Dpb11 and 9-1-1 Complex Act Redundantly in Promoting Checkpoint Activation after Replication Stress

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Following DNA damage or replication stress eukaryotic cells phosphorylate and activate the Rad53 (hChk2) checkpoint protein, which is responsible for maintaining genome stability in these challenging conditions. The activation mechanism of DNA Damage Checkpoint and Replication Checkpoint is different, but partially overlapping because most of checkpoint factors are shared between these pathways. For the activation of these two checkpoints the upstream kinase complex Mec1/Ddc2 (hATR/hATRIP) is required and both the PCNA-like complex and the replicative factor Dpb11 (hTopBP1) play a relevant role. We have previously shown that in UV damaged yeast cells, Dpb11 is required for the histone methylation-independent function of Rad9. Dpb11 has been recently reported to be able to stimulate in vitro Mec1 kinase activity. To better understand in vivo the function of Dpb11 in activating the apical kinase, we decided to study checkpoint activation after treatment with hydroxyurea (HU), a RNR inhibitor. In fact, HU induces Rad53 phosphorylation independently of the Rad9 adaptor protein allowing us to study the function of Dpb11 in the activation of Mec1 and its interplay with 9-1-1 complex (Ddc1-Mec3-Rad17). We show here that a *ddc1ΔΔdpb11-1* double mutant displays a Rad53 phosphorylation defect after HU treatment, similar to the one of a *mec1-1* mutant. Unexpectedly the double mutant also lacks the hyperphosphorylation of histone H2A, which is maintained in *mec1-1*. These observations suggest that Dpb11 and the PCNA-like complex act independently in promoting Mec1 and Tel1 activation. Moreover a similar phenotype can be observed in a *dpb4ΔΔddc1Δ* strain which carry a deletion of a non-essential subunit of DNA polymerase ε, suggesting that Dpb11 may be working together with Pol ε in this function.

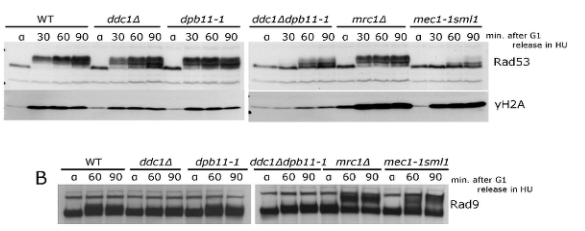

Fig.1 Ddc1 and Dpb11 cooperate for checkpoint activation after replication stress.
The indicated yeast strains were synchronized with α-factor in G1 and released in fresh medium containing 200 mM HU. At different time points after release Rad53, H2A and Rad9 phosphorylation was analysed. (A) The mutants *ddc1Δ* and *dpb11-1* are mildly defective in Rad53 phosphorylation, while the double mutant displays a greater defect, similar to the one of a *mec1-1* mutant. (B) Unexpectedly *ddc1Δdpb11-1* lacks H2A hyperphosphorylation characteristic of the *mec1-1* strain. Moreover whereas in both *mec1-1* and *mrc1Δ* Rad9 is hyperphosphorylated, indicating the activation of the DNA damage checkpoint, the double mutant *ddc1Δdpb11-1* lacks this Rad9 shift, suggesting either the absence or the incapacity to detect DNA damage.

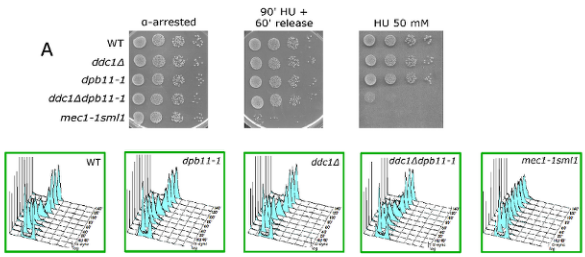

Fig.2 Mutant cells are proficient in replication fork stabilization after replication stress.
(A) The indicated yeast strains were tested for sensitivity to replication inhibition. Cells were synchronized with α-factor in G1, released and blocked in fresh medium containing 200 mM HU for 90 minutes and finally released in fresh medium to allow completion of DNA replication. Then serial dilutions were spotted onto YPD plates. In parallel the same strains were spotted onto YPD plates containing 50 mM HU. Similarly to the single mutants, *ddc1Δdpb11-1* is not sensitive to a HU pulse treatment. However when chronically exposed to replication stress, the double mutant becomes deeply sensitive. (B) The same strains, treated with HU as described before were also released in fresh medium supplemented with nocodazole. Every 20 minutes after the release, DNA content was measured by FACS analysis to evaluate the ability of the cells to restart replication. WT, *ddc1Δ*, *dpb11-1* and *ddc1Δdpb11-1* strains are able to duplicate their genome once released in HU-free medium, indicating the ability to maintain stalled replication fork in a competent state. These data suggest that the double mutant is proficient in replication fork stabilization after HU treatment.

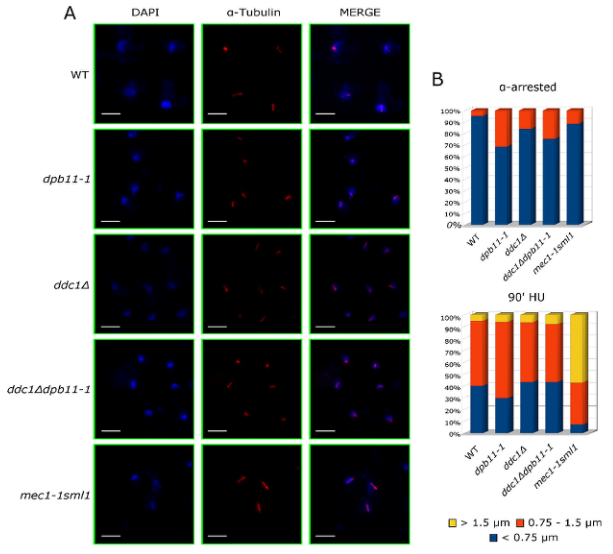

Fig.3 Mutant cells are proficient in the activation of replication checkpoint.
The indicated yeast strains were synchronized with α-factor in G1 and released in rich medium containing 200 mM HU to analyse spindle elongation. (A) 90 minutes after the release, nuclear morphology was visualized by DAPI staining and microtubules were visualized by indirect immunofluorescence using anti α-tubulin antibodies. Scale bars, 5 μm. (B) Spindle length in 250 cells for each sample was measured in the α-factor arrested samples and in the 90' HU samples. Data obtained are summarized in the two charts showing the percentage of cells belonging to the indicated spindle length classes. Under these conditions only the *mec1-1sml1* positive control showed spindle elongation, while *ddc1Δdpb11-1* is able to prevent it after replication stress, like the WT and the single mutants. These data suggest that the double mutant is proficient in replication checkpoint activation, preventing the entry into mitosis.

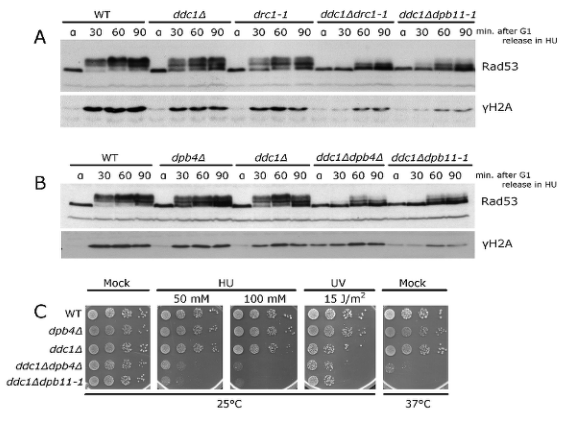

Fig.4 Dpb4 and Drc1/Sld2 are involved in the Dpb11-dependent branch of replication checkpoint activation.
The indicated yeast strains were synchronized with α-factor in G1 and released in fresh medium containing 200 mM HU. At different time points after release Rad53 and H2A phosphorylation was analysed. (A) The mutant *drc1-1* is mildly defective in Rad53 phosphorylation as published, while the double mutant *ddc1Δdrc1-1* has the same Rad53 phosphorylation level of *ddc1Δdpb11-1*. HU induced H2A phosphorylation shows a similar behaviour. (B) The same experiment was repeated analysing the mutant *dpb4Δ*. *dpb4Δ* is mildly defective in Rad53 phosphorylation, almost as the *ddc1Δ*, while the double mutant *ddc1Δdpb4Δ* displays a synergistic effect. HU induced H2A phosphorylation is greater in *ddc1Δdpb4Δ* than in *ddc1Δdpb11-1* probably because in *ddc1Δdpb4Δ* the histone is phosphorylated also in untreated conditions. (C) The same strains were also tested for sensitivity to replication inhibition, DNA damage or high growth temperature. The sensitivity to HU is synergic between *DDC1* and *DPB4*, but in damaging conditions Ddc1 plays the most important role. Interestingly the double mutant *ddc1Δdpb4Δ* is thermosensitive.

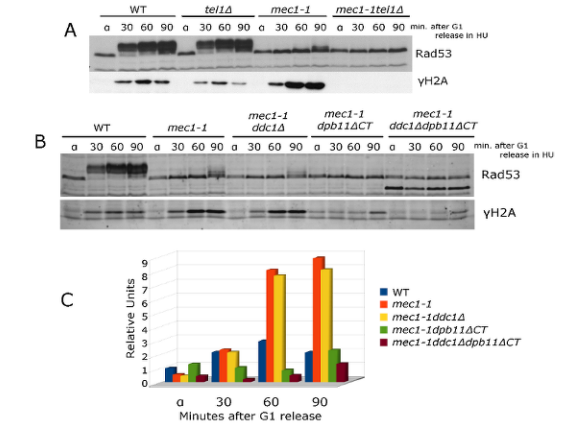

Fig.5 In the absence of Mec1, Dpb11 is required for Tel1 activation after replication stress.
The indicated yeast strains were synchronized with α-factor in G1 and released in fresh medium containing 200 mM HU. At different time points after release, Rad53 and H2A phosphorylation was analysed. Mutation in *SML1* to allow *mec1-1* viability is not indicated. (A) The HU induced H2A phosphorylation and the residual Rad53 activation, in the absence of Mec1 activity, depend on the Tel1 kinese. (B) The double mutant *mec1-1dpb11ΔCT* is completely defective in HU induced Rad53 and H2A phosphorylation, while the double mutant *mec1-1ddc1Δ* still shows these modification. (C) Quantification of H2A phosphorylation. The HU induced H2A phosphorylation in *mec1-1* strain depends on Dpb11 but not on Ddc1. These data suggest that, in a *mec1-1* background, Dpb11 is important for the activation of Tel1 after replication stress.

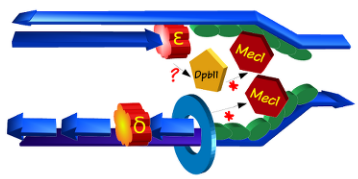

Fig.6 Possible model for Mec1 activation after replication stress.
To explain the redundant activity of Dpb11 and Ddc1 in activating Mec1 kinase we elaborated a model in which two distinct populations of Mec1 are activated independently either by 9-1-1 complex or Dpb11. Mec1 recruited on the lagging strand by ssDNA is likely activated by PCNA-like, that has a 5' DNA end suitabe for its loading. On the leading strand, without repriming events, there is no 5' end useful for 9-1-1 loading: in this situation Mec1 could be activated by Dpb11, probably recruited through an interaction with DNA polymerase ε.

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