Chapter 22
Cyclin E Deregulation and Genomic Instability

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Abstract Precise replication of genetic material and its equal distribution to daughter cells are essential to maintain genome stability. In eukaryotes, chromosome replication and segregation are temporally uncoupled, occurring in distinct intervals of the cell cycle, S and M phases, respectively. Cyclin E accumulates at the G1/S transition, where it promotes S phase entry and progression by binding to and activating CDK2. Several lines of evidence from different models indicate that cyclin E/CDK2 deregulation causes replication stress in S phase and chromosome segregation errors in M phase, leading to genomic instability and cancer. In this chapter, we will discuss the main findings that link cyclin E/CDK2 deregulation to genomic instability and the molecular mechanisms by which cyclin E/CDK2 induces replication stress and chromosome aberrations during carcinogenesis.

Keywords Cell cycle • Cyclin E • CDK2 • FBW7 • Replication stress • Chromosome aberration • Genomic instability • Fragile sites • Cancer

22.1 Introduction

Progression through the cell cycle is regulated by association of cyclin-dependent kinases (CDKs) with specific regulatory subunits known as cyclins. Oscillations in cyclin levels primarily dictate oscillations in CDK activity, ensuring the order and timing of cell cycle phases (Hochegger et al. 2008; Malumbres and Barbacid

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2009). The E-type cyclin family is composed of two proteins, cyclin E1 and cyclin E2, which exhibit high sequence similarity and are functionally redundant (Lew et al. 1991; Koff et al. 1991; Gudas et al. 1999; Lauper et al. 1998; Zariwala et al. 1998). Cyclin E levels are tightly regulated during normal cell cycles, accumulating at the G1/S transition and being completely degraded by the end of S phase (Koff et al. 1992; Dulic et al. 1992). Consistent with its expression pattern, cyclin E binds to and activates CDK2 to control S phase entry and progression (Koff et al. 1992; Dulic et al. 1992; Ohtsubo and Roberts 1993; Resnitzky et al. 1994). Cyclin E mRNA levels are mostly induced by E2F transcription factors (Ohtani et al. 1995; Geng et al. 1996), whereas cyclin E protein is degraded by the SCF^{Fbw7} ubiquitin ligase complex in a phosphorylation-dependent manner (Won and Reed 1996; Clurman et al. 1996; Strohmaier et al. 2001; Moberg et al. 2001; Koepp et al. 2001). Cyclin E/CDK2 activity is also controlled by the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip}$, and potentially other CDK-inhibitory proteins, which are able to bind to and inactivate the cyclin E/CDK2 complex (Harper et al. 1993; Gu et al. 1993; Xiong et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994; Reynaud et al. 1999).

Once activated, the cyclin E/CDK2 complex promotes the G1/S transition largely through phosphorylation and inactivation of the RB protein and the subsequent release of E2F transcription factors (Chellappan et al. 1991; Hinds et al. 1992; Dyson 1998; Harbour and Dean 2000). E2F proteins then promote S phase entry by regulating the expression of numerous genes required for DNA replication, such as the pre-replication complex components ORC1, CDC6, CDT1, and MCMs (Ohtani et al. 1996, 1998, 1999; Yan et al. 1998; Yoshida and Inoue 2004); the enzymes required for nucleotide and DNA synthesis, such as dihydrofolate reductase (DHFR), thymidine kinase (TK), and DNA polymerase α (Blake and Azizkhan 1989; Dou et al. 1994; Pearson et al. 1991); and the histone H2A (Oswald et al. 1996). Besides the RB protein, cyclin E/CDK2 directly phosphorylates and regulates other substrates required for S phase entry and progression, such as the DNA replication factors CDT1 and CDC6 (Liu et al. 2004; Mailand and Diffley 2005); the replication initiator Treslin (Kumagai et al. 2011); the activator of histone expression NPAT (Zhao et al. 2000; Ma et al. 2000); the transcription factors CBP/p300, E2F5, SMAD3, and MYC (Ait-Si-Ali et al. 1998; Morris et al. 2000; Matsuura et al. 2004; Hydbring et al. 2010); the centrosome proteins NPM, MPS1, and CP110 (Okuda et al. 2000; Tokuyama et al. 2001; Fisk and Winey 2001; Chen et al. 2002); and the DNA repair protein BRCA1 (Ruffner et al. 1999).

Regulation of E-type cyclins and the function of cyclin E/CDK2 in normal and aberrant cell cycles have been extensively reviewed elsewhere (Hwang and Clurman 2005; Caldon and Musgrove 2010; Siu et al. 2012). Here, we will focus on how deregulation of cyclin E/CDK2 causes replication stress and chromosome aberrations that may lead to genomic instability in cancer.

22.2 Cyclin E-Mediated Chromosome Instability

Several lines of evidence support the notion that cyclin E/CDK2 deregulation causes genomic instability. The initial finding that linked cyclin E to chromosome instability was the observation that constitutive cyclin E overexpression induced chromosome gains and losses in non-transformed rodent fibroblasts and human mammary epithelial cells, leading to aneuploidy (Spruck et al. 1999). Importantly, constitutive overexpression of cyclin D1 or cyclin A2 had no effect on the number of chromosomes in these cells. Later, it was shown that deletion of *FBXW7*, the gene encoding the F-box protein FBW7 involved in cyclin E recognition and degradation by the SCF^{Fbw7} E3 ubiquitin ligase complex, resulted in increased frequency of micronucleus formation, multipolar spindles, and eventually chromosome instability in colorectal cancer cells (Rajagopalan et al. 2004). Even though FBW7 is involved in the degradation of other oncoproteins, such as c-MYC, c-JUN, and NOTCH (Davis et al. 2014), downregulation of cyclin E was sufficient to revert micronucleus formation in FBW7-depleted cells (Rajagopalan et al. 2004). More recently, generation of a hyperactive *CDK2* knockin allele in a human colorectal cancer cell line that expresses high cyclin E-associated kinase activity also showed increased rates of micronucleus formation when compared to *CDK2* wild-type cells (Hughes et al. 2013). Together, this evidence supports a causal role for cyclin E in chromosome instability during carcinogenesis.

One of the proposed mechanisms to explain chromosome instability in cancers is centrosome amplification, which leads to the formation of merotelic attachments and eventually chromosome segregation errors (Fig. 22.1) (Godinho and Pellman 2014). Normal cyclin E/CDK2 activity is required to ensure initiation of centrosome duplication in *Xenopus* egg extracts (Hinchcliffe et al. 1999; Lacey et al. 1999). In mammalian cells, it is also clear that CDK2 activity is necessary for

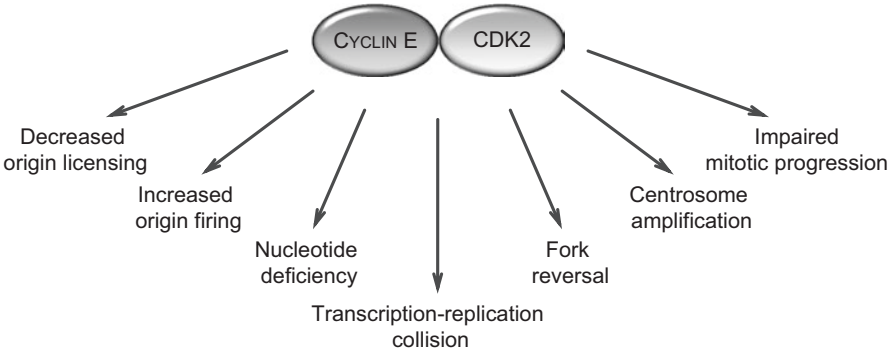

Fig. 22.1 Mechanisms of cyclin E-induced genomic instability. Cyclin E/CDK2 deregulation may cause impaired assembly of pre-replication complex, increased origin initiation, deficiency of nucleotide biosynthesis pathway, collisions between replication and transcription machineries, formation of aberrant replication intermediates, such as fork reversal, centrosome amplification, and impairment of mitotic progression and checkpoint function

centrosome duplication; however, it is still uncertain whether cyclin E or cyclin A plays a major role in CDK2-dependent centrosome duplication (Matsumoto et al. 1999; Meraldi et al. 1999; Hanashiro et al. 2008). Cyclin E overexpression alone does not efficiently induce centrosome amplification in mouse embryonic fibroblasts (MEFs), normal human fibroblasts, and epithelial cells (Spruck et al. 1999; Mussman et al. 2000; Kawamura et al. 2004). However, high levels of cyclin E synergize with loss of p53 function to induce centrosome amplification and chromosome instability in human cell lines and tumors (Mussman et al. 2000; Kawamura et al. 2004). Furthermore, MEFs from a hyperactive *CDK2* knockin mouse model, which show elevated cyclin E- and cyclin A-associated kinase activities, had an elevated number of centrosomes when compared to wild-type MEFs (Zhao et al. 2012).

Cyclin E/CDK2 localizes to centrosomes (Matsumoto and Maller 2004), where it phosphorylates and dissociates NPM protein, initiating separation of paired centrioles and duplication of centrosomes (Okuda et al. 2000; Tokuyama et al. 2001). It is therefore possible that deregulation of cyclin E/CDK2 kinase activity, in combination with other insults, impairs NPM release from centrosomes, leading to centrosome amplification and chromosome instability. Indeed, deletion of NPM causes aberrant mitotic figures with multiple centrosomes and aneuploidy in MEFs (Grisendi et al. 2005), and alterations in *NPM* are frequently observed in human cancers (Grisendi et al. 2006). As discussed above, the centrosome proteins MPS1 and CP110 are also directly phosphorylated by cyclin E/CDK2 (Fisk and Winey 2001; Chen et al. 2002) and therefore may represent potential targets for cyclin E-induced chromosome instability as well.

Another mechanism that drives chromosome instability in tumorigenesis is impairment of mitotic checkpoint function and progression through mitosis, which may cause chromosome missegregation and aneuploidy (Fig. 22.1) (Varetti et al. 2014). It has been shown that cyclin E overexpression delays progression through early stages of mitosis, leading to accumulation of cells in prometaphase and unaligned metaphase (Keck et al. 2007). Impairment of mitotic progression was caused by cyclin E/CDK2-mediated phosphorylation and inactivation of the APC/C adaptor protein CDH1 and subsequent accumulation of the APC/C^{Cdh1} ubiquitin ligase substrates cyclin B1 and securin, resulting in mitotic failure and polyploidy. In agreement, *FBXW7*-deficient cells, which have increased cyclin E-associated CDK2 activity, also exhibit increased levels of the APC/C substrates cyclin B1 and securin and accumulation of cells in prometaphase (Bailey et al. 2015). Interestingly, a genome-wide RNAi screen in these cells identified synthetic lethality with BUBR1, a spindle assembly checkpoint (SAC) protein, and high sensitivity to depletion of two other SAC components BUB1 and MPS1. These results suggest that cells with increased levels of cyclin E may depend on intact mitotic checkpoints for survival. Moreover, it has also been shown that cyclin E/CDK2 phosphorylates and prematurely activates the protein phosphatase CDC25C, leading to increased activity of the mitotic kinases cyclin B1/CDK1 and PLK1 and delayed mitotic progression (Bagheri-Yarmand et al. 2010).

22.3 Cyclin E-Mediated Replication Stress

Replication stress is characterized by the slowing or stalling of DNA replication forks, which may lead to fork collapse, DNA damage, and ultimately genomic instability (Zeman and Cimprich 2014). Activated oncoproteins and mutated tumor suppressors that drive sustained cellular proliferation cause replication stress and genomic instability, two events that are frequently observed in human cancers (Hills and Diffley 2014; Macheret and Halazonetis 2015). Indeed, this is the case for the oncoprotein cyclin E. Overexpression of cyclin E has been shown to cause replication stress, typified by slowed progression and premature termination of replication forks, DNA damage, and loss of heterozygosity at fragile sites (Bartkova et al. 2005, 2006; Bester et al. 2011). Cyclin E-mediated replication stress most likely is linked to elevated CDK2 kinase activity, as a *CDK2* hyperactive knockin allele was sufficient to delay replication fork progression, induce DNA damage, and increase micronucleus formation without cyclin E overexpression (Hughes et al. 2013). In a seminal series of articles, it has been proposed that oncogene-induced replication stress, including the oncoprotein cyclin E, activates the DNA damage response (DDR) pathway and leads to cell cycle arrest, cell death, and senescence, acting as an inducible barrier to tumor progression (Bartkova et al. 2005, 2006; Gorgoulis et al. 2005; Di Micco et al. 2006). Disruption of the DDR pathway facilitates cell proliferation and increases replication stress, leading to genomic instability in preneoplastic lesions.

The primary mechanism underlying cyclin E/CDK2-induced replication stress and genomic instability is interference of the nucleotide biosynthesis pathway (Fig. 22.1). Nucleotides are structural components of nucleic acids and therefore essential for a wide variety of biological processes, such as cell growth, DNA replication, and transcription (Lane and Fan 2015). Cyclin E overexpression, through disruption of the RB/E2F pathway, enforces cell proliferation of human fibroblasts with insufficient nucleotide levels (Bester et al. 2011). Nucleotide deficiency induced by cyclin E overexpression slowed replication fork progression and caused double-strand DNA breaks. Importantly, either exogenous supplementation of nucleosides or upregulation of nucleotide metabolism genes attenuated cyclin E-mediated replication stress and DNA damage. Consistent with this, replication stress in the form of impaired fork progression has been shown to generate structural as well as numerical chromosome instability during mitosis (Burrell et al. 2013).

Collisions between DNA replication and transcription machineries are another important source of replication stress (Fig. 22.1). Transcription complexes represent natural obstacles to the progression of replication forks, especially at fragile sites that contain extremely long genes (>800 kb), where replication forks have a high probability of encountering transcription complexes during the period of one cell cycle (Helmrich et al. 2011). Transcription-replication collisions may generate increased DNA topological tension and formation of R-loops (RNA-DNA hybrid structures), inducing replication fork stalling, DNA damage, and fragile site instability (Bermejo et al. 2012; Helmrich et al. 2013). Oncogenic events that interfere with the timing and location of DNA replication and transcription may increase

the probability of transcription-replication collisions. In cells overexpressing cyclin E, it has been shown that inhibition of transcription elongation attenuates replication stress and DNA damage (Jones et al. 2013). This study also showed that inhibition of replication initiation restores normal levels of fork progression in cyclin E-overexpressing cells, suggesting that increased replication initiation and transcription-replication collisions contribute to the replication stress upon high levels of cyclin E (Fig. 22.1). One potential consequence of transcription-replication collisions is the formation of aberrant replication intermediates, such as reversed replication forks (Neelsen and Lopes 2015). Consistently, it has been shown that cyclin E overexpression induces accumulation of reversed forks and chromosome breakage in human cells, suggesting that DNA topological stress also underlie cyclin E-mediated replication stress and genomic instability (Fig. 22.1) (Neelsen et al. 2013). Furthermore, it has been shown that cyclin E-induced collapsed forks may be processed and repaired by break-induced replication (BIR) repair, which generates copy number alterations, such as segmental genomic duplications (Costantino et al. 2014).

22.4 Genomic Instability in Cyclin E Mouse Models

Cyclin E is frequently overexpressed in human tumors, and its deregulation has been associated with poor prognosis and decreased survival of cancer patients (Scuderi et al. 1996; Porter et al. 1997; Iida et al. 1997; Erlanson et al. 1998; Fukuse et al. 2000; Muller-Tidow et al. 2001; Keyomarsi et al. 2002; Schraml et al. 2003). Overexpression of cyclin E in mouse models has been shown to induce mammary and lung carcinomas as well as hematopoietic malignancies, further supporting a causative role for cyclin E in carcinogenesis (Bortner and Rosenberg 1997; Karsunky et al. 1999; Geisen et al. 2003; Loeb et al. 2005; Smith et al. 2006; Ma et al. 2007; Minella et al. 2008; Siu et al. 2014).

Several tissue-specific transgenic and knockin mouse models have provided significant information on the role of cyclin E deregulation in genomic instability. A knockin mouse expressing a nondegradable form of cyclin E in MEFs showed increased chromosome breaks, translocations, and aneuploidy in a $p21^{-/-}$ background (Loeb et al. 2005). In this model, cyclin E overexpression also cooperated with p53 deficiency and RAS activation to cause cellular transformation, induce whole chromosome gains and losses, and accelerate lung carcinogenesis. Consistent with this, transgenic mice expressing either wild-type or degradation-resistant cyclin E in the lungs incurred multiple pulmonary adenocarcinomas with specific gains of chromosomes 4 and 6 (Ma et al. 2007). In mammary gland transgenic mouse models, cyclin E overexpression has been shown to induce p53 loss of heterozygosity and drastically increase tumor formation in a $p53^{+/-}$ background (Smith et al. 2006; Akli et al. 2007). Lastly, a knockin mouse with expression of nondegradable cyclin E in the hematopoietic stem cell compartment exhibited abnormal hematopoiesis, chromosome instability illustrated by chromosome gains

and losses, and decreased latency of T-cell malignancies in a $p53^{-/-}$ background (Minella et al. 2008; Siu et al. 2014). Again, p53 and p21 deficiencies were synergistic with cyclin E deregulation in promoting chromosome instability. Indeed, it has been shown that cyclin E-associated genomic instability is restrained by the p53/p21 pathway (Bartkova et al. 2005; Minella et al. 2002, 2007). Disruption of the inducible barrier established by the p53/p21 pathway may allow cyclin E overexpression to trigger genomic instability through some of the mechanisms discussed above, such as centrosome amplification and replication stress. Therefore, current mouse models support the notion that cyclin E deregulation contributes to tumorigenesis by promoting genomic instability in vivo.

22.5 Cyclin E Deregulation Promotes Replication Failure at Targeted Sites

We have discussed above the relationship between cyclin E and replication stress. Since cyclin E overexpression promotes replication stress and therefore slows replication fork progression (Bester et al. 2011; Jones et al. 2013; Liberal et al. 2012), we hypothesized that cells experiencing cyclin E deregulation might enter mitosis with incompletely replicated chromosomes. This in turn would lead to abnormal segregation and chromosomal damage during anaphase. Consistent with this, we observed that cyclin E-overexpressing non-transformed cells exhibited high levels of anaphase chromosomal anomalies such as bridged chromosomes and nonattached chromosomal fragments up to the size of the entire chromosome arms (Teixeira et al. 2015). If this observed chromosomal damage is a result of incompletely replicated chromosomal segments impairing segregation of sister chromatids, there are two obvious models that could account for the under-replication. Cyclin E-mediated replication stress could promote under-replication without any regional or feature specificity, or under-replication could occur at specific sites or regions possessing features that might sensitize them. To distinguish between these alternatives, we harvested cells blocked in mitosis immediately following cyclin E overexpression and analyzed their DNA by comparative genomic hybridization (CGH) array analysis. Indeed, a relatively small number of specific regions varying in size from approximately 200 to 100,000 base pairs had frequently failed to complete replication prior to entry into mitosis (Teixeira et al. 2015). Presumably, these under-replicated regions were responsible for the anaphase segregation anomalies we had observed in real time after cyclin E overexpression. Based on these observations, one would predict that these under-replicated regions would be included in deleted chromosomal segments subsequent to anaphase. We interrogated both mixed populations and single cells after cyclin E overexpression and found that deletion of these specific loci did indeed occur at high frequency (Teixeira et al. 2015). However, it appears that most cells carrying such deletions were incapable of clonal expansion, suggesting that checkpoint barriers eliminate cells with severely

damaged genomes, should surveillance mechanisms be intact. This presumably reduces the impact of cyclin E-mediated damage at the population level and is therefore protective from potentially oncogenic events (Bartkova et al. 2005). Indeed, the link between cyclin E deregulation and the p53 surveillance system has been discussed above.

22.6 Genomic Features Associated with Replication Failure

22.6.1 Late-Replicating Genomic Regions

The majority of under-replicated regions detected in our study have been annotated as large late-replicating domains (Teixeira et al. 2015; Weddington et al. 2008). These are, for the most part, heterochromatic regions with a paucity of replication origins. The fact that origins in these domains fire late during the replication cycle combined with the low density of origins provides a likely explanation for failure to complete replication under conditions of replication stress (Le Tallec et al. 2014; Ozeri-Galai et al. 2014). However, these properties alone cannot explain the specific locations and boundaries of the under-replicated regions, as they were relatively small compared to the larger domains and highly targeted to specific sites. Therefore, other features of these sites must be relevant.

22.6.2 Recombinational Hotspots/Translocation Breakpoints

A number of the sites have been annotated as recombinational hotspots or translocation breakpoints. A subset of these has been classified as fragile sites, as well. Both recombination and translocation are processes that are initiated by double-strand DNA breaks. Repair involving homologous sequences versus heterologous sequences containing microhomology determines the outcome (Berti and Vindigni 2016). Significantly, within this context, one characteristic of fragile sites is a tendency to experience double-strand breaks at abnormally high frequencies, presumably due to replication barriers within these sites (Le Tallec et al. 2014; Ozeri-Galai et al. 2014; Thys et al. 2015). These observations suggest that features of fragile sites might impede DNA replication under conditions of cyclin E-mediated replication stress leading to local replication failure. One feature of fragile sites suggested as causative for replication impairment and double-strand breaks is unusual DNA structures, such as palindromic sequences leading to formation of hairpins and loops (Thys et al. 2015; Ozeri-Galai et al. 2011). Such structured nonlinear DNA could easily explain why stressed replication forks might stall or collapse. However, this cannot completely explain the specificity of under-replicated sites under conditions of cyclin E overexpression, since only a small subset of fragile sites,

recombinational hotspots, and translocation breakpoints is affected. In addition, two recent studies showing that cyclin E overexpression causes instability at genomic regions with fragile site characteristics have given somewhat different results (Teixeira et al. 2015; Miron et al. 2015). Interestingly, among the susceptible genomic regions identified on each study (16 and 26, respectively), only one chromosome band was coincident for both (3q26). Since one study was carried out in mammary epithelial cells and the other in fibroblasts, a possible interpretation of the data is that cyclin E-mediated fragility may also be cell type specific, as has been shown previously in cells from different tissues (Le Tallec et al. 2011, 2013; Hosseini et al. 2013). In addition, not every under-replicated site was associated with fragile site features, suggesting factors unique to cyclin E-mediated replication stress must come into play (see below).

22.6.3 Low Origin Density and Licensing

Interrogating a number of databases of origin distribution in human cells compiled using diverse methodologies, we found that most of the under-replicated sites were located in chromosomal regions characterized by extremely low origin density (Teixeira et al. 2015). Under conditions of replication stress leading to fork collapse, the probable lack of nearby functional forks is likely to eliminate the most common mechanism for rescue of localized replication failure: processing of unreplicated DNA by an adjacent replicon (Letessier et al. 2011; Kawabata et al. 2011). However, it is likely that replication stress caused specifically by cyclin E overexpression compounds the logistical problems of completing the replicative cycle. This is because, in addition to replication stress, cyclin E overexpression impairs assembly of the pre-replication complex (Ekholm-Reed et al. 2004). Specifically, high cyclin E/CDK2 activity at the M/G1 boundary inhibits chromatin loading of MCM proteins, which constitute the primary replicative helicase. Based on investigation of the impact of direct MCM protein depletion, it is unlikely that this effect of cyclin E overexpression would alter DNA replication during an unperturbed replicative cycle. Very high percentages of individual MCM proteins can be depleted via RNAi-mediated silencing with no apparent effect on unperturbed replication (Ge et al. 2007; Ibarra et al. 2008). However, MCM-depleted cells are extremely sensitive to replication stress, as they fail to assemble backup origins. These origins are competent but normally remain dormant except under conditions of replication stress, when they are mobilized to rescue collapsed and/or poorly functioning replication forks (McIntosh and Blow 2012). Since it is probable that cyclin E overexpression via impaired MCM loading leads to a deficiency of backup origins, but also simultaneously causes replication stress, the problem of rescuing collapsed forks in origin-sparse regions is likely exacerbated, leading to replication failure.

22.6.4 Transcription-Replication Collisions

As stated above, one possible source of cyclin E-mediated replication stress is collision between the replication and transcriptional machineries. These encounters would be predicted to occur most frequently at fragile sites containing very long genes (>300 kb). Consistent with this, cyclin E overexpression produced copy number losses at two very long genes, *EPHA6* and *NCAM2*, in human mammary epithelial cells (approximate size of 935 kb and 545 kb, respectively) (Teixeira et al. 2015). In addition, transcriptional changes were observed at two other long genes, *DAB1* and *NRXN3*, in human fibroblasts (approximate size of 430 kb and 1.7 Mb, respectively) experiencing deregulated levels of cyclin E (Miron et al. 2015).

22.6.5 Sensitive DNA Structures

As alluded to the above, a number of the sites under-replicated after cyclin E overexpression correspond to fragile sites, a characteristic of which is the presence of nonlinear DNA structures expected to pose barriers to replication fork progression (Thys et al. 2015). However, most fragile sites were not represented as under-replicated sites in our analysis. One fragile site, nevertheless, is likely to be informative: FRA11B/G (Fechter et al. 2007; Burrow et al. 2009). This site on chromosome 11 is particularly interesting because it is the breakpoint for rearrangements in mixed-lineage leukemia (MLL); hence, the locus is referred to as MLL (Muntean and Hess 2012). It is also noteworthy that this site is also frequently deleted in breast cancer (see below). The under-replicated segment detected in our study was 4,331 bp, which contains part of the breakpoint cluster region (BCR) for MLL translocations in leukemia (Muntean and Hess 2012). We therefore scanned this region for DNA sequences predictive of the ability to form energetically favorable hairpin loop structures. Interestingly, two such structures were detected near the center of the segment separated by approximately 500 base pairs (Teixeira et al. 2015). To determine whether these structured DNA elements posed a barrier to replication under conditions of cyclin E-mediated replication stress, we cloned the segment containing them, with and without the palindromic sequences, into an episomal vector that replicates autonomously in mammalian cells. While both the control and palindrome-containing plasmids were well maintained in the absence of cyclin E overexpression, only the control plasmid was maintained when cyclin E was overexpressed. These data are consistent with the hypothesis that palindromic structures pose a barrier to replication, specifically under conditions of cyclin E-mediated replication stress (Teixeira et al. 2015). Therefore, two structural barriers to replication in close proximity may represent a feature that promotes sensitivity to cyclin E-mediated replication stress, although such structures are likely to be sensitive to other sources of replication stress as well (see below). However, it is worth noting that cyclin E overexpression has been determined to be associated with MLL

translocations in the context of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (Accordi et al. 2010).

22.7 Cyclin E and Genomic Instability in Breast Cancer

What does the observation that cyclin E overexpression promotes replication failure at a small subset of specific loci implicate for oncogenesis? The first question one might ask is are deletions in the chromosomal regions surrounding the under-replicated sites found in actual cancer? To address this, we interrogated a database of approximately 2000 breast tumors subjected to CGH array analysis (Teixeira et al. 2015; Curtis et al. 2012). As a surrogate for cyclin E overexpression, we employed copy number increases of the *CCNE1* locus, presumably due to gene amplification. This undoubtedly represents an underestimation of tumors overexpressing cyclin E and likely introduces experimental noise, as several posttranslational mechanisms have been shown to elevate cyclin E levels. Nonetheless, when *CCNE1* copy number increase was compared with copy number decrease at each of the under-replicated sites, a highly significant correlation was observed for many of them. This suggests that cyclin E overexpression is a driver of deletion at these sites. It should be noted that overall these specific sites experience deletions at relatively low frequency, and their detailed analysis is likely to yield clues concerning what characteristics constitute sensitivity specifically to cyclin E-mediated replication stress. On the other hand, some of the sites that were more frequently deleted in the data set did not show a significant correlation with cyclin E copy number increase. The MLL locus was one of these. Presumably, features of the MLL locus, specifically two likely hairpin loops in close proximity, render this site sensitive to multiple forms of replication stress, including but not exclusive to cyclin E. The second relevant question is whether these specific deletions have a direct role in oncogenesis. Unfortunately, at this point, we do not know the magnitudes or boundaries of deletions that occur surrounding these sites when they have not completed replication but are forced through anaphase. However, one might speculate that large deletions could drive oncogenesis by promoting loss of heterozygosity at tumor suppressor loci and deletions over fragile sites (Bignell et al. 2010). It is interesting to note that tumor suppressor genes have been identified on chromosomes 3q (Guo et al. 2002; Schwaenen et al. 2009; Thean et al. 2010) and 21q (Lee et al. 2003; Silva et al. 2003; Yamada et al. 2008), the arms where cyclin E-driven deletions occur in breast cancer.

In our study using immortalized non-transformed human mammary epithelial cells, it was clear that few cells that had sustained deletions after exposure to cyclin E overexpression were capable of expanding robustly and forming colonies. Although reassuring from a human health perspective, this observation raises the question of how cyclin E-driven deletions might get fixed in an expanding premalignant population. The answer probably lies in the fact that individual breast cancers when they present commonly possess more than 100 genetic modifications

(Nik-Zainal et al. 2016). At least some of these are likely to have been selected to override checkpoint barriers, thereby allowing clonal expansion of chromosomally damaged cells.

22.8 Relevance to Other Sources of Replication Stress

The discussion above has focused on mechanisms of genomic instability associated with cyclin E overexpression/deregulation (Fig. 22.1). However, other oncogenic events have been associated with replication stress, e.g., overexpression of c-MYC (Dominguez-Sola et al. 2007; Srinivasan et al. 2013; Rohban and Campaner 2015) or mutation of RAS (Di Micco et al. 2006; Miron et al. 2015; Maya-Mendoza et al. 2015). Although the mechanisms whereby these overexpressed or mutant proteins cause replication stress are likely to differ, one can infer that in severe instances, incompletely replicated genomes will enter mitosis with the end result being genomic instability.

A number of model system experiments support this idea. A hypomorphic allele of the mouse MCM helicase component MCM4, designated Chaos3, which promotes instability of the pre-replication complex (Kawabata et al. 2011), causes anaphase aberrations similar to what we observed for cyclin E overexpression. Although $MCM4^{Chaos3}$ does not appear to affect the number of origins fired, the number of dormant backup origins is reduced, and the number of stalled replication forks is increased, as it has been proposed for cyclin E overexpression. Presumably, intrinsic levels of replication stress during the normal replicative cycle require such backup origins in order to avoid under-replicated regions and the resulting aberrant anaphases. Interestingly, the $MCM4^{Chaos3}$ mouse is cancer prone (Shima et al. 2007), indicating that this type of chromosomal damage is directly linked to oncogenesis.

In yeast, the absence of the cohesin-like complex Smc5-Smc6 causes replication impairment at loci that contain replication barriers, such as the rDNA cluster (Torres-Rosell et al. 2007). Yet, cells progress through mitosis resulting in elevated rates of chromosomal nondisjunction, even though all checkpoints are intact. These results confirm that no robust checkpoint exists that can detect and respond to small quantities of unreplicated DNA in eukaryotes ranging from yeast to human (Mohebi et al. 2015; Koundrioukoff et al. 2013).

22.9 Conclusions

Elevated cyclin E has been shown to be associated with aggressive disease and poor outcome in at least some human malignancies. The link between cyclin E overexpression and genomic instability suggests a mechanism whereby cyclin E might promote oncogenesis. Our recent work showing that cyclin E overexpression promotes replication failure at a small number of specific loci and, as a consequence,

chromosomal damage following anaphase leaves some important unanswered questions relevant to oncogenesis. First and foremost is a detailed description of the genomic damage that occurs in individual cells. Such information will allow the assessment of whether the classic two-hit tumor suppressor model is applicable or whether more complex mechanisms apply such as amplifications and translocations. The second important issue to be resolved is whether cyclin E can serve as a prototype for other oncoproteins that cause replication stress and promote genomic instability. On the one hand, as outlined above, some of the modalities of cyclin E function in the context of the replication stress are likely to be unique. On the other, there are certain to be mechanistic commonalities. Only investigation of the pathways leading to replication stress and from replication stress to genomic alterations for other oncoproteins will resolve this question.

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