S phase block following MEC1^ATR inactivation occurs without severe dNTP depletion

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ABSTRACT

Inactivation of Mec1, the budding yeast ATR, results in a permanent S phase arrest followed by chromosome breakage and cell death during G2/M. The S phase arrest is proposed to stem from a defect in Mec1-mediated degradation of Sml1, a conserved inhibitor of ribonucleotide reductase (RNR), causing a severe depletion in cellular dNTP pools. Here, the casual link between the S phase arrest, Sml1, and dNTP-levels is examined using a temperature sensitive mec1 mutant. In addition to S phase arrest, thermal inactivation of Mec1 leads to constitutively high levels of Sml1 and an S phase arrest. Expression of a novel suppressor, GIS2, a conserved mRNA binding zinc finger protein, rescues the arrest without down-regulating Sml1 levels. The dNTP pool in mec1 is reduced by ~17% and GIS2 expression restores it, but only partially, to ~93% of a control. We infer that the permanent S phase block following Mec1 inactivation can be uncoupled from its role in Sml1 down-regulation. Furthermore, unexpectedly modest effects of mec1 and GIS2 on dNTP levels suggest that the S phase arrest is unlikely to result from a severe depletion of dNTP pool as assumed, but a heightened sensitivity to small changes in its availability.

KEY WORDS: Mec1, ATR, Sml1, RNR, dNTP, DNA replication, Replication arrest

INTRODUCTION

Budding yeast Mec1 belongs to the conserved ATM/ATR family of signal transducers involved in a range of processes, including DNA damage repair, checkpoint response, cell cycle regulation, and meiosis (Kato and Ogawa, 1994; Weinert et al., 1994; Abraham, 2001; Carballo et al., 2008). In addition, Mec1 and its mammalian counterpart ATR, are essential during unperturbed proliferation, whereby their inactivation leads to permanent DNA replication block followed by a fatal mitotic catastrophe in the respective organism (Brown and Baltimore, 2000; Casper et al., 2002; Cha and Kleckner, 2002; Eykelenboom et al., 2013).

The replication block in mec1 cells was proposed to stem from a defect in the Mec1-Rad53-Dun1 dependent removal of Sml1 at the onset of S phase (Zhao et al., 1998, 2001; Zhao and Rothstein, 2002). Sml1 is an inhibitor of the ribonucleotide reductase (RNR), which catalyses the rate limiting step in dNTP synthesis (Desany et al., 1998; Zhao et al., 1998, 2001; Zhao and Rothstein, 2002). Rad53, a homolog of mammalian CHEK2, is an essential downstream effector kinase of Mec1 (Allen et al., 1994; Matsuoka et al., 1998). Dun1 is another serine/threonine kinase and responsible for Sml1 phosphorylation and degradation (Zhao et al., 2001; Zhao and Rothstein, 2002). According to this view, the Mec1-Rad53-Dun1-dependent Sml1 removal and ensuing RNR activation would promote the dNTP production. In support for this view, it was shown that dNTP levels in mec1- or rad53-hypomorphs and a dun1Δ strain were reduced by as much as 46% compared to a MEC1 control strain (Zhao et al., 2001; Fasullo et al., 2010; Hoch et al., 2013).

Notably, however, nearly all analyses on a lethal mec1 allele [e.g. mec1Δ or mec1-kd (kinase dead)] have been performed in a strain background that was either deleted for SML1 or over-expressing RNR1, a requirement for maintaining viability of a mutant lacking Mec1’s essential function (e.g. Desany et al., 1998; Zhao et al., 1998). As a result, while it is clear that absence of Mec1 causes dNTP pool to decrease, the true extent of the reduction and whether it would be sufficient to account for the replication arrest remain elusive. Here, we addressed these questions utilizing a temperature sensitive mutant, mec1-4, which maintains its viability at permissive temperature in an otherwise wild-type background, circumventing the need to exogenously manipulate Sml1 and/or RNR activity (Cha and Kleckner, 2002).

RESULTS AND DISCUSSION

We began the analysis by performing a multi-copy suppressor screen for mec1-4 (Fig. S1). The screen identified GIS2 (glucose inhibition of gluconeogenic growth suppressor 2) as a novel suppressor (Fig. 1A): The only other suppressors identified were MEC1 and RNR1 (Fig. S1). GIS2 was originally isolated based its role in alternative carbon source utilization (Balcìunas and Ronne, 1999). Subsequently, it was shown to encode a conserved zinc finger protein, whose orthologs include the fission yeast Byr3, identified as a negative regulator of the RAS/PKA pathway (Wang et al., 1991) and CNBP/ZNF9, an essential mammalian protein, implicated in myotonic dystrophy type 2 (Rajavashishth et al., 1989; Liquori et al., 2001).

To rule out the possibility that GIS2 was an allele specific suppressor, we examined its effects on a different mec1 allele, mec1-40. While mec1-4 contains a single amino acid alteration in the conserved kinase domain, mec1-40 carries an alteration in the N-terminal HEAT (Huntington, elongation factor 3, protein phosphatase 2A, Tor1) repeat domain (Perry and Kleckner, 2003; E. Waskiewicz and R.C., unpublished results). Introduction of a multi-copy plasmid carrying GIS2 (pGIS2) also suppressed mec1-40 temperature sensitivity, demonstrating that the suppression was not allele-specific (Fig. S2A); however, it was not able to rescue a null (mec1Δ) or a kinase dead (mec1-kd) (data not shown). Notably, GIS2 did not rescue lethality conferred by temperature sensitive alleles of YCG1, TOP2, ESP1 or DBF4,
encoding for a condensin subunit, topoisomerase II, separase, or the regulatory subunit of Cdk7-Dbf4 kinase, respectively; thus, GIS2 is not a suppressor of general temperature sensitivity (Fig. S2B).

To test whether the GIS2 suppression was mediated by restoring Mec1’s function in response to replication stress or DNA damage, we assessed the effects of pGIS2 on sensitivity of mec1-4 to hydroxyurea (HU) or methyl methanesulfonate (MMS), respectively. GIS2 did not rescue the drug sensitivity (Fig. S2C), suggesting that the suppression was independent of the role of Mec1 in mediating responses to HU or MMS.

The effects of GIS2 on S phase progression were assessed. In a MEC1 strain carrying either pGIS2 or a control YEplac24 plasmid (pCont), genome duplication was initiated and completed within 40 min following α-factor arrest/release (Fig. 1B). A mec1-4 strain carrying pCont initiated genome duplication but failed to complete, in agreement with previous reports (Cha and Kleckner, 2002; Hashash et al., 2012). In contrast, DNA replication in the same mec1-4 strain carrying pGIS2 was completed by t=40 min. We infer that the GIS2 rescue of mec1 lethality is mediated by promoting efficient genome duplication, thereby averting the downstream fatal mitotic catastrophe.

To test whether the GIS2 suppression was dependent on the Mec1-Rad53-Dun1 pathway (Zhao et al., 2001; Zhao and Rothstein, 2002), we assessed the effects of GIS2 on a mec1-4 dun1Δ double mutant (Fig. 1C). Deletion of DUN1 shows synthetic growth defects with hypomorphic mec1 mutants (e.g. Zhao and Rothstein, 2002). Similarly, we observed synthetic interaction between DUN1 and mec1-4, whereby dun1Δ lowered restrictive temperature of a mec1-4 strain from 30°C to 27°C (Fig. 1C). Nevertheless, pGIS2 improved viability of a mec1-4 dun1Δ mutant at 27°C, indicating that the suppression did not require the Mec1-Rad53-Dun1 signalling.

Next, we assessed the effects of pGIS2 on steady-state Sml1 levels. During unchallenged proliferation, Sml1 undergoes S-phase- and MEC1/RAD53/DUN1-dependent downregulation (e.g. Zhao et al., 2001). As expected, we observed a notable reduction in the Sml1 levels in a MEC1 strain between t=10-30 and 70-90 min following an α-factor arrest/release, corresponding to the first and presumably the second round of S phase, respectively (Fig. 2; Fig. S3). A similar S-phase-dependent reduction in SmI1 levels was observed in a mec1-4 culture released at 23°C between t=40 and 80 min (Fig. 2). The notable delay in the timing is likely due to the lower temperature utilized to maintain viability of the mutant. The latter confirms that mec1-4 cells are proficient in promoting the S-phase-dependent Sml1 destruction at permissive temperature. At 30°C, however, Sml1 levels in the mutant continued to increase and were maintained at high levels despite the fact that...
the cells were in S phase (Fig. 2; Fig. S3). Introduction of pGIS2 promoted efficient genome duplication in the mec1-4 strain at 30°C (Fig. 2C). Remarkably, however, the Sml1 levels in the latter did not decrease, but increased, during genome duplication (Fig. 2). The current observation eliminates Sml1 downregulation as a mechanism underlying the GIS2 suppression. Furthermore, it demonstrates that the replication defect following Mec1 inactivation can be decoupled from Sml1 stabilization.

To test whether the GIS2 suppression might be mediated by an increase in RNR activity, we assessed its effects on RNRII transcription induction at the onset of S phase (Fig. 3A). In all strains, the level of RNRII transcripts following α-factor arrest/release peaked at the first time point, t=10 min. The levels in the WT and mec1-4+pGIS2 strains gradually decreased back to the basal level by 40 min (Fig. 3A), coinciding with the completion of bulk genome duplication in these cultures (Fig. 1B). Importantly however, pGIS2 did not increase the level or duration of RNRII mRNA induction the mec1-4 culture (Fig. 3A). We also assessed effects of pGIS2 on levels of Rnr1 protein as well as RNRI, RNRII, 3, and 4 transcripts, where no noticeable difference was observed (Fig. 3B; Fig. S3). Taken together, we conclude that the GIS2 suppression is not mediated by an increase in RNR expression.

In yeast, additional mechanisms of controlling dNTP production exist; for example, the dATP feedback inhibition of RNR and regulation of Rnrl, 2, 3, and 4 sub-cellular localization (Chabes et al., 2003; Yao et al., 2003; Lee et al., 2008). Instead of testing potential involvement of each of these mechanisms, we decided to directly assess the effects of GIS2 on dNTP pools. Following a temperature shift from 23°C to 30°C, both MEC1 and mec1-4 cultures exhibited a transient reduction in the dNTP levels followed by a recovery (Fig. 4A). The dNTP levels in a mec1-4 strain transformed with pCont were reduced to ∼93% of the control. Thus, the replication arrest and its rescue conferred by Mec1 inactivation and GIS2, respectively, are both accompanied by unexpectedly modest changes in dNTP levels. The current observations are reminiscent of a rad53 allele lacking key Mec1 phosphorylation sites, which was similarly shown to reduce dNTP pool by only ∼15% (Hoch et al., 2013).

dNTP levels in mec1- or rad53-hypomorphs, or a dunlΔ can be reduced by as much as 46% of a control (Zhao et al., 2001; Fasullo et al., 2010; Hoch et al., 2013). Notably, all of these mutants are viable, which indicates that that genome duplication is possible even when dNTP levels fall below those that we observed in mec1-4 cells. We also found that mec1-4 cells in a SK1 background had ∼30% higher dNTP levels compared to a MEC1 strain in a different genetic background of a comparably sized genome (∼17 Mb), which was used as a control in the above mentioned mec1-rad53- hypomorph studies (Fig. 4B; 390 pmoles vs 296 pmoles/10^8 cells). Taken together these observations strongly suggest that the dNTP pool in a mec1-4 strain would have been sufficient to support genome duplication under normal condition, and therefore was unlikely to be the sole cause of the arrest. The ratio among four different dNTPs in mec1-4 was comparable to MEC1 (Fig. 4C), ruling out the possibility that the replication block is due to imbalance in dNTP precursors (Kumar et al., 2010).

To the best of our knowledge, these are the first direct dNTP pool measurements in a strain expressing a lethal mec1 allele without altering Sml1 or RNR activity. While the results confirm a Mec1’s role in promoting dNTP synthesis, they reveal that loss of this function is unlikely to be a direct cause of the mec1 replication arrest. To date, ∼80 direct targets of Mec1 have been identified. They include components of the RPA complex (Rfa1 and Rfa2; Smolka et al., 2007), the GINS complex (Psf1; De Piccoli et al., 2012), and the MCM-helicase complex (Mcm4 and Mcm6; Randell et al., 2010), all of which are directly involved in DNA replication. Therefore, inactivation of Mec1 might result in a system-wide failure in genome duplication stemming from inability to phosphorylate key components of the replication machinery. Under such condition, DNA replication might become acutely sensitive to dNTP levels, whereby even a modest reduction in dNTP pool, which would not impair normal genome duplication, triggers a permanent arrest.

Most known suppressors of mec1 lethality are involved in dNTP synthesis or its regulation (e.g. Desany et al., 1998; Zhao et al., 1998; Tsapoina et al., 2011). In yeast, the rate of replication fork progression correlates with the dNTP pool size (Malinsky et al., 2001; Sabouri et al., 2008; Odsbu et al., 2009; Poli et al., 2012). Furthermore, elevated levels of dNTP can promote fork progression through DNA lesions that normally block its progression (Malinsky et al., 2001).
sensitive mec1-4 allele were previously described (Cha and Kleckner, 2002). Multi-copy suppressor screen for mec1-4 is described in Fig. S1. To obtain a synchronous culture for cell cycle analysis, cultures grown to mid-log phase at 23°C (permissive temperature for mec1-4) were arrested with 5 μg/ml α-factor for 3 h before being released to fresh YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose] media at the indicated temperature.

Fluorescence activated cell scan (FACS) analysis

Cells were fixed [40% (v/v) ethanol, 0.1 M sorbitol] for 3 h and incubated overnight at 37°C with RNase solution (50 mM Tris-HCl pH 7.5, 100 μg/ml RNaseA). The next day, the cells were treated with 500 μl of pepsin solution (50 mM HCl, 5 mg/ml pepsin) for 5 min or longer at room temperature being resuspended in 1 ml SYTOX solution (50 mM Tris-HCl pH 7.5, 1 mM SYTOX Green; Invitrogen, Molecular Probe). After an overnight incubation at 4°C, samples were analysed on a Becton Dickinson Flow Cytometer (Hashash et al., 2012).

Western blotting and antibodies

Whole cell extracts from ~10^10 cells were prepared from cell suspensions in 20% trichloroacetic acid (TCA) by agitation with glass beads. Precipitated proteins were solubilized in SDS-PAGE sample buffer and analysed by SDS-PAGE and western blotting. Antibodies for western blotting were mouse monoclonal α-p53 (1:1000; Abcam) and mouse monoclonal α-MYC (1:1000; NIMR, London, UK), α-tubulin (1:5000; NIMR, London, UK), and rat monoclonal α-HA (1:1000; NIMR, London, UK). Measurement of dNTP levels

NTP and dNTP extraction and quantification were performed as described (Jia et al., 2015).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.E., S.R., G.M., A.C and R.S.C. designed the experiments; C.E., S.R. and G.M. performed the experiments; C.E., G.M., A.C and R.S.C. interpreted the results. A.C and R.S.C. performed the experiments; C.E., G.M., A.C and R.S.C. interpreted the results. A.C and R.S.C. coordinated the study.

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Supplementary information

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MATERIALS AND METHODS

Yeast strains and media

All strains were of the SK1 background unless noted (Table S1). Hydroxylamine mutagenesis of MEC1 and isolation of the temperature sensitive mec1-4 allele were previously described (Cha and Kleckner, 2002). Multi-copy suppressor screen for mec1-4 is described in Fig. S1. To obtain a synchronous culture for cell cycle analysis, cultures grown to mid-log phase at 23°C (permissive temperature for mec1-4) were arrested with 5 μg/ml α-factor for 3 h before being released to fresh YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose] media at the indicated temperature.
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