Supplementary Material

Supplementary Material file consist of two sections: the supplementary results and supplementary material and methods. **Supplementary results section** consists of the presentation of the results obtained from transcriptional analyses and already presented in the main manuscript file, but here the calculation was done using the control form each time point as calibrator (Supplementary Figures 1-7). These results are also presented on the heat maps (Supplementary Figure 8). Also, supplementary results section contains the results of the protein expression analysis in the supernatant samples after the immunoprecipitation analysis of the MFN2 protein (Supplementary Figure 9). Besides, here we presented the results obtained in the ex vivo experiments in which spermatozoa from undisturbed control were stimulated with adrenaline for one hour and the mitochondrial membrane potential was measured (Supplementary Figure 10). Lastly, here we show the tables containing the variables loadings from principal component analysis used to graph Figure 11 (Supplementary Tables S1-S3). **Supplementary Material and Methods** section contains the key resource table (Supplementary Table S4), the tables related to the primer characteristics and Ct values (Supplementary Tables S5-S11), relative quantification of protein expression after immunoprecipitation analysis as well as the information about antibodies used in the research (Supplementary Table S12).

**Supplementary Results**

Relative expression of transcripts of mitochondrial biogenesis and functionality markers as well as markers of signaling pathways regulating mitochondrial dynamic and spermatozoa functionality calculated using the control group of the same time point as a calibrator.

Calculation of relative gene expression, using the control group of the same time point as a calibrator, showed the changes within each single time point. Transcriptional changes of mitochondrial dynamics and functionality markers in spermatozoa of acutely stressed adult rats are the most prominent at the ZT23 time point, compared to the Control of the same time point. All the followed mitochondrial biogenesis markers (except Ppara) and markers of mitochondrial fusion and architecture are significantly increased in spermatozoa from ZT23-1x3hMO group compared to ZT23-Control. In addition, Prkn, Cox4i1, Cytc and Ucp2 also increased, while Ucp1 and Ucp3 decreased, indicating significant transcriptional changes of mitochondrial autophagy and function at the ZT23 time point (Supplementary Figure S1, S2, S3, S4, S5). Also, transcription of the signalling molecules of cAMP and MAPK pathways (regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality) is the most significantly changed in the spermatozoa of acutely stressed adult rats at ZT23, compared to the Control at the same time point (Supplementary Figure S6, S7).
Supplementary Figure S1. Transcription of mitochondrial biogenesis markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of mitochondrial biogenesis. To analyze the effect of acute stress with the recovery period, animals were subjected to immobilization stress (IMO) for 3 hours, once, from ZT0 to ZT3 (1x3hIMO), and allowed to recover (1x3hIMO+R) for 0, 8, 14 and 20 hours after the IMO (ZT3, ZT11, ZT17, and ZT23; ZT0 is a time when the light turned on). In short, the rats were bound in a supine position to a wooden board by fixing the rats' limbs using thread, while the head motion was not limited. Unstressed, freely moving rats were present as a control group (Control) in each experiment. All the activities during the dark phase were performed under the red light. At the end of the experimental period control and stressed animals were quickly decapitated without anesthesia and trunk blood was collected. Individual serum samples were stored at -70 °C until they were assayed for androgens (testosterone + dihydrotestosterone; T+DHT) and corticosterone (CORT) levels. In each experiment, the control and stressed animals randomly divided into four time points groups, with up to 6 animals per time point. The sample size was checked by Power Analysis using G Power software (http://core.ecu.edu/psyc/wuenschk/Power.htm) according to previous results obtained by our group. The experiment was repeated two times. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S2. Transcription of mitochondrial fusion and architecture markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of mitochondrial fusion and architecture. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S3. Transcription of mitochondrial fission markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of mitochondrial fission. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S4. Transcription of mitochondrial autophagy markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of mitochondrial autophagy. Data bars are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at level p < 0.05: * vs. control group of the same time point.
Supplementary Figure S5. Transcription of mitochondrial functionality markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of mitochondrial functionality. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S6. Transcription of cAMP signaling markers regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of cAMP signaling pathway. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S7. Transcription of MAPK signaling markers in spermatozoa of acutely stressed adult rats is the most prominently changed at the ZT23 time point. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for analysis of the transcriptional profile of markers of MAPK signaling pathway. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p <0.05: * vs. control group of the same time point.
Supplementary Figure S8. Heat map analysis of transcriptional profile of mitochondrial dynamic and functionality markers (A) and signaling molecules regulating mitochondrial dynamics and functionality (B) in spermatozoa of acutely stressed adult rats with recovery at different time points (0hours-ZT3, 8hours-ZT11, 14hours-ZT17, 20hours-ZT23).

The relative fold changes in the genes expression for the before mentioned genes were calculated using the corresponding control group of the same time point as a calibrator to show the changes within each single time point (ZT3, ZT11, ZT17 and ZT23). Color from red to green indicates low to high expression. Relative protein expression of mitochondrial biogenesis and functionality.
markers as well as markers of signaling pathways regulating mitochondrial dynamic and spermatozoa functionality in spermatozoa samples after MFN2 immunoprecipitation analysis

Considering the relationship of mitofusin 2 expression and motility and cryoprotective potential of human spermatozoa (1), immunoprecipitation analysis of MFN2 protein was performed in spermatozoa samples of stressed animals with different recovery periods. Spermatozoa samples for immunoprecipitation analysis were lysed and concentration of proteins in each sample was estimated by Bradford method (set at concentration of 300 µg/ml). Pre-clearing of the lysate was done using normal goat serum (Santa Cruz Biotechnology, normal goat serum: sc-2043, (https://www.scbt.com)). After pre-clearing step, lysates were mixed with MFN2 antibody (Santa Cruz Biotechnology). Immunoprecipitated complexes with MFN2 antibody were recovered by protein G agarose bead slurry and supernatant was used for further protein analysis using Western blot method. Results show that there is no significant difference in protein expression of ERRα (Supplemental Figure 9A) and ERK1/2 (Supplemental Figure 9C) proteins in all analyzed groups compared to control group of ZT3 time point. Results show that there is significant decrease in 1x3hIMO+R group of ZT3 time point, and oppositely increase in 1x3hIMO+R group of ZT23 time point in the expression of NRF1 protein in supernatant after the immunoprecipitation of MFN2 protein in spermatozoa (Supplemental figure 9B). Relative expression of p38MAPK protein in supernatant after the immunoprecipitation of MFN2 protein, was decreased in stressed groups with recovery periods of 0, 8, 14 and 20 hours (ZT3, ZT11, ZT17 and ZT23), as well as in control groups of ZT11 and ZT17 time points, compared to control group of ZT3 time point (Supplemental Figure 9D). Since immunoprecipitation analysis show protein interaction between MFN2 and PRKAc, analysis for the PRKAc protein in the supernatant was performed. The results show that there is no PRKAc protein present in the supernatant sample after the immunoprecipitation analysis (Supplemental Figure 9E).
Supplementary Figure S9. Relative expression of ERRα, NRF1, ERK1/2 and p38 MAPK proteins as mitochondrial functionality markers as well as markers of signaling pathways regulating mitochondrial dynamic and spermatozoa functionality in spermatozoa samples after MFN2 immunoprecipitation analysis.

Relative expression of (A) ERRα, (B) NRF1, (C) ERK1/2 and (D) p38 MAPK are analyzed in supernatant after MFN2 immunoprecipitation analysis. Analysis of PRKAc (E) in supernatant after MFN2 immunoprecipitation analysis prove the interaction of the two analysed proteins shown in main manuscript file. Spermatozoa samples for immunoprecipitation analysis were lysed and concentration of proteins in each sample was estimated by Bradford method and set at concentration of 300 µg/ml. Pre-clearing of the lysate was done using normal goat serum. After pre-clearing step, lysates were mixed with MFN2 antibody. Immunoprecipitated complexes with MFN2 antibody were recovered by protein G agarose bead slurry and supernatant was used for further protein analysis using Western blot method. Data bars are mean ± SEM values of two independent in vivo experiments. Statistical significance was set at level p < 0.05: # vs. control group of the ZT3 time point, * vs. control group of the same time point.

Mitochondrial membrane potential (Δψ) measurement using TMRE fluorescent dye in adult capacitated and non-capacitated spermatozoa after one hour ex vivo adrenaline treatment

Three-months-old male Wistar rats were used for the experiment. Adult animals were not subject to any previous treatment. Spermatozoa were isolated from the caudal epididymides and were incubated in capacitated or non-capacitated medium for one hour. After the incubation period, capacitated and non-capacitated spermatozoa were treated ex vivo with adrenaline in dose of 10 µM for 3 hours.
To monitor the membrane potential of mitochondria ($\Delta \psi$) in spermatozoa, tetramethylrhodamine ethyl ester (TMRE) as fluorescent probe was employed according to the procedure published previously (2). Briefly, $1 \times 10^5$ spermatozoa were loaded in each well of a 96-well black plate, with eight replicates from each group. Spermatozoa in a 96-well plate were left for 3 hours in the incubator at 37 °C, to recover from the stimulation procedure. After that period medium was aspirated, TMRE fluorescent dye was added in a final concentration of 100 nM and incubated for 20 minutes at 37 °C. Spermatozoa were washed from TMRE fluorescent dye by aspiration and fluorescence was measured in 0,1% BSA-1xPBS solution (Ex/Em 550/590 nm). After the measurement, all wells were washed with 1xPBS solution and frozen at -20 °C until the measurement of protein concentration in each well by Bradford protein assay.
Supplementary Figure S10. The relative ratio of TMRE fluorescence (%) in adult spermatozoa after *ex vivo* adrenaline treatment.

Membrane potential of mitochondria (Δψ) in non-treated (basal) and adrenaline treated non-capacitated and capacitated spermatozoa was measured using Tetramethylrhodamine ethyl ester (TMRE) as fluorescent probe. TMRE fluorescence (%) is presented as ratio of TMRE fluorescence and protein concentration is each well.

For each group in the experiment eight replicates was measured. (A) TMRE fluorescence (%) per µg of proteins in each well. (B) TMRE fluorescence (%) calculated on the basal group of non-capacitated and capacitated spermatozoa. (C) TMRE fluorescence (%) calculated on the non-capacitated spermatozoa of basal group. (D) TMRE fluorescence (%) calculated on the non-capacitated group of adrenaline treated and non-treated (basal) spermatozoa.

Data bars are mean ± SEM values of one *ex vivo* experiment.
**Supplementary Table S1.** Variables loadings from principal component analysis. PC-principal component.

| Variable     | PC1     | PC2     | PC3     |
|--------------|---------|---------|---------|
| Ppargc1a     | 0.250532| -0.15211| -0.12788|
| Ppargc1b     | 0.225101| 0.202388| 0.072176|
| Tfam         | 0.254698| 0.013513| 0.027626|
| Nrf1         | 0.254442| 0.050704| 0.017779|
| Nrf2a        | 0.230169| 0.235813|-0.05402 |
| Ppara        | -0.22415| 0.095104|-0.10858 |
| Ppard        | 0.2604  | -0.01874| 0.038501|
| MtNdl        | 0.2596  | -0.00916| -0.08758|
| Mfn1         | 0.259605| 0.033005|-0.02459 |
| Mfn2         | 0.260528| 0.02916 | 0.057576|
| Opa1         | 0.260379| 0.061838|-0.04801 |
| Fis1         | 0.230016| -0.21855| 0.05477 |
| Drp1         | 0.193326| -0.11477| -0.34203|
| Pink1        | 0.008186| 0.084998| 0.695303|
| Prkn         | 0.122627| 0.247971|-0.09388 |
| Tfeb         | 0.229033| -0.00532| 0.332673|
| Cox4i1       | 0.210243| 0.261678|-0.21528 |
| Cox4i2       | -0.09302| 0.3472  | 0.00419 |
| Cytc         | -0.01923| 0.556862| 0.145128|
| Ucp1         | -0.15383| -0.3233 | 0.050703|
| Ucp2         | 0.195433| -0.2827 | 0.373116|
| Ucp3         | -0.20624| 0.2215 | 0.123077|
**Supplementary Table S2.** Variables loadings from principal component analysis. PC-principal component.

|     | PC1     | PC2     | PC3     |
|-----|---------|---------|---------|
| Adcy3 | -0.29879 | -0.07554 | 0.433935 |
| Adcy5 | -0.30231 | 0.142303 | 0.370199 |
| Adcy6 | -0.27565 | -0.27288 | -0.1054 |
| Adcy7 | -0.34729 | 0.141474 | -0.17697 |
| Adcy8 | 0.15907  | -0.59539 | 0.120152 |
| Adcy9 | -0.19952 | -0.36001 | -0.58736 |
| Adcy10 | 0.303708 | -0.14735 | 0.309543 |
| Prkaca | -0.32499 | -0.04482 | -0.19298 |
| Prkacb | -0.33611 | -0.05349 | 0.273228 |
| Prkar1a | -0.10587 | -0.59685 | 0.168749 |
| Prkar2a | -0.3434  | -0.01335 | 0.173834 |
| Prkar2b | -0.34301 | 0.111726 | -0.07994 |

**Supplementary Table S3.** Variables loadings from principal component analysis. PC-principal component.

|     | PC1     | PC2     | PC3     |
|-----|---------|---------|---------|
| Mapk1 | -0.32048 | 0.0333  | 0.451097 |
| Mapk3 | -0.37113 | 0.035601 | -0.24431 |
| Mapk6 | -0.35524 | -0.04676 | -0.24312 |
| Mapk7 | -0.17178 | 0.67431  | 0.265602 |
| Mapk8 | -0.28422 | -0.4551  | -0.1826 |
| Mapk9 | -0.35456 | 0.142737 | 0.022819 |
| Mapk11 | 0.206154 | 0.527175 | -0.61479 |
| Mapk12 | -0.34248 | 0.025731 | -0.3073 |
| Mapk13 | -0.31441 | 0.186037 | 0.254423 |
| Mapk14 | -0.37242 | -0.00804 | -0.19021 |
### Supplementary Materials and Methods

**Supplementary table S4.** Key resources table.

| Resource or reagent                              | Source                                         | Identifier                                      |
|--------------------------------------------------|------------------------------------------------|------------------------------------------------|
| **Experimental model and biological samples**    |                                                |                                                |
| *Wistar* rat                                     | LaRES and ChronAge Laboratories (DBE, Faculty of Sciences, University of Novi Sad, Serbia) | [http://wwwold.dbe.pmf.uns.ac.rs/en/nauka-eng/lares](http://wwwold.dbe.pmf.uns.ac.rs/en/nauka-eng/lares) |
| Primary culture of spermatozoa                   | Three months-old male rats                     | NA                                             |
| **Commercial Reagents/Assays**                   |                                                |                                                |
| GenElute Mammalian Total RNA Miniprep Kit         | Sigma Aldrich, Germany                         | [https://www.sigmaaldrich.com](https://www.sigmaaldrich.com) |
| DNase I (RNase-free) treatment                   | New England Biolabs, USA                       | [https://international.neb.com](https://international.neb.com) |
| High Capacity Kit for cDNA                       | Applied Biosystems/Thermo Fisher Scientific, USA | [https://www.thermofisher.com](https://www.thermofisher.com) |
| Power SYBR Green PCR Master Mix                   | Applied Biosystems/Thermo Fisher Scientific, USA | [https://www.thermofisher.com](https://www.thermofisher.com) |
| **Primers**                                      |                                                |                                                |
| Supplementary tables S5 to S11                    | This paper                                     | [www.ncbi.nlm.nih.gov/sites/entrez](http://www.ncbi.nlm.nih.gov/sites/entrez) |
| **Antibodies**                                   |                                                |                                                |
| Rabbit polyclonal anti-PGC-1 (H-300) antibody    | Santa Cruz Biotechnology Inc.                  | Cat # sc-13067                                 |
| Rabbit polyclonal anti-NRF1 (H-300) antibody     | Santa Cruz Biotechnology Inc.                  | Cat # sc-33771                                 |
| Rabbit polyclonal anti-NRF2 (C20) antibody       | Santa Cruz Biotechnology Inc.                  | Cat # sc-722                                   |
| Monoclonal anti-MFN2 (F-5) antibody              | Santa Cruz Biotechnology Inc.                  | Cat # sc-515647                                |
| Rabbit polyclonal anti-GAPDH (FL-335) antibody   | Santa Cruz Biotechnology Inc.                  | Cat # sc-25778                                 |
| Rabbit polyclonal anti-ERRα (H-80) antibody      | Santa Cruz Biotechnology Inc.                  | Cat # sc-66882                                 |
| Mouse monoclonal anti-PRKAc antibody             | Transduction LaboratoriesTM                     | Cat # 610981                                   |
| Rabbit polyclonal ERK1/2 p44/42 MAPK (ERK1/2)    | Cell Signaling Technology                      | Cat #9102                                      |
| Rabbit polyclonal p38 MAPK                       | Cell Signaling Technology                      | Cat #9212                                      |
| Goat polyclonal ACTIN (I-19)                     | Santa Cruz Biotechnology Inc.                  | Cat # sc-1616                                  |
| **Software**                                     |                                                |                                                |
**Supplementary table S5.** Primers sequences used for the real-time PCR analysis of molecular markers of mitochondrial biogenesis.

| Gene  | Accession code | Primers                                  | Primer length | Product length | AV Ct |
|-------|----------------|------------------------------------------|---------------|----------------|-------|
| Ppargc1a | NM_031347      | F: 5'-AGCCGTAGGCCAGGTATGACA-3'          | 22 bp         | 107 bp         | 30.88 |
|        |                | R: 5'-TGCTTGGCCCTTCCAGACTCCC-3'         | 22 bp         |                |       |
| Ppargc1b | NM_176075      | F: 5'-ACCTTCGGTGTTCCGAGACATG-3'         | 22 bp         | 81 bp          | 28.75 |
|        |                | R: 5'-GTGGAAGGAGGGCTTTAGTGC-3'          | 22 bp         |                |       |
| Tfam    | NM_031326      | F: 5'-TATAGTCTGTCGCCCGAGGGAT-3'         | 22 bp         | 125 bp         | 27.92 |
|        |                | R: 5'-AAGGCTGACAGGGGAGGTATG-3'          | 22 bp         |                |       |
| Nrf1    | NM_001100708   | F: 5'-GACCATCAGACGCAAGCA-3'             | 22 bp         | 136 bp         | 27.60 |
|        |                | R: 5'-ATGGGCTGACAGCGGAGGTATG-3'         | 21 bp         |                |       |
| Nrf2a   | NM_001108841   | F: 5'-AGCGGAACTGAACGTGGTG-3'            | 21 bp         | 84 bp          | 27.06 |
|        |                | R: 5'-GTGACTGCTGCTGGAATCCCGT-3'         | 22 bp         |                |       |
| Ppara   | NM_013196      | F: 5'-GTCTCTGGAAGAACGACAGC-3'           | 22 bp         | 110 bp         | 28.78 |
|        |                | R: 5'-TTACGCCAAAATGCACCACCG-3'          | 21 bp         |                |       |
| Ppard   | NM_013141      | F: 5'-ACGGGTAAAGGCGGGTCCATCTGC-3'       | 22 bp         | 109 bp         | 26.49 |
|        |                | R: 5'-TGCTCTGCTGCTGCCATGCAC-3'          | 23 bp         |                |       |
| mtNd1   |                | F: 5'-GCGTGGAGAGGAGATCAGGG-3'           | 20 bp         | 271 bp         | 18.14 |
|        |                | R: 5'-GGCAATGCTGTGGGAGGGT A-3'          | 20 bp         |                |       |
| Gapdh   | NM_017008      | F: 5'-TGGCAAGTATGATGACATCAAG-3'         | 25 bp         | 110 bp         | 27.18 |
|        |                | R: 5'-AGCCAGGATGCCCCATAG-3'             | 20 bp         |                |       |

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from the NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.

**Supplementary table S6.** Primers sequences used for the real-time PCR analysis of molecular markers of mitochondrial fusion and architecture.

| Gene  | Accession code | Primers                                  | Primer length | Product length | AV Ct |
|-------|----------------|------------------------------------------|---------------|----------------|-------|
| Mfn1  | NM_138976.1    | F: 5'-CCTTGATCATCACCTGCTGGTTC-3'        | 24 bp         | 143 bp         | 28.63 |
|        |                | R: 5'-CCTTGGCTGCTATGTGGTG-3'            | 21 bp         |                |       |
| Mfn2  | NM_130894.4    | F: 5'-GCAAGCCGACTTTGAGG-3'              | 20 bp         | 118 bp         | 27.35 |
|        |                | R: 5'-CAGATGAGCAGAACGTCCTCC-3'          | 23 bp         |                |       |
**Supplementary Material**

| Gene | Accession Code | Primers | Primer length | Product length | AV Ct |
|------|----------------|---------|---------------|---------------|------|
| **Opa1** | NM_133585.3 | F: 5'-AAAAGCCCTCCCAGTTCAGA-3'  
R: 5'-TACCCGCAGTGAAGAATCCT-3' | 21 bp  
22 bp | 101 bp  
26.14 | |
| **Gapdh** | NM_017008 | F: 5'-TGCCAAGTATGATGACATCAAGAAG-3'  
R: 5'-AGCCCGAGGATGCCCTTAGT-3' | 25 bp  
20 bp | 110 bp  
21.08 | |

Primer sequences were designed by using software Primer Express 3.0 (Applied Biosystems) and full gene sequences from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.

**Supplementary table S7.** Primers sequences used for the real-time PCR analysis of molecular markers of mitochondrial fission.

| Gene | Accession Code | Primers | Primer length | Product length | AV Ct |
|------|----------------|---------|---------------|---------------|------|
| **Fis1**  
NM_00110591 9.1 | F: 5'-ACGCCCTGCCGATTCTTCTC-3'  
R: 5'-GCAAACCGTCAATCTCTGCAC-3' | 20 bp  
20 bp | 108 bp  
29.75 | |
| **Drp1**  
NM_053655.3 | F: 5'-AGGTTGCCCGTGACAAATGA-3'  
R: 5'-CACAGGCATCACGCAAATGCG-3' | 20 bp  
20 bp | 94 bp  
27.81 | |
| **Gapdh**  
NM_017008 | F: 5'-TGCCCAAGTATGATGACATCAAGAAG-3'  
R: 5'-AGCCCGAGGATGCCCTTAGT-3' | 25 bp  
20 bp | 110 bp  
22.04 | |

Primer sequences were designed by using software Primer Express 3.0 (Applied Biosystems) and full gene sequences from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.

**Supplementary table S8.** Primers sequences used for the real-time PCR analysis of molecular markers of mitochondrial autophagy.

| Gene | Accession Code | Primers | Primer length | Product length | AV Ct |
|------|----------------|---------|---------------|---------------|------|
| **Pink1**  
NM_0010669 4.1 | F: 5'-CAAGCAAGTGTCTGACCCAC-3'  
R: 5'-GTTTCTCACACAGCGGATT-3' | 20 bp  
20 bp | 111 bp  
25.69 | |
| **Prkn**  
NM_020093.1 | F: 5'-CTTTCCAGCTCAAGGAAGTGG-3'  
R: 5'-CAGAGGCTATTTCTCGTGA-3' | 20 bp  
20 bp | 182 bp  
32.34 | |
| **Tfeb**  
NM_00102570 7.1 | F: 5'-CGCACACATTATGCGCCTGG-3'  
R: 5'-CTGTACAGCAGTGGCTAG-3' | 20 bp  
20 bp | 102 bp  
29.18 | |
| **Gapdh**  
NM_017008 | F: 5'-TGCCCAAGTATGATGACATCAAGAAG-3'  
R: 5'-AGCCCGAGGATGCCCTTAGT-3' | 25 bp  
20 bp | 110 bp  
22.04 | |

Primer sequences were designed by using software Primer Express 3.0 (Applied Biosystems) and full gene sequences from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.
**Supplementary table S9.** Primers sequences used for the real-time PCR analysis of molecular markers of mitochondrial functionality.

| Gene | Accession code | Primers | Primer length | Product length | AV Ct |
|------|----------------|---------|---------------|----------------|------|
| **Cox4i1** | NM_017202 | F: 5'-CGCTGAGATGAACAAGGGCACC-3'  
R: 5'-TCCCCAGATCAGCACAAGCGCA-3' | 22 bp  
21 bp | 93 bp | 21.88 |
| **Cox4i2** | NM_053472 | F: 5'-CACAGCCCAAAGGATGCTGCTA-3'  
R: 5'-TGTGCAGTAAGGCTCATCCCGC-3' | 22 bp  
22 bp | 105 bp | 32.19 |
| **Cytc** | NM_012839 | F: 5'-GCAAGCATAGACTGGACCAA-3'  
R: 5'-TTGTGGCATCTGTGTAAGAGAATC-3' | 22 bp  
25 bp | 88 bp | 23.56 |
| **Ucp1** | NM_012682 | F: 5'-TCAGCTCTTGTGCCTCGGGGTT-3'  
R: 5'-TGACACAGCTGGGTACATTG-3' | 21 bp  
22 bp | 114 bp | 29.96 |
| **Ucp2** | NM_019354 | F: 5'-ACGACCTCCCTTGGCCACTCAC-3'  
R: 5'-GGTACTGGGCAAGGCAGAT-3' | 22 bp  
22 bp | 117 bp | 22.07 |
| **Ucp3** | NM_013167 | F: 5'-TGCTCAACCCACGGATGTT-3'  
R: 5'-CTTGGGCGATGGTCTCTAGGCA-3' | 21 bp  
22 bp | 112 bp | 29.81 |
| **Gapdh** | NM_017008 | F: 5'-TGCCAAGTATGATGACATCAAGAG-3'  
R: 5'-AGGCCAGGGATGCCCTTATAG-3' | 25 bp  
20 bp | 110 bp | 21.13 |

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from the NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.
**Supplementary table S10.** Primers sequences used for the real-time PCR analysis of cAMP signaling elements.

| Gene   | Accession code | Primers                                                                 | Primer length | Product length | AV Ct |
|--------|----------------|-------------------------------------------------------------------------|---------------|----------------|-------|
| Adcy3  | NM_13079       | F: 5'-GCATCGAAACCTACCTCATCA-3'                                         | 21 bp         | 141 bp         | 31.76 |
|        |                | R: 5'-TGGGTCCTTGGTGCTCCTCA ATAA-3'                                     | 21 bp         | 105 bp         | 29.95 |
| Adcy5  | NM_022600      | F: 5'-AACAGGTGAAACGCAATGTC-3'                                          | 20 bp         | 99 bp          | 26.86 |
|        |                | R: 5'-CTCTGGGAGGTTGAGTT-3'                                              | 20 bp         | 123 bp         | 26.55 |
| Adcy6  | NM_012821      | F: 5'-CTGCCCTACGCTGTATGTC-3'                                           | 21 bp         | 105 bp         | 29.95 |
|        |                | R: 5'-GGAGTTGCTGCGGAAAGCT-3'                                            | 18 bp         | 123 bp         | 26.55 |
| Adcy7  | NM_053396      | F: 5'-TTCCGTGGTGTAACCGGT-3'                                             | 20 bp         | 123 bp         | 28.83 |
|        |                | R: 5'-GCCCTTGTGCGTCTCGTT-3'                                             | 20 bp         | 123 bp         | 28.83 |
| Adcy8  | NM_017142      | F: 5'-ATTGCCCTAGTGTTGACTA-3'                                            | 19 bp         | 113 bp         | 30.64 |
|        |                | R: 5'-GAAACTCTCAGCTGGGCT-3'                                             | 17 bp         | 113 bp         | 30.64 |
| Adcy9  | NM_001106980   | F: 5'-TCACCAAGCTAGCGCGCGG-3'                                            | 20 bp         | 124 bp         | 29.13 |
|        |                | R: 5'-GGGCTGTCACGCTGTCGTC-3'                                            | 20 bp         | 124 bp         | 29.13 |
| Adcy10 | NM_021684      | F: 5'-CCAGGACATCGTATCGGGAAGCT-3'                                       | 20 bp         | 113 bp         | 25.59 |
|        |                | R: 5'-ACCGGTTCGCGATCGCAAC-3'                                            | 20 bp         | 113 bp         | 25.59 |
| Prkaca | NM_00110922.1  | F: 5'-TCAGTGGACGCCAGGTCG-3'                                             | 21 bp         | 99 bp          | 25.59 |
|        |                | R: 5'-TCTGCCGCTCGGTGGCTG-3'                                             | 20 bp         | 99 bp          | 25.59 |
| Prkacb | NM_001077645   | F: 5'-GGGTCTAGGGGAACACGTCG-3'                                           | 20 bp         | 124 bp         | 26.98 |
|        |                | R: 5'-CCAGGCATTACTCGGGGAGGT-3'                                          | 22 bp         | 124 bp         | 26.98 |
| Prkar1a| NM_013181      | F: 5'-TGTCAGCGACCTGAGGT-3'                                              | 20 bp         | 112 bp         | 23.77 |
|        |                | R: 5'-AGTGACGCCGGAGCAGT-3'                                               | 20 bp         | 112 bp         | 23.77 |
| Prkar2a| NM_019264      | F: 5'-GCCGACCTCGTAGTGGTA-3'                                             | 20 bp         | 108 bp         | 26.81 |
|        |                | R: 5'-TGCTCTCGGTGAAAGTTCG-3'                                            | 20 bp         | 108 bp         | 26.81 |
| Prkar2b| NM_001030020   | F: 5'-CCATGCGGCTCAGGGAAGCT-3'                                           | 20 bp         | 107 bp         | 28.70 |
|        |                | R: 5'-GCAACATCGCGTAGGAGCAGC-3'                                          | 20 bp         | 107 bp         | 28.70 |
| Gapdh  | NM_017008      | F: 5'-TGCCAAGTATGAGTACATCAAGAAG-3'                                      | 25 bp         | 110 bp         | 21.12 |
|        |                | R: 5'-AGCCAGAGATGCCCTATAG-3'                                            | 20 bp         | 110 bp         | 21.12 |

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.
Supplementary table S11. Primers sequences used for the real-time PCR analysis of MAPK signaling elements.

| Gene   | Accession code | Primers | Primer length | Product length | AV Ct |
|--------|----------------|---------|---------------|----------------|-------|
| Mapk1  | NM_053842.1    | F: 5' - GTTCTGCACGCTGACCCAG-3'  
R: 5' - GCAAGGCAAGTCAGAGATC-3' | 21 bp          | 80 bp           | 26.12 |
| Mapk3  | NM_017347.2    | F: 5' - TCTTCTCAAGCTGCCCAT-3'   
R: 5' - ACATCCAAATCCCAACACACA-3' | 20 bp          | 60 bp           | 25.86 |
| Mapk6  | NM_031622.2    | F: 5' - CATTTGAACTGGCATGTCGTTT | 20 bp          | 62 bp           | 25.61 |
| Mapk7  | NM_001191547.1 | F: 5' - GCCCTTCCACTAGCCTTTT | 20 bp          | 62 bp           | 27.40 |
| Mapk8  | NM_053829.2    | F: 5' - TCAAGTCTCGTGATGATGATCGTTC | 23 bp          | 60 bp           | 27.26 |
| Mapk9  | NM_017322.1    | F: 5' - GGAGCCCGCTGCGATGAAAA-3'  
R: 5' - AGCCAGATGTCACCAGAGAAG-3' | 18 bp          | 57 bp           | 26.97 |
| Mapk10 | NM_001270544.1 | F: 5' - GGGCGCTGACCTGAATAACA | 20 bp          | 80 bp           | 29.84 |
| Mapk11 | NM_001109532.2 | F: 5' - GGATGTGTTCACTCCCGATGA | 20 bp          | 80 bp           | 26.23 |
| Mapk12 | NM_021746.1    | F: 5' - CTGGTCTGTGGCTGATCA-3'   
R: 5' - CCAGTTCCTGCGATGAT-3' | 21 bp          | 80 bp           | 27.04 |
| Mapk13 | NM_019231.2    | F: 5' - GCAGTCCTGCGATGAT-3'    
R: 5' - CCAGTCCTGCGATGAT-3' | 21 bp          | 80 bp           | 25.28 |
| Mapk14 | NM_031020.2    | F: 5' - TGCAAGTATGATGATGATGATGAT | 20 bp          | 110 bp          | 20.94 |
| Gapdh  | NM_017008      | F: 5' - TGCCAGATGATGATGATGATGAT | 25 bp          | 20 bp           | 20.94 |

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/sites/entrez). F - forward; R - reverse.

Relative quantification of protein expression after immunoprecipitation analysis

Spermatozoa samples for immunoprecipitation analysis were lysed in 1 ml buffer containing 20 mM HEPES, 10 mM EDTA, 2.5 mM MgCl2, 40 mM β-glycerophosphate, 1 mM DTT, 1% NP-40, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 µM aprotinin, 2 µM leupeptin and Phosphatase inhibitor cocktail tablets (cont. (1R, 2S, 3R, 6S)-1.2-dimethyl-3.6-epoxycyclohexane-1.2-dicarboxylic anhydride). Concentration of proteins in each sample was estimated by Bradford method and set at concentration of 300 μg/ml. Equal amount of protein in each sample (300 μg) was used for the immunoprecipitation. Pre-clearing of the lysate was done using 5 µl of normal goat serum (Santa Cruz Biotechnology, normal goat serum: sc-2043, (https://www.scbt.com)) mixed with 1 ml of lysate and incubated on ice for 1 hour. After the incubation 100 µl of bead slurry was added to each sample and incubated for 30 minutes at 4 °C with gentle agitation. Supernatant for the immunoprecipitation was collected after 10 minutes at 14 000 xg at 4 °C centrifugation. After pre-clearing process, lysates were mixed with MFN2 antibody (Santa Cruz Biotechnology) and incubated at 4 °C overnight with constant rotation. During additional overnight incubation at 4 °C with constant
rotation, immunoprecipitated complexes with MFN2 antibody were recovered by 80 µl of protein G agarose bead slurry and supernatant was used for further protein analysis by Western blot. Western blot analysis was done in the same manner as the analysis described in the main manuscript text and as described previously (1). Immune-reactive bands were detected using MyECL Imager (Thermo Fisher Scientific Inc.; https://www.thermofisher.com) and analyzed as two-dimensional images using Image J version 1.48 (http://rsbweb.nih.gov/ij/download.html). The optical density of images is expressed as volume adjusted for the background, which gives arbitrary units of adjusted volume. Normalization of the data was done using ACTIN protein expression, as the endogenous control. Immune-detection was performed with different antibodies (all details are listed in Supplementary Table S12).
### Supplementary table S12. The characteristics of the antibodies.

| Target | Name of Antibody | Antigen sequence | Manufacturer, catalog # | Mono- or polyclonal | Dil. used |
|--------|------------------|------------------|-------------------------|---------------------|-----------|
| PGC1   | PGC-1 (H-300): sc-13067 | Amino acids 1-300 mapping near the N-terminus of PGC-1 of human origin | Santa Cruz Biotechnology Inc. sc-13067 | Rabbit polyclonal antibody | 1:500 |
| NRF1   | NRF-1 (H-300): sc-33771 | Amino acids 204-503 mapping near the C-terminus of NRF-1 of human origin | Santa Cruz Biotechnology Inc. sc-33771 | Rabbit polyclonal antibody | 1:200 |
| NRF2   | NRF2 (C-20): sc-722 | Peptide mapping at the C-terminus of Nrf2 of human origin | Santa Cruz Biotechnology Inc. sc-722 | Rabbit polyclonal antibody | 1:100 |
| MFN2   | Mfn2 (F-5): sc-515647 | Amino acids 461-528 mapping within a cytoplasmic domain of Mfn2 of human origin | Santa Cruz Biotechnology Inc. sc-515647 | Mouse monoclonal antibody | 8 µg |
| ERRα   | ERRα (H-80): sc-66882 | Amino acids 81-160 mapping near the N-terminus of ERRα of human origin | Santa Cruz Biotechnology Inc. sc-66882 | Rabbit polyclonal antibody | 1:100 |
| PRKAc  | Purified Mouse Anti-PKA[C] | Amino acids 18-347 of PKA[Cα] of human origin. | BD Transduction Laboratories™ | Mouse monoclonal antibody | 1:500 |
| ERK1/2 | p44/42 MAPK (ERK1/2) Antibody #9102 | Polyclonal antibodies are produced by immunizing animals with a synthetic peptide (KLH-coupled) derived from sequence in the C-terminus of rat p44 MAP kinase. | Cell Signaling Technology #9102 | Rabbit polyclonal antibody | 1:500 |
| p38 MAPK | p38 MAPK Antibody #9212 | Polyclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to the sequence of human p38 MAPK. P38 MAPK antibody detects endogenous levels of total p38α, β or γ MAPK protein. | Cell Signaling Technology #9212 | Rabbit polyclonal antibody | 1:500 |
**ACTIN**

Actin (I-19): sc-1616

Polyclonal affinity purified antibody raised against a peptide mapping at the C-terminus of Actin of human origin. Santa Cruz Biotechnology Inc. sc-1616 Mw (Actin) = 43 kDa

Goat polyclonal antibody 1:100

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**GAPDH**

Anti GAPDH (FL-335) antibody

Amino acids 1-335 representing full length GAPDH of human origin. Santa Cruz Biotechnology Inc. sc-25778

Rabbit polyclonal antibody 1:150

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**References**

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