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Review

Running into problems: how cells cope with replicating damaged DNA

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Abstract

The ability of cells to fully and faithfully replicate DNA is essential for preventing genomic instability and cancer. DNA is susceptible to damage both in resting and in actively replicating cells. Thus, genome duplication necessarily involves replication of damaged DNA. The many mechanism cells use to avoid or overcome the problems of replicating an imperfect DNA template are discussed.

1. Introduction

Dividing cells are vulnerable to DNA damage from both internal and external sources. By converting relatively benign damage such as single-strand breaks or modified bases, into potentially cytotoxic double-strand breaks or fixed mutations, the act of DNA replication itself can exacerbate DNA damage. Thus, genomic integrity is especially vulnerable during the process of DNA replication. To cope with the need to complete replication in the presence of DNA damage, organisms have evolved with a number of mechanisms for replicating through or around damaged sites in ways that cause minimal genome instability. In addition, eukaryotes have evolved with a complex network of signal transduction pathways that slow DNA replication.

2. Checkpoint responses

In mammals there are two parallel pathways that respond to different types of DNA damage during replication. The first, described more than 20 years ago by Painter and Young, is defective in cells from patients with the hereditary, cancer prone disorder ataxia telangiectasia [1]. In normal human cells activation of ataxia telangiectasia mutated (ATM) depends on autophosphorylation and dissociation of ATM from dimeric units [2]. Within S-phase, the time in which DNA is replicated, ATM is required to prevent the firing of new origins of replication in the presence of double-strand breaks. A failure in this pathway results in the phenomenon of radiation resistant DNA synthesis (RDS), a phenotype that is shared with other cancer causing mutations that affect either damage-induced checkpoints or repair functions [3]. Even in normal cells, the presence of DNA damage does not completely block DNA replication. Forks that have already fired and are actively replicating DNA continue synthesis [4,5]. Thus, mechanisms that minimize damage

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when a replication fork reaches a fault in the template are essential to preserve genomic integrity. A second damage control pathway is regulated by the ATM related protein, ATR (ATM and Rad3 related). The ATM and ATR proteins are conserved members of a protein kinase family that includes DNA-PK (reviewed in [6]). In addition to a role in responding to double-strand breaks, the ATR pathway is activated by agents that directly interfere with replication fork progression such as hydroxyurea, thymdine, UV-light and DNA alkylating agents. ATR stably associates with a partner protein, ATRIP, that is required for function in vivo [7]. Recent works suggests that ATR-ATRIP is recruited to sites of damage through its association with the single-strand binding protein complex, RPA [8].

Directly downstream of ATM and ATR are two checkpoint kinases that are both activated by phosphorylation (reviewed in [9,10]). The two checkpoint kinases, Chk1 and Chk2 (which is known as Rad53 and Cds1 in budding and fission yeast, respectively) are well conserved at the level of sequence in eukaryotes. However current evidence suggest that they do not have equivalent function in all species. In budding yeast, the Rad53 protein kinase is an effector of both the DNA damage checkpoint and the replication checkpoint. Consistent with these dual roles, Rad53 is phosphorylated and activated both by agents that induce double-strand breaks and by agents that cause replicational stress. It is an essential gene with non-checkpoint functions [9]. Rad53 is required to resume DNA replication following transient replication blockage by nucleotide deprivation, polymerase dysfunction or DNA damage [11]. Recent analysis proves that preventing damage-induced DNA replication fork catastrophe is a primary function of the Mec1/Rad53 checkpoint pathway in budding yeast [5,12]. (For a more detailed review of the role of Rad53 and the S-phase checkpoint in budding yeast see the review by Longhese et al., in this issue). The function of Rad53 in replication resumption is explained, in part, by the observation that it is required to maintain stalled replication forks and to prevent their degeneration into abnormal structures [5,13]. In budding yeast, the second checkpoint kinase, Chk1, functions in parallel with Rad53 to prevent mitotic exit in response to DNA damage [14].

In fission yeast the Rad53 homologue, Cds1, is an effector of the replication checkpoint. Like Rad53, it is required to successfully resume replication after exposure to agents that deplete nucleotide pools [15,16]. However, unlike Rad53, Cds1 is not activated and does not mediate responses outside S-phase [17]. In fission yeast, Chk1 is the effector of the G2 damage-induced checkpoint and it does not respond to incompletely replicated DNA [18]. Neither Cds1 nor Chk1 is an essential gene in fission yeast, and even in the absence of both genes the organism is viable.

In human cells the Rad53/Cds1 structural homologue, Chk2 is rapidly activated in response to ionizing radiation at any stage of the cell cycle, but it is poorly activated in response to replicational blocks [19,20]. Chk2 phosphorylates key effectors of the mammalian checkpoint pathways, p53 [21,22] and the Cdc25 family of phosphatases [19,20], in vitro. However, phenotypic analysis of Chk2-deficient animals and cells shows that Chk2 is not required to slow DNA replication in the presence of DNA damage, nor is it required for recovery from replicational stress [23,24]. Data concerning the role of Chk2 in the G1 damage-induced checkpoint are not clear cut; it is likely that Chk2 has a role at low doses of irradiation, and that redundant pathways mask its contribution at higher doses [23–25].

By contrast, the mammalian Chk1 homologue is phosphorylated and activated in response to incomplete DNA replication [26]. Chk1 phosphorylates and inactivates Cdc25 in vitro and loss-of-function studies indicate an important role for Chk1 in both the G1 and the G2 damaged induced checkpoints [27–29]. The early embryonic lethality of Chk1−/− mice, and the death that occurs in Chk1 deficient murine cells, make it difficult to assess its function in specific checkpoint responses. Nevertheless, painstaking phenotypic analysis of Chk1−/− murine cells support the idea that it is required for the IR-induced G2 checkpoint and for recovery from replicational stress [28,29]. Disruption of Chk1 in somatic chicken (DT40) cells is not lethal, and Chk1 is required for damage-induced G2 arrest and for maintaining viable replication forks when DNA polymerase is inhibited [30]. The requirement for avian Chk1 in maintaining replication forks during replication arrest supports the conclusion of an earlier study in which a chemical inhibitor was used to show that Chk1 is needed to ensure that activation of late replication origins is blocked and arrested replication fork integrity is maintained when DNA

synthesis is inhibited in human cells [31]. Together, the loss-of-function studies in murine and avian cells provide compelling evidence for the importance of Chk1 in recovery from replicational stress in higher eukaryotes. Likewise, the Chk1 homologues in *Xenopus* and *Drosophila* are implicated in replicational stress responses [32,33]. Thus, a primary function of the yeast Rad53/Cds1 checkpoint kinases, recovery from replicational stress, appears to be regulated by the structurally unrelated Chk1 kinase. Perhaps when more is known of the mechanisms by which cells recover from replicational stress this paradox will be unraveled.

Phosphorylation and activation of the checkpoint kinases is dependent on a family of mediator proteins. The mediator proteins are a weakly conserved group of proteins, characterized as being large, acidic BRCA1 carboxy-terminal (BRCT) domain and fork head-associated (FHA) domain containing proteins that are essential for the phosphorylation and activation of the checkpoint kinases [9]. The founder member of the group, Rad9, is required for the radiation-induced activation of Rad53 [34]. A distantly related protein, Claspin, interacts with and is important for Chk1 activation in *Xenopus* and in human cells [35,36]. A protein named Mrc1 is important for the phosphorylation and activation of Cds1 and Rad53 in fission and budding yeast, respectively [37,38]. A model in which activation of the checkpoint kinases is both promoted and controlled by the mediator proteins is emerging in yeast. It is too early to say if the same model will hold in higher eukaryotes. The functions of other direct substrates of ATM and ATR involved in both sensing DNA damage, in amplifying the signal generated by DNA damage, and in repairing the damage are reviewed in [9].

An important function of the DNA damage checkpoint is to delay cell cycle progression until repair has been achieved. If cells are in G1 at the time damage is sustained, the checkpoint can prevent the initiation of S-phase until damage is repaired and thus the problems of replicating damaged DNA are avoided. p53, the primary regulator of the G1 damage checkpoint in mouse and human cells is both directly and indirectly regulated by ATM and ATR in response to DNA damage [39]. Following DNA replication, ATM and ATR are required to arrest cells prior to mitosis. As discussed above, Chk1 appears to be the main effector of the G2/M DNA damage checkpoint [27–29]. By delaying the onset of mitosis cells preserve the opportunity to use recombination repair and reduce the possibility of mis-segregating small chromosome fragments. Unlike the G1 to S, or G2 to M checkpoint, in which DNA damage imposes a complete block to cell cycle progression the S-phase DNA damage checkpoint merely slows the rate of replication in response to DNA damage; it does not halt replication. At first glance, the slowing of DNA replication might provide time for repair, reducing the incidence at which damage is encountered by a replication fork. However, the idea that replication is slowed to allow time for repair is a hypothesis. An equally plausible possibility is that damage-induced repair processes cause the slowing of DNA replication [40]. Cells may switch to a modified form of DNA replication, repair coupled-replication, which is more appropriate for replicating damaged DNA, but which is inherently slow. If this is the case, replication is not slowed to reduce the frequency at which forks hit damage, replication is modified to more appropriately copy a damaged template. The latter hypothesis is supported by recent evidence that damaged induced fork slowing is dependent on recombination repair protein XRCC3 in mammalian and avian cells [41]. Regardless of the mechanism by which fork progression is slowed, replication continues in the presence of DNA damage, and thus it is inevitable that active replication forks will sometimes encounter DNA damage. The remainder of this review focuses on the different mechanisms used to ensure that DNA replication is completed even in the presence of DNA damage. The order of the options listed below is not intended to imply any order by which these mechanisms might be used within cells.

3. Repair procedures that facilitate DNA replication

3.1. Option 1: repair the damage before the fork hits it

In principal, damage that is restricted to one strand of the duplex can be repaired by removal of the damaged region and copying of the information retained on the remaining strand. Nucleotide excision repair, both global and transcription coupled, efficiently removes bulky photoproducts such as UV-induced pyrimidine

dimers and 6–4 photoproducts that cause significant distortion to the DNA helix. Small chemical alterations are targeted by a similar "cut and patch" repair system known as base excision repair [42]. Because nucleotide and base excision repair rely on copying an undamaged strand, they are largely error free processes. Damage can be repaired prior to, during and following DNA replication. However, many base modifications do not cause significant helix distortion, and they are poorly recognized by the excision repair pathways. Because these damaged sites persist longer in the genome they create problems when encountered by an active replication fork. A third form of excision repair, mismatch repair removes mispaired nucleotides that result from DNA polymerase error, from slippage during replication or during recombination when similar, but not identical sequences may form a heteroduplex. Eukaryotic mismatch repair involves several homologues of the *E. Coli* MutS and MutL family that recognize the mismatch; both exonuclease and endonuclease activities are required to degrade the newly synthesized, damaged strand. Resynthesis, using the parental strand as template, is catalyzed by DNA polymerases δ and ε [43]. Defects in the mismatch repair system dramatically increase mutation rates and predispose individuals to hereditary non-polyposis colorectal cancer and to a variety of sporadic cancers [44].

3.2. Option 2: use a translesion polymerase to bypass the damage

If damaged nucleotides could be recognized and correctly decoded by DNA polymerase they would not block DNA replication. However, replication of the human genome requires the copying of 6×10^9 bases in 6–7 h. Even when this job is divided among ∼3000 active replication forks it still requires that each fork be moving at a rate of ∼50 bp/s. The evolution of DNA polymerases that are both fast enough and accurate enough to complete replication of the entire genome has probably been achieved at the cost of flexibility, and the replicative polymerases are unable to decode many of the common forms of damaged bases that result both from intrinsic and extrinsic damage. One way to get past damaged nucleotides is to use specialized polymerases called translesion or bypass polymerases [45]. These are a special class of DNA polymerase that can decode damaged nucleotides, but which lack the speed and specificity of the highly processive replicative polymerases. The remarkable capacity that translesion polymerases have for inserting the correct nucleotide opposite a damaged base, and thus for contributing to genomic stability is illustrated by the discovery that the variant form of xeroderma pigmentosum (XP) is caused by a defect in polymerase η, a translesion polymerase that preferentially inserts adenosine opposite thymidine containing lesions [46,47]. XP is a disorder, which in most cases is caused by a defect in the nucleotide excision repair process [42]. In patients with XP-variant, removal of bulky adducts (by nucleotide excision repair) is normal; it is the replication past remaining unrepaired damage that is defective.

Bypass polymerases have high error rates when using intact DNA as a template in vitro, and thus it appears that these activities need to be tightly regulated in vivo. The mechanism by which cells control the switch between replicative polymerases and the bypass polymerases is an area of considerable interest. One factor may be damage-induced modifications of PCNA. During DNA replication PCNA forms a trimeric ring that encircles DNA, creating a sliding clamp that helps to tether the replicative polymerases to the DNA and that promotes processive DNA synthesis [48]. PCNA also interacts with and enhances the activity of several bypass polymerases (reviewed in [49]). Damage-induced ubiquitination and sumosylation of PCNA has been shown to control switching between alternative repair processes that make use of different translesion polymerases [50]. Thus, it is possible that differential modification of PCNA enhances the recruitment of specialized repair promoting polymerases [51].

Another factor likely to influence polymerase switching involves the replacement of PCNA by the structurally homologous damage-specific sliding clamp. The checkpoint or damage-specific sliding clamp, known as the 9-1-1 complex, is composed of a trimer of related proteins that in mammalian cells are encoded by Rad9, Rad1 and Hus1. Modeling suggests that the three proteins form a structure that is similar to PCNA [52]. The 9-1-1 complex associates with DNA in a damage-induced manner [53], loading is promoted by the damage-specific clamp-loading complex of Rad17-RFC [54]. The recruitment of the 9-1-1 complex to sites of damage is crucial for efficient

checkpoint signaling as it promotes phosphorylation of key ATR substrates (reviewed in [55]). In addition, the observation that DinC, a translesion polymerase in fission yeast, associates with components of the damage-specific clamp loader suggests that the clamp loader plays a role in recruiting bypass polymerases to regions of damaged chromatin [56] (see the review by Kai and Wang in this issue). The full potential that the damage-specific clamp loader, the 9-1-1 sliding clamp, and modified forms of PCNA have for recruiting repair proteins to promote replication in the presence of damage remains to be explored.

3.3. Option 3: bypass through template switching

In theory, if a lesion is in the lagging strand, priming of synthesis at a new site downstream of the damaged region, would enable lagging-strand synthesis to continue. A single-strand gap, that has to be filled in, or fixed by recombination will be left behind the progressing fork, but the overall process of DNA replication would not be blocked. The mechanism by which lagging-strand synthesis is achieved is quite well understood, how it might be modulated in the presence of a damaged template is not [57]. When replication forks encounter lesions on the leading that halt polymerase activity, the forks stall. The inability to continually move forward is thought, in some cases, to result in the process of fork reversal that leads to the formation of a X-shaped structure, graphically named a chicken-foot, and structurally related to the four-branched structure known as a Holliday junction [58]. Regression of blocked forks can be driven non-enzymaticaly by the release of super-helical tension that accumulates ahead of a moving fork [59]. It can also be driven enzymatically, as in *E. coli* where fork regression and Holliday junction formation are promoted by the RecG DNA helicase [60]. Whether it is spontaneous or enzymatic, the process of fork reversal drives reassociation of the parental strands by displacement of the newly synthesized strands and involves pairing of the two daughter strands. At its simplest, fork reversal might allow the fork to be held stable while a synthesis-blocking lesion is removed. In the case where fork progression is interrupted not by DNA damage, but by the physical presence of protein complexes, perhaps the transcription machinery or repair apparatus ahead of the fork, fork regression might allow the replication fork to be held in a stable form while the physical block is removed.

An alternative use for a regressed replication fork, which enables cells to bypass damage, was proposed by Higgins et al. [61]. This model is based on the assumption that if synthesis is blocked by a lesion on the leading strand template then replication could continue past the equivalent point on the complementary lagging strand. The annealing of the parental strands by the process of fork regression and branch migration, would permit pairing of the nascent daughter strands, and because the nascent lagging strand is longer than the nascent leading strand it can act as template for extension. Migration of the Holliday junction back into the fork structure would re-establish semi-conservative base pairing and allow synthesis to resume past the point of damage. In this model damage is simply bypassed, presumably it can be repaired once the area has been fully replicated. The ability to bypass one or two damaged nucleotides provides an attractive hypothetical mechanism by which cells might efficiently complete replication in the presence of commonly occurring damage such as that caused by UV-light. Higgins et al., presented physical evidence for the operation of the model in the form of electron micrographs showing four pronged replication structures and the presence of daughter–daughter duplex more than 20 years ago [61]. However, the frequency at which these structures form, and whether they actually represent productive intermediates in a fork rescue process is a matter of conjecture.

3.4. Option 4: recombination repair

In each of the above mechanisms the replication fork can be thought of as having stumbled at a problem, but in all cases the replication machinery was maintained on the DNA duplex and replication was able to resume where it was interrupted. Template switching models allow bypass and resumption of DNA replication downstream of persistent damage. However, no amount of regression and reversion can replicate past a break in the template or passed duplex strands that are held together by interstrand cross-links. Thus, a mechanism of replication restart is needed to cope with fork blocking problems that cannot be, or are not, fixed in a timely manner. In bacteria, replication fork restart or recovery through

homologous recombination is the favored path for fork restoration under normal growth conditions [62]. Increasingly, the repair of replication forks through homologous recombination is seen as a major function of the eukaryotic recombination systems as well [63]. The process of homologous recombination requires a free double-strand end and an intact homologous duplex to act as the template for extension of the free end [64]. If an active replication fork encounters a single-strand break, the act of replication could itself generate a double-strand end. In cases where the backbone of the replication template is intact, but damage cannot be bypassed, cleavage of the regressed replication fork or Holliday junction is required to generate the free end. Deliberate release of a free double-strand end might be expected to increase the possibility of inaccurate recombination. Nevertheless, evidence that such pathways are commonly used both by prokaryotes and eukaryotes cells is accumulating. For example, Holliday junctions are formed from stalled replication forks in eukaryotes under conditions of replicational stress [13,61,65]. Whether they also occur in non-stressed cells is less clear; it is possible that they occur quite frequently but that endogenous Holliday junction resolvase activities are sufficient to rapidly process the junctions. In bacteria, the Holliday junction resolvase, RuvC, nicks opposing strands to generate an intact duplex and one free double-strand end [58]. Two activities that resolve Holliday junctions into linear duplex have been identified in human somatic cell extracts [66,67]. One, that is of unknown composition and is known as Resolvase A, behaves very much like the RuvABC complex in that it introduces symmetrical related nicks into strands of like polarity and associates with branch migration activities [67,68]. A second human Holliday junction resolvase activity was found associated with Mus81 [66,67]. Mus81 resolves Holliday junctions into linear duplexes by cleaving exclusively on strands of like polarity, but unlike Resolvase A or RuvC, Mus81 is not known to associate with branch migration activity. Given that there would be little advantage in branch migration in the context of replication fork recapture, the lack of associated branch migration activity makes Mus81 an attractive candidate to catalyze the cleavage of Holliday junctions formed at stalled replication forks.

In yeast, Mus81 mutants are hyper-sensitive to DNA damaging agents that impede DNA replication, and Mus81 mutation is lethal in combination with disruption of members of the RecQ helicase family, Rqh1 or Sgs1 [69,70]. In humans, mutations in the RecQ-like helicases, BLM and WRN, are associated with defective DNA replication, genomic instability and increased incidence of cancer [71]. BLM and WRN have both been demonstrated to branch-migrate Holliday junctions in vitro [72,73]. Thus, one explanation for the genetic interaction between Mus81 and the RecQ helicases is that the absence of the RecQ helicases leads to an accumulation of Holliday junctions and thus a greater dependence on Mus81 function to complete DNA replication. However, the phenotypes and biochemical studies of cells that lack WRN and BLM imply that these proteins have additional roles in replication and repair. In particular a number of RecQ helicases, including BLM, have been found to associate with DNA topoisomerase activities. Thus, the requirement of the RecQ helicases in DNA replication and genomic integrity may be explained by their role in promoting topoisomerase catalysed release of super-helical tension or decatenation of DNA [71].

Based on the phenotypes of fission yeast mutants of Mus81, the rescue of Mus81 deficiency by a prokaryotic Holliday junction resolvase, and in vitro evidence that Mus81-containing complexes resolve Holliday junctions in vitro, Boddy et al. proposed that Mus81 and the Mus81 binding protein, Eme1, are subunits of a nuclear Holliday junction resolvase [74]. A similar function is predicted for the human protein [66]. An alternative function for Mus81, based on its ability to cleave replication forks, was proposed to account for the role that Mus81 plays promoting replication in budding yeast [75,76]. Recent analysis of budding yeast mutants confirms that Mus81 acts downstream of the regressed replication fork and is not likely to directly cleave replication forks [77,78]. The observation that Mus81-associated endonuclease efficiently cleaves 3′ flaps in vitro suggests a role in trimming flaps that might arise following extension of a 3′ end during the process of synthesis-dependent strand annealing (SDSA) [67,75,78]. SDSA is an attractive model for double-strand break repair: Because it can be accomplished without forming a Holliday junction it could account for mitotic recombination without

cross-over [79]. However, models of SDSA are based on the presence of two double-strand ends generated by a break. In the case of replication restart, cleavage of the regressed fork would generate only one double-strand end, thus, replication fork recapture inevitably results in the formation of at least one Holliday junction [63,80]. Thus, one line of evidence that favors a role for Mus81 in Holliday junction resolution is that Mus81 mutants are sensitive to agents that interfere with replication but not that induce double-strand breaks [69,81]. Secondly, Mus81 deficiency is rescued by expression of a bacterial Holliday junction resolvase, RusA [74,78,82]. If Mus81 deficiency resulted in a failure to cleave a 3′ flaps generated by SDSA it would not lead to Holliday junction accumulation, and thus, one would not expect expression of a Holliday junction resolvase to rescue a defect in SDSA. This interpretation depends on knowing the specificity of RusA when it is expressed in vivo in yeast. The available data strongly support the assertion that RusA acts exclusively on Holliday junctions in vivo [82,83]. Thus, ability of RusA to rescue Mus81 defects coupled with biochemical evidence that Mus81-Eme1 resolves Holliday junction in vitro, suggests that Mus81-Eme1 resolves Holliday junctions in vivo. Nevertheless, an alternative function for Mus81 based on its in vitro preference for 3′ flaps and recombination rates analysis remains favored by others [78,84].

Once generated, by Holliday junction resolution or directly from a single-strand nick, the free double-strand end is acted upon by a 5′ to 3′ exonuclease to generate a region of single-strand DNA. The annealing of the resected single-strand region to homologous sequence in the intact duplex provides a template for DNA synthesis. In this respect, replication restart is similar to the repair of double-strand breaks through homologous recombination and depends on many of the same proteins [79]. Because resumption of replication depends on copying the intact sister chromatid, it can be both error free and non-recombinogenic. The potential for error, if it occurs, is that a similar but not identical region of the genome will be invaded and significant loss or duplication of information will occur. Perhaps, this risk is worth taking if the only other option is failure to complete replication.

4. Conclusion

Although it is clear that all of the above mechanisms contribute to the successful completion of DNA replication, their relative contributions in different organisms and in different levels of replicational stress remains to be determined. The observation that DNA replication is remarkably efficient and largely error free is testimony to the many powerful mechanisms the cell has used to overcome any obstacles that might prevent it from generating progeny that are a perfect copy of its imperfect self.

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