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Spatial and Translational Regulation of Exocyst Subunits by Cell Cycle in Budding Yeast

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Previous studies have shown that exocyst complex is located at polarized growth sites at different cell cycle stages in budding yeast. But how cell cycle and the cyclin-dependent kinase, Cdk1, regulate the distribution of exocyst complex on the plasma membrane and the protein level of each exocyst subunit is not clear.

Material/Methods: Using budding yeast as a research material, regulation of cell cycle and Cdk1 on exocyst localization on the plasma membrane and on level of each exocyst subunit were examined by methods of cell biology and molecular biology.

Results: Exocyst complex is located at growth sites on the plasma membrane in both budding and non-budding stages. Cdk1 activity is required for polarized distribution of exocyst complex in late G1, S and M phases, but not in cytokinesis stage. Cdk1 is not required for the assembly and localization of exocyst complex on plasma membrane. The protein level of Sec3 but not other exocyst subunits is regulated by the cell cycle.

Conclusions: Cdk1 activity is required for exocyst polarization before cytokinesis during the cell cycle progression, but not for its assembly and localization on the plasma membrane. Dynamic localization and protein level of the complex subunits are regulated by the cell cycle.

MeSH Keywords: **CDC2 Protein Kinase • Cell Cycle • Exocytosis**

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Background

Exocytosis is a type of vesicle trafficking that is often limited to specific areas of the plasma membrane [1]. The vesicles enveloping the cargoes detached from the donor membrane pass through cytoplasm, and then are tethered and fused to the receptor membrane. The transported cargoes are released into the extracellular matrix, and the membrane of the vesicles is incorporated into the plasma membrane, allowing the plasma membrane to replenish. The process of exocytosis is involved in many biological phenomena, such as embryogenesis, cell cycle, neuronal cell growth, cell polarization, cell migration and tumor invasion [2–6]. The uncontrolled regulation of exocytosis can cause various diseases such as epilepsy, hypertension, diabetes, and asthma [7].

Membrane trafficking involved in exocytosis regulates cell surface growth during the cell cycle [37]. There are many proteins involved in the process of exocytosis. The complex consisting of 8 structural and functional subunits – Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 – are called the exocyst complex [1,8–10]. The components of the exocyst complex are direct targets of many signaling molecules that modulate exocytosis of various physiological processes spatially or temporally, such as polarized cell growth, epithelial formation, neuronal branching, and cell migration [3,4,11–13].

Cyclin-dependent kinase 1 (Cdk1) is a major regulator of the cell cycle in yeasts and plays a central role in growth control during the cell cycle progression [14–16]. It has been shown that Cdk1 controls the polarized secretion and growth of cells. Luo et al. showed that mitotic phosphorylation of Exo84 by Cdk1 inhibits exocyst assembly and arrests cell growth in *Saccharomyces cerevisiae* [17]. In *Candida albicans*, however, Cdk1 phosphorylation of Exo84 regulates efficient hyphal extension but not exocyst assembly [18]. It was also reported that Cdk1 is required for polarized localization of the exocyst complex subunit, Sec3 [14,19]. However, whether Cdk1 affects the subcellular localization of other exocyst complex and how the exocyst complex is regulated by cell cycle temporally are unknown.

In this study we investigated the effect of the cell cycle and Cdk1 kinase on the localization, assembly, and protein level of exocyst subunits. We found that the exocyst complex is located at growth sites on the plasma membrane of cells in both the budding and non-budding stages. Although Cdk1 activity is required for polarized distribution of exocyst complex in late G1, S, and M phases, it is not required for the assembly and localization of the exocyst complex on the plasma membrane. We also found that the protein level of Sec3, but not other exocyst subunits, is regulated by the cell cycle. Our study indicates that dynamic localization and protein level of the exocyst complex subunits are regulated by the cell cycle.

Material and Methods

Plasmids and yeast strains

We used standard methods for yeast growth and genetic manipulations [20]. All strains used in this study are listed in Supplementary Table 1, and all plasmids used are listed in Supplementary Table 2. Yeast transformation was performed based on the lithium acetate method [21]. Briefly, about 2×10^6 units of cells grown to early log phase were collected in a 1.5-ml centrifuge tube and washed twice with 1 ml of distilled water. To prepare the transformation reaction, 240 μ l PEG 3350 (50% wt/vol), 36 μ l 1.0 M LiAc, 5 μ l 10 mg/ml ssDNA, and 0.1–10 μ g DNA were added in that order and the tube was vortexed until the cell pellet had been completely mixed. The suspended cells were incubated in a water bath pre-warmed to 42°C for 30 min. After that, 200 μ l of the transformation mix was plated onto SD medium. The plates were incubated at 25°C for 3–4 days to recover transformants.

Immobilization of yeast cells

The yeast cells were fixed prior to examination by fluorescence microscopy for subsequent manipulation and microscopy. About 2.5×10^6 of yeast cells were taken from liquid cell culture and put in a microcentrifuge tube. The cells were centrifuged at 8000 rpm for 1 min at room temperature, and the supernatant was discarded. Then, about 0.5 ml of methanol pre-cooled to -20°C was added to the centrifuge tube to resuspend the cells. The tubes containing cells were placed on ice for 10 min, and then centrifuged at 8000 rpm for 1 min at room temperature. The supernatant was discarded, and the resulting pellet was washed once with -20°C pre-cooled acetone. The cells were then washed 3 times with 4°C pre-cooled PBS and resuspended in 100 μ l of PBS. The fixed yeast cells can be directly placed under a fluorescence microscope or stored at 4°C for fluorescence microscopy.

Microscopy

Microscopy was conducted with a fluorescence microscope (OLYMPUS) equipped with a UPlanSApo 100X/1.40 oil immersion objective lens. Images were taken using Image-Pro Plus 7.0 acquisition software. Determination of the cell cycle stages was performed by surveying nuclear position and morphology using a MARS-tagged nuclear envelope protein (Nup57-MARS), as described previously [22]. The Nup57-MARS yeast strains harboring either Sec5-3GFP, Exo70-3GFP, or Exo84-3GFP on the chromosome were grown in synthetic complete SD medium overnight at 25°C. Cells were collected by centrifugation and resuspended in fresh SD medium.

To examine the co-localization of exocyst subunits, *cdk1-as* mutant cells carrying Sec3-tdTomato and Exo84-3GFP on the

chromosomal were grown to early log phase at 25°C, followed by either DMSO or 15µM 1NM-PP1 for 30 min. The cells were then fixed for fluorescence microscopy. The GFP signal was visualized with a 485/30-nm band-pass excitation filter, and a 525/25-nm band-pass emission filter. The tdTomato fluorescence was visualized with a 560/25-nm band-pass excitation filter, and a 600/50-nm band-pass emission filter.

Whole cell extract preparation

The published Hot-SDS protein extraction protocol was used, with slight modifications [23]. Briefly, 5 A₆₀₀ units of yeast cells were collected by centrifugation, washed once with distilled water, and then suspended in 1 ml pre-cold distilled water, followed by the addition of 1 ml 0.2 M NaOH. Samples were mixed and incubated at room temperature for 5 min. Cells were collected by centrifugation, resuspended in 1 ml 5X SDS sample buffer, and boiled at 100°C for 5 min. Samples were centrifuged, and protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories).

Western blot

We mixed 20 µl of whole cell extract with same protein concentration with 4 µl of 5X SDS loading buffer and boiled the mixture at 100°C for 5 min. Protein samples were then centrifuged at 4°C for several minutes and separated on 10% SDS-PAGE gel. Western blotting was performed using the anti-Sec3, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 antibody. The images were processed using Image J software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. One-way ANOVA was used for univariate analysis. P<0.05 was considered statistically significant.

Results

Exocyst complex is located at active growth sites in both budding and non-budding stages

Unlike t-SNARE proteins, which are distributed along the entire plasma membrane [24], the exocyst complex has been shown to be located at the bud tip of cells in budding stages, such as late G1, S, M, and cytokinesis [19]. Whether the exocyst exists in cells in the non-budding stage (early G1) is unknown, so we used the subcellular localization of Nup57 to indicate the location of the nuclear membrane and to determine the cell cycle in which the cells are located. Nup57 is an essential subunit of the nuclear pore complex (NPC), which functions as the organizing center of an NPC subcomplex. Determination

of the cell cycle stages was performed by examining nuclear position and morphology using the MARS-tagged nuclear envelope protein (Nup57-MARS), as described previously [22].

To investigate the subcellular localization of exocyst in different stages of the cell cycle in budding yeast, yeast strains carrying Nup57-MARS and 3GFP tagged Sec5 or Exo70 on the chromosome were made and used for fluorescence microscopy. A saturated overnight culture (SONC) was prepared and then released into fresh YPD liquid medium. The yeast cells at different stages of the cell cycle were fixed and examined under a fluorescence microscope. As shown in Figure 1A, the exocyst complex was located at polarized growth sites, as expected. We found that it was concentrated at one site on the plasma membrane in late G1 phase, located in the bud in S phase, or distributed on the plasma membrane of the daughter cells in M phase. During the cytokinesis, the complex was shifted to the bud neck of the cells. Interestingly, we found that the exocyst complex existed in early G1 cells, and was scattered on the plasma membrane. Cells in G1 phase are considered to be non-budding. This result suggests that exocyst is also required for growth of cells in non-budding stage, though the growth is isotropic.

To confirm that exocyst is located on the plasma membrane at both budding and non-budding stages, we also examined the localization of 3GFP tagged Sec3 and Exo84 in cell division cycle (*cdc*) mutants of *cdc28-4*, *cdc7-1*, *cdc20-1*, and *cdc15-2*, which are arrested early G1, S, metaphase, and anaphase at 37°C, respectively. As shown in Figure 1B, exocyst was discretely located on the plasma membrane in *cdc28-4* cells arrested in G1 phase, while the complex was mainly located to polarized growth sites in cells arrested in S, metaphase, and anaphase phases. Taken together, these results indicated that exocyst is located in the bud tip or on the plasma membrane, depending of the cell cycle.

Cdk1 controls polarized localization of exocyst before cytokinesis during cell cycle progression

Because Cdk1 is the master regulator of cell cycle progression, we wanted to know whether Cdk1 controls the subcellular localization of exocyst on the plasma membrane. It has been reported that Cdk1 is required for polarized localization of Sec3 in the bud tip [14,19]. As a landmark of exocytosis, localization of Sec3 is actin-independent, which is different from the rest of other 7 subunits of the complex. Whether Cdk1 regulates polarized localization of other exocyst subunits is unknown. To investigate the role of Cdk1 on the subcellular localization of exocyst, we used a *cdk1-as* mutant carrying 3GFP tagged Exo84 on the chromosome, which is a representative component of the exocyst complex. Cells with an analog-sensitive *cdk1* mutant (*cdk1-as*) are capable of encoding a mutant kinase specifically inhibited by 1NM-PP1 [25]. We treated

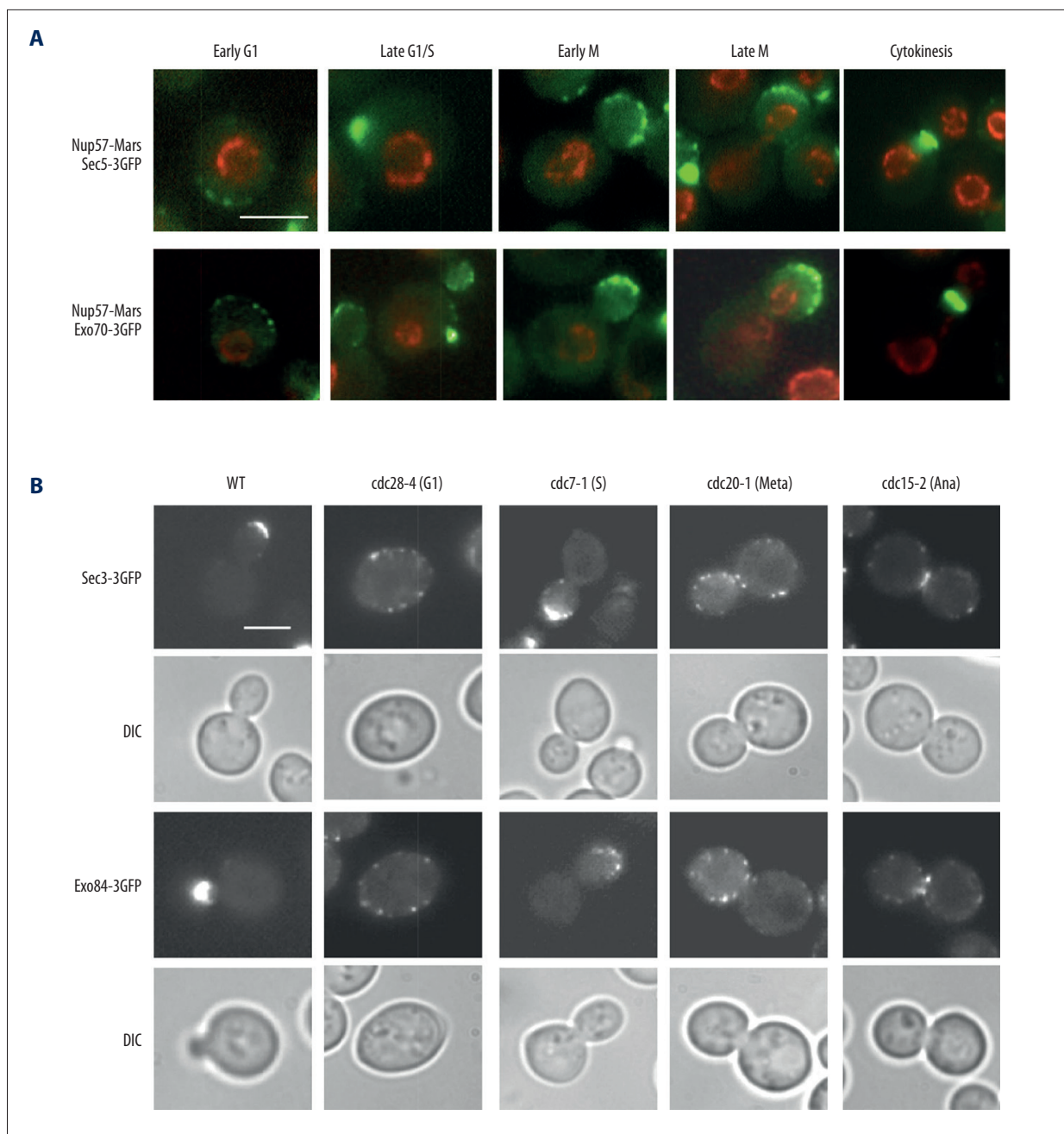


Figure 1. Localization of exocyst in different cell-cycle stages. **(A)** The yeast strains harboring both Nup57-MARS and Sec5-3GFP or Exo70-3GFP on the chromosome were grown to early log phase in synthetic complete (SC) medium overnight at 25°C. We dropped 2 μl of the suspension onto the agar pad for microscopy. Scale bar, 5 μm. **(B)** Wild-type and *cdc* mutants harboring 3GFP tagged Sec3 or Exo84 on the chromosome were cultured at 25°C to early logarithmic growth phase, and then shifted to 37°C for 90 min. The cells were fixed and examined by fluorescence microscopy. Scale bar, 5 μm.

wild-type cells and cells containing the analog-sensitive *cdk1* mutant (*cdk1-as*) harboring Exo84-3GFP with 15 μmol/L of 1NM-PP1 for 30 min to inhibit Cdk1 kinase activity. As a mock control, the wild-type and *cdk1-as* mutant cells were treated with DMSO for 30 min. Then, cells were fixed and examined by fluorescence microscopy. As known in Figure 2A, exocyst is

located to polarized growth sites in both wild-type and *cdk1-as* cells at the time when 1NM-PP1 was added into the media. After 30 min, exocyst was still located at the bud tip in cells treated with DMSO. In cells treated with 1NM-PP1, however, exocyst was scattered on the plasma membrane instead of concentrated in the bud tip (Figure 2A).

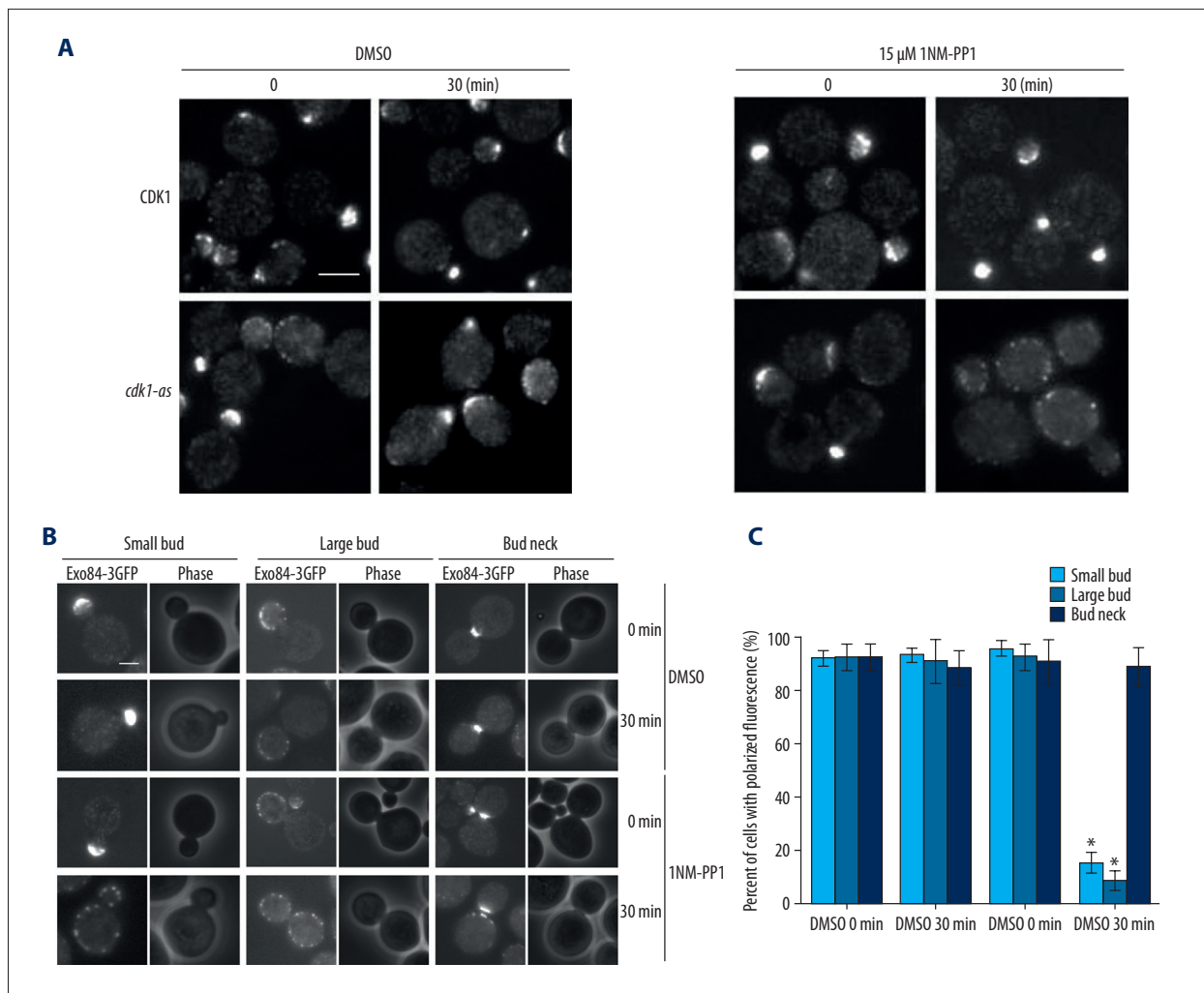


Figure 2. Cdk1 activity is required for polarized localization of exocyst. **(A)** Wild-type and *cdk1-as* mutant harboring 3GFP tagged Exo84 were treated with either DMSO or 15 μmol/L of 1NM-PP1 for 30 min. The cells were immobilized and examined by fluorescence microscopy. Scale bar, 5 μm. **(B)** *cdk1-as* mutant carrying Exo84-3GFP on the chromosome was treated with either DMSO or 1NM-PP1 for 30 min. The cells were fixed and examined by fluorescence microscopy. Scale bar, 5 μm. **(C)** Quantification of cells with polarized Exo84-3GFP from **(B)**. $p < 0.05$.

It has been reported that exocyst is located at polarized growth sites, including bud tip and bud neck, during budding stage and cytokinesis stage, respectively. To confirm in which stage(s) Cdk1 activity is required for polarized localization of exocyst, we further examined exocyst localization in cells either in budding or in cytokinesis stages. As shown in Figure 2B and 2C, 1NM-PP1 treatment disrupted the polarized localization of exocyst in cells with either small bud or large bud, while the localization at the bud neck in cells in cytokinesis stage was not affected.

CDC28 is the gene encoding Cdk1 kinase in budding yeast. At 37°C, *cdc28-4* mutant cells are arrested in G1 phase and the Cdk1 activity is completely inhibited. According to the results in Figure 1B, exocyst localization was not polarized in *cdc28-4* mutant, and Cdk1 activity was not required for the subcellular

localization of exocyst on the plasma membrane. We conclude that Cdk1 controls polarity of exocyst on the plasma membrane in cells of late G1, S, and M phases, but not for cells in early G1 phase or cytokinesis during cell cycle progression.

Cdk1 activity is not required for co-localization of exocyst subunits on the plasma membrane

It has been shown that exocyst assembly and cell growth rate are increased when Cdk1 activity is inhibited [17,26,27]. Based on these results, we speculated that the assembled exocyst complex is still located on the plasma membrane when Cdk1 activity is inhibited. To test this hypothesis, *cdk1-as* mutant cells harboring tdTomato tagged Sec3 and 3GFP tagged Exo84 on the chromosome were treated with either DMSO or 1NM-PP1

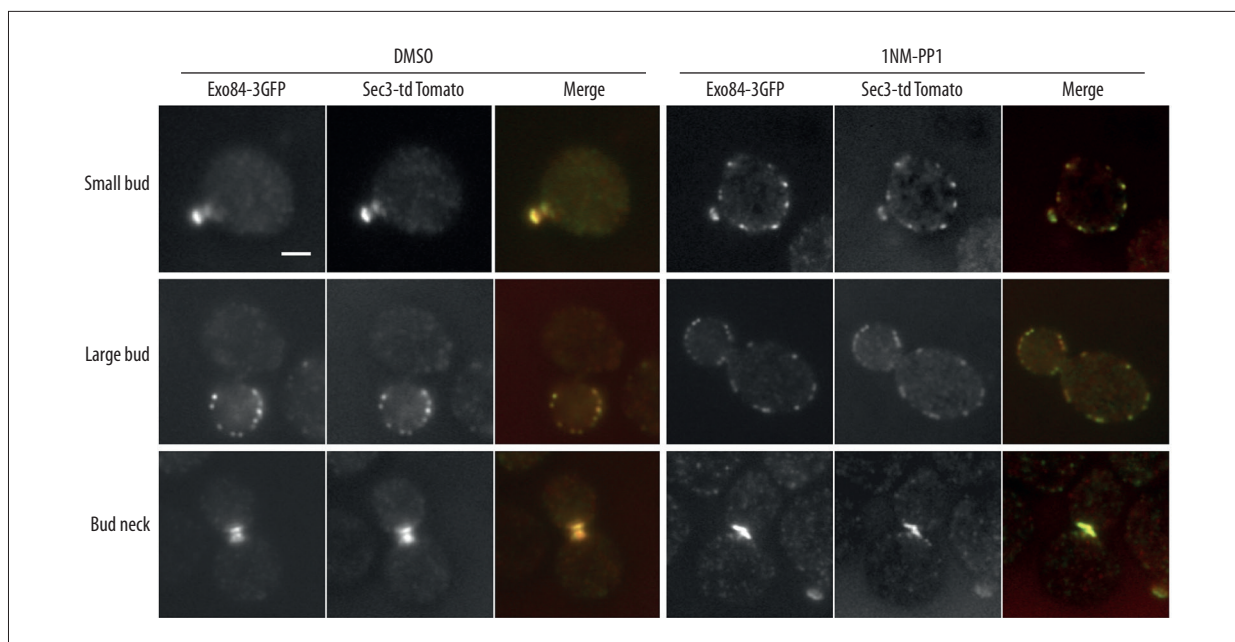


Figure 3. Exocyst components are co-localized when Cdk1 activity is inhibited. *cdk1-as* mutant cells carrying Exo84-3GFP and Sec3-tdTomato on the chromosome were cultured at 25°C to logarithmic growth phase, then treated with either DMSO or 15 $\mu\text{mol/L}$ 1NM-PP1 for 30 min. The cells were fixed and examined by fluorescence microscopy. Scale bar, 5 μm .

and then examined by fluorescence microscopy. As shown in Figure 3, Exo84-3GFP and Sec3-tdTomato were co-localized at the polarized growth sites on the plasma membrane in cells treated with DMSO. After being treated with 1NM-PP1 for 30 min, although Exo84-3GFP and Sec3-tdTomato were scattered on the plasma membrane as expected, these 2 proteins were still co-localized almost completely. This result is consistent with our previous data showing that Cdk1 activity is not required for exocyst assembly [17].

Sec3 protein abundance fluctuates in different cell cycle stages

Since exocyst is an octameric protein complex, the abundance of each subunit in cells may affect the assembly and localization of the whole complex. To test whether the protein level of exocyst subunits is also regulated by cell cycle progression, total protein was extracted from wild-type and the cell division cycle (*cdc*) mutants, *cdc28-4*, *cdc34-2*, *cdc7-1*, *cdc20-1*, and *cdc15-2*, arrested at different cell cycle stages at restrictive temperature. Each exocyst subunit was detected by Western blot using target specific antibodies. Sec5 was not examined because it lacks antibody. As shown in Figure 4, the protein expression level of most exocyst subunits is quite stable. The protein level of Sec3, however, started to increase at early G1 phase and peaked at late G1 phase. In S phase, the Sec3 protein level started to decrease, but its level was still similar to that in early G1 phase. The protein level continued to decrease at metaphase and anaphase, and the cells arrested in anaphase

showed the lowest Sec3 protein level. This result suggests that Sec3 plays a critical role during cell cycle progression.

Discussion

Regulation of polarized secretion and cell growth is an essential feature of cell cycle progression. The exocyst complex, specifically tethering post-Golgi vesicles to the plasma membrane prior to vesicle fusion, plays critical role in polarized secretion. Previously, some studies suggest that exocyst complex is controlled by the cyclin-dependent kinase Cdk1 in budding yeast [14,17–19], but the mechanism by which this complex is regulated by the cell cycle is unclear. The present study, for the first time, indicates that exocyst complex is localized at the plasma membrane in each stage of the cell cycle. We also showed that exocyst is an equilibrium between bud tip and plasma membrane modulated by Cdk1 activity. When Cdk1 is inhibited, more exocyst is found in the plasma membrane, but when Cdk1 is active, exocyst is concentrated to the bud tip. In addition, we found that the protein level of Sec3, but not other exocyst subunits, is regulated by the cell cycle. Our findings provide important clues to understand how the cell cycle regulates polarized secretion and cell growth.

The exocyst complex, located on either the bud tip or bud neck, is critical for polarized cell growth in budding yeast. However, whether the complex is involved in isotropic growth in early G1 phase after cytokinesis is unclear. Goranov's studies showed

that the effect of Cdk1 on exocyst localization is only limited to late G1, S, and M phases during cell cycle progression, but not in early G1 and cytokinesis, which is consistent with the observation that there is no Cdk1 activity in these 2 phases. The molecular mechanism of polarized localization of exocyst regulated by cell cycle at the bud neck is unknown.

As a landmark of exocytosis, Sec3 is considered to be a regulator of exocytosis through its function of N-terminal domain, which can bind to Cdc42, Rho1, and Phosphatidylinositol 4,5-bisphosphate [28–32]. It was also reported that degradation of Sec3 is involved in cellular wound healing [33]. Based on these results, we speculated that the protein level of Sec3 and other exocyst subunits are regulated by the cell cycle. However, in this study we found that only the protein level of Sec3, but not other exocyst components, was controlled by the cell cycle. Although the biological role of this phenomenon is unknown, it further confirms that Sec3 plays a critical role in regulation of exocytosis [19,34–36].

Conclusions

In this study, we found that exocyst complex is located at growth sites on the plasma membrane in every stage of the cell cycle progression. Cdk1 activity is required for exocyst polarization before cytokinesis, but not for the protein complex assembly. We also found that the protein level of one of the exocyst subunits, Sec3, is different in each cell cycle stage. Based on these findings, we conclude that the exocyst complex is spatially and translationally regulated by the cell cycle. Our findings provide important clues to understand how polarized cell growth in yeast is regulated by the cell cycle.

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Conflict of interest

None.

Supplementary Tables

Supplementary Table 1. Yeast strains and genotypes.

Strain	Genotype
NY1490	<i>Mat a ura3-52, leu2-3, 112, his3-200, trp1 Gal-, LA-</i>
GY3220	<i>Mat a ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1, can1-100, bar1-</i>
GY3587	<i>Mat a ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1, can1-100, bar1-, cdk1: cdk1-as</i>
W303	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 GAL+</i>
GY3685	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 GAL+ cdc7-1</i>
GY3687	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 GAL+ cdc20-1</i>
GY3656	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 MET- GAL cdc28-4</i>
GY3691	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 GAL+ cdc15-2</i>
GY3688	<i>MAT a ade2-1 his3-11,15 leu2-3 trp1-1 ura3 can1-100 GAL+ cdc34-2</i>

Supplementary Table 2. Plasmids used in this study.

Plasmid	Description
pG1656	pRS306-SEC3-3GFP, URA3, integration by cutting with BstE II
pG1657	pRS306-SEC5-3GFP, URA3, integration into SEC5 gene by cutting with AflII
pG1662	pRS306-EXO70-3GFP, URA3, integration by cutting with BstE II
pG1663	pRS306-EXO84-3GFP, URA3, integration by cutting with Bgl II
pGV268	pYM-MARS_KAN, template for PCR to create a C-terminal Mars fusion protein of Nup57
pG1414	pRS305-SEC3-tomatoRFP, LEU2, integration by cutting with BstAPI

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