Supplementary figures

Transcriptional and metabolic adaptation of neurons to the mitochondrial toxicant MPP+

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Overview:

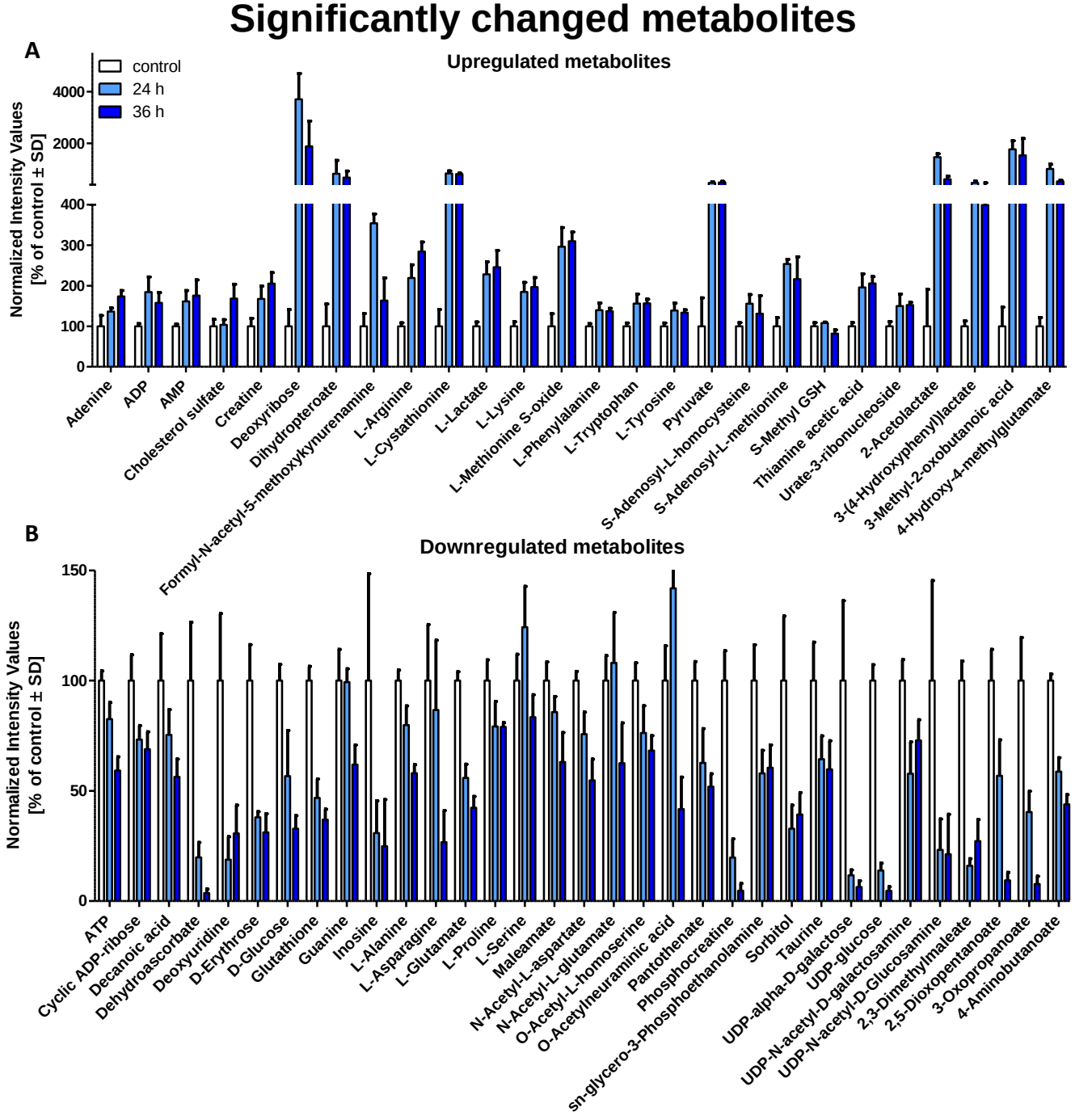

Figure S1

Overview of significantly altered metabolites determined during untargeted analysis with the accurate-mass Q-TOF LC-MS system

Cells were treated with 5 µM MPP^+ (control, 24 h or 36 h) in four independent experiments. After metabolite extraction, samples were run with the accurate-mass Q-TOF LC-MS system (Agilent, Santa Clara, CA). Metabolites were determined by the MassHunter Acquisition software (Agilent Technologies). 'Areas under the Curve' (AUC) for every peak/metabolite of the ion chromatogram were calculated and used as basis for a relative quantification. AUCs were normalized to the mean of the AUCs of control samples (untreated cells) of 4 independent experiments. Only metabolites that were significantly regulated (FDR adjusted p-value of ≤ 0.05) and that could be unambiguously identified, are displayed.

Example-peaks of significantly changed metabolites

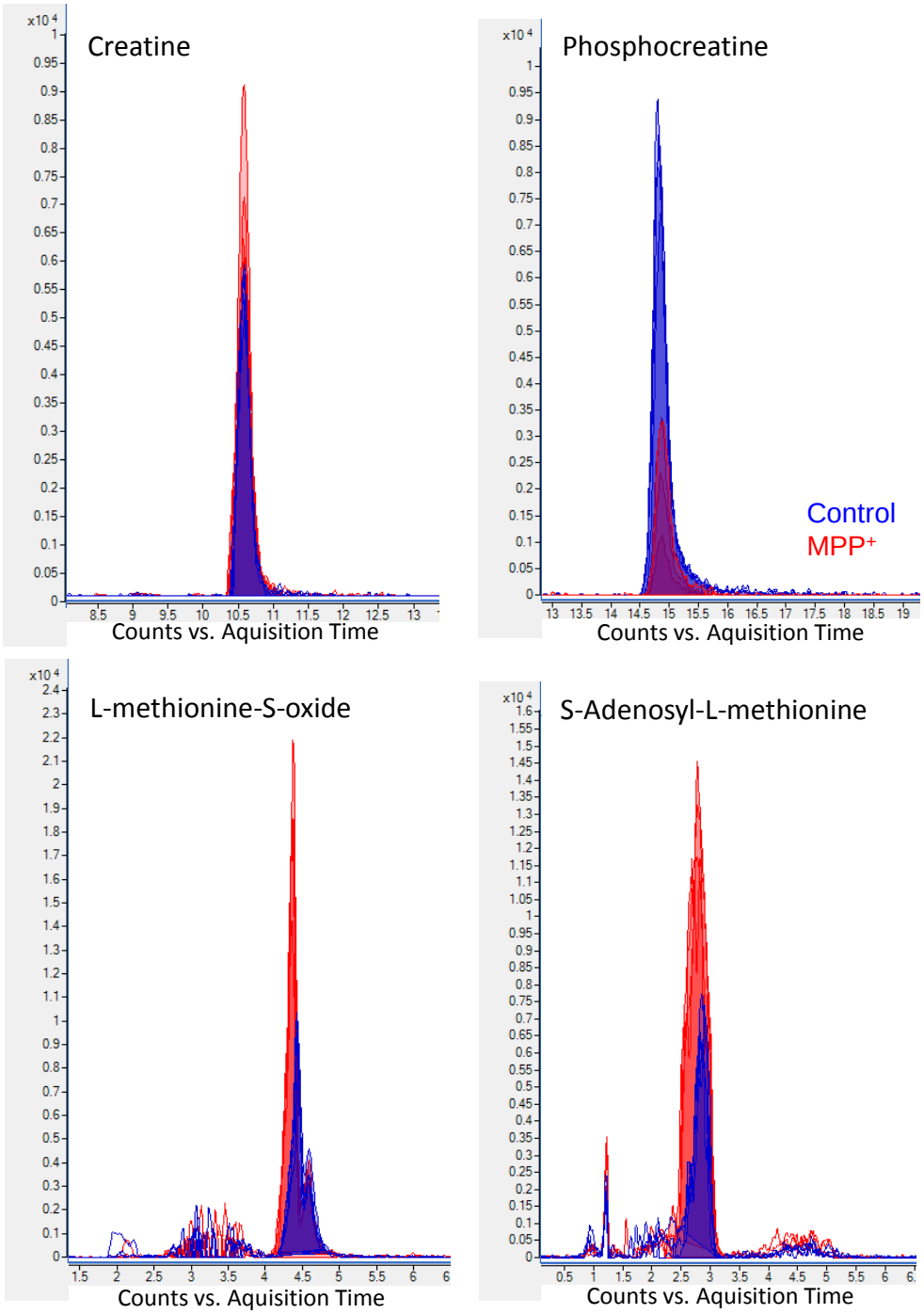

Figure S2

Example-peaks of metabolites affected by MPP$^+$ treatment

Cells were treated with MPP$^+$ or solvent for 24 h. Samples were run with the accurate-mass Q-TOF LC-MS system. Peak overlays are displayed by the Agilent MassHunter Acquisition software. The areas under the curve (integral of the peak curve) were used for quantification. Data are examples of typical primary data in a representative experiment.

Marker expression

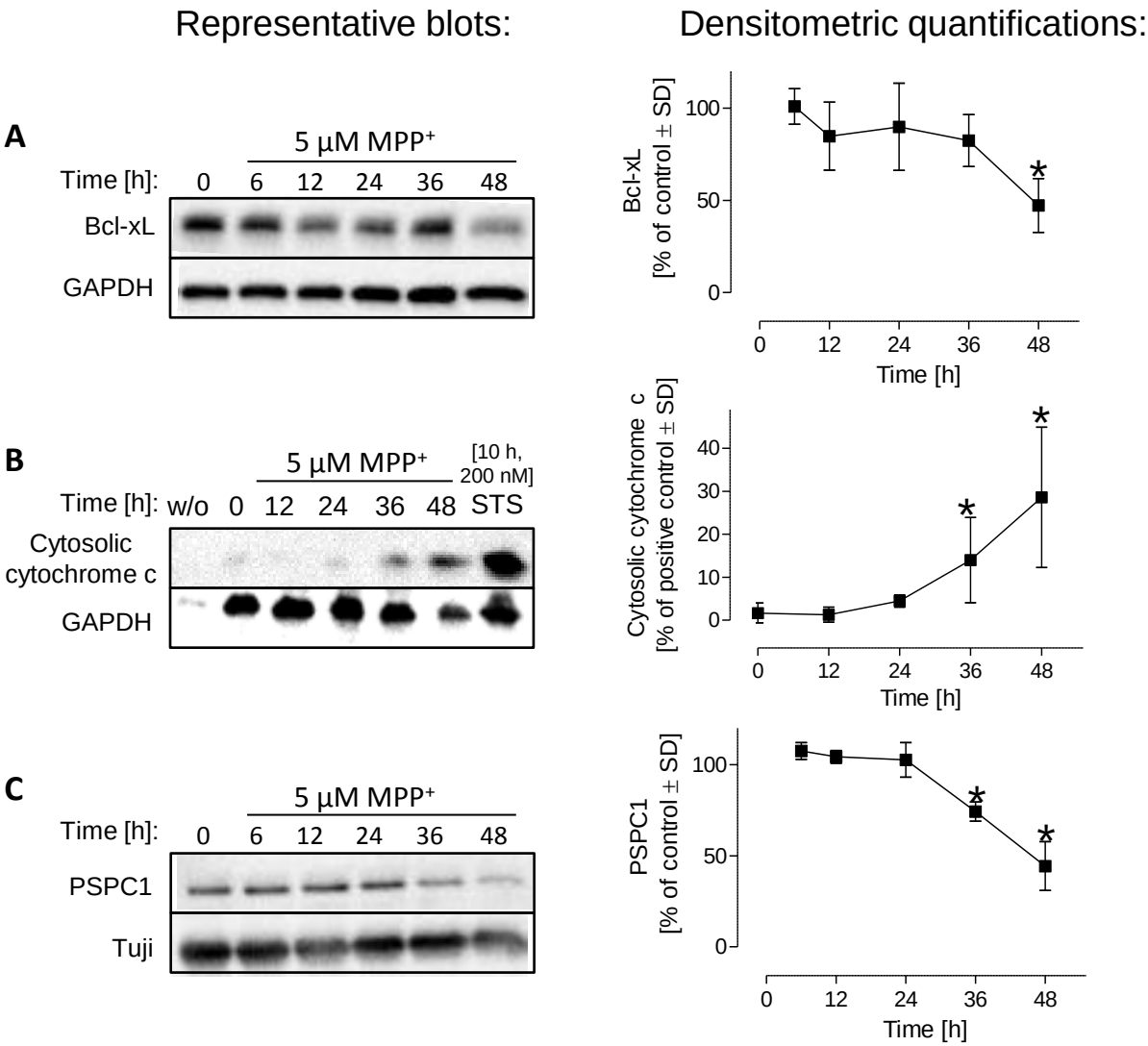

Figure S3

MPP+-induced changes of amount and location of apoptosis and cell stress-related proteins

LUHMES cell lysates were prepared at different times after exposure to MPP+ as illustrated in Fig. 1A. Proteins were quantified by western blot. To the left, representative blots are shown. To the right, densitometric quantifications of the respective proteins are displayed as means ± SD of 3 independent differentiations. Calibration was relative to loading control and untreated cell samples. A) Bcl-xl levels. B) The cytosolic cytochrome c levels were determined by extraction of soluble cytosolic proteins after permeabilisation of the outer cell membrane with 50 µg/ml digitonin. This procedure did not permeabilize the outer mitochondrial membrane (Single et al. 1998). Controls were healthy cells without digitonin (w/o), healthy cells with digitonin (0) and a positive control of cells exposed to 200 nM staurosporine for 10 h (STS). C) PSPC1 (paraspeckle component 1). *: $p < 0.05$.

Single, B., et al. (1998). "Simultaneous release of adenylate kinase and cytochrome c in cell death." Cell Death Differ **5**(12): 1001-1003.

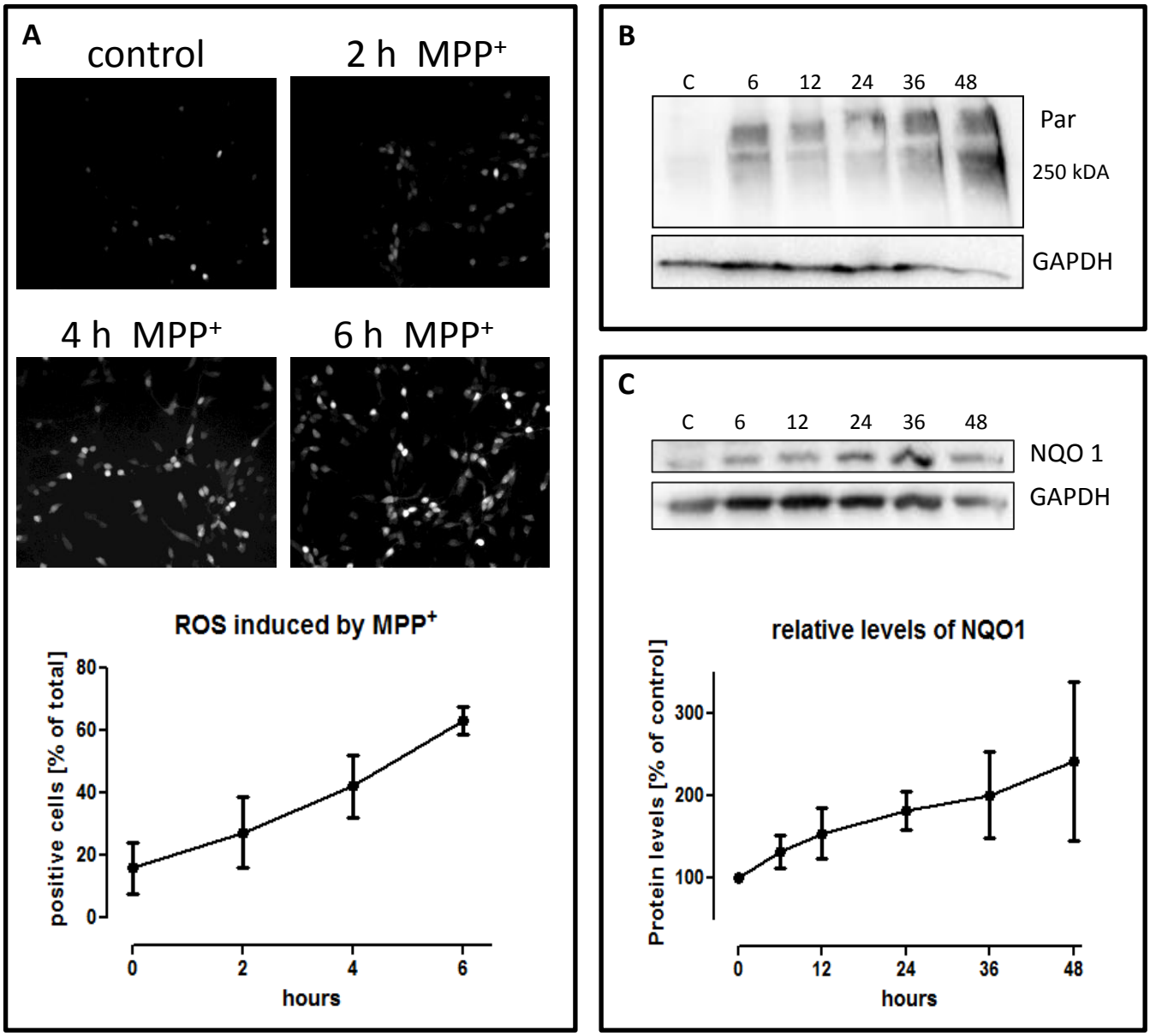

Figure S4:

Time course of MPP+ induced ROS generation

LUHMES were seeded at a density of $1.5*10^5$ cells/cm^2 at day 2. Medium was exchanged at day 5 and cells were treated with MPP+ at various times.

a) Images of live cell staining of LUHMES (day 6) cultured with 5 µM MPP+ for 2-6 h. Untreated control cells and treated cells were loaded for 45 minutes with a fluorescin-based ROS detection dye (ENZO, Total ROS detection kit, ENZ-51011), medium was replaced and cells were imaged immediately. Cells that showed fluorescence at 530 nm, when excited with 488 nm light were counted as positive for ROS production. Data are means ± SD of triplicates.

b) Whole cell lysates of LUHMES treated with 5 µM MPP+ for 0, 6, 12, 24, 36 and 48 h were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti PAR (H10 monoclonal AB from 10H hybridoma cells (1:300) (Kawamitsu, Hoshino et al. 1984)., mouse) primary antibody overnight and with anti-mouse (Jackson, 1:2500) secondary antibody for one hour. (n=3), before the Western blot was developed.

c) Whole cell lysates of LUHMES treated with 5 µM MPP+ for 0, 12, 24, 36, and 48 h, were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. For Western blot detection, membranes were incubated with anti NQO1 (Cell Signaling, 1:1000) primary antibody overnight and with anti-rabbit (GE Healthcare, IgG,1:5000) secondary antibodies for one hour. Protein levels (scans) were normalized to loading controls (GAPDH), and data are averages from 5 experiments.

PCA analysis of gene array data

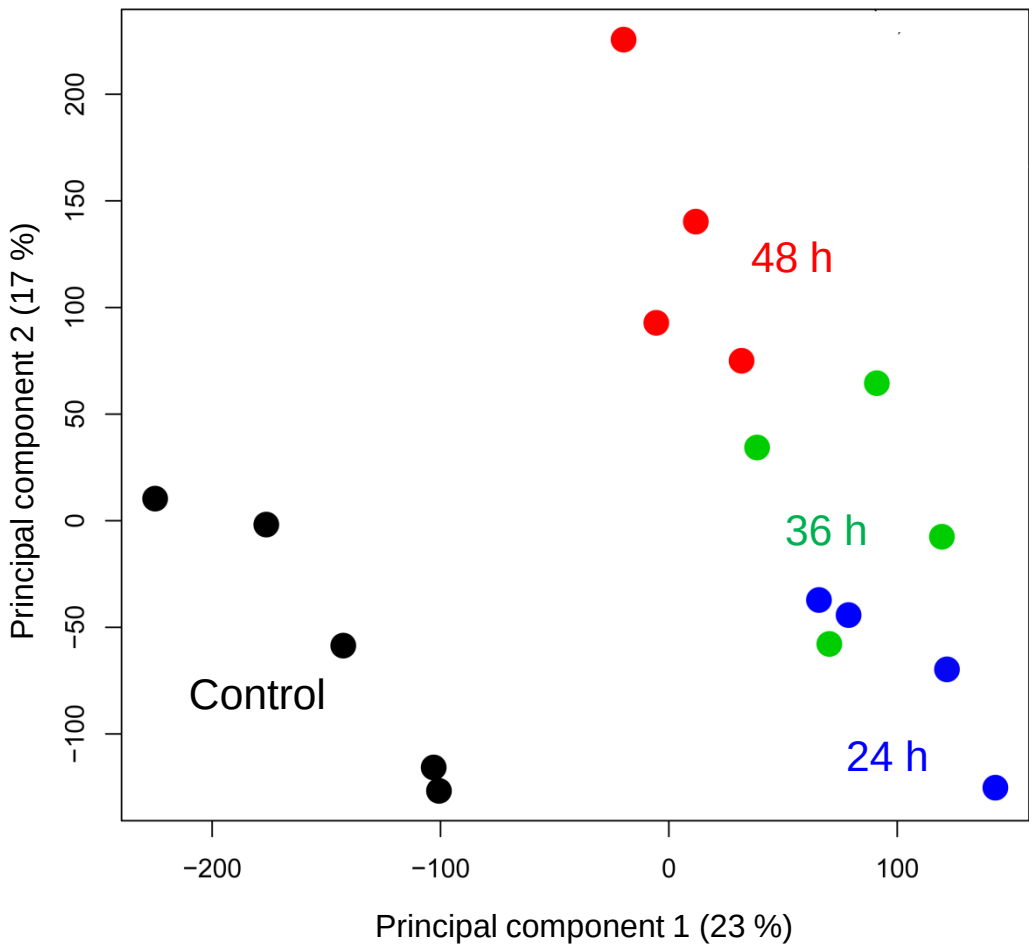

Figure S5
Principal component analysis (PCA) of regulated genes of MPP$^+$-treated LUHMES cells
Cells were treated with MPP$^+$ (5 µM) for different times and samples were prepared for microarray analysis as described in Fig. 1A. The signal of all probesets significantly regulated (FDR adjusted p-value of ≤ 0.05; fold change values ≥ 2) was used for principal component analysis (PCA). The first two dimensions of the respective data are displayed.

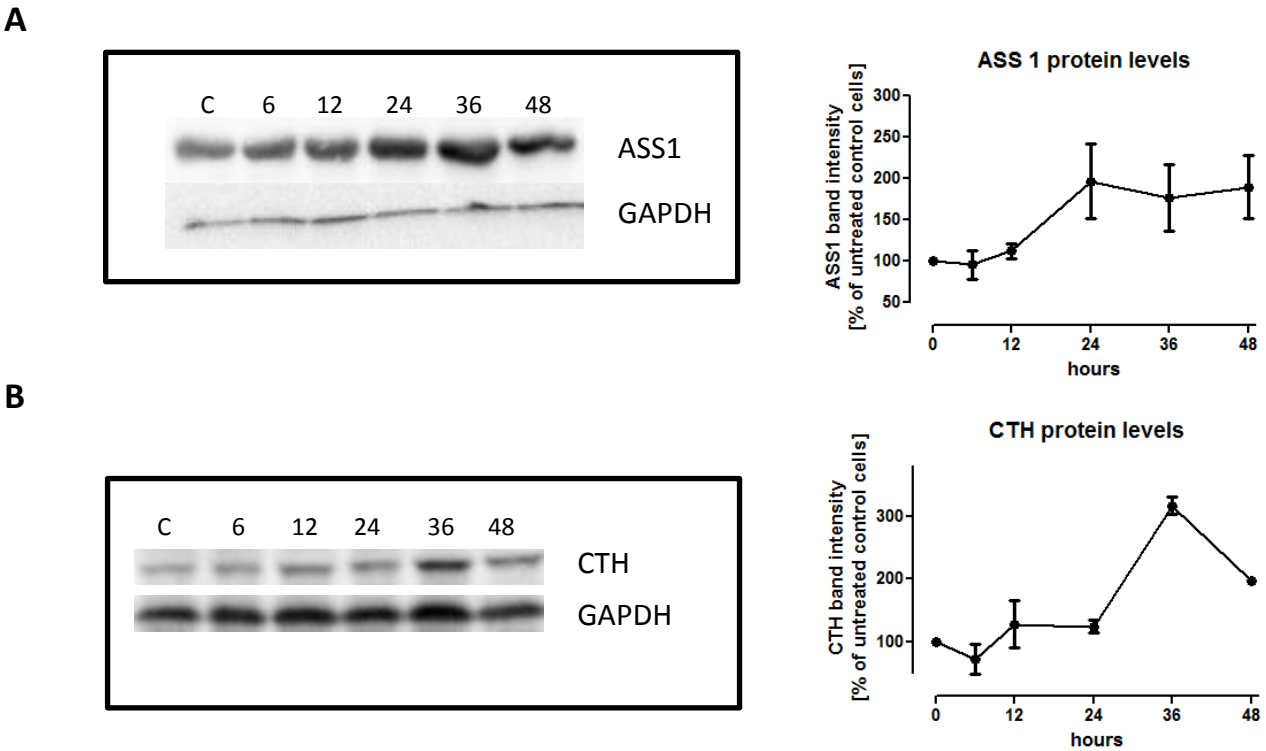

Figure S6:

Regulation of proteins involved in cellular thiol supply by MPP^+

LUHMES were seeded at a density of $1.5*10^5$ cells/cm^2 at day 2. Medium was exchanged at day 5 and cells were treated at various times with 5 µM MPP^+.

A) Whole cell lysates of LUHMES treated with 5 µM MPP^+ for 0, 12, 24, 36, and 48 h, were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti-ASS1 (Sigma Aldrich, mouse, 1:250) overnight and with anti-mouse (Jackson,1:5000)) secondary antibody for one hour. Protein amounts were quantified by a luminescence imager relative to the loading control (GAPDH). These relative protein amounts were then normalized to the amount of protein in control cells. Data are means ± SD of 5 determinations.

B) Whole cell lysates of LUHMES treated with 5 µM MPP^+ for 0, 12, 24, 36, and 48 h, were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with anti-CTH (Abcam, mouse, 1:250) overnight and with anti-mouse (Jackson,1:5000)) secondary antibody for one hour (n=3). Protein was quantified as in a.

C) Cells (350.000/well), treated with or without 5 µM MPP^+ for 0, 12 or 24 h, were washed 1× with Hank's balanced salt solution (HBSS), containing Ca^{2+}, pH 7.4. Then 200 µl containing 0.2 µCi/ml [^{14}C]-cystine (110 mCi/mmol stock solution; Perkin Elmer, Waltham, MA, USA) was added. The supernatants were removed after 20 min, cells were washed 3× gently with warm HBSS and then lysed with PBS/0.1% Triton X-100. Radioactivity in cell lysates was measured using a Beckman LS-6500 scintillation counter. Experiments were performed in parallel in the presence of a 100-fold excess (500 µM) of unlabelled (cold) cystine.

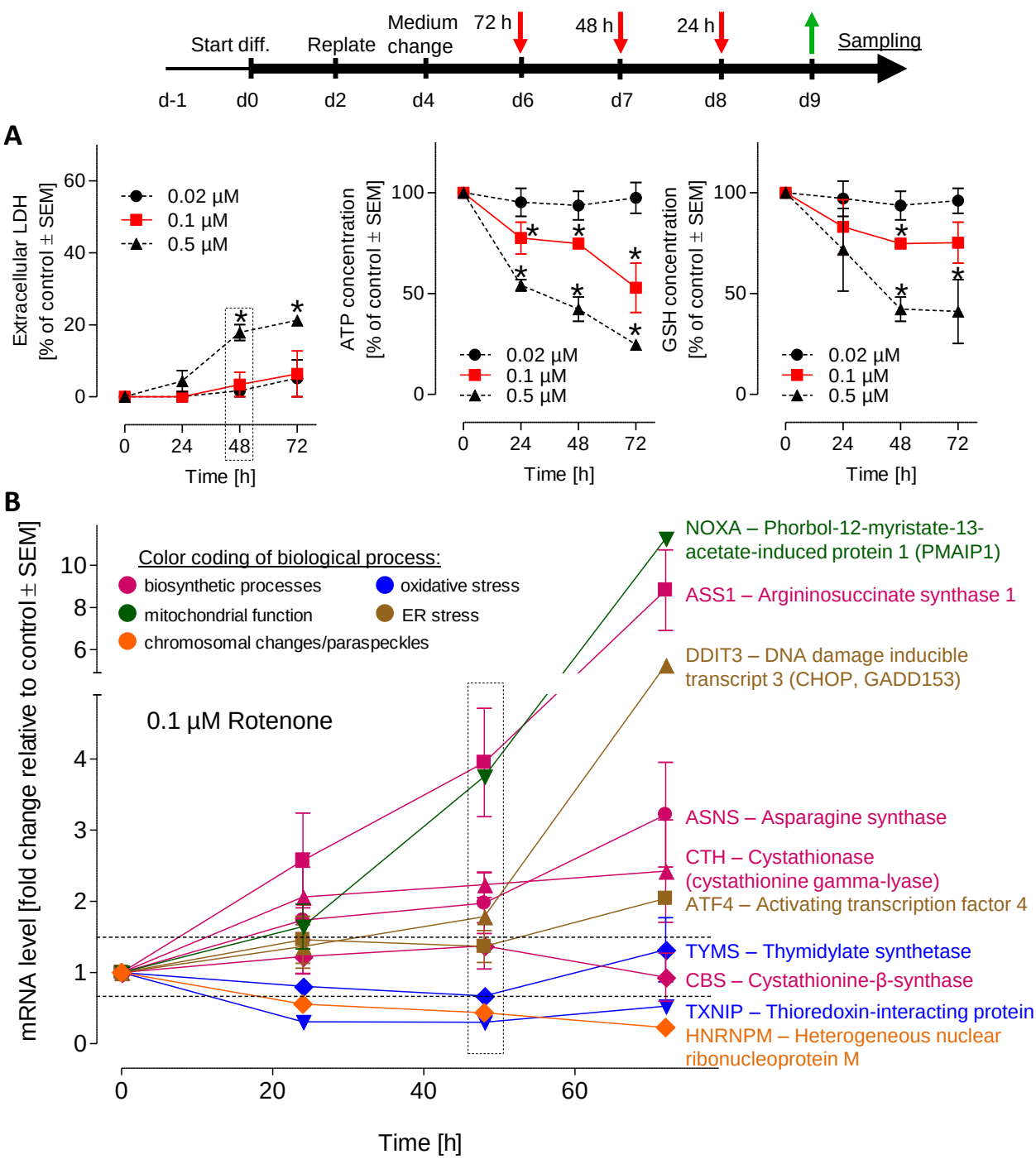

Figure S7

Transcriptional changes triggered by the complex I inhibitor rotenone in LUHMES cells

Cells were treated with different concentrations of rotenone (20, 100, 500 nM) for different times (24, 48, 72 h) and all samples were taken at day 9 (d9), as indicated in the treatment scheme. A) General cytotoxicity was evaluated by measurement of LDH release into the medium. GSH and ATP concentrations were determined in cell lysates. B) Several genes, which had been found to be regulated in the MPP^+ toxicity model, were examined. The mRNA from samples treated with 100 nM rotenone was quantified by RT-qPCR. All Data are means ± SEM of three independent experiments. They were normalized to untreated control. For easier comparison, the 48 h data for cytotoxicity and gene regulation are highlighted by a dashed box.

Combined Omics pathway-analysis

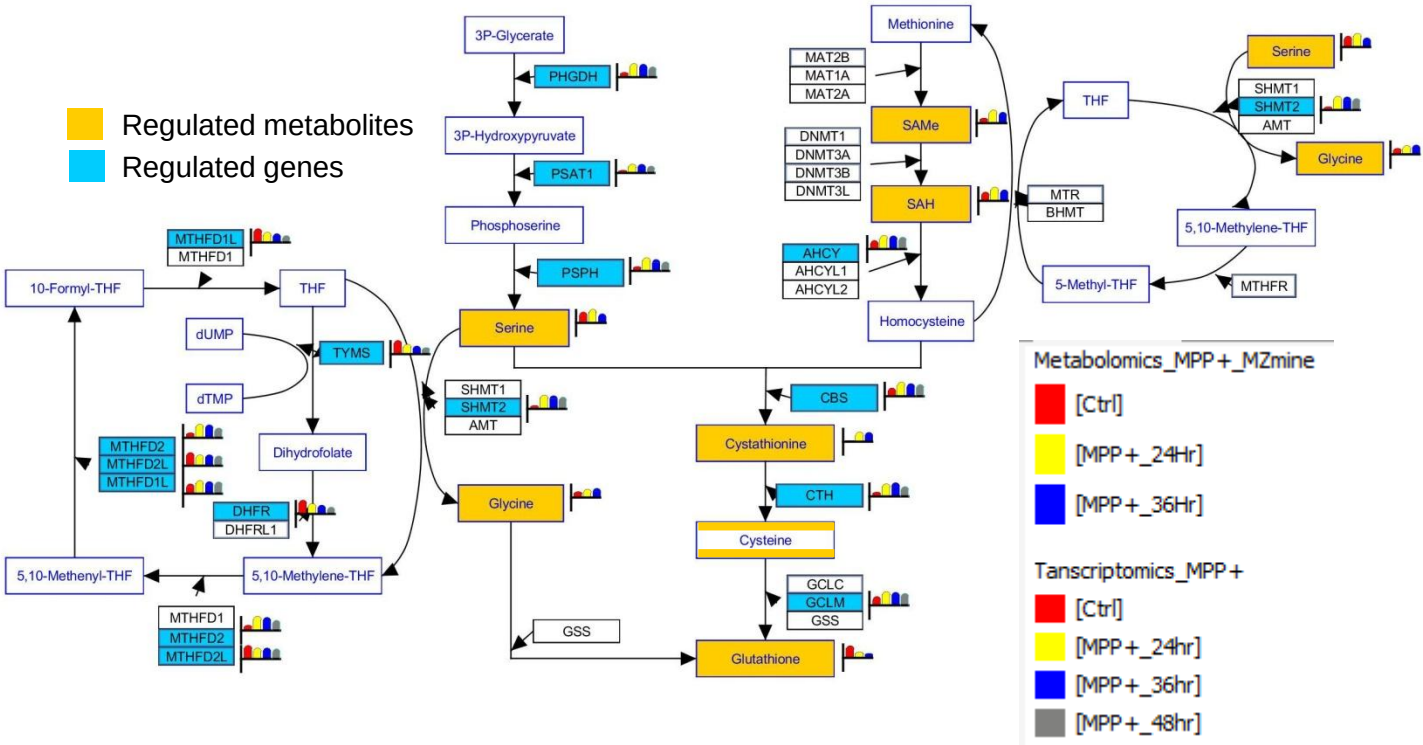

Figure S8

Integrated analysis of metabolomics and transcriptomics data to identify affected pathways

The pathway-analysis is based on transcriptomics and metabolomics data of the cells treated for 24 h with 5 µM MPP^+. The integration of both data sets for multi-omics analysis was performed using Mass Profiler Professional (MPP) software (version 12.6, Agilent Technologies). The MPP multi-omics capability allows two different omic experiments to be mapped and seen on the same pathway. Metabolites, identified by Q-TOF LC-MS and transcripts of the DNA microarray analysis were combined and reanalyzed together for pathway enrichment. A fold change cut-off for transcripts and metabolites of 1.5 and a significance threshold of 0.05 (FDR corrected) were used. WikiPathways served as pathway source. Differentially detected metabolites and genes are highlighted in color and have an adjacent heat strip for the relative abundances across the different conditions (red = control samples, yellow = 24 h samples, blue = 36 h samples, grey = 48 h samples). Regulated metabolites are indicated in yellow (cysteine was detected only in the targeted analysis); blue highlights regulated genes. (AHCY = adenosylhomocysteinase, AHCYL1 = adenosylhomocysteinase-like 1, AHCYL2 = adenosylhomocysteinase-like 2, AMT = aminomethyltransferase, BHMT = betaine—homocysteine S-methyltransferase, CBS = cystathionine-β-synthase, CTH = cystathionase, DHFR = dihydrofolatereductase, DHFRL1 = dihydrofolatereductase-like 1, DNMT1 = DNA (cytosine-5-)-methyltransferase 1, DNMT3A = DNA (cytosine-5-)-methyltransferase 3 alpha, DNMT3B = DNA (cytosine-5-)-methyltransferase 3 beta, DNMT3L = DNA (cytosine-5-)-methyltransferase 3-like, dTMP = deoxythymidine monophosphate, dUMP = deoxyuridine monophosphate, GCLC = glutamate-cysteine ligase, GCLM = glutamate-cysteine ligase, modifier subunit, GSS = glutathione synthetase, MAT2B = methionine adenosyltransferase II beta, MAT1A = methionine adenosyltransferase I alpha, MAT2A = methionine adenosyltransferase II alpha, MTHFD1 = methylenetetrahydrofolate dehydrogenase 1, MTHFD1L = methylenetetrahydrofolate dehydrogenase 1-like, MTHFD2 = methylenetetrahydrofolate dehydrogenase 2, MTHFD2L = methylenetetrahydrofolate dehydrogenase 2-like, MTHFR = methylenetetrahydrofolatereductase, MTR = methionine synthase, PHGDH = phosphoglyceratedehydrogenase, PSAT1 = phosphoserine aminotransferase 1, PSPH = phosphoserinephosphatase, ROS = reactive oxygen species, SHMT1 = serine hydroxymethyl-transferase 1, SHMT2 = serine hydroxymethyl-transferase 2, THF = tetrahydrofolate, TYMS = thymidylatesynthetase)

MPP+ induced cellular adaptations

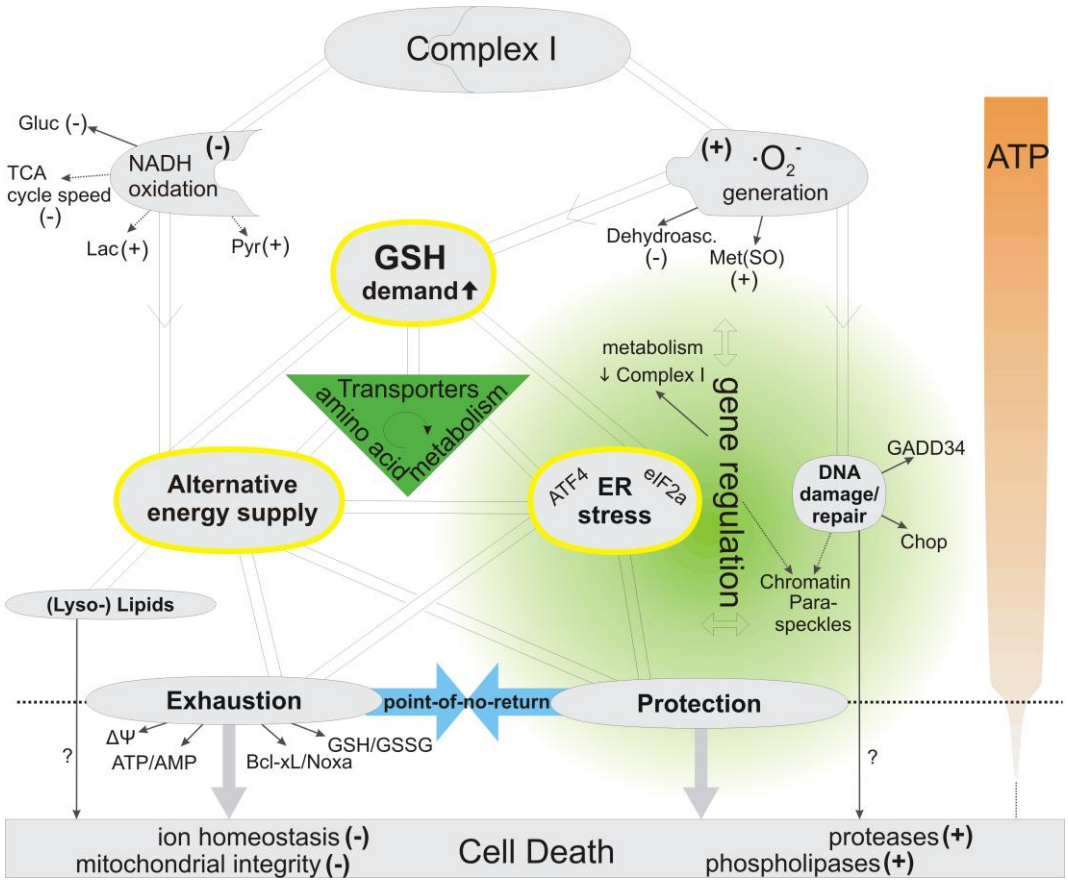

Figure S9

Overview of MPP+-induced adaptations in human dopaminergic neurons

The findings of this study (labeled here with *) have been incorporated into a network of adaptive regulations. The molecular initiating event of MPP+ toxicity is inhibition of mitochondrial respiratory chain complex I. Thus, NADH oxidation is hindered, and this leads to an arrest of the TCA cycle. The subsequent slowdown of the pyruvate dehydrogenase* leads to an accumulation of pyruvate* and lactate*. Cellular adaptations lead to increased glucose consumption* and usage of alternative ATP synthesis sources*. Complex I inhibition also leads to a higher O_2^-. production, as indicated by decreased levels of the antioxidant dehydroascorbate* and an increase in methionine sulfoxide*. The two primary events cause a general increase in GSH demand with adaptations on metabolite and transcriptome level*. Demand for alternative energy sources and GSH results in changed amino acid metabolism* and higher expression of their transporters*. Several of these changes contribute to a rise in ER stress, as indicated by phosphorylation of eIF2a* and increases of ATF4*. Many of the observed changes in gene regulation may be attributed to ATF-4 and related transcriptions factors. A DNA damage response* and altered lipid metabolism* suggest that many further cellular changes take place long before energy is depleted. At the point of no return the counter-regulation capacity of the cell is exhausted, ATP and GSH drop steeply*, programmed cell death pathways (NOXA ↑, PUMA ↑, Bcl-xL ↓) are activated* and loss of mitochondrial integrity ensues*.Pyr = pyruvate, Lac = lactate, Gluc = glucose, TCA = tricarboxylic acid cycle, Dehydroasc = dehydroascorbate, Met(SO) = methionine sulfoxide, GSH = reduced glutathione, GSSG = glutathione disulfide.

Antibodies

Figure 10– Antibodies used for western blot or immunostaining

Antigen	Antibody (supplier; clone)	Dilution	Blocking (5%)	Species
ATF4 (CREB-2)	Anti-ATF4(D4B8, cell signaling)	1:1000	BSA	rabbit
CTH	Anti-CTH (abcam)	1:250	BSA	mouse
GADD34	Anti-GADD34 (proteintech)	1:1000	BSA	mouse
eIF2a-p	Anti-eIF2a [pS52] (invitrogen)	1:1000	BSA	rabbit
PSPC1	Anti-PSPC1 (sigma)	1:200	Milk	rabbit
GAPDH	Anti-GAPDH (Sigma; Clone GAPDH-71.1)	1:5000	BSA	mouse
Bcl-xl	anti Bcl-xl (CellSignaling)	1:1000	Milk	rabbit
Cytochrome c	Anti-Cytochcrome c (BD Pharmingen; Clone 7H8.2C12	1:1000	Milk	mouse
anti-mouse	anti-mouse HRP antibody (Jackson Immuno Research)	1:2500		goat
anti-rabbit	anti-rabbit HRP antibody (GE Healthcare)	1:5000		goat

Primers

Figure 11– Primers used for RT-qPCR

Name	Forward sequence	Reverse Sequence
ASNS	GGGGCTTGGACTCCAGCTTG	GAGCCTGAATGCCTTCCTCA
ASS1	TGCTCCCTGGAGGATGCCTG	GTGTAGAGACCTGGAGGCGC
ATF2	AGAGCGAAATAGAGCAGCAG	CATGGCGGTTACAGGGCAAT
ATF4	GGCTGGCTGTGGATGGGTTG	CTCCTGGACTAGGGGGGCAA
CBS	TCCTGGGAATGGTGACGCTT	GTGCTGTGGTACTGGATCTG
CCNB	TGGATGTGCCCCTGCAGAAG	CAGTGACTTCCCGACCCAGT
CTH	TGGATGATGTGTATGGAGGTACAAACAGG	GCCTTCAATGTCAATCACCTTCTGGG
DDIT3	ATGGCAGCTGAGTCATTGCC	TCCTCAGTCAGCCAAGCCAG
DDIT4	AGTCCCTGGACAGCAGCAAC	AACTGGCTAGGCATCAGCAG
GADD34	GCATCACCCAGGCCCAGGAG	AGACGAGCGGGAAGGTGTGG
GAPDH	CACCATCTTCCAGGAGCGAGATC	GCAGGAGGCATTGCTGATGATC
HNRNPM	TGGTGTGGTGGTCCGAGCAG	GGACGCTCAGGAGGGAAGAA
MLF1IP	TTTGTAAGGCAGCCATCGCC	CTGTGGCTCTAACCGAAGCA
NOXA	CAGTGCCAACTCAGCACATTG	CGCCCAACAGGAACACATTGA
NQO1	TGGAGTCGGACCTCTATGCCA	CTTGTGATATTCCAGTTCCCCCTGC
PHGDH	AAC TCC AGG TGG TGG GCA GG	GCT CCC ATT TGC CGT CCT TC
PPA2	TGGAAAGCTACGCTATGTGG	GCTTCAGGATCATTCGCATTG
PSAT1	AGC TGC CGC ACT CAG TGT TG	AAC TGG CCG CAC CCA CCT CC
PSPC1	CAGCAGCGTGAGCAGGTTGA	CGCCGATGCTCCTCTTCATG
PSPH	CCC CGG CAT AAG GGA GCT GG	GCT GTT GGC TGC GTC TCA TC
SFPQ	TCAGGCAAATCTTTTGCGCC	CTCTCTTTGGCGCCTCATTT
SHMT2	CAACCTGGCACTGACTGCTC	GATGTCCGCGTGCTTGAAAG
SLC3A2	CAC CCT GCC AGG GAC CCC TG	GCC GCC GGA ACA AGG AAA GG
SLC7A11	GCA GCG TGG GCA TGT CTC TG	CAC AGC AGT AGC TGC AGG GC
TXNIP1	CATGGCGTGGCAAGAGCCTT	CTCAGAGCTGGTTCGGCTGG
TYMS	CAGCTTCAGCGAGAACCCAG	ACCTCGGCATCCAGCCCAAC