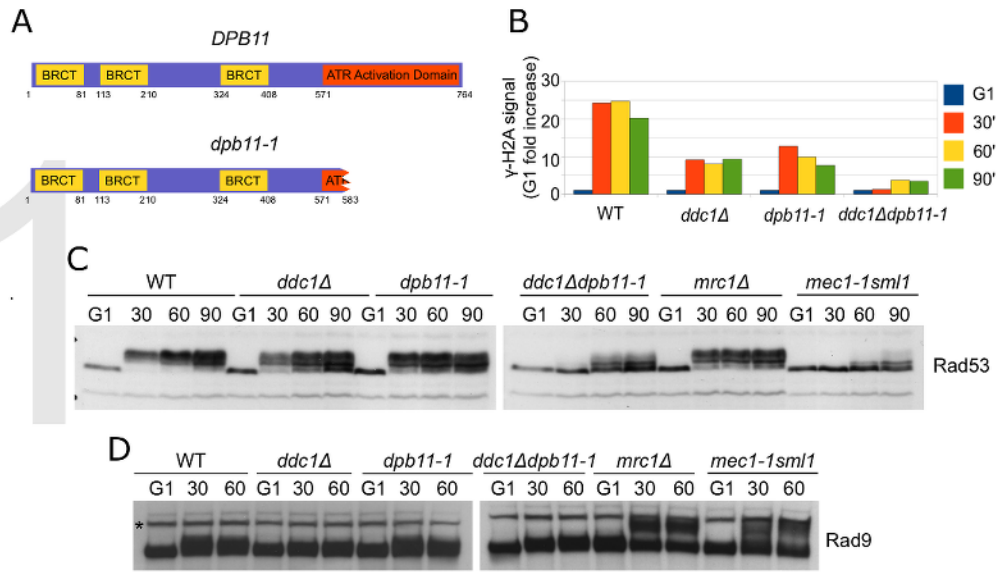
Dpb11 and 9-1-1 Complex Act Redundantly in Promoting Checkpoint Activation after Replication Stress

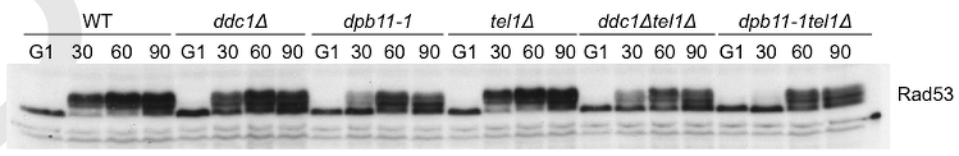
Fabio Puddu, Gabriele Piergiovanni, Paolo Plevani & Marco Muzi-Falconi

Department of Biomolecular Sciences and Biotechnology, University of Milano, Via Celoria 26, 20133 Milan, Italy, EU

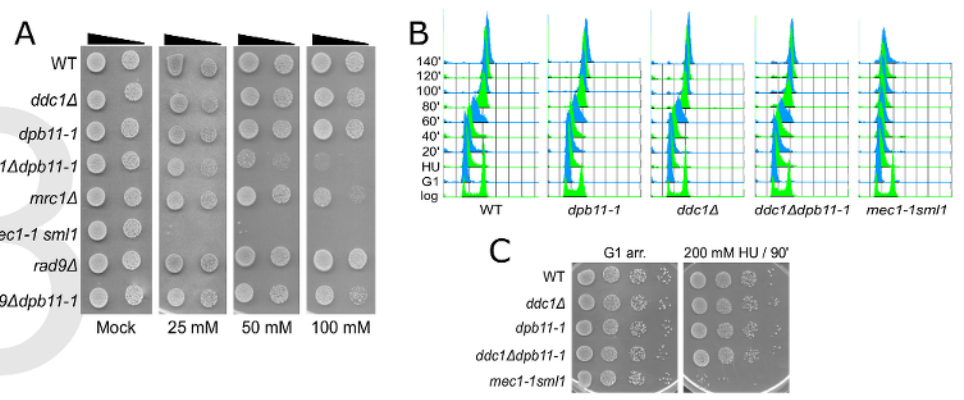
Following DNA damage or replication stress, yeast cells phosphorylate and activate the Rad53 (hChk2) checkpoint protein, which is responsible for maintaining genome stability in these challenging conditions. The activation mechanism of the DNA damage checkpoint and of the replication checkpoint is different, but partially overlapping because most of checkpoint factors are shared between these pathways. For the activation of the 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 also 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), an inhibitor of ribonucleotide reductase. 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 *ddc1Δdpb11-1* double mutant strain displays a Rad53 phosphorylation defect after HU treatment, similar to the one of a *mec1-1* mutant. Moreover, the double mutant also lacks phosphorylation of histone H2A. These observations suggest that Dpb11 and the PCNA-like complex act independently in promoting Mec1 activation. A similar phenotype can be observed in a *dpb4Δddc1Δ* strain carrying a deletion of a non-essential subunit of DNA polymerase ε, indicating that Dpb11 may be working together with Polε in this function.



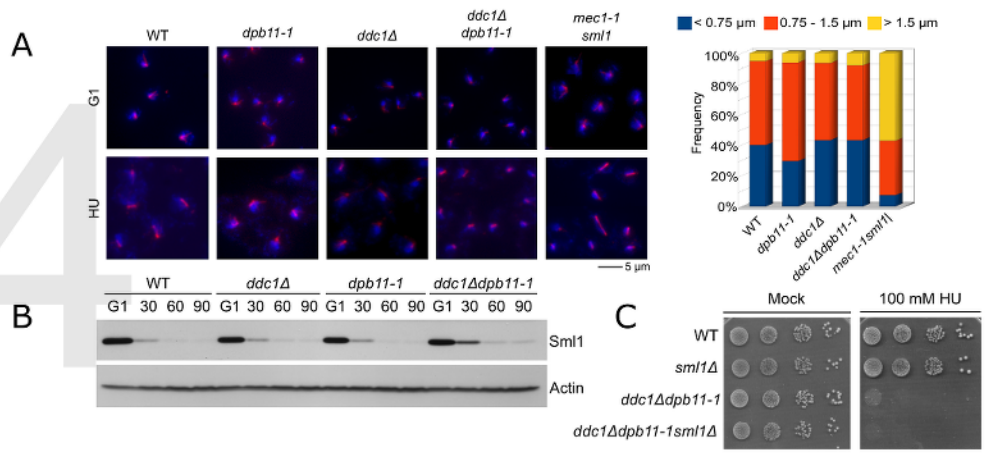
Ddc1 and Dpb11 independently promote Mec1 activation after replication stress
(A) Dpb11 contains an ATR activation domain in the C-terminus which is lacking in *dpb11-1* cells. The indicated yeast strains were synchronized in G1 and released in 200 mM HU. At different time points H2A **(B)**, Rad53 **(C)** and Rad9 **(D)** phosphorylation was analysed. The mutantions *ddc1Δ* and *dpb11-1* display a synthetic defect in H2A and Rad53 phosphorylation. Ddc1 and Dpb11 both play their role in the context of a physiological replication stress response as suggested by the absence of DNA damage checkpoint activation that was observed as the absence of Rad9 hyperphosphorylation.



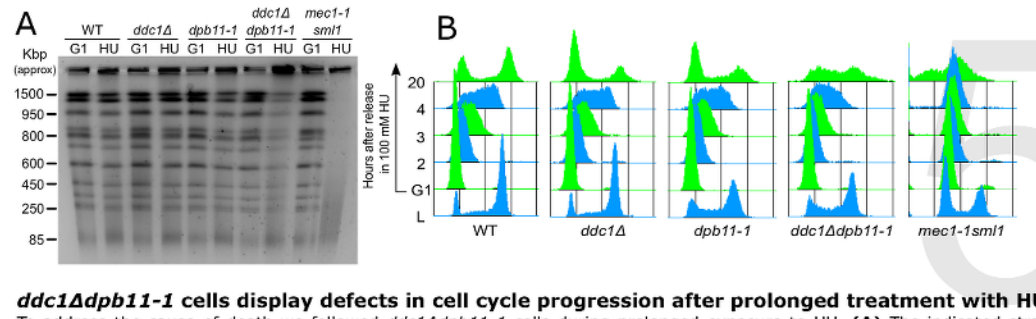
Ddc1-dependent and Dpb11-dependent Rad53 activation does not require Tel1
The indicated yeast strains were synchronized in G1 and released in fresh medium with 200 mM HU. At the indicated time-points after release Rad53 was analysed by western blot. Although delayed, *ddc1Δtel1Δ* and *dpb11-1tel1Δ* strains still show high levels of Rad53 phosphorylation, comparable to the one observed in the single mutants *ddc1Δ* and *dpb11-1* respectively. This result suggests that Tel1 is not required for the Ddc1-dependent or Dpb11-dependent rad53 activation.



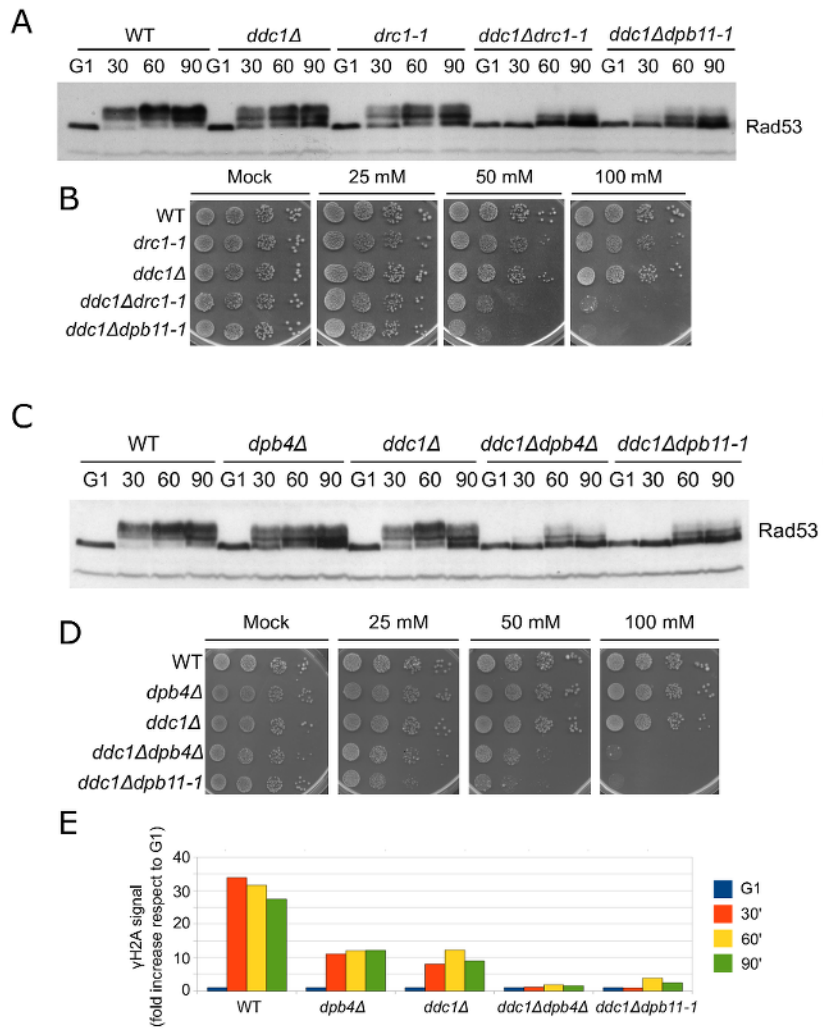
Inability to fully activate Mec1 does not impairs recovery from replication stress
The strains in figure were tested for sensitivity to replication inhibition and ability to recover from S-phase arrest **(A)** Serial dilutions of yeast cultures were spotted on YPD+HU plates. Differently form *rad9Δdpb11-1*, *ddc1Δdpb11-1* is extremely sensitive to HU, further confirming that in *dpb11-1* cells the role of Ddc1 during replication stress response is not the activation of the DNA damage checkpoint. **(B)** Cells were synchronized in G1, released in 200 mM HU for 90 minutes and finally released in YPD+nocodazole to allow completion of DNA replication. DNA content was measured by FACS analysis. Surprisingly *ddc1Δdpb11-1* cells are able to restart stalled replication forks and complete DNA replication. **(C)** Consistently double mutant cells are not sensitive if treated for 90 minutes with HU.



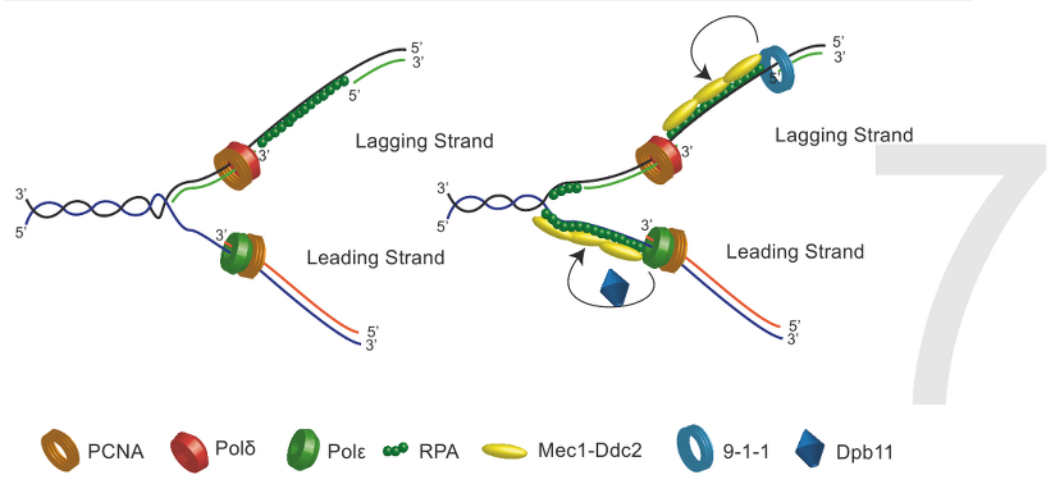
Cells incapable of full Mec1 activation are proficient in delaying mitosis and degrading Sml1
The indicated yeast strains were analysed for spindle elongation and Sml1 degradation in the presence of HU. **(A)** 90 minutes after G1 release in 200 mM HU, microtubules were visualized by indirect immunofluorescence (blue: DNA; red: tubulin). A quantification is shown in the right graph. In these conditions *ddc1Δdpb11-1* is able to prevent spindle elongation, like the WT, whereas the mutant *mec1-1* is not. These data suggest that the double mutant is proficient in replication checkpoint activation, preventing the entry into mitosis. **(B)** The same strains were tested for Sml1 degradation following a G1 release in 200 mM HU. Although delayed the double mutant is able to degrade Sml1. **(C)** Consistently deletion of *SML1* does not rescue the sensitivity of *ddc1Δdpb11-1*.



***ddc1Δdpb11-1* cells display defects in cell cycle progression after prolonged treatment with HU**
To address the cause of death we followed *ddc1Δdpb11-1* cells during prolonged exposure to HU. **(A)** The indicated strains were synchronized in G1 and released in YPD + 100 mM HU. Progression into the cell cycle was followed by FACS analysis. Double mutant cells display a slower S-phase and 20 hours after the release, double mutant cells fail to recover the FACS profile of an exponential culture. **(B)** PFGE analysis of yeast chromosomes extracted from cells exposes to 100 mM HU for 20 hours after G1 release. Differently from WT and single mutant cells, *ddc1Δdpb11-1* chromosomes are mostly retained in the well, suggesting the presence of uncompleted replicative structures. Differently from *mec1-1* cells, double mutant cells do not accumulate DNA fragmentation.



Sld2 and Polε subunit Dpb4 are required for Ddc1-independent Rad53 phosphorylation.
Dpb4 and Sld2 role in Ddc1-independent replication stress signaling was assayed monitoring checkpoint activation and sensitivity of the double mutants *ddc1Δdpb4Δ* and *ddc1Δdrc1-1*. **(A)** The indicated strains were synchronized in G1 and released in 200 mM HU. The mutant *drc1-1* is only mildly defective in Rad53 phosphorylation. Differently the double mutant *ddc1Δdrc1-1* has the same Rad53 phosphorylation defect of *ddc1Δdpb11-1*. **(B)** Consistently *drc1-1* and *ddc1Δ* mutations display a synergistic sensitivity to HU. **(C)** The indicated strains were treated as described before. The mutant *dpb4Δ* is partially defective in Rad53 phosphorylation, whereas *ddc1Δdpb4Δ* levels of Rad53 phosphorylation are comparable to the one of *ddc1Δdpb11-1*. **(D)** Moreover these two strains display a similar sensitivity to HU. **(E)** Also H2A phosphorylation is defective in *ddc1Δdpb4Δ*. Indeed almost no increase in the levels of γ-H2A could be observed when this strain was exposed to HU, suggesting that Ddc1 and Dpb4 are modulating Mec1 activity.



A possible model for Mec1 activation after replication stress.
To explain the redundant activity of Ddc1 and Dpb11-Sld2-Polε in activating Mec1 kinase we elaborated a model in which two distinct populations of Mec1 could be activated independently by the 9-1-1 complex or by Dpb11.
The population of Mec1 that is recruited on the lagging strand is likely activated by the 9-1-1 complex, that has a near 5' DNA end suitabe for its loading. Differently, on the leading strand, in the absence of repriming events, there is no 5' end that could be used for the loading: in this situation Mec1 could be activated by Dpb11, probably recruited through an interaction with DNA polymerase ε, which is the leading strand DNA polymerase.