DNA Replication Stress as a Hallmark of Cancer

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Abstract

Human cancers share properties referred to as hallmarks, among which sustained proliferation, escape from apoptosis, and genomic instability are the most pervasive. The sustained proliferation hallmark can be explained by mutations in oncogenes and tumor suppressors that regulate cell growth, whereas the escape from apoptosis hallmark can be explained by mutations in the TP53, ATM, or MDM2 genes. A model to explain the presence of the three hallmarks listed above, as well as the patterns of genomic instability observed in human cancers, proposes that the genes driving cell proliferation induce DNA replication stress, which, in turn, generates genomic instability and selects for escape from apoptosis. Here, we review the data that support this model, as well as the mechanisms by which oncogenes induce replication stress. Further, we argue that DNA replication stress should be considered as a hallmark of cancer because it likely drives cancer development and is very prevalent.
INTRODUCTION

Cancer is defined as an abnormal, uncontrolled growth of tissue. In terms of human health, cancer accounts for about one quarter of all deaths in developed countries and, therefore, is a major health issue (1). While there are significant differences between different types of cancers, there are also properties shared by practically all of them. These properties, referred to as hallmarks of cancer, include sustained proliferative signaling, evasion of growth suppressors, escape from apoptosis, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, evasion of immune surveillance, deregulated cellular energetics, genomic instability, and tumor-promoting inflammation (2–4). The number of cancer hallmarks has almost doubled since the term was originally introduced in 2000 by Hanahan & Weinberg (2), in part, reflecting our increased knowledge of cancer.

It is evident that certain hallmarks will be more central to cancer development than others. Identifying these hallmarks and their molecular basis could allow us to formulate a more general model for cancer development. Obviously, the molecular changes responsible for the indispensable hallmarks should be present in the vast majority of cancers. In the past few years, the genomes of thousands of cancers have been analyzed in great detail (5–11). Thus, it is now possible to better link molecular changes to specific hallmarks and also to gauge the importance of specific hallmarks on the basis of the frequency of the underlying molecular changes.

Here, we review recent findings in the field, arguing that DNA replication stress is a feature present in most cancers and, therefore, worthy to be included among the classical hallmarks. Thus, an emerging view of cancer progression proposes that oncogenes inducing sustained proliferation also induce DNA replication stress, and that two other hallmarks, escape from apoptosis and genomic instability, are a consequence of replication stress.

CANCER-DRIVER GENES

Seminal studies in the late 1970s and early 1980s have demonstrated the existence of cellular genes, termed oncogenes, that drive cancer development (12–14). A large number of oncogenes have been identified on the basis of two main criteria: being mutated in human cancers and being able to transform cells when mutated or overexpressed.

Oncogenes constitute one class of cancer-driver genes. A second class of cancer-drivers constitutes genes whose wild-type form suppresses cancer development (14). These genes, referred to as tumor suppressors, are inactivated by point mutations or deletions in human cancers. Most tumor suppressor genes are direct antagonists of oncogenes. For example, the protein product of CDKN2A interacts directly with and inhibits the protein product of the CDK4 oncogene (15–18).

Thus, specific pathways are important in cancer development, and genes that function in these pathways can be either oncogenes or tumor suppressors, depending on their precise function in the pathway.

There are multiple mechanisms by which oncogenes and tumor suppressor genes can be activated and inactivated, respectively. Point mutations (single nucleotide substitutions, SNSs) and somatic copy number alterations (SCNAs) represent two very common mechanisms (5–11). The genomes of thousands of human cancers have been analyzed, providing frequencies at which cancer-driver genes are targeted by each mechanism. In regard to SNSs, results from sequencing analysis of 3,281 tumors representing 12 common cancer types show that the TP53 (p53) tumor suppressor gene is targeted by SNSs in 42.0% of all cancers (8). PIK3CA, which encodes the catalytic subunit of PI3K, is a distant second with SNSs in 17.8% of all cancers, followed by PTEN, APC, VHL, and KRAS, which are mutated in 9.7, 7.3, 6.9, and 6.7% of all cancers,
The top 20 genes most frequently targeted by SNSs across 12 human cancer types, representing 3,281 cancers according to Reference 8. The bars are colored green for genes stimulating sustained cell proliferation (oncogenes), orange for genes inhibiting sustained cell proliferation (tumor suppressors), and red for genes that function in the DNA damage response pathway (a second class of tumor suppressors). The bar corresponding to NOTCH1 is colored blue because this gene functions as an oncogene or tumor suppressor depending on the cell type. For each gene, the percentage of cancers with SNSs targeting the specific gene is indicated. SNS: single nucleotide substitution.

respectively (Figure 1). Among the 20 cancer-driver genes that are most frequently targeted by SNSs in humans, three are oncogenes and 17 are tumor suppressors.

In contrast to SNSs, which target a single gene, SCNAs typically involve several genes. Analysis of thousands of SCNAs in 4,934 tumors representing the same 12 cancer types described above has led to the identification of peaks of amplified and deleted genomic regions (11). Among the ten most significantly amplified genomic regions, nine contain established oncogenes (Table 1). CCND1, which encodes cyclin D1, is the most significantly amplified gene, followed by EGFR, MYC, TERC, ERBB2, and CCNE1.

Unlike the genomic amplifications, not all genomic deletions target cancer-driver genes. In fact, only four of the ten most significantly deleted genomic regions encompass established tumor suppressor genes, which in order of statistical significance are CDKN2A/CDKN2B, STK11, ARID1A, and PTEN. The remaining six regions target very large genes (Table 2). These genes are large because they contain very large introns, whereas their mRNA is not unusually long. The tendency of deletions to target very large genes is not limited to the ten most significantly deleted regions. Among the top 70 recurrently deleted genomic regions, 22 target one of the 100 largest genes in the genome (11).

It has long been debated whether the very large genes, which are frequently targeted by deletions in cancer, are bona fide tumor suppressors (19–23). In various experimental models, including knockout mice, inactivation of these genes does not, in general, result in dramatic phenotypes as far as tumor development is concerned, unlike what is seen when bona fide tumor suppressors are inactivated. Further, whereas the deletions targeting well-established tumor suppressor genes are typically homozygous, the deletions targeting very large genes are typically hemizygous and, thus, a wild-type copy of the gene is retained (9). Another distinction relates to the frequency with
Table 1  Top ten recurrently amplified genomic regions in human cancers

<table>
<thead>
<tr>
<th>Amplification boundaries</th>
<th>Gene</th>
<th>Cancer-driver</th>
<th>Gene boundaries</th>
<th>Gene size (bp)</th>
<th>mRNA size (bp)</th>
<th>Fragile site</th>
<th>SNS frequency (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr1:11:69,464,719–69,502,928</td>
<td>CCND1</td>
<td>Onc</td>
<td>chr1:11:69,455,873–69,469,242</td>
<td>13,370</td>
<td>4,289</td>
<td>-</td>
<td>6.6e-08</td>
</tr>
<tr>
<td>chr7:55,075,808–55,093,954</td>
<td>EGFR</td>
<td>Onc</td>
<td>chr7:55,086,678–55,279,262</td>
<td>192,585</td>
<td>5,600</td>
<td>-</td>
<td>2.2e-15</td>
</tr>
<tr>
<td>chr8:128,739,772–128,762,863</td>
<td>MYC</td>
<td>Onc</td>
<td>chr8:128,748,315–128,753,680</td>
<td>5,366</td>
<td>2,366</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chr17:37,848,534–37,877,201</td>
<td>ERBB2</td>
<td>Onc</td>
<td>chr17:37,844,167–37,884,915</td>
<td>40,749</td>
<td>4,624</td>
<td>-</td>
<td>1.3e-06</td>
</tr>
<tr>
<td>chr19:30,306,758–30,316,875</td>
<td>CCNE1</td>
<td>Onc</td>
<td>chr19:30,302,805–30,315,224</td>
<td>12,420</td>
<td>1,947</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chr1:150,496,857–150,678,056</td>
<td>MCL1</td>
<td>Onc</td>
<td>chr1:150,547,027–150,552,214</td>
<td>5,188</td>
<td>4,085</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chr12:69,183,279–69,260,755</td>
<td>MDM2</td>
<td>Onc</td>
<td>chr12:69,201,952–69,239,324</td>
<td>37,373</td>
<td>7,495</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chr11:77,610,143–77,641,464</td>
<td>INTS4</td>
<td>-</td>
<td>chr11:77,589,766–77,705,771</td>
<td>116,006</td>
<td>3,149</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chr8:38,191,804–38,260,814</td>
<td>WHSC1L1</td>
<td>Onc</td>
<td>chr8:38,127,217–38,239,872</td>
<td>112,656</td>
<td>5,431</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table of the top ten recurrently amplified genomic regions in 4,934 tumors from 12 cancer-types and their associated gene according to Reference 11. The p-values for SNS frequency indicate if a gene is recurrently targeted by single nucleotide substitutions (SNSs) in human cancers. Onc, oncogene.

which well-established tumor suppressor genes and very large genes are targeted by SNSs. The frequencies of SNSs within the former genes in human cancers are several orders of magnitude higher than those within the very large genes (Table 2). As described below, very large genes map to common fragile sites (CFSs) and, therefore, the deletions targeting these genes may be explained on the basis of DNA replication stress (19, 24, 25).

MOLECULAR FUNCTIONS OF CANCER-DRIVER GENES

It is a reasonable assumption that mutations (SNSs, deletions, amplifications, translocations, etc.) in cancer-driver genes underlie the hallmarks of cancer. For most genes, it is possible to make a link with a specific hallmark; therefore, the frequencies by which genes are targeted in cancer can provide an indication of the importance of the corresponding hallmark. Along these lines, we matched the genes most frequently targeted by SNSs (20 genes), genomic amplifications (10 genes), and genomic deletions (10 genes) to a recently described list of cancer hallmarks (3, 8, 11).

It is evident that many genes frequently mutated in human cancers are linked to the two hallmarks relating to cell growth: sustained proliferative signaling and evasion of growth suppressors. We have argued in the past that these two hallmarks are in fact two sides of the same coin (26). For example, RB1, which encodes for pRb, has been assigned to the evasion of growth suppressors hallmark, whereas CCND1, which encodes for cyclin D1, would probably be assigned to the sustained proliferative signaling hallmark (3). Yet, both these genes function in the same molecular pathway as antagonists of each other (15–18). Accordingly, the hallmarks sustained proliferative signaling...
Table 2  Top ten recurrently deleted genomic regions in human cancers

<table>
<thead>
<tr>
<th>Rank</th>
<th>Deletion boundaries</th>
<th>Gene</th>
<th>Cancer-driver</th>
<th>Gene boundaries</th>
<th>Gene size (bp)</th>
<th>mRNA size (bp)</th>
<th>Fragile site</th>
<th>SNS frequency (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>chr9:21,865,498–22,448,737</td>
<td>CDKN2A</td>
<td>TS</td>
<td>chr9:21,967,751–21,994,490</td>
<td>26,740</td>
<td>1,251</td>
<td>-</td>
<td>4.4e-15</td>
</tr>
<tr>
<td>2</td>
<td>chr19:1,103,715–1,272,039</td>
<td>STK11</td>
<td>TS</td>
<td>chr19:1,205,798–1,228,434</td>
<td>22,637</td>
<td>3,276</td>
<td>-</td>
<td>2.5e-13</td>
</tr>
<tr>
<td>3</td>
<td>chr5:58,260,298–59,787,985</td>
<td>PDE4D</td>
<td>-</td>
<td>chr5:58,265,866–59,783,925</td>
<td>1,519,061</td>
<td>8,240</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>chr6:161,693,099–163,153,207</td>
<td>PARK2</td>
<td>-</td>
<td>chr6:161,768,590–163,148,834</td>
<td>1,380,245</td>
<td>4,073</td>
<td>FRA6E</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>chr2:139,655,617–143,637,838</td>
<td>LRP1B</td>
<td>-</td>
<td>chr2:140,988,996–142,889,270</td>
<td>1,900,275</td>
<td>16,531</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>chr8:2,079,140–6,262,191</td>
<td>CSMD1</td>
<td>-</td>
<td>chr8:2,792,875–4,852,328</td>
<td>2,059,454</td>
<td>14,340</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>chr1:7,829,287–8,925,111</td>
<td>CAMTA1</td>
<td>-</td>
<td>chr1:6,845,384–7,829,766</td>
<td>984,383</td>
<td>8,444</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>RERE</td>
<td>-</td>
<td>chr1:8,412,464–8,877,699</td>
<td>465,236</td>
<td>8,194</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>chr1:26,900,639–27,155,421</td>
<td>ARID1A</td>
<td>TS</td>
<td>chr1:27,022,522–27,108,601</td>
<td>86,080</td>
<td>8,585</td>
<td>-</td>
<td>1.5e-14</td>
</tr>
<tr>
<td>9</td>
<td>chr10:89,615,138–90,034,038</td>
<td>PTEN</td>
<td>TS</td>
<td>chr10:89,623,195–89,728,532</td>
<td>105,338</td>
<td>5,547</td>
<td>-</td>
<td>2.2e-15</td>
</tr>
<tr>
<td>10</td>
<td>chr16:7,812,958–79,627,770</td>
<td>WWOX</td>
<td>DERATED</td>
<td>chr16:7,813,310–79,246,567</td>
<td>1,113,258</td>
<td>2,505</td>
<td>FRA16D</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Table of the top ten recurrently deleted genomic regions in 4,934 tumors from 12 cancer types and their associated gene according to Reference 11. The p-values for SNS frequency indicate if a gene is recurrently targeted by single nucleotide substitutions (SNSs) in human cancers. TS, tumor suppressor.

and evasion of growth suppressors may be considered as a single sustained proliferation hallmark, to which more than half of the top 20 genes mutated in human cancer are linked (Figure 2).

The replicative immortality hallmark is also linked to genes frequently mutated in cancer. The TERC gene, from which the RNA component of the telomerase complex is transcribed, is within the top ten genes amplified in human cancer (Table 1), whereas the TERT gene, which encodes the reverse transcriptase subunit of telomerase, is within the top 20 amplified genes (11, 27, 28).

Another hallmark linked to frequently mutated genes is that of escape from apoptosis (Figure 2). We assign the TP53 gene, the most frequently mutated gene in human cancer (Figure 1), to this hallmark because TP53 induces apoptosis in response to DNA damage. However, TP53 can also induce senescence in response to DNA damage, and therefore cells with mutant TP53 will escape not only apoptosis but also senescence. Accordingly, we prefer to use the term escape from apoptosis/senescence for this hallmark. Other frequently mutated genes in cancer that can be linked to the escape from apoptosis/senescence hallmark are ATM, MDM2, and MCL1. ATM functions upstream of TP53 in the DNA damage response pathway (29–31). The MDM2 gene, which encodes a ubiquitin ligase that targets p53 for degradation (32), is among the ten most significantly amplified genes, as is the MCL1 gene, whose protein product inhibits the proapoptotic protein Bax (33).

Links between the genomic instability hallmark and genes frequently mutated in cancer have been a matter of controversy. It appears that almost all cancers have genomic instability...
Figure 2

Linkage of cancer hallmarks to the most frequent genetic mutations identified in human cancers (top 20 genes targeted by SNSs, top 10 genes focally amplified, and top 10 genes focally deleted) (3, 8, 11). The genes under the heading “Chromatin modifiers” could not be readily linked to a specific hallmark. Oncogenes are indicated by green letters, tumor suppressors by red letters, and NOTCH1 by blue letters. Note that only 4 of the top 10 focally deleted genomic regions contain cancer-driver genes and that only cancer-driver genes are shown in this figure. As shown in Table 2, the remaining 6 focally deleted genomic regions contain very large genes that are subject to deletions due to DNA replication stress. SNS, single nucleotide substitution; Ampl, amplification; Del, deletion.

(5, 26, 34–36), defined as a higher rate of acquisition of genomic changes per cell division compared to normal cells. In principle, mutations in DNA repair genes could have explained the genomic instability hallmark, but such mutations are not common in sporadic human cancers (26). Mutations in DNA damage checkpoint genes are also unlikely to explain the almost universal presence of genomic instability in cancer. Inactivation of TP53 does not lead to genomic instability under physiological conditions (37, 38). Mutations in ATM are frequent (compared to other genes) and can lead to genomic instability (39), but in absolute terms the frequency of ATM mutations is too low to explain why almost all cancers have genomic instability. As discussed below, the genes driving sustained proliferation may be the ones responsible for the genomic instability hallmark.

Among the remaining hallmarks, induction of angiogenesis can be linked to mutations in the VHL gene (40), and invasion and metastasis can be linked to mutations in NAV3, a gene regulating cell motility (41). However, the tumor promoting inflammation, avoiding immune destruction, and deregulated cellular energetics hallmarks do not appear to be directly linked to any of the genes that are among those most frequently mutated in human cancer (Figure 2).

Interestingly, there are several genes frequently mutated in human cancers that cannot be directly linked to a specific hallmark (Figure 2). These include MLL3, MLL2, ARID1A, PBRM1,
SET2, GATA3, and WHSC1L1, all of which have the capacity to modify chromatin structure. How mutations in these genes promote cancer development is not clear. Gene expression profiles, cell lineage specificity, and/or cell differentiation state might be affected (42). In turn, these changes could impact cell proliferation, induce metabolic changes, and/or promote invasion and metastasis.

ORDER OF ACQUISITION OF CANCER-DRIVER MUTATIONS AND CANCER HALLMARKS

Since the early 1990s, it has become clear that there is some order by which cancer-driver genes are mutated (43–46). Colon cancer is an ideal system to document this order because lesions representing various stages of cancer development, ranging from hyperplasia to metastatic cancer, can be isolated. The first event in the majority of colon cancers appears to be mutations that inactivate the APC gene, a negative regulator of Wnt signaling (43–47). Inactivation of both copies of the APC gene appears to be sufficient for development of adenomas with mild dysplasia (48). The APC mutations are followed by mutations in other growth controlling genes, notably KRAS, NRAS, GNAS, and AKT1, leading to the development of adenomas with moderate or severe dysplasia. Subsequently, inactivation of the TP53 tumor suppressor gene and mutations in other oncogenes, notably in PIK3CA, are associated with progression from adenoma to carcinoma (43–46, 48).

The bladder is another organ in which lesions representing various stages of cancer development can be isolated and studied. Analysis of early noninvasive lesions (Ta) and later mucosa- or muscle-invasive lesions (T1 or T2) reveal the presence of mutations in growth controlling genes, such as FGFR3 and HRAS, at early stages of cancer development followed by mutations in TP53 at later stages (49). Similar studies in other tumor types suggest that, generally, mutations in oncogenes precede TP53 mutations (50).

Extending these observations from gene mutations to cancer hallmarks, one may conclude that cancer hallmarks are acquired in a specific order. Thus, during cancer development, sustained proliferation would precede evasion from apoptosis/senescence because mutations in genes implicated in growth control precede mutations in TP53.

TP53 AS A BARRIER TO ONCOGENE-INDUCED DNA DAMAGE

An important question is why mutations in cancer-driver genes are acquired in a specific temporal order. The p53 tumor suppressor protein has a well-established function in the DNA damage response (29, 30). In fact, the primordial function of the p53 family might have been to induce apoptosis in germ cells following DNA damage (51–53). In somatic cells, p53 protein levels increase in response to DNA damage, leading to activation of target genes that induce cell cycle arrest, senescence, or apoptosis (54). The precise outcome depends on the cell type, the extent and type of DNA damage, and other less-well-defined parameters. Activation of p53 in response to DNA double-strand breaks (DSBs) is dependent on the checkpoint kinase ATM, itself encoded by a gene that is frequently inactivated in human cancer. In response to DNA damage, ATM phosphorylates p53, leading to its dissociation from the ubiquitin ligase Mdm2 and, consequently, to increased p53 protein levels (39). Similar to the TP53 and ATM genes, the MDM2 gene is frequently targeted in human cancers (11). Specifically, MDM2 is amplified, resulting in constitutive p53 degradation and a DNA damage checkpoint defect.

Taking into account that the ATM/TP53/MDM2 pathway responds to DNA DSBs, we and others proposed that DSBs are present in precancerous and cancerous cells (55–59). Indeed, colon adenomas, head and neck squamous cell hyperplasias and dysplasias, and noninvasive Ta bladder lesions, all of which are early precancerous lesions, have signs of a DNA damage response (DDR).
The presence of DNA damage in these lesions is not simply a feature of DNA replication. Normal, nontransformed cells, such as those present in the basal layer of the epidermis and in colon crypts, replicate as fast as or even faster than precancerous cells, yet do not exhibit a DDR (57, 58).

High-throughput sequencing analysis of colon adenomas and bladder Ta precancerous lesions reveals mutations almost exclusively in genes related to the sustained proliferation hallmark (48, 49). In turn, this implies that DNA damage is induced, either directly or indirectly, in response to deregulation of the genes that control cell proliferation. Supporting this argument, activation or overexpression of oncogenes in tissue culture cells leads to DNA damage within one cell cycle (57, 60–63). Further, in experimental models of human foreskin hyperplasias induced by ectopically expressing growth factors, a DDR is observed within three weeks of exposure to growth factors (58).

Thus, the model emerging so far suggests that the escape from apoptosis/senescence hallmark is a response to DNA damage induced by the genes linked to the sustained proliferation hallmark. Specifically, mutations targeting genes such as \textit{PIK3CA}, \textit{PTEN}, \textit{APC}, \textit{KRAS}, \textit{CDKN2A}, \textit{CCND1}, \textit{EGFR}, and \textit{MYC} that drive cell proliferation would also induce a DDR, leading to activation of the \textit{ATM}/\textit{TP53}/\textit{MDM2} pathway and p53-dependent induction of apoptosis or senescence. According to this model, the \textit{ATM}/\textit{TP53}/\textit{MDM2} pathway serves as a barrier to cancer progression, which needs to be overcome, as happens, for example, by SNSs targeting \textit{TP53} (55–58, 63, 64). Indeed, in human precancerous lesions, the presence of a DDR precedes the acquisition of \textit{TP53} mutations, arguing that DNA damage provides the selection pressure for \textit{TP53} inactivation. Further, precancerous lesions that retain functional p53 exhibit high levels of apoptosis or senescence, whereas lesions in which \textit{TP53} has been inactivated are mostly devoid of apoptotic or senescent cells, even though a DDR is still present (58).

The oncogene-induced DDR is observed whenever cells acquire the sustained proliferation hallmark, and it does not necessarily require the presence of activated oncogenes. For example, triple-knockout mouse fibroblasts, in which the genes encoding pRb, p107, and p130 are inactivated, do not require mitogens to proceed through the cell cycle, yet exhibit a robust DDR (65). Similarly, inactivation of the \textit{APC} tumor suppressor gene is sufficient to induce a DDR (66, 67). Thus, an oncogene-induced DDR can be observed either when oncogenes are activated or when tumor suppressors that regulate cell proliferation are inactivated. Due to lack of a better term, when we refer to oncogene-induced DNA damage, we also include the DNA damage induced when tumor suppressors that regulate cell proliferation are inactivated.

**DNA REPLICATION STRESS UNDERLIES ONCOGENE-INDUCED DNA DAMAGE**

Several observations suggest that oncogene-induced DNA damage is secondary to DNA replication stress. DNA replication stress is a term that broadly defines impediments in DNA replication and generally includes stalling and collapse of DNA replication forks (68, 69). During stalling, DNA replication is stopped, but the overall structure of the replisome stays intact; once the problem responsible for stalling is resolved, DNA replication can ensue. Depletion of nucleotides is one way to induce fork stalling. Fork collapse is characterized by a disruption of replisome integrity. Some or all of the proteins involved in replication may dissociate from the DNA template, and even the template itself may be processed to generate aberrant DNA structures. Although stalled and collapsed forks are clearly distinct entities, forks that have stalled for more than a few hours tend to collapse (70).

The ability of oncogenes to induce DNA replication stress has been well documented in tissue culture systems (62, 63). First, oncogene overexpression induces activation of the DNA replication stress checkpoint, as manifested by activation of ATR, a checkpoint kinase that is similar to ATM,
but which is activated specifically in response to replication stress. ATR phosphorylates several substrates, including the checkpoint kinase Chk1 and the single-stranded DNA binding protein RPA to regulate cell cycle progression and the stability of replication forks under stress (71). Overexpression of oncogenes in nontransformed cells also induces the emergence of nuclear foci containing single-stranded DNA. These foci are present exclusively in cells that are in S phase and are thought to arise when the activities of the DNA polymerase and the DNA replicative helicase are uncoupled. Foci containing 53BP1 and phosphorylated histone H2AX are also observed following oncogene activation indicating the presence of DNA DSBs in these cells. These foci map to nuclear regions that stain positively for PCNA, further linking their formation to DNA replication (63).

The induction of DNA DSBs following oncogene activation can also be detected by direct methods, such as pulse field gel electrophoresis of genomic DNA. Using this method, the oncogene-induced formation of DNA DSBs has been shown to be inhibited by aphidicolin, a DNA polymerase inhibitor, suggesting that the formation of DNA DSBs is dependent on DNA replication (63).

The effect of oncogene activation on DNA replication forks has also been studied at the single-molecule level by the DNA combing technique (72). Cells are labeled consecutively with two thymidine analogs (iodo- and chloro-deoxyuridine), and then genomic DNA is prepared and spread on glass surfaces in the form of DNA fibers. DNA segments that have incorporated the two analogs can be visualized by immunofluorescence, allowing the DNA replication forks to be classified as progressing/ongoing (incorporation of both analogs with no intervening gap in the DNA fiber), terminated (incorporation only of the analog that was administered first), or newly fired (incorporation only of the analog that was administered last). The lengths of the DNA fibers incorporating each analog can be used to measure replication fork speeds, and when multiple forks can be visualized on a single fiber, asymmetry in fork progression and interorigin distances can also be determined. Using this method, activated oncogenes have been shown to induce an increase in the fraction of terminated forks, a decrease in replication fork speed, an increase in fork asymmetry, and a decrease in interorigin distance (62, 63, 73–76). Slower fork speeds and fork asymmetry likely represent fork stalling and/or collapse, whereas the decrease in interorigin distance may represent compensatory firing of dormant origins and/or deregulated origin firing.

Electron microscopy, another method that allows study of DNA replication forks at the single molecule level, also confirms the ability of oncogenes to induce DNA replication stress. Oncogene overexpression induces the formation of aberrant DNA structures that may correspond to reversed DNA replication forks (77).

Certain regions in vertebrate genomes are particularly sensitive to the presence of DNA replication stress. These regions are referred to as CFSs and are prone to deletions or loss of heterozygosity (LOH) when cells are treated with DNA polymerase inhibitors or when nucleotide pools become depleted (78, 79). Overexpression of oncogenes in tissue culture model systems induces genomic deletions or LOH within CFSs that precisely mimic the genomic changes observed when these cells are treated with aphidicolin or hydroxyurea (62, 80, 81). As described below, CFSs are very frequently targeted in human precancerous lesions and cancers (57, 58, 62, 82), providing one of the best arguments in favor of the hypothesis that DNA replication stress is prevalent in human cancers and drives genomic instability.

**MECHANISMS BY WHICH ONCOGENES INDUCE DNA REPLICATION STRESS**

The mechanism or mechanisms by which oncogenes induce DNA replication stress have not been clearly defined. Yet, this is an important question and many promising leads have emerged from
the significant effort that has been invested to resolve this question. One should emphasize that it is possible that different oncogenes induce DNA replication stress via different mechanisms, and that even a single oncogene may induce replication stress by more than one mechanism.

The process of DNA replication involves discrete steps that occur at different phases of the cell cycle (83). Following binding of the origin recognition complex to DNA, prereplication complexes (pre-RCs) are assembled in G1 to render the origins competent for DNA replication (licensed origins). The pre-RCs mature into preinitiation complexes (pre-ICs), which fire during S phase, forming replisome progression complexes (RPCs). Broadly speaking, formation of the pre-RCs is inhibited by CDKs and, thus, origin licensing is restricted to the G1 phase of the cell cycle. In contrast, formation of the pre-ICs and of the RPCs is dependent on CDK activity and, thus, takes place at the G1/S transition and in S phase (83–86). Because oncogenes deregulate CDK activity, the steps described above could potentially be compromised in cancer cells.

One would think that it should be trivial to design experiments that elucidate which of the steps described above are compromised by oncogenes. In practice, however, this is not easy. First, our understanding of DNA replication at the molecular level is far from complete, and the system is too complex to be easily dissected. Second, the tolerance of the replication machinery to various perturbations is not well understood. Third, some of the available assays are surrogates for the process of interest. Nevertheless, despite these difficulties, progress has been made toward understanding how oncogenes induce DNA replication stress.

### Deregulation of Origin Licensing/Firing

One level at which oncogenes may act to induce DNA replication stress is origin licensing and/or firing. Both these processes are tightly controlled by CDK activity and many oncogenes target CDKs, either directly or indirectly (84–86). **CCNE1**, which is amplified in many human cancers and which encodes for cyclin E1, acts directly on Cdk2, whereas certain tumor suppressors, such as **CDKN2A** and **CDKN2B**, encode for CDK inhibitors (15, 87). Other oncogenes modulate CDK activity indirectly via growth signaling pathways (14).

In budding yeast, deregulation of CDK activity induces DNA replication stress. Thus, yeast could provide relevant mechanistic insights. In one study, overexpression of the G1 cyclin Cln2 induced DNA replication stress and genomic instability (88). The mechanism involved inhibition of pre-RC assembly (origin licensing). In a second study, deletion of the **SIC1** gene, a CDK inhibitor, induced DNA replication stress as revealed by reduced origin licensing and firing and increased interorigin distance, genomic instability, and mitotic defects probably stemming from failure to complete DNA replication (89). These effects were mediated by cyclins Clb5/6, which are active in S phase, rather than by Cln2, the G1 cyclin, because all phenotypes induced by **SIC1** deletion were rescued by Clb5/6 deletion. In yeast, expression of the licensing factor Cdc6 occurs in two waves corresponding to late mitosis and late G1, respectively (90). In the absence of **SIC1**, the length of the G1 phase is shortened and the second wave of licensing does not occur, presumably due to high CDK activity mediated by Clb5/6. Thus, replication stress in yeast lacking **SIC1** may arise from a defect in firing of the origins that are licensed in late G1.

Like **SIC1** deletion in yeast, overexpression of cyclin E in human cells shortens the G1 phase and induces DNA replication stress (57, 63, 73–75, 87, 91). Thus, one might anticipate that activated oncogenes or, at least, cyclin E might induce DNA replication stress by compromising origin licensing and consequently origin firing. Unfortunately, not all studies have supported this prediction.

In one study, overexpression of cyclin E in a human nasopharyngeal epidermoid carcinoma cell line resulted in a reduced number of nuclear BrdU foci in early S phase cells and in a reduction
in BrdU fluorescence intensity, suggesting the presence of a smaller number of DNA replication factories and a reduced rate of DNA synthesis at each factory (91). In parallel, there was reduced association of the Mcm helicase subunits Mcm4 and Mcm7 with chromatin during G1, suggesting a defect in origin licensing. However, association of other Mcm subunits, notably, Mcm3 and Mcm5, with chromatin was not impaired. Because origin licensing involves the loading of Mcm heterohexameric rings on chromatin, it is hard to see how loading of some subunits would be compromised, but not of others. The authors explained the discrepancy in loading on the basis of differences in kinetics. Recent results indicate that in yeast Mcm3,5,7 are loaded before Mcm2,4,6 (92). However, the kinetics of assembly of the various Mcm subunits in human cells remains to be determined.

Interestingly, overexpression of cyclin E in U2OS osteosarcoma cells gave opposite results from those reported with the nasopharyngeal carcinoma cells above (74). The number of IdU foci was higher in early S phase cells following cyclin E overexpression, and interorigin distance was slightly decreased, rather than increased, as would be expected if licensing was compromised. Further, depletion of the licensing factor Cdc6 by siRNA to inhibit origin licensing or inhibition of Cdc7 to inhibit origin firing suppressed the induction of DNA DSBs in cells overexpressing cyclin E. If cyclin E overexpression induces increased origin firing, as these experiments suggest, what could be the mechanism? Cyclin E-Cdk2 phosphorylates and stabilizes Cdc6 (93). This stabilization is apparently particularly important for origin licensing when quiescent cells are stimulated to reenter the cell cycle, and it may correspond to the second wave of Cdc6 expression in yeast. Thus, one could imagine that in cells overexpressing cyclin E, higher levels of Cdc6 might enhance origin firing. Other oncogenes, such as RAS and MOS, which induce DNA replication stress, also enhance Cdc6 protein levels, and ectopic expression of Cdc6 is sufficient to induce DNA replication stress (63). The MYC oncogene may also induce DNA replication stress by stabilizing Cdc6 (76). In conclusion, oncogenes are likely to induce DNA replication stress by affecting origin licensing and firing, but depending on the cellular context, origin licensing and firing may be upregulated or downregulated.

If cyclin E overexpression enhances origin licensing, how can this observation be reconciled with the studies in yeast showing that SIC1 deletion inhibits licensing? One possible explanation is that deletion of SIC1 leads to enhanced Clb5/6 activity, which are S phase-specific cyclins, whereas cyclin E functions at the G1/S transition. Thus, Clb5/6 will suppress origin licensing, whereas cyclin E, at least under certain conditions, may stimulate licensing via Cdc6 stabilization (93).

**Shortage of Replication Building Blocks**

A second level at which oncogenes can act to induce DNA replication stress is at the level of fork progression. A decrease in fork speed has been observed in cell systems in which oncogenes, such as KRAS and CCNE1, have been activated (62, 73–75). This is often accompanied by decreased interorigin distance (implying increased origin firing), fork asymmetry, and an increased number of DNA DSBs. A decrease in fork speed has also been observed in primary keratinocytes expressing the human papillomavirus oncogenes E6 and E7, which target the p53 and pRb tumor suppressor proteins, respectively (73). Interestingly, E6 and E7 also induced a decrease in deoxynucleotide levels. This decrease was apparently important for the changes in DNA replication dynamics and the induction of DNA damage, as addition of deoxynucleosides in the tissue culture media suppressed, albeit not completely, the decrease in replication fork speed and the induction of DNA DSBs (73). Exogenously added deoxynucleosides also rescued DNA replication stress in human fibroblasts overexpressing cyclin E. Interestingly, in both systems, overexpression of the MYC
oncogene led to an increase in endogenous deoxynucleotide levels and rescued DNA replication stress. From these results, the authors concluded that deregulated cell cycle entry by oncogenes is not accompanied by activation of all the pathways needed for proper progression through the cell cycle. For example, E6/E7 and cyclin E promote entry into S phase without activating nucleotide synthesis. Entry into the cell cycle with uncoordinated activation of pathways needed for DNA replication may explain why MYC induces DNA replication stress when overexpressed on its own (76, 94) but suppresses DNA replication stress when overexpressed in E6/E7-transformed cells (73).

The concept that DNA replication stress in cancer cells is due to a relative deficiency in building blocks needed for DNA replication has been further extended by studies of U2OS osteosarcoma cells treated with MK-1775, a chemical inhibitor of the Wee1 kinase (95). Wee1 inhibits CDKs and, therefore, Wee1 inhibitors can be used to rapidly induce CDK activity. In this regard, MK-1775 mimics oncogenic activation with the advantage of activating CDKs with faster kinetics than oncogenes. Treatment of U2OS cells with MK-1775 induced a profound decrease in replication fork speed and induction of DNA DSBs (95). Interestingly, despite the decrease in fork speed, the overall incorporation of the thymidine analog EdU per cell was increased. By dividing the total level of EdU incorporation per cell by the average fork speed, the authors calculated that Wee1 inhibition results in a threefold increase in the number of progressing forks. This very significant increase was accompanied by a decrease in deoxynucleotide levels, which apparently became limiting because the decrease in fork speed and the induction of DNA DSBs in this system were partially rescued by exogenously added deoxynucleosides (95). Thus, these results indicate that high levels of CDK activity induce DNA replication stress by increasing origin firing, which in turn depletes the nucleotide pools.

Nucleotides are not the only building blocks whose levels may decrease relative to the number of replication forks in cells overexpressing oncogenes. DNA replication in cells requires synthesis of histones and of many other chromatin-associated proteins, as well as of proteins present at the replisome (96). Thus, an increase in the number of progressing forks may result in a relative deficiency of any of these proteins, thereby explaining the decrease in replication fork speed. Suppression of new histone synthesis by targeting factors involved in histone biogenesis slows fork progression by at least twofold, suggesting that fork speed can be regulated by histone supply (97). However, DNA DSBs and activation of the replication checkpoint were observed only 48 h after inhibition of histone biosynthesis, suggesting that limited histone supply needs to be chronic to induce DNA replication stress (97).

Replisome proteins can also become limiting. In one study, U2OS cells were treated with the Wee1 inhibitor MK-1775 to enhance origin firing and, in parallel, fork progression was challenged with hydroxyurea (98). The large number of stalled forks in this setting consumed all the available RPA, leading to fork collapse and formation of DNA DSBs. Studies in yeast have also demonstrated that certain proteins involved in origin firing, such as Sld2, Sld3, Sld7, and Cdc45, can be limiting (99, 100). In fact, the limited availability of these proteins is functionally important, as it controls the timing of origin firing. Whether replisome proteins become limiting in cancer cells remains to be determined.

As discussed above, either uncoordinated activation of pathways needed for DNA replication or increased number of progressing forks could account for the relative lack of factors needed for DNA replication in cancer cells. However, an alternative possibility is that this relative lack may be secondary to a shortened G1 phase. Cells typically grow in size in G1 and have checkpoints to ensure that they do not enter S phase if their size is too small (101). TOR is involved in such a checkpoint, which, however, can be overcome by high levels of CDK activity (102). Overexpression of CCNE1, CCND1, or MYC and activation of RAS, RAF, or SRC drive premature entry into S
phase (75, 87, 91, 103–105), suggesting that cells start replicating their DNA with a smaller than normal capacity for protein synthesis. Under these conditions, the cells may be unable to sustain a normal rate of DNA replication.

**Interference Between Replication and Transcription**

DNA replication utilizes the same template as transcription, creating the potential for interferences between these two processes. Indeed, analysis of replication origins and transcription units across evolution suggests that organisms have evolved mechanisms to limit such conflicts (106). In *Escherichia coli*, transcription units are codirectional with progressing forks, suggesting the need to avoid head-on collisions (107). In yeast, highly transcribed genes are also replicated codirectionally with transcription (108). However, in human cells, the mapping of origins does not suggest a preference for codirectionality between transcription and replication (109). Nevertheless, human cells might regulate the spatio-temporal program of origin firing, so as to avoid conflicts between DNA replication and transcription (110–112). Oncogenes might disrupt this program either by affecting origin firing, slowing fork progression, or by changing the length of the cell cycle phases (shorter G1, longer S phase), resulting in genomic loci being transcribed and replicated at the same time.

As discussed above, CFSs are the genomic loci that are most susceptible to genomic rearrangements in the presence of oncogene-induced DNA replication stress (58, 62, 63, 82). CFSs correspond to very large genes (24, 25), and due to their large size, transcription of these genes spans the G1 and S phases of the cell cycle and can even extend into the next cell cycle (113). In turn, this means that transcription and replication occur concurrently on the same template. Analysis of aphidicolin-induced chromosomal breaks within five CFSs in two different cell lines revealed a perfect correlation between the presence of breaks and transcription. Further, the breakpoints mapped to the region of the gene being transcribed in S phase, suggesting that the breakpoints were due to conflicts between transcription and replication (113).

The role of transcription on oncogene-induced DNA replication stress was directly examined in a subsequent study utilizing U2OS cells overexpressing cyclin E (74). Short-term treatment of these cells with cordycepin, an RNA-specific chain terminator that inhibits transcription elongation, rescued slow fork progression and induction of DNA DSBs, although not completely. The rescue was attributed to alleviation of conflicts between transcription and replication because during the transient treatment of cells with cordycepin, cyclin E levels were not affected.

Interference between transcription and replication may also explain the presence of DNA replication stress at specific early-replicating regions of the genome, referred to as early-replicating fragile sites (ERFS) (114). ERFS were initially mapped as genomic loci prone to undergo DNA damage in cells that enter S phase in the presence of hydroxyurea. These same sites were then shown to be fragile when the *MYC* oncogene was overexpressed. ERFS correspond to highly transcribed regions of the genome and genetic manipulation of one such site so as to reduce expression rendered this site resistant to breakage.

How interference between transcription and replication leads to DNA replication stress is not well understood (106, 110). One possibility is collisions between the transcription and replication machineries. Alternatively, RNA:DNA hybrids formed during transcription might impede fork progression (115). Supporting this latter model, depletion of topoisomerase 1 (Top 1) mimics oncogene-induced DNA replication stress in the sense that it slows fork speed, decreases inter-origin distance, enhances fork asymmetry, and induces chromosomal breaks at CFSs (116). In this setting, Top1 is thought to release the DNA supercoiling that accompanies transcription and in so doing prevents the formation of nascent RNA:DNA hybrids.
PATTERNS OF GENOMIC INSTABILITY INDUCED BY DNA REPLICATION STRESS

One of the earliest observations linking DNA replication stress to genomic instability was the appearance of chromosomal breaks following treatment of cells with aphidicolin (78). These breaks were observed preferentially at specific chromosomal sites, termed CFSs. Subsequently, comparative genomic hybridization analysis revealed the presence of genomic deletions mapping to the CFSs, suggesting that the chromosomal breaks may result from an inability to complete DNA replication (19, 80, 117, 118).

Several models have been proposed to explain why DNA replication stress leads to genomic deletions preferentially within CFSs. One model proposes that CFSs are sites of interference between replication and transcription. As discussed above, CFSs correspond to very large genes that are transcribed throughout the cell cycle, implying that DNA replication cannot be dissociated temporally from transcription (113). Thus, under conditions of replication stress, the transcriptional machinery would collide with slowly progressing forks, even when transcription and replication are codirectional. Another model proposes that in CFSs the density of replication origins is low (119). Thus, when DNA replication is challenged, there would be a scarcity of dormant origins to rescue the replication defect. A third model proposes that CFSs correspond to genomic regions that are intrinsically difficult to replicate (120, 121). Thus, any further perturbation in DNA replication would result in an inability to replicate these genomic regions. Another contributing factor to the sensitivity of CFSs to DNA replication stress is the fact that CFSs are late-replicating regions (79, 122). Thus, any form of DNA replication stress that slows down replication could result in cells entering mitosis with the CFSs not having been fully replicated (123).

The presence of unreplicated DNA at CFSs and other genomic regions has the potential to also induce broad changes in chromosome structure and number by interfering with mitosis. Specifically, regions of unreplicated DNA may form ultrafine anaphase bridges, which interfere with chromosome segregation during mitosis, resulting in DNA damage in the next cell cycle and genomic instability (124, 125).

Another type of genomic change induced by DNA replication stress is tandem segmental genomic duplications. Such duplications are observed more frequently when DNA replication stress is induced by ionizing radiation (IR) or oncogenes than when it is induced by aphidicolin or hydroxyurea (75, 126). The difference may relate to the fact that IR and oncogenes induce fork collapse, whereas aphidicolin and hydroxyurea induce primarily fork stalling. Collapsed forks are processed into one-ended DNA DSBs, which are then repaired by a specific repair pathway, break-induced replication (BIR), which is a form of homologous recombination (127, 128). According to one model, BIR may produce tandem genomic duplications when the collapsed fork being repaired is near a progressing fork (75, 129).

In addition to changes in chromosome structure, DNA replication stress has the potential to induce SNSs. Stalling or collapse of replication forks results in DNA being single-stranded for protracted periods of time; during this time, damaged bases cannot be repaired efficiently, resulting in SNSs (48). Single-stranded DNA also arises during repair of collapsed replication forks by BIR because in BIR the two strands of DNA are not coordinately replicated. Thus, in yeast, replication by BIR is associated with a thousand-fold increase in mutation rates, as compared to normal replication (130, 131).

GENOMIC INSTABILITY PATTERNS IN HUMAN CANCERS

A comparison of the genomic instability patterns induced by DNA replication stress to those present in human cancers can help answer the question whether replication stress is a major
source of genomic instability in cancer. Other sources of DNA damage, such as reactive oxygen species (ROS) (132, 133), telomere dysfunction (134–136), and mitotic defects (137, 138), will, of course, also contribute to the genomic instability patterns present in cancers.

Not all genomic changes observed in human cancers are equally informative in terms of the underlying mechanisms leading to genomic instability. Mutations that drive cancer progression may be induced at a low frequency but be very prevalent in human cancers because they are selected. Mutations in oncogenes and tumor suppressors would fall into this category. It is almost evident that the loci containing these genes are not exceedingly more susceptible to mutagenesis than the rest of the genome. Yet, due to selection pressures, the p values for SNSs within these loci are very high (Tables 1 and 2). However, mutations that do not confer a selective advantage for growth, the so-called passenger mutations (5), are indicative of the mechanisms driving genomic instability. Thus, when considering the genomic instability patterns present in cancer, we focus on the passenger mutations. The distinction between cancer-driver and passenger mutations applies, of course, to all types of mutations, including SNSs and SCNAs, both of which are reviewed here.

At the chromosomal level, practically all cancers have changes in chromosome structure, which are typically accompanied by changes in chromosome number (35). Many of the changes in chromosome structure are SCNAs: amplifications (mostly duplications) and deletions (Figure 3). SCNAs can be recurrent or nonrecurrent. We consider these two classes separately because, as described below, many of the recurrent SCNAs involve oncogenes or tumor suppressors and, therefore, have been selected.

First, in regard to the recurrent SCNAs, three-fourths involve a chromosomal segment (focal SCNAs), whereas the remaining one-fourth involves an entire chromosome arm (arm-level SCNAs). The focal SCNAs can be further subdivided into those internal to the chromosome (four-fifths) and those that are telomere-bound (one-fifth) (11). The internal SCNAs encompass the ten most common amplifications and the ten most common deletions present in human cancers (11). Their distribution in the genome is presented in Tables 1 and 2. The most significantly amplified regions correspond to oncogenes and, therefore, their high recurrence rate can be explained by the selection advantage they confer. Accordingly, they are not very informative in regard to mutagenic mechanisms. Of the ten most common deletions, four map to tumor suppressor genes and, therefore, have also been selected. However, the remaining six map to very large genes, including CFSs (Figure 3). It is, indeed, remarkable that CFSs are subject to deletions with similar frequencies as tumor suppressor genes, even though the loss of CFSs is not thought to provide a selective advantage for growth. The high recurrence rate of focal deletions targeting very large genes and CFSs is not limited to the ten most common deleted regions. Of the top 49 recurrent internal chromosomal deletions, 21 target one of the 100 largest genes in the human genome (11). Taken together, these results suggest that DNA replication stress contributes significantly to the induction of focal deletions in human cancers (19).

Telomere-bound SCNAs scored lower on the list of significantly recurrent SCNAs than the internal SCNAs, in the sense that none of the ten most common focal amplifications or deletions present in human cancers are telomere-bound (Tables 1 and 2). Further, among 21 recurrent telomere-bound chromosomal deletions, only one targets one of the largest genes in the human genome and none targets a bona fide tumor suppressor gene. This indicates that the mechanisms leading to the establishment of telomere-bound SCNAs differ from those leading to the establishment of internal SCNAs (Figure 3). Telomere dysfunction can, of course, lead to the emergence of telomere-bound SCNAs via the proposed breakage-fusion-bridge cycle mechanism (134–136, 139, 140).

Not all chromosomal rearrangements observed in human cancers are recurrent. A list of nonrecurrent SCNAs in ovarian and breast cancers reveals that segmental head-to-tail tandem
duplications with microhomology junctions represent the most common type (141, 142). In one experimental system of oncogene-induced replication stress, repair of collapsed forks by BIR led to this type of duplication, suggesting that the duplications observed in human cancers are induced in response to fork collapse (75). Other common types of nonrecurrent SCNAs in ovarian and breast cancers are amplifications, deletions, translocations, and inversions (Figure 3).

Genomic instability in human cancers also occurs at the single nucleotide level. Depending on the sequence context, in which nucleotide changes occur, several mutational signatures have been identified (6). The most prevalent signature involves substitution of cytosines by thymines in the context of CpG dinucleotides. Because cytosines are often methylated in the context of CpGs, the mutations probably arise by spontaneous deamination of a methylated cytosine to a thymine (143).

Interestingly, the distribution of CpG to TpG substitutions in human cancers is not random. Very large genes contain twice as many CpG to TpG mutations per megabase of genomic DNA as...
the rest of the genome (48, 144–146). This distribution may be explained by the presence of DNA replication stress. The very large genes correspond to the late-replicating regions of the genome and the CFSs (24, 25, 79). Collapsed DNA replication forks at these regions could be repaired by BIR, which is mutagenic (130, 131). A second mechanism could relate to DNA persisting in a single-stranded form for protracted periods of time, as a consequence of fork stalling or collapse (48). During this time, methylated cytosines may become spontaneously deaminated to form thymines (143). In the context of single-stranded DNA, these thymines do not generate a base pair mismatch and are not repaired. In conclusion, the distribution of SNSs in human cancer genomes suggests that at least some of the SNSs in human cancers may arise as a consequence of DNA replication stress.

The two main signatures of genomic instability that are linked to DNA replication stress, chromosomal deletions and SNSs targeting very large genes and late-replicating regions, are also present in human precancerous lesions. In fact, the preference for genomic instability targeting these regions appears to be even higher in the precancerous lesions than in the advanced cancers, suggesting that DNA replication stress is an important driver for cancer development (48, 57, 58, 147).

SYNTHESIS AND CONCLUDING REMARKS

Our understanding of cancer has improved dramatically during the past few decades. One important contribution, representing the work of numerous researchers, is the identification of phenotypes and molecular changes that are shared by almost all cancers (2–4). In turn, this knowledge has permitted the formulation of models that try to explain cancer development. Within the context of this review, we have focused on oncogene-induced DNA replication stress and whether it can serve as a driver for cancer progression. Clearly, other drivers, such as dysfunctional telomeres, mitotic defects, and ROS, are also important for cancer development, but, here, we only briefly touched upon them because their roles in cancer progression have been recently reviewed by others (133, 137, 148).

The results reviewed here suggest that it may be possible to integrate cancer phenotypes and molecular changes to explain some aspects of cancer development (Figure 4). The first step in cancer development involves mutations in genes regulating cell growth (oncogenes and tumor suppressors). These mutations underlie the sustained proliferation hallmark. Interestingly, precancerous lesions exhibit DNA replication stress, which we propose should be considered as a hallmark of cancer because it is present in almost all cancers from the earliest stages. Given the ability of oncogenes to induce DNA replication stress in various model systems and the general absence of mutations in DDR genes in precancerous lesions, the mutations driving cell proliferation must also be responsible for the DNA replication stress hallmark (Figure 4). DNA replication stress and the ensuing DNA damage have two major consequences for the biology of the precancerous lesions. First, they contribute to the genomic instability hallmark, which can fuel cancer progression, and second, they activate a DDR, which activates ATM and TP53 (149).

A second step in cancer development involves suppression of the DDR. Activation of TP53 by DNA damage leads to apoptosis or senescence, thereby curtailing growth of the precancerous lesion. In turn, this selects for mutations conferring an escape from apoptosis/senescence hallmark (Figure 4). Mutations targeting the TP53, ATM, or MDM2 genes are primarily responsible for establishment of this hallmark because they can suppress DDR-induced apoptosis and senescence without compromising the DNA replication checkpoint per se, on which cancer cells rely to stabilize and repair their damaged replication forks. We note that, traditionally, the escape from apoptosis/senescence hallmark only referred to apoptosis, but DDR-induced senescence is also very prevalent in human precancerous lesions (63, 150).
The acquisition of the escape from apoptosis/senescence hallmark may be linked to progression of precancerous lesions to invasive cancers and to increased levels of genomic instability, as cells with excessive DNA damage are no longer eliminated by TP53-dependent apoptosis or senescence (63).

The sequence of events described here links some of the most pervasive hallmarks of cancer (sustained cell proliferation, DNA replication stress, genomic instability, and escape from apoptosis/senescence) to the most common mutations present in cancer (targeting the genes regulating cell growth and TP53; Figures 1 and 4). Of course, this model represents a simplified view of a specific aspect of early cancer development. Dysfunctional telomeres also contribute significantly to the genomic instability present in cancers and also provide selection pressure for inactivating TP53 (Figure 4). Further, this model does not attempt to explain the acquisition of the other hallmarks of cancer not shown in Figure 4. We hope that further work will eventually permit the translation of our newly acquired knowledge into effective therapies.

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