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Chromosomal Instability in Cancer Cells

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Chromosomal Instability in Cancer Cells

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Preface

The content of this book were triggered by the 2nd International Meeting on "Molecular based treatment of GI cancers", which was held in Göttingen, Germany, on March 1–2, 2013. The meeting was focused on exploring how genomic technologies, including gene expression profiling, the detection of genomic imbalances, and next-generation sequencing can be harnessed to identify molecular portraits of cancer with the goal to improve the treatment of patients, a goal that can also be summarized with the terms "personalized medicine" or "precision medicine". Ultimately, this will improve treatment outcome and quality of life. The meeting was organized by Drs. Michael Ghadimi, Clemens Hess, Matthias Dobbelstein (Göttingen), Josef Rüschoff (Kassel), and Thomas Ried (Bethesda, MD). The speakers included numerous internationally recognized leaders in their respective fields, who covered aspects of the role of the tumor microenvironment, microRNAs, the role of epigenetic modifications, and chromosomal instability and intratumor heterogeneity on treatment response. A round table discussion on how to best implement genomic information in clinical decision making completed the meeting. The meeting agenda can be retrieved at http://www.kfo179.de/en/kongress.html.

In the book issue presented here we invited articles that cover the role of DNA repair and chromosomal translocations, the use of mouse and yeast models to understand chromosomal instability and tumor progression, the role of telomere integrity and chromosome segregation errors for the emergence of specific genomic copy number alterations in solid tumors, the influence of the newly identified phenomenon of chromothripsis, and how aneuploidy influences the transcriptional equilibrium of cells. The meeting was dedicated to Prof. Heinz Becker, who, for many years lead the Department of Surgery at the University of Göttingen. Heinz Becker was one of the leaders of the German Rectal Cancer Study group, which established that neoadjuvant therapy of patients with rectal cancer reduces the risk of tumor recurrence compared to postoperative treatment. He was very supportive

of the research activities at the Department, which culminated in the establishment of a Clinical Research Unit supported by the Deutsche Forschungsgemeinschaft (http://www.kfo179.de/en/home.html).

We lost our friend and colleague Heinz Becker in 2014 and dedicate this issue to him.

Göttingen B. Michael Ghadimi
Bethesda Thomas Ried

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DNA Repair and Chromosomal Translocations

Stefan K. Bohlander and Purvi M. Kakadia

Abstract

The balance between DNA damage, especially double strand breaks, and DNA damage repair is a critical determinant of chromosomal translocation frequency. The non-homologous end-joining repair (NHEJ) pathways seem to play the major role in the generation of chromosomal translocations. The "landscape" of chromosomal translocation identified in malignancies is largely due to selection processes which operate on the growth advantages conveyed to the cells by the functional consequences of chromosomal translocations (i.e., oncogenic fusion proteins and overexpression of oncogenes, both compromising tumor suppressor gene functions). Newer studies have shown that there is an abundance of local rearrangements in many tumors, like small deletions and inversions. A better understanding of the interplay between DNA repair mechanisms and the generation of tumorigenic translocations will, among many other things, depend on an improved understanding of DNA repair mechanisms and their interplay with chromatin and the 3D organization of the interphase nucleus.

Keywords

DNA repair · Chromosomal translocations

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1 Introduction

The study of chromosomal translocations has played a pivotal role in the analysis of the genetic basis of cancers. In fact, it was the discovery of recurring chromosomal translocations in acute and chronic myeloid leukemia that conclusively showed for the first time that cancer is a genetic disease (Rowley 1973, 2001). Although we know for many recurring translocations in great detail what their functional consequences are and how they alter the regulatory circuits in a cell to convert it into a cancer cell, much less is known about the causes of chromosomal translocations and why we find very specific translocations occurring at high frequencies in certain malignancies.

This chapter will focus on the interplay between DNA repair and chromosomal translocations. It appears intuitive that translocations should be the results of DNA repair gone slightly wrong. But there is also evidence that the fusion genes or deregulated gene expression, which are the consequences of some translocations, might result in increased genomic instability and lead to the acquisition of additional genomic lesions including chromosomal translocations.

2 Genome Stability

Keeping a stable genome is an essential and non-trivial task, which is vital for every living organism. It appears obvious that the more stable a genome is, the better it is for the organism. So the best solution would be an absolutely stable genome. However, an absolutely stable genome is not as desirable as one might think at first glance, because a completely stable genome does not allow any evolutionary changes to occur. It will thus be necessary to allow a certain level of genomic instability that is suited for a given organism. The stability of the genome should be high enough to allow proper functioning and faithful reproduction of an organism and at the same time still allow evolutionary changes to occur. The stability of a genome is the result of the balance between DNA damage and repair (Fig. 1). It is quite "costly" for a cell or organism to maintain a stable genome. This cost comes in several forms like the energetic costs of repairing damaged DNA, which includes the synthesis and maintenance of the many proteins that are part of the sophisticated DNA repair machinery.

It is becoming more and more apparent that the fine tuning of genomic stability is not only a very important aspect of a species' evolutionary potential but that changing the level of genomic stability in tumor cells has a great influence on the "evolutionary" potential of a tumor and thus on its ability to adapt to different external challenges like chemotherapy or to its ability to find appropriate niches for growth in the body (metastases). Recent in-depth sequencing studies and careful evaluation of traditional molecular genetic and cytogenetic data have revealed many examples of astounding clonal heterogeneity in tumors and provided evidence that tumor development is governed by the evolutionary principle of selection of the fittest (Heselmeyer-Haddad et al. 2012; Yates and Campbell 2012). It is therefore not surprising that we find many tumors with elevated levels of genomic instability, which allow the tumors to evolve more rapidly.

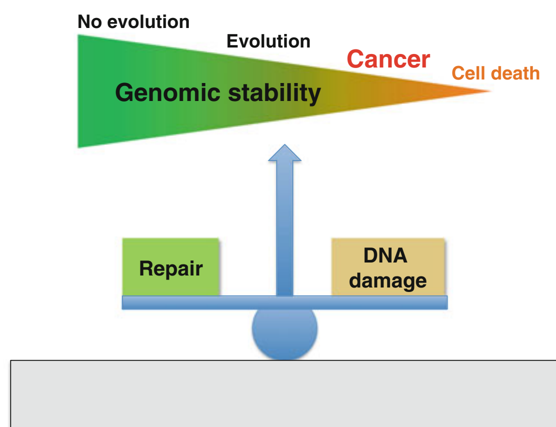

Fig. 1 The balance of DNA damage and DNA repair determines the stability of a genome

In this context, we need to take a close look at the factors that determine genomic stability and that are not only important for the generation of recurring chromosomal translocations but these factors can also be altered as a consequence of chromosomal translocations.

3 DNA Damage and Repair

3.1 Balance of DNA Damage and DNA Repair

The stability of a genome is determined by the balance of two factors (Fig. 1): (1) the rate of DNA damage and (2) the rate of DNA repair.

3.2 Sources of DNA Damage

There are many causes of DNA damage. Most commonly one thinks of DNA damaging external agents like ionizing radiation or ultra violet radiation, as well as chemicals or drugs like alkylating agents. However, spontaneous chemical reactions and cell internal sources of DNA damaging agents like reactive oxygen species (ROS) or S-adenosyl-methionine and errors in normal DNA metabolism (transcription and DNA replication) contribute considerably to the DNA damage load in a cell. It is estimated that between one thousand and one million DNA lesion occur in every human cell per day (Lodish et al. 2004) (Fig. 2).

It should be noted that a minimum of two DNA double-strand breaks (DSBs) is required for a chromosomal translocation to occur (Fig. 3). Certain cells, like

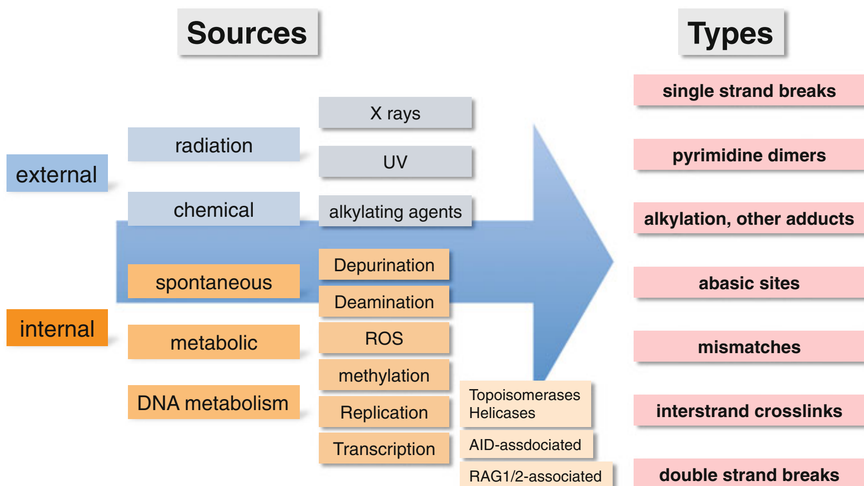

Fig. 2 Sources and types of DNA damage. Note that this is not an exhaustive list

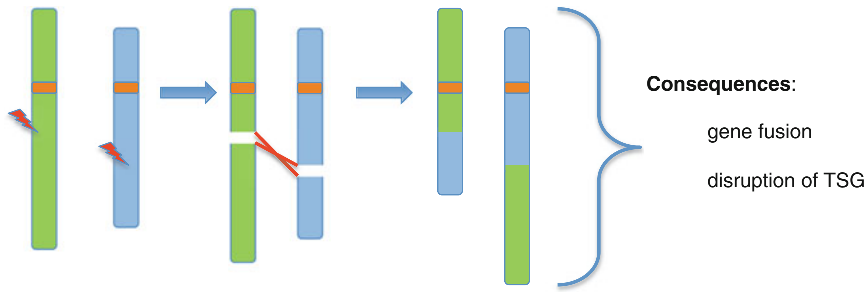

Fig. 3 Faulty repair of two DNA DSBs results in balanced chromosomal translation. The example shows two DSBs on two non-homologous chromosomes

developing B and T cells, will introduce directed DSBs into their genomes as part of the immunoglobulin or T cell receptor maturation process (Zhang et al. 2010). This process has been especially well studied both in terms of the mechanisms involved and in the context of its contribution to the formation of recurrent chromosomal translocations found in lymphoid malignancies.

DNA replication is a considerable source of DNA damage. DNA replication is an extremely complicated process which involves among many other steps the initiation of replication at specific origins of replication, the synthesis of two new DNA strands as a leading and a lagging strand and the ligation of the newly synthesized fragments (Okazaki fragments) of the lagging strands. At any of these steps problems can occur which might lead to abortive replication with replication fork stalling and possibly DNA strand breaks (Halazonetis et al. 2008). The fidelity with which the correct nucleotides are incorporated into the new DNA strands depends on the intracellular concentration of the individual deoxyribonucleotide triphosphates. Under certain conditions, like enhanced cellular proliferation, the local nucleotide pools can become depleted and nucleotides incorrectly incorporated at an increased rate (Bester et al. 2011). This in turn can lead to replication fork stalling, repair attempts, faulty repair and eventually also to DSBs, which might result in chromosomal rearrangements.

The most widely studied source of DSBs in the context of chromosomal translocations are the DNA breaks that are introduced by the RAG1/2 enzymes in the course of lymphoid cell differentiation (Zhang et al. 2010).

3.3 Types of DNA Damage and Their Source

As stated above, the most relevant type of DNA damage for the formation of a chromosomal translocation is a DSB. However, other types of DNA damage like abasic sites, base mismatches etc., can result in DSB if these lesions cannot be repaired properly or if the lesion (damaged nucleotide or strand cross-link) is encountered during DNA replication. We will briefly review these types of DNA lesions and then focus on DSBs (Fig. 2).

3.3.1 Uracil, Abasic Sites, 8-Oxoguanine, Single Strand Breaks, Pyrimidine Dimers, O-6-Methylguanine

Spontaneous deamination of a cytosine residue will lead to the generation of a uracil residue and the spontaneous depurination reaction will remove a guanine or adenine base from the DNA strand. Both, deamination and depurination will create an abasic site. It should be noted that these reactions occur spontaneously at an estimated rate of 5000 depurination and about 100 deamination reactions per human cell per day (De Bont and van Larebeke 2004).

Alkylating agents and oxygen radicals attack nucleotides at various places creating bulky DNA adducts or single stranded breaks, respectively. Oxygen radicals will also generate 8-oxoguanine. Alkylating agents are mostly of external origin while oxygen radicals can be derived both from external sources such as ionizing radiation and intracellular sources such as ROS generated by metabolic processes. Single strand DNA breaks are frequently generated due to radiation or ROS.

Photoproducts such as pyrimidine dimers are the result of direct UV radiation. They can lead to stalled replication forks and eventually to DSBs.

Guanine nucleotides are spontaneously methylated to O-6-methylguanine in a non-enzymatic reaction by the ubiquitous methyl group donor S-adenosyl-methionine (De Bont and van Larebeke 2004).

3.3.2 Replication Errors and Mismatches

DNA replication is a highly complex process and requires the interplay of many proteins that work together in the so-called replisome, which in eukaryotic cells contains at least three different DNA polymerases. While this process has a high fidelity, wrong nucleotides can be incorporated leading to mismatches. Replication through microsatellite repeats can easily lead to small insertions and deletions and the expansion or contraction of the repeat. Replication errors are more frequent when the intracellular nucleotide pool has become depleted as might be the case in a rapidly proliferating tumor (Bester et al. 2011).

The replisome also contains or attracts proteins that can detect replication errors and will then recruit repair proteins. Sometimes repair attempts are not successful and will result in DSBs (see below).

3.3.3 Double Strand Breaks (DSBs)

As mentioned above, DSBs, if not repaired faithfully, are the main source of chromosomal translocations. Several cell external and cell intrinsic causes for DSBs are well known. It is estimated that about 50 DSB occur every day in a normal human cell (Vilenchik and Knudson 2003) (Fig. 4).

Random DSBs Due to External Influences

There are many external agents that can lead to DSBs in a cell. The most well known are high-energy radiation in the form of gamma rays, alpha particles or beta rays. All these high energy radiation sources will either directly shatter DNA or lead to the formation of an ionization trail with highly reactive molecules such as ROS,

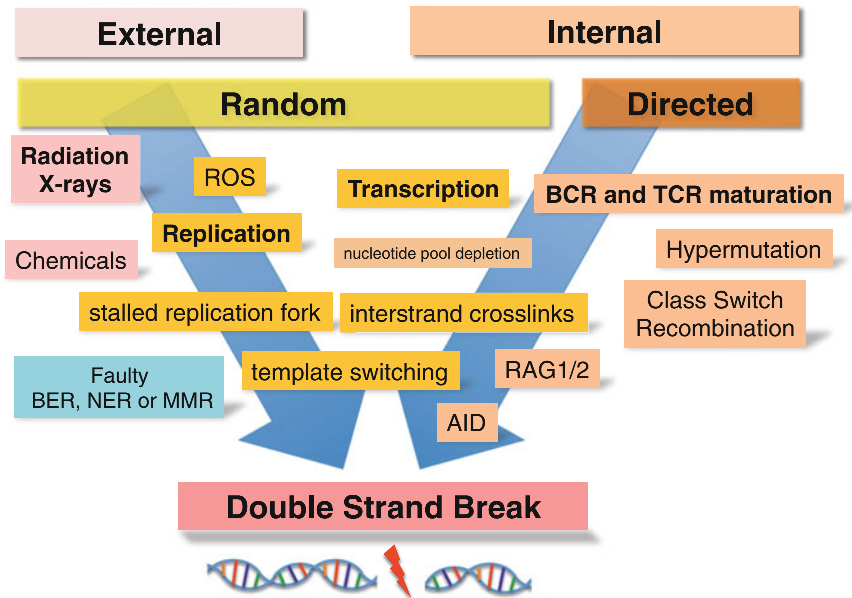

Fig. 4 Causes of DSBs. Abbreviations: *ROS* reactive oxygen species; *BER* base excision repair; *NER* nucleotide excision repair; *MMR* mismatch repair; *AID* activation-induced cytidine deaminase; *RAG1/2* recombination associated genes 1 and 2; *BCR* B cell receptor; *TCR* T cell receptor

which will in turn attack and react with DNA causing DSBs. The most effective radiation in producing DSBs are alpha particles, which deposit a high amount of energy in the form of ionization events over a very short distance in the cell.

In addition to leading directly to a DSB as a consequence of the action of ROS or a direct shattering of the DNA strands, radiation will also generate interstrand crosslinks which will cause a problem during replication (see below).

Random DSBs Due to Internal Influences

ROS are normally produced in a cell as the result of metabolic processes (Sallmyr et al. 2008). If ROS are not scavenged efficiently or produced at a higher rate due to increased metabolic activity, the likelihood that DSB are generated increases.

DNA replication can be a major source of DSB breaks. The replication fork stalls if it encounters a mismatch or an intrastrand crosslink. Attempts to repair mismatches generated during replication can lead to abortive repair by the mismatch repair pathway which will lead to DSBs. As mentioned above, the frequency of mismatches is increased if the nucleotide pools are depleted due to rapid proliferation causing the DNA polymerases to incorrectly incorporate nucleotides at a higher rate (Bester et al. 2011).

Directed DSBs Due to Internal Processes (e.g., Immunoglobulin Rearrangements)

Interestingly, there are several physiological processes which introduce DSBs "on purpose". These processes are required for the generation of genomic rearrangements at the immunoglobulin and the T cell receptor loci during the maturation of B and T cells, respectively (Dudley et al. 2005). The mechanisms that lead to these DSBs and their proper resolution in the form of productive rearrangements have been studied in great detail and their involvement in the generation of chromosomal translocations that drive lymphoid malignancies has been subject to intense scrutiny (Zhang et al. 2010).

RAG 1/2 Associated DSBs

The RAG1/2 recombination-associated endonucleases are responsible for introducing DSB to initiate the process of VDJ recombination at *IGH*, *TRB*, and *TRD* loci and the VJ recombination process at the *IGK*, *IGL*, *TRA* and *TRG* loci. The DSBs introduced by the RAGs are the first step in recombining one of many V (variable) segments with one of many J (joining) segments to create functional immunoglobulin or T cell receptor genes. In the case of the *IGH*, *TRB* and *TRD* loci a D (diversity) segment to J segment recombination precedes the V–J recombination for a full VDJ recombination event. The V(D)J recombination is completed by repairing or joining the DSBs with the classical nonhomologous end-joining pathway (C-NHEJ) (Zhang et al. 2010) (see below).

DSB are very dangerous for a cell because they compromise the integrity of its DNA. Since the purpose of these DSBs is to produce a functional gene, the RAG endonucleases do not cut randomly in the genome but are guided by specific recombination signal sequences (RSS), which consist of heptamer/nonamers that are separated either by a 12 bp or a 23 bp spacer (Tonegawa 1983). The RAGs only introduce DSB in two heptamer/nonamer sequences if one contains a 12 bp and the other a 23 bp spacer. Even though there is sequence specificity and several constraints in where DSBs can be introduced by the RAGs, some variations in the heptamer/nonamer sequences are tolerated. This means that DSBs can be introduced at other genomic loci that happen to have sequences which resemble the heptamer/nonamer sequences of the immunoglobulin and TCR loci. It is quite obvious that DSBs outside the immunoglobulin or TCR loci can lead to unintended and potentially dangerous chromosomal rearrangements if joined via the C-NEHJ pathway (Robbiani et al. 2009).

AID Associated DSBs

In addition to the diversity in the immunoglobulin and TCR genes generated by the V(D)J recombination process, more diversity is achieved by the process of somatic hypermutation (SHM). In SHM, mutations are introduced into the variable region exons of the immunoglobulin or TCR genes. The key enzyme responsible for SHM is the activation-induced cytidine deaminase (AID). AID deaminates cytidines in single-stranded DNA regions. The AID enzyme does not only introduce mutations

into the variable region exons (Li et al. 2004) but is also responsible for the DSB required for class switch recombination (CSR) (Muramatsu et al. 2000). Since AID needs single-stranded DNA, its activity is coupled to active transcription which requires the unwinding and melting of the DNA double strand. Thus, AID is targeted by active transcription to the switch region of the constant region exons (for CSR) or to the variable region exons (for SHM). The cytidine deamination events introduced by AID into the switch regions of the constant region exons are processed to DSB through the activity of proteins involved in DNA repair (Chaudhuri and Alt 2004; Di Noia and Neuberger 2007). What causes AID induced deamination events to lead to single nucleotide mutations and small indels at the variable region exons on the one hand and to DSB in the switch regions of the constant region exons on the other hand is currently unknown (Muramatsu et al. 2007). High throughput genome-wide translocation sequencing showed that AID-associated DSBs that occur outside the normal loci for CSR are more frequent in actively transcribed regions (Chiarle et al. 2011).

DSB Due to Normal Chromatin Movement and Processes (Topoisomerase and Helicase Action)

The nucleus is a very crowded place occupied by 46 linear DNA molecules with a total length of about 2 m crammed into a small space of just a few cubic micrometers (approximately 100–200 μm^3). Many processes in the nucleus like transcription, DNA replication and chromosome condensation require the unwinding of supercoiled DNA to relieve torsional stress and to allow replication and transcription factors to gain access to specific sites. Due to topological constraints it frequently becomes necessary to break one DNA double strand temporarily to pass another DNA strand through this gap. Introducing a DSB into a DNA molecule to pass another DNA strand through the gap and then repairing the gap is a very risky process and can easily lead to a faulty repair if another DSB is in the vicinity. Topoisomerases and helicases and a number of DNA repair proteins are involved in this process (Kaneko et al. 2004; Plank and Hsieh 2009; Vos et al. 2011).

To relieve torsional stress of a DNA molecule, e.g., during transcription, DNA helicases will introduce a nick in one strand of a dsDNA molecule, release the stress and then re-ligate the single-stranded nick. This process is also risky since it can result in a DSB (Carrasco et al. 2014).

3.4 Detecting DNA Damage

Any DNA damage in a cell has to be detected rapidly so that it can be repaired before the lesion is passed on to the daughter cells. One of the worst consequences of improperly DNA damage is cancer.

While many of the DNA lesions described above are detected by specific proteins and mechanisms, we will focus on the pathways in place to detect DSB, which are the predominant substrate for the generation of chromosomal translocations.

3.4.1 Detecting DSB and Signaling for Repair

It is absolutely crucial for cell survival to quickly detect any DSBs and repair them efficiently. A DSB generates two ends of a double stranded DNA molecule, which immediately attract the attention of several proteins. These proteins will then orchestrate the recruitment of additional DNA repair proteins. This is an extremely complicated process involving a great number of proteins, which is still under intense investigation. Several pathways and proteins that are working in this DSB surveillance network have been identified (Fig. 5).

Even though telomeres technically constitute ends of linear dsDNA molecules they are protected or hidden from the detection of the break surveillance proteins by their special structure and a large number of telomere-associated proteins (Slijepcevic and Al-Wahiby 2005).

Immediately after a DSB occurs, the surrounding chromatin undergoes extensive modifications which are accompanied by various posttranslational changes of histones and other proteins (poly(ADP-ribosyl)ation, ubiquitylation, sumoylation, acetylation, phosphorylation). These modifications also extend from the site of the DSB and lead to the recruitment of DNA repair proteins (Lukas et al. 2011).

Poly(ADP-ribosyl)ation

The first detectable event after a DSB is poly(ADP-ribosyl)ation (PAR) mediated by the PARP1-3 enzymes. PAR modifies lysine residues of the core histones tails. PAR of the histone tails leads to the recruitment of the chromatin remodelling complex NuRD/CHD4 and polycomb complexes (Chou et al. 2010).

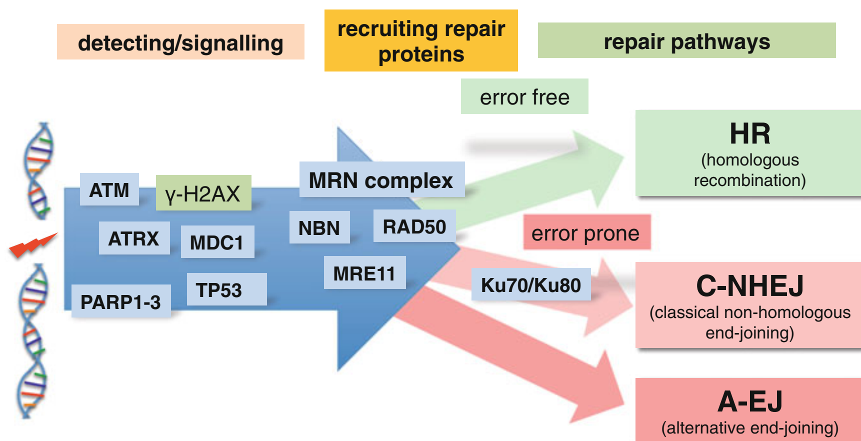

Fig. 5 Double strand breaks (DSBs): Detection, signalling, recruitment of repair proteins and repair pathways. The MRN complex consists of RAD50, NBN and MRE11. The repair pathways are divided into error free (*green*) and error prone (*red*)

Gamma H2AX (Phosphorylated Form of H2AX)

Another early event at a DSB is the phosphorylation of histone H2AX by ATM to form gamma-H2AX. Gamma-H2AX is recognized by its sensor MDC1 (mediator of DNA damage checkpoint protein1), which interacts among others, with the Nijmegen breakage syndrome protein, NBS1 (Goldberg et al. 2003). NBS1 in turn interacts with ATM and tethers it to the DSB to increase local gamma-H2AX concentration. MDC1 recruits a number of other proteins like RNF8 which subsequently leads to the recruitment of BCRA1, RAD18, PTIP (Pax transactivation domain interacting protein) and P53BP1 to the sites of DSBs (Lukas et al. 2011).

It should be noted that replication stress in the form of stalled replication forks also elicit gamma-H2AX formation via ATR (ATM and Rad 3-related) kinase activity (Wang et al. 2011).

TP53

The TP53 tumor suppressor gene plays an important role in the detection and signaling of DNA damage in a cell and orchestrating cellular responses to DNA damage like cell cycle control and apoptosis TP53 (Meek 2009).

53BP1

P53BP1 (P53 binding protein 1) can shield under-replicated DNA regions after the cell has gone through mitosis from the activity of DNA nucleases until the under-replicated DNA regions have been repaired. These under-replicated DNA regions, which are often found at common fragile sites, can become visible in mitosis as so-called ultrafine DNA bridges. 53BP1 nuclear bodies are found in the nuclei of cells that have just passed through mitosis (Lukas et al. 2011).

It should be noted that the exact molecular mechanisms that lead to DSB recognition are far from being completely understood and the description above offers only a glimpse of the complexity that is already known. These mechanisms also vary from cell type to cell type and also depend on the origin of the DSBs.

3.4.2 Signaling During Repair

Once the cell has detected one or more DSBs or other DNA lesions, it needs to repair the damage or make the decision that the damage is too extensive to be repaired, halt cell division or undergo programmed cell death. How this decision is made is not entirely clear. Rather than assessing the damage and making a ‘conscious’ decision that the damage is too extensive, the cell will attempt to repair the damage and while the repair process is in progress, the progression through the cell cycle will be blocked. Entering S phase, or even mitosis, with unrepaired DNA lesions can have catastrophic consequences.

Several well-known tumor suppressor genes function in pathways that prevent a cell from progressing through the cell cycle as long as there are unrepaired DNA lesions. These include the retinoblastoma gene (*RB*), the *TP53* gene and the *INK4A/B* genes as well as proteins that are more directly involved in DNA repair (Huen and Chen 2010; Sperka et al. 2012).

3.5 Repairing DNA Damage

Just as there are many different types of DNA lesions, there are many damage repair pathways that are very specific for certain lesions. We will only briefly mention these specialized repair pathways and then focus on the pathways involved in the repair of DSBs. However, the failure to properly repair other types of DNA damage such as mismatches, uracil bases, abasic sites, pyrimidine dimers or chemically modified bases (alkyl groups, O-6-methylguanine) can also result in DSBs (Fig. 6).

3.5.1 Base Excision Repair (BER)

Abasic sites, uracil bases, 8-oxoguanine and single strand breaks (check ssBreak repair) are efficiently repaired by the base excision repair (BER) process (Krokan and Bjørås 2013). This process involves the removal of the uracil by DNA glycosylases and then the removal of the sugar phosphate by the action of the AP endonuclease (AP for *ap*urinic and *ap*yrimidinic site) and a phosphodiesterase. This creates a one nucleotide gap in the DNA strand with the lesion, which is then filled by the action of DNA polymerase(s) and sealed by a DNA ligase (Krokan and Bjørås 2013).

3.5.2 Nucleotide Excision Repair (NER)

Bulky DNA adducts and pyrimidine dimers and many other types of lesions are repaired by the nucleotide excision repair (NER) process. The altered DNA strand is recognized by a multi-protein complex that introduces single strand nicks on

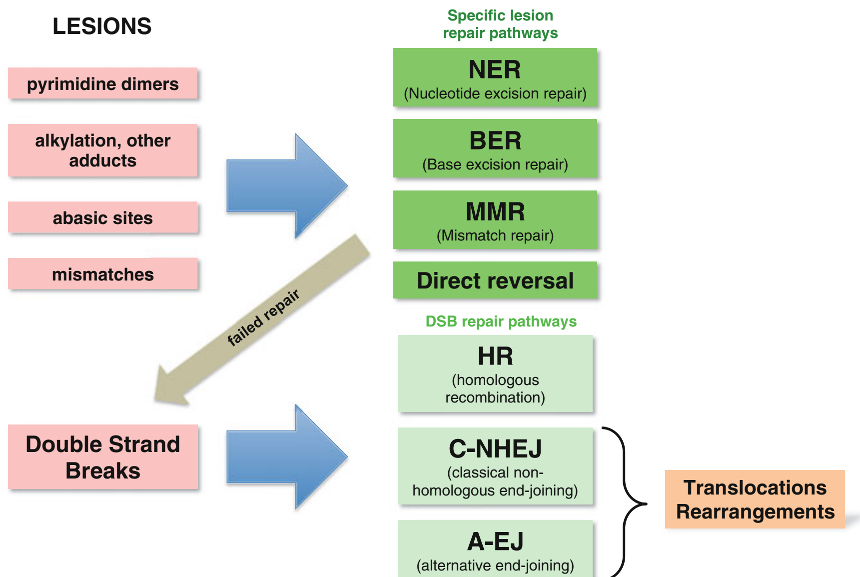

Fig. 6 Different types of DNA lesions and their repair pathways. Note that failed repair of a specific lesions might lead to a DSB which will then have to be repaired. Translocations and other rearrangements are mostly the result of C-NHEJ and A-EJ joining the incorrect DNA ends

either side of the lesion. The nicks are spaced 12 nucleotides apart in bacteria but more than 24 nucleotides apart in eukaryotes. The intervening fragment is removed by the action of a helicase and the resulting single strand gap is repaired by the action of polymerases and finally sealed by ligases. Mutations in NER pathway proteins, especially those that are involved in the initial recognition of the damaged DNA, are found in Xeroderma pigmentosa patients (Shuck et al. 2008).

3.5.3 Direct Reversal

Certain DNA lesion like O-6-methylguanine and thymidine dimers can also be repaired by *direct reversal* mechanisms. The methyl group of O-6-methylguanine is removed by the action of MGMT (Tano et al. 1990), and UV-induced pyrimidine dimers can be reversed by the action of the photolyases (Kneuttinger et al. 2014). Photolyases, which catalyze the direct reversion of thymidine dimers, are not functional in placental mammals (Lucas-Lledó and Lynch 2009).

3.5.4 Mismatch Repair

Mismatches and smaller insertions and deletions that arise frequently during DNA replication are efficiently repaired by the mismatch repair process (MMR). A multiprotein complex, containing the components of the MMR machinery and additional proteins, is loaded onto the freshly replicated DNA double strands, scans the DNA and recognizes distortions of the helical structure caused by base mismatches between the two strands. Once a distortion is detected the newly synthesized DNA strand is nicked by the endonuclease in the complex and the endonuclease exo1 removes several nucleotides including the mismatches in this strand. Then the gap in the strand is filled by the action of DNA polymerase delta and the repair is completed by the action of a DNA ligase (Li 2008). There are about 10 proteins involved in MMR and mutations in several of the genes coding for these proteins are found in hereditary cancer syndromes like HNPCC (Müller and Fishel 2002) (Fig. 6).

3.5.5 Double Strand Break Repair

Faulty repair in any of the repair pathways described above can result in DSBs, which can lead to chromosomal translocations. The cell will attempt to repair any DSB using one of three or four DSB repair pathways. DSB repair pathways can be subdivided into error-prone and error-free pathways. In this context, error-free has to be understood as theoretically error free or usually error free. After an error free repair, the nucleotide sequence of the repaired region will be identical to the nucleotide sequence before the DSB.

Homologous Recombination (HR)

Homologous recombination is a usually error free DSB repair pathway. This process operates in the S and G2 phase of the cell cycle because it requires an intact sister chromatid, which serves as the template for the DNA repair. The MRE11/RAD50/NBS protein complex is loaded onto the end of the DSBs where

the 5′-3′ exonuclease activity of MRE11 removes nucleotides from one of the strands of the free dsDNA end (Daley et al. 2013; Lammens et al. 2011; Williams et al. 2010). This leads to an exposed single-stranded 3′ DNA overhang, which is processed with the help of replication proteins A (RPA), RAD51, BRCA2 and several other proteins into the RAD51-ssDNA-nucleoprotein filament. The RAD51-ssDNA-nucleoprotein filament will then invade the dsDNA of the homologous site on the sister chromatid, which serves as a template for the error-free synthesis of the DNA across the DSB region (Pellegrini et al. 2002; Popp and Bohlander 2010; Yang et al. 2002). After the Holliday junctions are resolved, the error-free repair is completed. This repair pathway can result in sister chromatid exchange. The DSB repair by HR is a sterically very complicated process and involves, in addition to the proteins mentioned above, several other important proteins like RECQL2 (Werner Syndrome), BLM (RECQL3) (Bloom Syndrome), BRCA2 (familial breast cancer), RAD54, PALB2 (familial pancreatic cancer), FANCM, FANCC (Fanconi anemia) and others (Daley et al. 2013; Popp and Bohlander 2010). A number of these proteins are mutated in hereditary tumor or genome instability syndromes and are also somatically mutated in these tumors (Bunting and Nussenzweig 2013; Ellis et al. 1995; Jones et al. 2009; Meetei et al. 2003; Strathdee et al. 1992; Wooster et al. 1995; Yu et al. 1996).

Single Strand Annealing (SSA)

SSA is a variant of HR, a homology dependent mode of joining DSB. It operates between closely spaced direct repeats (less than 25 kbp apart) and usually results in the deletion of one copy of the repeat (Ivanov et al. 1996; Zhang et al. 2010).

BIR (Breakage Induced Replication Template Switching)

Another, mechanism to generate a translocation would be for the DNA replication machinery to switch to another chromosome as a template. This so-called breakage-induced replication (BIR) template switching can be induced by a break during DNA replication (Bunting and Nussenzweig 2013).

Classical Non-Homologous End Joining (C-NHEJ)

The C-NHEJ repair pathway for DSB is error-prone, which means that after the joining of the broken ends there will be, mostly smaller, deletions or insertions at the location of the former DSB (Rassool 2003; Roth and Wilson 1986). This process does not need an intact template strand from the sister chromatid and is therefore the major repair pathway for DSBs during the G1 and S phases of the cell cycle. The C-NHEJ pathway is also far more likely to join DSBs that do not belong together since it does not require homologous sequences to guide the repair process. It is, together with alternative end joining (A-EJ), the major repair pathway for removing DSB in the cells. C-NHEJ is used in V(D)J recombination at the immunoglobulin and T cell receptor loci for repairing the breaks that are generated in the process of somatic recombination and CSR.

To initiate the C-NHEJ process, the DSB DNA ends are bound by the KU70/80 proteins to prevent the drifting apart of the DSB ends (Soutoglou et al. 2007). Then

DNA-PKCs and the MRN complex (MRE11-RAD50-NBS) are recruited to the breaks. The MRN complex is crucial in all three DSB repair processes (HR, C-NHEJ and A-EJ). It executes and coordinates many of the activities that are required for DSB repair. For example, the RAD50 coiled-coil domains extend from the core MRN complex which occupies the DSB end to connect the DSB to the other break or the sister chromatid (Lammens et al. 2011).

The MRN complex is responsible for some resection at the DSB and then the broken DNA ends are joined together by the activity of the DNA ligase IV-XRCC4 complex (Roth and Wilson 1986).

Other important proteins involved in C-NHEJ are Artemis (Moshous et al. 2001) and Cerunnos (XLF) (Ahnesorg et al. 2006; Buck et al. 2006). Both proteins were identified because mutations in these factors lead to certain severe combined immunodeficiency syndromes, in which immunoglobulin recombination and TCR recombination are severely compromised.

Alternative End Joining Pathway (A-EJ) or Microhomology-Mediated End-Joining (MMEJ)

There are probably several A-EJ pathways. A-EJ is the repair of DSB in the absence of KU70/80, XRCC4 or Ligase 4 (Daley and Wilson 2005; Wang et al. 2003). The A-EJ pathway also relies on the MRN complex as the crucial component of the repair process (Popp and Bohlander 2010).

A-EJ repaired DSBs are characterized by the presence of stretches of micro-homologies of six to eight base pairs. In some models, A-EJ pathways are responsible for the majority of DSB repairs that resulted in chromosomal translocations (Boboila et al. 2010; Simsek and Jasin 2010).

In systems with inducible chromosomal translocations, the analysis of the breakpoints showed that microhomology-based mechanisms were responsible for only a minority of de novo translocations (Daley and Wilson 2005; Wang et al. 2003). Next generation sequencing studies also showed that less than a third of human germ line chromosomal translocations showed microhomologies at the breakpoints. The breakpoints showed more complicated structures with fragmentation of local DNA sequences, small inversions and deletions. This suggests that C-NHEJ rather than A-EJ contributes to a substantial fraction of human germline chromosomal translocations (Chiang et al. 2012).

4 Translocations

4.1 Translocations—the Tip of the Iceberg

It should be noted that there is now solid evidence that translocations between non-homologous chromosomes are rare compared to intrachromosomal rearrangements (Mahowald et al. 2009; Zhang et al. 2012) between loci that are closer to each other in the interphase nucleus than loci on different chromosomes (Campbell et al. 2008; Pleasance et al. 2010a, b; Stephens et al. 2009).

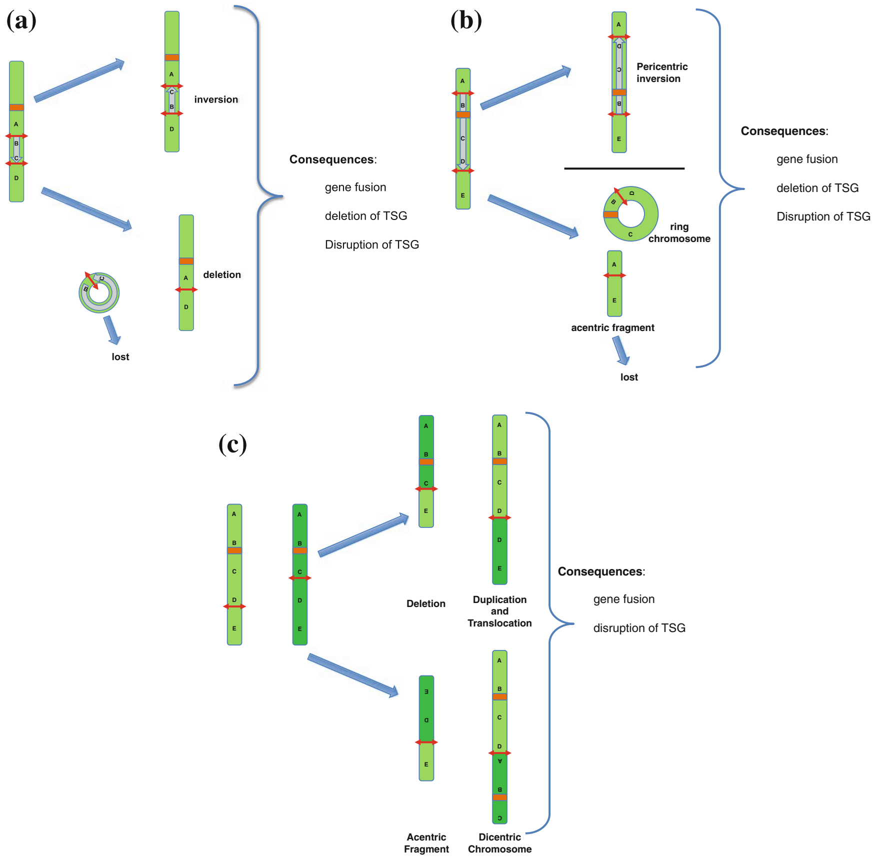

Fig. 7 Possible types of rearrangements after two DSBs depending on the location of the DSB. Note that not all possible constellations of two DSBs are shown. The case of two DSBs on two different chromosomes is shown in Fig. 3. Ring chromosomes and dicentric chromosomes, as well as acentric fragments are frequently mitotically unstable and lead to aneuploidies. **a** Two DSBs located on the same arm of the same chromosome. This can result in a paracentric inversion or a deletion. The acentric circular fragment will be lost during mitosis. The consequences of such rearrangements can be a gene fusion event (resulting in a fusion gene or deregulated gene expression) and/or the disruption or deletion of a gene (tumor suppressor gene). **b** Two DSBs located on different arms of the same chromosome. This will result in a pericentric inversion or in a ring chromosome and an acentric fragment which will be lost during mitosis. **c** Two DSBs located on the same arms of two homologous chromosomes. The result is either a deletion accompanied by a duplication or a dicentric chromosome and an acentric fragment

In addition to the classical, balanced translocation (like the t(9;22)(q34;q11) or the t(8;14)(q24;q32) (Dalla-Favera et al. 1982; Rowley 1973; Taub et al. 1982)), two DSBs can lead to a great variety of other chromosomal rearrangements (Fig. 7).

4.2 DSBs and the Resulting Chromosomal Rearrangements

Depending on the number of co-occurring DSBs and whether the DSBs occur on the same chromosome arm, on different chromosome arms of the same chromosome or on different chromosomes, different chromosomal rearrangements will result. It is quite obvious that the complexity and types of rearrangements increases rapidly with increasing number of co-occurring DSBs. We will only discuss a few scenarios to illustrate the increasing complexity of possible rearrangements as the number of DSB increases and that the most common rearrangements following DSBs have only become “visible” through new technical developments in sequencing, which allowed the analysis of whole tumor genomes at unprecedented resolution.

4.2.1 Consequences of Two DSBs

If two DSBs occur on the same arm of a chromosome there are four free dsDNA ends that can either be repaired in their original order or be joined in a different order, which will result in genomic rearrangements. There are only two possible outcomes of this non-correct joining: (1) Deletion: If the ends of the chromosome fragment between the two breaks are joined to form a circular DNA molecule and the two ends of the flanking fragments are joined, an interstitial deletion will be the outcome. Since the circle does not have a centromere, it will be lost in subsequent cell divisions. (2) Inversion: The fragment between the two breakpoints can be inverted resulting in a paracentric inversion (Fig. 7a).

If the two DSBs occur in different arms of the same chromosome and are joined in the incorrect order there are also only two outcomes but the consequences are more complicated. (1) If the ends of the fragment containing the centromere are rejoined, a ring chromosome will be produced. The fragments containing the telomeres might then join to form an acentric chromosome, which will be lost in subsequent cell divisions. The net result will be the deletion of the DNA sequences distal of the two breakpoints (i.e., the sequences towards the telomeres). (2) The alternative, incorrect order of joining the four DSB break ends will result in a pericentric inversion of the chromosome (Fig. 7b).

If two DSBs occur on the same arm of two homologous chromosomes and are joined incorrectly, translocations, interstitial duplications and deletions will result. Alternatively, an acentric fragment and a dicentric chromosome can form. Both are mitotically very unstable (Fig. 7c).

Finally, if two DSBs occur on different, non-homologous chromosomes and are joined in the incorrect order, two possible outcomes are observed:

1. A balanced chromosomal translocation occurs if the DSB ends are joined in the correct orientation with respect to their centromere to telomere orientation (Fig. 3). The ends on the fragments with the centromeres need to be joined to the ends on the fragments with the telomeres.
2. A dicentric chromosome and an acentric fragment will be generated if the two ends connected to a centromere and the two ends connected to the telomeres are

joined, respectively. Dicentric chromosomes are extremely unstable during cell division and are subject to additional breakage events while the acentric fragments will be lost during mitosis (see Fig. 7c, lower right half).

4.2.2 Consequences of Three and More DSBs

The number and types of possible rearrangements that can result if the ends from three DSB breaks are joined in an incorrect order is quite large. Three DSBs will generate six DNA ends. These six DNA ends can theoretically only be joined in three non-correct ways. However, the type of rearrangements that can result from three DSBs also depends on where these DSBs are located with respect to each other. For example, if the breaks are all on the same arm of the same chromosome the possible rearrangements are different from the situation in which all three DSBs are on different, non-homologous chromosomes. For example, if all three DSBs are on the same chromosomal arm, there can either be a deletion or an inversion rearrangement. If the three breaks occur all on different chromosomes, a three way balanced translocation can be one of the results. If two breaks occur on the same chromosome arm and the third break on a different chromosome, interchromosomal insertions will be observed.

If the number of DSB breaks increases, the number of possible rearrangements will increase in a non-linear fashion. The most extreme example of rearrangements resulting from multiple DSBs is a so-called ‘chromothripsis’ event. The complexity of chromothripsis events has only recently been discovered and required next generation sequencing approaches to decipher (Molenaar et al. 2012; Stephens et al. 2011). It should be noted that the exact causes of chromothripsis events are not entirely understood at the moment. One of the explanations would be a catastrophic event that produces many, local DSBs which are then repaired in the wrong order. There is also the possibility that locally disrupted DNA replication leads to a chain of microhomology-mediated template switching events (see below) (Crasta et al. 2012; Holland and Cleveland 2012) .

4.3 Steric Constraints on the Formation of Chromosomal Rearrangements

4.3.1 Arrangement and Mobility of Chromosomes in the Interphase Nucleus (Chromosome Territories)

In the interphase nucleus, chromosomes are not arranged randomly like spaghetti on a plate. Each chromosome occupies a so-called chromosome territory (CT) (Cremer and Cremer 2001) (Fig. 8). Chromosome territories are of complex shapes. Adjacent chromosome territories interdigitate with each other (Cremer and Cremer 2001). There is no fixed order of how CTs are arranged with respect to each other in the interphase nuclei of a given cell type (i.e., there is no rule that the CT of chromosome 1 should always make contact with the CT of chromosome 10, for example). The arrangement of CTs with respect to each other seems to be dynamic

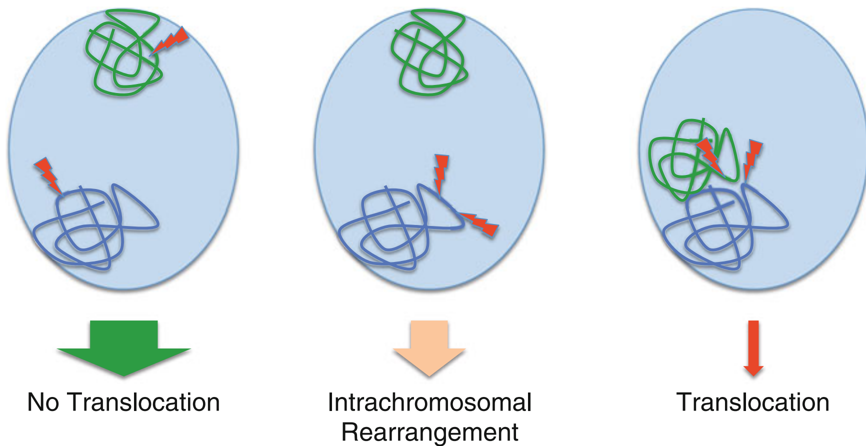

Fig. 8 Possible scenarios for the location of two simultaneously occurring DSBs in the interphase nucleus and the likely outcome. The width of the arrows correlates with the likelihood of the different outcomes. Only two chromosome territories (CTs) are shown as example. *Left* the two DSBs occur in different CTs which are far apart in the interphase nucleus. *Middle* two DSBs occur in the same chromatin domain on the same CT. *Right* two DSBs occur in different, adjacent CTs but closely spaced in intermingling chromosome domains

and also changes during cellular differentiation. There is a tendency of CTs of chromosomes with a high gene density to be located more centrally in the nucleus and the CTs of gene poor chromosomes to be found more frequently in the nuclear periphery (Cremer et al. 2001).

Interphase DNA is wrapped around an octamer of core histones which form the nucleosomes. The DNA wrapped around individual nucleosomes has the appearance of bead on a string and is the basic 10 nm chromatin fiber (Belmont 2006; Misteli 2010). In the interphase nucleus, the chromatin fiber is further compacted with linker histones and non-histone proteins and organized into so-called chromatin domains (CDs), containing on the order of 1 Mbp of DNA. CDs form the basic units of higher-order chromatin organization (de Graaf and van Steensel 2013). While the interphase chromatin is not held in place by a rigid matrix, there is evidence that the movement of the chromatin, and hence the DNA, is spatially confined. Thus, the two ends of a DSB are not free to move to any place within the nucleus but have been shown to move within a radius of approximately 1 μm (Soutoglou et al. 2007).

These steric constraints, namely the packaging of chromosomes in CT, the organization of the DNA into CD, and the resulting limited mobility of DSB ends have very important implications for the frequency and types of chromosomal rearrangements that can occur as a consequence of DSBs.

As stated above, a minimum of two DSBs is required for a chromosomal rearrangement to occur. In addition, these two DSBs have to occur at the same time. There are an estimated 50 DSBs per nucleus per day (Vilenchik and Knudson

2003). However, DSB repair is usually initiated within a few minutes of the breakage event. At that time, the broken ends are still very close together and have not moved apart very far because of the limited time available and the constraints imposed on the DNA by the higher order chromatin organization (Soutoglou et al. 2007). In order to rejoin the ends of one DSB (DSB-A) with the ends of another DSB (DSB-B), DSB-A and DSB-B have to occur close enough to each other in the interphase nucleus and at almost the same time. Even if two DSBs occur at the same time in the same nucleus but are separated by more than 1 μm, it is unlikely that the DNA ends can move far enough so that a rearrangement can occur (Fig. 8).

Considering these limitations, it is quite safe to assume that chromosomal rearrangements have a much higher chance to occur if the two originating DSBs occur in close spatial and temporal proximity. Therefore, it is quite obvious, considering the arrangement of DNA in the interphase nucleus, that two DSBs that are close enough to each other to lead to a rearrangement are much more likely to be located on the same chromosome and also within the same or neighboring CD. These considerations imply that intrachromosomal rearrangements that involve breaks that are less than 1 Mbp (DSBs in the same CD) or only a few Mbp apart (DSBs in neighboring CDs) are much more frequent than interchromosomal rearrangements (i.e., translocations). Data from recent high throughput sequencing experiments of tumor genomes are supporting these assumptions. About 80 % of all the rearrangements found in these studies were indeed intrachromosomal, with the majority involving breakpoints that were just a few Mbp apart (Wijchers and de Laat 2011) (Fig. 8).

4.4 Functional Consequences of Translocations

Before we ask the question of why specific translocations recur, we will briefly consider the functional consequences of genomic rearrangements that result from DSBs. For the sake of simplicity, we will focus on the functional consequences of translocations, but the same principles do apply to deletions, inversions and more complicated rearrangements.

4.4.1 Passenger Translocations

Some translocations found in tumors or as constitutional translocations do not appear to have any functional consequences. In the tumor setting, the term "passenger translocations" has been coined (Zhang et al. 2010). Whether a particular translocation is a true passenger translocation is very difficult to determine. The function and identity of many regulatory elements (locus control regions, enhancers, insulators) are still very poorly understood. These elements can influence gene expression over very large (up to 1 Mbp) genomic distances. In the context of haematological malignancies, any translocation that is non-recurring and does not lead to an obvious fusion transcript or is in the vicinity of a known oncogene or tumor suppressor gene is likely to be a passenger translocation.

4.4.2 Driver Translocations

Driver translocations are those translocations whose functional consequences are responsible or contribute to the malignant phenotype of a cell. There are two well recognized types of driver mutation: (1) those leading to a fusion gene and (2) those leading to the transcriptional deregulation of breakpoint adjacent genes (Fig. 9).

Fusion Genes

One of the consequences of a chromosomal translocations is the formation of a fusion gene which gives rise to a chimeric protein. In these cases, the two DSBs leading to the translocation occur in two gene loci, mostly in the introns. After the translocation, a fusion transcript is produced that contains in its 5′ part coding exons from the first gene and in its 3′ portion coding exons from the second gene. Only so-called in frame fusion transcript will lead to the translation of a chimeric protein, which consists of an N-terminal protein portion from the first gene and a C-terminal portion from the second gene. The best known example is the t(9;22)(q34;q11) translocation found in more than 90 % of all chronic myeloid leukemia cases (Ben-Neriah et al. 1986; de Klein et al. 1982; Rowley 1973), which gives rise to the well-known BCR/ABL fusion protein. Chimeric proteins like the RUNX1/RUNX1T1, CBFB/MYH11, PML/RARA and various MLL fusion

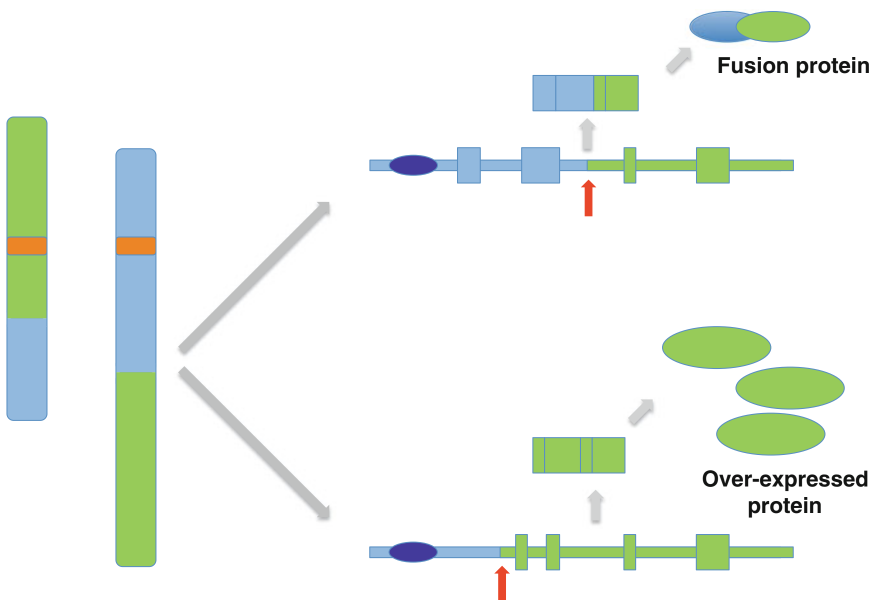

Fig. 9 The functional consequences of a balanced translocation. *Top* the formation of a fusion gene that leads to a fusion protein (*blue/green*). *Bottom* the formation of a fusion gene that leads to the deregulation (over expression) of a gene adjacent to the breakpoint (*green gene*). Boxes on the DNA strand denote exons and the ovals denote promoter or enhancer sequences. *Red arrows* point to breakpoints. Please note that this diagram only shows the situation at one of the two breakpoint junctions

proteins can be identified in about 16–20 % of acute myeloid leukemia cases (Mitelman et al. 2007) (Fig. 9, top right).

Deregulated Gene Expression

The second functional consequence of a chromosomal translocation is the juxtaposition of a strong enhancer element to an (onco)gene. This leads to the transcriptional deregulation, in most cases the overexpression, of the affected gene. The first example for this type of translocation was the t(8;14)(q24;q32) associated with Burkitt's lymphoma and leading to the overexpression of the MYC protooncogene through the IgH intron enhancer and/or locus control regions (Erikson et al. 1983; Madisen and Groudine 1994) (Fig. 9, bottom right).

The total number of known gene fusions, both those leading to a chimeric protein and those leading to deregulated gene expression is presently more than 2000 (http://cgap.nci.nih.gov/Chromosomes/Mitelman, queried May 2014). Just 7 years ago, a similar count listed only 328 gene fusions (Mitelman et al. 2007). This huge increase in the number of gene fusion in recent years is due to the widespread use of high throughput sequencing technologies in the study of tumor genomes.

Interestingly, translocations leading to chimeric proteins are mostly found in myeloid leukemia and some solid tumors whereas translocations that lead to deregulated gene expression are very common in lymphoid malignancies. Most of the translocations found in lymphoid malignancies involve one of the immunoglobulin loci or the T cell receptor loci. As discussed below, this is a consequence of faulty somatic rearrangements involving these loci.

Additional Mechanisms: Disrupting Tumor Suppressor Genes, Two Reciprocal Fusion Proteins

Even though the two mechanisms described above (fusion protein formation and oncogene overexpression) are considered the main mechanisms through which driver translocations exert their oncogenic potential, additional mechanisms might be relevant in certain translocations. Balanced translocation frequently do not only generate a single fusion protein but will often lead to the expression of two reciprocal fusion proteins. While in most instances one of these fusion protein is considered to be the main and sole driving force behind the malignant phenotype, this does not always appear to be the case. For example, there is experimental evidence that both fusion proteins contribute to malignant transformations in the case of acute promyelocytic leukemia (APL). Mice that express the PLZF/RARA and the RARA/PLZF fusion develop a more typical APL phenotype than mice that just express the PLZF/RARA fusion alone (He et al. 2000). For certain MLL fusion, the reciprocal fusion protein, X/MLL, also seems to play an important role like in the case of AF4/MLL (Bursen et al. 2010).

Another consequence of a reciprocal translocation that generates a fusion protein is the fact that the two gene loci involved in the translocation will not be able to serve their wildtype function. They will be "knocked-out" in the rearranged allele, resulting in hemizygosity for these genes. It has become very obvious, that the

disruption of one of the fusion partner is probably playing an important role in some translocations, especially in the case of the very frequent ETV6/RUNX1 fusions observed in childhood B-ALL. Many of these leukemias do not only have the ETV6/RUNX1 fusion but also have a deletion of the non-rearrangmed ETV6 allele leading to a complete loss of wild type ETV6 function (Bohlander 2005). In addition, there are hints that some fusion proteins might interfere with the tumor suppressor function of one of the fusion partner proteins they are composed of through protein protein interaction, like in the case of the INPP5D/ABL1 fusion (Kakadia et al. 2011).

Thus the "transformational impact" of a chromosomal rearrangement can be much stronger than that of a simple point mutation. A translocation can lead to the formation of a new oncoprotein and at the same time compromise the function of one (or even two) tumor suppressor genes.

4.5 Specific Mechanisms Causing Chromosomal Translocations

One of the key questions is whether there are any mechanisms or factors that favor the occurrence of chromosomal translocations.

4.5.1 Random DsDNA Breaks Followed by Faulty Repair via HR, NHEJ or A-EJ (MMEJ)

The analysis of the genomic DNA sequence at translocation breakpoints can provide insights into the mechanisms that led to the joining of the chromosomes. However, this analysis can give only very indirect information of what might have caused the DSB itself.

In most cells, except for cells of the lymphatic lineage, it appears that DSBs are caused by random events of the types discussed above (radiation, stalled replication forks, failed repair of other DNA lesions, etc.) and are then repaired through either the classical or alternative non-homologous end joining pathway (C-NHEJ or A-EJ). The repair via the NHEJ does not need any regions of homologies between the two DNA ends. A translocation breakpoint that was joined by the NHEJ pathway might have small deletions or the random addition of a few nucleotides at the breakpoint junction when the sequences are compared to the non-rearranged chromosome regions (Weinstock et al. 2006; Zhang et al. 2010).

4.5.2 Due to Faulty RAG1-2 or AID Action

It is well known that most of the DSBs that lead to chromosomal translocations in haematological malignancies of the lymphoid lineage are due to the off-target action of the RAG1/2 endonucleases or the activity of AID. As discussed above, the RAG1/2 enzymes target their endonuclease activity to special heptamer/nonamer consensus sequences. As this targeting is not 100 % accurate and restricted to the immunoglobulin loci or the T cell receptor loci, DSBs in other loci can occur. These off-target breaks are again repaired by the NHEJ pathway and an analysis of the

genomic breakpoint sequence will often identify heptamer/nonamer homologous sequences next to the breakpoint at the off target breakage site (Raghavan et al. 2001).

4.5.3 Chromothripsis

The occurrence of very complex chromosomal rearrangements involving one or just a few chromosomes is called chromothripsis (chromosome shattering). These events have just recently become amenable to analysis through whole cancer genome sequencing approaches (Stephens et al. 2011). It is likely that chromosthrispsis events originate from replication fork stalling and template switching (Lee et al. 2007) or alternatively from microhomology-mediated break-induced replication (Hastings et al. 2009) with multiple template switching events. The local nature of these events could be caused by this process being linked to micronuclei formation which is especially likely in situation where various cell spindle and/or anaphase checkpoint mechanisms are defective, like in *TP53*-mutated tumors (Forment et al. 2012; Holland and Cleveland 2012; Liu et al. 2011).

4.5.4 Shortened Telomeres

As a cell undergoes cell divisions, its telomeres will shorten with each division unless the telomerase enzyme is active. This holds true both for somatic cells as we age as well as for premalignant and malignant cells that proliferate even more rapidly. This process of "telomere erosion" can lead to state where the telomere repeats become so short that the usual proteins that protect the telomeres can not attach to the telomeres properly and protect them. As a consequence, telomeres become exposed and will be recognized as DNA breaks, and repair proteins will be recruited. Two telomeres can then be joined together via the NHEJ pathway. This will result in the formation of dicentric chromosomes. Dicentric chromosomes are mitotically unstable because the two centromeres in the chromosome might be pulled in opposite directions (Artandi and DePinho 2010). This will result in a chromatid break and additional cycles of repair and breakage, the so called fusion-bridge-breakage cycles, eventually resulting in multiple rearrangements and aneuploidies. Although this mechanism leads to genomic instability, it is less well documented as the cause of recurring chromosomal translocations. Recently, such a mechanism has been proposed to be responsible for a complicated amplification event of a megabase pair regions on chromosome 21 in childhood acute lymphoblastic leukemia (Li et al. 2014). The observed rearrangements resembled chromothrispsis events.

4.5.5 Template Switching During DNA Replication, Replication Stress

As also discussed above, DNA replication can be an abundant source of DSBs, which lead to chromosomal translocations (Halazonetis et al. 2008; Jackson and Loeb 2001). Especially under stress conditions such as nucleotide depletion (Bester et al. 2011), when the replication fork encounters damaged nucleotides and stalls

(Lee et al. 2007) or encounters regions that are difficult to replicate like fragile sites (Arlt et al. 2006; Barlow et al. 2013; Helmrich et al. 2011; Ozeri-Galai et al. 2012), DSBs or template switching can occur which lead to translocations or even to chromothripsis events (Forment et al. 2012).

4.5.6 Increased Rate of Chromosomal Translocations in Certain Mendelian Disorders

There is a small number of very rare Mendelian disorders that show an elevated incidence of chromosomal translocations and a high tumor incidence. The identification of the causative genes in these syndromes have, not surprisingly, uncovered genes that are involved in DSB repair. These findings underscore the importance of DSB break repair for keeping the number of chromosomal translocations at a low level. Since the different DSB repair pathways discussed above have several components in common, for example the MRN complex (MRE11, RAD50, NBS complex) is central to DSB repair via the HR, C-NEHJ, and A-EJ pathways (Popp and Bohlander 2010), the higher frequency of chromosomal rearrangements in several of these conditions can not be accurately attributed to defects in specific repair pathways. However, these disorder clearly demonstrate the importance of a well functioning repair process in keeping the number of chromosomal translocations low. These conditions include among others: Bloom Syndrome (Ellis et al. 1995), Werner Syndrome (Yu et al. 1996), Nijmegen Breakage Syndrome (Carney et al. 1998; Varon et al. 1998), Ataxia teleangiectasia (Mahowald et al. 2009), and Fanconi anemia. Fanconi anemia, which is characterized by bone marrow failure and the susceptibility to a broad range of liquid and solid tumors, is caused by mutations in more than 15 genes, including BRCA2/FANCD1, that are mainly involved in the repair of interstrand crosslinks and homologous recombination DSB repair (Kottemann and Smogorzewska 2013).

4.6 Why Do We See Certain Translocations Recurringly?

Considering that DSBs breaks are efficiently and quickly repaired in a cell and that the aberrant joining of two chromosomal ends is a rare events due to steric and temporal constraints, and also considering the lack of evidence for a specific 3D arrangement of interphase chromosomes, which might increase the frequency of certain translocations, the question arises: Why do we see recurring chromosomal translocations in tumors at all?

4.6.1 Selection Theory

The main driving force behind the recurrence of specific chromosomal translocations in malignancies is selection. The fusion protein or the overexpressed oncogene that results from certain chromosomal translocations will convey such a strong growth advantage to the cell harboring the translocation that its progeny will outcompete the other cells in the tissue. As discussed in more detail below, there is

evidence that some fusion genes or over-expressed genes resulting from chromosomal translocations actually increase genomic instability thereby accelerating the acquisition of additional genetic changes that enhance the malignant phenotype of the cell resulting in a positive "cancer evolution feedback loop". The selection of chromosomal translocation based on their functional consequences is a well-established phenomenon and has to be considered the main reason why we see recurring chromosomal translocations in tumors. For example, the expression of the BCR/ABL fusion protein in a hematopoietic stem cell will convey such a strong growth advantage to the progeny of this cell that the development of a rapid onset myeloproliferative syndrome is observed in murine model systems (van Etten 1993).

4.6.2 Factors Influencing the Sites of DSBs and the Efficiency of DSB Repair

However, the question arises whether there are factors or mechanisms that influence the frequency or location where chromosome translocations occur?

Two questions would be of interest in this context: (1) Are there factors that lead to more frequent DSB to occur at certain genomic loci? (2) Are certain genomic loci more likely to be joined through incorrect repair of two DSBs than others?

Transcriptional Status and Translocation Frequency

Transcription of DNA by RNA Pol II leads to the formation of a so-called R-loop, an area of the genome where the DNA becomes single-stranded. These single-stranded regions are more susceptible to DNA damage. The nascent RNA transcript can rehybridize with the template strand and cause strand breaks (Li and Manley 2006). Transcription is also required and facilitates the action of AID (activation-induced cytosine deaminase) to deaminate cytosine residues and generate abasic sites. This abasic site are further processed through enzymes of the BER machinery and result in nicks and eventually DSBs for CSR (Lin et al. 2012). However, since transcription is very widespread throughout the human genomes and recent transcriptome sequencing experiments have shown that a much greater proportion of the genome is being transcribed than previously thought (Djebali et al. 2012), it is difficult to imagine that transcription-associated DSBs play a major role in increasing the rate of DSBs at specific sites and would be able to greatly increase the rate at which specific genes are fused in translocation events.

Evidence for Regions Targeted by AID Being More Susceptible to Translocations

Although the action of AID (activation-induced cytosine deaminase) in the process of somatic hypermutation and CSR requires active transcription, there are additional factors that target AID to specific loci. It is well known that regions that are targeted by AID are more susceptible to translocations and that chromosomal translocations found in cells of the lymphoid lineage can be traced back to AID action. With the help of high throughput sequencing it could be shown that the DSB frequency

influences the likelihood that a certain genomic locus is joined in a translocation event with another locus. These studies showed that AID target sites were more likely to be involved in translocations than other sites in B cells (Chiarle et al. 2011; Klein et al. 2011). It can be assumed that similar mechanisms that increase the frequency of DSB at certain genomic loci also operate in non-lymphoid cells. However, it is poorly understood which genomic regions might be more prone to DSBs. Unusual DNA sequences like those found at fragile sites could be preferential sites for DSBs (Arlt et al. 2006; Barlow et al. 2013).

Evidence for Certain Drugs Causing Certain Translocations

There is some evidence that certain chemicals, like topoisomerase II inhibitors or naturally occurring bioflavonoids, can influence the location of chromosomal translocation breakpoints. The action of these compounds is thought to change the location of the DSBs to be closer to nuclear matrix attachments sites. Thus the genomic breakpoints in the MLL gene in therapy-related leukemias are closer to topoisomerase II binding sites than in de novo leukemia (Bode et al. 2000; Broeker et al. 1996; Rowley et al. 1997; Strick et al. 2000; Strissel et al. 1998).

Chromatin Status Plays a Role in DSB Repair

The transcriptional status of a genomic region is closely associated with a distinct interphase chromatin configuration, the more open euchromatin. It could be demonstrated that the initial response to DSBs, namely the accumulation of gamma-H2AX and recruitment of repair factors, is much faster in euchromatin and that DSB repair is accomplished faster in euchromatic regions (Cowell et al. 2007). Conversely, DSBs are repaired at a slower rate if they happen to occur in heterchromatin or transcriptionally inactive regions (Goodarzi et al. 2008; Kim et al. 2007).

It is very difficult to estimate the effect of the sometimes opposing influences of these various factors on the frequency and repair efficiency of DSB breaks. Euchromatic regions are more transcriptionally active and thus more susceptible to breakage. At the same time the recruitment of DNA repair proteins is faster, and DSBs are repaired more rapidly in euchromatic regions, thus minimizing the time in which a chromosomal translocation, involving the misguided repair with a second temporally and spatially matched DSB, could occur.

Chromatin plays an important and still not completely understood role in DSB repair. For example the chromatin protein HP1 is rapidly recruited to DSB and is required for efficient repair (Ayoub et al. 2009; Luijsterburg et al. 2009). Certain posttranslational histone modifications like histone 4 lysine 20 (Schotta et al. 2008) or histone 3 lysine 79 methylation (Lin et al. 2009) also influence genome stability. Another example of changes in epigenetic marks, in this case DNA methylation, that can lead to genetic instability and chromosomal translocations is the immunodeficiency-centromeric instability-facial anomalies syndrome 1 (ICF1: OMIM 242860). ICF1 is characterized by an increased frequency of whole arm translocations and other chromosome abnormalities (decondensation, chromatid breaks) involving heterochromatic regions of chromosomes 1, 9 and 16 in

peripheral blood lymphocytes. This syndrome is caused by mutations in the de novo DNA methyltransferase *DNMT3B*, which leads to DNA hypomethylation in the constitutive heterochromatin (Xu et al. 1999).

Model Systems to Study Translocations

Several model systems have been developed to study the factors that might contribute to the generation of specific, recurrent translocation events. Lin and colleagues demonstrated that genotoxic stress together with liganded androgen receptor were able to induce the TMPRSS2/ERG fusion (Lin et al. 2009). Bastus and colleagues could show that long term and high dosage androgen treatment of non-malignant prostate cell lines without genotoxic stress was sufficient for the formation of the TMPRSS2/ERG fusion (Bastus et al. 2010). A TMPRSS2/ERG fusion could only be observed in cell lines that expressed the androgen receptor. The androgen exposure apparently induced chromatin reorganization in the interphase nucleus which led to a more frequent colocalization of the *TMPRSS2* and *ERG* loci. It should be noted that both *TMPRSS2* and *ERG* are located just 3 Mbp apart on chromosome 21. Whether this rather spontaneous cell type specific induction of a recurrent rearrangement would also be possible for two gene loci that are located on different chromosomes or whether this phenomenon is due to the close proximity of both loci remains to be examined.

Gene replacement studies using the *MYC* genes clearly demonstrated that cellular selection based on the functional consequences of a gene fusion cannot be the only factor that leads to the occurrence of recurring translocations in certain tumors but that also other factors (e.g. sequences flanking a gene locus, chromatin configuration or transcriptional status) are playing a role in the generation of driver translocations (Gostissa et al. 2009). However, these factors might only be relevant in the lymphoid lineage where translocation breakpoint regions are enriched in degenerate heptamer–nonamer sequences that can act as aberrant targets for the RAG endonucleases.

Evidence for a Defined 3D Arrangement of Interphase Chromosomes?

As discussed above, there is no firm evidence that a defined 3D arrangement of certain chromosome territories with respect to each other exists in the interphase nucleus. More recent studies using high throughput sequencing approaches have shown that the key factors in determining translocation frequency are the frequency of DSBs and that translocation partner choice is dictated by the physical proximity of the DSB in the interphase, which in turn is mainly a function of the physical distance between two loci on the same chromosome (Bunting and Nussenzweig 2013; Mahowald et al. 2009; Zhang et al. 2012). Recent next generation sequence analyses of whole tumor genomes clearly demonstrated that there is an abundance of chromosomal rearrangements (small deletions and insertions) that involve chromosomal loci that are in close vicinity on the same chromosome (Campbell et al. 2008; Pleasance et al. 2010a, b; Stephens et al. 2009). It should be noted that classical cytogenetics analyses is not able to detect these rearrangements.

4.6.3 Translocations Have to Occur in the Right Cell Type

There is ample evidence that a single translocation event, which leads, for example, to the generation of a fusion oncogene, is not sufficient to transform a cell fully into a cancer cell. For example, the expression of the RUNX1/RUNX1T1 fusion protein is not sufficient to cause leukemia (Okuda et al. 1998). Even in models where two oncogenic drivers were introduced via retroviral transduction into hematopoietic cells in a murine bone marrow transplantation model, there was a very long latency until leukemia did develop (Schessl et al. 2005). These observations clearly suggest that in order to be eventually found in a tumor, a driver translocation has to occur in a stem cell or a similarly long-lived cell, which has a sufficiently long life expectancy to acquire additional transforming mutations.

The requirement for additional genetic lesions will also favor initiating driver translocations that increase the level of genetic instability. Some of the mechanisms employed by driver translocations which might be responsible for an increase in genetic instability are discussed in the following paragraphs.

4.7 Increased Genomic Instability as a Consequence of Translocations

A slight increase in genomic instability will favor the acquisition of the additional genetic lesions that are required for complete malignant transformation. These additional genetic lesions are not only restricted to chromosomal translocations but can be any change in the genome that leads to the activation of an oncogene, the inactivation of a tumor suppressor gene or other changes in gene activity that promote a malignant phenotype. Genetic stability is dependent on a delicate balance between DNA damage and DNA damage repair. It is advantageous for a tumor to shift the genomic stability rheostat slightly towards instability (Fig. 1). Too big a shift towards instability, however, will not be tolerated by the cells.

4.7.1 Increasing DNA Damage

There are several mechanisms by which driver translocations have been shown to increase the rate at which DNA damage is generated in a cell. Many fusion proteins, especially those leading to the activation of a tyrosine kinase, increase the cellular proliferation rate. An increased cellular proliferation places higher demands on nutrients and increases the rate of DNA replication. Increased DNA replication can lead to local or global depletion of the nucleotide pools, which in turns leads to an increased rate of DNA damage (Bester et al. 2011). Increased cellular proliferation goes hand in hand with an increase in energy requirement and also an increase in the production of ROS which are also damaging DNA (Nowicki et al. 2004; Sallmyr et al. 2008).

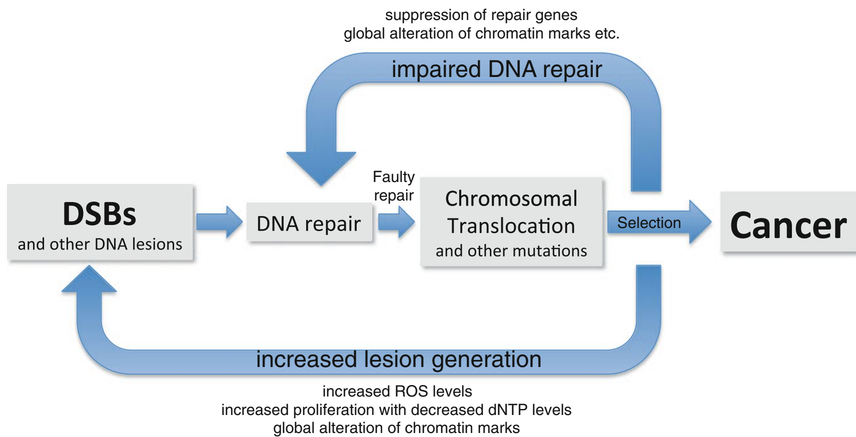

Fig. 10 Schematic representation of the positive feedback loops involving increased DNA lesion generation and impaired DNA repair process in the evolution to cancer

4.7.2 Changing DNA Repair Efficiency

There are several ways in which chromosomal translocation might influence the repair efficiency for DNA lesion. One is the downregulation of repair proteins and cell cycle checkpoint genes (Mulaw et al. 2012). The other avenue is to shift DSB repair to more error-prone pathways (Sallmyr et al. 2008).

It appears that certain fusion proteins like the CALM/AF10 fusion can also lead to genome-wide alterations in epigenetic marks that have the potential to increase genomic instability (Lin et al. 2009). There are probably several other, more subtle ways in which the rheostat of genomic stability can be reset in tumor cells.

Figure 10 summarizes the various mechanisms that might play a role in increasing genetic instability as a consequence of chromosomal translocations.

5 Open Questions and Perspectives

5.1 Open Questions

Of course, many open questions remain regarding the connection of DNA repair and chromosomal translocations. Next generation sequencing studies have recently uncovered many more rearrangements than had been detected by more conventional methods and shown that many translocations are far more complicated than previously thought (Chiang et al. 2012). These results will lead to new questions and technological advances (i.e. next generation sequencing, new imaging techniques) will undoubtedly help in answering these questions. A better understanding of the extremely complicated process of DNA damage repair will be required. In addition, we have to realize that we are very far from a comprehensive understanding of the 3D organization of the genome in the interphase nucleus and the

various processes that depend on and influence this organization (epigenetic modifications, chromatin dynamics, transcription, DNA replication, DNA damage repair, etc.) (Misteli 2010).

5.2 Preventing Translocations

Even though it might seem far-fetched, a better understanding of the mechanisms that lead to chromosomal translocations might one day help to develop strategies to prevent or to reduce the incidence of translocations. Bunting and colleagues showed that it was possible by depleting 53BP1 in *Brca1* mutated cells to promote DSB repair via the error free homologous recombination pathway instead of via the error prone non-homologous end joining pathways (Bunting et al. 2010).

5.3 Using Genetic Instability in Cancer Cells to Generate Synthetic Lethality

A more realistic goal might be to exploit the genetic instability of tumor cells to develop treatment strategies that are based on the concept of synthetic lethality, like the the use of PARP inhibitors in leukemias with compromised DSB repair or by targeting RAD52 (Cramer-Morales et al. 2013; Gaymes et al. 2009; Skorski 2008).

5.4 Intrachromosomal Driver Translocations

Next generation sequencing studies have shown that rearrangements due to DSBs between loci that are in the same or in adjacent chromatin domains are found much more frequently in tumors than translocations between loci on different chromosome arms which can be identified easily with traditional cytogenetics methods. It is very likely that many of these intrachromosomal rearrangements will just have passenger nature (passenger translocations or rearrangements). However, there will also be many driver rearrangments among these local rearrangements (e.g. the TMPRSS2/ERG fusion (Tomlins et al. 2005)). These intrachromosomal driver translocations might be much more frequent and much more varied than the traditional interchromosomal driver translocations. Since driver rearrangements are usually identified on the basis of recurrence one would expect that a much great number of tumor samples has to be analyzed to identify such low frequency intrachromosomal driver rearrangements.

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CINcere Modelling: *What Have Mouse Models for Chromosome Instability Taught Us?*

Judith E. Simon, Bjorn Bakker and Floris Foijer

Abstract

Chromosomal instability (CIN) is a process leading to errors in chromosome segregation and results in aneuploidy, a state in which cells have an abnormal number of chromosomes. CIN is a hallmark of cancer, and furthermore linked to ageing and age-related diseases such as Alzheimer's. Various mouse models have been developed to explore the role of CIN in ageing and cancer. While these models reveal only a modest contribution of CIN to the initiation of cancer, they also clearly show that CIN is a powerful accelerator of cancer in a predisposed background. Other than cancer, CIN also appears to provoke premature ageing in some of the CIN models. In this review, we discuss the phenotypes of the various available mouse models, what we have learnt so far, and importantly, also which questions still need to be addressed.

Keywords

Aneuploidy · Chromosomal instability · Cancer · Ageing · Mouse models

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1 Chromosomal Instability and Aneuploidy

During each cell division our genetic code is replicated, followed by symmetrical segregation of all chromosomes into the emerging daughter cells. Cancer cells occasionally exhibit errors segregating their chromosomes, a process known as chromosomal instability (CIN), leading to cells with abnormal numbers of chromosomes, a state defined as aneuploid. In addition to whole chromosome abnormalities, CIN can also lead to structural abnormalities such as amplifications, deletions or translocations, either through defects in the DNA damage machinery or as a direct result of chromosome missegregation events (Janssen et al. 2011). Although numerical and structural abnormalities frequently coincide, in this review we will focus on how mouse models have contributed to our understanding of the consequences of whole chromosome instability.

David von Hansemann was the first to report abnormal chromosome numbers in carcinoma samples in 1890, long before the relationship between chromosomes and the genetic code had been established (Hardy and Zacharias 2005; Bignold et al. 2006; Siegel and Amon 2012). Early in the 20th century, Theodor Boveri showed that aneuploidy leads to abnormal development or even death by injecting two sperms instead of one into sea urchin embryos. These observations led to the hypothesis that aneuploidy can lead to cancer or developmental defects (Boveri and Manchester 1995; Bignold et al. 2006; Boveri 2008, Ried 2009). Since then, many studies confirmed that CIN is a hallmark of human malignancies, affecting 2 out of 3 cancers (Duijf et al. 2013). More recently, aneuploidy has also been associated with ageing and age-related diseases (Faggioli et al. 2012). For instance, trisomy for chromosome 21 is frequently found in plaques in Alzheimer patients' brains (Iourov et al. 2009). Conversely, people with Down syndrome develop early onset Alzheimer's disease (Lai and Williams 1989), further emphasizing the relationship between trisomy 21 and neurodegenerative disease.

Although CIN has been associated with cancer for more than a century, we are only beginning to understand the consequences of CIN and aneuploidy at the cellular and molecular level. CIN is believed to accelerate the evolution of cancer cells by facilitating gain of oncogenes and loss of tumour suppressor genes. Paradoxically, when modelled in yeast strains (Torres et al. 2007) or mouse embryonic fibroblasts (MEFs) (Williams et al. 2008), aneuploidy appears to decrease rather than increase cell proliferation, suggesting that cancer cells find ways to cope with the adverse effects of aneuploidy. However, as transformation of aneuploid cells into aneuploid cancer cells can only occur in vivo by definition, animal models for CIN are essential to solve this paradox.

2 Provoking CIN In Vivo

Several processes that safeguard correct chromosome segregation have been targeted to engineer mouse models for CIN. Figure 1 shows a schematic overview of a large number of genes that have been targeted for this purpose. One of the first models specifically designed to study the in vivo consequences of CIN is the Mad2 knockout mouse, targeting the spindle assembly checkpoint (SAC) (Dobles et al. 2000). The SAC prevents missegregation of chromosomes by inhibiting metaphase to anaphase progression until all chromosomes are properly attached to kinetochores in a bi-oriented fashion. Defects of the SAC therefore result in flawed chromosome segregation, which makes the SAC an attractive target to model CIN in vivo. A second means to induce CIN in vivo is by interfering with kinetochore integrity, a protein structure that connects the centromeric DNA to the mitotic spindle. This has been done by removing structural components of the kinetochore (e.g., CenpB, CenpC) or alternatively, by stabilizing kinetochore-microtubule attachments through e.g., overexpressing Mad2 or Hec1 (Diaz-Rodríguez et al. 2008; Kabeche and Compton 2012). Centrosomes are the microtubule-organizing centres in the cell from which the mitotic spindle emanates (Ganem et al. 2007; Gordon et al. 2012). Abnormal centrosome numbers can either result in multipolar divisions or, when supranumeral centrosomes cluster, predispose for lagging chromosomes in mitosis (Ganem et al. 2009). Therefore, a third way to provoke CIN in vivo is by inducing centrosome amplification, e.g., through overexpression of Plk4 (Ko et al. 2005; Marthiens et al. 2013a). A fourth approach to induce CIN in vivo is by disrupting the cohesion complex, a ring like structure that holds the sister chromatids together during interphase. Cohesion defects have been modelled by abrogating components of the cohesion complex (e.g., SA1), but also by deregulating upstream players such as pRb (Coschi et al. 2010; Manning et al. 2010; Van Harn et al. 2010). Similarly, various other genes have been knocked out in the mouse which indirectly affect chromosome segregation.

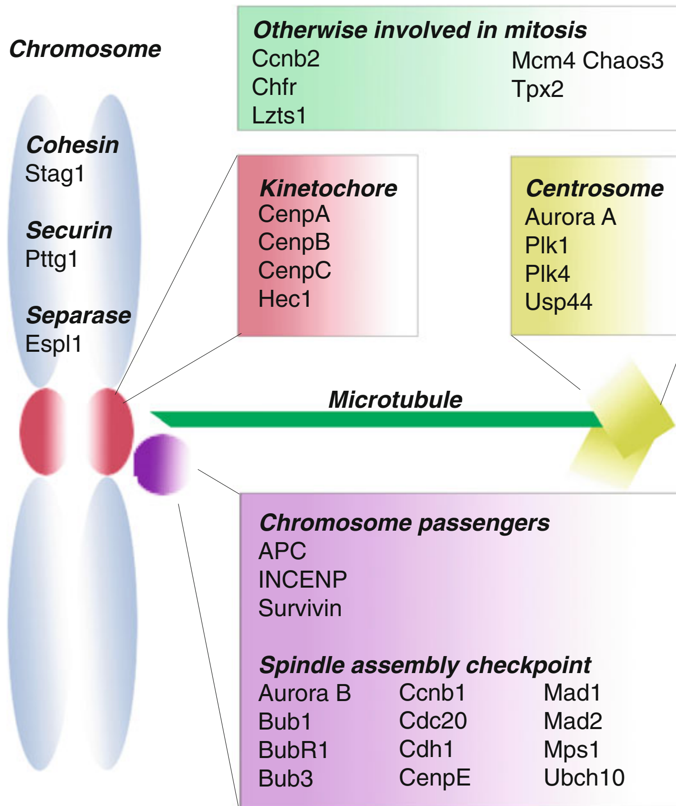

Fig. 1 Schematic overview of various genes targeted to provoke CIN in vivo

3 In Vivo Consequences of CIN

In the last two decades, a large number of mouse models for chromosome instability have been engineered. Hereunder, we summarize the findings from these models asking the following questions:

1. Is CIN a bona fide instigator of cancer?
2. Which genes collaborate with CIN in vivo converting aneuploid cells into aneuploid cancer cells?
3. What are other consequences of CIN in vivo?

4 Can CIN Initiate Cancer?

CIN has detrimental consequences for cells grown in vitro (Kops et al. 2004; Williams et al. 2008; Torres et al. 2008), yet, two out of three human tumours are aneuploid (Weaver and Cleveland 2006; Duijf et al. 2013). This raises the question whether CIN is an initiating factor in cancer, a facilitator or merely a side effect of tumorigenesis. In the vast majority of all models (see Fig. 1), full inactivation of the targeted genes resulted in early embryonic lethality. Although the time of embryonic death varied between genotypes (Table 1), embryos were typically lost before embryonic day 10, which presumably was the result of aneuploidy in the inner cell mass of the developing embryos (Dobles et al. 2000; Weaver and Cleveland 2006; Foijer et al. 2008; Holland and Cleveland 2009; Schvartzman et al. 2010). To circumvent embryonic lethality, phenotypes of heterozygous mice were monitored, or in some cases, conditional alleles were engineered. Even though tumour phenotypes have been reported for many of these models (Table 1) tumour incidence is relatively low, with in most cases fewer than 50 % of the mice developing cancer. Moreover, tumours only arise late in the life of the mice, with latencies typically ranging from 12 to 24 months (Table 1). The most frequent pathologies observed include lymphoma, lung and liver tumours. Furthermore, not all models develop spontaneous tumours, for instance in case of the Bub family members [Bub1, Bub3, Rae1 (Kalitsis 2000; Babu et al. 2003; Wang et al. 2004; Baker et al. 2006a; Jeganathan et al. 2007; Baker et al. 2009)]. There is no clear correlation between the severity of the tumour phenotypes and the mechanism that drove CIN in the mice (i.e. SAC mutation, cohesion defects, centrosome abnormalities etc.). Expression levels of the CIN-provoking genes on the other hand appear to be a better predictor of tumour incidence: phenotypes were most severe in cases where CIN-driving proteins were overexpressed to high levels [e.g. Mad2, Cyclin B1, Cyclin B2, Hec1, Plk4 (Ko et al. 2005; Sotillo et al. 2007; Baker et al. 2008; Diaz-Rodríguez et al. 2008)] possibly because the relative effect on protein expression (several folds overexpression) was more dramatic than in heterozygous mice, where protein levels were typically reduced $\sim$50 %. However, as tumour latency is high in these models as well, additional hits must be required for aneuploid cells to become malignant.

5 Does CIN Predispose to Cancer?

Exposure to carcinogens is a powerful tool to assess tumour predisposition in vivo. Given the relative weak tumour phenotypes of CIN mice, various CIN models were exposed to carcinogens (Table 1) to assess whether CIN is a powerful collaborator in transforming cells. Indeed, carcinogens aggravated the tumour phenotypes of some of the CIN mice, more than their control counterparts. For instance, when Mad1 heterozygous mice were treated with Vincristine (a microtubule-depolymerizing agent), 40 % of the mice developed mostly lung tumours, while no tumours were detected in control-treated mice (Iwanaga et al. 2007). Likewise, carcinogens

Table 1 List of various mouse models engineered to provoke CIN in vivo, with phenotypes and observed aneuploidy levels in vivo and in vitro where quantified

Group	Gene	−/−	±	Cancer predisposed (chemical or genetic collaboration)	Aneuploidy in tissues	Aneuploidy in MEFs	Implicated in human cancer (Schvartzman et al. 2010)	References
Spindle assembly checkpoint	AuroraB	EL	>60 %; 24 mo	Tumour suppression upon DMBA + TPA-induced (not sig.)	ND	ND		Fernández-Miranda et al. (2011)
	Bub1	EL (E6.5)	VNODD	DMBA-induced-57 %, $p53^{+/-}$; 16.6 mo	ND	ND		Jeganathan et al. (2007), Baker et al. (2009)
	Bub1 hypomorph	n/a	50 %; 12 mo	78 %, $p53^{+/-}$; 12 mo	ND	15 % (seg. defects)		Jeganathan et al. (2007), Baker et al. (2009)
	Bub3	EL (E6.5)	VNODD	DMBA-induced	10 % (splenocytes)	20 %		Kalitsis (2000), Babu et al. (2003), Kalitsis et al. (2005), Baker et al. (2006b)
	Bub3; Rae1	ND	VNODD	DMBA-induced	40 % (splenocytes)	40 %		Babu et al. (2003), Baker et al. (2006b)
	BubR1	EL (E6.5)	VNODD	DMBA-induced	Polyploidy in megakaryocytes	15 %	Yes	Wang et al. (2004), Baker et al. (2004)
	BubR1 hypomorph	n/a	Premature ageing	DMBA- and azoxymethane-induced	30 % (splenocytes)	35 %	Yes (MVA)	Baker et al. (2004)
	BubR1 overexpression	n/a	Delayed ageing	DMBA-induced, but decreased susceptibility than WT	1 % (splenocytes)	9 % (WT comparable)		Baker et al. (2013a)
	Ccnb1 (Cyclin B1) overexpression	n/a	>75 %; (lung, lymphoma, liver, lipoma)	~80 %, APC+/min; 40 % (WT comparable; skin), DMBA-treatment	hi 20 %, lo 12 % (splenocytes)	31 % (ctrl. 15 %)		Nam and van Deursen (2014)

(continued)

Table 1 (continued)

Group	Gene	−/−	±	Cancer predisposed (chemical or genetic collaboration)	Aneuploidy in tissues	Aneuploidy in MEFs	Implicated in human cancer (Schvartzman et al. 2010)	References
	Cdc20 AAA mutant (does not bind to Mad2)	EL (E12.5)	50 %; 24 mo	ND	35 % ($Cdc20^{AAA/+}$, splenocytes)	28 % ($Cdc20^{AAA/+}$ and 52 % of		Li et al. (2009)
	Cdh1	EL (E10.5)	17 % females – (mammary); mild brain abnormalities and altered behaviour	Tumour suppression upon TPA/DMBA treatment	ND	Increased (not quantified)		García-Higuera et al. (2008)
	CENPE	EL (<E7.5)	20 % (lung, spleen); 19–21 mo	Tumour suppression upon DMBA treatment or $p19^{Arf}$ loss	40 % (splenocytes)	20 % (up to 70 % at high passage)		Weaver et al. (2003, 2007)
	Mad1	EL	20 % (lung); 18–20 mo	Vinicristine-induced	ND	10 %		Iwanaga et al. (2007)
	Mad2	EL	30 % (lung); 18 mo	ND	ND	55 %		Dobles et al. (2000), Michel et al. (2001)
	Mad2 overexpression	n/a	50 % (lymphomas, lung and liver); 20 mo	DMBA-induced	Aneuploid tumours (not quantified)	50 %	Yes	Sotillo et al. (2007)
	Mps1 (T-cell restricted)	VVNOD	~50 % (lymphoma) 17 mo	100 %, $p53^{+/-}$; 5 mo	>90 % of cells aneuploid	ND		Foijer et al. (2014)
	Rae1	EL (E6.5)	No spont. tumorigenesis	DMBA-induced	10 % (splenocytes)	20 %		Babu et al. (2003), Baker et al. (2006b)
	Ubch10 overexpression	n/a	Expression level dependent: 40–80 % (lymphoma, lung adenoma, lipoma and liver and skin)	Yes, but not significantly different compared to wild type	4–19 % hi-lo, 5 mo (splenocytes); 52–64 % (lymphoma)	28–33 % (WT 13 %)	Yes	van Ree et al. (2010)

(continued)

Table 1 (continued)

Group	Gene	−/−	±	Cancer predisposed (chemical or genetic collaboration)	Aneuploidy in tissues	Aneuploidy in MEFs	Implicated in human cancer (Schvartzman et al. 2010)	References
Kinetochore	CENPA	EL (E6.5)	VNODD	ND	Chromosome missegregation in E6.5 CENPA$^{-/-}$ embryos	n/a		Howman et al. (2000)
	CENPB	VNODD	VNODD	ND	ND	ND		Hudson et al. (1998), Perez-Castro et al. (1998), Kapoor et al. (1998)
	CENPC	EL (E3.5)	VNODD	ND	Aberrant mitosis and micronuclei in early embryos	n/a		Kalitsis et al. (1998)
	Hec1 overexpression	n/a	13 % (lung), 26 % (liver); 67 wk, 60 wk	ND	ND	25 %	Yes	Diaz-Rodríguez et al. (2008)
Cohesion	Espl1 (separase)	EL (E6.5)	Epsl1$^{+/H}$; VNODD	86 % (lymphomas), p53$^{-/-}$; 4 mo—50 % (carcinoma), p53$^{+/-}$	57 % (splenocytes); 84 % (bone marrow)	ND	Yes	Mukherjee et al. (2011)
	Espl1 overexpression (mammary restricted)	n/a	80 % (mammary), 11 mo	100 % (mammary), p53$^{+/-}$; 14 mo	>80 % (mammary tumours)	ND		Mukherjee et al. (2013)
	Stag1 (exon 3 and 4, encoding SA1-cohesin subunit)	EL (between E12.5 to E18.5)	40–50 % (haematoma, lung, fibrosarcoma, liver, vascular, pancreas); 24 mo	Resistance to 3MC and DEN induced fibrosarcomas and liver tumours	40 % (fetal liver)	>70 %		Remeseiro et al. (2012)
	Pttg (securin)	Reduced testis, spleen and thymus weight.	n/a	Tumour protective, pRb$^{+/-}$	ND	15 % (WT 1 %)		Wang et al. (2001), Chesnokova et al. (2005)

(continued)

Table 1 (continued)

Group	Gene	−/−	±	Cancer predisposed (chemical or genetic collaboration)	Aneuploidy in tissues	Aneuploidy in MEFs	Implicated in human cancer (Schvartzman et al. 2010)	References
	Pttg (securin) overexpression	n/a	Enlarged pituitary; altered nuclear morphology	>80 % (pituitary), $pRb^{+/-}$; 10 mo	ND	ND		Abbud et al. (2005), Donangelo et al. (2006)
Chromosome passengers	APC/MIN	EL (<E8.5)	Intestinal tumours; 3 mo	ND	Aneuploidy and abnormal mitosis in crypt cells	Increased, not quantified		Su et al. (1992), Oshima et al. (1995), Rao et al. (2005), Caldwell et al. (2007)
	Incenp	EL (3.5–8.5)	VNODD	ND	Abnormal nuclear morphology hyperdiploid content in E3.5 embryos	n/a		Uren et al. (2000)
	Survivin	EL (6.5)	VNODD	ND	Giant nuclei in early embryos	n/a		Uren et al. (2000)
Centrosome	Aurora A overexpression (mammary restricted)	n/a	Increased p16 expression	45 %, $p53^{-/-}$ (mammary gland); 4.5 mo	ND	13.6 %		Zhang et al. (2004, 2008)
	Plk1	EL (E10.5)	27.5 % (lymphoma, lung); 12.5–17.5 mo	100 % (lymphoma, lung), $p53^{-/-}$	12 % (splenocytes)	ND		Lu et al. (2008)
	Plk4 overexpression (CNS restricted)	n/a	Microcephaly, 100 % post-natal lethality; <1 wk	100 % lethality, $p53^{-/}$; 5 mo	31.7 % centrosome amplification (neural stem cells); >60 % aneuploidy of chr. 18 in $p53^{-/-}$	ND		Marthiens et al. (2013b)

(continued)

Table 1 (continued)

Group	Gene	−/−	±	Cancer predisposed (chemical or genetic collaboration)	Aneuploidy in tissues	Aneuploidy in MEFs	Implicated in human cancer (Schvartzman et al. 2010)	References
	Usp44	VNODD	Usp44$^{+/-}$ 20 %, Usp44$^{-/-}$ 50 %; 15 mo (lung, liver, lymphoma, sarcoma)	n/a	8 %, 5 mo; 16 %, 15 %, 15 mo (splenocytes)	18 % (WT 13 %)	Yes	Zhang et al. (2012)
Otherwise involved in mitosis	Ccnb2 (Cyclin B2) overexpression	n/a	>70 % (lung, lymphoma, liver, lipoma); 14 mo	>80 %, APC$^{+/min}$; >80 % (lung), DMBA-treatment	18 % (splenocytes)	36 % (ctrl. 16 %)		Nam and van Deursen (2014)
	Chfr	VNODD	Chfr$^{-/-}$ 50 %; 20 mo	DMBA-induced	ND	25 %		Yu et al. (2005)
	Mcm4 Chaos3	Chaos$^{3/-}$ EL (E14.5)	Mcm4Chaos$^{3/+}$ (mammary); 12 mo	ND	ND	ND		Shima et al. (2007)
	Tpx2	EL (E8.5)	53 % (lymphoma, lung)	no	18.3 %, 16 wk; 27 %, 90 wk (splenocytes) 48.9 %, 90 wk (lymphomas)			Aguirre-Portolés et al. (2012)

Genes that have been used to generate CIN mouse models

EL embryonic lethal; *VNODD* viable, no overt developmental defects; *ND* not determined; *n/a* not applicable

(NMBA or DMBA) accelerated tumorigenesis in Lzts1-deficient and Chfr-deficient mice (Yu et al. 2005; Vecchione et al. 2007). Furthermore, even in CIN mice without a tumour phenotype (e.g., Bub1$^{+/-}$, Bub3$^{+/-}$, Rae1$^{+/-}$ and Bub3$^{+/-}$ Rae1$^{+/-}$), DMBA treatment had a stronger tumour promoting effect than on wild type mice (Baker et al. 2006b; Jeganathan et al. 2007). As carcinogens reduce tumour latency and increase tumour incidence in a CIN background, also these experiments indicate that additional mutations are required for a CIN cell to transform into a malignant cell.

6 Which Genes Collaborate with CIN in Cancer?

To test which genetic alterations collaborate with CIN in tumorigenesis, various CIN models were crossed into cancer-predisposed backgrounds. For instance, when CIN was combined with p53 heterozygosity, [Bub1, Espl1, Mps1 (Baker et al. 2009; Mukherjee et al. 2011; Baker et al. 2013b)] tumour incidence dramatically increased while tumour latencies decreased. In all reported cases, tumours had lost the remaining p53 wild type allele, indicating that full p53 loss and CIN synergize in tumorigenesis (Baker et al. 2009; Foijer et al. 2014). However, as CIN further increased tumour incidence of p53^{null} mice, CIN must have facilitated cancer formation through additional genomic alterations as well. Furthermore, CIN provoked by Bub1 hypomorphic alleles or Cyclin B1 overexpression accelerates tumours in a Apc^{min} background (Baker et al. 2008, 2009). However, in other tumour predisposed backgrounds (e.g., pRb or Pten heterozygousity) CIN has no effect on tumour incidence (Baker et al. 2009).

7 CIN as a Tumour Suppressor

In some cases CIN can also act in a tumour suppressive manner. For instance, CIN driven by SA1 heterozygosity delays 3-methyl-colanthrene (3-MC)-induced fibrosarcoma and diethyl-nitrosamine (DEN)-induced liver tumours (Remeseiro et al. 2012). Similarly, even though Cdh1$^{+/-}$ mice and CenpE$^{+/-}$ mice are more susceptible to spontaneous tumours, they are more resistant to carcinogenic insults than their wild type counterparts (Weaver et al. 2007; García-Higuera et al. 2008). Furthermore, CIN can also delay tumorigenesis in some genetically predisposed models, for instance by delaying p19^{Arf} or Pten loss-driven tumours (Weaver et al. 2007; Baker et al. 2009). Why then is CIN tumour promoting in one setting, but tumour suppressive in another? The answer might lie in the levels of CIN. CIN is quite toxic and provokes an ‘aneuploidy stress’ response in untransformed cells (Kops et al. 2004; Torres et al. 2007; Williams et al. 2008; Foijer et al. 2013). However, aneuploid cancer cells also exhibit this stress response (Dürrbaum et al. 2014; Foijer et al. 2014), suggesting that aneuploid cancer cells still suffer from the disadvantageous effects of CIN. Therefore, the levels of CIN occurring in premalignant cells could be a determining factor for the outcome. The fact that p19^{Arf} loss provokes aneuploidy itself fits with this hypothesis, as CenpE heterozygosity would

exacerbate CIN to a level that is toxic for cancer cells (Silk et al. 2013). However, further experiments are required to determine at what level CIN is beneficial for cancer cells and at what level the balance is tipped.

8 What Other Phenotypes Are Provoked by CIN?

There is increasing evidence that aneuploidy also occurs in untransformed tissues, with liver being the most well-known example. Up to half of both human and murine hepatocytes are aneuploid (Duncan et al. 2012a, b), but it is unclear why hepatocytes evolved to become aneuploid. One suggestion is that particular karyotypes are selected for during hepatotoxic insults, making the hepatocytes more resistant to injury (Duncan et al. 2012b). Other studies quantified over 30 % of normal human neuroblasts to be aneuploid (Rehen et al. 2001, 2005), which has been suggested to contribute to the plasticity of neurons (Kingsbury et al. 2005). However, when provoked in a random fashion, CIN appears to mostly have disadvantageous effects on brain functioning, as mice heterozygous for Cdh1 exhibit defects in neuromuscular coordination and learning (García-Higuera et al. 2008). The interfollicular epidermal cells in mouse skin on the other hand appear to cope surprisingly well with CIN as they tolerate full abrogation of the SAC provoked by Mad2 loss, which results in dramatic aneuploidy (Foijer et al. 2013). The hair follicle stem cells that reside in the same compartment do not cope at all and disappear, resulting in mice with functional skin, but without hair (Foijer et al. 2013). Together these data clearly indicate that CIN is tolerated by some cell lineages, but not others, underscoring the importance of in vivo modelling.

9 Linking Ageing and CIN In Vivo

Ageing is the time-dependent functional decline in the fitness of cells, organs and organisms. A common hallmark of ageing is genomic instability, as exemplified by genetic alterations in old blood cells (Forsberg et al. 2012; López-Otín et al. 2013). Some of the CIN mouse models also suggest a role for aneuploidy in ageing. For instance, BubR1 hypomorphic mice are not only prone to severe aneuploidization, but also display progeroid pathologies. Similar to BubR1, combined Bub3/Rae1 haploinsufficiency also results in a premature ageing phenotype, albeit less severe than the BubR1 hypomorphic mice (Baker et al. 2006b), MEFs isolated from BubR1 hypomorphic mice express various ageing-associated markers such as p53, p21, $p19^{Arf}$ and $p16^{Ink4a}$. Interestingly, when $p16^{Ink4a}$ positive cells are killed in vivo using a $p16^{Ink4a}$-promotor regulated suicide construct, ageing pathologies induced by a reduction of BubR1 protein levels are dramatically delayed (Baker et al. 2011). The pathologies observed in BubR1 hypomorphic mice mimic those of patients with multi-variegated aneuploidy (MVA), a disease that frequently coincides with mutations in *BUB1B*, the gene encoding BUBR1 (Hanks et al. 2004,

2006; Matsuura et al. 2006). Furthermore, BubR1 expression levels decline with age providing further evidence for a role of BubR1 in ageing (Baker et al. 2011) in mice. Even more striking, when BubR1 is overexpressed, a dose-dependent delay in the onset of ageing is observed, as well as protection against developing chemically-induced tumours (Baker et al. 2013a). As discussed above, in most tested cases overexpression of CIN-controlling proteins increases CIN and predisposes for cancer (Sotillo et al. 2007; Diaz-Rodríguez et al. 2008; Fernández--Miranda et al. 2011). Apparently, BubR1 is the exception that forms the rule, but future work should reveal whether BubR1 has a unique role in the SAC or whether it has additional roles that can explain the beneficial effects of an overdose of BubR1.

10 What Have We Learnt from Modelling CIN in the Mouse so Far?

As most tumours are aneuploid to some extent, CIN makes an attractive target for therapy. For this, understanding how CIN is signalled is crucial. A large number of mouse models have been engineered over the last 15 years specifically for this purpose, with a wide variety of phenotypes summarized in Table 1. Even though many of the targeted genes will have other roles than safeguarding faithful chromosome segregation, some common conclusions can be drawn from the cumulative data. The first important conclusion is that CIN alone is not sufficient for efficient tumourigenesis and that CIN alone mostly has disadvantageous effects on cell proliferation. This has important implications for therapy targeting aneuploid cancer, as discussed below. A second conclusion is that CIN facilitates tumourigenesis efficiently in some tumour-predisposed backgrounds, chemical or genetic. However, when CIN is aggravated and becomes too severe, it can actually suppress tumour formation in the mouse, which can also be exploited in cancer therapy. A third and perhaps the most important conclusion is that several unaddressed questions remain before we can develop therapeutic strategies targeting aneuploid cell progeny, some of which are discussed below. Although all models discussed here were designed to study the consequences of CIN in vivo, the majority mimic a situation that is not typically found in human cancers, as loss of genes that regulate chromosome segregations are rarely lost in human cancer (Schvartzman et al. 2010; Foijer 2012). Even though those models mimic chromosome missegregation and its consequences, overexpression of CIN-modulating genes is more common [e.g., Mad2 overexpression, which is seen in many tumours (Hernando et al. 2004; Sotillo et al. 2007)]. Possibly, mimicking the CIN-provoking mutations that are found in human cancers would result in a physiologically more relevant CIN level, thus adding to our understanding of CIN and its role in tumorigenesis. A lot can be learned about affected pathways from in vitro studies as well. For instance, Donnelly and colleagues have shown that increased HSF1 activity can play a facilitating

role in coping with aneuploidy-induced proteotoxic stress by regulating the gene expression of various heat shock proteins (Donnelly et al. 2014)

11 Questions that Need to Be Addressed

11.1 Which Mutations Make an Aneuploid Cell an Aneuploid Cancer Cell?

Some tumour suppressor genes, (e.g., p53) were found to accelerate the malignant transformation of aneuploid cells, but the mechanism behind this collaboration remains unclear. As CIN alone is a poor initiator of cancer, pathways that convert aneuploid cells in aneuploid cancer cells make up important therapeutic targets. So far, CIN-collaborating genes were picked in an 'educated guess' approach. However, to identify in an unbiased fashion the pathways that convert CIN cells into CIN cancer cells, (in vivo) genetic screens are required.

11.2 At What Rate Is CIN Tumorigenic and at What Levels Tumour Suppressive?

The effects of CIN across the various mouse models are diverse, but it is unclear why. It is inevitable that the levels of CIN are different among the various CIN models, but there is no clear correlation between the levels of aneuploidy and the resulting phenotype based on the available data. However, as the level of CIN might determine whether tumours are promoted or are suppressed (Silk et al. 2013), high resolution quantification of CIN will be crucial when targeted in therapy. Furthermore, even though aneuploidy is a hallmark of cancer, the actual rates of chromosome missegregation (i.e., the CIN rates) in human cancer are unknown. To quantify these, primary (tumour) cells need to be fully karyotyped at the single cell level at various stages. So far, most studies have relied on metaphase-spread based (spectral) karyotyping using dividing cell populations, such as primary MEFs or tumour cell lines. However, this technique cannot be applied to most primary tumour cells as they do not divide frequently (Mitchison 2012; McGranahan et al. 2012). A new, but costly approach to quantify karyotypes of single cells is next-generation sequencing (NGS) (Knouse et al. 2014; Bakker et al. 2015). However, to quantify aneuploidy, full coverage (or even multiple coverage) per cell is not a requirement. 1–2 % coverage per cell will be sufficient to quantify chromosome numbers for an individual cell, allowing sequencing libraries of many cells to be pooled in each sequencing lane. Single cell karyotyping will allow us to faithfully measure in vivo missegregation rates (i.e. CIN) by assessing subtle karyotype differences between cells within one tumour (karyotype heterogeneity) (Bakker et al. 2015). Such technology will allow us to determine at which rate CIN is tumorigenic or tumour suppressive in mouse models and what the CIN rates are in human primary tumours.

11.3 What Determines the Tissue Specific Response to CIN?

There is a marked difference as to how cell lineages respond to CIN. For instance, CIN is highly toxic to embryonic stem cells (Burds et al. 2005), but quite well tolerated by interfollicular epidermal cells (Foijer et al. 2013), hepatocytes and possibly neurons (Rehen et al. 2001, 2005; Kingsbury et al. 2005; Duncan et al. 2012a). As of yet, it remains unclear what determines this differential response. Possibly, some cell lineages such as stem cells, induce a stronger stress response upon aneuploidy, resulting in apoptosis or differentiation. Alternatively, aneuploidy-tolerating cells spend more time in pro-metaphase and therefore have more time to correct improper kinetochore-microtubule attachments, thus reducing the missegregation rates and therefore reducing aneuploidy to tolerable levels. Indeed some cell types tolerate at least some aneuploidy including neurons and hepatocytes. However, further in vivo experiments are required to assess which molecular pathways make up the response to aneuploidy at the tissue level and how the differential wiring of these pathways in different cell lineages determines the fate of aneuploid cells.

11.4 What Are the Molecular Mechanisms that Explain the Link Between CIN and Ageing?

Some of the CIN mouse models exhibit a premature ageing phenotype, most clearly knockout models of Bub family proteins (BubR1, Bub3/Rae1) (Baker et al. 2004, 2006b). Conversely, BubR1 transgenic mice show increased lifespan, clearly implicating BubR1 with ageing (Baker et al. 2013a). This data, together with the observation that BubR1 expression decreases with ageing in wild type animals, (Baker et al. 2004, 2006b), suggest that CIN may play a role in natural ageing. Why were phenotypes only described for Bub protein members? Possibly, (subtle) signs of premature ageing were overlooked in other CIN models, as these models were developed specifically to study the relationship between CIN and cancer and not ageing, (Ricke and van Deursen 2013). Indeed, a more detailed analysis of transcriptomes of Mad2^{null} epidermal cells suggests an ageing-like response in murine skin following SAC abrogation (Foijer et al. 2013), suggesting that CIN indeed provokes a premature ageing response in untransformed tissue. However, more detailed and high resolution mapping of CIN in ageing human tissues is required to confirm physiological relevance for a potential link between CIN and ageing. When this link is confirmed, the underlying molecular mechanisms that link CIN and ageing should be elucidated, employing exciting and possibly new, more human relevant CIN mouse models.

11.5 What Is the Potential of CIN-Targeting Therapy?

Aneuploidy is a hallmark of cancer and selectively killing aneuploid cells would therefore be a powerful means to treat cancer. The various mouse models for CIN have revealed that there are three possible outcomes for aneuploid cell progeny depending on the tissue affected: (1) cell death (e.g., in case of hair follicle stem cells), (2) cellular senescence (evidenced by premature ageing and upregulation of the senescence marker $p16^{Ink4a}$) and (3) tolerance of aneuploidy (Fig. 2). The latter outcome is the most dangerous, as proliferating aneuploid cells can further evolve into aneuploid cancer cells. Therefore, to target aneuploid cancer, those cells that tolerate aneuploidy will need to be forced to either commit suicide or senesce. There are multiple ways as to how such therapy could work, ranging from very broad spectrum to highly 'personalized' therapies. As discussed above, too much CIN is detrimental to cells (Silk et al. 2013). Therefore, further increasing CIN in aneuploid tumours could be a broad-spectrum way to target aneuploid cancer cells. Indeed, mild CIN renders cells more sensitive to therapeutics that exacerbate CIN such as low doses taxol (Janssen et al. 2009). However, the inherent risk to this therapy is

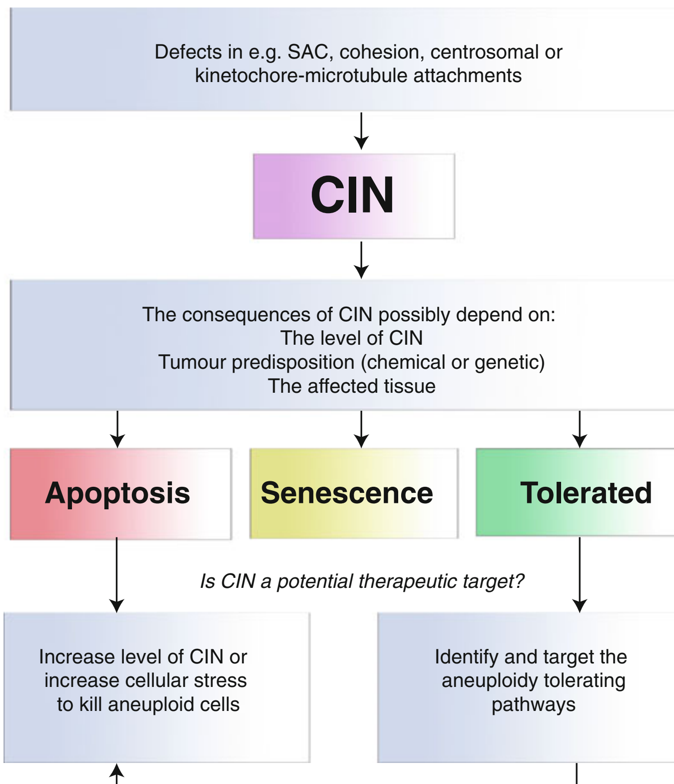

Fig. 2 Flowchart summarizing the in vivo consequences of CIN and therapeutic promise

that untransformed (non-CIN) cells will also be exposed to CIN and might convert into a new CIN tumour over time. A second approach of targeting CIN cells is by modulating the pathways that regulate cell fate following aneuploidization. In this approach, the pathways that result in cell death of (embryonic) stem cells following CIN are artificially activated in aneuploid cancer cells, resulting in cancer cell death. However, before feasibility of such therapy can be assessed, CIN-responsive pathways need to be mapped first. Instead of targeting aneuploidy-signalling pathways, therapy can also target the downstream consequences of CIN. For instance, one common response to aneuploidy is a deregulation of cellular metabolism, which affects untransformed cells as well as cancer cells (Williams et al. 2008; Torres et al. 2008; Foijer et al. 2014). The first proof of principle evidence for such therapy is just emerging. Recent studies are showing that energy stress inducer AICAR and the Hsp190 inhibitor 17-AAG selectively can kill aneuploid (cancer) cells by enhancing aneuploidy-induced stress (Tang et al. 2011; Ly et al. 2013; García Martínez et al. 2014). The next step to this will be to test whether this is also effective in vivo. A fourth ‘personalised medicine’ approach to tackle aneuploid cancer is by targeting the mutation that is driving CIN. One candidate for such therapy is Hec1, as it is frequently overexpressed in a variety of cancers. Indeed, inhibition of the Hec1/Nek2 pathway results in reduced tumour growth in a xenograft mouse model (Wu et al. 2008), providing proof of principle evidence for this approach. Similarly, gene products that collaborate with CIN in transformation can be targeted using molecular therapy. For the latter, we first need to identify candidate targets, for instance in in vivo genetic screens. However, for molecular therapy full sequencing of the tumour is a requirement. However, as sequencing costs are rapidly decreasing and the number of specific pathway inhibitors are rapidly increasing, this approach might become feasible within the near future.

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Telomere Dysfunction, Chromosomal Instability and Cancer

Jitendra Meena, K. Lenhard Rudolph and Cagatay Günes

Abstract

Telomeres form protective caps at the ends of linear chromosomes to prevent nucleolytic degradation, end-to-end fusion, irregular recombination, and chromosomal instability. Telomeres are composed of repetitive DNA sequences (TTAGGG)n in humans, that are bound by specialized telomere binding proteins. Telomeres lose capping function in response to telomere shortening, which occurs during each division of cells that lack telomerase activity—the enzyme that can synthesize telomeres de novo. Telomeres have a dual role in cancer: telomere shortening can lead to induction of chromosomal instability and to the initiation of tumors, however, initiated tumors need to reactivate telomerase in order to stabilize chromosomes and to gain immortal growth capacity. In this review, we summarize current knowledge on the role of telomeres in the maintenance of chromosomal stability and carcinogenesis.

Keywords

Telomerase · Genome stability · Senescence

Cagatay Günes and Karl Lenhard Rudolph equally contributed to this work.

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1 Telomeres and Telomerase

1.1 Telomeres Are Protective Caps at the Ends of Linear Chromosomes

Cells with linear chromosomes have to meet several challenges: (i) cellular repair mechanism have to discriminate between broken ends as a result of DNA damage and the ends of the chromosomes, (ii) the ends of chromosomes must be protected against degradation by nucleases, and (iii) as the conventional DNA polymerase cannot replicate the most extreme ends of chromosomes (end-replication-problem), loss of genetic material must be compensated by some end maintenance mechanism. Work by Hermann Muller and Barbara McClintock provided the first evidence that the ends of linear chromosomes must be capped by a specialized structure to prevent chromosome fusions (McClintock 1939, 1941; Muller 1938). Müller introduced the term 'telomere' to emphasize this specific function of chromosome ends. A telomere is functionally defined as a region of DNA at the molecular end of a linear chromosome that is required for replication and stability of the chromosome (Blackburn and Szostak 1984). It has become clear now that the telomeres are composed of short repetitive DNA-sequences and specific proteins, the shelterin, that bind this sequence to protect the ends of linear chromosomes (de Lange 2010).

Telomeric DNA consists of a tandem array of GT-rich repeats (e.g. TTGGGG in Tetrahymena and TTAGGG in humans and other vertebrates). The number of the repeats and consequently the length of telomeric DNA varies among species (Fig. 1) ranging from 36 nucleotides present at the ends of macronuclear chromosomes of ciliated protozoans (Klobutcher et al. 1981), ~300 bp in *Saccharomyces cerevisiae* (Zakian 1989) to ~150,000 bp in mice (Kipling and Cooke 1990). In human somatic cells telomeres consist of 7000–10,000 bp telomeric DNA and of about 20,000 bp in germ cells (Allshire et al. 1989; Moyzis et al. 1988).

One important feature of telomeres, which is conserved in all eukaryotes, is that they posses a protruding 3′ single-stranded overhang due to the mechanism of the lagging strand DNA replication (Makarov et al. 1997; McElligott and Wellinger 1997).

Group	Organism	Telomeric repeat
Vertebrates	Human, mouse, Xenopus	TTAGGG
Filamentous fungi	Neurospora	TTAGGG
Kinetoplastid protozoa	Trypanosoma, Crithidia	TTAGGG
Higher plants	Arabidopsis	TTTAGGG (300-700 repaets)
Ciliated protozoa	Tetrahymena, Oxytricha	TTGGGG or TTTTGGGG
Fission yeasts	Schizosaccharomyces pombe	TTAC(A)(C)G(1-8)
Budding yeast	Saccharomyces cerevisiae	G(2-3)(TG)(1-6)T (consensus)

Fig. 1 Telomere-DNA is an evolutionarily conserved GT-rich repetitive sequence. Basically all eukaryotes use GT-rich repetitive sequences at the ends of their chromosomes (telomeres). Despite considerable variation in telomere length and telomere-sequence, telomerase activity is the major telomere maintenance mechanism among the eukaryotes, with only few exceptions (*Drosophila melanogaster*). Modified from Meyne et al. (1989)

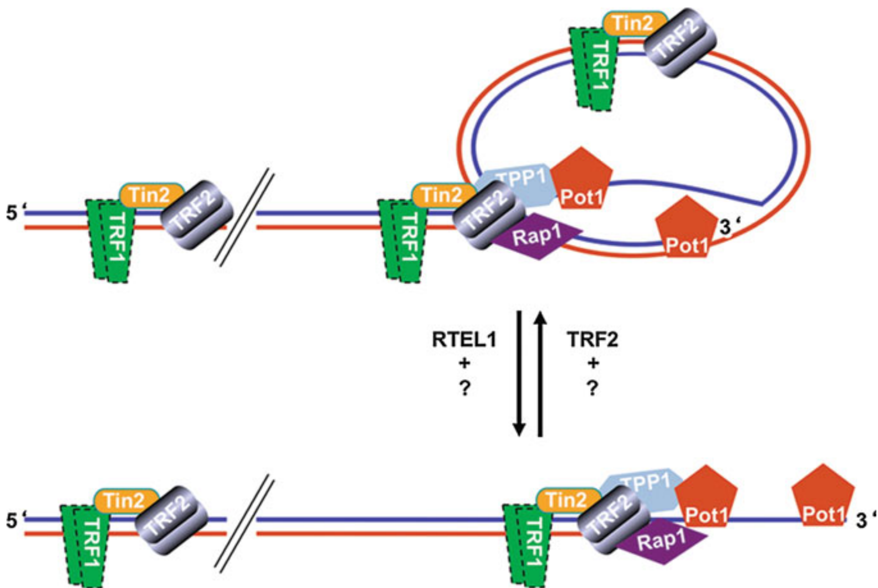

Fig. 2 Telomeres, Protein-DNA complexes at the ends of linear chromosomes, may form a lariat structure, the telomere-loop (T-loop). A schematic representation of the D-loop, T-loop structure at the chromosome ends. Lagging strand blue, leading strand red. The 3′-protruding end of the lagging strand may invade into the double stranded telomeric DNA and result in the displacement of the double strand (displacement-loop: D-loop). A large lariat structure can be observed at the telomeres (telomere-loop: T-loop). Several proteins have been localized to the telomeres. The number, temporal and spatial localization of these proteins at the telomeres is not completely understood. There is evidence that T-loop formation is facilitated by TRF2 (Doksani et al. 2013; Griffith et al. 1999) whereas RTEL1 helicase activity is required for the faithful T-loop resolution during replication (Vannier et al. 2012)

In mammalian cells, this single stranded overhang may fold back and invade into the preceding double stranded telomeric DNA to form a unique D-loop and T-loop structure (Griffith et al. 1999). Telomere looping may be a bona fide end protecting mechanism since it has been observed in mammals, plants and several lower eukaryotes (Cesare et al. 2003; Griffith et al. 1999; Munoz-Jordan et al. 2001; Murti and Prescott 1999). This special structure functions to seal the ends of chromosomes thus protecting them from hazardous cellular actions. In its absence, the 3′-overhang is simply occupied with specific telomere-binding proteins protecting the chromosome ends from DNA-damage.

To date a series of proteins have been described to be associated with telomeres (Fig. 2). In human cells, six proteins, TRF1, TRF2, TIN2, TPP1, RAP1 and POT1, form the shelterin complex and interact with several other proteins for telomere length regulation (de Lange 2005). Among the latter, proteins involved in DNA double-strand break repair (Ku-proteins) and non-homologous-end-joining (RAD50-NBS1-Mre11 complex) are found. It is not yet clear whether these proteins are present at the telomeres at all times or in a cell-cycle dependent manner. Among these proteins TRF1 and TRF2 form a platform for the binding and function of other telomere specific factors (Fig. 2).

1.2 Telomerase

Telomerase is a ribonucleo-protein complex with reverse transcriptase activity with conserved sequence homology to non-LTR and LTR reverse transcriptases (Shippen-Lentz and Blackburn 1990). The activity of telomerase is necessary to overcome the ‘end replication problem’. The human telomerase enzyme is composed of two essential components, the RNA component (TERC: Telomerase RNA) which acts as a template for reverse transcription (Blasco et al. 1995); and the catalytic subunit Telomerase reverse transcriptase (TERT) with the reverse transcriptase activity (Meyerson et al. 1997; Nakamura et al. 1997). In recent years, a number of additional factors, including dyskerin, TCAB1, NOP10 and TPP1 have been identified to be constantly or transiently associated with the telomerase complex and have important functions in telomerase recruitment to telomeres or subcellular localization of the telomerase complex (Cohen et al. 2007; Collins and Mitchell 2002; Nandakumar and Cech 2013; Venteicher et al. 2009; Zhong et al. 2011; Gonzalez et al. 2014).

Telomerase is active in a variety of tumor cell lines and transformed cells in culture but not in normal fibroblasts (Morin 1989) or embryonic kidney cells (Counter et al. 1992) and most somatic human tissues do not exhibit telomerase activity (Djojosubroto et al. 2003; Kim et al. 1994; Meyerson et al. 1997; Shay and Wright 1996; Weise and Gunes 2006). In human, telomerase activity is down-regulated during embryogenesis and cellular differentiation through repression of its catalytic subunit (Gunes et al.2000; Wright et al. 1996; Sirma et al. 2011). Due to

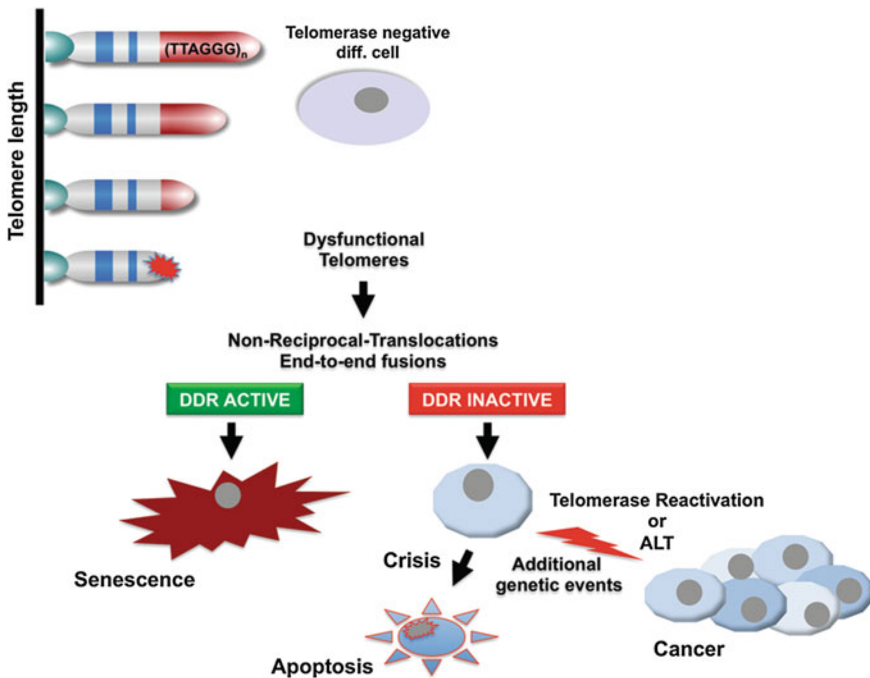

Fig. 3 Telomere hypothesis of senescence and cancer. Proliferation-dependent telomere shortening leads to telomere dysfunction, manifested by non-reciprocal-translocations and end-to-end fusions, resulting in the activation of DNA-damage checkpoints, and induction of senescence in telomerase negative, check-point proficient human cells. Checkpoint-deficient cells continue to proliferate experiencing further telomere shortening and eventually end up in crisis, characterized by apoptotic cell death, in the absence of a telomere maintenance mechanism. Activation of telomerase (or the ALT mechanism) is one of the key events to overcome crisis during tumourigenesis to stabilize telomere length and for the continuous proliferation of malignant cells

the lack of telomerase, telomeres shorten during aging in human tissues in vivo and telomere length sets a limit to the proliferative capacity of human fibroblasts (HFs) in vitro involving the p53 and Rb pathways (Chang and Harley 1995; Harley et al. 1990; Shay et al. 1991). In this line, cells devoid of these two major pathways exhibit extended life-span but telomeres continue to shorten until a ‘crisis’ checkpoint. Cells that survive the crisis checkpoint possess telomerase activity or activate an alternative mechanism of telomere maintenance (ALT) (Counter et al. 1992). Based on these observations, Allsopp et al. (1992) proposed a model for of telomere hypothesis of ‘cell ageing and immortalization’ (Fig. 3).

These observations together with the findings that telomerase activity can be detected in early human development but is absent in most normal somatic cells have led to the hypothesis that the down-regulation of telomerase activity in somatic cells may be a tumor-protective mechanism. In line with this hypothesis it

was shown that telomerase is required for tumorigenic conversion of primary human cells (Hahn et al. 1999a). In adult human tissues some cell types maintain weak but detectable telomerase activity or telomerase activity may be induced upon stimulation. These include bone marrow stem cells, germline cells in testes, activated peripheral blood lymphocytes, skin epidermis and intestinal crypt cells (Chiu et al. 1996; Hiyama et al. 1995, 1996; Morrison et al. 1996; Ramirez et al. 1997; Ravindranath et al. 1997; Ritz et al. 2005; Weise and Gunes 2009).

Although telomerase activity could be detected in the vast majority of human cancers, it is worth mentioning that about 10–15 % of human tumors do not express detectable levels of telomerase activity. Tumors that lack telomerase activity, maintain their telomere length via a recombination-based mechanism (ALT for Alternative Lengthening of Telomeres) (Bryan et al. 1997). Experimental data indicate that telomere maintenance is required for continuous tumor cell proliferation and tumor progression (Greenberg et al. 1999; Hahn et al. 1999b; Rudolph et al. 2001). The prominent occurrence of telomerase in human cancers and data from mouse models on its requirement for tumor progression motivated the development of telomerase inhibitors to suppress tumor growth in pre-clinical studies (Damm et al. 2001; Dikmen et al. 2005; Djojosubroto et al. 2005; Herbert et al. 2002; Kumar et al. 2013; Norton et al. 1996; Zahler et al. 1991). One of these inhibitors, a lipid-conjugated 13-mer oligonucleotide that is complementary to the RNA template of telomerase, thereby directly inhibiting telomerase activity is a promising candidate and has evaluated safety, tolerability and pharmacokinetics in Phase I clinical trials. This inhibitor, Imetelstat, was developed by Geron Inc. and is now being tested to treat Hematologic Myeloid Malignancies in Phase II clinical trials. As a potential drawback, experimental studies on mouse models showed that deletion of telomerase in tumors provokes the activation of ALT as an adaptive response in cancer cells (Hu et al. 2012). It is therefore essential to explore and understand the factors that control the ALT pathway.

2 Telomere Shortening Impairs Proliferation of Transformed Cells but Dysfunctional Telomeres Can Initiate Cancer Formation

The role of telomeres in human biology was unclear until the discovery of telomerase and subsequent demonstration that telomeres shorten during aging due to the end-replication problem (Greider and Blackburn 1985; Harley et al. 1990; Hastie et al. 1990). As discussed above, telomere shortening limits the proliferation capacity of human cells, referred to as 'Hayflick Limit'. At this stage, cells exhibit a 'cellular senescence' phenotype characterized by morphological changes and by the accumulation of aneuploidy, polyploidy and chromosomal fusions (Benn 1976; Saksela and Moorhead 1963; Thompson and Holliday 1975). Telomerase negative human cells that can overcome the senescence checkpoint by the expression of viral oncoproteins continue to accumulate chromosomal instability during the extended

proliferation period (Counter et al. 1992). These observations indicated a pivotal role of functional telomeres in genome stability and telomerase activity thwarted telomere shortening and genomic instability (Harley 1991).

Dysfunctional telomeres can result either from alterations in the telomere-associated proteins required for end-capping function, or from alterations that promote the gradual or sudden loss of sufficient repeat sequence necessary to maintain proper telomere structure. The identification of mammalian telomerase components in the mid 90s enabled to experimentally address the functional role of telomere shortening in aging and cancer formation in vivo (Blasco et al. 1997; Rudolph et al. 1999).

Telomerase knockout mice exhibit progressive shortening of telomeres resulting in loss of telomere capping function (also referred to as telomere dysfunction) in 3rd–6th generation of knockout mice. In vivo studies supported the observations from HFs that dysfunctional telomeres are recognized by the DNA-damage-response (DDR) machinery leading to activation of p53 and Rb dependent checkpoints inhibiting tumorigenesis in cancer mouse models (Chin et al. 1999; Greenberg et al. 1999). A formal experimental prove of telomere-dysfunction induced tumor suppression in vivo was provided by studies where overexpression of *c-Myc* oncogene in mice with short telomeres induced genomic instability as determined by increased end-to-end fusions, non-reciprocal translocations and anaphase bridges. These genomic instability induced senescence in the presence of wild-type p53 (Feldser and Greider 2007). In fact, the tumor suppressor function was dependent on the senescence-activation function of p53 (Cosme-Blanco et al. 2007). During aging or in the absence of functional checkpoints, however, (i.e., loss of p53) or by the co-expression of oncogenic mutations, telomere dysfunction promotes genomic instability and initiates tumorigenesis (Artandi et al. 2000; Chin et al. 1999; Rudolph et al. 1999, 2001). The studies with telomerase deficient mice also underpinned the need for telomere stability—either by activating telomerase or by the ALT mechanism—for continuous tumor cell proliferation in vivo (Begus-Nahrmann et al. 2012; Ding et al. 2012; Greenberg et al. 1999; Jaskelioff et al. 2009; Rudolph et al. 2001).

In human, telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities and therefore is a cancer predisposition factor (Gisselsson et al. 2001; Wu et al. 2003). The cellular basis of telomere dysfunction induced genomic instability is explained by chromosomal breakage-fusion-bridge (BFB) cycles (McClintock 1939, 1941). Persistent or transient telomere dysfunction in telomerase knockout mice can result in increased mutation rates and induce BFB-cycles resulting in gains and losses of chromosomes (Blasco et al. 1997; Hackett et al. 2001; Lee et al. 1998; Rudolph et al. 2001). Although BFB-cycles seem to be the major physiological outcome of dysfunctional telomeres, persistent telomere dysfunction can induce genomic instability via cytokinesis failure and tetraploidy (Davoli et al. 2010; Pampalona et al. 2012).

Progressive telomere shortening may also result from mutations in shelterin proteins and telomerase have been shown to be associated with human pathologies. Mutations in telomerase components (*TERT*, *TERC*, *DKC1*) telomerase associated

factors (*NOP10*, *NHP2*, *WRAP53*) or the shelterin components (*TRF1*, *TRF2*, *POT1*) forms a bigger portion of several human diseases, like dyskeratosis congenita, aplastic anemia, pulmonary fibrosis, malignant melanoma and late stage liver cirrhosis (Hartmann et al. 2011; Savage et al. 2008; Shi et al. 2014; Vulliamy et al. 2001, 2004, 2005; Walne et al. 2007, 2008; Yamaguchi et al. 2005, 2010; Zhong et al. 2011). Mutations in telomerase components result in reduced telomerase activity and accelerated telomere shortening and thus accelerated stem cell exhaustion with age, accompanied by an increased frequency of chromosomal breaks and chromosomal aberrations and increased risk for cancer formation (Calado et al. 2012).

Together, both, mice and human studies indicate that telomere dysfunction induced genetic instability occurs through persistent bridge-breakage events, leading to a continuous reorganization of the tumor genome. These findings also show that senescence and apoptosis induced by telomere dysfunction and p53 activation contribute to tumor suppression.

3 Activation of Checkpoints as a Consequence of Telomere Dysfunction

Due to their structure and shielding by shelterin components telomeres are protected from irregular repair activities. Studies on shelterin components have identified at least six different DNA damage repair pathways that protect telomeres from irregular recombination events (Martinez et al. 2012; Sfeir and de Lange 2012). The choice of the repair pathway is dependent on the type of DNA-damage and the cell type and dictates the cellular consequences in response to telomere dysfunction. Mammalian DSBs are repaired primarily by homologous recombination (HR) or non homologous end joining (NHEJ). Gene knockout studies have revealed that loss of the shelterin components TRF1 and TRF2 activates ATM/ATR signaling for NHEJ whereas dysfunctional telomeres due to loss of POT1 trigger ATR-signaling or the activation of homologous DNA repair. Activation of the classical (c-NHEJ) or alternative (alt-NHEJ) non homologous end-joining repair pathways involving MRN complex (MRE11, NBS and Rad50), DNA-PK and Lig4 (c-NHEJ) or Lig3 or CtIP (alt-NHEJ) (Rai et al. 2010) initiate end-to-end fusions but repair activities at dysfunctional telomeres leads to chromosomal fusions, which are not stable during the cell cycle and can be a source of genetic instability (d'Adda di Fagagna et al. 2004; Takai et al. 2003). Upstream protein kinases such as ataxia telangiectasia mutated (ATM) and ATR as well as the downstream protein kinases CHK1 and CHK2 are also involved in the 5′-end-resection at dysfunctional telomeres causing a G1 cell cycle arrest or the senescence response by activating the tumour suppressor p53 pathway. In the absence of p53BP, a target of the ATM kinase that accumulates at the sides of DNA damage and suppresses end-resection, the classical NHEJ pathway is inhibited and may direct the repair mechanism towards the homologous repair, resulting in increased recombination at dysfunctional telomeres, a phenotype

observed in telomerase negative, ALT-positive tumor cells (Dimitrova et al. 2008; Martinez et al. 2012).

Whether the same pathways are activated as a consequence of physiological telomere shortening remains to be shown but some data exist indicating that the alt-NHEJ is the major pathway to repair DNA damage at naturally occurring dysfunctional telomeres (Rai et al. 2010). The p16/INK4a-Rb pathway has been implemented to contribute to the detection of telomere-induced DNA damage, activating the senescence pathway and recent data show that p16/INK4a protects cells against dysfunctional telomere–induced ATR-dependent DDR in Pot1b deficient mice but the contribution of p16 remains still elusive yet (Shay et al. 1991; Wang et al. 2013). Elucidating the DDR pathways in response to physiological telomere dysfunction would be crucial to better understand the role of genomic instability to tumorigenesis during aging.

4 Telomere-Dysfunction and Induction of Senescence as a Tumor Suppressor Mechanism

As discussed above, telomere shortening is regarded as the main cause of telomere dysfunction leading to induction of replicative senescence in aging cells. There is now emerging evidence that the accumulation of telomeric DNA damage in response to DNA replication stress can also contribute to induction of senescence. The induction of this checkpoint involves abrupt induction of replication stress at telomeres, which appears to be independent of classical telomere shortening (Fig. 4).

Dysfunctional telomeres can be detected by the accumulation of telomere dysfunction-induced foci (TIF) at the telomeres (d'Adda di Fagagna et al. 2003; Takai et al. 2003). These foci include 53BP1 and phosphorylated H2AX (gamma-H2AX) at the dysfunctional telomeres. Interestingly, recent observations show the accumulation of persistent TIFs upon oncogene-induced senescence (OIS) or stress-induced senescence (Fumagalli et al. 2012; Hewitt et al. 2012; Suram et al. 2012). We recently showed that aneuploidy-induced senescence (AIS) involves replication stress and TIF formation at telomeres indicating that telomeres seem to mediate (AIS) (Meena et al. 2015). These new findings may provide a unifying mechanism for senescence as a general tumor suppressor mechanism whereby telomeres may converge different kinds of cellular stress in one pathway (Reviewed in Gunes and Rudolph 2012, 2013).

The biological basis for this function of telomeres as a sensor of replication defects may be due to their specific sequence composition and structure. Telomeres can form G-quadruplex structures (G4) by intra-molecular Hoogsteen G-G base pairs. G4 structures increase in a cell cycle dependent manner in human cells (Biffi et al. 2013) and preferentially form at the 3′-end of chromosomes (Tang et al. 2008), are highly stable. G4 structures are thought difficult to resolve during replication and may provoke replication fork stalling and chromosome fragility (Tarsounas and Tijsterman 2013). Fragile sites are particularly prone to

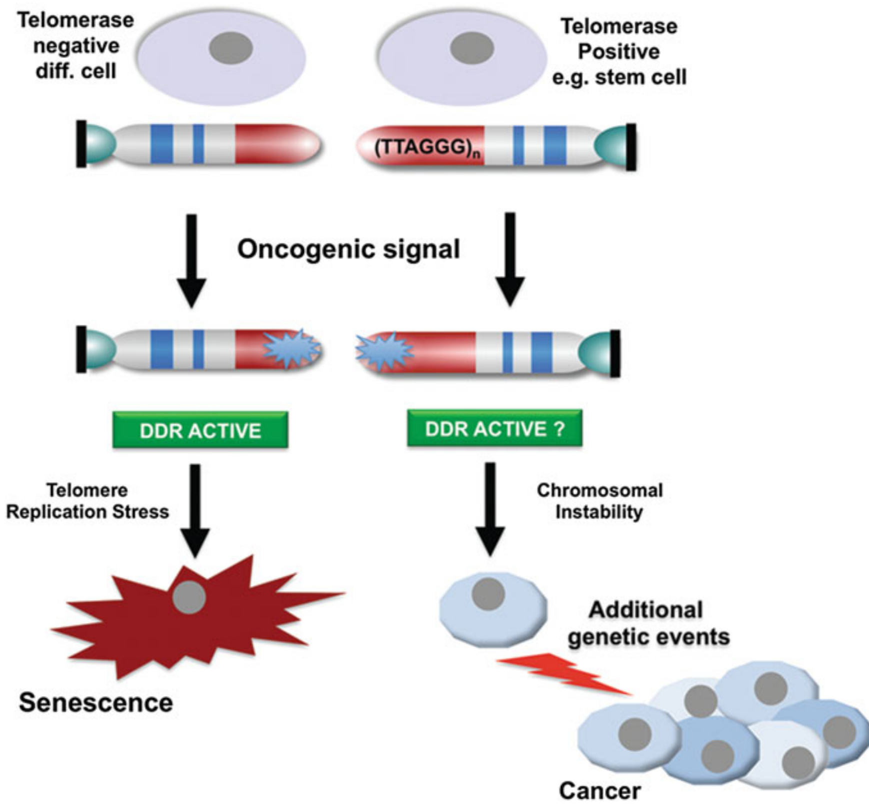

Fig. 4 Telomerase activity alleviates telomere replication stress and facilitates to overcome oncogene-induced senescence. Oncogene activation leads to abrupt accumulation of DNA damage at telomeres resulting in senescence and tumour suppression. Telomerase-positive stem cells could be resistant to oncogene-induced senescence and may be selected as the cell type of origin of tumour development

chromosomal breakage and recombination events as a result of replication stress (O'Keefe and Richards 2006). Replication stress can be induced by inappropriate proliferation signaling such as oncogene activation or loss of cell cycle inhibitors that deregulate transcription and generate DNA damage (Bermejo et al. 2012; Di Micco et al. 2006). Telomeres are difficult to replicate and may lead to fork stalling during replication upon inflated proliferation signals (Suram et al. 2012). Consistently, replication stress at telomeres and thus inefficient replication of telomeric DNA could attract DDR and induce the senescence checkpoints as a tumor suppressor mechanism. In cells defective in functional repair mechanisms or faithful telomere replication, however, dysfunctional telomeres can initiate genome instability.

5 TRF1 and Telomerase in the Context of Telomere Replication Stress

There is emerging experimental evidence that replication through difficult replicating sites requires coordinated action of telomerase activity, telomere binding proteins and specific helicases that are recruited to the telomeres for faithful replication. TRF1 plays a key role in this context. Loss of mammalian TRF1 or its fission yeast counterpart Taz1 leads to stalled replication forks and fragile telomere phenotype (Martinez et al. 2009; Miller et al. 2006; Sfeir et al. 2009). Importantly, MEFs from TRF1 deficient mice exhibited a premature senescence phenotype compared to their wild type counterparts; in the absence of cellular checkpoints, i.e., in cells expressing SV40-LT, the senescence phenotype was rescued but led to increased chromosomal instability (Martinez et al. 2009). At organismal level, mice lacking TRF1 in the stratified epithelia (TRF1flox/flox × K5-Cre transgenic bitransgenic mice) showed dysfunctional telomeres associated with skin hyperpigmentation and epithelial dysplasia but died perinatally. When these mice were crossed with p53 null mice, they could survive but exhibited an increase in squamous cell carcinoma. Together, these studies indicate that telomere replication is facilitated by the shelterin factor TRF1 to prevent replication fork stalling and that telomeric replication stress generates fragile telomeres that can instigate genomic instability and cancer.

Interestingly, BLM helicase, which is also able to bind and resolve G4 structures, interacts with TRF1 and is recruited to telomeres during replication in late S/G2 and cells lacking BLM accumulate dysfunctional telomeres and telomere-dependent chromosome fusions (Barefield and Karlseder 2012). RTEL1 is another helicase that facilitates faithful telomere replication, potentially by resolving the G-quadruplex structures at the T-loop (Vannier et al. 2012, 2013). Other helicases with G4 resolving activity include the recQ helicases WRN, RECQL4 and DNA2. DNA2 deficiency results in defective telomere replication, leading to elevated fragile telomeres, telomeres loss, and telomere DNA damage response (Lin et al. 2013). In the same line, it has recently been demonstrated that the activity of the Pif1 helicase, that can associate with telomerase, is required to open telomeric G4 structures and that the enzymatic activity of telomerase is crucial for this function indicating that the damage present at telomeres is repaired by telomerase (Chang et al. 2009; Mateyak and Zakian 2006; Paeschke et al. 2011). It remains speculative whether Pif1 activity precedes and facilitates telomere replication or it is required to resolve structures generated during replication. Studies in the ciliate *Stylonychia lemnae* indicate that telomerase recruitment by the telomere binding protein-ß, the homologue of the mammalian shelterin protein TPP1, facilitates unfolding G4-structures. However, the exact mechanisms how these helicases act to resolve telomeric G4 and their differential functions remain elusive.

Recent studies indicate that BRCA2 and RAD51 act in concert to heal fragile telomeres in mouse cells, probably by enabling the restart of replication at stalled replication forks that are processed by HR during the S-phase (Badie et al. 2010).

BRCA2 recruits RAD51 to the telomeres during replication in S-phase and both factors are required for maintenance of telomere length in mouse embryonic fibroblasts (MEFs). Consistently, MEFs lacking BRCA2 or RAD51 exhibited an increased fragility, telomere shortening and telomere dysfunction induced DNA damage foci (TIF) indicative of loss of telomere protection. Interestingly, telomerase positive cells showed higher fragility in the context of BRCA2 mice when compared to telomerase negative cells with shorter telomeres from late generation telomerase knockout cells. This result indicates that longer telomeres have a greater chance to accumulate fragile telomeres in the absence of repair mechanisms and in the presence of telomerase. In conclusion, the adult stem cells, the main cell type that retains telomerase activity in adult human tissues may represent the cell type of origin of cancer formation (Fig. 4).

Together, telomeres have a dual role in cancer formation. Telomere shortening and telomere replication stress in malignant cell clones serve as a tumor suppressor mechanism by activating senescence and or crisis checkpoints. In contrast, telomere shortening in aging tissues can also lead to an induction of chromosomal instability by promoting chromosomal fusion and fusion-bridge-breakage cycles. In addition, the inhibition of cell proliferation in aging tissues can also increase the selective pressure for clonal outgrowth of (pre-) malignant cell clones by changing the tissue environment and by impairing proliferative competition of non-transformed cells (Bilousova et al. 2005; Braig et al. 2014; Ju and Rudolph 2006). The influence of telomeres on tumor protection/tumor promotion may depend on the lifetime. Early in life when telomeres are long cancer protective effects of telomere shortening/ replication stress in malignant cell clones may be dominant. In contrast, tumor-promoting effects of telomere shortening may become dominant in aged tissue and tissues experiencing telomere shortening in response to chronic diseases such as liver cirrhosis in response to hepatitis or progressive stages of ulcerative colitis (Rabinovitch et al. 1999; Rudolph et al. 2009). It remains to be investigated whether targeting of senescence checkpoints in response to telomere shortening or telomere replication stress could lead to development of novel anti-cancer therapies and how these approaches affect tissue aging. Studies in mouse models indicate that it is possible to improve tissue maintenance without increasing cancer risk by inhibiting downstream checkpoint responses (Cdkn1a/p21) that limit proliferation of cells in response to telomere shortening (Choudhury et al. 2007). In addition, it was shown that p21 deletion can have anti-tumor effects in mouse models of leukemia or irradiated human tumor cells (Lazzarini et al. 2008; Viale et al. 2009; Waldman et al. 1996). It is possible that the tumor inhibiting effects of p21 deletion involve the increase in telomere replication stress in genomically instable tumor cells. Together, these studies suggest that it should be possible to define molecular targets that can improve both tissue maintenance and cancer protection in aging tissues.

Important areas of future research include the delineation of (i) distinct cellular stress factors that cause telomere replication stress, (ii) molecular mechanisms that are involved in the induction of replication stress, (iii) activation of checkpoints in

response to replication stress at telomeres, and (iv) mechanism how telomerase contributes to the suppression of telomere replication stress.

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Genetic Instability and Disease Prognostication

Timo Gemoll, Gert Auer, Thomas Ried and Jens K. Habermann

Abstract

Genetic instability is a striking feature of human cancers, with an impact on the genesis, progression and prognosis. The clinical importance of genomic instability and aneuploidy is underscored by its association with poor patient outcome in multiple cancer types, including breast and colon cancer. Interestingly, there is growing evidence that prognostic gene expression signatures simply reflect the degree of genomic instability. Additionally, also the proteome is affected by aneuploidy and has therefore become a powerful tool to screen for new targets for therapy, diagnosis and prognostication. In this context, the chapter presents the impact of genomic instability on disease prognostication occurring in human cancers.

Keywords

Genetic instability · Prognosis · Genomics · Proteomics · Cancer

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1 Genomic Instability and Disease Prognostication in Colorectal Cancer

Colorectal cancer is globally the fourth leading cause of cancer mortality, with about 1.2 million new cases and 608,000 deaths worldwide per year (Jemal et al. 2011). The incidence of colorectal cancer is higher in developed countries although the disease is rarely diagnosed before the age of 40. Most patients with R0 resection of node-negative CRC are cured of their cancer by surgery, but an unacceptable number of patients experience relapse due to regional recurrence or to distant metastasis, or both. Today, Dukes classification of CRC and the Tumor-Node-Metastasis (TNM) classification system for solid tumors are the routine staging systems and the basis to evaluate patient prognosis in CRC. However, in terms of prognosis it has been shown that genomic instability plays an important role as do various demographical, pathological and molecular characteristics: next to age (Kearney et al. 1993; Cascinu et al. 1996), tumor stage (Sun 2006; Gerling et al. 2010), tumor location (Zarbo et al. 1997), histopathological grade (Schillaci et al. 1990), disease free survival and overall survival (Garrity et al. 2004; Chen et al. 2002), several studies have shown that patients with aneuploid tumors had a worse outcome compared to patients with euploid tumors (Witzig et al. 1991; Sinicrope et al. 2006; Bosari et al. 1992). Similarly, Gerling et al. (2010) presented survival data of CRC patients showing that advanced stage but diploid carcinomas had a similar prognosis as compared with early stage tumors, but the outlook for aneuploid carcinoma is typically unfavorable, indicating that aneuploidy in CRC more strongly impacts on prognosis than the tumor stage itself.

In one of the most comprehensive meta-analyses of 10,126 patients, Walther et al. (2008) demonstrated that genomic instability is associated with worse prognosis in CRC and that it could be used to stratify patient prognosis, in addition to pathological staging: CRC patients with aneuploid tumor cells—quantified by either flow cytometry (n = 9,526 patients) or image cytometry (n = 600 patients)—appeared to have a poorer survival irrespective of their ethnic background, anatomical location and treatment with 5-fluoroucacil (5-FU)-based adjuvant chemotherapy. In line with this, Guastadisegni et al. (2010) confirmed the association between MSI and favorable prognosis. Thirty-one eligible studies reporting survival for 12,782 patients characterized for MSI indicated that MSI has the potential to be used in the clinical setting as a prognostic and predictive marker. Being part of the

meta-analysis, the study by Sinicrope et al. (2006) found that DNA ploidy was the strongest prognostic marker.

Interestingly, the pattern of chromosomal aneuploidy in sporadic (SCC) and ulcerative (UCC) colitis-associated colorectal carcinomas seems to be strinkingly conserved. Nevertheless, in a single cohort of 31 UCCs and 257 SCCs Gerling et al. (2010) associated the frequency of aneuploidy to clinical parameters and showed that UCCs have a higher frequency of aneuploidy compared to SCCs (100 % versus 74.6 %; $p < 0.006$). A logistic regression analysis assessed age, sex, UICC stage, T- and N-status, histologic tumor grading, underlying inflammation, and DNA ploidy status. Out of these features, only age and DNA ploidy status were significant contributing parameters, indicating both patients of higher age at diagnosis and patients with aneuploid malignancy have a poor survival prognosis. Additional logistic regression analysis comprising these two significant parameters only confirmed age [odds ratio (OR), 1.05; 95 % CI, 1.02–1.09; $p = 0.003$] and DNA ploidy (OR, 4.07; 95 % CI, 1.46–11.36; $p = 0.007$) to be independent prognostic parameters. Among those, DNA aneuploidy with an OR of 4.07 seemed to be the strongest independent prognostic marker for R0-resected colorectal cancer patients overall. The dominance of aneuploidy as an independent poor prognostic predictor in patients with SCC and UCC was further supported by the fact that patients with diploid tumors at advanced stages (UICC stage III/IV) did present a survival comparable to that of patients with aneuploid tumors at early stages. The latter finding might even suggest that the presence of aneuploid tumor cell populations may influence the patient's prognosis more dominantly than tumor stage. This was in part supported by Laubert et al. (2013) who could demonstrate that aneuploidy and elevated CEA levels, apart from increasing T category, could predict metachronous metastases and thus assist individual risk assessment.

In this context, other authors report a comparable incidence of DNA aneuploidy in SCC. Interestingly, the high incidence of aneuploidy is not restricted to late-stage lesions but is found in more than 50 % of stage I CRC tumors. This was evaluated on the basis of single tumor samples and did not take into account that the intra-tumor heterogeneity could lead to an underestimation of the true occurrence of chromosomal aneuploidy and genomic instability, respectively (Flyger et al. 1999; Bondi et al. 2009).

The essential etiologic element of CRC is widely accepted to lie in genetic changes of epithelial cells in the colonic mucosa. Morphologic changes from normal mucosa and adenomatous polyps to cancer with accumulation of genetic aberrations are well documented (Fearon and Vogelstein 1990). However, individual colorectal adenomas and carcinomas have different propensities to progress to malignancy. In this context, genome, transcriptome and proteome analysis with respect to DNA ploidy data may yield aneuploidy-associated biomarkers that could assess the individual progression risk to malignancy. On the genome/transcriptome level, fluorescence in situ hybridization (FISH) with specific probe sets was used to screen a total of 47 samples [centromere probes for chromosomes 17 and 18 (CEP17 and CEP18), *SMAD7* (SMAD family member 7; 18q21.1), *EGFR* (epidermal growth factor; 7p12), *NCOA3* (nuclear receptor coactivator 3; 20q12), *TP53* (Tumor protein

53; 17p13.1), *MYC* (v-myc avian myelocytomatosis viral oncogene homolog; 8q24.21), and *RAB20* (member RAS oncogene family; 13q34)]. These samples reflected different stages during colorectal cancer development and included 18 adenomas of patients without synchronous or subsequent carcinoma, 23 adenomas of carcinoma patients, and 6 matched carcinomas (Habermann et al. 2011a). In summary, Habermann et al. concluded that genomic instability in colorectal adenomas is reflected by genomic amplification of the oncogenes *EGFR*, *MYC*, *NCOA3*, and *RAB20*. For *NCOA3* it could be shown that a diploid signal count of that gene is associated with a longer adenoma recurrence-free observation time ($p = 0.042$).

On the proteome level, a comprehensive proteomic analysis of diploid and aneuploid colorectal cancer cell lines and clinical tissues was carried out (Gemoll et al. 2011). Two proteins, HDAC2 (histone deacetylase 2) and TXNL1 (thioredoxin-like 1), were not only significantly expressed in two-dimensional gel electrophoresis (2-DE) analysis and validated by Western blotting, but showed expression differences also in clinical samples, discerning aneuploid from diploid CRCs (Fig. 1). It seems that HDAC2 is overexpressed in colorectal cancer and associated with reduced survival (Ashktorab et al. 2009; Weichert et al. 2008). Furthermore, HDAC2 overexpression could be induced by a loss of the anaphase-promoting complex (APC), favoring the development of genomic instability. This is in line with the finding of HDAC2-overexpression in patients with aneuploid tumors by Gemoll et al. (2011). In contrast, TXNL1 is involved in the cellular response to sugar starvation stress and regulates the redox equilibrium in

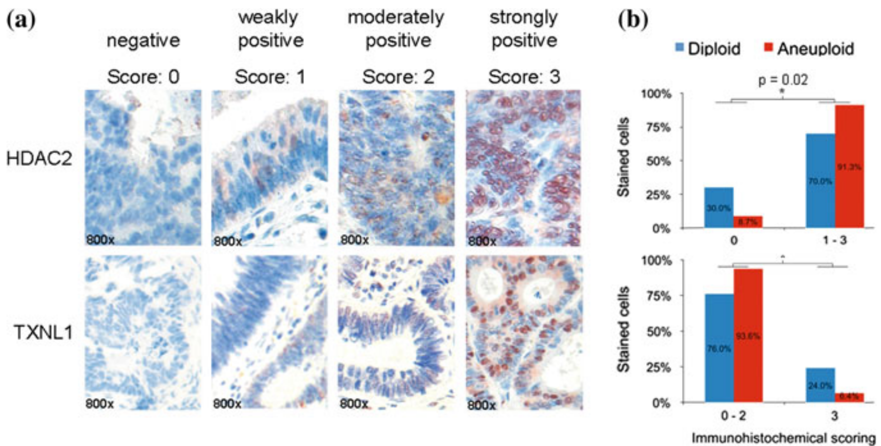

Fig. 1 **a** HDAC2 and TXNL1 immunohistochemical detection in colorectal cancer specimens based on a tissue microarray. Image examples are given at 800-fold magnification. **b** Tissue-microarray-based immunohistochemical evaluation of HDAC2 and TXNL1 comparing diploid versus aneuploid colorectal carcinoma specimens. Immunoreactivity was scored with “0” showing no positivity, “1” presenting up to 20 % immunopositive cells, “2” up to 50 %, and “3” above 50 % stained cells. *Bar plots* of the TMA analysis confirmed HDAC2 and TXNL1 as significantly (*asterisk*) differentially expressed proteins between diploid and aneuploid tumors. Figure adapted from Gemoll et al. (2011)

higher eukaryotes (Jimenez et al. 2006; Manandhar et al. 2009). TXNL1 binds to the transcription factor B-MYb and is overexpressed in diploid as compared to aneuploid carcinomas, thus potentially maintaining genomic stability (Gemoll et al. 2011). Interestingly, TXNL1 was also expressed at low levels in aneuploid endometrial malignancies (Gemoll et al. 2012).

2 Genomic Instability and Disease Prognostication in Breast Cancer

Breast cancer is one of the major causes of morbidity and mortality in females all over the world (Jemal et al. 2011). Despite the fact that tremendous progress has been achieved in chemotherapy and radiation therapy, breast cancer is still one of the most frequent malignancies with poor prognosis. The effects of independent prognostic factors for survival of breast cancer patients, including estrogen receptor/progesterone receptor (ER/PR) status, *HER2* gene amplification and/or overexpression, tumor size, lymph node status, histological grade, and age have been thoroughly recognized (Ferguson et al. 2013). Especially the tumor, node, and metastasis (TNM) system has been extensively used. However, breast cancer is a malignant disease with multiple driving factors involved, and it has been reported that molecular mechanisms may affect tumor growth and progression, thereby potentially limiting the prognostic value of the TNM system (Coradini and Daidone 2004; Song et al. 2013).

Aneuploidy is, in general, correlated to cell proliferation and poor differentiation but not disease stage (Silvestrini 2000). However, Fallenius et al. (1988) demonstrated that node positive non-aneuploid tumors exhibited a better survival than node negative but aneuploid tumors, indicating that ploidy in this study cohort was a stronger prognostic marker than node assessment.

In 2006, Kronenwett et al. (2006) introduced a new concept to measure a tumor cell population with high levels of clonal heterogeneity. The stemline-scatter-index (SSI) is computed with the sum of the proliferation index, the variance of the diploid G0/G1 peak, and the 5c exceeding rate (5cER). Primarily based on the ploidy classification by Auer et al. (1980), the SSI is able to divide cytometrically assessed diploid, tetraploid and aneuploid samples into genomically stable and unstable subtypes. A total of 890 invasive breast cancer patients with a mean follow-up of 8.9 years were evaluated by using this algorithm and showed a significantly better survival of genomically stable subtypes compared with the unstable subtype within each ploidy category ($0.04 < p < 0.004$).

To evaluate potential differences in gene expression patterns between genomically stable and unstable breast tumors, Habermann et al. (2009) examined 17 diploid genomic stable, 15 aneuploid genomic stable, and 16 aneuploid genomic unstable breast carcinomas. A 12-gene expression signature associated with genomic instability in breast cancer was defined and demonstrated a biological and prognostic value across multiple different cancer entities (Habermann et al. 2009;

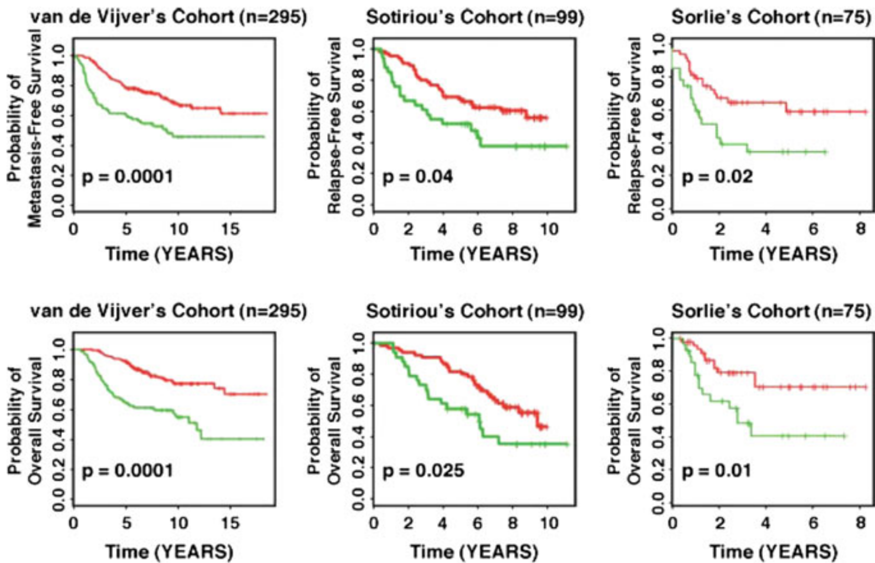

Fig. 2 Applying the 12-gene genomic instability signature for prediction of disease-free and overall survival in independent datasets using Kaplan-Meier analyses. The curves in red reflect carcinoma patients harboring the genomically stable signature, the curves in green represent patients with the one implying genomic instability. For all three examples, statistically significant association of genomic instability with shorter disease-free and overall survival was observed. Figure adapted from Habermann et al. (2011a, b)

Mettu et al. 2010). For breast cancer, genomic unstable carcinomas in patient cohorts from Sorlie et al. (2003), van de Vijver et al. (2002), Sotiriou et al. (2003) were associated with distinct shorter relapse-free survival and metastasis-free survival ($p < 0.04$; Fig. 2). All three studies were not analyzed regarding genomic stability/instability of the samples, initially. However, it was shown that the 12-gene signature is independent of clinicopathological factors such as lymph node status, the NIH criteria, the St. Gallen criteria, and grading used for breast cancer prognostication. In addition, gene sets of the MammaPrint® (van de Vijver et al. 2002; van't Veer et al. 2002) and Oncotype DX® (Paik et al. 2004) tests—two clinically used breast cancer prognostic gene expression signatures—were used to predict genomic instability: 84 % (MammaPrint®) and 91 % (Oncotype DX®) of all cases were correctly classified. Along this line, Swanton et al. (2009) corroborated the importance of genomic instability by showing a link between aneuploidy-associated gene expression and poor response to taxane, a microtubule-stabilizing (MTS) agent. A pre-therapeutic assessment of genomic instability could therefore even optimize treatment stratification.

In 2007, Yildrim-Assaf et al. (2007) published another example for histogram reclassification: Based on thresholds in the categories of 5cER (>10 aneuploid cells) and 9cER (>1 aneuploid cell), patients with node negative and positive breast cancers can be stratified into a high-risk subgroup with unfavorable prognosis. In

total, 370 breast cancer patients showed histology grade, lymph node status, and the above-mentioned binary DNA histogram classification to be the three strongest relapse predictors in a Cox multivariate analysis. The significance of rare-event nuclei (5cER and 9cER) was emphasized by the work of Sidoni et al. (2001) who examined fresh scrape smears from 599 breast carcinomas. According to their results, single cell aneuploidy is a marker for genetic instability with an increased risk of tumor recurrences despite otherwise favorable prognostic parameters. The data seem in accordance with the concept of progressive genetic evolutionary changes in solid tumors (Bartek et al. 1990) and with the unfavorable prognostic significance of DNA hypertetraploidy component as demonstrated in both image (Siitonen et al. 1993) and flow cytometry studies (Pinto et al. 1997).

3 Genomic Instability and Disease Prognostication in Other Cancers

The relationship between genomic instability and cancer prognosis has been explored across a range of cancer types. Next to breast and colorectal cancer (see above), several retrospective studies, summarized in Table 1, consistently associated genomic instability with poor prognosis and demonstrated that it provided additional prognostic information beyond conventional clinical parameters (McGranahan et al. 2012).

In endometrial cancer, genomic instability has been quantified by either image cytometry or flow cytometry (Evans and Podratz 1996). Next to traditional phenotypic variables, including stage, histologic grade and subtype, Britton et al. (1989, 1990) showed prognostic significance in univariate analysis of 256 and 203 endometrial carcinomas. A more detailed assessment revealed DNA ploidy as an independent prognostic factor by Ikeda et al. (1993). In 2002, Lundgren et al. (2002) published a study of relapse free survival following surgical treatment in 358 consecutive patients and found that DNA diploidy predicted disease free-survival. Likewise, prospective and multivariate studies successfully indicated the status of genomic instability as an independent prognostic variable (Susini et al. 2007; Wik et al. 2009). In this context, it seems that the grade of genomic instability correlates with a recurrent pattern of chromosomal imbalances and dominates specific gene and protein expression changes, irrespective of the histopathological subtypes in endometrial cancers. In order to identify the impact of chromosomal aberrations on protein expression, Gemoll et al. mapped genomic imbalances with associated gene and protein expression changes of endometrial cancer patients (Gemoll et al. 2012; Habermann et al. 2011b): Next to recurrent genomic imbalances of the chromosome arms 1q, 3q, 8q, 4q, and 15q, two proteins, AKR7A2 (aflatoxin B1 aldehyde reductase member 2) and ANXA2 (Annexin A2), showed translational alterations in consistence with transcriptional changes. While AKR7A2 is involved in the detoxification of aldehydes and ketones, there is evidence that ANXA2 facilitates

Table 1 Summary of flow and image cytometry studies relating to genomic instability in various cancer types

Cancer type	Method of measuring genomic instability	Number of patients	Outcome Shorter survival/poor prognosis of aneuploid/genomic instable tumors	Reference
Colorectal cancer	Flow cytometry	694	Higher survival rate of diploid tumors	Witzig et al. (1991)
	Flow cytometry	528	Higher survival rate of diploid tumors	Sinicrope et al. (2006)
	Image cytometry	213	Higher survival rate of diploid tumors without metastasis (Dukes'stage A & B)	Bosari et al. (1992)
	Image cytometry	288	Higher survival rate of diploid tumors; Aneuploidy strongest prognostic marker for CRC	Gerling et al. (2010)
	Flow and image cytometry	10,126	Higher survival rate of diploid tumors	Walther et al. (2008)
	Image cytometry	217	Higher survival rate of diploid tumors	Laubert et al. (2013)
	Genotyping of MSI markers	12,782	Higher survival rate of MSI tumors	Guastadisegni et al. (2010)
	Flow cytometry	163	Higher survival rate of diploid tumors	Flyger et al. (1999)
	Image cytometry	219	Higher survival rate of euploid tumors	Bondi et al. (2009)
	Image cytometry	47	Diploid signal count of *NCOA3* is associated with a longer adenoma recurrence-free surveillance	Habermann et al. (2011a, b)
	Image cytometry	78	Higher survival rate of diploid tumors; HDAC2 & TXNL1 marker for genomic stability	Gemoll et al. (2011)
Breast cancer	Image cytometry	227	Higher survival rate of diploid tumors	Fallenius et al. (1988)
	Image cytometry	890	Higher survival rate of genomically stable subtypes	Kronenwett et al. (2006)
	Image cytometry	112	Higher survival rate of diploid tumors	Auer et al. (1980)

(continued)

Table 1 (continued)

Cancer type	Method of measuring genomic instability	Number of patients	Outcome Shorter survival/poor prognosis of aneuploid/genomic instable tumors	Reference
	Image cytometry	48	12-gene signature predict degree of genomic instability and disease prognostification	Habermann et al. (2009), Mettu et al. (2010)
	Image cytometry	370	Lower survival rate of highly aneuploid tumors	Yildrim-Assaf et al. (2007)
	Image cytometry	599	Single cell aneuploidy as marker for genomic instability and biologic aggressiveness	Sidoni et al. (2001)
	Image cytometry	134	Lower survival rate of tumors with cancer cells with >5c DNA content	Siitonen et al. (1993)
	Flow cytometry	860	Hypertetraploidy as marker for biologic aggressiveness	Pinto et al. (1997)
Endometrial cancer	Flow cytometry	256 and 203	Higher survival rate of diploid tumors	Britton et al. (1989, 1990)
	Flow cytometry	76	Higher survival rate of diploid tumors	Ikeda et al. (1993)
	Image cytometry	358	Higher survival rate of diploid tumors	Lundgren et al. (2002)
	Flow cytometry	174	Higher survival rate of diploid tumors	Susini et al. (2007)
	Flow cytometry	363	Higher survival rate of diploid tumors	Wik et al. (2009)
Ovarian cancer	Flow cytometry	682	Higher survival rate of diploid tumors	Akeson et al. (2009)
	Image cytometry	284	Higher survival rate of diploid tumors	Kristensen et al. (2003)
	Image cytometry	47	Higher survival rate of diploid tumors	Kildal et al. (2004)
Large B-cell lymphoma	H&E staining	54	Lower survival rate of patients with chromosomal instability	Bakhoum et al. (2011)
Oral squamous cancer	FISH	77	Lower survival rate of patients with chromosomal instability	Sato et al. (2010)
Synovial sarcoma	CGH	22	Lower survival rate of patients with specific chromosomal instability	Nakagawa et al. (2006)

the reorganization of the extracellular matrix in physiological and pathological processes such as tumor invasion (Mai et al. 2000).

Furthermore, in a multivariate analysis of 682 and 284 ovarian cancers, genomic instability was found to be associated with a worse prognosis. Here, Akeson et al. (2009) determined age, stage, presence of residual tumor, histological subtype, CA125, and DNA ploidy status as univariate predictors of survival time. Along the same line, Kristensen et al. (2003) showed the predictive power of genomic instability in multivariate analysis with a hazard ration of 10.3. These findings are supported by the study of Kildal et al. (2004) that found clinical stage to be the strongest prognostic feature, followed by the extent of residual tumor, and DNA ploidy status.

Furthermore, studies in synovial and oral squamous cell carcinomas as well as diffuse B-cell lymphoma, have suggested that genomic instability is associated with poor prognosis (Mettu et al. 2010; Bakhoum et al. 2011; Sato et al. 2010; Nakagawa et al. 2006).

4 Conclusion

Genomic instability is a defining feature of human cancers. It has an impact on the expression levels of resident genes but in addition also on associated protein expression. Such aneuploidy-associated protein expression patterns could reveal novel diagnostic and therapeutic targets. The evidence for the selective contribution of genomic instability on prognosis is supported by several studies in which patients with aneuploid tumors had a worse outcome compared to patients with euploid tumors. Overall, the assessment of nuclear aneuploidy by image or flow cytometry could become a routine practice to assist in predicting individual cancer risk and in disease prognostication in solid tumors.

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Causes of Chromosomal Instability

Holger Bastians

Abstract

The majority of human cancer cells are highly aneuploid harboring chromosome numbers deviating from the modal number of 46. In cancer, aneuploidy is a consequence of an increased rate of whole chromosome missegregation during mitosis, a process known as chromosomal instability (CIN). In fact, CIN is a hallmark of human cancer and is thought to contribute to tumorigenesis, tumor progression, and the development of therapy resistance by providing a high genetic variability that might foster rapid adaptation processes. However, the molecular mechanisms that cause chromosome missegregation in cancer cells are still poorly understood. So far, several mechanisms underlying CIN have been proposed and some of them are indeed detectable in human cancer cells exhibiting CIN. Examples include, for instance, weakened spindle checkpoint signaling, supernumerary centrosomes, defects in chromatid cohesion, abnormal kinetochore-microtubule attachments and increased spindle microtubule dynamics. Here, the mechanisms leading to CIN in human cancer cells are summarized.

Keywords

Aneuploidy · CIN · Mitosis · Chromosome segregation

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1 Introduction

One of the most intriguing features of human cancer cells is the presence of highly abnormal and complex karyotypes that are characterized by both, structural and numerical aberrations. In fact, chromosomes from human cancer cells are prone to structural rearrangements that typically include translocation, inversions, amplifications and deletions of large parts of the chromosomes resulting in “structural aneuploidy” (Obe and Durante 2010). At the same time, most cancer cells exhibit “numerical aneuploidy”, which is defined as chromosome numbers that are not a multiple of the haploid chromosome number (Lengauer et al. 1998; Thompson et al. 2010; Holland and Cleveland 2009). Recent large-scale copy number analyses from more than 3000 cancer specimens have demonstrated that about 25 % of a typical cancer genome is affected by copy number alterations at a whole chromosome arm or entire chromosome level. In contrast, copy number alterations on a very short (focal) level affect only 10 % of a typical cancer genome indicating that the gain or loss of whole chromosomes or at least of chromosome arms reflects a major route to somatic copy number alterations in human cancer (Beroukhim et al. 2010).

It is obvious that numerical aneuploidy is the result of whole chromosome missegregation, which occurs during mitotic cell division. Mitotic chromosome missegregation as being a typical feature of human cancer cells has been proposed for a long time. In fact, the German pathologist David Paul von Hansemann (1858–1920) described already in 1890, just a few years after the discovery of chromosomes, that cancer cells frequently show abnormal mitotic figures associated with missegregation of the “hereditary material” (Hansemann 1890). Moreover, Hansemann was the first who postulated that alterations in the “hereditary material” of a normal cell might be responsible for the initiation of the “cancerous process”. Just a few years later, in

1914, the German zoologist Theodor Boveri published his seminal book “Zur Frage der Entstehung maligner Tumoren” (Concerning the origin of malignant tumors), in which he hypothesized that abnormal chromosome segregation during mitosis might be a key step towards tumorigenesis (Boveri 1914). Moreover, in his experiments, Boveri found that abnormal numbers of centrosomes can lead to chromosome missegregation, which in most cases is detrimental to a cell. Yet, only a “particular, incorrect combination of chromosomes” could give rise to “schrankenloser Vermehrung” (unlimited growth). In this regard, Boveri postulated the existence of cancer-promoting and cancer-inhibiting chromosomes. Today we would call them chromosomes harboring oncogenes and tumor suppressor genes, respectively. Thus, the intriguing observations made by Hansemann and Boveri were the seed for the definition of cancer as a genetic disease and set the stage for subsequent research on the role of whole chromosome missegregation as a cause for cancer.

2 Aneuploidy Versus Chromosomal Instability

In full agreement with the seminal work from Hansemann and Boveri, modern cytogenetic technologies for the detailed analyses of karyotypes that include, for instance, fluorescence-in situ-hybridization (FISH), spectral karyotyping and comparative genomic hybridization (CGH) clearly confirmed the highly frequent presence of numerical aneuploidy in human cancer cells (Lengauer et al. 1998; Camps et al. 2009; Lengauer et al. 1997). Moreover, along the hypothesis from Boveri, certain combinations of gains and losses of specific chromosomes can indeed frequently be detected, which is most likely the result of a long-term selection process in these cancer cells and not a consequence of missegregation of only particular chromosomes (Knutsen et al. 2010). Interestingly, the majority of cancer cells exhibit chromosome numbers in a diploid range from 40 to 60 chromosomes suggesting that only single chromosomes and not large parts of the chromosome content are missegregated in cancer cells over time (Holland and Cleveland 2009; Storchova and Kuffer 2008) (see also: http://cgap.nci.nih.gov/Chromosomes/Mitelman). In fact, severe missegregation of many chromosomes at a time would produce non-viable progeny, a situation that is even exploited for anti-cancer therapy where anti-mitotic drugs induce massive chromosome missegregation and cell death on purpose (for a review on anti-mitotic drugs and their mode of action see: (Kaestner and Bastians 2010)). It is important to emphasize that aneuploidy represents a current state of a karyotype and does not describe its dynamics. In principle, aneuploidy can be the result of a single chromosome missegregation event. This is for example the case for constitutional trisomies in humans, which are the result of a single non-disjunction event during meiosis (Nicolaidis and Petersen 1998). In contrast, high-grade aneuploidies in human cancer cells are the result of persistent chromosome missegregation during mitosis and this increased rate of gains and losses of whole chromosomes is refereed to as chromosomal instability (CIN). Thus, aneuploidy describes a state and CIN refers

to a process (Thompson et al. 2010; Holland and Cleveland 2009; Lengauer et al. 1997; Thompson and Compton 2008). This indicates that direct measurements of CIN in tumor tissues, which are usually available only upon fixation, is difficult and determining karyotype variability can only provide an indirect measure for CIN. In cancer cell lines, however, the rate of chromosome missegregation has been determined by live cell analyses and showed that a typical human cancer cells missegregate chromosomes on average every one to five mitoses, which is around 20–100 times higher than in a normal non-cancer cell and which leads to a karyotype evolvement over time (Lengauer et al. 1997; Thompson and Compton 2008). This has first been demonstrated in colon carcinoma cell lines where the karyotype evolvement from a single-cell-colony was followed over time. These initial studies by Vogelstein and colleagues revealed that a minority of colon cancer cells exhibit an increase in mutation rates (the so-called microsatellite instability (MIN/MSI) phenotype), but no gross development of whole chromosome aneuploidy whereas the majority of cancer cells develop numerical chromosome aberrations over time (Lengauer et al. 1997). Thus, these studies defining and detecting CIN in human cancer cells initiated the investigation of the mechanisms leading to whole chromosome missegregation and CIN in human cancer.

3 Chromosome Segregation During Mitosis

The proper and timely coordinated progression of mitosis is a prerequisite for faithful chromosomes segregation. Thus, it is important to briefly discuss the key regulatory steps required for proper chromosome segregation in order to understand what defects may account for CIN in cancer cells.

Upon accomplished DNA replication, the entry into mitosis requires the activation of the cyclin dependent kinase 1 (CDK1) bound to it regulatory subunits cyclin A and cyclin B. At the same time additional kinases such as polo-like kinase 1 (PLK1) and kinases of the Aurora family (Aurora-A and -B) are activated (Lens et al. 2010). Together, these kinases trigger the first steps of mitosis during prophase including the breakdown of the nuclear envelope, the condensation of the chromosomes and the separation of the two centrosomes, which act as the main microtubule organizing centers at the poles of the cell. In prometaphase, highly dynamic microtubules are building up the mitotic spindle and are responsible for capturing the kinetochores, protein structures that are assembled on centromeric DNA, in a stochastic search-and-capture mechanism (Kline-Smith and Walczak 2004). The back-to-back geometry of the two sister kinetochores (on the two sister chromatids) favor bi-orientation of the chromosomes (Loncarek et al. 2007). However, erroneous microtubule-kinetochore attachments can occur, but are usually corrected before the actual chromosome segregation occurs (Cimini et al. 2003). At this stage the chromosomes consist of two sister chromatids that are held together by cohesion protein complexes that are established already during DNA replication (Remeseiro and Losada 2013). The coordinated growth and shrinkage of microtubules bound to

kinetochores finally lead to a complete alignment of all chromosomes on a so-called metaphase plate. This stable state is associated with bi-orientation of all chromosomes and with the generation of tension across the two kinetochores. Only upon establishment of this stable state is the separation of the two sister chromatids triggered by the cleavage of the cohesion protein complexes by a protease called separase. Consequently, the two sisters are separated during anaphase (Stemmann et al. 2005). Once the sister chromatids arrive at the opposite poles, near the centrosomes, the nuclear envelope is reformed during telophase and finally the cytoplasm is cleaved by establishing an actin-myosin driven cleavage furrow during cytokinesis.

The progression through the different stages of mitosis requires the continuous activity of the CDK1, PLK1 and Aurora kinases, which act at different locations within the mitotic cells such as the centrosomes, the spindle, or at centromeres or kinetochores. Importantly, exit from mitosis is driven by inactivation of these key kinases, in particular of CDK1, which is inactivated by a highly regulated ubiquitin-proteasome dependent protein proteolysis of the cyclin B subunit. This protein destruction step requires a multi-subunit ubiquitin ligase called the “anaphase promoting complex” or “cyclosome” (APC/C). The APC/C is kept inactive during the early phases of mitosis and is activated once cells have achieved a stable metaphase state. The APC/C targets several proteins during mitosis, most notably cyclin B at the end of mitosis and a protein called securin at the metaphase to anaphase transition, which represents an important trigger for the onset of anaphase. Securin is an inhibitor of separase, the protease that cleaves and releases cohesion complexes from chromosomes. Before metaphase, the APC/C is inhibited and securin is stable, thereby inhibiting separase and the cohesion complexes are stably bound to the two sister chromatids holding them tightly together (Peters et al. 2008). Once the APC/C is activated at the metaphase to anaphase transition securin degradation is triggered, thereby allowing the activation of separase, which then cleaves the cohesion complexes and initiates the separation of the two sister chromatids (Teixeira and Reed 2013; Peters 2006).

4 The Mitotic Spindle Assembly Checkpoint

The APC/C is the key trigger for the onset of anaphase (by mediating the degradation of securin) and for the exit from mitosis (by mediating the degradation of cyclin B). Thus, it is conceivable that the ubiquitin ligase activity must only be activated after cells have successfully achieved complete chromosome alignment. This important regulation is provided by a signal transduction pathway known as the mitotic spindle assembly checkpoint (Lara-Gonzalez et al. 2012; Musacchio 2011). The checkpoint involves a number of proteins such as Mad1, Mad2, Bub1, BubR1, Bub3 and Msp1 that are specifically recruited to kinetochores that are not properly attached to spindle microtubules. The combined action of all these proteins and a number of other kinetochore components is needed to generate a so-called mitotic checkpoint complex (MCC) that contains BubR1, Bub3, and Mad2 and

which detaches from the kinetochore. The MCC binds to and sequesters cdc20, an essential activating subunit of the APC/C, throughout the cell and thus, prevents the activation of the APC/C ubiquitin ligase and the onset of anaphase. Hence, the MCC generated at unattached kinetochores is currently regarded as a principle inhibitor of the APC/C. It is remarkable that a single improperly attached kinetochore (out of 92) is sufficient to block the entire sister chromatid separation process, indicating that the inhibitory signal derived from a single kinetochore is strong enough to block the activation of the APC/C throughout the cell (Lara-Gonzalez et al. 2012; Musacchio 2011). This is obviously essential to preclude premature separation of sister chromatids in the early phases of mitosis.

5 Mitotic Mechanisms Underlying CIN in Human Cancer

Multiple mechanisms have been so far proposed to be involved in the generation of CIN in human cancer cells. The following paragraphs summarize the most important mechanisms, which are also depicted in Fig. 1.

5.1 Defects in the Mitotic Spindle Assembly Checkpoint

It is conceivable that an impairment of the spindle assembly checkpoint (SAC) results in premature sister chromatid separation, which gives rise to chromosome missegregation and aneuploidy (Lara-Gonzalez et al. 2012; Musacchio 2011). In fact, this has been clearly demonstrated by deleting one allele of *MAD2* in an otherwise chromosomally stable colon cancer cell line HCT116, which results in persistent chromosome missegregation and thus, is sufficient to cause CIN (Michel et al. 2001). Moreover, weakening the checkpoint in mice by partially reducing the expression of various SAC genes including *MAD1*, *MAD2*, *BUB1*, *BUBR1,* and *BUB3* results in premature separation of sister chromatids, chromosome missegregation and CIN. Importantly, the majority of these mouse models demonstrated, for the first time, that the experimental induction of aneuploidy can support tumorigenesis and thus, verified Hansemann´s and Boveri´s hypothesis of a causal relationship of aneuploidy and tumorigenesis (Ricke et al. 2008; Pfau and Amon 2012). However, although inactivating mutations in *BUB1* and *MAD2,* as well as reduced expression of *MAD2* has been found in some cancer cell lines (Cahill et al. 1998; Li and Benezra 1996; Wang et al. 2002), large scale sequencing of human tumors has not revealed a frequent rate of SAC mutations in human tumors (Greenman et al. 2007; Wood et al. 2007; Jones et al. 2008; Parsons et al. 2008; Sjoblom et al. 2006). In addition, a functionally weakened SAC appears not to be a frequent event in cancer cells exhibiting CIN (Tighe et al. 2001). These findings indicate that impairments of the SAC can cause CIN by allowing chromosome segregation to occur in the presence of unaligned chromosomes, but are rarely detected in human cancer.

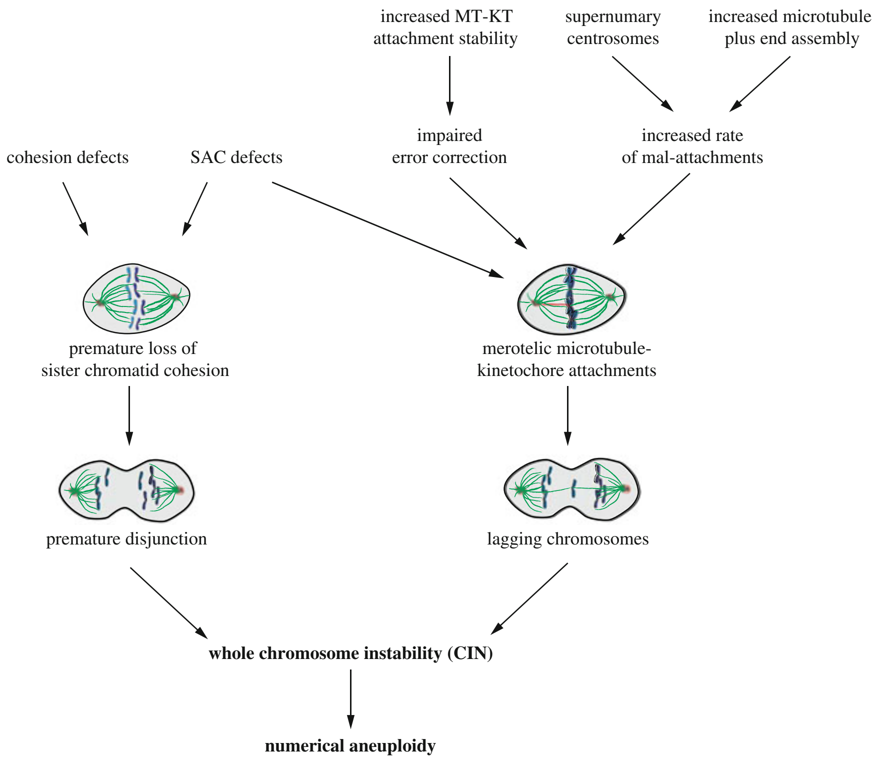

Fig. 1 Summary of the major mechanisms causing CIN in human cancer cells. The major defects leading to chromosome missegregation in human cancer cells are a premature loss of sister chromatid cohesion and the persistence of microtubule-kinetochore attachment errors that subsequently lead to the generation of lagging chromosomes. Various mechanisms can lead to these major causes of chromosome missegregation and the most important routes such as impaired chromatid cohesion, defects in SAC function, increased microtubule-kinetochore attachment stability, supernumerary centrosomes and increased microtubule plus end assembly are depicted

5.2 Sister Chromatid Cohesion Defects

Similar to an impairment of the SAC abnormal sister chromatid cohesion can also result in chromosome missegregation and CIN. Sister chromatids are held together by ring-shaped cohesin protein complexes, which consists of four different subunits including STAG1 or STAG2, RAD21, SMC1 and SMC3, which are loaded onto the chromosomes during DNA replication (Remeseiro and Losada 2013; Peters et al. 2008). In early mitosis the bulk of cohesin complexes dissociates from the chromosome arms while cohesion at the centromere is still maintained. The remaining cohesin complexes are released by cleavage of the RAD21 subunit by separase at the metaphase to anaphase transition. Dysfunction of any of the cohesin subunits can be expected to impair chromatid cohesion and may allow unscheduled separation of sister chromatids before full alignment on a

metaphase plate occurs. In fact, mutations in genes encoding for cohesin subunits, in particular in *STAG2,* have been identified in various human cancers including bladder cancer, colorectal cancer, glioblastomas or myeloid neoplasms (Solomon et al. 2011, 2013; Welch et al. 2012). These mutations are indeed associated with improper chromatid cohesion and are sufficient to mediate CIN. Similarly, heterozygous knockout of *STAG1* in mice also results in aneuploidy and supports tumorigenesis, but this effect might be mediated through incomplete telomere replication (Remeseiro et al. 2012). Since cohesin complexes also fulfill important functions outside of mitosis, e.g., for gene transcription (Remeseiro et al. 2013) it remains possible that cancer-associated mutations of cohesin genes might drive tumorigenesis through mechanisms independent of premature sister chromatid separation during mitosis.

5.3 Erroneous Microtubule-Kinetochore Attachments and Lagging Chromosomes

Albeit detectable, premature separation of sister chromatids occurs in human cancer at rather low rate. Thus, other mechanisms must be responsible for widespread chromosome missegregation events as seen in cancer cells. The most common phenotype observed in mitotic cancer cells and leading to chromosome missegregation is the presence of so-called lagging chromosomes during anaphase (Thompson and Compton 2008). Lagging chromosomes are the result of erroneous microtubule-kinetochore attachments that are not resolved before anaphase onset (Cimini 2008). Since the capture of kinetochores by microtubules during the early phases of mitosis is a largely stochastic process, it is conceivable that kinetochore mal-attachments occur at a regular basis. Those mal-attachments include mono-, syn-, and merotelic attachments that need to be corrected, in order to establish proper amphitelic attachments that are a prerequisite for proper chromosome alignment and segregation (Fig. 2). In contrast to mono- and syntelic attachments, merotelic attachments, characterized by kinetochores being attached to microtubules emanating from the two opposite poles at the same time, represents a particular problem for a mitotic cell. These attachments fully occupy the kinetochores and are not recognized by the SAC, thus allowing the onset of anaphase despite the presence of this erroneous kinetochore attachment (Cimini 2008). This results in one (or more) lagging sister chromatids(s) that cannot properly be segregated towards one pole of the cell and thereby, creates a pre-stage of chromatid missegregation (Fig. 2). Intriguingly, the presence of lagging chromosomes is most prevalent in cancer cells exhibiting CIN and it is assumed that this represents a major route to chromosome missegregation and aneuploidy in cancer (Thompson and Compton 2008; Cimini et al. 2001). However, the molecular mechanisms leading to the generation of erroneous kinetochore attachments might be various and are discussed below.

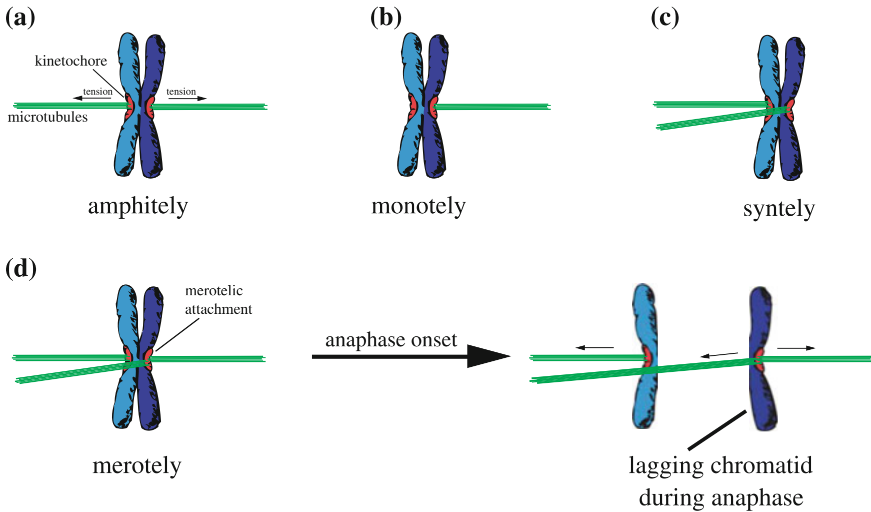

Fig. 2 Schematic depiction of erroneous microtubule-kinetochore attachments. **a** Amphitelic attachment. Both sister kinetochores are correctly attached in a bi-oriented manner to microtubules emating from the two opposite centrosomes. **b** Monotelic attachment. Only one sister kinetochore is attached to microtubules from one centrosome. **c** Syntelic attachment. Both sister kinetochores are attached to microtubules emanating from the same pole. **d** Merotelic attachment. Both sister kinetochores are attached to microtubules from the two opposite poles, but one kinetochore is bound to microtubules from both poles. After anaphase onset merotelic attachments cause the generation of a lagging chromatid, which cannot be properly segregated

5.4 Abnormal Microtubule-Kinetochore Attachment Stability

The interaction of microtubules with kinetochores during the early phases of mitosis is a highly dynamic process, during which finally around 20 microtubules are bound to one kinetochore. Erroneous attachments are resolved by de-stabilization of these interactions while correct attachments are stabilized. The cycles of attachment, release and re-attachment are called error correction and are required for achieving bi-oriented amphitelic kinetochore attachments for every chromosome (Nicklas and Ward 1994). The dynamic turnover of microtubules bound to kinetochores (so-called k-fibres) can be measured in live cells by determining fluorescence dissipation after photo-activation of PA-GFP-tubulin within mitotic spindles. These measurements revealed that the turnover of k-fibres is high in early mitosis ($t_{1/2}$ = 2–3 min) and low in metaphase ($t_{1/2}$ = 6–7 min) indicating that error correction is more active in early mitosis than in cells that have achieved full chromosome alignment (Kabeche and Compton 2013; Zhai et al. 1995). It is obvious that error correction takes place at the kinetochore-microtubule interface and therefore, kinetochore-based microtubule depolymerases are key to error correction. In fact, microtubule depolymerases of the kinesin-13 family such as MCAK

or Kif2B are directly involved in destabilizing erroneous kinetochore attachments, thereby releasing faulty attached kinetochores from the microtubule plus ends (Bakhoum et al. 2009a, b; Maney et al. 1998). In addition, other kinetochore proteins such as the Hec1-Ndc80 complex, which is frequently deregulated in human cancer, also contribute to the stabilization of the k-fibres (DeLuca et al. 2006). Most notably, the Aurora-B kinase, which localize to the inner centromere can directly regulate the localization and activity of MCAK and Kif2b and can thereby contribute to destabilization of k-fibres and to error correction (Cimini et al. 2006; Hauf et al. 2003; Knowlton et al. 2006; Andrews et al. 2004). Consequently, inhibition of Aurora-B or loss of Kif2b or MCAK causes hyper-stability of microtubule-kinetochore attachments leading to lagging chromosomes and chromosome missegregation. How a cell recognizes erroneous kinetochore attachments and how it can discriminate between proper and improper attachment is currently not entirely clear, but might be related to the fact that only proper amphitelic attachments are able to provide the basis for the generation of full tension across the two sister kinetochores. In this situation, the inner centromere-based Aurora-B kinase might be spatially separated from their substrates, namely the kinetochore-based microtubule depolymerases, which can then no longer de-stabilize the microtubule-kinetochore attachments (Liu et al. 2009).

Most intriguingly, hyper-stable microtubule-kinetochore attachments are detected in many cancer cells exhibiting CIN suggesting that erroneous kinetochore attachments cannot be properly resolved in those cancer cells (Bakhoum et al. 2009a, b). In fact, reduced turnover of microtubules bound to kinetochores correlates with the subsequent appearance of lagging chromosomes during anaphase and with an increased rate in chromosome missegregation in human cancer cell lines. Moreover, overexpression of *KIF2B* or *MCAK* results in de-stabilization of these hyper-stable attachments and is sufficient to suppress chromosome missegregation in cancer cells with CIN (Bakhoum et al. 2009b). These findings suggest that cancer cells might harbor an insufficiency of the error correction machinery, but so far no frequent alterations in genes known to play a role in error correction have been found. Genes such as *AURORA-B*, *KIF2B* or *MCAK* are found rarely or not at all mutated or inactivated in human cancer (Greenman et al. 2007; Wood et al. 2007; Jones et al. 2008; Parsons et al. 2008; Sjoblom et al. 2006). Nevertheless, other genes not necessarily directly involved in error correction might contribute to hyper-stable kinetochore attachments. An important example might be the tumor suppressor gene *APC*, which is frequently inactivated in human cancer and which was previously shown to be involved in mediating the interaction of microtubule plus tips with kinetochores (Fodde et al. 2001). Loss of *APC* results in hyper-stable kinetochore attachments, lagging chromosomes and whole chromosome missegregation, but this might also reflect the generation of erroneous kinetochore attachments per se (Bakhoum et al. 2009a). Similarly, overexpression of the SAC gene *MAD2* was found to be frequent in human cancer and instead of mediating hyper-activity of the mitotic spindle checkpoint, it was found to cause hyper-stable kinetochore attachments resulting in lagging chromosomes and aneuploidy (Kabeche and Compton 2012). Whether this also applies to other SAC genes remains to

be seen. Interestingly, a weakened spindle checkpoint can also be associated with the generation of lagging chromosomes. The molecular basis for this is still unclear, but one could argue that cells without a proper SAC might have a reduced time window for error correction before anaphase is initiated and thus, uncorrected kinetochore attachments can persist leading to the appearance of lagging chromosomes during anaphase.

5.5 Supernumerary Centrosomes

Given the fact that the presence of lagging chromosomes is a major phenotype of human cancer cells exhibiting CIN it is important to emphasize that there are at least two ways to achieve this important route to chromosome missegregation: (i) reduced error correction as discussed above leading to the persistence of unresolved erroneous kinetochore attachments. (ii) increased rate of the generation of erroneous kinetochore attachments that might simply overwhelm the cellular (in principle functional) error correction machinery.

As shown recently, the presence of supernumerary centrosomes is one important way to increase the rate of erroneous kinetochore attachments (Ganem et al. 2009; Silkworth et al. 2009). Since the days of Boveri, it is well known that cancer cells frequently exhibit more than two centrosomes. In fact, about 20–30 % of cancer cells might contain more than two centrosomes (Ghadimi et al. 2000). Moreover, as Boveri already realized supernumerary centrosomes can be a source for chromosome missegregation and it was assumed for many years that supernumerary centrosomes can give rise to multipolar mitotic spindles, which inevitably would result in highly unequal chromosome segregation. Indeed, multipolar spindles can be seen occasionally in cancer cells with supernumerary centrosomes, which further supported this assumption. However, Pellman, Cimini and collegues followed the fate of cancer cells after undergoing multipolar mitoses and it became clear that multipolar chromosome missegregation is not compatible with cell viability (Ganem et al. 2009; Silkworth et al. 2009). Instead, the majority of cells containing supernumerary centrosomes only transiently build up a multipolar spindle, which subsequently is re-organized into a bipolar spindle by clustering the supernumerary centrosomes together into two poles. In the end, most cancer cells with supernumerary centrosomes undergo bipolar mitoses, albeit with reduced fidelity. Interestingly, the transient formation of multipolar spindle intermediates followed by centrosomes clustering facilitates the generation of erroneous kinetochore attachments and consequently, leads to lagging chromosomes. Thus, centrosome clustering provides a plausible mechanism that explains the survival of cancer cells in the presence of more than two centrosomes. The transient spindle geometry abnormality arising from this explains the strong correlation between supernumerary centrosomes and CIN in human cancer (Ganem et al. 2009; Silkworth et al. 2009). Although a number of genes have been identified that are required for centrosome clustering in human cancer cells (Kwon et al. 2008) the detailed mechanism of how centrosome clustering is regulated is currently not well understood.

5.6 Increased Microtubule Plus End Assembly

Most recently, an increase in microtubule plus end assembly rates within mitotic spindles has been identified as a key trigger for CIN in human cancer cells (Ertych et al. 2014). In fact, this increase in microtubule plus end dynamics is sufficient to cause transient spindle geometry abnormalities that facilitate the generation of kinetochore mal-attachments in the presence of a fully functional error correction machinery. Importantly, restoration of proper microtubule plus end assembly rates cannot only suppress the transient spindle abnormalities and the generation of lagging chromosomes, but also restores chromosomal stability in otherwise chromosomally instable human cancer cells. This clearly establishes a causal relationship between increased spindle microtubule dynamics, lagging chromosomes, and CIN. Moreover, this particular phenotype appears to be highly frequent in human cancer and can be mediated by cancer-relevant genetic lesions such as amplification of *AURKA* [encoding for the centrosomal Aurora-A kinase; (Marumoto et al. 2005)] or the loss of the tumor suppressor genes *CHK2* and *BRCA1,* which have been previous implicated in the regulation of mitosis and for the maintenance of chromosomal stability (Stolz et al. 2010, 2011). However, the molecular mechanisms underlying the detected increase in microtubule plus end dynamics in cancer cells are still elusive. Nevertheless, the identification of this widespread mechanism leading to CIN further supports the notion that an increase in the rate of the generation of erroneous kinetochore attachments might overwhelm the cellular error correction capacity and thus, causes the persistence of lagging chromosomes during anaphase, finally leading to increased rates of chromosome missegregation (Ertych et al. 2014).

6 Abnormalities During Interphase Causing CIN

In addition to defects that arise during mitosis and directly causing chromosome missegregation it is conceivable that mechanisms that originate during interphase might also (indirectly) lead to chromosome missegregation. Some examples will be discussed in the next paragraphs.

6.1 Centrosome Amplification

As discussed, the presence of supernumerary centrosomes during mitosis causes transient spindle geometry abnormalities that facilitate the generation of erroneous microtubule-kinetochore attachments leading to chromosome missegregation. While the direct consequence of supernumerary centrosomes can clearly be seen during mitosis, supernumerary centrosomes originate from defects in the centrosome duplication cycle taking place during interphase (Meraldi and Nigg 2002). Centrosomes must duplicate only once per cell cycle and this process is initiated by

centriole duplication at the G1 to S phase transition concomitant to DNA replication. Several proteins including Cep192, HsSAS-6 and PLK4 among others are required for proper centriole duplication. Conceivably, over expression of these key drivers of centriole duplication results in centrosome overduplication and can be indeed found in human cancer (Anderhub et al. 2012). Similarly, the CDK2-cyclin E kinase activity is also involved in centrosome duplication and overexpression of cyclin E is sufficient to induce centrosome amplification (Hinchcliffe et al. 1999). Increased cyclin E levels are detected in human cancer cells and can be induced either by amplification of the *CYCLIN-E* gene locus or by inhibition of the ubiquitin-proteasome dependent proteolysis of cyclin E (Rajagopalan et al. 2004; Spruck et al. 1999). In fact, the latter requires a substrate recognition subunit of the SCF ubiquitin ligase called hCDC4/Fbw7, which is frequently mutated in human cancer leading to an unscheduled accumulation of cyclin E. Consequently, loss of hCDC4/Fbw7 in human tumors is associated with centrosome amplification and aneuploidy (Rajagopalan et al. 2004).

6.2 Alterations in Gene Transcription Affecting Mitosis

In human cancer, there are numerous examples of transcription factors that are deregulated. So far, the best studied examples include oncogenic transcription factors such as c-myc, c-jun, or c-fos or the tumor suppressor protein p53 that mainly contribute to a de-regulation of the G1 to S phase transition of the cell cycle. However, there are also many examples of de-regulated gene transcription that directly affects genes involved in mitotic processes. For example, the transcription of *MAD2* during G2 phase of the cell cycle is restrained by the repressor-element-1-silencing transcription factor (REST; also known as: neuron-restrictive silencing factor, NRSF) that must be degraded during G2 by the ubiquitin-proteasome pathway in order to allow the expression of proper levels of *MAD2* (Guardavaccaro et al. 2008). However, *REST* is frequently over expressed in, e.g., neuro- and medulloblastomas (Su et al. 2006) leading to reduced expression of *MAD2* resulting in an impairment of the checkpoint (Guardavaccaro et al. 2008). As discussed before, this can directly trigger chromosome missegregation in the presence of unaligned chromosomes.

MAD2 expression is also under control of the E2F transcription factors, which are negatively regulated by the retinoblastoma tumor suppressor protein (pRb). In fact, loss of *RB*, which is frequent in various types of cancer and associated with aneuploidy in tumors, can result in *MAD2* overexpression (Hernando et al. 2004), which, as discussed already above, can contribute to the generation of hyper-stable microtubule-kinetochore attachments leading to lagging chromosomes and missegregation (Kabeche and Compton 2012). In addition, loss of *RB* has also been implicated in altering the centromere geometry, thereby facilitating microtubule-kinetochore attachment errors and aneuploidy (Manning et al. 2010).

Similarly, the forkhead transcription factor FoxM1 is required for proper progression of mitosis and for the maintenance of chromosomal stability by regulating the expression of various key regulators of mitosis including Plk1, cyclin B, and Cenp-F. (Laoukili et al. 2005). Importantly, FoxM1 is frequently overexpressed in human cancer resulting in overexpression of several mitotic regulators during mitosis (Fu et al. 2008). However, the molecular consequences of elevated levels of, e.g., cyclin B during mitosis and on chromosome segregation are currently not clear.

6.3 Replication Stress as a Source for CIN

Most recently, impaired replication fork progression and replication stress during S phase has been implicated as a source for chromosome missegregation during mitosis (Burrell et al. 2013). In fact, it was shown that several genes located on chromosome 18q, which is subject to frequent loss in cancer cells exhibiting CIN, can act as CIN suppressor genes. Moreover, loss of these CIN suppressor genes results in replication stress leading to the generation of acentric chromosome fragments that are missegregated during mitosis. Importantly, the missegregation of chromosome fragments after replication stress might also cause structural chromosome abnormalities frequently found in aneuploid cancer cells. However, whether this mechanism can also account for whole chromosome missegregation is under debate (Bakhoum et al. 2014).

7 Conclusions

Aneuploidy in tumor cells evolves at rather low rates. Therefore, the underlying defects causing chromosome missegregation at low rates are expected to be subtle. This hampers the investigation of the molecular mechanisms leading to perpetual chromosome missegregation. Nevertheless, great progress has been made to understand how chromosome segregation is regulated and what defects might contribute to chromosome missegregation in cancer cells. It appears that the generation of lagging chromosomes, resulting from erroneous microtubule –kinetochore attachments, might be a major source for CIN in cancer. However, the routes leading to this intriguing phenotype are various and most likely, cancer cells employ different routes to achieve the same goal. It will still be a big challenge for future research to unravel the exact mechanisms contributing to CIN. This will be important to develop strategies that might be used to suppress CIN in order to prevent the high adaptation capability of human cancer cells that enables those cells to cope with environmental challenges and to develop therapy resistance.

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Patterns of Chromosomal Aberrations in Solid Tumors

Marian Grade, Michael J. Difilippantonio and Jordi Camps

Abstract

Chromosomal abnormalities are a defining feature of solid tumors. Such cytogenetic alterations are mainly classified into structural chromosomal aberrations and copy number alterations, giving rise to aneuploid karyotypes. The increasing detection of these genetic changes allowed the description of specific tumor entities and the associated patterns of gene expression. In fact, tumor-specific landscapes of gross genomic copy number changes, including aneuploidies of entire chromosome arms and chromosomes result in a global deregulation of the transcriptome of cancer cells. Furthermore, the molecular characterization of cytogenetic abnormalities has provided insights into the mechanisms of tumorigenesis and has, in a few instances, led to the clinical implementation of effective diagnostic and prognostic tools, as well as treatment strategies that target a specific genetic abnormality.

Keywords

Chromosomal aberrations · Copy number alterations · Genomic imbalances · Gene expression · Ploidy · Solid tumors · Colorectal cancer · Molecular cytogenetic

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1 Introduction

Cytogenetic abnormalities are a hallmark of cancer cells. Clonal chromosomal aberrations have been found in the majority of human tumor types, and their identification continues as a result of technical improvements in genome-wide assessment methodologies (Albertson et al. 2003). The increasing detection of such genetic changes allowed the description of specific disease entities. Furthermore, the molecular characterization of cytogenetic abnormalities has provided insights into the mechanisms of tumorigenesis and has, in a few instances, led to the clinical implementation of treatment strategies that target a specific genetic abnormality.

Chromosomal alterations in cancer are mainly classified into two broad groups: structural chromosomal aberrations, and numerical or copy number alterations. In fact, the detection of such alterations demands highly advanced scientific and technological methodologies. The first theories that cancer was a disease of the chromosomes were postulated by David von Hansemann back in the nineteenth century (Hansemann 1890), and later established by the work of Theodor Boveri in the 1920s (reviewed in Ried 2009). However, it was not until about 50 years ago that the history of cancer cytogenetics began after seminal contributions of Peter Nowell and David Hungerford with the finding of a small marker chromosome while studying cultured cells from patients with chronic myeloid leukemia (Nowell and Hungerford 1960). The application of conventional cytogenetic techniques in solid tumors has been extremely challenging, especially due to the difficulty in obtaining good quality metaphase chromosomes to generate banding-based karyotypes. In the last decades, numerous pioneering studies involving the hybridization of fluorescent-labeled probes led to the identification of both numerical and structural aberrations in solid tumors (Ried 2004). In the early nineteen-nineties, comparative genomic hybridization (CGH) was developed to measure genetic alterations across the cancer genome. Nevertheless, the resolution remained at the level of chromosome bands, and the description of genetic alterations based on this

technique was rather challenging. Thereafter, microarray-based technologies and advances in next-generation sequencing during the early 21st century have allowed the characterization of the landscape of genomic aberrations in almost any tumor type at the resolution of a single nucleotide.

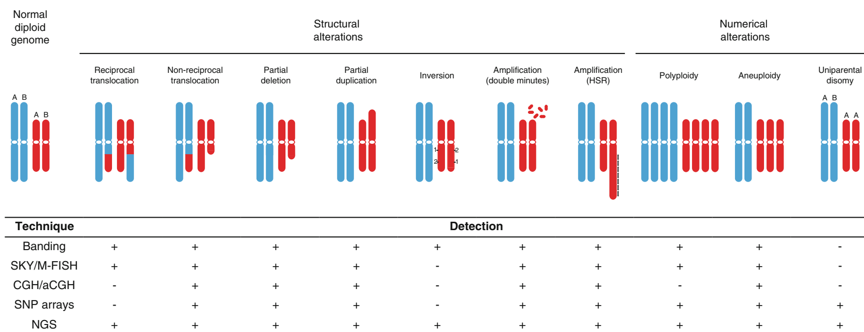
In this chapter, we discuss examples of the two main classes of chromosomal abnormalities, i.e., structural and copy number alterations, with a particular focus on the specific pattern of such alterations according to the tumor type, and the extent to which these alterations might provide information for the development of effective diagnostic and prognostic tools, as well as the implementation of predictive markers for anticancer therapies.

2 Methodological Approaches

Chromosome rearrangements and copy number alterations can be analyzed using a multitude of efficient, large-scale genomic technologies including chromosomal banding, fluorescence in situ hybridization (FISH), high-throughput CGH, loss of heterozygosity (LOH), and recently, next-generation sequencing (Fig. 1).

2.1 Identification of Chromosome Translocations

The forty-six human chromosomes were initially organized into a karyotype based solely on their size and the positioning of their centromere, to which the mitotic machinery attaches. In 1968, Caspersson and his colleagues developed a method for staining chromosomes with quinacrine mustard, which resulted in a banding pattern



Technique	Reciprocal translocation	Non-reciprocal translocation	Partial deletion	Partial duplication	Inversion	Amplification (double minutes)	Amplification (HSR)	Polyploidy	Aneuploidy	Uniparental disomy
	Detection									
Banding	+	+	+	+	+	+	+	+	+	-
SKY/M-FISH	+	+	+	+	-	+	+	+	+	-
CGH/aCGH	-	+	+	+	-	+	+	-	+	-
SNP arrays	-	+	+	+	-	+	+	+	+	+
NGS	+	+	+	+	+	+	+	+	+	+

Fig. 1 Schematic illustration of commonly observed numerical and structural chromosome alterations identified in solid tumors, and the methodologies capable of their detection. Detection of an alteration, however, is not necessarily synonymous with the ability to unambiguously determine the genomic origin of aberrant material. Chromosome banding, SKY/M-FISH and chromosome comparative genomic hybridization (CGH) are low resolution techniques, whereas array-based CGH (aCGH), single nucleotide polymorphism (SNP) arrays and next-generation sequencing (NGS) are much higher resolution methodologies. (Adapted from Albertson et al. 2003)

(Q-bands) that was unique to each chromosome pair (Caspersson et al. 1968). Shortly thereafter, a similar methodology was developed involving the treatment of the metaphase chromosome preparations with the enzyme trypsin followed by staining with Giemsa. The binding of this stain to A-T base pairs resulted in patterns of alternating "light" and "dark" bands, which became known as G-bands (Seabright 1971; Sumner et al. 1971). While this technique was advantageous for unequivocally identifying and organizing normal chromosomes, it proved to be an enormous advance in terms of demonstrating the complexity of the cancer genome. Cancer karyotypes usually show complex rearrangements, involving genomic regions of different chromosomal origin combining in the formation of derivative chromosomes. It remained extremely difficult, however, for cytogeneticists to determine the exact composition of these "marker" chromosomes based solely on G-banding.

In the late nineteen-nineties, the development of two sophisticated multi-colored fluorescence in situ hybridization (M-FISH) methods utilizing a combination of whole-chromosome painting probes for uniquely labeling each chromosome pair, i.e., spectral karyotyping (SKY) and M-FISH, represented an enormous leap forward in our understanding of the underlying complexity of cancer karyotypes (Schrock et al. 1996; Speicher et al. 1996). Although the resolution was limited to the level of an individual chromosome band, these techniques allowed the assessment of specific chromosome partners involved in both balanced and unbalanced translocations, as well as the visualization of previously unidentified cryptic aberrations (Veldman et al. 1997). Nevertheless, the requirement for high-quality chromosome metaphase spreads from primary solid tumors remained a challenge, especially for tumors that are difficult to culture or have a relatively slow rate of cell division.

The utilization of both chromosomal banding-based techniques and SKY/M-FISH resulted in a plethora of well-annotated karyotypes for most cancer types archived in the Mitelman Database of Chromosome Aberrations in Cancer (http://cgap.nci.nih.gov/Chromosomes/Mitelman). It was not until the post-genome era, with the introduction of the next-generation sequencing technology, which allowed the in silico alignment of paired short reads from the ends of fragments covering the whole genome, that genomic rearrangements, including balanced translocations or inversions, could be defined at the individual nucleotide level.

2.2 Identification of Copy Number Changes

In 1992, Kallioniemi and colleagues introduced a genome-wide screening technique, termed CGH, which allowed visualization of chromosomal imbalances without the need to prepare tumor metaphase chromosomes (Kallioniemi et al. 1992). Total genomic DNA isolated from the patient's tumor (i.e., test DNA) and from any other source of non-tumor tissue (i.e., reference DNA) were each labeled with a different fluorescent molecule, or fluorochrome. Equal amounts of labeled test and reference DNA were mixed and hybridized to normal lymphocyte metaphase chromosomes, which cytogeneticists were already capable of preparing for

high resolution banding (Yunis and Chandler 1978). Deviations from a 1:1 intensity ratio between the two fluorochromes along the length of each chromosome indicated the gain or loss of genomic material in the tumor sample relative to a normal non-tumorous reference sample. Because this technique still relied on the usage of metaphase chromosomes, the resolution remained limited to the size of an individual band, or about 5–10 Mb (Carter 2007). Later, metaphase chromosomes were replaced by increasingly shorter normal genomic DNA fragments, including YACs, BACs, or oligonucleotides representing the entire genome spotted onto glass slides (Pinkel et al. 1998; Solinas-Toldo et al. 1997). This array-based CGH, or aCGH, resulted in a much higher resolution and flexibility, limited only by the size and spacing of the DNA fragments that were arrayed. Using an automated calculation of the ratio between the intensities of the two fluorochromes for each spotted feature on the microarray, a dedicated software provides a detailed map of genomic gains and losses distributed across the genome. In contrast to conventional CGH, the analysis of aCGH data does not require previous knowledge to identify chromosome pairs based on G-banding, which makes this methodology much more universal and powerful. Further developments in the sensitivity of array technology resulted in the ability to detect differences in the hybridization efficiency of two DNA fragments that differed in a single nucleotide (Carter et al. 2012). In addition to copy number changes, these single-nucleotide polymorphism or SNP-based arrays were capable of providing information regarding the haplotype of each allele. If an individual has different alleles at a particular genomic locus, SNP arrays provide the opportunity to determine if one allele is preferentially lost in the tumor. In addition, such a LOH in the presence of two copies of the locus or chromosome enables the detection of somatically acquired uniparental disomies (Tuna et al. 2009). Thus, the extent to which one platform is more appropriate than another depends on the study design and the type of information one is looking for.

Undoubtedly, the major advantage of CGH and SNP arrays is the amenability of any cancer specimen to DNA extraction. There are, however, some caveats that must be considered when using these methodologies. First, some 60–70 % of tumor purity is strongly recommended to be able to identify single-copy genomic alterations, i.e., "contamination" with normal, non-cancerous cells is highly problematic. Second, these methodologies only take a snapshot of the tumor lifespan, so intrinsic tumor heterogeneity may potentially dilute out the intensity ratio of the main clonal population. Hence, the detection of low-level copy number events may be limited due to the natural presence of subpopulations with different DNA content. Third, the amount of tissue available from fresh tumor samples, particularly in the case of biopsies, is often limited. It would therefore be advantageous to be able to assess copy number alterations by extracting DNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections once histopathology has been performed. This remains challenging because of the low yield and poor integrity of the extracted DNA. Currently, very few groups have been able to successfully perform aCGH using a small number of cells isolated by microdissection from archival FFPE tissues sections (Al-Mulla 2011; Hirsch et al. 2012; Johnson et al. 2006; van Essen and Ylstra 2012) or from individual circulating tumor cells (Heitzer et al. 2013).

Recent incorporation of next-generation sequencing-based approaches led to the development of tools to infer copy number changes from whole-exome or whole-genome sequencing in a pretty reliable manner (Kendall and Krasnitz 2014). Compared with microarray experiments, these technologies not only allow the identification of alterations at the nucleotide level, but they also have the advantage that the signal intensity does not reach a point of saturation, thus they have a much higher dynamic range and a higher rate of detecting aberrations. High-coverage genome sequencing has been applied to detect clonal subpopulations to an unprecedented level (Gerlinger et al. 2014). Nevertheless, as the number of studies applying next-generation sequencing to large cohorts is limited by the costs, aCGH or SNP arrays remain the gold-standard methodology to assess copy number changes in solid tumors.

3 Structural Chromosomal Rearrangements in Solid Tumors

The study of chromosomal abnormalities in cancer underwent a paradigm shift with the discovery of the Philadelphia chromosome in patients with chronic myelogenous leukemia (CML) by Nowell and Hungerford (1960). The genomic composition of this aberrant chromosome was later determined by Janet Rowley to result from a balanced translocation between chromosomes 9 and 22, or t(9;22) (Rowley 1973). The staining of chromosomal preparations of cells at the metaphase stage with Giemsa was utilized to identify the t(9;22). This landmark discovery initiated the description of marker chromosomes in a plethora of human cancers. In leukemia and lymphoma, the application of this technique to identify aberrant chromosomes has led to improved treatment and clinical outcomes for many patients, and it is still being used for clinical assessment (Rampal and Levine 2013; Rowley 2008).

While chromosome translocations, inversions, and insertions are typically observed in cancer (Albertson et al. 2003), balanced translocations, in which material from both partner chromosomes is retained by the cell, are often identified in hematological malignancies (e.g., t(9;22) in CML or t(8;14) in Burkitt's lymphoma). The most likely explanation is that site-specific DNA recombination of antigen receptor genes is an essential physiological step for the development of mature B- and T-lymphocytes. Any error in the regulation of this process could result in the rearrangement of other genomic regions. Juxtaposition of a cellular proto-oncogene to an actively transcribed region of the genome has the potential to generate a cell with a growth and/or survival advantage. Thus, these aberrations tend to be causal in the development of hematological malignancies, and therefore drugs designed to target the resulting proteins have proven to be extremely effective.

In contrast, partial deletions, duplications, and unbalanced translocations (i.e., rearrangements in which genomic material is lost) are the most frequent chromosomal alterations identified in cancers of epithelial origin (Mitelman et al. 1997). Distinct patterns of recurrent chromosomal translocations in these tumors are extremely rare (Mitelman 2000). One explanation could be the difficulty of identifying and mapping

structural rearrangements in these karyotypically complex tumors. Although this issue is currently overcome by the usage of next-generation sequencing approaches, newly generated data have failed to demonstrate the relevance of such rearrangements in the development of epithelial cancers. Another reason for the rare occurrence of recurrent structural chromosome aberrations in carcinomas could be tissue-specific differences in the mechanisms responsible for their generation, such as the absence of site-specific recombination in non-lymphocytic cells.

The identification of recurrent translocations has only been described in a few solid tumor types. A number of approaches, including cancer outlier profiling analysis of gene expression signatures (Tomlins et al. 2005) and next-generation RNA and DNA sequencing, have demonstrated the presence of gene fusions that result in altered transcriptional expression or protein activity in some of the main epithelial cancer types, although at a frequency of only about 10 % (Mitelman et al. 2004, 2005, 2007; Giacomini et al. 2013). Prostate cancer (*TMPRSS2-ETV1*, *TMPRSS2-ETV4*, *TMPRSS2-ETV5*) (Kumar-Sinha et al. 2008), colorectal cancer (*VTI1A-TCF7L2*, *NAV2-TCF7L1*) (Bass et al. 2011; Cancer Genome Atlas Network 2012a), papillary thyroid carcinoma (*RET-NTRK1*) (Wells and Santoro 2009), papillary renal cell carcinoma (*PRCC-TFE3*) (Kauffman et al. 2014), and non-small-cell lung cancer (NSCLC) (*RET* and *ROS1*) (Oxnard et al. 2013; Shames and Wistuba 2014) are examples of solid tumors with chromosomal rearrangements generating gene fusions with biological and, potentially, clinical implications.

4 Cancer Ploidy and Chromosome Aberration Rates

Solid tumors with a chromosome number between triploid (n = 69) and tetraploid (n = 92) have been estimated to occur in some 30 % of all epithelial cancers (Storchova and Kuffer 2008). As it is unlikely that chromosome missegregation alone occurs at sufficiently high rates to explain how cancer cells achieve such pseudo-polyploid karyotypes during the tumor lifespan, it has been proposed that the cancer genome first undergoes a whole genome duplication event. This tetraploidization, being an unnatural event, is thought to be highly unstable, allowing for the development of structural abnormalities and selective loss of chromosomes until the genome somehow becomes stable again (Burrell et al. 2013). Tetraploidy and high levels of aneuploidy are often correlated with disease aggressiveness, poor prognosis and the generation of metastases (Camps et al. 2004; Gerlinger et al. 2012). In fact, ongoing rates of chromosome missegregation events define levels of genomic instability in several cancer types (Camps et al. 2005). In colorectal cancer (CRC), for instance, the rate of chromosomal instability is directly related to the mutational status of genes involved in the DNA mismatch repair pathway (Lengauer et al. 1998). Near-diploid colorectal tumors are mismatch repair deficient, whereas aneuploid tumors contain intact repair pathways and show higher rates of both numerical and structural chromosome alterations, features observed in the majority of human carcinomas (Lengauer et al. 1997). In the past, the only plausible

strategy to identify the total number of chromosomes per cell was to prepare and analyze metaphase chromosomes from dividing cells. Recently, application of SNP arrays in combination with specific analytical tools attempts to further define absolute allelic copy number changes allowing the determination of the tumor ploidy directly from tumor tissue (Van Loo et al. 2010; Carter et al. 2012).

5 Recurrent Low-Level Copy Number Alterations Among Different Cancer Types: Defining the Cancer Genome

Aneuploidy represents a ubiquitous feature of cancer cells of epithelial origin, and usually implies growth advantages, poor prognostication and shortened patient survival (Gordon et al. 2012; Holland and Cleveland 2009); therefore, gains and losses of chromosomes are positively selected throughout the tumor lifespan. As a result, most cancer genomes show a modal chromosome number far from the normal diploid genome of 46 chromosomes. Low-level copy number changes usually include genomic imbalances that affect the entire chromosome or a chromosome arm, regardless of parameters such as size or gene density. The identification of low-level copy number alterations by karyotyping, CGH and next-generation sequencing provides supporting evidence of a distinct pattern of genomic imbalances depending on the tumor's tissue of origin. In this section, we will describe some examples of the tumor-type specific distribution of copy number alterations.

CRC, being among the more amenable solid tumors to cytogenetic analyses, is one of the most well-studied cancer genomes. Bardi and colleagues systematically cultured colon cancer cells from primary specimens and reported extensive cytogenetic data on both the tumors and derived cell lines, plotting the results as chromosome maps of gains and losses (Bardi et al. 1993, 1995). Later, using conventional CGH, Ried and his colleagues described recurrent alterations in sporadic (i.e., non-hereditary) CRCs in which genomic gains affecting chromosomes 7, 8q, 13, and 20q occurred with frequencies upwards of 80 %, and genomic losses of chromosomes 4, 8p, 17p, and 18q were often observed (Ried et al. 1996). In addition, several reports have shown that some of these aberrations, mainly the gain of 7 and 20q, can already be observed in preneoplastic polyps (Habermann et al. 2011), and most, if not all, are still present in liver metastases of this disease and in in vitro models derived from primary tumors or metastasis (Camps et al. 2009; Platzer et al. 2002). The plethora of conventional and array-based CGH studies applied to map genomic imbalances in CRC convincingly confirmed these earlier results [reviewed in (Grade et al. 2006a)], supporting the idea of a genomic ID associated with CRC. These chromosomal aberrations, as highlighted in Fig. 2, actually accompany the genetic (mutational) and epigenetic events comprehensively described in The Cancer Genome Atlas (Cancer Genome Atlas Network 2012a), and serve as the basis for the CRC progression model published by Bert Vogelstein and Eric Fearon more than two decades ago (Fearon and Vogelstein 1990; Vogelstein et al. 1988).

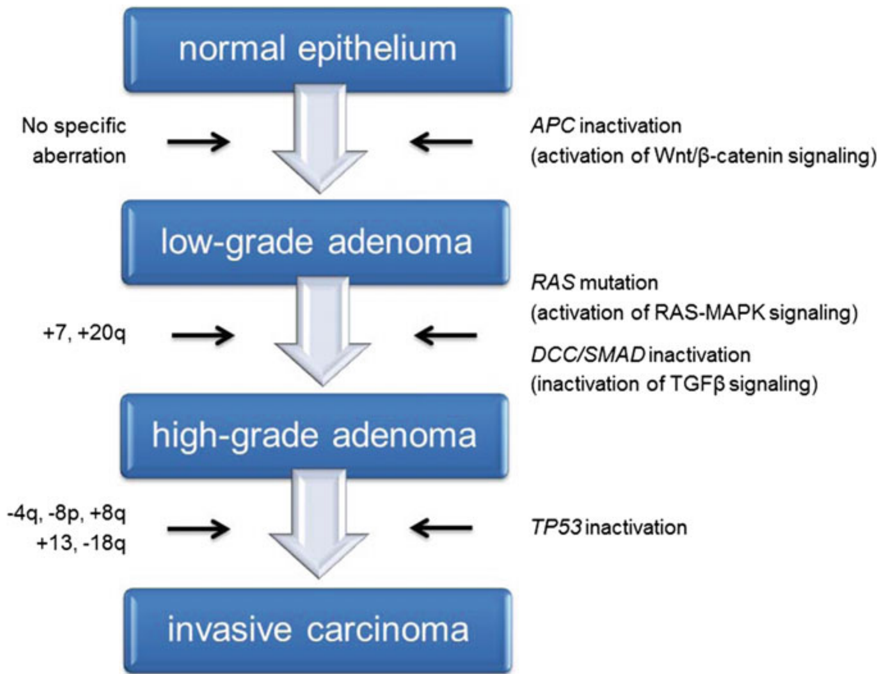


Fig. 2 Progression model of colorectal carcinogenesis. The progression of low-grade adenomas to high-grade adenomas is accompanied by gains of chromosomes 7 and 20q. Gains of chromosomes 8q and 13, as well as losses of chromosomes 4p, 8p and 18q, indicate transition into invasive carcinomas. These chromosomal aberrations, which are specific for colorectal cancer, accompany the genetic (mutational) changes observed at the level of individual genes that serve as the basis for the colorectal cancer progression model, referred to as the adenoma-carcinoma-sequence by Vogelstein and Fearon

It is interesting to note the high tissue-specificity of some of the genomic imbalances described above. For example, the gain of chromosome 13 is practically exclusive to colorectal neoplasms. Although it is not one of the earliest chromosome aneuploidies observed in adenomas, the incidence reaches some 70 % in carcinomas and metastases. The presence of several driver genes (i.e., *CDK8*, *CDX2*, *LNX2*, and *DIS3*), relevant for colorectal carcinogenesis and distributed along the length of this chromosome, favors the gain of the whole chromosome over a short focal amplification, and defines chromosome 13 as a CRC chromosome (Camps et al. 2013; de Groen et al. 2014; Firestein et al. 2008; Salari et al. 2012). Although not as exclusive as the gain of chromosome 13, the gain of chromosome arm 20q occurs in more than 60 % of CRC. Based on integrative genomic approaches, multiple putative oncogenes could drive the selection of this region of the genome in colorectal and other cancer types (Carvalho et al. 2009). Notably, rectal cancers exhibit mirroring genomic profiles compared to colon cancers (Grade et al. 2006b, 2009).

Additionally, besides specific focal events, similar patterns of gross genomic imbalances are observed in other gastrointestinal adenocarcinomas such as esophageal and gastric cancers (Dulak et al. 2012). In contrast, non-small cell lung carcinoma, prostate cancer, ovarian cancer, breast cancer, glioma and others display a loss of chromosome 13 (Di Fiore et al. 2013), as it also contains the well-known tumor suppressor gene *RB1* at 13q14, thus demonstrating that the mechanisms to instigate carcinogenesis depend on different driver genes in a tissue-of-origin specific fashion.

The fact that chromosomal gains and losses define specific tumor entities also applies to breast cancer, even though the picture is a bit more complex because of the heterogeneity of this disease (Kallioniemi et al. 1994; Pollack et al. 2002; Ried et al. 1995). With higher resolution CGH techniques, primary breast carcinomas could be discerned into three groups: (i) near-diploid tumors characterized by extra copies of chromosome arm 1q and losses of 16q, thus referred to as "1q/16q" tumors; (ii) aneuploid tumors defined by recurrent copy number gains of 8q and extensive chromosomal instability, named "complex"; and (iii) aneuploid tumors with frequent focal high-level amplifications, e.g., of the oncogenes *CCND1*, *MYC*, and *ERBB2*, also known as the "amplifier" group (Fridlyand et al. 2006). Integration of gene expression signatures defined five major breast cancer subtypes (basal-like, luminal A, luminal B, ERBB2, and normal breast-like) (Perou et al. 2000), and copy number alteration data showed that recurrent genomic aberrations differ between these subtypes and that stratification correlated with clinical outcome (Chin et al. 2006). In addition, recent studies that applied quantitative measurements of the nuclear DNA content to primary breast carcinomas unambiguously established that tumors with a higher degree of chromosome instability were associated with a worse prognosis (Habermann et al. 2009). Of note, the genomic profiling of serous ovarian cancer partially resembles that of basal-like breast carcinomas, including gains of 1q, 3q, 5p, and 8q, and losses of chromosome 4, 5q, 8p, and 13 (Cancer Genome Atlas Network 2012b; Cancer Genome Atlas Research Network 2013).

In a completely different cancer lineage, the application of molecular cytogenetic techniques also revealed that essentially all cervical carcinomas exhibit an extra copy of the long arm of chromosome 3 (Heselmeyer et al. 1996). The gain of 3q is already present in dysplastic precursors, and, in fact, the presence of this single cytogenetic aberration discerns those lesions that will eventually progress from those that will not (Heselmeyer et al. 1997). Deduced primarily from retrospective studies of Pap smears using interphase FISH with a series of probes for the enumeration of 3p, 3q and cellular ploidy, the gain of 3q determines the acquisition of invasiveness capacities, thereby demonstrating the dominant nature of this genomic imbalance in cervical cancer. One potential driver gene located on chromosome 3q is the human telomerase RNA component (*TERC*) gene, which is involved in maintaining the end of chromosomes, the shortening of which is associated with cellular senescence and aging (Heselmeyer-Haddad et al. 2005; Yin et al. 2012). However, one can not overlook that many other genes at chromosome 3q will also exhibit increased transcriptional activity, thus possibly contributing to cervical

carcinogenesis. In other tumors of squamous cell origin, such as head and neck or bladder squamous cell carcinoma, the gain of chromosome 3q is also a prevalent alteration. Other genomic imbalances commonly observed in these tumor subtypes include gains located at chromosome 1, 7, and 20, and losses located at 4, 11, 16, 17, and 19 (Cancer Genome Atlas Research Network 2014a).

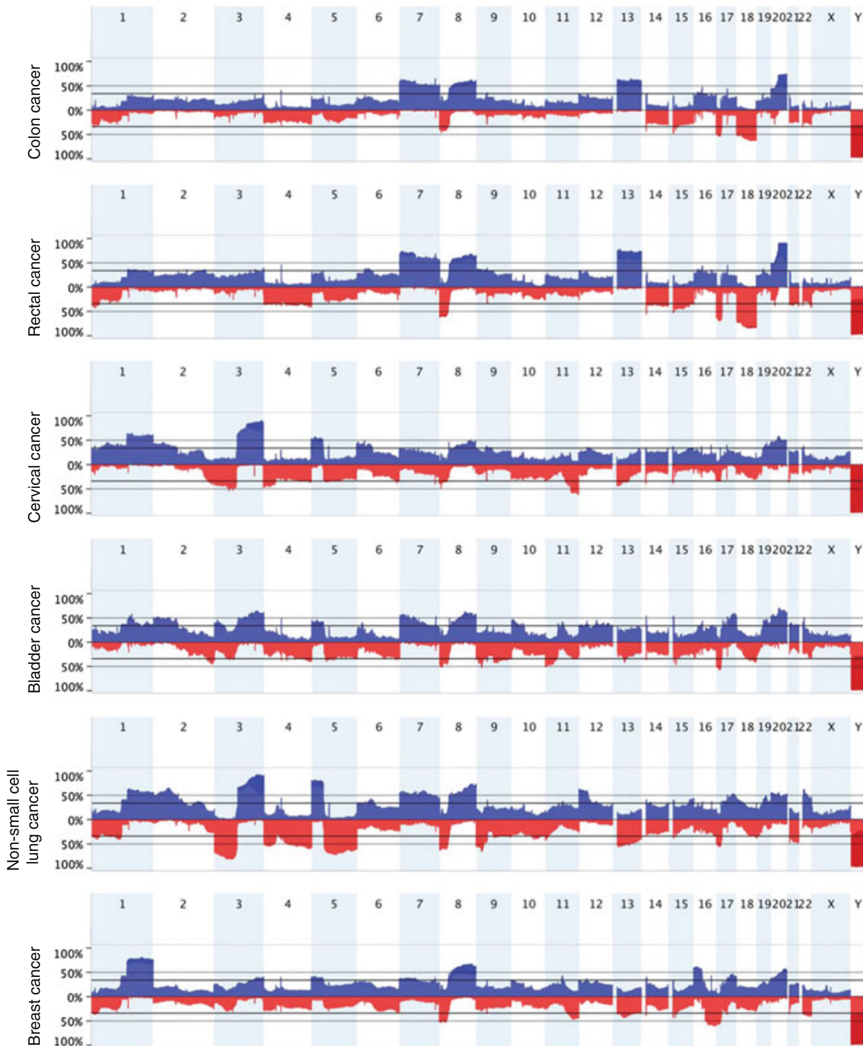


Fig. 3 Diagram of genomic profiles showing the most common gains and losses in colon, rectal, head and neck, bladder and breast cancer. Note the prevalence of specific genomic imbalances unique to each tumor type, thus illustrating the individual landscapes of copy number alterations. SNP array data were collected from The Cancer Genome Atlas (http://cancergenome.nih.gov/)

A catalog of recurrent copy number alterations has also been established for non-small cell lung carcinoma (NSCLC) using aCGH, with specific genomic imbalances including gains at 1q, 3q, 5p, and 8q, and losses at 3p, 8p, 9p, 13, and 17p (Tonon et al. 2005). Although driver genes on genomic sites of copy number changes are still under discovery-based approaches, integrative analysis comprising mutational profiling of NSCLC have identified *KRAS*, *BRAF*, *EGFR*, *MET,* and *FGFR1* as the main candidate driver oncogenes in this disease (Cancer Genome Atlas Research Network 2014b).

Altogether, these studies and others have shown that copy number alterations are tumor-type specific, and that they can be used for efficient tumor classification (Fig. 3). The examples of copy number changes described above suggest that tissue types arising from similar origin tend to share similarities as far as chromosomal gains and losses is concerned, as seen in cancer types of squamous cell origin (i.e., gain of 3q in cervical, bladder, head and neck, NSCLCs), cancers in reproductive organs, such as serous ovarian and serous-like endometrial carcinomas, and cancers affecting gastrointestinal tract tissues (i.e., esophageal, stomach, colon and rectal carcinomas). Comprehensive meta-analyses to understand how tissue or lineage specific transcriptional profiles have an influence on the determination of tumor-type associated signatures of genomic imbalances constitute a fundamental concept underlying the nature of somatic copy number alterations in cancer.

6 Consequences of Genomic Imbalances on Global Gene Expression

The presence of chromosome imbalances specific to the tissue of cancer origin may lead one to ask: What consequences does this additional genomic material have on the biology of cancer cells? The examples mentioned above suggest that recurrent low-copy number changes provide a selective advantage to the specific cell type to propagate indefinitely, often under sub-optimal metabolic conditions and in the presence of genomic and mitotic defects. In both normal and cancer cells, low-level copy number alterations, regardless if they naturally occur in tumors or are artificially induced in non-tumor cells, result in a massive transcriptional deregulation (Grade et al. 2006b, 2007; Upender et al. 2004) (Fig. 4). In contrast to what has been described in normal cells, aneuploidy-dependent transcriptional enhancement must have a positive impact on the growth of cancer cells (Tang and Amon 2013). In addition, most of the genes implicated in the pathogenesis are located in chromosomal regions selected to give growth advantage to the tumor cell (e.g., gain of *MYC* at 8q, loss of *TP53* at 17p and loss of *SMAD4* at 18q in CRC). The integrative strategy of looking at the transcriptional profile for all of the genes confined within regions recurrently involved in genomic imbalances has been extensively used to discover novel cancer genes, as well as to identify genes considered targets for cancer therapeutics (Camps et al. 2013). Therefore, minimal common regions of gains and losses are likely to contain driver genes whose dosage-related deregulation will be of high importance for carcinogenesis.

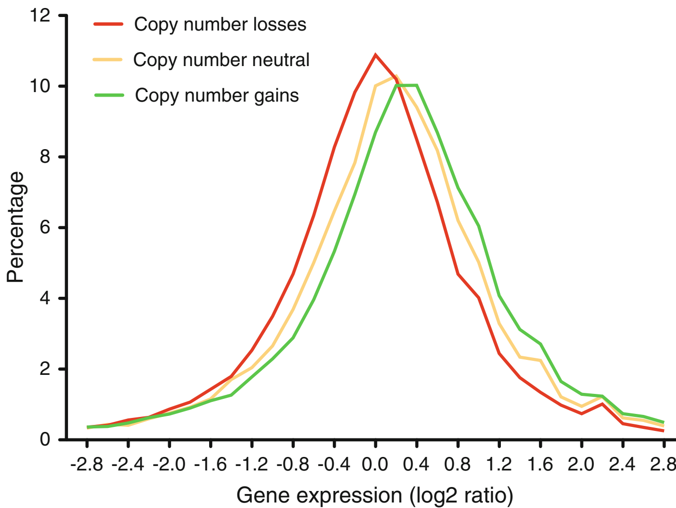


Fig. 4 Plot showing the correlation between copy number changes and gene expression from a set of colorectal cancers. In *yellow*, genomic segments that are copy number neutral; in *red*, genomic segments that show a copy number reduction; and in *green*, genomic segments that show a copy number gain. The Y-axis indicates the levels of gene expression in $\log_2$ ratio. (Adapted from Ried et al. 2012)

Nevertheless, it may also be possible that over-activation of proliferative genes, such as the oncogene *MYC*, impairs cell viability when expressed at "too-high" levels or when affecting the transcription of too many genes, as they induce a disequilibrium of the metabolic stoichiometry of the cancer cell (Sabo et al. 2014; Wahlstrom and Henriksson 2014). Regulation of driver gene expression and their molecular consequences must be critical to ensure cellular viability (Walz et al. 2014). This may be one of the reasons why several cancers do not show high-level amplifications of *MYC*, but rather show a low-copy number gain of chromosome arm 8q containing this gene to achieve the necessary balance in the amount of this transcription factor (Meyer and Penn 2008). However, one must acknowledge that in such scenarios not only *MYC* will be over-expressed, but many other genes as well. Thus the question arises as to which extent other genes, e.g., those already described in the literature as drivers, will determine the positive selection of genomic gains (and losses in the case of tumor suppressor genes). For example, the obvious candidate gene for the selection of the 8q gain is *MYC*, but due to the high recurrence of the low-copy gain of this entire arm, other genes may play a role to boost the cellular fitness. However, whether the transcriptional activation of genes that accompany the target gene of a whole or partial aneuploid chromosome has biological relevance for the cancer cell remains largely uninvestigated. In essence, rather than a single gene, genomic imbalances (i.e., aneuploidy) remarkably represent the driver event in cancer cells.

7 Focal Amplifications and Deletions Point to Driver Genes

7.1 Genomic Amplifications

The term genomic amplification is restricted to focal regions of the genome that are represented in multiple copies (Myllykangas and Knuutila 2006). Two cytogenetically distinct DNA structures have been found to harbor those amplified genomic strings. One type of structure consists of concatenation of a genomic region that has been duplicated numerous times, usually within that same chromosome. These structures fail to display typical banding patterns after trypsin-Giemsa staining and are known as homogeneously staining regions (HSR). The other type of structure consists of small-paired extra-chromosomal bodies known as double minute chromosomes or double minutes (DM) that have been shown to be circular DNA. DMs are basically acentric, atelomeric extra-chromosomal elements containing between 1 and 2 Mb of duplicated DNA that are present in tens to hundreds of copies in a single cell (Kuttler and Mai 2007; L'Abbate et al. 2014). The nature of DMs is still under investigation and it is not clear yet how DMs are inherited from cell to cell. One of the biological mechanisms that originate genomic amplifications is the breakage-fusion-bridge model. Briefly, this model was proposed by Barbara McClintock at the beginning of the last century (McClintock 1939) and is based on the cycling formation of uncapped DNA ends by consecutive DNA double strand breaks and subsequent repair by recombination-based mechanisms, leading to broad DNA amplification, progressive terminal deletions and an increase of genomic instability. These focal regions of high-level copy number change frequently contain oncogenes to promote carcinogenesis (Difilippantonio et al. 2002). The integrative analysis of aCGH and gene expression profiling in cancer allowed the discovery of numerous regions of amplifications in several cancer types, providing evidence for the existence of genes whose oncogenic function was unreported (e.g., Camps et al. 2013; Lockwood et al. 2008). High-throughput analyses of large cohorts of clinical samples resulted in the identification of some cancer lineages with a preference to amplify specific areas of the genome, while other cancer types accumulate low-copy number changes affecting whole chromosomes or chromosome arms (Zack et al. 2013).

Well-known examples of oncogenes that can be activated as a consequence of focal genomic amplification are *ERBB2* in breast cancer, *MYCN* in neuroblastoma, *MYC* in colon, esophageal, gastric, ovarian cancer and others, *CCND1* in bladder cancer, and *MDM2* and *CDK4* in well-differentiated and dedifferentiated liposarcoma, among others (Crago and Singer 2011). Cyclin D1 (*CCND1*), located at chromosome band 11q13, plays an important role in cell cycle regulation, binds to cyclin-dependent kinases (CDK4/6), and promotes phosphorylation of *RB1*, orchestrating progression through the G1 restriction point. This genomic location shows recurrent gene amplifications in several cancer types such as breast, head and neck, bladder, ovarian cancer, and others. Integration of aCGH and gene expression

profiling data suggest that *CCND1* is not the only target in this recurrent genomic amplification, but that there are other genes within this focal area whose expression might also be relevant for tumorigenesis, suggesting a synergistic effect between well-known driver oncogenes and other genes that are amplified in the same amplicon or in different regions of the genome. For example, *PPFIA1* amplification was found exclusively in *CCND1*-amplified breast cancers, suggesting that *PPFIA1* gene copy number changes represent *cis*-like events of *CCND1* amplification (Dancau et al. 2010). Co-amplification in *trans* at chromosomes 8p11–8p12 and 11q12–11q14, including *CCND1*, often occurs in breast tumors suggesting a transcriptional crosstalk between genes in the 8p and 11q amplicons, as well as their cooperation with major pathways of tumorigenesis (Kwek et al. 2009). As for probably the most relevant oncogene in human cancer, *MYC*, the co-amplified neighboring long non-coding RNA gene, *PVT1*, is essential for maintaining the functional expression of the *MYC* transcription factor (Huppi et al. 2008; Tseng et al. 2014). Another important oncogenic alteration involves focal amplifications of the *FGFR1* gene, located on chromosome 8p and encoding a membrane-bound receptor tyrosine kinase, in up to 20 % of squamous cell lung cancers (Dutt et al. 2011; Weiss et al. 2010).

7.2 Homozygous Deletions

Array CGH played a very important role in the discovery of disease-associated microdeletions with clinical impact, both in developmental-related delays and cancer (Shinawi and Cheung 2008). Perhaps even more important than genomic amplifications in cancer are homozygous deletions, which usually harbor tumor suppressor genes. Tumor suppressor genes are subjected to the two-hit model described by Knudson (1971), where one of the alleles is mutated either in the germline or somatically, while the other allele loses its function either by a second somatic deletion, an epigenetic modification, or by a somatically uniparental disomy event. Among the most frequent losses in human epithelial cancers are the homozygous deletions at 9p21 involving *CDKN2A* (also referred to as *p16*), a CDK4 inhibitor, which can also bind the p53-stabilizing protein MDM2 (Ozenne et al. 2010). The absence of functional *CDKN2A*, either by homozygous deletion, hypermethylation or mutation, contributes significantly to the tumor phenotype through the deregulation of CDK4 and p53, thereby inducing cell cycle G1 progression. *CDKN2A* is homozygously deleted or hypermethylated at high frequency in cell lines derived from tumors of lung, breast, brain, bone, skin, bladder, kidney, ovary, and lymphocytes (Weisenberger 2014; Gil and Peters 2006).

Analogous to *CDKN2A*, loss of function for the tumor suppressor gene retinoblastoma 1 (*RB1*) at 13q14 has similar effects on promoting G1 progression (Manning and Dyson 2011). As previously stated, homozygous deletions at 13q14 have prognostic significance in a variety of not only epithelial human cancers, but also hematological malignancies (Rowntree et al. 2002; Starostik et al. 1999). In

colon and rectal cancer, mutations and homozygous deletions of the adenomatous polyposis coli gene (*APC*) at 5q22, specially in those patients with familial adenomatous syndrome (FAP), but also in a large percentage of sporadic cancer, play a critical role in the release of cytosolic beta-catenin, enabling it to cross the nuclear membrane and transcriptionally activate the Wnt/β-catenin signaling pathway (Nathke 2004), which leads to increased cellular proliferation.

Of note is the high frequency of focal deletions affecting larger genes in the genome, such as *FHIT*, *WWOX*, *PTPRD*, *MACROD2*, *PARK* and others, as seen by analyzing somatic copy number alterations in primary tumors in across various cancer cohorts. Since the functional and clinical relevance of these deletions remains elusive, there is no solid evidence that these genes are indeed tumor suppressor genes. Nevertheless, it has been suggested that the genomic plasticity of the regions where these genes are located (e.g., the presence of fragile sites) might be a causative force for these events.

8 Implications for Clinical Practice

As extensively discussed above, chromosomal aberrations play a prominent and defining role in many human solid tumors. Due to the continuous evolution of genomic analysis technologies, cancer genomics has moved from being purely a descriptive enumeration of structural and numerical DNA aberrations, and is increasingly being applied to classify cancer entities into different subtypes (Garraway 2013; Garraway and Lander 2013; McClintock 1939; Tran et al. 2012). For example, screening for amplifications of the *MDM2* and *CDK4* genes enables classification of well-differentiated and dedifferentiated liposarcomas (Crago and Singer 2011). Furthermore, cancer genomics are employed to identify genetic alterations that can be targeted therapeutically, and therefore may guide clinicians in choosing rational, molecularly defined treatment strategies (Garraway 2013; MacConaill 2013; Tran et al. 2012).

Similar to the standard of care in hematologic malignancies, NSCLC is the prime example of a solid tumor that should undergo extensive molecular biomarker testing prior to starting any treatment (Rampal and Levine 2013). Apart from (activating) mutations in genes such as *EGFR*, *KRAS*, *BRAF*, *PIK3CA* or *DDR2*, which do not represent chromosomal aberrations, at least five activating alterations are clinically relevant: *ALK*, *RET*, and *ROS1* rearrangements, and *MET* and *FGFR1* amplifications (Li et al. 2013; Oxnard et al. 2013; Shames and Wistuba 2014). For *ALK*, which encodes a transmembrane tyrosine-kinase receptor, oncogenic fusion with one of its upstream activators, *EML4*, has been demonstrated (Soda et al. 2007). While advanced tumors with *EML4*-*ALK* rearrangements are insensitive to EGFR tyrosine-kinase inhibitors (TKIs) often used for treating NSCLC (Shaw et al. 2009), high initial responses have been observed upon treatment with the ALK inhibitor crizotinib (Kwak et al. 2010; Shaw et al. 2013). Nevertheless, acquired resistance to crizotinib frequently develops (Shaw and Engelman 2013), and evidence is

accumulating that this secondary resistance can be overcome with novel ALK inhibitors such as ceritinib (Shaw and Engelman 2014). Several different fusion partners of the gene encoding the tyrosine-kinase receptor ROS1 have been identified (Bergethon et al. 2012; Rimkunas et al. 2012; Takeuchi et al. 2012), and *ROS1*-rearranged tumors may also benefit from treatment with crizotinib (Bos et al. 2013).

Similarly, fusions between the tyrosine-kinase receptor gene *RET* have been reported with *KIF5B*, *CCDC6* or *TRIM33* (Ju et al. 2012; Kohno et al. 2012; Lipson et al. 2012; Takeuchi et al. 2012). Because several TKIs show at least some activity against the *RET* kinase, *RET*-rearranged tumors are amenable to targeted strategies as well (Oxnard et al. 2013). Another prominent alteration involves amplification of the membrane-bound tyrosine receptor gene *FGFR1*, which led to the development of FGFR TKIs (Dutt et al. 2011; Gavine et al. 2012; Weiss et al. 2010; Zhang et al. 2012). Interestingly, *MET* amplifications have been associated with acquired resistance of EGFR-mutated NSCLC to TKIs (Cappuzzo et al. 2009; Dziadziuszko et al. 2012; Toschi and Cappuzzo 2010). Because amplifications in the *MET* gene activate multiple signaling pathways, a number of agents targeting this transmembrane tyrosine-kinase receptor are in preclinical and clinical development (Sadiq and Salgia 2013). Unfortunately, however, it was quickly realized that targeted agents developed for adenocarcinoma, the most common type of lung cancer, were largely ineffective against squamous cell carcinoma, the second most common type of lung cancer.

Apart from NSCLC, there are many other examples of human cancers in which the presence of chromosomal aberrations influences treatment decisions (as stated above, gene mutations are not covered in this chapter). Historically, the detection of gene amplifications or protein overexpression of HER2 in breast cancers was the first example, followed by the clinical implementation of trastuzumab, a monoclonal antibody targeting HER2, which has been the standard of care for HER2-positive breast cancer for more than a decade (Giordano et al. 2014; Hudis 2007; Spector and Blackwell 2009). Since *HER2* amplifications and HER2 protein over expression can be detected in other solid tumors as well (Martin et al. 2014), strategies to target HER2 are increasingly being evaluated in non-breast related cancers (Kasper and Schuler 2014; Okines et al. 2011). Prominently, Bang and colleagues demonstrated improved survival rates for patients with HER2-positive advanced gastric and gastro-esophageal junction cancers that were treated with trastuzumab plus chemotherapy compared with chemotherapy alone (Bang et al. 2010). This led to the initiation of various clinical trials aimed at testing combinations of trastuzumab or other HER2-targeting agents with platinum/fluoropyrimidine-based chemotherapy for patients with HER2-positive cancers (Kasper and Schuler 2014; Okines et al. 2011). Very recent data indicate that targeting HER2 may represent a therapeutic strategy in patients with CRC as well (Conradi et al. 2013; Guan et al. 2014; Seo et al. 2014).

Anaplastic oligodendroglioma (AO) is another example of how a better understanding of the cancer genome influences clinical practice. Patients with co-deletions of chromosome arms 1p and 19q benefit from combined radiotherapy and chemotherapy compared to radiation therapy alone. Consequently, it is now

recommended to screen AO patients for these chromosomal aberrations (Anderson and Gilbert 2013; Polivka et al. 2014).

The last example that will be discussed here are the so called “soft tissue sarcomas” (STS). STS actually represent a heterogeneous group of different subtypes, which are relatively rare, genetically and biologically different, and vary in their treatment responses (Barretina et al. 2010). Interestingly, however, certain subtypes are sensitive to trabectedin, either as single agent or in combination with conventional chemotherapeutics. For instance, trabectedin has shown considerable activity in Ewing sarcoma, Leiomyosarcomas and in myxoid liposarcomas (Grohar and Helman 2013; Sharma et al. 2013). In myxoid liposarcomas, trabectedin appears to block the trans-activating ability of the fusion proteins FUS-CHOP or EWS-CHOP, which act as transcription factors in this disease (D’Incalci et al. 2014; Di Giandomenico et al. 2013). These proteins represent the causative abnormality and are the result of translocations that fuse the *CHOP* gene, located at 12q13.1–q13.2, with either *FUS*, located at 16p11.2 (t(12;16)), or *EWS*, located at 22q12.2 (t (12;22)).

Nevertheless, cancer genomics also poses several substantial and critical challenges (Buettner et al. 2013; Dienstmann et al. 2013; Garraway and Lander 2013; Wistuba et al. 2011), a few of which are listed below: *First*, even the most frequent alterations represent only about 20 % of NSCLC, with each individual rearrangement being rather rare. Even more, *EGFR* and *ALK* alterations predominantly occur in lung adenocarcinomas, while *FGFR1* amplifications prevail in squamous cell carcinomas (Cancer Genome Atlas Research Network 2012; Seo et al. 2012). So which of these aberrations should be tested? While there is consensus that molecular testing of advanced and metastatic NSCLC with adenocarcinoma histology should include *EGFR* mutations and *ALK* rearrangements, other targetable biomarkers are excluded (Dacic 2013). *Second*, and particularly problematic for NSCLC, is the fact that many tumors are either de novo resistant against molecular-based therapies, or acquire resistance relatively quickly during treatment (Camidge et al. 2014). Accordingly, simultaneous testing of multiple genetic/chromosomal aberrations would be meaningful. *Third*, in cases in which resistance develops, should an additional biopsy of the residual tumor be performed so that molecular testing can be repeated? Monitoring cancer genetics through genotyping of circulating tumor cells or DNA in the bloodstream, i.e., liquid biopsy sampling, might help to overcome this problem (Crowley et al. 2013; Diaz and Bardelli 2014). *Fourth*, in situations where multiple genetic aberrations should be analyzed simultaneously, is there enough material available? *Fifth*, the best detection method is still a matter of debate. Among “classical” techniques such as FISH and IHC, next-generation sequencing would be an alternative. Multiplex PCR testing would be another option to obtain maximum diagnostic information from limited tissue. *Sixth*, is molecular testing standardized and robust enough to serve as the basis for clinical decisions, i.e., the choice of therapeutic agents? *Finally*, and perhaps the most problematic as it affects all of the prior points, intratumor heterogeneity can infer with both the assessment and the interpretation of the tumor genomics landscape (Swanton 2012).

Despite these challenges and problems, recent technological advances and innovations now offer the possibility to comprehensively interrogate essentially every relevant genetic/chromosomal aberration within the genome of individual cancer patients. Molecular testing has already led to more accurate classification of cancer subtypes, and to better guidance with respect to clinical decision-making. Therefore, cancer genomics has finally come of age, from being a purely academic endeavor used for understanding the underlying tumor biology to soon becoming part of standard medical practice for informing clinical decisions in the therapeutic choices for patients with solid tumors.

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Yeast as Models of Mitotic Fidelity

Eduardo Torres

Abstract

Chromosome missegregation leads to aneuploidy which is defined as the cellular state of having a chromosome count that is not an exact multiple of the haploid number. Aneuploidy is associated with human diseases including mental retardation, neurodegenerative diseases and cancer. In addition, aneuploidy is the major cause of spontaneous abortions and its occurrence increases with aging. Therefore, it is important to understand the molecular mechanisms by which cells respond and adapt to aneuploidy. *Saccharomyces cerevisiae* has proven to be a good model to study the effects aneuploidy elicits on cellular homeostasis and physiology. This chapter focuses on the current understanding of how the yeast *S. cerevisiae* responds to the acquisition of extra chromosomes and highlights how studies in aneuploid yeasts provide insights onto the effects of aneuploidy in human cells.

Keywords

Aneuploidy · Reactive oxygen species · Proteotoxic stress · Genomic instability

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1 Introduction

Chromosome missegregation leading to aneuploidy during human development is almost always lethal. Only trisomies of chromosomes 13, 18 and 21 responsible for Patau's, Edwards and Down syndrome, respectively, survive to birth (Nagaoka et al. 2012). Down syndrome patients are the only ones that live past the first months of life. However, they suffer from several pathological conditions including mental retardation, stunted growth and increased risk for heart disease, diabetes and leukemias (Antonarakis et al. 2004; Hasle et al. 2000). The incidence of aneuploidy increases with aging and is frequently observed in neurons of Alzheimer's disease patients (Iourov et al. 2009, 2011). Aneuploidy in somatic cells has been observed but the fate of these cells or whether they play a role in human diseases or alternatively, in the physiology of healthy individuals is unclear (Duncan et al. 2012; Rehen et al. 2005; Yurov et al. 2007). Notably, aneuploidy is an almost universal feature of tumor cells, a hallmark of which is unlimited growth capacity (Holland and Cleveland 2009). Although the role of aneuploidy in cancer remains a topic of hot debate, it is evident that its occurrence and complexity increases with tumor progression and correlates with malignancy.

A major hurdle in studying the role of aneuploidy in cancer is the fact that significant karyotypic heterogeneity exists, not only among different tumors but also within cells of the same tumor (Albertson et al. 2003; Mosoyan et al. 2013). In addition, tumors and cancer cell lines not only display varying degrees of aneuploidy but also harbor other complex genomic rearrangements such as focal amplifications and deletions, chromosomal translocations and thousands of mutations (Beroukhim et al. 2010; Kandoth et al. 2013; Roschke et al. 2003; TCGA 2012). It is therefore difficult to pinpoint common characteristics or phenotypes of aneuploid cells. An important question that begs attention is whether specific signaling pathways or cellular processes are affected by aneuploidy. Addressing this question is important because exploiting aneuploidy-specific properties of cancer cells could lead to novel therapeutic approaches that would specifically target these cells while sparing the euploid counterparts.

Optimally, to study the effects of aneuploidy on cellular physiology, a series of isogenic cell lines characterized by distinct aneuploidies yet devoid of other genomic alterations is required. The budding yeast *Saccharomyces cerevisiae* provides such a system, and is therefore ideal to model aneuploidy. This chapter summarizes studies performed in aneuploid budding yeasts that demonstrate how aneuploidy disrupts cellular physiology and homeostasis. Because many cellular processes affected by aneuploidy in yeast are highly conserved in human cells, studies of aneuploid yeasts contribute significant insights into the role aneuploidy in human diseases.

2 Generating Aneuploid Yeasts in A Common Genetic Background

Saccharomyces cerevisiae consists of 16 or 32 chromosomes in the haploid ($1n$) or diploid state ($2n$), respectively. Several studies have shown that acquisition of an entire set of chromosomes, or polyploidy, is well tolerated in yeast (discussed elsewhere) (Andalis et al. 2004). Three different approaches have been utilized to generate aneuploid yeast strains in a homogeneous genetic background (Table 1). One approach utilizes chromosome transfer followed by selection to generate haploid yeast cells harboring an extra copy of a single chromosome (henceforth disomes) (Conde and Fink 1976; Hartwell et al. 1982). Indeed, a total of 13 out the possible 16 disomes were generated using this strategy (Torres et al. 2007). Because aneuploidy increases chromosomal instability (discussed below), aneuploid yeast strains generated by this method are maintained under selection. Interestingly, four additional karyotypic stable strains were obtained that carry one or two extra chromosomes in addition to the one that was selected for. To circumvent potential artifacts of selection in studying the effects of aneuploidy on cellular physiology, disomes can be grown in non-selective media for a small number of generations while maintaining their original karyotype. A second approach to generate aneuploid yeasts is through meiosis of triploid ($3n$) or pentaploid ($5n$) strains. Segregation of homologous chromosomes during meioisis I of these polyploid strains happens randomly, thus generating various aneuploidies. Aneuploid strains recovered from this method show reduced viability (15–50 %) and high genomic instability leading to heterogeneous populations (Parry and Cox 1970; Pavelka et al. 2010; St Charles et al. 2010; Zhu et al. 2012). Nonetheless, a total of 38 semi-stable aneuploid strains have thus far been recovered and their phenotypic properties analyzed (Pavelka et al. 2010). Of these, five strains were characterized in depth. Using a third approach missegregation of a single chromosome can be induced by centromere silencing (Anders et al. 2009; Reid et al. 2008). In haploid yeast, this method generates unviable monosomies and disomic strains. The latter strains were utilized to map genetic traits to specific chromosomes but other phenotypes were not analyzed. In diploids, centromere silencing of one of the homologous chromosomes generates trisomies ($2n + 1$) and monosomies ($2n - 1$). Of note,

Table 1 Methods to generate aneuploid yeast strains

Wild-type	Aneuploid strains	Method	Notes
Haploid $(1n)$16 chr	Monosomies $(n - 1)$	Centromere silencing (CS)	Inviable
	Disomies $(n + 1)$	CS, Chromosome transfer (CT) and meiois of 3n or 5n (RM)	12 disomes from CT characterized in depth
	Multiple disomies $(1n + x)$, $(x = 2 - 6)$	CT and RM	5 strains from RM characterized in depth
	Haploid $(n + \text{YAC})$	CT	Yeast artificial chromosome(YAC) with mammalian DNA
Diploid $(2n)$32 chr	Monosomies $(2n - 1)$	CS	Unstable, endoduplication
	Trisomies $(2n + 1)$	CS, CT and RM	
	Multiple trisomy $(2n + x)$, $(x = 2 - 8)$	RM	

monosomies recovered from this method were shown to be unstable as colonies from these strains were heterogeneous in size and endoduplication of the entire missing chromosome was frequently observed (Reid et al. 2008). Because monosomies are either lethal or unstable, studies of the effects of aneuploidy on cellular physiology have mainly focused on the consequences of one aspect of aneuploidy, i.e. the gain of extra chromosomes. In summary, in addition to being facile and quick, a major advantage of these approaches is that different aneuploid strains can be generated in an isogenic background, making it possible to assess the effects of aneuploidy on cellular physiology, in the absence of other genomic alterations.

3 Aneuploidy Hampers Cellular Proliferation

A simple hypothesis that could explain the detrimental effects of aneuploidy during human development and the uncontrollable proliferation of cancer cells is that aneuploidy leads to increased fitness and promotes proliferation at the cellular level. During development, these properties would allow aneuploid cells to circumvent regulatory mechanisms, such as senescence and apoptosis, required for normal development. In cancer, increased cellular proliferation and resistance to death are indeed hallmarks of the disease (Hanahan and Weinberg 2011). However, studies in aneuploid yeast suggest that the opposite occurs, at least under normal (non-stress) conditions; that is, without exceptions, acquisition of an extra chromosome inhibits

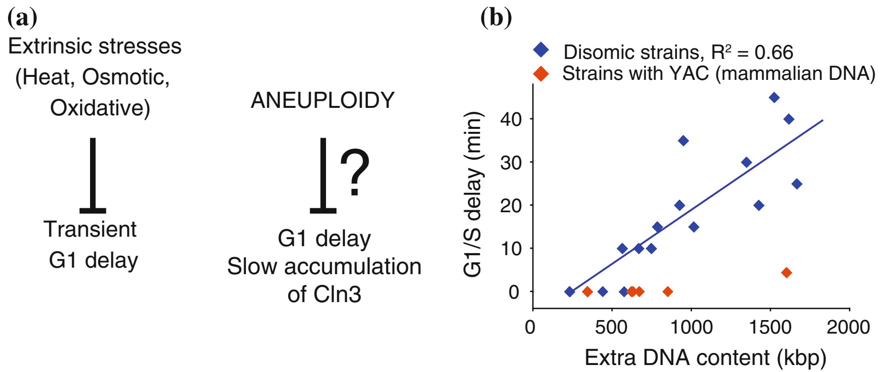

Fig. 1 Aneuploidy causes cell cycles delay in manner similar to extrinsic stresses (**a**), the G1 delays observed in aneuploid yeast strains correlate with the size of the extra chromosome they harbor (**b**)

cell cycle progression and lowers cell viability, independent of the identity of the gained chromosome (Fig. 1). Impaired proliferation of aneuploid yeasts is not simply due to the presence of extra DNA, as yeast strains harboring yeast artificial chromosomes (YAC) of comparable sizes to the yeast chromosomes harboring human or mouse DNA do not show cell cycle delays (Torres et al. 2007). These results indicate that the presence of the extra yeast genes and their products is responsible for the proliferation defects. Interestingly, the degree to which proliferation is affected in aneuploid yeast cells correlates with the number of open reading frames (ORF) encoded by the extra chromosome, underscoring the idea that aneuploidy is a problem of genomic imbalance. Earlier studies in plants and flies showed that the detrimental effects of acquiring extra chromosomes correlate with the size of the extra genomic material (Blakeslee et al. 1920; Lindsley et al. 1972). In humans, Chromosome 21 encodes the least number of proteins compared to other autosomes, possibly explaining why trisomy 21 is the only viable one.

The molecular mechanisms by which aneuploidy hampers cellular proliferation are not well understood. Studies of disomic yeasts synchronized in G1 revealed that the transition through G1/S-phase during cell cycle progression was the one most affected by aneuploidy (Torres et al. 2007). Subsequent studies of small G1 cells isolated by elutriation showed that disomic yeasts display a cell cycle entry delay due to a slowed accumulation of the G1 cyclin *CLN3* (Thorburn et al. 2013). Recently, acetyl-CoA was shown to control *CLN3* transcription by promoting the acetylation of histones present in its regulatory region raising the possibility that altered metabolism may be responsible for the cell cycle delays in aneuploid yeast (Shi and Tu 2013). However, in aneuploid yeast cells *CLN3* transcript levels do not seem to be affected, indicating that posttranscriptional mechanisms are responsible for the delayed Cln3 protein accumulation (Thorburn et al. 2013). It is noteworthy, that independent of the nature of extrinsic cellular stresses, yeast cells usually arrest in G1 (Belli et al. 2001; Lee et al. 1996; Rowley et al. 1993). This implies that the

same molecular mechanisms that arrest yeast cells in response to extrinsic stresses may play a role in mediating cellular responses to aneuploidy. Several pathways and factors control Cln3 protein levels during G1 including nutrients, Map kinase and Ras-cyclic AMP signaling pathways (Johnson and Skotheim 2013). Which of these pathways is/are affected by aneuploidy remains to be investigated. The effects on cell cycle progression in aneuploid yeast cells isolated from random meiosis have not been studied, yet 38 of such strains proliferate slower than their euploid counterparts (Pavelka et al. 2010).

Studies of aneuploid yeasts suggest that in the absence of other genomic alterations, aneuploidy inhibits proliferation of mammalian cells. Consistent with this hypothesis, embryonic fibroblasts from Down syndrome patients proliferate slower and accumulate less biomass than fibroblasts obtained from healthy individuals (Rosner et al. 2003; Segal and McCoy 1974). Similarly, primary mouse cells and immortalized human cell lines harboring an extra chromosome show impaired proliferation (Stingele et al. 2012; Williams et al. 2008). Interestingly, in a study that induced aneuploidy in near diploid cancer cell line by chromosomal missegregation, cell cycle arrest occurred in G1 in a manner dependent on p53 (Thompson and Compton 2010). This implied a possible role for this tumor suppressor, whose protein levels are induced by a myriad of cellular stresses (Zilfou and Lowe 2009), in the anti-proliferative effects of aneuploidy.

In summary, aneuploidy seems to inhibit cell cycle progression from yeast to humans, similar to other cellular stresses. However, the specific signaling pathways mediating this effect still remain to be explored. Nevertheless, a major implication of the aneuploidy studies described above is that other genetic alterations or environmental factors must exist to allow cancer cells to overcome the aneuploidy-induced growth inhibitory effects.

4 Transcriptional Responses to Aneuploidy

The fact that yeast strains harboring YAC with human or mouse DNA do not show cell cycle defects indicates that expression of the yeast genes present on the extra chromosome is responsible for the hampered proliferation of aneuploid yeasts. Indeed, several studies have reported an increase in the levels of mRNAs encoded by the extra chromosomal genes in aneuploid cells (Fig. 2). Gene expression analysis of 17 aneuploid strains generated by chromosome transfer and five aneuploid strains recovered from random meiosis showed that on average, levels of mRNAs encoded by genes on the additional chromosome proportionally increase with gene copy number (Pavelka et al. 2010; Torres et al. 2007). The correlation between gene copy number and mRNA expression is so strong that gene expression profiling alone can reveal changes in chromosome numbers (Fig. 2). For example, gene expression analysis of hundreds of yeast strains harboring a single gene deletion revealed widespread aneuploidy among them (22 of 290) (Hughes et al. 2000). Several studies also indicate that the correlation between increased gene

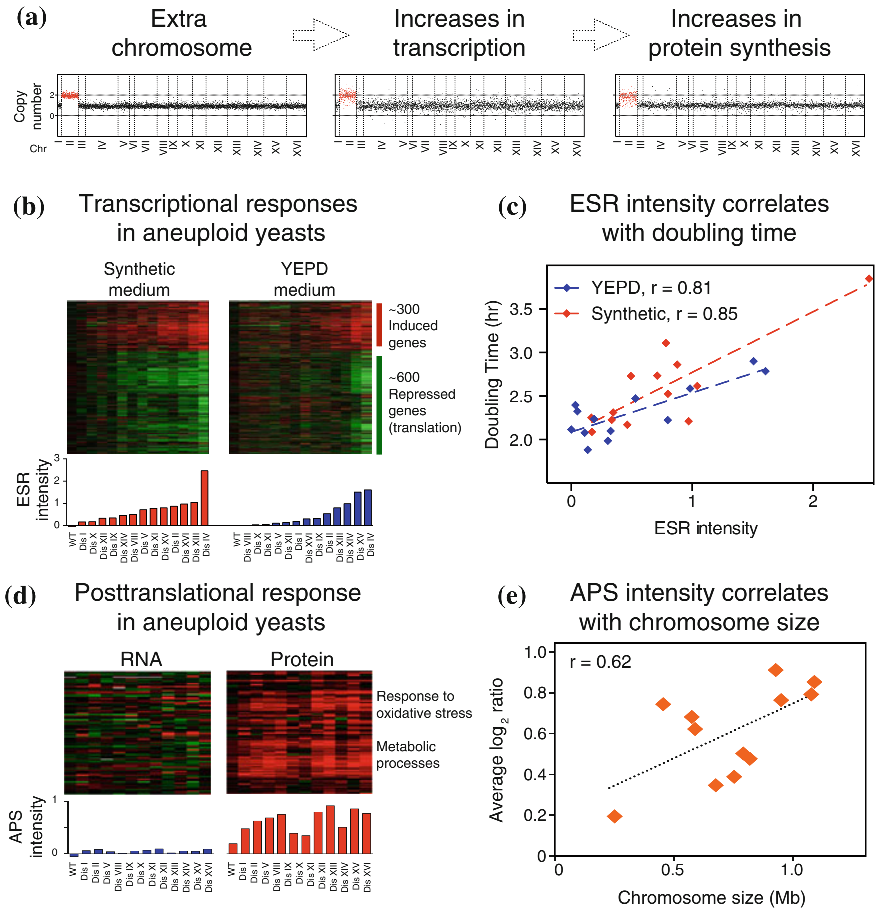

Fig. 2 **a** Relative DNA, mRNA and protein content of cells harboring an extra copy of chromosome II (disome II). Data obtained from Torres et al. (2007) and Dephoure et al. (2014). **b** Aneuploid elicits a transcriptional response similar to the environmental stress response. **c** The intensity of this response correlates with proliferation rate (*r* = Pearson correlation). Data obtained from Torres et al. (2007). **d** Aneuploid cells show an aneuploidy-specific protein signature (APS) associated with altered metabolism and redox stress. This signature is mediated by posttranslational mechanisms and its intensity correlates with the size of the extra chromosome present in aneuploid yeast strains (*r* = Pearson correlation)

copy number leading to changes in gene expression is also true in trisomic mouse embryonic fibroblasts, trisomic human cell lines and aneuploid cancer cells (Crawley and Furge 2002; Hyman et al. 2002; Stingele et al. 2012; Williams et al. 2008). Despite this correlation, the possibility that mRNA levels of a small number of genes do not scale up with gene copy number and are subject to dosage compensation through gene silencing or posttranscriptional mechanisms remains to be investigated. While genome-wide approaches are very powerful at revealing general

patterns of gene expression, a more gene-centric approach will be necessary to unveil the identity of such genes. Nonetheless, a major conclusion from these studies is that while dosage compensation mechanisms play a key role in regulating gene expression of sex chromosomes, such mechanisms do not seem to be activated upon acquisition of an extra copy of an autosome. In support of this hypothesis, the introduction of a single copy of the gene XIST, X-inactivation gene, on Chromosome 21 was shown to silence transcription and suppress several phenotypes, including deficits in proliferation in pluripotent stem cells of down syndrome patients (Jiang et al. 2013).

In addition to changes in mRNA resulting from increased gene copy number, aneuploidy elicits a transcriptional response similar to that of cells exposed to cellular stresses, such as heat shock, oxidative or osmotic stress, among others (Gasch et al. 2000) (Fig. 2b). This transcriptional signature referred to as the environmental stress response (ESR) encompasses approximately 600 downregulated and 300 upregulated genes. The common denominator between cellular responses to extrinsic stresses is transient cell cycle arrest. In fact, gene expression profiling of wild-type yeast grown in chemostats, where the proliferation rate can be adjusted by changing dilutions rates of different nutrients, showed a significant overlap between genes that respond to stress and those whose expression levels change as a function of proliferation rate (Brauer et al. 2008). For example, ribosomal genes are downregulated upon several stresses and change their expression levels as a function of proliferation rates. This raises the question of whether aneuploid cells display a common gene expression signature due to their impaired proliferation or to induced cellular stress. Because stress responses are tightly coupled with cell cycle arrest this is a difficult question to answer. Nonetheless, there is a strong correlation between the intensity of the ESR in disomic yeasts, measured as the weighted average of up-regulated and down-regulated genes, and cell cycle delays (Fig. 2c). Comparison of gene expression changes in disomic strains grown in selective medium versus rich medium indicates that the slow growth signature is ameliorated in rich media (Fig. 2b, c). Because the differences in proliferation rates between disomic strains and wild-type cells are less in rich media than in selective medium, the slow growth expression signature seems to be partly a function of proliferation. Consistent with this hypothesis, euploid cells harboring gene mutations that confer proliferation defects show similar gene expression patterns (Torres et al. 2007). The slow growth expression signature is also present in several aneuploid strains isolated from random meiosis, which proliferate slower than the euploid cells (Pavelka et al. 2010; Sheltzer et al. 2012). Importantly, similar patterns of gene expression have been observed in aneuploid fission yeast, plants, mouse, and human cells (Sheltzer et al. 2012). Altogether, these studies indicate that aneuploidy hampers cellular proliferation resulting in transcriptional changes similar to those observed in cells affected by extrinsic cellular stresses or in cells that experience hampered proliferation.

5 Aneuploidy Alters Proteome Content of The Cell

A key question that arises from transcriptional analysis is whether changes in mRNAs proportionally translate into changes in protein levels. Understanding how changes in gene expression affect the proteome content of aneuploid cells is of particular importance, as it could reveal novel cellular responses to aneuploidy mediated by posttranscriptional mechanisms.

Quantitative proteome analyses indicate that on average, increases in gene copy number lead to proportional increases in protein levels in all aneuploid strains analyzed (Fig. 2a). Importantly, these results are independent of the method used to generate the aneuploid strains, the growth conditions implemented, or mass spectrometry method utilized to quantify the proteomes. Utilizing stable isotope labeling of amino acids in cell culture (SILAC) and liquid chromatography-mass spectrometry, quantification of protein abundances in 12 different disomic strains revealed quantitative information for ~70–80 % of all verified open reading frames (ORFs) relative to wild-type cells (Dephoure et al. 2014). Specifically, it was shown that on average, the levels of proteins encoded by the additional chromosome increase by an approximate 2-fold. These results were reproduced when aneuploid cells were grown in rich media and their proteome quantified utilizing a different mass spectrometry approach referred to as isobaric tandem mass tag (TMT)-based quantitative mass spectrometry (Dephoure et al. 2014). A similar conclusion was reached using yet a another technique, multidimensional protein identification technology or MudPIT, to analyze the proteome content of five aneuploid cells isolated from random meiosis (Pavelka et al. 2010). Altogether, these analyses indicate that a major consequence of gaining an extra chromosome is increased protein synthesis. Therefore, gaining an extra chromosome leads not only to cellular imbalances due to the extra activity of the proteins encoded on the duplicated chromosome but also to increased burden on the protein quality control pathways including protein synthesis, folding and turnover (see below). In addition, because much of ATP utilization in the cell is devoted to protein synthesis, aneuploidy causes a significant demand for energy, leading to a disruption in cellular metabolic processes, independent of the identity of the chromosome gained.

6 Subunits of Macromolecular Complexes are Significantly Attenuated in Aneuploid Cells

Proteome quantification of disomic yeast strains revealed that despite the general correlation between increased gene copy number, mRNA and protein levels, approximately 20 % of the proteins encoded by the genes located on duplicated chromosomes do not scale up with copy number of their corresponding genes (Fig. 3) (Dephoure et al. 2014). Neither growth conditions nor the quantitative approach utilized affected the degree of attenuation, because quantification of protein levels in aneuploid cells grown in selective or rich media, that were

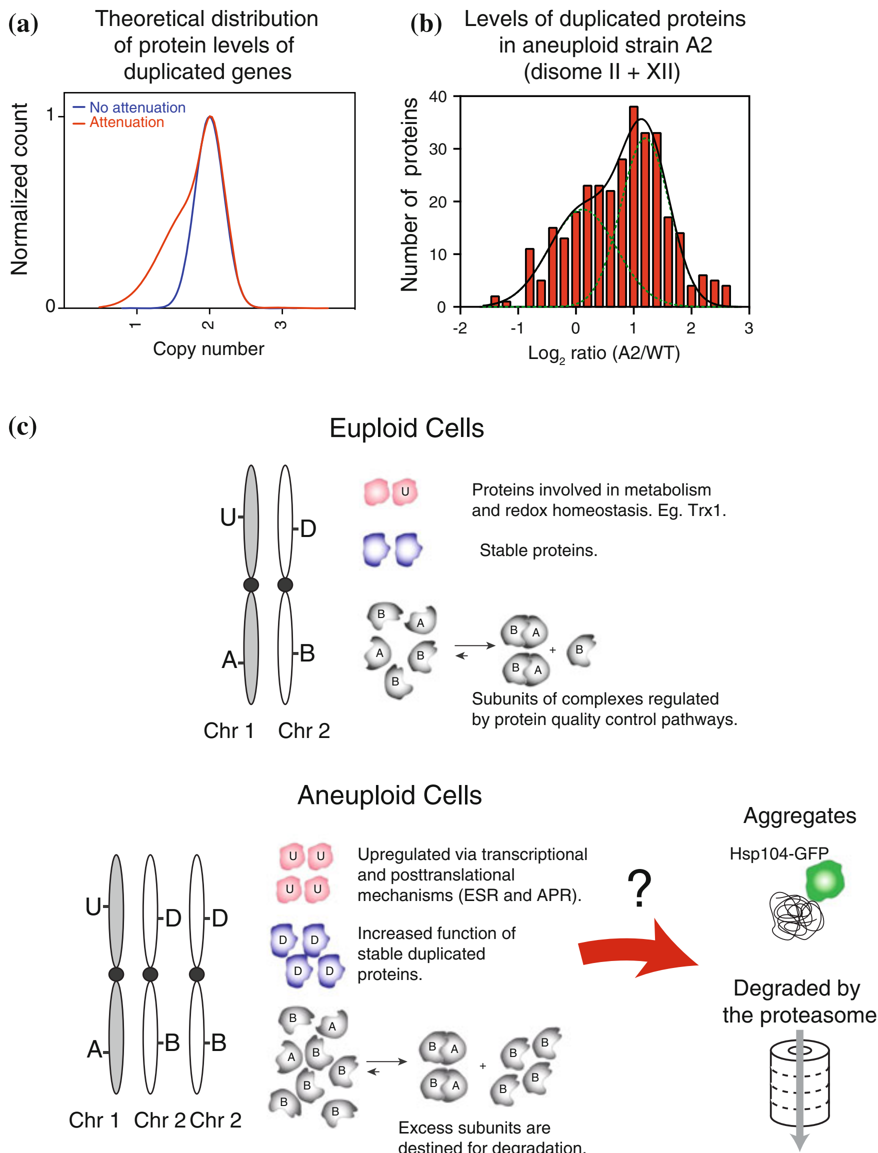

Fig. 3 **a** Theoretical distribution of protein levels upon gaining an extra chromosome. **b** Distribution of the levels of duplicated proteins in aneuploid strain *A2* (Pavelka et al. 2010). **c** Schematic of the consequences of increased protein synthesis in aneuploid cells. *U* represent proteins that are upregulated by transcriptionally (ESR) or posttranslationally (APS) mechanisms; *D* represent proteins whose levels proportionally correlated with gene copy number; *A* and *B* represent subunit of complex *AB*. Excess proteins could either form protein aggregates or be degraded by protein degradation pathways

analyzed via two distinct mass spectrometry approaches showed reproducible results. In addition, proteome analysis of five aneuploid strains generated by random meiosis showed that 23–38 % of duplicated proteins were significantly attenuated (Fig. 3b). These results indicate that although acquisition of an extra chromosome leads on average, to 2-fold increases in protein levels of the added genes, a large and statistically significant number of proteins do not increase proportionally with copy number. Interestingly, analysis of the identity of the attenuated proteins revealed that most of the attenuated proteins are subunits of macromolecular complexes. Enrichment of subunits of macromolecular complexes among the attenuated proteins was observed in every aneuploid strain analyzed, including 12 disomic strains generated by chromosome transfer and five aneuploid strains generate by random meiosis.

Protein attenuation of subunits of macromolecular complexes is mainly mediated by posttranslational mechanisms, most likely protein degradation. Indeed, ribosomal footprinting analysis of two disomic strains demonstrate similar translation efficiencies for the proteins encoded by the duplicated genes, independent of whether the cellular protein levels were attenuated (Dephoure et al. 2014; Thorburn et al. 2013). Nevertheless, translational control may play a role in the attenuation of a small subset of genes and remains to be investigated. In summary, these results provide direct evidence that a major consequence of aneuploidy is increased protein degradation.

A hypothesis that could explain the attenuation of the protein levels of duplicated genes is that protein stability of individual subunits is dependent upon their ability to be incorporated into multi-subunit molecular assemblies (Fig. 3c). Cells have evolved several mechanisms that facilitate complex assembly such as co-transcriptional regulation and dedicated chaperone systems that help stabilize unstable subunits so as to prevent their degradation [e.g. (Burgess and Zhang 2013; Kunjappu and Hochstrasser 2014; Xie and Varshavsky 2001)]. Recently, analysis of protein synthesis in *Escherichia coli* and yeast showed that subunits of macromolecular complexes are produced in equimolar amounts through similar synthesis rates (Li et al. 2014). Acquisition of an extra chromosome disrupts such modes of regulation, leading to the production of excess subunits that cannot be assembled into stable complexes, and are therefore degraded. Consistently, analysis of the ribosome and the nucleosome subunits indicate that these proteins are short-lived unless assembled into their proper complexes (elBaradi et al. 1986; Gunjan and Verreault 2003; Meeks-Wagner and Hartwell 1986; Tsay et al. 1988). In aneuploid yeast, analysis of protein levels of duplicated subunits of several macromolecular complexes showed a large range of subunit stabilities. Remarkably, almost every complex analyzed contains at least one or more subunits that are degraded when produced in excess. Conversely, most macromolecular complexes with the exception of the nucleosome and ribosome contain at least one subunit that appears to be stable on its own. These results indicate that for most complexes, a stable scaffold protein may be required for complex assembly. Importantly, attenuation of subunits of macromolecular complexes was also observed in immortalized human cell lines harboring extra chromosomes (Stingele et al. 2012). These results indicate

that, from yeast to humans, acquisition of extra chromosomes leads to increased synthesis of unstable proteins that can create a burden on the protein folding and degradation pathways of the cell.

7 Cellular Responses to Aneuploidy Are Mediated by Transcriptional and Posttranscriptional Mechanisms

Proteome analysis of aneuploid strains revealed that transcriptional changes in aneuploid yeast associated with the ESR/slow growth signature translate into changes in protein levels. As a result, downregulation of ribosomal genes, which is a major part of the ESR/slow growth response, leads to lowered ribosome levels in the aneuploid strains. Ribosomal footprinting of 2 disomic strains do not show any signs of impaired translation. Therefore, the decreased ribosomal protein levels observed in aneuploid cells while maintaining seemingly normal efficiencies of protein synthesis may account for the hypersensitivity of aneuploid cells to drugs that target the translation machinery.

In addition to changes in protein levels resulting from transcriptional changes, clustering analysis revealed an additional signature of upregulated proteins common among all aneuploid yeast strains analyzed (Fig. 2d, e) (Dephoure et al. 2014). This novel protein signature, termed the aneuploidy-specific protein signature (APS), was observed in 12 disomic strains generated by chromosome transfer and five aneuploid strains obtained from random meiosis. Gene ontology enrichment analysis revealed that the APS proteins are associated with cellular responses to oxidative stress and metabolic processes such as amino acid biosynthesis and cellular bioenergetics. Interestingly, the intensity of the APS as measured by the average increase of its 92 proteins, correlates with the size of the additional chromosome, indicating that it may be a direct consequence of the cellular imbalances resulting from the acquisition of an extra chromosome. Surprisingly, the corresponding mRNA transcripts for most of the upregulated proteins are not increased indicating that the upregulation in protein levels is mediated posttranscriptionally. These results indicate that aneuploidy causes alterations in redox homeostasis and metabolism (discussed below). An important question that remains to be investigated is to identify the molecular mechanism by which the APS proteins are upregulated. Ribosome footprinting analysis of disomic strains for chromosome V and XVI suggest that posttranslational mechanisms mediate upregulation of the APS proteins as no increases in translation efficiencies were detected. Therefore, posttranslational modifications may certainly play a role as most of the 92 APS proteins have been shown to be ubiquitinated or phosphorylated. Lastly, these results highlight the importance of proteomic studies to potentially identify novel biomarkers of aneuploid cells not detected by transcriptional analysis alone that could be exploited therapeutically for the detection as well as targeting of aneuploid cancer cells (Hanash and Taguchi 2010).

8 A Major Consequence of Aneuploidy is Increased Burden on the Protein Quality Control Pathways

Gaining extra chromosomes lead to increased protein synthesis, folding and turnover. As a result, aneuploid cells experience an increased burden on the protein quality control pathways leading to proteotoxic stress. Several lines of evidence support this hypothesis. Aneuploid cells are hypersensitive to high temperature, to translation inhibitors such rapamycin and cycloheximide, to the Hsp90 chaperone inhibitor geldanamycin, and to the proteasome inhibitor MG132. Introducing a conditional loss-of-function allele of the proteasome lid subunit Rpn6 encoding gene is synthetically lethal in two disomic strains and significantly decreases the proliferative abilities of all several other disomic strains. In addition, aneuploid yeast cells are prone to aggregate formation of endogenous proteins as well as ectopically expressed hard-to-fold proteins (Oromendia et al. 2012). Most of the signs of proteotoxic stress are present in aneuploid strains generated by chromosome transfer or recovered from random meiosis. Importantly, the identity of the endogenous proteins that are prone to aggregation is unknown. An interesting hypothesis that is yet to be investigated is that aggregate formation caused by increased synthesis of proteins encoded by the duplicated genes could lead to the sequestration of other abundant cellular proteins with essential housekeeping functions, thereby causing a disruption in cellular homeostasis (Olzscha et al. 2011).

Interestingly, despite the lack of enrichment for cellular processes associated with proteotoxic stress in the ESR/slow growth signature or the APS, several proteins involved in protein quality control are upregulated in all aneuploid strains analyzed. These include regulators of chaperone activity, protein folding, ubiquitination, and protein trafficking. The upregulation of these proteins is consistent with an increased demand of these processes in aneuploid cells but their functional consequences remain to be studied. In support of an increased demand for protein degradation, the proteasome levels in aneuploid strains showed a small but significant increase compared to wild-type cells.

Other evidence in support of proteotoxic stress being a major consequence of aneuploidy comes from the identification of genetic alterations that ameliorate the detrimental consequences of aneuploidy (Torres et al. 2010). Several mutations in genes that regulate protein turnover were identified in evolved aneuploid cells that show improved fitness. In particular, mutations that introduce early stop codons in the ubiquitin specific protease *UBP6* (see below), as well as mutations in two E3 ligases, *RSP5* and *UBR1,* and in *RPT1*, an essential subunit of the proteasome, were identified. These results indicate that several of these mutations may improve the fitness of aneuploid cells by helping them cope with protein imbalances associated with aneuploidy. Altogether, these studies in aneuploid yeast raise the possibility that aneuploid cancer cells experience profound proteotoxic stress. The increased reliance of aneuploid tumor cells on the protein turnover pathways provide the

rational for utilizing already approved proteasome inhibitors, such as Bortezomib, and point to the development of novel approaches to target protein degradation pathways in cancer cells.

9 Aneuploidy Alters Cellular Metabolism

Several lines of evidence indicate that metabolic processes are affected in aneuploid cells. Gaining an extra chromosome leading to more protein synthesis increases the demand for biomass production and ATP synthesis. In fact, increased burden on cellular bioenergetics may explain several phenotypes associated with aneuploidy. When compared to wild-type cells, disomic yeast strains exhibit increased glucose uptake yet achieve a growth plateau at a smaller population size (measured by optical density at 600 nm). Aneuploid strains lose viability after prolonged culturing in stationary phase, indicating that these cells cannot survive for long periods of time under low glucose conditions (Torres et al. 2007). In addition, intracellular ROS levels are elevated in disomic yeast strains, and proteomic analyses suggest that aneuploid cells respond to these increases by maintaining elevated levels of proteins associated with redox stress (Dephoure et al. 2014). However, the source of the elevated intracellular ROS levels remains to be determined. Interestingly, strains harboring YACs with human or mouse DNA do not show increases in ROS levels, indicating that increased DNA content is not responsible for this phenotype. One possibility is that increased protein translation, folding and turnover creates high demand for ATP, leading to an accumulation of ROS (Gorrini et al. 2013). Increased protein folding leading to endoplasmic reticulum stress could also contribute to ROS accumulation (Tu and Weissman 2002). Another yet not mutually exclusive possibility is that altered metabolism due to the upregulation of anabolic processes alters redox homeostasis in aneuploid cells (Gorrini et al. 2013). The fact that the average increase in levels of the APS, which includes ROS-associated proteins, strongly correlates with the size of the extra chromosome in the disomes, suggests that this response may be a direct consequence of the acquisition of extra genes. Importantly, the functional consequences of higher intracellular ROS levels urge the investigation as to whether it constitutes a mechanism through which aneuploidy induces genomic instability.

10 Aneuploidy Induces Genomic Instability

How frequently chromosomes are missegregated in vivo is an important and difficult question to address in humans. It is clear that the occurrence of aneuploidy varies depending on the tissue or cell type analyzed. Moreover, the fate of the aneuploid cells in vivo is unknown. Given that aneuploidy inhibits proliferation and lowers viability, aneuploid cells without further chromosomal alterations will most likely not hyperproliferate or survive. However, if they do survive, an important question that arises is whether aneuploidy on its own leads to increased genomic

instability thereby promoting other genomic alterations that might improve cellular fitness, and potentially lead to neoplastic transformation. Studies in yeast suggest that this is indeed a possibility, as aneuploid yeasts display increased genomic instability compared to wild-type cells.

Studies of disomic yeast strains generated by chromosome transfer showed that most of these strains displayed increased rates of gene mutations, chromosome losses or homologous recombination events compared to wild-type cells (Sheltzer et al. 2011). Aneuploid cells recovered from random meiosis also show high chromosomal instability as they tend to lose or gain chromosomes (Zhu et al. 2012). Whether recombination or mutation rates are also increased compared to euploid cells in the latter strains remains to be determined. Consistent with aneuploidy causing genomic instability, aneuploid yeast strains are sensitive to a variety of drugs that induce genotoxic stress. The mere presence of extra DNA does not seem to cause genomic instability in yeast because cells harboring YACs with human or mouse DNA do not show similar phenotypes. Nevertheless, the molecular mechanisms by which aneuploidy increases different aspects of genomic instability in each disomic strain remains unclear. Increases in intracellular ROS could certainly lead to increased genomic instability. Interestingly, the genomic instability traits observed in aneuploid yeast do not seem to correlate with the size of the extra chromosome, indicating that genomic instability could be due to aneuploidy-induced imbalances in protein stoichiometry. Proteotoxic stress caused by introducing toxic amino acid analogs, inhibition of the chaperone machinery or proteasome inhibition have been shown to induce genomic instability in yeast (Chen et al. 2012; Shor et al. 2013). Therefore, a plausible explanation for the increased genomic instability observed in aneuploid strains is altered metabolism and/or proteotoxic stress. Direct evidence could come from analyzing genomic instability phenotypes of aneuploid cells harboring genomic alterations that suppress proteotoxic or redox stress.

11 Genomic Alterations That Suppress Aneuploidy-Associated Phenotypes

Yeast cells grown under stress conditions usually evolve and acquire genomic alterations leading to improved fitness under that particular stress. For example, cells grown under low glucose conditions amplify their high affinity glucose transporters (Dunham et al. 2002). Cells grown in the presence of drugs acquire mutations in the target genes leading to resistance (Hill et al. 2013). Due to increased genomic instability, disomic strains grown in medium that select for the presence of the extra chromosome quickly evolve and improve their proliferation rates (Fig. 4). Karyotypic analysis of 52 evolved disomic yeast strains showed that the majority of evolved strains maintained the extra chromosome suggesting that genomic alterations other than chromosome loss are responsible for the improved proliferation. Consistent with increased recombination rates, eight strains lost parts of one copy of the duplicated chromosome while acquiring a duplication of parts of

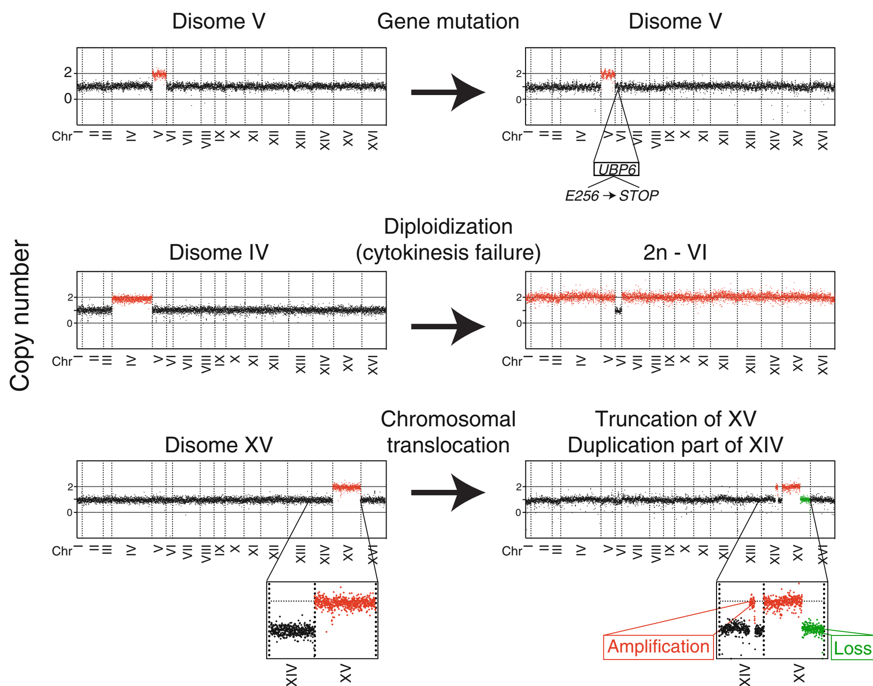

Fig. 4 Examples of genomic alteration promoted by aneuploidy. On the *left* the original karyotypes of disomes *V*, *IV* and *XV* are shown. On the *right*, karyotypes of evolved aneuploid strains that show improved cellular fitness (Torres et al. 2010)

other chromosomes. Interestingly, four descendants of cells harboring an extra copy of chromosome IV lost the entire copy of the additional chromosome and diploidized, suggesting that cytokinesis may also be affected by aneuploidy. Whole-genome sequencing of 12 evolved strains that kept the entire copy of the extra chromosome revealed 43 gene mutations were present in those strains. Several mutations that regulate protein turnover pathways and other cellular processes such as metabolism and transcription were also mutated. Altogether, these results indicate that aneuploidy leads to increased genomic instability and promotes the acquisition of mutations in genes that regulate cellular processes that likely play important roles in mediating the response to aneuploidy.

12 Increased Protein Turnover Suppresses Aneuploidy Associated Phenotypes

Among the mutations that improve the fitness of aneuploid yeast strains are loss-of-function mutations in the deubiquitinating enzyme *UBP6*. These mutations lead to increased proteasomal activity, thus enhancing cellular protein turnover (Hanna et al. 2006). Interestingly, they were identified in two different disomic

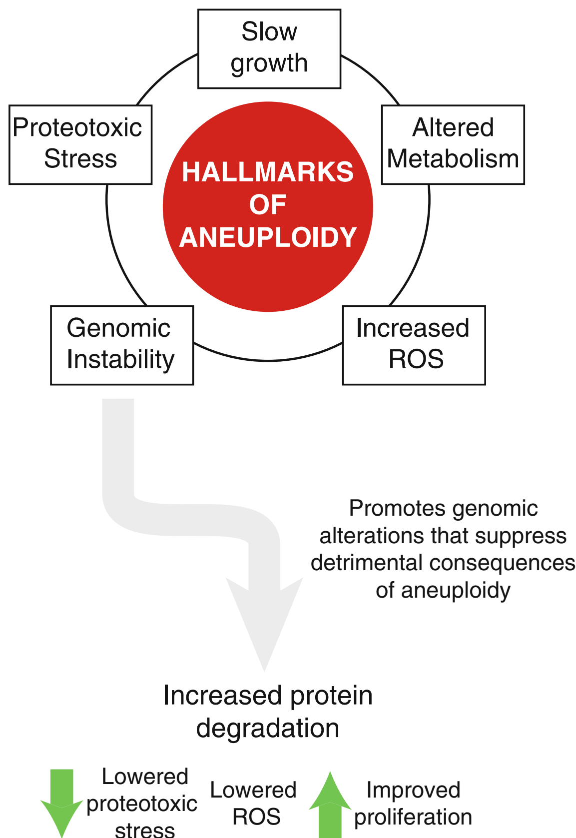

Fig. 5 Hallmarks of aneuploidy. Gaining an extra chromosomes disrupts cellular homeostasis and physiology. Aneuploidy causes a G1 delay due to cellular stress. Increased gene copy number leads to increase protein synthesis causing an increased burden on protein quality control pathways causing proteotoxic stress. Increased protein production demands higher energy utilization leading to altered cellular metabolism. Increased reactive oxygen species (ROS) and genomic instability are observed in almost every aneuploid strains. Genomic instability promotes the acquisitions of genomic alterations that suppress several phenotypes associated with aneuploidy. Increased protein turnover, due to the loss of *UBP6*, suppresses proteotoxic stress, lowers intracellular levels of ROS and improves the fitness of several aneuploid strains

strains, indicating that loss of *UBP6* function may affect aneuploid strains independent of the extra chromosome they carry. Indeed, deletion of *UBP6* was shown to improve the fitness of several aneuploid strains under standard growth conditions. Strikingly, proteome analyses showed that loss of function of *UBP6* results in significant attenuation in the levels of several of the most upregulated proteins in all aneuploid strains, independent of the identity of the extra chromosome. Gene expression analyses indicate that protein attenuation upon loss of *UBP6* is mediated posttranscriptionally. The detailed mechanisms by which loss of *UBP6* leads to

such gross protein attenuation of upregulated proteins are not understood. Nonetheless, a remarkable consequence of increased protein degradation mediated by the loss of *UBP6* is that several of the aneuploidy-associated phenotypes are suppressed in almost every aneuploid strain analyzed (Fig. 5). These include reduction of protein aggregate formation, suppression of proliferation defects in several aneuploid strains grown under standard conditions and in most strains grown under elevated temperatures, as well as the reduction in the levels of intracellular ROS and significant amelioration of the APS. These results provide strong evidence that proteotoxic stress is a major consequence of aneuploidy and indicate that targeting pathways that help cells cope with such stress could be exploited to specifically kill aneuploid cancer cells. Of high importance, identification of the direct targets of *UBP6* could shed light onto the molecular mechanisms by which aneuploidy-associated phenotypes can be suppressed. Interestingly, the human homologue of *UBP6*, *USP14*, shows loss of heterozygocity (LOH) in 33 % of humans cancers and is located next to the telomere of the short arm of Chromosome 18, one of the most frequently lost chromosomes in tumors (Bamford et al. 2004; Davoli et al. 2013). Therefore, the effect of *USP14* loss of function in human aneuploidy deserves further investigation.

13 Implications to Human Disease

In cancer, aneuploidy can certainly contribute to tumorigenesis by providing a mechanism through which cells acquire extra copies of oncogenes and/or lose tumor suppressors. Consistently, the distributions of tumor suppressor genes and oncogenes strongly correlate with patterns of aneuploidy and copy number variation in cancer cells (Davoli et al. 2013). Furthermore, specific chromosomal aneuploidies are frequently found in a given type of cancer (Gordon et al. 2012; Padilla-Nash et al. 2012). Studies in aneuploid yeast summarized in this chapter indicate, however, that gaining an extra chromosome comes at a high cost. Acquisition of an extra copy of a whole autosome disrupts cellular homeostasis and physiology in a manner similar to that of other stresses. Increased protein synthesis leading to proteotoxic stress and altered metabolism seem to be a key process driving much of the consequences aneuploidy has on cellular physiology. Aneuploid cancer cells must find ways to overcome such stress. Importantly, increases in intracellular ROS due to aneuploidy has been shown to occur in human cells following the induction of chromosome missegregation (Li et al. 2010). An important implication of increased ROS levels in aneuploid cells is that they could be a source of genomic instability in vivo, promoting genomic alterations that suppress aneuploidy-associated phenotypes and promote tumor evolution.

Although frequently discussed in the context of cancer, aneuploidy is a main cause of the detrimental symptoms in Down syndrome patients and likely plays an active role in neurodegenerative diseases. Studies in yeast suggest that proteotoxic stress may play an important role in both cases. The discovery that increased protein

degradation ameliorates several aneuploid-associated phenotypes provides a proof of principle and motivation to explore the potential of increased protein degradation as a therapeutic strategy to improve the symptoms of Down patients and prevent or delay the onset of Alzheimer's or Huntington's disease.

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The Diverse Effects of Complex Chromosome Rearrangements and Chromothripsis in Cancer Development

Mirjam S. de Pagter and Wigard P. Kloosterman

Abstract

In recent years, enormous progress has been made with respect to the identification of somatic mutations that contribute to cancer development. Mutation types range from small substitutions to large structural genomic rearrangements, including complex reshuffling of the genome. Sets of mutations in individual cancer genomes may show specific signatures, which can be provoked by both exogenous and endogenous forces. One of the most remarkable mutation patterns observed in human cancers involve massive rearrangement of just a few chromosomal regions. This phenomenon has been termed chromothripsis and appears widespread in a multitude of cancer types. Chromothripsis provides a way for cancer to rapidly evolve through a one-off massive change in genome structure as opposed to a gradual process of mutation and selection. This chapter focuses on the origin, prevalence and impact of chromothripsis and related complex genomic rearrangements during cancer development.

Keywords

Chromothripsis · Chromosomal rearrangement · Next-generation paired-end sequencing · Mutation signatures

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1 Signatures of Genetic Changes in Cancer Genomes

Genetic changes in cancer genomes can arise from a variety of mutational mechanisms that are enforcing specific mutation signatures (Alexandrov et al. 2013). Systematic evaluation of mutation signatures from large sets of cancer genomes has uncovered more than 20 distinct profiles, associated with age of cancer diagnosis, strand asymmetry in transcribed regions, defective homologous recombination and drug treatment (Alexandrov et al. 2013).

A very striking mutation pattern observed in a large array of cancer genomes involves local hypermutation characterized by C → T and C → G mutations at TpC sites (Alexandrov et al. 2013; Nik-Zainal et al. 2012). This phenomenon has been termed *kataegis*, which is Greek for thunderstorm. Kataegis occurs at sites of structural genomic rearrangements and is mediated through deamination of cytosine by APOBEC deaminases (Taylor et al. 2013). On the level of structural genomic rearrangements, a remarkably complex pattern of changes has been described, which involves reshuffling of tens to hundreds of genomic segments leading to massively rearranged chromosomes (Stephens et al. 2011). This phenomenon has been termed chromothripsis, Greek for chromosome (chromo) shattering (thripsis). Chromothripsis has now been found in many different cancer types and appears a major mutational force driving tumor development (Kloosterman et al. 2014). Here, we will discuss our current understanding of chromothripsis, its origin and prevalence in cancer, functional consequences and relation to other types of complex genome rearrangements identified in cancer genomes.

2 The Landscape of Structural Genomic Rearrangements in Cancer

Many cancer genomes harbor structural changes, ranging from small insertions/deletions (indels) to duplications, inversions, translocations and whole chromosome aneuploidies. The number of acquired structural changes in cancer genomes is typically smaller than for substitutions (Vogelstein et al. 2013). For

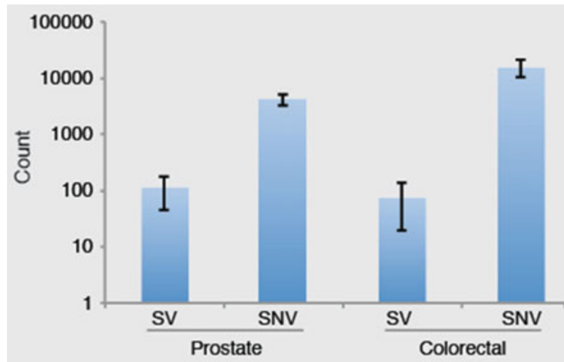

Fig. 1 Numbers of somatic structural variants (SV) and single nucleotide variants (SNVs) identified in prostate (Berger et al. 2011) and colorectal cancer (Bass et al. 2011)

example, prostate and colorectal tumors harbor 37 and 204-fold more single nucleotide changes than structural variants, respectively (Fig. 1).

Insight into structural genomic rearrangements in cancer has first been obtained through the identification of cytogenetically visible changes, such as whole-arm translocations. The Philadelphia translocation (t(9;22)) was the first structural variation observed in cancer (Rowley 1973). This translocation occurs in 95 % of chronic myelogenous leukemia (CML) cases and results in the formation of an oncogenic fusion gene between *BCR* and the tyrosine kinase gene *ABL1* (Shtivelman et al. 1985). These initial findings have triggered subsequent investigation of cytogenetic aberrations in cancer cells, culminating into the identification of hundreds of cancer gene fusions (Mitelman et al. 2007).

The emergence of array-based techniques such as comparative genomic hybridization (aCGH) and SNP-arrays (Box 1) has provided further crucial insight into the impact of chromosomal rearrangements in cancer genomes (Beroukhim et al. 2007). Pan-cancer analyses of thousands of datasets have uncovered genomic regions and genes that show significant change in copy number across different cancer types (Beroukhim et al. 2010; Zack et al. 2013), leading to the identification of novel cancer genes.

Patterns of structural rearrangements can be substantially refined by the use of next-generation sequencing technology. Paired-end sequencing strategies have been instrumental to detect both copy number changes and balanced rearrangements in the human genome simultaneously (Box 1) (Korbel et al. 2007). Application of this methodology to cancer genomes has led to the classification of somatic rearrangements across all size ranges and types at unprecedented resolution (Campbell et al. 2008) (Fig. 2). Sequencing data have shown that cancer genomes contain more somatic rearrangements than previously anticipated (Stephens et al. 2009). Also, rearrangement types may vary for different cancers, and even cancer subtypes. This is illustrated by the predominance of tandem duplications among the structural landscape of subsets of breast and ovarian cancers, whereas other subsets

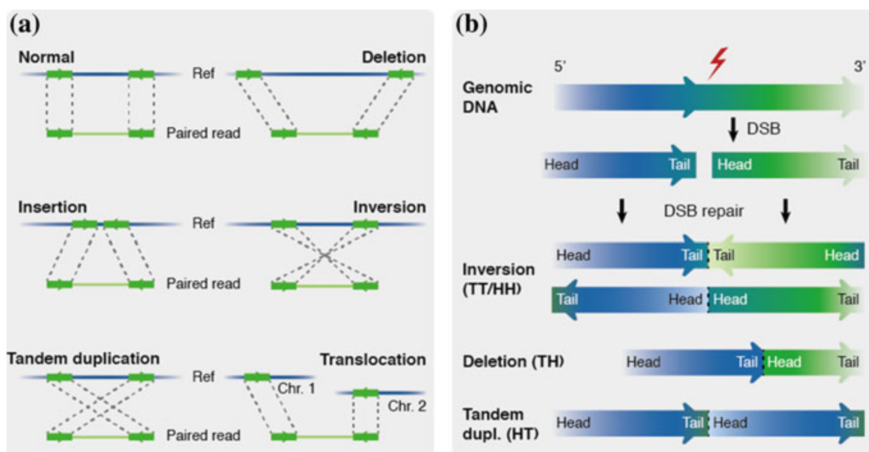

Fig. 2 The detection of genomic rearrangements from paired-sequencing data. **a** Individual reads are aligned to a reference genome and discordant reads indicate the presence of rearrangements (Box 1). Different SV types lead to different types of discordance; deletions show a larger distance between aligned reads compared to the original DNA fragment length, whereas insertions will be characterized by a smaller distance between aligned reads. In the case of an inversion and tandem duplication the order of the reads in the pair is swapped, for inversions the reads will map to the opposite DNA strand. In case of an inter-chromosomal translocation the reads of a read pair will map to different chromosomes. **b** Intrachromosomal rearrangements lead to a change in orientation of breakpoint junctions (Box 1). The orientation is indicated by using head (H), which indicates the 5′ end of a fragment (i.e. the lowest coordinate), and tail (T), indicating the 3′ end of a fragment (i.e. the highest coordinate). Different rearrangement types lead to different orientation conformations. The first letter corresponds to the fragment with the lowest coordinate and the second to the fragment with the highest coordinate. TH orientation of the breakpoint junction indicates the presence of a deletion, TT and HH indicate the presence of an inversion. HT (not TH) orientation indicates a tandem duplication, due to the fact that the first letter corresponds to the read with the lowest coordinate, which in the case of a tandem duplication is the second read in the pair

are mostly characterized by deletions (McBride et al. 2012). In addition, certain tumor types show a markedly increased number of structural changes (e.g., breast cancer and lung squamous cell cancer) when compared to others (kidney cancer) (Yang et al. 2013). Cancer genomes harbor an overrepresentation of complex changes, interchromosomal translocations and tandem duplications when compared to germline rearrangements (McBride et al. 2012; Campbell et al. 2010; Hillmer et al. 2011). Also, chromosomal rearrangements can arise throughout cancer development and metastasis, leading to the detection of both shared and lesion-specific rearrangements in several cancers (Campbell et al. 2010; Kloosterman et al. 2011a; Hoogstraat et al. 2014; Ding et al. 2010).

3 A Historical View on Complex Genome Rearrangements and Cancer Development

The complexity of some chromosomal aberrations in cancer was noted well before the introduction of high-resolution copy number and sequencing technologies. For example, the vast majority of leukemia's with a Philadelphia chromosome contain a simple translocation between chromosomes 9 and 22, but very complex variants involving up to five chromosomes have also been observed (Adhvaryu et al. 1988; Kadam et al. 1990; Rosson and Reddy 1988). In addition, BCR-ABL positive cases of leukemia without a karyotypically visible rearrangement have been described (Fitzgerald and Morris 1991). Careful analysis of the *BCR* and *ABL* loci in these cases has revealed complex exchanges of chromosome segments and it was suggested that this was not a result of serial changes, but rather a single concerted event, reminiscent of chromothripsis (Fitzgerald and Morris 1991). Furthermore, cytogenetic analysis already revealed that amplification of *MYCN* in neuroblastoma is a process involving complex genomic rearrangements (Nishi et al. 1992). Those studies suggested that the complex and heterogeneous rearrangements of *MYCN* in amplicons of neuroblastoma cell lines have preceded the amplification process. The amplification of oncogenes may involve the separate propagation of chromosome fragments by formation of episomes (double-minute chromosomes). Alternatively, oncogene-containing chromosome segments can form an array within a chromosome and appear as homogeneously staining regions (HSR) (Cowell 1982; Shimizu 2009). HSRs are thought to arise via breakage-fusion bridge (BFB) cycles, a process of chromosome fusion following telomere attrition (Shimizu et al. 2005; Cowell and Miller 1983). Sequencing of amplified regions in cancer genomes revealed a wide variety of configurations, including evidence for BFB cycles and integration of double minutes into the genome (Bignell et al. 2007).

Further evidence for the importance of complex genomic aberrations for cancer development was gained through cytogenetic studies that revealed recurrently affected regions, such as the identification of complex rearrangements on 12q13 in leiomyoma (Nilbert et al. 1989). The genes in this region are now known to be important drivers for this cancer type and the underlying complex rearrangements are frequently caused by chromothripsis. Osteosarcomas have a remarkably high frequency of complex chromosomal rearrangements, many of which are recurrent (Bayani et al. 2003). Notably, osteosarcomas form a cancer type with a very high frequency of chromothripsis (Stephens et al. 2011).

Altogether, early cytogenetic studies have shown the involvement of complex genomic rearrangements in cancer gene amplification, formation of cancer fusion genes and recurrent changes on specific chromosomal regions. It is very likely that at least some of these complex rearrangements resulted from chromothripsis. The precise structure of complex rearrangements, their differences and impact on cancer genes has only become apparent based on genome sequencing analysis (Box 1). This has led to the discovery of chromothripsis as a distinct and well-discernable entity among complex chromosome rearrangements.

4 Hallmarks of Chromothripsis in Cancer Genomes

A key feature of chromothripsis is the geographic localization of rearrangements within the genome and profound clustering of breakpoints on one or multiple parts of one or a few chromosomes. The highly localized character of chromothripsis is further emphasized by the fact that all rearrangements involve a single parental chromosome (Stephens et al. 2011; Korbel and Campbell 2013). A second hallmark is the presence of frequent oscillations between two copy number states due to the loss of fragments to the cell, with loss-of-heterozygosity (LOH) in the lower copy number (deleted) regions, but retainment of heterozygosity in regions with the

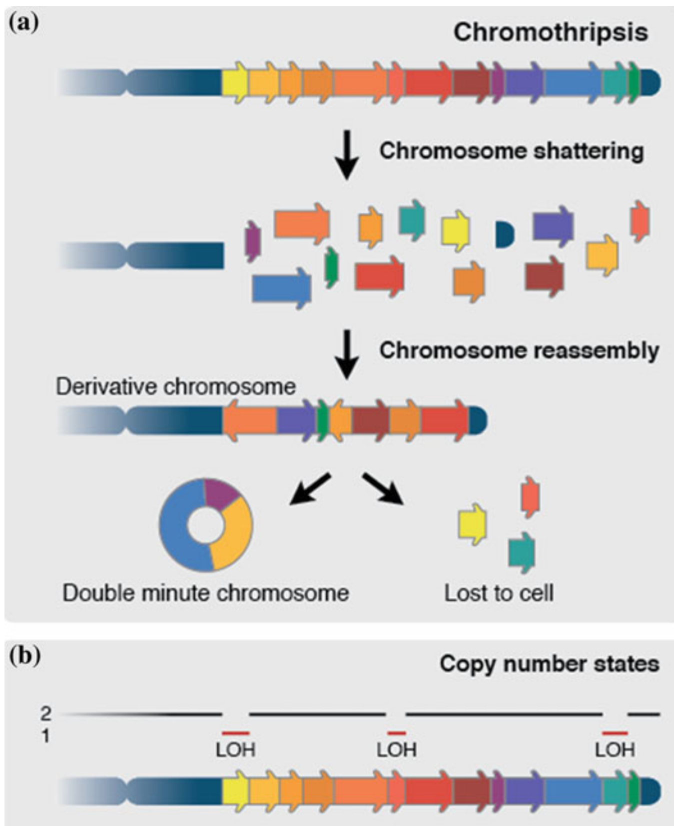

Fig. 3 Chromosome shattering and reassembly by chromothripsis. **a** One or multiple parts of one or a few chromosomes are shattered into pieces in a one-off catastrophic event. Chromosome are subsequently stitched back together in a random order by NHEJ, with some fragments being incorporated into a double-minute chromosome and others getting lost to the cell. **b** Chromothripsis typically leads to frequent oscillations between two copy number states in which the lower copy state shows loss of heterozygozity (LOH)

higher copy number (Stephens et al. 2011). Alternatively, three or more copy-number states can be observed. Chromosome fragments can be incorporated into a double-minute chromosome (Fig. 3), which can subsequently become amplified if it contains an oncogene (Stephens et al. 2011; Rausch et al. 2012). Additionally, partial chromosome duplication preceding or following chromothripsis may occur (Zhang et al. 2013). In those instances, three or more copy number states can be observed: one for lost fragments, one for retained fragments and one or more, high copy-number states for duplicated fragments and fragments of amplified double-minute chromosomes (Stephens et al. 2011; Rausch et al. 2012). As a third hallmark of chromothripsis, regions with the lower copy number are not caused by simple tail-to-head deletions, but by a series of complex breakpoint junctions that span the involved chromosomal regions. Fourth, all four possible intrachromosomal breakpoint orientations (Fig. 2b) are represented in approximately equal numbers. Finally, the overall chromosome configuration resulting from chromothripsis involves joined fragments that are often not located in the proximity of each other in the reference genome (Stephens et al. 2011).

These hallmarks of chromothripsis have led to a model, whereby one or multiple parts of one or more chromosomes are shattered into pieces and randomly stitched back together, with some parts getting lost to the cell, and others incorporated into a double-minute chromosome (Fig. 3). This results in massively rearranged chromosomes involving tens to hundreds of rearrangements. A lower limit for the number of breaks constituting a chromothripsis event is not defined. It could be that complex rearrangements constituted by just a few or many local breakpoints are both caused by the same molecular trigger (Kloosterman and Cuppen 2013).

Chromothripsis is not restricted to cancer, but also occurs in the germline, leading to severe congenital phenotypes (Kloosterman et al. 2011b, 2012; Chiang et al 2012). The nature of chromosome breakage and reassembly appears similar for cancer and germline, although germline chromothripsis rearrangements have substantially less breakpoints and display a more balanced state (Kloosterman et al. 2011b, 2012; Chiang et al 2012). Both differences are likely due to strong selective pressure against catastrophic DNA damage during embryonic development. Alternatively, they could reflect differences in chromothripsis mechanisms (Zhang et al. 2013; Kloosterman and Cuppen 2013).

5 The Mechanism Behind Chromothripsis

Although chromothripsis has been found in many different cancer types and appears a major mutational force driving tumor development, the precise origin of chromothripsis is only beginning to be understood. The vast majority of chromothripsis breakpoint junctions are characterized by microhomology of 2–4 nt (Stephens et al. 2011; Rausch et al. 2012; Morrison et al. 2014). This is consistent with repair of the DNA fragments by nonhomologous end-joining (NHEJ) (Moore and Haber 1996).

Several hypotheses and experimental data have shed light on possible causes of chromothripsis (Stephens et al. 2011; Zhang et al. 2013; Kloosterman et al. 2012; Holland and Cleveland 2012; Forment et al. 2012; Maher and Wilson 2012). The most compelling explanation concerns the formation of micronuclei that capture (part of) one or multiple chromosomes following chromosome segregation errors (Crasta et al. 2012; Hatch et al. 2013). This model for chromothripsis formation provides an elegant explanation for the clustering of rearrangements to one or a few chromosomes, because these are physically separated during DNA damage formation and repair, but can rejoin the other chromosomes in the nucleus during subsequent cell divisions (Crasta et al. 2012; Hatch et al. 2013). It has been shown that repair of DNA damage is defective and/or delayed in micronuclei due to defects in DNA damage response signaling (Crasta et al. 2012; Terradas et al. 2009). Chromosomes in micronuclei showed γ-H2AX foci, but lacked efficient recruitment of its downstream components of the DNA damage response, leading to extended persistence of γ-H2AX foci in the micronuclei (Crasta et al. 2012). Furthermore, DNA replication in micronuclei is asynchronous with the primary nucleus as many micronuclei show DNA replication in G2-phase (Crasta et al. 2012). Premature chromosome compaction (PCC) is a well-known mechanism that can lead to pulverization of chromosomes (Johnson and Rao 1970; Sperling and Rao 1974). If micronuclei enter mitosis before completion of DNA replication PCC can occur, leading to the occurrence of massive amounts of DNA double-stranded breaks (DSBs) (Crasta et al. 2012; Donley and Thayer 2013). This damage occurs in the first cell cycle following micronuclei formation (Crasta et al. 2012). Indeed, chromosome paintings of micronucleated cells demonstrated small fragments from one or two chromosomes (Crasta et al. 2012). A study by Hatch et al. (2013) uncovered a possible explanation for DNA damage to chromosomes contained in micronuclei. The authors showed a strong correlation between micronuclear envelope breakdown and the occurrence of massive DNA damage, although it remains unknown how nuclear envelope breakdown exactly leads to this damage (Hatch et al. 2013). Recent work has now demonstrated that chromosomes captured in micronuclei can undergo massive genomic rearrangements, including all the known hallmarks of chromothripsis as observed in cancer genomes (Zhang et al. 2015), thus providing experimental proof for a mechanism causing chromothripsis.

Although the micronucleus model has now been tested experimentally, other possible causes of chromothripsis may exist, including the influence of exogenous sources of DNA damage during mitosis. It has been suggested that free radicals and ionizing radiation can trigger chromothripsis rearrangements during mitosis when the chromosomes are highly condensed and DNA damage signaling is suppressed (Stephens et al. 2011; Zhang et al. 2011, 2013; Kloosterman and Cuppen 2013; Holland and Cleveland 2012; Maher and Wilson 2012). External damage occurring during this state of the cell cycle could possibly slice through multiple, closely located, segments of one or a few chromosomes, thereby explaining the localized character of chromothripsis (Stephens et al. 2011; Zhang et al. 2013).

Finally, it has been suggested that the occurrence of repeated BFB-cycles associated with telomere attrition could lead to chromothripsis rearrangements (Stephens et al. 2011; Sorzano et al. 2013). Telomere attrition leads to dicentric chromosomes, which form an anaphase bridge when the centromeres are pulled to opposite daughter cells during anaphase. The dicentric chromosome subsequently breaks at a random location between the two centromeres and fragments of this break are inherited by the daughter cells (Sorzano et al. 2013). It has been suggested that the dicentric chromosome may acquire the massive DNA damage seen in chromothripsis at the cleavage furrow during cytokinesis in a one-off event (Holland and Cleveland 2012) or that successive BFB-cycles, over the course of multiple cell divisions, are responsible for chromothripsis rearrangements (Sorzano et al. 2013).

Repeated BFB-cycles can explain the clustering of rearrangements near telomeres. However, BFB-cycles characteristically lead to multiple amplified copy number states, which is inconsistent with the two copy number states typically detected in chromothripsis. Furthermore BFB-cycles lead to an enrichment of inverted-type rearrangements, while rearrangement orientations in chromothripsis are randomly distributed (Bignell et al. 2007; Korbel and Campbell 2013; Zhang et al. 2013). A more plausible suggestion is the incorporation of lagging dicentric chromosomes into a micronucleus, rather than the nucleus of one of the daughter cells. In this way, telomere attrition may facilitate the occurrence of chromothripsis by inducing micronuclei formation (Holland and Cleveland 2012; Li et al. 2014). In acute lymphoblastic leukemia (ALL) the occurrence of dicentric chromosomes due to BFB-cycles or a germline Robertsonian translocation preceded chromothripsis, possibly through micronuclei formation after the formation of an anaphase bridge by the lagging chromosome (Li et al. 2014).

6 Other Types of Complex Genomic Rearrangements in Cancer Genomes

Prostate cancers exhibit patterns of complex rearrangements related to chromothripsis, characterized by chains of translocations and deletions, that occur in a highly interdependent manner (Fig. 4a). This phenomenon has been termed chromoplexy (from the Greek *pleko*, which means to weave or braid) (Baca et al. 2013). Chromoplexy chains typically contain three to over 40 rearrangements, involving one to up to ten chromosomes. A key characteristic is the occurrence of ‘deletion bridges’, small deletions between fusion junctions of translocation rearrangements in a subset of rearrangements, although chromoplexy in general seems to be a relatively copy-neutral process, when compared to chromothripsis (Zhang et al. 2013; Baca et al. 2013). Chromoplexy has been detected in 88 % of prostate tumors and frequently accounts for the dysregulation of prostate cancer genes, often disrupting multiple genes coordinately. Strikingly, two or more separate chromoplexy chains were detected in 63 % of prostate tumors (Baca et al. 2013). Where chromothripsis typically leads to a very high number of rearrangements in one event, multiple chromoplexy events can occur in successive cell cycles (Fig. 4b) (Baca et al. 2013).

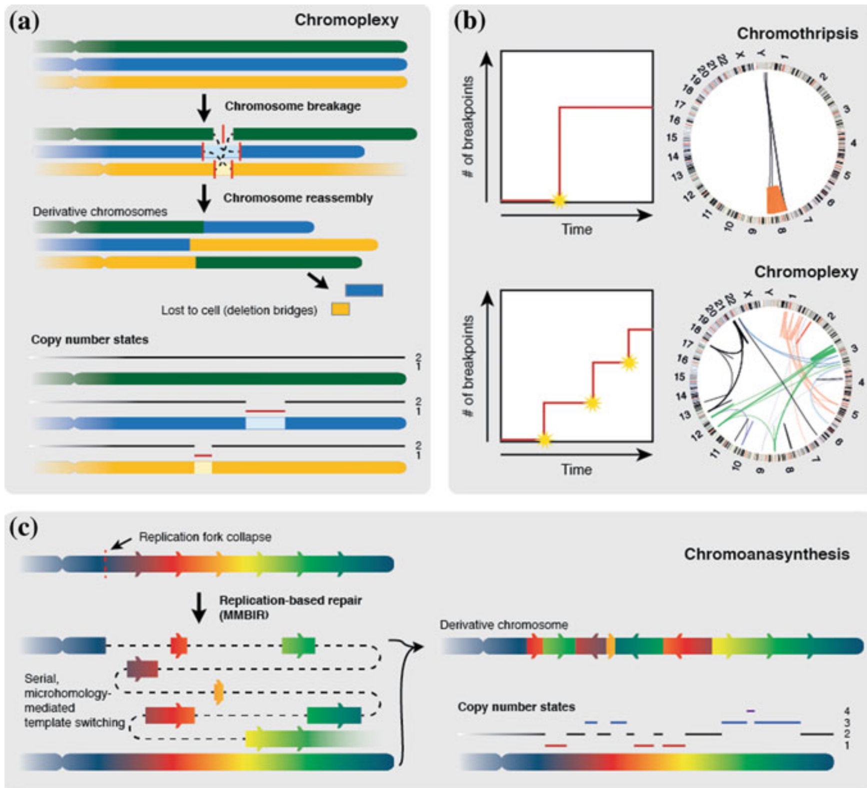

Fig. 4 Other mechanisms for complex rearrangements. **a** Chromoplexy. Multiple chromosomes undergo one or multiple DSBs (*solid lines* indicate breaks). Chained translocations are formed when chromosomes are reassembled with deletion bridges spanning translocations in some cases. In general, chromoplexy leads to fewer copy number alterations when compared to chromothripsis. **b** Differences between chromothripsis and chromoplexy. Chromothripsis generates a large number of breakpoints in a one-off catastrophic event. Chromoplexy leads to fewer breakpoints per event, but multiple chromoplexy chains can be formed in one cell in subsequent cell cycles. Due to this, chromothripsis usually leads to extreme clustering of breakpoints, whereas chromoplexy breakpoint clustering is less extreme. **c** Chromoanasynthesis is a replication-dependent process. Replication fork collapse leads to serial microhomology-mediated template switching leading to the formation of a derivative chromosome showing inversions and deletions, but also duplications and triplications of genomic regions

Chromoplexy rearrangements are usually not as highly localized as chromothripsis, as chromothripsis typically involves only one or a few chromosomes, whereas chromoplexy can involve up to ten chromosomes (Stephens et al. 2011; Baca et al. 2013). Furthermore, the total number of rearrangements in chromoplexy is smaller than for chromothripsis. Also, the relatively high number of (large) deletions seen in chromothripsis is not found in chromoplexy (Baca et al. 2013). Thus, chromoplexy could be regarded as a phenomenon involving rearrangements of intermediate complexity. In ETS + prostate cancer, well-known ERG fusions (e.g. TMPRSS2-

ERG) frequently result from chromoplexy (58 %) (Baca et al. 2013). This clearly emphasized the oncogenic capability of chromoplexy in prostate tumors.

Like chromothripsis, each chromoplexy chain is suggested to form during a one-off catastrophic event, which is supported by the fact that both ends of each break are involved in chained translocations. A mechanism for the occurrence of this phenomenon remains to be established, but chromoplexy in ETS + tumors involves highly transcribed regions, which are colocalized in interphase nuclei, suggesting a mechanistic coupling of chromosome rearrangement and transcription (Baca et al. 2013).

An alternative category of complex genomic rearrangements was described in patients with developmental delay and cognitive anomalies (Liu et al. 2011). Chromoanasynthesis (anasynthesis for reconstruction) is characterized by interspersed changes in copy number, including duplications, triplications and deletions, combined with translocations and inversions (Fig. 4c). Like chromothripsis, chromoanasynthesis rearrangements are highly localized, usually involving only a single chromosome. Breakpoint junctions of chromoanasynthesis rearrangements frequently show microhomology or templated sequence insertions (54–1542 bp) (Liu et al. 2011). Despite the similarities between chromothripsis and chromoanasynthesis there are marked differences concerning rearrangement clustering and copy number changes (Zhang et al. 2013; Kloosterman and Cuppen 2013; Holland and Cleveland 2012). Chromoanasynthesis is characterized by multiple copy number states on a single chromosome due to a combination of deletions, duplications and triplications, as opposed to chromothripsis, where deletions are frequently observed, but typically no duplications or triplications are found (Zhang et al. 2013; Liu et al. 2011). Furthermore, detailed analysis of chromoanasynthesis breakpoint junctions revealed that these lack the typical double-strand break signatures that are found in chromothripsis (Kloosterman and Cuppen 2013).

The templated insertions and microhomology found at breakpoint junctions in chromoanasynthesis rearrangements are a signature of replication-based mechanisms, specifically microhomology-mediated breakage-induced repair (MMBIR) (Liu et al. 2011; Carvalho et al. 2009; Zhang et al. 2009). MMBIR is initiated by a broken DNA replication fork and leads to the incorporation of templated sequences of rearranged segments through serial, microhomology-mediated template switching, explaining the occurrence of multiple copy number states (Fig. 4c) (Liu et al. 2011; Hastings et al. 2009).

7 Methods for Identificaton of Complex Genomic Rearrangements in Cancer Genomes

Despite the major technical advances in the past decades (Box 1), the detection of genomic rearrangements remains complex due to both technical issues related to next-generation DNA sequencing, such as short sequence reads and small library insert size, and biological factors, such as the occurrence of (highly) repetitive regions, overlapping SVs and the complexity of breakpoints. Although different

Table 1 Variation in criteria and methods used for chromothripsis and chromoplexy detection

Study (type)	Criteria	Technique (sample size)	Detection tool
Stephens et al. (2011) (Chromothripsis)	(1) Tens to hundreds of genomic rearrangements that show geographic localization within the genome (2) Pronounced clustering of breakpoints on chromosomes or chromosome arms (3) Rearranged regions show frequent oscillations between two and occasionally three copy number states (4) Regions with copy number 1 are caused by a series of complex rearrangements that span the involved region and not by simple deletions (5) Heterozygosity is retained in regions with higher copy number (6) The complex rearrangements represent all four intrachromosomal breakpoint orientations in approximately equal numbers (7) The two conjoined fragments of chromosomes at each breakpoint fusion are often not located in the proximity of each other on the reference genome	Paired-end sequencing, SNP array (776)	In-house SV/CNA detection tools

(continued)

Table 1 (continued)

Study (type)	Criteria	Technique (sample size)	Detection tool
	(8) Rearrangements involve a single parental copy of the chromosome (9) Double minute chromosome may form		
Magrangeas et al. (2011) (Chromothripsis)	Identical to criteria set by Stephens et al. (2011)	SNP array (764)	Partek Genomic Suite
Rausch et al. (2012) (Chromothripsis)	At least 10 changes in segmental copy number involving two or three copy-number states per chromosome	Paired-end/long-mate-pair sequencing, SNP array (605)	PEMer tool and in-house tools
Kim et al. (2013) (Chromothripsis)	(1) At least 10 CNAs localized to one chromosome (2) Occurrence of breakpoints is random e.g. the size of neighboring segment is similar	aCGH, SNP array (8227)	In-house statistic tool
Malhotra et al. (2013) (Chromothripsis)	Breakpoints are assigned to the same breakpoint cluster if: (1) Breakpoints are located within 100 kb of each other in the reference genome (2) Different loci share breakpoint calls, indicating interconnection between breakpoints Breakpoint clusters are defined as complex and resulting from a one-off event when: (3) No more than three copy number states are detected	Paired-end sequencing, de novo assembly (64)	HYDRA-MULTI and in-house tools

(continued)

Table 1 (continued)

Study (type)	Criteria	Technique (sample size)	Detection tool
	(4) A maximum of one amplified copy number state exceeding four predicted copies is present in the region and this is not a focal amplification of a contiguous amplified region. Chromothripsis: (5) At least 10 or more clustered breakpoints and copy number profiles are present that meet all four criteria described above.		
Zack et al. (2013) (Chromothripsis)	(1) Unexpectedly large number of CNAs on one chromosome compared to the CNA frequency of the sample (2) CNAs on a single chromosome are highly localized, discordant with what is expected by chance (3) CNAs lead to copy number changes of +1 or −1 and are non-overlapping	SNP array (4934)	In-house method, to be published
Cai et al. (2014) (Chromothripsis)	(1) Significant clustering of breakpoints (2) Segmental copy number changes	Paired-end/mate-pair sequencing, whole-genome sequencing, SNP array, aCGH (18,394)	Scan-statistic based algorithm
Govind et al. (2014) (Chromothripsis)	(1) Genome localization: low number of chromosomes involved in	Whole-genome sequencing (21)	Various tools for SV detection, ShatterProof for detection of chromothripsis

(continued)

Table 1 (continued)

Study (type)	Criteria	Technique (sample size)	Detection tool
	complex rearrangements (2) Chromosome localization: highly localized SV clustering on chromosome (3) Low number of copy number states, but high number of copy number oscillations (4) Translocation clustering: high number of localized translocations (5) Insertions at translocation breakpoints: high fraction of translocations where short insertions are found (6) High retained heterozygosity in areas between CNVs (7) Presence of a non-synonymous TP53 mutation (does currently not influence chromothripsis score)		
Baca et al. (2013) (Chromoplexy)	ChainFinder determines whether genomic rearrangements where formed in an interdependent manner and assigns breakpoints that where formed interdependently to the same (rearrangement) chain (e.g. cycle) (1) Two breakpoints are	NGS, paired-end sequencing (57)	dRanger algorithm for detection of SVs, ChainFinder for detection of chromoplexy

(continued)

Table 1 (continued)

Study (type)	Criteria	Technique (sample size)	Detection tool
	assigned to the same chain if the p-value for the independent generation of the breakpoints is rejected with a false-discovery rate $>10^{-2}$. (2) All scenarios are considered for one or more rearrangements in a cycle to occur independently. Only if each scenario is rejected with a family-wise error rate $<10^{-2}$, all rearrangements in a cycle are assigned to the same chain		

types and sizes of simple SVs can be detected relatively straightforward, the distinction of chromothripsis from other complex rearrangement types is challenging. Several bioinformatics tools have been developed to specifically detect chromothripsis or chromoplexy rearrangements (Baca et al. 2013; Cai et al. 2014; Govind et al. 2014; Malhotra et al. 2013; Kim et al. 2013). An essential part of these tools is their definition of criteria to distinguish each type of complex genomic rearrangement. These criteria are not entirely fixed across different studies, leading to differences in sensitivity for detection of complex rearrangement types (Table 1).

A new software tool, termed ShatterProof, was developed to standardize detection and quantification of chromothripsis (Govind et al. 2014). ShatterProof identifies highly mutated regions and subsequently scans these for chromothripsis based on seven variables (Table 1): (1) Genome localization: a high score indicates a much higher SV density on the investigated chromosome than on other chromosomes; (2) Chromosome localization: a high score indicates a high SV density in the region compared to the SV density of the total chromosome; (3) Aberrant CNVs: The presence of a low number of copy number states but a high number of copy number oscillations leads to a high score; (4) Translocation clustering: regions that show a high number of localized translocations are given a high score; (5) Insertions of translocation breakpoints: regions with a high fraction of translocations where short insertions are found receive a high score; (6) Amount of retained heterozygosity: regions that retained heterozygosity in areas between CNVs receive a high score; (7) TP53 mutation; the presence of non-synonymous

TP53 mutations is reported back, but does not influence the chromothripsis score. An overall chromothripsis score is determined per region and reported back to the user. The higher the overall score, the more chromothriptic the region is. Although this tool provides a standardized pipeline for detection of chromothripsis, ShatterProof uses SV calls generated by other tools that have varying outcomes, thereby also influencing the outcome of ShatterProof, affecting the robustness of the tool.

Other tools for chromothripsis detection required either genome sequencing data (Malhotra et al. 2013), or copy number profiles from arrayCGH or SNParray (Cai et al. 2014; Kim et al. 2013). The differences between each of these tools mainly concern assumptions on the numbers of breaks involved, the numbers of copy number states allowed and the clustering of breakpoints within chromosomes (Table 1).

Baca et al. (2013) developed an algorithm specifically for detection of chromoplexy chains, termed ChainFinder. This algorithm identifies genomic rearrangements and their associated deletions that appear to have arisen interdependently from combined analysis of somatic breakpoints junctions and segmented copy number profiles. In order to do so, ChainFinder first identifies potential deletion bridges and subsequently performs a statistical analysis of all closely neighboring breakpoint pair distances to identify chain-like rearrangement patterns. Next, ChainFinder determines the probability of observing the two detected fusion breakpoints independently (i.e., not formed in the interdependent manner as breakpoints formed in a chain). Only breakpoints fulfilling the criteria for interdependent formation are assigned to chains (Table 1). It has been suggested that ChainFinder may be used in a broader sense to distinguish chromoplexy and chromothripsis events from other non-associated rearrangements on the same chromosome or its other parental counterpart (Zhang et al. 2013).

8 Prevalence of Chromothripsis in Human Cancer

Since its initial discovery in 2011, chromothripsis has been observed in many cancer types. Current pan-cancer estimates of the frequency of chromothripsis range from 1 to 2 % (Stephens et al. 2011; Kim et al. 2013) to 5 % (Zack et al. 2013). However, at the level of individual cancer types marked differences have been observed (Fig. 5). These estimates are dependent on three main factors. First, the detection methods used in several studies have variable capacity to detect chromothripsis (Box 1). High-resolution sequencing will provide insights into copy-neutral rearrangements and smaller changes, whereas array-based methods only provide copy number measurements. As a result, a higher estimate for the frequency of chromothripsis is obtained from whole-genome sequencing data (Kloosterman et al. 2014). Second, since its initial discovery in 2011, several studies have used different criteria to define chromothripsis (Table 1). Comprehensive criteria for inference of chromothripsis have only recently been outlined in detail, but are applicable to next-generation sequencing data only (Korbel and

Fig. 5 Occurrence of chromothripsis in different tumor types

Campbell 2013). Third, biases with respect to sample selection have a strong influence on reported rates for chromothripsis and should be taken into account. Retinoblastoma tumors with a lack of mutations in *RB1* display a chromothripsis frequency of 27 % (3/11) (McEvoy et al. 2014), whereas, the fraction of chromothripsis in retinoblastoma in general is much lower (3/94) (McEvoy et al. 2014). Also, tumor cell lines show an overrepresentation of chromothripsis when compared to primary tumors (Cai et al. 2014).

A consistently high frequency of chromothripsis is displayed by genomes of glioblastoma as defined by copy number profiling [16 %, (Zack et al. 2013)] and genome sequencing [39 %, (Malhotra et al. 2013)]. In another study complex genomic changes in glioblastoma were also noted at a high frequency (Yang et al. 2013). In most tumors the complex changes were concentrated on genes known to drive glioblastoma formation, including gain of *EGFR* and loss of *CDKN2A*. High rates of chromothripsis were also found in bone cancers (25 %), and in most of these tumors multiple chromosomes are involved (Stephens et al. 2011).

Chromothripsis further appears to occur at high frequency in specific subtypes or selected groups of samples. A strong association has been found between chromothripsis and mutations in *TP53* in medulloblastoma (Rausch et al. 2012). Virtually all medulloblastoma of sonic-hedgehog subtype (SHH-MB) that are *TP53* mutated display chromothripsis, whereas SHH-MB without *TP53* mutations show no chromothripsis (Rausch et al. 2012). Several patients carried a germline *TP53* mutation, leading to Li-Fraumeni syndrome, characterized by a high risk for cancer development. Various other subtypes of medulloblastoma show much lower frequencies of chromothripsis (Rausch et al. 2012), but the association of *TP53* with chromothripsis has been confirmed in group 3 medulloblastoma as well (Northcott et al. 2012). The association between *TP53* mutation and chromothripsis has been further substantiated in acute myeloid leukemia, where 47 % of cases with *TP53* mutation harbor chromothripsis versus 1 % of cases without *TP53* mutation (Rausch et al. 2012). Chromothripsis in urinary bladder cancer also seems correlated with *TP53* mutation (Morrison et al. 2014). These findings suggests that the presence of *TP53* mutations in specific cancer types makes the cell more permissive for the occurrence of chromothripsis or provides a selective advantage for cells carrying chromothripsis rearrangements (Rausch et al. 2012; Zhang et al. 2013). However, the association between *TP53* mutation and chromothripsis is certainly not prominent in all cancers. Uterine leiomyomas contain chromothripsis with more than 20 breaks in 17 % of the cases (Mehine et al. 2013), while none of these carry *TP53* mutations. In fact, some of the leiomyomas harboring chromothripsis were benign tumors.

Genetic predisposition for chromothripsis was also demonstrated by the genomic analysis of childhood acute lymphoblastic leukemia with amplifications on chromosome 21 (iAMP21 ALL) (Li et al. 2014). Around 3 % of the patients with this type of ALL, carry a constitutional Robertsonian translocation, involving chromosome 15 and 21 (rob(15;21)c). Patients with rob(15;21)c have a 2700-fold increased risk for developing iAMP21 ALL. This is attributed to the dicentric Robertsonian chromosome, which may undergo aberrant chromosome segregation

during mitosis. Consequently, chromothripsis may occur following pulverization of the Robertsonian lagging chromosome in micronuclei (Crasta et al. 2012; Li et al. 2014). Interestingly, the creation of a dicentric chromosome by BFB-cycles in ALL iAMP21 in sporadic cases was followed chromothripsis by four out of five patients, indicating a predisposition to chromothripsis by the presence of a dicentric chromosome in this specific subtype of ALL (Li et al. 2014).

A final remarkable difference in chromothripsis rates in different subtypes of tumors is highlighted by a recent study on ependymoma, a tumor of the central nervous system (Parker et al. 2014). The authors show that virtually all ependymoma's of supratentorial origin contain chromothripsis leading to C11orf95–RELA fusions. In constrast, none of the posterior fossa ependymoma's show chromothripsis. These differences between the two types of ependymoma's are unexplained, but may suggest chromothripsis-predisposing cellular conditions for the supratentorial type.

9 Chromothripsis Can Drive Cancer Development by Establishing Oncogenic Lesions

The destructive nature of chromothripsis implies that it generally is not an advantageous event for cell survival. Yet, chromothripsis has been identified in a multitude of cancers suggesting that the maintenance of chromothripsis is due to a positive effect of the event on cancer cell survival.

Investigation of chromothripsis breakpoints has revealed clear effects on cancer genes through a variety of mechanisms. First, tumor suppressor genes are commonly disrupted. The *TP53* tumor suppressor gene is located on the p arm of chromosome 17 and is a frequent target of chromothripsis in a variety of tumors (Cai et al. 2014). Loss of *CDKN2A* is another recurrent aberration resulting from chromothripsis in glioblastoma (Yang et al. 2013), diffuse large B-cell lymphoma (Morin et al. 2013) and chronic lymphocytic leukemia (Stephens et al. 2011). Similar tumor-driving changes have been observed for chromoplexy in prostate cancer, where 46 % of the tumor specimens showed disruption of at least one tumor suppressor, among which *TP53*, *PTEN*, *CDKN1B*, *NKX3-1* and *RB1* (Baca et al. 2013). *RB1* has also been discovered as a recurrent target of chromothripsis in retinoblastoma samples without *RB1* point mutations (McEvoy et al. 2014).

A second mechanism by which chromothripsis may promote tumor development concerns the amplification of oncogenes. Although chromosomal regions affected by chromothripsis typically contain only two copy number states, some regions display high-level amplification within a chromothripsis context (Stephens et al. 2011; Rausch et al. 2012). This observation explained formation of double-minutes containing oncogenes, which are beneficial to the cancer cell if present in high numbers. Both *MYC* and *MYCN* are frequent targets of double-minute amplification as a result of chromothripsis in neuroblastoma and medulloblastoma (Rausch et al. 2012; Kim et al. 2013; Molenaar et al. 2012). Other targets of amplification by

chromothripsis involve the cell division kinase *CDK4* (neuroblastoma), the Sonic-Hedgehog signaling genes *GLI2* and *BOC* (Sonic-Hedgehog subtype of medulloblastoma) and *MDM2* (glioblastoma) (Rausch et al. 2012; Kim et al. 2013; Molenaar et al. 2012). Complex genomic rearrangements involving EGFR amplification in glioblastoma were also attributed to double-minute chromosome formation (Yang et al. 2013).

Third, chromothripsis involves the connection of multiple remote chromosomal fragments into a newly formed derivative chromosome. By chance each of the resulting junctions may result in the formation of novel fusion genes. Indeed, in-frame fusion genes are frequently formed due to chromothripsis. Those fusions may simply be a coincidence of the joining of two chromosome segments and lead to fusion genes without any role in tumor development. For example, the VCaP prostate cancer cell line contains an extreme example of chromothripsis involving 573 breakpoints on chromosome 5q (Alves et al. 2013). Eighteen of the breakpoints resulted in potential in-frame fusions, of which only five could be confirmed at the mRNA level. In general, the number of fusion genes created by chromothripsis does not appear to be higher than what is expected by chance. Furthermore, chromothripsis does not lead to a higher number of fusion genes than simple structural genomic rearrangements (Stephens et al. 2011; Alves et al. 2013). Several studies put forward strong evidence for the oncogenic capacity of some fusion genes caused by complex genomic rearrangements. An interesting example involves fusions of the non-coding gene *PTV1* in medulloblastoma (Northcott et al. 2012). *PTV1* is frequently co-amplified together with *MYC* and a large proportion of *MYC*-amplified medulloblastoma display *PTV1-MYC* fusions resulting from chromothripsis. The *PTV1* gene is a host for *miR-1204*, which is highly expressed in samples with *PTV1-MYC* fusions and antagonizing expression of *miR-1204* in a medulloblastoma cell line abrogates cell growth. In a recent study, whole genome sequencing was performed for ependymoma (Parker et al. 2014). A large fraction of the supratentorial subtype of ependymoma contains novel fusions between *C11orf95* and *RELA*. The latter gene is a transcription factor in the NF-κB pathway and translocates spontaneously to the nucleus as a result of fusion to C11orf95. Chromothripsis caused the *C11orf95-RELA* fusion in all examined tumors in this study.

A fourth effect of chromothripsis rearrangements may be the disturbance of gene expression either through direct gene deletion or by interference with gene regulation (Stephens et al. 2011). Such examples have been reported in uterine leiomyomas, where expression of *HMGA1* and *HMGA2* is upregulated as a result of chromothripsis breakpoints (Mehine et al. 2013). In addition the genomic region involving *COL4A5* and *COL4A6* was recurrently rearranged leading to increased expression of the nearby gene *IRS4*, a downstream target of insulin-like growth factor I, which is known to be involved in leiomyoma development (Mehine et al. 2013).

Finally, the cataclysmic nature of chromothripsis allows simultaneous targeting of multiple cancer genes (Fig. 6). Analysis of chromothripsis in medulloblastoma revealed amplification of both *MYCN* and *GLI2* on a single double minute

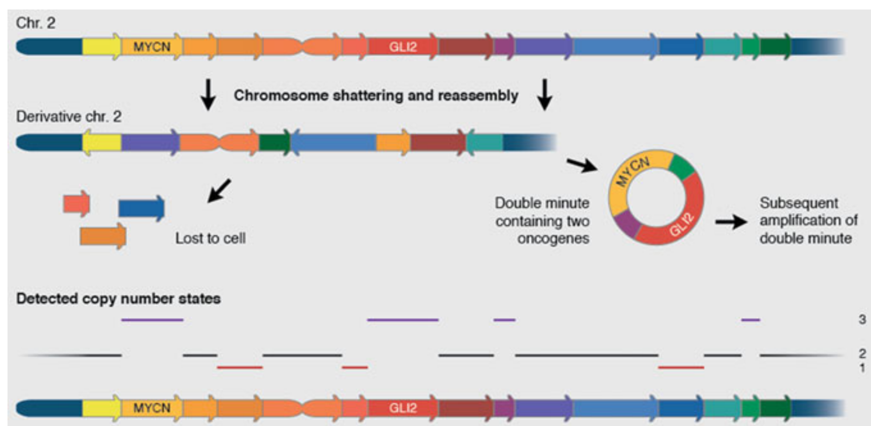

Fig. 6 Illustrative example of a double-hit by chromothripsis, based on the findings of Rausch et al. (2012). Chromothripsis leads to reshuffling of chromosome 2. During this event, a double minute chromosome is formed containing two known oncogenes, *MYCN* and *GLI2*. Subsequent amplification of the double minute chromosome -due to the presence of the oncogenes, which are beneficial to the cancer cell if present in higher numbers- leads to the presence of a third, highly amplified copy number state

chromosome resulting from chromothripsis (Rausch et al. 2012). Furthermore, several instances of double hits of cancer genes were caused by chromoplexy in prostate cancer, including formation of a *TMPRSS2-ERG* fusion gene together with disruption of the tumor suppressor *SMAD4* by a single chain of rearrangements. In exceptional cases three lesions were described that affected cancer genes, such as simultaneous disruption of *CDKN2A*, *WRN* and *FBXW7* in chordoma (Stephens et al. 2011).

Altogether, chromothripsis and other types of complex genome rearrangements are an important pan-cancer mechanism causing oncogenic lesions. Large-scale cancer genome analysis demonstrated that complex rearrangements are among the first rearrangements appearing in cancer genomes and may thus contribute to tumor initiation (Malhotra et al. 2013). Nevertheless, effects on cancer genes are not identified in all instances. Three neuroblastoma tumors were reported with chromothripsis on chromosome 5, without causing a notable tumor-driving lesion (Molenaar et al. 2012). In these cases chromothripsis may merely be a coincidental passenger event, apparently without a negative effect on cell survival. Such a scenario is underscored by the recent finding of temporal chromothripsis during the course of chronic lymphocytic leukemia development (Bassaganyas et al. 2013). This study indicated that a cancer subclone containing chromothripsis occurred as a secondary event years after initiation of disease. Subsequent rapid expansion of this subclone contributed to the aggressiveness, but the chromothripsis clone did not survive chemotherapeutic treatment and was not found in the relapse. Alternative explanations for the lack of oncogenic lesions resulting from chromothripsis could be the coupling of chromothripsis to a separate oncogenic mutation on the same

chromosome or yet unknown cellular consequences, such as global effects on chromosome structure and gene regulation.

10 Prognostic and Therapeutic Implications of Chromothripsis

With the overwhelming evidence that chromothripsis can promote formation of cancer-driving changes, the question is quickly raised as to whether chromothripsis represents a biomarker allowing clinical categorization of cancer patients. Several studies have now provided evidence for the association of chromothripsis with poor survival in different cancer types, including neuroblastoma, medulloblastoma, acute lymphoblastic leukemia and multiple myeloma (Rausch et al. 2012; Cai et al. 2014; Molenaar et al. 2012; Moorman et al. 2007; Magrangeas et al. 2011). It is unclear if chromothripsis in these tumors is only a biomarker associated with poor survival or whether the chromothripsis rearrangements themselves lead to more aggressive tumor growth. In the first case, chromothripsis may be a marker of severe genomic instability and a concomitant capacity of tumors to swiftly adapt as a response to changing environmental conditions. Consistent with this, association between chromosomal instability and poor clinical outcome has been observed in several human cancers (Carter et al. 2006). In the latter scenario, the actual genes affected by chromothripsis could result in a more aggressive disease course and lower survival. This option poses important opportunities for targeted therapy. For example, the RELA fusions observed in supratentorial ependymoma, which drive NF-κB signaling, may be an interesting drug target specifically associated to chromothripsis (Parker et al. 2014). Both scenarios sketched above may be true, but likely depend on the tumor type and environmental conditions. This is underscored by the observation of chromothripsis in some cases of benign uterine leiomyomas (Mehine et al. 2013). Also, a transient subclone of chronic lymphocytic leukemia containing chromothripsis has been described (Bassaganyas et al. 2013). In these instances, chromothripsis likely represents a passenger event with little impact on disease course and is therefore not a valuable biomarker. However, the routine detection of chromothripsis breakpoints provides new opportunities for monitoring disease status and progression. Cancer-specific breakpoints can be used for the design of PCR amplicons to track the presence of cancer rearrangement in blood plasma, enabling personal diagnostic strategies (Leary et al. 2010; McBride et al. 2010). Circulating tumor DNA in blood plasma can even be used for direct sequencing and interrogation of cancer-specific chromosome aberrations, thus allowing non-invasive detection of tumors and their genetic changes (Berger et al. 2012). The same methodology can be applied to oncogenic chromothripsis rearrangements.

11 Concluding Remarks

The discovery of chromothripsis has triggered a new field of research focused on understanding and discovery of complex genomic rearrangements in cancer. This has considerably changed our view on cancer evolution, which as we now think, can involve catastrophic events, in addition to a gradual pattern of mutation and selection. The field has now converged on three types of rearrangements, namely chromothripsis, chromoplexy and chromoanasynthesis. Although chromoanasynthesis displays distinct, replication-dependent genomic features, the difference between chromothripsis and chromoplexy is far less clear. Both phenomena appear to involve double-stranded DNA breaks in confined genomic regions and both lead to a typical pattern including deletions. Whether chromoplexy is a phenomenon, which is as widespread as chromothripsis is currently unclear. Chromoplexy may be a milder form of chromothripsis, generally involving fewer rearrangements. Due to stringent criteria for chromothripsis for the number of focal copy number changes (>10) (Table 1) chromoplexy events may have gone unnoticed in several studies. Furthermore, complex genomic changes in cancer genomes can be masked by simple variants, which have preceded or followed them. Whether chromothripsis and chromoplexy are truly two distinct phenomena or rather a more severe and milder display of the same event will likely be uncertain until the mechanism behind these phenomena is better understood, providing a more precise description of characteristics per event type. Until then, we may just speculate and come up with new models, which fit the experimental data, such as the recently proposed translocation-induced chromothripsis (Zhang et al. 2013). This model describes the occurrence of chromoplexy creating a platform for a chromothripsis event, possibly explaining the mechanism for chromothripsis involving multiple chromosomes. Whatever the mechanism, we do know that cancers may combine different complex changes, including chromothripsis, over consecutive cell divisions, altogether culminating into a chromosome configuration, which allows faster division, better survival and reduced death of the cancer cell.

Box 1: Detection of genomic rearrangements

Array CGH

Array CGH is a molecular cytogenetic method that compares a test sample to a reference sample by labeling DNA from both samples with different fluorophores. Labeled DNA is denatured and hybridized in a 1:1 ratio to a microarray containing specific cloned DNA fragments 100–200 kb in size. The ratio of test sample versus reference is determined for every fragment.

Deviations from the 1:1 ratio indicate the presence of a CNA in that specific region (Solinas-Toldo et al. 1997). Array CGH provides a quick, relatively affordable and genome-wide scan for CNAs, but is unable to detect copy neutral events.

SNP array
Unlike array CGH, SNP arrays are not performed by competitive hybridization; rather a pool of normal samples is used to estimate the expected intensity of a probe as a reference. A large number of SNPs spread out across the genome is tested. For each SNP, the probe intensity of the sample is compared to the reference. Like array CGH, SNP arrays are affordable and relatively fast and easy to analyze. An advantage of SNP arrays over array CGH is the determination of the allele frequency, which provides a percentage for the cells carrying the reference allele for every SNP (Conlin et al. 2010). In cancer research, the allele frequency is often used to determine the percentage of mosaicism of a sample, using bioinformatics tools such as Absolute (Carter et al. 2012).

Next generation sequencing (NGS)
Paired-end sequencing, is widely used for SV detection. Genomic DNA is fragmented to a specific size, varying from a few hundred bp up to 2–3 kb or even 10 kb in extreme cases. Paired reads from both ends of these fragments are generated and subsequently sequenced in a massively parallel fashion (Mardis 2009). In a typical sequencing run, 100 s of millions of short reads of 25–150 nucleotides are generated. De novo assembly, in which a genome is reconstructed from overlapping reads and subsequently compared to a reference genome to detect genomic rearrangements, is possible but highly complex due to the short read length that leads to fragmented assembled genomes. Instead, resequencing is normally applied, where paired sequencing reads are directly aligned to a reference genome. The vast majority of reads will map concordant, in which case the distance between the aligned reads is equal to the length of the original DNA fragment. Discordant reads indicate the presence of an SV, characterized by a difference in distance between the aligned reads and the length of the original DNA fragment (Fig. 2). Besides alignment distance another essential variable for SV detection is the orientation of the reads within the read pairs. The orientation of breakpoint junctions is indicated by using head (H), which indicates the 5′ end of a fragment, and tail (T), indicating the 3′ end of a fragment. Different rearrangement types lead to different orientation conformations (Fig. 2). Paired end sequencing also enables the detection of copy number events by analyzing read density. A deletion will lead to a decreased coverage for the deleted area when compared to the rest of the genome, a duplication to increased coverage. In order to reliably call an SV, multiple reads have to overlap a breakpoint, forming a cluster of discordant reads. The larger the number of reads overlapping the breakpoint, the more precise the breakpoint

location can be determined, from a relatively large breakpoint region up with low coverage to the determination of the breakpoint at the nucleotide level at high coverage. PCR and Sanger sequencing is generally used to validate breakpoints at high resolution.

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Consequences of Aneuploidy in Cancer: Transcriptome and Beyond

Milena Dürrbaum and Zuzana Storchová

Abstract
Cancer cells differ from normal healthy cells in multiple aspects ranging from altered cellular signaling through metabolic changes to aberrant chromosome content, so called aneuploidy. The large-scale changes in copy numbers of chromosomes or large chromosomal regions due to aneuploidy alter significantly the gene expression, as several hundreds of genes are gained or lost. Comparison of quantitative genome, transcriptome and proteome data enables dissection of the molecular causes that underlie the gene expression changes observed in cancer cells and provides a new perspective on the molecular consequences of aneuploidy. Here, we will map to what degree aneuploidy affects the expression of genes located on the affected chromosomes. We will also address the effects of aneuploidy on global gene expression in cancer cells as well as whether and how it may contribute to the physiology of cancer cells.

Keywords
Aneuploidy · Copy number alterations · Transcriptome · Proteome · Cancer

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1 Introduction

Accurate duplication of chromosomes and their equal segregation during cell division is essential for the maintenance of genomic stability and successful propagation of any organism on Earth. Failure to faithfully duplicate and segregate chromosomes leads to cell cycle arrest, cell death or to aneuploidy characterized by a karyotype that differs from multiples of the haploid set. Aneuploid karyotypes identified in cells can be distinguished into three main classes: somatic copy number variations that consist of gains or losses of chromosomal regions from one kilobase (1000 nucleotide bases) to several megabases in size, structural aneuploidies up to the size of a chromosome arm, and whole chromosomal aneuploidies, where cells gain or lose one or more entire chromosomes (Fig. 1).

Whereas copy number variations and possibly also segmental aneuploidy arise due to incorrect DNA replication and repair, whole chromosomal aneuploidy results from errors in chromosome segregation (reviewed in Gordon et al. 2012; Holland and Cleveland 2012). The propagation of aneuploid cells that have missegregated chromosomes is limited as they often arrest in the G1 phase directly following the erroneous mitosis (Thompson and Compton 2010; Li et al. 2010; Kuffer et al. 2013; Kumari et al. 2014). Even if aneuploid cells overcome this burden and continue to divide, their proliferation is often markedly impaired and they may suffer from additional detrimental changes (Torres et al. 2007; Williams et al. 2008; Stingele et al. 2012). The altered physiology of aneuploid cells is likely due to the large-scale changes in gene copy number and their expression. In humans, aneuploidy is associated with pathologies such as trisomy syndromes and cancer. Intriguingly, the effects of aneuploidy might become advantageous in adverse or rapidly changing environments (Pavelka et al. 2010; Lee et al. 2011). The molecular mechanisms underlying the cellular consequences of chromosome missegregation both acute, i.e., in daughter cells immediately after chromosome segregation errors, and chronic, i.e., during subsequent proliferation of aneuploid cells, remain incompletely understood.

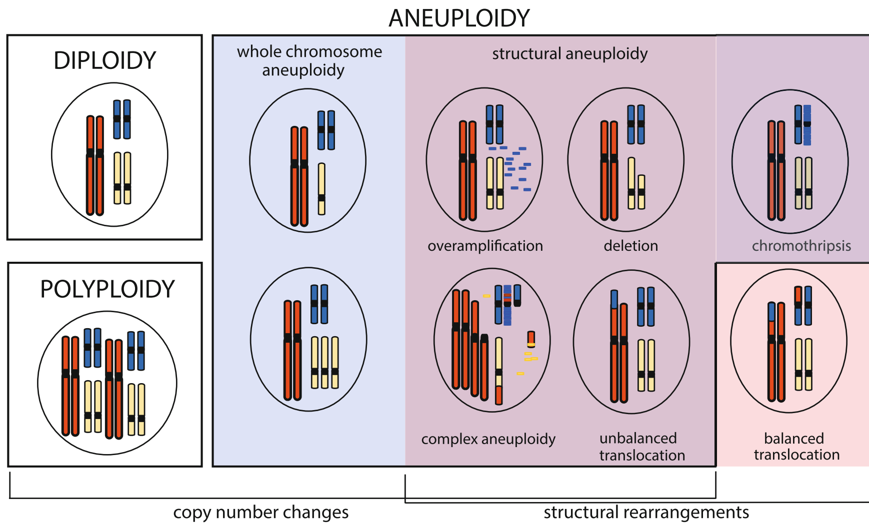

Fig. 1 Karyotype aberrations. Doubling the karyotype results in polyploidy, whereas loss or gain of one entire chromosome results in whole chromosome aneuploidy. Unbalanced structural rearrangements such as gain or loss of regions as large as chromosome arms and unbalanced translocations can cause structural aneuploidy. Chromotripsis, an event of complex genome rearrangements, is often accompanied by amplifications and deletions and therefore results in structural aneuploidy as well.

The chronic consequences of aneuploidy per se can only rarely be analyzed, because in many cases aneuploidy is accompanied by chromosomal instability (CIN), an ongoing elevated frequency in mitotic errors that leads to additional chromosome gains or losses in a significant proportion of cell divisions. Cancer cells with CIN missegregate a chromosome about once every one to five divisions, compared to rates of one chromosome per a hundred cell divisions in stable, diploid cell lines (Cimini et al. 1999; Thompson and Compton 2008). Being accompanied by other types of instabilities, whole chromosome CIN manifests in the complexity of karyotypes in cancer and high inter- and intratumor genomic heterogeneity (Burrell et al. 2013). Additionally, cancer cells display multiple genomic alterations, such as point mutations, and small rearrangements, such as insertions, deletions, duplications, inversions, amplifications and translocations. The ongoing CIN in cancer cells means that both acute and chronic effects of aneuploidy act in tumor cell lines simultaneously. Thus, studies of aneuploidy in these cells resemble studies of a continually changing creature, a Proteus of sorts, who defies to be captured in its true nature.

Deciphering the consequences of aneuploidy has been recently advanced by analysis of cells from embryos and patients with trisomy syndromes as well as by novel aneuploid model systems (Fig. 2). To complete the picture, models carrying mutations that interfere with chromosome segregation and thereby induce the CIN

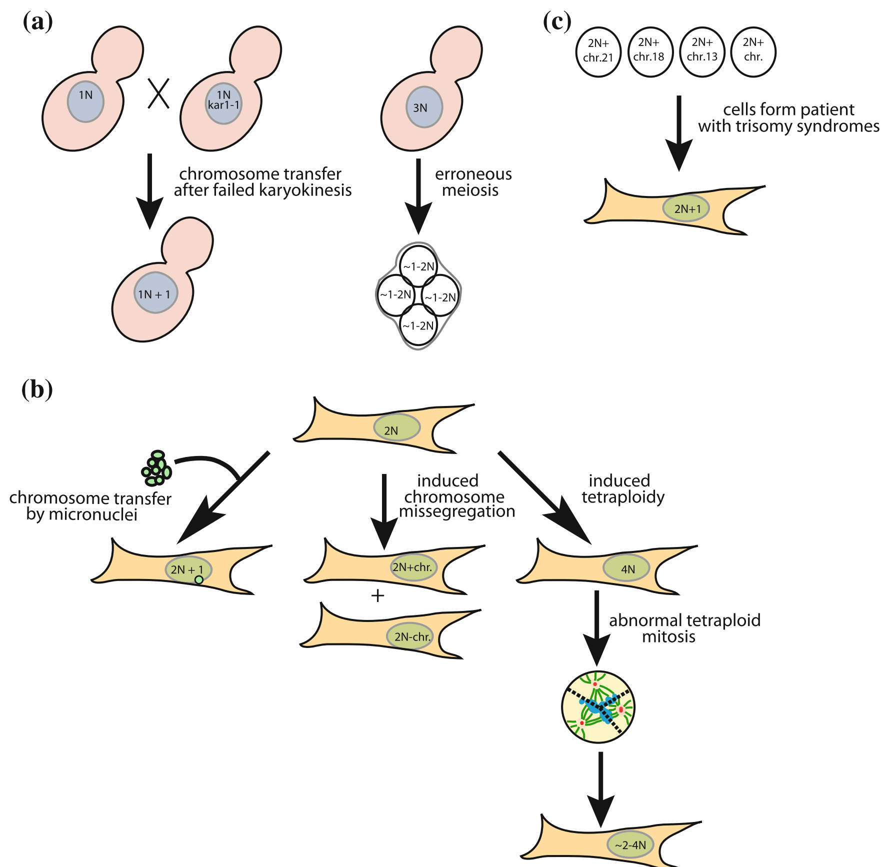

Fig. 2 Aneuploid model cell lines. **a** Yeast aneuploid model cell lines arise via chromosome transfer after failed nuclear fusion or from erroneous meiosis result in aneuploidy. **b** Mammalian aneuploid model cell lines of defined aneuploidy are generated by micronuclei-mediated chromosome transfer. Random aneuploidies arise from induced chromosome missegregation or via abnormal mitosis in tetraploid cells. **c** Cells derived from embryos or patients with trisomy syndromes present a source for cell lines with defined aneuploidy

phenotype were established (e.g. Sotillo et al. 2007; Weaver and Cleveland 2007; Baker et al. 2009 for a review see Ricke and van Deursen 2013). With the advent of microarray technology and mass spectrometry, transcriptome and proteome studies in both aneuploid model cell lines and cancers gave new insights into the response to aneuploidy. In our review we will focus on the consequences of aneuploidy in cancer and compare them with the recently characterized effects of aneuploidy in model systems. In the light of these findings we will discuss how these expression changes are linked to the physiological effects of aneuploidy.

2 Consequences of Aneuploidy on Transcriptome and Proteome

The fundamental characteristics of aneuploid phenotypes are determined by the impact of altered gene copy numbers on the transcriptome and proteome. In order to detect expression changes attributable directly to aneuploidy, multiple studies compared model cell lines with a defined aneuploidy to the parental diploid cell line. Yet, it should be noted that the quantitative analysis of transcriptome and proteome changes in aneuploid cells often suffers from severe technical difficulties. Part of the problem is that the quantitative change that needs to be determined is small, as the presence of one extra copy results in a 50 % expression increase. Thus, normalization strategies, used thresholding cut offs and statistical analysis may strongly affect the interpretation of the studies. The analysis of tumors is further complicated by the fact that they consist of cancer cells with heterogeneous karyotypes intermixed with a fraction of normal diploid cells (Mitelman 2013; Stevens et al. 2014). Thus, the transcriptome analysis determines the average gene expression changes affected by both the effects of gene copy number and the tumor heterogeneity. Despite these limitations, the global gene expression profiling markedly progressed our understanding of the cellular consequences of aneuploidy.

2.1 Transcriptome and Proteome Response to Model Aneuploidy

The majority of genes encoded on the supernumerary chromosomes are differentially expressed according to the gene copy numbers in most model aneuploid cells, such as in yeast (Torres et al. 2007; Chikashige et al. 2007), murine (Williams et al. 2008) and human cell lines (Stingele et al. 2012; Upender et al. 2004; Nawata et al. 2011), *Arabidopsis thaliana* (Huettel et al. 2008; Sheltzer et al. 2012) and maize (Birchler 2013). The gene expression largely scales with the copy number changes also in trisomies from patients' samples and mouse models: trisomy of chromosome 21 resulted in correlative expression change of the genes in both human and mouse models (Ait Yahya-Graison et al. 2007; Chou et al. 2008; Wang et al. 2011; Vilardell et al. 2011). Markedly, gene dosage compensation was found for 25 % to 50 % of the genes on the trisomic chromosome (Ait Yahya-Graison et al. 2007; Chou et al. 2008; Wang et al. 2011; Vilardell et al. 2011). In Drosophila the transcripts originating from aneuploid chromosomes are buffered towards the diploid levels (Stenberg et al. 2009; Zhang et al. 2010; Lundberg et al. 2012). This reflects the specificity of gene dosage compensation mechanisms in Drosophila rather than a general feature of aneuploidy (reviewed in Birchler 2013; Stenberg and Larsson 2011; Donnelly and Storchova 2014).

Global protein expression scales with the gene copy number changes as well, as the expression from the extra chromosome is increased by 1.6–1.9-fold in yeast and mammalian cells (Stingele et al. 2012; Pavelka et al. 2010; Torres et al. 2010).

However, initial small-scale analysis in disomic yeast strains determined that 13 out of 16 proteins were not differentially expressed as expected from the gene copy numbers (Torres et al. 2007). Genome-wide comparison revealed that around 20 % of the proteins encoded on supernumerary chromosomes did not show an enhanced expression in yeast as well as in human cell lines (Stingele et al. 2012; Torres et al. 2010). Intriguingly, the proteins whose abundance was compensated to euploid level were enriched for subunits of macromolecular protein complexes (Torres et al. 2007, 2010; Stingele et al. 2012). In concordance, global quantitative analysis demonstrated a significant reduction in the expression of protein complex core subunits encoded on the aneuploid chromosomes in 3 out of 5 yeast strains with a complex aneuploid karyotype (Pavelka et al. 2010). Moreover, other protein classes such as protein kinases and transcription factors are subjected to dosage compensation to restore the stoichiometric balance at least in human cell lines (Stingele et al. 2012 and our unpublished results). The mechanisms enabling the dosage compensation are not known, but it has been suggested that they may contribute to restoring the protein stoichiometry (Torres et al. 2007; Donnelly and Storchova 2014). In summary, gene and protein expression in model aneuploid cell lines generally scales according to the gene copy number, but specific protein classes are compensated towards normal diploid expression levels by an unknown mechanism.

2.1.1 Global Transcriptional Response to Aneuploidy

Aneuploidy affects the expression not only of the genes located on the supernumerary chromosomes, but also the expression of multiple other genes across the entire genome (Torres et al. 2007; Stingele et al. 2012; Upender et al. 2004; Sheltzer et al. 2012; Gemoll et al. 2014). This global gene deregulation can originate from at least two sources. First, transcriptional regulators that are located on the aneuploid chromosome regions and therefore present in altered copy numbers can affect the expression of genes on the other chromosomes. In this case, the response to aneuploidy should be largely dependent on the specific extra chromosome. Intriguingly, a uniform transcriptional response was identified in all aneuploidy model cell lines (Sheltzer et al. 2012; Dürrbaum et al. 2014). This rather suggests a second model, where the expression changes throughout the whole genome are driven by aneuploidy per se and the resulting protein imbalance triggers a specific cellular response that feeds back to the transcriptional regulation. The gene expression pattern in aneuploid yeast strains of different origins resembles the transcriptional pattern of a previously described yeast environmental stress response —ESR (Torres et al. 2007; Sheltzer et al. 2012). In particular, the enriched gene ontology terms for differentially expressed genes suggested aneuploidy-driven alterations in RNA processing and energy metabolism. Similarly, aneuploid mammalian cells of different origins show a uniform aneuploid pathway response pattern (Williams et al. 2008; Stingele et al. 2012; Sheltzer et al. 2012; Dürrbaum et al. 2014; Foijer et al. 2013). The aneuploidy response pattern is similar also in cultured amniocytes from trisomic pregnancies (Sheltzer et al. 2012). In detail, gene ontology terms such as endoplasmic reticulum (ER), Golgi apparatus, lysosomes

and vacuoles and membrane metabolism were consistently upregulated, whereas DNA and RNA metabolic pathways—e.g., DNA replication, repair, transcription and RNA splicing—were downregulated on the transcriptional level (Dürrbaum et al. 2014). These deregulated pathways were confirmed by proteome analysis of six different human aneuploid cell lines (Stingele et al. 2012).

What triggers these conserved changes? Recently, it was shown that aneuploidy affects molecular pathways linked to the maintenance of protein homeostasis (Torres et al. 2007; Stingele et al. 2012; Tang et al. 2011; Oromendia and Amon 2014). That is, gain of an extra chromosome may disturb cellular proteostasis by flooding the cellular system with proteins and exhausting the resources for their synthesis, thus causing proteotoxic stress (Donnelly and Storchova 2014; Oromendia and Amon 2014). As a consequence, protein degradation pathways might be activated to eliminate overexpressed proteins and accumulated misfolded proteins. Indeed, aneuploidy activates autophagy and upregulates annotations associated with lysosome, vacuoles and membrane metabolism (Dürrbaum et al. 2014; Tang et al. 2011; Stingele et al. 2013). In addition, the upregulation of ER and Golgi might be also attributed to autophagy, as these organelles serve as a membrane source for autophagosomes (Lamb et al. 2013). In concordance with the hypothesis that the transcriptional response reflects the cellular changes upon proteotoxic stress, autophagy inhibition in near- diploid HCT116 cells resulted in transcriptional changes similar to the aneuploid transcriptional response pattern (Dürrbaum et al. 2014) and aneuploid cells are sensitive to drugs triggering proteotoxic stress (Torres et al. 2007; Oromendia and Amon 2014). Taken together, the current data suggest that the aneuploid transcriptional response pattern arises as a direct consequence of proteotoxic stress due to karyotypic imbalance.

Another possibility is that the transcriptional pattern of aneuploid cells reflects their slower proliferation. Impaired proliferation and delayed progress through the G1 and S phases was observed in disomic yeasts as well as in trisomic and tetrasomic mammalian cell lines (Torres et al. 2007; Williams et al. 2008; Stingele et al. 2012). Two budding yeast strains carrying mutations in cell cycle regulatory factors that delay cell cycle progression show similar transcriptional changes as observed in response to aneuploidy (Sheltzer et al. 2012). Further, the differential transcription was changed when disomic and euploid yeast were grown at the same growth rate in the chemostat (Torres et al. 2007). However, slow proliferation cannot fully explain the aneuploidy response pattern, as complex human aneuploid cell lines exhibit the same transcriptional changes, yet their growth rate is similar to diploid cell lines (Dürrbaum et al. 2014). Moreover, similar expression changes were also identified in chromosomally unstable cancer cell lines of the NCI-60 panel that generally proliferate well (Roschke et al. 2008; Sheltzer 2013). This finding suggests that either both, CIN and aneuploidy, trigger a similar transcriptional response or that the aneuploidy response pattern is in fact a cellular response to CIN, as aneuploidy can promote CIN (Sheltzer et al. 2011, our unpublished data). Interestingly, despite the striking similarities among the aneuploid pathway response, there is no significant overlap of the individual deregulated genes between different aneuploidies (Williams et al. 2008; Gemoll et al. 2014; Dürrbaum et al. 2014). This

indicates that there are common physiological changes in response to aneuploidy, but the specific molecular determinants are diverse, likely as a consequence of heterogeneous karyotypes (Dürrbaum et al. 2014). Taken together, aneuploidy triggers activation and inhibition of certain pathways and these global changes likely influence all other physiological consequences of aneuploidy.

2.2 Cancer Signatures and Transcriptome Dynamics in Aneuploid Cancers

A longstanding interest of cancer research is to characterize the transcriptome and proteome changes of cancer tissues. This is a necessary step for classifying tumor subtypes and for identifying molecular contributors to tumor progression and biomarkers of tumor aggressiveness or treatment strategies (Sara et al. 2010; Eddy et al. 2010; Chibon 2013). The gene expression changes are often seen as a result of large-scale epigenetic remodeling or gene mutations. Recent studies of aneuploid model cell lines emphasized that global gene expression is also altered by aneuploidy. The relative contribution of aneuploidy to the cancer transcriptome remains a subject of ongoing investigations.

2.2.1 Correlation of mRNA and DNA Copy Number Changes in Aneuploid Cancers

In general, gene expression in regions with copy number variations as a result of aneuploidy is correspondingly altered in all types of cancer tissues. However, the estimations of the correlation between gene copy numbers and gene expression is strongly influenced by the methods used to integrate the DNA copy numbers with the gene expression data. In brief, there are three main approaches: (1) simple comparison of the fold changes and calculation of the percentages of genes with high/low copy number that is co-directional with mRNA expression changes; (2) gene-by-gene correlation of gene copy number with gene expression; and (3) correlation of the average gene expression with the averaged copy number across a chromosome arm or segment (Fig. 3; Table 1). Using the first approach the mRNA expression derived from trisomic chromosomes or more than twofold amplified regions is increased within a range from 1.14 to 3-fold compared to the normal diploid case (Schoch et al. 2005; Xu et al. 2010). This broad range already indicates that the results of a fold change comparison will vary according to the cutoffs applied. When no cutoffs were applied, 62.5–87 % of the genes on trisomic chromosomes in acute myeloid carcinoma showed increased expression (Schoch et al. 2005). Similar percentages were reported in breast cancer, when twofold amplified regions were considered (Pollack et al. 2002) and in colon cancer, when genes with the highest 20 % of expression were analyzed (Tsafrir et al. 2006). If considering only more than 2.5-fold amplified regions, 44 % of genes are correspondingly expressed in colon cancer (Hyman et al. 2002). In contrast, several studies report exceptions of amplified regions that do not show correlative changes

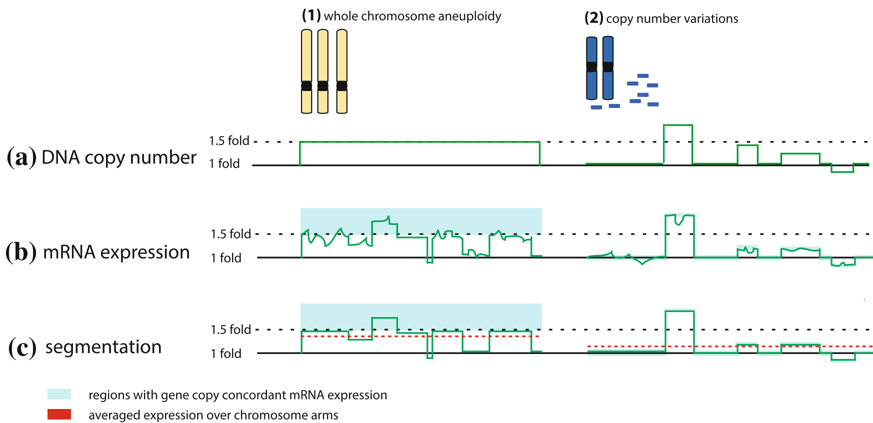

Fig. 3 Consequences of aneuploidy. Aneuploidy can arise from incidental chromosome missegregation or ongoing chromosomal instability. The acute response to chromosome missegregation, such as proteotoxic stress, growth defects, energetic stress and DNA damage, can activate cell cycle arrest or cell death. In a chronic response the aneuploid cells can adapt to the cellular stresses. Ongoing genomic instability generates genetic diversity and increases the adaptive potential. Cells adapt to aneuploidy by overcoming the adverse effects on i.e. proliferation due to growth-promoting mutations or by mutating the p53 signaling pathway

in gene expression (Tsafrir et al. 2006; Habermann et al. 2007; Taylor et al. 2010). For example, the loss of 8p is common in cancer, but a study in prostate cancer did not detect a correlative decrease in gene expression for all genes in the corresponding region (Taylor et al. 2010). A silencing of chromosomal amplifications in colon cancer has been also reported, but the results are based on a very stringent analysis; here, only 3.8 % of genes located in twofold amplified regions showed a twofold change in mRNA expression (Platzer et al. 2002).

In addition to the statistical method applied, sample size might influence the results; thus, studies calculating the gene-by-gene correlation as in (Holland and Cleveland 2012) over a large tumor sample set give more robust results. The correlation of median gene expression and copy number was consistently reported to be between 0.52 and 0.63 in colorectal cancer (Tsafrir et al. 2006; Grade et al. 2007). Analyses suggest that 10–40 % of the mRNA expression of five different types of tumors can be explained by copy number changes (Xu et al. 2010; Chin et al. 2006; Gu et al. 2008). Moreover, plotting the distribution of gene-by-gene expression and copy number correlation results in a normal-shaped curve with the mean shifted towards positive correlations (Xu et al. 2010; Pollack et al. 2002). The weak positive correlation becomes stronger by applying the approach described by Thompson and Compton (2010), i.e. correlating the average gene expression and copy number changes of a chromosome arm or applying a segmentation algorithm. This approach identified high correlation in three meta-studies on preexisting data (Gu et al. 2008; Ortiz-Estevez et al. 2011; Fontanillo et al. 2012). Moreover,

Table 1 Selection of recent results on correlation of gene copy number and gene expression in aneuploid cancers

Cancer tissue	Method	Results	Remarks	Publication
Comparison of fold changes				
Oral squamous cell carcinoma	Genes were divided into subgroups according to their level of copy number change and compared to the average gene expression fold change of the respective groups	Dosage-response relation between copy number changes and mRNA expression	20 samples	Xu et al. (2010)
Colorectal carcinomas	Upper/Lower 20 % of global mRNA and CGH levels were used as cut offs for comparison of expression levels and corresponding copy number changes	63 % of genes overexpressed showed also amplification; 62 % of downregulated genes showed also lower CGH (in lowest 20 %)	114 samples	Tsafrir et al. (2006)
Ovarian carcinomas	Analysis of 93 genes on chromosome 22	Correlation of gene copy number and expression changes	18 samples	Benetkiewicz et al. (2005)
Acute myeloid leukemia	Average expression on aneuploid/trisomic chromosome was calculated	1.14–1.27-fold increase in gene expression and 62.5–87 % of genes expressed higher than in diploid	7–12 samples per trisomy, 104 controls	Schoch et al. (2005)
Colon cancer liver/hepatic metastases	Twofold cut off for amplified regions on 7p, 8q, 13q, and 20q	3.8 % genes in the amplified regions with ≥2 fold change in mRNA expression; 90 % of genes showed a 0.5–2-fold change	23 samples, only 7p, 8q, 13q, and 20q	Platzer et al. (2002)
Breast cancer cell lines	2.5-fold cut off for amplified regions; overexpression defined as top 7 % of global expression levels	44 % genes in amplified regions showed overexpression; 10.5 % of overexpressed genes showed >2.5-fold copy number amplification	14 cell lines	Hyman et al. (2002)
Primary breast cancer	≥4-fold change cut off for highly amplified genes	62, 42 % of high amplified genes showed >2-fold, >4-fold increase in mRNA expression, respectively	44 samples	Pollack et al. (2002)

(continued)

Table 1 (continued)

Cancer tissue	Method	Results	Remarks	Publication
Gene-by-gene correlation of copy number and mRNA expression				
Oral squamous cell carcinoma	Gene-by-gene correlation of mRNA expression and copy number	Normal distribution of correlations with a peak shifted towards positive correlations. Significant correlation of 30 % of transcripts	20 samples	Xu et al. (2010)
Primary breast cancer, breast, pancreatic, prostate, lung cancer cell lines	Median of gene-wise correlation of mRNA expression and gene copy number	Copy number variations explain 12–40 % of expression changes	Metaanalysis of Pollack et al. (2002), Hyman et al. (2002), Heidenblad et al. (2005), Zhao et al. (2005), Kim et al. (2006)	Gu et al. (2008)
Colorectal carcinoma	Gene-by-gene correlation of mRNA expression and gene copy number	Correlation value: 0.52 colon carcinoma; 0.56 rectal adenocarcinoma	32 colon carcinomas, 17 rectal adenocarcinoma	Grade et al. (2006, 2007)
Primary breast cancer	Gene-by-gene correlation of mRNA expression and gene copy number	10 % of genome showed significant correlations	145 samples	Chin et al. (2006)
Colorectal carcinomas	Correlation of average mRNA and gene copy number expression over all samples for each probe	mRNA and CGH correlation $r = 0.69$	114 samples	Tsafrir et al. (2006)
Primary breast cancer	Gene-by-gene correlation of mRNA expression and gene copy number	Distribution of correlations showed normal- shaped curve with a peak shifted towards positive correlations	44 samples	Pollack et al. (2002)
Correlation of averaged copy number and mRNA expression over chromosome segments				
Glioblastoma multiforme	Gene expression and copy number for a chromosome segment defined in a segmentation algorithm	Positive correlations of ≥0.6 was found for 55 % of the human gene loci	64 samples	Fontanillo et al. (2012)
Gastric cancer	Correlation of average mRNA and gene copy number over samples of 20 Mb chromosome arm bins	Positive correlation of mRNA expression with gene copy number of the respective genomic region	64 samples	Fan et al. (2012)

(continued)

Table 1 (continued)

Cancer tissue	Method	Results	Remarks	Publication
Glioblastoma multiforme and acute lymphoblastic leukemia	Gene expression and copy number for a chromosome segment defined in a segmentation algorithm	Segmented gene expression and copy number significantly correlated with $r = 0.6$ and 0.19, respectively	Metaanalysis of Kotliarov et al. (2006), Bungaro et al. (2009)	Ortiz-Estevez et al. (2011)
Liver carcinoma	Transcriptome correlation map method (Stransky et al. 2006) was used to identify regions with strong concordance in gene expression and copy number changes	General strong association of gene expression and copy number changes	139 samples	Woo et al. (2009)
Colon carcinoma	Chromosome arm average gene expression compared to chromosomal copy number	Positive correlated shift in expression profiles	32 samples	Grade et al. (2007)
Neck squamous cell carcinoma	For each chromosome arm average Z-score gene expression was compared to average CGH; for arms 3p and 22q, averaged expression of smaller chromosomal regions was analyzed	9/39 arms showed concordance in the direction of average gene expression versus copy number, 4/39 was opposite; loss of 3p and gain of 22q were reflected in consistent expression changes	13 samples	Masayesva et al. (2004)

average gene expression along a chromosome has been shown to predict chromosomal aneuploidy (Hertzberg et al. 2007).

Taken together, the effect of aneuploidy on the transcriptome is less pronounced in cancer tissues compared to model aneuploid cell lines owing to the population heterogeneity as well as genetic and epigenetic changes that were selected throughout the tumor evolution. Thus, aneuploidy is only one of many factors influencing the gene expression. Despite the heterogeneity of the approaches and interpretations, there is a trend towards a weak but consistent correlation of chromosome copy number changes and abundance of transcripts in cancer, which is prone to noise at the gene level, but becomes robust when only distinct regions of copy number changes are considered. In future, single cell analysis of cancer cells may provide more insights into the transcriptome changes and their correlation with copy number changes within cancer cell populations.

2.2.2 Aneuploidy and the Cancer Proteome

There are only a few studies that have systematically investigated the influence of aneuploidy on the cancer proteome. Myhre et al. (2013) compared DNA copy number, mRNA and protein levels of 52 breast cancer-related proteins in order to determine at which level the protein expression in cancer is regulated. A large fraction of the analyzed proteins (48.07 %) exhibited no correlation to DNA or mRNA expression values, 28 and 26 % showed a correlation of DNA to mRNA and mRNA to protein, respectively, and 15 % showed a correlation on all three levels. However, the sample size is too small to assume a general genome-wide effect of gene copy number on protein expression. In-depth proteomic analysis of two breast cancer cell lines revealed only weak correlations of 0.22 and 0.28 between gene copy number and protein expression changes (Geiger et al. 2010). Comparison of average expression to average copy number of adjacent chromosome regions increased the correlation. These results indicate that the effect of copy number alterations on the proteome level is even smaller than the effect on the transcriptome level. However, it should be noted that protein levels are influenced not only by gene copy numbers, but also by regulation of transcription, translation and by protein stability. In fact, only 40 % of individual protein concentrations can be explained by mRNA abundance in normal eukaryotic cells (Vogel and Marcotte 2012), which may partially explain the rather minor effects of gene copy number on mRNA and protein expression observed in cancer samples. This also suggests that many gene copy number changes are not reflected on protein levels and extensive proteome analysis will be required to fully estimate the effects of copy number changes on cancer proteome.

2.2.3 Gene Expression Signatures and Aneuploidy in Cancer

Finding commonly deregulated genes associated with cancer subtypes, prognosis or response to treatment remains central to cancer research (reviewed in Chibon 2013). Since aneuploidy is mostly associated with poor prognosis (McGranahan et al. 2012), a predictive gene expression signature for aneuploidy might be a promising approach for tumor classification. A gene signature is a group of genes, for which the expression is associated with a characteristic feature of cancer such as prognosis, subtype, instability or aneuploidy (Chibon 2013). In most cases, gene signatures are derived from transcriptome data, where the gene expression is correlated with cancer features and genes with the highest correlation build the signature. A well-known signature is the CIN70 signature derived from the NCI60 cancer cell line panel of cells to characterize cells with high CIN (Carter et al. 2006). The signature consists of 70 genes strongly correlated with the “total functional aneuploidy” as a proxy of overall aneuploidy across the NCI60 cancer cell lines. Therefore, CIN70 correlates with both CIN and aneuploidy without clear distinction. CIN70 successfully predicts the clinical outcome in multiple cancers, as a high net expression of the 70 signature genes predicts poor prognosis in the majority of cancer samples tested (Carter et al. 2006; Birkbak et al. 2011; Muthuswami et al. 2013). Recently, it was suggested that the CIN70 signature does not correlate with

numerical heterogeneity in the NCI60 cancer cell line panel, but with proliferation (Sheltzer 2013). Nevertheless, the high predictive potential of CIN70 was confirmed. Moreover, a novel "HET70" gene signature, whose expression correlates with karyotype heterogeneity in the NCI60 panel was also shown to predict poor prognosis (Sheltzer 2013). In addition, more signatures, mostly for very specific cancer subtype classifications were published in recent years, all either based on or enriched for CIN genes (Chung et al. 2013; Ying et al. 2013; Szasz et al. 2013; Al-Ejeh et al. 2014). In uveal melanoma, a direct transcriptional comparison of high aneuploid and low aneuploid tumors, defined as tumors with >20 and <5 % of nondiploid chromosome arms, respectively, identified a 54-gene signature (Ehlers et al. 2008). Significant overlap of this 54-gene signature genes with genes upregulated in breast cancer, multiple myeloma, keratinocytes treated with UVB irradiation, and with cell cycle genes was found, but the prognostic relevance remains to be proven.

All above described gene signatures are linked to proliferative cellular processes such as replication, chromosome segregation (CIN70) and centrosome function, cell cycle regulation, DNA damage repair (54-gene signature), and the high net expression of the signatures are predictive. In contrast, these gene ontology terms are largely downregulated in model aneuploid cell lines. Accordingly, a gene signature (TRI70) associated with aneuploidy in model aneuploid cell lines predicted good prognosis (Sheltzer 2013), whereas aneuploidy in cancer is generally associated with poor prognosis (McGranahan et al. 2012). These results reflect a general reverse transcriptional pathway deregulation observed when comparing the transcriptome of aneuploid model cell lines and aneuploid cancers (Sheltzer 2013 and our unpublished results). We hypothesize that aneuploidy per se acts as a cellular stress factor and impairs essential cellular pathways. This results in a strong selection pressure for enhanced expression of the impaired factors. Thus, cells that gained mutations, gene amplification, additional aneuploidies and other changes that reverse the aneuploidy response may overcome the adverse effects. Along this line, comparison of different colorectal cancer stages revealed a selection for specific chromosomal imbalances throughout tumor progression that was accompanied by correlating transcriptional changes affecting an increasing number of cellular pathways (Habermann et al. 2007). In future, this hypothesis should be tested by determining the effects of the evolutionary adaptation to aneuploidy on transcriptome and proteome in aneuploid model cell lines and aneuploid tumors.

3 Consequences of Aneuploidy on Proliferation

In describing the cellular response to aneuploidy it is useful to distinguish the acute response to sporadic chromosome missegregation from the chronic consequences of aneuploidy (Fig. 4). In the acute response cells that missegregate their chromosomes often arrest their cell cycle or die (e.g., Thompson and Compton 2010; Li

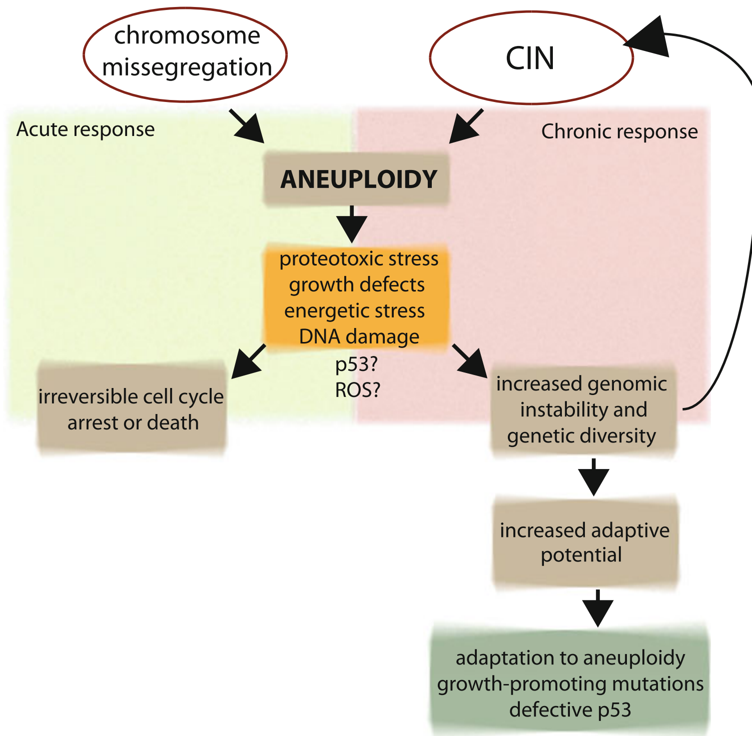

Fig. 4 Correlation of gene copy and mRNA expression. **a** Gain of a chromosome (*1*) or unbalanced structural changes (*2*) result in DNA copy number changes, such as 1.5 fold increase in case of trisomy. **b** As a result, mRNA expression levels change as well, but the percentage of genes with mRNA abundance in concordance with DNA copy numbers might vary largely and depends on the applied cut off (*light blue*; see method 1). **c** Segmentation algorithms or averaging of the expression values improves the correlation of gene copy number and gene expression by diminishing the signal noise at the single gene level (see method 3)

et al. 2010; Kuffer et al. 2013; Basu et al. 1998; Lanni and Jacks 1998; Dobles et al. 2000). The high frequency of cell cycle arrest after chromosome missegregation in higher eukaryotes suggests that not only chromosome loss but also chromosome gain is detrimental for cells. Missegregating cells accumulate the tumor suppressor p53 and the cyclin kinase inhibitor p21 in their nuclei in subsequent G1 (Thompson and Compton 2010; Li et al. 2010; Kuffer et al. 2013). Deletion of p53 or inhibition of the p38 mitogen-activated protein kinase (MAPK), which directly phosphorylates p53, abolishes the cell cycle arrest following chromosome missegregation (Thompson and Compton 2010). Thus, chromosome missegregation generates a p38 kinase–dependent stress response that activates p53 and triggers the cell cycle arrest. Other reports have described a role for the ataxia-telangiectasia mutated

(ATM) gene product and its activation by increased levels of reactive oxygen species (ROS) and possibly also by oxidative DNA damage (Li et al. 2010; Kuffer et al. 2013). The high levels of oxidative DNA damage and ROS generated in cells immediately upon chromosome segregation errors might arise due to increased energy metabolism in aneuploid cells or due to the response to protein folding defects. The transcriptional pattern of chronic aneuploidy is suggestive of increased oxidative metabolism, although there is no overlap with transcriptional changes observed in cells subjected to oxidative stress (Stingele et al. 2012; Dürrbaum et al. 2014). In Drosophila epithelial cells, the response to chromosome missegregation is characterized by the activation of the c-Jun N-terminal kinase (JNK) signaling cascade, which triggers apoptosis independently of p53 (Dekanty et al. 2012; Clemente-Ruiz et al. 2014). Both JNK pathway and p38 MAP kinase belong to the class of MAP kinases that are responsive to stress stimuli such as osmotic shock, inflammatory cytokines, translation inhibitors, heat shock and ER stress, growth factors and elevated ROS. However, it remains to be clarified whether the presence of a single extra chromosome can indeed cause oxidative or ER stress and how quickly this response commences.

Even if the progeny of abnormal mitoses survive, a marked impairment in cell cycle progression and proliferation is often observed in model aneuploid cells (Torres et al. 2007; Williams et al. 2008; Stingele et al. 2012). Cell lines derived from patients with trisomy syndromes or from the available mouse models suffer from impaired proliferation as well (Segal and McCoy 1974; Contestabile et al. 2009; Gimeno et al. 2014). Although the reasons for the growth delay remain unclear, three possible explanations have recently been proposed. First, proliferation might be affected by the type of aneuploidy, such as gains of chromosomes or chromosome arms carrying a gene whose overexpression impairs proliferation; indeed, aneuploidies of some chromosomes are lethal (Torres et al. 2007; Hassold et al. 2007; Thorburn et al. 2013). Second, the recently suggested proteotoxic stress caused by aneuploidy might compromise cell cycle progression and cell division, as has been shown experimentally in budding yeast, where as little as 0.1 % of misfolded proteins significantly reduce cellular proliferation and fitness (Geiler-Samerotte et al. 2011). Finally, as aneuploidy leads to increased levels of genomic and CIN (Sheltzer et al. 2011; Zhu et al. 2012), this might impair cellular proliferation.

Interestingly, cells with complex karyotypic changes appear to suffer less from the adverse effects of aneuploidy (Pavelka et al. 2010; Zhu et al. 2012). This is likely due to a selection process, in which only aneuploid cells with the most beneficial karyotype combinations survive and outgrow the other aneuploids. Thus, aneuploidy can also be advantageous for proliferation, if for example a gene supporting proliferation is carried on the extra chromosome or if growth suppressor has been lost. The advantageous effects are highly context-dependent. Chromosome-specific effects on immortalization capacity were determined in aneuploid MEFs (Williams et al. 2008). Trisomy of 13 or 19 allowed faster immortalization than diploid MEFs, whereas cells with trisomy of chromosome 1 or 16 failed to immortalize or immortalized slower, respectively. Since immortalization is an event

that requires multiple mutations in various genes leading to the increased proliferation, this finding could be due to chromosome-specific increased genomic instability observed in aneuploids. Indeed, immortalized MEFs showed an altered complex near-tetraploid karyotype (Williams et al. 2008). Similar effects can be observed in cancer as well; chromosomes 7, 12 and 20 that carry *EGFR*, *BRAF*, *SHH*, *KRAS*, *CDK4*, *MDM2*, *BCL2L1*, *E2F1* and *CDC25B* oncogenes are preferentially gained in tumor samples (Duijf et al. 2013). In a large-scale analysis on 4934 cancers, 140 recurrent regions of copy number alterations with 70 amplified regions and 70 lost regions were identified. These regions showed a remarkable percentage of oncogenes and tumor suppressors, respectively, among those were the oncogenes described above (Zack et al. 2013). In colorectal cancer, specific copy number alterations during cancer progression support the tumor development (Habermann et al. 2007). The interplay between negative and positive effects of altered gene copy numbers might be a major force in shaping the genome of cancer cells. Indeed, analysis of karyotype composition of more than 8.200 tumor-normal tissue pairs revealed that cumulative loss or gain of gene copy due to copy number variations shapes the composition of the cancer genome and can predict the complex pattern of aneuploidy or local copy number changes observed in cancers (Davoli et al. 2013). Taken together, copy number changes may have both advantageous and deleterious effects that depend on specific karyotypic compositions and on the environment.

Although beyond the scope of this review it is worth noting that spontaneous aneuploidy often occurs during in vitro expansion of stem cells (Peterson et al. 2011; Lund et al. 2012). This is likely due to the fact that the cell cycle regulation and the checkpoint linkage to apoptosis is attenuated in stem cells and thus aneuploid stem cells can readily proliferate (Mantel et al. 2007). Many of the chromosomal aberrations observed in stem cells are recurrent and can be found in both embryonic stem cells as well as in induced pluripotent stem cell (iPSC). These include trisomy 12, trisomy 8, and amplification of 20q11.21 (Martins-Taylor et al. 2011; Taapken et al. 2011). Similarly, human neural progenitor cells gain chromosome 7 and 19 in up to a quarter of the cells, reflecting selection for epidermal growth factor receptor (EGFR) overexpression in cells trisomic for chromosome 7 and 19, which was determined by immunofluorescence (Sareen et al. 2009). Although aneuploidy provides a growth advantage to the stem cells, as aneuploids successfully compete with their diploid counterparts in the culture, it also exerts negative effects. Human mesenchymal stem cells often gain an extra chromosome, but the trisomic lines underwent replicative senescence after 50–60 population doublings and never showed neoplastic changes (Estrada et al. 2013). Thus, depending on the cell type and culture conditions, aneuploidy in general or of a particular chromosome provides a growth advantage; however, in some context aneuploidy can also lead to delayed proliferation or premature senescence. In future it will be necessary to dissect which conditions lead to advantageous situation and which to adversity.

4 Aneuploidy and Its Link to Chromosomal Instability

4.1 Genomic Instability in Eukaryotic Aneuploid Model Cell Lines

The genome is subject to multiple changes during tumorigenesis due to the instability intrinsic to most cancers. Genomic instability can be divided into several distinct forms based on the scale of genomic changes: nucleotide instability due to point mutations, microsatellite instability, structural genomic/CIN, and whole CIN. These four types of instability frequently coexist in the same cells. Whether any of these instabilities can be driven by chromosomal imbalance alone has not been conclusively resolved. Therefore, analysis of cells from patients with constitutional trisomies as well as of defined aneuploidy models provides useful insights, as the isolated effects of aneuploidy can be analyzed.

Studies from patients with constitutional trisomy syndromes showed conflicting results likely caused by the fact that interphase fluorescence in situ hybridization (FISH) is prone to artifacts. In FISH analyses applied to interphase cells using enumeration probes for chromosomes 8, 15, and 16, the frequency of cells with altered copy number for each of these chromosomes showed a significant twofold increase in the 14 samples derived from trisomic patients compared to 14 samples derived from control subjects, with a strong bias to chromosome losses (Reish et al. 2011). Recent analysis that used two independent FISH probes simultaneously for chromosomes 2 and 17 (dual-color FISH) determined a two to threefold increase in levels of chromosome abnormalities in samples from trisomic patients in comparison to the normal population (Valind et al. 2013). This increase was not statistically significant and markedly below the increase observed in both chromosomally stable and chromosomally unstable cancer cell lines (DLD1 up to 10-fold, SW480 up to 30-fold, respectively) (Valind et al. 2013). One way of explaining this finding is that only embryos with either normal or near-normal levels of chromosome segregation errors survive. This notion is supported by the fact that the vast majority of embryos with trisomy do not survive until birth; for example, 80 % of trisomy 21 embryos die during embryogenesis (Biancotti et al. 2010). The second possibility is that aneuploidy, or at least trisomy, does not grossly impair chromosome missegregation. Intriguingly, trisomy syndromes are not associated with accelerated tumorigenesis; rather, the spectrum of tumors changes: there is an increased risk of acute lymphoblastic leukemia, acute myeloid leukemia and testicular germ cell tumors in trisomy 21 patients, and Wilms' tumor and hepatoblastoma in trisomy 18, whereas decreased risk of carcinoma in trisomy 21 patients has been identified to date (Ganmore et al. 2009).

Little is known about genomic instability in model aneuploid cell lines. Budding yeast that contain a single extra chromosome show a two to fourfold increase in mutation rates at two independent loci (Sheltzer et al. 2011). In addition, abnormalities such as DNA repair and recombination defects, indicated by increased Rad52-GFP foci and sensitivity to double-strand break-inducing drugs,

demonstrated the wide range of aneuploidy-induced genetic instabilities (Sheltzer et al. 2011). A genome-wide screening for genetic requirements of aneuploidy revealed that aneuploid yeasts can not survive without a functional homologous recombination pathway (Tange et al. 2012). Interestingly, the genomic instability phenotypes depend on the type of aneuploidy, since different disomies show different levels of genomic instability that do not correlate with the size of chromosomes (Sheltzer et al. 2011). Similarly, different combinations of aneuploid chromosome pairs resulted in a range of different CIN levels after propagation of random aneuploid yeast strains (Zhu et al. 2012).

Genomic instability was also reported in human aneuploid model cell lines: human embryonic stem cells carrying an extra copy of chromosome 8 accumulate low levels of additional chromosomal aberrations unrelated to chromosome 8 (Nawata et al. 2011), while unbalanced chromosome translocations were observed in renal carcinoma cell lines after trisomy of chromosome 3 was induced (Kost-Alimova et al. 2004). Chromosome-specific effects are also suggested by analysis of the diploid colorectal cancer cell line DLD1 with trisomies of chromosome 7 and 13 (DLD1 + 7, DLD1 + 13), the non-cancerous immortalized diploid epithelial cell line hTERT-RPE1 that had spontaneously gained chromosome 12 (RPE1 + 12), and primary cells derived from amniocentesis that are trisomic for chromosome 13 (AF + 13). Whereas DLD1 + 7, DLD1 + 13, and AF + 13 exhibited higher rates of chromosome missegregation, affecting only particular chromosomes, RPE1 + 12 cells displayed no increase in chromosome missegregation (Nicholson and Cimini, personal communication). The chromosome-specific effect was most apparent for trisomy of chromosome 13, as the increased abundance of the cytokinesis regulator *SPG20* (Spartin) on chromosome 13q13.3 caused cytokinesis failure in both DLD1 + 13 and AF + 13 cells (Nicholson and Cimini, personal communication). Both chromosome-specific and non-specific effects were observed by our group: tetrasomy of chromosome 5 and trisomy of chromosome 3 in the diploid colorectal cancer cell line HCT116 leads to a small, but significant increase in mitotic errors, whereas trisomy of chromosome 5 in HCT116 and trisomy of chromosome 21 in hTert-RPE1 did not affect chromosome segregation significantly (our unpublished results).

The molecular mechanisms underlying increased genome instability are currently unclear. Aneuploidy per se leads to downregulation of multiple pathways linked to DNA metabolism, such as DNA repair and replication, this may lead to insufficient DNA repair or to replication defects. Alternatively, increased metabolic requirements and oxidative stress might elevate the levels of DNA damage and chromosome missegregation. The genomic instability might be also due to an altered expression of a specific gene coding for an essential DNA repair factor located on a lost chromosome. Stoichiometric imbalances might also impair the molecular processes essential for maintenance of genomic stability. The importance of protein stoichiometry for the function of proteins has been previously demonstrated in this context. For example, correct stoichiometry of Mad1 and Mad2, proteins involved in the spindle assembly checkpoint, markedly affects checkpoint

functionality (Foijer et al. 2013; Zhu et al. 2012; Heinrich et al. 2013). Changes in stoichiometry may be responsible for CIN in complex aneuploid yeasts, as chromosomally stable aneuploid strains displayed a MAD2: MAD1 ratio of 1:1, whereas 40 % of the chromosomally unstable aneuploid budding yeast strains displayed a ratio closer to 0.5 (Zhu et al. 2012). Similarly, components of the minichromosome maintenance complex MCM2-7, a putative heterohexameric helicase required for DNA replication, show exquisite gene dosage sensitivity: already one hypomorphic allele (called Chaos3) that results in significantly decreased MCM4 protein levels triggers elevated chromosome missegregation and increased tumor formation in mice heterozygous for Mcm4^{Chaos3} (Shima et al. 2007). The effect of protein imbalance on genome integrity seems to scale with the degree of aneuploidy, since the degree of CIN is higher in aneuploids close to the diploid state than near haploid yeast strains (Zhu et al. 2012). Taken together, increased genomic instability is often detected in the aneuploid context, but the molecular processes involved in the maintenance of genomic stability that are impaired by abnormal chromosomal content remain to be determined.

4.2 Genomic Instability as a Consequence of Aneuploidy in Cancer

A study of somatic copy number changes by CGH in 3,131 cancer samples corresponding to 26 histological types found 25 % of the cancer genome to be affected by whole arm or whole-chromosome SCNAs, whereas 10 % is affected by focal SCNAs (Beroukhim et al. 2010). The investigators observed an average of 24 gains and 18 losses per cancer genome as well as a mean of 17 and 16 % of the genome affected by gain and deletions, respectively. In addition to aneuploidy, cancer genomes are largely unstable, which is probably best reflected in the cancer karyotype heterogeneity (Mitelman 2013; Lengauer et al. 1998; Heselmeyer-Haddad et al. 2012). Aneuploidy and genomic instability are closely intertwined and together they have been proposed as drivers for tumorigenesis (e.g., in Gordon et al. 2012; Williams et al. 2008; Lengauer et al. 1997, 1998; Duesberg et al. 1998; Storchova and Pellman 2004; Pfau and Amon 2012). The mechanisms of genomic instability in cancer have been extensively reviewed (Negrini et al. 2010; Thompson et al. 2010). However, little is known on whether aneuploidy directly contributes to genomic instability in cancer. Aneuploidy arises from ongoing genomic instability and it has been suggested that both can drive each other autocatalytically in a vicious cycle (reviewed in Potapova et al. 2013; Fig. 4). This is further supported by the observation that chromosome instability in cancer increases with the degree of aneuploidy (Zhu et al. 2012; Duesberg et al. 1998, 2004; Fabarius et al. 2003). Similar as in cell line models of aneuploidy, there are multiple mechanisms by which aneuploidy can promote genomic instability (Gordon et al. 2012). Yet, none of these above mentioned mechanisms have been convincingly demonstrated to date. It is beyond doubt that understanding the

molecular processes that contribute to genomic instability in aneuploid tumors will substantially add to our knowledge of tumorigenesis and might open new avenues to cancer treatments.

5 Additional Changes in Response to Aneuploidy in Cancer Cells

Tumors are characterized by altered signaling pathways that affect cellular metabolism. The metabolic alterations are usually seen as adaptations to the unique biochemical microenvironment, to provide the energy production and sufficient macromolecular biosynthesis and for maintenance of redox balance. Additionally, the identified metabolic alterations may not merely be an adaptation to malignancy, but could also be required for malignant transformation (for review see Cairns et al. 2011; Ward and Thompson 2012). A prominent metabolic phenotype observed in tumor cells is the Warburg effect (Vander Heiden et al. 2009). This effect is characterized by the use of glycolysis rather than mitochondrial oxidative phosphorylation as the main source of ATP even under normal oxygen-rich concentrations, resulting in abnormally high rates of glucose uptake into tumor cells and elevated production of the glycolytic metabolite lactate. Remarkably, model mammalian aneuploids also show striking metabolic changes (Williams et al. 2008; Stingele et al. 2012; Sheltzer et al. 2012). First, the expression of genes involved in oxidative metabolism and glycolytic pathways is elevated in model aneuploid cells (Torres et al. 2007; Williams et al. 2008; Stingele et al. 2012; Sheltzer et al. 2012; Dürrbaum et al. 2014). Additionally, metabolic features of the Warburg effect such as increased glucose uptake or elevated lactate production have been observed in trisomic MEFs (Williams et al. 2008). Model aneuploid cells are hypersensitive to metabolic inhibitors such as AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide), an analog of AMP that stimulates AMP-dependent protein kinase, and the sensitivity to AICAR correlates with the size of the extra chromosome (Tang et al. 2011). There were also greater growth inhibitory effects of AICAR observed in aneuploid cancer cell lines than in chromosomally stable cancer cell lines, suggesting that the sensitivity is indeed linked to aneuploidy and is not a general phenomenon of cancerous cells (Tang et al. 2011). The mechanism underlying the metabolic switch as well as what advantage it might provide to both cell line models of aneuploidy and aneuploid cancers remains unclear.

Aneuploidy may be tumor suppressive or tumor promoting by inducing non-cell-autonomous effects. Tumors are not a homogeneous mass of malignant cells, but consist of different, often highly heterogeneous subclones of cancer cells as well as of a variety of normal cell types, including fibroblasts, endothelial cells and pericytes of the tumor vasculature (Lorusso and Ruegg 2008; Chouaib et al. 2010; Augsten 2014; Moschetta et al. 2014). Moreover, the surrounding tissue and the context-specific interactions between cancer cells and normal tissues contribute to formation of a unique microenvironment that affects the growth and the cell-to-cell interactions within the tumor (Liotta and Kohn 2001). CIN can also

facilitate signalling in cancer cells that stimulate their own eradication by cytotoxic leukocytes or inhibit their growth facilitation by other cell types (Galon et al. 2006). One such signal could be the production of novel immunogenic proteins due to, for instance, mutations, translocations or changes of chromosome composition. Such proteins are also known as tumor-specific antigens and are actively sought after in order to develop cancer immunotherapies. Intriguingly, increased expression of inflammatory response factors was identified in human model aneuploid cells (Dürrbaum et al. 2014). Especially, MHC protein complex and genes associated with antigen processing were upregulated, which possibly increase peptide presentation and elevated immunogenicity. Interestingly, tetraploidy can induce translocation of calreticulin (CRT) to the plasma membrane surface response to ER stress and this in turn stimulates an anticancer immune response (Senovilla et al. 2012). The exposure of CRT on the surface of stressed and dying cancer cells facilitates their uptake by dendritic cells and the subsequent presentation of tumor-associated antigens to T lymphocytes. Accordingly, the growth of tetraploid cancer cells was restricted in immunocompetent mice, but not in immunosupressed mice, suggesting that cancer cells with increased DNA content are subjected to immunosurveillance (Senovilla et al. 2012). Since tetraploidy constitutes a genomically metastable state that leads to aneuploidy and genomic instability, it is possible that aneuploid cells with complex karyotypes derived from tetraploid cells might elicit a similar response. In contrast, human colon carcinoma DLD-1 + 7 cells (trisomy 7) did not display a constitutive ER stress response and the CRT translocation, suggesting that extensive changes in the karyotype composition are required to induce this type of response (Senovilla et al. 2012). Taken together, karyotype changes that effect gene expression might also alter the spectrum of peptide presentation, thus facilitating immunogeneity of aneuploid cells. Whether and how this contributes to immune and inflammatory response to cancer remains to be addressed.

6 Conclusion and Perspectives

Aneuploidy affects the cellular physiology on multiple levels. Recent genome-wide approaches provide new means to uncover the scope of the somatic copy number changes in cancer cells and to determine their effects on gene expression. Yet, it remains to be clarified how the aneuploidy-induced gene expression changes contribute to tumorigenesis and the physiological features of aneuploid cancer cells. Since aneuploidy impairs cell growth in most models, it is of interest to identify the pathways that lead to growth suppression as well as the adaptive changes that allow proliferation with an altered karyotype. Moreover, the effects of aneuploidy appear to be context-dependent, yet the specific determinants have not been identified. Finally, the recent identification of a common stress response to aneuploidy brings about the possibility that aneuploidy can be exploited therapeutically for the treatment of cancer. Yet, similar response has not been found in tissues from

aneuploid cell lines. Thus, in future it will be necessary to identify the differences between cellular response to aneuploidy in non-cancerous cells and in cancer tissues.

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