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Review Article

Reviewing the Modification, Interactions, and Regulation of the C-Terminal Domain of Topoisomerase IIα as a Prospect for Future Therapeutic Targeting

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Abstract

Topoisomerase II is a critical enzyme in the regulation of DNA topology during replication, transcription, and mitosis. While the fundamental mechanism is largely understood, the regulation and localization of topoisomerase II in the nucleus is less clear. The enzyme includes a 300 - 400 amino acid C-terminal domain that differs among various isoforms and species. For example, in humans, topoisomerase IIα (TOP2A) and IIβ (TOP2B) share around 30% amino acid identity in the C-terminus, whereas they are about 70% identical across the protein as a whole. Since these two enzymes play significantly different functional roles in human cells, it is of interest to determine the significance of the differences in the C-termini of TOP2A and TOP2B. Importantly, a number of protein-protein interactions and post-translational modifications have been mapped to this region in both isoforms. However, the functional roles of many of the modifications and interactions have not been identified. Therefore, this review sets out to explore the C-terminal domain of TOP2 in order to define the roles that this region plays in regulating function and localization of the enzyme in cells. It is possible that by exploring the C-terminal domain of TOP2A and comparing with that of TOP2B, researchers may be able to segregate regulation of the isoforms and design strategies that enable selective targeting of TOP2A for the treatment of cancer.

Keywords: *Topoisomerase II; Cancer; Pharmacology; Enzymology; DNA*

Abbreviations

BRCT: BRCA1 C-Terminus; CTD: C-Terminal Domain; TOP2: Topoisomerase II

Introduction

Over the last two decades, much progress has been made toward understanding cancer and developing more effective ways to target the disease. Yet, even with the completion of the human genome project, the ENCODE project, and the epigenetics roadmap project, there remains vast gaps in our knowledge of exactly how cancer develops in certain cases and how do we detect cancer earlier and remove it more effectively.

While new agents continue to be developed, there are many anticancer agents that have been in use for 30 - 40 years. Most of these classical agents target fundamental processes and enzymes involved in cell growth and division. For example, etoposide and doxorubicin

both impact the function of DNA topoisomerase II, which regulates DNA topology during critical cellular processes [1-3]. These agents act by using the enzyme to induce DNA damage.

Topoisomerase II in humans is part of a family of enzymes known as type IIA topoisomerases and includes two isoforms: topoisomerase IIα (TOP2A) and topoisomerase IIβ (TOP2B). These two enzymes are encoded by separate genes on different chromosomes and play distinct yet somewhat overlapping functions in the cell [1,2]. TOP2A is involved in unlinking sister chromatids that become catenated during replication and this appears to be an essential function for the α isoform [1,2]. TOP2B is involved in the regulation of chromatin topology during changes in gene expression and during transcription [1,2]. While both of these enzymes appear to be critical for development, growth, and survival in organisms, cultured cells are able to survive without TOP2B [1].

Unfortunately, topoisomerase II-targeted agents are associated with severe and life-threatening adverse events. For instance, etoposide is known to induce the strand breaks that may lead to rearrangements that are associated with acute myeloid leukemia [4]. Additionally, doxorubicin and other anthracyclines all have dose-limiting cardiotoxic effects [5,6]. In both of these cases, there are lines of evidence that point to a role for TOP2B in being involved in the adverse events [6,7]. Unfortunately, the clinically-approved pharmacological agents that target TOP2A and TOP2B cannot distinguish between either isoform [3]. NK314 is one agent that appears to show some selectivity but is not clinically approved [8]. Thus, while TOP2A may be an ideal target, it appears the effects of poisoning and inhibition on TOP2B may be contributing to some toxic and life-threatening side effects. It should be noted that it is unclear whether selective targeting of TOP2A will yield a treatment that is as efficacious as the available therapies.

To this end, researchers have been exploring ways to selectively target TOP2A [9,10]. These efforts generally appear to focus on targeting the enzyme in the catalytic core. It is with this in mind that the current review focuses on exploring TOP2A and provides summary information regarding its known domains, protein-protein interactions, and post-translational modifications. Within this review, we will focus largely on information related to the poorly-understood C-terminal domain (CTD) of the enzyme. We hypothesize that this information may be useful in developing novel strategies for selective targeting of TOP2A through the CTD rather than the core of the enzyme.

Overview of topoisomerase II structure and function

As mentioned above, TOP2A and TOP2B belong to the Type IIA subfamily of topoisomerases. These enzymes resolve knots and tangles in DNA using a transient double-strand break mechanism [11]. While the catalytic cycle has been reviewed elsewhere, the basic concept is that TOP2 generates a transient, enzyme-bound double-stranded break in one segment of DNA and passes an intact double-helix through the double-strand break [3]. Once the second helix is through the break, the first double-helix is ligated and both helices are released. This mechanism requires ATP and divalent metal ions (Mg^{2+}) [11]. Using this mechanism, TOP2 can resolve knots within DNA molecules and untangle intertwined DNA molecules [1].

TOP2 in eukaryotes is a homodimer where two protomers come together to form a functional enzyme. Each protein chain has multiple functional protein domains as seen in figure 1. The ATPase domain is found in the N-terminus at the “top” of the enzyme followed by what is known as the transducer domain, which facilitates communication between the ATPase domain and the cleavage/ligation domain. Next, the TOPRIM domain which bears metal-ion binding residues that are part of the cleavage and ligation reaction in the “middle” region of the enzyme [12]. The region following the TOPRIM is a DNA-binding region that also contains the active site tyrosine. The C-gate or “lower” portion of the enzyme follows this and loops down and back up to the sides. Finally, the CTD, whose structure or conformations is/are yet to be solved for the eukaryotic TOP2 enzymes, is thought to be along the sides of the enzyme perhaps wrapping around the outsides. Each of these sections will be briefly summarized below.

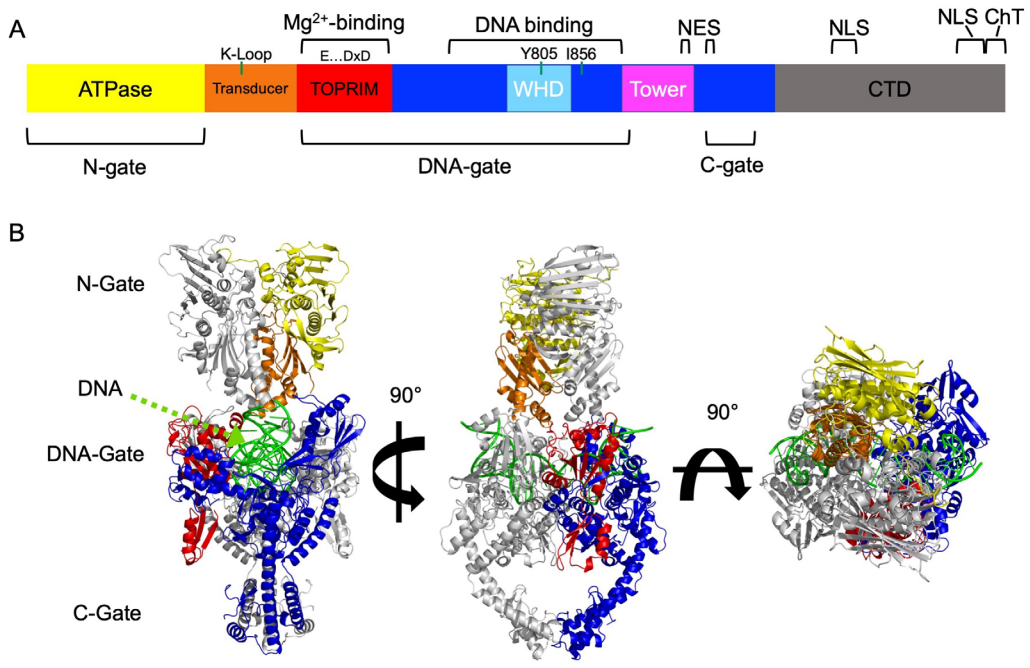

Figure 1: *Topoisomerase II Domain Structure and Homodimer Ribbon Diagram. A) The domain structure is mapped denoting the N-terminal ATPase (yellow), Transducer (orange), metal-ion binding TOPRIM (red), core DNA-binding (blue), and C-terminal domain (CTD, gray). Denoted on the map are several key features including the location of the three "gates", the K-Loop, the metal-binding Glu and Asp residues, the active site tyrosine (Y805), the Winged Helix Domain (WHD), the DNA intercalating Ile (I856), the Tower domain, the Nuclear Export Signals (NES), Nuclear Localization Signals (NLS), and Chromatin Tether Domain (ChT). B) Ribbon diagram of the crystal structure of yeast topoisomerase II missing the C-terminal domain and bound to a DNA segment (RCSB 4GFH). One protomer is colored to match the domain structure, the other monomer is in light gray. DNA "gate" segment (G-segment) is in green. Gate locations are denoted at left. Ribbon diagram was generated using Pymol.*

The ATPase domain is among the GHKL family of ATPases and binds and hydrolyzes ATP in a manner that coordinates with the catalytic process of the enzyme [13,14]. ATP binding appears to occur along with DNA binding and lead to "closure" of the N-terminal "gate" or N-gate of the enzyme [14-16]. Evidence from kinetic studies using yeast TOP2 suggest that hydrolysis may be sequential with one ATP being hydrolyzed along with strand passage and the second being hydrolyzed when after strand passage [14,15,17]. Structural data provide evidence that the ATPase domain may rotate during or after strand passage, which may prevent back-tracking of the double-helix through the DNA-gate [14].

The Transducer domain immediately follows the end of the ATPase domain and connects to the TOPRIM domain. This domain forms the sides or walls of the N-gate clamp during catalysis. A series of Lys residues, termed the K-loop (K342-K346, Figure 1) appears to be positioned to interact with the gate-segment DNA.[14] In addition, evidence indicates that this domain may play a role in communication between the ATPase domain and the cleavage-ligation domain.[16] In particular, the QTK loop (Q376, T377, K378) may help keep the N-terminal gate open when there is no ATP bound to the ATPase domain [16].

The TOPRIM (topoisomerase/primase) domain is the location of metal ion binding [12]. In particular, this region binds divalent cations, typically thought to be Mg^{2+}, that participate in DNA cleavage and ligation [11]. This domain contains a motif found in several

metal-ion-dependent enzymes that includes a nearly invariant Glu followed further down the sequence by an Asp-x-Asp (DxD) or an Asp-x-Asp-x-Asp (DxDxD) motif [12]. The TOPRIM together with the active site region serve as the DNA-gate or cleavage/ligation domain, as well. This region includes a DNA binding domain that encompasses the active site Tyr residue (Y805 in TOP2A) and the winged-helix Domain (WHD) [18]. The winged-helix is a helix-turn-helix motif found in a some DNA binding proteins where the "wings" are loops made from small beta-sheets which can interact with the minor groove.

Interestingly, the TOPRIM domain of one protomer interacts with the active site Tyr and WHD of the opposite protomer to form an active site. This symmetry provides an explanation for why the enzyme is unable to release DNA when the DNA gate is open: the TOPRIM and active site Tyr separate when the DNA gate opens, which prevents ligation from occurring until the gate closes. This region also includes the 'tower' domain that forms part of the DNA interaction interface with the G-segment [19].

Following the cleavage/ligation domain, long alpha helices loop down to the lower C-gate of the enzyme before looping back up. Structural evidence indicates that this portion of the enzyme opens to release the transported segment from the enzyme [20]. The α-helices that loop back up the sides of the enzyme connect with the C-terminal domain, which is the least-understood portion of the enzyme. It is this C-terminal domain that will be the focus of this review.

Overview of C-terminal domain features and functions

In human TOP2A, the C-terminus begins around amino acid 1172 and goes to the end of the protein. This region of over 350 amino acids contains some functional motifs that have been identified and is the site of dozens of post-translational modifications (Figure 2). It may also serve as the site of protein-protein interactions, but there are only a few confirmed examples of this so far, which will be discussed below.

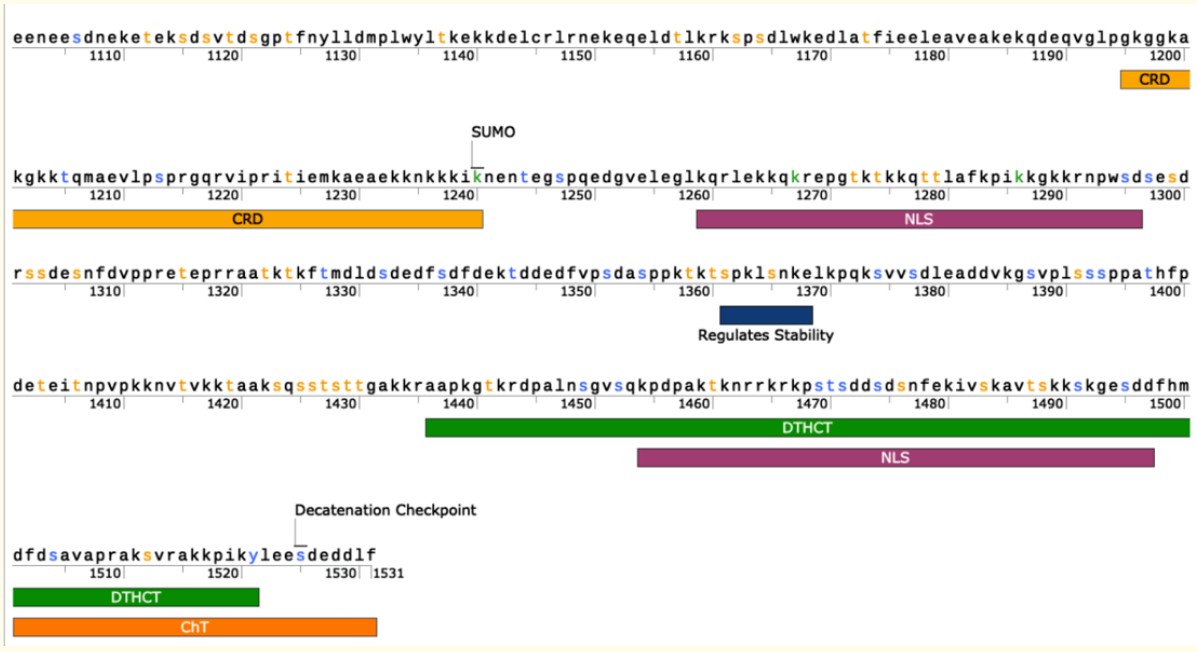

Figure 2: *Topoisomerase IIα C-terminal Domain. The key features of the C-terminal domain are mapped onto the sequence of TOP2A. Residues in blue are known to be modified in association with mitosis. Residues in orange may or may not have known functional consequences. Some residues suspected to be involved in sumoylation are highlighted in green. The C-terminal regulatory domain (CRD), Nuclear Localization Sequences (NLS), DNA Gyrase B, Topoisomerase IV, and HATPase C-Termini (DTHCT), and chromatin tether (ChT) domain are all denoted.*

Structure of the CTD

While the eukaryotic TOP2 CTD structure has not been solved, it is predicted to be a relatively unstructured region due to the "low complexity" sequence through that region [21]. The CTD of DNA gyrase (GyrA subunit), a bacterial type II topoisomerase has been solved [22]. The structure has been described as a propeller and participates in DNA wrapping around the enzyme [22]. However, this sequence is highly variable among type II topoisomerases, and it is unclear whether the bacterial structures are shared by the eukaryotes. As discussed below, the eukaryotic CTD is involved in DNA interactions, but the actual structure of this domain is less clear and may involve multiple conformations that could result from differential post-translational modifications as mentioned below. Interestingly, the CTD influences the ability of CTD to bind and cleave DNA [23]. Further, swapping the CTD regions of TOP2A and TOP2B leads to a change in DNA substrate preference, indicating a key role for this region in interacting with and "selecting" target regions on the DNA [24,25]. These findings bring about the intriguing possibility that the CTD could be targeted therapeutically in order to disrupt TOP2A function.

Nuclear localization sequences (NLS)

Studies of the NLS in TOP2A support the presence of two separate regions having NLS activity. The first is between 1259-1296, and the second is 1454-1497 [26,27]. Both of these have been examined by disruption and other molecular biology techniques to try to validate whether these can signal for import into the nucleus. The strongest NLS in TOP2A is the sequence at amino acids 1454 - 1497 [26,27]. Studies have found that all three basic residues and the Lys-Ser-Lys (1490 - 1492) in this sequence are required for NLS activity [27,28]. The other NLS at 1259-1296 is moderately functional and will permit TOP2A to localize to the nucleus even in the absence of the main NLS [26]. Separate studies have identified importin α1, α3 and α5 with having the ability to bind to TOP2 [29]. Interestingly, in the region before the C-gate (AA 1017 - 2018 and 1054 - 1066), there are also Nuclear Export Sequences (NES) that have been identified [30]. The literature supports an interaction of TOP2A with CRM-1 (exportin), but there is some debate about whether TOP2 is shuttled from the nucleus to the cytoplasm [29,31]. A series of studies demonstrate that inhibition of CRM-1 can prevent removal of TOP2 from the nucleus and sensitize cancer cells to TOP2-targeted anticancer agents [32].

C-terminal regulatory domain (CRD)

A 2014 study found that in a dynamic equilibrium, TOP2B can alternate between an active form when in the nucleoplasm and an inert form when in the nucleolus [33]. The mechanism of this switch in activity relies on RNA's binding to a region of the CTD from 1201 - 1250 in human TOP2B (1195 - 1240 in human TOP2A). The binding of RNA to this site inhibits the catalytic actions of TOP2B *in vitro*. The region involved in this RNA-tethering process was termed the C-terminal regulatory domain (CRD) and may be essential for the enzyme's accumulation in the nucleoli [33]. The differential locations and strengths of the NLS in topoisomerase isoforms may lead to their unique nuclear localization patterns. In yeast expressing mammalian TOP2A CTD and TOP2B CTD tagged with yellow fluorescent protein, TOP2A CTD was dispersed throughout the nucleus while TOP2B CTD was concentrated in a subnuclear compartment [34]. Importin- α isoforms have been shown to be involved in the transport of TOP2A and TOP2B to the nucleus from the cytoplasm [29].

Chromatin tether domain (ChT)

In 2013, researchers identified a region at the extreme C-terminus of TOP2A including residues 1,500 - 1,531 that appears to be very important, though possibly not absolutely required, for binding to chromatin [35]. Deletion of this region of the protein results in several defects in chromosomal processes. The ChT appears to be important for interaction with DNA and with histone H3 [35]. The ChT facilitates chromatin association and increases the residency time on mitotic chromosomes through an interaction with histone H3. The details of these interactions are still to be examined. Lane., *et al.* concluded that the ChT is essential for mitotic chromosome condensation and segregation and plays a role in the localization of the enzyme to chromosomes during mitosis [35].

DTHCT

One last domain is important to mention. A 2004 study into the domain structure of nucleolar proteins identified a region that spans 1435 - 1521 in human TOP2A [36]. This low complexity region was termed DTHCT because it has sequence features found in DNA Gyrase B, Topoisomerase IV, and HATPase C-Termini [36]. While this region has not been examined further in publications, it does overlap with the ChT mentioned above. However, it includes additional residues that are not part of the ChT. As discussed below, this region includes several key Ser and Thr that serve as phosphorylation sites in TOP2A, which appear to be associated with mitosis and chromosome segregation (Figure 2). Additional studies are needed to better understand the roles of this region in various proteins across different organisms.

Summary of key post-translational modifications in CTD

It has been known for some time that phosphorylation and other modifications impact the activity of TOP2 [37,38]. According to PhosphositePlus (Jan. 2020), there is evidence of approximately 208 distinct sites of post-translational modifications in TOP2A [39]. The modifications include a mix of phosphorylation, acetylation, ubiquitylation, and sumoylation (Figure 3). There are also a few sites with evidence of methylation. Ubiquitylation has been detected at many sites across the protein. However, in the CTD, phosphorylation accounts for the majority of post-translational modifications [38]. Out of the 208 sites identified along TOP2A as of this writing, 87 modifications occur in the CTD. Most of these modifications are poorly understood, with a substantial portion of the data arising from high-throughput screens [38]. In order to select modification sites of interest, we filtered the output from PhosphositePlus to include only modifications with a minimum of five references (see red line in figure 3B), which generally included at least one associated low-throughput study. Any sites meeting this threshold of evidence were examined further. The following section is a summary of studies which sought to characterize the nature and significance of some of the best-studied post-translational phosphorylation sites in the CTD.

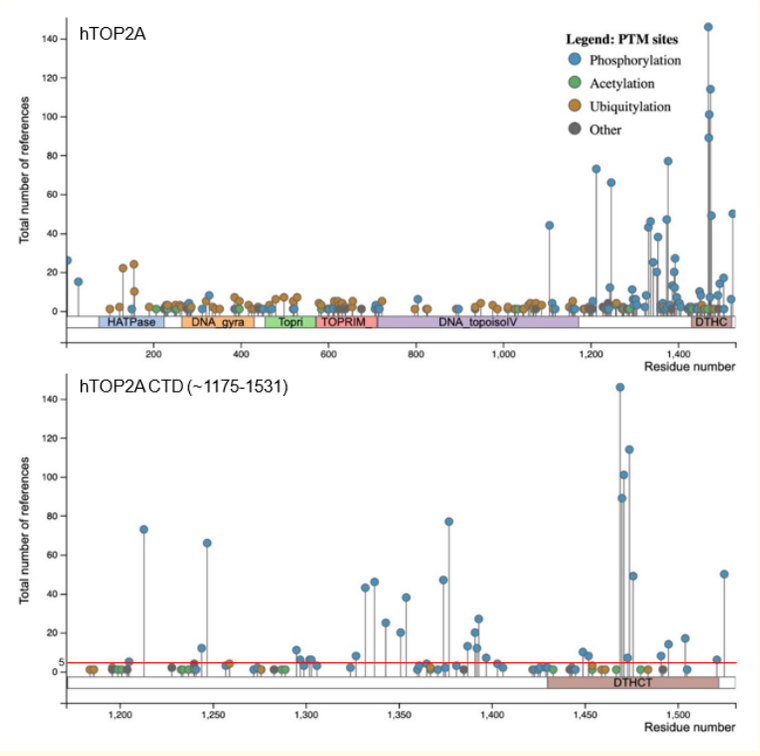

Figure 3: *Post-Translational Modification Sites on Human Topoisomerase II. Top panel: putative modifications across the entire TOP2A protein. Bottom panel: putative modifications in the C-terminal domain of TOP2A. Images were generated using PhosphoSitePlus at the following URL: https://www.phosphosite.org/proteinAction.action?id=2303.*

Modifications associated with mitosis

Considering TOP2A's increased presence in dividing cells and its hypothesized roles in mitosis, it is no surprise that many of the well-studied post-translational modifications are mitosis-specific. Of the phosphorylation sites found to be cell cycle dependent, several have been associated with the enzyme's activity during chromosome condensation and chromatid segregation.

Previously, it was shown that S1213 (previously referred to as S1212) is a mitosis-specific phosphorylation site in HeLa cells [40]. P34-cdc2 and MAP kinase phosphorylate several serine residues in the C-terminus *in vitro*, some of which are modified during G2/M phase in HeLa cells [40]. Of these phase-specific phosphorylation sites, S1213 was identified as a major phosphorylated position in cellular and purified systems [40]. It should also be noted that the MAP kinase ERK2 impacts TOP2A activity in a manner that does not require the kinase activity of ERK2 [41]. From immunofluorescence data, it has been determined that S1213-phosphorylated TOP2A relieves topological strain beginning at the chromosome arm, and moves to the centromere during metaphase chromosome condensation [42]. A more recent study offers insight into the mechanism by which S1213 influences the enzyme's localization to centromeres. The study characterized S1213 and S1525 (previously referred to as S1524) of TOP2A as *in vitro* CDC7 phosphorylation sites through a chemical genetics approach [43]. CDC7 is involved in replication stress response, and as a complex with DBF4 is essential in DNA replication origin firing. The study suggests that CDC7/DBF4 phosphorylates TOP2A at S1213 and S1525 in early S-phase, which may be involved in delaying TOP2A's localization to centromeres [43]. This finding is consistent with the previous results obtained by Ishida., *et al* [42].

A study by Antoniou-Kourounioti., *et al.* using HEK-293T cells suggest that S1213 and S1247 are substrates of cyclin-dependent kinase 1 (CDK1) [44]. Phosphorylation at these sites increase the efficiency that TOP2A is maintained at the centromere as the cell progresses towards anaphase. Additionally, the researchers argue that phosphorylated S1213 increases the protein's mobility, leading to lower residence time on mitotic chromosomes. Phosphorylation of the residues at T1244 and S1247 promote sumoylation at K1240, which in turn enhances TOP2A accumulation at centromeres during metaphase [44]. These results are consistent and expand on the earlier finding by Wells and Hickson that S1247 was primarily phosphorylated during the G2/M transition [40].

Using purified proteins and whole-cell lysates from HeLa cells, one study proposes that Pin1 promotes phosphorylation of TOP2A at S1213 by cdc2/cyclin B during the G2/M phase [45]. This is potentially achieved through a conformational change on TOP2A that is induced by Pin1 binding. Interestingly, the study found that Pin1 was required for effective TOP2A phosphorylation during mitosis [45]. The authors suggest that through this mechanism Pin1 plays a central role in chromosome condensation in mitosis by stimulating TOP2A's DNA-binding and chromosome condensation activities [45].

Polo-like kinase 1 (Plk1)-mediated phosphorylation at S1337 and S1525 arises in S-phase and peaks in mitosis, and was found to be required for normal sister chromatid segregation [46]. Phosphorylation at S1337 and S1525 by Plk1 in HeLa and HEK293 cells may stimulate TOP2A's catalytic activity by modifying at these sites [46].

A handful of other post-translational modifications in the C-terminus have been shown to correspond with the transition into mitosis and may regulate TOP2A's mitotic functions. Investigations into the mechanism of modification at these residues have revealed that some are controlled by multiple interacting proteins. Unlike the previously discussed modifications, however, the effects of cell cycle dependent phosphorylation at T1343, S1469, and S1525 on the enzyme's functions have not been determined. The dynamic relationships between various proteins and TOP2A during the G2/M transition provide valuable insight into the enzyme's complex regulatory network, and how the C-terminal domain is a key player in mediating isoform-specific functions.

T1343 has been shown to be phosphorylated in HeLa cells throughout the cell cycle, but phosphorylation at this site was increased during G2/M phase [47]. The researchers focused on T1343 as a target of casein kinase II (CKII). CKII also phosphorylates T1343 during

mitosis in whole mitotic cells and mitotic cell extracts [48]. Interestingly, the related enzymes, casein kinase Iδ and CKIε are able to phosphorylate TOP2A just outside the CTD at Ser-1106 [49]. There is evidence to support that Pin1 interacts with CKII and/or TOP2A to inhibit the phosphorylation of T1343 by CKII [50]. In order for Pin1 to inhibit TOP2A phosphorylation, both the protein binding activity of the Trp-Trp (WW) domain, which allows Pin1 to interact with target proteins in a phosphorylation-dependent manner, and the isomerase domain of Pin1 are required [50]. A different study demonstrates that Plk3 phosphorylates T1343 on TOP2A *in vitro* and in HEK293 cells, while Plk1 did not act on this residue [51].

The cell-cycle specific phosphorylation of S1469 and S1525 on TOP2A may be controlled by the differing specificities of CKII and protein phosphatase 2A (PP2A) [52]. The researchers had previously shown that CKII phosphorylates S1469 during mitosis, contributing to a mitosis-specific phosphoepitope of TOP2A but not altering the enzyme's catalytic activity [53]. During interphase, CKII is present in the nucleus along with TOP2A [48]. The kinase is able to phosphorylate S1469 and S1525, producing the mitotic MPM-2 phosphoepitope, which is able to be bound by the MPM-2 antibody [53]. The MPM-2 antibody recognizes a mitosis-specific phosphoepitope [54]. Since the phosphatase, PP2A, is also in the nucleus during interphase, its stronger substrate preference for S1469 prevents that site from remaining phosphorylated. Meanwhile, CKII has greater activity at S1525 than PP2A, so S1525 remains phosphorylated throughout interphase and early mitosis [52]. As the cell enters mitosis, PP2A exits the nucleus while CKII remains nuclear until pro-metaphase. These experiments, completed in HeLa cells and purified systems, provide evidence that the change in protein distribution during the onset of mitosis allows for CKII to phosphorylate both S1469 and S1525. Based on these findings, this system appears to regulate TOP2A's mitotic functions [52].

Wells., *et al.* identified that S1525 was a phosphorylation substrate of CKII, but this site was not found to be phosphorylated in a cell-cycle dependent manner as the researchers did not see a change in phosphorylation levels of S1525 across the G2/M transition in HeLa cells [40,55]. However, more recent studies have found conflicting evidence about S1525's phosphorylation status throughout the cell cycle.

For example, one study found that S1525 becomes phosphorylated primarily at the G2/M transition and may have a role in what has been named the "decatenation checkpoint" [56]. While a S1525A mutant retained the ability to decatenate in a purified system, cells displayed increased levels of pseudomitosis [56]. Researchers found that the S1525 residue is required to bind the enzyme to MDC1, a checkpoint protein, at the BCRT domain. The authors hypothesize that in response to chromosome entanglement, phosphorylated S1525 becomes exposed, which allows binding to MDC1, which may provide a means for activation of the decatenation checkpoint [56].

Other effects of phosphorylation

Still many of the phosphorylation sites in the C-terminus are not specific to mitosis. These modifications may play a diverse set of roles in modulating TOP2A. Various studies have identified sites whose phosphorylation status has effects on the protein's stability and degradation.

An analysis of TOP2A phosphorylated sites in HeLa cells revealed that phosphorylation at S1354 and S1361 were not cell-cycle dependent, but their dual phosphorylation inhibited cleavage by trypsin at an identified cleavage site between the two residues [40]. Simultaneous phosphorylation at these two sites may have cut off trypsin's access to the cleavage site, which may reflect the flexible nature of the C-terminal domain.

A study, using both *in vitro* and *in vivo* methods, implicated that the series of amino acids from S1361 to E1368 in the CTD participate in regulating the stability of TOP2A [57]. The researchers suggest that CKII phosphorylates S1365, acting as a priming kinase for GSK2β, which then phosphorylates S1361. A complex containing Csn5 and Fbw7, an E3 ubiquitin ligase, is then recruited to the resulting dually

phosphorylated residue, and facilitates its ubiquitin-dependent degradation [57]. The same study also indicated that this regulation does not appear to occur at the sequence between S1393 and T1397 under the conditions tested [57].

P38γ may enhance TOP2A's protein stability via phosphorylation at S1525, according to one study [58]. Interestingly, p38γ was activated by TOP2A-targeting drugs and phosphorylated S1525, preventing it from being degraded by the proteasome pathway. P38γ also increased TOP2A gene expression and may positively regulate the enzyme's expression in primary cancer cells.

Drawing from *in vitro* studies with p34-cdc2 and *in vivo* studies with MAPK as the acting kinases respectively, phosphorylation at S1393 and S1377 did not appear to be cell cycle-dependent [40]. S1377 is also phosphorylated both *in vitro* and *in vivo* by CKII [55].

Other post-translational modifications in the CTD

While phosphorylation sites dominate the CTD of TOP2A, several other residues have been identified as targets for acetylation, ubiquitylation, methylation, and sumoylation. Of these post-translational modification sites, very few of them have been studied for their effects. For example, sumoylation at K1240, K1267 and K1286 have been demonstrated in *Xenopus laevis* and K1240 in humans [44,59,60].

Ryu., *et al.* identified several sumoylated residues in 2015 using both an *in vitro* and yeast expression system [59]. They found that sumoylation at the three sites mentioned above promotes protein binding. The cell cycle checkpoint mediator Claspin contains two SUMO-interacting motifs (SIMs), which allow it bind to centromeres of mitotic chromosomes when TOP2A is sumoylated at K1235, K1276, and K1298 in *Xenopus laevis*, which corresponds to K1240, K1267, and K1286 in human TOP2A, respectively [59]. In another study, researchers using *Xenopus laevis* egg extracts showed that this modification is also involved in the centromeric localization of Haspin, the histone H3 kinase, and the phosphorylation of histone H3 at threonine 3 [60]. Additionally, Haspin and the TOP2A CTD sumoylation-dependent phosphorylation at H3T3 play a role in recruiting Aurora B from inner centromeres to kinetochore-proximal centromeres and the core of chromosome arms upon TOP2A catalytic inhibition [61,62]. Evidence suggests that catalytic inhibition of TOP2A activates a metaphase checkpoint during which the enzyme is sumoylated in the CTD, leading to the mobilization of Aurora B kinase. Recruitment of Aurora B in response to inhibited TOP2A eventually causes metaphase arrest in HeLa cells [62].

K1240 was also found by Antoniou-Kourounioti., *et al.* to be a significant sumoylation site in the CTD [44]. As discussed in the above section, sumoylation at K1240 is facilitated by phosphorylation at T1244 and S1247. This modification appears to play a role in the enzyme's centromeric localization during metaphase [44].

Summary

As seen from the brief survey above, most of the studies related to TOP2A CTD phosphorylation sites demonstrate effects in various stages of mitosis. While there are some examples of other impacts on activity or function, these appear to be the minority of the evidence found so far. It is also unclear why more of the modification sites have not been shown to have a clear role. While it is possible that some of the results in PhosphositePlus represent spurious or aberrant modifications, it is also possible that these sites operate in concert. Thus, studies examining changes at single positions may not be able to detect an impact on function. Whereas, studying multiple sites simultaneously may aid in understanding the roles and functions of these modifications. This may require careful and detailed site-directed mutagenesis and equally detailed and well-controlled experimental analysis to clarify the impact of these modifications. Also, this work may be needed to address and more clearly understand the role of protein-protein interactions in TOP2 function. This topic will be the focus of the next section.

Survey of known and putative protein-protein interactions

As seen above, TOP2A interacts with several kinases and other modifying proteins, but this appears to only be part of the interaction story of TOP2A. In reviewing the BioGrid database (BioGrid.org), dozens of protein-protein interactions are identified in low- and high-throughput screens [63]. As of early 2020, there were around 129 unique physical interactions documented between TOP2A and other proteins (Figure 4). Interestingly, TOP2B also displayed evidence of dozens of physical protein-protein interactions with ~46 proteins overlapping between the two isoforms. Compared with data from a 2009 review, the number of possible interaction partners for TOP2 has expanded significantly [1].

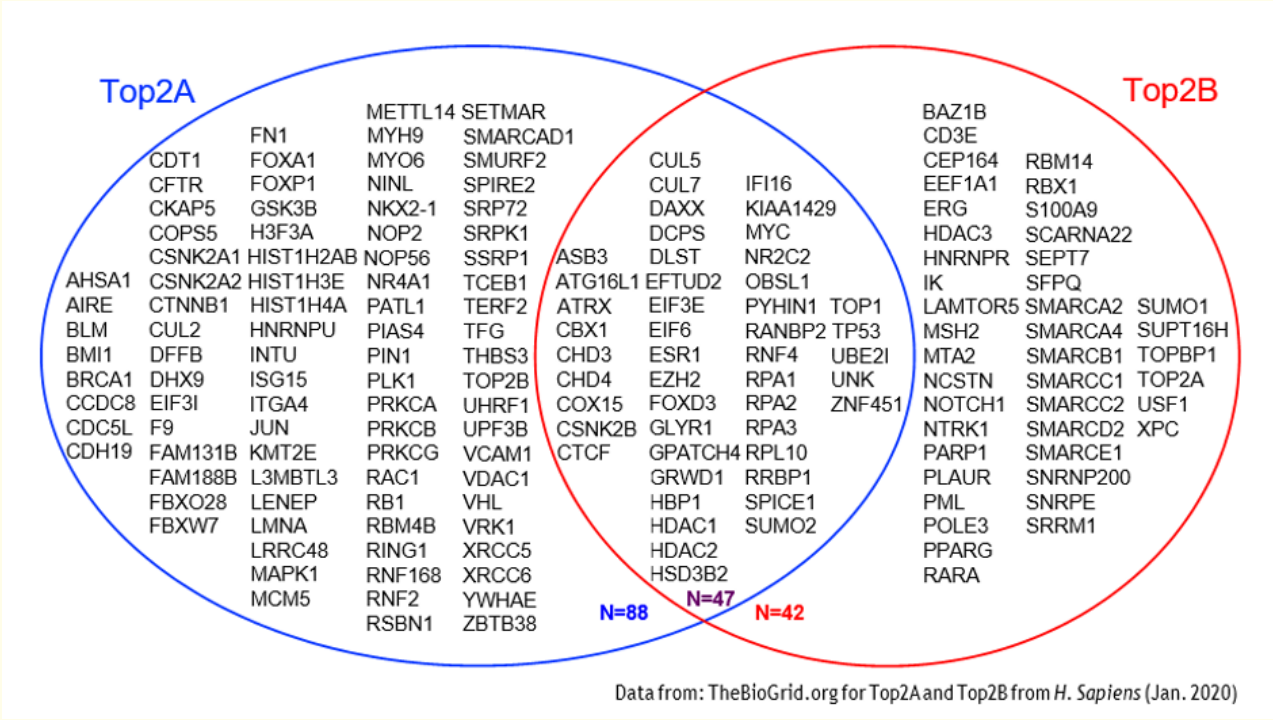

Figure 4: *Topoisomerase II Protein-Protein Interactions Catalogued in the BioGrid Database. Protein-protein interactions were compiled from the BioGrid database (TheBioGrid.org) for human TOP2A and TOP2B as of January 3, 2020. Proteins that interact with TOP2A are in the blue circle, while those that interact with TOP2B are in the red circle. The overlapping area represents proteins with evidence for interacting with both isoforms. Genetic interactions and some non-human protein interactions were excluded from this diagram.*

While the BioGrid data is helpful, it should be noted that a lack of evidence for an interaction between either TOP2A and TOP2B and a given protein does not always mean they do not actually interact. Further, not all of the putative interactions will be found to be biologically relevant. In addition, the available data often does not clarify which of these interactions occurs with the CTD of TOP2A. Only a very small portion of the proteins in the BioGrid list have detailed interaction information where the location and consequences of the interaction are fully characterized. In spite of this, it is informative to consider which proteins have been identified and to examine the functions and roles of those proteins. As seen in table 1, the proteins that interact with TOP2A but not TOP2B, according to current

evidence, fit into many of the expected categories and functions--replication, transcription, mitosis/chromosome segregation, DNA repair, etc. However, other functions were not necessarily predictable, yet were found to have representative proteins that have evidence of TOP2 interaction. What role or roles these interactions play in the regulation of TOP2 activity and/or the regulation of larger cellular processes remains largely unexplored.

Transcription/Chromatin:	Signaling and Phosphorylation:	Cell Structure, Surface, Transport, and Adhesion:	Ubiquitination/Sumoylation:	DNA Replication and Repair:
BMI1	CSNK2A1	CDH19	COPS5	BLM
FOXA1	CSNK2A2	CFTR	CUL2	BRCA1
FOXP1	CTNNB1	FN1	FAM188B (MINDY4)	CDT1
H3F3A	GSK3B	INTU	FBXO28	DFFB
HIST1H2AB	MAPK1	ITGA4	FBXW7	DHX9
HIST1H3E	NR4A1	LMNA	PIAS4	MCM5
HIST1H4A	PIN1	LRRC48 (DRC3)	RING1	TERF2
HNRNPU	PLK1	MYH9	RNF168	XRCC5
JUN	PRKCA	MYO6	RNF2	XRCC6
KMT2E	PRKCB	SPIRE2	SMURF2	
L3MBTL3	PRKCG	SRP72	VHL	
LENEP	RAC1	TFG		
METTL14	SRPK1	THBS3		
NKX2-1	VRK1	VCAM1		
RSBN1	YWHAE	VDAC1		
SETMAR				
SMARCAD1				
SSRP1				
TOP2B				
UHRF1				
ZBTB38				

Translation:	Cell Cycle/Mitosis:	Immune Function:	Chaperone:	Unknown Function:
EIF3I	CCDC8	AIRE	AHSA1	FAM131B
TCEB1/ELOC	CDC5L	F9		
NOP2	CKAP5	ISG15		
NOP56	NINL			
PATL1	RB1			
RBM4B				
UPF3B				

Table 1: *BioGrid putative protein-protein interactions unique to TOP2A.*

As mentioned in a previous section, several kinases and ligases have been implicated in modifying the CTD. Those will not be discussed again here. Instead, we will survey a few examples of proteins with known TOP2A CTD interactions.

CRM1 or Exportin-1 is a nuclear export receptor that pairs with Ran-GTP to escort proteins bearing a nuclear export signal (NES) out of the nucleus. While the NES in TOP2A are not in the CTD as has been defined, they are not far from it and clearly are important for the regulation of function. There are two identified NES sites that occur at the dimer interface (1017 - 1028 and 1054 - 1066), which may become accessible after phosphorylation of residues in the area and allow for binding by CRM1 [31]. Interestingly, it has been found that mutation of S1525 to Ala prevented nuclear export [32]. The authors suggest is due to the inability to phosphorylate this position, which they independently verified using an inhibitor to casein kinase II [32]. This evidence suggests that distal, C-terminal modifications can impact positions within the protein and regulate protein-protein interactions.

MDC1 (mediator of DNA damage checkpoint protein-1) is a checkpoint protein that contains a BRCT (BRCA1 C-Terminus) domain, which has been found to interact with P-S1525 and participate in what is called the “decatenation checkpoint” [56,64]. Disruption of this interaction via mutation of S1525 prevents the interaction of MDC1’s BRCT domain with TOP2A and also prevents the activation of the decatenation checkpoint [56]. This evidence suggests a major role for P-S1525 in nuclear export and checkpoint activation. Interestingly,

evidence from a previous study demonstrated that TOP2A also interacts with BRCA1 [65]. This interaction has also been more recently identified in a high-throughput screen [66]. Neither of these studies examined whether the BRCT domain in BRCA1 interacts with the CTD of TOP2A, but it seems likely that the interaction would likely be similar to that of TOP2A and MDC1, especially given the fact that the earlier study suggested a role in the decatenation checkpoint.

Histone H3.1 and H3.3 have both been shown to interact with the extreme CTD of TOP2A (~1501 - 1531), which includes the S1525 mentioned above [35]. This region has been termed the chromatin tether (ChT) domain by the authors of that study [35]. The ChT domain apparently mediates interactions with the histones mentioned above and DNA.

PLSCR1 (phospholipid scramblase 1) was initially discovered as a protein involved in phosphatidylserine externalization on the plasma membrane. Interestingly, this protein interacts with a specific region of the CTD (1432 - 1441) and stimulates decatenation [67]. The same study also identified an interaction with TOP2B. More recent work has identified a nuclease activity and demonstrated that PLSCR1 may have other roles in cells [68]. At this point, the exact role and impact of this interaction with either isoform of TOP2 needs further clarification.

Discussion and Implications

As discussed above, the CTD of TOP2A is the target of multiple modifications and interactions. The details and implications of many of these interactions remain to be elucidated. It is clear from the data above that the CTD of TOP2A is a critical, functional regulatory domain. This portion of the protein controls localization, impacts protein stability, facilitates protein and chromatin interactions, and serves as a recruitment site for proteins.

While these functions and roles have been elucidated, the nature of the interactions between the CTD of TOP2A and other protein partners has not been as clearly established. As noted above, some proteins have known interaction sites with TOP2A, but many of the suspected protein partners have no biochemical or structural data to indicate where on TOP2A they interact. This is further clouded by the fact that the CTD has not been crystallized for any of the eukaryotic TOP2 enzymes, which makes it difficult to identify structural motifs/interaction domains.

Additionally, secondary structure and disorder prediction algorithms show that the CTD of TOP2 is considered low complexity sequence and is expected to be highly disordered. Therefore, it may not be possible to crystallize the structure since it does not appear to take on strong secondary structural features. However, it may be possible that interactions with DNA, chromatin, or various protein partners cause the CTD to take on specific conformations. This remains to be demonstrated experimentally. If it is the case that interactions between the CTD and other molecules leads to more defined conformations, it may be possible to explore the nature of those interactions and to potentially develop probes or even inhibitors to promote or disrupt various interactions.

Another possibility to consider is that the disordered nature of the CTD may enable the protein to participate in other interactions in the nucleus that have yet to be explored, such as phase separation. It is possible that the disordered CTDs of TOP2A and TOP2B may facilitate phase separation interactions in the nucleus. These interactions, if they exist, may help clarify the how the roles of TOP2A and TOP2B are segregated in the nucleus.

Conclusion

Having surveyed the post-translational modifications and protein-protein interactions of the CTD of TOP2A, it is clear that the CTD is a functional regulatory domain of TOP2A. Presumably, this model may provide some foundation for understanding the CTD of other TOP2 enzymes. It is also important to note that many modifications and protein-protein interactions have not been characterized yet.

Thus, their roles and significance are unclear. Additionally, it will be enlightening to compare TOP2A and TOP2B to determine which interactions and modifications may play critical roles in the regulation of each isoform. This may serve as a way to identify strategies for isoform-specific targeting, which will be helpful in designing the next generation of TOP2-targeted agents.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

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