# DNA Repair Mechanisms and the Bypass of DNA Damage in *Saccharomyces cerevisiae*

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**ABSTRACT** DNA repair mechanisms are critical for maintaining the integrity of genomic DNA, and their loss is associated with cancer predisposition syndromes. Studies in *Saccharomyces cerevisiae* have played a central role in elucidating the highly conserved mechanisms that promote eukaryotic genome stability. This review will focus on repair mechanisms that involve excision of a single strand from duplex DNA with the intact, complementary strand serving as a template to fill the resulting gap. These mechanisms are of two general types: those that remove damage from DNA and those that repair errors made during DNA synthesis. The major DNA-damage repair pathways are base excision repair and nucleotide excision repair, which, in the most simple terms, are distinguished by the extent of single-strand DNA removed together with the lesion. Mistakes made by DNA polymerases are corrected by the mismatch repair pathway, which also corrects mismatches generated when single strands of non-identical duplexes are exchanged during homologous recombination. In addition to the true repair pathways, the postreplication repair pathway allows lesions or structural aberrations that block replicative DNA polymerases to be tolerated. There are two bypass mechanisms: an error-free mechanism that involves a switch to an undamaged template for synthesis past the lesion and an error-prone mechanism that utilizes specialized translesion synthesis DNA polymerases to directly synthesize DNA across the lesion. A high level of functional redundancy exists among the pathways that deal with lesions, which minimizes the detrimental effects of endogenous and exogenous DNA damage.

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DNA damage is induced by exposure to environmental agents and is generated spontaneously during normal cellular metabolism (reviewed by Friedberg *et al.* 2006). Reactive oxygen species (ROS) are an unavoidable by-product of aerobic metabolism and cause both base damage and strand breaks. Additional spontaneous cellular reactions include the hydrolytic loss of bases, especially purines, from the phosphodiester backbone, as well as the deamination and alkylation of bases. In humans, it has been estimated that up to 100,000 spontaneous DNA lesions are generated daily per cell (Hoeijmakers 2009). Environmental DNA-damaging agents include the ultraviolet (UV) component of sunlight, which generates cyclobutane pyrimidine dimers and oxidative base damage; ionizing radiation, which produces clusters of ROS that create double-strand DNA breaks; and base-damaging chemicals such as aflatoxins, benzo(a)pyrene, methyl chloride, and nitrosamines, which alter or destroy base-pairing capacity. Because DNA damage has the potential to inhibit and/or alter fidelity of replication and transcription, there is a need for diverse and highly accurate repair processes. There is also a need for bypass mechanisms that allow unrepaired damage to be tolerated if encountered during replication. An emerging theme in the past 20 years is that there is considerable overlap between the various repair and bypass pathways in terms of the cognate lesions that each can deal with. This functional redundancy is partially a reflection of the very high load of endogenous DNA damage and underscores the importance of these pathways in the maintenance of genome stability.

The first comprehensive review of yeast DNA-repair pathways was published as part of the 1981 Cold Spring Harbor yeast books (Haynes and Kunz 1981). Studies at that time had focused on identifying the genes involved in surviving treatment with UV light and ionizing radiation (*RAD* genes) and on using epistasis analysis to place the genes into discrete pathways. These early genetic studies identified three discrete pathways, with each being named for the gene whose mutation conferred the most severe phenotype. The *RAD3* epistasis group encodes components of the nucleotide excision repair pathway, which is the major pathway for repairing UV-induced lesions; the *RAD52* epistasis group encodes components of the homologous recombination pathway and is required for the repair of ionizing radiation-induced damage; and the relatively ill-defined *RAD6* postreplication repair pathway encodes components required for the bypass of damages that block replicative DNA polymerases. It should be noted that components of the other major DNA-damage repair pathway—base excision repair—were absent among the early *rad* mutants and that most were identified biochemically.

The second iteration of the Cold Spring Harbor yeast books was published in 1991, a time when the emphasis was on cloning (usually by functional complementation of the mutant phenotype) and sequencing *RAD* genes and on purifying the encoded proteins and defining their biochemical properties (Friedberg *et al.* 1991). The current review will focus on the progress made in the intervening 20 years, which has truly been astounding. The damage-reversal and excision-repair pathways that remove DNA damage will be summarized, with an emphasis on the roles that individual players have within the defined pathway. A major area of new focus will be the mismatch repair system, which is responsible for removing errors made during DNA replication. The only yeast mismatch repair gene known in 1991 was *PMS1*, and rapid progress has been made in identifying other mismatch repair components and unraveling their molecular mechanisms. In addition, recent studies indicate that ribonucleoside monophosphates are frequently incorporated into genomic DNA, and a pathway for their removal has been described. Finally, the postreplication repair pathway, which is a tolerance/bypass pathway rather than a true repair pathway, will be considered. The most significant advances with relation to this pathway have been the characterization of specialized translesion synthesis DNA polymerases and the discovery that post-translational modification of proliferating cell nuclear antigen (PCNA) regulates alternative mechanisms of lesion bypass. The repair of double-strand breaks, which occurs primarily via homologous recombination in yeast, is covered in another review in this series and will not be considered here. Importantly, all of these pathways exhibit high evolutionary conservation, with discoveries made in the budding yeast *Saccharomyces cerevisiae* serving as a paradigm for repair processes in higher eukaryotes.

## Direct Reversal of DNA Damage

The simplest and most accurate repair mechanism is the direct reversal of damage in a single-step reaction. Direct reversal, however, applies to only a very limited number of

DNA lesions. The enzymatic photoreactivation of a cyclobutane pyrimidine dimer (CPD), which is the major product of UVB and UVC radiation, by DNA photolyase is the prototype of this type of reaction. In addition to possessing a CPD-specific photolyase (Phr1), yeast also has a methyltransferase (Mgt1) that removes methyl groups from modified bases.

### *Phr1, pyrimidine dimer DNA photolyase*

The *PHR1* gene was identified through the isolation of a mutant unable to photoreactivate CPDs (Resnick 1969) and cloned by restoration of photoreactivation in a *phr1* mutant also deficient in nucleotide excision repair (Schild *et al.* 1984). Transcription of the *PHR1* gene is stimulated as a general response to DNA-damaging agents such as UVC radiation and alkylating agents (Sebastian *et al.* 1990). The Phr1 protein is a monomer that contains stoichiometric amounts of two noncovalently attached chromophores: a catalytic flavin adenine dinucleotide ($FADH^-$) and a methylenetetrahydrofolate (MTHF) "second chromophore" (G. B. Sancar 1985; A. Sancar 2008). Photoreversal occurs by a series of steps initiated when Phr1 binds to CPD-containing DNA in a light-independent reaction. The MTHF of bound Phr1 then absorbs a photon in the near-UV to visible wavelengths (300–500 nm) and transfers its excitation energy to $FADH^-$. Next, the photo-excited $FADH^-$ transfers an electron to the CPD to generate an unstable dimer radical anion. The CPD ring splits to restore DNA structure, and a reverse electron transfer restores the functional form of the flavin chromophore.

### *Mgt1, $O^6$-methylguanine/$O^4$-methylthymine DNA methyltransferase*

The mispairing of $O^6$-methylguanine ($O^6$-MeG) with T and $O^4$-methylthymine ($O^4$-MeT) with G gives rise to GC-to-AT and AT-to-GC transitions, respectively. The yeast Mgt1 protein, which reverses both types of damage, was identified biochemically (Sassanfar and Samson 1990), and the corresponding gene was cloned by functional complementation in methyltransferase-deficient *Escherichia coli* cells (Xiao *et al.* 1991). Mgt1 repairs $O^6$-MeG by a suicide reaction that irreversibly transfers the methyl group to a cysteine residue in the enzyme active site to generate *S*-methylcysteine (Sassanfar and Samson 1990; Xiao *et al.* 1991). Although the protein has much lower affinity for $O^4$-MeT than for $O^6$-MeG *in vitro* (Sassanfar *et al.* 1991), $O^4$MeT repair *in vivo* can be inferred because expression of Mgt1 in *E. coli* prevents methylation-associated AT-to-GC transitions (Xiao *et al.* 1991).

Spontaneous mutation rates in Mgt1-deficient strains are enhanced, suggesting the presence of a natural source of $S_N1$-type endogenous or environmental alkylating agents (Xiao and Samson 1992). As expected, disruption of *MGT1* enhances sensitivity to the killing and mutagenic effects of $S_N1$-type alkylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Xiao and Samson 1992). Transcription of *MGT1* is not enhanced by low concentrations of MNNG, suggesting the absence of a bacterial-type adaptive response (Xiao and Samson 1992). The level of Mgt1 is, however, regulated by the Ubr1/Rad6- and Ufd4/Ubc4-mediated protein degradation pathways; loss of both pathways confers hyperresistance to MNNG and hypersensitivity to Mgt1 overexpression (Hwang *et al.* 2009).

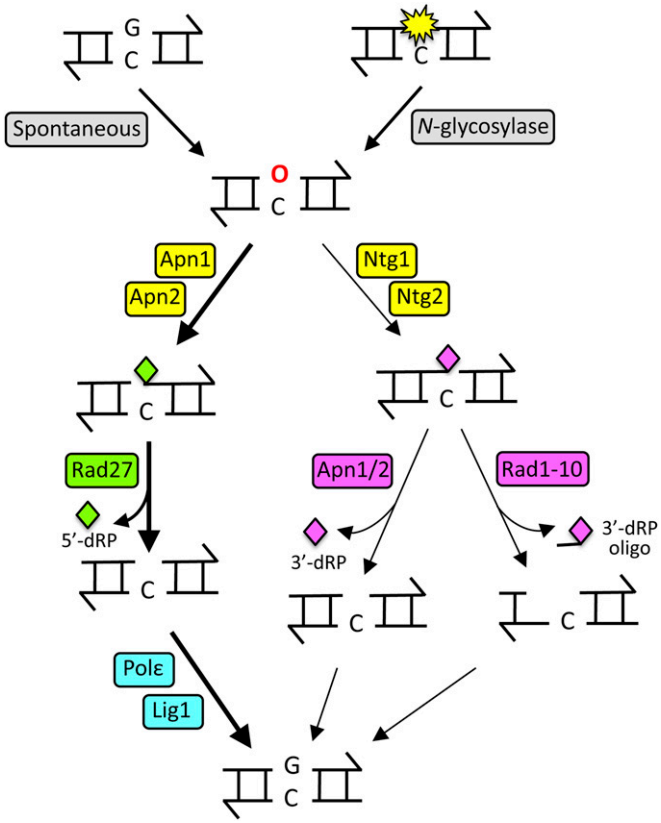


**Figure 1** The BER pathway. AP sites (red "O") are generated either by spontaneous base loss or a DNA *N*-glycosylase. Apn1 and Apn2 nick the backbone on the 5′ side of an AP site to initiate the major pathway for repair; the resulting 5′-dRP is removed by the Rad27 5′-flap endonuclease. AP-site processing can also be initiated by the Ntg1 or Ntg2 lyase, which nicks on the 3′ side of lesion. The resulting 3′-dRP can be removed by the 3′-diesterase activity of Apn1/Apn2 or as part of a Rad1-Rad10 generated oligonucleotide. Finally, the gap is filled by DNA Pol ε, and the backbone is sealed by DNA ligase 1.

## Base Excision Repair

The major endogenous DNA damages result from oxidative stress, hydrolysis, or deamination and are removed by the base excision repair (BER) pathway. BER requires the sequential action of five DNA-modifying activities: (1) a DNA *N*-glycosylase that releases the base from deoxyribose, (2) an endonuclease/lyase that nicks the DNA backbone at the resulting apyrimidinic/apurinic (AP) site, (3) a 3′- or 5′-phosphodiesterase that removes the remaining deoxyribose phosphate residue, (4) a DNA polymerase that fills the gap thus created, and (5) a DNA ligase to seal the remaining nick (Hoeijmakers 2001). The critical steps in the BER process are illustrated in Figure 1 and properties of the major BER factors are summarized in Table 1.

**Table 1 DNA *N*-glycosylases, AP endonucleases, and end-processing enzymes**

| Gene name (alternative) | Protein size (kDa) | Properties | Mammalian counterpart |
|---|---|---|---|
| *UNG1* | 40.5 | Monofunctional DNA *N*-glycosylase. Excision of uracil in ssDNA and dsDNA. Nuclear and mitochondrial. | UNG1/2 |
| *MAG1* (*MMS5*) | 34.3 | Monofunctional DNA *N*-glycosylase. Excision of 3-MeA, 7-MeG, HX, or 1-$N^6$-ethenoA from dsDNA. | AAG |
| *NTG1* (*SCR1*, *FUN33*, *OGG2*) | 45.5 | Bifunctional DNA *N*-glycosylase/AP lyase. Excision of oxidatively damaged pyrimidines and AP sites in dsDNA. Nuclear and mitochondrial. | NTH1 |
| *NTG2* (*SCR2*) | 43.8 | Bifunctional DNA *N*-glycosylase/AP lyase. Excision of oxidatively damaged pyrimidines and AP sites in dsDNA. Nuclear. | NTH1 |
| *OGG1* | 42.8 | Bifunctional DNA *N*-glycosylase/AP lyase. Excision of 8-oxoG, 8-oxoA, FapyG, and AP sites opposite C in dsDNA. Nuclear and mitochondrial. | OGG1 |
| *APN1* | 41.4 | AP endonuclease and 3′-phosphodiesterase. Incision of regular and oxidized AP sites. Excision of 3′-blocked ends. Nuclear and mitochondrial. | APE1 |
| *APN2* (*ETH1*) | 59.4 | AP endonuclease and 3′-phosphodiesterase. Incision of regular and oxidized AP sites. Excision of 3′-blocked ends. Nuclear. | APE2 |
| *TPP1* | 27.4 | DNA 3′-phosphatase. | PNPK |
| *TDP1* | 62.3 | Hydrolyzes phospho-tyrosyl bond. Repair of trapped topoisomerase I and II. | TDP1 |
| *RAD27* (*ERC11*, *RTH1*, *FEN1*) | 43.3 | 5′-Flap endonuclease. Excision of 5′-dRP after cleavage of AP sites by Apn1 or Apn2. | FEN1 |
| *HNT3* | 25.8 | Repair of abortive ligation product, 5′-AMP. | APTX |

ssDNA, single-strand DNA; dsDNA, double-strand DNA; 3-MeA, $N^3$-methyladenine; 7-MeG, $N^7$-methylguanine; HX, hypoxanthine; 1-$N^6$-ethenoA, 1,$N^6$-ethenoadenine.

### *DNA N-glycosylases*

BER is initiated by a DNA *N*-glycosylase that cleaves the *N*-glycosylic bond between the cognate damaged or unusual base and the sugar moiety to which it is attached. The reaction results in the release of a free base and the formation of an AP site, the central intermediate in the BER pathway (Figure 1). Five DNA *N*-glycosylases are present in yeast, and these fall into two classes: (1) monofunctional enzymes that only catalyze cleavage of the *N*-glycosylic bond (Ung1 and Mag1) and (2) bifunctional DNA *N*-glycosylases/AP lyases that catalyze both cleavage of the *N*-glycosylic bond and nicking of the phosphodiester backbone at AP sites (Ntg1, Ntg2, and Ogg1).

***Ung1, uracil-DNA N-glycosylase 1:*** Uracil in DNA arises either by cytosine deamination or through incorporation of dUTP in place of dTTP. Ung1 is highly specific for the removal of uracil in single- or double-strand DNA (Percival *et al.* 1989); it belongs to the UDG family, which includes the *E. coli* Ung protein as well as human UNG1/2 (Sousa *et al.* 2007). The *UNG1* gene was originally defined by mutations that allowed successful transformation of uracil-containing DNA into yeast (Burgers and Klein 1986) and was cloned by complementation using an *in vitro* assay (Percival *et al.* 1989). *UNG1* is cell-cycle regulated at the transcriptional level (Johnston and Johnson 1995), and mutant cells are sensitive to the killing effect of deaminating agents such as sodium bisulfite. Ung1-deficient cells exhibit a moderate spontaneous mutator phenotype (Burgers and Klein 1986), with a strong bias for the GC-to-AT transitions expected to result from cytosine deamination (Impellizzeri *et al.* 1991; Guillet *et al.* 2006). Ung1 localizes to the mitochondria as well as to the nucleus, and its loss additionally elevates mutations in mitochondrial DNA (Chatterjee and Singh 2001).

***Mag1, methylpurine-DNA N-glycosylase 1:*** The *MAG1* gene was identified by functional complementation in *E. coli*, and its disruption confers high sensitivity to the killing effects of alkylating agents such as methyl methanesulfonate (MMS) and MNNG (Chen *et al.* 1989). *MAG1* transcription is induced following exposure to low doses of alkylating agents (Chen *et al.* 1990), and the purified protein excises $N^7$-methylguanine and $N^3$-methyladenine (Chen *et al.* 1989, 1990; Berdal *et al.* 1990), as well as $N^3$-methylguanine, 1,$N^6$-ethenoadenine, and hypoxanthine from DNA (Saparbaev and Laval 1994; Lingaraju *et al.* 2008). Mag1 also releases normal bases, primarily guanine, at a slow rate from intact DNA (Berdal *et al.* 1998), and overexpression of the protein confers a strong spontaneous mutator phenotype in cells unable to efficiently repair AP sites (Glassner *et al.* 1998; Klapacz *et al.* 2010).

***Ntg1 and Ntg2, endonuclease III homologs:*** The e**N**donuclease **T**hree-like **G**lycosylase 1 (*NTG1*) and *NTG2* genes were identified based on homology of the encoded proteins to *E. coli* endonuclease III (Eide *et al.* 1996; Augeri *et al.* 1997). Each protein possesses a highly conserved helix-hairpin-helix DNA-binding motif, but only Ntg2 has an endonuclease III-like iron-sulfur (Fe-S) cluster (Eide *et al.* 1996; Augeri *et al.* 1997; You *et al.* 1998; Alseth *et al.* 1999). The Mms19 protein was recently identified as the protein

that delivers Fe-S clusters to Ntg2 and other key proteins involved in DNA metabolism (Stehling *et al.* 2012). Ntg1 has a positively charged N terminus that serves as a mitochondrial-targeting signal, and the protein localizes primarily to mitochondria (You *et al.* 1999). By contrast, Ntg2 localizes exclusively to the nucleus (You *et al.* 1999). An additional difference is that *NTG1* is inducible at the transcription level in cells exposed to oxidizing agents, whereas *NTG2* is not (Alseth *et al.* 1999).

Both Ntg1 and Ntg2 excise a variety of oxidized pyrimidines such as 5-hydrouracil, 5-hydroxycytosine, 5-6-dihydrothymine, and thymine glycol, as well as two purine lesions [formamidopyrimidine (Fapy)-Ade and Fapy-Gua] (Senturker *et al.* 1998). Neither Fapy-Ade nor Fapy-Gua, however, is a substrate for *E. coli* endonuclease III (Dizdaroglu *et al.* 1993). Ntg1 also excises 8-oxoguanine (8-oxoG) opposite guanine, but Ntg2 is not active against this lesion (Senturker *et al.* 1998). Both Ntg1 and Ntg2 are endowed with a robust AP lyase activity that incises DNA on the 3′-side of a regular AP site using a β-elimination reaction, yielding a single-strand break with a 3′-α,β-unsaturated aldehydic (3′-dRP) end (Figure 1) (Meadows *et al.* 2003).

Cells lacking both Ntg1 and Ntg2 are not unusually sensitive to the killing effects of MMS, γ-radiation, or $H_2O_2$, nor do they display a spontaneous mutator phenotype (Gellon *et al.* 2001). These data suggest alternative activities that can repair oxidized bases and AP sites in yeast. Indeed, inactivation of either the nucleotide excision repair (NER) or homologous recombination pathway in an *ntg1*Δ *ntg2*Δ double mutant results in a synergistic increase in sensitivity to the killing and mutagenic effects of $H_2O_2$ (Swanson *et al.* 1999; Gellon *et al.* 2001). The role of Ntg1 in the maintenance of mitochondrial DNA is controversial; both enhanced and reduced spontaneous mutation frequencies have been reported in an *ntg1*Δ background (Doudican *et al.* 2005; Phadnis *et al.* 2006).

***Ogg1, 8-oxoguanine-DNA N-glycosylase 1:*** Ogg1 is a bifunctional DNA *N*-glycosylase/AP lyase that, like Ntg1 and Ntg2, incises AP sites using a β-elimination reaction (Girard *et al.* 1997). Although it is the functional homolog of the *E. coli* Fpg (MutM) protein, Ogg1 has no sequence homology to its bacterial counterpart (Boiteux *et al.* 1987; Van Der Kemp *et al.* 1996). Instead, Ogg1 is a member of a superfamily of repair proteins that share a common ancestor with endonuclease III of *E. coli* and, in turn, with Ntg1 and Ntg2.

Ogg1 excises Fapy-Gua and 7,8-dihydro-8-oxoG from γ-irradiated DNA, whereas a wide range of other lesions, including oxidized pyrimidines and adenine lesions, are not substrates (Karahalil *et al.* 1998). The 8-oxoG *N*-glycosylase and AP lyase activities of Ogg1 are highly dependent on the identity of the base opposite the lesion, with the enzyme exhibiting a marked preference for cytosine (Girard *et al.* 1997). Although γ-irradiated DNA contains 8-oxoadenine (8-oxoA) opposite thymine, biochemical data suggest that Ogg1 exclusively excises 8-oxoA opposite cytosine (Girard *et al.* 1998). With respect to the *N*-glycosylase and AP lyase reactions, the catalytic mechanisms for Ogg1, Ntg1, and Ntg2 are very similar. The catalytic lysine residue of Ogg1 (K241) attacks the C1′ of the *N*-glycosylic bond between 8-oxoG and deoxyribose, releasing free 8-oxoG and yielding a covalent imino enzyme-DNA intermediate between Ogg1 and the C1′ of the abasic sugar moiety. A β-elimination reaction then produces a single-strand break with a 3′-dRP end and a restored Ogg1 protein (Boiteux *et al.* 2002). Although a crystal structure for yeast Ogg1 has not been reported, the strong sequence homology between the yeast and human proteins suggests that structural and mechanistic studies performed with human OGG1 most likely apply to yeast Ogg1 (Boiteux and Radicella 2000; Bruner *et al.* 2000; Bjoras *et al.* 2002; Fromme *et al.* 2004; Dalhus *et al.* 2009). Human OGG1, like most DNA *N*-glycosylases, binds the damaged strand and bends the DNA to flip the lesion into the active-site pocket.

Inactivation of *OGG1* does not lead to unusual sensitivity to DNA-damaging agents such as $H_2O_2$, γ-radiation, MMS, or UV radiation (Thomas *et al.* 1997). Mutants do, however, exhibit enhanced spontaneous and UVA-induced mutation rates (Thomas *et al.* 1997; Kozmin *et al.* 2005). There is a strong bias for GC-to-TA transversions in an *ogg1*Δ background, consistent with frequent insertion of A opposite a template 8-oxoG (Thomas *et al.* 1997; Ni *et al.* 1999; De Padula *et al.* 2004). Ogg1 additionally plays a role in the maintenance of telomere length homeostasis, reflecting repair of oxidized guanines in telomeric sequences (Lu and Liu 2010). Ogg1 localizes to both the nucleus and the mitochondria and plays an important role in the maintenance of mitochondrial as well as nuclear DNA (Singh *et al.* 2001; Vongsamphanh *et al.* 2006).

***GO network:*** In *E. coli*, MutT, MutM (Fpg), and MutY limit the mutagenic potential of 8-oxoG, and together are referred to as the "GO" network (Michaels and Miller 1992). Inactivation of either MutM or MutY results in a spontaneous mutator phenotype, which is characterized by specific accumulation of GC-to-TA transversions. MutM (Fpg) excises 8-oxoG opposite cytosine, whereas MutY releases adenine opposite 8-oxoG; together these two glycoslyases form the core of the GO network. The third component of the system is MutT, the loss of which is associated with AT-to-CG transversions. MutT hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, thereby cleansing the dNTP pool and preventing the incorporation of 8-oxo-dGMP opposite a template adenine. In *S. cerevisiae*, loss of Ogg1 generates only a moderate mutator phenotype, suggesting the occurrence of a MutY homolog that helps limit 8-oxoG-associated GC-to-TA transversions. Although lacking a MutY-type glycosylase that deals with 8-oxoG:A mispairs, *S. cerevisiae* nevertheless possesses a biological GO network that prevents the mutagenic action of endogenous 8-oxoG (Figure 2). This network involves the mismatch repair system, which serves a role analogous to that of MutY by removing adenine incorporated opposite a template 8-oxoG (Ni *et al.* 1999), as well as the Pol η

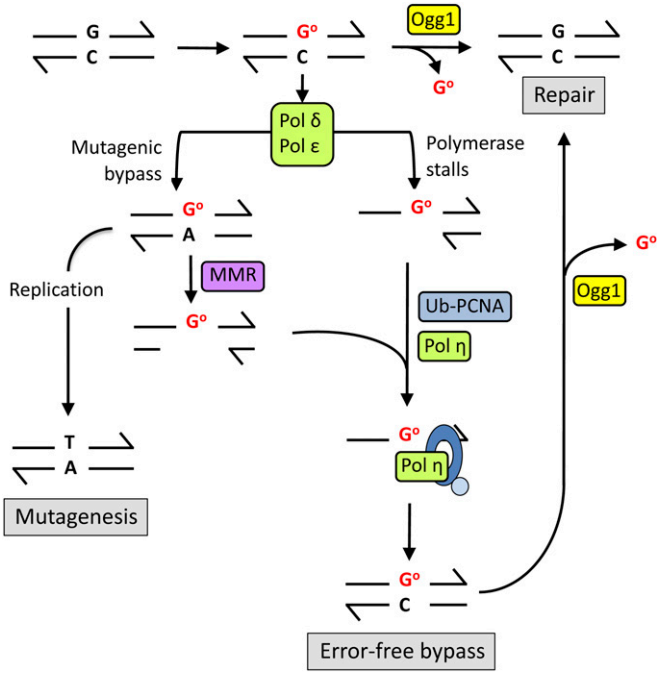


**Figure 2** The GO network. Reactive oxygen species attack guanine base-paired with cytosine to yield 8-oxoG ($G^O$). Ogg1 excises 8-oxoG from the DNA backbone, and the resulting AP site is repaired via BER ("repair"). If encountered during replication, local sequence context will determine whether Pol δ/ε stalls at or bypasses the $G^O$ lesion. If Pol δ/ε stalls at 8-oxoG during replication, Pol η is recruited by ubiquitinated PCNA (Ub-PCNA) and preferentially incorporates C opposite the lesion ("error-free bypass"). During bypass by Pol δ/ε, adenine is frequently inserted instead of cytosine to create a $G^O$:A mispair, which is recognized by the MMR machinery. The newly synthesized, A-containing strand is degraded to generate a single-strand gap containing the lesion, and C is incorporated opposite the lesion during a gap-filling reaction, which may involve Pol η. If not repaired, the $G^O$:A mispair will yield a GC-to-TA transversion at the next round of replication ("mutagenesis").

translesion synthesis DNA polymerase, which preferentially inserts C opposite a template 8-oxoG (Haracska *et al.* 2000). Yeast has no known functional homolog of the bacterial MutT protein, although homologs are present in other eukaryotes.

### *AP endonucleases*

AP endonucleases bind to AP sites in duplex DNA and nick the phosphodiester backbone immediately 5′ of the lesion. In addition, these enzymes process a variety of 3′-blocked termini that would otherwise block DNA polymerization and ligation. Yeast has two AP endonucleases—Apn1 and Apn2—with >95% of *in vivo* activity attributed to Apn1 (Popoff *et al.* 1990).

***Apn1, AP endonuclease 1:*** The *APN1* gene was identified through immunological screening of an expression library, and the encoded protein shares extensive homology with the *E. coli* endonuclease IV (Nfo) protein (Popoff *et al.* 1990). In addition to its canonical AP-endonuclease activity, which nicks DNA on the 5′-side of a regular or reduced AP site, Apn1 has a 3′-phosphodiesterase activity that excises 3′-blocking groups such as 3′-dRP, 3′-phosphoglycolate (3′-PGA), and 3′-phosphate (3′-P); it possesses a 3′-tyrosyl-DNA phosphodiesterase activity that contributes to the removal of covalently bound topoisomerase 1; and it has 3′-exonuclease activity (reviewed in Boiteux and Guillet 2004). Apn1 also has an endonuclease activity that nicks DNA on the 5′-side of oxidized bases, which initiates an alternative DNA-repair pathway referred to as nucleotide incision repair (Ischenko and Saparbaev 2002). Deletion of *APN1* confers moderate sensitivity to the killing effects of oxidizing or alkylating agents (Ramotar *et al.* 1991), and *apn1*Δ cells have a spontaneous mutator phenotype, accumulating mostly AT-to-CG events (Kunz *et al.* 1994). Apn1 is important for repairing alkylation damage in mitochondrial as well as nuclear DNA (Acevedo-Torres *et al.* 2009), and the protein localizes to both cellular compartments (Ramotar *et al.* 1993).

***Apn2, AP endonuclease 2:*** *APN2* was identified based on homology to exonuclease III of *E. coli* (Johnson *et al.* 1998; Bennett 1999), and its transcription is induced by MMS (Bennett 1999). Loss of *APN2* does not sensitize cells to the lethal or mutagenic effects of MMS or $H_2O_2$, but *apn1*Δ *apn2*Δ double mutants are extremely sensitive to both agents (Johnson *et al.* 1998; Bennett 1999). In addition to hydrolytic cleavage of DNA on the 5′-side of an AP site, Apn2 is also endowed with X′ to Y′ exonuclease and 3′-phosphodiesterase activities that remove 3′-blocking groups (Unk *et al.* 2000). Both activities are stimulated by physical interaction with the PCNA sliding clamp, which targets multiple DNA metabolic proteins to nicks/gaps in DNA (Moldovan *et al.* 2007).

***Origin, repair, and biological impact of endogenous AP sites:*** AP sites are abundant, endogenous DNA lesions (De Bont and Van Larebeke 2004; Swenberg *et al.* 2011) that can be lethal and mutagenic (Boiteux and Guillet 2004). Although highly sensitive to alkylating or oxidizing agents, cells lacking both Apn1 and Apn2 are viable and exhibit only a moderate spontaneous mutator phenotype (Johnson *et al.* 1998; Bennett 1999). This suggests either that AP sites are formed at a much lower rate *in vivo* than predicted or that backup repair activities manage the effects of persistent AP sites. Indeed, crossing deletions of candidate DNA-repair genes into an *apn1*Δ *apn2*Δ background revealed that loss of Rad1-Rad10, a complex best known for its nicking activity during NER, causes cell death (Guillet and Boiteux 2002). In addition, simultaneous inactivation of Apn1, Apn2, Ntg1, and Ntg2 results in a strong spontaneous mutator phenotype dominated by AT-to-CG transversions (Collura *et al.* 2012). Together, these data confirm that AP sites form under physiological growth conditions and that these sites impair cell viability and genetic stability when DNA repair is compromised.

Although there are likely multiple origins of endogenous AP sites, genetic studies suggest that most are linked to dUTP incorporation, which is normally limited by action of the Dut1 dUTPase (Guillet *et al.* 2006). If incorporated, however, the uracil is excised by Ung1 to create an AP site (Guillet and

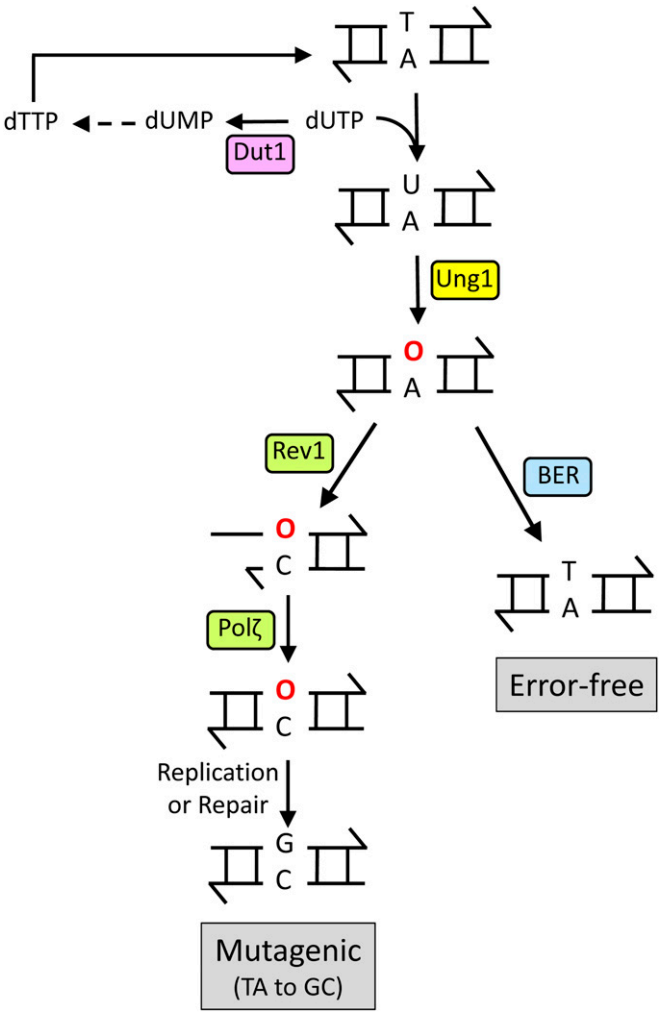


**Figure 3** Bypass of endogenous AP sites. dUTP levels in the nucleotide pool are reduced by Dut1 activity, thereby limiting the incorporation of dUMP into genomic DNA. Most endogenous AP sites (indicated by red "O") are generated by Ung1 removal of uracil in DNA. The resulting AP site can be repaired by the BER pathway or bypassed by the concerted action of Rev1, which usually inserts cytosine opposite the AP site, and Pol ζ, which extends the O:C terminus.

Boiteux 2003; Collura *et al.* 2012). As noted above, inefficient AP-site repair results in mostly AT-to-CG mutations, which presumably reflect bypass of dUMP-derived AP sites by the concerted action of the Rev1 and Pol ζ translesion synthesis DNA polymerases (Figure 3).

### *Single-strand break repair: "dirty end" processing factors*

To be a substrate for DNA polymerase and DNA ligase, single-strand breaks must have "clean" ends: a 3′-OH dNMP and a 5′-P dNMP. Genetic and biochemical studies have revealed that there is considerable redundancy with respect to the proteins that can generate such ends during BER (see Figure 1).

***Processing 3′-dirty ends:*** DNA *N*-glycosylases/AP lyases incise DNA on the 3′-side of a regular AP site, generating a single-strand break with an abasic 3′-dRP end (Boiteux *et al.* 2002). Other 3′-blocked ends include the 3′-PGA or 3′-P, formed when DNA is exposed to oxidative stress, as well as the covalent attachment of Top1 via a 3′-phosphotyrosyl linkage (Caldecott 2008). Whereas Tdp1 catalyzes the removal of 3′-Top1 (Pouliot *et al.* 1999), the major defenses against 3′-dRP, 3′-PGA, and 3′-P are the 3′-DNA phosphatase activity of Tpp1 and the 3′-phosphodiesterase activity of Apn1 and Apn2 (Vance and Wilson 2001a,b). Rad1-Rad10 also has a role in the release of all types of 3′-blocked termini at a single-strand break (Vance and Wilson 2002; Boiteux and Guillet 2004; Guzder *et al.* 2004). Finally, other structure-dependent endonucleases, such as Mus81-Mms4 and Slx1-Slx4, can participate in the removal of 3′-dirty ends (Guillet and Boiteux 2002; Deng *et al.* 2005).

***Processing 5′-dirty ends:*** Apn1 and Apn2 nick 5′ of AP sites, generating nicks with 5′-dRP ends. In mammalian cells, removal of 5′-dRP is accomplished either by the lyase activity of DNA polymerase β or by the 5′-flap endonuclease (FEN) activity of FEN1 (Hoeijmakers 2001). In *S. cerevisiae*, both DNA Pol4 (Bebenek *et al.* 2005) and Trf4 (Gellon *et al.* 2008) possess a 5′-dRP lyase activity, but whether either contributes to BER *in vivo* has not been clearly established (McInnis *et al.* 2002; Gellon *et al.* 2008). The high MMS and $H_2O_2$ sensitivity of yeast cells missing Fen1/Rad27 (Reagan *et al.* 1995; Hansen *et al.* 2000), and the suppression of the MMS sensitivity of *rad27*Δ by *APN1* deletion (Wu and Wang 1999), suggest that Rad27 is the main activity for removing 5′-dRP (see Figure 1).

The ligation intermediate 5′-AMP, which reflects an abortive attempt to ligate a nick, constitutes another important type of 5′-dirty end. In human cells, 5′-AMP is reversed by Aprataxin, the loss of which causes the severe neurodegenerative disorder ataxia-oculomotor apraxia 1 (Caldecott 2008). Hnt3 is an Aprataxin-like protein in *S. cerevisiae*, and it can repair 5′-AMP *in vitro* (Ahel *et al.* 2006). Although Hnt3-deficient cells are not unusually sensitive to the killing effect of $H_2O_2$ or MMS, simultaneous loss of Hnt3 and Rad27 results in a synergistic increase in $H_2O_2$ and MMS sensitivity, suggesting redundant roles in the repair of 5′-AMP (Daley *et al.* 2010).

### *DNA polymerase and DNA ligase*

The removal of 3′- and/or 5′-dirty ends by appropriate BER enzymes generates a small gap in DNA, which is then ready to be filled by a DNA polymerase (Figure 1). DNA polymerase ε (Pol ε) is the major source of BER-associated repair synthesis in nuclear extracts (Wang *et al.* 1993), and Pol2, the catalytic subunit of Pol ε, binds with high affinity to a BER intermediate *in vitro* (Sukhanova *et al.* 2011). Furthermore, the *pol2-16* allele, which impairs DNA polymerase activity, confers significant sensitivity to MMS, but not to UV radiation (Kesti *et al.* 1999). A role of other DNA polymerases such as polymerase δ (Pol δ) in BER has not been excluded, however. DNA repair is completed by the Cdc9

DNA ligase (Lig1), which uses an AMP-lysine intermediate susceptible to generating 5′-AMP damage (Tomkinson *et al.* 1992).

## Nucleotide Excision Repair

Components of the NER pathway belong to the *RAD3* epistasis group, and most were identified in genetic screens for mutants with enhanced UV sensitivity (Haynes and Kunz 1981). NER is characterized by an ability to remove a large number of structurally unrelated, helix-distorting lesions that interfere with base pairing and generally impair replication and transcription. This pathway is particularly relevant for preventing the lethal and mutagenic effects of environmental mutagens; cognate lesions include the UV-induced CPD and 6-4 photoproduct [(6-4) PP] as well as chemical carcinogen-induced bulky adducts (Cadet *et al.* 2005; Friedberg *et al.* 2006). Loss of NER in humans is associated with the disease xeroderma pigmentosum, which is characterized by an extreme sensitivity to sunlight and cancer predisposition (Friedberg *et al.* 2006). NER also removes bulky, endogenous oxidative DNA damage that results from intramolecular crosslinking between the C8 position of purines and the 5′ position of deoxyribose (Kuraoka *et al.* 2000). Finally, NER can provide an alternative mechanism to repair AP sites and oxidized bases (Scott *et al.* 1999; Swanson *et al.* 1999; Torres-Ramos *et al.* 2000; Gellon *et al.* 2001).

The NER pathway can be divided into two subpathways based on the initial lesion recognition step: global-genome NER (GG-NER) and transcription-coupled NER (TC-NER). In GG-NER, dedicated proteins directly recognize an initiating lesion, and complexes containing Rad7 and Rad16 are unique to this subpathway. By contrast, TC-NER is initiated when a lesion on the transcribed strand blocks RNA polymerase; unique to TC-NER are Rad26 and the Rpb9 subunit of RNA polymerase II (RNA Pol II). Following lesion recognition, the two subpathways converge and dual incisions are made flanking the damage. Although additional proteins are relevant *in vivo*, biochemical reconstitution of the dual incision reaction on naked, UV-irradiated DNA has identified six essential NER factors: Rad4-Rad23, Rad14, TFIIH, Rad1-Rad10, Rad2, and replication protein A (RPA) (Guzder *et al.* 1995b). *In vivo*, there may exist a preassembled "repairosome" that contains all NER factors (Svejstrup *et al.* 1995; Rodriguez *et al.* 1998), or, alternatively, NER factors may assemble either one-by-one or as part of discrete subcomplexes (Prakash and Prakash 2000). Recent studies in mammalian cells favor the sequential recruitment of DNA repair factors/subcomplexes during NER (Volker *et al.* 2001; Mocquet *et al.* 2008). Following the dual-incision reaction, a lesion-containing single strand of 25–30 nucleotides is released from the helix, the resulting gap is filled by DNA polymerase, and the remaining nick is sealed by ligase. The properties of proteins involved in NER are summarized in Table 2 and are detailed further below in relation to major steps of this repair pathway. Figure 4 presents a complete model of NER that illustrates lesion recognition during GG-NER; Figure 5 presents unique aspects of TC-NER.

### *Recognition of lesions during GG-NER*

The GG-NER pathway repairs lesions without regard to transcription status or associated chromatin structure and is initiated when a trimeric Rad4-Rad23-Rad33 complex senses distortion of the DNA helix (Figure 4A). Recruitment of Rad4-Rad23-Rad33 to sites of DNA damage may be facilitated by interactions between Rad4 and chromatin remodeling complexes such as Ino80 (Sarkar *et al.* 2010) and SWI/SNF (Gong *et al.* 2006). The Rad7-Rad16 complex is mandatory for GG-NER *in vivo*; as described in human cells, this complex, in association with alternative factors, may promote the recruitment of Rad4 through its high affinity for DNA damage and its E3 ubiquitin (Ub) ligase activity (Sugasawa *et al.* 2005). Similar to the analogous human complex, yeast Rad4-Rad23-Rad33 likely initiates the opening of an ~10-bp region around the lesion (Tapias *et al.* 2004).

***Rad4-Rad23-Rad33 and Rad34:*** Whereas Rad4 is essential for all NER, loss of either Rad23 or Rad33 only reduces repair. Simultaneous deletion of both proteins, however, generates a complete deficiency in NER (Den Dulk *et al.* 2006). Rad4 and Rad23 form a stable complex that specifically binds to UV-irradiated DNA *in vitro* (Jansen *et al.* 1998), and, as noted above, this complex is required for dual incision in the reconstituted system (Guzder *et al.* 1995b). Although Rad23 is unable to bind damaged DNA by itself, it stimulates Rad4-binding activity and prevents its degradation by the proteasome (Xie *et al.* 2004). In the crystal structure of a Rad4-Rad23-DNA-CPD complex, Rad4 inserts a β-hairpin through the DNA duplex, causing the two damaged base pairs to flip out of the helix; the damaged nucleotides are exposed to solvent whereas the undamaged ones contact Rad4. These structural data suggest that Rad4 recognizes damage through the sensing of thermodynamically unstable base pairs and thus provide an explanation for its broad substrate specificity (Min and Pavletich 2007). It should be noted that inactivation of Rad4 completely abolishes both GG-NER and TC-NER in yeast. In human cells, however, inactivation of the XPC homolog results in only an intermediate sensitivity to UV radiation, and functional TC-NER is retained (Venema *et al.* 1991; Verhage *et al.* 1994).

Rad33 is a an additional NER factor that binds directly to Rad4 (Den Dulk *et al.* 2006, 2008). Rad4 interacts with Rad23 and Rad33 through independent sites in its C-terminal region, and the roles of these proteins are presumably to modulate Rad4 activity and integrity (Den Dulk *et al.* 2006). Finally, the Rad34 protein has sequence homology to Rad4 and, like Rad4, interacts with Rad23. Available data suggest a role for Rad34 during NER that occurs in the RNA Pol I-transcribed ribosomal DNA genes (Den Dulk *et al.* 2005; Tremblay *et al.* 2008).

**Table 2 NER genes**

| Gene name (alternative) | Protein size (kDa) | Properties | Mammalian counterpart |
|---|---|---|---|
| *RAD4* | 87.2 | Forms a complex with Rad23 and Rad33 that binds damaged DNA. | XPC |
| *RAD23* | 42.4 | Forms a complex with Rad4 that binds damaged DNA. | HRAD23B |
| *RAD33* | 20.3 | Forms a complex with Rad4 that binds damaged DNA. | CEN2 |
| *RAD7* | 63.8 | Forms a complex with Rad16. | DDB1 |
| *RAD16* (*PSO5*) | 91.4 | Forms a complex with Rad7 that has ATP-dependent binding of damaged DNA, chromatin remodeling activity, and E3 ligase activity. | DDB2 |
| *RAD1* (*LPB9*) | 126.4 | Forms a complex with Rad10 that has structure-dependent endonuclease activity; incises DNA on the 5′-side of lesions. | XPF |
| *RAD10* | 24.3 | Forms a complex with Rad1. | ERCC1 |
| *RAD2* | 117.8 | Structure-dependent endonuclease; incises DNA on the 3′-side of lesions. | XPG |
| *RAD14* | 43.0 | Zinc-finger protein; binds damaged DNA. | XPA |
| *RFA1* (*BUF2*, *FUN3*, *RPA1*) | 70.3 | Component of heterotrimeric RPA, the yeast single-strand DNA binding protein. | RPA |
| *RFA2* (*BUF1*, *RPA2*) | 29.9 | Component of heterotrimeric RPA. | RPA |
| *RFA3* (*RPA3*) | 13.8 | Component of heterotrimeric RPA. | RPA |
| *RAD25* (*SSL2*, *LOM3*) | 95.3 | TFIIH subunit; DNA-dependent ATPase and X′ to Y′ helicase. | XPB |
| *RAD3* (*REM1*) | 89.8 | TFIIH subunit; DNA dependent ATPase and helicase with Y′ to X′ polarity. | XPD |
| *TFB1* | 72.9 | Core TFIIH component. | GTF2H1 |
| *SSL1* | 52.3 | Core TFIIH component. | GTF2H2 |
| *TFB2* | 58.5 | Transcription initiation factor IIB, a core TFIIH component. | GTF2H4 |
| *TFB4* | 37.5 | Transcription factor B subunit 4, a core TFIIH component. | GTF2H3 |
| *TFB5* | 8.2 | Core TFIIH component. | TTDA |
| *CDC9* (*MMS8*) | 84.8 | DNA ligase 1. | LIG1 |
| *RAD26* | 124.5 | DNA-dependent ATPase required for transcriptional bypass of lesions and for TC-NER. | CSB |
| *RAD28* | 58.2 | WD40 repeat protein of unknown function. | CSA |
| *RPB9* (*SSU73*) | 14.3 | Nonessential RNA Pol II subunit required for Rad26-independent TC-NER. | POLR21 |
| *POL2* | 255.7 | Catalytic subunit of replicative DNA Pol ε. | p261, POLE |
| *POL3* | 124.6 | Catalytic subunit of replicative DNA Pol δ; important for repair synthesis during NER. | p125, POLD1 |

***Rad7-Rad16-Abf1 and Rad7-Rad16-Cul3-Elc1:*** Rad7 and Rad16 are required for CPD removal from unexpressed sequences and for repair of UV-induced damage located on the nontranscribed strand of active genes (Verhage *et al.* 1994), indicating a specific role in the GG-NER subpathway (Hanawalt and Spivak 2008). Loss of either protein results in an intermediate level of UV sensitivity (Prakash *et al.* 1993). Rad7 and Rad16 form a stable complex in an ATP-dependent manner, and the complex binds with high affinity to UV-irradiated DNA (Guzder *et al.* 1997). Rad16 has homology to Snf2, the catalytic subunit of the SWI/SNF chromatin-remodeling complex, but the complex does not have detectable DNA helicase activity. Rad16 facilitates histone H3 acetylation after UV irradiation (Teng *et al.* 2008), however, and an effect of histone H3 methylation on NER at silenced loci has been reported (Chaudhuri *et al.* 2009). Although not required for dual cleavage of UV-irradiated DNA *in vitro*, the Rad7-Rad16 complex markedly stimulates the reaction; combining Rad4-Rad23 and Rad7-Rad16 synergistically enhances binding to UV-damaged DNA (Guzder *et al.* 1999). Finally, physical interaction between Rad4 and Rad7 allows the formation of a large lesion-recognition complex (Guzder *et al.* 1999). These data clearly point to a role for Rad7-Rad16 in DNA damage recognition during GG-NER, and other studies suggest an additional role for Rad7-Rad16 in post-incision events (Reed *et al.* 1998).

Purification of Rad7-Rad16 led to the identification of the essential protein autonomously replicating sequence binding factor 1 (Abf1) as the third component of a trimeric Rad7-Rad16-Abf1 complex. Abf1 plays a direct role in NER; *abf1* conditional mutants are defective in the removal of UV damage and exhibit high sensitivity to UV radiation (Reed *et al.* 1999). Rad7-Rad16-Abf1 can generate superhelical torsion in DNA *in vitro* (Yu *et al.* 2004), suggesting a model in which the complex binds to ABF1 sites in DNA in the absence of UV irradiation. Following UV irradiation, the complex uses ATP hydrolysis to translocate on DNA, generating conformational changes and eventually stalling at damaged sites to facilitate the recruitment of Rad4 (Guzder *et al.* 1998; Yu *et al.* 2009).

In addition to interacting with Rad4, Rad7-Rad16 interacts with Elc1-Cul3 to form a cullin-based E3 Ub ligase that promotes UV-dependent ubiquitination of Rad4 and other chromatin-associated proteins (Ramsey *et al.* 2004; Gillette *et al.* 2006). Rad7-Rad16-based complexes thus have multiple roles in lesion recognition that include binding to DNA damage, interacting with Rad4-Rad23-Rad33, driving conformational changes in DNA, and remodeling chromatin through acetylation and ubiquitination. Although essential for GG-NER *in vivo*, Rad7-Rad16 is not required for dual incisions in a reconstituted GG-NER system (Guzder *et al.* 1995b). Most likely, the *in vitro* levels of other repair factors

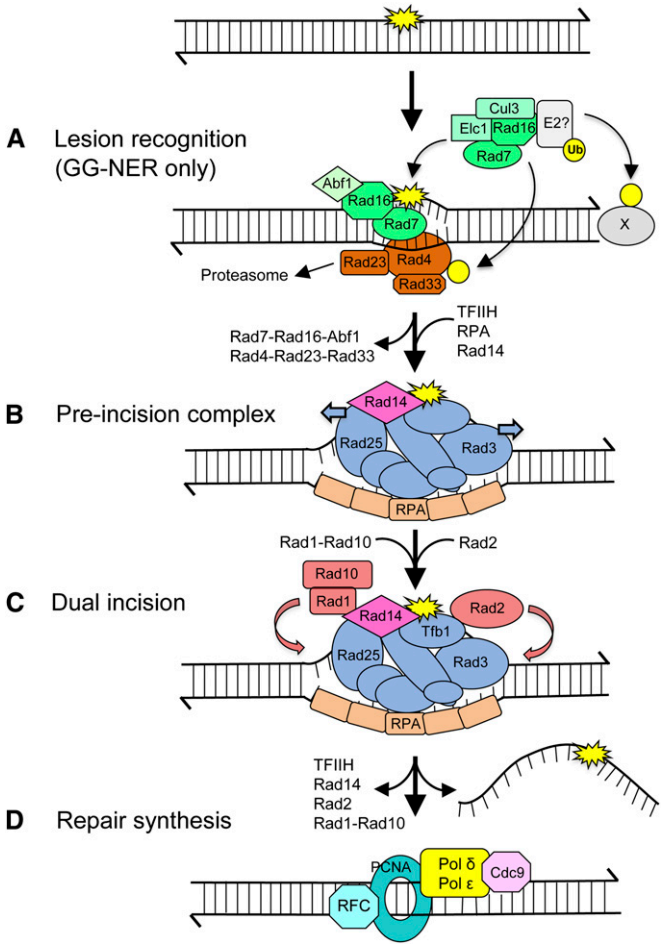


**Figure 4** The NER pathway. (A) During GG-NER, a helix-distorting lesion (yellow star) is recognized by Rad4-Rad23-Rad33 and Rad7-Rad16 complexes. Rad7-Rad16-Abf1 has chromatin-remodeling activity, whereas Rad7-Rad16-Elc1-Cul3-E2 has Ub ligase activity, which modifies Rad4 and additional factors ("X"). These reactions allow efficient recognition of lesions by Rad4, its proper positioning, and opening of the helix ~10 bp. (B) TFIIH (components are in blue), Rad14, and RPA are recruited to form a pre-incision complex that verifies the lesion and further unwinds DNA. Rad4-Rad23-Rad33 and Rad7-Rad16-Abf1 are released. (C) The structure-specific endonucleases Rad1-Rad10 and Rad2 are positioned to incise 5′ and 3′ of the lesion, respectively. (D) A lesion-containing oligonucleotide (25–30 nt) is released from the duplex, followed by repair synthesis and ligation.

and DNA damage are high enough to bypass the need for complexes that operate primarily at a chromatin level *in vivo*.

### *Formation of an open-structure and pre-incision complex*

During GG-NER, the DNA structure generated by Rad4-Rad23-Rad33 allows the recruitment and positioning of the TFIIH transcription factor, which extends opening of the helix using the ATPase/helicase activities of Rad3 and Rad25 (Figure 4B). The Rad4-Rad23-Rad33 recognition complex is released, and Rad14 and RPA are recruited to stabilize the pre-incision complex. In the case of TC-NER, TFIIH is already positioned at the lesion (see below). The presence of the lesion in DNA is then reassessed in a "verification step" mediated by TFIIH, RPA, and Rad14. RPA binds

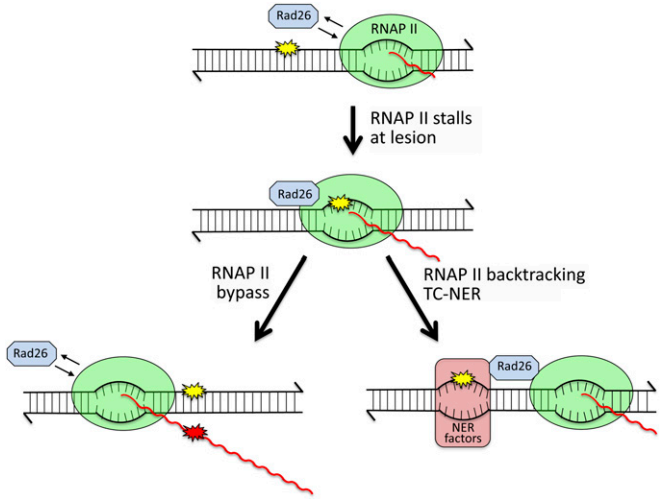


**Figure 5** Responses to stalling of RNA Polymerase II at a template lesion. Stalling of RNA Polymerase II (RNAP II) at a lesion (yellow star) in the transcribed strand of an active gene stabilizes its interaction with Rad26/CSB. Transcriptional bypass of a lesion that is a moderate block to RNAP II is promoted by Rad26/CSB. During such bypass, an incorrect rNMP (red star) can be inserted into the nascent mRNA (red wavy line), which may then specify a mutant protein ("transcriptional mutagenesis"). If the lesion is a strong block to RNAP II, Rad26/CSB and additional factors mediate the backtracking of polymerase, which exposes the lesion and promotes the recruitment of NER factors. Following lesion removal, transcription resumes without loss of the transcript.

the undamaged strand, whereas Rad14 binds to the DNA lesion (De Laat *et al.* 1999). In the absence of lesion verification, the NER reaction aborts before dual incision occurs (Sugasawa *et al.* 2001).

***TFIIH:*** TFIIH is an essential factor required for the initiation of transcription at RNA Pol II promoters, and it also is required for NER. A role of TFIIH in NER emerged when the *RAD3* and *RAD25* gene products were identified as components of TFIIH (Feaver *et al.* 1993). Rad3 is a DNA-dependent ATPase with Y′ to X′ DNA helicase activity (Sung *et al.* 1987; Harosh *et al.* 1989); the ATPase activity of Rad3 is essential for NER, but not for transcription (Feaver *et al.* 1993). Rad25 is also a DNA-dependent ATPase, but has DNA helicase activity with an opposite, X′ to Y′ polarity (Guzder *et al.* 1994; Sung *et al.* 1996). In contrast to Rad3, the ATPase/helicase activity of Rad25 is essential during both NER and transcription.

TFIIH is composed of 10 subunits that can be divided into two subcomplexes: a core TFIIH subcomplex with seven subunits (Rad25, Rad3, Tfb1, Tfb2, Ssl1, Tfb4, and Tfb5) and a CAK kinase complex composed of three subunits (Kin28, Ccl1, and Tfb3) (Egly and Coin 2011; Gibbons *et al.* 2012). The core TFIIH complex alone is highly active in NER (Svejstrup *et al.* 1995); only Tfb5 is dispensable for viability and for NER (Giglia-Mari *et al.* 2004; Ranish *et al.* 2004). Absence of Tfb5, however, results in poor growth, enhanced sensitivity to UV radiation, and greatly reduced NER activity *in vitro* (Ranish *et al.* 2004; Zhou *et al.* 2007).

The roles of Tfb1, Ssl1, Tfb2, Tfb4, and Tfb5 in NER are probably to structure the TFIIH core complex via protein–protein interactions. The catalytic role of TFIIH during NER primarily reflects the opposite polarities of the Rad3 and Rad25 ATPase/helicase activities, which unwind DNA flanking the lesion. In addition, TFIIH interacts with Rad4, Rad23, and RPA (Bardwell *et al.* 1994b; Guzder *et al.* 1995a). TFIIH is thus a pivotal component of NER because of its intrinsic helicase activities and its interactions with other essential NER components.

***Rad14:*** Rad14 contains a zinc-finger domain and binds with high affinity to UV-damaged DNA (Guzder *et al.* 1993). It is essential for incision of UV-damaged DNA in a reconstituted system (Guzder *et al.* 1995b) and is required for NER *in vivo*. Rad1-Rad10 forms a complex with Rad14 through a direct interaction between Rad1 and Rad14 (Guzder *et al.* 1996b); the biological significance of the interaction is suggested by the high UV sensitivity of mutant Rad1 proteins that are unable to interact with Rad14 *in vitro* (Guzder *et al.* 2006).

***RPA:*** RPA is the eukaryotic counterpart of the *E. coli* single-strand binding protein (SSB) and is composed of three subunits encoded by the *RFA1*, *RFA2*, and *RFA3* genes. It binds with high affinity to single-strand DNA and is indispensable during NER (Guzder *et al.* 1995b). RPA also binds to Rad14 and to TFIIH (see below), and these interactions are presumably important during NER (Huang *et al.* 1998).

#### *Dual incision*

Following formation of a pre-incision complex, the Rad2 and Rad1-Rad10 structure-dependent endonucleases are positioned to form a new complex with TFIIH, RPA, and Rad14 (Figure 4C). Because neither Rad2 nor Rad1-Rad10 has specificity for damaged DNA, each must be targeted through interactions with other proteins. Rad2 thus interacts with TFIIH via Tfb1 (Lafrance-Vanasse *et al.* 2012), and Rad1-Rad10 forms a complex with Rad14 (Guzder *et al.* 1996b). Rad2 makes an incision 2–8 nt from the lesion on the 3′ side, while Rad1-Rad10 makes an incision 15–24 nt from the lesion on the 5′ side (Evans *et al.* 1997). The lesion-containing oligonucleotide thus generated is released together with other NER factors. A recent study in human cells suggests that 5′ incision precedes 3′ incision (Staresincic *et al.* 2009).

***Rad1-Rad10 complex:*** Rad1 forms a stable complex with Rad10 that degrades circular single-strand DNA (Bailly *et al.* 1992; Tomkinson *et al.* 1993) and nicks supercoiled DNA, probably at transient, single-strand regions (Tomkinson *et al.* 1994). The major activity of Rad1-Rad10, however, is as a structure-dependent endonuclease that recognizes the junction between single- and double-strand DNA. It specifically removes unpaired 3′ tails by nicking within duplex DNA at a position 2–5 nt from the junction (Bardwell *et al.* 1994a; Davies *et al.* 1995; Rodriguez *et al.* 1996). In NER reactions reconstituted from purified proteins, Rad1-Rad10 is essential for the incision of UV-damaged DNA (Guzder *et al.* 1995b). The properties of purified Rad1-Rad10 are consistent with nicking of the damaged strand on the 5′-side of a lesion after the DNA helix has been locally unwound. It should be noted that the role of Rad1-Rad10 is not limited to NER; the complex is also important for removing 3′ dirty ends generated during BER (see above) and nonhomologous 3′ tails that arise during homologous recombination (Lyndaker and Alani 2009).

***Rad2:*** The Rad2 protein is endowed with an endonuclease activity that degrades circular, single-strand DNA and a Y′ to X′ exonuclease activity that digests single- or double-strand DNA (Habraken *et al.* 1993, 1994). Like Rad1-Rad10, Rad2 is a junction-specific endonuclease, but cleaves with opposite polarity. Rad2 thus removes 5′-overhanging tails and processes bubble structures by nicking duplex DNA 1 nt from a single- to double-strand junction (Habraken *et al.* 1995).

#### *Resynthesis and ligation*

In human cells, dual incision and repair synthesis are closely coordinated. Following incision to create a lesion-containing oligonucleotide, RPA and XPG (yeast Rad2) associate with the PCNA clamp and the replication factor C (RFC) clamp loader to form a platform for Pol δ (Mocquet *et al.* 2008); the recruitment of Pol δ is associated with release of the remaining NER factors (Figure 4D). In human cells, three DNA polymerases (Pol δ, Pol ε, and Pol κ) are involved in repair synthesis (Ogi *et al.* 2010; Lehmann 2011). Although poorly documented in yeast, data suggest that either Pol δ or Pol ε can carry out repair synthesis (Budd and Campbell 1995). The final ligation reaction is performed by DNA ligase 1, the product of the *CDC9* gene (Budd and Campbell 1995).

#### *TC-NER*

TC-NER promotes rapid, strand-specific removal of transcription-blocking lesions by targeting the NER apparatus to a stalled RNA Pol II complex (reviewed by Hanawalt and Spivak 2008). TC-NER was discovered in mammalian cells, where repair of CPDs is faster in the expressed *DHFR* gene than in transcriptionally silent downstream sequences (Bohr *et al.* 1985). Importantly, the more efficient repair of CPDs in expressed sequences specifically reflects the preferential removal of lesions from the transcribed DNA strand (Mellon *et al.* 1987). In yeast, CPDs at the transcriptionally active *MATα* locus are similarly repaired faster than those at the inactive *HMLα* locus (Terleth *et al.* 1990), but it was initially not clear whether this reflected a difference in chromatin structure or a specific role of transcription in repair. Preferential repair of CPDs in the transcribed strand (TS) relative to the nontranscribed strand (NTS) was subsequently demonstrated in the *RPB2* gene (Sweder and Hanawalt 1992).

As noted previously, all genes that are essential for GG-NER are required for TC-NER, with the exception of *RAD7* and *RAD16*, which are required only for repair of the NTS (Verhage *et al.* 1994). TC-NER, which is triggered when RNA Pol II stalls, has two subpathways: one dependent on the Rad26 protein and a second dependent on the Rpd9 subunit of RNA Pol II. A connection between TC-NER and mRNP biogenesis/export, which affects transcript elongation, has also emerged (Gaillard *et al.* 2007), with a screen of the yeast deletion collection for elongation defects revealing additional players within the two major TC-NER subpathways (Gaillard *et al.* 2009).

***Rad26 and Rad28:*** Genes specifically involved in TC-NER were first identified in humans, where their loss is responsible for Cockayne syndrome (CS). CSA- or CSB-deficient cells are very sensitive to UV, a property that allowed cloning of the human *CSA* and *CSB* genes (Troelstra *et al.* 1992; Henning *et al.* 1995). Based on sequence homology to the encoded proteins, yeast homologs were identified and the corresponding genes were named *RAD28* and *RAD26*, respectively (Van Gool *et al.* 1994; Bhatia *et al.* 1996). CSB and Rad26 share strong sequence homology, which includes the seven conserved motifs of DNA/RNA helicases in the SNF2 subfamily. Although both proteins exhibit DNA-dependent ATPase activity, neither has detectable helicase activity (Guzder *et al.* 1996a). In the case of CSB, the ATPase activity is important for *in vivo* function (Citterio *et al.* 1998). CSA/Rad28 is a WD40 repeat protein with no identified catalytic activity and is probably involved in protein interactions (Henning *et al.* 1995; Bhatia *et al.* 1996). In contrast to human CS cells, yeast strains lacking either Rad26 or Rad28 are not UV sensitive, which explains why the corresponding genes were not recovered in early mutant screens.

Analysis of strand-specific repair of CPDs demonstrated that repair of the TS is significantly delayed in *rad26*Δ mutants (Van Gool *et al.* 1994), but is not affected in *rad28*Δ cells (Bhatia *et al.* 1996). Although not evident in a *rad26*Δ single mutant, an effect of Rad26 on survival after UV irradiation can be observed in the absence of GG-NER, with a *rad16*Δ *rad26*Δ double mutant being more UV sensitive than a *rad16*Δ single mutant (Bhatia *et al.* 1996; Verhage *et al.* 1996). A *rad16*Δ *rad26*Δ double mutant, however, is still less UV sensitive than a completely NER-deficient strain such as *rad14*Δ, suggesting residual repair of CPDs on the TS by a Rad26-independent TC-NER subpathway (Verhage *et al.* 1996).

Recent yeast studies suggest that Rad26 may be associated with RNA Pol II during transcriptional elongation; its "recruitment" to the site of the lesion would thus result from stalling of RNA Pol II in an elongation mode (Malik *et al.* 2010). Furthermore, Rad26 is subject to Mec1-dependent phosphorylation, which enhances the rate of TC-NER of UV-induced damage *in vivo* (Taschner *et al.* 2010). Finally, Rad26 copurifies with Def1, a factor that is involved in the ubiquitination of RNA Pol II and leads to its degradation by the proteasome. Although Def1 does not affect TC-NER of UV-induced DNA damage, genetic data nevertheless suggest a connection between Def1 and NER (Woudstra *et al.* 2002; Reid and Svejstrup 2004).

***Model for Rad26-dependent TC-NER:*** The stalling of RNA Pol II at a lesion in the TS is presumed to stabilize the interaction with the Rad26 protein. As illustrated in Figure 5, the stalled complex has two alternative outcomes that depend on whether the lesion stalls the transcription machinery transiently or permanently. Inactivation of Rad26 results in a delay in messenger RNA (mRNA) synthesis, and data suggest that Rad26 can promote elongation through endogenous DNA damages such as 8-oxoG, 3-MeA, and AP sites (Lee *et al.* 2001, 2002; Yu *et al.* 2003). *In vitro*, some lesions can be efficiently bypassed by RNA Pol II with the help of elongation factors such as CSB, elongin, and TFIIS (Charlet-Berguerand *et al.* 2006). At a moderately blocking lesion, Rad26 is thought to promote bypass and thereby allow transcription to continue. Because such bypass may be associated with the incorporation of incorrect ribonucleotides into the corresponding mRNA, it can lead to the production of aberrant proteins via a process termed "transcriptional mutagenesis" (Bregeon and Doetsch 2011). At strongly blocking lesions, RNA Pol II permanently stalls without the possibility of bypass, and TC-NER is triggered. Rad26 initiates chromatin remodeling to attract additional NER factors, and, in humans, a CSA-containing E3 complex recruits XAB2, HMGN1, and TFIIS (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008). These latter factors allow backtracking of RNA Pol II without dissociation from the template, which exposes the lesion to the NER machinery. Following processing of the lesion by NER, transcription can be rapidly resumed.

It should be noted that the lack of CPD repair on the TS fails to explain the growth and neurological defects associated with CS, as these defects are absent in NER-defective patients (Hanawalt and Spivak 2008). In the absence of Rad26/CSB, permanent stalling of RNA Pol II at endogenous DNA lesions would significantly impair RNA synthesis and might generate a signal for apoptosis and cell death, which could account for the severe growth defects observed in CS patients (Hanawalt and Spivak 2008). In contrast to the situation in human cells, lesion-stalled RNA Pol II complexes might be disassembled by the replication machinery in rapid-cycling yeast cells, leading to only minor, if any, effects on cell survival.

***Rpb9 subpathway:*** Rpb9, a nonessential subunit of RNA Pol II, mediates a Rad26-independent TC-NER subpathway; *rpb9*Δ *rad26*Δ double mutants are completely defective in TC-NER (Li and Smerdon 2002). *rad16*Δ *rad26*Δ *rpb9*Δ triple-mutant cells are extremely sensitive to UV radiation, similar to a *rad1*Δ strain, and the removal of CPDs in both the TS and the NTS is abolished (Li and Smerdon 2002). These data suggest a direct or indirect recruitment of NER

**Table 3 MMR genes**

| Gene name (alternative) | Protein size (kDa) | Description | Mammalian counterpart |
|---|---|---|---|
| *MSH1* | 109.4 | Mitochondrial MutS homolog. | — |
| *MSH2* (*PMS5*) | 108.9 | Forms MutSα and MutSβ mismatch-recognition heterodimers with Msh6 and Msh3, respectively. | MSH2 |
| *MSH3* | 116.5 | Component of MutSβ, which recognizes small and large IDLs. | MSH3 |
| *MSH4* | 99.2 | Interacts with Msh5 to form the MutSγ heterodimer, which is meiotic-specific and binds to HJs. | MSH4 |
| *MSH5* | 102.2 | Interacts with Msh4 to form the MutSγ heterodimer. | MSH5 |
| *MSH6* (*PMS3*) | 140.1 | Component of MutSα, which recognizes base-base mismatches and small IDLs. | MSH6 |
| *PMS1* | 99.4 | Interacts with Mlh1 to form the MutLα heterodimer; has endonuclease activity. | PMS2 |
| *MLH1* (*PMS2*) | 87.1 | Interacts with Pms1 to form the MutLα heterodimer. | MLH1 |
| *MLH2* | 78.2 | Interacts with Mlh1 to form the MutLβ heterodimer; important for repair of some frameshift intermediates. | PMS1 |
| *MLH3* | 82.0 | Interacts with Mlh1 to form the MutLγ heterodimer; required for meiotic crossover formation and for repair of some frameshift intermediates. | MLH3 |
| *POL30* (*REV6*) | 28.9 | Subunit of PCNA homotrimer; required for MMR and interacts with MutSα, MutSβ, and MutLα. | PCNA |
| *EXO1* (*DHS1*) | 80.2 | Y′ to X′ Double-strand exonuclease. | EXO1 |

factors by Rpb9, similar to that described for Rad26-dependent TC-NER.

***TC-NER at AP sites:*** Involvement of NER in the removal of AP sites was inferred by the synergistic increase in the killing and mutagenic effects of MMS observed when both BER and NER are disabled (Swanson *et al.* 1999; Torres-Ramos *et al.* 2000). Recent results demonstrate that AP sites likely are not directly recognized by the NER machinery, but rather that their removal is via TC-NER that is triggered when RNA Pol II stalls at an AP site (Kim and Jinks-Robertson 2010).

## Additional Remarks Concerning NER Mechanisms

The specific models proposed in Figures 4 and 5 incorporate genetic data from yeast with biochemical data obtained primarily with purified human proteins. While the overall schemes presented for GG-NER and TC-NER are likely correct, details of the reactions will surely be modified with time. As noted, yeast Rad4 is absolutely required for the repair of CPDs in the TS, whereas human xeroderma pigmentosum group C (XPC) protein is not; inactivation of yeast Rad28 does not impair TC-NER, whereas human CSA cells are deficient in TC-NER; inactivation of Rad26 in yeast does not confer UV sensitivity, whereas CSB cells are highly sensitive to UV radiation; and yeast has an alternative Rpb9-dependent TC-NER subpathway that is absent in human cells.

Yeast studies dealing with repair at nucleotide resolution and in the context of chromatin have not been included here; these important aspects of NER have been recently reviewed (Waters *et al.* 2009; Reed 2011). Also not considered here is the checkpoint-signaling role of single-strand gaps created during NER (Giannattasio *et al.* 2010) and roles of post-translational protein modifications. Furthermore, it should be noted that the dominant role of the NER pathway in promoting survival following acute exposure to high levels of UV damage becomes secondary to that of the *RAD6* lesion-bypass pathway (see below) when cells are exposed to low levels of chronic damage (Hishida *et al.* 2009). Finally, while the NER pathway promotes genome integrity in dividing cells, it appears to be required for most UV-induced mutagenesis that occurs in nondividing yeast cells (Eckardt and Haynes 1977; James and Kilbey 1977).

## Mismatch Repair

The mismatch repair (MMR) pathway removes helical distortions that arise when errors are made during DNA synthesis or when non-identical duplexes exchange strands during recombination (reviewed by Harfe and Jinks-Robertson 2000c; Kunkel and Erie 2005; Hsieh and Yamane 2008). During replication, mismatch correction limits mutagenesis; during recombination, correction of mismatches within heteroduplex DNA intermediates generates gene conversion events. In addition to initiating mismatch removal during recombination, the MMR system monitors identity between interacting molecules, which can limit use of non-identical substrates as repair templates. Finally, loss of MMR sensitizes cells to the antimetabolite 5-fluorouracil (Matuo *et al.* 2010) and can alter resistance to DNA damage (Bertrand *et al.* 1998; Durant *et al.* 1999; Cejka *et al.* 2005). Importantly, the sensitivity of methyltransferase (Mgt1)-deficient yeast or mammalian cells to MNNG depends on functional MMR (Branch *et al.* 1993; Cejka *et al.* 2005). This is thought to reflect repetitive, futile attempts of the MMR machinery to repair the $O^6$-MeG:T mismatches generated during replication of damaged templates. The focus here will be on the replication-related role of the MMR machinery, and relevant genes are summarized in Table 3.

### *Bacterial paradigm*

The intellectual framework for eukaryotic MMR derives from the *E. coli* system, which contains three dedicated mutator or "Mut" proteins (reviewed in Modrich and Lahue

1996). A MutS homodimer binds mismatches, a MutL homodimer coordinates mismatch detection with downstream processing steps, and MutH nicks the nascent strand to initiate removal. Discrimination between nascent and template strands is provided by transient, hemi-methylation of DNA in the wake of replication, with MutH specifically nicking the new, unmethylated strand. The MutH-nicked strand is then degraded by the concerted action of a helicase (UvrC) and one of four single-strand exonucleases, DNA polymerase fills the gap, and ligase seals the remaining nick.

In eukaryotes, the single MutS and MutL homodimers are replaced with heterodimers, each of which has specialized functions. An explanation for the evolution of heterodimeric complexes in eukaryotes emerged when the crystal structure of the bacterial MutS homodimer revealed that it is a structural heterodimer (Lamers *et al.* 2000; Obmolova *et al.* 2000). The basic functions of the MutS and MutL homologs are highly conserved within the eukaryotic lineage, with an early link between MMR defects and the genetic instability characteristic of human nonpolyposis colorectal cancer coming from studies of dinucleotide repeat instability in yeast (Strand *et al.* 1993). A MutH-like protein is absent in eukaryotes, as well as in most bacteria, and methylation does not serve as a strand-discrimination signal during replication. The exact nature of this signal(s) and the mechanism of strand removal has yet to be fully resolved.

### *MutS homologs*

The yeast genome encodes six MutS homologs, four of which (Msh1, Msh2, Msh3, and Msh6) were identified by homology to bacterial MutS proteins (Reenan and Kolodner 1992a,b; New *et al.* 1993; Marsischky *et al.* 1996). Msh1 functions exclusively in the mitochondria (Mookerjee *et al.* 2005; Sia and Kirkpatrick 2005), while Msh2, Msh3, and Msh6 are important for maintaining nuclear genome stability. Deletion of *MSH2* completely disables mitotic and meiotic MMR (Reenan and Kolodner 1992b), but *msh3*Δ or *msh6*Δ mutants exhibit weaker phenotypes (New *et al.* 1993; Johnson *et al.* 1996b; Marsischky *et al.* 1996). A seminal observation was that an *msh3*Δ *msh6*Δ double mutant is phenotypically indistinguishable from an *msh2*Δ single mutant, leading to the proposal of functionally redundant, MutS-like complexes in which Msh2 partners with either Msh6 or Msh3 (Johnson *et al.* 1996b; Marsischky *et al.* 1996). These complexes are referred to as MutSα and MutSβ, respectively. Msh4 and Msh5 form a third, heterodimeric MutS-like complex known as MutSγ, which has meiotic-specific roles (Pochart *et al.* 1997).

***Recognition specificities of MutSα and MutSβ:*** Nuclear mismatch-recognition activity is shared between MutSα and MutSβ, with the specificities of the complexes being deduced by comparing the phenotypes of *msh6*Δ and *msh3*Δ single mutants, respectively, with those of an *msh2*Δ single mutant (Detloff *et al.* 1991; Marsischky *et al.* 1996; Luhr *et al.* 1998; Nicholson *et al.* 2000). The general consensus is that MutSα and MutSβ are specialized to remove base-base mismatches and large insertion-deletion loops (IDLs), respectively, but are largely redundant with respect to small (<4 nt) IDLs (reviewed by Harfe and Jinks-Robertson 2000c). Similar to bacterial MutS, MutSα efficiently initiates repair of all base-base mismatches with the exception of C:C (Detloff *et al.* 1991). As noted previously, MutSα repairs the mismatch generated when adenine is misincorporated opposite a template 8-oxoG (Ni *et al.* 1999) and is considered to be the functional homolog of the *E. coli* MutY DNA glycosylase (Figure 2). Finally, with regard to the "exclusive" role of MutSα in base-base mismatch removal, some transversions increase in an *msh3*Δ background (Harrington and Kolodner 2007), suggesting that MutSβ functionally replaces MutSα in some contexts.

Forward mutation assays have shown that IDLs are corrected more efficiently than are base–base mismatches (Marsischky *et al.* 1996; Yang *et al.* 1999; Lujan *et al.* 2012). Although there is functional redundancy between MutSα and MutSβ with respect to repairing small IDLs, the relative correction efficiencies of the two complexes can vary dramatically as a function of position of the extrahelical loop, primary sequence, and surrounding sequence context (Strand *et al.* 1995; Harfe and Jinks-Robertson 1999, 2000a; Marsischky and Kolodner 1999; Gragg *et al.* 2002). Early studies with tandem repeats suggested a 13- to 16-nt size limit for IDL recognition by MutSβ (Sia *et al.* 1997), but subsequent analyses suggest that larger loops also may be repaired (Kirkpatrick and Petes 1997; Harfe *et al.* 2000; Kearney *et al.* 2001). The removal of larger IDLs, as well as MutSβ-dependent removal of nonhomologous 3′ tails during homologous recombination (Sugawara *et al.* 1997; reviewed by Lyndaker and Alani 2009), involves the Rad1-Rad10 endonuclease complex, which is essential during NER. A second MMR- and NER-independent pathway for repairing large IDLs has been identified in cell extracts (Sommer *et al.* 2008), but its physiological relevance is unclear.

***Central role of ATP binding/hydrolysis:*** MMR is an ATP-dependent process, and a major area of emphasis has been the functional significance of ATP binding/hydrolysis by MutS complexes, which are Walker-type ATPases (reviewed by Kunkel and Erie 2005). *In vitro*, ATP destabilizes the interaction of MutSα with mismatches that are well repaired *in vivo*, but fails to destabilize binding to hairpins (Alani 1996), which are refractory to MMR (Nag *et al.* 1989). This key observation led to the proposal that ATP-dependent dissociation of MutSα from a mismatch licenses the downstream steps required for repair (Alani 1996).

Mutation of the conserved Walker ATP binding/hydrolysis motifs of Msh2 or Msh6 results in a null phenotype (Studamire *et al.* 1998; Drotschmann *et al.* 2002), and these alleles exert a dominant-negative effect (Studamire *et al.* 1998; Das Gupta and Kolodner 2000), which reflects an inability of MutSα to dissociate from mismatches (Hess *et al.* 2006).

Although the role of ATP hydrolysis has been controversial (Blackwell *et al.* 1998; Gradia *et al.* 1999; Junop *et al.* 2001), the current consensus is that ADP-ATP exchange converts a mismatch-bound MutSα complex into a sliding clamp whose movement does not require ATP hydrolysis (Mazur *et al.* 2006; Hargreaves *et al.* 2010).

***Structural studies of MutS complexes:*** Crystal structures of bacterial MutS (Lamers *et al.* 2000; Obmolova *et al.* 2000), human MUTSα (hMUTSα; (Warren *et al.* 2007) and hMUTSβ (Gupta *et al.* 2012) have revealed five conserved domains (Figure 6, A and B). Each complex has two channels, one of which accommodates duplex DNA; consistent with the structural asymmetry, only one subunit makes mismatch-specific contacts (Figure 6C). In the static crystal structures, DNA is sharply kinked at the position of the mismatch (Lamers *et al.* 2000; Obmolova *et al.* 2000). Dynamic DNA conformational changes have been detected by atomic force microscopy, however, when MutS binds to mismatched *vs.* homoduplex DNA, and these changes may be important for mismatch verification (Wang *et al.* 2003).

Bacterial MutS interacts with mismatches through critical phenylalanine and glutamic acid residues, and these residues are conserved only in Msh6. Mutation of the corresponding Phe337 or Glu339 of Msh6 results in a mutator phenotype and abrogates mismatch binding *in vitro* (Bowers *et al.* 1999; Drotschmann *et al.* 2001; Holmes *et al.* 2007). Msh2 has an aromatic amino acid at a position comparable to that of Phe337 in Msh6, and its mutation is without consequence (Bowers *et al.* 1999; Drotschmann *et al.* 2001). In Msh3, the conserved Phe and Glu are replaced with lysine; the corresponding *msh3-K187A,K189A* allele confers variable effects on IDL repair (Lee *et al.* 2007).

The crystal structure of hMUTSβ suggests two distinct modes of IDL recognition (Gupta *et al.* 2012), and two corresponding classes of *msh3* and *msh2* mutants have been identified: those defective only in the repair of small IDLs and those defective in repairing large IDLs and removing nonhomologous 3′ tails (Studamire *et al.* 1999; Dowen *et al.* 2010). Msh3 mismatch specificity can be imparted to yeast MutSα by replacing the Msh6 mismatch-binding domain (MBD) with the presumptive Msh3 MBD; the reverse domain swap, however, does not yield a functional complex (Shell *et al.* 2007b). A separation-of-function *msh2* allele that differentially affects MutSα and MutSβ provides additional evidence that these complexes sense and/or respond to mismatches differently (Lee *et al.* 2007; Kumar *et al.* 2011).

***MutSγ, the Msh4-Msh5 complex:*** Msh4 and Msh5 were identified in screens for meiotic-specific transcripts and recombination defects (Ross-MacDonald and Roeder 1994; Hollingsworth *et al.* 1995). Although a specific allele of *MSH5* was reported to enhance alkylation-damage tolerance (Bawa and Xiao 2003), there has been no confirmation of a mitotic role. Msh4 and Msh5 have overall homology to

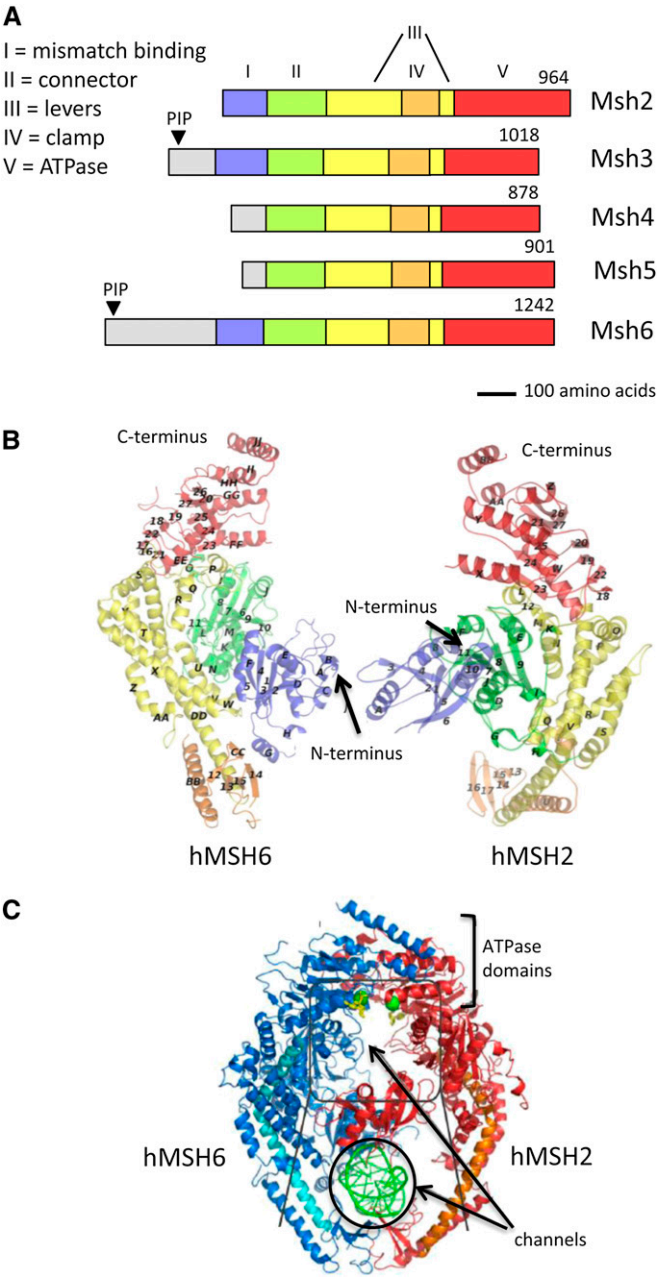


**Figure 6** Alignment of MutS homologs and MutSβ crystal structure. (A) Linear alignment of the yeast nuclear MutS homologs, with domains identified in the MutS crystal structure color-coded and indicated by Roman numerals (Obmolova *et al.* 2000). (B) Crystal structures of human MSH2 and MSH6, with domains colored as in A. (C) Crystal structure of hMUTSα with a mismatch, with DNA indicated in green. Crystal structures are from Warren *et al.* (2007).

Msh2, Msh3, and Msh6, but each is missing conserved domain I (Figure 6A), which separates the two channels in the MutS crystal structure (Figure 6, B and C). The Msh4-Msh5 complex thus is predicted to contain a single channel large enough to accommodate two DNA duplexes (Obmolova *et al.* 2000). hMSH4-MSH5 binds to progenitor and mature Holliday junctions (HJs) that connect recombining duplexes; upon addition of ATP, hMUTSγ forms an ATPase-independent

sliding clamp that embraces both duplexes (Snowden *et al.* 2004). Only the Walker A box of the yeast Msh5 protein has been shown to be required for meiotic crossover formation (Pochart *et al.* 1997), but both subunits bind ATP *in vitro* (Snowden *et al.* 2008). During meiosis, MutSγ is required for detection of the single-end invasion intermediates that mature into Holliday junctions (Börner *et al.* 2004).

### *MutL homologs*

The yeast genome encodes four MutL homologs: Mlh1, Mlh2, Mlh3, and Pms1. *PMS1* was the first eukaryotic MMR gene identified, and its name reflects an inability to repair mismatches in recombination intermediates (Williamson *et al.* 1985). Cloning revealed strong homology of Pms1 to bacterial MutL proteins (Kramer *et al.* 1989), a property that was exploited to identify the remaining MutL homologs (Prolla *et al.* 1994a; Crouse 1998). Mlh1 is the common component of three MutL-like complexes: Mlh1-Pms1, Mlh1-Mlh2, and Mlh1-Mlh3, which are referred to as MutLα, MutLβ, and MutLγ, respectively (Wang *et al.* 1999). MutLα interacts with MutSα and MutSβ to coordinate most MMR, and MutLγ partners with MutSγ to regulate meiotic crossover formation. In addition to being the common component of three MutL-like complexes, Mlh1 interacts with the Exo1 exonuclease during MMR (see below), the Ntg2 DNA *N*-glycosylase/lyase, and the Sgs1 helicase via a highly conserved binding site at its C terminus, which is referred to as S2 (Gellon *et al.* 2002; Dherin *et al.* 2009).

***MutLα, the Mlh1-Pms1 complex:*** Initial genetic analyses placed Mlh1 and Pms1 in a common pathway, and the proteins were shown to physically interact (Prolla *et al.* 1994b; Pang *et al.* 1997). The N-terminal region of MutL homologs is highly conserved and contains an ATPase domain; although required for dimerization, the C terminus has only weak sequence conservation (reviewed by Kunkel and Erie 2005). Crystal structures of the N-terminal domain of *E. coli* MutL (Ban and Yang 1998), human PMS2 (Guarne *et al.* 2001), and yeast Pms1 (Arana *et al.* 2010) have been solved, as has the crystal structure of the C-terminal domain of *E. coli* MutL (Guarne *et al.* 2004). A model for the intact MutL dimer has been proposed in which the N- and C-terminal domains are separated by a proline-rich linker, with the dimer containing a large central cavity (Guarne *et al.* 2004).

MutL proteins belong to the GHL family of ATPases (Ban and Yang 1998), and there is functional asymmetry between the MutLα subunits. ATP binding/hydrolysis by Mlh1 is essential for MMR, but only a weak mutator phenotype results from loss of ATP binding/hydrolysis by Pms1 (Tran and Liskay 2000). Consistent with the *in vivo* asymmetry, Mlh1 binds ATP with higher affinity than does Pms1, suggesting that ATP binding is likely sequential (Hall *et al.* 2002). In two-hybrid studies, the N-terminal fragments of Pms1 and Mlh1 interact only if ATP hydrolysis by both is blocked; additional elimination of ATP binding by either fragment, however, prevents the interaction (Tran and Liskay 2000). These data suggest that conformational changes are associated with the ATP hydrolysis cycle, and such changes in MutLα have been visualized by atomic force microscopy (Sacho *et al.* 2008). Finally, MutLα binds cooperatively to DNA (Hall *et al.* 2001), and mutational impairment of DNA binding is associated with a mutator phenotype (Hoffmann *et al.* 2003).

As noted previously, eukaryotes do not have a MutH-like protein that nicks mismatched DNA, and yet MMR is nick-directed (Constantin *et al.* 2005). This conundrum was partially resolved by the discovery that the hPMS2, which is the homolog of yeast Pms1, has a latent endonuclease activity (Kadyrov *et al.* 2006). Site-directed mutation of the endonuclease motif eliminates mitotic function of MutLα (Erdeniz *et al.* 2007; Kadyrov *et al.* 2007) without affecting its ATPase activity or ability to form a ternary complex with mismatched DNA and MutSα (Kadyrov *et al.* 2007). A similar endonuclease motif is present in Mlh3, but is absent in Mlh1 and Mlh2.

***Interaction of MutSα and MutSβ with MutLα:*** MutSα/MutSβ must interact with MutLα to initiate downstream steps of MMR, and ternary complexes with mismatched DNA have been described (Habraken *et al.* 1997, 1998). A domain of Msh2 required to mediate interaction with MutLα resides within the connector loop (see Figure 6); site-directed mutagenesis of this region abolishes the MutSα-MutLα interaction *in vitro* and is associated with a mutator phenotype (Mendillo *et al.* 2009). Whether this region of Msh2 is similarly important for MutSβ-MutLα interaction has not been examined. Recent studies, however, have identified a region of hMSH3 that facilitates ternary complex formation with hMUTLα and mismatched DNA, and this region overlaps a PCNA interaction peptide (PIP) (see below) motif at the extreme N terminus of hMSH3 (Charbonneau *et al.* 2009; Iyer *et al.* 2010). These data may explain why mutation of the Msh3 PIP domain disables MMR to a greater extent that does mutation of the Msh6 PIP box (Clark *et al.* 2000). In terms of the region of MutLα that mediates interaction with the MutS complexes, the N terminus and C terminus of hMLH1 are important for interaction with hMUTSα (Plotz *et al.* 2006) and hMSH3 (Charbonneau *et al.* 2009), respectively.

***MutLγ, the Mlh1-Mlh3 complex:*** Subtle phenotypes of *mlh3*Δ (as well as *mlh2*Δ) mutants have been reported in some frameshift-reversion assays, where the corresponding MutL complex seems to partner with MutSβ (Flores-Rozas and Kolodner 1998; Harfe *et al.* 2000). The most significant phenotype of *mlh3*Δ mutants, however, is a deficit in meiotic crossover formation, a phenotype shared with *mlh1*Δ, *msh4*Δ, and *msh5*Δ mutants (reviewed by Hoffmann and Borts 2004). Site-directed mutagenesis of the Mlh3 endonuclease domain results in phenotypes similar to that of a null

mutant (Nishant *et al.* 2008). A recent study demonstrated that MutLγ is the major meiotic HJ resolvase in yeast (Zakharyevich *et al.* 2012), thereby providing a direct explanation for crossover reduction in mutants. An attractive model is that MutSγ binds to and stabilizes HJs, which are then symmetrically nicked by MutLγ.

### *Other proteins important for MMR*

The *E. coli* paradigm suggests that repair of replication errors requires (1) a mechanism(s) to discriminate the nascent and template strands and (2) helicases and/or exonucleases to degrade the mismatch-containing segment. The only two proteins implicated unequivocally in these steps are the PCNA sliding clamp and the Exo1 Y′ to X′ exonuclease.

***PCNA sliding clamp:*** The PCNA sliding clamp is a donut-shaped homotrimer of the Pol30 protein; it encircles duplex DNA and is loaded/unloaded by RFC at primer-template junctions. Originally described as a processivity factor for replicative DNA polymerases, PCNA serves as a landing pad for multiple DNA-metabolic proteins, most of which interact with the intermonomer region of the clamp via a highly conserved PIP domain (reviewed by Moldovan *et al.* 2007). The involvement of PCNA in MMR was discovered through characterization of *pol30* missense alleles that have an associated mutator phenotype (Johnson *et al.* 1996a; Umar *et al.* 1996; Lau *et al.* 2002). PCNA interacts physically with MutSα (Clark *et al.* 2000; Flores-Rozas *et al.* 2000), MutSβ (Johnson *et al.* 1996a), and Mlh1 (Umar *et al.* 1996; Lee and Alani 2006). Both Msh3 and Msh6 (but not Msh2) have a PIP box near the amino terminus, and its mutation partially disables MMR (Clark *et al.* 2000; Flores-Rozas *et al.* 2000). Interaction of MutSα with PCNA stimulates mismatch binding *in vitro* (Flores-Rozas *et al.* 2000), and loss of the Msh6 PIP motif reduces colocalization with PCNA *in vivo* (Hombauer *et al.* 2011a). A potential PIP box in Mlh1 also has been identified, but whether this mediates interaction with PCNA is unclear (Lee and Alani 2006). In addition to its PIP domain, Msh6 contains an extended N-terminal domain (NTD) that is absent in other MutS proteins (Figure 6A) and forms an unstructured tether to PCNA (Shell *et al.* 2007a). The NTD of Msh6 additionally has nonspecific DNA-binding activity that is functionally important (Clark *et al.* 2007).

Interactions with PCNA are assumed to target MMR proteins to sites of new DNA synthesis and to contribute, at least partially, to strand discrimination (Umar *et al.* 1996). PCNA also may act as a scaffold to coordinate sequential steps during replication-error repair (Lee and Alani 2006). Finally, the tight link that PCNA provides between MMR and replication may circumvent the potential obstacle posed by nucleosomes that assemble behind the replication fork (Gorman *et al.* 2010).

***Exo1 exonuclease:*** Exo1 is double-strand-specific exonuclease that degrades single strands in the Y′ to X′ direction (for a review see Tran *et al.* 2004); its role in mismatch correction was first described in *Schizosaccharomyces pombe* (Szankasi and Smith 1995). Because Exo1 works on duplex DNA, there is no need for a helicase to unwind the nicked segment; accordingly, no helicases have been implicated in eukaryotic MMR. The budding yeast Exo1 interacts with Msh2 (Tishkoff *et al.* 1997a) and with Mlh1 (Tran *et al.* 2001), and it has been suggested to have a structural (Amin *et al.* 2001; Tran *et al.* 2001) as well as an enzymatic role during MMR (Sokolsky and Alani 2000). In contrast to the strong mutator phenotype of *msh2*Δ*/mlh1*Δ strains, *exo1*Δ mutants have only a weak mutator phenotype (Tishkoff *et al.* 1997a). The *exo1*Δ mutator phenotype reflects a role of Exo1 both during MMR and in the promotion of error-free damage bypass (Tran *et al.* 2007). Mutating the Mlh1-interacting peptide of Exo1 or the S2 site of Mlh1 results in a modest MMR-dependent mutator phenotype, which is greatly enhanced when combined with hypomorphic mutations of *MLH1* or *PMS1* (Tran *et al.* 2007; Dherin *et al.* 2009). Interestingly, mutating the PIP box of Msh6, which by itself causes only a weak mutator phenotype, completely eliminates MutSα-dependent MMR in an *exo1*Δ background (Hombauer *et al.* 2011a).

***Are there additional participants in MMR?:*** Because of the weak mutator phenotype of *exo1*Δ mutants and the existence of redundant Y′ to X′ and X′ to Y′ exonucleases in *E. coli* MMR, an early assumption was that there would be additional nucleases involved in eukaryotic MMR. To date, two candidates have emerged: the Rad27 flap endonuclease and X′ to Y′ exonucleolytic proofreading activities of the replicative DNA polymerases Pol δ and Pol ε. In the case of Rad27, its loss results in dinucleotide repeat instability comparable to that seen in *msh2*Δ mutants (Johnson *et al.* 1995), but whether this reflects a direct role in MMR has been questioned (Tishkoff *et al.* 1997b). An involvement of Pol δ and Pol ε in MMR has been inferred from genetic analyses of homopolymer run instability in exonuclease-defective *pol3-01* or *pol2-4* strains (Tran *et al.* 1999). As to whether there might be additional, as yet unidentified, players during MMR, no viable candidates have emerged despite multiple genetic screens (Jeyaprakash *et al.* 1994; Tran *et al.* 1999; Amin *et al.* 2001; Sia *et al.* 2001; Huang *et al.* 2003).

### *Putting it all together: the mechanism(s) of mismatch removal*

Interaction of MMR proteins with PCNA is a simple way to focus mismatch correction to regions of new DNA synthesis. How the initiating nicks are targeted specifically to the nascent strand and precisely how the nicked strand is removed remain to be fully elucidated, and differences may exist depending on whether an error arises during leading- *vs.* lagging-strand synthesis. Indeed, the MMR system more efficiently removes lagging- than leading-strand errors (Pavlov *et al.* 2003; Kow *et al.* 2007; Mudrak *et al.* 2009; Lujan *et al.* 2012). Most genetic and structure–function studies of

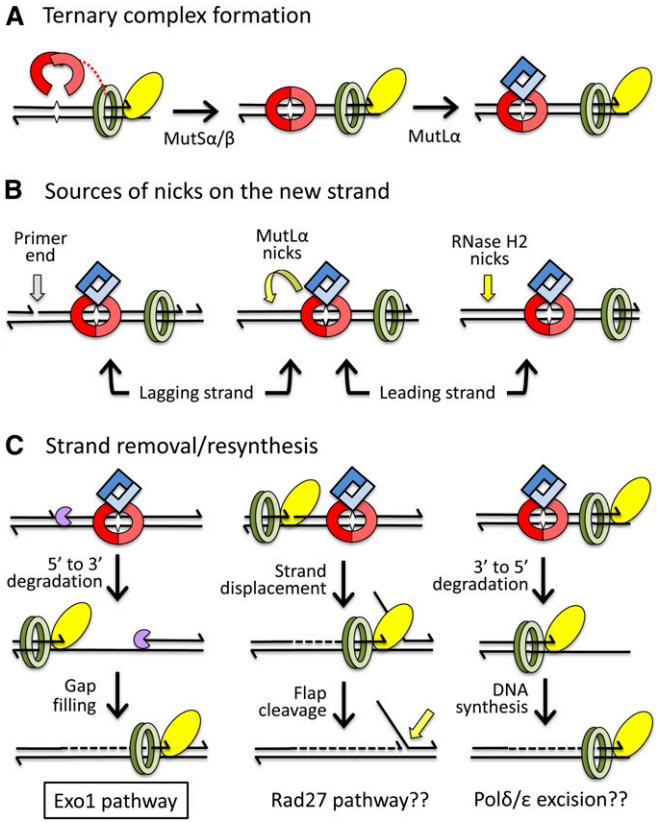


**Figure 7** Proposed mechanisms of MMR. (A) MutSα/β (the red clamp-like structure) is tethered to PCNA (green donut) during replication. Following mismatch recognition/verification by MutSα/β, MutLα (blue heterodimer) interacts to form a ternary complex. (B) . On the lagging strand of replication, the 5′ ends of Okazaki fragments can serve as an entry site for mismatch removal. On either the lagging or leading strand of replication, the asymmetry of PCNA can provide a strand-discrimination signal for MutLα-catalyzed nicking of the nascent strand. On the leading strand, additional nicks may be provided by the activity of RNase H2 at rNMPs incorporated by DNA Pol ε. (C) Removal of the mismatch-containing nicked strand from the 5′ direction can be accomplished by Exo1 (purple pacman). Additional possible removal mechanisms include strand displacement and 5′-flap removal by Rad27 or mismatch excision from the 3′ direction using the proofreading activity of Pol δ/ε (yellow oval). The resulting gap is filled (dashed lines) by DNA polymerase and sealed by DNA ligase. The 3′ ends of single strands are indicated by half-arrowheads.

eukaryotic MMR have been done in yeast, whereas most attempts to biochemically reconstitute the system have utilized purified human proteins. Below, recent results that provide insight into how MMR likely functions *in vivo* are discussed, and a model that incorporates current data is presented in Figure 7.

***Temporal and spatial control of MMR:*** The general assumption that, as in *E. coli*, MMR is temporally coupled to replication has been confirmed by limiting MutSα expression to specific phases of the cell cycle (Hombauer *et al.* 2011b). Although a low level of MMR has been detected in nondividing cells, it lacks strand discrimination, presumably because the normal link with replication is missing (Rodriguez *et al.* 2012). Such residual MMR, however, may provide a mechanism to introduce a potentially beneficial change into both strands of duplex DNA under stress conditions. A similar, noncanonical MMR mechanism may be relevant to somatic hypermutation of immunoglobulin genes and to methylation-associated mutagenesis in humans (Peña-Diaz *et al.* 2012).

Visualization of fluorescently tagged MMR proteins has revealed that MutSα foci localize to replication factories and that this requires interaction of Msh6 with PCNA (Hombauer *et al.* 2011a). The number of these foci is insensitive to the rate of replication errors, however, suggesting that MutSα is a constitutive component of replication factories. Although the formation of MutLα foci is, as expected, dependent on functional MutSα, foci are rarely coincident. In contrast to MutSα foci, the number of MutLα foci increases as a function of replication-error load, suggesting that MutLα foci reflect sites of active MMR. Taken together, these observations suggest that there may be more than one mode of mismatch recognition and that sites of active repair complexes likely contain superstoichiometric numbers of MutLα relative to MutSα complexes.

***Origins of nicks:*** The 5′ ends of Okazaki fragments are a natural source of nicks on the lagging strand of replication and provide an entry site for Exo1 to initiate Y′ to X′ removal of a mismatch-containing segment. Although this potentially solves the strand-discrimination problem during lagging-strand synthesis, there must be an additional mechanism(s) for generating strand-specific nicks during the relatively continuous synthesis of the leading strand (Figure 7B). The endonuclease activity of MutLα provides at least one source of nicks (Kadyrov *et al.* 2006, 2007; Erdeniz *et al.* 2007). In a purified *in vitro* system, interaction with PCNA is required to activate the endonuclease activity of hMUTLα, and the inherent asymmetry of PCNA with respect to the 3′ end dictates that the "correct" strand is nicked (Pluciennik *et al.* 2010). It also has been suggested that ribonucleotide monophosphates (rNMPs), which are preferentially incorporated into the leading strand during DNA synthesis (Nick McElhinny *et al.* 2010b), might provide an additional strand-discrimination signal, through either spontaneous hydrolysis or RNaseH2-dependent cleavage of the DNA backbone (Clark and Kunkel 2010).

***Mismatch removal:*** Exo1 is the only undisputed protein involved in degrading mismatch-containing DNA *in vivo*, and yet *exo1* null mutants do not have a strong mutator phenotype (Tishkoff *et al.* 1997a). At least in the purified human system, strand-displacement synthesis provides an alternative way to directionally remove a mismatch-containing segment if a 5′ nick is present (Figure 7C) (Kadyrov *et al.* 2009). If such a mechanism were to operate *in vivo*, one would expect Rad27 to be involved in removing the flap thus created. As noted previously, Rad27 was early implicated in yeast MMR (Johnson *et al.* 1995), but subsequent

work revealed that the mutation signature associated with its loss is very different from that associated with MMR defects (Tishkoff *et al.* 1997b). If Exo1 and Rad27 were to work in redundant pathways for mismatch removal from a 5′ nick, however, an involvement of Rad27 in MMR might have been masked. The inviability of the exoΔ1 *rad27*Δ double mutant, which is thought to reflect defective Okazaki fragment processing (Tishkoff *et al.* 1997a; Tran *et al.* 2002), precludes testing a redundant role of these proteins during MMR. It remains possible that mismatch removal could be effected during DNA synthesis by the 3′–5′ exonuclease activity of replicative DNA polymerases (Figure 7C).

## Ribonucleotide Excision Repair

Either RNase H1 or RNase H2 can degrade RNA transcripts that remain stably base paired with the DNA template (R-loops), but only RNase H2 has the ability to incise 5′ of a single rNMP imbedded in duplex DNA (reviewed by Cerritelli and Crouch 2009). The incorporation of rNMPs by DNA polymerases is restricted by an active-site pocket lid, which excludes sugars containing a 2′-OH group (Joyce 1997). Even so, it has been estimated that ~10,000 rNMPs are incorporated into a haploid yeast genome during each replication cycle, making rNMPs potentially the most abundant noncanonical base/lesion in genomic DNA (Nick McElhinny *et al.* 2010b). The absence of RNase H2 is associated with replication stress (Nick McElhinny *et al.* 2010a; Lazzaro *et al.* 2012) and with a distinctive, topoisomerase 1-dependent mutation signature (Kim *et al.* 2011a). In yeast cell extracts, the Rad27 flap endonuclease cooperates with RNase H2 to excise an rNMP from duplex DNA, with Rad27 nicking on the 3′ side of the RNase H2-incised rNMP (Rydberg and Game 2002). An entire ribonucleotide excision repair reaction has been reconstituted using purified yeast RNase H2, Rad27 (or Exo1), PCNA, the RFC clamp loader, Pol δ (or Pol ε), and DNA ligase I (Sparks *et al.* 2012). Defects in human RNase H2 are associated with the neuro-inflammatory disease Aicardi–Goutières syndrome (Crow *et al.* 2006), and RNase H2 is essential in the mouse (Reijns *et al.* 2012).

## Bypass of DNA Damage

DNA-damage bypass by the *RAD6* pathway allows DNA to be synthesized across lesions that otherwise block replicative DNA polymerases. This not only permits the completion of replication, but also allows generation of a complementary, undamaged strand that can be used as a template in a subsequent BER or NER reaction. Although not a true repair pathway, the *RAD6* pathway is commonly referred to as the post-replication repair (PRR) pathway, a name originally adopted to describe time-dependent changes in DNA fragment sizes following UV irradiation of NER-deficient cells (Di Caprio and Cox 1981; Prakash 1981).

There are error-free and error-prone subpathways of PRR, with gene assignment based (1) on whether loss results in an increase or decrease in induced mutagenesis, respectively, and (2) on epistatic survival relationships following treatment of single and double mutants with DNA damage (reviewed by Haynes and Kunz 1981). Many genes can be clearly assigned to the error-free or -prone component of PRR, but some reside in both subpathways. Such dual functions largely reflect roles of the encoded proteins in the post-translational modification of PCNA, which directs bypass into the alternative subpathways (reviewed by Moldovan *et al.* 2007; Ulrich and Walden 2010).

Elimination of the error-free PRR pathway results in a strong mutator phenotype, indicating that it is the major mechanism of lesion bypass. Error-free bypass involves a template switch to the newly synthesized strand of the sister chromatid, thereby allowing an undamaged, complementary strand to direct synthesis over the offending lesion. This process is intimately connected to homologous recombination, which is discussed in detail in another chapter and will be dealt with only peripherally here. The error-prone subpathway employs one or more specialized translesion synthesis (TLS) DNA polymerases, which catalyze DNA synthesis across damage that blocks replicative DNA polymerases. A list of the major genes involved in PRR is given in Table 4.

### *Components of error-free bypass*

The central players in the error-free subpathway of PRR are the Rad6-Rad18 complex, the Ubc13-Mms2 complex, and Rad5. Rad6 and Rad18 comprise one pair of E2-E3 ubiquitinating enzymes that modify PCNA, while Ubc13-Mms2 and Rad5 constitute a second E2-E3 pair. Rad6-Rad18 adds a single Ub moiety to PCNA, which then can be extended into a regulatory, poly-Ub chain by Rad5 and Ubc13-Mms2. Poly-Ub PCNA coordinates the downstream steps of template switching, the primary candidate mechanisms of which involve either reversal of a blocked replication fork or recombination-mediated invasion of the undamaged sister chromatid (Figure 8, A and B, respectively). Because mono-Ub of PCNA also is required for most TLS, Rad6-Rad18 is required for virtually all lesion bypass.

***Rad6-Rad18 complex:*** Early genetic studies placed *rad6* and *rad18* mutants in the same epistasis group, but *rad6* mutants had additional growth and sporulation defects (Haynes and Kunz 1981). Purification of Rad6 revealed an E2 Ub-conjugase activity *in vitro* (Jentsch *et al.* 1987), and the complex phenotypes of *rad6* mutants reflect its interaction with three different E3 Ub ligases: Ubr1, Bre1, and Rad18. Rad6-Ubr1 targets proteins for N-end rule degradation (Dohmen *et al.* 1991), Rad6-Bre1 modifies chromatin (Wood *et al.* 2003; Zimmermann *et al.* 2011), and Rad6-Rad18 adds a single Ub to PCNA monomers (Hoege *et al.* 2002).

**Table 4 PRR genes**

| Gene name (alternative) | Protein size (kDa) | Description | Mammalian counterpart |
|---|---|---|---|
| *RAD6* (*UBC2*, *PSO8*) | 19.7 | E2 Ub conjugase that forms a complex with Rad18 (also Bre1 and Ubr1) E3 Ub ligase; Rad18-Rad6 mono-ubiquitinates PCNA on K164. | RAD6A, RAD6B |
| *RAD18* | 55.2 | E3 Ub ligase that binds ssDNA; forms a complex with Rad6 to mono-ubiquitinate PCNA at K164. | RAD18 |
| *RAD5* (*REV2*, *SNM2*) | 134.2 | E3 Ub ligase involved in polyubiquitination of PCNA; ATPase activity. | HTLF, SHPRH |
| *MMS2* | 15.5 | Forms a heterodimeric complex with Ubc13; Ub conjugase involved in polyubiquitination of PCNA. | MMS2 |
| *UBC13* | 17.5 | Forms a heterodimeric complex with Mms2; Ub conjugase involved in polyubiquitination of PCNA. | UBC13 |
| *UBP10* (*DOT4*) | 88.5 | DUB for PCNA. | USP1 |
| *POL30* (*REV6*) | 28.9 | Subunit of PCNA homotrimer; post-translationally modified to direct PRR subpathways. | PCNA |
| *REV1* | 112.2 | dCMP transferase activity; required for Pol ζ-dependent lesion bypass. | REV1L |
| *REV3* (*PSO1*, *ANT2*, *RAD8*) | 173.0 | Catalytic subunit of Pol ζ. | REV3L |
| *REV7* | 28.8 | Accessory subunit of Pol ζ. | REV7 |
| *POL31* (*HUS1 SDP5*, *HYS2*) | 55.3 | Subunit of Pol δ and Pol ζ. | B-subunit (p66) |
| *POL32* (*REV4*) | 40.3 | Subunit of Pol δ and Pol ζ. | C-subunit (p50) |
| *RAD30* (*DBH1*) | 71.5 | Pol η TLS polymerase. | POL η |
| *UBC9* | 17.9 | E2 SUMO conjugase that SUMOylates PCNA. | UBC9 |
| *SIZ1* (*ULL1*) | 100.8 | E3 SUMO ligase that SUMOylates PCNA. | — |
| *SRS2* (*HPR5*, *RADH*) | 134.3 | 3′–5′ helicase; translocase that dismantles Rad51 nucleoprotein filaments. | PARI, FBH1 |

Rad18 has a zinc-coordinated RING finger domain, displays an ATPase activity, and binds to single-strand DNA (Bailly *et al.* 1994). Rad18 forms a stable complex with Rad6 and self-associates via a separate domain (Ulrich and Jentsch 2000); the significance, if any, of self-association is not known. Because mono-Ub of PCNA is required for TLS, loss of either Rad6 or Rad18 is associated with a reduction in induced mutagenesis. By contrast, *rad18*Δ strains exhibit a mutator phenotype in spontaneous mutation assays (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005). These opposing responses highlight a basic difference in how cells respond to acute *vs.* chronic damage (De Graaf *et al.* 2009; Hishida *et al.* 2009). Finally, Rad6-Rad18 has been implicated in the control of an inducible DNA-damage response similar to the SOS response of *E. coli* (Fu *et al.* 2008), but this role has been disputed (Davies *et al.* 2010).

***Rad5 protein:*** *RAD5* was cloned by complementation of the UV sensitivity of mutants, and the encoded protein contains the seven DNA-helicase motifs shared by the SNF2/SWI2 superfamily of ATPases (Johnson *et al.* 1992). The ATPase activity of Rad5 can drive regression of a model replication-fork structure *in vitro* (Figure 8A) (Blastyak *et al.* 2007), but whether it has this activity *in vivo* is not known. Rad5 self-associates and can interact simultaneously with Rad18 and Ubc13, allowing formation of a larger complex composed of Rad6-Rad18, Rad5, and Ubc13-Mms2 (Ulrich and Jentsch 2000). The domain of Rad18 required for self-association is coincident with that required for Rad5 interaction and is independent of its Rad6-interaction domain (Ulrich and Jentsch 2000). Whether a homodimer of the Rad6-Rad18 complex and/or of Rad5 has physiological function separate from that of the fully assembled complex is not known.

Although they are in the same epistasis group, *rad5*Δ mutants are less UV sensitive than *rad18*Δ mutants and generally are not defective in UV-induced mutagenesis (Johnson *et al.* 1992). This difference reflects the role of mono-Ub PCNA in promoting TLS (see below). While the major function of Rad5 is clearly as part of error-free PRR, a *rad5*Δ mutant is more sensitive to UV than an *mms2*Δ or *ubc13*Δ mutant (Ulrich and Jentsch 2000). Rad5 is necessary for the damage-induced reversion of some alleles (Gangavarapu *et al.* 2006) and a structural role for Rad5 during mutagenic bypass of defined lesions has been suggested (Pagès *et al.* 2008a).

Like *rad18*Δ mutants, *rad5*Δ strains have a spontaneous mutator phenotype that depends on TLS components. The mutator phenotype is abolished, however, in a *rad5*Δ *rad18*Δ double mutant (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005). The genetic data suggest that, in addition to working together to promote the error-free template switching, Rad18 and Rad5 each independently promote the TLS of a common spontaneous lesion(s). In the absence of either Rad5 or Rad18, template switching cannot occur, and damage is bypassed in a mutagenic manner by the remaining Rad18- or Rad5-dependent TLS pathway, respectively; when both Rad18 and Rad5 are absent, however, neither error-free nor error-prone bypass operates.

***Ubc13-Mms2 complex:*** *MMS2* was identified in a screen for mutations that enhance MMS sensitivity (Prakash and Prakash 1977), and epistasis analysis placed it in the error-free

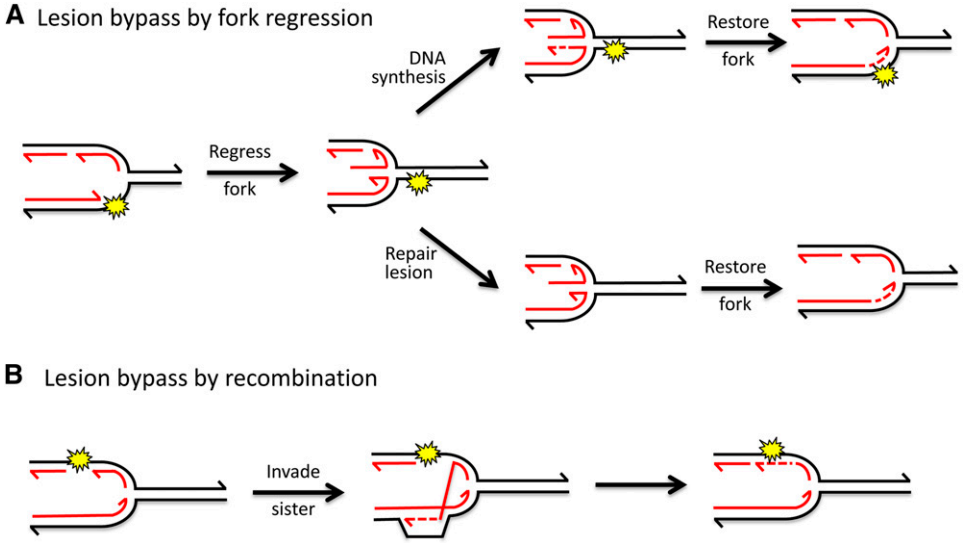


**Figure 8** Mechanisms of template switching during PRR. Alternative template-switch mechanisms are shown for a lesion blocking leading- *vs.* lagging-strand replication, but recombination and gap filling are possible on either strand. (A) Lagging-strand synthesis continues when leading-strand synthesis is blocked by a lesion. Helicase-driven reversal of the fork into a "chicken-foot" structure pairs the newly synthesized strands, with the more extensively extended lagging strand providing a template for additional leading-strand synthesis. Resetting of the fork places the lesion back into duplex DNA, where it can be repaired. (B) A lesion-associated gap on the lagging strand is filled when the blocked 3′ end invades the sister chromatid and uses it as template for additional DNA synthesis. Black and red lines are template and nascent DNA, respectively, and 3′ ends are indicated by the half-arrowheads; the red dotted lines indicate DNA synthesized during the bypass reaction. Yellow stars represent DNA lesions.

arm of PRR (Broomfield *et al.* 1998). Mms2 has homology to Ub-conjugating enzymes, but lacks catalytic activity (Broomfield *et al.* 1998; Xiao *et al.* 1999; Torres-Ramos *et al.* 2002). It forms a stable complex with the *bona fide* E2 conjugase Ubc13, and it is the Ubc13-Mms2 complex that promotes assembly of a regulatory poly-Ub chain on mono-Ub PCNA (Hofmann and Pickart 1999). Deletion of *UBC13* or *MMS2* produces similar phenotypes in terms of increased damage sensitivity and mutagenesis (Brusky *et al.* 2000; Ulrich and Jentsch 2000; Xiao *et al.* 2000).

### *Error-free PRR and recombination*

The existence of discrete PRR and homologous recombination (HR) pathways as defined in early epistasis analyses has become blurred. Even in the initial description of PRR as a mechanism that restores UV-damaged DNA to its full length, a partial requirement for the central recombination protein Rad52 was noted (Prakash 1981). Similarly, error-free bypass of staggered lesions on a plasmid absolutely requires Rad18 and is partially dependent on Rad52 (Zhang and Lawrence 2005). The complex relationship between HR and error-free PRR has been most recently studied using 2D gels, which allow the progression of individual replication forks to be followed in the presence of DNA damage. Fork stalling can be visualized, as well as X-shaped structures that reflect HJs and/or catenated sister chromatids. The formation of the X-structures requires Rad18, Rad5, and Ubc13-Mms2 and, additionally, the Rad51 strand-exchange protein (Branzei *et al.* 2008; Minca and Kowalski 2010). Genetic studies suggest that there are three distinct genetic pathways of replication-associated recombination: template switching to the sister chromatid promoted by Ubc13-Mms2, fork regression promoted by the Mph1 helicase, and a gap-filling reaction promoted by the Shu complex (Choi *et al.* 2010). Overall, current data suggest that HR (1) is important for the invasion step of a template switch that occurs in the context of an unbroken replication fork and generates catenated sister chromatids (pseudo-HJs) and (2) is important for the repair of double-strand breaks, which arise from fork collapse or chromosome breakage and generate true HJs.

### *Components of error-free and error-prone TLS*

A mutagenic subpathway of PRR initially was inferred through isolation of "reversionless" (*rev*) mutants in which the damage-induced reversion of auxotrophic markers was greatly reduced (Lemontt 1971). Of the *REV* genes thus identified, all have been characterized except that defined by *rev5*; only *REV1*, *REV3*, and *REV7* have retained their original names. Sequencing *REV3* revealed homology of the encoded protein to the B family of replicative DNA polymerases, leading to the key insight that specialized TLS polymerases uniquely able to replicate over DNA damage likely exist (Morrison *et al.* 1989). It should be noted that a complete TLS event requires two distinct steps: (1) insertion of a dNMP opposite the lesion and (2) extension of the resulting lesion:base mispair.

Yeast has three TLS polymerases: Pol ζ, which is composed of Rev3 and Rev7, and the Y-family polymerases Pol η, which is encoded by *RAD30*, and Rev1 (reviewed by Waters *et al.* 2009). The yeast and most of the ~15 mammalian TLS polymerases are dispensable; the only known essential enzyme in mammals is Pol ζ (Bemark *et al.* 2000; Esposito *et al.* 2000). Whether this reflects a direct requirement for Pol ζ is unclear, however, as the effects of its loss in chicken DT40 cells can be suppressed by additional loss of Pol η (Hirota *et al.* 2010). Although TLS polymerases are widely referred to as "error-prone," the fidelity of each relative to replicative polymerases is lesion specific. The current model is that most TLS polymerases are specialized to bypass one or a few naturally occurring lesions in a relatively

error-free manner. All, however, are notoriously error-prone on undamaged DNA, lack exonucleolytic proofreading activity, and are nonprocessive.

***Assaying TLS:*** The *in vivo* role of a TLS polymerase during lesion bypass can be determined by comparing survival and/or mutagenesis of wild-type and mutant cells following exposure to DNA damage. Because damaging agents indiscriminately generate multiple types of lesions genome-wide, however, it is difficult to link a specific lesion to the genetic readout. By contrast, transformation-based assays can assess the bypass of a single, defined lesion. Lesions have been engineered into gapped plasmids (Nelson *et al.* 2000; Gibbs *et al.* 2005), double-strand plasmids (Baynton *et al.* 1998, 1999; Pagès *et al.* 2008a,b), and single-strand plasmids (Zhao *et al.* 2006, 2010). Additionally, a single-strand oligonucleotide containing a defined lesion can be introduced into the genome by transformation, where it serves as a template in the subsequent round of DNA replication (Otsuka *et al.* 2002a,b, 2005; Kow *et al.* 2005; Bao and Kow 2009). Finally, *in vitro* assays with purified proteins afford the opportunity to assess the individual steps of TLS through manipulating primer-template combinations and/or the nucleotide pool. The efficiency of full bypass, the insertion specificity opposite the lesion, and/or the ability to extend a primer from a lesion:base mispair can thus be measured.

***Pol ζ, the Rev3-Rev7(-Pol31-Pol32) complex:*** *REV1*, *REV3*, and *REV7* are in the same epistasis group and are required for >90% of induced and at least 50% of spontaneous mutagenesis (reviewed by Lawrence 1994). The polymerase activity of Rev3 is stimulated by Rev7, and these proteins compose the core Pol ζ heterodimer (Nelson *et al.* 1996a). Although core Pol ζ can accomplish complete bypass (Nelson *et al.* 1996a; Stone *et al.* 2011), its primary activity *in vitro* is as an extender of nucleotides inserted opposite a lesion by another DNA polymerase (Johnson *et al.* 2000; Guo *et al.* 2001; Haracska *et al.* 2001b, 2003; Washington *et al.* 2004). In addition to DNA-damage bypass, Pol ζ is required for mutagenesis that occurs in strains with a defective replisome, indicating that an unpaired terminus need not involve a lesion (Northam *et al.* 2010). All four of the yeast B-family DNA polymerases contain an Fe-S cluster (Netz *et al.* 2012), which is emerging as a common feature of many DNA metabolic proteins.

Pol ζ is important in the bypass of a broad variety of lesions *in vivo*: methylated bases (Johnson *et al.* 2007), AP sites (Haracska *et al.* 2001b; Kim and Jinks-Robertson 2009), oxidative damage (Johnson *et al.* 2003), interstrand crosslinks (Grossmann *et al.* 2001; Sarkar *et al.* 2006), UV-induced lesions (Kozmin *et al.* 2003; Abdulovic and Jinks-Robertson 2006), and DNA-protein crosslinks (De Graaf *et al.* 2009; Grogan and Jinks-Robertson 2012). Although most studies have focused on the role of Pol ζ in tolerating nuclear DNA damage, it localizes to and is important in the mitochondria as well (Zhang *et al.* 2006; Kalifa and Sia 2007).

Most Pol ζ-dependent bypass of induced lesions is Rad18-dependent (see above), but separate Rad18- and Rad5-dependent pathways of spontaneous lesion bypass have been documented using forward and reverse mutation assays (Liefshitz *et al.* 1998; Cejka *et al.* 2001; Minesinger and Jinks-Robertson 2005). A multiple-mutation signature produced by Pol ζ in the latter assay indicates that synthesis is limited to tracts of ~10 nt *in vivo* (Harfe and Jinks-Robertson 2000b), which is consistent with the distributive behavior of TLS polymerases *in vitro*. The multiple-mutation signature has been particularly useful for following Pol ζ activity *in vivo* (Minesinger and Jinks-Robertson 2005; Sabbioneda *et al.* 2005; Abdulovic and Jinks-Robertson 2006; Minesinger *et al.* 2006; Lehner and Jinks-Robertson 2009; Grogan and Jinks-Robertson 2012) and has been recapitulated *in vitro* using undamaged templates (Zhong *et al.* 2006; Stone *et al.* 2009).

Cooperation between Pol ζ and the replicative DNA polymerase Pol δ during lesion bypass was early inferred through analysis of non-null catalytic (*pol3*) mutants (Halas *et al.* 1997) and by a dependence of Pol ζ-dependent bypass on the Pol32 subunit of Pol δ (Huang *et al.* 2000; Gibbs *et al.* 2005; Minesinger and Jinks-Robertson 2005; Hanna *et al.* 2007; Pagès *et al.* 2008b; Auerbach and Demple 2010). Recent data, however, have demonstrated that the noncatalytic Pol31 and Pol32 subunits of Pol δ are shared with Pol ζ and that the Pol ζ holoenzyme is actually a four-subunit complex (Baranovskiy *et al.* 2012; Johnson *et al.* 2012). Other proteins implicated in Pol ζ-dependent bypass include the Cdc7 kinase (Pessoa-Brandao and Sclafani 2004), the 9-1-1 checkpoint clamp (Sabbioneda *et al.* 2005), and the Rad24, Rad9, and Mec1 checkpoint proteins (Paulovich *et al.* 1998; Barbour *et al.* 2006; Hishida *et al.* 2009; Pagès *et al.* 2009).

***Rev1, a deoxycytidyl transferase:*** Rev1 generally is assumed to be required during Pol ζ-dependent lesion bypass *in vivo* (reviewed by Lawrence 1994), but there have been reports of differential Rev1 *vs.* Pol ζ requirements during gapped-plasmid repair (Baynton *et al.* 1999) for instability in *pol3-t* background (Mito *et al.* 2008) and in mitochondrial DNA stability (Zhang *et al.* 2006). The initial biochemical characterization of purified Rev1 revealed deoxycytidyl (dCMP) transferase activity during bypass of a template AP site (Nelson *et al.* 1996b), and a strong preference for dCMP insertion *in vitro* has been confirmed (Pryor and Washington 2011). Rev1 additionally has been defined as a G-template-specific DNA polymerase (Haracska *et al.* 2002). Indeed, the crystal structure of Rev1 with a template G or AP site reveals that each is evicted from the active-site pocket to allow pairing of the incoming dCTP with an arginine (Nair *et al.* 2005, 2011). Although it has been argued that Rev1 catalytic activity is not relevant for AP-site bypass *in vivo* (Haracska *et al.* 2001b; Pagès *et al.* 2008b), the

consensus is that dCMP is the predominant nucleotide inserted opposite AP sites and that most Pol ζ-dependent bypass of AP sites relies on Rev1 catalytic activity (Otsuka *et al.* 2002b; Auerbach *et al.* 2005; Gibbs *et al.* 2005; Kow *et al.* 2005; Kim *et al.* 2011b). According to the two-polymerase model, Rev1 inserts dCMP opposite a lesion, with Pol ζ providing extension activity (see Figure 3). Altogether, the data suggest a specialized, "error-free" role of Rev1 in bypassing guanines either damaged or lost from the DNA backbone.

In addition to AP-site bypass, Rev1 catalytic activity is relevant during bypass of ethenoadenine- (Zhou *et al.* 2010) and 4-nitroquinilone 1-oxide-induced damage (Wiltrout and Walker 2011a); bypass of other types of lesions, however, depends only on Rev1 presence (Nelson *et al.* 1996b; Zhou *et al.* 2010; Wiltrout and Walker 2011a). Rev1 thus has an essential structural role, as well a variable enzymatic role, during Pol ζ-dependent bypass. Rev1 interacts with both Rev7 and Rev3 (Acharya *et al.* 2005, 2006; D'Souza and Walker 2006), with the Pol32 subunit of Pol δ/ζ (Acharya *et al.* 2009), with Rad5 (Pagès *et al.* 2008a), and with mono-Ub PCNA (Wood *et al.* 2007; D'Souza *et al.* 2008). Recent work suggests that interaction of Rev1 with Rad5 is required for the noncatalytic function of Rev1 (Kuang *et al.* 2013). In contrast to mammalian REV1, which interacts with multiple Y-family DNA polymerases via a highly conserved C terminus (Guo *et al.* 2003), no interaction of yeast Rev1 with Pol η has been detected (Kosarek *et al.* 2008). Finally, Rev1 is hyper-phosphorylated during mitosis and after DNA damage, suggesting potential post-translational regulation of its activity (Sabbioneda *et al.* 2007).

***Pol η:*** The *RAD30* gene was identified by homology to the *E. coli* DinB polymerase (McDonald *et al.* 1997; Roush *et al.* 1998). *RAD30* is in the *RAD6-RAD18* epistasis group, and synergistic sensitivity to UV damage is seen upon additional loss of Rad5 or Rev1/Pol ζ (McDonald *et al.* 1997). Although loss of Pol η can have opposing, allele-specific effects on UV-induced reversion (McDonald *et al.* 1997; Roush *et al.* 1998), it generally is associated with an elevation in Pol ζ-dependent mutagenesis (*e.g.*, Johnson *et al.* 1999b; Abdulovic and Jinks-Robertson 2006). The TLS field was launched into prominence by the discovery that loss of human POL η is responsible for the variant, NER-proficient form of xeroderma pigmentosum (XP-V). *In vitro*, Pol η has the unique ability to bypass *cis-syn* thymine dimers in an error-free manner, thereby providing an explanation for why loss confers XP-V (Johnson *et al.* 1999a; Masutani *et al.* 1999).

Structural studies have revealed that the active-site pocket of Pol η is large relative to that of replicative DNA polymerases (Trincao *et al.* 2001). This facilitates bypass of bulky lesions, but also makes Pol η one of the most inaccurate TLS polymerases on undamaged DNA templates (Matsuda *et al.* 2000; Washington *et al.* 2001). In contrast to the rigid steric fit used by replicative DNA polymerases for dNTP discrimination, Pol η relies more on Watson–Crick hydrogen bonding. This latter property allows the error-free bypass of lesions such as thymine dimers, which retain base-pairing capacity (Washington *et al.* 2003; Alt *et al.* 2007).

Numerous *in vitro* studies have reported bypass of defined lesions by purified Pol η, but only three for which there is accompanying genetic data will be mentioned here: UV-induced lesions, AP sites, and 8-oxoG. Bypass of UV-induced *cis-syn* thymine dimers by Pol η is error-free (see above), as is that of the more distorting (6-4) CC or (6-4) TC (Yu *et al.* 2001). By contrast, Pol η preferentially inserts a G opposite the 3′-T of (6-4) TT *in vitro* (Johnson *et al.* 2001). This specificity can explain why UV-induced reversion of the *arg4-17* allele, which occurs primarily via T-to-C transitions, is reduced in a *rad30*Δ background (Zhang and Siede 2002). With regard to an AP site, bypass by Pol η is poor *in vitro*, presumably because the enzyme requires a template base opposite the incoming dNTP (Haracska *et al.* 2001c). No significant involvement of Pol η has been observed in a gap-filling assay (Gibbs *et al.* 2005) or during bypass of genomic AP sites (Otsuka *et al.* 2002b; Auerbach and Demple 2010; Kim *et al.* 2011b). With respect to 8-oxoG, Pol η has a strong preference for inserting C rather than A *in vitro* (Haracska *et al.* 2000; McCulloch *et al.* 2009), and there is a strong increase in 8-oxoG-associated mutagenesis upon deletion of the *RAD30* gene (Haracska *et al.* 2000; De Padula *et al.* 2004; Mudrak *et al.* 2009; Van Der Kemp *et al.* 2009). As noted previously, Pol η is part of the yeast GO network (Figure 2).

Pol η interacts with PCNA via a C-terminal PIP domain, and its activity is stimulated by PCNA *in vitro* (Haracska *et al.* 2001a). The PIP domain is essential for survival following UV irradiation (Haracska *et al.* 2001a) and for error-free 8-oxoG bypass (Mudrak *et al.* 2009). Pol η additionally has a Ub-binding (UBZ) domain that further facilitates interaction with mono-Ub PCNA and is required for *in vivo* function (De Padula *et al.* 2004; Parker *et al.* 2007; Pabla *et al.* 2008; Van Der Kemp *et al.* 2009). *In vitro*, mono-Ub PCNA regulates a polymerase exchange between Pol δ and Pol η during lesion bypass (Zhuang *et al.* 2008). Finally, Pol η itself is mono-Ub, and its UBZ domain is required for this as well (Parker *et al.* 2007). Mono-Ub of Pol η decreases in S phase or following UV irradiation, suggesting a regulatory role for this modification (Pabla *et al.* 2008).

### *Post-translational modification of PCNA*

Regulation of error-free *vs.* error-prone PRR through post-translational modification of PCNA was reported in a landmark study published in 2002 (Hoege *et al.* 2002). Following a sublethal dose of DNA damage, Rad6 and Rad18 are required to attach a single Ub moiety to lysine 164 (K164) of Pol30, which can be extended into a regulatory, K63-linked poly-Ub chain in the presence of Mms2, Ubc13, and Rad5. As noted previously, mono-Ub of PCNA promotes TLS (Stelter and Ulrich 2003) while poly-Ub is required for the error-free

template-switch pathway (Hoege *et al.* 2002). During S phase or following a very high dose of damage, however, a single small ubiquitin-like modifier (SUMO) moiety is attached to K164, and SUMO additionally is attached to K127 (Hoege *et al.* 2002). SUMOylation of PCNA requires the E2 SUMO conjugase Ubc9 and the Siz1 E3 SUMO ligase and helps promote error-free PRR (see below). PCNA also can be poly-SUMOylated (Parker *et al.* 2008; Windecker and Ulrich 2008), but the significance of this is not known. Ubiquitination of PCNA at K164 and its key role in promoting PRR is highly conserved among eukaryotes, but SUMOylation is not (Ulrich and Walden 2010). Although not relevant to PRR, ligase 1 defects trigger PCNA ubiquitination at K107; this requires Rad5 and Msm2-Ubc4 and is important in checkpoint activation (Das-Bradoo *et al.* 2010). The post-translational modifications to PCNA are summarized in Figure 9.

The *pol30-K164R* single-, *pol30-K127R* single-, and *pol30-K127R,K164R* double-mutant alleles have been widely used to assess how Ub and/or SUMO affects PRR (*e.g.*, Hoege *et al.* 2002; Pfander *et al.* 2005). One intriguing observation made in early studies was that a *pol30-K127R, K164R* double mutant is less sensitive to DNA damage than a *pol30-K164R* single mutant (Hoege *et al.* 2002). Survival of a *pol30-K164R* mutant also is increased by loss of the Srs2 X′–Y′ helicase (Hoege *et al.* 2002), consistent with the long-known suppression of *rad6* and *rad18* alleles by *srs2* alleles (Aboussekhra *et al.* 1989; Schiestl *et al.* 1990; Rong *et al.* 1991). The requirement of homologous recombination for *srs2* suppression of *rad6* (Schiestl *et al.* 1990), together with the hyper-recombination phenotype *srs2* mutants (Rong *et al.* 1991), suggested that one role of Srs2 is to limit recombination. Indeed, Srs2 has "strippase" activity that dismantles the Rad51 nucleoprotein filaments required for the strand-invasion step that initiates recombination (Krejci *et al.* 2003; Veaute *et al.* 2003; reviewed by Marini and Krejci 2010). The demonstration that SUMOylated PCNA physically interacts with the C terminus of Srs2 brought the genetic and biochemical observations into a coherent model in which SUMOylated PCNA recruits Srs2 to dismantle Rad51 nucleoprotein filaments, thereby preventing unrestrained/inappropriate recombination (Papouli *et al.* 2005; Pfander *et al.* 2005). In the absence of Srs2, PRR defects are thus partially rescued because damage is bypassed by the alternative, homologous recombination pathway.

In principle, the Pol30 monomers that compose the PCNA homotrimer can be differentially modified and/or interact with different proteins. With regard to the former, template switching appears to occur most efficiently when PCNA is simultaneously SUMOylated and polyubiquitinated (Branzei *et al.* 2008). Both modifications can exist on the same Pol30 monomer (Windecker and Ulrich 2008) and recent work suggests that Rad18 is actually a SUMO-targeted ubiquitin ligase (Parker and Ulrich 2012). With regard to the latter, replicative and TLS polymerases can potentially interact simultaneously with the clamp, providing a mechanism to effect bypass by switching between polymerases (the "tool

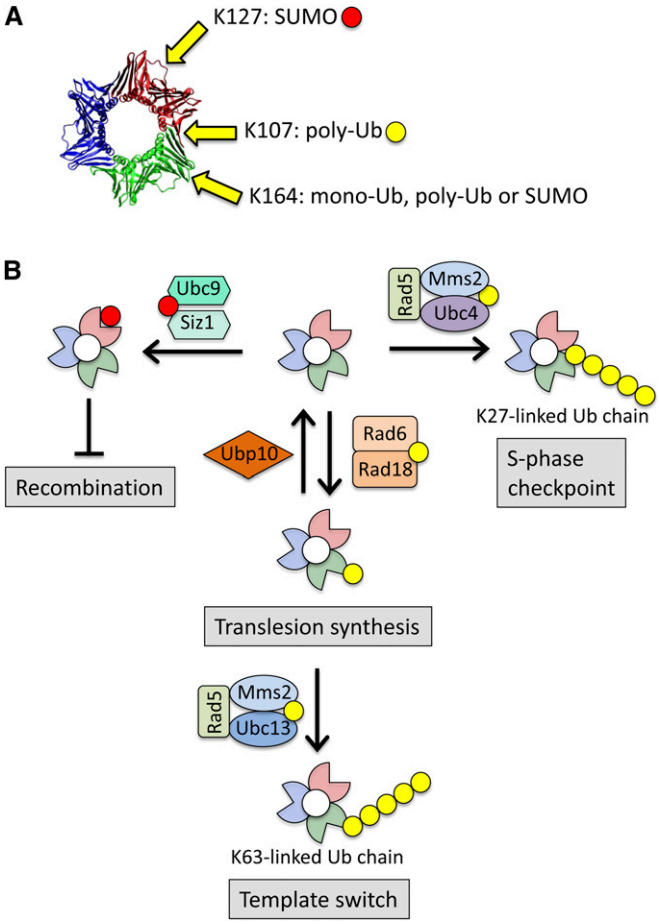


**Figure 9** Post-translational modifications to PCNA and PRR regulation. (A) Crystal structure of the human PCNA homotrimer with the subunits indicated in different colors (http://en.wikipedia.org/wiki/Proliferating_cell_nuclear_antigen). The approximate positions of the lysines (K) modified by Ub or SUMO (yellow and red circles, respectively) are indicated. (B) The proteins/complexes involved in modifying PCNA to direct the appropriate response are indicated and are described in the text.

belt" model). The crystal structure of a Ub-Pol30 fusion protein places the PIP-binding domain and Ub on the back face of the homotrimer (*i.e.*, the side away from the primer end), with Pol δ interacting on the front face (Freudenthal *et al.* 2010). The solution structure of Ub-PCNA indicates that the association of Ub with PCNA is dynamic, however, with Ub transitioning between different docking sites on the clamp (Tsutakawa *et al.* 2011). This suggests a model in which Ub holds a TLS polymerase in reserve on the back face of PCNA and then transitions it to a side position for lesion bypass.

*In vitro* data suggest that the switch from Pol η back to Pol δ during lesion bypass requires either a de-ubiquitinating enzyme (a "DUB") or unloading of Ub-PCNA by a clamp loader (Zhuang *et al.* 2008). Ubp10 has been biochemically identified as the DUB for PCNA, removing both mono- and di-Ub (Gallego-Sanchez *et al.* 2012). Elg1, which is part of an alternative clamp loader, promotes genome stability and interacts with PCNA, preferring the SUMOylated form of the clamp. Srs2 accumulates on DNA in the absence of Elg1, and

loss of Srs2 and Elg1 confers a synthetic fitness defect that is suppressed by blocking SUMOylation of PCNA (Parnas *et al.* 2010). These observations suggest that persistence of SUMO-PCNA on DNA is toxic and that Elg1 may be important either for removing SUMO from PCNA or for unloading SUMO-PCNA from DNA.

### *When and where does PRR occur?*

In *Xenopus* extracts, PCNA ubiquitination is triggered when the replicative DNA polymerase and helicase are uncoupled at a replication fork (Chang *et al.* 2006). This is expected to generate tracts of single-strand DNA bound by RPA; indeed, yeast RPA interacts with Rad18, and this interaction is required for damage-associated PCNA ubiquitination (Davies *et al.* 2008). PRR presumably evolved as a mechanism to circumvent problems that arise during replication, but whether bypass occurs directly at a stalled fork or as a gap-filling process behind the fork has not been resolved. During "discontinuous" lagging-strand synthesis, the constant repriming of Okazaki fragments provides a ready mechanism to generate gaps that could be subsequently filled by TLS or a template switch (Figure 8B). By contrast, the "continuous" nature of leading-strand synthesis led to an early assumption that bypass would have to occur directly at the fork to prevent replication arrest (Figure 8A). Following high levels of damage, however, gaps have been detected by electron microscopy on both arms of yeast replication forks (Lopes *et al.* 2006). In addition, cell-cycle-specific induction of key PRR proteins has demonstrated that PRR can be temporally separated from replication and hence need not occur directly at a stalled fork (Daigaku *et al.* 2010; Karras and Jentsch 2010). Even though PRR can occur completely outside the context of replication, this does not necessarily mean that this is how it normally occurs. It remains possible that there are inherent differences in how lesions are bypassed on the leading *vs.* lagging strand of replication, reflecting the relatively continuous and discontinuous modes, respectively, of new DNA synthesis (Gangavarapu *et al.* 2007; Minca and Kowalski 2010).

With regard to the specific timing of Pol ζ-dependent bypass, there is 50-fold more Rev1 in G2/M than in G1/S (Waters and Walker 2006), and this reflects primarily regulation via proteosomal degradation (Wiltrout and Walker 2011b). Rev3 and Rev7 levels, however, are constant throughout the cell cycle (D'Souza and Walker 2006). Maximal expression of Rev1 outside of S phase has led to the suggestion that most Pol ζ-dependent lesion bypass occurs via gap-filling reactions that occur well behind the replication fork. Consistent with this, spontaneous, Pol ζ-dependent lesion bypass is refractory to correction by the MMR machinery (Lehner and Jinks-Robertson 2009). The higher mutation rate of late-replicating DNA also is reduced in *rev1*Δ background (Lang and Murray 2011), consistent with the cell-cycle regulation of Rev1 and suggesting that there may be temporal separation of error-free and error-prone lesion bypass.

## Summary and Future Directions

There has been remarkable progress in unraveling the diverse mechanisms that deal with damage to the DNA double helix and with errors introduced during DNA synthesis. Genetic studies have elucidated the high degree of redundancy built into these systems, with the net effect being the maintenance of an extraordinarily stable genome in the face of constant internal and external assaults. Importantly, the lessons learned in yeast have been useful for understanding basic principles that operate in all eukaryotes and for understanding the origins of sporadic and inherited cancers. The focus here has largely been on the individual players involved in specific pathways and on underlying biochemical mechanisms. Understanding how the cellular environment affects repair/bypass mechanisms remains a future challenge: the composition and cellular locations of machines/factories, the roles of post-translational modifications, the effect of the cell cycle, and the roles of chromatin modifications. There are also expected to be novel connections between proteins and pathways that emerge from recently developed systems approaches. At least in yeast, however, genetic screens will continue to be a useful tool. There is much to keep us busy in the next 20 years, when perhaps even new and unanticipated pathways and mechanisms for promoting genome stability will be discovered.

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