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A homeodomain transcription factor regulates the DNA replication checkpoint in yeast

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Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia Rad3-related; DSC1, DNA synthesis control-like transcription factor complex; HRP, horse-radish peroxidase; HU, hydroxyurea; MBF, MluI cell cycle box binding factor; PCR, polymerase chain reaction; UV, ultraviolet

Checkpoints monitor the successful completion of cell cycle processes, such as DNA replication, and also regulate the expression of cell cycle-dependent genes that are required for responses. In the model yeast *Schizosaccharomyces pombe* G_1/S phase-specific gene expression is regulated by the MBF (also known as DSC1) transcription factor complex and is also activated by the mammalian ATM/ATR-related Rad3 DNA replication checkpoint. Here, we show that the Yox1 homeodomain transcription factor acts to co-ordinate the expression of MBF-regulated genes during the cell division cycle. Moreover, our data suggests that Yox1 is inactivated by the Rad3 DNA replication checkpoint via phosphorylation by the conserved Cds1 checkpoint kinase. Collectively, our data has implications for understanding the mechanisms underlying the coordination of cell cycle processes in eukaryotes.

Introduction

Cell cycle progression is highly ordered and is regulated by many mechanisms. For example, transcript profiling studies in model organisms such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* revealed that cell cycle progression is associated with the phase-specific expression of defined sets of genes.[1-6] In *S. pombe*, several hundred periodically-expressed genes are grouped into clusters according to the timing of their expression during the cell cycle.[1-3] Work from several groups identified transcription factors that regulate these different gene clusters (reviewed in ref. 7). For example, a G_1/S phase-specific gene cluster is regulated by the MBF (also known as DSC1) transcription factor complex, which consists of several proteins including Cdc10 and the DNA binding proteins Res1 and Res2.[7-11] Recently, two repressor proteins were identified that regulate MBF; Nrm1 and the homeodomain protein Yox1.[12,13] Thus, both the regulated activation and repression of transcription is utilized to determine the precise timing of specific gene expression during the cell division cycle. Interestingly, although there are some differences in their utilization, similar transcription factors, including MBF, the Yox1-related proteins Yox1 and Yhp1, and Nrm1, regulate the expression of genes at equivalent times in the cell division cycle in the distantly related *S. cerevisiae*.[7,13,14] Furthermore, MBF has been proposed to be analogous to E2F, which regulates mammalian G_1/S phase-specific gene expression. Thus, there is significant overlap in the proteins involved in regulating cell cycle-dependent gene expression from yeast to mammals. However, despite the identification of such transcription factors there is still much to learn about the mechanisms underlying their regulation.

Checkpoints maintain cell viability by ensuring accurate completion of cell cycle processes and by coordinating repair of damage. For example, in response to DNA damage or incomplete DNA replication, evolutionarily conserved checkpoint pathways are activated to arrest the cell division cycle, providing an opportunity to repair DNA damage and stabilise replication forks (reviewed in refs. 15 and 16). These particular checkpoints are mediated by a highly conserved subfamily of protein kinases, including ATM and ATR in mammalian cells and Rad3 in *S. pombe*, which act via downstream protein kinases Chk1 and Chk2 (Cds1 in *S. pombe*). In *S. pombe*, Rad3 primarily activates Chk1 in response to DNA damage, but activates Cds1 in response to DNA replication defects.[17,18] Both Chk1 and Cds1 cause cell cycle arrest through the inactivation of the cyclin-dependent kinase Cdc2.[19] Recently, it has become clear that cell cycle checkpoints also regulate the expression of specific cell cycle-dependent gene clusters. For example, inhibition of DNA replication in *S. pombe* by the ribonucleotide reductase inhibitor hydroxyurea (HU) leads to Rad3-mediated upregulation of

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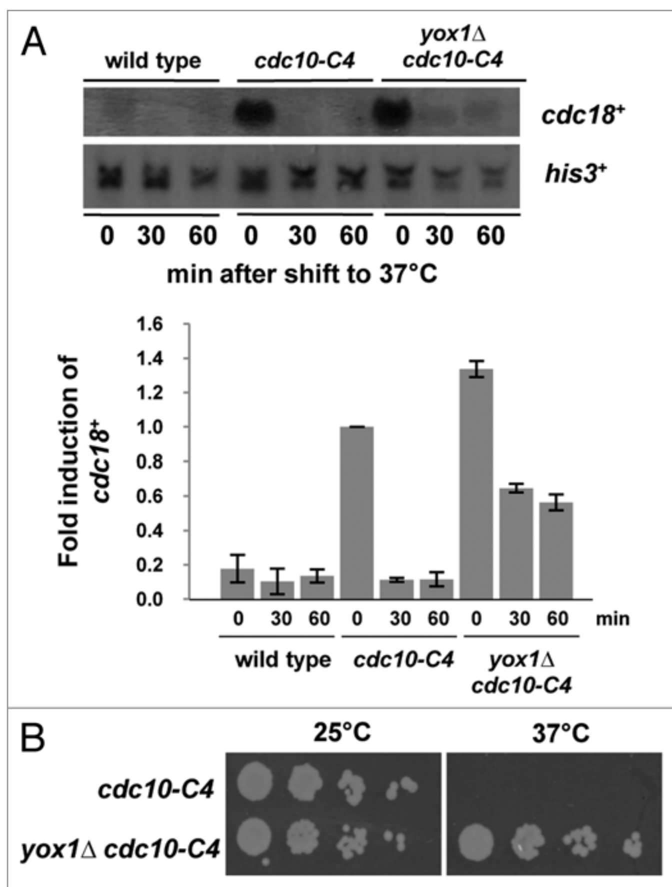

Figure 1. Deletion of *yox1+* suppresses a loss of function mutation of MBF. (A and B) Deletion of *yox1+* suppresses the phenotypes associated with *cdc10-C4* mutant cells, grown at the non-permissive temperature. (A) Wild-type (CHP429), *cdc10-C4* (SW27) and *yox1Δcdc10-C4* (FP36) cells were grown to mid-log-phase at 25°C, then shifted to 37°C for 0, 30 or 60 min. RNA was prepared from two independent experiments and analyzed by northern blotting with a probe specific for *cdc18+*. A representative northern blot is shown. RNA from the two independent experiments was quantified using *his3+* as a loading control and fold induction was calculated relative to the time-zero sample of the *cdc10-C4* strain. The error bars shown are ±SD. (B) 5 μl of 10-fold serial dilutions of mid-log-phase growing *cdc10-C4* (SW27) and *yox1Δcdc10-C4* (FP36) cells were plated onto YE5S agar and incubated at either 25°or 37°C for three days.

MBF-dependent gene expression.[3,20-22] Interestingly, one aspect of this regulation involves phosphorylation of the Nrm1 repressor by Cds1 which results in derepression of MBF-dependent gene expression.[13,23]

Here, we have further investigated the potential role(s) and regulation of the Yox1 homeodomain transcription factor in *S. pombe*. Consistent with previous work we find that Yox1 represses MBF-regulated gene expression. Excitingly, our data also indicates that inhibition of the repressor function of Yox1 by the Rad3-dependent checkpoint is essential for the response of cells to incomplete DNA replication. Indeed, Yox1 is phosphorylated in a Rad3- and Cds1-dependent manner upon checkpoint activation. Collectively, our data provide insight of the role of Yox1 in the regulation of MBF-dependent gene expression during the cell division cycle and, furthermore, reveal a new mechanism of regulation of gene expression by the ATM/ATR-related Rad3 cell cycle checkpoint pathway.

Results

Yox1 regulates MBF-dependent gene expression. Previous work established that Yox1 and Nrm1 act as repressors of MBF-dependent gene expression in *S. pombe*[12,13] and, moreover, several lines of evidence suggested that Yox1 and Nrm1 may act in the same pathway.[12] It has also been shown that MBF-dependent gene expression is specifically derepressed at the permissive temperature (25°C) in cells containing the temperature sensitive *cdc10-C4* allele, which encodes a C-terminal truncation of the MBF component Cdc10, and moreover that MBF-dependent gene expression decreases when these mutant cells are placed at the non-permissive temperature (>32°C).[9,24] Interestingly, Nrm1 is unable to bind the mutant Cdc10 protein, suggesting that the derepression of MBF-regulated genes at the permissive temperature is due to the inability of mutant Cdc10 to bind Nrm1.[23] Hence, to further examine the relationship between Yox1 and MBF activity, the function of Yox1 was investigated in cells containing the *cdc10-C4* allele. As expected the level of expression of the MBF-regulated *cdc18+* gene was found to be higher in *cdc10-C4* mutant cells, compared to wild-type cells, when grown at the permissive temperature (**Fig. 1A**). Furthermore, the levels of MBF-regulated *cdc18+* mRNA specifically decreased following a shift of *cdc10-C4* mutant cells to the non-permissive temperature (**Fig. 1A**). Interestingly, loss of Yox1 function appeared to at least partially reverse the reduction in *cdc18+* mRNA levels observed in *cdc10-C4* mutant cells at the non-permissive temperature (**Fig. 1A**). Moreover, consistent with this data, loss of Yox1 also suppressed the growth defects of *cdc10-C4* mutant cells at the non-permissive temperature (**Fig. 1B**). These results were not predicted by the observations that Nrm1 is unable to bind the Cdc10-C4 mutant protein and that Yox1 binding to MBF-regulated promoters is Nrm1-dependent.[12,23] Hence, in addition to confirming the key role of Yox1 in the regulation of MBF-dependent gene expression, our data suggests that Yox1 can function independently of Nrm1 to inhibit MBF activity.

Yox1 represses DNA replication checkpoint-dependent gene expression. Previous work demonstrated that MBF-regulated gene expression is upregulated in response to activation of the Rad3-dependent DNA replication checkpoint.[3,20-22] Hence, we next examined whether Yox1 plays any role(s) in Rad3 checkpoint-regulated expression of MBF-dependent genes. Cells lacking Rad3 function display increased sensitivity to hydroxyurea (HU) and ultraviolet (UV) light (**Fig. 2**).[25,26] Consistent with the function of Yox1 as a repressor of MBF-dependent gene expression during the cell division cycle, *yox1*Δ cells were found to be more resistant than wild-type cells to a high concentration (10 mM) of HU (**Fig. 2A**). Interestingly, we found that, similar to *yox1*Δ cells, *nrm1*Δ cells are also more resistant than wild-type cells to a high concentration of HU (**Fig. 2A**). However, in contrast to either single mutant, *yox1*Δ*nrm1*Δ cells have similar HU sensitivity to wild-type cells (**Fig. 2A**). This result is interesting

as it indicates that the increased HU resistance displayed by both single mutants is dependent on the presence of the other protein within these cells. Furthermore, these data are consistent with our analysis of the relationship between *yox1*$^+$ and *cdc10-C4* which suggested that Nrm1 and Yox1 can function independently of each other.

A previous study demonstrated that loss of Nrm1 partially rescues the increased HU sensitivity associated with loss of Rad3 function.[23] Hence, to test whether any similar relationship exists between Rad3 and Yox1, the sensitivities of *yox1*Δ*rad3*Δ and *rad3*Δ cells to HU were compared. Similar to Nrm1, loss of Yox1 function partially suppresses the increased HU-sensitivity of *rad3*Δ cells (**Fig. 2B**). Furthermore, similar effects were observed when *rad3*Δ and *rad3*Δ*yox1*Δ cells were exposed to UV light-induced damage (**Fig. 2C**). These data raised the possibility that inhibition of Yox1 by the Rad3 pathway is important for the response of cells to DNA replication stress. Hence, we next investigated the potential role of regulation of Yox1 in HU-induced expression of MBF-dependent genes. As expected, the levels of *cdc18*$^+$ mRNA increased in wild-type cells following HU treatment (**Fig. 3**). Consistent with the increased HU resistance associated with loss of Yox1 function (**Fig. 2A**), *cdc18*$^+$ mRNA levels were much higher in untreated *yox1*Δ cells and, moreover, displayed no further major increases following HU treatment (**Fig. 3**). Significantly, the high levels of *cdc18*$^+$ mRNA observed in *yox1*Δ cells were similar to the HU-induced levels of *cdc18*$^+$ mRNA in wild-type cells (**Fig. 3**). Hence, taken together, these data suggest that maximal HU-induced expression of *cdc18*$^+$ involves inhibition of Yox1 function by the Rad3 cell cycle checkpoint pathway.

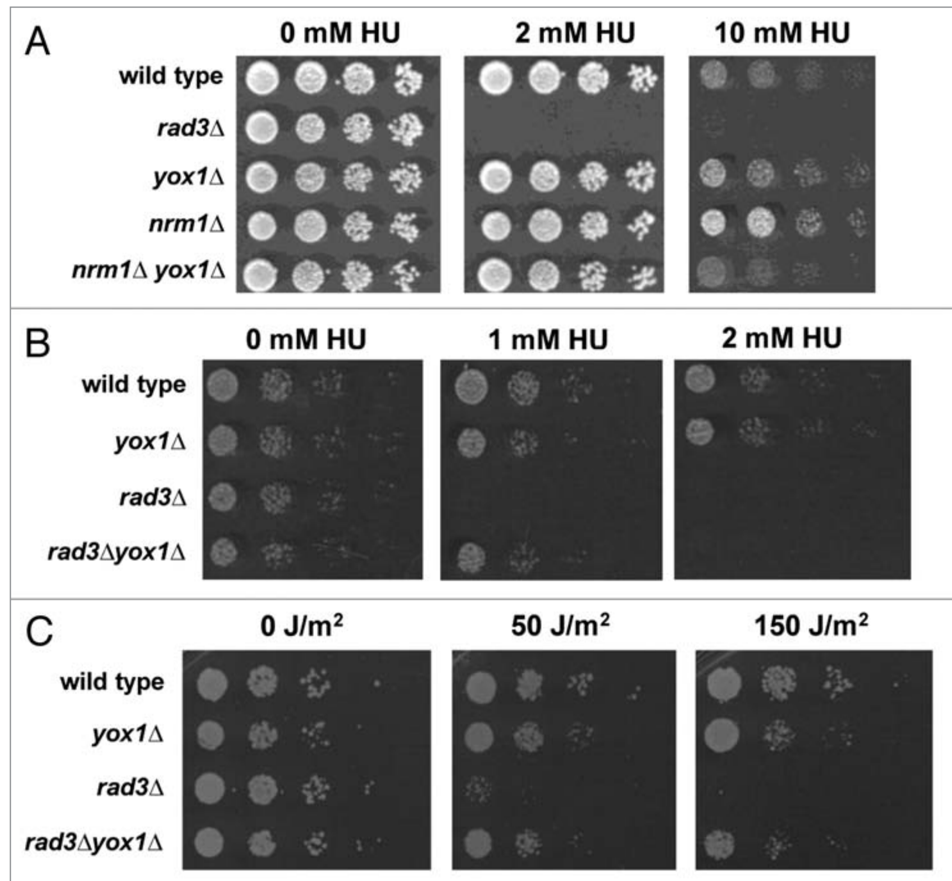

Figure 2. Loss of Yox1 function increases resistance to different stress conditions. (A) Deletion of either *yox1*$^+$ or *nrm1*$^+$, but not of both genes together, increases the resistance of *rad3*$^+$ cells to HU and (B and C) deletion of *yox1*$^+$ partially suppresses the increased sensitivities to HU and UV light associated with the *rad3*Δ mutation. ~5 μl of serial dilutions of either mid-log-phase growing wild-type (ED666), *yox1*Δ (FP0), *nrm1*Δ (SW804), *yox1*Δ*nrm1*Δ (SW805), *rad3*Δ (TH146) or *yox1*Δ*rad3*Δ (FP33) cells were plated onto either (A and B) YE5S agar supplemented with the indicated concentrations of HU or (C) YE5S agar and exposed to 0, 50 J/m^2 or 150 J/m^2 UV radiation. Plates were incubated at 30°C for 2–3 days.

Yox1 is regulated by the Rad3 cell cycle checkpoint pathway. To investigate whether Yox1 is regulated by the Rad3 cell cycle checkpoint pathway western blot analyses were performed on extracts isolated from HU-treated cells expressing Pk epitope-tagged Yox1 from the normal chromosomal locus. Interestingly, Yox1 protein levels were found to increase substantially in cells treated with HU and, furthermore, rapidly diminish following removal of HU (**Fig. 4A**). This increase in Yox1 protein levels is consistent with the observation that expression of *yox1*$^+$ is induced by HU.[3] Interestingly, HU treatment also stimulated the appearance of slower mobility forms of Yox1, the timing of which coincided with upregulation of MBF-dependent gene expression (**Fig. 4A**). Analysis of these slower mobility forms revealed that they represented HU-induced phosphorylation of Yox1 (**Fig. 4B**). Rad3 functions as a protein kinase hence we next investigated the potential role of Rad3 in this regulation of Yox1. Consistent with the essential requirement for Rad3 in HU-induced MBF-dependent gene expression, loss of Rad3 function inhibited the accumulation of Yox1 following HU treatment (**Fig. 5A**). However, importantly, these data also indicated that HU-induced phosphorylation of Yox1 is Rad3-dependent (**Fig. 5A**). It was possible that the regulation of Yox1 by Rad3 is indirect and indeed Rad3 activates downstream protein kinases such as Cds1. Hence, we next examined HU-induced phosphorylation of Yox1 in *cds1*Δ mutant cells. Similar to the effect of deletion of *rad3*$^+$, deletion of the *cds1*$^+$ gene also inhibited HU-induced phosphorylation of Yox1 (**Fig. 5B**). Collectively, these data suggest that the repressor activity of Yox1 is inhibited by Rad3 checkpoint pathway-dependent phosphorylation of the protein and moreover, that this regulation plays an important role in the HU-induced upregulation of MBF-dependent gene expression.

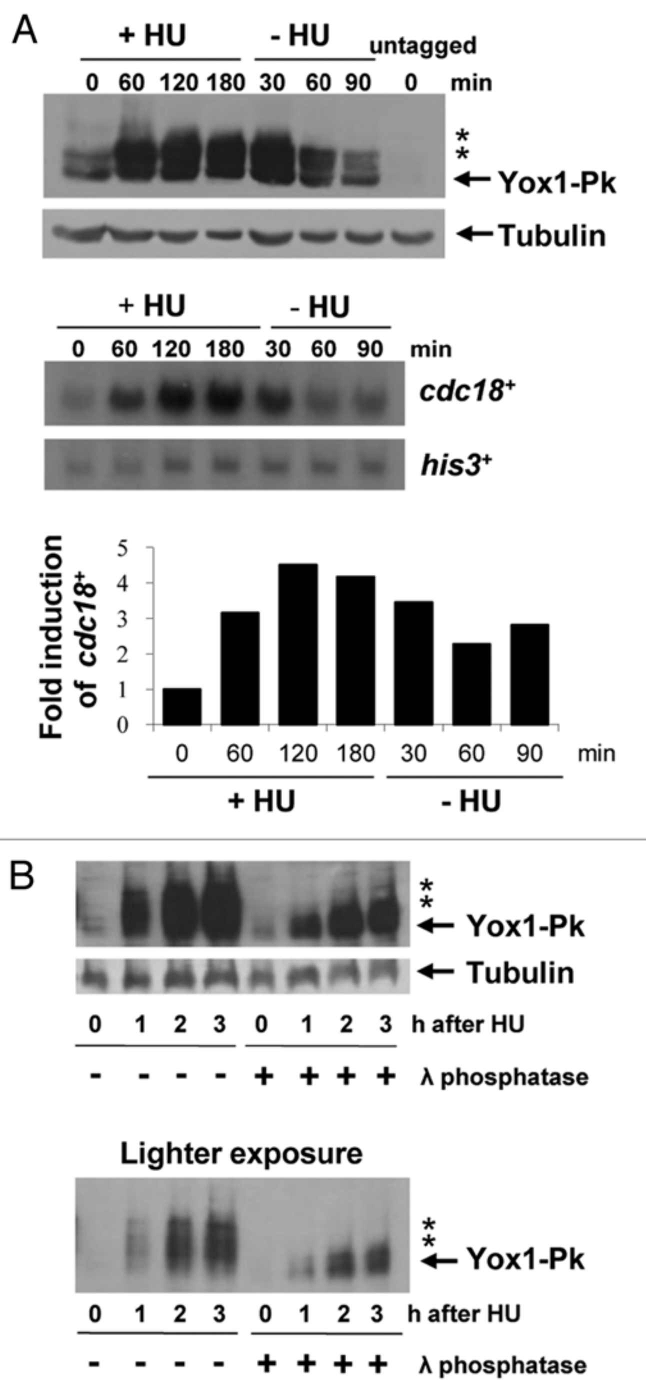

Figure 4 (Right). HU treatment stimulates the accumulation and phosphorylation of Yox1. Western blot and RNA analyses revealed that Yox1 accumulates and is phosphorylated in response to HU with the timing of maximal modification coinciding with maximal HU-induced upregulation of *cdc18*+ expression. (A) Cells (FP4) expressing Pk epitope-tagged Yox1 (Yox1-Pk) from the normal chromosomal locus were grown to mid-log-phase at 30°C and treated with 11 mM HU for 0, 60, 120 and 180 min (+HU), then HU was washed off, and the cells were incubated at 30°C for a further 30, 60 and 90 min (-HU). Extracts for RNA and protein analyses were collected at each time point. The northern blot was analyzed using a probe specific for *cdc18*+ and RNA was quantified using *his3*+ as a loading control. Fold induction was calculated relative to the time-zero sample. In the western blot analysis extract isolated from mid-log-phase growing wild-type (CHP429-untagged) cells was used as a negative control. (B) Extracts isolated from cells expressing Yox1-Pk (FP4), grown to mid-log-phase and treated with 11 mM HU for 0, 1, 2 or 3 h, were incubated either with or without λ phosphatase. In (B) a lighter exposure is included for comparison. (A and B) Western blots were probed with anti-Pk antibody to visualise Yox1-Pk and with anti-tubulin antibody to determine loading. Yox1-Pk is indicated and asterisks indicate slower mobility, phosphatase-sensitive forms of the protein.

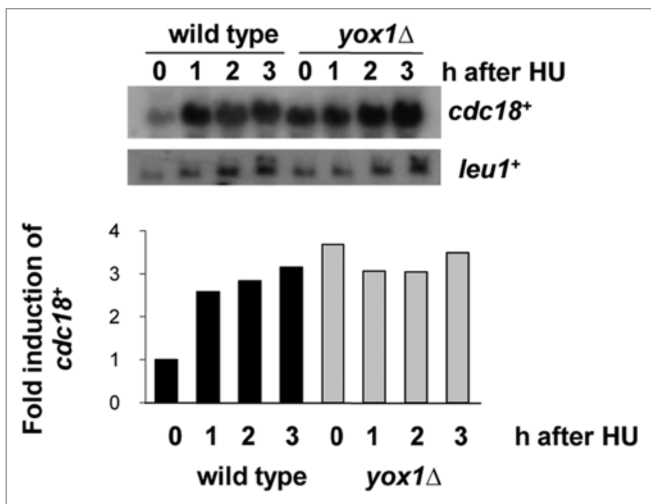

Figure 3. Loss of Yox1 function results in maximal HU-induced MBF-dependent gene expression. Analyses of RNA isolated from mid-log-phase growing wild-type (ED666) and *yox1*Δ (FP0) cells, treated with 11 mM HU for the indicated times, revealed that loss of Yox1 function leads to maximal HU-induced expression of the MBF-dependent *cdc18*+ gene. The northern blot was analyzed with a probe specific for *cdc18*+ and RNA was quantified using *leu1*+ as a loading control. Fold induction was calculated relative to the time-zero sample of the wild-type strain.

Discussion

In this paper we have identified new roles and mechanisms of regulation of the Yox1 homeodomain protein in *S. pombe*. In agreement with previous studies Yox1 was found to act as a repressor of MBF-dependent gene expression. However, our data suggests that Yox1 can act independently of the Nrm1 repressor to regulate MBF-dependent gene expression. Furthermore, our data indicates that regulation of Yox1 by the Rad3 DNA replication checkpoint is essential for upregulation of MBF-dependent gene expression and cell survival in response to replication stress. Hence, regulation of Yox1 coordinates the timing of expression of MBF-dependent genes to ensure the orderly progression of the cell cycle and to determine the appropriate response of cells to replication stress.

In contrast to other genes, the expression of MBF-dependent genes is derepressed in cells expressing the temperature sensitive mutant version of Cdc10 encoded by the *cdc10-C4* allele when grown at the permissive temperature.[24] Furthermore, this expression is inhibited when *cdc10-C4* mutant cells are placed at the non-permissive temperature.[24] The basis for this derepression and subsequent loss of gene expression is not known. Here, we found that deletion of *yox1*+ suppresses the temperature sensitive growth phenotype associated with the *cdc10-C4* allele. Moreover, loss of

Yox1 function at least partially suppressed the reduction of expression of the MBF-dependent *cdc18*$^+$ gene that occurs in *cdc10-C4* mutant cells at the non-permissive temperature. Hence, these data suggest that the repression of MBF-dependent gene expression in *cdc10-C4* mutant cells is Yox1-dependent. Previous work suggested that Yox1 and Nrm1 act in the same pathway to repress MBF-dependent gene expression.[12] Furthermore, it has been demonstrated that Nrm1 is unable to bind to the Cdc10 protein encoded by the *cdc10-C4* allele and that Yox1 binding to MBF-regulated promoters is Nrm1-dependent.[12,23] However, the observation that loss of Yox1 function can suppress the *cdc10-C4* mutation at the non-permissive temperature suggests that Yox1 may also act independently of Nrm1. Indeed, this conclusion is consistent with our findings that loss of either Nrm1 or Yox1, but not both together, increases the resistance of *rad3*$^+$ cells to HU. Interestingly, transcript profiling studies established that *yox1*$^+$ expression is regulated by MBF[3] and, similar to other MBF-regulated genes, we find that *yox1*$^+$ expression is derepressed in *cdc10-C4* mutant cells grown at the permissive temperature (data not shown). However, in contrast to *cdc18*$^+$, the levels of *yox1*$^+$ RNA were maintained in *cdc10-C4* mutant cells at the non-permissive temperature (data not shown). Hence, these data raise the possibility that this maintenance of *yox1*$^+$ RNA may be at least partly responsible for the observed repression of MBF-dependent gene expression in *cdc10-C4* mutant cells at the non-permissive temperature.

In eukaryotes the presence of replication stress triggers the DNA replication checkpoint, which, when activated, regulates the cell cycle and induces the appropriate response pathways to overcome the stress and repair damage. In *S. pombe* a key response to replication stress is the upregulation of MBF-dependent gene expression and this requires the activities of the mammalian ATM/ATR-related Rad3 kinase and the mammalian Chk2-related Cds1 kinase. Phosphomimetic mutations of proposed Cds1 phosphorylation sites in the MBF component Cdc10 are sufficient to induce MBF-dependent transcription.[22] Additionally, Cds1 has been proposed to phosphorylate the MBF co-repressor Nrm1 in order to promote its dissociation from promoters, and therefore derepress target gene expression.[23] The regulation of both Nrm1 and Cdc10 in this manner appears to represent a redundant mechanism, whereby the phosphorylation of either protein by Cds1 is sufficient for the checkpoint-mediated elevation of MBF-dependent gene expression. Here, we find that loss of Yox1 partially rescues the replication stress defect associated with loss of Rad3 function, suggesting that checkpoint-dependent inhibition of Yox1 repressor function is also essential for the normal replication stress-dependent activation of MBF-regulated gene expression (**Figs. 2–6**). Indeed consistent with this model, no further major increases

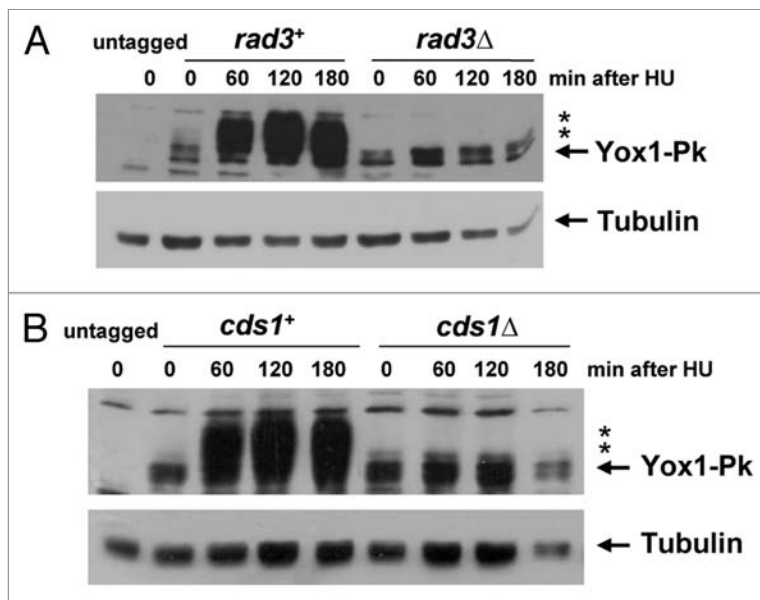

Figure 5. HU-induced phosphorylation of Yox1 is both Rad3- and Cds1-dependent. Western blot analyses of extracts isolated from mid-log-phase growing (A and B) *rad3*$^+$*cds1*$^+$Yox1-Pk (FP4), (A) *rad3*ΔYox1-Pk (FP34) and (B) *cds1*ΔYox1-Pk (FP75) cells, treated with 11 mM HU for the indicated times, revealed that phosphorylation of Yox1 is both Rad3- and Cds1-dependent. Yox1 was visualized with anti-Pk antibody and anti-tubulin antibody was used to determine loading. Extract isolated from mid-log-phase growing wild-type (CHP429-untagged) cells was used as a negative control. Yox1-Pk is indicated and asterisks indicate slower mobility forms of the protein.

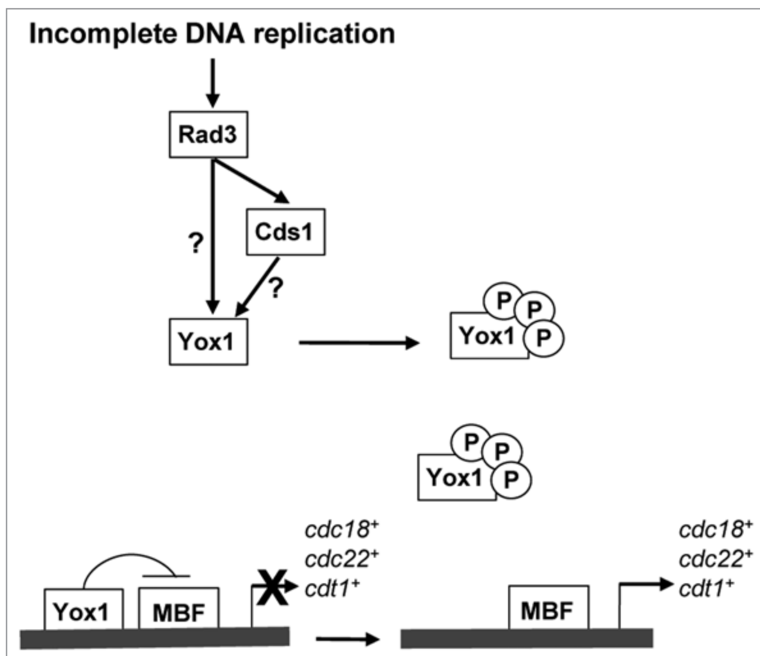

Figure 6. Model for the regulation of Yox1 by the Rad3 DNA replication checkpoint. During the cell division cycle Yox1 functions as a repressor of MBF-dependent gene expression. However, Yox1 is also regulated by the Rad3 cell cycle checkpoint in response to incomplete DNA replication. In the model activation of the Rad3 checkpoint pathway stimulates Rad3/Cds1-dependent phosphorylation of Yox1, inhibiting its repressor function, which leads to derepression of MBF-dependent gene expression.

Table 1. A list of the *S. pombe* strains used in this study

Strain	Genotype	Source
CHP429	*h^- leu1-32 ura4-D18 his7-366 ade6-M216*	Gift from C. Hoffman
CHP428	*h^+ leu1-32 ura4-D18 his7-366 ade6-M216*	Gift from C. Hoffman
ED666	*h^+ leu1-32 ura4-D18 ade6-M210*	Bioneer
FP0	*h^+ leu1-32 ura4-D18 ade6-X yox1::KanMX*	Bioneer
FP4	*h^- leu1-32 ura4-D18 his7-366 ade6-M216 $yox1^+$::3xPk::$ura4^+$*	This study
FP33	*$h^?$ leu1-32 ura4-D18 ade6-X rad3::$ura4^+$ yox1::KanMX*	This study
FP34	*h^- leu1-32 ura4-D18 ade6-X rad3::$ura4^+$ $yox1^+$::3xPk::$ura4^+$*	This study
FP36	*$h^?$ cdc10-C4 leu1-32 ade6-M216 yox1:: KanMX*	This study
FP75	*h^- leu1-32 ura4-D18 cds1::$ura4^+$ $yox1^+$::3xPk::$ura4^+$*	This study
FP96	*h^+ leu1-32 ura4-D18 cds1::$ura4^+$*	This study
FY7448	*h^- leu1-32 ura4-D18 cds1::$ura4^+$*	Yeast Genetic Resource Center, Japan
SW27	*h^- cdc10-C4 leu1-32 ade6-M216*	Lab Stock
TH146	*h^- leu1-32 ura4-D18 ade6-X rad3::$ura4^+$*	Gift from Tim Humphrey
SW804	*h^+ leu1-32 ura4-D18 ade6-X nrm1::KanMX*	Bioneer
SW805	*$h^?$ leu1-32 ura4-D18 ade6-X yox1::KanMX nrm1::KanMX*	This study

$h^?$ indicates an undetermined mating type and *ade6-X* indicates that the mutant allele is either *ade6-M210* or *ade6-M216*.

in MBF-dependent gene expression occurs in *yox1Δ* cells treated with HU and, significantly, both Rad3 and Cds1 are essential for replication stress-induced phosphorylation of Yox1. Since the timing of Rad3/Cds1-dependent phosphorylation of Yox1 coincides with the upregulation of MBF-dependent gene expression, we suggest that this phosphorylation of Yox1 is essential for dissociation of Yox1 from MBF target promoters and activation of gene expression (**Fig. 6**). In this case the regulation of Yox1 would mirror that of Nrm1 and, together with our results suggesting that Yox1 and Nrm1 act in separate pathways to regulate the response of cells to HU, may represent an additional mechanism for the control of gene expression in response to replication stress.

Together with previous studies our work suggests that regulation of Yox1 influences MBF-dependent gene expression during the cell cycle and upon activation of the mammalian ATM/ATR-related Rad3 cell cycle checkpoint. Interestingly, the role of homeodomain proteins as regulators of the cellular response to damage may be conserved. For example, expression of the homeodomain transcription factor Nkx3.1 facilitates the activation of ATM and ATR, and Nkx3.1 is downregulated in the majority of human primary prostate cancers.[27] In addition, overexpression of the human homeodomain protein HoxA5 in *S. cerevisiae* causes the upregulation of components of the mismatch repair (MMR) system that are important for the detection and repair of DNA damage.[28] Interestingly, it has also been proposed that the E2F family of transcription factors in mammalian cells is functionally analogous to MBF. Thus, further study of the conserved aspects of G_1/S phase/DNA replication checkpoint-regulated gene expression, including investigation of links with homeodomain proteins, will be important to provide insight into the coordination of cell cycle processes and DNA damage responses in eukaryotes.

Materials and Methods

Yeast strains and growth conditions. The *S. pombe* strains used in this study are listed in **Table 1**. Standard EMM (minimal) media containing the appropriate supplements and YE5S (rich) media were used for the growth of all yeast strains and yeast cells were transformed as described previously in references 29 and 30. Cells were grown at 30°C except cells containing the *cdc10-C4* allele, which were grown as described in the text.

Plasmids and strain construction. To construct the FP4 strain, expressing Yox1 tagged at the C-terminus with 3xPk epitopes from the normal chromosomal locus, first the full-length *$yox1^+$* gene, excluding the start and stop codons, was amplified by PCR using the oligonucleotide primers YoxPiF (AAA ACT GCA GAG TCT TTC TGA TTC TCC ATC) and YoxPK3 (GCG CGG ATC CGT GAT CAT TGC ATT GTT G) and CHP429 genomic DNA as template. The PCR product was digested with *Bam*HI and *Pst*I, and ligated with *Bam*HI/*Pst*I-digested pRIP42PkC[31] to create pRIP42*yox1*-PkC. Finally, pRIP42*yox1*-PkC was linearized by digestion with *Xba*I and introduced into CHP429 cells.

Several strain constructions were from crosses. Strain FP33 was from a cross between TH146 and FP0; strain FP34 was from a cross between FP4 and TH146; strain FP36 was from a cross between FP0 and SW27; strain FP96 was from a cross between FY7448 and CHP428; strain FP75 was from a cross between FP4 and FP96; strain SW805 was from a cross between SW804 and a strain containing a deletion of the *$yox1^+$* gene. Strain constructions were confirmed by PCR, DNA sequencing and western blot analysis as appropriate.

Sensitivity tests. Cells to be tested were grown to a concentration of approximately 2 x 10^6 cells/ml, then subjected to serial dilution before being spotted onto YE5S plates containing the indicated concentrations of HU and incubated at 30°C. To test UV radiation sensitivity, 5 μl of 10-fold serial dilutions were spotted onto YE5S plates, which were then exposed to the indicated dose of UV radiation using a UV Stratalinker (Stratagene). Plates were incubated at 30°C in the dark.

RNA analyses. RNA was isolated and northern blotting performed as described previously in reference 32. Gene-specific probes were generated by PCR and labeled with [α-^{32}P] dCTP using the Prime-a-Gene® Labeling System (Promega). Membranes were quantified using a phosphorimager (Bio-imaging analyzer Fujifilm Bas-1500) and Tina 2.0 software (Raytest).

Protein analyses. Protein extracts were prepared as described previously using lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM imidazole, 0.1% leupeptin, 0.1% pepstatin A, 1% aprotinin, 1% phenylmethylsulfonyl fluoride, 0.2% Na_3VO_4, 5% NaF).[33] Proteins to be

phosphatase-treated were prepared in lysis buffer without phosphatase inhibitors. 400 U λ phosphatase were then added to 40 μg protein and incubated for 15 min at 37°C. All protein samples were analyzed by 8–13% SDS-PAGE and transferred onto Protran® nitrocellulose transfer membranes. Membranes were incubated overnight with anti-Pk primary antibody (Serotec) at a concentration of 1:1,000, and then for 1 h with HRP-conjugated secondary antibody (Sigma-Aldrich) at a concentration of 1:200. Protein was visualized by ECL detection (GE Healthcare). To confirm even loading blots were stripped and reprobed with 1:1,000 anti-tubulin antibody (CRUK).

Acknowledgements

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