Deleting the Yeast Phospholipase D Spo14 Augments Homotypic Vacuole Fusion

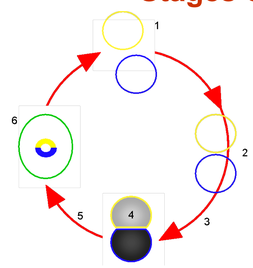
Sabit Ejub, Matthew L. Starr, and Rutilio A. Fratti

Department of Biochemistry, School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign

Abstract

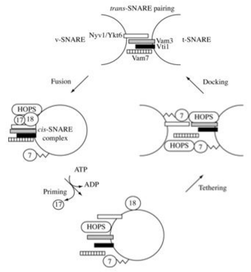
Membrane fusion is a necessary process that allows eukaryotic cells to carry and deliver cargo between organelles. The mechanisms and machinery involved in membrane fusion are conserved throughout eukaryotes, so the yeast vacuole provides an ideal model system to study. Yeast vacuole fusion proceeds through a series of stages that include priming, tethering, docking, and fusion. Phosphatidic acid (PA) is a glycerophospholipid present throughout eukaryotic organelle membranes. The conversion of PA to diacyglerol (DAG) has been shown to play an important role in the activity and recruitment of vacuole fusion protein factors during the priming and tethering stages. Increased PA levels at the vacuole have been shown to significantly inhibit each of these stages. In yeast, phospholipase D (Spo14) catalyzes the hydrolysis of phosphatidylcholine into choline and PA making it a protein of interest in our system. Spo14p activity has been shown to play a vital role in numerous cellular pathways, including signal transduction, membrane trafficking, and regulation of mitosis. Here we aim to identify a role for Spo14 activity in the regulation of yeast vacuolar membrane fusion. To date, we have demonstrated that deleting *SPO14* causes a marked increase in vacuole fusion that may be attributed to a decrease in phosphatidic acid levels at the yeast vacuole.

Stages of Fusion

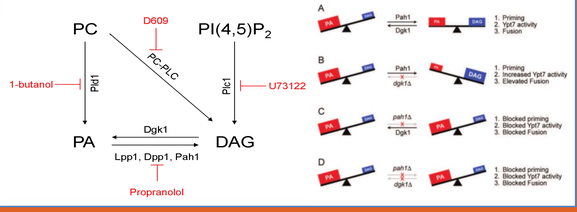


1. **Priming**: cis-SNAREs disrupted by Sec17/18
2. **Tethering**: Ypt7-dependent contact
3. **Docking**: Vacuoles drawn together
4. **Vertex formation**: Fusion factors become enriched at vertices and **release of calcium**
5. **Lipid mixing**
6. **Content mixing**

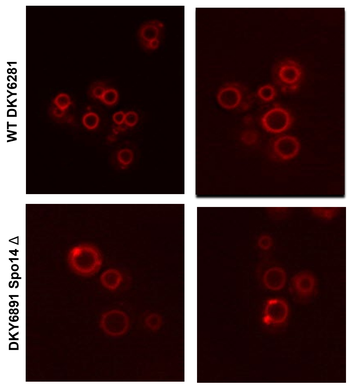
Fusion Schematic



Membrane Lipid Modification



Vacuole Morphology and Spo14 Localization



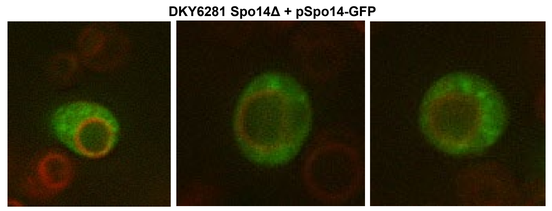
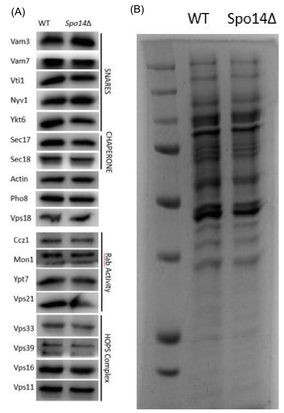


Figure 1: Vacuole morphology is consistent between wild-type DKY6281 and *spo14Δ*. Staining was done using a pulse-chase with the vacuole specific dye FM4-64. Wild-type and *spo14*Δ vacuole morphologies show no significant difference and no apparent fusion defect. There are specific regions in which the spo14Δ vacuoles show increased FM4-64 staining.

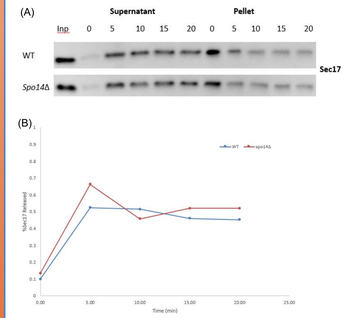
Figure 2: GFP-tagged Spo14 localizes away from the vacuole. Spo14 is potentially shown to localize in smaller endosomal sites rather than the vacuole.

Vacuole Protein Content

Figure 3: Protein content at the vacuole unchanged in spo14Δ. (A) Analysis of fusion components. Vacuoles were isolated from wild-type BJ3505 and BJ3505 spo14Δ (5µg protein) and immunoblotted with antibodies against the indicated proteins. These fusion components include: SNAREs, SNARE chaperones, GTPases, and proteins associated with the homotypic fusion and protein sorting (HOPS) complex. (B) Analysis of overall vacuole protein content of BJ3505 and Spo14 using Coomassie Blue staining.



Sec18 Priming



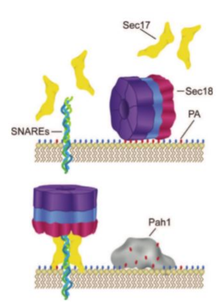
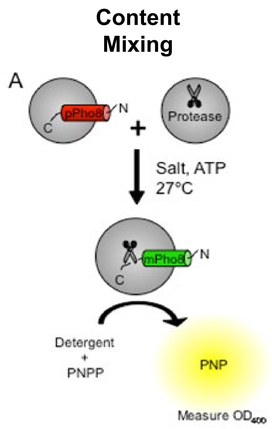


Figure 4: Sec18p priming is unaltered in spo14Δ. (A) Vacuoles from DKY6281 and spo14Δ yeast were assayed for priming activity as detected by the release of Sec17p from the vacuole membrane fraction. Fusion reactions of 3µg of vacuoles were used. Vacuoles were pelleted by centrifugation at the indicated time-points and proteins in the supernatant fraction were resolved by SDS–PAGE and imaged by western blot. (B) Percentage of Sec17p release was normalized and quantified to input values.

Vacuole Fusion



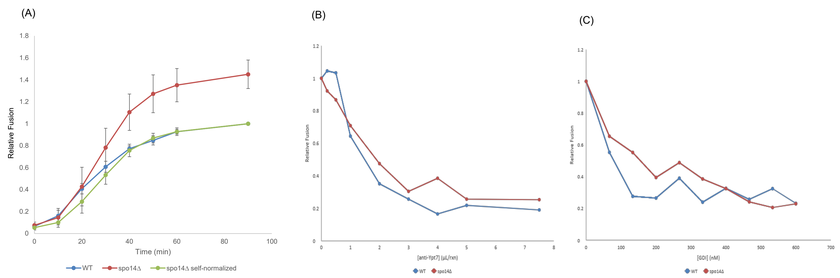
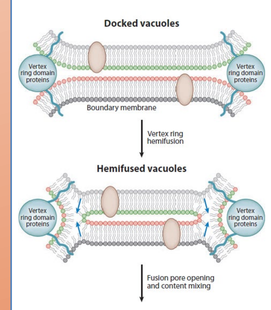


Figure 5: Spo14Δ shows an increase in fusion, but shows similar response to fusion inhibitors. (A) Fusion reactions were performed using wild-type and spo14Δ vacuoles. Reactions were incubated at 27C for the indicated times and assayed for Pho8 activity. The green trace represents spo14Δ fusion when normalized to its own maximum. This reveals the rate of spo14Δ vacuole fusion vs wild type. (B) Fusion reactions were performed with treatment of anti-Ypt7 in a dose response manner. Increased levels of the Ypt7 inhibitor were used and relative fusion was measured at the end of the 90 minute reaction. Ypt7 is a GTP-binding protein of the Rab family and interacts with the HOPS complex to bring membranes close enough together for SNARE proteins to engage each other and dock. (C) Fusion reactions were performed with treatment of guanosine nucleotide dissociation inhibitor (GDI). GDI binds to GDP-bound Rab GTPases and extracts them from the membrane.

Lipid Mixing



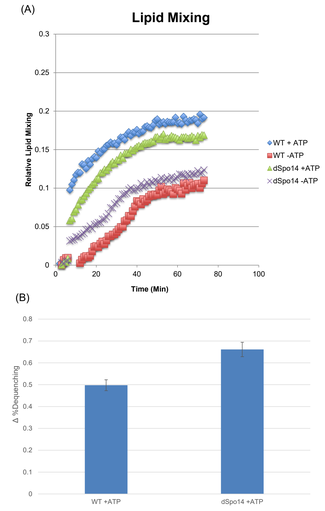


Figure 6: Spo14Δ shows an increase rate of lipid mixing. (A) Lipid mixing assay were performed with wild-type and spo14Δ DKY6281 vacuoles. Reporter vacuoles (2 µg) labeled with Rh-PE were incubated with 16 µg of unlabeled vacuoles. Fluorescence (λex= 544 nm; λem= 590 nm) was measured every 60 seconds for 80 minutes. (B) Raw lipid mixing data was quantified to show percent differences in rhodamine dequenching between strains.

Conclusions

- **Spo14Δ shows a 50% increase in vacuole content mixing**
- **Sec18p priming is not altered in the *spo14Δ***
- **Content of various membrane fusion associated proteins is not affected in the *spo14Δ***
- **Indication that the *spo14Δ* has overall increased lipid mixing**

Future Directions

- **Measure vacuole and whole cell phosphatidic acid content using thin-layer chromatography (TLC)**
- **Measure vacuole fusion of WT and *spo14Δ* using a standard inhibitor panel**
- **Create *spo14Δdgk1Δ* and measure effects on membrane fusion and fusion factors**
- **Measure SNARE complex formation using GST-Vam7 pulldown assay to compare WT and spo14Δ vacuoles**

Acknowledgments

Thanks to all of the graduate laboratory staff for their friendship and mentorship, and special thanks to Dr. Fratti for his continued support and guidance over these past years. This research was supported by a grant from the National Institutes of Health (R01-GM101132) to RAF.