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Supplemental Information

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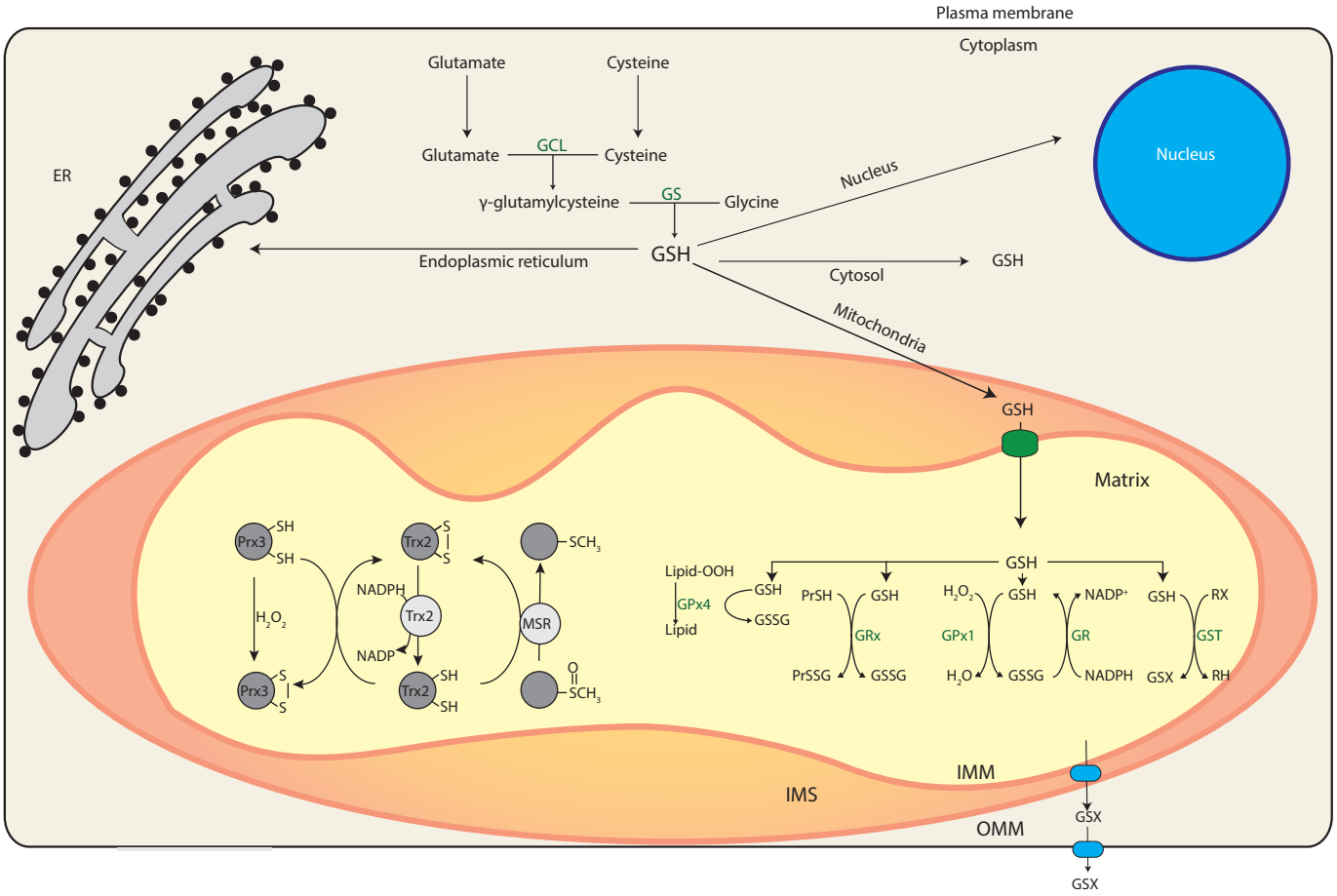
Figure S1

a


Plasma membrane
Cytoplasm
Glutamate
Cysteine
GCL
γ-glutamylcysteine
GS
Glycine
GSH
Endoplasmic reticulum
Nucleus
Cytosol
Mitochondria
ER
Matrix
IMM
IMS
OMM
GSX
Prx3
Trx2
NADPH
NADP
MSR
Lipid-OOH
GPx4
Lipid
PrSH
PrSSG
GRx
GPx1
GR
NADP+
GST
RX
RH

b

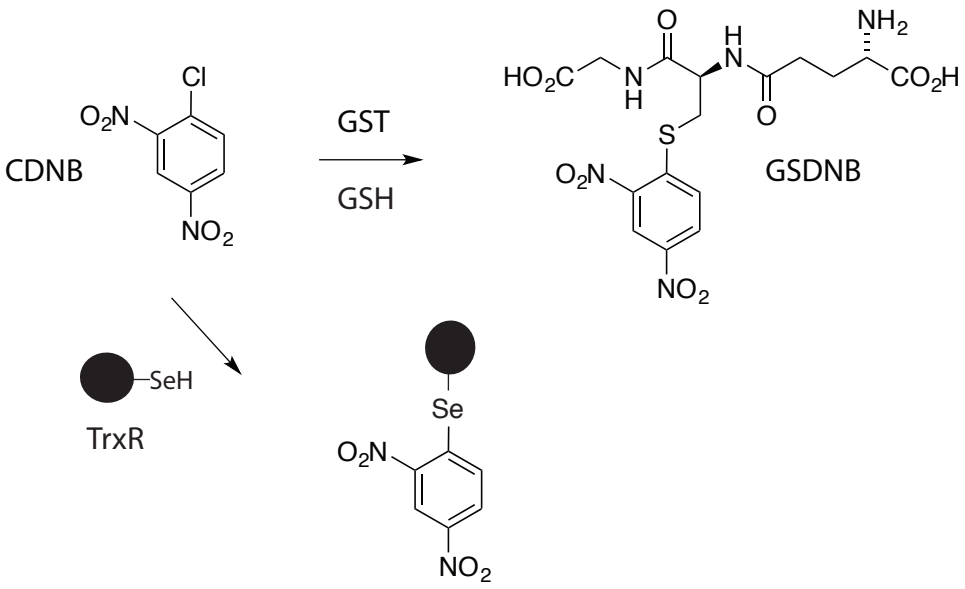

CDNB
GST
GSH
GSDNB
TrxR
SeH
Se

Figure S1. Mitochondrial GSH and Trx systems (*Related to Figure 1*)

Mitochondrial thiol redox homeostasis. (**a**) Glutathione (GSH) is synthesised in the cytosol and is then slowly transported into mitochondria. Within mitochondria GSH degrades H_2O_2 and other peroxides by glutathione peroxidases (GPXs) 1 and 4 and the glutathione disulfide (GSSG) generated is recycled by glutathione reductase (GR), driven by the mitochondrial NADPH pool. GSH also recycles oxidised protein thiols in conjunction with glutaredoxin 2 (Grx2) and detoxifies electrophiles and xenobiotics through glutathione S-transferases (GSTs), with the resulting GSH conjugates being exported from mitochondria by ABC transporters. Thioredoxin-2 (Trx2) is a small dithiol protein that maintains other protein dithiols reduced. Doing so converts Trx2$(\text{-SH})_2$ to its disulfide (Trx2-SS) which is then reduced back to Trx2$(\text{-SH})_2$ by thioredoxin reductase 2 (TrxR2) driven by the mitochondrial NADPH pool. Trx2 also provides electrons to peroxiredoxin 3 (Prx3) to degrade H_2O_2 and to methionine sulfoxide reductases (Msr) to reverse methionine oxidation. (**b**) Reaction of CDNB with GSH catalysed by GSTs to form 1-*S*-glutathionyl-2,4-dinitrobenzene (GSDNB) and with the active site selenol of TrxR. OMM; outer mitochondrial membrane. IMM; inner mitochondrial membrane. IMS; intermembrane space. γGCL; glutamate cysteine ligase. GS; glutathione synthetase.

Figure S2

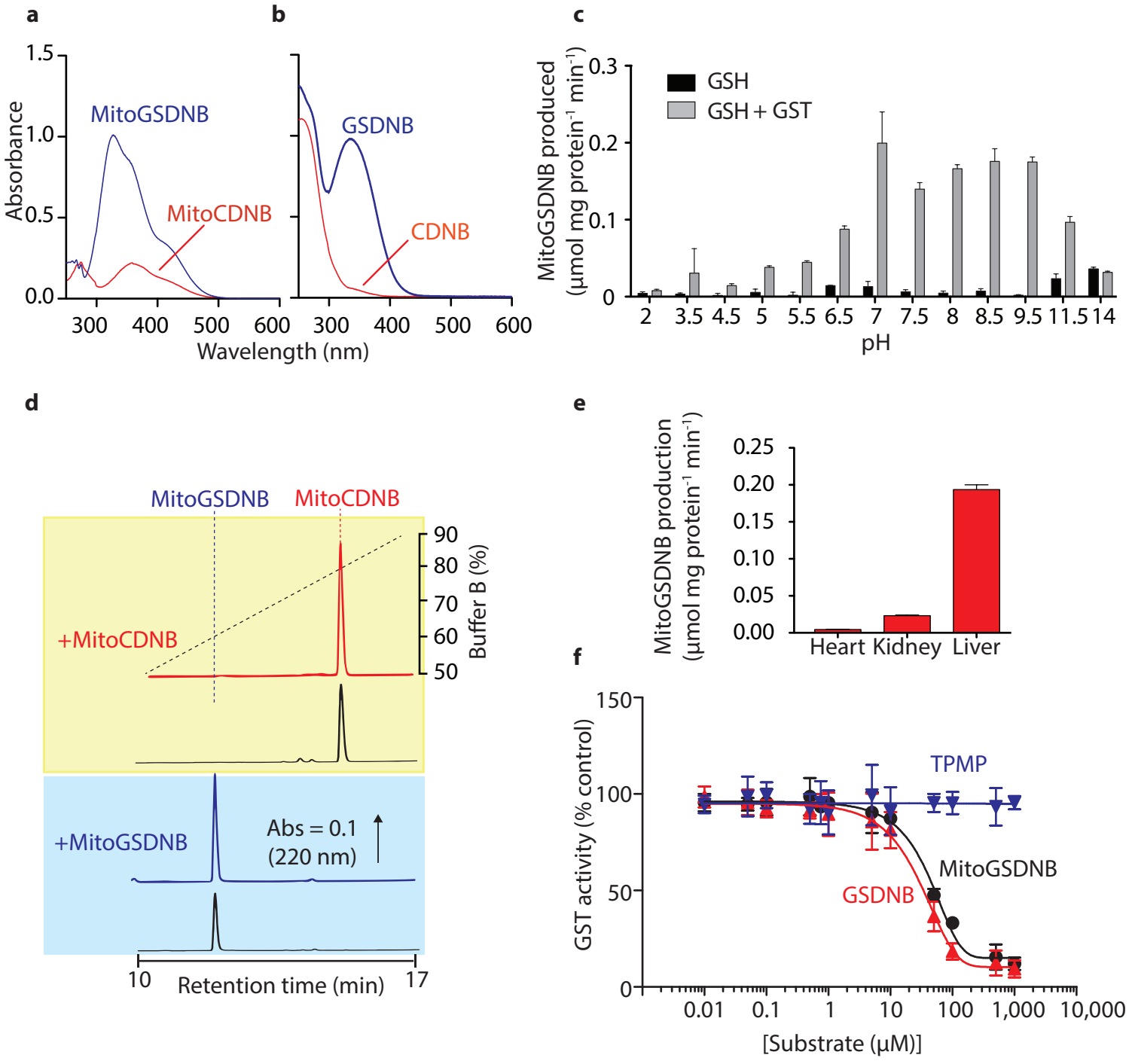

a
b
c
Absorbance
MitoGSDNB
MitoCDNB
GSDNB
CDNB
Wavelength (nm)
MitoGSDNB produced (µmol mg protein-1 min-1)
GSH
GSH + GST
pH
d
MitoGSDNB
MitoCDNB
+MitoCDNB
+MitoGSDNB
Abs = 0.1 (220 nm)
Buffer B (%)
Retention time (min)
e
MitoGSDNB production (µmol mg protein-1 min-1)
Heart
Kidney
Liver
f
GST activity (% control)
TPMP
MitoGSDNB
GSDNB
[Substrate (µM)]
g
Thioredoxin
Control
MitoCDNB
CDNB + MitoCDNB
Anti-Trx
Anti-TBTP
h
Peroxiredoxin
Control
MitoCDNB
CDNB + MitoCDNB
Anti-Prx
Anti-TBTP

Figure S2. Reactivity of MitoCDNB (*Related to Figure 2*)

Reactivity of MitoCDNB. (**a**) UV-Vis spectra of 100 μM MitoCDNB (red) or MitoGSDNB (blue) in KCl buffer. (**b**) UV-Vis spectra of 100 μM CDNB (red) or GSDNB (blue) in KCl buffer. (**c**) pH dependence of MitoCDNB reactivity with GSH. MitoCDNB (10 μM) and GSH (1 mM) were incubated at different pH values at 37 °C without (black), or with GST-κ (100 μg; grey) and MitoGSDNB production measured at 328 nm over 30 min. (**d**) RP-HPLC identification of MitoGSDNB. Samples from Figure 2b (black lines) were spiked with 20 nmol MitoCDNB (red) or MitoGSDNB (blue) and re-analysed by RP-HPLC. (**e**) MitoCDNB transformation by GST in different tissues. Mitochondrial matrix fractions (100 μg protein) from rat liver, kidney or heart were incubated with GSH and MitoCDNB as in (**c**) and MitoGSDNB production calculated. Data are means ± S.E.M, N = 3. (**f**) Inhibition of GST by GSDNB (red), MitoGSDNB (black) and TPP (blue) assessed by measuring GST (100 μg)-mediated conjugation of GSH (1 mM) to acrolein (1 mM) in PBS in the presence of increasing concentrations of compounds. The activity of GST in the absence of other compounds was 6.84 ± 0.6 μmol acrolein-GSH/min/mg protein (mean ± S.E.M, n = 3). (**g**, **h**) Alkylation of Trx1 (**g**) and Prx1 (**h**) by MitoCDNB. Trx1 (20 μg) or Prx1 (20 μg) was incubated with 20 μM MitoCDNB (MitoCDNB), 20 μM CDNB for 5 min at RT followed by 20 μM MitoCDNB for 10 min at RT (CDNB + MitoCDNB) or 0.1 % EtOH in PBS (control) for 10 min at RT. The protein was then analysed by western blotting with anti-Trx1 or anti-Prx1 antibodies and then reprobed with anti-TPP antiserum.

Figure S3

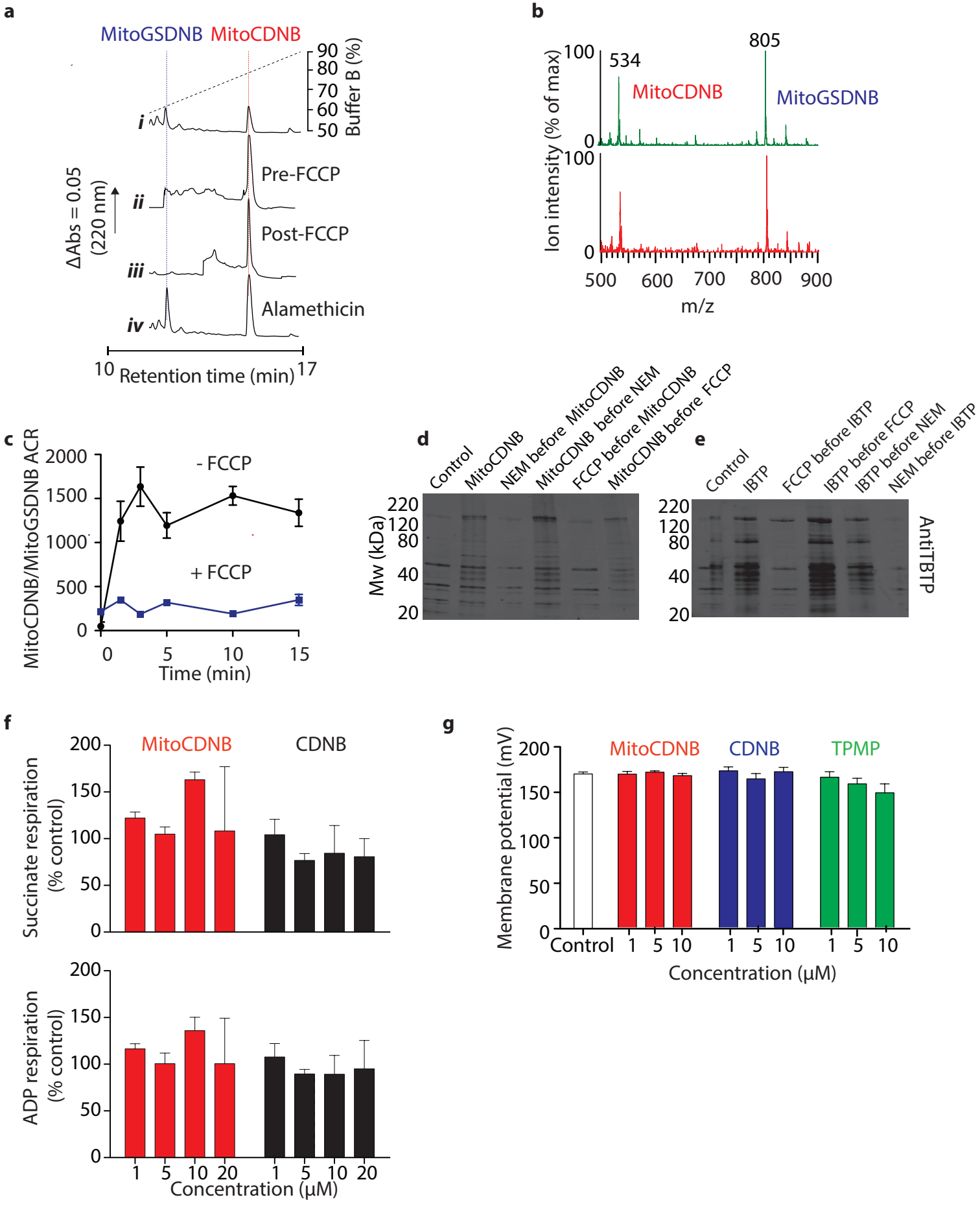

a
MitoGSDNB
MitoCDNB
90
80
70
60
50
Buffer B (%)
i
Pre-FCCP
ii
Post-FCCP
iii
Alamethicin
iv
ΔAbs = 0.05
(220 nm)
10
Retention time (min)
17
b
100
534
805
MitoCDNB
MitoGSDNB
0
100
0
Ion intensity (% of max)
500
600
700
800
900
m/z
c
MitoCDNB/MitoGSDNB ACR
2000
1500
1000
500
0
- FCCP
+ FCCP
0
5
10
15
Time (min)
d
Control
MitoCDNB
NEM before MitoCDNB
MitoCDNB before NEM
FCCP before MitoCDNB
MitoCDNB before FCCP
220
120
80
40
20
Mw (kDa)
e
Control
IBTP
FCCP before IBTP
IBTP before FCCP
IBTP before NEM
NEM before IBTP
220
120
80
40
20
AntiTBTP
f
MitoCDNB
CDNB
200
150
100
50
0
Succinate respiration
(% control)
200
150
100
50
0
ADP respiration
(% control)
1
5
10
20
1
5
10
20
Concentration (μM)
g
MitoCDNB
CDNB
TPMP
200
150
100
50
0
Membrane potential (mV)
Control
1
5
10
1
5
10
1
5
10
Concentration (μM)

Figure S3. Interactions of MitoCDNB with mitochondria (*Related to Figures 2 and 3*)
(**a**) Analysis of mitochondrial supernatants following incubation with MitoCDNB. Rat liver mitochondria were incubated as described in Figure 2h, and the supernatants analysed by RP-HPLC. (**b**) Mass spectrometric analysis of MitoCDNB reaction products within mitochondria. Mitochondria were incubated with 10 μM MitoCDNB for 5 min, pelleted by centrifugation and then extracted and analysed by mass spectrometry. The upper trace shows all m/z values detected while the lower trace is a scan of precursors that generate an m/z of 183 upon fragmentation. (**c**) Accumulation ratio (ACR) for MitoCDNB in mitochondria. Mitochondria were incubated with MitoCDNB ± FCCP, then pelleted and the MitoCDNB in the supernatant and pellets analysed by RP-HPLC and used to calculate the ACR assuming a mitochondrial volume of 0.6 μl/mg protein. (**d, e**) Binding of MitoCDNB and IBTP to mitochondrial proteins. MitoCDNB (**d**, 10 μM) or IBTP (**e**, 10 μM) was incubated with rat liver mitochondria (1 mg protein/mL) for 5 min with further addition of 1 mM NEM or 2 μM FCCP before or after MitoCDNB/IBTP. Mitochondria were then pelleted by centrifugation, and analysed by Western blotting using an anti-TPP antiserum. (**f**) Acute effects of MitoCDNB on mitochondrial respiration. Respiration by rat liver mitochondria (1 mg protein/mL) was determined as a percentage of vehicle control after addition of succinate (10 mM, top) and 2 mins later after addition of ADP (75 μM, bottom). The respiration rates for control incubations were 140.2 ± 9.2 (ADP rate) and 229.3 ± 13.2 (succinate rate) pmol/s/mg protein. (**g**) Acute effects of MitoCDNB on mitochondrial membrane potential. Rat liver mitochondria (1 mg protein/ml) were incubated with [^{3}H]-TPMP with MitoCDNB, MitoGSDNB or TPMP. Mitochondria were pelleted and both pellets and supernatants assessed for radiolabel content and the membrane potential calculated. All data are means ± S.E.M of N = 3.

Figure S4

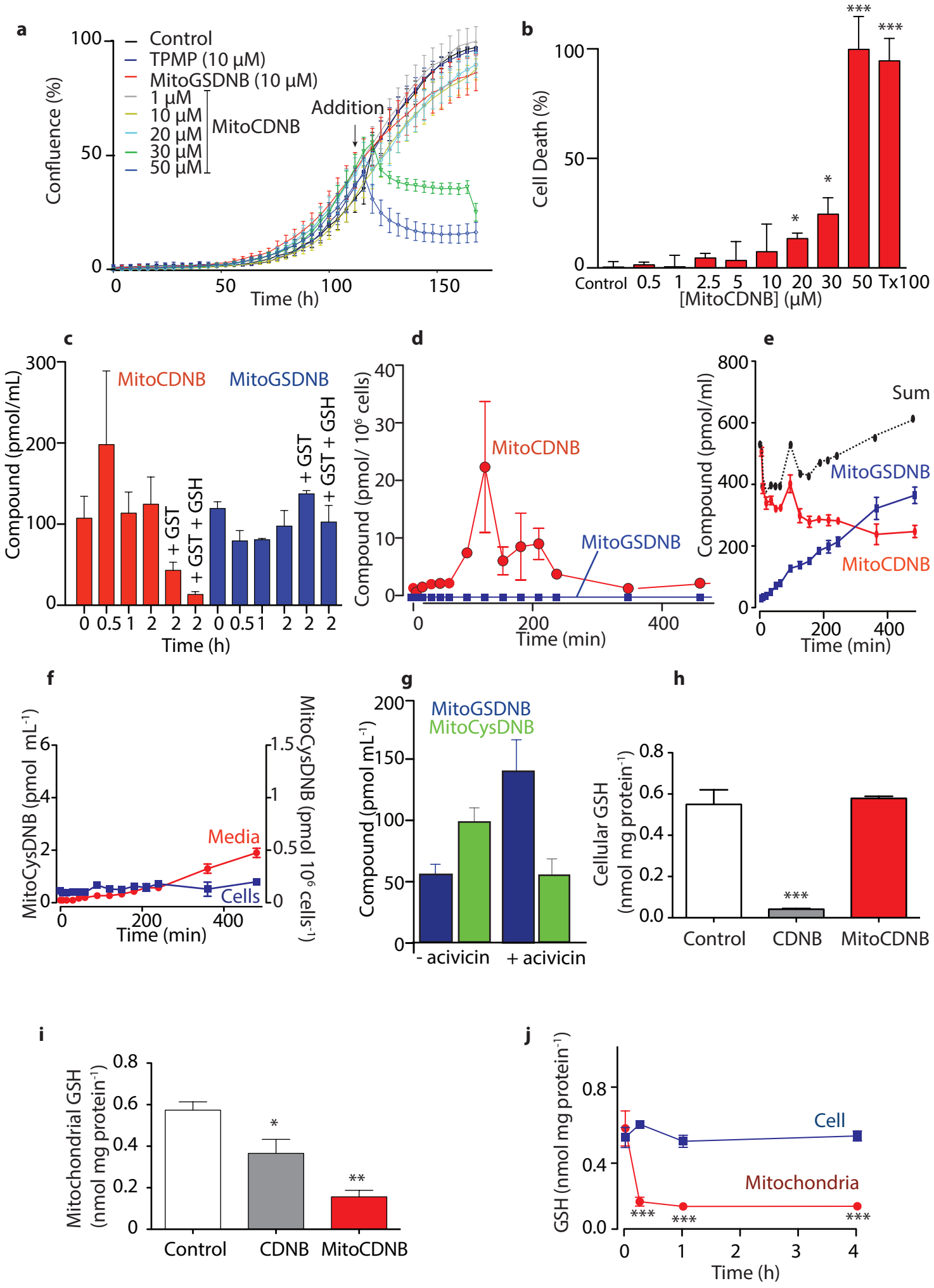

a
Confluence (%)
Control
TPMP (10 µM)
MitoGSDNB (10 µM)
1 µM
10 µM
20 µM
30 µM
50 µM
MitoCDNB
Addition
Time (h)
b
Cell Death (%)
Control
[MitoCDNB] (µM)
Tx100
c
Compound (pmol/mL)
MitoCDNB
MitoGSDNB
+ GST
+ GST + GSH
Time (h)
d
Compound (pmol/ 10⁶ cells)
MitoCDNB
MitoGSDNB
Time (min)
e
Compound (pmol/ml)
Sum
MitoGSDNB
MitoCDNB
Time (min)
f
MitoCysDNB (pmol mL⁻¹)
MitoCysDNB (pmol 10⁶ cells⁻¹)
Media
Cells
Time (min)
g
Compound (pmol mL⁻¹)
MitoGSDNB
MitoCysDNB
- acivicin
+ acivicin
h
Cellular GSH (nmol mg protein⁻¹)
Control
CDNB
MitoCDNB
i
Mitochondrial GSH (nmol mg protein⁻¹)
Control
CDNB
MitoCDNB
j
GSH (nmol mg protein⁻¹)
Cell
Mitochondria
Time (h)

Figure S4. Metabolism of MitoCDNB by cells (*Related to Figures 3 and 4*)

(**a**) Effect of MitoCDNB on C2C12 cell growth was analysed using an Incucyte system. Data are means ± S.E.M, N= 4 biological replicates, with each biological replicate condition repeated eight times. (**b**) Effect of MitoCDNB on HepG2 cell viability. Cells were incubated with MitoCDNB for 10 min before addition of propidium iodide (1 mg/ml) and were then analysed using flow cytometry. Cell death was compared to vehicle controls (0.1 % EtOH) and 100 % cell death was generated with 5 % (v/v) Triton-X100 (Tx100). (**c**) Stability of MitoCDNB and MitoGSDNB in culture medium. MitoCDNB (1 μM) or MitoGSDNB (1 μM) were incubated in 1 mL DMEM supplemented with 10 % FBS, with further additions of GSH (10 mM), or GSH and 100 μg GST-κ where indicated. MitoCDNB metabolites were then quantified by LC-MS/MS. (**d, e**) Uptake and metabolism of MitoCDNB by C2C12 cells. C2C12 cells were incubated with 10 μM MitoCDNB, then the cells and the culture medium were analysed by LC-MS/MS to determine the MitoCDNB and MitoGSDNB levels and the sum of MitoCDNB and MitoGSDNB. (**f**) Generation of MitoCysDNB by C2C12 cells. MitoCDNB (10 μM) was incubated with C2C12 cells as described in **d** and **e**, above. Cells and media were then extracted, analysed by LC-MS/MS and levels of MitoCysDNB quantified. (**g**) Breakdown of MitoGSDNB by GGT. HepG2 cells were incubated in 1 mL DMEM + 10 % FBS ± acivicin (500 μM) for 30 min. MitoGSDNB (1 μM) was then added and media assessed for presence of MitoCysDNB or MitoGSDNB by LC-MS/MS after 120 min. (**h**) GSH depletion in cells by MitoCDNB. C2C12 cells were incubated for 1 h with MitoCDNB (10 μM), CDNB (10 μM) or vehicle (0.1 % EtOH) and then isolated and total GSH levels measured. (**i**) Depletion of mitochondrial GSH in cells by MitoCDNB. C2C12 cells were incubated as in (**h**), mitochondria were isolated and GSH levels measured. (**j**) Time course of GSH depletion by MitoCDNB. C2C12 cells were incubated with MitoCDNB (10 μM) or vehicle (0.1 % EtOH) and at the indicated times either total cellular or mitochondrial GSH was measured. Data are means ± S.E.M. N = 3-4. ***p < 0.001, *p < 0.05, **p < 0.01 relative to control, or time = 0 (**j**).

Figure S5

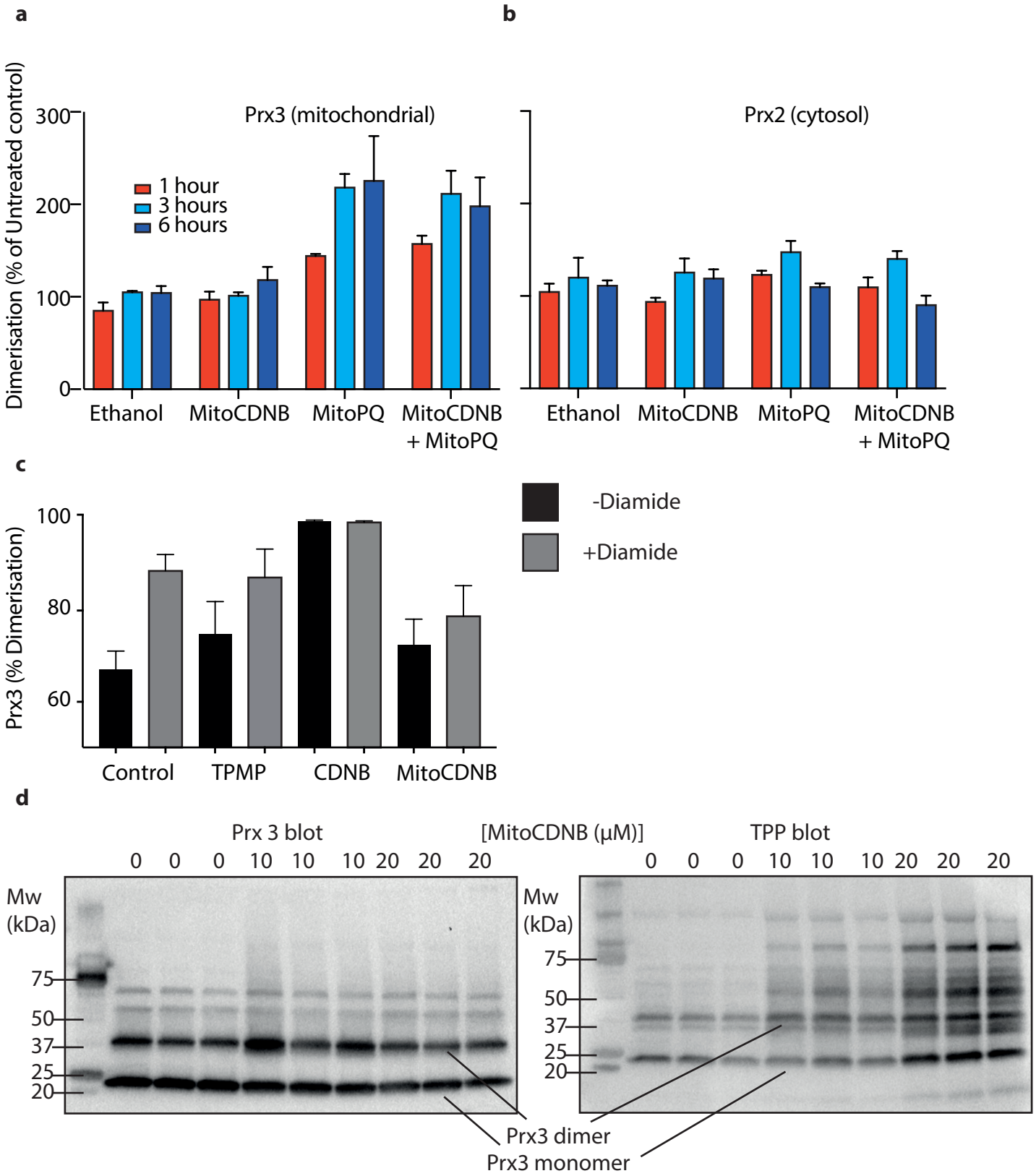

a
b
Dimerisation (% of Untreated control)
300
200
100
0
Prx3 (mitochondrial)
1 hour
3 hours
6 hours
Ethanol
MitoCDNB
MitoPQ
MitoCDNB + MitoPQ
Prx2 (cytosol)
Ethanol
MitoCDNB
MitoPQ
MitoCDNB + MitoPQ
c
Prx3 (% Dimerisation)
100
80
60
-Diamide
+Diamide
Control
TPMP
CDNB
MitoCDNB
d
Prx 3 blot
[MitoCDNB (μM)]
TPP blot
0 0 0 10 10 10 20 20 20
Mw (kDa)
75
50
37
25
20
Prx3 dimer
Prx3 monomer

Figure S5. Effect of MitoCDNB on peroxiredoxin dimerization (*Related to Figure 4*)
(**a**) Effect of C2C12 cells were treated with MitoPQ (5 μM), MitoCDNB (10 μM), both together, or an EtOH carrier control and then assessed for dimerization of Prx2 **(a)** and Prx3 **(b)**. Means ± SEM n = 3. (**c**) Effect of diamide on Prx3 dimerization. C2C12 cells were treated with 5 μM MitoCDNB, CDNB or TPMP plus an EtOH control for 1 h then further incubated for 10 min ± 250 μM diamide and Prx3 dimerisation assessed. (**d**) Western blotting of Prx3 and MitoCDNB. Rat liver mitochondria (1 mg protein/ml) were incubated with rotenone and succinate, and various concentrations of MitoCDNB for 30 min at 37°C. Then mitochondria were pelleted and 50 μg protein was separated by SDS-PAGE and analysed by western blot using an anti-Prx3 antibody (1:500) and then re-probed using anti-TPP antiserum (1:1,000), and visualised by ECL.

Figure S6

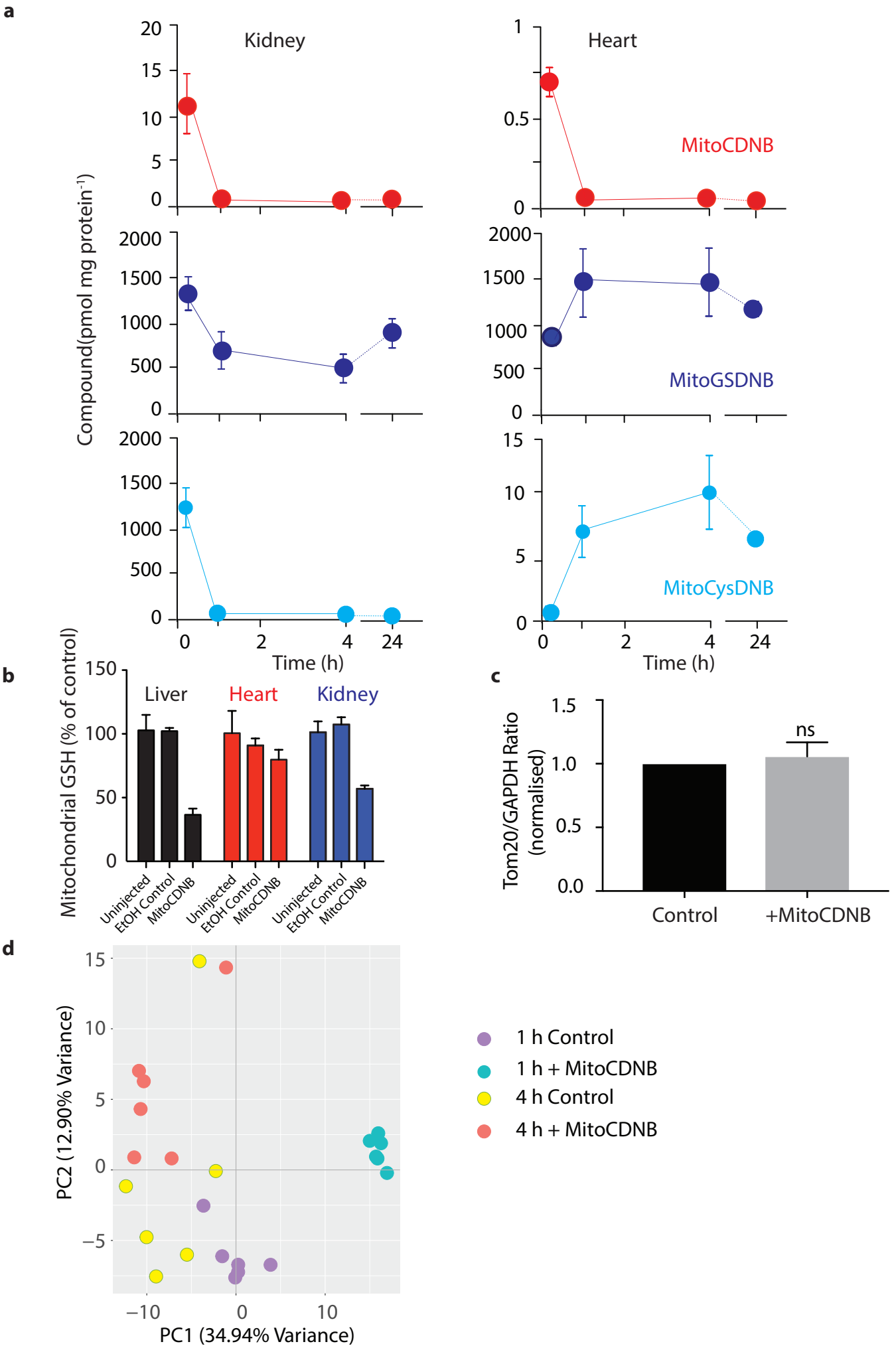

a
Kidney
Heart
MitoCDNB
MitoGSDNB
MitoCysDNB
Compound(pmol mg protein-1)
Time (h)
b
Liver
Heart
Kidney
Mitochondrial GSH (% of control)
Uninjected
EtOH Control
MitoCDNB
c
Tom20/GAPDH Ratio (normalised)
ns
Control
+MitoCDNB
d
PC2 (12.90% Variance)
PC1 (34.94% Variance)
1 h Control
1 h + MitoCDNB
4 h Control
4 h + MitoCDNB

Figure S6. Distribution and effects of MitoCDNB and its metabolites *in vivo* and in cells (*Related to Figures 5 and 6*)

(a) Mice were injected with MitoCDNB (5 mg/kg) and at the indicated times whole kidney or heart tissue were extracted and levels of MitoCDNB, MitoGSDNB and MitoCysDNB quantified by LC-MS/MS. (**b**) Comparison of mitochondrial GSH levels in in liver, heart and kidney mitochondria isolated from mice that received no injection, or 24 h after injection of vehicle (5 % EtOH) or MitoCDNB (5 mg/kg). Date are means ± SEM, N = 4. The absolute levels of GSH in the mitochondria from the control tissues were: liver = 3.71 ± 0.6; kidney 2.14 ± 0.52; heart 2.36 ± 0.1 (nmol GSH/mg protein). (**c**) Effect of MitoCDNB on mitochondrial content. C2C12 cells were incubated ± 10 μM MitoCDNB for 4 h and then analysed by western blotting for content of GAPDH and TOM20, which were quantified by Image Studio Lite. The ratio of TOM20 to GAPDH determined and the value in the MitoCDNB sample was normalised to that of the control. Data are means ± SEM of 3 separate incubations. (**d**) Principal component analysis of effect of MitoCDNB on RNA expression levels. Mice (n = 6 in each condition) were administered MitoCDNB (5 mg/kg) or carrier by tail vein injection and then the liver was isolated 1 and 4 h after injection and the transcriptomes analysed by RNA seq and subjected to principal component analysis using the top 500 most variably expressed genes.