Competition, collaboration and coordination – determining how cells bypass DNA damage

Julian E. Sale
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK
jes@mrc-lmb.cam.ac.uk

Journal of Cell Science 125, 1633–1643
© 2012. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.094748

Summary

Cells must overcome replication blocks that might otherwise lead to genomic instability or cell death. Classical genetic experiments have identified a series of mechanisms that cells use to replicate damaged DNA: translesion synthesis, template switching and homologous recombination. In translesion synthesis, DNA lesions are replicated directly by specialised DNA polymerases, a potentially error-prone approach. Template switching and homologous recombination use an alternative undamaged template to allow the replicative polymerases to bypass DNA lesions and, hence, are generally error free. Classically, these pathways have been viewed as alternatives, competing to ensure replication of damaged DNA templates is completed. However, this view of a series of static pathways has been blurred by recent work using a combination of genetic approaches and methodology for examining the physical intermediates of bypass reactions. These studies have revealed a much more dynamic interaction between the pathways than was initially appreciated. In this Commentary, I argue that it might be more helpful to start thinking of lesion-bypass mechanisms in terms of a series of dynamically assembled 'modules', often comprising factors from different classical pathways, whose deployment is crucially dependent on the context in which the bypass event takes place.

Key words: DNA damage tolerance, Translesion synthesis, Homologous recombination, Mutagenesis

Introduction

Genome duplication is carried out by a finely tuned molecular machine that works with astonishing rapidity and accuracy. However, the polymerases at its core are geared to operate on a pristine DNA template, a situation that is rarely found for long in practice. DNA is continuously damaged by insults from both endogenous and exogenous sources, leading to base lesions that block the replicative polymerases. Despite the existence of highly efficient repair mechanisms, damaged bases are still inevitably encountered during replication, which can result in replication fork arrest (reviewed by Branzei and Foiani, 2010). Attempting excision repair of the lesion at this point is risky because, by the time the polymerase has stalled, the lesion is present in single-stranded DNA, the duplex having been unwound by the replicative helicase. Thus, an excision step would result in the formation of a double-strand break (DSB) and collapse of the replication fork, a potentially much more dangerous situation that creates a potent substrate for undesirable genomic rearrangements. Although it has been suggested that some bacteria, notably *Escherichia coli*, can reverse the replication fork to bring the lesion back into double-stranded DNA for safe excision (Courcelle et al., 2003; McGlynn and Lloyd, 2000), there is not a great deal of evidence that this approach is conserved in eukaryotes. Instead, an important strategy for dealing with polymerase-stalling lesions, which is found in all branches of life, is to use specialised mechanisms to replicate the lesion before attempting excision repair: these are collectively known as DNA damage tolerance mechanisms (reviewed by Branzei and Foiani, 2010; Sale et al., 2012) and are also sometimes referred to as post-replication repair (because the replication of damaged DNA was originally associated with the filling of gaps left after bulk replication had been completed, although it is important to note that the word 'repair' in this context refers to the gaps, not the lesions).

DNA damage tolerance can proceed through one of two broad mechanisms (Fig. 1A). In the first, termed translesion synthesis (TLS), the stalled replicative polymerases are replaced by specialised TLS DNA polymerases (Table 1) that are able to replicate directly across the lesion (Sale et al., 2012). They are able to do this because their active sites have a larger volume than those of the replicative polymerases and are therefore more able to accommodate damaged or distorted templates (reviewed in Yang, 2003). The trade off for this facility is an increased risk of mutagenesis. TLS is mutagenic for two reasons. First, damaged bases are often miscoded, for example 8-oxoguanine can pair with deoxyadenosine (dA) (Friedberg et al., 2006). Second, TLS polymerases have an intrinsically lower fidelity owing to their lower ability to discriminate the correct base (Beard et al., 2002) and their lack of exonuclease proofreading activity (Sale et al., 2012). In the second general mechanism, the stalled polymerase makes use of an alternative, undamaged template (Branzei, 2011). Most frequently this is the newly synthesised daughter strand on the sister chromatid. This so-called 'template switching' mechanism is, therefore, in contrast to TLS, accurate. Many aspects of its proposed molecular mechanism closely resemble classical homologous recombination, but, as discussed below, the two can be distinguished genetically. Failure of DNA damage tolerance mechanisms frequently results in formation of DSBs owing to collapse of the replication fork, and in this situation homologous recombination is needed to reinitiate replication (Fig. 1A).

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There are a number of excellent recent reviews on the mechanisms of DNA damage tolerance (e.g. Branzei, 2011; Ulrich, 2009; Sale et al., 2012) so in this Commentary, I first begin with an overview of the classical pathways of lesion bypass, before focusing on the interactions between these under the three headings 'Competition', 'Collaboration' and 'Coordination'. In Competition, I explore the classical model and its limitations; in Collaboration, I consider recent evidence that reveals significant fluidity between the boundaries of the classical pathways. I suggest that this moves us towards a more context-specific way of thinking about the mechanisms by which simple DNA lesions are bypassed. Finally, in Coordination, I discuss how replication of complex DNA lesions and clusters of DNA damage require the regulated deployment of a series of mechanisms.

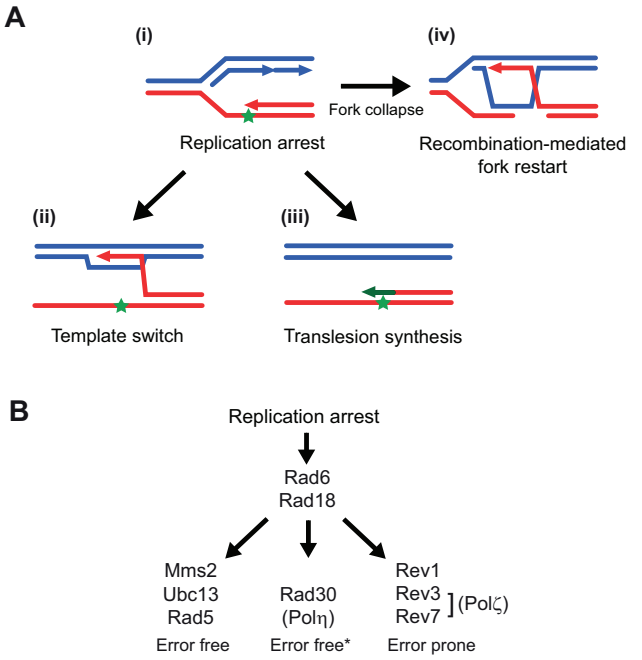

Fig. 1. Competition between pathways in the Rad6 DNA damage tolerance epistasis group. (**A**) A simplified representation of the mechanisms of TLS and template switching. Following replication fork arrest at a DNA lesion (green star) (i) the lesion can be replicated using an alternative DNA template, most commonly the newly synthesised daughter strand on the sister chromatid (ii), or specialised DNA polymerases can directly replicate the lesion by TLS (iii). If the template strand is incised the fork will collapse and this will require recombination to re-establish the fork (iv). Note (ii) illustrates just one of the possible models of template switching (also see the main text and Fig. 3). (**B**) The classical view of the core of the Rad6 pathway of DNA damage tolerance in *S. cerevisiae*. The pathways depends on the actions of Rad6 and Rad18, which are epistatic to components of the 'error prone' and 'error free' modes of bypass. For simplicity, the scheme does not include the alleles of polδ (Torres-Ramos et al., 1997) and PCNA [pol30-46, (Torres-Ramos et al., 1996)] that first suggested that these genes were involved in damage tolerance, nor the antirecombinogenic helicase Srs2 (also see the main text and Table 1). Although often used in the early literature the terms 'error prone' and 'error free' pathways are not always informative. Rad30 (also known as pol*η*) is apparently required for an error-free pathway (denoted 'error free*' in the figure). However, this principally applies to the replication of cyclobutane pyrimidine dimers, the common UV lesion, which mechanistically it does by TLS.

The core pathways of lesion bypass in eukaryotes

Much of our understanding of the molecular mechanisms of damage tolerance comes from genetic studies in the budding yeast *Saccharomyces cerevisiae.* Here, the majority of DNA damage tolerance is accounted for by genes of the *RAD6* epistasis group (Table 1). In essence, the *RAD6* group genes act in two subpathways (Fig. 1B), both of which require the action of Rad6 and of a second protein Rad18 (Bailly et al., 1994; Prakash, 1981). The first pathway contains the translesion DNA polymerases, Rev1, Polζ (comprising the Rev3 catalytic subunit and the smaller, possibly regulatory, subunit Rev7) and Pol*η* (Rad30). The second pathway accounts for the error-free 'template switch' mode of bypass and comprises Rad5, Ubc13 and Mms2 (reviewed by Broomfield et al., 2001; Lehmann, 2000). Early studies also obtained evidence for the involvement of the core replication machinery in the form of proliferating cell nuclear antigen (PCNA; the sliding clamp that tethers replication and repair proteins to the DNA) (Torres-Ramos et al., 1996) and the replicative DNA polymerase, polδ (Torres-Ramos et al., 1997). In addition, Rad52, the eponymous founder of the homologous recombination epistasis group, can also mediate some post-replication repair (Prakash, 1981). However, for many years the molecular mechanism underlying the Rad6 group remained a significant puzzle because, with the exception of the TLS polymerases, the core factors are involved in protein ubiquitylation (Box 1) (Bailly et al., 1997; Hofmann and Pickart, 1999; Jentsch et al., 1987; Ulrich and Jentsch, 2000).

In 2002, a groundbreaking study brought together these apparently diverse functions of the Rad6 group by showing that the key target of the ubiquitylation activity of the pathway is PCNA (Pol30 in yeast) (Hoege et al., 2002). That work, together with many subsequent studies, led to a working model for the Rad6 group. The formation of single-stranded DNA, which at sites of stalled replication is coated with the single-strand-binding protein replication protein A (RPA), leads to the recruitment of the E3 ubiquitin ligase Rad18, which, acting in concert with Rad6 (an E2 ubiquitin conjugating enzyme), monoubiquitylates lysine (K) 164 of PCNA (Fig. 2) (Davies et al., 2008; Haracska et al., 2004; Hoege et al., 2002; Stelter and Ulrich, 2003). The monoubiquitin installed by Rad6 and Rad18 then stimulates TLS by promoting the association of the specialised TLS DNA polymerases with PCNA through the interaction between ubiquitin and the ubiquitin-binding domains, which are located within their C-terminal regions (Bienko et al., 2005). Thus, the ubiquitylation of PCNA couples the persistent exposure of single-stranded DNA that follows replication fork arrest to lesion bypass, thereby ensuring that the potentially damaging activities of the TLS polymerases are only deployed at distressed forks.

The ubiquitin moiety at K164 of PCNA can be extended with a K63-linked ubiquitin chain by the E3 ligase Rad5, which acts together with the E2 enzyme Ubc13 and E2-like protein Mms2 (Hoege et al., 2002; Hofmann and Pickart, 1999; Ulrich and Jentsch, 2000). Although such polyubiquitylation of PCNA has been genetically linked to 'template switching' (Hoege et al., 2002), the exact function of these chains in promoting strand exchange remains a key unanswered question. Rad5 also possesses an ATPase activity that is necessary for its role in damage tolerance (Gangavarapu et al., 2006) and which is linked to its helicase activity, which in vitro can promote reversal of fork-like structures (Blastyak et al., 2007).

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Table 1. The Rad6 epistasis group

S. cerevisiae	*H. sapiens*	Function
Rad6	RAD6A, RAD6B	E2 ubiquitin conjugating enzyme (Jentsch et al., 1987); works with the E3 ubiquitin ligase Rad18 in DNA damage tolerance (Bailly et al., 1994)
Rad18	RAD18	Main E3 for PCNA monoubiquitylation (Hoege et al., 2002); works with Rad6; human protein also binds polη (Watanabe et al., 2004)
Ubc13	UBC13	E2 ubiquitin conjugating enzyme; involved in a number of other processes in the cell, notably the double strand break response where it works with the E3 Rnf8 (Huen et al., 2007)
Mms2	UBE2V2	E2-like protein; binds to Ubc13 and restricts chain formation to K63 linkage but has no catalytic activity of its own (Hofmann and Pickart, 1999)
Rad5	SHPRH, HLTF	E3 for PCNA polyubiquitination (Hoege et al., 2002); RAD5 also has ATP-dependent helicase activity (Blastyak et al., 2007). HLTF can monoubiquitinate PCNA (Lin et al., 2011)
Rev1	REV1	Y-family polymerase; deoxycytidyl transferase (Nelson et al., 1996a); has a key role in coordinating TLS as its C-terminus interacts with PCNA and the other TLS polymerases (Guo et al., 2003; Ross et al., 2005)
Rev3	REV3	Forms a complex with Rev7 to create the B-family polymerase, polζ with Rev3 being the catalytic subunit (Nelson et al., 1996b); has a key role in TLS, notably extension of a mismatched primer terminus created by incorporation opposite a lesion
Rev7	REV7	Small subunit of polζ (Nelson et al., 1996b); function unclear but probably regulatory; mediates binding to Rev1 (Murakumo et al., 2001)
Rad30	Polη, Polι	Y-family TLS polymerase. Polη (also known as RAD30A in vertebrates) is important for error-free bypass of UV-induced cyclobutane pyrimidine dimers (Johnson et al., 1999; Masutani et al., 1999) A second Rad30 homologue (RAD30B or Polι) is present in most, but not all, vertebrates; its restrictive active site makes it extremely error-prone on template deoxythymidine (dT), but helps in the accurate replication of 8-oxoguanine (Vaisman and Woodgate, 2001)
–	Polκ	Y-family polymerase; not conserved in budding yeast but related to *E. coli* DNA polymerase IV (dinB). Mediates efficient bypass of abasic sites and bulky dG adducts such as benzo[*a*]pyrene diol-epoxide (BPDE) but with a propensity for −1 frameshifts (Ohashi et al., 2000)
Srs2	?	Antirecombinogenic helicase that can displace Rad51 filaments; a direct vertebrate homologue is not obvious, but a number of vertebrate helicases have been shown to perform similar antirecombinogenic functions, including RECQL5 (Hu et al., 2007), RTEL (Barber et al., 2008) and PARI (Moldovan et al., 2011)

Box 1. Ubiquitylation

Ubiquitin is a 76-amino-acid protein that can be covalently attached to accessible lysine residues on other proteins (Pickart, 2001). It can also be attached to itself to form chains through any of its seven lysine residues or its N-terminus to form linear chains. Ubiquitin is produced from a number of loci in yeast and human cells both as linear multimers and as fusions with ribosomal proteins. Ubiquitin monomers are produced by cleavage with ubiquitin C-terminal hydrolases. Ubiquitin–protein conjugation begins with the activation of ubiquitin by the attachment of its C-terminal glycine residue to a sulfhydryl group in the E1 ubiquitin activating enzyme. The ubiquitin is then transferred to the active site of one of the 30–40 known E2 ubiquitin conjugating enzymes by a transthioesterification reaction. Finally, transfer of the ubiquitin to its target is mediated by an E3 ubiquitin ligase, of which there are several hundred in the mammalian genome. E3 ligases fall into two groups, those containing a HECT domain and those containing a RING domain (Pickart, 2001). The principal E3 ligases involved in damage tolerance all fall into the latter category, and they play a crucial role in substrate recognition and assisting the transfer of the ubiquitin directly from the E2. Ubiquitylation has many diverse consequences for protein function. It is best known for its role in promoting protein degradation by the proteasome (through formation of K48-linked chains), but it can also be used to alter protein–protein interactions by interfering with existing binding interfaces or creating new binding surfaces, thereby promoting dynamic changes in protein complex formation (Grabbe et al., 2011; Welchman et al., 2005). However, the precise role played by ubiquitylation in many aspects of the DNA damage response, including DNA damage tolerance, remains poorly understood, particularly the role of the different topologies of ubiquitin chain (Ulrich and Walden, 2010).

These mechanisms are broadly conserved in vertebrates. PCNA is monoubiquitylated by RAD18 in response to DNA damage, and this step is important for the recruitment of translesion polymerases (Bienko et al., 2005; Kannouche et al., 2004). However, there is good evidence that PCNA ubiquitylation can also be mediated by activities other than RAD18 (Simpson et al., 2006; Terai et al., 2010; Zhang et al., 2008), including one of the vertebrate homologues of Rad5, helicase-like transcription factor (HLTF) (Lin et al., 2011). Vertebrate PCNA is also polyubiquitylated (Chiu et al., 2006), which is, in part, mediated by the Rad5 homologues, SHPRH (for SNF2, histone-linker, PHD and RING finger domain-containing helicase) and HLTF (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2008; Unk et al., 2006). However, the loss of SHPRH and HLTF appears to result in only rather subtle effects on damage tolerance in mice (Hendel et al., 2011; Krijger et al., 2011a). It is currently not clear whether this means that PCNA polyubiquitination is less important in vertebrate lesion bypass than it is in yeast or that other mechanisms, such as homologous recombination, can efficiently substitute.

K164, and the nearby K127, of PCNA can also be modified by conjugation of the ubiquitin-related protein SUMO (Hoege et al., 2002; Stelter and Ulrich, 2003). In budding yeast, PCNA SUMOylation acts to inhibit classical homologous recombination by recruiting the helicase Srs2, which is able to displace the recombinase Rad51 from DNA, allowing Rad18-dependent template switching to proceed (Papouli et al., 2005; Pfander et al., 2005). However, although PCNA SUMOylation has been observed in *S. pombe* (Frampton et al., 2006) and a number of vertebrate species (Arakawa et al., 2006; Leach and Michael, 2005), the conservation of PCNA SUMOylation as a means of regulating recombination is unclear.

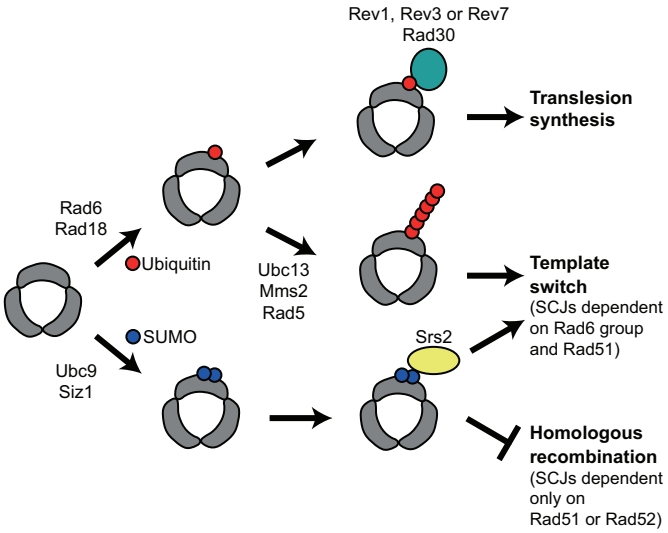

Fig. 2. The PCNA switchboard in *S. cerevisiae*: regulation of competition between bypass pathways. The DNA sliding clamp PCNA is a trimer, but for clarity only one subunit is shown as being modified. In practice, the PCNA trimer could have three different modifications simultaneously, either the same modification on all three subunits or any combination. PCNA can be modified at K164 with either ubiquitin, which is added by Rad6 and Rad18, or by SUMO, which is added by Ubc9 (the SUMO E2) and Siz1 (a SUMO E3 ligase) (Ulrich, 2009). Monoubiquitin facilitates the recruitment of the TLS polymerases to PCNA through interaction with the ubiquitin-binding domains found in Rev1 and Rad30 (polη) and hence promotes TLS (Bienko et al., 2005). Alternatively extension of the monoubiquitin with a K63-linked chain promotes template switching by a poorly understood mechanism (Hoege et al., 2002). SUMOylation of PCNA at K164 in *S. cerevisiae*, recruits the antirecombinogenic helicase Srs2 that displaces Rad51 from single-stranded DNA and prevents the initiation of classical recombination (Papouli et al., 2005; Pfander et al., 2005). SCJ, sister chromatid junction.

Competition

The classical model of lesion bypass (Fig. 1) envisages a series of pathways competing to process a lesion with post-translational modifications of PCNA regulating this competition (Fig. 2). The sequential action of RAD6 and RAD18 to monoubiquitylate PCNA, before ubiquitin chain extension by UBC13, MMS2 and RAD5 (Hoege et al., 2002; Parker and Ulrich, 2009), provides a common intermediate, monoubiquitylated PCNA, that allows the decision between TLS and template switching to be made. Thus, either monoubiquitylated PCNA recruits a TLS polymerase, or it becomes polyubiquitylated and promotes template switching. However, as discussed below, in vertebrates, TLS DNA polymerases can be recruited independently of PCNA ubiquitylation (Hendel et al., 2011; Szüts et al., 2008) and thus the simple model of a genome-wide competition between bypass pathways that is regulated by PCNA modification becomes inadequate. Instead, the notion of competition between pathways needs to be qualified by the context in which lesion bypass is taking place. I now consider three examples of such context and explore how context might influence pathway competition.

Timing of lesion bypass

The first way in which bypass events can be distinguished is by when they take place relative to the passage of the replication fork. There has been a long debate over this issue. Early models envisaged the formation of post-replicative gaps opposite DNA lesions that are subsequently sealed (Rupp and Howard-Flanders, 1968; Rupp et al., 1971). However, alternative models for bypass that takes place at the fork thereby maintaining fork progression were also proposed (Higgins et al., 1976). These ideas gained further ground with evidence for a recombination mechanism in *Escherichia coli* that did not depend on daughter strand gaps but that could be used to repair forks stalled by UV damage (Courcelle et al., 1997). By the time the catalytic activity of the TLS polymerases had been identified (Woodgate, 1999), most models proposed envisaged bypass taking place at the fork. So, what is the evidence that distinct mechanisms do indeed operate at forks and at gaps?

Evidence to suggest that bypass at the fork and at post-replicative gaps could be distinguished genetically initially came from work in the avian cell line DT40 (Edmunds et al., 2008). In DT40 cells, PCNA ubiquitylation does not have the same central role in the control of TLS as it does in yeast, as double mutants lacking both PCNA ubiquitylation and either REV1 or Polκ, are more sensitive to DNA damage than either of the single mutants (Arakawa et al., 2006; Edmunds et al., 2008; Okada et al., 2002; Ross et al., 2005). PCNA ubiquitylation and REV1 also have independent roles in mutagenesis in DT40 cells (Arakawa et al., 2006; Szüts et al., 2008). We proposed an explanation for these observations by suggesting that PCNA ubiquitylation and REV1 defined the temporally distinct pathways of bypass at the fork and at gaps behind the fork (Edmunds et al., 2008). We suggested that at the fork TLS proteins could be recruited independently of PCNA ubiquitylation by the C-terminal domains of REV1, which binds both PCNA and the other TLS polymerases (Guo et al., 2003; Ohashi et al., 2004; Ross et al., 2005; Tissier et al., 2004). A similar division of labour might also exist in mammalian cells. A REV1-dependent mode of TLS is seen at the fork (Jansen et al., 2009) and PCNA ubiquitylation, which has been proposed to largely control post-replicative gap filling (Niimi et al., 2008), has recently been shown to be unessential for TLS (Hendel et al., 2011; Krijger et al., 2011b). This suggests that REV1-dependent TLS might well be able to operate independently of PCNA ubiquitylation. However, in mice REV1 also plays a clear role in post-replicative gap filling (Jansen et al., 2009), indicating that REV1 could have both PCNA-ubiquitylation-dependent and -independent roles.

Location of the lesion on the leading versus lagging strand

The location of a lesion on the leading or lagging strand template might also have a significant influence on how it is bypassed. The formation of post-replicative gaps will be more likely on the lagging strand owing to the inherently discontinuous nature of lagging strand replication. There is also some emerging genetic evidence of distinct bias in pathway usage at leading and lagging strand blocks. In the case of TLS, REV1 might be particularly important in replicating lesions and structures present on the leading strand template (Sale et al., 2009; Sarkies et al., 2010). Likewise, the role played by Rad5 in template switching in yeast has been proposed to be strand-specific, with Rad5 required for processing blocked leading strands, and Rad52-dependent recombination operating at blocked lagging strands (Gangavarapu et al., 2007).

The nature of the lesion itself

The chemical and structural nature of the lesion is frequently an important determinant of the mechanism employed to repair it. For

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instance the 8-oxoguanine lesion mentioned above can be specifically recognised and excised by the DNA glycosylase OGG1 (van der Kemp et al., 1996). Such selectivity is also apparent in TLS, with particular polymerases, or combinations of polymerase, being deployed at specific lesions (Shachar et al., 2009). For example, UV-induced cyclobutane pyrimidine dimers appear to be efficiently, and fairly specifically, replicated by DNA polymerase η (Johnson et al., 1999; Masutani et al., 1999). However, it remains unclear to what extent the polymerase selection exerted by a lesion is mediated by specific protein–protein or protein–DNA interactions and to what extent it is simply stochastic. An interesting further example of specialisation for different lesions has recently been documented in the template-switching pathway. The two vertebrate Rad5 homologues, SHPRH and HLTF, are differentially required for bypassing DNA damage that results from either UV, which requires HLTF, or the DNA-adducting agent methylmethane sulphonate, which requires SHPRH (Lin et al., 2011).

These examples illustrate that consideration of the context of a lesion is important for interpreting the potential competition between alternative pathways that could replicate it. In the next section, I consider how recent evidence has led to the distinction between the classically defined pathways becoming increasingly blurred.

Collaboration

As experimental techniques to examine the intermediates and outcomes of DNA lesion bypass reactions have advanced, it has become clear that the rather static view of the bypass pathways provided by classical genetics is too limited. In this section, I will examine the evidence for crosstalk between the classical bypass pathways (Fig. 3A).

Crosstalk between classical homologous recombination and template switching

Many models of template switching resemble classical homologous recombination (Fig. 1), and the involvement of recombination proteins in template switching was anticipated by some of the earliest genetic analyses (reviewed in Broomfield et al., 2001). It has become increasingly clear that this mechanistic resemblance is more than superficial. Recent evidence from experiments using two-dimensional electrophoresis to analyse the Rad18-dependent template switch reaction has shown that the formation of key intermediates also depends on core recombination proteins, notably Rad51 (Branzei et al., 2008; Minca and Kowalski, 2010; Vanoli et al., 2010). Yeast cells deficient in the RecQ family helicase Sgs1 accumulate recombination structures, or X-molecules, during normal growth and more so following treatment with DNA-damaging agents such as methylmethane sulphonate (Liberi et al., 2005). Although the formation of these structures depends on the recombinase Rad51 (Liberi et al., 2005), it also requires Rad18 and Rad5 when PCNA can be SUMOylated, but not in the absence of PCNA SUMOylation (Branzei et al., 2008). This suggests that Rad18 and Rad5 are only needed to promote bypass in certain contexts, which are possibly defined by the presence of SUMOylated PCNA.

As well as RAD51 being required for the formation of RAD18-dependent template switch intermediates, RAD18 also appears to modulate classical RAD51-dependent recombination, notably the frequency at which crossovers are formed (Simpson et al., 2006; Szüts et al., 2006). The involvement of RAD18 in homologous recombination appears to be mediated by its recruitment to DSBs through its zinc finger domain, which binds break-associated polyubiquitin chains (Huang et al., 2009). In this context, RAD18 is proposed to act as an adaptor protein, independently of its ubiquitin ligase activity, bridging break-induced ubiquitylation with the recruitment of the recombination mediator protein RAD51C (Huang et al., 2009). The extent to which Rad18 is directly involved homologous recombination in yeast is less clear. However, Rad18-deficient *S. cerevisiae* exhibit sensitivity to ionising radiation that is independent of K164 of PCNA (Chen et al., 2005) which supports the possibility of a role for Rad18 in processing DSBs that is independent of PCNA ubiquitylation. In yeast, Rad5 has also been implicated in DSB repair (Chen et al. 2005), again a role that is independent of its ubiquitin ligase function. Furthermore, there is evidence that both the fission yeast (*S. pombe*) and plant (*Arabidopsis thaliana*) homologues of Rad5 are also involved in recombination (Chen et al., 2008; Doe et al., 1993). However, it is not clear that this putative role for Rad5 in recombination is maintained in the vertebrate Rad5 homologues SHPRH and HLTF (Unk et al., 2010).

Crosstalk between template switch and TLS

The original screen for yeast mutants that exhibit impaired UV-induced mutagenesis identified the *rev2* mutant (Lemontt, 1971a), together with *rev1* and *rev3*, which subsequently were shown to be TLS polymerases (Nelson et al., 1996a; Nelson et al., 1996b). *rev2*, it turns out, is allelic with *rad5* (Game and Cox, 1971). Although Rad5 is now best known as a key factor in the 'error-free' branch of the Rad6 post-replication repair pathway (Johnson et al., 1992), Lemontt had suggested that Rev1 and Rev2 (Rad5) act in parallel pathways that both generate a common intermediate that is processed by Rev3 to generate mutations (Lemontt, 1971b), clearly implicating Rev2 (Rad5) in TLS. However, subsequent results from analyses of the role of Rad5 in mutagenesis appeared to depend on the reporter allele used (Lawrence and Christensen, 1978). Recently, the ability to analyse the bypass of specific lesions at a known site in plasmids has allowed an unambiguous demonstration of the role of Rad5 in TLS in both *S. cerevisiae* (Pages et al., 2008; Zhang and Lawrence, 2005) and *S. pombe* (Coulon et al., 2010). Intriguingly, this role appears to be independent of Mms2 and Ubc13 in *S. cerevisiae* and, furthermore, does not require either the helicase or ubiquitin ligase activity of Rad5 (Pages et al., 2008), suggesting that Rad5 has a structural role. Supporting this hypothesis, Pagès et al. demonstrated a physical interaction between *S. cerevisiae* Rad5 and Rev1 (Pagès et al., 2008). However, in *S. pombe*, the role of the Rad5 homologue, Rad8, in TLS does require Mms2 and Ubc13, suggesting a different mode of action in this organism (Coulon et al., 2010). Moving further up the evolutionary scale to mammalian cells, recent evidence suggests that the Rad5 homologue HLTF can help suppress UV mutagenesis by enhancing PCNA ubiquitylation and the recruitment of polη (Lin et al., 2011). However, another recent study found that there is no impact on TLS in shuttled plasmids in murine cell lines where both the Rad5 homologues (SHPRH and HLTF) have been disrupted (Hendel et al., 2011). Thus, the evidence for a conservation of the role of Rad5 and its homologues in coordinating TLS is patchy and might, as seen

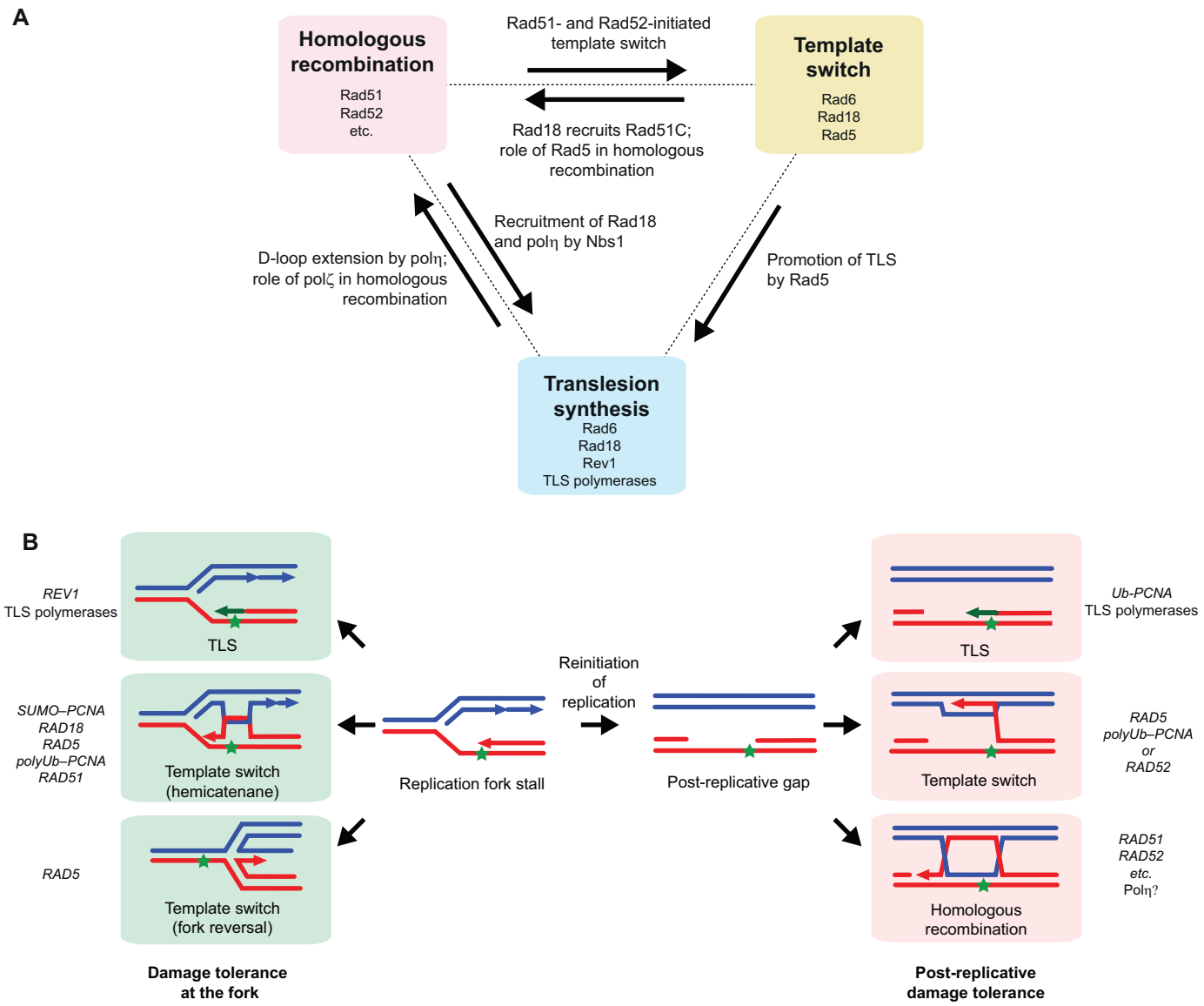

Fig. 3. Collaboration between homologous, template switching and TLS. (**A**) Summary of interactions between the three main classically defined pathways operating at stalled forks. Each is shown with the key proteins that represent the pathway within the coloured boxes. Genetic and mechanistic overlaps discussed in the main text are indicated next to the arrows. (**B**) A modular approach to replicating damaged DNA. Instead of simple genetic pathways, combinations of bypass proteins work together in discrete mechanistic ensembles, or 'modules'. The green boxes represent mechanisms that act at the fork. The modules shown in pale pink act at post-replicative gaps that are formed remotely from the replicative helicase by reinitiation of DNA synthesis downstream of the block. Key genes acting in each 'module' are indicated alongside each box. The situations illustrated are necessarily limited and do not, for instance, cover the different requirements that might be dictated by contexts such as the lesion being on the leading or lagging strand or the nature of lesion itself (see main text for further discussion). polyUb, polyubiquitylation.

in the initial studies on the *rad5* yeast mutants, be highly dependent on context.

Crosstalk between TLS polymerases and recombination

A causal relationship between recombination and mutagenesis was established by examination of the repair of a chromosomal break that was induced by the HO endonuclease in *S. cerevisiae* (Strathern et al., 1995). This revealed that DNA synthesis associated with the recombinational repair of the break is error prone compared with that of normal replication. Subsequent work showed that these errors are dependent on Rev3, although Rev3 is not required for the recombination reaction itself (Holbeck and Strathern, 1997). Both Rev3 and Rev1 are enriched at DSBs (Hirano and Sugimoto, 2006) and although their recruitment does not require PCNA ubiquitylation, it does depend on functional mitosis entry checkpoint protein 1 (MEC1), the yeast homologue of the ataxia telangiectasia and Rad3-related protein (ATR) checkpoint kinase. This role for Rev1 and Rev3 might be conserved as homologues of both proteins have been implicated in homologous recombination in vertebrates (Okada et al., 2005; Sharma et al., 2011; Sonoda et al., 2003).

Parallel investigations have suggested that pol*η* has a role in extending Rad51-coated primer termini following the strand-invasion step of homologous recombination (McIlwraith et al.,

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2005), an activity that could not be replaced by other specialised polymerases or polδ. Furthermore, pol*η* interacts with the Rad51 recombinase (McIlwraith et al., 2005). Supporting this model, Kawamoto and colleagues showed that pol*η*-deficient DT40 cells display a decrease in gene conversions that are induced by DSBs or abasic sites at the immunoglobulin loci (Kawamoto et al., 2005). However, the impact of pol*η* disruption is much less pronounced than that of core recombination factors, such as RAD54 (Bezzubova et al., 1997) or the Rad51 paralogues (Sale et al., 2001). Furthermore, neither pol*η*-deficient DT40 cells nor cells derived from xeroderma pigmentosum variant (XP-V) patients, which carry a mutation in pol*η* (Masutani et al., 1999), exhibit sensitivity to ionising radiation. In fact, XP-V-derived cells exhibit an increased UV-induced sister chromatid exchange (Cleaver et al., 1999) suggesting that failure to bypass UV-induced dimers results in an increased reliance on recombination-mediated resolution of arrested or collapsed forks. Thus, despite a considerable amount of evidence implicating TLS polymerases in recombination, further work is needed to clarify their mechanistic role, which again might be heavily dependent on the context of the recombination reaction.

In summary, recent studies have demonstrated that the boundaries between the classically defined genetic pathways of DNA damage bypass are more blurred than was initially appreciated. This should probably not come as a surprise given that the mechanisms of damage bypass have evolved to deal with a wide range of different problems rather than to fit neatly into simple pathways that would make our understanding more straightforward! However, these observations do mean that it becomes more important to consider bypass mechanisms on a fork-by-fork basis, rather than as a single process operating on an entire population of replication forks or, indeed, cells. Taking this idea to its logical conclusion would suggest that we could dispense with the idea of distinct pathways altogether and replace them with permutations of various factors in order to describe the process in terms of discrete mechanistic ensembles or ‘modules’ (Fig. 3B). To bypass simple replication blocks, a single ‘module’ might suffice, but the replication of more complex lesions or clusters of DNA damage would require the carefully coordinated deployment of a series of ‘modules’ and it is this coordination that I will consider in the final section.

Coordination

The concept of a modular approach to processing blocked replication forks suggests that there is the need for coordination to ensure that factors are deployed in the correct order, particularly when dealing with complex lesions or clusters of damage in large genomes. To illustrate this idea, I will first consider how the Fanconi anaemia group of proteins coordinate the repair and replication of interstrand crosslinks in vertebrate cells. I will then explore the dual role of NBS1, which is deficient in the autosomal recessive chromosome instability disorder Nijmegen breakage syndrome (NBS), in both recombination and TLS (Yanagihara et al., 2011) and speculate how this might provide a platform for coordination of the two processes in some circumstances.

Interstrand crosslinks can arise from exposure to bifunctional DNA-adducting agents, such as the chemotherapeutic agents cisplatin and mitomycin C (Noll et al., 2006), and from endogenous sources such as naturally generated aldehydes (Langevin et al., 2011; Stone et al., 2008). They are highly cytotoxic as they inhibit transcription and replication and, unlike lesions involving only one strand, cannot be replicated simply by the damage tolerance mechanisms discussed above. Thus, to avoid continued replication arrest or the formation of potentially lethal DSBs, vertebrate cells coordinately deploy a series of mechanisms to bypass these lesions (reviewed in Deans and West, 2011). Important aspects of the molecular mechanism of replication-dependent interstrand crosslink repair have recently been revealed by a series of elegant experiments in which the replication of a plasmid containing a site-specific crosslink has been monitored in *Xenopus* egg extracts (Knipscheer et al., 2009; Long et al., 2011; Raschle et al., 2008). A summary of the mechanism as it is presently understood is shown in Fig. 4.

How is this complex set of processes choreographed? There is now very strong evidence of a pivotal role for the multiprotein complex known to be mutated in the human genetic condition Fanconi anaemia (reviewed by Kee and D’Andrea, 2010). It has been known for many years that cells from Fanconi anaemia patients are particularly sensitive to interstrand crosslinking agents (Sasaki and Tonomura, 1973). Furthermore, genetic evidence has shown that the Fanconi anaemia pathway cooperates with both homologous recombination factors and the TLS polymerases REV1 and Polζ in conferring resistance to interstrand crosslinking agents (Niedzwiedz et al., 2004). At the centre of the Fanconi anaemia complex is a multisubunit ubiquitin ligase comprising at least eight components, which has two key targets in the related proteins Fanconi anaemia complementation group D2 (FANCD2) (Garcia-Higuera et al., 2001) and Fanconi anaemia complementation group I (FANCI) (Smogorzewska et al., 2007). Ubiquitylation of these proteins is significantly enhanced in response to fork arrest caused by an interstrand crosslink, a step necessary for effective interstrand crosslink repair (Knipscheer et al., 2009). Depleting FANCD2 from the *Xenopus* egg system results in failure of both the nucleolytic incisions and TLS across the lesion (Knipscheer et al., 2009), suggesting that the Fanconi anaemia proteins are involved in the early steps of this reaction. Intriguingly, although FANCD2 and FANCI appear to operate upstream of the recombinational repair of the incised strand, they are not needed for loading of RAD51, but instead for the initial nucleolytic incisions (Knipscheer et al., 2009; Long et al., 2011). How the Fanconi anaemia pathway promotes these incisions is still unclear, but it appears probable that it involves the scaffold protein SLX4, which has recently been shown to be mutated in the FANCP complementation group and, hence, is now also known as FANCP (Kim et al., 2011; Stoepker et al., 2011). SLX4 binds to a number of nucleases, including SLX1, MUS81–EME1 and ERCC1–XPF (Munoz et al., 2009), all of which have been implicated in interstrand crosslink repair, although complementation analysis of FANCP or SLX4-deficient fibroblasts indicates that the interaction with ERCC1–XPF, but not with SLX1, is important in mediating resistance to mitomycin C (Crossan et al., 2011). Thus, the replication of interstrand crosslinks in vertebrates requires the careful orchestration of endonucleolytic incision, REV1-dependent TLS and RAD51-dependent homologous recombination, with the Fanconi anaemia complex apparently acting as principal conductor to ensure these ‘modules’ are deployed in the correct order.

NBS1 is part of the MRE11 complex comprising NBS1, MRE11 and RAD50 (reviewed by Stracker and Petrini, 2011). This complex has a key role in the DNA damage response by

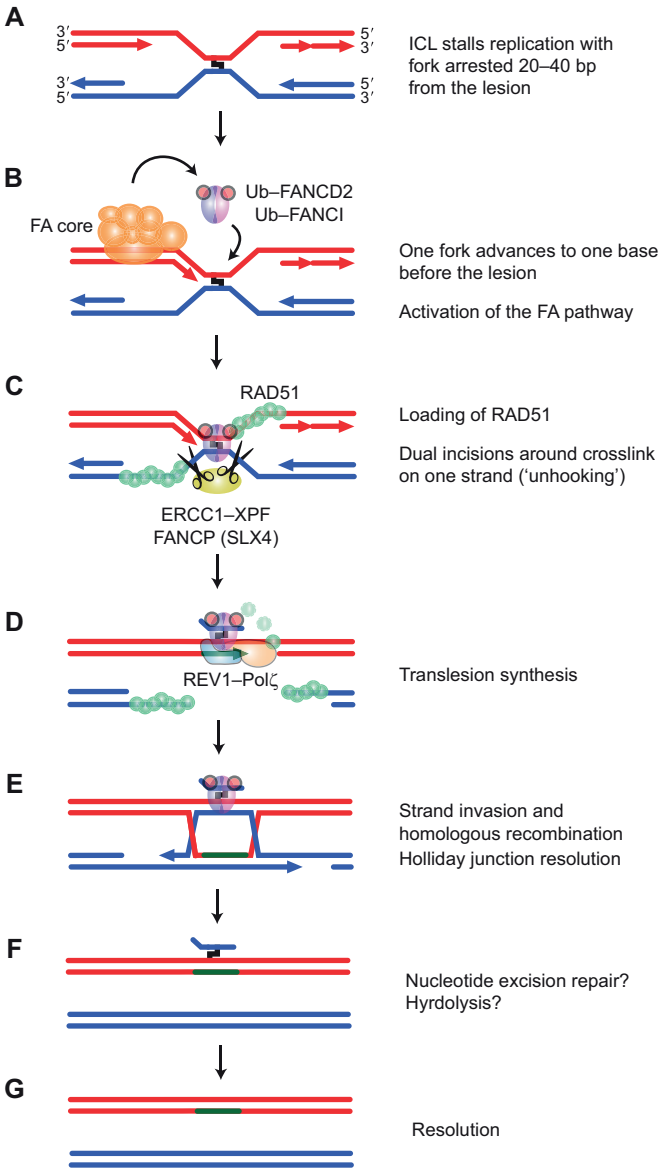

Fig. 4. Model for replication-dependent interstrand crosslink repair in vertebrates and its coordination by the Fanconi anaemia proteins. (**A**) In the system used by Walter and colleagues, a site-specific interstrand crosslink resides on a plasmid that it replicated in a *Xenopus* cell extract (Raschle et al., 2008). The replication forks move from the origin around the plasmid until they arrest ~20–40 nucleotides away from the crosslink (Raschle et al., 2008). (**B**) One of the forks then advances to one base before the crosslink. The Fanconi anaemia (FA) pathway recognises the lesion and is 'activated' by ubiquitylation (red circle) of FANCD2 and FANCI (pink and purple ovals) by the Fanconi anaemia core complex (orange cluster) (Knipscheer et al., 2009). (**C**) RAD51 (green circles) is loaded onto the single-stranded DNA around the crosslink prior to incisions being made either side of the lesion on one strand (Long et al., 2011). This step depends on the presence of FANCD2. The incision step is likely to be regulated by the nuclease complex ERCC1-XPF (Niedernhofer et al., 2004) coordinated by FANCP (SLX4) (yellow oval) (Crossan et al., 2011; Kim et al., 2011; Stoepker et al., 2011). (**D**) The now 'unhooked' crosslink can be bypassed by TLS, likely by REV1 with Polζ (blue and orange ovals) (Okada et al., 2005; Raschle et al., 2008). This step also requires FANCD2. (**E**) The remaining broken duplex can now be repaired by homologous recombination (Long et al., 2011). (**F**) The remainder of the crosslink is removed from the duplex either by nucleotide excision repair or hydrolysis. (**G**) Both duplexes are restored. The green line represents the tract of DNA synthesised by the TLS polymerases.

coordinating checkpoint activation through ataxia telangiectasia mutated (ATM), stabilisation of damaged replication forks and initiation of homologous recombination. However, a recent paper has shown that NBS1 is also important for the recruitment of RAD18 and for effective polη-dependent TLS (Yanagihara et al., 2011). NBS1 contains a domain in its C-terminus, alongside those responsible for it interaction with MRE11 and ATM, that interacts with RAD18. Although NBS1 and RAD6 compete for the same binding interface in the RAD18 molecule, the fact that RAD18 is a homodimer allows for a simultaneous interaction with both NBS1 and RAD6. Functionally, NBS1 is required for efficient deployment of polη and this is likely to explain the UV sensitivity of NBS1-deficient cells. Importantly, the UV sensitivity is not due to defective recombination because cells harbouring an NBS1 mutant that cannot bind MRE11 are sensitive to ionising radiation but not UV. Although the most straightforward explanation for this newly discovered role for NBS1 is that it is parallel and independent from its role in recombination, it is tempting to speculate that NBS1 provides a platform for the coordination of recombination and TLS. Such coordination might be particularly important for dealing with clustered DNA damage, such as that created by ionising radiation, which is characterised by a combination of DNA breaks and base lesions in close proximity. Similar to the replication of interstrand crosslinks, replication of clustered damage is likely to require the coordinate deployment of several of the 'modules' discussed above, and understanding this coordination will be an important area of future work.

Conclusions and perspectives

We have moved from an apparently well-ordered view of lesion bypass laid out by the early genetic studies to a much more fluid model that has been revealed by recent experiments. The existence of multiple, intersecting mechanisms not only provides robustness to ensure safe bypass of lesions but also allows a modular approach to enable the bypass of complex lesions. A number of key issues remain to be resolved, some of which are highlighted below.

The first concerns the function of PCNA polyubiquitylation because the exact role of the K63-linked ubiquitin chains that form on PCNA during damage bypass remains a mystery. Do they simply provide a docking station for key reactants in the process, thereby locally concentrating them? Another possibility is that they have a role in maintaining the necessary apposition of the sister chromatids. Or do they have a more active role in 'remodelling' the stalled fork (Goldfless et al., 2006)? And if so, what is the nature of this process? The interaction of the bypass pathways with chromatin is another rather unexplored area. Recent evidence suggests that the timing of lesion bypass can directly affect the ability of cells to accurately propagate transcriptional states (Sarkies et al., 2012; Sarkies et al., 2010). Use of post-replicative gap-filling can result in DNA synthesis that is uncoupled from the supply of histones displaced ahead of the advancing fork, and these histones carry modifications that contribute to the specification of transcriptional states. This provides a potential mechanism by which a defect in DNA damage tolerance at the fork can give rise not only to genomic instability but also to widespread changes in gene expression, a feature of many cancers (Sarkies and Sale, 2012). It is also probable that the chromatin structure itself influences DNA damage tolerance pathways, and evidence for such interactions is

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starting to emerge (Falbo et al., 2009). A final question is whether DNA damage tolerance pathways have potential as therapeutic targets. Recent data (Doles et al., 2010; Xie et al., 2010) provide some of the first clear evidence that targeted inhibition of DNA damage tolerance pathways holds promise as a chemotherapeutic approach in cancer. Answers to these questions will therefore not only further our understanding of the mechanism of DNA damage tolerance and its regulation but also, hopefully, provide new insights into how cancer arises and how it can be controlled.

Acknowledgements

I would like to thanks members of my lab for discussions and comments on the manuscript and Gerry Crossan, Juan Garaycoechea and K. J. Patel for advice on the interstrand crosslink repair model.

Funding

Work in my lab is funded by the Medical Research Council, Association for International Cancer Research and The Fanconi Anemia Research Fund.

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