Erv14 family cargo receptors are necessary for ER exit during sporulation in *Saccharomyces cerevisiae*

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Summary

Sporulation of *Saccharomyces cerevisiae* is a developmental process in which four haploid spores are created within a single mother cell. During this process, the prospore membrane is generated de novo on the spindle pole body, elongates along the nuclear envelope and engulfs the nucleus. By screening previously identified sporulation-defective mutants, we identified additional genes required for prospore membrane formation. Deletion of either *ERV14*, which encodes a COPII cargo receptor, or the meiotically induced *SMA2* gene resulted in misshapen prospore membranes. Sma2p is a predicted integral membrane that localized to the prospore membrane in wild-type cells but was retained in the ER in *erv14* cells, suggesting that the prospore membrane morphology defect of *erv14* cells is due to mislocalization of Sma2p. Overexpression of the *ERV14* paralog *ERV15* largely suppressed the sporulation defect in *erv14* cells. Although deletion of *ERV15* alone had no phenotype, *erv14 erv15* double mutants displayed a complete block of prospore membrane formation. Plasma membrane proteins, including the t-SNARE Sso1p, accumulated in the ER upon transfer of the double mutant cells to sporulation medium. These results reveal a developmentally regulated change in the requirements for ER export in *S. cerevisiae*.

Supplementary material available online at
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Key words: Prospore membrane, Yeast, Sporulation, ER, Erv14p

Introduction

In response to starvation, cells of the yeast *Saccharomyces cerevisiae* undergo meiosis and form spores (Byers, 1981). In this developmental process, each haploid nucleus is encapsulated by a newly generated double membrane, termed the prospore membrane (Moens and Rapport, 1971; Byers, 1981). After completion of prospore membrane formation, spore wall materials including β-glucan, chitosan and dityrosine are deposited into the lumen of the prospore membrane to generate mature spores (Lynn and Magee, 1970).

Prospore membrane formation is initiated at the onset of the Meiosis II on the cytosolic face of the spindle pole body (SPB) where precursor vesicles coalesce to make a double membrane structure (Neiman, 2005). During sporulation, plasma membrane proteins are delivered to the prospore membrane, indicating that Golgi-derived vesicles are targeted to the prospore membrane in this developmental stage (Moens and Rapport, 1971; Neiman, 1998). Much of the molecular machinery for secretory vesicle fusion at the plasma membrane also participates in prospore membrane extension (Neiman, 1998). Prospore membrane formation, however, also involves sporulation-specific proteins including a multiprotein complex formed on the SPB and a unique SNARE protein (Neiman, 1998; Knop and Strasser, 2000; Bajgier et al., 2001). One example is *SMA2,* a meiotically induced gene encoding a predicted multi-membrane-spanning protein. In sporulation, *sma2Δ* cells undergo meiotic nuclear divisions but cannot make visible spores (Rabitsch et al., 2001). The Don1 protein, which is normally found at the leading edge of the prospore membrane, is mislocalized in *sma2Δ* cells, suggesting that *sma2Δ* mutants have prospore membrane defects (Rabitsch et al., 2001).

Transmembrane proteins destined for the plasma membrane enter the secretory pathway at the endoplasmic reticulum (ER), and are then packaged into COPII vesicles for delivery to the Golgi apparatus (Antonny and Schekman, 2001). These secretory proteins are sorted from ER-resident proteins and selectively incorporated into the COPII vesicles (Barlowe, 2003). Erv14p is a transmembrane protein that cycles between the ER and early Golgi and is involved in COPII cargo selection (Powers and Barlowe, 1998; Powers and Barlowe, 2002). In the ER, Erv14p physically interacts with COPII components as well as its cargo protein Axl2p (Powers and Barlowe, 2002). Axl2p is an integral plasma membrane protein that is required for the axial budding pattern of haploid cells (Roemer et al., 1996). In the absence of Erv14p, Axl2p is not incorporated into COPII vesicles, resulting in the accumulation Axl2p in the ER. As a consequence of the failure of Axl2p to reach the cell surface, *erv14Δ* haploid cells display a non-axial budding pattern (Powers and Barlowe, 1998). An *ERV14* paralog *ERV15* is also present in the *S. cerevisiae* genome, although deletion of *ERV15* has no reported phenotype and overexpression of *ERV15* could not rescue the *erv14* budding defect (Powers and Barlowe, 1998). This family of ER cargo receptors is

evolutionarily conserved and in *Drosophila* an Erv14p family protein, Cornichon, is required for the delivery of a transforming growth factor (TGF)-α-like protein, Gurken, to the oocyte surface during oogenesis (Roth et al., 1995; Bokel et al., 2006).

Although *erv14Δ* haploid cells display a budding defect, *erv14Δ* diploid cells are defective in sporulation (Powers and Barlowe, 1998). This phenotype is not related to Axl2p transport, because overexpression of *AXL2* suppresses the budding but not the sporulation defect (Powers and Barlowe, 1998) and *axl2Δ/axl2Δ* cells have no sporulation deficiency (Enyenihi and Saunders, 2003). In this paper, we report that the sporulation defect of *erv14Δ* mutants is due to improper prospore membrane growth. This defect is partially rescued by overexpression of *ERV15,* and *erv14Δ erv15Δ* double mutant cells display a complete block to prospore membrane formation. Several proteins whose transport from the ER is independent of *ERV14* and *ERV15* during vegetative growth become trapped in the ER in the mutant cells during sporulation. These data indicate that the requirements for ER exit are altered during sporulation in yeast.

Results

Screening for prospore membrane formation mutants

To further our understanding of the molecular basis for prospore membrane formation, additional genes involved in this process were identified from among a collection of non-essential genes previously reported to be required for meiosis and sporulation (Enyenihi and Saunders, 2003). Deletions in 45 genes that were known to have severe sporulation defects but for which the basis for the defect had not yet been characterized were examined (Table 1). To visualize the prospore membrane, these deletion diploids were transformed with a plasmid expressing a green fluorescent protein (GFP) gene fusion to *SPO20*$^{51\text{-}91}$ (Nakanishi et al., 2004). The diploids were then sporulated and prospore membranes were observed by fluorescence microscopy. The criteria for a mutant in prospore membrane formation were that: (1) the cells grew well vegetatively; (2) the cells underwent meiosis efficiently; and (3) the cells displayed abnormal prospore membranes. Of the 45 mutants, eight met these criteria: *erv14Δ*, *gcs1Δ*, *mso1Δ*, *rcy1Δ*, *sma1Δ*, *sma2Δ*, *sec22Δ* and *vps13Δ* (Table 1). Of these mutants, three, *MSO1*, *SMA1* and *GCS1*, have recently been reported to have prospore membrane defects (Knop et al., 2005; Riedel et al., 2005; Connolly and Engebrecht, 2006), validating our screen. The remaining mutants affecting prospore membrane growth were characterized further.

Table 1. Mutants screened for prospore membrane formation deficiency

No defect	Poor or no growth in SD or YPA	Poor or no meiosis	Fragmented DAPI staining in meiosis	Prospore membrane formation defects
bim1	*pep12*	*cdc10*	*bub3*	*erv14*
hsl7	*pep3*	*mon1*	*dep1*	*gcs1*
slg1	*pep7*	*npr2*	*doa1*	*mso1*
tps2	*ppa1*	*pkh2*	*doa4*	*rcy1*
ubc11	*shp1*	*sac7*	*nem1*	*sec22*
ydr070c	*sod2*	*tps1*		*sma1*
	uba3	*ubi4*		*sma2*
	vma2	*vac8*		*vps13*
	vma6	*vam3*		
	vma13	*vam6*		
	yll033w	*vam7*		
	ydr126w	*vps30*		
	vps41			
	ypt7			

VPS13 is required for prospore membranes to reach normal size

Analysis of GFP-Spo20$^{51\text{-}91}$ in *rcy1Δ*, *sec22Δ*, and *vps13Δ* cells revealed a very similar phenotype. All three of these mutants displayed small, round prospore membranes that often failed to capture nuclei (Fig. 1). Sec22p is a SNARE protein involved in ER-Golgi vesicle trafficking (Liu and Barlowe, 2002) and Rcy1p and Vps13p participate in protein cycling between the endosome and the late Golgi (Brickner and Fuller, 1997; Wiederkehr et al., 2000). The known

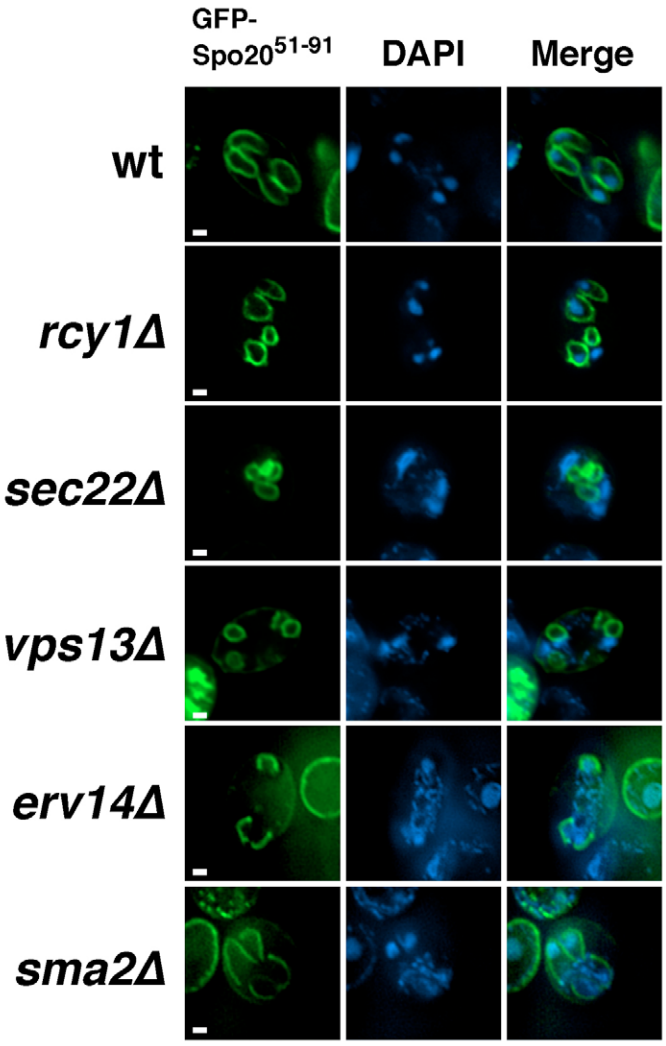

Fig. 1. Prospore membrane formation mutants. Wild-type (AN120), *rcy1Δ/rcy1Δ* (31221), *sec22Δ /sec22Δ* (35177), *vps13Δ /vps13Δ* (HI29), *erv14Δ /erv14Δ* (HI26) and *sma2Δ /sma2Δ* (HI44) cells were transformed with pRS426-G20 and the localization of the prospore membrane marker GFP-Spo20$^{51\text{-}91}$ was observed in late Meiosis II cells. *sec22Δ/sec22Δ* and *rcy1Δ/rcy1Δ* mutants are BY4743 background, the others are in the SK-1 background. The corresponding DNA images are shown (DAPI). Bars, 1 μm.

functions of these proteins suggest that the prospore membrane deficiency could be an indirect consequence of secretory pathway defects occurring prior to vesicle delivery to the prospore membrane.

Sporulation deficiencies are sometimes variable in different strain backgrounds (Connolly and Engebrecht, 2006). Therefore, we attempted to make *rcy1*, *sec22* and *vps13* disruptants in the SK-1 background. However, *sec22Δ* and *rcy1Δ* mutants were not viable in the SK-1 background (H.N., unpublished observations). A *VPS13* deletion caused the same phenotype in the SK-1 background as seen in the BY4743 background (Fig. 1) and the more efficient sporulation of SK-1 allowed for a quantitative analysis of the *vps13* phenotypes (Table 2). Several parameters were examined including the number of prospore membranes per cell, the frequency of nuclear capture by the prospore membrane and the size of the prospore membranes. The average number of prospore membranes present per tetranucleate (i.e. post-meiotic) cell was much lower in *vps13Δ* cells than in the wild type. Moreover, only about a quarter of the prospore membranes in *vps13Δ* cells appear to capture nuclei as judged by comparison of the GFP and DAPI fluorescence. Finally, the longest diameter of the prospore membrane was a little more than half that of wild-type cells, indicating that prospore membranes, when present, are smaller in *vps13* cells. Taken together, these results detail a profound defect in membrane formation and growth in the *vps13Δ* mutant.

ERV14 and *SMA2* are required for morphologically normal prospore membranes

The localization of GFP-Spo20$^{51-91}$ in *erv14Δ* and *sma2Δ* cells revealed a slightly different defect than that seen in the *rcy1Δ*, *sec22Δ* and *vps13Δ* mutants. In both the *sma2Δ* and the *erv14Δ* mutants, a fraction of the cells displayed aberrant, boomerang-shaped prospore membranes (Fig. 1). Quantitative analysis of the membrane defects of these mutants in the SK-1 background also indicated that *sma2Δ* and *erv14Δ* cells display similar defects (Table 2). Although the number of prospore membranes per cell and the frequency with which these membranes capture nuclei is greater in these mutants than in *vps13Δ*, it is still significantly worse than that in wild-type cells. Unlike, *vps13Δ*, 10-20% of the prospore membranes display an abnormal boomerang-shaped morphology in *sma2Δ* and *erv14Δ* cells. *SMA2* is a meiotically induced gene encoding a protein with four predicted membrane-spanning regions and our observations are consistent with an earlier report that the leading edge protein Don1p is more broadly distributed in *sma2Δ* mutants (Rabitsch et al., 2001). Erv14p is a cargo receptor that is required for certain proteins to be packaged into COPII vesicles and exit the ER (Powers and Barlowe, 1998; Powers and Barlowe, 2002). The abnormal phenotypes of *sma2Δ* and *erv14Δ* were seen in both BY4743 and SK-1 backgrounds. These results suggest that Sma2p and Erv14p are required for the proper shaping of the prospore membrane.

Table 2. Quantification of prospore membrane defects in *vps13Δ*, *sma2Δ* and *erv14Δ*

Relevant genotype*	Number of PrSM†	PrSM capturing nucleus (%)‡	Size of PrSM (μm)§	Boomerang shape PrSM¶
Wild type	3.7	100	2.95	0
vps13Δ/vps13Δ	2.2	26.1	1.60	0
sma2Δ/sma2Δ	2.4	41.5	1.58	17.6
erv14Δ/erv14Δ	3.1	67.5	1.71	11.4

*Only cells that had completed Meiosis II, as judged by DAPI staining were scored for the analysis.

†For each strain the number of prospore membranes present per cell for over 200 cells were counted.

‡The percentage of prospore membranes that encapsulated a nucleus, as judged by DAPI staining is shown. Over 200 individual prospore membranes were scored for each strain.

§The longest diameter of over 200 prospore membranes was measured in each strain. The average diameter is given.

¶The percentage of prospore membranes displaying a boomerang morphology, as in Fig. 1, is shown. Over 200 prospore membranes were analyzed in each strain.

Overexpression of *SMA2* weakly suppresses the sporulation deficiency of *erv14Δ*

The known cargo of Erv14p, Axl2p, is not necessary for sporulation (Enyenihi and Saunders, 2003), indicating that the sporulation phenotype is probably due to the transport defect of some other cargo. In theory, the cargo should be: (1) non-essential for vegetative growth; (2) a transmembrane protein localized to the prospore membrane; and (3) necessary for proper prospore membrane extension. The *erv14Δ* and *sma2Δ* mutants have similar prospore membrane defects (Fig. 1).

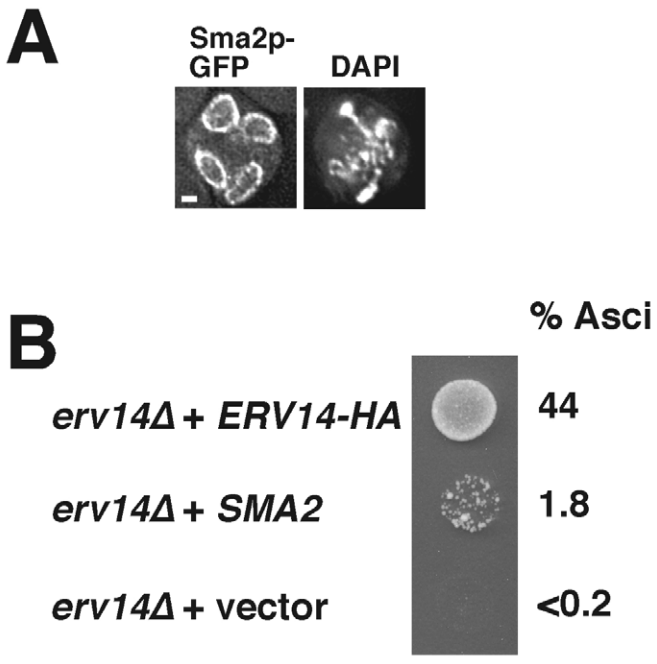

Fig. 2. Overexpression of *SMA2* partially suppresses the sporulation defect of an *erv14Δ* mutant. (A) Sma2p-GFP localizes to the prospore membrane. Sma2p-GFP expressed from pRS424-SPO20pr-SMA2-GFP was observed in a sporulating wild type cell (AN120). DNA was stained with DAPI (DAPI). Bar, 1 μm. (B) Overexpression of *SMA2* restores sporulation to the *erv14Δ* mutant. *erv14Δ/erv14Δ* (H126) cells harboring pRS314-ERV14HA, pRS424-SPO20pr-SMA2 or empty vector, were sporulated and 3×10^5 cells were spotted onto a YPD plate, exposed to ether vapor and then incubated at 30°C.

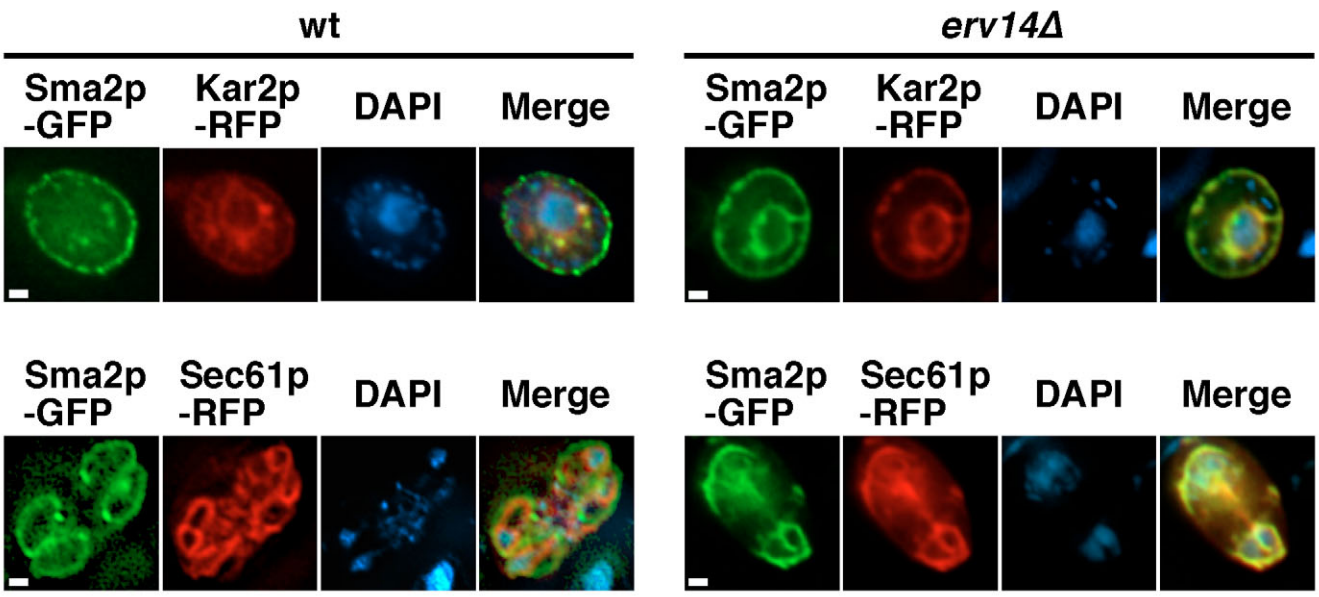

Fig. 3. Sma2p-GFP accumulates in the ER in vegetative and sporulating *erv14Δ* cells. Wild-type (AN120) and *erv14Δ/erv14Δ* (HI26) cells were transformed with pRS424-SSO1pr-Sma2-GFP and YEp352-GAPII-KmRFP (Kar2-RFP), or with pRS424-SPO20pr-Sma2-GFP and pRS426-SPO20pr-SEC61-mRFP, and the localization of Sma2-GFP and the RFP-tagged ER markers was observed in vegetative and sporulating cells. The corresponding DNA images are shown (DAPI). Bar, 1 μm.

Moreover, a Sma2p-GFP fusion protein localized to the prospore membrane (Fig. 2A). Thus, Sma2p seemed a good candidate for Erv14p cargo. Consistent with this idea, overexpression of *SMA2* weakly but reproducibly suppressed the sporulation defect of the *erv14Δ* mutant (Fig. 2B). By contrast, overexpression of *ERV14* did not improve sporulation in a *sma2* strain (Y.S., unpublished data), consistent with the idea that *ERV14* effects sporulation through *SMA2*. These data suggest that Sma2p might require Erv14p for exit from the ER in sporulating cells.

Sma2p-GFP accumulates in the ER in vegetative *erv14Δ* mutant

If Sma2p is a cargo protein of Erv14p, then the protein should accumulate in the ER in *erv14Δ* mutants. To examine this possibility, Sma2p-GFP was expressed ectopically in vegetative cells using the *SSO1* promoter. In wild-type cells, Sma2p-GFP displayed a diffuse cytoplasmic fluorescence in the majority of cells but was occasionally seen as dots at the cell periphery (Fig. 3). However, in 100% of the *erv14Δ* cells, Sma2-GFP colocalized with the ER marker RFP-HDEL (Fig. 3). Next, to examine transport of Sma2p-GFP from the ER in sporulating cells, an RFP fusion to the ER membrane protein Sec61p (Deshaies et al., 1991) under control of the sporulation-specific *SPO20* promoter was constructed. During Meiosis II, this fusion was found in rings surrounding the DAPI-stained chromatin of the forming haploid nuclei as well as in a reticular structure near the middle of the cell. In wild-type cells, this pattern was distinct from the prospore membrane localized Sma2-GFP fusion (Fig. 3). By contrast, in *erv14Δ* cells, the fluorescence pattern of the Sma2p-GFP fusion perfectly matched that of Sec61p-RFP (Fig. 3). Thus, Sma2p accumulates in the ER in sporulating as well as vegetative *erv14Δ* cells. These data indicate that *ERV14* is required for exit of Sma2p from the ER.

A paralog of *ERV14* can rescue the *erv14Δ* sporulation defect

SMA2 overexpression only weakly suppressed the *erv14Δ* sporulation defect, suggesting that other proteins important for sporulation might also require *ERV14* for transport from the ER. To search for such factors, a multi-copy suppressor screen was used to identify additional cargo proteins necessary for sporulation. Out of 62,000 *erv14Δ* transformants carrying a multi-copy genomic library, 69 were capable of sporulation. PCR analysis revealed that 62 of the suppressor plasmids contained *ERV14*. From the remaining colonies, three different plasmids that reproducibly rescued the sporulation deficiency were recovered. Sequence analysis revealed that all three of these plasmids contained a yeast chromosomal region including the *ERV15* gene (H.N., unpublished result). A DNA fragment containing only the *ERV15* gene also rescued the sporulation deficiency (Fig. 4). Thus, *ERV15* is a multi-copy suppressor of *erv14Δ*. The amino acid sequence of Erv15p is 63% identical to that of Erv14p, suggesting that Erv15p is a functional homologue to Erv14p, rather than a cargo protein. Although overexpression of *ERV15* does not rescue the Axl2p transport defect of *erv14Δ* in vegetative cells (Powers and Barlowe, 1998), Erv15p can substitute for Erv14p in sporulation.

Deletion of *ERV15* exacerbates the *erv14Δ* phenotype

That *ERV15* can partially rescue the *erv14Δ* sporulation defect suggests that *ERV15* might normally function in sporulation as well. Consistent with previous reports (Deutschbauer et al., 2002; Enyenihi and Saunders, 2003; Marston et al., 2004), we found that homozygous *erv15Δ* diploids showed no

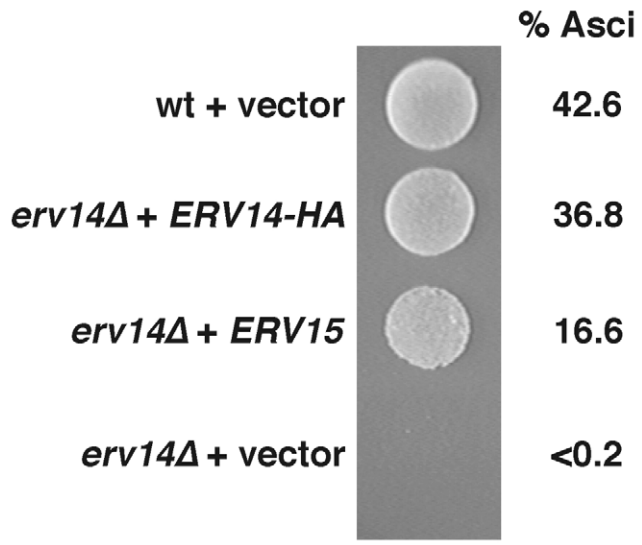

Fig. 4. Overexpression of *ERV15* suppresses the sporulation defect of an *erv14Δ* mutant. Wild-type (AN120) cells harboring the empty vector pRS424, or *erv14Δ/erv14Δ* (H126) cells harboring pRS314-ERV14HA, pRS424-ERV15 or empty vector were sporulated and 3×10^5 cells were spotted onto a YPD plate, exposed to ether vapor and incubated at 30°C.

sporulation defect and had normal prospore membranes (Y.S., unpublished data). However, when prospore membranes were analyzed in *erv14Δ erv15Δ* double mutant diploids, these cells were found to have a more profound sporulation defect than the *erv14Δ* strain alone. Specifically, using the peripheral membrane protein GFP-Spo20$^{51\text{-}91}$ as a marker, prospore membranes were completely absent from these cells, rather, small foci of GFP-Spo20$^{51\text{-}91}$ fluorescence were seen in the cytoplasm (Fig. 5). When Meiosis II SPBs were examined using Mpc54-RFP as a marker, 43% of the SPBs were found to have associated GFP-Spo20$^{51\text{-}91}$ foci. This is reminiscent of the phenotype seen in *sso1* mutant cells where prospore membrane precursor vesicles accumulate on the SPBs (Nakanishi et al., 2006).

ERV14/*ERV15* are required generally for ER exit during sporulation

The stronger phenotype of the *erv14Δ erv15Δ* double mutant strain led us to examine the localization of additional plasma membrane and prospore membrane proteins in these cells. The plasma membrane ATPase Pma1p, the t-SNARE Sso1p, and the dityrosine transporter Dtr1p (Serrano et al., 1986; Aalto et al., 1993; Felder et al., 2002) as well as Sma2p were expressed as GFP fusions either under the control of the *TEF2* promoter in vegetative cells or under the *SPO20* promoter in sporulating cells. The localization of these four proteins was examined in wild type, *erv14Δ*, *erv15Δ* and *erv14Δ erv15Δ* diploids. Examples of the results for GFP-Sso1p are shown in Fig. 6. In all four strains, GFP-Sso1p localized to the plasma membrane during vegetative growth. In wild-type and *erv15Δ* cells, GFP-Sso1p was found in the prospore membrane during sporulation. Similarly, in *erv14Δ* cells, GFP-Sso1p was localized predominantly to the abnormal prospore membranes, although significant background fluorescence that appeared to colocalize with the ER marker could also be seen. That this localization of GFP-Sso1p in the *erv14Δ* strain does represent prospore membranes was demonstrated by colocalization with the prospore membrane marker RFP-Spo20$^{51\text{-}91}$ (Fig. 6, bottom panel). However, in the *erv14Δ erv15Δ* double mutant cells, although GFP-Sso1p trafficked normally to the plasma membrane during vegetative growth, GFP-Sso1p expressed during sporulation accumulated in the ER. These results define a sporulation-specific requirement for the Erv14/15 proteins in the ER exit of Sso1p.

The Pma1p and Dtr1p proteins displayed similar behavior to Sso1p (Fig. 7). Their transport from the ER was completely insensitive to *ERV14/ERV15* in vegetative cells. During sporulation, their ER exit was influenced to varying extents by deletion of *ERV14*, but strongly inhibited in the absence of both *ERV14* and *ERV15*. By contrast, Sma2p required *ERV14* for exit from the ER under either growth condition. These results suggest there are two distinct requirements for the Erv14 family proteins. Some specific proteins such as Sma2p may require these cargo receptors for transport under all conditions, but during prospore membrane formation there appears to be a more general requirement for these proteins to promote exit from the ER.

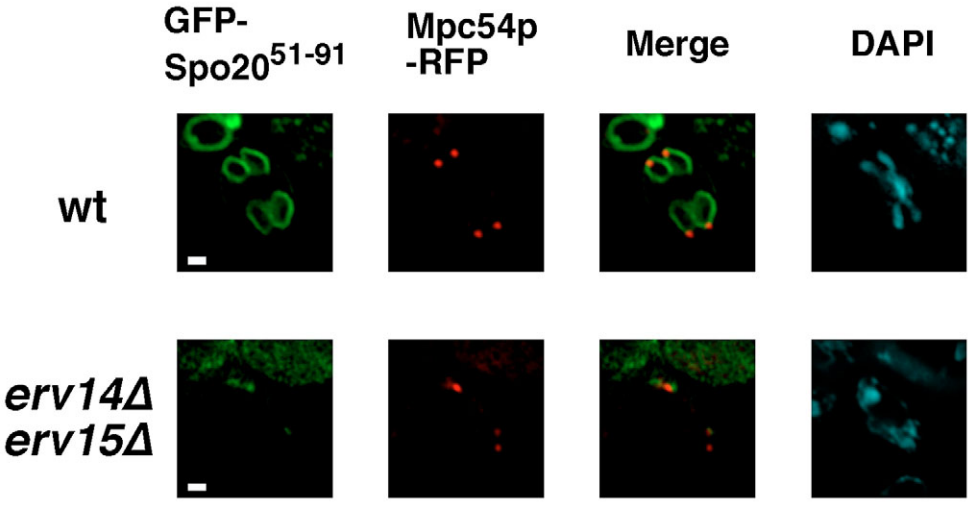

Fig. 5. Prospore membrane formation is blocked in *erv14Δ erv15Δ* double mutants. Wild-type (AN120) and *erv14Δ/erv14Δ erv15Δ/erv15Δ* (YS232) cells were transformed with pRS426-G20 and pRS304-MPC54-RFP, and the localization of the prospore membrane marker GFP-Spo20p$^{51\text{-}91}$ and the SPB marker Mpc54-RFP were observed in sporulating cells. Bars, 1 μm.

Discussion

Erv14 family proteins have been characterized as receptors to facilitate the movement of specific cargo molecules into COPII-coated vesicles and out of the ER. Erv14p has been shown to play such a role for the Axl2 protein in haploid yeast cells and a similar relationship between the Erv14 family member cornichon and the TGF alpha protein gurken has been described in *Drosophila* (Bokel et al., 2006). Our studies on the sporulation defect of *erv14Δ* mutants suggest that this family of proteins may have two functions, as receptors for specific cargo molecules and a more general function in ER exit as well.

Strains lacking *ERV14* display prospore membrane defects including a significant frequency of boomerang-shaped membranes. This morphological phenotype is also seen in strains lacking *SMA2*, which encodes an integral prospore membrane protein. Sma2p requires *ERV14* for its transit from the ER both in sporulating cells and when it is expressed ectopically in vegetative cells. Thus, the behavior of Sma2p is consistent with it being a specific cargo for Erv14p and the failure to transport Sma2p can account for the similar morphology of the prospore membrane in the two mutants.

In a screen for high copy suppressors of the *erv14Δ* sporulation defect, we identified the *ERV14* paralog, *ERV15*. Genetic analysis suggests that these two genes have overlapping functions in sporulation: deletion of *ERV14* produces abnormal prospore membranes, deletion of *ERV15* reveals no phenotype, and simultaneous removal of both genes causes a complete block to prospore membrane formation. Analysis of the localization of three plasma membrane or prospore membrane proteins revealed that the failure in prospore membrane formation in the double mutant appears to be the result of a more general block in ER exit. This requirement for the Erv proteins is developmental-stage specific. Pma1p, Sso1p and Dtr1p all localize normally in vegetative *erv14Δ erv15Δ* cells; only when these cells are sporulated do the proteins accumulate in the ER.

Work on GPI-anchored proteins has revealed distinct routes of COPII-mediated exit from the ER for GPI-anchored and non-GPI-anchored proteins (Morsomme and Riezman, 2002; Morsomme et al., 2003). It might be that for the non-GPI anchored proteins there also exists more than one class of COPII vesicle. Some proteins, such as Sma2p or Axl2p are restricted to the Erv14/15 class vesicles, recruited there by specific interactions with Erv14p, whereas other proteins such as Pma1p or Sso1p either use an alternative class of vesicle or may exit via multiple vesicle types. Thus, mutation

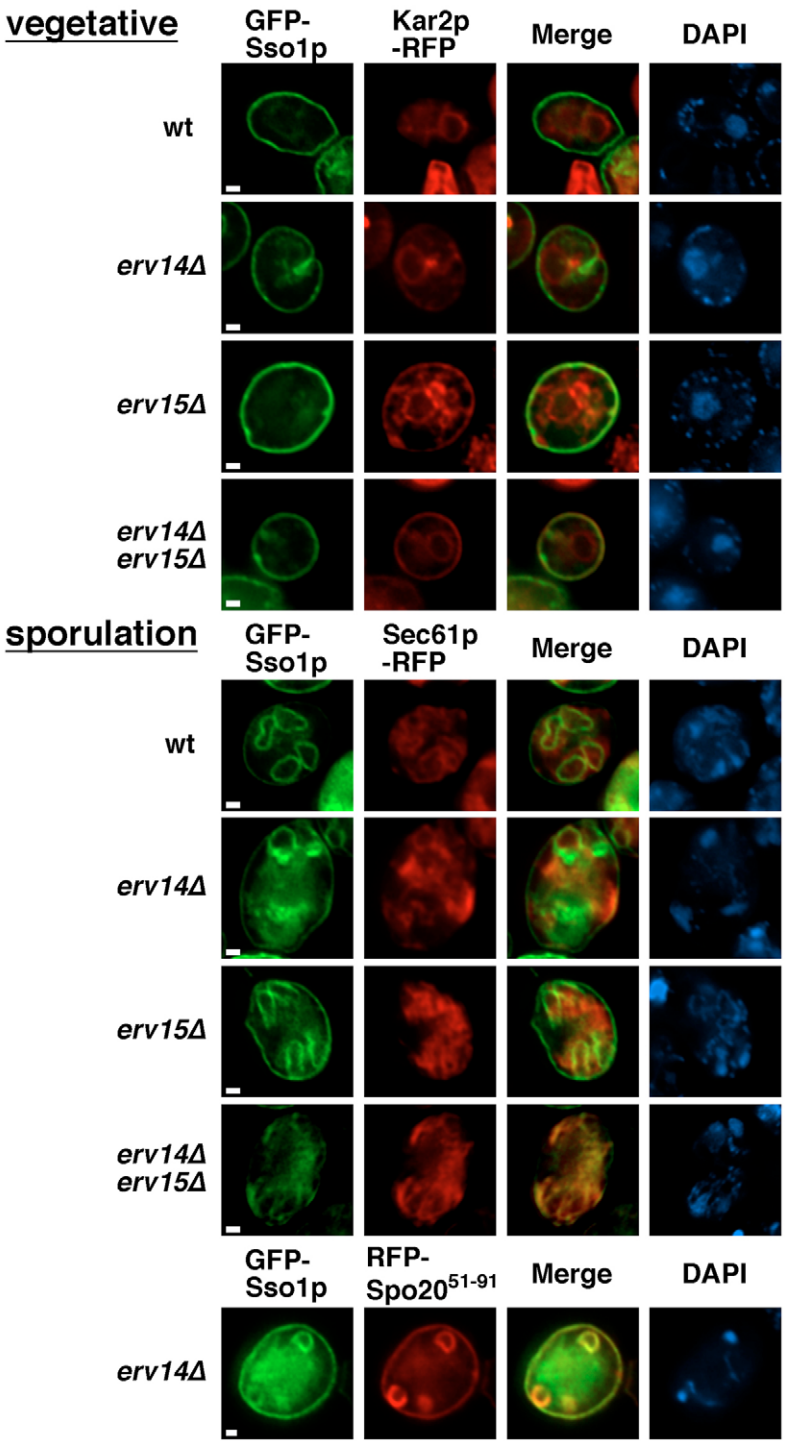

Fig. 6. GFP-Sso1p accumulates in the ER in sporulating *erv14Δ erv15Δ* double mutants. The localization of GFP-Sso1p was examined in vegetative growth and sporulation in wild-type (AN120), *erv14Δ* (HI26), *erv15Δ* (YS229) and *erv14Δ erv15Δ* (YS232) strains. For analysis of vegetative cells, GFP-Sso1p was expressed under the control of *TEF2* promoter from pRS424-TEF2pr-GFP-SSO1+3′ and the ER was labeled with Kar2-RFP expressed from YEpGAPII-KmRFP. In sporulating cells, GFP-Sso1p and Sec61p-RFP were expressed from sporulation-specific promoters in plasmids pRS424-SPO20pr-GFP-SSO1+3′ and pRS426-SPO20pr-SEC61-mRFP, respectively. In the bottom panel, localization of Sso1p to the prospore membrane in *erv14Δ* (HI26) cells was examined using the prospore membrane marker RFP-Spo20$^{51-91}$. The corresponding DNA images are shown (DAPI). Bar, 1 μm.

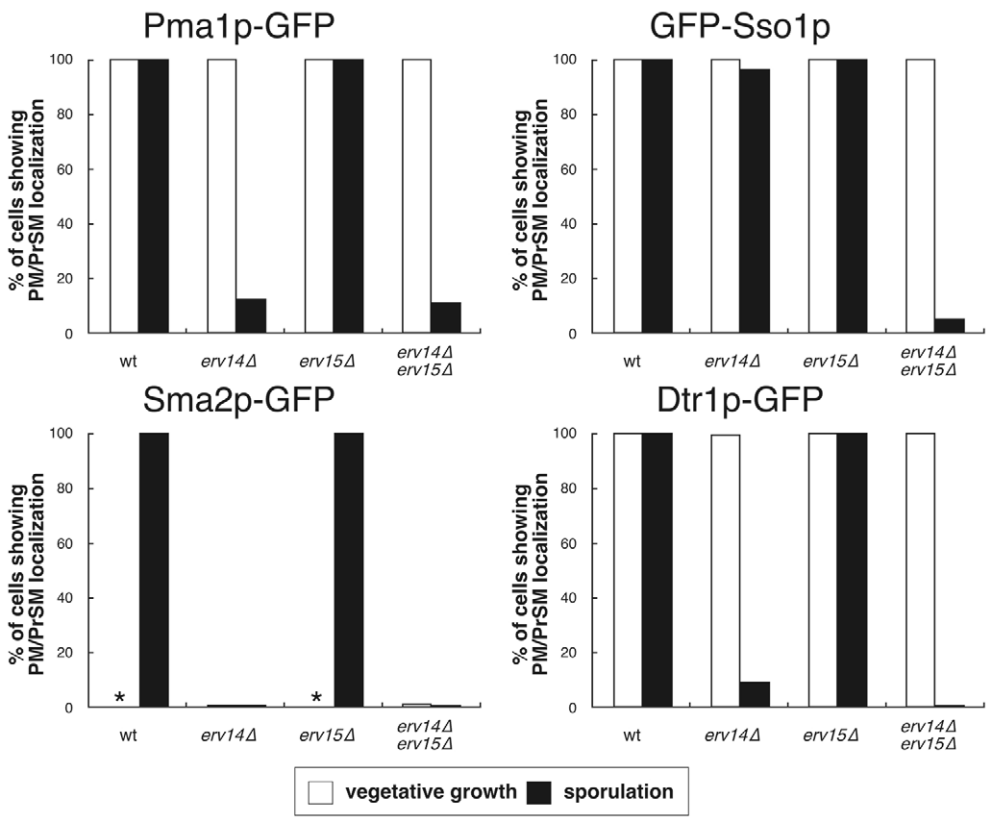

Fig. 7. Multiple proteins require *ERV* genes for localization to the prospore membrane during sporulation. Localization of Pma1p, Sso1p, Dtr1p and Sma2p during vegetative growth and sporulation in wild-type (AN120), *erv14Δ* (HI26), *erv15Δ* (YS229) and *erv14Δ erv15Δ* (YS232) strains was quantified using GFP fusions and ER markers as in Fig. 6. All the GFP fusions were expressed vegetatively under control of the *TEF2* promoter and during sporulation under control of the *SPO20* promoter except Dtr1p-GFP, which was expressed from its native promoter. The ER markers used were Kar2-RFP in vegetative cells and Sec61p-RFP in sporulating cells. For each strain at least 100 cells were examined and the percentage of cells showing localization of the GFP fusion to the plasma membrane during vegetative growth (open bars) or prospore membrane during sporulation (black bars) are shown. Asterisks in the Sma2-GFP graph indicate that the Sma2-GFP was mainly found diffusely throughout the cytoplasm in wild-type and *erv15Δ* cells during vegetative growth.

of *ERV14* and *ERV15* has no significant effect on the trafficking of the latter proteins in vegetative cells. However, we suggest that during sporulation, one or more of these alternative vesicle types is absent and the Erv14/15 vesicles now become essential for the exit of a much larger spectrum of proteins. The large number of different proteins trapped in the ER during sporulation of the *erv14Δ erv15Δ* double mutant is hard to reconcile with a model in which the Erv proteins function solely as receptors for specific cargo molecules. Rather, it suggests that these proteins might also play a more general role in the generation of a particular class of COPII vesicles. A similar dual role for Erv14p has been proposed based on genetic and biochemical studies that indicate Erv14p is required for efficient COPII vesicle formation (Powers and Barlowe, 2002).

In addition to *erv14Δ*, this work demonstrates that the sporulation defects of *vps13Δ*, *sec22Δ* and *rcy1Δ* mutants are due to a failure to properly form prospore membranes. The products of these genes participate in ER-Golgi or endosome-Golgi transport steps. The prospore membrane phenotypes of these mutants could result from a general slowing of vesicle traffic. That is, the simultaneous formation of four prospore membranes may require greater flux through the secretory pathway than can be accommodated in these mutants. For example, in *S. pombe*, several of the *spo* mutants defective in generating forespore membranes, the *S. pombe* equivalent of the prospore membrane, proved to be hypomorphic alleles of constitutive ER-Golgi transport functions (Nakase et al., 2001; Nakamura-Kubo et al., 2003). The temperature-sensitive growth phenotype of *sec22Δ* strains (Liu and Barlowe, 2002) suggests that the secretory pathway is not fully functional in this mutant and is consistent with the possibility that the sporulation phenotype results from a general slowing of anterograde transport.

Alternatively, one or more of the mutants could reveal a sporulation-specific alteration of the secretory pathway, as in the *erv14Δ* mutant. In *S. pombe*, sorting nexin homologues involved in sorting of proteins to the vacuole are relocalized to the forespore membrane during sporulation, suggesting the existence of a recycling pathway between the forespore membrane and the late Golgi (Koga et al., 2004). It may be that Vps13p and Rcy1p are similarly involved in protein recycling during prospore membrane extension. The shape and size of prospore membranes in these mutants are reminiscent of those in *arf1* cells (Rudge et al., 1998). Arf proteins are involved in several events in intracellular vesicle trafficking and a specific function of Arf1p in prospore membrane formation has been suggested by the finding that an *arf1Δ arf2Δ* double mutant harboring an *arf1-myc* allele arrests sporulation with small prospore membranes (Rudge et al., 1998). The possibility that Vps13p, Rcy1p and/or Arf1p participate in a recycling pathway unique to the prospore membrane is an intriguing one.

Differentiation of higher cells often involves coordinated rearrangements of the endomembrane system (Arvan and Castle, 1998; Spritz, 1999; Towler et al., 2004). Similarly, the generation of the prospore membrane requires a developmentally regulated alteration of the late secretory pathway such that post-Golgi vesicles first fuse with each other and then the prospore membrane, rather than the plasma membrane (Neiman, 1998). The data presented here indicate that the early secretory pathway is also altered during

sporulation so that the Erv14 and Erv15 proteins are now required for the ER exit of a broad array of proteins. It will be of interest to determine how these changes at different ends of the secretory pathway are coordinated.

Materials and Methods

Yeast strains and media

Unless otherwise noted, standard media and genetic methods were used (Rose and Fink, 1990). 1% potassium acetate medium contains 1% potassium acetate and 10 μg/ml of adenine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, uracil and valine. *S. cerevisiae* strains used in this study are listed in supplementary material Table S1. HI26 (*erv14Δ/erv14Δ*), HI29 (*vps13Δ/vps13Δ*), HI44 (*sma2Δ/sma2Δ*), and YS229 (*erv15Δ/erv15Δ*) were constructed by targeted deletion of the appropriate gene in the haploids AN117-4B and AN117-16D (Neiman et al., 2000), and the resulting haploids were mated to form homozygous diploid mutants. To construct YS232 (*erv14Δ/erv14Δ, erv15Δ/erv15Δ*), the *erv14Δ* AN117-4B derivative was crossed to the *erv15Δ* AN117-16D strain, sporulated, and dissected. Mating of two *erv14Δ erv15Δ* segregants created YS232. For the gene deletions, pFA6a-His3MX6 or pFA6a-kanMX6 (Longtine et al., 1998) was used as a template to amplify the DNA cassettes using the primers HNO401 and HNO402, HNO411 and HNO412, HNO601 and HNO602, and YSO109 and YSO110 to disrupt *ERV14*, *VPS13*, *SMA2* and *ERV15*, respectively. *sec22Δ/sec22Δ* and *rcy1Δ/rcy1Δ* in the BY4743 background were purchased from Research Genetics (now Invitrogen, San Diego, CA).

Plasmids

Plasmids used in this study are listed in supplementary material Table S2. pRS314-ERV14HA was generated by cloning the *Kpn*I-*Sac*I fragment containing an *HA* fusion to *ERV14* from pRS316-ERV14HA (Powers and Barlowe, 1998) into similarly digested pRS314 (Sikorski and Hieter, 1989). pRS424-ERV15 was created by cloning the *Bam*HI-*Bam*HI fragment from a plasmid obtained in the multi-copy suppressor screen described below into pRS424 (Christianson et al., 1992). This plasmid includes the *ERV15* open reading frame with 729 bp of upstream and 258 bp of downstream flanking sequences. pRS424-SSO1pr-SMA2-GFP was constructed by first replacing the *TEF2* promoter in pRS424-TEF (Mumberg et al., 1995) with a *Sac*I and *Spe*I fragment carrying the *SSO1* promoter (600 bp upstream of the *SSO1* coding region) prepared by PCR using HNO441 and HNO442 as primers to generate pRS424-SSO1pr. The *SMA2* open reading frame was amplified from yeast genomic DNA using HNO551 and HNO552 as primers, digested by *Xba*I and *Hin*dIII, and ligated into similarly digested GFP-C-Fus (Niedenthal et al., 1996) to generate a *SMA2-GFP* fusion. This *GFP* fusion was then cut out with *Xba*I and *Xho*I, and cloned into *Spe*I and *Xho*I sites of pRS424SSO1pr. pRS424-SPO20pr-SMA2-GFP was constructed in two steps. First, the *SPO20* promoter region (731 bp upstream of the *SPO20* coding region) was amplified by PCR using HNO631 and HNO632 as primers. This PCR product was digested with *Sac*I and *Xba*I and cloned into similarly digested pRS424-TEF (Mumberg et al., 1995) to make pRS424-SPO20pr. An *Xba*I and *Xho*I fragment containing the *SMA2-GFP* fusion from pRS424-SSO1pr-SMA2-GFP was then cloned into the *Spe*I and *Xho*I sites of pRS424-SPO20pr. To generate pRS424-SPO20pr-SMA2, the *SMA2* gene was amplified by PCR using HNO551 and HNO672 as primers and yeast genomic DNA as template, digested with *Xba*I and *Xho*I, and cloned into similarly digested pRS424-SPO20pr. pRS426-R20 was constructed by replacing the *GFP* coding region in pRS426-G20 (Nakanishi et al., 2006) with the gene for monomeric red fluorescent protein (RFP)(Campbell et al., 2002) using *Eco*RI and *Xba*I sites. The *RFP* gene was amplified by PCR using pTiKmRFP (Gao et al., 2005) as a template and HNO941 and HNO942 as primers. pRS426-SPO20pr-SEC61-mRFP was constructed by first amplifying monomeric *RFP* using YSO33 and HNO944 and cloning the PCR fragment between the *Hin*dIII and *Xho*I sites of pRS426-TEF (Mumberg et al., 1995). The *SEC61* gene was then amplified using HNO901 and HNO902 as primers and cloned into the *Spe*I and *Bam*HI sites of the resulting plasmid to create a *SEC61-RFP* fusion. Finally, the *TEF2* promoter was replaced with *SPO20* promoter from pRS424-SPO20pr using a *Sac*I-*Spe*I fragment. pRS426-SPO20pr-GFP-SSO1+3′ was constructed in two steps. First, the *SSO1* gene was amplified by PCR using ANO185 and HNO541 as primers, digested with *Xba*I and *Xho*I, and cloned into similarly digested GFP-N-Fus (Niedenthal et al., 1996). The resulting plasmid was then digested with *Eco*RI and *Xho*I, and the fragment carrying the *GFP-SSO1* fusion was cloned into similarly digested pRS424-SPO20pr. pRS424-TEFpr-GFP-SSO1+3′ was constructed by cloning an *Eco*RI-*Xho*I fragment containing the *GFP-SSO1* fusion from pRS426-SPO20pr-GFP-SSO1+3′ into similarly digested pRS424-TEF. pRS414-TEFpr-SMA2-GFP was constructed by cloning an *Xba*I-*Xho*I fragment from pRS424-SSO1pr-SMA2-GFP into similarly digested pRS414-TEF (Mumberg et al., 1995). pRS414-TEFpr-DTR1-GFP was constructed in two steps. First, an *Eco*RI and *Xho*I fragment from GFP-C-Fus containing the GFP coding region was cloned into similarly digested pRS426-TEF to make pRS426-TEF-GFP. Next, the *DTR1* gene was amplified by PCR using pRS424-DTR1-GFP as a template (Nakanishi et al., 2006), and HNO532 and HNO721 as primers, then digested with *Eco*RI and cloned into similarly digested pRS426-TEF-GFP. To construct pRS416-TEFpr-PMA1-GFP and pRS424-SPO20pr-PMA1-GFP, first the *PMA1* gene was amplified by PCR using HNO761 and HNO762 as primers and yeast genomic DNA as a template, and cloned into pRS426-TEF-GFP using *Spe*I and *Hin*dIII to create pRS426-TEFpr-PMA1-GFP. Then a SpeI-XhoI fragment containing the GFP fusion to PMA1 was cloned into pRS416-TEF (Mumberg et al., 1995) or pRS424-SPO20pr, to generate pRS416-TEFpr-PMA1-GFP and pRS424-SPO20pr-PMA1-GFP, respectively. YEp352GAPII-KmRFP was a gift from X. D. Gao (University of Hokkaido, Hokkaido, Japan). pRS424GAPII-KmRFP was created by cloning a *Bam*HI fragment containing the promoter region of *TDH3* and the region encoding a fusion of Kar2 and monomeric RFP to a C-terminal HDEL signal from YEp352GAPII-KmRFP into *Bam*HI site of pRS424. Sequences of all the oligonucleotides used in these constructions are available upon request.

Sporulation assays

BY4743 background strains harboring pRS426-G20 plasmid were sporulated as follows. Yeast cells were grown overnight in SD-ura medium. 100 μl of the cultures were shifted into 5 ml of 1% YPAcetate medium and grown overnight. The cells were harvested and resuspended into 1% potassium acetate medium at 3×10^7 cells/ml. Prospore membranes were observed at 30 hours after shifting to 1% potassium acetate medium in the fluorescence microscope. Sporulation efficiency was determined after 50 hours in the light microscope. Sporulation assays for SK-1 background strains were performed essentially as described (Neiman, 1998). After overnight incubation, the percent sporulation was determined by observation in the light microscope. For ether test, strains were sporulated at 30°C and 10 μl of cultures were spotted onto YPD plate. The plate was inverted over a paper filter soaked with 4 ml of ethyl ether for 15 minutes and then removed and incubated at 30°C for 2 days.

Multi-copy suppressor screening

HI26 was transformed with a 2 μ *URA3* yeast genomic DNA library (a gift or R. Sternglanz, SUNY Stony Brook) and transformants were selected on SD –ura plates. The transformants were replica plated onto YPD medium, grown overnight at 30°C, replica plated onto SPO medium, and incubated at 30°C for 2 days. The colonies were then replica plated back onto YPD medium and treated with ether vapor. After 24 hours incubation at 30°C, colonies surviving the ether treatment were selected as primary candidates. These candidates were retested on SPO plates and sporulation was verified under the light microscope. Plasmids were recovered from the cells that regained sporulation ability as follows. 100 μl of overnight SD-ura culture was shifted into 5 ml of YPD and cells were grown at 30°C for 5 hours. 3 ml of the culture was harvested and suspended in 30 μl STES buffer (0.2 M Tris-HCl pH 7.6, 0.5 M NaCl, 0.01 M EDTA and 1% SDS). An approximately equal amount of acid-washed glass beads were added and vortexed for 3 minutes. 200 μl of water and phenol:chloroform (1:1) were added and vortexed for 2 minutes. The aqueous phase was ethanol precipitated and the pellet was suspended in 100 μl water. These preparations were then subjected to PCR analysis using HNO403 and HNO404 to examine whether the plasmids contain the *ERV14*. Those that did not contain the *ERV14* were transformed into XL2-Blue (Stratagene, La Jolla CA) and plasmids were recovered. These plasmids were transformed into HI26 and those that reproducibly rescued the sporulation deficiency were sequenced using the primers ANO244 and ANO245.

Microscopy

For direct detection of GFP or RFP fluorescence, cells were fixed with 3.7% formaldehyde for 10 minutes and then placed in mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Images were acquired using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) with a Zeiss mRM Axiocam and deconvolved using Zeiss Axiovision 3.1 software.

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