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Debulking of topoisomerase DNA-protein crosslinks (TOP-DPC) by the proteasome, non-proteasomal and non-proteolytic pathways

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Abstract

Topoisomerases play a pivotal role in ensuring DNA metabolisms during replication, transcription and chromosomal segregation. To manage DNA topology, topoisomerases generate break(s) in the DNA backbone by forming transient enzyme-DNA cleavage complexes (TOPcc) with phosphotyrosyl linkages between DNA ends and topoisomerase catalytic tyrosyl residues. Topoisomerases have been identified as the cellular targets of a variety of anti-cancer drugs (*e.g.* topotecan, irinotecan, etoposide and doxorubicin, and antibiotics (*e.g.* ciprofloxacin and levofloxacin). These drugs, as well as other exogenous and endogenous agents, convert the transient TOPcc into persistent TOPcc, which we refer to as topoisomerase DNA-protein crosslinks (TOP-DPC) that challenge genome integrity and lead to cell death if left unrepaired. Proteolysis of the bulky protein component of TOP-DPC (debulking) is a poorly understood repair process employed across eukaryotes. TOP-DPC proteolysis can be achieved either by the ubiquitin-proteasome pathway (UPP) or by non-proteasomal proteases, which are typified by the metalloprotease SPRTN/WSS1. Debulking of TOP-DPC exposes the phosphotyrosyl bonds, hence enables tyrosyl-DNA phosphodiesterases (TDP1 and TDP2) to access and cleave the bonds. In this review, we focus on current knowledge of the protease pathways for debulking TOP-DPC and highlighting recent advances in understanding the mechanisms regulating the proteolytic repair pathways. We also discuss the avenues that are being exploited to target the proteolytic repair pathways for improving the clinical outcome of topoisomerase inhibitors.

Keywords

Topoisomerases; DNA-protein crosslinks; Ubiquitin-proteasome pathway; SPRTN; SUMOylation

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

1. Introduction

DPC and especially those generated by topoisomerases (the TOP-DPC discussed in this review) are a current focus of scientific research due to their prevalence as environmental DNA damage and source of human diseases including cancers and neurodegenerative disorders. They are also a fundamental molecular mechanism for widely used antibacterial and anticancer therapies, which include not only topoisomerase inhibitors (fluoroquinolones, camptothecins, etoposide, doxorubicin to name a few) but also for DNA alkylating agents, platinum derivatives and 5-azacytidine derivatives.

The excision repair of TOP-DPC can be divided in three successive processes (outlined in red in Fig. 1): detection, debulking and excision. The debulking is generally carried out by proteolytic digestion leading to topoisomerase polypeptides remaining covalently linked to the DNA terminus (5'-DpC or 3'-DpC). This is the focus of our review (Sections 4 and 5). An alternative cellular topoisomerase unfolding pathway has recently been discovered and will be discussed in Section 6. The excision pathways for TOP-DpC (DNA-peptide-crosslinks) and non-proteolyzed TOP-DPC by nucleases have been detailed in a complementary review [1] and will only be discussed in Section 9. Both DNA Repair reviews are complementary.

2. DNA-protein crosslinks (DPC) classification

Covalent attachments of proteins to DNA are among the most frequent and potentially detrimental DNA modifications. DPC represent steric blocks for DNA transactions such as replication and transcription and their translocating helicases and polymerases. In addition, TOP-DPC are always associated with DNA strand breaks, a significant proportion of which are DNA double-strand breaks (DSB) that are also among the most detrimental DNA damaging lesions.

DPC have been classified in two main groups: chemical crosslinks and enzymatic crosslinks [2,3] and in four types based on the mechanisms of their formation and on their chemical structure [4]. Type 1 DPC are not associated with DNA breaks and are both chemical and enzymatic (Fig. 2). They are typically produced by chemical bridges between a base and a nearby chromatin protein as in the case of formaldehyde, acetaldehyde, 1–3 butadiene and heavy metals (chromium and platinum-based chemotherapies including cisplatin, carboplatin or oxaliplatin) [5]. They are also produced enzymatically by DNA incorporation of the anticancer drug 5-azadeoxycytidine, which forms DNA methyltransferase [DNMT] crosslinks. The other DPC are "terminal DPC" in which the DPC is at the end of a DNA break. Type 2 DPC are initiated by a break produced by DNA repair. A typical example is the crosslinking of DNA polymerase β (POLB) at the 5'-end of a break made by AP endonuclease during base excision repair (AP-lyase activity toward 5'-deoxyribose phosphates [5'-dRP]) [6] or the 3'-DPC made by PARP1 [7] (reviewed in [5]). Type 3 DPC are formed by the covalent attachment of type IB topoisomerases (tyrosyl catalytic residues for TOP1 or TOP1MT) or as repair intermediate with the covalent attachment tyrosyl-phosphodiesterase 1 (histidine catalytic residue for TDP1) to the 3'-phosphodiester-end of the broken DNA. Type 4 DPC are formed by covalent attachment of a type IA or

IIA topoisomerase (tyrosyl catalytic residues for TOP2α, TOP2β, TOP3α, TOP3β) to the 5'-end of the broken DNA or by the physiological action of the type VI topoisomerase and meiotic recombinase SPO11 as it breaks DNA to form double-strand breaks with its catalytic tyrosine covalently attached to the 5'-end of the DNA by tyrosyl-phosphodiester linkage (Fig. 1d and e).

Hence, DPC can be classified based on 4 criteria: 1/ whether they are chemical, which can be either direct with a bond between the DNA and the protein as in the case of 5-azadeoxycytine crosslinks to DNMT or indirect through chemical bridges (heavy metal in the case of chromium or cisplatin) [5]; 2/ whether they are enzymatic (Fig. 2) and associated with a break in the DNA backbone (*i.e.* protein-associated strand breaks [PASB]) as in the case of POLB, topoisomerases and TDP1; 3/ whether they are self-reversible as in the case of POLB, topoisomerases and TDP1 because they represent transient catalytic intermediates; and 4/ whether they are intrinsically irreversible as in the case of the 5-azacytosine-DNMT and SPO11 DPC.

3. Topoisomerase DNA-protein crosslinks (TOP-DPC)

All living cells possess topoisomerases and humans have 6 enzymes encoded by different genes: TOP1, TOP1MT, TOP2A, TOP2B, TOP3A and TOP3B. Topoisomerases have been referred to as the "magicians of the DNA world" [8,9] because they change the topology of the genome without affecting the DNA sequence and without requiring the assistance of protein cofactor [10].

The multiple topological manipulations carried out by topoisomerases include the relaxation of DNA to avoid supercoiling with the generation of plectonemic and toroidal DNA structures, as well as the removal and introduction of knots and catenanes. The DNA relaxation function is common to all topoisomerases and is critical during DNA replication and transcription to avoid both DNA overwinding (positive supercoils, which antagonizes DNA strand separation) ahead of translocating helicase complexes and DNA underwinding (negative supercoils promoting alternate DNA structures including R-loops, guanosine quartets, cruciforms and z-DNA) in the wake of translocating helicases complexes (TOP1 and TOP2 enzymes). The DNA decatenation function is essential following replication as the daughter DNA molecules are intertwined because of the helical structure of the DNA (TOP2 and TOP3 enzymes). The biological function of knots remains hypothetical [10,11].

Topoisomerases form TOP-DPC to break the DNA backbone and carry out their functions. Hence, TOP-DPC have the following characteristics [10]:

i. Each DPC is coupled with a DNA break generated by the formation of a topoisomerase tyrosyl-phosphodiester bond at one end of the break (Fig. 2); the polarity of the covalent linkage is a characteristic of the enzyme class: 5'-linkage for the type IA (TOP3α, TOP3β) and type IIA enzymes TOP2α and TOP2 β) and 3'-linkage for the type IB enzymes (TOP1 and TOP1mt). Hence TOP-DPC are protein-linked DNA breaks (PLDB) [also referred to as PASB (protein-associated strand breaks)]. We can also refer to them as terminal breaks,

which are 3'-DPC for TOP1 and TOP1MT, and 5'-DPC for TOP2α, TOP3β, TOP3α, TOP3β and SPO11 (Fig. 2c–e)

ii. A significant fraction of the TOP-DPC breaks are double-strand breaks (DSB) (reviewed in [10]) because the topoisomerases form dimeric complexes and cleave the opposite DNA strands in concert in the case of the type IIA enzymes (TOP2α and TOP2β) and SPO11. TOP1 single-strand breaks (SSB) are also converted into single-ended DSB by replication run-off [12] or replication fork breakage after fork reversal [13,14]. In addition, TOP1 SSB can be converted to DSB by transcription-mediated R-loop formation [15] or when they form opposite to a preexisting nick (Table 1) [16–20].

iii. Under normal conditions, with the exception of SPO11, TOP-DPC are intrinsically transient and self-reversible. In other words, once the DNA realigns itself across the break, the tyrosyl covalent bond is eliminated by the nucleophilic attack of the free hydroxyl end of the DNA across the break as it rejoins the DNA backbone and releases the active topoisomerase molecule. These catalytic intermediates are referred to as the topoisomerase cleavage (or cleavable) complexes (TOPcc).

iv. The TOP-DPC (TOP1-DPC, TOP1MT-DPC, TOP2α-DPC, TOP2β-DPC, TOP3α-DPC and TOP3β-DPC) are likely to be the most frequent DPC at any given time in the cell because of the ubiquitous functions of the 6 human topoisomerases as they form constantly and rapidly reversible TOPcc. Circumstantial evidence for the ubiquitous nature of TOP-DPC is the evolution of tyrosyl-DNA phosphodiesterases (TDP1 and TDP2) as specialized enzymes to remove abortive TOP-DPC [21] and the fact that genetic inactivation of TDP1 and TDP2 cause clinical syndromes (SCAN1 and SCAR23 due to the accumulation of TOP-DPC in neurons [22–27]).

v. TOP-DPC do not form randomly in the genome as they are associated with the diverse functions of the topoisomerases at specific genomic sites during DNA replication, transcription and chromatin remodeling [10]. Moreover, topoisomerases tend to cleave DNA at preferred sequences (albeit with limited sequence selectivity, which allows them to carry out their genome-wide functions [28–32].

vi. TOP-DPC are trapped by widely used anticancer drugs, by DNA structural alterations and by post-translational modifications of the topoisomerase polypeptides. A main mechanism for the trapping of TOP-DPC involves the misalignment of the broken ends within the TOPcc because of the presence of a drug molecule between the broken DNA ends of the TOPcc or because of the misalignment of the ends by DNA alterations (Table 1 and see below).

4. Induction of trapped (irreversible/abortive) TOP-DPC

Any TOPcc is a potentially irreversible TOP-DPC. Table 1 details the broad range of conditions leading to trapped TOP-DPC, which are also referred to as abortive TOPcc.

Matching references are included in the right column of the table. TOP3-DPC have not been included. We have recently reported their existence upon mutation of TOP3B [33].

The most studied pharmacological and therapeutic generators of TOP-DPC are small cytotoxic molecules used as anticancer agents and also used as chemical probes to study the repair of TOP-DPC. These drugs are highly specific for either TOP1 or TOP2 as they form biochemical contacts at the enzyme-DNA interface that prevent DNA rejoining. They are commonly referred to as TOP poisons [34]. The camptothecins selectively poison TOP1cc whereas etoposide and its derivative teniposide only poison both TOP2α and TOP2β but not TOP1 enzymes. Other agents that poison TOPcc have additional DNA effects, which are generally considered as their primary mechanism of action; among them, cisplatin, which distorts the DNA helical structure by forming intra-strand crosslinks or the antimetabolites gemcitabine and cytarabine, which poison TOP1cc by altering the bases incorporated into DNA during replication (Table 1 and references therein).

Chemical and physical carcinogens are a common source of TOP-DPC as they form DNA base adducts (benzo[*a*]pyrene, aldehydes, nucleophiles), crosslinks (ultraviolet [UV] and ionizing radiations [IR]) and DNA breaks. A recent review gives an detailed overview of the environmental DPC [5].

Endogenous metabolic and DNA repair processes can generate TOP-DPC as in the case of oxygen radicals (reactive oxygen species [ROS]), aldehydes, abasic sites, mismatches, DNA breaks and ribonucleotide incorporation during DNA synthesis (Table 1).

Independent studies have also revealed the presence of TOP-DPC during normal transcription, especially during transcriptional activation by hormones (such as androgens and estrogens) and cellular stress pathways. TOP2βcc have been mapped to promoters and TOP1cc to enhancers. The conceptual question is whether these TOP-DPC are byproducts of the normal TOPcc that adapt the DNA to the transcription processes or whether they are promoted by some unknown factor to anchor the promoters or enhancers to scaffolding nuclear structures [10,35].

Evidence for endogenous TOP-DPC is their accumulation and the development of genomic instability in mice and patients lacking the TOP-DPC repair enzymes, TDP1 and TDP2, which are the tyrosyl-DNA phosphodiesterases that excise TOP1-DPC and Spartan (SPRTN) (SprT-like domain at the N-terminus), a metalloprotease, which proteolyzes DPC during DNA replication [35–40].

5. Debulking the TOP-DPC by the ubiquitin-proteasome pathway (UPP)

5.1. Introduction to the UPP

The 26S proteasome is a large (~2.5–2.6 MDa) and highly sophisticated protease complex that degrades intracellular proteins in both the cytoplasm and nucleus [41–43]. The ATP-driven proteasome-mediated degradation plays a pivotal role in modulating a wide array of cellular processes such as cell cycle progression, transcription, signal transduction, response to cellular stress, immune response as well as DNA damage response and repair [44]. The

26S proteasome comprises two sub-assemblies: the 20S core particle (CP), a hollow cylinder in which proteins are degraded by a chymotrypsin-like protease, a trypsin-like protease and a peptidyl-glutamyl peptide-hydrolyzing (PHGH) protease. At the top and bottom of the core particle are two 19S regulatory particles (RP) that bear multiple ATPase and ubiquitin binding sites that recognize, deubiquitylate, unfold and transfer the substrates to the CP for destruction (Fig. 3) [42].

Proteasomal degradation requires the prior covalent addition of polyubiquitin chains to substrate proteins (ubiquitylation) to direct those ubiquitylated substrates to the proteasome [45]. Ubiquitin (Ub) is a 76 amino acid, 8.5 kDa polypeptide conserved in all eukaryotes. Ubiquitylation is a three-step enzymatic cascade catalyzed sequentially (Fig. 3). First, a ubiquitin activating enzyme (E1) forms a thioester bond between a cysteine of the E1 protein and the C-terminal glycine of ubiquitin in an ATP-dependent reaction. This activation allows one of ~40 ubiquitin-conjugating enzymes (E2s) to catalyze the transfer of Ub from E1 to the active cysteine of E2. In the final step, one of ~600 ubiquitin ligases (E3s) recognizes the target protein and transfers the Ub moiety from the E2 to the substrate by creating an isopeptide bond between a ε-NH2 of a lysine residue of the substrate and the C-terminal glycine of Ub (Fig. 3a). In human, the majority of ubiquitin ligases belongs to the family of RING (Really Interesting New Gene) finger domain proteins, which bind proteins and transfer Ub moieties from the E2 to their targets [46]. RING ligases can act as monomers (TRIM41, c-CBL), homodimers (RNF4), heterodimers (BRCA1-BARD1, RINGA/Bmi1), as multisubunit ligases (APC/C), or in complex with a Cullin family member (Cullin-RBX1/2 ligase complexes also referred to as CRL [Cullin Ring Ligases]) [47].

Ub molecules can form various polymeric chains (polyubiquitylation) with different lysine residues *via* isopeptidyl linkages in an iterative manner [48]. These chains are linked through one of the seven lysines (K6, K11, K27, K29, K33, K48 and K63) as well as the N-terminal methionine residue (M1) of Ub [49]. Ub chains linked *via* K48 target proteins for proteasome-mediated degradation [50] (Fig. 3b). K63-linked polyubiquitylation is not associated with proteasomal degradation and has roles in many cellular events including endocytic trafficking, inflammation and DNA repair. The function of other lysine linkages remains largely unknown. Ubiquitylation can be reversed by a class of deubiquitylating enzymes (DUB), which release ubiquitin from substrate proteins by cleaving the isopeptidyl linkage [51]. In addition to the proteasomal DUB subunit PSMD14 (Fig. 3b), which are required to recycle Ub and allow the proteolytic degradation of the polyubiquitylated substrates, there are approximately 79 known DUBs in human cells. They are classified into two main categories: cysteine proteases and zinc metalloproteases [52]. Reversal of ubiquitin modifications by DUB is associated with ubiquitin maturation and editing. Once a polyubiquitin chain is detached from its target protein, it is broken down into single ubiquitin molecules by a DUB to replenish the ubiquitin pool [53].

Ubiquitylation and the UPP perform regulatory roles in the repair of virtually all types of DNA damage [54] including direct or reversal repair by O6-methylguanine DNA methyltransferase (MGMT) [55], mismatch repair (MMR) [56], base-excision repair (BER) [57], nucleotide-excision repair (NER) [58,59], DSB repair and post-replication repair (PRR) by translesion synthesis (TLS) [60], as well as DNA interstrand crosslink (ICL)

repair by the Fanconi anemia (FA) pathway [61] and DPC repair [62]. In addition, poly- and mono- ubiquitylation of histones play critical roles in response to DSB by recruiting DNA repair complexes to the damage sites and facilitating cell cycle arrest [63].

5.2. UPP in the repair of TOP1-DPC

The first study suggesting the implication of the UPP for TOP1-DPC showed that CPT induces a time- and concentration-dependent decrease of cellular TOP1 protein [64]. A follow-up study demonstrated that CPT-induced TOP1 downregulation is a result of the 26S proteasome-mediated degradation as a resistance mechanism to CPT [65]. Treatment with CPT reduced cellular TOP1 by 75 % in the mouse mammary carcinoma cell line ts85 (temperature-sensitive for ubiquitin-activating enzyme) within 2–4 hours. TOP1 was ubiquitylated within minutes of CPT treatment at permissive temperature but not at the restrictive temperature for its thermolabile E1 [65]. MG-132 and lactacystin, specific inhibitors of the 26S proteasome, prevented destruction of cellular TOP1, suggesting that the UPP degrades TOP1 in response to TOP1 trapping [65]. A subsequent study [66] demonstrated that cell lines defective in the proteasomal degradation of TOP1 were hypersensitive to CPT, implying that the UPP-mediated degradation of TOP1 is a resistance mechanism to CPT in human cancer cells. Additional studies show that inhibition of ubiquitylation and of the proteasome markedly enhance the levels of drug-induced TOP1-DPC [67,68] and delay the removal of the DPC [68], further demonstrating that the UPP targets TOP1-DPC.

Two processes, transcription and replication trigger the ubiquitylation and proteasomal degradation of TOP1-DPC (Fig. 4a, b). In the first, TOP1-DPC block the translocating RNA polymerase II (Pol II) elongation complex, which elicits a signal for the recruitment of the UPP and the degradation of both the TOP1-DPC and the large subunit of Pol II Rpb1. This transcription-coupled degradation of TOP1-DPC was proposed to expose TOP1-occluded SSB, enabling the recruitment of PARP1 and the repair of the broken DNA [69]. The second cellular process initiating the UPP-mediated degradation of TOP1-DPC is the collision of translocating DNA polymerase complexes (replisomes) with TOP1-DPC (Fig. 4a) [12,14]. CPT-induced cytotoxicity against cancer cells is well established to arise from replication fork collisions [12,14,70–72], and γH2AX, ATM-Ser1981 phosphorylation, Chk2-Ser33/35 phosphorylation, RPA2 phosphorylation, which are markers of DSB are readily induced by CPT in replication-dependent manner [73,74]. Inhibition of the UPP abolishes the induction of all these DSB characteristics, indicating that ubiquitin-mediated proteasomal degradation removes the bulky DPC thereby exposes the otherwise TOP1-concealed DNA breaks to activate DNA damage responses [75]. Implicit from these findings is a model wherein the ongoing replication fork is arrested by TOP1-DPC, which in turn triggers the UPP-mediated repair of TOP1-DPC, leading to the formation of DSB that comprises TOP1-concealed break and the leading strand where replication fork runs off [12].

To date, several E3 ligases have been implicated in the UPP for TOP1-DPC (Table 2). The first reported was Cullin3 (CUL3), the scaffolding protein of CRL3 following the discovery that CPT-resistant human cancer cell lines exhibit enhanced TOP1-DPC degradation and high expression of CUL3 [67]. Downregulation of CUL3 not only enhanced TOP1-DPC but

also reversed CPT resistance, indicating that CRL3 promotes TOP1-DPC clearance and CPT resistance. A follow-up study confirmed this conclusion by showing that depletion of CUL3 attenuated CPT-induced DSB signals [75]. Apart from CUL3, CUL4B, the scaffolding protein of CRL4 complexes has been linked to TOP1-DPC degradation. Patient-derived lymphoblastoid cell lines deficien for CUL4B were found to display increased sensitivity to CPT and delayed degradation of TOP1 in response to CPT [76]. BRCA1 another ubiquitin ligase has also been reported to ubiquitylate TOP1 in a DNA-PKcs-dependent manner [77,78]. The proposed model is that DNA-PKcs phosphorylates TOP1 at Ser10, which in turn evokes BRCA1 to ubiquitylates TOP1 [78].

Although the “division of labor” between ubiquitin ligases for the repair of TOP1-DPC is presently unclear, it is plausible that different E3 ligases are employed in a context-dependent manner. For example, given the role of CRL4 in replication licensing by degrading the CDT1 and CDKN1A ($p21^{WAF1}$) in a PCNA-dependent manner [79,80], it is conceivable that CRL4 may be primarily recruited to ubiquitylate TOP1-DPC upon the stalling of replication forks.

5.3. UPP in the repair of TOP2-DPC

Proteasomal degradation of TOP2 was first reported in adenovirus E1A-infected human carcinoma cells [81,82] with the finding that TOP2α was degraded by the proteasome during adenovirus E1A-induced apoptosis. Teniposide was later shown to induce ubiquitin-dependent degradation of TOP2 [83]. The proteasome was found to preferentially degrade TOP2β over TOP2α [84], and inhibition of transcription but not replication prevented teniposide-induced TOP2β degradation, in line with the cardinal role of TOP2β in regulating transcription [10]. A follow-up study in colorectal cancer cell lines found that TOP2α also undergoes proteasome-mediated degradation upon treatment with teniposide, and that TOP2α degradation is blocked by transcription but not replication inhibitors [85]. These findings suggest that interference with transcription triggers the degradation of both TOP2α and TOP2β by the UPP (Fig. 4a; Table 2). TOP2 proteasomal degradation that interfere with replication remained unexplained from this set of results. Also, etoposide-induced TOP2α- and β-DPC were found significanty increased by ubiquitylation and proteasome inhibitors [68,86], and their clearance was blocked UPP inhibition, indicating a direct role of the UPP in the degradation of TOP2-DPC for repair. The TOP2 catalytic inhibitor ICRF-193 (bisdioxopiperazine), which traps TOP2 on DNA in a closed clamp conformation after the breaks are sealed, was also reported to arrest transcription and induce TOP2β degradation [84], supporting the model wherein TOP2 trapping rather than TOP2-linked DSB blocks advancement of RNA Pol II and triggers the UPP. Accordingly, proteasome inhibitors effectively sensitize tumor cells to etoposide and other TOP2 inhibitors [87,88] and improve the efficacy of etoposide in the refractory HT-29 xenograft [87].

Several ubiquitin E3 ligases have been implicated in the ubiquitylation of TOP2α-DPC (Table 2), of which BMI1/RING1A was the first identified for human TOP2α-DPC degradation by ubiquitylation upon exposure to etoposide [85]. APC/C-Cdh1 has also been shown to be required for ubiquitylation and proteasomal degradation of etoposide-induced

TOP2α-DPC in human cells [89]. Yet, the mechanism that determine which Ub ligase processes TOP2-DPC remains elusive and warrants further investigations.

5.4. SUMOylation of TOP1-DPC and TOP2-DPC for UPP

Ubiquitin-like proteins (UBLs) act in parallel yet connected pathways with ubiquitin [90]. The Small Ubiquitin-like Modifiers (SUMOs), one of the most studied UBL families, includes four members: SUMO-1, SUMO-2, SUMO-3 and SUMO-4 [91]. Like ubiquitin, SUMO modifications are covalently attached to substrate proteins through an enzymatic cascade catalyzed by E1/E2/E3 enzymes. The two E1 enzymes are SUMO-Activating Enzymes Subunit 1 and 2 (SAE1 and SAE2), an-dUBC9 is the only known SUMO E2 conjugating enzyme in eukaryote. In general, most SUMOylated proteins contain a consensus motif Ψ-K-x-D/E, in which Ψ is a hydrophobic residue, K (lysine), which is used for SUMO conjugation, x indicates any amino acid residue, and D/E either aspartic or glutamic acid, respectively [92]. SUMOylation regulates a wide range of biological processes including subcellular transport, protein-protein interactions, transcriptional regulation, cell cycle progression, apoptosis as well as DNA damage responses and repair (DDR) [91].

SUMOylation is involved in different cellular contexts of DDR [93]. It plays key roles in BER, NER and DSB repair by facilitating the assembly of repair protein complexes at DNA damage foci and by regulating their activities and interactions [94–99]. Recently, SUMO-2/3 modfications have been shown to signal the ACRC/GCNA family that belongs to the family ofSprT proteases (see next section) to target the SUMOylated DPC in response to formaldehyde in *C. elegans* and human germ cells [100].

Most relevant to our review, SUMOylation is also a signal for the UPP [101]. It recruits the ubiquitin E3 ligase RING-finger protein 4 (RNF4) to SUMOylated proteins, which enables their Ub-mediated proteasomal degradation [102,103]. RNF4 is a SUMO-Targeted Ubiquitin Ligase (STUbL) (Fig. 4c) recruited to SUMOylated substrate proteins by its 3 SIMs (SUMO Interacting Motifs). Its RING-finger domain belongs to the zinc finger (Znf) domain family. It interacts with E2 enzyme(s), Ub and substrates [104]. RNF4 co-localizes with SUMO-1 in PML (ProMyelocytic Leukemia) nuclear bodies [105] and ubiquitylates PML proteins that have been modified by poly-SUMO2/3 chain [102,103,106].

Ubiquitylation by RNF4 is coordinated with the PIAS-type SUMO ligases [96,97,107], which contain a SP-RING domain that structurally resembles the classic RING finger domain of Ub E3 ligases [108]. Unlike RING finger domain proteins that chelate two zinc ions *via* cystine and histidine residues to interact with the E2 enzymes, the SP-RING domain requires a single zinc ion for the coordination [109]. The SAP (SAF-A/B, Acinus and PIAS) domain of PIAS SUMO E3 enzymes acts as a DNA binding domain. PIAS4- and Ubc9-mediated SUMOylation of DDR proteins such as RPA, 53BP1 and BRCA1 induces their recruitment to DNA damage sites [95,110,111]. Also, PIAS1, 4-mediated SUMOylation at the DSB foci is a prerequisite for RNF4-dependent ubiquitylation of DDR proteins [97], which ensures proteasome-mediated proteolysis of RPA and BRCA1 for DNA repair [97,107]. The disassembly of MDC1 by RNF4 is a prerequisite for the recruitment of

downstream DDR factors [112]. Indeed, the removal of RPA is required for the loading of RAD51 and BRCA2 on resected DNA for HR-dependent DSB repair [96].

Human TOP1 can be modified by SUMO-1 both *in vitro* and *in vivo* [113–115], and CPT stimulates the SUMO-1 modification of TOP1 [113]. TOP1 SUMO-1 modification also drives the delocalization of TOP1 from nucleoli and nucleoplasm upon exposure to TOP1 inhibitors [116,117], but the consequences of the delocalization remain unknown. In yeast *S. pombe*, SUMOylation engages the ubiquitylation pathway. The SUMOylation of TOP1 by Pli1 (the ortholog of vertebrate Siz/PIAS) recruits the STUbL Slx8 (the RNF4 ortholog) [118,119].

TOP2 SUMOylation drives TOP2 to centromeres for sister chromatid decatenation in yeast and Xenopus laevis, indicating a role of SUMOylation in regulating TOP2 activity and preserving segregation fidelity under unperturbed condition [120,121]. SUMO-1 modifications of human TOP2 have also been observed in response to teniposide, implying SUMOylation as a response mechanism against TOP2-DPC [122]. The TOP2 catalytic inhibitor ICRF-193 also stimulates SUMO-1 modification of TOP2 [113], suggesting that the trapping of TOP2 (either covalent or non-covalent) on DNA rather than TOP2-mediated DNA lesions induces TOP2-DPC SUMOylation.

We have recently extended these results by showing that SUMOylation engages the ubiquitin-mediated proteasomal degradation of both TOP1- and TOP2-DPC in both *S. cerevisiae* and human cancer cells [68] (Fig. 4c). TOP1-DPC, TOP2α-DPC and TOP2β-DPC in spite of their structural differences (Fig. 2) are similarly and promptly modified by SUMO-2/3, SUMO-1 and Ub in a sequential order, which leads to their proteasomal degradation. We identified PIAS4 as the SUMO ligase and RNF4 as the STUbL in human cells and Siz1 as the SUMO ligase and Slx5-Slx8 as the STUbL in yeast. The SUMOylation and SUMO-dependent ubiquitylation of TOP-DPC were found replication/transcription-independent [68] (Fig. 4c). One conceivable model for the SUMO-dependent TOP-DPC repair could be that prolonged residence of topoisomerases on chromatin engages the SUMO system for repair. Another explanation is that SUMO may play a role in dissociating non-covalent DNA-bound topoisomerases from chromatin and the failure to release the covalent DNA-bound topoisomerases by SUMO triggers the UPP for destruction.

Given the distinction between TOP1, TOP2α and β in terms of their sizes, catalytic mechanisms and functions, one could imagine that TOP1- and TOP2-DPC are targeted by different ubiquitylation pathways for the repair in different cellular contexts. By contrast to the cullin-RING pathways [67,76,123], the PIAS4-RNF4 axis appears able to engage on both TOP1- and TOP2-DPC independently of DNA transactions and DDR (Fig. 4c). Similarly, we recently found that TOP3β-DPC are directly ubiquitylated by the E3 RING ligase TRIM41 though its recruitment to the TOP3β-DPC [33].

As discussed below (Section 6), in addition to their role in initiating TOP-DPC ubiquitylation, a recent study reported that SUMO-2/3 modifications of TOP2-DPC and binding of ZNF451 (ZATT), a zinc-finger-containing SUMO ligase, changes the conformation of TOP2α-and β-DPC [124,125], which enables TDP2 to gain access to

the TOP2-DNA junction through its SUMO-interacting motif (SIM) and to carry out the phosphotyrosyl bond cleavage without requiring prior proteolysis.

6. Debulking of TOP-DPC by non-proteasomal proteases

Mounting evidence show the existence of non-proteasomal pathways for the debulking of TOP-DPC (Table 3; Fig. 1). Unlike the UPP, which digests ubiquitylated TOP-DPC by multiple incisions through its chymotryptic, tryptic and post-acidic subunits inside the proteasome, the non-proteasomal proteases digest the DPC *in situ*. Also, their catalytic mechanism is different as they are metalloproteases (SPRTN/Wss1), serine (FAM111A) and aspartate (Ddi1) proteases (Table 3).

6.1. Spartan (SPRTN/Wss1)

SPRTN (Wss1 in *S. cerevisiae*) is the most studied non-proteasomal metalloprotease for TOP-DPC and general DPC. It was initially reported in yeast that the metalloprotease Wss1 (Weak suppressor of smt3) removes TOP1-DPC and formaldehyde (FA)-induced DPC to protect cells against CPT and FA, respectively [126]. Wss1 was shown to cleave TOP1 and other DNA-binding proteins *in vitro* using its proteolytic activity in a DNA-dependent manner [126]. Genome-wide screen revealed that loss of Wss1 is synthetic lethal with loss of TDP1, and that such synthetic lethality could be reversed by deletion of TOP1 implying the natural occurrence of endogenous TOP1-DPC (see Section 3). Accordingly, yeast cells lacking both Tdp1 and Wss1 accumulate Top1-DPC and exhibit gross genomic rearrangements as well as hypersensitivity to CPT [126]. A concomitant study [127] using a plasmid containing a site-specific DPC (using the DNMT HpaII) identified a replication-coupled protease pathway that repairs the DPC independently of the proteasome in Xenopus egg extract. Subsequently, SPRTN (SprT-like domain at the N-terminus) was identified as the replication-coupled protease for DPC repair in metazoan [128–132].

SPRTN and its yeast functional ortholog Wss1 both contain a N-terminal metalloprotease domain: the SprT domain in SPRTN and the WLM domain in Wss1 [133] (Fig. 5a). However, the SprT domain of SPRTN only shares the active HEXXH motif with the WLM domain of Wss1 (up to 13 % sequence similarity) [134]. SPRTN was initially described as a PCNA interacting protein involved in the regulation of translesion synthesis, preventing UV-induced mutagenesis during replication [135–141]. Later studies demonstrated the importance of the SPRTN protease activity towards TOP-DPC as well as DPC with intact DNA backbone (see Fig. 2a) by showing that the loss of SPRTN in mammalian cells resulted in a failure to repair FA-induced DPC as well as TOP1-DPC and TOP2-DPC [128–132,142]. Biochemical studies confirmed that SPRTN cleaves a range of DPC substrates including TOP1 and TOP2α using its catalytic glutamic acid residue that coordinate a metal Zn^{2+} cofactor [128,132].

SPRTN binds single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) and other DNA structures [62,130–132]. However, SPRTN was found to digest substrates only in the presence of ssDNA but not dsDNA [130,131], implying that a ssDNA segment adjacent to the DPC must be present for SPRTN to efficiently act on the DPC.

SPRTN deficiency in mice results in TOP1-DPC accumulation in the liver at an early age and promotes liver tumorigenesis [129], suggesting a role of SPRTN as tumor suppressor. In human, SPRTN deficiency is the cause of Ruijs-Aalfs syndrome, an autosomal recessive disorder with premature aging, chromosome instability and early onset of hepatocellular carcinoma [143,144].

The mechanisms that control SPRTN protease activity towards DPC remain poorly understood. One reported regulatory mechanism is that dsDNA binding of SPRTN induces its autocleavage thereby preventing undesired cleavage of chromatin-associated proteins under normal conditions [128,131,132,134]. ssDNA binding of SPRTN, on the other hand, primarily stimulates both autocleavage and substrate cleavage [131]. The ssDNA segment adjoining the DPC site was recent found to be generated by accessory DNA helicase RTEL1, which enables the CMG helicase to bypass a DPC on the leading strand template and allows SPRTN to repair the DPC [145]. SPRTN is mono-ubiquitylated under unperturbed condition and de-ubiquitylated upon exposure to DPC-inducing agents. This deubiquitylation appears required for the binding of SPRTN to DNA, implying an ubiquitin switch mechanism controlling the recruitment of SPRTN to chromatin [131]. Future investigations to identify the E3 ligase and particularly the deubiquitylating enzyme for controlling this ubiquitin switch are warranted [146]. Chk1 also appears to phosphorylate SPRTN upon its release from replicative chromatin, which also promotes the recruitment of SPRTN to chromatin for DPC repair and may explain the integration of SPTN activity with the replication stress response [147].

Echoing the finding that SUMOylation primes TOP-DPC for proteasome-dependent degradation by recruiting RNF4 in human cells and Slx5-Slx8 in yeast [1], Wss1 also targets SUMOylated substrates through its c-terminal SUMO-interacting motifs in yeast [148,149] (Fig. 5A). In contrast, in vertebrates, SPRTN does not contain SIMs but instead bears a ubiquitin-binding zinc finger domain (UBZ) [136,137], implying that SPRTN may recognize and target ubiquitylated DPC. The difference between SPRTN and Wss1 is also evidenced by the inability of SPRTN to complement wss1Δ yeast cells [128]. A very recent study, however, shows that SPRTN clears SUMOylated (SUMO-1) DPC during replication in human cells [150]. Due to the lack of SUMO-interacting domain, it remains unclear how SPRTN is recruited by SUMOylation of the DPC.

6.2. ACRC/GCNA

ACRC (acidic repeat-containing protein), also known as GCNA (germ cell nuclear antigen-1), is a metalloprotease that repairs DPC in metazoan in parallel with SPRTN. ACRC is highly expressed during sexual cycles in germ cells while maintaining low levels in somatic and most cancer cells (http://discover.nci.gov/cellminercdb) [151,152]. Phylogenetic analyses showed that ACRC is a SPRTN-related protease carrying the SprT domain [153]. ACRC is recruited to SUMOylated DPC through its SIMs upon FA treatment, [100]. In *C. elegans*, the ACRC protein ortholog GCNA-1 appears crucial for the cells to withstand DPC-inducing agents [100]. Two recent studies [154,155] provide evidence supporting the role of ACRC/GCNA in limiting DPC accumulation and preventing replication stress in metazoans by showing that ACRC repairs TOP2-DPC in Drosophila while suppressing copy number

variation in human germ cell tumors [155]. GCNA colocalizes with TOP2 on condensed chromosomes during mitosis in *C. elegans* [155] and suppresses Spo11-dependent DNA damage *via* its SprT protease activity during meiosis in Drosophila [154], implying a role of ACRC/GCNA in repairing both TOP2- and SPO11-DPC (see Fig. 2).

6.3. Ddi1

Ddi1 (DNA-damage inducible 1), a yeast aspartate protease harboring the retroviral protease-like (RVP) domain, acts as an alternative pathway to Wss1-dependent DPC repair in budding yeast [146,156]. Simultaneous inactivation of Ddi1 and Wss1 shows synergistic effect on CPT-induced cytotoxicity [146]. DDi1 is also implicated in the repair of TOP2-DPC based on the finding that the *Ddi1*Δ cells are hypersensitive to etoposide. Ddi1 appears to function in parallel to Wss1, as the hypersensitivity to etoposide was greatly enhanced in cells lacking both Ddi1 and Wss1 [146]. The recruitment of Ddi1 for DPC repair appears, like Wss1 to dependent on replication. Yet, Ddi1 was recently found to proteolyze polyubiquitylated substrates presumably *via* its ubiquitin-interacting UBA domain [157], raising the possibility that Ddi1 targets ubiquitylated DPC whereas Wss1 is recruited by DPC SUMOylation in yeast. Further studies are warranted to determine whether DDI1 and DDI2, the human homologs of Ddi1, also act as proteases for both TOP1- and TOP2-DPC.

6.4. FAM111A

The serine protease FAM111A (FAMily with sequence similarity 111 member A) removes TOP1-DPC and PARP1-DNA complexes using its trypsin-like domain to ensure replication fork progression in human cells [158]. FAM111A-deficient cells not only accumulate TOP1-DPC and PARP1-induced DNA damage but are also hypersensitive to CPT and to the PARP inhibitors niraparib and talazoparib [158]. The role of FAM111 in TOP1-DPC repair during replication is highly reminiscent of SPRTN. Moreover, both FAM111A and SPRTN bind ssDNA and undergo autocleavage. One difference between FAM111A and SPRTN is the inability of SPRTN to prevent replication fork stalling from PARP1-DNA complexes, raising the possibility that FAM111A targets chromatin-bound proteins that blocks forks regardless of the chemical properties of the protein-DNA interface whereas SPRTN may only target the covalent DPC during replication.

7. Non-proteolytic debulking of TOP-DPC

Early biochemical studies of the tyrosyl-DNA phosphodiesterases established that neither TDP1 and TDP2 can effectively hydrolyze the topoisomerase tyrosyl phosphodiester covalent bonds when the TOP-DPC are in their native conformation unless the TOP-DPC are first heat-denatured [159,160]. These experiments suggested that the tyrosyl-DNA bonds, which are concealed in the TOP-DPC could be made accessible by unfolding the topoisomerases, and that the proteolytic pathways reviewed above were not the only cellular mechanism for debulking TOP-DPC.

The biological evidence for such a pathway in human cells was provided by a landmark study showing that the binding of ZATT^{ZNF451} (a prototypical member of the SUMO E3/E4 ligase family) to TOP2α and TOP2β and the SUMOylation of TOP2α and TOP2β promotes

the binding of TDP2 to the TOP2-DPC and the release of the SUMOylated topoisomerases with formation of clean (protein-free) DSB [125]. This initial discovery and a recent follow-up study show that ZATT^{ZNF451} binding to TOP2α and TOP2β does not require etoposide treatment and that SUMOylated (SUMO2- but not SUMO1-modified) TOP2α and TOP2β are bound to TDP2 and ZATT^{ZNF451} even under baseline conditions (without drug treatment) in HEK293 F cells [125,161]. Notably, based on survival studies and DSB repair (using γH2AX), the ZATT^{ZNF451} pathway appears to overlap only partially with the TDP2 and proteasome pathways, indicating the redundancy of the different TOP2-DPC repair pathways even within a single cell type (human HEK293 F and murine MEF cells) [125,161].

The ZATT^{ZNF451} pathway is not involved in TOP1-DPC [125] in spite of the well-established SUMOylation of the TOP1-DPC [1,68,113]. SUMO-1 modifications that cap the TOP1- and TOP2-DPC SUMO2/3 chains in response to TOP inhibitors [68] may prevent the recruitment of TDP2 to SUMO2 and to the TOP-DPC.

Although this ZATT^{ZNF451} pathway appears well-defined for TOP2α- and TOP2β-DPC, it is likely that it is coordinated with the other debulking pathways, as recently demonstrated by the recruitment of TDP2 to TOP2-DPC both by its “split-SIM” motifs and its N-terminus UBA motif [125,161,162] and the lack of ZATT^{ZNF451} ortholog in *C. elegans* [125]. It remains unknown whether there exists a ZATT^{ZNF451} functional equivalent for TOP1-DPC repair to obviate the need for a proteolytic debulking step.

8. p97/VCP/Cdc48 as a mediator for the debulking of TOP-DPC

p97, also called Cdc48 in yeast or valosin-containing protein (VCP) in higher eukaryotes, is a conserved AAA + ATPase [163–166] known for extracting client proteins from their environment and facilitating their proteasomal degradation by inducing conformational alteration in the proteins through ATP hydrolysis [166,167]. p97 plays a crucial role in disassembly and rearrangement of chromatin-associated protein complexes to ensure DNA replication and DNA repair processes to preserve genome integrity [167]. As an enzymatic complex, p97 cooperates with a variety of protein cofactors that associate with its N-terminal domain or C-terminus *via* distinct binding motifs/domains to determine substrate specificity and obtain additional enzymatic activities [167].

Cdc48 had been shown to facilitate the activity of Wss1 for the processing SUMOylated substrates in yeast [126], but it remained unknown whether p97/VCP played a direct role in the repair of TOP-DPC until a recent study showed that p97 inhibition leads to an accumulation of TOP1-DPC in human cells [168]. The study identified TEX264, a gyrase inhibitory-like protein, as a p97 cofactor that recruits p97 to SUMO-1 modified TOP1-DPC to facilitate the SPRTN-dependent proteolysis [168]. Given the role of p97 in unfolding their client proteins, it can be speculated that p97 remodels TOP1-DPC on chromatin and exposes their cleavage sites, thereby promoting SPRTN activity towards the DPC (Fig. 1).

p97 is also known to collaborate with the CRL ubiquitin ligases to promote proteasomal degradation of the CRL-ubiquitylated proteins through its cofactors that contain various

ubiquitin binding domains [169]. This raises the possibility that p97 may also play a role in facilitating the CRL-dependent degradation of TOP-DPC (Fig. 1). Future studies are warranted to interrogate whether p97 enables TDPs to act on the phosphotyrosyl bonds of TOP-DPC through conformational remodeling as another proteolysis-independent mechanism in addition to ZATT^{ZNF451}.

9. Pathway choice for TOP-DPC debulking

To suppress topoisomerase-induced genomic instability, cells have evolved multiple proteolytic mechanisms to efficiently cope with TOP-DPC. Yet, how the cell dictates the pathway choice between proteasome, non-proteasomal proteases and non-proteolytic pathways (Fig. 1) is an important remaining conundrum. A very recent study in Xenopus egg, shows that the proteasome specifically targets polyubiquitylated DNA methyltransferase HpaII-DPC for degradation whereas SPRTN digests the unmodified counterpart during replication [62]. Yet, the replication-associated protease SPRTN is probably not relevant in non-replicative cells as a repair mechanism for TOP-DPC. As mentioned, it has been established that TOP1-DPC as well as TO2β-DPC are subjected to ubiquitylation and targeted by the proteasome upon their detection by RNA polymerase II [69,84], implying the key role of the ubiquitin-proteasome pathway for the repair of TOP-DPC in the context of transcription both in non-replicative and proliferating cells.

Emerging evidence suggests a role of SUMOylation as an early defense mechanism against TOP-DPC and general DPC [68,100]. It is also clear that SUMOylation of topoisomerases is readily detected under physiological conditions [68,125]. Moreover, the SUMOylation of TOP-DPC has recently been demonstrated in yeast as well as human cells to serve as a signal for the ubiquitylation of TOP-DPC [68]. Whether the responsive SUMOylation of TOP-DPC and general DPC plays a role in recruiting SPRTN remains to be clarified. One study in *C. elegans* and human cells observed that SUMOylation was not required for the enrichment of SPRTN on chromatin for the repair of DPC induced by formaldehyde [100] whereas a more recent study found that SPRTN targets TOP1-DPC for proteolysis upon SUMO-1 modification of the DPC [150], imply a specific role of SUMOylation in regulating the SPRTN-dependent repair of TOP1-DPC.

It is likely that the debulking pathway choice is dependent on cell proliferation and cell cycle molecular component (whether cells are actively replicating such as germ cells or quiescent such as neurons). Cellular differentiation is also critical, and most importantly the extent and levels of TOP-DPC. Indeed, cells may use some pathways for endogenous and low levels of physiological TOP-DPC (associated with transcription, replication or recombination) and additional pathways for acute and high levels TOP-DPC such as those induced by anticancer TOP1 and TOP2 poisons and acute environmental exposure to DNA toxins (see Table 1).

10. Excision of the residual TOP-DpC following proteolysis

The excision step for TOP-DpC (Fig. 1) is reviewed in a complementary publication [1] and will only be summarized and elaborated upon here. Due to the unproteolyzable tyrosyl phosphodiester bond left by the debulking proteases, specialized mechanisms are entailed

for the clearance of the remaining DNA-bound topoisomerase peptide (DpC) (Fig. 1). As indicated above, TDP1 and TDP2 serve as precise excision mechanisms to hydrolyze the tyrosine-DNA bond following the debulking step [21]. In addition to the TDP hydrolytic pathway, cells use endonucleases such as MRE11 to remove the TOP-DPC by trimming the DNA strand covalently attached to the TOP-DPC (Fig. 1).

10.1. TDP1

TDP1 belongs to the phospholipase D (PLD) superfamily; it is highly conserved from yeast to humans [170,171] and hydrolyzes the 3' phosphotyrosyl bond of TOP1-DPC as well as other types of 3'-blocking lesions [21]. TDP1 also exhibits activity against 5'-phosphotyrosyl linkages, albeit much weaker than does TDP2 [160,172], suggesting a role of TDP1 as backup excision mechanism for repairing TOP2-DPC [173]. The resulting "clean" DNA termini with a 3'-phosphate is then further processed, leading to restoration of the DNA by non-homologous end joining (NHEJ) or homologous recombination (HR) [21,174,175]. Given that the phosphotyrosyl bond is completely buried by the bulky DNA-bound topoisomerase, it is conceivable that additional steps are required to unveil the TOP1-occluded bond for access by TDP1. Indeed, *in vitro* studies have shown that TDP1 act on proteolyzed or denatured TOP1-DPC but not native, full-length TOP1-DPC [176,177], indicating that exposure of the phophotyrosyl bond by proteolysis by the proteasome or other proteases or possibly unfolding of the TOP-DPC are critical for the action of TDP1 [176].

In yeast, Wss1 and Tdp1 compensate each other in the repair of Top1-DPC and only the *Δtdp1Δwss1* double-mutant displays elevated Top1-DPC and hypersensitivity to CPT [126]. Although it appears plausible that Wss1 could debulk Top1-DPC to allow Tdp1 to process the remaining Top1-DpC, the redundant roles of Wss1 and Tdp1 in Top1-DPC repair raise the possibility that Tdp1 could be act on denatured full-length TOP1-DPC without the need for proteolysis in yeast or that Tdp1 can be coupled with another protease, such as Ddi1 [146] (see Section 5.2). By contrast, in mammals, a couple of studies [129,132] suggest that SPRTN and TDP1 both work in the same repair pathway of TOP1-DPC as depletion of TDP1 in SPRTN-depleted cells does not cause further hypersensitization and TOP1-DPC accumulation compared to SPRTN knockout alone. The degree of sensitization to CPT in SPRTN depleted mammalian cells is similar to that of TDP1 depleted cells [129,132].

Following the debulking of TOP1-DPC either by the proteasome or SPRTN, the role of NER nucleases ERCC1-XPF has been invoked in the repair of TOP1-DPC in parallel with the TDP1 hydrolysis pathway [178]. Thus, it is conceivable that TDP1 hydrolyzes 3′-phosphotyrosyl bond linked to DNA, leaving a 3′ phosphoryl nick DNA that is eventually repaired by the BER pathway (XRCC1, polynucleotide kinase 3′-phosphatase, POLB and LIGIII). Alternatively, ERCC1-XPF and RPA remove 3′-phosphotyrosyl bound peptides to generate a gap for POLD, FEN1 and LIG1, which are NER factors [179].

Although Tdp1 has been shown to excise Top2-DPC in yeast [180] and DT40 cells [172], and to also excise 5'-DpC in biochemical assays [172,177], studies in human lymphoma cells indicate a lack of involvement in TDP2 knockout TK6 cells [181]. Hence, further

studies are warranted to clarify the relevance of TDP1 for TOP2-DPC in different models and circumstances.

10.2. TDP2

Yeast does not express TDP2 and only Tdp1, which appear to perform the function of TDP2 for the Top2-DPC [180]. The polarity of TDP2 is opposite to TDP1; it primarily acts on 5'-phosphotyrosyl linkages for the repair of TOP2-DPC and TOP3-DPC [1,33,182]. In human cells, TDP2 appears to display activity toward the 3'-counterpart, albeit less efficient than TDP1 [172,173]. Yet, genetic studies indicate that TDP2 acts as a backup pathway for TOP1-DPC in the absence of TDP1 [173,181] Akin to TDP1, TDP2 preferentially excises TOP2-DPC fragments after proteolysis or denatured TOP2-DPC *in vitro* [183], suggesting a role of proteolytic debulking as a precondition for the activity of TDP2, presumably by unveiling the 5' tyrosine-DNA bond.

As mentioned in Section 6, ZATT (ZNF451) is a SUMO E3 ligase that induces conformational change in TOP2-DPC by either direct binding or SUMO-2/3 modification, enabling TDP2 to gain access to the 5' tyrosyl bond without the debulking of the TOP2 adduct [125]. Although SPRTN depleted human cells are sensitive to ETP [128,132] to a similar extent to TDP2 depleted cells, the genetic interaction of SPRTN and TDP2 as well as TDP1 still needs to be further investigated. Also, how SPRTN and TDPs (TDP1 and TDP2) are coordinate with each other in counteracting TOP-DPC is still unclear and warrants further investigations.

10.3. MRE11

The Mre11-Rad50-Nbs1 nuclease complex has been demonstrated to play an important role in processing TOP2- as well TOP1-DPC in parallel to TDPs [1,184,185]. It has been proposed that Mre11, in cooperation with its functional partner CtIP, incise the DNA segment adjoining TOP-DPC, imlying that the exposure of the phosphotyrosyl bond is not required for Mre11 to remove TOP-DPC. This model is supported by evidence that Nbs1 promotes Mre11/Rad50 catalyzed endo- and exonucleolytic cleavage of DNA containing 5'-adducts to generate clean double-strand break ends [186] as well as the fact that Mre11 processes the covalent DNA-bound SPO11, a TOP2-like protein that mediates meiotic recombination, without the need for proteases to digest the bulky SPO11 adduct [187,188].

In yeast, the lack of both TDP1 and MRE11 renders cells substantially more sensitive to CPT than either single mutant where the nuclease dead MRE11 shows mild sensitivity and TDP1 KO are insensitive to CPT [189]. In mammalian cells, endogenous TOP2-DPC accumulate in MRE11-deficient cells, but not in TDP2-deficient cells [190]. However, deletion of TDP2 in cells that lack MRE11 markedly increases DNA damage, etoposide sensitivity and TOP2-DPC accumulation [190]. It appears that the clean DSB that are generated by both TDP2- and MRN-dependent repair of TOP2-DPC are ultimately channeled into the NHEJ pathway in vertebrates [190,191]. A very recent study reported that Mre11 is recruited by the ubiquitin E2 enzyme UBC13-mediated K63 polyubiquitylation for the repair of etoposide-induced TOP2-DPC in G1 cells [192]. The substrates of the ubiquitylation were nevertheless not identified in the study. Given that both TOP1- and

TOP2- DPC can be modified by ubiquitin through K63 linkage [68], it will be important to determine whether UBC13 specifically targets TOP-DPC for the ubiquitylation to employ the Mre11-dependent repair.

11. Concluding remarks

Many of the pathways described here for the TOP-DPC are common to other DPC, such as the proteasomal and SPRTN pathway. Using topoisomerase inhibitors is a powerful and convenient approach to discover novel pathways for the debulking of DPC prior to their proteolytic processing. While our understanding of the repair mechanisms for TOP-DPC has increased markedly in the last years, many pressing questions remain. In particular, it is undetermined whether the repair machineries are recruited to the sites of TOP-DPC, and/or whether TOP-DPC are relocalized (presumably by chromatin remodeling) to regions (repair factories) with high abundance of the repair proteins together with the proteasome. The mechanisms that determine the division of labor between all the identified protease and debulking pathways also remain largely unknown. The hierarchy, interplay and redundancy of the protease pathways therefore warrant future exploration.

Apart from DPC formed by TOP1 and TOP2, the biology and repair of TOP3-DPC (Induction/detection/debulking and excision) formed by the type IA topoisomerases has only begun to be investigated. While we recently reported a common pathway involving the proteasome and TDP2 and a specific UPP with the E3 ligase TRIM41 for the repair of TOP3β-DPC [33], it is yet unclear whether additional pathways are involved for TOP3β-DPC, and whether TOP3α forms DPC under physiological conditions or in response to exo/endogenous agents and how such TOP3-DPC are processed.

Understanding the debulking pathways in the context of the DDR pathways will help rationalize combination therapies such as combining topoisomerase inhibitors with inhibitors of the proteasome, which are FDA-approved for a small population of cancer patients, or ubiquitin or SUMO or p97/VCP inhibitors, which are clinical development. The question will be to define the synthetic lethality, which is likely due to the redundancy and parallel pathways for the debulking of TOP-DPC.

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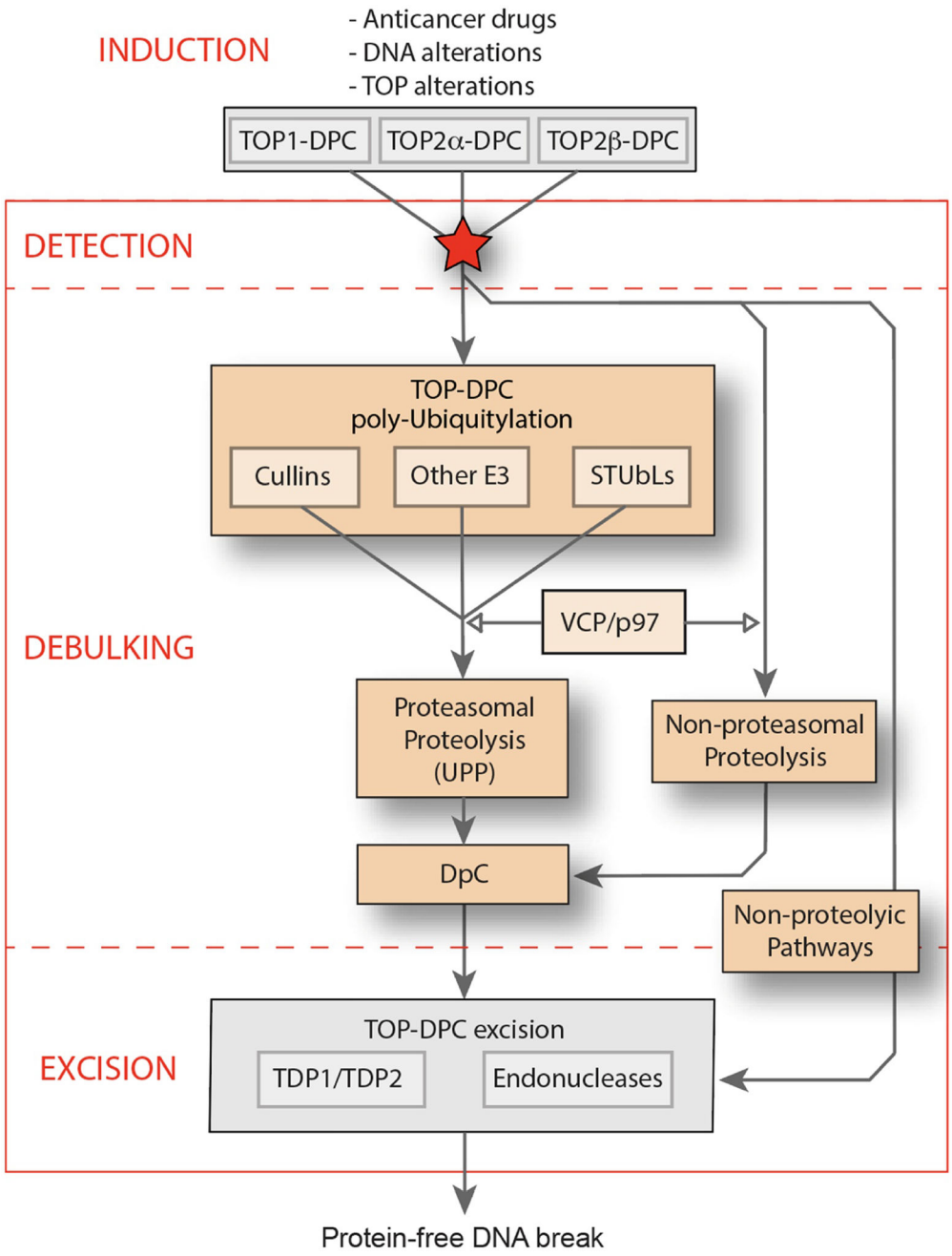

Fig. 1. Pathways for the debulking of TOP-DPC.

Overall scheme for the induction and excision repair of TOP-DPC. The ubiquitin proteasomal pathway (UPP) is represented in the middle with the different ubiquitin pathways for TOP-DPC (Cullins, SUMO-targeted ubiquitin ligases [STUbLs]). The non-proteasomal pathway is examplifed by Spartan/Wss1. Both the proteasomal and non proteasomal generate DNA peptide crosslinks (DpC). The non-proteolytic pathways shown as the arrow at right includes the endonuclease pathway (examplified by MRE11) and the ZATT^{ZNF451} pathway, which unfolds TOP2-DPC and primes their hydrolytic removal by TDP2 [125]. TOP-DPC excision is detailed in a separate issue of DNA Repair [1].

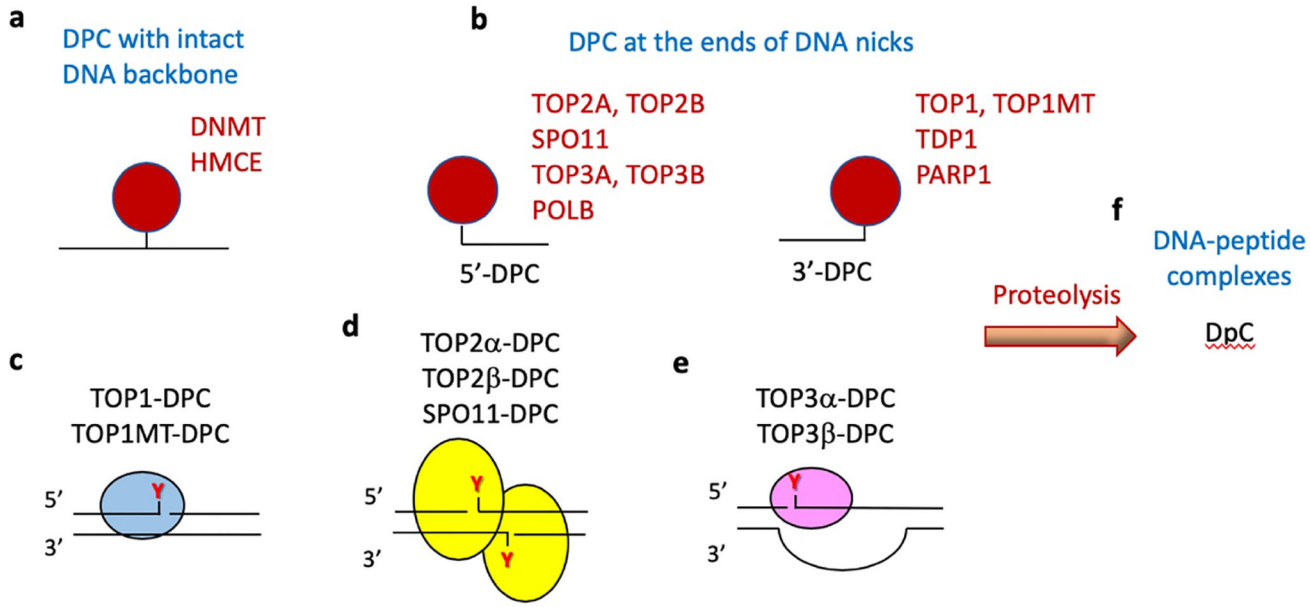

Fig. 2. Architecture and polarity of TOP-DPC in the context of the enzymatic DPC.

Pathological enzymatic DPC result from abortive enzymatic reactions. Depending on the structure of the DNA at the DPC site, enzymatic DPC can be classified in two groups: (**a**) DPC with intact DNA backbone as in the case of DNMT (DNA methyltransferase) covalently linked to 5-azacytosine incorporated into the DNA or HMCES (5-hydroxymethylcytosine binding ES specific) at AP sites in single-stranded DNA, and (**b**) DPC at the end of a nick, or terminal DPC, which can be subclassified as 5'-DPC and 3'-DPC depending whether the DPC is at the 5'- or 3'-terminus of the DNA (see text for details). DNA polymerase β (POLB) forms 5'-DPC whereas tyrosyl-DNA-phosphodiesterase 1 (TDP1) and poly(ADPribose)polymerase 1 (PARP1) form 3'-DPC. TOP-DPC belong to both the 5'- and 3'-DPC subgroups as they generate transient covalent protein-linked DNA breaks (PLDB), termed topoisomerase cleavage complexes (TOPcc) at either the 5'- or 3'-end depending on the topoisomerase. Abortive (trapped) TOPcc form stable TOP-DPC at the end of the broken DNA. **c**. TOP1 and TOP1MT cleaves one strand of duplex DNA with covalent linkage through their active tyrosine (Y) to the 3'-end. **d**. TOP2 enzymes (TOP2α [TOP2A] and TOP2β [TOP2B]) and the meiotic recombination topoisomerase-like SPO11 act as homodimers and cleave duplex DNA with covalent linkage through their active tyrosine (Y). **e**. TOP3 enzymes (TOP3α (TOP3A) and TOP3β [TOP3B]) act as monomers. They reversibly cleave exposed single-stranded DNA regions of the DNA double helix by covalent linkage to the 5'-end using Mg^{2+}. Little is known about the repair of TOP3-DPC (see Fig. 4 and text for details).

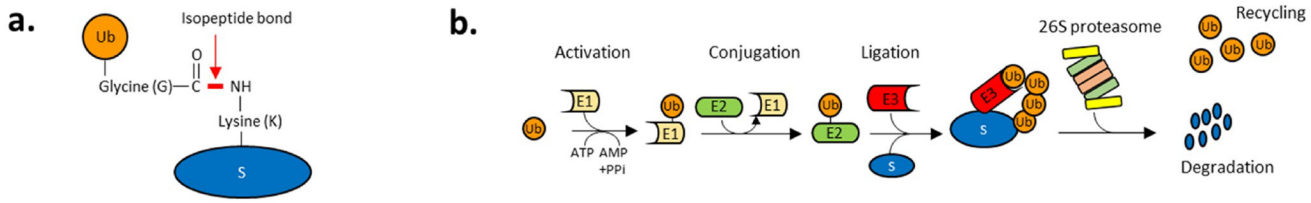

Fig. 3. The ubiquitin-proteasome system (UPP).

a. Ubiquitin (Ub) is a small protein (~76 amino acid) forming a covalent isopeptide bond between its c-terminal glycine carboxyl group and the NH2 group of a lysine residue on either substrate proteins or ubiquitin itself. **b**. Substrate ubiquitylation is achieved in three steps: substrate proteins are processed by ATP-activated enzymatic cascades with ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ub is first activated by an E1 in an ATP-dependent manner. The E1 catalyzes the acyl-adenylation of the C-terminus of an ubiquitin molecule using ATP. The activated Ub is then conjugated to E1 through a thioester bond between the c-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group (activation). E2 enzymes transfers the Ub molecule from E1 to the active site cysteine of an E2 *via* a trans(thio)esterification reaction (conjugation). E3 ligases allow the attachment of the Ub molecule from E2 to a substrate protein (S) by catalyzing the formation of an isopeptidyl linkage between a lysine residue of the substrate and the c-terminal glycine of the ubiquitin (ligation). Lysine 48-linked polyubiquitylation targets substrates to the 26S proteasome. The 19S regulatory particle of the proteasome docks the ubiquitylated substrate, unfolds its and deubiquitylates the substrate to recycle ubiquitin and transfer it to the 20S core particle where 3 proteolytic subunits degrade the substrate (see Fig. 4).

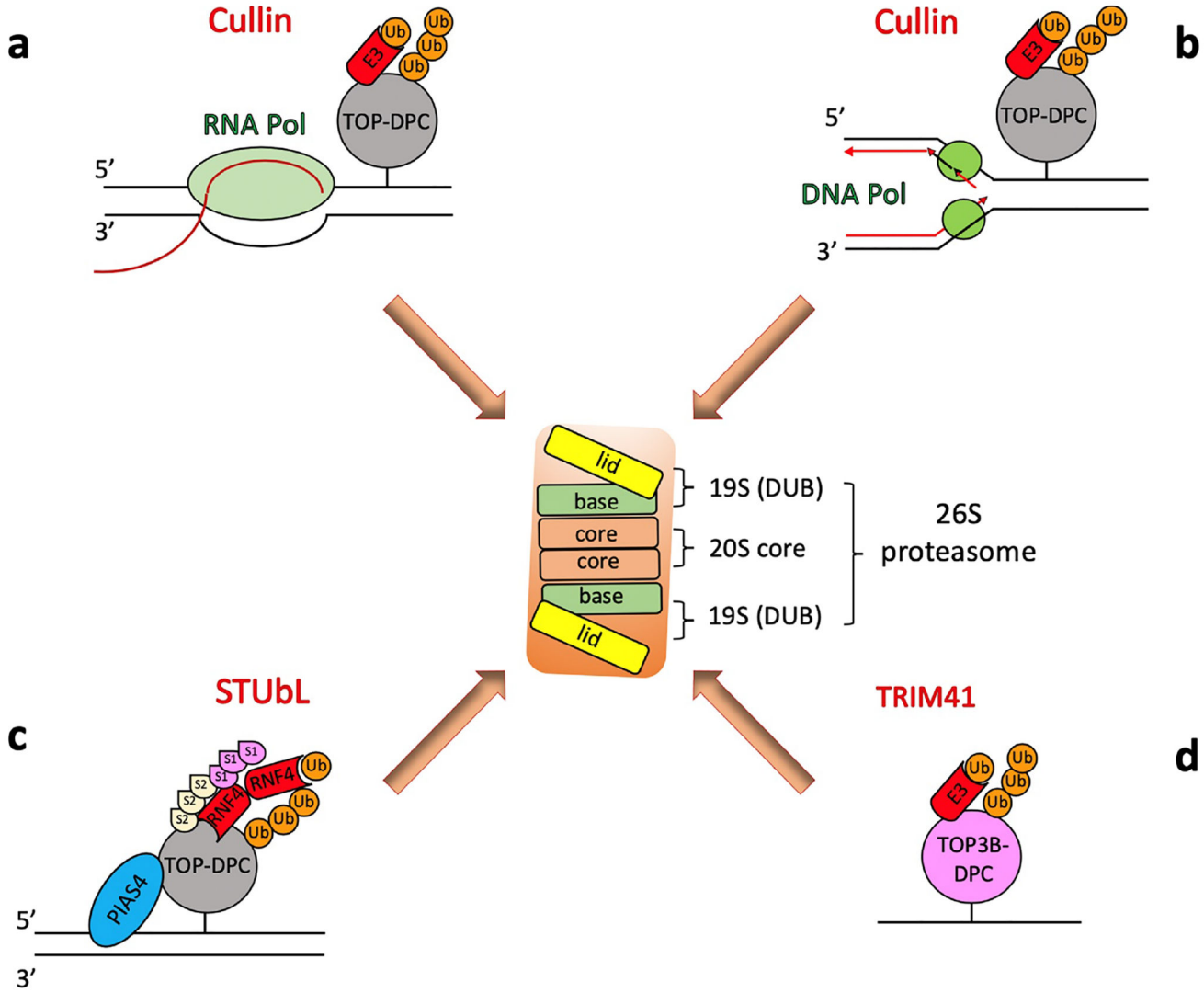

Fig. 4. Known UPP repair pathways for TOP-DPC.

a-b. DNA translocating complexes including transcription (a) and replication (b) trigger the ubiquitylation and subsequent proteasomal degradation of TOP-DPC. Cullin-RING ligases have been implicated as the E3 for both TOP1- and TOP2-DPC. **c**. An alternative ubiquitylation pathway is by STUbL (SUMO-targeted Ub ligases), which are recruited by SUMOylation of the TOP1- and TOP2-DPC. SUMOylation-dependent ubiquitylation is catalyzed by SUMO ligase PIAS4 and STUbL RNF4 in human cells and Siz1 and Slx5/Slx8 in yeast. **d**. Recent studies show that TOP3β-DPC are directly ubiquitylated by the E3 ligase TRIM41 (see text for details and reference).

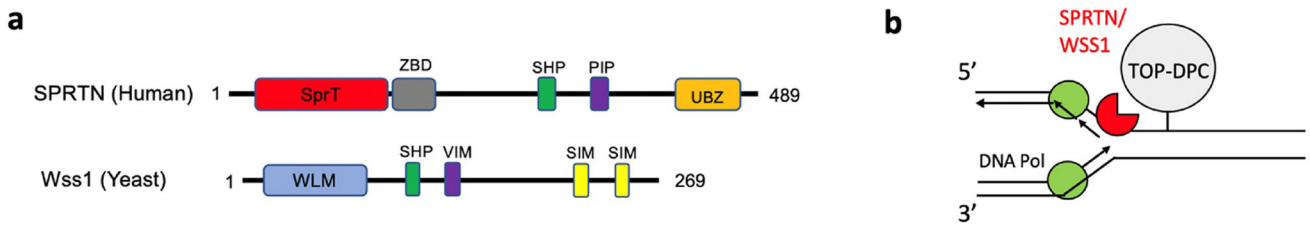

Fig. 5. Repair of TOP-DPC by the non-proteasomal metalloproteases SPRTN/Wss1.

a. Domain/motif architecture of human SPRTN and its yeast ortholog Wss1. ZBD, Zinc binding domain; PIP, PCNA interaction peptide; SHP, p97 or VCP-binding motif; SIM, SUMO interaction motif; SprT, the metalloprotease domain similar to that of the *Escherichia coli* SprT protein; UBZ, ubiquitin-binding zinc finger; VIM; VCP interaction motif; WLM, Wss1- like metalloprotease domain Schematic of the repair of TOP-DPC by SPRTN/Wss1. **b**. As a component of the replisome, SPRTN/WSS1 is activated by its binding to ssDNA. It targets TOP-DPC as well as general DPC for proteolysis in association with replication.

Table 1

Inducers of TOP-DPC including anticancer drugs, common DNA alterations and transactions, carcinogenic physical agents and metabolic products and DNA repair defects.

	Inducers	Molecular mechanism	TOP1-DPC	TOP2-DPC	References
Anticancer drugs	TOP1 and TOP2 poisons	Interfacial inhibition with a single drug molecule bound at the DNA break-topoisomerase interface	• Camptothecins: Topotecan, Irinotecan, Belotecan, Onivyde, Enhertu and other ADCs • Indenoisoquinolines: LMP400 (Indotecan), LMP776 (Indimitecan), LMP744)	• Non-intercalators (Etoposide, Teniposide) • DNA intercalating agents: Amsacrine, Anthracyclines: Doxorubicin, Daunorubicin, Epirubicin, Idarubicin: Mitoxantrone	[193,194,195,196]
	Platinum salts	DNA distortion	Cisplatin, carboplatin	Unknown	[197]
	Antimetabolites	DNA distortions; ribonucleotide incorporations	Hydroxyurea, Gemcitabine, Cytarabine, Fluorodeoxyuridine	Unknown	[19,20,160,198,199,200,201,202,203]
	Others	Distortion of DNA minor groove	Hoechst 33342 Nogalamycin Actinomycin D	Actinomycin D	[204,205,206,207,208,209]
Carcinogens	Food & environmental products	DNA distortions & topoisomerase alterations	Trapping by carcinogenic base damage, exocyclic adducts, crosslinks, benzo[*a*] pyrene & crotonaldehyde adducts	Trapping by flavone derivatives, tea and wine products & carcinogenic base adducts	[5,203,210,211,212,213,214,215,216,217,218,219,220,221]
	UV adducts & IR-induced DNA damage	DNA distortions, base alterations & breaks	Trapping by DNA alterations	Trapping by DNA alterations and enzymatic inhibition	[222,223,224] [16,17,18,218,225]
Endogenous metabolites & DNA alterations	ROS - aldehydes	DNA distortions	DNA alterations	DNA alterations	[215,219,226,227]
	Abasic sites, mismatches, DNA breaks	DNA distortions	DNA distortions	DNA distortions	[16,17,18,19,20,218,219,225,228,229,230]
	Ribonucleotide incorporation in DNA	Chemical reactivity & DNA distortions	Trapping of flanking TOP1cc	Trapping of TOP2cc	[19,20,160,198,199,200]
Transcription	Unknown	Unknown	Enhancers	Promoters	[35,36,37,38,39,40]
DNA repair defects	Genetic alterations	Lack of TOP-DPC excision	TDP1, SPRTN, RNase H2 and ATM deficiencies	TDP2 deficiency and MRE11	[23,25,27,35,129,231,232,233,234]

Table 2

Ubiquitin ligases for proteasomal degradation of TOP-DPC.

Ubiquitin Ligase	Detection/signal	Substrate	TOP-DPC Inducing Agent	References
Cullin 3-RING ligase 3 (CRL3)	Unknown	TOP1-DPC	CPT	[67]
BRCA1	Transcription and serine 10 phosphorylation of TOP1 by DNA-PK	TOP1-DPC	CPT	[77,235]
Cullin 4B-RING ligase (CRL4B)	Unknown	TOP1-DPC	CPT	[76]
Slx5-Slx8 heterodimer	SUMOylation in yeast *S. cerevisiae* by Siz1	TOP1- and TOP2-DPC	CPT, etoposide and amsacrine	[68,119,236]
RNF4 homodimer (Slx5-Slx8 ortholog)	SUMOylation in human by PIAS4	TOP1- and TOP2-DPC	CPT and etoposide	[68]
$SCF^{\beta\text{-}TrCP}$ (SKP1-Cullin 1-F box protein (β-TrCP)	Phosphorylation of TOP2β in its degron motif	TOP2β-DPC	Teniposide (VM26)	[123]
Bmi1-Ring1A Heterodimer	Unkown	TOP2α-DPC	Teniposide (VM26)	[85]

Table 3

Non-Proteasomal Proteases for TOP-DPC.

Proteases	Organisms	TOP1-DPC	TOP2-DPC	Notes	References
SPRTN	Human Mouse	Yes	Yes	*In vitro* and *in vivo* for TOP1-DPC (human, mouse) and TOP2-DPC (human)	[128,129,132]
Wss1	Yeast	Yes	Unknown	*In-vitro* and *in-vivo* evidence for TOP1-DPC after CPT treatment, in parallel with TDP1.	[126]
GCNA/ACRC	*C. elegans*, mouse, *Drosophila melanogaster*, human	Unknown	Yes	Selective for germline and early embryo across species. (C. *elegans, Drosophila melanogaster* and mouse)	[154,155]
Ddi1	Yeast	Yes	Unknown	*In vivo*	[146,156]
FAM111A	Human	Yes	Unknown	*In vivo* for TOP1-DPC	[158]