Review

More forks on the road to replication stress recovery

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High-fidelity replication of DNA, and its accurate segregation to daughter cells, is critical for maintaining genome stability and suppressing cancer. DNA replication forks are stalled by many DNA lesions, activating checkpoint proteins that stabilize stalled forks. Stalled forks may eventually collapse, producing a broken DNA end. Fork restart is typically mediated by proteins initially identified by their roles in homologous recombination repair of DNA double-strand breaks (DSBs). In recent years, several proteins involved in DSB repair by non-homologous end joining (NHEJ) have been implicated in the replication stress response, including DNA-PKcs, Ku, DNA Ligase IV-XRCC4, Artemis, XLF and Metnase. It is currently unclear whether NHEJ proteins are involved in the replication stress response through indirect (signaling) roles, and/or direct roles involving DNA end joining. Additional complexity in the replication stress response centers around RPA, which undergoes significant post-translational modification after stress, and RAD52, a conserved HR protein whose role in DSB repair may have shifted to another protein in higher eukaryotes, such as BRCA2, but retained its role in fork restart. Most cancer therapeutic strategies create DNA replication stress. Thus, it is imperative to gain a better understanding of replication stress response proteins and pathways to improve cancer therapy.

Keywords: DNA repair, genome instability, cancer therapy

Introduction

Replication of genetic information is a fundamental biological process that is common to all living organisms from microorganisms such as simple viruses to the most complex multicellular organisms on the Earth (Machida et al., 2005). DNA repair systems protect cells from damage caused by numerous intrinsic and extrinsic threats, thereby helping to maintain genome integrity. The genome is particularly vulnerable to change during DNA replication, and a growing body of evidence supports a nexus between replication and repair factors that act in complex enzymatic and signaling networks in response to DNA damage-induced replication stress. Many proteins have been identified during the last decade that function to stabilize and restart stalled replication forks, and restart collapsed forks. Most of these proteins have well-defined roles in homologous recombination (HR) repair of DNA double-strand breaks (DSBs) and/or DNA damage checkpoint signaling. For detailed information about the core HR and checkpoint proteins that function in the replication stress response, the reader is directed to several excellent recent reviews (Cimprich and Cortez, 2008; Branzei and Foiani, 2009; 2010; Budzowska and Kanaar, 2009; Ciccia and Elledge, 2010; Petermann and Helleday, 2010; Smits et al., 2010; Warmerdam et al., 2010). In this review, we briefly survey current replication stress paradigms, and highlight recent evidence that implicates other DNA repair factors in the mammalian replication stress response, including DNA-PKcs, Ku and DNA Ligase IV (LigIV), which were originally identified as key factors in non-homologous end-joining (NHEJ), Metnase, a recently discovered NHEJ factor, and two less-well-characterized HR factors, RPA and RAD52.

Cellular replication stress responses

The accurate duplication and distribution of DNA to daughter cells is regulated by a network of coordinated processes, including proofreading, DNA repair, cell cycle checkpoints, chromosome decatenation and chromosome segregation. DNA is subject to many forms of damage caused by intrinsic and extrinsic factors, including metabolic reactive oxygen species, ionizing radiation (IR), UV light, genotoxic chemicals and topoisomerase poisons (Hanks et al., 2004). Cells are particularly vulnerable to DNA damage during DNA replication because virtually all forms of DNA damage block DNA replication, causing replication stress (Branzei and Foiani, 2010). Replication fork stalling is also caused by hydroxyurea (HU) which depletes nucleotide pools, the Topoisomerase I inhibitor camptothecin, the Topoisomerase IIα (TopoIIα) inhibitor etoposide and the DNA polymerase inhibitor aphidicolin. DNA repair and S-phase checkpoint systems form a network that responds to replication stress and is critical for maintaining genome stability and cancer suppression. S-phase hypersensitivity to DNA damage is

the basis for the majority of current cancer chemo- and radiotherapeutics, which cause DNA damage directly or indirectly (Hayden, 2008; Hutchinson, 2010; McNeely et al., 2010).

Cells respond to stalled forks in several ways. Uncoupling of the replicative helicase from DNA polymerase leads to accumulation of single-stranded DNA (ssDNA) bound by RPA, which is a major signal for downstream events, including fork repair (Petermann and Helleday, 2010) and checkpoint activation (Branzei and Foiani, 2009; Budzowska and Kanaar, 2009). The replisome at stalled forks is stabilized by proteins that function in DNA repair and the DNA damage checkpoint response, including RPA, ATR-ATRIP, ATM, BLM and INO80 (Zou et al., 2006; Davies et al., 2007; Shimada et al., 2008; Budzowska and Kanaar, 2009); the action of these proteins may preserve the fork structure while the damage is repaired. Alternatively, error-prone translesion synthesis polymerases may be recruited to monoubiquitinated proliferating cell nuclear antigen (PCNA), allowing lesion bypass in a damage tolerance pathway (Moldovan et al., 2007; Niimi et al., 2008). If a stalled fork is not restarted in a timely manner, it may convert to unusual DNA structures and collapse to one-ended DSB ('double-strand end'—DSE). When a replication fork encounters a single-strand break in a template strand, this may result in direct fork collapse to a DSE. Analogous to their action at frank DSBs (i.e. DSBs directly induced by radiation or nucleases), ATM and ATR are recruited to collapsed forks and phosphorylate histone H2AX (γH2AX) in the vicinity of DSEs (Ward and Chen, 2001), activating checkpoint and repair processes (Downey and Durocher, 2006; Chanoux et al., 2008).

The replication stress (intra-S) checkpoint involves stepwise activation of damage sensor, transducer and effector proteins (Budzowska and Kanaar, 2009) (Figure 1). ssDNA bound by RPA at stalled forks recruits ATR through an ATRIP–RPA interaction. ATR activation depends on RAD17 (plus Rfc2–5) loading of the RAD9-HUS1-RAD1 complex (9-1-1; a PCNA-like scaffold and processivity factor) through a RAD9–RPA interaction. RAD9 recruits TopBP1, an essential factor for ATR activation. ATR phosphorylates RAD17, which recruits Claspin to be phosphorylated by ATR. Phosphorylated RAD17-Claspin (along with TIM and its partner Tipin) promotes ATR phosphorylation/activation of Chk1 which phosphorylates effector proteins that stabilize stalled forks, repair collapsed forks and prevent late origin firing—presumably to prevent further encounters of forks with DNA damage (Ciccia and Elledge, 2010; Kemp et al., 2010). However, there is both recent and older evidence indicating that broken forks stimulate replication initiation at adjacent, dormant origins, presumably to complete replication of sequences that were not replicated by the broken fork (Taylor and Hozier, 1976; Doksani et al., 2009).

HR: a primary replication fork restart pathway

HR is often described as 'error-free', but HR involving single-strand annealing (SSA) or crossovers can result in large-scale genome rearrangements including deletions, inversions and translocations (Weinstock et al., 2006; Shen and Nickoloff, 2007). HR is under strict regulation: levels vary during the cell cycle (Shrivastav et al., 2008; Ciccia and Elledge, 2010) presumably to balance the benefits of local error-free repair of DSBs and restart of blocked replication forks, while minimizing 'risky' HR (crossovers, SSA). HR factors also produce and resolve unusual structures at stalled forks, such as 'chicken feet' (Gangloff et al., 2000). HR occurs spontaneously at relatively high frequencies compared with point mutagenesis (10^{-5} vs 10^{-8}–10^{-9}). The large-scale genetic changes resulting from HR are important drivers of protein evolution and tumor evolution (Strout et al., 1998; Deininger and Batzer, 1999; Kolomietz et al., 2002).

HR plays a major role in restarting stalled and collapsed forks, and this role is essential in higher eukaryotes (Sonoda et al., 1998; Budzowska and Kanaar, 2009). Most spontaneous HR occurs during DNA replication as a consequence of replication forks encountering DNA damage (Arnaudeau et al., 2001; Saleh-Gohari et al., 2005). In addition to core HR proteins

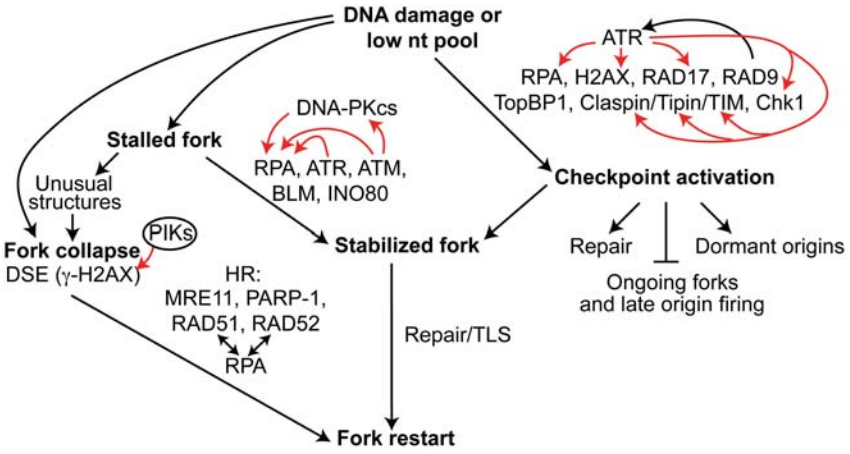

Figure 1 Key proteins and pathways in the replication stress response network. Black arrows/bars indicate activation/suppression pathways or interactions between proteins; red arrows indicate some of the known phosphorylation events by indicated PIKs. Note that several proteins, such as RPA and ATR, have roles in more than one pathway in this network.

(RAD51 and its paralogs, RAD54, BRCA1/2, BLM), replication stress-induced HR depends on PARP-1 dependent recruitment of MRE11 (in the MRN complex with RAD50 and NBS1) (Bryant et al., 2009).

At stalled forks, HR catalyzes template switching of a blocked replicating strand to the undamaged sister chromatid where DNA synthesis and a second template switch bypass the blocking lesion. When lesions occur in repeated sequences, invasion can occur in or out of register, with the latter producing detectable genetic rearrangements (Figure 2). Thus, high levels of (detectable) HR using linked repeat reporter systems reflect a type of 'error-prone' HR that is not necessarily a desirable outcome, nor do such HR events necessarily correlate with increased survival after replication stress (Saintigny et al., 2001; Lundin et al., 2002) (M. Shrivastav, A.K.A., C.A., N. Sharma and J.A.N., unpublished results). Similarly, at collapsed forks, HR mediates strand invasion of the 3′ end of a DSE into sister chromatids, a process termed break-induced replication (BIR) (Figure 3) (Llorente et al., 2008). In yeast, BIR is known to replicate entire chromosome arms (>100 kb), leading to large-scale loss of heterozygosity, and can occur by relatively efficient Rad51-dependent, or inefficient Rad51-independent mechanisms (Malkova et al., 2001; Davis and Symington, 2004; Krishna et al., 2007; Lydeard et al., 2007). As in the strand-switching mechanisms shown in Figure 2, BIR can also give rise to detectable HR-mediated rearrangements in repeated regions. Because HR in repeated regions can lead to rearrangements, genome stability depends on accurate repeat alignment during HR; suppressing these rearrangements is critical in mammalian genomes that comprise at least 50% repetitive sequences. It is well established that HR between repetitive sequences, like Alu elements, is a significant source of genome instability in cancer and other diseases (Deininger and Batzer, 1999; Kolomietz et al., 2002; Elliott et al., 2005). Moreover, defects in the HR proteins BRCA2 and BLM specifically shift repair toward genome-altering HR mechanisms (SSA and crossovers, respectively), and predispose to highly penetrant, early onset cancer syndromes (Tutt et al., 2001; Wu and Hickson, 2003). Thus, accurate HR is essential for maintaining genome stability and tumor suppression, particularly in cells experiencing replication stress.

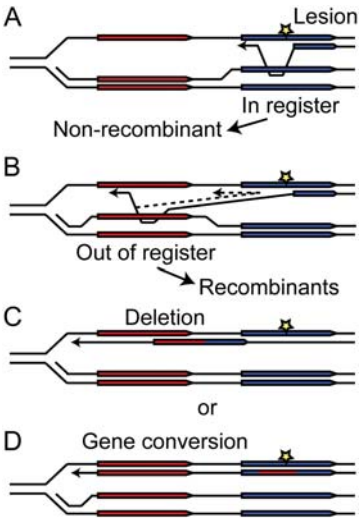

Figure 2 Replication-associated HR can occur with or without genetic rearrangement. A lesion in one repeat (blue) blocks replication which restarts by template switching in register (**A**), an undetectable error-free HR event, or out of register (**B**) which can produce detectable rearrangements. After the out of register template (red) is copied, a second template switch yields repeat deletion (solid arrow in **B** gives intermediate in **C**), or gene conversion (dashed arrow in **B** gives intermediate in **D**). Similar events can occur with blocked leading or lagging strand. The heteroduplex intermediates in **C** and **D** are subject to mismatch repair which can fix (make permanent) the genetic changes or restore the parental configuration, or heteroduplex may go unrepaired and segregate during the next mitosis, producing one daughter cell with a deletion or gene conversion and one daughter cell with the original parental configuration. Note that lesion is not repaired; these are HR-mediated lesion bypass mechanisms.

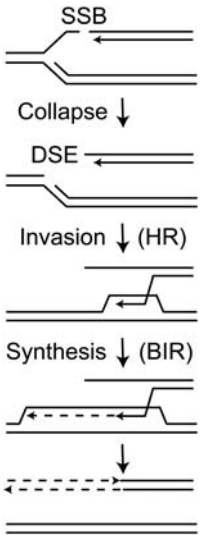

Figure 3 Break-induced replication rescues collapsed forks. A DSE at a collapsed fork can invade the sister chromatid and DNA synthesis can extend the broken end to the end of the chromosome. Synthesis of the second strand completes the replication process.

A conserved role for mammalian RAD52 in HR-mediated replication fork restart?

Yeast Rad52 (yRad52) has essential roles in HR repair of frank DSBs, acting as a mediator of yeast Rad51, and it also has key roles in replication-associated HR (Lisby et al., 2001; 2003; Liberi et al., 2005; Lettier et al., 2006). yRad52 and mammalian RAD52 show significant amino acid sequence similarity (Shen et al., 1995), and similar biochemical activities (Benson et al., 1998; West, 2003), suggesting a conserved function through evolution. However, knockout of *RAD52* reduces HR-mediated gene targeting efficiency in mouse ES cells by only 30–40%, it has little effect on radiation resistance (Rijkers et al., 1998), and unlike yeast, RAD51 focus formation in mammalian cells does not require RAD52 (van Veelen et al., 2005). *RAD52* knockout in chicken DT40 cells

reduces gene targeting to a greater degree (3- to 10-fold), but it does not change radioresistance (Yamaguchi-Iwai et al., 1998). These results suggest that mammalian RAD52 functions have been supplanted by another RAD51 mediator, such as BRCA2.

A recent study focused on the functional interactions between human RAD52, RAD51 and the BRCA2-interacting protein BCCIP (Wray et al., 2008). Although co-immunoprecipitation assays revealed that RAD51 interacts with BCCIP and with RAD52, there was no detectable interaction between BCCIP and RAD52. Wray et al. (2008) found that RAD51 co-localizes with BCCIP in nuclear foci early after IR, and with RAD52 later, but there was little co-localization of BCCIP and RAD52. Interestingly, RAD52 foci are more prevalent after replication stress induced by HU than after direct DSB induction by IR. Fluorescence recovery after photo-bleaching showed that RAD52 diffusion is reduced to a greater extent by HU than IR (Wray et al., 2008). Reduced RAD52 diffusion, and reduced focus formation, reflects RAD52 functions in multimeric complex formation and association with chromatin at sites of DNA damage. These results are consistent with a model in which BCCIP/BRCA2-dependent repair of DSBs by HR is an early response to IR-induced DNA damage, with RAD52-dependent HR occurring later to restart blocked or collapsed replication forks. Although further studies are required to firmly establish a role for mammalian RAD52 in replication-associated HR, the evidence to date suggests that mammalian RAD52 may have lost its role in frank DSB repair but retained its role in HR-mediated replication fork restart.

NHEJ protein roles in the replication stress response

Several reports provided early evidence that NHEJ factors are involved in the replication stress response. CHO V3 cells (DNA-PKcs null) were found to be sensitive to the replicative-stress agents campothecin (Arnaudeau et al., 2001), HU and thymidine (Lundin et al., 2002), although not nearly as sensitive as cells lacking key HR proteins such as XRCC3. Based on these results, these authors concluded that replication fork restart was primarily mediated by HR, and that NHEJ played a lesser role in survival during replication stress. They further suggested that this lesser role could be direct, i.e. via NHEJ of DSEs arising at adjacent collapsed forks. However, because forks are often widely spaced, this process would result in large-scale deletions or other types of genome rearrangements. NHEJ has been proposed to mediate replication stress-associated, large-scale genome rearrangements due to transient shifts from HR toward NHEJ (Hastings et al., 2009). An alternative explanation is that DNA-PKcs promotes HR by phosphorylating HR factors and via crosstalk with ATM and ATR (Yajima et al., 2006; Shrivastav et al., 2009, Ciccia and Elledge, 2010). This is perhaps more appealing because it avoids problems associated with NHEJ of DSEs, and because it was later shown that cells lacking DNA-PKcs also down-regulate ATM (Peng et al., 2005; Shrivastav et al., 2009), and ATM has well-established roles in both fork stabilization and HR (Ciccia and Elledge, 2010). However, not surprisingly, the complexities of the replication stress response are refractory to such tidy explanations, as defects in other NHEJ proteins, including Ku, and the LigIV binding partner XRCC4, also confer sensitivity to replication stress (Saintigny et al., 2001). Yeast Ligase IV, however, reportedly has no role in DNA replication or RAD52-dependent HR (Teo and Jackson, 1997). Other than DNA-PKcs, none of these NHEJ proteins has known signaling roles, and the XRCC4-LigIV complex, in particular, is known to catalyze the ligation step of NHEJ, again suggesting a direct role for NHEJ in promoting survival during replication stress, although not necessarily through NHEJ of distant DSEs. In the following sections, we review recent studies aimed at defining the mechanisms by which specific NHEJ factors are involved in the replication stress response.

DNA-PKcs: signaling (and repair?) roles in the replication stress response

ATR, ATM and DNA-PKcs are upstream PI3-like kinases (PIKs) in the DNA damage response. PIKs show a degree of differential activation under various stress conditions and differences in target proteins, but PIKs also show significant functional overlap and crosstalk. For example, ATM and DNA-PKcs are both activated by DSBs, they phosphorylate at least six shared targets with known roles in HR (including H2AX, RPA and c-abl), and both ATR and ATM regulate DNA-PKcs via phosphorylation (Baskaran et al., 1997, Burma et al., 2001; Kastan et al., 2001; Chan et al., 2002; Kitagawa et al., 2004; Chen et al., 2005; Yajima et al., 2006; Chen et al., 2007; Shrivastav et al., 2009).

Phosphorylated DNA-PKcs localizes to replication centers following replication stress (Chen et al., 2005) and as noted above, DNA-PKcs-defective cells are hypersensitive to replication stress (Arnaudeau et al., 2001; Saintigny et al., 2001; Lundin et al., 2002; Yajima et al., 2006; Shimura et al., 2007). Although early studies gave conflicting results on whether DNA-PKcs phosphorylates RPA in response to DNA damage (Boubnov et al., 1995; Fried et al., 1996), the bulk of the evidence is consistent with such a role (Shao et al., 1999; Wang et al., 2001; Block et al., 2004; Cruet-Hennequart et al., 2006; Anantha et al., 2007; Cruet-Hennequart et al., 2008; Stephan et al., 2009). DNA-PKcs also regulates the stability of histone mRNA abundance through phosphorylation of the RNA helicase UPF1, linking DNA-PKcs to histone synthesis and thus DNA replication (Kaygun and Marzluff, 2005; Muller et al., 2007). DNA-PKcs deficiency suppresses new origin firing and stalls existing replication forks in stressed conditions (Shimura et al., 2007). The persistence of γH2AX foci and induction of Rad51 foci following replication stress in DNA-PKcs-defective human cells (Shimura et al., 2007) are consistent with prior observations of enhanced spontaneous HR (between direct repeats) in DNA-PKcs-defective CHO cells (Allen et al., 2002). Since spontaneous HR is a consequence of replication fork collapse (Saleh-Gohari et al., 2005), this result suggests that DNA-PKcs suppresses HR during fork restart. Thus, DNA-PKcs defective cells display both hyper-HR and sensitivity to replication stress, showing that HR, at least

as measured with direct repeats, does not correlate with resistance to replication stress. The hyper-HR seen in DNA-PKcs-defective cells suggests that DNA-PKcs may promote HR accuracy during fork restart. One hypothesis is that HR accuracy is regulated through DNA-PKcs phosphorylation of RPA.

Post-translational modifications of RPA in response to replication stress

RPA is an essential, trimeric ssDNA binding protein comprising 14, 32 and 70 kDa subunits. RPA has well-characterized roles in normal replication, HR and in the replication stress response including fork stabilization, fork restart and checkpoint activation (Wold, 1997; Fanning et al., 2006; Zou et al., 2006; Budzowska and Kanaar, 2009). RPA activity is modulated during the cell cycle and in response to replication stress through CDK, ATM, ATR and DNA-PKcs-dependent phosphorylation events (Shao et al., 1999; Oakley et al., 2003). RPA forms foci in response to etoposide, and these foci co-localize with newly replicated DNA but not with PCNA or Ligase I, indicating that etoposide-induced replication stress causes dissociation of the replication machinery. This dissociation of core replication proteins is dependent on ATR, Chk1 and NBS1, but not on DNA-PKcs nor ATM, but RPA focus formation is independent of all three PIKs (Montecucco et al., 2001; Rossi et al., 2006). The chromatin-bound RPA32 is hyperphosphorylated in response to etoposide, and this phosphorylation is strongly dependent on ATR (Rossi et al., 2006). The changes in subcellular location and/or post-translational modification of RPA and other replicative and repair proteins are thought to reflect a switch from a normal replication mode to a repair mode (Sharma et al., 2004; Fanning et al., 2006; Rossi et al., 2006).

In vitro and *in vivo* analyses identified five specific residues in each of the 32 and 70 kDa RPA subunits that are phosphorylated in response to replication stress caused by aphidicolin or HU (Nuss et al., 2005). The MRN complex co-localizes with RPA at sites of HU- or UV-induced damage, and the DNA damage-induced MRN-RPA interaction is regulated by phosphorylation and dephosphorylation of subunits of both complexes (Robison et al., 2004). Subsequent work with camptothecin, etoposide and MMS showed differential responses of MRN and RPA to these agents: MRN foci were detected with all agents but MRN co-localization with RPA was only detected in response to etoposide, suggesting a model in which MRN–RPA interactions occur only when relatively long stretches of ssDNA are produced (Robison et al., 2005). Recent work focusing on the physical interaction between RPA and MRN revealed that MRN binding to the N-terminus of RPA14 and subsequent phosphorylation of RPA32 were crucial to the DNA-damage response (Oakley et al., 2009). ATR-dependent hyperphosphorylation of RPA32 following HU treatment requires the NBS1 subunit of MRN (Manthey et al., 2007), and NBS1 appears to be important, but not essential, for ATR-dependent RPA32 hyperphosphorylation after etoposide (Rossi et al., 2006). In addition to extensive regulation by phosphorylation, protein phosphatase 2A-dependent dephosphorylation of key RPA32 residues (Ser21 and Thr33) is necessary to overcome the ATR/ATM-regulated DNA-damage checkpoint, promote efficient fork restart, and re-entry into the cell cycle (Feng et al., 2009). Although it is clear that RPA phosphorylation has general importance in the replication stress response, there is much to be learned about the specific roles of various RPA phosphorylation and dephosphorylation events in aspects such as fork stabilization, checkpoint activation, fork restart efficiency and the accuracy of replication-stress-associated HR events, all of which contribute to genome stability and cell survival after damage. The importance of RPA phosphorylation in cancer chemotherapy is underscored by the fact that cisplatin-resistant head and neck squamous cell carcinoma cell lines display significantly higher levels of RPA phosphorylation compared with cisplatin-sensitive cell lines (Manthey et al., 2010).

Roles for Ku, Artemis and XLF in the replication stress response

Ku, comprising a Ku70–Ku80 heterodimer, is a core NHEJ factor that binds broken DNA ends, protects ends from nucleolytic attack and recruits DNA-PKcs to ends, forming the active DNA-PK holoenzyme that promotes NHEJ. Ku likely plays an important role in DNA replication, as it associates with mammalian origins of replication and replication-related proteins including DNA polymerases, TopoIIα, RF-C, the origin recognition complex and PCNA (Matheos et al., 2002; Rampakakis et al., 2008). After replication stress, Ku70 associates with γH2AX foci in a DNA-PKcs independent manner, but resolution of these foci (presumably reflecting DSE and/or DSB repair) depends on DNA-PKcs (Shimura et al., 2007). Through its physical interaction with the XRCC4-LigIV complex, Ku increases the rate of DNA ligation (Ramsden and Gellert, 1998; Nick McElhinny et al., 2000); this role is also independent of DNA-PKcs. Ku80-defective mammalian cells display reduced proliferation due to impaired initiation of DNA synthesis (Rampakakis et al., 2008), prolonged S-phase following DNA damage (Zhou et al., 2002), and sensitivity to replication stress (Saintigny et al., 2001). Although *Ku* mutations typically confer sensitivity to replication stress, mutation of *Ku70* and other NHEJ factors in hyper-recombinogenic chicken DT40 cells confers resistance to camptothecin (Adachi et al., 2004). In *Schizosaccharomyces pombe*, mutation of *pku70* or *pku80* increases sensitivity to replication stress, and sensitivity is further enhanced by a second mutation in *rqh1* (Miyoshi et al., 2009), a homolog of mammalian *BLM*, which functions in HR (including Holliday junction processing) and replication fork stabilization and restart (Wu and Hickson, 2003; Davies et al., 2007; Shimura et al., 2008). Similar results are seen in *Saccharomyces cerevisiae* mutants lacking BLM and Ku homologs (Sgs1 and yKu) (Ui et al., 2005; Yamana et al., 2005). Expression of RusA, a bacterial Holliday junction resolvase, partially suppresses the HU sensitivity of *rqh1* mutant cells (Doe et al., 2002). These results suggest that 'toxic' HR intermediates are likely contributors to HU-induced lethality in *rqh1* mutants. *Schizosaccharomyces pombe pku70* or *pku80* mutants are also further sensitized to replication stress by mutation of *mus81*,

suggesting that stalled replication forks in *pku* mutants are resolved by Mus81 endonuclease (Miyoshi et al., 2009).

These fork resolution pathways appear to be highly conserved through evolution. In mammalian cells, Mus81 introduces DSBs in response to replication stress in a BLM-dependent manner, and these DSBs are proposed to convert unusual structures at stalled replication forks to forms that permit restart (Shimura et al., 2008). Interestingly, these DSBs are repaired in a DNA-PKcs (and presumably Ku) dependent manner (Shimura et al., 2007). However, the precise fork structures subject to DSB induction, and the DSB repair products are still a mystery. Nonetheless, these results suggest that Ku suppresses the formation of replication intermediates that require resolution by BLM and other factors. This role for Ku may be in stabilization of stalled forks since Ku80 prevents the dissociation of PCNA from chromatin (Park et al., 2004). Ku also promotes proper loading of DNA replication licensing factors at origins, including Orc1 and Orc4 (Rampakakis et al., 2008). Ku may regulate the association of other replication factors, in addition to PCNA, with stalled forks, thereby enhancing fork restart.

Artemis and XLF (also called Cernunnos) are accessory NHEJ factors with specialized roles in DSB repair. Artemis is a nuclease that processes hairpins formed during V(D)J recombination, and a fraction of DNA ends produced by IR, preparing ends for re-joining (Lobrich and Jeggo, 2005). XLF interacts with and promotes XRCC4-LigIV ligase activity (Ahnesorg et al., 2006). Following replication stress, Artemis-deficient cells fail to form RPA and RAD51 foci in G2 cells, and they do not display stress-induced (HR-mediated) sister chromatid exchanges (Beucher et al., 2009). Artemis is phosphorylated by ATR after replication stress, and a phosphorylation site mutant shows delayed S-phase progression following replication stress (Wang et al., 2009). Artemis, in complex with DNA-PKcs, has many *in vivo* substrates including ssDNA and ssDNA–dsDNA transitions (Gu et al., 2010) that are present at replication forks. Defects in the NHEJ factor XLF increase sensitivity to aphidicolin, delay resolution of γH2AX foci, increase Chk1 activation, and enhance fragile site instability, but do not affect replication fork progression (Schwartz et al., 2009). Although the defects in RPA and RAD51 focus formation and sister chromatid exchange suggest that Artemis regulates replication fork restart by affecting strand resection (and hence HR), roles for Artemis or XLF in NHEJ-mediated fork processing cannot be ruled out.

Metnase: another NHEJ factor that promotes replication fork restart

Metnase is a fusion protein with protein methylase (SET) and nuclease domains that arose in anthropoid primates (Cordaux et al., 2006). In addition to its methyl transferase and nuclease activities (Roman et al., 2007), Metnase promotes DNA integration (Lee et al., 2005; Williamson et al., 2008a,b), interacts with and enhances TopoIIα-dependent chromosome decatenation (Williamson et al., 2008a,b; Wray et al., 2009a,b) and promotes NHEJ (Lee et al., 2005; Hromas et al., 2008). siRNA knockdown of Metnase in human cells slows proliferation and greatly enhances sensitivity to replication stress (De Haro et al., 2010). In the absence of replication stress, Metnase knockdown does not affect DNA replication fork progression; however, after a brief (1 h) HU treatment, Metnase knockdown confers a dramatic defect in replication fork restart, indicating that Metnase has an early role in the replication stress response. In addition, with longer HU treatments that cause fork collapse and induction of γH2AX foci, the speed at which these foci are resolved is proportional to Metnase expression level, arguing for a late role as well. This late role is likely to involve stimulation of NHEJ, but is also possible that its early roles depend on its interactions with the NHEJ machinery. Metnase stimulation of NHEJ requires functional SET and nuclease domains (Lee et al., 2005), but it is not yet known if both domains are also required to stimulate replication fork restart. An intriguing possibility is that the Metnase nuclease functions similar to Mus81-BLM, inducing DSBs at unusual structures that arise at stalled replication forks. Metnase also interacts with RAD9 in the 9–1–1 complex, and PCNA, suggesting additional possible roles in fork stabilization via activation of the ATR, 9–1–1, Chk1 checkpoint pathway or PCNA stabilization (De Haro et al., 2010), as noted for Ku.

Summary and perspectives

Stalled and collapsed replication forks pose significant threats to cell viability and genome stability. Although segments of DNA that fail to be replicated if a stalled or collapsed fork cannot be restarted may be rescued by replication from an adjacent fork, this may not always be possible, such as at regions near telomeres that lack replication origins. Forks are stalled by most DNA lesions but some DNA sequences that are difficult to replicate cause forks to stall during every round of DNA replication; such sequences include repetitive elements such as telomeric repeats, G-quadraplex DNA, fragile sites and fork block sequences in ribosomal DNA arrays. Considerable evidence indicates that HR proteins play a primary role in restarting stalled and collapsed replication forks, but a growing body of evidence implicates NHEJ proteins in this critical process. Given the significant cross-regulation of HR and NHEJ pathways in DSB repair (Pierce et al., 2001; Allen et al., 2002; Shrivastav et al., 2008), it is possible that HR and NHEJ proteins cooperate in fork restart. On the other hand, it is equally likely (and not mutually exclusive) that HR and NHEJ proteins function in redundant fork restart pathways. Because of the critical importance of replication fork restart for cell survival and genome integrity, it is not difficult to imagine the evolution of redundant fork restart pathways. Defining such redundancy may provide important information leading to improved replication stress-mediated cancer therapeutic strategies that exploit synthetic lethal relationships in tumor cells (Helleday et al., 2008).

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