Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint

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Following genotoxic insults, eukaryotic cells trigger a phosphorylation based signal transduction cascade known as DNA damage checkpoint response. Full checkpoint activation involves loading onto damaged DNA of an apical kinase and several downstream factors. Chromatin modifications contribute to the recruitment of checkpoint proteins. In budding yeast, methylated H3-K79 provides a binding site for the checkpoint factor Rad9, allowing signal transduction to the Rad53 effector kinase. Loss of Dot1, the specific histone methyltransferase, prevents H3-K79 methylation leading to a checkpoint defect in the G1 phase of the cell cycle and to a partial reduction of checkpoint activation in mitosis. This suggests that another pathway can contribute to Rad9 recruitment to damaged DNA in M phase. In order to identify this second pathway, we sought for mutants that abolished the checkpoint response to UV irradiation in mitosis, when combined with a *dot1Δ* mutation. We show that the replication factor Dpb11 is the keystone of this second pathway. A *dot1Δdpb11-1* double mutant cannot activate Rad53 when M cells are irradiated and is very sensitive to UV treatment. We suggest that Dpb11 is held in proximity of damaged DNA through an interaction with Mec1-phosphorylated 9-1-1 complex. Indeed, the physical interaction between Ddc1 and Dpb11 requires a functional Mec1 kinase, and a *ddc1-T602A* non phosphorylable mutant mimics a *dpb11-1* mutation when combined with a *DOT1* deletion. Moreover, this point mutation in Ddc1 prevents DNA damage-induced phosphorylation of Dpb11.

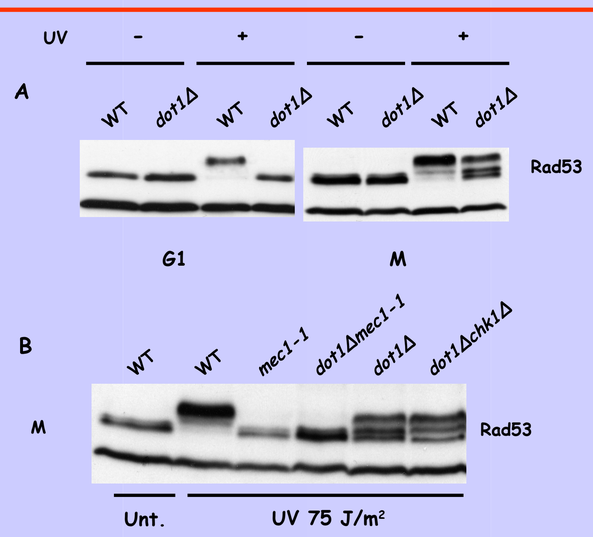

Figure 1. G2/M residual Rad53 phosphorylation in the absence of the histone pathway for Rad9 recruitment depends upon Mec1. (A) WT and *dot1Δ* cells were arrested either in α-factor (G1) or nocodazole (M) and UV irradiated. 30 minutes later checkpoint activation was monitored looking at Rad53 phosphorylation. *dot1Δ* strain, which lacks H3-K79 methylation, is defective in G1 checkpoint, but retains the ability to activate G2/M checkpoint. (B) The indicated strains were arrested in nocodazole and UV irradiated. Rad53 phosphorylation was analyzed 30 minutes after treatment. In the absence of H3-K79 methylation, checkpoint activation depends completely upon Mec1.

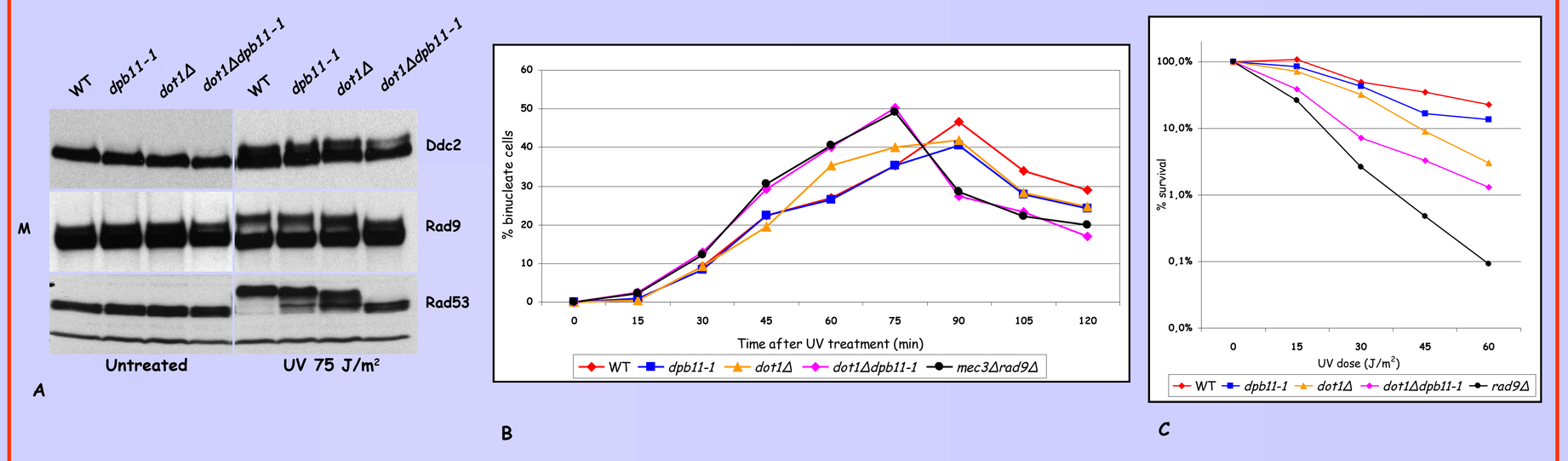

Figure 2. UV induced G2/M checkpoint activation in the absence of *DOT1* depends upon Dpb11.
(A) WT, *dpb11-1*, *dot1Δ*, *dot1Δdpb11-1* strains, carrying Ddc2 and Rad9 tagged proteins were UV irradiated. 30 minutes after treatment, TCA protein extracts were prepared and phosphorylation of checkpoint substrates was monitored by SDS-PAGE and western blotting. The double mutant *dot1Δdpb11-1* does not phosphorylate Rad53 and Rad9, while Ddc2 (Mec1-activity marker) appear to be normally phosphorylated. (B) G2/M UV checkpoint assay. The indicated strains were synchronized with nocodazole, UV irradiated with 40 J/m2 and released into the cell cycle. Kinetics of nuclear division was monitored counting the number of binucleated cells at the indicated time points. WT and single mutant strains delay the anaphase entry. Instead *dot1Δdpb11-1* displays the same kinetics of a checkpoint defective strain (*mec3Δrad9Δ*). (C) UV survival assay. The indicated strains were grown to stationary phase, then diluted and plated. Plates were irradiated with different UV dosages and survival was assayed counting the number of colonies formed after 3 days of growth. *dot1Δ* and *dpb11-1* exhibit synergistic effects on sensitivity to UV but the double mutant is not as sensitive as a *rad9Δ* strain.

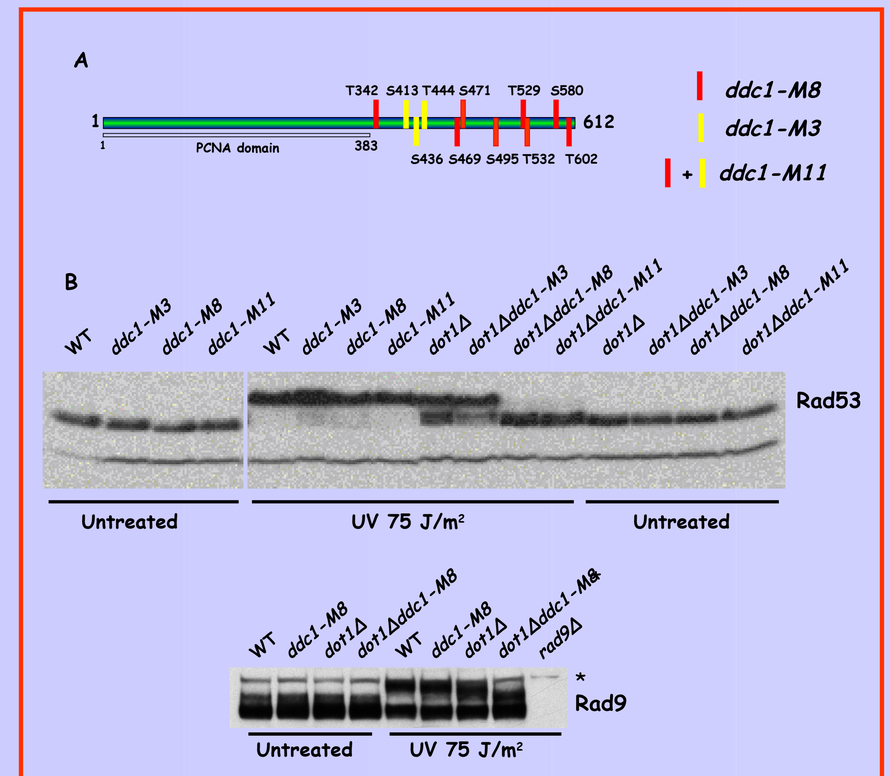

Figure 3. Dot1 and phosphorylation of Ddc1 are required for the establishment of an effective UV response. (A) Outline of the Cdc28 (yellow) and Mec1 (red) putative phosphorylation target sites in Ddc1. Cdc28 and Mec1 target sites were mutated to alanine in *ddc1-M3* and *ddc1-M8* strains respectively. *ddc1-M11* contains the combination of all these mutations. (B) The indicated strains were arrested in nocodazole and either UV irradiated or mock treated. Rad9 and Rad53 phosphorylation was analyzed 30 minutes after irradiation. Protein extract from a *rad9Δ* strain was loaded on the same gel in order to identify the α-Rad9 cross reacting band, indicated with an asterisk. Mec1 target sites in Ddc1 are required for the *DOT1*-independent pathway for Rad9 and Rad53 phosphorylation.

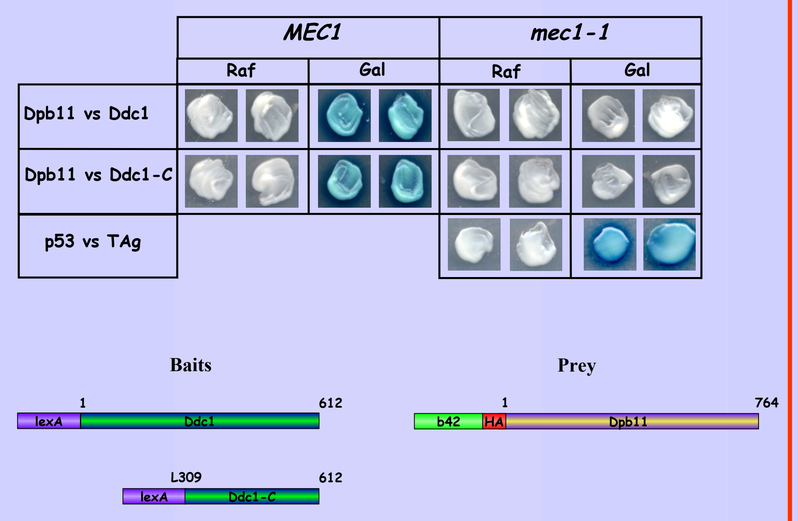

Figure 4. The interaction between Dpb11 and Ddc1 requires a functional Mec1 kinase. Plasmids pFP1 (pJG4-5-*DPB11*) and pFP2 (pEG202-*DDC1*) were co-transformed with pSH18-34, a β-gal reporter plasmid, either in *MEC1* or *mec1-1* yeast cells. A similar strategy was adopted for pFP4 (pEG202-*ddc1-C*) which carries only the C-terminal fragment (309-612) of *DDC1*, containing the eleven putative Cdc28/Mec1 phosphorylation target sites. To assess two-hybrid interaction these strains were patched on X-gal plates containing either raffinose (Dpb11 prey repressed) or galactose (Dpb11 prey expressed) as carbon source. After 2 days the plates were analyzed. The interaction between p53 and TAg was tested in the *mec1-1* strain as positive control.

Figure 5. Ddc1 T602 is required for Rad53 and Rad9 phosphorylation in the absence of *DOT1* and belongs to the same pathway of *DPB11*.
(A) The indicated strains were arrested in nocodazole and subjected to UV irradiation or mock treated. Rad53 and Rad9 phosphorylation was analyzed 30 minutes after UV treatment. Protein extract from *rad9Δ* was loaded on the same gel in order to identify the α-Rad9 cross reacting band, indicated with an asterisk. Ddc1 threonine 602 is the only residue required for *DOT1*-independent checkpoint activation. (B) WT, *ddc1-T602A*, *dpb11-1*, *ddc1-T602A dpb11-1* were arrested in nocodazole and UV irradiated; the level of Rad53 phosphorylation was assayed 30 minutes after the treatment. Ddc1 threonine 602 and Dpb11 work on the same pathway leading to Rad53 phosphorylation.

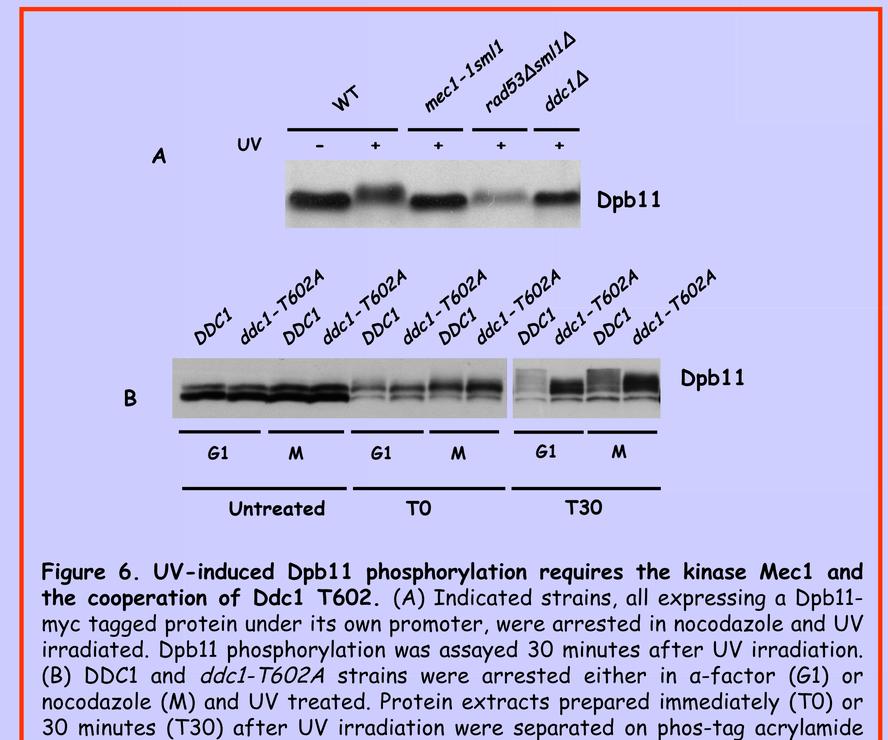

Figure 6. UV-induced Dpb11 phosphorylation requires the kinase Mec1 and the cooperation of Ddc1 T602. (A) Indicated strains, all expressing a Dpb11-myc tagged protein under its own promoter, were arrested in nocodazole and UV irradiated. Dpb11 phosphorylation was assayed 30 minutes after UV irradiation. (B) DDC1 and *ddc1-T602A* strains were arrested either in α-factor (G1) or nocodazole (M) and UV treated. Protein extracts prepared immediately (T0) or 30 minutes (T30) after UV irradiation were separated on phos-tag acrylamide gels, to increase separation of the phosphorylated forms. The two images represent different exposure times. The Ddc1 point mutation greatly reduces Dpb11 phosphorylation.

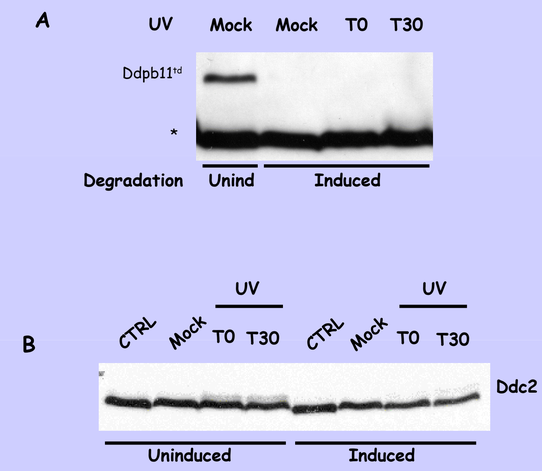

Figure 7. Dpb11 is required for rapid and full hyperphosphorylation of Ddc2. (A) Protein extracts from *dpb11td* strain, in which a degron cassette is fused at the N-terminus of Dpb11 were prepared in different condition to assess the presence of the Dbp11-degron protein; cells were coltured at 28°C in YP+raffinose (Mock Unind.) then shifted to the degradation medium at 37°C (Mock Induced), UV irradiated and shifted back to 28°C (T0: sample taken immediately; T30: 30 minutes later). Presence of Dpb11-degron was assessed using α-HA antibodies. (B) The experiment was repeated in the same conditions in parallel with a strain carryng also a WT copy of *DPB11* under the control of its own promoter. Ddc2 hyperphosphorylation was monitored by SDS-PAGE and Western Blot. When Dpb11 is not present in the cell, the hyperphosphorylation of Ddc2 is almost completely lost.

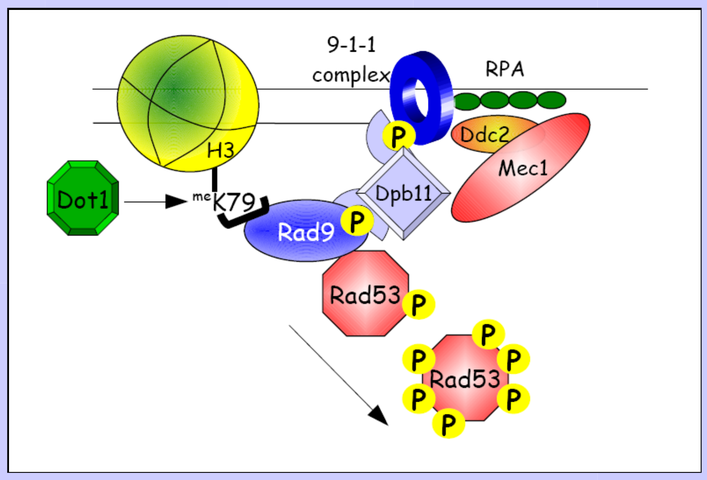

Figure 8. Possible model for Dpb11 function in UV-induced DNA damage checkpoint. Dpb11 is recruited to sites of DNA damage through the interaction of its C-terminal BRCTs with the 9-1-1 subunit Ddc1, phosphorylated by Mec1 on T602. Once recruited it plays a double role in checkpoint activation: firstly it enhances Mec1 kinase activity and secondly, in G2/M, it participates with H3-K79 in Rad9 recruitment, likely through an interaction of its N-terminal BRCTs with a Cdc28 phosphorylated residue on Rad9. Full Mec1 activity and tight Rad9 recruitment allows a rapid and full phosphorylation of Rad53 which correlates with checkpoint activation.