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The LAMMER Kinase Homolog, Lkh1, Regulates Tup Transcriptional Repressors through Phosphorylation in *Schizosaccharomyces pombe**[S]

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Disruption of the fission yeast LAMMER kinase, Lkh1, gene resulted in diverse phenotypes, including adhesive filamentous growth and oxidative stress sensitivity, but an exact cellular function had not been assigned to Lkh1. Through an *in vitro* pull-down approach, a transcriptional repressor, Tup12, was identified as an Lkh1 binding partner. Interactions between Lkh1 and Tup11 or Tup12 were confirmed by *in vitro* and *in vivo* binding assays. Tup proteins were phosphorylated by Lkh1 in a LAMMER motif-dependent manner. The LAMMER motif was also necessary for substrate recognition *in vitro* and cellular function *in vivo*. Transcriptional activity assays using promoters negatively regulated by Tup11 and Tup12 showed 6 or 2 times higher activity in the Δ*lkh1* mutant than the wild type, respectively. Northern analysis revealed derepressed expression of the *fbp1*$^+$ mRNA in Δ*lkh1* and in Δ*tup11*Δ*tup12* mutant cells under repressed conditions. Δ*lkh1* and Δ*tup11*Δ*tup12* mutant cells showed flocculation, which was reversed by co-expression of Tup11 and -12 with Ssn6. Here, we presented a new aspect of the LAMMER kinase by demonstrating that the activities of global transcriptional repressors, Tup11 and Tup12, were positively regulated by Lkh1-mediated phosphorylation.

LAMMER kinases are found in all eukaryotes, where they possess an almost identical catalytic subdomain that is essential for kinase activity (1), suggesting that function is conserved across greatly diverged organisms (2). LAMMER kinase identity is pronounced among higher eukaryotes in the "EHLAMMERILG" motif in a kinase subdomain, but some amino acids are substituted in fungi in this motif (2–9). The LAMMER motif was recently reported to be essential for kinase activity and localization but not for binding of substrates *in vitro* (9). All of the LAMMER family kinases possess dual specificity kinase activity, including serine/threonine and tyrosine kinase, and autophosphorylation activities (1, 5, 8, 10, 11).

A number of studies have reported that the LAMMER kinases are involved in mRNA splicing through regulating splicing factors, such as serine/arginine-rich (SR)[2] proteins, in humans (7, 12), fruit flies (2, 13, 14), and plants (6, 9). Although no evidence for splicing factor/LAMMER kinase interaction has been presented in yeasts, Kns1 (LAMMER kinase from *Saccharomyces cerevisiae*) phosphorylates and interacts with mammalian SR protein (ASF/SF2, SRp20/X16, and RBP1) *in vitro* (11), suggesting that LAMMER kinases of yeast, like those of plants and animals, may phosphorylate and interact with SR proteins *in vivo*.

LAMMER kinases may play a role in the regulation of cellular processes in addition to that of alternative splicing. Clk1 activates ERK-1, ERK-2, and pp90RSK (15). Clk1 and Clk2 phosphorylate and activate the tyrosine phosphatase PTP-1B (16), suggesting that they are involved in signaling. DOA and PK12 phosphorylate major histones (H1, H2A, H2B, H3, and H4) (11). According to a recent study, DOA is observed on all four *Drosophila* chromosomes, in a manner similar to many general chromatin factors (17), meaning that the LAMMER kinase might be involved in gene expression by regulating splicing factors or chromatin factors.

Although a cellular function for LAMMER kinase in fungi has not yet been described, the *lkh1* deletion mutant of *Schizosaccharomyces pombe* reveals various phenotypes, including abnormality in cell division (3, 18); flocculation in liquid medium and filamentous and adhesive growth on agar (3); and sensitivity to oxidative stress (19, 20). Recently, we found that phosphorylation by Lkh1 is required for the activation of Csx1, which protects mRNAs encoding the transcription factor, Atf1, from exonuclease-mediated decay under oxidative conditions (20). This suggests that Lkh1 may play important roles in regulating gene expression in *S. pombe*; however, the mode of Lkh1 action remains to be determined. In order to elucidate the cellular function of the Lkh1, an *in vitro* pull-down screen using maltose-binding protein-tagged cLkh1 was carried out, and Tup12 was identified as an Lkh1-interacting protein.

S. cerevisiae Tup1 is a global transcriptional repressor that interacts with Ssn6 (21–23). The Tup1-Ssn6 complex is involved in the repression of some genes regulated by glucose, oxygen radicals, DNA damage, and other cellular stresses (24–28). Repression via this complex is controlled by a variety of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S3.

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[2] The abbreviations used are: SR, serine/arginine-rich; EMM, Edinburgh synthetic minimal medium; PVDF, polyvinylidene difluoride; GST, glutathione *S*-transferase; GFP, green fluorescent protein.

DNA-binding proteins, such as transcriptional regulators, histones, histone deacetylase, and the components of RNA polymerase II holoenzyme (29–33). The repression of target genes has been reported to occur by two different mechanisms. First, the Tup1-Ssn6 complex interferes directly with the interaction between general transcription machinery components (33–35). Second, the Tup1-Ssn6 complex mediates repression by altering the local chromatin structure (29, 30, 36, 37). Tup1 interacts directly with the amino-terminal tail domain of histone H3 and H4 *in vitro* (38). The Tup1-Ssn6 complex is functionally conserved throughout the fungi, and there are related corepressors in higher eukaryotes, like HIRA, TLE, and Groucho (39, 40), that are functionally and structurally homologous to yeast corepressors.

The fission yeast *S. pombe* has two redundant counterparts of *S. cerevisiae* Tup1, which are Tup11 and Tup12 (41, 42). They are involved in transcriptional repression of the *fbp1*$^{+}$ gene, which encodes fructose-1,6-*bis*-phosphatase (41, 43), and the *cta3*$^{+}$ gene, which encodes the cation-transporting P-type ATPase (44). Tetramers consisting of only Tup11 or Tup12 interact with Ssn6 proteins and regulate different programs of gene expression (45). Binding studies showed that Tup11 interacts with the *S. cerevisiae* transcription factor Matα2 and with histones H3 and H4 (43). Tup11 also interacts with Fep1 (46, 47), a transcription factor that represses the expression of *fio1*$^{+}$, *fip1*$^{+}$, and *frp1*$^{+}$ genes, which regulate iron concentration (47, 48). As the *TUP1* deletion mutant of *S. cerevisiae* (49), the *tup11tup12* double deletion mutant shows pleiotropic phenotypes, including flocculation in liquid medium, defective mating, and defective stress response (41, 45), which mirror those of the *lkh1* deletion mutant (3). Based on this and the fact that Tup12 was isolated as Lkh1-interacting protein, we investigated a functional link between Lkh1 and Tup12 as well as Tup11 in gene expression regulation.

We show that LAMMER kinase, Lkh1, directly interacts with and phosphorylates the transcriptional repressors Tup11 and Tup12. To our knowledge, this is the first report on the upstream regulation of Tup repressors in yeasts.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—All *S. pombe* strains used in this study are listed in supplemental Table S1. YES and Edinburgh synthetic minimal medium (EMM) with appropriate supplements were used as the rich medium and the selective medium, respectively (50). Cells were grown at 30 °C. Standard techniques for fission yeast molecular genetics were used following Moreno *et al.* (50, 51). The ultracompetent cell method and the lithium acetate method were applied to transform *Escherichia coli* and *S. pombe* cells, respectively (52).

Plasmid Construction—To express GST-Tup11 or GST-Tup12, fusion proteins in fission yeast, the *tup11*$^{+}$ and *tup12*$^{+}$ genes were PCR-amplified with gene-specific primer sets (supplemental Table S2; for *tup11*$^{+}$, T11-a6 and Tup11-b7; for *tup12*$^{+}$, T12-a7 and T12-b7), and amplicons were cloned into pESP-1 (Stratagene) at the BamHI site separately. After construction, the pESP-*tup11*$^{+}$ and pESP-*tup12*$^{+}$ plasmids were confirmed by restriction mapping and partial sequencing.

Plasmid pREPLacZ was constructed by cloning a 3,065-bp SalI-SmaI fragment of *LacZ* amplified by PCR with specific primer sets (LacZ-a1 and LacZ-b1) into SalI-SmaI-digested pREP82. This plasmid was used for construction of the *LacZ* fusion with the gene-specific promoter. The *fip1*$^{+}$ and *fbp*$^{+}$ promoter regions were amplified by PCR with gene-specific primer sets (*fip1*$^{+}$, pfip-a2 and pfip-b1; *fbp1*$^{+}$, fbp-a1 and fbp-b1) and were cloned into the pREPLacZ vector separately. pREPLacZ-p*fip1*$^{+}$ contained the *fip1*$^{+}$ promoter region up to −1,143 bp from the start codon, and the pREPLacZ-p*fbp*$^{+}$ contained the *fbp*$^{+}$ promoter region up to −2.5 kb from the start codon.

In order to express Tup11 or Tup12 in *E. coli*, BamHI-XhoI fragments of *tup11*$^{+}$ and *tup12*$^{+}$ from pT-*tup11*$^{+}$ and pT-*tup12*$^{+}$, the T-vectors containing PCR-amplified *tup11*$^{+}$ and *tup12*$^{+}$, were subcloned into the corresponding restriction site of the pET28a and pET32a (Novagen) separately. DNA fragments for the full-length Lkh1 (*flkh1*$^{+}$) and the catalytic domain alone (*clkh1*$^{+}$) were amplified by PCR with gene-specific primer sets (lkh1-a3/lkh1-b2, and lkh1-a4/lkh1-b2) and cloned into pET28a and pGEX4T-1, respectively.

Using PCR-mediated mutagenesis (53), DNAs for kinase-inactive and LAMMER motif deletion mutant were constructed. DNA for the kinase-inactive mutant was made by overlapping PCR with PCR products amplified by point-mutated primer sets (lkh1-a7/lkh1-b9 and lkh1-a9/lkh1-b7). The overlapping PCR product, in which Arg^{391} (A<u>G</u>G) was substituted for Lys^{391} (A<u>A</u>G), was cloned into the p42GFP vector. DNA for the LAMMER motif deletion mutant was PCR-amplified using gene-specific primers (lkh1-a7/lkh1-b10 and lkh1-a10/lkh1-b7) and was cloned into p42GFP vector. In order to produce the GST fusion form of the Lkh1 catalytic domain (GST-cLkh1), the LAMMER motif-deleted Lkh1 catalytic domain (GST-cLkh1$^{\Delta LA}$), and kinase-inactive Lkh1 catalytic domain (GST-cLkh1^{K391R}) in *E. coli*, PCR products amplified by primers (lkh1-a4/lkh1-b2) using p42GFP-*lkh1*$^{+}$, p42GFP-*flkh1*$^{\Delta LA+}$, and p42GFP-*flkh1*$^{K391R+}$ as templates, respectively, were cloned into pGEX4T-3.

Purification of Tup11, Tup12, and cLkh1—*E. coli* (BL21) transformants containing *tup11*$^{+}$ and *tup12*$^{+}$ on bacterial expression vectors (pET28a and pET32a), respectively, were grown in Luria-Bertani with 50 μg/ml kanamycin or 100 μg/ml ampicillin at 37 °C until A_{600} = 0.8. The cultures were induced for 1 h at 37 °C and 4 h at 30 °C with 1 mM isopropyl thio-β-D-galactoside and then harvested. Cell pellets suspended in 10 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 1 mM phenylmethylsulfonyl fluoride) and sonicated (Sonic & Materials Inc.) on ice for 5 min. The lysates were centrifuged at 14,000 × *g* for 20 min. Nickel columns loaded with soluble lysates were washed with 10 volumes of binding buffer and 5 volumes of washing buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and then eluted with five volumes of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9).

For purification of GST fusion proteins, bacterial cells containing *flkh1*$^{+}$, *clkh1*$^{+}$, *clkh1*$^{\Delta LA}$, and *clkh1*^{K391R} on pGEX4T-3, respectively, were grown in Luria-Bertani with 100 μg/ml of ampicillin at 37 °C until A_{600} = 0.8. The cultures were induced

for 1 h at 37 °C and overnight at 18 °C with 1 mM isopropyl thio-β-D-galactoside and then harvested by centrifuging at 14,000 × g for 10 min. Pellets were suspended in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, sonicated on ice for 5 min, and centrifuged at 14,000 × g for 20 min. Soluble lysates were mixed with glutathione-Sepharose beads (Elpis) and incubated at 4 °C for 1 h by rotation with a mixer (Seoulin Bioscience). The beads isolated by centrifugation were washed with 1 ml of phosphate-buffered saline 10 times.

GST Pull-down Assay—For interaction between Lkh1 and Tup11 (or Tup12), GST- or GST-cLkh1-bound glutathione-Sepharose beads were mixed with His-tagged Tup11 or Tup12 soluble lysates (200 μg) induced in bacteria cells. After reaction at 4 °C for 1 h with rotation, beads were harvested by centrifugation and washed with washing buffer (20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.1% Triton X-100) 10 times. Precipitates were resolved on 10% PAGE and transferred to PVDF membrane. Interaction between cLkh1 and Tup11 (or Tup12) was confirmed by Western analysis using an anti-His antibody (Sigma). In order to test the effect of the LAMMER motif on the cLkh1 and Tup interaction, GST-cLkh1 (1 μg), GST-cLkh1$^{\Delta LA}$ (1 μg), and GST-cLkh1^{K391R} (1 μg) were mixed with His-tagged Tup11 or Tup12 soluble lysates (200 μg) and then examined as described above.

In Vitro Kinase Assay—Trx-His tagged Tup11 (or Tup12), GST-fLkh1, GST-cLkh1, GST-cLkh1$^{\Delta LA}$, and GST-cLkh1^{K391R}, purified as described above, were used for kinase assays with Tup11 and Tup12 putative substrates. The GST fusion form of Lkh1 (100 ng) was mixed with Trx-His-tagged Tup11 or Trx-His-tagged Tup12 (1 μg) in 30 μl of kinase buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol) with 10 μCi of [γ-P^{32}]ATP and then incubated at 30 °C for 30 min. The reaction was stopped by adding 6 μl of 5 × SDS-PAGE sample buffer. After separation on 10% SDS-PAGE, proteins were transferred to PVDF membrane and visualized by autoradiography. Trx-His-tagged Hsp70 was included in the assay as a substrate negative control.

β-Galactosidase Assay—Wild-type and mutant cells containing pREPLacZ-pfbp were cultured overnight under repressing conditions (8% glucose) in EMM. Cells were washed twice with sterile water and subcultured in EMM medium under repressing (8% glucose) or derepressing conditions (0.1% glucose, 3% glycerol). Cells containing pREPLacZ-p*fip1*$^+$ were cultured overnight in EMM, washed twice with sterile water, and subcultured in EMM medium in the presence and absence of additional $FeCl_3$. Cultures were grown to a final cell density of 1×10^7 cells/ml and then harvested by centrifugation. Cell lysates prepared from harvested cells were used for a galactosidase activity assay as described previously (54).

Purification of His-tagged Lkh1 Complex and Immunoblotting—*S. pombe* cells cultured to log phase in EMM with supplements were washed once with sterile water and resuspended in binding buffer (50 mM imidazole, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM Na_3Vo_4, 10 mM NaF, 0.1% Nonidet P-40). The cells were treated with glass beads, and cell extracts (1 mg) were incubated with nickel-charged resin (Elpis) for 2 h at 4 °C. Resins were harvested by microcentrifugation for 10 s. After washing nickel-charged resins three times with binding buffer, proteins were eluted with SDS-PAGE loading buffer at 95 °C for 5 min and then resolved on 10% PAGE and transferred to PVDF membrane. Lkh1 and Tup11 (or Tup12) were identified by anti-His antibody (H1029, Sigma) and anti-FLAG antibody (F3165, Sigma), respectively.

Phosphatase Treatment—Precipitates of the GST-Tup12 complex from GST pull-down were washed three times with binding buffer (phosphate-buffered saline containing 10 mM NaF, 1 mM Na_3VO_4) and two times with calf intestinal alkaline phosphatase buffer (50 mM Tris-HCl, pH 9, 1 mM $MgCl_2$) and were resuspended in 50 μl of the calf intestinal alkaline phosphatase buffer containing 50 units of calf intestinal alkaline phosphatase (Takara, Japan) in the presence or absence of phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4) and incubated for 2 h at 30 °C. The phosphatase reaction was stopped by the addition of 15 μl of 5× SDS-PAGE sample buffer and boiling for 1 min. Western blot analysis was performed as described above.

Northern Blot—Total RNAs were prepared with the RNeasy minikit (Qiagen, Germany) as recommended by the manufacturer's instructions. Twenty μg of total RNAs were separated on a 1% agarose gel containing formaldehyde and transferred onto a Hybond-N membrane (Amersham Biosciences). Gene-specific probes were prepared from PCR-generated fragments and labeled with [α-^{32}P]dCTP using a random priming kit (Stratagene). The signal was visualized by exposing the filter to x-ray film.

Reverse Transcription-PCR—Cells of ED665, Δ*lkh1*, Δ*tup11*, Δ*tup12*, and Δ*tup11*Δ*tup12* strains were grown in YES medium at 30 °C for 13 h to mid-log phase (3×10^7 cells/ml). Three μg of RNAs prepared with the RNeasy Mini kit (Qiagen) were subjected to DNase treatment (New England BioLabs), and Maloney murine leukemia virus (U.S. Biochemical Co.) for cDNA synthesis. Reverse transcription-PCRs using an SPCP16A4.07c-specific primer set (flo1-a1, TGGAACGGTTCAGCTACCTAT; flo1-b1, AATTGGCTTATTAGCTCCAGC) and actin-specific primer set (act-a1, TATCCCGGTATTGCCGATCGT; act-b1, TTAGAAGTACTTACGGTAAAC) were performed as described previously (20).

RESULTS

Lkh1 Interacts with Tup11 and Tup12 in Vitro—Pull-down screening with the maltose-binding protein-tagged catalytic domain of Lkh1 was carried out to identify the interacting partners. This approach led to the identification of Tup12 as an interacting protein by subsequent mass spectrometry (data not shown). The deletion of genes encoding Tup proteins causes flocculation of *S. cerevisiae* (49) and *S. pombe* (38) in a liquid medium and filamentous growth of *Candida albicans* (55), which mirrors the effects of *lkh1* deletion in *S. pombe* (3). Binding assays using the GST-cLkh1 fusion protein and His_6-tagged Tup12 confirmed the interaction between cLkh1 and Tup12 *in vitro* (Fig. 1*B*). Because the fission yeast has two homologues of the *S. cerevisiae* Tup1 (Tup11 and Tup12), the possibility of interaction between Lkh1 and Tup11 was also investigated. Tup11 does interact with GST-cLkh1 (Fig. 1*A*). These data indicate that both Tup11 and Tup12 are *in vivo* binding partners of the *S. pombe* LAMMER kinase, Lkh1.

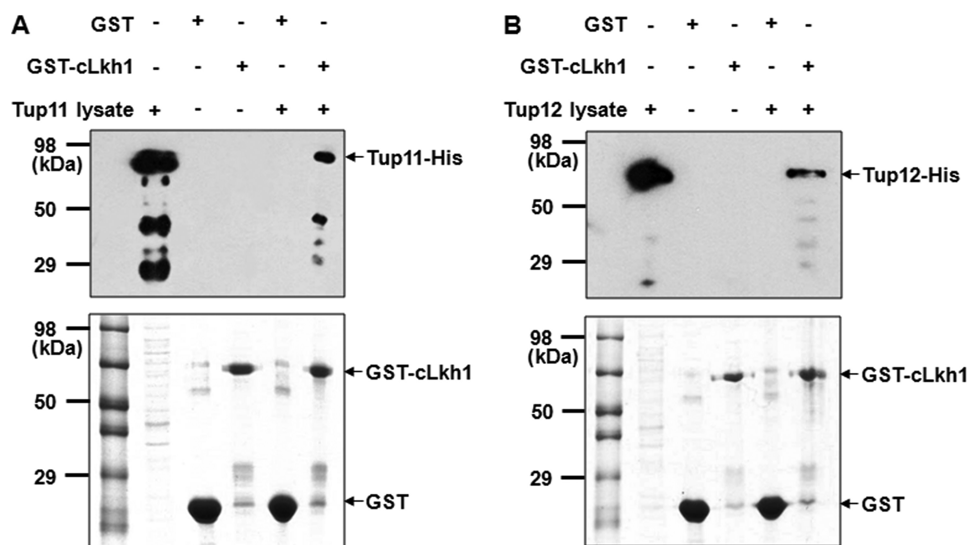

FIGURE 1. ***In vitro* interaction between Tup11/12 and Lkh1.** Soluble proteins containing His_6-tagged Tup11 (*A*) and Tup12 (*B*) were mixed with GST (*lanes 2*) or GST-cLkh1 (catalytic domain) (*lanes 3*), incubated at 4 °C for 1 h, and then collected by centrifugation. The precipitates were resolved on 12% PAGE and transferred to PVDF membranes. Tup11 (*A*, *top*) and Tup12 (*B*, *top*) were detected by anti-His antibody. The membranes were stained with Ponceau S (*bottom panels*).

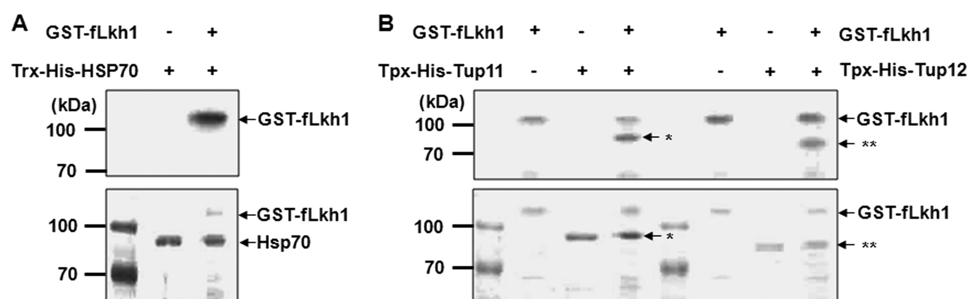

FIGURE 2. **Phosphorylation of Tup11 and Tup12 *in vitro*.** The Trx-His_6 fusion form of Tup11 and Tup12 and the Hsp70 recombinant proteins (1 μg) were purified and mixed with GST-fLkh1 fusion protein in the presence of [γ-^{32}P]ATP, respectively. After incubation at 30 °C for 30 min, the reaction 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 Trx-His-tagged Hsp70 (*A*, *top*), which was used as a negative control, and Trx-His-tagged Tup11 (*) and Tup12 (**) (*B*, *top*) was obtained. The gels were stained with CBBR-250 solution (*A* and *B*, *bottom panels*).

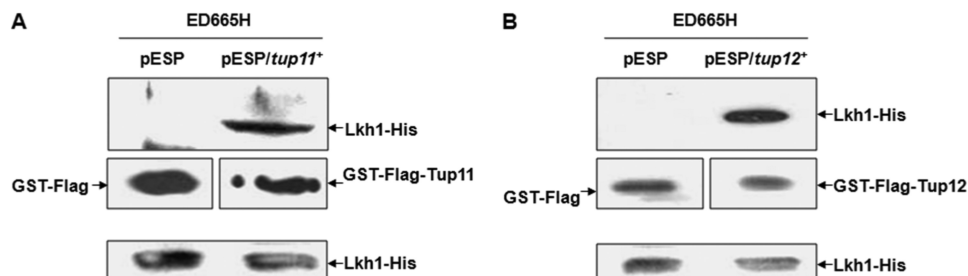

FIGURE 3. **Co-precipitation of Lkh1 with Tup11 and Tup12 *in vivo*.** ED665H cells containing chromosomal His_{12}-tagged Lkh1 and GST-FLAG-tagged Tup11 (or Tup12) were grown in EMM medium. Total protein extracts (1 mg) were subjected to precipitation with glutathione-Sepharose beads. The precipitates were resolved by 12% SDS-PAGE and transferred to PVDF membrane. Tup11 (*A*), Tup12 (*B*), and Lkh1 were detected by anti-FLAG antibody (*middle panels*) or anti-His antibody (*top panels*), respectively. Equal amounts of total proteins were precipitated with nickel-charged beads, resolved by 12% SDS-PAGE, transferred to PVDF membranes, and then Western blotted with anti-His antibody to ensure the expression of Lkh1 proteins (*A* and *B*, *bottom panels*).

Lkh1 Phosphorylates Tup11 and Tup12 in Vitro—All LAMMER family kinases possess dual specificity kinase activities, including serine/threonine and tyrosine kinase activities and autophosphorylation activity (1, 5, 8, 10). Their major substrates are known to be SR proteins (7, 11), which regulate RNA splicing by modulating RNA-RNA and RNA-protein interactions (13). The *S. cerevisiae* LAMMER kinase, Kns1, has been found to phosphorylate and interact with mammalian SR proteins (ASF/SF2, SRp20/X16, and RBP1) *in vitro* (7) and to interact with the dual specificity phosphatase Sdp1 by yeast two-hybrid screening (56). In *S. pombe*, however, the *in vivo* substrates of the LAMMER kinase Lkh1 have not been reported with the exception of the recently identified Csx1, which binds to and stabilizes *atf1*$^+$ mRNAs under oxidative conditions (20). Since *in vitro* binding studies revealed the interaction of Lkh1 with Tup11 and Tup12, we examined if Lkh1 can phosphorylate these proteins, which are transcription repressors, *in vitro*. As shown in Fig. 2, Lkh1 exhibited a high level of autophosphorylation activity similar to other eukaryotic LAMMER family kinases reported previously (20), but it exhibited no activity toward the unrelated protein Hsp70 (Fig. 2*A*), which was used as a negative control. In the autoradiogram, phosphorylation of Tup11 and Tup12 by Lkh1 is evident (Fig. 2*B*, *top*). The reduction in the mobility of both of the Tup proteins in SDS-PAGE after reaction with Lkh1 (Fig. 2*B*, *bottom*) supports phosphorylation by Lkh1. These data suggested that Tup11 and Tup12 may be the *in vivo* substrates of Lkh1.

Lkh1 Interacts with and Phosphorylates Tup11 and Tup12 in Vivo—Because the *in vitro* interaction between Tup11/12 and Lkh1 was determined, the *in vivo* interaction between these proteins was investigated. In order to perform co-precipitation experiments *in vivo*, strain ED665H, in which chromosomal loci encoding Lkh1 had been modified to express His_{12}-tagged Lhk1 under the endogenous promoter, was transformed with pESP-derived vectors with *tup11*$^+$ (or *tup12*$^+$) to produce a FLAG-tagged GST fusion form of each Tup protein under the control of the *nmt1* promoter. Pull-down assays with glutathione-Sepharose beads using extracts from cells producing His-tagged Lkh1 and FLAG-tagged GST-Tup11 revealed co-precipitation of GST-Tup11 with Lkh1, but

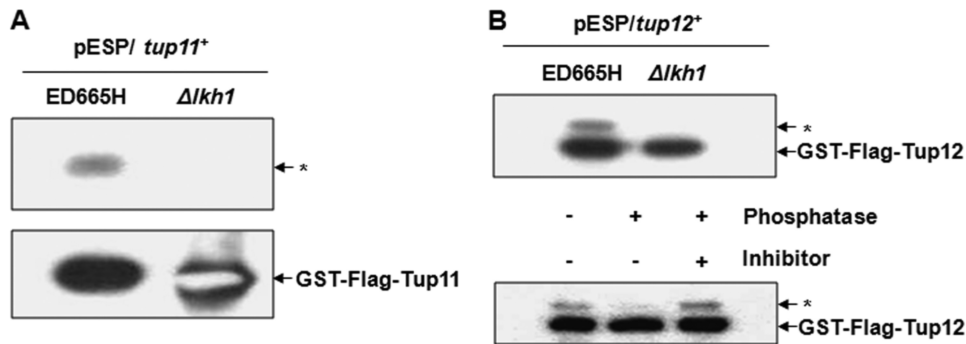

FIGURE 4. **Phosphorylation of Tup11 and Tup12 *in vivo*.** *A*, total proteins extracted from ED665H and Δ*lkh1* cells expressing FLAG-tagged GST fusion Tup11 were mixed with glutathione beads and incubated at 4 °C for 2 h. The mixtures were precipitated by centrifugation, and Western blotting was performed by anti-FLAG antibody (*bottom*) and anti-phosphoserine antibody (*top*), respectively. *B*, the precipitates from total proteins of ED665H and Δ*lkh1* cells expressing the FLAG-tagged GST fusion form of Tup12 combined with glutathione-Sepharose beads were resolved by 10% SDS-PAGE and transferred to PVDF membrane. Western blotting was performed with an anti-FLAG antibody (*B*, *top*). The FLAG-tagged GST fusion form of Tup12 purified by GST pull-down from ED665H cells was treated with alkaline phosphatase in the presence and absence of phosphatase inhibitors, and then Western blotting was performed with anti-FLAG antibody (*bottom*).

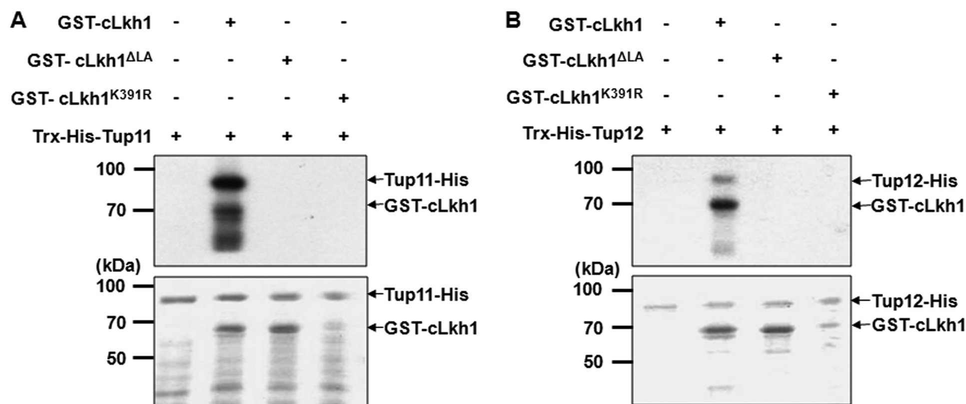

FIGURE 5. **Requirement of LAMMER motif for kinase activity of Lkh1 toward Tup11 and Tup12.** The catalytic domain of Lkh1 (cLkh1) and mutants were assayed for kinase activity using Trx-His-tagged Tup11 and Tup12 as substrates, respectively. GST-cLkh1 (*lane 2*), GST-cLkh1$^{\Delta LA}$ (*lane 3*), and GST-cLkh1^{K391R} (*lane 4*) were mixed with His-tagged Tup11 (*A*) and Tup12 (*B*), respectively. After the reaction at 30 °C for 30 min, the reaction was stopped by the addition of SDS-PAGE sample buffer. Reaction samples were resolved on 10% PAGE in duplicate. One was transferred to PVDF membrane, and then autoradiography of phosphorylated Tup11 (*A*, *top*) and Tup12 (*B*, *top*) was obtained. The other gel was stained with CBBR-250 solution (*A* and *B*, *bottom panels*).

FLAG-tagged GST and His-tagged Lkh1 did not co-precipitate (Fig. 3*A*, *top* and *middle*). The expression of His-tagged Lkh1 in ED665H cells containing pESP and pESP/*tup11*$^+$ was identified by a pull-down assay using nickel-charged beads (Fig. 3*A*, *bottom*). Lkh1 was now co-precipitated with Tup11 on the nickel-charged beads (data not shown). In pull-down assays using extracts from the ED665H cells producing His-tagged Lkh1 and FLAG-tagged GST-Tup12, Lkh1 was identified in the protein complex precipitated by glutathione-Sepharose beads (Fig. 3*B*). These results show that an intracellular protein-protein interaction occurs between Lkh1 and Tup.

Because Tup1 of *S. cerevisiae* is known to be phosphorylated (57), and the recombinant Tup11 and Tup12 of *S. pombe* were phosphorylated by Lkh1 *in vitro*, we decided to examine whether Lkh1 can phosphorylate Tup11 and Tup12 *in vivo*. The electrophoretic mobility of the Tup proteins was analyzed by a GST pull-down assay and subsequent immunoblot analysis using cell extracts from the wild-type and *lkh1* deletion mutant expressing the FLAG-tagged GST-Tup11 (or GST-Tup12). The Tup11 protein did not show a lower electrophoretic mobility band in either the wild-type or the *lkh1* deletion mutant (data not shown). On the other hand, Tup12 showed a lower electrophoretic mobility band (Fig. 4*B*, *top*), indicating the possibility that Tup12 might be phosphorylated by Lkh1 *in vivo*. If phosphorylation was responsible for lower electrophoretic mobility in SDS-PAGE, then the removal of phosphates should result in the migration of each protein as a single band on SDS-PAGE. Tup11 and Tup12, purified by GST pull-down using wild-type cells containing FLAG-tagged GST-Tup11 (or GST-Tup12), were treated with phosphatase in the presence and absence of a phosphatase inhibitor. Phosphatase treatment did not result in any apparent change in the electrophoretic mobility of Tup11 (data not shown) but resulted in the loss of the Tup12 band with lower mobility (Fig. 4*B*, *bottom*), indicating that the Tup12 band with lower electrophoretic mobility was produced by Lkh1 phosphorylation. Because change in the mobility of Tup11 was not apparent by phosphatase treatment, phosphorylation of Tup11 was confirmed by Western blot analysis of the FLAG-tagged GST fusion form of Tup11 with anti-phosphoserine antibody after pull-down with glutathione-Sepharose beads. As shown in Fig. 4*A*, phosphorylation of Tup11 was not observed in the *lkh1* deletion mutant but was observed in wild-type cells (Fig. 4, *top*). These data suggested that Lkh1 directly binds to Tup11 and Tup12 and then may regulate the activities of these transcription repressor proteins through phosphorylation.

LAMMER Motif Is Required for Substrate Binding and Phosphorylation—By comparison with the three-dimensional structures available for other kinases, the LAMMER motif is predicted to be located at the α-helix below the substrate binding cleft (1), suggesting the possibility that the LAMMER motif is required for substrate recognition and kinase activity. A point mutation in this motif in PK12, a plant LAMMER kinase, affects kinase activity and subnuclear localization but not substrate binding (9). In order to investigate these potential properties in Lkh1, the LCMMEK residues in the LAMMER motif were deleted to generate a Lkh1$^{\Delta LA}$ mutant protein, and arginine was substituted for lysine 391 in subdomain II to generate a kinase-inactive mutant, Lkh1^{K391R}. The kinase activity of the mutant proteins was assayed using Tup11 and Tup12 as substrates (Fig. 5). GST-cLkh1 showed autophosphorylation activity, as

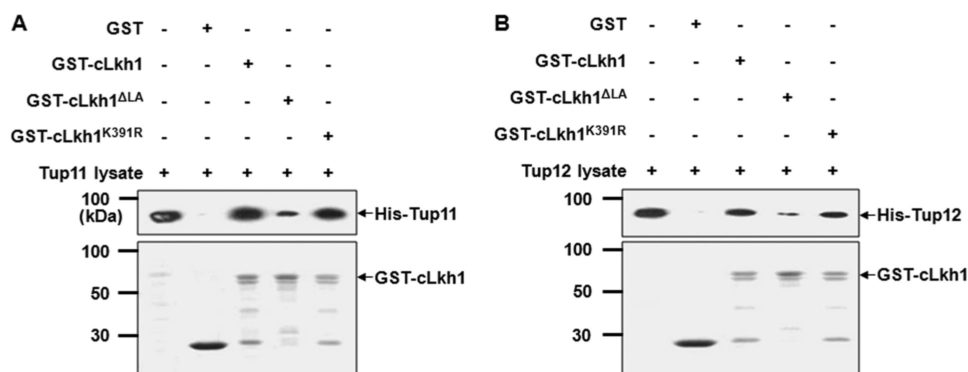

FIGURE 6. **Requirement of the LAMMER motif for interaction between Lkh1 and Tup11 (or Tup12).** Soluble proteins containing recombinant His_6-tagged Tup11 and Tup12 were mixed with GST (3 μg; *lane 2*), GST-cLkh1 (1 μg; *lane 3*), GST-cLkh1$^{\Delta LA}$ (1 μg; *lane 4*), and GST-cLkh1^{K391R} (1 μg; *lane 5*), respectively. After incubation at 4 °C for 1 h, proteins were precipitated by centrifugation. The precipitates were resolved on 12% PAGE in duplicate. One was transferred to PVDF membranes, and then Tup11 (*A*, *top*) and Tup12 (*B*, *top*) were detected by Western blotting using anti-His antibody, respectively. The other gel was stained with CBBR-250 staining solution (*A* and *B*, *bottom panels*).

TABLE 1
Effect of *lkh1* deletion on p*fbp1-lacZ* expression

Relevant genotype[a]	β-Galactosidase activity[b]	
	Repressed	Derepressed
	units/mg	
Wild type	281.4 ± 13.5	1082.9 ± 26
Δ*lkh1*	1758.5 ± 46.2	3160 ± 127.1
Δ*tup11*	377.7 ± 33.5	2853.3 ± 70
Δ*tup12*	1682.9 ± 57.8	3207.4 ± 47.9
Δ*tup11*Δ*tup12*	2060.7 ± 76.5	3631.1 ± 119.5
Δ*lkh1*Δ*tup11*Δ*tup12*	2525.9 ± 74.9	4291.8 ± 151.2

[a] All of the strains have the pREPLacZ-p*fbp*$^+$ plasmid.
[b] β-Galactosidase activity was determined from three independent cultures, as described under "Experimental Procedures." The average ± S.E. represents specific activity/mg of soluble protein.

reported previously (20), and phosphorylated the recombinant Tup proteins, whereas neither the point mutant protein, GST-cLkh1^{K391R}, nor the LAMMER motif-deleted mutant protein, GST-cLkh1$^{\Delta LA}$, exhibited autophosphorylation or other kinase activities (Fig. 5, *A* and *B*, *top*).

Because mutation in this domain results in the abolishment of Lkh1 kinase activity, we determined whether the LAMMER motif is required for binding of substrates by examining the interaction between the mutant forms of Lkh1 and Tup11/12 *in vitro*. As shown in Fig. 6, GST-cLkh1 and GST-cLkh1^{K391R} strongly interacted with both of the recombinant Tup proteins; however, GST-cLkh1$^{\Delta LA}$ retained ~30% of the substrate-binding activity in comparison with that of GST-cLkh1 and GST-cLkh1^{K391R} (Fig. 6, *A* and *B*, *top*). Although the LAMMER motif of PK12, the tobacco LAMMER kinase, is not required for binding to the substrate, SR protein (atSRp34/SR1), but is required for the kinase activity (9), the results presented here suggest that the LAMMER motif of Lkh1 in fission yeast plays an important role in substrate recognition and phosphorylation.

It is also of interest that the mutant forms of Lkh1 could not reverse the flocculation phenotype of the *lkh1* deletion mutant when GFP fusion forms of mutant fLkh1, fLkh1$^{\Delta LA}$, and fLkh1^{K391R} were overexpressed in *lkh1* deletion mutant cells (supplemental Fig. S1). The GFP fusion form of wild-type fLkh1 formed one or two speckles in the nucleus, but the GFP fusion form of the mutants showed abnormality in subnuclear distribution (supplemental Fig. S2, *middle*). In contrast to the effect on flocculation and subnuclear distribution of speckles, the GFP fusion form of the mutants exerted an effect on a different function in the *lkh1* deletion mutant (*i.e.* on cell length regulation); the kinase-inactive mutant fLkh1^{K391R} made the cells a little bit longer, and the fLkh1$^{\Delta LA}$ mutant made the cells shorter than the *lkh1* deletion mutant cells transformed with the GFP fusion form of wild-type fLkh1 (supplemental Fig. S2, *left*). These results indicate that the LAMMER motif itself is required for intracellular functions of Lkh1 other than substrate recognition and phosphorylation.

Expression of fbp1$^+$ Is Derepressed in the lkh1 Deletion Mutant under Repressed Conditions—Recent studies have shown that Tup11 and Tup12 negatively regulate the expression of *fbp1*$^+$, the gene encoding fructose-1,6-bisphosphatase, in *S. pombe*. Transcription of *fbp1*$^+$ is regulated by the glucose concentration (41, 58). In high glucose conditions, cells respond to the extracellular glucose by activating cAMP-dependent protein kinase (59) and repressing *fbp1*$^+$ expression (60). In low glucose conditions, cells activate the stress-activated protein kinase pathway, resulting in the derepression of *fbp1*$^+$ transcription (61). Tup11 and Tup12 repress *fbp1*$^+$ transcription by altering the chromatin structure (58); however, the proteins regulating the activity of Tup11 and Tup12 have not yet been identified in yeasts. Because our results show that Lkh1 directly interacted with and phosphorylated Tup11 and Tup12 *in vivo*, we determined whether Lkh1 can affect the expression of *fbp1*$^+$. We first performed β-galactosidase assays using a p*fbp-LacZ* reporter in wild-type, single (Δ*lkh1*, Δ*tup11*, and Δ*tup12*), double (Δ*tup11*Δ*tup12*), and triple deletion mutants (Δ*lkh1*Δ*tup11*Δ*tup12*) under repressed (8% glucose) and derepressed conditions (0.1% glucose and 3% glycerol) (Table 1). *fbp1*$^+$ expression was only weakly derepressed in the deletion mutant of *tup11*$^+$ but was highly derepressed in the deletion mutant of *lkh1*$^+$ or *tup12*$^+$. Double and triple deletion mutants showed 7.3- and 8.9-fold increases in *fbp1-lacZ* expression under repressed conditions as compared with wild-type cells. In order to test the intracellular relationship between Lkh1 and the Tup proteins in *fbp1*$^+$ expression, we examined *fbp1*$^+$ expression in single (Δ*lkh1*, Δ*tup11*, and Δ*tup12*), double (Δ*tup11*Δ*tup12*), and triple deletion mutants (Δ*lkh1*Δ*tup11*Δ*tup12*) by Northern blot analysis under repressed and derepressed conditions (Fig. 7). Analogous to the results of the β-galactosidase activity assay, the expression of *fbp1*$^+$ was derepressed under repressed conditions in the *lkh1* deletion mutant. *fbp1*$^+$ expression was also derepressed under repressed conditions in the Δ*tup12*, Δ*tup11*Δ*tup12*, and triple deletion mutants but not in the wild-type and the Δ*tup11* mutant. These results indicate that Lkh1 modulates *fbp1*$^+$ expression mainly by activating the repressor activity of Tup12.

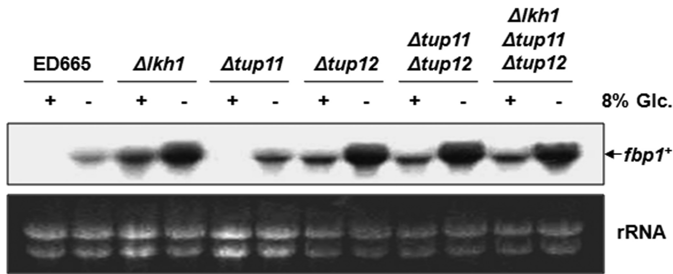

FIGURE 7. **Transcription of *fbp1*$^+$ gene in Δ*lkh1*, Δ*tup11*, and Δ*tup12* mutants.** Total RNA samples were prepared from ED665 (*lanes 1* and *2*), Δ*lkh1* (*lanes 3* and *4*), Δ*tup11* (*lanes 5* and *6*), Δ*tup12* (*lanes 7* and *8*), Δ*tup11*Δ*tup12* (*lanes 9* and *10*), and Δ*lkh1*Δ*tup11*Δ*tup12* (*lanes 11* and *12*) grown in repressing (8% glucose; *lanes 1*, *3*, *5*, *7*, *9*, and *11*) or derepressing (0.1% glucose and 3% glycerol; *lanes 2*, *4*, *6*, *8*, *10*, and *12*) conditions. Each RNA sample (30 μg/lane) was separated on 1% agarose gel in the presence of formaldehyde, blotted on nylon membrane, and hybridized with an [α-^{32}P]CTP-labeled PCR product corresponding to the coding region of the *fbp1*$^+$ gene.

TABLE 2
Effect of gene deletion on p*fip1-lacZ* expression

Relevant genotype[a]	β-Galactosidase activity[b]	
	EMM	EMM + $FeCl_3$
	units/mg	
Wild type	4.27 ± 0.60	2.63 ± 0.24
Δ*lkh1*	7.92 ± 1.49	4.37 ± 0.14
Δ*tup11*	4.37 ± 0.12	3.12 ± 0.11
Δ*tup12*	4.95 ± 0.08	3.27 ± 0.19
Δ*tup11*Δ*tup12*	8.55 ± 1.12	8.76 ± 1.45
Δ*lkh1*Δ*tup11*Δ*tup12*	10.85 ± 0.41	10.57 ± 0.73

[a] All of the strains have the pREPLacZ-p*fip*$^+$ plasmid.
[b] β-Galactosidase activity was determined from three independent cultures as described under "Experimental Procedures." The average ± S.E. represents specific activity/mg of soluble protein.

Iron Response Genes Are Up-regulated in the lkh1 Deletion Mutant—In the fission yeast, *tup11*$^+$ and *tup12*$^+$ are also known to repress the expression of *fip1*$^+$, *fio1*$^+$, and *frp1*$^+$ genes, which are involved in the response to iron concentrations, through interaction with the iron-responsive transcription factor Fep1 (46, 47). Deletion of either *tup11*$^+$ or *tup12*$^+$ is not sufficient to eliminate the iron-mediated repression of *fio1*$^+$ (47), suggesting that *tup11*$^+$ and *tup12*$^+$ are functionally redundant in down-regulating the expression of the iron transport genes. Therefore, we determined whether Lkh1 is required for the expression of iron transport genes by investigating the expression in the *fip1*$^+$-*LacZ* reporter system. β-Galactosidase activity in the *lkh1* deletion mutant was 1.6- and 1.8-fold higher than that in the wild type grown in EMM with or without $FeCl_3$, respectively (Table 2). As is the case for *fbp1*$^+$ expression, β-galactosidase activity also increased in the Δ*tup11*Δ*tup12* mutants and the triple mutants (Δ*lkh1*Δ*tup11*Δ*tup12*) but not in the Δ*tup11* or Δ*tup12* single deletion mutants. Reverse transcription-PCR analysis of *fio1*$^+$, for which expression was repressed by iron present in the YES medium, revealed that it was derepressed by *lkh1* deletion and by *tup11tup12* double deletion (supplemental Fig. S3). These results suggest that Lkh1 negatively modulates the expression of iron response genes, *fip1*$^+$ and *fio1*$^+$, by regulating both Tup11 and Tup12.

tup11tup12 Double Deletion Mutant Shows Flocculation—Previously, we had reported that an *lkh1*$^+$ *S. pombe* null mutant showed cation-dependent flocculation in liquid culture (3). The *TUP1* deletion mutant of *S. cerevisiae* also flocculates in cation- and pH-dependent manners (49). These observations led us to examine flocculation in the *tup* deletion mutants. As shown in Fig. 8, the *tup11* deletion mutant did not show a flocculation phenotype, but the *tup12* deletion mutant showed flocculation, although the flocculation was slightly lower than that of the *lkh1* deletion mutant (Fig. 8*A*). Double mutants, regardless of *lkh1* deletion (Δ*tup11*Δ*tup12* and

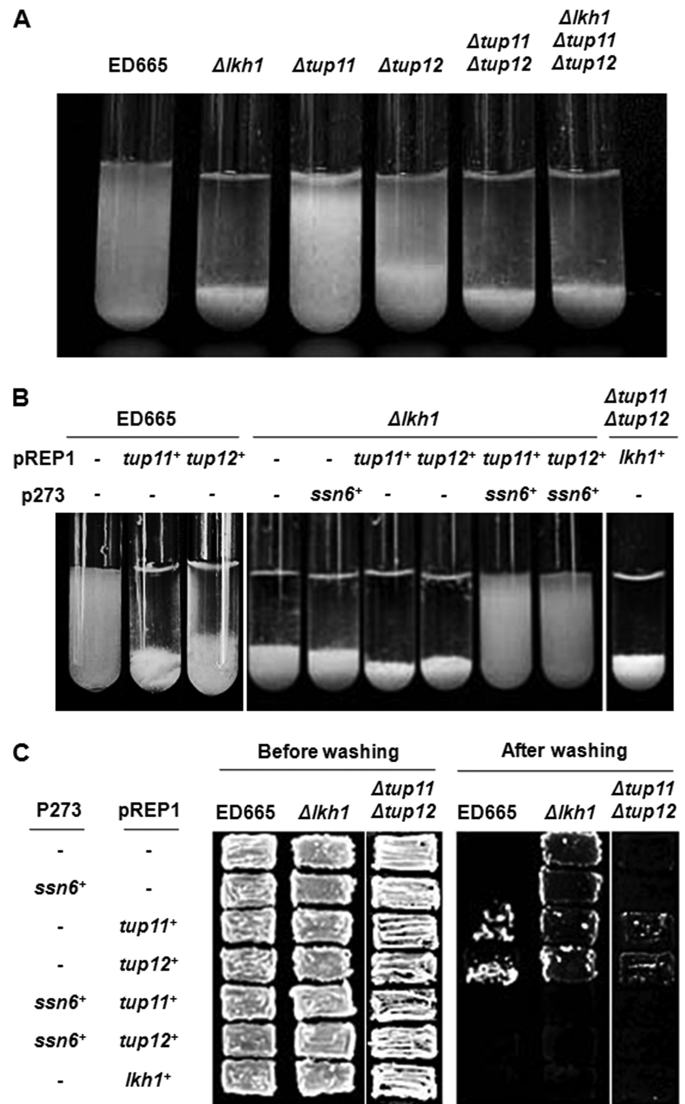

FIGURE 8. **Flocculation and adhesive growth of deletion strains.** *A*, Lkh1-independent flocculation of Δ*tup11*Δ*tup12* mutants. The cells of ED665, Δ*lkh1*, Δ*tup11*, Δ*tup12*, Δ*tup11*Δ*tup12*, and Δ*lkh1*Δ*tup11*Δ*tup12* were grown to early stationary phase (~10^8 cells/ml) in YE medium and washed with 10 mM EDTA and excess water. $CaCl_2$ was added to the cell suspension to initiate the flocculation reaction. *B*, reversion of the flocculating phenotype of *lkh1* deletion mutants by overexpression of Tup11 or Tup12 with Ssn6. Δ*lkh1* cells were transformed by pREP1/*tup11*$^+$ and p273/*ssn6*$^+$ (*lane 6*), and pREP1/*tup12*$^+$ and p273/*ssn6*$^+$ (*lane 7*), respectively. pREP2/*lkh1*$^+$ was introduced into *tup11tup12* double deletion mutant cells (*lane 8*). Wild-type (containing pREP1 and p273 vectors; *lane 1*), Δ*lkh1* (containing pREP1 and p273 vectors; *lane 2*), Δ*lkh1* (containing pREP1 and p273/*ssn6*$^+$; *lane 3*), and transformants were grown to early stationary phase (~10^8 cells/ml) in EMM medium and washed with 10 mM EDTA and excess water. $CaCl_2$ was added to the cell suspension to initiate the flocculation reaction. Photographs were taken 1 min after the initiation of the flocculation reaction. *C*, reversion of the adhesive phenotype of *lkh1* deletion mutant by overexpression of Tup11 or Tup12 with Ssn6. ED665, PHM1K, and Δ*tup11*Δ*tup12* cells were transformed by pREP1/*tup11*$^+$ and/or p273/*ssn6*$^+$. After culturing for 3 days in an EMM agar plate containing adenine, the plate was washed with tap water.

Δ*lkh1*Δ*tup11*Δ*tup12*), showed almost the same level of flocculation activity as the *lkh1* deletion mutant (Fig. 8*A*). These results indicated that flocculation in the *tup11tup12* deletion mutant was affected by Lkh1. Therefore, we determined whether the flocculation phenotype of the *lkh1* deletion mutant was reversed by overexpression of Tup proteins. Interestingly enough, overexpression of Tup11 and Tup12 did not reverse but rather increased flocculation in liquid medium in the case of the *lkh1* deletion mutant as well as in the case of the wild type. However, co-expression of spSsn6 reversed the flocculation caused by the overexpression of both Tup11 and Tup12 in the *lkh1* deletion mutant, but the expression of Lkh1 in the *tup11tup12* double deletion mutant did not reverse the flocculation phenotype (Fig. 8*B*). We had also reported that $lkh1^+$ disruption caused filamentous adhesion growth of *S. pombe* cells on agar surface (3). Analogous to the results from flocculation tests, overexpression of Tup11 and Tup12 did not reverse the adhesion of the *lkh1* deletion mutant but rather increased adhesion in the case of the wild type as well as in the case of the Δ*tup11*Δ*tup12* double deletion mutant. However, co-expression of spSsn6 reversed the adhesion caused by the overexpression of both Tup11 and Tup12 in all strains tested (Fig. 8*C*). These results suggest that the Tup11/12 protein complex alone might activate transcription, but the Tup11/12 protein complex with Ssn6 might repress the expression of a flocculin gene(s); further, these results also suggest that the activity of the Tup11/12-Ssn6 repressor complex is regulated by Lkh1.

DISCUSSION

We present a new function of LAMMER kinase: the regulation of gene expression by phosphorylation of and interaction with the *S. pombe* general transcription repressor proteins Tup11 and Tup12. The repressors are important for controlling the expression of many genes involved in a wide range of physiological processes.

The interaction between Lkh1 and Tup11/12 was demonstrated through *in vitro* and *in vivo* binding assays. Tup1 and Ssn6 are known phosphoproteins in *S. cerevisiae* (57), but no kinase has been reported to phosphorylate Tup1. *Drosophila* Gro/TEL1, a homolog of Tup1, is phosphorylated at serine 239 by PKC2, which affects neuronal differentiation (62). The LAMMER kinase Lkh1 phosphorylated the *S. pombe* counterparts of ScTup1, Tup11, and Tup12, *in vitro* and *in vivo* (Figs. 2 and 4). Notably, the amount of Tup12 phosphorylated by Lkh1 *in vivo* appears to be small (Fig. 4*B*). Although rigorous confirmation of this issue would demand further experimental evidence, this might be caused by the culture condition of the cells used. Because Tup proteins act as transcriptional regulators for responses to stresses such as salt, heat shock, and oxidative stress (45), phosphorylation of these proteins would be increased under stressed conditions. Cells used for the pull-down assay in Fig. 4*B*, however, were grown in the absence of stress.

Comparisons with three-dimensional structures of other kinases revealed that the LAMMER motif is located at the α-helix below the substrate-binding cleft (1), and it may be important for the kinase activity and substrate recognition. For example, the $PK12^{RAQ}$ mutant, which is altered in the conserved LAMMER motif, loses its kinase activity and shows aberrant localization in the nucleus, but it does not lose its substrate-binding ability (9); this suggests that the LAMMER motif is required for kinase activity and subnuclear localization. A kinase-inactive form ($Lkh1^{K391R}$) and a LAMMER deletion form ($Lkh1^{LA\Delta}$) of Lkh1 were constructed and tested to determine whether the LAMMER motif is essential for the kinase activity and for binding to substrate. As shown in Figs. 5 and 6, mutant forms did not possess kinase activity for Tup11 and Tup12 but retained 30% of the binding ability to Tup11 and Tup12. These results indicated that the LAMMER motif of Lkh1 is important for kinase activity and substrate recognition. In contrast to the wild type, the GFP fusion form of mutants showed abnormal distribution of Lkh1 in the nucleus and a different effect on the regulation of cell size and morphology. Therefore, the autophosphorylation activity and LAMMER motif of Lkh1 are also important for subnuclear localization of Lkh1 in the nucleus and for the function of Lkh1 in cell development (supplemental Fig. S2). Although the deletion mutants of the yeast LAMMER kinases Kns1 and Lkh1 are viable, no mutant Lkh1s were able to rescue the flocculation phenotype of the *lkh1* deletion mutant, suggesting that this motif is essential for Lkh1 function *in vivo*. The differential role of the LAMMER motif in Lkh1 and PK12 might be explained by the fact that LAMMER motifs in higher eukaryotes are more conserved than those in lower eukaryotes. Variation within the LAMMER motif might offer substrate specificity.

LAMMER protein kinases affect the gene expression by regulating RNA splicing, which is modulated by RNA-RNA and RNA-protein interactions (11). DOA plays important roles in activating the *hedgehog* signaling pathway by stabilizing transcription factors in *Drosophila* (11). Lkh1 phosphorylated and interacted with transcription repressor Tup11/12 *in vitro* and *in vivo* (Figs. 1 and 3). As shown in Fig. 7, the expression of $fbp1^+$, known to be repressed by Tup11/12 (41, 58), was derepressed in the *lkh1* deletion mutant, indicating that Lkh1 modulates the gene expression via protein-protein interactions and Tup substrate phosphorylation. In *S. cerevisiae*, Tup1 directly interacts with the amino-terminal domains of histones H3 and H4 (38), recruits histone deacetylase (37), and promotes repressive chromatin structure (23). In *S. pombe*, the involvement of Tup11/12 in the repression of $fbp1^+$ is mediated by repressive chromatin structure (58). DOA, PK12, and CLK2 phosphorylate the proteins that form the chromatin structure, namely histones H1, H2A, H2B, H3, and H4 (11) and a highly basic protein, P1 protamine, which plays a crucial role in the condensation of sperm chromatin (11). Recently, DOA was observed to be continuous across all four *Drosophila* chromosomes, in a manner similar to that of many general chromatin factors (17). These results suggest the involvement of LAMMER in the formation of repressive chromatin structure.

Although Lkh1 interacted with and phosphorylated both of the Tup proteins, Tup12 appeared to be the major substrate of Lkh1. As shown in Fig. 7, the expression of $fbp1^+$ in the *tup11* deletion mutant was still repressed, similar to the wild type, whereas it was derepressed in *lkh1* and *tup12* deletion mutants under repressive conditions. In addition, the flocculation phenotype observed in *lkh1* deletion mutant was also observed in

the *tup12* deletion mutant but not in the *tup11* deletion mutant (Fig. 8*A*). Therefore, Tup12 could act as a major cooperative partner for gene regulation by Lkh1. It is noteworthy that genes differentially affected by the deletion of $tup11^+$ and $tup12^+$ have recently been reported (45).

The overexpression of Tup11/12 in *lkh1* deletion mutant and in the wild-type cells enhanced the flocculation ability in liquid medium and adhesive growth on agar (Fig. 8, *B* and *C*). Co-expression of Ssn6 with Tup11/12, however, reversed the effects of Tup11/12 overexpression (Fig. 8*C*). These results indicate that without corepressor Ssn6, Tup11/12 may activate the expression of genes involved in flocculation and adhesion. Additionally, the overexpression of Tup11/12 may dilute out the corepressor protein(s), which is required for the formation of an active repressor complex with Tup11/12. Further investigations are needed to elucidate these issues. Although the co-expression of Tup11/12 with Ssn6 in *lkh1* deletion mutant cells rescued the flocculation phenotype, the introduction of $lkh1^+$ into the Δ*tup11*Δ*tup12* mutant could not reverse the flocculation phenotype (Fig. 8); this indicates that Tup11/12 represses the expression of the genes related to flocculation, and it is regulated by the LAMMER kinase Lkh1 via phosphorylation. It should be noted, however, that the regulation of Tup repressor activity would not be completely Lkh1-dependent, and the involvement of a factor(s) other than Lkh1 could not be excluded. Although further experimental evidence is required, our postulation could be supported by our recent results from the reverse transcription-PCR analysis of $cta3^+$ expression, which was increased in the Δ*tup11*Δ*tup12* double deletion mutant but decreased in the *lkh1* deletion mutant.[3]

LAMMER family kinases are reported to be involved in gene expression by regulating splicing; however, we presented a novel cellular function for the LAMMER kinase by showing that fission yeast Lkh1 affects gene expression through the phosphorylation of transcription repressors Tup11 and Tup12. Rigorous confirmation of this issue, however, would require further studies involving the identification and substitution of a Lkh1-mediated phosphorylation site(s) in the Tup proteins and investigation of the effect of a substitution(s) on the Tup-mediated expression of target genes, such as $fbp1^+$. Further investigation of Lkh1 function in the regulatory mechanism for Tup-containing transcriptional repressor complexes will elucidate Tup-mediated regulation of gene expression and will provide an insight into the diversity of the functions of the counterparts in higher eukaryotes.

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