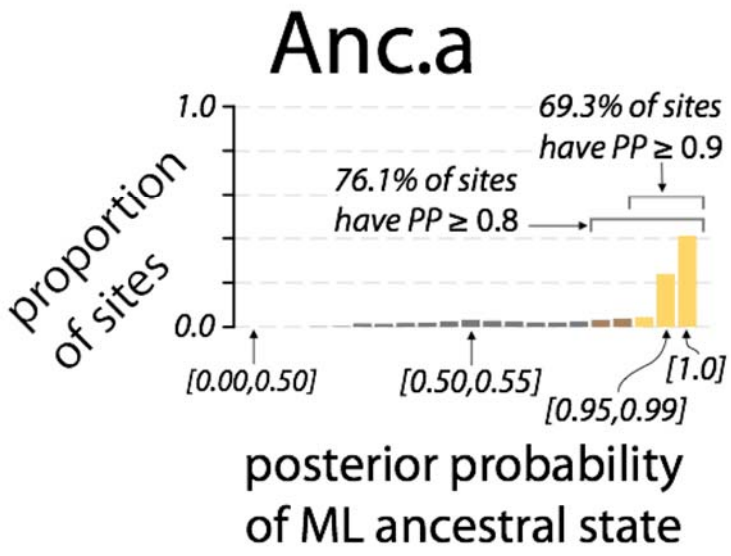
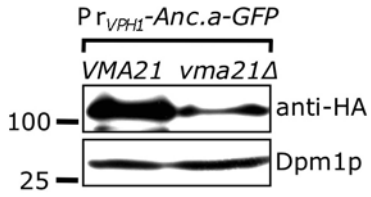
**Supplemental Figure S1**



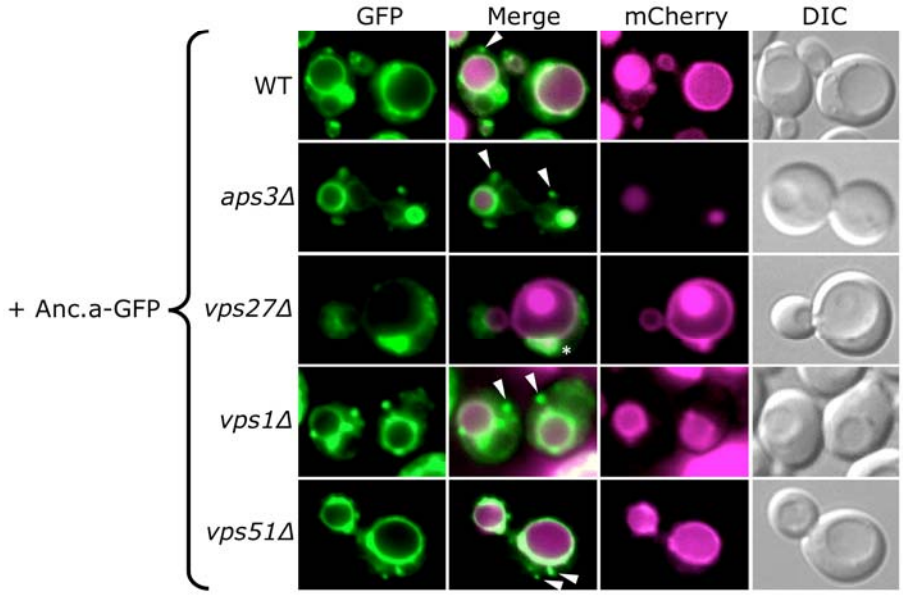

**Supplemental Figure S1**: Support for the Anc.a reconstruction was characterized by binning 5%-sized bins, and counting the proportion of total amino acid sites within each bin.

**Supplemental Figure S2**




**Supplemental Figure S2**: Stability of Anc.a protein is dependent on the presence of the V-ATPase assembly factor, Vma21p. Whole cell extracts were prepared from yeast expressing Anc.a-GFP under control of the *VPH1* promoter (Pr$_{VPH1}$-*Anc.a-GFP*; GFY256), and a strain expressing Anc.a-GFP deleted for *VMA21* (Pr$_{VPH1}$-*Anc.a-GFP vma21Δ*; GFY300). Western blot analysis was performed using anti-HA and anti-Dpm1p (loading control) antibodies. The size of the nearest molecular marker (kDa) is shown.

**Supplemental Figure S3**




**Supplemental Figure S3:** Deletion of various trafficking factors does not alter Anc.a localization in yeast. Yeast deleted for *VPS27* ("class E" vesicular protein sorting gene), *APS3* (AP-3 complex), *VPS1* (dynamin-related GTPase), or *VPS51* (Golgi-associated retrograde protein complex) were transformed with a vector expressing Pr$_{VPH1}$-*Anc.a-GFP* (shown as Anc.a-GFP; pGF246) and a vector expressing mCherry-ALP (pGF242) and visualized by fluorescent and DIC microscopy. White arrows indicate the presence of distinct fluorescent puncta that do not colocalize with mCherry-ALP signal. An asterisk denotes the aberrant multivesicular body in *vps27Δ* cells. The wild-type control is yeast strain SF838-1Dα.

Supplemental Table S1
Subunit a sequences used for reconstruction of Anc.a

| GenBank protein sequences used to generate phylogeny of Anc.a |
|---|
| A.capsulatus_EEH08148 |
| A.capsulatus_EER39772 |
| A.capsulatus_XP_001544538 |
| A.clavatus_XP_001271993 |
| A.dermatitidis_EEQ85687 |
| A.fumigatus_XP_751699 |
| A.nidulans_predicted_XP_663210 |
| A.niger_NP_984223 |
| A.oryzae_predicted_XP_001820188 |
| A.terreus_XP_001213128 |
| A.thaliana_NP_179736 - |
| B.fuckeliana_predicted_XP_001559630 |
| C.albicans_EEQ44988 |
| C.albicans_predicted_XP_712251 |
| C.albicans_predicted_XP_712308 |
| C.cinerea.okayama_predicted_XP_001839899 |
| C.globosum_XP_001225834 |
| C.immitis_XP_001247773 |
| C.neoformans_XP_570271 |
| D.discoideum_Q54E04 - |
| D.hansenii_CAG86124 |
| E.bieneusi_XP_001827782 |
| F.neoformans_AAK81705 |
| G.zeae_predicted_XP_380994 |
| M.globosa_predicted_XP_001732767 |
| M_grisea_predicted_XP_361473 |
| N.crassa_predicted_XP_956054 |
| N.fischeri_predicted_XP_001266930 |
| P.anserina_XP_001909914 |
| P.brasiliensis_EEH20928 |
| P.brasiliensis_EEH40703 |
| P.brasiliensis_EEH45553 |
| P.chrysogenum_CAP97862 |
| P.marneffei_predicted_XP_002151272 |
| P.nodorum_predicted_XP_001796790 |
| P.tritici-repentis_XP_001931738 |
| S.japonicus_XP_002171793 |
| S.pombe_NP_594219 |
| S.sclerotiorum_predicted_XP_001587166 |
| T.stipitatus_XP_002341761 |

Sequences are labeled with the first letter of their genus and the full name of their species followed by their GenBank accession ID number.

Supplemental Table S2
Robustness to uncertainty for Anc.a sequence

| Strain | 100 mM $Ca^{2+}$ | 3.5 mM $Zn^{2+}$ | Strain | 100 mM $Ca^{2+}$ | 3.5 mM $Zn^{2+}$ |
|---|---|---|---|---|---|
| WT | +++++ | +++++ | WT | +++++ | +++++ |
| *vph1*Δ *stv1*Δ | - | - | *vph1*Δ *stv1*Δ | - | - |
| Anc.a | ++++ | +++ | Anc.a | ++++ | +++ |
| G25S | ++++ | +++ | S268T | ++++ | +++ |
| N46K | ++++ | +++ | D274N | ++++ | +++ |
| E76D | ++++ | +++ | E278D | ++++ | +++ |
| I82L | ++++ | +++ | S279T | ++++ | +++ |
| E83D | ++++ | +++ | V302I | ++++ | +++ |
| A90S | ++++ | +++ | T337A | ++++ | +++ |
| K99Q | ++++ | +++ | E347D | ++++ | +++ |
| D111N | ++++ | +++ | E352D | ++++ | +++ |
| E119Q | ++++ | +++ | E361K | ++++ | +++ |
| R120K | ++++ | +++ | K364R | ++++ | +++ |
| A134T | ++++ | +++ | Q392R | ++++ | +++ |
| G144A | ++++ | +++ | S449T | ++++ | +++ |
| T148S | ++++ | +++ | I459V | ++++ | +++ |
| E150D | ++++ | +++ | K484E | ++++ | +++ |
| Q160A | ++++ | +++ | Q496E | ++++ | +++ |
| E165D | ++++ | +++ | I562V | ++++ | +++ |
| S171A | ++++ | +++ | V611I | ++++ | +++ |
| D183E | ++++ | +++ | V632I | ++++ | +++ |
| E218D | ++++ | +++ | H649N | ++++ | +++ |
| E233Q | ++++ | +++ | H650R | ++++ | +++ |
| A236S | ++++ | +++ | Q653E | ++++ | +++ |
| K240R | ++++ | +++ | S666A | ++++ | +++ |
| D252N | ++++ | +++ | D678E | ++++ | +++ |
| F256Y | ++++ | +++ | V747A | ++++ | +++ |
| D261E | ++++ | +++ | I750F | ++++ | +++ |

50 residues from the entire Anc.a sequence were chosen at random to represent the 134 independent sites with posterior probabilities of less than 80%. Single amino acid substitutions to Anc.a were introduced using a modified Quikchange protocol (Zheng *et al.*, 2004). Vectors were transformed into *vph1Δ stv1Δ* yeast and tested on rich media containing 100 mM $Ca^{2+}$ or rich media containing 3.5 mM $Zn^{2+}$. Alternative Anc.a genes were under control of the *STV1* promoter.