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Nucleocytoplasmic transport of Alp7/TACC organizes spatiotemporal microtubule formation in fission yeast

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Ran GTPase activates several target molecules to induce microtubule formation around the chromosomes and centrosomes. In fission yeast, in which the nuclear envelope does not break down during mitosis, Ran targets the centrosomal transforming acidic coiled-coil (TACC) protein Alp7 for spindle formation. Alp7 accumulates in the nucleus only during mitosis, although its underlying mechanism remains elusive. Here, we investigate the behaviour of Alp7 and its binding partner, Alp14/TOG, throughout the cell cycle. Interestingly, Alp7 enters the nucleus during interphase but is subsequently exported to the cytoplasm by the Exportin-dependent nuclear export machinery. The continuous nuclear export of Alp7 during interphase is essential for maintaining the array-like cytoplasmic microtubule structure. The mitosis-specific nuclear accumulation of Alp7 seems to be under the control of cyclin-dependent kinase (CDK). These results indicate that the spatiotemporal regulation of microtubule formation is established by the Alp7/TACC–Alp14/TOG complex through the coordinated interplay of Ran and CDK.

Keywords: cyclin-dependent kinase; microtubule; nuclear transport; Ran GTPase; TACC–TOG

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INTRODUCTION

Ran GTPase is widely known as the universal regulator of nucleocytoplasmic transport (Ohno *et al*, 1998). Ran predominantly exists in its GTP form in the nucleus owing to the activity of the guanine nucleotide exchanging factor (RanGEF/RCC1). In general, cargo proteins that contain a nuclear localization signal (NLS) are recognized by the nuclear import factor Importin-α. Once the cargo enters the nucleus, the Importin complex uses the GTP-bound Ran that is concentrated in the nucleus to unload the cargo. Nuclear export is regulated in the opposite direction by Exportin/Crm1. The Ran system is also essential for mitotic spindle formation. Interestingly, the molecular mechanisms by which Ran exerts its influence on nuclear transport and microtubule formation are identical in principle (Karsenti & Vernos, 2001; Hetzer *et al*, 2002; Zheng, 2004; Kalab & Heald, 2008). The 'target' molecules for Ran, which promote mitotic spindle assembly, are captured by Importin. Around the chromosomes, however, Ran-GTP exists at high concentration, which induces dissociation of the targets, thereby leading to microtubule formation around the chromosomes. Several such targets for Ran have been identified in higher eukaryotes, including TPX2, NuMA and HURP.

By contrast, the only target identified so far in yeast is Alp7/transforming acidic coiled-coil (TACC) protein (also known as Mia1) of the fission yeast *Schizosaccharomyces pombe* (Sato & Toda, 2007). The *alp7* mutant shows mitotic defects such as impaired spindle formation and chromosome mis-segregation (Oliferenko & Balasubramanian, 2002; Sato *et al*, 2003, 2004). TACC forms a conserved microtubule-associated protein (MAP) complex with the tumour overexpressed gene (TOG) subunit (Gergely, 2002). In fission yeast, Alp7/TACC interacts with a TOG orthologue Alp14 and localizes to the cytoplasmic microtubules during interphase, whereas it localizes to the spindle pole body (SPB; a yeast centrosome equivalent), nuclear spindle microtubules and kinetochores during mitosis. This mitosis-specific nuclear

localization of Alp7 might suggest that Ran targets Alp7 only during mitosis. To understand how and when the Alp7–Alp14 complex is targeted by Ran and localizes to the nucleus, we sought to examine the behaviour of Alp7 and Alp14 during the cell cycle.

RESULTS AND DISCUSSION

Alp7 localizes to the nucleus only during mitosis (Fig 1A). There are two main possible ways to achieve mitosis-specific nuclear accumulation of Alp7: (i) the nuclear entry of Alp7 is inhibited during interphase but potentiated during mitosis, and (ii) the nuclear entry of Alp7 is not restricted to mitosis, but it is only during mitosis that Alp7 is retained in the nucleus. To discriminate between these two possibilities, we used leptomycin B (LMB), an inhibitor of the nuclear export factor Exportin/Crm1 (Kudo *et al*, 1998). Cells bearing Alp7–YFP (yellow fluorescent protein) with the SPB marker Cut12–CFP (cyan fluorescent protein) and the microtubule marker mRFP-Atb2 (monomeric red fluorescent protein (RFP)-tagged α2-tubulin) were prepared and LMB was added. Within 1 h, nuclear accumulation of Alp7–YFP was observed, although cells were still in interphase, as judged from their microtubule structures (Fig 1B). This suggests that Alp7 undergoes nucleocytoplasmic shuttling—Alp7 enters the nucleus during interphase but is immediately exported to the cytoplasm by Exportin/Crm1. Alp7 was also identified independently in a genome-wide screen for proteins that accumulate in the nucleus in an LMB-dependent manner (Matsuyama *et al*, 2006).

In general, TACC is required for the localization of TOG to the mitotic centrosome. This is also true for fission yeast, as Alp7/TACC is essential for the localization of Alp14/TOG to mitotic SPBs (Sato *et al*, 2004). As an NLS is present in Alp7 (Sato & Toda, 2007), the role of the Alp7/TACC subunit might be to import the Alp14/TOG subunit into the nucleus. To explore this possibility, Alp14 was visualized together with CFP–tubulin in the *alp7-RARA* (R122A/R124A) mutant, in which the intrinsic NLS has been made inactive (Sato & Toda, 2007). Alp14–rRFP co-localized with Alp7-RARA–GFP (green fluorescent protein) to the cytoplasmic microtubules even 1.5 h after LMB addition, at which time wild-type Alp7–GFP and Alp14–rRFP (refoldable RFP) had accumulated in the nucleus (Fig 1C). These results verify that the nuclear entry of Alp14/TOG is dependent on a functional NLS in Alp7/TACC.

Many of the cargo proteins that are exported by Exportin/Crm1 contain a nuclear export signal (NES), which consists of a characteristic cluster of leucine residues. To identify the NES in Alp7, we performed a domain analysis by deleting the amino-terminal or carboxy-terminal part of Alp7. Deletion of the C-terminal 45 amino-acid residues caused constitutive accumulation of Alp7 in the nucleus, irrespective of the cell-cycle stage, which suggests that NES activity resides in this region (Alp7-ΔC45–GFP; Fig 2A,B). This region contains two putative NES-like sequences with clustered leucine and hydrophobic residues (Leu 430-Leu 440 and Leu 454-Leu 470; supplementary Fig S1A online). To delineate the NES sequence, Leu 433 and Leu 435, Leu 461 or other hydrophobic residues were mutated to alanine. The Alp7-L433A/L435A–GFP protein behaved in a manner similar to wild-type Alp7–GFP (supplementary Fig S1B,C online). Furthermore, the Alp7-L454A–, Alp7-M457A– and Alp7-V462–GFP proteins localized normally to the cytoplasmic dots with Alp14–rRFP (supplementary Fig S2 online). By sharp contrast, the Alp7-L461A–GFP construct led to Alp7 accumulation mostly in the nucleus during interphase, although localization to the cytoplasmic microtubules was still observed (Fig 2C; supplementary Figs S1D and S2 online), indicating that the intrinsic NES activity of Alp7 was significantly impaired.

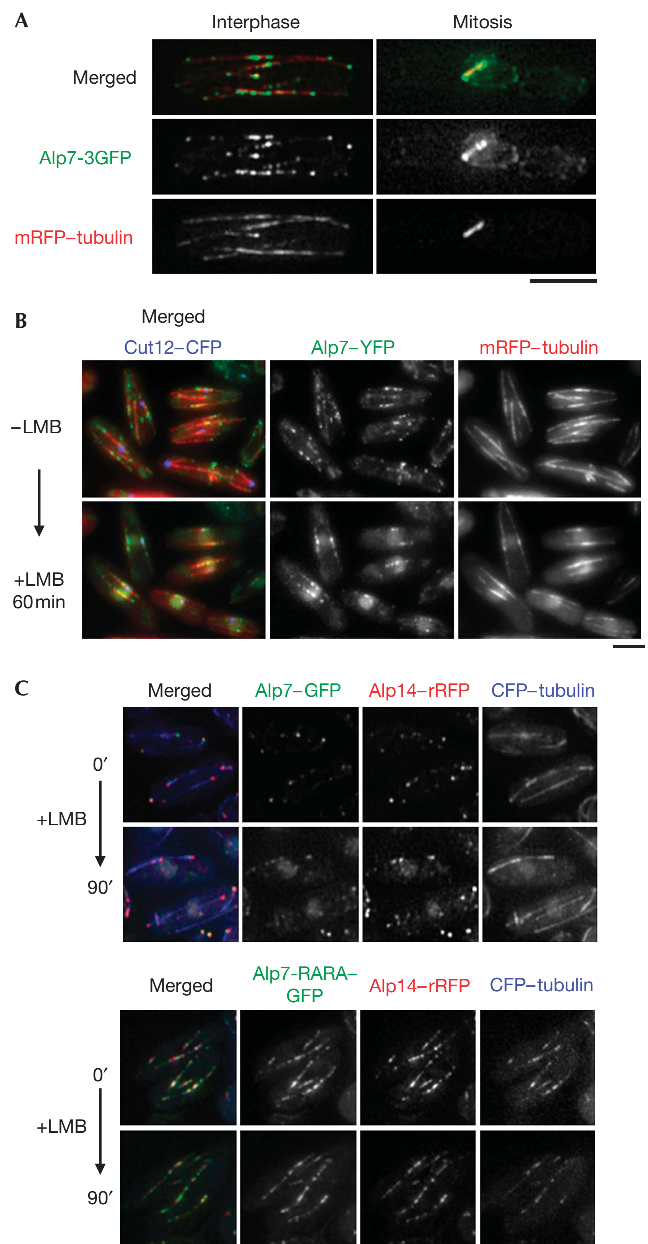

Fig 1 | Alp7 undergoes nucleocytoplasmic shuttling. (**A**) Localization of Alp7–3GFP together with monomeric RFP (mRFP)–tubulin during the cell cycle. Nuclear accumulation was observed only during mitosis (right). (**B**) Alp7 accumulated in the nucleus in response to LMB treatment. Alp7–YFP cells expressing Cut12–CFP (an SPB marker) and mRFP–tubulin were prepared for time-lapse imaging. (**C**) Cells expressing Alp7–GFP or Alp7-RARA–GFP with Alp14–rRFP (refoldable RFP) and CFP–tubulin (Atb2) were cultured. Example pictures at 0 and 90 min of a time-lapse imaging experiment are shown. Scale bars, 5 μm. CFP, cyan fluorescent protein; GFP; green fluorescent protein; LMB, leptomycin B; RFP, red fluorescent protein; SPB, spindle pole body; YFP, yellow fluorescent protein.

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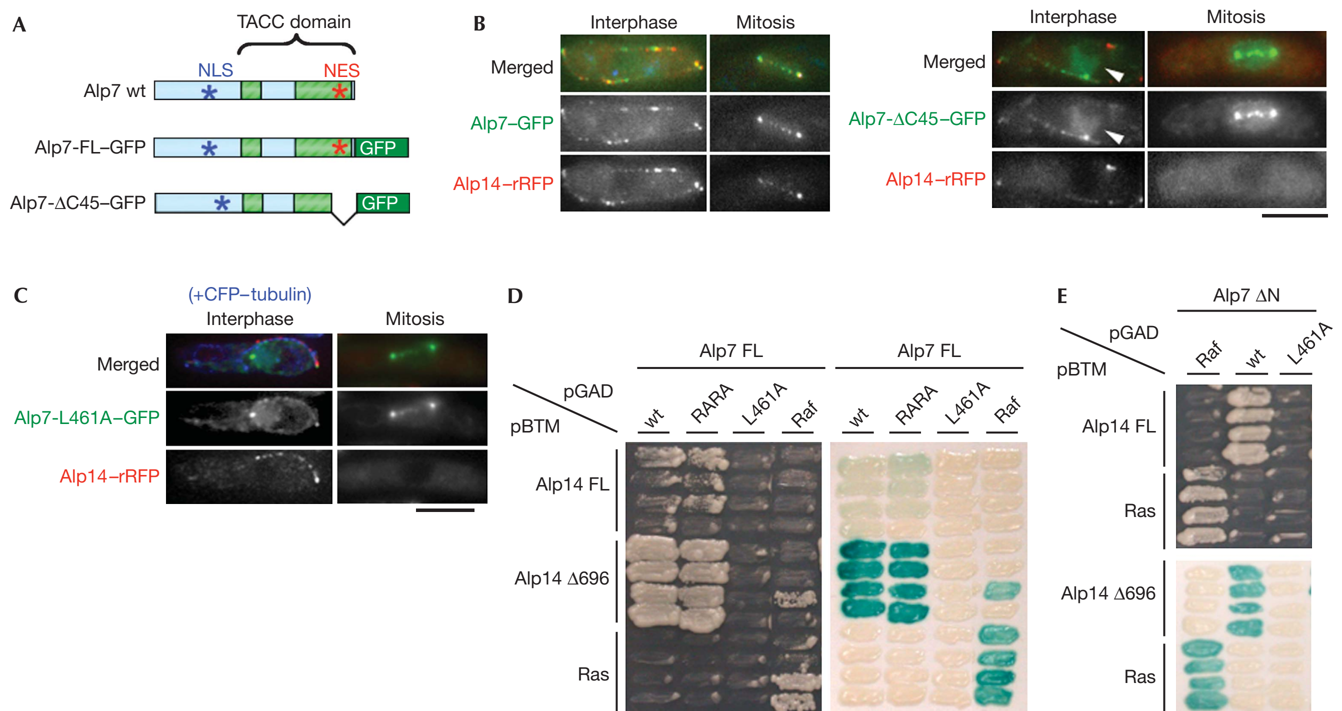

Fig 2 | The nuclear export signal-defective mutant Alp7-L461A does not interact with Alp14. (**A**) Domains of Alp7. Strains expressing GFP fusion proteins with either full-length Alp7 (Alp7FL–GFP) or Alp7 that lacks the C-terminal 45 amino acids (Alp7-ΔC45–GFP) were created. (**B**) Localization of Alp7-ΔC45–GFP. In wild type (left), Alp7–GFP co-localized with Alp14–rRFP along the cytoplasmic microtubules (interphase), mitotic SPBs and spindle microtubules (mitosis). By sharp contrast, Alp7-ΔC45–GFP (right) accumulated in the nucleus during interphase (arrowheads), whereas Alp14–rRFP did not during any stage of the cell cycle. (**C**) Alp7-L461A did not co-localize with Alp14–rRFP in the nucleus. Representative interphase and mitotic cells are shown. Scale bars, 5 μm. (**D**,**E**) Alp7-L461A and Alp14 do not physically interact in the yeast two-hybrid assay. A shorter fragment of Alp14 (Alp14 Δ696) and Alp7 (Alp7 ΔN) showed a stronger interaction with Alp7 and Alp14, respectively (Sato *et al*, 2004). An NLS mutant of Alp7, Alp7-RARA, did not abrogate the interaction with Alp14. GFP; green fluorescent protein; NES, nuclear export signal; NLS, nuclear localization signal; rRFP; refoldable red fluorescent protein; SPB, spindle pole body; TACC, transforming acidic coiled-coil; wt, wild type.

As this domain is located in the TACC domain, the L461A mutation might also abrogate the function of Alp7. In fact, the *alp7-L461A* mutant showed few microtubule bundles in the cytoplasm (supplementary Fig S1D online), which is reminiscent of the *alp7Δ* mutant (Sato *et al*, 2004; Zheng *et al*, 2006). Consistent with this, Alp7-L461A failed to complement either the hypersensitivity of the *alp7Δ* mutant to thiabendazole (TBZ; a microtubule depolymerizing drug) or growth at high temperature (supplementary Fig 1E online). Thus, the *alp7-L461A* mutant lost its intrinsic NES activity and its ability to organize cytoplasmic microtubules.

Next, we sought to determine why Alp7-L461A was not functional. Given that our previous study had shown that the C-terminal TACC domain containing Leu 461 is responsible for Alp14 binding (Sato *et al*, 2004), we looked for an interaction between Alp7-L461A and Alp14. Intriguingly, Alp14 co-localized neither with Alp7-ΔC45 nor with Alp7-L461A, which is particularly evident during mitosis (Fig 2B,C). Even during interphase, Alp14 localization to interphase microtubules was greatly compromised (Fig 2B,C). These observations raised the possibility that the interaction between Alp7-L461A and Alp14 was impaired. A yeast two-hybrid assay showed that this was indeed the case (Fig 2D). The short C-terminal fragment of Alp14 (Alp14 Δ696) and the Alp7 TACC fragment, which lacks the N terminus (Alp7 ΔN), showed elevated affinities for full-length Alp7 and Alp14, respectively (Sato *et al*, 2004). Alp7-L461A did not, however, interact even with Alp14 Δ696 (Fig 2D). Similarly, introduction of the L461A mutation into Alp7 ΔN (Alp7 ΔN-L461A) abolished the interaction with both full-length Alp14 and Alp14 Δ696 (Fig 2E). Collectively, these results show that L461 has a dual function in Alp7 function: it is a part of the NES, and it is involved in the interaction with its binding partner Alp14/TOG. Alternatively, Alp7 might need to interact with Alp14 to be exported to the cytoplasm. Alp7 did not, however, accumulate in the nucleus in the *alp14Δ* mutant, which excludes the possibility that Alp14 functions as a nuclear export factor for Alp7 (supplementary Fig S3 online).

Alp7 shuttles between the cytoplasm and the nucleus but is retained to the cytoplasmic microtubules during interphase and to the nuclear spindle during mitosis. It is, therefore, possible that the microtubule structure *per se* might function as an anchoring platform. The absence of cytoplasmic microtubules did not, however, cause nuclear accumulation of Alp7 (supplementary Fig S4 online). Thus, interphase microtubule structures do not function as tethering devices. We then questioned whether altering the localization of Alp7 would, in turn, affect microtubule

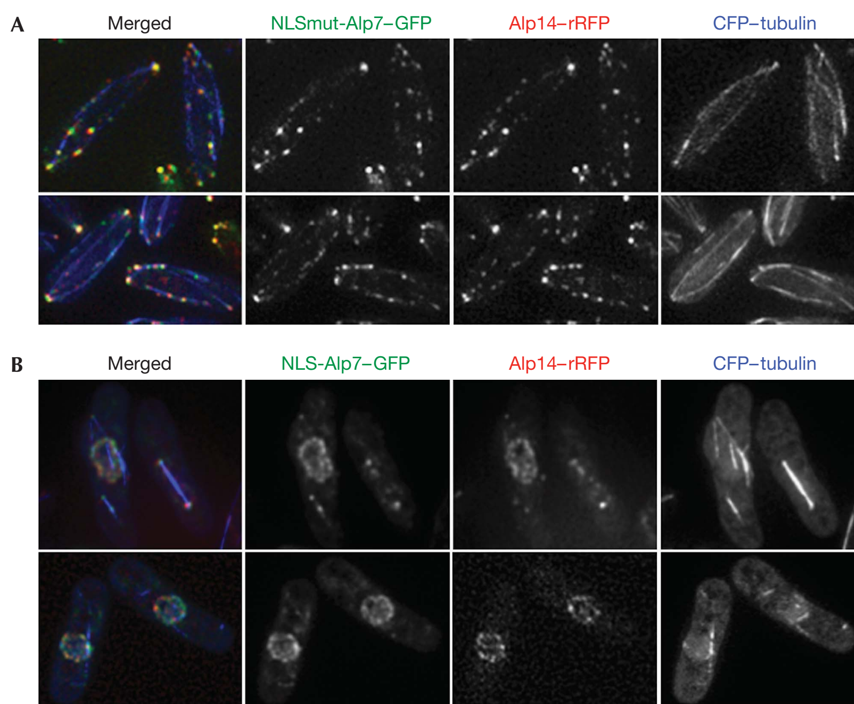

Fig 3 | Nuclear export of Alp7 is essential for maintaining cytoplasmic microtubule integrity. Cells expressing Alp14–rRFP and CFP–tubulin together with either **(A)** NLSmut-Alp7–GFP or **(B)** NLS-Alp7–GFP were observed. **(A)** Alp7–GFP fused with NLSmut (an inactive NLS) behaved in a manner similar to wild-type Alp7–GFP. **(B)** When NLS-Alp7–GFP was expressed, Alp14–rRFP also accumulated in the nucleus. Note that cytoplasmic microtubules were short and fragmented, leading to bent cell morphology. Scale bar, 5 μm. CFP, cyan fluorescent protein; GFP; green fluorescent protein; NLS, nuclear localization signal; rRFP, refoldable red fluorescent protein.

structure. Therefore, we forced Alp7 to accumulate in the nucleus by adding a robust canonical NLS sequence to the N terminus of this protein (PKKKRKV; NLS-Alp7). On fusing an inactive NLS peptide (PAAARKV) to Alp7 (NLSmut-Alp7) as a control, the protein co-localized with Alp14–rRFP to the cytoplasmic microtubules (Fig 3A), as was the case for wild-type Alp7. By contrast, NLS-Alp7–GFP accumulated in the nucleus and recruited Alp14 to the nucleus (Fig 3B). It should be noted that in these cells cytoplasmic microtubules were short and fragmented (Fig 3B). This is probably because the Alp7–Alp14 complex was sequestered in the nucleus, which caused a shortage of this complex in the cytoplasm that led to the subsequent disorganization of the microtubules; this is reminiscent of the *alp14* deletion mutant (Garcia *et al*, 2001). The shorter cytoplasmic microtubules seen in NLS-Alp7–GFP-containing cells were distinct from those observed in the NES-defective *alp7-ΔC45* (or *alp7-L461A*) mutant (Fig 2B,C), in which the nuclear accumulation of Alp7 was also observed but Alp14 was still localized to the cytoplasmic microtubules. This difference suggests that Alp14 on the cytoplasmic microtubules exerts its ability to stabilize microtubules independently of the Alp7/TACC subunit. Alternatively, some population of Alp7 might still associate with Alp14 in the Alp7-ΔC45 (or *alp7-L461A*) mutant on the microtubules, thereby providing the activity to stabilize microtubules. Whichever is the case, these results show that nuclear export of the Alp7–Alp14 complex is essential for maintaining the cytoplasmic microtubule structure. Therefore, nucleocytoplasmic shuttling of the Alp7–Alp14 complex is required for cell cycle-dependent microtubule assembly, interphase cytoplasmic microtubule arrays and nuclear mitotic spindles.

Then what determines the cytoplasmic and nuclear retention of Alp7 during interphase and mitosis, respectively? Two possible, albeit not mutually exclusive, situations might occur on entry into mitosis: (i) the acceleration of nuclear import, and (ii) the inhibition of nuclear export. To distinguish between these two possibilities, the signal intensity of GFP fluorescence from wild-type or truncated Alp7–GFP fusion proteins, the latter of which had a defective NES, was quantified during both interphase and mitosis. A line was drawn along the long axis of a cell, along which the signal intensity of Alp7–GFP was measured. Nuclear accumulation of the full-length Alp7–GFP (Alp7FL–GFP) signal clearly occurred during mitosis but not during interphase, which validates these measurements (Fig 4A). Importantly, the nuclear intensity along the section of the NES-lacking Alp7-ΔC45–GFP, which was detectable during interphase, did not increase on mitotic entry (Fig 4B), although total nuclear volume might have slightly increased. Hence, instead of the NLS, NES activity might be responsible for the mitosis-specific nuclear accumulation of Alp7.

To verify the downregulation of NES during mitosis, LMB was added to both interphase and mitotic cells, and Alp7–YFP signals were quantified as described above (Fig 4C). As LMB blocks nuclear export, this experiment should evaluate whether nuclear export of Alp7 is active during mitosis. Although the Alp7–YFP signal was increased in the interphase nucleus in response to the addition of LMB (Fig 4C, left), it was not augmented further in the

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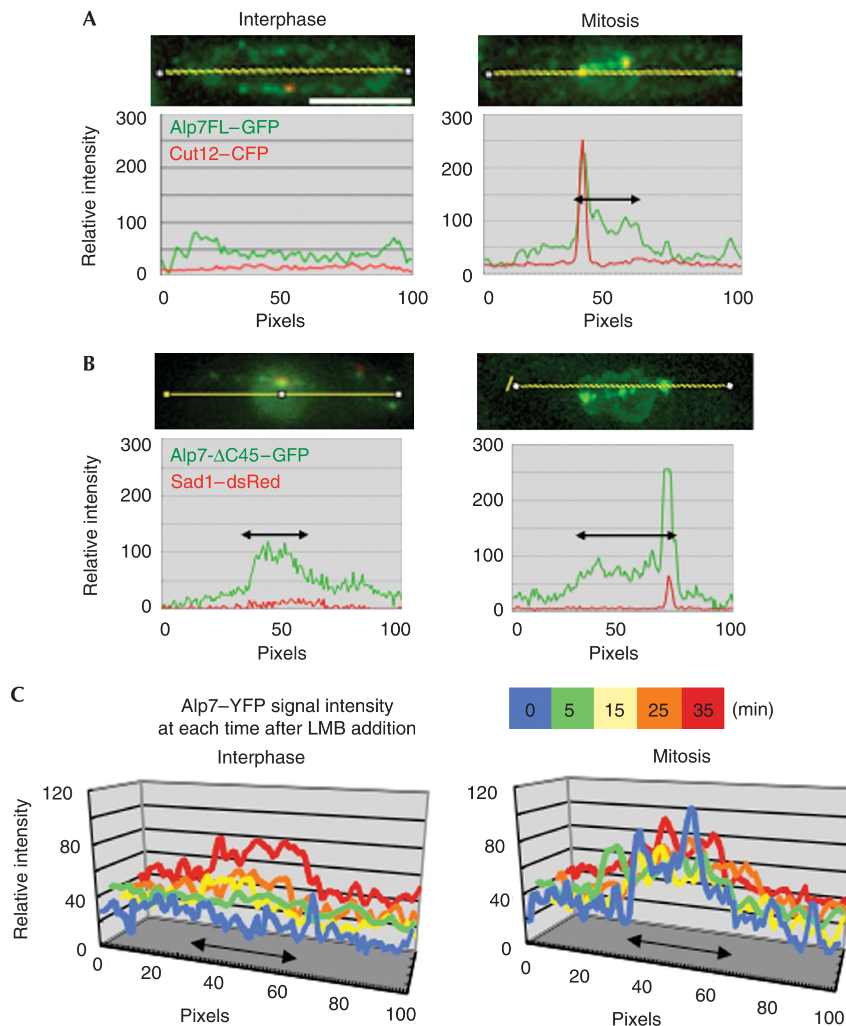

Fig 4 | Nuclear export signal inhibition during mitosis leads to Alp7 accumulation in the nucleus. (**A**,**B**) Representative images showing the intensity of (**A**) full-length Alp7 (Alp7FL–GFP) or (**B**) Alp7-ΔC45–GFP during interphase (left) and mitosis (right). Relative signal intensity was measured along the length of the yellow line. Signals from SPB marker proteins (Cut12–CFP or Sad1–dsRed) were also quantified for comparison. The number of cells quantified was as follows: four interphase and six mitotic wild-type cells, and 14 interphase and 8 mitotic Alp7-ΔC45 cells; the analysis of each representative cell is shown. In (**B**), Alp7-ΔC45–GFP seems to localize to, in addition to SPBs, foci between the two separated SPBs, the reason for which is currently unknown. Scale bar, 5 μm. (**C**) Time-lapse profiles of Alp7–YFP after LMB addition during interphase (left) and mitosis (right). Signal intensity was measured along the long axis, as in (**A**), on LMB addition. Signal intensity at each time point (0, 5, 15, 25 and 35 min) was drawn in colour as indicated. CFP, cyan fluorescent protein; GFP, green fluorescent protein; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; SPB, spindle pole body; YFP, yellow fluorescent protein.

mitotic nucleus (Fig 4C, right). This strongly suggests that nuclear export of Alp7–YFP does not occur during mitosis. We conclude that the inhibition of NES activity leads to Alp7 accumulation in the nucleus in a mitosis-specific manner, although we cannot completely exclude the possibility that activation of the NLS, as well as inhibition of the NES, occurs. It should be noted that the NES coincides with the Alp14-binding site (Leu 461). It is, therefore, tempting to speculate that Alp14 and Crm1 might compete with one another for Alp7 binding. If so, an increased affinity between Alp14 and Alp7 in the nucleus might be able to inhibit nuclear export of the Alp7–Alp14 complex by blocking the accessibility of Crm1. Further studies are required to test the competition model between Alp14 and Crm1.

Which factor inactivates the NES of Alp7 during mitosis? Possible candidates are mitotic kinases. Cdc2/Cdc13 (CDK/cyclin B) is localized at mitotic SPBs and spindles (Alfa *et al*, 1990; Decottignies *et al*, 2001). Given this localization, we speculated that CDK might well be such a regulator. To assess this possibility, we observed Alp7–GFP in temperature-sensitive *cdc2* or *cdc13* mutants, but the ambiguous localization of Alp7 in these conditional mutants hampered our ability to draw a solid conclusion (data not shown). As an alternative approach, the analogue-sensitive *cdc2-as* mutant (Dischinger *et al*, 2008) was used. Addition of the analogue 1NM-PP1 to inhibit CDK caused a significant loss of nuclear Alp7 during mitosis (Fig 5; supplementary Fig S5 online). Alp7 in particular was lost from the

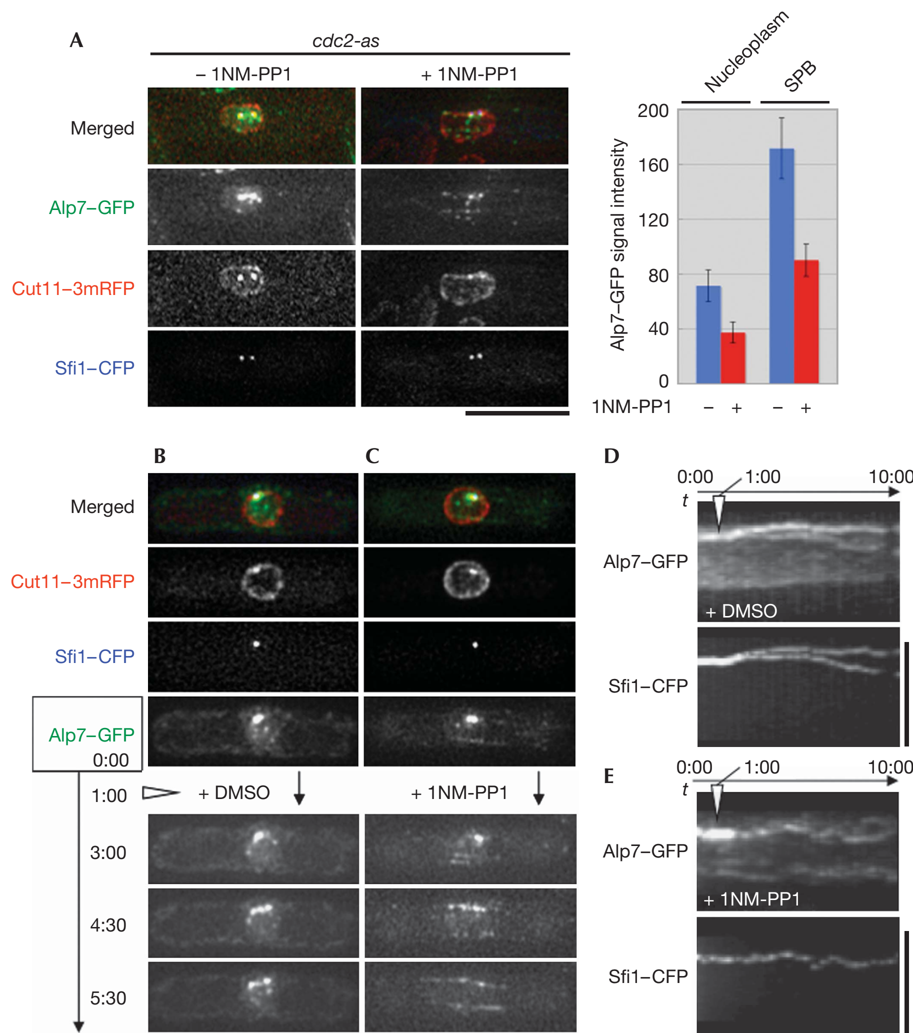

Fig 5 | Cyclin-dependent kinase activity is required for nuclear accumulation of Alp7 during mitosis. (**A**) The analogue-sensitive *cdc2-as* mutant expressing Alp7–GFP, Cut11–3mRFP and Sfi1–CFP was cultured in the absence and presence of the ATP analogue 1NM-PP1 (1 μM) for 2 h, and nuclear localization of Alp7–GFP in pre-anaphase cells (note that SPBs are recognized as two separate dots) was observed. Average signal intensity of Alp7–GFP in the 6 × 6 pixels in the nucleoplasm and at the SPB was quantified ($n = 7$ for −1NM-PP1; $n = 6$ for +1NM-PP1). Error bars, s.d. (**B–E**) Time-lapse imaging of Alp7–GFP dispersion in response to 1NM-PP1. The same strain used in (**A**) was cultured in the absence of 1NM-PP1 and time-lapse imaging of Alp7–GFP in early mitotic cells was started ($t = 0$ m:00 s; note that Cut11–3mRFP is concentrated at the SPB). At 1:00, DMSO, as control, (**B**,**D**) or 1NM-PP1 (**C**,**E**) was added to the sample. 1NM-PP1 caused dispersion of Alp7–GFP from the nucleus (5:30). (**D**,**E**) Kymographs showing nuclear Alp7–GFP and Sfi1–CFP signals on Cdc2 inactivation during mitosis. Rectangular regions around the nucleus were compressed and aligned over the course of time (for 10 min). Alp7–GFP signals were dispersed from SPBs and the nucleoplasm in response to 1NM-PP1. Arrowheads indicate the timing of drug addition. Scale bars, 5 μm. CDK, cyclin-dependent kinase; CFP, cyan fluorescent protein; DMSO, dimethyl suphoxide; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; SPB, spindle pole body.

nucleoplasm and spindle microtubules, although a certain amount of Alp7 remained at the SPBs. These results indicate that the nuclear accumulation of Alp7 requires CDK activity. Experiments using the *cdc13* shut-off mutant (*cdc13*s.o.) during meiotic prophase also supports the requirement of CDK activities for nuclear accumulation of Alp7 (supplementary Fig S6 online).

Is there any reason for Alp7 to enter the nucleus during interphase, apparently without a specific purpose? We envisage that in fission yeast the system to accelerate or decelerate the velocity of nuclear import depending on the cell-cycle stage might not be well developed or advantageous. Instead, the fission yeast might have evolved to devise the NES, the activities of which are specifically downregulated during mitosis, which is under the control of CDK. Kinesin-8 Klp5 and Klp6 shuttle between the nucleus and the cytoplasm; as is the case for Alp7 and Alp14, they also localize to cytoplasmic microtubules during interphase and to the nuclear spindle during mitosis (Unsworth *et al*, 2008). Furthermore, the protein phosphatase Clp1/Flp1 is retained in the cytoplasm during late mitosis by the Sid2 kinase and the 14-3-3 proteins (Chen *et al*, 2008). It would be intriguing to address

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how these proteins establish nuclear and cytoplasmic retention in a cell cycle-dependent manner.

Ran maintains the nucleocytoplasmic balance of microtubule formation through the transport of Alp7 (and Alp14). CDK shifts this nucleocytoplasmic shuttling towards nuclear retention on entering mitosis. Our findings here, therefore, have revealed that Ran, Exportin and CDK coordinate the spatiotemporal regulation of microtubule formation in which Alp7/TACC, together with Alp14/TOG, is a crucial component. It is still unknown how CDK affects NES activity, as no consensus sites for phosphorylation by CDK were found around the NES of Alp7. Further studies are awaited to clarify the molecular mechanism by which CDK regulates the mitotic nuclear retention of Alp7–Alp14.

METHODS

Strains and plasmids. Detailed methodologies about the construction of strains and plasmids are described in the supplementary information online. Strains used in this study are listed in supplementary Table S1 online.

Microscopy and LMB treatment. Experimental procedures have been described previously (Sato & Toda, 2007; Sato *et al*, 2009). Briefly, we used the DeltaVision-SoftWoRx system (Applied Precision, Issaquah, WA, USA). Signal intensity of fluorescent proteins was quantified using ImageJ (National Institutes of Health). For LMB experiments, images for the first time point were taken without LMB (for 0 min). LMB was then added at a final concentration of 100 ng/ml (Kudo *et al*, 1998; Sato *et al*, 2001).

Note added in proof. After this work was accepted, the following paper appeared that reports nucleocytoplasmic shuttling of Alp7, similar to our work. Ling YC, Vjestica A, Oliferenko S (2009) Nucleocytoplasmic shuttling of the TACC protein Mia1p/Alp7p is required for remodeling of microtubule arrays during the cell cycle. *PLoS ONE* **4**: e6255.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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