Mec1/ATR regulates the generation of single-stranded DNA that attenuates Tel1/ATM signaling at DNA ends

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Abstract

Tel1/ATM and Mec1/ATR checkpoint kinases are activated by DNA double-strand breaks (DSBs). Mec1/ATR recruitment to DSBs requires the formation of RPA-coated single-stranded DNA (ssDNA), which arises from 5'–3' nucleolytic degradation (resection) of DNA ends. Here, we show that *Saccharomyces cerevisiae* Mec1 regulates resection of the DSB ends. The lack of Mec1 accelerates resection and reduces the loading to DSBs of the checkpoint protein Rad9, which is known to inhibit ssDNA generation. Extensive resection is instead inhibited by the Mec1-ad mutant variant that increases the recruitment near the DSB of Rad9, which in turn blocks DSB resection by both Rad53-dependent and Rad53-independent mechanisms. The *mec1-ad* resection defect leads to prolonged persistence at DSBs of the MRX complex that causes unscheduled Tel1 activation, which in turn impairs checkpoint switch off. Thus, Mec1 regulates the generation of ssDNA at DSBs, and this control is important to coordinate Mec1 and Tel1 signaling activities at these breaks.

Keywords checkpoint; double-strand break; Mec1; resection; Tel1

Subject Categories DNA Replication, Repair & Recombination

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Introduction

The response to DNA double-strand breaks (DSBs) in eukaryotes is governed by DNA damage checkpoint signal-transduction pathways, whose key players include the *Saccharomyces cerevisiae* protein kinases Mec1 and Tel1, whose mammalian orthologs are ATR and ATM, respectively (Ciccio & Elledge, 2010). Mec1/ATR recruitment to sites of damage is mediated in part through its association with Ddc2/ATRIP (Paciotti *et al.*, 2000; Cortez *et al.*, 2001; Nakada *et al.*, 2005; Chen *et al.*, 2007), whereas Tel1/ATM is directly activated by

the MRX (Mre11-Rad50-Xrs2)/MRN (Mre11-Rad50-Nbs1) complex, which is required to recruit Tel1/ATM to the DSB ends (Nakada *et al.*, 2003; Falck *et al.*, 2005; Lee & Paull, 2005; You *et al.*, 2005; Berkovich *et al.*, 2007).

Once Mec1/ATR and/or Tel1/ATM are activated by damaged DNA, their checkpoint signals are propagated through the protein kinases Rad53 and Chk1 (vertebrate Chk2 and Chk1, respectively). Activation of these downstream kinases requires conserved mediator proteins, including the BRCT-domain-containing protein Rad9 and its *S. pombe* and metazoan orthologs Crb2 and 53BP1, respectively (Gilbert *et al.*, 2001; Sweeney *et al.*, 2005; Usui *et al.*, 2009). Recruitment of Rad9 to chromatin involves multiple pathways. Rad9 is already bound to chromatin in unperturbed cells and this binding requires the Rad9 Tudor domain and methylation of histone H3 at lysine 79 (Javaheeri *et al.*, 2006; Grenon *et al.*, 2007; Hammet *et al.*, 2007; Granata *et al.*, 2010). Rad9 binding to the sites of damage is further strengthened by an interaction between its BRCT domain and histone H2A that has been phosphorylated at serine 129 (γH2A) (Javaheeri *et al.*, 2006; Toh *et al.*, 2006; Hammet *et al.*, 2007). Similarly, 53BP1 binding to DSBs is facilitated by phosphorylation of the histone variant H2AX at serine 139 (Iwabuchi *et al.*, 2003; Ward *et al.*, 2003).

Mec1/ATR and Tel1/ATM respond to different types of DNA structures. In fact, blunt DSB ends and DNA ends with short single-stranded DNA (ssDNA) tails are the preferred substrates for Tel1/ATM binding (Shiotani & Zou, 2009), while Mec1/ATR recruitment requires the formation of RPA-coated ssDNA arising from 5'–3' nucleolytic degradation (resection) of the DSB ends (Zou & Elledge, 2003). This resection is catalyzed by the concerted action of nucleases and helicases (Longhese *et al.*, 2010). In *S. cerevisiae*, the MRX complex has been shown to initiate DSB end resection by acting in concert with Sae2 (Ivanov *et al.*, 1996; Clerici *et al.*, 2005). Subsequent extensive nucleolytic degradation of the DSB ends relies on two pathways, one of which is dependent on the 5'–3' exonuclease Exo1, while the other depends on the RecQ helicase Sgs1 that acts in conjunction with the single-strand-specific nuclease Dna2 (Mimitou & Symington, 2008; Zhu *et al.*, 2008). DSB end resection is also negatively regulated by Rad9 (Lydall & Weinert, 1995; Lazzaro *et al.*, 2008).

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Once recruited to DSBs, Tel1/ATM facilitates activation of Mec1/ATR (Adams *et al*, 2006; Jazayeri *et al*, 2006; Myers & Cortez, 2006), possibly because it promotes production of ssDNA that transforms the DSB ends from Tel1/ATM substrates to Mec1/ATR substrates (Mantiero *et al*, 2007; Shiotani & Zou, 2009). As the single-stranded 3' overhangs increase in length, they in turn attenuate Tel1/ATM activation, thereby promoting a switch from a Tel1/ATM- to a Mec1/ATR-dependent checkpoint (Mantiero *et al*, 2007; Shiotani & Zou, 2009). The importance of this Tel1/ATM to Mec1/ATR switch in the response to DSBs remains to be determined.

Interestingly, Mec1 itself might regulate the generation of 3'-ended ssDNA at DNA ends by acting on positive and negative regulators of DSB resection. In fact, Mec1-dependent phosphorylation of Sae2 is important for Sae2 function in this process (Baroni *et al*, 2004; Cartagena-Lirola *et al*, 2006). Furthermore, Mec1 phosphorylates histone H2A on Ser129 (Downs *et al*, 2000), and this phosphorylation appears to inhibit DSB processing (Eapen *et al*, 2012). Finally, Mec1 activates the Rad53 checkpoint kinase, which in turn counteracts DSB resection by phosphorylating and inhibiting Exo1 (Jia *et al*, 2004; Morin *et al*, 2008). However, whether Mec1 dysfunction has any consequence on DSB resection is unknown, and the possible outcomes are difficult to envisage, as the Mec1-dependent phosphorylation events influence the activity of both positive and negative regulators of this process.

Once DSBs are repaired, the checkpoint signal is extinguished and cells resume cell cycle progression (recovery). However, yeast cells can escape an extended checkpoint arrest and re-enter the cell cycle even with an unrepaired DSB (adaptation) (Sandell & Zakian, 1993; Toczyski *et al*, 1997). Noteworthy, resection-defective *S. cerevisiae* mutants, such as *sae2Δ*, *fun30Δ* or *sgs1Δ* mutants, are also adaptation-defective, i.e. they fail to turn off the checkpoint in response to an unrepaired DSB (Clerici *et al*, 2006; Eapen *et al*, 2012). This finding raises the possibility that resection of DNA ends is required not only to activate a Mec1-dependent checkpoint, but also to extinguish the checkpoint signal.

To better understand the role of Mec1 in DSB resection and the interplays between resection and checkpoint activation/inactivation, we searched for and characterized an adaptation-defective *mec1-ad* mutant that was impaired in resection but proficient in checkpoint activation. By investigating the consequences on DSB resection of either the presence of the Mec1-ad variant or the absence of Mec1, we provide evidence that Mec1 regulates the generation of ssDNA at DSB ends. In addition, we show that DSB resection is important not only for Mec1 activation, but also for attenuating Tel1 signaling in order to allow proper termination of the checkpoint response.

Results

Isolation of *mec1* mutants unable to turn off the checkpoint

Mec1 might directly regulate the generation of ssDNA at the broken ends, possibly by acting on positive and negative regulators of DSB resection, but the consequences of Mec1 inactivation on this process are unknown. To gain insights into this issue, we searched for *mec1* mutants that were defective in resection but proficient in checkpoint activation. We took advantage of the well-established notion that a single irreparable DSB triggers a

Mec1-dependent G2/M cell cycle arrest (Pelliccioli *et al*, 2001). Wild-type cells then adapt to this checkpoint and form microcolonies after a 12–15 h arrest, whereas adaptation-defective mutants remain arrested at G2/M as large budded cells (Sandell & Zakian, 1993; Toczyski *et al*, 1997; Pelliccioli *et al*, 2001). As resection-defective mutants fail to adapt to the checkpoint (Clerici *et al*, 2006; Eapen *et al*, 2012), we screened for *mec1* mutants that cannot turn off the checkpoint after generation of a single irreparable DSB. To this end, we used a *mec1Δ sml1Δ* JKM139 derivative strain, where the lethal effect of *MEC1* deletion is suppressed by the lack of *Sml1* (Zhao *et al*, 1998). In this strain, expression of the HO endonuclease coding region from a galactose-inducible promoter leads to generation at the *MAT* locus of a single DSB that cannot be repaired by homologous recombination (HR), because the homologous donor loci *HML* or *HMR* are deleted (Lee *et al*, 1998). The N-terminal and the C-terminal *MEC1* coding regions were amplified by mutagenic PCR. Then, co-transformation of the *mec1Δ sml1Δ* strain with the PCR products and a centromeric plasmid containing part of the *MEC1* gene allowed reconstruction of the *MEC1* ORF on the plasmid by gap repair (Fig 1A). Scoring 3000 transformant clones at the microscope for the ability to form microcolonies on galactose-containing plates (Fig 1A) allowed identification of five transformants that were still arrested as large budded cells after 20 h in galactose. The one with the strongest phenotype was called *mec1-ad* and chosen for further characterization. Sequencing of the whole wild-type and mutant *MEC1* coding regions revealed that the *mec1-ad* allele carried multiple base pair substitutions causing the four amino acid changes D310G, K697T, Y944F and E961K in the Mec1 mutagenized N-terminal region (Fig 1B).

To assess more quantitatively the adaptation defect caused by the *mec1-ad* allele, we generated a stable *mec1-ad* mutant by substituting the chromosomal *MEC1* gene with the *mec1-ad* allele, followed by spotting G1-arrested cell cultures of the stable mutant on galactose-containing plates. As expected when the checkpoint is activated, most wild-type and *mec1-ad* cells arrested at 2-cell dumbbell stage within 4 h after HO induction (Fig 1C). Then, nearly all the wild-type cells formed microcolonies with more than 2 cells within 24 h, whereas most *mec1-ad* cells remained arrested at the 2-cell dumbbell stage (Fig 1C). Failure to adapt of these mutant cells correlated with their inability to turn off the Rad53-mediated checkpoint. In fact, when galactose was added to exponentially growing cell cultures of the same strains, Rad53 phosphorylation, which is required for Rad53 activation as a kinase, became detectable in both cell cultures about 4 h after HO induction (Fig 1D). Then, it decreased in wild-type cells 12–14 h after galactose addition, when cells resumed cell cycle progression, whereas it persisted longer in *mec1-ad* cells that were defective in re-entering the cell cycle (Fig 1D, and data not shown). Thus, *mec1-ad* cells are defective in adaptation to the checkpoint induced by a single irreparable DSB.

We then investigated whether the *mec1-ad* mutant fails to turn off the checkpoint also in response to DNA damaging agents. Rad53 phosphorylation persisted longer in *mec1-ad* than in wild-type cells both after transient exposure to camptothecin (CPT) (Fig 1E), which traps covalent topoisomerase 1-DNA cleavable complexes, and in the presence of the DNA alkylating agent methyl methanesulfonate (MMS) (Fig 1F). By contrast, both the amount and the kinetics of

Rad53 phosphorylation were very similar in *mec1-ad* and wild-type cells released from G1 arrest in the presence of the DNA replication inhibitor hydroxyurea (HU) (Fig 1G). Accordingly, *mec1-ad* cells

were more sensitive than wild-type cells to CPT and MMS, but not to HU (Fig 1H). Given that the cytotoxicity of CPT and MMS has been linked to the generation of replication-mediated DSBs

(Pommier, 2006; Ray Chaudhuri et al, 2012), these results suggest that *mec1-ad* mutant cells are specifically defective in turning off a DSB-induced checkpoint.

The difference in turning off the checkpoint between wild-type and *mec1-ad* cells might be due to differences in the amounts and/or intrinsic kinase activity between Mec1 and the Mec1-ad variant. We therefore constructed strains where the chromosomal wild-type *MEC1* or *mec1-ad* coding regions were fused with Myc epitopes. Western blot analysis with anti-Myc antibodies revealed similar amounts of Mec1-Myc and Mec1-ad-Myc in *MEC1-MYC* and *mec1-ad-MYC* protein extracts (Fig 1I). Moreover, equivalent amounts of immunoprecipitated Mec1-Myc or Mec1-ad-Myc phosphorylated with very similar efficiencies the known artificial substrate of the ATM-kinase family PHAS-I (Phosphorylated Heat and Acid Stable protein) (Fig 1J). Thus, the inability of the *mec1-ad* cells to turn off the checkpoint cannot be simply explained by an increase in Mec1-ad amount or kinase activity compared to wild-type Mec1.

The *mec1-ad* mutant is defective in resection and repair of a DSB

As resection-defective mutants fail to adapt to the checkpoint (Clerici et al, 2006; Eapen et al, 2012), we investigated whether the *mec1-ad* mutant was defective in the generation of 3'-ended ssDNA at the HO-induced DSB. Because ssDNA is resistant to cleavage by restriction enzymes, we directly monitored ssDNA formation at the HO-induced DSB by following the loss of SspI restriction fragments by Southern blot analysis under alkaline conditions, using a ssRNA probe annealing on one side of the break (Fig 2A). Mec1-ad specifically impaired extensive resection of DNA ends. In fact, resection was unaffected by the *mec1-ad* mutation next to the DSB (r1-r3 in Fig 2B and C), but it was severely reduced in *mec1-ad* cells compared to wild-type at 5.9, 6.5, 8.9 and 15.8 kilobases from the DSB site (r4-r7 in Fig 2B and C).

Repair of a DSB made between tandem DNA repeats occurs by single-strand annealing (SSA), which requires nucleolytic degradation of the 5' DSB ends to reach the complementary DNA sequences that can then anneal (Fishman-Lobell et al, 1992), and break-induced

replication (BIR), by which the ssDNA generated at one repeat invades the other repeat and copies the distal part of the chromosome (Jain et al, 2009). SSA usually out-competes BIR, which is a kinetically slow mechanism (Jain et al, 2009). To assess the physiological relevance of the resection defect displayed by *mec1-ad* cells, we asked whether the Mec1-ad variant can affect repair of a DSB flanked by direct repeats. To this end, we introduced the *mec1-ad* allele in strain YMV80, which carries two tandem *leu2* repeats located 25 kb apart, with a recognition site for the HO endonuclease adjacent to one of the repeats (Fig 2D; Vaze et al, 2002). This strain also harbors a *GAL-HO* construct for galactose-inducible HO expression. When we monitored DSB repair kinetics by Southern blot analysis of KpnI-digested DNA with a *LEU2* probe, accumulation of the repair product was severely reduced in *mec1-ad* cells compared to wild-type (Fig 2E and F). Consistent with an inability of *mec1-ad* cells to repair this DSB, the amount of Rad53 phosphorylation after HO induction was increased and persisted longer in YMV80 *mec1-ad* cells than in wild-type (Fig 2G). Moreover, YMV80 *mec1-ad* cells formed colonies on galactose-containing media less efficiently than wild-type cells (Fig 2H).

While both SSA and BIR contribute to repair the DSB in YMV80 strain, the SSA becomes the predominant repair mode in YMV45 strain, where the distal *leu2* repeat is only at 4.6 kb from the DSB (Supplementary Fig S1A) (Jain et al, 2009). When we monitored repair of the HO-induced DSB in YMV45 derivative strains, accumulation of the repair product was delayed in *mec1-ad* cells compared to wild-type (Supplementary Fig S1B and C), but this delay was not sufficient to affect viability in *mec1-ad* cells (Supplementary Fig S1D). Although we cannot exclude that Mec1-ad also affects BIR when the homologous DNA repeat is at 25 kb from the DSB, the distance-dependent effect of Mec1-ad on DSB repair indicates that Mec1-ad affects SSA by impairing extensive DSB end resection.

When a DSB occurs, MRX and Sae2 initiate resection of the 5' strand, while extensive resection of the DSB ends relies on two pathways, which depend on Sgs1 and Exo1, respectively (Mimitou & Symington, 2008; Zhu et al, 2008). The *mec1-ad sae2Δ*, *mec1-ad exo1Δ* and *mec1-ad sgs1Δ* double mutants all exhibited

Figure 1. Screening for adaptation-defective *mec1* mutants.

- The *MEC1* coding region was amplified by PCR under mutagenic conditions. The PCR products and a *LEU2* centromeric plasmid containing part of the *MEC1* gene were co-transformed into a *mec1Δ sml1Δ leu2* JKM139 derivative strain to allow reconstruction of the whole *MEC1* coding region by gap repair (see Supplementary Information). *Leu*⁺ transformant clones were then scored at the microscope for the ability to form microcolonies on galactose-containing plates, where an irreparable HO cut is induced at the *MAT* locus.
- Schematic representation of the Mec1 protein. Vertical lines point out the position of the amino acid substitutions caused by the *mec1-ad* mutations. The Ddc2-interaction region and the domains conserved among the PI-kinases are indicated.
- YEPR G1-arrested cell cultures of wild-type JKM139 and otherwise isogenic *mec1-ad* cells were plated on galactose-containing plates (time zero). At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of large budded cells and of cells forming microcolonies of more than two cells.
- Exponentially growing YEPR cultures of the strains in (C) were transferred to YEPRG (time zero), followed by western blot analysis with anti-Rad53 antibodies.
- Exponentially growing cells of the strains in (C) were incubated for 1 h with 50 μM CPT. Cultures were then released into fresh medium, and samples were taken at the indicated times to detect Rad53 by western blot analysis. exp, exponentially growing cells.
- Exponentially growing cell cultures of the strains in (C) were arrested in G1 with α-factor (αf) and then released from the pheromone block in YEPD containing 0.015% MMS (F) or 50 mM HU (G), followed by western blot analysis with anti-Rad53 antibodies.
- Exponentially growing cell cultures of the strains in (C) were serially diluted (1:10) and each dilution was spotted out onto YEPD plates with or without CPT, MMS or HU.
- Western blot analysis with anti-MYC antibody of protein extracts from exponentially growing cell cultures expressing MYC-tagged Mec1 and Mec1-ad proteins. Coomassie staining was used as a loading control.
- Kinase assays and western blot analysis were performed on equal amounts of anti-MYC immunoprecipitates of protein extracts from exponentially growing cell cultures of the strains in (I).

Source data are available online for this figure.

more severe repair defects than any of the corresponding single mutants (Supplementary Fig S2), suggesting that the Mec1-ad variant functions in both Exo1- and Sgs1-mediated resection pathways.

Mec1-ad is robustly associated to DSB ends and increases the efficiency of γ H2A generation

Although Mec1 and Mec1-ad showed similar kinase activity *in vitro* (Fig 1J), the resection defect of *mec1-ad* cells might be due to a specific impairment of Mec1-ad in phosphorylating and activating positive regulators of DSB resection. Alternatively, Mec1-ad might possess an increased ability to phosphorylate and activate negative regulators of the same process.

Among the positive regulators of DSB resection, Mec1 is known to phosphorylate and activate Sae2 (Baroni et al, 2004; Cartagena-Lirola et al, 2006). As *mec1-ad* cells phosphorylated Sae2 at wild-type levels after HO induction (Supplementary Fig S3), their resection defect is not caused by reduced Sae2 phosphorylation. This result is consistent with the finding that *mec1-ad* and *sae2 Δ* cells are impaired in different resection pathways, as a *mec1-ad sae2 Δ* double mutant showed a more severe SSA defect than each single mutant (Supplementary Fig S2A and B).

Among the negative regulators of DSB resection, Mec1 is known to phosphorylate histone H2A at serine 129 (γ H2A) (Downs et al, 2000), and γ H2A generation appears to inhibit the resection rate (Eapen et al, 2012). We found that γ H2A increased in level and persisted longer in *mec1-ad* cells than in wild-type after DSB formation (Fig 3A and B), indicating that the Mec1-ad variant phosphorylates H2A more efficiently than wild-type Mec1. If the resection defect of *mec1-ad* cells were dependent on enhanced γ H2A formation, it is expected to be suppressed by the *hta1-S129A* allele, which encodes a H2A variant where Ser129 is substituted by a non-phosphorylatable alanine residue. This was indeed the case, because *mec1-ad hta1-S129A* cells accumulated the longest ssDNA products at the HO-induced DSB more efficiently than *mec1-ad* cells (Fig 3C and D). It is worth pointing out that, as *S. cerevisiae* histone H2A is encoded by both the *HTA1* and the *HTA2* genes, all the strains we used for this analysis also carried the *HTA2* deletion.

As Mec1-ad and wild-type Mec1 did not significantly differ from each other in either the amount or the intrinsic kinase activity

(Fig 1I and J), the increased efficiency of H2A phosphorylation might be due to enhanced ability of Mec1-ad to bind/persist at DNA ends. Strikingly, when we analyzed Mec1-ad recruitment at DSBs by chromatin immunoprecipitation (ChIP) and quantitative real time PCR (qPCR), the amount of Mec1-ad bound at the HO-induced DSB end was higher than that of wild-type Mec1 (Fig 3E). Altogether, these data suggest that the robust Mec1-ad association to the DSB increases the efficiency of γ H2A generation, which in turn impairs extensive DSB resection.

Rad9 prevents extensive DSB resection in *mec1-ad* cells by both Rad53-dependent and Rad53-independent mechanisms

It is known that γ H2A recruits to the sites of damage the checkpoint protein Rad9 (Javaheri et al, 2006; Toh et al, 2006; Grenon et al, 2007; Hammet et al, 2007; Granata et al, 2010), which counteracts 3'-ended ssDNA generation at DSB ends (Lydall & Weinert, 1995; Lazzaro et al, 2008). We then hypothesized that the more efficient γ H2A generation in *mec1-ad* cells might increase Rad9 persistence at DSBs, thus preventing extensive resection. Consistent with a role of Rad9 in inhibiting DSB resection in *mec1-ad* cells, the lack of Rad9 completely suppressed the resection defect of *mec1-ad* cells. In fact, resection in *mec1-ad rad9 Δ* cells was as fast as in *rad9 Δ* cells, where resection was markedly increased compared to wild-type (Fig 4A and B). Furthermore, the amount of Rad9 bound at 1.8 kb and 5.4 kb from the HO-induced DSB was higher in *mec1-ad* cells than in wild-type (Fig 4C). This binding still depended on γ H2A formation, as Rad9 association to the DSB in *mec1-ad hta1-S129A* double mutant was reduced to the extent observed in the *hta1-S129A* single mutant (Fig 4D).

The increased Rad9 binding in *mec1-ad* cells was not a general consequence of the *mec1-ad* resection defect. In fact, enhanced Rad9 occupancy at the HO-induced DSB was not observed in either *sae2 Δ* mutant cells, which are impaired in initiation of resection (Clerici et al, 2006), or *exo1 Δ sgs1 Δ* mutant cells, which lack long range resection (Mimitou & Symington, 2008; Zhu et al, 2008) (Fig 4E). Rather, *exo1 Δ sgs1 Δ* cells showed reduced Rad9 binding to the DSB (Fig 4E), likely due to poor γ H2A phosphorylation and Mec1/Rad53 activation (Fig 4F) caused by the low amount of ssDNA which is formed at *exo1 Δ sgs1 Δ* DSB ends (Mimitou & Symington, 2008; Zhu et al, 2008).

Figure 2. Extensive DSB resection is defective in *mec1-ad* cells.

- A System used to detect DSB resection. Gel blots of SspI-digested genomic DNA separated on alkaline agarose gel were hybridized with a single-stranded RNA MAT probe that anneals to the unresected strand. 5'-3' resection progressively eliminates SspI sites (S), producing larger SspI fragments (r1 through r7) detected by the probe.
- B, C Exponentially growing YEPR cell cultures of wild-type JKM139 and isogenic *mec1-ad* strain were arrested in G2 with nocodazole and transferred to YEPRG at time zero in the presence of nocodazole and were analysed for (B) ssDNA formation as described in (A). Resection products were analysed by densitometry (C). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (B).
- D Schematic representation of the YMV80 chromosome III region where the HO-cut site is flanked by homologous *leu2* sequences that are 25 kb apart. HO-induced DSB formation results in generation of a 2.5 kb DNA fragment (HO-cut) that can be detected by Southern blot analysis of KpnI-digested genomic DNA with a *LEU2* probe. DSB repair generates a 3.5 kb DNA product (product).
- E–G Exponentially growing YEPR cell cultures carrying the system described in (D) were arrested in G2 with nocodazole and transferred to YEPRG at time zero in the presence of nocodazole and analysed at the indicated times after HO induction by (E) Southern blot using KpnI-digested genomic DNA, (F) densitometric analysis of the product band signals in (E), and (G) Western blot analysis with anti-Rad53 antibodies. In (F), the intensity of each band was normalized with respect to a loading control (not shown). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (E).
- H Exponentially growing YMV80 wild-type and isogenic *mec1-ad* cells were serially diluted (1:10) and each dilution was spotted out onto YEPD and YEPRG plates.

Source data are available online for this figure.

Figure 3. The lack of γ H2A suppresses the resection defect of *mec1-ad* cells.

A Western blot analysis with anti- γ H2A and anti-H2A antibodies of protein extracts at the indicated times after HO induction.

B Densitometric analysis of the γ H2A signals in (A) normalized to total H2A.

C, D Exponentially growing YEPR cell cultures of wild-type JKM139 and otherwise isogenic derivative strains were transferred to YEPRG at time zero. All the strains carry also the deletion of the *HTA2* gene. DSB resection at the *MAT* locus was analyzed as described in Figure 2A (C). Densitometric analysis of the resection products (D). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (C).

E Exponentially growing YEPR *MEC1-MYC* and *mec1-ad-MYC* cell cultures were transferred to YEPRG. Relative fold enrichment of Mec1-Myc or Mec1-ad-Myc fusion proteins at the indicated distances from the HO cleavage site was determined after CHIP with anti-Myc antibodies and subsequent qPCR analysis. Plotted values are the mean values $\pm$ s.d. from three independent experiments.

Source data are available online for this figure.

Figure 4. Impairment of resection by Mec1-ad involves increased Rad9 persistence at DSBs.

A, B Exponentially growing YEPR cell cultures of wild-type JKM139 and otherwise isogenic derivative strains were transferred to YEPRG at time zero. DSB resection at the *MAT* locus was analyzed as described in Figure 2A (A). Densitometric analysis of the resection products (B). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (A).

C–E Exponentially growing YEPR cultures of strains expressing a fully functional Rad9-HA fusion protein were transferred to YEPRG. Relative fold enrichment of Rad9-HA at the indicated distance from the HO cleavage site was evaluated after ChIP with anti-HA antibodies and qPCR analysis. Plotted values are the mean values $\pm$ s.d. from three independent experiments.

F, G Western blots of protein extracts after HO induction were probed with specific antibodies for the indicated proteins.

Source data are available online for this figure.

After DSB formation, Rad9 is required to phosphorylate and activate the checkpoint kinase Rad53, which is known to inhibit resection at uncapped telomeres through phosphorylation and inhibition of Exo1 (Jia *et al*, 2004; Morin *et al*, 2008). However, the finding that *RAD9* deletion can suppress the resection defects of *exo1Δ* cells (Lazzaro *et al*, 2008) indicates that Rad9 acts also independently of Rad53. As Rad53 phosphorylation was higher in *mec1-ad* cells than in wild-type (Fig 1D) and this phosphorylation was abolished by deleting *RAD9* (Fig 4G), we asked whether Rad9 blocks DSB resection in *mec1-ad* cells by acting through Rad53. We could not use *rad53Δ* cells to address this point, because they show growth defects even when the lethal effect of *RAD53* deletion is suppressed by the lack of Sml1 (Zhao *et al*, 1998). Thus, we substituted the chromosomal wild-type *RAD53* gene with the kinase-deficient *rad53-K227A* allele that did not affect cell viability in the absence of DNA damaging agents (Fay *et al*, 1997). Because expression of the Rad53-K227A mutant variant causes both wild-type and *mec1-ad* cells to progress through cell cycle even after DSB formation (Supplementary Fig S4), *HO* expression was induced by galactose addition to nocodazole-arrested cells that were kept arrested in G2 with nocodazole. Resection of the HO-induced DSB was more efficient in *rad53-K227A* mutant cells than in wild-type cells (Fig 5A and B). Furthermore, the long resection products accumulated earlier in *mec1-ad rad53-K227A* than in *mec1-ad* cells (Fig 5A and B), indicating that Rad53 activation contributes to prevent resection in the presence of Mec1-*ad*.

As shown above, *RAD9* deletion completely suppressed the resection defect of *mec1-ad* cells. If Rad9 were inhibiting DSB resection in *mec1-ad* cells by acting exclusively through Rad53, *rad53-K227A* should be epistatic to *mec1-ad* in DSB resection, and therefore the resection rate should be similar in galactose-induced *mec1-ad rad53-K227A* and *rad53-K227A* cells. Furthermore, as Exo1 has been shown to be the only target of Rad53 in preventing DSB resection (Jia *et al*, 2004), a *mec1-ad exo1Δ* double mutant should be as defective in resection as the most defective single mutant. Indeed, Rad9 appears to block DSB resection in *mec1-ad* cells by both Rad53-dependent and Rad53-independent mechanisms. In fact, while *rad9Δ* and *rad9Δ mec1-ad* cells resected the DSB with similar kinetics (Fig 4A and B), resection in *mec1-ad rad53-K227A* cells was not as fast as in *rad53-K227A* cells (Fig 5A and B), indicating that Rad53 inactivation is not sufficient to bypass the inhibitory effect of Mec1-*ad* on resection. Furthermore, consistent with a more severe SSA defect in the *mec1-ad exo1Δ* double mutant than in each single mutant (Supplementary Fig S2C and D), *mec1-ad exo1Δ* cells accumulated the resection products less efficiently (Fig 5C and D) and exhibited a higher sensitivity to CPT than each single mutant (Fig 5E).

The lack of Mec1 accelerates resection and reduces Rad9 binding at DSBs

The effect of the Mec1-*ad* variant on DSB resection might reflect a functional role of Mec1 in regulating nucleolytic processing of DSBs. We therefore monitored the kinetics of DSB resection in *mec1Δ sml1Δ* cells. *HO* was expressed by galactose addition to nocodazole-arrested cells that were kept arrested in G2 with nocodazole for the subsequent 4 h, because the lack of Mec1 causes cells to progress through cell cycle even after DSB formation. Strikingly, DSB resection was accelerated by the absence of Mec1, as *mec1Δ sml1Δ* cells

accumulated the long resection products earlier than wild-type (Fig 6A and B). Consistent with a previous study (Naiki *et al*, 2004), cells lacking Mec1 showed reduced Rad9 binding to the DSB ends compared to wild-type (Fig 6C), suggesting that accelerated DSB resection in these cells might be due to this reduced Rad9 association.

Interestingly, resection in *mec1Δ* cells was not as efficient as in *rad9Δ* cells (Fig 6A and B). One possibility is that the absence of Mec1 does not completely abolish Rad9-mediated inhibition of DSB resection. Alternatively, Mec1 might promote the activity of proteins, such as Sae2, which contribute to resect DSBs. This second hypothesis is supported by the finding that resection in a *mec1Δ rad9Δ* double mutant was as fast as in the *mec1Δ* single mutant (Fig 6A and B).

EXO1 overexpression suppresses the adaptation defect of the *mec1-ad* mutant

Mec1 is known to be recruited to 3'-ended ssDNA arising during 5'–3' nucleolytic degradation of the DSB ends (Zou & Elledge, 2003). Although the generation of 3'-ended ssDNA is defective in *mec1-ad* cells, these cells fail to turn off the checkpoint after generation of either repairable or irreparable DSBs. As other mutants defective in DSB resection are unable to adapt to the checkpoint triggered by an unrepaired DSB (Eapen *et al*, 2012), we hypothesized that reduced resection allows the checkpoint signal to persist. To test this hypothesis, we asked whether *EXO1* overexpression can suppress the inability of *mec1-ad* cells to turn off the checkpoint by restoring DSB resection. The presence of a 2μ high copy number plasmid carrying the *EXO1* gene caused a markedly increased resection in *mec1-ad* cells compared to the empty vector (Supplementary Fig S5). Strikingly, when G1-arrested cell cultures were spotted on galactose-containing plates to induce HO, 80% of *mec1-ad* cells overexpressing *EXO1* formed microcolonies within 24 h, whereas most *mec1-ad* cells carrying the empty 2μ vector remained arrested as large budded cells (Fig 7A). Furthermore, when galactose was added to exponentially growing cell cultures of the same strains, Rad53 hyperphosphorylated forms decreased after 10–12 h in *EXO1* overexpressing *mec1-ad* cells, whereas they persisted much longer in *mec1-ad* cells carrying the empty vector (Fig 7B). Finally, high copy number *EXO1* suppressed the sensitivity to CPT and MMS of *mec1-ad* cells (Fig 7C). These data suggest that the inability of *mec1-ad* cells to turn off the checkpoint signal is due to their resection defect. On the other hand, *EXO1* overexpression did not reduce Rad9 binding in *mec1-ad* cells (Fig 7D), further supporting the above finding that increased Rad9 recruitment to the DSB in *mec1-ad* cells is not a consequence of their resection defect.

TEL1 deletion suppresses the adaptation defect caused by *mec1-ad* and by other mutations affecting resection

The ability of Tel1 to activate the checkpoint is disrupted when the DSB ends are exposed to 5'–3' nucleolytic degradation (Mantiero *et al*, 2007). This observation raises the possibility that the persistent checkpoint signaling in *mec1-ad* cells might be due to unscheduled Tel1 activation. We first spotted G1-arrested cell cultures on galactose-containing plates, in order to investigate whether *TEL1* deletion can suppress the adaptation defect of *mec1-ad* cells. This was indeed

Figure 5. Rad9 impairs resection in *mec1-ad* cells by acting both dependently and independently of Rad53.

- A, B YEPG G2-arrested cell cultures of wild-type JKM139 and otherwise isogenic derivative strains were transferred to YEPG at time zero in the presence of nocodazole. DSB resection at the *MAT* locus was analyzed as described in Figure 2A (A). Densitometric analysis of the resection products (B). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (A).
- C, D Exponentially growing YEPG cell cultures of wild-type JKM139 and otherwise isogenic derivative strains were transferred to YEPG at time zero. DSB resection at the *MAT* locus was analyzed as described in Figure 2A (C). Densitometric analysis of the resection products (D). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (C).
- E Exponentially growing cell cultures of the strains in (C) were serially diluted (1:10) and each dilution was spotted out onto YEPD plates with or without CPT.

Figure 6. The lack of Mec1 accelerates DSB resection and decreases Rad9 persistence at DSBs.

- A, B YEPR G2-arrested cell cultures of wild-type JKM139 and otherwise isogenic strains were transferred to YEPRG at time zero in the presence of nocodazole. All the strains carry also the deletion of the *SML1* gene. DSB resection at the *MAT* locus was analyzed as described in Figure 2A (A). Densitometric analysis of the resection products (B). Plotted values are the mean values $\pm$ s.d. from three independent experiments performed as in (A).
- C YEPR G2-arrested wild-type and *mec1Δ* cell cultures expressing a fully functional Rad9-HA fusion protein were transferred to YEPRG at time zero in the presence of nocodazole. Relative fold enrichment of Rad9-HA at the indicated distance from the HO cleavage site was evaluated after ChIP and qPCR analysis. Plotted values are the mean values $\pm$ s.d. from three independent experiments.

Figure 7. Overexpression of *EXO1* suppresses the adaptation defect of *mec1-ad* mutant cells.

- A** YEPR G1-arrested cell cultures of wild-type JKM139 and otherwise isogenic *mec1-ad* cells containing a 2 μ plasmid, either empty or carrying the *EXO1* gene, were plated on galactose-containing plates. At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of large budded cells and of cells forming microcolonies of more than 2 cells.
- B** Exponentially growing YEPR cultures of the strains in (A) were transferred to YEPRG. Western blots of protein extracts prepared at the indicated times were probed with anti-Rad53 antibodies.
- C** Exponentially growing cell cultures of wild-type JKM139 and otherwise isogenic derivative strains were serially diluted (1:10) and each dilution was spotted out onto YEPD plates with or without CPT or MMS.
- D** Exponentially growing YEPR *mec1-ad* cells containing a 2 μ plasmid, either empty or carrying the *EXO1* gene, and expressing a fully functional Rad9-HA fusion protein were transferred to YEPRG. Relative fold enrichment of Rad9-HA at the indicated distance from the HO cleavage site was evaluated after ChIP and qPCR analysis. Plotted values are the mean values $\pm$ s.d. from three independent experiments.

Source data are available online for this figure.

Figure 8. Tel1 is responsible for checkpoint persistence in *mec1-ad* cells.

- A YEPR G1-arrested cell cultures of wild-type JKM139 and otherwise isogenic mutant strains were plated on galactose-containing plates. At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of large budded cells and of cells forming microcolonies of more than 2 cells.
- B–D Exponentially growing YEPR cultures of strains with the indicated genotypes were transferred to YEPRG at time zero. Western blot of protein extracts at the indicated times after HO induction were probed with specific antibodies for the indicated proteins. Densitometric analysis of γ H2A signals normalized to total H2A is shown in (D).
- E Exponentially growing YEPR cell cultures expressing a fully functional Rad9-HA fusion protein were transferred to YEPRG. Relative fold enrichment of Rad9-HA at the indicated distance from the HO cleavage site after ChIP analysis and subsequent qPCR. Plotted values are the mean values $\pm$ s.d. from three independent experiments.
- F YEPR G1-arrested cell cultures of wild-type JKM139 and otherwise isogenic strains were plated on galactose-containing plates and monitored at 8 and 24 h to determine the frequency of cells forming microcolonies of more than 2 cells (adapted cells).

Source data are available online for this figure.

the case, as *mec1-ad tel1 Δ* cells formed microcolonies on galactose more efficiently than *mec1-ad* cells (Fig 8A). Furthermore, when galactose was added to exponentially growing cell cultures of the same strains, both Rad53 phosphorylated forms (Fig 8B) and γ H2A (Fig 8C, D), which persisted longer in *mec1-ad* than in wild-type cells, disappeared in *mec1-ad tel1 Δ* double mutant cells within 12–14 h after HO induction. Thus, Tel1 activation is responsible for the inability of *mec1-ad* cells to turn off the checkpoint.

By contrast, Tel1 activation does not account for the resection defect of *mec1-ad* cells. In fact, although the lack of Tel1 slightly affected DSB resection (Supplementary Fig S6; Mantiero *et al*, 2007), very similar resection kinetics were observed in *mec1-ad tel1 Δ* and *mec1-ad* cells (Supplementary Fig S6). Furthermore, *TEL1* deletion did not reduce either Rad9 binding to the DSB (Fig 8E) or the high amounts of phosphorylated Rad53 and γ H2A that we detected in *mec1-ad* cells during the first 8–10 h of DSB induction (Fig 8B–D).

Interestingly, *sae2 Δ* , *dna2 Δ* , *sgs1 Δ* and *exo1 Δ sgs1 Δ* mutants, which are all defective in DSB resection, fail to adapt to the checkpoint triggered by an unrepaired DSB (Fig 8F; Clerici *et al*, 2006; Eapen *et al*, 2012). Furthermore, *sae2 Δ* cells showed prolonged Tel1-dependent checkpoint activation after treatment with DNA damaging agents (Usui *et al*, 2001). These data raise the possibility that activation of a Tel1-dependent checkpoint is not specific for the *mec1-ad* mutant, but rather it is a general consequence of resection defects. In fact, when G1-arrested cell cultures were spotted on galactose-containing plates to induce HO, *sgs1 Δ tel1 Δ* and *dna2 Δ tel1 Δ* double mutants formed microcolonies more efficiently than *sgs1 Δ* and *dna2 Δ* single mutants (Fig 8F), indicating that Tel1 activation is responsible for the persistent cell cycle arrest of these DSB resection-defective mutants. Unfortunately, we cannot assess whether the adaptation defect of *sgs1 Δ exo1 Δ* double mutant cells depends on Tel1, because *sgs1 Δ exo1 Δ tel1 Δ* triple mutant cells had severe growth defects (data not shown). It must be pointed out that, because Dna2 is essential for cell viability, *dna2 Δ* cells were kept viable by the *pif1-M2* mutation, which impairs the ability of Pif1 to promote formation of long flaps that are substrates for Dna2 (Budd *et al*, 2006).

Increased persistence of MRX at DSBs maintains Tel1-dependent checkpoint signaling

We found that Tel1 persisted longer in *mec1-ad* cells than in wild-type at 0.6 and 1.8 kb from the HO cut site (Fig 9A), suggesting that this increased association to DNA may account for Tel1 unscheduled activation. Tel1 recruitment to the DSB requires

the MRX complex (Nakada *et al*, 2003; Lisby *et al*, 2004), and the lack of Mre11 suppressed the adaptation defect of *mec1-ad* cells (Fig 9B). Moreover, Mre11 persisted longer in *mec1-ad* cells than in wild-type at 0.6 and 1.8 kb from the HO cut site (Fig 9C). Detection of Mre11 binding at these distance from the HO cleavage site was not influenced by loss of input DNA (and therefore of the PCR signal) due to DSB resection. In fact, the Mre11 ChIP signals have been normalized for each time point to the input signal that, in any case, decreased with similar kinetics in both wild-type and *mec1-ad* cells (Fig 9D).

The data above suggest that a slowing down of DSB resection might cause unscheduled Tel1 activation by favoring the persistence of DNA structures that are efficiently bound by MRX. Consistent with this hypothesis, *EXO1* overexpression, which counteracts Tel1 activation in *mec1-ad* cells (Fig 7), decreased Mre11 recruitment to the HO-induced DSB (Fig 9E). In addition, galactose-induced *sgs1 Δ* and *exo1 Δ sgs1 Δ* cells, which are both resection- and adaptation-defective (Fig 8F; Mimitou & Symington, 2008; Zhu *et al*, 2008), showed increased MRX association at the DSB ends compared to wild-type (Fig 9F).

We then asked whether Tel1 activation is directly linked to the amount of MRX bound near the DSB by investigating if increasing the MRX binding without impairing resection might prevent adaptation by eliciting a Tel1-dependent signaling. The MRX complex is known to compete for binding to DNA ends with the Ku70/Ku80 heterodimer, whose lack increases Mre11 binding at the DSB without reducing the resection rate (Lee *et al*, 1998; Clerici *et al*, 2008). Consistent with a previous study (Lee *et al*, 1998), *ku70 Δ* cells were unable to adapt to the DSB-induced checkpoint (Fig 9G). Strikingly, *TEL1* deletion completely suppressed the adaptation defect of *ku70 Δ* cells, as a *ku70 Δ tel1 Δ* double mutant formed microcolonies on galactose-containing plates as efficiently as wild-type cells (Fig 9G). We can conclude that Tel1 activation is dictated by the amount of MRX at the DSB ends.

Discussion

RPA-coated ssDNA is the key structure that enables the Mec1/ATR kinase to recognize DSBs (Zou & Elledge, 2003). Mec1/ATR activation is coupled with ssDNA-dependent loss of ATM/Tel1 activation, suggesting that DSB resection promotes a switch from a Tel1/ATM- to a Mec1/ATR-dependent checkpoint (Mantiero *et al*, 2007; Shiotani & Zou, 2009; Gobbin *et al*, 2013). By analyzing DSB resection in the presence of the adaptation-defective Mec1-ad variant versus

the lack of Mec1, we show here that Mec1 regulates the generation of ssDNA at DSBs. In addition, we provide evidence that DSB resection not only generates a substrate for Mec1 binding, but it also allows the termination of the checkpoint response by attenuating Tel1 signaling activity.

We find that the lack of Mec1 accelerates the generation of ssDNA at DSB ends, whereas the Mec1-ad variant impairs extensive resection. Our data indicate that Mec1 regulates ssDNA production at the DSB ends by regulating the activity of Rad9, which is known to inhibit ssDNA formation at broken DNA ends. In fact, eliminating Rad9 or decreasing its binding to the DSB by preventing γ H2A formation suppresses the resection block of *mec1-ad* cells. Moreover, Mec1-ad enhances the amount/persistence of Rad9 at the DSB ends by increasing the efficiency of H2A phosphorylation, whereas the lack of Mec1 reduces Rad9 recruitment at the same DNA ends. Notably, although DSB resection in *mec1 Δ* cells is accelerated compared to wild-type, it is not as efficient as in *rad9 Δ* cells. This finding, together with the observation that *RAD9* deletion does not further increase ssDNA generation in *mec1 Δ* cells, indicates also a positive role for Mec1 in DSB resection.

The difference between wild-type Mec1 and Mec1-ad cannot be ascribed to differences in their amounts or intrinsic kinase activity. Rather, Mec1-ad is more robustly associated than wild-type Mec1 to the DSB ends. This increased persistence might improve the efficiency of γ H2A phosphorylation, which in turn makes chromatin more accessible to Rad9 loading. The Mec1-ad variant carries 4 amino acid substitutions and we were not able to ascribe the *mec1-ad* phenotype to any specific amino acid change. Interestingly, the *mec1-ad* mutations are located in the N terminal part of Mec1 that contains the interaction domain for Ddc2, which helps Mec1 recruitment to RPA-coated ssDNA (Wakayama *et al*, 2001; Nakada *et al*, 2005). Whether these mutations increase the ability of Mec1 to directly interact with RPA and/or Ddc2 remains to be determined.

How can Rad9 prevent extensive DSB resection in *mec1-ad* cells? Rad9 is required to activate the checkpoint kinase Rad53, which is known to limit ssDNA generation by inhibiting Exo1 (Jia *et al*, 2004; Morin *et al*, 2008). Although we cannot exclude the possibility that Mec1-ad might directly activate Rad53, Rad9 seems to inhibit ssDNA production in *mec1-ad* cells both by activating Rad53 (and thereby inhibiting Exo1) and by some other Rad53-independent mechanism (s). How Rad9 can partially block resection independently of Rad53 is unknown, but it is tempting to speculate that Rad9 may have a role in inhibiting the activity of nucleases/helicases either directly or indirectly, by influencing chromatin structure.

Which are the physiological consequences of the resection defect in *mec1-ad* cells? We observe a persistent checkpoint activation in *mec1-ad* cells after generation of either a repairable or an irreparable DSB. Surprisingly, although the Mec1-ad variant is more strongly associated to DSBs than wild-type Mec1, the checkpoint persistence in *mec1-ad* cells is due to Tel1 signaling activity. Activation of a Tel1-dependent checkpoint appears to be a general consequence of impaired DSB resection, as other resection-defective mutants, such as *dna2 Δ* and *sgs1 Δ* , prevent checkpoint turning off by activating Tel1 (Fig 8F; Eapen *et al*, 2012). This unscheduled Tel1 activation seems to be directly linked to the persistence of MRX at the DSB ends. In fact, *mec1-ad* cells, as well as the resection-defective *sgs1 Δ* and *exo1 Δ sgs1 Δ* mutants, show increased MRX persistence at the DSB ends compared to wild-type.

Furthermore, the lack of Ku, which increases the amount of MRX bound to the DSBs without reducing the resection rate (Clerici *et al*, 2008), causes persistent Tel1-dependent checkpoint signaling (Fig 9G). As the mammalian counterpart of MRX has been shown to bind ds/ssDNA junctions (Duursma *et al*, 2013), we speculate that resection generates ds/ssDNA structures with long ssDNA that inhibits Tel1 signaling because they are not efficiently bound by MRX. A similar mechanism has been proposed for mammalian ATM, where generation of DSBs with long ssDNA tails has been shown to attenuate ATM activity by decreasing MRN binding at DSBs (Shiotani & Zou, 2009).

The finding that checkpoint persistence in *mec1-ad* cells is due to Tel1 and not to Mec1 signaling suggests that cells possess a mechanism for Mec1 inactivation, whereas Tel1 can escape this inhibition. Therefore, the role of DSB resection in attenuating MRX/Tel1 signaling activity is important to ensure that the checkpoint is turned off even when the damage is not repairable, in order to preserve cell viability at the expense of potential mutations. Modulation of MRX/Tel1 activity could be important not only at DSBs but also during DNA replication. In fact, while MRX/Tel1 activation should be limited at DSBs to allow checkpoint switch off after a time window, it might be relevant at the replication forks, where resection is not extensive and both MRX and Tel1 are important to prevent accumulation of cruciform DNA intermediates and inverted repeat fusions (Doksani *et al*, 2009; Kaochar *et al*, 2010).

Altogether these data support a model where Mec1 is loaded on RPA-coated ssDNA and phosphorylates H2A, which contributes to load Rad9 on the DSB ends (Fig 9H). Once recruited, Rad9 promotes Rad53 activation and limits the amount of ssDNA generated at the DSB ends by both Rad53-dependent and Rad53-independent mechanisms, thus providing a negative feedback loop on Mec1 activity. As Mec1 is required for H2A phosphorylation, this Rad9-mediated inhibition of Mec1 can in turn keep under control the amount of DSB-bound Rad9. A defect in DSB resection caused by either dysfunctions of the resection machinery or Rad9 excess at DSBs leads to a persistent checkpoint by prolonging MRX occupancy at DSBs that in turn can promote sustained Tel1 signaling. Thus, the checkpoint machinery appears to regulate ssDNA generation at the DSB ends, and this control mechanism ensures a proper checkpoint response by controlling the balance between Mec1 and Tel1 activities.

Materials and Methods

Yeast strains

Strain genotypes are listed in Table S1. Strains JKM139, YMV80 and YMV45 were kindly provided by J. Haber (Brandeis University, Waltham, MA, USA). A plasmid carrying the *hta1-S129A* allele, kindly provided by D. Durocher (University of Toronto, Toronto, ON, Canada), was used to substitute the chromosomal *HTA1* gene in *hta2 Δ* strains. Strains carrying *MEC1-MYC* and *mec1-ad-MYC* alleles have been constructed as described in Paciotti *et al* (2000). The *pif1-M2* mutation was introduced into JKM139 derivative strains as described in Schulz & Zakian (1994). Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR) or 2% raffinose and 3% galactose (YEPRG).

Figure 9. MRX persistence at DNA ends increases Tel1 signaling.

- A Exponentially growing YEPR cell cultures expressing a fully functional Tel1-HA fusion protein were transferred to YEPRG. Relative fold enrichment of Tel1-HA at the indicated distance from the HO cleavage site after ChIP analysis and subsequent qPCR. Plotted values are the mean values $\pm$ s.d. from three independent experiments.
- B YEPR G1-arrested cell cultures of wild-type JKM139 and *mec1-ad* and *mec1-ad mre11Δ* isogenic strains were plated on galactose-containing plates. At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of large budded cells and of cells forming microcolonies of more than 2 cells.
- C–F Exponentially growing YEPR cell cultures of JKM139 derivative strains with the indicated genotypes and expressing a fully functional Mre11-Myc fusion protein were transferred to YEPRG. Relative fold enrichment of Mre11-Myc at the indicated distance from the HO cleavage site was evaluated after ChIP and subsequent qPCR analysis (C, E, F). Plotted values are the mean values $\pm$ s.d. from three independent experiments. Input DNA used for the ChIP analysis in (C) normalized to the ARO site (D).
- G YEPR G1-arrested cell cultures of wild-type JKM139 and otherwise isogenic strains with the indicated genotypes were plated on galactose-containing plates and analyzed as in (B).
- H Working model for Mec1-mediated regulation of ssDNA generation (see text for details).

DSB resection and repair

Double-strand break formation and repair in the YMV45 and YMV80 strains were detected as described in Clerici *et al* (2005). DSB end resection at the *MAT* locus in JKM139 derivative strains was analyzed on alkaline agarose gels as described in Trovesi *et al* (2011). Quantitative analysis of DSB resection was performed by calculating the ratio of band intensities for ssDNA and total amount of DSB products.

ChIP analysis

Chromatin immunoprecipitation analysis was performed as described in Viscardi *et al* (2007). Input and immunoprecipitated DNA were purified and analyzed by qPCR using a Biorad MiniOpticon. Data are expressed as fold enrichment at the HO-induced DSB over that at the non-cleaved *ARO1* locus, after normalization of each ChIP signals to the corresponding input for each time point. Fold enrichment was then normalized to the efficiency of DSB induction.

Other techniques

Immunoprecipitation and kinase assay were performed as described in Baldo *et al* (2008). Rad53 was detected by using anti-Rad53 polyclonal antibodies (AB104232) from Abcam. γ H2A was detected by using anti- γ H2A polyclonal antibodies kindly provided by W.M. Bonner (NIH, Bethesda, USA). H2A was detected using anti-H2A polyclonal antibodies (Active Motif).

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

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Author contributions

Conceived and designed the experiments: MC, CT, MPL. Performed the experiments: MC, CT, AG. Analysed the data: MC, CT, GL, MPL. Wrote the paper: MPL, GL.

Conflict of interest

The authors declare that they have no conflict of interest.

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