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Lsm1 promotes genomic stability by controlling histone mRNA decay

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Lsm1 forms part of a cytoplasmic protein complex, Lsm1-7-Pat1, involved in the degradation of mRNAs. Here, we show that Lsm1 has an important role in promoting genomic stability in *Saccharomyces cerevisiae*. Budding yeast cells lacking Lsm1 are defective in recovery from replication-fork stalling and show DNA damage sensitivity. Here, we identify histone mRNAs as substrates of the Lsm1-7-Pat1 complex in yeast, and show that abnormally high amounts of histones accumulate in *lsm1*Δ mutant cells. Importantly, we show that the excess of histones is responsible for the *lsm1*Δ replication-fork instability phenotype, since sensitivity of *lsm1*Δ cells to drugs that stall replication forks is significantly suppressed by a reduction in histone gene dosage. Our results demonstrate that improper histone stoichiometry leads to genomic instability and highlight the importance of regulating histone mRNA decay in the tight control of histone levels in yeast.
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Introduction

The regulation of mRNA turnover is a powerful way to control gene expression. In eukaryotes, mRNAs are primarily degraded by two redundant decay pathways, namely the 3′–5′ and 5′–3′ (Garneau *et al*, 2007). In *Saccharomyces cerevisiae*, a required initial step in both pathways is the shortening of the poly(A) tail, or deadenylation, which results in the production of oligoadenylated mRNA from polyadenylated mRNA. After deadenylation, transcripts can be degraded by either the 5′–3′ or the 5′–3′ pathways (Anderson and Parker, 1998; He *et al*, 2003). In the 3′–5′ pathway, transcripts are further deadenylated and then degraded by a multisubunit 3′–5′ exonuclease complex, known as the exosome (Mitchell *et al*, 1997), that has been recently shown to have also endonucleolytic activity (Lebreton *et al*, 2008). In the 5′–3′ decay pathway, poly (A) shortening of the mRNAs triggers the removal of the 5′ cap by the Dcp1/Dcp2 decapping enzyme complex, which then permits the 5′–3′ exonucleolytic degradation of the mRNA by the Xrn1p exonuclease (Larimer *et al*, 1992; Beelman *et al*, 1996). Decapping is a critical control point in this pathway, and multiple accessory factors have been described to stimulate decapping (Coller and Parker, 2004). These factors include the Lsm1-7-Pat1 complex (Bouveret *et al*, 2000; Tharun *et al*, 2000), the DEAD box helicase Dhh1 (Coller *et al*, 2001) and the proteins Edc1, Edc2, Edc3 and Scd6 (Dunckley *et al*, 2001; Decourty *et al*, 2008). Deletion of any of these factors results in the stabilization of multiple reporter mRNAs, although the relative contribution of each of these proteins to the stability of a particular mRNA is not known.

The Lsm1-7-Pat1 complex is made of seven Sm-like proteins, Lsm1 through Lsm7 and the protein Pat1 (Bonnerot *et al*, 2000; Bouveret *et al*, 2000; Tharun *et al*, 2000). Lsm1 is the key subunit and distinguishes this complex from the related Lsm2–Lsm8 that interacts with U6 snRNAs and functions in pre-mRNA splicing in the nucleus (Mayes *et al*, 1999; Salgado-Garrido *et al*, 1999; He and Parker, 2000; Tharun, 2009b). The Lsm1-7-Pat1 complex is highly conserved from yeast to humans and interacts with other factors involved in the 5′–3′ pathway, such as Dhh1, Dcp1 or Xrn1 (Tharun *et al*, 2000), in cytoplasmic foci known as P-bodies (Sheth and Parker, 2003). *In vitro* analysis of the Lsm1-7-Pat1 purified from yeast showed that the complex has the intrinsic ability to bind preferentially to the 3′ ends of oligoadenylated mRNAs over polyadenylated mRNAs (Tharun, 2009a). Another interesting observation is that, among the deadenylated RNAs, the complex has a strong binding preference for mRNAs carrying a U-tract over those that do not (Chowdhury *et al*, 2007), implying that mRNAs with 3′-terminal U-tracts will be further stabilized in the absence of a functional complex. Interestingly, it has been recently shown in human cells that at the end of S-phase, or when DNA replication is inhibited, histone mRNAs acquire 3′-terminal oligo(U) tracts in a process known as oligouridylation (Mullen and Marzluff, 2008). Lsm1-7-Pat1 then recognizes and binds to the oligo (U) tail and possibly leads to the recruitment of decay factors of both 5′–3′ and 3′–5′ pathways. This post-transcriptional modification of histone mRNAs is very important to avoid the toxic effect of an excess of histones in human cells (Marzluff and Duronio, 2002). In *S. cerevisiae*, mRNA uridylation has not been detected (Rissland and Norbury, 2009), and the lowering of histone levels after inhibition of replication is achieved through a combination of histone gene repression and histone degradation (Gunjan *et al*, 2005). Rad53, a protein kinase involved in several crucial aspects of the DNA damage response (Gunjan and Verreault, 2003), has been demonstrated to mediate histone degradation in yeast. However, the contribution of Lsm1-7-Pat1 complex to the maintenance of proper histone levels in yeast has not been determined.

Using a genome-wide screen in *S. cerevisiae*, we identified the *lsm1*Δ mutant as hypersensitive to DNA-damaging drugs. In this study, we address the role of Lsm1 in preventing DNA

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damage, and the relationship of this function to its role in mRNA degradation. We demonstrate that Lsm1 promotes the stability of replication forks after stalling induced by DNA alkylation, nucleotide depletion or in natural pause sites in the DNA. Importantly, we show that the stability is maintained by the essential role that Lsm1 exerts in the control of histone mRNA levels. The implications of these findings for genomic stability and cancer are discussed.

Results

Deletion of LSM1 results in hypersensitivity to DNA-damaging drugs and accumulation of double-strand breaks

Using a genome-wide screen in *S. cerevisiae*, we identified the mutant *lsm1*Δ as hypersensitive to trabectedin, a DNA-binding drug that causes replication-dependent double-strand breaks (DSBs) (Herrero *et al*, 2006; Guirouilh-Barbat *et al*, 2008). We then examined the sensitivity of *lsm1*Δ to other DNA-damaging agents, methyl methanesulfonate (MMS), campthotecin (CPT), phleomycin (Phl) and hydroxyurea (HU), which have been reported to induce DSBs directly or indirectly (Koy *et al*, 1995; Petermann *et al*, 2010). *lsm1*Δ cells were highly sensitive to MMS, CPT and HU and moderately sensitive to Phl (Figure 1A).

Hypersensitivity to genotoxic drugs could be the result of defects in the activation of DNA damage checkpoints or the accumulation of DNA damage due to a higher incidence, or defects in DNA repair. To test the first possibility, we analysed the phosphorylation state or Rad53, the major DNA damage checkpoint effector kinase of *S. cerevisiae* (Sanchez *et al*, 1999), after treatment with different drugs. Rad53 was phosphorylated, and so activated, in both *lsm1*Δ and wild-type cells following exposure to MMS, Phl or HU (Figure 1B). In fact, compared with the wild-type strain, in the *lsm1*Δ mutant we observed a further increase in the degree of Rad53 activation after treatment with HU, as revealed by the stronger intensity of the Rad53 phosphorylated form. Our results indicate that Lsm1 is not necessary for the activation of DNA damage checkpoint. In order to analyse the putative accumulation of DNA damage in *lsm1*Δ cells, we examined the formation of Rad52-YFP foci after treatment with MMS. In normal cells, Rad52 shows a diffuse nuclear localization; when DSBs occur, Rad52 relocalizes and forms discrete foci. Each Rad52 focus represents a centre of recombinational repair capable of processing multiple DNA lesions (Lisby *et al*, 2001). We found that the number of cells exhibiting Rad52 foci was higher in the *lsm1*Δ mutant compared with the wild-type strain (Figure 1C), even in the absence of DNA damage, suggesting either a higher incidence of DSBs in *lsm1Δ* cells or defects in their repair.

DSBs can be repaired by two mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). First, we investigated whether Lsm1 is involved in either pathway by combining the deletion of *LSM1* with mutations conferring defects in HR, such as *rad52*Δ, or NHEJ, such as *yku80*Δ. Deletion of *RAD52* highly increased the sensitivity of *lsm1*Δ cells to the DNA-damaging drugs MMS, HU (Figure 2A) and Phl (data not shown), suggesting that Lsm1 affects a pathway other than HR. Moreover, we found that *lsm1*Δ cells required the presence of Rad52 not only to repair exogenous DNA damage, but also for proper growth (Figure 2B), which suggests a higher incidence of DSBs occurring in the *lsm1*Δ strain. To confirm that Lsm1 is not involved in DSB repair, we took advantage of the HR reporter system shown in Figure 2C (Frank-Vaillant and Marcand, 2002). In this system, a DSB can be induced by the galactose-inducible HO endonuclease. In HR-competent cells, the cleaved MAT locus in chromosome III recombines with the homologous sequence of chromosome V and cells can survive in a galactose-containing medium. In contrast, HR defective strains are unable to form colonies under the same conditions. The *lsm1*Δ cells were able to grow in the galactose-containing medium, which confirmed that Lsm1 is not involved in DSB repair by HR.

To analyse whether *lsm1*Δ cells are affected in the repair of DSBs by NHEJ, we employed an *in vivo* plasmid repair assay (Boulton and Jackson, 1996) (Figure 2D). Initially, we assayed the ability of the strains under study to repair a DSB with 5′-overhanging ends produced by digestion with *Eco*RI.

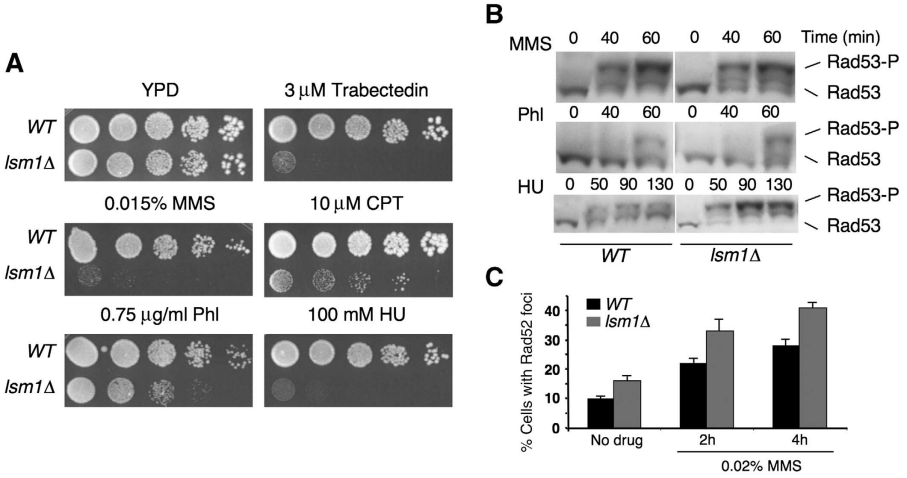

Figure 1 *lsm1*Δ cells are hypersensitive to DNA damage, proficient to activate the DNA damage checkpoint and accumulate DNA DSBs. (**A**) Sensitivity of wild-type and *lsm1*Δ cells to different drugs. (**B**) Phosphorylation of Rad53 in response to DNA damage. Cells were treated with 0.033% MMS, 2 μg/ml Phl or 0.2 M HU for the indicated time periods. (**C**) Percentage of cells containing Rad52-YFP foci in wild-type and *lsm1Δ* strains untreated or treated with 0.02% MMS for 2 and 4 h.

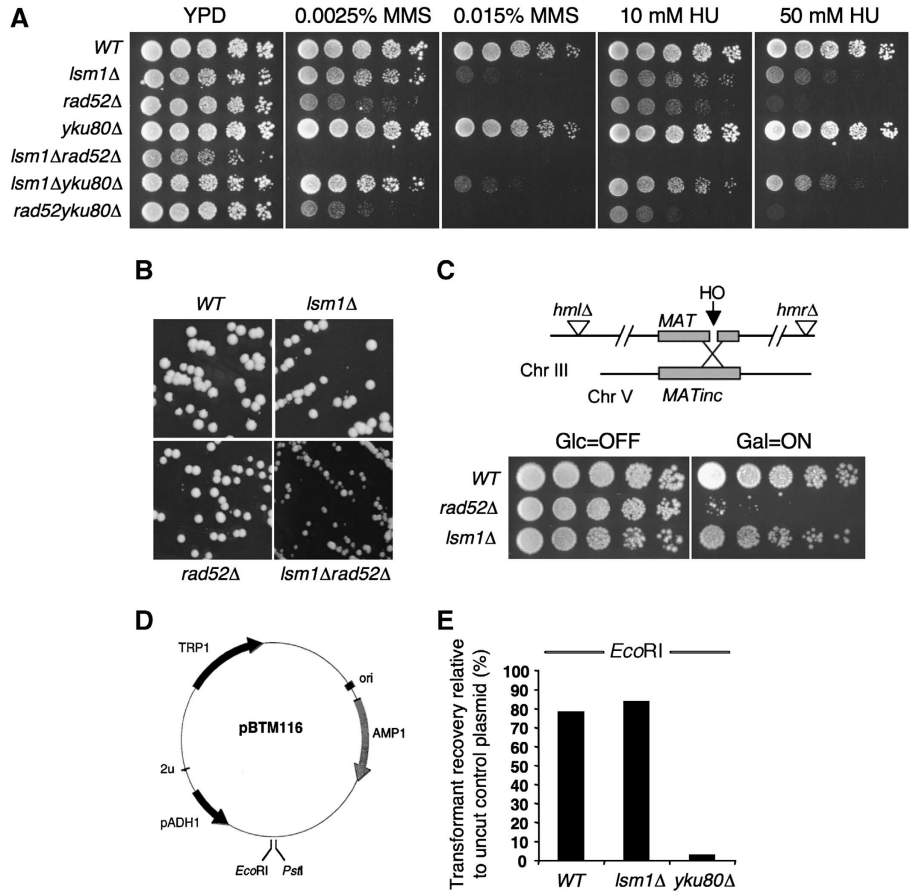

Figure 2 *lsm1*Δ cells are competent to repair DSBs by HR and NHEJ. (**A**) Sensitivity of different mutants to DNA-damaging drugs. (**B**) Colonies formed by different strains in YPD medium after 2 days of growth at 30°C. (**C**) Schematic representation of the strain GA2321 used to monitor HR-dependent repair of HO endonuclease-induced DSB. In this strain, both *HML* and *HMR* loci are deleted and a non-cleavable copy of the *MAT* locus is integrated on chromosome V. Expression of HO endonuclease is controlled by the GAL1,10 promoter. Cells were grown overnight in a raffinose-containing medium and then plated on media containing galactose (HO ON) or glucose (HO OFF). (**D**) Plasmid map of the vector pBTM116 used to monitor NHEJ repair. (**E**) Percentage of transformant recovery. Each strain was transformed with equal amounts of linear or uncut plasmid, and the number of colonies obtained with the linearized plasmid was normalized to the obtained from uncut plasmid.

As shown in Figure 2E, transformant recovery in wild-type and *lsm1*Δ cells was very similar in both cases around 80%. As expected, *yku80*Δ cells, used as a control, exhibited very low transformant recoveries. Similar results were obtained for the repair of 3′-overhanging ends generated by the enzyme *Pst*1 (data not shown). Taken together, our results indicate that *lsm1*Δ cells are competent to repair DSBs by both HR and NHEJ, and, therefore, accumulation of DSBs seemed to be due to a higher incidence of DNA damage.

Lsm1 is necessary to maintain replication-fork stability

HR is important not only for the repair of DSB, but also for the recovery of stalled or collapsed replication forks (Herzberg *et al*, 2006; Lambert *et al*, 2007). The strong requirement of HR for proper growth of *lsm1*Δ cells, together with their hypersensitivity to MMS and HU, that cause replication-fork slow down or stalling, suggests that Lsm1 may be defective in the stabilization of, or progression through, stalled replication forks. To test this hypothesis, we first monitored S-phase progression in wild-type and *lsm1*Δ strains in the presence or absence of MMS by flow cytometry (Figure 3A). Cells were synchronized in G1 with α factor and then released into fresh medium, without drug or with 0.033% MMS. At this concentration, MMS reduces the rate of replication-fork progression in wild-type cells (Tercero and Diffley, 2001). In the absence of MMS, both wild-type and *lsm1*Δ cells reached a 2C DNA content in approximately 1 h (data not shown). However, *lsm1*Δ was found to be defective in S-phase progression in the presence of MMS (Figure 3A, grey line) compared with the wild-type strain (dark line). This defect resulted in loss of viability as shown in Figure 3B. We then analysed activation and inactivation of Rad53. Wild-type and *lsm1*Δ cells were synchronized in G1 and released into medium containing 0.033% MMS for 1 h to activate Rad53 (Figure 3C). MMS was then washed out and cells were released into drug-free medium. Rad53 remained activated much longer in *lsm1*Δ cells than in the wild-type strain. Moreover, we found an increased phosphorylation of H2A in *lsm1*Δ cells after treatment with MMS, suggesting a higher incidence of DSBs in the *lsm1*Δ strain (Figure 3C).

Completion of DNA replication upon treatment with MMS in S-phase was monitored by pulsed field gel electrophoresis (PFGE). This technique allows distinction between linear chromosomal DNA, which enters the gel, from DNA containing replication bubbles, which is trapped inside the agarose plugs and stays in the loading well. Cells were arrested in G1, washed and treated with MMS as described above (Figure 3C). Samples were taken at 50, 90, 120 and 150 min

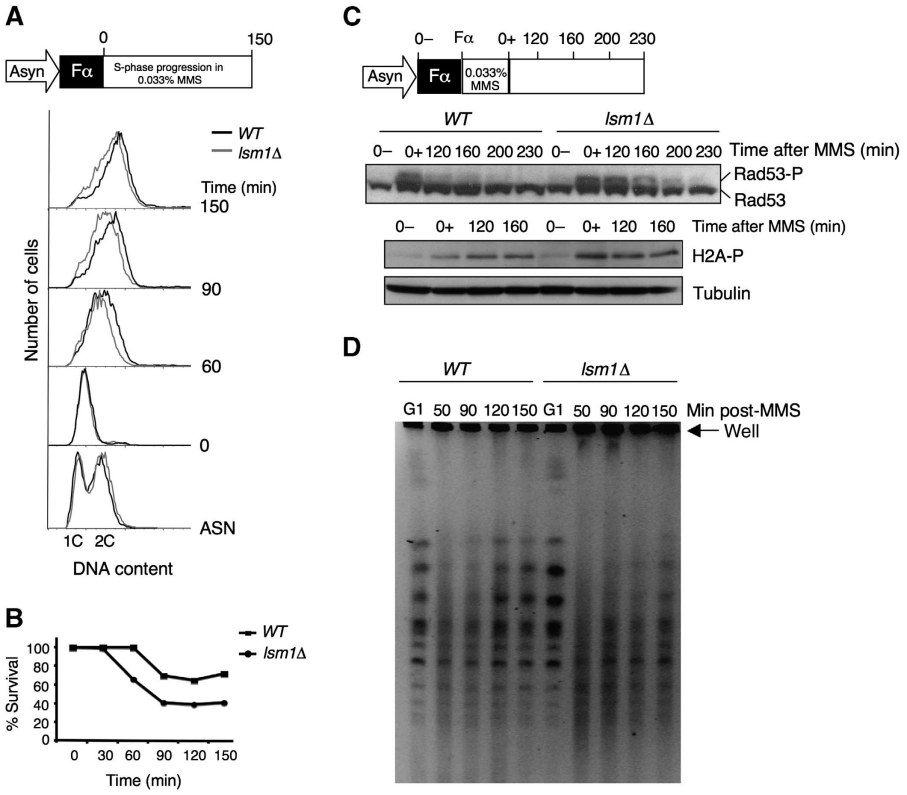

Figure 3 Lsm1 is necessary for the recovery of replication-fork stalling induced by MMS. (**A**) Schematic representation of the experiment and FACS analysis of the S-phase progression in wild-type and *lsm1Δ* cells in the presence of 0.033% MMS. (**B**) Percentage of survival after treatment with 0.033% MMS. (**C**) Schematic representation of the experiment and Rad53 checkpoint kinase activation and histone H2A phosphorylation upon recovery from MMS damage in wild-type and *lsm1Δ* cells. (**D**) Analysis of completion of DNA replication by PFGE. Cells were treated as in (**C**) and samples were collected at the indicated points, embedded in agarose plugs and analysed by PFGE.

after release from MMS treatment. In both wild-type and *lsm1*Δ cells, intact chromosomal DNA was separated as individual bands in G1-arrested cells, whereas most of the DNA was retained in the loading well 50 min after drug exposure in S-phase (Figure 3D). Chromosomal DNA re-entered the gel approximately 2 h after drug release in wild-type cells. In contrast, most of the DNA from *lsm1*Δ cells remained in the well throughout the recovery period, which clearly revealed a defect in the completion of DNA replication after MMS treatment.

Next, we determined whether Lsm1 was also important for recovery after replication stalling induced by nucleotide depletion. Thus, we assessed the activation status of Rad53 in wild-type and *lsm1*Δ strains both after 3 h of treatment with HU and following drug removal (Figure 4A). Compared with the wild-type strain, in the *lsm1*Δ mutant, we observed a further increase in the degree of Rad53 activation after treatment with HU, as revealed by the stronger intensity of the Rad53 phosphorylated form (Figure 4A, time 0). Prolonged Rad53 checkpoint kinase activation upon removal of HU was also evident. This result indicated that the *lsm1*Δ strain suffered more DNA damage that the wild-type strain after treatment with HU. The persistence of Rad53 phosphorylation also suggested that the *lsm1*Δ strain exhibits a delay in recovery from replication stress. We, therefore, analysed the DNA content of wild-type and *lsm1*Δ strains after release from an HU-induced cell-cycle arrest (Figure 4B). As expected, *lsm1*Δ cells exhibited a clear delay in progression through S-phase as compared with the wild-type strain.

Finally, we looked at the effect of *LSM1* deletion on the integrity of DNA replication forks arrested at natural pause sites. Fork pausing at the rDNA array induces HR, which results in the accumulation of extra-chromosomal circles (ERCs) (Sinclair and Guarente, 1997). Deletion of *FOB1*, encoding a protein required for replication-fork blocking, suppresses the elevated pausing, reducing DSB occurrence and ERC formation (Defossez *et al*, 1999). The consequences of replication-fork instability can thus be measured at the rDNA locus through the quantification of ERCs. Genomic DNA was obtained from wild type, *lsm1*Δ, *fob1*Δ and ERCs were detected by Southern blot using an rDNA probe. *lsm1*Δ cells contained higher levels of ERCs compared with the wild type, which clearly revealed a higher incidence of DSBs occurring at the rDNA locus of the *lsm1*Δ strain. As expected, ERCs were nearly undetectable in the *fob1*Δ strain. As a positive control, we used *rtt101*Δ that has been previously shown to accumulate ERCs (Luke *et al*, 2006).

These results suggested that Lsm1 is required for the stabilization of replication forks that move through damaged and paused DNA sites, which is supported by the analysis of synthetic genetic interactions of *lsm1*Δ with *mms4*Δ and *mus81*Δ, two genes involved in processing of stalled replication forks (Osman and Whitby, 2007). If deletion of *LSM1* results in the accumulation of stalled replication forks, we expected an *lsm1*Δ strain to require *MMS4* or *MUS81* for proper growth. Figure 4D shows that *lsm1*Δ *mus81*Δ and *lsm1*Δ *mms4*Δ double mutants form smaller colonies than single mutants, confirming our expectations.

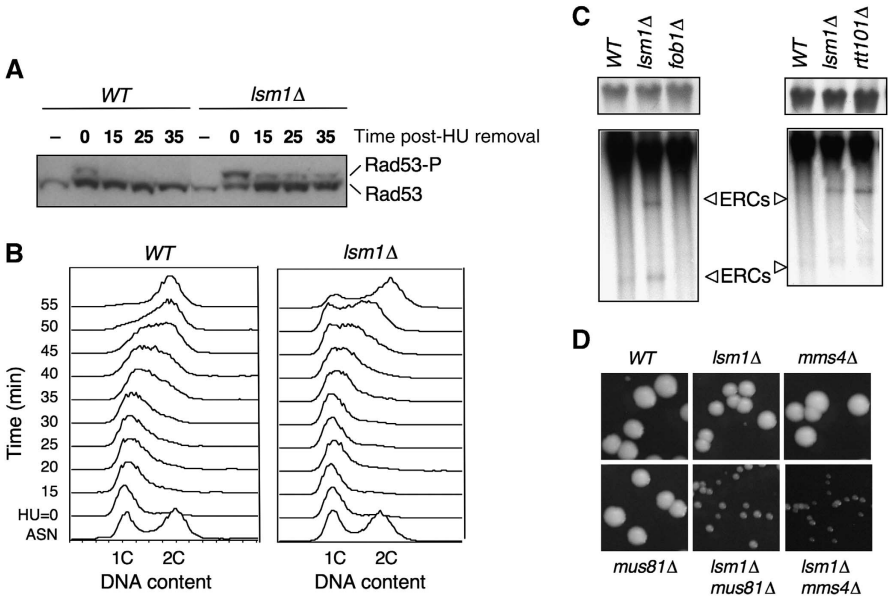

Figure 4 Lsm1 is required for the recovery of replication-fork stalling induced by nucleotide depletion (HU) and at natural pause sites. (**A**) Prolonged Rad53 checkpoint kinase activation upon removal of HU in *lsm1*Δ cells. The phosphorylation state of Rad53 was determined by western blot at the indicated time points. (**B**) Cell-cycle progression after treatment with HU is delayed in *lsm1Δ* cells. DNA contents of *WT* and *lsm1*Δ cells were determined by FACS analysis of mid-log phase cultures (ASN), cells synchronized in S-phase after treatment with 200 mM HU and at the indicated times after removal of the drug. (**C**) *lsm1*Δ cells accumulate ERCs. ERCs were detected by Southern blot hybridization using a radioactive rDNA probe. A shorter exposure of the signal corresponding to the rDNA array is shown in the upper panel. (**D**) Synthetic fitness interaction between *lsm1Δ, mms4*Δ and *mus81*Δ mutants.

Inactivation of the 3′–5′ (exosome) pathway in the lsm1Δ mutant enhances hypersensitivity to DNA-damaging drugs

Since Lsm1 is involved in mRNA degradation, we hypothesized that in its absence some proteins that interfere with replication-fork progression would be stabilized leading to the formation of aberrant DNA structures that need to be resolved by HR and replication re-start pathways. If our hypothesis is correct, we expected that a higher stabilization of the putative replication-fork interfering protein(s) would exacerbate the phenotype of *lsm1*Δ. As mentioned above, there are two general pathways for the degradation of mRNAs, the 5′–3′ and the 3′–5′. Disruption of both pathways leads to loss of viability as revealed by the synthetic lethality of mutants in components of the exosome, *ski2*Δ or *ski3*Δ, with the 5′–3′ exonuclease *xrn1*Δ (Anderson and Parker, 1998). However, since deletion of *LSM1* does not cause a complete block of decapping, *lsm1*Δ is not synthetically lethal with any of the *ski* mutations (He and Parker, 2001), although the double mutant probably presents stronger defects in mRNA degradation. A double mutant *lsm1Δ ski2*Δ was, therefore, obtained, and sensitivity to different DNA-damaging drugs analysed. Our results clearly showed that inactivation of the 3′–5′ pathway greatly increased the sensitivity of *lsm1*Δ cells to DNA damage (Figure 5A).

Lsm1 controls histone mRNA levels

To uncover the substrates of Lsm1 whose stabilization resulted in the instability of stalled replication forks, we took advantage of the recent findings in human cells indicating that LSM1 is required for histone mRNA degradation at the end of S-phase and following replication stress (Mullen and Marzluff, 2008). We tested the following questions: (i) Are histone mRNAs targets of degradation by the Lsm1 complex in yeast? (ii) Do *lsm1*Δ cells accumulate free histones? (iii) Is the putative excess of histones in *lsm1Δ* cells responsible of their replication-fork instability? To answer the first question, we analysed the abundance and decay rates of different histone mRNAs in wild-type, *lsm1*Δ, *ski2*Δ and *lsm1*Δ *ski2*Δ strains. Cells were collected from the exponential phase of growth and at different times upon inhibition of transcription with 1–10 phenanthroline. We found that deletion of *LSM1* increased the stability of all histone mRNAs analysed four- to six-fold (Figure 5B), whereas deletion of *SKI2* affected histone mRNA stability to a lesser extent (two-fold). Double mutant *lsm1*Δ *ski2*Δ exhibited a strong accumulation of histone mRNAs (6–10-fold). These results indicated that in budding yeast, degradation of histone mRNAs takes place mainly by the 5′–3′ degradation pathway and to a lower extent by the 3′–5′ degradation pathway.

At this point, we were interested in determining how many transcripts were upregulated in the *lsm1*Δ strain. For this purpose, we performed microarray analysis. Of a total of 5766 genes present in the microarray, 2888 genes were upregulated in the *lsm1*Δ compared with the wild type (Supplementary Table S3). Consistent with the northern blots, all the histones genes were upregulated.

Maintenance of a short half-life of histone mRNAs must be very important for proper regulation of histone levels when DNA replication is blocked. To test this hypothesis, we measured histone H3 mRNA degradation in wild-type and *lsm1*Δ cells following HU treatment. As shown in Figure 5C, histone H3 mRNA decay was severely impaired in *lsm1*Δ compared with the wild-type strain.

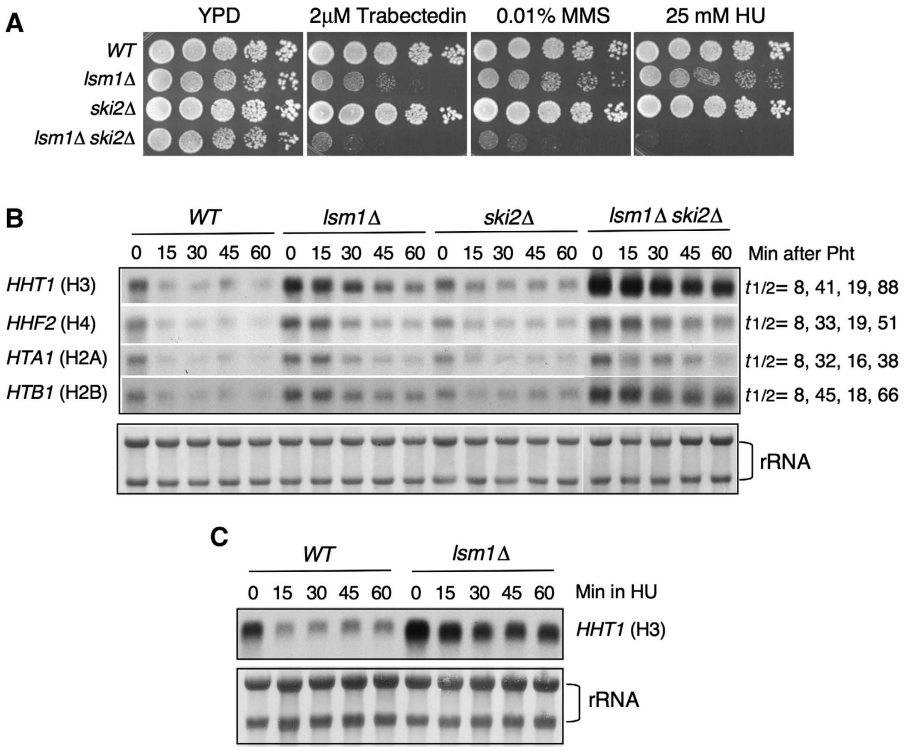

Figure 5 Sensitivity to DNA-damaging drugs and histone mRNA levels in mRNA decay mutants. (**A**) Deletion of *SKI2* increases sensitivity of *lsm1Δ* cells to DNA damage. Five-fold serial dilution of cultures of the indicated mutants were spotted on YPD or YPD containing different drugs at the indicated concentrations. (**B**) Lsm1 controls histone mRNA levels. Total RNA from the indicated strains were separated on a agarose formaldehyde gel and sequentially hybridized with different histone probes. Histone mRNAs was quantitated by densitometry analysis using Image J. The amount of mRNA was plotted against time to determine the half-lives of histone mRNAs. (**C**) Histone H3 mRNA levels after treatment with 0.2 M HU. Total rRNA stained with methylene blue was used as loading controls.

lsm1Δ cells are defective in the degradation of overexpressed histones

Histones are very stable proteins. However, excess histones that are not packaged into chromatin are degraded very rapidly. In *S. cerevisiae*, this degradation is dependent upon the catalytic activity of Rad53 (Gunjan and Verreault, 2003). Since *lsm1*Δ accumulates histone mRNAs, we reasoned that this strain might be accumulating an excess of free histones, even in the presence of a functional Rad53. We, therefore, performed a histone degradation assay as described previously (Gunjan and Verreault, 2003). Strains expressing HA-tagged histone H3 were arrested in G1 to prevent overexpressed histones being packaged into chromatin. Histone H3 overexpression was then induced by addition of galactose. Transcription repression was mediated by switching half of the cultures to a glucose-containing medium. Cycloheximide was added to the other half of the cultures to repress protein synthesis. As shown in Figure 6A, histone degradation is severely delayed in *lsm1*Δ cells compared with the wild type without cycloheximide. This defect must be due to the translation of stabilized histone mRNAs, since histone protein degradation occurs normally in the *lsm1*Δ strain treated with cycloheximide.

lsm1Δ cells are hypersensitive to histone overexpression

It has been previously shown that *rad53*Δ cells, exhibiting defects in histone degradation, are sensitive to histone overexpression (Gunjan and Verreault, 2003). If Lsm1 is indeed important to avoid an excess of free histones in the cell, we expected *lsm1*Δ cells to be also hypersensitive to histone overexpression. Wild-type, *lsm1*Δ and *lsm1*Δ *ski2*Δ strains were transformed with plasmids encoding histone genes under the control of the *GAL1* promoter or with an empty vector. Transformants were then grown in a raffinose-containing medium and serial dilutions of the cell cultures were plated on glucose (promoter OFF) or galactose (promoter ON)-containing media. As shown in Figure 6B, the growth of wild-type cells was not affected by histone H3 overexpression and was minimally affected by overexpression of histones H2A–H2B or H4. In contrast, *lsm1*Δ cells were found extremely sensitive to overexpression of the four core histones (Figure 6B) as also was *lsm1Δ ski2*Δ (data not shown).

A reduction in histone H3–H4 or H2A–H2B gene dosage suppresses the sensitivity of lsm1Δ cells to DNA-damaging drugs

The results shown above indicate that Lsm1 exerts an essential role in the maintenance of proper histone levels. We next wondered whether this function was related to the role of Lsm1 in promoting replication-fork stability. If this was the case, we expected *lsm1*Δ phenotypes to be alleviated by a reduction in histone gene dosage. Each of the four core histones, H3, H4, H2A and H2B, is encoded by a pair of nearly identical genes, *HHT1-HHT2, HHF1-HHF2, HTA1-HTA2* and *HTB1-HTB2*, respectively. It has been previously shown that wild-type cells lacking the gene pair *HHT2-HHF2* grow surprisingly well because *HHT1-HHF1* alone probably

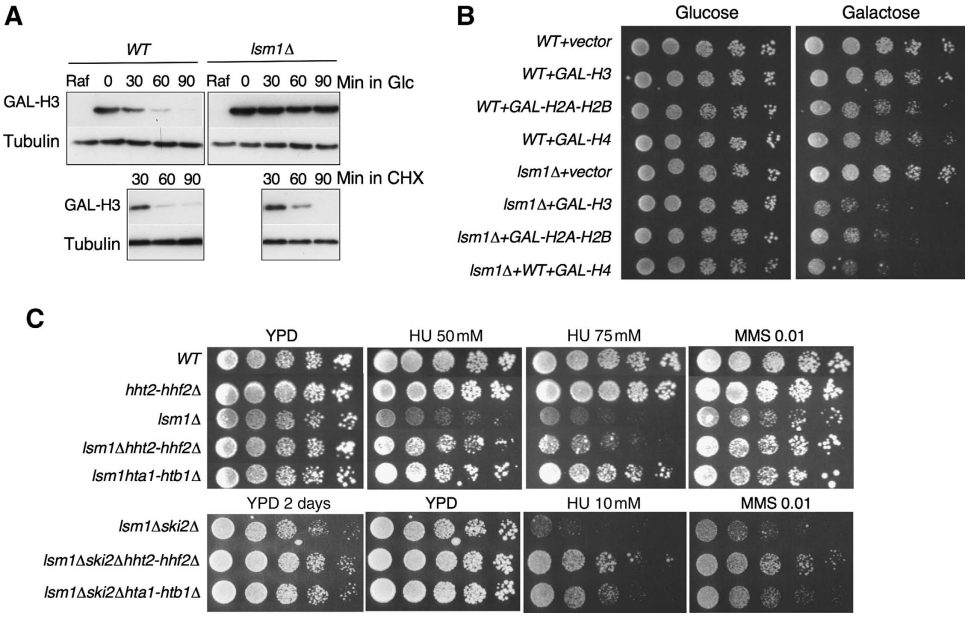

Figure 6 *lsm1*Δ cells accumulate free histones that make replication forks sensitive to drug-induced replication-fork stalling. (**A**) Degradation of overexpressed histones is impaired in *lsm1*Δ cells. Wild-type and *lsm1*Δ strains carrying the 2 μ-*URA3-GAL-HA-HHT2* plasmid (pGAL-H3) were grown overnight in minimal medium lacking uracil and with raffinose as a carbon source. Cells were synchronized in G1 with α factor for 2 h and galactose was added to the medium for 1 h. Half of the cultures were then collected and transferred to a glucose-containing medium, and the other half was transferred to the same medium containing galactose, but with the addition of cycloheximide at 35 μg/ml. Samples were taken every 30 min after switching to glucose or cycloheximide. (**B**) Effect of histone overexpression on the growth of WT, *lsm1*Δ and *lsm1*Δ *ski2*Δ strains. Strains transformed with empty vector or with plasmids expressing histones H3, H4 or H2A–H2B under the control of GAL promoter were grown overnight in minimal medium without uracil and with 2% raffinose as a carbon source. Five-fold serial dilutions of each strain were plated on the same medium, either with glucose or galactose, as carbon source. The plates were incubated for 4 days at 30°C. (**C**) Deletion of either *hht2-hhf2* or *hta1htb1* histone gene pairs significantly suppresses the DNA damage sensitivity of the *lsm1*Δ and *lsm1*Δ *ski2*Δ strains.

provides sufficient amounts of histones for nucleosome assembly (Gunjan and Verreault, 2003). However, deletion of the *HTA1-HTB1* gene pair causes loss of viability (Libuda and Winston, 2006). Recently, Libuda and Winston (2010) performed a genome-wide screen in *S. cerevisiae* looking for mutations that suppressed the loss of viability caused by *HTA1-HTB1* deletion. Interestingly, *LSM1*, *LSM6* and *LSM7* were found among the genes that increased viability. Based on these data, we decided to lower the dose of histone H3–H4 in wild-type and *lsm1*Δ cells and H2A–H2B in *lsm1*Δ cells, and analyse the effect on the sensitivity to drugs that cause replication-fork stalling. Deletion of *HHT2-HHF2* in the wild-type strain had no effect in the sensitivity of the strain to MMS or HU. In contrast, we found that deletion of either *HHT2-HHF2* or *HTA1-HTB1* histone genes pairs significantly suppressed the DNA damage sensitivity of the *lsm1*Δ and *lsm1Δ* ski2Δ strains to both MMS and HU. Moreover, a lower dose of histone H3–H4 or H2A–H2B in the *lsm1Δ* ski2Δ strain also suppressed its slow growth phenotype. Our results strongly indicate that DNA damage sensitivity of the *lsm1*Δ mutants is mostly due to the harmful effect of excess histones and that the Lsm1-7-Pat1 complex controls the levels of histone mRNAs in the cell.

Discussion

Histone synthesis and degradation is tightly coupled to DNA synthesis in order to ensure correct chromatin assembly during S-phase. In this study, we have identified Lsm1 as a new factor that contributes to genomic stability in the yeast *S. cerevisiae* by promoting histone mRNA decay.

In wild-type cells, treatment with MMS activates the DNA damage checkpoint, which results in a slowing down of replication-fork progression and inhibition of origin firing (Tercero and Diffley, 2001). However, these effects are transient and replication continues once the damage is repaired or bypassed. In contrast, *lsm1*Δ cells prolong the activation of the S-phase DNA damage checkpoint and are defective in completing chromosome replication after treatment with MMS. The same phenotype is observed when replication-fork progression is blocked by treatment with HU. Moreover, replication-fork stalling induced by HU results in an increased DNA damage in the *lsm1*Δ cells compared with the wild-type strain, as revealed by the stronger intensity of the Rad53 phosphorylated form. These phenotypes indicate that Lsm1 is required to maintain the stability of stalled replication forks.

HR is an important mechanism, not only for the repair of DSBs, but also for the re-start of stalled or collapsed replication forks (Herzberg *et al*, 2006; Lambert *et al*, 2007). However, this is a double-edged sword, since unscheduled HR also leads to increased recombination and genomic instability (Tourriere and Pasero, 2007). We found that *lsm1*Δ cells are fully proficient to repair DNA DSBs, but accumulate this type of lesion, even in the absence of exogenous DNA damage. Moreover, *lsm1*Δ mutant cells required the HR protein Rad52 for proper growth, exhibited a recombination frequency three times higher than the wild-type strain (Supplementary Figure S1) and had a strong requirement for Mus81–Mms4, a complex involved in the

rescue of stalled replication forks (Osman and Whitby, 2007), for proper growth. These observations indicate that Lsm1 has a role in the maintenance of replication forks that stall during normal S-phase. In accordance with this conclusion, deletion of *LSM1* also results in the instability of replication forks that encounter natural impediments in the DNA, such as replication-fork barriers in the rDNA locus.

Several lines of evidence suggest that the role of Lsm1 in promoting genomic stability is related to its function in the degradation of mRNAs. First, it has been demonstrated that Lsm1 is mostly a cytoplasmic protein (Tharun *et al*, 2000). Therefore, the possibility of exerting a direct role in the maintenance of genomic stability in the nucleus is unlikely. Second, some other non-essential members of the Lsm1-7-Pat1 complex were found in our genome-wide screen of mutants hypersensitive to trabectedin, such as *lsm6*Δ and *pat1*Δ. Third, the inactivation of the second route of mRNA decay, the 3′–5′ pathway, in the *lsm1*Δ mutant highly increased its sensitivity to DNA-damaging drugs. Based on these evidences, we hypothesized that in the absence of Lsm1 some proteins that interfere with replication-fork progression would be stabilized, leading to the formation of aberrant DNA structures that need to be resolved by HR and replication re-start pathways.

The Rad53 protein monitors excess histones and targets them for degradation by ubiquitylation-dependent proteolysis (Gunjan and Verreault, 2003; Singh *et al*, 2009). This mechanism prevents the accumulation of free histones when replication slows down or stops. Excess histones accumulating in *rad53*Δ result in slow growth, DNA damage sensitivity and chromosome loss. These phenotypes are significantly supressed by a reduction in histone gene dosage. Another way to control histone levels in *S. cerevisiae* is transcriptional repression mediated by the Hir proteins (Sherwood *et al*, 1993). In animal cells, regulation of histone synthesis occurs both transcriptionally and post-transcriptionally (Sittman *et al*, 1983; Ye *et al*, 2003; Kaygun and Marzluff, 2005; Mullen and Marzluff, 2008). Post-transcriptional regulation consists of rapid degradation of histone mRNAs, both after inhibition of replication and at the end of a normal S-phase. Histone mRNAs are the only mRNAs in mammalian cells that are not polyadenylated. Instead, they end in a stem-loop structure that is recognized by the stem-loop binding protein (SLBP). It has been shown that histone mRNA degradation in human cells requires the protein Upf1, which interacts with the SLBP at the 3′ end of histone mRNAs after treatment with HU (Kaygun and Marzluff, 2005). Recent results obtained by Mullen and Marzluff (2008) postulate that the recruitment of Upf1 results in the recruitment of terminal uridilyl transferases that carry out the oligouridylation of histone mRNAs. Interestingly, these authors show that the Lsm1-7 complex recognizes and binds to the uridylated 3′ end of the mRNAs, possibly triggering the recruitment of decay factors. In *S. cerevisiae*, histone mRNAs do not terminate in a stem loop and both SLBP and uridylating enzymes are absent (Gunjan *et al*, 2005). However, we show here that Lsm1 is also very important for the control of histone mRNA levels in yeast. Deletion of *LSM1* results in a strong stabilization of histone mRNAs, which leads to the accumulation of abnormally high amounts of histones in the cell. It is possible that the Lsm1-7-Pat1 complex ensures a very short half-life of histone mRNAs by recognizing U-tracts that are present in the 3′ ends of different histone mRNAs, since it has been shown that the Lsm1-7-Pat1 complex has a strong binding preference for mRNAs carrying a U-tract over those that do not (Chowdhury *et al*, 2007). This short half-life of the histone mRNAs is very important for the proper regulation of histone levels by the known transcriptional (Hir proteins) and post-translational (Rad53) mechanisms. Lsm1 influences yeast histone levels in such a way that deletion of *LSM1* leads to the accumulation of histones, even in the presence of a functional Rad53 and intact transcriptional regulation mechanisms. In line with the notion that Lsm1 exerts a strong control over histone levels, we found that the double mutant *lsm1*Δ *rad53*Δ is lethal, as are *lsm1*Δ *hir1*Δ and *lsm1*Δ *hir2*Δ, as revealed by genome-wide synthetic lethal interactions (Pan *et al*, 2006).

Mammalian histone mRNAs are regulated both at the level of transcription and post-transcriptionally (Sittman *et al*, 1983). Unlike their mammalian counterparts, yeast histone mRNAs were traditionally thought of being regulated primarily at the level of transcription due to the very short half-lives of these transcripts, particularly in response to replication arrest, while any contribution of post-transcriptional regulation of histone mRNAs was dependent upon the 3′ ends of the histone mRNAs (Lycan *et al*, 1987). Later, it was suggested that the poly-A polymerases Trf4/5 and the exosome was having a role in post-transcriptional regulation of histone mRNAs in yeast (Reis and Campbell, 2007). More recently, Lsm4 (Mazzoni *et al*, 2005) and Lsm1 have been implicated in the histone regulation in yeast (Palermo *et al*, 2010) which, together with our findings, strongly suggest that the traditional view that yeast histone mRNAs are largely regulated at the level of transcription needs to be revised to accommodate the important contribution of post-transcriptional mechanisms in their regulation. This would suggest that despite the significant differences in the structure of yeast and mammalian histone mRNAs, and in the mechanistic details of their regulation, the overall principles governing the regulation of yeast and mammalian histone mRNAs are essentially the same, indicating that yeast is an excellent model for the study of histone regulation and its impact on genomic stability.

It has been demonstrated that accumulation of free histones in *rad53*Δ cells leads to chromosome loss, a form of genomic instability. Here, we report that *lsm1Δ* cells accumulate DNA DSBs, known to induce genomic instability, are hyper-recombinant and exhibit instability at the rDNA loci. Since the Lsm1-7-Pat1 complex is highly conserved throughout evolution, we anticipate that defects in this complex will also lead to genomic instability in higher eukaryotes. In this regard, a genome-wide screen recently carried out by Paulsen *et al* (2009) identified many mRNA processing factors involved in this process. The gene encoding Lsm6, a subunit of the Lsm1-7 complex, was among those identified.

Interestingly, it has been reported that many cancers present alterations in the human *LSM1* gene (Takahashi *et al*, 2002; Fraser *et al*, 2005; Streicher *et al*, 2007; Watson *et al*, 2008). The molecular mechanisms by which alterations of *LSM1* lead to neoplastic transformation remain largely unknown. Our results suggest that malignant transformation could be related to the role of Lsm1 in the maintenance of genomic stability through the regulation of histone mRNA levels. Future experiments will be carried out to verify this hypothesis.

Materials and methods

Yeast strains and media

Yeast strains and plasmids used in this study are listed in Supplementary Tables SI and SII. Standard yeast media and growth conditions were used.

Spot assays for analysing sensitivity to DNA-damaging agents

Yeast strains were grown in YP medium, unless otherwise specified, until they reached exponential growth. Cells were harvested by centrifugation and adjusted to an OD_{600} of 0.2.5 μl of undiluted cell culture and 1/5 serial dilutions of each cell culture were spotted onto plates containing different drugs at the indicated concentration. Plates were incubated at 30°C for 3 days.

Protein extracts and western blots

Protein extracts were obtained by TCA precipitation as described previously (Foiani *et al*, 1994). For Rad53 detection, protein extracts were run in 7.5% SDS–PAGE gels, transferred to nitrocellulose and incubated with anti-Rad53 antibodies (Santa Cruz Biotechnology, Inc) at 1/500 for 3 h. Equivalent loading in each lane was confirmed by Ponceau staining. Anti-goat antibodies were used as secondary antibodies at 1:3000 dilution and incubated for 1 h at room temperature. For detection of HA-tagged H3, extracts were resolved in 12% SDS–PAGE gels and probed with mouse anti-HA 12CA5 antibodies (Roche Applied Sciences) at 1/3000. For detection of tubulin, mouse anti-tubulin antibodies (a gift of Dr Keith Gull) were used in a 1/3000 dilution. The immunoblots were developed using ECL™ western blotting detection reagents (Amersham, UK).

Detection of Rad52-YFP

Rad52-YFP foci from exponentially growing cells transformed with plasmid pWJ1213 were visualized by fluorescence microscopy using a Leica DM6000B microscope controlled by the MetaMorph software. Three independent experiments counting 300 cells each time were performed for each strain and condition.

NHEJ assays

The plasmid repair assay for NHEJ was performed as previously described (Boulton and Jackson, 1996). Briefly, plasmid pBTM116 was digested with the appropriate restriction enzyme and then heat inactivated by treatment at 65°C for 20 min. The same amount of uncut or linearized plasmid DNA was used to transform each strain with the lithium acetate method. Diluted samples were plated on minimal media lacking the appropriate amino acids, and colonies were counted after incubation at 30°C for 4–5 days. Percentage of transformants relative to uncut plasmid determined the efficiency of NHEJ.

Frequency of recombination

The frequency of HR was determined by using the plasmidic inverted repeat system SU. This system is based on two truncated *leu2* alleles that share a 0.6-kb internal fragment. HR between the repeats leads to the inversion of the intervening sequence and the formation of an *LEU2* wild-type copy (Prado and Aguilera, 1995). Spontaneous recombination frequencies were obtained by fluctuation test as the median value of six independent colonies as described previously (Prado and Aguilera, 1995).

Fluorescence-activated cell sorting analysis

Samples of 2×10^7 cells were collected by centrifugation, fixed in 70% ethanol and processed for flow cytometry as described previously (Hutter and Eipel, 1979). A Becton Dickinson (Mountain View, CA) FACSCalibur was used to determine the DNA content.

Pulse-field gel electrophoresis

For PFGE analysis, 6×10^8 cells were washed with 1 ml of 10 mM Tris, 50 mM EDTA and 0.1% sodium azide, pH 8. Agarose plugs containing chromosomal DNA from 5×10^7 cells were prepared as described previously (Lengronne *et al*, 2001). Electrophoresis was performed for 24 h at 6 V/cm with a switch time of 60–120 s in 0.5 × TBE at 14°C. The gels were stained with 0.5 g/ml ethidium bromide for 20 min, destained in deionized water for 20 min and then photographed.

Extra-chromosomal circles

For the detection of ERCs, genomic DNA was extracted and purified using the DNAeasy Blood and tissue kit from Quiagen. Equal amounts of DNA (1.5 μg) were separated on a 0.7% agarose gel at 1 V/cm for 24 h. rDNA species were detected by Southern blot using a radioactive rDNA fragment, *RDN25*.

RNA extraction and northern blots

Total RNA from cells was obtained following the recommendations of the RNAeasy Mini kit from Qiagen. A total of 5 μg of each sample were separated on a agarose formaldehyde gel and northern blotting was carried out following the protocol of the ExpresssHybTM Hybridization Solution (BD Biosciences). Probes corresponding to different mRNAs were obtained by PCR and labelled with [α-32P]dCTP. The Rediprime II Random Prime labelling System kit (Amersham, UK) was used.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: ABH and SM conceived and designed the experiments. ABH performed the experiments and analysed the data. ABH and SM wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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