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Genome-Scale Genetic Interactions and Cell Imaging Confirm Cytokinesis as Deleterious to Transient Topoisomerase II Deficiency in Saccharomyces cerevisiae

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ABSTRACT Topoisomerase II (Top2) is an essential protein that resolves DNA catenations. When Top2 is inactivated, mitotic catastrophe results from massive entanglement of chromosomes. Top2 is also the target of many first-line anticancer drugs, the so-called Top2 poisons. Often, tumors become resistant to these drugs by acquiring hypomorphic mutations in the genes encoding Top2. Here, we have compared the cell cycle and nuclear segregation of two coisogenic hypomorphic mutations in the genes encoding Top2. Here, we have compared the cell cycle and nuclear segregation of two coisogenic Saccharomyces cerevisiae strains carrying top2 thermostensitive alleles that differ in their resistance to Top2 poisons: the broadly-used poison-sensitive top2-4 and the poison-resistant top2-5. Furthermore, we have performed genome-scale synthetic genetic array (SGA) analyses for both alleles under permissive conditions, chronic sublethal Top2 downregulation, and acute, yet transient, Top2 inactivation. We find that slowing down mitotic progression, especially at the time of execution of the mitotic exit network (MEN), protects against Top2 deficiency. In all conditions, genetic protection was stronger in top2-5; this correlated with cell biology experiments in this mutant, whereby we observed destabilization of both chromatin and ultrafine anaphase bridges by execution of MEN and cytokinesis. Interestingly, whereas transient inactivation of the critical MEN driver Cdc15 partly suppressed top2-5 lethality, this was not the case when earlier steps within anaphase were disrupted; i.e., top2-5 cdc14-1. We discuss the basis of this difference and suggest that accelerated progression through mitosis may be a therapeutic strategy to hypersensitize cancer cells carrying hypomorphic mutations in TOP2.

KEYWORDS topoisomerase II top2-4 and top2-5 synthetic genetic array analysis mitotic exit network cytokinesis plasma membrane abscission anaphase bridges Cdc14

Upon chromosome replication, topological intertwining arises between sister chromatids. These intertwines often become interlocked.
Top2 is essential for cellular viability. In unicellular eukaryotes and bacteria, the study of Top2 functions has been largely facilitated by the availability of conditional alleles. In the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe early studies showed that inactivation of Top2 by means of thermosensitive (ts) alleles leads to a mitotic catastrophe as determined by a sudden loss of viability once the cells reach anaphase (Holm et al. 1985; Uemura and Tanagida 1986). In agreement with a role in removing sister chromatid catenations, Top2 inactivation yielded cells with DAPI-stained anaphase bridges and broken chromosomes once cells completed cytokinesis (DiNardo et al. 1984; Uemura and Yanagida 1984; Holm et al. 1985, 1989; Uemura and Tanagida 1986). In the case of S. cerevisiae, all these studies were carried out with two ts alleles isolated in independent screens, top2-1 and top2-4. Both alleles yield Top2-ts proteins sensitive to poisons. In the same screen where top2-4 was isolated, top2-5 was also obtained (Holm et al. 1985). Later, top2-5 was shown to be resistant to poisons and served as a key tool to understand the mechanism of action of this class of clinical drugs (Jannatipour et al. 1993; Perego et al. 2000). Nevertheless, the cell cycle of the top2-5 strain was not characterized and has been assumed to be equivalent to that of the top2-4 strain.

Here, we have revisited the cell cycle progression of cells expressing the broadly used top2-4 allele and compared its behavior to a coisogenic top2-5 strain. In addition, we have performed a genome-scale synthetic genetic array (SGA) analysis for these two top2-ts alleles. We show that top2-5 goes faster through the cell cycle and gathers more genetic interactions related to mitotic progression than top2-4. In addition, we show that execution of the mitotic exit network (MEN) has specific deleterious effects on sublethal downregulation of Top2-5, and that this correlates with destabilization of anaphase bridges by cytokinesis.

**MATERIALS AND METHODS**

**Yeast strain construction, cell cycle experiments, and fluorescence microscopy**

All the strains used in this work are listed in Supplemental Material, Table S1 in File S1 together with their relevant genotypes. C-terminal tagging with GFP/RFP variants or an auxin-based degron system, gene deletions and ts allele transfers were carried out using standard PCR methods as described before (Tong et al. 2001; Janke et al. 2004; Nishimura et al. 2009).

Most strains were grown overnight in air orbital incubators at 25°C in YPD media before every experiment. Cell cycle time course experiments and fluorescence microscopy were performed as described before (Quevedo et al. 2012; Silva et al. 2012; Garcia-Luis and Machin 2014). Briefly, asynchronous cultures of MATª haploids were adjusted to OD600 = 0.3 and then synchronized in G1 at 25°C for 3 hr by adding 50 ng/ml (bar1Δ strains) or 5 µg/ml (BAR1 strains) of α-factor (F6901, Sigma-Aldrich). The G1 release was induced by washing the cells twice in YPD and resuspending them in fresh media containing 0.1 mg/ml of pronase E (81750, Sigma-Aldrich). Next, the culture was incubated at 37°C for 4 hr and samples were taken every 30 min for direct visualization under a Leica DMI6000 fluorescence microscope. DNA was stained using DAPI (32670, Sigma-Aldrich) at 4 µg/ml final concentration after keeping the cell pellet 24 hr at −20°C. In the experiments performed to visualize ultrafine anaphase bridges (UBFs), a synthetic complete medium containing 100 µg/ml adenine (SC+ Ade) was used instead of YPD and images were taken with a Zeiss AxioImager Z1 fluorescence microscope.

Plasma membrane (PM) abscession and zymolyase digestion were employed to address progression of cytokinesis (Norden et al. 2006; Mendoza et al. 2009; Quevedo et al. 2012). For membrane abscission, the PM reporter 2-PH-GFP (two GFP-fused pleckstrin homology domains of phospholipase C from Rattus norvegicus) was used. When this reporter was not available, PM was stained with 5 µg/ml Hoechst 33258 (94403, Sigma-Aldrich) for either 5 or 15 min at 37°C before processing for fluorescence microscopy. Aside from the DNA, Hoechst dyes have great affinity for the lipid bilayer where they also become strongly fluorescent (Shapiro and Ling 1995). Zymolyase treatment was performed as described before (Quevedo et al. 2012). Briefly, samples were taken from the culture, fixed with 5% formaldehyde for 1 hr at 37°C, and then washed twice with PBS and once with 1 M sorbitol in 50 mM KPO4, pH 7.5. Finally, the sample was split in two; one half was treated with 0.2 mg/ml zymolyase 20T (E1005, Zymo Research) in the above sorbitol buffer containing 4 mM β-mercaptoethanol for 20 min at 37°C, whereas the other half was treated in the same conditions but without zymolyase (mock control).

For the time-lapse movies, an asynchronous culture was concentrated by centrifugation to three OD600 equivalents and plated on YPDA (YPD, agar 2% w/v). Patches were made from this plate and mounted on a microscope slide. They were incubated at 37°C in high humidity chambers to avoid drying of the agarose patch. Photos were taken every 30 or 60 min for 6 hr in order to minimize both photobleaching and cell damage by the excitation light.

For clonogenic assays, ~300 cells (as calculated from OD600 measurements) from an asynchronous culture were seeded on two YPD plates. One plate was incubated at 25°C for 3 d, whereas the other was first preincubated at 37°C for 6 hr before being transferred to 25°C. Proportion of survivors was calculated from the fraction of colonies that grew after the 37°C preincubation normalized to the number of colonies on plates continuously grown at 25°C. The same principle of temperature incubations was employed in the spot dilution assay. In this case, the asynchronous culture was first normalized to OD600 = 1, then 1:10 serial diluted, and finally spotted on YPD plates (~5 µl per spot) with a 48-pin replica plater (R2383, Sigma-Aldrich).

Variation between independent experiments was evaluated by SEM. Comparisons between mean values for different strains or conditions were performed by the unpaired two-sided student’s t-test. When percentages were calculated for selected cell phenotypes observed by microscopy, exact binomial 95% confidence intervals (CI95) were also included as error bars in order to inform about the accuracy of such observation. When percentages are mentioned in the main text, CI95 is indicated afterward in parentheses. Cells from at least two independent experiments were pooled for such calculations.

**Synthetic genetic array analyses**

SGA was performed as described before (Tong et al. 2001; Baryshnikova et al. 2010; Li et al. 2011). In order to make the strain arrays (described in detail in Figure S1 in File S1), we first replaced the Top2 locus in the haploid MATa strain Y7092 with our query top2-ts alleles attached to the selection marker natMX4 (resistance to nourseothricin). For consistency, we also attached the natMX4 marker to our reference Top2 Y7092 strain. The new natMX4 strains were then mated with the MATa mutant collections (4322 knockout strains for nonessential genes plus 1231 strains with thermosensitive alleles for essential genes). These panels of MATa strains bear the kamMX4 marker (resistance to G418) at the mutated locus. Diploids were selected on YPD plates containing both nourseothricin and G418, and later sporulated and selected for MATa haploids containing both markers. Once the Top2, top2-4, and top2-5 arrays were constructed they were replicated on to plates with the same medium used in the MATa selection and exposed to the different temperature regimes described in the Results section.

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The image analysis, processing, and fitness scoring of the arrays were done using SGAtools (http://sgatools.ccb.cbrc.utoronto.ca/about) (Wagih et al. 2013). Gene Ontology (GO) enrichment analysis was performed using the Generic GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder) of Princeton University (Boyle et al. 2004). Networks were made with Cytoscape v3.3.0 (http://www.cytoscape.org/cy3.html) (Shannon et al. 2003).

Data availability
Strains are available upon request. File S1 contains additional material and methods, four supplemental figures, six supplemental tables, and legends for the SGA files included in File S2. File S2 is a zip compressed file which contains 12 Microsoft Excel files with raw and processed data for each SGA analysis.

RESULTS
Three nuclear segregation patterns can be revealed by live microscopy of top2-4 and top2-5 cells
We started this work by revisiting fluorescence microscopy time course experiments in the original top2-4 and top2-5 ts strains (Holm et al. 1985). As a control, we also included the TOP2 reference wild-type allele in the same genetic background. We first engineered the strains in order to label the histone H2A (HTA2 gene) with GFP. This strategy allowed us to complement the histone time course experiments with fluorescence videomicroscopy of the nuclear DNA without adding DNA intercalating dyes. We further labeled Rad52 with RedStar2 to assess DNA damage upon inactivation of Top2. Rad52 forms nuclear foci to repair DSBs through the homologous recombination repair pathway (Lisby et al. 2001).

All strains were arrested in G1 at the permissive temperature (25°C) for 3 hr and then released at 37°C to follow the progression through a synchronous cell cycle. As expected, the TOP2 control cycled normally after the release, with segregation of the nuclear masses taking place very quickly at 90–120 min and no signs of DNA damage after that (Figure 1A, left panels). Separation between the segregated histone-labeled masses was clear and often laid close to the cell poles. Hereafter, we refer to this segregation phenotype as long-distance binucleated (LD-binucleated) and grouped cells within this phenotype provided that the distance between the split masses was >1 μm. From 150 min onwards, the TOP2 strain split the daughter from the mother and asynchronously entered a second cell cycle. In contrast to TOP2, the top2-4 mutant exhibited a phenotype of cells stuck in anaphase after 4 hr, in which ~75% of the cells were in a “dumbbell” state (i.e., the bud as big as the mother) and ~35% (50% of dumbbells) had two very close nuclear masses as determined by histone labeling (Figure 1A, central panels; Figure 1, B and C). We refer to this abnormal form of nuclear segregation as short-distance binucleated (SD-binucleated, <1 μm of separation) (Figure 1D). Unexpectedly, the actual presence of chromatin anaphase bridges (CABs, i.e., stretched histone-labeled DNA across the bud neck, Figure 1D) was low, with a SD-binucleated:CAB ratio of 5:1. The presence of SD-binucleated was constant from 150 min onwards. Coinciding with this change in the nuclear morphology, a steady increase of cells with Rad52 foci was also observed (up to 35% of cells by 240 min). In the case of top2-5, we observed a quicker G1-S entry and a different mix of cell morphologies by the end of the time course (Figure 1A, right panels; Figure 1B). Thus, we found a decrease of the dumbbell category from 150 min and the presence of “threesomes,” where the mother cell has rebudded, in up to 25% of the population, a percentage that was higher than that of top2-4 (Figure 1B). Furthermore, there was only a transient peak of SD-binucleated at 120 min (Figure 1A, right panels), with <10% of the cells having this morphology after 4 hr (Figure 1B). Likewise, Rad52 foci abruptly rose from 120 min, reaching 60% of all cells by 180 min, twice as many as in top2-4 (Figure 1A, lower panels).

We next complemented the time course experiments with videomicroscopy aimed to follow up single cells throughout their first and second cell cycles (up to 6 hr). In order to do so, we filmed on agarose patches an asynchronous population of cells at the restrictive temperature and analyzed those cells that were in G1 (unbudded) at the time of the temperature shift. First, we filmed the TOP2 strain as a control and found normal cell cycle progression for up to two generations for the mother and one generation for its first daughter (Figure 2A, upper chart). We also found little indication of cells undergoing arrest in G1 (categories 1, 5 and 9), in G2/M (categories 3, 7 and 11), or present- ing CABs (categories 4, 8 and 12). By contrast, we found that top2-ts cells starting in G1 struggled to rebud in the second cell cycle (Figure 2A, mid and lower charts), with only a minority of cells that had done so by 6 hr (~20% of top2-4 and top2-5 mothers; i.e., the sum of the orange bar values from morphological categories 6–13). As in the case of the time course with liquid cultures, the cell cycle on agarose patches was slightly quicker in top2-5 than in top2-4 (drop of category 1).

Videomicroscopy allowed us to gain insights about the time that cells spent with the SD-binucleated and CABs phenotypes (Figure 2B). Thus, around 5–10% of top2-ts cells had these phenotypes in two continuous frames (i.e., aberrant segregation phenotypes lasted >1 hr). Although the low percentage and limited number of single cells analyzed precluded a definitive assessment, these long-lasting incomplete segregations occurred in top2-4 cells more often. A close look at cells while transiting through anaphase (30 min frame intervals) confirmed the long-lasting nature of these “short distance” nuclear morphologies, as opposed to those cells that quickly ended up in the LD-binucleated morphology (Figure 2C). To confirm that the SD-binucleated phenotype was a proper split of nuclear masses rather than a mere relocalization of histones, we took samples and stained the DNA with DAPI. In all cases, the DAPI signal overlapped with the H2A-GFP (Figure 2D).

Finally, the use of live cell imaging also allowed us to visualize other striking aberrant anaphases. Although these phenotypes occurred in <1% of filmed cells, both G1 and early S phase (small bud) at the time of the temperature shift, they show remarkable instances of uncoupling between the nuclear division and the cell cycle in top2-ts (e.g., rebudding before splitting the chromatin bridge) (Figure S2 in File S1).

Cytokinesis progression correlates with the shape of the segregating nuclear mass in top2-ts mutants
Both the presence of dynamic SD-binucleated phenotypes and the low percentage of CABs were surprising for top2-ts, taking into account that depletion of Top2 is considered the prototypical model to elicit anaphase bridges. This led us to study top2-ts anaphases in more detail. First, we wondered about the completion of cytokinesis in these mutants. Previous reports have suggested that top2-4 CABs delay cytokinesis (Mendoza et al. 2009). This delay would explain the observed accumulation of dumbbells and threesomes, but it would also predict an easy visualization of CABs due to the gross defects in sister chromatid resolution.

Cell wall digestion has often been used to check whether mother and daughter cells have finished cytokinesis yet not completed septum formation. We performed this digestion on top2-ts cells that were well into anaphase or beyond; i.e., 4 hr after the G1 release at 37°C (Figure 3A). Strikingly, dumbbells remained as such, although more than half of the threesomes were split in two (one budded and one un budded cell,
whose proportions thus became slightly higher upon digestion). This result was somewhat expected for top2-4, based on the higher prevalence of SD-binucleated dumbbells and previous reports (Mendoza et al. 2009); however, it was surprising for top2-5 since dumbbells were mostly LD-binucleated and were steadily being split into two by 4 hr (Figure 1, A and B). Hence, we decided to complete the analysis of top2-5 by directly checking abscission of the PM at the bud neck. In order to do so, we engineered the original top2-5 strain to express the PM marker 2xPH-GFP. Interestingly, abscission (i.e., PM resolution in two at the bud neck) had taken place in at least 50% of the dumbbells and all threesomes 4 hr after the G1 release (Figure 3, B and C). As for the other 50% of dumbbells that fell into a preabscission category, half of them had a full contracted furrow and the other half an open neck; i.e., PM ingression at the cleavage furrow was absent or just partial. Interestingly, CABs were only visible in cells with an open cleavage furrow, although most of these cells were just mononucleated (~1:2 ratio). By contrast, SD-binucleated cells always had either a contracted or a resolved PM (Figure 3B, photos).

**Chromatin anaphase bridges in top2-ts are stabilized by preventing mitotic exit**

Since there is PM ingestion in top2-ts mutants, we next decided to check what the nuclear morphology looks like if ingestion is fully abrogated. Cytokinesis is executed by the MEN, which also makes possible the telophase-to-G1 transition (Jaspersen et al. 1998; Meitinger et al. 2012; Weiss 2012). We chose two broadly-used ts alleles for essential genes involved in MEN to prevent cytokinesis, cdc15-2 and cdc14-1. At 37°C, both mutants block cells in telophase with an open cleavage furrow (Bembenek et al. 2005); however, there are important differences between them. Cdc14 is the key MEN player, but it also has physiological roles unrelated to cytokinesis in early anaphase. Thus, Cdc14 gets activated twice in anaphase, first by the so-called FEAR
By contrast, full segregation of the histone signal was seen in all cdc15-2. This synergistic nuclear segregation defect in top2-5 cdc14-1 was worse; we observed that most of the nucleus was in the bud (Figure 4D).

We found that TOP2 cdc15-2 and top2-4 survived slightly better than top2-5 in the restrictive regime (Figure 4A and B), whereas there was no difference for top2-5 cdc14-1. In order to address the nuclear morphology, we arrested these strains in telophase, together with the corresponding TOP2 cdc15-2/cdc14-1 controls. We found that top2-5 cdc15-2 could not resolve the CAB (Figure 4C). By contrast, full segregation of the histone signal was seen in all TOP2 cdc15-2 cells. In the case of top2-5 cdc14-1, the nuclear segregation was worse; we observed that most of the nucleus was in the bud (Figure 4D). This synergistic nuclear segregation defect in top2-5 cdc14-1 may account for the lack of genetic suppression of top2-5 despite cdc14-1 also blocking cytokinesis (see Discussion chapter). Overall, these two double mutants demonstrate that the top2-5 strain gives rise to actual CABS and that the contraction of the cytokinetic furrow quickly split this CAB apart (see below).

We also made a top2-4 cdc15-aid strain. We used cdc15-aid (Cdc15 depletion by auxin addition rather than temperature shift) because it was difficult to phenotypically confirm top2-4 cdc15-2 as top2-4 alone got stuck as dumbbells (Figure 1). We also observed CABS when both Top2-4 and Cdc15-aid were depleted [33% (24±4%)]. In this case, most CABS had a SD-binucleated appearance (Figure S3 in File S1).

**Ultrafine anaphase bridges are also split apart in top2-5**

A final cell imaging analysis we performed was to check UFBs in top2-5 and address their fate after membrane abscission. These UFBs comprise mysterious forms of DNA that connect segregated nuclei and are refractory to classical DNA dyes and histone labeling (Chan et al. 2007). UFBs can, however, be detected with specific proteins that interact with them in anaphase; one such protein in yeast is Dpb11 (Germann et al. 2014). Thus, we looked at a top2-5 strain that had been triple-labeled.
In order to weigh the overall negative contributions of mitotic exit and Top2 inactivation and cytokinesis as deleterious enhancers of transient nonessential genes (4322 knockout strains) and a collection of ts alleles two collections of yeast mutants: the haploid gene deletion collection of CABs during the drop of the latter in the cells. Since there was no transient enrichment of UFBs relative to that UFBs are likely to be severed by PM abscission in this mutant. Moreover, we found a relocalization of Dpb11 to foci, which was specific for the top2-5 strain (Figure 5C). This finding further indicates that DNA at the top2-5 anaphase bridges is broken at the time of PM abscission (Germann et al. 2011).

Synthetic genetic array analysis confirms mitotic exit and cytokinesis as deleterious enhancers of transient Top2 inactivation

In order to weigh the overall negative contributions of mitotic exit and cytokinesis in each top2-ts allele, we carried out an SGA analysis with two collections of yeast mutants: the haploid gene deletion collection of nonessential genes (4322 knockout strains) and a collection of ts alleles for essential genes (1231 ts strains) (Tong et al. 2001; Giaever et al. 2002; Li et al. 2011). SGA allows screening for genetic interactions in S. cerevisiae by comparing the fitness of the different mutant combinations (i.e., single mutants in the collections vs. double top2-ts/collection mutants). In addition, we decided to perform the SGA analysis in three different conditions that modify Top2 activity.

In our first analysis, we grew all the strains constantly at the permissive temperature (25°C), and compared the collection of top2-4 and top2-5 double mutants with the corresponding TOP2 counterparts as references. Thermosensitive alleles are expected to have a mild reduced fitness at the permissive temperature and, in our case, we indeed detected genetic interactions at 25°C, 139 in top2-4 and 167 in top2-5 (Figure 6A, Table S2, and Table S3 in File S1). Eighty-four interactions were shared between top2-4 and top2-5, and these interacted with both alleles in a similar way, either positively or negatively (Figure 6B and Table S4 in File S1). Among them was top1Δ, which is well known to have synthetic sickness with top2-ts alleles (Kim and Wang 1989). However, most of the observed genetic interactions were positive, which is typically a sign of suppressive pathways.

Next, we sought to study the changes that increasing the temperature would produce in the observed genetic interactions. We opted for two different incubations: one set of arrays was grown at semipermissive temperature (30°C) for 2 d, and another set was incubated at 37°C for 6 hr, and then shifted to 25°C to allow growth of survivors. This transient restrictive regime gives enough time to complete one cell cycle without Top2 on solid media (Figure 2A), and indeed allowed us to observe the cdc15-2 protection of top2-5 (Figure 4A). In both cases we used the top2-ts arrays that were grown at 25°C as controls to compare colony sizes. Thus, we obtained a large number of new interactions that, unlike...
shock treatment and putative roles of Top2 during chromosome bioenergetics and autophagy (Table 1). This could be related to the heat interactions revealed common negative interactions at 37°C, and Table S6 in File S1). Ontological classiﬁcation during constant growth at 25°C, were mostly negative (Figure 6C, Table S5, and Table S6 in File S1). Ontological classification of signiﬁcant interactions revealed common negative interactions at 37°C × 6 hr with bioenergetics and autophagy (Table 1). This could be related to the heat shock treatment and putative roles of Top2 during chromosome reshaping and transcription reprogramming to cope with this stress (Pommier et al. 2016). Importantly, this classiﬁcation also spotlighted a high number of positive interactions between top2-5 grown at 30°C and/or 37°C × 6 hr and thermosensitive alleles related to mitotic progression, especially anaphase/telophase progression (Figure 6D and Table 1). Many of the genes belong to the MEN, including CDC15, whereas others are related to the cytoskeleton or rDNA metabolism, which is known to undergo important modiﬁcations during anaphase (Machín et al. 2016). To a lesser extent, top2-4 was also enriched in mitotic division alleles when incubated at 37°C × 6 hr, some of which overlap with those of top2-5 (e.g., STU1, CDC10, and MOR2) (Table S5 and Table S6 in File S1).

**DISCUSSION**

In this work we have presented single-cell biology studies and large-scale genetic interaction data that strongly support that the survival of a yeast cell transiently depleted from Top2 is negatively correlated with the commitment to execute cytokinesis at the end of mitosis. To a great degree, our data conﬁrm results by others who have previously explored such a possibility (Baxter and Difﬂey 2008). Importantly, we have classiﬁed the different aberrant nuclear morphologies seen in top2-ts anaphases and correlated them with the degree of completion of cytokinesis.

In addition, we have found many positive genetic interactions between top2-ts and alleles or mutations that delay different stages of the cell cycle (Figure 6, Table 1, Table S2, Table S3, Table S4, Table S5, and Table S6 in File S1). We reason that such positive interactions, measured as improved ﬁtness on a SGA, are better explained by: (i) fewer cells with CABs reaching the point-of-no-return (i.e., cytokinesis) during the transient 37°C × 6 hr shift; and (ii) the additional cell cycle delay allowing the reduced Top2 activity to end up resolving catenations, especially in the case of steady incubations at 25°C and 30°C. Again, these positive interactions support previous works that claimed that blocking the cell cycle in G1 or G2/M protects against transient Top2 depletion (Holm et al. 1985; Thomas et al. 1991).

Finally, our work also put forward some intriguing questions about why downregulation of Cdc14, the trigger of exit from mitosis, does not protect against Top2 deﬁciency. Last but not least, we uncovered surprising differences between the two top2-ts alleles used in this work, top2-4 and top2-5.

![Figure 4 The mitotic exit network (MEN) destabilizes top2-mediated chromatin anaphase bridges (CABs). (A) Spot dilution assay to compare survivability of different combinations of the top2-5, cdc14-1, and cdc15-2 alleles to a transient temperature shift to 37°C. All strains are HTA2-GFP bar1Δ derivatives from the corresponding TOP2 and top2-5 coisogenic strains. (B) Clonogenic assay to compare survivability of the top2-5, cdc15-2, and top2-5 cdc15-2 strains to a transient 6 hr shift to 37°C (mean ± SEM, n = 5). (C) The cdc15-2 HTA2-GFP and top2-5 cdc15-2 HTA2-GFP strains were synchronized in G1 at the permissive temperature (25°C) and then released into a synchronous cell cycle at 37°C for 4 hr. Samples were taken at the end of the experiment and analyzed by ﬂuorescence microscopy. On the left, representative microscope ﬁelds for each strain. On the right, bar chart of cells with CABs (error bars are CI95). Open arrowheads point to examples of short CABs (~1/3 of observed CABs in top2-5 cdc15-2). Filled arrowheads point to examples of long CABs (~2/3 of observed CABs). The asterisk highlights the complex strung chromatin bridges seen in ~50% of the long CABs. (D) The cdc14-1 HTA2-GFP and top2-5 cdc14-1 HTA2-GFP strains were treated as in (C). On the left, representative microscope ﬁelds for each strain. On the right, bar chart of nuclear morphologies in dumbbells (error bars are CI95). Arrows point to cells where the bulk of the nucleus migrated to the bud, whereas open arrowheads point to CABs. Arrows and arrowheads point exactly at the bud neck. The bar corresponds to 5 μm. BF, bright ﬁeld.
On the nature and fate of the anaphase bridges that result from depleting Top2

We started this work by revisiting earlier works from Botstein’s and Sternglanz’s laboratories on yeast cells cycling without Top2 activity (DiNardo et al. 1984; Holm et al. 1985). These studies reported that S. cerevisiae had no terminal phenotype at the top2-ts restrictive temperature. Rather, a mixture of un budded and large-budded (dumbbell) cells was the final outcome of Top2 inactivation. In many dumbbell cells, failure in chromosome segregation was evident due to the presence of DAPI-stained anaphase bridges. They assumed that there were two classes of cells coming from a top2-ts G1-synchronized culture: those which get stuck as dumbbells with major defects in nuclear segregation, and those which complete cytokinesis and cell separation. In light of the un budded progeny they observed, they concluded that the latter had gone through a devastating mitotic catastrophe. In general, our data fully support this conclusion (profiles of cell morphologies in Figure 1 and Figure 2). The most shocking difference is the relatively low percentage of visible anaphase bridges, which we here refer to as CABs since we used histone-labeled DNA (H2A-GFP). Of note, we also observed a small fraction of anaphase bridges when staining with either DAPI or Hoechst and, in addition, H2A-GFP and DAPI signals perfectly colocalized (Figure 2B). Instead of CABs, we often distinguished very close split nuclear masses just across the neck (SD-binucleated). The distance between the split signals was normally <1 μm. It is likely that this phenotype has been referred to as “anaphase bridge” in previous works. Notably, a similar phenotype was described before for a strain that depletes Top2 through a degron system and referred to as “cut” phenotype (Baxter and Difl ey 2008). Besides, a similar terminal phenotype occurs in mouse topo IIα−/− embryos and in a significant fraction (~1/3) of epithelial cells treated with Top2 catalytic inhibitors (Wheatley et al. 1998; Akimitsu et al. 2003). Outstandingly, we have been able to correlate this SD-binucleated phenotype to ingestion of the cleavage furrow at the cytokinetic plate. On the one hand, we observed that CABs were only visible when ingestion was absent (Figure 3B). On the contrary, SD-binucleated required either full contraction or resolution of the PM at the neck (absision). On the other hand, when we blocked MEN in top2-ts by depleting Cdc15, and hence we also blocked cleavage furrow ingestion, the SD-binucleated morphology was absent and a CAB was seen instead (Figure 4C and Figure S4 in File S1). As mentioned already, the large-scale screen for genetic interactions also supports that commitment to execute cytokinesis is deleterious for top2-ts (Figure 6, Table 1, Table S2, Table S3, Table S4, Table S5, and Table S6 in File S1).

An intriguing question is what happens to the CAB during PM ingestion and abscission. Two alternative hypotheses were possible: (i) the CAB is broken through cytokinesis, or (ii) the CAB becomes a histone- and DAPI-invisible UFB. Notably, a recent report has shown that thin channels of nuclear material are formed between the mother and the daughter cell when Top2 is depleted (Amaral et al. 2016). These channels go through cells that appear to have completed PM abscission, are fully surrounded by septum, and might be wide enough to accommodate UFBs. These channels might also explain why we observed PM abscission in many top2-ts dumbbells but were unable to split them upon zymolyase treatment (Figure 3, A and B). We have tried to address the fate of CABs during PM ingestion by triple-labeling the PM, the CAB (H2A-GFP), and the UFB (Dpb11-RFP). Our data do support that most CABs (and UFBs) are broken apart at the time of PM abscission (Figure 5). Thus, there was no change in the CAB/UFB ratio during the drop of anaphase bridges by PM abscission in top2-5. In addition, Dpb11 relocalized to foci which, together with the formation...
of Rad52 foci (Figure 1A), strongly points toward DSBs taking place in top2-5 late anaphase. Since PM abscission, CAB/UFB disappearance, and Dpb11 foci accumulation could all be prevented in the top2-5 cdc15-2 double mutant, we favor the hypothesis that all kinds of anaphase bridges are broken as a consequence of cytokinesis. Nevertheless, the alternative hypotheses of (i) Dpb11 relocalizing from UFBs to DNA repair foci upon CAB breakage or (ii) the UFB becoming too narrow for the current limits of fluorescence microscopy cannot be ruled out entirely and deserve further investigation in the future, especially in the light of the aforementioned channels.

Altogether, we propose a model where cells depleted from Top2 always form CABs at anaphase (Figure 7). These CABs can be massive and might often prevent the mitotic spindle from pulling the segregating sister chromatids away from each other, hence the short distances that separate the splitting masses across the bud neck. Alternative possibilities for such short distances are feasible but unlikely; e.g., Top2 is known to play no roles during mitotic spindle enlargement (Andrews et al. 2006). Importantly, these CABs do not prevent cleavage furrow ingression, which quickly changes them into SD-binucleated (Figure 3B). Remarkably, cells depleted from the condensin subunit Brn1, which are thought to give rise to top2-equivalent anaphase bridges, do not delay execution of cytokinesis either (Cuylen et al. 2013), and only a short delay of 15 min was seen for karyokinesis in anaphase bridges restricted to the rDNA (Quevedo et al. 2012). What happens after full membrane ingression is less clear. However, DNA damage seems apparent (Figure 1 and Figure 5), suggesting that DNA is severed as reported previously (Holm et al. 1989; Baxter and Diffley 2008). Again condensin depletion leads to similar results (Cuylen et al. 2013). Strong support for this model was obtained when we blocked MEN and cytokinesis (i.e., cdc15-2) in top2-5 strains. Thus, CABs were stabilized in the top2-5 cdc15-2 double mutant and no signs of DNA damage were detected (Figure 1 and Figure 5). Strikingly, though, even

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**Figure 6** SGA analyses identify the MEN as deleterious enhancers of Top2 downregulation. (A) The z-scores of the genetic interactions detected at permissive temperature (steady growth at 25°C) are plotted. The top2-ts arrays of mutants were compared with the TOP2 arrays as a control. 5553 alleles were screened in total. Positive and negative genetic interactions that meet the cutoffs (>-2 or <-2) are indicated. (B) Network of the genes that interact with top2-4 and top2-5. Green background encircles the 84 shared genes between the two top2-ts. Pink nodes indicate the positive interaction between top2-5 and genes involved in the aggregation and bonding of cellular components (GO: 0071844). (C) The numbers of genetic interactions identified during steady growth at 30°C and after 6 hr incubation at 37°C are shown. Circle intersections depict number of genetic interactions shared between both treatments. (D) Heat map of top2-5 SGA scores (those with P < 0.05). Yellow, positive interactions; blue, negative interactions; black, no interaction. The 25°C Column represents the z-score obtained comparing top2-5 vs. TOP2 at 25°C, whereas the 30°C and 37°C × 6 hr columns compare top2-5 at these temperature regimes vs. top2-5 at 25°C. MEN, mitotic exit network; Ck/Cs, cytokinesis/cell separation; Skln, cytoskeleton; rDNA, ribosomal DNA metabolism.
et al. (Kalitsis plays a key role in preventing CABs throughout all life kingdoms. The condensin complex also Top2 is not the only universal player needed to avoid the occurrence of bridge resolution through the FEAR network. On the putative synergistic role of Cdc14 in anaphase.

| SGA group | Gene Ontology | P-value |
|-----------|---------------|---------|
| top2-4 – 30° (pos. int.) | Carbohydrate biosynthetic process (GO:0016051) | 0.04323 |
| top2-4 – 37° x 6h (pos. int.) | APC-dependent ubiquitin-dependent protein process (GO:0031145) | 0.00681 |
| | Cell division (GO:0051301) | 0.00706 |
| top2-4 – 37° x 6h (neg. int.) | Macropolythaphagy (GO:0034262) | 0.03361 |
| top2-5 – 30° (pos. int.) | Macromolecular complex subunit organization (GO:0043933) | 0.00115 |
| | Organelle assembly (GO:0070925) | 0.04773 |
| top2-5 – 37° x 6h (pos. int.) | Cell division (GO:0051301) | 1.49E-05 |
| | Mitotic cell cycle process (GO:1903047) | 0.00017 |
| | Mitotic cell cycle (GO:0000278) | 0.00033 |
| | Mitotic nuclear division (GO:0007067) | 0.00035 |
| | Mitotic cell cycle phase transition (GO:0044772) | 0.0006 |
| | Cell cycle phase transition (GO:0044770) | 0.00068 |
| | Cell separation after cytokinesis (GO:0000920) | 0.02562 |
| | Nuclear division (GO:0000280) | 0.03884 |
| | Regulation of cell division (GO:0051302) | 0.04929 |
| top2-5 – 37° x 6h (neg. int.) | Macropolythaphagy (GO:0034262) | 0.00048 |
| | Cellular respiration (GO:0045333) | 0.00067 |
| | Oxidative phosphorylation (GO:0006119) | 0.00238 |
| | Generation of precursor metabolites and energy (GO:0006091) | 0.00301 |
| | Phosphorylation (GO:0016310) | 0.01924 |
| | Autophagy (GO:0006914) | 0.02062 |
| | Homoserine metabolic process (GO:0009092) | 0.02391 |
| | Sister chromatid biorientation (GO:0031134) | 0.02391 |
| | Energy derivation by oxidation of organic compounds (GO:0015980) | 0.04017 |

The corresponding top2-ts double mutant arrays were grown at 25° x 2 d, 30° x 2 d, and 6 hr x 37° + 25° x 2 d. The genetic positive interactions (pos. int.) and negative interactions (neg. int.) refer to the comparison between the downregulating temperature regimes and steady growth at 25°.

Table 1 Significant biological processes that genetically interact with top2-ts in different downregulating regimes

Gene Ontology (GO) enrichment analyses were done using the Generic GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder). GO terms that contained > 1500 genes were discarded, as they are usually too general. Similarly, redundant GO terms with < 10 genes were discarded.

P-values were computed using a hypergeometric distribution, and adjusted with the Bonferroni correction.

A complete block of MEN yielded only partial recovery when Top2-5 was reactivated (Figure 4A). In our study, this recovery was slightly smaller than the one previously reported (Baxter and Diffley 2008), likely due to the fact that we incubated the cells at 37° for a longer period. It is probable that the partial recovery relates to cells restoring Top2 and Cdc15 functions at the same time after the 37–25° shift, although the possibility of CABs becoming irreversible upon a long absence of Top2 should not be ruled out completely. Indeed, we have shown before that rDNA bridges in cdc14-1 eventually become challenging to resolve (Machín et al. 2006; Quevedo et al. 2012).

On the putative synergistic role of Cdc14 in anaphase bridge resolution through the FEAR network

Top2 is not the only universal player needed to avoid the occurrence of CABs during the mitotic cell division. The condensin complex also plays a key role in preventing CABs throughout all life kingdoms (Kalitsis et al. 2017). Previous works have placed condensin and Top2 within the same pathway to accurately remove sister chromatid catenations in S. cerevisiae (Baxter et al. 2011; Charbin et al. 2014). The master mitotic phosphatase Cdc14 controls resolution of sister chromatids by acting on overall transcription and thus favoring condensin localization onto DNA, conditions that are especially critical for the highly transcribed rDNA array (Machín et al. 2016). This Cdc14 control over condensin takes place in early anaphase and is possible because Cdc14 is transiently activated there through the FEAR network. Cdc14 also plays an essential role for exit from mitosis at late anaphase through its second activation by MEN. The kinase Cdc15 is critical for Cdc14 activation by MEN but not by the FEAR network (Stegmeier and Amon 2004). In this work, we have seen two important differences when the top2-5 allele was combined with ts mutants for the CDC14 and CDC15 genes. First, top2-5 cdc15-2 could partly rescue the reduced fitness of top2-5, whereas top2-5 cdc14-1 did not (Figure 4, A and B). This was confirmed in the SGA analysis, where cdc15-1 (cdc15-2 was not present) also alleviated top2-5; whereas two out of the three included cdc14-ts alleles were neutral (cdc14-1 and cdc14-2; just cdc14-3 emerged as a suppressor). Second, top2-5 cdc15-2 led to CABs that resembled the morphology of the SD- and LD-binucleated nuclear masses in top2-5; i.e., addition of a bridge to these morphologies was the only difference (Figure 4C). Nevertheless, top2-5 cdc14-1 gave a missegregation pattern that was clearly worse than either ts allele alone. In most cases the nuclear mass failed to split entirely, yet it localized within the daughter cell (Figure 4D). This phenotype is reminiscent of...
cells depleted of separase, the protease that breaks sister chromatid proteinaceous cohesion at the anaphase onset (McGrew et al. 1992). Taking into account that cdc14-1 strains are also known to mistakenly segregate the nucleus to the daughter cell (Ross and Cohen-Fix 2004), the synergistic nuclear segregation defect in cdc14-1 could carry intrinsic and variable properties in terms of cell cycle progression, commitment to enter anaphase, etc. (Andrews et al. 2006). One of the first surprising findings was that there was a difference in terms of resistance to the clinically used Top2 poisons within the same top2-ts group where top2-4 was isolated. Thus, top2-5 proved to be resistant to poisons even at permissive temperature (Jannatipour et al. 1993). This fact led us to include cosogenic top2-4 and top2-5 strains in this work. Surprisingly, we found marked differences between them. In general, both top2-ts alleles shared many similarities: (i) they failed to arrest in G2/M; (ii) they formed short-lived CABs, which could be stabilized by deleting Cdc15; (iii) they had DNA damage coinciding with anaphase progression; (iv) they led to two classes of split nuclear masses in anaphase (SD- and LD-binucleated); and (v) the immediate progeny often failed to bud again, and when they did, budding was restricted to the mother. The differences were more related to the timing of the cell cycle events and relative proportions of several phenotypes (Figure 1). It is important to highlight that these differences were more obvious in time course experiments carried out in liquid cultures than when we filmed single cells on agarose patches (Figure 2). In the time course experiments, top2-5 progressed through the cell cycle faster than top2-4. It also became stalled as dumbbells less frequently than top2-4. Likewise, SD-binucleated occurred less frequently in top2-5. Since top2-5 had a quicker G1-S transition in cultures relative to cells filmed on agarose patches, the most trivial explanation for these differences lay in this G1-S transition, making later phenotypes appear earlier and evolve into others quicker. As for the reason behind this difference, we can hypothesize about two origins. On the one hand, the difference may relate to the genetic history of these strains through the numerous passes over the years. Taking into account that it has been shown that Top2-5 only keeps 33% of normal Top2 activity at 25°C (Jannatipour et al. 1993), we believe that the top2-ts strains might be genetically unstable at 25°C, which would in turn boost the probability of top2-4 and top2-5 being genetically different despite their cosogenic origins. On the other hand, there might be an intrinsic difference between the top2-ts alleles. In support of this second hypothesis we have the fact that the top2-5 allele still gathered more genetic interactions than top2-4 when transferred to the SGA background. It is difficult to speculate on the causes of such an intrinsic difference. There is only a missense mutation in the protein encoded by top2-4, a proline-to-glutamine change at position 820 (P820G), whereas Top2-5 has three in a cluster: R883P, R885I, and M887I (Thomas et al. 2006). In both cases, mutations lay in the gyrase A motif near the catalytic center of the enzyme (Y782). Although it is plausible that the three mutations of top2-5 render the enzyme less active than Top2-4, more work is needed to conclusively explain intrinsic differences upon the 37°C shift. Lastly, an interesting possibility is that differences may correlate with the poison-resistant nature of Top2-5. This latter scenario would have important health-related implications and, therefore, it is worth testing in future works.

**Conclusions and perspectives**

In this work, we have characterized the consequences of depleting yeast cells from Top2 using two top2-ts alleles that differ in their resistance to chemotherapeutic Top2 poisons. As previously reported, the first cell cycle takes place with normal kinetics until anaphase, when top2-ts forms anaphase bridges. We have shown that these bridges are quickly split apart by PM abscission and can be maintained if mitotic exit is blocked. Finally we provide genetic evidence that stabilization of these bridges improve survivability upon transient Top2 inactivation. Since transient inactivation of targets is of the utmost importance to predict cell response to pharmacological drugs and also uncover adjuvant treatments, our results point toward upregulation of mitotic exit as a
putative target to synergistically promote cell death upon Top2 down-regulation/mutation in cancer cells.

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