Topoisomerase II: Characterization of the Topoisomerase II Checkpoint and the Classic *top2-4* mutant allele

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DEDICATION

This dissertation is dedicated to the strong women in my family. My mother and grandmothers have been a guiding light in my life and in my education. They are intelligent, educated, and driven women who have been an example and a motivation for me through my entire life.

ABSTRACT

Topoisomerase II (Topo II) is an essential, highly conserved enzyme that is a major component of mitotic chromosomes. Topo II functions as a homodimer to perform an ATP-dependent strand-passage reaction. In this reaction, Topo II transiently cuts one dsDNA molecule to allow the passage of a second dsDNA molecule. This cycle resolves knots in the DNA (catenanes) that form during replication and this resolution is essential for mitosis. Progression through the cell cycle in the absence of Topo II results in mitotic catastrophe (HOLM *et al*. 1985). The Clarke lab identified and provided genetic evidence that there is a Topo II checkpoint in budding yeast (ANDREWS *et al.* 2006).

Failure of the Topo II checkpoint results in aneuploidy and reduced cell viability (ANDREWS *et al.* 2006). Unexpectedly, by using DNA damage checkpoint components Andrews *et al*. found that the Topo II checkpoint does not enforce a G_2/M delay, but rather requires a subset of spindle checkpoint proteins. The Topo II checkpoint is different from the spindle checkpoint in that it is not activated when the spindle is damaged or when there is a lack of tension. Additionally, it does not delay cell cycle progression through Pds1-dependent inhibition of Esp1/separase (ANDREWS *et al.* 2006). Thus, the Topo II checkpoint is distinct from other known checkpoints.

Due to the recent discovery of the Topo II checkpoint there is little known about what the checkpoint monitors or what is required for checkpoint signaling. To begin to answer these questions, we needed a way to look at a single cell cycle in the absence of Top2. Top2 is essential in cells; it cannot simply be deleted. Instead, a Top2 degron system was constructed in *Saccharomyces cerevisiae*.

Using the Top2 degron we found that the checkpoint is dependent upon the presence of Top2 protein in the cell during the cell cycle. Under these conditions, the

chromosomes should have abnormal condensation, kinetochore biorientation should be perturbed, and catenations should persist. Surprisingly, despite all this, the checkpoint is unable to sense the disturbance when there is no Top2 protein present.

The Top2 degron system allows for the expression of Top2 mutants, which would normally be inviable. We found that mutants that affect the ability of Top2 to open its DNA gate to allow T-segment transport also activate the checkpoint. However, mutants that affect the strand-passage cycle at a later step do not activate the checkpoint. This is the first step in our mechanistic understanding of what the checkpoint monitors.

In our attempt to begin to understand what is required for Topo II checkpoint activation, we found that the C-terminal tail of Top2 is necessary. Previous research has shown that this tail can be deleted without disturbing the strand-passage reaction or completion of the cell cycle (CARON *et al.* 1994 and JENSEN *et al.* 1996). However, our research shows that the checkpoint cannot be activated when the tail is deleted and that overexpression of the tail interferes with checkpoint activation. The tail is highly post-translationally modified (ALGHISI *et al.* 1994 and BACHANT *et al.*2002) and most of these modifications have no known function. We hypothesize that the tail might be used to initiate Topo II checkpoint signaling and that it could provide a docking site for the initiating proteins.

Finally, we more closely examine a well-used temperature sensitive mutant allele of *top2*, *top2-4*. This mutant was first identified and characterized by Holm *et al.* in 1985. It has since been extensively used as a null form of *top2*, when grown above its restrictive temperature. We found that this mutant does not behave as a null. Not only is the Top2-4 protein stable above restrictive temperature, but it also has a dominant effect on the ability of Top2-B44 to activate the Topo II checkpoint.

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BACKGROUND

THE CELL

Greek philosophers from the 3rd and 4th century were the first to debate cellular theory, or atomism as it was then called. The idea was that all matter is composed of indivisible subunits, which only differ from each other in shape and size and not in composition. This theory remained only a philosophical debate until the microscope was invented in the late 1500s. The microscope allowed for the first visualization of this subunit and was called a cell[1] by Robert Hooke in 1664. Prior to the microscope, scientists were limited to what they could observe with unaided eyes or at most a hand magnifying glass. The invention of the microscope revealed an entirely new world of discovery and allowed scientists to determine and characterize the building blocks of organisms. Naturally, this new technology came with controversy and debate. These primitive microscopes were known for producing halos, refractory images, and other optical illusions. Due to these challenges and commonly held misconceptions cellular theory was not generally accepted until the 1830s, more than 150 years after Robert Hooke's first description.

While the identification, description, and debate of all sorts of microscopic material was ongoing throughout the 18th and 19th centuries, many scientists were beginning to ask how new cells were formed. In 1802, C.F. Brisseau-Mirbel proposed three modes for cell formation: (1) new cells were formed at the surface of old ones, (2) they were formed between the adjacent walls of old cells or (3) they were formed within old cells and were 'birthed'. Importantly, Mirbel's argument was that new cells arise from old cells. This view

[1] However, using his microscope Hooke only observed the dead cells of cork and also confused true cells with the vascular structures of plants. Nonetheless, his term is still used today.

was in direct contrast to many German scientists such as L.C. Treviranus (1779-1864). This group of scientists believed that new cells arise from subcellular particles that were already present within the old cell.

The debate continued despite the observation of binary fission[2] in microscopic, single-celled species reported as early as 1744 by Arthur Trembley (Figure 1). His observations were generally accepted, but binary fission was thought to be the exception and not the rule for how new cells were formed, and certainly not something that occurred in higher organisms. In fact, binary fission in multi-celled organisms was not reported until the 1830s by B.C. Dumortier and Hugo Mohl and even these men doubted if binary fission would be seen in animal cells.

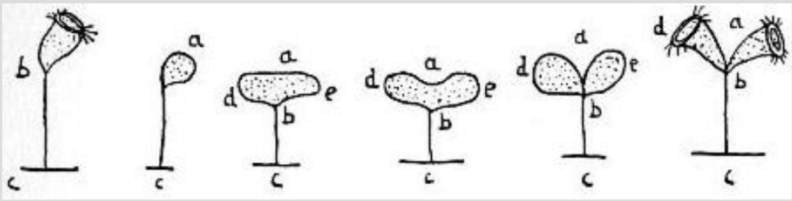


Figure 1. Abraham Trembley's illustration of binary fission in *Synedra* first described in a 1766 letter to Count Bentinck.

While the validity of binary fission as the mode of cell formation was being debated, the microscopists were continuing to report their observations with the use of ever more powerful and accurate microscopes. While the nucleus was likely first observed by Franz Bauer in the 1790s, it was not named and recognized as a legitimate structure found in most cells until Robert Brown's paper in 1833. Even then, the importance of the nucleus was not recognized until much later when scientists were able to observe nuclear envelope breakdown, chromosome condensation, and partitioning of the chromosomes

[2] Binary fission was a term originally applied to what we now call cellular division. In the earliest context it referred to how new cells arise due to the splitting in two of a previously formed cell.

and ultimately identified chromosomes[3] as responsible for genetic traits. These leaps and bounds came in the late 1800s and early 1900s through the work of a number of scientists including Walther Flemming, Carl Rabl, Eduard Strasburger, and Theodor Boveri, to name only a few.

THE CELL CYCLE

It was during this exciting time that the cell cycle was first observed and named. Of course, at this time the details and important function behind the cell cycle were unknown; however, the descriptions of cell cycle events were incredibly accurate. The first descriptions and illustrations of the cell cycle were by Wilhelm Hofmeister, using plant cells, in 1848 and 1849 (Figure 2) and Robert Remak in 1858. Although neither grasped the biological significance of their findings they were able to see and accurately describe prophase, metaphase, and anaphase[4].

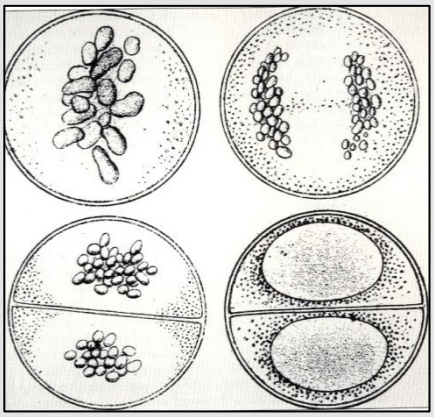


Figure 2. Wilhelm Hofmeister's illustration of mitotic stages. Metaphase, upper left; anaphase, upper right; telophase, lower left; reconstitution of the nuclear envelope, lower right

The cell cycle is the duplication of the cellular contents followed by equal division so that one cell becomes two exact duplicates of itself. While the cell cycle varies in its details between organisms, the fundamental task of the cell cycle is to accurately pass on genetic information to new cells. To do this, the cell must faithfully replicate its DNA, and then segregate chromosomes so that the old cell and the new cell each receive one

[3] Chromosome was first seen in print in 1888, used by H.W.G. Waldeyer-Harz. It is now defined as a structure composed of a very long DNA molecule and associated proteins that carry the hereditary information of an organism.

[4] Prophase, metaphase, and anaphase were originally used by Strasburger in 1884 and will be described below.

complete copy of the genome[5]. Replication and division describe two phases of the cell cycle S phase and M phase, respectively. The other two phases are known as G_1 and G_2 phase. These gap phases allow time for the cells to grow and double their mass of proteins and organelles and are also when checkpoints[6] have the opportunity to act.

Bringing the pieces together, the cell cycle begins in G_1 (Figure 3). At this point cells are not actively going through the cell cycle. If extracellular conditions are favorable and the proper signals are received, the cell will enter S phase and begin to replicate its genome. Once replication has been faithfully completed the cell will enter G_2. These three phases: G_1, S, and G_2 collectively define the period called interphase[7].

Once interphase is completed and the genome has been accurately copied, the cell is ready

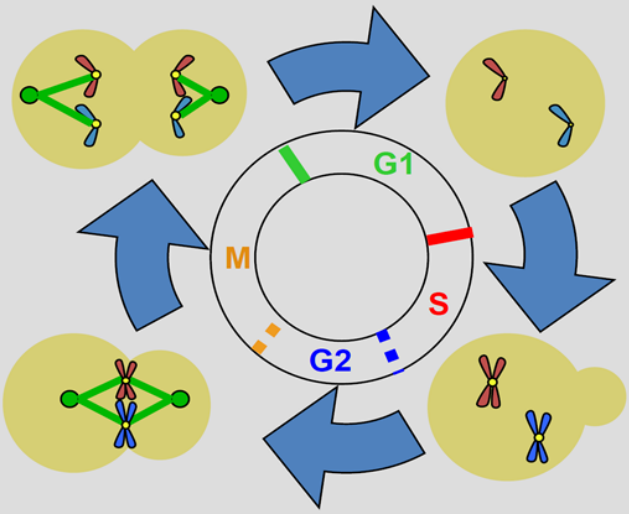


Figure 3. The cell cycle in budding yeast. The cell cycle begins in G_1 with unreplicated chromosomes (top right). In yeast, G_1 cells are unbudded. Cells progress into S phase, where chromatids are replicated and a small bud forms. Replication results in the formation of catenanes, which help hold the sister chromatids together. As the cell cycle continues into mitosis the bud enlarges. The sister chromatids are aligned on the metaphase plate by attachment to spindle bodies (green circles) via microtubules (green lines). The sister chromatids are pulled apart in anaphase and move to the poles of the cell during telophase. Catenations must be resolved prior to sister chromatid segregation in anaphase. Following cytokinesis, one cell cycle is completed resulting in two cells that are each in G_1 with an identical and complete copy of the genome.

[5] Genome refers to the total genetic information contained within a cell; specifically, DNA is the carrier of this information.
[6] Checkpoints will be discussed in detail in the next section. Briefly, checkpoints provide the cell time to fix problems that occur during the cell cycle.
[7] Interphase was first used by H. Lundegårdh in 1913 to distinguish between the apparently inactive phase that a cell undergoes during the period between mitoses (interphase) and the resting cell that does not enter mitosis (G_0).

to go through the dramatic events of mitosis[8] (Figure 4). Mitosis is broken down into six stages: prophase, prometaphase, metaphase, anaphase, telophase[9] and cytokinesis[10]. During prophase the sister chromatids[11] condense. Condensation is followed by prometaphase, during which the newly condensed sister chromatids begin to align along the spindle equator of the due to their attachment to the microtubules of the mitotic spindle. This final alignment of chromosomes is called the metaphase plate and is the morphological hallmark of cellular entrance into metaphase. Once all sister chromatids are properly aligned the cell enters anaphase. In this stage, sister chromatids are synchronously separated, one sister chromatid into each forming cell. Upon arrival of the sister chromatids at the opposite poles, decondensation begins and the spindle begins to breakdown in telophase. The last stage is cytokinesis, during which the cytoplasm is divided in two by a pinching between the cells.

In *Saccharomyces cerevisiae*, there are some species-specific differences in cell cycle stages. In contrast to many eukaryotes, *S. cerevisiae* does not have a defined G_2 phase. Instead, events that are separated in other species occur simultaneously. Specifically, the mitotic spindle begins to form during late S phase and due to the maintenance of the nuclear envelope in *S. cerevisiae* the spindle forms within the nucleus. This means that in *S. cerevisiae* the events that normally occur in prometaphase of mitosis actually occur during late S phase, so that the cell enters mitosis with uncondensed chromosomes, but these chromosomes are already attached to microtubules.

[8] Mitosis is a term coined by Walther Flemming in 1882. It is now defined as the division of the nucleus of a eukaryotic cell, involving condensation of the DNA into visible chromosomes to form two identical sets.
[9] Telophase first appeared in print in 1884 by Martin Heidenhain and refers to the third stage of mitosis.
[10] Cytokinesis is the division of the cytoplasm into two and is the final stage of mitosis.
[11] Sister chromatids are two identical copies of chromatin (the DNA and protein that make up the nucleus) connected most prominently at a centromere (the region of DNA where the mitotic spindle attaches).

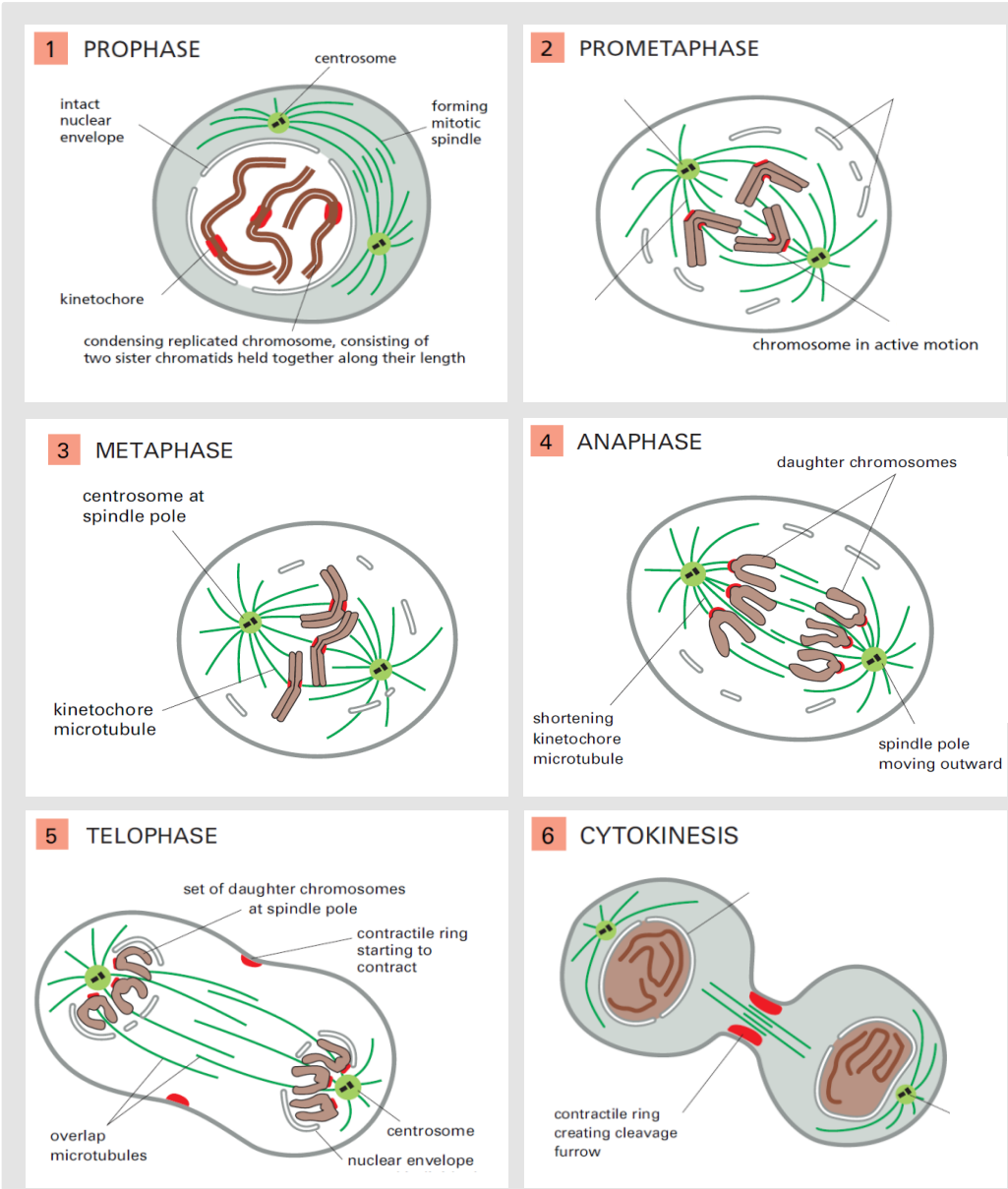


Figure 4. An overview of Mitosis. (1) Prophase is characterized by the condensation of the closely associated sister chromatids. (2) Prometaphase (not distinct in *S. cerevisiae*) starts with nuclear envelope breakdown. Chromosomes attach to spindle microtubules via kinetochores to begin actively moving (3) Metaphase is defined as the alignment of the sister chromatids on the metaphase plate at the spindle equator. (4) At anaphase, sister chromatids separate synchronously and each is slowly pulled toward the spindle pole it is attached to. (5) Telophase is typified by the elongated spindle and the arrival of the chromosomes at the poles. (6) Cytokinesis completes mitosis by dividing the cytoplasm through the pinching off of the cells in the middle at the contractile ring. (Modified from Alberts et al. 2008)

An advantage of using yeast to study cell cycle events is that scientists can visualize progression through the cell cycle by monitoring budding (Figure 3). In yeast, a small bud begins to form coincidentally with S phase. This allows scientists to determine when the cell has entered the cell cycle and is replicating its genome. This bud will continue to enlarge as the cell continues through the cell cycle. By the time the yeast cell reaches anaphase, the bud should be slightly smaller than the mother cell. Using this phenotype we can determine cell cycle entrance and approximate the cell cycle phase.

CELL CYCLE CHECKPOINTS

The gap phases, G_1 and G_2, of the cell cycle are more than just a time to grow. These periods provide time for the cell to monitor internal and external conditions to ensure they are suitable for continuation through the cell cycle. This monitoring is termed a checkpoint (HARTWELL and WEINERT 1989). There are several checkpoints present in the cell which ultimately ensure accurate segregation of the genome. All checkpoints monitor a particular process in the cell. When defects are detected the activated checkpoint halts progression through the cell cycle. This pause allows the cell time to correct the defect. Once the checkpoint is satisfied the cell cycle continues. Each checkpoint ensures the fidelity of cell division and ultimately the accurate replication and division of the genome.

There are several known checkpoints that control entrance and progression through mitosis. The DNA-damage checkpoint functions to inhibit mitotic progression when the DNA is damaged, thus preventing the inheritance of damaged DNA (Figure 5a) (CLARKE and GIMENEZ-ABIAN 2000). Secondly, the spindle checkpoint monitors the proper attachment of kinetochores to the microtubules making up the mitotic spindle (Figure 5b). In the case of improper attachments, the spindle checkpoint is active to inhibit

progression into anaphase of mitosis (CLARKE and GIMENEZ-ABIAN 2000). Lastly, the Topoisomerase II checkpoint ensures that all chromosomes are completely decatenated[12] so that chromosomes can segregate properly during anaphase of mitosis (CLARKE and GIMENEZ-ABIAN 2000).

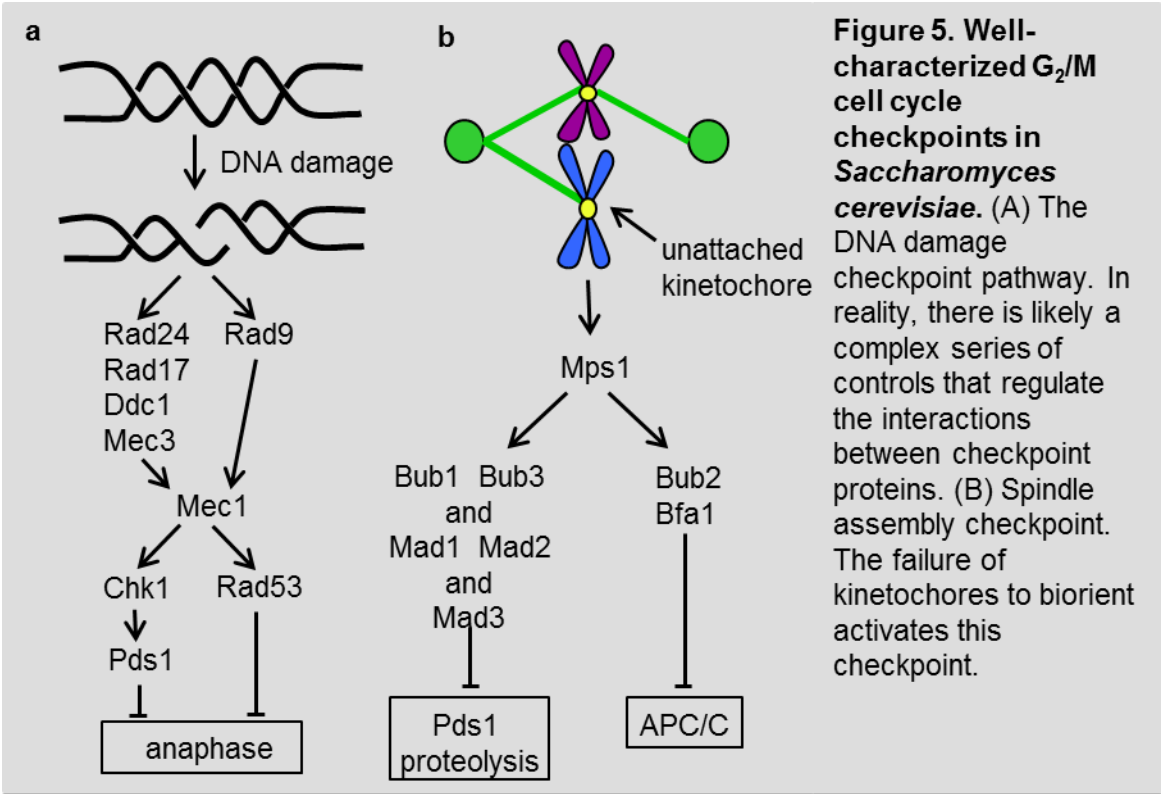


Figure 5. Well-characterized G_2/M cell cycle checkpoints in *Saccharomyces cerevisiae*. (A) The DNA damage checkpoint pathway. In reality, there is likely a complex series of controls that regulate the interactions between checkpoint proteins. (B) Spindle assembly checkpoint. The failure of kinetochores to biorient activates this checkpoint.

[12] Catenations are knots in the DNA that arise due to the replication of double helical DNA. These catenanes are resolved by a process called decatenation. Catenations will be discussed in further detail in the next section.

TOPOISOMERASE II

To put into context a description of the Topoisomerase II checkpoint, it is important to appreciate the function and catalytic cycle of the enzyme itself. DNA Topoisomerase II is an essential, highly conserved enzyme that performs the indispensable function of removing knots in DNA. Knots are the result of the replication of plectonemic,[13] double helical DNA during S phase (Figure 6) (WANG 2009). Watson and Crick's 1953 *Nature* paper proposing the now famous double helical structure of DNA was immediately met with doubters. One such doubter was Max Delbrück, who felt that the resulting entangling of the DNA following replication was "insuperable" or impossible to overcome. How could highly entangled (catenated) DNA molecules be separated for chromosome segregation? These doubts were completely reasonable; it was more than twenty years before we were to discover Nature's answer to this problem.

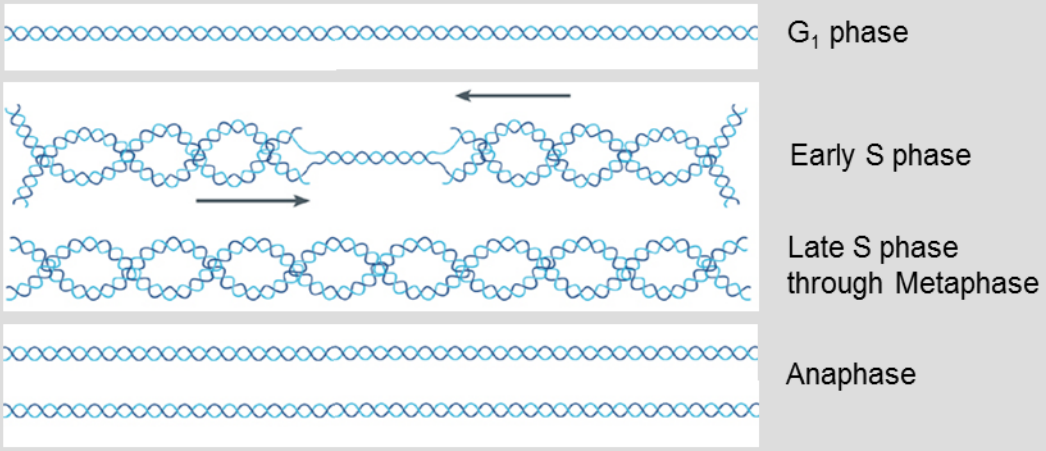


Figure 6. Catenation state throughout the cell cycle. A single copy of the genome is present in G_1 phase. As the cell replicates the double helical DNA in S phase the resulting sister chromatids are catenated. Catenations remain present through metaphase, but are resolved prior to anaphase when sister chromatids segregate. (modified from Nitiss J. 2009)

[13] Plectonemic is a structure with intertwined lines and was first used in 1941 by C.L. Huskins in a discussion of chromosome structures.

In the 1970's, Kiyoshi Mizuuchi and Howard Nash were studying another enzyme, phage λ integrase, when they discovered a new enzymatic activity. This activity appeared when *Escherichia coli* cell extract was added to the reaction. This led them, in 1976, to team up with Martin Gellert to purify this new activity. In so doing, they discovered a Type II DNA topoisomerase in *E. coli* called DNA gyrase (GELLERT M 1976).

While DNA gyrase was the founding member of this new family of enzymes, it was discovered that a representative of this family is present in every living cell. The required characteristic for inclusion into the Type II DNA topoisomerase family is the ability to make double stranded cuts in a DNA helix allowing for the transport of a second, double helical segment of DNA through the cuts, followed by religation of the initial strand. This quintessential characteristic is what makes Type II DNA topoisomerases so fascinating. How does this enzyme make a double stranded cut in DNA, resolve knots between sister chromatids and yet cause no DNA damage? The answer lies in its elegant and intriguing enzymatic activity, called the strand-passage reaction (SPR).

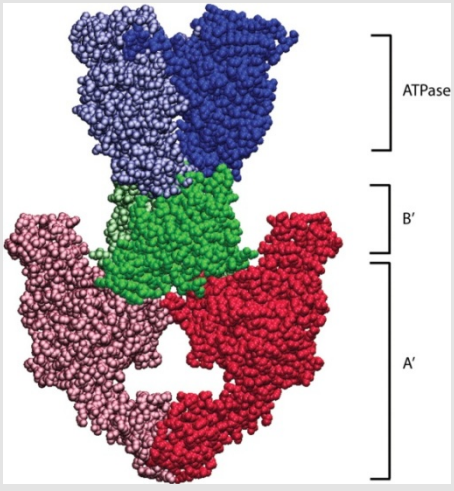


Figure 7. A possible representation of the Top2 enzyme. A composite of structures of *Saccharomyces cerevisiae TOP2* (PDB 1PVG and 1BGW) computationally combined together. ATPase domains, blue, form the N-gate. The central B′-subfragment forms the DNA gate (green). The C-terminal A′ portion (red) encloses a hole large enough to accommodate T-segment DNA, which is thought to exit through the C-gate. (Collins, T.R.L. *et al.* 2009)

To look in detail at the SPR, we will examine the Type II DNA topoisomerase in *S. cerevisiae*, *TOP2*. The protein encoded by the *TOP2* gene (Top2) functions as a homodimer (Figure 7). This complex is composed of three gates, two linking regions between gates, and a flexible C-terminus. The N-terminal portion of Top2 contains the first gate. It is an ATPase domain that undergoes a conformational change to tightly dimerize in the presence of ATP (ROCA and WANG 1992; WIGLEY DB 1991). The ATPase domain is followed by a transducer domain which is thought to transduce ATP binding and hydrolysis signals to the second gate, the DNA gate.

The DNA gate itself is composed of several domains (Figure 8). The TOPRIM domain interacts with one Mg^{2+} per monomer. This interaction positions conserved acidic residues within the TOPRIM domain to promote the formation of the covalent Top2/DNA complex (DONG and BERGER 2007). TOPRIM is followed by the winged-helix domain (WHD). The WHD contains the active site tyrosyl residue (Y782) which is essential for cleaving DNA. This residue uses

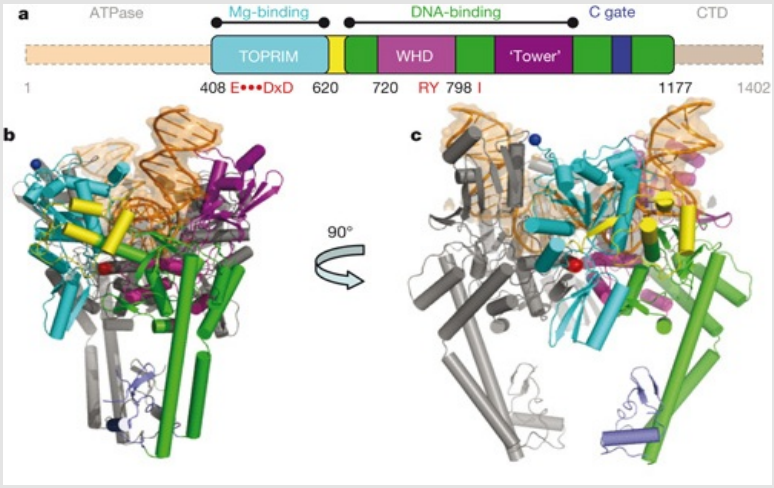


Figure 8. Crystal structure of the DNA gate of *TOP2*. (A) Domain arrangment. TOPRIM, cyan; linker domain, yellow; WHD, magenta; tower domain, dark purple; C-gate, blue-grey; and auxiliary scaffolding, green. Regions not included in the crystal structures are faded. The catalytic tyrosine and acompanying arginine (RY), and DNA bending isoleucine (I) are labled. (b,c) Cartoon of the DNA gate. One protomer is colored grey; the other is colored as shown in panel **a.** DNA is shown in orange. Spheres indicate the locations of the catalytic tyrosine (red) and the amino termini (blue). (modified from Dong and Berger 2007)

transesterification so that the oxygen of the active site tyrosine attacks a phosphoryl group present in the backbone of DNA to form the DNA-protein covalent bond (BERGER *et al.* 1996) (Figure 9). The last domain contained within the DNA gate is called the Tower domain. In crystallography studies it was recently seen that this domain functions as a buttressing element for the DNA extending out of the enzyme (DONG and BERGER 2007). The tower makes polar and electrostatic contacts with the phosphate backbone to assist in the formation of the 75° bend that helps position the DNA near the active site tyrosines (BERGER *et al.* 1996). These three domains (TOPRIM, WHD, and tower) are all contained within the DNA gate and are responsible for making extensive protein contacts with the G-segment[14], cutting and opening the G-segment, followed by closing and religation of the G-segment.

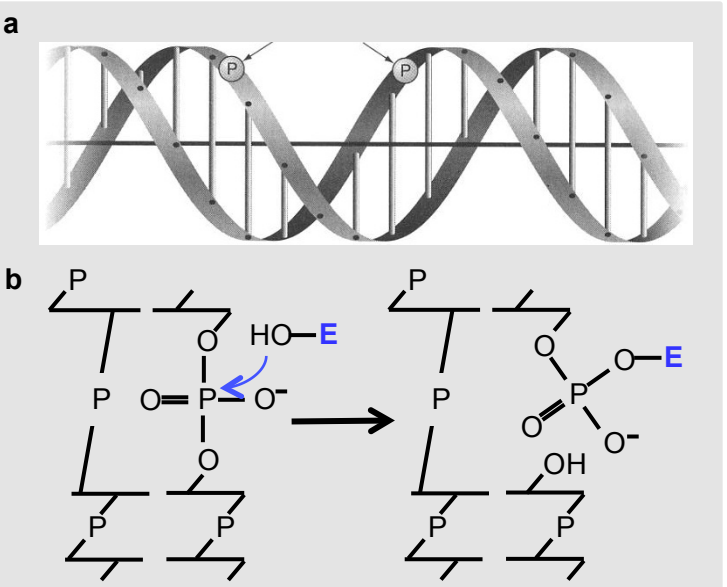


Figure 9. Top2 makes a double strand break in the DNA by transesterification. (a) DNA double helix depicting the approximate locations of two phosphorus atoms (P). (b) Depiction of the transesterification reaction. The hydroxyl group on the enzyme (E) attacks the phosphorous atom (P) in the DNA backbone to break an O-P bond in the DNA strand and simultaneously form an O-P bond between the DNA and the Top2 protein. (modified from Wang, J.C. 2009)

The DNA gate is followed by a coiled-coil element that connects the DNA gate to the C-gate. This element provides flexibility in the opening of the DNA gate (BERGER *et al.*

[14] G-segment or gated-segment is the double-stranded piece of DNA that is bound and cut by the Top2 enzyme.

1996). The C-gate is capable of opening once a T-segment[15] has passed through the DNA gate. The C-gate's major role is in stabilizing the dimerization of Top2 when one of the other gates is open and preventing a T-segment from traversing the dimer in the reverse direction.

As mentioned above, functional Top2 exists as a dimer and so combined has two ATPase domains. Coordination of ATP hydrolysis with large scale conformational changes of the Top2 protein is a focus of intense study. Based on current research, we know that closing/dimerization of the N-gate requires each ATPase domain to bind an ATP (Figure 10a). If N-gate closure results in T-segment trapping then the SPR begins (Figure 10). Binding of ATP and trapping of a T-segment promotes, but is not required for the cutting of the G-segment. However, hydrolysis of one ATP is required for opening of the DNA gate, meaning ATP hydrolysis is required to move apart the cut DNA ends. Consequently, G-segment cutting can be distinct from DNA gate opening, which results in the separation of the G-segment ends. ATP hydrolysis is transmitted through the linker region to the DNA gate, because hydrolysis of an ATP causes that linker region to swing, forming a more open cavity (WEI *et al.* 2005). Opening of the gate promotes T-segment transport through the DNA gate and into the cavity between the DNA gate and the C-gate. It is thought that closure of the DNA gate following T-segment transport, results in steric hindrance that forces the C-gate to open, allowing T-segment release. For the entire Top2 dimer to reset, the last ATP must be hydrolyzed and the products released to allow N-gate opening.

[15] The T-segment is the double-stranded piece of DNA that is trapped by the N-gate and passed intact through the enzyme and cut G-segment.

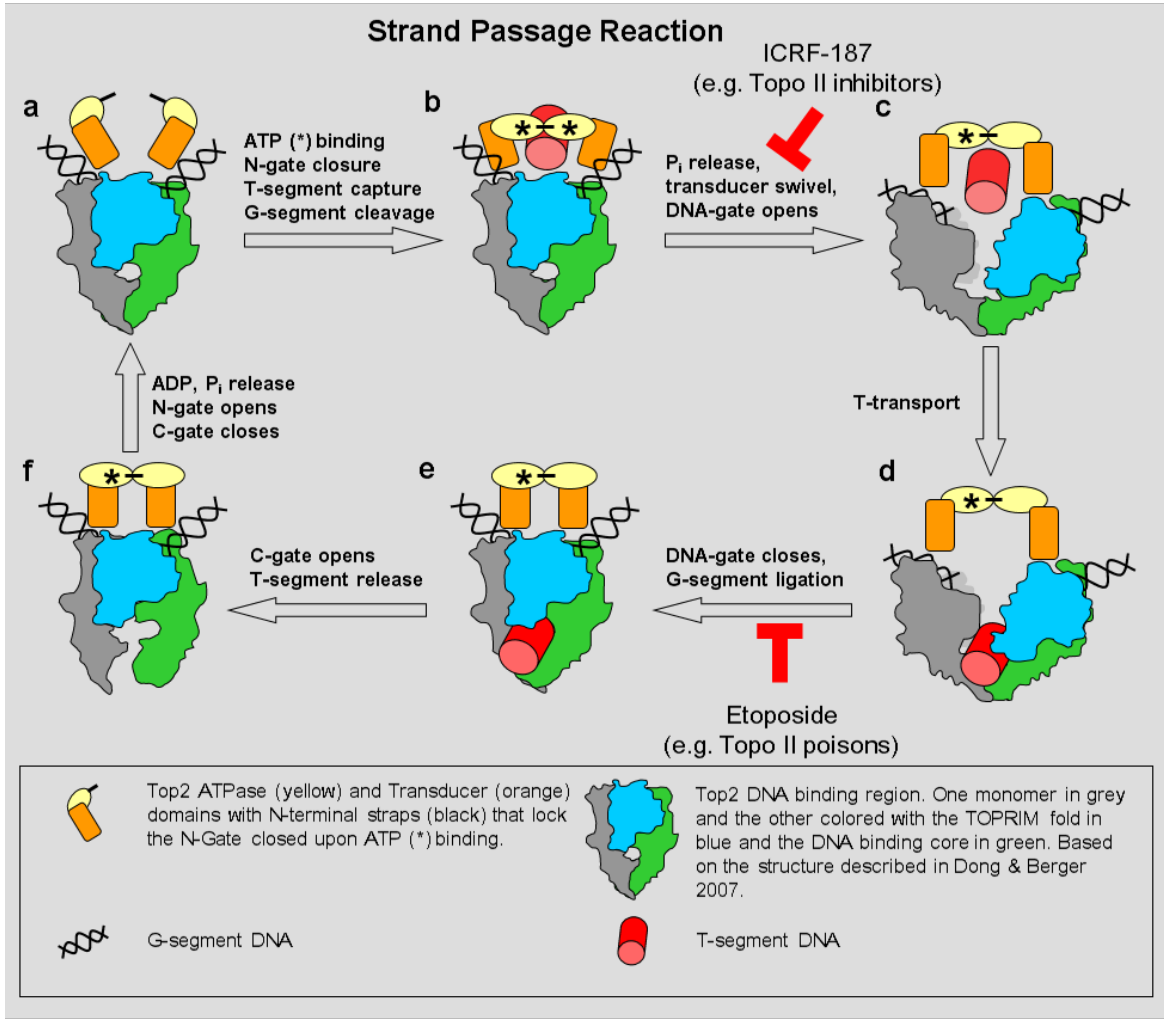


Figure 10. Cartoon of the Strand Passage Reaction and the effects of Topo II targeting compunds. (a) Top2 homodimer bound to G-segment DNA. N-Gate is open in the absence of bound nucleotide. (b) Binding of one ATP molecule to each monomer is required for N-Gate closure. If a T-segment is captured, then G-segment cleavage is thought to be coupled with N-Gate closure, in order for T-segment accommodation. (c-d) Hydrolysis of one ATP promotes conformation changes that swivel the Transducer domain, opening the DNA-Gate and leading to T-transport. Topo II inhibitors, like ICRF-187, inhibit ATP hydrolysis preventing T-transport. (e) DNA-Gate closure leads to G-segment re-ligation. Topo II poisons, like etoposide, prevent G-segment ligation trapping the DNA-gate in an open conformation. (f) C-Gate opening allows T-segment release. Hydrolysis of the second ATP and release of the hydrolysis products permits N-Gate opening.

A recent paper has theorized on the relationship between ATP hydrolysis and Top2 enzymatic activity (BATES *et al.* 2011). The use of ATP hydrolysis in the SPR has long puzzled scientists since the catalytic reaction of Top2 does not have an energetic cost; in fact the reaction is energetically favorable. This has left investigators wondering why the SPR of Top2 is coupled to ATP hydrolysis.

The authors recognize that Top2 must have strong protein:protein interfaces to allow for stable protein dimerization. This dimerization is what prevents Top2 from forming double strand breaks after it cleaves DNA. Without these strong interactions a Top2 monomer covalently bound to DNA would move away from its other half. However, to allow for T-segment transport each of these protein:protein interfaces must at some point weaken and become labile. Recent evidence has begun to provide some clues. Hydrolysis of one ATP and the resulting release of inorganic phosphate appears to precede and accelerate the transport of the T-segment (BAIRD *et al.* 1999; SCHOEFFLER and BERGER 2008). Meanwhile, structural studies of Top2 homologs in other species have shown that binding of nucleotide, hydrolysis and phosphate release likely transmit conformational changes to the DNA gate through the transducer domain (DONG and BERGER 2007) (SCHOEFFLER and BERGER 2005). The authors propose that ATP binding and hydrolysis result in conformational changes that weaken the protein:protein interface in the DNA gate and strengthen the N-gate interface. This allows for the DNA gate to open, but the enzyme:DNA complex to remain intact, thus preventing double strand breaks, due to the strength of the N-gate interaction.

THE TOPOISOMERASE II CHECKPOINT

The entire process described above is the strand-passage reaction (SPR) of Topoisomerase II. This unique enzymatic activity allows for the untangling of replicated DNA prior to sister chromatid segregation in anaphase. Mitotic catastrophe results when cells progress through the cell cycle, in the absence of Top2. This catastrophic mitosis is the result of persistent catenations that continue to hold the DNA together, thus preventing the cell from equally segregating its genome. Based on this observation, it has long been proposed that a checkpoint exists that delays mitosis when the decatenatory activity of Top2 is perturbed (DOWNES *et al.* 1994). However, unlike other checkpoints which have been vigorously researched using genetic approaches, the proposed Topoisomerase II checkpoint appeared to be absent in lower eukaryotes, hindering such studies. Classic yeast *top2* mutants progress through mitosis without delay, thus impeding efforts to genetically characterize the Topoisomerase II checkpoint. This impediment was removed in 2006

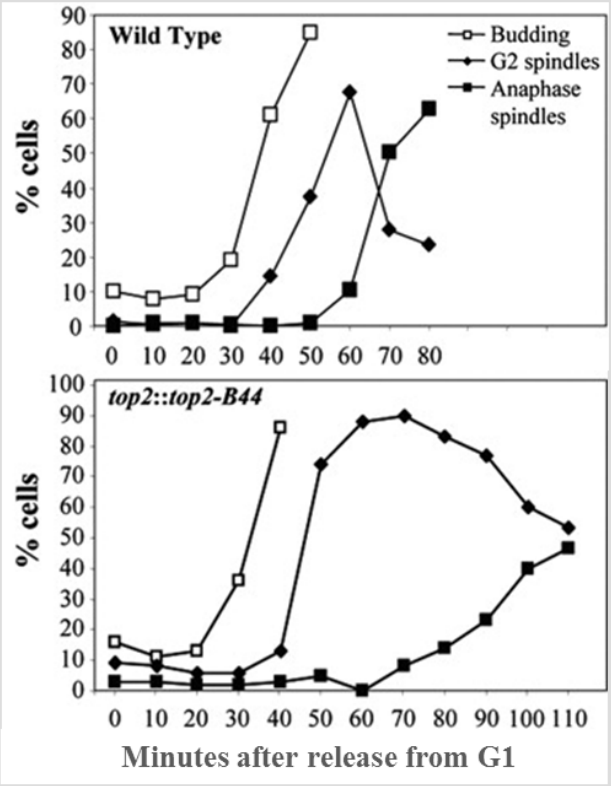


Figure 11. *top2-B44* cells delay in G_2/M. *top2-B44* cells delay with G_2 spindles, while wild type cells progress into anaphase. Each yeast strain was synchronized in G1 and then released. Budding (open squares), G2 spindle (diamonds), and anaphase spindles (closed squares) were scored. (Modified from Andrews C.A. *et al.* 2006)

when a new *top2* mutant, *top2-B44*, was identified and found to delay in G_2/M up to three times the length of G_2/M in wild-type *TOP2* cells (ANDREWS *et al.* 2006) (Figure 11).

top2-B44 was extensively genetically characterized. The checkpoint activated in this strain was found to be unique from both the DNA damage checkpoint and the spindle checkpoint. It does not require DNA damage checkpoint proteins, such as Rad53 and Mec1, to delay in G_2/M (ANDREWS *et al.* 2006). The lack of involvement of DNA damage checkpoint proteins was surprising. Critics of the Topoisomerase II checkpoint in humans have long believed that inhibitors that activate the human version of the Topo II checkpoint, the G_2 decatenation checkpoint, are actually causing difficult to detect levels of DNA damage (ADACHI *et al.* 2004; DOMINGUEZ *et al.* 2001; HAJJI *et al.* 2003; MIKHAILOV *et al.* 2002). The results from Andrews *et al.* suggest that there is a Topo II checkpoint that is independent of DNA damage checkpoint proteins.

However, Andrews *et al.* found that *top2-B44* requires some spindle checkpoint proteins (Mad1, Mad2, Mad3, and Ipl1) to delay in G_2/M. Yet, the Topo II checkpoint is genetically distinct as it does not enforce the checkpoint through Pds1. Pds1, an anaphase inhibitor, is the only known target of the spindle checkpoint. The only other known mechanism that regulates the onset of nuclear division is Swe1-dependent inhibition of the Cdc28 kinase. Swe1 was shown to be dispensable for Topo II checkpoint activation in *top2-B44* cells. These data begin to describe a novel and distinct G_2/M checkpoint that is activated by a mutant form of *top2*. However, we are left with many questions as to what the checkpoint monitors, how the checkpoint signaling cascade is initiated, what proteins are involved in the checkpoint cascade, and how is cell cycle progression arrested?

HUMAN AND YEAST TOPOISOMERASE II CHECKPOINT

Yeast and humans have a large number of proteins and processes that are conserved between species. Nowhere is this more evident than in proteins and events that occur during the cell cycle. For this reason, yeast have been and are extensively used to study processes that affect humans. Moreover, yeast are able to exist as a haploid and therefore numerous genetic and biochemical techniques are much easier done in yeast.

Saccharomyces cerevisiae, a single-celled eukaryote, has a Topoisomerase II checkpoint that when activated results in a delay in G_2/M. Humans are multi-celled eukaryotes and their cells arrest in response to Topo II inhibitors. In contrast to yeast, human cells arrest in either G_2 phase or metaphase. As mentioned earlier, *S. cerevisiae* cells do not have a distinct G_2 phase and instead proceed from S phase into mitosis. Due to the relatively recent discovery of the Topo II checkpoint, it is unknown whether *S. cerevisiae* have just one of the two human Topo II checkpoints or whether the yeast checkpoint is a combination of the two checkpoint pathways in human cells.

STEM CELLS, CANCERS AND THE TOPOISOMERASE II CHECKPOINT

While there is still much to know about the Topo II checkpoint from a mechanistic point-of-view, scientists have begun to uncover its biological significance to human health. Bladder and lung cancer cell lines are deficient in the human G_2 Topo II checkpoint, also known as the G_2 decatenation checkpoint (DOHERTY *et al.* 2003; NAKAGAWA *et al.* 2004). Both studies showed that these cells proceed through mitosis in the presence of Topo II inhibitors. In addition, lung cancer cell lines with an impaired G_2 decatenation checkpoint exhibit an increased sensitivity to Topo II inhibitors. The increased sensitivity of cells that lack a checkpoint provides a potential treatment

strategy for lung cancers and possibly other cancers that retain a functional checkpoint. If the checkpoint can be inactivated then these cancers can be treated with a lower dose of drug, thus mitigating side effects.

In addition to lung and bladder cancers, the G_2 decatenation checkpoint was found to be inefficient in mouse embryonic stem cells and mouse neural progenitor cells (DAMELIN *et al.* 2005). Damelin *et al.* showed that, in general, undifferentiated cells are deficient for the G_2 decatenation checkpoint. These cells do not respond to Topo II inhibition by arresting in G_2, but rather enter mitosis and attempt to segregate their entangled chromosomes, resulting in aneuploid daughter cells. They showed that differentiation of these same progenitor cells resulted in an increase in G_2 decatenation checkpoint efficiency. This is of particular importance as clinical use of stem cells[16] for human therapy becomes more common. Stem or progenitor cells do not normally undergo so many rounds of proliferation. Forcing these cells to proliferate without adequate defense mechanisms, such as the G_2 decatenation checkpoint, amplifies the consequences of their inherent cell cycle deficiencies. The *ex vivo* expansion of stem cells risks the introduction of mutations into the genome, which ultimately means exposing patients to mutant cells. In order to produce safe stem cell therapies it will be essential to know the components of the Topo II checkpoint.

Currently, there is a theory that the lack of a G_2 decatenation checkpoint in stem cells might result in the formation of cancer stem cells[17] (DAMELIN and BESTOR 2006). Specific types of mutations and aberrant chromosomes are expected to result from a deficient G_2 decatenation checkpoint. These include the gain and/or loss of whole

[16] Stem cells are undifferentiated cells that are capable of producing daughter cells that can differentiate into a specialized cell.
[17] Some researchers believe that cancer stem cells are the result of normal stem cells becoming mutated. These mutant stem cells would result in the inheritance of the mutation(s) in their descendants.

chromosomes and chromosome fragments, and the initiation of chromosome rearrangements. These types of abnormalities have been detected in human and mouse embryonic stem cells (DAMELIN *et al.* 2005).

Loss-of-heterozygosity (LOH) events, that are most commonly found in stem cells, are due to chromosome nondisjunction and could result from G_2 decatenation checkpoint deficiency in stem cells (CERVANTES *et al.* 2002). It is postulated that these events could give rise to cancer stem cells. Understanding the deficiencies of cancer stem cells could provide new chemotherapeutic targets that could eliminate the most dangerous, but smallest number of cancer cells, the cancer stem cells.

TOPOISOMERASE II TARGETING COMPOUNDS

There are a number of natural and synthetic compounds that target bacterial and eukaryotic Topoisomerase II family members. While many compounds target bacterial Topoisomerase II (DNA gyrase) and are commonly used as antibiotics, such as coumermycin A and novobiocin, there are also a number of Topoisomerase II compounds that target both yeast and human homologs. These compounds are most commonly used as anticancer drugs and many have been isolated from natural sources. For example, the fungus *Streptococcus peucetius* is the source for several anthracycline drugs. Etoposide and teniposide are plant metabolites. These products would be effective tools in Nature's warfare and would provide both defense and offense for the organism. However, these compounds are not only to be found in fungi and exotic plants, they are also found in the everyday foods that we eat. Soy, coffee, wine, tea, cacao, and some fruits and vegetables are known to contain Topoisomerase II poisons (HENGSTLER *et al.* 2002).

Topoisomerase II target compounds come in two classes: poisons and inhibitors (Figure 10). Topo II poisons, such as etoposide, target the topoisomerase-DNA complex, meaning they trap the enzyme and DNA in a conformation that has cut DNA (NITISS 2009). This generally results in the eventual degradation of the enzyme which leaves behind a double stranded break in the DNA. These drugs are known to activate the DNA damage checkpoint.

This is in contrast to Topo II inhibitors, like ICR-187, which target the N-terminal ATPase domain and prevent catalytic turnover of the ATP. These inhibitors, while preventing ATP hydrolysis, do allow the enzyme to complete one strand passage reaction, but ultimately result in the locking of the N-terminal gate due to the inability to hydrolyze the ATP (CLASSEN *et al.* 2003; ROCA *et al.* 1994). This type of compound provided the first evidence in mammals for a G_2 checkpoint that monitors Topo II activity (KALWINSKY *et al.* 1983).

This theorized checkpoint was thought to be distinct because evidence suggested that Topo II inhibitors induced G_2 delays without causing DNA strand breaks (CREIGHTON and BIRNIE 1969; DOWNES *et al.* 1994; TANABE *et al.* 1991). However, controversy over the presence or lack of DNA damage has muddled conclusions about a G_2 checkpoint that monitors Topo II activity. Some labs have suggested that Topo II inhibitors do in fact, cause DNA damage, most likely as an indirect consequence of perturbed decatenation (ADACHI *et al.* 2004; DOMINGUEZ *et al.* 2001; HAJJI *et al.* 2003; MIKHAILOV *et al.* 2002). This objection has mostly been laid to rest with recent data suggesting that when Topo II inhibitors are used at clinically relevant concentrations and times, DNA damage is not observed (LUO *et al.* 2009; SKOUFIAS *et al.* 2004).

In summary, the Topoisomerase II checkpoint is mechanistically uncharacterized. Further knowledge of this checkpoint will provide meaningful insight into novel cancer therapies. Also, an understanding of this checkpoint could allow for the improvement of its function in stem cells. This improvement could result in stem cell therapy that is of less risk to patients.

This work begins to characterize the Topoisomerase II checkpoint in yeast. I will provide evidence that the Topoisomerase II checkpoint monitors a specific stage in the strand passage reaction to determine if the checkpoint should be activated and that checkpoint activation requires the C-terminal end of Top2. Finally, I will show data that draws attention to misconceptions about a classic *top2* allele.

CHAPTER I

MOLECULAR MECHANISM FOR TOPOISOMERASE II CHECKPOINT ACTIVATION

While the evidence supports the existence of a Topo II checkpoint, there remains debate over the nature of the activating event of the checkpoint, leading to the observed G_2/M cell cycle arrest. One possibility is that the checkpoint senses dysfunctional Topo II directly. Alternatively, it could function through the well-characterized DNA damage and/or spindle checkpoint mechanisms. These well-known checkpoints could indirectly monitor the aberrant topological changes in DNA or DNA breaks that can result from dysfunctional Topo II activity.

To address if DNA breaks lead to activation of a checkpoint, it was previously shown that yeast strains containing the *top2-B44* hypomorphic allele activate the G_2/M delay without DNA breakage (ANDREWS *et al.* 2006). In *top2-B44* cells, Rad52 foci were present at low levels similar to wild type and there was no Rad53 phosphorylation shift, suggesting the DNA damage checkpoint is not activated (ANDREWS *et al.* 2006). Additionally, kinetochores correctly attach to the mitotic spindle in *top2-B44* cells, resulting in proper biorientation of chromosomes (ANDREWS *et al.* 2006). Chromatin condensation was also found to occur at the appropriate time in *top2-B44* cells (Figure 12), providing evidence that the spindle assembly checkpoint is not responsible for the G_2/M delay. Based on these data there is no evidence that DNA breaks or aberrant DNA topology leads to checkpoint activation in *top2-B44* cells. For this reason, we sought to determine if cells directly monitor Topo II activity to determine if a checkpoint should be activated.

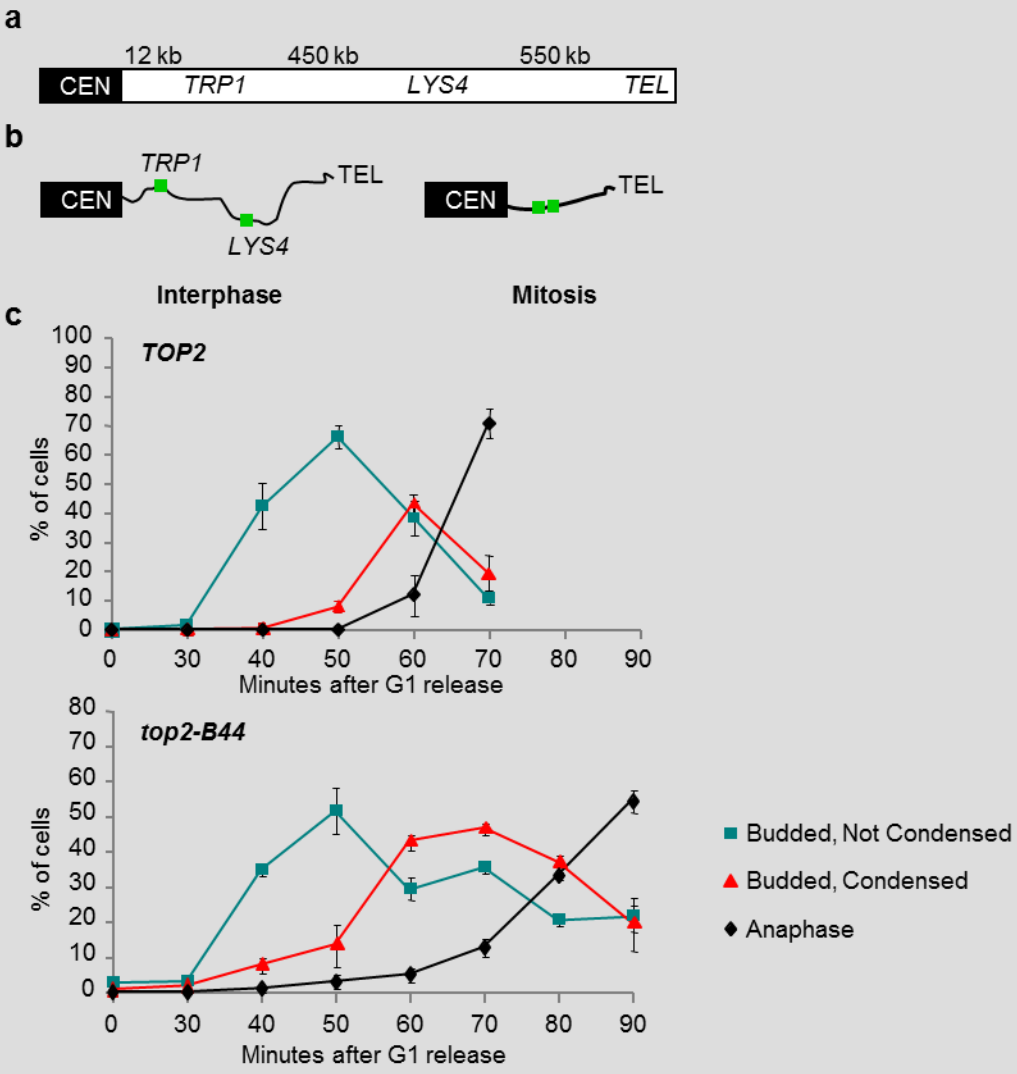


Figure 12. *top2-B44* does not have a chromosome condensation defect. (a-b) Schematic description of the chromosome condensation assay, based on Vas *et al.* 2007. LacO tandem repeats integrated at two loci (*TRP1* and *LYS4*) on the right arm of chromosome IV are visualized by LacI-GFP. In interphase, loci are seen as separated foci (green squares), while in mitosis the foci coalesce upon chromosome condensation. (c) Cell cycle analysis of chromosome condensation in wild type and *top2-B44* strains. Anaphase (black line) in wild type cells occurs with normal timing, while anaphase in *top2-B44* cells, was delayed due to checkpoint activation. Chromosome condensation occurs in *top2-B44* cells at similar time and to a similar level as *TOP2* cells. (Lane, A. unpublished)

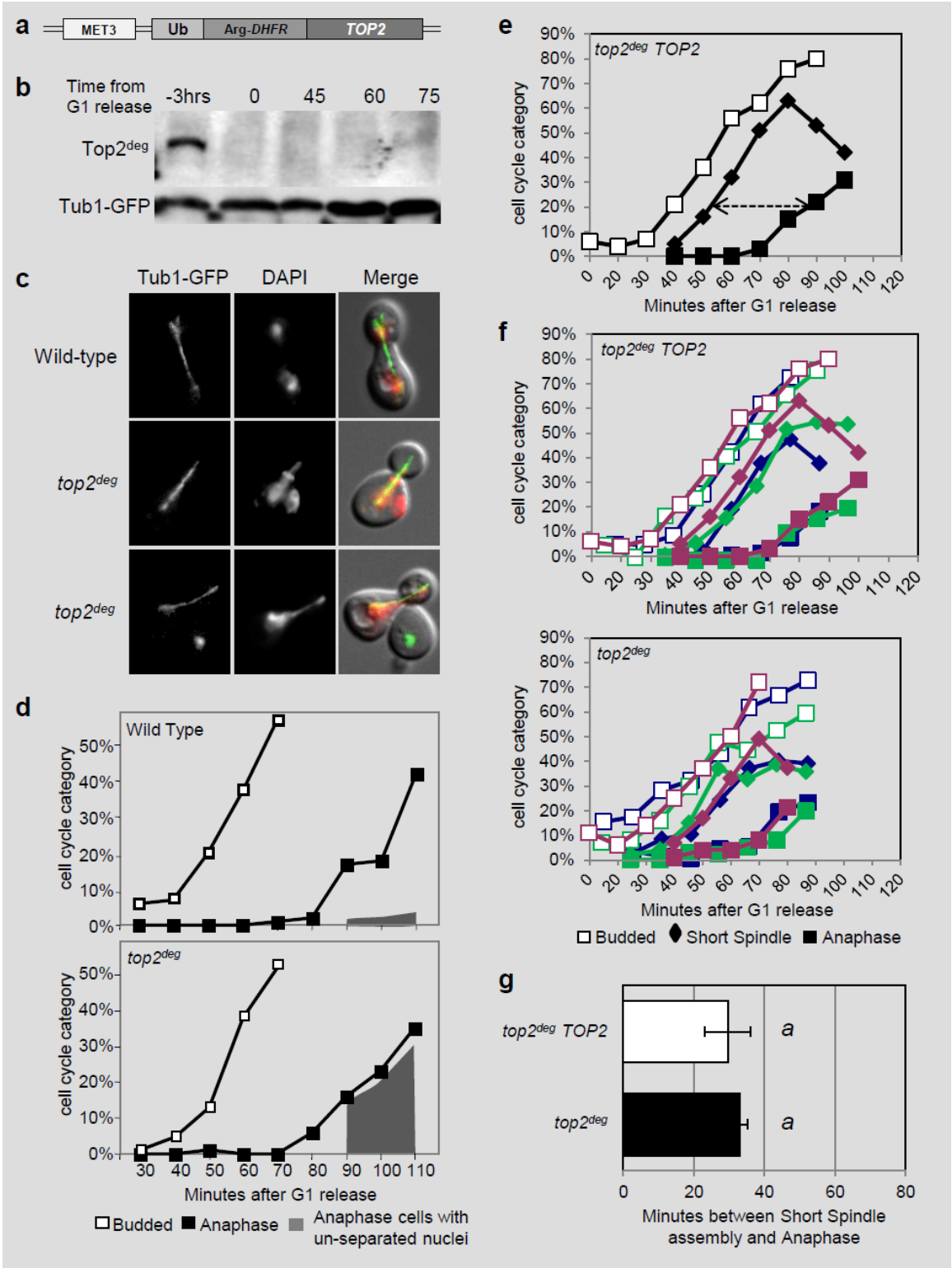
To do this determination, a system was needed in which the endogenous Top2 could be eliminated from the cell, leaving only an additional mutant *top2* present. However, *TOP2* is an essential gene and deletion results in lethality. Instead, a temperature-sensitive allele, *top2-4* (HOLM et al. 1985), that is inviable at 30°C was tested to determine if it would suffice for these experiments. This allele has been used since the 1980s and was assumed to be null above its restrictive temperature. Temperature-sensitive mutants, in yeast, generally become unstable and the protein unfolds at higher temperatures, resulting in their targeting for degradation. However, as will be discussed in Chapter 3, *top2-4* while lethal above its restrictive temperature is not degraded and was found to have a dominant negative activity.

RESULTS

Construction and Characterization of a Top2 degron system

Since *top2-4* proved not to be ideal for our study; we instead constructed a *top2* degron mutant (*top2^{deg}*). This system controls the expression and degradation of Top2 in yeast. The degron constructed is expressed from the regulatable *MET3* promoter (Figure 13a) and also contains the classic degron fusion (Ubiquitin-Arg-DHFR) (DOHMEN *et al.* 1994). This fusion to wild-type *TOP2* promotes destabilization of protein folding at high temperature and is the target of the E3 ubiquitin ligase, Ubr1 (DOHMEN *et al.* 1994). In

Figure 13 (next page). In yeast, checkpoints are not activated due to aberrant DNA topology. (a) Schematic of *top2^{deg}* allele under tight transcriptional control and encoding a thermo-labile Top2^{deg} protein. (b) Western blot of Top2^{deg}. Temperature and carbon-source shifts (Table 1) promote efficient degradation (Tub1-GFP, loading-control). (c-d) Micrographs and quantification of failed nucleus segregation (DAPI) in anaphase cells with elongated spindles (Tub1-GFP) after Top2^{deg} was degraded in G1 cells and the subsequent cell cycle analyzed. Shaded region (*d*) indicates fraction of anaphase nuclei that were not segregated. (e-g) Interval between spindle assembly and anaphase (*e*, arrow) is not significantly different with wild type Top2 or after Top2^{deg} degradation in G_1 (*f*). Green/blue/purple: three experimental repeats (*f*).


a
MET3
Ub
Arg-DHFR
TOP2
b
Time from G1 release
-3hrs
0
45
60
75
Top2deg
Tub1-GFP
c
Tub1-GFP
DAPI
Merge
Wild-type
top2deg
top2deg
d
Wild Type
top2deg
cell cycle category
Minutes after G1 release
Budded
Anaphase
Anaphase cells with un-separated nuclei
e
top2deg TOP2
cell cycle category
Minutes after G1 release
f
top2deg TOP2
top2deg
cell cycle category
Minutes after G1 release
Budded
Short Spindle
Anaphase
g
top2deg TOP2
top2deg
a
a
Minutes between Short Spindle assembly and Anaphase

addition, a second copy of *UBR1* is expressed under control of the inducible *GAL1-10* promoter. In this way the degron can be stably expressed at 26°C in media lacking methionine, so that the promoter is on and the protein is stable. To rapidly degrade Top2^{deg} protein, the cells are grown in media containing methionine, galactose is added to turn on *UBR1* expression, and cultures are shifted to 35°C to promote Top2^{deg} unfolding and degradation (Table 1). Using this method Top2^{deg} protein levels were undetectable in yeast cells synchronized in G_1 (Figure 13b), which allowed us to study the effects of progression through a subsequent cell cycle in the absence of Top2 (FURNISS *et al.* 2009b).

Table 1. Conditional expression and degradation of Top2^{deg}.

Steps to Inactivate Degron	Conditions	Top2-degron transcription	Gal-Ubr1 transcription	Protein folding
1	Overnight culture SR-Met 26°C	ON	OFF	Stable
2	YPR 26°C + αF	OFF	OFF	Stable
3	4% Galactose added to culture	OFF	ON	Stable
4	Culture shifted to 35°C	OFF	ON	Unstable
5	Wash and release into YPG at 35°C	OFF	ON	Unstable

If degradation of the Top2^{deg} protein is successful, then cells should reach G_2 unable to resolve catenated DNA that formed during DNA replication. When these cells enter anaphase they should fail to segregate their chromosomes. This outcome was observed in ~90% of anaphase cells examined (Figure 13c and 13d), indicating G_2 was reached with the extensive DNA topological conditions expected from cells deficient for Top2.

Despite these gross errors in chromosome segregation and DNA topology, anaphase onset was not delayed under these conditions (Figure 13e-g). Cells were synchronized in G_1 under the conditions described in Table 1. Upon release into the cell

cycle spindle morphologies, visualized by GFP-Tub1, were monitored to determine cell cycle stage (FURNISS *et al.* 2009a). G_1 cells are identified due to their single bright dot, with small projections; these are the spindle pole body and astral microtubules. As the cell continues into S phase a small bud forms and the spindle pole body duplicates, but as it is not separated remains a single, bright spot. When the spindle bodies separate in G_2/M a thick, bright bar with a dot on either end is observed. This is the 2 μm mitotic spindle. This bar elongates to around 7-10 μm in anaphase. When elongation occurs the spindle gets lighter due to a lower density of microtubules present in this region resulting from the shortening of the kinetochore microtubules. Finally, in telophase the spindle begins to breakdown, and the spindle bodies are found close to the poles of the mother and daughter cells.

Based on these results, we conclude that centromeric chromatin defects, which result from hyper-DNA catenation present at the centromeres, are not sufficient to activate any pre-anaphase checkpoint, including the spindle checkpoint. This is consistent with a previous study which indicated that Top2 deficiency in yeast does not delay mitotic progression (BAXTER and DIFFLEY 2008) and a second study in human cells in which Topo IIα was depleted and no checkpoint activation was observed (BOWER *et al.* 2010). To ensure the Topo II checkpoint could be activated in the degron system we introduced the *top2-B44* allele under endogenous control. We then asked if the checkpoint is activated in a cell cycle where $Top2^{deg}$ is degraded in G_1 so only Top2-B44 is present. In this situation anaphase initiation was delayed, indicating checkpoint activation (Figure 14). Having established a functional system for determining the mechanism of checkpoint activation, we asked whether the aberrant activity of Top2-B44 was initiating a Mad2-dependent checkpoint, as previously determined. The strain,

top2^{deg} *top2-B44 mad2Δ,* was no longer able to delay in G_2/M (Figure 14). This confirmed that the degron system is capable of activating the Topo II checkpoint as the observed G_2/M delay was still Mad2 dependent.

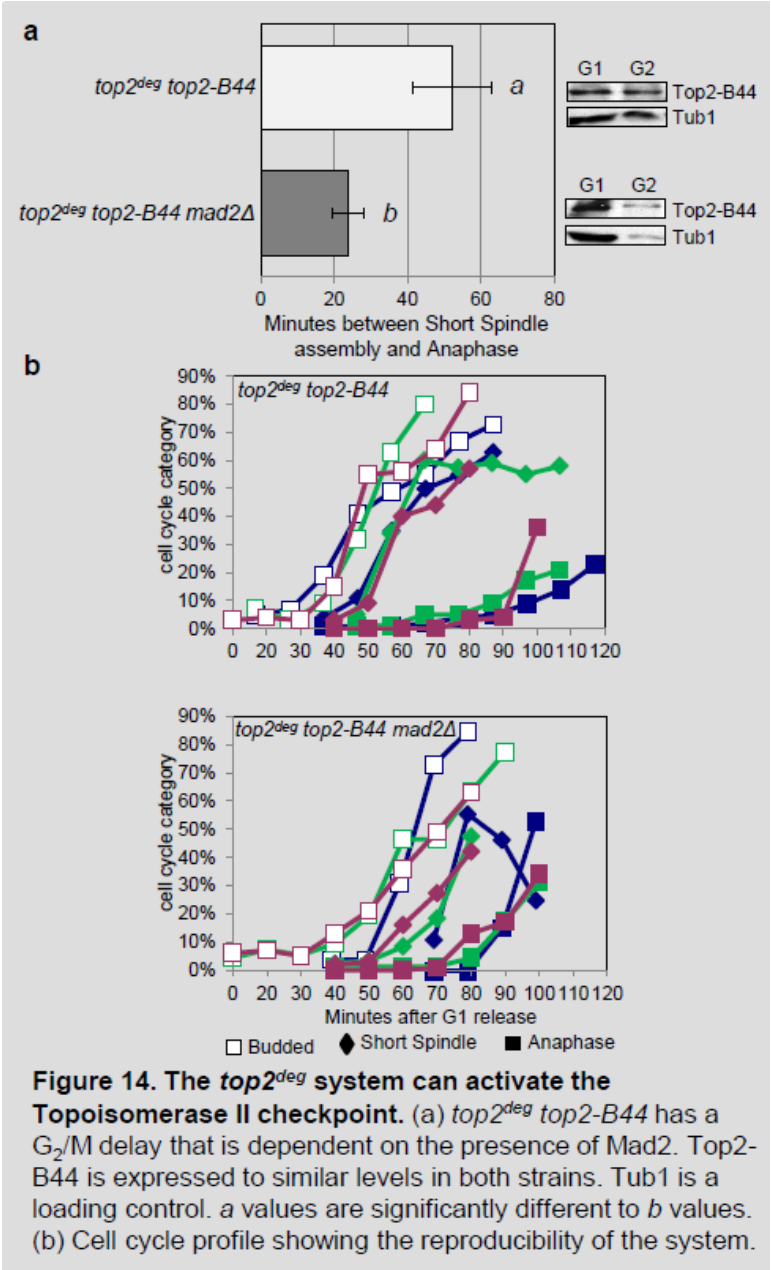


Figure 14. The *top2*^{deg} system can activate the Topoisomerase II checkpoint. (a) *top2*^{deg} *top2-B44* has a G_2/M delay that is dependent on the presence of Mad2. Top2-B44 is expressed to similar levels in both strains. Tub1 is a loading control. *a* values are significantly different to *b* values. (b) Cell cycle profile showing the reproducibility of the system.

We have shown that hyper-catenated DNA is not sufficient for cell cycle arrest, but that Top2-B44 is capable of checkpoint initiation. There are two possibilities that would explain activation of the checkpoint in *top2-B44* cells. Abnormal Top2-B44 protein structure could be detected, resulting in checkpoint activation. Alternatively, cells could directly monitor the SPR enzyme cycle. To begin to distinguish between these possibilities, we used SPR mutants that have been previously characterized structurally or biochemically (Figure 15).

Figure 15 (next page). Strand Passage Reaction mutants analyzed.

K651A has greatly reduced affinity for G-segment DNA and thus does not perform appreciable strand passage reactions *in vivo* and cannot support viability. *In vitro*, relaxation of supercoiled DNA can be detected at very low levels, indicating that Top2^{K651A} can, albeit with a very limited capacity, perform the SPR and therefore overall folding of the enzyme is not abolished.

Y782F lacks the active site tyrosine and therefore cannot cut G-segment DNA. It can bind to the G-segment and undergo rounds of ATP binding/hydrolysis as well as N-gate opening and closure. It is predicted to lack the ability to capture a T-segment due to space constraints within the N-terminal orifice of the enzyme in the absence of G-segment cleavage.

G144I cannot bind nucleotide and therefore cannot lock the N-Gate closed. For this reason it is unlikely to capture a T-segment and since T-segment capture stimulates G-segment cleavage, it has much reduced cleavage activity. *In vitro*, however, cleavage activity has been measured and given this event, the enzyme may sample conformations normally associated with T-transport even in the absence of ATP hydrolysis and T-segment capture.

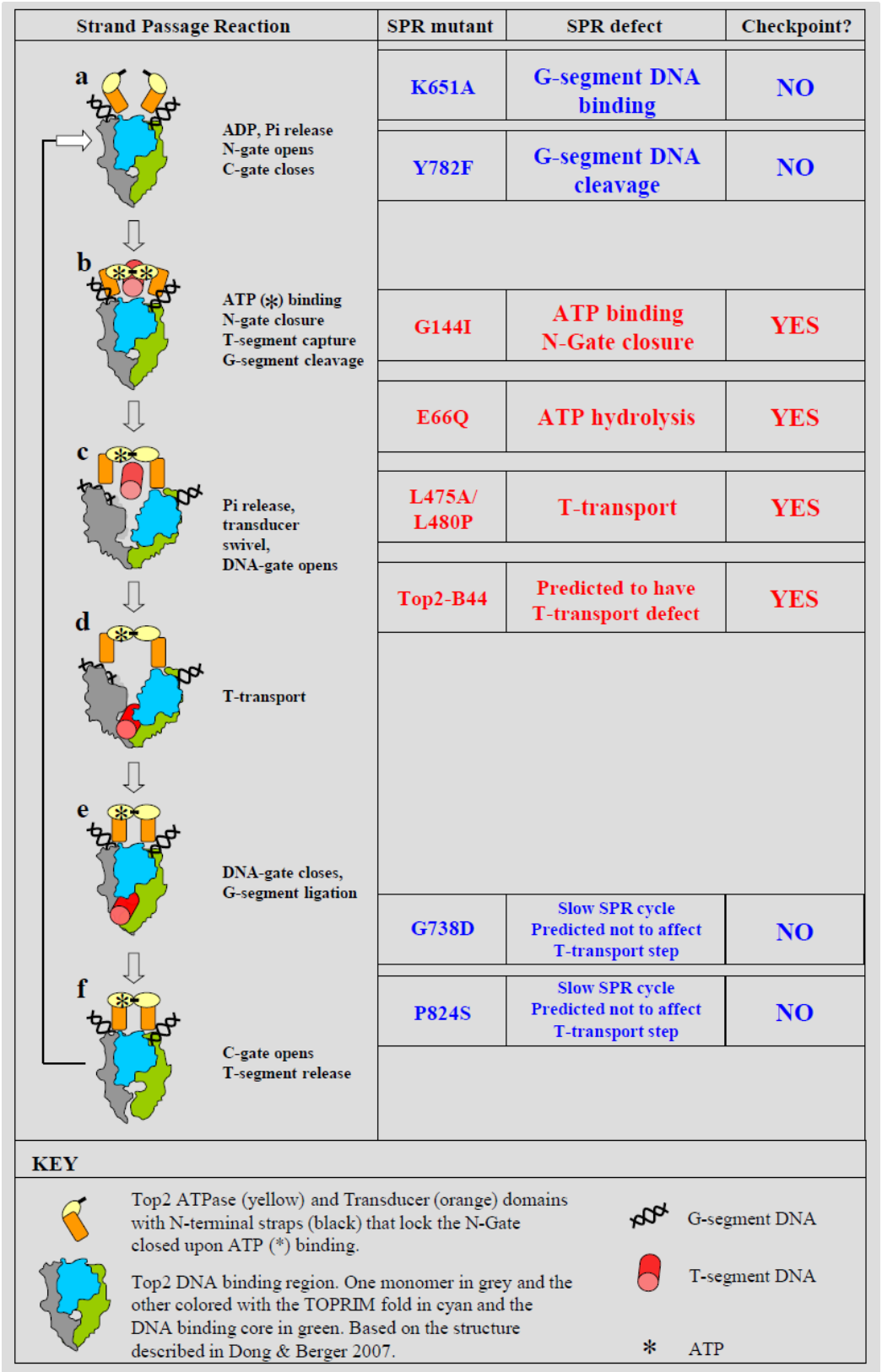
E66Q has 200-fold reduced ATP hydrolysis activity and therefore inefficiently performs conformation changes that promote T-transport, including DNA-Gate opening. *In vitro* studies indicate that inefficient T-transport is followed by SPR arrest after release of the T-segment. A second SPR cycle is not possible because the N-Gate cannot open without release of the hydrolysis products.

L475A/L480P has wild type ATP hydrolysis activity but T-transport occurs at a much reduced rate.

Top2-B44 is predicted to be defective in T-transport. The mutated residue is positioned where the TOPRIM-fold lies adjacent to the DNA binding domain. The mutation is predicted to affect communication between these regions.

G738D and P824S are positioned in the C-gate portion of the enzyme. These mutants have a much reduced rate of the SPR but are predicted to not affect the T-transport steps associated with DNA-gate opening, but rather would affect a later step of the SPR.

Right column, Indicates if checkpoint activation occurs upon expression of each mutant at endogenous levels in yeast cells depleted of Top2^{deg} during G1.


Strand Passage Reaction
SPR mutant
SPR defect
Checkpoint?
a
ADP, Pi release
N-gate opens
C-gate closes
K651A
G-segment DNA binding
NO
Y782F
G-segment DNA cleavage
NO
b
ATP (*) binding
N-gate closure
T-segment capture
G-segment cleavage
G144I
ATP binding
N-Gate closure
YES
E66Q
ATP hydrolysis
YES
c
Pi release,
transducer
swivel,
DNA-gate opens
L475A/
L480P
T-transport
YES
Top2-B44
Predicted to have
T-transport defect
YES
d
T-transport
e
DNA-gate closes,
G-segment ligation
G738D
Slow SPR cycle
Predicted not to affect
T-transport step
NO
f
C-gate opens
T-segment release
P824S
Slow SPR cycle
Predicted not to affect
T-transport step
NO
KEY
Top2 ATPase (yellow) and Transducer (orange) domains with N-terminal straps (black) that lock the N-Gate closed upon ATP (*) binding.
Top2 DNA binding region. One monomer in grey and the other colored with the TOPRIM fold in cyan and the DNA binding core in green. Based on the structure described in Dong & Berger 2007.
G-segment DNA
T-segment DNA
ATP

Top2-B44 must associate with the DNA to activate the checkpoint

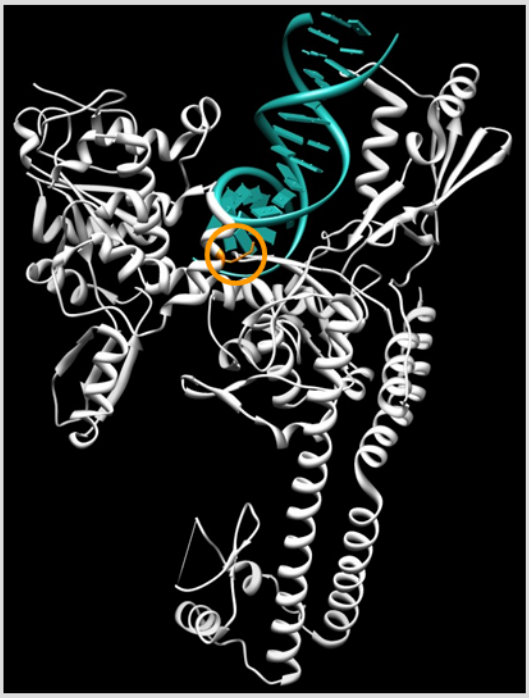
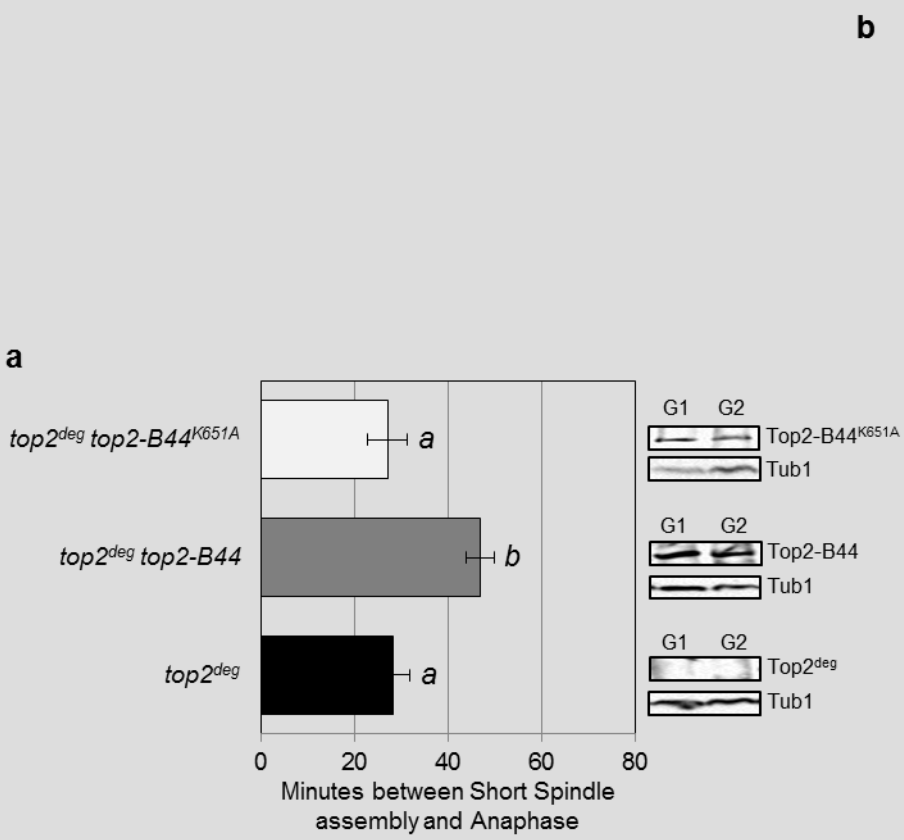


Figure 16. Crystal structure of the DNA-gate and upper C-gate of one Top2 monomer. The G-segment is shown in teal and the K651 residue is highlighted in orange.

First, we asked whether Top2-B44 only needs to be physically present in the cell, in order for the potential aberrant protein structure to be detected by the checkpoint machinery. To do this we introduced a K651A mutation into *top2-B44*. K651 is a conserved residue found in a small, flexible linker region located between the highly structured TOPRIM and WHD domains (Figure 16). These domains form a deep, positively charged groove which contains the G-segment DNA (DONG and BERGER 2007). From previous studies we know that Top2^{K651A} has an extensive reduction in its affinity for DNA (LIU and WANG 1999) and cannot support cell viability. Despite these abnormalities and inviability, the K651A substitution is not predicted to destabilize Top2 protein structure overall. This prediction is supported by the stability of Top2-B44^{K651A} *in vivo*, suggesting that the protein is correctly folded (Figure 17 Western). Using the *top2^{deg}* strain, when Top2-B44^{K651A} was present at equal levels to Top2-B44 in control strains, a pre-anaphase delay was not induced (Figure 17), suggesting that the Topo II checkpoint machinery can only activate the checkpoint when Top2-B44 is associated with DNA. Top2's essential function is at the DNA. One possible explanation for this result is that the checkpoint monitors the defective SPR of Top2-B44.



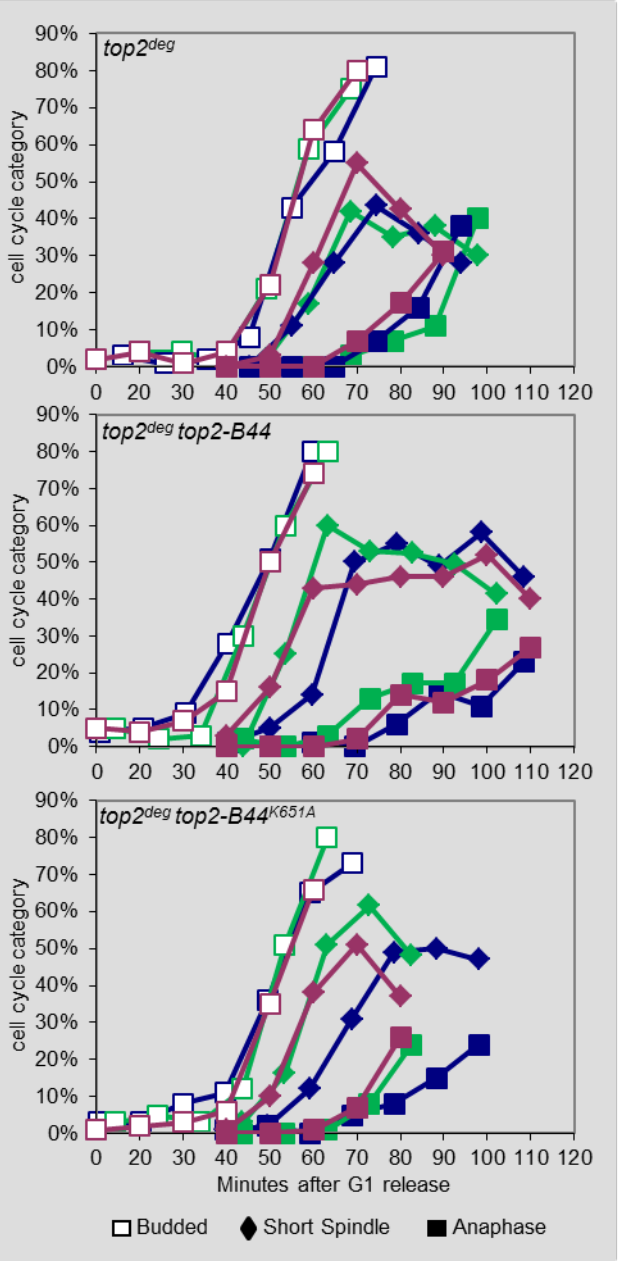


Figure 17. Topoisomerase II checkpoint activation requires Top2 to associate with the DNA. (a) Bars represent the time spent in G_2/M. Western blots show each Top2 mutant relative to Tub1 control at G_1 and G_2. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different. (b) Cell cycle analysis of each strain.

The Strand Passage Reaction must be initiated for checkpoint activation

To probe the possibility that the enzymatic cycle of Top2 is monitored by the checkpoint we asked if SPR initiation is required for checkpoint activation. Each subunit in the homo-dimer of Top2 has a catalytic tyrosine that is required for phosphodiester linkage with DNA (LIU and WANG 1998) (Figure 18a). This residue is required for cutting the DNA (Figure 9). Introducing the Y782F mutation into *TOP2* results in an enzyme that is not only unable to cleave the G-segment DNA, but is also unable to capture a T-segment. It is thought that there is not enough room between the N-gate and DNA gate, if both are closed, to fit a DNA double helix, so a Top2^{Y782F} mutant would be unable to trap a T-segment (DONG and BERGER 2007). Despite its inability to trap a T-segment, its ability to bind ATP and close the N-gate is unaffected, and so it goes through a futile cycle of N-gate opening and closure (Figure 18b). Therefore, Top2^{Y782F} arrests its enzymatic cycle bound to the G-segment, but before the SPR can be initiated (LIU and WANG 1998).

Cell cycle analysis of degron cells expressing Top2^{Y782F} at endogenous levels revealed a lack of checkpoint response (Figure 18c and 19). This reinforces the previous data suggesting that lack of Top2 activity, resulting in abnormal DNA topologies, does not activate the checkpoint. It also supports the model that a Top2 enzyme incapable of SPR initiation does not activate checkpoint signaling. In further support of this we found that a Top2-B44^{Y782F} enzyme, expressed at endogenous levels, is incapable of triggering checkpoint activation (Figure 18b and 19). We conclude that the defective enzymatic cycle of Top2-B44 is required for checkpoint activation, possibly due to a defective step in the SPR being monitored.

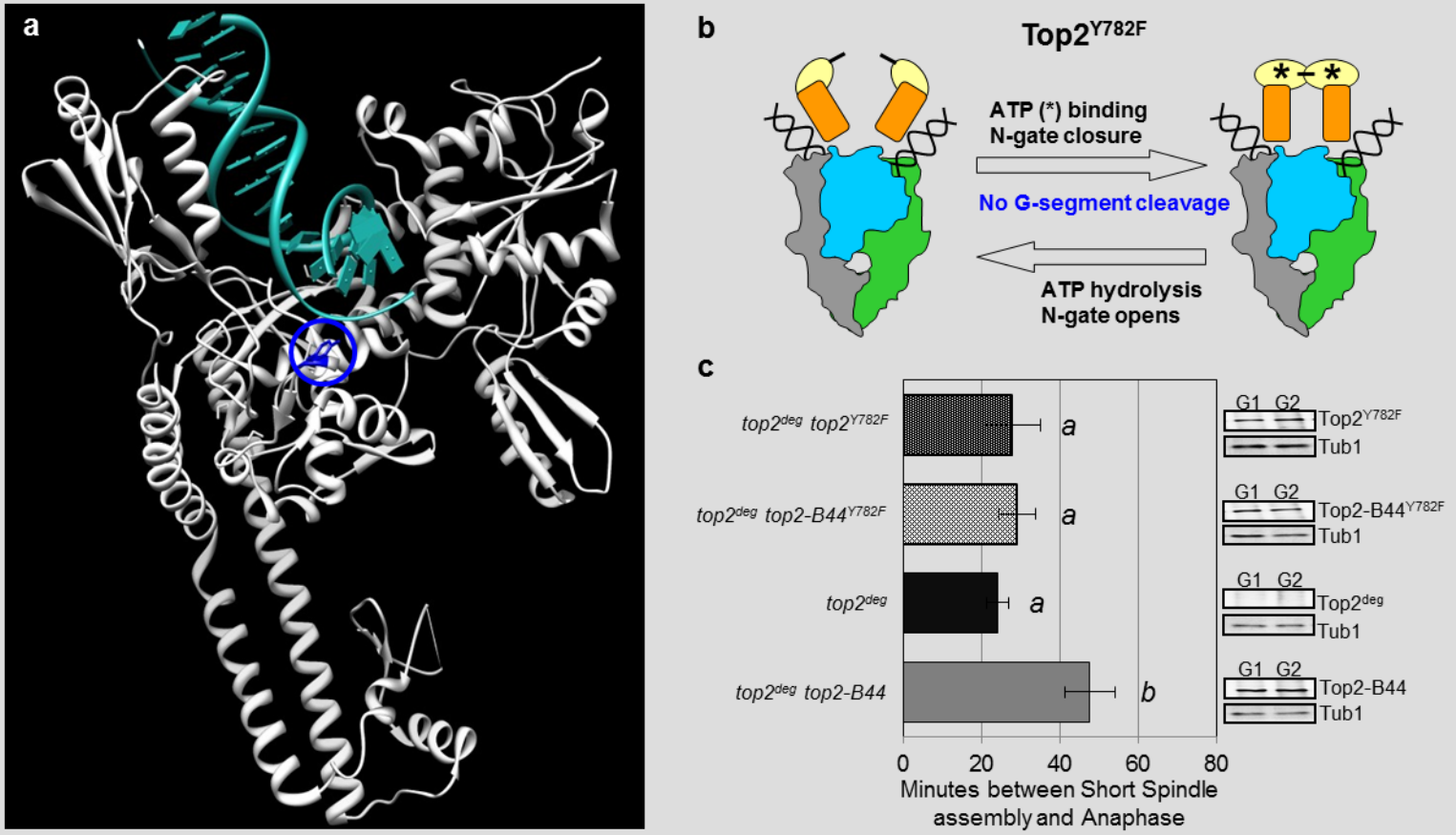


Figure 18. Initiation of the strand passage reaction is required for activation of the Topoisomerase II checkpoint. (a) Crystal structure of the DNA-gate and upper C-gate of one Top2 monomer. DNA is in teal and the catalytic tyrosine (Y782) is highlighted in blue. (b) Cartoon depicting the catalytic defect in Top2^{Y782F} which cannot cleave G-segment DNA and so has non-productive cycles of ATP hydrolysis and N-gate closure/opening. (c) Bars represent the time spent in G_2/M. Western blots show each Top2 mutant relative to Tub1 control at G_1 and G_2. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different.

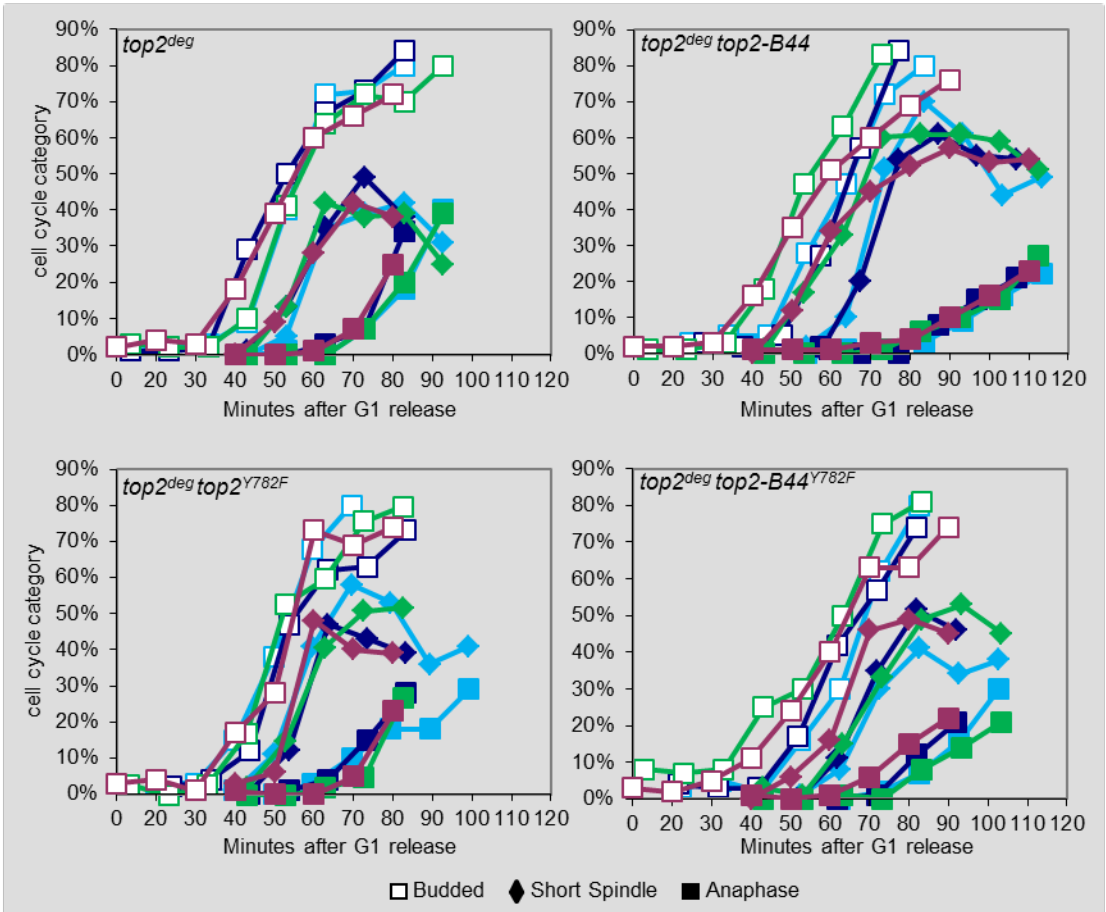


Figure 19. Cell cycle analyses illustrating that initiation of the strand passage reaction is required for activation of the Topoisomerase II checkpoint

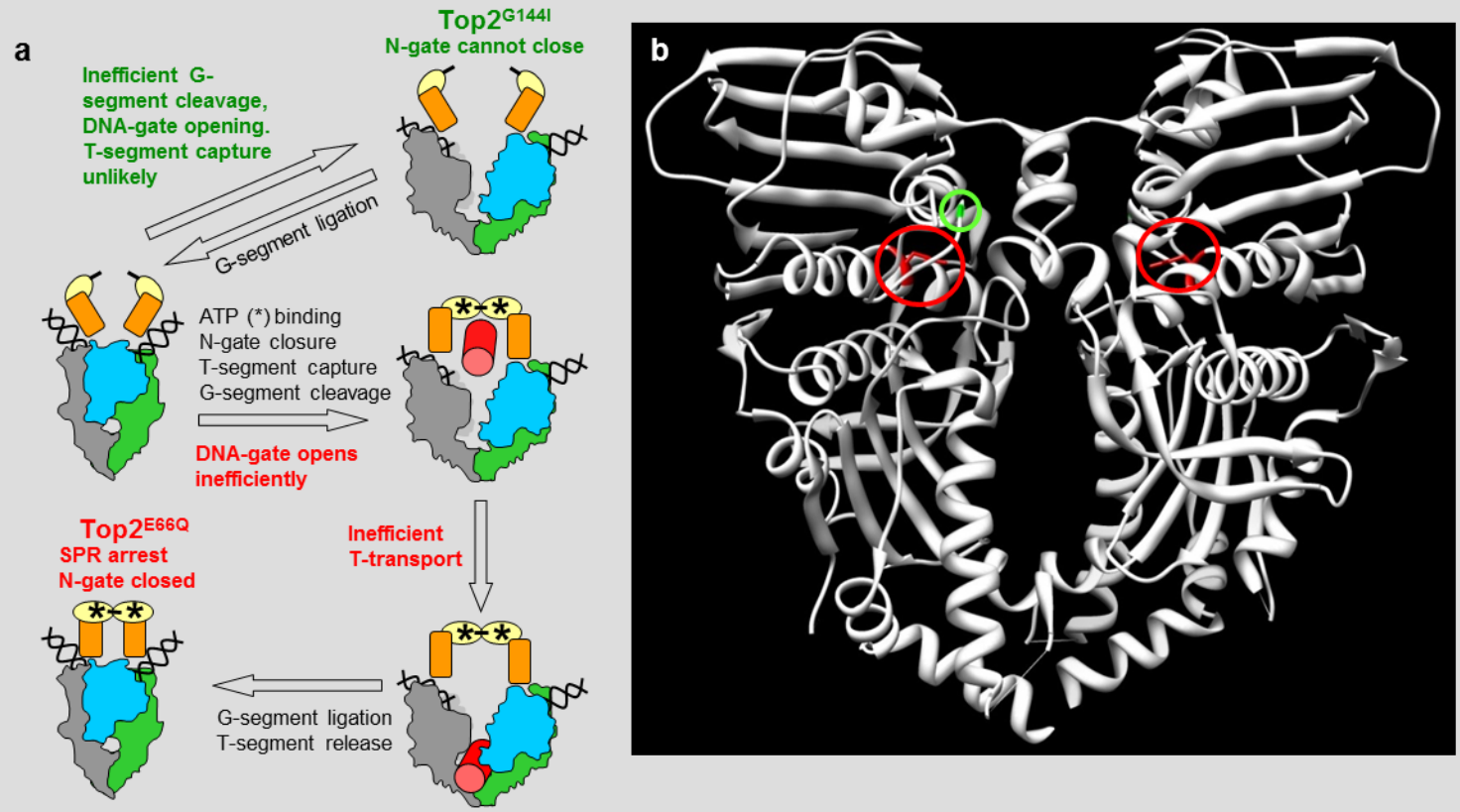


Figure 20. Mutations in the ATPase domain interfere with T-segment transport. (a) Cartoon illustrating the catalytic defects in Top2^{G144I} which cannot bind ATP (green) and Top2^{E66Q} which cannot hydrolyze ATP (red). (b) Crystal structure of the ATPase region of Top2 shown as a homodimer. G144 is highlighted in green and E66 is highlighted in red. The G144 residue on the right monomer is not visible in this image.

Strand Passage Reaction mutants reveal a stage of the enzymatic cycle that is monitored by the Topoisomerase II checkpoint

Due to the inability of Top2 mutants containing the Y782F mutation to cleave the G-segment, and therefore trap a T-segment, we wanted to know if T-segment capture and N-gate closure were required for checkpoint activation. A *top2-G144I* mutant that cannot bind ATP and so cannot close the N-gate, but can inefficiently cleave the G-segment was used (LINDSLEY and WANG 1991) (Figure 20). Remarkably, Top2^{G144I} was capable of activating a checkpoint similar in length to Top2-B44 (Figure 21a and 22a). When Top2^{G144I} was prevented from initiating the SPR by introducing the Y782F mutation, just

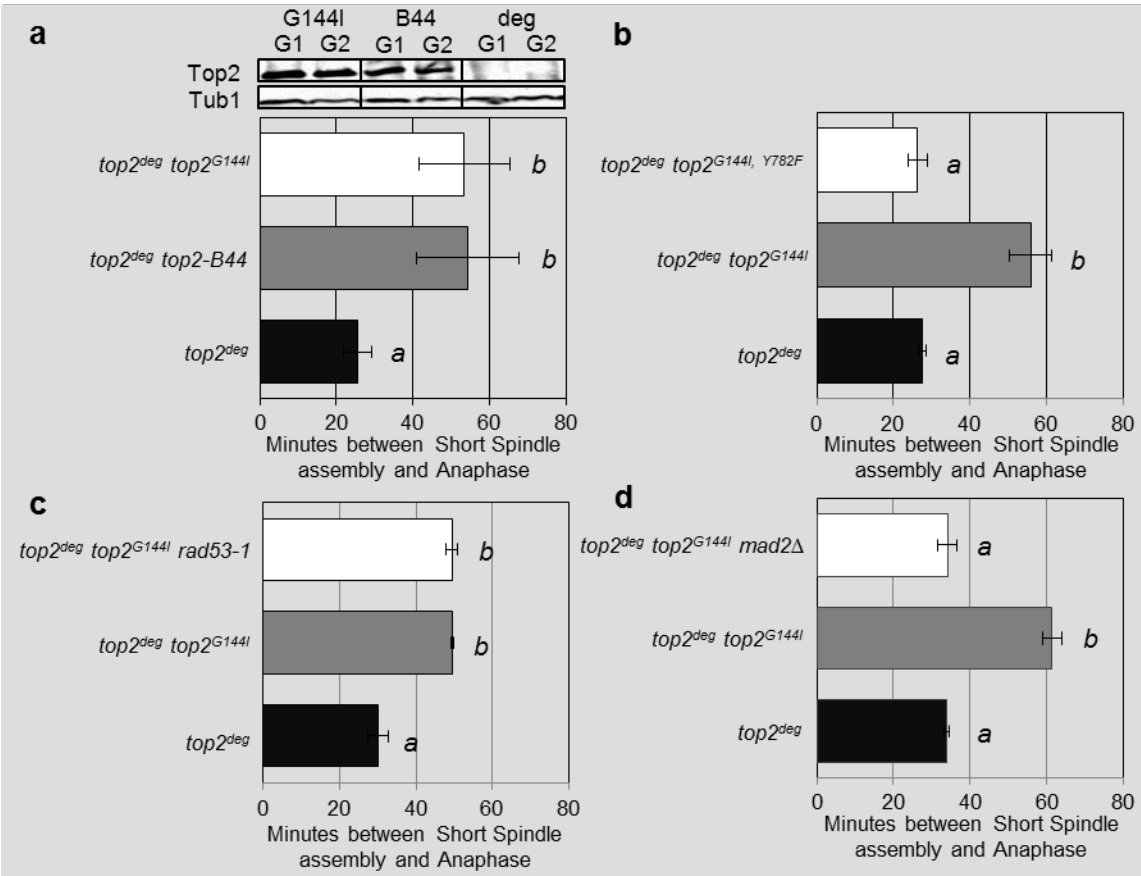


Figure 21. Top2 nucleotide binding defects activate checkpoint signaling. (a-d) Bars indicate the amount of time spent in G_2/M. Western blots show each mutant relative to Tub1 at G_1 and G_2. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different.

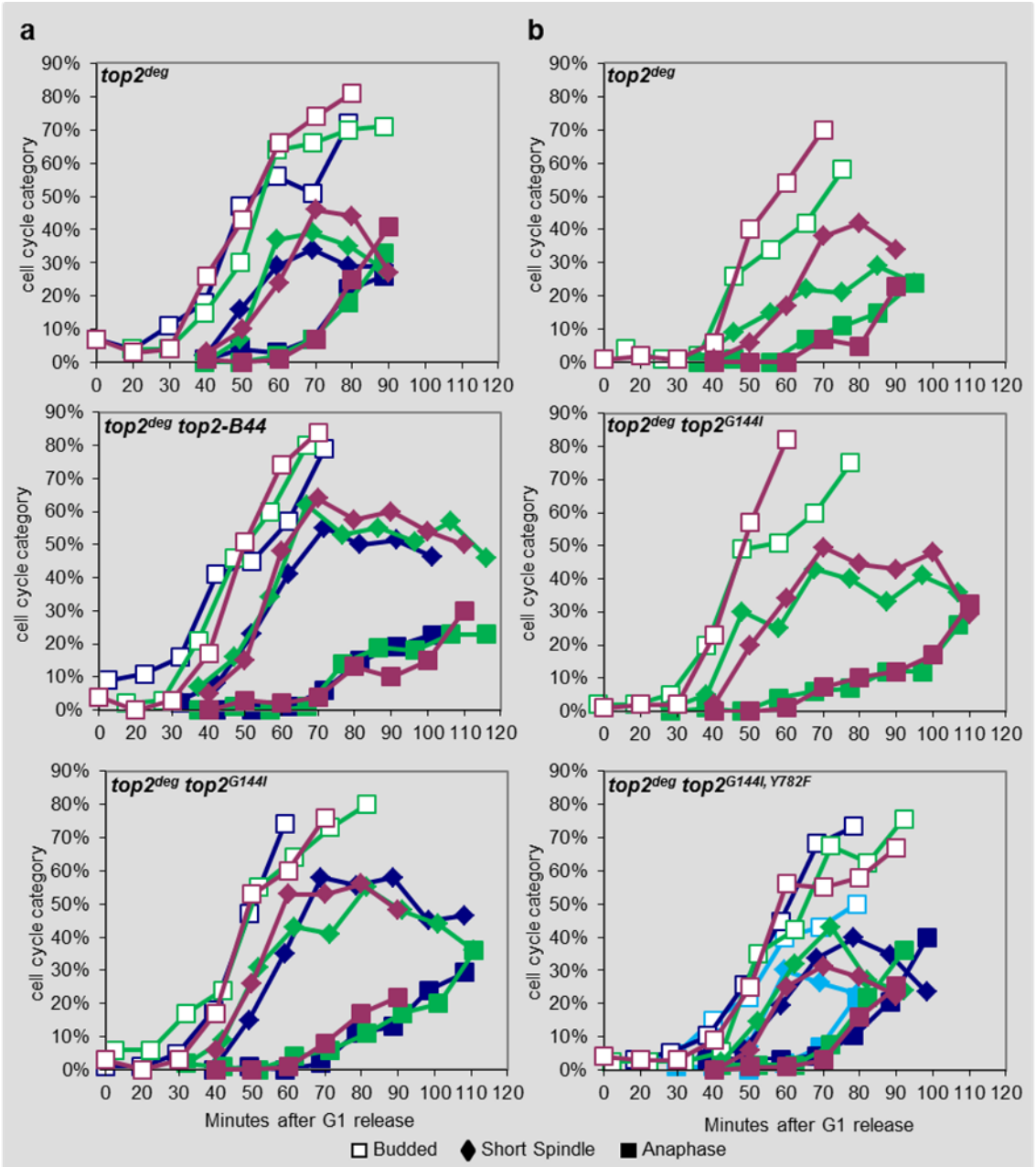


Figure 22. Cell cycle analyses depicting the requirement of SPR initiation to activate the checkpoint in Top2 ^{G144I} mutants.

as in Top2-B44^{Y782F}, it was no longer capable of checkpoint activation (Figure 21b and 22b). This indicates that DNA cleavage is required for Topo II checkpoint activation, but that N-gate closure in which a T-segment is captured is not.

Top2^{G144I} is not thought to induce DNA breakage or activate the DNA damage checkpoint, based on biochemical and structural predictions. In fact, Top2^{G144I} has a reduced incidence of DNA cleavage activity. The reduction is the result of inefficient T-segment capture which is caused by its inability to bind ATP and close the N-gate

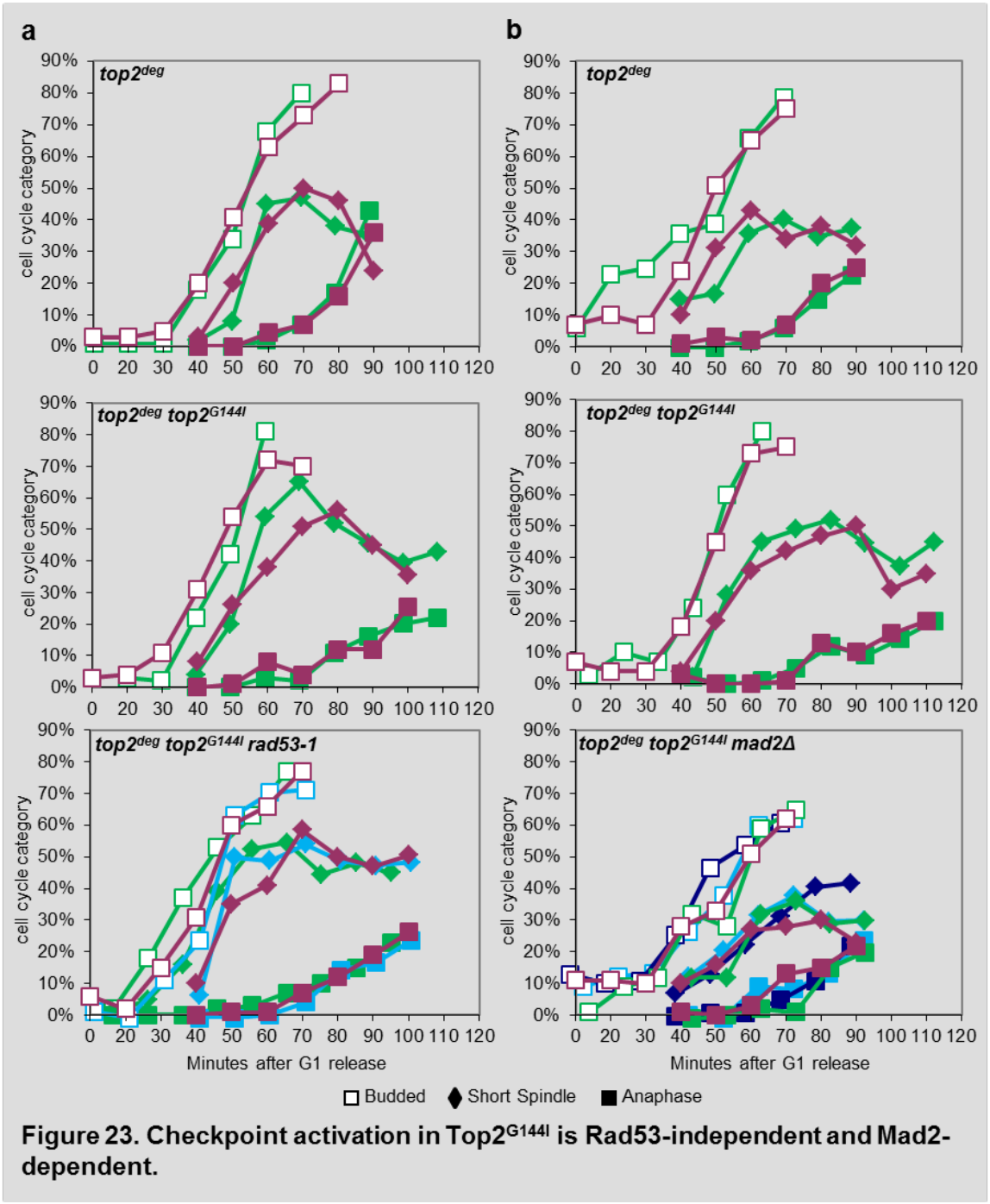


Figure 23. Checkpoint activation in Top2^{G144I} is Rad53-independent and Mad2-dependent.

(LINDSLEY and WANG 1993). Despite this we wanted to ensure that Top2^{G144I} is activating the Topo II checkpoint. We found that checkpoint activation by Top2^{G144I} is independent of the DNA damage checkpoint kinase Rad53 (Figure 21c and 23a). In contrast, double mutants of *top2*^{G144I} combined with a deletion of *MAD2* were defective for checkpoint activation (Figure 21d and 23b). The dependence on Mad2, but independence from Rad53 for checkpoint activation was therefore the same between *top2-B44* and *top2*^{G144I}. This similarity provides further support that both mutants activate the same checkpoint.

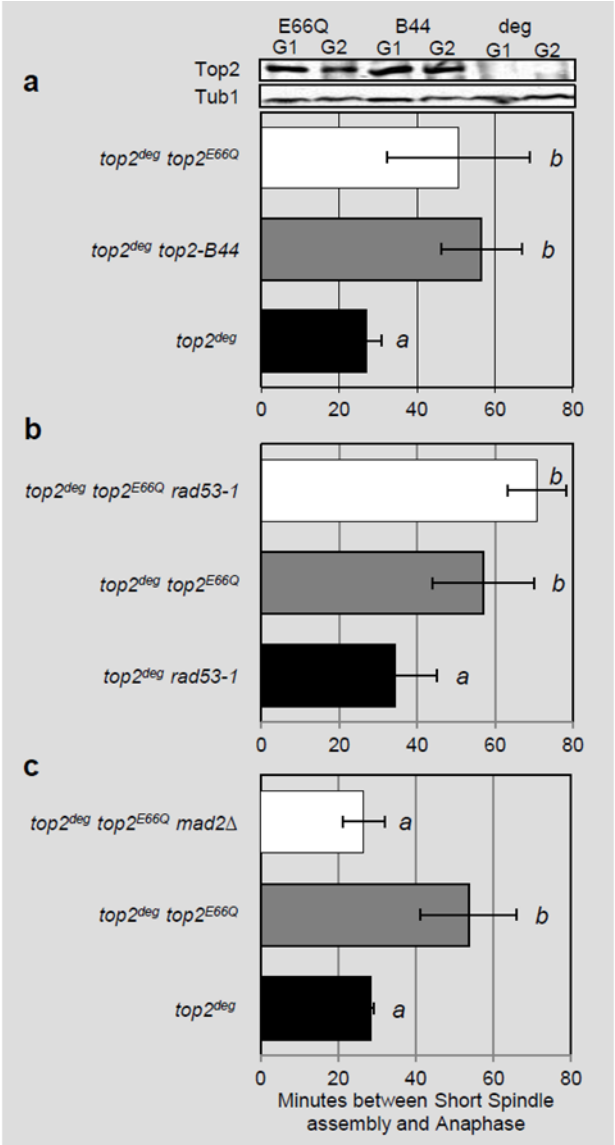


Figure 24. Top2 ATP hydrolysis defects activate checkpoint signaling. (a-c) Western blots show each mutant relative to Tub1 at G_1 and G_2. Bars indicate the amount of time spent in G_2/M. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different.

Top2^{E66Q} is a mutant that can bind ATP, but cannot hydrolyze it (BAIRD *et al.* 1999). This means it is similar to Top2^{G144I}, in that it cannot hydrolyze ATP, but for different reasons (Figure 20). Top2^{G144I} cannot hydrolyze ATP because it cannot bind it and therefore cannot close the N-gate. This is in contrast to Top2^{E66Q} which can bind ATP and close the N-gate, but cannot hydrolyze the ATP. This loss in hydrolysis means Top2^{E66Q} cannot

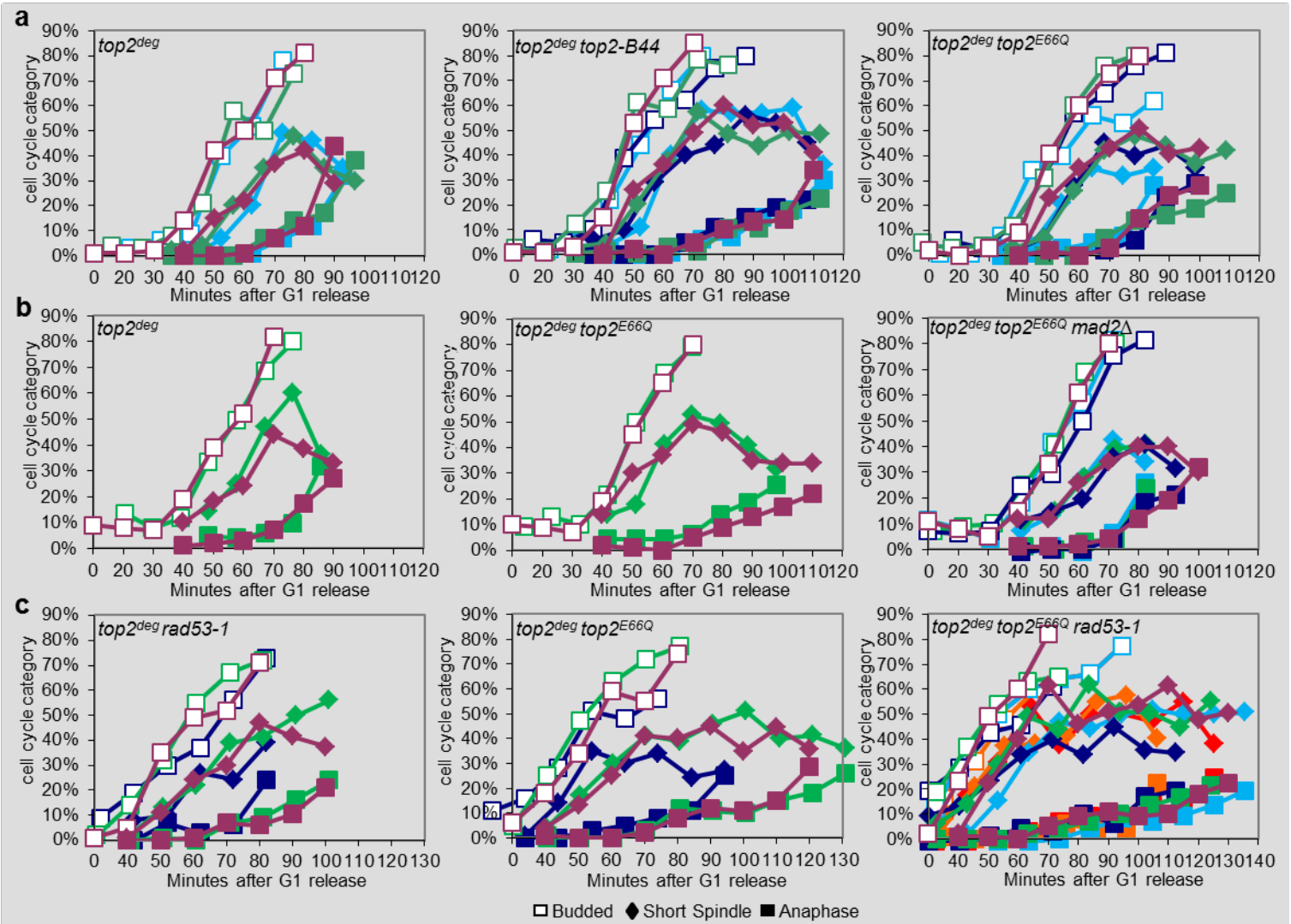
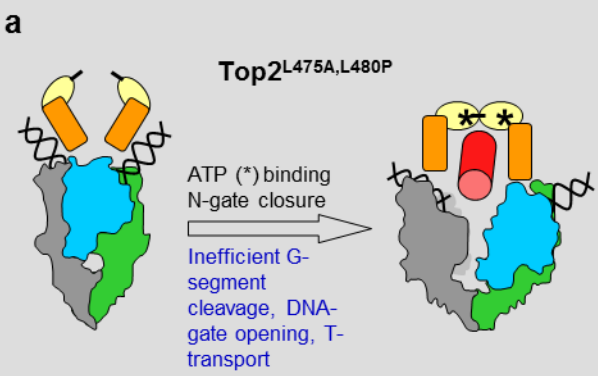


Figure 25. Cell cycle analyses depicting checkpoint activation by Top2^{E66Q} is Mad2-dependent and Rad53-independent.

swing the linker region and therefore cannot open the DNA gate efficiently. The two mutants differ in their ability to close the N-gate and in their capability of opening the DNA gate. However, they both have defects in ATP hydrolysis and are defective in T-segment transport and both activate a Rad53-independent, Mad2-dependent checkpoint (Figure 24 and 25).

To test if perturbed ATP hydrolysis is monitored by the checkpoint we examined a third mutant, *top2*$^{L475A/L480P}$. This mutant encodes an enzyme which is proficient for ATP hydrolysis, but still has defective T-segment transport (WASSERMAN and WANG 1994) (Figure 26). We found that this mutant had a G_2/M delay that was similar to the previous mutants and that this checkpoint was also Mad2-dependent and Rad53-independent, suggesting that the Topo II checkpoint is activated by inefficient T-segment transport (Figure 27 and Figure 28).



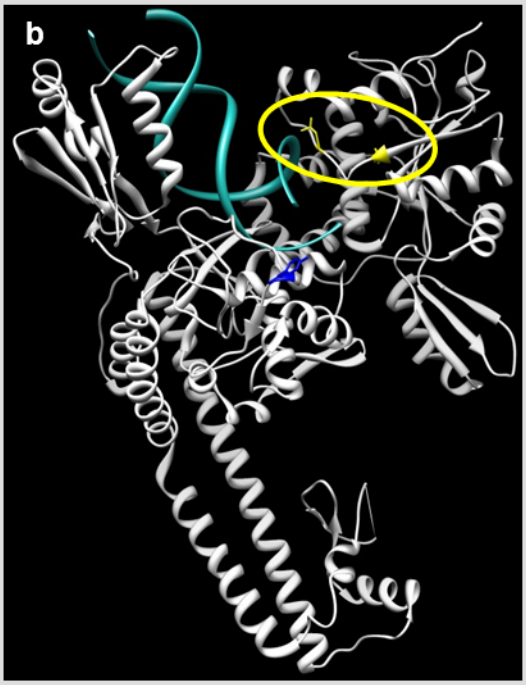


Figure 26. Top2^{L475A,L480P} has inefficent T-segment transport. (a) Cartoon describing the catalytic defects of Top2^{L475A,L480P} . This mutant while capable of binding and hydrolyzing ATP and closing the N-gate has inefficient G-segment cleavage and therefore defective T-segment transport. (b) A monomer of the DNA gate and upper C-gate region of Top2. L475 and L480 are highlighted in yellow. DNA is shown in teal and the catalytic tyrosine is shown in blue.

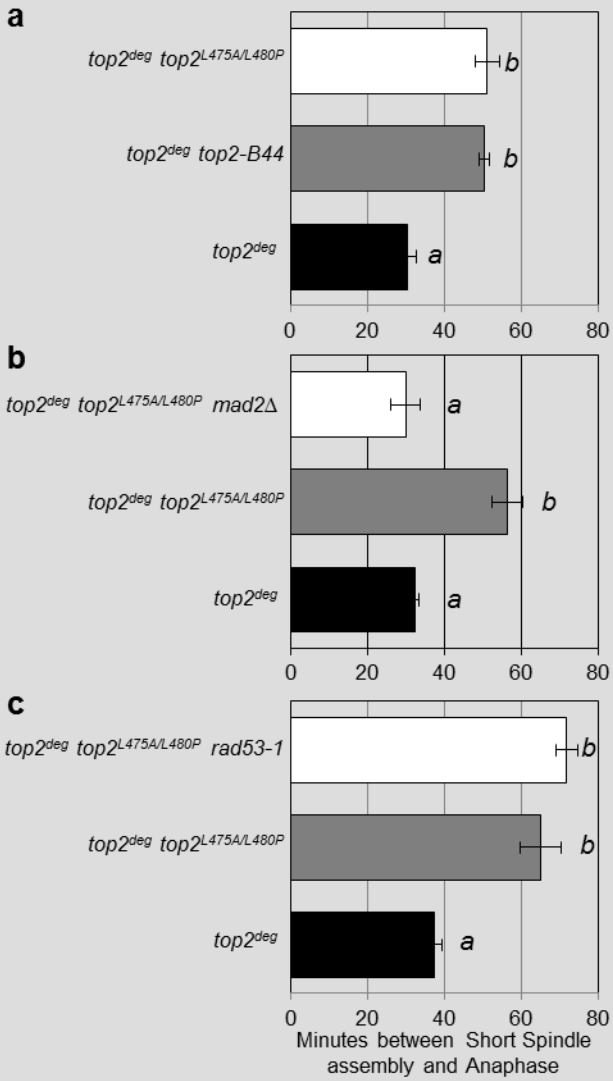


Figure 27. Inefficient DNA transport activates checkpoint signalling. (a-c) Western blots show each mutant relative to Tub1 at G_1 and G_2. Bars indicate the amount of time spent in G_2/M. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different.

We compared the data from these mutants to two mutants that are located C-terminal to the DNA gate, *top2*^{G738D} and *top2*^{P824S}. These mutants are known to have an overall reduced rate of strand passage, but are not predicted to have a defect in T-segment transport (LIU *et al.* 1994) (Figure 29a). We found that these mutants do not activate a checkpoint and proceed through the cell cycle without delay (Figure 29b&c and 30).

Based on the results from the mutants discussed in this section, we conclude that checkpoint activation does not require: (1) a defect in N-gate opening or closure, or (2) a defect in ATP binding or hydrolysis. Moreover, a reduced rate of strand-passage is not sufficient for checkpoint activation. Together, these mutants indicate that the checkpoint monitors the conformational changes associated with the T-segment transport step of the SPR (Figure 31). While Top2-B44 has not been

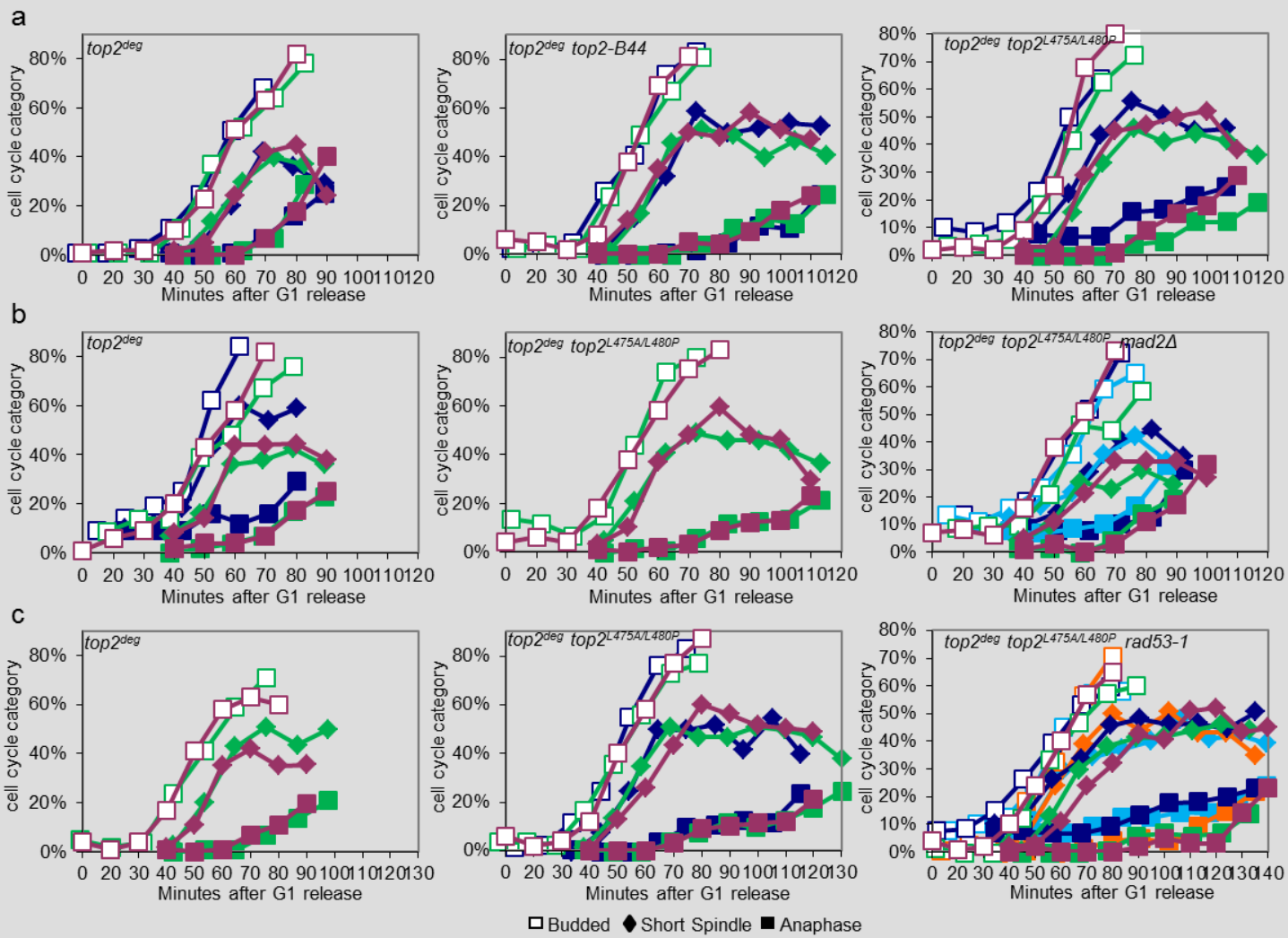


Figure 28. Cell cycle analyses depicting checkpoint activation by Top2$^{L475A,\ L480P}$ is Mad2-dependent and Rad53-independent.

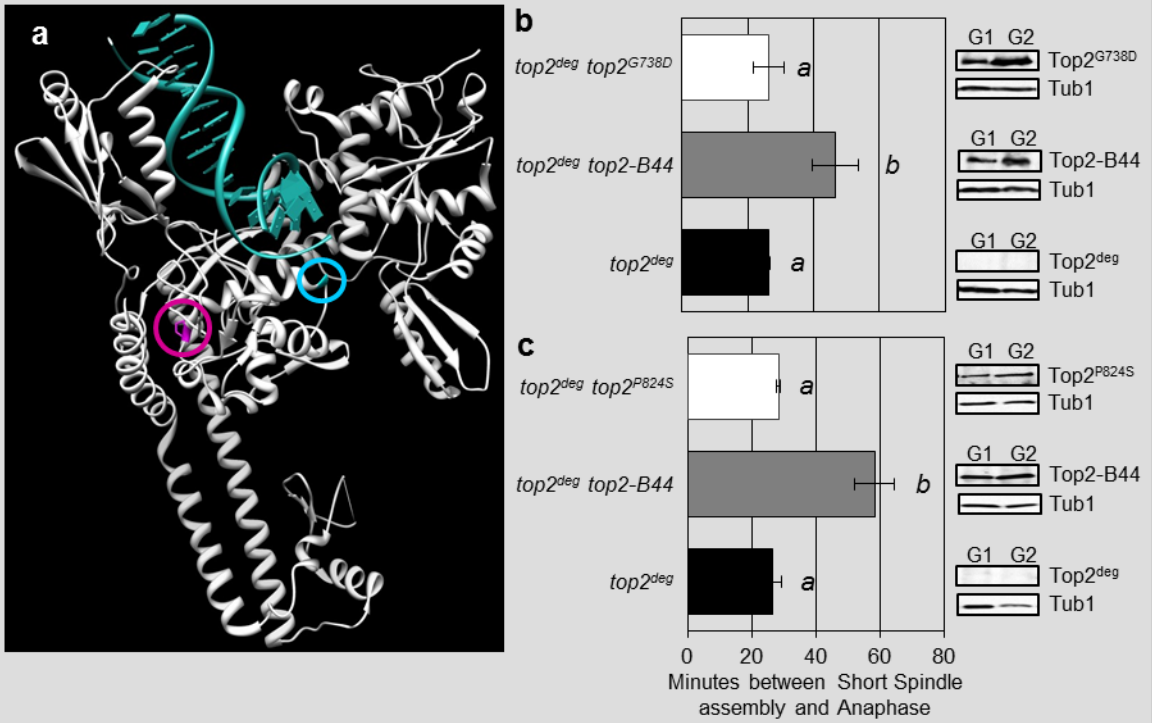


Figure 29. Top2 mutants with a slow SPR do not activate a checkpoint. (a) A monomer of the DNA gate and upper C-gate region of Top2. G738D is highlighted in light blue and P824S is highlighted in magenta. DNA is shown in teal. (b-c) Western blots show each mutant relative to Tub1 at G_1 and G_2. Bars indicate the amount of time spent in G_2/M. *a* values are significantly different to *b* values. Strains with the same letter are not significantly different.

characterized biochemically, it is also predicted to have a reduced rate of T-segment transport, which further supports our conclusion. Its mutation, F977L, is located between the TOPRIM fold and the core of the DNA-binding region of the enzyme (Figure 32). This suggests this mutant affects the positioning of the G-segment within the positively charged groove and/or might perturb communication between these regions which is believed to coordinate G-segment cleavage and T-segment transport.

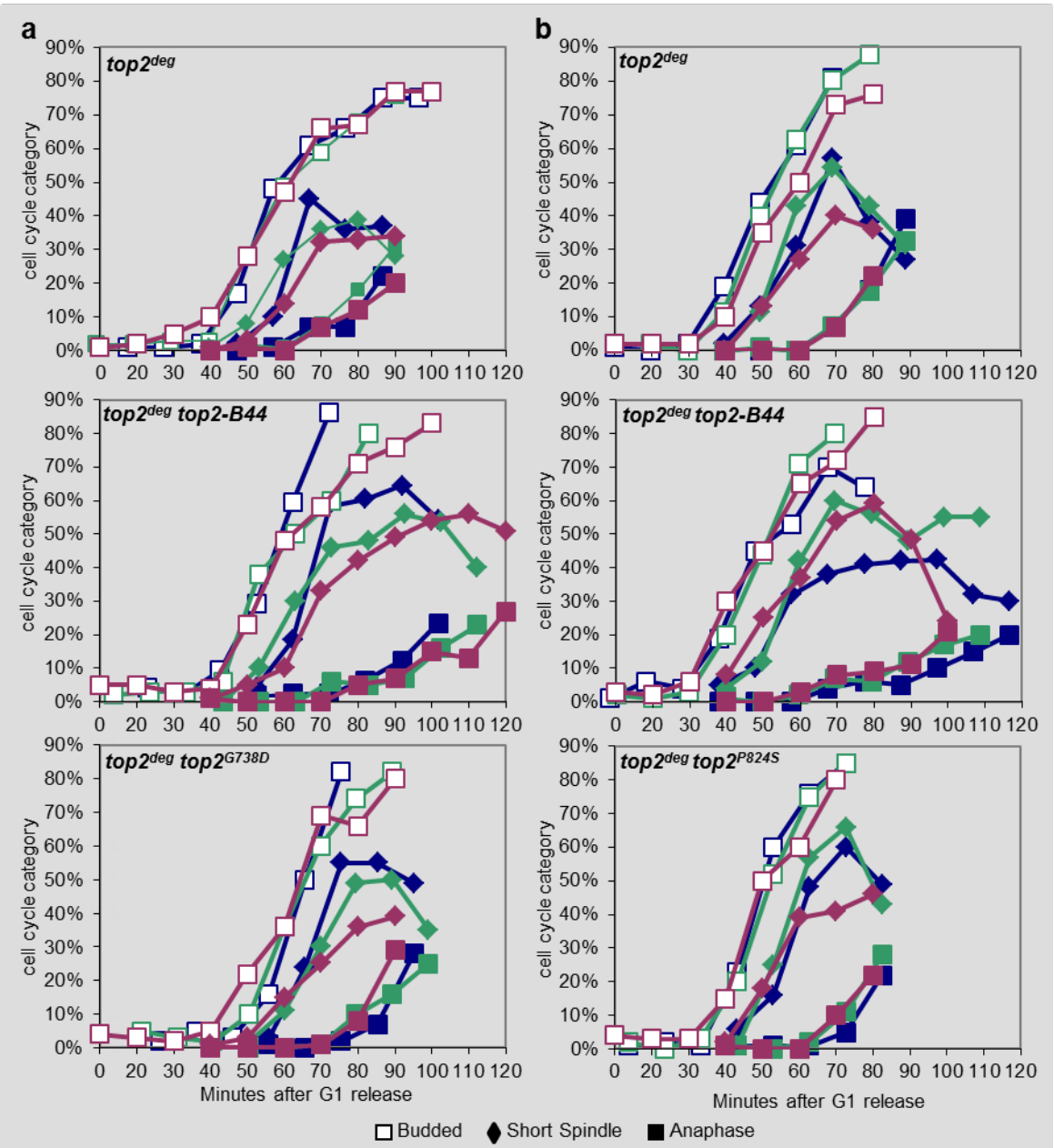


Figure 30. Cell cycle analses illustrating the lack of checkpoint activation in Top2^{G38D} and Top2^{P824S}.

DISCUSSION

Evidence presented here indicates that the previously identified Topo II checkpoint is not activated as an indirect consequence of perturbed Top2 activity. The lack of cell cycle delay after Top2^{deg} was depleted in G_1 indicates that DNA topology defects, including

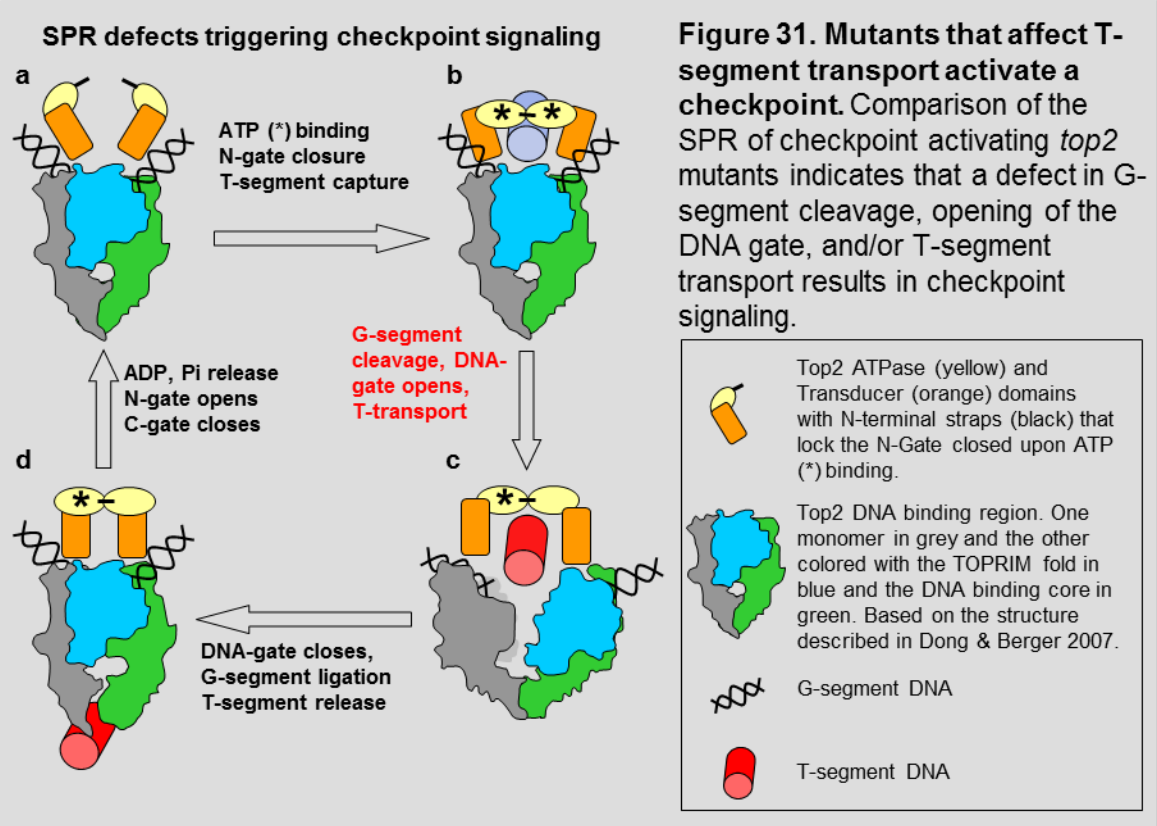


Figure 31. Mutants that affect T-segment transport activate a checkpoint. Comparison of the SPR of checkpoint activating *top2* mutants indicates that a defect in G-segment cleavage, opening of the DNA gate, and/or T-segment transport results in checkpoint signaling.

hyper-catenation, are not sufficient in yeast for the spindle checkpoint or other checkpoints to be activated (Figure 15). Instead, the mechanism of checkpoint activation appears to require direct monitoring of the SPR of Top2. In conjunction with previous data (ANDREWS *et al.* 2006), these results establish that the Topo II checkpoint is distinct from other checkpoints.

Previously identified and characterized checkpoints detect physical perturbations such as DNA breaks or defective microtubule-kinetochore attachments. This is in contrast to the Topo II checkpoint described here, in which an enzymatic activity is monitored. Evidence is abundant that the SPR of Top2 is directly monitored by cells to ensure that sister chromatids are properly separated prior to anaphase. This difference suggests a novel class of cell cycle checkpoint. The Topo II checkpoint, rather than monitoring a physical structure, appears to monitor an enzyme activity. Due to the

intimate nature of the DNA structure and Top2 enzyme activity we cannot specifically conclude that the cell is monitoring the enzymatic activity versus the unique DNA structure associated with Top2. Rather, we would argue that they are really two sides of the same coin. The very specific structure of the DNA that occurs during the SPR would not arise in the cell without the enzymatic activity of Top2. Similarly, Top2 would not undergo the conformation changes of the SPR without the DNA. Either way, the Topo II checkpoint is intimately related to the enzyme cycle of Top2, which in and of itself is unique. Other checkpoints are not associated with the function of any single protein and rather monitor a physical structure that can result from a number of different situations. This dependence on a single enzyme makes the Topo II checkpoint unique.

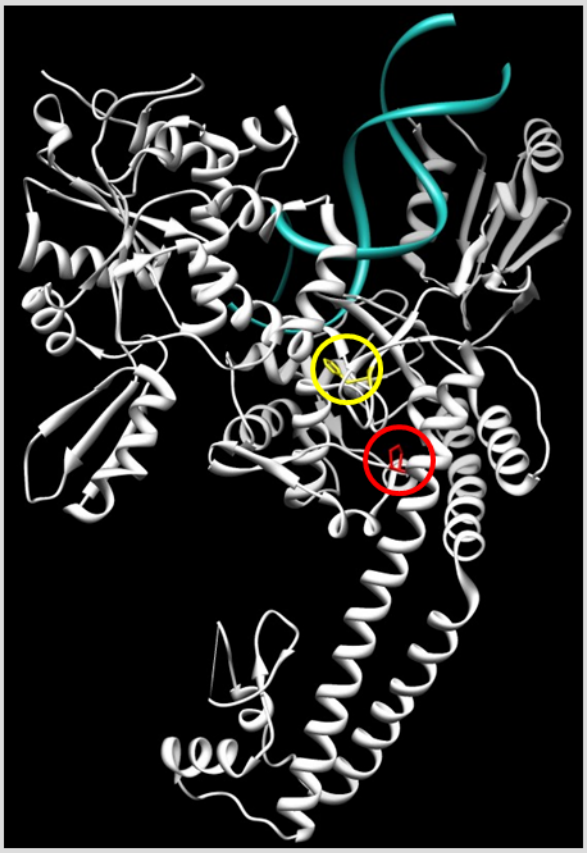


Figure 32. Crystal structure of Top2 depicting the residues mutated in Top2-4 and Top2-B44. A monomer of the DNA gate and upper C-gate region of Top2. The mutated residue in *top2-4*, P821, is highlighted in red. F977, the residue mutated in *top2-B44*, is highlighted in yellow. DNA is shown in teal.

The mutants that activate the Topo II checkpoint are similar in their overabundance of specific SPR intermediates (defects in T-segment transport) that may form a structural platform for signaling cascade complex assembly. We suggest that checkpoint proteins can bind to Topo II when specific steps in the SPR are either delayed or locked. In Chapter II,

evidence will be provided to suggest this assembly might occur in the unstructured C-terminal region (CTR) of Top2 (this region is not to be confused with the C-gate of Top2). The CTR is a sequence of about 200 amino acids that does not yield a defined structure by X-ray crystallography and is therefore thought to be flexible. The CTR is located C-terminal to the C-gate and when deleted does not affect the SPR of Top2 (CARON *et al.* 1994).

While the presented research has answered many questions about the Topo II checkpoint there are still questions about the checkpoint signaling complex. It is interesting to speculate that the enzymatic monitoring of Top2 by the Topo II checkpoint could have evolved due to the remarkable abundance of naturally occurring Topo II poisons and inhibitors that interrupt the SPR (HENGSTLER 2002; NITISS 2009). As mentioned previously, Topo II poisons lead to an accumulation of a Top2-DNA covalent complex, such that the DNA is cleaved, thus causing extensive dsDNA breaks and activating the DNA damage checkpoint (NAM *et al.* 2010). This is in contrast to Topo II inhibitors that do not trap the enzyme when the DNA is cleaved. The bisdioxopiperazine class of inhibitors (e.g. ICRF-187 or ICRF-193) traps the enzyme after the N-gate has closed, but prevents ATP hydrolysis. This type of drug mimics $Top2^{E66Q}$ in which ATP is bound, but not hydrolyzed. Similar to $Top2^{E66Q}$, bisdioxopiperazines activate the Topo II checkpoint (DOWNES *et al.* 1994). It seems that the cell has evolved a way to cope with toxins that can attack the essential function of Topoisomerase II by activating either the DNA damage or Topo II checkpoints.

CHAPTER II

The C-terminal Region of Top2 plays a role in cell viability and Topoisomerase II checkpoint activation

The previous chapter focused on the Strand Passage Reaction of Topoisomerase II and showed several crystal structures and space filling models. While these images are informative they ignore the most C-terminal region (CTR) of Top2. Yeast Topoisomerase II is 1,428 amino acids long. However, the CTR has not been solved by crystallography and is presumed to be unstructured as structural information for this region is lacking. We do know that the CTR, specifically the last 208 amino acids, are dispensable for the essential, enzymatic function of Top2 (CARON *et al.* 1994). This truncated version of Top2 ($Top2^{1-1220}$) is capable of complementing *top2* temperature sensitive strains and can decatenate plasmids *in vivo* (CARON *et al.* 1994). This result was further supported by another study examining $Top2^{1-1236}$ (JENSEN *et al.* 1996). These authors found this mutant enzyme supported viability, was capable of decatenation *in vitro*, and properly localized to the nucleus (JENSEN *et al.* 1996). The C-terminal region has the lowest sequence conservation between species, but is highly post-translationally modified in all species examined (GEISS-FRIEDLANDER and MELCHIOR 2007; LOU *et al.* 2005)

One such modification is SUMO conjugation (small ubiquitin-like modifier). SUMO is a small protein of only about 100 amino acids in length. SUMOylation may affect protein stability, localization, or activity of proteins because the addition of SUMO alters protein surfaces. This alteration can either promote or disrupt interactions with proteins, DNA, and/or other macromolecules (GEISS-FRIEDLANDER and MELCHIOR 2007). Top2 has three regions in the CTR that match the consensus sequence for SUMO modification. Within

those regions are five potential lysines for SUMO modification. Mutation of these five lysines to arginines prevents the addition of SUMO and results in a chromosome cohesion defect at yeast centromeres (BACHANT *et al.* 2002). Additionally, a decrease in the faithfulness of chromosome transmission results when the yeast E3 SUMO ligases, Siz1 and Siz2, are lost (TAKAHASHI *et al.* 2006).

Human Topoisomerase IIα is also known to be modified by SUMO. In HeLa cells, studies have shown that depletion of a SUMO E3 ligase, PIASγ, results in a loss of Topo IIα centromere localization (DIAZ-MARTINEZ *et al.* 2006). Further, binding of Topo IIα to DNA stimulates SUMOylation of Topo IIα at lysine 660 in human cells. This SUMOylation inhibits Topo IIα decatenation activity (RYU *et al.* 2010). Ryu *et al.* suggest that SUMOylation of Topoisomerase IIα regulates decatenation at centromeres. This regulation promotes centromere cohesion until sister chromatids are ready to segregate in anaphase.

In addition to SUMO, Topoisomerase II is post-translationally modified by ubiquitin. There is a large body of research on the role of Topoisomerase II ubiquitination in mammalian cells; however, little has been done in yeast. From the mammalian research we know that Topoisomerase IIα is ubiquitinated in a BRCA1-dependent manner (LOU *et al.* 2005). When cells are deficient for BRCA1 they have lagging chromosomes in anaphase of mitosis, similar to cells treated with the Topo II inhibitor, ICRF-193. Studies done *in vitro* by Lou *et al.*, using nuclear extracts deficient for BRCA1, resulted in defective DNA decatenation. In support of this, ubiquitination through a BRCA1-dependent pathway was shown to correlate with higher DNA decatenation activity (LOU *et al.* 2005). These data show that post-translational modification of Topo IIα by ubiquitination plays a role in the regulation of enzymatic activity.

Topo IIα is known to also be ubiquitinated by Bmi1/Ring1A. In contrast to BRCA1-dependent ubiquitination, drug induced proteasomal degradation of Topo IIα is induced by the E3 ubiquitin-ligase activity of Bmi1/Ring1A (ALCHANATI *et al.* 2009). Upon exposure to VM26 (teniposide), Topo IIα is degraded in a Bmi1/Ring1A, ubiquitin-dependent manner. When ubiquitination is inhibited, the cells become more sensitive to VM26. These instances of ubiquitination and SUMOylation illustrate the significant and diverse functional effects of post-translational modification of Topo IIα.

On top of SUMOylation and ubiquitination, Topoisomerase II is extensively phosphorylated in its CTR. This phosphorylation is primarily the result of Casein Kinase II (CKII) activity. A number of CKII phosphorylation sites in the CTR have been identified (ALGHISI *et al.* 1994). These authors showed that Top2 is inactive when it is completely dephosphorylated. However, activity can be restored by the addition of CKII.

Curiously, the truncated version of Top2 (1-1220) retains activity *in vitro* and *in vivo*, despite the loss of the phosphorylated CKII residues. This is in contrast to a de-phosphorylated full-length Top2, which is nearly inactive (ALGHISI *et al.* 1994), suggesting that the CTR could function as a negative regulatory domain. Additionally, while C-terminal truncations of Top2 support viability in yeast and can decatenate DNA *in vitro*, not all truncations fully complement null mutants, which opens the possibility that the C-terminus has a secondary role (CARON *et al.* 1994).

Another difference between CTR truncation and loss-of-phosphorylation mutants was their differing response to Topoisomerase II poisons. Truncated versions of Top2 were found to increase drug resistance to etoposide and teniposide (Topoisomerase II poisons), while hypo-phosphorylated mutants had sensitivities similar to wild-type Top2 (VASSETZKY *et al.* 1996). Drug resistance, to Topoisomerase II poisons, is usually

associated with a reduced expression of Topoisomerase II or a reduced level of enzymatic activity. Both outcomes result in fewer strand-breakage events and so lessen the number of protein-DNA covalent complexes that can be trapped by the drug.

A possible explanation for these contrary results may lie in the fact that the hypo-phosphorylated mutants only had some of the phosphorylation sites mutated. They may have retained enough phosphorylation to not affect the overall activity of the enzyme. Therefore, this lack of an effect on enzymatic activity would not change the phosphorylation mutant's drug resistance from that seen with the wild-type enzyme. In contrast, the truncation mutant, Top2$^{1\text{-}1236}$, could have reduced activity. For a cell to be viable, it does not require high levels of Topoisomerase II activity. It is possible the Top2 truncation mutants might have enough activity to support viability, but that the activity is reduced enough to increase the cell's drug resistance.

In an effort to characterize the Topoisomerase II checkpoint a major goal is to identify proteins involved in the checkpoint signaling pathway, specifically the initiating proteins. Based on previous work, we hypothesized that the CTR of Top2 could function as a checkpoint signaling platform[18]. This region is not required for the SPR, is flexible, and is highly post-translationally modified. These characteristics make the CTR a promising candidate as the checkpoint signaling platform.

RESULTS

The C-terminal Region of Top2-B44 is required for viability

If the CTR is the checkpoint platform, as we predict, then deletion of the CTR should abolish the checkpoint in checkpoint activating *top2* mutants, such as *top2-B44*. Thus we

[18] Platform is defined as the structure that assembles proteins at a lesion, which allows the cell to perceive that lesion and respond by triggering a checkpoint cascade. For example, in the Spindle Assembly Checkpoint the platform is unattached kinetochores.

generated CTR truncation mutants of *TOP2* and *top2-B44* at amino acid 1236. These mutant forms of *top2*, on a plasmid, were transformed into a yeast strain that had its endogenous *TOP2* deleted and had a wild-type version present on a *URA3* plasmid. Transformants were transferred to media containing the drug 5-FOA. This drug selects against cells producing their own uracil, meaning yeast are selectively pressured to lose the *URA3* plasmid containing *TOP2*. Following 5-FOA drug selection only yeast containing a functioning Topoisomerase II are viable. We found that all cells transformed with *top2*$^{\Delta CTR}$ were able to grow on media containing 5-FOA, while those transformed with *top2-B44*$^{\Delta CTR}$ were unable to form colonies. Yeast transformed with *top2*$^{\Delta CTR}$ were able to lose the *TOP2* plasmid and survive with only Top2$^{\Delta CTR}$ present in the cell. However, cells were inviable when only *top2-B44*$^{\Delta CTR}$ was present, suggesting the CTR was required for *top2-B44* to support viability, but not in the case of *TOP2*.

These plasmids were then individually transformed into *TOP2/top2Δ* diploid yeast. Transformed diploids were induced to undergo meiosis and the resulting asci were dissected to obtain haploids. Half of the spores resulting should be *TOP2* and the other half should be *top2Δ*. Spores that have their endogenous *TOP2* deleted would need a functional Top2 supplied to be viable. We found that 45% of *top2Δ* yeast are able to grow when the only Topoisomerase II present was Top2$^{\Delta CTR}$. This is in contrast to 2% of viable spores recovered when Top2-B44$^{\Delta CTR}$ was the only Topoisomerase II present. These results further support the previous evidence suggesting that the CTR of Top2-B44 has a function that is required for viability.

The discrepancies in viability for cells expressing *top2*$^{\Delta CTR}$ in the 5-FOA (100%) versus sporulation assays (45%) are likely a result of experiment design. In the first case, a colony of yeast is transferred to selective 5-FOA media. These yeast initially

have Top2 protein present in all of the cells and are then pressured to eliminate the *pTOP2* plasmid. This means that as the cells continue to divide they will slowly lose the wild-type Top2 as it gets degraded and remain with only the mutant form. This type of experiment is less stringent than the sporulation experiment which requires a single cell, with no wild-type *TOP2* genes present and only the mutant *top2*$^{\Delta CTR}$ form expressed following meiosis, to replicate and divide to form a visible colony. A possible explanation for the 2% viable cells in the *top2-B44*$^{\Delta CTR}$ sporulation assay could be due to recombination in the diploids as they progress through mitosis. It is possible that haploid spores have obtained advantageous mutations that allow them to grow and form colonies.

These data suggesting that the Topo II checkpoint plays a role in cell viability are consistent with previous work. Andrews *et al.* showed that when the Topo II checkpoint is lost in *top2-B44* strains, through deletion of *MAD2* or *MAD1*, a reduced viability results.

Checkpoint activation requires the CTR of Top2-B44

We have established that the CTR is required for viability in *top2-B44* cells, but not in *TOP2* cells. To determine if the CTR plays a role in the Topo II checkpoint, both mutants were individually transformed into the degron strain, *top2*^{deg}. The length of G_2/M was assayed to determine if Top2-B44$^{\Delta CTR}$ is capable of activating a checkpoint after depletion of Top2^{deg} in G_1 arrested cells. We found that Top2$^{\Delta CTR}$ and Top2-B44$^{\Delta CTR}$ both proceed through the cell cycle with timing similar to Top2 (Figure 33). This suggests that deletion of the CTR in the normally checkpoint active Top2-B44 results in a loss of checkpoint activation.

These data and the previous viability results provide evidence that not only is the CTR of Top2-B44 required for viability, but also for checkpoint activation. Combined, these data indicate a role for the Topo II checkpoint in cell survival. This information supports the findings of Andrews *et al.* 2006 that the Topo II checkpoint prevents aneuploidy and cell death. Together, these results further implicate the Topo II checkpoint as a protector of the cell's genome.

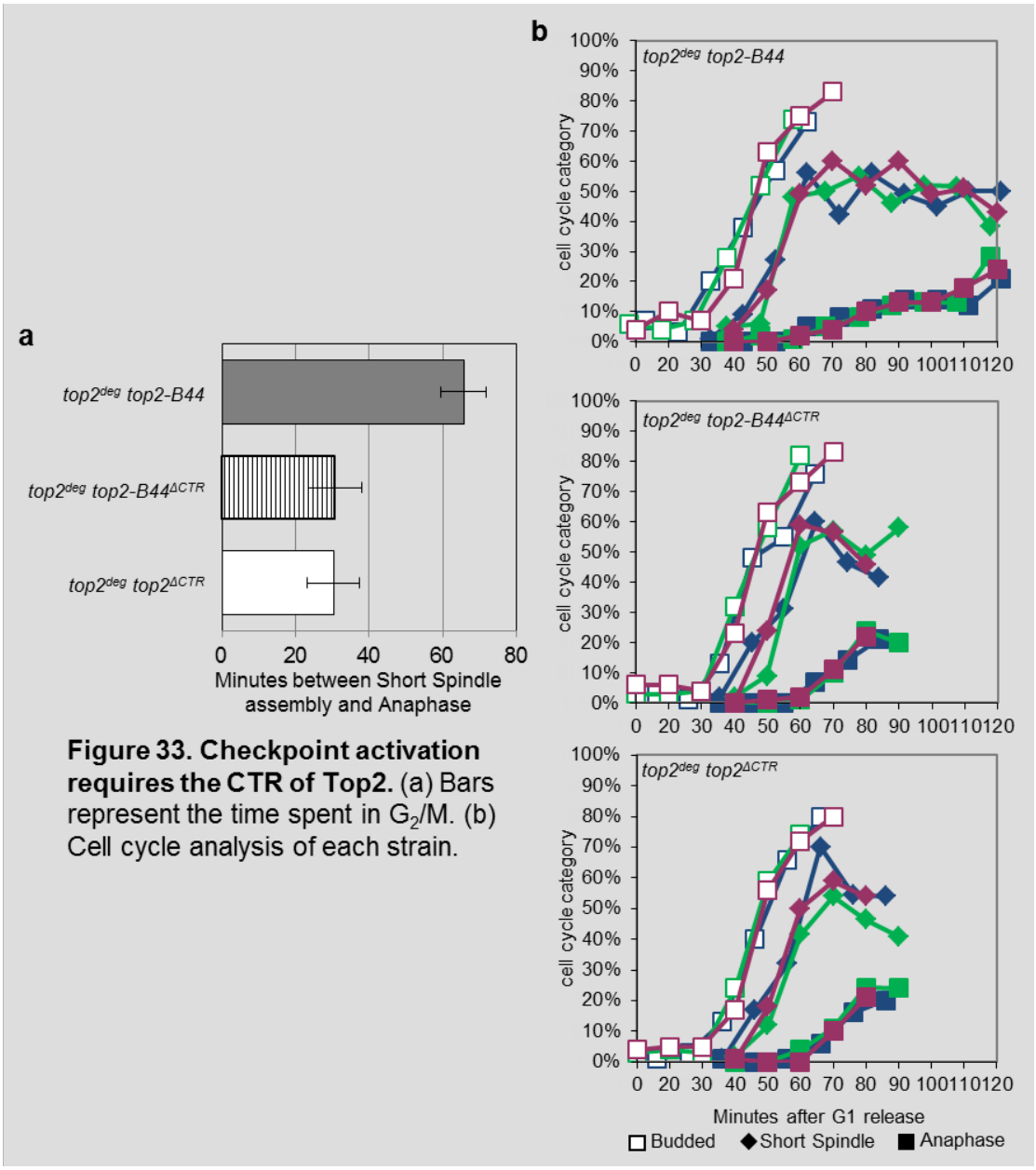


Figure 33. Checkpoint activation requires the CTR of Top2. (a) Bars represent the time spent in G_2/M. (b) Cell cycle analysis of each strain.

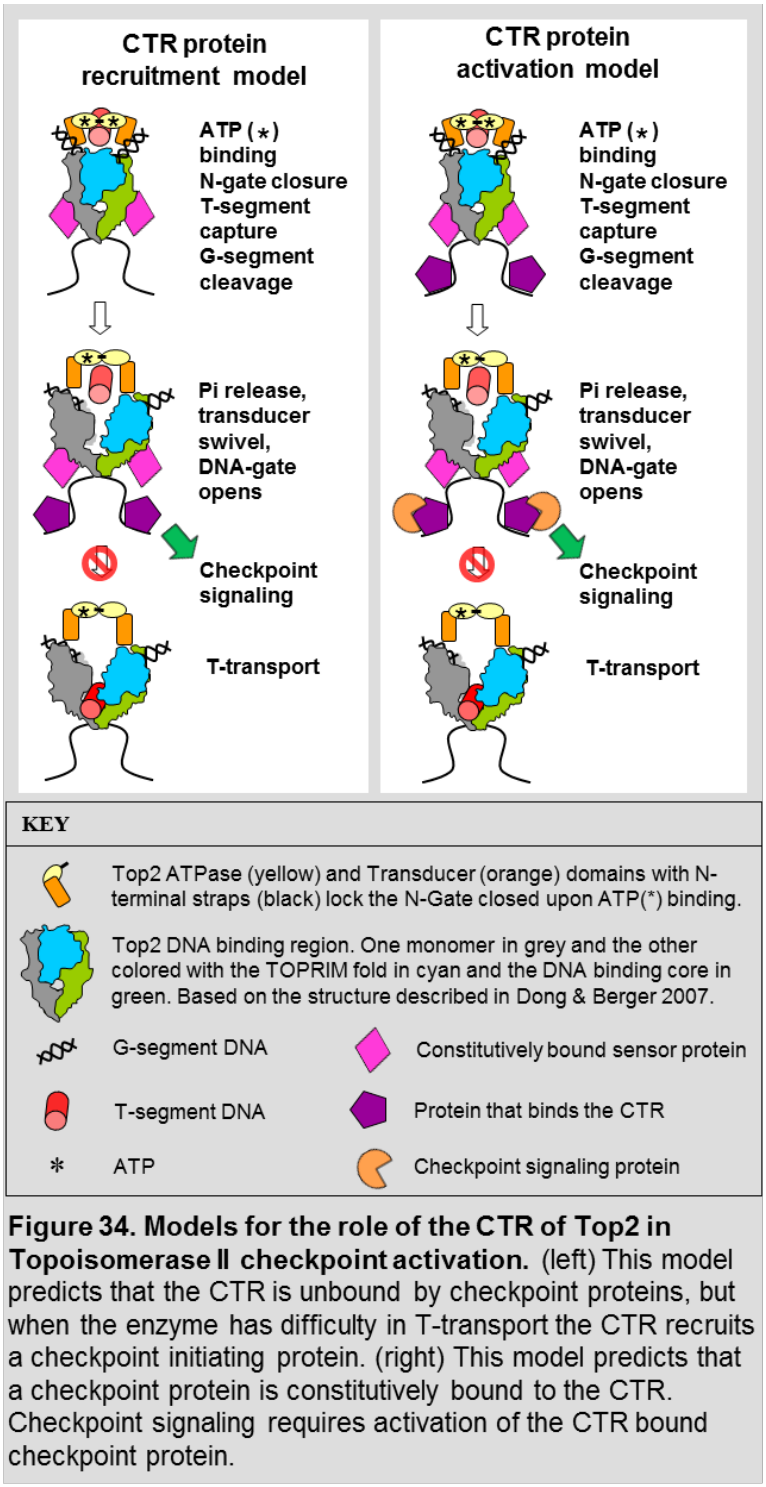


Figure 34. Models for the role of the CTR of Top2 in Topoisomerase II checkpoint activation. (left) This model predicts that the CTR is unbound by checkpoint proteins, but when the enzyme has difficulty in T-transport the CTR recruits a checkpoint initiating protein. (right) This model predicts that a checkpoint protein is constitutively bound to the CTR. Checkpoint signaling requires activation of the CTR bound checkpoint protein.

Overexpression of the CTR diminishes the G_2/M delay in *top2-B44* cells

If the CTR of Top2 is required for checkpoint activation because it functions as the checkpoint platform, as we proposed, and binds checkpoint signaling initiator proteins, then overexpression of the CTR alone could function to sequester some of these initiator proteins (Figure 34). If such initiating factors do not signal in this context, then the result may be abrogation of checkpoint signaling in *top2-B44* cells. To test these predictions we transformed *top2-B44* cells with empty plasmid or plasmid encoding Gal-CTR. The Gal-CTR plasmid has the CTR of *TOP2* (Met-1236-1428) under the control of

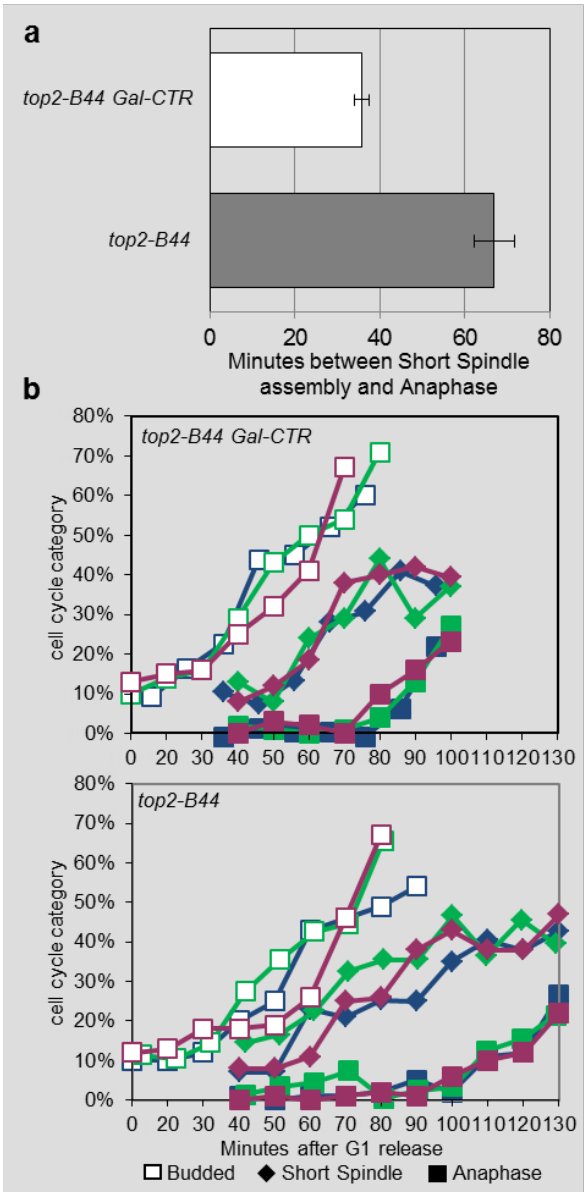


Figure 35. Checkpoint activation is lost when the CTR of Top2 is overexpressed. (a) Bars represent the time spent in G_2/M. (b) Cell cycle analysis of each strain.

the Galactose promoter for inducible overexpression. So to not affect cell viability by loss of checkpoint activation, transformants were selected for the plasmid, but under conditions in which the CTR was not expressed.

We found a loss of G_2/M delay when CTR expression was induced during G_1 synchronization (Figure 35). CTR overexpression resulted in the time spent in G_2/M to be reduced by about half, or approximately the length of wild-type G_2/M. This suggests that the overexpressed CTR is capable of binding checkpoint proteins and sequestering them from the full-length Top2-B44 protein, supporting the hypothesis that the CTR of Top2 plays a role in checkpoint activation through the binding of checkpoint proteins.

DISCUSSION

There are several interesting points to be made regarding these experiments. In our original model, the CTR of Top2 was hypothesized to recruit a protein when the checkpoint is activated (Figure 34). In Chapter I, we discussed the requirement for Top2-

B44 to be bound to the DNA and capable of beginning the SPR for it to signal checkpoint activation. The CTR, on its own, would not have SPR activity. Based on this, it would not be predicted to assemble complexes capable of signaling, but the data suggest that the CTR on its own is able to bind checkpoint proteins. However, unpublished results from our lab (Andrew Lane) suggest that the CTR of human Topoisomerase IIα is capable of interacting with DNA on its own. Similar work has not yet been done using the CTR of yeast Top2 and as these sequences are the most highly divergent of the enzyme, it is difficult to extrapolate from humans to yeast.

Second, our original hypothesis was that Top2-B44 transmits the need for checkpoint activation to the CTR. Most likely, this would seem to be the result of a sensor protein that detects the aberrant activity of Top2-B44, and would then recruit checkpoint initiator proteins to the CTR. If this is the case, then the CTR on its own would not be capable of this effect, which is at odds with the experimental finding that overexpression of Gal-CTR diminishes the G_2/M delay. However, it has commonly been noted that overexpression of mutant proteins lacking activation or interacting signals will still be able to bind proteins simply due to their overexpression. It is conceivable that this is the case for CTR overexpression.

Alternatively, it is possible the CTR of Top2 is constantly bound by a protein that is required for checkpoint activation. In Top2-B44, the combination of this unknown protein and another protein which is recruited to the CTR would result in checkpoint activation. In contrast, when the CTR is overexpressed it sequesters this constitutively bound protein so there is less available for binding the CTR of Top2-B44 and so the G_2/M delay is reduced. One thing in common with all these theories is a relevant interaction at the CTR, which could be investigated biochemically, genetically, or by yeast-two-hybrid.

CHAPTER III

Evidence that *top2-4* acts dominantly to disrupt chromosome segregation

Research on Topoisomerase II in yeast has been active since the late 1970s and 1980s. During that period the first temperature-sensitive mutants of *TOP2* were constructed and characterized (HOLM *et al.* 1985). At that time, the role of Type II Topoisomerases in the cell remained mysterious as little work had been done *in vivo*. To begin to determine the effect Top2 had on the cell, researchers constructed temperature sensitive mutants of *TOP2* using an integrative replacement/disruption method (SHORTLE *et al.* 1984). In this method, a vector containing a fragment of the gene of interest is mutagenized (a nick is introduced, enlarged and repaired by an error-prone process (SHORTLE *et al.* 1982) and integrated into the yeast genome at the endogenous location (Figure 36). If a single crossover occurs between a point mutation and the deleted end of the gene, then the mutation will be recombined into the intact copy of the chromosome, while the wild-type copy is disrupted. Excision of the plasmid, by growth on 5-FOA, results in a single chromosomal copy of the gene of interest, now bearing a mutation. This method was employed for the construction of the *top2-4* temperature sensitive yeast strain (HOLM *et al.* 1985).

Characterization of this strain in Holm *et al.* 1985 was limited to morphological observations. The authors discovered that temperature sensitive mutants of *TOP2* do not exhibit a uniform arrest when grown asynchronously at restrictive temperature. Instead, an asynchronous population will arrest with an approximately equal number of unbudded and large budded cells. This was surprising as the previously characterized cell cycle mutants would arrest at a single cell cycle phase when the cells were grown at

restrictive temperature (HARTWELL *et al.* 1973). This synchronous arrest indicated when the mutant protein was required for the continuation of the cell cycle. Due to the arrest in multiple cell cycle stages in *top2-4* cells, researchers were unable to conclude when Top2 function was required.

However, *top2* mutants do become inviable when allowed to traverse the cell cycle under restrictive conditions (HOLM *et al.* 1985). Inviability at restrictive temperature can be relieved when cells are arrested in G_1 and prevented from entering the cell cycle. This rescue was the first suggestion that the lethality of *TOP2* mutants resulted from a failure specific to the cell cycle (HOLM *et al.* 1985). The authors discovered that Top2 is required at the time of mitosis because if mitotic entrance under restrictive conditions is prevented, then lethality is rescued. So, loss of Top2 prior to mitosis is not lethal but functional Top2 is required for viability during mitotic progression.

Further papers continued to characterize the effects of *top2-4* strains grown at the non-permissive temperature. An increase in chromosome breakage and non-disjunction was found in *top2-4* cells growing above the restrictive temperature (HOLM *et al.* 1989). Specifically, this breakage is apparent on the long arms of chromosomes (around 300kb) (SPELL and HOLM 1994). The authors speculate that this might be due to the ability of catenations to be resolved passively on short arms but the long arms of chromosomes retain some catenations and are therefore prone to breakage. While these data indicate the persistence of cohesion between sister chromatids in *top2-4* cells, it does not provide definitive evidence that there is no Top2-4 protein present in the cell.

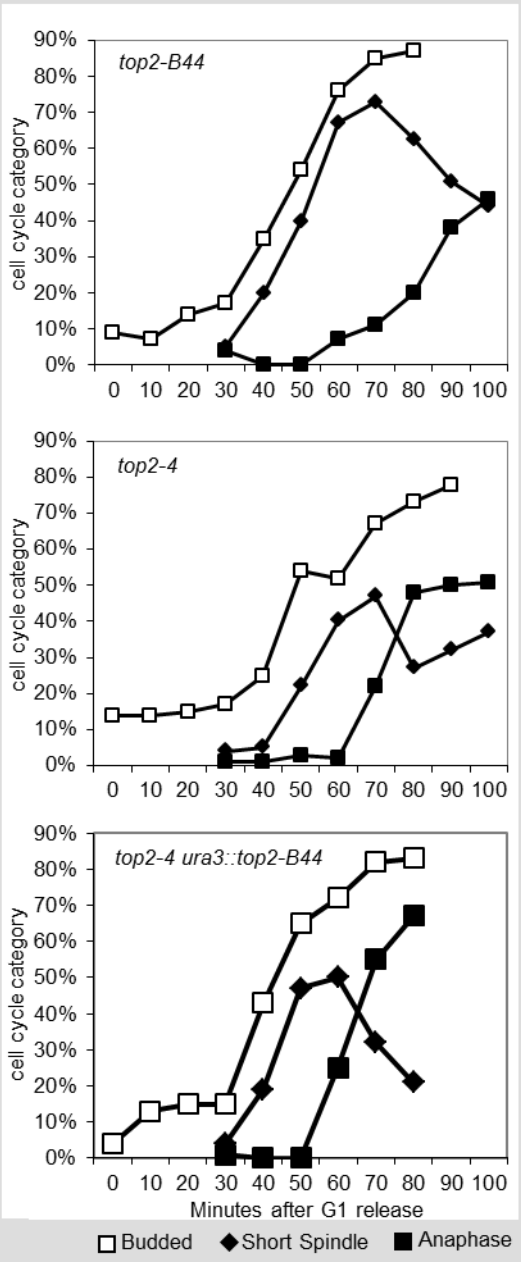


Figure 36. Top2-B44 cannot activate the Topo II checkpoint when Top2-4 is co-expressed. Cell cycle analysis of each strain.

While the initial paper and further papers worked out many answers for questions related to the function of Top2 in the cell, they did not determine what happened to Top2-4 above the restrictive temperature. Specifically they did not determine the stability of the Top2-4 protein or its ability to decatenate DNA *in vitro*. As such, there is no data on the consequences to the Top2-4 protein when cells are grown under restrictive conditions. Yet, because temperature sensitive strains are usually the result of the mutated protein becoming unstable at high temperatures, it was assumed this was the case for Top2-4 protein and so this strain was presumed to be null at restrictive temperatures. Based on these data, our hypothesis was that this strain could be used to test for the ability of *top2* mutants to activate the checkpoint. Before this could be done we needed to ensure that *top2-4* functioned as a null.

RESULTS

Top2-B44 does not activate a checkpoint in the presence of Top2-4

To determine if the *top2-4* strain could be used as a null, we transformed *top2-B44* on a plasmid into the strain and compared the cell cycle of the transformants to *top2-B44* and *top2-4* single mutant strains. *top2-B44* is temperature sensitive at 37°C, while *top2-4*

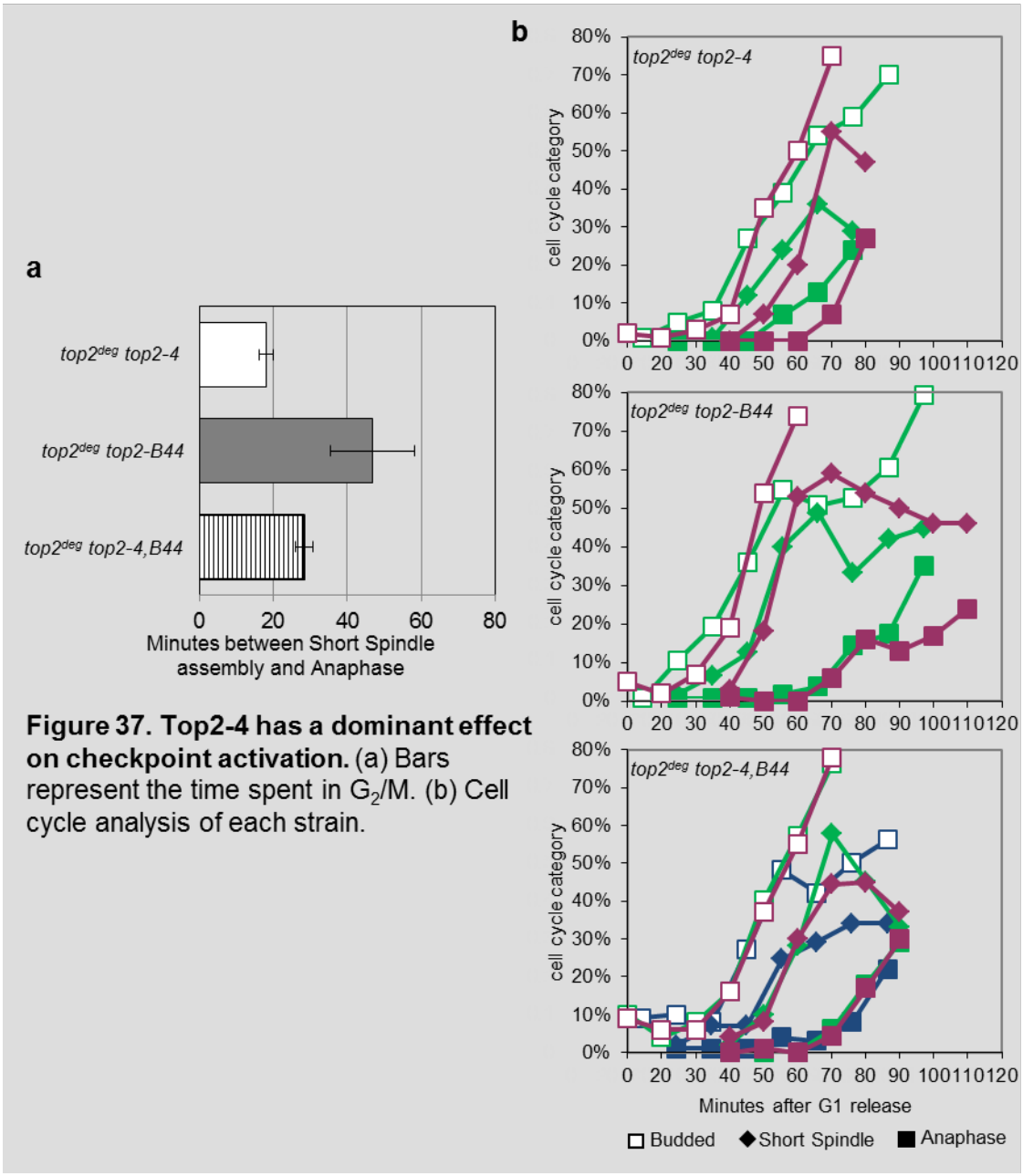


Figure 37. Top2-4 has a dominant effect on checkpoint activation. (a) Bars represent the time spent in G_2/M. (b) Cell cycle analysis of each strain.

is temperature sensitive at 30°C. Double mutants were synchronized in G_1 and shifted to 35°C so that Top2-4 is inactivated, and presumably degraded, while Top2-B44 is still present. Under these conditions the only Topoisomerase II present should be Top2-B44 and a checkpoint should be activated. This was not the case and the double mutants proceeded through the cell cycle without delay (Figure 36), meaning, the *top2-4 top2-B44* double mutant did not activate the Topo II checkpoint. This was surprising and was our first clue that the *top2-4* strain at restrictive temperature does not function as a null as previously presumed.

Instead Top2-4 appears to have a dominant effect on checkpoint function, as the G_2/M delay is lost in the double mutants. Also, we found that while *top2-4* strains are dead when grown at 30°C and *top2-B44* strains are dead above 37°C, the double mutant *top2-4 ura3::top2-B44* is temperature sensitive at an intermediate 35°C temperature, further suggesting the possibility that Top2-4 has a dominant effect.

To further support the dominant role of Top2-4, a *top2* mutant that contains both mutations in a single allele (*top2-4,B44*) was constructed. All three mutant forms (*top2-4, top2-B44*, and *top2-4,B44*) were individually expressed in the degron background. When the cell cycle was monitored to assay for checkpoint activation we observed that the compound mutant allele, *top2-4, B44*, was unable to delay in G_2/M (Figure 37). This again illustrates the dominance of the *top2-4* mutation over the *top2-B44* mutation.

***top2-4* remains stable above restrictive temperatures**

To determine if this effect could be the result of Top2-4 protein stability we examined the protein directly. We observed that Top2-4 is stable above its restrictive temperature. A *top2-4-GFP* strain was grown for 4 hours at 37°C and protein samples were taken every hour and analyzed by Western blotting. The protein was stable despite the cells

being grown well above their restrictive temperature of 30°C (Figure 38a). To further examine Top2-4 stability, the cells were examined under the microscope after four hours of growth at 37°C to determine if GFP fluorescence remained. Intense Top2-4-GFP nuclear-like staining was observed (Figure 38b), further supporting the conclusion that Top2-4 remains present. These data and the loss of checkpoint activation in *top2-4 top2-B44* strains, provide conclusive evidence that Top2-4 does not function as a null above restrictive temperatures.

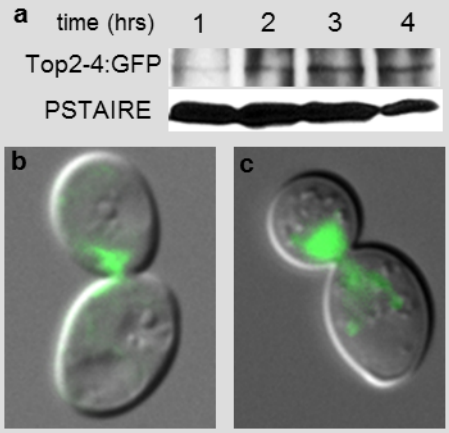


Figure 38. Top2-4 protein is stable above restrictive temperature. (a) *top2-4* cells were grown at 37°C for four hours. Protein samples were taken every hour. PSTAIRE is a loading control. (b&c) *top2-4:GFP* was grown at 37°C for four hours and cells were examined under the microscope. Intense nuclear staining was observed after both 0 hrs (b) and 4 hrs (c) of growth at 37°C.

***top2-4* prevents proper DNA segregation**

Based on the ability of Top2-4 to affect the checkpoint activation of Top2-B44 and the temperature sensitivity of *top2-4 ura3::top2-B44* cells, we wanted to determine if the persistence of Top2-4 affects DNA segregation. To answer this question we used the *top2* degron system to control the presence of functional Top2. We tested two strains; the degron only strain (*top2*^{deg}) and the experimental strain, *top2*^{deg} *top2-4*. In this experiment, the cells were synchronized in G_1 under permissive conditions for both *top2*^{deg} and *top2-4* (Figure 39a). Each strain was then released into the cell cycle under the same, permissive conditions, so that S phase was initiated and completed with functional Top2^{deg} present. This was followed by the addition of nocodazole to arrest cells in early metaphase, while functional Top2^{deg} remains present. Once the cells were arrested, they were shifted to conditions where Top2^{deg} transcription

is inhibited and any protein is degraded. These conditions, specifically the shift in temperature to 37°C, also inactivate any Top2-4 protein. At this stage the cells were arrested in metaphase, with either no Top2 (as is the case for *top2*^{deg} strains) or with only non-functional Top2-4 present. At this point they are no longer able to resolve any

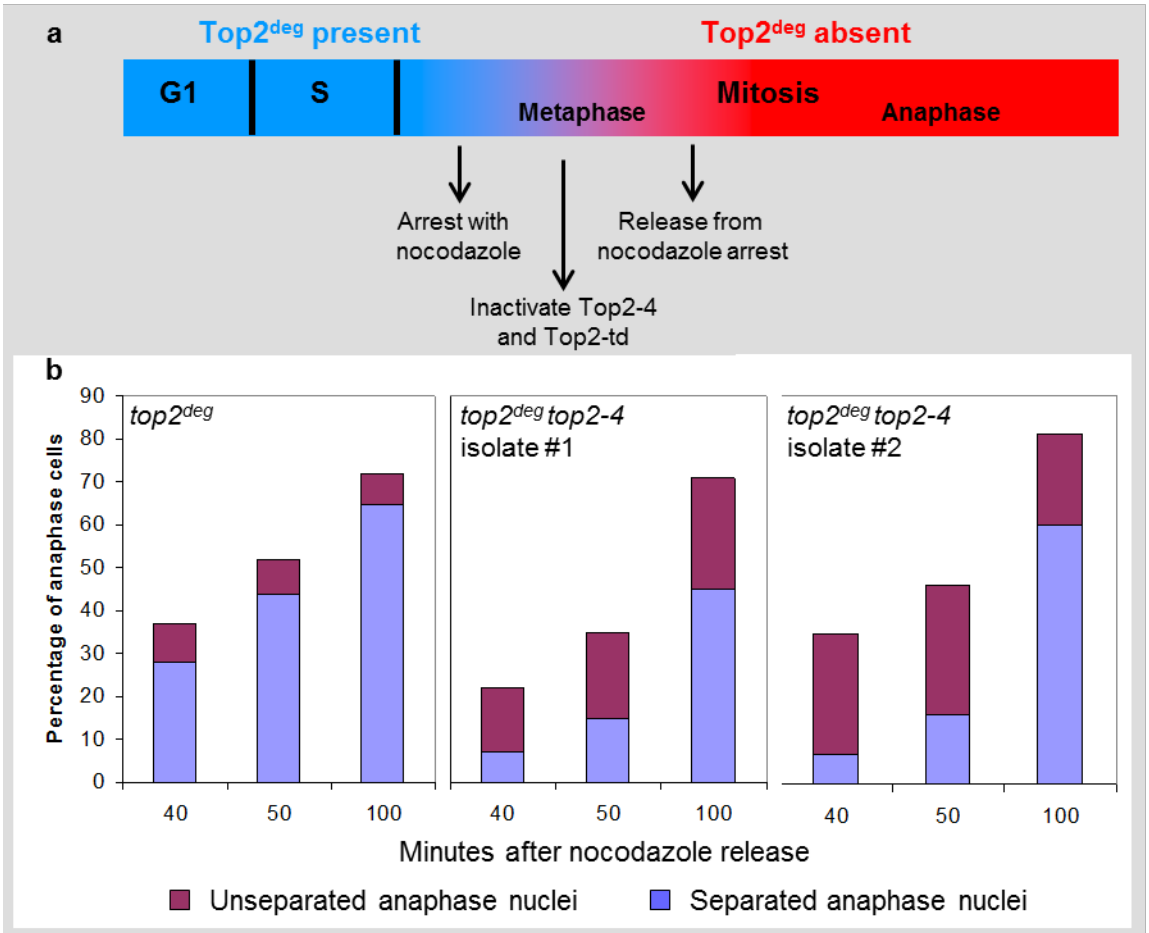


Figure 39. Top2-4 prevents proper sister chromatid segregation. (a) A schematic depicting the experiment. Cells stably express functional Top2^{deg} and Top2-4 during G_1 arrest, achieved using α factor. Cells are released into the cell cycle still expressing functional Top2^{deg} and Top2-4. Metaphase arrest is achieved using nocodazole. During metaphase arrest cells are treated so that Top2^{deg} expression is turned off and protein is degraded and Top2-4 becomes non-functional. Nocodazole is removed and cells are allowed to complete mitosis without any functional Top2. (b) Bar represents the number of anaphase cells at that particular time point. Color inside the bar represents the anaphase cells with either unseparated (abnormal) nuclei or separated (normal) nuclei. Two graphs on the right are from the same experiment, but using two isolates.

catenations that may remain. The cells are then released from the nocodazole-induced metaphase arrest and allowed to progress into anaphase. We then observed the phenotype of these anaphase cells 40, 50, or 60 minutes after release.

Normal versus abnormal anaphase was determined as described previously in Chapter I Figure 13. The segregation of the DNA in anaphase cells was determined by observing the phenotype of the nuclei in cells with anaphase spindles. In the control strain, *top2*^{deg}, we found that the majority of anaphase cells experience a normal anaphase in which the nuclei appear evenly separated into the daughter cells (Figure 39b). This, in itself, is a novel finding as it suggests that Top2 has resolved all catenations prior to its degradation in metaphase and so is not required for anaphase. This is novel as data from human cells has been unclear as to the requirement of Top2 during the stages of mitosis.

The normal anaphase in *top2*^{deg} strains is in contrast to the *top2*^{deg} *top2-4* strains. We found that when these cells attempt to segregate their DNA in the presence of inactivated Top2-4 they undergo an abnormal anaphase (figure 39b). We find the DNA is unevenly stretched and not evenly and clearly segregated. This suggests that while Top2-4 is inactive, in that separate DNA is no longer obtained, it is still having an effect on the cell through the prevention of proper sister chromatid separation.

The previous data has provided evidence that the temperature-sensitive allele *top2-4*, while unable to support viability above restrictive temperatures, does not function as a null allele under these conditions. Instead, Top2-4 protein remains stable at high temperatures and exerts an at least semi-dominant effect on other *top2* alleles. Additionally, it seems to affect sister chromatid separation or segregation during anaphase.

DISCUSSION

One explanation for these results is that Top2-4, while unable to effectively allow for separation of sister chromatids, might still bind DNA. If it were to do this, as some of these experiments suggest, it could function like a cohesin ring and be a protein clamp to hold two sister chromatids together. This possibility is extremely interesting as it suggests Top2-4 may act as a C-gate mutant, in which the C-gate is incapable of opening *in vivo*. A C-gate mutant has yet to be identified. The Top2-4 mutation (P821Q) is C-terminal to the catalytic tyrosine (Y782F) and closer to the second hole between the DNA gate and the C-gate (Figure 32). Based on its position, this mutation is unlikely to affect the DNA gate region and therefore most likely does not affect cutting and opening of the G-segment, but it could affect the C-gate and the release of the transported T-segment.

Of even greater interest is to go back and re-examine the extensive number of papers using *top2-4* that presume it functions as a null. Due to the dimerization of Top2, its stability at high temperatures complicates experiments using different *top2* mutants since they will dimerize with Top2-4. We have shown that the presence of both Top2-4 and Top2-B44 in the same strain has a phenotypic effect in the loss of checkpoint activation and the change in temperature sensitivity. Some research using this allele may remain unaffected by these results. For example, human Topoisomerase IIα and a variety of mutant variants have been introduced into yeast to examine their effect. This research might be unaffected as yeast Top2 and human TopoIIα do not stably dimerize (JENSEN *et al.* 2000). However, the stability of the Top2-4 enzyme and its effect on sister chromatid segregation, as presented here, could mean that a re-examination of the

results and the interpretation of virtually every experiment done where this mutant is present is still required.

To further probe the effect this mutant has on cells it will be important to determine the decatenation state of chromosomes. To determine if Top2-4 functions as a protein clamp, holding otherwise decatenated chromosomes together, we could examine the decatenation state of chromosomes using contour-clamped homogeneous electric field (CHEF) pulsed-field gels. Additionally, other *TOP2* mutants with *in vitro* biochemically characterized effects could be examined in conjunction with *top2-4* to determine if these effects are altered in the presence of Top2-4.

FUTURE

There are still many questions to pursue in the characterization of the Topoisomerase II checkpoint. We have provided convincing evidence that the Topoisomerase II checkpoint monitors the changes associated with G-segment cutting, opening of the DNA gate, and transport of the T-segment. To further narrow the precise mechanism of checkpoint activation will require the identification of new mutants that can separate these three stages.

A major focus for further research will be in the identification of Topo II checkpoint proteins. One step of this process will be to determine the role the CTR of Top2 plays in the Topo II Checkpoint. Especially important is the identification of proteins that bind the CTR of Top2. One method is to perform a yeast-two-hybrid (Y2H) analysis using full length Top2, Top2-B44, and truncation versions of both. Using this analysis, we can distinguish checkpoint proteins from other Top2 binding proteins (Top2-B44 vs. Top2-

B44$^{\Delta CTR}$ and Top2). Additionally, we might identify novel Top2 binding proteins and also identify those that specifically bind the CTR of Top2 during a normal cell cycle (Top2 vs Top2$^{\Delta CTR}$).

Alternatively, a candidate approach can be taken to identify proteins involved in the checkpoint. There are a few proteins known to interact with Top2 that hold promise. First, using a Y2H approach the CTR of Top2 (1118-1428) was found to interact with Sgs1 (WATT *et al.* 1995), a DNA helicase that is the yeast homolog of human BLM and WRN. Both of these helicases were shown to affect the role of human Topoisomerase II (SO *et al.* 2007) and the human Topoisomerase II checkpoint (ADACHI *et al.* 2004),

A second candidate, Yra1, was found to interact with Top2 in two separate screens (HO *et al.* 2002; KROGAN *et al.* 2006). Yra1 is annotated to be a RNA binding protein that is required for export of mRNA from the nucleus. However, Yra1 was also identified as an interaction partner for Dia2, an F-box protein involved in protein degradation (SWAMINATHAN *et al.* 2007). Yra1 associates with chromatin and binds replication origins. Separation of function mutants were characterized that separated Yra1's role in mRNA export from its role in Dia2 related activities. It may thus play a novel role in Top2 function.

Finally, Dun1 is a serine-threonine kinase involved in the DNA damage checkpoint and post-replicative DNA repair. It was found to interact with Top2 in a high-throughput mass spectrometric protein complex identification screen (HO *et al.* 2002). These proteins (Sgs1, Yra1, Dun1) could be individually knocked out or mutated in *top2-B44* strains. Cell cycle progression could be analyzed in the resulting double mutants to see if the checkpoint was still activated. If the checkpoint is lost in these double mutants, it would suggest that the candidate protein is involved in the Topo II Checkpoint. We could

then do co-immunoprecipitation using Top2-B44 and Top2-B44$^{\Delta CTR}$ to determine if it specifically binds the CTR of Top2.

While the study of the Topo II checkpoint remains important and necessary, I firmly believe that the characterization of the Top2-4 protein will be of significant use to the Topoisomerase II community. The knowledge of how the stable protein functions above its restrictive temperature and what activity it contains will be of importance in reviewing all previous work done using strains containing the *top2-4* mutant allele, as well as any future work. We know that *top2-4* cells are inviable when grown above the restrictive temperature, but is this a result of a loss of decatenation or the potential protein clamp function of stable Top2-4? To continue this pursuit it would be important to answer two questions. First, does Top2-4 retain any decatenation activity above the restrictive temperature? And second, does it function as a protein clamp?

To answer the first one, we could synchronize *top2^{deg}* and *top2^{deg} top2-4* cells in G_1 while destabilizing Top2^{deg} and inactivating Top2-4 proteins. Cells would then be released into media containing nocodazole to arrest them prior to metaphase of mitosis. Samples taken of G_1 cells and mitotic cells could be run on a CHEF gel and compared. *top2^{deg}* cells will have gone through the cell cycle without any Top2, meaning their replicated DNA should be fully catenated. These cells should exhibit an altered chromosome migration pattern. We can then compare this to *top2^{deg} top2-4* cells which have stable but non-functional Top2-4 protein present. If these cells are truly incapable of decatenation they should have a similar pattern to *top2^{deg}* cells. Alternatively, if Top2-4 is capable of decatenation, but remains on the DNA like a protein clamp these cells should have a normal chromosome migration pattern because all protein is removed during sample preparation.

CONCLUSIONS

The Topoisomerase II checkpoint, while only relatively recently identified, will prove to play a prominent role in future research and in human therapies. The abundance of natural and synthetic Topoisomerase II inhibitors and the role they play in human health and therapies emphasizes the importance of this research. Before we can design new therapies or completely understand the ones in current use, we must understand the pathway that they affect. To this end we have shown that the Topoisomerase II checkpoint requires SPR initiation, does not monitor ATP hydrolysis or N-gate closure specifically, and is not activated in response to a general slowing of the enzymatic cycle. Rather, the checkpoint explicitly monitors the step of the SPR where the T-segment is transported through the opened DNA gate.

Additionally, we have also shown that the CTR of Topoisomerase II is required for checkpoint activation. As this region is not required for the essential enzymatic function of Topoisomerase II and is unstructured but highly post-translationally modified it is the ideal candidate to be the Topoisomerase II checkpoint platform. This breakthrough will allow for the identification of proteins involved in the Topo II checkpoint, specifically beginning with the sensor and initiating proteins.

Finally, we have found that a classic *top2* mutant, *top2-4*, does not function as presumed and in fact could have dominant effects on cellular phenotypes. This discovery is of particular importance to the Topoisomerase II field as this mutant has been used as a null form of Top2 for over 25 years. This discovery will cause the field to re-examine their results and conclusions.

MATERIALS and METHODS

Yeast strains and plasmids

The yeast strains used in this study are haploid derivatives of BF264-15 15DU. Yeast strains were modified according to standard yeast genetic approaches (BURKE 2000). Plasmids were mutagenized by site-directed mutagenesis according to Liu and Naismith (LIU and NAISMITH 2008).

Construction of *top2^{deg}*

TOP2 with degron sequence was cut out with XbaI/NotI and ligated into TopoTA. This fragment was then cut out of TopoTA with NotI and ligated into pRS405-*MET3*, that was also cut with NotI. The pRS405-*MET3-degron-TOP2* plasmid was transformed into a diploid strain, *TOP2/top2*Δ*::KAN^R* (DCY 2140). Diploid transformants were induced to sporulate and were dissected. Tetrads were patched onto dextrose containing media that is lacking in methionine (SD-Met). Tetrads were then replicated onto SD-Met media at 30°C and 37°C, SD-Met plus G418, SD-Met-Leu, and YPD media to identify haploids that are *top2*Δ and contain *MET3-degron-TOP2* (DCY 3040). This strain was then crossed to a strain containing *UBR1* under the control of the *GAL1* promoter (DCY 2695). Diploids were induced to sporulate and were dissected. Tetrads were then replicated onto YPG at 30°C and 37°C, SD-Met plus G418, SD-Met-Leu, synthetic media containing galactose and lacking methionine (SG-Met), and SD-Met at 30°C and 37°C. Spores were selected that were dead on YPG and grew on SD-Met at 30°C, SD-Met plus G418, and SD-Met-Leu.

Growth conditions to inactivate Top2^{deg}

Step 1, cells were grown to an OD of 2.0-6.0 overnight at 26°C in synthetic raffinose medium lacking methionine and tryptophan. Step 2, for synchrony, cells were diluted to OD 0.2 in rich raffinose medium at 26°C with α factor (concentration varying from 1:2200 to 1:3500 of a 1mg/ml stock). After 1h, galactose was added to a final concentration of 4% (Step 3). Following another 30 min growth the temperature was raised to 35°C (Step 4). After a further 30 min growth (2 hours total with α factor) α factor was washed off with water that was pre-warmed to 35°C. The cells were released into YPG at 35°C (Step 5).

Construction of *top2* mutants

top2-B44$^{\Delta CTR}$ was constructed by cutting plasmid 426 with AvrII and KasI followed by gap repair using primers 507 &508, resulting in plasmid 525. To ensure as little mutation as possible 525 and 426 were cut with KasI and SacII. The small fragment of 525, containing the deletion, and the large fragment of 426 were gel purified and ligated together.

Strand passage reaction initiation mutants were constructed similarly (571,573,609). Initial plasmids were cut with BmtI and NsiI and the desired (larger) fragment was gel purified. F136 was also cut with BmtI and NsiI and the desired (smaller) fragment was gel purified. The two purified fragments were ligated together and transformed into bacteria.

Strand passage reaction mutants were performed similarly. *pRS414-TOP2* was mutagenized by PCR with oligos containing the desired mutations. E66Q used primers

597 and 598, G144I primers 599 and 600, L475A, L480P primers 601 and 602, G738D primers 603 and 604, P824S primers 605 and 606.

Determination of G_2/M period

G_2/M period was determined as previously described (ANDREWS *et al.* 2006; FURNISS *et al.* 2009a). Briefly, at each time point and for each strain, at least 100 cells were scored for spindle and budding morphology. The G_2/M cell cycle interval was determined by plotting cell cycle categories against time. G_2/M was defined as the period after 20% of cells had formed a G_2 spindle until 20% of cells formed an anaphase spindle. Each experimental strain was analyzed a minimum of three times. The data are aligned relative to bud emergence since this signals cell cycle entry and is the variable parameter of cell cycle progression following release from G_1 synchrony.(ANDREWS *et al.* 2006)

Microscopy

Spindle morphologies were visualized using *TUB1-GFP* (STRAIGHT *et al.* 1997) as described previously (FURNISS *et al.* 2009a). Nuclear morphologies were visualized using a method from Juan Martinez (Purdue University). Cells were grown under the conditions specified previously and were collected (500 µl) by centrifugation. The resulting pellet was washed once with 1 ml 1X PBS, resuspended in 1.4 ml of filter-sterilized 4 % p-formaldehyde solution (4 % p-formaldehyde, 3.5 % sucrose in water), and incubated for 20 min at room temperature. Cells were then centrifuged at 3000 *g* and the pellet was washed with 1 ml wash buffer (1.2 M sorbitol, 100 mM potassium phosphate, pH 7.5). Cells were resuspended in 1 ml 1x PBS containing 5 µl of 1 mg/ml DAPI solution and incubated at room temperature, in the dark, for 3 min. Cells were

washed once with 1X PBS and resuspended in the remaining 1X PBS after decanting. Anaphase cells were identified based on spindle morphology and were scored for separated or un-separated nuclei. During a normal mitosis, anaphase cells go through an intermediate stage where the nuclei are stretched, but have not separated completely (this accounts for the low percentage of anaphase cells in wild-type with un-separated nuclei).

Biochemistry

Proteins were extracted by collecting 15mls of cells and resuspending them in 1 mL 0.25M NaOH and 1% BME solution. Resuspension was placed on ice for 10 min. 160 µl of 50% TCA was added, solution was inverted, and placed on ice for 10 min. Cells were pelleted 14,000 *g* for 10 min at 4^{o}C. Supernatant was poured off and the pellet was resuspended in 1 mL of ice-cold acetone. Cells were pelleted again and supernatant was poured off. The cell pellet was dried for 3 min at 55^{o}C with the cap open. The dried cell pellet was resuspended in 100 µl of 2X protein-loading buffer and neutralized with 5 µl of 1M Tris base. Western blots were performed using the following antibodies: 1:1000 dilution of anti-Top2 (TopoGen) and a 1:2500 dilution of anti-GFP (Clontech). Secondary antibody, HRP-conjugated goat anti-rabbit (Pierce) was used at 1:5000 and HRP-conjugated goat anti-mouse IgG (Invitrogen) was used at 1:5000. Pierce© ECL Western Blotting Substrate and SuperSignal© West Pic Chemiluminescent (Thermo Scientific) was used to visualize protein.

Statistical Analysis

Values for G2/M from the yeast cell cycle analysis were analyzed using One-Way Anova, using the Tukey HSD post-hoc analysis (significance p=0.05).

Table 2. List of strains used.

Strain	Number	Genotype
top2^{deg}	4075	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414 (TRP1)*
top2^{deg} TOP2	4051	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-TOP2 (TRP1)*
top2^{deg} top2-B44	4053	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2-B44 (TRP1)*
top2^{deg} top2-B44 mad2Δ	4081	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) mad2::KAN pRS414-top2-B44 (TRP1)*
top2^{deg} top2-B44^{K651A}	4269	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2-B44^{K651A} (TRP1)*
top2^{deg} top2^{Y782F}	4284	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2^{Y782F} (TRP1)*
top2^{deg} top2-B44^{Y782F}	4285	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2-B44^{Y782F} (TRP1)*
top2^{deg} top2^{G144I}	4276	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2^{G144I} (TRP1)*
top2^{deg} top2$^{G144I, Y782F}$	4355	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2$^{G144I, Y782F}$(TRP1)*
top2^{deg} top2^{G144I} mad2Δ	4359	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) mad2::KAN pRS414-top2^{G144I} (TRP1)*

Strain	Number	Genotype
top2^{deg} top2^{G144I} rad53-1	4359	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) rad53-1 pRS414-top2^{G144I} (TRP1)*
top2^{deg} top2^{E66Q}	4330	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2^{E66Q} (TRP1)*
top2^{deg} top2^{E66Q} mad2Δ	KFY147	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) mad2::KAN pRS414-top2^{E66Q} (TRP1)*
top2^{deg} top2^{E66Q} rad53-1	4357	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) rad53-1 pRS414-top2^{G144I} (TRP1)*
top2^{deg} rad53-1	4383	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) rad53-1 pRS414 (TOP2)*
top2^{deg} top2$^{L475A/L480P}$	4331	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2$^{L475A/L480P}$ (TRP1)*
top2^{deg} top2$^{L475A/L480P}$ mad2Δ	KFY148	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) mad2::KAN pRS414-top2$^{L475A/L480P}$ (TRP1)*
top2^{deg} top2$^{L475A/L480P}$ rad53-1	4385	*MATa bar1Δ top2::KAN leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) rad53-1 pRS414-top2$^{L475A/L480P}$ (TRP1)*
top2^{deg} top2^{G738D}	4277	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2^{G738D} (TRP1)*
top2^{deg} top2^{P824S}	4279	*MATa bar1Δ top2::KAN^{R} leu2::Met3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3:Tub1-GFP (URA3) pRS414-top2^{P824S} (TRP1)*

Strain	Number	Genotype
top2^{deg} top2-B44$^{\Delta CTR}$	4244	*MATa bar1Δ top2::KAN^{R} leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) pRS414-top2-B44$^{\Delta CTR}$ (TRP1)*
top2^{deg} top2$^{\Delta CTR}$	4246	*MATa bar1Δ top2::KAN^{R} leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) pRS414-top2-B44$^{\Delta CTR}$ (TRP1)*
top2-B44	4249	*MATa bar1Δ top2::top2-B44 (URA3, KAN^{R}) Tub1-GFP^{um} pRS425 (#137)*
top2-B44Gal-CTR	4251	*MATa bar1Δ top2::top2-B44 (URA3, KAN^{R}) Tub1-GFP^{um} pJBN69 (#528)*
top2-4 ura3::top2-B44	2927	*MATa bar1Δ ura3::top2-B44 (URA3) Tub1-GFP^{um}*
top2-4	2299	*MATa bar1Δ top2-4 ura3::HIS3:Tub-GFP (URA3)*
top2-B44	2769	*MATa bar1Δ top2::top2-B44(KAN^{R}) Tub1-GFP*
top2^{deg} top2-4	4191	*MATa bar1Δ top2::KAN^{R} leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) pRS414-top2-4 (TRP1)*
top2^{deg} top2-4,B44	4193	*MATa bar1Δ top2::KAN^{R} leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) pRS414-top2-4,B44 (TRP1)*
top2-4-GFP	F94	*MATa bar1Δ top2-GFP (URA3, KAN^{R})*
top2^{deg} cdc15-2	4257	*MATa bar1Δ top2::KAN^{R} leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) cdc15::cdc15-2*
top2^{deg} cdc15-2 top2-4 isolate #1	4266	*MATa bar1Δ top2::top2-4 leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) cdc15::cdc15-2*
top2^{deg} cdc15-2 top2-4 isolate #1	4268	*MATa bar1Δ top2::top2-4 leu2::MET3-Ub-DHFR-HA-TOP2 (LEU2) Gal-myc-UBR1 (HIS3) ura3::HIS3-TUB1-GFP (URA3) cdc15::cdc15-2*

Table 3. List of plasmids.

Plasmid number	Genetic information
292	*AMP^{R} pRS414-TOP2 (TRP1)*
426	*AMP^{R} pRS414-top2-B44 (TRP1)*
429	*AMP^{R} pRS405-pMET3 (LEU2)*
444	*AMP^{R} TopoTA-top2 degron (URA3)*
446	*AMP^{R} pRS405-MET3-top2 degron (LEU2)*
522	*AMP^{R} pRS414-top2-4 (TRP1)*
525	*AMP^{R} pRS414-top2$^{\Delta CTR}$ (TRP1)*
528	*AMP^{R} p2μM LEU2 ADH-GAL4ad-HA-TOP2 CTD (obtained from Jeff Bachant pJNB69)*
530	*AMP^{R} p2μM TRP1ADH-GAL4dbd-HA-TOP2 CTD (obtained from Jeff Bachant pMN002)*
532	*AMP^{R} pRS414-top2-B44$^{\Delta CTR}$ (TRP1)*
555	*AMP^{R} pRS414-top2-4,B44 (TRP1)*
571	*AMP^{R} pRS414-top2^{Y782F} (TRP1)*
573	*AMP^{R} pRS414-top2-B44^{Y782F} (TRP1)*
578	*AMP^{R} pRS414-top2^{G738D} (TRP1)*
589	*AMP^{R} pRS414-top2^{G144I} (TRP1)*
592	*AMP^{R} pRS414-top2^{P824S} (TRP1)*
596	*AMP^{R} pRS414-top2^{E66Q} (TRP1)*
609	*AMP^{R} pRS414-top2^{G144I,YF} (TRP1)*
610	*AMP^{R} pRS414-top2$^{L475A/L480P}$ (TRP1)*
AV62	*AMP^{R} pRS414-top2-B44^{K651A} (TRP1)*
F136	*pFA6 Gal-HA-top2^{Y782F}*

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APPENDIX

Table 4. Statistical significance of strains tested from Chapter I.

Strain 1	Strain 2	Significance
top2^{deg} TOP2	*top2^{deg}*	.423
top2^{deg} top2-B44	*top2^{deg} top2-B44 mad2Δ*	.022
top2^{deg} top2-B44	*top2^{deg}*	.002
	top2^{deg} top2-B44^{K651A}	.001
top2^{deg}	*top2^{deg} top2-B44*	.002
	top2^{deg} top2-B44^{K651A}	.932
top2^{deg} top2-B44^{K651A}	*top2^{deg} top2-B44*	.001
	top2^{deg}	.932
top2^{deg} top2-B44	*top2^{deg}*	.000
	top2^{deg} top2^{Y782F}	.002
	top2^{deg} top2-B44^{Y782F}	.003
top2^{deg}	*top2^{deg} top2-B44*	.000
	top2^{deg} top2^{Y782F}	.812
	top2^{deg} top2-B44^{Y782F}	.621
top2^{deg} top2^{Y782F}	*top2^{deg} top2-B44*	.002
	top2^{deg}	.812
	top2^{deg} top2-B44^{Y782F}	.985
top2^{deg} top2-B44^{Y782F}	*top2^{deg} top2-B44*	.003
	top2^{deg}	.621
	top2^{deg} top2^{Y782F}	.985
top2^{deg} top2-B44	*top2^{deg}*	.011
	top2^{deg} top2^{G144I}	.924
top2^{deg}	*top2^{deg} top2-B44*	.011
	top2^{deg} top2^{G144I}	.017
top2^{deg} top2^{G144I}	*top2^{deg} top2-B44*	.924
	top2^{deg}	.017
top2^{deg}	*top2^{deg} top2^{G144I}*	.001
	top2^{deg} top2$^{Y782F, G144I}$	.915
top2^{deg} top2^{G144I}	*top2^{deg}*	.001
	top2^{deg} top2$^{Y782F, G144I}$	.000
top2^{deg} top2$^{Y782F, G144I}$	*top2^{deg}*	.915
	top2^{deg} top2^{G144I}	.000

Strain 1	Strain 2	Significance
top2^{deg}	*top2^{deg} top2^{G144I}*	.000
	top2^{deg} top2-G144I mad2Δ	.987
top2^{deg} top2^{G144I}	*top2^{deg}*	.000
	top2^{deg} top2-G144I mad2Δ	.000
top2^{deg} top2-G144I mad2Δ	*top2^{deg}*	.987
	top2^{deg} top2^{G144I}	.000
top2^{deg}	*top2^{deg} top2^{G144I}*	.001
	top2^{deg} top2^{G144I} rad53-1	.001
top2^{deg} top2^{G144I}	*top2^{deg}*	.001
	top2^{deg} top2^{G144I} rad53-1	.997
top2^{deg} top2^{G144I} rad53-1	*top2^{deg}*	.001
	top2^{deg} top2^{G144I}	.997
top2^{deg} top2-B44	*top2^{deg}*	.016
	top2^{deg} top2^{E66Q}	.479
top2^{deg}	*top2^{deg} top2-B44*	.016
	top2^{deg} top2^{E66Q}	.083
top2^{deg} top2^{E66Q}	*top2^{deg} top2-B44*	.479
	top2^{deg}	.086
top2^{deg}	*top2^{deg} top2^{E66Q}*	.034
	top2^{deg} top2^{E66Q} mad2Δ	.943
top2^{deg} top2^{E66Q}	*top2^{deg}*	.034
	top2^{deg} top2^{E66Q} mad2Δ	.015
top2^{deg} top2^{E66Q} mad2Δ	*top2^{deg}*	.943
	top2^{deg} top2^{E66Q}	.015
top2^{deg} rad53-1	*top2^{deg} top2^{E66Q}*	.016
	top2^{deg} top2^{E66Q} rad53-1	.000
top2^{deg} top2^{E66Q}	*top2^{deg} rad53-1*	.016
	top2^{deg} top2^{E66Q} rad53-1	.051
top2^{deg} top2^{E66Q} rad53-1	*top2^{deg} rad53-1*	.000
	top2^{deg} top2^{E66Q}	.051

Strain 1	Strain 2	Significance
top2^{deg} top2-B44	*top2^{deg}*	.000
	top2^{deg} top2$^{L475A/L480P}$	.468
top2^{deg}	*top2^{deg} top2-B44*	.000
	top2^{deg} top2$^{L475A/L480P}$	.000
top2^{deg} top2$^{L475A/L480P}$	*top2^{deg} top2-B44*	.468
	top2^{deg}	.000
top2^{deg}	*top2^{deg} top2$^{L475A/L480}$*	.004
	top2^{deg} top2$^{L475A/L480}$ mad2Δ	.759
top2^{deg} top2$^{L475A/L480}$	*top2^{deg}*	.004
	top2^{deg} top2$^{L475A/L480}$ mad2Δ	.001
top2^{deg} top2$^{L475A/L480}$ mad2Δ	*top2^{deg}*	.759
	top2^{deg} top2$^{L475A/L480}$	.001
top2^{deg} rad53-1	*top2^{deg} top2$^{L475A/L480}$*	.000
	top2^{deg} top2$^{L475A/L480}$ rad53-1	.000
top2^{deg} top2$^{L475A/L480}$	*top2^{deg} rad53-1*	.000
	top2^{deg} top2$^{L475A/L480}$ rad53-1	.067
top2^{deg} top2$^{L475A/L480}$ rad53-1	*top2^{deg} rad53-1*	.000
	top2^{deg} top2$^{L475A/L480}$	.067
top2^{deg} top2-B44	*top2^{deg}*	.006
	top2^{deg} top2^{G738D}	.006
top2^{deg}	*top2^{deg} top2-B44*	.006
	top2^{deg} top2^{G738D}	.999
top2^{deg} top2^{G738D}	*top2^{deg} top2-B44*	.006
	top2^{deg}	.999
top2^{deg} top2-B44	*top2^{deg}*	.000
	top2^{deg} top2^{P824S}	.000
top2^{deg}	*top2^{deg} top2-B44*	.000
	top2^{deg} top2^{P824S}	.864
top2^{deg} top2^{P824S}	*top2^{deg} top2-B44*	.000
	top2^{deg}	.864