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Supplemental Information

ATP-Dependent Lon Protease Controls Tumor

Bioenergetics by Reprogramming

Mitochondrial Activity

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SUPPLEMENTAL TABLE

Supplemental Table 1, related to Figure 5.

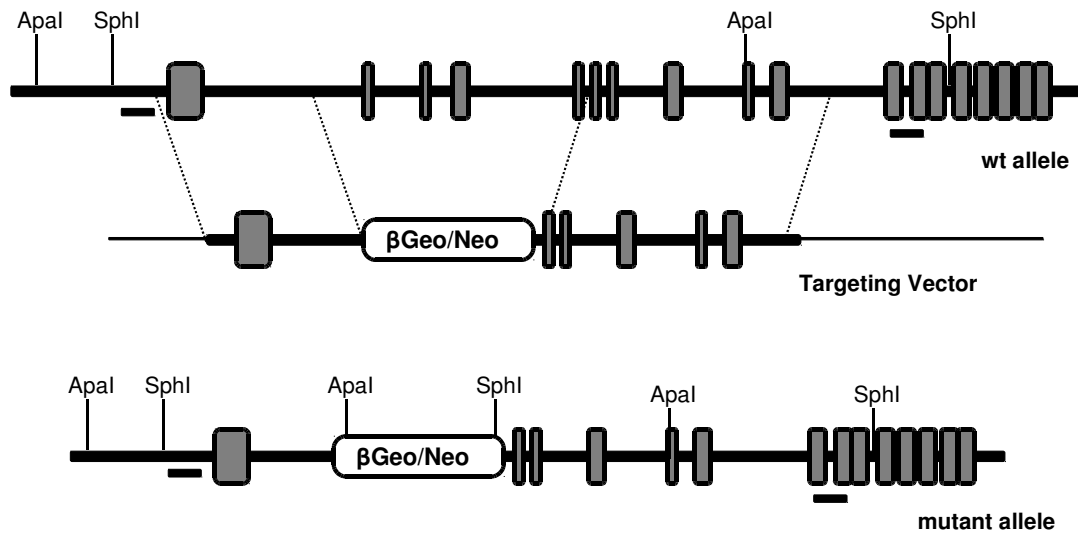
Table S1. Comparison of mitochondrial activities of individual and associated OXPHOS complexes, measured as specific activity (IU/mg) and relative to the citrate synthase activity.

		pLKO1	shLon		pMX	LON	
Specific activity (IU/mg)	CI	1.00 ± 0.05	0.66 ± 0.04	***	1.00 ± 0.09	1.23 ± 0.19	
	CII	1.00 ± 0.03	0.93 ± 0.09		1.00 ± 0.02	0.75 ± 0.04	**
	CI+III	1.00 ± 0.08	0.84 ± 0.17		1.00 ± 0.06	0.76 ± 0.03	**
	CII+III	1.00 ± 0.08	0.73 ± 0.08	**	1.00 ± 0.04	0.67 ± 0.07	***
	COX	1.00 ± 0.11	0.96 ± 0.16		1.00 ± 0.11	0.69 ± 0.11	**
	CS	1.00 ± 0.09	0.95 ± 0.07		1.00 ± 0.10	1.16 ± 0.18	
Specific activity relative to CS	CI	1.00 ± 0.10	0.70 ± 0.07	***	1.00 ± 0.06	1.16 ± 0.18	
	CII	1.00 ± 0.11	0.99 ± 0.14		1.00 ± 0.11	0.72 ± 0.15	**
	CI+III	1.00 ± 0.08	0.88 ± 0.13		1.00 ± 0.10	0.72 ± 0.15	**
	CII+III	1.00 ± 0.10	0.77 ± 0.10	***	1.00 ± 0.11	0.66 ± 0.21	**
	COX	1.00 ± 0.08	1.01 ± 0.12		1.00 ± 0.03	0.70 ± 0.15	**

Data shown correspond to three independent experiments carried out in triplicated. Statistical significance was performed by analysis of variance (ANOVA). Paired differences were assessed by the post hoc Fisher's protected least significant difference test (PLSD). CI: complex I; CII: complex II; CIII: complex III; COX: complex IV; CS: citrate synthase. ** $P < 0.01$; *** $P < 0.001$.

Figure S1 related to Figure 1; Quiros et al.

A



B

Stage	Number of mice				Total
	+/+ (25%)	+/- (50%)	-/- (25%)	undeterm.	
3.5 dpc	43	67	25	24	159
7.5 dpc	23	33	11	3	70
8.5 dpc	12	31	1	3	47
9.5 dpc	7	16	0	1	24
At weaning	96	181	0	0	277

Figure S1. (A) Schematic representation of wild-type and mutant *Lonpl* alleles, and the targeting vector. The exons are indicated with boxes, the bold solid lines represent introns and the thin solid lines indicate the plasmid backbone in the targeting vector. (B) Analysis of embryos derived from intercrossing of *Lonpl* heterozygous mice shows an embryonic lethality at 8.5 dpc. Genotypes were determined by PCR analysis.

Figure S2 related to Figure 3; Quiros et al.

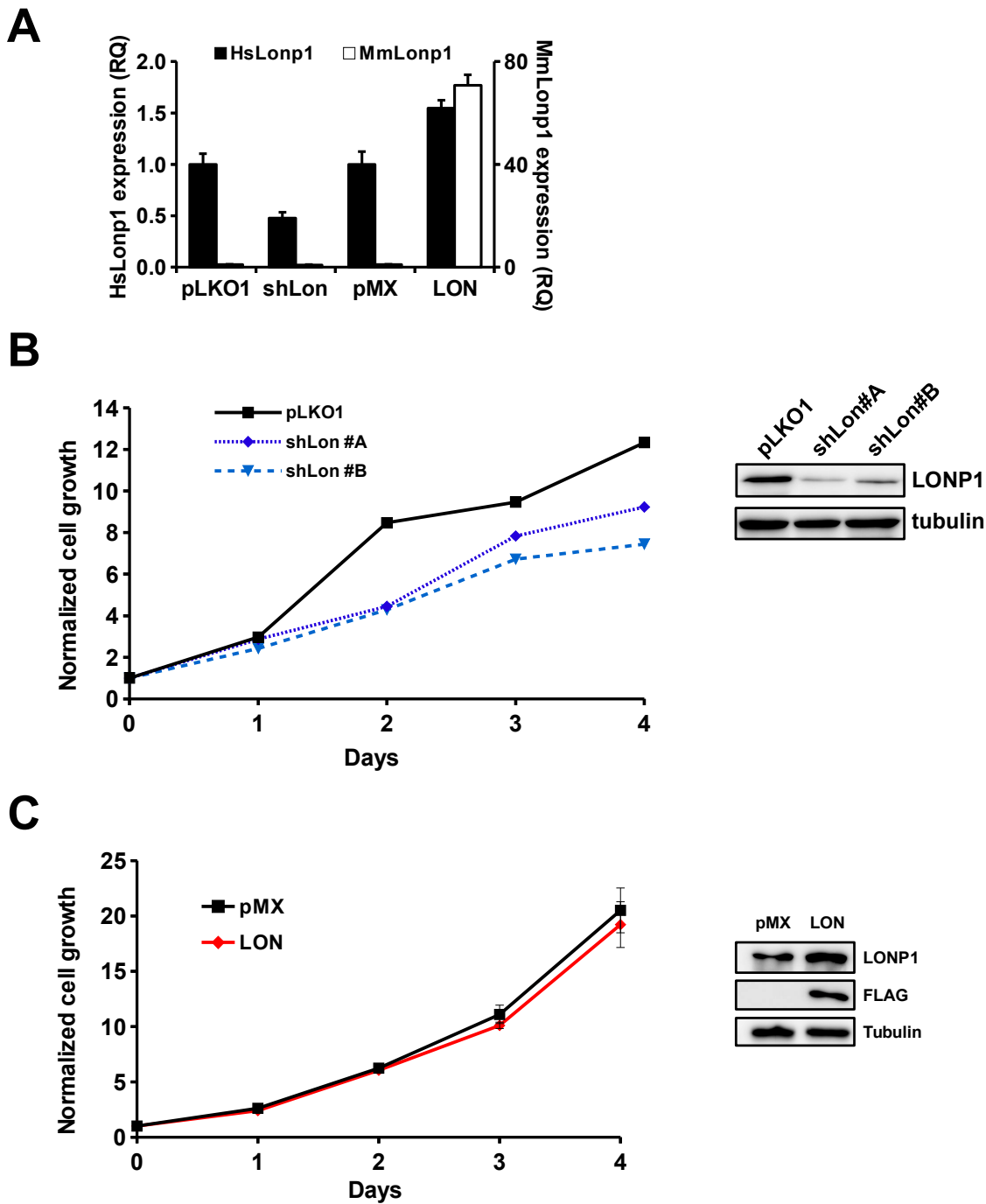


Figure S2. (A) qPCR analysis of HCT116 colon cancer cells transduced with *LONP1* shRNA vectors (shLon) or with the empty vector as a control (pLKO1), as well as with pMX expressing murine *LONP1* (LON) and the empty vector (pMX). qPCR analysis was carried out using two different *LONP1* probes to detect endogenous (HsLonp1) and exogenous (MmLonp1) *LONP1* expression values. (B) MTT and Western-blot analysis of HCT116 colon cancer cells transduced with two different single shRNA against *LONP1*. (C) MTT and Western-blot analysis of DLD-1 colon cancer cells transduced with a *LONP1*-flagged construction and the empty vector pMX.

Figure S3 related to Figure 4; Quiros et al.

A

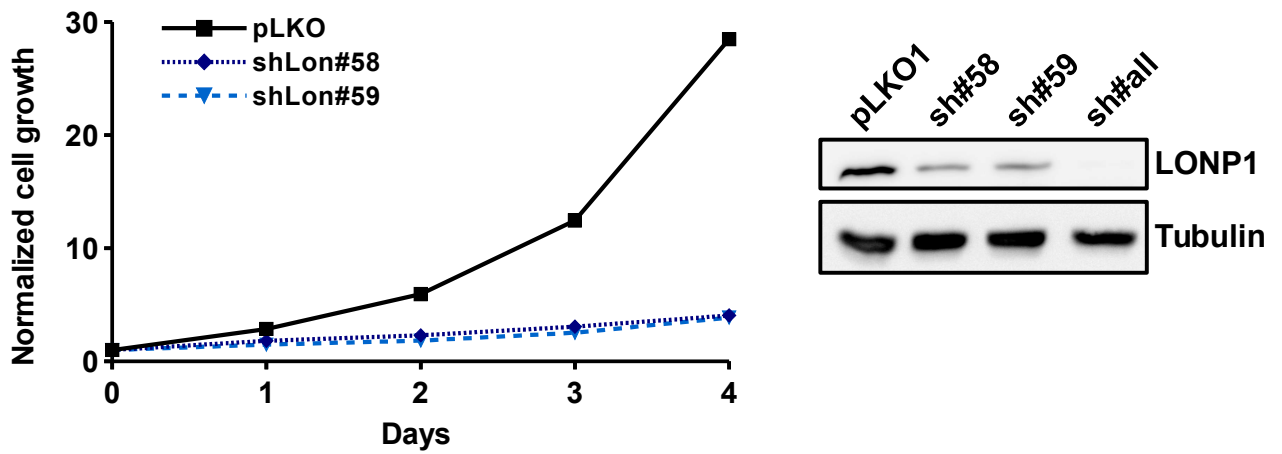


Figure S3. (A) MTT and Western-blot analysis of B16F10 melanoma cells transduced with two different single shRNA against LONP1, and the complete set of shRNAs. Notice the effect in the use of complete set compared to the use of single shRNA to decrease LONP1 levels.

Figure S4 related to Figure 5; Quiros et al.

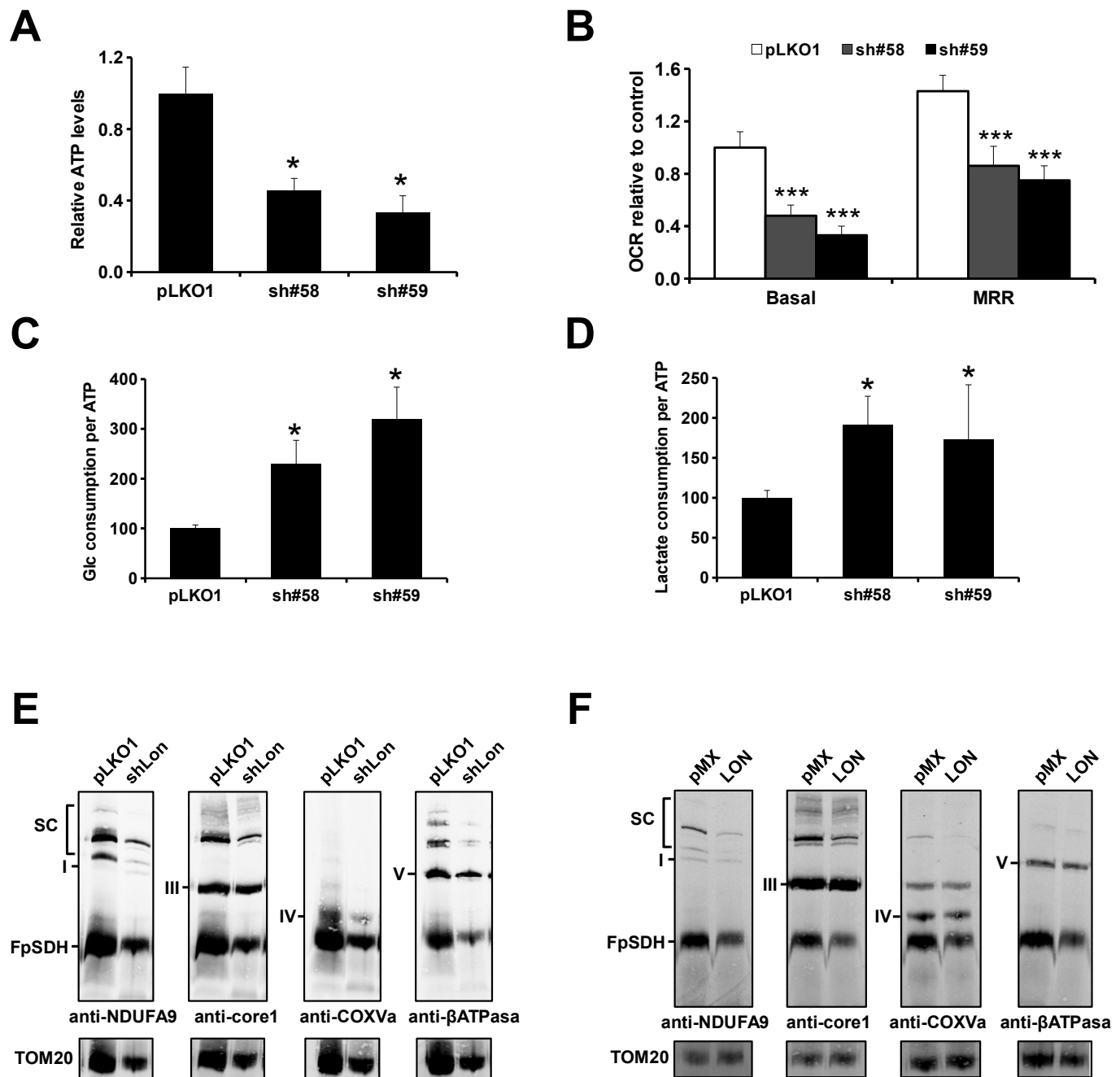


Figure S4. B16F10 melanoma cells with knockdown of LONP1 using two single shRNAs (sh#58 and sh#59). (A) Relative ATP levels of the analyzed cells. (B) Oxygen consumption rate (OCR) in basal conditions and after stimulation with FCCP, MRR (maximal respiration rate), represented as OCR per cell relative to each control cell. (C) Percentage of glucose consumption and (D) lactate production during 48 h, relative to control. BNGE immunoblots showing distribution of CI, CIII, CIV and CV free and in supercomplexes detected with indicated antibodies in each case, as well as FpSDH (CII) using TOM20 as loading control, in (E) pLKO1 and shLon cells, and (F) pMX and LON cells. All experiments were carried out in triplicate in at least three independent experiments. Bars represent means \pm SEM. * $P < 0.05$, *** $P < 0.001$.

Figure S5 related to Figure 6; Quiros et al.

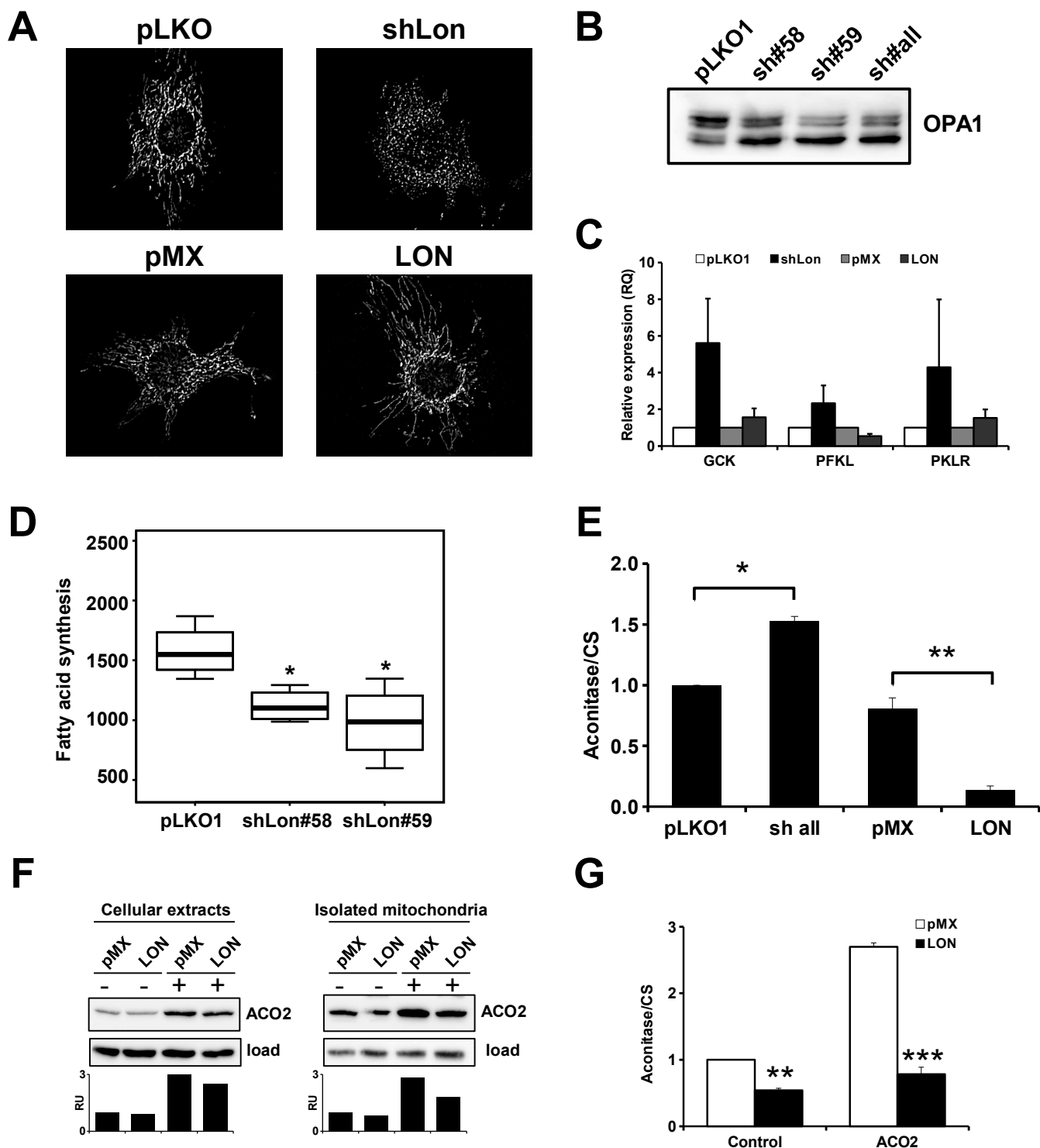


Figure S5. (A) Representative pictures of mitochondrial morphology from pLKO1, shLon, pMX and LON cells. Mitochondria were visualized after transfection with a vector containing mito-DsRed2. (B) Western-blot analysis of OPA1 processing in B16F10 melanoma cells with knockdown of LONP1 using two single shRNAs (sh#58 and sh#59) and the complete set of shRNAs (sh#all). (C) Relative expression levels of glycolytic genes, glucokinase (GCK), phosphofructokinase (PFKL) and pyruvate kinase (PKLR) in the indicated cells. (D) Fatty acid synthesis rate measured as ^{14}C incorporation into cellular lipids represented as relative units (RU). (E) Aconitase (ACO2) activity relative to citrate synthase activity. (F) Western-blot analysis and densitometry quantification of ACO2 levels in control (pMX) and *LONP1*-overexpressing cells (LON), determined in cellular extracts and isolated mitochondria, after transfection of ACO2 cDNA. (G) ACO2 activity relative to citrate synthase activity of cells previously described. All experiments were carried out in triplicate in at least three independent experiments. Bars represent means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal studies and carcinogenesis protocols

For xenograft studies, 2×10^6 HCT116 cells infected with different constructs of pMX or shRNA vectors were injected subcutaneously into the flanks of six-week-old athymic Nude-Foxn1^{nu/nu} mice (Charles River). Tumor size was measured twice a week with a caliper and tumor volume was determined using the formula: $V=0.4 \times A \times B^2$, where A is the largest and B is the smallest dimension of the tumor. Significant differences were assessed by a linear mixed-effects model. For colon carcinogenesis protocol, 6-week-old mice were injected intraperitoneally with 10 mg/kg AOM (azoxymethane; Sigma-Aldrich, St. Louis, MO, USA). 4 days after AOM administration, dextran sodium sulfate (DSS; MP Biomedicals) at 2% was administered in the drinking water for five consecutive days. Thereafter, mice received reverse osmosis water. Two DSS cycles more were administered with intervals of 16 days on water between cycles at 2% and 1.5% of DSS respectively. During the course of the experiment, mice were monitored for body weight, rectal prolapse, diarrhea, and macroscopic bleeding as indicative of clinical score (Neufert et al., 2007; Wirtz et al., 2007). Ten days after the last DSS cycle, mice were euthanized and colons were resected, flushed with phosphate-buffered saline (PBS) and measured in length. Colons were fixed in 4% paraformaldehyde (PFA) and transversal sections were hematoxylin and eosin (H&E) stained. Number of tumors was count and grade of inflammatory lesions was analyzed in each mice. Skin carcinogenesis was induced using DMBA/TPA protocol. Briefly, 8-week-old mice backs were shaved and treated those 2 days later with a single application of DMBA followed by bi-weekly applications of TPA for 12 weeks. The number and size of papillomas per mouse was recorded weekly. Two weeks after the end of TPA treatment, mice were sacrificed and papillomas were removed, fixed in 4% PFA and subsequently stained with H&E to analyze the grade of lesions. For lung metastasis experimental model, mice were anesthetized and 25,000 B16F10-luc2 tumor cells (100 μ l) were injected through the jugular vein. Mice were sacrificed after 21 days and lungs were collected for histological analysis. Serial sections of the lung (at least 10 sections spaced 100 μ m) were stained with H&E, and metastatic foci were counted. Metastases were classified in small (<10 cells), medium (between 10 and 50 cells), and large (>50 cells). For histological analysis, tissues were fixed in 4% PFA in PBS and stored in 70% ethanol. Fixed tissues were embedded in paraffin by standard procedures. Blocks were sectioned (5 μ m) and stained with H&E.

Immunostaining

Normal and tumor colorectal samples were obtained from the tumor bank of the Instituto

Universitario de Oncología del Principado de Asturias (IUOPA) and used to evaluate LONP1 expression. The automated system DISCOVERY® (Ventana Medical Systems) was used to carry out the immunohistochemical protein detection. Sections were deparaffinized and rehydrated in EZ Prep® (Ventana Medical Systems) for 20 min. Antigen retrieval was done by heating (CC1 HCl-Tris buffer solution, pH 9.0) (Ventana Medical Systems). Endogenous peroxidase activity was blocked with H₂O₂ solution (Inhibitor®, Ventana Medical System) for 4 min. Samples were incubated with primary antibody at 37 °C: polyclonal anti-LONP1 (Atlas Antibodies). Slides were incubated with the secondary antibody (OmniMap® Ventana Medical Systems) for 30 min at room temperature. Then the samples were visualized with 3-3' -diaminobenzidine (DAB). Finally, samples were counterstained with hematoxylin, dehydrated and mounted in Entellan (Merck). Sections were photographed (20X) under a light microscope (Nikon-Eclipse 80i).

RNA preparation

Cells were washed twice with PBS and immediately homogenized in Trizol (Life Technologies) and processed in the same day through alcohol precipitation according to the manufacturer's instructions. RNA pellets were then washed in cold 80% ethanol and stored at -80 °C until further use. Following re-suspension of RNA in nuclease-free water (Life Technologies), the samples were quantified and evaluated for purity (260/280 nm ratio) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Real-time quantitative PCR

cDNA was synthesized using 2 µg of total RNA, 0.14 mM random hexamer primer, 0.2 mM of each deoxynucleoside triphosphate and Superscript II reverse transcriptase (Life Technologies). Quantitative PCR (qPCR) was performed using TaqMan® gene expression assay (Life Technologies) or Power SYBR® Green PCR Master Mix (Life Technologies), following manufacturer's instructions, using an Applied Biosystems 7300HT Real-Time PCR System. As an internal control, gene expression was normalized to the β-actin gene using TaqMan® gene expression assay. Relative expression is represented as relative quantification, using RQ value ($RQ=2^{-\Delta\Delta C_t}$).

Viral package and cell infection

Lentiviruses and retrovirus were packaged in HEK-293T cells using a VSVG-based and pCL-Ampho package system respectively, kindly provided by Dr. J.M. Silva (Columbia University,

New York, USA). Cells were transfected using TransITs-LT1 Transfection Reagent (Mirus Bio LLC) and a mixture of 2 µg of the desired plasmid and 1 µg of each viral helper, following manufacturer's instructions. Transfection medium was removed 24 h after transfection and fresh medium was added to the plate. Cell supernatants were collected at 24 and 48 h and filtered through a 0.45-µm sterile filter. Cells were seeded in 6-well plates at 20–30% confluence 24 h before infection. The following day, 1 ml of viral supernatant supplemented with 5 mg/ml of polybrene (Millipore) was added to growing cells. This step was repeated twice and cells were left to recover for 24 h in growth media before puromycin selection (2 µg/ml).

Proliferation assay

To quantify cell proliferation, a Cell Titer 96 Non Radioactive cell proliferation kit was used following manufacturer's instructions (Promega Corp.). Briefly, HCT116 and B16F10 cells infected with the corresponding vectors were seeded into 96-well plates at a density of 5×10^3 cells per well (100 µl) and incubated at 37°C, 5% CO₂ for 4 days. Cell proliferation was quantified by measuring the conversion of a tetrazolium salt into formazan in living cells. At the desired time points (0 h, 24 h, 48 h and 72 h), 15 µl of Dye solution was added into each well (n=5) and cells were incubated at 37 °C for 2 h. Then, 100 µl of solubilization/stop mixture was added into each well. After 1 h of incubation at 37 °C, absorbance was measured at 570 nm with a Power Wave XS Microplate reader (Biotek). Then, each point was normalized to time 0 h and mean ± SEM was calculated and represented. Statistical significance was assessed using a non-parametric Mann Whitney-Wilcoxon test.

Western blotting

Cultured cells were washed twice with 1× PBS and lysed in RIPA buffer containing 50 mM Tris buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1 % SDS, 10 mM EDTA, and complete protease inhibitor cocktail (Roche Applied Science). Once homogenized, lysed cells were centrifuged at 13,000 g at 4 °C for 10 min, and supernatants were collected. The protein concentration of the supernatant was evaluated by bicinchoninic acid technique (BCA protein assay kit; Pierce Biotechnology Inc.). Protein samples (15 µg) were loaded into SDS-polyacrylamide gels. After electrophoresis, gels were electrotransferred onto PVDF membranes, blocked with 5% nonfat dried milk in TBS-T (TBS with 0.05% Tween-20) and incubated with primary antibodies following the commercial instructions. After 3 washes with TBS-T, membranes were incubated with the corresponding secondary antibody in 1.5% milk in TBS-T, and developed with Immobilon Western

Chemiluminescent HRP substrate (Millipore) in a LAS-3000 Imaging System (Fujifilm). Antibodies against LONP1 were obtained from Atlas Antibodies; anti- α -tubulin and anti- β -actin were from Sigma-Aldrich; anti-Flag, anti-AMPK α , anti-phospho-AMPK α (Thr172), anti-Bcl-2 and anti-ACO2 were from Cell Signaling; and anti-OPA1 was from BD Bioscience.

Mitochondrial analysis

We quantified mtDNA by real-time PCR using an Applied Biosystems 7300HT Real-Time PCR System and Power SYBR® Green PCR Master Mix (Life Technologies) as previously described (Moreno-Loshuertos et al., 2006). Briefly, total DNA was used as a template and amplified with specific oligodeoxynucleotides for *mt-Co2* and *Sdha*. We calculated the mtDNA copy number per cell using *Sdha* amplification as a reference for the nuclear genome. Mitochondrial membrane potential, ROS levels, and mitochondrial morphology were determined as previously described (Quirós et al., 2012). Aconitase activity was determined as previously described (Gardner et al., 1994). Briefly, 10 μ g of mitochondria were used to measure aconitase activity following the linear increase in absorbance at 340 nm during 60 min at 25 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.4), 6 mM sodium citrate, 0.2% Triton X-100, 0.6 mM MnCl₂, 0.2 mM NADP and 2 U/mL of NADP-dependent isocitrate dehydrogenase.

Lactate, glucose and ATP measurements

Extracellular lactate levels were measured by a colorimetric assay and glucose levels were determined using the Accu-Chek II glucometer (Roche Diagnostics). Briefly, 5 x 10⁴ cells were seeded in triplicates into 24-well plates for 48 hours in the presence or absence of 500 μ M of CoCl₂. After that, media were collected and the content of glucose and lactate was determined, normalizing to cell number and subtracting the time zero content of each one. Lactate measurement was performed in a hydrazine/glycine buffer (0.5 M glycine, 0.4 M hydrazine, pH 9.0), containing 17 mg/ml NAD and 11.4 mg/ml lactate dehydrogenase (LDH). The absorbance due to formation of NADH was monitored in a Power Wave XS Microplate reader (Biotek) at 340 nm during 1 h at 25 °C. The presence of lactate on samples was correlated with the lactate concentrations from a standard curve. Cellular ATP levels were determined using the ATP determination kit (Molecular probes, Invitrogen) according to the instructions of the manufacturer. Briefly, cells were collected by trypsinization and lysed using passive lysis buffer (PLB) (Promega). The lysates were centrifuged at 12,000 g at 4 °C for 5 min, and supernatants were collected. The protein concentration of the supernatant was evaluated by BCA protein assay kit (Pierce Biotechnology). We used 1 μ g of lysed material for ATP determination with a D-luciferin/firefly luciferase reaction

mix, and luminescence was measured in a Luminometer TD20/20 and compared to a freshly prepared ATP standard curve.

Electrophoretic analysis of mitochondrial supercomplexes assembly

Mitochondrial membranes were isolated from 5×10^6 cells as previously described (Nijtmans et al., 2002). Mitochondrial membrane proteins were applied and run on a 3-13% first dimension gradient blue native gel electrophoresis (BNGE) as previously described (Schägger, 1995). After electrophoresis, the complexes were electroblotted onto PVDF membranes and sequentially probed with specific antibodies against complex I, anti-NDUFA9; complex III, anti-core1; complex IV, anti-COX5a; complex II, anti-Fp; complex V, anti-F1-ATPase (ATP5B), all from Abcam and anti-Tom20 from Santa Cruz.

Spectrophotometric analysis of mitochondrial complexes

Measurements of mitochondrial complexes enzymatic activities were assessed individually in the spectrophotometer as described elsewhere (Birch-Machin and Turnbull, 2001). Briefly, NADH-dehydrogenase activity rotenone sensitive (CI activity) was measured at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 25 mM K_2HPO_4 , 5 mM MgCl_2 , 3 mM KCN, 2.5 mg/ml BSA, pH 7.2; 0.13 mM NADH, 0.13 mM UQ1 and 0.2 $\mu\text{g/ml}$ antimycin A. Rotenone sensitivity was measured under the same conditions adding 5 μM rotenone. Succinate dehydrogenase activity (CII activity) was measured at 600 nm ($\epsilon = 19.2 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 25 mM K_2HPO_4 , 5 mM MgCl_2 , 3 mM KCN, 2.5 mg/ml BSA, pH 7.2; 0.03 mM DCPIP (dichlorophenol indophenol), 10 mM succinate, 2 $\mu\text{g/ml}$ antimycin A and 5 μM rotenone. Cytochrome c oxidase activity (COX; CIV activity) was measured at 550 nm ($\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 100 mM K_2HPO_4 pH 7.4 and reduced cytochrome c (1 mg/ml, freshly made). NADH cytochrome c oxido-reductase activity (CI+III) was measured at 550 nm ($\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 25 mM K_2HPO_4 , 5 mM MgCl_2 , 3 mM KCN, 2.5 mg/mL BSA, pH 7.2; 0.2 mM KCN, 0.1 mM cytochrome c and 0.1 mM NADH. Succinate cytochrome c oxido-reductase activity (CII+III) was measured at 550 nm ($\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 25 mM K_2HPO_4 , 5 mM MgCl_2 , 3 mM KCN, 2.5 mg/ml BSA, pH 7.2; 0.2 mM KCN, 0.1 mM cytochrome c, 3 mM succinate and 5 μM rotenone. Citrate synthase activity (CS) was measured at 412 nm ($\epsilon = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$) in a mix containing 10 mM Tris-HCl pH 8, 0.023 mg/ml acetyl CoA, 0.1 mM DTNB (5,5-dithio-bis-2-nitrobenzoic acid), 0.25 mM oxalacetate and 0.1% Triton X-100.

Fatty acid synthesis assay

Fatty acid synthesis was determined as previously described (Sabbisetti et al., 2009), with some modifications. Briefly, cells were plated in 6-well plate at 3×10^5 cells per dish and incubated overnight. The next day, cells were incubated with 3 μCi of $[1-^{14}\text{C}]$ acetate during 4 h. After that, cells were harvested and resuspended in 500 μL of PBS, and 20 μL aliquots were taken to assess protein content. Then, cells were pelleted and lipids were extracted following Folch method, adding 1 mL of chloroform:methanol (2:1) with occasional vortexing. After 20 min incubation, 200 μL of PBS were added, the samples were mixed and centrifuged, and the lower phase was counted for ^{14}C using scintillation counter. Each experiment was performed in triplicates and normalized with protein content.

Protein digestion and iTRAQ labeling

Protein digestion was performed as described (Bonzon-Kulichenko et al., 2011), with minor modifications. The resulting soluble protein extracts from cell lysates or mitochondria-enriched samples were run on a SDS-PAGE gel (10% resolving gel and 4% stacking gel) at 50V. The electrophoresis was stopped when the front dye had barely passed into the resolving gel, ensuring concentration of all proteins into a unique band. Staining was performed using GelCode[®] Blue Stain Reagent (Thermo Scientific). Gel pieces were cut into cubes (2 mm). For the protein digestion, modified porcine trypsin (Promega) was added at a final ratio of 1:20 (trypsin-protein). Digestion proceeded overnight at 37 °C in 100 mM ammonium bicarbonate, pH 8.8. The resulting tryptic peptides were extracted twice by 1 h incubation at room temperature in 100% acetonitrile and 5% formic acid and dried-down. Samples were desalted with C18 RP cartridges and vacuum dried. The concentration of peptides was determined by measuring amide bonds in protein chains with the Direct Detect[®] Spectrometer (Millipore). A total of 200 μg of each peptide mixture were labeled with different iTRAQ tags according to the manufacturer protocol. *LONPI*-overexpressed derived samples (LON) were labeled with 115 iTRAQ tag, while the 117 iTRAQ tag was used to label the *LONPI*-knockdown samples (shLon). The iTRAQ tags 114 and 116 were used to label the corresponding controls, respectively. After labeling, the samples were vacuum dried and finally dissolved in 1% trifluoroacetic acid for both desalting and iTRAQ reagent excess removal in reversed-phase C-18 cartridges.

Identification and quantification of proteins

Tryptic peptide mixtures were subjected to nano-liquid chromatography coupled to mass spectrometry for protein identification and quantification. Peptides were separated on a C-18 reversed phase (RP) nano-column (75 μm I.D. and 50 cm, Acclaim PepMap100, Thermo Scientific)

and analyzed in a continuous acetonitrile gradient consisting of 0-30% B in 240 min, 50-90% B in 3 min (B = 90% acetonitrile, 0.5% acetic acid). A flow rate of ca. 200 nL/min was used to elute peptides from the RP nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on a Q-Exactive mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution=70000) followed by the MS/MS spectra from most intense fifteen parent ions were analyzed along the chromatographic run (272 min). Dynamic exclusion was set at 30 s. For protein identification, tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer 1.4.0.288 (Thermo Fisher Scientific). All MS/MS samples were analyzed using SEQUEST™ (Thermo Fisher Scientific). Searching engines were set up to search Mouse_human keratin.fasta (119,718 entries). All searchings were performed assuming full trypsin digestion. Two mixed cleavages were allowed, and an error of 300 ppm or 15 ppm was set for full MS or MS/MS spectra searches, respectively. Oxidation in M, and deamidation in Q or N were selected as dynamic modifications. Database searching results were analyzed using the probability ratio method (Martínez-Bartolomé et al., 2008) and FDR was calculated using a decoy database and the refined method (Navarro and Vázquez, 2009). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Statistical analysis of quantitative proteomics data were performed using the QuiXoT package, an in-house developed software based on a statistical model previously described (Jorge et al., 2009). In this model, the accuracy of individual quantifications is taken into account by expressing protein abundance changes in units of standard deviation, using the standardized variable z_q , to which we also refer as the z-score.

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