

# Chromosomal rearrangements in cancer

## Detection and potential causal mechanisms

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**Abbreviations:** aCGH, array comparative genomic hybridization; ALK, anaplastic lymphoma kinase; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATM, ataxia telangiectasia mutated; ATRA, all-trans-retinoic acid; BCR, breakpoint cluster region; BIR, break-induced replication; BLM, Bloom Syndrome mutated; BRCA, breast cancer; CGH, comparative genomic hybridization; cCGH, conventional CGH; CML, chronic myelogenous leukemia; CNV, copy number variation; DSB, DNA double strand break; EF-PRR, error free-postreplication repair; EML4, echinoderm microtubule-associated protein like 4; EPT, extra pericentromeres and telomere; ETO, Eight-Twenty One oncoprotein; FISH, fluorescence *in situ* hybridization; FOX, forkhead box protein; HLTf, helicase-like transcription factor; HTGTs, high-throughput genome-wide translocation sequencing; HR, homologous recombination; LCR, low copy number repeat; LSP, locus specific probe; MLL, mixed lineage leukemia; NGS, next-generation sequencing; NHEJ, nonhomologous end joining; NSCLC, non-small cell lung cancer; PAX, paired box; PRR, post replication repair; PML, promyelocytic leukemia; RARA, retinoic acid receptor alpha; ROS1, c-ros oncogene 1; SKY, spectral karyotyping; SNP, single nucleotide polymorphism; TLS, translesion synthesis; WGS, whole genome sequencing

Many cancers exhibit chromosomal rearrangements. These rearrangements can be simple, involving a single balanced fusion that preserves the proper complement of genetic information, or complex with one or more fusions that disrupt this balance. Recent technological advances have improved our ability to detect and understand these rearrangements, leading to speculation about potential causal mechanisms such as defective DNA double strand break repair and faulty DNA replication. A better understanding of these potential cancer-causing mechanisms will lead to novel therapeutic regimens to fight cancer. This review describes technological advances in methods used to detect simple and complex chromosomal rearrangements, cancers that exhibit these rearrangements, potential mechanisms for rearrangement of chromosomes, and intervention strategies designed specifically against fusion gene products and causal DNA repair/synthesis pathways.

although such translocations often disrupt regulation of the genes involved. Other rearrangements are complex, with multiple fusions at a single locus and do not maintain the normal complement of genetic information. Such rearrangements have the potential to cause cancer if they mutate a tumor suppressor gene or activate an oncogene<sup>2</sup> and can also contribute to tumor heterogeneity and clonal evolution as mechanisms for metastasis and drug resistance.<sup>3,4</sup> Thus, chromosomal rearrangements are important in cancer etiology. This review will first describe recent advancements in technologies that drove the discovery and characterization of translocations. Next, we describe cancers that exhibit simple balanced translocations or more complex rearrangements. Finally, we review potential mechanisms that lead to chromosomal rearrangements and present possible intervention strategies.

### Introduction

Accumulation of genetic mutations can contribute to cancer development, progression, and metastasis.<sup>1</sup> Some rearrangements are simple balanced translocations that result from a single fusion and preserve the proper complement of genetic information,

### Technologies for the Discovery and Evaluation of Genomic Rearrangements

The technological advances summarized in Table 1 have enabled the detection of chromosomal rearrangements in cancer cells. Chromosomal rearrangements in cancer were first identified in the early 50s by karyotype analyses based on Giemsa (G-banding) or reverse Giemsa banding (R-banding). G-banding is a technique that produces a visible display of condensed chromosomes whereas R-banding produces bands that are complementary to Giemsa bands.<sup>5,6</sup> This technology allowed the observation of whole chromosomes during metaphase and provided the basis for the hypothesis that tumorigenesis is a genetic disease based on the realization that alteration of chromosome structure is a frequent event in cancer. However, this technology did not effectively identify the specific chromosomal locations or structures involved in complex rearrangements.

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**Table 1.** Techniques for identification of chromosomal rearrangements

Technique	Procedure	Purpose	Limitations
Giemsa (G)-banding	MSP with a banding pattern used to identify chromosomes	Identify chromosome number and structural rearrangements	Resolution < 10 Mb, not sensitive enough to detect small inversions
Reverse giemsa (R)-banding	MSP with a banding pattern complementary to G-banding	Identify chromosome number and structural rearrangements	Resolution ~10 Mb, not sensitive enough to detect small inversions
Fluorescence in situ hybridization (FISH)	Probes anneal to specific regions such as pericentromere and telomere in MSPs	Identify chromosome number, rearrangements, and specific structures such as pericentromeres and telomeres	Allows analysis of only 4 fluorochromes at one time
Spectral karyotyping (SKY)	Sophisticated FISH using a combination of paint probes targeting all chromosomes in a single hybridization	Identify all chromosomes and visualize complex structural alterations	Resolution 5–10 Mb, not sensitive enough to detect small inversions
Comparative genomic hybridization (CGH)	Hybridize test DNA to reference DNA to identify copy number variation	Identify deletions and amplifications	Resolution depends on the density of the probes, does not allow detection of inversions and balanced translocations
Whole genome sequencing (WGS)	Sequencing the entire genome	Analysis of sequence at the fusion site at single nucleotide resolution	Costly and requires intensive data analysis
High-throughput genome-wide translocation sequencing (HTGTS) and translocation capture sequencing (TC-Seq)	Sequencing applied to sites of induced DSBs in B cells	Identify large numbers of translocations	Requires skilled personnel for library preparation, costly and requires intensive data analysis

**Abbreviations:** DSB, double-strand break; MSP, metaphase spread

To address this issue, the technique of fluorescence in situ hybridization (FISH) was developed in the late 70s. FISH greatly improved the resolution of standard karyotyping by providing tools for visualizing specific loci on metaphase chromosomes. In addition, FISH allowed the quantitative analysis of chromosomal alterations in interphase cells, thus providing a technique for analyzing non-dividing cells. Refinements and improvements of the technology have been ongoing and approximately 40 different applications of FISH have been reported to date.<sup>7</sup> Although several of these applications specifically address unique biologic questions, others are widely employed for the analysis of chromosomes in cancer cells and are routinely used for the clinical evaluation of patient samples.

In general, the most commonly used methods of FISH require the generation of a locus-specific probe (LSP) targeting a gene of interest (usually an oncogene or a tumor suppressor gene) that is labeled with one fluorochrome, combined with one or more reference probes (a subcentromeric probe mapping to the same chromosome as the LSP of interest or a probe mapping to a region flanking the LSP) that is labeled with a different fluorochrome. A variety of probes can be used in different combinations depending on the specific loci of interest, allowing precise and highly detailed analysis of chromosome alterations including visualization of chromosome breakpoints, copy number alterations (gains and losses), and inversion of chromosomal regions. FISH is a cost-effective approach that has the advantages of allowing analysis at the single cell level and facilitating characterization of genomic regions that are notoriously difficult to study with other approaches (e.g., structural alterations that map to repetitive regions such as pericentromeres and telomeres).

Spectral karyotyping (SKY) is a more sophisticated FISH approach that requires access to a spectra cube (interferometer).<sup>8,9</sup> SKY is based on the combinatorial use of paint probes targeting all chromosomes in a single hybridization, providing an exceptional ability to visualize complex structural alterations. The application of SKY to the analysis of human and murine samples has been instrumental in advancing the field of molecular cytogenetic analysis and gaining a better understanding of the complexity of chromosome alterations in cancer. It has allowed refinement of the complexity of previously known breakpoints and better characterization of cases that were difficult to resolve because of poor spreads or contracted metaphase chromosomes, highly rearranged karyotypes with numerous marker chromosomes, or subtle chromosomal aberrations.<sup>10-12</sup> SKY has also been proven to be a powerful tool in the analysis of murine chromosomes. The use of genetically engineered mice as model systems of human cancer has fueled the need for better methods of cytogenetic analysis because of the challenges associated with studying murine chromosomes that are similar in size and are acrocentric.<sup>13</sup> As a result, the characteristic banding pattern typically used for the identification of human chromosomes is less helpful in mice, making karyotyping very difficult. Furthermore, at the present time there are few cytogeneticists trained in karyotyping mouse chromosomes. There are more than 160 reports describing the use of SKY to characterize the karyotype of a wide range of murine models. In our experience, SKY has been an extremely valuable tool for analyzing structural alterations resulting in complex rearrangements in murine chromosomes.<sup>14,15</sup> In one specific application, SKY has been used to resolve the chromosomes involved in the formation of dipericentrics and chromosomes with extra pericentromeres and telomeres (EPTs). The

pericentromere is the region surrounding the centromere, which is a highly complex structure important for chromosome segregation during mitosis. The pericentromere is more easily detected than the centromere using FISH, hence the term dipericentric. Dipericentric chromosomes, also called dicentric chromosomes, have a pericentromere at each end of the chromosome and none in the middle. EPTs are complex structural alterations involving segmental duplications with extra centromeres and telomeres at the poles of a chromosome as well as in the middle of the chromosome.<sup>15</sup> However, although SKY is well suited for the analysis of complex alterations it has a limited resolution of 10 Mb and is therefore not suitable for fine mapping of breakpoints.

Comparative genomic hybridization (CGH), which was developed during the 90s, can be used to better define breakpoints.<sup>16</sup> Conventional CGH (cCGH), originally designed for hybridization on metaphase chromosomes, makes use of differentially labeled test DNA and reference DNA that are co-hybridized with chromosomes to identify regions of copy number variation (CNV). To overcome limitations of the resolution provided by karyotyping and cCGH the technique was adapted to matrix-based hybridization protocols in array CGH (aCGH).<sup>17</sup> A large variety of matrices is now available, offering different levels of resolution and dynamic ranges (from one to several fold changes in copy number, as frequently observed in cancer cells). aCGH, often combined with single nucleotide polymorphism (SNP) arrays, is routinely used in clinical settings to diagnose prenatal and intellectual disabilities. Several array designs are available for the cancer genome (e.g., Agilent 400K CGH/SNP and Affymetrix CytoScan and OncoScan FFPE arrays). Such arrays are used in cancer clinical genomics to stratify lymphoma patients; for example, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), large B-cell lymphoma (DLBCL), and mantle cell lymphoma (MCL). In basic research aCGH is extensively applied to analyze complex rearrangements. For example, Carvalho and colleagues applied a custom-designed 44 k microarray to study genomic segments at the methyl CpG binding protein 2 (*MECP2*) and the proteolipid protein 1 (*PLP1*) loci that are characterized by a common genomic organization, duplication-inverted triplication-duplication.<sup>18</sup> By mapping the junctions of breakpoints in a cohort of 9 subjects they provided evidence supporting the mechanism leading to the formation of a triplicated segment embedded within a duplication: duplication-inverted triplication-duplication complex rearrangements are formed by the combination of homology-directed break-induced replication (BIR) with microhomology-mediated BIR or non-homologous end joining (NHEJ) that involves replication fork repair after stalling and template switching (discussed below). A major limitation of aCGH is the inability to detect balanced translocations; in addition, as the technique is based on analysis of bulk DNA (DNA isolated from a pool of cells) it has restricted sensitivity to detect mosaic alterations (more than 20% of the population of cells must present the same alteration to enable detection).

Next-generation sequencing (NGS) approaches, including whole genome sequencing (WGS), promise to further advance our understanding of complex chromosomal alterations by

precise mapping of breakpoints and junctions, thus enabling unprecedented sensitivity and resolution of structural and mutational changes. Improvements in WGS have allowed the discovery of complex chromosomal rearrangements in solid tumors.<sup>19</sup> Furthermore, although these translocations were initially thought to be simple and balanced, deep sequencing has revealed that some are in fact quite complicated. WGS has revealed genomic architecture that is susceptible to complicated rearrangements and has narrowed down the sequence flanking the genomic alterations so that breakpoints can be identified. The single-nucleotide resolution of WGS enables mapping of chromosome regions too small for analysis by FISH or aCGH. A combination of WGS, SNP arrays, and FISH approaches were used in the discovery of the phenomenon of chromothripsis (or chromosome shattering), which shows a complicated pattern of multiple fusions at a single location.<sup>20</sup> In the past few years WGS has been instrumental in revealing new causative mechanisms of chromosomal breaks and rearrangements.

High-throughput genome-wide translocation sequencing (HTGTS) and translocation capture sequencing (TC-Seq) were applied to identify translocation junctions in B cells upon induction of DNA double-strand breaks (DSBs) at specific locations in the genome.<sup>21,22</sup> These studies identified a large number of translocations, revealing a marked association between transcription start sites and translocation targets. The majority of translocation junctions were formed through end joining with short microhomologies. Although most cells repair induced DSBs by rejoining the ends without causing major genomic rearrangements, a significant fraction of cells join the induced DSB ends to endogenous DSBs elsewhere in the genome, creating intra- and interchromosomal rearrangements. Translocations occur more frequently on the chromosome carrying the induced break and preferentially target transcribed chromosomal regions, even up to 50 Mb away.

The application of NGS sequencing to study chromosome alterations in cancer opens new and exciting directions of study but has certain challenges. Although the price of sequencing is decreasing dramatically, the cost of sequencing a mammalian genome remains high when considering that analysis of several samples is usually required to address biological questions. Moreover, the analytical tools essential for WGS analysis in cancer samples are not yet fully developed. For example, in the case of chromothripsis, automated methods designed to detect or annotate high throughput sequencing data are not available. To address these limitations, several new technologies are being developed. One such method is ShatterProof, which uses structural variation calls (translocations, copy number variations, short insertions, and loss of heterozygosity) and performs robust statistical tests to accurately predict the presence and location of chromothriptic events.<sup>23</sup> Another limitation of applying WGS studies to cancer cells is the need for high coverage ( $> 30 \times$ ) to allow characterization of clonal heterogeneity by tumor biopsy. Despite these limitations, advances in technology have greatly improved our ability to identify and characterize chromosomal rearrangements, thus presenting the opportunity to address causal factors.

**Table 2.** Examples of simple balanced rearrangements found in cancers

Fusion partners	Breakpoint	Cancer	Defect	Targeted therapy
Philadelphia chromosome: breakpoint cluster region with c-abl (BCR-ABL1)	t(9;22)(q34;q11)	CML, ALL, AML	Tyrosine kinase activation Balanced	Imatinib
Anaplastic lymphoma kinase gene with echinoderm microtubule-associated protein like 4 (ALK-EML4)	Inv(2)(p21;p23)	NSCLC	Tyrosine kinase activation Balanced	Crizotinib
c-ros oncogene 1 (ROS1) with multiple genes		NSCLC, cholangiocarcinoma, glioblastoma multiforme, gastric adenocarcinoma	Tyrosine kinase activation Balanced and non-balanced	Crizotinib
AML1/ETO	t(8;21)(q22;q22)	AML	Aberrant recruitment of epigenetic modifiers affecting normal myelomonocytic development Balanced	General chemotherapy (cytarabine and anthracycline)
Promyelocytic leukemia with retinoic acid receptor $\alpha$ (PML-RARA)	t(15;17)(q22;q21)	AML	Nuclear receptor signaling and PML body assembly Balanced	ATRA and arsenic oxide (AS203)
Mixed lineage leukemia (MLL)-unclassified partners		AML	Four types of unclassified fusion partners: (1) nuclear proteins, (2) cytoplasmic proteins, (3) histone acetyltransferases, (4) septins Balanced	ATRA
Paired box with Forkhead box (PAX3-FOXO1)	t(2;13)(q36;q14)	ARMS	Transcriptional activation Balanced	Thapsigargin
PAX7-FOXO1	t(1;13)(p36;q14)	ARMS	Transcriptional regulation Balanced and non-balanced	Targeting downstream pathways
FOXO3-MLL	t(6;11)(q21;q23)	Leukemia and ARMS	Transcriptional regulation Usually balanced	ATRA
FOXO4-MLL	t(X;11)(q13;q23)	Leukemia and ARMS	Transcriptional regulation Usually balanced	ATRA
FOXP1-PAX5	t(3;9)(p13;p13)	Lymphoblastic lymphoma	Transcriptional regulation Balanced and non-balanced	None

**Abbreviations:** ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ARMS, alveolar rhabdomyosarcoma; ATRA, all-trans-retinoic acid; CML, chronic myelogenous leukemia; NSCLC, non-small cell lung cancer.

The technologies described above have been instrumental for the mapping of genomic alterations, especially in the cancer genome where chromosomal alterations characterize many solid tumors and hematologic malignancies. As a result, specialized databases compiling a detailed inventory of chromosomal alterations have been developed. The most comprehensive of these are the Mitelman Database (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>), which contains all published chromosome aberrations in neoplastic disorders with clinical features of more than 64,000 cancers, and the Catalogue of Somatic Mutations in Cancer (COSMIC) database,<sup>24</sup> which currently reports the characterization of 327 types of gene fusions identified in more than 60,000 cancer cases (accessed May 5, 2014). Data available at this time indicate that gene fusions occur in approximately

15% of all solid cancer cases analyzed, which is less frequent than the reported rate in hematological malignancies (16.2%). Chromosomal rearrangements might be found in more tumors as high throughput NGS sequencing becomes more widespread and affordable.

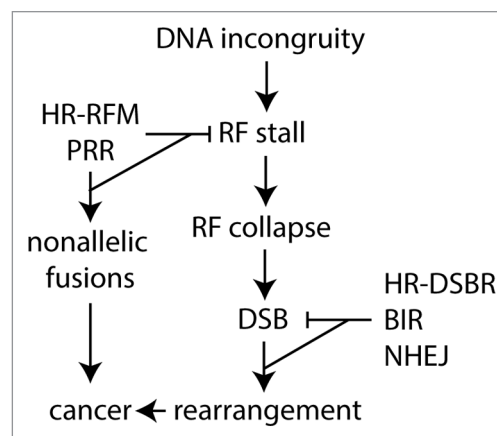
### Simple and Complex Rearrangements Found in Cancers

The presence of simple and balanced rearrangements (summarized in Table 2) can increase cancer risk. Simple rearrangements have no or limited alterations at the fusion ends whereas balanced rearrangements fuse chromosomes without losing genetic



information. Their role in cancer etiology was first described in 1960 with the discovery of the Philadelphia chromosome in patients with various forms of leukemia.<sup>25,26</sup> The Philadelphia chromosome arises from a head-to-tail fusion of the breakpoint cluster region (BCR) with the gene encoding the c-abl (*ABL1*) proto-oncogene tyrosine kinase to generate the *BCR-ABL* fusion transcript. The *BCR-ABL* fusion generates a constitutively active tyrosine kinase that can transform cells and inhibit apoptosis induced by a variety of agents.<sup>27</sup> Activation of protein kinases is seen in other gene fusions. For example, anaplastic lymphoma kinase (ALK) can be activated by an inversion that fuses the *ALK* gene with the echinoderm microtubule-associated protein like 4 (*EML4*) gene. This rearrangement occurs in 2–5% of non-small cell lung cancers (NSCLCs).<sup>28</sup> C-ros oncogene 1 (ROS1) receptor tyrosine kinase (conserved with ALK) is also activated in approximately 1% of NSCLCs and in other cancers (cholangiocarcinoma, glioblastoma multiforme, gastric adenocarcinoma) after fusion with a variety of genes.<sup>29–31</sup> In addition to the Philadelphia chromosome, a variety of other balanced translocations are commonly observed in hematologic malignancies.<sup>19</sup> For example, 10% of all cases of acute myeloid leukemia (AML) exhibit one of the following three fusions: (1) the N-terminal DNA-binding domain of AML1 with the Eight-Twenty One oncoprotein (ETO); (2) the promyelocytic leukemia (*PML*) gene with the retinoic acid receptor  $\alpha$  (*RARA*); and (3) the mixed lineage leukemia (*MLL*) gene with one of several partners.<sup>32</sup> The AML-ETO fusion causes aberrant recruitment of epigenetic modifiers, thus affecting normal myelomonocytic development, whereas *PML-RARA* affects both nuclear receptor signaling and *PML* body assembly. In addition, the transactivation domains of the forkhead box (*FOX*) gene family fuse with the DNA binding domains of a variety of genes to produce a fusion transcript that encodes an unregulated transcription factor. Such fusions include paired box (*PAX*) with *FOXO1*<sup>33–35</sup> and *MLL* with either *FOXO3* or *FOXO4*. These translocations have been found in alveolar rhabdomyosarcoma and leukemia.<sup>36,37</sup> Furthermore, *PAX3-FOXO1* is associated with an aggressive phenotype and poor prognosis.<sup>38</sup> The presence of fusion proteins that activate similar oncogenic pathways suggests that cancers of different molecular and cellular origin share common pathogenetic mechanisms determined by the transcriptional effector properties of the forkhead protein subfamily.<sup>39</sup> Thus, simple and balanced chromosomal translocations can result in fusion proteins that enable cancer development.

More complex rearrangements are also found in cancer. These rearrangements have multiple joins at the fusions and can involve more than two chromosomes, and result in a change in genetic content as well as a change in the chromosomal linear structure. Complex genomic rearrangements have been reported in lymphoma.<sup>40–45</sup> Complex chromosomal translocations leading to gene fusions are also found in solid cancers,<sup>19</sup> including whole arm translocations and isochromosomes in head and neck squamous cell carcinoma,<sup>46</sup> complex genomic rearrangements including inversions in pancreatic cancer,<sup>47</sup> and rearrangements including tandem duplications in breast cancers.<sup>48,49</sup> Palindrome structures were also found in cells derived from colon cancer,



**Figure 1.** Replication fork maintenance and double strand break repair can either suppress or cause chromosomal rearrangements. BIR, break-induced replication; DSB, double strand break; DSBR, double strand break repair; HR, homologous recombination; RF, replication fork; NHEJ, nonhomologous end joining; PRR; post replication repair; RFM, replication fork maintenance.

breast cancer, and embryonal rhabdomyosarcoma, and in primary medulloblastomas.<sup>50</sup> Furthermore, rearrangements that have undergone chromothripsis have been identified, with tens to hundreds of fusions mapping to a single chromosomal location.<sup>20,51,52</sup> Chromothripsis has been detected in as many as 3% of cancers (and more might be found with wider application of WGS) and occurs in a wide range of cancers including leukemia, medulloblastoma, melanoma, glioma, sarcoma, bone cancer, colorectal cancer, renal cancer, and thyroid cancer.<sup>53</sup> It appears that chromosomal disruption and pulverization might be linked to the generation of micronuclei as a consequence of mitotic errors.<sup>54</sup> The discovery and understanding of these complex rearrangements is continuing to advance as technology improves yet the causal mechanisms are still not understood. Defects in the repair of DNA DSB repair and faulty DNA synthesis have been proposed as mechanisms underlying these rearrangements.<sup>40</sup>

### Double Strand Break Repair Pathways that Influence Chromosomal Rearrangements

Multiple pathways are responsible for DNA DSB repair, including nonhomologous end joining (NHEJ), homologous recombination (HR), and break-induced replication (BIR). These pathways are important for maintaining chromosomal integrity. Conversely, their faulty application has the potential to cause chromosomal rearrangements (Fig. 1).

The NHEJ pathway repairs DSBs without a homologous DNA template; therefore, it is the predominate repair pathway during  $G_1$  but also can be involved during  $S/G_2$ .<sup>55</sup> NHEJ components include the KU heterodimer composed of KU70 and KU80, which binds to DNA ends together with the kinase DNA-PK<sub>cs</sub>. The XRCC4/DNA ligase IV heterodimer subsequently ligates the DNA ends. Loss of any of these proteins results in a severe DSB repair defect that causes hypersensitivity

to clastogenic agents and defective V(D)J recombination during lymphocyte development.<sup>56,57</sup> NHEJ-deficient cells are predisposed to chromosomal alterations and telomere joining events that result in chromosome fusions.<sup>58,59</sup> In contrast, NHEJ itself can also rearrange chromosomes by joining noncontiguous ends, as occurs in Fanconi anemia cells.<sup>60,61</sup> This is a particularly interesting find, because the Fanconi anemia pathway maintains replication forks in association with HR.<sup>62</sup> Similarly, NHEJ enables chromosomal translocations in cells mutant for breast cancer 1 (BRCA1), breast cancer 2 (BRCA2), and ataxia telangiectasia mutated (ATM),<sup>63,64</sup> which are all tumor suppressors important for HR. Theoretically, NHEJ-mediated joining of chromosome ends after telomere deletion would result in dicentric products of two chromosomes. Similarly, NHEJ could join the ends of sister chromatids to generate a palindromic chromosome. Thus, NHEJ repairs DSBs to suppress rearrangements but can also inappropriately join ends to yield rearrangements.

There are also alternative pathways to the classic KU-dependent NHEJ that have the potential to rearrange chromosomes.<sup>65</sup> This alternative-NHEJ is poorly understood, but is known to be error prone because it frequently changes the DNA ends found at the joins.<sup>66,67</sup> Furthermore, alternative-NHEJ can utilize microhomology to imprecisely join ends (microhomology-mediated end joining or MMEJ). Microhomologies are often found at the fusion joins of translocations in human cancer cells, implicating MMEJ as an enabler of cancer development and progression.<sup>68</sup> Thus, alternative end joining pathways also have the potential to rearrange chromosomes.

Homologous recombination pathways repair DSBs using a homologous template for fidelity, and HR is therefore restricted to S/G<sub>2</sub> phases of the cell cycle. The RecA recombinase RAD51 forms a filament on 3' single DNA strands to induce annealing to the sister chromatid template.<sup>69</sup> The recombination mediator BRCA2 assists in the formation and stability of the RAD51 filament. The RecQ helicase Bloom Syndrome mutated (BLM)<sup>70,71</sup> regulates unwanted recombination that occurs through the dissolution of Holliday junctions.<sup>72-75</sup> Both BRCA2 and BLM suppress cancer, thereby demonstrating that deficient or unregulated HR is mutagenic. Expression of mutant RAD51 (K133A) in mammalian cells causes a large number of chromosomal rearrangements, some of which are highly complex.<sup>15</sup> The K133A mutation prevents binding of ATP to the highly conserved Walker A motif and disables the ability of RAD51 to induce topological changes in duplex DNA in an ATP-dependent manner. This mutant is highly toxic when expressed in proliferating cells. In addition, normally functioning HR can also rearrange chromosomes if the invading strand anneals to a homologous but nonallelic substrate, which creates a potential problem with repeated sequences.

Break-induced replication repairs one-ended DSBs or gaps during replication.<sup>76,77</sup> A single strand at the DSB or gap invades a complementary strand to initiate DNA synthesis that has the potential to proceed to the end of the chromosome, causing loss of heterozygosity. BIR in yeast is dependent on Rad52 and the Pol32 subunit of DNA polymerase delta, demonstrating its dependence on DNA synthesis. In mammals, the Pol32 ortholog, POLD3, is required for cell cycle progression and processive

DNA synthesis in cells undergoing replicative stress induced by cyclin E overexpression. Cyclin E overexpression results in BIR-mediated, POLD3-dependent segmental duplications.<sup>78</sup> POLD3-mediated BIR facilitates genomic amplifications of up to 200 kb. Thus, BIR can also cause chromosomal rearrangements.

## DNA Synthesis Pathways that Influence Chromosomal Rearrangements

As inappropriate NHEJ, HR, and BIR can result in chromosomal rearrangement, pathways that suppress DSB generation during DNA synthesis would decrease the need to engage these potentially mutagenic pathways (Fig. 1). DSB formation can occur when a replication fork encounters an incongruity in DNA such as secondary structure, damage, a protein adduct, or a transcription bubble.<sup>79</sup> These impediments have the potential to stall or collapse replication forks. Whereas a stalled fork is simply a temporary pause, a collapsed fork has lost the replisome and can lead to fork failure or “floundering” if not properly resolved.<sup>40</sup> A myriad of proteins can process unresolved collapsed forks with the potential formation of intermediate structures such as a regressed fork (chickenfoot), a hemicatenane, and a single-strand gap.<sup>73,80-82</sup> These structures are susceptible to formation of one-ended DSBs that will become two-ended DSBs at the point where forks converge, thus invoking DNA repair pathways. To avoid fork collapse, the nascent strand could simply switch templates to the complementary sister chromatid to bypass the DNA incongruity. In such circumstances, the lesion or alternative structure is bypassed but not corrected. Lesion bypass mechanisms appear to be important for replication fork maintenance but are not well understood.

It is possible that components of the HR pathway facilitate lesion bypass. In yeast, HR suppresses blocked replication forks through a template exchange mechanism<sup>83</sup> and in mammals a RAD51-dependent pathway responds to stalled replication forks without a DSB.<sup>84</sup> RAD51 was also purified from the nascent replication strand before formation of DSBs.<sup>85</sup> Furthermore, the RAD51 K133A mutant shows impaired replication fork restart.<sup>15</sup> RAD51 overexpression and enhanced RAD51 filament stability also rescue nascent strand degradation in *FANCD2*-mutant cells.<sup>62</sup> Moreover, after nucleotide depletion, BRCA2 protects nascent replication strands from degradation and enables replication fork restart.<sup>62,86,87</sup> BLM similarly enables replication fork recovery. Moreover, RAD51, BRCA2, and BRCA1 minimize long-tract gene conversion in response to stalled forks, but not DSBs.<sup>88</sup> The response of a RAD51-mediated pathway to stalled replication forks is consistent with a potential template switch mechanism.

Postreplication repair (PRR) is a lesion bypass pathway that suppresses broken replication forks to maintain genome stability. The term PRR is actually a misnomer because the lesion is not repaired but bypassed, and this pathway may also be called lesion bypass or DNA damage tolerance. There are two branches to PRR that are best understood in yeast.<sup>89,90</sup> The first branch is translesion synthesis (TLS), which bypasses lesions simply by

exchanging a high fidelity replication polymerase with a translesion synthesis polymerase. TLS is induced by monoubiquitination of PCNA lysine 164 (K164) by the E2/E3 ubiquitinase RAD6/RAD18. A translesion polymerase then replaces the processive polymerase to bypass the lesion. Some TLS polymerases have low stringency and introduce mismatches at the base lesion, and as a consequence are error prone.<sup>91</sup> TLS is conserved from yeast to mammals. The second branch of PRR is error-free postreplication repair (EF-PRR), which bypasses the lesion through a template switch. EF-PRR is induced by monoubiquitination of PCNA lysine K164 by RAD6/RAD18 followed by polyubiquitination of the previously monoubiquitinated K164 by the E2/E3 ubiquitinase UBC13-MMS2/RAD5. EF-PRR is poorly understood in yeast and even more elusive in higher eukaryotes. However, RAD6/RAD18 and UBC13-MMS2 are conserved from yeast to mammals suggesting conservation of function. In addition, mammals possess two functional yeast RAD5 orthologs: helicase-like transcription factor (HLTF) and SNF2 histone-linker PHD-finger RING-finger helicase (SHPRH). Both HLTF and SHPRH mediate PCNA polyubiquitination and suppress chromosomal alterations<sup>92,93</sup>; however, they are not redundant because they impart lesion specificity.<sup>94</sup> Furthermore, HLTF has DNA translocase activity that reverses regressed forks<sup>95</sup> and can form a D loop independent of RAD51 and RAD54,<sup>96</sup> both of which suggest a mechanism for strand switching. Thus, EF-PRR in mammals likely suppresses replication fork anomalies to minimize fork collapse.

Template switch pathways have been mostly studied in bacteria and yeast by observing the switch between nonallelic repeats that result in a chromosomal alteration. In bacteria, a template switch was observed between inverted<sup>97,98</sup> and direct<sup>99</sup> repeats and found to be independent of the *recA* recombinase. In yeast, a template switch between repeats occurred during BIR.<sup>100</sup> Faulty template switching was also observed between inverted repeats during replication that required RAD59, but not RAD51.<sup>101</sup> In fission<sup>102</sup> and budding<sup>103,104</sup> yeast, replication-based pathways without a DSB fuse inverted repeats to generate palindromic chromosomes. In fission yeast, HR was found to be primarily responsible for fusing repeats after replication forks were experimentally induced to stall at a reporter. In budding yeast, a genetically undefined pathway was found to be responsible for spontaneous inverted repeat fusion and this process was suppressed by HR and EF-PRR. Thus, template switch-mediated repeat fusion is observed in bacteria and yeast, although the causal pathways for spontaneous fusion are not fully understood.

Mammalian cells are potentially vulnerable to template switch-mediated rearrangements because of the high number of repeats found in the genome. Approximately 11% of the human genome is composed of 300-bp Alu repeat elements and an additional 5% includes larger repeat regions called low copy number repeats (LCRs).<sup>105</sup> Furthermore, pericentromeres, centromeres, and telomeres are composed of repeats that are dispersed in the chromosomal arms.<sup>106-109</sup> These repeats are potential substrates for rearrangements.<sup>105</sup> Replication forks are prone to stalling in areas dense with repeats such as telomeres,<sup>110</sup> tRNA genes,<sup>111,112</sup> and triplet repeats.<sup>113,114</sup> Inverted repeats can form hairpins that

have the potential to stall replication forks and cause chromosomal rearrangements in yeast<sup>115</sup> and stimulate PCNA polyubiquitination in human cell-free extracts.<sup>116</sup> Rearrangements are found at palindromic structures in humans,<sup>117</sup> suggesting that they originate from a homology-based mechanism combined with defects in DSB repair<sup>20</sup> or DNA synthesis.<sup>118-120</sup> DNA repeats pose a particular problem for replication forks because they can form cruciforms, hairpins, triplex H-DNAs, left handed Z-DNAs, and slipped strand S-DNAs.<sup>121</sup> Repeat-induced chromosomal rearrangements are common. Copy number variation is a spontaneous change in the number of DNA segments that occurs between repeats.<sup>44,120</sup> Moreover, monozygotic twins display different DNA CNV profiles, demonstrating plasticity involving repeats.<sup>122</sup> CNV is a common event that is important for murine<sup>123</sup> and primate<sup>124,125</sup> evolution. Palindromic chromosomes are also found in human cells<sup>50,117</sup> and the involvement of repeats at the rearrangement joins suggests a DNA synthesis mechanism such as faulty template switch or fork stalling and template switching.<sup>40,45</sup> The etiology of these structural variations is not known, but could involve defects in DSB repair or DNA synthesis.

Recently, we described two template switch pathways in wild type mouse embryonic stem cells that fused inverted repeats to generate unstable multipericentric chromosomes.<sup>14</sup> The level of sequence identity within the repeats determined pathway choice: exposure to  $\gamma$ -radiation enhanced the fusion of identical but not mismatched repeats whereas exposure to UV light had the opposite effect. Thus, genotoxic exposure delineated two pathways that fuse repeats based on sequence identity. Even though these are distinct pathways, both appear to fuse repeats as a consequence of replication fork stalling rather than DSB repair since fusion of both identical and mismatched repeats was spontaneous (a DSB was not induced) and enhanced by hydroxyurea-induced replication fork stalling (hydroxyurea depletes nucleotides to stall forks).

We hypothesized that HR enabled identical repeat fusion because HR repairs damage caused by hydroxyurea and  $\gamma$ -radiation but not UV light<sup>84,126</sup> and only identical repeats contain sufficient homology for HR.<sup>127</sup> Repeat fusion was tested in BLM-deficient cells which exhibit unregulated HR.<sup>128</sup> We found that BLM-deficient cells exhibited enhanced fusion of identical, but not mismatched, repeats and that reducing the level of either RAD51 or BRCA2 substantially reduced identical repeat fusion in these cells.<sup>14</sup> The existence of a BLM-regulated pathway that depends on RAD51 and BRCA2 and fuses identical, but not mismatched, repeats is consistent with HR.

We hypothesized that EF-PRR fuses mismatched repeats because EF-PRR, as measured by PCNA ubiquitination, processes damage induced by UV light but not by  $\gamma$ -radiation.<sup>93,129</sup> Repeat fusion was tested in RAD18-mutant cells<sup>130</sup> which do not efficiently ubiquitinate PCNA<sup>14</sup> and should therefore be defective in EF-PRR. We found that lack of RAD18 slightly decreased identical repeat fusion, possibly reflecting a subtle role in HR.<sup>131-133</sup> In contrast, loss of RAD18 almost completely ablated mismatch repeat fusion implicating a role for EF-PRR.<sup>14</sup> Moreover, mutation of a nonprocessive 3'→5' exonuclease, TREX2, ablated

mismatch repeat fusion but enhanced identical repeat fusion, clearly delineating these pathways. TREX2 appears to be epistatic with RAD18 with regard to PCNA ubiquitination and both are important for replication fork maintenance. These observations are consistent with an epistatic relationship for RAD18 and TREX2 mediating mismatch repeat fusion through EF-PRR.

Both the BLM-regulated fusion of identical repeats and the RAD18/TREX2-mediated fusion of mismatched repeats generate very complicated chromosomal rearrangements that include dipercentric and EPTs.<sup>14</sup> We propose that a DNA synthesis pathway causes dipercentric as a result of a template switch that bypasses a hairpin followed by replication to the telomere. Subsequent breakage-fusion-bridge cycles cause further alterations as chromosomes segregate during mitosis. Interestingly, using a combination of SKY and LSPs on interphase nuclei we showed that a repeat fusion reporter was amplified, mobile, and located in micronuclei. Thus, template switch mechanisms have the potential to generate extrachromosomal double minutes that enhance cancer development and progression and cause resistance to chemotherapeutic agents.<sup>134</sup> These repeat fusion pathways could influence cancer etiology and drug effectiveness.

### Targets for Cancer Therapy

Simple balanced chromosomal rearrangements generate gene fusions that can be exploited for cancer therapy. Cancers with rearrangements that activate kinases might be susceptible to small molecule inhibitors,<sup>135</sup> for example the tyrosine kinase inhibitors imatinib and crizotinib are for cancers possessing the BCR-ABL fusion protein-positive cancers<sup>136</sup> and for EML4-ALK and ROS1-positive NSCLC,<sup>28,137</sup> respectively. Another example is the use of all-trans-retinoic acid (ATRA) for PML patients with the PML/RARA fusion. Retinoic acids are key players in myeloid differentiation and act through their agonistic nuclear receptors (RAR  $\alpha$ /RXR) to modulate the expression of target genes. The PML-RARA fusion generates an oncoprotein that blocks granulocytic differentiation, and ATRA is thought to modulate cellular differentiation that is dependent on PML-RAR $\alpha$  proteolysis.<sup>138</sup> Patients that become resistant to ATRA are treated with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), which exerts its therapeutic effect by promoting degradation of the PML/RARA protein that drives the growth of acute promyelocytic leukemia cells.<sup>139</sup> ATRA is also able to overcome the differentiation block induced by MLL chimeric proteins in acute promyelocytic leukemia and therefore constitutes the standard of care for induction therapy in this disease. In addition to kinase inhibitors, thapsigargin, a sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPases (SERCA) inhibitor, targets the PAX3-FOXO1 fusion. Thapsigargin activates AKT to alter cytosolic calcium levels by blocking the ability to pump calcium into the sarcoplasmic and endoplasmic reticulum, thereby inhibiting the fusion of autophagosomes with lysosomes and ultimately causing endoplasmic reticulum stress and cell death.<sup>140,141</sup>

Thus, the fusion products from simple balanced translocations represent good therapeutic targets for the treatment of cancer.

In addition to targeting fusion proteins, future cancer therapies could attack the pathway that compensates for the primary DNA repair defect that initially caused the cancer. The compensatory pathway is an attractive target because it would attack the tumor on multiple fronts. The first front would take advantage of synthetic sickness/lethality.<sup>142</sup> A drug that targets the compensatory pathway would enhance sensitivity to commonly used cytotoxic drugs that cause DNA damage. Such a drug would be especially effective if the compensatory pathway is adept at bypassing the cytotoxic drug-induced lesions.<sup>143</sup> In addition, this class of drug would have a good therapeutic index for cases where only the cancer, but not the patient, is mutant for the primary pathway as a result of loss of heterozygosity. The second front would prohibit further genome modification.<sup>144</sup> The compensatory pathway is often mutagenic because it is overused and under-regulated in an attempt to atone for the primary defect. In addition, lesion bypass pathways such as TLS are often error prone.<sup>143</sup> Thus, a drug that targets the compensatory pathway would suppress mutations that might otherwise enhance cancer development and metastasis and lead to drug resistance. These novel drugs would be especially attractive when lesion bypass mechanisms compensate for DNA repair defects because such mechanisms are nonessential for cell and organism viability,<sup>145,146</sup> unlike NHEJ<sup>56,147-149</sup> and HR.<sup>150,151</sup> Thus, a drug that attacks a compensatory pathway has the potential to both enhance sensitivity to genotoxic therapeutics and reduce further mutations.

### Summary

Chromosomal rearrangements frequently found in cancers are proposed to facilitate cancer development, progression, metastasis, and drug resistance. Cytogenetics and NGS have revealed simple and complex chromosomal rearrangements in many cancer cells with more expected as throughput increases. Knowledge of such rearrangements will enable drug development. Many drugs that target fusion proteins generated from simple rearrangements are currently used in treatment of cancers. Novel drug strategies could be developed that attack compensatory pathways to enhance the efficacy of current cytotoxic drugs through synthetic sickness/lethality and suppress continued rearrangements that would otherwise facilitate cancer progression, metastasis, and drug resistance. Thus, a better understanding of cancer-causing chromosomal rearrangements will enable the development of novel anticancer regimens.

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