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The DNA Replication Checkpoint Directly Regulates MBF-Dependent G_1/S Transcription▽

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The DNA replication checkpoint transcriptionally upregulates genes that allow cells to adapt to and survive replication stress. Our results show that, in the fission yeast *Schizosaccharomyces pombe*, the replication checkpoint regulates the entire G_1/S transcriptional program by directly regulating MBF, the G_1/S transcription factor. Instead of initiating a checkpoint-specific transcriptional program, the replication checkpoint targets MBF to maintain the normal G_1/S transcriptional program during replication stress. We propose a mechanism for this regulation, based on in vitro phosphorylation of the Cdc10 subunit of MBF by the Cds1 replication-checkpoint kinase. Replacement of two potential phosphorylation sites with phosphomimetic amino acids suffices to promote the checkpoint transcriptional program, suggesting that Cds1 phosphorylation directly regulates MBF-dependent transcription. The conservation of MBF between fission and budding yeast, and recent results implicating MBF as a target of the budding yeast replication checkpoint, suggests that checkpoint regulation of the MBF transcription factor is a conserved strategy for coping with replication stress. Furthermore, the structural and regulatory similarity between MBF and E2F, the metazoan G_1/S transcription factor, suggests that this checkpoint mechanism may be broadly conserved among eukaryotes.

In response to inhibition of DNA replication, the replication checkpoint arrests the cell cycle, stabilizes replication forks, and regulates transcription (3, 5). The transcriptional branch of the checkpoint response upregulates genes thought to be important for cells to survive prolonged replication arrest and to synthesize extra deoxynucleotides. A commonly used trigger of the replication checkpoint is treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU), which prevents deoxynucleotide synthesis, thus preventing DNA replication. HU treatment activates a conserved checkpoint signaling pathway (3). In the fission yeast *Schizosaccharomyces pombe*, the central checkpoint kinase Rad3 (the homolog of the budding yeast Mec1 and metazoan ATM and ATR) activates the S-phase checkpoint effector kinase Cds1 (the homolog of the budding yeast Rad53 and Dun1, also called Chk2 in mammals), which regulates the downstream targets of the checkpoint (3). In the budding yeast *Saccharomyces cerevisiae*, Rad53 and Dun1 are thought to control transcription mainly through the Rfx1/Crt1 transcriptional repressor, which regulates a replication and DNA damage checkpoint-specific transcriptional program (21). However, the Rfx1 regulatory circuit is not evolutionarily conserved and it is unclear how the replication checkpoint regulates transcription in other eukaryotes.

During a normal fission yeast cell cycle, G_1/S transcription is regulated by the MBF transcription factor, also known as DSC1, and its negative regulator Nrm1 (1, 10). MBF—including the proteins Cdc10, Res1, Res2, Rep1, and Rep2—and Nrm1 are conserved in budding yeast, which also contains the paralogous SBF-Whi5 transcription factor-repressor proteins (9–11). Furthermore, the MBF/SBF transcription factors and the Nrm1/Whi5 repressors are analogous to the E2F and Rb proteins of metazoans (8, 9, 11). In each of these systems, expression outside of G_1/S phase is repressed by binding of the repressor to the transcription factor and expression during G_1/S phase is allowed by displacement of the repressor (9–11, 19). Although the details of regulation of these systems differ, in many cases phosphorylation of either the transcription factor or the repressor is sufficient to activate transcription. As an example, cell cycle-regulated phosphorylation of either SBF or Whi5 is sufficient to displace Whi5 and activate SBF-dependent transcription (9). Likewise, phosphorylation of either Rb or E2F suffices to activate E2F transcription in mammalian cells (13, 33).

The fission yeast G_1/S transcriptional program comprises about 20 genes expressed in an MBF-dependent manner (25, 28, 30). These genes, even those without obvious checkpoint functions, are also expressed in response to HU treatment (2, 7, 30). This correlation led us to hypothesize that the HU-induced DNA replication checkpoint might regulate transcription by directly regulating MBF. Our hypothesis makes three testable predictions: (i) all MBF-dependent G_1/S transcripts should be upregulated in response to HU in a checkpoint-dependent manner; (ii) mutations in MBF components that affect G_1/S transcription should similarly affect checkpoint-dependent transcription; and (iii) Cds1, the replication-checkpoint effector kinase, should directly regulate MBF activity.

MATERIALS AND METHODS

Strains and cell culture. The following strains were used: yFS104 (*h*$^+$ *leu1-32 ura4-D18*), yFS105 (*h*$^-$ *leu1-32 ura4-D18*), yFS163 (*h*$^-$ *leu1-32 ura4-D18 ade6-210*

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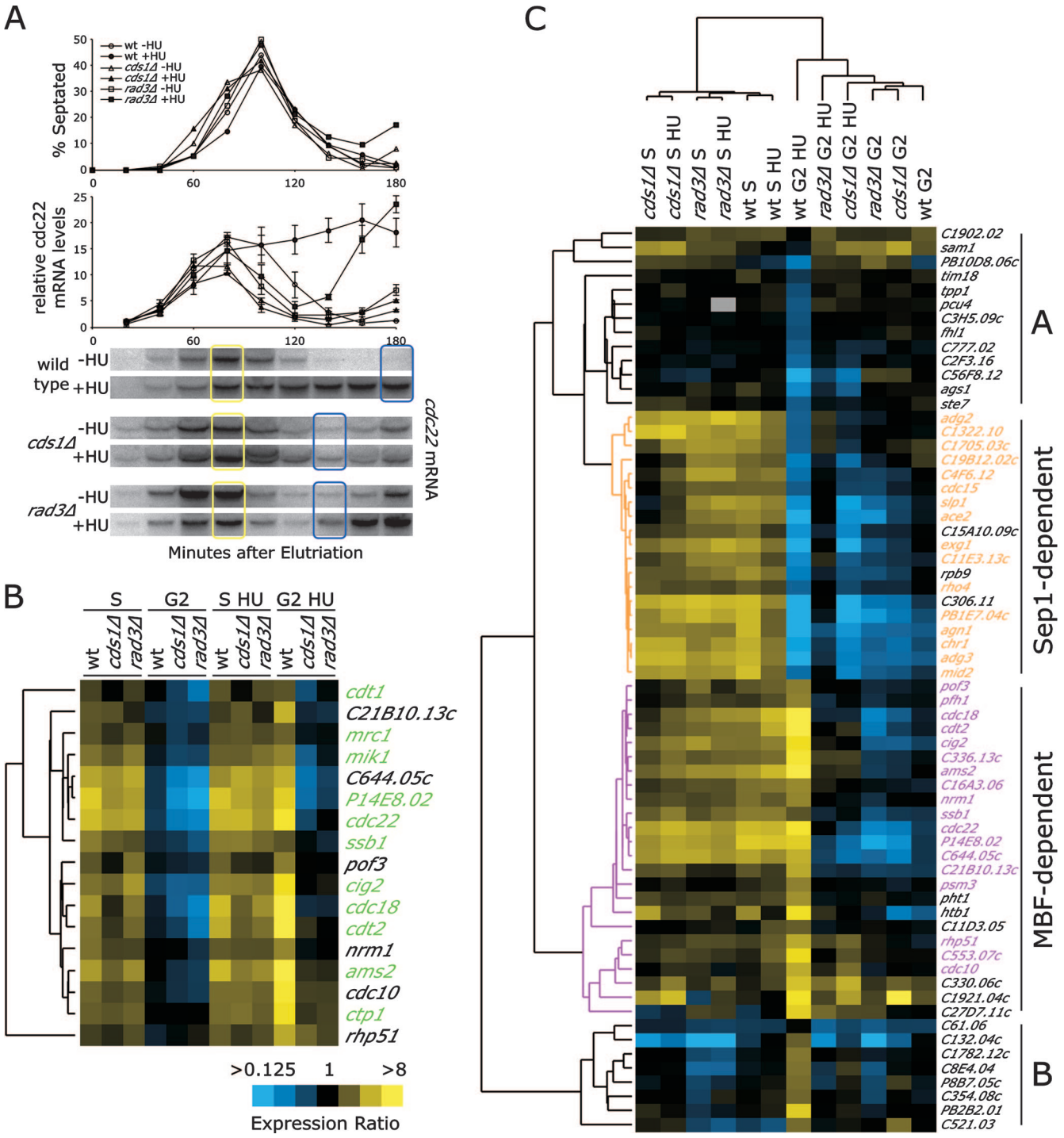

FIG. 1. All MBF-dependent G_1/S transcripts are upregulated by the replication checkpoint. (A) Wild-type (yFS105), *cds1*Δ (yFS199), and *rad3*Δ (yFS189) cells were synchronized in early G_2 phase by centrifugal elutriation and followed through a synchronous cell cycle in the presence or absence of HU. Samples were taken every 20 min for RNA isolation and visual inspection of septation. Northern blots were probed with *cdc22*, stripped, and reprobed with *adh1* as a loading control. The quantitations represent the mean and SEM of the results of three experiments, normalized to the wild-type 20-min time point, except for those for *cds1*Δ, which represent the mean and variance of the results of two experiments. The boxes indicate the points taken for microarray analysis; yellow boxes represent the G_1/S time points and blue boxes represent the G_2 time points. HU-treated *rad3*Δ cells enter a second round of the cell cycle earlier than untreated cells because, having failed to replicate, they have half as much DNA as wild-type cells; because the size at which cells divide is determined by the DNA/cytoplasm ratio and because they lack the replication checkpoint G_2 arrest, HU-treated *rad3*Δ cells divide when they are half the size of untreated cells. (B) Averaged microarray data from two experiments for each set of conditions were clustered to identify transcripts whose levels were elevated in G_1/S phase and were also elevated in the presence of HU in a Rad3-dependent manner. Only the 17 genes meeting those criteria are displayed. The ratio of expression of each gene in the experimental sample to that in an asynchronous control sample is color coded from bright blue to bright yellow. The genes in green type were identified as MBF-dependent genes in two of three previous studies (25, 28, 30). The increase in induction shown by the results of

res1::*ura4*), yFS189 (*h*$^-$ *leu1-32 ura4-D18 ade6-704 rad3*::*ura4*), yFS199 (*h*$^-$ *leu1-32 ura4-D18 cds1*::*ura4*), yFS397 (*h*$^+$ *leu1-32 ura4-D18 nmt1:cds1-D312E:leu1 mik1-13Myc:kanMX6*), yFS252 (*h*$^-$ *ura4-D18 res2*::*ura4*), yFS257 (*h*$^-$ *leu1-32 ade6-M216 cdc10-C4*), yFS502 (*h*$^+$ *leu1-32 ura4-D18 cdc10-8A:kanMX6*), yFS526 (*h*$^-$ *leu1-32 ura4-D18 cdc10:kanMX6*), yFS500 (*h*$^+$ *leu1-32 ura4-D18 cdc10-S720E:kanMX6*), yFS527 (*h*$^+$ *leu1-32 ura4-D18 cdc10-T723E:kanMX6*), yFS528 (*h*$^+$ *leu1-32 ura4-D18 cdc10-2E:kanMX6*), yFS493 (*h*$^+$ *leu1-32 ura4-D18 nrm1*::*kanMX6*), yFS642 (*h*$^+$ *leu1-32 ura4-D18 cdc10-2E:kanMX6 nrm1*::*kanMX6*), yFS532 (*h*$^-$ *leu1-32 ura4-D18 cdc10:kanMX6 rad3*::*ura4*), yFS531 (*h*$^-$ *leu1-32 ura4-D18 cdc10:kanMX6 cds1*::*ura4*), yFS530 (*h*$^-$ *leu1-32 ura4-D18 cds1*::*ura4 cdc10-2E:kanMX6*), and yFS529 (*h*$^+$ *leu1-32 ura4-D18 rad3*::*ura4 cdc10-2E:kanMX6*).

Cells were grown in yeast extract with supplements at 30°C (18). Cells were synchronized by centrifugal elutriation (18). The S and G_2 time points for the wild-type, *cds1*Δ, and *rad3*Δ array experiments whose results are shown in Fig. 1 were chosen as the points of highest and lowest *cdc22* expression in pilot Northern blotting time courses. The G_2 time points are earlier for the checkpoint mutants, because HU-treated *rad3*Δ cells (and, to a lesser extent, *cds1*Δ cells) enter a second round of the cell cycle earlier than untreated cells. This early division occurs because, having failed to replicate, the treated cells have half as much DNA as the untreated cells. Because the size at which cells divide is determined by the DNA/cytoplasm ratio and because they lack the replication checkpoint G_2 arrest, the treated cells divide at half the size of the untreated cells. HU sensitivity was assayed by spotting threefold serial dilutions of cells onto YES plates supplemented with 0, 1, or 3 mM HU and photographing growth after 7 days.

Site-directed alleles of *cdc10* were made by oligonucleotide-mediated mutagenesis. A 3′ fragment of *cdc10* was amplified with CD42 (TATAGACTAGT ACTTCGATCGAAGAACAGAAAAGT) and CD43 (GACTAGTCGGAATT CCCGACTGTTCTTAGCGGCGTATCGG) and cloned into pFA6a-KanMX6 (35). Mutants, along with the 3′ kanamycin resistance marker, were amplified using CD44 (GATGCTGACGCTCCTTTTACTGTC) and PP184 (TTCTTTTT CTGTGGCCTCGCTTTCAAGCTGTCATGGACATGCACTGTGAGTCACT CCGTAAAACTAACTTATCTGTGAAGATCTGTTTAGCTTGCCTCGT) and transformed into yFS104 or yFS105. Accurate integration was confirmed by PCR and sequencing.

RNA analysis. RNA was prepared for Northern blot analysis and microarray analysis as previously described (25). Northern blots were probed with random-prime-labeled *cdc22*, stripped, and reprobed with *adh1*. *cdc22* levels were normalized to that of *adh1*, and then all time courses were normalized to those of the asynchronous wild-type controls included on all gels, such that the 20-min time point for the wild-type time course was set to 1. Microarray analysis was carried out as previously described, using 3′-biased, open reading frame PCR product-spotted arrays (25). Briefly, total RNA from experimental samples was reverse-transcribed with a poly(dT) primer, labeled with Cy3, and cohybridized with similarly prepared asynchronous, wild-type, Cy5-labeled cDNA. Experiments were performed twice, with between two and eight replicate spots per gene per experiment. All raw data are available at ArrayExpress (www.ebi.ac.uk/arrayexpress). Hierarchical clustering was performed with Cluster (16) and visualized with Java TreeView (jtreeview.sourceforge.net). All quantitations are given as means ± standard errors of the means (SEM) except as noted.

To identify the 17 genes shown in Fig. 1B, we clustered the data from the 24 wild-type (yFS105), *rad3*Δ (yFS189), and *cds1*Δ (yFS199) experiments (two experiments each for the G_1/S and G_2 phases with or without HU) for all 821 genes that exhibited an at least twofold increase in signal level in at least four experiments. Of these, only one cluster of 18 genes showed high-level expression in all S-phase samples and in wild-type HU-arrested samples and low-level expression in all G_2 and checkpoint-mutant HU-arrested samples. Of those genes, one was a tRNA deaminase downstream of *nrm1* on the opposite strand and was excluded as a likely read-through artifact. To identify the 64 genes in Fig. 1C, we filtered the same 24 experiments for all genes whose results showed a greater-than-twofold difference (either up or down) in signal level between wild-type G_2 and wild-type HU-arrested cells (to find HU-induced transcripts) and a greater-than-twofold difference in signal level between wild-type HU-arrested and *rad3*Δ HU-arrested cells (to find checkpoint-dependent transcripts). We did not include G_1/S comparisons in this filter, because we did not want to exclude potential genes that might be checkpoint regulated but not G_1/S regulated.

In vitro kinase assay. The Cdc10 C-terminal 61 codons were PCR amplified and cloned as a glutathione *S*-transferase (GST) fusion into the pGEX-3X BamHI and EcoRI sites; site-directed mutations were made by oligonucleotide-directed mutagenesis and verified by sequencing. Cds1 in vitro kinase assays were performed as previously described (22). Briefly, GST-Cdc10, expressed in *Escherichia coli* and purified on glutathione beads, was incubated for 15 min at 30°C with γ-[^{32}P]ATP and Cds1 immunopurified from HU-treated *S. pombe*. Labeled protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

All MBF transcripts are upregulated by the replication checkpoint. To test our first prediction, we used a whole-genome open reading frame microarray to assay the replication checkpoint transcriptional response. Previous work had shown that the entire MBF-regulated transcription cluster is induced by HU treatment but had not addressed the role of the checkpoint (30). To do so, we synchronized wild-type, *rad3*Δ cells—which lack the central checkpoint kinase—and *cds1*Δ cells—which lack the replication-checkpoint effector kinase activated by Rad3—and followed the cultures through a synchronous cell cycle in the absence or presence of HU (Fig. 1A). We followed the levels of the *cdc22* G_1/S transcript, encoding the large subunit of ribonucleotide reductase, by Northern blotting. Samples were collected from time points in which the untreated cells were in S phase or G_2, and RNA was labeled and hybridized to microarrays. Using hierarchical clustering, we identified 17 genes that were upregulated at least twofold in G_1/S phase and also upregulated at least twofold in response to HU in a Rad3-dependent manner (Fig. 1B). All of these genes are MBF-dependent transcripts, defined as those genes expressed at the G_1/S phase in an MBF-dependent manner in one of three published fission yeast cell cycle transcription experiments (25, 28, 30). Furthermore, of the 14 core G_1/S MBF-dependent transcripts, defined as those identified in at least two of the three data sets, 11 were identified as transcriptional targets of the replication checkpoint; the other 3 were excluded from the cluster because of the low amplitude of their signals. These results suggest that the entire MBF transcriptional program is upregulated by the checkpoint. Similar results have been recently reported (7).

We also wanted to determine whether any non-G_1/S genes are regulated by the checkpoint in response to the presence of HU. We identified 64 genes whose transcript levels showed a Rad3-dependent change of at least twofold in response to HU. Hierarchical clustering identified four groups of genes (Fig. 1C). One of these groups consisted mainly of the MBF-dependent transcripts described above. Another group consisted

these experiments is lower that shown in panel A because the comparison is to asynchronous cells, which have higher levels of MBF-dependent transcripts than the G_2 cells used as a baseline in panel A. wt, wild type. (C) The same data were filtered to identify transcripts that showed a Rad3-dependent change of at least twofold in response to HU. The resulting 64 transcripts were clustered, revealing the four groups described in the text. The genes in orange type are those previously identified as Sep1-dependent genes; those in purple type have been identified as MBF-dependent genes (25, 28, 30). In this analysis, the experiments were clustered in order to emphasize that the S-phase samples for a given gene in the Sep1- and MBF-dependent clusters behaved similarly, as did the checkpoint mutant G_2 samples. In contrast, those in clusters A and B show less change in transcript levels and less correlation within the S-phase and G_2 samples.

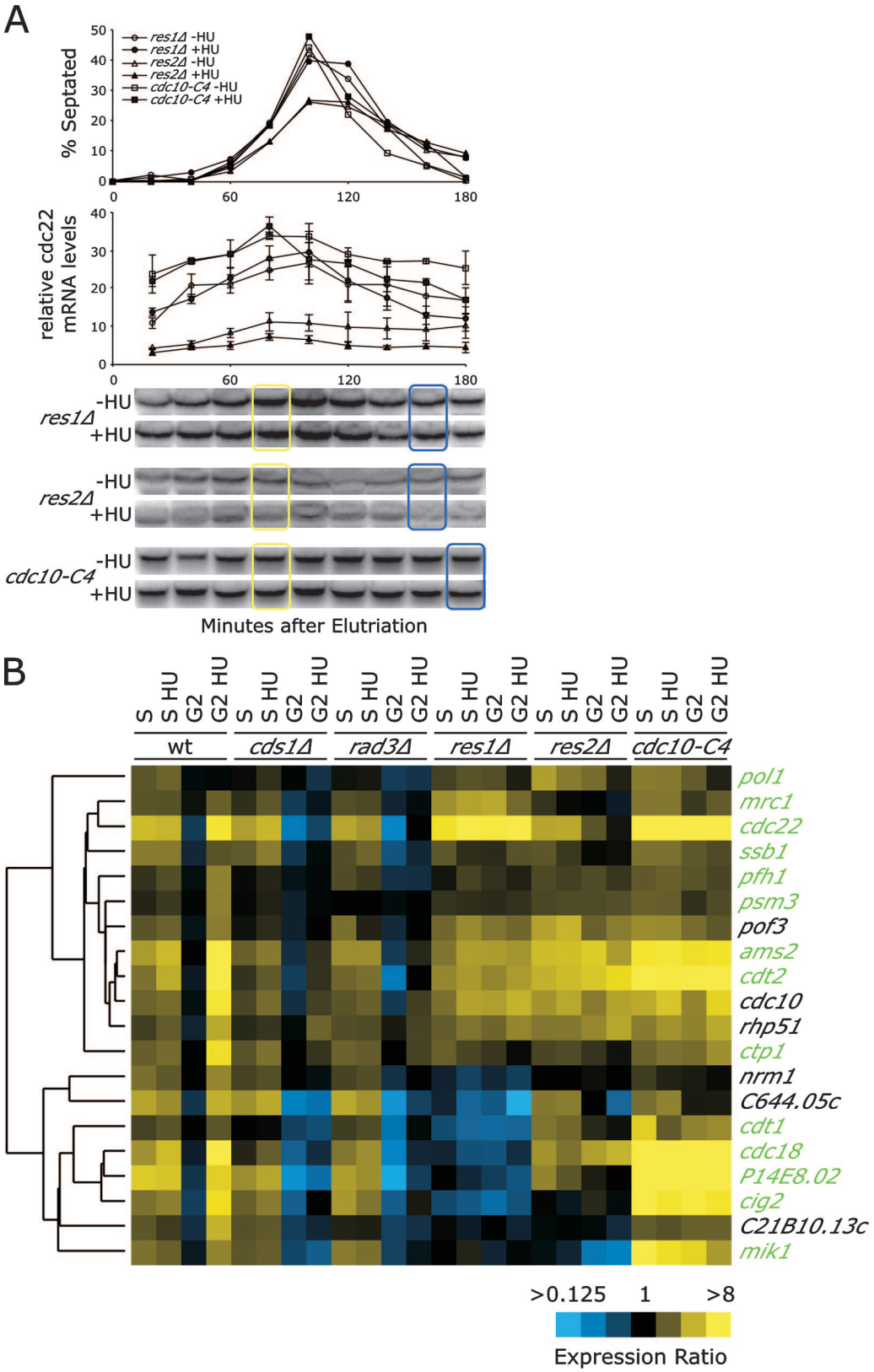

FIG. 2. Mutations in subunits of MBF affect checkpoint-dependent transcription. (A) Cell cycle and *cdc22* transcriptional profiles of *res1*Δ (yFS163), *res2*Δ (yFS252), and *cdc10-C4* (yFS257) cultures were analyzed as described for Fig. 1A. The quantitations for *res1*Δ represent the mean and SEM of the results of three experiments; the quantitations for *res2*Δ and *cdc10-C4* represent the mean and variance of the results of two experiments. (B) Data for the 14 core MBF-dependent genes (green type) and the 6 other MBF-dependent genes identified in Fig. 1B were found to be clustered across all of the represented experiments. wt, wild type.

mainly of Sep1-dependent transcripts. These genes, which encode proteins involved in mitosis and cytokinesis, are upregulated during mitosis (25, 28, 30). Presumably, they are indirectly downregulated by the replication checkpoint as a consequence of the G_2 arrest, which prevents cells from entering mitosis. This conclusion is bolstered by the fact these genes are also downregulated in *cds1*Δ cells, which arrest in G_2 phase due to Rad3-dependent activation of Chk1 (another checkpoint effector kinase), but not in *rad3*Δ cells, which fail to arrest in G_2 phase and therefore septate even in the presence of HU (4). The latter two groups, labeled A and B in Fig. 1C, comprise genes that are either high or low in wild-type, HU-treated G_2 cells and largely unchanged under all other conditions. We suspect that these signals are spurious for three reasons: (i) the clusters are not enriched in genes of any particular biological functions; (ii) these genes show lower signal amplitude and higher variance compared to the HU-treated sample results than genes in the other clusters (2.3-fold ± 1.8-fold for clusters A and B versus 4.9-fold ± 1.6-fold for the other two clusters), suggesting that they represent a low level of noise in our analysis; and (iii) only 3 of the 21 genes were identified by Chu et al. as HU regulated (7). From these results, we conclude that the replication checkpoint does not directly regulate the levels of any genes other than those of the MBF-dependent transcripts.

Mutations in MBF affect checkpoint-dependent transcription. To test our second prediction—that mutations in MBF components affecting G_1/S transcription should similarly affect checkpoint-dependent transcription—we examined the checkpoint response in cells lacking Res1 or Res2, redundant DNA-binding subunits of MBF, and in cells carrying *cdc10-C4*, a dominant activating allele of the essential Cdc10 subunit of MBF (24). We found that all three alleles greatly reduced cell cycle regulation of the MBF-dependent genes, reducing the amplitude of *cdc22* regulation from over 15-fold in wild-type cells to about 2-fold in the mutants, with *res2*Δ cells showing constitutively low levels and *res1*Δ and *cdc10-C4* showing constitutively high levels, as is consistent with previous results (7, 36) (Fig. 2A). As predicted by our hypothesis, *cdc22* was not checkpoint regulated in any of the mutant strains. We saw a similar lack of checkpoint regulation across the MBF-dependent transcripts by microarray analysis (Fig. 2B). These results show that the checkpoint is unable to regulate transcription in the absence of a functional MBF transcription factor and suggest that MBF activity is regulated by the checkpoint.

Cds1 phosphorylates Cdc10 at specific C-terminal sites in vitro. Our final prediction is that Cds1 directly regulates MBF activity, possibly through direct phosphorylation. Our attention was drawn to the 61 C-terminal amino acids deleted in *cdc10-C4* (24). Since removal of this sequence constitutively activates Cdc10, we imagined that inhibitory Cds1-dependent phosphorylation of the sequence could similarly activate MBF. Furthermore, the sequence contains seven serines and a threonine capable of being phosphorylated by Cds1. To test whether this region is a potential Cds1 substrate, we expressed it as a GST fusion in *E. coli* and used it as an in vitro kinase substrate for Cds1 immunopurified from HU-arrested fission yeast. We found that HU-activated Cds1 efficiently phosphorylated the wild-type fusion protein but failed to do so when serine 720 (S720) was mutated to alanine (Fig. 3A). Approximately 85% of in vitro phosphorylation requires S720; the rest requires S732 and/or S736. As is consistent with these results, S720 and S732 are the only serines in the C terminus found in R-x-x-S motifs, which are favored by Cds1-related kinases (26, 31).

Phosphomimetic mutations of Cdc10 are sufficient to induce constitutive MBF transcription. We turned to genetics to investigate the in vivo relevance of Cdc10 C-terminal phosphorylation in the checkpoint. We made a series of site-directed mutant constructs to test whether phosphorylation is necessary or sufficient for checkpoint regulation of MBF in vivo. These mutations were used to replace the wild-type copy of *cdc10* at its endogenous locus. To test whether Cdc10 C-terminal phosphorylation is necessary, we mutated all seven serines and the threonine to alanine or glycine. We found that this allele, *cdc10-8A*, exhibited no significant defect in checkpoint regulation of transcription (Fig. 3B, C, and D). We suspect that this lack of phenotype was due to redundant phosphorylation of Cdc10 and its repressor Nrm1, as discussed below.

To test whether Cdc10 C-terminal phosphorylation is sufficient for checkpoint regulation of transcription, we made serine-to-glutamate mutations, which imperfectly mimic phosphorylation. We analyzed a variety of mutant combinations and obtained positive results with S720, the major in vitro phosphorylation site, and T723, which is in the context of T-x-x-D, a putative Cds1 recognition motif (15). We expect mutations that mimic checkpoint phosphorylation to cause constitutive expression of MBF-dependent transcripts. Indeed, the double S720E T723E mutant, which we call *cdc10-2E*, and *cdc10-8E*, in which all eight potential phosphorylation sites are changed to glutamate, both showed constitutive *cdc22* and overall MBF-dependent transcript levels comparable to wild-type checkpoint-induced levels (Fig. 3B, C, and D and unpublished results). Furthermore, the levels of *cdc22* and overall MBF-dependent transcripts in *cdc10-2E* were not markedly increased by HU treatment. The single S720E mutation showed no significant constitutive increase in MBF-dependent transcript levels; the single T723E mutation showed a partial, approximately twofold increase in MBF-dependent transcript levels in the absence of HU (Fig. 3B, C, and D). From these results we conclude that phosphorylation of both S720 and T723 is most likely sufficient for checkpoint regulation of transcription.

The lack of a checkpoint-transcriptional phenotype for *cdc10-8A* and the constitutive checkpoint-transcriptional phenotype of *cdc10-2E* are consistent with the possibility of redundant phosphorylation of Cdc10 and Nrm1, either of which would suffice to disrupt Nrm1 inhibition of Cdc10 and activate MBF-dependent transcription. This model predicts that Nrm1 should have no function in *cdc10-2E* cells. To test this prediction, we compared *cdc22* transcript levels in *cdc10-2E*, *nrm1*Δ, and *cdc10-2E nrm1*Δ cells in both synchronous and asynchronous cultures. As predicted, *cdc10-2E* and *nrm1*Δ showed equivalently high levels of *cdc22* transcripts and the deletion of *nrm1* in a *cdc10-2E* background did not further elevate *cdc22* levels (Fig. 3E and data not shown).

***cdc10-2E* confers resistance to HU.** The replication checkpoint has three known functions: the role investigated here in maintaining the G_1/S-phase transcriptional program, a well-understood role in preventing mitosis, and a less-well-understood role in stabilizing stalled replication forks (3). To inves-

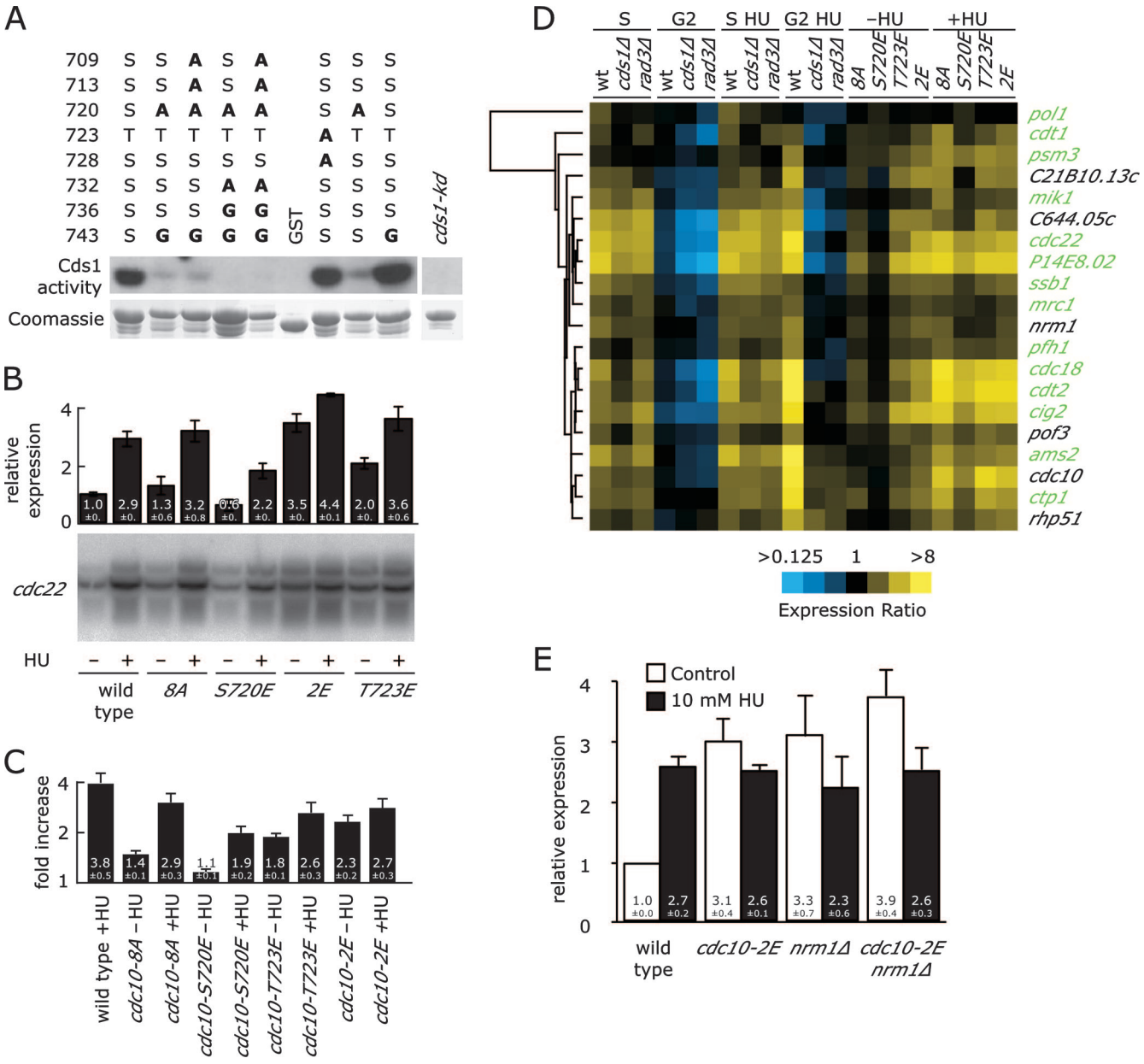

FIG. 3. Phosphomimetic substitutions of Cds1 phosphorylation sites in the C terminus of Cdc10 suffice to promote the checkpoint transcriptional program. (A) The 61 C-terminal amino acids of Cdc10 fused to GST, and the indicated S-to-A and S-to-G mutations, were used as in vitro substrates for Cds1 kinase immunopurified from wild-type (yFS105) or *cds1-D312E* (yFS397, *cds1* kinase dead [*cds1-kd*]) cells treated for 4 h with 10 mM HU. (B) Northern analysis of *cdc22* transcript levels in asynchronous wild-type (yFS105), *cdc10-8A* (yFS502), *cdc10-S720E* (yFS500), *cdc10-2E* (yFS528), and *cdc10-T723E* (yFS527) cells left untreated or treated with 10 mM HU for 4 h. The quantitation data represent the mean and SEM of the results of between three and six experiments, normalized to *adh1* and untreated wild-type results. The amplitude of the induction shown is lower than that shown in Fig. 1A because the data are normalized to asynchronous wild-type cells instead of to G_2 wild-type cells. (C) Quantitation of the array data shown in panel D. Average expression levels of the 20 genes relative to those of asynchronous wild-type cells are shown. (D) Data for the 20 genes examined as described for Fig. 2B were clustered across the all of the represented experiments. The data represent the averages of the results of two independent experiments. wt, wild type. (E) Northern analysis of *cdc22* transcript levels in asynchronous wild-type (yFS105), *cdc10-2E* (yFS528), *nrm1*Δ (yFS528), and *cdc10-2E nrm1*Δ (yFS528) cells left untreated or treated with 10 mM HU for 4 h.

tigate the importance of checkpoint-mediated transcription relative to the other functions, we built strains that lacked all three functions (*rad3*Δ) or that lacked just the transcription and fork stabilization functions (*cds1*Δ) and induced constitutive checkpoint signaling in these backgrounds with *cdc10-2E*. We tested these strains for sensitivity to moderate levels of HU and found that elevated levels of MBF-dependent transcripts made cells significantly more resistant to this treatment (Fig. 4). Specifically, the restoration of checkpoint-induced levels of MBF-dependent transcripts allowed *rad3*Δ cells to survive in the presence of 1 mM HU and *cds1*Δ cells to survive at 3 mM HU, conditions under which they cannot survive normally.

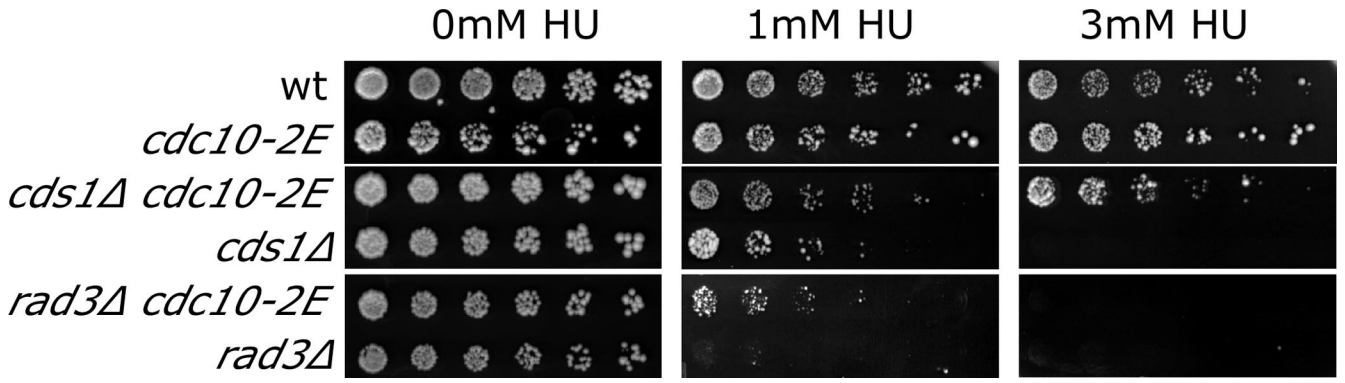

FIG. 4. The role of checkpoint-dependent transcription in surviving replicative stress. Wild-type (yFS526), *cdc10-2E* (yFS528), *cds1Δ44* (yFS531), *cds1Δ44 cdc10-2E* (yFS530), *rad3Δ44* (yFS532), *rad3Δ44 cdc10-2E* (yFS529) cells were threefold serially diluted on to YES plates supplemented with 0, 1 or 3 mM HU and photographed after 7 days of growth.

These results indicate that checkpoint-mediated transcriptional response plays an important adjunct role in the survival of replicative stress, in addition to the known role of the checkpoint in the maintenance of replication fork stability and the prevention of premature mitosis (14, 17).

DISCUSSION

Our results demonstrate that the fission yeast replication checkpoint regulates the MBF G_1/S transcription factor to maintain the normal G_1/S transcriptional program during replication stress. This conclusion is supported by three lines of evidence. First, all MBF transcripts and only MBF transcripts are upregulated by the checkpoint in response to HU arrest (Fig. 1). Second, mutations in the Res1, Res2, and Cdc10 MBF subunits affect checkpoint-regulated transcription in the same manner in which they affect normal G_1/S transcription (Fig. 2). Third, phosphomimetic mutations of sites of in vitro Cds1 phosphorylation, in the allele we call *cdc10-2E*, cause constitutive G_1/S transcription in vivo (Fig. 3). In addition, restoring a sustained high level of G_1/S transcription with *cdc10-2E* to *rad3*Δ and *cds1*Δ cells, which normally lack checkpoint-induced transcription, modestly increases their resistance to HU, demonstrating the in vivo relevance of the response (Fig. 4). Chu et al. reported that the overexpression of the MBF Rep2 subunit partially rescues the HU sensitivity of *cds1*Δ and *rad3*Δ cells, much as expression of *cdc10-2E* does (7). We speculate that overexpression of Rep2 may phenocopy the constitutive expression seen in *cdc10-2E* and suppress HU sensitivity by the same mechanism.

Our results also shed light on the roles of the Res1 and Res2 DNA binding subunits of MBF. Res1 and Res2 have been proposed to be activating and repressing subunits, respectively (2). Our array data show that the situation is more complicated, with each protein required for the activation and repression of a different subset of genes (Fig. 2B). For instance, *cdc22* is upregulated in *res1*Δ cells, while *cdc18* is downregulated.

Our results suggest that Cds1 regulates MBF by phosphorylating the C terminus of Cdc10; however, we have been unable to detect such phosphorylation in vivo. To detect checkpoint-dependent in vivo phosphorylation of Cdc10, we tried a number of approaches, including Western blot analysis with a variety of one-dimensional polyacryamide gel electrophoresis systems under a variety of conditions, two-dimensional isoelectric focusing–sodium dodecyl sulfate-polyacryamide gel electrophoresis, phosphoepitope affinity purification, and mass spectroscopy, all with only negative results. In particular, although we have been able to detect phosphorylation of Cdc10, we have found no evidence for checkpoint-dependent phosphorylation or C-terminal-specific phosphorylation. We believe two factors may have contributed to our inability to detect in vivo checkpoint-dependent phosphorylation of Cdc10. The first is that Cdc10 is multiply phosphorylated throughout the cell cycle; therefore, the addition of one or two extra checkpoint-dependent phosphates may not greatly affect its overall phosphorylation state or its mobility on a polyacrylamide gel (32). The second is that sites on Cdc10 phosphorylated by Cds1 during the checkpoint activity may also be phosphorylated during normal S phase. Use of normal regulatory phosphorylation sites by checkpoint kinases is certainly the case for Cdc25 in the G_2 DNA damage checkpoint. The sites phosphorylated on Cdc25 during the G_2 checkpoint are the same sites used by other kinases to regulate the G_2/M transition in the normal cell cycle (27). Therefore, G_2 checkpoint activation does not increase the phosphorylation of Cdc25, it merely maintains normal G_2 phosphorylation in a checkpoint-dependent manner. If Cdc10 were regulated in a similar manner, we would not expect to see a checkpoint-dependent change in phosphorylation.

Our analysis of *cdc10-2E* suggests that phosphorylation of Cdc10's C terminus is sufficient to activate G_1/S transcription. However, the fact that replication checkpoint control of MBF is intact in *cdc10-8A*, which cannot be phosphorylated by Cds1 on its C terminus, shows that such phosphorylation is not necessary for checkpoint regulation. We hypothesize that phosphorylation of either Cdc10 or the Nrm1 MBF repressor, which binds to and inhibits MBF in G_2 phase (10), is sufficient for checkpoint regulation of MBF. In the paralogous SBF-Whi5 system, Whi5 repression of SBF is relieved by CDK phosphorylation, but phosphorylation of either Whi5 or SBF is sufficient to disrupt binding (9, 11). Likewise, Cds1 phosphorylates Nrm1 in response to the presence of HU (12), which may suffice for checkpoint regulation in the *cdc10-8A* cells. This model suggests that, while not necessary for checkpoint

regulation, phosphorylation of Cdc10 is sufficient for checkpoint regulation through disruption of the binding and inhibition of MBF by Nrm1. As is consistent with this model, *cdc10-2E* and *nrm1*Δ have similar and nonadditive transcriptional phenotypes (Fig. 3E).

Our analysis of the role of MBF at the fission yeast replication checkpoint reveals a very different regulatory logic from that employed by budding yeast. The major transcriptional response to replication stress in budding yeast is a checkpoint-specific, Rfx1-dependent induction of the *RNR* genes, which are induced to 10-fold-higher levels during an HU arrest than during a normal S phase (21). This apparently budding-yeast-specific transcriptional response is in marked contrast to the fission yeast strategy of simply maintaining normal G_1/S transcription and may explain why budding yeast can tolerate 10-fold-higher levels of HU than fission yeast. However, results of genomic analysis of the budding yeast replication stress response and recent work using the budding yeast Nrm1 suggest that MBF is a conserved checkpoint target in yeast (10, 12, 20). Therefore, the checkpoint regulation of MBF may be conserved in budding yeast but largely obscured by the more dramatic Rfx1-dependent response.

The fission yeast regulatory mechanism places some genes under checkpoint control that have no obvious checkpoint function, such as the origin-licensing genes *cdc18* and *cdt1*. But it also assures the continued expression of the three genes known to be important for the survival of replication stress: *cdc22*, encoding the large subunit of ribonucleotide reductase; *mik1*, encoding a mitosis-inhibiting kinase (6, 29); and *mrc1*, encoding the Cds1 mediator (34). In addition, several other genes that have plausible roles in replication stress are also regulated, including *ssb1*, encoding the large subunit of the RPA single-strand DNA binding protein; *rph51*, encoding the Rad51 recombinase; *ctp1*, encoding a subunit of the MRN recombinational repair complex (23); and *pfh1*, encoding a putative repair helicase.

It has long been recognized that the G_1/S transcription factors—MBF in fission yeast, MBF and SBF in budding yeast, and E2F in metazoa—share common domain structures, but the similarity of their cell cycle regulation characteristics has only recently been appreciated (8–11). In particular, MBF appears to be regulated by Nrm1 in much the same way that E2F is regulated by Rb. Because HU treatment of primary human cells causes extensive phosphorylation of Rb and because Chk2, the human homolog of Cds1, can phosphorylate and activate E2F, we propose that checkpoint regulation of transcription through Nrm1/Rb-MBF/E2F is likely be conserved across eukaryotes (13, 33).

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REFERENCES

1. **Bähler, J.** 2005. Cell-cycle control of gene expression in budding and fission yeast. Annu. Rev. Genet. **39:**69–94.
2. **Baum, B., J. Wuarin, and P. Nurse.** 1997. Control of S-phase periodic transcription in the fission yeast mitotic cycle. EMBO J. **16:**4676–4688.
3. **Boddy, M. N., and P. Russell.** 2001. DNA replication checkpoint. Curr. Biol. **11:**R953–R956.
4. **Brondello, J. M., M. N. Boddy, B. Furnari, and P. Russell.** 1999. Basis for the checkpoint signal specificity that regulates Chk1 and Cds1 protein kinases. Mol. Cell. Biol. **19:**4262–4269.
5. **Carr, A. M.** 1997. Control of cell cycle arrest by the Mec1sc/Rad3sp DNA structure checkpoint pathway. Curr. Opin. Genet. Dev. **7:**93–98.
6. **Christensen, P. U., N. J. Bentley, R. G. Martinho, O. Nielsen, and A. M. Carr.** 2000. Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis. Proc. Natl. Acad. Sci. USA **97:**2579–2584.
7. **Chu, Z., J. Li, M. Eshaghi, X. Peng, R. K. Karuturi, and J. Liu.** 2007. Modulation of cell cycle-specific gene expressions at the onset of S phase arrest contributes to the robust DNA replication checkpoint response in fission yeast. Mol. Biol. Cell **18:**1756–1767.
8. **Cooper, K.** 2006. Rb, whi it's not just for metazoans anymore. Oncogene **25:**5228–5232.
9. **Costanzo, M., J. L. Nishikawa, X. Tang, J. S. Millman, O. Schub, K. Breitkreuz, D. Dewar, I. Rupes, B. Andrews, and M. Tyers.** 2004. CDK activity antagonizes Whi5, an inhibitor of G_1/S transcription in yeast. Cell **117:**899–913.
10. **de Bruin, R. A., T. I. Kalashnikova, C. Chahwan, W. H. McDonald, J. Wohlschlegel, J. R. Yates, P. Russell, and C. Wittenberg.** 2006. Constraining G_1-specific transcription to late G_1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback. Mol. Cell **23:**483–496.
11. **de Bruin, R. A., W. H. McDonald, T. I. Kalashnikova, J. R. Yates, and C. Wittenberg.** 2004. Cln3 activates G_1-specific transcription via phosphorylation of the SBF bound repressor Whi5. Cell **117:**887–898.
12. **de Bruin, R. A. M., T. I. Kalashnikova, J. A. Wohlschlegel, C. Chahwan, J. Yates, P. Russell, and C. Wittenberg.** 5 August 2008. The DNA replication checkpoint promotes G_1/S transcription by inactivating the MBF repressor Nrm1. Proc. Nat. Acad. Sci. USA. doi:10.1073/pnas.0801106105.
13. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C. M. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085–1095.
14. **Desany, B. A., A. A. Alcasabas, J. B. Bachant, and S. J. Elledge.** 1998. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. **12:**2956–2970.
15. **Durocher, D., I. A. Taylor, D. Sarbassova, L. F. Haire, S. L. Westcott, S. P. Jackson, S. J. Smerdon, and M. B. Yaffe.** 2000. The molecular basis of FHA domain: phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell **6:**1169–1182.
16. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA **95:**14863–14868.
17. **Enoch, T., and P. Nurse.** 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell **60:**665–673.
18. **Forsburg, S. L., and N. Rhind.** 2006. Basic methods for fission yeast. Yeast **23:**173–183.
19. **Frolov, M. V., and N. J. Dyson.** 2004. Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J. Cell Sci. **117:**2173–2181.
20. **Gasch, A. P., M. Huang, S. Metzner, D. Botstein, S. J. Elledge, and P. O. Brown.** 2001. Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p. Mol. Biol. Cell **12:**2987–3003.
21. **Huang, M., Z. Zhou, and S. J. Elledge.** 1998. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell **94:**595–605.
22. **Kai, M., H. Tanaka, and T. S. Wang.** 2001. Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures. Mol. Cell. Biol. **21:**3289–3301.
23. **Limbo, O., C. Chahwan, Y. Yamada, R. A. de Bruin, C. Wittenberg, and P. Russell.** 2007. Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol. Cell **28:**134–146.
24. **McInerny, C. J., P. J. Kersey, J. Creanor, and P. A. Fantes.** 1995. Positive and negative roles for cdc10 in cell cycle gene expression. Nucleic Acids Res. **23:**4761–4768.
25. **Oliva, A., A. Rosebrock, F. Ferrezuelo, S. Pyne, H. Chen, S. Skiena, B.**

Futcher, and J. Leatherwood. 2005. The cell cycle-regulated genes of *Schizosaccharomyces pombe*. PLoS Biol. **3:**e225.

26. **O'Neill, T., L. Giarratani, P. Chen, L. Iyer, C. H. Lee, M. Bobiak, F. Kanai, B. B. Zhou, J. H. Chung, and G. A. Rathbun.** 2002. Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. J. Biol. Chem. **277:**16102–16115.
27. **Peng, C. Y., P. R. Graves, S. Ogg, R. S. Thoma, M. J. Byrnes III, Z. Wu, M. T. Stephenson, and H. Piwnica-Worms.** 1998. C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. Cell Growth Differ. **9:**197–208.
28. **Peng, X., R. K. Karuturi, L. D. Miller, K. Lin, Y. Jia, P. Kondu, L. Wang, L. S. Wong, E. T. Liu, M. K. Balasubramanian, and J. Liu.** 2005. Identification of cell cycle-regulated genes in fission yeast. Mol. Biol. Cell **16:**1026–1042.
29. **Rhind, N., and P. Russell.** 2001. Roles of the mitotic inhibitors Wee1 and Mik1 in the G_2 DNA damage and replication checkpoints. Mol. Cell. Biol. **21:**1499–1508.
30. **Rustici, G., J. Mata, K. Kivinen, P. Lió, C. J. Penkett, G. Burns, J. Hayles, A. Brazma, P. Nurse, and J. Bähler.** 2004. Periodic gene expression program of the fission yeast cell cycle. Nat. Genet. **36:**809–817.
31. **Seo, G. J., S. E. Kim, Y. M. Lee, J. W. Lee, J. R. Lee, M. J. Hahn, and S. T. Kim.** 2003. Determination of substrate specificity and putative substrates of Chk2 kinase. Biochem. Biophys. Res. Commun. **304:**339–343.
32. **Simanis, V., and P. Nurse.** 1989. Characterization of the fission yeast $cdc10^+$ protein that is required for commitment to the cell cycle. J. Cell Sci. **92:**51–56.
33. **Stevens, C., L. Smith, and N. B. La Thangue.** 2003. Chk2 activates E2F-1 in response to DNA damage. Nat. Cell Biol. **5:**401–409.
34. **Tanaka, K., and P. Russell.** 2001. Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. Nat. Cell Biol. **3:**966–972.
35. **Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:**1793–1808.
36. **Whitehall, S., P. Stacey, K. Dawson, and N. Jones.** 1999. Cell cycle-regulated transcription in fission yeast: Cdc10-Res protein interactions during the cell cycle and domains required for regulated transcription. Mol. Biol. Cell **10:**3705–3715.