

**Supplemental Information**

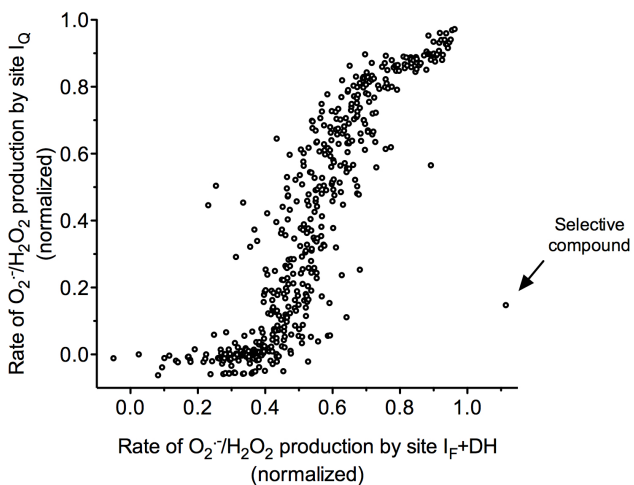
**Suppressors of Superoxide-H<sub>2</sub>O<sub>2</sub> Production at Site**

**I<sub>Q</sub> of Mitochondrial Complex I Protect against Stem**

**Cell Hyperplasia and Ischemia-Reperfusion Injury**

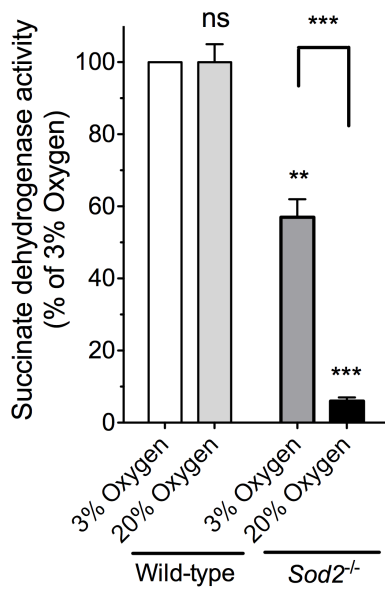
**Martin D. Brand, Renata L.S. Goncalves, Adam L. Orr, Leonardo Vargas, Akos A. Gerencser, Martin Borch Jensen, Yves T. Wang, Simon Melov, Carolina N. Turk, Jason T. Matzen, Victoria J. Dardov, H. Michael Petrassi, Shelly L. Meeusen, Irina V. Perevoshchikova, Heinrich Jasper, Paul S. Brookes, and Edward K. Ainscow**

## SUPPLEMENTAL INFORMATION



**Figure S1. Comparison of the extent of suppression of superoxide/ $H_2O_2$  production from site  $I_Q$  and site  $I_F+DH$  in isolated rat skeletal muscle mitochondria. Related to Table 1.**

Data are for the 501 compounds with  $IC_{50}$  against site  $I_Q < 0.32 \mu M$  identified in Step 4 in Table 1, assayed at  $10 \times IC_{50}$  against site  $I_Q$  in parallel assays of site  $I_Q$  and site  $I_F+DH$  (step 5 in Table 1). The one compound selective for site  $I_Q$  is highlighted.



**Figure S2. Succinate dehydrogenase activity in astrocytes from wild-type and *Sod2*<sup>-/-</sup> mice. Related to Experimental Procedures and Fig. 3.**

Data are scaled to wild-type, 3% oxygen and are means  $\pm$  SE (n = 6). Statistics compare values to wild-type, 3% oxygen or as indicated. ns, not significant; \*\*p < 0.01; \*\*\*p < 0.001 by repeated measures ANOVA followed by Tukey's post-hoc test.

**Table S1. Small molecule screening data. Related to Table 1.**

Category	Parameter	Description
Assay	Type of assay	In vitro biochemical assay using intact isolated rat skeletal muscle mitochondria and an Amplex UltraRed-based H <sub>2</sub> O <sub>2</sub> detection system.
	Target	The site in mitochondrial complex I that generates superoxide/H <sub>2</sub> O <sub>2</sub> during reverse electron transport; notionally the ubiquinone-binding site (site I <sub>Q</sub> of Complex I, NADH:ubiquinone oxidoreductase; EC 1.6.5.3).
	Primary measurement	Endpoint measurement of H <sub>2</sub> O <sub>2</sub> produced from site I <sub>Q</sub> driven by succinate and inhibited by rotenone.
	Key reagents	Amplex UltraRed (Life Technologies), Superoxide dismutase (Sigma), Horseradish peroxidase (Sigma).
	Assay protocol	Described in "Supplemental Experimental Procedures" under "Ultra high-throughput primary screens: sites III <sub>Qo</sub> , I <sub>Q</sub> and II <sub>F</sub> , and site I <sub>F</sub> +DH".
	Additional comments	Parallel screens of superoxide/H <sub>2</sub> O <sub>2</sub> produced from the outer ubiquinone binding site of complex III (site III <sub>Qo</sub> of the cytochrome bc <sub>1</sub> complex; EC 1.10.2.2), the flavin binding site of complex II (site II <sub>F</sub> of succinate dehydrogenase; EC 1.3.5.1) and the flavin binding site of complex I (site I <sub>F</sub> of NADH:ubiquinone oxidoreductase; EC 1.6.5.3) plus 2-oxoacid dehydrogenases were used to identify hits selective only for superoxide/H <sub>2</sub> O <sub>2</sub> production by site I <sub>Q</sub> . For details of all assays in 96-well format see Orr, A.L. et al. (2013) Free Rad. Biol. Med. 65, 1047-1059 and Orr, A. L. et al. (2015) Nat. Chem. Biol. 11, 834-836
Library	Library size	635,000
	Library composition	The GNF Academic Screening Collection consists of a collection of compounds that were selected after applying proprietary algorithms designed to select for optimal compound properties and eliminate undesirable functional groups.
	Source	Composed from multiple sources.
Screen	Format	1536-well plates.
	Concentration(s) tested	10 μM, 1% v/v DMSO.
	Plate controls	DMSO vehicle as negative control.
	Reagent/compound dispensing system	GNF Systems Washer Dispenser for reagents; GNF Systems Pintool for compounds.
	Detection instrument and software	BMG Labtech PHERAstar Plus microplate reader (λ <sub>ex</sub> = 540 nm, λ <sub>em</sub> = 590 nm); Data was analyzed and hit selection performed using GNF proprietary software.
	Assay validation/QC	Median Z' score = 0.19, Median assay window = 7.1.
	Correction factors	Signal normalized to median signal across plate.
	Normalization	Endpoint measurements normalized as percent of DMSO controls.
Post-HTS analysis	Hit criteria	Hits in the I <sub>Q</sub> assay were first selected based on >50% inhibition. Compounds that also had <50% effect in the III <sub>Qo</sub> and <45% inhibition in the II <sub>F</sub> assay were chosen as selective suppressors of superoxide/H <sub>2</sub> O <sub>2</sub> production by site I <sub>Q</sub> and tested further.
	Hit rate	5.5% (34,714/635,000).
	Additional assay(s)	Additional counterscreens and secondary screens outlined in "Experimental Procedures" and "Supplemental Experimental Procedures" and summarized in Table 1.
	Confirmation of hit purity and structure	LC-MS from powder supply at reconfirmation.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES, related to Experimental Procedures.

### Screening

In contrast to Orr et al. (2015), where we selected only for suppressors of site III<sub>Qo</sub>, compounds were chosen for follow-up (step 1 in Table 1) if the inhibition in the I<sub>Q</sub> assay was greater than 50% whereas inhibition in the II<sub>F</sub> assay was less than 45% and inhibition in the III<sub>Qo</sub> assay was less than 40%. This initial selection yielded 34,714 compounds. To decrease this number, hits were assigned by chemical structure into clusters sharing a Tanimoto similarity greater than 80%. The three most active compounds in each cluster were then selected (step 2 in Table 1), yielding 13,555 compounds for confirmation.

These compounds were retested in triplicate in the initial screening assays and also tested for mitochondrial uncoupling or inhibition, measured by fluorescence of the mitochondrial membrane potential-sensitive dye tetramethylrhodamine methyl ester (TMRM), and in a viability assay in HEK-293 cells sensitized to mitochondrial inhibition by growth in glucose-free, galactose-containing medium. Compounds were chosen as selective against site I<sub>Q</sub> if they suppressed the median H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub> more than 70%, with less than 20% change in III<sub>Qo</sub>, II<sub>F</sub> and TMRM assay signals, and less than 20% toxicity in the HEK-293 viability assay (step 3 in Table 1). Compounds were also excluded if more than one other compound in the chemical cluster showed toxicity. This selection yielded 3680 compounds.

The compounds were then run in an eight-point dose-response to determine the IC<sub>50</sub> against H<sub>2</sub>O<sub>2</sub> production in the I<sub>Q</sub> assay (step 4 in Table 1). 501 compounds had an IC<sub>50</sub> of less than 0.32 μM. These compounds were then tested at 10 x IC<sub>50</sub> against site I<sub>Q</sub> in 96-well format in a panel of eight assays measuring inhibition of mitochondrial H<sub>2</sub>O<sub>2</sub> production from each of seven different superoxide/H<sub>2</sub>O<sub>2</sub>-producing sites - I<sub>Q</sub>, I<sub>F</sub>+DH, O<sub>F</sub>, P<sub>F</sub>, III<sub>Qo</sub> (low and high succinate), II<sub>F</sub>, and G<sub>Q</sub> plus two assays of mitochondrial membrane potential, Δψ<sub>m</sub>, using TMRM fluorescence (with either succinate plus rotenone or glutamate plus malate as substrates) (step 5 in Table 1). From this panel screening only one compound was found to be selective for inhibition of superoxide/H<sub>2</sub>O<sub>2</sub> production from site I<sub>Q</sub>. Notably, for the triaged compounds there was a clear relationship between inhibition in the I<sub>Q</sub> assay and inhibition in the site I<sub>F</sub>+DH assay (Supplemental Figure S1), indicating that the site I<sub>F</sub>+DH assay is a sufficient discriminator of off-target effects.

To find more compounds that were selective for suppression of H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub>, all 850 compounds that had an IC<sub>50</sub> of less than 1 μM at step 4 in Table 1 were tested for inhibition of site I<sub>F</sub>+DH (step 6 in Table 1). The assay was run at a single concentration of 10 μM in triplicate and identified an additional 511 compounds that caused less than 20% inhibition in the I<sub>F</sub>+DH assay and had an IC<sub>50</sub> against site I<sub>Q</sub> of less than 1 μM. These compounds were then re-tested in 96-well assays for inhibition of superoxide/H<sub>2</sub>O<sub>2</sub> production by sites I<sub>Q</sub> and I<sub>F</sub>+DH at a concentration of 5 μM (step 7 in Table 1). Only 14 compounds suppressed the I<sub>Q</sub> assay more than 45%, whilst causing less than 20% inhibition in the I<sub>F</sub>+DH assay. These 14 compounds, plus the original compound, were then tested in a selectivity panel of six assays analyzing H<sub>2</sub>O<sub>2</sub> production from five mitochondrial sites: I<sub>Q</sub>, I<sub>F</sub>+DH, II<sub>F</sub>, G<sub>Q</sub> and III<sub>Qo</sub> at 0.5 and 5 mM succinate (single concentration, >5 x IC<sub>50</sub> against site I<sub>Q</sub>) (step 8 in Table 1). From this panel eight compounds (including the original compound) gave greater than 50% inhibition of superoxide/H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub>, with a two-fold or greater activity in the I<sub>Q</sub> assay than any other assay.

These eight compounds were representatives of specific compound classes, since the original hit selection (step 2 in Table 1) had clustered the molecules according to similarity and the most active three compounds in each class had been taken forward for confirmation. To confirm the activity of these compound classes and repopulate our hits, we selected the other molecules in the compound clusters of the eight candidate suppressors of site I<sub>Q</sub> that had been originally identified in the primary screen (step 9 in Table 1), resulting in the selection of 123 compounds (including the original eight compounds). These compounds were then tested in dose-response for their activity in the I<sub>Q</sub>, I<sub>F</sub>+DH and III<sub>Qo</sub> assays (eight-point, duplicate or triplicate determinations, maximum compound concentration 10 μM) (step 10 in Table 1). As before, compounds were defined as being non-specific if they caused an inhibition in the I<sub>F</sub>+DH or III<sub>Qo</sub> assay of >20%. 48 compounds across six compound classes were identified as having an IC<sub>50</sub> for I<sub>Q</sub> suppression of less than 10 μM and no effect >20% in the other assays.

The 48 compounds were then tested in a selectivity panel of eight assays against five sites (I<sub>Q</sub>, I<sub>F</sub>+DH, III<sub>Qo</sub>, G<sub>Q</sub> and II<sub>F</sub>) (step 11 in Table 1). 19 compounds from four compound classes were selective for site I<sub>Q</sub>.

Because a major criterion was lack of interference with energy metabolism, we measured resting and ADP-stimulated respiration in isolated mitochondria as a stringent test of oxidative phosphorylation (Step 12 in Table 1). Of the remaining 19 hits, one was unavailable for further testing and nine compounds across two classes had no

unwanted effect (Fig. 2B,C). Compounds from the other two classes, related to those that caused effects on respiration, had non-specific effects in the I<sub>F</sub>+DH assay that were more pronounced in compounds with a higher potency against site I<sub>Q</sub>, whereas compounds in the two classes not showing effects on respiration showed no correlation between on-target and off-target activity in the other H<sub>2</sub>O<sub>2</sub> assays.

These 10 compounds were assigned the names S1QELs, with numbering reflecting their compound class (Fig. 1). Notably, one of these classes, S1QEL1, contained compounds related to the compound identified in the original hit selection and triage (step 5 in Table 1). S1QEL2.1, the member of the second compound class with the lowest IC<sub>50</sub> against site I<sub>Q</sub> (Fig. 1G) was not available for retesting and was not examined further, leaving six compounds from class 1 (S1QELs 1.1-1.6) and three compounds from class 2 (S1QELs 2.2-2.4) (Fig. 1). These compounds were selective for site I<sub>Q</sub> (Fig. 2A), had relatively high affinity (IC<sub>50</sub> values are given in Fig. 1), and did not affect respiration or oxidative phosphorylation in isolated skeletal muscle mitochondria (Fig. 2B,C) or HEK-293 cells (Fig. 2D), or growth of HEK-293 cells at less than 20 x IC<sub>50</sub> against site I<sub>Q</sub> (Fig. 2E) (except for S1QEL2.4, which decreased cell growth at ~2 x IC<sub>50</sub> and was not examined further; S1QEL2.3 was not tested in this assay).

#### **Ultra high-throughput primary screens: sites III<sub>Qo</sub>, I<sub>Q</sub> and II<sub>F</sub>, and site I<sub>F</sub>+DH**

The rationale and general design of the primary 1536-well assays for production of superoxide/H<sub>2</sub>O<sub>2</sub> from sites III<sub>Qo</sub>, I<sub>Q</sub>, and II<sub>F</sub> (Step 1 in Table 1) and site I<sub>F</sub>+DH (step 7 in Table 1) are detailed elsewhere (Orr et al., 2013; Orr et al., 2015). Freshly isolated rat muscle mitochondria were assayed in KHEB medium (containing 120 mM KCl, 5 mM HEPES, 1 mM EGTA, and 0.3% (w/v) bovine serum albumin), using individual media for assay of each site. Media contained Amplex UltraRed (50 μM), superoxide dismutase (5 U • ml<sup>-1</sup>), and horseradish peroxidase (1 U • ml<sup>-1</sup>) plus one of the following sets of components to drive H<sub>2</sub>O<sub>2</sub> production predominantly from a single mitochondrial site in each assay: site I<sub>Q</sub>, 5 mM succinate alone; site III<sub>Qo</sub>, 5mM succinate with 2.5 μM antimycin A and 4 μM rotenone; site II<sub>F</sub>, 1 mM succinate with 2.5 μM antimycin A, 4 μM rotenone and 2 μM myxothiazol; site I<sub>F</sub>+DH, 5 mM glutamate plus 5 mM malate with 4 μM rotenone. Each assay was run on a different 1536-well microplate. To each well, 4 μl of assay medium was added followed by 50 nl of 1 mM test compound in DMSO. Assays were initiated by the addition of 1 μl mitochondria (final concentration 0.2 mg protein • ml<sup>-1</sup>). The final screening concentration of the test compounds was 10 μM. Plates were incubated for 15 min at room temperature before fluorescence of the resorufin product of Amplex UltraRed oxidation was read on a BMG Labtech PHERAstar Plus microplate reader (λ<sub>ex</sub> = 540 nm, λ<sub>em</sub> = 590 nm). Fluorescence intensity was normalized to the intra-plate median signal observed for each assay plate.

#### **High-throughput secondary screens: Δψ<sub>m</sub> and cell viability**

Compounds were tested for effects on mitochondrial membrane potential, Δψ<sub>m</sub> (Step 3 in Table 1) as previously described (Orr et al., 2013; Orr et al., 2015). 4 μl of KHEB medium containing 2.5 μM of the potentiometric dye tetramethylrhodamine methyl ester (TMRM, Life Technologies), 5 mM glutamate and 5 mM malate was added to each well of a 1536-well microtiter plate. 50 nl of 1 mM test compound in DMSO was added and the assay was initiated by addition of 1 μl mitochondria (to give 0.2 mg protein • ml<sup>-1</sup>). The final screening concentration of the test compounds was 10 μM. The plates were incubated for 10 min at room temperature then TMRM fluorescence was read on a BMG LabTech PHERAstar Plus microplate reader (λ<sub>ex</sub> = 540 nm, λ<sub>em</sub> = 590 nm). Fluorescence intensity was normalized to the intra-plate median signal observed for each assay plate. Compounds that altered the fluorescent signal more than ± 30% (approximately 3 standard deviations) were removed from further testing.

The effect of compounds (10 μM) on viability and growth of HEK-293T cells cultured in glucose-free Dulbecco's Modified Eagle Medium (DMEM) containing 10% v/v fetal bovine serum, 2 mM pyruvate, 2 mM glutamine and 20 mM galactose, was assessed after 72 h exposure (GalacTox, Step 3 in Table 1) by measuring total ATP with standard procedures (Cell Titer Glo, Promega). Replacing glucose with pyruvate, glutamine and galactose enforces reliance on mitochondrial over glycolytic ATP production and is particularly useful for uncovering mitochondrial toxicities of candidate drugs (Swiss et al., 2013). The average effect of compounds was normalized to the intraplate median signal. Compounds that lowered viability >20% were removed from further testing (Step 3 in Table 1). HEK-293T cells were from ATCC and regularly tested for mycoplasma contamination.

Recently, we identified CN-POBS as a suppressor of electron leak to O<sub>2</sub> specifically at site I<sub>Q</sub> without affecting energy metabolism (Orr et al., 2013). However, CN-POBS has relatively low affinity and off-target effects at the upper end of its effective range, making it inferior to the S1QELs identified here. Retrospective analysis revealed that CN-POBS was present in the 635,000-compound library screened here and appeared as an initial hit (Step 1 in Table 1). However, CN-POBS analogs with greater potency were also identified, so CN-POBS itself was dropped at

step 2 in Table 1. Further testing showed that these CN-POBS analogs inhibited cell growth, so they were dropped at step 3 in Table 1.

### **Initial dose-response screens: sites III<sub>Qo</sub> and I<sub>Q</sub>**

Using the protocol described above for the ultra high-throughput primary screen, the remaining compounds were tested in a dose response for inhibition of H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub> (Step 4 in Table 1). Each compound was tested in duplicate at eight doses between 5 nM and 10 μM. Endpoint fluorescence values were normalized to intraplate DMSO wells.

### **Expanded rescreens: nine assays covering five sites of superoxide/H<sub>2</sub>O<sub>2</sub> production and two assays of Δψ<sub>m</sub>**

Expanded rescreens at 10 x IC<sub>50</sub> (determined versus site I<sub>Q</sub> superoxide/H<sub>2</sub>O<sub>2</sub> production) against sites I<sub>F</sub>+DH, I<sub>Q</sub>, III<sub>Qo</sub> (at 0.5 mM and 5 mM succinate), II<sub>F</sub> (0.1 mM succinate), and G<sub>Q</sub> (Step 8 in Table 1), and also against Δψ<sub>m</sub> (Step 5 in Table 1); at 5 μM against sites I<sub>F</sub>+DH, and I<sub>Q</sub> (Step 7 in Table 1), or at 10 μM against sites I<sub>F</sub>+DH, I<sub>Q</sub> (0.5 mM succinate and 5 mM succinate), III<sub>Qo</sub> (0.5 mM succinate, 5 mM succinate, and 4 mM succinate plus 1 mM malonate), II<sub>F</sub> (using palmitoyl carnitine as substrate), and G<sub>Q</sub> (Step 11 in Table 1) were performed as described previously (Orr et al., 2013; Orr et al., 2015).

The expanded rescreens were in 96-well format in a total volume of 100 μl. Two assays for Δψ<sub>m</sub> were driven by 5 mM glutamate plus 5 mM malate or 5 mM succinate plus 4 μM rotenone. Nine site-specific assays for H<sub>2</sub>O<sub>2</sub> production included the following additions: site I<sub>F</sub>+DH, 5 mM glutamate plus 5 mM malate and 4 μM rotenone; site I<sub>Q</sub>, 0.5 mM succinate or 5 mM succinate; site III<sub>Qo</sub>, 0.5 mM succinate or 5 mM succinate or 4 mM succinate plus 1 mM malonate, all plus 4 μM rotenone and 2.5 μM antimycin A; site II<sub>F</sub>, 0.1 mM succinate plus 4 μM rotenone or 15 μM palmitoyl carnitine, each plus 2.5 μM antimycin A and 2 μM myxothiazol; site G<sub>Q</sub>, 25 mM glycerol 3-phosphate plus 4 μM rotenone, 1 mM malonate, 2.5 μM antimycin A, and 2 μM myxothiazol. Endpoint fluorescence readings after 30 min were normalized to vehicle (DMSO) and known inhibitor controls as described (Orr et al., 2013; Orr et al., 2015) and expressed as % change from DMSO. Inhibitor controls for sites I<sub>F</sub>+DH, I<sub>Q</sub>, III<sub>Qo</sub>, II<sub>F</sub>, and G<sub>Q</sub> were respectively: 20 mM aspartate, 1 μM FCCP (plus 10 mM malonate at Step 11 in Table 1), 2 μM myxothiazol, 10 mM malonate, and 1 μM FCCP.

### **Mitochondrial respiration**

Using mitochondria freshly isolated from rat skeletal muscle, respiration on 5 mM succinate plus 4 μM rotenone or 5 mM glutamate plus 5 mM malate in a mannitol- and sucrose-based medium (Seahorse MAS buffer (Rogers et al., 2011)) containing 0.3% (w/v) fatty acid-free bovine serum albumin was measured using a Seahorse XF24 at 2 μg mitochondrial protein per well for succinate and 10 μg mitochondrial protein per well for glutamate plus malate as described previously (Orr et al., 2014; Rogers et al., 2011) (Fig. 2B,C; Step 12 in Table 1). Baseline rates with substrate only (state 2) were measured in the presence of DMSO vehicle or compound, followed by injection of 5 mM ADP (phosphorylating state 3) then 1 μg • ml<sup>-1</sup> oligomycin (non-phosphorylating state 4o). Each respiratory substrate was tested on a different plate and each S1QEL was tested at least three times with each substrate. S1QELs were added to a final concentration of 10 μM with succinate as substrate (with myxothiazol as the positive control), or (at 20 x IC<sub>50</sub> against superoxide/H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub>) with glutamate plus malate as substrate (with rotenone as the positive control).

Respiration of mitochondria freshly isolated from *Drosophila* on 10 mM pyruvate plus 10 mM proline or 10 mM glycerol 3-phosphate plus 2 μM rotenone was assayed in KHE medium (120 mM KCl, 5 mM HEPES and 1 mM EGTA, pH 7.2) supplemented with 5 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub> and 0.3 % (w/v) fatty acid-free bovine serum albumin. Oxygen consumption rates were measured in a Seahorse XF24 at 2.5 μg mitochondrial protein per well with glycerol 3-phosphate or 5 μg mitochondrial protein per well with pyruvate plus proline as substrate. The temperature controller of the XF24 was turned off, enabling assay at 28 °C. Respiration was measured in the presence of DMSO vehicle or S1QELs at 10 μM with glycerol 3-phosphate as substrate, or 20 x IC<sub>50</sub> against superoxide/H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub>, with pyruvate plus proline as substrate.

### **Cellular respiration**

Basal and uncoupled respiration rates of HEK-293 cells were measured in the presence of pyruvate, glutamine and galactose. 15,000 cells were seeded per well of Seahorse V7-PS plates and grown for 48 h in glucose-free DMEM with 20 mM galactose, 4 mM pyruvate, 2 mM glutamine, 10% (v/v) fetal bovine serum, 1 U • ml<sup>-1</sup> penicillin and 100 μg • ml<sup>-1</sup> streptomycin. ~24 h before the assay, cells were incubated with fresh glucose-free DMEM with 20 mM galactose and 4 mM pyruvate. Cells were rinsed in prewarmed assay medium (120 mM NaCl, 7 mM KCl, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM NaHCO<sub>3</sub>, 2.4 mM Na<sub>2</sub>SO<sub>4</sub>, 20 mM galactose, 4 mM pyruvate, 2 mM glutamine, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 0.3% (w/v) bovine serum albumin and 40 mM TES, pH 7.4) and equilibrated for ~30 min at

37°C before measurement of respiration (Fig. 2D). S1QELs or vehicle control (DMSO) were injected via port A to a final concentration of 20 x IC<sub>50</sub> against superoxide/H<sub>2</sub>O<sub>2</sub> production by site I<sub>Q</sub> in isolated mitochondria, and measurements were taken every 30 – 60 min for 200 min. Respiration was then uncoupled by adding 10 µM FCCP with 2 µg • ml<sup>-1</sup> oligomycin. Rates were normalized to the basal rate before compound addition.

### **Succinate dehydrogenase activity and protein levels in primary astrocytes**

Primary astrocytes were isolated from *Sod2*<sup>+/+</sup> homozygous “wild-type” mice on a CD1 background (and in Supplemental Fig. 2 were compared to those from sibling *Sod2*<sup>tm1Cje</sup> null mice, “*Sod2*<sup>-/-</sup> mice” (Melov et al., 1999)). Dissected brains without meninges, olfactory bulb and cerebellum were digested in Hibernate A-Ca (Brainbits) containing 10 U/ml papain (Worthington #3176), 0.5 mM EDTA, 1 mM cysteine, 55 µM β-mercaptoethanol and 10 µM EUK-134 (Eukarion) at 37°C for 45 min. Brains were triturated in Hibernate A-Ca supplemented with 10% (w/v) fetal bovine serum, 2 U/ml DNase I and 10 µM EUK-134, then passed through a 40 µm cell strainer. Cell suspensions were washed twice in complete cell culture medium comprising DMEM/F12 (with glutamax, pyruvate and high glucose; Gibco) supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were cultured with 10 µM EUK-134 at 3% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C until banking at P1 (passage 1) or P2. EUK-134 was used for its anticipated protective effect, but had no such effect, so was omitted after banking. Cells were passaged after shaking off microglia (280 rpm, 3-6 h), by trypsinization (0.25%) and scraping if needed. Experiments used banked aliquots of two independent cell preparations up to P4. Astrocytes were plated into the center 60 wells of 96-well microplates (Corning 3340) coated with poly-D-lysine (0.1 mg/ml, 3 h to overnight; for five-day experiments) or polyornithine (0.1 mg/ml, 2 h) followed by laminin (10 µg/ml, 2 h; for three-day experiments) at 5×10<sup>3</sup> cells per well. S1QELs or S3QELs were added one day after plating, in complete culture medium, at 1:1000 DMSO, and no media were changed until assay. For experiments at 3% O<sub>2</sub> the incubator door was not opened for the five days duration to guard against oxygen spikes.

To measure succinate dehydrogenase activity *in situ*, culture plates were fixed with 1% (w/v) ice cold paraformaldehyde in PBS (pH 7.4) for 5 min (Andersen and Hoyer, 1973). Plates were washed twice and frozen at -80 °C for 10 min then incubated in TRIS-HCl buffer (200 mM; pH7.2) with 1 mg/ml nitro blue tetrazolium (NBT), 5 mM MgCl<sub>2</sub>, 200 µM phenazine methosulfate, 0.5 mM NaN<sub>3</sub>, 2 µM rotenone, 2 µg/ml Hoechst 33342 and 125 mM succinate (Na), at 37°C on a rocker for 45 min (Bancroft, 1996). The reaction was stopped by layering Optiprep under the reaction mixture, which also mitigated refraction of light by cells during densitometry. Recordings were made on a Nikon Ti-Eclipse Perfect Focus System fully motorized epifluorescence microscope, equipped with a Cascade 512B camera (Photometrics, Tucson, AZ), an S-Fluor 10× air lens, a Lambda LB-LS17 Xe-arc light source and 10-3 filter wheels (Sutter Instruments, Novato, CA) and an MS-2000 linear encoded motorized stage (ASI; Eugene, OR), controlled by NIS Elements 4.2 (Nikon, Melville, NY). 3×3 tiled images (1536×1536 pixels; 1.6 µm/pixel) were recorded as 520/35 nm (center/bandwidth of bandpass filter) bright field and Hoechst 33342 fluorescence channels (excitation: 390/40 nm, dichroic: 409 nm, emission: 460/80 nm) in 60 wells plus three blank wells without cells. Optical density (OD) images were calculated using Image Analyst MKII (Image Analyst Software, Novato, CA) using the blank wells to cancel effects of inhomogeneity in illumination, but zero optical density was defined as the local background in OD images. To normalize to cell density, average optical density was calculated only in 16 µm wide rings around nuclei detected by segmentation of the Hoechst 33342 images. Succinate dehydrogenase activity was expressed as OD of experimental wells minus a succinate-free control.

To measure expression levels of succinate dehydrogenase subunit B (SDHB) and ATP synthase β, the sample was further fixed using 4% (w/v) paraformaldehyde in PBS at room temperature for 20 min, and incubated in PBS containing 3% (w/v) bovine serum albumin, 1% (v/v) Triton X100, 5 mM NaN<sub>3</sub> with 1:200 anti-SDHB rabbit polyclonal (Sigma, HPA002868), and 1:1000 anti-ATP synthase β subunit mouse monoclonal (Sigma A9728) antibodies at 37°C for 2 h. Plates were incubated with anti-rabbit-IgG Alexa647 (1:500; Thermo Fisher, #A21244) and anti-mouse-IgG Alexa488 (1:500; Thermo Fisher, #A21202) at room temperature for 1 h then re-imaged in PBS as above but adding fluorescence detection channels for Alexa488 (excitation: 472/30 nm, dichroic: 495 nm, emission: 520/35 nm) and Alexa647 (excitation: 628/40 nm, dichroic: 650 nm, emission: 692/40 nm). Images were analyzed in Image Analyst MKII as above by using local background subtraction and gated averaging of fluorescence intensities in the perinuclear areas. Immunofluorescence intensity was expressed after subtraction of intensities measured in controls lacking primary antibody.

OD and immunofluorescence intensity data were statistically analyzed in Microsoft Excel and Graphpad Prism. Experimental repeats were defined as assay plates that were treated independently. Significance was tested using repeated measures ANOVA considering that assay plates are independent but conditions within individual assay



plates are not independent. EC<sub>50</sub> values (μM, with SE of the fit) were S1QEL1.1: 0.15 ± 0.04; S1QEL1.2: 0.02 ± 0.01; S1QEL2.3: > 8.7 (Fig. 3B).

#### **Aconitase and citrate synthase activity in primary astrocytes**

To measure total cellular aconitase and citrate synthase activities, primary astrocytes were cultured as above, but in 6-well plates at 3×10<sup>5</sup> cells per well. Treatments were applied in 1:1000 DMSO for 5 days in a 20% O<sub>2</sub> tissue culture incubator. Aconitase activity was assayed as described (Miwa and Brand, 2005) with modifications. Briefly, the assay buffer comprised TRIS/HCl (50 mM; pH7.4), 0.6 mM MnCl<sub>2</sub> and 0.1% v/v Triton X100. Cultures were lysed in assay buffer on ice and further permeabilized by freeze-thawing three times. Aconitase activity was assayed fluorimetrically in a BMG LabTech PHERAstar FS platereader, using half-area glass-bottomed 96-well plates in bottom readout mode, in assay buffer supplemented with 0.76 U/ml isocitrate dehydrogenase (Megazyme Inc., Chicago, Illinois), 5 mM Na-citrate and 0.2 mM NADP<sup>+</sup> at 30°C. The rate of NADPH formation was measured between 10 and 30 min after the start of the incubation by excitation at 360 nm and emission at 460 nm. Citrate synthase activity was assayed by chromogenic product formation between CoA-SH and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB). Briefly, assay buffer was supplemented with 0.1 mM DTNB, 0.3 mM acetyl-CoA and 0.5 mM Na-oxaloacetate. The rate of product formation was followed between 1 and 4 min by measuring absorbance at 412 nm. For both assays, 1/10 of the cell lysate (~ 8 μg protein) was assayed in a total volume of 50 μl. Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

#### **Supplemental References (not cited in main text)**

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