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

Fabio Puddu, Magda Granata, Lisa Di Nola, Alessia Balestrini, Gabriele Piergiovanni, Federico Lazzaro, Michele Giannattasio, Paolo Plevani & Marco Muzi-Falconi

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli studi di Milano, Via Celoria 26, 20133 Milano, Italia.

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 checkpoint factor Ddc1 is the keystone of this second pathway. A ddc1-T602A double mutant cannot activate Rad9 when M cells are irradiated and is very sensitive to UV treatment. We suggest that Ddc1 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-phosphorylatable mutant mimics a ddc1Δ deletion. Moreover, this point mutation in Ddc1 prevents DNA damage-induced phosphorylation of Dpb11.

Figure 1. 62% M residual Rad53 phosphorylation in the absence of the histone pathway for Dsb1 recruitment depends upon Mec1. (A) WT and dot1Δ cells were arrested either in a-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 Rad53 in M phase. (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 62% M checkpoint activation in the absence of DOT1 depends upon Dpb11. (A) WT, ddc1-M11, dot1Δ, ddc1-M8, ddc1-C and Cdc2 were UV irradiated and shifted to 30°C (T0: sample taken immediately after UV dose) and incubated for 30 minutes after treatment. TCA protein extracts were prepared and probed with a phospho-specific antibody against Rad53. Untreated control in panel A shows the level of Rad53 phosphorylation in G1. (B) UV Cdc2 check point assay. The indicated strains were synchronized with nocodazole, UV irradiated (Mock Unind.) then shifted to 37°C (T0: sample taken immediately after UV dose) and single mutant strains delay theaphase entry. Untreated strain displays the same kinetics of a checkpoint defective strain (mec1-1 sml1Δ). (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 ddc1-M11 exhibit synthetic genetic lethality 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) functional domains in Ddc1. Cdc28 and Mec1 interact directly in M phase. (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 reaction band, indicated with an asterisk. Rad9 target sites in Ddc1 are required for the DOT1-independent pathway for Rad9 and Rad53 phosphorylation.

Figure 4. The interaction between Ddc1 and Ddc1 requires a functional Mec1 kinase. Plasmids pPyF6 (pJG9-S-DBP1) and pPY2 (pEG202-DDC1) were co-transformed into the indicated strains with Ddc1-C tagged proteins. The vector alone (Δ) was transformed into a mec1Δ strain. Rad53 phosphorylation in Ddc1-C was monitored by Western blotting. Untreated control in panel A shows the level of Rad53 phosphorylation in G1. (B) Rad53 phosphorylation was analyzed 30 minutes after treatment. The indicated strains were arrested in nocodazole and UV irradiated; the level of Rad53 phosphorylation in Ddc1-C was monitored by Western blotting. Untreated control in panel A shows the level of Rad53 phosphorylation in G1. (C) Rad53 phosphorylation was analyzed 30 minutes after treatment. The indicated strains were arrested in nocodazole and UV irradiated; the level of Rad53 phosphorylation in Ddc1-C was monitored by Western blotting. Untreated control in panel A shows the level of Rad53 phosphorylation in G1.

Figure 5. Ddc1 T602 is required for Rad53 and Rad9 phosphorylation in the absence of DOT1 and between the same pathway of DRE1. (A) UV induced Dcc1 phosphorylation requires the kinase Mec1 and the cooperation of Ddc1 T602. (B) The indicated strains, all expressing a Dpb11-α-hemaglutinin tagged protein under its own promoter, were arrested in nocodazole and UV irradiated. Phosphorylated Dcc1 was detected in Ddc1α-hemaglutinin tagged cells cultured at 28°C or in nocodazole in the absence of UV irradiation. Ddc1α-hemaglutinin tagged cells were arrested in nocodazole and UV irradiated and probed with an α-hemaglutinin antibody. (C) UV induced Dcc1 phosphorylation requires the kinase Mec1 and the cooperation of Ddc1 T602. (D) The indicated strains were arrested in nocodazole and UV irradiated. Phosphorylated Dcc1 was detected in Ddc1α-hemaglutinin tagged cells cultured at 28°C or in nocodazole in the absence of UV irradiation. Ddc1α-hemaglutinin tagged cells were arrested in nocodazole and UV irradiated and probed with an α-hemaglutinin antibody. (E) UV induced Dcc1 phosphorylation requires the kinase Mec1 and the cooperation of Ddc1 T602. (F) The indicated strains were arrested in nocodazole and UV irradiated. Phosphorylated Dcc1 was detected in Ddc1α-hemaglutinin tagged cells cultured at 28°C or in nocodazole in the absence of UV irradiation. Ddc1α-hemaglutinin tagged cells were arrested in nocodazole and UV irradiated and probed with an α-hemaglutinin antibody.

Figure 6. UV-induced Dcc1 phosphorylation requires the kinase Mec1 and is blocked by a ΔDbp11 strain. The indicated strains, all expressing a Dpb11-α-hemaglutinin tagged protein under its own promoter, were arrested in nocodazole and UV irradiated. Phosphorylated Dcc1 was detected in Ddc1α-hemaglutinin tagged cells cultured at 28°C or in nocodazole in the absence of UV irradiation. Ddc1α-hemaglutinin tagged cells were arrested in nocodazole and UV irradiated and probed with an α-hemaglutinin antibody.

Figure 7. Dcc1 is required for rapid and full hyperphosphorylation of Ddc2. (A) Protein extracts from ddc1-M8 strain, in which a degradation tag is fused to the N-terminus of Dcc1, were prepared in different conditions to examine the effect of the ddc1-M8 allele on the hyperphosphorylation of Ddc2. Protein extracts were prepared immediately (T0) or 30 minutes (T30) after UV irradiation and fractionated on a TCA-precipitated gel. α-Acetyl phosphate was used as an antibody. (B) Protein extracts from ddc1-M8 strain were prepared immediately (T0) or 30 minutes (T30) after UV irradiation. α-Acetyl phosphate was used as an antibody. (C) Protein extracts from ddc1-M8 strain were prepared immediately (T0) or 30 minutes (T30) after UV irradiation. α-Acetyl phosphate was used as an antibody.