschiedene Zelllinien, in denen unter definierten Bedingungen OIS ausgelöst werden konnte, angelegt und zu unterschiedlichen Zeitpunkten mit den vorgenannten Methoden untersucht. Die Ergebnisse zeigen, dass OIS zu Chromosomenveränderungen und zur Instabilität des Genoms führt. Damit könnte OIS ein wichtiger Mechanismus bei der Entstehung von malignen Erkrankungen sein.

Schlüsselwörter: chromosomale Instabilitäten, Fragile Site, OIS (oncogene induced senescence), Aneuploidie, CGH (comparative genomic hybridization)

Abstract

Mitotic origin and subsequent consequences of CNVs

The majority of solid tumors exhibit chromosomal instability characterized by gains or losses of chromosomes or fragments of chromosomes. The causes of chromosomal instability and the mechanisms leading to chromosomal alterations remain unclear. There is evidence that certain regions in the genome, the “fragile sites” – might play an important role. Fragile sites occur in each genome and can break under certain conditions or build constructions that are visible in chromosomal analysis.

The aim of this study is a detailed analysis of a special mechanism, the “oncogene induced senescence (OIS), in particular with regard to their possible role in tumorigenesis. As demonstrated in a study conducted by an italian work group, OIS can cause stress during DNA replication. This stress can lead to changes at fragile sites or at sites of replication origin that can modify the number of the affected regions. Copy number changes (CNCs) within the genome can be detected by FISH (flourescence in situ hybridization) or by array-CGH (array-comparative genomic hybidization). In order to investigate numeric changes at a high resolution, different cell lines in which OIS can be triggered under certain circumstances, were established and analyzed at different points in time, using the above mentioned methods. The results demonstrate that OIS lead to chromosomal changes and to instability of the genome. Consequently, OIS could represent an important mechanism in the genesis of malign diseases.

Keywords: chromosomal instability, fragile site, OIS (oncogene induced senescence), aneuploidy, CGH (comparative genomic hybridization)

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

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Table of contents

1. Introduction and objectives

This chapter gives an overview about the background of the project. Different research questions have been addressed with different experiments therefore the background information and the chapter materials and methods, results, and discussion are divided into four smaller chapters each related to an experiment. Defined terms are copy number variants (CNVs), chromosomal instability (CIN), and fragile sites (FS). The chapter addresses their causes and effects and gives a short overview of the linkage between DNA damage response (DDR) and oncogene-induced senescence (OIS). Furthermore the connection between Fanconi anaemia and Bloom's syndrome protein in the DDR pathway are discussed.

1.1. Part 1 – Giemsa staining experiment

This chapter explains types of chromosomal instability and cellular mechanisms preventing and repairing breaks or damage of the DNA. The aim of this experiment was to prove the chromosomal stability of tumor cell lines. Single cells have been isolated and grown for three to four weeks till the cells reach a specific density. From a small part of the cells another row of single cells was isolated, termed "subclones" and from the other part metaphase slides were prepared and a giemsa staining was done. The process including isolation of single cells, culturing, preparation of metaphase slides, and the giemsa staining was repeated three times. At the end karygrams from three "generations" all grown from one single cell could be determined. Fig. 1 gives an overview of the project outline.

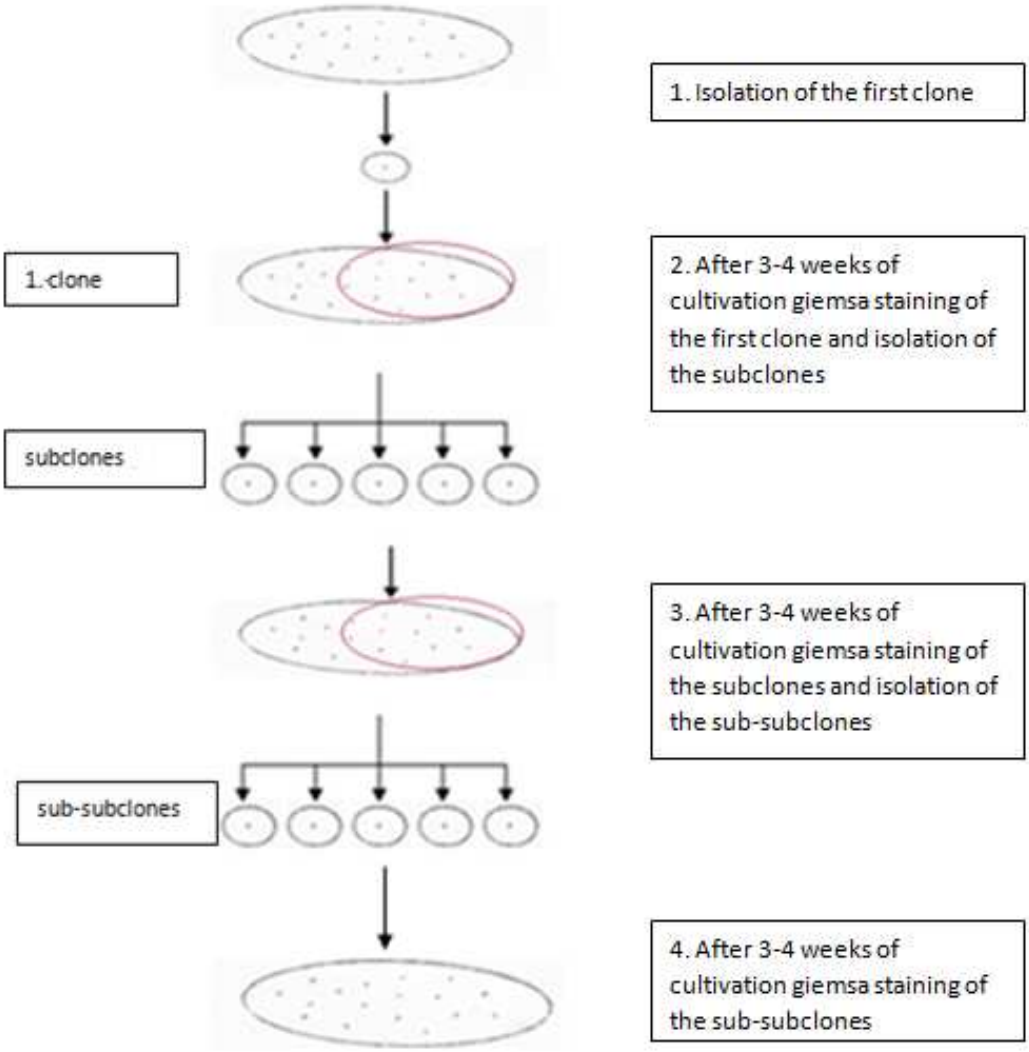

Fig. 1 Project outline of the giemsa staining experiment: 1. Isolate one single cell from the total cell population, let them proliferate for three to four weeks. 2. Isolate ten subclones from the population of the first clone, prepare metaphase slides of the first clone and do a giemsa staining of the prepared metaphases of the first clone. 3. After another three to four weeks the cell density of the subclones is high enough to isolate from each subclone another ten sub-subclones. Furthermore a giemsa staining on prepared metaphases slides of the subclones is done. 4. At least after cultivation of the sub-subclones a giemsa staining of each cell population is done.

More than 50 years ago, in 1956, a diploid human cell was determined having 46 chromosomes which was fundamental for the initiation of the field of human cytogenetics. [1] The human chromosome consists of 22 pairs of autosomes and one pair of gonosomes which determines if the human develop to a male or to a female. [2]

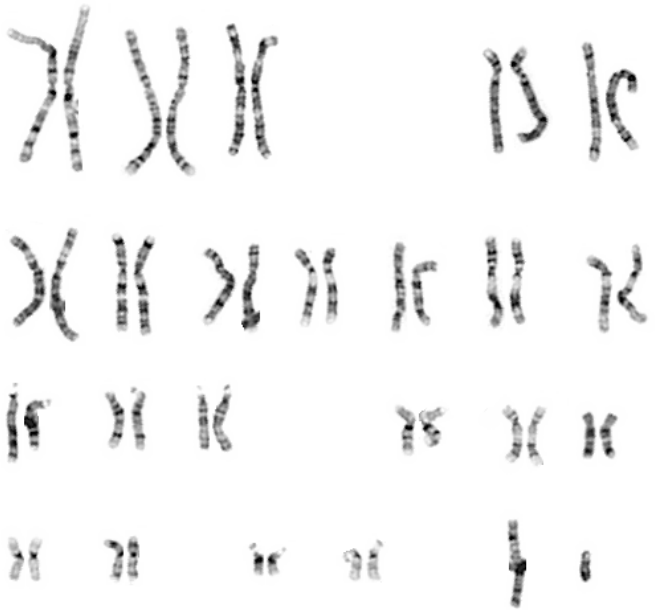

Fig. 2 Human karyotype: A normal human karyotype of a male person [Reprinted from Online Biology Book, http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookhumgen.html. 2007]

Giemsa staining is a technique to colour the whole chromosomes with darker or less dark bands, see Fig. 2. The staining allows the identification of larger chromosomal abnormalities such as gains and losses of chromosomal fragments or whole chromosomes.

1.1.1. Abnormalities in the DNA – causes and consequences

The attack on the DNA from numerous exogenous agents such as radiation, chemicals, and endogenous sources like free radicals which are generated during essential metabolic processes may cause breaks in the DNA. An accumulation of DNA damage or/and a permanent change results in tumor development, apoptosis, cell growth arrest, or impaired cell functions. The human system includes several mechanisms to prevent and correct failures in the DNA. Some of the repair mechanisms need a correct complementary strand as a template to repair the damage, e.g. nucleotide excisions repair (NER) and mismatch repair (MMR). In the case of DNA double strand breaks the failure is on both strands at the same location, NER or MMR would not work because there is no template given. Defects in the repair mechanism lead to diseases and patients have a higher predisposition to suffer from cancer. E.g. a defect of the NER pathway induces Xeroderma pigmentosum in humans, a disease characterized by sun sensitivity of the skin and predisposition to skin cancer. Two main forms of genomic instability are associated

with tumorigenesis. On the one hand there is the mutational instability (MIN) arising from changes in the DNA sequence, point mutations or small deletions and on the other hand there are chromosomal instabilities (CIN) arising from improper rearrangement of chromosomes. [3] E.g. 80 - 85 % of colorectal cancers show a CIN phenotype. Geigl et al. specified the term of MIN. In his paper MIN is described to be the same as microsatellite instabilities (MSI). Microsatellite consists of repeating DNA sequences of one to six base pairs in length, those sequences can be shorten or lengthen if there are defects in the DNA repair mechanism. [4]

Fig. 3 illustrates the causes and consequences of DNA double strand breaks (DSB).

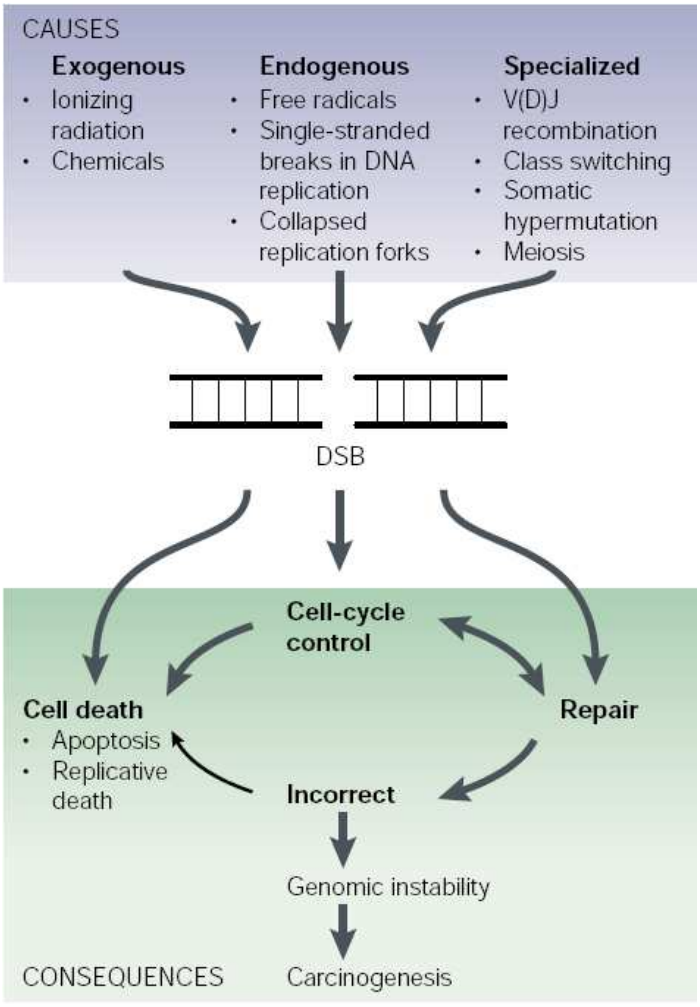

Fig. 3 Causes and consequences of DNA double strand breaks: DNA Double Strand breaks (DSB) are caused by exogenous, endogenous and special cellular processes. Cells have evolved several mechanisms to repair the damaged DNA. If neither apoptosis or, cell death mechanisms, nor the repair is successful it results in an incorrect DNA sequence which leads to genomic instability and carcinogenesis.
[Reprinted from Van Gent et al., 2001]

Copy number variants (CNVs) occur in human and other mammalian cells. They are an important component of genomic variations. More than 1.300 CNVs are known in the healthy population as heterozygous or homozygous deletions or duplications. [5] Chromosomal rearrangements causing deletions, insertions or translocations of genetic materials often result in the expression of altered gene products with an oncogenetic potential or the loss of function of tumor suppressor genes. [6] Copy number variations and sub microscopic copy number changes cause developmental and genetic disorders like mental retardation, autism, epilepsy, psychiatric disorders, skeletal defects, cancer cells, and others. [5]

The major mechanisms which lead to copy number variants (CNVs) and disease related copy number changes (CNCs) are meiotic unequal crossing over or non-allelic homologous recombination (NAHR). Both mechanisms are mediated by flanking repeated sequences or segmental deletions or duplication. Other mechanisms which are involved in the process of developing CNCs and CNVs are non-homologous end-joining (NHEJ) or aberration of replication of a mitotic cell through an error in the DNA damage response (DDR) and the homologous recombination (HR) pathway. [5] Environmental agents and chemicals including caffeine, alcohol, and cigarette smoke increase the development of instabilities in the DNA, these agents lead to breaks in fragile sites and cause tumor progression. [6]

Mechanisms causing defects in the DNA are explained on the following pages:

Homologous recombination (HR)

Homologous recombination (HR) is a mechanism where nucleotide sequences are exchanged between two identical strands of DNA. The HR-process involves physical breakage and rejoining of DNA. HR is a common mechanism to repair DNA double strand breaks and promote genomic diversity. [11] Programmed recombination between allelic sequences on homologous chromosomes occurs once per generation during the meiotic cell divisions which are limited to develop germ cells and in mitosis to repair DNA damages. [8]

The regulation of the HR takes place in the S- and G_2-phases of the cell cycle, because sister chromatids are readily available. Sister chromatids are an ideal HR

template. They consist of an identical copy of a chromosome. Bischop and Schiestl reported that 30 to 50 % of induced breaks are repaired by HR. This repair mechanism can be stimulated by a variety of DNA damaging agents. [11]

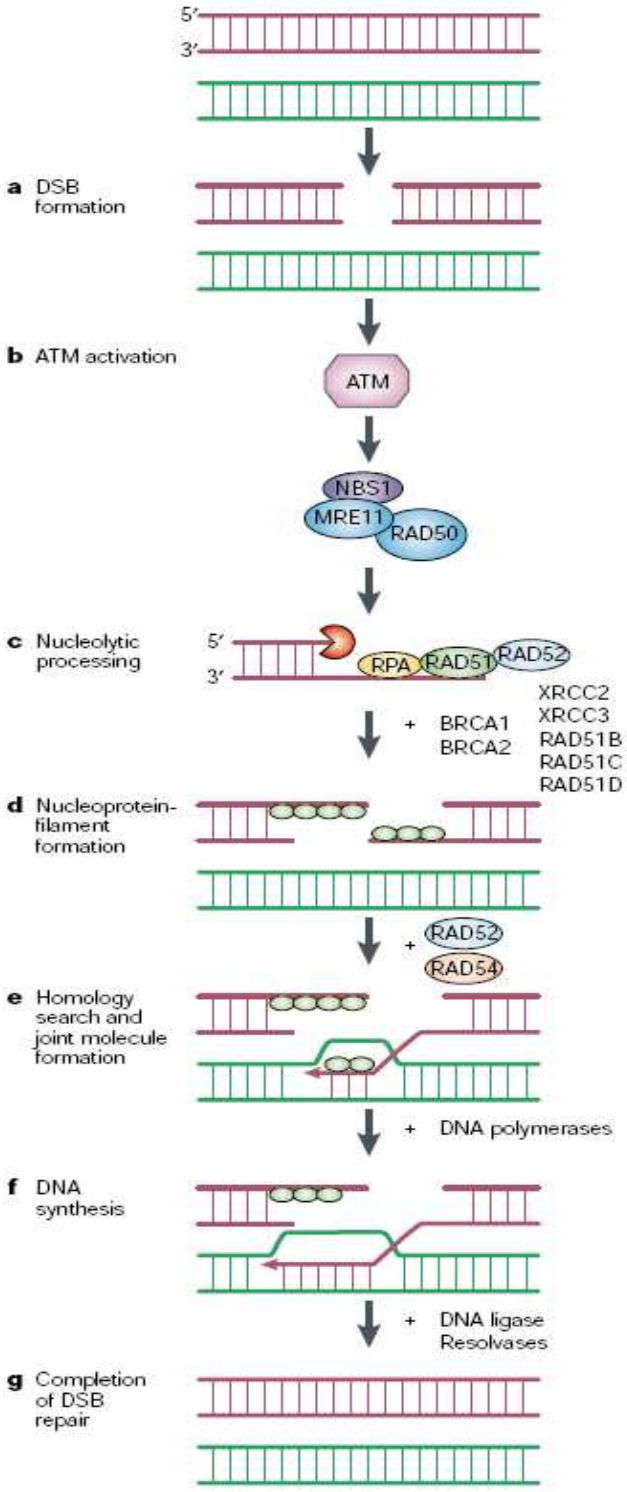

Fig. 4 Model of HR: HR used to repair DNA DSB. Steps from the DSB identifidation to the repair: ATM activation, nucleolytic processing, nucleoproteinfilament formation, homology joint molecule formation, DNA synthesis, and Completion of the DSB repair [Reprinted from van Gent et al., 2001]

Crossing over

Crossing over is a process during the meiosis, where segments of a chromosome switch places. In the case of equal crossing over the alleles e.g. “A” and “B” on the chromosomes “4A” and “4B” switch places. After this process chromosome 4A contains allele B and chromosome 4B contains allele 4A. The entire segment of the allele switches place with another allele. In unequal crossing over not the whole segment switches place, e.g. allele B leaves a piece behind on chromosome 4B and the rest switches place with allele 4A. The result is a shorter segment B on chromosome A and a larger segment, including whole allele A and parts of allele B, on chromosome B. [7]

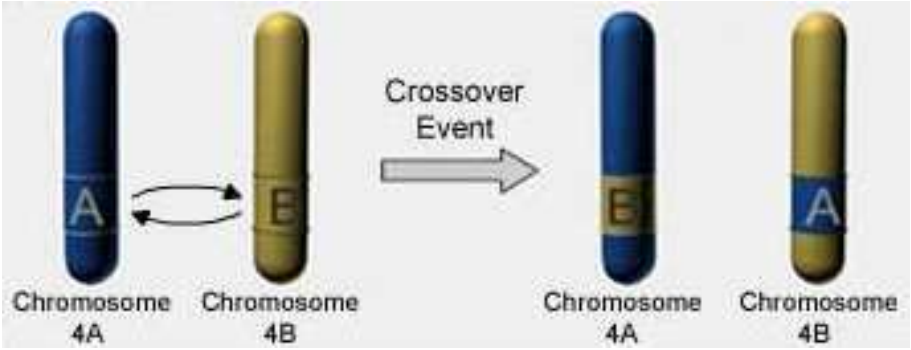

Fig. 5 Model of equal crossing over: The gene A on chromosome 4A changes completely the place with the gene B on 4B. [Reprinted from HOPES, 2004]

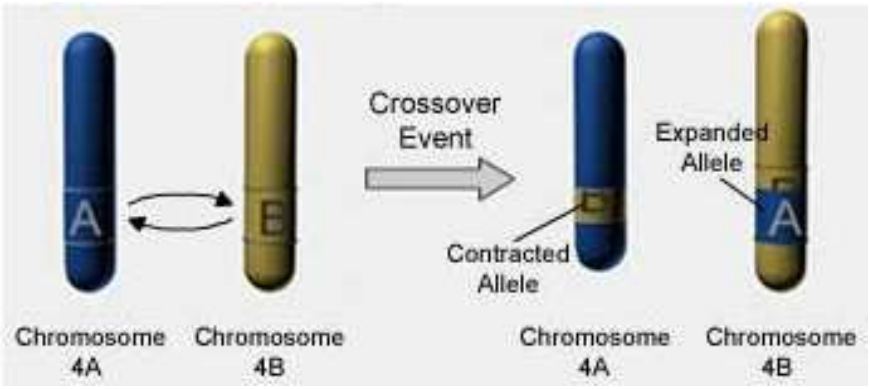

Fig. 6 Model of unequal crossing over: The gene A changes completely the place on chromosome 4B with gene B but the gene B do not change fully the place so that there is a reduced form of gene B on chromosome 4A and on chromosome 4B there is the whole gene A and a part of gene B. [Reprinted from HOPES, 2004]

Non-allelic homologous recombination (NAHR)

An incorrectly splicing of segmental duplications leads to non-allelic homologous recombination (NAHR). NAHR crossover can result in disease related chromosomal rearrangements e.g. deletions – male infertility, or duplication – Charcot Marie Tooth disease. [8]

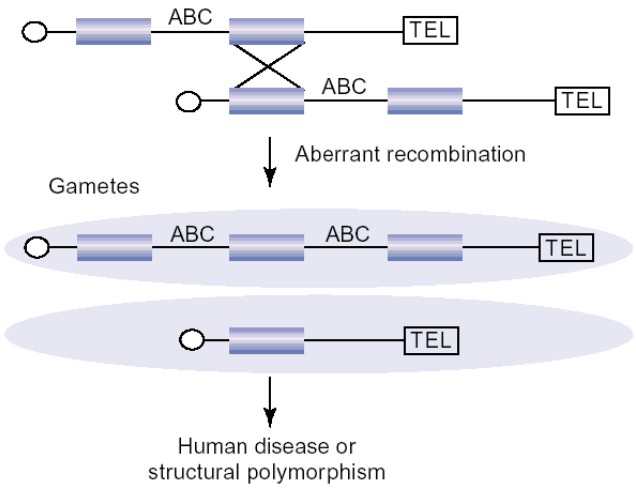

Fig. 7 Model of NAHR: NAHR between blocks of segmental duplications lead to microdeletion and microduplication of the unique region. If sensitive genes are part of the duplicated region diseases can be caused. [Reprinted from Eichler Lab, 2006]

Non-homologous end-joining (NHEJ)

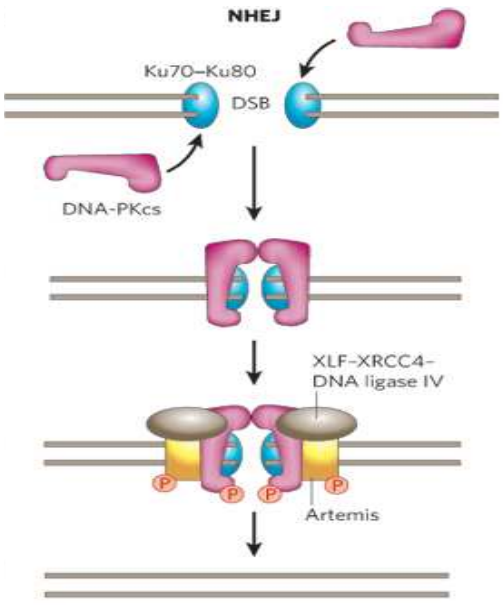

Fig. 8 Model of the NHEJ: The Ku dimer (Ku70-Ku80) and DNA-PKcs recognize a DNA double strand break. The ends are synapsed and the DNA PKcs and Artemis are phosphorylated, and DNA ends are processed by a complex containing XLF and XRCC4 and DNA ligase IV.
[Reprinted from Downs et al., 2007]

The main role of NHEJ is the pathway for repairing non-replication associated breaks, induced for example by ionizing radiation. The mechanism is initiated by the binding of a special heterodimer (Ku heterodimer) to a double stranded DNA end. Through a special complex and DNA ligase the DNA ends are proceeding. Cells with lacking NHEJ proteins arrest at cell cycle checkpoints. [9-10]

1.1.2. Aneuploidy and tumorigenesis

The chromosome number of human solid tumors typically ranges from 40 to 60 and in special cases 70 or more chromosomes. The exact procedure behind gaining and losing of extra chromosomes during the cell division is unclear. A high rate of chromosome missegregation in aneuploid tumor cells causes phenotypic changes which contribute to tumor cell evolution and pose therapeutic challenges. It may occur that chromosomal differences are the reason for changes in the growth properties of metastatic cells compared with the solid tumor. [12] In principle there are two ways known to become aneuploid:

1) Errors in the cell division lead to an alteration of the number of intact chromosomes and results in “whole chromosome aneuploidy”.
2) Chromosomal rearrangement like deletions or translocations which arises from breaks in the DNA result in “segmental aneuploidy”.

Segmental aneuploidy is a cause for tumorigenesis. E.g. telomere dysfunction and inactivated checkpoints can through fusion bridge-breakage cycles result in segmental aneuploidy. [4] Arlt et al. found out that aphidicholin induced replication stress causes a high frequency of copy number changes (CNCs) e.g. submicroscopic deletions and duplications. [5]

1.2. Part 2 – Fluorescence in situ hybridization (FISH) experiment

The second experiment demonstrates that oncogene induced senescent cells with an impaired DNA damage response show an increased number of firing replication origins compared to normal cells. The aim was to prove that RAS expressing cells show more signals of replication origin than normal cells. This was proofed with a FISH analysis. A fluorescently labelled DNA probe of gene of interest is hybridized on cells of interest which are fixed on a glass slide. The FISH technique allows visualization of chromosomal and nuclear locations of specific DNA regions through a microscope. Fig. 9 illustrates the steps of the FISH technique.

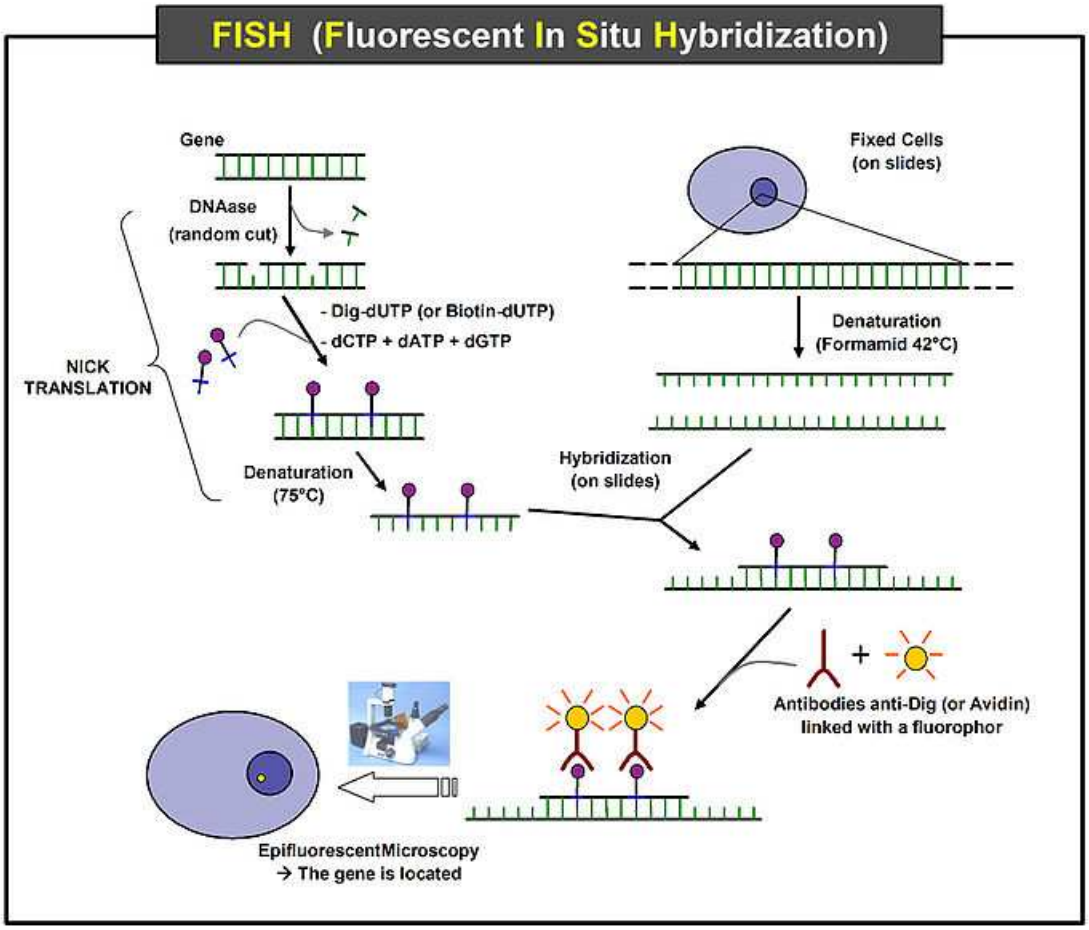

Fig. 9 Scheme of the FISH technique: Genes of interest are random cut with a DNAse and via Nick Translation labeled nucleotides are integrated into the DNA double strand. A heat denaturation results in a labeled single strand. Before the hybridization starts the cells which are fixed on a glass slide are also denatured so that the labeled probe can be hybridized on the slide and it binds on the complementary strand. The binding is detected via fluorescent labeled antibodies with a fluorescent microscope. [Reprinted from Wikipedia, 2007]

1.2.1. Cellular senescence

Cellular senescence is a fundamental aspect of cell behaviour and was first described in the work of Hayflick in 1961. [13 - 14] Cellular senescence is considered as safeguard mechanism which may prevent aged or abnormal cells from further expansions. [13] Senescence is divided into two parts, replicative and stress induced senescence. Senescent cells slow down their proliferation rate after a period of rapid proliferation and undergo a dramatic morphologic change e.g. increased volume and loss of shape. Senescent cells undergo changes in nuclear structure, gene expression, protein processing, and metabolism. Observations have shown that a variety of stressors can induce a senescent phenotype therefore senescence is suggested to be a general cellular response mechanism. Senescence of cells might cause tissue aging but a breakdown of this mechanism can lead to cancer genesis. The ectopic expression of the catalytic subunit of the telomerase enzyme hTERT (human Telomerase Reverse Transcriptase) halts the erosion of telomers and prevents the entrance into replicative senescence. Human fibroblast cell lines are the best studied example of a cell type in which the cause of senescence is attributed to critical telomere attrition, their senescence can usually be prevented by expression of hTERT. [14]

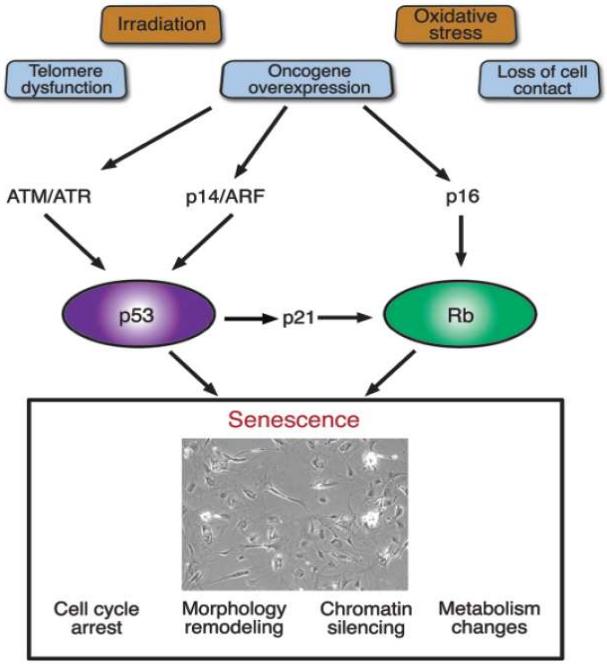

Fig. 10 Senescence as a general stress response program: Irradiation and oxidative stress cause telomere dysfunction, oncogene overexpression and loss of cell contact. This leads to senescence which is characterized by cell cycle arrest, morphology remodelling, chromatin silencing, and metabolism changes.
[Reprinted from Ben-Porath, Weinberg, 2004]

Fig. 10 illustrates that a variety of physiologic stresses like telomere dysfunction, oncogene overexpression, or loss of cell contact lead to the onset of cellular senescence. The stress is the reason for the stimulation of signal pathways which funnelled down to the activation of p53 protein, Rb protein or both. This activation process is the linkage between senescence and DNA damage response (DDR), p53 can be activated through the DDR signalling pathway by the ataxia teleangiectasia (ATM), ATR (ATM and Rad3 related protein kinase) protein kinases, or p14/ARF protein. The Rb protein is activated by the p16 protein or p21 protein, which is a target of p53. The stress induced effects on p53 or Rb depend on the stress signal. If the process is activated the cell undergoes functional and morphological changes. [14]

The connection pathway between senescence and DNA damage response (DDR) is defined but what is DDR? DDR is the answer of a cell to DNA double helix damage. The function of DDR is to prevent or arrest the duplication and portioning of damaged DNA into daughter cells to impede the propagation of corrupted genetic information and it coordinates cellular efforts to repair DNA damage and maintain genome integrity. [15] DDR is associated with early tumorigenesis. Cell proliferation and transformation induced by oncogene activation are restrained by cellular senescence. Studies show that in normal human cells senescence triggered by the expression of an activated oncogene is a consequence of the activation of a robust DDR. The expression of oncogene such as RAS causes senescence. Oncogene induced senescence (OIS) is characterized by the formation of senescence associated heterochromatic foci. RAS expression affects a strong proliferation burst. It is suggested that oncogene activation leads to an increased number of active replicons. [16] Verification of this thesis is done by fluorescence activated cell sorting (FACS) and fluorescence in situ hybridization (FISH).

The identification of RAS as a human oncogene and activated RAS proteins which show to be capable of transforming immortalized rodent cells was in 1982. Fifteen years later, in 1997 studies show that activated RAS cells trigger an initial wave of proliferation, followed by an irreversible growth arrest, known as cellular senescence, and a concomitant accumulation of p53 and p16 proteins. It was suggested that RAS-induced senescence could be bypassed by inactivating the pathway of the proteins Rb and p53. The data supported that one of the main functions of cellular

senescence is to suppress tumor development by preventing the progression of benign lesions in the absence of additional cooperating mutations. Mechanisms to prevent and suppress tumorigenesis are apoptosis, “cell death”, and senescence. Apoptotic cells are physically eliminated. Senescent cells stall in S-phase of the mitotic cycle and show an augmented number of active replicons and exhibit defects in DNA replication fork progression, resulting in an activation of ATR and ultimately ataxia teleangiectasia (ATM). There exist many mechanisms to prevent tumor development but what must happen to induce tumorigenesis? [17]

Fig. 11 shows the steps which happen after the appearance of oncogenic stress and the result of senescent cells. These cells prevent the tumorigenesis.

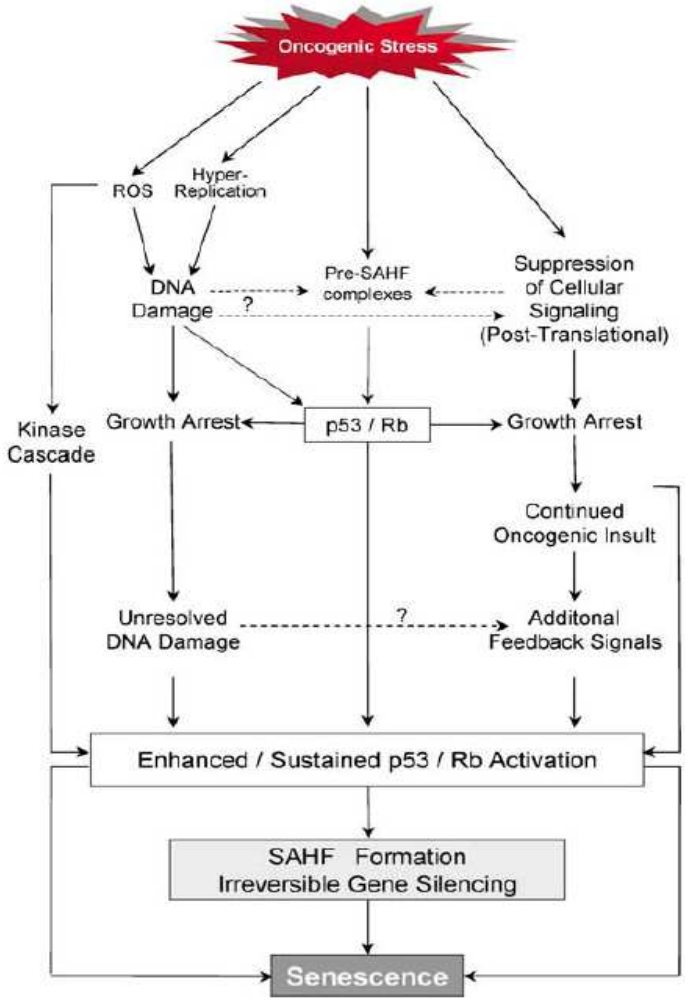

Fig. 11 Model of integrated OIS (oncogene induced senescence): Senescence regulating signals are ROS (Reactive Oxygen Species), replicating stress suppression of cellular signalling and chromatin remodelling via SAHF (Senescence Associated Heterochromatic Foci). This figure illustrates how these signals cooperate together. All senescence triggers can activate Rb and p53. In response to physiological oncogenic stress caused by specific genetic alterations, multiple pathways may be required to cooperate to achieve a threshold and/or sustained activation of Rb and p53. Once this occurs, chromatin remodelling may “lock in” growth arrest. [Reprinted from Courtois-Cox et al., 2008]

1.3. Part 3 – Immunohistochemical staining experiment

The third experiment illustrates that the protein FANCD2 which plays a role in the DNA damage response (DDR) pathway can be associated to the occurrence of fragile sites and tumorigenesis. The aim was to adapt an immunohistochemical staining protocol for the protein FANCD2. Stressed cells, like tumor cells or senescent cells should have an increased number of FANCD2 signals per cell. An adaption of the protocol to tissue section should give an overview of positive FANCD2 staining associated with tumor development.

This chapter explains the interaction of Fanconi anaemia and Bloom's syndrome protein during the DNA damage response (DDR) pathway. DDR is a signal transduction pathway that coordinates cell cycle transitions, DNA replication, DNA repair, and apoptosis. If there is any defect in the DDR pathway cells with a defect in the DNA are able to divide without apoptosis or repair. This leads to the induction of chromosomal associated diseases.

DNA damage is an umbrella term and it can take different forms e.g. base modification, strand breaks, interstrand cross-links, and other lesions. The human genome generated multiple defence mechanisms over years of evolution. Studies show that the BRCA1 associated complex, known as BASC, is a key for the recognition of DNA damages. This complex contains many human disease genes e.g. BLM (Bloom's syndrome), ATM (ataxia teleangiectasia), BRCA1 (breast cancer), and mismatch-repair proteins (hereditary non-polyposis colon cancer – HNPCC). The FA pathway is linked to the BRCA1 complex and part of the BASC complex. [18]
Literature shows that the protein of the Fanconi anaemia FANCD2 and FANCI are associated with common fragile sites (CFS). These loci are interlinked through BLM associated ultrafine DNA bridges. It is described, that there has to be a relation between the pathway of Fanconi anaemia and the Bloom's syndrome or/and other proteins which are involved in the DNA damage repair such as ATM, BRCA1, and BRCA2. Bloom's syndrome (BLM) is a chromosomal disorder associated with growth retardation, sunlight sensitivity, and cancer predisposition. [19]

Fanconi anaemia (FA) was first described by Guido Fanconi, a paediatrician, in 1927. [21] FA is an autosomal recessive disease which is characterised by congenital abnormalities, defective haemopoiesis, and FA patients suffer from a high risk of developing acute myeloid leukaemia and several solid tumors. Fanconi anaemia is caused by mutations in one of the 13 different Fanconi anaemia genes. [20] The identified FA genes are part of caretaker genes of cancer, which prevent the accumulation of mutations and chromosome aberrations. They constitute the corresponding proteins, the "FANC/BRCA" pathway. Eight of the 13 FA genes accumulate together and form the FANC core complex, a large nuclear complex. The assignment of the FANC core complex is to monoubiquitylate FANCD2 and FANCI. The monoubiquitylation takes place during the S-phase of the cell cycle and in response to DNA damage. Monoubiquitylated FANCD2 and FANCI are proteins which are involved in the DNA repair mechanism. [21]

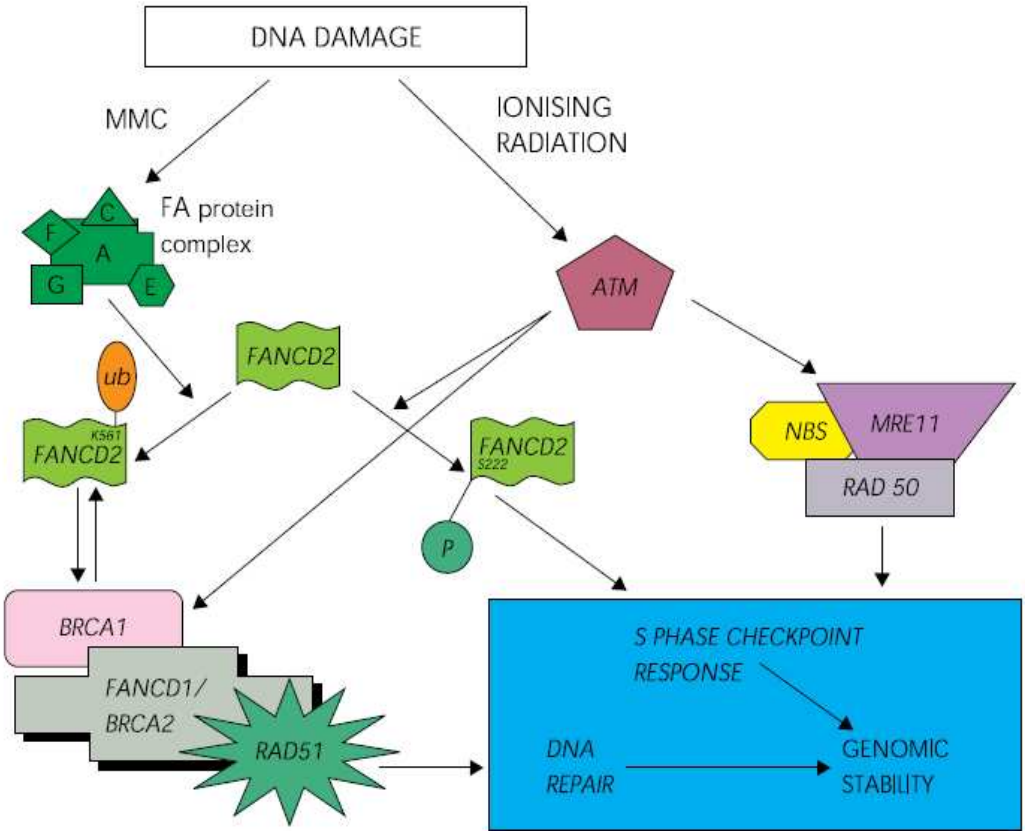

Fig. 12 Schematic diagram of the Fanconi anemia complex [Reprinted from Tschikowitz, Hodgson, 2003]

Fig. 12 shows the interaction of the Fanconi anaemia core complex with other proteins in the DNA damage response (DDR) pathway. Grompe defines FANCD2 as a monoubiquitylated protein at lysine 561 in response to DNA damage. This ubiquitination of FANCD2 leads to a co localization of FANCD2 and BRCA1 in nuclear foci. It is pointed out that a disruption of the Fanconi anaemia pathway blocks the ubiquitination and the effects are cells which are hypersensitive to DNA cross-linking agents. The monoubiquitination of lysine 561 is necessary for resistance to DNA cross-links. [18] If a DNA damage caused by cross-linking agents like mitomycin C (MMC) occurred it activates the Fanconi anemia complex. This activation leads to monoubiquitination of FANCD2 which interacts with BRCA1 to affect DNA repair. Damages which are caused by ionising radiation activate the ataxia telangiectasia (ATM) which phosphorylates FANCD2 at the position serine 222. The phophorylation of serine 222 independently affects the ability of cells to arrest in the DNA synthesis in response to ionizing radiation. The phosphorylated FANCD2 coordinates in addition to other proteins such as NBS (Nijmegen breakage syndrome gene), MRE11 (a double strand break repair protein), and RAD50 (DNA binding substance which regulates the metabolism of nucleosid and nucleotides) the S-phase checkpoint response. The translation point of the NBS gene plays a central role in the complex with MRE11 and RAD50 and is an important part for the double strand breakage repair and control of the cell cycle. [18, 20]

The defect protein in Bloom's syndrome, BLM, is a DNA helicase belonging to the highly conserved RecQ family. [22] The main function of the BLM protein is at the DNA replication fork disrupted by chemical substances or by DNA synthesis inhibitors such as aphidicholin (APH). [19] BLM appears to act in S-phase to prevent excessive and inappropriate recombination events. The protein BLM interacts with RAD51, a key protein in the DNA double strand break repair pathway, which mediates the homologous recombination (HR). [22 - 23] BLM and RAD51 interact directly via residues in the N- and C-terminal domain of BLM. RAD51 catalyses early steps of HR. The pairing of homologous sequences and DNA exchange of DNA strands to form a "holliday junction recombination intermediate". RecQ helicases such as BLM can bind to and disrupt those recombination intermediates. Loss of BLM would rise to an excessive recombination. [23]

Immunofluorescence staining gives an evidence for the existence of ultra fine bridges (UFBs) many of them originate from the centromere in anaphases. UFBs in normal cells provide the evidence for the existing linkage between DNA and centromeres. FANCD2 foci stained by immunofluorescence were found in BLM cell lines. Studies showed FANCD2 foci localized in pairs on the chromatids, one on each sister locus. The FANCD2 partner protein was similarly localized. The formation of FAND2/I sister foci is dependent on an intact Fanconi anaemia core complex and monoubiquitination of FANCD2 but independent from other proposed downstream Fanconi anaemia factors, including FANCJ and FANCD1 (known as BRCA2), ATM, ATR, and checkpoint kinases. Replication inhibitors such as mitomycin C (MMC), hydroxyurea, and aphidicholin (APH) induce FANCD2 foci. [19]

FANCD2 expression is not only induced by stress some normal adult and fetal tissue express FANCD2. The highest expression was found in spermatocytes, fetal oocytes, and in germinal centre cells of the spleen, tonsil, lymph nodes, and in the squamous epithelia of cervix, oropharynx, tonsil, and larynx. No FANCD2 expression was observed in bone, brain, kidney, colon, endometrium, gall bladder, lung, parotid, prostate, adult bone marrow, liver, endocrine glands like adrenal gland, pituitary gland, and thyroid, and heart muscular, muscle, and myometrium. A detailed list of FANCD2 expression in adult tissue is part of the appendix. Tissue specific FANCD2 expression studies help to understand the role of the Fanconi anaemia (FA) in haematopoiesis and carcinogenesis of different tissues and organs, in FA patients and non-FA persons. [24] In accordance to the study of Hölzel et al. an increased number of FANCD2 foci under stress may be a good pre screen for breaks in fragile sites e.g. in colon tissue.

1.4. Part 4 – Custom made array experiment

The fourth experiment did not come to an end because the time available for the thesis was limited. The aim of this experiment was to create a custom made array, which includes common fragile sites (CFSs), which are related to tumorigenesis. The array should be a method to detect deletions or duplications in regions of fragile sites. It should figure out if the variations in the CFSs are a common mechanism in tumorigenesis.

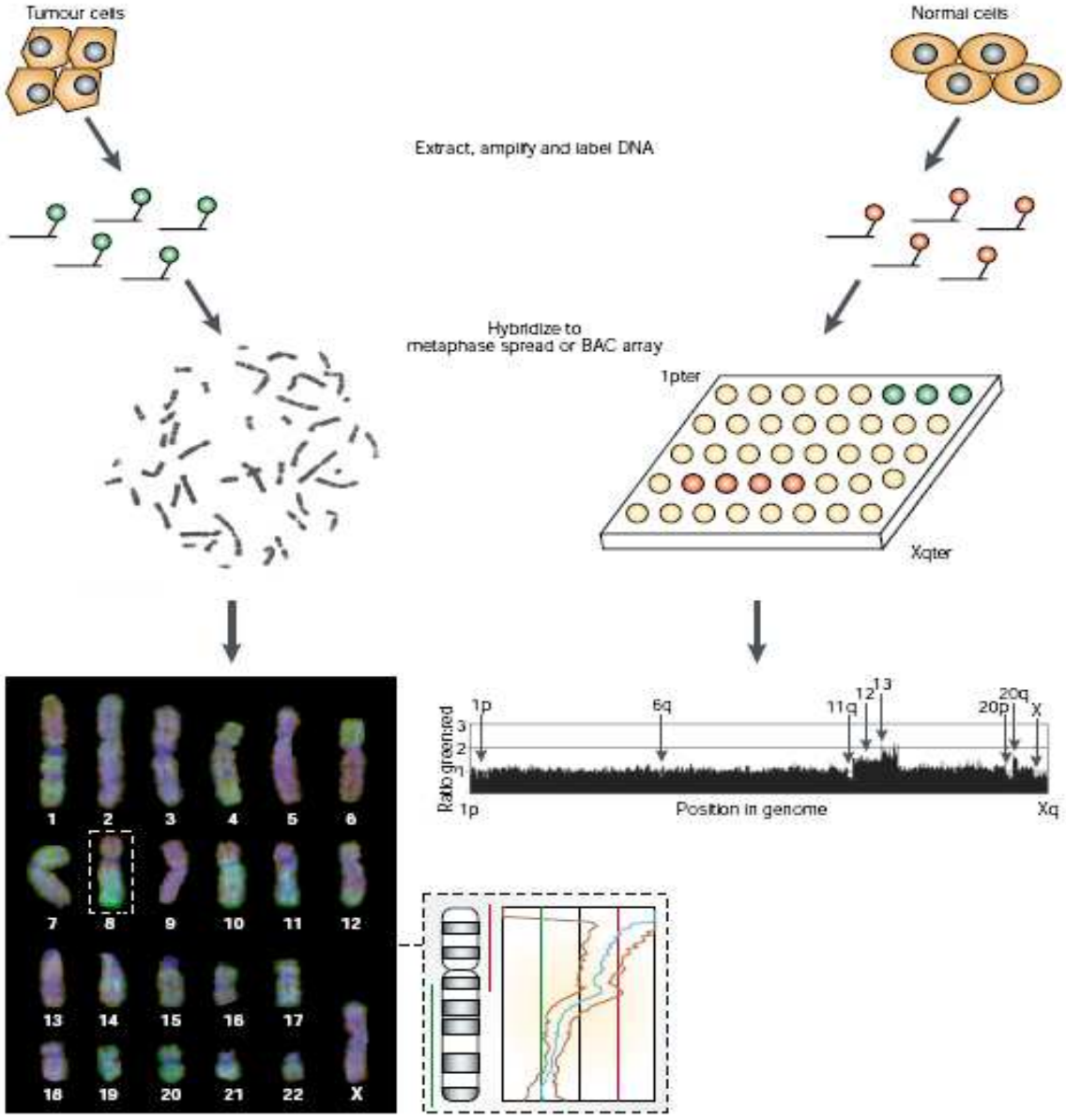

Fig. 13 Array comparative genome hybridization: A sample DNA (green) and a reference DNA (red) are differentially labelled with fluorochromes. The labelled DNAs are mixed together and hybridized on a BAC (bacterial artificial chromosome) array, which includes thousands of small DNA spots distributed all over the genome. The two DNAs compete for a binding partner on the array. Through laser scanning at specific nm space, specific for each dye, the spots on the array got coloured. Green Spots show up an extra copy in the sample DNA, there must be a duplication, red spots characterize a deletion in the sample DNA because more reference DNA bound on the array, and yellow spots stand for a balanced genome. [Reprinted from Trask, 2002]

Fragile sites are regions which are especially susceptible to breaks and gaps under stress. They are classified into two groups rare and common, depending on their frequency in the population. Rare fragile sites occur in less than five percent of the population, and are inherited in a Mendelian manner. [6] Common fragile sites (CFSs) are “hot spots” for increased sister chromatid exchanges. CFSs show a high rate of translocations and deletions in somatic cell hybrid systems. [25]

The first mapped and most common fragile site is FRA3B. FRA3B is located on chromosome three and includes the FHIT gene which spans approximately 900 k. Rare fragile sites are characterized by large di- or trinucleotide repeats e.g. FRAXA. [25]

Fragile Site	**Location**	**Associated genes**
FRA2G	2q31	IGRP, RDHL, LRP2, others
FRA3B	3q14.2	FHIT
FRA4F	4q22	GRID2
FRA6E	6q21	PARKIN, MAP3K4, LPA, others
FRA7E	7q21.11	LEP
FRA7G	7q31.2	CAV1, CAV2, TESTIN, MET
FRA7H	7q32.3	None identified
FRA9E	9q32-33.1	PAPPA, ROD1, KLF4, others
FRA16D	16q23.3	WWOX
FRAXB	Xp22.3	STS

Tab. 1 Most common fragile sites, their location and associated genes [Reprinted from Glover, 2006]

Tumor types in which fragile site instability exists include breast, lung, digestive tract, kidney, adenocarcinoma, and myeloma. It is suggested that genes which are in the region of fragile site may be tumor suppressor genes because they play a role in tumor progression. [25]

Common fragile sites (CFSs) correlate with chromosomal breakpoints in tumors and were shown to play a role in the in vivo occurrence of deletions, translocations, gene amplification, and integration of foreign DNA. [26]

The real process behind the mechanism of fragile site breakage is unclear. Studies show that a deficiency of a protein which is involved in the cell cycle checkpoint increase breakages at fragile sites. [6] In that way the kinases ataxia teleangiectasia (ATM) and ATR were studied. Both work overlapping in the checkpoint pathway in response to DNA double strand breaks and replication stress. Glover show that ATM deficient cells work as normal control cell in presence of aphidicolin (APH) but ATR deficient cells are very sensitive to aphidicolin. After the aphidicolin treatment those cells show a highly significant increase in gaps and breaks at fragile sites. [25]

2. Methods

In this chapter methods which were used during the project are described and materials and chemicals are listed.

2.1. Cell culture

A very important assumption for the work in the cell culture is a sterile working place. The applicant has to wear a coat and gloves and the hands as well as the surface of the lamina, has to be disinfected before and after the working process.

Materials and chemicals:

- Culture Flasks (75 cm² and 25 cm²)
- Culture dishes (6 cm and 3,5 cm)
- Pipettes (10 ml and 5 ml)
- Pipettus
- Falcons (15 ml)
- Centrifuge
- Incubator (37 °C)
- Lamina
- Cryotubes
- Trypsin-EDTA (Invitrogen, cat. no. 616260)
- Phosphate buffered saline (PBS) (Sigma Aldrich, cat. no. P5368)
- Penecillin/Streptomycin (PS) (PAA Laboratories, cat. no. P01007-0596)
- Fetal bovine serum albumin (FBS) (PAA Laboratories, cat. no. A15109-1513)
- Dimethyl sulfoxide (DMSO) (Merck, cat. no. 025250)
- L-Glutamine (PAA Laboratories, cat. no. M00406-1872)
- Media (see Tab. 2)

Media	Cell line	Provider of media
DMEM Dulbecco's Modified Eagle Medium + 10 % FBS (fetal bovine serum albumin) + 1 % PS (Penicillin/Streptomycin)	HCT116 BJ cell line	Invitrogen cat. no. 41966
Mc Coy's 5A +10 % FBS (fetal bovine serum albumin) + 1 % PS (Penicillin/Streptomycin)	HT29	SIGMA Aldrich cat. no. M9309
DMEM + 15 % FBS (fetal bovine serum albumin) + 1 % PS (Penicillin/Streptomycin) + 2 mM L-Glutamine	HGMDFN090	Invitrogen cat. no. 41966
RPMI1640 + 10 % FBS (fetal bovine serum albumin) + 1 % PS (Penicillin/Streptomycin) + L-Glutamine	HeLa	SIGMA Aldrich cat. no. M3817

Tab. 2 List of used media with additional substances according to the cell line

2.1.1. Splitting Cells and change the media

1. The used media was sucked off.
2. In the case of changing the media fresh media was added after aspiration of the old one.
3. For splitting the cells an enzyme, Trypsin-EDTA, was added to the culture flask, 3 ml of the enzyme to a 75 cm² flask and 1,5 ml to a 25 cm². Trypsin is an enzyme which cleaves peptides on the C-terminal side of lysine and arginine amino acid residues.
4. The cells were incubated at 37 °C for about 5 minutes. This helps the enzyme to remove the cells from the bottom of the flask.
5. When the trypsinization process is completed the cells are in suspension and appear rounded. To inhibit further enzyme activity fresh media containing fetal bovine serum albumin (FBS) was added.
6. Cells were resuspended by gently up and down pipetting strokes.
7. The cell suspension could be divided 1:3 to 1:8, the splitting rate differed from cell lines. E.g. BJ cell lines were divided 1:3 and HT29 1:6
8. After the splitting process the culture flask were placed back to the 37 °C incubator.

2.1.2. Freeze the cells

1. The first steps including the trypsinization process and resuspending were the same like described above in 2.1.1., steps one to five.
2. The cell suspension was transferred into a 15 ml falcon and centrifuged for 10 minutes by 1.500 rpm.
3. After the centrifugation the supernatant was removed.
4. The cell pellet was resuspended with 95 % fetal bovine serum albumin (FBS) and 5 % dimethyl sulfoxide (DMSO) and portioned into cryotubes, 200 µl cell suspension/cryotube.

2.1.3. Cell lines

Cell lines which were used during the project are: HeLa, HCT116, HT29, BJ PD40, BJ shp53 RAS, BJ shp53 pBABE, BJ hTERT shp53 RAS, BJ hTERT shp53 pBABE, HGMDFN090. Some of the used cell lines have been in culture at the Institute of Human Genetics e.g. HeLa, HCT116, HT29 and others e.g. HGMDFN090 had to be ordered. All cells from the BJ cell line came from a collaborator laboratory from IFOM-IEO Campus in Milan Italy. Culture condition for the cell lines was a 37 °C incubator with 5 % CO_2.

HT29 are cells from a colorectal adenocarcinoma from a 44 year old female Caucasian. This cells are treated with Mc Coy's 5A media plus 10 % FBS and 1 % PS (penicillin/streptomycin).

The donor of **HCT116** cells is an adult male who suffers from colorectal cancer. The media consists of a DMEM media plus 10 % FBS and 1 % PS.

HGMDFN090 is a dermal fibroblast cell line isolated from a 37 years and 10 month old female, the donor has no clinical effects but she is the mother of a child who suffers from progeria. Progeria is a disease which is characterized by a rapid aging in the childhood. The death of progeria patients is mostly caused by heart diseases between the age of 8 and 21. HGMDFN090 was obtained from the Progeria

Research foundation. For culturing a DMEM media with 15 % FBS, 1 % PS and 2 mM L-Glutamine was needed.

HeLa are cells from a cervical cancer taken from Henrietta Lacks. HeLa cells were treated with a RPMI 1640 media which includes 10 % FBS (fetal bovine serumalbumin), 1 % PS (penicillin/streptomycin) and 2 mM L-Glutamine.

BJ cells are normal fibroblast cells isolated from the foreskin of a newborn male. The growth media consists of DMEM media plus 10 % FBS and 1 % PS. **BJ PD 40** is a negative control cell line. **BJ shp53 pBABE** and **BJ shp53 RAS** are transduced with shp53. The “pBABE” cell line is RAS negative. The cell line contains an empty RAS vector and functioned as positive control cell line. The “RAS” cell line express the oncogene RAS. This is the cell line of interest to determine the oncogene induced senescence (OIS). **BJ hTERT shp53 pBABE** and **BJ hTERT shp53 RAS** are additionally transfected with hTERT (human Telomerase Reverse Transcriptase). hTERT rescue the cells for some time from the process of senescence.

2.2. Preparation of metaphase and interphase slides

Metaphase and interphase slides were used for the immunohistochemical staining, giemsa staining and FISH experiments. If it is necessary to test if the labelled probe e.g. by the FISH experiment bind specific, metaphase slides would help. Furthermore chromosomal analysis is only possible on metaphase slides.

2.2.1. Interphase slides

Materials and Chemicals:

- Pipettes (10 ml and 5 ml)
- Pipettus
- Pipettes + tips
- Incubator
- Falcons (15 ml)
- Slide board + filter
- Centrifuge

- Glass slides
- Warming plate
- Trypsin-EDTA (Invitrogen, cat. no. 616260)
- Fresh media (see Tab. 2)
- Phosphate buffered saline (PBS) (Sigma Aldrich, cat. no. P5368)

1. The first steps were the same as described in 2.1.2. steps one to four.
2. The cell pellet was washed three times with phosphate buffered saline (PBS) and diluted after washing in PBS. The cell density should not be too high because then it may happen that the cells accumulate and this may result in a complication of the signal analysis.
3. Labelled glass slides were placed in the slide board, a filter was put upon, and the slide board was closed. The filled slide board was placed into the centrifuge.
4. Between approximately 500 and 800 µl of the cell suspension, depending on the density of the solution, were transferred in the middle of the filter.
5. The program of the centrifuge was: 3 minutes at 1.000 rpm without break. A non break program was necessary because otherwise the cells would accumulate at one side and would not cover the whole provided surface.
6. The liquid was removed.
7. Slides were dried on a warming plate at 37 °C and for future experiments they were stored at –20 °C.

2.2.2. Metaphase Slides

Materials and Chemicals:

- Pipettes (10 ml and 5 ml)
- Pipettus
- Pipettes + tips
- Lamina
- Incubator (37 °C)
- Falcons (15 ml)
- Centrifuge

- Glass slides
- Warming plate
- Microscope
- Glass Pasteur pipettes
- “Nunc” tubes
- Colcemid (PAA Laboratories, cat. no. J01-003)
- Trypsin-EDTA (Invitrogen, cat. no. 616260)
- Fresh media (containing fetal bovine serum albumin (FBS), see Tab. 2)
- 0,8 % hypotonic solution (tri-sodiumcitrat-dihydrat)
- Fixing solution “fixativ” (methanol:aceticacid, 3:1)

1. The basis of the preparation of metaphase slides is a culture flask with approximately 60 to 70 % of density. The cells should be in a good proliferation phase.
2. One or two drops of colcemid were added for overnight incubation or 10 µl colcemid per millilitre media for minimum three hours of incubation. Colcemid is toxic and depolymerise microtubules. It limits the formation of microtubules thus cells arrest in the metaphase. The duration of incubation depends on the cell line.
3. After the colcemid incubation the media was sucked off and a trypsinization process described in 2.1.1., steps three to five, followed.
4. The cell suspension was transferred to a “nunc” tube and centrifuged for 10 minutes by 1.500 rpm.
5. The supernatant was removed and the cell pellet was dissolved in 8 ml 0,8 % hypotonic solution, tri-sodiumcitrat-dihytrat, and incubated for 20 to 30 minutes at 37 °C. The hypotonic solution helps to bounce the cells.
6. The tube was centrifuged for 10 minutes by 1.500 rpm.
7. The supernatant was sucked off, but not all approximately 1 ml was left above the pellet. Vortexing and tapping the tube on the table surface helped to dissolve the pellet which is important for further procedure. To fix the cells between 4 to 6 ml “fixing solution” (methanol:aceticacid, 3:1) were added very slowly to the cell pellet. Starting with a few drops according to carefully mixing and vortexing. Everything which is not dissolved will be fixed.

8. A centrifugation step for 10 minutes by 1.200 rpm followed.
9. The supernatant was removed and 4 to 6 ml “fixing solution” were added.
10. Same centrifugation program as described in step 8.
11. Steps 9 and 10 were repeated. To be sure that all cells were fixed.
12. After the fixation process the supernatant was removed and the cell suspension was diluted in the “fixing solution”.
13. The suspension was dropped on the labelled glass slides. If the solution looks milky four drops are enough for one slide.
14. The slides were dried on a warming plate.
15. Metaphase slides were stored in 70 % ethanol in the fridge by 2 - 8 °C.

2.3. Part 1 - Giemsa staining experiment

Giemsa staining is used to detect chromosomal instability like loss or gain of whole chromosomes or bigger segments. For the study on the chromosomal stability three “generations” of two tumor cell lines were considered. All grew from one single cell. Two protocols for isolating single cell colonies and single cells were tried. The outline of this experiment which simplifies the understanding is illustrated in Fig. 1.

Materials and chemicals:

- Well Plates (96-, 24-, 12-, and 6 well)
- Culture dishes (10 cm)
- Culture plate (6 cm, 10 cm)
- Cloning Cylinders (diameter 6, 8, and 10 mm)
  (Bel-Art Products, cat. no. 18982)
- Pipettus
- Pipettes (10 ml and 5 ml)
- Pipettes + tips
- Falcons (15 ml)
- Centrifuge
- Incubator (37 °C and 60 °C)
- Microscope
- Silicone grease

- Phosphate buffered saline (PBS) (Sigma Aldrich, cat. no. P5368)
- Media (see Tab. 2)
- Trypsin-EDTA (Invitrogen, cat. no. 616260)
- Ultrapure bovine serum albumin (BSA) [50 mg/ml]
  (Invitrogen, cat. no. AM2616)
- 2 x Saline-sodium citrate buffer (SSC) diluted from 20 x SSC
  Recipe for 1 l 20 x SSC stock solution:
  - 175,32 g Sodium chloride
  - 8,23 g Sodium di hydrate
- Staining buffer
- Giemsa staining solution
- Bi distilled water
- Ethanol (Merck, cat. no. 8187602500)
- Immersion liquid (Leica, cat. no. 11513859)

2.3.1. Methods to isolate single cells and single cell colonies

Two methods for isolating single cells were tested. On the hand a procedure which allows the isolation of single cell colonies and on the other hand isolation of single cells.

2.3.1.1. Isolation of cell colonies with cloning cylinders

This protocol was adapted from the protocol of Sigma Aldrich “Use of Corning Cloning Cylinders for Harvesting Cell Colonies”.

1. The cells of interest needed to be cultivated in a very low density in a 10 cm culture plates.
2. If the colony grew for a few days depending on the cell line, the isolation could start. The colony should include enough cells to transfer and culture them in a multi well plate.
3. The media was sucked off and the culture plate was rinsed twice with PBS.
4. A cloning cylinder was carefully pressed into silicone grease. The silicone grease had to be only on the bottom of the cloning cylinder. The cylinder was

set on a colony which was chosen before under the microscope. To be sure that the connection between cloning cylinder and culture plate was leak-proof a gently pressure was needed.

5. 100 µl Trypsin-EDTA were added to the cylinder. The trypsinization process was considered under the microscope. If the enzyme leaked or if the cylinder slided over the cell while positioning the cylinder, and silicone grease was distributed over the colony of interest, this method did not work.
6. If the trypsinisation was successful the cell colony could be transferred to a new culture plate with fresh media. For smaller colonies 12 well plates were preferred and for larger one 6 well plates are possible.

2.3.1.2. Isolation of single cells with a pipette tip

1. Cells of interest were trypsinized as described in chapter 2.1.1. steps three to five.
2. The cell suspension was transferred to a 15 ml falcon and centrifuged for 10 minutes at 1.500 rpm.
3. After centrifugation the cell pellet was diluted in PBS.
4. 8 µl of 1 % bovine serum albumin (BSA) were distributed on the bottom of a 6 cm culture plate. BSA reduces the binding of the cell to the culture plate surface.
5. 1 ml PBS and a few drops of the PBS cell suspension were added to the 6 cm culture plate. The density of the cells had to be very low otherwise an isolation of single cells was not guaranteed.
6. The culture plate was placed under the microscope and with a calm hand and a small tip (10 µl pipette) single cells were transferred to a 96 well plate. In each well of the 96 well plate 80 µl media were provided.

2.3.2. Giemsa staining protocol

1. The prepared metaphase slides, process described in 2.2.2. had to alter for two days at room temperature or for one hour at 60 °C.
2. Altered slides stayed overnight in 2 x SSC at 60 °C.

3. On the next day the slides were stained with a solution containing 20 ml staining buffer, 76 ml bi distilled water, and 4 ml concentrated giemsa staining solution. Slides were stained between 6 to 8 minutes. After the staining the slides were shortly rinsed with distilled water.
4. The back side of the slide was cleaned with ethanol to get rid of the giemsa staining solution and the staining was evaluated under the microscope. If the cells and metaphases are pink or lightly violet coloured the staining is optimal.
5. Stained slides were stored at room temperature.

2.4. Part 2 – Fluorescence in situ hybridization (FISH) experiment

Fluorescence in situ hybridization is a method to detect structure and segmental positions on the chromosomes with a labelled probe.

Materials and chemicals:

- Pipettes + tips
- PCR reaction tube
- Thermo cycler
- Thermomixer (42 °C and 78 °C)
- Vortexer
- Centrifuge
- Gel electrophoresis apparatus
- Microscope
- Prepared metaphase slides (see 2.2.2.)
- Diamond graze
- Water bath (37 °C and 72 °C)
- Cuvettes
- Eppendorf tubes (1,5 ml)
- Freezer (-70 °C)
- Fixo gum
- Cover glass slide (24 x 24 mm)
- Humid chamber
- Nick Translation Kit (Vysis, cat. no. 32-801300) (see Tab. 3 and Tab. 5)

- Insert containing centromere of chromosome 11
- BAC clone containing the origin of replication lamin B2
- 100 bp molecular ruler [100 µg/ml] (Bio-Rad Laboratories, cat. no. 170-8202)
- Biozym LE Agarose (Biozym, cat. no. 840004)
- Ethidiumbromid [10mg/ml] (Sigma, cat. no. 7637)
- 2 x Saline-sodium citrate buffer (SSC) diluted from 20 x SSC
- 20 x Saline-sodium citrate buffer (SSC) pH 7

  Recipe for 1 l 20 x SSC stock solution:
  - 175,32 g Sodium chloride
  - 8,23 g Sodium di hydrate
- 1 M Hydrogene chloride (HCl)
  - 8,7 ml 37 % HCl
  - 91,3 ml bi distilled water
- 0,01 M Hydrogene chloride (HCl) diluted from 1 M HCl
- Pepsin
- Phosphate buffered saline (PBS) (Sigma Aldrich, cat. no. P5368)
- Ethanol row (70 %, 85 %, and 100 %) at room temperature
- Ethanol row (70 %, 85 %, and 100 %) on ice
- 70 % formamide/2 x SSC pH 7,0
- 3 M sodium acetate (NaAc) pH 5,2
- 100 % Ethanol (Merck, cat. no. 8187602500)
- Human Cot-1 DNA [1 mg /ml] (Invitrogen, 15279-101)
- Hybridization buffer (Vysis, cat. no. 410574)
- Wash solution 1
  - 950 ml bi distilled water
  - 20 ml 20 x Saline-sodium citrate buffer (SSC)
  - 1,8 ml Tween 20 (Sigma Aldrich, cat. no. P7949)
- Wash solution 2
  - 840 ml bi distilled water
  - 10 ml 20 x Saline-sodium citrate buffer (SSC)
  - 6 ml Tween 20 (Sigma Aldrich, cat. no. P7949)

- 4',6-diamidino-2-phenylindole (DAPI) staining solution
    - 10 ml 4 x SSC
    - 2 % Tween 20
    - 1,5 µl DAPI concentrated solution
- Immersion liquid (Leica, cat. no. 11513859)

2.4.1. Labelling of probes

Two probes were labelled, one centromere probe of chromosome 11 which was labelled with Spectrum Orange and a BAC clone containing the origin lamin B2, which is located on chromosome 11. The BAC clone was labelled with Spectrum Green.

For the designing of the labelled centromere probe an insert amplifikation reaction was used. Reagents which were used for this reaction are listed in Tab. 3.

10 x PCR buffer	2,0 µl
5 mM $MgCl_2$	0,6 µl
5 mM dNTPs	1,0 µl
W1 %	1,0 µl
pUCF	0,4 µl
pUCR	0,4 µl
H_2O	14,4 µl
Taq polymerase	0,2 µl
Insert (centromere ofchromosome 11) diluted 1:10	2,0 µl

Tab. 3 Reagents used for the amplification reaction [Reprinted from the Nick Translation Kit]

All reagents, except the Taq polymerase, were added together in one PCR reaction tube and carefully mixed and vortexed. The correct volume of the Taq polymerase was carefully added to the tube and the solution was mixed through up- and down pipetting strokes. The PCR reaction tube was placed into the thermo cycler and the PCR program, see Tab. 4, was started.

Temperature	Time
95 °C	5 minutes
95 °C	45 seconds
66 °C	45 seconds
72 °C	1 minute
72 °C	5 minutes
8 °C	forever

Tab. 4 Program of the amplification reaction

For labelling the Nicktranslation Kit from Vysis was used. The labelling was pipetted on ice. Components for this reaction, see Tab. 5. Components were pipetted together in a PCR reaction tube and briefly vortexed and centrifugated before the Nicktranslation enzyme was added.

Nuclease free water	17,5 µl – x µl
1 µg extracted DNA (BAC clone or centromerprobe from the insert amplification)	x µl
0,2 mM Spectrum Green or Orange	2,5 µl
0,1 mM dTTP	5,0 µl
dNTP Mix (dAGC)	10,0 µl
10 x Nick translation buffer	10,0 µl
Nicktranslation enzyme	10,0 µl

Tab. 5 Reagents used for the Nick Translation reaction [Reprinted from the Nick Translation Kit]

The reaction tube was placed into a thermo cycler and the program, see Tab. 6, was started.

Temperature	Time
15 °C	6 hours
70 °C	10 minutes
8 °C	forever

Tab. 6 Program for the Nick Translation Kit

To control the labelling the probes and a 100 bp marker were applied on a 2 % agarose gel. After one hour running by 120 V the size of the probes could be estimated under UV light. The majority from the DNA smear was located around 300 bp. The isomeric structures of the fluorophores unicorporated Spectrum Green migrated to the top of the gel. Unicorporated Spectrum Orange migrated down the gel.

2.4.2. Preparation of slides for FISH

1. The hybridization field, the field in which the cells of interest were located was marked with a diamond graze.
2. Under the microscope the time of pepsin digestion for each slide was estimated. Cells which were lightening and surrounded with a lot of plasma, needed a longer digestion than those who appeared grey under the microscope.
3. Each slide was equilibrated for 2 minutes in 2 x SSC at room temperature.
4. Slides were digested with pepsin in 0,01 M HCl at 37 °C in a water bath. The digestion was stopped by putting the slides for a few seconds in a 37 °C PBS.
5. The slides were washed two times for 2 minutes in PBS at room temperature.
6. The washing was followed by an ascending alcohol row (70 %, 85 %, and 100 %) to dehydrate the slides. Each slide stayed 2 minutes in each solution.
7. The slides were dried for approximately 30 minutes.
8. Dried slides were denatured in a preheated 70 % FFA/SSC solution by 70 to 72 °C in the water bath for exactly 2 minutes.
9. The slides were transferred to an ice cold ascending alcohol row (70 %, 85 %, and 100 %) to dehydrate the slides. Each slide stayed 2 minutes in each solution.
10. Before the slides got in contact with the labelled probe they had to dry.

2.4.3. Preparation of probes for FISH

1. For the labelled probe for one FISH experiment following reagents, see Tab. 7, were pipetted together in a 1,5 ml Eppendorf tube.

Chromosome 11 labelled probe (dilution 1:10)	13 µl
BAC clone (containing Lamin B2)	13 µl
Human Cot-1 DNA	10 µl
100 % EtOH	75 µl
3 M NaAc	3 µl

Tab. 7 Labelled probe mixture used for the FISH experiment

2. The tube was placed for one hour at –70 °C for precipitation of the DNA

3. Then the reaction was centrifuged for 30 minutes by 13.000 rpm and 4 °C.
4. The supernatant was carefully removed and the pellet was washed with 70 % ethanol.
5. The following centrifugation step is the same as described in step 3.
6. The DNA pellet was diluted in 8 µl hybridization buffer and to improve the dilution the tube was placed on a thermomixer for 30 minutes at 42 °C
7. The transfer of the labelled probe to another thermomixer preheated at 78 °C was used to denature the probe.

2.4.4. Application of the labelled probes on the prepared slides

1. 8 µl of the labelled probe were applied to the marked region on the glass slide.
2. The field was covered with a cover slip glass (24 x 24 mm). To assure that the whole field is covered with the probe it should be free from bubbles.
3. The cover slip was encircled with a fixo gum to avoid dry out of the probe.
4. The hybridization process was done over night in a humid chamber in a 37 °C water bath. In this time the labelled probe could bind to the denatured material on the glass slide.

2.4.5. Washing slides after hybridization

In this experiment the probes were direct labelled therefore the washing process was without incubation of any antibodies or fluorophores.

1. Before starting the washing process started the cover slip glass and the fixo gum had to be removed.
2. Each slide was placed for 2 minutes in a pre heated 72 °C wash solution 1.
3. The slide was transferred to wash solution 2 at room temperature and stayed there for 1 minute.
4. Incubation for 1 minute at room temperature in 2 x SSC followed.
5. The slides were stained for 50 seconds in 4’,6-diamidino-2-phenylindole (DAPI). DAPI is fluorescent stain which is able to pass through the cell membrane and label fixed and lived cells. DAPI was used to visualize the cell core and metaphases on the slides under the fluorescence microscope.
6. The analysis of the signals was done with a fluorescence microscope.

2.5. Part 3 – Immunohistochemical staining experiment

This chapter describes the method of FANCD2 immunohistochemical staining on meta- and interphase slides, and on formalin fixed and paraffin embedded tissue sections.

Materials and Chemicals:

- Prepared metaphase, interphase, and paraffin fixed tissue slices
- Pipettes + tips
- Vectashield
- Cover glasses (24 x 60 mm)
- Eppendorf tubes (1,5 ml)
- Humid chamber
- Fluorescence microscope
- Water bath (95 °C)
- Container
- Phosphate buffered saline (PBS) (Sigma Aldrich, cat. no. P5368)
- Tris buffered saline (TBS) pH 7,4
    - 50 mM Tris HCl
    - 150 mM NaCl
- 1% bovine serum albumine/phosphate buffered saline (BSA/PBS)
- 0,5 % Triton X-100 (Calbiochem, cat. no. 648466-50ML)
- Aphidicholin (APH) (Baack Laborbedarf, cat. no. APP7633A)
- 4 % Paraformaldehyd/PBS pH 7,0
- Ultra V-Block (Labvision, cat. no. TA-125-UB)
- Antibody Diluent with Background Reducing Components (DAKO GmbH, cat. no. S302283)
- Primary antibody (Rabbit polyclonal to FANCD2, ABCAM, cat. no. ab2187)
- Secondary antibody (Donkey Anti Rabbit IgG Alexa Fluor 488 [2 mg/ml], Invitrogen, cat. no. A21206)
- Ethanol (100 %, 95 %, 70 %, and 50 %) (Merck, cat. no. 8187602500)
- Xylol (100 %) (Roth GmbH, cat. no. 9716.1)
- Xylol/Ethanol (1:1)

- 4',6-diamidino-2-phenylindole (DAPI)staining solution
  - 10 ml 4 x SSC
  - 2 % Tween 20
  - 1,5 µl DAPI concentrated solution
- Citrate buffer pH 6,0
  - 10 mM sodium citrate
  - 0,05 % Tween 20 (Sigma Aldrich, cat. no. P7949)
- Bi distilled water
- Immersion liquid (Leica, cat. no. 11513859)

2.5.1. Immunohistochemical staining procedure for meta- and interphase slides

This protocol was used for aphidicholin and non aphidicholin treated HeLa cells to test the adaption of the technique and to determine if a stress induction leads to an increase of FANCD2 signals. This protocol was applied on BJ PD40, BJ hTERT shp53 pBABE, and BJ hTERT shp53 RAS. The aim was to prove that oncogene expression leads to senescence which is associated to damages in the DNA and those may be detected with a FANCD2 staining.

1. The slides were put into pre warmed 37 °C PBS to equilibrate.
2. The second step was different for interphase and metaphase slides:

   A) **Interphase slides**: To fix cells on the interphase slides, they were incubated in 4 % paraformaldehyd/PBS pH 7 for 10 minutes at room temperature. In the last minute a few drops of 0,5 % Triton X 100 were added to permeabilize the cell membrane.

   B) **Metaphase slides**: Metaphase slides were fixed during the preparation step, described in 2.2.2.. A fixation with paraformaldehyd was not necessary. To permeabilize the cell membrane the slides were incubated for 1 minute with a few drops of Triton X 100. Triton X 100 facilitates the entry of the antibody.
3. The slides were washed four times for 7 minutes in PBS at room temperature, to get rid of the paraformaldehyd and Triton X 100.
4. A blocking with Ultra V-Block for 5 minutes instead of the usual blocking substance BSA increased the specificity of the staining.

5. After the blocking step, incubation with the primary antibody (Rabbit Anti FANCD2, ABCAM) for one hour at room temperature followed. The antibody was diluted 1:600 in an Antibody Diluent with Background Reducing Components. The slides were covered with a cover slip glass to avoid drying.
6. The slides were washed in Ultra V-Block/PBS diluted 1:5, four times for 7 minutes at room temperature. The addition of Ultra V-Block to the PBS in the washing process improved the signal quality and reduced the background.
7. The secondary antibody (Donkey Anti Rabbit IgG Alexa Fluor 488, Invitrogen) was diluted 1:600 in 1 % BSA/PBS. Incubation of the second antibody for one hour in the dark. As mentioned in step 5 the slides were covered with cover slip glasses to avoid drying.
8. The slides were washed four times for 7 minutes in PBS to get rid of all unspecific bound secondary antibodies.
9. For the detection of the interphases and metaphases the slides were stained for 45 seconds in DAPI, embedded in vectashield and covered with a cover slip glass.
10. Stained slides were stored at 4 °C. Storage reduces signal quality.

The way to obtain a functioning protocol was time consuming. During the adaption process different protocols were tried and parameters were changed. The main problem was a high background which leads to the suggestion that the staining may be unspecific.

To adapt the immunohistochemical staining protocol different parameters were changed:

- Time, degree, and temperature of permeabilization
- Time of the washing steps
- With/without fixation with 4 % paraformaldehyd/PBS pH 7
- Dilution of primary and secondary antibodies (1:200, 1:400, 1:600, 1:800)
- Blocking solution (1 % BSA, 5 % BSA, and Ultra V-Block)
- Without primary antibody to check if the secondary antibody binds unspecific

- Dilution of the primary antibody in Antibody Diluent with Background Reducing Components instead of 1 % BSA
- Washing with PBS/0,2 % Tween 20
- Washing with PBS/0,2 % Triton X 100
- With/without pepsin digestion

2.5.2. Immunohistochemical staining procedure for paraffin tissue sections

The aim was to apply the adapted FANCD2 protocol, seen in 2.5.1., on formalin fixed and paraffin embedded tissue sections. This method should give an overview of tumor types which have a failure in the DNA damage response (DDR). If there is a failure in the DDR the protein FANCD2 cannot be modified and the FANCD2 signals increase. Before the staining process started the paraffin fixed tissue sections had to undergo two additional steps deparaffinisation and antigen retrieval.

2.5.2.1. Deparaffinisation

This process includes a deparaffinisation with xylol followed by a rehydration with a descending alcohol row to improve the accessibility of the tissue section.

1. Each slide was put two times for 3 minutes in 100 % xylol.
2. Afterwards the slides were transferred to a 1:1 solution of 100 % xylol and 100 % ethanol.
3. The next step was a descending alcohol row:
    - Two times for 3 minutes in 100 % ethanol
    - 3 minutes in 95 % ethanol
    - 3 minutes in 70 % ethanol
    - 3 minutes in 50 % ethanol
4. To finish the deparaffinisation the slides were rinsed with cold water.

2.5.2.2. Antigen retrieval

Antigen retrieval improves the signalling. It breaks the protein cross links formed through the formalin fixation. In this step hidden antigen sites were uncovered. The used method was the “heat induced antigen retrieval”.

1. The prepared citrate buffer was pre heated in a container in a water bath to approximately 90 to 95 °C. The slides were placed in this buffer for 20 minutes.
2. The container including the buffer and the slides was put on the surface of the working space for 20 minutes to cool down.
3. The slides were cooled for 5 minutes with distilled water and rinsed two times for 2 minutes with distilled water

2.5.2.3. Staining Protocol

The staining protocol was the same as described above in 2.5.1.. The results were not sufficient, the protocol had to be adapted to the tissue sections. Parameters which were changed during the adaption process are:

- Permeabilisation in PBS and 0,25 % Triton X 100 for 10 minutes
- Blocking in TBST/1 % BSA and 10 % normal serum
- Incubation with the primary antibody in PBS/1 % BSA overnight at 4 °C
- Incubation with the primary antibody in TBS/1 % BSA overnight at 4 °C
- Washing with TBS/0,025 % Triton X 100
- Washing with TBS

2.6. Part 4 – Custom made array experiment

A custom made array with common fragile sites, origin of replication, and LOHs was designed. It was necessary to choose which cells should be applied to the array. A pre screen method, an isolation technique for single cells, and a DNA isolation protocol were decided.

2.6.1. Design of the custom made Array

As mentioned the array should include common fragile sites (CFSs). The sequences had to be searched. A detailed list of the location of each CFS, start and end positions, and genes located in this region is part of the appendix. More information about the position of LOHs and origin of replication are available in the appendix.
If all sequences of interest were found they were put together and a custom made array was ordered from Agilent.

2.6.2. Cell selection for the array

A problem which occurred was the selection of cells which should be applied on the custom made array. An array analysis is expansive and the cells of interest needed to be pre screened in advance to get sure, that there are fragile sites in the DNA. Two methods were considered on the one hand RT-PCR and on the other hand FANCD2 staining. Because of the slow growth of the cells no pre screen method was applied on the cells of interest.

2.6.2.1. Pre screen via RT-PCR

The first suggestion was a pre screen with a RT-PCR. Possible RT primers for this experiment were designed. Additional information about the location and sequence of the primer can be found in the appendix. One primer pair was designed for each common fragile site (CFS) region. The main steps for the primer design are:

1. The positions of the CFSs were found with the UCSC genome Browser (Genome Bioinformatics Groups of UC Santa Cruz, http://genome.ucsc.edu)

2. From the obtained results a gene, which is known to be located in the region of the CFS was decided. Genes are coloured and written in blue.
3. The genomic sequence of the gene is necessary for the design of the primers. The primer3 tool (http://frodo.wi.mit.edu/primer3) was used to search primers in the whole genomic sequence. The product size ranged from 95 to 105 bp, the primer size ranged from 18 to 27 bp, and the melting temperature of the primer should be in the region of 58 °C and 63 °C with one degree of difference. The tool picked up primers which fulfil the conditions above.
4. To check if the primer were working in a correct way a UCSC In Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr) was done. The forward and reverse primers of each primer pair had to be submitted. If the product size of the In Silico PCR and the Primer3 tool were the same the received primer product had to be blated to get sure that the product is unique in the genome.
5. For the blat the Human Blat Search Tool from UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat) was used. This tool submitted the primer product with the whole human genome and displayed a list with the percentage of identity, position, and the product size. The optimal case is one product with 100 % identity. Otherwise the primers do not bind specifically and the results of the RT-PCR are not satisfying.

2.6.2.2. Pre screen via FANCD2 staining

The immunohistochemical method was considered as pre screen method for the array experiment. Literature demonstrated that FANCD2 signals are increased in genetic material associated with tumorigenesis. For this experiment the adapted protocol from Part 2.5. was the basis.

Pre screen methods should be done on cell populations grown from one single cell to obtain a pure culture. Genomic changes may not be the same in the whole cell population. Therefore single cells from the BJ PD40, BJ hTERT shp53 pBABE, BJ hTERT shp53 RAS, HGMDFN090 with/without aphidicholin (APH) treatment, HCT116, HT29, and HeLa cell line were isolated according to the protocol described in 2.3.1.2. HGMDFN090 was used as human fibroblast control cell line.

The APH treatment involved an incubation period of 72 hours with 0,3 µM APH followed by a 3 days recovery period. Afterwards single cells were isolated.

The cells were isolated, but not pre screened because the proliferation rate was low and the time available for the thesis was limited. At the end of my internship the cultivated cells were delivered to my successor.

2.6.3. Isolation of genomic DNA

The isolation and the following steps of the array experiment were not done because of time limitation. The amount of cells which are needed for isolation genomic DNA and to do an array was estimated with the cell line HCT116 and HT29. Cells were isolated via two different protocols on the one hand with a Billatest Kit and on the other hand with a Quiagen protocol.

2.6.3.1. Billatest DNA Tissue Kit (Bilatec AG) protocol

The principle of this kit is the use of magnetic beads which allows the isolation of high yield and pure DNA. The DNA binds to the magnetic beads through the whole process of washing thus the remove of the supernatant is simplified through the application of the magnetic separator. The elution buffer separated the DNA from the magnetic beads the purified high yield isolated DNA thus is included in the elution buffer.

Materials and Chemicals:

- Magnetic seperator
- Thermomixer
- Eppendorf tubes (1,5 ml)
- Pipette + tips
- RNAse [10 mg/ml]
- Proteinkinase K [10 mg/ml]
- Billatest DNA Tissue Kit (Billatec AG, cat. no. 110402) including: lysis buffer, magnetic beads, binding buffer, washing buffer A, washing buffer B, washing buffer C, elution buffer

1. The cell pellet was overnight digested with 100 µl of lysate buffer containing proteinkinase K on a thermomixer at 56 °C and 600 rpm. E.g. reagents used for 5 samples 500 µl lysis buffer and 12,5 µl proteinkinase K [10mg/ml] were needed. If the lysis overnight was not successful, more proteinkinase K was added.
2. To get rid of the RNA a RNAse digestion with 2 µl RNAse for 5 minutes at room temperature followed.
3. After the two digestion processes 263 µl binding buffer and 75 µl magnetic beads were added to the suspension and carefully mixed through up and down pipetting. The mixture was added to the lysate and mixed through 10 pipetting strokes.
4. The sample was incubated 10 minutes at room temperature. In this time the DNA binds to the magnetic beads.
5. To separate the DNA – magnetic beads complex from the supernatant the tube was put for 2 minutes in a magnetic separator and the supernatant was removed.
6. The next two steps were washing processes with buffer A and buffer B. Both followed the same procedure. 500 µl of the washing buffer were added to the sample mixed through 15 pipetting strokes, the tube was put into the magnetic separator, and the supernatant was removed.
7. After removing buffer B, the tube stayed in the magnetic separator and 1 ml of washing buffer C was added for exactly 90 seconds.
8. Buffer C was carefully removed and the beads were resuspended in 25 µl elution buffer and incubated for 10 minutes at 55 °C.
9. To improve the pureness of the DNA the tube was placed two times for 2 minutes into the magnetic separator and the eluate which includes the DNA was transferred to a fresh tube.

2.6.3.2. Isolation of Genomic DNA from Tissue (Qiagen – QIAamp DNA Micro Handbook)

The principle of the Qiagen Kit is a bind-wash-elute procedure done with special QIAamp MinElute columns and centrifugation steps. DNA binds to the membrane of the QIAamp MinElute column through the washing process. The elution buffer is able to transfers the DNA from the membrane to the collection tube.

Materials and Chemicals:

- Vortexer
- Thermomixer
- Centrifuge
- Eppendorf tubes (1,5 ml)
- Pipette + tips
- Ethanol 100% (Merck, cat. no. 8187602500)
- RNAse [10 mg/ml]
- QIAamp DNA Micro Kit (Qiagen, cat. no. 56304) including: QIAamp MinElute columns, collection tubes (2ml), buffer ATL, buffer AL, buffer AW1, buffer AW2, buffer AE, carrier RNA, and proteinkinase K

1. To digest the cell pellet 180 µl ATL buffer and 20 µl proteinkinase K were added after puls vortexing for 15 seconds the overnight incubation on a thermomixer at 56 °C and 600 rpm started.
2. If the proteinase K digestion was successful a RNAse digestion, described in 2.6.3.1. step 2, followed.
3. 200 µl AL buffer were added and the suspension was mixed 15 seconds by puls vortexing.
4. 200 µl of 100 % ethanol were added and mixed by puls vortexing, and the suspension was incubated for 5 minutes at room temperature. The solution should be homogenous.
5. The QIAamp MinElute column was put in a collection tube and the homogenous solution was transferred from the 1,5 ml Eppendorf tube to the QIAamp MinElute column without wetting the rim. The lid of the tube was carefully closed and the sample was centrifuged for 1 minute at 8.000 rpm.

6. Adjacent the flow-through was discarded, the QIAamp MinElute column was placed in a new capture tube, and 500 µl buffer AW1 were added following by a centrifugation step for 1 minute at 8.000 rpm.
7. The following step is the same like step 6 just indeed of the 500 µl buffer AW1 500 µl buffer AW2 were added.
8. After discarding the flow-through the QIAamp MinElute column was transferred to a new capture tube and centrifuged for 3 minutes at 14.000 rpm to dry the membrane.
9. To eluate the DNA from the filter the QIAamp MinElute column was placed in a new 1,5 ml Eppendorf tube, 20 µl AE buffer were added, and incubated for 5 minutes at room temperature. The sample was centrifuged for 1 minute at 14.000 rpm to transfer the isolated DNA to the bottom of the Eppendorf tube.

2.6.4. Isolation of single cells for the array

Cells from the BJ PD40, BJ hTERT shp53 pBABE, BJ hTERT shp53 RAS, HCT116, HT29, and aphidicholin treated and non aphidicholin treated HGMDFN090 cell line were isolated with the protocol described in 2.3.1.2. and cultured in multi well plates.

3. Results

3.1. Part 1 - Giemsa staining experiment

The Giemsa staining was used to get information about the chromosome stability of two known tumor cell lines, HT29 (colon carcinoma cell line) and HCT116 (colorectal carcinoma).

HT29

The results of the HT29 cell line show that the first single clone is very unstable the number of chromosomes varies from 69 to 75. Problems in culturing the cells lead to a stop of the experiment with the staining of the subclones. In the last period of the experiment a bacterial contamination occurred in the HT29 cell line, to get rid of the contamination a higher penicillin/streptomycin concentration was added to the media, it was not successful. In order to keep the whole cell culture free of bacterial the contaminated cells, materials, and media had to be sterilised immediately.

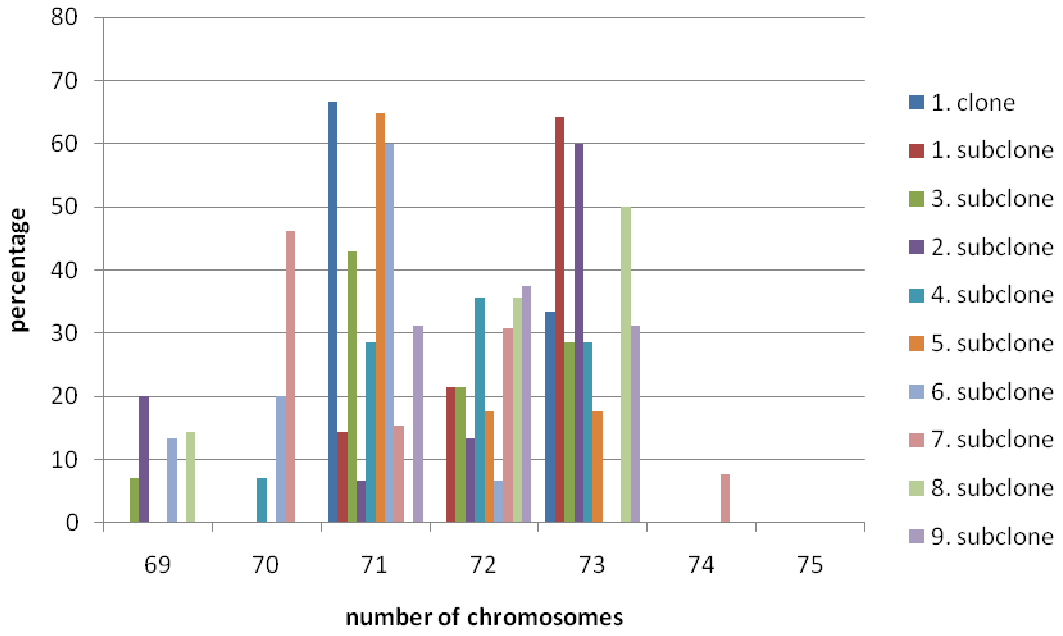

Fig. 14 Experimental data of the giemsa staining of the HT29 cell line: the data is taken from the second experiment, the first clone and the nine isolated subclones.

Fig. 14 illustrates the dissemination of the numbers of chromosomes in the first clone and isolated subclones. There is no preferred chromosome number, but the majority of analysed metaphase spreads has between 70 and 73 chromosomes.

HCT116

The experimental data of HCT116 shows that this cell line is stable in the first clone, in the subclones, and in the sub-subclones. The main part of HCT116 around 80 to 90 % has 45 chromosomes. A few percentages have a decreased or increased chromosome number (45 +/- 1). Fig. 15 to Fig. 17 illustrate graphs with a reduced data from the HCT116 giemsa staining experiment.

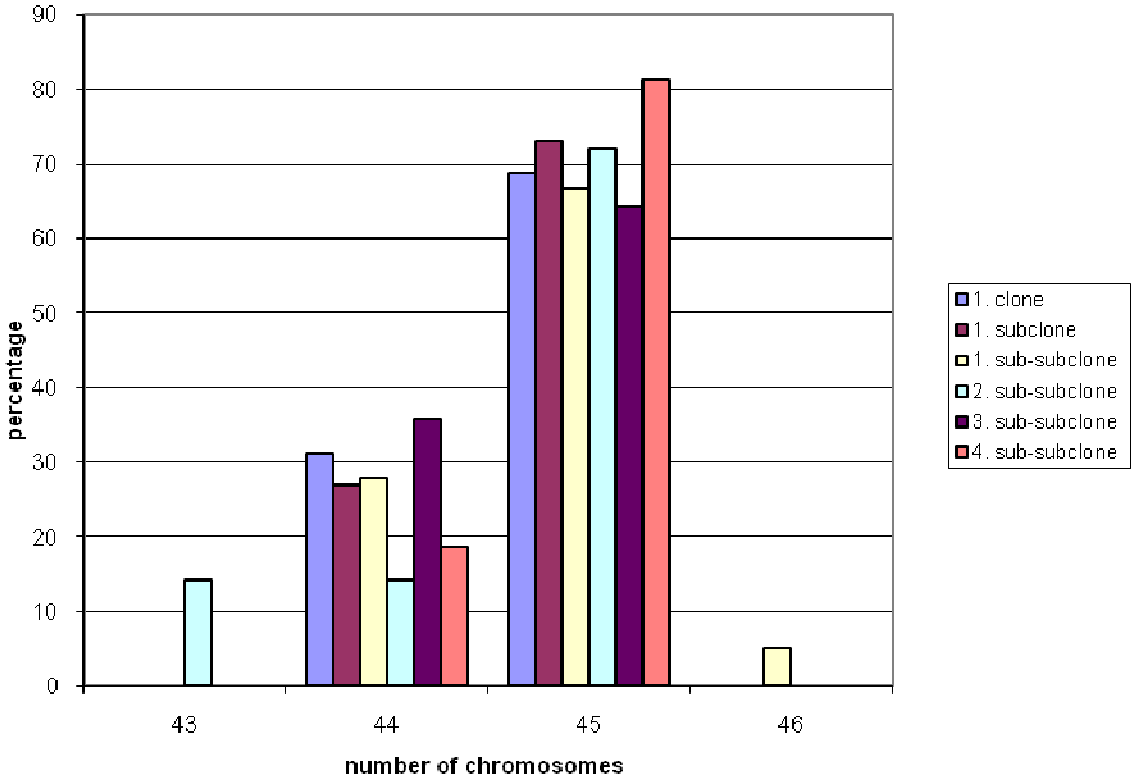

Fig. 15 Experimental data of the giemsa staining experiment of the HCT116 cell line: The data is from the fourth experiment, the first clone, the first subclone and four sub-subclones.

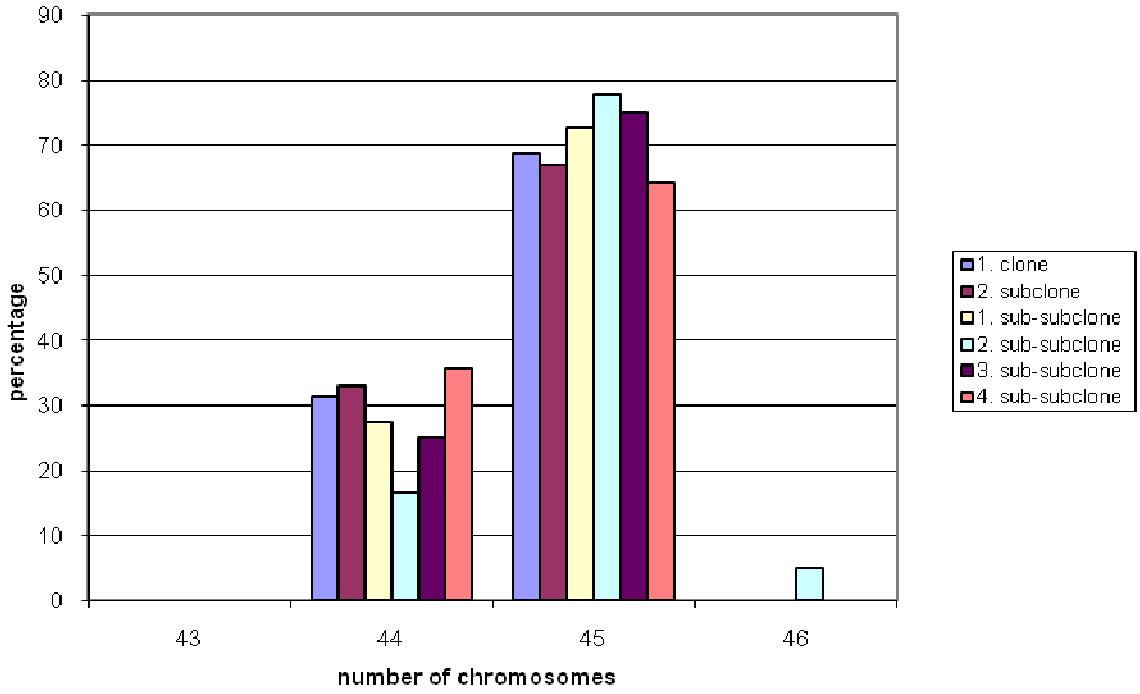

Fig. 16 Experimental data of the giemsa staining experiment of the HCT116 cell line: The data is from the fourth experiment, the first clone, the second subclone and four sub-subclones.

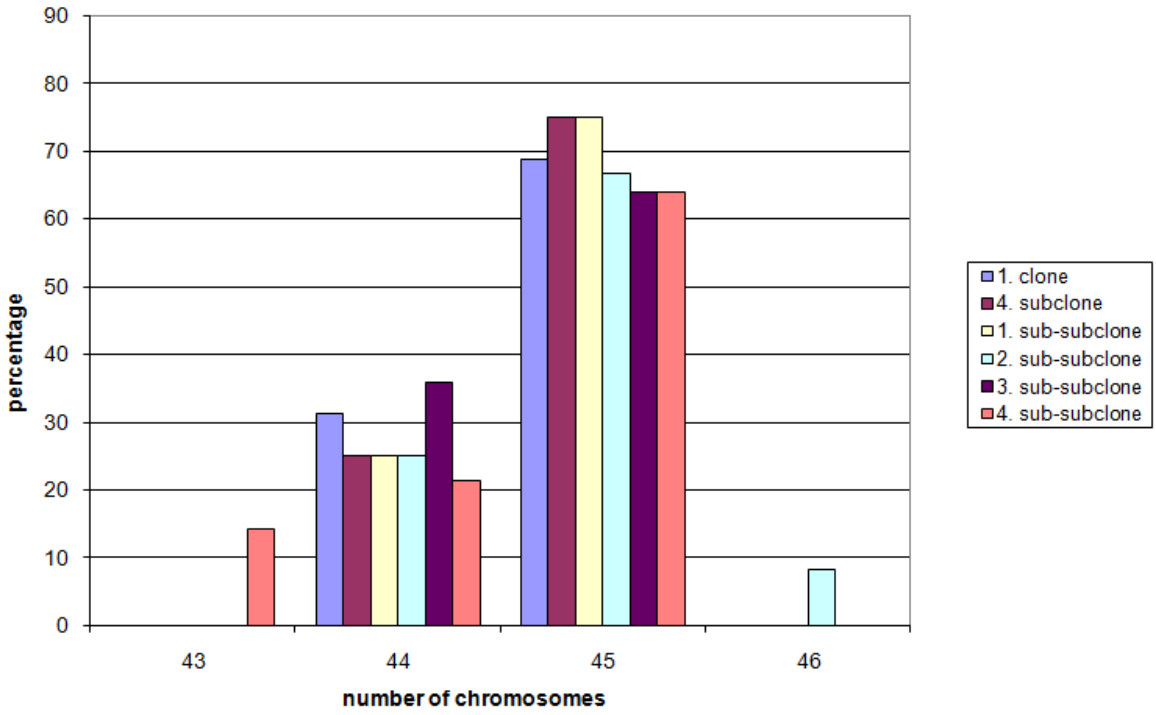

Fig. 17 Experimental data of the giemsa staining experiment of the HCT116 cell line: The data is from the fourth experiment, the first clone, the fourth subclone and four sub-subclones.

3.2. Part 2 – Fluorescence in situ hybridization (FISH) experiment

The FISH experiment was done on normal human fibroblast cells, BJ PD40, BJ shp53 RAS, and BJ shp53 pBABE cells. This experiment shows that oncogene expression leads to oncogene induced senescence (OIS). OIS cells have an increased number of polyploid cells. The thesis should confirm the study of di Micco et al. which describes that oncogenic RAS expression leads to an increased number of active replicons which displays in multiple DNA origin of replication signals in the FISH analysis.

The results of the FISH experiment are:

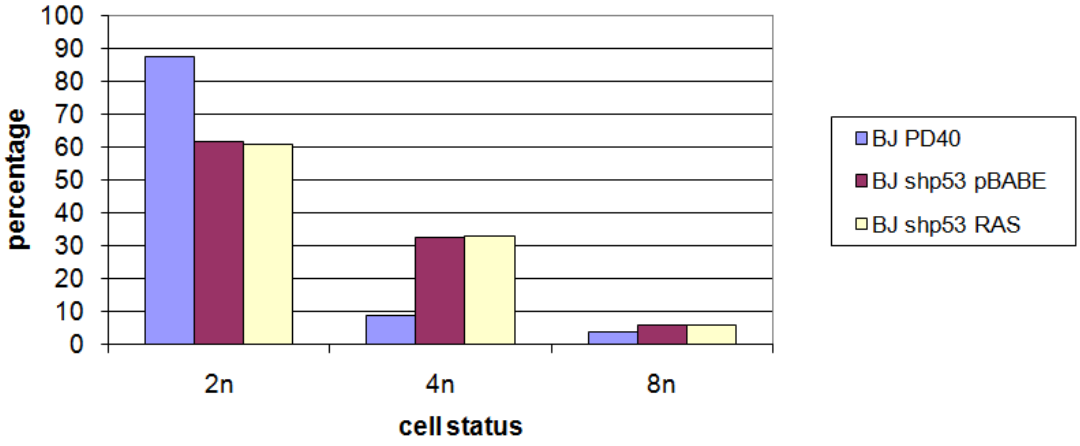

Fig. 18 Results of the FISH experiment of BJ PD40, BJ shp53 pBABE, and BJ shp53 RAS cells. 2n = diploid, 4n = tetraploid, 8n = polyploid

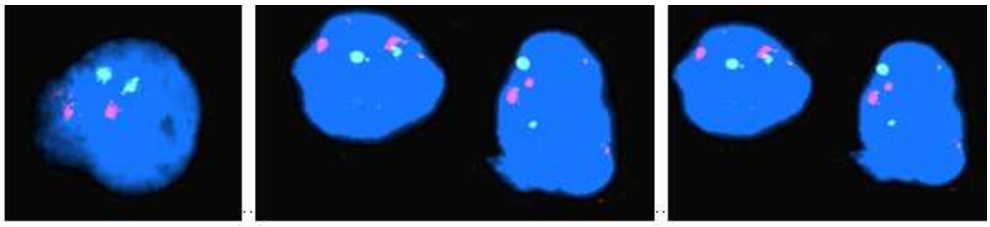

Fig. 19 Fluorescence pictures of PD40 cells: green labelled signals are the centromeres on chromosome 11 and red labelled signals are those from the origin of replication LAMIN B2 which is also located on the chromosome 11.

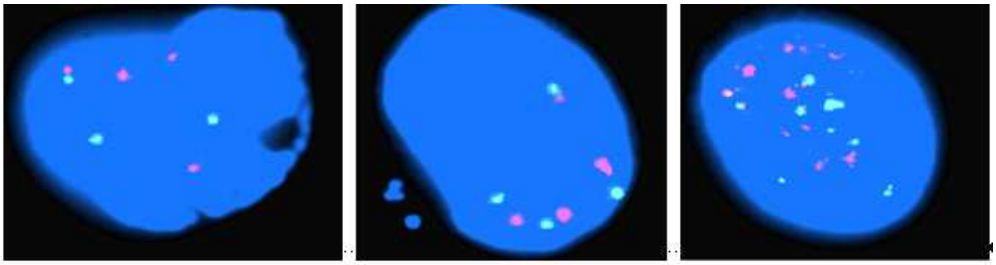

Fig. 20 Fluorescence pictures of BJ shp53 RAS cells: green labelled signals are the centromeres on chromosome 11 and red labelled signals are those from the origin of replication LAMIN B2 which is also located on the chromosome 11. RAS expressing cells are bigger and show a change in the morphology.

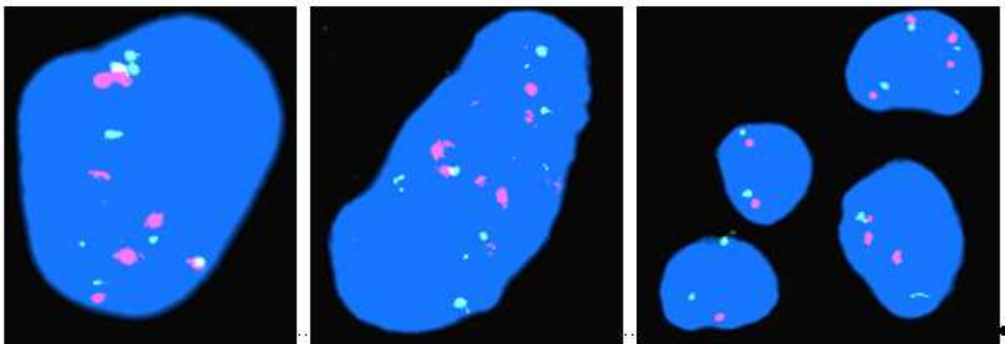

Fig. 21 Fluorescence pictures of BJ shp53 pBABE cells: green labelled signals are the centromeres on chromosome 11 and red labelled signals are those from the origin of replication LAMIN B2 which is also located on the chromosome 11. From the number of signals pBABE cells are in the middle of PD40 and RAS, just some of the pBABE cells show a change in the cell shape and morphology.

As explained RAS causes senescence of cells. Cells from the normal control cell line BJ PD40 are to 87,7% diploid. RAS expression and oncogene induced senescence (OIS) can be seen as reason for the different percentage of diploid, tetraploid, and octoploid cell status.

In the RAS expressing cell line 33 cells with a tetraploid status are detected. 20 of these cells are really tetraploid, 4 signals of the replication origin lamin B2 and 4 signals of the centromere probe were detected. 13 cells have 2 signals of the centromere and 4 signals of lamin B2. As mentioned a firing of DNA replication origins more than once per cell cycle induces DNA damage response (DDR) in senescent cells. In 13 % of the tetraploid RAS cells the DDR is induced. 6 % of the RAS senescent cells show more than 4 signals for both the replication origin and the centromere. These cells are polyploid and the number of polyploid cells increases with the senescence.

To proof the data from the FISH experiment a FACS analysis of the fibroblast cell lines was done. The applicant of this analysis was a scientist of a collaborator institute.

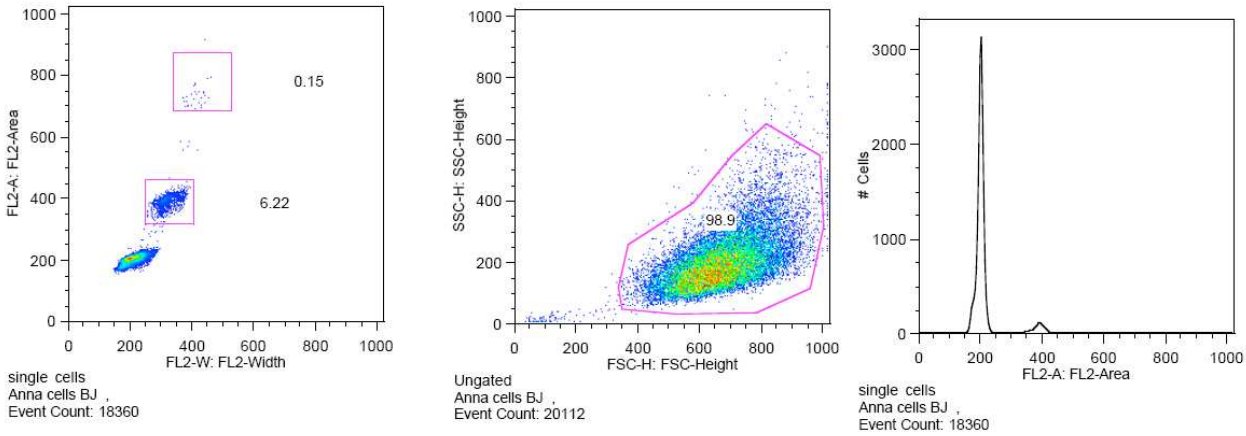

Fig. 22 FACS results of the BJ PD40 cell line: The histogram pointed out that nearly all of the measured cells are in the diploid phase and only a not mentionable part is tetraploid.

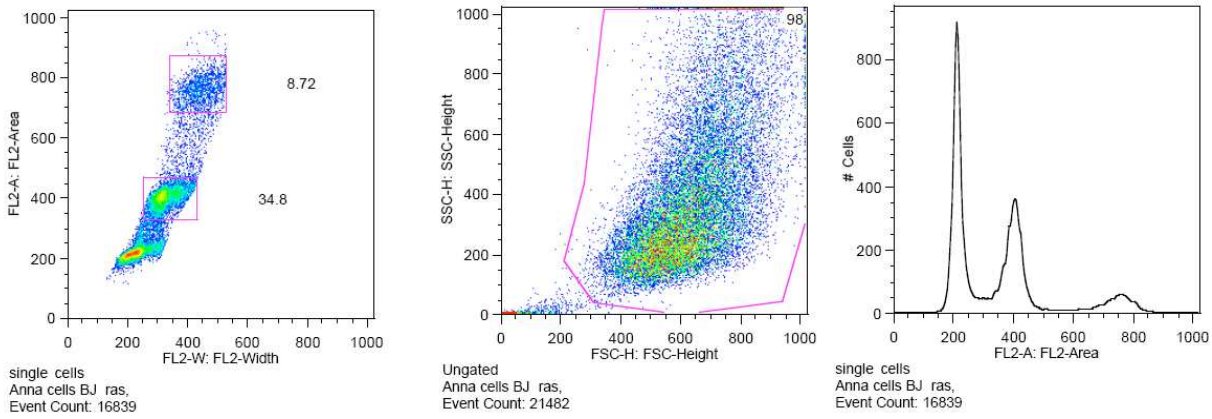

Fig. 23 FACS results of the BJ shp53 pBABE cell line: The histogram illustrates that the major part of the cells are in the diploid phase, some are tetraploid and just a small amount is in the polyploidy phase.

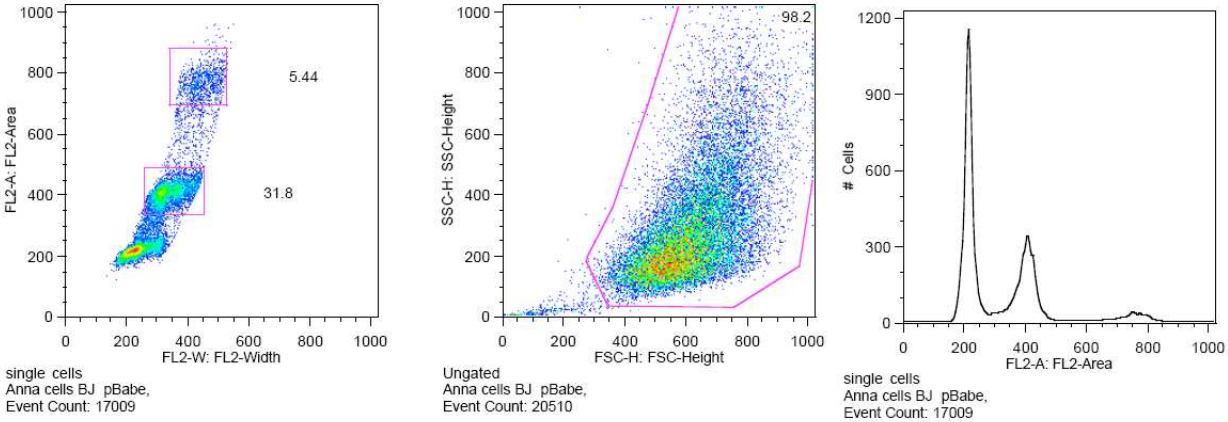

Fig. 24 FACS results of the BJ shp53 RAS cell line: The histogramm is similar to the one of the BJ shp53 pBABE cell line and it shows that a lot of cells are still in the diploid status but there exist a higher amount in the tetraploid and polyploid phase.

The comparison of the FISH and the FACS analysis show the same result:

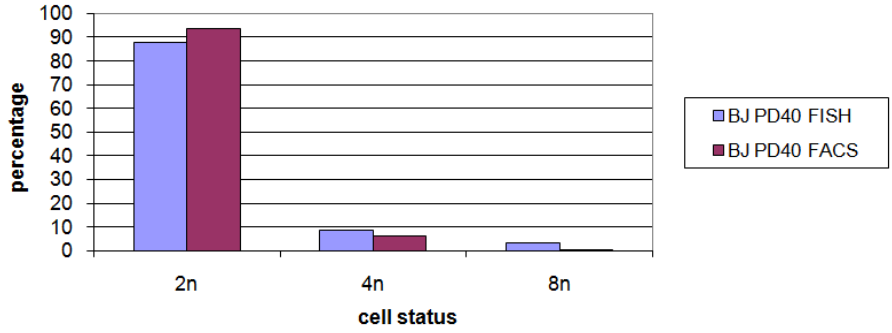

Fig. 25 FISH vs. FACS analysis of BJ PD40 cells
2n = diploid, 4n = tetraploid, 8n = polyploid

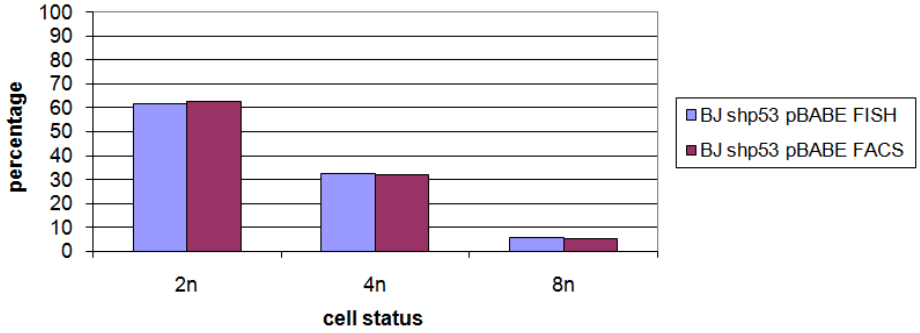

Fig. 26 FISH vs. FACS analysis of BJ shp53 pBABE cells
2n = diploid, 4n = tetraploid, 8n = polyploid

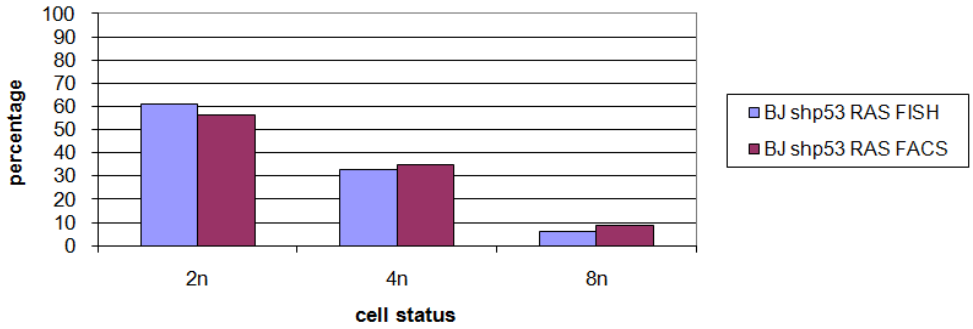

Fig. 27 FISH vs. FACS analysis of BJ shp53 RAS cells
2n = diploid, 4n = tetraploid, 8n = polyploid

Fig. 25 to Fig. 27, comparison of FISH and FACS analysis, show that in both experiments the data of the pBABE and RAS cell line are nearly identical. Around 60 % of the RAS and pBABE cell line are diploid, approximately 30 % are tetraploid and less than 10 % are polyploid. The control cell line BJ PD40 is to approximately 90 % diploid. This analysis shows no mentionable changes in the control cell line.

3.3. Part 3 - Immunohistochemical staining experiment

The immunohistochemical staining of the protein FANCD2 was applied on HeLa cells, human fibroblast cell lines, BJ PD40, BJ hTERT shp53 pBABE, and BJ hTERT shp53 RAS and on formalin fixed and paraffin embedded tissue sections.

3.3.1. FANCD2 staining of APH and non APH treated HeLa cells

HeLa cells are used as control cell line. One part was treated with aphidicholin (APH), a selective DNA polymerase inhibitor, and the other part was used as non treated control group. The aim was to show that an induced stress, through aphidicholin treatment, increases the numbers of FANCD2 signals per cell.

spots/cell	with APH [counted: 100]	without APH [counted: 100]
0		63
1	3	27
2	8	10
3	7	
4	18	
5	8	
6	30	
7	6	
8	19	
9	0	
10	1	

Tab. 8 Data from the FANCD2 staining of aphidicolin and non aphidicolin treated HeLa cells

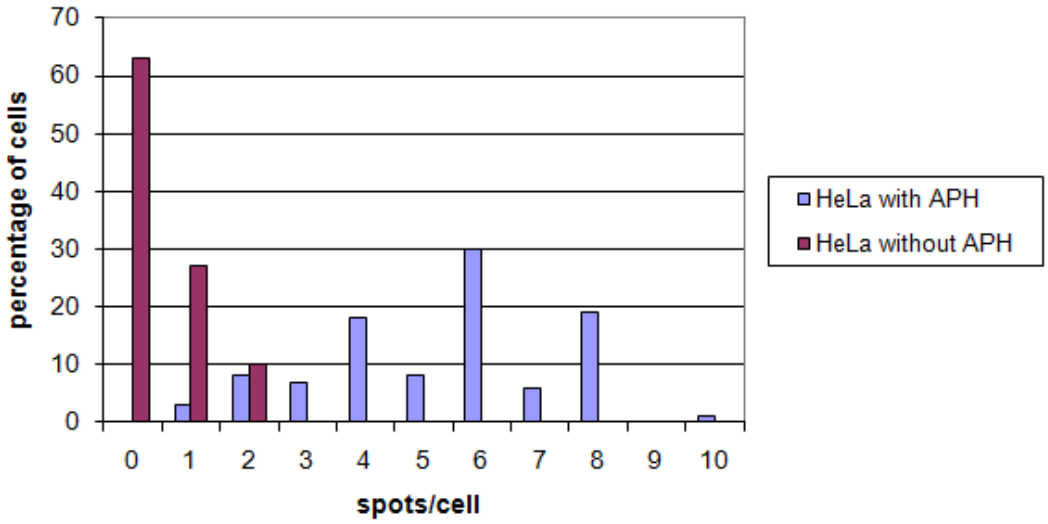

Fig. 28 FANCD2 staining of aphidicolin and non aphidicolin treated HeLa cells: On the horizontal bar the number of counted signal spots per cell are printed and on the vertical one there is the percentage.

Fig. 28, FANCD2 staining of APH treated and non APH treated HeLa cells, illustrates that the major part of the non treated HeLa cells have around zero or one signal. 10 out of 100 counted cells have two signals, see Tab. 8. Fig. 28 shows how effecient a 24 hours aphidicholin treatment followed by a two days recovery period is. Treated HeLa cells have more FANCD2 signals per cell about four to eight signals per cell were counted. Some cells have only one, two, or three signals that may be the result of the recovery period. During this time period the cell has the possibility to repair and refresh itself from the induced stress. Fig. 29 and Fig. 30 show some microscope pictures of HeLa cells with and without aphidicholin treatment.

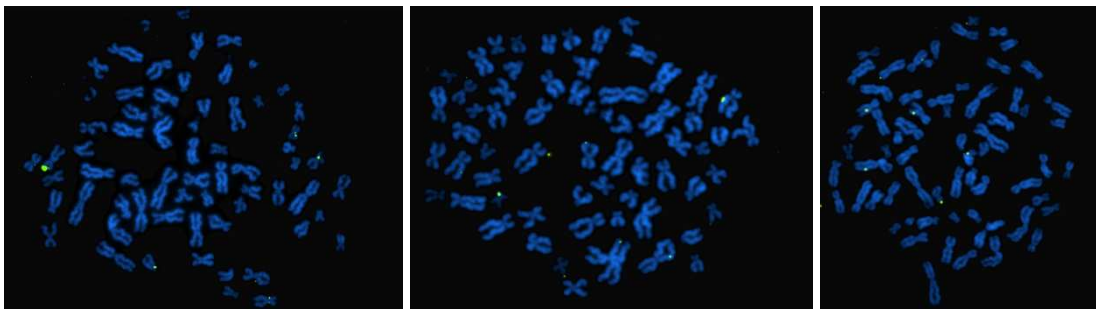

Fig. 29 FANCD2 foci on aphidicholin treated Hela metaphases. Hela cells were treated with 0,4 µM aphidicolin for 24 hours

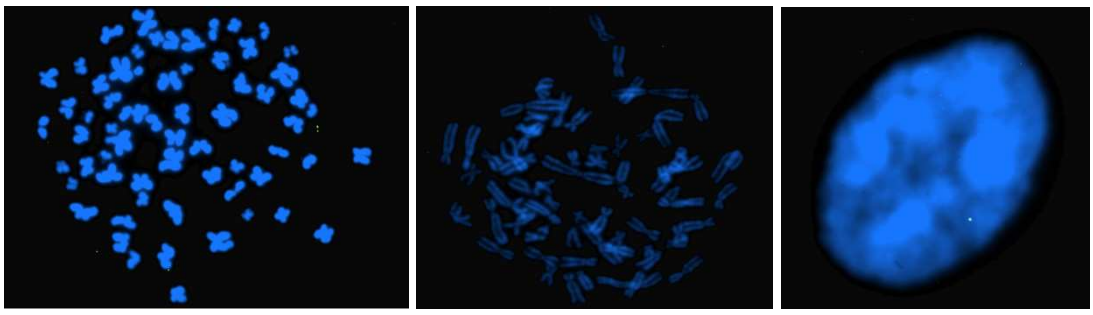

Fig. 30 Meta- and interphases with FANCD2 foci of non aphidicholin treated Hela cells

3.3.2. FANCD2 staining of BJ PD40, BJ hTERTshp53 pBABE, and BJ hTERT shp53 RAS cells

To imagine the consequence of senescence in RAS expressing cells a FANCD2 staining with prepared metaphase slides of human fibroblast cell lines BJ PD40, BJ hTERT shp53 pBABE, and BJ hTERT shp53 RAS was done.

	BJ PD40	**BJ hTERT shp53 pBABE**	**BJ hTERT shp53 RAS**
signal/cell	number of cells	number of cells	number of cells
0	9	0	0
1	5	7	0
2	8	6	0
3	2	3	0
4	0	4	0
5	0	4	0
6	0	0	2
7	0	0	3
8	0	0	11
9	0	0	4
10	0	0	1
11	0	0	1

Tab. 9 Data from the FANCD2 staining of human fibroblast cell lines, BJ PD40, BJ hTERT shp53 pBABE, and BJ hTERT shp53 RAS

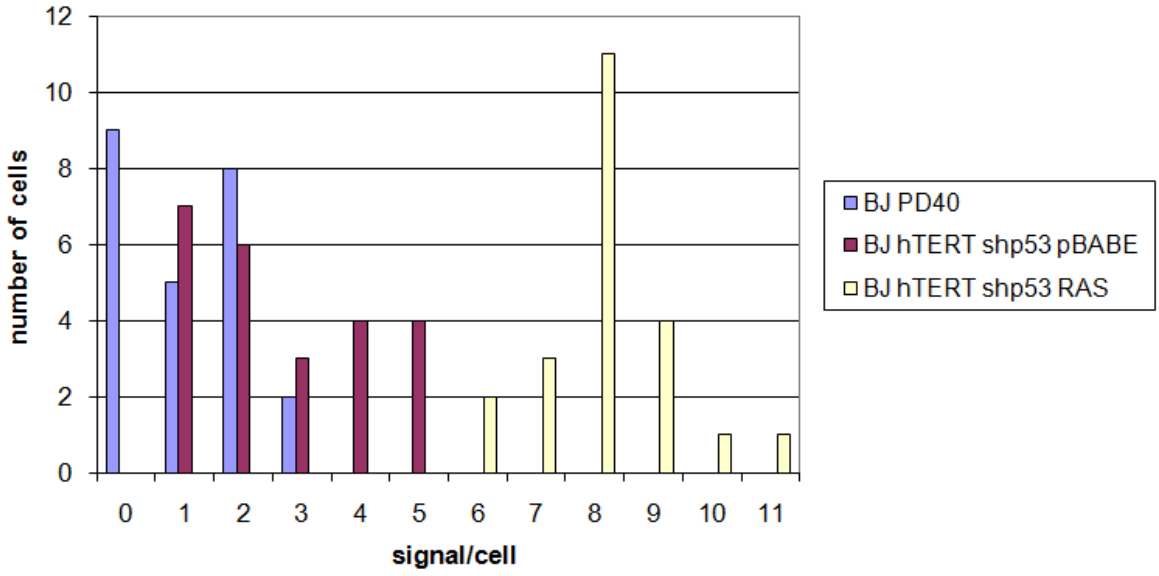

Fig. 31 FANCD2 staining of BJ PD40, BJ hTERT shp53 pBABE and BJ hTERT shp53 RAS

The results are as expected. Fig. 31 shows that the normal fibroblast cell line, BJ PD40 has between zero and two FANCD2 signals per cell. BJ hTERT shp53 pBABE, the cell line with the empty RAS vector, which is the positive control cell line

has between one and five FANCD2 signals per cell. BJ hTERT shp53 RAS, the RAS expressing cell line, has plus minus eight FANCD2 signals per cell. The list of detailed results can be seen in Tab. 9 and graphical demonstrated in Fig. 31. As mentioned oncogene expression leads to senescence which is detectable by senescence associated DNA damage foci. Studies show that DDR is induced by DNA replication origins which are firing more than once per cycle, which occurs in the BJ hTERT shp53 RAS cell line. Senescence caused by the expression of RAS leads to DNA damages which are according to an increased number of FANCD2 signals. The higher number of signals in the pBABE cell line is caused by the insertion of the empty RAS vector thereby changing the normal progression of the cell.

3.3.3. FANCD2 staining of paraffin tissue sections – Lymph nodes and colon cancer

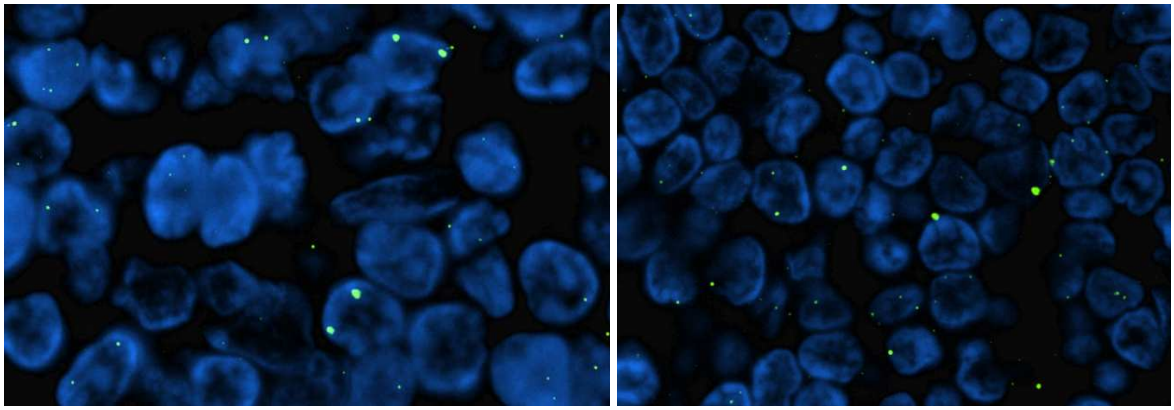

Fig. 32 FANCD2 staining of sections of formalin fixed and paraffin embedded lymph nodes

Lymph nodes are organs which express in their normal way of function the protein FANCD2. Zero to two signals are detectable and the germ center of the lymph nodes show four or more signals. The staining has a high background but the signals are so strong that they are detectable after reducing the background.

The staining of colon tissue showed very weak signals and high background. The protocol was adapted but it did not result in satisfying results. Pictures are not printed in this paper.

3.4. Part 4 – Custom made array experiment

Because of limited time available for the thesis the array experiment could not be finished.

The custom made array was designed. The array includes the region of common fragile sites (CFSs), replication origins, and LOH sites. Tables with the explicit regions can be found in the appendix.
Possible primers for the RT-PCR were designed. A list of designed primers is part of the appendix. It was not sure if a RT-PCR would be a good method to qualify the probes which have fragile sites. Therefore primers were not ordered. The isolation of single cells and culturing started.

3.4.1. Results of the test DNA isolation

From two cell lines HT29 and HCT116 two fractions, the first contained 250.000 cells and the second contained 500.000 cells were used for the DNA isolation experiment.

3.4.1.1. Results of the Billatest Kit

	HT29 250.000 cells	**HT29 500.000 cells**	**HCT116 250.000 cells**	**HCT116 500.000 cells**
ng/µl	143	271	67,6	164
260/280	1,85	1,66	1,75	1,85
260/230	1,51	1,30	1,13	1,87

Tab. 10 Results from the DNA isolation with the Billatest Kit

3.4.1.2. Results of the Qiagen Kit

	HT29 500.000 cells	**HCT116 500.000 cells**
ng/µl	92,69	69,13
260/280	1,83	1,82
260/230	1,98	1,39

Tab. 11 Results from the DNA isolation with the Qiagen Kit

Tab. 10 and Tab. 11 show the DNA concentration and measured ratios of 260/280 and 260/230. Higher concentration is obtained with the Billatest Kit. The result is that 250.000 cells and isolation by using the Billatest protocol yield in enough DNA amount and quality to do an array experiment.

4. Discussion

4.1. Part 1 – Giemsa staining experiment

There exist many different tumor types therefore a detection of aberrations on the DNA level may be a chance to identify potential harmful lesions at an early stage. The aim was to investigate the changes of the karyotype of two tumor cell lines and to verify if the karyotype is stable.

The karyotype of two tumor cell lines was investigated over 12 weeks of proliferation starting from one single cell. The hypothesis was that changes which occur in the subclones, termed “second generation”, may be temporarily and the sub-subclones, “third generation”, may be more identical to the first clone indicating chromosomal instability. If this occurs the cell line has a temporarily unstable karyotype. A closer look at Fig. 15 to Fig. 17, which show the experimental data of the giemsa staining of the HCT116, affirm that the HCT116 cell line has a stable near diploid karyotype. Due to a bacterial contamination of the HT29 single cell clones the karyotypes could not be established of the “third generation”. Only results of the first clone and subclones are available. The question if the karyotype of the HT29 cell line is stable could not be answered with this experiment. No significant results which define if aberrations which do not lead to a growth advantage cause instability and cell death are available. HT29 cell line shows a completely different distribution of the chromosomes and is more unstable than HCT116.

Tumorigenesis is a consequence of changes in the DNA. But is an aneuploid karyotype necessary for tumorigenesis? Thompson and Compton published that solid tumors can be highly aneuploid they acquire whole extra chromosomes or segmental extra parts of one chromosome. [12] The mechanism how cells gain or lose chromosomes is currently unknown. The important failure has to happen during the cell division. Abdel-Rahman et al. published in his study that genomic instability is a consequence of defects in apoptotic pathways. Mismatch repair defects with a replication error, a “RER+” phenotype which is characterized by microsatellite instability was found in 15 percent of sporadic colorectal carcinomas. “RER+” cell lines such as HCT116 typically have a stable near diploid karyotype. “RER–“ cell

lines like HT29 are unstable in chromosome numbers and structures. [27] The results from Abdel-Rahman et al. are identical to those of mine experiments. Both pointed out that the HCT116 is stable in chromosome number and structure and the HT29 is instable.

Yuen and Desai postulated that there is a connection between chromosomal instability (CIN) and aneuploidy. The result was that CIN in cancer cell lines undergo mitotic arrest in response to spindle damage and that the checkpoint pathway is functioning properly. [28] They showed that a loss of spindle checkpoint function is lethal to cells but a partial defect could underlie a CIN phenotype. A failure during the cell cycle is the reason for changes in the chromosome number and structure.

Additional methods and technical advices which should have been used to improve the outcome of the experiment:
Freeze an aliquot of the cell population after isolation of the subclones and sub-subclones. This may allow starting the experiment from a “safe point”, after the occurrence of contamination.
All used liquid materials in the cell culture should be portioned, one aliquot for one day. Thereby a delay of a contamination through the used media would not be given. This would affirm that the parameters of the used media like the pH-value would not change like they may do in succession of storing an opened bottle over a long time.
A critical point is the isolation of single cells with a pipette under the microscope. As consequence of a non 100 percent sterile microscope working space, the microscope was not placed into the lamina, it may happen that a contamination occurs. It would be better to place the microscope into the lamina to minimize the contact of the biological material with the surrounding.
To improve the statement of this experiment detailed information about the karyotype would have been necessary. In order to analyze numerous gains, losses, and translocations in the unstable HT29 cell line a great expertise in karyotyping would be required.
The conclusion of this experiment is that defects which cause genomic instability are selective for each tumor cell line. During the evolution of tumors some acquire one defect and other tumor cell lines acquire more independent defects.

4.2. Part 2 – Fluorescence in situ hybridization (FISH) experiment

The hypothesis was that oncogene induced senescent human fibroblast cells have an increased number of active replication origins. The aim was to adapt a functioning protocol and compare the signals of a labelled centromere probe and a fluorescent labelled replication origin, lamin B2, in human fibroblast cell lines.

Studies show that oncogene induced senescence (OIS) can be triggered by 2 mechanisms on the one hand by activated oncoproteins like RAS and on the other hand by the loss of tumor suppressor proteins. [29] Cellular senescence can be seen as a safeguard mechanism which may prevents aged or abnormal cells from further proliferations. Results, in chapter 3.2., show that oncogene induced senescence results in an increased tetra- and polyploid status. The expression of RAS in human fibroblast cell lines leads to oncogene induced senescence (OIS) which causes a change in shape of cells and senescence associated DNA damage foci. [16]

The consequence of the study of di Micco et al. is that in cells which have a functioning DNA damage response (DDR) the expression of oncogenes leads to cellular senescence. Di Micco and colleagues published that DNA replication is crucial for DDR induced by oncogenes. They found out that oncogene expression in cells which are not able to undergo DNA replication does not trigger a detectable DDR. [16] Oncogene induced DDR and senescence are dependent on DNA replication. Ben-Porath and Weinberg reported that a breakdown of this division restricting mechanism can result in cancer development. [14]

The overall results of my experiments are similar to those of di Micco et al.. The difference is the percentages of counted cells with more than two signals of the origin of replication lamin B2. Di Micco et al. described pBABE cells which have to 100 percent two signals of lamin B2 and oncogene induced senescent cells (OIS), RAS cells, showing to more than 50 percent two signals, around 30 percent have four signals and approximately 15 percent have more than four signals of the origin of replication.

My data show that both cell line pBABE and RAS show approximately the same results, around 60 percent are diploid, 30 percent have four signals of the origin of replication, and less than 10 percent have more than four signals of lamin B2. The results of my FISH experiment are identical to the results of the FACS analysis which was done on the same cell population.

The hypothesis which did not come out clear in my study is the difference between the BJ shp53 RAS and the BJ shp53 pBABE cell line. Both cell lines show an increased number of tetra and polyploid cells. A transfection of an empty vector into a cell has effects on the cell function and behaviour. Cells pass through changes which lead to a similar phenotype than seen in oncogene induced senescent (OIS) cells.

The results of the RAS cells are similar to the study of di Micco et al. but those of the pBABE cells differ. Possible reasons for the difference are: My experiments were done on cells with a higher passage number. It could be possible that through long cultivation additional breaks in the control cell line occurred. The cells in my experiment were not synchronised. A part of the pBABE cells seen with four signals may be in mitosis. Di Micco et al. did not apply a labelled centromere probe on the slides of the FISH experiment, the data just show signals of the lamin B2 signals. In contrast in my experiments the labelled probe of lamin B2 and a labelled centromere probe on the same chromosome was applied. It was possible to distinguish cells which are in proliferation from those who have an active replication of origin firing more than once per cell cycle. The results show that senescent cells have an augmented number of active replicons firing more than once per cell cycle. Fig. 19 to Fig. 21, microscopic pictures of the FISH analysis of the human fibroblast cell lines, show that a mentionable part of the tetraploid cells have two signals of the centromere but 4 signals per cell of the lamin B2 origin of replication.

Curtois-Cox et al. reported that senescent cells stall in S-phase and have an augmented number of active replicons and exhibit defects in DNA replication fork progression, resulting in an activation of ATR and ultimately ATM (ataxia teleangiectasia). [17]

Why has oncogene induced senescence (OIS) response emerged in evolution? And why can a process which stops proliferation but allows the cell to live and perform its physiological function seen as benefit?

Braig and Schmitt defined that OIS may occurs when the driving mitogenic oncogene is expressed at supraphysiological levels. They show that DNA damaging agents e.g. cellular stress, radiation or toxic substances results in the absence of an intact apoptotic response in the arrest of proliferation. [13] There are not only positive aspects associated with OIS. A negative aspect is that with advancing age a gradual accumulation of long lived senescent cells is manifested. [30]

To conclude senesce markers have been identified in several in vivo lesions e.g. human melanocytic nevi, murine lung adenomas, human dermal neurofibromas, human and murine prostatic adenomas, murine pancreatic intraductal neoplasias, and early murine melanomas. A variety of studies connect cancer types with OIS. To understand OIS will be a first step to understand kinetics and to evaluate cancer risks.

4.3. Part 3 – Immunohistochemical staining experiment

FANCD2 staining allows proving if the DNA damage response (DDR) of the cell works or not. The modified protein FANCD2 is a part of the DDR. Damages of the cell as consequence of tumorigenesis or cellular stress may result in a failure in the DDR pathway. This failure is the reason for a non modified FANCD2 protein which results in a positive FANCD2 staining.

The hypothesis was that gene products defective in the Bloom's syndrome (BLM) and in the Fanconi anaemia syndrome (FANC A-N) are associated with a multienzyme complex called "BRAFT". The functional crosstalk between the Bloom's syndrome and Fanconi anaemia pathway in suppressing tumor development is unclear, this complex is associated with chromosomal instability and common fragile site (CFS) loci. The aim was a screening of tumor cell lines, oncogene induced senescent cells, and formalin fixed and paraffin embedded tissue sections to get information about FANCD2 loci and the associated information like changes in the region of CFS loci or chromosomal instability.

The staining shows that normal unstressed cells have on average zero to one spot per cell but stressed cells or cells in which a vector is introduced show up more signals. This suggestion was proved with specifically stressed cells, stressed by an aphidicholin (APH) treatment. APH induces FANCD2 foci in interphase micronuclei. Chan et al. reported that the function of BLM at the DNA replication forks is disrupted by chemical adducts or DNA synthesis inhibitors like aphidicholin. [19]

The experiments from Chan and colleagues confirm my results from Fig. 28 which show the FANCD2 staining of APH treated and non APH treated HeLa cells. In their paper a similar experiment is discussed and the result is that aphidicholin (APH) treated cells show six to eight FANCD2 sister foci per cell and on average less than one signal per cell was found in untreated cells. [19]

My results show that non APH treated cells have on average zero to one FANCD2 signal per cell which is according to Chan et al. Fig. 28 show that an APH induction

increases the number of FANCD2 signals per cell. The lower number of signals in stressed cells may be the reason of the recovery period after the stress induction.

The FANCD2 staining of the human fibroblast cell lines, seen in Fig. 31 demonstrated that BJ PD40 cell line behaves similar to a non APH treated cell line. pBABE cell line has an increased number of signals. The reason for the increase of the signals may is the insertion of the empty RAS vector to the cell. The vector leads to failure in the DNA damage response (DDR) pathway of the cell. In a functioning DDR FANCD2 is monoubiquitylated. A failure in the DDR can disturb the modification, resulting in an increase of FANCD2 signals per cell. RAS expressing cell line show between six and eleven signal, which is a consequence of the oncogene induced senescence. A comparison of Fig. 28 and Fig. 31 demonstrate that damages induced by the oncogene expression are higher than those induced by aphidcholin stress.

Chan et al. reported that detected FANCD2 forms tightly linked paired sister foci signals, ultra fine bridges (UFBs), which seem to be located at common fragile site (CFS) regions. Analysis of the FANCD2 staining of metaphase spreads show that FANCD2 sister foci are located on chromosome arms and not at the centromere region. That is the reason for the suggestion that FANCD2 sister foci are established during the S-phase and persist into mitosis. [19] Obvious these bridges could not be seen in all of the stained cells in my experiments.

Studies pointed out that FANCD2 is expressed in normal human tissue e.g. stomach, testis, lymph node, spleen, tonsils, and more. [24] This finding and the suggestion that the FANCD2 foci is located to the CFS loci were the main aspects, why the staining was chosen as possible technique to identify cells which include fragile sites. FANCD2 positive staining is associated with the location of common fragile sites and chromosomal instability. The occurrence of ultra fine bridges between FANCD2 sister foci obtain pathological character and the protein is adjudged to play an important role in the DDR. These are reasons why scientists may concentrate more and more on FANCD2 in combination with the tumorigenesis. FANCD2 may be a pre screen method for tissue sections and it may be able to detect possible harmful lesions at an early stage.

4.4. Part 4 – Custom made array experiment

Because limited time was available for the thesis it was not possible to finish the array experiment. The aim was to create a custom made array which includes the common fragile sites (CFSs), replication origins, and LOH sites. The custom made array should be a method to identify fragile site associated tumor.

The array has been ordered but no array experiment has been done, because the experiment is very expensive and it was not totally clear how to identify the cells which include common fragile sites to put just those on the array. The isolated single cells grew very slowly in the multi well plate. The success rate of isolating single cells with pipetting was around 50 percent. In order of a low cell density it was not possible to isolate enough DNA to do a RT-PCR, prepare interphase slides, and do an array.

Possible identification methods are a RT-PCR and a FANCD2 staining. RT primers were designed which are located in the region of common fragile sites. The regions of the fragile sites span a part of the DNA which includes several mega base pairs and the primer product extend approximately 100 bp. The success rate of the detection of all gaps and breaks of the common fragile site (CFS) with one primer pair per CFS loci seemed to be low.

Another suggested method was an immunohistochemical FANCD2 staining with the cell line which was likely to be included in the array experiment. Studies show that common fragile sites are prevalent in tumorigenesis. Thus FANCD2 is part of the DNA damage response (DDR) pathway it seemed to be a perfect way.

To detect very small copy number changes and changes which are not present in every cell, it is necessary to start the culture with one single cell to obtain a pure culture. The growth of one single cell in a very small culture plate is slow. The culturing process was time consuming. It needed weeks to get enough cells to proceed to subsequent analysis.

Two methods for isolating a single cell were tested: On the one hand “Isolating single cell colonies via cloning cylinders” and on the other hand “Isolating single cells with a pipette”. The cloning cylinders were too big, they cover even if the cell population was cultured in a low density, more than one colony. It was not possible to isolate only one single cell colony. The advantage of this method would have been that colonies are isolated and no single cell. The culturing would not be so time consuming. The second method needed an expertise and a very calm hand. Otherwise cells may be disturbed with the pipette or more than one single cell were isolated. The disadvantage of this method is the time consumption because the process of isolation and the culturing is time consuming. It was necessary to isolate more cells than needed, because not all cells will start growing. The success rate was around 50 percent. Possible reasons for the low success rate are that old cells or cells which may be disturbed through the isolation process would not grow in the culture plate. The advantage is if you precede carefully it results in numerous cells proliferate from the same “mother cell”.

Recent studies from my successor show: A RT-PCR, with the primer suggested in this thesis, applied on two pure cell population of the BJ hTERT shp53 RAS cell line, was not possible to identify changes in the common fragile site (CFS) region as significant. The result was a variety of changes which are in the normal range. The same two pure cell population were applied on a 60 k array to screen the genetic material and detect gains and/or losses. Results show variations in the region of CFSs. The two pure cell populations show one deletion on chromosome 9, one has one duplication on chromosome 10 and the other one has a deletion on chromosome 13. All those chromosomal instabilities are not detectable in the DNA of a traditional cell culture.

5. Outlook

This thesis was done to investigate if chromosomal instability (CIN) is an early event in tumorigenesis and if it is a common mechanism in cells with oncogene induced stress. The protocols for the fluorescence in situ hybridization (FISH) and the immunohistochemical staining had to be adapted. This process was very time intensive and is now used as basis for further researches. The custom made array to investigate abnormalities in the region of common fragile sites (CFSs) was designed and will be used in further experiments. The slow growth of the cell lines was the reason why no array experiment was done within the thesis.

Recent experiments of my successor show deletions and duplications, in the single cell colonies established during my thesis, which were not detectable in the DNA of traditional cell culture experiments. These results approve that the investigation of single cell colonies has a great benefit over the investigation of whole cell cultures as aberrations occur randomized and are not present in all cells. Array-CGH in combination with immunostaining and FISH analysis has the great potential to connect abnormalities in the CFS loci, tumorigenesis, and oncogene induced senescence (OIS).

List of figures

List of tables

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Appendix

Appendix A: Start and end positions of common fragile sites

Appendix B: Start and end position of the origin of replication

Appendix C: Start and end position of the LOH sites

Appendix D: List of designed RT-PCR primers

Appendix E: List of adult human tissue and organs expressing FANCD2

Appendix A: Start and end position of common fragile sites:

Fragile Site	Publication	Start (Clon)	Ende (Clon)	Start bp	Ende bp
FRA2G	Limongi et al.: **Characterization of the human fragile site FRA2G.** Genomics 2003, Vol. 81, 93 - 97	**AC009475**	**AC009967**	169,206,756	170,442,781
FRA3B	Ohta et al.: **The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carsinoma associated t(3;8) breakpoint, is abnormal in digestive tract cancers.** Cell 1996, Vol. 84, 587 - 597	**D3S2977**	**D3S1480**	60,134,763	60,936,724
FRA4F	Rozier et al.: **Characterization of a conserved aphidicolin-sensitive common fragile site at human 4q22 and mouse 6C1: possible association with an inherited disease and cancer.** Oncogene 2004, Vol. 23, 6872 - 6880	**AC112695 (RP11-9B6)**	**AC106881 (RP11-710C12)**	93,421,437	96,746,233
FRA6E	Denison et al.: **Characterization of FRA6E and its potential role in autosomal recessive juvenil parkinsonism and ovarian cancer.** Genes, Chromosome & Cancer 2003, Vol. 38, 40 - 52	**D6S1581**	**D6S1719**	160,096,758	166,089,914
FRA6F	Morelli et al.: **Cloning and characterization of the common fragile site FRA6F harboring a replicative sequence gene and frequently deleted in human tumors.** Oncogene 2002, Vol. 21, 7266 - 7276	**D6S1698**	**D6S266**	111,322,396	113,889,649
FRA7E	Zlotorynski et al.: **Molecular basis for expression of common and rare fragile sites.** Molecular and Cell Biology 2003, Vol. 23, 7143 - 7151	**AC0003988**	**AC006151**	79,720,563	84,727,200
FRA7G	Huang et al.: **FRA7G extends over a broad region: coincidence of human endogenous retroviral sequences (HERV-H) and small polydispersed circular DNAs (spcDNA) and fragile sites.** Oncogene 1998, Vol. 16, 2311 - 2319	**AC002066 D7S522**	**D7S486**	115,770,525	115,782,190
FRA7H	Mishmar et.al.: **Molecular characterization of a common fragile site (FRA7H) on human chromosome 7 by the cloning of a simian virus 40 integration site**", Proceeding of the National Academy of Science of the United States of America 1998, Vol. 95, No. 14, 8141 - 8146	**D7S786**	**Cosmid clone 62D21–1.1**	129,871,046	130,384,418
FRA9E	Callahan et al.: **Characterization of the common fragile site FRA9E and its potential role in ovarian cancer.** Oncogene 2003, Vol. 22, 590 - 601	**D9S1832**	**D9S154**	108,602,202	118,480,851
FRA16D	Ried et al.: **Common chromosomal fragile site FRA16D sequence: identification of the FOR gene spanning FRA16D and homozygous deletions and translocation breakpoints in cancer cells.** Human Molecular Genetics 2000, Vol. 9, 1651 - 1663	**AC009044**	**AC009129**	76,668,997	77,860,116
FRAXB	Arlt et al.: **Molecular characterization of FRAXB and comparative common fragile site instability in cancer cells.** Genes, Chromosome & Cancer 2002, Vol. 33, 82 - 92	**DXS1130**	**DXS237**	6,878,144	7,658,235

Appendix B: Start and end position of the origin of replication

	Publication	Start bp	End bp	chromosome number
MYC (2)	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	128,803,756	128,812,118	chromosome 8
		128,815,102	128,833,477	chromosome 8
Lamin B2 (2)	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	2,361,076	2,384,805	chromosome 19
		2,394,564	2,397,191	chromosome 19
Hämoglobin B	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	5,184,279	5,211,028	chromosome 11
		5,217,893	5,226,050	chromosome 11
FRAXA (7)	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	146,633,555	146,647,759	chromosome X
		146,696,368	146,702,766	chromosome X
		146,706,006	146,708,160	chromosome X
		146,714,362	146,719,459	chromosome X
		146,728,517	146,753,686	chromosome X
		146,780,483	146,782,891	chromosome X
		146,811,457	146,820,884	chromosome X
HPRT	Gerhardt et al.: **Identification of New Human Origins of DNA Replication by an Orogin Trapping Assay.** Molecular and Cell Biology 2006, Vol.26, No.20, 7731 - 7746	133,377,140	133,533,599	AC004383 Chromosome X
LAMIN B2	Gerhardt et al.: **Identification of New Human Origins of DNA Replication by an Orogin Trapping Assay.** Molecular and Cell Biology 2006, Vol.26, No.20, 7731 - 7746	2,359,751	2,401,466	AC011522 chromosome 19
Or10	Gerhardt et al.: **Identification of New Human Origins of DNA Replication by an Orogin Trapping Assay.** Molecular and Cell Biology 2006, Vol.26, No.20, 7731 - 7746	43,129,820	43,250,377	AC099795 Chromosome 1
Or6	Gerhardt et al.: **Identification of New Human Origins of DNA Replication by an Orogin Trapping Assay.** Molecular and Cell Biology 2006, Vol.26, No.20, 7731 - 7746	55,578,149	55,750,146	AC026120 Chromosome 12
Or III	Gerhardt et al.: **Identification of New Human Origins of DNA Replication by an Orogin Trapping Assay.** Molecular and Cell Biology 2006, Vol.26, No.20, 7731 - 7746	119,455,414	119,604,515	AC108095 Chromosome 5

Chr. 22	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	28,957,459	28,978,040	chromosome 22
		28,994,037	29,049,598	chromosome 22
		29,059,018	29,076,714	chromosome 22
		29,104,370	29,166,999	chromosome 22
		29,211,414	29,297,403	chromosome 22
		29,311,351	29,385,760	chromosome 22
		29,415,895	29,487,581	chromosome 22
		29,520,964	29,531,047	chromosome 22
		29,543,406	29,545,057	chromosome 22
		29,547,524	29,551,472	chromosome 22
		29,603,147	29,605,216	chromosome 22
		29,622,826	29,627,694	chromosome 22
		29,645,234	29,646,154	chromosome 22
		29,650,788	29,661,603	chromosome 22
		29,666,419	29,672,302	chromosome 22
		29,674,829	29,679,764	chromosome 22
		29,685,712	29,699,547	chromosome 22
		29,708,952	29,711,353	chromosome 22
		29,802,008	29,817,039	chromosome 22
		29,823,896	29,900,021	chromosome 22

Chr. 22	Lucas et al.: **High-throughput mapping of origins of replication in human cells.** EMBO reports 2007, Vol. 8, No. 8, 770 - 777	29,912,611	29,917,072	chromosome 22
		29,925,441	29,932,712	chromosome 22
		29,967,694	29,971,195	chromosome 22
		29,978,784	29,979,761	chromosome 22
		29,987,590	29,999,992	chromosome 22

Appendix C: Start and end position of the LOH sites

LOH Site	Publication	Start bp	End bp	chromosome number
TP53	Bartkova et al.: **DNA damage response as a candidate ant-cancer barrier in early human tumorigenesis.**. Nature 2005, Vol. 434, 864 - 870	7,505,822	7,531,588	17
ATM	Bartkova et al.: **DNA damage response as a candidate ant-cancer barrier in early human tumorigenesis.**. Nature 2005, Vol. 434, 864 - 870	107,598,769	107,745,036	11
CHK2	Bartkova et al.: **DNA damage response as a candidate ant-cancer barrier in early human tumorigenesis.**. Nature 2005, Vol. 434, 864 - 870	27,413,731	27,467,822	22
CHK1	Bartkova et al.: **DNA damage response as a candidate ant-cancer barrier in early human tumorigenesis.**. Nature 2005, Vol. 434, 864 - 870	125,000,246	125,051,360	11

Appendix D: List of designed RT-PCR primers

Fragile Site	Region	forward Primer	reverse Primer	Ident.	Size	Gene	T_m forward	T_m reverse
FRA2G	chr2: 169,206,756 - 170,442,781	TGGCTCAGATGAACGTCAAG (169883524 - 169883543)	acttctttgcgacaggttgg (169883449 - 169883468)	100%	95bp	LRP	59,98 °C	60,29 °C
FRA3B	chr3: 60,134,763 - 60,936,724	aagccattatgggacaggtg (60610704 - 60610723)	ggatagcagggtagcagcag (60610620 - 60610639)	100%	104bp	FHIT	59,81 °C	60 °C
FRA4F	chr4: 93,421,437 - 96,746,233	tctccaaacacctgcacatc (93736588 - 93736607)	ggggaaaggaccaagctaag (93736671 - 93736690)	100%	103bp	GRID2	59,68 °C	60,07 °C
FRA6E	chr6: 160,096,758 - 166,089,914	tccctggattttggtgaaag (163605254 - 163605273)	ggggtctcctctggataagc (163605339 - 163605358)	100%	105bp	PARCRG	59,9 °C	60,04°C
FRA6F	chr6: 111,322,396 - 113,889,649	ggccctacctgtctttcctc (112625170 - 112625189)	agctgatctggtggatttgg (112625093 - 112625112)	100%	97bp	LAMA4	60,07 °C	60,07 °C
FRA7E	chr7: 79,720,563 - 84,727,200	ggttcttggcttcagctttg (81831140 - 81831159)	tggactgggtctgattctcc (81831065 - 81831084)	100%	95bp	CACNA2D1	59,99 °C	60,05 °C
FRA7G	chr7: 115,770,525 - 115,782,190	ACTCCTACAGCCACCACAGC (115927121 - 115927140)	tcacCTTGAGATGCGAGTTG (115927206 - 115927225)	100%	105bp	CAV2	60,33 °C	59,98 °C
		tcaataccagcaccatgagc (115926583 - 115926602)	Agggaaccttgtggttaggc (115926662 - 115926681)	100%	99bp	CAV2	59,68 °C	60,36 °C
FRA7H	chr7: 128,848,390 - 130,384,418	AGAGGACATGgtgcgttttc (129632439 - 129632458)	aacccaggacagatcagcag (129632362 - 129632381)	100%	97bp	TMEM209	60,12 °C	60,26 °C
FRA9E	chr9: 108,602,202 - 118,480,851	gggtagccggatctaggaag (117961408 - 117961427)	cagcaactcaagaggggaag (117961491 - 117961510)	100%	103bp	PAPPA	60,05 °C	59,98 °C
		tgagctgccactcagtgaac (117961780 - 117961799)	tggaccttgattttgccttc (117961862 - 117961881)	100%	102bp	PAPPA	60,19 °C	60,05 °C
FRA16D	chr16: 76,668,997 - 77,860,116	tgacaatccttgcccctaag (76826863 - 76826882)	cgctgaagatgcagacagag (76826945 - 76826964)	100%	102bp	WWOX	60,07 °C	59,88 °C
FRAXB	chrX: 6,878,144 - 7,658,235	GGAGTTTCCATCCCATGTTG (6978194 - 6978213)	CTGGGCTTCGTTTCTGTCTC (6978112 - 6978131)	100%	102bp	HDHD1A	60,17 °C	59,99 °C
		gttctggcttcttggcagac (6995515 - 6995534)	gagcagctctccatcaaagg (6995430 - 6995449)		105bp	HDHD1A	60 °C	60,1 °C
		tgacttcccggagtggtaag (7196028 - 7196047)	ccatcatgctgatgtcttgg (7196106 - 7196125)		98bp	STS	60,1 °C	60,07 °C

Ident. … Identity

T_m ..... melting temperature

Appendix E: List of adult human tissue and organs expressing FANCD2

Reprinted from Hölzel et al.: **FANCD2 protein is expressed in proliferating cells of human tissues that are cancer-prone in Fanconi anaemia.** Journal of Pathology 2003, Vol. 201, 198-203

Tissue	Adult	Cell type
Placenta	+	Cytotrophoblastic cells
Fallopian tube	+	Epithelium
Breast	+	Duct epithelium cells
Pancreas	+	Exocrine cells
Rectum	+	Proliferating basal cells
Small intestine	+	Proliferating basal cells
Stomach	++	Proliferating neck cells
Trachea	+	Respiratory epithelium
Ovary	++	Granulose/theca cells
Testis	+++	Spermatocytes
Lymph node	++	Germinal centre cells
Spleen	++	Germinal centre cells
Tonsil	+++	Germinal centre cells
Thymus	+	T-lymphoblasts
Cervix	++	Proliferating parabasal cells
Oesophagus	+	Proliferating parabasal cells
Larynx	++	Proliferating parabasal cells
Oropharynx	++	Proliferating parabasal cells
Tonsil	++	Proliferating parabasal cells

+ ……. low expression
++ …… intermediate expression
+++ …… high expression