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Research Article

RESEARCH ARTICLE

Activation of Rab GTPase Sec4 by its GEF Sec2 is required for prospore membrane formation during sporulation in yeast *Saccharomyces cerevisiae*

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One sentence summary: Sec2 depletion during sporulation causes a Sec4 targeting defect, leading to defects in prospore membrane formation and spore walls, caused by insufficient targeting of downstream effector to the prospore membrane.
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ABSTRACT

Sec2 activates Sec4 Rab GTPase as a guanine nucleotide exchange factor for the recruitment of downstream effectors to facilitate tethering and fusion of post-Golgi vesicles at the plasma membrane. During the meiosis and sporulation of budding yeast, post-Golgi vesicles are transported to and fused at the spindle pole body (SPB) to form a *de novo* membrane, called the prospore membrane. Previous studies have revealed the role of the SPB outer surface called the meiotic outer plaque (MOP) in docking and fusion of post-Golgi vesicles. However, the upstream molecular machinery for post-Golgi vesicular fusion that facilitates prospore membrane formation remains enigmatic.

Here, we demonstrate that the GTP exchange factor for Sec4, Sec2, participates in the formation of the prospore membrane. A conditional mutant in which the *SEC2* expression is shut off during sporulation showed sporulation defects. Inactivation of Sec2 caused Sec4 targeting defects along the prospore membranes, thereby causing insufficient targeting of downstream effectors and cargo proteins to the prospore membrane. These results suggest that the activation of Sec4 by Sec2 is required for the efficient supply of post-Golgi vesicles to the prospore membrane and thus for prospore membrane formation/extension and subsequent deposition of spore wall materials.

Keywords: sporulation; *Saccharomyces cerevisiae*; Rab GTPase

INTRODUCTION

Diploid cells of *Saccharomyces cerevisiae* undergo meiosis and sporulation in response to nitrogen starvation in the presence of a non-fermentable carbon source (Neiman 2011). Four haploid nuclei are engulfed by *de novo* membrane structures, called prospore membranes, and subsequently covered by rigid spore wall structures. Spores await germination until external

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Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
AN117-4B	*MATα ura3 leu2 trp1 his3Δsk arg4-NspI lys2 ho::LYS2 rme1::LEU2*	(Neiman *et al.* 2000)
AN117-16D	*MATa ura3 leu2 trp1 his3Δsk lys2 ho::LYS2*	(Neiman *et al.* 2000)
AN120	*MATa/MATα ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3*	(Neiman *et al.* 2000)
YSY38-2	AN120, but homozygous *kanMX6-CLB2pr-3HA-SEC2*	This study
YSY57	AN120, but homozygous *natNT2-CLB2pr-3HA-SEC2*	This study
HI3	AN120, but homozygous *sso1::HIS3MX6*	(Nakanishi *et al.* 2006)

environmental cues signal nutrient rich conditions (Neiman 1998). In this developmental process, the prospore membrane is formed from the cytoplasmic face of the spindle pole body (SPB) which is embedded within the nuclear membrane and equivalent to the centrosome in multi-cellular eukaryotes. During meiotic progression, at the onset of meiosis II, the outer plaque of the SPB is modified to become the meiosis-specific structure, called the meiotic outer plaque (MOP), which serves as a platform for the fusion of post-Golgi secretory vesicles. Each prospore membrane extends along the nuclear envelope as meiosis progresses and then each haploid nucleus is completely engulfed by the scission of the prospore membrane. This scissioning process allows the inner and outer prospore membranes to separate completely and spore wall materials are deposited within the lumen of these prospore membranes to produce the rigidity needed to survive starvation conditions (Neiman 2011).

During vegetative growth of *S. cerevisiae*, post-Golgi vesicles are targeted to the growing plasma membrane site where Sec4, a Rab GTPase, functions as a molecular switch to recruit its downstream effectors for the tethering and fusion of vesicles (Guo *et al.* 1999). Rab GTPase cycles from a cytoplasmic, inactive GDP-bound state to a membrane-bound, active GTP-bound state via its endogenous GTP exchange factor (GEF) activity (Walch-Solimena, Collins and Novick 1997). Once Sec4 is activated by Sec2 (a Sec4-specific GEF), the GTP-bound form of Sec4 is targeted to the surface of the secretory vesicles and recruits a vesicle-associated, octameric tethering complex dubbed the exocyst (Walch-Solimena, Collins and Novick 1997). Earlier works suggested that exocysts are composed of two subcomplexes: a vesicle-associated subcomplex which is recruited toward the vesicle membrane via a direct interaction of Sec15 with Sec4 and a plasma membrane-associated subcomplex which is probably downstream of the Rho1/Cdc42-mediated pathway (Zajac *et al.* 2005). Thus, initial membrane contact is governed through a complex formation of the vesicle- and plasma membrane-associated subcomplexes after which docked vesicles are fused to the plasma membrane through SNARE-mediated fusion machinery (He and Guo 2009). Recent studies have suggested that exocysts carry an open-handed conformation composed of a stable rod-shaped complex and may undergo conformational change in response to various stimuli (Heider *et al.* 2016; Picco *et al.* 2017).

In sporulating cells, initiation of prospore membrane formation requires tethering and fusion of post-Golgi vesicles at the MOP during the onset of meiosis II. Sec4 Rab GTPase was originally thought to mediate the docking and fusion of post-Golgi vesicles at the MOP to form a new prospore membrane (Neiman 1998). Indeed, *sec4* temperature-sensitive mutants (*sec4ts*) have been reported to show a sporulation-defective phenotype that fails to form the prospore membrane at restrictive temperature (Neiman 1998) and GFP-Sec4 localises adjacent to the MOP in an *SSO1* deletion mutant (*SSO1* encodes the plasma membrane t-SNARE) resulting in the accumulation of vesicles on the MOP surface (Mathieson *et al.* 2010). However, in spite of these results, the regulation of Sec4 GTPase activity during sporulation remains unclear. The GEF domain of Sec2, which is required for exchange activity on Sec4, resides at the N-terminus of Sec2 (1–160 amino acids) and its remaining 590 amino acids are important for associating with secretory vesicles through affinity for upstream Rab GTPase, Ypt32 and phosphatidylinositol 4-phosphate (PI4P), the latter two existing specifically on the vesicles (Ortiz *et al.* 2002; Medkova *et al.* 2006). A temperature-sensitive allele, sec2-59, which lacks the C-terminal vesicle associating domain but contains an intact GEF domain, did not show any sporulation defects at restrictive temperature (Yang and Neiman 2010; A Neiman personal communication). Moreover, our previous observation has shown that the function of the MOP is more than a scaffold for vesicle fusion as Mpc54, one of the MOP components, is required for the recruitment of Sec4 and its effector, exocyst proteins (Mathieson *et al.* 2010). These findings suggest that the Sec4-mediated vesicle fusion machinery in sporulating cells differs from that of vegetatively growing cells. Furthermore, in *Schizosaccaharomyces pombe*, one of the MOP components, Spo13, functions as a GEF for Ypt2 which is equivalent to Sec4 in *S. cerevisiae* (Yang and Neiman 2010). However, there is no homology between Spo13 and any component of the MOP in *S. cerevisiae*.

To elucidate Sec4 regulation during sporulation, we analysed the function of Sec2 during sporulation by constructing a conditional mutant in which *SEC2* expression was controlled by use of the developmental stage-specific promoter *CLB2* (Lee and Amon 2003; Pablo-Hernando *et al.* 2007). Sec2-depleted cells were defective in sporulation though meiotic progression was normal. Further analysis revealed that membrane formation during sporulation was aberrant possibly due to insufficient supply of membrane materials required for prospore membrane formation that would progress to defects in the formation of the spore wall's elaborate structure. The region required for vesicle targeting within Sec2 was important for prospore membrane localisation but was not required for prospore membrane formation. Altogether, the control of Sec4 GTPase by its GEF Sec2 is indispensable for both prospore membrane and spore wall formation during sporulation.

MATERIAL AND METHODS

Yeast strains and media

Standard methods and media were used (Burke, Dawson, Sterns). The strains used in this study are listed in Table 1. All strains in this study were derived from the fast-sporulating SK-1 strain background (Kane and Roth 1974). Gene disruption and

Table 2. Plasmids used in this study.

Name	Description	Source
pFA6a-natNT2-CLB2pr-3HA	PCR template	This study
pRS424-SEC2	*TRP1*, 2μ, *SEC2*	This study
pRS424-SEC2^{L104A}	*TRP1*, 2μ, *SEC2* ^{L104A}	This study
pRS424-SEC4	*TRP1*, 2μ, *SEC4*	This study
pAFS91	*URA3*, integration, *HIS3pr-GFP-TUB1*	(Straight *et al.* 1997)
pRS424-R20	*TRP1*, 2μ, *mRFP-SPO20*$^{51-91}$	(Suda *et al.* 2007)
pRS426-R20	*URA3*, 2μ, *mRFP-SPO20*$^{51-91}$	(Nakanishi *et al.* 2007)
mod_pRS303-P$_{TEF1}$-mKate2-SPO20$^{51-91}$	*HIS3*, integration, *mKate2-SPO20*$^{51-91}$	(Nakamura *et al.* 2017)
pRS304-MPC54-RFP	*TRP1*, integration, *MPC54-RFP*	(Suda *et al.* 2007)
pRS314-TDH3pr-GFP-SEC2	*TRP1*, *CEN*, *TDH3pr-GFP-SEC2*	This study
pRS314-ADH1pr-GFP-SEC4	*TRP1*, *CEN*, *ADH1pr-GFP-SEC4*	This study
pRS424-SPO20pr-GFP-SSO1+3′	*TRP1*, 2μ, *SPO20pr-GFP-SSO1+3′*	(Nakanishi *et al.* 2007)
pRS414-TEF2pr-DTR1-GFP	*TRP1*, *CEN*, *TEF2pr-DTR1-GFP*	(Nakanishi *et al.* 2007)
pRS424-SPO20pr-GFP-SEC15	*TRP1*, 2μ, *SPO20pr-GFP-SEC15*	This study
pRS424-SPO20pr-GFP-SEC2	*TRP1*, 2μ, *SPO20pr-GFP-SEC2*	This study
pRS424-SPO20pr-GFP-SEC2$^{1-160}$	*TRP1*, 2μ, *SPO20pr-GFP-SEC2*$^{1-160}$	This study
pRS424-SPO20pr-GFP-SEC2$^{1-374}$	*TRP1*, 2μ, *SPO20pr-GFP-SEC2*$^{1-374}$	This study
pRS424-SPO20pr-GFP-SEC2$^{1-508}$	*TRP1*, 2μ, *SPO20pr-GFP-SEC2*$^{1-508}$	This study
pRS314-SEC2-FLAG-6xHIS	*TRP1*, *CEN*, *SEC2-FLAG-6xHIS*	This study
pRS314-SEC2$^{1-160}$-FLAG-6xHIS	*TRP1*, *CEN*, *SEC2*$^{1-160}$*-FLAG-6xHIS*	This study
pRS314-SEC2$^{1-160, L104A}$-FLAG-6xHIS	*TRP1*, *CEN*, *SEC2*$^{1-160, L104A}$*-FLAG-6xHIS*	This study
pRS314-SEC2$^{1-374}$-FLAG-6xHIS	*TRP1*, *CEN*, *SEC2*$^{1-374}$*-FLAG-6xHIS*	This study
pRS314-SEC2$^{1-508}$-FLAG-6xHIS	*TRP1*, *CEN*, *SEC2*$^{1-508}$*-FLAG-6xHIS*	This study

insertion were done by PCR-mediated gene replacement (Longtine *et al.* 1998) and verified by PCR. The PCR cassettes of kanMX6-CLB2pr-3HA or natNT2-CLB2pr-3HA were amplified from pRK69 (Kamieniecki, Liu and Dawson 2005) or pFA6a-natNT2-CLB2pr-3HA and inserted at the 5' end of the ORF of *SEC2* both in AN117-4B or AN117–16D and resulting haploids were mated to generate YSY38-2 and YSY57, respectively.

Plasmids

The plasmids used in this study are listed in Table 2. Briefly, pFA6a-natNT2-CLB2pr-3HA was generated by the replacement of kanMX6 in pRK69 with natNT2 from pFA6a-natNT2 (Janke *et al.* 2004), and pRS424-SEC2 was made by cloning of DNA fragments, including *SEC2* ORF and its 5', 3' UTR region, into the EcoRI-XhoI site of pRS424. For pRS424-SEC4, construction was similar to pRS424-SEC2. L104A substitution in pRS424-SEC2 was generated by homologous recombination in yeast cells. The promoter region of *ADH1* and the 3' UTR of *CYC1* were amplified from gDNA of YPH499 while the GFP coding sequence amplified from pFA6a-GFP-kanMX6 was inserted at the SacI-XbaI, XhoI-KpnI and XbaI-SpeI sites of pRS314 to generate pRS314-ADH1pr-GFP-Nterm. *SEC4* was amplified and cloned into the SpeI-XhoI site of pRS314-ADH1pr-GFP-Nterm to generate pRS314-ADH1pr-GFP-SEC4. The promoter region of *SPO20* and the 3' UTR of *CYC1* were amplified from gDNA of YPH499 and GFP coding sequence amplified from pFA6a-GFP-kanMX6 was inserted at the SacI-XbaI, XhoI-KpnI and XbaI-SpeI sites of pRS424 to generate pRS424-SPO20pr-GFP-Nterm. *SEC15* was amplified and cloned into the SpeI-PstI site of pRS424-SPO20pr-GFP-Nterm to generate pRS424-SPO20pr-GFP-SEC15. *SEC2* truncations were amplified and similarly cloned into pRS424-SPO20pr-GFP-Nterm or pRS314-FLAG-6xHIS to generate pRS424-SPO20pr-GFP-SEC2, -SEC2$^{1-160}$, -SEC2$^{1-374}$ and -SEC2$^{1-508}$ as well as pRS314-SEC2-FLAG-6xHIS, -SEC2$^{1-160}$-FLAG-6xHIS, -SEC2$^{1-160, L104A}$-FLAG-6xHIS, -SEC2$^{1-374}$-FLAG-6xHIS and -SEC2$^{1-508}$-FLAG-6xHIS. *GFP-SEC2* was cloned into pRS314 under the control of the *TDH3* promoter.

Sporulation assays

Induction of sporulation was performed essentially as described previously (Neiman 1998). Sporulation efficiency was determined by visualisation of DNA with 4′6-diamidino-2-phenylindole (DAPI) and under a light microscope. For ethanol testing, strains were sporulated at 30˚C and incubated in 30% ethanol for 40 min before plating onto YPD (1% yeast extract, 2% peptone, 2% dextrose) plates.

Microscopy

Differential interference contrast (DIC) images were obtained by using a BX52 microscope (Olympus, Japan) with an Orca ER CCD camera (Hamamatsu Photonics, Japan) and processed using iVision-Mac software (BioVision Technologies, Exton, PA). Confocal fluorescence microscopy was performed using super-resolution confocal live imaging microscopy that we developed by combining a spinning-disk confocal scanner (Yokogawa Electric, Japan), cooled image intensifiers (Hamamatsu Photonics, Japan) and EM-CCD cameras (Hamamatsu Photonics, Japan). Deconvolution was performed using Volovity software (Perkin Elmer, MA). Conventional fluorescence microscopy was performed using a BZ-X710 (Keyence, Japan) and as previously described (Ishihara *et al.* 2009). Electron microscopy for sporulating cells were performed essentially as described previously (Okamoto *et al.* 2012).

Immunoblotting

Protein analysis of 3xHA-Sec2 was conducted by western blotting. Cells were induced to sporulate and total protein was prepared by bead beating in 1x Laemmli buffer. Proteins were

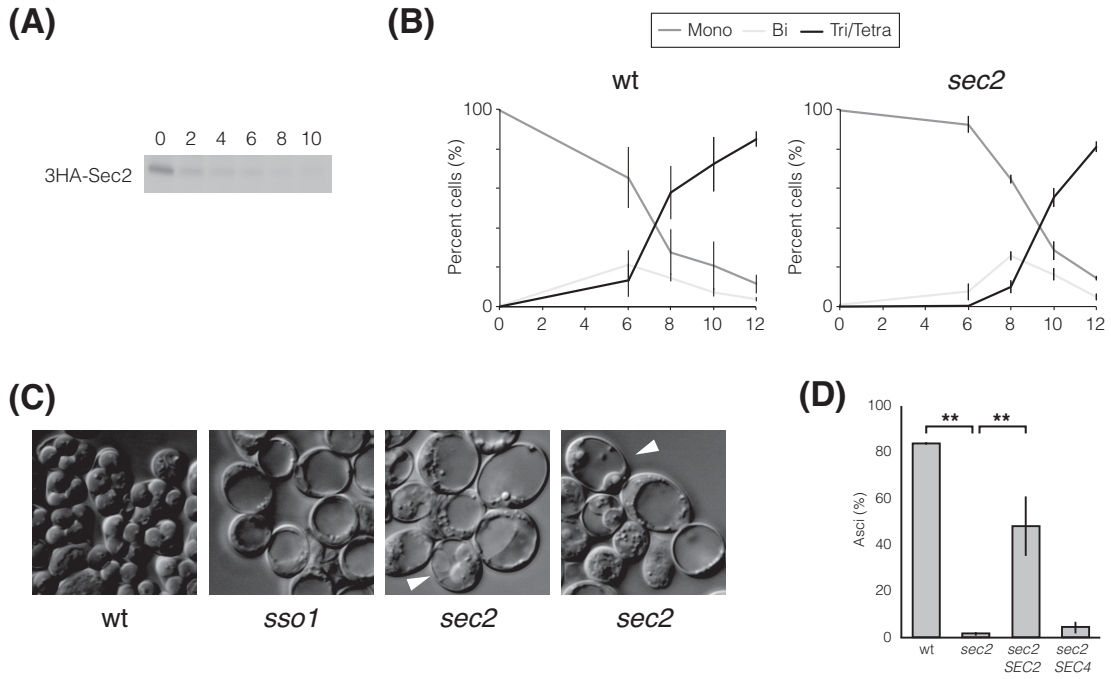

Figure 1. *CLB2pr-SEC2* exhibits defects in sporulation. (**A**) *CLB2pr-SEC2* cells were sporulated and 3HA-Sec2 proteins were detected by immunoblotting. The time after the transfer to sporulation medium are shown in hours. (**B**) Meiotic progression of the cells was observed by DAPI staining. Dark grey, light grey and black lines represent mono-, bi- and tri/tetra-nucleate cells, respectively. More than 200 cells were tested in three independent experiments. Horizontal lines at each time points represent standard error. (**C**) Bright field images of spores in wild-type, *sso1* and *CLB2pr-SEC2* are shown. Arrowheads indicate immature spores. (**D**) Wild-type, *CLB2pr-SEC2* and *CLB2pr-SEC2* expressing either *SEC2* or *SEC4* were sporulated for 24 h and observed by DIC microscopy. Sporulation efficiency is shown as the mean ± the standard deviations. More than 100 cells were examined in three independent experiments. **, $P < 0.01$ (Tukey–Kramer test).

analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by western blotting with anti-HA antibody (12CA5). Bands were visualised by horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G secondary antibody and a luminol-based system (GE Healthcare, UK).

RESULTS

Sec2 is required for sporulation

A previous study revealed that the temperature-sensitive *sec2-59* mutant does not show any defects in sporulation at restrictive temperature (Yang and Neiman 2010). However, to address the question of the requirement of Sec2 for the formation of the prospore membrane and sporulation, we constructed a conditional mutant in which the upstream promoter region of *SEC2* was replaced with the *CLB2* promoter that allows the normal expression of downstream *SEC2* in vegetative growth but represses it during sporulation. In this conditional mutant (*CLB2pr-SEC2*), Sec2 expression was significantly reduced after 2 h and undetected after 6 h of incubation in the sporulation medium (Fig. 1A). Meiotic progression in this mutant was confirmed using DAPI staining. Although *CLB2pr-SEC2* cells showed delayed entry into meiosis I and II compared to wild-type cells, tetra-nucleated cell formation efficiency was almost comparable (Fig. 1B). Spore morphology of *CLB2pr-SEC2* cells was analysed via light microscopy and although the mature spore was clearly observed in wild-type cells and no apparent spores were seen in *sso1* cells, only a slight number of immature monads and dyads were observed in *CLB2pr-SEC2* cells (Fig. 1C). Expression of Sec2 rescued the sporulation defect of *CLB2pr-SEC2* cells (Fig. 1D, 48.1% sporulation by SEC2 versus 1.7% sporulation by empty vector). We could not detect any suppression by *SEC4* expression (4.4% sporulation). These results suggest that *SEC2* expression during sporulation is required for spore formation.

CLB2pr-SEC2 causes a spore wall-defective phenotype

Given that *CLB2pr-SEC2* cells formed a slight number of immature spores, we analysed whether the deposition of spore wall materials in these cells is defective or not. If spores are properly formed and rigid spore walls are constructed, spores show a resistance to ethanol. To this end, equal numbers of cells with spores from wild-type and *CLB2pr-SEC2* cells were exposed to ethanol. Spores from *CLB2pr-SEC2* cells were sensitive to ethanol and did not survive (Fig. 2A), suggesting that immature spores observed in *CLB2pr-SEC2* cells are defective in spore wall deposition. Next, we confirmed the spore wall defect observed in *CLB2pr-SEC2* cells directly by transmission electron microscopy (TEM). Wild-type and *CLB2pr-SEC2* cells were induced to sporulate for 24 h and were subjected to TEM analysis. In wild-type cells, a thick spore wall layer was present, confirming that cells formed mature spores (Fig. 2B, wt). However, in *CLB2pr-SEC2* cells, the morphologies of the spore wall within the ascus were aberrant. An example of these aberrant spore walls is shown in Fig. 2B in which two immature spores, one with relatively thin layers of the spore wall and the other with almost complete loss of spore wall materials, are formed (Fig. 2B, *sec2*). These observations suggest that the sporulation defect in *CLB2pr-SEC2* cells is related, at least in part, to the defect in spore wall deposition.

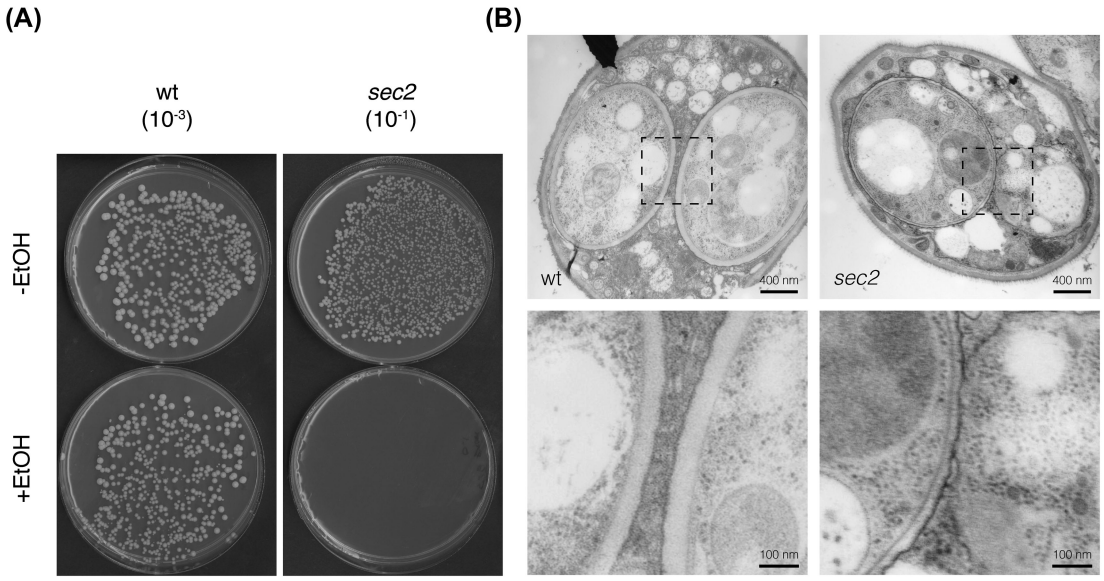

Figure 2. Spore wall defect in *CLB2pr-SEC2*. (**A**) Wild-type and *CLB2pr-SEC2* were sporulated and equal number of the spores, which was calculated by sporulation efficiency, were treated with 30% ethanol and plated onto YPD. Numbers represent OD_{600} unit cells used in this experiment. (**B**) Representative examples of TEM image of asci in wild-type and *CLB2pr-SEC2*. Higher magnification of images shown in the upper panels is shown in the lower panels.

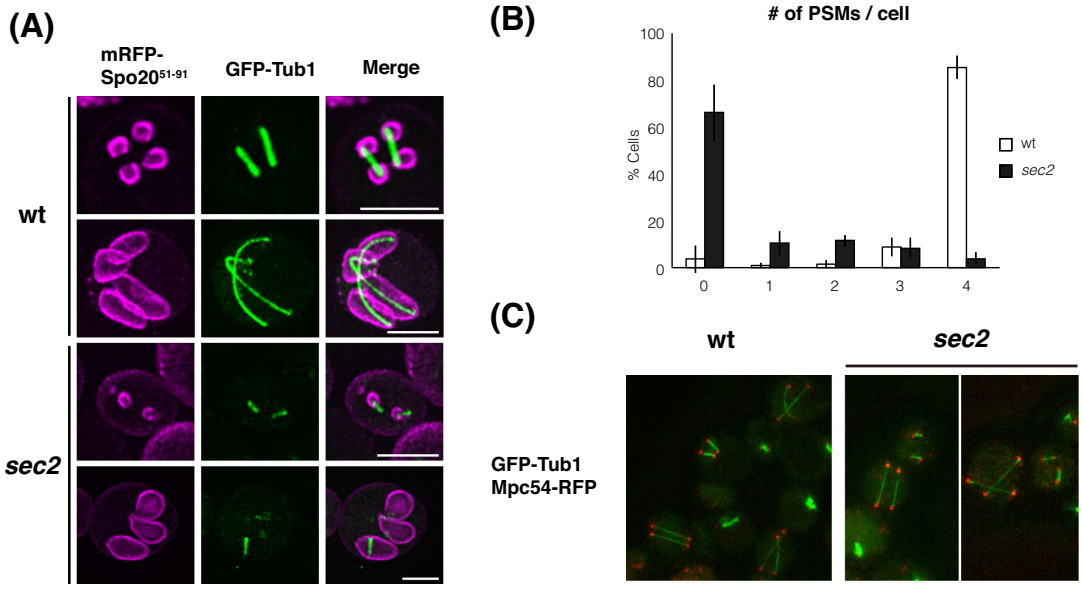

Figure 3. Prospore membrane formation defect in *CLB2pr-SEC2*. (**A**) Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ and GFP-Tub1 were sporulated and observed by confocal microscopy for prospore membrane and meiotic spindle. (**B**) Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ were sporulated and DNA was visualised by DAPI. The numbers of prospore membrane per cells were counted and distribution of the cells is shown as the mean ± the standard deviations. More than 100 cells were examined in four independent experiments. (**C**) Wild-type and *CLB2pr-SEC2* cells expressing Mpc54-RFP and GFP-Tub1 were sporulated and observed for MOP and spindle. Scale bars, 4 μm.

Prospore membrane formation was aberrant in *CLB2pr-SEC2*

Analogous to the fusion defect of post-Golgi vesicles to the plasma membrane in *sec2ts* and *sec4ts* (Salminen and Novick 1987; Nair *et al.* 1990), fusion defects in vesicles at the MOP of the SPB in *CLB2pr-SEC2* could occur, leading to the defect of prospore membrane formation indicative of sporulation deficiency. Therefore, we examined whether prospore membranes are properly formed in the *CLB2pr-SEC2* mutant. The prospore membrane was visualised by the expression of a Spo20 protein fragment that includes an amphipathic helical domain fused to mRFP both in wild-type and *CLB2pr-SEC2* cells. In wild-type cells, prospore membranes were formed at the end of the meiotic spindles (where the MOP is assembled) and were then progressively shaped as horseshoe-like, tubular and round morphologies (Ishihara *et al.* 2009; Fig. 3A, wt). In *CLB2pr-SEC2* cells, however, prospore membrane growth was aberrant and the number of prospore membranes per ascus was reduced (Fig. 3A, *sec2*). Counting of the prospore membrane in tetra-nucleated cells stained with DAPI showed that more than 80% of wild-type cells have four prospore membranes per cell, but only 10% of

CLB2pr-SEC2 cells could form four prospore membranes per ascus (Fig. 3B).

Prospore membrane formation defects observed in *CLB2pr-SEC2* cells prompted us to examine the assembly of the MOP because it has been reported to be a prerequisite for prospore membrane formation (Neiman 2005). Mpc54-RFP was expressed together with GFP-Tub1 both in wild-type and *CLB2pr-SEC2* cells and their localisation during sporulation was determined by fluorescence microscopy. In 94.3% of *CLB2pr-SEC2* cells, Mpc54-RFP signals were found to localise to the ends of each meiotic microtubule (>2 μm), comparable to wild-type cells (94.4%; Fig. 3C), suggesting that the MOP is properly assembled in this mutant. These results suggest that the *SEC2* expression during sporulation is important for prospore membrane formation.

Vesicle fusion machinery at the plasma membrane facilitates prospore membrane formation in sporulating cells

Rab GTPase Sec4 and its GEF Sec2 are targeted to the growing site of the plasma membrane for the fusion of post-Golgi vesicles during vegetative growth in *Saccharomyces cerevisiae* (Walch-Solimena, Collins and Novick 1997; Ortiz *et al.* 2002; Medkova *et al.* 2006). It was suggested that prospore membrane formation is driven by the fusion of precursor vesicles and that Sec4 functions in this early event of prospore membrane formation (Neiman 1998; Mathieson *et al.* 2010). Therefore, we examined the localisation of Sec2 and Sec4 in sporulating cells. Sec2-GFP and GFP-Sec4 were expressed both in wild-type and *CLB2pr-SEC2* cells and their localisation was observed by fluorescence microscopy. Sec2-GFP was functional because expression of Sec2-GFP rescued the sporulation defect of *CLB2pr-SEC2* cells (data not shown). Sec2-GFP was localised beneath the prospore membrane in the middle and late phase of sporulation in wild-type cells (Fig. 4A). GFP-Sec4 signals were also seen near the prospore membrane as marked by mRFP-fusion of Spo20$^{51-91}$ in wild-type cells whereas its localisation in *CLB2pr-SEC2* cells was diffused all over the cells and only faint signals were occasionally seen near the prospore membrane (Fig. 4B and Fig. S1A, Supporting Information).

Sec15, an exocyst component and a downstream effector of Sec4, has been reported to localise at the site of vesicle fusion for the prospore membrane (Mathieson *et al.* 2010). Thus, we next analysed whether observed Sec4-targeting defects in *CLB2pr-SEC2* cells could also affect its downstream effector Sec15. GFP-Sec15 signal was found on and around both the growing and rounded prospore membrane marked by mRFP-fusion of Spo20$^{51-91}$ in wild-type cells. By contrast, the signal was reduced on the growing prospore membrane and was not obvious at the rounded prospore membrane in *CLB2pr-SEC2* cells (Fig. 5 and Fig. S1B, Supporting Information).

These observations suggest that the vesicle fusion machinery at the plasma membrane in vegetatively growing cells similarly facilitates prospore membrane formation in sporulating cells and that failures in vesicle fusion machinery might result in the observed defects in *CLB2pr-SEC2* cells.

Cargo targeting is required for prospore membrane formation in *CLB2pr-SEC2* cells

The defects in *CLB2pr-SEC2* cell fusion machinery caused sporulation blockage with a reduced number of cells forming a prospore membrane. This led us to examine whether known

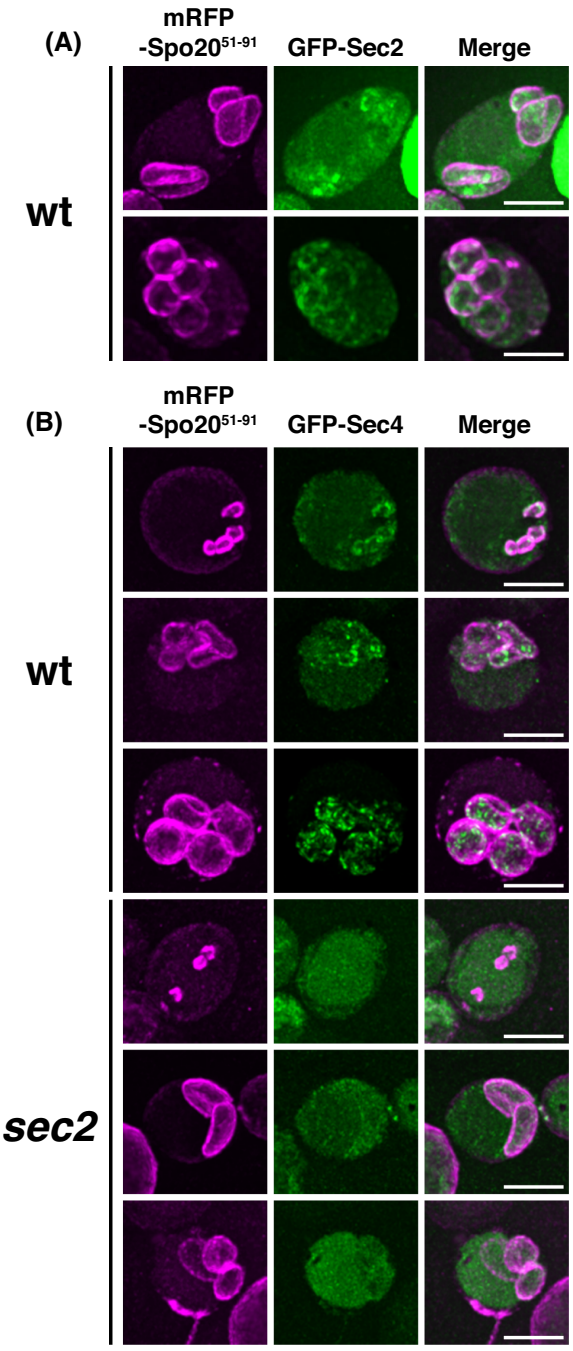

Figure 4. Localisation of Sec2 and Sec4 during sporulation. (**A**) Wild-type cells expressing mRFP-Spo20$^{51-91}$ and GFP-Sec2 were sporulated and observed by confocal microscopy. Representative cell is shown. (**B**) Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ and GFP-Sec4 were sporulated and observed by confocal microscopy. Scale bars, 4 μm.

cargo proteins required for prospore membrane formation and/or spore wall deposition are precisely targeted to the site of vesicle fusion.

The t-SNARE Sso1 and dityrosine transporter Dtr1 have been reported to localise onto the prospore membrane and to be required for the fusion of vesicles to make a SNARE complex with Spo20-Snc1/2 and for the translocation of spore wall component dityrosine through the prospore membrane (Neiman, Katz and Brennwald 2000; Felder *et al.* 2002). These proteins

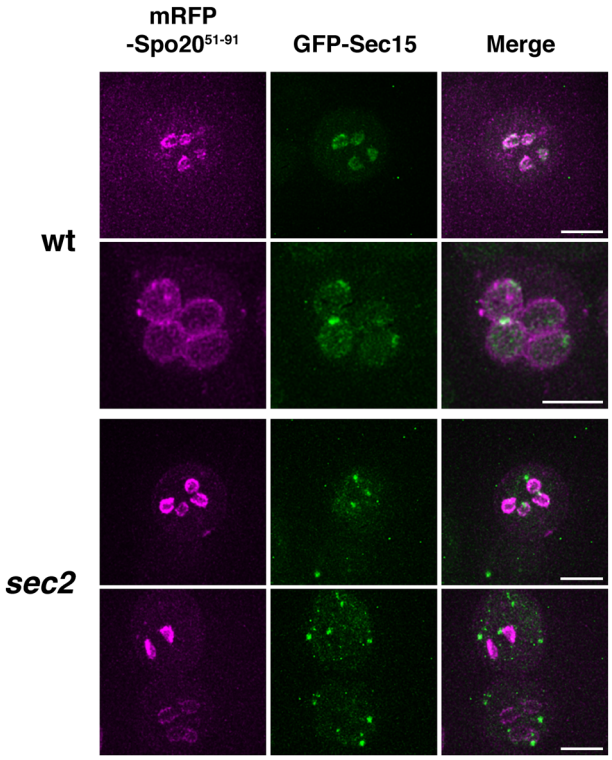

Figure 5. Targeting defect of Sec15 in *CLB2pr-SEC2*. Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ and GFP-Sec15 were sporulated and observed by confocal microscopy. Scale bars, 4 μm. Representative examples of localisation of GFP-Sec15 and mRFP-Spo20$^{51-91}$ in wild-type and *CLB2pr-SEC2* are shown.

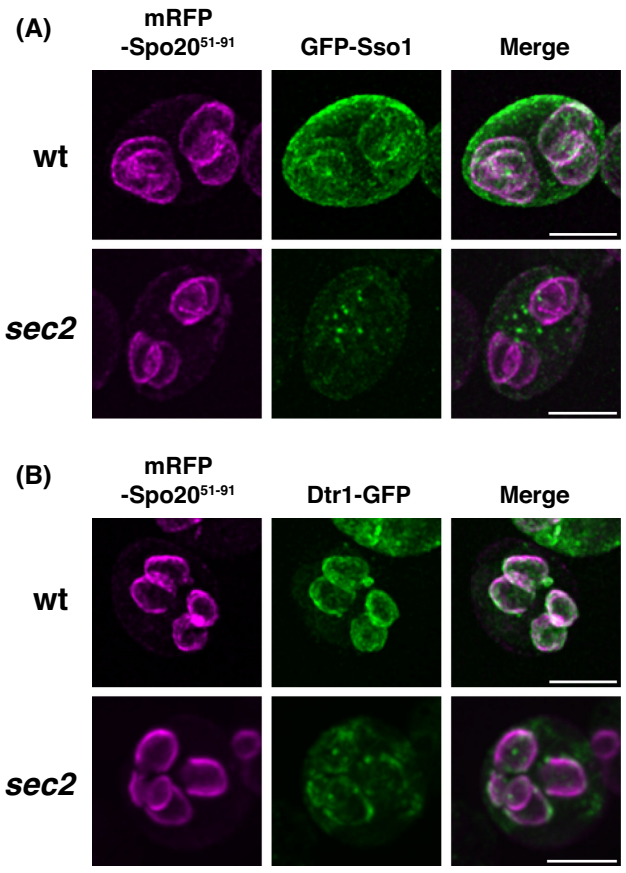

Figure 6. Localisation of cargos in *CLB2pr-SEC2*. (**A**) Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ and GFP-Sso1 were sporulated and observed. (**B**) Wild-type and *CLB2pr-SEC2* cells expressing mRFP-Spo20$^{51-91}$ and Dtr1-GFP were sporulated and observed. Scale bars, 4 μm.

were expressed as GFP fusions and their localisation was examined in wild-type and *CLB2pr-SEC2* cells. As reported previously, GFP-Sso1 and Dtr1-GFP in wild-type cells were enriched in the prospore membrane which was marked with mRFP fusion-tagged Spo20$^{51-91}$ (Fig. 6A and B, wt). In *CLB2pr-SEC2* cells, the signals for GFP-Sso1 and Dtr1-GFP were relatively weak, and in some cases the signal was not observed in the prospore membranes. (Fig. 6A and B, *sec2*). Similar results were obtained in *CLB2pr-SEC2* cells that showed defects in the formation of four prospore membranes per ascus (Fig. S2A and B, Supporting Information). Although observed weak signals for these cargo proteins could be due to the reduced level of proteins, it might reflect defects in efficient delivery of these proteins toward the prospore membrane. These results suggest that the important function of Sec2 is in the proper formation of prospore membrane, possibly through the activation of Sec4 GTPase, and any anomalies in this would presumably translate to spore wall deposition defects.

Functional analysis of Sec2 domains in spore formation

Regions of Sec2 required for interaction with upstream Rab GTPase, Ypt32 and PI4P, both of which are important for Sec2 localisation onto the post-Golgi vesicles, lie downstream of the GEF domain (Ortiz *et al.* 2002; Medkova *et al.* 2006; Mizuno-Yamasaki *et al.* 2010). Yang and Neiman have shown that a C-terminal deletion of Sec2 lacking these regions was able to confer sporulation, suggesting that the GEF domain of Sec2 is sufficient to develop spore formation (Yang and Neiman 2010). Thus, we extended our analysis to determine Sec2 domains required for its localisation during sporulation and for the suppression of sporulation defects observed in *CLB2pr-SEC2* cells. Various Sec2-deletions were therefore expressed from CEN-based, low copy number plasmids in cells. We found that the expression of Sec2$^{1-160}$ was sufficient to suppress the sporulation defects in *CLB2pr-SEC2* cells at a level equivalent to that of full-length Sec2 (Fig. 7A, 30.6% sporulation by full-length Sec2 versus 31.2% sporulation by Sec2$^{1-160}$). Introduction of an L104A substitution that has been reported to reduce GEF activity (Sato *et al.* 2007) to Sec2$^{1-160}$ showed no suppression of sporulation defects in *CLB2pr-SEC2* cells (Fig. 7A, 6.9% by Sec2$^{1-160, L104A}$). Unfortunately, no apparent signal was detected for these GFP-fused constructs during sporulation. When these constructs were expressed from a 2 μm-based plasmid, full-length Sec2 only showed a faint prospore membrane pattern but an apparent prospore membrane pattern was observed for GFP-Sec2$^{1-508}$ (Fig. 7B). Further C-terminal deletions of Sec2, GFP-Sec2$^{1-374}$ and GFP-Sec2$^{1-160}$ that lack PI4P or Ypt32 binding showed diffuse cytoplasmic patterning in tetra-nucleated cells. These results suggest that the GEF activity, but not the membrane-targeting of Sec2, is indispensable to drive prospore membrane formation.

DISCUSSION

A previous study proposed the involvement of the MOP in exocyst recruitment and prospore membrane formation (Mathieson *et al.* 2010) but details of the post-Golgi vesicular machinery for membrane fusion remained unclear to this point. The lack of

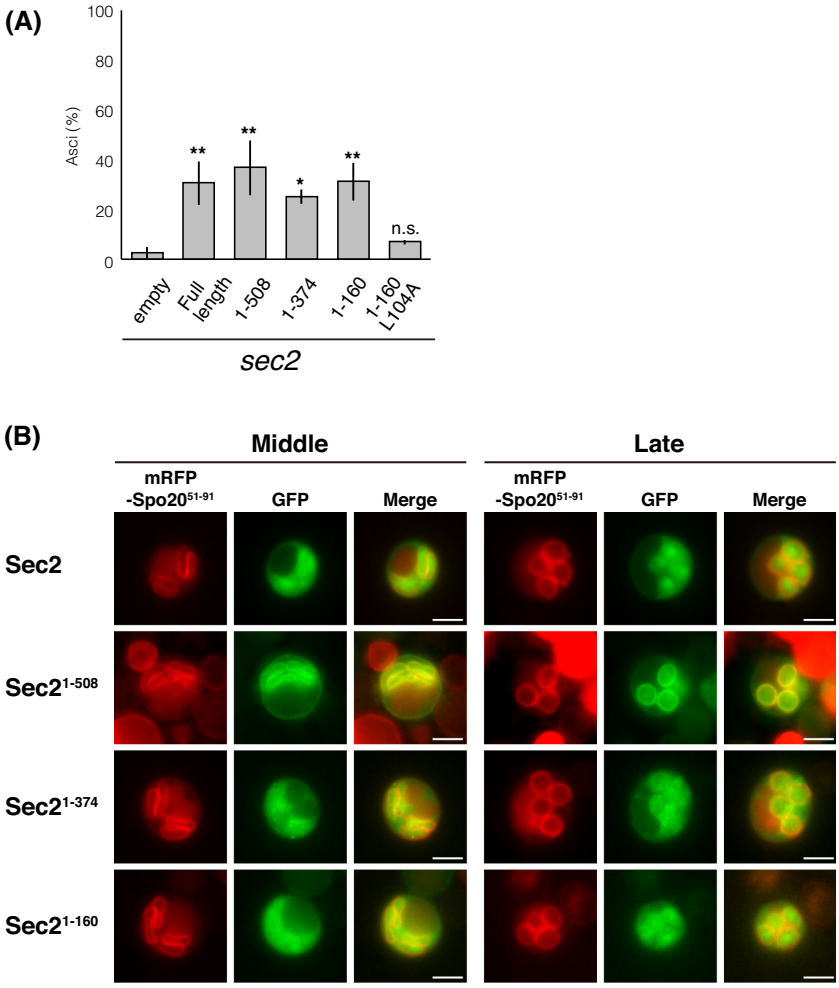

Figure 7. Analysis of Functional domain of Sec2. (**A**) *CLB2pr-SEC2* expressing CEN-Sec2-deletion constructs were sporulated for 24 h and observed by microscopy. Sporulation efficiency is shown as the mean ± the standard deviations. More than 200 cells were examined in three independent experiments. **, $P < 0.01$, *, $P < 0.05$, n.s., not significant (Tukey–Kramer test). (**B**) Wild-type and *CLB2pr-SEC2* cells expressing GFP-fusion Sec2 deletions from 2μ plasmid and mRFP-Spo20$^{51-91}$ were sporulated and observed. Scale bars, 4 μm.

sporulation defects in *sec2ts* led us to construct a conditional mutant of Sec2 and examine Sec4 regulation during sporulation. Here, we provide evidence that Sec2-mediated Sec4 activation is required for prospore membrane formation.

Phenotype of *CLB2pr-SEC2*

In vegetative and sporulating cells, failures in post-Golgi fusion result in the accumulation of vesicles around the vesicular fusion site, visible as clustering of vesicles in the buds of *sec2ts* and *sec4ts* at restrictive temperature and numerous vesicles clustered on the MOP structure in *sso1* during sporulation (Salminen and Novick 1987; Nair *et al.* 1990; Nakanishi *et al.* 2006). However, in our *SEC2* shut-off mutant, we could not detect the accumulation of vesicles around the MOP structure under fluorescent microscopy. Instead, we observed a partial defect in prospore membrane formation, the phenotypic consequence of which might be caused by incomplete transcription shut off of *SEC2* or a relatively longer half-life of the Sec2 protein. Alternative approaches such as the auxin-inducible degron could be useful to avoid ambiguities in the analysis of essential genes during sporulation (Nishimura *et al.* 2009). Nonetheless, as numbers of prospore membranes per ascus apparently decreased in *CLB2pr-SEC2*, Sec2 might be implicated in the initiation of prospore membrane formation. Another possibility for the partial defect of *CLB2pr-SEC2* is that the vesicular targeting mechanisms mediated by Sec2-Sec4 likely contribute to, but are not essential for, prospore membrane formation.

Our previous study revealed that the MOP functions in the process of vesicular fusion and is required, at least in part, for the recruitment of the exocyst to the prospore membrane formation site (Mathieson *et al.* 2010). In the present study, Sec15, an exocyst component, was unable to localise properly to observed prospore membranes in *CLB2pr-SEC2*. Thus, Sec2-Sec4 also contributes to the recruitment of exocysts to the vesicular fusion site during prospore membrane formation, and since the MOP is covered with the nascent prospore membrane after its initial formation, it is possible that exocyst recruitment is mediated firstly by the MOP then followed up by Sec2-Sec4. In any case, detailed mechanisms for each component of the exocyst complex in the formation of the prospore membrane are next to be clarified.

Normally, simultaneous progression of the formation of four prospore membranes should be achieved within the cells wherein septin structure and leading edge complex may func-

tion as coordinators. Destabilisation of these components has been shown to cause defects in prospore membrane growth, leading to a reduced number of prospore membranes (Moreno-Borchart *et al.* 2001; Lam *et al.* 2014; Heasley and McMurray 2016). As a similar defect was observed in *CLB2pr-SEC2*, it could be argued that sufficient vesicular supply ensures the proper growth of prospore membranes. In this scenario, certain cargo proteins, including Sso1, could be inefficiently delivered thus shifting at least some of the blame to intracellular logistics.

Requirement of Sec2 activity for prospore membrane formation

The *CLB2pr-SEC2* mutant showed partial sporulation defects with a slight number of immature spores. Observed sporulation defects were suppressed by the expression of Sec2 but not by Sec4, implying that *SEC2* transcriptional shut-off resulted in an insufficient threshold of Sec2 proteins to activate Sec4. Another possibility is that Sec2 could have functions other than as a GEF for Rab GTPase. Recent observation of Rabin8, the mammalian homolog of Sec2, has shown a GEF activity-independent role for neurite outgrowth (Homma and Fukuda 2016). However, our analysis using various deletion constructs of Sec2 revealed that C-terminal truncations of Sec2 could suppress defects regardless of their localisation, and the introduction of a GEF activity-deficient mutation abolished construct suppression. These findings suggest that Sec2 GEF activity is essential for sporulation and that requirements for a proper cellular distribution of this activity might not be completely strict. Diverse functions of Sec2 homologs in higher eukaryotes could have therefore been acquired due to selective evolutionary pressures.

Sec2-Sec4 localisation during prospore membrane formation

During prospore membrane growth, fluorescence signals for Sec2 and Sec4 accumulated inside of growing membranes, reminiscent of our previous observation in which the Golgi-localised proteins predominantly segregate into the space in between the nuclear membrane and prospore membrane from the early onset of meiosis II (Suda *et al.* 2007). Although we have only observed Sec2 and Sec4 in overexpression conditions, these findings may provide evidence that the post-Golgi vesicles could be segregated into the forming prospore. Detailed future analyses from super resolution microscopy, such as structure illumination microscopic systems, could help to elucidate the site of vesicle fusion for the prospore membrane.

Spore wall defect and cargo delivery

Immature spores found in *CLB2pr-SEC2* cells indicate that the strain is defective in construction of rigid spore walls and this was confirmed by the ethanol sensitivity. Further electron microscopic observation showed heterogeneity in deposition failures of spore wall materials within the *CLB2pr-SEC2* asci. Several proteins required for spore wall deposition were reported as possible cargo proteins in the post-Golgi vesicles (Neiman 2011) and although their deliveries toward the prospore membrane could be affected, we found that they were not significantly disturbed in *CLB2pr-SEC2*. Thus, the characteristic spore wall defects in *CLB2pr-SEC2* cells might be the logistical consequence of inefficient membrane material supply to the prospore membrane.

Collectively, our analysis suggests that Sec2 and its cognate Rab Sec4, the key components for membrane trafficking of post-Golgi vesicles, are required for prospore membrane formation. Although these details await clarification together with the observation of Rab GTPase function in the formation of forespore membrane in fission yeast sporulation and ciliary membrane formation in ciliogenesis (Chiba *et al.* 2013; Imada and Nakamura 2016), our work will contribute to the understanding of molecular mechanisms for *de novo* membrane formation within the cells.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflict of interest. None declared.

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