Causes of Genome Instability

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replication stress, DNA damage response, chromosomal rearrangements, anaphase bridges, fragile sites, transcription-replication collisions

Abstract

Genomes are transmitted faithfully from dividing cells to their offspring. Changes that occur during DNA repair, chromosome duplication, and transmission or via recombination provide a natural source of genetic variation. They occur at low frequency because of the intrinsic variable nature of genomes, which we refer to as genome instability. However, genome instability can be enhanced by exposure to external genotoxic agents or as the result of cellular pathologies. We review the causes of genome instability as well as how it results in hyper-recombination, genome rearrangements, and chromosome fragmentation and loss, which are mainly mediated by double-strand breaks or single-strand gaps. Such events are primarily associated with defects in DNA replication and the DNA damage response, and show high incidence at repetitive DNA, non-B DNA structures, DNA-protein barriers, and highly transcribed regions. Identifying the causes of genome instability is crucial to understanding genome dynamics during cell proliferation and its role in cancer, aging, and a number of rare genetic diseases.

INTRODUCTION

Cells use a number of mechanisms to preserve the genome from the mutagenic action of genotoxic agents and to guarantee faithful chromosome duplication and transmission to the offspring. In addition to DNA damage repair, cells monitor replication to minimize errors of DNA synthesis. In eukaryotes, cell-cycle checkpoints guarantee coordination of DNA synthesis and DNA repair with cell division. Genome instability is mainly due to sporadic replication or repair errors but can also take place in response to developmental or environmental signals, as occurs in meiosis, and antigen receptor and immunoglobulin gene diversification in T and B cells. However, high levels of instability can be induced by external genotoxic stress or can be the result of a cellular pathology. The key role of genome instability in tumorigenesis and a number of rare cancer-prone genetic diseases, as well as its potential risks in stem cell–based therapies, has put it at the center of cancer biology and biomedical research.

Depending on the mechanisms involved, genome instability can result in (*a*) mutations, including point mutations and microsatellite contractions and expansions caused by erroneous or error-prone DNA synthesis, defective nucleotide or base excision repair (NER/BER), or mismatch repair (MMR); (*b*) variations in chromosome number caused by failures in the chromosome segregation apparatus or the mitotic checkpoint, also termed chromosome instability (CIN); and (*c*) other types of genetic alterations, including gross chromosomal rearrangements (GCRs), copy number variants (CNVs), hyper-recombination, and loss of heterozygosity (LOH) (3). These genetic alterations are in most cases initiated by single-stranded DNA (ssDNA) gaps or double-strand breaks (DSBs) generated as a consequence of replication stress and cover events mediated by homologous recombination (HR), including break-induced replication (BIR), and nonhomologous end-joining (NHEJ) mechanisms. Such instability events represent the most extended form of instability and can also be associated with mutations and chromosome loss. Here, we review the causes of this instability, with emphasis on the cellular processes involved and the *cis* structural and functional chromosomal features exacerbating instability. Our aim is to provide a comprehensive view of genome instability and its central role in uncontrolled cell proliferation.

Mitotic checkpoint: a quality-control mechanism that blocks anaphase entry until all chromosomes are properly duplicated and attached to the mitotic spindle

Gross chromosomal rearrangements (GCRs): genome reorganizations that include gene amplification, repeat expansions, translocations, inversions, insertions, deletions, and duplications

Loss of heterozygosity (LOH): loss of one of the two alleles of a diploid by deletion, gene conversion, or chromosome loss

Homologous recombination (HR): DSB repair pathways involving identical or nearly identical sequences of DNA as templates

Break-induced replication (BIR): DSB repair subpathway in which one DNA strand end invades a dsDNA that is used as template for replication

Nonhomologous end-joining (NHEJ): DSB repair pathways ligating DNA ends without the use of homologous DNA templates

REPLICATION DYSFUNCTION AS A MAJOR SOURCE OF INSTABILITY

Genome instability may result from failures at different steps of the DNA cycle, from replication to segregation. However, failures in DNA replication and the DNA damage response (DDR) are the most common causes. DNA replication is the most vulnerable cellular process during cell-cycle progression, and it is tightly controlled at stages from initiation to termination. Once-per-cell-division genome duplication is controlled at the steps of loading and activation of the replicative DNA helicase at replication origins (23). In eukaryotes, during G1-phase, the origin recognition complex (ORC) acts together with the Cdc6 ATPase and the Cdt1 protein to load an inactive double hexamer of the MCM2-7 complex, the catalytic core of the replicative DNA helicase (**Figure 1**). As cells enter S-phase, the cyclin-dependent kinase (CDK) and the Dbf4-dependent kinase (DDK) promote helicase activation and replisome assembly (125) by stimulating the recruitment of the GINS complex and Cdc45 to the inactive MCM2-7 double hexamer, resulting in the formation of the active Cdc45-MCM-GINS helicase. Rereplication is prevented by inhibiting reloading at origins of the MCM2-7 helicase core. This is achieved in some species by CDK phosphorylation of MCM2-7 and its loading factors and in others by direct inhibition of Cdt1 by the Geminin protein (125). Once replication has initiated, forks may undergo either transient pausing or a longer delay referred to as fork stalling. The replisome usually remains

associated with stalled forks (48) so that replication could restart without major consequences once the obstacle has been removed. However, a persistent fork arrest might lead to a DSB in one of the nascent sister double-stranded DNAs (dsDNAs) (**Figure 1**) or to fork regression generating a Holliday junction or chicken-foot structure (173, 181) (**Figure 2*a***), a potential major source of genome instability. It seems that in these cases, the replisome may be disassembled prior to breakage and restart (38).

Low Replication-Initiation Density

Eukaryotic chromosomes have a distribution of licensed origins that exceeds the number of active replication origins in S-phase. As a consequence, many licensed origins remain dormant, and thus constitute a backup group that can be activated to compensate for replication defects (9, 22). Reduced efficiency of origin licensing is a major source of genome instability. For example, yeast cells that lack the Sic1 CDK inhibitor initiate replication from fewer origins, increasing the distance between replication forks. These mutants have an extended S-phase, accumulate ssDNA, and show a strong increase in GCRs and chromosome loss (107). Similarly G1-cyclin Cln2 overexpression induces GCRs by reducing the loading of the MCM2-7 helicase core (189). Thus, precocious activation of CDKs limits MCM2-7 loading, reducing replication origin licensing and leading to instability.

Various results suggest that in mammals, low-density initiation may also lead to unfinished replication that is responsible for genome instability. Thus, human cells depleted of excess origin-bound MCM2-7 replicate normally but are hypersensitive to replicative stress and checkpoint inactivation, and under these conditions they undergo micronucleus formation, implying chromosome fragmentation (86). In addition, mouse embryonic fibroblasts carrying an *MCM4* mutant allele, which reduces MCM2-7 stability and the number of dormant origins, are highly susceptible to chromosome breaks induced by the replicative polymerase inhibitor aphidicolin (177). These cells contain an increased number of stalled forks, even in unchallenged S-phase, and show unfinished replication intermediates when entering M-phase, leading to chromosome segregation defects that are associated with tumorigenesis (93). Therefore, in low origin-density regions, there are not enough licensed origins that can be activated to compensate for fork stalling (**Figure 2*b***). Breakage could occur later in G2/M as a consequence of under-replicated DNA entering M-phase (see below).

Untimely Initiation Causing Re-replication

Untimely replication can also lead to genome instability by a different mechanism, namely by loading of the MCM2-7 helicase core outside of the G1-phase. Re-replication generated by Cdt1 deregulation in *Xenopus* egg extracts leads to DNA damage checkpoint activation and small fragments of dsDNA, the structure of which suggests generation by head-to-tail replication fork collisions at multifork structures (44) (**Figure 2*c***). Consistent with this, in budding yeast local DNA amplification induced by deregulation of Cdc6 and MCM2-7 occurs at Ty and long terminal repeat (LTR) retrotransposon regions via Rad52-dependent recombination, suggesting that deregulation of replication initiation enhances the formation of DSBs (73). Similarly, untimely initiation of replication in G1 cells caused by overexpression of a phosphomimetic form of the Sld2 replication-initiation factor increases GCRs. This effect is enhanced by increasing the loading of MCM2-7 via the simultaneous overexpression of Cdc6, favoring the idea that replication reinitiation causes the rearrangements (188). Therefore, re-replication caused by untimely initiation seems to enhance the probability of fork breakage. Consistent with this, the *c-MYC* oncogene, overexpression of which induces genome instability and replication stress, physically interacts

DNA damage response (DDR): complex network of DNA repair and DNA damage checkpoint pathways that coordinates cellular processes to deal with DNA lesions

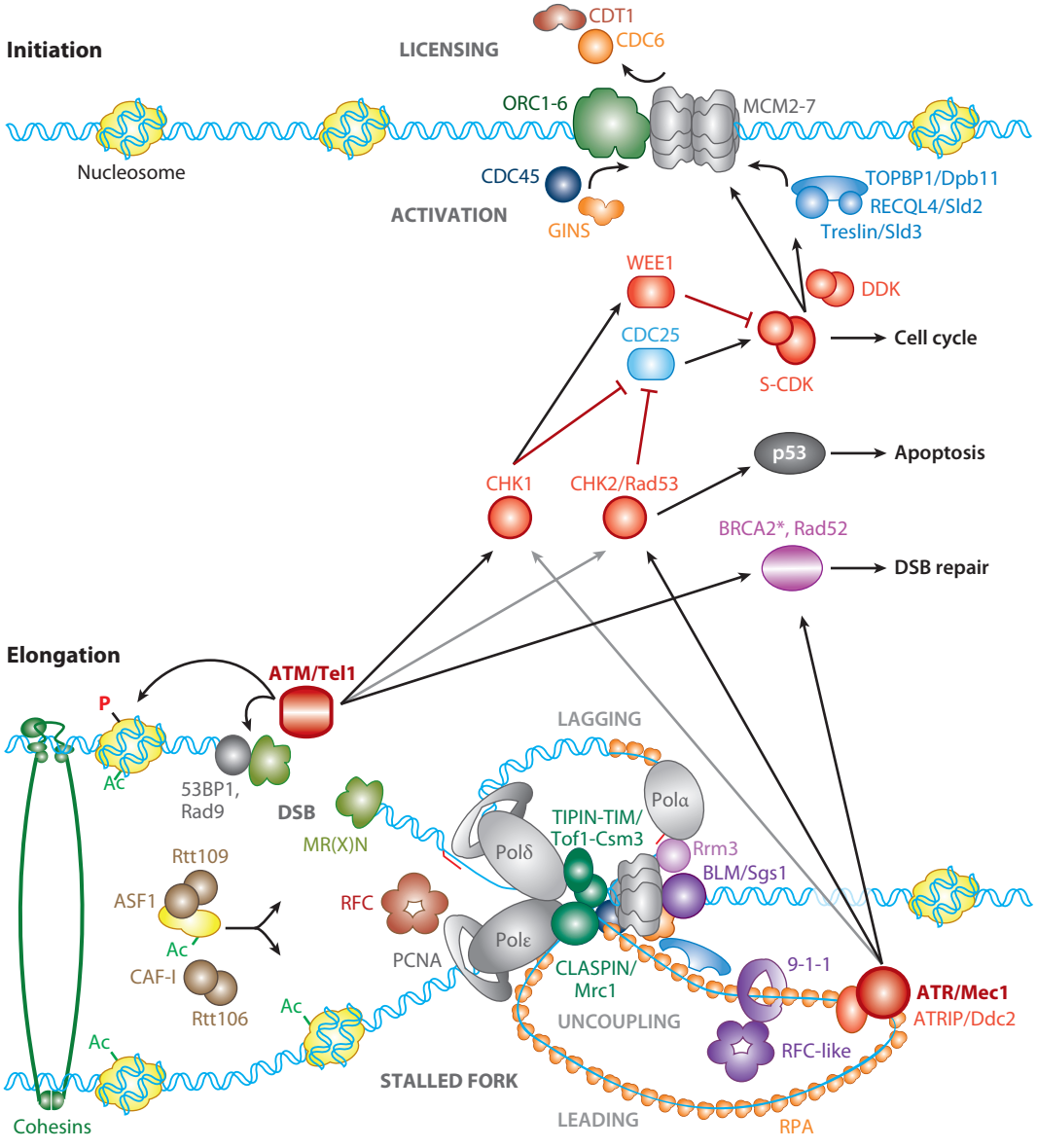
Holliday junction: cruciform structure formed by two duplexes of DNA

Licensed origin: origins loaded with the origin recognition complex and the MCM2-7 hexamer that are waiting to be charged with GIN and Cdc45 to initiate replication

with MCM2-7 and promotes additional origin firing, resulting in DNA damage and checkpoint activation (53). Reinitiation of replication forks could therefore cause multifork structures (**Figure 2*c***) that would enhance the probability of replication fork collisions and possible breakages that would in turn promote GCRs.

Faulty Replication Fork Progression

In the past two decades, evidence has accumulated that indicates that defective progression of replication forks can lead to DSBs or ssDNA gaps and chromosome fragility, sister-chromatid exchange (SCE),



hyper-recombination, GCRs, or chromosome loss, which can all be detected by a varied number of techniques (**Figure 3*a–i***). Initial hints that a tight genetic control of replication serves to suppress genome instability came from the hyper-recombination phenotypes of replication mutants of *Escherichia coli* (123, 213) and *Saccharomyces cerevisiae* (4, 74, 209). Since then, many studies, in everything from microorganisms to mammals, have identified additional mutations that affect fork progression and that increase mitotic recombination and GCRs (**Table 1**) (see **Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**) (3). These include mutations in the *RAD27* gene, encoding a flap endonuclease involved in Okazaki fragment processing, and in the *RFA1* gene, encoding the single-strand binding subunit of replication protein A (RPA) in budding yeast (34, 192, 198). Defective replication may generate DSBs responsible for both recombination and GCR events (**Figure 3*e–b***). Consistent with this, most of these mutants arrest or are delayed in S-phase and, as has been shown for *rad27* mutants, viability is dependent on DSB repair proteins, such as Rad52 or the MR(X)N complex (45). Conversely, GCRs in these mutants are further increased by simultaneous inactivation of MRX, as is the case of the *rfa1-t33* alleles, which is consistent with the accumulation of DSBs in replication protein A (RPA)-deficient cells (33). The relevance of replication factors in maintaining genome integrity in mammals is evidenced by CIN and the predisposition to cancer evident in mice with mutations in MCM4 or in genes encoding RPA subunits (177, 206) as well as similar mutant cell lines from human patients (**Table 1**; see **Supplemental Table 1**).

Dysfunction of replication factors can generate replicative stress similar to that caused by replication inhibitors, including hydroxyurea, which inhibits ribonucleotide reductase (RNR), leading to a depletion of

MR(X)N: yeast Mre11-Rad50-Xrs2 complex or human MRE11-RAD50-NBS1 complex involved in DSB recognition and processing and checkpoint activation

Supplemental Material

Figure 1

Regulation of DNA replication in eukaryotes and elements controlling genome integrity acting in *trans*. Origin licensing requires the hexameric origin recognition complex ORC1-6 to recruit the inactive DNA helicase complex, MCM2-7, via the action of Cdc6 ATPase and Cdt1 protein. Origin activation depends on the activation of MCM2-7 by the cyclin-dependent kinase (CDK) and the Dbf4-dependent kinase (DDK), which promote loading of the replisome, and requires several factors: Cdc45, GINS, RECQL4/Sld2, Treslin/Sld3, and TOPBP1/Dbp11. Together with the MCM2-7-Cdc45-GINS helicase, the leading-strand DNA Polε and lagging-strand Polδ and Polα, the trimeric clamp PCNA (proliferating cell nuclear antigen) and pentameric clamp-loader replication factor C (RFC) constitute the basic replisome. The MCM2-7 helicase is phosphorylated by DDK, allowing loading of Cdc45 and CLASPIN/Mrc1 together with Tof1 and Csm3 (TIPIN and TIM), which, along with the Sgs1 RecQ helicase, monitor replication fork progression to prevent fork collapse and reactivate replication. Fork stalling can result in the uncoupling of leading- and lagging-strand synthesis, generating a long stretch of replication protein A (RPA)-bound single-stranded DNA (ssDNA) that activates the ATR/Mec1 kinase via ATRIP/Ddc2. If a double-strand break (DSB) is generated (for simplification, shown in the lagging strand), the ATM/Tel1 kinase is activated. Upon activation, ATM/Tel1 and ATR/Mec1 PI3KKs phosphorylate several effectors to activate the replication/DNA damage checkpoint and arrest the cell cycle. Downstream effector kinases CHK1 and CHK2/Rad53 inhibit CDK in part by Cdc25 phosphatase inactivation and by favoring WEE1 inhibitory phosphorylation of CDK. In addition, the p53 pathway responsible for transcription of genes required for cell-cycle arrest or the apoptotic response is activated as a last resource. However, histone H2AX is phosphorylated by PI3KKs, acting as a signal to maintain repair factors, such as MR(X)N, BRCA2, etc., at chromatin regions flanking DSBs. These repair factors are also phosphorylated by PI3KKs to amplify the checkpoint signal and to promote the activation of the appropriate repair pathways. When a fork stalls, the Rfc1 subunit of RFC can be replaced by RAD9/Rad17, forming an alternative clamp loader that would deposit the 9-1-1 (RAD9-RAD1-HUS1/Rad17-Mec3-Ddc1) complex instead of PCNA. The 9-1-1 complex in coordination with TOPBP1/Dpb11 activates ATR/Mec1 to promote repair (for simplicity 9-1-1 has not been drawn at the junction of double-stranded DNA and ssDNA). Cohesins transiently associate with the replication fork and, under replicative stress, accumulate at forks, channeling broken forks to repair with the sister chromatid. During replication, nucleosomes are assembled into the newly synthesized DNA strands. This depends on H3K56 acetylation (requiring Asf1 and Rtt109 histone acetyltransferase), which stimulates the binding of H3-H4 to the CAF-I and Rtt106 chaperones responsible for incorporating H3(K56ac)-H4 into DNA (see Reference 125 and references therein). Genes and proteins are referred to by their "human/*S. cerevisiae*" names, unless the two names are the same or no obvious homolog is yet identified. Gray arrows represent minor regulations.

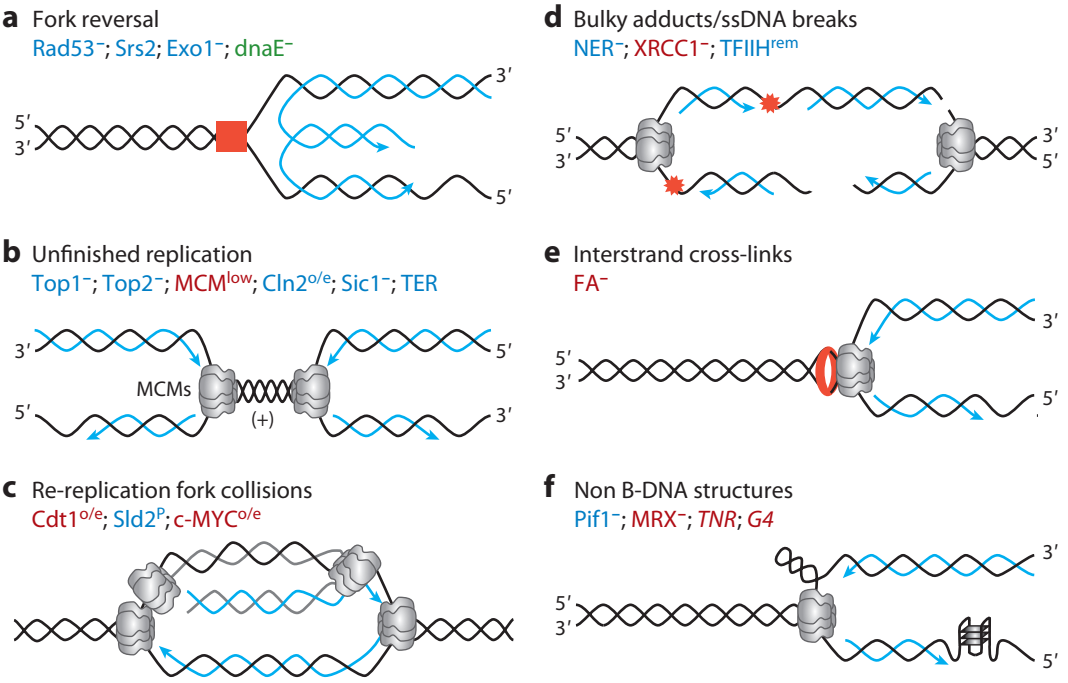


Figure 2

Cis-elements affecting genome instability. Site-specific structures that can compromise replication fork progression in *cis* leading to either single-stranded DNA (ssDNA) gap accumulation or double-strand breaks (DSBs) responsible for instability events. (*a*) Holliday junction or chicken-foot structures generated by fork regression. (*b*) Unreplicated DNA regions caused by low density replication initiation or failures in replication termination. (*c*) Replication fork collisions and breakage caused by untimely re-replication. (*d*) Unrepaired bulky adducts or ssDNA nicks generated as DNA repair intermediates. (*e*) Interstrand cross-links. (*f*) Secondary structures formed in ssDNA. Defects or specific elements leading to these structures are indicated: mammals (*red*), yeast (*blue*), and bacteria (*green*). Abbreviations: FA, Fanconi anemia; G4, G-quadruplexes.

dNTPs in cells, and aphidicolin, which inhibits DNA polymerase. Replication stress can either enhance interruption of DNA synthesis and stalling of the replication fork (**Figures 1** and **2**) or diminish replication fork restart, leading to the accumulation of DSBs (**Figure 3*a–d***). In yeast, it has been shown that replication fork speed is modulated by dNTP availability, as hydroxyurea causes a sharp transition to slow-moving forks, whereas RNR upregulation enhances fork progression (157). However, an improperly repaired lesion or DNA adduct can block fork progression (**Figure 2*d***), and this is likely the basis for genome instability induced by UV irradiation or the alkylating agent methyl-methanesulfonate, or by deficiencies in NER or BER. An example is provided by the *rem* alleles of budding yeast. Isolated as semidominant hypermutators and hyper-recombinators, these alleles map to the gene encoding the Rad3 subunit of TFIIH, which is involved in NER (140). In this mutant, the ssDNA gap generated after DNA-fragment excision appears not to be efficiently filled, leading to the breakage of the replication fork that encounters the gap and making viability dependent on the recombination functions Rad52 and MRX (141) (**Figure 2*d***).

The Fanconi anemia pathway illustrates the cellular response to replication fork stalling at damaged DNA (139). This pathway regulates replication-mediated removal of interstrand cross-links (**Figure 2*e***) and stabilizes and protects stalled forks from exonucleolytic

Table 1 A selection of eukaryotic proteins with a role in the maintenance of genome stability[a]

Mammals	Yeast	Function	Genome instability
Replication			
CDC45	Cdc45	Replication initiation	GCR, CIN
CDC6	Cdc6	Replication initiation	HR
Claspin	Mrc1	Replication and intra-S-phase checkpoint	GCR, TNR
EGL1	Egl1	RFC-like	HR, GCR, CIN, SCE
FANCM	Mph1	DNA helicase	GCR
FBXL2	Dia2	SCF F-box protein	HR, GCR
FEN1	Rad27	Flap endonuclease	HR, GCR
hDNA2	Dna2	Helicase/nuclease	GCR, CIN
LIGI	Cdc9	DNA ligase I	HR
MCM2-7	Mcm2-7	Replicative DNA helicase	HR, GCR
ORC3-5	Orc3-6L	Origin replication complex	GCR
PCNA	Pol30	Replication and checkpoint mediator	HR
PIF1	Pif1	RNA-DNA helicase	GCR, Tel fusion
POLA1-PRIM1 and 2	Pol1-Pri1 and 2	Polymerase α-primase complex	HR, GCR
Polε	Pol2	DNA polymerase ε	GCR
Polδ	Pol3/Cdc2	DNA polymerase δ	HR
RFC1-5	Rfc1-5	Clamp loader and checkpoint sensor	HR, GCR
RPA1-3	Rfa1-3	ssDNA coating and checkpoint signaling	GCR
TIM	Tof1	S-phase checkpoint	GCR, TNR
TIPIN	Csm3	S-phase checkpoint	TNR
TOP1	Top1	Topoisomerase I	HR
TOP2A and B	Top2	Topoisomerase II	HR
TOPBP11	Dbp11	Replication and checkpoint mediator	GCR
–	Rrm3	DNA helicase	HR
DNA damage checkpoint			
9-1-1 complex	Ddc1-Mec3-Rad17	PCNA-like complex mediator	GCR, TNR
ATM	Tel1	Checkpoint kinase	HR, GCR
HST4ATR-ATRIP	Mec1-Dcd2	Checkpoint kinase	HR, GCR, TNR
CHK1	Chk1	Checkpoint effector kinase	GCR
CHK2	Rad53	Checkpoint effector kinase	GCR, TNR
RAD17	Rad24	RFC-like S-phase checkpoint	HR, GCR, TNR
–	Rad9	Checkpoint mediator	HR, GCR
Chromatin			
ASF1A and B	Asf1	Chaperone	HR, GCR
CAF-I complex	CAF-I complex	Chaperone	HR, GCR
H2AX	H2A	DNA damage signaling	GCR
HMG1	Spt2	Chromatin remodeling	GCR, CIN
HST3 and HST4	Hst3 and Hst4	Histone deacetylases	HR, TNR
INO80 complex	Ino80 complex	Chromatin assembly	GCR, CIN

(*Continued*)

Table 1 (*Continued*)

Mammals	Yeast	Function	Genome instability
p300/CBP	Rtt109	Histone acetylase	HR, GCR, TNR
SIN3	Sin3	Histone deacetylase	CIN, TNR
SIRT2	Sir2	Histone deacetylase	HR, CIN
Postreplicative repair			
HLTF & SHPRH	Rad5	Helicase	GCR
POLκ	–	Translesion synthesis polymerase	[CIN]
POLτ	–	Translesion synthesis polymerase	CIN
RAD18	Rad18	E3-ubiquitin ligase	HR, GCR
REV1	Rev1	Translesion synthesis (dCMP transferase)	GCR
REV3L	Rev3-7	Translesion synthesis polymerase	GCR, SCE, TNR
Double-strand break repair			
BLM	Sgs1	DNA helicase	HR, GCR, SCE, TNR
BRCA1	–	Recombination and checkpoint	GCR, CIN
BRCA2	–	Recombination and cross-link repair	GCR, CIN
CtIP	Sae2	DSB resection	GCR
FBH1	Srs2	DNA helicase	HR, TRN
KU70-KU80	YKu70-YKu80	Nonhomologous end joining	GCR, CIN, Tel fusion
LigIV	Dnl4	Nonhomologous end joining	GCR
MRN complex	MRX complex	DSB recognition/processing	HR, GCR, TNR
MUS81	Mus81	Structure-selective endonuclease	GCR
RAD51	Rad51	Homologous pairing	LOH
RAD51B-D, XRCC2-3	Rad57 and 59	Rad51 ssDNA annealing paralogs	GCR
RAD52	Rad52	Recombination ssDNA annealing	GCR
RAD54	Rad54	Recombination dsDNA modification	GCR
TOP3A-B	Top3	Topoisomerase III	HR, GCR
WNR	Sgs1	DNA helicase	GCR
XRCC4	–	Nonhomologous end joining	CIN, Tel fusion
Telomere			
POT1 and TTP1	Cdc13	Telomere capping	HR, GCR
TRF1	–	T-loop binding shelterin	Tel fragility
TRF2	–	T-loop binding shelterin	GCR, Tel fusion
mRNA biogenesis			
ASF/SF2	–	premRNA splicing	GCR
CSTF3	Rna14	mRNA 3′-end processing	HR
FIP1L1	Fip1	mRNA 3′-end processing	CIN
SETX	Sen1	RNA-DNA helicase	CIN
TREX-2	THSC/TREX-2	mRNA export	HR
THO complex	THO complex	mRNP biogenesis	HR
UAP56-ALY	Sub2-Yra1	mRNP biogenesis and export	HR

(*Continued*)

Table 1 (*Continued*)

Mammals	Yeast	Function	Genome instability
Others			
APTX	Hnt3	DNA 5′ AMP hydrolase	GCR, SCE
EXO1	Exo1	Exonuclease	GCR, CIN
FANCA-N	–	Fanconi anemia pathway	HR, GCR
MLH1	Mlh1	Mismatch repair	HR, GCR
MSH2	Msh2	Mismatch repair	GCR
PDXK	Bud16	Pyridoxal kinase (Pdxk)	GCR
SMC5 and SMC6	Smc5 and Smc6	DNA repair and cohesion	GCR

[a]The table lists proteins in which mutations have been shown to increase homologous recombination (HR), gross chromosomal rearrangements (GCRs), chromosomal instability (CIN), sister chromatid exchanges (SCEs), tri-nucleotide repeat expansions and contractions (TNR), telomere fusions (Tel fusion), or fragile telomeres (Tel fragility). A phenotype inside brackets ([ ]) indicates that it is caused by overexpression of the protein. For further details and references see **Supplementary Table 1**. Abbreviations: DSB, double-strand break; PCNA, proliferating cell nuclear antigen; RFC, replication factor C complex; SCF, Skp1-Cdc53/Cullin-F-box.

degradation. The Fanconi anemia complex coordinates the action of different repair pathways. It contains the BRCA2 (FANCD1) DSB repair factor and interacts with the BLM-TOP3-RRM1,2 helicase-topoisomerase complex and RPA via the FANCM subunit. In cells of Fanconi anemia patients, interstrand cross-link agents, such as cisplatin and mitomycin C, increase SCEs, breaks, chromosomal aberrations, and missegregation (**Table 1**; see **Supplemental Table 1**). Interestingly, chicken DT40 cells lacking FANCM/Mph1 helicase, when treated with camptothecin, accumulate stalled forks that cannot restart. In this case, full replication is ensured by activation of neighboring dormant origins (170), which enables complete replication after fork stalling.

In summary, conditions that affect the function of the replisome and replication-associated factors generate replication stress, which increases the chances that a replication fork undergoes persistent stalling. This leads to the accumulation of ssDNA gaps or DSBs, which in turn can generate an instability, such as a GCR (**Figure 3*g*,*h***). However, breaks that occur as a consequence of replication fork stalling are not restricted to S-phase and may also occur in G2- or M-phase. The timing and nature of the break can determine the repair mechanism and genome instability event.

S-Phase Checkpoint Dysfunction

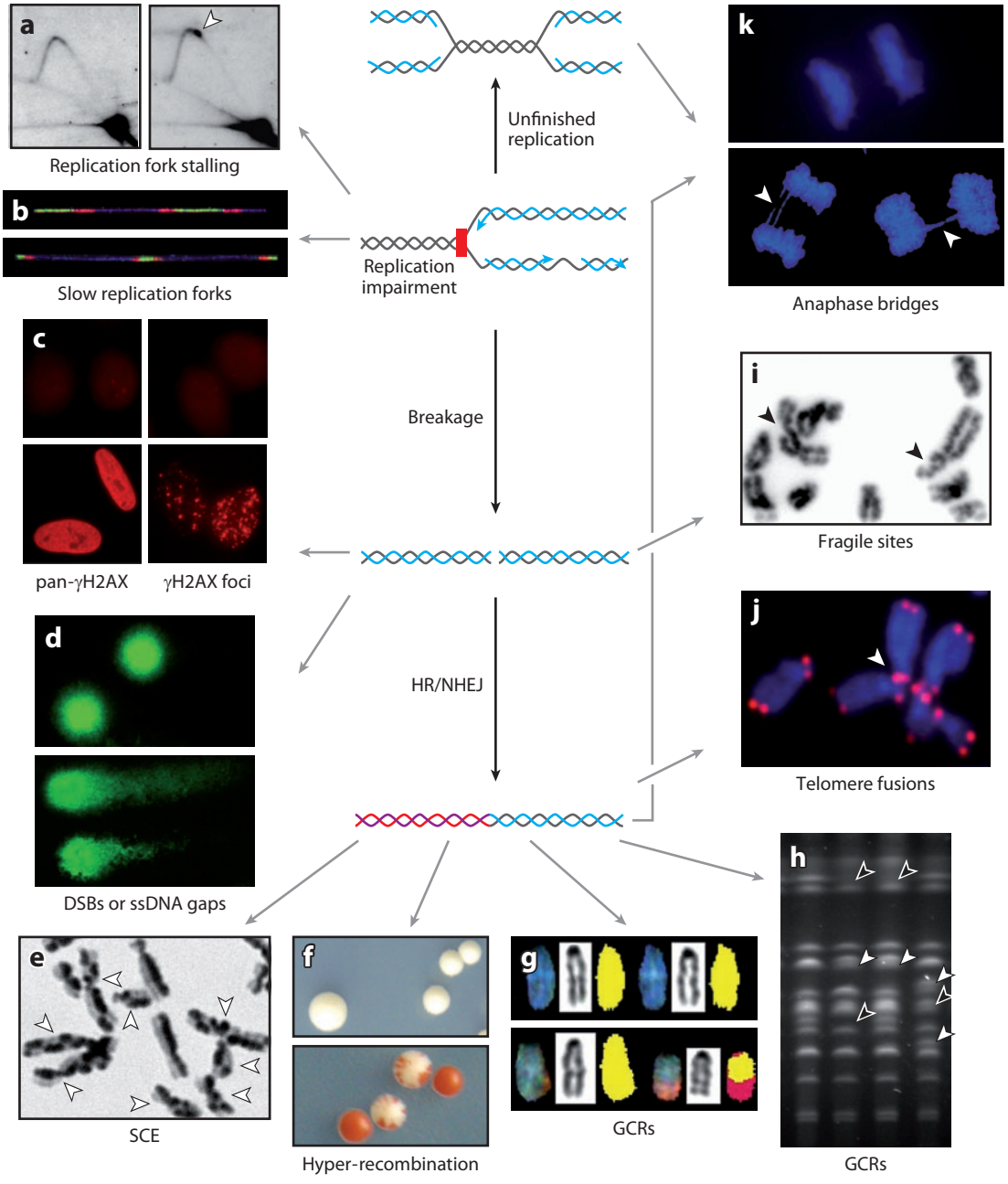
Supplemental Material

During replication fork progression, if the lagging-strand polymerase is blocked, DNA synthesis still proceeds through the next Okazaki fragment without major consequences. In contrast, leading-strand polymerase blockage causes uncoupling between lagging- and leading-strand synthesis at the fork (153). In this case, the replicative DNA helicase might proceed ahead of the polymerase, allowing replication fork reactivation past the damage, as has been shown in *E. coli*–derived in vitro systems (78, 211). As a consequence, long stretches of RPA-bound ssDNA accumulate (**Figure 1**). In eukaryotes, RPA-bound ssDNA and DSBs generated after fork stalling activate the S-phase checkpoints. These guarantee genome integrity by delaying the cell cycle and promoting replication fork restart and repair before chromosome segregation (14). The first protein kinases acting in checkpoint activation, ataxia telangiectasia–mutated (ATM)/Tel1 and ATM-related (ATR)/Mec1, regulate the selection and timing of replication in a damage-dependent and -independent manner. Both kinases regulate many effectors by phosphorylation, including downstream effector kinases CHK1 and CHK2/Rad53, which slow down replication and inhibit late origin firing

S-phase checkpoints: refers to the three checkpoints acting during S-phase: the replication, intra-S-phase, and S-M checkpoints

via the inhibition of CDKs (14) (**Figure 1**). As a consequence, origin firing is deactivated and replisomes cannot be re-recruited (176).

Activation of ATR/Mec1 and CHK2/Rad53 kinases inhibits mitosis, slows down replication, stabilizes the replication fork, blocks activation of late-firing origins, and regulates nucleases, thus preventing aberrant replication and recombination intermediates (36, 37). As a result, inactivation of the S-phase checkpoint genes causes genome instability from yeast to mammals (7, 145, 154, 185), and many cancer-prone



inheritable diseases are due to checkpoint defects (**Table 1**; see **Supplemental Table 1**). In yeast, mutations in genes that encode Rfc5, Dbp11, Mec1, Rad53, Chk1, or Sgs1 cause as much as a 200-fold increase in GCRs (145). The majority of these GCRs are deletions with telomere additions, but translocations with microhomologies at the breakpoints are also formed (146). Dysfunctions in the S-phase checkpoint compromise maintenance of the replisome integrity, thereby leading to breakage and GCRs (28, 30). Similarly, in human cells ATM-deficiency enhances retrotransposition of L1 elements that lack endonuclease activity and must depend upon spontaneous DSBs for retrotransposition (40). Therefore, evidence from different systems and organisms shows that the replication and S-phase checkpoints safeguard replication fork stability and prevent DSB formation. If a DSB does occur, its timing and the mechanism used for its repair determine the type of heritable genome alteration generated.

Defective Nucleosome Assembly and Remodeling

In eukaryotes, DNA is organized into chromatin, the basic components of which are the nucleosomes. Nucleosome remodeling has been shown to be critical in many aspects of genome dynamics (see **Supplemental Table 1**). DNA replication progression is strictly dependent on de novo nucleosome assembly in the nascent DNA strands, which is regulated by histone modifications. Thus, the Lys56 of newly synthesized histone H3 is acetylated (126), permitting its incorporation into nucleosomes by the joint action of histone acetyltransferase Rtt109 and chaperones Asf1, CAF-I, and Rtt106 (**Figure 1**). Interestingly, DNA damage checkpoint activation causes Mec1-dependent retention of H3K56ac in the DNA, implying a role for H3K56ac in DDR (126). Notably, deregulation of nucleosome assembly causes replication and checkpoint defects, resulting in recombinogenic DSBs and ssDNA gaps (210) as well as hyper-recombination (160). Yeast strains that contain nonacetylatable H3K56R histones, or lack the histone acetyltransferase Rtt109 or the histone chaperones Asf1, CAF-1, and Rtt106, or that express low levels of histone H4 or that have dysfunctional nucleosome remodelers, all show DNA damage foci, hyper-recombination, GCRs, or repeat contractions, providing evidence that improper nucleosome assembly also results in replication dysfunction and triggers genome instability (**Table 1**). The recent observation that disruption of chromatin assembly

Nucleosome: chromatin subunit containing 147 bp of DNA wrapped around a histone octamer composed of two units of H2A, H2B, H3, and H4

Supplemental Material

Figure 3

Intermediates and chromosome structural alterations, as observed by different techniques. (*a*) Replication fork stalling, as monitored by 2D-gel electrophoresis and Southern analysis in yeast (for details about the technique, see Reference 161). (*b*) Slower human replication forks covering shorter DNA synthesis tracks, as determined by incorporation of IdU and CldU via DNA combing (52), which permits visualization of the process of replication on DNA fibers. (*c*) Accumulation of double-strand breaks (DSBs) or replicative stress, as inferred by γH2AX foci or by γH2AX pan staining, respectively, in human cells. (*d*) DSBs or ssDNA (single-stranded DNA) gaps as seen directly by nuclear "comet tails" via single-cell electrophoresis assays in human cells (52). (*e*) Sister-chromatid exchanges (SCEs), as determined by Giemsa staining in human cells (207). (*f*) Hyper-recombination, as determined by colony sectoring in yeast (5). (*g*) Gross chromosomal rearrangements (GCRs), as determined by spectral karyotyping in mouse cells (118). (*h*) Translocations, as visualized by pulse-field gel electrophoresis in yeast (168). (*i*) Fragile sites, as detected by mitotic spreads in human cells (109). (*j*) Telomere fusions, as determined by CO-FISH (chromosome-orientation fluorescent in situ hybridization) in mouse cells (124). (*k*) Anaphase bridges, presumably resulting from unfinished replication, dicentric chromosomes, and sister-chromatid nondisjunction, as detected by fluorescence microscopy in mouse cells. Arrows indicate the specific structural alterations referred to in each panel; in panel h, closed and open arrows indicate the position where the translocated or missing parental chromosome migrates or should migrate, respectively. When necessary, a normal control is shown on top of the panel, with the exception of panel a, which is shown on the left. Detailed descriptions of each technique can be found in the references provided. Photos are from the laboratories of A. Nussenzweig (*g*), A. Losada (*k*), M. Blasco (*j*), L. Tora (*i*), and ours (*all others*). Abbreviations: HR, homologous recombination; NHEJ, nonhomologous end-joining.

affects the size and distribution of Okazaki fragments suggests that this instability might be caused by dysfunctional lagging-strand processing (179). Nevertheless, given the additional role of chromatin in DNA repair, it cannot be excluded that, in some cases, the genome instability associated with dysfunctional nucleosome assembly may be due to defects in DSB repair (6) (see below).

FAILURES IN POSTREPLICATIVE REPAIR AND HOMOLOGOUS RECOMBINATION

When a replication fork encounters a DNA adduct, cells induce DNA damage tolerance mechanisms that allow completion of replication. Adducts can be bypassed by postreplicative repair via translesion polymerases (either faithful or error-prone) or via error-free template switching using the sister chromatid (64, 105). Postreplicative repair guarantees genome stability by allowing completion of replication (albeit at the expense of mutations), and its deregulation can result in recombinogenic DSBs. Thus, mammalian cells lacking Polη, REV1, or Polζ show increased DSBs, HR, chromosomal aberrations, and/or aneuploidy (127, 175), and yeast deficient in postreplicative repair pathways shows high levels of mitotic recombination and GCRs (**Table 1**; see **Supplemental Table 1**). However, upregulation of translesion polymerases, such as mammalian Polθ and Polκ, impairs replication and results in DSBs and chromosomal abnormalities (**Table 1**; see **Supplemental Table 1**). It has been proposed that translesion polymerases may compete with replicative polymerases but hinder replication because of their poor processivity (90, 156).

HR is the major pathway for the repair of DSBs generated during replication. HR is necessary for the restart of broken forks and, in the absence of postreplicative repair, as an alternative way of bypassing lesions that block DNA synthesis. HR is active during S- and G2-phases, when sister-chromatid recombination is possible (81). If HR is dysfunctional, DSBs may cause different kinds of genome instability, reflecting the mechanism and template used for repair. GCRs (**Table 1**; see **Supplemental Table 1**) can result from NHEJ or microhomology-mediated BIR between two heterologous chromosomes (**Figure 4**). Consistent with this, marked increases in GCRs have been reported in yeast deficient in HR because of mutations in Rad52, Rad51, or the MRX complex (144, 146), and in mice with an RPA dysfunction that abolishes recombinational DSB repair (206). One of the best-studied cases of genome instability in human and yeast is that caused by inactivation of the BLM/Sgs1 helicase involved in Holliday junction dissolution (81) (**Table 1**; see **Supplemental Table 1**). In humans, this leads to greatly elevated frequency of SCEs (**Figure 3*e***). Interestingly, in human BLM$^{-/-}$ cells the structure-selective endonucleases MUS81-EME1, SLX1-SLX4, and GEN1 are necessary for SCE and genome instability (207).

SCE is controlled by specific factors, including chromatin modifiers such as histone acetylases and deacetylases (142) (**Table 1**; see **Supplemental Table 1**), as well as cohesin. In yeast, elimination of cohesin reduces SCE but increases damage-induced recombination between homologous chromosomes (39, 41). Cohesin transiently associates with replicating regions (**Figure 1**), and in response to replicative stress it accumulates at replication forks in a Rad50-dependent manner (193). Failure to restart broken forks by recombination with the sister chromatid can be a source of GCRs (**Figure 4**) if repair uses sequences from other regions of the genome.

SITE-SPECIFIC HOTSPOTS OF GENOME INSTABILITY

Genome instability does not originate randomly throughout genomes but occurs more frequently at specific regions that we refer to as hotspots. Deciphering the nature of these hotspots and how they are expressed is crucial to understanding genome instability.

Supplemental Material

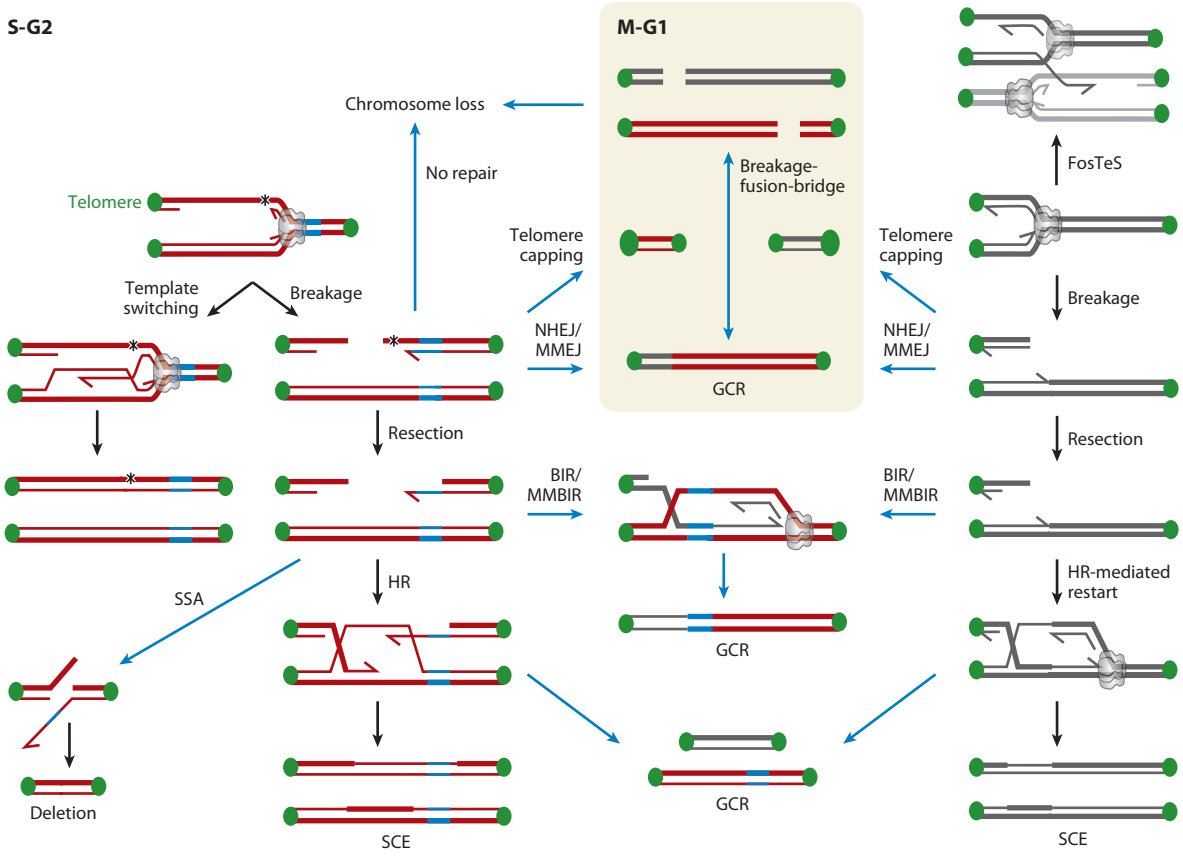


Figure 4

Mechanisms of genome instability involving double-strand breaks (DSBs) or gaps. A simplified summary of the possible mechanisms used by cells to resume replication after replication fork stalling at the lagging (*left*) or leading (*right*) strand, as well as the alternative mechanisms responsible for different chromosome alterations, is shown. All repair events shown take place during S- and G2-phases, but the yellow background groups events initiated by breaks that can also occur during M- and G1-phases. Heterologous chromosomes are shown in dark gray and red to better illustrate the gross chromosomal rearrangements (GCRs) occurring between them. In some cases, only one of several possible outcomes is shown for simplicity, but more are possible. Telomeres are indicated as green spots. Newly synthesized DNA is shown as a thin line and template strands as thick lines. The blue DNA segment represents a hypothetical sequence in the red chromosome, homologous to the dark gray chromosome, that can act as a template for break-induced replication (BIR) or microhomology-mediated BIR events. The light gray chromosome (*top right*) represents the homolog of the dark gray chromosome used in FosTeS (fork stalling and template switching). Blue and black arrows indicate repair events that either lead or do not lead, respectively, to the genome instability events shown. Abbreviations: HR, homologous recombination; NHEJ, nonhomologous end joining; SCE, sister-chromatid exchange.

DNA Repeats

DNA repeats, such as trinucleotide repeats (TNRs), long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), LTRs, and retrotransposons, constitute one of the most important natural elements that is prone to instability. Tandem repeats can undergo long expansions, which are linked to a number of neurological diseases (166). Repeat expansion normally originates in tandem repeats with repeat units from 2- to 64-bp long, including AT- and GC-rich micro- and minisatellites in human cells. Repeat expansion may be caused by replication slippage, MMR, or BER (131, 155), or by DSBs or ssDNA

Supplemental Material

gaps generated by faulty replication (87, 172, 178), which may be caused by the ability of the repeats to form non-B DNA structures (155) (**Figure 2*f***) (see below). In *E. coli*, yeast, and human cells, long tandem repeats can be difficult to replicate and can undergo fork stalling in an orientation-dependent manner (92, 129, 135). Furthermore, increased contractions and/or expansions are seen in yeast and/or human cells defective in replication functions, such as DNA polymerases, PCNA, FEN1/Rad27, Tof1, Csm3, CLASPIN/Mrc1, Ligase, Srs2, and Sgs1 (**Table 1**; see **Supplemental Table 1**). These contractions and/or expansions could occur either by fork stalling and template switching (FoSTeS), in which the nascent 3′-end strand switches to another repeat and uses it as template for further DNA synthesis (76), or by unequal SCE repair if the replication fork breaks (**Figure 4**).

Fragile Sites and Slow Replication Zones

Fragile sites were defined cytologically as metaphase chromosomal regions showing constrictions or breaks after mild replication inhibition (**Figure 3*i***) (57). Some are common and present in all human genomes, and others are rare, found in only 5% of individuals. There are no specific or unique DNA sequences associated with them, but many common fragile sites are composed of flexible DNA helixes containing interrupted runs of AT-rich repeats, whereas rare fragile sites represent expanded DNA repeat regions consisting mainly of CGG repeats or AT-rich minisatellite repeats (65, 80). Fragile sites represent up to 80% of the GCR breakpoints observed in early-stage tumors or precancerous cells, including SCEs, translocations, deletions, and integration sites of oncogenic viruses (16, 150). At those fragile sites containing DNA repeats with the potential to form non-B DNA structures, fragility could in principle reflect structure formation that delays replication fork progression (see below), but the extent to which this actually contributes to fragility is unclear. In many cases, replication initiation does not occur within a region of up to 700 kb surrounding the site; two such examples are *FRA3B*, which contains numerous LINEs, SINEs, LTRs, and transposons, and *FRA16D*, which is the most active human common fragile site but which is devoid of micro- or minisatellite sequences (104, 109, 149). Initiation-poor fragile sites may fail to complete replication before M-phase, when the breaks would become visible (108). Interestingly, fragility is cell type–specific, as some of the sites are fragile in lymphocytes but not in fibroblasts and vice versa (109). In each case, fragile sites correlate with replication exclusion regions, suggesting that chromatin status and gene expression patterns may influence replication and therefore fragility (see below).

Delayed replication fork progression through mammalian fragile sites can be recapitulated in *S. cerevisiae* (99). Thus, a region containing 105 CGG repeats of the Fragile X site, integrated in the yeast genome, causes a delay in fork progression that is strongest in *tof1*Δ and *mrc1*Δ cells (204). Checkpoint functions may contribute to repeat stability, as yeast fragility is increased in *mec1*Δ, *rad9*Δ, or *rad53*Δ mutants (100), and inhibition of ATR causes a 5–20-fold increase (28). Fragility is also observed when DSB repair factors, such as *BRCA1*, *RAD51*, DNA-PKcs, and ligase IV, are depleted (10, 171). Fragile-site stability is also compromised when chromosome condensation is prematurely induced by calyculin A, suggesting that fragile sites may contain incompletely duplicated sequences in cells exiting S-phase (59). Mild aphidicolin-mediated replication stress also causes chromosome breakage at fragile sites, which has been linked to failures in activation of additional origins (150). Therefore, some fragile sites correspond to regions that are difficult to replicate or that have low fork density, so that under conditions of replicative stress and S-phase checkpoint inactivation, these sites become hotspots for chromosome breakage and GCR breakpoints. Fragility has also been observed in B lymphocytes at early replication sites that colocalize with highly expressed gene clusters

and are enriched for repetitive elements and CpG dinucleotides (13). Stability of these sites depends on the ATR checkpoint kinase.

Identification of fragility hotspots in *S. cerevisiae* has confirmed the relationship between fragility and replication fork progression dysfunction. *mec1* cells accumulate stalled forks and associated chromosome breakage in regions termed replication slow zones, which coincide with replication termination regions, in which forks converge late in S-phase (30). However, breakage in *mec1*Δ cells could also be mediated by internal chromosomal stress as a consequence of mitotic chromosome condensation, as condensin subunits and topoisomerase II are required for break accumulation (75). Reduced levels of DNA polymerase α cause elevated levels of chromosome translocation and loss that involve recombination between head-to-head Ty elements (106). In S-phase checkpoint mutants, clusters of tRNA genes, capable of stalling forks, become breakpoints in translocations, chromosome truncations, and integration of retrotransposons and mitochondrial DNA (1). tRNA genes and/or Ty elements are enriched near 17 early origin-proximal regions that exhibit premature replication fork arrest and are prone to breakage in the absence of S-phase checkpoint activity (164).

Therefore, in organisms ranging from yeast to humans, fragility is found to be enriched at particular sites with varied structural and functional features that make them difficult to replicate (**Figure 2**). Fragility may be originated by different features, including non-B DNA structures, low-density replication-initiation zones, and transcription (see below), which can additively impair replication fork progression. Presumably, the more of these features that are present in a particular region, the more likely this region would be to exhibit fragility.

Non-B DNA Structures, G-Quadruplexes, and Telomeres

DNA repeats and other types of sequences with the potential to form non-B DNA structures, such as Z-DNA, triplex DNA (H-DNA), hairpins, cruciforms, and G-quadruplexes, seem to be responsible for the instability observed in hotspots (**Figure 2*f***). This may reflect not just possible effects on replication fork progression but also the ability to serve as substrates for specific nucleases. For example, fragile site–like GAA/TCC-expanded repeats in budding yeast strongly stimulate DSBs and GCRs in an MMR nuclease-dependent manner (95). It has been proposed that during replication, GAA repeats form triplex/H-DNA structures, which are a substrate for efficient cleavage by MMR nucleases. In addition, quasipalindromic Alu sequences integrated in the yeast genome stimulate ectopic recombination, suggesting that they are potential sites for DSBs. It is suggested that, in this case, capped hairpins are formed at these sites that, if not cleaved by MRX and Sae2 nucleases, generate inverted chromosome duplications upon replication (119), which in turn could potentially break later during mitosis (see below).

Two paradigmatic cases of non-B DNA structures that are fragility hotspots are G-quadruplexes and eukaryotic telomeres. The human *CEB1* minisatellite, which bears the G4 signature motif that confers the ability to form quadruplex structures, is unstable in yeast cells. Instability is only observed if the G-rich sequence is the template for leading-strand replication (121). The yeast Pif1 helicase, which binds and unwinds G-quadruplex in vitro, prevents such expansions (165). In *pif1*Δ cells, replication slows down at regions that bear the G4 signature motif (152). Interestingly, the human FANCJ helicase is required for stability of regions bearing the G4 motif (120). Therefore, instability at these sequences may reflect the requirement for a DNA helicase to resolve the non-B structure.

Telomeres are composed of G-rich TTAGGG repeats and can form G-quadruplex structures. Shelterins are key factors in preventing telomere instability. They stabilize T-loops, which protect telomere ends from being recognized by the DDR machinery (46). The human shelterin subunits TRF1 and TRF2 bind to double-stranded telomeric

G-quadruplexes: non-B DNA structures formed by G-quartets, planar structures of guanines associated through Hoogsteen base pairing, stacked on top of each other

T-loop: structure formed by hybridization of the telomere-end ssDNA into the telomeric dsDNA region

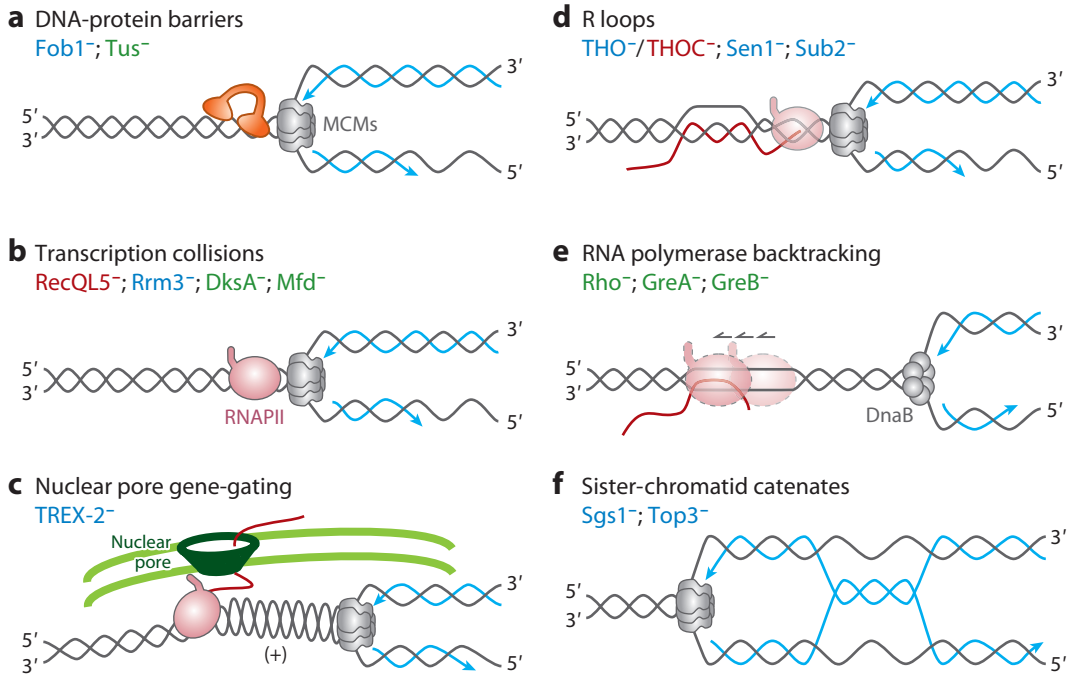


Figure 5

Elements affecting genome instability. (*a*) DNA-protein barriers, (*b*) transcription, (*c*) gene-gating to the nuclear pore, which could limit the release of the torsional stress caused by transcription-replication collisions, (*d*) R-loop accumulation, (*e*) defective backtracking of a stalled RNA polymerase, and (*f*) DNA hemicatenates formed as sister-chromatid X-shaped junctions resulting from HR-mediated rescue of replication fork breakage. Defects or specific elements leading to these structures are indicated: mammals (*red*), yeast (*blue*), and bacteria (*green*).

repeats, whereas POT1 binds to single-stranded telomeric repeats. Loss of POT1 activates ATR (47), whereas loss of TRF2 activates ATM, causing γ-H2AX and 53BP1, focus accumulation, hallmarks of DSBs, and MRN recruitment (187). TRF2 loss also leads to chromosome-to-chromosome fusion formation (**Figure 3*k***), which in turn is reduced by depleting ATM, 53BP1, or MRN (47, 51). In contrast, loss of TRF1 results in replication fork stalling, ATR activation, and accumulation in each chromosome of additional telomeric signals away from the chromosome end (124, 174). Thus, telomeres of TRF1-deficient cells resemble fragile sites and, indeed, aphidicolin treatment or ATR inhibition enhances fragility (124, 174). TRF1 has been proposed to recruit G4 helicases BLM and RTEL, which could resolve G-quadruplexes to facilitate replication of TTAGGG repeats (174, 199). Consistent with this, depletion of both helicases induces telomere fragility and is epistatic to TRF1 deletion (174).

IMPACT OF DNA PROTEIN BARRIERS AND TRANSCRIPTION ON REPLICATION

DNA Protein Barriers to Replication Fork Progression

DNA protein barriers are transient natural obstacles that in most cases cause a polar block to replication, arresting replication in only one direction to avoid collisions with the transcription machinery. As a consequence, replication termination or specific genome transactions could be facilitated (**Figure 5*a***). An example

of a DNA protein barrier is the Tus protein bound to the Ter sequences in *E. coli* (82), which terminates replication by inhibiting the DnaB replicative helicase. In eukaryotes, the polar replication fork block (RFB) site in yeast rDNA genes is mediated by the Fob1 protein (97) and prevents frontal collision between RNA polymerase I and replication forks (26). Other examples include the polar replication termination sequence 1 (*RTS1*) at the *mat* locus of fission yeast (43) and the tRNA gene sites that block replication in a polar manner (50), although the latter may also be related to transcriptional activity. In contrast to hydroxyurea-stalled forks, replication forks transiently stalled at DNA protein barriers do not activate the replication checkpoint (27, 195), suggesting that under normal conditions no significant accumulation of long ssDNA stretches or DSBs occurs at DNA protein barriers.

When taken out of their normal context or under replicative stress conditions, DNA protein barriers can produce persistent replication fork stalls that are responsible for breakage and rearrangements (**Figure 5*a***). In *E. coli* constructs in which a fork is blocked bidirectionally by two Ter sites, replication is rescued by HR, leading to hyper-recombination, and viability becomes dependent on the RecA and RecBC DSB repair functions, suggesting that DSBs accumulate under conditions of persistent stalling (84, 134). A similar phenomenon occurs when high numbers of *lacO* repeats are inserted into the human genome and the *lac* repressor is expressed (88). The impact of DNA protein fork barriers has been extensively studied in fission yeast, using DNA sequences containing two inverted *RTS1* copies (102). These inverted *RTS1* sequences are highly unstable, showing increased recombination and GCRs. Rad22/Rad52 is recruited to these sites of replication fork stalling, and promotes fork restart by a mechanism that involves recombination-dependent template switching, leading to chromosome duplications (101). Taken together, data from different systems reveal that DNA protein barriers can become a potential breakage hotspot under conditions of replication stress.

Transcription-Replication Collisions

Transcription has a global effect on genome integrity because it enhances mutation and recombination in bacteria (55, 201), yeast (94, 191, 202), and mammalian cells (148). A paradigmatic example of this is the yeast rDNA *HOT1* sequence, which is an RNA polymerase I transcriptional enhancer that stimulates ectopic recombination in a transcription-dependent manner (202) via DSB intermediates (203).

Transcription-associated recombination (TAR) is mediated by replication. In budding yeast, transcription driven by an S-phase promoter, but not by a G1-specific promoter, induces recombination between DNA repeats (161). In that study, instability was clearly seen in conditions favoring head-on collisions between DNA and RNA polymerases but was less frequent in conditions favoring codirectional collisions (**Figure 5*b***). The potential for head-on collisions to cause instability was initially inferred from the orientation of highly expressed *E. coli* genes, which matches the direction replication fork progression along the circular *E. coli* chromosome, and this inference has been confirmed experimentally (63). Inversion of the *E. coli rrn* operon from a codirectional to a head-on orientation makes cell viability strictly dependent on DNA repair by RecBC (49). However, a genome-wide analysis of RNA polymerase II occupancy in yeast suggests that transcription may be an obstacle to the progression of replication forks regardless of orientation (12). It is possible that a fork traveling in the same direction as an elongating RNA polymerase may not stall that strongly or that the transient RNA-DNA hybrid formed as an intermediate of RNA synthesis may serve to reinitiate codirectional but not head-on replication (158, 159).

Evidence for the replication dependence of TAR in mammals has been provided in Chinese hamster cells, in which replication

R-loop: three-strand nucleic acid structure formed by an RNA-DNA hybrid and the displaced ssDNA of the original DNA duplex

stress generated by thymidine, which slows down replication fork progression, enhances TAR at the *HPRT* gene (70). Furthermore, it has been recently reported that cyclin E overexpression induces TAR and that chemical inhibition of RNA synthesis reverses part of the replication stress generated by cyclin E (91).

Replication fork pausing and stalling has been seen in head-on collisions between RNA and DNA polymerases in phages, *E. coli*, and yeast (61, 115, 136, 161). Specific factors function to prevent or resolve fork stalling, as was originally observed with the T4-phage dda helicase in vitro (18). Other relevant factors include the *E. coli* helicases DinG, Rep, and UvrD (24), the stringent response regulators ppGpp and DksA, the GreA and Mfd proteins (190, 196), *S. cerevisiae* Rrm3 (11, 12, 161) and *S. pombe* Pif1 helicases (169), and the human RecQL5 helicase (110). Mutations in all of these factors increase pausing of a replication fork transiting a transcribed region and/or enhance instability in a transcription-dependent manner (**Figure 5*b***). The difference of these results from those identifying γ-H2AX clusters in eukaryotes at repressed RNA polymerase II genes (186) might indicate that breaks or ssDNA gaps originate differently or at different times in the cell cycle.

Although elongating or paused/stalled RNA polymerases can constitute physical barriers that block the replication fork progression, increased positive superhelical density generated during head-on collisions between the converging replication fork and RNA polymerase II may also contribute to fork stalling and collapse. Consistent with this, genome-wide ChIP-chip analysis in budding yeast has revealed that Top2 accumulates at regions in which replication forks encounter highly transcribed genes (20) (**Figure 2*b***).

Transient formation of ssDNA facilitated by negative supercoiling and chromatin changes associated with transcription may also make transcribed DNA more accessible to genotoxic agents (2). Consistent with this, genotoxic agents, such as methyl-methanesulfonate or 4-nitroquinoline-1-oxide, synergistically enhance recombination with transcription in yeast (66). Therefore, even though replication stress may still be the ultimate cause of transcription-mediated genome instability when replication forks encounter externally induced damage, transcription may enhance fragility by facilitating exposure of ssDNA regions to genotoxic agents.

Recent observations suggest that the impact of transcription on genome integrity may also be related to nuclear architecture and genome localization. Highly transcribed yeast genes seem to migrate to the nuclear periphery, where they are anchored to the nuclear pore to facilitate RNA export (**Figure 5*c***). It has been proposed that under replication stress, activation of the Rad53-dependent DNA damage checkpoint would release the putatively stalled or collapsed fork from the nuclear pore. This, in turn, would allow free DNA rotation, resolution of topological constraints, and replication fork restart, as has been observed in a study of THO and THSC/TREX-2 mRNP biogenesis and export mutants in yeast (21). This study suggests that nuclear positioning may be an important contributor in modulating genome instability. Evidence for a related phenomenon has also been provided in studies of the rDNA region in yeast (see sidebar, The Yeast rDNA as a Paradigm of Genome Instability).

Cotranscriptional R-Loops

Studies of yeast mutants affecting the conserved THO complex, which is involved in mRNP biogenesis and export, have indicated that cotranscriptional R-loops are an important mediator of transcription-associated instability (85). In these mutants, a putatively suboptimal mRNP assembly facilitates R-loop formation by hybridization between the DNA template and the nascent RNA exiting the elongating RNA polymerase (**Figure 5*d***). Additional evidence has accumulated in yeast and mammals, revealing that a number of mRNA processing defects increase different forms of instability, as determined by γ-H2AX foci,

hyper-recombination, or chromosome loss, in an R-loop-dependent manner (2, 111, 154, 184, 205).

R-loops also accumulate in yeast mutants of the senataxin ortholog Sen1, a DNA-RNA helicase, causing an increase in TAR (137). Thus, R-loops can form under conditions in which mRNA processing is not affected. Correlation between replication fork pausing or stalling and R-loops has been provided in *E. coli*, *S. cerevisiae*, *Caenorhabditis elegans*, and human cells (24, 29, 52, 68, 197, 208). Interestingly, yeast Sen1 helicase accumulates at the sites of transcription-replication head-on collisions, where it prevents fork collapse and breakage, presumably by resolving an RNA-DNA hybrid (8). However, in *E. coli*, backtracking of RNA polymerase appears to be required for the formation of transcription- and R-loop-dependent breaks (58) (**Figure 5*e***). This suggests that transcriptional arrest could generate structures that compromise genome instability, as may also be the case for yeast THO/TREX mutants (85).

Transcriptional and cotranscriptional R-loops may determine some hotspots for genome instability (2), among them TNRs (72, 113) and fragile sites (79), as well as sites of programmed instability, such as class switch recombination (CSR) in vertebrate B-cells. CSR initiates in the G-rich S-regions of the *Ig* genes in a transcription-dependent manner via R-loops (212) that are stabilized by G-quadruplexes putatively forming at the displaced G-rich strand (56). Activation-induced cytidine deaminase (AID), which acts specifically on ssDNA, might deaminate cytidines at the transiently displaced ssDNA, creating dU residues that are converted into DSBs by BER and/or MMR and that are responsible for CSR occurring via NHEJ (32, 122). Importantly, AID can induce DSB-mediated translocations between the *Ig* gene S-region and *c-MYC*, and these translocations are responsible for Burkitt's lymphoma (163, 167). Consistent with this, NHEJ-mediated translocations are stimulated when AID is expressed in yeast THO mutants that form R-loops (168). Moreover, the RNA exosome, a protein complex that controls cotranscriptional RNA quality, promotes both AID action at the S-regions and CSR, suggesting that the exosome could remove the RNA from a cotranscriptional RNA-DNA hybrid and thereby expose the template strand to AID (17, 60). Cotranscriptional R-loops can be favored by *cis* features such as G-richness or G-quadruplexes (19), and under suboptimal mRNP biogenesis or processing R-loops could form extensively throughout transcribed open reading frames (68). Therefore, R-loops can in principle cause genome instability by perturbing replication fork progression and blocking DNA synthesis as well as by enhancing DNA susceptibility to damaging agents and enzymes (2).

THE YEAST rDNA REPEAT AS A PARADIGM OF GENOME INSTABILITY

The budding yeast rDNA region consists of multiple and highly expressed rDNA repeats. It constitutes a region at risk for instability, whose integrity is assured at different levels: (*a*) The Fob1-dependent replication fork barrier limits replication fork–transcription collisions (97); (*b*) it is likely that RNA polymerase I transcription compromises rDNA stability via R-loop formation because Top1 and Top2 stabilize the rDNA repeats (35) and R-loops have been shown to accumulate in *top1*Δ cells (60); (*c*) genetic evidence suggests that transcription contributes to cohesin dissociation at the rDNA, allowing unequal SCE to be a major threat to rDNA stability (98); (*d*) rDNA is stabilized by silencing that is mediated by the Sir2-containing chromatin complex, which likely impedes access to HR factors (71), even though this is not sufficient to explain the low levels of recombination events; (*e*) the nucleolus excludes HR proteins, such as Rad52 (194); and (*f*) inner nuclear membrane (INM) proteins such as Heh1 and Nur1, which are responsible for tethering the rDNA to the INM, are also required for silencing and rDNA stability (133). Therefore, the high transcription activity and multiple repeat structure of the rDNA make this region a specialized substrate for a varied battery of mechanisms aimed at controlling replication fork stalling, mitotic recombination, and nuclear positioning, which are major causes of genome instability that potentially act throughout the genome.

Class switch recombination (CSR): site-specific rearrangement occurring between two S-regions of immunoglobulin genes via NHEJ in B-lymphocytes

ANAPHASE BRIDGES AND CHROMOSOME BREAKAGE AT M-PHASE

Anaphase bridges: DNA "strings" connecting two nuclei during chromosome segregation and representing nonproperly resolved recombination or replication intermediates or dicentric chromosomes

Breakage-fusion-bridge: chromosome rearrangement mechanism that involves the fusion and breakage of chromosomes following the loss of a telomere

DSBs, formed as a consequence of replication stress, can occur as late as M-phase. The observation that chromosomes trapped in the cell cleavage furrow during cytokinesis triggers the DSB ATM-CHK2 checkpoint pathway and that these DSBs lead to chromosomal aberrations in mammalian cells supports this view (89).

A particularly relevant structure that might be linked to M-phase breaks is the anaphase bridge, which may represent a step in the breakage-fusion-bridge cycle leading to GCRs (**Figures 3*k*** and **4**). Dicentric chromosomes formed by telomere fusion in humans have been shown to form anaphase bridges (200). Ultrafine anaphase bridges have been defined as types of anaphase bridges that may represent ssDNA fibers produced by sister-chromatid separation failure. Human BLM-Top3-Rmi1,2 complex associates with ultrafine anaphase bridges, which are flanked by FANCD2 and FANCI spots, regardless of whether chromosomes are broken (31, 147), suggesting a role in sister-chromatid disjunction. Interestingly, yeast BLM/Sgs1 and its SUMO-ligases Ubc9 and Mms21 are required to resolve DNA hemicatenates that result from Rad51-dependent sister-chromatid X-shaped junctions formed in the wake of the replication fork (25, 112) (**Figure 5*f***). Failure to resolve sister junctions may contribute to mitotic chromosome breakage and missegregation observed in BLM and Fanconi anemia patient cell lines. Anaphase bridges have also been detected under conditions of replication stress in fission yeast and human cells containing nonprogrammed replication fork barriers (88, 180). In fission yeast *pfh1* mutants, in which DNA seems to remain partially unreplicated without being detected by the checkpoint machinery, X-shaped structures, anaphase bridges, and associated chromosome fragmentation and missegregation can also be observed (183).

Anaphase bridges have been extensively studied in mammals, and it is likely that anaphase bridges represent intermediates of DSB-mediated genome instability arising as a consequence of replication failures. Although they have not been extensively studied in yeast, anaphase bridges may cause some of the genome instability seen in yeast. Examples include *sic1*Δ mutants (107), strains accumulating inverted-repeat chromosome duplications (138, 151), and possibly the incomplete replication termination observed in *top2* mutants. Top2 is necessary for chromosome segregation in mitosis and for preventing DSBs during cell division (83). As forks approach replication-termination sites, Top2 becomes critical to guarantee resolution of the supercoils accumulated between the two converging forks; in addition, *top2* mutants accumulate DSBs, as inferred from γ-H2A foci (62) (**Figure 2*b***). It would be interesting to see whether anaphase bridges may form in these situations. At this point, we do not know whether specific endonucleases cleave anaphase bridges. However, the observation that the activity of the structure-selective endonuclease GEN1/Yen1 is restrained until mitosis (128) opens the possibility that this enzyme could resolve anaphase bridges.

CELL PHYSIOLOGY AND METABOLISM IN GENOME INSTABILITY

Cell Physiology and Metabolism

A number of physiological conditions and metabolic defects can trigger genome instability. One example is oxygen metabolism. Budding yeast mutants that lack the Tsa1 peroxiredoxin, a thiol-specific peroxidase whose main role is to sense and scavenge reactive oxygen species, display GCRs and inviability in conjunction with mutations in DSB, and postreplicative repair genes, such as *MRE11*, *RAD51*, and *RAD6*, likely due to the over-accumulation of breaks and DNA lesions that block replication. Consistent with reactive oxygen species being responsible for these phenotypes, they are suppressed during anaerobic growth (162). Also, a Hog1-kinase-dependent

signal-transduction mechanism prevents instability under conditions of osmotic stress in yeast, and this may represent an additional example by which cell physiology can influence genome integrity (54). In mammals, the Fanconi anemia pathway specifically repairs acetaldehyde-mediated DNA damage, and the Aldh2 aldehyde-catabolizing enzyme is essential for embryonic development of *Fancd2−/−* mice (103).

The observation that the loss of mitochondrial DNA leads to a cellular crisis mediated by cell-cycle arrest that causes LOH in yeast has uncovered a novel dependence of genome stability on iron-sulfur cluster proteins. Genome instability is linked to downregulation of nonmitochondrial iron-sulfur cluster biogenesis that presumably reduces the level or activity of iron-sulfur cluster-containing proteins. Importantly, the Mms19 protein, previously identified as having a role in DNA repair, is a member of the cytosolic iron-sulfur cluster protein-assembly factor, which physically interacts with DNA replication and repair proteins, such as FANCJ, XPD, Pol∂, and DNA2 (67, 182). Thus, genome integrity might be indirectly compromised by defects in the metabolism and/or synthesis of replication and repair factors. It is possible that after more genome-wide searches (for examples, see References 7, 154) and as our knowledge of the mechanisms of genome instability increases, more cytosolic and metabolic functions will be identified as part of the global puzzle of cellular functions important for genome integrity.

Aging and Cancer

Aging, like cancer, may well combine many of the physiological and metabolic features triggering genome instability. Thus, it has been shown in budding yeast that as mother cells age, they display an increase of up to 100-fold in the frequency of LOH (132). As aging is a complex process, involving not only DNA damage accumulation but also damage of proteins, it is possible that aging-associated instability has multiple causes (116). Age-induced LOH is not the result of chromosome loss due to nondisjunction but is caused by mitotic recombination, likely generated by DNA damage, suggesting that aged cells have faulty replication (132). The observation that aging is accompanied by a progressive decline in rDNA stability supports this view (114). Strong evidence for a link between aging and replicative stress has been provided by ATR-deficient mice (143). Retrotransposition may be another contributor to chronological aging because yeast mutations that reduce Ty retrotransposition also reduce aging-associated LOH (130).

Tumorigenesis represents a specific cell condition with an intimate link to genome instability. It has been shown that early tumor cells show constitutive activation of the ATM-CHK2-p53 checkpoint pathway and that expression of proto-oncogenes, such as cyclin E and Cdc25, generates permanent replicative stress that may be the common basis of genome instability and tumorigenesis (15, 69). Therefore, dysfunction of proteins that directly or indirectly affect replication, in turn causing replication stress, may promote or stimulate tumorigenesis. Understanding the connection between tumorigenesis and replication stress and instability is important not only to understand the molecular basis of cancer but to evaluate the potential of using replication stress and checkpoint dysfunction to define specific targets in cancer therapy.

Chromothripsis

The extremely high levels of instability observed in a subpopulation of aggressive human cancers, termed chromothripsis, represent a novel form of instability, the cause of which is still uncertain, but which is likely linked to a particular type of cell stage or differentiation. Chromothripsis refers to a catastrophic event that might occur more frequently in cancer and aged cells and in which chromosomes undergo multiple chromosome fragmentation and rejoining, mainly by NHEJ, which leads to multiple GCRs (96, 117). Different mechanisms

have been proposed, including chromosome pulverization caused by replication and repair defects occurring in micronuclei resulting from errors in mitosis (42). However, it is too early to know the causes of this phenomenon. Understanding the molecular basis of chromothripsis will certainly shed light on our knowledge of genome instability and its consequences.

SUMMARY POINTS

1. Replicative stress generated by either faulty replication or checkpoint dysfunction is the major source of genome instability. This results in GCRs, chromosome fusions, and chromosome fragmentation and loss.
2. The S-phase checkpoint works to maintain replication fork integrity, avoiding breakage that is potentially responsible for genome instability. However, replicative stress can produce a persistent fork stalling. This may either uncouple replication fork progression and DNA synthesis, leading to long stretches of ssDNA, or evolve into a DSB or fork regression than can compromise genome integrity.
3. DSBs resulting from replication fork stalling and collapse are likely to occur during S- and G2-phases, but they may also occur during M-phase in association with anaphase bridges, which in turn are caused by unfinished replication, hemicatenates formed behind the fork, sister-chromatid nondisjunction, or dicentric chromosomes.
4. The type of genome instability and GCR depends on the initial causative event (an ssDNA gap or a DSB), the timing of its occurrence during the cell cycle (G1-, S-, G2-, or M-phase), and the mechanism involved in its repair (using or not using DNA homology).
5. Many breaks and genome instability events occur at specific hotspots that correspond to regions difficult to replicate or to low-replication fork density regions. These hotspots can contain non-B DNA structures, DNA protein barriers, highly transcribed regions, low-replication density zones, and DNA repeats.
6. Genome instability and replicative stress are common features of cancer and precancerous cells and aging. Identifying the causes of genome instability is key to understand tumorigenesis and aging as well as for the application of successful and risk-free stem cell–based therapies.

FUTURE ISSUES

1. Are the different types of genome instability events generated via ssDNA gaps or DSBs? Do DSBs occur during G1-phase, S-phase, late G2-phase, or anaphase?
2. How is a stalled replication fork processed? Is there a collapse of the replication machinery involving replisome disassembly associated with fork stalling? Is the breakage of a stalled fork enzymatically mediated, as, for example, via the Mus81 endonuclease?
3. What are the molecular structures of anaphase bridges? Are they resolved by nucleases? How and when is the break occurring in anaphase processed?

4. What is the role of chromatin structure and remodeling in preventing genome instability? Is it by regulating nucleosome assembly during replication, or does it also have a more direct role in the choice and efficiency of DNA repair and replication restart after replication fork stalling?
5. What are the *cis* elements responsible for chromosome fragility, and how do they potentiate fragility in each case? Is it just by delaying replication fork progression and/or impeding replication termination, or do they have additional roles as targets of specific nucleases or in defining the mechanisms of fork restart or the type of DSB repair mechanism used? Do noncoding RNAs, chromatin structure, and nuclear position play a role in chromosome fragility?
6. What are the mechanisms and physiological relevance of mitotic catastrophes such as chromothripsis? Do they follow the same expression pattern and mechanism as genome instability events that occur under replicative stress?

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