DNA damage regulates direct association of TOR kinase with the RNA polymerase II–transcribed HMO1 gene

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ABSTRACT The mechanistic target of rapamycin complex 1 (mTORC1) senses nutrient sufficiency and cellular stress. When mTORC1 is inhibited, protein synthesis is reduced in an intricate process that includes a concerted down-regulation of genes encoding rRNA and ribosomal proteins. The Saccharomyces cerevisiae high-mobility group protein Hmo1p has been implicated in coordinating this response to mTORC1 inhibition. We show here that Tor1p binds directly to the HMO1 gene (but not to genes that are not linked to ribosome biogenesis) and that the presence of Tor1p is associated with activation of gene activity. Persistent induction of DNA double-strand breaks or mTORC1 inhibition by rapamycin results in reduced levels of HMO1 mRNA, but only in the presence of Tor1p. This down-regulation is accompanied by eviction of Ifh1p and recruitment of Crf1p, followed by concerted dissociation of Hmo1p and Tor1p. These findings uncover a novel role for TOR kinase in control of gene activity by direct association with an RNA polymerase II–transcribed gene.

INTRODUCTION
Mechanistic target of rapamycin (TOR) kinase is an evolutionarily conserved serine/threonine protein kinase that may be incorporated into two functionally distinct complexes, mTORC1 and mTORC2 (Loewith and Hall, 2011). In Saccharomyces cerevisiae, mTORC1 contains either Tor1p or Tor2p in complex with Kog1, Lst8, and Tco89, and the complex is sensitive to rapamycin, which inhibits mTORC1 activity and mimics responses to nutrient limitation (Loewith et al., 2002; Wedaman et al., 2003). In contrast, mTORC2 contains Tor2p and is generally insensitive to rapamycin (Wullschleger et al., 2005). mTORC1 appears to be constitutively localized to the vacuole, which is a significant nutrient reservoir, rationalizing this cellular localization (Sturgill et al., 2008; Binda et al., 2009). While mTORC1 is active under nutrient sufficiency and promotes growth by phosphorylating downstream targets and controlling their cellular localization, nutrient starvation and rapamycin treatment results in a significant reduction in protein synthesis, in part by blocking initiation of translation (Barbet et al., 1996; Hardwick et al., 1999; Shamji et al., 2000; Huber et al., 2009). In addition, decreased mTORC1 activity is also associated with various stress conditions, including DNA damage (Reiling and Sabatini, 2006; Urban et al., 2007; Workman et al., 2014; Desantis et al., 2015). The cell cycle arrest that accompanies unrepaired DNA damage must be coordinated with metabolic arrest, and this process requires mTORC1 (Shen et al., 2007; Budanov and Karin, 2008; Dulic, 2013).

Two of the downstream targets of mTORC1, the AGC kinase Sch9p and the transcription factor Sfp1p have been implicated in activating expression of ribosomal protein (RP) genes and genes whose products are involved in ribosomal biogenesis (Ribi genes); together, these genes may account for ∼50% of transcriptional initiation by RNA polymerase (Pol) II (Rudra and Warner, 2004; Martin et al., 2006). In contrast, regulation of some Pol I– and Pol III–transcribed genes may be direct; Tor1p has been shown to bind to the yeast RNA Pol I– and III–transcribed 35S and 5S rRNA genes to activate transcription, whereas nutrient limitation and rapamycin treatment results in dissociation of Tor1p and its exit from the
nucleus (Li et al., 2006; Wei et al., 2009). On the RNA Pol III–transcribed 5S rDNA (ribosomal DNA encoding rRNA), chromatin-bound mTORC1 is responsible for regulating phosphorylation of the negative regulator Maf1 (Wei et al., 2009). Direct regulation of transcription by TOR kinase is conserved in mammals, where mTOR was shown to interact with the Pol III transcription factor TFIIC (Kantidakis et al., 2010; Tsang et al., 2010).

The yeast high-mobility group protein Hmo1p has been implicated in communicating mTORC1 signaling to downstream target genes (Hall et al., 2006; Xiao and Grove, 2009; Panday and Grove, 2017). It binds RP gene promoters and rDNA, and its presence is required for efficient down-regulation of gene activity when mTORC1 is inhibited (Gadal et al., 2002; Hall et al., 2006; Berger et al., 2007). On many RP gene promoters, Hmo1p assembles the transcription factors Forkhead-like 1 (Fhl1p) and Interacts with Fhl1p (Ifh1p) (Hall et al., 2006). A DNA sequence motif found in genes to which Fhl1p and Ifh1p bind, the IFHL motif is required for maximal RP gene transcription (Wade et al., 2004). Hmo1p and Fhl1p mutually promote binding of the other protein; it has been proposed that Fhl1p remains associated and recruits Ifh1p to promote transcription, whereas inhibition of mTORC1 results in nuclear export of phosphorylated Corepressor with Fhl1p (Crf1p), which displaces Ifh1p, resulting in repression of gene activity (Jorgensen et al., 2004; Martin et al., 2004; Schwalder et al., 2004; Wade et al., 2004; Rudra et al., 2005; Zhao et al., 2006). Because Hmo1p promotes binding of Fhl1p, its absence results in an attenuated response to rapamycin. Repression of RP gene expression was also reported to result in dissociation of Hmo1p and a concomitant ~20-base pair upstream shift of the +1 nucleosome that buries the transcription start site within the nucleosome (Knight et al., 2014; Reja et al., 2015).

Hmo1p also localizes to its own promoter, and it negatively regulates its own expression. HMO1 promoter activity is likely stimulated by Fhl1p, as evidenced by reduced expression in hmo1Δ cells upon mutation of the IFHL site, which is located ~605 base pairs upstream of the translational start (Figure 1A) (Xiao et al., 2011). Consistent with this inference, HMO1 promoter activity is reduced in the presence of rapamycin, and this response is attenuated in hmo1Δ. Because Fhl1p binds the HMO1 promoter, centered on the IFHL site, it is conceivable that the mechanism of the HMO1 gene repression that occurs on inhibition of mTORC1 is similar to that proposed for RP genes.

We show here that Tor1p binds directly to the HMO1 gene to activate expression, particularly in the absence of Hmo1p. Persistent induction of DNA double-strand breaks (DSBs) or inhibition of mTORC1 by rapamycin results in a Tor1p-dependent reduction in HMO1 mRNA levels that is accompanied by fast displacement of Ifh1p and subsequent recruitment of Crf1p, followed by eviction of Hmo1p and Tor1p from the region surrounding the HMO1 transcriptional start. Our data suggest that Tor1p controls gene activity by direct association with the HMO1 gene, indicating that direct regulation of gene activity by promoter-bound TOR kinase includes RNA Pol III–transcribed genes.

RESULTS
Tor1p activates HMO1 expression
Using reporter constructs in which the lacZ gene encoding β-galactosidase is under control of the HMO1 promoter, we previously reported that absence of Hmo1p results in increased promoter activity, whereas overexpression of Hmo1p leads to repression by comparison to wild-type cells (Xiao et al., 2011). Considering that Hmo1p is implicated in coordinating responses to mTORC1 inhibition by rapamycin on rRNA, RP, and HMO1 genes, we created a tor1Δ strain and measured HMO1 promoter activity (Figure 1B). As previously reported, increased promoter activity was observed on deletion of HMO1 (~2.4-fold), indicating that Hmo1p represses its own expression (Figure 1B). In a tor1Δ background, deletion of HMO1 likewise resulted in increased promoter activity (~2.2-fold increase in promoter activity in the hmo1Δtor1Δ strain compared with tor1Δ). Deletion of TOR1 reduced gene activity, particularly in cells deleted for HMO1. Measurement of HMO1 transcript levels in wild-type (WT) and tor1Δ cells confirmed the modestly reduced HMO1 expression in tor1Δ cells (~20%; Figure 1D). This suggests that Tor1p directly or indirectly functions to activate the HMO1 promoter, particularly in the absence of Hmo1p. The data also suggest that Hmo1p-mediated repression of the HMO1 promoter activity occurs regardless of Tor1p.

If HMO1 promoter activity were controlled by mTORC1 activity, and because both Tor1p and Tor2p may be components of mTORC1, the inability of Tor2p to compensate for loss of Tor1p was somewhat unexpected. A cognate site for Fhl1p was previously identified ~605 base pairs upstream of the HMO1 start codon (IFHL; Figure 1A); Fhl1p was shown to bind preferentially to this genomic location, and its binding was shown to depend on Hmo1p (Xiao et al., 2011). Mutation of this site resulted in failure to activate the HMO1 promoter in the absence of Hmo1p, and deletion of TOR1 did not reduce gene activity (Figure 1C). Taken together, these data suggest that Tor1p-mediated activation of the HMO1 promoter requires the IFHL site and that Hmo1p represses activation by factor(s) that also require the IFHL site.

FIGURE 1: Activity of the HMO1 promoter depends on Tor1p and the IFHL site. (A) Region upstream of HMO1 ORF (black). The IFHL site (red) is centered ~605 base pairs upstream of the HMO1 start codon and followed by three predicted Reb1p binding sites. Positions of amplicons used in ChIP shown below. (B) Activity of HMO1 promoter using lacZ reporter constructs. Activity is reported in Miller units. (C) Activity of HMO1 promoter using lacZ reporter constructs in which IFHL site is mutated. Activity is reported in Miller units. (D) HMO1 expression in WT and tor1Δ cells. The mRNA levels are reported relative to IPP1 and calculated using the ΔCt method. Data represent mean and SD from three independent experiments.

Tor1p binds the HMO1 gene and is required for reduced expression after persistent DSB
Persistent DNA damage is associated with an mTORC1–dependent reduction in metabolic activity. Hmo1p is also linked to the DNA damage response because it is
FIGURE 2: Reduced HMO1 expression after DSB induction requires Tor1p. (A, D) HMO1 expression in WT and tor1Δ cells before (Ctrl; gray bar) and after induction of HO endonuclease expression by addition of galactose (orange bars); HO endonuclease creates a DSB at the MAT locus. DNA repair is induced by addition of glucose (gray bars). Expression levels are normalized to that of control cells. Three independent experiments were performed. Error bars represent SD. (B, E) Representative gel images illustrating expression of HMO1 and IPP1 (reference gene) in WT and tor1Δ cells, respectively. (C, F) Western blot corresponding to expression data shown directly above using antibody to the FLAG tags. GAPDH or actin expression levels were used as internal loading controls, and the blots are representative of three independent experiments.

Efficiency of DSB induction. WT (solid line) and tor1Δ cells before (Ctrl; gray bar) and after induction of HO endonuclease (dashed line) were collected before and after induction of DSB formation. Error bars represent SD from three measurements.

FIGURE 3: Efficiency of DSB induction. WT (solid line) and tor1Δ cells (dashed line) were collected before and after induction of HO expression by addition of galactose. Primers spanning the break site were used for amplification. Relative Ct values are reported (using primers amplifying a fragment of POL5 as reference). An increase in relative Ct value reflects reduced amount of template (a greater extent of DSB formation). Error bars represent SD from three measurements.

FIGURE 4: Reduced HMO1 expression after inhibition of mTORC1 activity by rapamycin requires Tor1p. (A, B) HMO1 expression in wild-type (WT) and tor1Δ cells before (Ctrl; gray bars) and after addition of rapamycin (olive bars). Three independent experiments were performed. Error bars represent SD.
Tor1p dissociated from the HMO1 gene after prolonged exposure to rapamycin (Figure 8). The dissociation of Tor1p is consistent with reports that it exits the nucleus after nutrient deprivation or rapamycin treatment (Li et al., 2006).

Repression of HMO1 is associated with differential binding of Ifh1p and Crf1p

While HMO1 mRNA levels were significantly reduced after 30 min of DSB induction (Figure 2, A and B), eviction of Hmo1p and Tor1p was not observed until 2 h after addition of galactose to induce expression of HO endonuclease, when gene activity was minimal (Figures 6 and 7). Fhl1p binds the IFHL site located ~605 base pairs upstream of the HMO1 start codon (Xiao et al., 2011). We therefore investigated whether regulation of HMO1 expression might depend on the Fhl1p-associated factors Ifh1p and Crf1p, focusing on primer pairs Up2 and Up1 that amplify segments that flank the IFHL site (Figure 1A). While Fhl1p remained bound after 2 h of DSB induction (Figure 9A), induction of a DSB for 30 min resulted in significantly reduced Ifh1p binding and a concomitant increase in binding of Crf1p (Figure 9, B and C). Similarly, inhibition of mTORC1 by rapamycin resulted in reduced Ifh1p binding and increased occupancy of Crf1p, whereas Fhl1p binding was unaltered (Figure 10). Consistent with previous reports that HMO1 promoter activity by rapamycin is attenuated in the absence of Hmo1p (Xiao et al., 2011), little binding of Fhl1p or Ifh1p was detected in an hmo1Δ strain (unpublished data).

The increase in binding of corepressor Crf1p and the loss of the activator Ifh1p observed after 30 min of DSB induction and after 1 h of rapamycin treatment (Figures 9 and 10) correlates with reduced HMO1 mRNA levels (Figures 2 and 4) and with reduced HMO1 promoter activity upon addition of rapamycin (determined by β-galactosidase activity of constructs in which the lacZ gene is under control of the HMO1 promoter; Xiao et al., 2011). An interpretation consistent with these data and the reported roles of Crf1p and Ifh1p in controlling RP gene expression is that recruitment of RNA Pol II is compromised as a consequence of changing dynamics of Crf1p and Ifh1p binding. To address this inference experimentally, we performed a kinetic analysis of RNA Pol II binding by comparison with the kinetics of Crf1p and Ifh1p binding. On RPL30, a marked reduction in mRNA levels was seen 15 min after inhibition of mTORC1 with rapamycin in an hmo1Δ strain (unpublished data).

resulted in eviction of Hmo1p, but only after 2 h of DSB induction (Figure 6). Tor1p was also evicted after 2 h, but most efficiently in the vicinity of the HMO1 start site (Up1) and within the coding region (open reading frame [ORF]; Figure 7). Similarly, both Hmo1p and increased occupancy of Crf1p, whereas Fhl1p binding was unaltered (Figure 10). Consistent with previous reports that Hmo1p and Fhl1p binding is interdependent and that repression of HMO1 promoter activity by rapamycin is attenuated in the absence of Hmo1p (Xiao et al., 2011), little binding of Fhl1p or Ifh1p was detected in an hmo1Δ strain (unpublished data).

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galactose, as expected (Figure 11B, top row), but it was almost undetectable 10 min after DSB induction. Thus the dynamics of transcription factor binding is consistent with a failure to recruit RNA Pol II effectively if Ifh1p is absent; in addition, our data show that Crf1p recruitment occurs after dissociation of RNA Pol II from the HMO1 promoter, and Pol II was detectable only with primers amplifying a fragment of the ORF after 10 min (Figure 11C).

Cell cycle progression is also affected by the activity of mTORC1. To verify that changes in transcription factor binding were not a consequence of a cell cycle arrest, we used flow cytometry to assess cell cycle progression after addition of either galactose or rapamycin to asynchronous cells. As shown in Figure 12, no apparent change was observed 10 min after addition of galactose to induce DSB, the time at which both Ifh1p and RNA Pol II have left the HMO1 gene. After 2 h of DSB induction, the time at which both Hmo1p and Tor1p were seen to dissociate, an accumulation of cells in G1 was observed. We infer that binding of Ifh1p, Crf1p, and RNA Pol II is unrelated to cell cycle progression, whereas it is conceivable that dissociation of Hmo1p and Tor1p is a consequence of a G1 delay.

Arrest in G1 has been reported to occur after >6 h of incubation with rapamycin, primarily due to impaired protein synthesis, and a G2/M defect was reported to occur on inhibition of mTORC1 by rapamycin due to mTORC1-mediated regulation of polo-like kinase Cdc5 (Fingar and Blenis, 2004; Bernstein et al., 2007; Nakashima et al., 2008). We also noted a delay of G2/M progression after addition of rapamycin; however, a similar delay was
observed in tor1Δ cells, consistent with the expectation that Tor2p can substitute for Tor1p as a component of mTORC1 (Figure 13). In addition, these data indicate that changes in HMO1 mRNA levels on deletion of TOR1 are not due to changes in cell cycle progression compared with WT cells.

**Excess Hmo1p displaces Reb1p but not Tor1p**

The HMO1 promoter also features binding sites for the transcriptional regulator Reb1p (Figure 1A), which often binds near nucleosome-depleted regions (Hartley and Madhani, 2009). No change in Reb1p occupancy was observed after 30 min of DSB induction (Figure 14A), suggesting that Reb1p is not involved in mediating the initial decrease in gene expression. However, prolonged DSB induction (2 h) resulted in reduced Reb1p binding; the timing of reduced Reb1p binding corresponds to dissociation of Hmo1p and Tor1p (Figures 6 and 7). On the basis of the potential contribution of Reb1p to transcriptional activation, we monitored binding of Reb1p in cells in which HMO1 is expressed under control of the strong GAL1 promoter. Overexpression of Hmo1p resulted in modestly reduced Reb1p binding (Figure 14B), suggesting that displacement of Reb1p by excess Hmo1p may contribute to the reduced HMO1 promoter activity observed.
DISCUSSION

Tor1p associates with the HMO1 gene to activate transcription

mTORC1 controls transcription by all three nuclear RNA polymerases. In yeast, mTORC1 associates to a significant extent with the limiting membrane of the vacuole (the primary nutrient reservoir in yeast and the equivalent of lysosomes in higher eukaryotes), where it phosphorylates one of the main downstream targets, Sch9p, during nutrient sufficiency to activate downstream functions (Urban et al., 2007; Jin et al., 2014). Inactivation of mTORC1 has been linked to reduced expression of RP genes by a mechanism in which mTORC1 promotes phosphorylation of Crf1p by the cytoplasmic serine/threonine protein kinase Yak1p, which leads to localization of phosphorylated Crf1p to the nucleus, where it replaces Fhl1p. In addition, direct phosphorylation of the transcription factor Sfp1p by mTORC1 promotes its nuclear localization and binding to RP gene promoters, whereas rapamycin treatment results in Sfp1p dephosphorylation and its export from the nucleus (Jorgensen et al., 2004; Marion et al., 2004; Lempiäinen et al., 2009).

By contrast, TOR kinase from both yeast and mammals has been shown to associate directly with the RNA Pol I– and Pol III–transcribed rRNA genes in a nutrient-dependent manner, perhaps functioning to phosphorylate specific transcription factors or components of the transcription machinery. Our data suggest that TOR kinases likewise have a direct role in control of RNA Pol II–transcribed genes. Tor1p associates directly with the HMO1 gene (Figure 5, B and C), and it activates transcription, provided the IFHL site is present (Figure 1, B and C). While the mechanism by which Tor1p is recruited to the HMO1 gene is unclear, we note that HMO1 promoter activity is reduced in the absence of Tor1p, regardless of the presence of Hmo1p; we therefore infer that Tor1p is unlikely to be recruited by direct interaction with Hmo1p. Absence of Hmo1p results in significantly reduced binding of Fhl1p to the IFHL site (Xiao et al., 2011); however, we cannot rule out that residual Fhl1p binding may be sufficient to recruit Tor1p. It is also probable that the IFHL site is required not for Tor1p recruitment but for binding of a target for the TOR kinase.

During nutrient sufficiency, expression of HMO1 is repressed by Hmo1p, with overexpression of Hmo1p associated with further repression (Figure 1B; Xiao et al., 2011). Because binding of the transcription factor Reb1p is reduced under conditions of Hmo1p overexpression (Figure 14B), we speculate that accumulation of excess Hmo1p on the HMO1 gene may displace Reb1p and therefore contribute to
decreased gene activity, perhaps by forcing a repressive localization of the +1 nucleosome. This negative-feedback loop may ensure that cellular levels of Hmo1p remain optimal; because Hmo1p stabilizes chromatin, excess Hmo1p may result in slower rates of chromatin remodeling and, therefore, compromised cellular function. That Reb1p dissociates after prolonged DSB induction when HMO1 expression levels are very low is consistent with its role as an activator (Figure 14A).

**Tor1p is required for decreased HMO1 expression during stress**

Both DSB induction and treatment with rapamycin results in reduced HMO1 mRNA levels (Figures 2 and 4). The initial reduction in mRNA levels upon DSB induction correlates with reduced Ifh1p binding and recruitment of Crf1p, and the dissociation of Ifh1p is followed by dissociation of RNA Pol II, suggesting that Ifh1p is a required activator for HMO1 expression (Figures 9 and 11A and B). The delayed binding of Crf1p would be consistent with the need to import phosphorylated Crf1p into the nucleus. A similar response is also associated with rapamycin-mediated reduction in gene expression (Figures 10 and 11C). Notably, the reduced HMO1 mRNA levels characteristic of cells experiencing a DSB or rapamycin treatment are not seen in tor1Δ cells. This suggests that inhibition of a cytoplasmic (vacuolar) mTORC1 pool in which either Tor1p or Tor2p may be incorporated is unlikely to be sufficient for the observed change in mRNA levels. Assuming that inhibition of cytoplasmic mTORC1 is required for Crf1p phosphorylation and its accumulation in the nucleus as previously proposed (Martin et al., 2004; Schawalder et al., 2004), the failure to elicit a decrease in mRNA levels in the absence of Tor1p is intriguing and may suggest a need for promoter-bound kinase in maintaining the phosphorylation state of key transcription factors, thus rationalizing the binding of Tor1p to the HMO1 gene. Such transcription factors could include Sfp1p; Sfp1p is mainly localized in the nucleus and may suggest a need for promoter-bound kinase in maintaining the phosphorylation state of key transcription factors, thus rationalizing the binding of Tor1p to the HMO1 gene. Such transcription factors could include Sfp1p; Sfp1p is mainly localized in the nucleus during balanced growth, and it interacts directly with mTORC1, which was interpreted to suggest the existence of a nuclear pool of mTORC1 (Lempiainen et al., 2009).

Repression of RP gene expression leads to dissociation of Hmo1p, while Fhl1p remains bound, an event that results in a movement of the +1 nucleosome, suggesting that Hmo1p is important for correct assembly of the preinitiation complex (Reja et al., 2015). Similarly, the dissociation of both Hmo1p and Tor1p from the HMO1 gene correlated with the lowest level of HMO1 expression (Figures 2, 6, and 7). These observations reveal a previously unappreciated function of TOR kinase in direct control of transcription by RNA Pol II, and they raise the possibility that control of RP or Ribi genes may likewise involve promoter-bound TOR kinase.

**MATERIALS AND METHODS**

**Strains and plasmids**

All yeast strains were derived from WT strain DDY3, which is isogenic to W303-1a (MAT a Ade2 his3-11 leu2-3,112 lys2-1 trp1-1 ura3-1 can1-100; Simms et al., 2008). The strain deleted for HMO1 (hmo1Δ) and strains expressing FLAG-tagged Hmo1p or Fhl1p were described previously (Xiao et al., 2011). Strains expressing FLAG-tagged Ifh1p, Crf1p, and Tor1p were created by amplification of the sequence encoding the 3xFLAG-tag and the KanMX6 marker using primers carrying homology to the target genes. PCR products were used for transformation of DDY3 (or hmo1Δ in case of Ifh1-FLAG), followed by
selection for resistance to G418. Transformants were confirmed by PCR, and expression of tagged proteins was verified by Western blotting using antibody to the FLAG-tag (F1804; Sigma). Strains tor1Δ and hmo1Δtor1Δ were created by amplification of the TRP1 marker from plasmid pRS424 (Sikorski and Hieter, 1989), and the PCR product was used to transform DDDY3 and hmo1Δ cells, followed by selection for tryptophan prototrophy and verification of transformants by PCR and sequencing.

Plasmids pGWt carrying the wild-type HMO1 gene under control of the GAL1 promoter, pHiacZ in which lacZ is expressed under control of the HMO1 promoter, and pHiacZm in which lacZ is expressed under control of the HMO1 promoter lacking the IFHL site were previously described (Xiao et al., 2011). Plasmid expressing HO endonuclease was a gift from J. Haber (Brandeis University) and plasmid expressing FLAG-tagged Reb1p was a gift from D. Donze (Louisiana State University; Wang and Donze, 2016).

High-efficiency transformation

Cells were grown in yeast extract, peptone, dextrose (YPD) at 30°C to OD600 ~0.8, and the pelleted cells were washed with 1X phosphate-buffered saline (PBS); resuspended in 1X Tris, EDTA, and lithium acetate buffer (TEL); and left on a nutator overnight at room temperature. Cells were pelleted and resuspended in 100 μl 1X TEL per 10 ml culture and incubated at room temperature for 30 min. One hundred microliters of competent cells, 10 μl of carrier DNA, and 1 μg of plasmid DNA were mixed and incubated for 30 min. Seven hundred microliters of 40% polyethylene glycol (PEG) in 1X TEL were added to each tube and incubated at room temperature for 60 min without shaking. Eighty-eight microliters of dimethyl sulfoxide (DMSO) was added to each tube, and the cells were subjected to heat shock at 42°C for 45 min. The cells were spun at 8000 rpm for 30 s, and pellets were washed with 300 μl water and resuspended in 400 μl water. Two hundred microliters was plated on synthetic defined (SD) drop-out media.

β-Galactosidase assay

Cells were grown overnight in selective media and used to inoculate a fresh culture, which was grown to mid-log phase. The OD600 was recorded, and the cells were harvested immediately. An aliquot (1.5 ml) of the culture was centrifuged, the cell pellet was washed with Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0), and the cells were resuspended in 300 μl of Z buffer. One hundred microliters of the cell suspension was transferred to a fresh microcentrifuge tube, and the cells were lysed by freezing/thawing. Z buffer (0.7 ml) containing 0.3% β-mercaptoethanol was placed in reaction tubes and blanks, 160 μl of freshly prepared 4 mg/ml 2-nitrophenyl-β-D-galactoside (ONPG) in Z buffer was added, and samples were incubated at 30°C. Reactions were terminated by addition of 0.4 ml of 1 M Na2CO3, and the elapsed time was recorded. Reactions were clarified by centrifugation for 10 min at 14,000 rpm, and A420 was measured. The β-galactosidase activity was calculated using the following equation: β-galactosidase (Miller) units = 1000 × OD420/(t × V × OD600), where t is elapsed time and V is assayed culture volume (0.5 ml).

RNA isolation and in vivo gene expression

Cells were grown at 30°C to OD600 ~0.8. One milliliter of culture was removed to extract RNA. Cells were mixed with ice-cold diethyl pyrocarbonate (DEPC)-treated water and centrifuged, and the pellet was frozen at −80°C. Total RNA was isolated using the illustra RNAspin Mini Isolation kit (GE Healthcare). Contaminating DNA was removed using Turbo DNase (Ambion), and absence of DNA was verified by PCR. RNA was quantified using NanoDrop (Thermo Scientific). The cDNA was prepared from 500 ng total RNA using 1X AMV reverse transcriptase buffer with 1 mM MgCl2, 1 mM dNTP, and 10 U of AMV reverse transcriptase (New England Biolabs) in a total reaction volume of 25 μl. The mixture was incubated at 42°C for 1 h. A ViaA7 (Applied Biosystems) was used for quantitative PCR (qPCR) using Taq polymerase for amplification and SYBR Green I (Sigma) for detection. For analysis of expression after DSB induction or rapamycin addition, the data were normalized to the expression level in untreated control; expression of IPP1 (inorganic pyrophosphatase) was used as a reference, and relative expression in WT and tor1Δ cells was calculated using the ΔCt method. Each experiment was repeated three times, and average and SD are reported.

ChIP and PCR analysis

Yeast cells were grown at 30°C in 2% raffinose-containing yeast, peptone (YP) or in synthetic defined (SD) drop-out media to OD600 ~1.0. A 100 ml culture aliquot was saved as the uninduced sample. Where indicated, galactose was added to the remaining culture to a final concentration of 2% to induce expression of HO endonuclease, or rapamycin was added at a final concentration of 200 ng/ml to inhibit mTORC1, and cells were collected at the indicated times. For repressing HO expression and prevent further DNA damage, 2% glucose was added and cells were harvested at indicated times for ChIP assay. Cells were fixed with 1.0% formaldehyde and incubated at room temperature for 20 min with gentle shaking. Cells were lysed by vortexing with glass beads for 40 min at 4°C in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, and 0.1% sodium deoxycholate) containing protease inhibitors peptatin A (1 μg/ml), leupeptin (1 μg/ml), and phenylmethylsulfonyl fluoride (PMSF, 100 μM). For shearing chromatin into fragments of a predominant size of ~500 base pairs, the lysate was sonicated six times for 10 s each at 25% amplitude with intermittent chilling of the samples on ice. Sheared chromatin was then divided into 100 μl aliquots for ChIP. The lysate was precleared using protein G-Sepharose beads (GE Healthcare) to reduce non-specific binding to the beads. For immunoprecipitation, 5 μl of anti-FLAG antibody (F1804; Sigma) or anti-RNA Pol II CTD (O95-952; EMD Millipore) was used. PCR products were analyzed on 1.4% agarose gels and stained with ethidium bromide. Quantitative reverse transcriptase PCR (qRT-PCR) was conducted using an ABI ViiA 7 sequence-detection system and SYBR Green for detection. Four primer pairs amplifying different regions of the HMO1 gene were used (Figure 1A), and primer pairs amplifying segments of control loci 18S rDNA, MAT, POL5, KRE5, and IPP1 (Panday et al., 2015; Panday and Grove, 2016) were included where indicated. Data were normalized to corresponding input control at each time point. Each experiment was repeated three times, and average and SD are reported.

Efficiency of DSB induction

For determining the efficiency of DSB induction, qPCR was performed with primer pairs flanking the break site. Cells were grown at 30°C to an optical density at 600 nm of 1.0. An aliquot of cells was removed and used as no-damage control. Galactose was added to the remaining culture to a final concentration of 2% to induce DSB, and cells were collected after 1, 2, 3, and 4 h. Genomic DNA was isolated and used as template for PCR amplification. qPCR was conducted using an ABI ViiA 7 Real Time PCR system and SYBR Green for detection. Experiments were repeated three times, and data are reported as mean with SD. Primer sequences were previously reported (Panday et al., 2015).
Flow-cytometric analysis of cell cycle progression

For determining the impact of rapamycin on the cell cycle, WT and tor1Δ cells were grown in YPD media to OD_{600} ~0.8 cells, at which time rapamycin was added (200 ng/ml). Cells were collected at 0 min (before addition), 10 min, 30 min, 1 h, and 2 h. For assessment of the impact of DSB induction on the cell cycle, WT and tor1Δ cells, both transformed with plasmid expressing HO endonuclease, were grown in 2% raffinose-containing SD dropout media (2%). At OD_{600} ~0.6, at which time galactose was added (2%) to induce DSB. After induction, cells were collected at 0 min (before induction), 10 min, 30 min, 1 h, and 2 h.

Cell pellets (10^7 cells) were washed with water and centrifuged. For fixing, 3.5 ml of 100% ethanol was added with vortexing to avoid aggregation, and cells were kept at room temperature for 1 h. The fixed cells were centrifuged and washed with 3.5 ml sodium citrate buffer. Cells were centrifuged and pellets were resuspended in 0.5 ml RNase solution (2 mg/ml RNase A in 50 mM Tris, pH 8.0, and 15 mM NaCl). Resuspended cells were kept at 50°C for 2 h. After addition of 20 μl proteinase K (20 mg/ml), cells were incubated for 1 h at 50°C. A 20 μl aliquot of SYBR-Green I (1 X SYBR-Green I in 50 mM Tris, pH 7.4) was added, and cells were incubated overnight at 4°C. After sonication (6 s, 10% power), samples were acquired on a FACScan flow cytometer (BD Biosciences, San Jose, CA) configured for SYBR1 fluorescence measurements. Forward and side scatter measurements and fluorescence measurements for DNA replication were made using linear amplification. A total of 30,000 cells per sample were analyzed using CellQuest graphics software (BD Biosciences). A dot plot of SYBR1 width versus SYBR1 surface area was analyzed for the resolution of aggregates by gating. Single cell–derived histograms were used to examine DNA cell cycle components, G1 and G2.

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