

Sensing of Replication Stress and Mec1 Activation Act through Two Independent Pathways Involving the 9-1-1 Complex and DNA Polymerase ϵ

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Abstract

Following DNA damage or replication stress, budding yeast cells activate the Rad53 checkpoint kinase, promoting genome stability in these challenging conditions. The DNA damage and replication checkpoint pathways are partially overlapping, sharing several factors, but are also differentiated at various levels. The upstream kinase Mec1 is required to activate both signaling cascades together with the 9-1-1 PCNA-like complex and the Dpb11 (hTopBP1) protein. After DNA damage, Dpb11 is also needed to recruit the adaptor protein Rad9 (h53BP1). Here we analyzed the mechanisms leading to Mec1 activation *in vivo* after DNA damage and replication stress. We found that a *ddc1Δdpb11-1* double mutant strain displays a synthetic defect in Rad53 and H2A phosphorylation and is extremely sensitive to hydroxyurea (HU), indicating that Dpb11 and the 9-1-1 complex independently promote Mec1 activation. A similar phenotype is observed when both the 9-1-1 complex and the Dpb4 non-essential subunit of DNA polymerase ϵ (Pol ϵ) are contemporarily absent, indicating that checkpoint activation in response to replication stress is achieved through two independent pathways, requiring the 9-1-1 complex and Pol ϵ .

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Introduction

The DNA replication machinery can experience various types of stress during S phase. This can happen when the replisome encounters DNA lesions that hinder its progression, while traversing slow replication zones corresponding to genomic regions difficult to replicate [1] or when encountering replication fork barriers [2]. Replication stress can also be induced by inhibiting ribonucleotide reductase (RNR) with hydroxyurea, which causes a global replication arrest by reducing the dNTPs pools [3].

Under replication stress conditions, eukaryotic cells trigger a signaling cascade, known as the replication checkpoint, which, in budding yeast, culminates with the phosphorylation of Rad53 [4]. This protein kinase is essential for the activation of the molecular mechanisms required to cope with replication arrest: it promotes stabilization of stalled replication forks and allows DNA replication re-start after removal of the blocking agent [5,6,7,8]. Rad53 is also responsible for inducing the transcription of RNR genes by inhibiting the transcriptional repressor Crt1 and promoting the degradation of the RNR inhibitor Sml1 [9,10]. Finally, Rad53 prevents the firing of late replication origins [11] and restrains spindle elongation thus preventing mitosis [12,13,14].

The DNA damage and replication checkpoints are genetically distinct pathways; however, they are partially overlapping since they share several of the factors involved. In fact, replication stress

activates Mec1, the same apical kinase triggered by DNA damage, which is recruited to RPA-covered ssDNA by its binding partner Ddc2 [15]. After damage, Mec1 phosphorylates the Rad9 adaptor protein, which has been loaded onto DNA via chromatin-dependent and -independent pathways: the former requiring methylation of H3-K79 and the latter depending on the 9-1-1 complex and Dpb11 [16,17,18,19,20]. Phosphorylated Rad9, in turn, recruits Rad53, which becomes hyperphosphorylated in a Mec1-dependent manner. Differently, in the case of HU-induced checkpoint activation, the Rad9 adaptor protein is dispensable and its function is performed by Mrc1, a constitutive member of the replisome complex [21,22].

It is now clear that following genotoxin treatments, primary lesions are generally recognized by specific repair factors that process them to generate ssDNA regions, which elicit the DNA damage response. On the other hand, the actual mechanism acting in the activation of the replication stress response is poorly understood. In budding yeast, it has been suggested that replication proteins may be involved in sensing blocks of the replication fork. Indeed, in addition to Dpb11, the initiation factor Sld2/Drc1 and Pol ϵ itself are required for efficient checkpoint activation in response to HU treatment, although the corresponding mutants are only mildly sensitive to the drug [23,24,25].

Sld2 is an essential CDK1 target required for initiation of DNA replication. Its phosphorylation and subsequent interaction with Dpb11 is essential for the loading of Pol ϵ and the firing of

Author Summary

The maintenance of genome stability is an essential process which needs a careful control. Indeed, the checkpoints are surveillance mechanisms sensing alterations in the integrity of the genome and preventing the replication and segregation of defective DNA molecules. The DNA integrity checkpoint is a signal transduction cascade conserved from yeast to man, and the apical factors in the pathway are protein kinases, called Mec1/Tel1 in *Saccharomyces cerevisiae* and ATR/ATM in mammals. DNA integrity can be challenged by lesions caused by a variety of chemical/physical agents, or by replication stress caused by special DNA structures, or by a limited supply of deoxyribonucleotides (dNTPs). The mechanisms leading to checkpoint activation in response to DNA damage are better understood compared to the processes leading to activation as a consequence of replication stress. We investigated the mechanisms required for Mec1 activation in response to dNTPs depletion caused by hydroxyurea treatment. We found that Mec1 activation occurs through two independent pathways: one acting through the PCNA-like 9-1-1 complex and the second through Dpb11 and DNA polymerase ϵ . The existence of these two pathways suggest a model possibly reflecting a DNA strand specificity in the detection of replication stress.

replication origins [26,27]. Pol ϵ consists of four subunits: Pol2 and Dpb2 are essential for cell viability while Dpb3 and Dpb4 appear to be non-essential. These last two factors contains a histone-like fold motif and are also implicated in transcriptional regulation [28,29]. The Pol ϵ holoenzyme is composed of two structurally distinct domains: a globular domain, made of the N-terminus of the catalytic Pol2 subunit and a tail-like domain containing the other three factors, bound to the Pol2 C-terminus [30,31]. The catalytic subunit contains an N-terminal polymerase domain followed by a C-terminal region, where the checkpoint-defective mutations of POL2 map [24]. Surprisingly, deletion of the polymerase domain does not cause cell lethality, whereas the checkpoint domain is essential for cell viability [32].

It has been established that in response to DNA damage, the 9-1-1 clamp is loaded onto the 5' primer-template junction adjacent to RPA-coated ssDNA [33,34]. In higher eukaryotes, 9-1-1 then recruits TopBP1 which, through an interaction with ATRIP, stimulates the ATR kinase activity [35,36,37,38]. Recent work in yeast demonstrated that Mec1 activation can proceed also through a 9-1-1-dependent, but Dpb11-independent pathway, mediated by an activation domain present in the Ddc1 subunit of the 9-1-1 complex [39]. Indeed, it has been reported that *S. cerevisiae* 9-1-1 can directly activate the Mec1-Ddc2 kinase *in vitro* [40]. The *in vivo* balancing between these two pathways has been recently studied, following Rad53 phosphorylation [39], which is influenced not only by Mec1 activation, but also by the Rad9 mediator [39]. To determine directly the relative contributions of Ddc1 and Dpb11 to Mec1 activation in different cell cycle phases, and particularly in response to replication stress, we analyzed a direct target of Mec1 kinase, histone H2A, whose phosphorylation is not dependent upon Rad9.

In this study we found that, in G1 yeast cells, Mec1 activation induced by UV irradiation completely depends on the 9-1-1 dependent pathway, whereas Dpb11 only plays a minor role. Conversely, in response to replication stress, Mec1 activation is achieved through two independent pathways which rely on the 9-1-1 complex and Dpb11, respectively. At least one of these two

pathways is necessary to efficiently activate Mec1 and to allow cell growth in the presence of HU. Finally, we provide evidence that the DNA polymerase ϵ complex and Sld2 are required to establish the 9-1-1 independent branch of Mec1 activation and we suggest that this could reflect strand-specificity in detecting replication stress.

Results

UV-induced Mec1 activation requires 9-1-1 and the C-terminal tail of Dpb11

We have previously shown that, in M phase, Dpb11 is required to recruit the Rad9 adaptor protein to UV-damaged DNA in a pathway that is parallel to that controlled by histone modifications [16,20]. Dpb11 was also found to stimulate Mec1 kinase activity *in vitro* and this function appears to be modulated by its interaction with the 9-1-1 complex [41,42]. To dissect the Mec1-activation role of Dpb11 *in vivo* and to determine the relative contribution of Dpb11 and 9-1-1 to this mechanism in different cell cycle phases, we analyzed histone H2A phosphorylation as an assay for Mec1 activity. After UV damage H2A is phosphorylated directly on serine 129 (γ H2A) by Mec1 kinase; indeed *mec1-1* mutant cells fail to phosphorylate H2A after DNA damage and a strain deleted in *TEL1*, coding for a second sensor-kinase, does not show any significant reduction in γ H2A levels (Figure S1A and S1B).

We used a yeast strain carrying a C-terminal deletion of Dpb11 (*A583_764*) encoded by the *dpb11-1* allele, which removes almost entirely the ATR Activation Domain (AAD) and a strain carrying the deletion of *DDC1*, the gene encoding the 9-1-1 subunit involved in Mec1 activation [40]. WT, *dpb11-1*, *ddc1 Δ* and *ddc1 Δ dpb11-1* cells were arrested in G1 with α -factor and in M phase with nocodazole and UV irradiated. As it is shown in Figure 1A, histone H2A is extensively phosphorylated after UV treatment in G1 and this damage-dependent modification requires the presence of a functional 9-1-1 complex, while the contribution of the AAD domain of Dpb11 is only minor. The quantification of the signal (shown in the lower panel of Figure 1A), indicates that the level of phosphorylated histone H2A (γ H2A) in *dpb11-1* is \sim 50% of that found in WT cells.

In M phase cells the basal level of phosphorylated H2A-S129 is much higher (Figure S1C), and this likely influences the magnitude of the increase measured after UV-irradiation. In these conditions, Dpb11 plays a minimal role, if any, in H2A phosphorylation and also *DDC1* deletion reduces γ H2A only partially (\sim 50%) (Figure 1B). However, the residual H2A phosphorylation observed in a *ddc1 Δ* mutant strain is lost when *TEL1* is deleted, (Figure 1C). On the other hand, deletion of *TEL1* in the *dpb11-1* background does not significantly influence H2A phosphorylation (Figure S1D).

9-1-1 and Dpb11 act independently in signaling replication stress to the Mec1 kinase

To further elucidate the balancing between 9-1-1-dependent and Dpb11-dependent Mec1 activation in S phase, we decided to analyze this process after replication stress induced by HU. This allowed us also to minimize the side effects due to the involvement of Dpb11 in Rad9 recruitment because, during HU treatment, Rad9 does not become hyperphosphorylated and is not expected to play any role in checkpoint activation [22]. WT, *dpb11-1*, *ddc1 Δ* and *ddc1 Δ dpb11-1* cells were synchronized in G1, released into fresh medium supplemented with 200 mM HU, and checkpoint activity was monitored by measuring Rad53 phosphorylation (Figure 2A). Differently from what found in G1 and G2 cells, strains lacking either a functional 9-1-1 complex or the Dpb11 C-terminal region were fully able to phosphorylate Rad53. In these

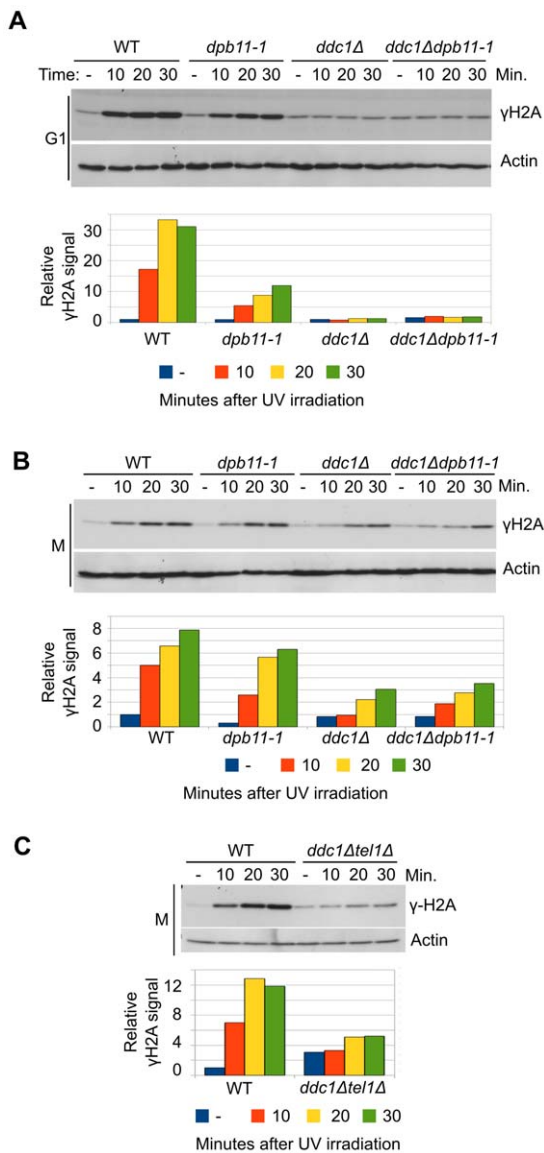


Figure 1. UV-induced Mec1 activation requires the 9-1-1 complex and the Dpb11 C-terminus. (A) K699 (WT), YFP20 (*dpb11-1*), YAN21/8d (*ddc1Δ*), YFP62/1d (*ddc1Δdpb11-1*) and YMIC5A3 (*mec1-1*) strains were grown to mid-log phase, arrested in G1 with α -factor and subjected to UV irradiation. At the indicated time-points, protein extracts were prepared and separated by SDS-PAGE. Mec1 activation was assayed by western blotting monitoring γ H2A and α -actin was used as loading control. A quantification of H2A phosphorylation is shown in the lower panel. The values indicate the fold increase respect to the WT untreated sample. The *mec1-1* mutation is functionally equivalent to a null mutation (B) The strains in panel A were arrested in M phase with nocodazole and subjected to the same treatment. Analysis and quantification of H2A phosphorylation was carried out as described above. (C) Strains K699 (WT) and YFP223 (*ddc1Δ tel1Δ*) were arrested in M phase with nocodazole and UV irradiated. At the indicated time-points Mec1 activation was assayed by western blotting monitoring γ H2A. A quantification of the signal corresponding to H2A-S129 is shown in the lower panel. The values indicate the fold increase respect to the WT untreated samples. doi:10.1371/journal.pgen.1002022.g001

experimental conditions, *ddc1Δ dpb11-1* double mutant cells showed a very severe defect in Rad53 phosphorylation, similar to that found in a Mec1-defective strain. These results suggest that a *dpb11-1 ddc1Δ* double mutation virtually abolishes UV-induced

Mec1 activation differently from what previously reported [39]. In addition, the double mutant strain showed synthetic lethality on HU plates (Figure 2B and [43]). To confirm that the *dpb11-1* and *ddc1Δ* mutations directly affect Mec1 activity, we monitored γ H2A levels in the same conditions. As shown in Figure 2C, the *ddc1Δ* and *dpb11-1* mutations showed a synthetic defect in the ability to phosphorylate H2A-S129 (Figure 2D).

Although displaying a severe defect in Rad53 phosphorylation, *ddc1Δdpb11-1* still displays a residual low level of phosphorylated Rad53, which may be dependent upon a residual Mec1 activity. However, Figure 2E and Figure S2A show that the residual Rad53 phosphorylation in the double mutant is instead due to Tel1. Indeed, an additional mutation eliminating Tel1 function completely abolishes Rad53 phosphorylation in a *dpb11-1 ddc1Δ* strain and strongly sensitizes cells to HU treatment, as shown in Figure S2B. These findings further support the hypothesis that Mec1 cannot become activated in response to replication stress in the absence of both Ddc1 and Dpb11-AAD.

To verify the possibility that in *dpb11-1* mutant cells an unscheduled, Ddc1-dependent, DNA damage response is triggered as a consequence of the inability to properly activate the replication stress response, similarly to what happens in a *mec1Δ* strain [22], we monitored DNA damage checkpoint activation looking at Rad9 hyperphosphorylation. As shown in Figure 2F, differently from what found in the *mec1Δ* control strain, no Rad9 hyperphosphorylation was detectable in *ddc1Δ*, *dpb11-1* single or double mutant strains. Consistently, *rad9Δdpb11-1* double mutant cells are far less sensitive than the *ddc1Δdpb11-1* strain to HU treatment (Figure 2B and [43]).

Low levels of Rad53 activity are sufficient to prevent replication fork breakdown and premature entry into mitosis

Rad53 kinase activity is required to stabilize stalled replication forks [7]. To verify whether the increased HU sensitivity of *ddc1Δdpb11-1* double mutant cells was due to their inability to fully activate Rad53 and thus to stabilize the replisomes, we performed a recovery assay. Briefly, WT, *dpb11-1*, *ddc1Δ*, *ddc1Δdpb11-1* and *mec1-1smi1* mutant strains were blocked in G1, released and exposed to HU for 90 minutes; cells were then washed and shifted into fresh medium lacking HU and allowed to recover. As shown in the control strain *mec1-1 smi1*, when Rad53 activity is impaired, cells transiently exposed to HU lose the ability to resume DNA synthesis and complete DNA replication once the drug has been removed ([6] and Figure 3A). Unexpectedly, we found that not only *dpb11-1* and *ddc1Δ* single mutant cells, but also the double mutant strain, which has a severe Rad53 hyperphosphorylation defect, were able to recover from the HU treatment with a WT kinetics (Figure 3A). Moreover, with lower HU concentrations, *ddc1Δ dpb11-1* cells were capable of completing a round of DNA replication, as demonstrated by the re-entering of the replicated chromosomes in a pulsed-field gel system (Figure 3B).

Another marker of checkpoint activation by HU is the arrest of cell cycle, preventing mitosis. When exposed to HU, checkpoint mutants fail to delay the onset of mitosis and display elongated spindles [14]. To address the hypothesis that *ddc1Δ dpb11-1* cells may die as a consequence of a premature mitosis, we measured spindle length 90 minutes after HU addition. *ddc1Δ dpb11-1* double mutant cells prevent spindle elongation in the presence of HU, a process which is clearly defective in a *mec1-1* mutant strain (Figure S3A), suggesting that the replication checkpoint can delay mitotic entry in the double mutant [10].

In agreement with all these data, the HU sensitivity of *ddc1Δ dpb11-1* double mutant cells can be observed only to chronic

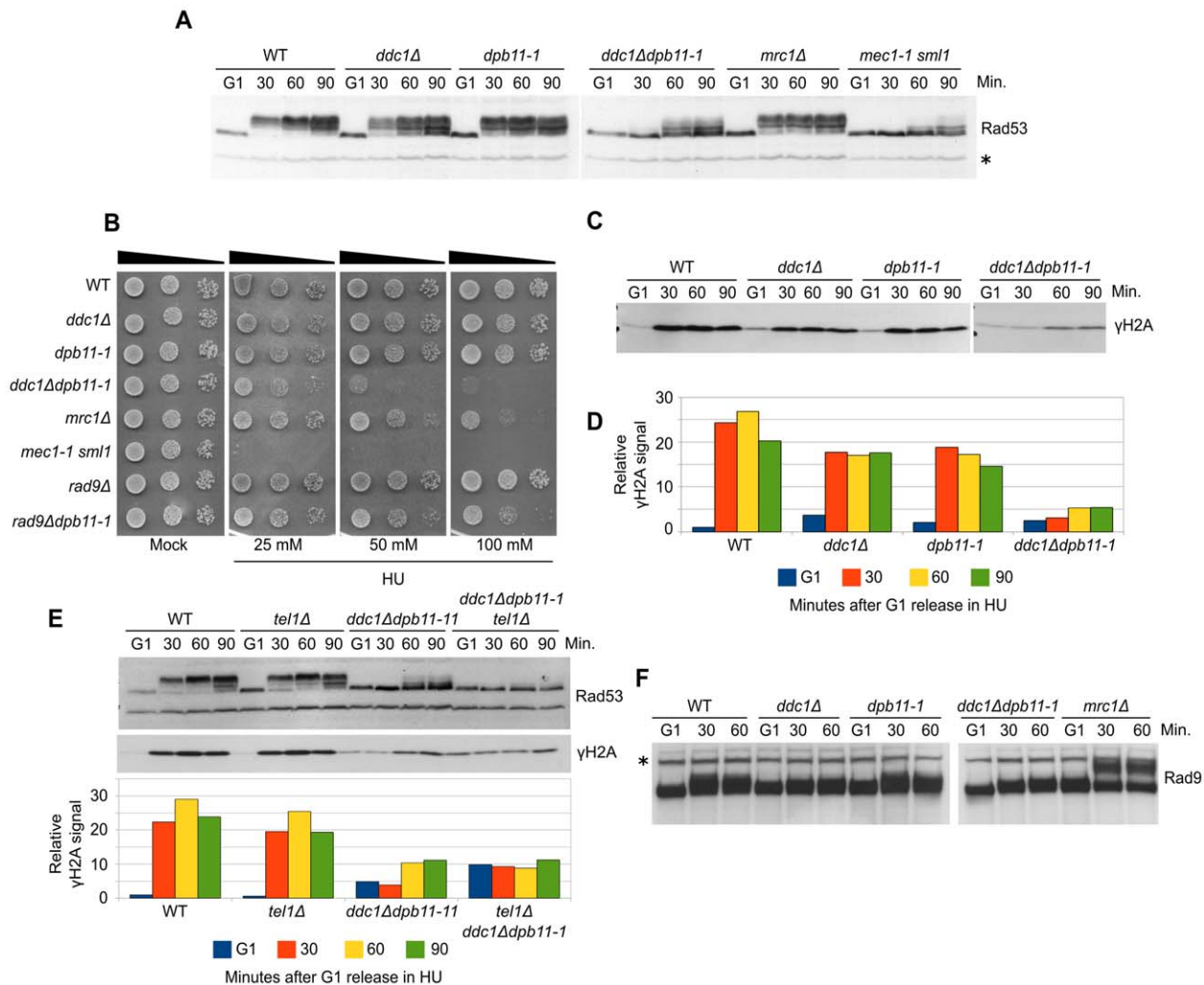


Figure 2. Dpb11 and 9-1-1 independently activate Mec1 after replication stress. (A) K699 (WT), YFP20 (*dpb11-1*), YAN21/8d (*ddc1* Δ), YFP62/1d (*ddc1* Δ *dpb11-1*), YFP125/6d (*mrc1* Δ) and YMIC5A3 (*mec1-1*) strains were grown to mid-log phase, synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. At the indicated time-points, protein extracts were prepared and separated by SDS-PAGE. Rad53 activation was assayed as the phosphorylation-dependent shift of the protein. (B) 10^2 – 10^4 cells from overnight cultures of the strains analyzed in panel A and YFP74 (*rad9* Δ) and YFP161/5C (*rad9* Δ *dpb11-1*) were spotted on YPD plates supplemented with HU at the indicated concentrations. Cell survival was assayed after 2–7 days. (C) The same filter in panel A was probed for Mec1 activation by analyzing the level of histone H2A phosphorylation. (D) Quantification of γ H2A in the experiment shown in panel C, using α -Rad53 cross-reacting band as loading control. The values indicate the fold increase respect to the WT G1 sample. (E) Strains K699 (WT), YMIC6C3 (*tel1* Δ), YFP62/1d (*ddc1* Δ *dpb11-1*) and YFP230 (*ddc1* Δ *dpb11-1**tel1* Δ) were synchronized in G1 and released into fresh medium supplemented with 200 mM HU. At the indicated time-points Rad53 and H2A phosphorylation were assayed by western blotting. A quantification of the signal is shown in the lower panel, using α -Rad53 cross-reacting band as loading control. The numbers indicate the fold increase respect to the WT G1 sample. (F) The same extracts of panel A were probed with α -Rad9 antibodies to determine the extent of Rad9 hyperphosphorylation. In all the relevant panels the loading control is indicated by an asterisk. doi:10.1371/journal.pgen.1002022.g002

exposure to the drug, while it is virtually undetectable if cells are transiently exposed to HU (Figure 3C).

The inability to fully activate Rad53 causes defects in the control of RNR induction

ddc1 Δ *dpb11-1* mutant cells exhibit extremely low levels of Mec1 and Rad53 activation and, despite being sensitive to exposure to even low concentrations of HU (Figure 2B), they do not show some of the most common phenotypes observed in replication checkpoint defective cells. To better characterize the sensitivity to the drug, we monitored cell growth in the presence of 100 mM HU. The single and double mutant *ddc1* Δ *dpb11-1* yeast strains were synchronized in G1, released into fresh medium supplemented with HU and cell cycle progression followed by FACS

analysis. The double mutant *ddc1* Δ *dpb11-1* showed a small delay in progressing through S-phase in the presence of HU, compared to WT and single mutant cells. Significantly, at late times (20 hours) after the release, a large fraction of double mutant cells appeared to be arrested at different stages of S-phase, while WT and single mutant cells had regained a FACS profile with 1C and 2C peaks (Figure 4A). Consistently, PFGE analysis of genomic DNA prepared from the various strains 20 hours after release from HU showed that in *ddc1* Δ *dpb11-1* double mutant cells most of the DNA fails to enter the gel, suggesting the presence of branched intermediates (Figure 4B, 4C). It is important to note that, differently from what found in a *mec1-1* strain, the *ddc1* Δ *dpb11-1* strain did not accumulate cells with a <1C DNA content, or low molecular weight DNA fragments (Figure 4A–4C) indicating a

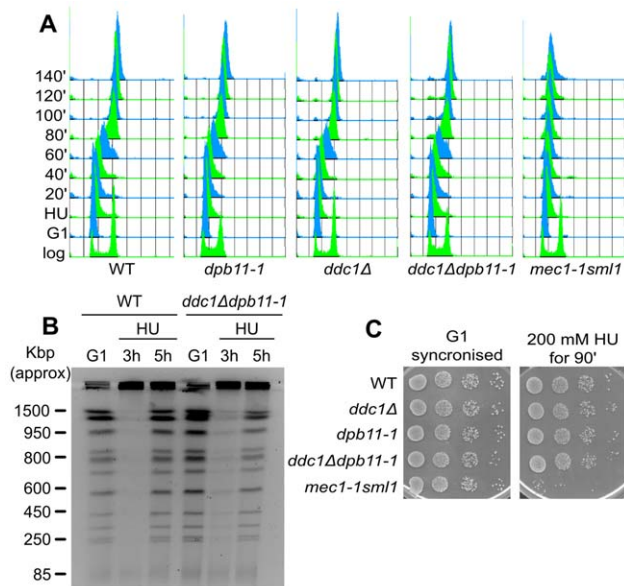


Figure 3. Low levels of Rad53 activity are sufficient to prevent replication fork breakdown. (A) HU recovery assay: K699 (WT), YFP20 (*dpb11-1*), YAN21/8d (*ddc1Δ*), YFP62/1d (*ddc1Δdpb11-1*) and YMIC5A3 (*mec1-1*) were synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. 90 min later cells were transferred to fresh YPD + nocodazole and allowed to resume DNA replication. Progression into S phase was monitored by FACS analysis. (B) The indicated strains were synchronized in G1 with α -factor and released into 100 mM HU + nocodazole. 3 and 5 hours later cells were harvested and total DNA was analyzed by Pulse Field Gel Electrophoresis (PFGE). (C) The strains in panel A were synchronized in G1 and released in YPD supplemented with 200 mM HU. 90 min later 10-fold serial dilution were prepared and spotted onto YPD plates. The same was done with the G1-synchronised cultures as control. doi:10.1371/journal.pgen.1002022.g003

correct segregation of chromosomes. Altogether, these findings may suggest that *ddc1Δ dpb11-1* cells are unable to counteract the effect of HU by upregulating ribonucleotide reductase (RNR). Indeed, Rad53 regulates both the timely degradation of the RNR inhibitor Sml1 and the inactivation of Crt1, which represses the transcription of RNR genes [9,10]. Consistently with this interpretation, *ddc1Δ dpb11-1* cells show a modest delay in Sml1 degradation and, more significantly, *CRT1* deletion suppresses, although not completely, the sensitivity of the double mutant strain to HU (Figure 4D, 4E).

Sld2 and DNA polymerase ϵ are required for Ddc1-independent checkpoint activation

Sld2/Drc1 and Pole ϵ participate in replication checkpoint signaling [24,25]. Moreover, these factors were recently found to be part of the same pre-loading complex, together with Dpb11 and GINS [44]. An interesting possibility is that Sld2 and Pole ϵ exert their checkpoint function by controlling Dpb11-mediated Mec1 activation. To address this hypothesis we combined the *drc1-1* allele with the *DDC1* deletion. As it is shown in Figure 5A, similarly to what reported above for Dpb11, Sld2 also acts in a pathway that is parallel to that involving Ddc1; indeed, residual Rad53 phosphorylation present in *ddc1Δ* cells depends on Sld2. Moreover, *drc1-1* cells do not show hyperphosphorylation of Rad9 in response to HU treatment, excluding the possibility of a secondary DNA damage response (Figure S4A). In agreement with these data, deletion of *DDC1* displays a synergistic sensitivity to

HU when combined with the *drc1-1* mutation and the HU sensitivity of the double mutant strain is very similar to that observed for *ddc1Δdpb11-1* cells (Figure 5B).

The checkpoint function of Pole ϵ appears to reside in the C-terminal domain of Pol2, which is bound, either directly or indirectly, by the three smaller subunits Dpb2, Dpb3 and Dpb4 and by Dpb11 [31,45]. To assess if Pole ϵ participates in the Dpb11 signaling branch via its minor subunits, we deleted *DPB4* in combination with the *DDC1* deletion. Figure 5C shows that Rad53 phosphorylation is severely impaired in the double mutant *ddc1Δdpb4Δ*, closely resembling the phenotype of a *ddc1Δdpb11-1* mutant. The same effect is measured by testing H2A phosphorylation in HU-treated samples (Figure 5D). The signals obtained for each time-point are quantified with respect to the signal detected in G1-arrested cells, in order to compensate for the higher basal level of γ H2A observed in *ddc1Δdpb4Δ* double mutant cells in the absence of any treatment. Moreover, no unscheduled DNA damage checkpoint activation occurs, since no Rad9 phosphorylation is detected in *dpb4Δ* or *dpb4Δ ddc1Δ* cells treated with HU (Figure S4B). Finally, the *ddc1Δdpb4Δ* strain shows an HU sensitivity similar to that found in *ddc1Δdpb11-1* cells (Figure 5E).

Discussion

Apical checkpoint kinases (Mec1/Tel1 in budding yeast, ATR/ATM in humans) convert a structural signal coming from damaged DNA to a phosphorylation-based signaling cascade, and a large amount of work has been devoted to clarify the underlying mechanisms. Initially, the attention was focused on the recruitment of these kinases to damaged DNA [15], based on the assumption that binding to damaged chromatin sites would lead to their activation. More recently, the finding that Dpb11/TopBP1 stimulates Mec1 activity suggests a more complex scenario [40,41,42].

In vitro data obtained in *Xenopus* egg and mammalian cell extracts demonstrate the ability of TopBP1 to increase Mec1 kinase activity [35,38]. The significance of this TopBP1 function does not appear to be specific for multicellular eukaryotes, since an interaction between Rad4/Cut5 and the checkpoint sensor kinase Rad3-Rad26 has also been found in *S. pombe* [46,47]. More recently, in *S. cerevisiae* cells, Dpb11 has been demonstrated to contain an ATR activation domain (AAD), which is sufficient to promote Mec1 activation *in vitro* [41,42]. These findings apparently contradict a previous observation that Mec1 can normally phosphorylate Ddc2 in a *dpb11-1* mutant, lacking part of the AAD, after UV damage in M phase [16], while in our hands *DDC1* deletion prevents Ddc2 phosphorylation (unpublished observation). Two explanations can be envisaged: in *dpb11-1* mutant cells, Mec1 activity may be sufficient to phosphorylate Ddc2, while being defective towards other substrates; alternatively, Dpb11 may play only a marginal role in response to UV irradiation in M phase. We favored the second hypothesis because *dpb11-1* mutant cells are mildly sensitive to UV irradiation and are proficient in the G2/M checkpoint; moreover, the 9-1-1 complex has also been identified as an activator of Mec1 *in vitro* [39,40] and may play a prominent role in M phase. If this assumption is correct, Dpb11 could play a role in Mec1 activation in response to a different kind of damage or in other cell cycle phases. Interestingly, it was demonstrated that the *dpb11-1* temperature-sensitive mutant is defective in checkpoint activation after replication stress caused by HU treatment at the restrictive temperature (36°C), while it is only mildly sensitive to the drug at permissive temperature ([23,25] and Figure 2B).

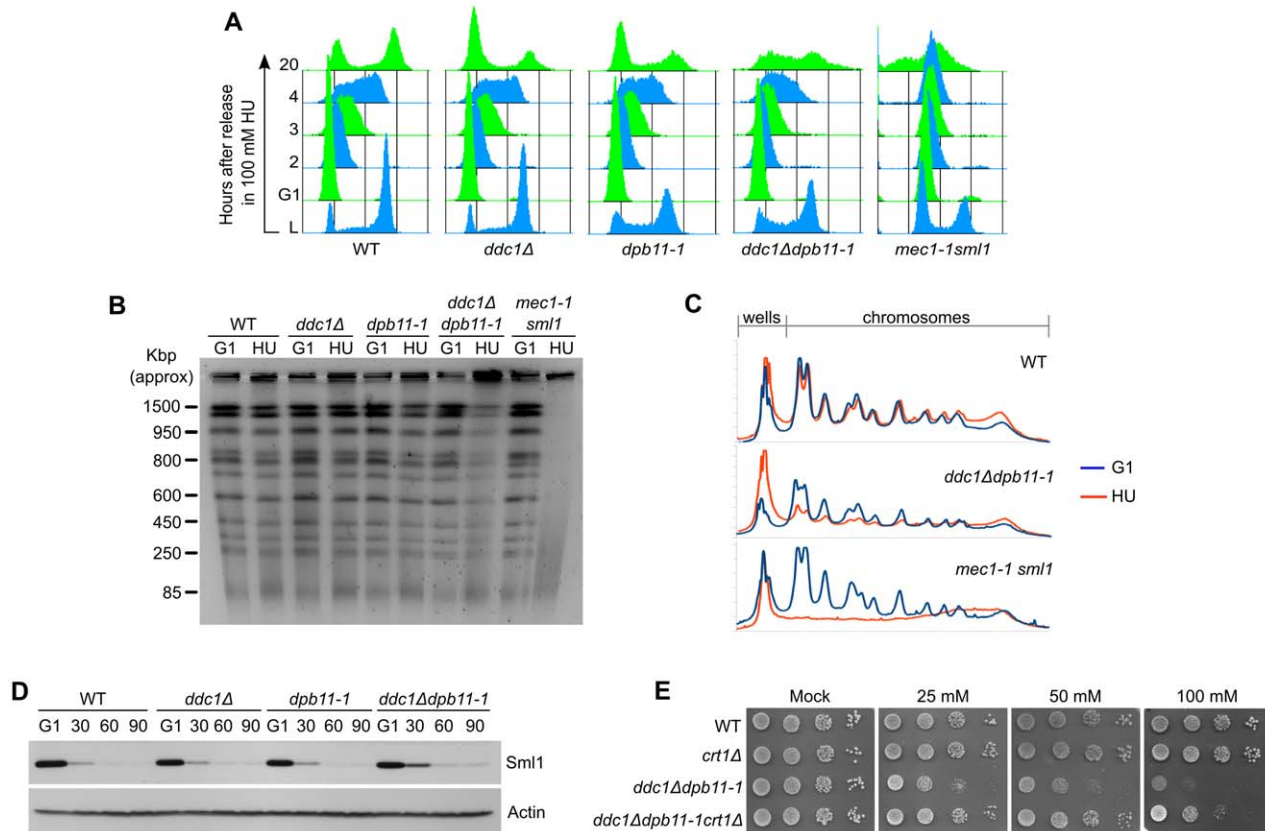


Figure 4. The inability to fully activate Rad53 causes defects in the control of RNR induction. (A) K699 (WT), YFP20 (*dpb11-1*), YAN21/8d (*ddc1Δ*), YFP62/1d (*ddc1Δdpb11-1*) and YMIC5A3 (*mec1-1*) were synchronized in G1 with α -factor and released into fresh medium supplemented with 100 mM HU. At the indicated time-points after the release, progression into the cell cycle was monitored by FACS analysis. (B) DNA extracted from the G1-arrested cells and from the cells released for 20 hours in HU was separated by PFGE and stained with ethidium bromide. (C) A plot representing the intensity profile of the significant gel lanes in panel B. (D) K699 (WT), YFP20 (*dpb11-1*), YAN21/8d (*ddc1Δ*) and YFP62/1d (*ddc1Δdpb11-1*) strains were synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. At the indicated time-points protein extracts were prepared and probed with α -Sml1 antibodies to measure the levels of Sml1 and with α -actin antibodies as a loading control. (E) Ten fold serial dilutions of overnight cultures of strains K699 (WT), YFP328 (*crt1Δ*), YFP62/1d (*ddc1Δdpb11-1*) and YFP330 (*ddc1Δdpb11-1crt1Δ*) were spotted on YPD plates supplemented with HU at the indicated concentration. Survival was assayed by monitoring cell growth after 6 days.

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To better understand the process of Mec1 activation *in vivo* after DNA damage or replication stress, we analyzed the relative functions of the two putative Mec1 activators: Dpb11 and the 9-1-1 complex. We extended our previous analysis by monitoring, in different cell cycle phases, a direct target of Mec1 kinase (histone H2A) as marker of Mec1 activity. We found that, both in G1 and in M phase, the 9-1-1 complex is absolutely required for Mec1 activation in response to UV treatment, while the contribution of Dpb11 AAD is only partial (~50%) and restricted to G1. These *in vivo* findings are in agreement with the current activation model inferred from *in vitro* biochemical data [39], indicating that 9-1-1 can stimulate Mec1 through both Dpb11-dependent and -independent pathways in G1 (Figure 6, left). Differently, in M phase, the ATR activation domain of Dpb11 is dispensable for full Mec1 activation, which relies mainly on the presence of 9-1-1 (Figure 6, right). In fact, the residual UV-induced H2A phosphorylation detectable in the *ddc1Δ* strain, is dependent upon the Tel1 kinase (Figure 1). Different requirements for Mec1 activation in G1 and in M phase may reflect differences in CDK-controlled processing of DNA filament ends to generate the substrate detected by checkpoint factors [48,49].

To complete studying of the pathways leading to Mec1 activation in different cell cycle stages, we analyzed the contribution of Dpb11 and Ddc1 to Mec1 activation in S phase cells challenged with replication stress. HU decreases the cellular concentration of dNTPs available for DNA synthesis and yeast cells respond by activating the replication checkpoint.

In vivo analysis of the phosphorylation state of two Mec1 substrates, H2A and Rad53, indicates that Dpb11 and 9-1-1 participate in Mec1 activation in response to HU treatment independently of each other in two parallel pathways. The possibility that *dpb11-1* may cause problems to the replication process triggering a DNA damage response mediated by the 9-1-1 complex, similarly to what happens in *mrc1Δ* cells [22], seems unlikely. In fact, the Rad9 DNA damage-specific adaptor does not become hyperphosphorylated in both *dpb11-1* and *ddc1Δ* single mutants. In agreement with such observation, *rad9Δdpb11-1* cells are much less sensitive to HU than *ddc1Δ dpb11-1* cells (Figure 2 and [43]).

We report that the HU sensitivity of *ddc1Δ dpb11-1* strain is not due to replication fork collapse or premature elongation of the mitotic spindle (Figure 3 and Figure S2), two phenotypes characteristic of mutants defective in the replication checkpoint

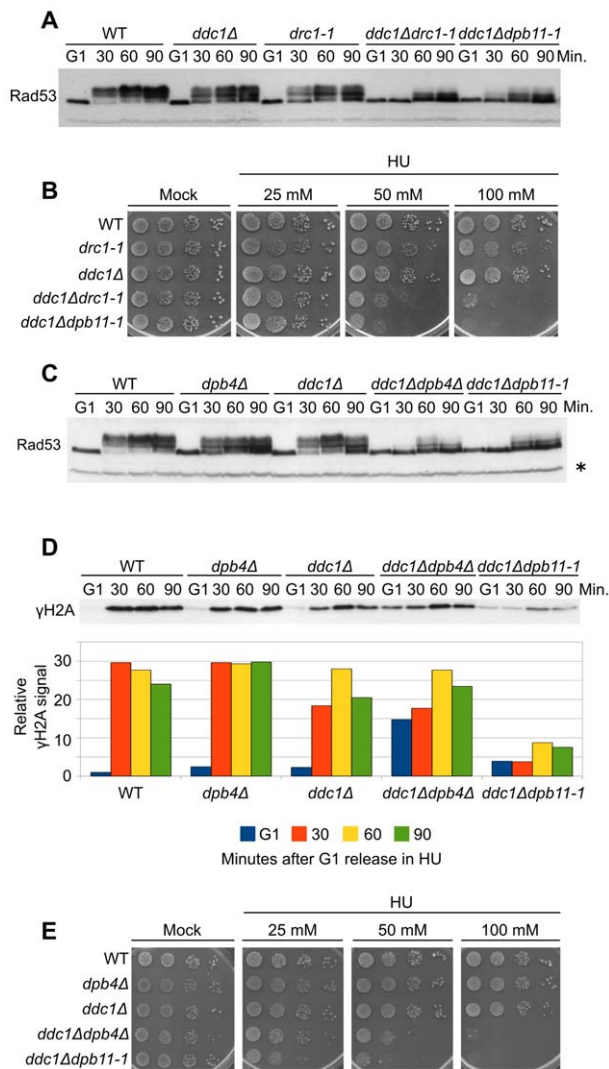


Figure 5. Pol ϵ associated proteins are involved in the 9-1-1-independent checkpoint signaling branch. (A) Strains K699 (WT), Y799 (*drc1-1*), YAN21/8d (*ddc1 Δ*), YFP218/1a (*ddc1 Δ drc1-1*) and YFP62/1d (*ddc1 Δ dpb11-1*) were cultured to mid-log phase, synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. At the indicated time-points Rad53 phosphorylation was assayed by SDS-PAGE and Western blotting. (B) Ten-fold serial dilutions of overnight cultures of the strains in panel A were spotted on YPD plates supplemented with HU at the indicated concentration. Survival was assayed by monitoring cell growth after 6 days. (C) Strains K699 (WT), YFP167/1a (*dpb4 Δ*), YAN21/8d (*ddc1 Δ*), YFP206/1a (*ddc1 Δ dpb4 Δ*) and YFP62/1d (*ddc1 Δ dpb11-1*) were cultured to mid-log phase, synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. At the indicated time-points, protein extracts were prepared and separated by SDS-PAGE. Rad53 phosphorylation was assayed by western blotting. (D) The same filter was probed for Mec1 activity by testing histone H2A phosphorylation. A quantification of γ H2A, using α -Rad53 cross reacting band as loading control is shown in the lower panel. The values indicate the fold increase respect to the WT G1 sample. (E) Ten-fold serial dilutions of overnight cultures of the strains in panel C were spotted on YPD plates supplemented with HU at the indicated concentration. Cell survival was assayed monitoring cell growth after 6 days. In all the relevant panels the loading control is indicated by an asterisk. doi:10.1371/journal.pgen.1002022.g005

[7,12]. Accordingly, the HU sensitivity of *ddc1 Δ dpb11-1* double mutant cells, differently from that of a *mec1-1sml1* strain, is not detectable in the case of transient HU treatment. This

observation suggests that another Rad53 function activated by the replication checkpoint, and different from that responding to temporary fork arrest, is essential for sustaining growth in the constant presence of hydroxyurea. Indeed, *ddc1 Δ dpb11-1* double mutant cells grown in the presence of HU show defects in completing replication and accumulate replication intermediates. Moreover, *ddc1 Δ dpb11-1* cells are unable to counteract the effect of HU by upregulating ribonucleotide reductase. Interestingly, *CRT1* deletion partially suppresses HU sensitivity of the double mutant strain (Figure 4E).

To obtain more insights on the pathways leading to Ddc1-dependent and Dpb11-dependent activation of replication checkpoint and to identify possible mechanisms specific for lagging or leading strand fork arrest, we analyzed mutants in the genes coding for proteins that are known to be involved in leading strand replication. During initiation of DNA replication, Dpb11 interacts with both Sld2 and Sld3 in a phosphorylation-dependent manner, a process that is required for origin firing [26,27]. Moreover, temperature sensitive *drc1-1* strains, mutated in Sld2, display the same checkpoint-deficient phenotype of *dpb11-1* cells, when treated with HU at the non-permissive temperature, (Figure 5 and [25]). We tested whether Sld2 functions with Dpb11 in the same 9-1-1-independent pathway for Mec1 activation. Combining the *drc1-1* allele with the *DDC1* deletion, we found that *ddc1 Δ drc1-1* double mutant cells display the same Rad53 phosphorylation defect and the same HU sensitivity of a *ddc1 Δ dpb11-1* strain, indicating that Mec1 activation by Dpb11 also requires Sld2 (Figure 5).

Mutants in the Pol2 C-terminus, the enzyme replicating the leading strand [50], are defective in the establishment of the replication checkpoint [24,50] and this protein region of Pol2 was suggested to be involved in its interaction with other three Pole subunits: the essential Dpb2 protein and the non-essential Dpb3 and Dpb4 subunits [31,45,51]. Disruption of the *DPB4* gene in a *ddc1 Δ* background leads to identical phenotypes to the one observed in *ddc1 Δ dpb1-1* and *ddc1 Δ drc1-1*, strongly suggesting that the 9-1-1-independent pathway involves leading strand replication factors. The observations that Dpb11 acts directly on Mec1 activity [41,42] and that, in the *dpb11-1* mutant, Pole seems to be normally loaded onto replication origins [52], strongly suggest that Dpb4, and possibly Sld2, function upstream of Dpb11 during checkpoint signaling. Unfortunately, it is impossible to perform a complete formal epistatic analysis as the *dpb11-1* mutation also affects replication initiation and deletion of *DPB4* or mutations in *SLD2* are synthetic lethal when combined with the *dpb11-1* allele [28,53].

In conclusion our data suggest that during exposure to hydroxyurea, two independent pathways sense replication stress and signal for Mec1 activation. The first pathway depends on 9-1-1, which is known to be loaded at the 5' of primer-template junctions, when RPA covers ssDNA ahead of the primer [34]. During unchallenged DNA replication these structures are normally formed on the lagging strand as a consequence of discontinuous DNA synthesis, and rapidly removed by refilling polymerase activity. Inhibition of DNA polymerization by HU likely stabilizes the 5' DNA end providing the structure required for 9-1-1 loading. On the other hand, the higher processivity of leading strand synthesis makes it likely that the nearest 5' end will be far away from the site of polymerase stalling, where ssDNA is generated and the Mec1-Ddc2 complex should be recruited. The absence of such structure could prevent the 9-1-1-dependent Mec1 activation. In this case a pathway requiring the leading strand factors Dpb4, Dpb11 and Sld2 becomes relevant to induce Mec1 activation (Figure 6, center). The hypothesis that Pole, Sld2

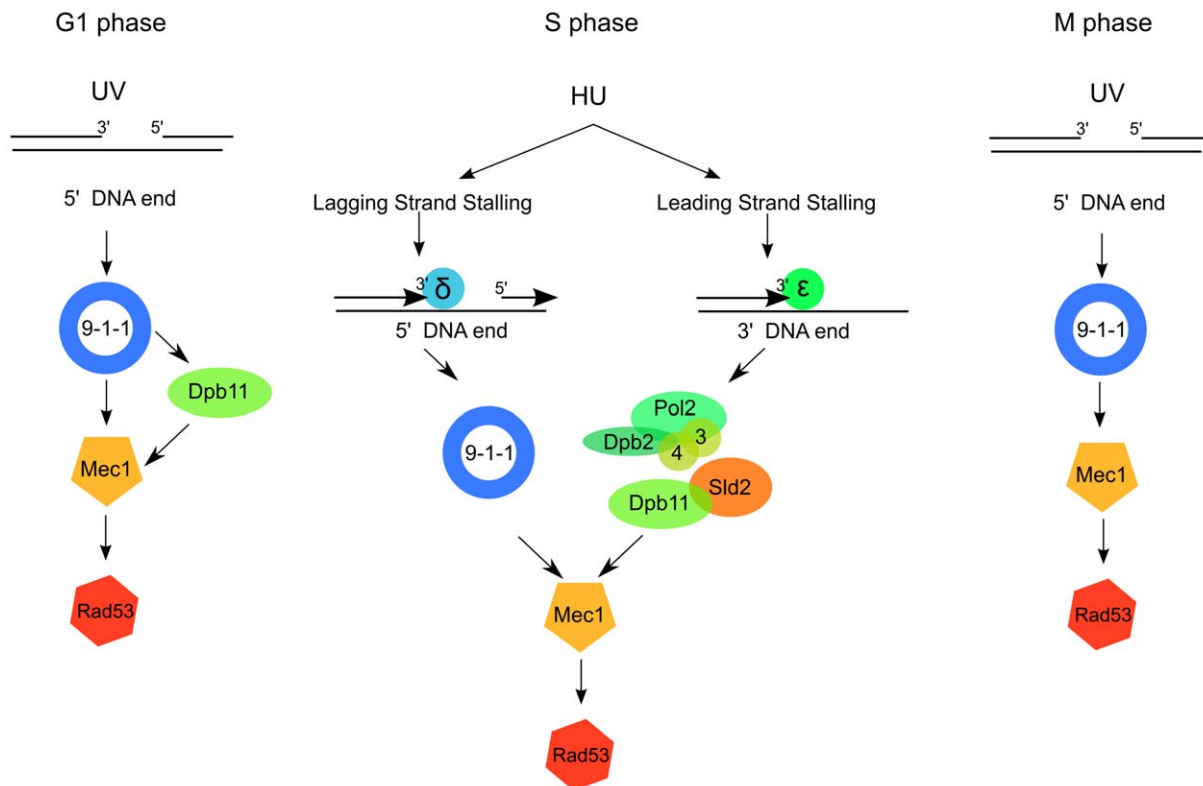


Figure 6. A model for 9-1-1 and Dpb11 function in Mec1 activation. After UV irradiation in G1, Mec1 is activated by the 9-1-1 complex both directly and through the Dpb11 C-terminus (right); in M phase Mec1 activation is achieved mainly through the 9-1-1 complex, independently of Dpb11 (left). In S phase Dpb11 and the 9-1-1 complex signal replication stress to Mec1 independently from each other, likely because the detection of replication stress occurs independently on the leading and lagging strands. 9-1-1 complex could signal replication stress on the lagging strand, where the 5' ends necessary for its loading are generated as the result of discontinuous replication. Dpb11, instead, could signal replication stress on the leading strand together with the interacting Pol ϵ .
doi:10.1371/journal.pgen.1002022.g006

and Dpb11 work together in sensing replication stress is supported by the recent finding that an unstable complex containing Dpb11, Sld2, Pole and GINS is formed at the beginning of S-phase [44]. Moreover, the demonstration that under unstressed conditions Pole acts on the leading strand while Pol δ works on the lagging strand [50,54] supports the hypothesis that Pole and its interacting subunits may function in sensing replication stress on the leading strand, while the 9-1-1 complex may be more important to detect lagging strand fork arrest. Additional work will be needed to confirm this model and to identify the mechanisms leading to Dpb11 recruitment at the sites of replication fork stalling, since Dpb11 appears to co-localize with Pole during initiation of DNA replication, but not during elongation [52].

Materials and Methods

Yeast strains

All of the strains used in this work are derivatives of W303 (K699 [*MATa ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3*]) and are listed in Table 1. Deletion strains were generated by using the one-step PCR system [55] or by genetic crossing.

Cell cycle synchronization and HU treatment

Cells were grown overnight at 25°C to a concentration of 5×10^6 cells/ml and arrested in G1 with 5 μ g/ml α -factor for

three hours. 60 ml of cultures were spun and resuspended in the same volume of YPD supplemented with HU (200 mM or 100 mM, depending on the experiment). 20 ml samples were taken every 30 minutes after the release. In the case of untreated samples cells were released in fresh YPD +10 μ g/ml nocodazole and every 5 minutes samples were taken for SDS-PAGE and FACS analysis.

Cell cycle arrest and DNA damage treatments

Cells were grown in YPD medium at 25°C to a concentration of 5×10^6 cells/ml and arrested with nocodazole or α -factor (20 μ g/ml). 50 ml of cultures were spun, resuspended in 500 μ l of sterile water, and plated on a Petri dish (14-cm diameter). Rapidly, a 15 ml untreated sample was taken. Plates were irradiated at 75 J/m² and cells were resuspended in 50 ml of YPD + nocodazole or α factor. Three 15 ml samples were taken every 10 minutes after irradiation.

SDS page, western blot, and quantification

Trichloroacetic acid protein extracts [56] were separated by SDS-PAGE; for the analysis of Rad9 phosphorylation, NuPAGE Tris-Acetate 3–8% gels (Invitrogen) were used following the manufacturer's instructions. Western blotting was performed with anti-Rad53, anti-H2A-S129 (Abcam #15083), anti-Actin (Sigma #A2066), anti-Sml1 and anti-Rad9 antibodies, using standard techniques. Values of phospho-H2A levels were obtained by quantifying the signal in the corresponding lanes using Quantity

Table 1. Strains used in this work.

Name	Relevant Genotype	Reference
K699	<i>MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1, can1-100</i>	K.Nasmyth
YAN21/8d	(K699) <i>ddc1Δ::kanMX6</i>	A. Nespoli
YFP20	(K699) <i>dpb11-1</i>	Puddu, 2008
YFP62/1d	(K699) <i>ddc1Δ::kanMX6 dpb11-1</i>	Puddu, 2008
YMIC5A3	(K699) <i>mec1-1 sml1</i>	M. Giannattasio
YFP125/6d	(K699) <i>mrc1Δ::HIS3</i>	This Work
YFP167/1a	(K699) <i>dpb4Δ::HIS3</i>	This Work
YFP206/1a	(K699) <i>ddc1Δ::KanMX6 dpb4Δ::HIS3</i>	This Work
Y799	(K699) <i>drc1-1</i>	Wang & Elledge, 1999
YFP218/1a	(K699) <i>ddc1Δ::KanMX6 drc1-1</i>	This Work
YFP74	(K699) <i>rad9Δ::HIS3</i>	This Work
YFP161/5C	(K699) <i>rad9Δ::HIS3 dpb11-1</i>	This Work
YFP328	(K699) <i>crt1Δ::HIS3</i>	This Work
YFP330	(K699) <i>ddc1Δ::KanMX6 crt1Δ::HIS3 dpb11-1</i>	This Work
YMIC6C3	(K699) <i>tel1Δ::KanMX6</i>	M. Giannattasio
YFP223	(K699) <i>ddc1::KanMX6 tel1Δ::HIS3</i>	This Work
YFP225	(K699) <i>dpb11-1 tel1Δ::HIS3</i>	This Work
YFP230	(K699) <i>ddc1::KanMX6 dpb11-1 tel1Δ::HIS3</i>	This Work

doi:10.1371/journal.pgen.1002022.t001

One software (BioRad) and normalizing it, first on the loading controls and then on the level of phospho-H2A in the untreated/G1-arrested sample of each strain.

Immunofluorescence

1 ml of a 5×10^6 cells/ml culture were fixed overnight at 4°C with fixation buffer (3,7% formaldehyde, 0,1 M K-phosphate pH 6,4, 0,5 mM MgCl₂). Cells were then washed three times with wash buffer (0,1 M K-phosphate pH 6,4, 0,5 mM MgCl₂), one time with spheroplasting solution (1,4 M sorbitol, 0,1 M K-phosphate pH 6,4, 0,5 mM MgCl₂) and resuspended in 200 μl of the same solution. Spheroplasts were prepared using 5 μl of 10 mg/ml Zymolyase at 37°C. Spheroplasts were washed with the same solution and used to prepare multi-well immunofluorescence slides which were incubated overnight with α-tubulin antibody (YOL1/34, Seralab) diluted 1:100 in PBS-5%BSA.

Sensitivity assay

HU plates were prepared by serial dilutions of the 2 M stock solution. Plates containing 25 mM, 50 mM and 100 mM HU were prepared. Overnight grown cultures were diluted to 1×10^6 cell/ml, then 10-fold serial dilutions were prepared and 10 μl of the suspensions were spotted on HU plates, which were incubated at 25°C. Images were taken 2 to 7 days later.

Pulsed field gel electrophoresis

Agarose plugs containing yeast chromosomes were prepared as described previously [57]. These were incubated overnight at 37°C in 0.5 ml/plug TE containing 1 mg/ml RNaseA. After extensive washes with Wash Buffer (10 mM Tris-HCl pH 7.5 50 mM EDTA), plugs were loaded on 1% agarose gel and sealed in the wells with a solution of 1% LMP agarose in TBE 0.5X. Gels were run at 4°C for 24 h at 165 V, with 60 seconds pulses for 12 h and 90 second pulses for 12 h, using an Amersham Gene Navigator system.

Supporting Information

Figure S1 H2A is phosphorylated during the cell cycle. (A) Strains K699 (WT) and YMIC5A3 (*mec1-1 sml1*) were arrested in G1 with α-factor and UV irradiated. At the indicated time-points Mec1 activation was assayed by western blotting monitoring γH2A, using actin as loading control. (B) Strains K699 (WT) and YMIC6C3 (*tel1Δ*) were arrested in M phase with nocodazole and UV irradiated. At the indicated time-points Mec1 activation was assayed by western blotting monitoring γH2A and using actin as loading control. (C) K699 (WT) was synchronized in G1 with α-factor and released into fresh medium. Every 10 min samples of the culture were withdrawn and progression into the cell cycle was monitored by FACS analysis. H2A phosphorylation was monitored by western blotting. (D) Strains K699 (WT), YMIC6C3 (*tel1Δ*), YFP20 (*dpb11-1*) and YFP225 (*tel1Δdpb11-1*) were processed as in B. (TIF)

Figure S2 Tel1 contributes minimally to Rad53 phosphorylation in the absence of either Ddc1 or Dpb11-AAD. (A) Strains K699 (WT), YAN21/8d (*ddc1Δ*), YFP20 (*dpb11-1*), YMIC6C3 (*tel1Δ*), YFP223 (*tel1Δddc1Δ*), YFP225 (*tel1Δdpb11-1*) were synchronized in G1 with α-factor and released in fresh medium supplemented with 200 mM HU. At the indicated time-points Rad53 phosphorylation was assayed by SDS-PAGE and western blotting. (B) Ten fold serial dilutions of overnight cultures of strains K699 (WT), YMIC6C3 (*tel1Δ*), YFP62/1d (*ddc1Δdpb11-1*), YMIC5A3 (*mec1-1*) and YFP230 (*ddc1Δdpb11-1tel1Δ*) were spotted on YPD plates supplemented with HU at the indicated concentration. Survival was assayed by monitoring cell growth after 6 days. (TIF)

Figure S3 Low levels of Mec1 activity are sufficient to delay mitosis following S-phase arrest. (A) K699 (WT), YFP20 (*dpb11-1*),

YAN21/8d (*ddc1Δ*), YFP62/1d (*ddc1Δdpb11-1*) and YMIC5A3 (*mec1-1*) were synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. 90 min later precocious ingression into mitosis was monitored measuring spindle elongation by indirect immunofluorescence. Representative pictures are shown (blue = DNA; red = tubulin). Spindle length of 200 cells for each sample was measured and the frequencies of the different spindle length classes are shown in the lower panel. (TIF)

Figure S4 Hydroxyurea does not elicit a DNA damage response in the *ddc1-1* and *dpb4Δ* mutants. (A) Strains K699 (WT), Y799 (*ddc1-1*), YAN21/8d (*ddc1Δ*), YFP218/1a (*ddc1Δddc1-1*) and YFP62/1d (*ddc1Δdpb11-1*) were grown to mid-log phase, synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. After 90 min Rad9 phosphorylation was assayed by SDS-PAGE and western blotting. (B) Strains K699 (WT), YFP167/1a (*dpb4Δ*), YAN21/8d (*ddc1Δ*),

YFP206/1a (*ddc1Δdpb4Δ*) and YFP62/1d (*ddc1Δdpb11-1*) were grown to mid-log phase, synchronized in G1 with α -factor and released into fresh medium supplemented with 200 mM HU. 90 minutes later protein extracts were prepared and separated by SDS-PAGE. Rad9 phosphorylation was assayed by SDS-PAGE and western blotting.

(TIF)

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Author Contributions

Conceived and designed the experiments: FP PP MM-F. Performed the experiments: FP GP. Analyzed the data: FP GP PP MM-F. Wrote the paper: FP PP MM-F.

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