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A novel role for the alcohol sensitive ring/PHD finger protein Asr1p in regulating cell cycle mediated by septin-dependent assembly in yeast

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ABSTRACT

Septin is a conserved eukaryotic family of GTP-binding filament-forming proteins with functions in cytokinesis and other processes. It has been suggested that the dynamic assembly of septin, including the processes from septin initially localizing to the presumptive bud site to the septin collar finally splitting into two cells, coordinates closely with the checkpoint response of cell cycle. Here, we discovered that over-expression of Alcohol sensitive Ring/PHD finger 1 protein (Asr1p) in *Saccharomyces cerevisiae* triggered the Swe1p-dependent cell cycle checkpoint for a G2/M transition delay, and this G2/M transition delay was caused by the septin defect. Since it was shown that Asr1p affected actin dynamics through the interaction with Crn1p and *crn1* should be epistatic to *asr1* in the regulation of actin, the gene knockout of *crn1* in the Asr1p over-expression strain restored the defects in septin and cell cycle along with the disordered actin dynamics. Our investigation further showed that the disturbed septin assembly caused by abnormal Asr1p lead to the abnormal localization of the checkpoint proteins such as Gla4/PAK and Cdc5/Polo, and finally triggered the Swe1p-dependent G2/M transition arrest. Additionally, the Ring finger/PHD domains of Asr1p were illustrated to be required but not sufficient for its role in septin. Taken together, our current data suggested a close relationship in the assembly between septin and actin cytoskeleton, which also partially explained how actin cytoskeleton participated in the regulation of the checkpoint of G2/M.

1. Introduction

Septin is a conserved GTP-binding protein family with seven members, including Cdc3, Cdc10, Cdc11, Cdc12, Shs1/Sep7, Spr3 and Spr28. These septin proteins assemble into a specialized structure, a septin ring, at the mother-bud neck in a filamentous collar when cell division begins. During this process, septin proteins are first recruited at the potential bud site as unorganized septin patches, and transforms into the septin ring within minutes. A cortical septin ring forms in the late G1 phase before bud emergence. Then the septin ring expands into a collar (also called an hourglass) spanning the whole bud neck. Once cytokinesis occurs, the septin collar splits into two distinct rings, marking the division site at both the mother and daughter sides [1–3]. Much evidence suggests that the septin collar at the bud neck serves as a scaffold for a variety of proteins which are involved in the checkpoint response of cell cycle to coordinate cellular morphogenesis with nuclear progression [4–6]. For example, the morphology checkpoint in the G2/M phase is mediated by stabilization of the Swe1p protein kinase, which causes inactivation of complexes of Cdc28p and mitotic cyclins [7–9]. It has been suggested that the localization of Swe1p as well as the other negative regulators to the daughter side of the neck depends on the formation of septin rings [8,9]. Perturbations of the septin collar at the bud neck lead to the disturbance of the Cla4/PAK and Cdc5/Polo location in the neck, and then the phosphorylation and degradation of Swe1p by Cla4p and Cdc5p is delayed [10–12]. Therefore, the dynamic of septin plays an important role in coordinating with the checkpoint responses and cell cycle.

The septin organization and assembly at the cortex is highly regulated during the cell cycle. Several proteins are known to affect septin-ring formation in yeast. For example, the Rho-family GTPase Cdc42p and its activating factor (GEF) Cdc24p have been reported to be required for the polarized assembly of both the actin

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cytoskeleton and the septins at the beginning of the cell cycle [13–17]. However, actin and the septins appear to be independent of each other for their organization at the presumptive bud site [2,18–20]. The actin cytoskeleton, one of the most important components of the cell cytoskeleton, participates in almost every cellular process, such as cell locomotion, endocytosis, vesicle and organelle transport, cytokinesis, polarized cell growth, vegetative reproduction through budding, and sexual reproduction through mating and meiosis. One example comes from an investigation of myosin 2 (Myo2p), one of the important components for yeast polarized growth. In 2005, McNulty et al. reported that the mutation of Myo2p led to the disturbance of the actin dynamic, which was responsible for the Swe1p-dependent G2/M cell cycle delay as well as abnormal cytokinesis and bud formation [21]. Additionally, Kimberly et al. also found that the Swe1p-dependent G2/M cell cycle arrest caused by the actin dynamic defect could be restored when Zds2p was over-expressed [22]. Though much experimental evidence has suggested that actin cytoskeleton participates in the regulation of the checkpoint of G2/M, the detailed model and its underlying mechanisms remain unclear.

In our previous investigation, we demonstrated the involvement of Asr1p in actin dynamics as well as actin-based cellular movements in *Saccharomyces cerevisiae* (data not published). It was also observed a severe defect of septin collar in Asr1p over-expression strain that bearing the disordered actin cytoskeleton with the disappearance of the actin patches. Our current data further demonstrated that the perturbation of septin organization changed the localization of Cla4p and Cdc5p and eventually induced the formation of abnormally elongated buds accompanying the Swe1p-dependent cell cycle delay.

2. Materials and methods

2.1. Yeast strains, plasmids and genetic manipulations

The *S. cerevisiae* strains used in this study are listed in Table S1. Construction of deletion alleles was carried out by modifying the target gene in the haploid strain BJ5457 using a PCR-based one-step gene replacement technique [23]. The successful deletion target genes were validated by PCR with the genomic DNA as templates. The overproduction of Asr1p was carried out by transforming a GAL1-*Asr1* plasmid in BJ5457. The display of septin and gene location was accomplished by green fluorescence using the plasmid of pDDGFP-2. The templates and the oligonucleotide primers employed in PCR reactions are shown in Table S2.

2.2. Media and growth conditions

Yeast strains were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or synthetic complete media (SM) lacking the appropriate aminoacids at 30 °C. Induction of GFP-tagged or Asr1p-tagged proteins was accomplished by inoculating a single yeast clone into SM lacking uracil or tryptophan and containing 2% glucose. After growth for 1–2 days, a certain proportion of the sample was collected and washed in SM, then re-inoculated in fresh SM without uracil and with 2% raffinose and 2% galactose as carbon sources instead of glucose. After 4–8 h, the glucose repression was overcome. The cells were finally visualized after transfer into the raffinose-galactose containing medium for 4–24 h [24].

2.3. Microarray analysis

Gene expression profiles between wild type and Δ*asr1* strains were analyzed using the Yeast Genome 2.0 array and Affymetrix GeneChip 3000 TG System. Total RNA extraction, fragmentation and hybridization were carried out at the Tianjin Biochip Corporation (TBC, China).

2.4. Fluorescence microscopy

Staining of the actin cytoskeleton was performed basically as described previously [25]. Briefly, cells were grown to exponential phase at 30 °C and then fixed with 3.75% formaldehyde at room temperature for 30 min. Cells were incubated with 5uM FITC-conjugated phalloidin (Sigma–Aldrich) for 90 min, washed, and resuspended in phosphate-buffered saline buffer. The stained cells or the cells containing GFP gene fusions were observed using a Nikon E800 microscope with a 100-oil immersion objective and an AxioVision imaging system, and photographed using a Nikon DS-5Mc color camera. NIS-Elements F 2.30 software was utilized for image analysis (Instruments Europe B.V., Düsseldorf, Germany).

2.5. Flow cytometry

In flow cytometric analysis, at least 10^7 cells were fixed in ice-cold 70% ethanol at 4 °C until needed. The fixed cells were resuspended in 50 mM sodium citrate with 1 mg/ml RNAse A (Sigma) at 37 °C for 4 h, and then incubated with 1 mg/ml proteinase K (Sigma) at 50 °C for 1 h. Cells were sonicated and incubated in the dark with Sytox Green with a final concentration of 1 μM (Invitrogen) at room temperature for 45 min. Samples were analyzed by flow cytometry on a FACS can single-laser fixed-alignment benchtop analyzer with an emission of 488 nm.

3. Results

3.1. Over-expression of Asr1p leads to the delay of G2/M transition

We conducted a microarray assay to investigate the gene expression profiles of the wild type strain and an *asr1*-depletion strain Δ*asr1*. The microarray data showed that a total of 176 genes were up-regulated in the Δ*asr1* strain when compared to the wild type strain. Among them, 34 genes are related to the regulation of the cell cycle (Table S3), suggesting a potential perturbation existed in the cell cycle.

To confirm the microarray results that Asr1p contributes to the regulation of the cell cycle, we successfully constructed the *asr1* over-expression strain again (Fig. S1), and further investigated the phenotype of the cell cycle. As identified in many previous publications, the generation of abundant elongated-bud cells is a distinct phenotype to determine whether the cells undergo cell cycle delay [21,22], hence we surveyed the bud morphologies and counted the proportion of elongated-bud cells at different time points after induction by galactose. Though the Δ*asr1* strain showed up-regulation of a large panel of cell cycle genes, the deletion of *asr1* had no effect when compared to the wild type strain. On the contrary, it was observed that the elongated-bud phenotype emerged in the Asr1p over-expression strain (Fig. 1A). Quantitative analysis showed that this severe abnormality in the *asr1* over-expression strain remained from 4 h to 24 h after induction ($P \leq 0.001$; Fig. 1B). Furthermore, we also examined the cell cycle by flow cytometry. Comparing to the normal distribution of cells with one copy (1C) and two copies (2C) of DNA in the negative control having a blank vector of pYES3/CT, the Asr1p over-expression cells exhibited a skewed distribution and nearly all had twice the amount of DNA (Fig. 1C). Taken together, our results suggested that the cells over-expressing Asr1p had a significantly delayed G2/M transition.

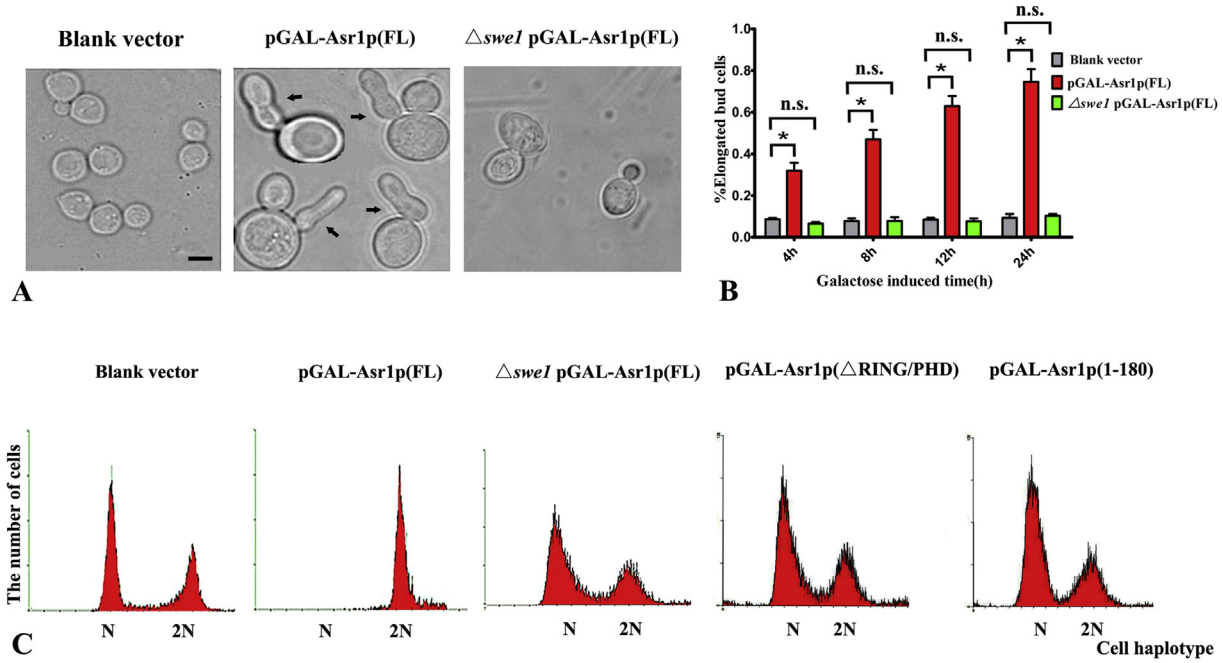

Fig. 1. Over-expression of *asr1* led to the elongated-bud accompanied with the G2/M cell cycle delay, but the abnormal phenotypes in both cell morphology and cell cycle could be reversed by the depletion of gene *swe1*. (A) The cell morphologies were observed in the negative control with blank vector, Asr1p over-expression strain and Asr1p over-expression in the Δ*swe1* background. Bar, 5 μm. The cells with elongated-bud morphology are marked with black arrowheads. (B) The quantitative analysis to elongated-bud cells in the control with blank vector, Asr1p over-expression strain and Asr1p over-expression in the Δ*swe1* background. Means ± s.d., n = 3. *P* values were determined by unpaired t test. $^{*}P \leq 0.001$. (C) Flow cytometry analysis of DNA content for the negative control with blank vector, Asr1p over-expression strain, Asr1p over-expression in the Δ*swe1* background, pGAL-Asr1p (ΔRING/PHD) and pGAL-Asr1p (1-180) mutants.

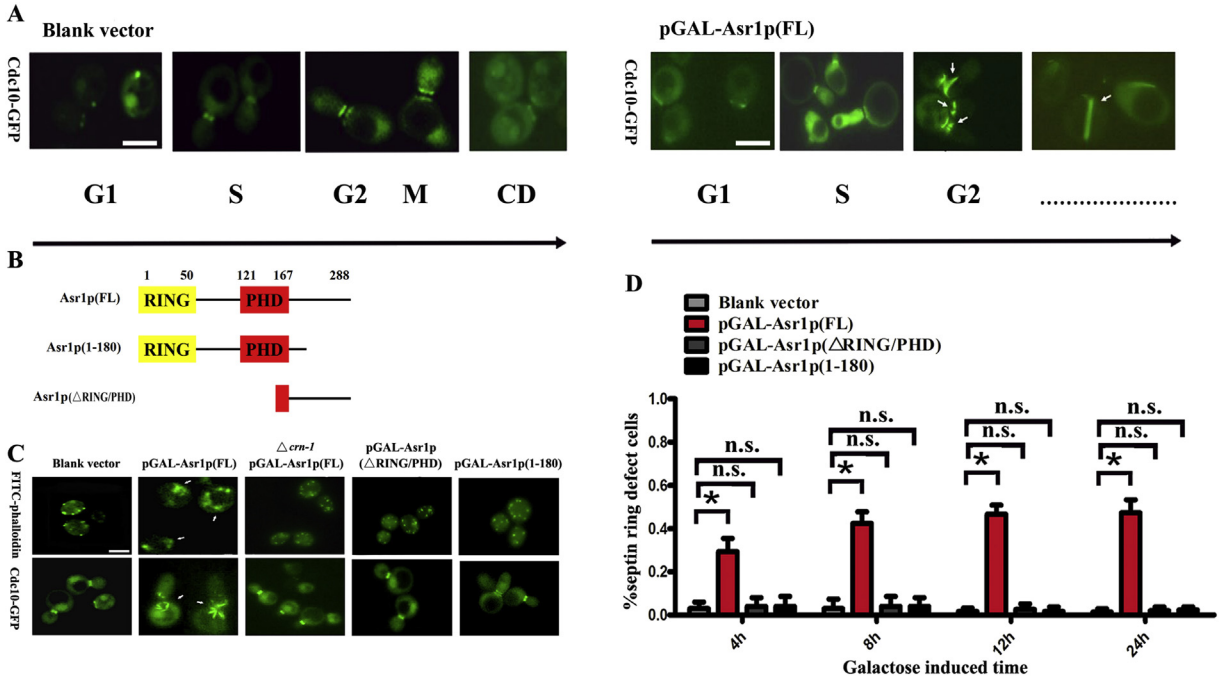

Fig. 2. Septin structure defect was caused by the overproduction of Asr1p in *S. cerevisiae*, and the Ring finger/PHD domains are required for the roles of Asr1p. (A) Septin dynamic during cytokinesis in the negative control with blank vector and Asr1p over-expression strain. Cdc10p-GFP was employed as a marker to observe the septin ring dynamic. The defects of septin are marked with white arrowheads. (B) Schematic representation of functional domains in Asr1 proteinis characterized by the presence of a RING finger domain (1–50) and a PHD domain (121–167). (C) Actin abnormality caused by Asr1p is responsible for septin ring defect. Actin was stained with FITC-conjugated phalloidin in the negative control with blank vector, Asr1p over-expression strain, Asr1p over-expression in the Δ*crn1* background, pGAL-Asr1p (ΔRING/PHD) and pGAL-Asr1p (1–180) mutants. Meanwhile, the septin structure was observed using Cdc10p-GFP as a marker in the same strains above. The defects in either actin or septin were marked with white arrowheads. Bar, 5 μm. (D) Quantitative analysis of the septin-defective cells in the same strains of figure C. *P* values were determined by unpaired t test. $^{*}P \leq 0.001$.

3.2. Asr1p over-expression leads to septin ring defect

Our previous experimental evidence demonstrated that an obvious defect of the actin cytoskeleton would be observed when *asr1* was over-expressed, suggesting that Asr1p is involved in actin dynamics (Fig. S2A and B). Therefore, we examined the possibility that the abnormal actin cytoskeleton altered by the expression of Asr1p could compromise septin structure. Cdc10p-GFP was employed as a marker to observe the septin ring dynamic. In negative control cells that had a blank vector of pYES3/CT, the dynamic assembly of septin kept coincident with the cell cycle from G1 to S phase. As the bud emerged, septin expanded into an hourglass-shaped collar at the cortex of mother-bud neck. When cytokinesis occurred, the septin hourglass split into two distinct rings that partitioned to the cell cortex of both the mother cell and the daughter cell (Fig. 2A, left panel). In the Asr1p over-expression strain, though unorganized septin patches were initially recruited to the cell cortex and a septin ring soon formed at the presumptive bud sites at G1 phase, the over-expression of Asr1p caused obviously morphological defects in septin rings at later stages after G2 phase: the failure of the septin collar to split into two distinct rings, as well as the delay of septin disappearance (Fig. 2A, right panel; Fig. 2C). Quantitative analysis also showed a significant increase in the percentage of cells that had abnormal septin organization ($^{*}P \leq 0.001$; Fig. 2D). From those data, it has been suggested that the gene *asr1* participates in the regulation of septin assembly in *S. cerevisiae*.

3.3. Defect of septin causes the abnormal localization of Cla4p and Cdc5p

The septin collar at the bud neck has been revealed to serve as a scaffold for a variety of proteins, including the vital proteins involved in the checkpoints response of cell cycle. The two kinases, Cla4/PAK and Cdc5/Polo are localized at the septin collar of the bud neck and responsible for stepwise phosphorylation and down-regulation of Swe1p [10–12]. Meanwhile, the failure of Swe1p degradation retards the timely mitotic entry [7–9]. Thus, we hypothesized that the defect of septin in the *asr1* over-expression strain would influence the localization of Cla4p and Cdc5p, and finally led to the G2/M arrest. To verify this assumption, we expressed Cla4p-GFP and Cdc5p-GFP in the negative control strain with a blank vector of pYES3/CT and the Asr1p over-expression strain, respectively. As expected, the two proteins Cla4p and Cdc5p of the negative control were recruited to the cortex of the mother-bud neck in the morphogenesis checkpoint response (Fig. 3, upper panel). However, compared with the unperturbed cells, Cla4p and Cdc5p in the Asr1p over-expression strain mainly remained in the cytoplasm throughout the whole cell cycle, rather than being specifically recruited to the cortex of the mother-bud neck (Fig. 3, lower panel). Therefore, our results suggested that the septin defect caused by *asr1* overproduction disturbed the location of Cla4p and Cdc5p in the morphogenesis checkpoint response.

3.4. G2/M cell cycle delay upon the overproduction of Asr1p is Swe1p-dependent

The morphology checkpoint in the G2/M phase is mediated by stabilization of the Swe1p protein kinase [7–9], and our experimental data from the microarray assay also showed 6 up-regulated genes with more than twofold were involved in the function related to Cdc28p (AXL2, ICY2, SWE1, PDS1, SSK2, TOS2) (Table S4). To examine whether the G2/M cell cycle arrest was Swe1p-dependent, we deleted Cdc28p-inhibitory kinase gene *swe1* again in the *asr1* over-expression strain (Fig. S1). It was observed that the elongated-bud phenotype and cell cycle delay were largely suppressed by deleting the gene *swe1*. Comparable to the Asr1p over-expression strain in which the elongated buds were observed during the range of 4 h–24 h, the number of cells having the elongated-bud phenotype was less than 10% of the proportion at each of the same time points when the *swe1* gene was knocked out, i.e. similar to the population in the wild type strain (Fig. 1A and B). Next, we examined the cell cycle by flow cytometry. Compared to the Asr1p over-expression strain that had almost twice the normal amount of DNA and exhibited a skewed distribution, the cells with *swe1* gene

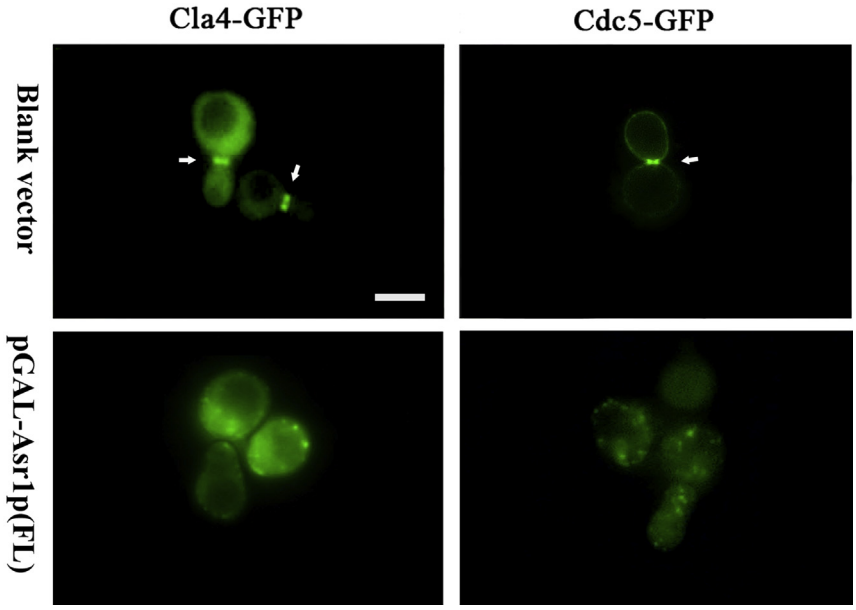

Fig. 3. Abnormal localization of Cdc5p and Cla4p was caused by the overproduction of Asr1p. The methods of cell cultivation and GFP observation were the same as mentioned above. Compared to the negative controls with Cdc5p and Cla4p localizing to the cortex of the mother-bud neck, the localization of Cdc5p and Cla4p changed and mostly remained in the cytosplasm in Asr1p over-expression strain. Bar, 5 μm.

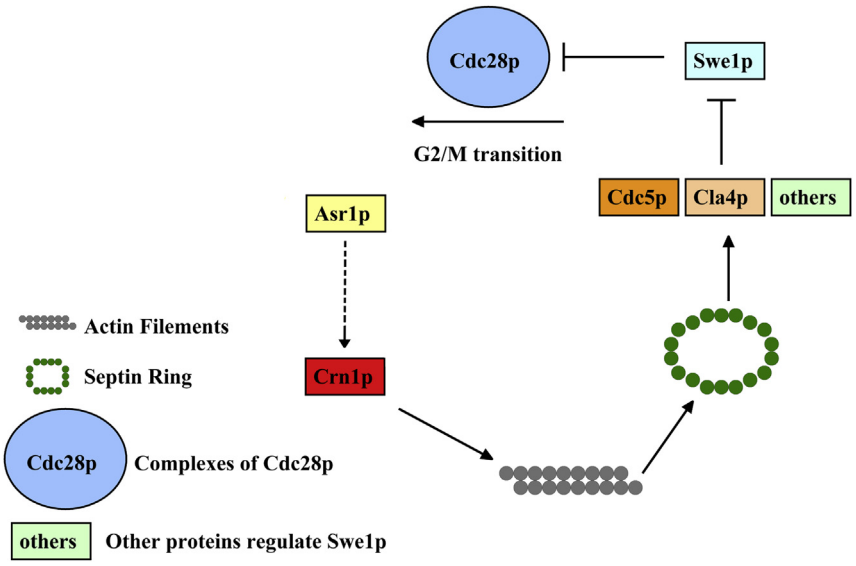

Fig. 4. The model of Asr1p triggers the morphogenesis checkpoint of the G2/M cell cycle. In this model, actin abnormality caused by Asr1p leads to septin collar defect. This septin defect disturbs the location of Cla4p and Cdc5p to the cortex of the mother-bud neck, fails to phosphorylate and degrade Swe1p, and subsequently triggers the G2/M transition morphology checkpoint.

knockout in the background of *asr1* over-expression had a nearly normal distribution with one copy (1C) reappearing (Fig. 1C). Thus, we conclude that G2/M cell cycle delay upon the overproduction of Asr1p is Swe1p-dependent.

Furthermore, we performed a qPCR to compare the mRNA expressional levels of *swe1* between the Asr1p over-expression strain and the negative strain with a blank vector. Our results showed similar levels of *swe1* mRNA between those two strains at the four different time points (4 h, 8 h, 12 h, and 24 h) after induction by galactose (Fig. S3). It thus suggests the G2/M transition arrest should not be attributed to the changes of *swe1* mRNA levels.

3.5. Disordered actin is the main reason for the defects of septin ring

Asr1p has been suggested to be involved in actin dynamics via interacting with the conserved actin-associated protein Crn1p, and thus the disordered actin cytoskeleton in *asr1* over-expressing cells could also be rescued by knockout of *crn1* gene (Fig. S2C). So, our question was what would happen to the septin ring when the normal actin dynamic was restored. To answer this question, we transformed the Δ*crn1* deletion mutant with the pGAL-*asr1* plasmid to over-express Asr1p in the Δ*crn1* background and then observed the phenotype of septin. It was demonstrated that the defects of septin disappeared and nearly all cells formed normal septin in the presumptive bud site (Fig. 2C). Therefore, the result supported our hypothesis that the disordered actin caused by Asr1p over-expression was at least partially responsible for the defects of septin.

3.6. The ring finger/PHD domains are required for the roles of Asr1p

Asr1p has been reported to have two Ring finger/PHD domains, which are important to mediate the interactions with other proteins [26]. To understand whether the Ring finger/PHD domains are required for its roles in septin, we over-expressed the truncated Asr1p protein: Asr1p (1–180) mutant containing the first and second RING finger/PHD domains and Asr1p (ΔRING/PHD) that lacked the RING finger/PHD domain (Fig. 2B and Fig. S1), and further investigated the septin structure and actin dynamics. Compared to the cells over-expressing the full length Asr1p, no obvious defects in actin dynamics and septin structure were observed when both of the truncated Asr1p proteins over-expressed (Fig. 2C and D). We also examined the cell cycle by flow cytometry, and our results showed that either Asr1p (1–180) or Asr1p (ΔRING/PHD) had a nearly normal distribution with one copy (1C) and two copies (2C) of DNA (Fig. 1C). These results suggest that the Ring finger/PHD domains should be required but not sufficient for the roles of Asr1p in septin.

4. Discussion

Asr1p is a unique protein in *S. cerevisiae* mentioned relatively few times in the literature. It was first suggested to participate in the alcohol response by transitioning its localization from the cytoplasm to the nucleus [26,27]. Recently, Daulny et al. reported that it functions as a RING finger ubiquitin-ligase of RNA polymerase II and thus inactivates RNA polymerase II [28]. Additionally, it is also involved in the ubiquitination of calmodulin in yeast [29]. In our investigation, it was shown that Asr1p influences actin dynamics by interacting with the conserved actin-associated protein Crn1p, and furthermore that *crn1* is epistatic to *asr1* in the regulation of actin (Fig. S2). Meanwhile, actin dynamics have also been posited to participate in the assembly or disassembly of septin, but without validated experimental evidence [30,31]. Septin assembly has been suggested to be a strict process consisting of the activation of a variety of proteins [13–17], and this is the first report that the E3 ubiquitin-ligase Asr1p can influence septin assembly and induce arrest of the G2/M cell cycle in a Swe1p-dependent manner.

From our current data, we clearly observed the obvious defects in both septin and actin structures when Asr1p over-expression was achieved. We speculated that the disordered actin dynamics might be responsible for the disturbed septin. Our hypothesis was at least partially supported by the experimental evidence that the reversal of the actin defect by the knockout of the *crn1* gene in the Asr1p over-expression strain also restored the phenotype of septin. In this case, the failure of the septin assembly also disturbed the location of Cla4p and Cdc5p to the cortex of the mother-bud neck,

then influenced the phosphorylation and degradation of Swe1p, and eventually perturbed the cell cycle with the G2/M transition arrest. Based on our current data, it was hypothesized that the dynamics of the actin cytoskeleton influence the assembly of septin, and the perturbations of the septin at the bud neck lead to the disturbance of the location of the negative regulators of Swe1p and thus prevent its degradation. The accumulation of Swe1p caused by the septin collar defect subsequently accelerates the inactivation of complexes of Cdc28p and mitotic cyclins, triggering the morphogenesis checkpoint following the G2/M transition arrest (Fig. 4).

In conclusion, our study is the first report to suggest the involvement of Asr1p in septin dynamics and the direct relationship between the assembly of actin and septin. The underlying mechanism of how actin affects septin assembly requires further elucidation.

Conflict of interest

All of the authors, including Wei Zou, Jinyuan Yan, Ninghui Zhao, Shanzhuang Niu, and Xiaowei Huang, declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “A Novel Role for the Alcohol Sensitive Ring/PHD Finger Protein Asr1p in Regulating Cell Cycle Mediated by Septin-Dependent Assembly in Yeast”.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.113.

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