Supplemental Materials

Molecular Biology of the Cell

Ling et al.
**Supplemental Methods**

*Electron Microscopy*

Temperature sensitive yeast strains, CBY924/926, were grown overnight at 25°C in synthetic media, then switched to 37°C for two hours. 10 OD units were harvested on a 0.45 um Millipore filter and were processed for electron microscopy as described previously (Chen et al., 2012).

*Recombinant Protein Expression and Purification*

Rosetta (DE3) cells transformed with protein expression plasmids were grown at 37°C to an OD$_{600}$ of 0.4. The bacteria were then shifted to 15°C and protein expression was induced by the addition of 0.5 mM IPTG for 16 hours. GST fusion proteins were purified from Rosetta (DE3) cells with glutathione sepharose 4B (GE Healthcare) according to the manufacture instructions. His-tag fusion proteins were purified from Rosetta (DE3) cells with Ni-NTA agarose (Qiagen) according to the manufacture instructions. Purified protein concentrations were determined by the Bradford assay (BioRad).

*Protein Binding Assay*

For 10xHis-Osh4 and GST-Sec4 binding experiments, glutathione sepharose beads immobilized with GST, GST-Sec4, GDP loaded GST-Sec4$^{S34N}$ or GTP loaded GST-Sec4$^{Q79L}$ were incubated with 10xHis-Osh4 in 1x PBS buffer containing 1mg/ml bovine serum albumin (BSA), 1 mM DTT and 1 mM MgCl$_2$. 
The total volume of incubation was 400 µL. After incubation for 60 min at room temperature, the beads were washed with 1x PBS buffer containing 1 mM DTT and 1 mM MgCl₂ three times. Bound proteins were analyzed by SDS-PAGE and immunoblotting. The same protocol was used to test 10xHis-Osh4 and GST-Ypt32 binding.

**Subcellular Fractionation of Yeast Cell Lysates**

Yeast cells were grown to an OD₆₀₀ of 0.5-0.8 and shifted to appropriate temperatures for 1hr. Cells were harvested and spheroplasted with zymolase 100T. Spheroplasts were lysed mechanically in the lysis buffer (1xPBS, 5mM EDTA, pH=8.0 with protease inhibitors added) by using a homogenizer (Wheaton). Unbroken cells were cleared by centrifuging the lysates at 300x g for 5 min at 4 °C. The supernatants were then centrifuged at 13,000x g for 10 min at 4 °C. The pellets were dissolved in the sample buffer as the P13 fraction. The supernatants from the P13 spin were further centrifuged at 100,000x g for 60 min at 4 °C in the Beckman ultracentrifuge. The pellets were dissolved in the sample buffer as the P100 fraction and the supernatants were collected as the S100 fraction. Proteins in the S100 fraction were precipitated by TCA and finally dissolved in the sample buffer for SDS-PAGE analysis.
Supplemental Figure 1. Osh proteins regulate secretion in yeast cells

(A) Vesicles accumulate in the osh1-7Δ/CEN osh4ts cells at 37°C. osh1-7Δ/CEN OSH4 cells and osh1-7Δ/CEN osh4ts cells were processed for thin section electron microscopy analysis as described in experimental procedures. Electron micrographs of osh1-7Δ/CEN OSH4 cells and osh1-7Δ/CEN osh4ts cells at 37°C are shown. Scale bar, 500 nm.

(B) Sec15-3xGFP and Sec3-3xGFP are mislocalized in osh1-7Δ/CEN osh4ts cells at the non-permissive temperature. osh1-7Δ/CEN OSH4 cells and osh1-7Δ/CEN osh4ts cells were grown overnight at 25 °C in a synthetic medium containing 2% glucose and then shifted to 37°C for 1 hour. Cells shown are representative of over 100 cells observed. Scale bar, 5 μm.

Supplemental Figure 2. Subcellular fractionation of Osh4p in wild type, sec4ts or pik1ts cells.

(A) Distribution of Osh4-3xGFP in different subcellular fractions in wild type, sec4ts or pik1ts cells at indicated temperatures. Cell lysates were subjected to differential centrifugation yielding three different factions as described in supplemental materials and methods. The P13 fraction is enriched in plasma membrane, endoplasmic reticulum and mitochondria. The P100 fraction is enriched in Golgi, vacuole and secretory vesicles. The S100 fraction is enriched in cytoplasmic proteins. Osh4-3xGFP was detected with anti-GFP antibody. 10% of the whole cell lysates were loaded as input.

(B) Quantification of Osh4-3xGFP in the S100 fraction in wild type, sec4ts or pik1ts cells at indicated temperatures. Two independent experiments were performed. The intensity of the protein bands was quantified using ImageJ. The percentage of Osh4-3xGFP in the S100 fraction was calculated. The mean and SD of two experiments are shown.

Supplemental Figure 3. Sec4 does not directly recruit Osh4 to secretory vesicles

(A) Osh4-3xGFP localization does not change in cells expressing high levels of Sec4p from an episomal plasmid (NRB170). Cells shown are representative of over 100 cells observed. Scale bar, 5 μm.

(B) Sec4p does not interact with Osh4p in vitro. 10xHis-Osh4 was incubated with glutathione sepharose beads with immobilized GST, GST-Sec4, GDP or GTP loaded Sec4 mutants. Bound proteins were analyzed by SDS-PAGE and immunoblotting. 0.5% of 10xHis-Osh4 was loaded as input.
(C) Ypt32p does not interact with Osh4p in vitro. 10xHis-Osh4 was incubated with glutathione sepharose beads with immobilized GST, GST-Ypt32, GTP or GDP loaded GST-Ypt32. Bound proteins were analyzed by SDS-PAGE and immunoblotting. 0.5% of 10xHis-Osh4 was loaded as input.

Supplemental Figure 4. PI4P regulates, but is not sufficient to determine, Osh4’s localization
Osh4-3xGFP localization in wild type cells, sac1Δ cells and inp52Δ inp53Δ cells. Scale bar, 2 μm.

Supplemental Figure 5. Osh4 regulates the distribution of PI4P, but not PI(4,5)P₂, in cells
(A) Localization of GFP-PHᵀᵃᶠᶠᵖ¹ and mCherry-Sec4 in wild type or osh4Δ cells. Cells shown are representative of over 100 cells observed. Scale bar, 2 μm.

(B) Localization of mCherry-PHᵀᵃᶠᶠᵖ¹ and Sec7-GFP in wild type or osh4Δ cells. Cells shown are representative of over 100 cells observed. Arrowheads indicate a mCherry-PHᵀᵃᶠᶠᵖ¹ positive small bud that does not contain Sec7-GFP. Scale bar, 2 μm.

(C) Localization of the PI(4,5)P₂ probe GFP-2xPHᴾᴸ𝐶 in wild type cells, osh4Δ cells, sac1Δ cells and inp52Δ inp53Δ cells. Cells shown are representative of over 100 cells observed. Scale bar, 5 μm.

Video S1 Movie of the PI4P probe in wild type cells. PI4P probe is GFP-2xPH(Osh2) as described in manuscript.

Video S2 Movie of the PI4P probe in osh4delta cells. PI4P probe is GFP-2xPH(Osh2) as described in manuscript.
Ling et al., Supplemental Figure 1

A

osh1-7Δ/CEN OSH4 37°C

0.25 vesicles/μm²

osh1-7Δ/CEN osh4Δ 37°C

1.72 vesicles/μm²

B

osh1-7Δ/CEN OSH4 25°C
Sec3-3xGFP
Sec15-3xGFP

osh1-7Δ/CEN OSH4 37°C 1hr
Sec3-3xGFP
Sec15-3xGFP

osh1-7Δ/CEN osh4Δ 25°C
Sec3-3xGFP
Sec15-3xGFP

osh1-7Δ/CEN osh4Δ 37°C 1hr
Sec3-3xGFP
Sec15-3xGFP
Ling et al., Supplemental Figure 2

A

|          | 25°C |           |          | 37°C 1hr |           |          |
|----------|------|-----------|----------|---------|-----------|----------|
|          | In   | P13       | P100     | S100    | In        | P13      | P100     | S100    |
| WT       |      |           |          |         |           |          |          |         |
| sec4<sup>ts</sup> |      |           |          |         |           |          |          |         |
| pik1<sup>ts</sup> |      |           |          |         |           |          |          |         |

![Western Blot Images]

B

![Bar Graph]

% of Osh4-3xGFP in soluble fraction

WT 25°C WT 37°C sec4<sup>ts</sup> 25°C sec4<sup>ts</sup> 37°C pik1<sup>ts</sup> 25°C pik1<sup>ts</sup> 37°C
Ling et al., Supplemental Figure 3

A

Sec4
Normal expression

Sec4
Over-expression

Osh4-3xGFP

DIC

B

Input

GST

GST-Sec4

GST-Sec4 (mutants) + GDP

GST-Sec4 (mutants) + GTP

GST-Sec4 (mutants)

GST

10xHis-Osh4

C

Input

GST

GST-Ypt32

GST-Ypt32 + GTP

GST-Ypt32 + GDP

GST-Ypt32

GST

10xHis-Osh4
Ling et al., Supplemental Figure 4

WT  sac1Δ  inp52Δ inp53Δ

Osh4-3xGFP

DIC
Ling et al., Supplemental Figure 5

A

| DIC | GFP-PH<sub>质膜</sub> | mCherry-Sec4 | Merged |
|-----|---------------------|--------------|--------|
| WT  | ![WT DIC](image)     | ![WT GFP-PH](image) | ![WT mCherry-Sec4](image) | ![WT Merged](image) |
| osh4Δ | ![osh4Δ DIC](image) | ![osh4Δ GFP-PH](image) | ![osh4Δ mCherry-Sec4](image) | ![osh4Δ Merged](image) |

B

| DIC | mCherry-PH<sub>质膜</sub> | Sec7-GFP | Merged |
|-----|--------------------------|----------|--------|
| WT  | ![WT DIC](image)         | ![WT mCherry-PH](image) | ![WT Sec7-GFP](image) | ![WT Merged](image) |
| osh4Δ | ![osh4Δ DIC](image) | ![osh4Δ mCherry-PH](image) | ![osh4Δ Sec7-GFP](image) | ![osh4Δ Merged](image) |

C

| WT  | osh4Δ | sac1Δ | inp52Δ | inp53Δ |
|-----|-------|-------|--------|--------|
| GFP-2xPH (PLC) | ![WT GFP-2xPH](image) | ![osh4Δ GFP-2xPH](image) | ![sac1Δ GFP-2xPH](image) | ![inp52Δ GFP-2xPH](image) | ![inp53Δ GFP-2xPH](image) |
| DIC | ![WT DIC](image) | ![osh4Δ DIC](image) | ![sac1Δ DIC](image) | ![inp52Δ DIC](image) | ![inp53Δ DIC](image) |