Supporting Information for:

Restored physiology in protein-deficient yeast by a small molecule channel

Alexander G. Cioffi,[†,‡] *Jennifer Hou* [†,‡] *Anthony S. Grillo,* [†,§] *Katrina A. Diaz,* [†,‡] *and Martin D. Burke*[*,†,‡,§]

[†]Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-6789

[‡]Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 South Matthews Avenue, Urbana, IL 61801

[§]Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Matthews Avenue, Urbana, IL 61801

Materials and Methods

Yeast cell lines and growth conditions. Wild-type *S. cerevisiae* (ATCC 9763), and trk1Δtrk2Δ *S. cerevisiae* (SGY 1528) were maintained with yeast peptone adenine dextrose (YPAD) growth media consisting of 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 0.015 g/L adenine hemisulfate salt (final potassium concentration = 10 mM). For solid media, 20 g/L agar was added to this same mixture. To culture trk1Δtrk2Δ *S. cerevisiae*, additional potassium was added as potassium chloride (100 mM KCl). The media was adjusted to pH 5.0 using citric acid.[1] After autoclave sterilization, dextrose was subsequently added as a sterile 20% w/v solution in water (dextrose solutions were filter-sterilized using a 0.22 μm filter). Liquid cultures were incubated at 30 °C on a rotary shaker (200 rpm). Solid cultures were maintained at 30 °C in an incubator. For solid media containing AmB (AK Scientific), the media was allowed to cool for 15 minutes before addition of AmB at a final concentration of 125 nM. AmB was added to the media as a solution in DMSO. Plates were examined for growth after 24-48 hours of incubation.

Disc diffusion assay. The assay was performed using a previously described protocol.[2]

Broth microdilution assay. The protocol was adapted from the minimum inhibitory concentration assay described in the Clinical and Laboratory Standards Institute document M27-A2[3] with the following modifications: trk1Δtrk2Δ yeast were grown in high potassium (100 mM) YPAD liquid media overnight and transferred to normal potassium YPAD liquid media containing vehicle or the indicated concentration of AmB or C35deOAmB.

Tetraethylammonium block assay. The broth microdilution assay was performed with the addition of 125 nM AmB and the indicated concentration of tetraethylammonium to the normal

potassium YPAD media and the indicated concentration of tetraethylammonium to the high potassium (100 mM) YPAD media.

$^{86}Rb^{+}$ uptake assay. The procedure for $^{86}Rb^{+}$ uptake was adapted from Mulet and coworkers.[4] Overnight yeast cultures were grown as described above. The supernatant was poured off, and the cells were resuspended in sterile water. This wash was repeated two more times. The cells were then resuspended in 40 mL of the potassium starvation/uptake buffer (50 mM succinic acid, 2% glucose, adjusted to pH 5.5 with Tris). After 3 hour incubation in the potassium starvation buffer, cells were centrifuged and washed twice with sterile water. Cells were resuspended in 1 mL of the potassium starvation/uptake buffer. Cell concentration was determined using an INCYTO Neubauer disposable hemocytometer, and wild type *S. cerevisiae* and trk1Δtrk2Δ cells were diluted to the same cell density ($3x10^7$ cells/mL). 700 µL of the yeast suspension was added to a 1.5 mL Eppendorf tube. After a 5 minute pre-incubation in the potassium starvation/uptake medium, AmB or C35deOAmB was added (final concentration 3 µM) as a DMSO solution. $^{86}RbCl$ (1.1 µCi) was then added to the reaction mixtures. Reaction mixtures were vortexed to ensure a homogeneous solution. At the indicated times, the uptake reaction was stopped by taking a 100 µL aliquot from the reaction mixture and diluting with 10 mL of ice-cold 20 mM $MgCl_2$. Cells were then collected via vacuum filtration through a 0.45-µm-pore-size nitrocellulose filter (Millipore HAWP). Cells were washed with two 15 mL aliquots of 20 mM ice-cold $MgCl_2$. Moist filters were transferred to plastic vials for measuring radioactivity. Radioactivity was monitored using a Perkin Elmer Wizard^2 automatic gamma counter. Results are reported in counts per minute as an average of three biological replicates.

Cell viability assay. Assay was adapted from Corliss and coworkers.[5] Wild type and trk1Δtrk2Δ cells were inoculated in high potassium (100 mM KCl) YPAD. Starter cultures were grown at 30

°C for 14-15 hours. Cells were then cultured in high potassium YPAD at 30 °C for 2-2.5 hours. 100 µL of a 0.25 mg/mL propidium iodide (PI, Sigma-Aldrich P4864) solution was prepared. After 2-2.5 hours, cells were centrifuged at 800g for 5 minutes, at 23 °C. The supernatant was decanted, cells were resuspended in 40 mL of high potassium YPAD, vortexed, and centrifuged. The wash step was repeated. After pouring off the supernatant, cells were resuspended in 15 mL of high potassium YPAD, vortexed, and diluted to an OD_{600} of 0.5. To set one (WT, WT + PI, trk1Δtrk2Δ, and trk1Δtrk2Δ + PI) and set two (WT + PI + AmB, trk1Δtrk2Δ + PI + AmB), 26.4 µL of DMSO were added to 1 mL aliquots of WT and trk1Δtrk2Δ, and incubated at 30 °C for 30 minutes. A 1% w/v low gelling temperature agarose (Sigma-Aldrich A9414) was prepared in normal potassium (10 mM) YPAD. When set one incubation completed, cells were pulse centrifuged, supernatant was removed, and resuspended in normal potassium YPAD to wash. Cells were centrifuged and resuspended in 250 µL of normal potassium YPAD. Afterwards, 1 µL of 0.25 mg/mL PI dye was added to appropriate samples and 250 µL of 1% agarose was added. Samples were plated onto microscope slides with cover slips and were incubated in humidity chambers for 24 hours at 30°C. For set two, cells were resuspended in normal potassium YPAD containing 125 nM AmB instead, to wash. Cells were resuspended in 250 µL of normal potassium YPAD containing 250 nM AmB before adding to 1% agarose (to obtain 125 nM AmB final). Confocal microscope (Zeiss LSM700) images were taken at 40x where ~15 random images were taken per slide, per treatment group, per experiment. The total number of PI stained cells was subtracted from the total cells recorded, and divided by total cells to give % viability. At least 200 cells were recorded per treatment group and 4 independent sets of data were obtained.

Doubling time and sustainable restoration of cell growth assay. Wild type and trk1Δtrk2Δ yeast were treated as described in the broth microdilution assay described above, with the trk1Δtrk2Δ yeast treated with 125 nM AmB. The OD_{600} was measured every hour for 24 hours, including a reading at time zero. Wild type yeast were then streaked onto normal potassium YPAD agar plates and AmB-rescued Trk1Δtrk2Δ yeast were streaked onto AmB-containing (125 nM) normal potassium YPAD agar plates. These agar plates were then incubated at 30 °C for about 48 hours. The same procedure was then repeated for over 42 days, measuring the max OD_{600} and doubling time every three days. Doubling time was determined using the following equation: $T_d = (t_2-t_1) \times [\log(2)/\log (q_2/q_1)]$, where t_2 and t_1 represent the time at the two points and q_2 and q_1 represent the OD_{600} values in the exponential phase of growth (OD_{600} from 0.2 to 0.6).

Preparation of amphotericin-ergosterol complex. This complex was prepared and validated as previously described.[6]

Sensitivities to chemical inhibitors assay. This was performed similar to the broth microdilution assay with the following changes: prior to harvesting cells, small molecules AmB, nystatin A1 (Riedel-de-Haen), candicidin (TOKU-E), or mepartricin B (Santa Cruz Biotechnology) and chemical inhibitors nocodazole (Sigma-Aldrich), ebselen (Cayman Chemical),[7] or bafilomycin B1 (Santa Cruz Biotechnology)[8] were prepared as stock solutions in DMSO. Saturated cell cultures were centrifuged for 5 minutes at 1000g. The supernatant was poured off, and cells were resuspended in sterile MilliQ water. Cells were centrifuged again, and supernatant was poured off. The wash step was repeated. The supernatant was poured off, and cells were resuspended in normal potassium YPAD. Cells were diluted with normal potassium YPAD to an OD_{600} of 0.01. Next, an appropriate volume of AmB, nystatin A1, candicidin, or

mepartricin B was added to give a final rescuing concentration of 125 nM, 1000 nM, 8 nM, and 8 nM, respectively. A control was prepared by adding the corresponding volume of DMSO to trk1Δtrk2Δ cells. 195 uL of WT + small molecule, 195 uL of trk1Δtrk2Δ + small molecule, and 195 uL of trk1Δtrk2Δ + DMSO were added to a 96 well plate. Next, 5 µL of DMSO or chemical inhibitor was added to each well, with each concentration tested in triplicate for both WT and trk1Δtrk2Δ. The plate was covered and incubated at 30 °C for 24 hours. A BioTek Synergy H1 Hybrid Reader was used to measure the OD_{600}. Utilizing GraphPad PRISM, data were fitted by nonlinear regression, inhibition dose response, variable slope (four parameters) to yield EC_{50} values with SEM. For statistical analysis, EC_{50} values from the two treatment groups were compared by unpaired t-test.

Supplementary Figure 1

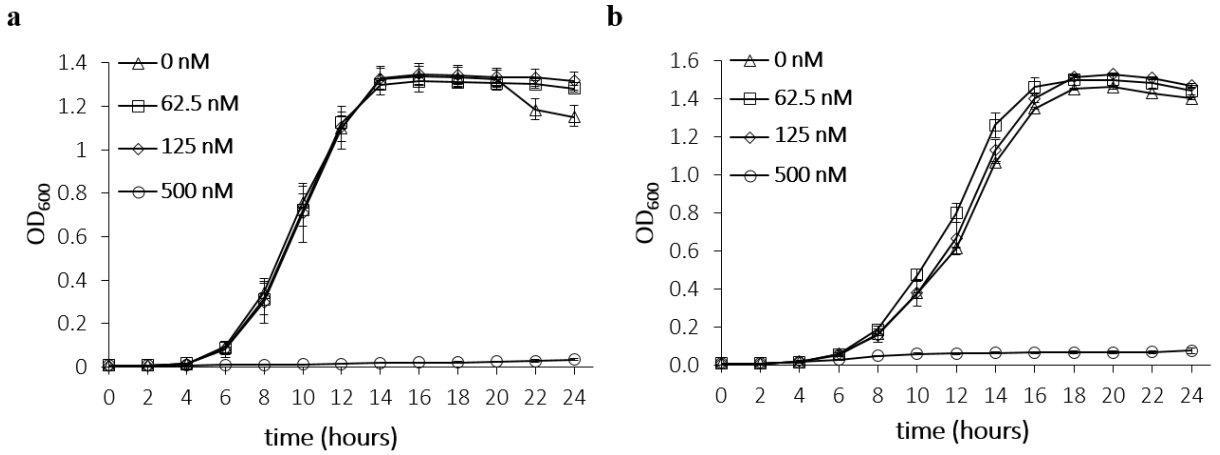

Figure S1. AmB does not cause growth stimulatory effects. (a) AmB does not cause growth stimulatory effects in wild type cells grown in the presence of 100 mM KCl. Curves represent AmB concentrations of 0 nM, 62.5 nM, and 125 nM. No growth is observed at or above the minimum inhibitory concentration of 500 nM AmB. (b) AmB does not cause growth stimulatory effects on trk1Δtrk2Δ cells grown under the same permissive conditions (100 mM KCl). Curves represent AmB concentrations of 0 nM, 62.5 nM, and 125 nM. No growth is observed at or above the minimum inhibitory concentration of 500 nM AmB.

Supplementary Figure 2

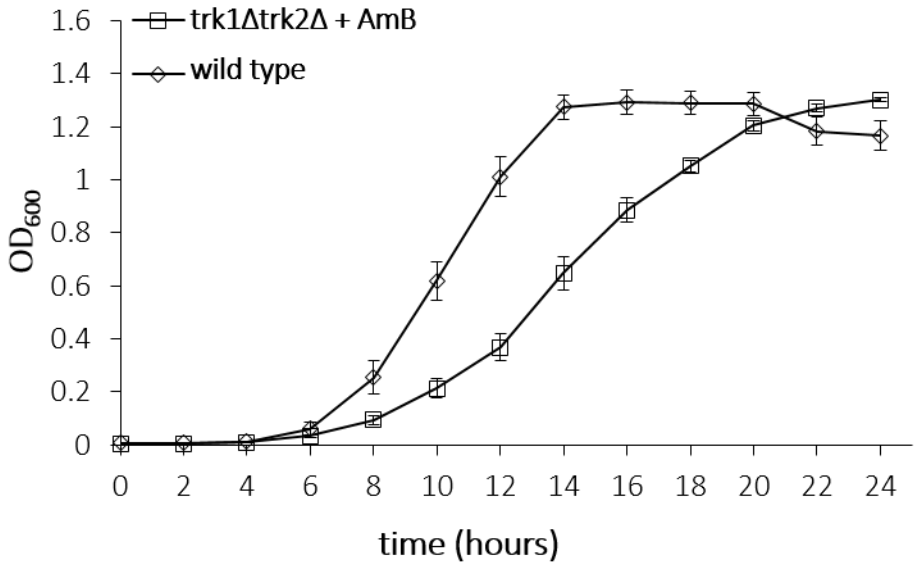

Figure S2. Growth curves for wild type and AmB-rescued mutant. Wild type and AmB-rescued (125 nM) trk1Δtrk2Δ yeast growth curve for 24 hours. The doubling time for wild type and AmB-rescued trk1Δtrk2Δ yeast was 86 ± 2.4 minutes and 144 ± 2.8 minutes, respectively.

Supplementary Figure 3

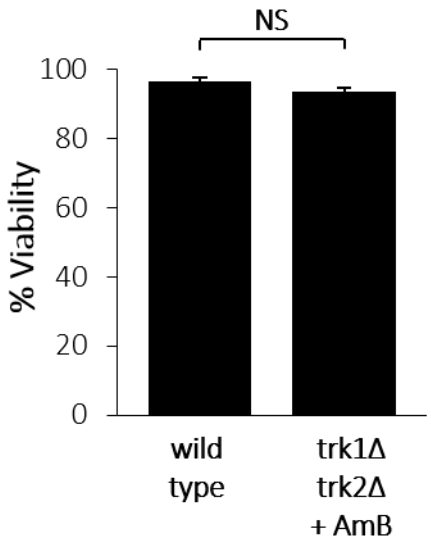

Figure S3. AmB-treated trk1Δtrk2Δ cells show no decrease in cell viability compared to wild type as judged by propidium iodide staining.

Supplementary Figure 4

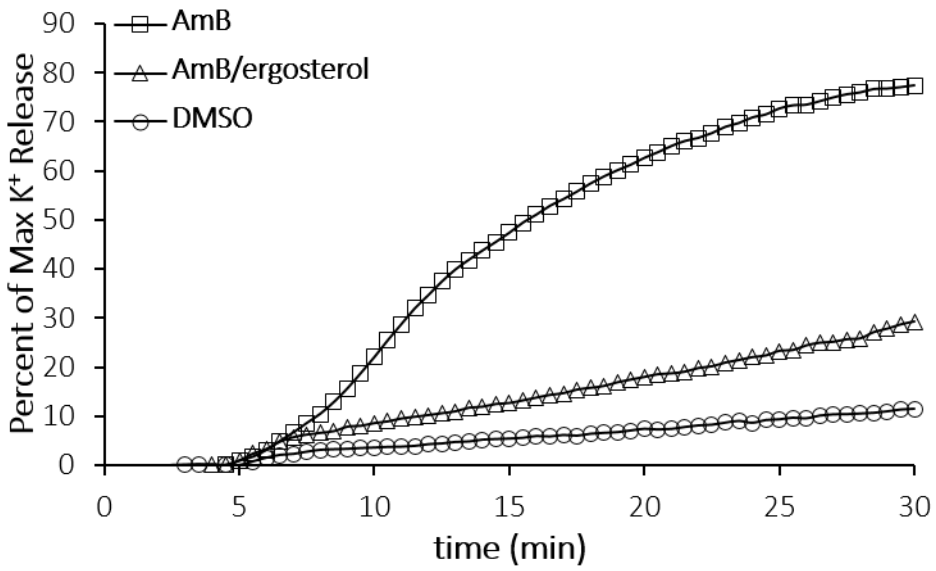

Figure S4. AmB readily permeabilizes wild type *S. cerevisiae* compared to DMSO vehicle. A pre-formed AmB-ergosterol complex still permeabilizes yeast cells. Experiment was performed in triplicate. Plot is representative of each replicate.

Supplementary References

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