DNA replication checkpoint promotes G_1-S transcription by inactivating the MBF repressor Nrm1

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Edited by John B. Gurdon, University of Cambridge, Cambridge, United Kingdom, and approved June 3, 2008 (received for review February 3, 2008)

The cell cycle transcriptional program imposes order on events of the cell-cycle and is a target for signals that regulate cell-cycle progression, including checkpoints required to maintain genome integrity. Neither the mechanism nor functional significance of checkpoint regulation of the cell-cycle transcription program are established. We show that Nrm1, an MBF-specific transcriptional repressor acting at the transition from G_1 to S phase of the cell cycle, is at the nexus between the cell cycle transcriptional program and the DNA replication checkpoint in fission yeast. Phosphorylation of Nrm1 by the Cds1 (Chk2) checkpoint protein kinase, which is activated in response to DNA replication stress, promotes its dissociation from the MBF transcription factor. This leads to the expression of genes encoding components that function in DNA replication and repair pathways important for cell survival in response to arrested DNA replication.

cell cycle | *Schizosaccharomyces pombe* | ATR | Cds1 | CHK1

Cell proliferation in all organisms depends on the faithful temporal execution of the events of the cell division cycle. Periodic expression of large families of genes during the cell cycle is one of the primary cellular mechanisms imposing orderly progression of cell cycle events. The transcriptional regulatory network is, in turn, a target for internal and environmental signals including checkpoints that can restrain cell cycle progression.

Cell cycle checkpoints typically serve two functions. First, they make the execution of cell cycle events contingent upon satisfaction of specific criteria. Second, they facilitate satisfaction of those criteria and promote conditions for cell cycle progression. When these checkpoint responses fail, cells are at risk of increased mutation, chromosome rearrangement, and chromosome loss, phenomena that are associated with transformation and malignancy. The importance of this regulation is illustrated by the fact that the mechanism that halts cell cycle progression in the presence of incomplete DNA replication and DNA damage is mediated by an evolutionarily conserved subfamily of protein kinases including ATM and the closely related protein ATR in mammals, Rad3 in fission yeast (*Schizosaccharomyces pombe*), and Mec1 in budding yeast (*Saccharomyces cerevisiae*) (1–4). These protein kinases exert their effects largely through checkpoint effector protein kinases: Chk1 and Chk2 in mammals, Cds1 and Chk1 in fission yeast, and Rad53 in budding yeast. These checkpoint effectors arrest cell division by phosphorylating cell cycle regulatory proteins. They also regulate gene expression, presumably by phosphorylating transcription factors, to ensure a sufficient supply of proteins whose activity is necessary for recovery from arrested DNA replication.

In fission yeast, the Cds1 protein kinase is activated primarily in response to stalled or collapsed DNA replication forks during S phase, whereas Chk1 is primarily activated in response to DNA damage outside of S phase (5, 6). Persistent expression of G_1-S-specific genes occurs in cells arrested in S phase with incompletely replicated DNA (7) and depends on functional Cds1 (8). We report that Cds1 phosphorylates the MBF-associated corepressor Nrm1 in response to replication fork stalling, thereby activating G1/S gene expression.

Results

Nrm1 Binds G_1-S Promoters via MBF. In fission yeast, regulation of G_1-S transcription depends upon MBF, a heteromeric transcription factor that is composed of Cdc10 and two sequence-specific DNA-binding proteins, Res1 and Res2, each of which recognize the Mlu1 cell cycle box (MCB) sequence motif (9). MBF's repressor activity outside of G_1-S depends on its interaction with the transcriptional corepressor, Nrm1 (10). Nrm1 is itself regulated by MBF and associates with it to constrain transcription to G_1-S via negative feedback. This role of Nrm1 is conserved between the distantly related fission yeast and budding yeast. Mass spectrometry-based MultiDimensional Protein Interaction Technology (MuDPIT) (11) analysis of affinity-purified Res2 and Nrm1 confirmed that Nrm1 is a component of MBF (Fig. 1*A*). Nrm1 interacted with Cdc10 but not with Res1 in the absence of Res2 [supporting information (SI) Figs. S1 and S2] and, conversely, deletion of Res1 abrogated the interaction between Nrm1 and Res2 (Fig. S3). These results are consistent with Nrm1 binding MBF through the Cdc10 component. Furthermore, the *cdc10-C4* mutant, which eliminates the C-terminal 61 aa of Cdc10 (12), abrogated MBF-binding to Nrm1 (Fig. S1). Despite the loss of the C terminus, the Cdc10-C4 protein retained the ability to bind to the well established MBF target promoter *cdc22*$^+$. However, Nrm1 failed to bind to this promoter in that mutant (Fig. 1*B*), and the mutant cells constitutively expressed MBF target genes throughout the cell cycle (7, 12). These data support our observation that Nrm1 is required for repression of MBF-regulated promoters outside of G_1-S phase (10).

Consistent with the requirement for Nrm1 for repression of G_1-S transcription as cells enter S phase, the timing of Nrm1 accumulation and association with the MBF target promoter *cdc22*$^+$ during the cell cycle is coincident with inactivation of MBF-regulated transcription (Figs. 1*C* and 2*E*). Furthermore, binding of Nrm1 to the MBF target promoters *cdc22*$^+$ and *cdc18*$^+$ depends on its DNA binding component Res2 (Fig. 1*D*). This is despite the capacity of Nrm1 to bind to Cdc10 without Res2 (Fig. S1). Thus, Nrm1 binding to DNA appears to require intact MBF.

Author contributions: R.A.M.d.B., C.C., P.R., and C.W. designed research; R.A.M.d.B. and T.I.K. performed research; A.A., J.W., and J.R.Y. contributed new reagents/analytic tools; R.A.M.d.B., P.R., and C.W. analyzed data; and R.A.M.d.B. and C.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0801106105/DCSupplemental.

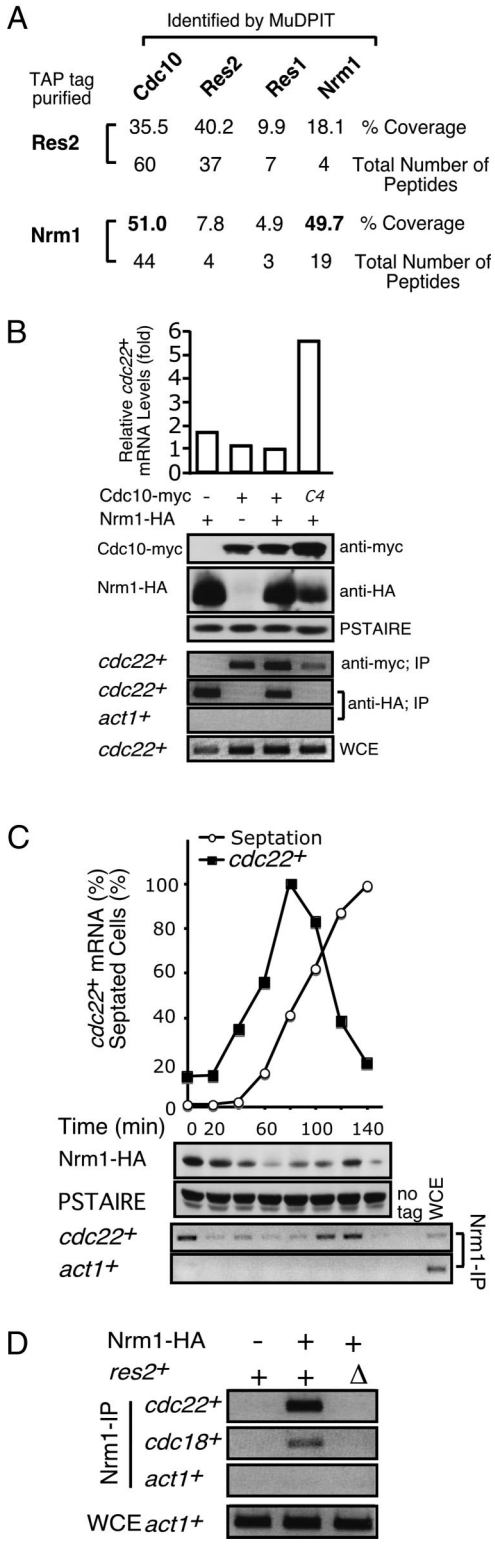

Fig. 1. Binding of Nrm1 to transcriptionally repressed MBF-regulated genes through the Cdc10 component of MBF during the G_1-S transition. (*A*) Number of spectra derived from peptides having a mass consistent with peptides predicted for Cdc10, Res2, Res1, or Nrm1 (Total Number of Peptides), and percentage coverage (% coverage) identified by MudPIT analysis of tandem affinity purification (TAP; Calmodulin Binding Peptide-TEV protease cleavage site-protein A) tag immunopurified protein Res2 or Nrm1. (*B*) Abundance of *cdc22*$^+$ mRNA (MBF-dependent; *Top*), Cdc10, and Nrm1 proteins (Cdc10–13xmyc and Nrm1–3xHA; *Middle*; loading control anti-PSTAIRE) in whole cell extract, and EtBr detection of PCR amplified Chromatin Immunoprecipitation (ChIP) signals (*Bottom*) of *cdc22*$^+$ (MBF-dependent) and *act1*$^+$ (MBF-independent) promoter DNA by Cdc10-myc or Nrm1-HA (anti-myc; IP and anti-HA; IP), and WCE signals of *cdc22*$^+$. (*C*) Abundance of *cdc22*$^+$ mRNA (filled squares) and septation index (open circles; G_1 exit correlates with septation) in *cdc25-22* cells released from G_2/M phase arrest by temperature shift (36.5°C to 25°C) are shown as percentage of highest amount (100%). Shown are abundance of Nrm1 protein (*Middle*) and EtBr detection of PCR amplified ChIP (*Bottom*) signals of *cdc22*$^+$ (MBF-dependent) and *act1*$^+$ promoter DNA (MBF-independent) by Nrm1-HA (Nrm1-IP) from the same experiment. Immunoprecipitation from a strain containing untagged genes (no tag) was negative, and whole cell extract (WCE) was used as a positive control. (*D*) EtBr detection of PCR amplified ChIP signals of *cdc22*$^+$, *cdc18*$^+$ (MBF-dependent), and *act1*$^+$ (MBF-independent) promoter DNA by Nrm1–3xHA (Nrm1-IP) in *res2*$^+$ and *res2*Δ cells.

Nrm1 Dissociates from Promoters in Response to DNA Replication Stress. The role of Nrm1 in the regulation of MBF target genes positions it as a potential target through which the DNA replication checkpoint could activate transcription at the G_1-S transition. We therefore investigated whether Nrm1 function is regulated in response to DNA replication fork arrest resulting from treatment of cells with hydroxyurea (HU). Because *nrm1*$^+$ is an MBF target, Nrm1 accumulated in HU-treated cells (Fig. 2*A*). However, it accumulated as a slower migrating form that did not bind to promoters (Fig. 2 *A*, *C*, and *E*, and Fig. 3*A* and *B*). Conversely, release of cells from HU arrest resulted in a shift to the faster migrating form, and binding to promoters was restored, which coincided with repression of MBF-regulated transcription (Fig. 2*A*). Phosphatase treatment of Nrm1 isolated from HU-arrested cells indicated that the slower migrating bands are a consequence of phosphorylation (Fig. 2*B*). Furthermore, in HU-treated cells the phosphorylated Nrm1 was reduced in complexes immunoprecipitated with myc-tagged Res2 (Fig. 2*C*). That response is not restricted to arrest in response to HU, because MMS and camptothecin, additional agents that block replication fork progression during S phase, also led to transcriptional induction and Nrm1 phosphorylation (Fig. 2*D*). These data suggest that Nrm1 function may be inactivated by phosphorylation in cells with arrested DNA replication, leading to expression of MBF-regulated genes outside of G_1-S phase.

To more fully characterize the phosphorylation of Nrm1 and its dissociation from promoters in response to HU, we analyzed those responses over the course of the cell cycle in cells synchronized by centrifugal elutriation (Fig. 2*E*, *Left*). Both HU-treated and untreated cells progressed into G_1 phase activating G_1-S transcription. However, whereas transcription in untreated cells decreased as cells progress through S phase and septate, transcription remained active in HU-treated cells (Fig. 2*E Left Top* graph). The persistence of transcriptional activity in the HU-treated cells was associated with phosphorylation of Nrm1 and failure of Nrm1 to bind to MBF target promoters. As expected, the HU-treated cells arrested in the cell cycle as indicated by the absence of septated cells at 240 min (Fig. 2*E Left Bottom* graph). This is consistent with the hypothesis that hyperphosphorylation of Nrm1 in response to HU is associated with activation of the DNA replication checkpoint, which occurs only upon entry into S phase. Furthermore, Nrm1 phosphorylation is associated with its failure to bind MBF-regulated promoters and repress transcription.

Nrm1 Phosphorylation Depends on the Checkpoint Protein Kinases. Despite the presence of many potential cyclin-dependent kinase (CDK) target sites in Nrm1, it is unlikely that Cdc2 is a source of Nrm1 phosphorylation in response to HU. First, Cdc2 activity is low during checkpoint arrest because of the inhibition of its activating phosphatase Cdc25 by the DNA replication checkpoint kinase Cds1 (3). Furthermore, Cdc2 activity is not required for MBF-dependent

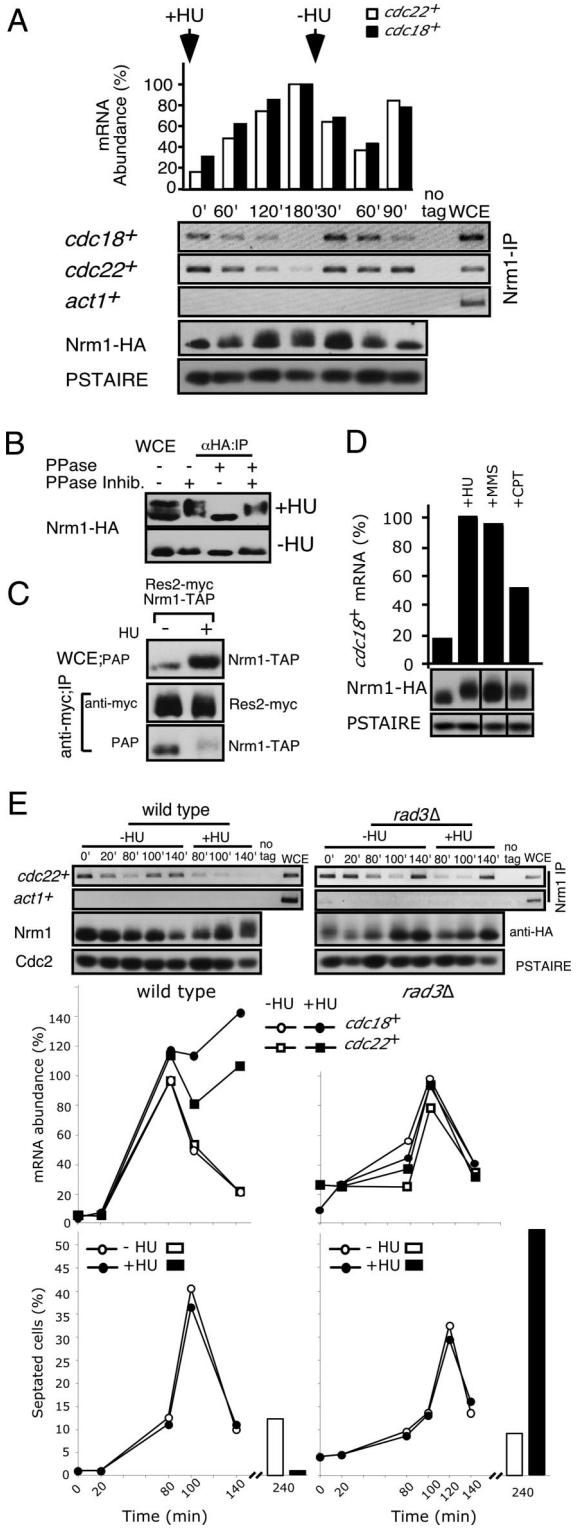

Fig. 2. Nrm1 regulation by phosphorylation in response to HU treatment. (*A*) *cdc18*$^+$ (filled squares) and *cdc22*$^+$ (open squares) mRNA levels (*Top*), EtBr detection of PCR amplified ChIP signals (*Middle*) of *cdc18*$^+$, *cdc22*$^+$, and *act1*$^+$ promoter DNA by Nrm1-HA (Nrm1-IP), and Nrm1 protein levels (Nrm1-HA; *Bottom*; loading control PSTAIRE) during HU arrest (12 mM HU; 0′-180′) and released (-HU; 30′-90′). (*B*) Nrm1-HA from whole cell extract (WCE). Nrm1-HA immunoprecipitated (antiHA-IP) from cells treated with 12 mM HU for 3.5 h (+HU) or untreated (-HU) was treated with lambda phosphatase (PPase) in the presence or absence of phosphatase inhibitors (PPase Inhib). (*C*) Whole cell extract (WCE; *Top*) and anti-myc immune complexes (anti-myc;IP; *Bottom*) containing Res2 tagged with 13xmyc epitops along with Nrm1 containing the tandem affinity purification (TAP) tag from untreated cells (-HU) and cells treated with 12 mM HU for 3.5 h (+HU) probed with anti-myc to detect Res2–13xmyc or anti peroxidase-anti-peroxidase (PAP) to detect Nrm1-TAP. (*D*) Nrm1 protein levels (Nrm1-HA; *Middle*; loading control PSTAIRE) and *cdc18*$^+$ mRNA amount (% of highest) in cells untreated and treated with HU (+HU; 12 mM), MMS (+MMS; 0.03%), and camptothecin (+CPT; 5 μM) for 3.5 h. (*E*) Small, unseptated wild type cells and *rad3*Δ cells were isolated by centrifugal elutriation and allowed to progress synchronously through the cell cycle in the absence or presence of HU (+HU; 12 mM). The abundance of Nrm1 protein and PCR-amplified *cdc22*$^+$ (MBF-dependent) and *act1*$^+$ (control) promoter DNA fragments generated from ChIP of Nrm1-HA (Nrm1-IP). Immunoprecipitation from a strain without tagged genes (no tag) is provided as a negative control, and whole cell extract (WCE) is provided as a positive control. Abundance of *cdc22*$^+$ mRNA (*Top* graphs; shown as percentage of highest amount in untreated cells) and septation index (*Bottom* graphs) from the same experiment. The bar graph (*Bottom* graphs) represents septation at 240 min, the expected time for the second round of septation in cycling cells.

transcriptional activation in response to HU (13). In fact, it has been suggested that Cdc2 associated with the cyclin Cig2 plays a role in the repression of MBF-regulated transcription as cells enter S phase via phosphorylation of Res1 (14).

To establish whether phosphorylation and inactivation of Nrm1 in response to DNA replication arrest depends on the DNA replication checkpoint pathway, we analyzed Nrm1 function in HU-treated cells carrying mutations in the checkpoint protein kinases. In HU-treated *cds1*Δ mutants, Nrm1 phosphorylation was reduced, whereas promoter binding was substantially increased, which correlated with the inability of *cds1*Δ mutants to fully induce MBF-dependent transcription (Fig. 3*A*). Thus, regulation of Nrm1 in response to HU is at least partially dependent on Cds1. The incomplete effect of *cds1*Δ on Nrm1 phosphorylation may be explained by the activation of Chk1 in the absence of Cds1 (15–17). Indeed, when we repeated the same experiment with a *chk1*Δ *cds1*Δ mutant or a *rad3*Δ mutant that does not activate Chk1 or Cds1, we observed a further increase in Nrm1 binding to promoters and repression of MBF-dependent gene expression. These effects were accompanied by a decrease in Nrm1 phosphorylation in both mutants relative to that in HU-treated *cds1*Δ mutants (Fig. 3 *A* and *B*).

Our prior analysis of the effect of HU on cells synchronously traversing the cell cycle revealed that the effect of HU on Nrm1 phosphorylation and promoter binding was restricted to S phase, which is consistent with it occurring in response to activation of the DNA replication checkpoint. To further evaluate the role of the checkpoint signaling pathway in the transcriptional response, we monitored the phosphorylation and promoter binding of Nrm1 in *rad3*Δ mutants during the cell cycle, both in the presence and absence of HU (Fig. 2*E Right*). We found that untreated wild type and *rad3*Δ cells exhibited periodic transcription of MBF targets, repressing transcription as cells progressed into S phase. Consistent with that pattern of transcription, Nrm1 remained unphosphorylated and bound to MBF-regulated promoters. However, unlike HU-treated wild type cells, Nrm1 behaved identically in *rad3*Δ mutants treated with HU, and transcription was repressed in a timely manner. As expected, *rad3*Δ cells showed similar levels of septation to those of untreated wild type cells, whereas HU-treated *rad3*Δ cells showed increased septation at 240 min. Thus, inactivation of the checkpoint signaling pathway abrogated Nrm1 phosphorylation and transcriptional repression. Overall, these data suggest that regulation of the cell cycle transcriptional program in response to DNA replication arrest is likely to involve checkpoint-dependent phosphorylation of Nrm1.

Both *cds1*Δ and *rad3*Δ mutants were highly sensitive to HU (Fig. 3*D*). Interestingly, overexpression of the MBF target *rep2*$^+$, a putative MBF activator (18), suppresses the HU-sensitivity of *cds1*Δ and *rad3*Δ mutants (8). We hypothesized that a failure to

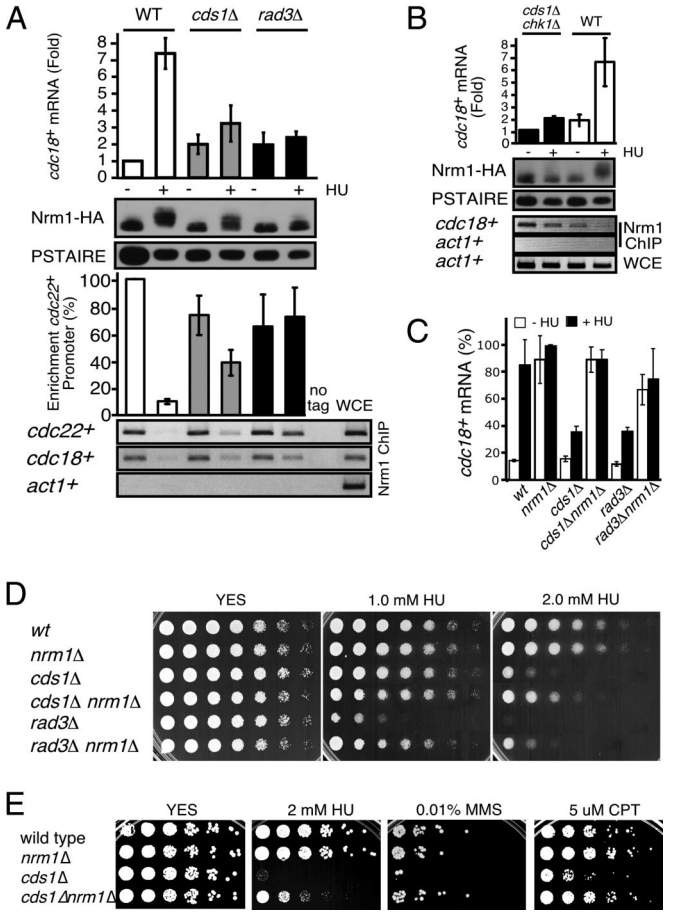

Fig. 3. Regulation of MBF-dependent transcription through DNA replication checkpoint-dependent phosphorylation of Nrm1. (*A* and *B*) Nrm1 protein levels (Nrm1-HA; *Middle*; loading control PSTAIRE) and EtBr detection of PCR amplified signals of *cdc18*$^+$, *cdc22*$^+$, and *act1*$^+$ promoter DNA from ChIP analysis by Nrm1-HA (Nrm1-IP; *Bottom*). *cdc18*$^+$ mRNA amount (*Top*) as fold compared with amount in untreated wild type. Wild-type (wt; open bar), *cds1*Δ (gray bar) and *rad3*Δ (black bar) (*A*), and *cds1*Δ *chk1*Δ (black bar) (*B*) in cells untreated (−HU) and treated with HU (+HU; 12 mM for 3.5 h). (*A*) *cdc22*$^+$ promoter DNA quantified by qPCR shown as bar graphs (above) shown as percentage of the highest amount of promoter DNA detected in this experiment (100%). (*C*) Abundance of *cdc18*$^+$ mRNA in wild-type (wt), *nrm1*Δ, *cds1*Δ, *rad3*Δ, *nrm1*Δ *cds1*Δ, and *nrm1*Δ *rad3*Δ cells untreated (-HU; open bars) and HU-treated (12 mM HU; +HU; filled bars) are shown as a percentage of the highest amount of expression observed in this experiment (100%). Error bars in *A*–*C* represent SD of the values obtained by RT-PCR of triplicate samples. (*D*) Serially diluted with three volumes of medium of wt, *nrm1*Δ, *cds1*Δ, *rad3*Δ, *nrm1*Δ *cds1*Δ, and *nrm1*Δ *rad3*Δ cells spotted on YES plates (*Left*), and YES plates containing 1.0 mM (*Middle*) or 2.0 mM (*Right*) of HU. (*E*) Serially diluted with five volumes of medium of wt, *nrm1*Δ, *cds1*Δ, and *cds1*Δ *nrm1*Δ cells spotted on YES plates (*Left*), and YES plates containing 2.0 mM HU, 0.01% MMS, or 5 μM camptothecin (*Right*).

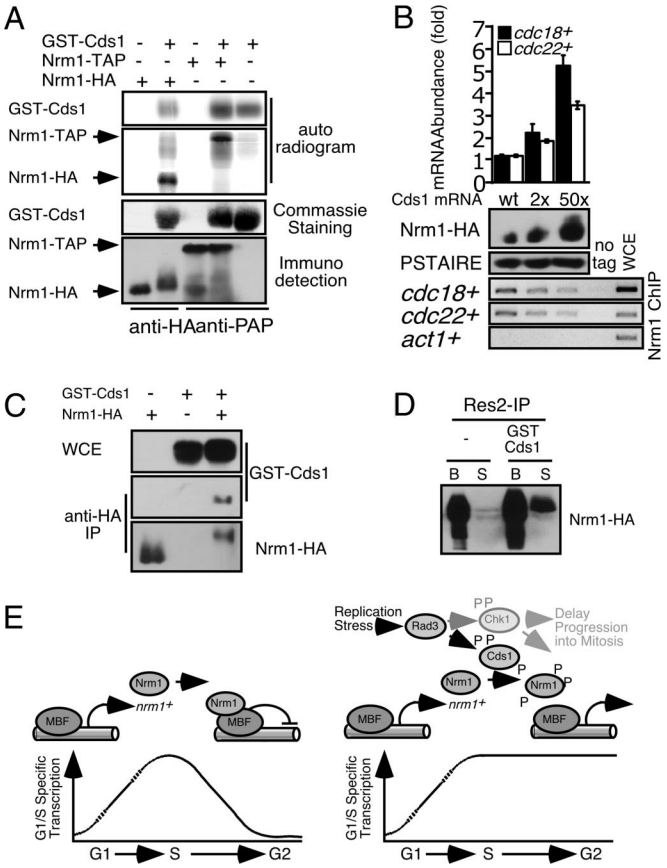

Fig. 4. DNA Replication Checkpoint Promotes G_1-S Transcription in response to stalled DNA replication forks by inactivating the MBF Repressor Nrm1. (*A*) Second and fourth panel from top: Nrm1–3xHA (*Left*) and Nrm1-TAP (*Right*) prepared under denaturing conditions. Arrows indicate Nrm1 by immunodetection (*Bottom*), and Nrm1 phopshpo-species by autoradiogram (second from top). *Top*: Protein kinase activity of Cds1 determined via autophosphorylation. Exposure of autophosphorylation is 1/1000 of that used to detect Nrm1. Third panel from top: Cds1 detection by Commassie staining. (*B*) Amount of *cdc18*$^+$ (filled bars) and *cdc22*$^+$ (open bars) mRNA (*Top*) in wt and cells constitutively expressing (2×) and overexpressing Cds1 (50×). Also shown are Nrm1 protein levels (Nrm1-HA; *Middle*) and EtBr detection of PCR amplified ChIP signals of *cdc18*$^+$, *cdc22*$^+$, and *act1*$^+$ promoter DNA by Nrm1-HA (Nrm1-IP; *Bottom*). Error bars represent SD of the values obtained by RT-PCR of triplicate samples. (*C*) WCE (*Top*) and anti-HA-IP (*Bottom*) containing Nrm1–3xHA, GST-Cds1, or both. (*D*) Anti-myc ProteinA beads containing anti-myc-purified MBF/Nrm1 (Res2-IP) prepared from cells expressing Res2–13xmyc and Nrm1–3xHA. After treatment, the bound fraction (*B*) and the released fraction (*S*) analyzed by anti-HA to detect Nrm1-HA. (*E*) Model. During the G_1-S transition Nrm1 represses MBF dependent transcription (*Left*). During DNA replication stress the ATR homolog, Rad3 phosphorylates and activates Cds1 (and Chk1), which phosphorylates and inactivates Nrm1 leading to transcriptional induction of MBF targets (*Right*).

regulate Nrm1, and, therefore, activation of MBF transcription might contribute to the HU sensitivity of these checkpoint mutants. Consistent with that hypothesis, inactivation of Nrm1 in *cds1*Δ and *rad3*Δ mutants restored high amounts of MBF-regulated transcription (Fig. 3*C*). In turn, this correlates with increased HU resistance relative to that in similar mutants with wild-type *nrm1*$^+$ (Fig. 3*D*). The increased resistance to the RNR-specific inhibitor HU is not merely the result of high levels of the MBF target *cdc22*$^+$, which encodes a RNR subunit, because the sensitivity of cells to replication stress induced by MMS and camptothecin was also suppressed by inactivation of Nrm1 (Fig. 3*E*). Thus, the DNA replication checkpoint pathway may facilitate survival of replication arrest by inactivation of Nrm1 that, in turn, allows for sustained activation of G_1-S transcription.

Nrm1 is Phosphorylated by the DNA Replication Checkpoint Kinase Cds1. We evaluated the capacity of Nrm1 to act as a direct target for Cds1 in an *in vitro* kinase assay with purified GST (GST) tagged Cds1 (Fig. 4*A*). We detected incorporation of γ-^{32}P from ATP into both Nrm1 fused to a tandem affinity purification (TAP) tag or to a three hemagglutinin (3xHA) tag. Using the smaller HA epitope tag, we detected a quantitative electrophoretic shift of Nrm1 to a slower mobility species. Because Nrm1-HA was not phosphorylated when incubated with cata-

lytically inactive Cds1 (Fig. S4*A*), we concluded that Nrm1 is likely a direct target for phosphorylation by the Cds1 kinase.

We hypothesized that phosphorylation of Nrm1 by Cds1 promotes its dissociation from MBF. In an effort to establish the requirement for Nrm1 phosphorylation for its release from the transcription complex, we have mapped HU-inducible phosphorylation sites on Nrm1, achieving up to 60% peptide coverage by mass spectrometry (AA and JY, unpublished). Conversion of those sites to alanine resulted in little or no effect on Nrm1 phosphorylation by Cds1 *in vitro* and only a small effect on the mobility of the mutant protein in response to HU *in vivo* (Fig. S4 *B* and *C*). Consistent with those results, we observed at most a 25% reduction in the extent of induction of transcription of MBF targets by HU (data not shown). Although these results are consistent with a contribution of the mutated phosphorylation sites to regulation of Nrm1 activity, they suggest that other phosphorylation sites in Nrm1 might contribute to the regulation of the Nrm1/MBF interaction. In addition, phosphorylation of Cdc10 and/or Res1/2 subunits may contribute to, or may be redundant with, Nrm1 phosphorylation for dissociation of the Nrm1/MBF complex. Redundancy of G_1 control is well documented including a role for phosphorylation of the SBF component Swi6 in the Cln3/CDK dependent dissociation of the Whi5 repressor from SBF (19, 20). In fact, the MBF components Cdc10 of fission yeast and Swi6 of budding yeast are phosphorylated by the checkpoint protein kinases Cds1 and Rad53, respectively (N. Rhind, personal communication and ref. 21). Based on these observations, we suggest that other phosphorylation sites in Nrm1, or in other MBF components that are targeted by Cds1, also play a role in dissociation of Nrm1 from MBF.

As an alternative approach to establish the requirement for Cds1-dependent phosphorylation for the release of Nrm1 from the MBF complex, we carried out a series of experiments to show that Nrm1 phosphorylation *in vivo* and disassociation from MBF *in vitro* directly depends on Cds1. We first evaluated the capacity of overexpressed Cds1 to phosphorylate Nrm1 in the absence of checkpoint activation. Cds1 expressed from the *nmt1* promoter led to a dose-dependent increase in Nrm1 phosphorylation. This effect correlated with decreased levels of Nrm1 at MBF-dependent promoters and an increased expression of MBF target genes (Fig. 4*B*). These results are consistent with the finding that Cds1 interacts directly with Nrm1 under those conditions (Fig. 4*C*). Not unexpectedly, that interaction, although specific, is difficult to detect. Finally, to show that Cds1-dependent phosphorylation of Nrm1 directly leads to dissociation from MBF, Nrm1-MBF complexes were immunopurified from cells expressing Res2–13xmyc and Nrm1–3xHA, and the resulting Sepharose-bound complexes were treated with purified soluble GST-Cds1 kinase *in vitro* (Fig. 4*D*). Cds1 treatment led to phosphorylation of a portion of the MBF-associated Nrm1 and release into the supernatant. In contrast, the unphosphorylated Nrm1 was retained on the beads in both the treated and untreated samples. Nrm1-MBF complexes incubated with either GST alone or GST-Cds1 in the absence of ATP were unable to promote release of Nrm1 into the supernatant (Fig. S5). We conclude that Cds1-dependent phosphorylation of Nrm1 is sufficient to promote dissociation of Nrm1 from MBF consistent with its dissociation from promoters in response to DNA replication stress *in vivo*.

Discussion

Stalling of eukaryotic DNA replication forks by nucleotide depletion is known to activate the expression of genes involved in DNA replication and repair. However, a mechanism for transcriptional activation was lacking. We provide a mechanism for activation of G_1-S transcription by the DNA replication checkpoint in response to DNA replication stress in the fission yeast (Fig. 4*E*). We show that Nrm1, a corepressor of MBF dependent G_1-S transcription, is a target of the DNA replication checkpoint kinase Cds1. Cds1, activated by the ATR homolog Rad3, phosphorylates Nrm1 leading to its dissociation from MBF-regulated promoters and transcriptional induction of MBF-dependent transcription, many of which encode DNA replication and repair proteins. The finding that Chk1 kinase can at least partially substitute for Cds1 in the absence of Cds1 and that it is likely to play a role in the activation of MBF-regulated genes by DNA damage during G_2 phase (22) suggests that this may be a general feature of the DNA structure checkpoints.

Nrm1 participates in a negative feedback response that appears to be conserved throughout evolution. Checkpoint-dependent inactivation of Nrm1, which is involved in repressing its own expression, allows rapid down regulation of transcription once cells recover from a DNA replication arrest. *E. coli* LexA mediates similar feedback regulation during the SOS response (reviewed in ref. 23), whereas budding yeast Crt1 and mammalian Mdm2, a negative regulator of p53, mediate feedback regulation of transcription during recovery from the DNA structure checkpoint response (24–26). This conservation suggests that the transcriptional response initiated by DNA damage and replication stress is harmful once those problems have been rectified. Although the defects provoked by persistent transcription remain to be established, we find that overexpression of MBF targets during the mitotic cell cycle as a result of inactivation of Nrm1 (10) leads to genome instability (R.dB., C.W., C. Dovey, and P.R., unpublished). This is consistent with an important role for repression of G_1-S transcription outside of the G_1 phase and, perhaps, after recovery from DNA replication stress. Future research will reveal whether rapid down regulation of the transcriptional response mediated by the DNA structure checkpoints is generally important to maintain genome stability.

Recent studies suggest that the regulation of G_1-S transcription by the DNA structure checkpoints is a conserved response to genotoxic stress in eukaryotes. These studies provide one mechanism by which that response can be implemented. Our preliminary studies indicate that a similar mechanism is used by budding yeast. In those cells, regulation of G_1-S genes is mediated by two transcription factors, MBF and SBF. The timely repression of MBF, but not SBF, transcripts during the G_1-S transition requires binding of ScNrm1 (10). We find that, like in fission yeast, the subset of G_1-S genes regulated by MBF is induced by the DNA replication checkpoint via inactivation of ScNrm1 (R.dB. and C.W., unpublished).

G_1-S transcriptional regulation in mammals depends on the E2F family of transcription factors and their regulators, the Rb family members. Several recent studies indicate a role for E2F in a DNA damage checkpoint suggesting a central role for E2F and their regulators in cell cycle progression and genome integrity (27). The degree of conservation from yeast to human of both the G_1-S transcription network (28) and the DNA replication checkpoint response suggest that regulation of G_1-S transcription by the DNA replication checkpoint also may be conserved in mammals. Because the G1-S transcription factors and their regulators are putative targets of cell cycle checkpoints that regulate genomic stability, they are expected to play a central role in the avoidance of DNA damage and chromosomal aberrations, phenomena that directly contribute to tumorigenesis. Consequently, understanding the mechanisms governing regulation of G_1-S gene expression in response to genotoxic stress may provide new insights into the genesis and treatment of human cancer.

Methods

Strains. The strategy of Rigaut *et al.* (29) was used to append a tandem affinity purification (TAP) tag to *res2*+ and *nrm1*+ (carboxyterminus) at their endogenous loci. The PCR method of Bahler *et al.* (30) was used to disrupt and tag (carboxyterminus) *cdc10*+, *res1*+, *res2*+, and *nrm1*+ at their endogenous

locus. The *nrm1*+-HA ORF was cloned by PCR into the pCR 2.1-TOPO vector (Invitrogen). Phosphorylation site mutations were introduced by PCR, resulting in amino acid substitutions S9A, T11A, T55A, S57A, T116A, S174A, T236A, and S237A. Nrm1^{8A}-HA was integrated at the endogenous locus by homologous recombination. A description of strains used is provided in Table S1.

TAP Affinity Purification and MudPIT Analysis. TAP purification and analysis was carried out as described by Boddy *et al.* (31). Multidimensional protein identification technology (MudPIT) was carried out as described by McDonald and Yates (32).

Coimmunoprecipitation. Immunoprecipitations were carried out using TAP purification buffers. Immunoprecipitated proteins were resolved by 12.5% SDS/PAGE. Peroxidase-Anti-Peroxidase (PAP) Soluble Complex was used as an antibody to detect TAP tagged proteins.

Cell Synchronization. A population of small G_2-synchronized cells was obtained by centrifugal elutriation as described in ref. 33 except that the experiment was done at room temperature, the sonication step was omitted, and cells were grown in YES medium throughout the experiment. Temperature sensitive *cdc25-22* cells were arrested in G_2/M by incubation at 35.5°C for 3.5 h and subsequently released into the cell cycle at 25°C.

Real-Time PCR and RT-PCR. Total RNA was isolated using the Rneasy Plus kit (Qiagen). The iQ SYBR Green supermix (Bio-Rad) was used for quantitative PCR on ChIP samples, and the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) was used for RT-PCR experiments. Reactions were run on the Chromo-4 Real-Time PCR Detector (Bio-Rad) using standard PCR and RT-PCR conditions. Data were analyzed using MJ Opticon Monitor Analysis Software 3.0.

ChIP Analysis. Chromatin immunoprecipitation was performed as described Flick *et al.* (34).

***In Vitro* Kinase Assay.** Soluble, GST, Cds1, and kinase dead Cds1 (Cds1KD) protein was purified from cultures expressing GST, N-terminal GST-tagged *cds1*+ or N-terminal GST-tagged and C-terminal HIS-HA-tagged *cds1*KD+ under the control of the *nmt1*+-promoter by the GST purification method described by Boddy *et al.* (15). Anti-HA and PAP immunoprecipitates from cells of *nrm1*+, Nrm1–3xHA, or Nrm1-TAP fusion protein that were prepared under denaturing conditions, were used as substrates. Kinase assays were carried out as described by Deshaies and Kirschner (35). Reactions were resolved by 12.5% SDS/PAGE. Phosphorylation of Nrm1 was analyzed using a PhosphorImager (Molecular Diagnostics). *In vitro* kinase assay on MBF was carried out as described above. MBF was purified from cells of Res2–13xmyc and Nrm1–3xHA fusion protein by anti-myc purification.

ACKNOWLEDGMENTS. The authors thank M. Guaderrama for technical support and thank members of the TSRI Cell Cycle Group for helpful discussion. This work was supported by USPHS grant GM059441 to C.W. and grants GM59447 and CA77325 to P.R.

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