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Septin-associated protein kinase Gin4 affects localization and phosphorylation of Chs4, the regulatory subunit of the Baker's yeast chitin synthase III complex

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ABSTRACT

Chitin is mainly formed by the chitin synthase III complex (CSIII) in yeast cells. This complex is considered to be composed of the catalytic subunit Chs3 and the regulatory subunit Chs4, both of which are phosphoproteins and transported to the plasma membrane by different trafficking routes. During cytokinesis, Chs3 associates with Chs4 and other proteins at the septin ring, which results in an active CSIII complex. In this study, we focused on the role of Chs4 as a regulatory subunit of the CSIII complex. We analyzed the dynamic localization and interaction of Chs3 and Chs4 during cell division, and found that both proteins transiently co-localize and physically interact only during bud formation and later in a period during septum formation and cytokinesis. To identify unknown binding partners of Chs4, we conducted different screening approaches, which yielded several novel candidates of Chs4-binding proteins including the septin-associated kinase Gin4. Our further studies confirmed this interaction and provided first evidence that Chs4 phosphorylation is partially dependent on Gin4, which is required for proper localization of Chs4 at the bud neck.

1. Introduction

Research of the past decades demonstrated that the baker's yeast, *Saccharomyces cerevisiae*, is a powerful model to analyze the genes involved in chitin biosynthesis. In particular, the functions of three genes that encode the chitin synthases *Chs1*, *Chs2* and *Chs3*, have been intensively studied in this system (Cabib et al., 1993; Ruiz-Herrera and Ortiz-Castellanos, 2010). While Chs1 functions in cell wall repair, Chs2 forms the chitin of the primary septum, and Chs3 is required to form chitin for the lateral cell wall and a chitin ring at the bud neck during cell division (Cabib et al., 1996). Chs3 is the catalytic subunit of the chitin synthase complex III (CSIII), which produces the majority of chitin deposited in the yeast cell wall. It forms di- or oligomeric complexes *in vivo* (Gohlke et al., 2017; Sacristan et al., 2013). Intracellular trafficking of Chs3 is under tight control, which facilitates specific regulation of CSIII activity to maintain cell wall integrity (Levin, 2011). The export of Chs3 from the endoplasmic reticulum (ER) is controlled by the ER chaperone Chs7 and the palmitoyltransferase Pfa4, which both prevent the formation of high-molecular mass aggregates at the ER (Lam et al., 2006; Trilla et al., 1999). Trafficking from the trans-Golgi-network (TGN) to the plasma membrane involves the exomer complex that includes the essential component Chs5 and four paralogues, Chs6, Bch1, Bch2 and Bud7, which are collectively termed Chs5-Arf1-binding Proteins (ChAPs) (Sanchatjate and Schekman, 2006; Santos and Snyder, 1997; Trautwein et al., 2006; Ziman et al., 1996; Ziman et al., 1998). Furthermore, Chs3 circulates between endosomal reservoir vesicles called chitosomes and the plasma membrane in a cell-cycle dependent manner (Bartnicki-Garcia, 2006; Chuang and Schekman, 1996; Santos and Snyder, 1997; Ziman et al., 1996). This reservoir of Chs3 is maintained by endosomal recycling, which depends on clathrin-coated vesicles (CCVs), the adaptor protein 1 (AP-1) complex, Golgi-localized, gamma-ear-containing, ARF-binding (GGA) proteins, and the epsin-like proteins (Robinson, 2015). In addition, Arcones et al. (2016) recently demonstrated that the reservoir is filled by recycling through the retromer complex, which is coordinated by the Rab family GTPase Ypt7 (Purushothaman et al., 2017). The retromer consists of a trimer of Vps35, Vps29 and Vps26, which is required for cargo selection, and the dimer of Vps5 and Vps17, which mediates vesicle formation (Seaman, 2012). Finally, the reservoir may also be directly replenished by AP-2 dependent endocytosis in a similar way as recently shown for the Mid2 protein (Chapa-y-Lazo et al., 2014). Notably, different regions within the cytosolic, N-terminal domain of Chs3 appear to mediate the distinct

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trafficking steps (Weiskoff and Fromme, 2014). The general model of intracellular Chs3 trafficking predicts exclusion of Chs3 from vacuolar degradation, although it is unclear how Chs3 evades its destruction. Arcones et al. (2016) have also shown that Chs3 is target of N-terminal ubiquitination by the Rsp5/Art4 complex; though specific subpopulations of Chs3 could be tagged for vacuolar degradation and this mechanism may contribute to multifarious regulation of CSIII.

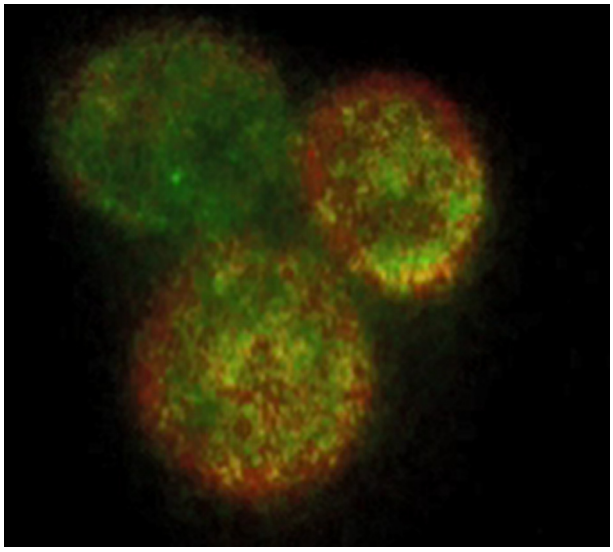
The regulatory subunit of the CSIII complex is Chs4, which interacts with N-terminal, cytosolic domains of Chs3. Chs4 is the only known activator of the CSIII complex, although the precise mechanism of CSIII activation is currently unknown. It is a CaaX protein and hence prenylated at the C-terminus, a process that is required for membrane association of Chs4 and chitin synthase activity (Meissner et al., 2010). Chs4 is also required for tethering Chs3 to the bud neck, because it interacts with the septin-binding protein Bni4 (Reyes et al., 2007; Sacristan et al., 2012). Bni4 also recruits the catalytic subunit of protein phosphatase 1 (Glc7) to the bud neck in a temporal and spatial restricted manner (Larson et al., 2008). As Bni4 is phosphorylated by multiple kinases including Slt2 and/or Kss1 (Perez et al., 2016), coordinated phosphorylation appears to be important for septum assembly during cytokinesis (Larson et al., 2008). Phosphorylation is also involved in regulating trafficking of Chs3 from internal storage vesicles to the plasma membrane, a process that involves activation of Rho1 and Pkc1 (Valdivia and Schekman, 2003). Although Chs3 is phosphorylated as indicated by mass spectrometric analyses of the yeast phosphoproteome (Albuquerque et al., 2008; Li et al., 2007; Swaney et al., 2013), the substitution of the corresponding S/T residues in the N-terminal region of Chs3 did not reveal obvious phenotypes (Sacristan et al., 2013). Thus, the role of phosphorylation in regulating Chs3 trafficking and activity is not completely understood.

In this study, we analyzed the dynamic localization and interaction of Chs3 and Chs4 during cell division, and found that both proteins colocalize and interact only at two distinct phases at the bud neck. Recently, it has been shown that Hof1 interacts with Chs4 to inhibit Chs3 activity during secondary septum formation (Oh et al., 2017). Here we show that colocalization of both proteins at the bud neck is an early and concurrent event before cytokinesis starts. We further identified hitherto unknown interaction partners of Chs4 using three independent but complementary approaches. Among the identified proteins were several kinases, of which only Gin4 locates at the bud neck. We demonstrate that Gin4 interacts with Chs4 *in vitro* and *in vivo*. Genetic deletion of Gin4 resulted in elongated bud phenotypes as well as altered phosphorylation and mislocalization of Chs4, which indirectly changes chitin levels in the cell wall.

2. Results and discussion

2.1. Spatial and temporal localization of the CSIII complex at the bud neck

Chs3 and Chs4 have been independently localized in the course of cell division, but so far no co-localization studies have been performed that would allow parallel determination of the spatial and temporal localization of individual CSIII subunits during cell division. Therefore, we generated yeast strains that co-express $Chs3^{GFP}$ and ^{mCherry}Chs4 fusion proteins and performed life cell fluorescence imaging to monitor the dynamics of CSIII assembly and disassembly (Fig. 1 and Movie 1). When the incipient bud forms, $Chs3^{GFP}$ fluorescence signals become detectable at the bud site. Also ^{mCherry}Chs4 concentrates at the incipient bud site. However, it is also found equally distributed throughout the lateral plasma membrane. Both signals co-localize at the bud neck of small-budded cells between 5 and 20 min. While the bud size increases, the ^{mCherry}Chs4 signal gradually diminishes at the bud site and steadily increases within the plasma membrane of the daughter cell where it is equally distributed. Ten minutes later (at 30 min), it is almost completely absent from the bud neck. $Chs3^{GFP}$ remains at the bud neck until it becomes first detectable in intracellular vesicles at 15 min. The intracellular signals become stronger over time until $Chs3^{GFP}$ has disappeared from the bud neck in medium-budded cells at 35 min and is then found exclusively in the vesicle stores. During this time, the Chs4 signal increases continuously in the cytosol and then in the plasma membrane of mother cells. At 55 min, however, the Chs4 signals begin to disappear from the plasma membranes and reappear at the bud neck of large-budded cells, almost simultaneously with the arrival of $Chs3^{GFP}$, which re-localizes from internal vesicles to the bud neck. Both proteins co-localize at the bud neck until the end of cytokinesis. Finally, Chs3 and Chs4 begin to disappear from the bud neck at 75–80 min. While Chs3 steadily accumulates in small-sized internal vesicles, Chs4 is detectable again at the lateral membranes. Based on this information, we propose that CSIII complexes occur at two distinct time periods during cytokinesis, during incipient bud formation within the first 10 min, and for a short time period of 10 min after primary septum formation has been completed.



Movie 1. While Chs3 and Chs4 proteins evidently take different trafficking routes to the plasma membrane, they both appear at the bud neck at two distinct time points during cell division. They first encounter during incipient bud formation, when the production of the chitin ring starts (Sanz et al., 2004). However, while the chitin ring grows with increasing bud size, Chs4 disappears from the bud neck and is then mainly found associated with the plasma membrane of the daughter cell. Contrarily, Chs3 continues to stay at the bud neck for at least additional 15 min before it is endocytosed and sorted back into chitosomes, which is consistent with the results from previous studies (Zanolari et al., 2011; Ziman et al., 1998). Therefore, the Chs3-Chs4 interaction observed in this first phase may be not necessary to maintain Chs3 at the bud neck or required to sustain chitin synthesis during further growth of the chitin ring. Both, Chs3 and Chs4 reappear at the bud neck again shortly before primary septum formation by Chs2 is expected to occur and stay associated at the bud neck during cytokinesis. This finding is in line with the model postulated recently by (Oh et al., 2017) suggesting that the binding of Hof1 to the Sel1-like repeats of Chs4 inhibits Chs3 activity during primary septum formation. According to this model, degradation of Hof1 finally releases Chs4, which then activates Chs3 and allows secondary septum formation (Oh et al., 2017). After 75–80 min Chs4 leaves the bud neck first before Chs3 follows shortly after. Eventually, the release of Chs4 may allow mobilization of Chs3 and its lateral diffusion to sites of endocytosis. Taken together, the interaction between Chs3 and Chs4 is of transient nature, highly dynamic and regulated by many proteins in a cell cycle dependent manner. The dynamic shuttling of Chs4 between the plasma membrane of the daughter cell and the bud neck during cell division strikingly resembles that of the small rho-like GTPase Cdc42, which is a