



Review

Repair of and checkpoint response to topoisomerase I-mediated DNA damage

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Abstract

Topoisomerase I (Top1) catalyzes two transesterification reactions: single-strand DNA cleavage and religation that are normally coupled for the relaxation of DNA supercoiling in transcribing and replicating chromatin. A variety of endogenous DNA modifications, potent anticancer drugs and carcinogens uncouple these two reactions, resulting in the accumulation of Top1 cleavage complexes. Top1 cleavage complexes damage DNA and kill cells by generating replication-mediated DNA double-strand breaks (DSBs) and by stalling transcription complexes. The repair of Top1-mediated DNA lesions involves integrated pathways that are conserved from yeasts to humans. Top1-mediated DNA damage and cell cycle checkpoint responses can be studied biochemically and genetically in yeast and human cells with known genetic defects. Defects in these repair/checkpoint pathways, which promote tumor development, explain, at least in part, the selectivity of camptothecins and other Top1 inhibitors for cancer cells.

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1. Introduction

DNA topoisomerases exist in all living organisms. In humans, there are 6 topoisomerase genes coding for nuclear topoisomerase I (Top1), mitochondrial topoisomerase I (Top1mt) [1], topoisomerases II α and β , and topoisomerases III α and β (reviewed in [2,3]). Nuclear Top1 is essential for animals as knockout are not viable in flies [4] and mice [5]. Top1 is however dispensable both in budding yeast *Saccharomyces*

cerevisiae (YSC) [6] and fission yeast *Saccharomyces pombe* (YSP) [7]. A critical function of Top1 is to relax supercoiled DNA in transcribing and replicating chromatin. Top1 may also play roles in DNA repair and recombinations [8,9].

DNA topoisomerases are the targets of antimicrobial and anticancer drugs, and mammalian Top1 is the selective target of camptothecins [10–12]. Top1 cleavage complexes (see Section 2) are also produced by endogenous and exogenous DNA lesions (for review see [13]), including UV-induced base modifications, guanine methylation and oxidation, polycyclic aromatic carcinogenic adducts [14], base mismatches,

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abasic sites, cytosine arabinoside or gemcitabine incorporation [15] and DNA nicks. Cleavage complexes can produce DNA damage after collisions of replication forks and transcription complexes. These lesions, and in particular replication fork collisions, need to be repaired for cell survival.

This review focuses on the DNA repair and checkpoint pathways elicited by Top1-mediated damage in nuclear DNA and the conservation of these specific DNA repair and cell cycle checkpoint pathways from yeast to humans. Some of the review is speculative in order to provide a basis for further investigations. We will concentrate on the genes and corresponding pathways that determine the cellular responses/repair of Top1 cleavage complexes both in yeasts and mammalian cells. The biochemistry and cellular biology of

Top1, the development of non-camptothecin Top1 inhibitors, and the apoptotic pathways elicited by Top1 cleavage complexes have been recently reviewed elsewhere and will only be mentioned here [2,3,13,16–19].

2. Abnormally high frequency of Top1 cleavage complexes can be generated by endogenous and exogenous agents

Top1 break sites are normally ubiquitous in the genome as a consequence of the normal activity of the enzyme. Top1 relaxes DNA supercoils, which build up ahead of replication and transcription complexes, by inducing transient single-strand breaks. This allows rotation of the DNA double helix around the intact

Table 1
Exogenous and endogenous factors producing Top1 cleavage complexes

Anticancer drugs ^a	M ^b	R ^c	Notes	References
Camptothecins	T	r	Highly selective and specific	[17]
Indolocarbazoles (NB-506)	T	r	In clinical development	[19]
Actinomycin D	T	r	Other effects: DNA, RNA polymerase	[17]
Hoechst	T	r	Other effects: DNA minor groove binding	[19]
Triple helix camptothecin conjugates	T	r	Sequence specific major groove binding	[260]
Indenoisoquinolines	T	r	Developed by Cushman and Pommier	[19]
Phenanthridines and analogs	T	r	Developed by LaVoie and Liu	[19]
Ecteinascinidin 743	T	r	N2-dG alkylation; NER poison	[19]
Cytosine arabinoside	T	r	Other effects: blocks DNA synthesis	[261,262]
Gemcitabine	T	r	Other effects: blocks DNA synthesis	[15]
Endogenous DNA lesions [13]				
Single base mismatches	T	r	Polymerase and mismatch defects	[13,263]
Mismatched loops	T	icc	Mismatch deficiencies	[263]
Abasic sites	T	icc	AP sites; base excision repair	[263]
8-Oxoguanosine	B	r	Free radicals: oxidative lesions	[22]
5-Hydroxycytosine	?	r	Free radicals: oxidative lesions	[22]
Single-strand breaks	T	icc	Free radicals; base excision repair	[27]
Cytosine methylation	F+T	r	Physiological	[264]
Triple helix formation	F+T	r		[23]
Exogenous DNA lesions [13]				
UV lesions	?	?	Dimers and 6,4-photoproducts	[265,266]
IR-induced DNA breaks	T	icc	Both single- and double-strand breaks	[27]
<i>O</i> ⁶ -Methylguanine	T	r	Produced by alkylating drugs (MNNG)	[267]
<i>O</i> ⁶ -dA-Benzo[<i>a</i>]pyrene adducts	T	icc	Intercalated carcinogenic adducts	[268]
<i>N</i> ² -dG-Benzo[<i>a</i>]pyrene adducts	F	icc	Minor groove carcinogenic adducts	[14,269]
<i>N</i> ² -dG-Benzo[<i>c</i>]phenanthrene adducts	T	r	Intercalated carcinogenic adducts	[14]
<i>N</i> ⁶ -Ethenoadenine	T	r	Carcinogenic vinyl adduct	[270]

^a For detailed review on non-camptothecin inhibitors (see [19]).

^b Mechanism for Top1 cleavage complex production. T: trapping of the Top1 cleavage complexes (i.e. inhibition of religation) (see Fig. 3B); B: enhancement of binding; F: enhancement of the forward (cleavage) reaction.

^c Reversibility of the Top1 cleavage complexes. r: reversible; icc: irreversible cleavage complexes.

strand and, once the DNA is relaxed, Top1 religates the breaks and regenerates intact duplex DNA. Biochemically, each Top1-mediated DNA break results from a nucleophilic attack from the enzyme catalytic tyrosine (human Tyr723) (for review see [2,3]) (see Fig. 3B). Covalent Top1-cleaved DNA intermediates are referred to as “cleavage complexes”. Under normal conditions, Top1 cleavage complexes are very transient and not detectable. The religation step is much faster than the cleavage (forward) step (see biochemical scheme in Fig. 3B).

A variety of conditions (summarized in Table 1) increase the frequency of Top1 cleavage complexes by inhibiting the religation (annotated “T” in Table 1), which is commonly referred to as “trapping” of Top1 cleavage complexes. Such trapping is generally due to a misalignment of the 5'-hydroxyl DNA end that needs to act as a nucleophile toward the enzyme tyrosyl-DNA phosphodiester bond for reversing the Top1 cleavage complex (see Fig. 3B; for details see [20]). Enhancement of Top1 binding rather than religation inhibition has been demonstrated for 8-oxoguanine [21,22]. Position-specific enhancement of forward rate was recently observed with triple helix-forming oligonucleotides [23].

3. Cellular lesions induced by Top1 cleavage complexes

3.1. DNA damage resulting from Top1 cleavage complexes

Top1 cleavage complexes are normally readily reversible after camptothecin removal, and short exposures to camptothecins (for less than 1 h in cell culture) are relatively non-cytotoxic [24–26]. Persistent drug exposure is required for effective cell killing, as Top1 cleavage complexes are converted into DNA lesions by cellular metabolism. Fig. 1 shows several mechanisms that convert reversible Top1 cleavage complexes into DNA damage (irreversible Top1 covalent complexes) by displacing the cleaved 5'-OH end so that it cannot be religated. Collisions between transcription and replication complexes are shown in panels (B) and (C), respectively. These lesions and the cellular consequences of transcription and replication inhibition will be discussed in the next section (Section 3.2).

Panels (D)–(G) (Fig. 1) show how preexisting DNA lesions can generate irreversible Top1 cleavage complexes, commonly referred to as “suicide complexes” (strand breaks in panels (D) and (E); base lesions in panel (F)) (see Table 1) (for review see [13]). The production of suicide complexes can be enhanced by Top1 poisons [27]. Accordingly, camptothecins and ionizing radiations act synergistically [28]. Also, at high camptothecin concentrations, two Top1 cleavage complexes may form on opposite strands, generating a DNA double-strand break (DSB) [29] (Fig. 1G).

3.2. Replication versus transcription

In most cancer cells [25,30,31] and budding yeast [32], camptothecin cytotoxicity appears primarily related to replication-mediated DNA lesions. However, protection by the DNA polymerase inhibitor, aphidicolin, is generally limited to the lowest (sub-micromolar) doses of camptothecin [26,29,33,34]. These dose-dependent effects are associated with differences in gene expression patterns [35] and cell cycle responses. Low camptothecin doses produce reversible G2 delay whereas higher doses result in S-phase delay and G2 arrest [36]. Replication-independent cytotoxicity can be observed in non-dividing cells, such as neurons [33] and normal lymphocytes (personal data, unpublished). Moreover, the XRCC1-defective CHO EM9 cells (see Section 4.3) remain hypersensitive to camptothecin when DNA replication is blocked [37,38], suggesting that specific pathways repair transcription-associated DNA lesions.

3.3. Replication inhibition by Top1 poisons

Camptothecin inhibits DNA synthesis rapidly and for several hours after drug removal [24,25,39]. The inhibition is initiated by collisions between replication forks and trapped Top1–DNA cleavage complexes (Fig. 1C), as demonstrated in Simian Virus 40 [40,41] and the human ribosomal DNA (rDNA) locus [42]. Replication fork collisions are generated when the Top1 cleavage complexes are on the leading strand for DNA synthesis. Replication proceeds up to the 5'-end of the Top1-cleaved DNA, a process referred to as “replication run-off” (Fig. 1C) [42]. The 5'-termini of the DSBs are rapidly phosphorylated in

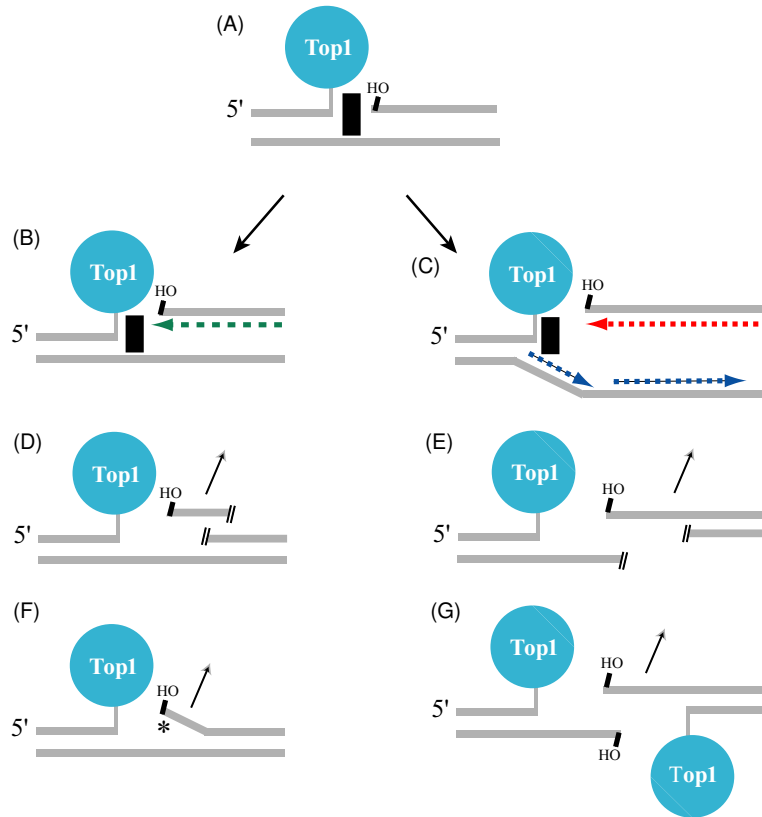


Fig. 1. Conversion of Top1 cleavage complexes into DNA damage by displacement of the 5'-hydroxyl at the end of the cleaved strand by DNA replication, transcription, or preexisting DNA lesions. (A) Schematic representation of a Top1 cleavage complex trapped by camptothecin (black rectangle). Top1 is covalently bound to the 3'-end of the broken DNA. The other end is a 5'-hydroxyl (OH). (B) Conversion of the cleavage complex into a covalent Top1–DNA complex by a colliding transcription complex (the RNA is shown in green). (C) Conversion of the cleavage complex on the leading strand into a covalent Top1–DNA complex by a colliding replication fork (the leading replication is shown in red; the lagging replication in blue). (D and E) Formation of a suicide complex by a single-strand break on the same (D) or the opposite (E) strand from the Top1 scissile strand. (F) Formation of an irreversible Top1 cleavage complex by a base lesion (*) (abasic site, mismatch, oxidized base, ...) at the 5'-end of the cleavage site (see [13]). (G) Formation of a double-strand break at two Top1 cleavage sites close to each other.

vivo, by the kinase activity of polynucleotide kinase phosphatase (PNKP) (see Figs. 3 and 5) (Fig. 1C) [42]. Replication-mediated DSBs are strand specific, as they are not detectable on the lagging strand in rDNA [42]. The repair of these replication-mediated DSBs is markedly more efficient in rDNA [42] than in the overall genome [43]. This differential repair might be due to the unique structure of rDNA, which consists of approximately 200 tandem repeats, to its telomeric location at the ends of the short arms of 5 of the human chromosomes, and to its unique location within nucleoli.

The persistent inhibition of DNA synthesis (for up to 8 h) following camptothecin removal [39] is due to the activation of an S-phase checkpoint [39], including inhibition of thymidine kinase [44]. This S-phase checkpoint is due to a decrease in DNA replication [45], primarily at the level of initiation [46]. It is currently unclear whether this inhibition corresponds to origins that normally fire late in S-phase, similar to the S-phase checkpoint induced by aphidicolin [47,48]. Checkpoint activation prevents cells from entering mitosis with damaged DNA and provides additional time for DNA repair. Furthermore,

replication fork arrest prevents the generation of new collisions. Inhibition of the S-phase checkpoint by 7-hydroxystaurosporine (UCN-01) has marked cytotoxic synergy with camptothecins [49]. UCN-01 inhibits both protein kinases Chk1 [50,51] and Chk2 [52]. This observation is important, since UCN-01 is used in clinical cancer chemotherapy. Because the synergism is more pronounced in cells with defective p53 [49], it is attractive to propose clinical trials associating camptothecin derivatives and UCN-01.

3.4. Transcriptional effects of Top1 poisons

Camptothecin is a potent inhibitor of both nucleolar (rRNA) and nucleoplasmic (mRNA) transcription [24,53–55]. This inhibition is primarily due to transcription elongation blocks by trapped Top1 cleavage complexes (Fig. 1B) [56–59], which is a high probability event since Top1 is associated with transcription complexes (for review see [17]). In vitro assays demonstrated that transcription complexes can convert Top1 cleavage complexes into irreversible strand breaks by the elongating RNA polymerase (see Fig. 1B) [29,60].

The transcription response to Top1 inhibition is locus- and cell-type dependent [61]. In the Chinese hamster dihydrofolate reductase (*DHFR*) gene, camptothecin stimulates RNA synthesis from promoter-proximal sequences, while transcription from promoter-distal sequences is reduced, indicating that camptothecin stimulates initiation while inhibiting elongation [62]. In human cells, transcription inhibition by camptothecin is not uniform [63]. While camptothecin causes a strong holdback of the endogenous *c-MYC* gene at the P2 promoter, it produces minimal effect on an episomal *c-MYC* gene or on the basal transcription of the *HSP70* and *GAPDH* genes [63]. It has minimal effect on transcription complexes at most of the rRNA promoters and on *7SK RNA* transcription by RNA polymerase III. Thus, the effects of camptothecin are gene dependent.

Transcription inhibition recovers rapidly following camptothecin treatment [55,62]. Interestingly, Cockayne syndrome cells, which are deficient in transcription recovery following DNA damage and in transcription-coupled nucleotide excision repair (NER), are hypersensitive to camptothecin [43], suggesting the importance of transcription-coupled DNA

repair for cellular response to top1-mediated DNA damage.

Inhibition of Top1 catalytic activity might also inhibit transcription by producing an accumulation of positive supercoils upstream from the transcribing RNA polymerase complexes [63,64] and by compacting chromatin domains [63,65]. The transcriptional effects of camptothecins could also be related to two other functions of Top1. First, Top1 is known to regulate transcription initiation by binding to TATA binding proteins, repressing basal transcription and enhancing transcription activation independently of its DNA nicking-closing activity [66–68]. Second, Top1 activates RNA splicing by acting as a specific kinase for RNA splicing factors from the SR family such as SF2/ASF [69–71], and by binding to RNA splicing factors PSF/p54 [72,73]. Camptothecin and NB-506, a non-camptothecin Top1 poison [19], block this Top1 SR kinase activity in vitro [69,74].

Top1 cleavage complexes can also activate cellular transcriptional stress responses. Camptothecin produces an elevation of transcription factors, including p53 [75], AP1 (*c-fos* and *c-Jun*) [63,76,77] and NF- κ B [78,79]. Microarray analyses demonstrate that many genes are rapidly upregulated following camptothecin treatment [36] in a p53-dependent and p53-independent manner [35].

4. Repair of Top1 covalent complexes

The various lesions resulting from the conversion of reversible Top1 cleavage complexes into DNA damage (schematized in Fig. 1B–G) are sometimes referred to as “suicide complexes” or “dead-end covalent complexes”. They are characterized by a covalently-linked Top1 molecule at the 3'-end of the break. On the 5'-end of the break, the cleaved strand is generally (except for single-strand removal, Fig. 1D, and base lesions, Fig. 1F) associated with a complementary strand. In the case of transcription-mediated Top1 suicide complexes (Fig. 1B) the resulting double-strand termini are DNA–RNA hybrids, whereas in the case of replication-mediated suicide complexes (Fig. 1C), the termini are DNA duplexes formed between the template and the newly synthesized leading strands (see Section 3.3). In the case of Top1 suicide complexes resulting from cleavage

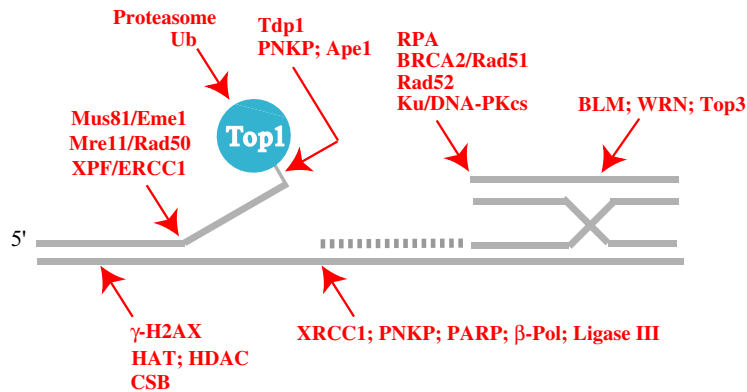


Fig. 2. Schematic representation of the repair pathways for Top1-mediated DNA damage. Processing of the 3'-end implicates Tdp1, PNKP (Ape1) following ubiquitin (Ub)-mediated Top1 proteolysis (see Fig. 3 and Section 4.1). 3'-processing can also be carried out by at least 3 different 3'-flap endonuclease complexes: Rad1/Rad10 (XPF/ERCC1 in humans), Mre11/Rad50/Xrs2 (Nbs1 is the human ortholog for Xrs2), and Mus81/Mms4 (Mus81/Eme1 in humans) (see Fig. 4 and Section 4.2). Processing of the 5'-end of the DNA implicates both homologous recombination (HR) (BRCA2, RAD52, RAD51) and non-homologous-end-joining (NHEJ) (Ku and DNA-PK). Resolution of Holliday junctions implicates the RecQ helicase (BLM, WRN) in association with Top3 (see Fig. 6 and Sections 4.4 and 5.5). Gap filling can be carried out by the base-excision pathway (XRCC1, PNKP, PARP, β -polymerase, ligase III) (see Fig. 5 and Section 4.3). Chromatin remodeling involves histone modifications (phosphorylation of H2AX (γ -H2AX), acetylation under the control of histone acetyltransferases (HAT) and histone deacetylases (HDAC)). Cockayne syndrome B (CSB) remodels chromatin in conjunction with DNA repair and transcription.

complexes in nicked DNA (Fig. 1E) or from neighboring cleavage complexes on opposite strands (Fig. 1G), a staggered DSB is formed.

Thus, the repair of Top1-mediated DNA damage requires the removal of the Top1 covalent complex, the repair of the DNA (or DNA–RNA) double-strand termini, and replication fork restart. The repair pathways and their schematic sites of action are represented in Fig. 2. Top1 can be removed by tyrosyl DNA phosphodiesterase (Tdp1) and 3'-flap endonucleases (XPF/ERCC1; Mre11/Rad50; Mus81/Eme1) (see Sections 4.1 and 4.2). Two additional mechanisms not shown in Fig. 2 can reverse Top1 cleavage complexes. First, Top1 can religate a non-homologous DNA strand bearing a 5'-hydroxyl end, which results in non-homologous recombinations (HRs) [80]. This property is shared by the vaccinia Top1 and has been proposed for the repair of replication-mediated DSB [81]. Vaccinia Top1-mediated DNA religation is commercially used for cloning (TOPO[®] Cloning, Invitrogen Life Technology, Carlsbad, CA). The repair of gaps in the DNA and of DSBs at the 5'-end of the damaged DNA involves the XRCC1 and the homologous and non-homologous DSB repair pathways (see Sections 4.3 and 4.4). Some of the known chromatin

rearrangement pathways associated with repair are listed at the bottom left of Fig. 2 (see Section 5.6).

4.1. Processing of the 3'-ends of Top1 covalent complexes by Tdp1 and PNKP

Nash and coworkers [82] discovered the *TDP1* gene and showed that Tdp1 catalyzes the cleavage of the covalent bond between the Top1 catalytic tyrosine and the 3'-end of the DNA [83] (Fig. 3A). Hydrolysis of the tyrosyl–DNA phosphodiester linkage generates a 3'-phosphate (Fig. 3A and C), which is further processed by a 3'-phosphatase, such as PNKP (or by Ape1).

Tdp1 belongs to the phospholipase D superfamily [84] of phospholipid hydrolyzing enzymes. Tdp1 is ubiquitous and highly conserved in eukaryotes. Tdp1 is physiologically important since a mutation in the enzyme causes a neurological disorder called spinocerebellar ataxia with axonal neuropathy (SCAN1) [85]. Human Tdp1 is a monomeric protein composed of two similar domains related by a pseudo-two-fold axis of symmetry. The catalytic site of each domain contains three conserved residues (HKD motif) [86,87] critical for Tdp1 activity [84]. A recent structure of

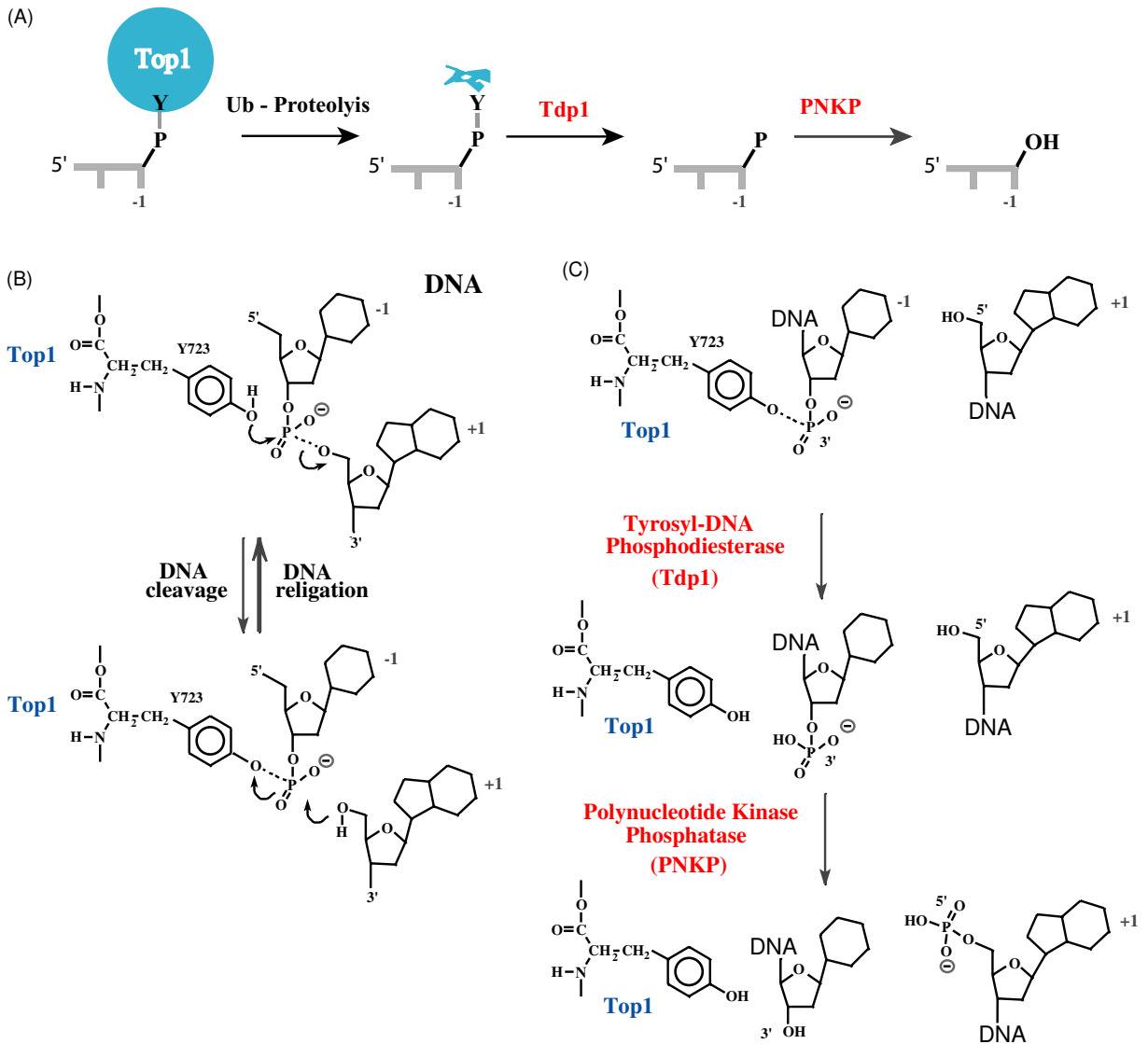


Fig. 3. Repair of Top1 covalent complexes by the Tdp1-PNKP pathway. (A) Schematic diagram for the successive actions of Tdp1 and PNKP. Tdp1 requires Top1 to be degraded (by the ubiquitin–proteasome pathway) to be active. (B) The two transesterifications catalyzed by Top1. DNA religation (reverse step) is much faster than the cleavage reaction (forward step), as indicated by the thickness of the arrows. (C) When the 5'-hydroxyl end of the broken DNA is too far to act as a nucleophile in the reverse reaction shown in panel (B), then Tdp1 hydrolyzes the tyrosyl–phosphodiester bond, regenerating a tyrosyl end on the Top1 polypeptide and leaving a 3'-phosphate end on the DNA. PNKP can hydrolyze this 3'-phosphate and phosphorylate the 5'-end of the broken DNA, which is now a substrate for DNA polymerases and ligases.

Tdp1 bound to a tyrosine-containing peptide demonstrate that the alterations in the structure of both the DNA and the Top1 are required for binding [88]. The DNA needs to be single-stranded and the Top1 re-

duced to a short polypeptide folded differently from the native Top1 [88]. The specificity of Tdp1 for processing 3'-tyrosyl–DNA but not 5' complexes, suggests that Tdp1 belongs to a pathway specific for

the repair of Top1–DNA adducts. However, Tdp1 can also remove 3′-phosphoglycolate generated by oxidative DNA damage, suggesting a broader role for Tdp1 [89].

Both the structure of the DNA segment bound to Top1 [82,90] and the length of the Top1 polypeptide chain determine Tdp1's biochemical activity [90]. Optimum Tdp1 activity requires: (1) a DNA segment consisting of at least a few nucleotides [90] that would bind in Tdp1's positively charged groove [86]; (2) an exposed phosphotyrosyl bond at the Top1–DNA junction; a tyrosyl group linked to the 3′-end of a nick is a poor substrate [91], indicating that Tdp1 acts after the 5′-end of the broken DNA has been either digested or displaced to provide access to the 3′-phosphotyrosyl bond; and (3) a short Top1 polypeptide segment, as the effectiveness of Tdp1 decreases as the length of Top1 polypeptide chain is extended [90]. In fact, Top1 needs to be proteolyzed for efficient Tdp1 activity [83,88,90] (Fig. 3A). As discussed in Section 5.1, Top1 ubiquitination and degradation have been observed following camptothecin treatment [92,93].

The 3′-phosphate ends generated by Tdp1 need to be hydrolyzed to a 3′-hydroxyl for further processing by DNA polymerases and/or ligases. In budding yeast, this 3′-phosphatase activity is carried out by the DNA 3′-phosphatase Tpp1 [94] and by the two functionally overlapping multifunctional apurinic (AP) endonucleases, Apn1 and Apn2 [95]. Apn1 is the homolog of *E. coli* endonuclease IV and represents the major AP endonuclease in budding yeast. Apn2 (also called Eth1) belongs to the second family of AP endonuclease (the *E. coli* exonuclease III family), which includes the human AP endonuclease, Ape1. Simultaneous inactivation of Tpp1, Apn1 and Apn2 (a to a lesser extent inactivation of Tpp1 and Apn1) is required to confer sensitivity to camptothecin [96], indicating the functional redundancy of the 3′-phosphatase pathways (Fig. 4A). Interestingly, the hypersensitivity of the *tpp1 apn1 apn2* triple mutant is rescued by inactivation of Tdp1 [96], consistent with the view that in the absence of Tdp1, budding yeast uses the Rad1/Rad10 pathway for removal of the Top1 covalent complexes (Fig. 4A) (see Section 4.2). The 3′-phosphatase homologs of Tpp1 are Pnk1 in fission yeast [97] and PNKP in human cells [94,98,99] (Fig. 3) (see Section 4.1). In addition

to their 3′-phosphatase activity, both Pnk1 [97] and PNKP [98,99] possess 5′-kinase activity (see Fig. 3C), which is missing for Tpp1. Tpp1 as well as Apn1 and Apn2 [95] are epistatic to Tdp1 (i.e. they function in the same pathway) (Fig. 4A). Another level of redundancy has recently been shown between Tdp1 and Apn1 or Ape1. Indeed, purified Apn1 or Ape1 are capable of removing 3′-tyrosyl lesions from 3′-recessed and nicked DNA substrates, which are poorly processed by Tdp1 [95,100]. In yeast, the tyrosyl phosphodiesterase activity of Apn1 is probably not relevant for the repair of Top1-mediated DNA lesions [95].

There is no Tdp1 inhibitor reported to date besides vanadate and tungstate, which have been used as phosphate mimetic in co-crystal structures [101]. It would, however, be important to develop Tdp1 inhibitors for cancer chemotherapy in association with camptothecins. The anticancer activity of Tdp1 inhibitors may prove to depend on the presence of genetic abnormalities, since camptothecin hypersensitivity in Tdp1-defective yeast is conditional for deficiencies in the checkpoint (Rad9) and 3′-endonucleases (Mus81/Eme1) pathways (Fig. 4A) [82,95,102]. A Rad9 defect in a Tdp1-deficient background confers marked camptothecin sensitivity [82], and it is tempting to speculate that Tdp1 is primarily required when the G2 checkpoint is deficient as in the case of the yeast *RAD9* mutant. These alternative Rad9-dependent pathways probably operate in G2-arrested cells by recombination (see Section 4.3). A second group of conditional genes (with respect to Tdp1 deficiencies) includes three sets of genes from the 3′-flap endonuclease pathway: Rad1/Rad10, Mre11/Rad50, and Mus81/Eme1. Mutation in each of these genes renders Tdp1-deficient cells highly sensitive to camptothecin (Fig. 4A; see below).

4.2. Endonuclease cleavage of Top1–DNA covalent complexes by the 3′-flap endonucleases: Rad1/Rad10, Mre11/Rad50/Nbs1 and Mus81/Eme1

Studies in genetically altered yeast strains demonstrate the existence of alternative pathways beside Tdp1/PNKP for removing the Top1 covalent complexes [95,102]. At least 3 endonuclease complexes can cleave damaged DNA 3′ from DNA lesions. The preferential substrates for these 3′-flap endonucleases

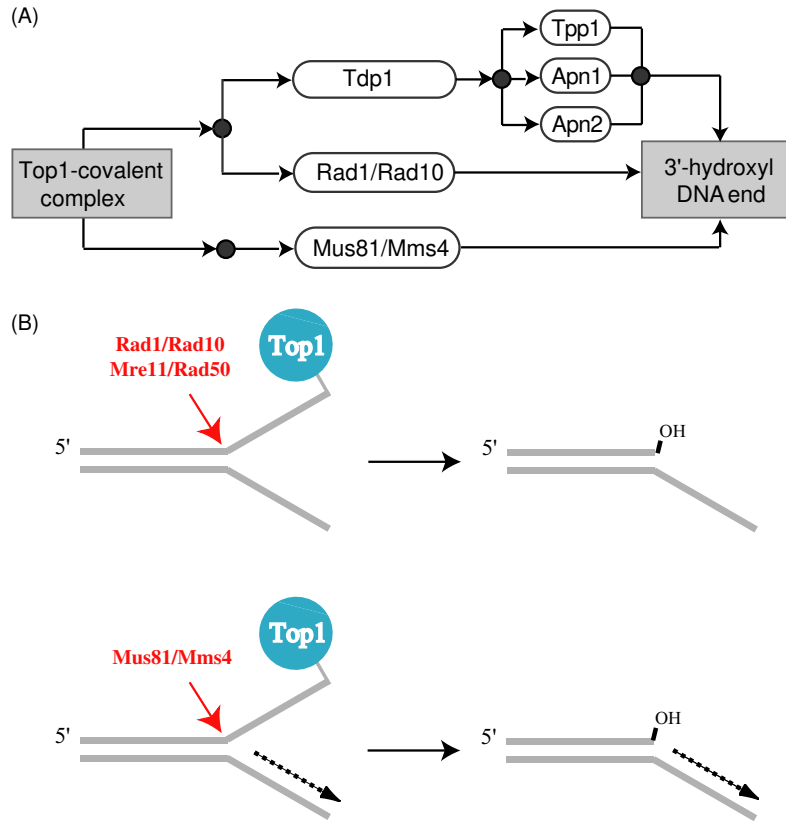


Fig. 4. Repair of Top1 covalent complexes by the 3'-endonuclease pathways. (A) Schematic representation of the genetic pathways implicated in the removal of the Top1–DNA covalent complexes. (B) Differential substrate requirements for Rad1/Rad10 (budding yeast orthologs for human XPF/ERCC1—see Table 3 and Section 4.2), Mre11/Rad50, and Mus81/Mms4 (budding yeast ortholog of human and fission yeast Mus81/Eme1—see Table 3 and Section 4.2). Both Rad1/Rad10 and Mre11/Rad50 require the DNA to be single-stranded opposite to the 3'-flap, suggesting that gap repair should follow their action. By contrast, Mus81/Mms4 requires the DNA to be double-stranded opposite to the 3'-flap as in collapsed replication forks. Mre11/Rad50/Nbs1 is not shown because its checkpoint and recombination functions contribute to cellular response in addition to its nuclease activity [95].

are described in Fig. 4B, and their genetic relationships are proposed in Fig. 4A.

Rad1/Rad10 (the human ortholog is the nucleotide excision repair 3'-endonuclease XPF/ERCC1) and Tdp1/PNKP appear to function in parallel and redundant pathways, whereas Mus81/Mms4 functions in parallel (Fig. 4A) [95,102]. Like Tdp1, Rad1/Rad10 requires a single-stranded gap between the 3'-end to be processed and the 5'-end of the DNA (Fig. 4B) [103], suggesting that Tdp1 and Rad1/Rad10 share common substrates. Such gapped DNA substrates are also common with the XRCC1 pathway (see Section 4.3). Similarly, the Mre11/Rad50/Xrs2 (MRX) (the human

orthologs are Mre11/Rad50/Nbs1 [MRN]) complex preferentially cleaves gapped substrates (Fig. 4B) and hairpin structures [104]. However, the MRN complex also possesses checkpoint functions that probably contribute to the normal response to camptothecin [95,102].

Mus81/Mms4 (the ortholog of budding yeast Mms4 is Eme1 in humans and fission yeast—see Tables 3 and 4) preferentially cleaves broken replication forks and requires the presence of duplex DNA near the 3'-end to be processed (Fig. 4B) [103,105,106]. Mus81-deficient yeasts are highly sensitive to camptothecin (Tables 3 and 4) [102,103,105,107] (Fig. 4A).

4.3. Possible role of the XRCC1/PARP/PNKP/ β -polymerase/ligase III complex

XRCC1, poly(ADP-ribose) polymerase (PARP), β -polymerase, ligase III, PNKP [108,109], and Ape1 [110] form base excision repair (BER) complexes. We recently found that Tdp1 is associated with XRCC1 (Fig. 5), indicating a connection between the XRCC1 pathway and the repair of both transcription- and replication-associated DNA damage induced by Top1 cleavage complexes [38].

PARP is a relatively abundant nuclear protein containing a zinc finger motif functioning as a nick-sensor. It binds to double- and single-stranded DNA breaks generated exogenously or by enzymatic nick-

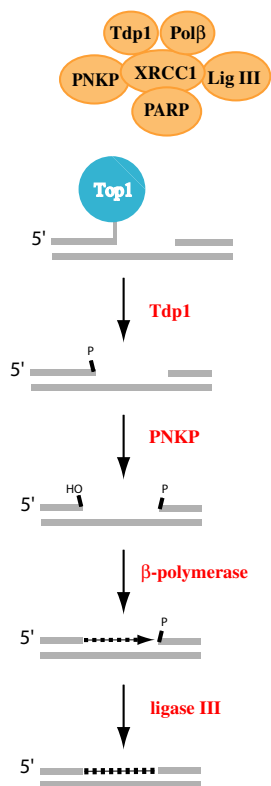


Fig. 5. Proposed repair of a Top1 covalent complex by the XRCC1-dependent pathway. The XRCC1 complex including the associated repair enzymes is shown at the top. Tdp1 hydrolyzes the Top1–DNA-phosphotyrosyl bond. PNKP hydrolyzes the resulting 3'-phosphate and phosphorylates the 5'-hydroxyl. β -Polymerase fills the gap and ligase III seals the DNA. Ape1 can also form complexes with XRCC1 and hydrolyze the 3'-phosphate.

ing during BER (reviewed in [111,112]). Binding of PARP to nicked DNA stimulates PARP to catalyze the transfer of successive units of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD), resulting in transient covalent binding of large, negatively charged, poly(ADP-ribose) polymers to macromolecular acceptors, including DNA processing enzymes, chromatin and PARP itself [112,113]. Poly(ADP-ribosylation) alters the structure and function of the acceptors and marks the beginning of the DNA repair process. Although Top1 is one of the poly(ADP-ribose) acceptors, the functional consequences of the PARP–Top1 interaction are not well-understood. While Top1 poly(ADP-ribosylation) inhibits Top1 activity [114–116], PARP binding activates Top1 [117].

Several observations implicate PARP in the cellular response to and repair of Top1 cleavage complexes: (1) PARP is activated in camptothecin-treated cells [118]; (2) PARP-deficient Chinese hamster V79 cells [119,120] and PARP-knockout mouse fibroblasts are hypersensitive to camptothecin (Table 2), and exhibit slow repair of Top1-induced DNA lesions (Barceló and Pommier, unpublished); (3) PARP inhibitors such as 3-aminobenzamide [121] or NU1025 [122] sensitize cells to camptothecins; and (4) increased PARP levels are associated with camptothecin resistance [123].

XRCC1 has no enzymatic activity but functions as a scaffolding factor for the enzymes required for BER, including PNKP [108]. XRCC1 is implicated in the repair of Top1 cleavage complexes, as: (1) CHO XRCC1-mutant EM9 cells are hypersensitive to camptothecin [37,38,124] (Table 2); (2) XRCC1 complementation in EM9 cells restores camptothecin resistance and enhances the repair of Top1-induced single-strand breaks and Tdp1 activity [38]; and (3) camptothecin-resistant cell lines show increased XRCC1 levels, and transfection of XRCC1 increases camptothecin resistance [125].

Fig. 5 proposes a scheme in which both Tdp1 and PNKP are physically and functionally associated with the XRCC1 complex [38]. After removal of the Top1–DNA complex by Tdp1, PNKP processes the DNA ends for β -polymerase and ligase III action. PARP's nick-sensor function could serve in a damage survey mechanism to recruit XRCC1 repair complexes to the sites of Top1-associated DNA damage.

Table 2
Genetic alterations sensitizing mammalian cells to Top1 poisons

Genes	Functions	References
ATM	Protein kinase from the PI(3)K family; implicated in DSB response	[155,157,158,271]
NBS1	Scaffolding protein forming a complex with Mre11 and Rad50 (MRN complex); DSB repair and recombination pathways	[156,185]
DNA-PKcs	Protein kinase from the PI(3)K family; implicated in DSB response	[39,189,272]
ATR	Protein kinase from the PI(3)K family; implicated in replication stress	[200]
WRN	Helicase from the RecQ family involved in genomic stability	[231–233]
BLM	Helicase from the RecQ family involved in genomic stability	[234]
XRCC2	One of the five Rad51 paralogs: Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3; implicated in DNA strand exchange/homologous recombination	[124,272,273]
XRCC3	One of the five Rad51 paralogs; implicated in DNA strand exchange/homologous recombination	[272]
Rad51C	One of the five Rad51 paralogs; implicated in DNA strand exchange/homologous recombination	[274]
BRCA2	Involved in Rad51 loading; homologous recombination	[245]
BRCA1	DNA damage response; TC–NER	[244]
XRCC1	BER	[37,38,124,125]
PARP	BER	[119,275]
CSA/CSB	TCR/BER	[43]
γ -H2AX	Core histone; phosphorylated in response to DSB	[173]
p53/p21	Checkpoints; apoptosis	[242,276]
Bcl-2	Apoptosis	[277]

Abbreviations—ATM: ataxia telangiectasia mutant; ATR: ataxia telangiectasia and Rad3-related; BER: base excision repair; BLM: Bloom syndrome; CSA/CSB: Cockayne syndrome complementation groups A and B; DNA-PKcs: DNA-dependent protein kinase catalytic subunit; DSB: DNA double-strand breaks; NER: nucleotide excision repair; PARP: poly(ADP-ribose) polymerase; PI(3)K: phosphatidylinositol 3 kinase; TCR: transcription-coupled repair.

The absence of PARP may hinder XRCC1 function, which could explain that nuclear extracts from PARP- and XRCC1-deficient cells exhibit low activity for Tdp1, PNKP, and β -polymerase [38] (Barceló and Pommier, unpublished).

4.4. 5'-End processing: repair of Top1-associated replication-mediated DNA double-strand breaks

The Top1-induced DSB generated by replication fork collisions can be processed both by homologous recombination (Rad52/51) and non-homologous end-joining (NHEJ) (Ku/DNA-PK). Tables 2–4 demonstrate the involvements of the HR and NHEJ pathways, as well as of the MRN pathway, which functions both for HR and NHEJ. PNKP is also probably implicated since the 5'-end of the replication-mediated DSB is rapidly phosphorylated in camptothecin-treated cells [42].

Fig. 6 shows two possible pathways for the repair and restart of replication forks following Top1-induced DNA damage. In the pathway shown in

panel (A), Tdp1 (see Section 4.1) or Mus81/Eme1 (see Section 4.2) would remove the Top1 covalent complexes. Gap repair (see below Section 4.3 and Fig. 5) would ligate the upstream portion of the template strand for leading strand synthesis with a newly synthesized Okazaki fragment. The repair of the double-strand break would proceed by homologous recombination following 5'-end resection (the corresponding nuclease has not been identified). This pathway is the classical break-induced replication model proposed by Haber and coworkers [126,127]. The resulting 3'-single-stranded DNA segment would serve to initiate homologous recombination by the Rad51/Rad52 pathway. Involvement of both the NHEJ and HR (Rad51/Rad52) pathways for the repair of Top1-mediated DNA damage in mammalian cells is supported by the hypersensitivity to camptothecin of cells deficient in these pathways (Tables 2–4, and references therein) and by the induction of HR repair by camptothecin in mammalian cells [128].

An alternative pathway is shown in panel (B), which is initiated by replication fork regression (RFR)

Table 3
Genetic alterations conferring hypersensitivity to topoisomerase I poisoning in budding yeast

Budding Yeast (YSC)			Function	Humans		
Gene	Effect	References		Gene	Effect	References
RAD52/51			homologous recombination (HR)			
RAD52 ^d	HS	[102,107,187,188,278,279]	Strand annealing	RAD52	?	
RAD51	HS	[102,107,188]	RecA homolog: strand invasion	RAD51C	HS	[274]
RAD55	HS	[102,107]	Strand annealing, exchange	XRCC2	HS	[124,272,273]
RAD57	HS	[102,107]	Strand annealing, exchange	XRCC3	HS	[272]
RAD54	HS	[102]	ATPase			
MMS1	S	[280]	Replication repair/epistatic Rad52			
RAD59	MS	[102]	Rad52-related recombination			
MRX (MRN)			3'-nuclease/checkpoint (HR + NHEJ)			
MRE11	HS	[95,102,187]	MRX/N complex; nuclease	MRE11	?	
RAD50	HS	[107,188]	MRX/N complex; scaffold	RAD50	?	
XRS2	HS	[107]	MRX/N complex; signaling	NBS1	HS	[156,185]
Mus81/Mms4 (Mus81/Eme1)			3'-flap endonuclease			
MUS81	MS	[102,103,107]	3'-Flap endonuclease with Mms4	MUS81	?	
MMS4	MS	[102,103]	Partner for Mus81 nuclease	EME1	?	
Tdp1-PNKP			3'-end processing			
TDP1	CS ^b	[95,102]	Tyrosyl-DNA phosphodiesterase	TDP1	?	
TPP1	CS ^{b,c}	[96,102]	Polynucleotide 3'-phosphatase	PNKP ^b	?	
APN1	CS ^{b,c}	[95,102,188]	AP endonuclease (endo IV family)			
APN2	CS ^{b,c}	[95,102]	AP endonuclease (exo III family)	APE1	?	
Rad1/Rad10 (XPF/ERCC1)			3'-endonuclease			
RAD1	CS ^b	[95,102,188]	3'-Flap endonuclease with Rad10	XPF	NS	
RAD10	CS ^b	[95,102]	Partner for Rad1	ERCC1	NS	
Rad27 (FEN1)			5'-endonuclease			
RAD27	MS	[102]	5'-Flap endonuclease	FEN1	?	
RecQ/Top3			helicases/topoisomerase			
SGS1	MS	[103,107]	Top3-associated helicase	WRN	HS	[231–233]
SRS2	MS	[102]	Rad51-associated helicase	BLM	HS	[234]
TOP3	S	[102,103]	Replication/recombination topoisomerase	TOP3 α		
TOP3 β	?					
9–1–1			("PCNA-like") clamp			
DDC1	MS	[102]	Replication/Repair clamp; "9–1–1"	RAD9	?	
RAD17	MS	[107,188,205]	Replication/Repair clamp; "9–1–1"	RAD1	?	
MEC3	MS	[107]	Replication/Repair clamp; "9–1–1"	HUS1	?	
RAD24	MS	[187]	Clamp loader for 9–1–1	RAD17	?	
Sensor PI(3)K-related protein kinases						
MEC1	HS	[187,188]	PI3LK checkpoint sensor kinase	ATR	HS	[200]
DDC2	?		Partner for MEC1	ATRIP	?	
TEL1	S	[187]	PI3LK checkpoint sensor kinase	ATM	HS	[155,157,158,271]
			PI3LK checkpoint sensor kinase	DNA-PK	HS	[39,272]
Transducer protein kinases; BRCT proteins						
RAD53	MS	[188]	Checkpoint effector kinase	CHK2	?	
RAD9	MS	[187,188]	Adaptor for checkpoint kinases	MDC1	?	
				BRCA1	HS	[244]

Table 3 (Continued)

Budding Yeast (YSC)			Function	Humans		
Gene	Effect	References		Gene	Effect	References
Replication						
CDC45	S	[281]	Initiation of DNA replication	CDC45L	?	
POL32	MS	[107]	Small subunit for Pol δ	TEX14	?	
TRF4	S	[253]	DNA polymerase	POLS	?	
DPB11	S	[281]	Replication initiation/checkpoint	TOPBP1	?	
RAD6	MS	[107,188,282]	Post-replication repair; Ub conjugate	RAD6A, B	?	
RAD18	S	[107,188]	Post-replication/repair; loads Rad6	RAD18	?	
Chromatin						
HTA1/2	S	[187]	Histone H2A	H2AX	S	[173]
HHF1/2	S	[255]	Histone H4	H4	?	
GCN5	S	[254]	Histone H3 acetyltransferase	PCAF	?	
YNG2	S	[254]	Histone H4 acetyltransferase	ING1-5	?	
ESA1	S	[255]	Histone H4 acetyltransferase	MYST1/HAT	?	
ASF1	S	[102]	Chromatin assembly	ASF1B	?	
MCD1	S	[253]	Chromatin cohesion	RAD21	?	
CTF4	MS	[102]	Chromatid cohesion and segregation	AND-1	?	
Transcription						
HPR1	S	[107]	Transcription and recombination	MGC5350	?	
SFP1	S	[107]	Transcription factor	REQ	?	
CCR4	S	[107]	Transcription	KIAA1194	?	
BUR2	S	[107]	Cyclin partner for Bur1	Cyclin H	?	
RPB9	S	[107]	RNA polymerase subunit	POLR21	?	
MPH1	MS	[107,282]	RNA helicase	MPH1	?	
Ubiquitin						
UBC9	S	[283]	Ubiquitin ligase	UBE2I	?	
DOA4	S	[283]	Ubiquitin hydrolase			

Abbreviations for effects: HS, S, MS, and CS correspond to hypersensitivity, sensitivity, moderate sensitivity, and conditional sensitivity, respectively.

^a The Rad52 epistasis group includes the RAD50, 51, 52, 54, 55, 57, 59, MRE11 and XRS2 genes.

^b Tdp1 deficiency results in HS only in the presence of Rad1/Rad10 deficiency [95,102]; conversely Rad1 deficiency does not confer hypersensitivity to CPT [103] unless the Tdp1–Apn1 pathway is defective [95]. Tpp1, Apn1 + Apn2 + Tpp1 need to be inactivated to confer full camptothecin hypersensitivity [96] (see Fig. 3A).

^c PNKP possesses both 3'-phosphatase and 5'-kinase activities, whereas the yeast ortholog, Tpp1 only possesses 3'-phosphatase activity. Neither Apn1, Apn2 or Tpp1 possess AP endonuclease activity [96].

[129,130]. During RFR, annealing of the newly replicated leading and lagging strands forms a DNA cruciform (four stranded junction), commonly referred to as a “chickenfoot” because of its morphology [130] (Fig. 6B). This reverse movement probably involves protein complexes promoting DNA strand exchange and annealing (duplex formation). Rad51, the eukaryotic equivalent of the bacterial RecA protein forms nucleoprotein filaments, and Rad52 promotes strand invasion and annealing between homologous DNA sequences [129,131–134]. BRCA2 (which is FANCD1 [135]) has recently been shown to promote Rad51 loading and HR [136,137]. Although positive

supercoiling ahead of the blocked replication fork could also promote branch migration [129,138], this mechanism appears uniquely unless the DNA fails to undergo free rotation at the Top1 break site. Once the DNA downstream from the Top1 cleavage complex has been reannealed, the Top1 cleavage complex could reverse without intervention of repair enzymes since the 5'-hydroxyl end of the DNA could be aligned with the Top1–DNA phosphotyrosyl bond (Fig. 6B). It is also plausible that Tdp1 could remove Top1 and that the resulting gap could be repaired by the BER pathway (see Section 4.3 above). Following the repair/removal of the Top1 cleavage complex, the fork

Table 4

Genetic alterations conferring hypersensitivity to topoisomerase I poisoning in fission yeast

Fission yeast (YSP)			Function	Humans		
Gene	Effect	References		Gene	Effect	References
Rhp54	HS	[105]	Homologous recombination (HR)	RAD52	?	
Rhp55	LS	[105]	Homologous recombination (HR)	XRCC2	HS	[124,272,273]
Rhp22A	LS	[105]	Homologous recombination (HR)	XRCC3	HS	[272]
Rhp51	MS	[105]	RecA homolog; Rad52 epistasis G	RAD51C	HS	[274]
Rad50	HS	[105]	MRX/N complex; scaffold	RAD50	?	
Mus81	HS	[105]	3'-Flap endonuclease with eme1	MUS81	?	
Eme1	HS	[105]	Partner for mus81 nuclease	MUS81	?	
RusA	Rs ^a	[105]	HJ resolvase			
Pnk1	S ^b	[97]	Polynucleotide kinase phosphatase	PNKP	?	
Rqh1	MS	[102]	Top3-associated helicase	WRN	HS	[231–233]
				BLM	HS	[234]
Chk1	MS	[284,285]	Checkpoint effector kinase	CHK1	?	
Swi1	HS	[286]	Mating-type switching	TIMELESS		

^a rusA suppresses hypersensitivity of *Mus81/Eme1*⁻ but does not reverse sensitivity of *rqh1*⁻; rusA also suppresses the lethality of double mutants for *Mus81/Eme1 + rqh1* [105]. RusA expressed in budding yeast partially suppresses hypersensitivity to CPT in Mms4-deficient cells [103].

^b *Pnk1*⁻ cells are hypersensitive to CPT in the absence of additional defects, indicating difference from budding (see footnote 'a') and importance of this pathway in fission yeast, which like mammals possesses a gene that has both 3'-phosphatase and 5'-kinase activity [97].

would restart after unwinding of the cruciform. This unwinding could be carried out by the RecQ helicases BLM (Bloom) and WRN (Werner). In the absence of these helicases, "chickenfoot" structures would need to be resolved by recombination, which might explain the high frequency of sister chromatid exchanges in Bloom syndrome (BLM) cells (for recent review see [139]).

5. Molecular pathways implicated in the cellular responses to Top1 cleavage complexes; determinants of response and resistance with potential clinical relevance

Cellular responses to Top1 poisons determine both tumor response and host toxicity. Efficient repair (see Section 4) is probably coupled with checkpoint activation. Cell cycle arrest would have two beneficial consequences: (1) it would give time for the repair of DNA damage; and (2) it would prevent further replication-dependent DNA damage. Both the S-phase and the G2 checkpoints, as well as the p53/p21 pathways are activated by Top1-mediated DNA damage

[49,75]. Because cell cycle checkpoints are connected to the apoptosis machinery, it is likely that extensive DNA damage activates apoptosis by involving the same DNA damage sensors and checkpoints [140]. Thus, an exciting challenge is to elucidate the relationships between sensor proteins, checkpoints, DNA repair and apoptosis. Integration of these pathways should explain the cellular determinants of response to Top1 poisons. The following section will review some of the cellular pathways/response elicited by Top1 poisons, and how defects in these pathways can sensitize tumors to camptothecins. Details on the roles of Chk2, c-Abl, and the stress kinase (JNK/SAPK) pathways can be found in a recent review [140]. Fig. 7 shows a schematic flowchart diagram for some of the checkpoint pathways activated by Top1-mediated DNA damage.

5.1. Ubiquitination, sumoylation and proteolysis of Top1

Top1 is rapidly degraded in normal peripheral-blood mononuclear cells [141–143] and some cancer cell lines [92,93,144] exposed to camptothecins. Top1

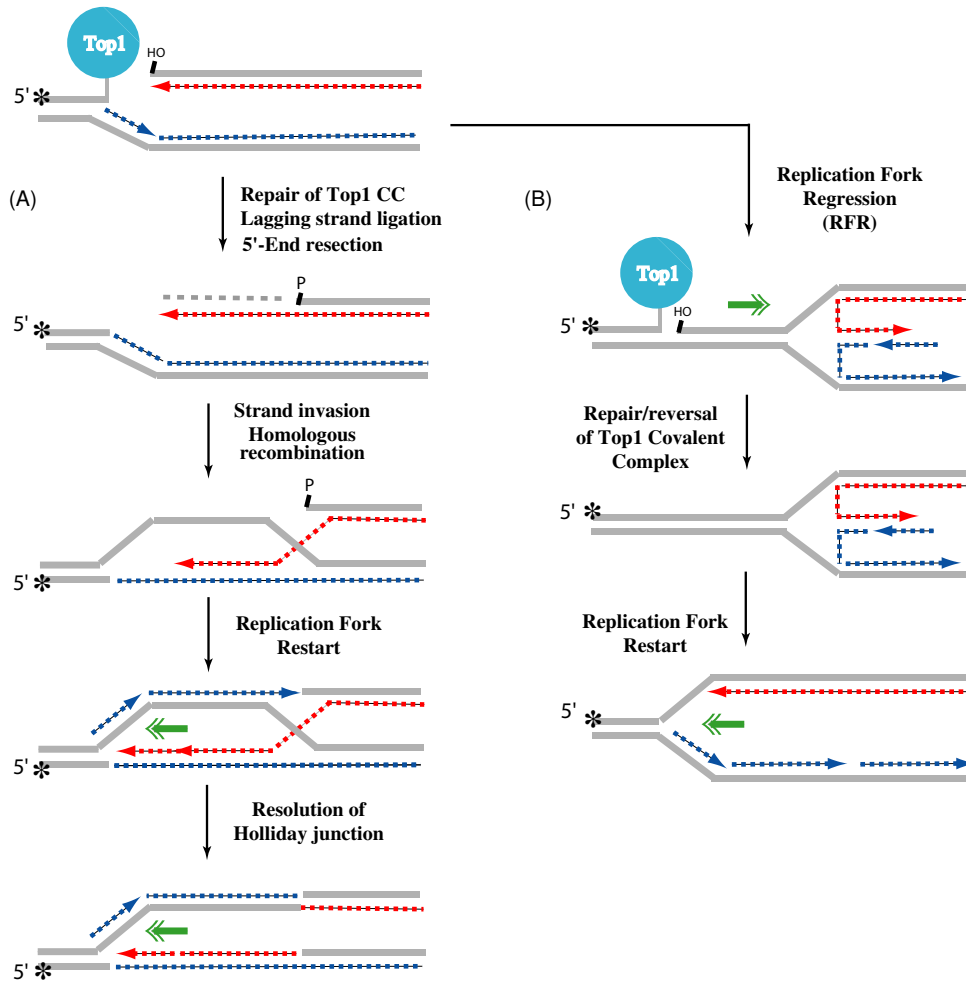


Fig. 6. Schematic representation of the proposed repair of Top1-mediated DNA replication-induced DSBs. (A) The Top1–DNA covalent complex is removed and the lagging strand ligated to restore one duplex. The 5′-end is first digested, leading to the formation of a 3′-single-stranded DNA segment that can act as a substrate for homologous recombination. (B) Replication fork regression allows the normal religation of the Top1 cleavage complex and lead to the formation of a “chicken foot”, which is equivalent of a Holliday junction. Replication fork restart requires melting of the “chicken foot” or resolution of the corresponding Holliday junction by the RecQ helicase/Top3 pathway (see Section 5.5).

degradation is deficient in some leukemiae [143,145] and following oncogenic transformation [142,143], suggesting that lack of Top1 degradation contributes to the selectivity of camptothecins for tumors. Top1 degradation is a response to transcription blocks and is replication independent [143,146]. It is abolished by inhibitors of the 26S proteasome, and ubiquitin–Top1 conjugates have been detected in cells treated with camptothecin, suggesting that Top1

is degraded by the ubiquitin/26S-proteasome pathway [93]. Degradation is nuclear [144] and specific for the hyperphosphorylated forms of Top1 that are associated with transcription [147], suggesting that collisions between RNA polymerase II complexes and Top1 cleavage complexes (see Fig. 1B) trigger Top1 ubiquitination [148] and subsequent degradation of Top1 by the 26S proteasome [143].

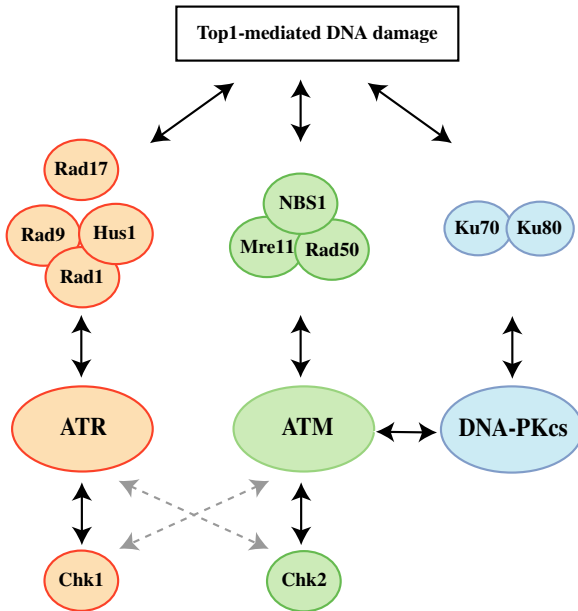


Fig. 7. Checkpoint pathways induced by Top1-mediated DNA damage. Sensor protein complexes that bind to damaged DNA are at the top. Sensor PI(3)kinases, ATM, ATR, and DNA-PK are in the middle. The effector kinases Chk1 and Chk2 are shown downstream from the PI(3)kinases.

Top1 degradation may serve two purposes: (1) confer cellular tolerance to camptothecin and protect normal cells; and (2) prepare for the excision of the Top1-covalent complexes by Tdp1, as Tdp1 requires Top1 to be proteolyzed for hydrolyzing the Top1–DNA bond [83,90] (see Section 4.1 and Fig. 2). Top1 down-regulation is correlated with camptothecin resistance in cell lines [93], and prevention of Top1 degradation by the 26S proteasome inhibitor MG132 enhances camptothecin-induced apoptosis [142]. Accordingly, synergy was recently reported between camptothecins and the clinically used proteasome inhibitor PS-341 [149].

Camptothecins also induce small ubiquitin-like modifier (SUMO-1; also SUMO-2/3) conjugation to Top1 [148,150,151]. Sumoylation is an early and transient response to camptothecin. Human small ubiquitin-like modifier, also named Ubl1, PIC1, GMP1, SMT3, or sentrin is an 11 kDa protein with 18% sequence similarity to ubiquitin. Sumoylation mimics the classical ubiquitination pathway. The first step is activation of SUMO, the second, transfer of

SUMO to the conjugation enzyme, and the last step, ligation of SUMO to its target protein (for review see [152]). Sumoylation employs a distinct set of E1, E2, E3 and protease enzymes. Ubc9 is the only E2 enzyme identified for SUMO-1 whereas a dozen E2 enzymes have been identified for ubiquitin in yeast. Top1 sumoylation shares some characteristics with ubiquitination. Both modifications take place at lysine residues (K117 and K153 for sumoylation of human Top1 [148]) and are independent of DNA replication [93,150]. However, they appear to differ in the following ways: (1) mutation of the K117 and K153 residues abrogates sumoylation without affecting ubiquitination [148]; (2) sumoylation is specific for dephosphorylated Top1 [146]; (3) it is effective in both normal and tumor cells [150]; (4) it is not linked to Top1 degradation; and (5) Top1 sumoylation is markedly enhanced independently of camptothecin treatment in cells expressing a catalytically inactive Top1 (the Y723F) [148]. Top1 sumoylation may competitively inhibit Top1 ubiquitination and degradation since the same lysine residues are used for both modifications. Top1 sumoylation may also modulate the cellular location, function [151] and/or enhance the activity of Top1 [148]. Thus, it is tempting to propose that ubiquitination and sumoylation have opposite effects: sumoylation by activating Top1 (via cellular relocation), and ubiquitination by inactivating Top1 (via proteolysis). Consistently, Top1 sumoylation has been proposed to enhance camptothecin-induced apoptosis [148]. However, Ubc9-defective yeast cells are hypersensitive to camptothecin suggesting that sumoylation of downstream target from Top1 also contribute to the cellular responses to Top1-mediated DNA lesions [150].

5.2. The ataxia telangiectasia mutated (ATM), Mre11/Rad50/Nbs1 and Chk2 pathways

The ataxia telangiectasia mutated (ATM) gene product is a central component of the DSB checkpoint pathways [153]. ATM is activated by autophosphorylation and inhibition of dimerization in response to chromatin modifications [154]. Cells from patients with ataxia telangiectasia (AT) are characterized by their failure to arrest DNA replication in response to DNA damage (“radioresistant DNA synthesis” (RDS) phenotype). AT cells are highly sensitive to camptothecin

[155,156] (Table 2). Increased sensitivity to camptothecin is also observed in cells deficient for the ATM ortholog in Chinese hamster [157,158] and in yeast (Tel1, Table 3).

The importance of ATM stems from the fact that it regulates most of the checkpoint and repair pathways. ATM phosphorylates p53 [159–161], Chk2 [162], Nbs1 [163–166], BRCA1 [167], 53BP1 [168], and histone H2AX [169] (for review see [153]). After phosphorylation/activation of ATM, many checkpoint proteins, including Nbs1, Mre11, BRCA1, and 53BP1 co-localize in nuclear foci following ionizing irradiation [170] and cooperate in the ionizing radiation-induced S-phase checkpoint [171]. AT cells are also deficient in activating NF- κ B following camptothecin treatment [172], suggesting that multiple ATM-dependent pathways are implicated in the cellular response to camptothecin. However, AT cells are not deficient in H2AX phosphorylation in response to camptothecin [173].

Mutations of the *NBS1* gene (mutated in Nijmegen breakage syndrome) result in an AT-related phenotype with radio-resistant DNA synthesis [174]. The Nbs1 gene product functions as a heterotrimer with the Mre11 and Rad50 gene products (MRN complex) [175], which forms foci at double-strand break sites [174], probably in association with other proteins including mismatch repair factors (MSH2, MSH6, MLH1), BRCA1, the Bloom syndrome protein, replication factor C (RFC) and ATM [176,177]. These large protein complexes have been named BRCA1-associated genome surveillance complexes (BASC) [177]. MRN also forms nuclear foci with H2AX in response to camptothecin-induced replication-mediated DSBs [173].

As described in Section 4.2, the MRN complex possesses a nuclease activity and could process the DNA ends for repair/recombination reactions [178,179]. The link between the MRN complex and the S-phase checkpoint pathway was recently strengthened by the finding that an AT-like disorder (ATLD) (including radioresistant DNA synthesis) is caused by mutations in the Mre11 gene [180]. Because the DNA binding of the Mre11 complex does not require ATM [165,181], it seems plausible that binding of the MRN complex to DSB activates and possibly recruits ATM, which could then phosphorylate Nbs1 [163,164,166,182,183], and activates the S-phase checkpoint [163,184]. These ob-

servations suggest the existence of a regulatory loop between the MRN complex, ATM, and the S-phase checkpoint.

AT cells [155,156] and NBS cells [156,185] are hypersensitive to camptothecin (Tables 2 and 3), indicating the importance of the MRN-ATM pathway for cellular response to camptothecin. Furthermore, camptothecin treatment induces phosphorylation of Nbs1 and BRCA1 [186]. This pathway is conserved in budding yeast, as mutations for the *TEL1* (ATM homolog) [187], *MRE11* [95,102,187], *RAD50* [107,188], or *XRS2* (Nbs1 homolog) [107] genes confer camptothecin hypersensitivity (Table 3).

5.3. The RPA and Ku-DNA-PKcs pathways

Camptothecin-induced replication-mediated DSB induce phosphorylation of the middle-size subunits of the human single-strand DNA binding protein (RPA2) by DNA-dependent protein kinase (DNA-PK) [39]. Like ATM, the catalytic subunit of DNA-PK (DNA-PKcs) belongs to the PI(3)kinase family. DNA-PKcs functions with the heterodimer of Ku proteins (Ku70/80) that bind to the ends of the DSB and activate the kinase activity of DNA-PKcs. DNA-PKcs-deficient cells (human glioblastoma MO-59-J cells [39] and neurons from SCID mice [189]) cells are hypersensitive to camptothecin (see Table 2). Moreover, the MO-59-J cells are defective in DNA synthesis inhibition following camptothecin treatment [39], suggesting that DNA-PK regulates the S-phase checkpoint. RPA2 has been proposed as one of the effectors in this pathway [39]. Although the exact roles of RPA2 phosphorylation remain to be elucidated, RPA2 is essential for stabilizing single-stranded DNA during replication, repair, and homologous recombinations [134]. An intriguing observation is that the cell cycle checkpoint abrogator UCN-01 inhibits RPA2 phosphorylation by acting upstream from DNA-PK [39]. Based on the recent findings that UCN-01 inhibits both Chk1 [50,51] and Chk2 [52], it is possible that “cross-talks” exist between the Chk1/Chk2 and DNA-PK pathways (Fig. 7). Furthermore, “cross-talks” probably exist between the ATM and DNA-PK pathways since ATM can be directly activated by DNA-PK [190].

5.4. The ATR-ATRIP, hRad9–hHus1–hRad1 (9–1–1), and Chk1 pathways

RPA is also required for activation of the Rad17, hRad9–hHus1–hRad1 (9–1–1), ATR pathway [191] and for ATR-dependent S-phase checkpoint activation [192].

ATR (ataxia telangiectasia and Rad 3-related) functions in the S-phase checkpoint probably in connection with the 9–1–1 complex (for review see [193]). ATR is with ATM and DNA–PKcs, a member of the PI(3)kinase family. By contrast to ATM, ATR is an essential gene [194], and ATR-deficient cells accumulate large numbers of replication-associated DNA breaks [195]. Recently, splicing mutations affecting expression of ATR have been shown to result in Seckel syndrome, which shares similarities with Nijmegen breakage and ligase IV syndromes [196]. ATR functions in close physical and functional association with ATR interacting protein (ATRIP), the ortholog of the yeast checkpoint genes Rad26 (fission yeast) and DDC2 (budding yeast) (see Table 3) [197]. Although the three PI(3)kinase pathways (ATM, ATR, DNA–PK) exhibit some degree of redundancy, ATM and DNA–PK are primarily activated by DSBs, whereas ATR–ATRIP are more specifically activated by replication- and UV-mediated DNA damage. The ATM and ATR pathways also have differential substrate specificity. ATM preferentially activates Chk2, whereas ATR preferentially activates Chk1 [193,198] (Fig. 7). Recent studies demonstrate that in ATR-kinase dominant-negative cells (ATR-kinase dead; ATR/kd) [199], phosphorylation of Chk1 in response to Top1 poisons is not observed, both S-phase and G2 checkpoints are abrogated, and the cytotoxicity of topotecan is enhanced [200] (Table 3). Thus, it is likely that ATR is critically involved in S-phase checkpoint activation in response to Top1-mediated DNA damage. ATR could exert its S-phase and G2-checkpoint functions by activating Chk1, which in turn phosphorylates and promotes the degradation of Cdc25A in response to camptothecin [198]. ATR also controls H2AX phosphorylation and the recruitment of the MRN complex to the damaged replication sites in response to camptothecin [173] (see Section 5.7).

The ATR, 9–1–1, and Chk1 pathways are probably closely connected (Fig. 7) because, in fission yeast, Rad1, Hus1, and Rad9 are essential for

Chk1 activation [201–203], and in human cells, the ATR-associated protein (ATRIP) is required for phosphorylation of hRad17 in response to DNA damage [197].

In humans and fission yeast, the group of checkpoint proteins, Hus1, Rad1, Rad9, and Rad17 are required to block entry into mitosis when DNA replication is inhibited or in the presence of damaged DNA (for review see [193,204]). The budding yeast orthologs are Mec3, Rad17 and Ddc1 for Hus1, Rad1 and Rad9, respectively (Table 3), indicating the conservation of the DNA integrity/checkpoint pathways from yeasts to humans. The budding yeast ortholog for Rad17 is Rad24, and strains defective for these genes are hypersensitive to camptothecin (Table 3) [102,107,187, 188,205].

Hus1 interacts with Rad1 and Rad9 [206–209]. In human cells, the “9–1–1” complex [210] interacts with hRad17 [211] and proliferating cell nuclear antigen (PCNA) [212]. Human Rad17 is homologous to RFC1 (the largest subunit of the pentameric Replication Factor C) and Hus1, Rad1 and Rad9 are structurally related to PCNA [213], suggesting mechanistic similarities between the 9–1–1/Rad17 pathway and components of the normal replicative DNA polymerase complex. Rad17 in a complex with RFC2–5 (equivalent of clamp loader RFC) and the 9–1–1 complex could act as a sliding clamp for DNA polymerase (~PCNA) [213,214]. A recent study suggests that translesion DNA polymerases such as Pol zeta and Din B may be recruited by the 9.1.1 complex [215]. It is therefore assumed that Rad17 and the 9–1–1 complex act as sensors for DNA damage and that Rad17 loads the 9–1–1 complex onto damaged DNA at arrested replication forks [192,193]. Recently, Rad17 was found to be an essential gene controlling replication [216].

In camptothecin-treated cells, Hus1 and Rad1 become hyperphosphorylated, and Rad9 becomes firmly anchored to nuclear components in association with Hus1 and the hyperphosphorylated form of Rad1 [217]. Hus1 is an essential gene whose inactivation results in genomic instability and massive apoptosis in mice [218]. p21 inactivation is required for viability of Hus1-deficient cells, and *Hus1*–/–*p21*–/– cells display a unique sensitivity to hydroxyurea and UV, but only slightly increased sensitivity to ionizing radiation [218].

5.5. The RecQ (Bloom and Werner syndrome) pathways

The RecQ pathway, in association with Top3 [219], is important for: (1) faithful chromosome segregation during anaphase [220]; (2) meiotic recombinations [221]; (3) possibly unwinding replicating DNA [222]; (4) resolution of stalled replication forks [223]; and (5) replication forks restart after their collapse [224,225] (resolution of Holliday junctions in Fig. 6 B and C) [224,225].

The RecQ pathway is highly conserved. The *E. coli* ortholog is RecQ, and the yeast orthologs are Sgs1 in budding yeast (Table 3), and Rqh1 in fission yeast (Table 4). Slow growth suppressor 1 (Sgs1) was identified as a suppressor of the slow growth phenotype of Top3 mutants [221]. Sgs1 mutants exhibit hyper-recombination and defects in chromosome segregation [226]. Sgs1 interacts with both Top2 and Top3 [226–228]. There are 5 RecQ orthologs in humans. Mutations in 3 of them, BLM, WRN and RecQ4 lead to human diseases (Bloom, Werner and Rothmund–Thompson syndromes, respectively [229]) characterized by premature ageing and increased cancer incidence. Although BLM, WRN and Sgs1 proteins are similar in length, and *sgs1* mutant can be partially rescued by BLM and WRN [230], these three proteins share little homology outside their helicase domain [230]. By contrast to BLM, WRN cells do not show increased sister chromatid exchanges.

WRN [231–233] and BLM [234] cells (Table 2), and yeast cells deficient for Sgs1 [102,103,107] (Table 3) or Rqh1 [102] (Table 4) are hypersensitive to camptothecin. WRN protein forms distinct nuclear foci in response to replication-mediated DNA damage induced by camptothecin [235]. These WRN foci co-localize with RPA and with Rad51 foci partially, implying cooperative functions between the RecQ/Top3 pathway and the homologous recombination pathways in response to Top1-mediated DNA damage [235] (see Fig. 6). WRN also binds to Ku70/80, which stimulate its exonuclease activity [236,237], suggesting a possible regulatory function on the NHEJ pathway as well.

Crosstalk exists between the RecQ and the ATR and ATM pathways. Phosphorylation of BLM by ATR is required for formation of MRN foci in the presence of stalled replication forks [238,239]. Phosphorylation of

BLM by ATM at T99 is also required for the cellular response to DNA damage [240]. The known camptothecin sensitivity of both AT and BLM-defective cells makes it important to investigate the connections between ATR, ATM and BLM in response to Top1-mediated DNA damage.

5.6. The p53, BRCA1 and Fanconi anemia (FA) pathways

Although mutations in the p53 pathway are the most common defects in human cancers, p53-deficiencies do not translate into hypersensitivity to camptothecin in cultured cancer cells [241]. However, transfection of the E6 papilloma virus ubiquitin ligase, which degrades p53, sensitizes both colon and breast human carcinoma cells to camptothecin [242]. Camptothecin-induced p53 elevation is replication-dependent [75] and, by contrast to ionizing-radiation-induced p53 elevation, is preserved in AT cells [243], indicating that this p53 response is independent of ATM. Because of the diversity of the p53 downstream targets that either induce apoptosis or cell cycle arrest or enhance DNA repair [140], it is likely that the outcome of p53 deficiencies is conditional on the cellular context.

BRCA1- and BRCA2-deficient cells are hypersensitive to camptothecins [244,245], which is logical considering the key roles of BRCA1 and BRCA2 in DNA repair, HR (see Section 4.4), checkpoint response [132], and genomic stability. BRCA1 is probably one of the human functional analogs of Rad9 in budding yeast. As for BRCA1, Rad9 mutants are hypersensitive to camptothecin [187,188] (see Table 3). The other Rad9 human functional analogs include MDC1, 53BP1, and Nbs1. These BRCT-containing proteins may serve to present potential substrates for the checkpoint PI(3)kinases, ATM and ATR. BRCA1 is connected to the Fanconi anemia (FA) pathway. BRCA1 serves as a mono-ubiquitin ligase for one of the Fanconi proteins, FANCD2 [246]. BRCA2 was also recently identified as another Fanconi protein, FANCD1 [132,135]. Thus, the BRCA and FANCD pathways are closely connected.

The sensitivity of Fanconi anemia cells to camptothecin is controversial. Saito and coworkers found that FA cells are hypersensitive to camptothecin while their Top1 gene is normal [247]. By contrast, two

independent studies found no difference in sensitivity to camptothecin [248,249]. Discrepancies might be due to the existence of 7 complementation groups for Fanconi anemia [132], and to the fact that the cell lines previously used belonged to different complementation groups [250].

5.7. The chromatin remodeling pathways (CSA/CSB, γ -H2AX, histone acetylation)

Recently, chromatin changes and histone modifications have been shown to contribute to DNA repair and cell cycle checkpoint responses. Tables 2–4 list chromatin modifications that sensitize to camptothecin.

It has been known for some time that Top1 cleavage complexes induced by camptothecin induce the disassembly of nucleosomes, resulting in DNA relaxation [65,251]. Cockayne syndrome B (CSB) acts as a chromatin remodeling factor [252]. CSB cells are hypersensitive to camptothecin (Table 1) and accumulate abnormally high levels of DSBs in nascent DNA [43]. Yeast mutants defective for chromatin assembly and cohesion (TRF4, MCD1/SCC1, CTF4) are also hypersensitive to camptothecin [102,253], indicating the importance of chromatin remodeling for the repair of Top1-mediated DNA lesions.

The basic structural chromatin unit is the nucleosome, consisting of 150 bp of DNA wrapped around the histone octamer. Ser139-phosphorylated histone H2AX (referred to as γ -H2AX) is rapidly accumulated in response to DSBs [169], including those generated by Top1 cleavage complexes in replicating DNA [173]. γ -H2AX could alter chromatin structure to allow access to DNA repair factors, and it could function in checkpoint activation in association with other proteins that co-localize in nuclear foci, such as the MRN complex, BRCA1, and BLM [169]. Cells from H2AX knockout mice are hypersensitive to camptothecin [173,187] and fail to form MRN foci in response to camptothecin [173]. The H2AX kinases in response to replication-mediated DSBs are primarily ATR and DNA-PK, whereas ATM is primarily involved in γ -H2AX formation in non-replicating DNA [173]. Thus, γ -H2AX might link chromatin structure and PI(3)kinase checkpoint pathways in mammalian cells.

Histone acetylation facilitates chromatin opening and transcription. Deficiencies in histone H3 and H4

acetylation in GCN5 and ESA1 mutants, respectively, and in ASF1 mutants sensitize yeast cells to S-phase genotoxic agents including camptothecin [102,254] (Table 3). Similarly, mutations in wild-type H4 acetylation sites shows camptothecin hypersensitivity, defects in NHEJ repair and in replication-coupled repair. Both pathways require the ESA1 histone acetyl transferase (HAT), which is responsible for acetylating H4 tail N-terminal lysines, including ectopic lysines that restore repair capacity to a mutant H4 tail [255], suggesting a role for histone acetylation in DNA replication, repair, recombination, and genomic integrity during replication. These observations are relevant to the fact that histone acetylation modifiers are in clinical trials and will be tested in association with camptothecins.

6. Summary and conclusions

The repair of Top1-mediated DNA damage involves multiple apparently redundant pathways (see Fig. 2). Excision of the Top1–DNA adducts can be effected by at least 4 pathways including the phosphodiesterase phosphatase Tdp1/PNKP pathway (Section 4.1), and the three recently identified 3'-flap endonuclease pathways (Rad1/Rad10, Mre11/Rad50, Mus81/Mms4) (Section 4.2). The DNA breaks associated with Top1 covalent complexes can be repaired by at least 4 other pathways including the XRCC1 (BER) pathway (Section 4.3), the two DSB pathways (NHEJ and HR) (Sections 4.4 and 5.3), and the RecQ helicase/Top3 pathway (Section 5.5). Recent evidence suggests that HR repair seems to be more important than NHEJ for the repair of replication fork-associated DSBs induced by Top1 cleavage complexes [256]. Two additional mechanisms have been demonstrated in vitro. First, Top1 cleavage complexes can be reversed by illegitimate recombinogenic repair, whereby Top1 at a DNA break transfers the covalently held strand to a 5'-hydroxyl DNA end other than the original strand at the break. Such reactions have been extensively studied in vitro for the mammalian [257] and the vaccinia Top1 enzymes. Their recombinogenic potential might explain the mutagenicity of Top1 cleavage complexes and camptothecins. Second, it has been shown that human [258] and vaccinia [259] Top1s can catalyze autocleavage of the covalent adduct in the presence

of a suitably active nucleophile such as hydrogen peroxide, which can be produced in vivo by some cells during stress. Although there is in vivo data on this mechanism, it is worth mentioning the intrinsic repair capacity of Top1. The redundancy for the repair pathways involved in the processing of Top1 cleavage complexes might only be apparent because it is likely that each pathway functions preferentially for certain types of lesions generated under specific conditions. For instance transcription-mediated lesions (Fig. 1B) and base mismatches (Fig. 1F) might be repaired primarily by the XRCC1 pathway, whereas replication-mediated DSBs (Fig. 1C) might rely on the Mus81/Mms4, RecQ helicases/Top3, and HR pathways. It is also likely that more than one pathway can repair the same lesions. Such redundancy (see Section 4.2 and Fig. 2) should provide biological robustness, i.e. in the absence of one pathway, another could remove the top1 covalent complexes. The conservation of the repair pathways from humans to yeasts supports the view that endogenous Top1-mediated DNA lesions occur under physiological conditions (see Table 1).

The conditional sensitivity of Tdp1-deficient yeasts in the context of either Rad9 or Rad1/Rad10 deficiencies (Section 4.2 and Fig. 4A), suggests that it might be possible to develop selective therapeutic strategies against specific tumors. A majority of tumors are deficient for DNA repair (for instance, mismatch repair) and/or for cell cycle checkpoint proteins (such as p53, pRb, BRCA1, FANC). Thus, Tdp1 inhibitors may selectively potentiate camptothecins in XPF/ERCC1-deficient cells and in tumors deficient for BRCA1, MDC1, TOPBP1, 53BP1, or Nbs1 (the human Rad9 orthologs). It is rather likely that the relative selectivity of camptothecins (and other cytotoxic DNA damaging agents) for cancers is due to preexisting deficiencies (in DNA repair or/and cell cycle checkpoint or/and survival pathways), which by themselves provide selective growth advantages for tumor development.

Camptothecins are clearly active in a subset of patients. They are currently prescribed based on the histological origin of the tumors (colon, ovarian, lung, ...) irrespective of their individual molecular profile. Genetic defects promoting tumor development are likely to determine the cancer sensitivity to cytotoxic agents, such as Top1 inhibitors. Diagnosing such

defects and relating them to specific drug responses should provide the rationale for using cytotoxic drugs in individual patients. Thus, it remains critical to further characterize which molecular defects selectively sensitize cells to camptothecins and to the novel Top1 inhibitors presently in development, and to further define the molecular profiling of tumors.

Yeast is a powerful tool to elucidate molecular pathways, and the viability of Top1-deficient cells in yeast can be used to validate Top1 as the primary target for the pathways elicited by camptothecin or other potential novel Top1 poisons. Most current studies are being performed in budding yeast (Table 3). Additional studies in fission yeast, which until now has been less used for camptothecin studies, should provide further insights into the cellular response pathways to Top1-mediated DNA damage (Table 4). Finally, the availability of murine knockout models, human cell lines from patients with rare genetic diseases, and knockdown cells (by siRNA) will undoubtedly provide opportunities for rationale drug use and design based on the presence of specific molecular defects in the target cancer cells.

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