The DNA Damage and the DNA Replication Checkpoints Converge at the MBF Transcription Factor

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Running head: DDR Represses G1-to-S Transcription

Abbreviations: MBF, MluI Binding Factor; MMS, Methyl methanesulfonate; DDR, DNA damage response;
ABSTRACT

In fission yeast cells, Cds1 is the effector kinase of the DNA replication checkpoint. We have previously shown that when the DNA replication checkpoint is activated, the repressor Yox1 is phosphorylated and inactivated by Cds1 resulting in the activation of the MBF-dependent transcription. This is essential to re-initiate DNA synthesis and for a correct G1-to-S transition. Here we show that Cdc10, which is an essential part of the MBF core, is the target of the DNA damage checkpoint. When fission yeast cells are treated with DNA damaging agents, Chk1 is activated and phosphorylates Cdc10 at its carboxy-terminal domain. This modification is responsible for the repression of MBF-dependent transcription through induced release of MBF from chromatin. This inactivation of MBF is important for survival of cells challenged with DNA damaging agents. Thus, Yox1 and Cdc10 couple normal cell cycle regulation in unperturbed conditions and the DNA replication and DNA damage checkpoints on a single transcriptional complex.

INTRODUCTION

Genomic integrity is constantly threatened by many processes that take place in any living cell. Reactions like transcription and DNA replication or the exposure to external or internal damaging agents suppose for the cell an increased risk of rearrangements in DNA or single nucleotide substitutions, defects that are the hallmark of cancer cells (Elledge, 1996; Ciccia and Elledge, 2010). In order to maintain genomic integrity, all eukaryotes have developed a highly conserved mechanism to detect, signal and repair damage in DNA, known as the DNA damage response (DDR) (Hartwell and Weinert, 1989; Elledge, 1996; Rhind and Russell, 1998). When DNA replication is challenged, cells activate a DNA replication checkpoint blocking cell cycle progression until they are able to overcome the replication defects (Murakami and Okayama, 1995; Boddy and
Russell, 1999). Similarly, in response to damage to DNA, cell cycle must be arrested through the DNA damage checkpoint (Rhind and Russell, 2000). In the fission yeast *Schizosaccharomyces pombe* there are two effector kinases, Cds1 and Chk1, which are activated by the DNA replication and the DNA damage checkpoint, respectively. The main aim of these kinases is to block cell cycle progression before cells enter into mitosis; they do so by phosphorylating and inhibiting the phosphatase Cdc25, what fully prevents activation of Cdc2 (Walworth *et al.*, 1993; Furnari *et al.*, 1997).

In fission yeast, activation of Cds1 in response to a DNA replication defect, also invokes a transcriptional response that ultimately increases the concentration of the deoxynucleotides required to complete DNA synthesis. This response is achieved by activating the transcription factor MBF (Dutta *et al.*, 2008), which in a normal – unperturbed– cell cycle is responsible for the transcription of a set of genes that are required for the S phase of the cell cycle (Lowndes *et al.*, 1992). MBF, which is the functional homologue of mammalian RB/E2F, is a high molecular weight complex whose core elements are the product of the Start gene cdc10, and Res1 and Res2 which form a heterodimeric DNA-binding domain (Simanis and Nurse, 1989; Lowndes *et al.*, 1992; Tanaka *et al.*, 1992; Miyamoto *et al.*, 1994; Obara-Ishihara and Okayama, 1994; Ayte *et al.*, 1995). Under replicative stress, the activation of the MBF-dependent transcription is the consequence of the phosphorylation of several components of the MBF complex, including Cdc10 (Dutta *et al.*, 2008), the co-repressor Nrm1 (de Bruin *et al.*, 2008), the repressor Yox1 (Caetano *et al.*, 2011; Gomez-Escoda *et al.*, 2011; Purtill *et al.*, 2011) and the co-activator Rep2 (Nakashima *et al.*, 1995; Chu *et al.*, 2007; Chu *et al.*, 2009). Specifically, phosphorylation of Yox1 by Cds1 disrupts the binding of Yox1 to the MBF complex, activating MBF-dependent transcription. Mutants in which Yox1 cannot be phosphorylated lack the proper transcriptional response under replicative
stress (Gomez-Escoda et al., 2011). In this work, we demonstrate that there is also a direct link between the DNA damage checkpoint and the MBF complex, which, contrary to what happens after the activation of the DNA replication checkpoint, is responsible of inactivating the transcription of the S phase genes. This is achieved by direct phosphorylation of Cdc10 at serine-720 and serine-732 by the effector kinase, Chk1.

RESULTS

Cdc10 is targeted by the DNA damage checkpoint.

While investigating the effect of the DNA replication checkpoint on the regulation of the transcription factor MBF, we noticed that when cells were treated with hydroxyurea (HU) on a Yox1 mutant background (Yox1.SATA) that cannot be phosphorylated by the DNA replication checkpoint effector kinase, Cds1, MBF-dependent induction of transcription was abrogated (Fig. 1A) (Gomez-Escoda et al., 2011). Under these conditions, the core MBF element, Cdc10, was released from chromatin (Fig. 1B), in parallel to the release of the repressor Yox1 (Fig. 1C). We could observe this release independently of the presence of Cds1, since in cells lacking Cds1, Cdc10 was also released when they were treated with HU, and to a similar extent than in the Yox1.SATA cells. Unexpectedly, this release of Cdc10 was abrogated in the absence of Chk1 (both Δchk1 and Δchk1Δcds1 strains) or when Chk1 could not be activated in cells that lack the sensor kinase (Δrad3 strain). Interestingly, we could observe that Chk1 was phosphorylated (which is a hallmark of its activation) when either Yox1.SATA or Δcds1 cells were treated with HU, pointing to the fact that in these specific genetic backgrounds both the DNA replication and DNA damage checkpoint were activated by HU (Fig. 1D).
It was previously shown that besides Yox1, other components of the MBF complex (Nrm1 and Cdc10) could be phosphorylated by Cds1 when cells were under replicative stress (de Bruin et al., 2008; Dutta et al., 2008). However, our previous results (Figure 1) pointed to the possibility that some MBF components could also be targeted by Chk1 specifically when the DNA damage checkpoint was activated. To further investigate the signaling from this checkpoint to the MBF factor, we treated fission yeast cells with different DNA damaging agents, like MMS or γ-irradiation (Fig. 2A). Indeed, both damaging agents were able to induce the release of Cdc10 from two of the better characterized MBF-dependent promoters, cdc18 and cdc22. To determine if Cdc10 was released alone or with other components of the MBF complex, we decided to test for co-immunoprecipitation between Cdc10 and Res2, which contains the DNA binding activity of the MBF complex on its amino-terminal region (Miyamoto et al., 1994; Obara-Ishihara and Okayama, 1994). As shown in Fig. 2B, the interaction between Cdc10 and Res2 was well preserved, if not improved, after treating fission yeast cells with MMS. In fact, both Res1 and Res2 are released from chromatin after the treatment with MMS (Fig. 2C), pointing to the possibility that the core elements of MBF (Res1, Res2 and Cdc10) was released as a complex from chromatin (and not as individual components), after the DNA damage checkpoint was induced.

The effect of the DNA damage checkpoint on MBF is dose-dependent.

To further characterize the response to MMS, we treated cells with increasing concentrations of the drug (from 0.002% to 0.1%) for 60 minutes. At the lower doses, we could not observe any noticeable effect on Cdc10, since it remained bound to the canonical promoters that we tested. In fact, Cdc10 was not released from chromatin unless cells were treated with higher MMS concentrations (0.05% and up) (Fig. 3A).
and Yox1), we could clearly observe that both proteins were released from chromatin (and consequently from the MBF complex) already at the lower MMS concentrations (Fig. 3A). This effect on Nrm1/Yox1 paralleled a noticeable induction of the transcription of the MBF genes at low MMS concentrations (Fig. 3B). We could detect a further release of both Yox1 and Nrm1 when we treated cells with higher MMS concentrations, which paralleled with the release of Cdc10 described above. This second wave of Nrm1 and Yox1 release at higher concentrations correlates with a repression of MBF-dependent transcription at higher MMS doses (Fig. 3B). To separate both events (Cdc10 release at higher concentrations and Nrm1/Yox1 release at lower concentrations) and if the DNA damage checkpoint was indeed able to induce a release of the MBF complex from chromatin (and its consequent down-regulation of the MBF-dependent transcription), we decided to repeat the MMS treatment in a strain lacking the repressor system (Δnrm1Δyox1 background strain). These cells, which have an already induced transcription of the MBF-dependent genes as their basal steady-state, were exposed with increasing MMS concentrations for 1 hour. As shown in Fig. 3C, a clear repression of the two MBF-dependent genes (cdc18 and cdc22) was observed. To determine whether release of the MBF complex from chromatin was due to cell death, we measured the viability of the cells during the timing of the treatment. As shown in Fig. 3D and Supplementary Fig. S1, the MMS concentrations used (and even higher concentrations) barely affect cell viability during the time of the treatment.

Next, we wanted to further characterize the signaling from the DNA damage checkpoint to the MBF complex. To confirm that the release of Cdc10 was exclusively due to the activation of the DNA damage checkpoint (and that the DNA replication checkpoint was not involved in this release), we analyzed the binding of Cdc10 and Yox1 to cdc18 and cdc22 promoters in cells with impaired signaling in each or both of
these checkpoint signaling pathways after treatment with MMS. As shown in Fig. 4A, the release of Cdc10 that we observed in cells lacking Cds1 was similar to the observed in wild type cells. However, in cells lacking either Chk1 or the upstream activating kinase, Rad3, Cdc10 was not released after the treatment with MMS. Under these concentrations of MMS, Yox1 release from chromatin paralleled the release of Cdc10, pointing to the possibility that it was the MBF complex as a whole that was released from chromatin when only the DNA damage checkpoint was induced (Fig. 4B).

**Chk1 phosphorylates Cdc10 and inactivates MBF-dependent transcription.**

We then decided to focus on the possibility that Cdc10 itself could be a direct target of Chk1. In fact, Cdc10 has already been described as a target for Cds1, although no clear phenotype has been associated to Cdc10 mutants in the residues that are phosphorylated *in vitro* by Cds1 (Dutta et al., 2008), that is ser-720 and thr-723. Since Cds1 and Chk1 can phosphorylate similar target sequences (O'Neill et al., 2002; Seo et al., 2003; Xu and Kelly, 2009), we set out to determine whether Cdc10 was *in vitro* a bona-fide target for Chk1 phosphorylation.

Cdc10 has 4 putative sites that can be phosphorylated by Chk1 (ser-563, thr-603, ser-720 and ser-732). While the first two are in close proximity to the ankyrin domain, which mediates protein-protein interactions, the last two residues are in the C-terminal region of Cdc10, which is essential for loading the Yox1/Nrm1 repressor system onto chromatin (Supplementary Fig. S2). In fact, and as a first approach, we noticed that Cdc10 release after MMS treatment was not observed in a strain that lacks the last 61 amino acids of Cdc10, *cdc10-C4* (Figure 5A). Thus, we decided to focus on this carboxy-terminal domain of Cdc10 as a potential substrate of Chk1 phosphorylation. In fact, in our *in vitro* Chk1 kinase assays, a Cdc10 construct lacking the last 61 amino
acids was not phosphorylated. Conversely, a construct containing only the carboxy-
terminal 61 amino acids (and thus containing the last two putative phosphorylation
sites) was consistently phosphorylated (Fig. 5B-C). When serines 720 or 732 were
mutated to alanine, the extent of phosphorylation was diminished. Furthermore, in the
double mutant, Cdc10 phosphorylation by Chk1 was completely abolished (Fig. 5C). It
is worth noting that these phosphorylation sites are partially different from the described
as Cds1-phosphorylation sites in Cdc10, where the authors noticed that only when both
ser-720 and thr-723 were mutated to glutamic acid, MBF-dependent transcription was
induced (Dutta et al., 2008). In fact, in this mutant background, the Nrm1/Yox1
repressor system is unable to bind the MBF complex (data not shown). Next, to
determine if in vivo Chk1 was able to phosphorylate Cdc10 on serines 720 and 732, we
used an anti-phospho-serine antibody. As shown in Fig. 5D, Cdc10 is phosphorylated
when cells were treated with MMS. However, when serines 720 and 732 were replaced
by alanines, we were unable to detect this phosphorylation. In fact, and confirming the
notion that the DNA damage checkpoint could be regulating the MBF complex, we
were able to detect direct interaction between Chk1 and Cdc10 by co-
immunoprecipitation (Fig. 5E)

To test whether Cdc10 phosphorylation by Chk1 is essential for the in vivo
regulation of Cdc10/MBF binding to its target promoters upon activation of the DNA
damage checkpoint, we introduced the serine-to-alanine mutations in fission yeast,
replacing the endogenous copy of cdc10. When treated with MMS, the strains that
carry single mutations (including those next to the ankyrin domain) were responding in
a similar manner to a wild type strain, that is, Cdc10 was released from its target
promoters (Fig. 6A and supplementary Fig. S3). However, in a strain that carries the
double mutation S720AS732A (herein Cdc10.2A) and that cannot be phosphorylated in
vitro and in vivo by Chk1, the release of Cdc10 was impaired from cdc18 promoter after the treatment with MMS (Fig. 6A). Interestingly, we could observe only a small effect on the regulation of its binding activity to cdc22 promoter, pointing to the fact that Chk1 might differentially regulate the binding of Cdc10 to only a subset of MBF-dependent genes. Similar effect was observed when cells were irradiated (Fig. 6B).

To test the consequences of the Chk1-mediated regulation of Cdc10 binding to chromatin, we measured the effect on transcription. As expected, when a strain in which the two Chk1 phosphorylation sites were mutated to alanine (Cdc10.2A) was treated with increasing doses of MMS, cdc18 transcription was steadily maintained, while cdc22 decreased to a similar extent as in the wild type strain counterpart (Fig. 6C, compare with Fig. 3C). This different response between two MBF-dependent genes, led us to expand our set of analyzed genes and in a more quantitative manner by RT-qPCR. As shown in Fig. 6D, while phosphorylation of Cdc10 by Chk1 is responsible for the downregulation of cdc18 and cdt2, regulation of cdc22 and mik1 might be mediated by some other overlapping mechanisms. Finally, we hypothesized that a strain, in which the transcriptional response of the DNA damage checkpoint was abolished, should have survival problems when confronted with a damaging agent, like MMS. As can be observed in Fig. 6E, a strain carrying the double mutation (Cdc10.2A) was sensitive to MMS. Interestingly, this strain has a wild type level of survival when confronted with drugs that block DNA replication (HU), indicating that these two residues are not regulated by the DNA replication checkpoint.
DISCUSSION

The MBF complex is an essential transcription factor that fission yeast cells need for the normal and controlled expression of the S phase transcription program. When DNA replication is challenged (i.e. after treating cells with HU), fission yeast cells activate their effector kinase (Cds1) and, among many other effects, are able to maintain a high level of MBF-dependent transcription (Gomez-Escoda et al., 2011). Since ribonucleotide reductase (Cdc22) is the target of HU and its expression is directly regulated by MBF (Lowndes et al., 1992), hyperactivation of the complex might help to overcome the block to DNA replication inflicted by the drug. Similar processes have been described in the distantly related budding yeast (de Bruin et al., 2008; Bastos de Oliveira et al., 2012; Travesa et al., 2012) and might be conserved to some extent in higher eukaryotes. Yox1, the repressor of the MBF complex, is the main MBF target of fission yeast Cds1 (Aligianni et al., 2009; Gomez-Escoda et al., 2011). It has recently been described that Yox1 phosphorylation by Cds1 results in its inactivation (Caetano et al., 2011; Gomez-Escoda et al., 2011; Ivanova et al., 2011; Purtill et al., 2011). We now report here that the DNA damage checkpoint exerts a new layer of control on the MBF complex. However, instead of exerting a positive effect on MBF, Chk1, the effector kinase of the DNA damage checkpoint, is responsible of inactivating MBF-dependent transcription (Fig. 7). This is achieved by direct phosphorylation of one of the core components of the MBF complex, Cdc10, at two different sites on its carboxy-terminal domain. This phosphorylation induces the exit of Cdc10 from the chromatin and thus the repression of the transcription of the MBF-dependent genes. Interestingly, low doses of MMS are able to induce MBF dependent transcription (probably through Cds1-dependent phosphorylation of Yox1) whilst high doses repress the same set of genes by directly phosphorylating Cdc10. In fact, under such severe damage there is no
active MBF complex associated with the corresponding promoters since Res1 and Res2 are also released from chromatin (Fig. 2B and 2C). Our hypothesis is that cells that have to cope with severe DNA damage must stop any attempt to initiate DNA synthesis which will worsen its situation; this is achieved by switching off the S phase transcriptional program. However, fission yeast cells sense discrete or minor DNA damage (low MMS concentration, HU) at least partly as a block to DNA synthesis, activating the DNA replication checkpoint. Consequently, these cells need to maintain activated the transcriptional S phase program until they manage to fully complete the duplication of its genome. In conclusion, MBF would be doubly targeted by the DNA replication and the DNA damage checkpoints with an outcome that goes in opposite directions: while the DNA damage checkpoint targets Cdc10 and causes a repression, the DNA replication checkpoint phosphorylates Yox1 and induces an activation of transcription. Interestingly, while all the MBF-dependent genes are induced upon a challenge to DNA replication (Dutta et al., 2008; Gomez-Escoda et al., 2011), only a subset of them seems to be under the control of the DNA damage checkpoint (Fig. 6). We do not know how this is achieved, but it has been long known that not all MBF-dependent genes are regulated in the same manner; for example, in synchronized cultures, transcription of cdc18 is induced in anaphase, while induction of cig2 takes place later during the G1-to-S transition (Baum et al., 1997). Thus, the differential regulation of the MBF-dependent genes by the DNA damage checkpoint may be due to intrinsically differences in the chromatin structure of the two groups of MBF dependent genes; alternatively, we have not excluded that other components or regulators of the MBF complex can be overlapping targets for Chk1 that play a role only in a subset of MBF-dependent genes. Further work will be required to characterize this differential regulation.
While up-to-now in higher eukaryotes it has not been demonstrated a clear link between the DNA replication checkpoint and the regulation of the expression of S phase genes, previous reports have insinuated a connection between the DNA damage checkpoint and E2F, which, at some extent, is the functional homologue of fission yeast MBF and budding yeast MBF/SBF (Stevens et al., 2003; Inoue et al., 2007; Zalmas et al., 2008). Initially it was reported that E2F-1 was phosphorylated and activated in response to DNA damage, resulting in cells being directed to apoptosis (Stevens et al., 2003). However, a recent report demonstrated that irradiation might also cause phosphorylation of Rb (by Chk1/2) on a site that is also phosphorylated by CDK/cyclins in unperturbed cell cycle. Intriguingly, phosphorylation of Rb on this site induces repression of E2F-dependent transcription (Inoue et al., 2007). We propose that the checkpoint regulation of transcription through Cdc10 might be distantly conserved across eukaryotes, with the same final outcome (repression of transcription after DNA damage), but using highly divergent mechanisms: while in higher eukaryotes the phosphorylation tethers the repressor (Rb) to the transcription factor (E2F-1), in fission yeast decreases the binding of the transcription factor to its cognate promoters. It will be interesting to know whether similar mechanisms are conserved in the distantly related budding yeast.

MATERIALS AND METHODS

Strains and media. All S. pombe strains are isogenic to wild type 972h-. The strains used in this work are listed in the supplementary information. Media were prepared as previously described (Moreno et al., 1991). HU (10mM), MMS and γ-irradiation treatment were carried out on midlog grown cultures (3-4x10^6 cells/ml) in YE5S media. Liquid cultures were treated with HU for 4 hours and to MMS for 60 minutes, unless where indicated. To analyze sensitivity to HU and MMS on plates, S. pombe strains
were grown in liquid YE5S media to an OD$_{600}$ of 0.5. Cells were then diluted in YE5S and spotted onto YE5S media agar plates. Plates were incubated at 30°C for 3–4 days.

**Viability assays.** For viability tests, cells were grown in liquid YE5S media to an OD600 of ~0.3 and the cultures were treated with MMS. For propidium iodine staining, cells were centrifuged, washed twice with PBS, and incubated with 3 µg/ml of the dye for 40 min on ice in darkness. For the phloxine B staining, cells were incubated with 5 µg/ml of the dye for 2 h with shaking at 30°C in darkness, centrifuged and washed twice with PBS. 10,000 cells from each sample were scanned using channel FL3 for propidium iodine and channel FL2 for phloxine B with FACSCalibur.

**Protein extraction.** Extracts were prepared in NET-N buffer [20 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1mM dithiothreitol (DTT), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg/ml aprotinin, protease inhibitor cocktail (Sigma), 2 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na$_3$VO$_4$), 2 mM β-glycerophosphate]. Cells were broken with glass beads in a BioSpec Minibeadbeater. Immunoprecipitations (1 to 3 mg of whole-cell lysate) were performed with 10 µl of protein G sepharose, previously crosslinked with α-HA monoclonal antibody. Immunoprecipitates were washed after 1 hour of incubation three times with NET-N buffer and resolved in SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibody. In Figure 5D, the detection of phosphorylated Cdc10 was performed using purified mouse anti-PhosphoSerine/Threonine MAb (BD Biosciences cat. no. 612548).

**In vitro Chk1 kinase assay.** Substrates were prepared as GST fusion proteins in *E. coli* as described (Dutta *et al.*, 2008). Protein extracts (300 µg) from MMS-treated cultures of a strain with HA-tagged Chk1 were immunoprecipitated as described (Ayte *et al.*, ...
followed by three washes with NET-N buffer and one wash with kinase buffer (10mM Heps pH7.5, 20mM MgCl₂, 4mM EGTA, 2mM DTT). Immunoprecipitates were incubated in kinase buffer containing 2µg of substrate and 10µCi of [γ-³²P]ATP for 30 min at 30°C. Labeled proteins were resolved in 12% SDS-PAGE and detected by autoradiography.

**Gene expression analysis.** RNA extraction was performed as described (Moldon et al., 2008) and 10 µg of extracted RNA were loaded on agarose gels and analyzed by northern blot. cdc18, cdc22, and tfb2 probes contained the complete ORFs of the genes. For the RT-qPCR, RNA was digested with DNase I for 30 min at 37°C, phenol extracted, and precipitated. Eight micrograms of total RNA was denatured at 65°C for 10 min and then chilled on ice. Reverse transcriptase reactions were carried out (60 min at 42°C, 30 min at 52°C, and 3 min at 94°C) following the manufacturer's guidelines (Promega) in the presence or absence of the enzyme. One microliter of the cDNA was used in the RT-qPCR with specific oligonucleotides.

**Chromatin immunoprecipitation.** ChIP experiments were performed as described (Moldon et al., 2008). All the experiments were plotted as the average of at least three different biological replicates ± SD and represented as relative binding respect to untreated wild type cells to facilitate the comparison between different strains.
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**FIGURE LEGENDS**

**Figure 1. Cdc10 is targeted by the DNA Damage Response.** (A) Total RNA was prepared from untreated (-) or HU-treated (+) cultures of wild type (WT) and Yox1.SATA (SATA) cells and analyzed by hybridization to the probes indicated on the left. *rRNA* is shown as loading control. (B) Loading of Cdc10 on *cdc22* and *cdc18* promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or HU-treated (10 mM HU, 4h at 30°C) cultures of wild type (WT), Yox1.SATA (SATA), ∆cds1, ∆chk1, ∆cds1∆chk1 or ∆rad3 cells. Endogenous Cdc10 is HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA. (C) The same chromatin extracts were analyzed for Yox1 binding with polyclonal
antibodies anti-Yox1. (D) Phosphorylation level of endogenous Chk1-HA in native extracts prepared from untreated (−) or HU-treated (+) cultures of wild type (WT), Yox1.SATA (SATA) or Δcbs1 strains. Proteins were resolved in an 8% SDS–PAGE and anti-HA western blotted to detect Chk1.
Figure 2. The DNA Damage Response releases intact MBF from its target promoters. (A) Loading of Cdc10 on cdc22 and cdc18 promoters was measured by ChIP analysis with polyclonal antibodies anti-Cdc10 of chromatin extracts isolated from untreated or treated with MMS (0.1% for 1h at 30ºC) cultures of a wild type strain (left panel) or from irradiated cells (100 Gy) (right panel). (B) The interaction between Cdc10 and Res2 is preserved in MMS-treated cells. Extracts (2.5 mg) from wild type (WT) and Cdc10-HA strains (with or without MMS treatment for the time indicate on top) were immunoprecipitated with α-HA antibody and analyzed for the presence of Res2 and Cdc10 with specific antibodies (monoclonal anti-Res2 and anti-HA, respectively). (C) Loading of Res1 (left) or Res2 (right) on cdc22 and cdc18 promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated with MMS (0.1% for 1h at 30ºC) cultures of a Res1-HA or Res2-HA strain, respectively.
Figure 3. Chk1 effect on Cdc10 is MMS-concentration dependent. (A) Loading of Cdc10, Yox1 and Nrm1-HA on cdc22 and cdc18 promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated with the indicated MMS concentrations (1h at 30°C) cultures. Cdc10 and Nrm1 are HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA, while Yox1 was determined with polyclonal antibodies anti-Yox1. (B) Total RNA was prepared from untreated (-) or MMS-treated cultures of wild type cells and analyzed by hybridization to the probes indicated on the left. rRNA is shown as loading control. (C) Total RNA was prepared from untreated or MMS-treated (increasing doses) cultures of Δyox1Δnrm1 cells, and analyzed by hybridization with the probes indicated on the left.
rRNA is shown as loading control. (D) Cell viability is unaffected at the used range of MMS concentrations. Viability test of wild type cells treated with different concentrations of MMS, using PI or Phloxin B to measure viable cells, was performed by FACS analysis.
Figure 4. Cdc10 is released from chromatin upon DNA damage. (A) Loading of Cdc10 on cdc22 and cdc18 promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30°C) cultures of wild type (WT), Δcds1, Δchk1, Δcds1Δchk1 or Δrad3 cells. Endogenous Cdc10 is HA tagged and the levels of binding were quantified on anti-HA immunoprecipitated DNA. (B) The same chromatin extracts were analyzed for Yox1 binding with polyclonal antibodies anti-Yox1.
Figure 5. Cdc10 Ser-720 and Ser-732 are phosphorylated by Chk1, inactivating MBF-dependent transcription. (A) Chk1 signals MBF through the C-terminal region of Cdc10. Loading of Cdc10 on cdc22 and cdc18 promoters was measured in untreated or MMS-treated (0.1% MMS, 1h at 25ºC) cultures of WT and cdc10-C4 strain by ChIP. The average of three individual experiments (±s.d.) is plotted. (B) Amino acid sequence of the Cdc10 region that is phosphorylated by Chk1. The phosphorylation consensus is indicated at the bottom. (C) Chk1 in vitro kinase activity (in arbitrary units) was assayed using GST, wild type (WT) Cdc10 or the Cdc10 mutants indicated on top as substrates. Coomassie staining of the gel is shown at the bottom. (D) Cdc10 phosphorylation was determined on extracts prepared from untreated (unt.) or MMS-treated (0.1%) cells, either from wild type (Cdc10) or Cdc10.2A strains. Immunoprecipitates were analyzed by Western blot with anti-phosphoserine (α-P-Ser) or anti-Cdc10 antibodies (α-Cdc10). (E) Extracts from the tagged strains indicated on top, untreated (unt) or treated with 0.1% MMS for 60 minutes were immunoprecipitated with α-Myc antibody and analyzed for the presence of Chk1 and Cdc10 with specific
antibodies (HA and Myc, respectively). Left panels, Western blot of the whole cell extracts used in the immunoprecipitations.
Figure 6. Cdc10 phosphorylation after DNA damage is essential for viability. (A) Loading of Cdc10 on cdc22 and cdc18 promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or treated (0.1% MMS, 1h at 30°C) cultures of wild type Cdc10 (WT) or the mutants indicated at the bottom. (B) Phosphorylation of S720 and S732 after IR induce the release of Cdc10 from chromatin. Loading of Cdc10 on cdc22 and cdc18 promoters was measured by ChIP analysis of chromatin extracts isolated from untreated or IR (100Gy) cultures of wild type (WT) and Cdc10.2A cells. The average of three individual experiments (±s.d.) is plotted. (C) Total RNA was prepared from untreated or MMS-treated (increasing doses) cultures of a cdc10.2AΔyox1Δnrm1 strain, and analyzed by hybridization with the probes indicated on the left. rRNA is shown as loading control. (D) RNA was prepared from wild-type (Cdc10) or Cdc10.2A cells exponentially growing or treated with 0.1% MMS for 1 hour. cdc18, cdt2, cdc22 and mik1 was quantitated by RT-qPCR. Results are shown as
fold induction over untreated wild type cells as the average of three individual experiments (±s.d.). (E) Survival was performed by spotting $10^1$ to $10^5$ cells of the indicated strains (in a $Δyox1Δnrml$ background) onto YE5S plates in the absence or presence of MMS or HU. Plates were incubated at 30°C for 3-4 days.
Figure 7. Cartoon depicting a model for the integration of the DNA damage and the DNA replication checkpoint on the MBF complex. Upon replicative stress, fission yeast cells activate the effector kinase Cds1. Among its targets, the repressor Yox1 is phosphorylated, which no longer can bind the MBF complex alleviating the transcriptional repression of genes required for DNA synthesis. Upon DNA damage, the effector kinase Chk1 phosphorylates Cdc10, which is a core component of the MBF complex. The outcome of this phosphorylation is, contrary to what happens under replicative stress, the release of Cdc10 from its target promoters and the repression of MBF-dependent transcription.