Correlation between checkpoint activation and *in vivo* assembly of the yeast checkpoint complex Rad17-Mec3-Ddc1.

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ABSTRACT

Rad17-Mec3-Ddc1 forms a PCNA-like complex that is required for the DNA damage response in *Saccharomyces cerevisiae* and acts at an early step of the signal transduction cascade activated by DNA lesions. We used the *mec3-dn* allele, that causes a dominant negative checkpoint defect in G1 but not in G2, to test the stability of the complex *in vivo* and to correlate its assembly and disassembly with the mechanisms controlling checkpoint activation. Under physiological conditions, the mutant complex is formed both in G1 and G2 although the mutant phenotype is detectable only in G1, suggesting that is not the presence of the mutant complex *per se* to cause a checkpoint defect. Our data indicate that the Rad17-Mec3-Ddc1 complex is very stable and it takes several hours to replace Mec3 with Mec3-dn within a wild type complex. On the other hand, the mutant complex is rapidly assembled when starting from a condition where the complex is not pre-assembled, indicating that the critical factor for the substitution is the disassembly step rather than complex formation. Moreover, the kinetics of mutant complex assembly, starting from conditions in which the wild type form is present, parallels the kinetics of checkpoint inactivation, suggesting that the complex acts in a stoichiometric way, rather than catalytically.

INTRODUCTION

The DNA damage checkpoint represents a set of genetically controlled surveillance mechanisms required to control cell cycle progression in response to genotoxic stress and to preserve genome integrity (reviewed in Refs. 1-6). Many of the key players of the DNA damage checkpoint were identified by genetic studies in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and they have functional and structural counterparts in human cells, indicating that this response has been highly conserved during evolution (4,7). In the last ten years, a number of studies have provided a working model on the organization of the DNA damage checkpoint pathway, although very little is known on the biochemical properties of critical checkpoint proteins. The checkpoint response is generally considered as a signal transduction cascade: DNA damage is sensed by specific factors thus generating a signal that, through the action of specific protein kinases, is transmitted to effectors whose final action is going to impinge on the cell cycle machinery.

In *S. cerevisiae*, the most recent view of the initial stage of the checkpoint response indicates that two protein complexes, Mec1-Ddc2 and Rad17-Mec3-Ddc1, are recruited near a DNA lesion (1). The recruitment of the Mec1-Ddc2 complex is independent on any other checkpoint factor, while loading of the Rad17-complex requires the function of the Rad24-complex (8,9). Mec1 belongs to the PI-3 protein kinase family, while Ddc2 (also called Lcd1 and

Pie1) appears to mediate DNA binding (10,11); their human homologues are called, respectively, ATR and ATRIP (12). Rad17, Mec3 and Ddc1 (the human homologues are, respectively, hRad1, hHus1 and hRad9) form a heterotrimeric complex structurally related to PCNA (13-15). Rad24 (the human homologue is hRad17) shares structural and functional similarity with the large subunit (called Rfc1) of the RFC complex and can be purified associated with the Rfc2-5 subunits (16). In a way that is still not understood, binding of these checkpoint factors to damaged DNA leads to activation of the Mec1 kinase that will phosphorylate a number of targets, including Ddc2, Ddc1, Rad9 and Rad53. Likely through the interaction with some of the checkpoint proteins listed above, Rad9 acts as a scaffold protein leading to the recruitment of the dual specificity Rad53 protein kinase, the yeast counterpart of human Chk2 (17,18). It has been suggested that Rad9 works as a solid state catalyst by increasing the local concentration of Rad53, thus leading to Rad53 autophosphorylation (17). Full Rad53 modification causes a change in the electrophoretic mobility of the protein that is often used as a biochemical marker of checkpoint activation (19). The targets of Rad53 and the molecular details leading to a temporary arrest of the G1/S and G2/M transitions or to the slowing down of DNA synthesis are still poorly defined. Checkpoint activation seems to modulate Cdk1 activity (19), and it has been suggested that Rad53 inactivates the Swi4-Swi6 complex (20). The best candidate to act as an effector controlling the G2/M transition is the anaphase inhibitor Pds1, since *pds1* mutants fail to arrest in G2 in response to DNA damaging agents and

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Pds1 is phosphorylated and regulated in a Mec1-dependent manner (21).

Finally, the DNA replication machinery itself, or factors controlling the

stability of replication forks, are the likely targets of the intra-S checkpoint

response, since the physical structure of replication intermediates is strongly

affected in checkpoint mutants (22,23).

We have recently identified a mutant version of *MEC3* that inactivates the

DNA damage checkpoint in G1 and partially compromises the intra-S

response, while leaving the G2/M checkpoint intact (24). The mutation

responsible for this peculiar phenotype (*mec3-dn*) uncovers differences in the

requirement for checkpoint activation in various phases of the cell cycle and is

caused by a modification of the N-terminus of Mec3. Mec3-dn is recognizable

through a modification of its electrophoretic mobility and, in the present

manuscript, Mec3-dn has been used to test *in vivo* the kinetics of formation of

the Rad17-complex and to gain some insight on the link between its assembly

and checkpoint activation. Surprisingly, the mutant complex is physically

present both in G1 and in G2, although the mutant phenotype is observed only

in G1, indicating that the presence of a mutant complex *per se* is not sufficient

to cause a checkpoint defect. Substitution of wild-type Mec3 with Mec3-dn in

the Rad17 heterotrimeric complex is very slow, while formation of the mutant

complex proceeds much faster if the subunits are initially disassembled,

indicating that the Rad17-complex is stable *in vivo*.

EXPERIMENTAL PROCEDURES

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Strains and Plasmids. Strain K699 (Mat*a ade2-1 trp1-1 leu2-3,112 his3,11,15 ura3 can1-100*) was an original gift from K. Nasmyth. The *MEC3* wild type gene was replaced with the *mec3-dn* mutant allele to generate YLL352 (26). YLL334, a K699 derivative carrying a HA-tagged chromosomal copy of *DDC1* and its Δ*mec3* derivative, YLL335, were generous gifts of M.P. Longhese. Strain YSH4 was derived from K699, by tagging the chromosomal copy of the *RAD17* gene with a 6His-3HA epitope. The vector plasmid pNB187 and its derivatives pNB187-Mec3 and pNB187-Mec3-dn, where the expression of *MEC3* and *mec3-dn* is driven by the *GAL1* promoter have been previously described (24). Standard yeast genetic techniques and media have been already described (26)

Cell cycle arrest and treatments with genotoxic agents. Logarithmically growing cells were blocked in G1 with 20 μg/ml α-factor, in S phase with 0.2 M hydroxyurea (HU) and in G2 with 20 μg/ml nocodazole. Cell cycle arrests were verified by estimating the percentage of budded/unbudded cells and by FACS analysis. When indicated yeast cultures were treated with 1μg/ml 4-Nitroquinoline oxide (4NQO) or with 200 μg/ml zeocine for 15 min. or with 60 J/m^2 UV-light as described (25). Cell cycle synchronization and checkpoint function analysis were performed as previously described (25,26).

Protein extracts, immunoprecipitation analysis and *in situ* kinase assay. Native extracts were obtained as described (25). For phosphorylation analysis,

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cell extracts were prepared by Trichloro Acetic Acid (TCA) treatment and

analyzed as previously described (19). All blots were stained with Sypro Ruby

(Molecular Probes) and analyzed in a Typhoon (Amersham Pharmacia) to

verify similar loading in all wells. Assay of Rad53 kinase activity after SDS-

PAGE electrophoresis and *in situ* renaturation was carried out as described

(19). Native extracts were immunoprecipitated following published procedures

(25).

RESULTS

Mec3-dn causes a checkpoint defect only in G1, but substitutes wild type

Mec3 in the Rad17-complex both in G1 and in G2. During the construction

of a two-hybrid bait plasmid for Mec3, we found that expression under the

ADH1 promoter of a *lexA-9MYC-MEC3* gene was causing a dominant negative

checkpoint defective phenotype in G1, but not in G2 (24). Characterization of

that peculiar mutant allele has been useful: i) to strengthen the order of

function of checkpoint proteins in the signal transduction cascade activated by

DNA damage, ii) to reveal differences in the requirements for checkpoint

activation in various phases of the cell cycle, iii) to uncover a role for Tel1 in

Rad53 phosphorylation in G2 (24). The availability of a mutant form of Mec3

that is distinguishable from the wild type protein on Western blots, allowed us

to test the kinetics of formation of the heterotrimeric Rad17-Mec3-Ddc1

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complex *in vivo* and to gain some insight on the correlation between complex

formation and checkpoint activation.

The specific G1 checkpoint defect caused by overexpression of the *lexA-9MYC-MEC3* fusion gene is recapitulated by replacement of the wild type copy

of the *MEC3* gene with a *9MYC-MEC3* fusion (called *mec3-dn*). As shown in

Figure 1A, checkpoint activation, measured by monitoring the level of Rad53

phosphorylation, is completely abolished in the *mec3-dn* strain in response to

4NQO treatment in G1-blocked cells. Conversely, the checkpoint is still

activated in *mec3-dn* G2-arrested cells as indicated by partial phosphoryaltion

of Rad53 and by proper G2/M arrest (data not shown). This finding indicates

that expression at physiological level of a NH$_2$-tagged version of Mec3 is

sufficient to cause a specific G1 checkpoint defect. The inability to

phosphorylate Rad53 in the G1 phase can be observed in response to different

DNA damaging agents (Figure 1B), and the defect found in the *mec3-dn* strain

is undistinguishable from that caused by overexpression of the *mec3-dn* allele

in a wild type genetic background (data not shown).

Overexpressed *mec3-dn* might titrate away from the Rad17-complex either

subunits of the complex itself or other partners. However, we consider this

possibility quite unlikely since the same phenotype is observed in the *mec3-dn*

strain expressing the mutant protein at physiological levels. Moreover,

overproduction of wild type *MEC3* does not cause any defect in Rad53

phosphorylation in response to DNA damage (data not shown). Therefore, it

was possible that Mec3-dn could substitute wild type Mec3 in the Rad17-

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complex in G1 but not in G2, thus explaining the specific cell cycle phase defect in checkpoint activation observed in the mutant strain.

To test this hypothesis we expressed *mec3-dn* under the control of an inducible *GAL1* promoter in a wild type background carrying a HA-tagged version of Rad17 and we performed co-immunoprecipitation experiments to monitor the structure of the heterotrimeric complex. As shown in Figure 2A, wild type Mec3 was present in the complex when cells were transformed with an empty vector, while it was the mutant protein that co-immunoprecipitated with Rad17 if *mec3-dn* was overexpressed. This finding demonstrates that overexpression of *mec3-dn* disrupts the interaction between Rad17 and wild type Mec3 and sequesters Rad17. On the other hand, when we looked at Ddc1 in similar experiments, it became clear that expression of *mec3-dn* did not affect the Rad17-Ddc1 association (Figure 2B). Since this interaction is known to require the presence of Mec3 (see Fig. 5A), these results suggest that Mec3-dn likely replaces the wild type protein in the Rad17-complex. This assumption was confirmed by showing that both Rad17 and Ddc1 are co-immunoprecipitated with the mutant form of Mec3 (Figure 2C). Altogether, these results may offer an explanation for the defective checkpoint activation in G1 cells expressing *mec3-dn*: in fact, the net result is the substitution of a wild type Rad17-complex with a mutant complex, that is likely deficient in some function required for activation of the checkpoint pathway. However, when we repeated the same experiments in G2-arrested cells where the expression of *mec3-dn* does not cause any evident checkpoint defect, we found that also at this cell cycle stage

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Mec3-dn substitutes the wild type protein giving rise to a mutant Rad17-complex (Figure 2A). Control experiments on exponentially growing cells and in cells blocked in G1, S and G2 confirmed that the mutant complex is present at all cell cycle stages and that Mec3-dn is not modified to any detectable extent by genotoxic treatment (Figure 2D). In conclusion, these findings suggest that the formation of a mutant complex *per se* is not sufficient to cause a Rad53 phosphorylation defect, but rather underlines different requirements for checkpoint activation in G1 and in G2.

Expression of *mec3-dn* for several hours is required to inactivate the DNA damage checkpoint in a wild type genetic background.

To gain some insight on the kinetics of Rad17-complex formation and to try to correlate it with checkpoint activation, we took advantage of the conditional GAL1-*mec3-dn* mutant. After an overnight induction with galactose, cells were arrested in G1 and Rad53 phosphorylation was analyzed after treatment with 4NQO. As shown in Figure 3A (lanes 7-9), if Mec3-dn is produced in galactose-containing medium for 18 hours before α-factor arrest and 4-NQO treatment, we observed a complete absence of Rad53 activation. On the contrary, in G1-arrested cells that were kept in α-factor and induced for only 3 hours with galactose, we found that Rad53 was normally phosphorylated (lanes 4-6), even though *mec3-dn* was expressed at a level comparable to that found after 18 hours of induction. This result might be explained by assuming that the kinetics of substitution of the wild type form with Mec3-dn is slow, or it may be related to the fact that, in the latter experiment, the mutant protein is

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produced only in the G1 phase of the cell cycle. If assembly of the Rad17-complex is occurring in a cell cycle phase other than G1, production of Mec3-dn exclusively in G1 might not cause any detectable checkpoint defect.

We addressed this possibility by producing Mec3-dn for the same amount of time in G1-arrested and in cycling cells. As outlined at the top of Figure 3B, exponentially growing cells were synchronized by α-factor treatment under galactose-inducing conditions. Half of the culture was then kept blocked in G1, while the remaining of the cells were allowed to go through a complete cell cycle before arresting them again in G1 followed by 4NQO treatment. At the indicated times (a to f), samples were taken to monitor the stage of the cell cycle by FACS analysis and to test checkpoint activation by assaying for Rad53 phosphorylation. As shown in Figure 3B (compare Rad53 phosphorylation in lanes c and f of the Mec3-dn samples), Rad53 is normally phosphorylated in cells that produced Mec3-dn only in G1-arrested cells or in cells that have been going through a complete cell cycle in the presence of the mutated form. Moreover, the level of Mec3-dn under the two experimental conditions is essentially identical. In conclusion, it is necessary to express *mec3-dn* for quite a long time (more than 6 hours) in the presence of wild type Mec3 before observing any G1 checkpoint defect, and passage through the cell cycle is not critical for checkpoint activation.

We then directly tested the substitution kinetics of wild type Mec3 with Mec3-dn and compared it with checkpoint activity. *mec3-dn* was expressed under the control of the *GAL1* promoter in a yeast strain carrying an HA-tagged version

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of *RAD17*; the kinetics of Mec3-dn substitution in the Rad17-complex was measured in anti-HA immunoprecipitates at the indicated time intervals (Figure 4, top). Under the experimental conditions used for *GAL1*-induced expression of *mec3-dn* , it takes approximately 12 hours to fully substitute Mec3-dn in the wild type complex. The last 2 hours of galactose induction were performed after α-factor addition to synchronize the cells in G1 and test the effect of Mec3-dn substitution on checkpoint function by assaying *in situ* the Rad53 kinase activity (19). As shown in the bottom of Figure 4, the timing of checkpoint inactivation correlates quite well with the kinetics of substitution of wild type Mec3 with Mec3-dn within the complex.

Replacement of wild type Mec3 by Mec3-dn is limited by slow dissociation kinetics.

The results described so far indicate that in the presence of wild type Mec3 it takes several hours to substitute Mec3-dn within the Rad17-complex and to inactivate the checkpoint. This finding can be ascribed to a lower affinity of the mutant protein for the other subunits of the heterotrimeric complex or to slow dissociation kinetics or both.

MEC3 is not an essential gene, but a *mec3•* strain is sensitive to DNA damaging agents and is checkpoint deficient at all cell cycle stages (26). We took advantage of the fact that in a *mec3•* strain the Rad17-complex is fully disassembled (Figure 5A) to test whether the timing of assembly and checkpoint activation was different when expressing the wild type or the mutant form under the control of the *GAL1* promoter. To this end, *mec3•* cells

were arrested in G1 or in G2 and the *GAL1* promoter was induced for 2 hours. As it is shown in Figure 5B, after 2 hours of induction in galactose-containing medium, expression of wild type Mec3 is capable to activate Rad53 phosphorylation both in G1- and in G2-arrested cells. Conversely, also under these experimental conditions, we failed to detect any Rad53 phosphorylation in response to DNA damage when Mec3-dn was produced in G1, while Rad53 modification was clearly detectable when expression of *mec3-dn* was induced in G2-arrested cells. We then directly tested whether, under the experimental conditions described above, Mec3 and Mec3-dn were associated with the other complex subunits. As it is shown in Figure 5C, both in G1 and in G2, wild type Mec3 and Mec3-dn were assembled within the Rad17-complex after 2 hrs of galactose induction, and this association is not influenced by the 4NQO treatment (data not shown). We compared the affinities of the Mec3 and Mec3-dn proteins for the other subunits of the complex, by performing coimmunoprecipitation experiments at earlier time-points. Figure 5D shows that, as early as 20 minutes after galactose addition, both Mec3 and Mec3-dn are fully assembled in the heterotrimeric complex. These results indicate that, starting from a situation where the Rad17-complex is disassembled, the timing of assembly of wild type or mutant complex is similar, suggesting that Mec3-dn does not have a lower affinity for the other complex subunits compared to wild type Mec3. Since it takes a much longer time to substitute Mec3 with Mec3-dn starting from an assembled complex compared to the situation observed in *mec3•* cells where the complex is disassembled, the limiting factor

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for the kinetics of Mec3 substitution is likely to be the disassembly step of the Rad17-complex.

DISCUSSION

Genetic studies indicate that Rad17-Mec3-Ddc1 in *S.cerevisiae* and the homologous factors Rad1-Hus1-Rad9 in *S.pombe* and multicellular eukaryotes act at an early stage of the DNA damage checkpoint pathway (1,3,4,8,9). It is clearly established that these proteins interact with each other to form an heterotrimeric complex and they share amino acid sequence similarities to the replication factor PCNA (14,15). PCNA has a characteristic ring-like structure capable to encircle a double-stranded DNA molecule and functions as a sliding clamp to stimulate the processivity of replicative DNA polymerases in eukaryotic cells (27). Loading of PCNA onto DNA is an ATP-driven process that requires the action of the pentameric complex RFC (28). In silico predictions suggested that Rad17-Mec3-Ddc1 and its homologs in other eukaryotes form a PCNA-like structure: indeed a ring-like structure has been observed by transmission electron microscopy for the human PCNA-like complex (29,30). However, crystallographic analysis will be required to firmly establish the structure of the complex and its interaction with DNA. Interestingly, loading of the PCNA-like complex near a DNA lesion in *S. cerevisiae* requires the action of an RFC-like factor, where the large Rfc1 subunit is substituted by the *RAD24* gene product (8,9). Again, the structure of

the RFC-like complex has been visualized by E.M. analysis and resembles that of replicative RFC (29,30). Very recently the two yeast complexes have been purified and it has been shown that the RFC-like complex is required to load the PCNA-like factor onto DNA where it is acting as a sliding clamp (40). The same does not appear to be true for the human counterparts purified from baculovirus-infected cells, indicating that either the proteins purified from insect cells are not fully active or that the human proteins require additional factors for activity (31). Alternative PCNA- and RFC-like complexes seem to exist in eukaryotic cells (32), indicating that these complexes may play specialized roles in different DNA transactions or they may mediate a number of protein-protein interactions, since PCNA seems to act as a landing platform for a variety of proteins involved in DNA metabolism (33).

It is not known whether the different types of PCNA- and RFC-like complexes are always present as separated entities or they exist in a more dynamic interplay, perhaps competing for common or similar subunits. For instance, we and others have shown that in *S. cerevisiae* and in *S.pombe* an hierarchy of interactions exists among the three subunits of the PCNA-like complex which was not predicted by the PCNA paradigm where each monomer plays an equivalent role (25,34). Howeover, the same does not appear to be true for the human PCNA-like complex since *in vitro* reconstitution studies indicate that each protein has a binding site for the other two partners (35). We took advantage of the peculiar structure of Mec3-dn and of the specific phenotype caused by this mutation to ask some basic questions on the stability of the

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PCNA-like complex, and to correlate its assembly and disassembly with the mechanisms controlling checkpoint activation. In this manuscript we demonstrated that the *mec3-dn* allele is causing a checkpoint defective phenotype in G1 and not in G2 not only when it is overexpressed, but also when the mutant form is produced at physiological level under the control of its own promoter. However, a mutant complex is formed and present also in G2, indicating that is not the presence of the mutant complex *per se* that is causing a cell cycle specific checkpoint defect. Therefore, either the PCNA-like complex interacts with different partners in G1 and in G2, or the *mec3-dn* allele is a hypomorphic mutant unrevealing a different threshold for checkpoint activation at various cell cycle stages. The data presented here also indicate that the PCNA-like complex is very stable, since it takes several hours to substitute Mec3-dn within a wild-type complex. On the other hand, the mutant complex is fully assembled in less than two hours starting from a condition where the complex is not pre-assembled, indicating that the critical factor for Mec3-dn substitution is the disassembly step rather than its formation. The kinetics of mutant complex assembly starting from conditions in which the wild type form is present seems to parallel the kinetics of checkpoint inactivation, suggesting that the complex acts in a stoichiometric way, rather than catalytically. Genetic studies indicated that the PCNA-like complex could be involved in processing DNA lesions (36) and an exonuclease activity has been found associated with certain subunits of the complex from human cells (37,38). We failed to detect such an activity when the yeast PCNA-like

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complex was purified to homogeneity from baculovirus infected cells (M. Minuzzo et al, in preparation). Therefore, we favor the hypothesis that the major role of the PCNA-like complex is to recruit other proteins onto DNA, including various Mec1 substrates and/or factors involved in DNA repair and translesion DNA synthesis.

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FIGURE LEGENDS

Figure 1. Expression of *mec3-dn* abolishes Rad53 phosphorylation in G1, while have only a partial effect in G2, leaving the checkpoint response intact. **A.** Rad53 phosphorylation was monitored by Western blotting on protein extracts prepared from strains K699 (*MEC3*) or YLL352 (*mec3-dn*). Extracts were prepared from cells blocked with α-factor (G1) or nocodazole (G2), with (+) or without (-) treatment with 4NQO. **B.** Protein extracts were prepared from cells arrested in G1 or G2 as described above, but treated with UV-light or zeocin, a drug causing double-strand breaks.

Figure 2. The Mec3 mutant protein replaces the wild type form in the Rad17 complex in G1 and also in G2. YSH4 cells, in which the *RAD17* chromosomal gene was tagged with a 6His-3HA epitope, expressing *mec3-dn* on plasmid pNB187-Mec3-dn or carrying the empty vector (pNB187) were arrested in G1 and G2. Native extracts were immunoprecipitated with the 12CA5 anti-HA (**A, B**) or the 9E10 anti-Myc antibodies (**C**) as previosly described (25). Recovered proteins were analyzed by Western blotting with Mec3-specific, Ddc1-specific or 12CA5 antibodies, as indicated. **D.** YSH4 cells exponentially growing or arrested in G1, S and G2, as described in the Experimental Procedures, were treated with 4NQO where indicated. Proteins were immunoprecipitated with anti-HA antibodies from crude extracts and analyzed by Western blotting with anti-Myc antibodies. The result of a control co-immunoprecipitation experiment performed on exponentially growing no-tagged K699 cells (Exp) is also shown. The total amount of Ddc1 and Rad17 present in the extracts is not

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affected by expression of Mec3 or Mec3-dn, as evaluated by control western blotting.

Figure 3. In a wild type genetic background replacement of Mec3 with Mec3-dn is slow. **A.** K699 cells were transformed with the vector plasmid pNB187 or with pNB187-Mec3-dn, where the expression of *mec3-dn* is driven by the *GAL1* promoter. Protein extracts analysed in lanes 5-6 were prepared from cells arrested by α-factor treatment in galactose-containing medium for 3 hrs. Samples analyzed in lanes 8-9 where induced overnight in galactose before cell cycle arrest in G1 by α-factor addition. Where indicated, cells were treated for 15 min with 4NQO before extract preparations. Western blots were probed with anti-Rad53 and anti-Myc antibodies. **B.** K699 cells transformed with plasmid pNB187 (vector) or with pNB187-Mec3-dn (*mec3-dn*), exponentially growing in raffinose containing medium, were arrested in G1 by α-factor treatment and concomitant galactose addition. After 3 hrs, half of the culture was released from the α-factor block and allowed to go through a complete cell cycle (3 hrs) in galactose-containing medium, while the other half of the culture was kept blocked in G1 for 3 more hrs by adding a fresh α-factor aliquot. The scheme of the experiment is shown at the top of the panel and the cell samples used for FACS (middle) or Western blot analysis (bottom) are indicated by letters (a to f). Western blots were probed with anti-Rad53 and anti-Myc antibodies.

Figure 4. Substitution kinetics of Mec3 with Mec3-dn parallels checkpoint inactivation. YSH4 cells, carrying a HA-*RAD17* gene and transformed with plasmid pNB187-Mec3-dn, were grown in raffinose-containing medium and then induced with galactose for the indicated times. The last 2 hrs of induction were performed after α-factor addition to arrest cell in G1, and to test the effect of Mec3-dn substitution on checkpoint activity after 4NQO treatment. Protein extracts prepared from cells taken at the indicated times were co-immunoprecipitated with anti-HA antibodies. The substitution kinetics of Mec3 with Mec3-dn was tested by probing the Western blots with anti-Mec3 antibodies. At the same time points Rad53 autophosphorylation activity was assayed on TCA protein extracts (bottom panel), after SDS-PAGE electrophoresis followed by *in situ* Rad53 renaturation (see Experimental Procedures).

Figure 5. The kinetics of assembly of Mec3 and Mec3-dn is similar starting from a disassembled complex. **A.** Protein extracts were prepared from strains K699 (wild type), YLL334 (K699 with HA-*DDC1*), YLL335 (*Δmec3* with HA-*DDC1*). Proteins were immunoprecipitated with anti-HA antibodies and the presence of Ddc1 and Rad17 were visualized on Western blots, with anti-HA and anti-Rad17 antibodies, respectively. **B.** YLL335 cells transformed with plasmids pNB187 (vector), pNB187-Mec3-dn (*mec3-dn*) or pNB187-Mec3 (*MEC3*) were arrested in G1 or in G2 and, where indicated, the expression of *MEC3* or *mec3-dn* was induced for 2 hrs by galactose addition with or without 4NQO treatment. Checkpoint activation was tested by monitoring the extent of

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Rad53 phosphorilation on Western blots. **C.** Protein extracts prepared from cells taken after 2 hrs of galactose induction and 4NQO treatment were immunoprecipitated with anti-HA and probed with anti-Mec3 antibodies. **D.** Extracts from an experiment similar to the one described in B were immunoprecipitated with anti-HA antibodies at the indicated timepoints, and analyzed with anti-Mec3 antibodies. In this particular experiment, expression of Mec3 was 2-3 times higher than that of Mec3-dn, as seen by control western blotting. Samples at 120 minutes were 2X overloaded.

figure1

A

B

figure2

A

IP Rad17-HA

B

IP Rad17-HA

C

IP Mec3-dn

D

figure3

A

	vector			mec3-dn			mec3-dn		
4NQO	-	-	+	-	-	+	-	-	+
gal induction	0	18h	18h	0	3h	3h	0	18h	18h

} Rad53

— Mec3-dn

1 2 3 4 5 6 7 8 9

B

exp	αf		a	b	c
exponential raffinose	→ α factor galactose	→ - αf →	cell cycle →	α factor →	4NQO
	↘ + αf			→	4NQO
		d		e	f

vector   mec3-dn

	vector					mec3-dn				
	f	a	b	c	d	f	a	b	c	d

} Rad53

— Mec3-dn

1 2 3 4 5 6 7 8 9 10

figure4

Hours after Gal induction

0   2   6   12 16 20

Western blot — Mec3-dn
— Mec3

In situ assay   Rad53
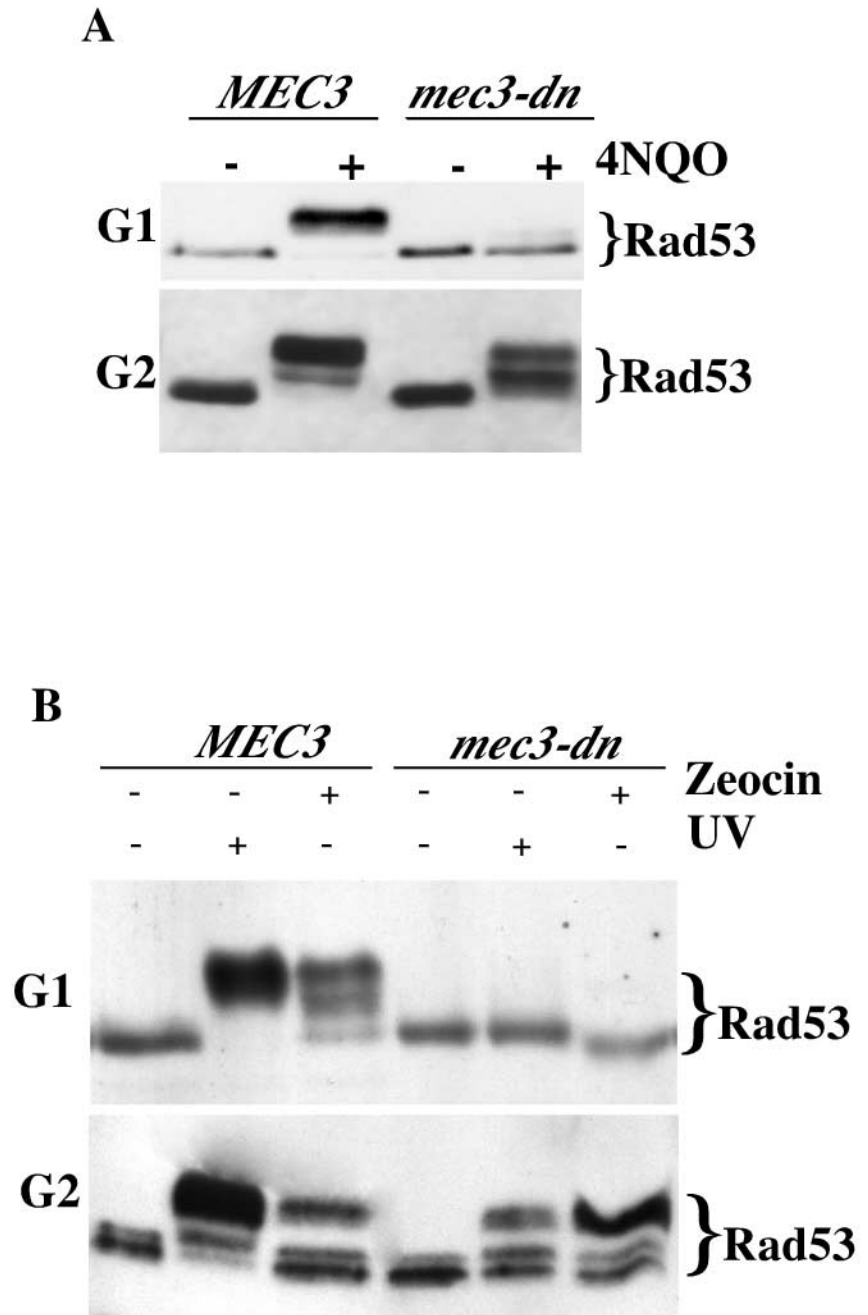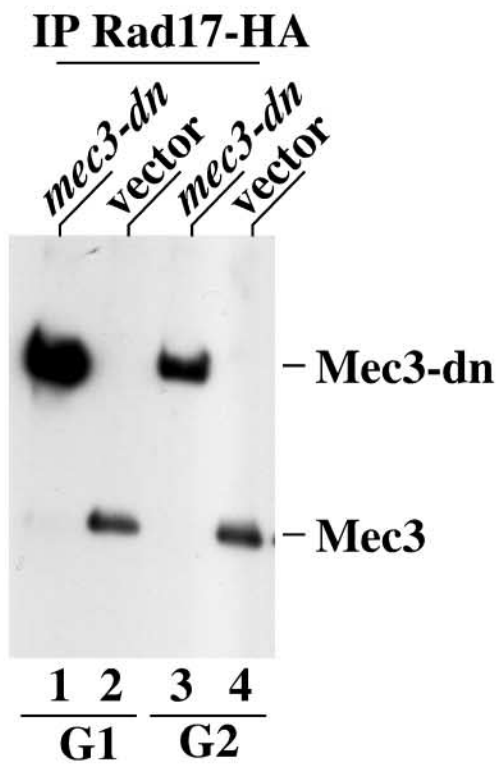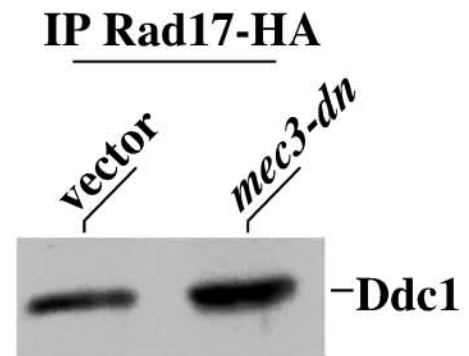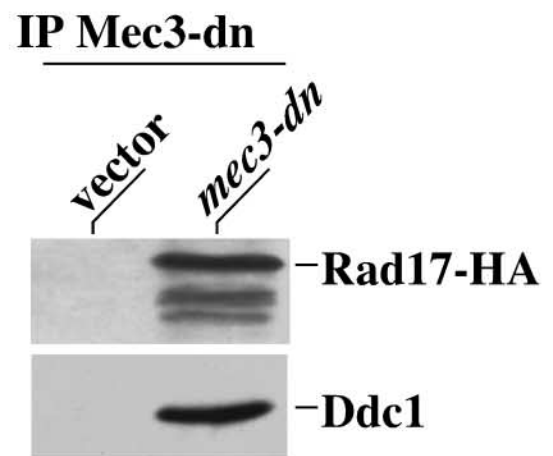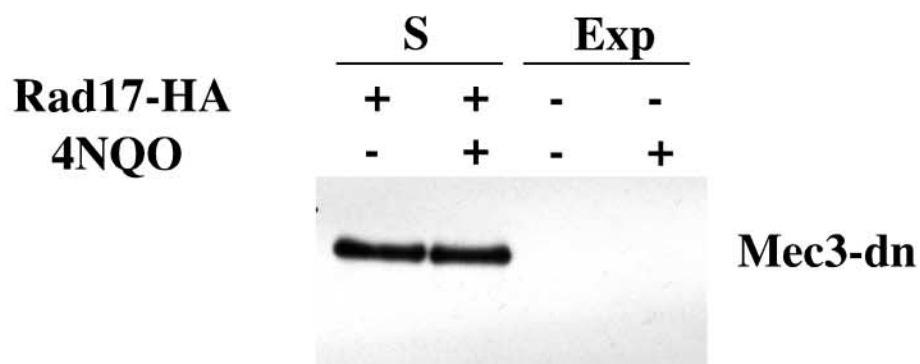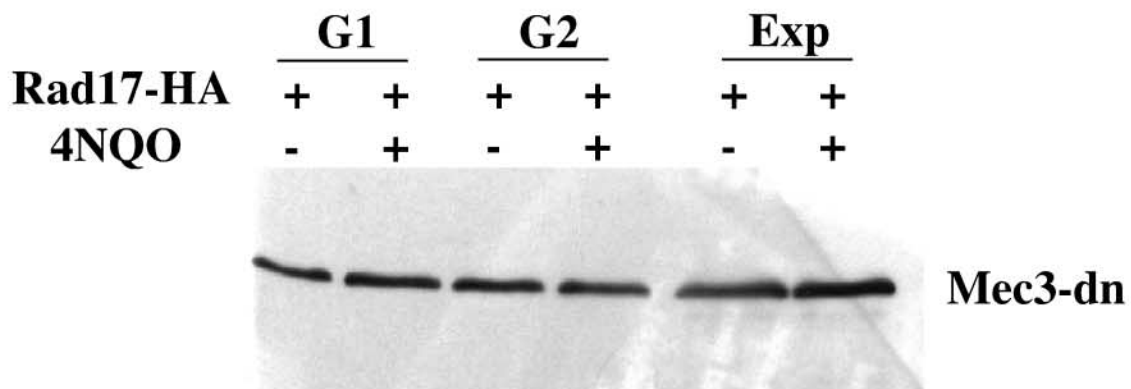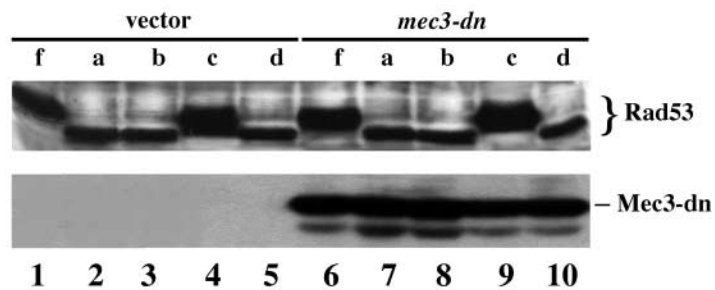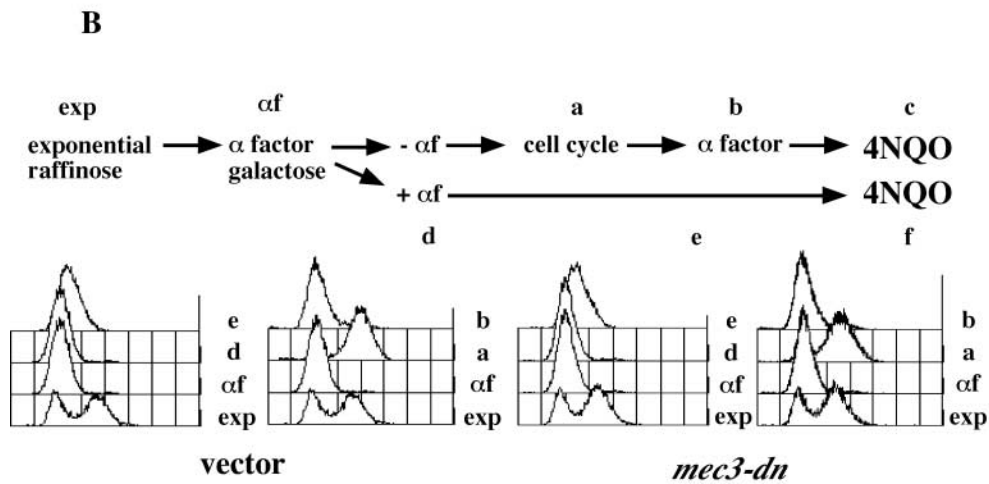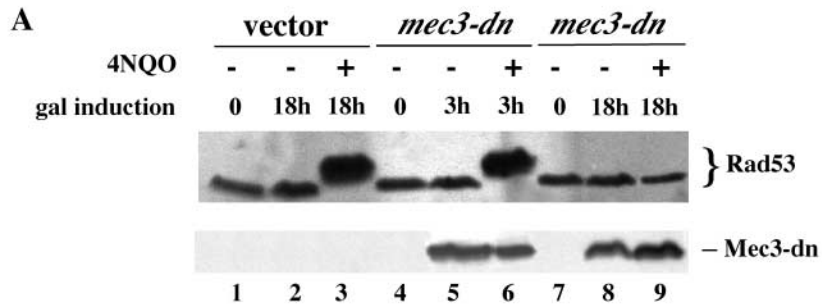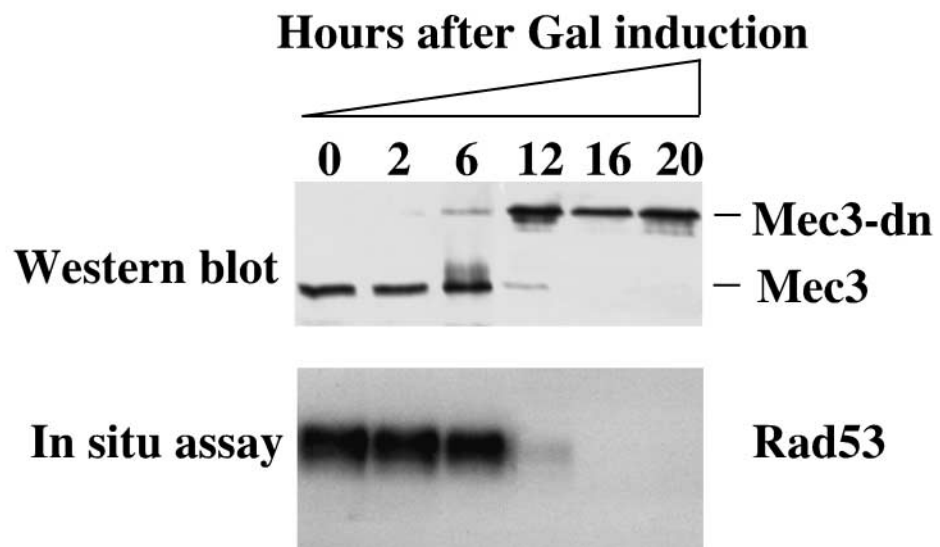
figure5

A

B

C

D
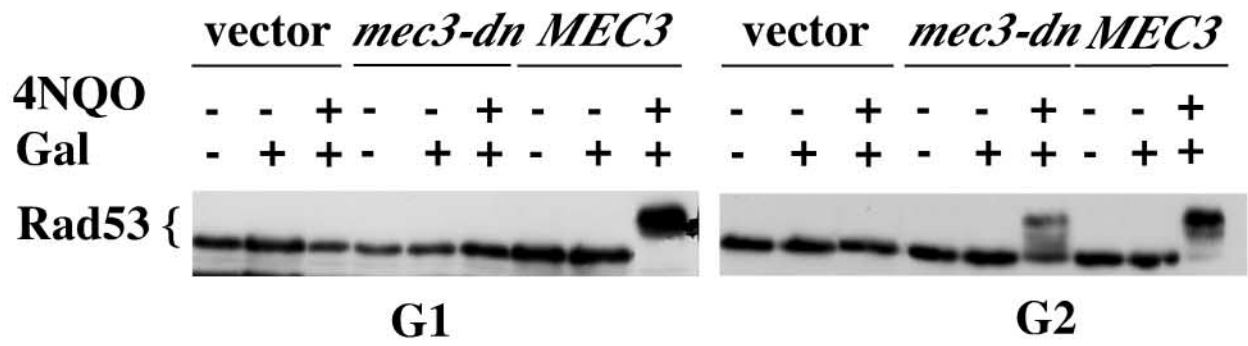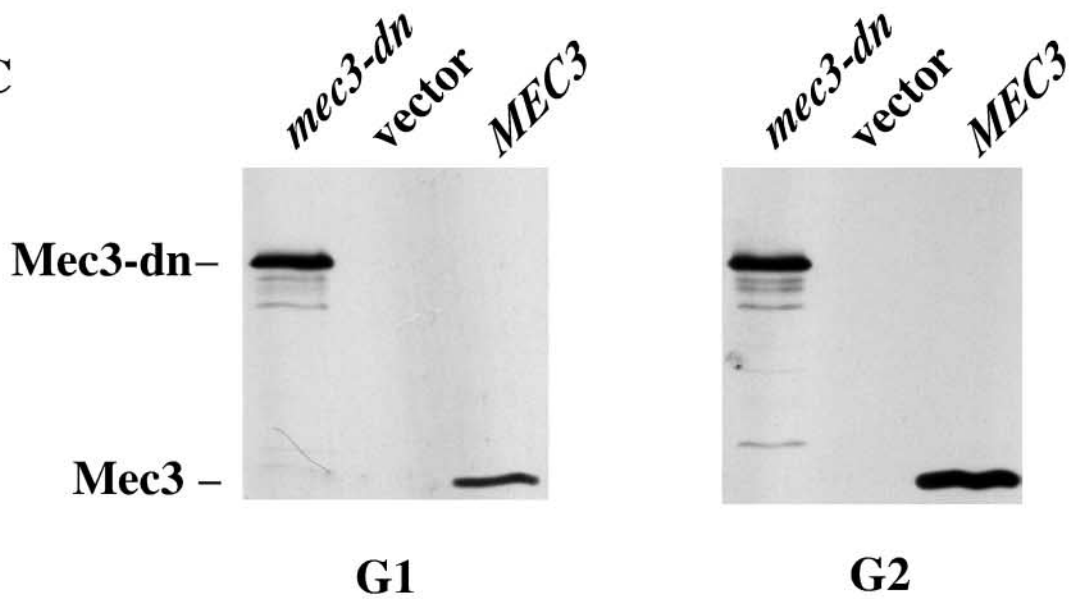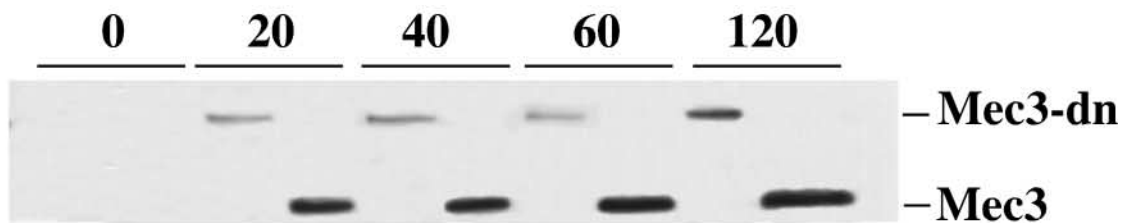
Correlation between checkpoint activation and in vivo assembly of the yeast checkpoint complex Rad17-Mec3-Ddc1

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