Mitochondrial-Derived Compartments Facilitate Cellular Adaptation to Amino Acid Stress

Max-Hinderk Schuler[1#], Alyssa M. English[1#], Tianyao Xiao[1], Thane J. Campbell[1], Janet M. Shaw[1], and Adam L. Hughes[1,2,*]

[1]Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT, 84112, USA

[2]Lead Contact

[#]These authors contributed equally to this work

*Correspondence: hughes@biochem.utah.edu

PAPER SUMMARY

Amino acids are essential building blocks of life. However, increasing evidence suggests that elevated amino acids cause cellular toxicity associated with numerous metabolic disorders. How cells cope with elevated amino acids remains poorly understood. Here, we show that a previously identified cellular structure, the mitochondrial-derived compartment (MDC), functions to protect cells from amino acid stress. In response to amino acid elevation, MDCs are generated from mitochondria, where they selectively sequester and deplete SLC25A nutrient carriers and their associated import receptor Tom70 from the organelle. Generation of MDCs promotes amino acid catabolism, and their formation occurs simultaneously with transporter removal at the plasma membrane via the multi-vesicular body (MVB) pathway. Combined loss of vacuolar amino acid storage, MVBs and MDCs renders cells sensitive to high amino acid stress. Thus, we propose that MDCs operate as part of a coordinated cell network that facilitates amino acid homoeostasis through post-translational nutrient transporter remodeling.

STAR METHODS

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Adam Hughes (hughes@biochem.utah.edu). All unique/stable reagents generated in this study are available from the Lead Contact without restrictions.

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact without restrictions.

Data and Code Availability

- Metabolomics data have been deposited at and are publicly available as of the date of the publication. Accession numbers are listed in the Key Resources Table. All other data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Yeast Strains

All yeast strains are derivatives of *Saccharomyces cerevisiae* S288c (BY) (Brachmann et al., 1998). Strains expressing fluorescently tagged *TOM70*, *TIM50*, *TOM20*, *ILV2, TCD2, BAP2, OAC1, ODC1, MTM1, YMC2* and/or AID*-6xFLAG/6xFLAG tagged *VMA2* from their native loci were created by one step PCR-mediated carboxy-terminal endogenous epitope tagging using standard techniques and oligo pairs listed in Table S2. Plasmid templates for fluorescent epitope tagging were from the pKT series of vectors (Sheff and Thorn, 2004). Plasmids used for AID*-6xFLAG/6xFLAG tagging and integration of GPD-OsTir1 into the *LEU2* locus are described below. Correct integrations were confirmed by a combination of colony PCR across the chromosomal insertion site and correctly localized expression of the fluorophore by microscopy. Deletion strains for *TOM70*, *TOM71*, *TOM20*, *GEM1, PEP4, ILV2, BAT1* and/or *BAT2, VPS27* and/or *DID4* were generated by one step PCR-mediated gene replacement with the indicated selection cassette using standard techniques and oligo pairs listed in Table S2. Plasmid templates for gene replacement were from the pRS series of vectors (Sikorski and Hieter, 1989). Correct insertion of the selection cassette into the target gene was confirmed by colony PCR across the chromosomal insertion site. Yeast strains constitutively expressing *TOM70, TOM20, TIM50* or the indicated mitochondrial carrier protein from the GPD promoter were generated by integration of the expression cassette into yeast chromosome I (199456-199457). Plasmids for integration of the GPD-driven expression cassette are described below. Correct insertion of the expression cassette into chromosome I was confirmed by colony PCR across the chromosomal insertion site.

Wild-type yeast strain AHY4706, which was rendered prototrophic with pHLUM (see below) to prevent complications caused by amino acid auxotrophies in the BY strain background, was used to quantify amino acid dependencies of MDC formation and for analysis of whole cell metabolite levels. *Wild-type* yeast strains AHY5082, AHY7053, AHY7620, AHY8529, AHY8531, AHY6257, AHY10448, AHY10466, AHY10468 and AHY10470 were used for super resolution and/or time-lapse imaging. AHY1447 and AHY10546 were used for indirect immuno fluorescence analysis. *Wild-type* and *gem1Δ* yeast strains AHY4706, AHY4057, AHY6259, AHY7802, AHY10448, AHY10554, AHY10466, AHY10552, AHY10468, AHY10548, AHY10470, AHY10550, AHY7808, AHY7816, AHY7804 and AHY7806 were used for quantification of MDC-dependent removal of proteins from mitochondria and protein enrichment in the MDC. Metabolite analysis was performed in AHY4706, AHY9666, BY4741, AHY4230, AHY9873 and AHY10141. Growth assays were performed with BY4741, AHY4230, AHY9873, AHY10141, AHY9913 and AHY10145. A complete list of all strains used in this manuscript can be found in the Key Resources Table.

Yeast Cell Culture and Media

Yeast cells were grown exponentially for 15 hours at 30 ºC to a maximum density of 6×10^6 cells/mL before the start of all experiments described in the paper, including MDC and spot assays. This period of overnight log-phase growth was carried out to ensure vacuolar and mitochondrial uniformity across the cell population and is essential for consistent MDC activation. Cells were cultured as indicated in media containing high amino acids (1 % yeast extract, 2 % peptone, 0.005 % adenine, 2 % glucose) or low

amino acids (0.67 % yeast nitrogen base without amino acids, 2 % glucose, supplemented nutrients 0.074 g/L each adenine, alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, myo-inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, uracil, valine, 0.369 g/L leucine, 0.007 g/L para-aminobenzoic acid). For growth in medium lacking all amino acids (0.67 % yeast nitrogen base without amino acids, 2 % glucose), cells were cultured in low amino acid medium and then shifted to medium containing no amino acids at time of drug treatment. Where casamino acids were added to low or no amino acid media, casamino acids were added at time of drug treatment to a final concentration of 2 %. For single amino acid re-addition experiments, individual amino acids were added to medium containing low amino acids at the time of drug treatment. All amino acids were added to a final concentration of 20 mg/mL, except indicated otherwise, with the exception of cysteine and tyrosine, which were added at final concentrations of 5 mg/mL and 1 mg/mL respectively, due to toxicity and/or solubility issues. Leucine and methionine catabolites were added at a final concentration of 10 mM at the time of drug treatment. Drugs were added to cultures at final concentrations of concanamycin A (500 nM), cycloheximide (10 μg/mL), rapamycin (250 nM), torin1 (5 μM), antimycin A (40 μM), FCCP (10 μM), oligomycin (10 μM), H_2O_2 (10 μM), $CoCl_2$ (1 mM), tunicamycin (5 μg/ml) and BPS (250 μM). Iron was added to cultures as $(NH_4)_2Fe(SO_4)_2(H_2O)_6$ at a final concentration of 2 mM.

Method Details

Extraction of Whole Cell Metabolites from Yeast

For analysis of whole cell metabolite levels in high amino acid media, cells were grown exponentially in high amino acid media for 15 hours to a maximum density of 6×10^6 cells/mL, resuspended in fresh media to a maximum density of 1.85×10^7 cells/mL, and treated for three hours with the indicated drugs. 5×10^7 total yeast cells were harvested by centrifugation for two minutes at 5000 ×g, washed once with ice cold water, and cell pellets were shock frozen in liquid nitrogen. Glucose tracing experiments were performed as described above, but cells were resuspended in high amino acid media containing 2 % $^{13}C_6$-Glucose (Cambridge Isotope Laboratories). For analysis of whole cell metabolite levels in low amino acid media supplemented with leucine, cells were grown exponentially in low amino acid media for 15 hours to a maximum density of 6×10^6 cells/mL, resuspended in low amino acid media supplemented with 10 mg/ml leucine to a maximum density of 1.85×10^7 cells/mL and treated for three hours with ConcA. 5×10^7 total yeast cells were harvested by fast quenching into four volumes of 100 % methanol at -40 °C (final 80 % methanol), pelleted by centrifugation for two minutes at 5000 ×g in a pre-cooled rotor at -20 °C, washed once with -40 °C cold 80 % methanol, and cell pellets were shock frozen in liquid nitrogen.

Whole cell metabolites were extracted from yeast cell pellets as previously described with slight modifications (Canelas et al., 2009). Briefly, 0.4 µg of the internal standard succinic-d_4 acid (Sigma Aldrich 10907HD) was added to each sample. Subsequently, 5 mL of boiling 75% EtOH were added to each cell pellet, followed by vortex mixing and incubation at 90°C for five minutes. Cell debris were removed by centrifugation for three minutes at 7000 ×g and supernatants were transferred to new

tubes and dried *en vacuo*. Pooled quality control samples were made by removing a fraction of collected supernatant from each sample and process blanks were made using only extraction solvent and no cell culture pellet.

GC-MS Analysis

GC-MS analysis was performed with an Agilent 5977B GC-MS MSD-HES or an Agilent 7200 GC-QToF-MS both using an Agilent 7693A automatic liquid sampler. Dried samples were suspended in 40 µl of a 40 mg/ml O-methoxylamine hydrochloride (MOX) (MP Bio #155405) in dry pyridine (EMD Millipore #PX2012-7) and incubated for one hour at 37 °C in a sand bath. 25 µl of this solution was added to auto sampler vials. 60 µl of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA with 1%TMCS, Thermo #TS48913) was added automatically via the auto sampler and incubated for 30 minutes at 37 °C. After incubation, samples were vortexed and 1 µl of the prepared sample was injected into the gas chromatograph inlet in the split mode with the inlet temperature held at 250°C. A 10:1 split ratio was used for analysis of the majority of metabolites. For those metabolites that saturated the instrument at the 10:1 split concentration, a split of 100:1 was used for analysis. The gas chromatograph had an initial temperature of 60°C for one minute followed by a 10°C/min ramp to 325°C and a hold time of 5 minutes followed by a 100°C/min ramp to 375°C. A 30-meter Phenomenex Zebron AB-5HT with 5m inert Guardian capillary column was employed for chromatographic separation. Helium was used as the carrier gas at a rate of 1 ml/min.

Data was collected using MassHunter software (Agilent). Metabolites were identified and their peak area was recorded using MassHunter Quant. This data was

transferred to an Excel spread sheet (Microsoft, Redmond WA). Metabolite identity was established using a combination of an in-house metabolite library developed using pure purchased standards, the NIST library, and the Fiehn library. Values for each metabolite were normalized to the internal standard in each sample and are displayed as fold change compared to the control sample except otherwise indicated in the figure legend. For isotope tracer analysis the area under the curve for each isotope was extracted using MHQuant software (Agilent). This data was exported as a.csv file and isotopically corrected using an in house modified version of DeuteRater (Naylor et al., 2017). All error bars show the mean ± SE from *N* = three to four biological replicates analyzed in the same GC-MS run.

REFERENCES

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132. 10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2.

Canelas, A.B., ten Pierick, A., Ras, C., Seifar, R.M., van Dam, J.C., van Gulik, W.M., and Heijnen, J.J. (2009). Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. Anal Chem *81*, 7379-7389. 10.1021/ac900999t.

Naylor, B.C., Porter, M.T., Wilson, E., Herring, A., Lofthouse, S., Hannemann, A., Piccolo, S.R., Rockwood, A.L., and Price, J.C. (2017). DeuteRater: a tool for quantifying peptide isotope precision and kinetic proteomics. Bioinformatics *33*, 1514-1520. 10.1093/bioinformatics/btx009.

Sheff, M.A., and Thorn, K.S. (2004). Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. Yeast *21*, 661-670. 10.1002/yea.1130.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.