Molecular Biology of the Cell
Vol. 19, 4310–4318, October 2008

The THP1-SAC3-SUS1-CDC31 Complex Works in Transcription Elongation-mRNA Export Preventing RNA-mediated Genome Instability

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Submitted April 7, 2008; Revised July 18, 2008; Accepted July 22, 2008
Monitoring Editor: Marvin P. Wickens

The eukaryotic THO/TREX complex, involved in mRNP biogenesis, plays a key role in the maintenance of genome integrity in yeast. mRNA export factors such as Thp1-Sac3 also affect genome integrity, but their mutations have other phenotypes different from those of THO/TREX. Sus1 is a novel component of SAGA transcription factor that also associates with Thp1-Sac3, but little is known about its effect on genome instability and transcription. Here we show that Thp1, Sac3, and Sus1 form a functional unit with a role in mRNP biogenesis and maintenance of genome integrity that is independent of SAGA. Importantly, the effects of ribozyme-containing transcription units, RNase H, and the action of human activation-induced cytidine deaminase on transcription and genome instability are consistent with the possibility that R-loops are formed in Thp1-Sac3-Sus1-Cdc31 as in THO mutants. Our data reveal that Thp1-Sac3-Sus1-Cdc31, together with THO/TREX, define a specific pathway connecting transcription elongation with export via an RNA-dependent dynamic process that provides a feedback mechanism for the control of transcription and the preservation of genetic integrity of transcribed DNA regions.

INTRODUCTION

The nascent mRNA is cotranscriptionally coated with proteins that assure RNA integrity, nuclear export, and downstream cytoplasmic steps. Formation of a mature ribonucleoprotein particle (mRNP) competent for export requires the correct coupling of transcription with mRNA processing steps such as 5′-end capping, splicing, 3′-end cleavage and polyadenylation (Aguilera, 2005; Buratowski, 2005; Cole and Scarcelli, 2006). Incorrectly spliced or 3′-end processed transcripts are retained within the nucleus, providing evidence that mRNA maturation and export are linked to each other (Lei and Silver, 2002). Any failure in the formation of an export-proficient mRNP would cause nuclear RNA retention and could trigger nuclear mRNA decay in association with the nuclear pore complex (NPC; Galy *et al.*, 2004) and with transcription (Andrulis *et al.*, 2002). Importantly, recent reports in yeast, *Drosophila* and human lymphocytes have revealed that dynamically regulated genes are recruited to the nuclear periphery when transcription is activated (Casolari *et al.*, 2004; Faria *et al.*, 2006; Mendjan *et al.*, 2006; Ragoczy *et al.*, 2006; Taddei *et al.*, 2006). It has been suggested that the activation of genes in the nucleus is linked to specialized NPCs that in turn facilitate efficient mRNA export (Blobel, 1985). Supporting this hypothesis, gene positioning at the proximity of the NPC has been shown to be dependent on factors involved in transcription initiation and mRNA export (Cabal *et al.*, 2006; Dieppois *et al.*, 2006; Kurshakova *et al.*, 2007; Chekanova *et al.*, 2008; Köhler *et al.*, 2008).

mRNP formation seems to play a key role not only in gene expression but also in other cellular processes such as the maintenance of genome integrity. An example of this connection between mRNP formation and genetic integrity is provided by the THO complex of *Saccharomyces cerevisiae*, a conserved four-protein complex composed of stoichiometric amounts of Tho2, Hpr1, Mft1, and Thp2 (Chavez *et al.*, 2000), which is recruited to active chromatin in vivo (Strasser *et al.*, 2002; Zenklusen *et al.*, 2002). Null mutations of any component of THO lead to similar phenotypes of transcription impairment and RNA export defects (Chavez *et al.*, 2000; Strasser *et al.*, 2002), the most intriguing phenotype being their transcription-associated hyper-recombination. Analysis of yeast THO mutants has led to the idea that transcription-associated recombination (TAR) may be a consequence of transcriptional-elongation impairment (Aguilera and Gomez-Gonzalez, 2008). One major cause of this phenomenon is the cotranscriptional formation of R-loops (DNA-RNA hybrids) formed behind the elongating RNAPII (Huertas and Aguilera, 2003). In the current view, the THO complex would participate in cotranscriptional formation of export-competent mRNPs during transcription elongation preventing R-loop formation. The observation that depletion of the *ASF/SF2* splicing factor in chicken DT40 cells and human HeLa cells also lead to genomic instability linked to R-loop formation indicates that a number of mRNA-processing enzymes may contribute to prevent the formation RNA-depen-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–04–0355) on July 30, 2008.

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dent structures that may trigger genome instability (Li and Manley, 2005).

THO forms, together with the RNA export proteins Sub2/UAP56 and Yra1/Aly, a larger and conserved complex termed TREX (Strasser *et al.,* 2002; Rehwinkel *et al.,* 2004). Interestingly, yeast mutants of *SUB2* and *YRA1* are synthetic-lethal with THO mutations and also lead to hyper-recombination and gene expression defects (Fan *et al.,* 2001; Jimeno *et al.,* 2002; Strasser *et al.,* 2002). Furthermore, mutations in the genes of the Mex67-Mtr2 export factor, the Nab2 hnRNP or the NPC-associated Thp1 and Sac3 proteins also confer hyper-recombination and gene expression defects (Gallardo and Aguilera, 2001; Jimeno *et al.,* 2002; Gallardo *et al.,* 2003), even though this is not a general feature of mRNA-processing mutations (Luna *et al.,* 2005). Despite some similarities there are important differences between THO and Thp1 and Sac3. Thus, Sub2 overexpression suppresses THO mutants, but it inhibits growth of the Thp1 mutant. Also, Nab2 overexpression suppresses the Thp1 mutant but has no effect on THO mutants (Jimeno *et al.,* 2002; Gallardo *et al.,* 2003). Notably, in contrast to THO, Thp1 and Sac3 associate with nucleoporins at the nuclear basket and mediate export of mRNPs (Fischer *et al.,* 2002; Lei *et al.,* 2003). In addition, Thp1 and Sac3 are found in association with Cdc31 centrin (Gallardo *et al.,* 2003; Fischer *et al.,* 2004), which functions in the duplication of microtubule-organizing centers, and with Sus1, a small protein conserved from yeast to humans recently identified as a novel component of SAGA histone-modification complex involved in transcription initiation (Rodriguez-Navarro *et al.,* 2004; Zhao *et al.,* 2008). The observation that Sus1 is involved in the SAGA-dependent histone H2B deubiquitylation and maintenance of normal H3 methylation levels (Köhler *et al.,* 2006) and that Thp1, Sac3, Sus1, and Ada2, a bona fide component of SAGA, act in the repositioning and dynamic motility of SAGA-dependent loci, to the nuclear periphery upon transcriptional activation (Cabal *et al.,* 2006; Kurshakova *et al.,* 2007; Chekanova *et al.,* 2008) suggests the possibility that Sus1 could be a bridge protein between transcription, via SAGA, and mRNA export.

Given all these observations, the question emerging is how RNA export factors, such as Thp1 and Sac3 control genome integrity and whether they are functionally related to THO/TREX, which is physically bound to active chromatin and does not seem to be located at the nuclear periphery. Another emerging question is whether or not the main function of Sus1 is linked to transcription initiation as part of the SAGA complex. Here we show that Thp1, Sac3, and Sus1 form a functional unit with a role in transcription elongation that is independent of SAGA and is linked to RNA export. Our data reveal that the Thp1-Sac3-Sus1-Cdc31 (THSC) complex, together with THO/TREX, define a specific pathway connecting transcription elongation with nuclear export by an RNA-mediated dynamic process. This provides a feedback mechanism for the control of transcription that guarantees genetic stability of highly transcribed DNA regions.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains used are listed in Supplemental Information Table S1 . Plasmids pRS316L, pRS316LYΔNS (Prado *et al.,* 1997), pRS314L-*lacZ*, pRS314GL-*lacZ* (Piruat and Aguilera, 1998), pCM184-LAUR (Jimeno *et al.,* 2002), pCM189-LEU2 (Gonzalez-Barrera *et al.,* 2002), pGCYC1-402 (Rondon *et al.,* 2003b), pGL-*rib*^{m}, pGL-*Rib*$^{+}$, pPHO5-rib^{m}-lacZ, pPHO5-Rib^{+}-lacZ, pGAL:RNH1 (Huertas and Aguilera, 2003) p416-GAL1 (Mumberg *et al.,* 1994), and p413GALAID (Gomez-Gonzalez and Aguilera, 2007) were described previously. Plasmid Ptet-SUB2 containing the complete *SUB2* coding sequence under control of the *tetO* promoter was constructed by inserting the 1.3-kb BamHI *SUB2* fragment obtained by PCR using the primers 5′-ATC GCG GAT CCA TGT CAC ACG AAG GTG AA-3′ and 5′-CGC GCG GAT CCT TAA TTA TTC AAA TAA GT-3′ into pCM189 (Gari *et al.,* 1997).

Chromatin Immunoprecipitation

For chromatin immunoprecipitation (ChIP) experiments, strains were grown in synthetic complete medium (SC) 2% glycerol-2% lactate to an OD_{660} of 0.5. The culture was split in two, and one-half was supplemented with 2% glucose (repressed transcription) and the other with 2% galactose (activated transcription). Samples were taken after 4 h, and ChIP assays were performed as described (Hecht and Grunstein, 1999). Monoclonal anti-Rpb1-CTD antibody 8WG16 (Berkeley Antibody Company, Richmond, CA) and protein A-Sepharose were used for RNAPII immunoprecipitation. The GFX purification system (Amersham, Indianapolis, IN) was used for the last DNA purification step. We used the PCR of the intergenic region at positions 9716–9863 of chromosome V as a negative control. Real-time quantitative PCR and calculations of the relative abundance of each DNA fragment were performed as described (Huertas *et al.,* 2006).

In Vitro Transcription Elongation Assays

Transcription elongation was assayed in yeast whole cell extracts (WCEs) in vitro. WCEs were prepared from yeast cells grown in rich medium YEPD at 30°C to an OD_{600} of 1, and the reactions were carried as described previously (Rondon *et al.,* 2003b).

Recombination and Mutation Analysis

Recombination and mutation frequencies of the monocopy centromeric plasmids pRS316L, pRS316LYΔNS, pRS314GL-*lacZ*, pRS314L-*lacZ*, pGL-rib^{m}, and pGL-Rib^{+} described earlier were obtained as the average of three to four median frequencies from two different transformants each and for each genotype tested. Median frequencies were obtained as previously described (Santos-Rosa and Aguilera, 1994) from six independent colonies per transformant.

Miscellaneous

Northern analyses were performed according to standard procedures with ^{32}P-radiolabeled probes. Probes used were described previously (Chavez *et al.,* 2000). RNA analyses for the Rib^{+} and rib^{m} constructs were performed as described in Huertas and Aguilera (2003).

RESULTS

Overexpression of SUB2 Inhibits Growth of Mutants of Thp1, Sac3, and Sus1, and Other mRNP Factors But Not of SAGA Mutants

We have previously reported that overexpression of Sub2, a component of TREX, strongly inhibited growth of *thp1*Δ cells (Gallardo *et al.,* 2003). Here we used this feature to obtain new insights into the functional relationship of Thp1 and Sac3 and other proteins involved in mRNP biogenesis. We placed the *SUB2* gene in a centromeric plasmid under the control of the Tet promoter, which allowed the expression of the gene in the absence of doxycycline (Ptet-SUB2), and transformed different mutants of mRNP biogenesis factors (Figure 1A). Figure 1B shows that growth of mutants of the THSC complex as *sac3*Δ and *sus1*Δ were affected when *SUB2* was overexpressed. The results suggest that Thp1, Sac3, and Sus1 act as a unit that functionally interacts with Sub2. We also analyzed two *cdc31* mutants, (*cdc31-1* and *cdc31-115*); *cdc31-1* is defective in spindle pole body (SPB) duplication, cell integrity and morphogenesis (Sullivan *et al.,* 1998), whereas *cdc31-115* is not affected in SPB duplication (Ivanovska and Rose 2001) but has an mRNA export defect (Fischer *et al.,* 2004). Overexpression of Sub2 did not have any effect on neither of these mutants.

Next, we analyzed the effect of *SUB2* overexpression on 22 mutants in genes encoding proteins with a function in nuclear 5′-end cap binding, transcription termination, 3′-end cleavage and polyA+ tail addition, mRNA degradation, mRNA export, and genes encoding mRNA-associated proteins involved in other nuclear processes. As can be seen in

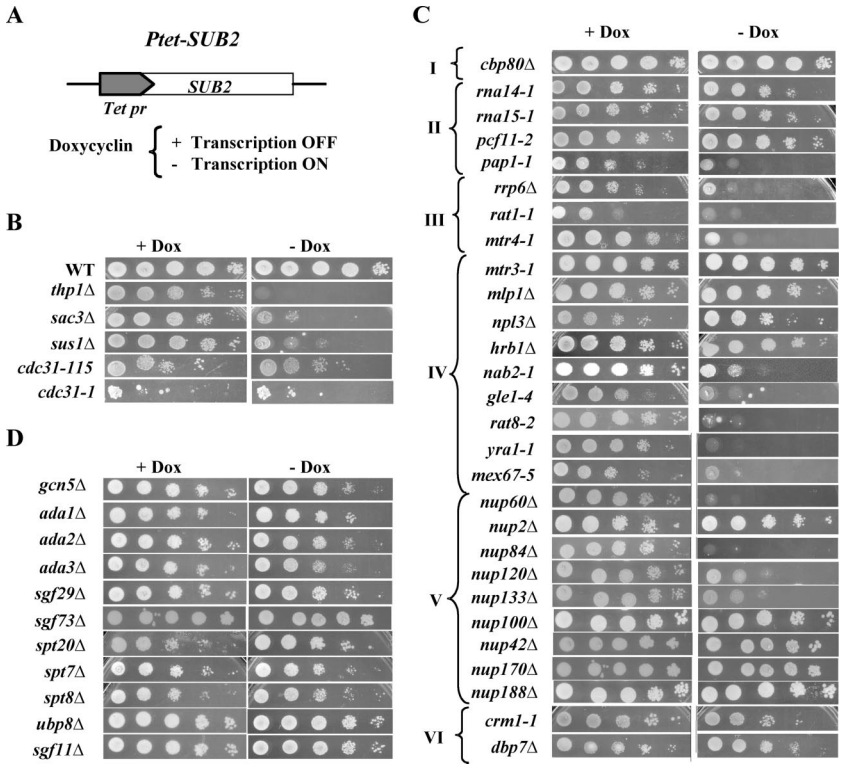

Figure 1. Effect of the overexpression of *SUB2* on mutants affected in different steps of mRNP biogenesis and export. (A) Scheme of the plasmid *Ptet-SUB2*, containing the *SUB2* gene under the *Tet* promoter, which is expressed in the absence of doxycycline. (B) Growth of wild-type W303–1A (WT), WFBE046 (*thp1*Δ), Y03517 (*sac3*Δ), Y17455 (*sus1*Δ), and *cdc31-115* and *cdc31-1* strains transformed with the plasmid *Ptet-SUB2*. Transformants were spotted as 10-fold serial dilutions on selective medium with and without doxycycline (5 μg/ml). (C) Effect of the overexpression of *SUB2* in mutants affected in RNA metabolism. I, nuclear cap binding complex; II, 3′-end processing and termination; III, nuclear mRNA degradation; IV, mRNA export and RNA processing steps; V, nucleoporins; and VI, protein nuclear export and others. (D) Effect of the overexpression of *SUB2* in SAGA mutants. Photographs were taken after 3 d at 30°C, except for the *nab2–1* mutant, which grows slowly and needed 5 d to form colonies without doxycycline.

Figure 1C overexpression of SUB2 inhibited growth not only of Thp1, Sac3, and Sus1 mutants, but also of mutants of genes involved in other steps of mRNP biogenesis: polyadenylation (*pap1-1*), mRNA stability (*rrp6*Δ, *rat1-1*, and *mtr4-1*), and mRNA export (*mex67-5*, *yra1-1*, *nab2-1*, *gle1-4*, and *rat8-2*). Nevertheless, it had no effect in mutants of other nuclear processes, such as protein transport (crm1-1) and the processing of other RNA species (*dbp7*Δ) and in mutants in genes encoding mRNA binding proteins (*npl3*Δ). Sub2 overexpression inhibited growth of a number of mutants including those in nucleoporins that interact with Thp1 and Sac3 (*nup60*Δ) and in nucleoporins of the Nup84 complex (*nup84*Δ, *nup133*Δ, and *nup120*Δ), but not of other nucleoporins (*nup2*Δ, *nup100*Δ, *nup188*Δ, and *nup170*Δ; Figure 1C). In addition, Sub2 overexpression inhibits growth of a mutant of Nab2, an hnRNP (heterogeneous nuclear ribonucleoprotein) that interacts genetically with Thp1 (Gallardo *et al.*, 2003; Figure 1C). Therefore, our results suggest that the growth-defect phenotype caused by overexpression of *SUB2* is specific to a subset of mRNP biogenesis and export factors that could define a particular pathway.

Because Sus1 was identified as part of the SAGA histone acetylase complex (Rodriguez-Navarro *et al.*, 2004) and Sub2 overexpression inhibited growth of *sus1*Δ mutants, we asked whether Sub2 overexpression also affected SAGA mutants. Notably, we did not observe growth inhibition in mutants of different representative genes of the functional and structural modules of SAGA (Figure 1D). This suggests that despite the association of Sus1 with SAGA, a function of Sus1 is directly related to Thp1 and Sac3 in mRNP biogenesis and export rather than SAGA.

*sus1*Δ *But Not SAGA Mutants Confers Transcription-dependent Hyper-Recombination*

As *SUS1* and *CDC31* encode proteins that have been shown to copurify with Thp1 and Sac3 (Fischer *et al.*, 2002; Gallardo *et al.*, 2003; Rodriguez-Navarro *et al.*, 2004), we wondered whether their mutations also lead to increased TAR. Representative mutants of the different modules of SAGA were included in the study. For the analysis of transcription-dependent recombination, we used the plasmid-based system LYΔNS based on 0.6-kb *leu2* repeats in which transcription has to proceed through a long and GC-rich intervening sequence. In this system *thp1*Δ and *sac3*Δ lead to an increase in recombination of two- to three orders of magnitude above wild-type levels (Gallardo and Aguilera, 2001; Gallardo *et al.*, 2003). As control, we used the L system, identical to LYΔNS but without intervening sequences between the *leu2* repeats and which is not significantly affected by *thp1*Δ. Here we show that whereas *sus1*Δ mutant showed a clear increase in recombination in LYΔNS (5.5-fold) but no effect in the L system, *ubp8*Δ, *sgf11*Δ, *spt7*Δ, *spt8*Δ, *gcn5*Δ, and *spt20*Δ mutants showed low recombination levels in both the L and LYΔNS systems (Figure 2A). Besides, none of the Cdc31 centrin mutants analyzed (*cdc31-1* and *cdc31-115*) showed hyper-recombination, neither at 30°C (Figure 2A) nor at restrictive temperature 34°C (data not shown). Consistent with Sub2 overexpression data (Figure 1), these results suggest that Sus1 share functions with Thp1 and Sac3 in the maintenance of genetic integrity. Although we found no hyper-recombination in the *cdc31* mutants tested, whether or not Cdc31 has a related or more distant role to the other subunits of the THSC complex would need to be addressed with specifically selected *cdc31* alleles, given that Cdc31 is the only essential subunit of this complex.

Altogether, the data suggest that the hyper-recombination phenotype of *sus1*Δ is transcription-dependent. To demonstrate this, we determined the effect of *sus1*Δ on recombination in the L-*lacZ* and GL-*lacZ* systems carrying 0.6-kb *leu2* direct repeats flanking the *lacZ* open reading frame under conditions of low (*GAL1* promoter in 2% glucose), medium (*LEU2* promoter), and high levels of transcription (*GAL1*

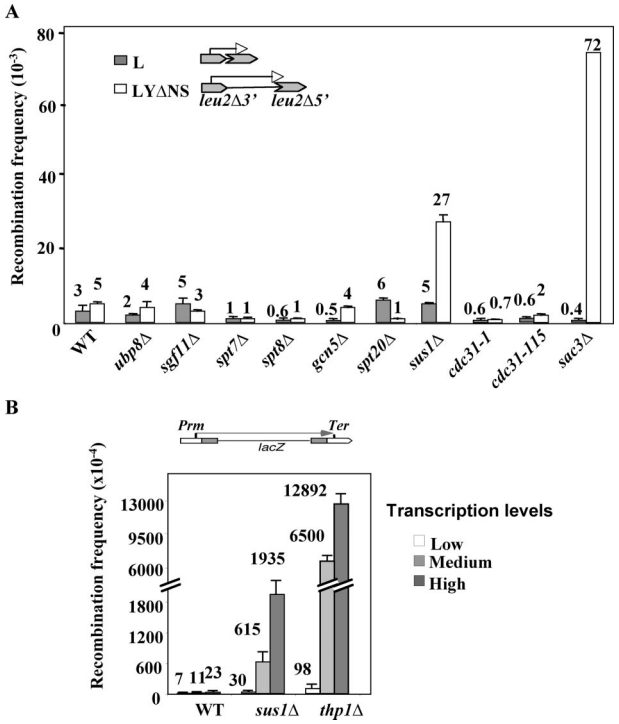

Figure 2. Recombination analysis of *sus1* and SAGA mutants. (A) Recombination was analyzed in the plasmidic recombination systems L and LYΔNS. A small diagram of each system used (not drawn to scale) is shown. Repeats are shown as gray boxes, and gray arrows indicate relevant transcripts produced by the constructs. Recombinants were selected as Leu^+. All mutants were analyzed together with their isogenic WT strains. Median and SD are shown. (B) Recombination analysis of *sus1*Δ *and thp1*Δ mutants. The frequency of Leu^+ recombination was determined in the plasmid-borne systems L-*lacZ* and GL-*lacZ*. Recombination frequencies are plotted as a function of the transcription levels. Low transcription refers to the GL-*lacZ* systems in strains cultured in 2% glucose; medium refers to L-*lacZ* in 2% glucose, and high to GL-*lacZ* in 2% galactose (for more details, see Supplemental Figure S1).

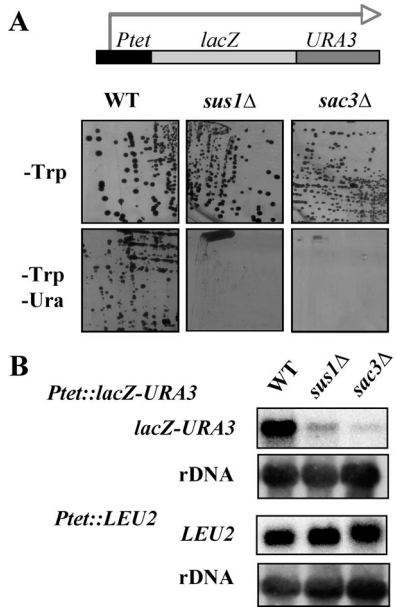

Figure 3. Transcription analysis of *sus1*Δ strain. (A) Analysis of the capacity of BYSU-3B (*sus1*Δ) and TY13517–1D (*sac3*Δ) strains carrying the *Ptet::lacZ-URA3* fusion construct (plasmid pCM184–LAUR) to form colonies on SC-Trp-Ura after 3 d at 30°C. (B) Northern analysis of the expression of the *Ptet::lacZ-URA3* and *Ptet-LEU2* fusion constructs (plasmid pCM189-LEU2). RNA was isolated from midlog phase cultures grown in SC-Trp. As a ^{32}P-labeled DNA probe, we used the 3-kb BamHI *lacZ* fragment, the ClaI-EcoRV *LEU2* internal fragment, and an internal 589-base pair 25S rDNA fragment obtained by PCR.

promoter in 2% galactose). As can be seen in Figure 2B and Supplemental Figure S1, the higher the levels of transcription the stronger the increase in recombination. The results demonstrate that hyper-recombination in *sus1*Δ is mainly transcription dependent as has been described for *thp1* and *sac3* mutants.

Transcription Elongation Is Impaired in THSC Mutants In Vivo, But Only Slightly In Vitro

We have previously reported that *thp1*Δ and *sac3*Δ mutants are defective in transcription through high G+C content genes like *lacZ* (Gallardo *et al.*, 2003). To test whether this is also the case of *sus1*Δ, we analyzed gene expression in the LAUR expression system (Jimeno *et al.*, 2002) containing a 4.15-kb *lacZ-URA3* translational fusion under the control of the *Tet* promoter. As can be seen in Figure 3A, *sus1*Δ and *sac3*Δ cells, carrying the LAUR system, were unable to form colonies on synthetic complete medium lacking uracil (SC-Ura-Trp), indicating that they did not express the *lacZ-URA3* fusion. Consistently, they did not produce β-galactosidase activity (data not shown). Northern analysis shows that whereas wild-type cells could express this construct properly, *sus1*Δ and *sac3*Δ mutants showed a reduction in mRNA accumulation (Figure 3B), similar to that observed for *thp1*Δ (Gallardo *et al.*, 2003). Such a reduction was not caused by an impairment of transcription initiation at the *Tet* promoter because mRNA accumulation of the *LEU2* gene under the *Tet* promoter (pCM189-LEU2 expression system) was the same in *sus1*Δ and *sac3*Δ as in the wild type (Figure 3B). We conclude, therefore, that *sus1*Δ lead to similar gene-expression defects as those of *thp1*Δ and *sac3*Δ mutants.

The impairment of *lacZ* expression and the transcription-associated recombination phenotypes of *sus1*Δ, *thp1*Δ, and *sac3*Δ suggest that transcription elongation may be impaired, as was previously shown for THO mutants (Rondon *et al.*, 2003b). To determine whether THSC mutants were impaired in transcription elongation we used our previously reported in vitro system containing two G-less cassettes (Rondon *et al.*, 2003a), in which transcription-elongation efficiency is determined in whole cell extracts (WCEs) by the values of the ratio of accumulation of the downstream (376-nt long) versus the upstream (84-nt long) G-less RNA fragments (Figure 4; see *Materials and Methods*). We assayed in vitro transcription elongation in *thp1*Δ, *sac3*Δ, and *sus1*Δ. In addition, we included in our analysis two mutants of SAGA: *ada2*Δ, impaired in histone acetylation, and *ubp8*Δ, mutated in the Ubp8-Sgf11 deubiquitinylating enzyme, shown to control binding of Sus1 to SAGA (Köhler *et al.*, 2006), because recently both have been reported to be linked to transcription elongation (Govind *et al.*, 2007; Wyce *et al.*, 2007). As can be seen in Figure 4, the transcription-elongation efficiencies of *thp1*Δ, *sac3*Δ, *sus1*Δ, *ada2*Δ, and *ubp8*Δ were above 80% of the wild-type values, in some cases close to wild-type levels, whereas in the THO mutants used as controls (*tho2*Δ and *hpr1*Δ) efficiencies were 60% or lower. As transcription is coupled with mRNA export and THSC is

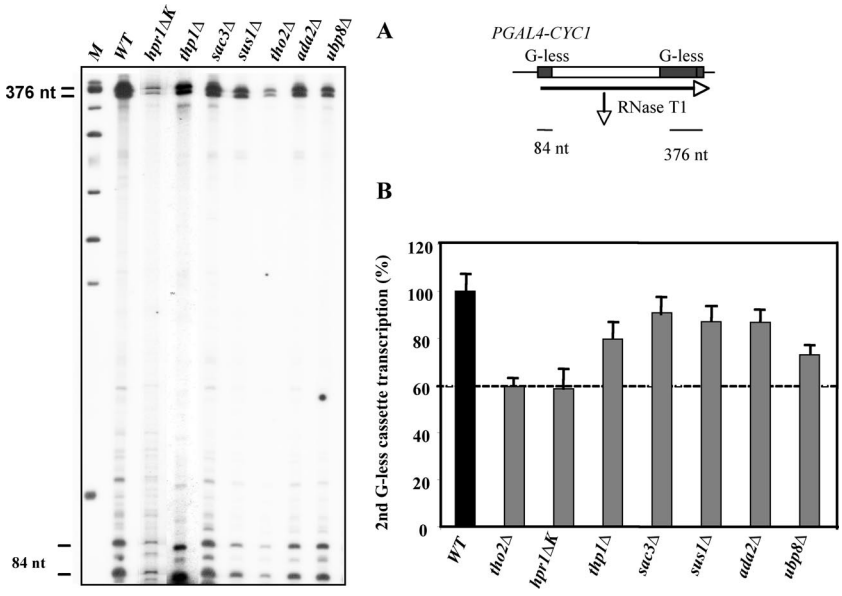

Figure 4. In vitro transcription elongation of THSC and SAGA mutants. (A) Scheme of the two G-less cassette system of plasmid pGCYC1-402 used for the analysis of in vitro transcription elongation. RNase T1 treatment of the mRNA driven from the *GAL4-CYC1* promoter, which is activated by purified *Gal4-VP16*, renders two fragments corresponding to the G-less cassettes. (B) In vitro transcription assays of WCEs from BY4741 (WT), Y02937 (*tho2*Δ), SChY58a (*hpr1ΔK),* Y01764 (*thp1*Δ), Y03517 (*sac3*Δ), Y17455 (*sus1*Δ), Y04282 (*ada2*Δ), and Y00809 (*ubp8*Δ) strains. M is the marker. Each reaction was stopped after 30 min, treated with RNaseT1, and run in a 6% PAGE. Transcription reactions were made at 23°C with WCEs obtained from cells grown at 30°C. Two bands from each G-less cassette were obtained, probably due to incomplete action of RNaseT1. Efficiency of transcription elongation was determined as the percentage of total transcripts that reached the 376-nt G-less cassette with respect to the transcripts that covered the 84-nt cassette. Radioactivity incorporated into the G-less cassettes was quantified in a Fuji FLA3000 (Tokyo, Japan) and normalized wi'th respect to the C content of each G-less cassette. The mean and SD of three independent experiments are shown.

located at the nuclear periphery in association with the NPC, it is possible that the effect of the THSC complex on transcription is relevant when coupled to the NPC, and not in cell extracts in which coupling is disrupted.

Next, we analyzed RNAPII elongation in vivo. RNAPII recruitment was assayed by ChIP at the 8-kb long *YLR454w* gene fused to the *GAL1* promoter (Mason and Struhl, 2005). RNAPII occupancy was determined at a 5′ and a 3′ region of *YLR454w* in *thp1*Δ *and sac3*Δ mutants, using *hpr1*Δ as a control. Figure 5 shows that the presence of RNAPII at the 3′-end of the gene was reduced with respect to the 5′-end to 51 and 73% in *thp1*Δ and *sac3*Δ mutants, respectively. These values were similar to those of *hpr1*Δ, indicating that the RNAPII elongation is decreased in THSC mutants in vivo.

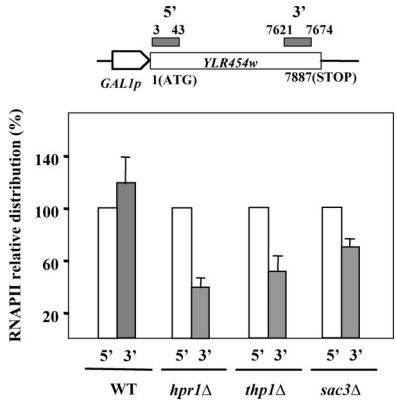

Figure 5. RNAPII occupancy at the *GAL1-YLR454w* gene in THSC mutants. ChIP analyses in wild-type, *hpr1*Δ, *thp1*Δ, and *sac3*Δ strains carrying the GAL1p::*YLR454w* fusion construct located at the endogenous *YLR454w* chromosomal locus. Strains used were WT-YLR454 (WT), WHYL.2A (*hpr1*Δ), WThpYL-1D (*thp1*Δ), and BSAC-4A (*sac3*Δ). The scheme of the gene and the PCR-amplified fragments are shown. The DNA ratios in regions 5′ and 3′ were calculated from the amounts of regions 5′ and 3′ relative to the amounts of the intergenic region. The recruitment data shown are referred to the value of the 5′ region taken as 100%. ChIPs were performed from three independent cultures, and quantitative PCRs were repeated three times for each culture. Error bars, SDs.

The Transcription and Hyper-Recombination Phenotypes of THO/TREX and THSC Mutants Are Mediated by the Nascent mRNA

The transcription impairment and hyper-recombination phenotypes of THO mutants have been shown to be dependent on the nascent mRNA (Huertas and Aguilera, 2003). To determine whether this was also the case for THSC and also Sub2 mutants, this last component of the TREX complex, we analyzed transcription in the previously described Rib$^+$ and rib^m constructs (Figure 6A, Supplemental Figure S2), in which a *PHO5-Rib-lacZ* transcriptional fusion containing either a wild-type (Rib$^+$) or a mutated (rib^m) hammerhead ribozyme sequence was placed under the control of the *GAL1* promoter (Huertas and Aguilera, 2003). In both constructs, a 2.2-kb-long mRNA is synthesized, but in the Rib$^+$ construct the active hammerhead ribozyme cleaves the transcript, leading only to a short 0.6-kb-long mRNA fragment downstream of the ribozyme. We analyzed transcription in *thp1*Δ, used as representative mutant of the THSC complex, and in *sub2*Δ, *hpr1*Δ, and *tho2*Δ as representative mutants of THO/TREX. Northern analyses revealed that the *thp1*Δ mutant was suppressed in the Rib$^+$ construct, as it was the case for *hpr1*Δ, *tho2*Δ, and *sub2*Δ mutants. In such mutants only 30–40% of the transcription efficiency of the wild type can be observed because this is the maximum level of transcription reached with these types of constructs (Garcia-Rubio *et al.*, 2008).

Next, we asked whether ribozyme cleavage, together with RNase H overexpression, was also capable of suppressing hyper-recombination of THSC and other mRNP biogenesis mutants. Hyper-recombination was assayed with the GL-*Rib*$^+$ and GL-*rib*^{m} repeats systems containing *PHO5*, followed by active ribozyme Rib$^+$ or inactive ribozyme rib^m sequences between 0.6-kb-long *leu2* direct repeats, respectively (Figure 6B). We have previously reported that the major suppression of hyper-recombination of the *hpr1*Δ strains was obtained when the ribozyme was active (GL-*Rib*$^+$) in the presence of highly expressed RNAse H that would remove the RNA chain of a putatively formed R-loop (Huertas and Aguilera, 2003). Here we show that *hpr1*Δ, *tho2*Δ, *sub2*Δ, and *thp1*Δ strains carrying the GL-*Rib*$^+$ con-

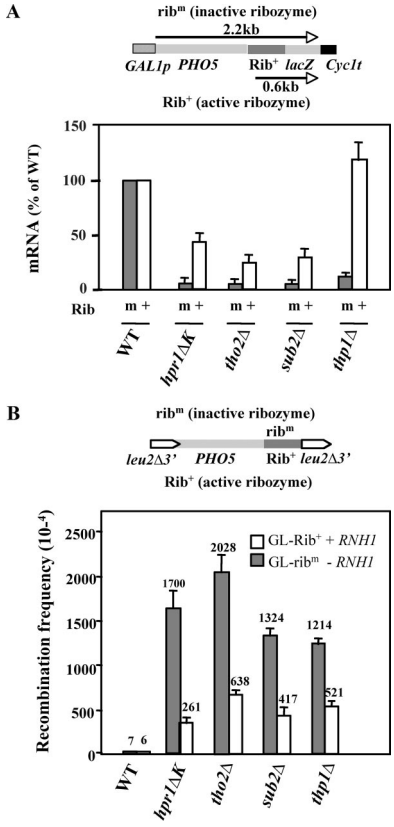

Figure 6. Nascent mRNA dependency of the transcription defect and hyper-recombination of mRNP biogenesis and export mutants. (A) Steady-state analyses of transcription of the *rib*^{m} and *Rib*$^+$ fusions in W303–1A (WT), SChY58a (*hpr1*Δ*K*), RK2–6C (*tho2*Δ), DLY23 (*sub2*Δ), and WFBE046 (*thp1*Δ) strains. The *PHO5-rib*^{m}*-lacZ* (*rib*^{m}) and *PHO5-Rib*$^+$*-lacZ* (*Rib*$^+$) transcriptional fusions were under the *GAL1* promoter. They contain an inactive or active (respectively) synthetically made 52-base pair ribozyme (*Rib*), followed by a 266-base pair fragment of the U3 gene to prevent the cleaved RNA from degradation, and the 369-base pairs PvuII 3′-end *lacZ* fragment at the UTR of *PHO5* (position +1405). Samples where taken after galactose addition at 0 or 120 min. One representative experiment is shown in Supplemental Figure S2. Results from 120-min samples were quantified and normalized with the endogenous U3 signal and are represented as the percentage value with respect to wild type taken as 100%. The average of three different experiments is plotted. (B) Recombination frequencies in W303–1A (WT), SChY58a (*hpr1*Δ*K*), RK2–6C (*tho2*Δ), DLY23 (*sub2*Δ), and WFBE046 (*thp1*Δ) cells containing the recombination systems GL-*Rib*$^+$ (□) and GL-*rib*^{m} (▨) are shown. All experiments were performed in 2% galactose to allow expression of the direct repeats. Lack (−*RNH1*) or overexpression (+*RNH1*) of RNase H1 was achieved with either p416-GAL1 or the multicopy plasmid pGAL-RNH1 carrying *RNH1* under the *GAL1* promoter, respectively. Recombination frequencies are the median value of six independent cultures. The average median value of 2–4 experiments and SD are shown.

struct and overexpressing RNaseH1 had significantly reduced recombination frequencies compared with those of GL-*rib*^{m}, as was previously shown for *hpr1*Δ mutants (Huertas and Aguilera, 2003). Altogether the results suggest that not only in THO complex, as shown with *hpr1* and *tho2* mutants, but also in TREX, as shown with *sub2*, and THSC, as shown with *thp1*, both the transcription impairment and hyper-recombination are dependent on the nascent mRNA.

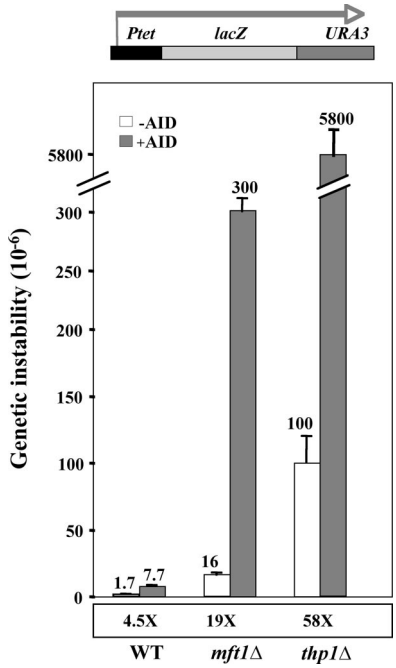

Figure 7. Spontaneous and AID-induced mutation frequencies in wild-type, *mft1*Δ, and *thp1*Δ mutants. Analyses of the genetic instability (mutation and recombination) phenotype in W303–1A (WT), WHMK-1A (*mft1*Δ), and WFBE046 (*thp1*Δ) strains, using the *Ptet*::*lacZ-URA3* (pCM184-LAUR) fusion construct. Ura$^-$ mutants are selected in synthetic medium (SC) with FOA. The human *AID* gene, present in p413GALAID, was overexpressed in 2% galactose medium. Median value of genetic instability frequency and SD of 3–4 different fluctuation tests are shown.

THSC Inactivation Strongly Enhances the Mutator Ability of Human Activation-induced Cytidine Deaminase Protein

Activation-induced cytidine deaminase (AID) is a specific B-cell enzyme essential for immunoglobulin (Ig) somatic hypermutation and class switching that acts in vitro on single-stranded DNA, one of its in vivo targets being the S regions of Ig genes, in which R-loops are formed (Muramatsu *et al.*, 2000; Revy *et al.*, 2000; Okazaki *et al.*, 2002). We have recently reported that the heterologous overexpression of human AID is able to strongly induce both mutation and recombination in yeast THO mutants (Gomez-Gonzalez and Aguilera, 2007). This is explained by the fact that R loops formed in THO mutants leave the nontranscribed chain (NTS) as single-strand DNA (ssDNA), thereby increasing accessibility to AID. Consistently, in *mft1*Δ cells expressing AID, mutations were 10-fold higher in the NTS, whereas such a strand bias was not observed in the wild type (Gomez-Gonzalez and Aguilera, 2007).

We wondered, therefore, whether THSC inactivation by *thp1*Δ, stimulated the action of AID, as an indirect manner to assess whether R-loops also formed in THSC mutants. We used the LAUR system. In this assay Ura$^-$ colonies are selected in SC-FOA. As can be seen in Figure 7, AID expression increases the frequency of Ura$^-$ colonies fivefold in wild-type cells and 19-fold in *mft1* cells, consistent with previously reported data (Gomez-Gonzalez and Aguilera, 2007). Noteworthy, the effect of AID was not specific to THO mutants but was also observed in *thp1*Δ mutant, in which AID increased mutations 58-fold. Such an increase was not seen in *spt4*Δ strains and others mutants in factors involved in transcription such as Spt6 and Rpb2 (data not shown).

Therefore, we can conclude that in THSC mutants, there is a transcription-dependent transient accumulation of ssDNA that facilitates AID action. This is consistent with the presence of R-loops that would leave the nontranscribed chain as single-stranded, as in THO mutants (Gómez-González and Aguilera 2007).

DISCUSSION

We show here that Thp1, Sac3, and Sus1 form a functional unit with a role in mRNP biogenesis and maintenance of genomic integrity in the cell that is independent of the main function of SAGA, but it is dependent on the nascent mRNA molecule. The THSC complex acts in the mRNP biogenesis pathway together with THO, Sub2, and the Mex67-Mtr2 export factor. We propose that eukaryotic transcription elongation is controlled by an RNA-export–associated feedback mechanism that prevents RNA-mediated genome instability.

THO contributes to the formation of an optimal mRNP, presumably facilitating the assembly of RNA-binding proteins onto the nascent mRNA such Sub2 or Yra1. A defective THO complex would lead to failure in this process that in turn would create suboptimal mRNP that would not be competent for export and would contribute to inhibit transcription elongation and to trigger recombination, with the concomitant formation of an R loop (Huertas and Aguilera, 2003). Here we show that THO is not the only complex in the absence of which, failures of transcription-RNA export coupling causes genome instability. Hyper-recombination and mRNA accumulation defects were previously observed in mutants of Sub2, Mex67, Thp1, and Sac3 (Jimeno *et al.,* 2002; Gallardo *et al.,* 2003), but whether mutations in these genes led to transcription-elongation impairment in an RNA-dependent manner was not known.

It is worth noticing that Sus1 is part of two different protein complexes, THSC and SAGA, and has been proposed to act as a bridge between mRNA export and transcription (Rodriguez-Navarro *et al.,* 2004). Recent data suggest that Sus1 could function in histone acetylation and transcription in a SAGA-dependent manner and is necessary for RNA export (Köhler *et al.,* 2006; Zhao *et al.,* 2008). Nevertheless, our results suggest that Sus1 plays an important role as part of the THSC complex in RNA biogenesis/export. This is deduced from the observations that overexpression of Sub2 inhibited the growth of *sus1*Δ cells, because *thp1*Δ and *sac3*Δ and other RNA export factor mutants, such as *pap1, mex67, yra1, nab2, gle1,*and *dbp5,* and nucleoporin mutants such as *nup60*, *nup84, nup133,* and *nup120*, but not any of the SAGA mutants tested, including *ubp8* and *sgf11*, encoding the closest partners of Sus1 in the SAGA complex (Köhler *et al.,* 2006) and *sgf73*, mutated in the Sgf73 subunit that mediate recruitment of Thp1-Sac3 to SAGA (Köhler *et al.,* 2008). It is likely that overexpression of Sub2 leads to an aberrant mRNP structure causing an irreversible block of mRNP biogenesis and export and hence growth inhibition in THSC mutants deficient in RNA export. Besides, *sus1*Δ confers a reduction in mRNA accumulation of *lacZ* similar to *thp1*Δ and *sac3*Δ, whereas this transcription defect is not observed in *ubp8*Δ and *sgf11*Δ mutants, the two SAGA subunits functionally linked to Sus1 (data not shown). Furthermore, *sus1*Δ mutants share the in vivo transcription impairment phenotype and transcription-dependent hyper-recombination of *thp1*Δ and *sac3*Δ mutants; whereas SAGA mutants show wild-type recombination phenotypes (Figure 2A and Supplemental Figure S3). Altogether, these data suggest that Sus1 forms a functional unit with Thp1 and Sac3 (THSC) with a role in mRNP biogenesis independent of SAGA. Nevertheless, and as it happens with some subunits of other protein complexes, such as Tex1 of TREX or Mft1 of THO (Luna *et al.,* 2005), the relevance of Sus1 in THSC seems to be lower than that of Thp1 and Sac3, according to the milder phenotypes of *sus1* mutants. This would be in agreement with the recent work on Sgf73 and the THSC complex that indicates that this factor mediates the recruitment of Thp1 and Sac3 to SAGA and their stable interaction with Sus1-Cdc31 (Köhler *et al.,* 2008).

The similarity of transcription and recombination phenotypes of THSC mutants with those of THO and the observation that THSC plays a role in maintaining the nuclear pore localization of genes (Cabal *et al.,* 2006, 2008; Kurshakova *et al.,* 2007) opens up the possibility that THSC could also bind to active chromatin in a transcription-dependent manner. Nevertheless, so far we have been unable to show that Thp1-Sac3 is recruited to active chromatin (data not shown). The observation that THSC mutants have a weak effect on transcription elongation in vitro compared with THO mutants is consistent with a role of THSC in mRNP biogenesis that would be coupled to its function at the nuclear pore. Our in vitro assays have been performed with WCEs in which nuclear envelops are disrupted and the DNA substrate is added independently and apart of NPCs. Concordantly, the effect of a complex that interacts with the nuclear pore as THSC (Fischer *et al.,* 2002) may not be properly detected with this in vitro assay, but it can be observed in vivo assays performed with intact cells. So far binding of Sus1 to *GAL1* promoter has been reported, suggesting that tethering of the DNA to the nuclear pore via THSC could be via promoters, regardless of its transcriptional state (Rodriguez-Navarro *et al.,* 2004; Cabal *et al.,* 2006; Kurshakova *et al.,* 2007), but this binding does not explain its in vivo transcription-elongation impairment.

A key result to understand the specific transcription phenotypes of the mutants of this process is provided by the analysis of the effect in different mutations of THO subunits, Sub2 and Thp1, on ribozyme-containing transcription and recombination assays and on hyper-mutation caused by human AID. Our study reveals that mutants in these proteins lead to a DNA structure susceptible to the action of human AID, as was recently shown in THO mutants (Gomez-Gonzalez and Aguilera, 2007). This, together with the observation that AID acts preferentially on ssDNA (Chaudhuri *et al.,* 2003) is consistent with formation of R-loops in these mutants, as has been shown for *hpr1*Δ (Huertas and Aguilera, 2003) and the S regions of Ig genes where AID acts (Yu *et al.,* 2003). Therefore, in contrast to the idea that THO could be a unique factor acting at transcription sites with a role preventing the interaction of nascent RNA with the DNA, other factors acting downstream on mRNP biogenesis and export has similar effect. This implies a feedback mechanism by which improperly formed mRNPs, presumably stacked at the nuclear pore, have a backward effect promoting transcription impairment and genetic instability. It is possible that THSC-malfunction disrupts both RNA export and mRNP assembly, causing transcription elongation impairment via a mechanism similar to that occurring in THO mutants and has yet to be deciphered.

Alternative mechanisms can explain the peripheral location of activated genes. These may involve either promoter-interacting proteins such as SAGA components or mRNP biogenesis and processing factors such as the RNA export factor Mex67 or the Mlp1 factor involved in mRNA surveillance (Dieppois *et al.,* 2006; Chekanova *et al.,* 2008). Our data suggests a model in which the biogenesis of mRNPs ends in

the localization of the transcribed DNA at the proximity of the NPC could be via the subsequent action of THO, Sub2-Yra1, Mex67-Mtr2, and THSC in a transcription- and RNA-mediated manner. This process would be independent of SAGA and would prevent the generation of suboptimal mRNPs that could react with DNA, compromising genome integrity. The recent observation that the Mex67 export factor is recruited to chromatin in a transcription and THO-dependent manner (Gwizdek *et al.*, 2006) provides a new scenario in which Mex67 may also be loaded onto the mRNP during transcription to allow its subsequent export through the NPC. This direct connection may explain the transcription defects and hyper-recombination phenotype of *mex67-5* mutants previously reported (Jimeno *et al.*, 2002). Our results, therefore, define a specific pathway that controls the fate of the mRNA from the site of transcription to the nuclear pore as a key process in the maintenance of genome integrity. Presumably, these protein factors from THO to THSC may function on the nascent RNA in a dynamic process starting on the DNA and finishing up in close proximity to the NPC. To unravel why THSC has a feedback effect in transcription elongation and genome integrity will help understand how different nuclear processes are interconnected and the key function of these mRNP biogenesis and export factors in maintaining genome integrity.

ACKNOWLEDGMENTS

We thank E. Hurt (Universität Heidelberg, Heidelberg, Germany) and M. Rose (Princeton University, Princeton, NJ) for yeast *cdc31* mutants, S. Jimeno for helpful comments on the manuscript, and D. Haun for style supervision. This work was supported by grants from the Spanish Ministry of Science and Education (SAF2003-00204 and BFU2006-05260) and Junta de Andalucía (CVI102 and CVI624). C.G.-A. and B.G.-G. were the recipients of (Formación Profesorado Universitario) Ph.D. training grants from the Spanish Ministry of Science and Education.

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