RNA polymerase II contributes to preventing transcription-mediated replication fork stalls

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Abstract

Transcription is a major contributor to genome instability. A main cause of transcription-associated instability relies on the capacity of transcription to stall replication. However, we know little of the possible role, if any, of the RNA polymerase (RNAP) in this process. Here, we analyzed 4 specific yeast RNAPII mutants that show different phenotypes of genetic instability including hyper-recombination, DNA damage sensitivity and/or a strong dependency on double-strand break repair functions for viability. Three specific alleles of the RNAPII core, *rpb1-1*, *rpb1-S751F* and *rpb9Δ*, cause a defect in replication fork progression, compensated for by additional origin firing, as the main action responsible for instability. The transcription elongation defects of *rpb1-S751F* and *rpb9Δ* plus our observation that *rpb1-1* causes RNAPII retention on chromatin suggest that RNAPII could participate in facilitating fork progression upon a transcription-replication encounter. Our results imply that the RNAPII or ancillary factors actively help prevent transcription-associated genome instability.

Keywords DNA damage response; DNA replication; double-strand break repair; genome instability; RNAPII

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; DNA Replication, Repair & Recombination; Transcription

DOI 10.15252/embj.201488544 | Received 20 March 2014 | Revised 29 September 2014 | Accepted 8 October 2014 | Published online 1 December 2014

The EMBO Journal (2015) 34: 236–250

Introduction

Genome integrity is essential for cell cycle progression, development and differentiation. In addition to the DNA lesions occurring spontaneously or induced by external genotoxic agents, cellular processes such as DNA replication, repair, recombination or transcription could affect the stability of the genome (Aguilera & Gómez-González, 2008). Consequently, cells have developed a complex network to coordinate these processes and guarantee genome integrity and cell proliferation. Deficiencies in this coordination result in a variety of diseases ranging from severe genetic disorders to cancer predisposition and accelerated aging. Conflicts between replication and transcription can cause an increase in DNA breaks as a

consequence of replication fork (RF) stalling and collapse leading to recombination and chromosome rearrangements in a process termed transcription-associated recombination (TAR) (Gaillard *et al*, 2013b). The fact that replication is the major source of recombinogenic DNA breaks and that recombination with the sister chromatid represents the major double-strand break (DSB) repair pathway during the S/G2 phase of the cell cycle supports this model (Aguilera & García-Muse, 2012). Consistently, TAR is mainly seen when transcription occurs in the S-phase and has been related to RF progression impairment caused by transcription (Prado & Aguilera, 2005; Aguilera & Gómez-González, 2008; Merrikh *et al*, 2012). Collisions between the replisome and the transcription machinery could lead to DNA breaks that would rely on recombinational repair to allow RF restart (Branzei & Foiani, 2010; Labib & De Piccoli, 2011).

An increasing number of studies have tried to understand the role of replication and recombination factors in transcription-associated genome instability both in prokaryotes and in eukaryotes (Kim & Jinks-Robertson, 2012; Gaillard *et al*, 2013b). The way by which bacteria deal with collisions between the replication and transcription apparatuses has been extensively studied, since co-directional collisions between the replisome and the RNAP are inevitable. This is due to the fact that the bacterial genome contains only one replicon; the rate of replication is much faster than that of transcription, and replication is not limited to one defined cell cycle phase. *Escherichia coli* contains three helicases, Rep, UvrD and DinG, which might promote replication of DNA bound to proteins such as the RNAP. Destabilization of transcription complexes can suppress the viability defect observed in *repΔC33 ΔuvrD* cells, indicating that the Rep–DnaB interaction facilitates resolution of transcription–replication conflicts (Atkinson *et al*, 2010). Moreover, DinG, Rep and UvrD are essential for efficient replication across highly transcribed regions *in vivo* (Baharoglu *et al*, 2010; Boubakri *et al*, 2010). Backtracking of RNAP at an obstacle has also been implicated in genome instability mediated by transcription–replication collisions (Dutta *et al*, 2011). RNAP mutants that reduce the frequency of RF stalling have been described in bacteria (McGlynn & Lloyd, 2000; Trautinger & Lloyd, 2002). It has been suggested that such mutants form less stable complexes with template DNA, thus decreasing the probability of collisions with replisomes.

In eukaryotes, several factors have been described as being involved in TAR, which in a number of cases proved to be a result from collisions between transcription and replication. These include

RNA-binding proteins and others with a role in transcription elongation, mRNP biogenesis, processing and/or export (Aguilera & García-Muse, 2013). As in bacteria, DNA helicases are involved in replication through obstacles that need to be bypassed to allow replication resumption. In yeast, one such helicase is Rrm3 (Ivessa *et al*, 2003), which is important in solving transcription–replication collisions (Prado & Aguilera, 2005; Azvolinsky *et al*, 2009). However, other elements can contribute to TAR, including the formation of R-loops, structures formed by RNA–DNA hybrids and the displaced DNA strand (Aguilera & García-Muse, 2012), or the local negative supercoiling transiently occurring behind the RNAP (Gaillard *et al*, 2013b). In such cases, the formation of single-stranded DNA (ssDNA), more susceptible to damage, would be facilitated (Schmidt *et al*, 2006; Kim *et al*, 2007). R-loops seem to occur naturally, but their accumulation is greatly enhanced in cells deficient in different steps of RNA processing and mRNP biogenesis and export. These deficiencies cause high levels of genome instability as detected by hyper-recombination, DNA damage accumulation or chromosome loss from yeast to humans (Jimeno *et al*, 2002; Huertas & Aguilera 2003; Li & Manley, 2005; Paulsen *et al*, 2009; Domínguez-Sánchez *et al*, 2011; Mischo *et al*, 2011; Wahba *et al*, 2011; Castellano-Pozo *et al*, 2012; Stirling *et al*, 2012). R-loops impair transcription elongation and RF progression (Wellinger *et al*, 2006; Tous & Aguilera, 2007; Gan *et al*, 2011) likely due to the ability of the R-loop and/or a putatively associated RNA polymerase to become a roadblock for transcription and replication (Drolet *et al*, 1995; Huertas & Aguilera, 2003).

Despite the increasing evidence that transcription may be one of the main obstacles to RF progression as a source of genome instability (Gaillard *et al*, 2013b), little is known about the factors implicated. Here, we asked whether the transcription machinery itself has a direct role in genome instability. We searched for specific mutations of different RNAPII subunits in *Saccharomyces cerevisiae* that caused genome instability and a strong dependency on DSB repair functions for viability. We analyzed three mutations *rpb1-1*, *rpb1S751F* and *rpb9Δ* with these phenotypes. Our results demonstrate that RNAPII by itself can become an obstacle to replication and can help solve or prevent the RF stalling and collapse responsible for genome instability.

Results

Genetic interactions between RNAPII and DNA repair mutants

To investigate whether the RNAPII itself might play a role in the origin of genome instability, we analyzed the sensitivity of a selected number of known RNAPII mutants (Supplementary Table S1) to several DNA damaging drugs. In the presence of hydroxyurea (HU), which inhibits the ribonucleotide reductase causing a depletion of dNTPs that impair replication progression, and methylmethanesulfonate (MMS), an alkylating agent that can indirectly cause DNA breaks, the selected RNAPII mutants showed different degrees of growth defects (Supplementary Fig S1A). Next, we asked whether or not there were genetic interactions between the RNAPII mutants and those of homologous recombination (HR) such as *rad52* and *mre11* (Fig 1 and Supplementary Fig S1B). The strongest interaction was observed in the tetrad analysis of the crosses of *rad52Δ* and *mre11Δ* with *rpb1-S751F*, where we did not find viable

Figure 1. Genetic interactions of RNAPII mutations with DNA repair mutations.

- A** Viability of single and double mutants of RNAPII and *rad52Δ*, *mre11Δ* and *cdc44-8*. Tenfold serial dilutions of double mutants obtained by genetic crosses were tested for growth in plain SC medium or supplemented with 10 mM HU or 0.005% MMS.
- B** Tetrad analysis of *rpb1-S751F*, *rad52Δ* and *mre11Δ* crosses. Δ indicates double mutants that fail to grow.
- C** Tetrad analysis of *rpb1-S751F* crossed by *pol32Δ* and *rad51Δ*. Δ indicates triple mutants that fail to grow.
- D** HU and MMS sensitivity of different mutant combinations of *rpb1-1*, *rpb1-S751F*, *pol32Δ* and *rad51Δ*.

Data information: Cells were cultured for 3 days at 30°C.

double mutants (Fig 1B), consistent with the sick phenotype used to isolate this mutant in a *rad52* background (F. Malagón, pers. comm.) (Strathern *et al*, 2013). However, *rpb1-S751F* was tolerated in both *rad51Δ* and *pol32Δ* backgrounds. As we have previously shown that Rad51 and Pol32 control two overlapping outcomes of Rad52-dependent HR events (Moriel-Carretero & Aguilera, 2010; Muñoz-Galván *et al*, 2013b), we tested whether *rpb1-S751F rad51Δ pol32Δ* triple mutants were viable or not. Indeed, they were not, suggesting that these mutants accumulate replication-born DNA breaks that cannot be repaired in the absence of Rad51 and Pol32.

Also, *rpb1-1* and *rpb2-10* strains showed strong growth defects in the absence of HR functions. The *rpb9Δ* mutant was highly sensitive

to HU and in the absence of Mre11 presented a severe growth defect in MMS. Additionally, the four mutants, in combination with the replicative clamp loader *RFC1* allele *cdc44-8*, essential in recombinational DSB repair (Holmes & Haber, 1999), showed a reduced-growth phenotype in low concentrations of HU and MMS. An extended analysis of *rpb1-1* in combination with mutations in other HR repair genes such as *rad50Δ*, *xrs2Δ*, *rad51Δ*, *rad54Δ*, *mus81Δ*, *pol32Δ*, *sgs1Δ* or *dia2Δ* confirmed the need of DSB repair for viability of *rpb1-1* (Supplementary Fig S1C). Taking into account these results, we selected *rpb1-1*, *rpb1-S751F*, *rpb2-10* and *rpb9Δ* for further analyses.

RNAPII mutants show increased DSBs and genetic instability

The need of HR functions for viability of *rpb* mutants under replication stress suggests that they may accumulate DSBs. We tested this possibility by Western blot against the phosphorylated form of histone H2A (H2A-P), a molecular marker for DSBs, in cells with and without HU treatment. In *rpb1-1* cells, an H2A-P signal was detected in the absence of HU (Fig 2A), confirming that breaks occur spontaneously at high frequency. Importantly, this signal was exacerbated in HU. In the other mutants, the H2A-P signal was stronger than in the WT in the presence of HU, but not detectable in its absence. This tendency of all four *rpb* mutants to accumulate DSBs was confirmed by analyzing the accumulation of Rad52 foci, a marker for DSB repair centers (Lisby et al, 2001). RNAPII mutations increased the percentage of cells with Rad52 foci, and these were further increased in thermo-sensitive mutants *rpb1-1*, *rpb1-S751F* and *rpb9Δ* at 37°C (Fig 2B). Therefore, DNA breaks accumulate in these RNAPII mutants and are processed into a recombinogenic intermediate.

Next, we asked whether recombination was enhanced in the mutants. We used the plasmid-based pTINV system, carrying inverted repeats of truncated *leu2* fragments, and the chromosomal *leu2-k::ADE2-URA3::leu2-k* direct-repeat system (Gómez-González et al, 2011b) (Fig 2). A significant increase of *Leu*⁺ recombinants could be seen in *rpb1-1* cells with respect to WT in both systems (Fig 2C and D), whereas the other mutants show recombination levels similar to WT. We then used a number of plasmid-based recombination systems to study TAR. These were the *L-lacZ* and *GL-lacZ* systems carrying 0.6-kb *leu2* direct repeats flanking *lacZ* under the *LEU2* or the *GAL1* promoter, respectively. Recombination can be analyzed in these systems under conditions of low (*GAL1p* in 2% glucose), medium (*LEU2p*) and high transcription (*GAL1p* 2% galactose) (Gómez-González et al, 2011b). As can be seen in Fig 2E, the higher the levels of transcription, the stronger the increase in recombination in *rpb1-S751F*. *rpb9Δ* showed a clear hyper-recombination phenotype that increased with transcription, which is particularly interesting since both mutants have defects in transcription elongation (Hemming et al, 2000; Strathern et al, 2013). No difference was observed with the *L-lacZ* systems in *rpb1-1* mutant. This could be due to a lower efficiency of HR leading to detectable recombination products, which are different for each assay (Gómez-González et al, 2011b). Interestingly, although the recombinational behavior of *rpb1* mutants and *rpb9Δ* was heterogeneous, they all cause a hyper-recombination phenotype that was mainly transcription-dependent. In addition, all mutants, with the exception of *rpb2-10*, showed a significant increase in plasmid loss when *lacZ* was transcribed, as determined with pGAL-LacZ (Fig 2F). In

Figure 2. Genetic instability in RNAPII mutants.

- A Western blot against the phosphorylated form of histone H2A in the presence of 100 mM HU in asynchronously growing WT and RNAPII mutant cells. Ponceau staining is shown as a loading control.
- B Rad52 foci formation at 30 and 37°C.
- C Recombination frequencies between inverted repeats in WT and RNAPII mutants measured with the plasmid-born pTINV system. Recombinants were selected as *Leu*⁺.
- D Recombination frequencies using the direct-repeat chromosomal system *leu2-k::ADE2-URA3::leu2-k* in WT and RNAPII mutants. Deletion events were detected and quantified in media containing 5-fluorotic acid (FOA) and could be directly visualized as red sectors.
- E Recombination frequencies between plasmid-born direct repeats in WT and RNAPII mutants. The frequency of *Leu*⁺ recombination was determined in the 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.
- F Mitotic stability of centromeric plasmid pGAL-lacZ in RNAPII mutants. Stability was analyzed in galactose after 23 generations. A small diagram of each system (not drawn to scale) is shown.

Data information: statistically significant differences with respect to WT according to Student's *t*-test ($P < 0.05$).

Source data are available online for this figure.

summary, despite the heterogeneity of phenotypes, genetic instability increased in *rpb1-1*, *rpb1-S751F* and *rpb9Δ*, whereas *rpb2-10* was poorly or not affected.

Among the mutants studied, viability of *rpb1-1* and *rpb1-S751F* shows the strongest requirements for HR functions, in particular under replication stress. Then, we analyzed the effect of *mre11Δ*, which abrogates the early steps of DSB repair, on the genome instability phenotype of *rpb1-1*. Inverted-repeat recombination increased in the *rpb1-1 mre11Δ* double mutant above the single mutants, whereas Rad52 foci accumulated at lower levels with respect to the single *mre11Δ* cells (Fig 3A and B). This suggests that unrepaired DNA breaks are channeled into a single-strand annealing pathway of recombination in the absence of an active MRX complex, avoiding Rad52 foci accumulation. As expected, this pathway was less effective provided that the Rad52-dependent HR pathway was also impaired. Consistently, *rpb1-1 mre11Δ* double mutants were highly sensitive to HU (Fig 3C).

These results are consistent with the idea that *rpb1* mutants generate more DNA breaks in a transcription-dependent manner. So we wondered whether this was also dependent on R-loops as it occurs in the *hpr1Δ* mutant, in which R-loops are reduced by RNH1 overexpression (Huertas & Aguilera, 2003), but this was not the

case. As can be seen in Fig 3D, *rpb1-1* and *rpb1-S751F* levels of Rad52-foci were not reduced by RNH1 overexpression.

DNA replication is impaired in *rpb1* and *rpb9* mutants

Next, we analyzed whether DNA replication was affected in these mutants by different means. First, we synchronized cells in G1 with α -factor and monitored growth at different times after G1 release under replicative stress. A clear arrest in G1/early S-phase was observed in *rpb1-1*, *rpb1-S751F* and *rpb9Δ*, whereas only a slight delay was observed in *rpb2-10* (Fig 4A). In the absence of HU, a similar tendency of S-phase delay was observed (Supplementary Fig S2).

Consequently, we directly analyzed the effect of the *rpb* mutations in RF progression by 2D gel electrophoresis. A scheme of the migration pattern of replication intermediates is shown (Fig 4B). Replication through the *SPF1* (*YELO31w*) gene of chromosome V lying close to the early replication origin *ARS508* (Gómez-González et al, 2009) in G1-synchronized cells released into S-phase under low replication stress (40 mM HU) revealed a clear delay in RF progression in *rpb1-1*, *rpb1-S751F* and *rpb9Δ* cells. Y-arcs persisted much longer in the mutants than in WT cells (Fig 4C). In addition, replication initiation was significantly delayed; whereas the Y-arc is fully visible in WT cells after 20 min, it was absent in the three mutants. However, the *rpb2-10* mutant was not affected either in initiation or in RF progression, since the 2D electrophoresis profile was the same as that of WT (Fig 4C). The results, consistent with the FACS analysis, evidence that RF progression is impaired in the mutants showing genome instability. Interestingly, 2D gel electrophoresis of *rpb1-1* reveals an accumulation of asymmetric X molecules not observed either in WT cells or in the other mutants tested. These structures could in principle be a consequence of two convergent forks unable to terminate properly, but this seems unlikely given the distance of the fork coming from the other side. The other possibility was that formation of X-structures depended on HR factors such as Rad51. FACS analysis revealed that the double-mutant *rpb1-1 rad51Δ* shows a profile similar to that of *rpb1-1*, indicating that *rpb1-1* was epistatic to *rad51Δ* (Supplementary Fig S3A). The 2D gel electrophoresis confirmed this epistatic relationship and revealed that the X-structures disappear in the double-mutant *rpb1-1 rad51Δ*. Therefore, the replication defect observed in *rpb1-1* leads to the accumulation of Rad51-dependent recombination intermediates (Supplementary Fig S3B).

Next, we assayed whether this was general to other regions all over the genome, for which we focused the rest of the study on the *rpb1* alleles, which altered the largest and essential subunit of RNAPII. First, we analyzed the kinetics of bromodeoxy-uridine (BrdU) incorporation using conveniently modified strains harboring the human TK gene via chromatin immunoprecipitation (ChIP) using anti-BrdU antibody. Two regions were analyzed in which transcription of the ORF was convergent to replication originated from its closest ARS. As can be seen in Fig 5A, BrdU incorporation not only was observed at later times than in WT cells, suggesting a delayed initiation as observed by 2D electrophoresis, but the speed of incorporation was slower, as can be deduced from a much lower slope of the incorporation profile of the *SPF1* gene. This reduction in the speed was also observed at *YFL034W*, located near a late replication origin (Fig 5A). We could detect

Figure 3. Requirement of HR functions for viability of *rpb1* mutants.

- A Recombination frequencies measured with the pTINV system in WT, *rpb1-1*, *mre11Δ* and *rpb1-1 mre11Δ*.
 B Rad52 focus formation in asynchronously growing cells at 30°C.
 C Viability after different times in 10 mM HU. The same cultures without HU were used as a control. The average values and standard deviations from three independent experiments are shown.
 D Rad52 focus formation in asynchronously growing cells expressing normal or high levels of RNH1.

Data information: * $P < 0.05$ (Student's *t*-test). Other details as in Fig 2.

Figure 4. Defects in replication fork (RF) progression in RNAPII mutants.

- A FACS analysis of cell cycle progression in *rpb* mutants. Cells were synchronized in G1 with α -factor and monitored at different times after release in the presence of 40 mM HU.
- B Scheme of the migration pattern of the bubble-, single Y-, double Y- and X-shaped replication intermediates in 2D gel electrophoresis.
- C Slowdown of RF progression through a chromosomal region. 2D gel electrophoresis of replication intermediates of 4.3-kb PstI DNA fragments from WT and RNAPII mutants in the presence of 40 mM HU at different times after G1 release. A black arrow indicates accumulation of long Y molecules, and a white one indicates the specific accumulation of X molecules.

Source data are available online for this figure.

replication impairment both under natural conditions in the absence of replication stress and with low concentration of HU. We could see that the effect was more severe in *rpb1-S751F* cells than in the *rpb1-1* mutant.

To assay whether RF progression was impaired all over the genome, we analyzed RF progression by DNA combing under low

replication stress. This technique offers the advantage of analyzing replication all over the genome rather than in a specific region. Representative DNA fibers are shown in Supplementary Fig S4. Consistent with both the 2D gel electrophoresis and BrdU incorporation, RF velocity was significantly reduced in both *rpb1-1* and *rpb1-S751F* cells (Fig 5B). Importantly, the distance between active origins was reduced from 161 to 105–106 kb on average from the WT to the two *rpb1* strains, suggesting that slower RFs are compensated for by additional origin firing and confirming that the major effect of the *rpb1* mutations analyzed is in RF progression (Fig 5B).

Altogether, our results indicate that the *rpb1* and *rpb9* mutants studied are impaired in replication, which can clearly be seen at regions actively transcribed.

The DNA damage response is delayed in *rpb1* mutants in the presence of genotoxic agents

DNA replication impairment is a major source of DNA lesions that can compromise genome integrity. Under these circumstances, the cellular DNA damage response (DDR) becomes essential. To test whether the delay in replication recovery implied either a failure in DDR activation or a longer time needed by the cells to repair the damage, we analyzed the kinetics of Rad53 phosphorylation after HU treatment (Fig 6A). Neither *rpb1-1* nor *rpb1-S751F* showed spontaneous detectable levels of Rad53-P, implying that the damage accumulated is not sufficient or its nature is not appropriate to activate the DDR. Interestingly, both *rpb1* mutants were able to respond to external damage by activating the Rad53 checkpoint, which can be visualized by high levels of Rad53-P after HU treatment. In G1-synchronized WT cultures treated with 100 mM HU for 1 h and subsequently released from G1 in HU-free media Rad53, phosphorylation was not visible after 40 min of release. However, Rad53 remained phosphorylated after 100–120 min in both *rpb1* mutants. Therefore, mutant cells take longer to remove the HU-induced damage, maintaining Rad53 activated for a more extended length of time, as can be seen by quantification of Rad53-P (Fig 6A).

Next, we tested the ability of the *rpb1* mutants to survive in combination with different *rad53* mutations such as the null *rad53Δ* (together with *sml1Δ*), *rad53-21*, which cannot delay the cell cycle in the presence of damage (Desany et al, 1998), or *rad53-1*, unable to avoid late origin activation under replication stress (Santocanale & Diffley, 1998). Whereas *rpb1-1* was lethal in combination with *rad53Δ*, *rpb1-S751F* showed a synthetic growth phenotype (Supplementary Fig S5A). In a *rad53-21* background, *rpb1-1* growth was heavily impaired, whereas the effect was fairly mild in *rad53-1*. The effect on *rpb1-S751F* was pretty mild. HU enhanced the phenotypes, but the differences between the mutants were similar as without HU (Supplementary Fig S5B). However, both *rpb1-1* and *rpb1-S751F* in combination with *rad53-1* were arrested in the G1/early S-phase (Supplementary Fig S5C). The results indicate that *rpb1-1* requires a proper DDR for viability whereas this demand is less for *rpb1-S751F*, consistent with an accumulation of recombinogenic DNA breaks and X-structures.

Finally, we assayed whether altered DDR of the *rpb1* mutants could affect the capacity of replication restart after MMS, as a way to explain the general impairment of RF progression in these

Figure 5. Effect of *rpb1-1* and *rpb1-S751F* on replication velocity.

A Analysis of replication by BrdU incorporation. Cells were synchronized in G1 with α -factor and released in the presence of BrdU. Samples were taken at the indicated times and processed to measure BrdU incorporation by ChIP analysis. Replication was analyzed in two DNA regions, one covering the early origin ARS508 and the SPF1 gene and the other the late origin ARS603 and the YFLO34W gene.

B DNA combing analysis of replication. Graphs indicate the distribution of BrdU track length (kb) at 60 and 120 min after α -factor release in the presence of 40 mM HU, the replication speed (kb/min) determined with the two time points analyzed and the inter-origin distance (kb) at 60 min after release. The median test was applied.

mutants. This was assayed by pulsed-field gel electrophoresis (PFGE), provided that only fully replicated chromosomes are able to enter the gel, whereas non-fully replicated chromosomes remain in the well as a consequence of the RF-containing branched chromosomes (Moriel-Carretero & Aguilera, 2010). Both *rpb1-1* and *rpb1-S751F* took longer to resume replication after MMS exposure

(Fig 6B). Altogether, our results suggest that *rpb1* mutants show a replication impairment likely due to the incapacity of the RF to restart replication at WT efficiency after encountering obstacles.

Global gene expression analyses of the *rpb* mutants

Next, we wondered whether mutations in RNAPII might affect expression of multiple genes. We performed microarray expression analysis in *rpb1-1* and *rpb1-S751F* mutants and in *rpb2-10*, used as a control. The global expression profiles of *rpb1-1* and *rpb1-S751F* were different from each other and different from the *rpb2-10* mutant (Fig 7A), suggesting that a secondary effect on global gene transcription does not explain the similar phenotypes of genome instability and replication impairment of *rpb1-1* and *rpb1-S751F*. Basic statistical analyses of the average length, G+C content and expression levels of up- and down-regulated genes revealed that both up- and down-regulated genes were shorter in *rpb* mutants than the genome average. Moreover, up-regulated genes were significantly richer in G+C and with lower expression in the 3 mutants analyzed ($P < 0.001$) (Supplementary Fig S6A). Instead, down-regulated genes were G+C poorer in *rpb1-S751F*, G+C richer in *rpb2-10* and with lower expression levels in *rpb1-1* and *rpb2-10* than in WT cells.

We focus the analysis on the data intersection of *rpb1-1* and *rpb1-S751F* mutants. From all RNAPII-transcribed genes, we selected those whose expression levels in the mutants were significantly different from the WT ($P \leq 0.05$). A twofold change cutoff relative to the WT yielded 645 genes in *rpb1-1* and 638 in *rpb1-S751F* (Fig 7B). From these, 77 genes were down-regulated in both *rpb1* mutants. In this subgroup, expression was reduced in several genes with a potential role in DDR (Supplementary Table S2), but the effect was not common to all of them and was too low as to explain the common phenotypes reported in this study for *rpb1* mutants. To confirm this, we selected 4 genes whose expression was decreased in both *rpb1* mutants: *HTL1*, a component of the RSC chromatin remodeling complex whose expression decreased twofold and sixfold in *rpb1-1* and *rpb1-S751F*, respectively; *NAT4*, an *N*- α -acetyl-transferase responsible for acetylation of the N-terminal residues of histones H4 and H2A (Song et al, 2003), whose expression was reduced 4.7-fold in *rpb1-1* and 2.4 in *rpb1-S751F*; *IRC4*, whose null mutant had been reported to increase the levels of spontaneous Rad52 foci (Alvaro et al, 2007) and whose expression was reduced 14-fold in *rpb1-1* and 2.6-fold in *rpb1-S751F*; and *SUS1*, a component of the SAGA histone acetylase and TREX-2 complexes, involved in transcription activation, elongation and mRNA export (Rodríguez-Navarro et al, 2004; González-Aguilera et al, 2008), whose expression decreased 2.3- and 2.7-fold in each mutant. We found that both *nat4* Δ and *htl1* Δ recombination and Rad52 foci levels were similar to the WT strain (Supplementary Fig S7A) whereas neither *IRC4* nor *SUS1* (*sus1* Δ) overexpression rescues the DNA damage sensitivity observed in *rpb1* mutants (Supplementary Fig S7B and C).

In addition, we analyzed *RNR3*, the large subunit of ribonucleotide-diphosphate reductase, which is strongly induced by DNA damage (Domkin et al, 2002) and whose expression was enhanced 2.8-fold in *rpb1-S751F*. *RNR3* overexpression enhances growth of WT and both *rpb1* mutants analyzed in HU or MMS (Supplementary Fig S7D).

These results exclude the possibility that a change in gene expression of a number of specific genes could be the major cause

Figure 6. Delayed repair of DNA damage in *rpb1* mutants.

A Dephosphorylation of Rad53 after exposure to HU. WT, *rpb1-1* and *rpb1-S751F* cells were synchronized in G1 with α -factor. After G1 release, cells were exposed to 100 mM HU for 1 h, washed to remove HU and cultured in HU-free medium for the indicated periods. Western blot against Rad53, signal quantification (ratio Rad53-P/total Rad53 protein) and FACS profiles are shown.

B DNA repair of MMS damage. Chromosome VII species revealed by hybridization with *ADE5,7* probes in pulsed-field gel electrophoresis (PFGE). Asynchronously growing WT and *rpb1* cells were exposed to 0.1% MMS for 30 min, washed to remove MMS and cultured in MMS-free medium for the indicated periods to allow repair. Repair was calculated as the percentage of FLC appearing after MMS treatment. The FLC signal was quantified with respect to the total signal of each lane. Data show the mean and SD of 3 independent experiments. NLC, nonlinear chromosome. FLC, full-length linear chromosome. CF, chromosome fragment resulting from DNA breaks.

Source data are available online for this figure.

of replication impairment and genome instability in *rpb1-1* and *rpb1-S751F*. Indeed, Gene Ontology analyses did not reveal any relevant functional class of genes involved in the maintenance of genome integrity that was equally affected by *rpb1-1* and *rpb1-S751F* (Supplementary Table S3). Altogether, the results suggest that there is neither a common profile of gene expression nor genes whose gene expression alterations could reasonably explain the genome instability phenotypes of the *rpb1* mutants by themselves. This supports the idea that a major part of the effect of *rpb1-1* and *rpb1-S751F* on genome instability is due to a direct role of

Figure 7. Analysis of global gene expression in RNAPII mutants.

- A** Hierarchical clustering of genes whose expression level is altered with respect to WT in *rpb* cells.
- B** Venn diagrams representing the overlap between genes whose expression level is up- or down-regulated with respect to the WT strain in *rpb1-1* and *rpb1-S751F*.
- C** Retention of RNAPII in the actively transcribed *PMA1* gene in RNAPII mutants. ChIP analysis of RNAPII in asynchronously growing WT, *rpb1-1*, *rpb1-S751F*, *rpb2-10* and *rpb9Δ* cells at the *PMA1* gene. The scheme of the gene and the PCR-amplified fragments are shown. Recruitment values were calculated from the amount of DNA present in each region, normalizing IP/input ratios, and relative to the amount of DNA of the intergenic region. Error bars indicate the standard deviation (SD).
- D** Northern analysis of the expression of *PMA1* gene. RNA was isolated from mid-log phase cultures grown in YPD-rich medium. As ³²P-labeled DNA probes, an internal 776-bp *PMA1* fragment and an internal 513-bp *SCR1* fragment obtained by PCR were used. Bars indicate the SD of 2 independent experiments.

Source data are available online for this figure.

the RNAPII itself and is consistent with the fact that hyper-recombination is, indeed, observed at transcribed DNA regions.

RNAPII retention at chromatin in *rpb1-1* mutant

Next, we wondered whether RNAPII accumulated at higher levels in the transcribed chromatin in the different RNAPII mutants analyzed, since this could reflect the incapacity of RNAPII to be released efficiently from the transcription site as a possible cause of genome instability. We analyzed the profile of RNAPII distribution at the actively transcribed *PMA1* gene by ChIP (Fig 7C). An increase of RNAPII retention in the transcribed region of *PMA1* with respect to the WT could be seen in *rpb1-1* cells. This retention is not specific of the *PMA1* gene; it is also observed in *SPF1* and *PDC1* genes in *rpb1-1* respect to WT (Supplementary Fig S8). Despite the fact that the four mutants studied were affected in transcription (Fig 7D and Supplementary Fig S8B), only *rpb1-1* showed an increased retention of RNAPII on genes.

Genome-wide occupancy of Rrm3 in *rpb1-1* cells as a marker of replication impairment

Since the *rpb1* mutants with transcription-dependent genome instability share a defective RF progression (Figs 4 and 5), we asked how one representative *rpb* mutant was affected in replication throughout the whole genome. Therefore, we selected *rpb1-1* for Rrm3-FLAG ChIP-chip analyses. Rrm3 is required for the progression of the RF through obstacles in the DNA, and its accumulation at specific DNA sites has been used to identify RF pauses (Ivessa et al, 2000, 2003). ChIP-chips performed in asynchronous cultures showed an increase of Rrm3 all over the genome in *rpb1-1* compared to WT (Fig 8 and Supplementary Fig S9). The hits of Rrm3 occupancy were clearly enhanced in telomeres in *rpb1-1* (Fig 8A and Supplementary Fig S9), implying that natural RF pause sites are stronger or more frequent at chromosome ends in *rpb1-1* cells. Finally, Rrm3 binds to less replication origins in *rpb1-1*, consistent with the delay in replication initiation as detected by 2D gel electrophoresis and BrdU incorporation (Supplementary Table S4).

There was a high coincidence between the ORFs enriched in Rrm3 in *rpb1-1* and WT cells although the difference between the

Figure 8.

Figure 8. Replication–transcription relationship.

- A Analysis of Rrm3 recruitment in WT and *rpb1-1*. Rrm3 IP histogram bars in the y-axis show the average signal $\log_2$ ratio of loci enriched in the immunoprecipitated fraction along the indicated regions in the x-axis. When the ratio fulfills the *P*-value criteria ($P < 0.01$) it is shown in red.
- B Composite profile of Rrm3 occupancy across the average ORF plotted as Rrm3 percentage of significant ChIP hits per segment. Each gene was divided into 10 equivalent segments from the start and end coordinates (segments 2–10). Two additional segments of the same size were considered upstream (5') and downstream (3') of each ORF (segments 1 and 12, respectively). The average signal $\log_2$ ratio for Rrm3 significant hits that map on each segment was plotted.
- C Composite profile of Rrm3 occupancy across the average ARS plotted as Rrm3 percentage of significant ChIP hits per segment. Each ARS was divided into 11 equivalent segments from the start and end coordinates. The average signal $\log_2$ ratio for Rrm3 significant hits that map on each segment was plotted.
- D Model for a role of RNAPII as a source of genetic instability. Conflicts between transcription and replication machineries affect replication progression causing DSBs at high levels under exogenous replicative stress, which demands the HR machinery for the repair and restart of RFs. RNAPII would have a function facilitating RF progression upon a transcription–replication encounter. This function would be affected in the *rpb1* and *rpb9* mutants analyzed here, causing RF progression impairment and genome instability.

two strains is significant (Supplementary Table S4). The subset of ORFs with high Rrm3 occupancy in *rpb1-1* is richer in G+C content and shorter in length than the average WT cells ($P < 0.001$) (Supplementary Fig S10A), which are coincident with the features of genes with higher mRNA levels in yeast (Marin *et al*, 2003). Rrm3 accumulates all over the length of genes in both WT and *rpb1-1* cells. The binding profiles show a tendency to increase toward the 3'-end (Supplementary Fig S10B), similar to transcription-dependent hyper-recombinant mutants defective in mRNP biogenesis (Gómez-González *et al*, 2011a).

In addition, we took advantage of microarray expression data and ChIP-chip analyses to study the correlation between replication impairment and gene expression. As can be seen in Supplementary Fig S11A, Rrm3 enrichment correlates with gene expression levels, even though these refer to asynchronous cultures and not to S-phase transcription, supporting the idea that replication obstacles occur preferentially at highly transcribed genes. This correlation is still more evident when we compare genes up- or down-regulated in *rpb1-1* (Supplementary Fig S11B).

Next, we analyzed the results taking into account the average of the significant signals in ORFs and ARSs all over the genome. For this, we divided the sequence of each ORF of the yeast genome into 10 equivalent segments (segments 2–10) plus two additional segments of the same size upstream (5') (segment 1) and downstream (3') of each ORF (segment 12). In a similar way, each ARS sequence was divided into 11 equivalent segments. Then, we calculated the average signal $\log_2$ ratio for Rrm3 hits mapping on each segment of all ORFs or ARSs, respectively, and these values were plotted. Interestingly, we see an increment of Rrm3 retention throughout ORFs and ARSs in *rpb1-1* respect to WT (Fig 8B and C). Since a major occupancy of Rrm3 at ORFs may be a consequence of transcription–replication collisions, our data are consistent with the idea that RNAPII itself participates in preventing such collisions.

Discussion

We provide genetic evidence that specific RNAPII mutants, *rpb1-1*, *rpb1-S71F* and *rpb9Δ*, show an increase in genetic instability as detected by hyper-recombination, DNA damage sensitivity, plasmid loss and/or a dependency on DSB repair functions for viability. These RNAPII mutants display RF progression impairment that could be compensated by additional origin firing. DNA damages accumulate in these mutants under replicative stress that activate the Rad53-mediated checkpoint, but that are repaired with considerable

delay. Genome-wide Rrm3 occupancy analysis and experiments showing that the *rpb1-1* mutant specifically causes a major retention of RNAPII at the site of transcription suggest that RNAPII can participate actively in facilitating the progression of colliding RFs, presumably by contributing to its own release from the site. Altogether, the data indicate that RNAPII contributes to maintain genome stability by distinct manners and without involving high R-loop accumulation.

rpb transcription elongation mutants have a differential effect on genome integrity

From the two specific mutations of the largest RNAPII subunit Rpb1 that cause genome instability, the *rpb1-1* mutation (G4622A) maps in the H region of Rpb1, a highly conserved amino acid region, which is important for the selection of the transcription start site (Nonet *et al*, 1987; Scafe *et al*, 1990). The *rpb1-1* mutation is temperature sensitive and inactivates RNAPII *in vivo*, causing a quick shutdown of mRNA synthesis after a shift to the non-permissive temperature (Nonet *et al*, 1987; Schroeder *et al*, 2000), even though the mutant form of RNAPII could still be seen at the transcription sites (Kim *et al*, 2010). No appreciable decrease of Rpb1 levels has been detected after this temperature shift (Tardiff *et al*, 2007). Also, suppressors of *rpb1-1* map in the conserved segment I of *RPB2*, suggesting an interaction between region H of *RPB1* and region I of *RPB2* (Martin *et al*, 1990).

rpb1-S751F maps in the F region, involved in transcription elongation (Braberg *et al*, 2013). The mutant Rpb1-S751F protein, instead, undergoes elevated transcriptional slippage (Strathern *et al*, 2013). Transcriptional slippage occurs when RNAPs exhibit higher inherent error rates during elongation usually due to misincorporation of the wrong nucleotide in combination with failure of the intrinsic correction mechanisms like that mediated by the exonuclease activity of RNAPII. Indeed, *rpb1-S751F* has reduced transcription levels compared to WT. The effect of the Rpb1-S751F bulky substitution on elongation is consistent with the close proximity of Ser751 to the active center of RNAPII (Strathern *et al*, 2013). Both *rpb1-1* and *rpb1-S751F* are MMS sensitive, but the sensitivity of the latter is clearly stronger.

The *rpb2-10* mutation maps in the H region linked to nucleotide binding. The mutant is sensitive to 6-azauracil (6-AU) and impaired in transcription elongation (Lennon *et al*, 1998), but genetic stability is poorly affected. Interestingly, *rpb2-4* also maps in the same region and causes sensitivity to 6AU, but its defect in transcription elongation is weaker (Powell & Reines, 1996) whereas *rpb2-7*, which maps in the A region, is the most sensitive to 6AU and does not present

transcription elongation impairment either (Powell & Reines, 1996). None of these mutants have a clear genome instability phenotype. The differential DDR phenotypes of the mutants suggest that, although transcription elongation impairment can be associated with genome instability, there is no strict correlation with the DDR defect. Indeed, analysis of transcription factor mutants affecting elongation is consistent with this conclusion (Rondon *et al*, 2003; Rondón *et al*, 2004; Luna *et al*, 2005). Despite the different mutants affected in transcription elongation that cause genome instability, our data suggest that the elongation defect is not sufficient to trigger genome instability.

The Rpb9 subunit is not essential for mRNA synthesis in yeast. During transcription initiation, Rpb9 modulates the selection of the transcription start site, recruiting TFIIE and interacting with TFIIF. During the elongation process, Rpb9, together with TFIIS, is required to stimulate the nascent transcript cleavage activity intrinsic to RNAPII (Van Mullem *et al*, 2002). The *rpb9Δ* mutant shows a strong sensitivity to 6-AU or mycophenolic acid (MPA) that can be suppressed overexpressing TFIIS (Hemming *et al*, 2000) and a transcription elongation defect (Tous *et al*, 2011). Rpb9 is also involved in transcription-coupled repair (TCR), which has led to the suggestion that the transcription elongation function of Rpb9 likely plays a role in backtracking the RNAPII stalled at a lesion, by coordinating RNAPII with TFIIS (Li *et al*, 2006). In addition, Rpb9 functions in ubiquitylation and degradation of RNAPII in response to UV-induced DNA damage (Chen *et al*, 2007). Although the TCR defect of *rpb9Δ* might in principle contribute to its genetic instability, the fact that *rad26Δ* TCR-defective mutants do not show hyper-recombination (González-Barrera *et al*, 2002) argues against such a possibility. Also, *rpb9Δ* is highly sensitive to HU, whereas this is not the case for *rad26Δ* strains (Gaillard *et al*, 2009).

Replication fork progression impairment as the cause of genome instability

Our data reveal that the *rpb1-1*, *rpb1-S751F* and *rpb9Δ* mutations increase genome instability, whereas this is not the case for *rpb2-10*. Interestingly, 2D gel electrophoresis shows that in *rpb1-1*, *rpb1-S751F* and *rpb9Δ*, long Y-shaped molecules accumulated toward the end of the descending simple Y-arc as a result of the movement of the RFs away from the restriction fragment containing *ARS508* (Fig 4). This is consistent with a slowdown of RFs and supports that replication failures occurring in the three mutants analyzed are responsible for genome instability. The same phenotype was previously described in *hpr1Δ* mutants (Gómez-González *et al*, 2009), implying that under replicative and transcription stress cells respond with the pausing or stalling of replication. Consistently, recombinogenic intermediates can be detected in *rpb1-1* in 2D gels, whereas no RF progression impairment was observed in *rpb2-10* mutants (Fig 4).

We believe that the *rpb1* and *rpb9* mutations analyzed induce instability by creating transcription intermediates that impair RF progression (Aguilera & García-Muse, 2012). The need of the recombinational DSB repair functions MRX, Rad52 and Rfc1 for proper viability of *rpb1-1* and *rpb1S751F* mutants is consistent with the idea that the replication impairment leads to DSBs that need to be repaired via homologous recombination; otherwise, cells die. Such breaks promote two different types of recombination events that

allow the restart of RFs, one Rad51- and the other Pol32-dependent, consistent with previous results in *rad3-102* (Moriel-Carretero & Aguilera, 2010) and histone deacetylase mutants (Muñoz-Galván *et al*, 2013a) that also impair RF progression causing genome instability.

Replication origins fire more often in *rpb1-1* and *rpb1-S751F* mutants than in WT cells, as determined by DNA combing (Fig 5). This may be a mechanism to counteract the slower RF progression in these mutants.

RNAPII function contributes to the prevention of transcription–replication conflicts

The molecular mechanisms by which a defective RNAPII or transcription elongation could lead to RF progression impairment and genome instability seem to be multiple. A lower capacity of the RNAPII to be released from the DNA after a premature transcription termination due to an elongation failure or after finding an obstacle, such as a DNA lesion, can increase the probability of a collision with the RF or its collapse. This explanation would fit for *rpb1-1*, in which RNAPII accumulates at higher levels than in WT cells at the ORFs analyzed, but not for *rpb1S751F* and *rpb9Δ*, which show the same RNAPII occupancy as WT cells. Therefore, an elongation failure, regardless of whether or not linked to a less efficient release of the RNAPII from the DNA, can increase genome instability. In this sense, analysis of the Rrm3 occupancy along the genome of *rpb1-1* showing no large increase in the number of hits with respect to WT cells suggests that the mutant form of the RNAPII does not enhance the probability of collisions or RF stalling. It is possible that the main effect of the mutated RNAPII is a reduced ability to resolve RF stalls, increasing the probability of their collapse. This could explain why Rrm3 accumulates preferentially in transcribed DNA regions (Fig 8) as occurs in WT cells (Azvolinsky *et al*, 2009) and that RF progression in RNAPII mutants is strongly impaired as determined by 2D electrophoresis, BrdU incorporation and DNA combing. This effect differs from that of other transcription mutants with a clear transcription-dependent genome instability phenotype as is the case of THO mutants, impaired in transcription elongation and RNA export, in which Rrm3 is accumulated at higher levels and at more ORFs than in WT cells (Gómez-González *et al*, 2011a). Indeed, transcription-associated genome instability can often be associated with the accumulation of R-loops (Aguilera & García-Muse, 2012) as it is the case of THO mutants (Huertas & Aguilera, 2003). However, since RNase H1 overexpression does not suppress genome instability in *rpb1* mutations, we rule out that an increase in R-loops is a major determinant of RF progression impairment observed in the mutants. Indeed, the lack of effect of null mutations or overexpression of genes deregulated in *rpb1-1* and *rpb1S751S* strains (*HTL1*, *NAT4*, *IRC4*, *SUS1* and *RNR3*) on DDR and genome instability is consistent with the idea that major effect of the *rpb* mutations is direct. In this sense, it is worth noting that *RNR3* overexpression diminished the sensitivity of the *rpb* mutants to replicative stress, as occurs in wild-type cells, consistent with the general conclusion that these mutations generate transcription-mediated RF progression failures as a source of instability.

Our results suggest that the RNAPII helps in minimizing the effects of natural transcription–replication collisions on genome integrity. This could occur by either facilitating the release of the

RNAPII from chromatin after a putative collision with the RF or by preventing RF collapse, a function that could be defective in *rpb1-1* mutants. The high RNAPII retention at ORFs could increase the probability of replication collisions. In addition, since selected elongation factors associate with RNAPII in a transcription-dependent manner in *rpb1-1* mutants (Tardiff *et al*, 2007), *rpb1-1* could also affect recruitment or turnover of factors involved in transcription or chromatin remodeling that could promote conflicts between the transcription and replication machineries. In this sense, it is possible that specific chromatin modifications associated with RNAPII elongation (Selth *et al*, 2010) or the topological constraints serve as a signal to prevent the replisome from entering a transcribed region and colliding with the RNAPII, or that a defective elongation causes a TCR failure leading to DNA lesions that perturb replication and can become DSBs (Belotserkovskii *et al*, 2013; Gaillard & Aguilera, 2013a). Deregulation of gene gating to the nuclear pore can also contribute to the instability of transcribed DNA (Bermejo *et al*, 2012). However, our identification of different RNAPII mutants with impact on RF progression indicates that eukaryotic RNAPII has evolved to minimize the possible impact of transcription–replication conflicts in genome instability, with important implications for the genetic origin of cancer.

Materials and Methods

Strains and plasmids

They are described in Supplementary Tables S5 and S6.

All experiments were performed at 30°C with few exceptions indicated in figure legends.

Analysis of recombination, Rad52-YFP foci and plasmid loss

Recombination frequencies were obtained as the median values of three fluctuation tests performed with six independent yeast colonies one from each transformant analyzed. The frequency of plasmid loss was calculated as the median frequency of six independent cultures grown on non-selective rich medium for 23 generations as previously described (Chavez & Aguilera, 1997). Spontaneous Rad52-YFP foci from S-G2 mid-log-phase cells bearing the plasmid pWJ1344 were visualized with a Leica DC 350F microscope (Lisby *et al*, 2001).

Western blot

Phosphorylated H2A and β -actin were detected by Western blot of TCA-extracted proteins separated in 15% PAGE using the ab15083 (Abcam) and ab8226 (Abcam) antibodies, respectively. For signal detection, SuperSignal West Pico system was used (Pierce). Detection of Rad53 and β -actin was accomplished by Western blot analysis of TCA-precipitated proteins separated in 4–20% Criterion TGX gradient PAGE (Bio-Rad). Antibodies sc-20169 (Rad53; Santa Cruz Biotechnology) and ab8224 (β -actin; Abcam) were used. For quantification, secondary antibodies conjugated to IRDye 680CW or 800CW (LI-COR) were used; the blot was scanned in an Odyssey IR scanner and analyzed with Image

Studio 2.0 software (LI-COR). For phosphorylation signal quantification, the ratio between Rad53-P versus total Rad53 protein signal was calculated.

DNA combing

DNA combing was performed as described (Bianco *et al*, 2012) in mid-log phase cell TK-containing strains synchronized in G1 with α -factor and released in the presence of 40 mM HU and 200 μ g/ml BrdU. DNA fibers were extracted in agarose plugs after 60 and 120 min with BrdU as previously described (Duch *et al*, 2013).

Pulsed-field and 2D gel electrophoresis

PFGE and 2D gel electrophoresis and hybridization were performed as described (Gómez-González *et al*, 2009; Moriel-Carretero & Aguilera, 2010).

RNAPII and BrdU chromatin immunoprecipitation

RNAPII ChIPs were performed as described (Gonzalez-Aguilera *et al*, 2011) using the monoclonal anti-Rpb1-CTD antibody 8WG16 (Berkeley Antibody Company, Richmond, CA, USA) and Dynabeads protein A (Invitrogen). The intergenic region at positions 9,716–9,863 of chromosome V was used as a negative control. BrdU ChIPs were performed in TK-containing strains synchronized in G1 with α -factor and released in the presence of 200 μ g/ml BrdU (Sigma) using anti-BrdU antibody (Medical & Biological Laboratories). As negative control we used the region between coordinates 240,032 and 243,611 of chromosome V. Primers used are indicated in Supplementary Table S7.

Microarray gene expression analysis

Global gene expression by microarray analysis was performed using total RNA isolated from yeast cells grown on YPD-rich medium at 30°C to mid-log phase by mechanical disruption using the RNeasy Midi kit (Qiagen). Microarray data analysis was performed in triplicate using the Affymetrix platform. RNA quality was confirmed with the Bioanalyzer H (Agilent technology). Synthesis, labeling and hybridization of cRNA to GeneChip Yeast Genome 2.0 Arrays covering 5,841 genes of *S. cerevisiae* were performed following Affymetrix protocols. Probe signals were captured and processed with GeneChip Operating Software 1.4.0.036 (Affymetrix), and the resulting CEL files were reprocessed using the Robust Multichip Average (RMA) normalization (Irizarry *et al*, 2003). Fold change ($\log_2$) values (M) and their FDR-adjusted *P*-values were calculated with LIMMA (linear models for microarray analysis) (Smyth, 2004) using the affyGUI interface (Wettenhall *et al*, 2006). Statistical analysis was performed using R language and the packages freely available from the 'Bioconductor Project' (<http://www.bioconductor.org>). Fold change cutoffs were analyzed at 95% confidence levels (FDR-adjusted *P*-values, 0.05). The expression profile of mutants was compared with that of its isogenic wild-type strain cultured in the same conditions. Genes showing at least twofold expression change were considered as altered and selected for further bioinformatics analysis. The expression data can be accessed at Gene Expression Omnibus (GSE55223). GO annotations were obtained with FatiGO,

available in Babelomics (<http://babelomics.bioinfo.cipf.es>). Hits with P -value < 0.05 were selected.

ChIP-chip experiments

Saccharomyces cerevisiae high-density oligonucleotide tiling microarrays from Affymetrix that allow an analysis of yeast chromosomes at a 300-bp resolution, each of the 300-bp region being covered by at least 60 probes, were used. ChIP-chip of asynchronously growing cells was carried out basically as described (Bermejo *et al*, 2009; Gómez-González *et al*, 2011a,b). Data were analyzed using a modified version of the Affymetrix Tiling Array Suite software (TAS), which produces a signal and the change P -value per probe position taking into account the probes localized within a given bandwidth around the inspected probe. The ChIP-chip data can be accessed at Gene Expression Omnibus (GSE55184). Chromosomal distribution of the signals was analyzed by binding clusters, defined as ranges within the chromosome under the following conditions: estimated signal (IP/SUP-binding ratio) positive in the whole range; P -value < 0.01 , minimum run of 100 bp and maximum gap of 250 bp. Results were visualized with the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu/>). Mapping of binding clusters into Stanford Genome Database genomic features (www.yeastgenome.org) was performed using specific Perl scripts. To visualize the distribution of binding sites along features, ORFs and ARS were divided into equivalent segments from the start and end coordinates as previously described (Gómez-González *et al*, 2011a,b; Santos-Pereira *et al*, 2013).

Miscellanea

Cell cycle synchronization, flow cytometry analysis (FACSCalibur; Becton-Dickinson), Northern and other molecular assays were performed following standard procedures.

Supplementary information for this article is available online: <http://emboj.embopress.org>

Acknowledgements

We would like to thank F. Malagón, S. Rodríguez-Navarro, J. Strathern and R.A. Young for kindly providing reagents; F. Malagón for pointing us that *rpb1S751F* showed a synthetic growth defect with *rad52*; P. Domínguez (Microscopy Unit, CABIMER) for technical assistance in confocal microscopy; E. Andújar and M. Pérez (Genomics Unit, CABIMER) for technical support with microarray and ChIP-chip experiments; Antonio Marín for providing bioinformatics tools for analysis of genome-wide data; U. Galindo for technical assistance; and D. Haun for style supervision. Research was funded by grants from the Spanish Ministry of Economy and Competitiveness (Consolider 2010 CSD2007-0015 and BFU2010-16372), the Junta de Andalucía (CVI4567) and the European Union (FEDER). IF-A and JL-B were recipient of predoctoral training grants from the Spanish Ministry of Economy and Competitiveness and the Instituto Carlos III, respectively.

Author contributions

IF-A performed most of the experiments; JL-B and MLG-R performed part of the characterization of *rpb1-S751F*. IF-A and AA designed the experiments and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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