CDK-dependent phosphorylation of Alp7–Alp14 (TACC–TOG) promotes its nuclear accumulation and spindle microtubule assembly

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ABSTRACT As cells transition from interphase to mitosis, the microtubule cytoskeleton is reorganized to form the mitotic spindle. In the closed mitosis of fission yeast, a microtubule-associated protein complex, Alp7–Alp14 (transforming acidic coiled-coil–tumor overexpressed gene), enters the nucleus upon mitotic entry and promotes spindle formation. However, how the complex is controlled to accumulate in the nucleus only during mitosis remains elusive. Here we demonstrate that Alp7–Alp14 is excluded from the nucleus during interphase using the nuclear export signal in Alp14 but is accumulated in the nucleus during mitosis through phosphorylation of Alp7 by the cyclin-dependent kinase (CDK). Five phosphorylation sites reside around the nuclear localization signal of Alp7, and the phosphodeficient alp7-5A mutant fails to accumulate in the nucleus during mitosis and exhibits partial spindle defects. Thus our results reveal one way that CDK regulates spindle assembly at mitotic entry: CDK phosphorylates the Alp7–Alp14 complex to localize it to the nucleus.

INTRODUCTION Microtubules dramatically change their organization throughout the cell cycle (Kirschner and Mitchison, 1986; Hagan and Hyams, 1988; Hayles et al., 1994). Cytoplasmic arrays of microtubules are formed during interphase but are disassembled and reorganized into the spindle microtubule upon mitotic entry. A small GTPase of the Ras superfamily, Ran, plays an essential role in the spatiotemporal microtubule reorganization (Dasso, 2001; Clarke and Zhang, 2008). Ran is known to dictate the nucleocytoplasmic transport of a number of biomolecules. Guanosine diphosphate (GDP)–bound Ran predominantly exists in the cytoplasm but is converted to the GTP form (GTP-Ran) in the nucleus (Bischoff and Ponstingl, 1991; Matsumoto et al., 1999, 2001; Nachury et al., 2001; Kalab et al., 1999). They are called spindle assembly factors (SAFs), which are ”cargo” proteins regulated by Ran-GTP (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001; Koffa et al., 2006; Sillje et al., 2006). They are released from the nuclear import complex in the Ran-GTP–rich area...
around chromosomes after nuclear envelope breakdown, which induces microtubule assembly around chromosomes (Karsenti and Vernos, 2001).

The fission yeast Schizosaccharomyces pombe undergoes a closed mitosis in which the nuclear envelope remains intact. Persistence of the nuclear envelope enables the Ran-GTP–dependent transport to remain as active during mitosis as during interphase (Matsusaka et al., 1998; Sato and Toda, 2007; Nakazawa et al., 2008). At the same time, this means that spindle microtubules must be assembled in the compartmentalized nucleus of the yeast. The spatial barrier necessitates that several MAPs be imported into the mitotic nucleus to promote spindle assembly. This suggests that the Ran-dependent system for microtubule assembly in open-mitosis organisms might have evolved from closed-mitosis organisms such as yeast. To investigate how the spindle is formed at mitotic onset, it is tempting to focus on the primitive Ran system in yeast, particularly on how SAFs overcome the nuclear envelope as a spatial barrier and accomplish their mitosis-specific enrichment to the spindle.

The orthologue of the human transforming acidic coiled-coil (TACC) protein, Alp7 (also called Mia1), undergoes nucleocytoplasmic shuttling, accumulates in the nucleus during mitosis, and localizes to spindle microtubules (Oliferenko and Balasubramanian, 2002; Sato et al., 2003), as well as to kinetochores in both mitosis and meiosis (Kakui et al., 2013; Tang et al., 2013). Alp7 has a nuclear localization signal (NLS) at the N-terminal part and is a crucial target of Ran-GTP–dependent microtubule formation (Sato and Toda, 2007, 2010). Thus Alp7 is a crucial and unique SAF that accumulates in the nucleus, and we have focused on the mechanism for Alp7 nuclear accumulation.

The significance of the nuclear function of Alp7 is demonstrated by the alp7-RARA mutant, which has mutations in the intrinsic NLS. Alp7-RARA fails to enter the nucleus and causes severe mitotic defects, such as an abnormal monopolar spindle, indicating that nuclear import of Alp7 during mitosis is indispensable for spindle integrity. The accumulation of Alp7 in the nucleus is influenced by cyclin-dependent kinase (CDK; Sato et al., 2009a), suggesting that nuclear accumulation of Alp7 is a key step linking cell-cycle control by CDK to spindle assembly.

It is also known that TACC proteins interact with their binding partner, tumor overexpressed gene (TOG; also known as XMAP215, the Xenopus orthologue), through the C-terminal TACC domain. The TACC–TOG complex is required for microtubule organization from spindle poles in various eukaryotes. TOG controls localization of TOG to the spindle microtubules and poles (Cullen and Ohkura, 2000; Lee et al., 2001; Bellanger and Gónczy, 2003; Le Bot et al., 2003; Srayko et al., 2003; Sato et al., 2004; Peset and Vernos, 2008). S. pombe has two TOG proteins, Dis1 (Nabeshima et al., 1995) and Alp14 (also called Mtc1; Garcia et al., 2001; Nakaseko et al., 2001). Both proteins have two conserved TOG domains, which consist of paddle-like HEAT repeats and are required for gripping the tubulin dimer (Neuward and Hirano, 2000; Ohkura et al., 2001). Dis1/TOG localizes to microtubules, spindle pole bodies (SPBs; the yeast centrosome equivalent), and mitotic kinetochores (Nabeshima et al., 1995; Nakaseko et al., 2001). Dis1 is phosphorylated by CDK, which is essential for translocating Dis1 from spindle microtubules to kinetochores (Nabeshima et al., 1995; Aoki et al., 2006). TOG proteins in most eukaryotes interact with TACC proteins (Peset and Vernos, 2008), but the TACC counterpart that interacts with Dis1 has not been found.

The Alp14-deletion mutant (alp14A) shows disorganized and fragile microtubules (Garcia et al., 2001; Nakaseko et al., 2001), indicating its role in microtubule stabilization. In line with this, TOG proteins, including Alp14, are predominantly located in dynamic plus ends of microtubules and catalyze microtubule polymerization using TOG domains (Brouhard et al., 2008; Al-Bassam and Chang, 2011; Al-Bassam et al., 2012; Widlund et al., 2011). Alp14 (TOG) interacts with Alp7 (TACC) through the C-terminal domain of Alp14 (Sato et al., 2004). The localization dependence of Alp14 on Alp7 implies that Alp7 regulates the localization, whereas Alp14 controls the dynamics of microtubules as the Alp7–Alp14 complex. It is therefore necessary to consider whether Alp14 could be responsible for nuclear accumulation of the Alp7–Alp14 complex. In this study, we construct and analyze the localization of a series of mutants of Alp7 and Alp14 throughout the cell cycle to identify the mechanism responsible for mitosis-specific nuclear localization.

RESULTS

Domain analysis of Alp14

It is necessary to learn how the Alp7–Alp14 complex is imported to the nucleus and exported to the cytoplasm in order to clarify the molecular machinery of the temporal regulation of the nuclear accumulation. We previously demonstrated that Alp7 has the NLS in its N-terminal half through truncation analyses of Alp7 (Sato and Toda, 2007). We next sought to identify the nuclear export signal (NES) of the Alp7–Alp14 complex.

Regarding the NES, we previously found that the mutant protein Alp7-L461A accumulates in the nucleus even during interphase (Ling et al., 2009; Sato et al., 2009a). Although residues around L461 could be good candidates for the NES, the Alp7-L461A mutant protein failed to associate with Alp14 (Sato et al., 2009a). We therefore could not exclude the possibility that L461 is a binding site for Alp14, and the binding partner Alp14 might have a real NES sequence. To test this possibility, we constructed seven truncation mutants that contained a short version of alp14 genes with the green fluorescent protein (GFP) gene fused to the C-terminus and expressed them from the endogenous promoter. The localization was observed together with mCherry-Atb2, a marker for microtubules. As seen before, Alp14-GFP localized to cytoplasmic microtubules during the interphase of wild-type (WT) cells, and in mitosis, it accumulated in the nucleus and localized to spindle microtubules (Garcia et al., 2001; Nakaseko et al., 2001; Figure 1A).

Three truncation mutant proteins that lack the N-terminal region (∆TOG, ∆TOG1, and ∆TOG2) also localized to cytoplasmic and spindle microtubules. ∆TOG-GFP lacking the entire TOG domains was able to localize to cytoplasmic microtubules during interphase and to spindle microtubules during mitosis (Figure 1A), although its localization pattern appeared distinct from that of WT, possibly because the overall microtubule array was disorganized in this mutant. The ∆TOG mutant showed severe sensitivity to both high temperature and thiabendazole (TBZ), meaning that this mutant lost its function (Figure 1B). Neither ∆TOG1 (lacking the first TOG domain) nor ∆TOG2 (lacking the second TOG domain) showed temperature sensitivity. ∆TOG2 showed TBZ sensitivity, whereas ∆TOG1 did not. The sensitivity in ∆TOG2 may be due to structural inflexibility caused by an artificial connection between the TOG1 and the C-terminal domains. Alternatively, as reported previously (Al-Bassam et al., 2012), TOG2 is more important than TOG1 for Alp14 function. Thus one TOG domain is dispensable for microtubule formation and maintenance.

The behavior of C-terminal truncation mutants confirmed the importance of the C-terminal region for localization to microtubules (Sato et al., 2004). The C-terminal end (amino acids 696–809) interacts with Alp7 (Sato et al., 2004). ∆C1 and ∆C2, which lack the
FIGURE 1: Domain analysis of Alp14 reveals NES activity in the middle region. (A) Seven alp14 truncation mutant genes were fused with the GFP gene and expressed from the native alp14 promoter: TOG1 (Δ1–244), TOG2 (Δ245–500), ΔTOG (Δ1–500), ΔC1 (Δ696–809), ΔC2 (Δ501–809), ΔtubBD (Δ642–695), and Δmid (Δ501–641). Wild type and alp14 mutants were grown at 25°C and visualized together with mCherry-Atb2, a microtubule marker. Alp7BD, Alp7-binding domain; tubBD, tubulin-binding domain. Right, localization of Alp14 (wild-type and mutant proteins) to microtubules (MT), in interphase (I), and in mitosis (M). +, localized; –, not localized; C, cytoplasm; N, nucleus. Scale bar, 5 μm.

(B) Three truncated mutants (ΔTOG, ΔC1, and ΔC2) exhibited temperature and TBZ sensitivities. The 1/10 serial dilutions of wild-type cells (WT; top), the alp14-deletion mutant (second from top), and alp14 truncation mutants were spotted on rich medium (YE5S) incubated at 26.5 or 36°C and YE5S plate containing TBZ (10 μg/ml) incubated at 26.5°C.
Alp7 interaction domain, but not the microtubule binding domain, is crucial for this association; and the domain 501–641 has NES activity. L615 is critical for the NES activity of Alp14

Because the constitutive nuclear localization seen in the alp14-∆mid mutant indicates a lack of NES, we next tried to narrow down the region. Seven truncation mutants that lack narrow regions in 501–641 were created to pinpoint the crucial region for the NES activity. Each mutant lacks ~20 residues sequentially in 501–641 and was fused with GFP to observe its localization with mCherry-Atb2. As shown in Figure 2A, six mutant proteins localized to cytoplasmic microtubules during interphase in the same way as the wild-type Alp14-GFP. The ∆tubBD mutant showed little sensitivity to high temperature or TBZ (Figure 1B), implying that the ∆tubBD protein might be loaded into microtubules in vivo via interaction with Alp7.

Of interest, the last mutant, Alp14-∆mid (∆501-641)-GFP, accumulated in the nucleus not only during mitosis but also during interphase (Figure 1A). The ∆mid mutant has no temperature or TBZ sensitivity (Figure 1B).

Taken together, the results show that TOG domains are dispensable for the association of Alp14 with microtubules; the Alp7 interaction domain, but not the microtubule binding domain, is crucial for this association; and the domain 501–641 has NES activity.

**L615 is critical for the NES activity of Alp14**

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CDK regulates TACC nuclear accumulation

... within 601–620. To confirm that NES activity resided in this region, we fused these 20 residues with the reporter protein glutathione S-transferase (GST)–GFP and expressed and the resulting fusion protein GST-GFP-14NES in WT cells. As shown in Figure 2B, GST-GFP exists in both the nucleus and the cytoplasm. Quantification of the average GFP fluorescence intensity in the nucleus and the cytoplasm verified that the nuclear GST-GFP intensity was 1.24-fold greater than the cytoplasmic intensity (Figure 2C). In contrast, the GST-GFP-14NES fusion protein predominantly localized to the cytoplasm (Figure 2B), and the nuclear/cytoplasmic signal ratio of GST-GFP-14NES dropped to approximately one-third that of GST-GFP (Figure 2C). This demonstrates that 601–620 of Alp14 retains the NES activity.

Having confirmed that the 601–620 region is sufficient for the NES activity of Alp14, we further pursued the critical residue for the NES activity in this region. There are four hydrophobic residues in 601–620 (leucines L603, L608, L615, and L620; Figure 3A), each of which was converted to alanine by mutagenesis. Three mutants (L603A, L608A, and L620A) behaved normally, but Alp14-L615A-GFP showed nuclear accumulation during interphase. Thus the L615 residue is critical for the NES activity of Alp14.

We next observed localization of the TACC protein Alp7 when the mutant TOG protein Alp14-L615A-GFP accumulated in the nucleus during interphase. Alp7 was then tagged with mCherry and endogenously expressed in the alp14-L615A-GFP mutant. Like Alp14-L615A-GFP, Alp7-mCherry also accumulated in the nucleus throughout the cell cycle (Figure 3B). Cytoplasmic microtubule organization appeared almost normal in the mutants lacking NES activity (alp14-Δmid, Δ601-620, and L615A), even though most of the Alp7–Alp14 complex accumulated in the nucleus (Figures 1A, 2A, and 3, A and B), indicating that some fraction of the proteins still localized to the cytoplasm.

**Figure 3:** Leucine 615 is critical for the NES activity of Alp14. (A) Four point mutants of Alp14-GFP were observed at 25°C. Each mutant (L603A, L608A, L615A, and L620A) has single–amino acid conversion (leucine to alanine) in 601–620. (B) Strains expressing Alp7-mCherry with Alp14-GFP (WT) or Alp14-L615A-GFP were observed at 25°C. (C) The expression level of Alp14-L615A-GFP and its interaction with Alp7-myc were examined using coimmunoprecipitation assay followed by Western blotting. Alp7-myc was immunoprecipitated, and Alp14-GFP and Alp14-L615A-GFP were detected with the anti-GFP antibody. –, untagged strain; +, myc-tagged strains. (D) A model describing how the Alp7–Alp14 complex shuttles between the nucleus and the cytoplasm in interphase. Alp7 and Alp14 shuttle as a complex: NLS in Alp7 is responsible for the nuclear import of the complex, and NES in Alp14 is in charge of exporting the complex to the cytoplasm. Exp, exporin.
The protein expression levels of Alp14-L615A-GFP and its affinity to Alp7 were comparable to those of Alp14-WT-GFP (Figure 3C). It is notable that Alp14 failed to enter the nucleus in the NLS-deficient alp7-RARA mutant (Sato et al., 2009a), indicating that the nuclear entry of the Alp7–Alp14 complex relies on the NLS in Alp7. Taken together, these results suggest that Alp14 and Alp7 shuttle between the nucleus and the cytoplasm as a complex during interphase and that Alp7 contributes to importing Alp14 in the nucleus, whereas the NES in Alp14 is responsible for exporting the complex during interphase (Figure 3D).

Alp7Δ61-116-GFP shows reduced nuclear accumulation

Having clarified how the nuclear import and export of the Alp7–Alp14 complex is achieved, we next sought to illuminate the molecular mechanism of how the complex accumulates, specifically during mitosis. We then searched for mutants of Alp7 and Alp14 that do not exhibit nuclear accumulation during mitosis. First, we suspected the possibility that the NES activity of Alp14 is inhibited during mitosis in order to achieve the nuclear accumulation of Alp7–Alp14.

To this end, we created a number of truncation mutants of Alp14, but none of them lost mitosis-specific nuclear accumulation except the C-terminally-deleted mutants that could not bind to Alp7 (Sato et al., 2004; Figure 1A and unpublished data). Next we tested the possibility that Alp14 is phosphorylated by CDK, possibly leading to inhibition of NES activity. We then performed an in vitro phosphorylation assay, but we did not find any hint of Alp14 phosphorylation by CDK (Supplemental Figure S1A). Because Alp14 possesses seven consensus sequences of CDK phosphorylation, we made the alp14 mutant by substituting the seven serine and threonine residues with alanine (alp14-7A-GFP, Supplemental Figure S1B). The localization of Alp14-7A-GFP, however, was normal (Supplemental Figure S1, B and C), reducing the possibility of phosphorylation-dependent regulation of localization.

Because we did not find any evidence for phosphorylation-based localization control of Alp14, we next focused on analyses of the binding partner, Alp7. We constructed truncation mutants of Alp7 tagged with GFP and observed with mCherry-Atb2 as a microtubule marker (Figure 4A). It was already shown that the C-terminal coiled-coil domain (TACC domain) of Alp7 is crucial for interaction with Alp14 and that the N-terminal region contains the NLS (Sato et al., 2004; Sato and Toda, 2007). The truncated protein lacking the entire TACC domain was located in both the nucleus and the cytoplasm (Sato et al., 2004; Sato and Toda, 2007), possibly because of passive diffusion. To block effects of diffusion, we constructed the mutant alp7Δ210-418-GFP, which lacks the middle C-terminal domain, with the far end (419–474) corresponding to the Alp14-binding domain left intact (Figure 4A and unpublished data). The mutant protein accumulated in the mitotic nucleus, although it did not localize to microtubules. This indicates that the middle region (210–418) of Alp7 does not contribute to mitosis-specific nuclear accumulation.

We then tested whether the critical element for mitosis-specific nuclear accumulation resides in the N-terminus. Of interest, we found that Alp7Δ61-116-GFP was reduced in nuclear accumulation during mitosis and during localization to the spindle, whereas other mutants behaved normally, in the same way as wild-type cells (Figure 4A). This indicates that the N-terminal region 61–116 right before the NLS (117–126) is responsible for mitotic nuclear accumulation of Alp7.

Our previous results demonstrated that mitotic nuclear accumulation of Alp7 is controlled by CDK (Sato et al., 2009a). Taking these results together, we hypothesized that CDK phosphorylates Alp7, which causes mitotic accumulation of Alp7–Alp14. To test whether Alp7 is phosphorylated in vivo, we prepared a mitotic extract from the cold-sensitive mutant of β-tubulin, nda3-KM311 (Toda et al., 1983; Umesono et al., 1983; Hirao et al., 1984), and purified Alp7-GFP expressed therein via immunoprecipitation followed by SDS–PAGE gel mobility shift assay. As shown in Figure 4B, Alp7-GFP was detected as a double band. The higher band with low mobility in the gel shifted to the lower band when treated with λ protein phosphatase. Addition of a phosphatase inhibitor canceled the mobility shift (Figure 4B), suggesting that the double band reflects phosphorylation of some fraction of Alp7 in mitotic cells.

Five residues in the N-terminus of Alp7 are phosphorylated by CDK during mitosis

To test whether Alp7 is directly phosphorylated by CDK, we performed an in vitro kinase assay using Alp7 peptides arrayed on a sheet (Figure 5A). Twenty-residue peptides placed every two residues from the N-terminus to the C-terminus of Alp7 were designed to cover the entire length of the Alp7 protein sequence with overlaps, and each kind of peptide was placed on a membrane. When the membrane with arrayed peptides was incubated with Cdc2-GST purified from mitotic cells and radioactive ATP, phosphorylation occurred mainly in two clusters of the Alp7 N-terminus array (clusters 1 and 2, Figure 5A).

The first cluster corresponds to amino acids 43–74, and the second cluster corresponds to 97–126, which includes the NLS (117–126). These clusters contain a number of serine/threonine residues, four sites of which (S50, S51, T100, T116) appear to be highly phosphorylated, judging by the assay. The second cluster includes two consensus phosphorylation sites (TP; T100 and T116) near the NLS. The N-terminal half of Alp7 (1–209) has one more consensus site (T131), which is slightly separated from these two clusters but right after NLS. We suspected possible phosphorylation to these five sites, and to test this, we purified the phosphodeficient recombinant proteins GST-Alp7-5A and GST-Alp7 (WT) from Escherichia coli (Figure 5B). The in vitro phosphorylation assay of these proteins, using Cdc2-GST purified from yeast and radioactive ATP, revealed that CDK phosphorylates GST-Alp7 but not GST-Alp7-5A (Figure 5C). Thus Alp7 is phosphorylated in vitro by CDK in the N-terminus, particularly at five serine or threonine residues (S50, S51, T100, T116, and T131).

To investigate whether these sites are phosphorylated in vivo, we prepared lysates from cells expressing Alp7-GFP or Alp7-5A-GFP from both asynchronous and mitotically arrested nda3-KM311 cells. We then resolved the cell extracts on the SDS–PAGE gel, followed by mobility shift assays with the anti-GFP antibody. We found mobility shifts of Alp7-GFP in the M-phase–arrested extract (17°C; Figure 5D), whereas we recognized no band shifts for Alp7-5A-GFP in either the asynchronous or the mitotic extracts (Figure 5D). This strongly indicates that five residues in the N-terminus of Alp7 are phosphorylated in vivo and that phosphorylation only occurs during mitosis. Taking all of the evidence together, we conclude that CDK phosphorylates five serine/threonine residues in the N-terminus of Alp7 during mitosis.

Alp7-5A-GFP retained nuclear import activity but caused reduction of mitotic accumulation

Next we examined whether Alp7-5A-GFP accumulates normally in the nucleus during mitosis. We replaced the chromosomal alp7+ gene with the mutant gene alp7-5A-GFP and observed its localization. Strikingly, nuclear accumulation during mitosis decreased more in Alp7-5A-GFP than with Alp7-GFP (WT; Figure 6A). Quantitative
assays for the nuclear/cytoplasmic ratio of Alp7-GFP verified that the ratio of Alp7-5A-GFP remarkably decreased compared with that of Alp7-WT-GFP (Figure 6B). The defects of Alp7-5A were not due to protein instability or loss of interaction with Alp14, judging from Western blotting and communoprecipitation assays (Figure 6C). The cytoplasmic retention of Alp7-5A-GFP may reflect loss of ability for Alp7 in mitosis-specific nuclear accumulation, as expected. It is possible, however, that this could have been due to complete loss of NLS activity caused by the point mutations created near NLS (Figure 6A). To examine the NLS activity of the 5A mutant, we added leptomycin B (LMB), an inhibitor of the nuclear export factor Crm1/exportin (Kudo et al., 1998), to living cells expressing Alp7-GFP and Alp7-5A-GFP and monitored the behavior (Figure 6, D and E). Alp7-GFP (WT) started to accumulate in the nucleus 30 min after LMB addition and finished accumulation in 90% of the interphase nuclei within the next 10 min (Figure 6, D and E). Alp7-5A-GFP started to accumulate in the nucleus 40 min after LMB addition, and it accumulated in >90% of interphase nuclei within the next 10 min. The timing of initial nuclear accumulation was delayed, but the kinetics of accumulation was comparable in those two strains (Figure 6E). These results suggest that Alp7-5A retains the ability for nuclear import, although its efficiency is slightly decreased.

We also found that the alp7-5A mutant exhibits temperature sensitivity at 36°C, which was suppressed by an addition of a canonical classical NLS derived from a T antigen of SV40 (Figure 6F). This suggests that defects in alp7-5A are due to loss of nuclear accumulation but not to other types of functional retardation. To investigate the effect of Alp7-5A on microtubule organization, we
Alp7 binds to importin α/Cut15

It is known that the nuclear import of proteins containing a classical NLS depends on importin α. *S. pombe* has two importin α proteins, Cut15 and Imp1 (Matsusaka et al., 1998; Umeda et al., 2005). We next investigated the interaction between Alp7 and Cut15 using the yeast two-hybrid system. We found that the N-terminal half of Alp7 containing the NLS (Alp7-N-WT) interacted with Cut15-80-522, which is believed to lack the importin-β–binding domain at the N-terminus, as well as the C-terminal unstructured region (Figure 7, A and B; Conti et al., 1998; Umeda et al., 2005). We also detected an interaction of Cut15-80-522 with Alp7-N-5A, although the interaction was slightly decreased, whereas the phosphomimetic protein Alp7-N-5E interacted with Cut15-80-522 as strongly as WT did. This suggests that N-terminal phosphorylation sites of Alp7 affected the interaction between Alp7 and Cut15 at least in budding yeast cells.

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FIGURE 6: The phosphodeficient alp7-5A mutant displays spindle defects. (A) Localization of Alp7-GFP (WT) and Alp7-5A-GFP observed at 25°C with Cut11-3mRFP as a nuclear envelope maker. I, interphase; M, mitosis. Nuclear accumulation in mitotic cells was reduced in the alp7-5A-GFP mutant (white arrowhead). (B) The ratio of the nuclear GFP intensity to the cytoplasmic intensity was quantified in both mitotic alp7-GFP and alp7-5A-GFP cells. \( n = 15 \) cells, \(* p = 5.35 \times 10^{-35} < 0.0001 \) (Student’s t test). (C) Alp7-5A-GFP interacts with Alp14-HA. Cell extracts were prepared from cells expressing Alp14-HA with either Alp7-WT-GFP or Alp7-5A-GFP. Alp7-GFP and Alp7-5A-GFP were immunoprecipitated with anti-GFP antibody, and Alp14-HA was detected with anti-hemagglutinin antibody. –, untagged Alp7 was expressed; +, Alp14-HA was expressed. (D) alp7-GFP (WT) and alp7-5A-GFP strains were observed with 10-min intervals after addition of leptomycin B (200 ng/ml), a Crm1/exportin inhibitor. (E) Time-course kinetics of population of cells in which GFP fluorescence is accumulated in the nucleus or which GFP fluorescence was recognized on cytoplasmic microtubules. In alp7-5A-GFP, the timing of nuclear accumulation of GFP signal was delayed \( \sim 10 \) min compared with WT. (F) Temperature sensitivity of the indicated strains was tested by spotting cells in 1/10 serial dilution. (G) Localization of Alp7-GFP and Alp7-5A-GFP in metaphase cells observed with mCherry-Atb2 at 36°C. Abnormally extended cytoplasmic astral microtubules were observed in alp7-5A-GFP cells (arrowheads). (H) A typical example of alp7-5A-GFP cells with mitotic defects at 36°C. Monopolar spindles and abnormally extended cytoplasmic microtubules are observed.
Our truncation analyses show that the C-terminal part of Alp14 is critical for localization of the MAP to the microtubule lattice (Figure 1A). Al-Bassam et al. (2012) performed plasmid-based domain analyses in which they expressed several Alp14 mutant fragments by plasmids in the \( \Delta \) alp14 mutant. From their analysis, it remained unclear whether the C-terminal part of Alp14 (\( \Delta \)TOG) controls its localization. The C-terminal part of Alp14 controls its localization (1978).

DISCUSSION

How Alp7–Alp14 is transported during the cell cycle

We propose a model for the mechanism of Alp7–Alp14 nucleocytoplasmic transport in Figure 7C. Alp7–Alp14 shuttles between the nucleus and the cytoplasm throughout the cell cycle, and the balance of NLS in Alp7 and NES in Alp14 determines the localization. During interphase, the NES of Alp14 dominates the localization of Alp7–Alp14. On mitotic entry, CDK phosphorylates serine/threonine residues close to the NLS of Alp7, in which phosphorylation enhances the interaction between Alp7 and Cut15. Then NLS activity prevails over NES activity, and, as a result, the Alp7–Alp14 complex accumulates in the nucleus and localizes to spindle microtubules to promote spindle assembly.

The C-terminal part of Alp14 controls its localization

Our truncation analyses show that the C-terminal part of Alp14 is critical for localization of the MAP to the microtubule lattice (Figure 1A; Sato et al., 2004). Al-Bassam et al. (2012) performed plasmid-based domain analyses in which they expressed several Alp14 mutant fragments by plasmids in the \( \Delta \) alp14 mutant. From their analyses, it remained unclear whether the C-terminal part of Alp14 (\( \Delta \)TOG) localizes to microtubule plus tips. The present study’s observation is based on integrated genes with GFP expressed at the endogenous level and succeeded in visualizing the localization of Alp14-\( \Delta \)TOG. We found that Alp14-\( \Delta \)TOG-GFP is able to localize to microtubules, but the localization pattern is distinct from that of wild-type Alp14-GFP. Namely, Alp14-\( \Delta \)TOG-GFP seems to localize to the microtubule lattice rather than the plus tip, where wild-type Alp14-GFP preferentially localizes (Figure 1A). Al-Bassam et al. (2012) also suggested that Alp14 harboring mutations in the second TOG domain (TOG2) failed to rescue the loss of microtubule bundles in the \( \Delta \)alp14 mutant. Our \( \Delta \)TOG2 did not show growth defects at 36°C but did exhibit hypersensitivity to TBZ like \( \Delta \)p14A. This suggests that TOG1 of Alp14, even without TOG2, retains the function to some extent, although it is compromised under some conditions.

Alp7 is phosphorylated by CDK

Our previous study demonstrated that localization of Alp7 is under control by CDK, but it remained unclear how the localization was
regulated. Global phosphoproteomic assays to search for phosphor-
ylation sites have been conducted and so far identify only serine 17
as a phosphorylation site (Wilson-Grady et al., 2008; Beltrao et al.,
2009). In this study, a peptide-array–based in vitro kinase assay re-
vealed that the N-terminus of Alp7 is phosphorylated by CDK. A
further kinase assay successfully pinpointed the phosphorylation
sites: five serine or threonine residues around the NLS. Mobility shift
assays using S. pombe protein extracts from asynchronous and mi-
totically arrested cells confirmed that these five residues are phos-
phorylated in vivo and only during mitosis (Figure 5D). These results
significantly point out the missing link of the cell cycle and Ran-
GTP–dependent microtubule formation.

Localization of other MAPs is also controlled by CDK. The other
TOG in fission yeast, Dis1, is phosphorylated by CDK (Aoki et al.,
2006). Phosphorylation of Dis1 by CDK is believed to enhance Dis1’s
association with kinetochores and/or its removal from the microtubu-
ule lattice during metaphase (Aoki et al., 2006). The phosphoedefi-
cient mutant protein Dis1-6A, in which six consensus sites of CDK
phosphorylation were substituted with alanine, localized to the spin-
dle in the nucleus (Aoki et al., 2006), suggesting that phosphoryla-
tion by CDK did not affect the nuclear localization of Dis1. It is also
of note that Dis1 has no canonical classic NLS, and a TACC counter-
part may not exist for Dis1. We therefore speculate that distinct
molecular systems operate for the nucleocytoplasmic transport of
Dis1.

Ase1 (human PRC1) is a microtubule-bundling factor (Pellman
et al., 1995; Loiodice et al., 2005; Yamashita et al., 2005), and its
nuclear entry is promoted by CDK (Fu et al., 2009), although it re-
 mains uncharacterized how the nuclear entry is enhanced. Ase1 in-
teracts with Klp9, the kinesin-6 family protein, to recruit Klp9 to
the spindle midzone for spindle elongation in anaphase, and the inter-
action is inhibited by CDK’s phosphorylation of Klp9 until anaphase
onset (Fu et al., 2009). Association of nuclear and spindle–associ-
ated protein (NuSAP) with the spindle in metaphase is also inhibited
by CDK’s phosphorylation of NuSAP (Chou et al., 2011). In those
cases, phosphorylation by CDK exerts inhibitory effects on the inter-
action between the MAPs and spindle microtubules. This study pro-
vides a molecular mechanism by which phosphorylation by CDK
serves to enhance nuclear accumulation of a MAP complex and its
association with spindle microtubules, thereby promoting spindle
assembly in the initial stage of mitosis.

Microscopy and LMB treatment
Cells were grown in yeast extract medium supplemented with ade-
 nine, uracil, leucine, histidine, and lysine (YES) at 25°C and ob-
served in minimal medium + N with supplements at 25°C (Figures
1A, 2, A and B, 3, A and B, 4A, and 6, A and D) or at 36°C (3 h;
Figure 6, G and H). For experiments in Figure 6D, LMB was added
at the final concentration of 200 ng/ml immediately after acquisition
of images at the first time point, followed by time-lapse imaging. To
acquire images, the DeltaVision SoftWoRx system (GE Healthcare,
Little Chalfont, UK) was used as described previously (Sato et al.,
2009b). Images were acquired as serial sections along the z-axis and
stacked using the Quick projection algorithm in SoftWoRx. The cap-
tured images were processed with Photoshop CS5, version 15.0
(Adobe, San Jose, CA).

Quantification of signal intensity
Fluorescence intensity was measured in Figures 2C and 6B and Sup-
plemental Figure S1C. Average fluorescence intensities of GFP in
6 × 6 pixels in the nucleus, the cytoplasm, and the background
(extracellular region) were quantified using the Data Inspector com-
mand of the SoftWoRx. Background intensity was subtracted from

CDK controls mitosis-specific nuclear accumulation of the
Alp7–Alp14 complex
The alp7-5A mutant exhibited defects in bipolar microtubule forma-
tion at the restrictive temperature. Addition of an external NLS res-
cued the temperature-sensitive alp7-5A mutant, indicating that the
defect in the alp7-5A mutant lies in an ability to localize to the nu-
cleus. Our microscopy indeed demonstrated that Alp7-5A-GFP
failed to accumulate to the nucleus during mitosis. Alp7-5A-GFP,
however, accumulated to the nucleus when LMB was added to in-
hbit nuclear exclusion. This is in sharp contrast to the NLS-deficient
mutant protein Alp7-RARA-GFP, which did not accumulate to the
nucleus in the presence of LMB (Sato and Toda, 2007; Sato et al.,
2009a). We therefore conclude that Alp7-5A-GFP retains the ability
to enter the nucleus but fails to accumulate efficiently, and phosphi-
rlation at these sites is required to shift the balance of Alp7–Alp14
shuttling from the cytoplasm to the nucleus.

It is possible that phosphorylation by CDK enhances the NLS
activity of Alp7 to accomplish nuclear accumulation in mitosis. Two-
hybrid analysis in budding yeast cells implied that the interaction
between Cut115/importin α and Alp7 was weakened in the presence
of 5A mutations. Thus we speculate that the NLS activity of Alp7-5A
is kept at the minimal level irrespective of cell cycle stage.

Klp5 and Klp6, fission yeast kinesin-8 family proteins that form a
heterodimer, are known to undergo nucleocytoplasmic shuttling
(Unsworth et al., 2008). Of interest, Klp5 and Klp6 each have an
NLS, and they migrate to the nucleus independently, despite their
dependence on localization to spindle microtubules. In addition,
both Klp5 and Klp6 have CDK phosphorylation consensus se-
quences close to their NLS sequences. It would be valuable to ana-
lyze whether those NLS are also enhanced by phosphorylation, as in
the Alp7–Alp14 complex. Temporal enhancement of NLS might be
a universal mechanism for nucleocytoplasmic shuttling proteins that
change their localization in a temporal manner.

MATERIALS AND METHODS
Yeast genetics and strains
Strains used this study are listed in Supplemental Table S1. We used
standard methods for yeast genetics and gene tagging (Moreno
et al., 1991; Bähler et al., 1998; Sato et al., 2005). For visualization of
mCherry-tubulin and cyan fluorescent protein (CFP)–tubulin, we
used Z2-mCherry-atb2(1-2-tubulin)-hph and Z2-CFP-atb2-nat strains,
respectively. As previously described for Patb2-GFP-atb2-kan (Kakui
et al., 2013), those strains were made as follows: Patb2-mCherry-
atb2-hph and Patb2-CFP-atb2-nat DNA fragment was prepared and
integrated into gene-free region adjacent to zfs1+ on the chromo-
some II as an extra copy of endogenous atb2+. Truncation mutants
were made as follows: construction of truncated alp7 and alp14
genes was done on plasmids harboring alp7+ and alp14+ genes,
respectively. Those truncated DNA fragments were amplified with
PCR and transformed into S. pombe cells to integrate them into the
original locus of the gene. The truncation mutants therefore do not
possess the wild-type allele of the gene and express the mutant
genes from the native promoter. For reporter protein assay (Figure
2B), genes for fusion proteins GST-GFP and GST-GFP-14NES (resi-
dues 601–620 of Alp14 were added to the C-terminus of GST-GFP)
were placed under the adh1 promoter (Patb2) on the plasmid
pFA6α containing the hph marker gene. The resulting plasmids con-
taining Padh1-GST-GFP-hph and Padh1-GST-GFP-14NES-hph were
linearized and integrated into the arg1 gene locus of the strain ex-
pressing Cut11-3mRFP as a nuclear envelope marker.
nuclear intensity and cytoplasmic intensity, and then the ratio of the nuclear to the cytoplasmic intensity was calculated.

**Protein extraction, immunoprecipitation, and λ-phosphatase treatment**

For Figures 3C and 6C, cells were collected and lysed with glass beads in HB buffer (25 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 15 mM MgCl₂, 5 mM ethylene glycol tetraacetic acid [EGTA], 60 mM β-glycerophosphate, 0.1 mM Na orthovanadate, 15 mM p-nitrophenyl phosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2% NP-40, complete protease inhibitor [Roche, Penzberg, Germany]), and the supernatant after centrifugation was collected as cell extract. Cell extract and protein G-coupled Dynabeads (Life Technologies, Waltham, MA) were mixed with the anti-Myc antibody (9E10; Sigma-Aldrich, St. Louis, MO; Figure 3C) or the anti-GFP antibody (A11122; Life Technologies; Figure 6C) and then incubated for 1.5 h at 4°C for immunoprecipitation. After that, beads were washed and resuspended with HB buffer containing the SDS sample buffer and boiled. Proteins were resolved on SDS-PAGE gels (10% acrylamide gel), followed by Western blotting with monoclonal anti-GFP (1:400; 7.1 and 13.1; Roche), anti-hemagglutinin (16B12; Life Technologies), and anti-α-tubulin (B5-1-2; Sigma-Aldrich) antibodies.

For Figure 4B, the alp7-GFP nda3-KM311 (the cold-sensitive β-tubulin mutant) strain was cultured at 30°C for 18 h and then shifted to 17°C for 6.5 h in the YE5S liquid medium. Cells were then collected and lysed with glass beads in HB buffer, and the supernatant after centrifugation was collected as cell extract. Cell extract was incubated with the anti-GFP antibody (A11122; Life Technologies) and protein G-coupled Dynabeads for 1.5 h at 4°C to immunoprecipitate Alp7-GFP. After the incubation, beads were washed with HB buffer and resuspended in λ-phosphatase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EGTA, pH 8.0, 2 mM dithiothreitol, 0.01% Brij-35, 2 mM MnCl₂). Then λ-protein phosphatase (New England Biolabs, Ipswich, MA) with or without phosphatase inhibitor (10 mM EGTA, 10 mM Na orthovanadate, 20 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate) was added, and samples were incubated for 20 min at 30°C. After that, phosphatase buffer was removed, and the pellet was resuspended with HB buffer followed by SDS sample buffer and boiled. Proteins were resolved on the SDS–PAGE gel (10% acrylamide gel; mono: bis = 29:8:0:2), followed by Western blotting with the anti-GFP monoclonal antibody (1:400; 7.1 and 13.1; Roche).

For Figure 5D, strains alp7-GFP nda3-KM311 and alp7-5A-GFP nda3-KM311 were cultured at 30°C for 18 h and then shifted to 17°C or kept at 30°C for 6 h in the YE5S liquid medium. Harvested cells were lysed with glass beads in HB buffer. Cell extracts were separated through SDS–PAGE (8% acrylamide gel supplemented with the final concentration of 50 μM Phos-Tag [Wako, Osaka, Japan]) and followed by Western blotting with anti-GFP monoclonal antibody (1:400; 7.1 and 13.1; Roche).

**Preparation of CDK and GST-fused proteins**

For Figure 5A, the nda3-KM311 strain was cultured at 30°C for 18 h and then shifted to 17°C for 13 h in YE5S to induce mitotic arrest. Cells were collected and lysed with glass beads in HB buffer. Cell extracts were incubated with agarose-conjugated p13/suc1 (Millipore, Billerica, MA) for 1.5 h at 4°C. After washing with HB buffer, the pellet was resuspended with HB buffer and used as the CDK solution.

For the assay in Figure 5C, The Cdc2-GST fusion protein was purified from S. pombe cells using a GST pull-down method. First, the cdc2’ gene was tagged with the GST gene using the standard method (Bährler et al., 1998) in the nda3-KM311 strain. The resultant strain, nda3-KM311 cdc2-GST, was cultured at 30°C for 18 h and then shifted to 17°C for 13 h. Cell extraction was done as described. Crude extracts were then incubated with glutathione Sepharose 4B (GE Healthcare) for 1.5 h at 4°C. After washing with HB buffer, the pellet was resuspended with HB buffer and used as the CDK solution. Activity of the purified Cdc2-GST was confirmed by an in vitro kinase assay using histone H1 (Calbiochem, San Diego, CA) as a substrate.

To express recombinant GST–Alp7-WT and GST–Alp7-5A in E. coli BL21 (DE3) cells, coding sequences for alp7* and alp7-5A were amplified with PCR and cloned into the GST-containing vector pGEX-4T (GE Healthcare).

**In vitro kinase assay using a peptide array or recombinant substrates**

For the kinase assay using a peptide array (Figure 5A), peptides were synthesized and spotted onto a cellulose membrane. This membrane was activated by treatment with 50% ethanol and 10% acetic acid for 1 h at room temperature and then washed with phosphate-buffered saline (PBS). The membrane was incubated for 2 h in the phosphorylation buffer (10 mM Tris-HCl, pH 8.0, 50 μM ATP, 100 μM MgSO₄, 100 μg/ml bovine serum albumin [BSA]; Ohta et al., 2012). For the kinase reaction, we added 0.84 μM (10 μCi) [γ-32P]ATP and CDK solution (Cdc2 purified from S. pombe cells) and incubated for 3 h. After the incubation, the membrane was washed with PBS and dried, and radioactivity was detected with autoradiography.

For the in vitro kinase assay using recombinant substrates (Figure 5C), CDK solution (Cdc2-GST purified from S. pombe cells) and substrates (GST-Alp7-7WT and GST-Alp7-5A) were incubated in HB buffer containing 20 μg/ml BSA and 200 μM ATP at 30°C for 30 min in the presence of 50 μM (1.5 μCi) [γ-32P]ATP. Reactions were terminated by addition of SDS sample buffer. Proteins were separated with SDS–PAGE, and the gel was stained with Biosafe-Coomassie (Bio-Rad, Hercules, CA), followed by autoradiography.

**Yeast two-hybrid assay**

Plasmids used in two-hybrid assays are listed in Supplemental Table S2. Genes encoding Alp7-N(1-209), Alp7-N(1-209)-Sa, and Alp7-N(1-209)-5E were amplified with PCR and cloned into the vector pGBK7T (Clontech, Mountain View, CA) and used as bait. The gene for Cut15-80-522 was similarly cloned into the vector pGADT7 (Clontech) and used as prey. Plasmids pGBK7T-T (cloned T-antigen) and pGADT7-5S (cloned p53) provided by Clontech were used as control. The Saccharomyces cerevisiae strain AH109 was cultured in the liquid medium YPDA (rich in yeast extract) and transformed with the plasmids. Transformants were grown on the synthetic medium SC lacking both leucine and tryptophan (SC–L–W). As selective media, SC plates lacking histidine and containing 5 mM of 3-amino-1,2,4-triazole (–His+3AT) and SC plates lacking histidine and adenine (–His–Ade) were used.

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CDK regulates TACC nuclear accumulation

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