Supporting Information

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SI Text

Immunofluorescence Microscopy of Yeast Cells. Cells were prepared for immunofluorescence according to ref. 1 except that only Zymolase 20T was used for spheroplasting. For immunostaining, a mouse monoclonal antibody against ADP-ATP carrier and a rabbit polyclonal antiserum against PhoE were used as primary antibodies. Pictures were taken by using a Zeiss Axiovert 200 M microscope.

In Vitro Import Assay. Radio-labeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine after in vitro transcription from pGEM4 vector encoding the chimeric protein pSu9(1–69)-DHFR. Import experiments were performed according to published procedures (2).

Drop-Dilution Assay. For dilution assays, yeast cells were grown to an OD_{600} of 1.0 in synthetic medium, diluted in 10-fold increments, and then 5 μl of each dilution were spotted onto solid media with different carbon sources.

1. Nasmyth K, Adolf G, Lydall D, Seddon A (1990) The identification of a second cell cycle control on the HO promoter in yeast: Cell cycle regulation of SW15 nuclear entry. *Cell* 62:631–647.
2. Habib SJ, Waizenegger T, Lech M, Neupert W, Rapaport D (2005) Assembly of the TOB complex of mitochondria. *J Biol Chem* 280:6434–6440.

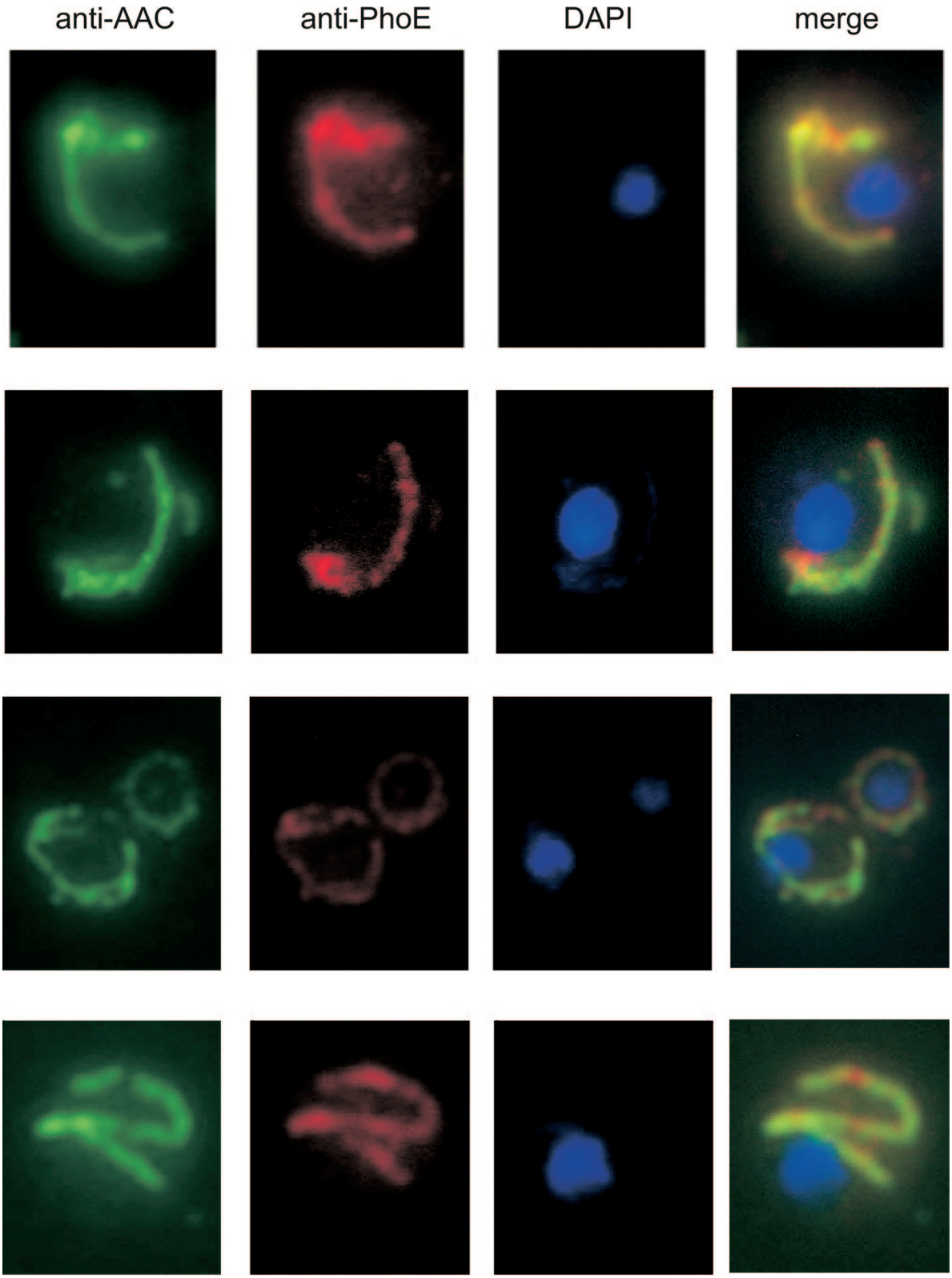

Fig. S1. PhoE colocalizes with mitochondria. Fluorescence images of yeast cells expressing PhoE at high levels were taken after fixation and staining with DAPI (DNA, blue), antibodies against PhoE (red), and ADP-ATP carrier (mitochondrial marker protein, green).

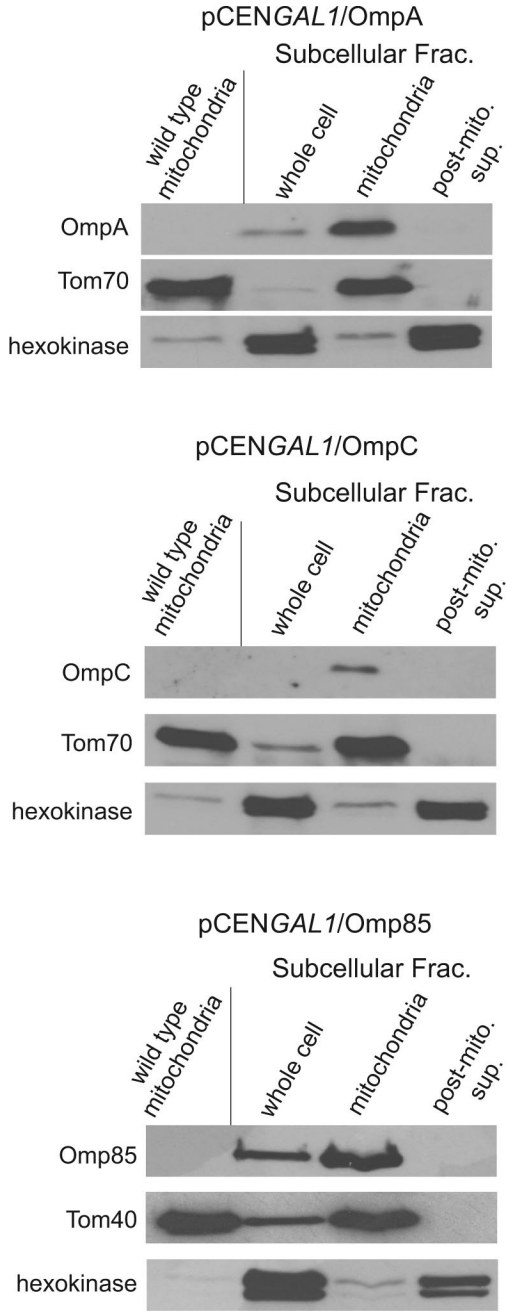

Fig. S2. Bacterial β-barrel proteins are targeted to mitochondria in yeast cells. Whole-cell lysate of cells expressing OmpA, OmpC, or Omp85 and fractions corresponding to mitochondria and postmitochondrial supernatant were analyzed by SDS/PAGE and immunodecoration with antibodies against the bacterial protein, the mitochondrial proteins Tom70 or Tom40, and a control marker protein for the cytosol (hexokinase). Mitochondria isolated from wild-type untransformed cells were coanalyzed as a control.

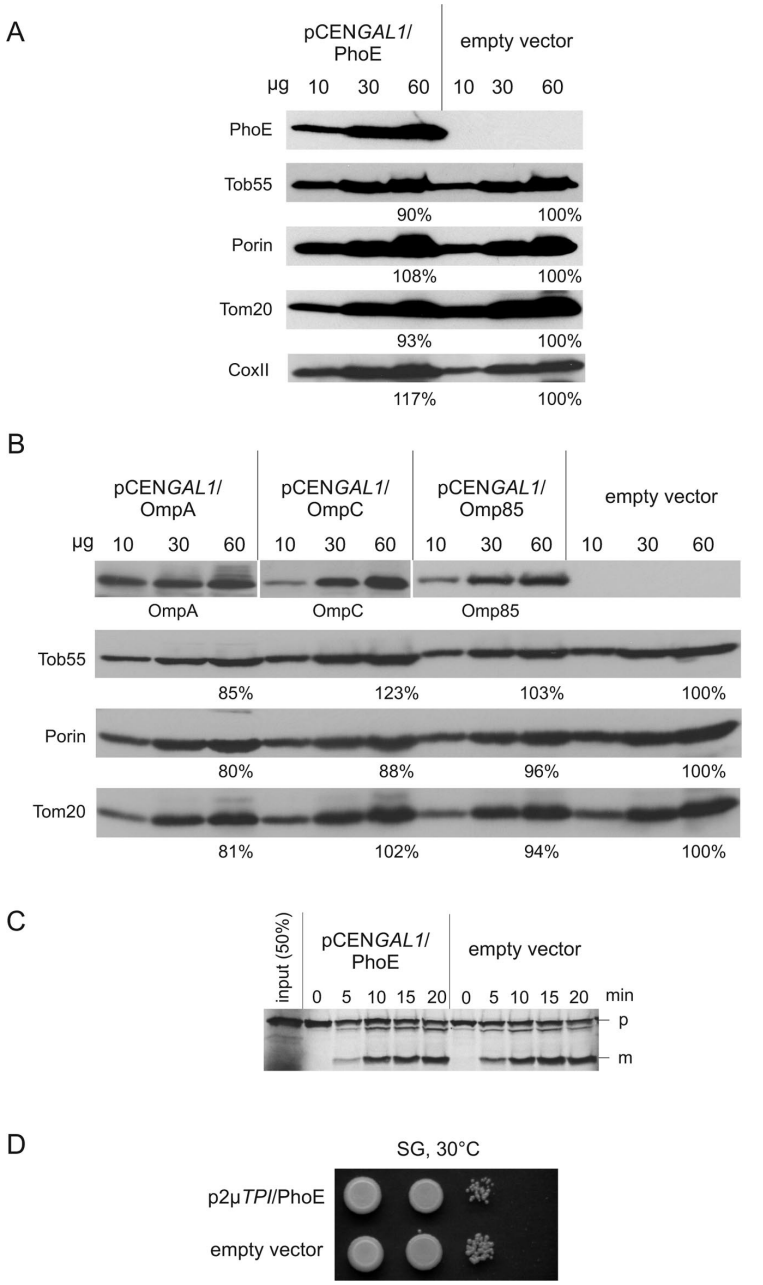

Fig. S3. Bacterial outer-membrane proteins do not interfere with mitochondrial functions. (*A*) Steady-state levels of endogenous mitochondrial β-barrel proteins are unaffected in cells expressing PhoE at high levels. The indicated amounts of mitochondria isolated from cells transformed with either a plasmid encoding PhoE or an empty plasmid were analyzed by SDS/PAGE and immunodetection with antibodies against PhoE, mitochondrial β-barrel proteins (Tob55 and porin), a signal-anchored protein of the outer membrane (Tom20), and a protein of the inner membrane (CoxII). The bands corresponding to 60 μg of mitochondria were quantified and the intensity of the band corresponding to mitochondria isolated from cells transformed with an empty plasmid was defined as 100%. (*B*) Steady-state levels of endogenous mitochondrial β-barrel proteins are unaffected in cells expressing OmpA, OmpC, or Omp85 at high levels. The indicated amounts of mitochondria isolated from cells transformed with either a plasmid encoding the indicated bacterial protein or an empty plasmid were analyzed by SDS/PAGE and immunodetection with antibodies against the bacterial proteins, mitochondrial β-barrel proteins (Tob55 and porin), and a signal-anchored protein of the outer membrane (Tom20). The bands corresponding to 60 μg of mitochondria were quantified and the intensity of the band corresponding to mitochondria isolated from cells transformed with an empty plasmid was defined as 100%. (*C*) Overexpression of PhoE does not reduce the capacity of isolated mitochondria to import precursor proteins in vitro. Mitochondria were isolated from cells harboring either plasmid-encoded PhoE or empty plasmid. Radiolabeled precursors of the matrix-destined protein pSu9 (1–69)-DHFR were incubated with the isolated mitochondria for the indicated time periods. The precursor and mature forms of pSu9 (1–69)-DHFR are indicated as p and m, respectively. (*D*) Overexpression of PhoE does not interfere with growth on a nonfermentable carbon source. Cells harboring either a multicopy plasmid encoding PhoE under the control of the *TPI* promoter or empty plasmid as control were tested by drop dilution assay for their ability to grow on synthetic glycerol-containing medium (SG) at 30 °C. Of note, to examine growth on various carbon sources, the *GAL* promoter in the plasmids expressing the bacterial proteins was replaced by the constitutive *TPI* promoter.

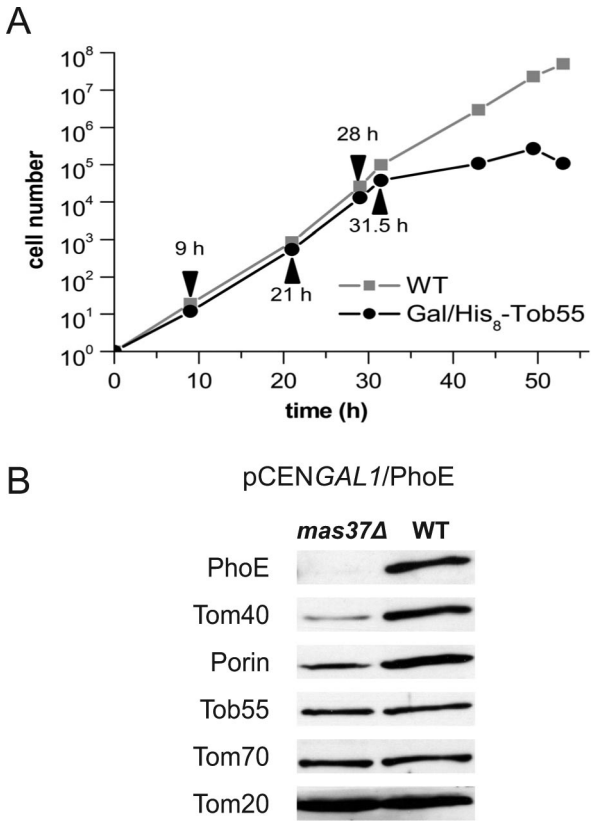

Fig. S4. Steady-state levels of PhoE are highly reduced when the function of the TOB complex is impaired. (*A*) Down-regulation of Tob55 affects cell growth. Cells from wild-type strain or from a strain expressing Tob55 under the control of the *GAL10* promoter (Gal/His_8-Tob55) were shifted from galactose- to glucose-containing medium at time 0. Relative cell number of the cultures was evaluated upon measuring the optical density at the indicated time points. (*B*) Mitochondria isolated from wild-type and *mas37*Δ cells transformed with a plasmid-encoding PhoE were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins.

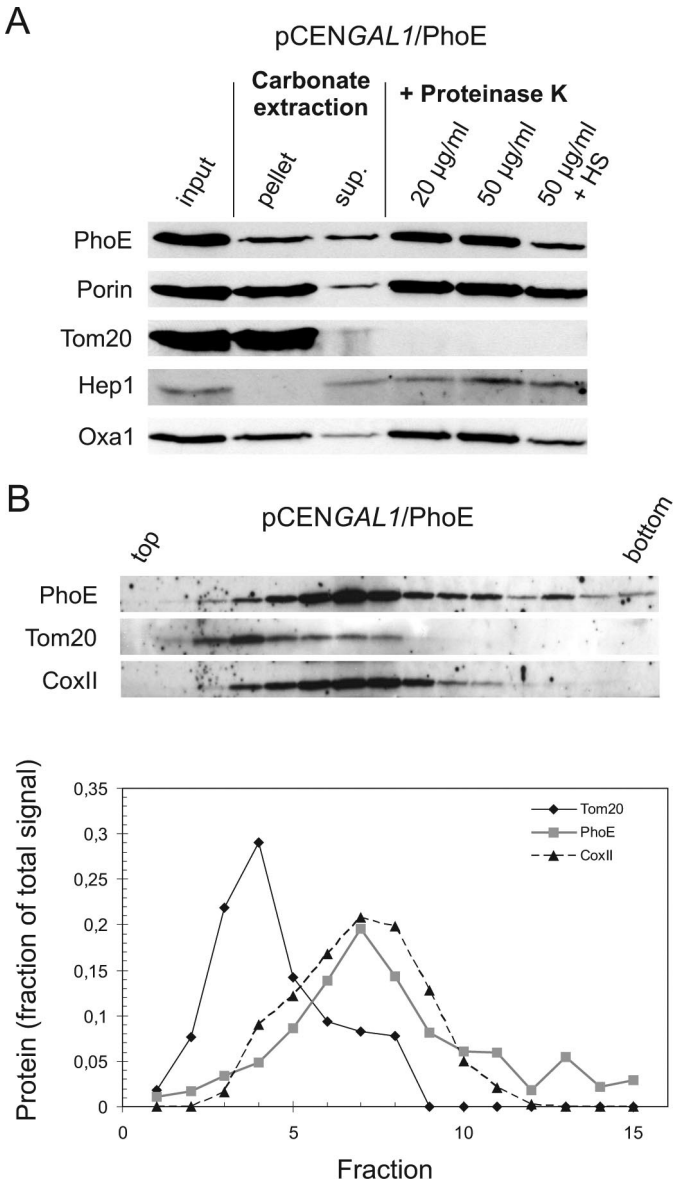

Fig. S5. Overexpressed PhoE is not properly inserted into mitochondrial membranes. (*A*) Assessment of membrane integration and assembly of PhoE. Mitochondria isolated from PhoE-overexpressing cells were loaded directly on SDS/PAGE gel (input) or were subjected first to alkaline extraction and then centrifuged to discriminate between membrane proteins (pellet) and soluble proteins in the supernatant (sup.). Additional aliquots of mitochondria were left intact or were treated by hypoosmotic swelling (HS) before their incubation with the indicated amounts of proteinase K. Proteins were analyzed by SDS/PAGE and immunodecorated with antibodies against the indicated proteins. Porin, protein embedded in the outer membrane; Tom20, outer membrane protein exposed to the cytosol; Hep1, soluble matrix protein; Oxa1, an inner membrane protein exposed to the intermembrane space (IMS). Of note, Hep1 was not affected by the protease treatment, demonstrating the intactness of the inner membrane under these conditions. (*B Upper*) At high expression levels, PhoE is not localized to a distinct membrane. Mitochondria from cells expressing PhoE under the *GAL1* promoter were submitted to hypoosmotic shock and subsequent disruption by sonication. Vesicles were then separated by a sucrose gradient centrifugation. Proteins from fractions were precipitated with trichloroacetic acid, analyzed by SDS/PAGE, and immunodecoration with antibodies against PhoE, the outer membrane protein Tom20, and the inner membrane protein CoxII. (*B Lower*) The bands corresponding to the detected proteins were quantified by densitometry. The relative amount in each fraction is presented as the portion from the total amount of this protein.

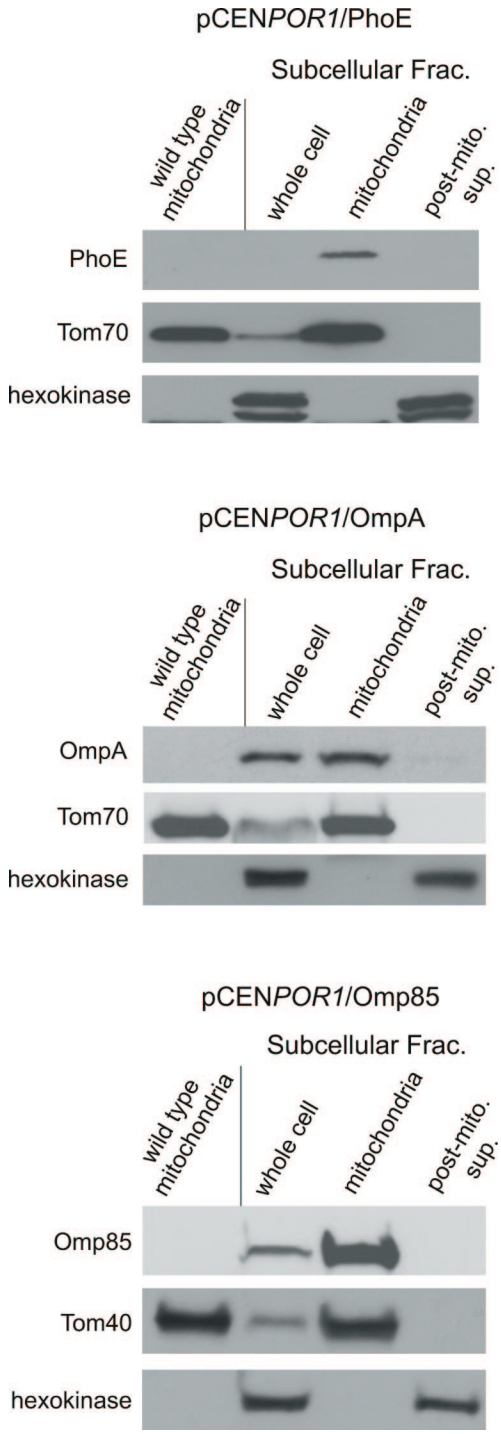

Fig. S6. Bacterial β-barrel proteins expressed at low levels are targeted to mitochondria in yeast cells. Whole-cell lysates of cells expressing the indicated bacterial protein under the control of the *POR1* promoter and fractions corresponding to mitochondria and postmitochondrial supernatant were analyzed by SDS/PAGE and immunodecoration with antibodies against the bacterial β-barrel protein, the mitochondrial proteins Tom70 or Tom40, and a control marker protein for the cytosol (hexokinase). Mitochondria isolated from wild-type, untransformed cells were coanalyzed as a control for the specificity of the antibodies.

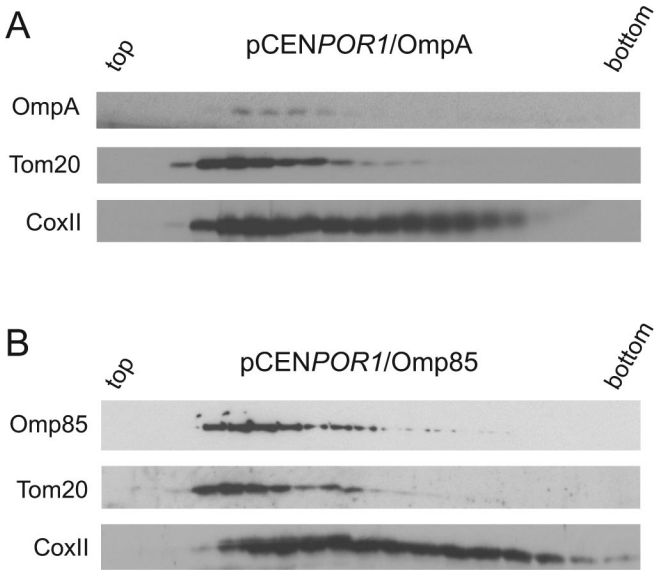

Fig. S7. OmpA and Omp85 are sorted to the mitochondrial outer membrane when expressed at low levels. Mitochondria from cells expressing OmpA (*A*) or Omp85 (*B*) under the *POR1* promoter were submitted to hypoosmotic shock and subsequent disruption by sonication. Inner- and outer-membrane vesicles were separated by sucrose density gradient centrifugation. Proteins from the collected fractions were analyzed by SDS/PAGE and immunodecoration with antibodies against either OmpA or Omp85, the outer membrane protein Tom20, and the inner membrane protein CoxII.

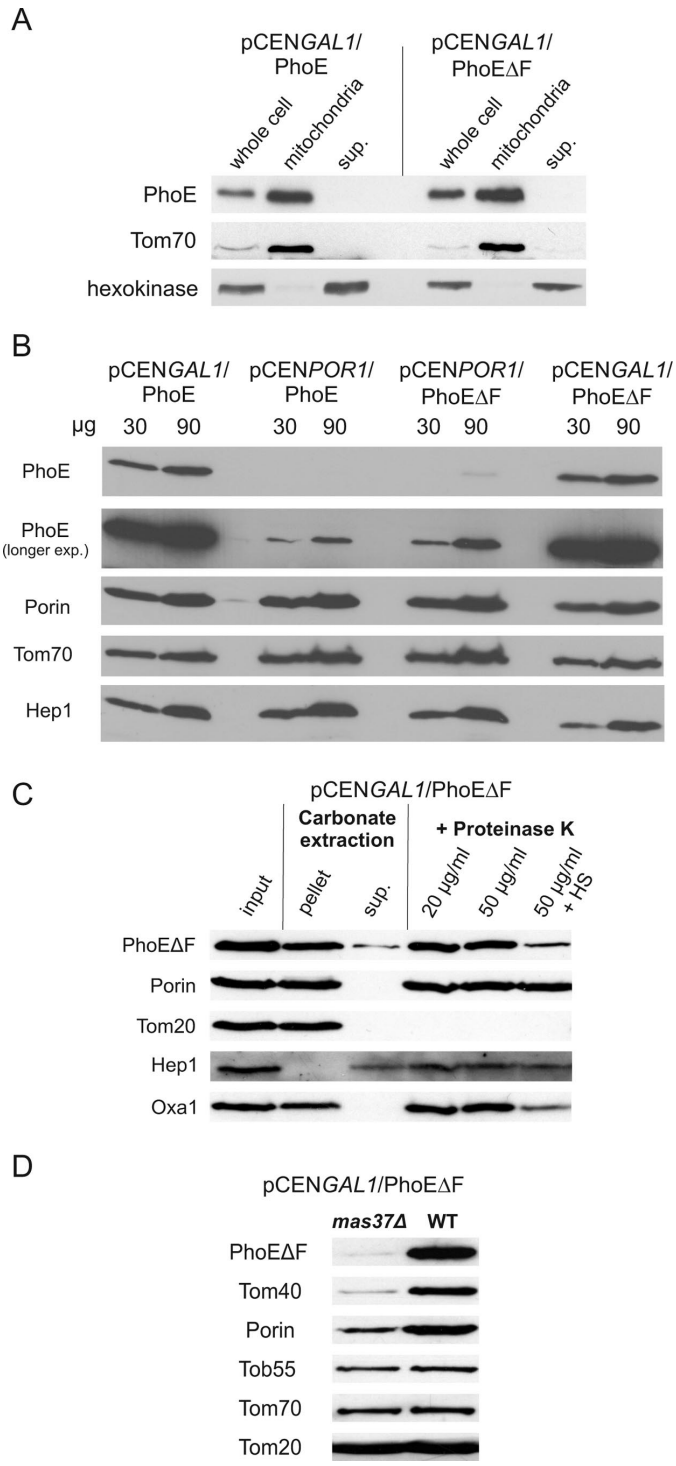

Fig. S8. PhoEΔF is imported into mitochondria but does not insert properly into the outer membrane. (*A*) PhoE variant lacking the C-terminal Phe residue (PhoEΔF) is targeted to mitochondria. Cells expressing full-length PhoE or PhoEΔF were treated and analyzed as described in Fig. S2. (*B*) The indicated amounts of mitochondria isolated from cells transformed with the specified plasmid were analyzed by SDS/PAGE and immunodetection with the indicated antibodies. A longer exposure of the immunodecoration with the antibody against PhoE is presented. (*C*) Mitochondria isolated from PhoEΔF-overexpressing cells were loaded directly on SDS/PAGE gel (input) or were subjected first to alkaline extraction and then centrifuged to discriminate between membrane proteins (pellet) and soluble proteins in the supernatant (sup.). Additional aliquots of mitochondria were left intact or were treated by hypoosmotic swelling (HS) before their incubation with the indicated amounts of proteinase K. Proteins were analyzed by SDS/PAGE and immunodecorated with antibodies against the indicated proteins. Porin, protein embedded in the outer membrane; Tom20, outer membrane protein exposed to the cytosol; Hep1, soluble matrix protein; OxaI, an inner membrane protein exposed to IMS. (*D*) Mas37 is required for import of PhoEΔF into mitochondria. Mitochondria were isolated from either wild-type or *mas37*Δ cells transformed with the plasmid expressing PhoEΔF under the *GAL1* promoter. Mitochondria were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and the indicated mitochondrial proteins.

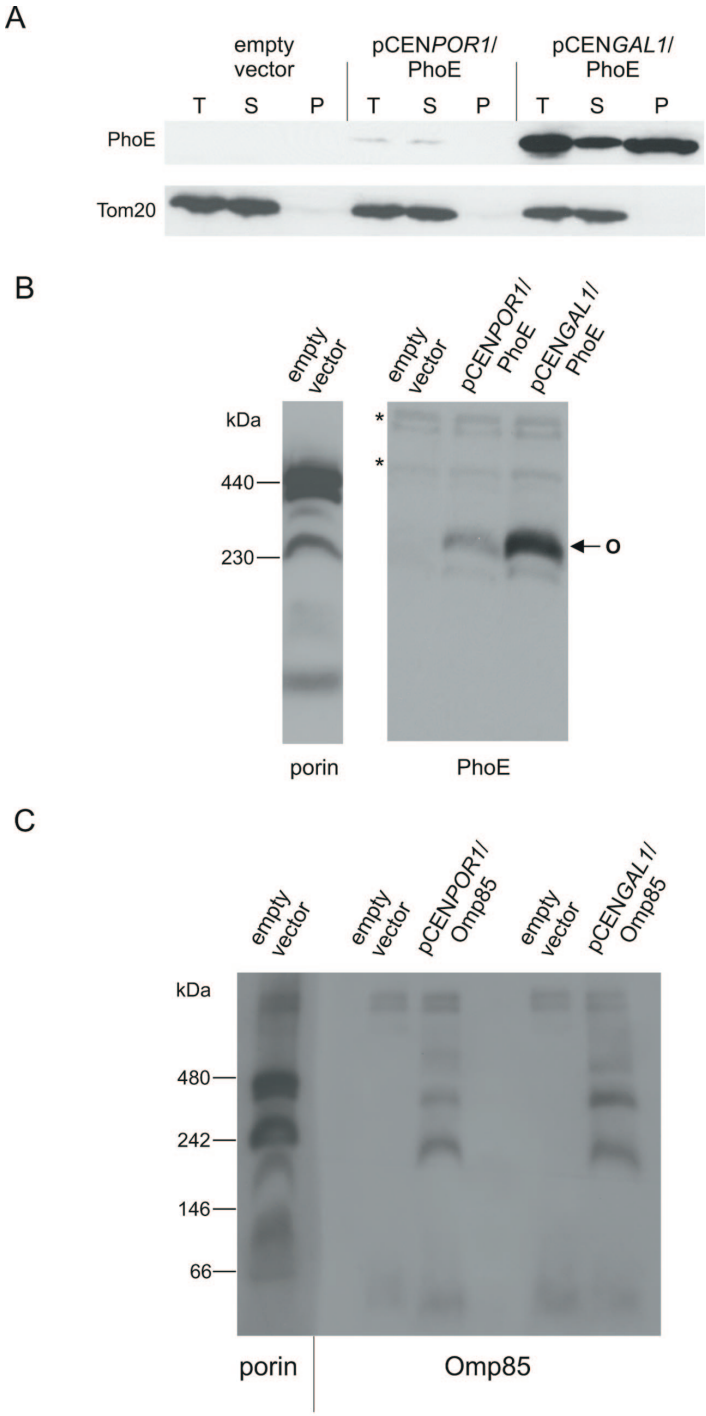

Fig. S9. PhoE and Omp85 form oligomers when expressed in yeast cells. (*A*) Mitochondria were isolated from cells transformed with empty plasmid and from cells expressing PhoE under the control of either *POR1* or *GAL1* promoters. The organelles were solubilized in a buffer containing 1% digitonin (T, Total) and the lysate was separated by centrifugation (36,700 × *g* for 10 min at 4 °C) to soluble fraction in the supernatant (S) and insoluble material in the pellet (P). Samples were analyzed by SDS/PAGE and immunodecoration with antibodies against PhoE and Tom20. (*B*) The supernatant fractions from *A* were analyzed by a 6–13% gradient BN/PAGE and immunodecoration with antibodies against PhoE or porin. The oligomeric form of PhoE and bands with which the antibody against PhoE cross-reacts are indicated with O and an asterisk, respectively. The migration of mitochondrial porin in this system is presented for comparison. Of note, the PhoE oligomer migrates similarly to one of the oligomeric forms of mitochondrial porin. This migration behavior excludes the possibility that the detected band represents folded PhoE monomer. (*C*) Mitochondria were isolated from cells transformed with empty plasmid and from cells expressing Omp85 under the control of either the *GAL1* or the *POR1* promoters. The organelles were solubilized in a buffer containing 1% digitonin. After a clarifying spin as in *B*, the supernatant fractions were analyzed by a 6–13% gradient BN/PAGE and immunodecoration with antibodies against Omp85. Porin oligomeric structures are immunodecorated for comparison.