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Mec1 is activated at the onset of normal S phase by low dNTP pools impeding DNA replication

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SUMMARY

The Mec1 and Rad53 kinases play a central role during acute replication stress in budding yeast. They are also essential for viability in normal growth conditions, but the signal that activates the Mec1-Rad53 pathway in the absence of exogenous insults is currently unknown. Here, we show that this pathway is active at the onset of normal S phase because dNTP levels present in G_1 phase may not be sufficient to support processive DNA synthesis and impede DNA replication. This activation can be suppressed experimentally by increasing dNTP levels in G_1 phase. Moreover, we show that unchallenged cells entering S phase in the absence of Rad53 undergo irreversible fork collapse and mitotic catastrophe. Together, these data indicate that cells use suboptimal dNTP pools to detect the onset of DNA replication and activate the Mec1-Rad53 pathway, which in turn maintains functional forks and triggers dNTP synthesis, allowing the completion of DNA replication.

KEYWORDS:

DNA replication, S-phase checkpoint, dNTP synthesis, cell cycle, budding yeast

INTRODUCTION

The integrity of the genome is at risk during the S phase of the cell cycle when the cell must duplicate faithfully all of its genetic information before mitosis. This is achieved through the sequential activation of thousands of replication origins, distributed along the chromosomes following a defined replication timing program (Fragkos et al., 2015). A new copy of the DNA is synthesized by replication forks, which progress bidirectionally from the replication origins until they converge with other forks (Burgers and Kunkel, 2017; Dewar and Walter, 2017). During this process, the forks may encounter obstacles that cause them to stall (Gaillard et al., 2015; Techer et al., 2017; Zeman and Cimprich, 2014). This replication fork stalling, commonly referred to as replication stress (RS), activates checkpoint signaling pathways. The best characterized of these signaling pathways is the DNA damage checkpoint, which arrests cell cycle progression in response to DNA breaks until lesions are repaired (Weinert and Hartwell, 1988). Stressed replication forks, by contrast, are detected by the DNA replication checkpoint, which acts in multiple ways to maintain genome integrity (Saldivar et al., 2017). Failure to build an efficient RS response leads to genomic instability, which can fuel tumorigenesis (Kotsantis et al., 2018; Macheret and Halazonetis, 2015).

The mechanism by which cells detect and signal stalled forks has been characterized extensively in budding yeast (Pardo et al., 2017) and it is highly conserved among eukaryotes (Saldivar et al., 2017). Briefly, obstacles to replication on the DNA template result in accumulation of single-stranded DNA (ssDNA) at replication forks. This ssDNA is coated with replication protein A (RPA) and recruits the Ser/Thr kinase Mec1 (known as ATR in vertebrates) through its partner Ddc2 (ATRIP in vertebrates) (Rouse and Jackson, 2002; Zou and Elledge, 2003). Mec1 phosphorylates multiple targets near blocked forks, including histone H2A (H2AX in vertebrates) (Redon et al., 2003; Rogakou et al., 1999). Mec1 also activates the effector kinase Rad53 (the functional homologue of CHK1 in vertebrates) to transduce the checkpoint response (Sanchez et al., 1999) and prevent fork collapse (Desany et al., 1998; Tercero et al., 2003). In addition, the Mec1-Rad53 pathway slows down DNA replication (Bacal et al., 2018; Santocanale and Diffley, 1998; Shirahige et al., 1998), prevents premature entry into mitosis (Clarke et al., 2001) and promotes dNTP synthesis by multiple mechanisms that increase the activity of ribonucleotide reductase (RNR), including the Dun1-dependent degradation of the RNR inhibitor Sml1 (Zhao et al., 1998).

In budding yeast, quantitative mass spectrometry analyses have shown that Mec1 is functional during normal S phase but does not activate Rad53 (Bastos de Oliveira et al., 2015; Lanz et

al., 2018). Rad53-independent roles of Mec1 include activation of replication origins (Randell et al., 2010), *de novo* telomere addition (Zhang and Durocher, 2010) and the response to proteotoxic stress (Corcoles-Saez et al., 2018). Likewise, checkpoint-independent functions of Rad53 have been identified during S phase, including degradation of excess histones (Gunjan and Verreault, 2003). Together, these data suggest that the canonical Mec1-Rad53 checkpoint pathway is not functional during normal DNA replication. Yet, Mec1 and Rad53 are both essential for cell viability (Weinert, 1997) and the lethal phenotype of *mec1*Δ and *rad53*Δ mutants can be suppressed by deleting *SML1* (Zhao et al., 1998). These observations led to the proposal that the Mec1-Rad53 pathway triggers the degradation of Sml1 at the onset of S phase to promote dNTP synthesis (Zhao et al., 2001; Zhao and Rothstein, 2002), but the signal that activates it has remained unknown.

Here, we show that the Mec1-Rad53 pathway is activated by a spontaneous replication stress resulting from limiting dNTP pools upon initiation of DNA replication. This transient activation of the Mec1 pathway triggers dNTP synthesis through the Rad53-dependent degradation of Sml1 and the upregulation of RNR activity. Importantly, this pathway also prevents fork collapse and premature entry into mitosis during this transient dNTP shortage. These data indicate that cells do not anticipate the G_1/S transition by producing dNTPs in late G_1 phase but rather use nucleotide shortage and fork arrest as a physiological signal to activate dNTP synthesis.

RESULTS

The Mec1-Rad53 pathway is active during normal S phase

Replication origins fire sequentially throughout S phase and slight differences in Rad53 activity have major effects on the extent of 'early' and 'late' replication origin firing (Poli et al., 2012). To determine whether the Mec1-Rad53 pathway is active during normal S phase, we monitored repression of late replication origins as a readout of checkpoint activation (Crabbé et al., 2010). Wild-type, *mec1-1 sml1-1* and *rad53-11 Saccharomyces cerevisiae* cells were arrested in G_1 phase by using α-factor and then released synchronously into S phase in the presence of BrdU to label ongoing DNA synthesis (**Fig. 1A**). As a positive control, we also treated the cells with 200 mM hydroxyurea (HU) to block elongation. The BrdU-labelled DNA was then immunoprecipitated and hybridized to tiling arrays to analyze origin firing, as described previously (Crabbé et al., 2010). In HU-treated wild-type cells, BrdU incorporation was detected at early origins (open arrowheads), but not at late origins (filled arrowheads; **Fig. 1B**), as illustrated for chromosome 14, reflecting checkpoint activation of the Mec1-Rad53 pathway. By contrast, BrdU incorporation was detected at both early and late origins in *mec1-1 sml1-1* and *rad53-11* mutants, indicating their inability to repress late replication origins. Of note, the BrdU peaks were wider in *mec1-1 sml1-1* mutants than in wild-type and *rad53-11* cells because the *sml1-1* mutation prevents inhibition of RNR by Sml1 and thus the dNTP pools are larger in these cells in G_1 phase, so the replication forks are able to progress further in the presence of HU (Poli et al., 2012). In untreated wild-type cells, BrdU incorporation was detected at early origins 20 minutes after their release from G_1 phase arrest and at late origins 10 minutes later (**Fig. S1B**), as previously described (Müller et al., 2014). In *mec1-1 sml1-1* and *rad53-11* mutants, BrdU incorporation at late origins was increased compared to wild-type cells (**Fig. 1B**). When expressed relative to wild-type levels, the ratio of BrdU incorporation was close to 1 for all early origins, but increased at late origins proportionally to the time of origin activation (**Fig. 1C, D**). These data indicate that although early origins fire with similar kinetics in the three strains, late origins are activated prematurely in the *mec1-1 sml1-1* and *rad53-11* mutants. This confirms and extends earlier studies that analyzed only two late origins (Santocanale and Diffley, 1998; Shirahige et al., 1998). We obtained similar results by monitoring BrdU incorporation **(Fig. S1B–D)** and DNA copy number variation **(Fig. S1E–G)** by deep sequencing (Fang et al., 2017; Müller et al., 2014). Interestingly, we saw no premature activation of late origins in the absence of the DDC mediator Rad9 **(Fig. S1B–D)**, indicating that Rad53 is not activated by DNA damage during

normal DNA replication. Together, these data indicate that the Mec1-Rad53 pathway is active during normal S phase and delays the activation of late origins, independently of the DDC.

Mec1 is transiently activated around early replication origins

To identify regions of the yeast genome that could activate Mec1 during normal S phase, we used chromatin immunoprecipitation coupled to NGS (ChIP–seq) to analyze the genome-wide distribution of the Mec1-binding protein Ddc2 and the histone H2A phosphorylated on serine 129 by Mec1 (γ-H2A). The detection of Ddc2 allows the localization of Mec1, whereas γ-H2A indicates Mec1 activity. To position these signals relative to forks, the distribution of Cdc45, a component of the replication helicase, was also mapped by ChIP–seq and variations in DNA copy number were followed by NGS (Müller et al., 2014). Wild-type cells expressing epitope-tagged Cdc45 or Ddc2 were released from a G_1 arrest, as above, and samples were collected in G_1 phase and at the indicated times after release, during the entire S phase (**Fig. 2A, B**). In G_1 phase, Ddc2 was enriched at tRNA genes and at highly expressed genes enriched in the RNAPII subunit Rpb3 (**Fig. S2A–C**), which corresponds to documented ChIP artefacts (Teytelman et al., 2013). By contrast, γ-H2A was absent from these highly expressed genes (high Rpb3 levels), presumably because of the fast histone turnover at these loci; γ-H2A was detected only at a subset of repressed genes, as reported earlier (Szilard et al., 2010). To focus on S phase-specific profiles, ChIP–seq signals from G_1-phase samples were subtracted from those from S-phase samples. In a typical experiment, Cdc45 peaks were detected at early origins 20 minutes after release from G_1 phase arrest (**Fig. 2C**). At 30 minutes, new peaks were detected at late origins and the Cdc45 peaks at early origins split in two due to bidirectional fork progression, as seen by analyzing the signals over 40 kb regions centered on replication origins (**Fig. 2D**). Interestingly, narrow Ddc2 and γ-H2A peaks (5–10 kb) centered on early origins appeared at 30 minutes and had disappeared 10 minutes later (**Fig. 2C, D**), indicating transient activation and inactivation of Mec1 around 30 minutes after release from G_1 phase arrest. Similar kinetics of Mec1 activation and inactivation can also be seen on heat maps of Ddc2 and γ-H2A ChIP signals at intervals centered on origins and sorted according to their replication time (**Fig. S2D**). Interestingly, Ddc2 and γ-H2A peaks did not overlap with Cdc45 peaks, which is consistent with the accumulation of RPA-coated ssDNA gaps behind forks under replication stress conditions (Bacal et al., 2018; García-Rodríguez et al., 2018). To confirm that Ddc2 enrichment reflects the recruitment of Mec1 to RPA-coated ssDNA upon early origins firing, we also immunoprecipitated RPA and an HA-tagged version of Mec1 (Mallory and Petes, 2000) and monitored their enrichment at four early origins by

ChIP-qPCR in G_1-arrested cells and after release for 30 minutes into S phase **(Fig. S2E)**. This analysis confirmed that RPA and Mec1 accumulate near early origins during normal replication to ~15-20% of the levels detected in HU-arrested cells **(Fig. S2F)**. Importantly, we also confirmed that H2A phosphorylation at these loci was essentially mediated by Mec1 and not by Tel1 as γ-H2A levels at early origins was not affected by the deletion of *TEL1* (**Fig. S2G and S2H**). Finally, the transient nature of Mec1 activation was confirmed by plotting variations in Ddc2 and γ-H2A levels at all early-, mid- and late-replicating regions of the genome (**Fig. 2E**). Together, these data show that Mec1-Ddc2 is recruited and transiently activated at early replicating regions during a normal S phase.

Mec1 activation does not correlate with highly transcribed regions

To investigate the origin of the stress causing Mec1 activation during normal DNA replication, we determined the percentages of various genome annotations that overlapped with the Ddc2 and γ-H2A peaks (**Fig. 3A**). This analysis showed that both Ddc2 and γ-H2A were enriched at early replication origins and centromeres. Ddc2 was also detected at tRNA genes and γ-H2A was enriched at ORFs. Since highly expressed genes interfere with fork progression (Azvolinsky et al., 2009; Szilard et al., 2010), we hypothesized that Mec1 might be activated by conflicts between replication and transcription. To assess this possibility, we monitored by ChIP–seq of G_1 and S phase cells the distribution of Rpb3 as an indicator of total RNAPII and Rpb1–pS2 as an indicator of active RNAPII and plotted their distributions relative to Ddc2 and γ-H2A. This analysis revealed that the levels of Ddc2 and γ-H2A at origins correlated neither with increased levels of active RNAPII (Rpb1–pS2, $R<0.1$; **Fig. 3B**) nor with total RNAPII (Rpb3, $R<0.2$; **Fig. 3B** and **S3A**). Moreover, the presence of tRNA genes in the vicinity of early origins did not correlate with the enrichment in Ddc2 and γ-H2A (**Fig. 3C**). This result was further confirmed by plotting Ddc2 and γ-H2A enrichment at origins relative to RNAPIII (Rpo31) levels (Cutler et al., 2018) (**Fig. S3B**). Together, these data indicate that the transient Mec1 activation observed at early replicating regions does not correlate with high transcription and is probably not caused by replication-transcription conflicts. These results are fully consistent with a recent study reporting no detectable replication pausing at highly transcribed genes by RNAPII in wild-type budding yeast cells (Osmundson et al., 2017).

Mec1 activation follows entry into S phase with limiting dNTP pools

We compared the distribution of γ-H2A during normal S phase to the pattern observed in cells exposed to HU, a potent RNR inhibitor (Nyholm et al., 1993). Strikingly, we observed that the distribution of γ-H2A (**Fig. 4A**) and its intensity around early origins were highly similar and correlated in the two cell populations (R=0.9; **Fig. 4B, C**), suggesting that Mec1 may be activated by a transient shortage of dNTP upon entry into S phase. To investigate this possibility, wild-type cells were released synchronously into S phase from a G_1 arrest and were collected every three minutes to measure variations in dNTP levels (**Fig. 4D, E**). Interestingly, levels of the dNTPs did not increase before the onset of S phase (arrow). Levels of dATP and dGTP rather increased after the beginning of DNA synthesis and Sml1 degradation (arrow), 15–18 minutes after release from the α-factor block (**Fig. 4E–G**). In particular, dATP levels were relatively low in G_1 phase compared to the other dNTPs **(Fig. 4E)** and were fully exhausted in HU-treated cells (**Fig. S4**). Together, these data suggest that cells do not prepare for DNA replication by building dNTP pools before activating early origins, but rather enter S phase with suboptimal dNTP levels. DNA polymerases are probably unable to efficiently utilize dNTPs at these low concentrations and would stall and activate the Mec1-Rad53 pathway.

Mec1 activation in normal S phase is suppressed by increasing dNTP levels

To test whether activation of Mec1 in early S phase is due to limiting amounts of dNTPs, we artificially increased the levels of dNTPs by overexpressing RNR components just before the G_1–S transition. To this end, we constructed a strain in which *SML1* was deleted (*sml1*Δ), to remove the inhibitor of RNR, and additional copies of the four *RNR* genes were expressed under the control of a galactose-inducible promoter. When induced by the addition of 2% galactose, the excess RNR activity in G_1 phase cells prevented entry into S phase (**Fig. S5A**), as described previously (Chabes and Stillman, 2007); however, by optimizing the conditions of induction, we were able to find conditions in which the cells progressed through S phase with normal kinetics (**Fig. 5A, B**). The *sml1*Δ mutation alone, before induction of the *RNR* genes, increased the amounts of individual dNTPs by 2–12-fold in G_1 phase cells (**Fig. 5C**). Induction of the *RNR* genes further increased dNTP pools by 4–35-fold in G_1 phase and in S phase cells 30 minutes after release from G_1 arrest (**Fig. 5C**). Remarkably, these increased dNTP levels reduced to background levels the accumulation of γ-H2A at four early origins (**Fig. 5D**) and globally at the genome-wide level (**Fig. 5E**). It also induced a premature activation of late origins, which is consistent with the fact that cells do not activate Mec1 and

Rad53 (**Fig. S5B**). Finally, the accumulation of γ-H2A in early S phase and its suppression by increased dNTP levels were also observed in the absence of Tel1 (**Fig. S5C and S5D**). Together, these findings are consistent with a model in which low dNTP levels at the onset of normal S phase activate Mec1.

Suboptimal dNTP pools in early S induces a global replication blockage in *rad53Δ* cells

The data presented above suggest that cells enter S phase with low dNTP pools, activate the Mec1-Rad53 pathway upon fork pausing and increase RNR via the Rad53-dependent degradation of the RNR inhibitor Sml1. To determine the fate of *rad53Δ* cells entering S phase in the presence of Sml1, we set out to overexpress *SML1* with a galactose-inducible promoter in *rad53*Δ cells released synchronously from G_1 phase (Zhao et al., 2001). Since *rad53Δ* cells grow more slowly than wild-type cells due to the toxicity of free histones (Gunjan and Verreault, 2003; Zhao et al., 2001), one copy of histone H3 and H4 genes (*hht2*Δ *hhf2*Δ) was deleted to suppress this slow growth phenotype without affecting nucleosome assembly (Gunjan and Verreault, 2003; Maya Miles et al., 2018). Control, *pGAL-SML1* and *rad53Δ pGAL-SML1* cells were arrested in G_1 phase with α-factor and *SML1* was overexpressed with the addition of galactose before releasing cells synchronously into S phase (**Fig. 6A**). As expected, *rad53Δ pGAL-SML1* cells remained blocked in early S phase because the lack of Rad53 makes them unable to degrade Sml1 (**Fig. 6B, C**). By contrast, Sml1 was rapidly degraded in *pGAL-SML1* cells, which entered G_2 phase 90 minutes after release from G_1 and accumulated again Sml1 in the following cell cycle (**Fig. 6B, C**). Induction of *SML1* in G_1-arrested cells induced a proportional reduction of the four dNTP levels. Interestingly, the relative amounts of dCTP, dTTP and dGTP were also similar upon release into S phase, but the relative amount of dATP was lower in *rad53*Δ *pGAL-SML1* cells (**Fig. 6D**, arrows). These data suggest that dATP levels could become limiting for DNA replication when cells are unable to degrade Sml1 in early S phase.

To characterize the consequences of suboptimal dNTP pools on DNA synthesis in the absence of Rad53, we next measured variations in DNA content in *pGAL-SML1* and *rad53Δ pGAL-SML1* cells by NGS as described (Müller et al., 2014). In control cells, DNA content increased at 213 early origins 45 minutes after release from the G_1 arrest, reflecting the initiation of DNA synthesis (**Fig. 6E**, **S6A**) and forks progressed by 6.3 kb on average (**Fig. 6F**). Local variations in DNA content were no longer detected at 90 minutes (**Fig. S6A**), indicating that replication is complete. In *rad53Δ pGAL-SML1* cells, initiation was detected at

179 early origins but elongation progressed more slowly than in control cells (2.2 vs 6.3 kb; **Fig. 6E**). At 90 minutes, forks did not progress further than 4.5 kb from early and late origins (**Fig. 6F**), as observed in HU-arrested *rad53-11* mutants (Poli et al., 2012). Persistent fork arrest in these cells was accompanied by a strong accumulation of γ-H2A 45 and 90 minutes after release from G_1-phase arrest (**Fig. 6E, S6B**), reminiscent of that observed in HU-arrested forks (**Fig. 4A**). By contrast, *pGAL-SML1* cells completed genome duplication in 90 minutes and γ-H2A was only transiently detected in early-replicated regions (**Fig. S6B**), as seen in wild-type cells (**Fig. 2C**). Together, these data indicate that the inability of *rad53Δ* cells to increase dNTP levels at the onset of S phase causes an irreversible arrest of replication forks.

Loss of viability in *rad53Δ* cells is caused by fork collapse and premature entry in mitosis

The degradation of the RNR inhibitor Sml1 depends on Dun1, a protein kinase activated by Rad53 (Andreson et al., 2010; Zhao and Rothstein, 2002). Unlike *MEC1* and *RAD53*, however, *DUN1* is not an essential gene (Zhao and Rothstein, 2002). These observations suggest that Mec1 and Rad53 have other functions during a normal S phase besides regulating dNTP levels. To investigate these other possible functions, we assessed the impact of *SML1* expression on the progress of *rad53Δ*, *mec1Δ* and *dun1Δ* mutants through S phase. To this end, *pGAL-SML1*, *rad53Δ pGAL-SML1 mec1Δ pGAL-SML1* and *dun1Δ pGAL-SML1* cells were arrested in G_1 phase by using α-factor and were released into S phase after induction of *SML1* expression with galactose (**Fig. 7A**). Samples were collected at the indicated times, plated on glucose-containing medium to turn off *SML1* expression, and the number of colonies was counted as a measure of cell survival (**Fig. 7B**). Overexpression of Sml1 in the *dun1Δ* mutant blocked S phase progression, as in *rad53Δ* and *mec1Δ* cells (**Fig. 7C**) but did not affect cell viability (**Fig. 7B**). We assume that cell viability in *dun1Δ* mutants was mediated by Mec1 and Rad53, the latter being phosphorylated at 120 minutes to the same extent as in HU-arrested cells (**Fig. 7D**). When *SML1* expression was turned off, *dun1Δ pGAL-SML1* cells were able to complete S phase, despite the persistence of detectable Sml1 levels. This is consistent with the fact that overexpression of a poorly degradable Sml1 derivative, sml-4SA, slows entry into S phase (Andreson et al. 2010). By contrast, *rad53Δ pGAL-SML1* and *mec1Δ pGAL-SML1* cells remained blocked with a nearly 1C DNA content (**Fig. 7E**), presumably because they were unable to maintain the integrity of forks under low dNTP conditions. These data indicate that unlike *mec1Δ* and *rad53Δ* cells, *dun1Δ* cells can

tolerate a transient depletion of dNTP pools in early S phase and can also complete S phase at a slow rate despite the presence of Sml1, as long as Mec1 and Rad53 are present to protect stalled forks.

To determine whether cells progress through the cell cycle in the absence of Sml1 degradation, we next monitored the levels of the mitotic cyclin Clb2 in *rad53Δ pGAL-SML1*, *mec1Δ pGAL-SML1* and *dun1Δ pGAL-SML1* cells released from G_1 arrest. Despite the fact that they failed to complete DNA replication, *rad53Δ*, *mec1Δ* and *dun1Δ* mutants accumulated Clb2 with similar kinetics as control cells expressing *pGAL-SML1* (**Fig. 7D**). We therefore asked whether these cells would progress through mitosis with partially replicated chromosomes. In budding yeast, there is no checkpoint to prevent cells with unreplicated DNA from entering mitosis (Torres-Rosell et al., 2007). Nevertheless, Rad53 and the mitotic spindle assembly checkpoint (SAC) prevent chromosome segregation in anaphase until the completion of DNA replication (Magiera et al., 2014; Palou et al., 2017).We used fluorescence microscopy to follow the segregation of nuclei during the time-course of this experiment by expressing mCherry-Pus1, a protein that marks the whole nuclear compartment (Siniossoglou et al., 1998). Two hours after the release of the cells from G_1 phase arrest, we saw normal segregation of nuclei only in the control *pGAL-SML1* cells (**Fig. 7F, G**). At the same time point, the *rad53*Δ *pGAL-SML1* cells had deformed nuclei, including nuclei of unequal sizes in mother and daughter cells and anaphase bridges (**Fig. 7F, G** and **Fig. S7**). These observations indicate that the SAC does not completely impede anaphase completion in *rad53*Δ *pGAL-SML1* cells. In *dun1*Δ *pGAL-SML1* cells, by contrast, the chromosomes had not segregated into mother and daughter cells at 120 minutes, consistent with Rad53 preventing the completion of anaphase (**Fig. 7D, F, G**). After inhibiting *SML1* expression in *rad53*Δ *pGAL-SML1* and *dun1*Δ *pGAL-SML1* cells by the addition of glucose, only *the dun1*Δ *pGAL-SML1* cells fully replicated their DNA and correctly segregated their chromosomes (**Fig. 7E–G**); most *rad53*Δ *pGAL-SML1* cells failed to complete S phase and had severe segregation defects (**Fig. 7E–G**).

We then asked whether the failure of *rad53*Δ *pGAL-SML1* cells to complete S phase and chromosome segregation might be the cause of the loss of viability observed above (**Fig. 7B**). To this end, we assayed the formation of nuclear foci of Rad52–GFP, as a marker of DNA damage and ongoing DNA repair by recombination. We observed a >10-fold increase in the percentage of cells with Rad52 foci in *rad53*Δ *pGAL-SML1* cells relative to the control *pGAL-SML1* and *dun1*Δ *pGAL-SML1* cells after release from G_1 phase arrest (**Fig. 7H**). These foci

were detected as early as 45 minutes after release from G_1, before cells attempted to separate their chromosomes (**Fig. 7C**). This suggests that the formation of Rad52 foci first occurs at blocked forks. Both the fraction of *rad53*Δ *pGAL-SML1* cells with Rad52 foci and the number of foci per cell further increased after addition of glucose and progression through mitosis, suggesting further chromosome fragmentation during the course of this mitosis (**Fig. 7E, F, H**). Altogether, these data indicate that the essential roles of Rad53 during normal S phase are to maintain the integrity of replication forks and prevent the segregation of partially replicated chromosomes after the initiation of DNA replication with limiting dNTP pools, which occurs spontaneously at the onset of S phase.

DISCUSSION

We used budding yeast as a model to investigate how the Mec1^{ATR} pathway is activated during normal S phase. We show that Mec1 and Rad53 delay the activation of late origins of replication even in the absence of exogenous RS. This function does not depend on the DDC mediator Rad9, indicating that the Mec1-Rad53 pathway detects paused forks in unperturbed cells independently of the presence of DNA damage. To identify regions of the yeast genome that activate the Mec1-Rad53 pathway during normal DNA replication, we mapped the distribution of Ddc2 and γ-H2A on DNA in cells synchronously passing through S phase in the absence of drugs. Time-resolved ChIP–seq experiments revealed that Mec1 is transiently activated at the onset of S phase in short regions (5–10 kb) centered on early replicating origins. These regions contain a high density of tRNA and highly expressed RNAPII genes that have been previously described to cause replication stress because of conflicts between replication and transcription (Azvolinsky et al., 2009; Ivessa et al., 2003; Stirling et al., 2012; Szilard et al., 2010). We found no significant correlation, however, between Mec1 activation and transcription levels at these loci, indicating that the burst of Mec1 activation occurring at the onset of S phase is caused by another type of stress.

The distribution of γ-H2A in untreated cells in early S phase was very similar to that observed in HU-treated cells in which RNR is inhibited and therefore dNTP levels are low. We hypothesized, therefore, that Mec1 might be activated by replication occurring under a suboptimal concentration of dNTPs. Consistent with this hypothesis, we found that cells entered S phase with low dNTP pools, which were not sufficient to sustain the activity of the hundreds of forks generated from early origins. Moreover, the accumulation of γ-H2A at early origins was totally suppressed by artificially increasing dNTP levels before releasing cells into S phase. Finally, impeding dNTP *de novo* synthesis in *rad53*Δ cells blocked the progression of replication forks shortly after their activation, even though dNTP pools were not exhausted. Together, these data indicate that the Mec1-Rad53 pathway is activated by critically low dNTP levels that do not permit optimal DNA replication, similar to the effect of HU (**Fig. 8**). Thus, bulk dNTP synthesis occurs *after* the activation of early origins.

Our finding that Mec1 is activated at the onset of S phase by low dNTP levels raises the question of how eukaryotic cells coordinate the production of dNTPs with DNA synthesis. Since high dNTP levels are detrimental to the fidelity of DNA replication (Ganai and Johansson, 2016; Pai and Kearsey, 2017), nucleotide pools are maintained at levels that are limiting for the progress of the replication fork (Davidson et al., 2012; Poli et al., 2012;

Stodola and Burgers, 2016). In budding yeast, dNTPs present in late G_1 phase are sufficient only to replicate 10–15% of the genome and are exhausted within minutes upon activation of early origins (Poli et al., 2012). Cells cannot anticipate entry into S phase by producing large dNTP pools in G_1 phase as they interfere with the initiation of replication (Chabes and Stillman, 2007). The production of dNTPs must therefore occur concomitantly with the activation of early origins, but the mechanism responsible for this coordination had remained unclear. Our data shed ~~new~~ light on this mechanism by explaining how the Mec1-Rad53 pathway could regulate RNR activity during normal S phase.

RNR is the rate-limiting enzyme for dNTP production (Giannattasio and Branzei, 2017; Sanvisens et al., 2013). Its activity is regulated at the G_1/S transition by various transcriptional and posttranscriptional mechanisms (Bastos de Oliveira et al., 2012; Elledge and Davis, 1990; Niida et al., 2010; Wu et al., 2018). In addition, RNR activity is upregulated by the Mec1-Rad53 pathway in response to DNA damage via the degradation of the RNR repressors Crt1, Dif1 and Sml1 (Huang et al., 1998; Lee et al., 2008; Zhao and Rothstein, 2002). Since deletion of *SML1* or overexpression of *RNR* genes rescues the lethality of *mec1* and *rad53* null alleles (Desany et al., 1998; Zhao et al., 1998), it has been proposed that the Mec1-Rad53 pathway activates RNR during normal S phase by promoting the Dun1-dependent degradation of Sml1 (Zhao et al., 2001; Zhao and Rothstein, 2002). However, the signal that triggers the Mec1–Rad53–Dun1 cascade in the absence of exogenous stress has remained unknown. Our finding that low dNTP pools impede DNA replication at the onset of the S phase provides this missing link and defines a simple and robust mechanism ensuring that dNTPs are produced at the very moment when they are needed.

Our data indicate that the Mec1–Rad53–Dun1 pathway plays a critical role at the onset of S phase by coordinating RNR activity with the firing of early replication origins. Yet, this model does not explain why the *MEC1* and *RAD53* genes are essential for viability, whereas *DUN1* is not. Mec1 and Rad53 should therefore have another important role during normal growth that is not shared by Dun1. We have found that the Mec1-Rad53 pathway delays the activation of late origins during a normal S phase, but this function is not essential for viability (Zegerman and Diffley, 2010).

As a matter of fact, the most important function of the Mec1-Rad53 pathway under acute RS conditions is to maintain the integrity of stalled forks to ensure that they will be able to resume DNA synthesis once the stress is relieved (Tercero et al., 2003). Remarkably, we have found that Rad53, but not Dun1, is also essential to prevent fork collapse in untreated cells.

These data indicate that Rad53 is not only required to stimulate dNTP synthesis at the onset of S phase but also to maintain the ability of cells to recover from dNTP shortage (**Fig. 8**). Unlike in HU-arrested cells, the activation of Rad53 during normal S phase is very transient, explaining why it has remained undetected (Bastos de Oliveira et al., 2015; Hoch et al., 2013). Importantly, we have observed that *rad53*Δ cells bearing under-replicated and damaged chromosomes progress through mitosis. Normally, the spindle assembly checkpoint (SAC) prevents the segregation of chromosomes with unreplicated centromeres, which impedes the correct kinetochores attachment. Since centromeres are linked to early origins in budding yeast (Fang et al., 2017), these observations suggest that cells lacking Rad53 are still able to replicate their centromeres before reaching anaphase, allowing them to bypass the SAC. The incapacity of these cells to properly segregate their chromosomes is causing even more genomic instability. These data illustrate the many critical functions of the Mec1-Rad53 pathway to ensure proper DNA replication and subsequent mitosis in normal growth conditions.

An important question that remains to be addressed is to what extent this mechanism is conserved in vertebrates. Unlike in yeast, dNTP pools do not increase after DNA damage in mammalian cells (Hakansson et al., 2006; Técher et al., 2016; Techer et al., 2017). Yet, ATR promotes the expression of the RNR subunit *RRM2* in late G_1 phase (Buisson et al., 2015) and prevents its degradation in response to DNA damage (D'Angiolella et al., 2012). Moreover, mice with mutations in *ATR* and with an extra copy of the *RRM2* gene show reduced RS at common fragile sites, which is consistent with the conclusion that ATR prevents spontaneous genomic instability by controlling dNTP pools (Lopez-Contreras et al., 2015). It is therefore likely that similar mechanisms operate in all eukaryotes to coordinate dNTP supply, fork stability and DNA synthesis.

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AUTHOR CONTRIBUTIONS

B.P., P.P., A.L., R.F., A.P. and A.C. conceived and planned the study. R.F., A.P., S.S., A.B., R.L. and B.P. conducted the experiments. R.F., I.P. and C.R. performed the bioinformatic analyses. M.S. and K.G. performed NGS experiments. B.P. and P.P. wrote the manuscript and all the authors reviewed it.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. The Mec1-Rad53 pathway represses late origins during a normal S phase.

(A) Wild-type (wt), *rad53-11* and *mec1-1 sml1-1* cells grown at 25°C in YPD medium were released synchronously from G_1 phase arrest for 20, 30 or 40 minutes in the presence of BrdU to label nascent DNA. Cells were also released for 60 minutes in medium containing 200 mM HU to block DNA synthesis.

(B) BrdU-labelled DNA was immunoprecipitated and hybridized to Affymetrix tiling arrays. Enrichment of replicated DNA fragments relative to input DNA (signal log ratio) is shown for a representative region on chromosome 14. Open arrowheads, early origins; filled arrowheads, late origins. *, transposon. Bracketed numbers indicate the range of exponents on the y axis.

(C) Scatter plot of BrdU incorporation at all replication origins (+/- 2.5 kb) in *rad53-11* and *mec1-1 sml1-1* mutants relative to wild-type cells, plotted against the time of origin activation (replication time) (Yabuki et al., 2002).

(D) Levels of BrdU incorporation in *rad53-11* and *mec1-1 sml1-1* mutants relative to wild-type cells expressed for groups of origins sorted according to their time of replication. The mean replication time for each bin is indicated on the x axis. ***, $P<0.0001$; ns: not significant, Mann–Whitney rank sum test.

Figure 2. **Mec1 is recruited to early replicating regions and phosphorylates H2A**

(A) Wild-type cells were synchronized in G_1 phase with α-factor and then released from G_1 phase arrest by the addition of Pronase to degrade the α-factor. Samples were recovered at the indicated times.

(B) Analysis of DNA content by flow cytometry.

(C) Distribution of Cdc45 (black), Ddc2 (orange) and γ-H2A (blue) was analyzed by ChIP–seq. A representative region on chromosome 15 is shown. Protein signals are expressed as a ratio of IP to input after subtraction of the corresponding signals from G_1 phase cells. Open arrowheads, early origins; filled arrowheads, late origins. Bracketed numbers indicate the range of exponents on the y axis.

(D) Average profiles of Cdc45, Ddc2 and γ-H2A across 40 kb regions centered on annotated replication origins (n=386).

(E) Variations of total Ddc2 and γ-H2A levels at early-, mid- and late-replicating regions during S phase.

Figure 3. Ddc2 and γ-H2A enrichment at early replicating regions does not correlate with high transcription levels.

(A) Percentage of genomic annotations overlapping with Ddc2 (orange) and γ-H2A (blue) signals in early S phase.

(B) Intensity of Ddc2 and γ-H2A signals plotted against the levels of Rpb1-pS2 and Rpb3 across 5 kb intervals centered on 192 early (blue) and 192 late (grey) origins.

(C) Scatter plots of Ddc2 and γ-H2A signals across 5 kb intervals centered on replication origins either containing (grey dots) or not containing (black dots) tRNA genes (tDNA) plotted against replication time (Trep). Statistical analysis of bins of early origins grouped according to their time of replication indicates that the presence of a tDNA (grey bars) does not increase Ddc2 or γ-H2A binding. Ns, not significant, Mann–Whitney rank sum test.

Figure 4. Entry into S phase is followed by a spontaneous reduction of dNTP pools.

(A) ChIP–seq analysis of the distribution of γ-H2A in wild-type cells 30 minutes after release from G_1 phase arrest (S_{30}) or after 60 minutes in medium containing 200 mM HU. A representative region on chromosome 15 is shown. Open arrowheads, early origins; filled arrowheads, late origins. Bracketed numbers indicate the range of exponents on the y axis.

(B) Scatter plot of γ-H2A levels in cells released from G_1 phase arrest in the presence or the absence of HU. Blue, early origins (n=192); grey, late origins (n=192). R, Spearman correlation coefficient.

(C) Average profiles and heat maps of γ-H2A levels across 60 kb regions centered on 192 early (blue) and 192 late (red) origins.

(D) Wild-type cells were arrested in G_1 phase with α-factor and released synchronously into S phase. Samples were collected every 5 minutes after release and DNA content was analyzed by flow cytometry.

(E) Analysis of intracellular concentrations of dNTPs at the indicated times after release from G_1 phase arrest.

(F) Western blot analysis of Sml1 protein levels after releasing cells from G_1 phase arrest. Tubulin is shown as a control for loading.

(G) Variation of DNA content (grey) and Sml1 protein levels (black) after release from G_1 phase arrest. Error bars correspond to the standard deviation of three independent experiments. DNA content is derived from flow cytometry data. Sml1 levels were determined by western blotting and were normalized to tubulin.

Figure 5. Experimental increase of dNTP levels suppresses Mec1 activation.

(A) Wild-type and p*GAL-RNR sml1Δ* cells were synchronized in G_1 phase before induction of *RNR* gene expression with 1% galactose and 1% sucrose. Cells were then released from G_1 phase arrest and collected at the indicated times.

(B) Analysis of DNA content by flow cytometry.

(C) Analysis of intracellular concentrations of dNTPs in G_1 phase before and after *RNR* induction and 30 minutes after release (S_{30}). Mean values and SD for two independent experiments are shown.

(D) ChIP–qPCR analysis of γ-H2A enrichment near four representative early origins in wild-type and p*GAL-RNR*1 *sml1Δ* cells in G_1 phase and after release from G_1 phase arrest. Means and SD for four independent experiments are shown.

(E) ChIP–seq analysis of γ-H2A levels at 192 early origins in wild-type and p*GAL-RNR sml1Δ* cells collected 30 minutes after release from G_1 phase arrest. Origins have been divided in two bins according to their replication time (Trep). ***: $p<0.001$, Mann–Whitney rank sum test.

Figure 6. Rad53 absence in normal S phase induces a strong block of replication forks associated with permanent activation of the S phase checkpoint.

(A) Control p*GAL-SML1* and *rad53*Δ p*GAL-SML1* cells in an *hht2*Δ *hhf2*Δ background were synchronized in G_1 phase and *SML1* was overexpressed by addition of 2% galactose. Cells were released from G_1 phase arrest and samples were taken at the indicated times.

(B) Analysis of DNA content by flow cytometry.

(C) Western blot analysis of Sml1 protein levels. Tubulin was used as a control for loading.

(D) Analysis of intracellular concentrations of dNTPs in G_1 phase before and after *SML1* induction and at the indicated times after release from G_1 phase arrest. Mean values and SD for two independent experiments are shown. Arrows point to reduced dATP levels in *rad53*Δ p*GAL-SML1* cells.

(E) Variations in DNA copy number (ratio of S/G_1 reads) and γ-H2A (ChIP-seq signal) in control p*GAL-SML1* and *rad53*Δ p*GAL-SML1* cells. A representative region on chromosome 15 is shown. Open arrowheads, early origins; filled arrowheads, late origins. Bracketed numbers indicate the range of exponents on the y axis.

(F) Distance covered by individual replication forks in control p*GAL-SML1* and *rad53*Δ p*GAL-SML1* cells released synchronously into S phase for the indicated times. The length of replicated tracks is derived from DNA copy number variations displayed in panel (E). Median distances and numbers of active origins are indicated. n/a indicates that distances could not be determined for control p*GAL-SML1* cells after 90 minutes due to completion of DNA replication.

Figure 7. *SML1* expression in *rad53*Δ cells induces fork collapse and mitotic catastrophe.

(A) p*GAL-SML1*, *rad53*Δ p*GAL-SML1*, *mec1*Δ p*GAL-SML1* and *dun1*Δ p*GAL-SML1* cells were synchronized in G_1 phase and *SML1* was overexpressed by addition of galactose before release from G_1 phase arrest. Glucose was added after two hours to repress *SML1* expression.

(B) Analysis of cell survival after induction of *SML1* for the indicated times.

(C) Analysis of DNA content by flow cytometry.

(D) Western blot analysis of Rad53, Clb2 and Sml1 levels in cells treated as indicated in (a) or after exposure to 200 mM HU for 120 minutes in S phase (HU). Tubulin and Ponceau staining were used as controls for loading.

(E) Analysis of DNA content by flow cytometry of *rad53*Δ p*GAL-SML1*, *mec1*Δ p*GAL-SML1* and *dun1*Δ p*GAL-SML1* cells that were incubated for 120 minutes in galactose-containing medium to induce *SML1* overexpression, then washed and placed in glucose-containing medium to recover from *SML1* overexpression.

(F) Fluorescence microscopy of nucleus segregation at various times after release from G_1 phase arrest of p*GAL-SML1*, *rad53*Δ p*GAL-SML1* and *dun1*Δ p*GAL-SML1* cells expressing mCherry–Pus1 (red) as a marker of the nucleus and Rad52–GFP (yellow) as a marker of foci of DNA repair. Representative images are shown for the three strains and for *rad53*Δ p*GAL-SML1* and *dun1*Δ p*GAL-SML1* cells released for 90 minutes in glucose-containing medium. Scale bar, 5 µm.

(G) Quantification of normal and abnormal segregation from images as in (f).

(H) Percentage of cells containing Rad52–GFP foci.

Figure 8. Models of dNTP pool regulation during S phase. See text for details.

(A) Classical model: Cells anticipate the requirement of high dNTP pools in S phase by overexpressing RNR subunits in late G_1 and by degrading the RNR inhibitor Sml1 in early S phase. The degradation of Sml1 depends on the Mec1-Rad53 pathway, but the mechanism that activates this pathway during normal DNA replication is unknown.

(B) Revised model: Cells enter S phase with suboptimal dNTP pools, which are not sufficient to sustain the activation of hundreds of early origins upon entry into S phase. This leads to the pausing of replication forks, the transient activation of the Mec1-Rad53 pathway and the degradation of Sml1, allowing dNTPs to reach optimal levels.

STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Philippe Pasero (philippe.pasero@igh.cnrs.fr). All unique strains and reagents generated in this study are available from the Lead Contact without restriction. Requests for strains and reagents donated by other laboratories should be directed to the specific laboratory from which they were received.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All *S. cerevisiae* strains used are haploid and are derived from W303 (see Table S1).

METHOD DETAILS

Cell growth and synchronization

Yeast strains used in this study are listed in the Yeast Strains table. Yeast strains were freshly thawed from frozen stocks and grown at 25°C using standard yeast genetics practices. Gene deletion and tagging were performed as described previously (Longtine et al., 1998). Cells were grown in YPD medium at 25°C unless otherwise stated. They were synchronized in G_1 phase with 8 µg/ml α-factor for 180 minutes and were released from G_1 phase arrest by the addition of 75 µg/ml Pronase and 20 mM citrate phosphate buffer (pH 5.6) in the presence or the absence of 200 mM HU. For overexpression of *SML1* and RNR subunits, cells were grown overnight in medium containing 2% raffinose. Three hours after addition of α-factor, the medium was supplemented for 1 hour with 2% galactose (*SML1*) or 1% galactose and 1% sucrose (RNR).

Analysis of DNA content by flow cytometry

Four hundred and fifty µl of culture samples at 10^7 cells/ml were diluted in 1 ml of 100% ethanol. Cells were centrifuged for 1 minute at 16.000 RCF and resuspended in 50 mM sodium citrate buffer containing 10 µl of RNase A (20 mg/ml, Qiagen 76254) for 2 hours at 50°C. Then, 10 µl of proteinase K (Sigma, P6556) were added for 2 hours at 50°C. Aggregates of cells were dissociated by sonication. Thirty µl of cell suspension were incubated with 170 µl of 50 mM sodium citrate buffer containing 0.5 µM Sytox Green (Invitrogen). Data were acquired on a MACSQuant Analyzer (Miltenyi Biotec) and analyzed

with FlowJo software. Two to four independent biological replicates were performed per sample.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described (Lengronne et al., 2004) with minor modifications. One billion ($1x10^9$) cells were crosslinked for 30 minutes with 1% formaldehyde (Sigma F8775) at room temperature on a shaking device. Fixation was quenched by addition of 0.25 M glycine (Sigma G8898) for 5 minutes under agitation. Cells were washed three times with cold TBS1X (4°C). Dry pellets were frozen and stored at -20°C. Cell pellets were resuspended in lysis buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate) supplemented with 1 mM PMSF, phosphatase inhibitor and anti-protease (cOmplete Tablet, Roche, 505649001) and lysed by bead beating (MB400 U, Yasui Kikai, Osaka). The lysate (WCE, Whole Cell Extract) volume was brought to 3 ml with cold lysis buffer and sonicated with a Q500 sonicator (Qsonica; 12 cycles: 15 sec ON, 45 sec OFF, amplitude 50). Forty µl of input material was saved for qPCR or sequencing analysis. 180 µl of Dynabeads Protein A (DPA) were washed three times and resuspended in 1 ml of PBS, 0.5% BSA, 0.1% Tween and incubated with specific volumes of antibodies (40 µl of anti-PK - Anti-V5 tag, AbD Serotec, MCA1360G; 4 µl of anti-H2A-S128 - AV137; 40 µl of anti-HA - Santa Cruz, SC-7392), on a rotating wheel for two hours at 4°C. Antibody-coupled Dynabeads were washed three times with 1 ml of PBS, 0.5% BSA, 0.1% Tween, added to 2.7 ml of WCE and incubated on a rotating wheel at 4°C overnight. Beads were then collected on a magnetic rack. They were washed on ice with cold solutions: twice with Lysis buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate), twice with Lysis buffer + 0.36 M NaCl (50 mM HEPES-KOH pH7.5, 360 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate), twice with Wash buffer (10 mM Tris-HCl pH8, 0.25 M LiCl, 0.5% IGEPAL, 1 mM EDTA, 0.1% sodium deoxycholate) and once with TE (10 mM Tris-HCl pH8, 1 mM EDTA). Antibodies were uncoupled from beads with 120 µl of Elution Buffer (50 mM Tris-HCl pH8, 10 mM EDTA, 1% SDS) for 10 min at 65°C. Eluates were incubated with 120 µl of TE containing 0.1% SDS at 65°C for 6 hours to de-crosslink then 130 µl of TE containing 60 µg RNase A (Sigma, R65-13) were added and the samples were incubated for 2 hours at 37°C. Proteins were digested by addition of 20 µl of proteinase K (Sigma, P6556) at 20 mg/ml and incubated for 2 hours at 37°C. Fifty µl of 5M LiCl were added to the DNA before purification by two rounds of extraction with phenol : chloroform :

isoamyl alcohol (25:24:1; Sigma, P2069) and precipitation by addition of 100 mM sodium acetate (Sigma, S2889), 26 µg/ml of glycogen (Roche, 10901393001) and two volumes of 100% ethanol overnight at -20°C. Samples were centrifuged for 45 min at 16,000 RCF at 4°C, washed with cold 70% ethanol and centrifuged again for 15 min at 16,000 RCF at 4°C. DNA pellets were dried and resuspended in 300 µl of H_20 prior to qPCR reactions or in 25 µl prior to deep-sequencing. The qPCR reactions were performed in a LightCycler480 (Roche). IP/Input ratios were calculated and qPCR results were normalized on negative zones: for yH2A ChIP-qPCR Dop1.

BrdU–IP–chip, BrdU–IP–seq, ChIP–seq and NGS-based analysis of DNA copy number

Immunoprecipitation and processing of BrdU-labelled DNA in BrdU–IP–chip and BrdU–IP–seq experiments were performed as described previously (Poli et al., 2012; Yoshida et al., 2014). For NGS-based assays, sequencing libraries were prepared by using the ThruPLEX DNA–seq Kit (Rubicon Genomics). NGS was performed on a HiSeq4000 sequencing system (Illumina). Single-end reads of 50 bp were aligned to the *S. cerevisiae* genome (2011) with Bowtie2, allowing only perfect matches. Log ratio on input were generated with Bamcompare and displayed using IGB v8.2 (Nicol et al., 2009). Average profiles and heat maps were prepared with Deeptools2 on 60 kb regions centered on replication origins. ChIP–seq scores expressed as RPKM (reads per kilobase per million mapped reads) were calculated by using Bedtools on 2 kb windows centered on replication origins.

Protein extracts and western blotting

TCA precipitation was performed as described previously (Longhese et al., 1997). Extracts were resolved by SDS–PAGE (Biorad) and then transferred to nitrocellulose membranes by using an Invitrogen system. The Rad53 shift due to phosphorylation was detected with a rabbit polyclonal antibody (gift of C. Santocanale, Galway) and tubulin was detected with the YOL1/ 34 antibody (Abcam; #ab6161). Sml1 was detected with a rabbit polyclonal antibody (Agrisera AB, Sweden, #AS10 847). Clb2 was detected with a rabbit polyclonal antibody (Santa Cruz Biotechnology, y-180. Rnr1 was detected with a rabbit polyclonal antibody (Agrisera, AS16 3639).

Survival test

One hundred cells were spread onto YPD plates and incubated at 30°C for 2 days. Survival rates were determined after 2 days by counting colonies.

Microscopy

Five hundred µl of cells were centrifuged for 1 minute at 16,000 RCF then resuspended in 10 µl of YPD medium. Four µl of the cell suspension were spread between slide and coverslip and used for microscopy analysis. Pictures were taken using a Zeiss AxioImager Z2 fluorescence microscope with a 63x objective. One hundred cells per sample were counted with the FIDJ plugin cell counter to determine the percentage of cells with Rad52 foci and chromosome segregation defects.

Analysis of intracellular dNTP pools

Thirty-five ml of cells at $1x10^7$ cells/ml were harvested on nitrocellulose membranes and suspended immediately in an ice-cold mixture of 12% TCA and 15 mM $MgCl_2$. The cells were vortex-mixed for 15 min at 4°C and then centrifuged at 14,000 rpm for 1 min at 4°C. The supernatant was neutralized with a freon–trioctylamine mix and analyzed as described previously (Jia et al., 2015).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All statistical tests and numbers of biological replicates are listed in the figure legends. To compare statistical significance between mean values of biological replicates, two-tailed unpaired t tests were used. To compare statistical significance between two distributions, the Mann-Whitney rank-sum test was used. All statistical tests were performed with GraphPad Prism 7.

DATA AND CODE AVAILABILITY

The sequencing data generated in this study have been deposited with the Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE136605. Unprocessed gel images presented in this manuscript can be found at DOI:10.17632/4n27v8xkpg.2

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