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RESEARCH ARTICLE

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# LAMMER Kinase Modulates Cell Cycle by Phosphorylating the MBF Repressor, Yox1, in *Schizosaccharomyces pombe*

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**ABSTRACT**

Lkh1, a LAMMER kinase homolog in the fission yeast *Schizosaccharomyces pombe*, acts as a negative regulator of filamentous growth and flocculation. It is also involved in the response to oxidative stress. The *lkh1*-deletion mutant displays slower cell growth, shorter cell size, and abnormal DNA content compared to the wild type. These phenotypes suggest that Lkh1 controls cell size and cell cycle progression. When we performed microarray analysis using the *lkh1*-deletion mutant, we found that only four of the up-regulated genes in the *lkh1*-deletion were associated with the cell cycle. Interestingly, all of these genes are regulated by the Mlu1 cell cycle box binding factor (MBF), which is a transcription complex responsible for regulating the expression of cell cycle genes during the G1/S phase. Transcription analyses of the MBF-dependent cell-cycle genes, including negative feedback regulators, confirmed the up-regulation of these genes by the deletion of *lkh1*. Pull-down assay confirmed the interaction between Lkh1 and Yox1, which is a negative feedback regulator of MBF. This result supports the involvement of LAMMER kinase in cell cycle regulation by modulating MBF activity. *In vitro* kinase assay and NetPhosK 2.0 analysis with the Yox1$^{T40,41A}$ mutant allele revealed that T40 and T41 residues are the phosphorylation sites mediated by Lkh1. These sites affect the G1/S cell cycle progression of fission yeast by modulating the activity of the MBF complex.

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Lkh1; LAMMER kinase; Yox1; MBF; cell cycle; phosphorylation

## 1. Introduction

LAMMER kinase is a dual-specificity kinase, which can act as both a tyrosine kinase and a serine/threonine kinase [1]. LAMMER kinases in various yeasts and filamentous fungi also share similar functions [2]. In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), Lkh1 plays roles in growth, flocculation, oxidative stress response, cell cycle, and sexual differentiation [3–6]. In the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), Kns1 is involved in adhesive filamentous growth, temperature sensing, and tRNA biogenesis [7–9]. In the dimorphic pathogenic fungus *Candida albicans* (*C. albicans*), Kns1 is involved in morphogenesis, response to cell wall- and DNA-damaging stress, cell cycle, and virulence [10]. In filamentous fungi *Aspergillus nidulans* (*A. nidulans*), opportunistic human pathogen *Aspergillus fumigatus* (*A. fumigatus*), and plant pathogen *Magnaporthe oryzae* (*M. oryzae*), LAMMER kinase is required for various biological processes including asexual development, sexual development, cell cycle regulation, cell wall biosynthesis, and virulence [11–14]. Sexual development and cell cycle are affected by the deletion of LAMMER kinase in the plant pathogen *Ustilago maydis* (*U. maydis*) [15]. While the mouse CDC2-like kinase (CLK) family contains four conserved isoforms as LAMMER kinase homologs, there are three CLKs in humans [16]. Analysis of breast and prostate tumors shows aberrant splicing in human CLK2 genes, suggesting that the misexpression of LAMMER kinase can cause certain cancer with defects in cell cycle regulation [17].

In *S. pombe*, the *lkh1*-deletion (Δ*lkh1*) strain has a defect in cell growth, resulting in shorter cell length compared to the wild type (WT) strain. Additionally, the Δ*lkh1* strain shows a reduced response to DNA replicative stresses [18,19]. In the cell cycle, *S. pombe* coordinates its cell size at the end of the G1 and G2 phases [20]. The Δ*lkh1* strain

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has a very short G1 phase and quick progresses to the S phase [6], suggesting that Lkh1 is required for the regulation of the G1/S phase.

Rum1, a cyclin-dependent kinase (CDK) inhibitor, plays an important role in cell cycle arrest [21]. Lkh1 phosphorylates the T110 residue of Rum1, which can induce CDK activity [6]. The G1/S phase transition, including CDK activation, is also regulated by the Mlu1 cell cycle box-binding factor (MBF) in yeast and human cells [22]. Transcriptional changes during the G1/S phase depend on the corepressors, negative regulator of MBF target 1 (Nrm1) and yeast homeodomain protein (Yox1). Both are MBF targets and are involved in negative feedback regulation [23]. The MBF-dependent transcription is maintained at a high level by the Cdc2 kinase-Cdc13 cyclin B complex during the G1/S phase [24]. Also, Nrm1 is required for the activation of Yox1, which represses the MBF-dependent transcription at the end of the S phase [25].

In this study, we investigated whether Lkh1 plays a direct or indirect role in inhibiting MBF activity. Our results suggested that Lkh1 is involved in the transcriptional regulation of MBF-dependent factors, as well as Nrm1 and Yox1, which act as negative regulators of MBF activity. We found that Lkh1 can interact with Yox1 and phosphorylate threonine 40 and threonine 41 (T40 and T41) of Yox1. To investigate the effects of phosphorylation at T40 and T41, a non-phosphorylated form of Yox1 was generated. The non-phosphorylated form of Yox1 showed increased expression of MBF-dependent genes and regulator genes, as well as increased flocculation. These phenotypes were similar to those observed in the Δ*lkh1* strain.

## 2. Materials and methods

### 2.1. Strains and growth conditions

*S. pombe* strains were grown in YES and Edinburgh synthetic minimal medium (EMM) with appropriate supplements at 30 °C [26]. For the construction of the Nrm1-His and Yox1-His tagged plasmids, the *nrm1* and *yox1* genes were amplified and cloned into pET28a (Sigma-Aldrich, Massachusetts, US). These plasmids were introduced into *Escherichia coli* BL21 strains for *in vitro* protein expression. The BL21 strain expressing the GST-tLkh1 fusion protein was obtained from a previous study [5]. To generate the Δ*yox1*-pREP81/Yox1$^{T40,41A}$ strain, PCR-mediated mutagenesis was performed following the manufacturer's protocols (In-Fusion kit, Clontech, California, US). PCR products amplified using point-mutated primer sets were cloned into pREP81. The pREP81/Yox1 or pREP81/Yox1$^{T40,41A}$ plasmids were introduced into *S. pombe* ED668Y(Δ*yox1*) strain (Bioneer, Daejeon, KR). All primers used in this study are listed in Table S1.

For the flocculation assay, cells were grown to the early stationary phase in YES media and then washed with 10 mM EDTA followed by excess water. $CaCl_2$ was added to the cell suspension to initiate the flocculation reaction. Cell suspension was transferred to Petri dishes for the detection of floc formation [27]. For determination of flocculation activity, the optical density ($OD_{600}$) of the upper layer of the cells was carefully measured as the initial cell density ($A_{t0}$). After 30 min of settling, the $OD_{600}$ of the upper layer was measured as the end cell density ($A_{t30}$). Cell sedimentation rate was calculated as $V = 1 - A_{t30}/A_{t0}$ [28].

### 2.2. RNA preparation, cDNA synthesis, and quantitative PCR (qRT-PCR)

The *S. pombe* strains were grown in EMM [4]. After harvesting, the cells were homogenized using 600 µm-sized glass beads (Sigma-Aldrich). Total RNAs were extracted using Trizol according to the manufacturer's protocols (Invitrogen, Massachusetts, US). cDNA was synthesized using extracted RNA, oligo-dT, and M-MLV reverse transcriptase (ELPIS Biotech, Daejeon, KR). qRT-PCR was performed using a TOPrealTM qPCR 2X PreMIX kit (Enzynomics, Daejeon, KR). Primers used for the qRT-PCR were listed in Table S1.

### 2.3. Purification of proteins from E. coli BL21

BL21 strains containing Nrm1- or Yox1-expressing plasmids (pET28a/*nrm1* or pET28a/*yox1*, respectively) were grown in Luria-Bertani (LB) medium with 50 µg/ml kanamycin. For the purification of GST fusion proteins, BL21 strains containing the Lkh1-expressing plasmid (pGEX4T-1/*lkh1*) were grown in LB medium supplemented with 40 µg/ml ampicillin and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) [5].

### 2.4. Pull-down assay

A pull-down assay was performed using a modified method as previously described [5]. In brief, GST- or GST-cLkh1-bound beads were mixed with His-tagged Nrm1 or Yox1 soluble lysates (200 µg) at 4 °C for 1 h. After washing and centrifugation, the precipitates were resolved on a 10% polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chicago, US). The interaction between the GST-tagged protein and His-tagged proteins was confirmed by Western analysis using an anti-His antibody (Sigma-Aldrich). The gels were stained

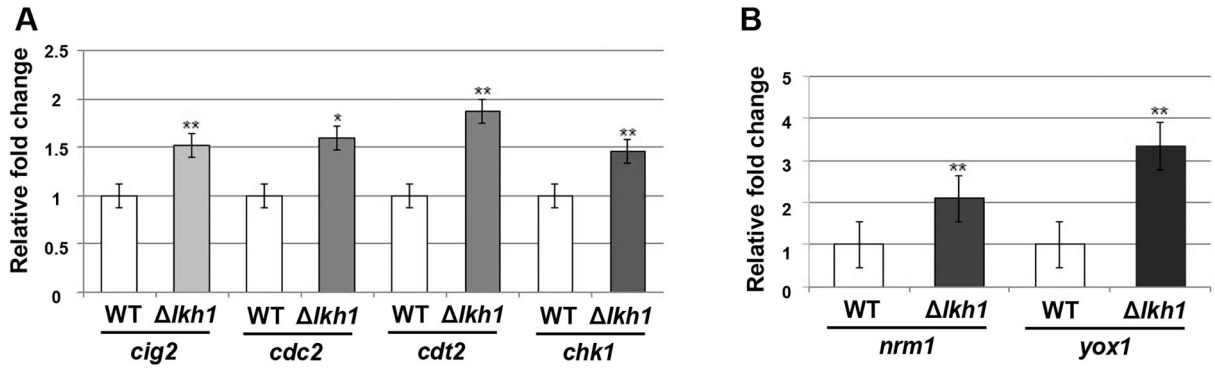


Figure 1. Deletion of *lkh1* increased expression of the genes associated with MBF activity. Total RNAs were prepared from the WT and the Δ*lkh1* strains and used for synthesis of cDNA. qRT-PCR was performed. (A) Expression of the MBF-dependent genes, *cig2, cdc2, cdt2,* and *chk1*. (B) Expression of the genes for negative regulators of MBF complex, *nrm1* and *yox1*. $*p < 0.05$; $**p < 0.01$.

with Coomassie Brilliant Blue R-250 (ThermoFisher, Massachusetts, US).

### 2.5. In vitro kinase assay

The GST fusion form of Lkh1 (100 ng) was mixed with His-tagged Yox1 (200 ng) in 30 µl of kinase buffer (25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol) with 10 µCi of [$\gamma$-$P^{32}$] ATP and incubated at 30 °C for 1 h [5]. The reaction was stopped by adding SDS-PAGE sample buffer. Protein samples were electrophoresed on a 10% SDS-PAGE gel, transferred onto PVDF membranes, and visualized using autoradiography.

### 2.6. In-gel digestion and LC-MS/MS analysis

In-gel protein digestion analysis was performed as previously described with some modifications [29]. A slice of SDS-PAGE gel with the target protein(s) was incubated with trypsin (Promega, Madison Wisconsin, US) and washed with a solution containing 25 mM acetonitrile and 25 mM ammonium bicarbonate (pH 7.8). Rehydration was conducted in 25 mM ammonium bicarbonate with 20 ng of trypsin at 37 °C for 20 h. Tryptic peptides were prepared by incubating them in 20 µl of 50% (v/v) acetonitrile with 0.1% (v/v) formic acid at 30 °C for 40 min. The Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) analysis was performed according to the modified method [30]. To identify the predicted phosphorylation sites in yeast, NetPhosK 2.0 was used [31].

## 3. Results

### 3.1. Deletion of lkh1 increased the transcription of the MBF-dependent genes

In a previous study, we performed microarray analysis using both the WT and Δ*lkh1* strains [32]. Among the cell cycle-related genes showing differential expression, the MBF-dependent genes (*cig2*, *cdc22*, *cdt2*, and *chk1*) exhibited significantly increased expression in the Δ*lkh1* strain compared to the WT strain. To validate the microarray data, we conducted qRT-PCR to assess the expression of the four MBF-dependent genes. In the Δ*lkh1* strain, these genes showed increased expression (Figure 1(A)). We hypothesized that Lkh1 negatively regulates MBF-dependent genes, either directly or indirectly. There are two major repressors for MBF activity, Nrm1 and Yox1 [23,33], which bind to MBF and lead to transcriptional repression as cells exit G1 phase. Next, we examined the effect of *lkh1* deletion on the expression of the *nrm1* and *yox1* genes. These genes were highly expressed in the Δ*lkh1* strain (Figure 1(B)), suggesting that Lkh1 affects the expression of the MBF-dependent genes by regulating the transcription of upstream repressor genes, *nrm1* and *yox1*.

### 3.2. Lkh1 interacted with and phosphorylated Yox1

We further investigated the protein-protein interaction between the repressors and Lkh1. A pull-down assay was carried out to identify the interacting partner. Tagged proteins were purified from *E. coli* BL21 strains containing each expression plasmid and co-incubated. After washing, the precipitates with glutathione-sepharose beads were analyzed using SDS-PAGE and Western blotting using an anti-His antibody. Lkh1 interacted with Yox1 but not Nrm1 (Figure 2(A,B)). Since the data revealed an interaction between Lkh1 and Yox1, we performed *in vitro* kinase assay to examine whether Lkh1 can phosphorylate Yox1. Lkh1 can phosphorylate serine/threonine and tyrosine, and undergo autophosphorylation [34]. In the autoradiogram, evidence of Yox1 phosphorylation by Lkh1 was observed (Figure 2(C)). To identify the phosphorylation sites in Yox1 by Lkh1, the phosphorylated protein sample in the SDS-PAGE was analyzed using peptide mass fingerprinting (data not shown), and NetPhosK 2.0 was used. From these analyses, T40 and T41 in the Yox1 homeodomain

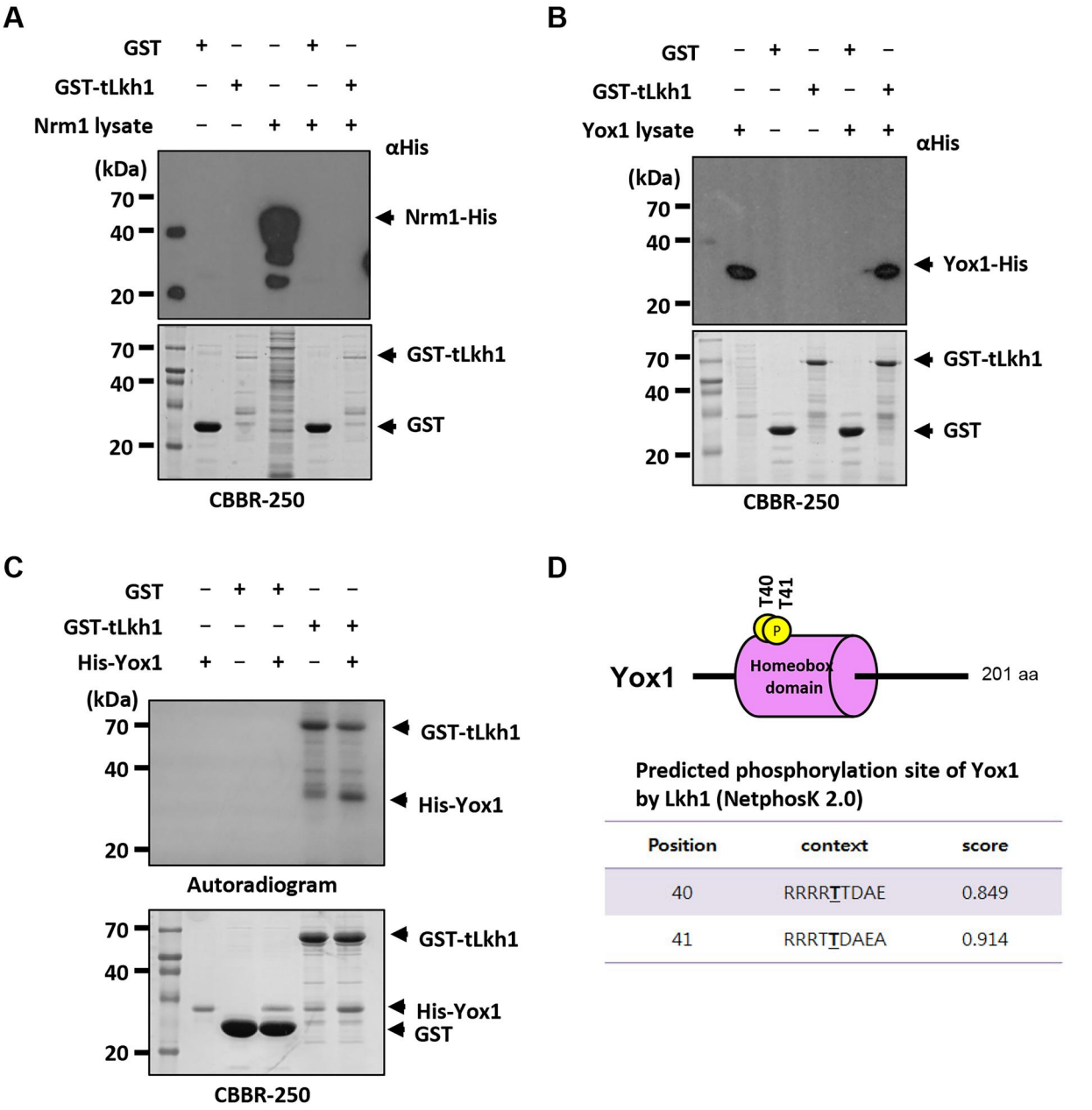


**Figure 2.** Lkh1 interacted with and phosphorylated Yox1 at T40 and T41. (A) *in vitro* interaction between Lkh1 and Nrm1. (B) *in vitro* interaction between Lkh1 and Yox1. (A-B) Soluble proteins containing His(6x)-tagged Nrm1 and Yox1 were mixed with glutathione sepharose bead containing GST or GST-tLkh1 fusion proteins for 1 h at 4 °C. Precipitates were resolved on 10% SDS-PAGE with duplication. One was stained with CBBR-250 (bottom) and another was transferred to PVDF membraned for Western blotting with anti-His antibody (top) (C) *In vitro* kinase assay using recombinant Lkh1 and Yox1. Phosphorylation of Yox1 by Lkh1. His(6x)-tagged Yox1 was purified and mixed with glutathione sepharose bead containing GST or GST-tLkh1 fusion proteins in the presence of [γ-$P^{32}$] ATP. After incubation at 30 °C for 1 h, the reaction was stopped by the addition of SDS-PAGE sample buffer. The kinase reaction samples were resolved on 10% PAGE and then transferred to PVDF membranes. Autoradiography of His-tagged Yox1 and GST-tLkh1 were obtained (top). Lkh1 has auto-phosphorylation activity. The gels were stained with CBBR-250 (bottom). (D) Predicted phosphorylation sites of Yox1 by Lkh1. Yox1 has homeobox domain (34-92 amino acids). NetphosK 2.0 was used to analysis predicted phosphorylated sites of Yox1 by Lkh1. T40 and T41 are located in homeodomain.

were identified as the predicted phosphorylation sites by Lkh1 (Figure 2(D)).

### 3.3. Non-phosphorylated $Yox1^{T40,T41A}$ increased the transcription of the MBF target genes

Next, we generated the pREP81/$Yox1^{T40,T41A}$ plasmid containing the non-phosphorylated Yox1 by replacing both threonine residues with arginine residues at positions 40 and 41. This plasmid was introduced into the Δ*yox1* strain. The pREP81/Yox1 plasmid was introduced into Δ*yox1* strain as a control group. The *S. pombe* strain expressing non-phosphorylated $Yox1^{T40,T41A}$ showed increased expression of MBF-dependent genes (*cig2*, *cdc22*, *cdt2*, and *chk1*) and MBF repressor genes (*nrm1* and

*yox1*) (Figure 3), which exhibited a similar pattern to that shown in the Δ*lkh1* strain (Figure 1). These data suggest that phosphorylation at T40 and T41 by Lkh1 is required for the role of Yox1 in transcriptional repression.

### 3.4. Non-phosphorylated Yox1$^{T40,T41A}$ showed a partial flocculation phenotype

Increased flocculation in the Δ*lkh1* strain is a remarkable phenotypic characteristic [5], which led us to examine flocculation in the WT, Δ*lkh1*, and *yox1* mutant strains. Interestingly, the Δ*yox1* strain exhibited increased flocculation, similar to the Δ*lkh1* strain. However, the Δ*yox1*-pREP81/Yox1 strain episomally producing Yox1 did not exhibit the flocculation phenotype (Figure 4). Although there is a difference in degree, the Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strain exhibited flocculation. These data suggest that phosphorylation at T40 and T41 in Yox1 is involved in the regulation of flocculation.

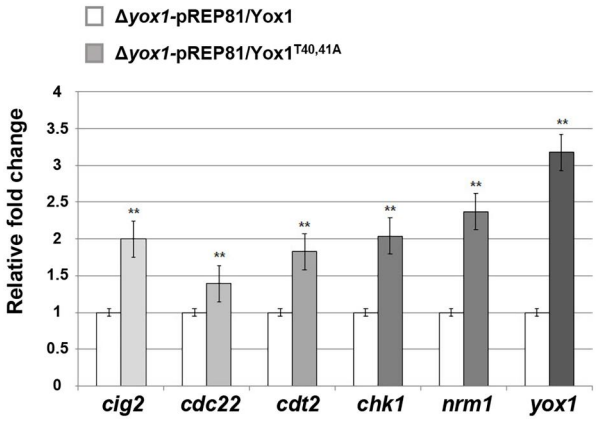


**Figure 3.** Non-phosphorylated Yox1$^{T40,T41A}$ increased expression of the genes associated with MBF activity. Total RNAs were prepared from the Δyox1-pREP81/Yox1 and the Δyox1-pREP81/Yox1$^{T40,T41A}$ strains and used for synthesis of cDNA. qRT-PCR was performed. (A) Expression of the MBF-dependent genes, *cig2, cdc2, cdt2,* and *chk1*. (B) Expression of the genes for negative regulators of MBF complex, *nrm1* and *yox1*. $^{**}p < 0.01$.

## 4. Discussion

In eukaryotes, LAMMER kinases have a role in cell cycle regulation [2]. In *S. pombe*, Lkh1 phosphorylates the CDK-inhibitor Rum1 for cell cycle regulation [6]. The Δ*lkh1* strain grows with a shorter cell length [18], which has been considered a consequence of a shorter G1 phase due to the failure of phosphorylation of Rum1 by Lkh1 [6].

This study showed that Lkh1 has an alternative pathway to regulate G1/S transition by negatively regulating the transcription of MBF-associated genes. MBF binds to DNA and activates the transcription of genes that are necessary for entering the S phase [35]. Both microarray analysis [32] and qRT-PCR data showed the MBF-dependent genes, such as *cig2*, *cdc2*, *cdt2*, and *chk1*, were highly expressed in the Δ*lkh1* strain (Figure 1(A)). The expression of the gene encoding negative regulators, Nrm1 and Yox1, was also increased in the Δ*lkh1* strain (Figure 1(B)). Taken together, these data suggest that Lkh1 is required for the transcriptional regulation of the genes involved in the G1/S transition.

Yox1 and Nrm1 are negative feedback regulators of MBF activity [23,33]. We identified Yox1 as an interaction partner of Lkh1, but not Nrm1 (Figure 2(A,B)). T40 and T41 in Yox1 were identified as phosphorylation sites by Lkh1 (Figure 2(C,D)). Both the Δ*lkh1* strain and the Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strain showed increased expression of MBF-dependent genes (Figure 1 and 3), suggesting that phosphorylation at

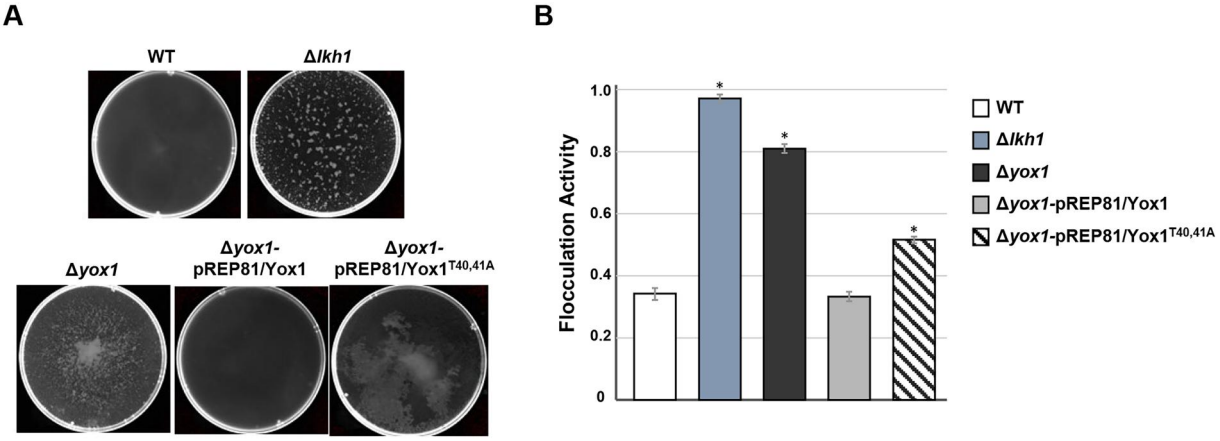


**Figure 4.** The WT, Δ*lkh1*, Δ*yox1* and Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strains showed increased flocculation. Cells of the WT, Δ*lkh1*, Δ*yox1*, Δ*yox1*-pREP81/Yox1 and the Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strains were grown in YES media to early stationary phase and washed with 10 mM EDTA and excess water. $CaCl_2$ was added to the cell suspension to initiate the flocculation reaction. (A) Cell suspension was transferred to Petri dishes and photographed. (B) The flocculation activity was determined by measuring the sedimentation rate as the optical density ($OD_{600}$) of the upper layer of the cell suspension after 30 min of settling. $^{*}p < 0.05$.

T40 and T41 by Lkh1 is necessary for Yox1's negative regulatory function during normal cell cycle. It has been known that Yox1 is a target of Cdk1 *in vitro*, but whether Cdk1 directly controls the activity of Yox1 has not been studied [36]. Here, for the first time, we report the novel phosphorylation sites of Yox1 that control MBF activity during the normal cell cycle by Lkh1.

Yox1 has roles in normal cell cycle regulation as well as in response to DNA replicative stress [37]. In DNA replicative stress, phosphorylation of Yox1 at S114 and T115 by a checkpoint kinase (human Chk2) Cds1 induces the dissociation of Yox1 from promoters, thereby maintaining the activity of G1/S phase transcriptional program [37]. The Δ*lkh1* strain showed susceptibility to hydroxyurea (HU), which induced DNA replicative stress [19]. The Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strain showed sensitivity to HU and methyl methane sulfonate (data not shown). LAMMER kinase is involved in the response to DNA replicative stress in other eukaryotes, such as *C. albicans* and *M. oryzae*, as well as in genome stability in *U. maydis* [10,14,15]. When the subcellular localization of Yox1 was investigated, we observed that non-phosphorylated Yox1$^{T40,T41A}$ resulted in a decreased nuclear to cytoplasmic ratio of ∼0.6, compared to Yox1 with the nuclear to cytoplasmic ratio of ∼1.4 (data not shown). These data suggest that phosphorylation at T40 and T41 is associated with the translocation of Yox1 to the nucleus. Taken together, the results suggest that Lkh1 is required for the localization and activation of Yox1, which regulates the cell cycle under both normal and DNA replicative stress conditions in *S. pombe*.

Flocculation is a mechanism for yeast to survive under adverse growth conditions [38]. Both the Δ*lkh1* strain and the Δ*yox1* strain exhibited flocculation in liquid media (Figure 4). The Δ*yox1*-pREP81/Yox1$^{T40,T41A}$ strain exhibited flocculation, albeit to a lesser extent than the Δ*lkh1* strain and the Δ*yox1* strain, suggesting the phosphorylation of Yox1 at T40 and T41 by Lkh1 is also involved in flocculation. However, it is possible that other regulatory mechanisms may also contribute to this process.

Consequently, phosphorylation of Yox1 at T40 and T41 by Lkh1 plays a role in cell cycle regulation and, to some extent, in flocculation. To understand the role of Lkh1 in DNA replicative stress, further investigation is needed to identify Lkh1-mediated phosphorylation sites in Yox1. This will provide insight into the genesis and treatment of human cancer.

## Author contributions

Joo-Yeon Lim analyzed and organized the data, and wrote and revised the manuscript. Kibum Park, Je-Hoon Kim, Jieun Lee, and Songju Shin performed the experiments and data analysis. Hee-Moon Park designed the experiments, analyzed the data, and revised the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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