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INVOLVEMENT OF EVOLUTIONARILY PLASTIC REGIONS IN CANCER ASSOCIATED CHR3 ABERRATIONS

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To my family

ABSTRACT

A functional test to identify tumor antagonizing regions on chromosome 3 (chr3), called the Elimination Test, was developed in our group. It is based on microcell mediated transfer of human chr3 into mouse or human tumor cells and analysis of the monochromosomal hybrids after their growth *in vivo*. We identified three regions on 3p14-p22, which were frequently lost in derived tumors (common or frequently eliminated regions). In order to understand the role of these regions in tumor development, we continued the study following two leads: analysis of breakpoint clusters to identify and characterize possible instability features and search for tumor suppressor genes within the deleted regions.

First, we identified and characterized a common eliminated region 1 (CER1) homologous sequence in mouse, where it was divided into two syntenic blocks on chromosome 9. In these blocks, the gene order and content was maintained with exception of two mouse gene duplications. Comparative analysis helped us to characterize five previously not identified mouse genes (Kiss et al. 2002). A more extensive comparative study of CER1 showed that its border regions are characterized by evolutionary plasticity: synteny breaks in several species, recent tandem gene duplications, retroposed pseudogene insertions, and horizontal evolution of the genes. Thus we showed that the cancer associated breakpoint regions have features of evolutionary plasticity. These results and other publications from our group suggested structural instability at the borders of eliminated regions identified by the Elimination Test (Darai et al. 2005) (Kost-Alimova et al. 2003; Kost-Alimova et al. 2004).

As a next step, in order to analyze rearrangements of entire chr3 in human tumor cells, we developed and compared two high resolution methods, array-CGH and mpFISH. We proved that although our 1Mb chr3 BAC/PAC array could identify single copy number changes even in pentaploid cells, mpFISH provided a more accurate analysis in the dissection of complex karyotypes at high ploidy levels. In heterogeneous or normal cell contaminated samples the most precise analysis can be made by mpFISH due to its ability to give information at single cell level (Darai-Ramqvist et al. 2006). Using high resolution methods we analyzed ten carcinoma cell lines and identified two new hot spots of tumor breakpoints at 3p12-p13 and 3q21. These tumor breakpoint regions carried large segmental duplications, retrotransposable elements and satellite repeats, which participated in recent primate evolution and, as we suggest, are associated with structural chromosomal instability (CIN). CIN is an ongoing dynamic process. Therefore in order to prove that the instability at the breakpoint regions characterizes structural CIN phenotype and it is required for tumor development and progression, dynamic analysis of the tumors must be done. This may elucidate the mechanism of tumor development; and may help to develop CIN phenotype markers useful in choice of consequent treatment.

Following the second lead of our study, we have analyzed a putative tumor suppressor gene *LIMD1*, which is located within the deleted central part of CER1. We found that it binds specifically to pRb and suppresses E2F driven transcription. A tumor

suppressor effect of this gene was proven in *in vitro* and *in vivo* experiments, as well as in tumor biopsies (Sharp et al. 2004). In another part of the study we analyzed in details chr3 rearrangements in human renal cell carcinoma and nasopharyngeal carcinoma derived monochromosomal (chr3) hybrids and showed that aneuploid tumors maintain a mandatory chromosomal segment balance with stringency concerning no gain of 3p14-21 and no loss of 3q26-27. We concluded that the mechanism of tumor suppression by chr3 transfer is based on the alternative quantitative model. According to this model the tumor cell does not tolerate an increased dosage of the relevant gene or segment, and the lost part can be either of normal cell derived exogeneous or tumor derived endogenous origin (Kost-Alimova et al. 2007).

LIST OF PUBLICATIONS

I. H Kiss, E Darai, Cs Kiss, M Kost-Alimova, G Klein, JP. Dumanski, S Imreh. Comparative human/murine sequence analysis of the common eliminated region 1 from human 3p21.3. *Mammalian Genome, 2002, Nov;13(11):646-55*.

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III. TV. Sharp, F Munoz, D Bourboulia, N Presneau, E Darai, HW Wang, M Cannon, DN. Butcher, AG. Nicholson, G Klein, S Imreh, and C Boshoff. LIM domains-containing protein 1 (LIMD1), a tumor suppressor encoded at chr3p21.3, binds pRB and represses E2F-driven transcription. *PNAS, 2004, November 23, vol. 101, no. 47, 16531–16536.*

IV. E Darai-Ramqvist, T Diaz de Ståhl, A Sandlund, K Mantripragada, G Klein, J Dumanski, S Imreh, M Kost-Alimova. Array-CGH and multipoint FISH to decode complex chromosomal rearrangements. *BMC Genomics. 2006 Dec 29;7:330.*

V. M Kost-Alimova, E Darai-Ramqvist, W Lung Yau, A Sandlund, L Fedorova, Y Yang, I Kholodnyuk, Y Cheng, M Li Lung, E Stanbridge, G Klein, S Imreh. Mandatory chromosomal segment balance in aneuploid tumor cells. *BMC Cancer. 2007 Jan 26;7:21.*

VI. E Darai-Ramqvist, A Sandlund, G Klein, S Imreh, M Kost-Alimova. Characterisation of cancer-associated frequent break regions on human chromosome 3. *manuscript*.

CONTENTS

LIST OF ABBREVIATIONS

Chr3	Chromosome 3
ET	Elimination test
MCH	Microcell hybrid
CER1=C3CER1	Common eliminated region 1
FER	Frequently eliminated region
CRR	Commonly retained region
CGH	Comparative genomic hybridization
FISH	Fluorescence in situ hybridization
mpFISH	Multipoint fluorescence in situ hybridization
M-FISH	Multiplex/multicolor fluorescence in situ hybridization
CIN	Chromosomal instability
MIN	Microsatellite instability
SD	Segmental duplication
TSG	Tumor suppressor gene
LOH	Loss of heterozygosity
HD	Homozygous deletion
FRA	Fragile site
SCID	severe common immunodeficiency
PCR	Polymerase chain reaction
moMCH	Human chr3/mouse fibrosarcoma microcell hybrids
huMCH	Human chr3/human renal cell carcinoma microcell hybrids
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
kb	Kilobase
Mb	Megabase
Myrs	Million years
BFB	Breakage-fusion-brigde
DM	Double minute
DSB	Double strand break
NHEJ	Non-homologous end joining
HR	Homologous recombination
PAC	P1 artificial chromosome
BAC	Bacterial artificial chromosome
CBR	Conservation breakpoint region
NES	Nuclear export signal
ANILFR	Average inter-locus fluorescent ratio
TBSD	Tumor breakpoint segmental duplication
SNP	Single nucleotide polimorfism
LIMD1	LIM domain containing 1 gene
E2F	Transcription factor activating adenovirus E2 gene
pRB	Retinoblastoma protein
FHIT	Fragile histidine triad gene

LTF/LF	Lactotransferrin/lactoferrin gene
LRRC2	Leucin reach repeat containing 2 gene
RIS1	RAS-induced senescence 1 gene
TMEM7	Transmembrane protein 7 gene
FYCO1	FYVE and coiled-coil domain containing 1 gene
LZTFL1	Leucine zipper transcription factor like 1 gene
CCR	Chemokine receptor gene
ZNF	Zink finger gene
RCC	Renal cell carcinoma
HERV	Human engogenous retrovirus
LTR	Long terminal repeat

Introduction

The nineteenth century discovery that all cells of an organism descend from the fertilized egg led to the realization that tumors are not foreign bodies but growth derived from normal tissues. The comparatively disorganized tissue architecture of tumors pointed toward cancer being a disease of malfunctioning cells. Tumors can be benign (localized and non-invasive) or malignant (invasive and metastatic). The metastases spawned by malignant tumors are responsible for almost all deaths from cancer.

Cancers seem to develop progressively, with tumors showing different grades of abnormality along the way from benign to metastatic. Benign tumors may be hyperplastic (normal but have excessive number of cells) or metaplastic (displacement of normal cells by normal cell types not usually encountered at that site). Tumors that breach the basement membrane and invade underlying tissue are malignant. An even further degree of abnormality is metastasis, the seeding of tumor cells to different sites in the body. Metastasis requires not only invasiveness but also such newly acquired traits as motility and adaptation to foreign environments (Weinberg 2007).

By the late 1970s different lines of evidence concerning cancer genes coalesced into a relatively simple idea. The genomes of mammals and birds contain a group of proto-oncogenes, which function to regulate normal cell proliferation and differentiation. Alterations of these genes affect either the control of their expression or the structure of their encoded proteins. This can lead to excessive activation of growth promoting genes, which appear in cancer cells as activated oncogenes. Once formed, such oncogenes proceed to drive the cell multiplication and play a central role in the pathogenesis of cancer.

Many of these cellular genes were originally identified because of their presence in the genome of rapidly transforming retroviruses, such as the Rous sarcoma virus, avian erythrovirus and Harvey sarcoma virus (Weinberg 2007). Subsequently transfection experiments revealed the presence of potent transforming genes in the genomes of cells that have been transformed by exposure to chemical carcinogens and cells derived from spontaneously arising human tumors. These tumor cells had no association with retrovirus infections. However, the oncogenes that they carried were found to be related to those carried by transforming retroviruses. This meant that a common set of proto-oncogenes could be activated by two alternative routes: retrovirus acquisition or somatic mutation.

The somatic mutations leading to proto-oncogene activation could be divided into two categories: those that caused changes in structure of encoded proteins (structural), and those that led to elevated, deregulated expression of these proteins (regulatory). Mutations affecting structure included point mutations affecting e.g. *RAS* proto-oncogene, and chromosomal translocations that yielded fusion genes such as *BCR-ABL*. Elevated expression could be achieved in human tumors by gene amplification or chromosomal translocations, such as those that place the *MYC* gene under the regulation of the immunoglobulin enhancer sequences (Weinberg 2007).

At the cellular level, the cancer phenotype is usually recessive (see also chapter 1.1). This indicates that the loss of genetic information is responsible for at least a part of cancer cell phenotype. Loss of functionally important genetic information is attributable to the loss of tumor suppressor genes (TSG), which are often present in the genomes of cancer cells as inactive, noll alleles. Consequently, TSG loss usually affects cell phenotype only when both copies of such gene are lost physically or functionally in a cell. The loss of TSG function can occur either through genetic mutation or epigenetic silencing of genes via promoter methylation. Inactivation of one copy of TSG (by mutation or methylation) may be followed by other mechanism that facilitate loss of heterozygosity (LOH) at the TSG locus, and may involve loss or rearrangement of chromosomal regions that harbours the gene.

TSGs regulate cell proliferation through many mechanisms. Their only common characteristic is that their loss increases the likelihood that the cell will undergo neoplastic transformation. When mutant, defective copies of a TSG are inherited in the germ line, the susceptibility to cancer often increases.
TSGs may be classified in two major groups:
a) gatekeepers, involved in governing the dynamics of cell proliferation;
b) caretakers, responsible for the maintenance of genome integrity.
The loss of TSGs may occur far more frequently during the development of a tumor than the activation of proto-oncogenes (Weinberg 2007).

Chr3 aberrations in solid tumors
Aberrations of chr3 occur in the majority carcinomas (about 60%- Mitelman database). Chromosome deletions are frequent on 3p, usually being terminal deletions with breakpoints starting from 3p11-p12. In contrast 3q usually was found in increased copies or harbouring amplifications, mostly at 3q26-qter region (see figures 1, 2, 3). ((http://cgap.nci.nih.gov/Chromosomes/Mitelman), and (http://www.ncbi.nlm.nih.gov/sky/)).

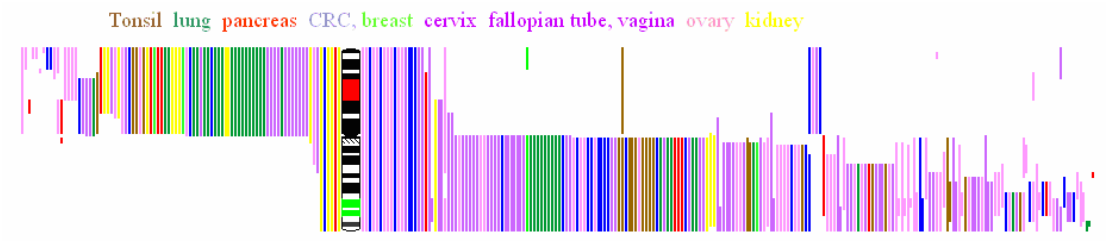


Figure 1. CGH data of chr3 losses and gains in human solid tumors/cell lines

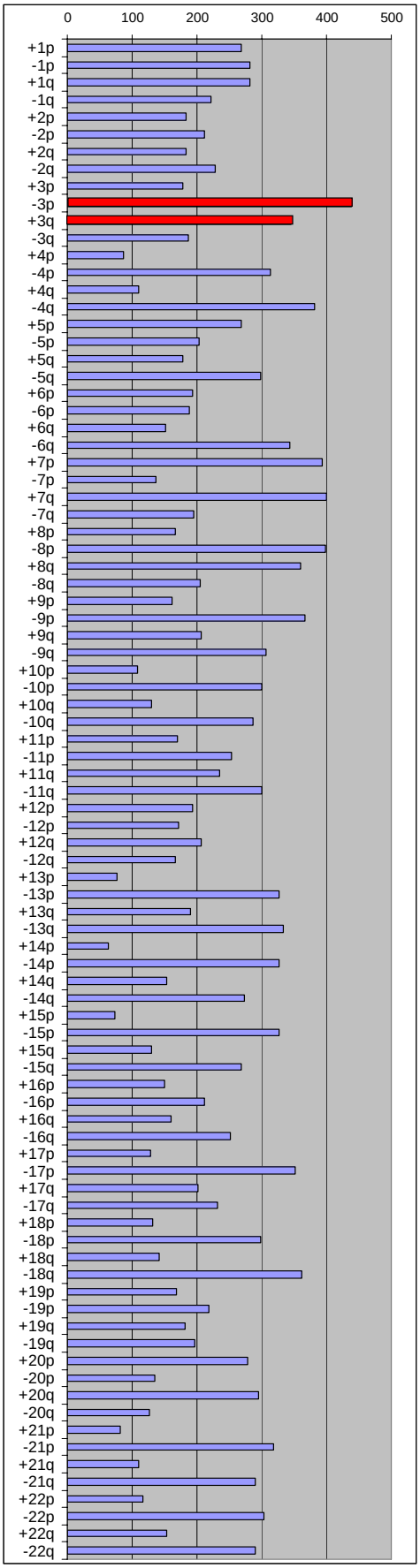


Figure 2. Chromosome arm loss/ gains in carcinomas (Based on Mitelman and CGH/SKY database)

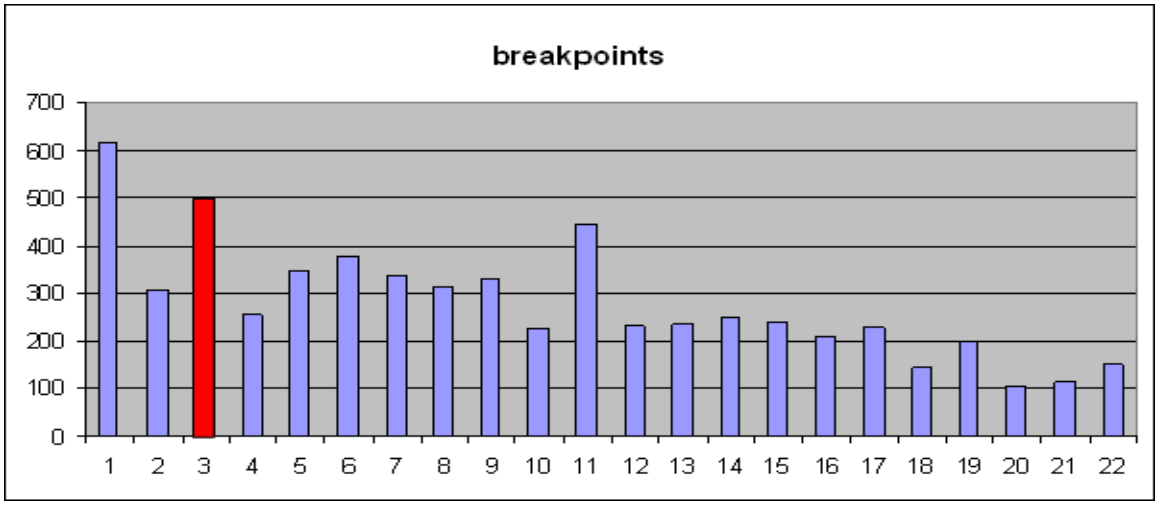


Figure 3. Chromosomal breakpoints in carcinomas (Based on Mitelman and CGH/SKY database)

The short arm of chr3 carries frequent and often extensive deletions in many malignancies, including lung, kidney, ovarian, and breast carcinomas. Interstitial deletions including 3p21 have been described in 23 different types of tumors. Band 3p21 is often interstitially deleted in mesotheliomas, lung adenocarcinomas, and in breast, kidney, ovary, pancreas, and gastric carcinomas; in neuroblastomas; germ cell tumors of the testis, chronic lymphoproliferative and myeloproliferative disorders, myelodysplastic syndrome, and non-Hodgkin lymphomas (Mitelman et al. 1997; Mitelman et al. 1997; Mitelman 2000). Homozygous deletions (HD) have been described for nine 3p locations (Imreh et al. 2003). Overlapping HDs were found in the FRA3B/FHIT region (3p14.2) in multiple solid tumor types and in 3p21 for two distinct regions (LC5 and LUCA regions) in lung tumors. There are significant changes also on the long arm of chr3. In different tumors 3q was found in increased copy number, with gain of the whole 3q or its terminal segment (see figure 2).

1. Elimination test identifies non-randomly lost and retained regions on chr3

1.1. Tumor suppression by somatic cell fusion

Tumor suppression as a biological phenomenon has been discovered in late 1960s when the groups of Henry Harris and George Klein demonstrated that fusion of a non-malignant, normal cell with a malignant one leads to loss of tumorigenicity in the resultant somatic cell hybrid (Harris et al. 1969) (see figure 4). This meant that malignancy is a recessive trait, and it may be linked to a recessive gene, like in many already known genetic diseases that follow Mendelian inheritance. Although earlier somatic cell hybrid work suggested the opposite, the dominancy of a malignant phenotype, the Harris–Klein team proved that chromosome losses occurred in such hybrids. It was an obvious assumption that those lost chromosomes may harbour tumor

suppressor genes. Thus, the resulting conclusion was that normal and tumor cell derived somatic cell hybrids have tumorigenicity suppressed phenotype until the normal complement is maintained intact. The same theory proved to be valid for single normal chromosome transfer into malignant cells. Microcell mediated chromosome transfer (MMCT) into malignant cells has shown that single chromosomes suppressed tumorigenicity in the microcell hybrids (MCHs). One of these was chr3 (Shimizu et al. 1990). Both in somatic cell hybrids and in MCHs the ultimate evidence was provided by the tumor suppressor test: loss of tumorigenicity after inoculation into syngeneic or immunosuppressed mice.

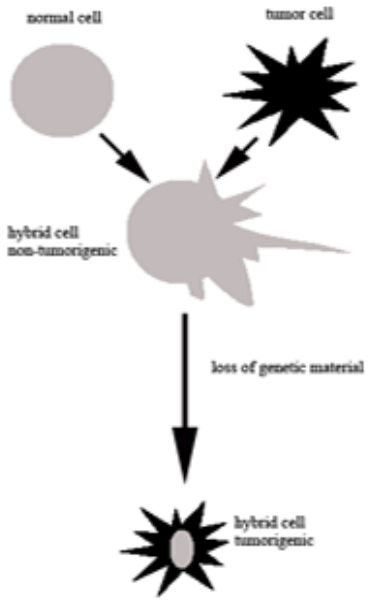


Figure 4. Suppression of tumorigenecity by somatic cell fusion

1.2. Elimination test

Based on the above mentioned results, in an effort to find tumor suppressor gene(s) in sporadic tumors we started to study chr3 containing MCHs. A functional test called "Elimination test" has been developed in our lab (Imreh et al. 1994). Human chr3/mouse fibrosarcoma MCHs were inoculated into SCID mice. The MCHs contained intact chr3 or chromosomes that had smaller or larger deletions, but all including 3p21. Obtaining suppression was not the only task. The aim was to build panels of tumors (tumorigenic segregants of initially suppressed hybrids). The fate of the transferred chromosome was followed with cytogentical and molecular methods. The deleted chromosomes were recovered in the SCID derived tumors as inoculated, even after serial in vivo passages. In contrast, the intact chr3 has been fragmented during in vitro/in vivo growth and multiple chimeric mouse human fragments have been identified. PCR marker analysis helped us to detect if a certain region was non-randomly missing from all tumors. (see figure 5)

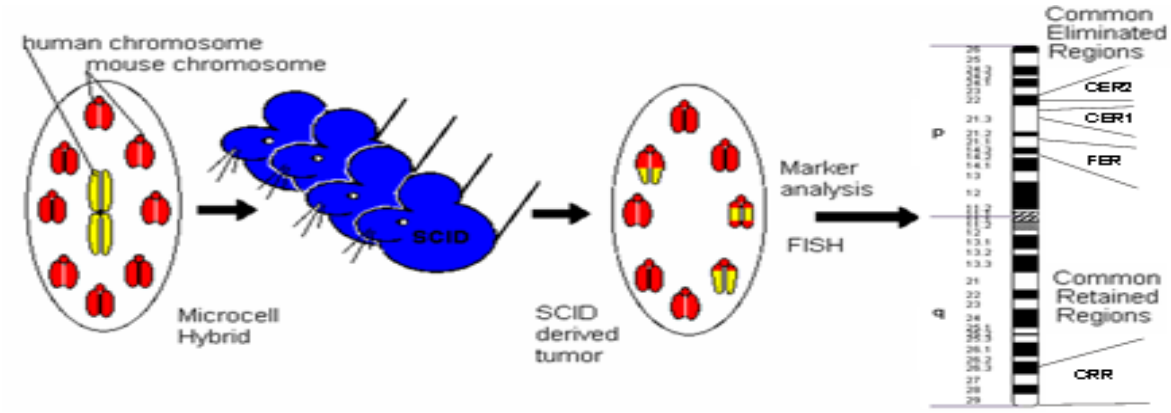


Figure 5. Scheme of the Elimination test

1.3. Chr3 regions involved in tumor development based on MCH studies

Analyzing large panel of different MCH tumor segregants that carried a human chr3 on a mouse fibrosarcoma A9 background (moMCHs) by cytogenetic and molecular methods, several commonly eliminated regions on 3p, and one commonly retained region on 3q were identified. The first region deleted (eliminated) from all tumors spanned 40 cM including a 3p21-p25 segment (Imreh et al. 1994). Further tumor panel analyses gradually restricted the eliminated region to 7 and than 1.6 cM (Kholodnyuk et al. 1997) and (Szeles et al. 1997). It was designated "common eliminated region 1" (CER1) and later registered by the EMBL Nomenclature Committee under the name of "chr3 common eliminated region" (C3CER1) (Kiss et al. 1999). In the frame of the first 40 cM deletion 3 distinct eliminated regions were described, the above mentioned CER1 on 3p21.3 (Mb 43.32–45.74), CER2 on 3p21.3-p22 (Mb 37.83–39.06) and FER at 3p14.3-p21.2 (Mb 50.6–58.1) (Imreh et al. 2003), (Kholodnyuk et al. 1997), (Kholodnyuk et al.) and (Kiss et al. 2002).

Our group have also shown that a 3q26-qter region (CRR) has been commonly retained even after several consecutive SCID passages (Imreh et al. 1997; Szeles et al. 1997). CRR was regularly retained in 85/92 human/murine MCH-derived tumors (Imreh et al. 1997; Kholodnyuk et al. 1997; Szeles et al. 1997; Yang et al. 1999). It contains several genes that are known or suspected to contribute to oncogenesis in a positive fashion. They include *FIM3, EVI1, BCL6, ETS1, ERM*, and the RNA subunit of telomerase (*hTR*). It is noteworthy in this connection that 3q gains were regularly observed by CGH in ovarian, cervical, and lung carcinomas and were correlated with a relatively poor prognosis (Blegen et al. 2003; Janssen et al. 2003).

As mentioned above, CER1, 2 and FER (see figure 5) have been identified in moMCHs. Our group tested also the validity of the elimination test on MCHs that contained a transferred chromosome on human tumor background (huMCHs). A normal human chr3 was transferred into the non-papillary renal cell carcinoma (RCC) line KH39 (Yang et al. 1999). KH39 was chosen as the recipient because 3p losses occur in virtually all RCCs and KH39 is monoallelic for all chr3 markers (uniparental

disomy). During SCID tumor growth of these huMCHs the entire short arm of chr3 was lost. In one huMCH (YYK1), the transferred chr3 was rearranged and contained deletions inside CER1 and FER. This chromosome remained unchanged in YYK1 derived tumors as shown by metaphase and interphase FISH, together with polymorphic marker analysis (Yang et al. 2001). The *LTF* gene (localized at the centromeric part of CER1) was disrupted by a pericentric inversion and made the centromeric border of CER1. The telomeric border of CER1 was similar in the huMCHs and moMCHs, located around the D3S1029 marker.

1.4. Candidate tumor suppressor genes in CER1

In order to identify candidate tumor suppressor genes within the commonly deleted region on our moMCH and huMCH system, we have focused on CER1 at 3p21.3. Spanning over 2 Mb in 3p21.3 with a telomeric breakpoint cluster around D3S1029, centromeric breakpoint in *LTF* (huMCH) or *LRRC2* gene (moMCH), CER1 is a gene rich segment that contains 33 genes. We will present briefly some strong candidate TSGs, some of them may already be classified as tumor suppressors: *LF, LIMD1* and *RIS1*, (Kiss et al. 1999; Yang et al. 1999; Kiss et al. 2001; Yang et al. 2001; Barradas et al. 2002; Kiss et al. 2002; Darai et al. 2005).

Lactoferrin (*LF*) or lactotransferrin (*LTF*) is located at or near to the centromeric border of CER1. It was present and expressed in vitro, but it was eliminated from all moMCH derived tumors analyzed in our goup until 1999 (Yang et al. 1999). Later, in six exceptional tumors derived from three different hybrids that maintained the PCR positivity for CER1, the *LF* expression has been found as eclipsed. In vitro, 5-aza-2′-deoxycytidine (5-aza-dC) treatment restored the *LF* expression however (Kholodnyuk et al. 2006). In huMCHs, where CER1 was shorter, *LF* was either eliminated or interrupted by a pericentric inversion (Szeles et al. 1997). *LF* is an 80 kDa iron binding glycoprotein, abundant in colostrum and it is a multifunctional gene. For testing the possibility that the presence and/or expression of *LF* is responsible for, or contribute to CER1 elimination, a PAC that contained the entire *LF* gene with its own promoter, and an *LF*-cDNA were transfected into the mouse fibrosarcoma line A9. Fourteen SCID-derived tumors from two independent PAC and two independent cDNA transfectants were analyzed. *LF* expression decreased or eclipsed in all tumors. Promoter methylation and/or rearrangement of the insertion site may be responsible for human *LF* down-regulation in transfected mouse sarcoma cells (Yang et al. 2003). There are numerous studies by different groups delivering evidence that *LF* is a TSG. It was reported to inhibit the growth of methylcholantrene induced mouse fibrosarcoma cells and of v-ras-transformed NIH3T3 cells in vitro and also reduced the experimental metastases of melanoma cells in mice in vivo (Bezault et al. 1994). Bovine LF can inhibit intestinal polyposis, colon, esophagus, and lung carcinogenesis and metastasis in rodents (Yoo et al. 1997; Ushida et al. 1998; Ushida et al. 1999). A bovine lactoferrin-derived peptide, lactoferricin-B, induced apoptosis and G1 arrest in a T-cell leukemia line (Yoo et al. 1997). LF inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma and head and neck cancer (Damiens et al. 1999; Xiao et al. 2004). LF is also an angiogenesis inhibitor. Oral administration of bovine LF inhibits VEGF165-mediated angiogenesis in the rat (Norrby et al. 2001; Oh et al. 2004; Shimamura et al. 2004; Varadhachary et al. 2004; Mader et al. 2005). *LF* upregulates the human p53 gene through induction of NF-kappaB activation cascade

(Oh et al. 2004). Bovine LF selectively induces apoptosis in human leukemia and carcinoma cell lines (Mader et al. 2005). Recent studies suggest that oral LF potentates also conventional chemotherapy (Varadhachary et al. 2004).

Ras-induced senescence 1 (*RIS1*) was found and cloned by Manuel Serranos group, when they tried to identify genes specifically upregulated in Ras-senescent human fibroblasts. Along a series of half a dozen genes including p21, this novel gene could play a role in senescence interfering with pathways inducing mitotic abnormality (Barradas et al. 2002).

LIM domain containing gene 1, *LIMD1*, was discovered and cloned in our group during the mapping and sequencing process of CER1 (Kiss et al. 1999). *LIMD1* belongs to the zyxin family of proteins, with role in cell adhesion, exerting also signalling functions adhesion site, transcriptional regulation and cellular differentiation during development. *LIMD1* has three LIM domains (zinc-finger protein interaction domains) at its C-terminus. We have strong evidence for the suppressor function of *LIMD1*, which is part of the papers discussed in this thesis. (For more details see Results and Discussion (Sharp et al. 2004)).

There are multiple additional tumor suppressor candidates in CER1. One of them **transmembrane protein 7 (*TMEM7*)** was also cloned by our group (Kiss et al. 2002). It is expressed specifically in the liver, and the encoded protein shares substantial sequence homology with human and mouse 28 kDa interferon-α (IFN-α responsive protein). On the basis of these observations, Popescu's group started to investigate the possible role of *TMEM7* in the development of hepatocellular carcinoma (HCC). *TMEM7* expression was lost or low in a subset of HCC cell lines and restoration of its expression reduced cell proliferation and tumorigenicity in nude mice. Interestingly, treatment of highly invasive HCC cell lines with IFN-α resulted in a marked increase in *TMEM7* expression as well as in inhibition of cell migration. These observations implicate loss of *TMEM7* expression in hepatocarcinogenesis and may have therapeutic applications in liver cancer (Xiaoling Zhou et al., unpublished).

We have reasons to search further for tumor inhibitory activity of CER1 genes ***LZTFL1, FYCO1*** (Voorhoeve et al. 2006) and ***LRRC2*** that were also cloned by our group (see (Imreh et al. 2003) for a review). Together the above data suggest that we may have reasons to consider that CER1 is a region with multiple tumor suppressors that are functionally connected.

2. Chromosome rearrangements in tumors. Chromosomal instability

Genetic or genomic instability refers to a series of genetic changes occurring at an accelerated rate in a cell population derived from the same early clone (Bayani et al. 2007). If we consider that the expected rate of mutations/gene/cell division is in the order of $2x10^{-7}$ (Renan 1993), than we can conclude that genomic instability is certainly an increase in the rate of mutations, enabling the cell to adapt to the changing selective pressure.
Genomic instability is classified in two major types: MIN – microsatellite instability and CIN chromosomal instability (Rajagopalan et al. 2003). Although MIN and CIN involves distinct mechanisms, recent data suggest that there is some overlap between the two mechanisms (Gorringe et al. 2005; Stewenius et al. 2005).
MIN involves simple DNA base changes due to different DNA repair process defects including base excision repair, mismatch repair and nucleotide excision repair (Kolodner 1995; Modrich 1997).

CIN is characterized mainly by abnormal karyotypes having both numerical and structural chromosomal rearrangements. It is characterized by continuous high rate of de novo numerical and structural rearrangements of chromosomes (Lengauer et al. 1997; Lengauer et al. 1998). (Weinberg 2007) (chapter12). Thus, CIN can be divided into two subtypes: numerical CIN and structural CIN (see figure 6). Defects in mitotic segregation can lead to numerical CIN, manifested by aneuploidy or changes such polisomy. Structural aberrations may be consequences of aberrant DNA repair pathway, can be facilitated by genomically unstable regions such as fragile sites, segmental duplications, specific sequence architecture (GC/AT content, specific simple repeats), and may be associated with epigenetic modifications or certain specific genetic events (e.g. breakage-fusion-bridge cycles, telomere dysfunctions); (See figure 8).

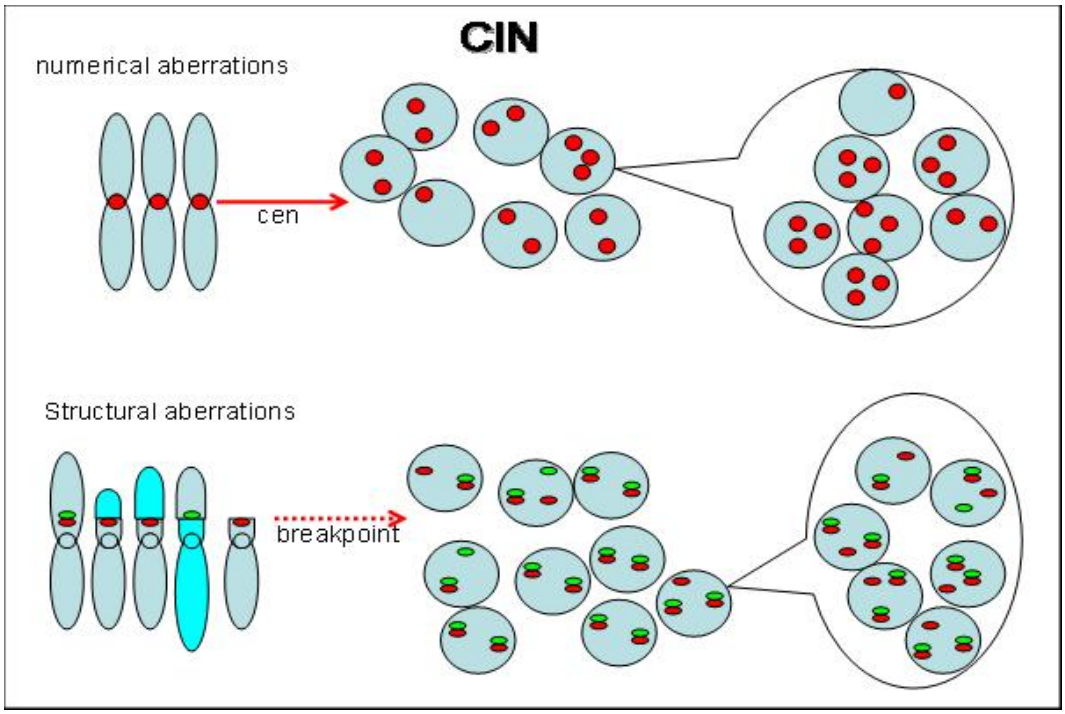


Figure 6. Scheme of numerical and structural CIN phenotype (as seen by FISH analysis)

2.1. Involvement of genomic sequence architecture in chromosomal instability

The observation that specific rearrangement "hot spots" of chromosomal regions are associated with different inherited diseases and cancer, led to the investigation of sequences that may increase DNA rearrangement rate leading to CIN (Aplan 2006).

Specific simple repeats

ALU repeat elements occur on average once each 4000 bp in the human genome and misspairing between the repeats undergo frequently recombination and can be responsible for deletions or duplications and genetic disease (Burt 2002; Kolomietz et al. 2002).

Centromeric repetitive sequences contain different satellite DNA repeats having very high level of sequence homology, which may be involved in translocations and facilitate a CIN phenomenon called "jumping translocations". These aberrations have been defined as non-reciprocal translocations involving a donor chromosome arm or a segment fused to several different recipient chromosomes (Lejeune et al. 1979; Sawyer et al. 1998). Structural chromosomal rearrangements in osteosarcoma tumors and cell lines showed 30% of breakpoint at the pericentromeric regions (Bayani et al. 2003), they were frequent in prostate cancer (Beheshti et al. 2000; Vukovic et al. 2007), multiple myeloma (Sawyer et al. 1998) and in many other cancer types (Kost-Alimova et al. 2004) (see figure 1).

Inverted repeats are specific repetitive sequences, usually AT rich, which have the possibility to generate large DNA palindromes (hairpin/cruciform structures) mediating double strand breaks leading to translocations. Several studies showed that AT-rich repeats contribute to genomic instability by increasing the rate of translocations (Leach 1994; Tapia-Paez et al. 2000; Kurahashi and Emanuel 2001; Tapia-Paez et al. 2001). A recent study done by Tanaka et al indicated that the location of palindromes in the cancer genome serves as a structural platform that supports also subsequent gene amplification (Tanaka et al. 2006).

Fragile sites

Fragile sites are chromosomal regions, spanning from 50kb to up to 4 Mb (Becker et al. 2002), which are prone to break and they are frequently sites of elevated sister chromatid exchange (Glover and Stein 1987), translocations and deletions in tumors (Glover and Stein 1987; Boldog et al. 1997), gene amplifications (Coquelle et al. 1997) or integration sites for oncogenic viruses (Rassool et al. 1991; Mishmar et al. 1998; Thorland et al. 2000). These observations suggest that unstable regions within common fragile sites may be predisposed to chromosomal brakeage and rearrangements during tumor growth (Popescu 2003; Glover 2006).

The most "fragile/break prone" site in humans is FRA3B at 3p14.2 deleted in a variety of histologically different cancers including renal cell carcinoma, lung cancer, pancreas and cervix carcinomas (Rabbitts 1994) and it was frequently involved in our MCH derived tumors in ET (Kholodnyuk et al. 2000).

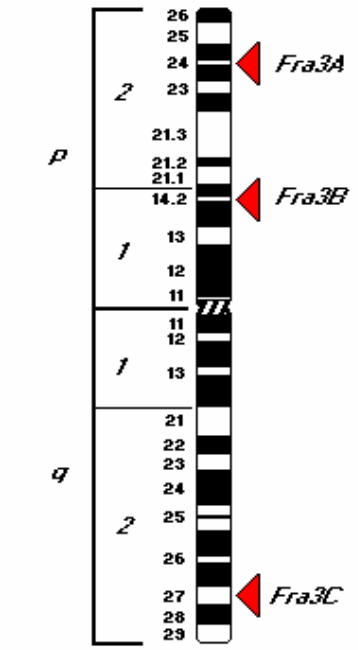


Figure 7. Chr3 fragile sites

This region contains the *FHIT* (Fragile Histidine Triad) gene, shown to be aberrantly expressed in various cancers including lung and cervical cancer (Holschneider et al. 2005) and hepatocellular carcinomas.
Other highly expressed fragile sites in lymphocytes include FRA16D (16q23), FRA6E (6q26), FRA7H (7q32.3) and FRAXB (Xp22.3) (Glover et al. 1984).

Fragile sites usually are located at light G-bands rich in GC and ALU repeats. They are centered around CpG islands and frequently associated with more relaxed DNA conformation and may contain genes (Smith et al. 2007). It was also shown that FRA's are late replicating, providing opportunity for breakage and recombination (Bayani et al. 2007).

Segmental duplications

Segmental duplications are low copy repeats ranging from 1 kb to a few hundred kilobases and their copy number is between two and around 50. They map to many chromosomes and are enriched within pericentromeric and subtelomeric regions. These large blocks of highly homologous regions generated by duplication are targets to structural chromosomal rearrangements increasing instability. Homologous recombination between segmental duplications during meiosis can lead to recurrent chromosomal rearrangements, a large class of human genetic diseases. In addition to meiotic events, low copy repeat sequences may be implicated also in mitotic chromosomal rearrangements (e.g. events on chr 15 were associated with phobic disorders and anxiety (Eichler 2001; Eichler 2001). Recently several studies suggested that low copy number mediated non-allelic homologous recombination (NAHR) is a major mechanism for human disease (Stankiewicz and Lupski 2002; Stankiewicz et al.

2003; Stankiewicz et al. 2004). Thus, segmental duplications can be regarded as mutational hot spots that contribute to large-scale genomic rearrangement events both in disease and in normal population. The genome architecture resulting from the size, orientation and arrangement of segmental duplications was shown to be responsible for genomic instability (Lupski 1998; Emanuel and Shaikh 2001; Stankiewicz and Lupski 2002).

2.2. Nuclear organization

Chromosomes are thought to occupy distinct territories in the interphase nucleus (Cremer et al. 2006; Zinner et al. 2006). This positioning of the chromosomes may play role in assuring accurate expression patterns and promoting DNA rearrangement within and between chromosomes. The sub-nuclear localization of a locus might have impact on the formation of chromosome translocations. Proximity effects are important in determining the translocation partners, since closely located chromosome loci have a higher likelihood to undergo illegitimate rejoining once chromosome breaks have occurred (Parada et al. 2002; Meaburn et al. 2007).

Centromeres and pericentromeric regions are associated with CIN not only by their repeat containing structure but also by their special position in the interphase nucleus which may contribute to increasing their propensity to rearrange (Bayani et al. 2007). Centromeres were reported to cluster in so called ”chromocenters” in mammalian cells. Mai et al (Mai and Garini 2005; Mai and Garini 2006) reported that telomeres also occupy a specific spatial orientation in the 3-dimentional interphase nucleus with distinct differences in normal and tumor cells. They show also that telomeres under *cMYC* overexpression formed disorganized aggregates within the nucleus. The formation of nuclear aggregates is considered to increase the likelihood of CIN events.

2.3. Epigenetic changes

Epigentics refers to a set of self-perpetuating modifications of DNA and nuclear proteins that produce lasting alterations in chromatin structure as a direct consequence, and meiotically and mitotically heritable changes in gene expression as an indirect consequence (Gericke 2006). It was proposed that DNA breakage and repair, chromatin remodelling and transcription share several key molecules in a number of epigenetically related pathways (Bassal and El-Osta 2005). There is an evident correlation between fragile site induced instability and epigenetic modification, based on the dependence of FRA expression on replicative delay associated with chromatin remodelling (Gericke 2006).

Hypomethylation of the genome with chromatin opening, results in increased mutation rate, including deletions and chromosomal copy number changes leading to structural instability (Chen et al. 1998; Eden et al. 2003). A causal link was proposed between hypomethylation and aneuploidy in human colorectal cancer cell lines (Lengauer et al. 1997) and primary colon tumors (Rodriguez et al. 2006). CIN is evident in hypomethylated centromeric regions of blood metaphase cells of patients with ICF syndrome (Xu et al. 1999). The same phenomena of decondensed pericentromeric heterochromatin by hypomethylation of DNA from the centromeric repeat regions of chr1 in multiple myeloma, permitted the recombination of similar centromeric repeats from different other chromosomes, and increased the incidence of structural chromosomal rearrangements in these tumor cells (Sawyer et al. 1998). The nature of

genomic damage associated with DNA hypomethylation and subsequent carcinogenesis is still unknown, however.

2.4. Specific genetic events

Breakage-fusion-bridge cycles

A mechanism called breakeage-fusion-bridge (BFB) has been described as a cycle, involving chromatid breaks and fusions, triggered by dicentric and ring chromosome rupture during anaphase resolution. This self-perpetuating process gives rise to amplifications (HSRs, DM,etc), complex chromosomal rearrangements, inverted repeats, interstitial deletions and duplications. As a result of this dynamic process, kayotypic heterogeneity is shown in pancreatic, ovarian, oral squamous carcinomas, osteosarcomas, leiomyofibromas and malignant fibrous histocytomas (Gisselsson et al. 2000; Gisselsson et al. 2001).

Telomere dysfunction

Several groups described an association between BFB cycles, structural chromosomal instability and telomere length (Gisselsson 2001; Gisselsson et al. 2001; Vukovic et al. 2007). Telomeres are complex nucleoprotein structures at the ends of linear chromosomes responsible for the chromosome end integrity. In the absence of telomerase or other dysfunction of telomere capping function, telomeres dysfunction, progressively shorten and promotes genomic instability, and eventually leads to cell death (crisis). Several studies showed a relationship between telomere length and karyotypic complexity (inverted repeats, BFB cycles, anaphase bridges, etc) (Gisselsson et al. 2000; Gisselsson 2003; Gisselsson et al. 2004; Gisselsson and Hoglund 2005; Stewenius et al. 2005; Vukovic et al. 2007).

Aberrant DNA repair

Defects in DNA repair pathways in cancer cells are likely to increase both the frequency and complexity of genomic rearrangements (Hoeijmakers 2001). DNA repair for CIN tumors typically utilizes homologous recombination (HR) or/and non-homologous end joining (NHEJ) pathways when resolving double strand breaks (DSB). In both above mentioned pathways, chromosomal deletions or translocations may result when broken ends from different chromosomes are indiscriminately fused together by error-prone repair mechanisms. It was shown that defects of various proteins involved in both pathways may increase the frequency of neoplasia and can elevate the extent of CIN (Hoeijmakers 2001).

Mitotic segregation errors

It is well known that mitotic segregation errors are the driving mechanisms for numerical CIN and clonal aneuploidy. Any error in alignment along the metaphase plate, segregation and migration to the appropriate poles and cytokinesis may cause improper cell division, resulting in the normal execution of apoptotic pathways. Malignant transformation of the cells confers the ability to escape normal apoptotic pathways, permitting the survival of abnormal daughter cells. There are many studies discussing the factors affecting mitotic segregation errors leading to chromosomal aneuploidy and CIN. Such events are e.g. centrosome duplication (observed in many different tumors: osteosarcomas, ovarian, breast, prostate carcinomas), chromosome

cohesion defects, improper chromosome attachment to the spindle microtubules, failure in cytokinesis and cell cycle/mitotic checkpoint defects (Gisselsson 2005; Kops et al. 2005).

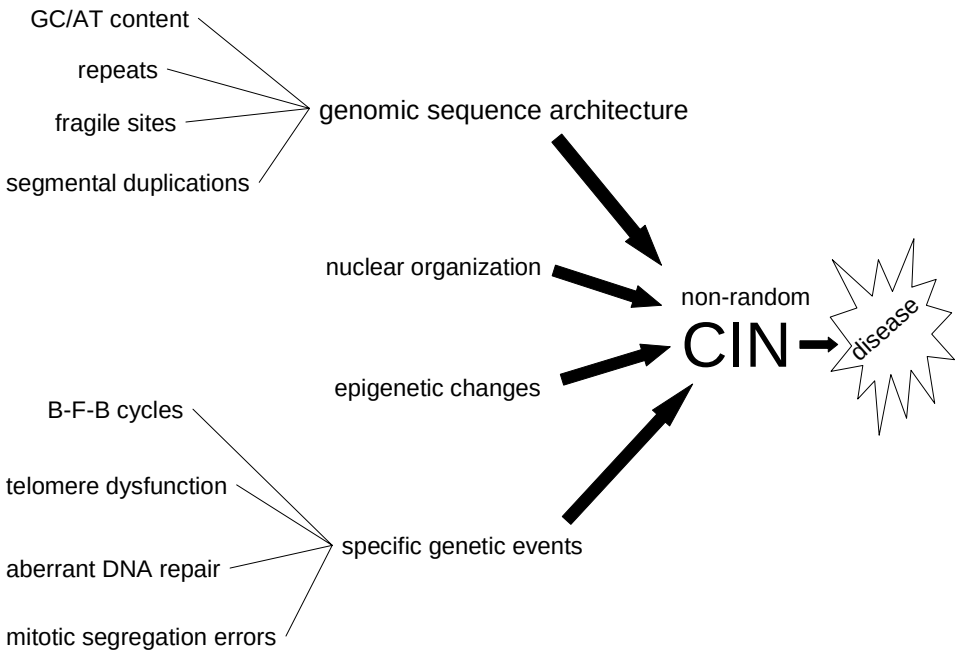


Figure 8. Chromosomal instability

3. Chromosomal rearrangements in evolution. Segmental duplications

Chromosome studies on different species reveal a wide variation in karyotypes. The great variation of karyotypes among vertebrates initially led to the expectation that the chromosomes of different orders had been shuffled beyond recognition. Recently the comparative genomics approaches changed this view highlighting a significant conservation between vertebrates (Andersson 1996; (Burt 2002). More recent submicroscopic resolution due to genomic-sequence availability suggests an abundance of small rearrangements and reusage of rearrangement breakpoints during mammalian evolution, which is inconsistent with the model of random breakage. This suggests that chromosomal breaks tend to reoccur at "hot spots" on mammalian chromosomes. Other evidence supporting non-randomness is that several other types of rearrangements, like movement of centromeres and segmental duplications, tend to occur at particular sites (Coghlan et al. 2005).

Differences identified by comparison of karyotypes between species are products of chromosomal rearrangements contributing to evolution. Within a certain species, these changes may lead to genetic disease and cancer.

There are two types of chromosome rearrangements described in evolution:
a) Intrachromosomal rearrangements like tandem duplications as the most frequent mechanism for gene duplication. As a result, a duplicated gene may accumulate mutations and become inactive, loose its function and become pseudogene; or may diverge and gain or modify its function contributing to gene innovation and gene family expansion. Other intrachromosomal rearrangements are deletions, inversions or transposition of segments.
b) Interchromosomal rearrangements are reciprocal translocations and Robertsonian fusion between centromeres. Duplication of genes between chromosomes for example by chromosome translocations, may lead to new gene functions by requiring new genomic environment (vicinity of regulatory elements).

Chromosomal rearrangements during evolution are thought to be a result of chromosome mispairing between homologous sequences at different sites in the genome and subsequent translocations. At a gross level the distribution of chromosomal breaks seem to be random, but at the molecular level chromosomal exchange must be a non-random process based on distribution of homologous sites, such as repeats of members of gene families (Burt 2002). In vertebrates SINE elements like ALU repeats and LINE (L1) elements are thought to play important role in evolution. It was also shown that the reused synteny breakpoints have strong association with segmental duplications in primate evolution (Bailey and Eichler 2006).

Segmental duplications or low copy repeats represent about 5% of the human genome and they emerged during the last 35 Myrs of primate evolution. They show >90% identity, often contain intron-exon structures of known genes and tend to localize at pericentromeric and subtelomeric regions (Kost-Alimova and Imreh 2007) See figure 9. The SDs have experienced extraordinary rates of structural change and rapid gene innovation in great apes and human genomes (Samonte and Eichler 2002; Courseaux et al. 2003; Nahon 2003).

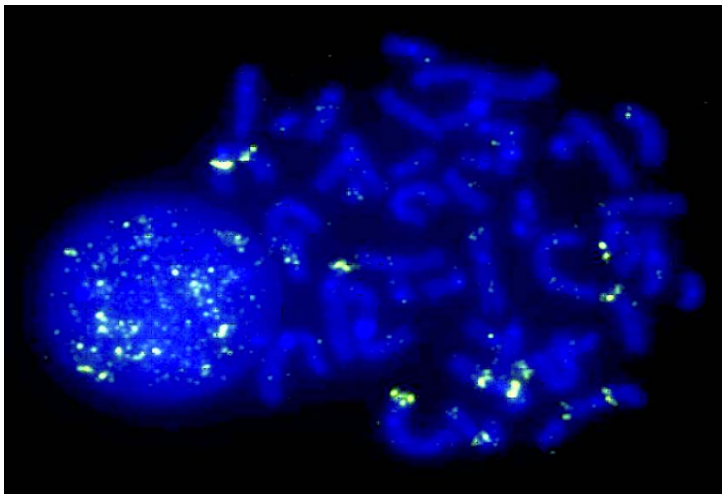


Figure 9. Segmental duplications in normal human cells

The idea that duplications might have provided the substrate for non-allelic homologous recombination has been recently proposed for human chr 7 and 18 (Dennehey et al. 2004; Muller et al. 2004), other primate chromosome rearrangements

(Locke et al. 2003) and nearly all recent, large-scale chromosome inversions in the human that occurred in the last 25 Myr (Murphy et al. 2005). It was shown before that far more human-mouse evolutionary chromosomal breakpoints contain segmental duplications that would be expected by chance. All these results provide evidence implicating segmental duplications as a major mechanism of chromosomal rearrangement in recent primate evolution (Kost-Alimova and Imreh 2007).

The evolutionary activity, observed at the breakpoint regions, including reuse, increased gene density, SD accumulation and the emergence of centromeres and telomeres, suggests the existence of a number of regions in the mammalian genome that may be disrupted by these various dynamic processes. See figure 10.

Literature data provides evidence that ”hot spot” regions rich in SD at 17p11.2-p12, are rearranged not only during evolution, but also in a variety of different constitutional and cancer-related structural chromosome aberrations (Chen et al. 1997; Reiter et al. 1998; Stankiewicz et al. 2001; Barbouti et al. 2004; Stankiewicz et al. 2004; Murphy et al. 2005; Selzer et al. 2005).

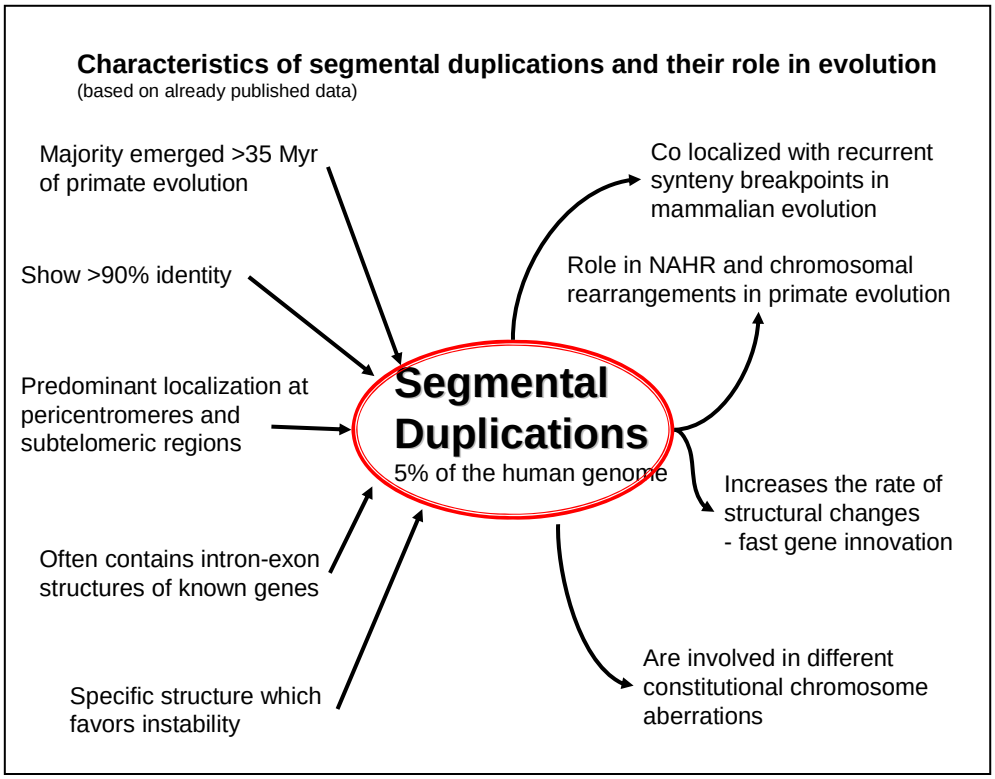


Figure 10. Characteristics of segmental duplications and their role in evolution

Aims of the study

The general aim of this study was to identify correlations between tumor breakpoint regions and evolutionarily plastic sequences on chr3. These may support the hypothesis that a region's instability may predispose certain chromosome sequences to break during tumor development. This instability at the tumor breakpoint regions may contribute to the elimination of chromosomal sequences of considerable size driven by selection for tumor growth (e.g elimination of tumor suppressor genes).

The more specific aims were:

1. To analyse CER1 sequences in relation to tumor breakpoint regions and evolutionary plasticity

1.1. To identify and characterize the mouse orthologous CER1 (paper I)

1.2. To identify and characterize evolutionary plastic features of the breakpoint cluster regions at CER1 borders (paper II)

1.3. To identify the selective factors behind CER1 loss by searching for TSG genes within CER1 (paper III)

2. To analyse the entire chr3 sequence in relation to tumor breakpoint regions and evolutionary plasticity

2.1. To develop new high resolution methods of chr3 analysis in tumors and compare their ability to decode complex chromosomal rearrangements (paper IV)

2.2. To identify the hot spots of chr3 breaks in tumors and characterize their role in evolution (paper VI)

2.3. To identify the selective factors behind the chr3 rearrangements in cancer (paper V)

Materials and Methods

1. Cell Lines

(I, II, III, V) MCH A9Hytk3 and MCH 903.1 containing a single cytogenetically intact human chr3 on mouse fibrosarcoma A9 background (Imreh et al. 1994; Cuthbert et al. 1995), were used as chr3 donors for microcell mediated chromosome transfer as described (Saxon and Stanbridge 1987).

Hygromycin resistance gene carrying chr3 from MCH A9Hytk3 was transferred into nonpapillary (clear cell) RCC cell line KH39. Microcell hybrid lines were selected and propagated in vitro with 400 µg/ml Hygromycin B (Sigma, St. Louis, MO). Four MCHs, designated YYK1, 2, 3 and 4, were obtained from four different fusion events (Yang et al. 2001).

Geneticin resistance gene carrying chr3 from MCH 903.1 was transferred into the poorly differentiated NPC cell line Hone 1. MCHs were selected and propagated in vitro with 400 µg/ml Geneticin (G418) (Sigma, St. Louis, MO). MCH 4.8 was used for our study.

Six-week old SCID mice were used for inoculations (10^6 cells/site). Mice were observed for tumor formation once a week up to 20 weeks. The tumors were excised under sterile conditions, explanted and expanded.

(IV, VI) Nine cancer cell lines were analyzed. They were selected according to their ploidy level and chr3 rearrangements determined by chr3 painting and metaphase chromosome counts. Seven renal cell carcinoma cell lines: A498, UOK115, UOK125, UOK147, TK164 (Yano et al. 1988; Gnarra et al. 1994; Tomita et al. 1996; Lo and Huang 2002), CAKI1 (ATCC catalog No.HTB46) and CAKI2 (ATCC catalog No.HTB47); one small cell lung cancer cell line U2020 (Sundaresan et al. 1998; Heppell-Parton et al. 1999; Heppell-Parton et al. 1999) with an interstitial homozygous deletion on 3p12.3 and a human nasopharyngeal carcinoma cell line HONE1 (Lo and Huang 2002) were used. All cell lines were cultured on IMDM medium with 10% fetal calf serum, 1% penicillin-streptomycin and 1% Glutamine. For the array experiments total genomic DNA was isolated using GeneElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Germany). For the mpFISH experiments cells were treated with 20µg/ml colcemid for 3-4 hrs to obtain metaphase chromosomes. After treatment with hypotonic solution cells were fixed in methanol: acetic acid (3:1) following standard protocols.

2. Transfections

The mouse fibrosarcoma cell line A9, was maintained in Iscove's modified Eagle's medium containing 10% fetal calf serum.
For transfections we have used RP6-3307 containing full length *LIMD1* gene in a blasticidin selectable pPAC4 vector. Fifty to 70% confluent monolayer A9 cells were transfected with a mixture of 12 µg PAC DNA and 10 µl Lipofectamine (Life Technologies) per well, in six well plates according to standard protocols. The

transfectants were selected on blasticidin (1-2 μg/ml). Two positive clones were chosen and expanded in vitro for inoculation into SCID mice. All transfectants were analysed by fluorescent in situ hybridization (FISH), DNA and RNA content.

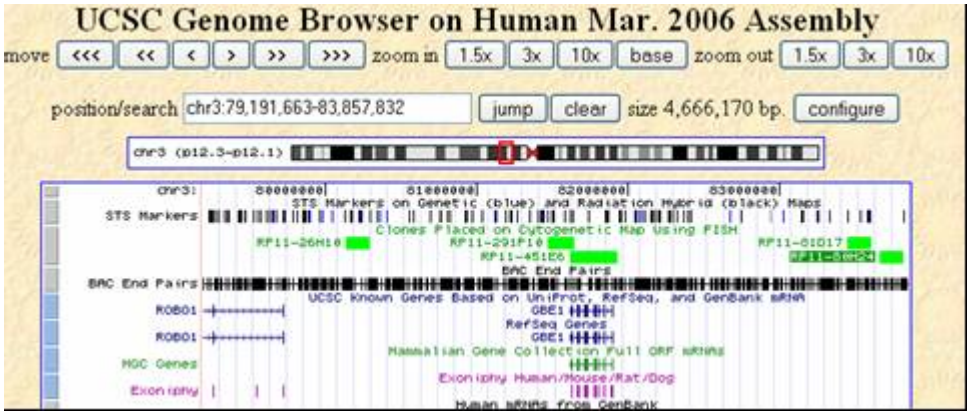
3. Tumor formation in SCID mice

(III) *LIMD1*-PAC transfectants and A9 fibrosarcoma cell line, as control, were chosen for mouse inoculation. One million A9 and *LIMD1*-PAC transfectants were inoculated subcutaneously into 6 week-old SCID mice. The mice were observed for tumor formation once a week up to 6 weeks.

The two positive *LIMD1*-PAC clones were inoculated in two series into 4 mice/clone, 3 inoculation sites/mouse. A9 cells were inoculated as controls at 1 site/mouse. Tumor growth was monitored once a week. After 6 weeks, the tumor take incidence was 8 out of 12 and 10 out of 12 respectively. Tumor growth suppression was observed at the tumor sites inoculated with *LIMD1*-PAC transfectants in all 8 SCID mice except one.

4. BAC/PAC clones

(IV,V,VI) A set of 179 BAC/PAC clones was selected according to several considerations:
(i) DNA from all these clones was commercially available (2x96 deep well blocks) from BACPAC Resources Center, CHILDREN'S HOSPITAL OAKLAND, Oakland, USA ;
(ii) all clones were FISH mapped, at least partially sequenced and their localization was approved using UCSC database ;
(iii) all clones were chr3 specific and covered the whole chromosome with a resolution of ~1Mb.



5. PCR reactions

(I) The Kiaa0028 cDNA sequence was verified with primers 1-8 (Table 1 in paper I) using mouse kidney cDNA library as a template. Primers 9-12 were used for the confirmation of the Xtrp3s1 cDNA sequence from mouse kidney cDNA library. Marathon RACE was performed to obtain the 5' and 3' end of the Xtrp3s1 cDNA using Marathon-ready kidney cDNA library (Clontech, # 7452-1). Primer 15 or 17 were used with AP1 primer in the primary reaction and primer 16 or 18 with AP2

primer in the nested reaction. Primer 13 and 14 were used for the sequencing of the obtained Marathon Xtrp3s1 fragment. Different parts of the Fyco1 gene were amplified with primers 19-26 from mouse kidney cDNA library. Primer 27-30 were used for the verification of the Tmem7 cDNA sequence. Marathon RACE was performed from Marathon-ready liver cDNA library (Clontech, # 7451-1) with primers 33 or 35 together with AP1 primer and primers 34 or 36 together with AP2 primer during the characterization of the Tmem7 gene. Primer 31 and 32 were used for the sequencing of the obtained Marathon Tmem7 fragment. Primer 37 and 38 were used for the amplification of a part of the Lrrc2 cDNA from mouse kidney cDNA library. The conditions of Marathon RACE PCR were according to the recommendations of the supplier. PCR amplified cDNA fragments were isolated in low melting point agarose and sequenced as described previously (Seroussi et al. 1998).

6. Northern blot

(I) Mouse 8-Lane Northern Blot (Clontech, #7762-1) was hybridized with a mouse Kiaa0028 (primer 5-6, bp), Xtrp3s1 (primer 11-12, bp), Fyco1 (primer 25-26, bp), Tmem7 (primer 27-28, bp), Lrrc2 (primer , bp) and β-actin cDNA probes, in separate experiments. Probe labeling, hybridization and washing were performed according to standard protocols (Feinberg and Vogelstein 1984; Sambrook and Gething 1989).

7. Sequence Data

(I,II) Human sequence data were downloaded from Human genome Project working Draft, May 2004 UCSC (http://genome.ucsc.edu)**.** The Ensembl server was also used to download the Homo sapiens, Fugu rubripes, Drosophila melanogaster Caenorhabditis elegans sequences (http://www.ensembl.org). The chicken, mouse, rat, and dog sequences were taken form USCS database (http://genome.ucsc.edu).

8. Segmental and gene duplication identification

(I,II) To identify segmental duplications we used the Human April 2003 (hg15) assembly (http://genome.ucsc.edu/)**,** Segmental Duplications Track from UCSC server. Cut off values were at least 1 kb of total sequence aligned (containing at least 500 bp non-RepeatMasked sequence) and at least 90% sequence identity.
For identification of close paralogs, we used BLAT search, which finds sequences corresponding to min. 20 amino acids with 80% and greater similarity (http://genome.cse.ucsc.edu). Each output sequence was analyzed, and if it was corresponding to a known gene in Genome Browser on Human May 2004 presented in UCSC Genome Bioinformatics Site, we have accepted this gene as close paralog.
For each CER1 gene we identified protein families with number of members using the Family view programs from the Ensembl server (http://www.ensembl.org/Homo_sapiens/familyview). Protein families were generated using the MCL (Markov CLustering) package available at http://micans.org/mcl/. The application of MCL to biological graphs was initially proposed by Enright A.J. at al.
For identification of tandemly duplicated gene clusters within CER1, we used PipMaker, which identifies similar parts in two sequences of more than 100bp with

at least 70% nucleotide identity. Then all CER1 protein sequences were compared using ClustalW**,** a program for alignment of multiple nucleotide or protein sequences on Bioinformatics Application Service Provider (BioASP) server (http://services.bioasp.nl/blast/cgi-bin/clustal.cgi). The program uses three steps to achieve this. First it constructs all pairwise alignments, which are then transformed into a guide-tree. This tree is used in the final step, which consists of a progressive alignment algorithm to achieve the multiple sequence alignment. ClustalW optionally generates a Phylogenetic Tree view on the alignment as well. ClustalW is fast and excels in aligning multiple sequences, which share a fair amount of overlap (~30%) among each other.

9. Identification of synteny breaks

(II) A 5 Mb human chr3 sequence between 43 - 48 Mb positions was taken from the Genome Browser, Human May 2004 presented in UCSC Genome Bioinformatics Site (http://genome.cse.ucsc.edu). In order to identify syntenic sequences we used mouse, chicken, dog and rat Alignment Net Data represented in this Genome Browser. These data show the best alignment chain for every part of the human genome, they are useful for finding orthologous regions and for studying genome rearrangements. Chains are derived from BLAST Alignments and sorted so that the highest scoring chain in the genome comes first. The program was developed at the University of California at Santa Cruz by Jim Kent. Mouse sequences were from Mouse mm5 assembly. The May 2004 *Mus musculus* draft genome data were obtained from the Build 33 assembly by NCBI. Rat sequences: Rat rn3 assembly The rn3 June 2003 *Rattus norvegicus* genome assembly is based on version 3.1 produced by the Atlas group at Baylor Human Genome Sequencing Center (HGSC) as part of the Rat Genome Sequencing Consortium. Chicken sequences were taken from Chicken galGal2 assembly. The galGal2 February 2004 *Gallus gallus* draft sequence was produced by the Genome Sequencing Center at the Washington University School of Medicine in St. Louis. Dog sequences were obtained from Dog canFam1 assembly**.** The canFam1 July 2004 *Canis familiaris* whole genome shotgun (WGS) assembly v1.0 was sequenced and assembled by the Broad Institute of MIT/Harvard and Agencourt Bioscience. The chain alignments spanning more than one gene have been verified, comparing corresponding sequences between human and other species using PipMaker.

In order to determine synteny breakpoint frequency at chromosomal regions surrounding CER1, we analyzed 50 Mb of human 3p between Mb positions 1 and 50 using mouse, chicken, dog and rat alignment Net data represented in UCSC Genome Browser on Human May 2004 Assembly. Conserved segments in this browser are represented as continuous (including 2 or more genes) alignments between human and other genomic sequences. The maximum number of synteny breaks between species may be estimated using these data. We considered synteny breakpoints if the chain alignment was broken and the neighbouring sequence of two or more genes represented another chain or gap in conservation. Based on this analysis we found in a 50 Mb of chr3 sequence, 26 breakpoints in comparison with chicken, 9 in comparison with mouse and 4 in comparison with dog. Therefore the density of breakpoints per Mb of human sequence in average is 0.52 in human-chicken synteny, 0.18 in human-mouse, and 0.08 in human-dog synteny.

10. Ortholog identification with two-way BLAST in low organisms and with phylogenetic methods

(II) The CER1 gene coded protein sequences were used for database searches by the BLASTP program (http://www.ensembl.org/Multi/blastview). We performed two-way BLASTP: each three first best hits for the CER1 genes, which coincide to a known gene in the different species, was ran back against the human database. In BLAST searches we enabled high filtering for low complexity and used Blosum 62 matrix. The cut off E-value for ortholog pairs was set to 1e-5. Pairs of sequences that were one another's best matches in their respective genomes were considered to be potential orthologs.

(I,II) Exactly the same sequences from the different species and the same two-way method were used for BLASTP search and for phylogenetic methods. We performed amino acid sequence alignments using the multi-processor version 1.81 of ClustalW (www.cmbi.kun.nl/bioinf/tools/clustalw.shtml), with Blosum 30 protein weight matrix and the output visualized by the version 3.21 of BOXSHADE program, written by K. Hofmann and M. Baron (www.ch.embnet.org/software/BOX_form.html), and in predicted phylogenetic tree format (http://services.bioasp.nl/blast/cgi-bin/clustal.cgi). For phylogenetic tree prediction another program was also used: the TreeTop - Phylogenetic Tree Prediction program (www.genebee.msu.su/services/phtree_reduced.html) that uses the alignments produced by ClustalW. Genes, showing one-to-one relationship, were considered to be potential orthologs.

11. Conserved element search

(I,II) Repetitive sequences were filtered out from genomic sequence using the RepeatMasker web server (ftp.genome.washington.edu/cgi-bin/RepeatMasker). Comparative sequence alignment was done using PipMaker and MultiPipMaker (bio.cse.psu.edu/pipmaker), (bio.cse.psu.edu/cgi-bin/multipipmaker) which produces percent identity plot (PIP) and dot-plot using the human sequences as reference, with the options "search for both strands" and "chaining".

12. Fluorescence in Situ Hybridization (FISH).

(IV,V,VI) The slides for FISH were prepared using standard techniques from the cells treated with colcemide, hypotonic solution and fixed in methanol: acetic acid (3:1).
(V) DNA from CER1 specific PAC RP6-123i13 clone (Yang et al. 1999) was isolated using Qiagen columns (QIAGEN, Inc., Hilden, Germany) and labelled with biotin-dUTP (BIO-Nick Translation Mix, Roche Molecular Biochemicals, Mannheim, Germany). Double color FISH was performed as described (Fedorova et al. 1997) using chr3 specific painting probe labelled with FITC (Cambio, Cambridge, UK), combined with biotin labelled RP6-123i13. The biotin-labelled probe hybridization was detected with Cy3 conjugated streptavidin (Amersham Biosciences, GE

Healthcare Worldwide). In case of analysis of Hone1 derived cells, we applied second round of FISH using chr2 and chr12 specific painting probes labelled with Cy3 and biotin (Cambio, Cambridge, UK). The biotin-labelled probe hybridization was detected with Cy5 conjugated streptavidin (Amersham Biosciences, GE Healthcare Worldwide)

A fluorescence microscope (Leitz-DMRB, Leica, Heidelberg, Germany) equipped with a Hamamatsu C 4800 cooled CCD camera (Hamamatsu, Herrsching, Germany) was used to analyse at least 20 metaphases and a minimum of 100 nuclei in each sample. For four-probe FISH, we captured the same metaphases after the first and after the second FISH round. Using Adobe Photoshop 7.0 (Adobe Systems, San Jose, Calif., USA) we superimposed the two images giving to different probes different artificial colors.

13. mpFISH

(IV,V,VI) Metaphase chromosomes and interphase nuclei were used from methanol:acetic acid (1:3) fixed cells. We analysed a minimum of 20 metaphases in each sample using FISH with chr3 specific painting probe labelled with FITC (Cambio, Cambridge, UK). In each metaphase, we identified all labelled normal and marker chromosomes.

Using multi-channel pipette 5µl of fixed cell suspension was applied to the slide to obtain 10 hybridization fields marked on the back-side of the slide. The slides were pre-treated with pepsin and prefixed before denaturation. As probes 200 ng of commercial BAC/PAC DNA (see BAC/PAC clones) was labelled with nick-translation either with biotin-dUTP or digoxigenin-dUTP (Nick Translation Mix; Roche Molecular Biochemicals, Mannheim, Germany). Two-colour mpFISH in microvolumes (1µl) was performed on 10 sites/slide under 9x9 mm cover slips. The hybridization technique was used as described previously (Fedorova et al. 1997). The biotin labelled probes was detected with Cy3 conjugated streptavidin (Amersham Biosciences, GE Healthcare Worldwide) and digoxigenin labelled probes with FITC conjugated anti-digoxigenin antibodies (Roche Molecular Biochemicals Mannheim). Between 100-200 interphase nuclei and 10-20 metaphase plates were analysed for each sample using a fluorescence microscope (Leitz-DMRB, Leica, Heidelberg, Germany) equipped with a Hamamatsu C 4800 cooled CCD camera (Hamamatsu, Herrsching, Germany) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, Calif., USA).

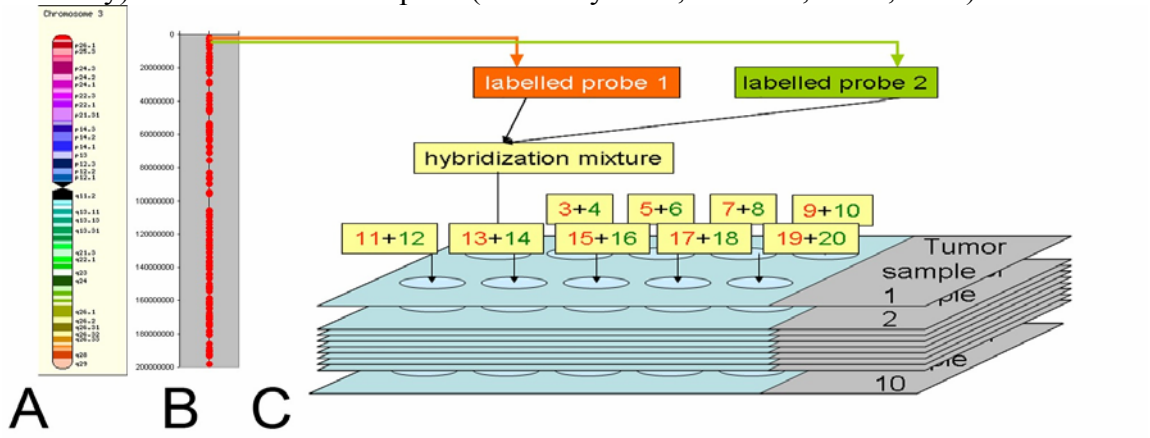


Figure 11. Scheme of mpFISH

14. Array-CGH

(IV,VI) DNA labelling, hybridization and post-hybridization processing, scanning and image analysis were performed as previously described (Buckley et al. 2002). A pool of peripheral-blood-derived DNA from 10 normal females was used as normal reference DNA for all hybridizations performed. In brief, 2 µg of test DNA and 2 µg of reference DNA were differentially labelled by random priming using Cy3-dCTP (PA53021, GE Healthcare, Piscataway, NJ) and Cy5-dCTP (PA55021, GE Healthcare). These were then mixed with 100 µg of Cot-1 DNA (Roche, Basel, Switzerland) and hybridized to the array. Image acquisition was performed using the GenePix 4000B scanner (Axon Instruments Inc., Union City, CA). Analysis of spot intensities was carried out using GenePixPro image analysis software (Axon Instruments). The average and the coefficient of variation of fluorescence ratios for each measurement point were calculated. Data points displaying a coefficient of variation greater than 5% between at least two of the replica spots were discarded from further analysis. The average of fluorescence ratios from autosomal controls was used in the normalization of data in each hybridization experiment. The ANILFR (Average Normalized Inter Locus Fluorescent Ratio) values were calculated in order to assess the inter-locus variation, representing region(s) on the array. The NFR (Normalized Fluorescence Ratio) for successfully scored loci from a certain, continuous region on the array, which corresponded to mpFISH identified regions, was used to calculate the ANILFR value and the standard deviation.

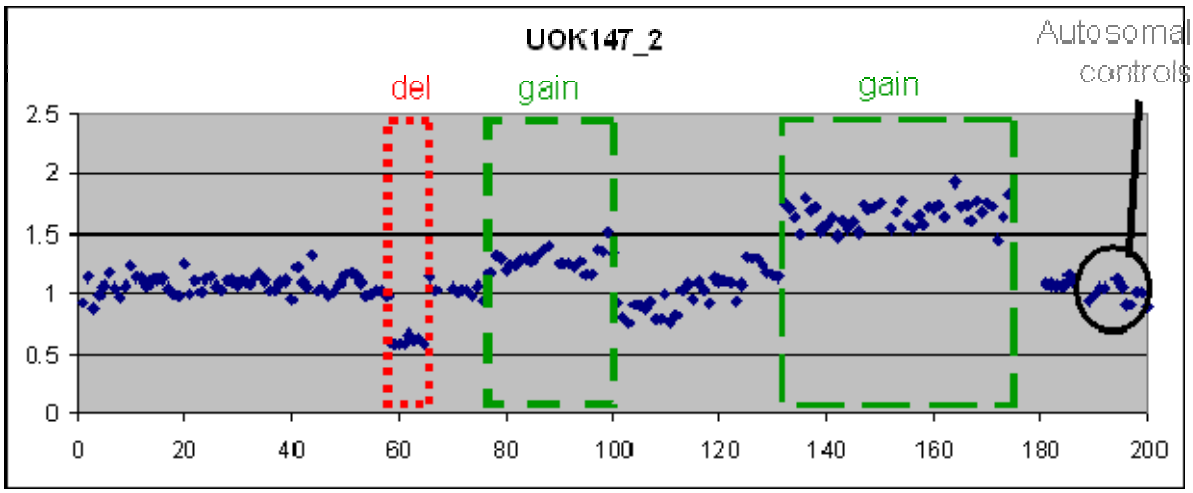


Figure 12. Array-CGH of UOK147 renal cell carcinoma cell line

Results and Discussion

1. Analysis of CER1 sequence in relation to tumor breakpoint regions and evolutionary plasticity

FISH analysis performed previously in our group identified 12 mouse loci that were orthologous to CER1 and surrounding 3p21 regions. It was shown that CER1 splits into two segments in mouse containing a mouse/human conservation breakpoint region (CBR). Several breaks occurred in tumors within the region surrounding the CBR, and this sequence has features that characterize unstable chromosomal regions: deletions in yeast artificial chromosome clones, late replication, gene and segment duplications, and pseudogene insertions. Sequence analysis of the 3p12-p22 region revealed that other cancer-associated deletions (regions eliminated from monochromosomal hybrids carrying an intact chr3 during tumor growth and homozygous deletions found in human tumors) co-localized non-randomly with mouse/human CBRs and were characterized by an increased number of local gene duplications and mouse/human conservation mismatches (single genes that do not match into the conserved chromosomal segment) (Kost-Alimova et al. 2003). These findings encouraged more detailed analysis of CER1 sequence in relation to tumor breakpoint regions and evolutionary plasticity.

1.1. Identification and characterization of the mouse orthologous CER1 region (paper I)

A large scale sequencing project performed in our lab, determined 1,32 Mb sequence of CER1 (Kiss et al. 2002), which was used for comparative analysis. Mouse orthologs of CER1 genes were identified using Celera database. This search revealed that CER1 corresponded to two distinct syntenic blocks on chromosome 9F in mouse.

We found high conservation in the order and structure of the 17 CER1 genes and identified five novel mouse genes located within the syntenic blocks: *Kiaa0028, Xtrp3s1, Fyco1* and *Lrrc2*. The mouse *Tmem7* gene had two transcripts of different size, which were expressed mainly in the liver. The human and the mouse TMEM7 proteins were not conserved within their C-termini. However, both proteins contained predicted transmembrane domains at their C-terminal parts. Lack of conservation of the human pseudogenes confirmed their retroposition.

Not only gene contents, but also GC content of the entire human and mouse region was similar 43.54% and 42.405%, respectively. RepeatMasker identified a striking discrepancy in the number of repetitive elements.

In the mouse sequence we could identify two new genes that were generated by duplication: *Xtrp3* that was generated by duplication of *Xtrp3s1,* and *Cmkbr1l1* derived from *Cmkbr1,* appeared after human-mouse divergence. In CER1 we noticed the presence of a large cluster of chemokine receptor genes (CCRs), and constructing their phylogeny - tree, we could hypothesize that all chemokine receptor genes arose from

intrachromosomal duplications during evolution. Supporting evidence was the clustering of three pairs of genes: *CCR9- STRL33; CCR1- CCR3* and *CCR2- CCR5* in both species. They are closest relatives and sit next to each other on the chromosomes in human and mouse.

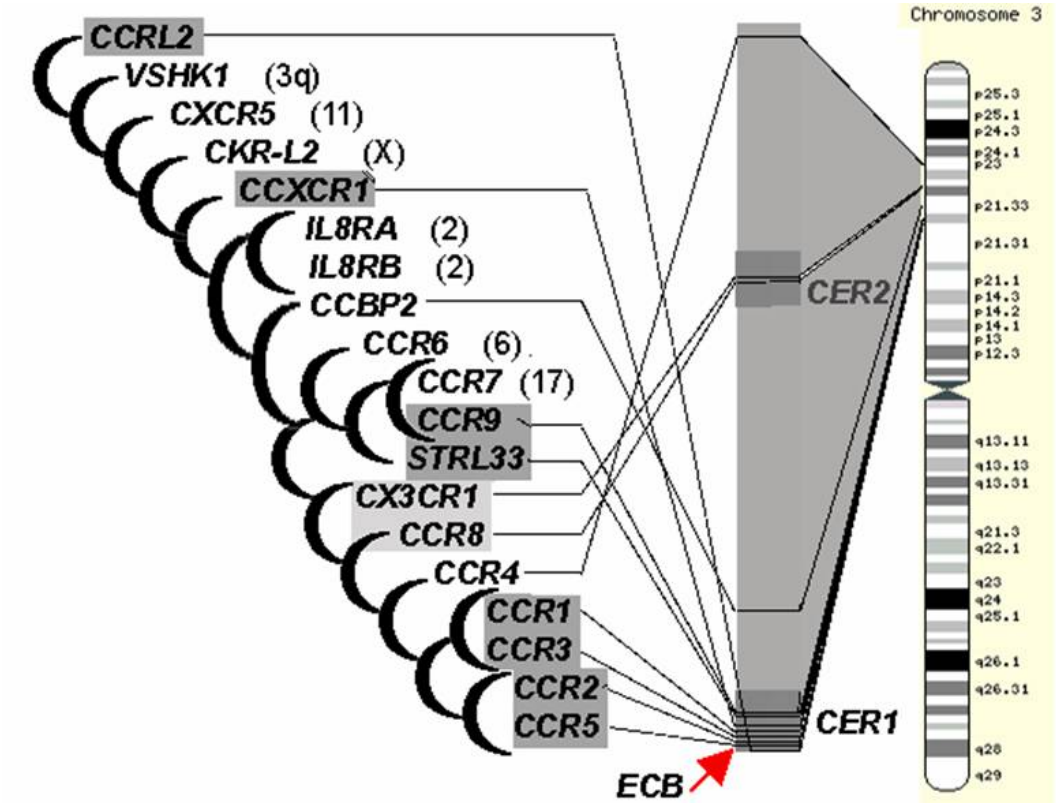


Figure 13. The family tree of the chemokine receptors in the human genome. ECB – evolutionary breakpoint region

In conclusion, this comparative analysis of CER1 identified the mouse orthologous region divided in two parts with the gene content and positions highly conserved between species. We found two new gene duplications in mouse (*Xtrp3s1* and *Cmlbr1*) and five novel mouse genes (*Kiaa0028, Xtrp3s1, Fyco1, Tmem7* and *Lrrc2*) were identified and characterized.

1.2. Evolutionary plasticity at the CER1 breakpoint cluster regions (paper II)

After the comparative study of CER1 between human and mouse, a more thorough evolutionary study was performed analyzing orthologous sequences in Caenorhabditis elegans, Drosophila melanogaster, Fugu rubripes, Gallus gallus, Mus musculus, Rattus norvegicus and Canis familiaris.

Examining the presence of duplication at the borders of CER1 we could show that there are more close paralogous genes at the borders (CCR family and the ZNF family

genes) then in the central part. These gene duplications were associated with increased frequency of retroposed pseudogene insertions.

As next step, we analyzed the conservation of CER1 in different species. We found four independent synteny breaks within the CER1 telomeric breakpoint cluster region, when compared human, dog and chicken genomes, and two independent synteny breaks within the CER1 centromeric breakpoint cluster region, when compared human, mouse and chicken genomes suggesting a non-random involvement of tumor breakpoint regions in chromosome evolution. Phylogeny analysis indicated that all genes from both CER1 breakpoint cluster regions underwent horizontal evolution in mammals, with formation of new genes, expansion of gene families and pseudogene insertions. In contrast CER1 middle region contained evolutionarily well conserved solitary genes and minimal amount of retroposed genes.

The coincidence of evolutionary plasticity with CER1 breakpoints suggested that regional structural instability at the CER1 borders plays an important role in both evolutionary and cancer associated chromosome rearrangements. Well-conserved central CER1 gene block is deleted as relatively large segment in tumors.

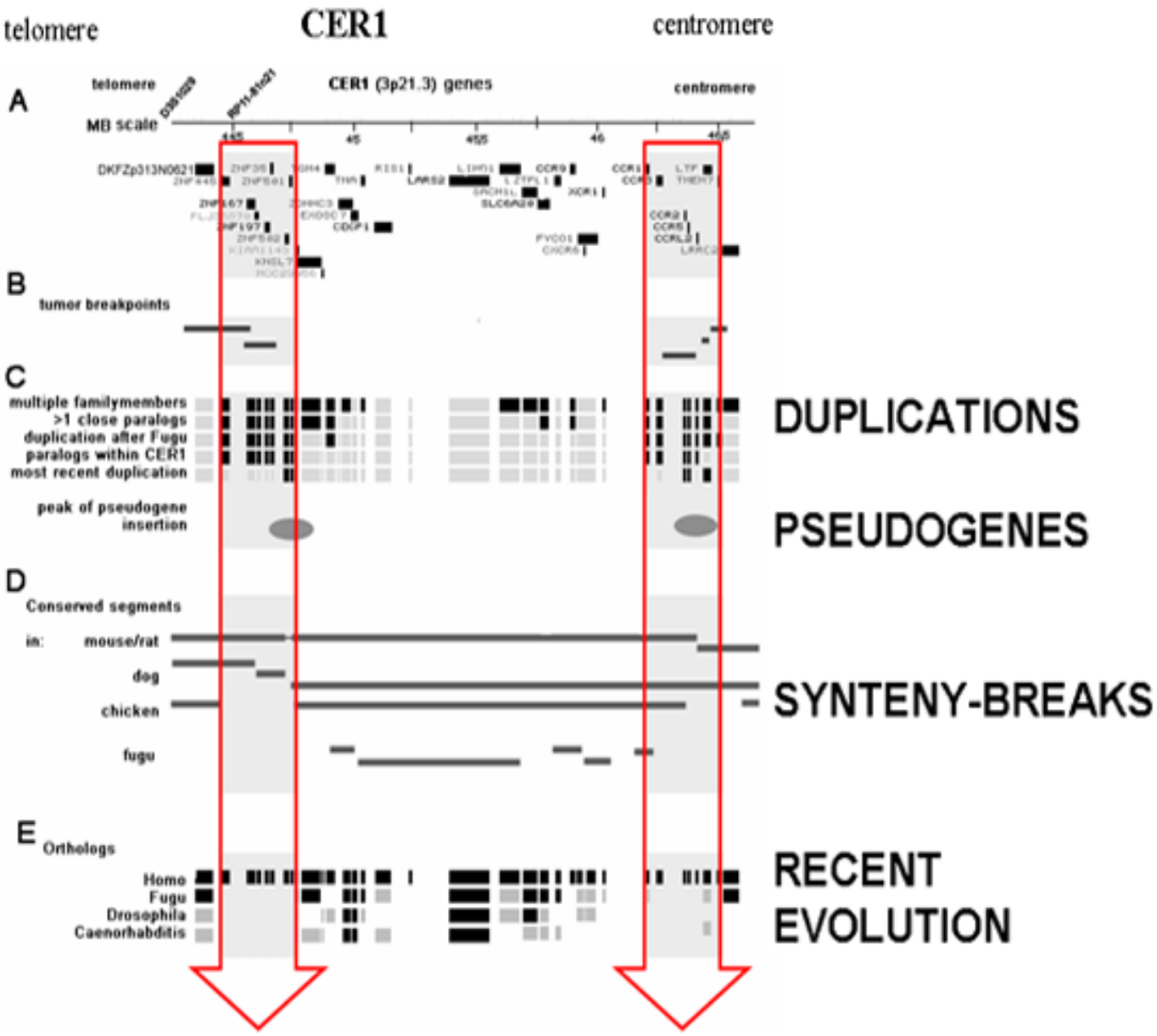


Figure 14. Overview of evidence for evolutionary plasticity of CER1 border regions; these regions are highlighted with red arrows throughout the figure.

A -Transcriptional map of CER1. Megabase positions, reference sequence genes and reference markers are indicated. **B** -CER1 breakpoints in tumors. In tumors derived from human chr3/ mouse ibrosarcoma MCHs we found breakages proximal to marker D3S1029 at the telomeric, and within LRRC2 gene at the centromeric border. In tumors originated from human chr3/ human MCHs the breakpoints were proximal to FISH clone RP11-81n21 and between CCR1 and LF gene at the telomeric and pentromeric border respectively. **C** -Gene duplications at the tumor breakpoint regions of CER1. Black boxes indicate: genes having multiple family members in human in the first line; genes having more than one close paralogs in the second line; genes, which were duplicated after human/fugu divergence in the third line; genes having close paralogs within CER1 in the fourth line; most recent duplications in the fifth line. Dark gray ovals indicate clustering of pseudogenes. **D** -Synteny breakpoints in mouse and rat, dog, chicken and fugu coincide with CER1 tumor breakpoints. Horizontal black bars show syntenic blocks. **E** -CER1 central part is well conserved even in fish and invertebrates. Black boxes indicate well-defined orthologs. Grey boxes indicate ambiguous orthologs.

1.3. Candidate TSGs within CER1 (paper III)

Our studies on CER1 were concerning not only the structure and instability features of this region, but we tried to identify the gene(s) that were providing the cells with selective growth advantage associated with their loss during tumor development. One of these is *LIMD1*, a gene identified and characterized earlier in our group. *LIMD1* is a member of LIM domain containing protein family that plays role in intracellular signalling, transcriptional regulation and cellular differentiation during development.

In this paper we showed that LIMD1 binds pRb in vitro and in vivo. A yeast two-hybrid screen identified LIMD1 as a specific pRB-interacting protein. These results were confirmed by an in vitro pull-down assay and by co-immunoprecipitation. The interaction was specific to pRB, since other member of the retinomblastoma family (p130) did not bind in vivo. The pRB binding site on *LIMD1* was localized to aa 404-442.

It was also shown that LIMD1 is actively exported from the nucleus to the cytoplasm and it contains a nuclear export signal (NES) located at aa 54-134, that facilitates cytosolic/nuclear shuttling. A nuclear import signal is located to the LIM domains.
Knowing that pRb represses the activity of E2F transcription factors, and the fact that LIMD1 binds specifically to pRb and shuttles to the nucleus, we analysed whether LIMD1 represses E2F1 driven transcription. We showed that it may repress E2F transcription both in a pRb dependent and independent way. The pRB independent suppression can be due to a LEM domain, which may function by interfering with chromatin-remodelling proteins.
Expression array showed that *LIMD1* down-regulated 85,9% of the *E2F1* responsive genes.

Transfection experiments confirmed tumor growth antagonizing role of *LIMD1*. It inhibited cell proliferation and repressed colony formation. In vivo studies performed using SCID mice showed a tumor suppressor activity of *LIMD1* in A9 fibrosarcoma cells. Furthermore, *LIMD1* reduced significantly the development of lung metastasis in athymic mice. Lung cancer cell lines as well as malignant lung tissue samples showed considerably decreased *LIMD1* mRNA levels.

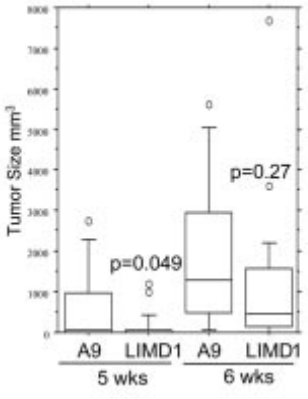


Figure 15. LIMD1 reduces growth rate in the mouse A9 fibrosarcoma model. Cumulative results of tumor size of mouse fibrosarcoma A9 and A9-LIMD1 P1-derived artificial chromosome (PAC) transfectants (LIMD1) versus weeks postinjection. Circles represent outliers.

In conclusion, *LIMD1* is a candidate tumor suppressor gene, which binds pRB and represses *E2F* driven transcription. In the case of lung carcinomas pRb mutation is not an early event, thus early *LIMD1* loss by e.g CER1 deletion, may change pRB regulation and be a critical early step in lung cancer development.

2. Analysis of the entire chr3 sequence in relation to tumor breakpoint regions and evolutionary plasticity

In order to generalise and comfirm our findings on CER1 region, we decided to analyse the entire chr3. This study became possible due to the development of new high resolution technologies of chromosome analysis, and due to the availability of new data on human and other mammalian genomes.

2.1. Development of new high resolution methods of chr3 analysis in tumors (paper IV)

Recently, several high-resolution methods of chromosome analysis have been developed. It is important to compare these methods and to select reliable combinations of techniques to analyze complex chromosomal rearrangements in tumours. In this study we have compared array-CGH (comparative genomic hybridization) and multipoint FISH (mpFISH) for their ability to characterize complex rearrangements of human chr3 in tumour cell lines. We have used 179 BAC/PAC clones covering chr3 with an approximately 1 Mb resolution to analyze nine carcinoma lines.

To identify precisely chr3 fragments in the rearranged tumor chromosomes we developed a modified FISH method, called multipoint-FISH, mpFISH. MpFISH can be imagined as a "macro-array FISH", which is the reciprocal of micro-array CGH. While in array-CGH the BAC/PAC DNA is spotted in micro-dots on the slide and the tumor DNA is hybridized to it, in mpFISH the tumor nuclei and chromosomes are fixed and the different BAC/PAC DNAs are hybridized to multiple areas on the slide (in our practice usually 10) in pairs of two (or more) color labeled probes.

Using these two high resolution methods on nine carcinoma cell lines (from near-diploid to near-pentaploid), we identified a total of 53 chr3 fragments possessing copy numbers from 0 to 14. MpFISH results from the BAC/PAC clones and array-CGH were generally concordant. In most cases the changes in copy number seen on array-CGH profiles reflected cumulative chromosome rearrangements. Most of them were the result of unbalanced translocations. Three chr3 aberrations detected by mpFISH were not detected by array-CGH: two insertions and one balanced translocation. Meanwhile each copy number change on the array profile could be related to a specific chromosome aberration detected by metaphase mpFISH.

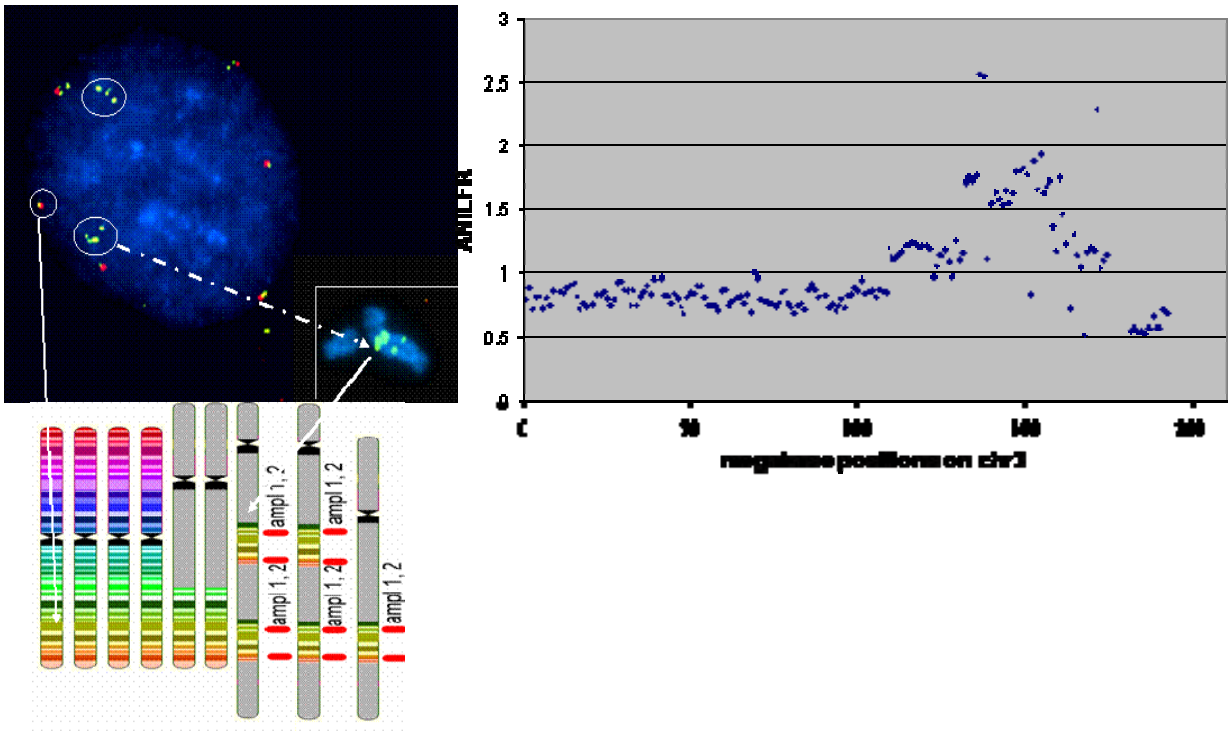


Figure 16. CAKI2, a renal cell carcinoma cell line, multipoint FISH on interphase and metaphase (probes from 3q28 – reconstitution of the amplification) **and array-CGH results**

Analysis of the correlation between real copy number from mpFISH and the average normalized inter-locus fluorescence ratio (ANILFR) value detected by array-CGH demonstrated that copy number is a linear function of parameters that include the variable, ANILFR, and two constants, ploidy and background normalized fluorescence ratio.
Although our chr3 BAC/PAC array could identify single copy number changes even in pentaploid cells, mpFISH provided a more accurate analysis in the dissection of complex karyotypes at high ploidy levels.

2.2. Identification of the hot spots of chr3 breaks in tumors and their role in evolution (paper VI manuscript)

In line with our earlier results, indicating associations between cancer associated breakpoints in microcell hybrids and human-mouse synteny breaks, we decided to conduct a detailed analysis of 10 carcinoma cell lines. The aim was to describe with high resolution the chr3 changes in tumor cell lines, and to verify the association between evolutionarily plastic and cancer associated break-prone regions.

In this paper we have analyzed ten carcinoma cell lines by several methods. CGH and M-FISH showed that chr3 is involved in rearrangements about two times more frequently than an average chromosome. Analysis of chr3 using mpFISH detected 54 breaks, out of which 27 were breakpoints of unbalanced translocations, 14 interstitial deletions, 9 duplications, 2 balanced translocations, 2 insertions. Based on FISH

experiments, three groups with different karyotype complexity emerged. The high complexity karyotype cell lines were characterized by breaks clustered around known fragile sites and previously unidentified tumor-break-prone regions (TBRs) at 3p12-p13 and 3q21 (around 75 and 130 Mb positions on human chr3 sequence). Looking at the structure of these TBRs we found specific, large (> 100 kb) segmental duplications at chr3:75, 127 and 131 Mb positions. These were homologous to each other and to particular sites on other chromosomes. Comparing our data with the Mitelman database of chromosome rearrangements in cancer we have found that the chromosomal bands adjacent to these duplications were frequently broken in human carcinomas.

Interstitial 3p13, 3q21, 7q22 and 11q13 regions are among the most involved break-prone sites in the human genome. Therefore we call these SDs Tumor-Breakpoint-Region Segmental Duplication (TBR SD). Together with the rearrangements within fragile sites, the TBR SD breaks caused the vast majority of chromosomal changes in moderate and high "complexity karyotype" cell lines.

It was already known in the literature that segmental duplications have an important role in mammalian and especially primate evolution. Comparative analysis of genomes of different species showed that breaks within TBR SDs occurred repeatedly during evolution. When the random breakpoints were re-used by breaks during mammalian evolution in 30% of the cases, the TBR SDs were re-used in almost 70% of cases, positioning them among the "hottest spots" of evolution. They appeared to play an important role in recent primate evolution. They were involved in three out of four primate-specific chr3 inversions.

Instability of the TBRs during evolution was exhibited in multiple rearrangement events, including high transposition activity, duplications, deletions and inversions. SNP was increased also. High number of LTR repeats and Retroposed Genes showed that the chromatin structure here is attractive for integration of retroelements. A specific LTR called HERVE, which was always combined with satellite repeats SATR1 and SATR2, flanks the TBR SDs. This specific location at the borders of the duplication blocks, suggests the use of this repeat in TBR SD duplication and transposition and proposes this structure as a "putative instability element".

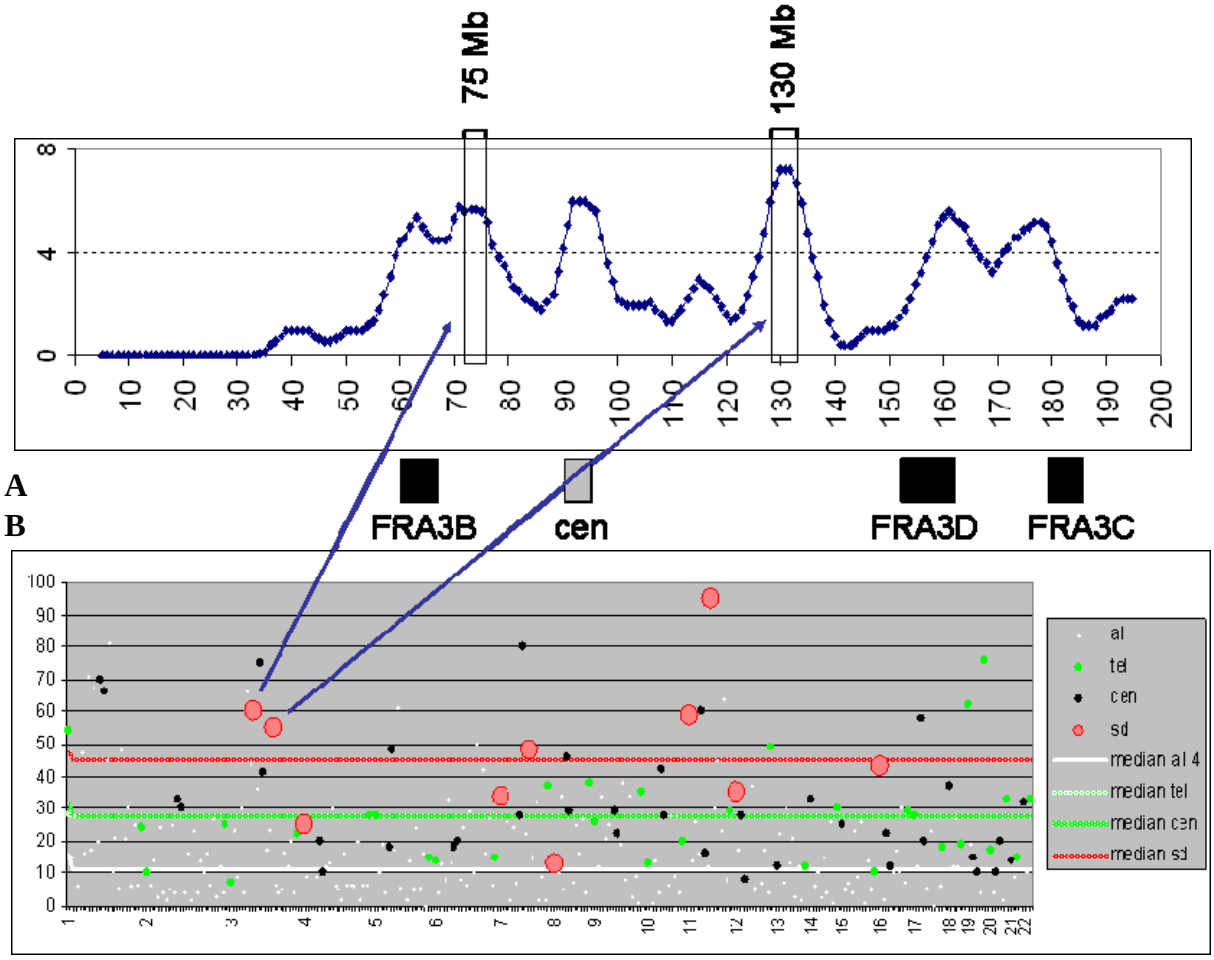


Figure 17. Frequency of breaks in carcinomas
A - breaks along chr3 based on our mpFISH analysis on 10 carcinoma cell lines;
B - frequency of breaks along the whole genome based on the Mitelman catalogue of chromosome rearrangements in carcinomas

The TBR SD contains genes of FAM86 family that were expanded by the duplications during primate evolution. FAM86 genes are characterised by presence of S-adenosyl-L-methionine-dependent methyltransferases domain. The various aspects of the role of DNA methylation in a number of cellular processes in eukaryotes including gene regulation and differentiation are well documented. Therefore expansion of such gene family could have an important role in primate evolution. TBR SDs are often deleted from chr3 in tumor cell lines, suggesting that reduction of the genes' methyltransferase activity could be advantageous for tumor growth.

In conclusion, identification and analysis of chr3 regions that were most frequently involved in tumor-related breaks showed that, by playing an important role in recent primate evolutionary chromosome plasticity, they are extending this feature to the somatic cancer cells. We propose that the mechanism of the instability within these regions involves the specific chromatin structure, which is related to the presence of segmental duplications and the described "putative instability element" composed of LTR and satellite repeats.

2.3. Chr3 rearrangements establish mandatory chromosomal segment copy number balance in tumor (paper V)

What is the advantage to rearrange chromosomes by breaks within the TBR SD regions?

We have mentioned already that there are genes of FAM86 family within the duplications. Change of their function is very likely to participate in tumor growth associated pathways. Alternatively or simultaneously, genes that are distant from TBR SD may be recurrently lost or gained by the TBR SD unbalanced translocations. We have shown that the rearrangements within the TBR SDs lead to manifestation of certain pattern of chr3 segmental imbalance, which most frequently involve 3p13-pter loss and 3q22-qter gain. Our last paper tried to answer the question whether such imbalance is essential for tumor growth:

1. Is a tumor dependent on its aneuploid segment copy number equilibrium, like a normal cell is dependent on its diploid balance?
2. Would one copy number shift influence tumor growth?
3. Is this copy number threshold valid just for certain regions or it is more generally valid for all chromosomal segments?

To answer these questions we used KH39 (renal cell carcinoma cell line) and Hone1 (nasopharyngeal carcinoma cell line), their monochromosomal chr3 hybrids and their SCID derived tumors.

The aim of this study was to distinguish between a qualitative and a quantitative model of tumor suppression.

According to the qualitative model a damaged or deleted tumor suppressor gene would be restored by the transfer of a normal chromosome. If so, suppression would be released only when the corresponding sequences of the exogenous normal chromosome are lost or inactivated.

According to the second quantitative model the tumor cell would not tolerate an increased dosage of the relevant gene or segment. To restore the copy number quantity set by the tumor the excedentary sequences will be lost regardless if they are endogenous or exogenous.

We analyzed the clones of KH39 and Hone1 derived hybrids after in vitro growth at different time points (0, 3, 6, 9 weeks,) as well as their SCID mice derived tumors. The control KH39 and Hone1 did not show any cytogenetic changes. The MCH Δ3/KH39 that had an extra chr3 copy with 3p21 deletions retained its dominant clone during 9 weeks of in vitro propagation and following in vivo growth. In contrast, the MCHs #3/KH39 and #3/Hone1, which had extra copies of chr3 with intact CER1 and FER, exhibited notable changes. After 9 weeks of in vitro growth, clones that have lost all supernumerary 3p21 copies took over having the best growth advantage. Clones with

one or no supernumerary 3p21 copies expanded in the in vivo derived #3/KH39 and #3/Hone1 tumors as well.

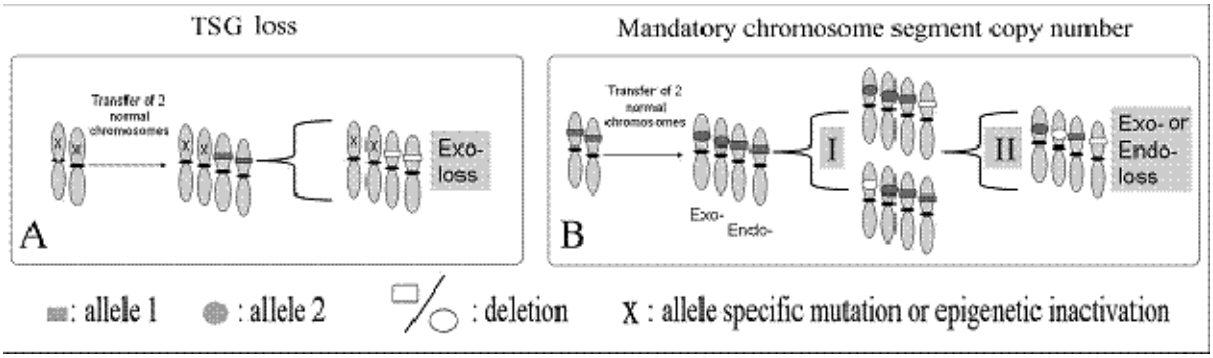


Figure 18. Chromosomal changes, expected on the basis of the "TSG loss" and the "mandatory chromosomal segment copy number" models, respectively.
A - The putative TSG is inactivated in the recipient tumor cell. Following the transfer of normal chromosomes, only the loss of exogenous TSG copies would provide the cell with selective growth advantage. **B -** The recipient tumor cell must maintain equilibrium with regards to the total copy number of certain chromosomal segments. Transfer of supernumerary chromosomes is followed by the restoration of the original balance by a multi-phase expansion of clones, losing first one, then the other extra-copy of the critical regions irrespectively of their endo- and exogenous origin.

Analyzing the changes of chr3 constitution during MCHs in vitro and in vivo growth, we have found that chromosomes, which contained 3p21, were preferentially lost compared to the chromosomes that did not have it. This suggests that the rigidity of the initial copy number is different for different chromosomal segments. Except CER1 there was a previously reported frequently eliminated region (FER) at 3p14.3-p21.2 that was part of all "lost", but none of the "retained chr3 segments". This region is most resistant to copy number gain.

In contrast, 3q26.3-q27 (Common Retained Region, CRR) was present preferentially in the "retained chr3 segments" being thus most tolerant to copy number gain.

These results indicate that aneuploid tumor cells are dependent on a precise balance that they have evolved with regard to some chromosomal segments, but not others.

Completing the cytogenetic results, LOH experiments with polymorphic microsatellite markers close to FER showed that exogeneous normal cell derived, or endogenous tumor derived, chromosome segments were lost with similar probability. This confirms our theory of the quantitative model of suppression.

In conclusion, we demonstrated in this paper that the in vitro and in vivo losses affecting the 3p14.3-p21.2 segment in tumor cells with an introduced exogenous

normal human chr3 may alternatively target the exogenous or an endogenous chr3. This shows a dosage effect and speaks against any qualitative model, e.g. mutational inequality between the tumor derived and the normal chromosome. The intolerance to this dosage change does not apply to 3q where the increased copy number is readily maintained and apparently favored. Therefore the most frequent chr3 changes, found in our carcinoma cell lines after breakages within TBR SDs, consisting of 3p13-pter losses and 3q22-qter gains, may create a FER/CRR copy number balance that is favorable for tumor growth.

Concluding remarks and future perspectives

In our group a functional test called the Elimination Test was developed in order to identify tumor antagonizing regions on chr3 microcell hybrids and identified CER1 on 3p21.3. In order to understand CER1 role in tumor development, we continued the analysis of this region following two leads:

1. We continued the search for tumor suppressor genes located in CER1

2. We analyzed CER1 breakpoint regions to identify and characterize specific instability features of these sequences.

Comparative genomics helped us to find that CER1 was divided into two syntenic blocks on mouse chromosome 9. On these blocks the gene order and content was maintained; the synteny breakpoint was located within the region containing the cluster of chemokine receptor (CCR) genes. These genes, together with the other cluster of ZNF genes, are located at the borders of CER1.

These cancer associated breakpoint regions defining CER1 are characterized by evolutionary plasticity: recent tandem duplications, retroposed pseudogene insertions and horizontal evolution of the genes located here.

In parallel with describing instability features at tumor breakpoint sites we analyzed a putative tumor suppressor gene *LIMD1*, which is located in the central part of CER1. We found that it binds specifically to pRb and suppresses E2F driven transcription. A tumor suppressor effect of this gene was proven in *in vitro* and *in vivo* experiments as well as in real tumors.

This study and other publications from our group (Kost-Alimova et al. 2003) showed evolutionary plasticity and other features of instability at the borders of eliminated regions (CER1, CER2 and FER) identified by the ET in the chr3 MCH system. As a next step we intended to analyze rearrangements of entire chr3 with high resolution methods and investigate the instability features at the breakpoint regions. In paper IV, we describe the development and comparison of two high resolution methods (array-CGH and mpFISH).

We proved that although our 1Mb chr3 BAC/PAC array could identify single copy number changes even in pentaploid cells, mpFISH provided a more accurate analysis in the dissection of complex karyotypes at high ploidy levels. In heterogeneous or normal cell contaminated samples the most precise analysis can be made by mpFISH due to its ability to give information at single cell level. Using these high resolution methods we analysed ten carcinoma cell lines and identified two new hot spots of tumor breakpoints at 3p12-p13 and 3q21. These tumor breakpoint regions are characterized by specific features of instability and participated in recent primate evolution.

By the described high resolution methods we could also describe in great details the chr3 rearrangements in KH39 and Hone1 monochromosomal (chr3) hybrids and

tumors derived from them. We could show that aneuploid tumors maintain a mandatory chromosomal segment balance with stringency concerning no gain of 3p14-21 and no loss of 3q26-27. The mechanism of suppression is based on the alternative quantitative model, According to this model the tumor cell does not tolerate an increased dosage of the relevant gene or segment, and the lost part can be either of normal cell derived exogeneous or tumor derived endogenous origin.

Structural chromosomal instability is an ongoing dynamic process. Therefore in order to prove that the instability at the breakpoint regions characterizes structural CIN phenotype and is required for tumor development and progression, dynamic analysis of the tumors must be done. This may elucidate the mechanism of tumor development and may help to develop CIN phenotype markers useful in choice of consequent treatment.

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