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Mechanisms to repair stalled Topoisomerase II-DNA covalent complexes

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ABSTRACT

DNA topoisomerases regulate the topological state of DNA, relaxing DNA supercoils and resolving catenanes and knots that result from biological processes such as transcription and replication. DNA topoisomerase II (TOP2) enzymes achieve this by binding DNA and introducing an enzyme-bridged DNA double-strand break (DSB) where each protomer of the dimeric enzyme is covalently attached to the 5' end of the cleaved DNA via an active site tyrosine phosphodiester linkage. The enzyme then passes a second DNA duplex though the DNA break, before religation and release of the enzyme. However, this activity is potentially hazardous to the cell, as failure to complete religation leads to persistent TOP2 protein-DNA covalent complexes which are cytotoxic. Indeed, this property of topoisomerase has been exploited in cancer therapy in the form of topoisomerase poisons which block the religation stage of the reaction cycle, leading to an accumulation of topoisomerase-DNA adducts. A number of parallel cellular processes have been identified that lead to removal of these covalent TOP2-DNA complexes facilitating repair of the resulting protein-free DSB by standard DNA repair pathways. These pathways presumably arose to repair spontaneous stalled or poisoned TOP2-DNA complexes, but understanding their mechanisms also has implications for cancer therapy, particularly resistance to anti-cancer TOP2 poisons and the genotoxic side effects of these drugs. Here we review recent progress in the understanding of the processing to TOP2 DNA covalent complexes., The basic components and mechanisms plus the additional layer of complexity posed by the post-translational modifications that modulate these pathways.

SIGNIFICANCE STATEMENT

Multiple pathways have been reported for removal and repair of TOP2-DNA covalent complexes to ensure the timely and efficient repair of TOP2-DNA covalent adducts to protect the genome. Post-translational modifications such as ubiquitination and SUMOylation are involved in the regulation of TOP2-DNA complex repair. Small molecule inhibitors of these post translational modifications may help to improve outcomes of TOP2 poison chemotherapy, for example by increasing TOP2 poison cytotoxicity and reducing genotoxicity, but this remains to be determined.

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INTRODUCTION

Double strand DNA breaks (DSBs) are highly lethal DNA lesions. Because of this, the generation of DSBs (for example by radiation therapy or treatment with DSB-inducing drugs) is an effective anticancer strategy. This includes drugs targeting DNA topoisomerase II (TOP2) called TOP2 poisons, which exploit the intrinsic ability of TOP2 to induce DSBs as part of its normal reaction mechanism. Both pathways involved in the repair of DSBs, namely homologous recombination (HR) repair and non-homologous end joining (NHEJ) require "clean" DNA ends, yet the clinically relevant DSBs induced by TOP2 poisons contain blocked DNA ends covalently linked to a TOP2 protein adduct at the 5' end. Further processing is therefore required to remove TOP2 adducts (known as TOP2-DNA covalent complexes) from DNA and produce clean, protein-free DSBs for repair. Better understanding how TOP2-DNA covalent complexes are processed may improve therapy with TOP2 poisons by increasing cytotoxicity and reducing genotoxicity, the latter of which occurs following misrepair of TOP2 poison-induced DSBs. This review summarises our current understanding of TOP2-DNA covalent complex repair, and how this is regulated by post-translational modifications such as SUMOylation and ubiquitination.

DNA TOPOISOMERASE II AND TOP2 POISONS

TOP2 poisons are anticancer agents which induce cytotoxic DSBs by interfering with the normal TOP2 reaction mechanism. In the absence of a TOP2 poison, TOP2 resolves topological problems in DNA (such as supercoils, knots and catenanes) which can otherwise inhibit many DNA-dependent processes such as DNA replication, transcription and chromosome segregation. This is achieved by passing one DNA strand through an enzyme-bridged DSB. The enzyme-bridged DSB is induced upon the nucleophilic attack of the phosphate backbone of DNA by the TOP2 active site tyrosine, which creates a break in the DNA strand whilst simultaneously forming a covalent 5'-phosphotyrosyl linkage between TOP2 and the 5'- end of DNA (Deweese and Osheroff, 2009). This intermediate of the TOP2 reaction cycle is termed the TOP2-DNA covalent complex, which facilitates strand passage through the DSB without initiating an unnecessary DNA damage response. Following strand passage, TOP2 religates the DSB in an error-free manner, and the TOP2-DNA complex is rapidly reversed as TOP2 completes its reaction cycle and dissociates from the DNA, while also concealing the break from recognition by the DNA damage response machinery. However, in the presence of a TOP2 poison like etoposide or mitoxantrone, religation of the TOP2-mediated DSB is inhibited, and the TOP2 protein remains covalently bound to the DNA. In contrast TOP2 inhibitors such as ICRF-193, inhibit the catalytic activity of TOP2 without inducing covalent complexes. TOP2-DNA covalent complexes can also be induced

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by endogenous events leading to abortive TOP2 reactions. For example, alterations in DNA can cause misalignment of the broken DNA ends, thus inhibiting religation of the DSB (Sun et al., 2020). The ensuing protein-DNA adduct can stall elongating polymerases, leading to inhibition of replication and transcription and potentially cell death (D'Arpa et al., 1990). Alternatively, the TOP2-DNA covalent complex is processed to protein-free DSBs which may also lead to cell death following a canonical DNA damage response. NHEJ repair is especially important for the repair of TOP2-mediated DNA damage, as knockout of NHEJ proteins such as KU70/80, Ligase IV or DNA-PKcs significantly increases the sensitivity of cells to TOP2-targetting agents such as etoposide and ICRF-193, but not Topoisomerase 1 (TOP1)-targeting agents like camptothecin (Adachi et al., 2003; Maede et al., 2014). In some cases, aberrant NHEJ repair leads to the generation of leukaemogenic chromosome translocations (Cowell et al., 2012; Olmedo-Pelayo et al., 2020).

Drug stabilised TOP2-DNA complexes pose a significant challenge to the DNA repair machinery, as the enzyme-linked DSB remains buried within the core of the bulky TOP2 adduct. Studies have shown that TOP2-linked DSBs themselves do not activate DNA-PK (Mårtensson et al., 2003; Muslimović et al., 2009) and thus further processing and the removal of the TOP2 protein is required before DSB repair can occur. A multitude of TOP2-DNA covalent complex processing mechanisms have been described, yet the factors influencing when or why a particular pathway is employed remain unclear. Better understanding how these lesions are processed may help improve TOP2 poison therapy by reducing drug resistance and TOP2 poison genotoxicity.

PROTEOLYTIC MECHANISMS

One potential mechanism of TOP2-DNA complex repair is the proteolytic removal of the TOP2 protein adduct, which leaves behind smaller peptide fragments which can then be removed in a further end-polishing step prior to DSB repair, such as direct cleavage by the 5'-phosphodiesterase, TDP2. Two major proteases have been implicated in the degradation of TOP2-DNA covalent complexes, namely the proteasome and SPRTN.

Proteasomal degradation of TOP2-DNA covalent complexes

Exposure to the TOP2 poisons etoposide and teniposide induces the degradation of both TOP2 isoforms (TOP2A and TOP2B) in human cells (Alchanati et al., 2009; Ban et al., 2013; Fan et al., 2008; Lee et al., 2016; Mao et al., 2001; Sunter et al., 2010). The TOP2 poison-induced proteolysis of TOP2 is reduced in the presence of the proteasome inhibitor MG132, indicating that degradation is partly proteasomal. In support of this, siRNA knockdown of 26S proteasome assembly chaperones such as POMP and p28 also inhibits the etoposide-induced degradation of TOP2B (Ban et al., 2013). The 26S proteasome mediates the

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degradation of both cytosolic and nuclear protein substrates and consists of a 20S proteolytic core and a 19S regulatory subunit. The 19S subunit facilitates both the unfolding of protein substrates (required to translocate the protein into the narrow proteolytic core) and the regulation of proteasomal degradation by ubiquitination. As such, the 19S subunit also contains ubiquitin binding sites and deubiquitinases, which help target ubiquitinated proteins for degradation (Liu and Jacobson, 2013). While earlier studies involved the study of both unbound TOP2 and DNA-bound TOP2 complexes (usually through western blotting techniques), more recent studies have employed other methods such as the TARDIS assay or ICE assay to specifically measure levels of drug-stabilised TOP2-DNA covalent complexes in the presence and absence of a proteasome inhibitor. These studies have shown that proteasomal inhibition specifically increases levels of TOP2-DNA complexes following etoposide or teniposide treatment (Alchanati et al., 2009; Fan et al., 2008; Sciascia et al., 2020), and slows the removal of covalently bound TOP2A and TOP2B complexes from DNA (Lee et al., 2016; Swan et al., 2020).

Consistent with the notion that removal of the covalent TOP2 adduct is required to activate a DNA damage response, proteasome inhibition also reduces the etoposide-induced phosphorylation of histone H2AX (Mao et al., 2001; Sciascia et al., 2020; Swan et al., 2020; Zhang et al., 2006). Phosphorylation of histone H2AX is one of the primary events to occur in response to a DSB (Paull et al., 2000), suggesting that proteasome inhibition prevents the conversion of TOP2-DNA covalent complexes to protein-free DSBs. It has also been suggested that proteasome inhibition prevents further processing of the TOP2-DNA covalent complex and instead promotes the spontaneous reversal of TOP2-DNA complexes, which occurs as etoposide dissociates from the DNA after drug washout (Sciascia et al., 2020). Co-treatment of cells with a TOP2 poison and a proteasome inhibitor such as MG132 also reduces the appearance of other etoposide-induced DNA damage signals, including phosphorylation of ATM, Chk1, and Chk2 (Fan et al., 2008; Zhang et al., 2006).

Interestingly, a number of studies suggest that the proteasomal processing of TOP2A- and TOP2B- DNA covalent complexes is at least partially transcription-dependent (Fan et al., 2008; Mao et al., 2001; Tammaro et al., 2013; Xiao et al., 2003; Zhang et al., 2006). This is unlikely to be due to the expression of a specific gene product, as inhibition of protein synthesis does not impede the TOP2 poison-induced degradation of TOP2B (Mao et al., 2001; Xiao et al., 2003). Instead, it is thought that TOP2 degradation is induced upon collision of the TOP2-DNA covalent complex with elongating RNAPII. Indeed, treatment with etoposide leads to the accumulation of hyperphosphorylated RNAPII, indicative of transcription arrest (Ban et al., 2013). It is conceivable that the processing of TOP2-DNA complexes will also be induced upon collision with DNA polymerases or helicases during

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replication, and indeed some studies also report a replication-dependent component of TOP2-DNA complex processing (Fan et al., 2008; Tammaro et al., 2013; Yan et al., 2016).

SPRTN-dependent degradation of TOP2

SPRTN is another protease which has been implicated in the degradation of TOP2-DNA covalent complexes, as well as other protein-DNA crosslinks induced by camptothecin and formaldehyde (Lopez-Mosqueda et al., 2016; Vaz et al., 2016). While many protein-DNA crosslinks are partly repaired by NER or HR repair, SPRTN was recently identified as the protease required for the processing of crosslinked protein adducts in human cells, in a protease-dependent but proteasome-independent mechanism which was previously identified in yeast and xenopus (Duxin et al., 2014; Stingele et al., 2014). Unlike the proteasome, SPRTN is a metalloprotease related to the Wss1 protease in yeast (Stingele and Jentsch, 2015), which associates with the replisome to facilitate the clearance of obstructing protein-DNA adducts ahead of the replication fork (Vaz et al., 2016). Consequently, cells lacking SPRTN accumulate both endogenous and exogenous protein-DNA crosslinks and are hypersensitive to protein-DNA crosslink-inducing agents (Stingele et al., 2016; Vaz et al., 2016). Specifically, cells lacking SPRTN display increased sensitivity to etoposide and increased levels of etoposide-induced TOP2-DNA covalent complexes, indicating that drug-stabilised TOP2-DNA covalent complexes are also processed by SPRTN (Lopez-Mosqueda et al., 2016; Vaz et al., 2016). In support of this, SPRTN co-precipitates with TOP2 *in vivo* (Vaz et al., 2016), and cleaves TOP2 in the presence of ubiquitin *in vitro* (Lopez-Mosqueda et al., 2016). This could be consistent with a replication-dependent but proteasome-independent mechanism of TOP2-DNA complex processing described by others (Tammaro et al., 2013).

VCP/p97 as another factor involved in proteolytic removal of TOP2-DNA covalent complexes

VCP/p97 is a protein segregase known to facilitate both proteasome-dependent and SPRTN-dependent degradation of protein substrates. VCP/p97 utilitises its AAA ATPase activity to induce conformational changes in target proteins, which leads to protein unfolding and denaturation. This can aid the extraction of proteins from specific cellular structures which may in turn lead to their degradation by SPRTN or the proteasome.

VCP/p97 and the proteasome

The 26S proteasome is comprised of a 20S catalytic subunit and a 19S regulatory subunit, the latter of which facilitates the translocation of ubiquitinated protein substrates into the narrow 20S core. Specifically, the 19S subunit contains AAA ATPases and deubiquitinases

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which unfold and deubiquitinate target proteins prior to their degradation, respectively. In addition to the 19S hexameric ATPase ring, the proteasome is known to associate with the VCP/p97 AAA ATPase which is also thought to facilitate proteasomal degradation by protein unfolding (Besche et al., 2009; Isakov and Stanhill, 2011). VCP/p97 is increasingly recognised as another important mediator of the ubiquitin-proteasome system, required for the proteasomal degradation of many, but not all, ubiquitinated proteins (Dai and Li, 2001; Heidelberger et al., 2018).

In addition to protein unfolding at the proteasome, VCP/p97 facilitates proteasomal degradation by extracting ubiquitinated proteins from cellular structures such as the endoplasmic reticulum (ER-associated degradation) or chromatin (chromatin-associated degradation). This is particularly apparent during the DNA damage response where VCP/p97 is required for the timely removal of specific DNA repair proteins from chromatin such as Ku70/80, L3MBTL1, DNA-PKcs and Rad51-Rad52 (Acs et al., 2011; Bergink et al., 2013; Jiang et al., 2013; van den Boom et al., 2016). VCP/p97 is also required for the proteasomal degradation of stalled RNA polymerase II (RNAPII) following UV-induced DNA damage (He et al., 2017; Lafon et al., 2015; Verma et al., 2011).

Notably, previous studies suggest that additional AAA ATPases (such as those associated with RNAPII) are required for the proteasomal degradation of TOP2-DNA covalent complexes after etoposide treatment (Ban et al., 2013), and Wei et al show that there is an accumulation of ubiquitinated TOP2A in cells depleted of Cdc48 (the yeast homolog of VCP/p97) (Wei et al., 2017). Interestingly, small molecule inhibition of VCP/p97 slows the removal of both TOP2A- and TOP2B- DNA covalent complexes from chromatin following etoposide treatment, in a manner similar to inhibition of ubiquitination or the proteasome and epistatic to the proteasome pathway (Swan et al., 2021). Consistently, VCP/p97 inhibition also reduces etoposide-induced phosphorylation of histone H2AX, suggesting that VCP/p97 is involved in the processing of TOP2-DNA covalent complexes to protein-free DSBs. Further studies are required to investigate the potential role of VCP/p97 in the proteasomal processing of TOP2-DNA covalent complexes.

VCP/p97 and SPRTN

VCP/p97 is also implicated in the extraction and degradation of proteins by SPRTN, which directly binds and recruits VCP/p97 via a conserved SHP box motif (Mosbech et al., 2012; Stingele et al., 2014). For example, SPRTN recruits VCP/p97 to monoubiquitinated PCNA during replication stress, where it helps to extract TLS polymerase Pol n for degradation, and prevents excessive translesion synthesis at DNA damage sites (Davis et al., 2012; Mosbech et al., 2012). Interestingly, SPRTN is also implicated in the proteolysis of both drug-induced

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and endogenous TOP1-DNA covalent complexes (Maskey et al., 2017; Vaz et al., 2016) in a manner that also involves VCP/p97 (Balakirev et al., 2015; Fielden et al., 2020; Nie et al., 2012; Stingele et al., 2014). Like TOP2, TOP1 can form genotoxic TOP1-DNA covalent complexes following treatment with anticancer drugs such as camptothecin (CPT), and sensitivity of cells to CPT is significantly increased following depletion of VCP/p97 or SPRTN.

Like the 3'-tyrosyl phosphodiesterase TDP1, which is known to cleave trapped TOP1 covalent complexes from DNA, it is thought that the substrate binding groove of SPRTN is too narrow to facilitate binding of bulky TOP1 adducts (Fielden et al., 2020; Li et al., 2019). It is therefore likely that processing or remodelling of the TOP1 adduct is first required before it can be proteolysed by SPRTN, which may involve the AAA ATPase activity of VCP/p97 (Fielden et al., 2020; Stingele et al., 2014). Fielden et al. identify a specialised complex containing VCP/p97 and the VCP/p97 cofactor TEX264 which is required for the clearance of TOP1-DNA covalent complexes (but not other protein-DNA adducts) by SPRTN (Fielden et al., 2020). While it is possible that VCP/p97 may also facilitate the SPRTN-dependent proteolysis of TOP2-DNA covalent complexes, this remains to be investigated.

TYROSYL DNA PHOSPHODIESTERASE 2 (TDP2)

TDP2 is a 5'-phosphodiesterase which directly cleaves the covalent linkage between trapped TOP2 complexes and DNA (Ledesma et al., 2009; Zeng et al., 2011), producing a clean and ligatable DSB for NHEJ repair (Gómez-Herreros et al., 2013; Schellenberg et al., 2016). TDP2 has been shown to be particularly important for the repair of TOP2-mediated DSBs during transcription and in response to androgens, which can otherwise lead to oncogenic chromosome translocations and genome instability (Al Mahmud et al., 2020; Gómez-Herreros et al., 2017). However, due to inaccessibility of the 5'-phosphotyrosyl linkage within the TOP2 active site, TDP2 alone cannot remove the TOP2-DNA complex until it has been proteolysed or denatured (Gao et al., 2014; Lee et al., 2018; Schellenberg et al., 2012). For example, proteasomal degradation of the TOP2 adduct leaves behind a small 5'-phosphotyrosyl peptide which can then be directly removed by TDP2 prior to NHEJ repair (Gao et al., 2014). It is conceivable that proteolysis via the proteasome or SPRTN could facilitate TDP2-dependent repair of the remaining 5'-phosphotyrosyl adduct. Alternatively, the ZATT SUMO ligase was identified as an additional factor which can facilitate the direct removal of TOP2-DNA covalent complexes by TDP2 in a proteasome-independent manner (Schellenberg et al., 2017; Zagnoli-Vieira and Caldecott, 2017). Specifically, it is proposed that SUMOylation of the TOP2-DNA covalent complex and binding of ZATT leads to a

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conformational change in TOP2, negating the need for TOP2 proteolysis and enabling direct access of TDP2 to the phosphodiester bond for cleavage.

NUCLEOLYTIC MECHANISMS

Alternatively, ligatable ends can be produced by nucleolytic processing of the TOP2-DNA complex, whereby the TOP2 protein is removed through cleavage of the adjacent DNA by a nuclease like Mre11. The ssDNA endonuclease activity of Mre11 is implicated in the resection of blocked DNA ends, which induces a nick in the 5' strand close to the 5' adduct (Garcia et al., 2011). This allows the 3'-5' exonuclease digestion of DNA by Mre11, consistent with its 3'-5' exonuclease activity *in vitro*, and creates a gap for loading of the 5'-3' exonucleases Exo1 and DNA2 (Wang et al., 2017). The endonuclease and exonuclease activity of Mre11 is known to be stimulated by another endonuclease, CtIP, in yeast and human cells (Anand et al., 2016; Cannavo and Cejka, 2014; Deshpande et al., 2016; Sartori et al., 2007). Interestingly, DNA2 may be required for the initiation of resection at clean DSBs, suggesting distinct initiation pathways for clean versus blocked DNA ends (Paudyal et al., 2017).

Evidence suggesting that Mre11 can remove covalently linked 5' adducts from DNA ends comes from study of the TOP2-like protein, Spo11. Spo11 induces enzyme linked DSBs during meiosis to facilitate meiotic recombination. In contrast to TOP2, the Spo11-induced DSB is not religated, and the covalently linked 5'-Spo11 adduct must be removed to allow recombination between broken strands (Neale et al., 2005). Mre11/CtIP is required for the removal of Spo11 adducts from DNA ends in *S. Cerevisiae* (Moreau et al., 1999; Neale et al., 2005), and the removal of Rec12 (the Spo11 homolog) in *S. pombe* (Milman et al., 2009; Rothenberg et al., 2009). Various studies have since shown that 5'-TOP2 DNA adducts are also removed in an Mre11/CtIP-dependent manner in yeast and human cells. *S. Pombe* Mre11 mutants deficient in Spo11 removal are hypersensitive to TOP-53 (an etoposide derivative), and display increased levels of drug-induced TOP2-DNA complexes compared to wild-type cells (Hartsuiker et al., 2009). A number of studies have also shown an increase in levels of drug-induced TOP2-DNA complexes and drug sensitivity in Mre11 or CtIP depleted cells, including chicken and human cells (Aparicio et al., 2016; Hamilton and Maizels, 2010; Hoa et al., 2016; Lee et al., 2012; Nakamura et al., 2010; Takeda et al., 2016). Once the TOP2 adduct is removed, etoposide-induced DSBs can then be resected by DNA2 (Tammaro et al., 2016). Interestingly, depletion or inhibition of Mre11 leads to the accumulation of TOP2-DNA complexes even in the absence of a TOP2 poison, indicating that Mre11 is important for the removal of endogenously trapped TOP2-DNA complexes

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(Hoa et al., 2016; Lee et al., 2012). This suggests that Mre11 preserves genome stability not only through HR repair but also by processing abortive TOP2 reactions.

Studies in mammalian cells have shown that BRCA1 is required for the removal of both endogenous and drug-induced 5'-TOP2 adducts by Mre11/CtIP (Aparicio et al., 2016; Morimoto et al., 2019; Nakamura et al., 2010; Sasanuma et al., 2018). Aparicio et al show that the association of BRCA1 with chromatin was significantly greater after etoposide treatment than after the induction of endonuclease-induced "clean" DSBs, and depletion of BRCA1 increases levels of etoposide-induced TOP2-DNA complexes similarly to CtIP or Mre11 depletion (Aparicio et al., 2016). Specifically, the interaction between BRCA1 and CtIP was required for the CtIP-dependent removal of etoposide-induced TOP2-DNA complexes. Notably, BRCA1 is also implicated in the degradation of TOP1 and TOP2B following CPT or etoposide treatment, respectively (Sordet et al., 2008; Xiao and Goodrich, 2005).

Despite its well-known role in HR, Mre11-mediated processing of TOP2-DNA complexes can lead to NHEJ repair of the TOP2-induced DSB in G0/G1 arrested cells, where resection does not occur due to absence of a homologous sister chromatid prior to DNA replication (Akagawa et al., 2020; Quennet et al., 2011). Sasunama et al also demonstrate that BRCA1 is required for the NHEJ repair of DSBs with TOP2 adducts, but not for the NHEJ repair of clean breaks (Sasanuma et al., 2018). The Mre11-dependent repair of etoposide-induced DSBs is epistatic with Ku but not TDP2, indicating that Mre11-dependent repair facilitates NHEJ in a manner distinct from the TDP2 pathway (Hoa et al., 2016). This suggests that NHEJ repair of etoposide-induced DSBs can occur via at least two different mechanisms: one involving the TDP2-dependent removal of proteolysed or denatured TOP2 adducts (Gómez-Herreros et al., 2013), and one involving the Mre11/CtIP/BRCA1-dependent removal of TOP2 adducts (Liao et al., 2016). It is unclear whether in vivo nucleolytic removal by the Mre11/CtIP/BRCA1 pathway can occur without prior processing of the TOP2 protein adduct, or if nucleolytic processing could provide an alternative "end polishing" step after TOP2 proteolysis or denaturation, similar to TDP2. However, MRE11 is able to remove TOP2A from TOP2A-DNA complexes in vitro in the absence of prior proteolytic processing (Lee et al., 2012).

ROLE OF POST-TRANSLATIONAL MODIFICATIONS

In addition to the diversity of pathways described above, the complexity of TOP2-DNA covalent complex repair is further expanded by additional layers of regulation achieved through post-translational modifications like ubiquitination and SUMOylation. Both are implicated in a number of these pathways, as summarised below.

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Ubiquitination in TOP2-DNA covalent complex repair

Ubiquitin is a small 76 amino acid peptide which is conjugated to the lysine residues of target proteins in a 3 step cascade involving an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligating enzyme. This culminates in an isopeptide bond between ubiquitin and the target lysine residue, which is readily reversed by enzymes known as deubiquitinases. The conjugation of ubiquitin to target proteins (i.e. ubiquitination) regulates many cellular processes, such as protein-protein interactions and proteasomal degradation. Ubiquitin itself contains 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) which can also be ubiquitinated, forming polyubiquitin chains with different topologies and functions (Komander, 2009). Typically, the conjugation of K11- or K48-linked ubiquitin chains to a target protein is associated with the degradation of the ubiquitinated protein by the proteasome, while K63-linked chains are frequently involved in protein-protein interactions (Akutsu et al., 2016; Komander and Rape, 2012). Thus, ubiquitination helps to regulate the timely recruitment and removal of repair proteins in various signalling pathways, including the DNA damage response (Jackson and Durocher, 2013).

In addition to the well-established role of ubiquitin in the regulation of proteasomal degradation, ubiquitination is also involved in the regulation of SPRTN-dependent proteolysis. A large proportion of SPRTN is constitutively monoubiquitinated but is rapidly deubiquitinated upon the induction of genotoxic protein-DNA adducts by formaldehyde (Stingele et al., 2016; Zhao et al., 2021). Two deubiquitinase enzymes, namely VCPIP1/VCIP135 and Usp7, were recently shown to activate SPRTN-dependent proteolysis. While VCPIP1/VCIP135 is proposed to regulate binding of SPRTN to DNA (Huang et al., 2020; Stingele et al., 2016), Usp7 increases SPRTN activity by preventing its inactivation by autocatalytic cleavage (Zhao et al., 2021). SPRTN also contains a UBZ domain which facilitates the recruitment of SPRTN to ubiquitinated protein-DNA adducts (Davis et al., 2012; Mosbech et al., 2012). Therefore, ubiquitination regulates protein degradation not only by signalling proteins for their destruction by the proteasome, but also by regulating SPRTN-dependent proteolysis.

TOP2 ubiquitination

As TOP2-DNA covalent complexes are processed both via proteasome- and SPRTN-dependent mechanisms, a number of studies have also investigated the role of ubiquitination in TOP2-DNA covalent complex repair. While some discrepancies exist in the literature (Ban et al., 2013; Mao et al., 2001), recent studies have shown that the removal of TOP2A- and TOP2B- DNA adducts, and the subsequent appearance of TOP2 poison-induced DSBs, is partly ubiquitin-dependent and epistatic with the proteasomal pathway (Swan et al., 2020).

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This seems to be mediated in part by the polyubiquitination of TOP2 by the E3 ubiquitin ligase BMI1/RING1A and the SUMO-targeted ubiquitin ligase RNF4, which leads to proteasomal degradation (Alchanati et al., 2009; Sun et al., 2019; Swan et al., 2020). Indeed, a number of E3 ubiquitin ligases have been shown to ubiquitinate TOP2 in various stress conditions, such as glucose starvation, HDAC inhibition and ICRF-193 treatment, which leads to the proteasomal degradation of TOP2 (Chen et al., 2011; Isik et al., 2003; Yun et al., 2009). Indeed, constitutive monoubiquitination by the Smurf2 ubiquitin ligase may protect TOP2 from polyubiquitin-dependent proteasomal degradation (Emanuelli et al., 2017). The ubiquitin-proteasome system is also known to regulate levels of TOP2A during the cell cycle (Eguren et al., 2014; Salmena et al., 2001).

While numerous ubiquitination sites have been identified on TOP2A and TOP2B (Kim et al., 2011), other studies have been unable to detect the polyubiquitination of TOP2A or TOP2B in the presence or absence of etoposide (Ban et al., 2013). Instead, Ban et al propose a mechanism of TOP2B-DNA complex processing by the proteasome which does not require prior ubiquitination of TOP2. In this model, TOP2B is proteasomally degraded following collision of the TOP2B-DNA complex with elongating RNAPII, where the proteasome is recruited to TOP2B via RNAPII-associated 19S AAA ATPases rather than a polyubiquitin signal (Ban et al., 2013).

Ubiquitination may also be required for the modification of other proteins involved in TOP2-DNA complex repair. For example, TDP2 contains an N terminal ubiquitin binding domain (UBA-like domain) which is required for TDP2 activity and the TDP2-dependent repair of etoposide-induced TOP2-DNA covalent complexes (Rao et al., 2016). While it was later shown that the TDP2 UBA domain binds K27- and K63-linked polyubiquitin chains, the TDP2 UBA does not bind ubiquitinated TOP2 and the ubiquitinated target required for TDP2-dependent repair remains unknown. It is speculated that a ubiquitin-dependent interaction involving the TDP2 UBA domain may induce a conformational change in the TDP2 active site, or may mediate interactions with ubiquitinated histones at DNA damage sites (Kawale and Povirk, 2018; Rao et al., 2016; Schellenberg et al., 2020).

Ubiquitination, and specifically the E2 ubiquitin conjugating enzyme Ubc13, has also been recently implicated in the Mre11-dependent processing pathway. Expression of a catalytically inactive Ubc13 mutant leads to the accumulation of etoposide-induced DSBs in a manner that was epistatic to the Mre11 pathway but independent of the TDP2 pathway (Akagawa et al., 2020). Ubc13 is known to mediate K63-linked ubiquitination at DNA damage sites, leading to the recruitment of various repair proteins including BRCA1 and Rap80. Akagowa et al showed that Ubc13 also mediates the recruitment of Mre11 to

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etoposide-induced TOP2 adducts (as well as other IR-induced “dirty” DNA ends). While the ubiquitinated target also remains unknown, Ubc13 (together with Rap80) was required for the etoposide-induced interaction between Mre11 and BRCA1, and subsequent activation of the Mre11 nuclease.

It is well known that other post-translational modifications such as SUMOylation or phosphorylation can promote protein ubiquitination, for example by inducing changes in chromatin conformation or the recruitment of ubiquitin ligases. For example, phosphorylation of TOP2A in response to HDAC inhibition (first by casein kinase II and then by glycogen synthase kinase 3β), leads to the recruitment of the ubiquitin ligase Fbw7 and ubiquitin-dependent proteasomal degradation (Chen et al., 2011). Phosphorylation of TOP2A is also required for its ubiquitination by BRCA1, which does not lead to proteasomal degradation but to increased decatenation activity (Lou et al., 2005). This emphasises the importance of other post-translational modifications in the regulation of TOP2 ubiquitination. Indeed, SUMOylation of TOP2 can directly lead to the RNF4/ubiquitin-dependent proteasomal degradation of etoposide-induced TOP2-DNA complexes, as discussed further below (Sun et al., 2019).

SUMOylation in TOP2-DNA covalent complex repair

Like ubiquitin, SUMO is conjugated to the lysine residues of target proteins in a 3-step catalytic cascade involving an E1, E2 and E3 enzyme. There are three isoforms of SUMO in human cells (four including the tissue specific SUMO-4); SUMO-1, SUMO-2 and SUMO-3 (Geiss-Friedlander and Melchior, 2007). However, SUMO-2 and SUMO-3 share 97% homology and are often referred to as SUMO-2/3. Like ubiquitin, SUMO can form polySUMO chains, although this is restricted to SUMO-2/3 and not SUMO-1. Interestingly, polySUMO chains are recognised by a class of E3 ubiquitin ligases known as SUMO-targeted ubiquitin ligases (STUbLs). STUbLs, (RNF4 or RNF111 in mammalian cells), contain a SUMO interacting motif (SIM) which binds non-covalently to SUMO and recruits the ubiquitin ligase to SUMOylated proteins (Geoffroy and Hay, 2009). In this way, SUMOylation of proteins, including SUMOylated TOP2-DNA covalent complexes, can lead to their ubiqiuitin-dependent degradation by the proteasome.

A number of studies have shown that TOP2 is SUMOylated in response to etoposide, teniposide and the TOP2 inhibitor ICRF-193 (Agostinho et al., 2008; Isik et al., 2003; Lee et al., 2018; Mao et al., 2000; Schellenberg et al., 2017; Sun et al., 2019), and this seems to occur prior to TOP2 ubiquitination (Isik et al., 2003; Sun et al., 2019). It is now known that SUMOylation of TOP2 by the ZATT E3 ligase facilitates the direct removal of TOP2-DNA covalent complexes by TDP2, as discussed above (Schellenberg et al., 2017). However,

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SUMO is also implicated in other relevant pathways which may affect TOP2-DNA covalent complex processing and repair, such as the SPRTN-dependent pathway (Vaz et al., 2020) and the recently described RNF4/ubiquitin-dependent proteasomal pathway. Following the SUMOylation of TOP2 by the E3 SUMO ligase PIAS4, RNF4 is recruited to TOP2 via its SIM domain and polyubiquitinates TOP2 with K48-linked chains, thereby leading to degradation of TOP2 by the proteasome (Sun et al., 2019). Indeed, knockdown or knockout of RNF4 increases levels of TOP2-DNA complexes and reduces the appearance of etoposide-induced γH2AX levels in a manner which is epistatic with the proteasomal processing pathway (Sciascia et al., 2020; Sun et al., 2019). However, levels of etoposide-induced histone H2AX phosphorylation were higher in RNF4$^{-/-}$ MEFs compared with proteasome-inhibited cells, consistent with the involvement of other ubiquitin ligases in the ubiquitin/proteasome-dependent pathway (Sciascia et al., 2020), such as BMI1/RING1A. Therefore, in addition to the ZATT/TDP2 pathway, SUMOylation of TOP2 can also lead to the proteasomal degradation of TOP2 adducts through RNF4-dependent polyubiquitination of TOP2.

CONCLUSION

Numerous pathways have now been described which facilitate the removal and repair of TOP2-DNA covalent complexes (Figure 1). The existence of multiple redundant pathways is now known, these ensure the timely and efficient repair of TOP2-DNA covalent adducts, thereby maintaining genome stability. However, it is not known what determines which repair pathway is used. Recent work also emphasises the important role of post-translational modifications such as ubiquitination and SUMOylation in the regulation of TOP2-DNA complex repair. There remain many unanswered questions about the timing and function of these post-translational modifications. Whether modulation of the removal and repair of TOP2-DNA covalent complexes with small molecule inhibitors will help to improve outcomes of TOP2 poison chemotherapy, for example by increasing TOP2 poison cytotoxicity and reducing genotoxicity remains to be determined in preclinical model systems.

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Footnotes

Author contributions

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legend

Figure 1: Schematic showing the routes to process TOP2-DNA covalent complexes to protein free double strand breaks suitable for DNA repair.

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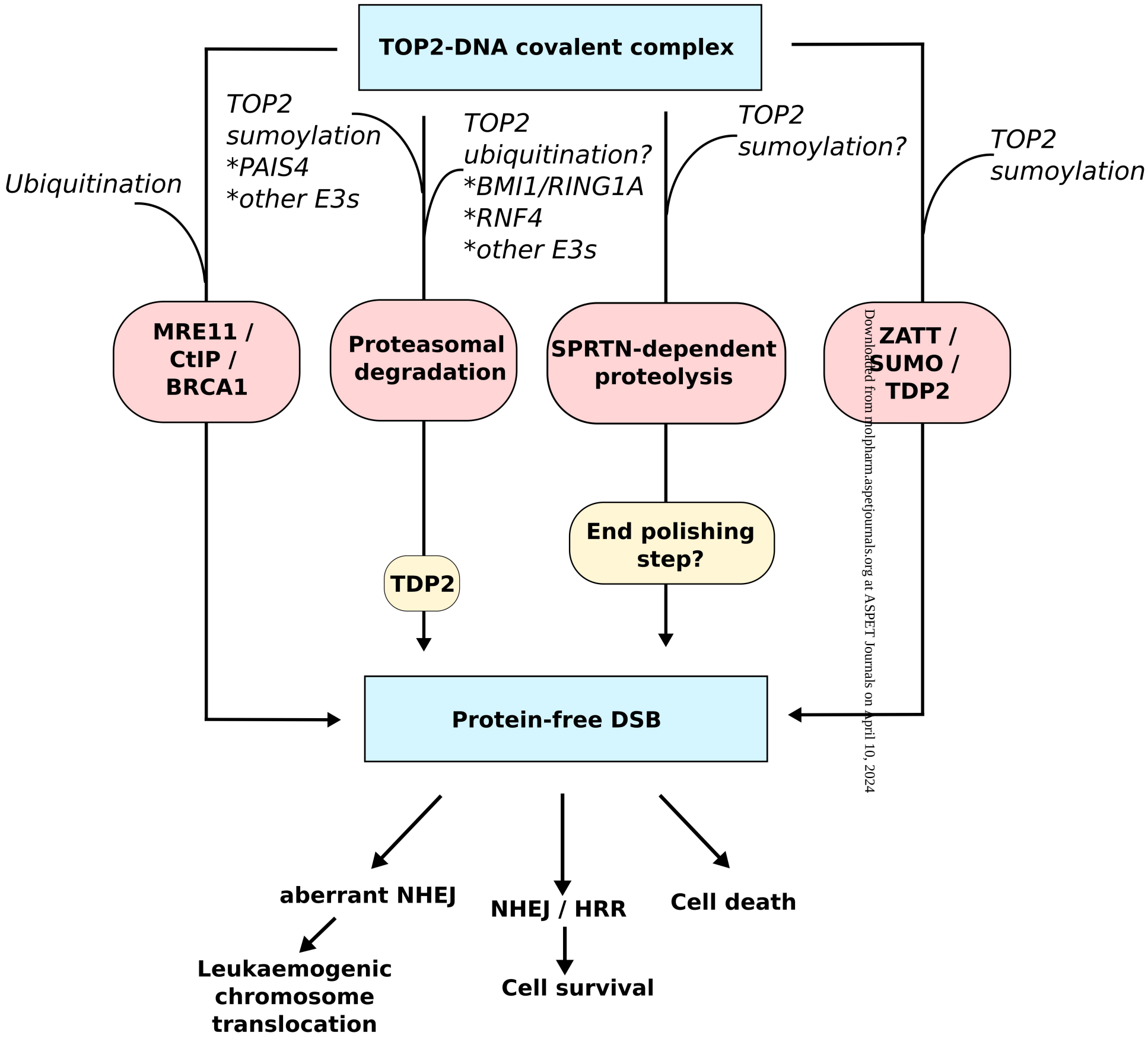

Figure 1