Supplement to: A. E. Medvedev, O. A. Buneeva, A. T. Kopylov, O. V. Tikhonova, M. V. Medvedeva, L. N. Nerobkova, I. G. Kapitsa, and V. G. Zgoda, Brain Mitochondrial Subproteome of Rpn10-Binding Proteins and Its Changes Induced by the Neurotoxin MPTP and the Neuroprotector Isatin (ISSN 0006-2979, *Biochemistry (Moscow)*, 2017, Vol. 82, No. 3, pp. 330-339)

MATERIALS AND METHODS

Materials. Isatin, MPTP, DTT, iodoacetamide, trypsin, Tris (hydroxymethyl) aminomethane, urea, ammonium hydrocarbonate, sodium chloride, Triton X-100, glycerol, bacitracin, phenylmethylsulfonyl fluoride (PMSF), 4-vinylpyridine, deoxycholic acid sodium salt triethylammonium bicarbonate, Coomassie Brilliant Blue G-250, and deprenyl were purchased from Sigma (USA), [^{14}C]2-phenylethylamine hydrochloride was from Amersham (UK), acetonitrile was from Fisher Chemical (UK), formic acid, trifluoroacetic acid (TFA), and 2-propanol were from Fluka (USA), TCEP (tris-(2-carboxyethyl)-phosphine) was from Pierce (USA), sequencing grade modified trypsin was from Promega (USA), and 10 kDa acetate cellulose filters were from Sartorius Stedium Biotech (Germany). Proteasome 19S Rpn10/S5a subunit (human), (recombinant) (GST-tag) (agarose immobilized) was from Enzo Life Sciences (USA). C-18 spin columns were from Pierce, Amicon Ultracel-10K centrifugal filter units were from Millipore (USA), and Acclaim PepMap® RSLC C18 column (150 mm × 75 μm, 2 μm particle size, 100 Å pore size) was from Dionex (Rockford).

Preparation of brain mitochondrial fraction and assay of MAO B activity. After analysis of behavioral reactions, animals were decapitated. Mouse brains were dissected and homogenized in the isolation medium containing 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer, pH 7.5, using an Ultra-Turrax T 10 homogenizer at a low speed, to obtain 30% w/v homogenate. Brain mitochondrial fraction was isolated by differential centrifugation as described in [27]. MAO B activity was assayed radiometrically using 0.005 mM [^{14}C]phenylethylamine as substrate [28]. Mitochondrial MAO B activity assayed with this substrate concentration demonstrated sensitivity to inhibition by "diagnostic" MAO B inhibitor deprenyl and also to isatin.

Affinity fractionation of Rpn10-binding proteins. Agarose resin with the immobilized proteasome Rpn10 subunit (0.5 ml) was washed five times with TBS (dissolved in 10 ml and centrifuged). The mitochondrial preparation (5 mg/ml protein, TBS, 1% Triton X-100) was added to the agarose slurry up to suspension (1 : 1), taking into consideration the additions of glycerol, PMSF, and bacitracin (final concentrations 5% (v/v), 1 mM, and 0.2 mg/ml, consequently). The resulting suspension was incubated overnight at 4°C with gentle stirring. After that, the agarose was washed eight times with TBS (using Eppendorf Centrifuge 5415 R (1 min, 50*g*)) up to the absence of protein in the solution (measured by OD_{280}). Affinity elution was performed with a column (0.5 ml) at room temperature and flow rate 0.2 ml/min; bound proteins were eluted using TBS containing 6 M urea. Eluates (10 column volumes) were diluted four-fold in TBS and then concentrated using Amicon Ultracel-10K centrifugal filter units (Millipore). After sample concentration to 250 μl, 20 ml of TBS was added and again the preparations were concentrated to 250 μl.

In parallel, we evaluated nonspecific binding of brain mitochondrial lysates using a control batch of cyanogen bromide-activated agarose prepared exactly as the affinity sorbent but without addition of the affinity ligand.

Sample preparation for mass spectrometric analysis, liquid chromatography, and high-resolution mass spectrometry. After affinity separations, fractions of Rnp10 mitochondrial proteins were desalted using C-18 spin columns as recommended by the manufacturer. C-18 resin was activated by 200 μl of 50% solution of acetonitrile and then equilibrated sequentially three times by 200 μl of 5% acetonitrile with 0.1% TFA. Proteins were acidified by TFA to 0.1% final concentration, loaded onto the C-18 resin in 100 μl final volume, and centrifuged at 400*g* for 90 s. The collected flow-through was loaded again and centrifuged under the same conditions. Resin with bound proteins was washed sequentially two times by 200 μl of 5% acetonitrile with 0.1% TFA. Bound proteins were eluted from the resin by 200 μl of 70% acetonitrile with 0.1% TFA, and the collected eluates were dried under vacuum at 30°C for 60 min.

Protein-denaturing solution was prepared from 5% acetonitrile and 0.1% of deoxycholic acid sodium salt buffered in 75 mM triethylammonium bicarbonate (Sigma). Dried proteins were resuspended in 100 μl of denaturing solution and reduced by addition of 1 μl of 0.5 M TCEP (Tris-(2-carboxyethyl)-phosphine) to 5 mM final concentration and incubated for 20 min at 60°C. After cooling the protein samples, they were alkylated by addition of 1.8 μl of 10% 4-vinylpyridine solution in 30% 2-propanol to 17.5 mM final concentration, the reaction lasting 30 min at 37°C. Proteins were digested overnight at 37°C using sequencing grade modified trypsin at ratio 1 : 50 (w/w). Trypsinolysis was terminated by addition of formic acid to 1% final concentration, and the resulting digest was centrifuged at 12,000*g* for 10 min at room temperature to sediment the deoxycholic acid precipitate.

The mixture of peptides was purified using spin filtration via 10 kDa acetate cellulose filters, dried under vacuum at 30°C, and resuspended in 20 μl of 0.3% formic acid for LC-MS analysis.

The chromatography separation was carried out on a Dionex Ultimate 3000 system comprised of a RSLC Nano pump, a column equipped with a switch valve, and an autosampler (Dionex, USA). An Acclaim PepMap® RSLC C18 column (150 mm × 75 μm, 2 μm particle size, 100 Å pore size) was used in combination with an enrichment C18 PepMap®100 μ-precolumn (300 μm i.d. × 5 mm, 5 μm particle size, 100 Å pore size). Mobile phase A (composed of 0.08% formic acid, 0.015% TFA in water) and mobile phase B (composed of 0.08% formic acid, 0.015% TFA in 80% acetonitrile) were used for analytical separation in the elution gradient at a flow rate of 0.3 μl/min. Mobile phase C (composed of 0.08% formic acid, 0.015% TFA in 2% acetonitrile) was used for sample loading onto the enrichment μ-precolumn at flow rate 10 μl/min. Linear gradient elution was applied starting from 2.5% of mobile phase B and increasing to 45% of mobile phase B for the first 45 min. The column was washed with 85% of mobile phase B for 7 min at flow rate 0.45 μl/min, followed by subsequent equilibration of the system with 2.5% of mobile phase B for the next 12 min. Before the next sample run, the enrichment column and the analytical column were re-equilibrated with mobile phase C and 2.5% of mobile phase B for 10 min, respectively.

A high-resolution Q Exactive (Thermo Scientific, USA) mass spectrometer was used for LC-MS analysis. The instrument was operated in the positive ionization mode and was equipped with the NSI ion source. The capillary voltage was set at −2000 V, and ion-transfer tube temperature was 260°C. Precursor ions were surveyed in the range 450-1300 m/z with AGC target value 1e6 ions or maximum integration time of 210 ms at resolution R = 70K. The isolation window for precursor ions was set as ±2 Th. A high-energy collision-induced dissociation (HCD) activation type was applied for fragmentation of the isolated precursor ions, and HCD cell pressure was 7.5 mTorr. The normalized collision energy was adjusted to 27 eV and ramped ±20% over the duty cycle. Fragment ions were registered in the ultra-high-field orbitrap mass analyzer with resolution R = 17.5K in a scan range with the first fixed mass as 110 m/z. The AGC was set with a target value to 2e5 ions or maximum integration time for 125 ms in top-N20 mode. Apex triggering (from 10 to 20 s), dynamic exclusion for 10 s and active on-analysis calibration by using lock-masses were employed. Ions with charge state z = 1+ and z = 7+, 8+ and >8+ were excluded from analysis.

Data analysis. The raw data files were entered into MASCOT for identification of proteins. MASCOT search engine version 2.4 was run against the UniproKB/Swiss-Prot human proteins database. Pyridylethylation of cysteines was set as fixed modification, and methionine oxidation and N-terminal cyclization of glutamine were variable modifications. Trypsin was chosen as the cleavage enzyme with up to two missed cleavages allowed, peptides precursor tolerance ±10 ppm, and fragment ions mass tolerance ±0.05 Da. Search results were percolated using decoy reverse database and results of identification were adjusted to FDR less or equal to 1%. The criteria of positive identification were set as follows: minimum score of 30, at least three positive identifications from three different runs.

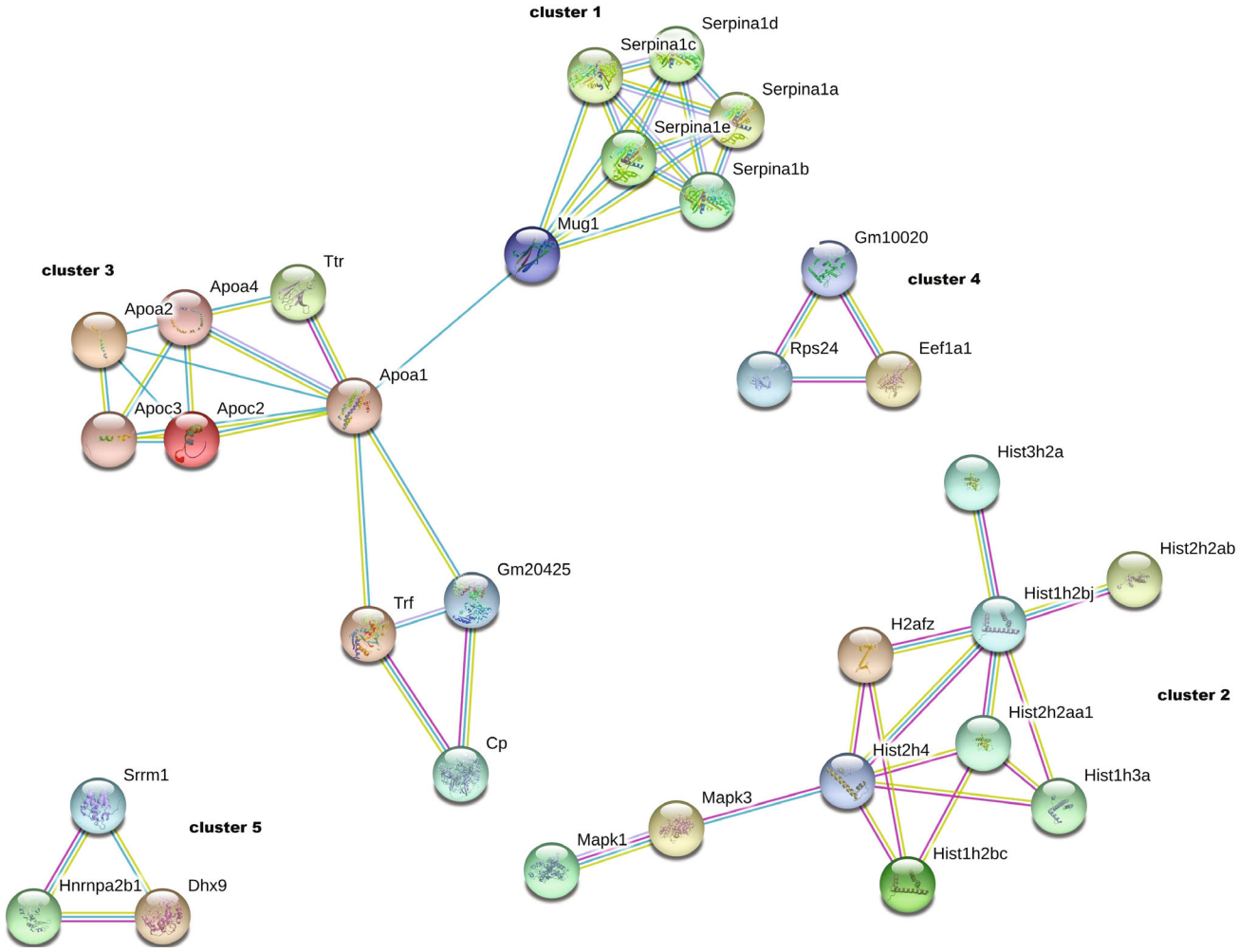

Fig. S1. Interaction clusters of Rpn10-binding proteins of brain mitochondrial fraction of control mice.

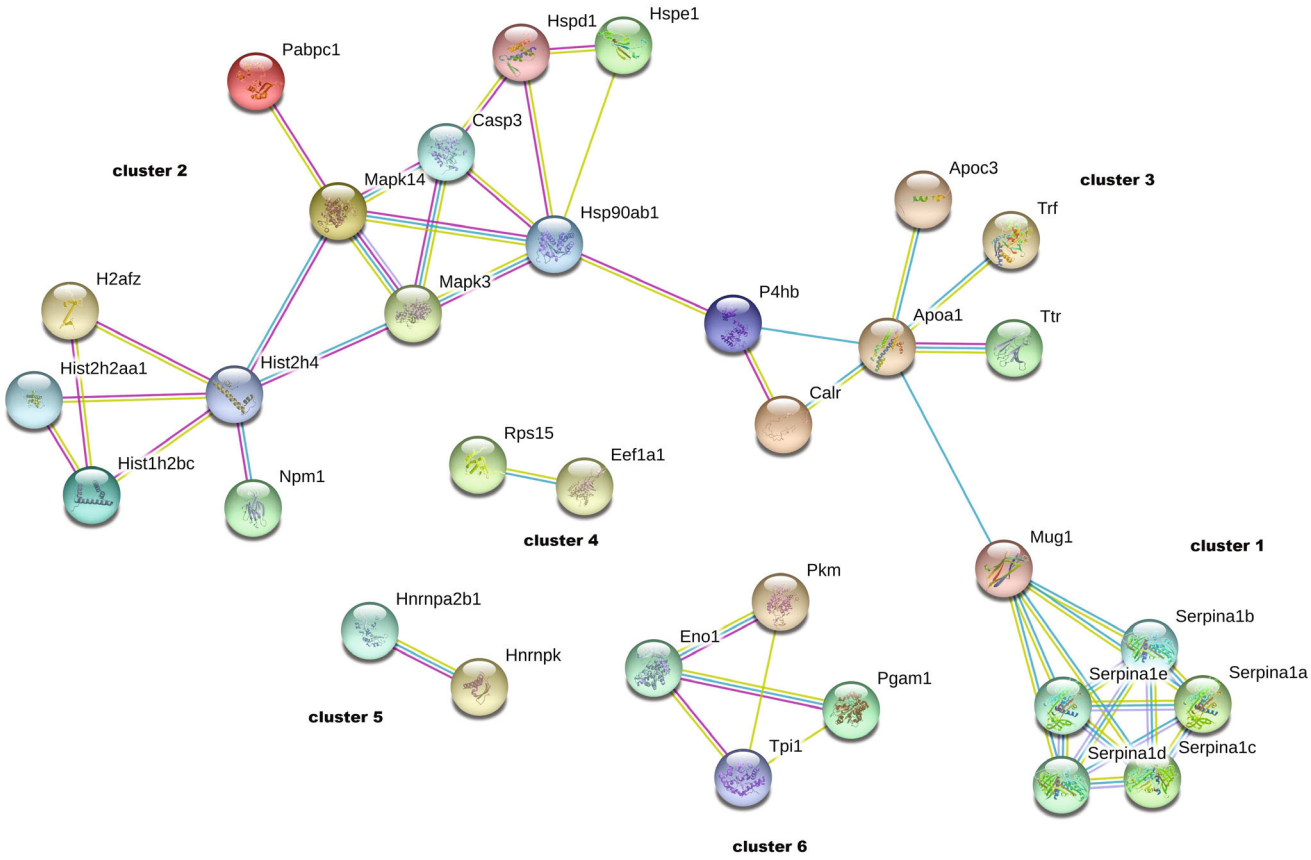

Fig. S2. Interaction clusters of Rpn10-binding proteins of brain mitochondrial fraction of MPTP-treated mice.

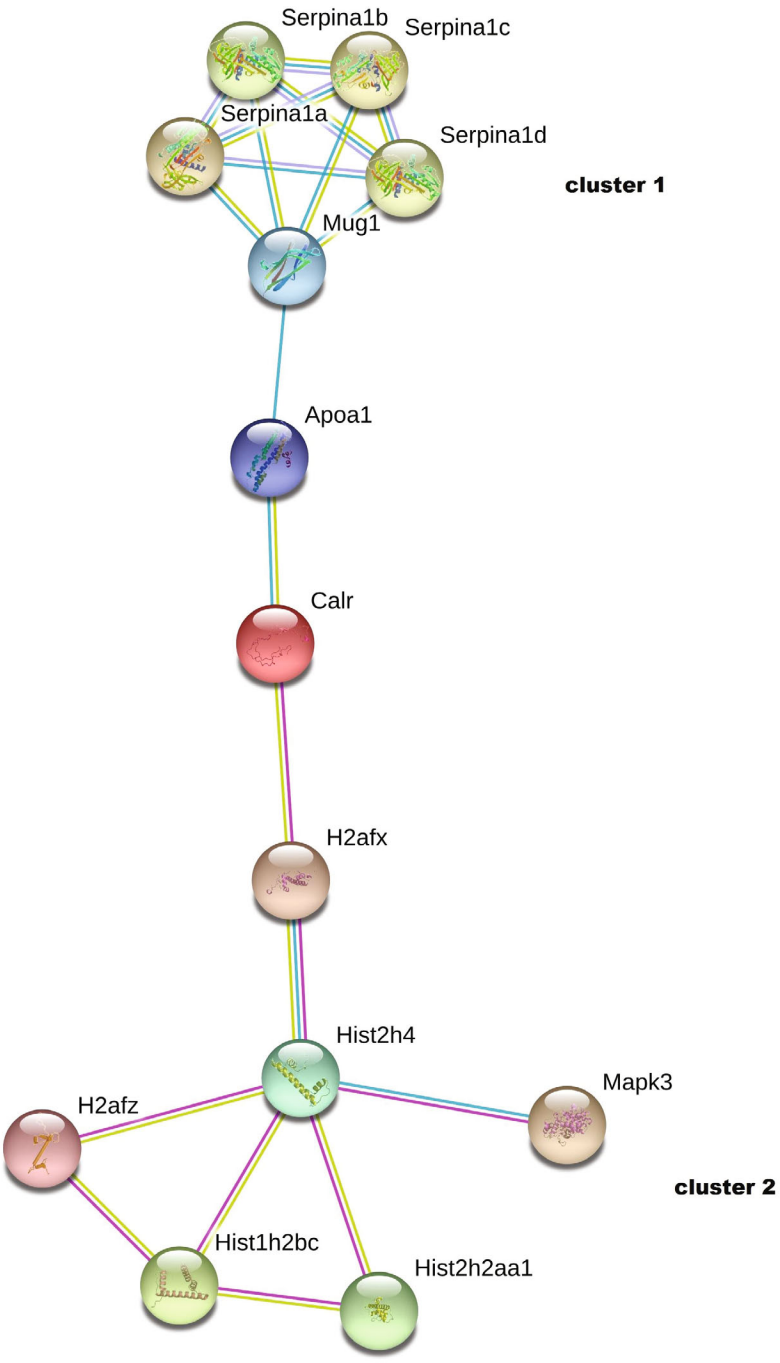

Fig. S3. Interaction clusters of Rpn10-binding proteins of brain mitochondrial fraction of isatin-treated mice.

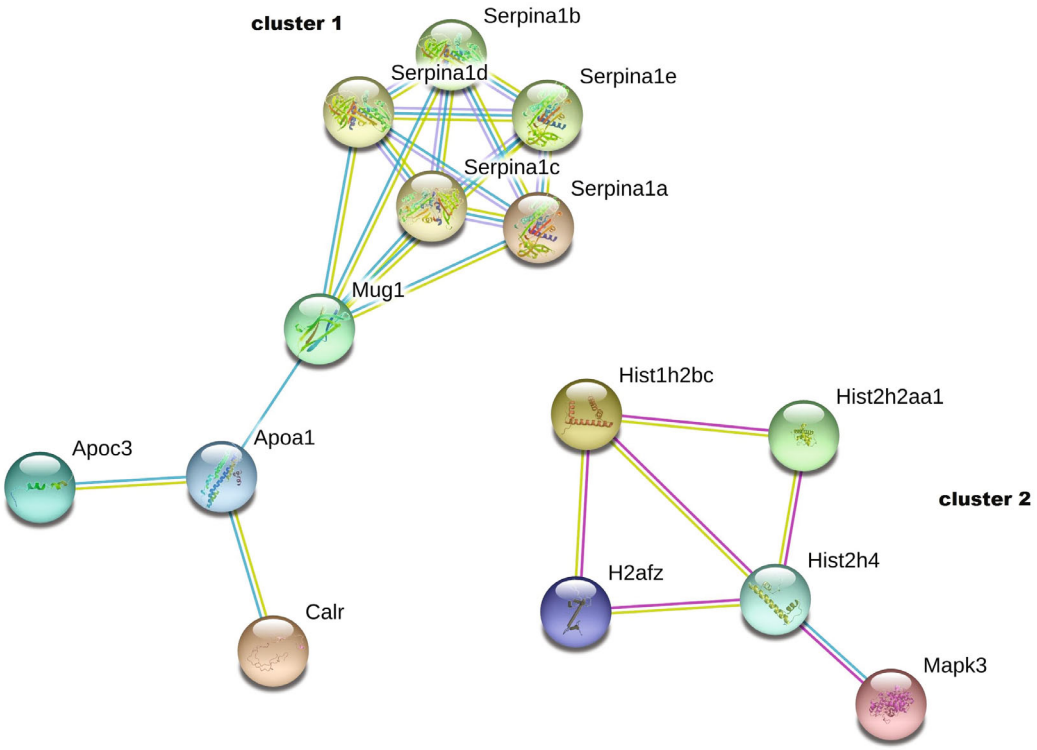

Fig. S4. Interaction clusters of Rpn10-binding proteins of brain mitochondrial fraction of mice treated with isatin and MPTP.

Table S1. Rpn10-binding proteins of control mice (brain mitochondrial fraction) identified in addition to the constitutive pool of proteins

No.	UniProt accession number	Protein name	Gene name	Protein function	Biological process	Interaction cluster ($C \geq 0.7$)
16	P45591	cofilin-2	Cfl2	actin binding	actin filament organization	
17	Q80TA9	ectopic P granules protein5 homolog	Epg5	autophagy	autophagy	
18	P68433	histone H3.1	Hist1h3a	DNA binding	nucleosome assembly, chromatin silencing	2
19	Q8CGP0	histone H2B type3-B	Hist3h2bb	DNA binding, nucleic acid binding	nucleosome disassembly	
20	Q8CGP2-2	isoform 2 of histone H2B type 1-P	Hist1h2bp	DNA binding, nucleic acid binding	nucleosome assembly	
21	Q61147	ceruloplasmin	Cp	ferroxidase activity, oxidoreductase activity, chaperone binding	ion transport, oxidation-reduction process	3
22	A2CG35	Ras-related protein Rab-12	Rab12	GTP binding	small GTPase mediated signal transduction	
23	Q6GYP7	Ral GTPase activating protein subunit alpha-1	Ralgapa1	GTPase activator activity	activation of GTPase activity, regulation of transcription	
24	D3Z5G7	carboxylic ester hydrolase	Ces1b	hydrolase activity	n.d.	
25	P23953	carboxylesterase 1C	Ces1c	hydrolase activity	n.d.	
26	Q3UW12	cyclic nucleotide-gated cation channel alpha-4	Cnga4	ion channel activity	ion transport	
27	P06728	apolipoprotein A-IV	Apoa4	lipid binding	lipid transport, phospholipid efflux	3
28	P09813	apolipoprotein A-II	Apoa2	lipid binding	lipid transport, phospholipid efflux	3

29	Q05020	apolipoprotein C-II	Apoc2	lipid binding	lipid transport, phospholipid efflux	3
30	E9Q0B5	protein Fcgbp	Fcgbp	n.d.	n.d.	
31	E9QNN1	ATP-dependent RNA helicase A	Dhx9	nucleic acid binding, hydrolase activity	RNA processing*	5
32	E9Q035	protein Gm20425	Gm20425	nucleotide binding	small GTPase mediated signal transduction	
33	D3Z0Y2	peroxiredoxin-6	Prdx6	oxidoreductase activity	cell redox homeostasis	
34	A6X935	inter-alpha-trypsin inhibitor heavy chain 4	Itih4	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	
35	E9QAZ2	ribosomal protein L15	Gm10020	structural constituent of ribosome	translation	4
36	P62849	40S ribosomal protein S24	Rps24	structural constituent of ribosome	translation	4
37	Q61838	pregnancy zone protein	Pzp	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	
38	D3YYR8	serotransferrin	Trf	n.d.	n.d.	3
39	P07309	transthyretin	Ttr	protein binding	transport	3
40	E9PVS1	inter-alpha-trypsin inhibitor heavy chain	Itih3	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity, hyaluronan metabolic process	
41	D3YZ68	elongation factor 1-alpha1	Eef1a1	GTP binding	translation	4
42	P63085	mitogen-activated protein kinase 1	Mapk1	MAP kinase activity	MAPK cascade	2
43	O88569	heterogeneous nuclear ribonucleoprotein A2/B1	Hnrnpa2b1	nucleic acid binding, RNA binding	mRNA processing	5
44	F6T4M4	serine/arginine repetitive matrix protein 1	Srrm1	n.d.	mRNA processing	5

45	E9QP56	apolipoprotein C-III	Apoc3	phospholipid binding, lipase inhibitor activity	phospholipid efflux	3
46	Q00898	alpha-1-antitrypsin 1-5	Serpina1e	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	1
47	E9PVG8	protein 9530053A07Rik	9530053A07Rik	n.d.	n.d.	
48	Q64522	histone H2A type 2-B	Hist2h2ab	DNA binding	chromatin silencing	2

Note: Here and in other tables: * Biological process assigned by similarity with the basic (reviewed status in UniProt) protein form; n.d., not designated.

Table S2. Rpn10-binding proteins (brain mitochondrial fraction) of MPTP-treated mice identified in addition to the constitutive pool of proteins

No.	UniProt accession number	Protein name	Gene name	Protein function	Biological process	Interaction cluster (C ≥ 0.7)
16	O89053	CORONINA1	Coro1a	actin binding	actin cytoskeleton organization	
17	P70677	caspase 3	Casp3	aspartic-type endopeptidase activity	apoptotic process, proteolysis	2
18	P0C0S6	histone H2A.Z	H2afz	DNA binding	chromatin silencing	2
19	H7BXC3	triosophosphate isomerase	Tpi1	catalytic activity	glycolytic process	6
20	Q9DBJ1	phosphoglycerate mutase 1	Pgam1	phosphoglycerate mutase activity, hydrolase activity	glycolytic process	6
21	P52480	pyruvate kinase isoform M1/M2	Pkm	pyruvate kinase activity	glycolytic process	6
22	P47811	mitogen-activated protein kinase 14	Mapk14	MAP kinase activity	MAPK cascade	2
23	Q6R5F8	protein GM10258	Gm10258	n.d.	n.d.	
24	Q5SQB0	nucleophosmin	Npm1	nucleic acid binding	regulation of apoptotic process, cell proliferation, cell cycle*	2
25	F6ZAX1	polyadenylate binding protein 1	Pabpc1	nucleic acid binding, RNA binding	n.d.	2
26	Q61703	inter-alpha-trypsin inhibitor heavy chain H2	Itih2	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	
27	Q3U4I7	pyridine nucleotide disulfide oxidoreductase domain-containing protein 2	Pyroxd2	oxidoreductase activity	oxidation-reduction process	
28	G3UWV6	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	Agpat1	transferase activity	phospholipid biosynthetic process	

29	Q64433	10 kDa HSP10 mch	Hspe1	chaperone binding, ATP binding,	protein folding	2
30	P09103	protein disulfide-isomerase	P4hb	protein disulfide isomerase activity	protein folding	2, 3
31	P11499	heat shock protein HSP90 beta	Hsp90ab1	protein binding, nucleotide binding	protein folding, regulation of protein ubiquitination	2
32	B2M1R6	heterogeneous ribonucleoprotein K	Hnrnpk	nucleic acid binding	regulation of transcription from RNA polymerase II promoter	5
33	A2AD03	Rab proteins geranylgeranyl transferase component A1	Chm	oxidoreductase activity, Rab GTPase binding	small GTPase mediated signal transduction	
34	P62843	40S ribosomal protein S15	Rps15	structural constituent of ribosome	translation	4
35	G3X914	cullin 5	Cul5	ubiquitin protein ligase binding	ubiquitin-dependent protein catabolic process	
36	Q61838	pregnancy zone protein	Pzp	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	
37	D3YYR8	serotransferrin	Trf	n.d.	n.d.	3
38	P07309	transthyretin	Ttr	protein binding	transport	3
39	E9PVS1	inter-alpha-trypsin inhibitor heavy chain	Itih3	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity, hyaluronan metabolic process	
40	D3YZ68	elongation factor 1-alpha1	Eef1a1	GTP binding	translation	
41	O88569	heterogeneous nuclear ribonucleo-protein A2/B1	Hnrnpa2b1	nucleic acid binding, RNA binding	mRNA processing	5

42	E9QP56	apolipoprotein C-III	Apoc3	phospholipid binding, lipase inhibitor activity	phospholipid efflux	3
43	Q00898	alpha-1-antitrypsin 1-5	Serpina 1e	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	1
44	E9PVG8	protein 9530053A07Rik	9530053A07Rik	n.d.	n.d.	
45	Q64522	histone H2A type 2-B	Hist2h2ab	DNA binding	chromatin silencing	2
46	Q61233	plastin2	Lcp1	actin binding, protein binding, GTPase binding	regulation of intracellular protein transport	
47	P14211	calreticulin	Calr	ubiquitin protein ligase binding, glycoprotein binding, mRNA binding	response to endogenous stimulus, negative regulation of protein metabolic process	3
48	P63038	60 kDa heat shock protein mch	Hspd1	ubiquitin protein ligase binding, nucleotide binding, ATP binding	protein folding, response to drug	2
49	P17182	alpha enolase	Eno1	RNA binding, GTPase binding, phosphopyruvate hydratase activity	glycolytic process	6
50	G3UX57	calmodulin	Calm3	calcium ion binding	regulation of enzyme activity*	

Table S3. Rpn10-binding proteins (brain mitochondrial fraction) of isatin-treated mice identified in addition to the constitutive pool of proteins

No.	UniProt accession number	Protein name	Gene name	Protein function	Biological process	Interaction cluster (C ≥ 0.7)
16	A2ANY6	protein Mdn1	Mdn1	ATP binding	ribosomal large subunit assembly	
17	P27661	histone H2A-X	H2afx	DNA binding	chromatin silencing, DNA repair	2
18	P00186	cytochrome P450 1A2	Cyp1a2	oxidoreductase activity, monooxygenase activity	response to drug, drug metabolism	
19	Q8CIM7	cytochrome P450 2D26	Cyp2d26	oxidoreductase activity, monooxygenase activity	oxidation-reduction process, response to drug	
20	Q9QY81	nuclear pore membrane glycoprotein 210	Nup210	protein dimerization activity	mRNA transport, protein transport	
21	A2AWH7	pyruvate dehydrogenase protein X component, mitochondrial	Pdhx	transferase activity	metabolic process	
22	Q3TTA7	isoform 2 of E3 ubiquitin-protein ligase	Cblb	ubiquitin-protein transferase activity	protein ubiquitination, regulation of signaling	
23	Q61233	plastin2	Lcp1	actin binding, protein binding, GTPase binding	regulation of intracellular protein transport	
24	P14211	calreticulin	Calr	ubiquitin protein ligase binding, glycoprotein binding, mRNA binding	response to endogenous stimulus, negative regulation of protein metabolic process	1, 2
25	P63038	60 kDa Heat shock protein mch	Hspd1	ubiquitin protein ligase binding, nucleotide binding, ATP binding	protein folding, response to drug	

Table S4. Rpn10-binding proteins (brain mitochondrial fraction) of mice treated with isatin and MPTP identified in addition to the constitutive pool of proteins

No.	UniProt accession number	Protein name	Gene name	Protein function	Biological process	Interaction cluster (C ≥ 0.7)
16	Q569L8	centromere protein J	Cenpj	tubulin binding, protein binding	centriole replication	
17	Q9EP84	G-protein coupled receptor kinase 6	Grk6	protein serine/threonine kinase activity, nucleotide binding, ATP binding	protein phosphorylation, signal transduction	
18	F6T4M4	serine/arginine repetitive matrix protein 1	Srrm1	n.d.	mRNA processing	
19	E9QP56	apolipoprotein C-III	Apoc3	phospholipid binding, lipase inhibitor activity	phospholipid efflux	1
20	Q00898	alpha-1-antitrypsin 1-5	Serpina1e	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	1
21	E9PVG8	protein 9530053A07Rik	9530053A07Rik	n.d.	n.d.	
22	Q64522	histone H2A type 2-B	Hist2h2ab	DNA binding	chromatin silencing	2
23	P14211	calreticulin	Calr	ubiquitin protein ligase binding, glycoprotein binding, mRNA binding	response to endogenous stimulus, negative regulation of protein metabolic process	1
24	P63038	60 kDa heat shock protein mch	Hspd1	ubiquitin protein ligase binding, nucleotide binding, ATP binding	protein folding, response to drug	
25	P17182	alpha enolase	Eno1	RNA binding, GTPase binding, phosphopyruvate hydratase activity	glycolytic process	
26	G3UX57	calmodulin	Calm3	calcium ion binding	regulation of enzyme activity*	