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Genome-Organizing Factors Top2 and Hmo1 Prevent Chromosome Fragility at Sites of S phase Transcription

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SUMMARY

Specialized topoisomerases solve the topological constraints arising when replication forks encounter transcription. We have investigated the contribution of Top2 in S phase transcription. Specifically in S phase, Top2 binds intergenic regions close to transcribed genes. The Top2-bound loci exhibit low nucleosome density and accumulate γH2A when Top2 is defective. These intergenic loci associate with the HMG protein Hmo1 throughout the cell cycle and are refractory to the histone variant Htz1. In *top2* mutants, Hmo1 is deleterious and accumulates at pericentromeric regions in G2/M. Our data indicate that Top2 is dispensable for transcription and that Hmo1 and Top2 bind in the proximity of genes transcribed in S phase suppressing chromosome fragility at the M-G1 transition. We propose that an Hmo1-dependent epigenetic signature together with Top2 mediate an S phase architectural pathway to preserve genome integrity.

INTRODUCTION

Genome stability during chromosome replication can be challenged by drugs affecting fork progression, intra-S DNA damage, oncogene activation, and transcription (Aguilera and Gomez-Gonzalez, 2008; Branzei and Foiani, 2008; Di Micco et al., 2006; Prado and Aguilera, 2005). The mechanisms and pathways preserving the integrity of replicating chromosomes have been widely studied (Branzei and Foiani, 2008; Cha and Kleckner, 2002; Myung and Kolodner, 2002; Schmidt and Kolodner, 2006; Smith et al., 2005). Certain chromosomal loci are fragile (Casper et al., 2002; Lemoine et al., 2005), but the physiological and pathological transitions occurring at these fragile sites are still unclear. Prokaryotic genomes have evolved to avoid transcription-replication clashing by placing coding sequences at the leading strands of replication forks (Rocha, 2004). In eukaryotes specialized networks deal with transcription-replication interference when collisions occur. Those regions experiencing clashes between replication and transcription often slow down the forks and trigger recombination events (Deshpande and Newlon, 1996; Prado and Aguilera, 2005). Dedicated *r*eplication *f*ork *b*arriers (RFB) block fork advance opposite to RNA polymerase I-mediated transcription, thus allowing those forks progressing codirectionally with transcription to complete replication of the rDNA locus (Brewer and Fangman, 1988; Torres et al., 2004). When forks clash with transcribed units that are codirectional with fork movement, the replisome can utilize the 3′ end of the RNA species for repriming a DNA chain downstream (Pomerantz and O'Donnell, 2008). Specialized DNA helicases assist fork progression when replication clashes with RNA polymerase II- and III-dependent transcription (Ivessa et al., 2003; Prado and Aguilera, 2005; Scholes et al., 2001).

We identified a subpopulation of DNA topoisomerase II (Top2) localizing to intergenic regions in S phase. The S phase specificity of those Top2 clusters prompted us to suggest that Top2 alleviates the topological problems generated on transcribed regions during replication (Bermejo et al., 2007). Top2 is a type II topoisomerase that catalyzes the passage of two independent segments of DNA through another (Champoux, 2001; Wang, 2002) and is implicated in higher-order chromatin organization (Gasser et al., 1986; Li et al., 1999; Varga-Weisz et al., 1997). Intrachromosomal looping may facilitate transcription initiation through the establishment of productive interactions between distant regulatory elements, transcription factors, and chromatin-remodeling complexes (Schneider and Grosschedl, 2007). Gene looping, taking place between initiator and terminator regions, has been proposed to facilitate polymerase recycling and to increase transcription rates (Ansari and Hampsey, 2005; O'Sullivan et al., 2004).

Chromosomal architecture also regulates replication dynamics. Cells experiencing replication stress enhance replicon

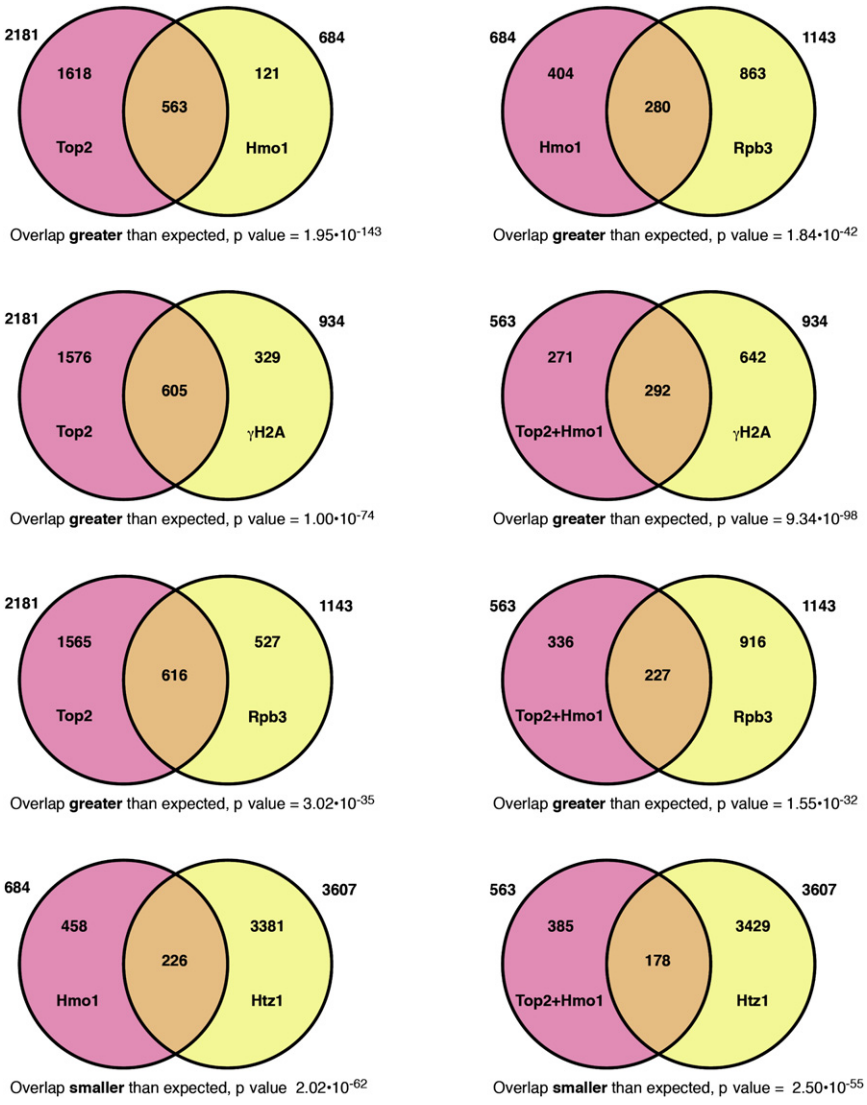


Figure 1. Genome-wide Protein-Binding Correlations

Overlap of candidate promoters bound by different targets of the experiments and assessment of their statistical significance. We considered as candidate promoters the 500 bp upstream each of the 5769 genes in *Saccharomyces* Genome Database (SGD), even where this region is overlapped by exons of other genes. When a promoter was overlapped by 30% of its size by a cluster, we considered the association significant. The absolute numbers shown in the diagrams are severely affected by the parameters used to define the clusters and thus should be considered relevant only to assess the statistical significance of the changes with respect to what would be the randomly expected. Lower- or upper-bound one-tailed exact fisher test p values are reported per each pair of targets; Top2+Hmo1 means genes bound by both.

firing by epigenetically priming higher-order chromosomal loops, and a DNA topoisomerase II mechanism has been involved in replicon resetting at mitosis through remodeling of chromosomal loops (Courbet et al., 2008; Lemaitre et al., 2005). Similarly, *h*igh *m*obility *g*roup (HMG) proteins bind DNA with low sequence specificity and act as chromatin architectural factors (Stros et al., 2007b; Thomas and Travers, 2001). They have affinity for DNA with a distorted conformation but can also induce changes in the structure of the DNA helix. HMG proteins have also been implicated in transcription regulation and maintenance of chromosomal integrity (Thomas, 2001; Sikdar et al., 2008).

The yeast HMGB protein Hmo1 modulates chromatin structure and transcription of certain RNA pol II transcribed genes through mechanisms that are still elusive (Berger et al., 2007; Hall et al., 2006; Kasahara et al., 2007; Lu et al., 1996). Hmo1 can bind four-way DNA junctions with high affinity and substitute for histones to organize rDNA transcribed units (Kamau et al., 2004; Merz et al., 2008). HMGB1, a human Hmo1-related protein, exhibits high affinity for distorted DNA structures like four-way junctions, cisplatin-modified DNA, or hemicatenated DNA loops (Bianchi et al., 1989; Gaillard and Strauss, 2000; Hughes et al., 1992). HMGB1 physically interacts with and stimulates TopoIIα activity on catenated DNA structures (Stros et al., 2007a).

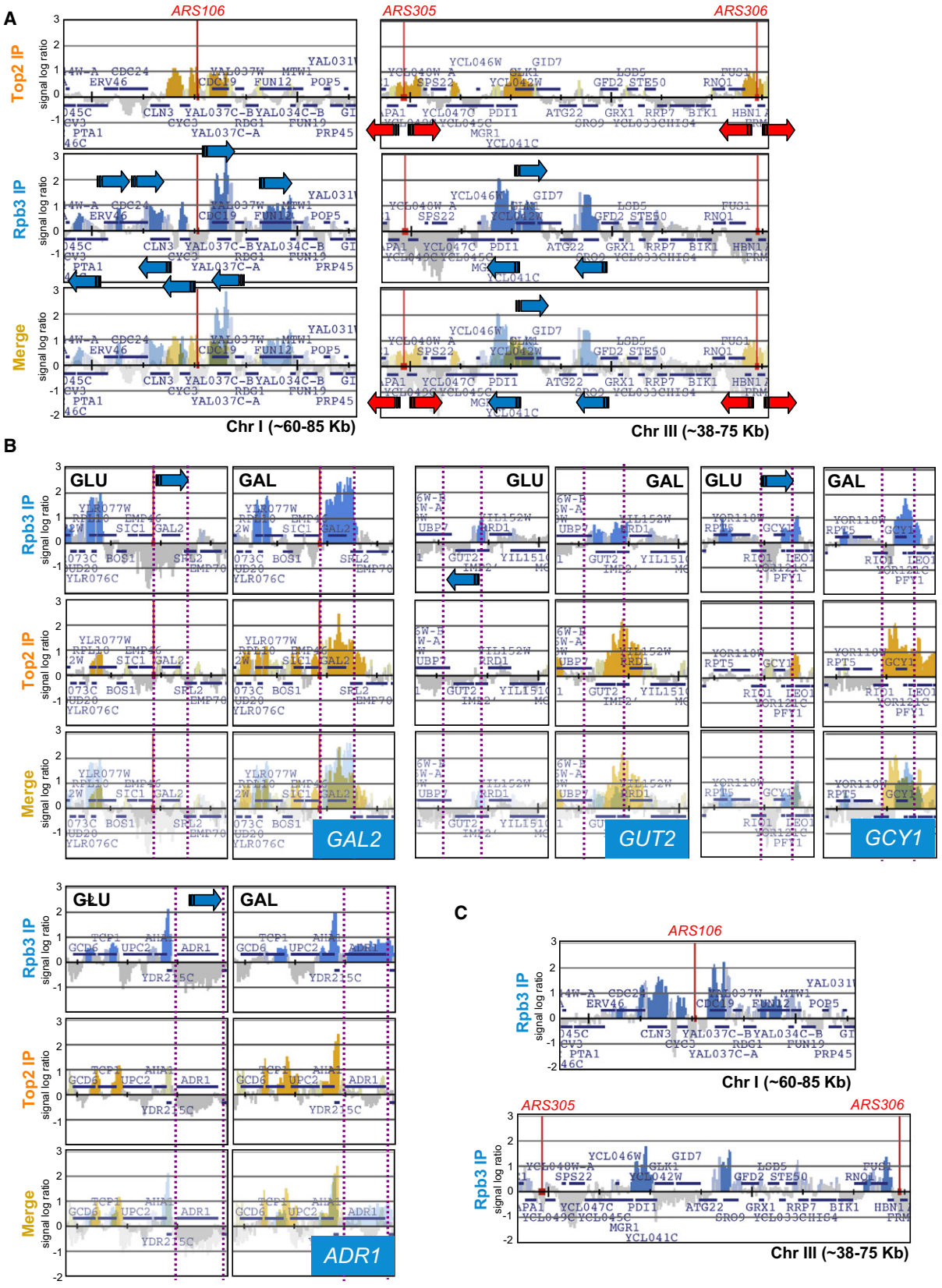
Here we show that yeast Top2 binding at intergenic loci during replication correlates with a fraction of RNA polymerase II genes transcribed in S phase. The intergenic regions bound by Top2 accumulate γH2A when Top2 activity is attenuated. Hmo1 localizes at the Top2 intergenic regions even in G1 and G2/M, when Top2 is not present. *HMO1* ablation alleviates certain phenotypes of *top2* mutants and Hmo1 protein exhibits an abnormal chromosomal distribution when Top2 is not functional.

Our data unmask a chromosome architectural pathway, mediated by Top2 and Hmo1, that protects chromosome integrity likely by topologically coordinating DNA replication and transcription.

RESULTS

DNA Topoisomerase II Associates with Regions Transcribed by RNA pol II during S Phase

Top2 does not form obvious clusters in G1 and enriches at pericentromeric regions in G2/M (Figure S1 available online and data not shown). We performed a genome-wide computational analysis of the S phase Top2 and RNA polymerase II Rpb3 subunit clusters (Supplemental Statistical Analysis and Figure 1). Ninety-one percent of Top2 peaks close to mRNA-encoding genes (we will refer to these clusters as iTop2 [intergenic


A
ARS106
ARS305
ARS306
Top2 IP
Rpb3 IP
Merge
signal log ratio
Chr I (~60-85 Kb)
Chr III (~38-75 Kb)
B
GLU
GAL
GAL2
GUT2
GCY1
ADR1
C
ARS106
ARS305
ARS306
Chr I (~60-85 Kb)
Chr III (~38-75 Kb)

Top2]). iTop2 preferentially binds to promoters and transcription termination regions, whereas it is excluded from exons. The iTop2 clusters also correlate with promoters shared by divergently transcribed genes. Rpb3 enriched at certain mRNA genes (Figures 2A and S2). We found a significant correlation between the Top2 clusters and Rpb3 clusters ($p = 4.5 \cdot 10^{-18}$; the significance of the correlations is computed as the actual distribution in respect to randomly simulated positions). The correlation is also evident when Top2-bound promoters were compared with Rpb3-associated promoters ($p = 3.01 \cdot 10^{-35}$) (Figure 2A and Supplemental Statistical Analysis). A large fraction of Rpb3 clusters were not enriched for Top2. We note that most of the Top2-free Rpb3-enriched regions do not randomly distribute; rather they are clustered into large (30–90 Kb) chromosomal domains (Figure S2). Hence, fork unrelated Top2 clusters associate with a subpopulation of mRNA genes that are transcribed in S phase, whereas other chromosomal regions, although experiencing extensive transcription, seem refractory to Top2 association.

To investigate the relationship between the iTop2 clusters and RNA pol II-driven transcription, we scored the S phase Top2-Rpb3 profiles in cells grown in galactose, which modulates the expression of a set of genes involved in carbon source metabolism. Rpb3 profiles of cells grown in glucose and galactose were comparable, with no obvious changes in the majority of mRNA-encoding genes (Figure 2B and data not shown). A subset of genes showed a differential Rpb3 binding in glucose versus galactose (Figure 2B and data not shown). Those galactose-inducible genes (*GAL2*, *GUT2*, *GCY1*, and *ADR1)* exhibited Rpb3 enrichment in galactose but not in glucose (Figure 2B). The changes in Rpb3 distribution were mirrored by Top2 distribution (Figure 2B). Hence, the S phase iTop2 recruitment correlates with the transcriptional status of galactose-regulated mRNA genes.

Rpb3 clusters were also present in G1, where Top2 is not detected (Figure 2C and data not shown). When we investigated whether the S phase Rpb3 clusters were affected when shifting *top2-1* mutants to the restrictive temperature, we failed to visualize significant differences between *top2* and wild-type (WT) strains (Figure 3A). Moreover, the genome-wide analysis of RNA levels in WT and *top2* cells did not reveal significant differences in expression either at iTop2 genes (Figure 3B) or at genes located at Top2-free regions (Figure 3C). Hence, Top2 is not needed to license the transcriptional program. This is consistent with the observations that Top2 activity is not required to resolve the topological constraints associated with transcription (Brill and Sternglanz, 1988; Wang, 2002).

The Top2 Intergenic Regions Accumulate γH2A in *top2* Mutants

We analyzed fork progression through three independent Top2-bound regions—*PDI1/GLK1*, *AGP1/KCC4*, and *ADP1/PGK1*—(Figure S3) by 2D gels. All three regions exhibited accumulation of Y-shaped replication intermediates owing to passive replication by forks emanating from adjacent replication origins (Figure S3). A small fraction of X-shaped sister chromatid junctions could also be visualized.

We conclude that replication can proceed through these regions without major impediments. We note that the presence of spots with higher intensity on the Y arc may result from occasional and transient fork pausing (Figure S3). We failed to detect by 2D gels a dramatic accumulation of pausing-related replication intermediates in *top2* mutants (data not shown) thus arguing against a primary role of iTop2 in assisting fork progression at these regions.

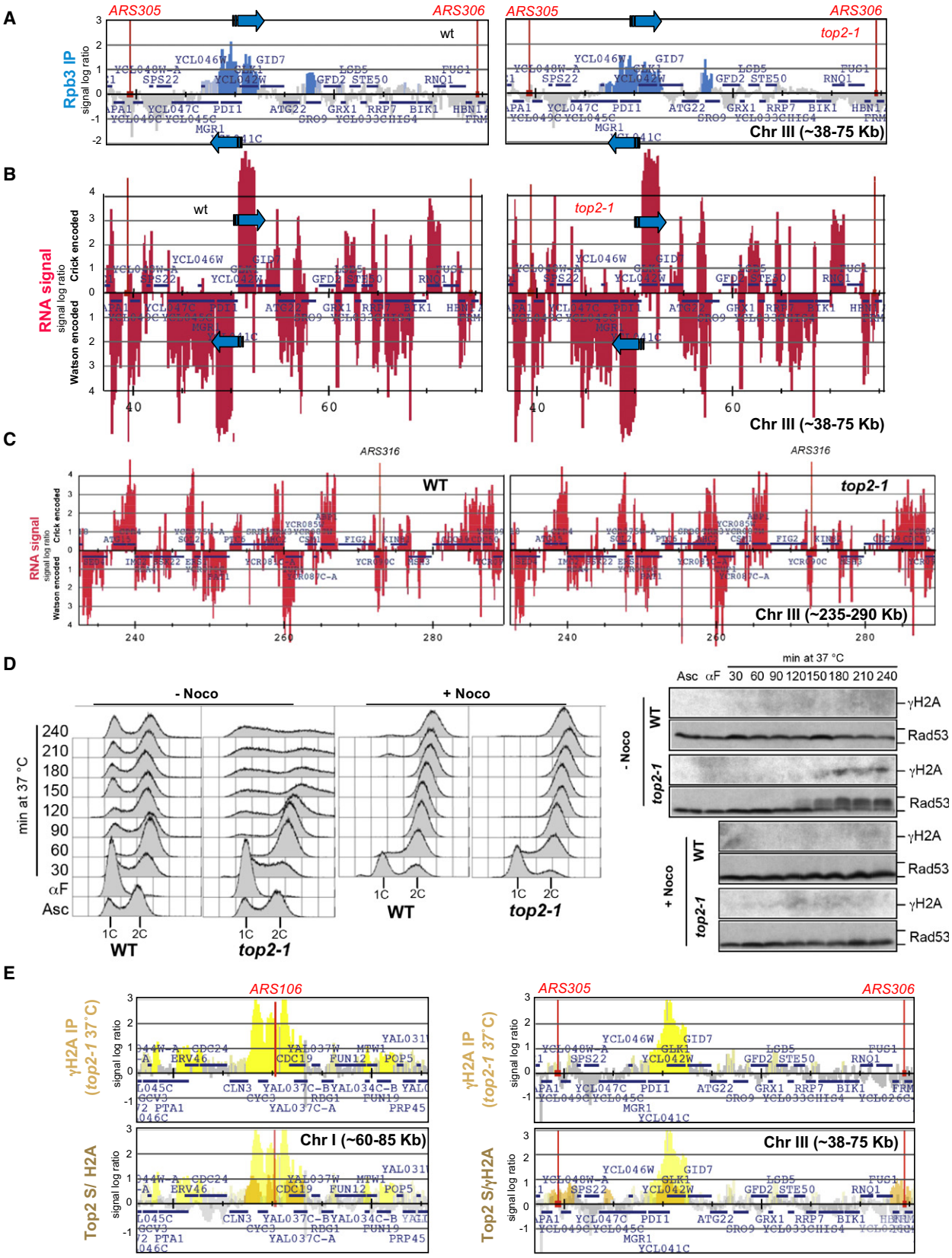
We then addressed whether Top2 plays any role in maintaining the integrity of these intergenic regions. Cells deficient in Top2 activity experience chromosome missegregation and DNA breaks perhaps due to the mechanical strain generated when segregating entangled and/or abnormally condensed sister chromatids (Holm et al., 1989; Spell and Holm, 1994). DNA breaks in *top2* mutants depend on the previous passage through S phase at the nonpermissive temperature and cell division (Bermejo et al., 2007) (Figure S4). Accumulation of DNA lesions is accompanied by phosphorylation of histone 2A on Ser129 (γH2A), close to the breaks (Lydall and Whitehall, 2005; Vidanes et al., 2005), and Rad53 checkpoint kinase phosphorylation (Sanchez et al., 1996). Wild-type and *top2-1* cells were arrested in G1 at the permissive temperature and released into cell cycle at the nonpermissive temperature with or without nocodazole (Figure 3D). Phosphorylated γH2A and Rad53 were detected in *top2* mutants, but not in WT cells, at 120–150 min after G1 release. These events were concomitant with cell division. When chromosome segregation was prevented in *top2* cells by nocodazole, the accumulation of phosphorylated γH2A and Rad53 was suppressed (Figure 3D). We then analyzed the genome-wide distribution of γH2A in *top2* mutants. *top2-1* cells were released from G1 at 37°C, and samples were taken upon cell division at 150 min for ChIP on Chip analysis (Figures 3E and S5). Eighty-six percent of the γH2A peaks localized to intergenic regions (Table S2 in Supplemental Statistical Analysis), while 76% mapped within 500 bp upstream or downstream of protein-encoding genes. Sixteen percent of the promoter regions were bound by γH2A (Table S5 in Supplemental Statistical Analysis). The Top2-bound regions showed a significant

Figure 2. Top2 Binding to Intergenic Regions Correlates with RNA pol II Transcription

(A) Top2-10×Flag/Rpb3-9×PK (CY8592) cells were released from G1 in the presence of 0.2 M HU. Samples were collected after 1 hr and processed for parallel ChIP with antibodies specific to the Flag and PK epitopes. Orange (Top2-IP) and blue (Rpb3-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated regions in log2 scale. The x axis shows chromosomal coordinates. Merging of Top2 and Rpb3 signals is shown. Positions of *ARS* elements are indicated. The horizontal bars mark the positions of the indicated ORFs. Blue and red arrows indicate the directions of the transcription machinery at genes significantly bound by Rpb3 or the replication forks emanating from active early replication origins, respectively.

(B) Top2-10×Flag/Rpb3-9×PK (CY8592) cells were released from G1 into 0.2 M HU with glucose (GLU) or galactose (GAL) and processed for parallel ChIP. Blue arrows indicate transcription direction of ORFs showing differential Rpb3 binding (marked by vertical dashed lines).

(C) Rpb3-9×PK (CY8519) cells were arrested in G1 and processed for ChIP.


A
ARS305
ARS306
wt
top2-1
Rpb3 IP
signal log ratio
Chr III (~38-75 Kb)
B
RNA signal
Crick encoded
Watson encoded
C
ARS316
WT
Chr III (~235-290 Kb)
D
- Noco
+ Noco
min at 37 °C
Asc αF 30 60 90 120 150 180 210 240
γH2A
Rad53
WT
top2-1
E
ARS106
γH2A IP (top2-1 37°C)
Top2 S/ H2A
Chr I (~60-85 Kb)
Top2 S/γH2A

enrichment for γH2A binding in Top2-deficient cells ($p = 1.2 \cdot 10^{-297}$) (Table S3 in Supplemental Statistical Analysis), suggesting that Top2 prevents aberrant DNA transitions, likely DNA breaks, at these genomic locations. A subpopulation of promoters accumulated γH2A in *top2* cells at chromosomal regions that do not exhibit Top2 binding in S phase cells (Figure S5). This observation suggests that either Top2 influences the architecture and integrity of large chromosomal domains or it acts at those regions later on in the cell cycle to prevent aberrant transitions. We note that γH2A modification might result not only from DSB formation in *top2* cells but also from nicks and gaps (Marti et al., 2006; Redon et al., 2002). Top2 clusters showed significant correlation with regions of low nucleosome density ($p = 2.07 \cdot 10^{-58}$) (Mavrich et al., 2008) (Figure 4A and Table S2 in Supplemental Statistical Analysis), thus suggesting that Top2 clusters associate with a particular chromatin signature.

Hmo1 Functionally Interacts with Top2

The yeast HMG protein Hmo1 binds nucleosome-free sites and is involved in chromatin organization and promoter-specific gene transcription (Hall et al., 2006; Kasahara et al., 2007; Merz et al., 2008). We analyzed Hmo1 chromosomal localization in S phase-arrested cells (Figure 4B). Consistent with previous observations in asynchronous cells (Kasahara et al., 2007), we observed Hmo1 peaks mostly, but not exclusively, at promoters (Figure 4B). We found a significant correlation between the Hmo1 clusters and the S phase Top2-bound regions ($p = 7.4 \cdot 10^{-205}$) (Figures 3B and S6 and Table S3 in Supplemental Statistical Analysis). The Top2-Hmo1-bound promoters correlated with transcribed genes ($p = 1.55 \cdot 10^{-32}$) (Figure 1), as scored by Rpb3 binding. The Hmo1-bound promoters represent 25% of the genes bound by Rpb3 in S phase (Figure 1). We found a significant correlation between the promoters bound by both Hmo1 and Top2 in S phase cells and the γH2A clusters in Top2-deficient cells ($p = 9.33 \cdot 10^{-98}$) (Figures S6 and 1). Hmo1 exhibited the same genomic profiles in G1, S, and G2/M cells (Figure 4C).

These data suggest that the Hmo1 clusters might be related to a particular architectural organization of certain promoters, which might also influence Top2 binding in S phase, and chromosome fragility in *top2* mutants. We also scored the chromosomal distribution in S phase of Htz1, a histone variant that marks two well-positioned nucleosomes in the majority (63%) of yeast mRNA promoters (Guillemette et al., 2005; Zlatanova and Thakar, 2008) (Table S5 in Supplemental Statistical Analysis). Peaks of Htz1 enriched at most promoters, with no apparent correlation with the gene transcriptional status, as indicated by Rpb3 enrichment (data not shown). There is a significant inverse correlation between the promoters bound by both Top2 and Hmo1 and those bound by Htz1 ($p = 2.5 \cdot 10^{-55}$) (Figures 3D and 1). These observations suggest that Htz1 and Hmo1 binding are mutually exclusive at promoters and that their genomic profiles might influence Top2-mediated chromosome transitions in S phase.

We then analyzed the genomic distribution of Top2 in *hmo1Δ* cells and that of Hmo1 in *top2* mutants. *hmo1Δ* cells were released from G1 into HU and the Top2 clusters were scored. We failed to detect a significant difference in Top2 distribution between *hmo1Δ* and WT cells (Figure 5A). This result suggests that Hmo1 is not needed for recruiting Top2 at the intergenic regions, although it does not exclude the possibility that Hmo1 is part of a larger protein complex that attracts Top2 at intergenic regions and/or that Hmo1 is redundant with other HMG proteins, considering the ability of HMG box proteins to bind similar structures (Agresti and Bianchi, 2003; Thomas and Travers, 2001). We tested the reciprocal dependency. *top2* mutants were released from G1 at the restrictive temperature in the presence of HU and scored for Hmo1 clusters. We failed to detect a significant difference in Hmo1 distribution between *top2* and WT cells (Figure 5B). This result suggests that Hmo1 recruitment at intergenic regions does not require Top2. This is also consistent with the observation that Hmo1 binds the intergenic regions in G1, prior to the association of Top2. Top2 distribution in G2/M-arrested cells differs from that in S phase (data not shown). We therefore tested whether Hmo1 distribution was affected in *top2* mutants released from G1 at the restrictive temperature in the presence of nocodazole (Figure 5C). We found that Hmo1 specifically enriched at pericentromeric regions in *top2* but not in WT cells (Figures 5C and S7). Hence, in the absence of a functional Top2, pericentromeric regions generate DNA structures that recruit Hmo1.

The observation that Top2 is not required for Hmo1 association but, rather, prevents the accumulation of Hmo1 at pericentromeric regions raises the possibility that Hmo1 contributes to some of the phenotypes observed in *top2* cells. Alternatively, Hmo1 may play a beneficial role in a *top2* mutant background in protecting the cells from even more severe phenotypes. To test these possibilities, we ablated *HMO1* in two different *top2* alleles and analyzed the double mutant phenotypes. While WT,

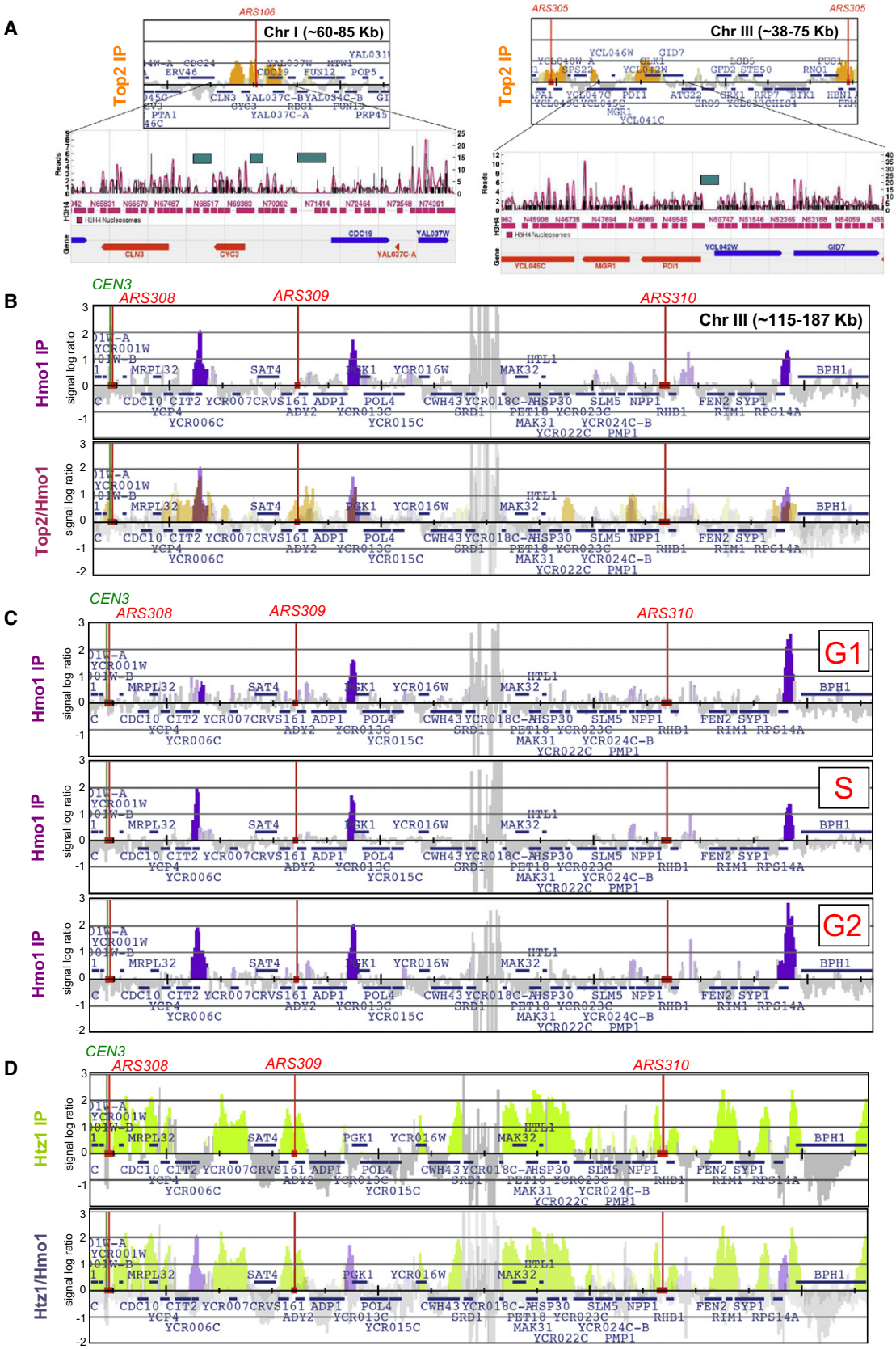
Figure 3. Transcription Analysis and γH2A Binding in *top2* Mutants

(A) Rpb3-9×PK (CY8519), *top2-1*/Rpb3-9×PK (CY8641) cells were released from G1 at 37°C into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the PK epitopes. Blue (Rpb3-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated region on chromosome III in log2 scale.

(B and C) Wild-type (SY2209) and *top2-1* (CY8423) cells were released from G1 at 37°C in the presence of 0.2M HU. Samples were collected after 1 hr, and total RNA was extracted, in vitro translated, and hybridized to Crick and Watson-strand arrays. Magenta (RNA enrichment) histogram bars in the y axis show the average signal ratio of loci in the Crick (upwards) and Watson (downwards) along the indicated regions on chromosome III on log2 scale. (B) refers to the same panels in Figure 2A. (C) refers to the iTop2-free region in Chr III (marked in Figure S2).

(D) Wild-type and *top2-1* cells were released from G1 at 37°C without (−Noco) or with (+Noco) nocodazole (15 μg/ml). Samples were collected at the indicated time points for FACS analysis, TCA protein precipitation, and immunodetection using Ser129 phosphorylated H2A (γH2A) and Rad53 antibodies.

(E) *top2-1* cells were released from G1 at 37°C, collected after 150 min, and processed for ChIP with antibodies specific to γH2A modification. Yellow (γH2A-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated regions in log2 scale. Merging of the γH2A signal and Top2 signal in S phase cells is shown. Positions of *ARS* elements are indicated.


A
Chr I (~60-85 Kb)
Top2 IP
ARS106
Chr III (~38-75 Kb)
Top2 IP
ARS305
ARS305
B
Chr III (~115-187 Kb)
CEN3
ARS308
ARS309
ARS310
Hmo1 IP
Top2/Hmo1
signal log ratio
C
G1
S
G2
D
Htz1 IP
Htz1/Hmo1

top2, *hmo1Δ*, and *top2hmo1Δ* cells were viable at the permissive temperature of 25°C, *top2-1* and *top2-4* cells exhibited growth defects when grown at the nonpermissive temperatures (32°C for *top2-1* and 30°C for *top2-4* mutations) (Figure 6A). Conversely, *top2-1 hmo1Δ* and *top2-4 hmo1Δ* mutant cells were able to grow at the same temperatures (Figure 6A), indicating that *HMO1* deletion partially suppresses the temperature sensitivity of *top2* mutations. This result suggests that the presence of Hmo1 might be deleterious in cells growing with limiting levels of Top2 activity. We then tested whether *hmo1Δ* was also able to suppress the chromosome segregation defects and DNA-damage checkpoint activation of *top2* mutants (Figure 6B). Wild-type, *hmo1Δ*, *top2-1*, and *top2-1hmo1Δ* cells were arrested in G1 and released into S phase at 32°C, which represents the lowest nonpermissive temperature for *top2-1* cells. Cell-cycle progression was monitored by fluorescence-activated cell sorting (FACS) analysis and checkpoint activation by western blotting of Rad53 and γH2A (Figure 6B). Under these experimental conditions, WT and *hmo1Δ* cells cycled normally, showed a 2C DNA content 60 min after G1 release, divided by 120 min, and progressively lost synchronicity (Figure 6B). Conversely, *top2-1* cells exhibited aberrant DNA contents after cell division, ranging from <1C to >1C by 120 min, indicative of unbalanced chromosome segregation (Baxter and Diffley, 2008; Holm et al., 1989). In the following time points, their cellular DNA content increased, indicating that *top2-1* mutants were able to undergo a second round of DNA replication. The aberrant FACS profile of *top2-1* cells was partially attenuated in *top2-1 hmo1Δ* mutants, which after cell division by 120–150 min showed the typical G1 DNA content. Moreover, *top2-1 hmo1Δ* cells were able to cycle at the nonpermissive temperature.

We did not detect phosphorylated forms of γH2A and Rad53 in WT or *hmo1Δ* cells, indicating that these genetic backgrounds do not experience damage under these experimental conditions. *top2-1* cells accumulated γH2A at 120 min concomitantly with the time of division (Figure 6B). Rad53 phosphoisoforms were only detected at 240 min when cells were about to complete a second round of replication, perhaps reflecting the time of DNA-break processing and ssDNA accumulation. *top2-1 hmo1Δ* cells did not accumulate γH2A after the first cell division by 120 min but did 90 min later, thus indicating that a population of cells still experience DNA-break formation. Moreover, Rad53 phosphorylation was barely detectable. We then analyzed DSB formation by pulse field gel electrophoresis (PGFE) in WT, *hmo1*, *top2*, and *hmo1top2* cells (Figure 6C). While in WT and *hmo1* cells only a discrete band corresponding to chromosome III was detected, a population of faster-migrating fragments representing broken chromosomes became apparent in *top2* cells by 150 min. Broken chromosome detection was reduced in *top2hmo1* cells as compared to *top2* mutants.

We conclude that *HMO1* deletion partially suppresses the chromosomal segregation defects and DNA-break formation leading to the accumulation of checkpoint signals in *top2* mutants.

Altogether, these data are consistent with the existence of a chromatin architectural pathway, mediated by Top2 and Hmo1, that acts to preserve the integrity of certain genomic regions.

DISCUSSION

Top2 at Intergenic Loci

In G2/M, Top2 is no longer associated with the intergenic regions. It is possible that, when forks pass through the intergenic regions, Top2 is dislodged, thus implying that its function is no longer needed. S phase Top2 enrichment correlates with transcribed genes. In most of the cases, the levels of transcription of genes close to the iTop2 clusters do not significantly vary throughout the cell cycle. Moreover, Rpb3 binding and the transcription program are not affected in Top2 mutants. These observations imply that transcription per se does not require Top2. However, a role for Top2 in facilitating transcription has been suggested at specific promoters (Collins et al., 2001; Ju et al., 2006). Our data do not rule out a possible contribution for Top1 in transcription in the absence of a functional Top2.

As Top2 has been implicated in DNA looping (Li et al., 1999), one possibility is that iTop2 contributes to the formation of architectural domains containing one or more transcribed units. Top2 might deal with the difficult topological context specifically in S phase when transcription has to face incoming forks. However, our data suggest that in *top2* mutants fork progression across the intergenic regions is not affected. Since Top1 and Top2 can resolve the same topological substrates and partially substitute for each other (Champoux, 2001; Wang, 2002), it is possible that in the absence of a fully functional Top2, Top1 (that is fork associated) deals with the topological problems arising when forks collide with transcription units. While the attenuation of Top2 activity does not seem to directly impinge on transcription or fork progression, it might have important implications for S phase events that might influence chromosome integrity later

Figure 4. Chromatin Signatures of Top2-Bound Intergenic Regions

(A) Nucleosome positioning maps (modified form http://h2az.atlas.bx.psu.edu/) of 10 Kb regions containing intergenic Top2 clusters. Green horizontal bands mark chromosomal segments presenting low nucleosomal density.

(B) Hmo1-6×PK (CY8516) cells were released from G1 into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the PK epitopes. Purple (Hmo1-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated region on chromosomes III in log2 scale. The x axis shows chromosomal coordinates. Positions of early firing origins on the chromosomal region and *CEN3* are indicated. The horizontal bars mark the positions of the indicated ORFs. Hmo1 and Top2 S phase signals merging is shown.

(C) Hmo1-6×PK cells were released from G1 (G1) in S phase or G2/M by treatment with 0.2 M HU for 1 hr (S) or 15 μg/ml Nocodazole for 3 hr (G2). Samples were collected and processed for ChIP.

(D) Htz1-3×Flag (SY2512) cells were released from G1 into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the Flag epitopes. Green (Htz1-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated region on chromosomes III in log2 scale. Merging of Htz1 and Hmo1 S phase signals is shown.

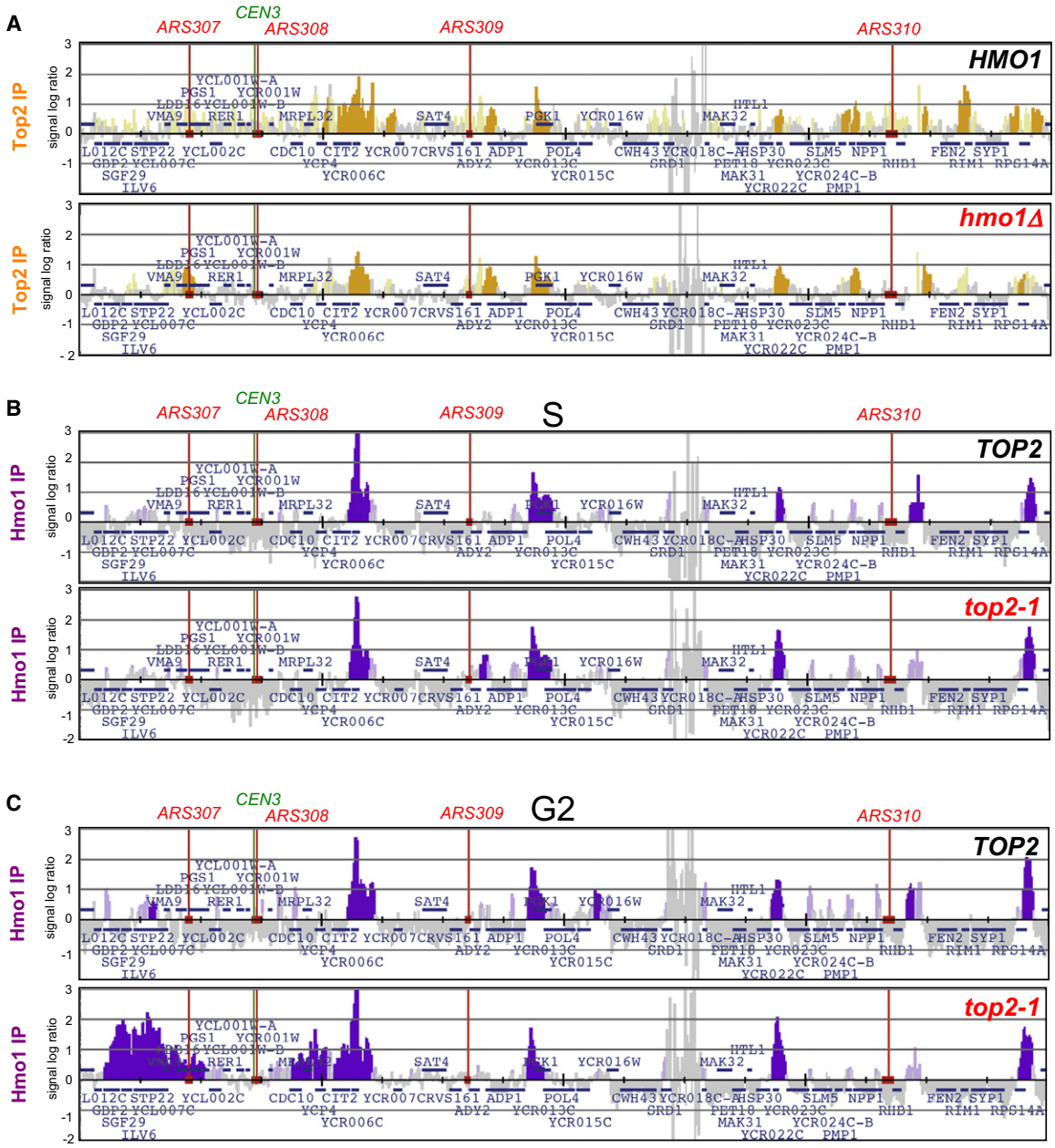


Figure 5. Hmo1 Binding Is Altered in *top2* Mutants

(A) *HMO1*/Top2-10×Flag (CY7315) and *hmo1Δ*/Top2-10×Flag (CY8478) cells were released from G1 into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the Flag epitopes. Orange (Top2-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along the indicated region on chromosome III in log2 scale.

(B) *TOP2*/Hmo1-6×PK (CY8516) and *top2-1*/Hmo1-6×PK (CY8599) cells were released from G1 at 37°C into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the PK epitopes.

(C) *TOP2*/Hmo1-6×PK and *top2-1*/Hmo1-6×PK cells were released from G1 at 37°C and arrested in G2/M by treatment with 15 μg/ml Nocodazole. Samples were collected after 3 hr and processed for ChIP.

in the cell cycle. It has been suggested that Top2 might prevent abnormal S phase entanglement between sister chromatids (perhaps through catenation mediated by the unscheduled action of type I topoisomerases), which might cause DNA breakage during chromosome segregation (Bermejo et al., 2008). We found that the γH2A sites coincide with the Top2 intergenic clusters. This observation may suggest that the intergenic regions represent hot spots not only for chromosome fragility at

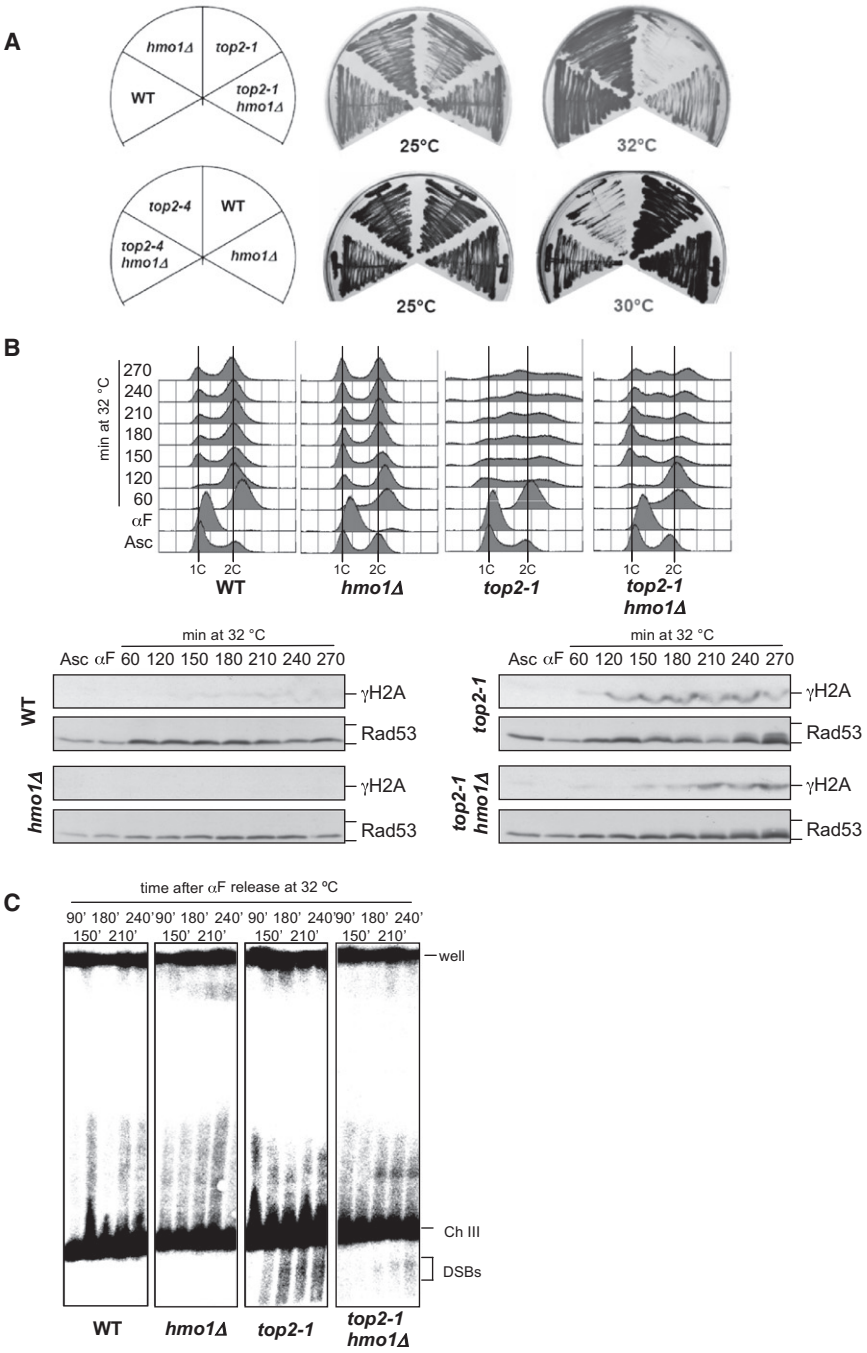


Figure 6. *HMO1* and *TOP2* Interact Genetically

(A) Wild-type (SY2209), *hmo1Δ* (CY8476), *top2-1* (CY8423), *top2-4* (CY8425), *top2-1 hmo1Δ* (CY8475), and *top2-4 hmo1 Δ* (CY8510) cells were streaked on YPD plates and grown for 3 days at the indicated temperatures.

(B) Wild-type, *hmo1Δ*, *top2-1*, and *top2-1 hmo1Δ* cells were released from G1 at 32°C. Samples were collected at the indicated time points for FACS analysis and TCA protein precipitation and immunodetection using Ser129 phosphorylated H2A (γH2A) and Rad53 antibodies.

(C) Wild-type, *hmo1Δ*, *top2-1*, and *top2-1 hmo1Δ* cells were released from G1 at 32°C. Samples were collected at the indicated time points and genomic DNA was extracted in agarose plugs. Chromosomes were separated by PFGE and analyzed by Southern blotting using *ARS305* probe. The positions of the well, intact (Ch III) and broken-fragment (DSBs) chromosome III signals are indicated.

the M-G1 transition but also for sister chromatid entangling during S phase.

Hmo1 Collaborates with Top2 in Controlling Chromosome Integrity

We speculate that an epigenetic mechanism dependent on the binding of Hmo1 and on Htz1 exclusion facilitates the establishment of high-order architectural structures in S phase with the help of Top2. This hypothesis is supported by observations indicating that HMGB1 stimulates the activity of TopoIIα in vitro (Stros et al., 2007a) and that Hmo1 itself has been involved in DNA looping (Kamau et al., 2004). Top2 recruitment also occurs in the absence of Hmo1. This observation does not rule out the possibility that Hmo1 contributes to create the proper chromatin context to facilitate Top2 action, perhaps by maintaining the intergenic regions at a low nucleosomal density (Merz et al., 2008). The relative positioning of Htz1 and Hmo1 in G1, which seem mutually exclusive, might also facilitate the selection of those chromosomal regions that undergo Top2-mediated topological transitions in S phase. While the iTop2 clusters are S phase specific, Hmo1 persists at the intergenic locations throughout the cell cycle perhaps reflecting the need to constantly keep the intergenic regions in a certain chromatin state. When Top2 activity is attenuated, Hmo1 accumulates at pericentromeric regions in G2/M. Attenuated Top2 activity causes DNA catenation that prevents efficient sister chromatid separation (DiNardo et al., 1984; Holm et al., 1989). The genomic locations causing catenation and the exact nature of the catenated structures are unknown. However, Top2 has been involved in cohesion at centromeric regions (Bachant et al., 2002) and centromeric regions have been proposed to undergo intrachromosomal looping (Warsi et al., 2008; Yeh et al., 2008). We speculate that in *top2* mutants type I topoisomerases act at pericentromeric regions and occasionally convert Top2 substrates into interlocked catenated structures (Bermejo et al., 2008) that are further stabilized by Hmo1. A key point is whether the intergenic locations that

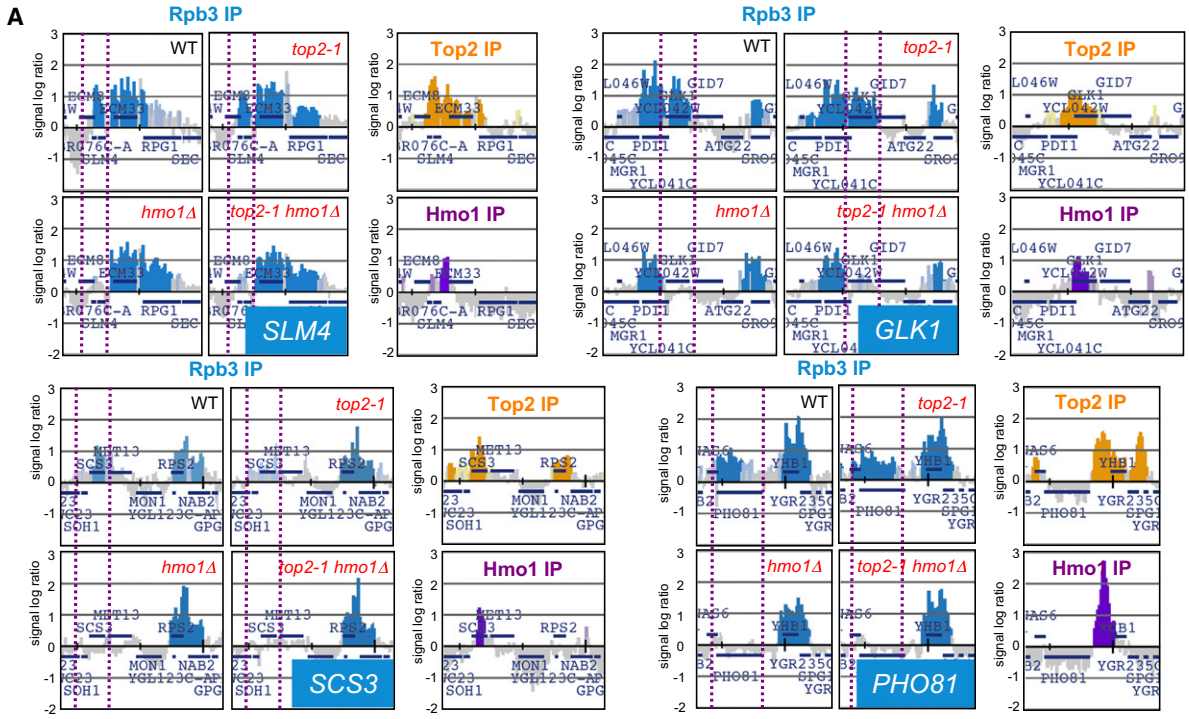


Figure 7. Hmo1 Influences Transcription and, Together with Top2, Coordinates the S Phase Architectural Chromosomal Context

(A) Hmo1 influences transcription of divergent genes. Rpb3-9×PK (CY8519), *top2-1*/Rpb3-9×PK (CY8641), *hmo1Δ* /Rpb3-9×PK (CY8640), and *top2-1 hmo1Δ*/Rpb3-9×PK (CY8642) cells were released from G1 at 37°C into 0.2 M HU. Samples were collected after 1 hr and processed for ChIP with antibodies specific to the PK epitopes. Blue (Rpb3-IP) histogram bars in the y axis show the average signal ratio of loci significantly enriched in the immunoprecipitated fraction along

recruit Hmo1 and Top2 physiologically undergo catenation. Our results show that indeed, in WT cells, replication forks at intergenic regions exhibit low abundant cruciform sister chromatid junctions resembling those hemicatenane-like structures forming in response to intra-S damage and accumulating in *sgs1* and *top3* mutants (Liberi et al., 2005; Mankouri and Hickson, 2006). Intriguingly, *HMO1* interacts with *SGS1* and *TOP3* (Berger et al., 2007; Gadal et al., 2002), and Hmo1 affects the metabolism of the hemicatenane-like structures accumulating at damaged forks (V.G.-H., M.F., and Dana Branzei, unpublished data). Hence one possibility is that Hmo1 somewhat positively influences the formation/accumulation of transient catenated molecules arising in S phase in WT cells at intergenic regions and that in *top2* mutants these structures pathologically accumulate also at pericentromeric regions. The hypothesis that the abnormal accumulation of catenated structures in *top2* mutants might be further stabilized/protected by Hmo1 could also explain the segregation defects of *top2* cells and the fact that Hmo1 is deleterious in this genetic background.

A Model for Hmo1- and Top2-Mediated Chromosome Architecture Integrating Replication, Transcription, and Sister Chromatid Cohesion

Based on our data and previous observations, we speculate that Hmo1 marks in G1 those nucleosome-free genomic regions that undergo S phase Top2-mediated looping. This would result in the topological insulation of transcription units in S phase (Blasquez et al., 1989; Zlatanova and van Holde, 1992). Top2 acting at the base of the DNA loops (Cockerill and Garrard, 1986; Earnshaw and Heck, 1985; Gasser et al., 1986) organized in a duplex juxtaposition state (resembling a precatenane crossing) might thus optimize transcription by creating the local conditions to concentrate transcription factors and facilitate polymerase recycling and transcription rates (Ansari and Hampsey, 2005; O'Sullivan et al., 2004; Schneider and Grosschedl, 2007). DNA looping might also assist transcription at specific intergenic locations experiencing divergent transcription. Indeed, Top2 and Hmo1 have a significant affinity for these sites (this work; Eivazova et al., 2009; Saitoh and Laemmli, 1994; Stros and Muselikova, 2000). Moreover, in S phase *hmo1Δ* mutants, 12% of the genes that are bound by Hmo1 in WT cells exhibited an almost complete depletion of RNA polymerase binding (e.g., *SLM4*, *GLK1*, *SCS3*, and *PHO81*; Figure 7A). Out of these transcriptional defective genes, 67% were associated to a divergent and actively transcribed open reading frame (ORF) (Figure 7A and data not shown). In the case of *top2* mutants, transcription at these sites is apparently normal (Figure 7A) although γH2A clusters accumulate. These observations may suggest that Top2 and Hmo1 modulate the S phase architectural chromosomal context at certain intergenic regions to protect genome integrity and in the meantime to facilitate transcription of those genes presenting a divergent arrangement.

Moving forks carry fork-associated Top1 and Top2. According to the current view, whereas Top2 might be implicated in precatenane resolution behind the forks (Bermejo et al., 2007; Lucas et al., 2001; Wang et al., 2008), Top1 might occasionally act to resolve supercoiling in front of the forks (Postow et al., 2001; Wang, 2002). It is unknown how forks can deal with DNA loops on the template. One possibility is that replication across DNA loops may generate interlocked sister chromatid junctions (Figure 7B). This hypothesis is consistent with the following observations: (1) hemicatenane-like structures can be visualized at replication forks (Lopes et al., 2003); (2) HMG proteins associate with hemicatenanes; (3) nucleosome-deprived DNA bridges can be visualized at anaphase in mammalian cells and associate with BLM (the human ortolog of Sgs1) and TopIIIα (Baumann et al., 2007; Chan et al., 2007); and (4) both *SGS1* and *TOP3* genetically interact with *HMO1* (Berger et al., 2007; Gadal et al., 2002).

The scenario described above should provide a framework for further discussion on epigenetic architecture imprinting, on replicon dynamics, and on the mechanisms connecting replication, transcription, and sister chromatid cohesion.

EXPERIMENTAL PROCEDURES

S. cerevisiae Strains

Yeast strains are listed in Table S1.

ChIP on Chip and Genome-wide Transcription Analyses

S. cerevisiae oligonucleotide microarrays were provided by Affymetrix. The Chip on chip analysis was carried out as described (Bermejo et al., 2007; Katou et al., 2006, 2003), employing anti-Flag monoclonal antibody M2 (Sigma-Aldrich), anti-PK SV5-Pk1 antibody (AbD Serotec), and anti-gH2A antibodies (a gift from Alain Verreault). For genome-wide transcription analysis, total RNA was isolated using the RNeasy Mini Kit (QIAGEN). Synthesis of cDNA, IVT amplification, and labeling were carried out following the Affymetrix GeneChip Expression Anaysis protocol. Labeled probes were hybridized to Affymetrix *S. cerevisiae* whole-genome tiling R and F arrays. Genomic profiles of all the proteins studied can be accessed from http://bio.ifom-ieo-campus.it/supplementary/Bermejo_et_al_CELL_2009.

Two-Dimensional Gel Analysis of Replication Intermediates

In vivo psoralen-crosslinking and 2D gel analysis were carried out as described (Gasser et al., 1996; Lopes et al., 2003).

the regions containing the indicated ORFs (marked by vertical dashed lines) in log2 scale. Maps of Top2 (orange) and Hmo1 (purple) S phase signal enrichment at these regions are shown.

(B) A model for Top2- and Hmo1-mediated topological transitions. We speculate that Top2 and Hmo1 orchestrate replication and transcription through the formation of architectural domains. Top1 acts on positive supercoiling generated ahead of replication forks by DNA unwinding (Wang, 2002). Top2 might mediate DNA looping at transcribed regions (Blasquez et al., 1989; Zlatanova and van Holde, 1992) (I). When forks approach the loop, single-strand DNA stretches (Sogo et al., 2002) can be juxtaposed to precatenane-like crossings generating triplex structures at the base of the loop (II). Top2 might then be dislodged and Top1 could catalize single-strand passages at the triplex junction (Zechiedrich and Osheroff, 1990) thus forming intrachromosomal catenation (III). Catenated structures could be then stabilized by Hmo1 (Bianchi et al., 1989) (IV) and converted into sister chromatid bridges after replication fork passage (V). In a Top2-defective context Top1 could generate more substrates for Hmo1, thus causing massive DNA entangling. DNA breaks might then arise when chromosome segregation takes place.

FACS and Western Blot Analysis

FACS analysis, protein extract, SDS/PAGE electrophoresis, and western blotting were performed as described (Pellicioli et al., 1999). Anti-Rad53 EL7 antibodies have been described (Bermejo et al., 2007). Phospho-S129 Histone H2A polyclonal antibodies (Abcam) were used to score γH2A phosphorylation.

Pulse-Field Gel Electrophoresis

DNA plugs were prepared as described (Lengronne et al., 2001). Yeast chromosomes were separated by PFGE (Gene Navigator System, Amersham) and electrophoresis was performed for 15 hr at 200V with 60 s pulses, followed by 9 hr with 90 s pulses, in TBE 0.5× at 14°C.

Statistical Methods

Evaluation of the significance of protein cluster distributions within the different genomic areas and protein-binding correlations was performed by confrontation to the model of the null hypothesis distribution generated by a Montecarlo-like simulation. The significance of correlation of binding at common promoters between different experimental targets was scored using a one-tailed Fisher's exact test (see Supplemental Statistical Analysis for further details).

ACCESSION NUMBERS

Microarray data can be obtained from the Gene Expression Omnibus with accession number GSE16258.

SUPPLEMENTAL DATA

Supplemental Data include one table, seven figures, and a Supplemental Statistical Analysis document and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00722-3.

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