Functional model of metabolite gating by human voltage-dependent anion channel 2

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SUPPORTING INFORMATION

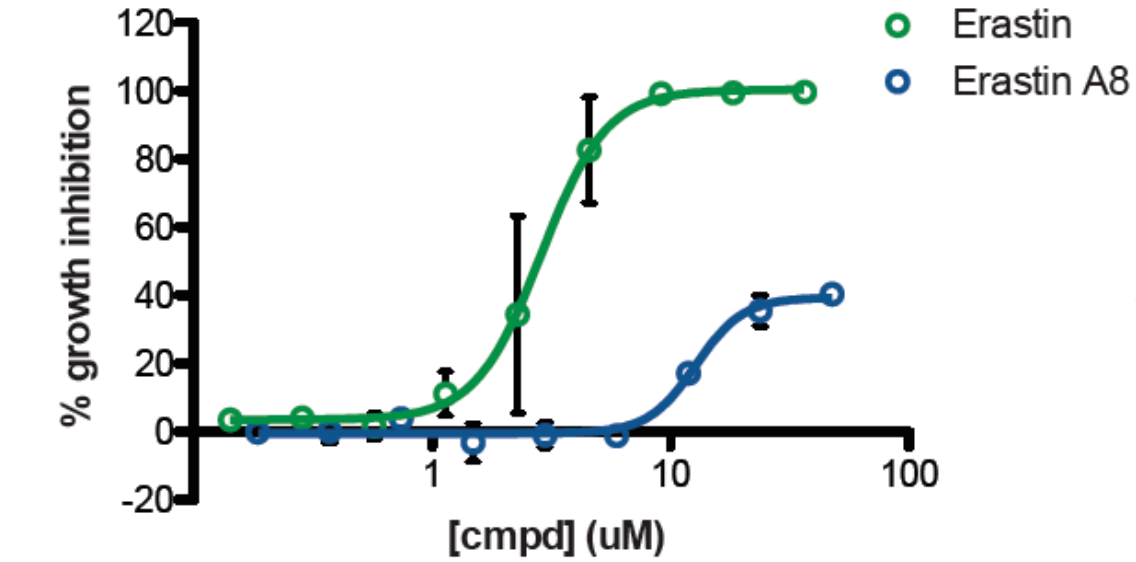
A Rapid Report to

Biochemistry

Erastin is lethal to mutant-Ras-expressing cells

In BJ/TERT/LT/ST/RAS^{V12} tumor cells, treatment with erastin for 24 hours results in dose-dependent lethality (EC_{50} ~ 3 µM.) Treatment with erastin A8 for 24 hours causes minimal cell death over the same concentration range.

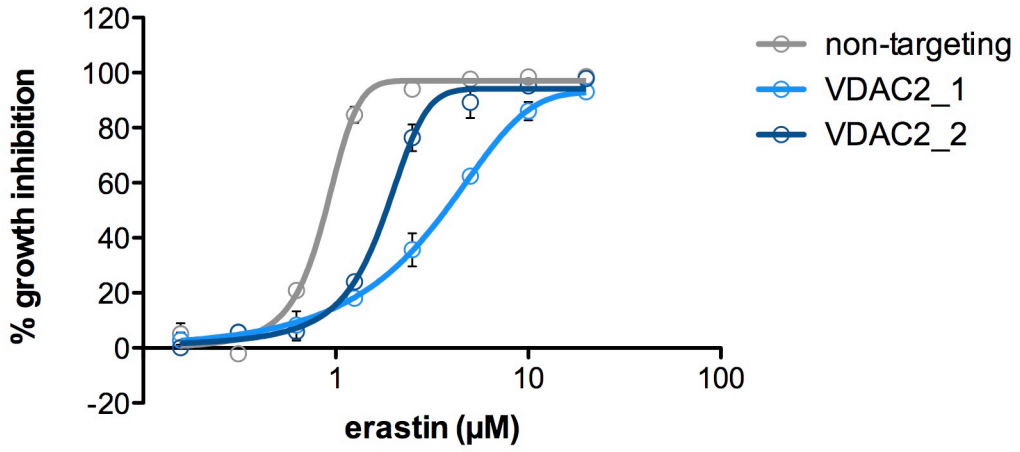
S1.



S1. Erastin, but not erastin A8, inhibits the growth of mutant-Ras-expressing BJ/TERT/LT/ST/RAS^{V12} fibroblasts.

Cell death by erastin is prevented by depletion of VDAC2 expression in Calu-1 lung carcinoma cells

S2



S2. Lentiviral shRNA attenuation of VDAC2 expression in K-Ras mutant Calu-1 tumor cells reduces erastin-induced death.

Cloning of ΔhVDAC2

Full-length hVDAC2 was cloned into, and expressed from, pDEST-17 (Invitrogen) as previously described (Yagoda et al.) To delete the twenty N-terminal amino acids of hVDAC2, the following primers were used:

Forward primer:	5'-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTC **CTT** GGC AAA GCT GCC AGA GAT-3'
Reverse primer:	5' – GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA AGC CTC CAA CTC CAG GGC – 3'

The forward PCR primer was designed to anneal to the VDAC2 cDNA template beginning 60 base pairs in from the start codon. Both primers also contained *attB* recombination sites in the flanking DNA. PCR was performed using pENTR-VDAC2 as template. The PCR product was purified and recombined into pDONR-221 (Invitrogen) according to the manufacturers instructions. The template for ΔhVDAC2 was subsequently recombined into pDEST-17 (Invitrogen) for expression and purification. Conditions for expression and purification were the same as for full-length hVDAC2.

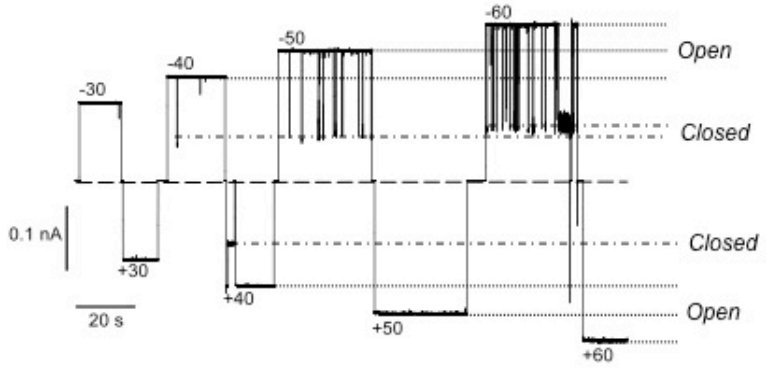
Expression and purification of natural abundance hVDAC2 and ΔhVDAC2

Bacterial cultures (BL21(DE3) E. *coli* transformed with either pDEST17-hVDAC2 or pDEST17-ΔhVDAC2 vector) were grown in 4 x 1 liters LB broth containing 50 mg/L ampicillin to an absorbance of 0.6 (λ=600 nm). Protein expression was induced by 0.4 mM IPTG and 0.2% w/v *L*-Arabinose overnight. Cultures were harvested by centrifugation at 6,000g for 10 min. The pellet was washed with distilled H_2O and resuspended in buffer (20% sucrose, 0.6% Triton-X 100, 5 μg/ml lysozyme) and incubated at 25° C for 10 min. The lysate was then sonicated 2 x 30 s and centrifuged at 15,000 g for 20 min. The pellet was resuspended in resuspension buffer (6 M guanidine-HCl, 100 mM NaCl, 50 mM Tris (pH 8.0)) and incubated for 1 h at 25° C with rotation. The suspension was then centrifuged (20 min, 15,000g), and the supernatant diluted with 100 mM NaCl, 50 mM Tris buffer (pH 8.0) to reach a guanidine-HCl concentration of 4.5 M. The solution was loaded at 4˚C on a Ni-NTA Superflow column (Qiagen) pre-equilibrated with five volumes of resuspension buffer. The column was washed with five

column volumes of wash buffer (100 mM NaCl, 50 mM Tris buffer (pH 8.0), 4.5 M guanidine- HCl), followed by a washing step with wash buffer, containing 25 mM imidazole (low imidazole wash). The protein was refolded on the column after the low-imidazole wash in a buffer containing 0.4% LDAO, 0.1M NaCl and 50mM Tris pH 8.0. The column was washed in three column volumes of the refolding buffer and eluted with 50 mL refolding buffer containing 250 mM imidazole.

Single channel recordings through hVDAC2 demonstrate typical conductance and voltage-gating behavior

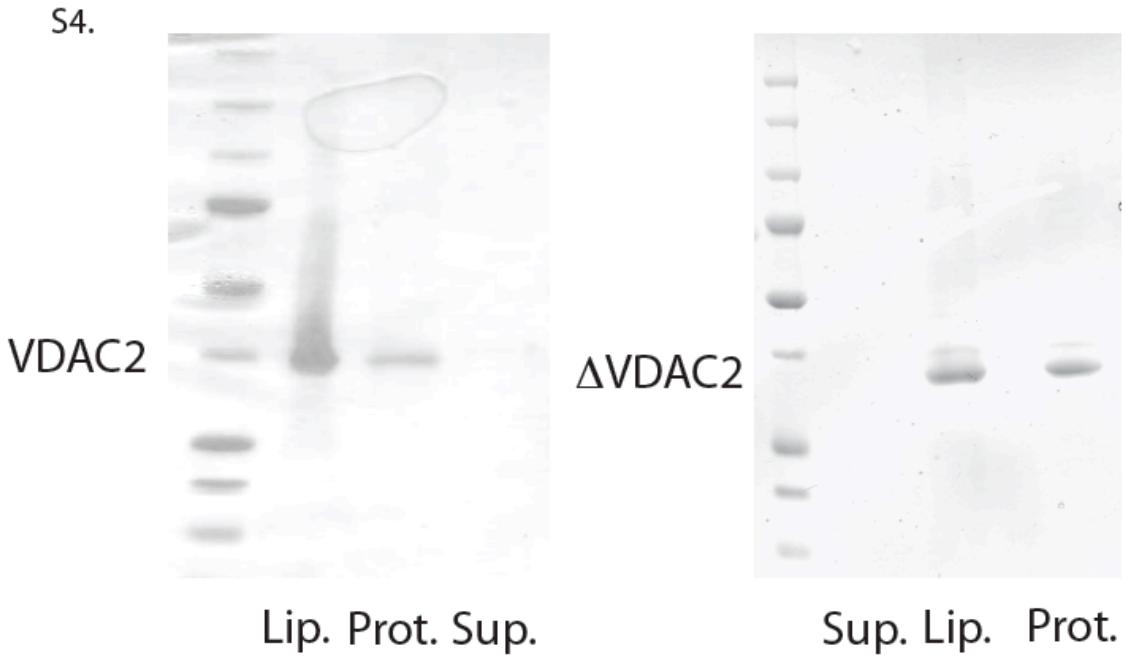
S3



S3. Current trace through a single hVDAC2 channel reconstituted into the planar lipid membrane from LDAO solution. Typical single channel conductance of 4.1nS in 1 M KCl at pH 7.4 and voltage gating at negative potentials is seen. Channel gates under applied voltages > - 50 mV. Applied positive and negative potentials are indicated. Dashed line indicates zero-current level; dotted lines indicate the conductance of the channel in its open state and dashed-and-dotted lines in its closed states. Current records were filtered by using average times of 10 ms. Bilayer membrane was formed from DPhPC.

Precipitation of hVDAC liposomes for ssNMR and NADH gating experiments

Purified, refolded hVDAC2 or ΔhVDAC2 protein was diluted in a 1:1 mass ratio with 50 mM Tris buffer (pH 7.5), 100 mM NaCl, 1 mM DTT, 2 mg/ml phosphatidylcholine, 0.5 mg/m phosphatidylserine, 0.25 mg/ml cholesterol, and 1% LDAO to a total volume of 1 mL per dialysis chamber. The sample was dialyzed into 2 L of dialysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT) for 12 hours, after which the dialysis buffer was changed. We continued dialyzing for 2 days, changing dialysis buffer every 12 h. Precipitate would appear typically after 36 hours, usually containing ≥95% of the protein. The precipitate was subsequently analyzed by PAGE to monitor incorporation of the protein (Figure S4).

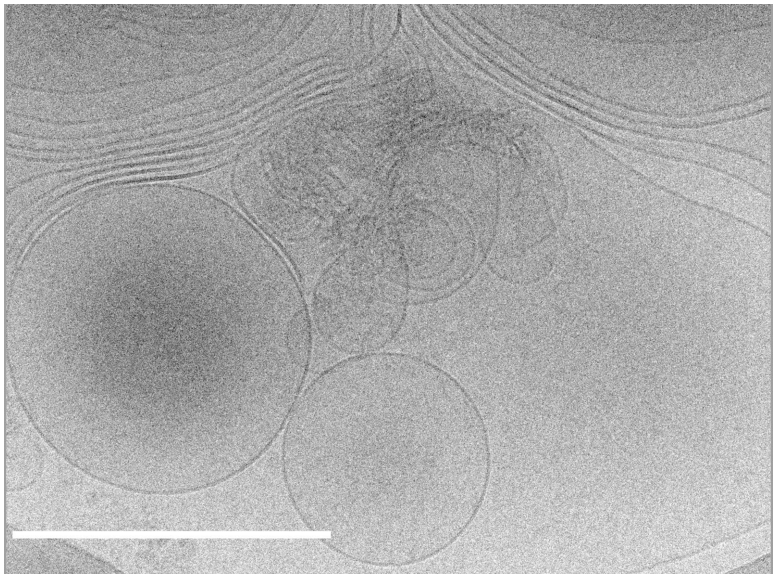


S4. PAGE gel of recombinant, purified hVDAC2 and ΔhVDAC2 before and after incorporation into liposomes. Prot. indicates stock of purified, recombinant protein, Lip.

indicates resuspended liposomes. Sup. shows the complete absence of protein from the supernatant of the precipitate.

Imaging of Liposomes in Precipitate

To determine the presence of liposomes in the precipitate, samples of precipitated hVDAC2 and precipitated, sonicated ΔhVDAC2 were subjected to Cryo-EM imaging. The analysis revealed the presence of mainly unilamellar liposome vesicles with diameters between 50-300 nm. The fraction of multilamellar liposomes and average diameter increased following sonication (Figure S5.)



S5. CryoEM imaging of ΔhVDAC2-containing liposomes.

Expression and purification of hVDAC2 and ΔhVDAC2 for solid state NMR

Bacterial cultures (BL21DE3, E. *coli*, pDEST17 vector) were grown in 4 x 1 l LB broth containing 50 mg/L ampicillin to an absorbance of 0.6 (λ=600 nm), transferred into 2 x 1 L minimal medium, isotopically enriched with $^{13}C_6$-glucose (4.0 g/l), and $^{15}NH_4Cl$ (1.5 g/l).

Protein expression was induced by 0.4 mM IPTG and 0.2% w/v *L*-Arabinose over night. Cultures were harvested by centrifugation at 6,000g for 10 min. The pellet was then washed with distilled H_2O and resuspended in buffer (20% sucrose, 0.6% Triton-X 100, 5 *μg/*ml lysozyme) and incubated at 25℃ for 10 min. The lysate was then sonicated for 2 x 30 s and centrifuged at 15,000 g for 20 min. The pellet was resuspended in resuspension buffer (6 M guanidine-HCl, 100 mM NaCl, 50 mM Tris (pH 8.0)) and incubated for 1 h at 25℃. The suspension was then centrifuged (20 min, 15,000 g), and the supernatant diluted with 100 mM NaCl, 50 mM Tris buffer (pH 8.0) to reach a guanidine-HCl concentration of 4.5 M. The solution was loaded at 4˚C on a Ni-NTA Superflow column (Qiagen) pre-equilibrated with five volumes of resuspension buffer. The column was washed with five column volumes of wash buffer (100 mM NaCl, 50 mM Tris buffer (pH 8.0), 4.5 M guanidine- HCl, 25 mM imidazole). The concentrated hVDAC2 remains in solution while it is unfolded in the presence of guanidine. The protein was precipitated as described for the liposome swelling experiments, then dialyzed for 4 more days with the same dialysis buffer to remove all traces of guanidine

Solid State NMR Samples and Setup

All experiments shown in Fig.2 were measured on a 750 MHz Bruker spectrometer at the New York Structural Biology Consortium (NYSBC), with support from Ansgar Siemer, Yisong Tao and Boris Itin (NYSBC). The experiments were performed on a triple resonance 4 mm probe at a set temperature of -13.5°C. The actual sample temperature was higher than 0 °C, due to r.f. heating during ^{1}H-decoupling and heating from magic

angle spinning. The spinning speed for all MAS experiments was 14 kHz. To avoid sample heating arising from high salt concentrations, we soaked a 50 µL aliquot of the precipitate in distilled H_2O over night. The washed hVDAC2 pellet was packed in a 12 µL Bruker rotor (8.3 mg wet sample mass). The humidity of the protein pellet was not controlled. For the erastin-bound sample, the remainder of the washed aliquot was incubated with excess erastin over night, re-pelleted, and packed in a 4mm Bruker rotor (8 mg wet sample mass).

We acquired four spectra (1794x768, 8 transients each) over a total time of 36 h, and used a 5 s pulse delay to limit sample heating. We observed almost no detuning during ^{1}H-decoupling during acquisition. For each sample we acquired four ^{13}C-^{13}C-DARR spectra, with a cross-polarization (CP) ^{1}H-^{13}C contact time of 500 ms, a CP ^{1}H-power level of 64 kHz, and a ^{13}C-power level to 50 kHz. We acquired 4 x 8 h ^{13}C-^{13}C-DARR spectra (1794 x 768 points) and a spectral width of 42.016 kHz in the direct and the indirect dimension, with a 5 s pulse delay between acquisitions. The spectra were externally referenced to adamantane, then added and processed with Topspin 2.1 software, using 60Hz exponential line broadening in the direct and the indirect dimension, and zero-filling to 8192 points.

Expression and purification of hVDAC2 and ΔhVDAC2 for solid state NMR of liposomes prepared for NADH-gating assay

Bacterial cultures (BL21DE3, E. *coli*, pDest17 vector) were grown in 4 x l LB broth containing 50 mg/L ampicillin to an absorbance of 0.6 (λ=600 nm), transferred into 2 x 1 L minimal medium, isotopically enriched with $^{13}C_6$-glucose (4.0 g/l), and $^{15}NH_4Cl$ (1.5 g/l). Protein expression was induced by 0.4 mM IPTG and 0.2% w/v *L*-Arabinose over night. Cultures were harvested by centrifugation at 6,000 g for 10 min. The pellet was washed with distilled H_2O and resuspended in buffer (20% sucrose, 0.6% Triton-X 100, 5 μg/ml lysozyme) and incubated at 25℃ for 10 min. The lysate was then sonicated for 2 x 30 s and centrifuged at 15,000 g for 20 min. The pellet was resuspended in resuspension buffer (6 M guanidine-HCl, 100 mM NaCl, 50 mM Tris (pH 8.0)) and incubated for 1 h at 25℃. The suspension was centrifuged (20 min, 15,000 g), and the supernatant diluted with 100 mM NaCl, 50 mM Tris buffer (pH 8.0) to reach a guanidine-HCl concentration of 4.5 M. The solution was loaded at 4˚C on a Ni-NTA Superflow column (Qiagen) pre-equilibrated with five volumes of resuspension buffer. The column was washed with five column volumes of wash buffer (100 mM NaCl, 50 mM Tris buffer (pH 8.0), 4.5 M guanidine- HCl, 25 mM imidazole). The protein was refolded on the column after the low-imidazole wash in a buffer containing 0.4% LDAO, 0.1 M NaCl and 50 mM Tris pH=8.0 and well as 0.1 mg/ml cholesterol. The protein was eluted in the same buffer containing 250 mM imidazole.

The lipid mix (20mg phosphatidylcholine, 5 mg phoshphatidylserine, 2 mg cholesterol (water soluble) was dissolved in 12.5 mL 1% LDAO, 50 mM Tris pH 8.0, 0.1 M NaCl dialysis buffer (50mM Tris pH 7.5, 0.1M NaCl, 1mM DTT). Protein was mixed 1:1 with

lipid mix and dialyzed into 2 liters dialysis buffer. The precipitation was collected in a dialysis chamber. The protein was dialyzed in batches for 12- 16 hours at 4°C, with a total volume of 12 mL. The dialysis buffer was exchanged (2L) for continued dialysis for the next 48-72 hours. The uniformly $^{13}C^{15}N$-labeled protein isolated from 4L of minimal medium was precipitated as indicated for the NADH-gating assays and yielded a colorless, dense pellet of 150 µL, after centrifugation in a bench top ultracentrifuge (13,000 g, 2 h). VDAC2 spectra were acquired at a set VT temperature of -40 °C and a spinning rate of 9kHZ on a 400 MHz Varian Infinity spectrometer equipped with a triple resonance HXY 4 mm probe referenced to adamantane (Figure S6). We acquired ^{13}C-^{13}C-DARR spectra, with 32 transients each, a mixing time of 20ms and a spectral width of 50.0 kHz in the direct and the indirect dimension. The spectra were summed up and processed with NMRpipe and analyzed with SPARKY (see Figure S6).

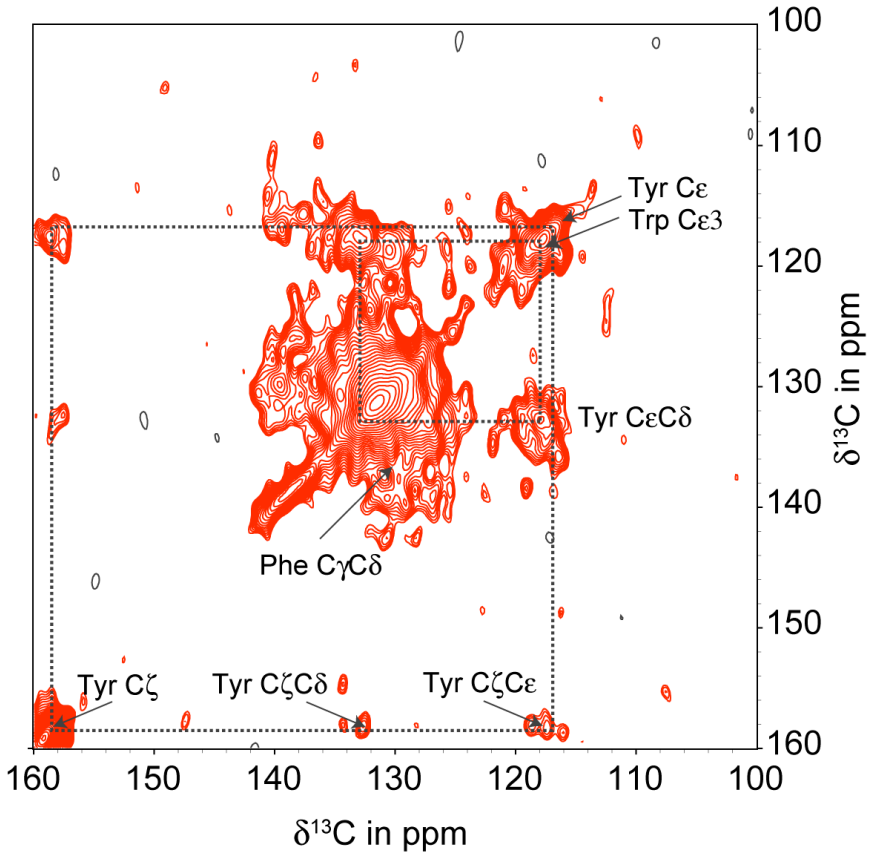
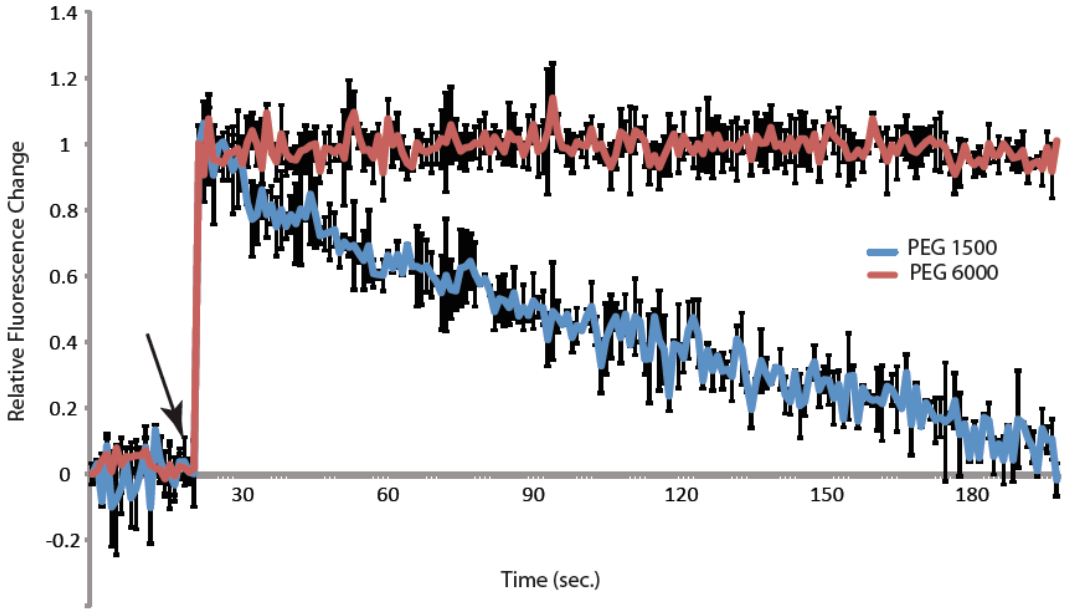


Figure S6 Excerpt of the aromatic region of the ^{13}C-^{13}C-DARR spectrum of VDAC2 at 400MHz (acquired at 9Khz spin rate and a mixing time of 20 ms and 80KHz ^{1}H decoupling at -40°C set VT temperature) shown in red contours. VDAC2 has 31 aromatic residues, Tyr, Trp and Phe. The observed cross peaks correspond well with shifts predicted for ^{13}C shifts of the aromatic ring in Tyr and Phe; dashed lines represent the connectivity within the side chain for Tyr (average shifts in the Biological Magnetic Resonance Data Bank: Tyr ^{13}Cg 127± 4 ppm, ^{13}Cd 132±5 ppm, ^{13}Ce 118 ±4 ppm, ^{13}Cz, 151±27 ppm, Phe: ^{13}Cg 135± 19 ppm, ^{13}Cd 131±4 ppm,).

Liposome swelling assays

Expression and purification of hVDAC2 was performed as stated above up to the low imidazole wash step. Subsequently, unfolded hVDAC2 was eluted from the column with the same buffer, with an imidazole concentration of 250 mM. The eluate was concentrated in an Amicon concentrator to 5% of the initial volume.

To prepare liposomes for osmolyte swelling assays, the protein was refolded in the presence of lipids. The concentrated protein was diluted in a 1:1 volume ratio with 50 mM Tris buffer (pH 7.4), 100 mM NaCl, 1 mM DTT, 5 mg/ml PC, 1.25 mg/m PS, 0.25 mg/ml cholesterol, and 1 % LDAO, to a total volume of 2 mL. The sample was dialyzed into 150 mL dialysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT). The dialysis buffer was changed twice over the course of one hour, until the formation of colorless, fluffy precipitate could be observed. Protein incorporation into the liposomes was observed by SDS-PAGE gel, confirming the presence of pure hVDAC2 in the precipitate, while no protein could be detected in the supernatant. Precipitated hVDAC2 was dialyzed overnight into double-distilled water with 1 mM KCl ad 1 mM $CaCl_2$, then resuspended in the same buffer to a protein concentration of 1 mg/ml. The sample was homogenized by gentle vortexing, then a 30µl aliquot was diluted to 1050 µL with double-distilled water. Light scattering by the sample was measured using a PTI fluorimeter (Photon Technologies International) at 420 nm. A baseline was measured for 1 min, then 30 µl of osmolyte (1M PEG1500 or 0.1M PEG6000) was added, and the light-scattering measured over the course of ten minutes. We observed initial shrinking and re-swelling with PEG1500, but no re-swelling in PEG6000 samples (Figure S7).

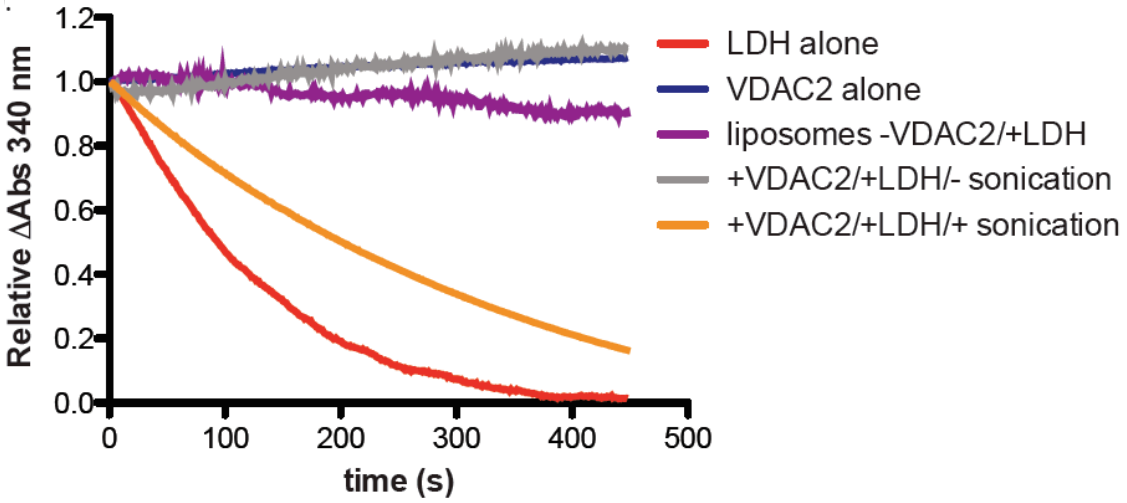


S7. The response of hVDAC2-containing liposomes to osmotic pressure changes induced with nonelectrolytes. Light-scattering was monitored at 420nm to show that PEG 1500 induces shrinking and re-swelling, while PEG 6000, incapable of entering through the VDAC pore does not induce reswelling. Osmolytes were added when indicated by arrow.

NADH gating assays

The assay makes use of the reaction catalyzed by lactate dehydrogenase (LDH), an enzyme that converts pyruvate and NADH to lactate and NAD^+. We introduced bovine heart LDH (Sigma) into VDAC-containing liposomes by sonication. Precipitated liposomes containing ~1mg of VDAC2 were pelleted by centrifugation in a 4°C tabletop centrifuge at 10,000 rpm for 10 min. The pellet was resuspended in 500 µL PBS, then mixed with 20 units LDH. The sample was sonicated for 12 sec on a low power setting (36-40 J of energy output) using a Branson Sonifier, after which the liposomes were spun down as before, resuspended in 500 µL of cold PBS and spun again. The washing step was repeated twice. The liposomes were centrifuged one more time, and the pellet was resuspended in 250 µL PBS per mg of protein.

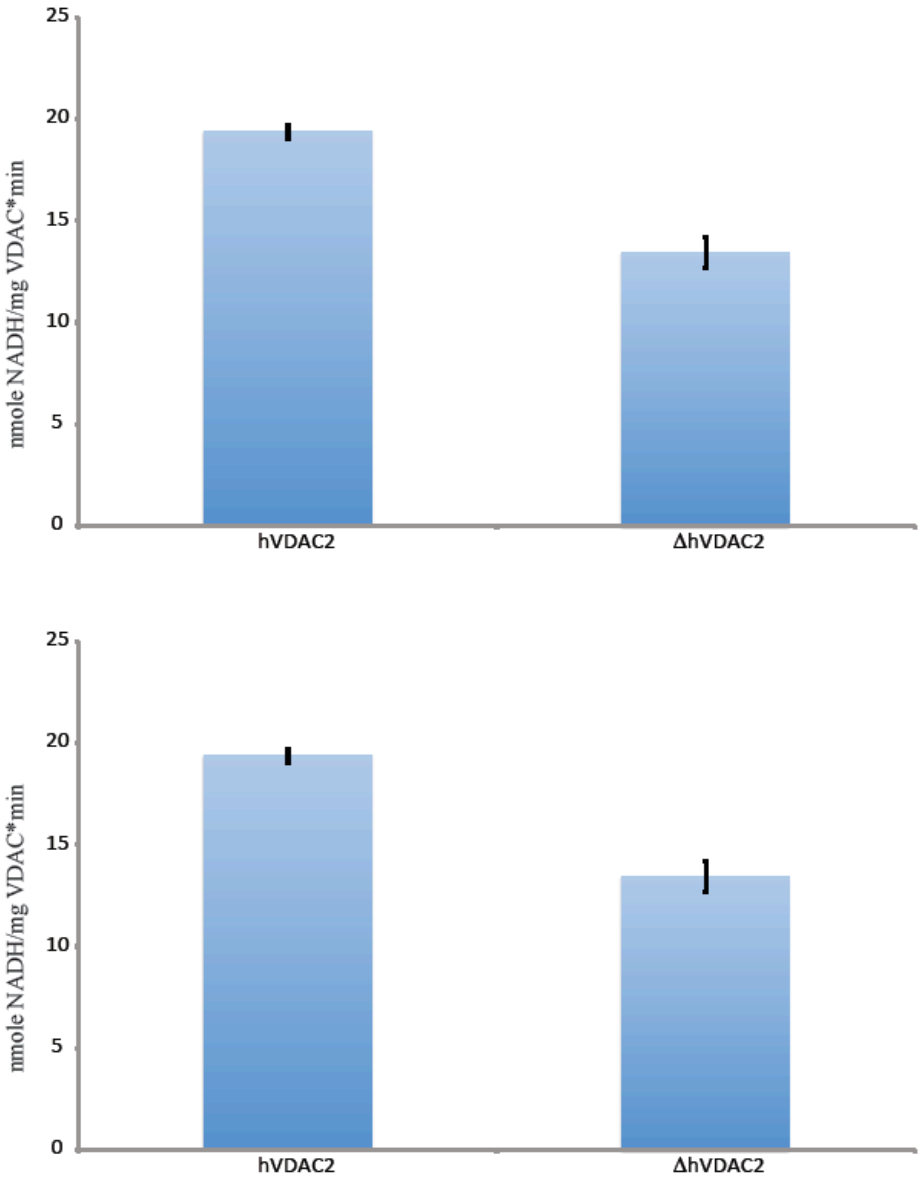
For the NADH-gating assay, we added 10-15 µL precipitate to 500 µL PBS buffer supplemented with 30 µL of 100 mM sodium pyruvate and 0.5 µL DMSO, to control for the amount of DMSO added with erastin or erastin A8. The solution was transferred in to a quartz cuvette and placed in a spectrophotometer (Beckman-Coulter, System Gold 168). After monitoring the baseline for 30 seconds, NADH was added to a final concentration of 84.6 µM, and absorbance was monitored at 340 nm (Figure S8).



S8. LDH is incorporated into liposomes, enabling detection of NADH gating. LDH in solution (red) rapidly degrades NADH, while liposomes containing hVDAC2 alone do not (blue). hVDAC2 liposomes incubated with LDH but not sonicated do not show NADH oxidation after LDH is removed by washing (gray). hVDAC2 liposomes incubated with LDH and sonicated show NADH oxidation even after excess LDH removal by washing, indicating LDH incorporation. Empty liposomes sonicated with LDH show very little NADH oxidation (purple) indicating that VDAC2 is responsible for NADH gating in hVDAC2 liposomes.

NADH transport in the liposomes is limited by the throughput of hVDAC2 channels, and thus is the rate-limiting step in the NADH oxidation reaction. To monitor erastin and erastin A8's effect on hVDAC2, we calculated reaction rates based on the steady-state rate of NADH depletion in the first two minutes. These reaction rates showed that

hVDAC2 liposomes were 44.2±2.4% more permeable to NADH than ΔhVDAC2 (Figure S9). These values are about 2-fold lower than those reported by Xu. et al in isolated yeast mitochondria containing a knock-in of murine VDAC2, which may be due to isoform specificity or simply a difference in model systems.



S9. ΔVDAC2 shows lower NADH gating activity than the wild-type.