

Mitochondrial inorganic polyphosphate (polyP) is a potent regulator of mammalian bioenergetics: a proteomics and metabolomics study

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ABSTRACT

Inorganic polyphosphate (polyP) is an ancient, ubiquitous, and well-conserved polymer which is present in all the studied organisms. It is formed by individual subunits of orthophosphate which are linked by structurally similar bonds and isoenergetic to those found in ATP. While the metabolism and the physiological roles of polyP in some organisms, including bacteria and yeast, have already been described, the exact role of this polymer in mammalian physiology still remains poorly understood. In these organisms, polyP shows a co-localization with mitochondria, and its role as a key regulator of the stress responses, including the maintenance of appropriate bioenergetics, has already been demonstrated by our group and others. Here, using Wild-type (Wt) and MitoPPX (cells enzymatically depleted of mitochondrial polyP) SH-SY5Y cells, we have conducted a comprehensive study of the status of cellular physiology, using proteomics and metabolomics approaches. Our results suggest a clear dysregulation of mitochondrial physiology, especially of bioenergetics, in MitoPPX cells when compared with Wt cells. Interestingly, the effects induced by the enzymatic depletion of polyP are similar to those present in the mitochondrial dysfunction that is observed in neurodegenerative disorders and in neuronal aging. Based on our findings, the metabolism of mitochondrial polyP could be a valid and innovative pharmacological target in these conditions.

INTRODUCTION

Inorganic polyphosphate (polyP) is an evolutionary conserved polymer, which is wide spread across all studied organisms, from bacteria to mammals [1, 2]. It is composed of multiple monomers of orthophosphate linked by high-energy phosphoanhydride bonds, similar to those present in ATP [1]. The concentration and length of polyP has shown to be variable in different subcellular locations and organisms [3]. In mammalian cells, polyP is usually formed by a couple of hundred monomers of orthophosphate, and it is found within the micromolar range [3]. The metabolism of polyP in these cells still remains poorly understood, even though a study showing the effects of the mitochondrial F_0F_1 -ATP synthase in the synthesis and degradation of the polymer in the presence of mitochondrial respiration substrates and phosphates has been recently published [4]. However, additional pathways independent of the ATP synthase are probably also involved in the metabolism of polyP in mammalian cells [5, 6]. In bacteria and yeast, the metabolism of the polymer is well-described. For example, it is known that polyP is converted into inorganic phosphate by the exopolyphosphatase (PPX) enzyme [1, 2].

PolyP has been proposed as a key molecule in the cellular stress response in a wide range of organisms, including mammals [7-9]. Accordingly, polyP has an important regulatory role in many cellular processes that are commonly dysregulated in neurodegenerative disorders, such as generation of reactive oxygen species (ROS), inflammation, apoptosis, energy metabolism, calcium signaling, and protein homeostasis [10-18]. In mammalian cells, polyP has been found in the cytoplasm, the nucleus, associated with membrane proteins [3], in the extracellular space [19], and in different organelles such as acidocalcisomes and mitochondria, where the levels of the polymer correlate with those of ATP [12, 20]. In fact, polyP synthesis is dependent on the metabolic status of the cell [21, 22] and the inhibition of the mitochondrial F_0F_1 -ATP synthase by oligomycin completely blocks the production of polyP [22]. The authors of this study hypothesized that the action of polyP in mitochondrial metabolism could be exerted either through its direct participation in the production of ATP or through the regulation of the enzymatic activity of the ATP synthase that works as a reservoir of phosphoryl groups, readily available under cellular stress [22].

Moreover, we have already demonstrated the role of polyP in the regulation of the intra-mitochondrial levels of free calcium, which are closely related to mitochondrial metabolism and ATP generation [11, 23]. In fact, intra-mitochondrial calcium signaling activates mitochondrial dehydrogenases, which leads to the increased levels of NADH and ATP [24], present in stress response and in aging [25, 26].

Mitochondrial function, including oxidative phosphorylation (OXPHOS) regulation, is especially relevant to high-energy cells, such as neurons [27]. Changes in energy metabolism can compromise brain function and be components in the etiopathology of multiple diseases, including neurodegenerative disorders, such as Parkinson's (PD) and Alzheimer's disease (AD) [28-30]. Moreover, mitochondrial bioenergetics is in close equilibrium with mitochondrial dynamics, mitophagy, and apoptosis, among other key processes within the organelle [31-33]. Therefore, the dysregulation of one of these processes will deleteriously affect the other processes. Lastly, increased ROS production, a well-known consequence of dysfunctional mitochondrial bioenergetics, is also a common aspect of neurodegeneration [31, 34]. Interestingly, our research group has recently investigated the impact of mitochondrial polyP in the regulation of mammalian OXPHOS [35]. By using HEK293 cells that are enzymatically depleted of mitochondrial polyP (MitoPPX), we demonstrated a significant shift from OXPHOS to glycolysis, globally affecting mitochondrial physiology.

Here, we conducted a comprehensive proteomics and metabolomics study of the role of mitochondrial polyP in SH-SY5Y cells, a cellular model widely used for experimental research in the field of neurodegeneration [36]. Our goal is to better understand the contribution of mitochondrial polyP to the molecular mechanisms that regulate cellular bioenergetics. To achieve this, we used Wild-type (Wt) and MitoPPX SH-SY5Y cells. Proteomics and metabolomics approaches were conducted to identify phenotypic differences between the cell lines, particularly focused on mitochondrial metabolism and bioenergetics. A comprehensive analysis of the obtained data was then conducted. Our data suggested that the enzymatic depletion of mitochondrial polyP has a deleterious impact on various metabolic pathways, including mitochondrial bioenergetics,

where several subunits of the Electron Transfer Chain (ETC) as well as metabolites, are altered. These integrated proteomic and metabolomics profiles could bring new insights into the specific role(s) of mitochondrial polyP in mammalian cells, as well as contribute to finding new therapeutic approaches using mitochondrial polyP as a valid target against dysfunctional organelle in neurodegenerative disorders.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle medium (DMEM):F12; 4',6-diamino-2-phenylindole (DAPI); penicillin-streptomycin; G418; lipofectamine; trypsin; alkaline phosphatase; Pierce BCA protein assay kit; Pierce ECL western blotting substrate; phosphoric acid; heat-inactivated fetal bovine serum (FBS); acetonitrile (ACN); methanol; ammonium acetate; Pierce Halt Protease and Phosphatase Inhibitor Cocktails; and acetic acid Optima LC-MS grade were purchased from Thermo Fisher Scientific (Waltham, MA, US). Phosphate-Buffered Saline (PBS); β -mercaptoethanol; tris(hydroxymethyl)-1,3-propanediol hydrochloride (TRIS-HCl); glycerol; Bovine Serum Albumin (BSA); phenylmethylsulfonyl fluoride (PMSF); Tween-20; triethylammonium bicarbonate buffer (TEAB); dimethyl sulfoxide (DMSO); iodoacetamide (IAA); yeast enolase protein; and potassium chloride were obtained from Sigma-Aldrich (San Louis, Missouri, US). All the materials and reagents used in the Western Blotting experiments, including secondary antibodies, polyvinylidene (PVDF) membranes, fat-free milk, protein ladders, and polyacrylamide-precast gels were obtained from BioRad (Hercules, California, US). Primary antibodies anti-OXPHOS, anti-PPX and anti- β -actin were obtained from Abcam (Cambridge, UK), and anti-eGFP from Cell Signaling (Danvers, MA, US). Deionized water was obtained using an 18 M Ω Milli-Q from EMD Millipore Corporation (Billerica, Massachusetts, US).

Cell growing and maintenance

Wt SH-SY5Y cells were obtained from the American Type Culture Collection (Manassas, Virginia, US), and grown following the instructions of the provider, as previously done [37-40]. Specifically, we used DMEM:F12 media,

supplemented with 20 units/mL penicillin-streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (FBS). Cells were grown in a humidified cell culture incubator, under a 5% CO₂ atmosphere at 37°C until around 80% optimal confluence was reached, as previously reported [37, 38]. MitoPPX cells were generated adapting the protocol from [11] to SH-SY5Y neuroblastoma cells. 500,000 cells were plated in 6-well plate. 24 hours later, the cells were transiently transfected using lipofectamine and a DNA construct containing the sequence for the mammalian expression of a mitochondrial targeting sequence (MTS), green fluorescent (eGFP) protein, and PPX [11]. After 24 hours, 0.5 mg/mL of Geneticin (the selection antibody) were added to the cells for two weeks. Subsequently, individual clones were selected, transferred into a 96-well plate and amplified. MitoPPX cells were grown and maintained in the presence of Geneticin to assure the expression of the construct.

PPX enzymatic assay

The binding between polyP-DAPI shifts the wavelength of emission of DAPI up to 550 nm [41]. Therefore, this is a widely used method to assay the presence of polyP in various organisms, including mammalian cells [20, 41]. Using this principle, the enzymatic activity of MitoPPX cells was assayed following the protocol previously published in [35].

Protein extraction and quantification

Cells were plated and grown to 90% confluency. Afterwards, cells were scrapped on ice-cold PBS 1x, and centrifuged at 1000 rpm for 5 minutes at 4°C. Pellets were re-suspended in lysis buffer (300mM NaCl, 50mM Tris-HCl pH 7.5, 1% TritonX-100) and shaken for 15 minutes at 4°C. Cell suspensions were centrifuged at 13000 rpm for 5 minutes at 4°C, and supernatants were directly frozen at -80°C. Protein content was measured through the bicinchoninic acid colorimetric (BCA) assay following manufacturer's instructions. Aliquots of the samples were prepared and stored at -80°C for further Western Blotting validation.

Western Blotting

Western Blotting analysis were conducted as previously published [26, 42, 43]. 20 µg of protein per sample was separated using 12% Mini-Protean TGX Precast Gel, and transferred into PVDF membranes. Non-specific protein binding was blocked using 5% fat-free milk and 0.1% Tween-20 in PBS 1x for 1 hour. Membranes were then hybridized with the specific primary antibodies overnight at 4°C (PPX 45 kDa; eGFP 25 kDa; CV-ATP5A 54 kDa; CIII-UQCRC2 48 kDa; CII-SDHB 29 kDa; CIV-COXII 22 kDa; CI-NDUFB8 18 kDa; all of them at 1:1000 dilution). The levels of protein expression were normalized using β-actin content (47 kDa; 1:1000). The signal was detected using Pierce ECL Substrate kit following manufacturer's instructions and the Gel Doc XR Image System from BioRad (Hercules, California, US). Finally, the intensity of the signal was quantified by densitometry analysis using ImageJ software from NIH (Bethesda, Maryland, US).

Proteomics assay

Wt and MitoPPX cells were scraped, washed with cold PBS, and immediately preserved at -80°C. Cells were shipped overnight on dry ice for further protein profiling at the Department of Genome Science of University of Washington (Seattle, Washington, USA).

Protein lysis and digestion

Cell pellets were resuspended in 100 µl of 5% SDS, 50 mM Triethylammonium bicarbonate (TEAB), 2 mM MgCl₂, and 1x Pierce Halt protease and phosphatase inhibitor cocktail, vortexed and briefly probe sonicated. Protein concentration was measured with a BCA assay. Homogenate of 50 µg was added to a process control of 800 ng of yeast enolase protein, which was then reduced with 20 mM DTT, and alkylated with 40 mM of IAA. Lysates were then prepared for S-trap column (Protifi, Long Island, New York, US), binding by the addition of 1.2% phosphoric acid and 350 µl of binding buffer (90% methanol, 100 mM TEAB). The acidified lysate was bound to the column incrementally, followed by three wash steps with binding buffer to remove SDS, three wash steps with 50:50 methanol:chloroform to remove lipids, and a final wash step with binding buffer. Trypsin (1:10) in 50mM TEAB was then added to the S-trap column for digestion at 47 °C for 1 hour. Hydrophilic peptides were then eluted

with 50 mM TEAB and hydrophobic peptides were eluted with a solution of 50% acetonitrile in 0.2% formic acid. The elute was pooled, speed vacuumed and resuspended in 0.1% formic acid. A heavy labeled Peptide Retention Time Calibrant (PRTC) mixture (Pierce, Waltham, Massachusetts, US) was added to each sample.

Proteomic profiling by LC-MS

1 µg of each sample with 150 femtomole of PRTC were loaded onto a 30 cm fused silica picofrit (New Objective, Littleton, Massachusetts, US) 75 µm column and 3.5 cm 150 µm fused silica Kasil1 (PQ Corporation, Malvern, Pennsylvania, US) frit trap loaded with 3 µm Reprosil-Pur C18 (Dr. Maisch, Ammerbuch, Entringen, Germany) reverse-phase resin analyzed with a Thermo Easy nano-LC 1200. The PRTC mixture was used to assess quality of the columns before and during analysis. Four of these quality control runs were analyzed prior to any sample analysis and then after every six to eight sample runs, another quality control run was analyzed.

Buffer A was 0.1% formic acid in water and Buffer B was 0.1% formic acid in 80% acetonitrile. The 40-minute QC gradient consists of a 0 to 16% B in 5 minutes, 16 to 35% B in 20 minutes, 35 to 75% B in 1 minute, 75 to 100% B in 5 minutes, followed by a wash of 9 minutes and a 30-minute column equilibration. The 110-minute sample LC gradient consists of a 2 to 7% for 1 minutes, 7 to 14% B in 35 minutes, 14 to 40% B in 55 minutes, 40 to 60% B in 5 minutes, 60 to 98% B in 5 minutes, followed by a 9-minute wash and a 30-minute column equilibration. Peptides were eluted from the column with a 50 °C heated source (CorSolutions, Ithaca, New York, US) and electrosprayed into a Thermo Orbitrap Fusion Lumos Mass Spectrometer with the application of a distal 3 kV spray voltage. For the quality control analysis, a cycle of one 120,000 resolution full-scan mass spectrum (350-2000 m/z) followed by data-independent MS/MS spectra on the loop count of 76 data-independent MS/MS spectra using an inclusion list at 15,000 resolution, AGC target of 4e5, 20 seconds maximum injection time, 33% normalized collision energy with a 8 m/z isolation window. For the sample digestion, first a chromatogram library of 6 independent injections is analyzed from a pool of all samples within a batch. For each injection, a cycle of one 120,000 resolution full-scan mass spectrum with a

mass range of 100 m/z (400-500 m/z, 500-600 m/z...900-1000 m/z) followed by data-independent MS/MS spectra on the loop count of 26 at 30,000 resolution, AGC target of 4e5, 60 seconds maximum injection time, 33% normalized collision energy with a 4 m/z overlapping isolation window. The chromatogram library data generated from a pooled samples was used to detect proteins from individual quantitative sample runs. These individual runs consist of a cycle of one 120,000 resolution full-scan mass spectrum with a mass range of 350-2000 m/z, AGC target of 4e5, 100 ms maximum injection time followed by data-independent MS/MS spectra on the loop count of 76 at 15,000 resolution, AGC target of 4e5, 20 seconds maximum injection time, 33% normalized collision energy with an overlapping 8 m/z isolation window. Application of the mass spectrometer and LC solvent gradients are controlled by the ThermoFisher XCalibur data system (Waltham, Massachusetts, US).

Proteomics data analysis

Thermo RAW files were converted into mzML format using Proteowizard (version 3.0.20064; Palo Alto, CA, US) using vendor peak picking and demultiplexing [44]. Chromatogram libraries were created by analyzing the 6 narrow window gas phase fractionated runs on the pool sample using default settings (10 ppm tolerances, trypsin digestion, HCD b- and y-ions) of EncyclopeDIA (version 0.9.5) using a Prosit predicted spectra library [45] based on Uniprot human canonical FASTA background (April 2019) as described previously [46]. Prosit library was created using the settings 1 missed cleavage, 33% Normalized Collision Energy (NCE), charge states of 2 and 3, m/z range of 396.4 to 1002.7, and a default charge state of 3. The resulting chromatogram library empirically corrected the on-column chromatographic retention times and the product ion intensities and only included peptides detected in the pooled sample [46].

The individual quantitative analyses were analyzed using EncyclopeDIA with the Chromatogram library generated from the narrow window gas phase fractionated data. EncyclopeDIA was set to require a minimum of three quantitative ions and filtering peptides at a 1% False Discovery Rate (FDR) using Percolator 3.01 [47]. The output of the EncyclopeDIA Quant Report was imported into Skyline (version 20.1.9.234; Fairfield, Ohio, US) with the human

uniprot FASTA as the background proteome to map peptides to proteins. We used a library of predicted spectra and retention times. In Skyline, data was normalized to the total ion current (TIC) and unique peptides were summed to protein TAF (total area fragment) quantities. If a peptide mapped to more than one protein, Skyline selected the first protein on the list. A csv file of unique protein TAFs for each replicate was exported. The Skyline documents and raw files for DIA library generation and DIA sample analysis are available at Panorama Public (ProteomeXchange ID: PXD028185; access URL: <https://panoramaweb.org/MitoPPX.url>).

Metabolomics assay

Sample preparation

Cells were prepared as for the proteomics assay, and shipped over night on dry ice for metabolite profiling at the University of Washington's Northwest Metabolomics Research Center (Seattle, Washington, US).

Aqueous metabolites were extracted using a protein precipitation method [48]. Cell samples were first homogenized in 200 μ L purified deionized water at 4 $^{\circ}$ C, and then 800 μ L of methanol containing $^{13}\text{C}_6$ -glucose and $^{13}\text{C}_2$ -glutamate (reference internal standards used to monitor sample preparation) were added. Afterwards samples were vortexed, stored for 30 minutes at -20 $^{\circ}$ C, sonicated in an ice bath for 10 minutes, centrifuged for 15 min at 14,000 rpm and 4 $^{\circ}$ C, and then 600 μ L of supernatant was collected from each sample. Lastly, recovered supernatants were dried on an Eppendorf Vacufuge (Brinkmann Instruments, Westbury, NY, US) and reconstituted in 1 mL of LC-matching solvent containing $^{13}\text{C}_2$ -tyrosine and $^{13}\text{C}_3$ -lactate (reference internal standards used to monitor instrument performance). Protein pellets that were left over from the sample preparation were used for BCA protein assay

Metabolite profiling by LC-MS

Targeted LC-MS metabolite analysis was performed on a duplex-LC-MS system composed of two Shimadzu Nexera XR LC-20AD pumps, CTC Analytics PAL HTC-xt temperature-controlled auto-sampler (Shimadzu, Kyoto, Japan) and AB Sciex 6500+ Triple Quadrupole MS equipped with ESI ionization source (AB

Sciex, Framingham, MA, US) [49]. UPLC pumps were connected to the auto-sampler in parallel to allow two independent chromatography separations: while one column performed separation and MS data acquisition in ESI+ ionization mode, the other column was equilibrated for sample injection, chromatographic separation and MS data acquisition in ESI- mode. Samples were injected on two identical Waters XBridge BEH Amide XP analytical columns (-2.5 μm , 130 Å, 2.1 x 150 mm) (Waters Corporation, Milford, MA, US). Each chromatography separation was 18 minutes (total analysis time per sample was 36 minutes). MS data acquisition was performed in multiple-reaction-monitoring (MRM) mode. LC-MS system was controlled using AB Sciex Analyst 1.6.3 software (AB Sciex, Framingham, MA, US).

Metabolomics data analysis

Measured MS peaks were integrated using AB Sciex MultiQuant 3.0.3 software. The LC-MS assay targeted 363 metabolites (plus 4 spiked reference internal standards). In addition to the study samples, two sets of quality control (QC) samples were used to monitor the assay performance as well as data reproducibility. One QC [QC(I)] was a pooled human serum sample used to monitor system performance over extended time and the other QC [QC(S)] was a pooled study sample, which was used to monitor data reproducibility. Each QC sample was injected per every 10-study samples. The data were well reproducible with a median CV of 5.86 %. Generated MS data were normalized with BCA total protein count.

Statistical analysis

For the proteomics data, we used TIC-normalized protein TAF quantities for the downstream analysis. Data were run in two batches. There were 5,847 proteins detected in run batch1 and 5,600 proteins detected in run batch2. We included 4,232 proteins with < 20% missingness in both batches for the imputation. We used a quantile regression approach for the imputation of left-censored missing data (QRILC), which has been suggested as the favored imputation method for left-censored Missing Not At Random (MNAR) data [50]. This was implemented in the R imputeLCMD package. We fitted a linear model to the protein level data to detect the genotype group differences while adjusting for run batch and

sample collection day in the model using the Bioconductor limma package [51]. The limma package uses empirical Bayes moderated statistics, which improves power by 'borrowing strength' between proteins in order to moderate the residual variance [52].

For the targeted metabolomics assay we performed a median normalization where we adjusted the data, so all samples have the same median value of the metabolite abundance post log₂ transformation. Only metabolites with < 20% missingness and a coefficient of variation (CV) < 20% in the pooled sample QC data were included in further analysis. Out of the possible 363 metabolites that the assay could detect, 151 metabolites passed these filtering criteria, which were included in the imputation step. We used the same imputation method as described above for the proteomic data. We fit a linear model to the imputed data to detect the genotype group differences while adjusting for collection day and protein amount in the model using the Bioconductor limma package.

For the unsupervised learning, the batch effect and sample weight were removed using the limma removeBatchEffect function prior to the PCA (principal component analysis) and clustering analysis. We used MetaboAnalyst (v 5.0) to perform univariate methods (T-test), multivariate analysis (PCA) and hierarchical clustering (Euclidean distance and Ward's linkage) [53].

Pathway analysis

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, California, US), was used to analyze the proteomics and metabolomics data for canonical pathways, upstream regulators, and disease and function analysis using the list of differentially expressed proteins (log₂FC cutoff of 1.5 and FDR<0.001) and metabolites (FDR<0.05). Protein and metabolite symbols were used as identifiers. All molecules were overlaid onto a global molecular network contained in the Ingenuity Knowledge Base. Networks of network-eligible molecules were algorithmically generated based on their connectivity. The functional analyses identified the canonical pathways, upstream regulators and biological functions and diseases that were most significant to the data set. A right-tailed Fisher's exact test was used to calculate the raw p-values. The z-score was used to predict the activation or inhibition state of the expression

pattern of molecules in our datasets. Canonical pathways, upstream regulators and biological functions and diseases which were likely activated (based on the pattern of differentially abundant proteins or metabolites) were presented in orange (positive z-score) and those that were likely inhibited were presented in blue (negative z-score).

RESULTS

SH-SY5Y cells overexpressing the PPX enzyme in mitochondria showed decreased levels of polyP

SH-SY5Y MitoPPX cells successfully expressed eGFP and the PPX enzyme, while these proteins were not observed in the Wt samples (Figure 1A). In addition, the activity of the PPX enzyme was assayed through the measurement of the cellular levels of exogenous polyP, using DAPI fluorescence as previously described [41]. A significant decrease of cellular polyP was observed in MitoPPX cells after 12 hours of incubation with exogenous polyP, when compared with Wt (Figure 1B). This indicated the functional activity of the overexpressed PPX enzyme in MitoPPX cells.

Pathway analysis of proteomics and metabolomics data

The global effects of the enzymatic depletion of mitochondrial polyP were investigated through the IPA software. We focused our studies especially in those pathways which relate to mitochondrial metabolism since the physiology of the organelle has shown to be seriously affected in neurodegeneration. Each dataset was analyzed separately and later integrated via meta-analysis. A complete summary of each IPA analysis (Proteomics, Metabolomics and Meta-analysis) can be accessed in the Supplementary Information.

Differential protein expression

Proteomics analysis led to the annotation of 4,232 proteins with less than 20% missingness in all samples. Out of 4,214 mapped proteins in IPA, we identified 405 differentially expressed proteins in MitoPPX cells compared to Wt ($\log_2FC < 1.5$ and $FDR < 0.001$), of which 386 were upregulated, whereas 19 were downregulated. The top 25 most significantly expressed proteins with a T-test p -value < 0.05 were depicted in a heat map (Figure 2A). PCA demonstrated

a clear discrimination between the two groups (in red MitoPPX cells and in green Wt cells; Figure 2B).

Differential metabolite expression

Out of 363 metabolites, the metabolomics profiling in IPA resulted in the detection of 151 metabolites with less than 20% missingness in all samples, and 81 abundant metabolites were significantly different in MitoPPX compared to Wt (FDR<0.05). T-test identified 52 upregulated and 36 downregulated abundant metabolites, with the top 25 significant metabolites (p-value<0.05) shown in the heat map (Figure 2C). PCA showed two clear populations (in red MitoPPX cells and in green Wt cells; Figure 2D).

Identification of canonical pathways

The most significant dysregulated pathways in the proteomics analysis included assembly of RNA polymerase II complex (raw p-value<0.001), androgen signaling (raw p-value<0.001), DNA double-strand break repair by non-homologous end joining (raw p-value<0.01), telomere extension by telomerase (raw p<0.01), glucocorticoid receptor signaling (raw p-value<0.01), and amyloid processing (raw p-value<0.05) (Figure 3A). In the metabolomics analysis, the most significant dysregulated pathways comprised tRNA changing, L-carnitine biosynthesis, purine nucleotides degradation II – aerobic, super pathway of citrulline metabolism, and super pathway of methionine degradation (all of them raw p-value<0.001) (Figure 3B).

Interestingly, both proteomics and metabolomics analysis showed the differential expression of the OXPHOS pathway in MitoPPX cells, compared to Wt samples (raw p-value<0.05 and <0.001, respectively). Proteomics data indicated that the variation in OXPHOS occurred at different subunits of the ETC complexes (Table 1 and Figure 4A) which was accompanied by alterations in the expression of ATP, ADP, NADH and NAD⁺ metabolites, all of them directly involved in OXPHOS (Table 2, Figure 4B). These results were supported by IPA prediction of decreased OXPHOS activity in the MitoPPX cells, when compared to Wt, proposed in both proteomics (negative z-score = -1.34; Figure 3A) and metabolomics datasets (negative z-score = -1). The dysregulation of the tricarboxylic acid (TCA) cycle observed in the

metabolomics data (Figure 3B), with increased succinate and decreased oxoglutarate, also suggested a global effect of polyP depletion on mitochondrial function and energetic metabolism (Figure 4C).

Analysis of upstream regulators

We performed upstream regulator analysis (URA) in IPA software to explore the potential upstream regulators of the protein and metabolite regulatory networks affected by changes in gene expression.

From 308 predicted upstream regulators of different molecule types identified in the proteomics analysis, only 11 were further considered based on $FDR < 0.05$ (Table 3 and Supplementary Information). The transcriptional regulator KDM5A (lysine-specific demethylase 5A), a histone demethylase, was increased in MitoPPX cells within our data set, compared to Wt ($\log_2 F_0.203$) and appears as one of the predicted upstream regulators (negative z-score = -0.632, $FDR < 0.05$) of ten proteins, four of them being dysregulated subunits of the ETC complexes in MitoPPX cells: COX7A2-complex IV, NDUFA4-complex I, SDHC-complex II and COQ7 (Figure 5A). In addition, KDM5A was a predicted upstream regulator of two other mitochondrial proteins: mitochondrial catalase, an enzyme involved in the regulation of ROS production; and TOMM22, a translocase of the outer mitochondrial membrane in charge of the recognition and translocation of cytosolically synthesized mitochondrial preproteins (Figure 5A).

The metabolomics analysis resulted in 1013 upstream regulators, with 69 $FDR < 0.001$ (Supplementary Information). Creatine, an endogenous compound in neuronal cells which is also involved in bioenergetics, was decreased in MitoPPX cells within our data set, compared to Wt ($\log_2 FC = -0.019$). This metabolite was predicted to be an upstream regulator of 7 metabolites (positive z-score = 2.383), including phosphocreatine, AMP and ATP (Figure 5B). In addition, SIX1 (Homeobox protein SIX1) and HDAC 11 (histone deacetylase 11) were predicted as upstream regulators (positive z-score=2.714 and 2.200, respectively). SIX1 is a transcription factor predicted to lead to decreased levels of 2-oxoglutaric acid and the increase of AMP and ATP (Figure 5B). Also, HDAC11 is predicted to lead to increased succinate levels (Figure 5B).

Analysis of diseases and functions

We also applied the 'disease and function analysis' from IPA to examine the biological context of proteomics and metabolomics alterations in MitoPPX and Wt cells (Supplementary Information). In our proteomics analysis, the top five significant diseases or functions were cancer (raw p value range: 4.8E-29 - 5.46E-03), organismal injury and abnormalities (raw p value range: 4.8E-29 - 5.46E-03), endocrine system disorders (raw p value range: 2.98E-20 - 4.12E-03) gastrointestinal disease (raw p value range: 2.17E-13 - 4.78E-03); and DNA replication, recombination, and repair (raw p value range: 1.1E-07 - 2.56E-03). Cancer was expected as the top significant disease because SH-SY5Y cells are immortalized cell lines. Neurological disease is within the top ten affected 'disease and functions', showing 283 proteins involved. In our metabolomics analysis, the top five significant 'disease or functions' are cellular growth and proliferation (raw p-value range: 5.27E-23 - 2.74E-03), organismal development (raw p-value range: 5.27E-23 - 2.74E-03), inflammatory disease (raw p-value range: 1.02E-15 - 2.74E-03), inflammatory response (raw p-value range: 1.02E-15 - 2.74E-03), organismal injury and abnormalities (raw p-value range: 1.02E-15 - 2.74E-03). Within all the detected 'disease and function', ten of them were related to mitochondria and affected in neurodegeneration. The most significant included dysfunction of mitochondria, permeabilization of mitochondria, and respiration of mitochondria (all of them FDR<0.001; Figure 6).

Meta-analysis

Next, we combined the proteins and metabolites differentially expressed together into an IPA analysis. The meta-analysis of both proteomics and metabolomics assays suggested altered canonical pathways associated with abundant metabolites and proteins in MitoPPX cells (Supplementary Information). The most significant pathways found included asparagine biosynthesis I, L-carnitine biosynthesis, tRNA charging, lipoate biosynthesis and incorporation II, and purine ribonucleosides degradation to ribose-1-phosphate (all of them raw p-value<0.001). The meta-analysis further supported the decreased OXPHOS pathway in MitoPPX cells (negative z-score = -1.76, Figure 7), observed in both the proteomics and metabolomics study, as well as the change in TCA (positive z-score=2.00, Figure 7) observed in the metabolomics analysis. In addition, the meta-analysis revealed the presence of

mitochondrial dysfunction in MitoPPX cells, when compared with Wt samples (z-score = NaN, Table 4). Using the 'Molecule Activity Predictor' (MAP) option in IPA software, we predicted up- and downstream properties/activities of molecules participating in mitochondrial function (Figure 8). These included an increase in the glutathione levels (GSH), probably to counteract the potential increased oxidative stress in MitoPPX cells, as well as an increase of TRX2, which is a mitochondrial protein important for the control of mitochondrial ROS homeostasis, leading to an increase of NADP⁺ levels (Figure 8).

Moreover, we identified 57 different upstream regulators with FDR set at 0.05 (Supplementary information). The analysis distinguished 40 regulators as genes, RNAs or proteins, such as the previously mentioned SIX1 (activation z-score = 2.714, FDR<0.001) and HDAC11 (positive z-score = 2.200, FDR<0.01). Additionally, IPA predicted another upstream regulated protein, UCP1 (uncoupling protein 1; positive z-score = 2.027, FDR<0.01).

Finally, the meta-analysis showed top five most significant dysregulated pathways as cancer (raw p value range: 1.66E-18 – 2.95E-03), organismal injury and abnormalities (raw p value range: 1.66E-18 – 2.95E-03), cellular growth and proliferation (raw p value range: 8.04E-13 – 2.44E-03), organismal development (raw p value range: 8.04E-13 – 2.68E-03), and endocrine system disorders (raw p value range: 2.77E-11 – 2.68E-03). In line with metabolomics data, we identified several significantly affected mitochondrial-related pathways, such as dysfunction of mitochondria, release of mitochondrial DNA, respiration of mitochondria, and activation of mitochondria (all of them FDR<0.05; Supplementary information).

Western Blotting validation

The expression of some subunits of the ETC complexes was assayed using Western Blotting in our Wt and MitoPPX SH-SY5Y cells. Decreased protein expression of NDUFB8-complex I and COXII- complex IV subunits were observed in MitoPPX cells, compared to Wt. These results further confirmed our data obtained from the proteomics and metabolomics assays, corroborating the important role of polyP in the regulation of the ETC function (Figure 9).

DISCUSSION

A wide range of mitochondrial alterations have been described in the etiopathology of neurodegenerative diseases, including AD and PD [54, 55], as well as in aging [56]. These alterations include but are not limited to dysregulation of the ETC complexes, which will ultimately affect OXPHOS functionality, increasing ROS levels and unbalancing the cellular antioxidant system [57-59]. However, the exact molecular basis of the mechanism(s) underlying mitochondrial dysfunction still remain mostly unknown. PolyP has been described as a key molecule in the cellular stress response, including the regulation of several mitochondrial functions, such as energy metabolism - including calcium homeostasis, and protein homeostasis [11, 12, 35, 60-65]. However, the exact molecular mechanism explaining the effects of polyP in the regulation of mitochondrial function are still unclear, especially under stress conditions, such as in neurodegeneration. To our knowledge, this is the first time that a comprehensive study of the effects of polyP on the proteome and the metabolome of mammalian cells has been conducted. Specifically, in the present study, a large number of protein expression and metabolite abundance alterations were described in SH-SY5Y cells that were enzymatically depleted of mitochondrial polyP (MitoPPX). These findings highlighted the importance of polyP in maintaining the proper cellular function, either through direct or indirect regulation of all proteins and metabolites which are differentially expressed in MitoPPX cells.

The number of differentially expressed mitochondrial proteins and the different abundance of mitochondrial metabolites observed in MitoPPX cells showed the clear impact that enzymatical depletion of mitochondrial polyP has in the metabolism of the organelle. Two of the main dysregulated mitochondrial metabolic pathways identified by IPA software were OXPHOS and TCA. Specifically, ETC complex I, complex II, and complex IV showed differential protein expression of some of their subunits, leading to a potential alteration of their activity. Moreover, altered levels of succinate and oxoglutarate evidenced the dysregulation of the TCA. In fact, the observed increased succinate and ETC complex II subunits could indicate a blockage of this complex. In addition, further evidence that demonstrated that mitochondrial metabolism was affected, were increased levels of ATP, ADP, NAD, and NADH in MitoPPX cells. These

findings reinforced the importance of polyP in the maintenance of mitochondrial and cellular function. One plausible mechanism explaining this differential pattern of protein and metabolites presence in MitoPPX cells could be the reduced chaperoning effects due to the lack of polyP in our model [62-64]. Interestingly, the chaperoning ability of the cells is critical in the activation of the stress response. In fact, the lack of mitochondrial polyP could promote the activation of the cellular stress response in MitoPPX cells. As previously mentioned, the crucial role played by polyP in the regulation of the stress response has already been demonstrated [3, 66-68]. This explanation is supported by our proteomic analysis data, showing increased expression of some antioxidant enzymes (including several peroxiredoxins, thioredoxins, SOD1 and catalase), as well as of proteins involved in the regulation of the cellular stress response (including KEAP, Kelch-like ECH-associated protein 1). This entire cellular stress environment could further deleteriously affect mitochondrial metabolism.

The observed effects of polyP could also be at least partially mediated by the direct interaction between the polymer and the altered proteins within the OXPHOS and TCA which were identified in our analysis. In contrast to the mentioned increased expression of several antioxidant enzymes, mitochondrial SOD2 expression was decreased in MitoPPX cells, which hints at this enzyme as a potential target of polyP. Mitochondrial SOD2 is a superoxide dismutase enzyme dependent on manganese, which is known to form complexes with polyP [62]. This property suggests a role for polyP in the detoxification process mediated by SOD2. Although we did not show direct evidence of increased ROS levels in these MitoPPX cells, the regulatory effects of the polymer on ROS have already been demonstrated in other organisms [62].

As previously mentioned, our data showed that ETC complexes I and IV were affected in MitoPPX cells. Interestingly, some authors have described deficiencies in the enzymatic activity of these same ETC complexes in neurodegenerative disorders, including AD and PD [57, 58, 69]. Additionally, some of these authors have evidenced an overall alteration of oxygen consumption as a consequence of these deficiencies in the ETC, which was also demonstrated by previous work conducted in HEK293 cells [35]. In the

context of neurodegenerative disorders, the effects of polyP in the dysregulation of complexes I and IV of the ETC could be further amplified by the action of the polymer on the amyloidogenic process [60, 64]. According to the literature, the lack of polyP could trigger increased toxicity of the amyloids, which could further affect the expression of ETC complexes I and IV, and thus the regulation of OXPHOS. However, further research should be conducted to specifically address the effects of the lack of mitochondrial polyP in a scenario that mimics amyloidosis, as well as the molecular mechanisms underlying the plausible observed effects in this scenario.

Thanks to our analysis, we were able to identify several upstream regulators of these changes in IPA analysis. For example, KDM5A was identified to regulate mitochondrial proteins (Figure 5A), confirming that the lack of polyP could be involved in the decrease of KDM5A leading to the decrease of ETC subunits (COX7A2-complex IV and NDUFA4-complex I), and the increase of catalase and TXN2 (both participating in the antioxidant defense). Interestingly, KDM5A is a well-known regulator of the expression of mitochondrial proteins, especially of those involved in mitochondrial respiration [70]. Furthermore, SIX1, a transcriptional factor described to regulate mitochondrial apoptosis [71], was predicted to lead to decreased levels of 2-oxoglutaric acid and increased ATP levels. These alterations could be the potential cause underlying the TCA abnormalities observed in MitoPPX cells. Finally, creatine was identified to upregulate ATP and phosphocreatine among others. Creatine is an endogenous chemical converted into phosphocreatine, which is used to produce new ATP under certain circumstances (i.e. during high-intensity exercise) [59]. Moreover, the protective role of creatine in maintaining mitochondrial physiology is also known [72], and it has been demonstrated that the dietary supplementation with this molecule could improve cellular bioenergetics and mitochondrial function, therefore decreasing neuronal cell death in neurodegeneration [73, 74]. In addition, IPA predicted three upregulated proteins, SIX1, UCP1, and HDAC 11, all of them involved in the upstream regulation of mitochondrial proteins and metabolites, as well as mitochondrial bioenergetics [75-80]. In general, the 'upstream regulators' analysis hinted at a cellular compensatory response in MitoPPX cells, in which

increased presence of proteins involved in cell stress response, including ATP generation, was present, when compared with the Wt samples.

This study has some limitations. Our data was not sufficient to clarify the exact molecular mechanisms by which mitochondrial polyP was involved in the regulation of bioenergetics within the organelle and therefore, in the regulation of mitochondrial physiology. In fact, the present proteomics and metabolomics approaches were not focused on the investigation of these mechanisms but to obtain a snapshot of mitochondrial metabolism. Further research is needed to better understand the role of polyP within mitochondria, especially in the context of neurodegeneration. However, this proteomics and metabolomics analysis bring new insights towards elucidating the role of polyP in the regulation of mitochondrial physiology in mammalian cells. Moreover, targeting the metabolism of polyP could provide us with novel therapeutic approaches for diseases where mitochondrial dysfunction has been broadly described as an early and triggering event, such as in neurodegenerative disorders.

AUTHOR'S CONTRIBUTION

Conceived and designed the study: MG-M, MM, DR, and MES. Collected the data: MG-M. Contributed data or analysis tools: MG-M, PU, FCN, JRA, VH, ES, GM, and MJP. Performed the analysis and wrote the paper: MG-M, FCN, JRA, MJP, and MES.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Dr. Maria E. Solesio Torregrosa (m.solesio@rutgers.edu). Proteomics data (skyline documents and raw files for DIA library generation and DIA sample analysis) has been deposited in Panorama Public (ProteomeXchange ID: PXD028185. Access URL: <https://panoramaweb.org/MitoPPX.url>). The full data set from the metabolomics assay can be found at Supplementary Information.

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TABLES

Table 1. List of the five altered proteins of the OXPHOS pathway in MitoPPX cells compared to Wt. ETC components show significant differences in their expression levels ($\log_2FC < 0$ means decreased expression and $\log_2FC > 0$ means increased expression) in MitoPPX cells, compared to Wt. Alterations in these proteins allowed the IPA software to predict a decrease in the OXPHOS system. The 'Expected' column indicates the state that protein is predicted to have if the pathway were activated. FDR: False Discovery Rate

Symbol	Gene Name	UniProt	Log2FC	FDR	Expected
COX6C	Cytochrome c oxidase subunit 6C	P09669	-2.67	2.40E-21	Up
COX7A2	Cytochrome c oxidase subunit 7A2	P14406	-2.31	1.89E-21	Up

COX7C	Cytochrome c oxidase subunit 7C	P15954	-1.54	2.78E-07	Up
NDUFA4	NDUFA4 mitochondrial complex associated	O00483	-2.00	3.82E-17	Up
SDHC	Succinate dehydrogenase complex subunit C	Q99643	2.61	3.38E-06	Up

Table 2. List of the four altered metabolites involved in the OXPHOS pathway in MitoPPX cells compared to Wt. Metabolites which are significantly different ($\log_2FC < 0$ means decreased expression and $\log_2FC > 0$ means increased expression) in MitoPPX cells compared to Wt. Alterations in these metabolites allowed the IPA software to predict a decrease in the OXPHOS system. The 'Expected' column indicates the state that protein is predicted to be in if the pathway were activated. FDR: False Discovery Rate

Symbol	Human Metabolome Database (HMDB)	Log2FC	FDR	Expected
Adenine-riboflavin dinucleotide	HMDB01248	-1.13	7.78E-05	Up
ATP	HMDB00538	0.91	2.69E-02	Up
NADH	HMDB01487	4.70	8.55E-06	Down

Succinic acid	HMDB00254	3.26	5.61E-13	Down
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Table 3. Analysis of upstream regulators generated from protein changes between MitoPPX and Wt cells identified using Pathway Analysis software.

The 11 predicted upstream regulators with a FDR set at 0.05 are listed below. The activation z-score can be used to infer likely activation states of the upstream regulators based on the direction of protein abundance change in the dataset, i.e. a negative activation z score indicates that the upstream regulator is downregulated in MitoPPX cells compared to Wt. NP indicates no prediction of activation status was generated by the IPA software.

Upstream Regulator	Activation z-score	FDR	Target molecules in dataset
APBB1	0.762	5.97E-03	ACTA2, EGFR, TAGLN, TYMS, VLDLR
CST5	-2.828	5.97E-03	ADSL, AHNAK, BRIX1, EXOC3, MALSU1, MSN, PDCL3, PITRM1, PPAN, PRKACB, ACTA2, EGFR, VIM
GLIPR2	NP	1.66E-02	ACTA2, EGFR, VIM
HNF4A	0.751	1.66E-02	AAMDC, ABCF3, ACTA2, AHNAK, ARFGAP1, ARFIP2, AS3MT, ASNS, C11orf58, CCDC25
miR-382-5p (miRNAs w/seed AAGUUGU)	NP	2.37E-02	SEPTIN3, TAGLN, VIM

MIR143-145a	NP	2.37E-02	ACTA2, DES, TAGLN
KDM5A	-0.632	4.31E-02	COQ7, COX7A2, GPT2, MCAT, MSTO1, NDUFA4, PDE3A, SDHC, TOMM22, TXN2
XAV939	NP	4.31E-02	AHNAK, CSRP1, DES, DHX36, IGFBP2
TP53	1.751	4.31E-02	A2M, ACOT11, ACTA2, ALDH1B1, ASNS, ATG7, BICD2, BID, CHEK1, CNN2
MED28	NP	4.85E-02	ACTA2, CNN2, TAGLN
miR-145-5p (and other miRNAs w/seed UCCAGUU)	-1.851	4.96E-02	ACTA2, AHNAK, C11orf58, CCDC25, NDUFA4, TAGLN, UNG

Table 4. List of the proteins and metabolites found altered in the mitochondrial dysfunction pathway in MitoPPX cells compared to Wt (meta-analysis). Table shows increase (Expr Log ratio) or decrease (-Expr Log Ratio) of different proteins and metabolites involved in the canonical pathway of mitochondrial dysfunction in the meta-analysis. FDR: False Discovery Rate

Symbol	Uniprot/Human Metabolome Database (HMDB)	Log2FC	FDR
Adenine-riboflavine dinucleotide	HMDB01248	-1.13	7.78E-05

ATP	HMDB00538	0.91	2.69E-02
COX6C	P09669	-2.67	2.40E-21
COX7A2	P14406	-2.31	1.89E-21
COX7C	P15954	-1.54	2.78E-07
Glutathione disulfide	HMDB03337	0.99	2.54E-03
NADH	HMDB01487	4.70	8.55E-06
NDUFA4	O00483	-2.00	3.82E-17
SDHC	Q99643	2.61	3.38E-06
TXN2	Q99757	2.45	1.76E-09

FIGURE LEGENDS

Figure 1. SH-SY5Y neuroblastoma cells overexpressing

exopolyphosphatase (PPX) enzyme. A) Significant Western Blot image confirming the expression of PPX (70 kDa together with eGFP protein) in the MitoPPX cells. **B)** Enzymatic assay demonstrating the activity of the PPX enzyme in our MitoPPX SH-SY5Y samples. After 12 hours of treatment with exogenous polyP, the levels of the polymer were decreased in MitoPPX cells, compared to Wt. These decreased levels were comparable to the levels seen in the positive control, which was conducted with alkaline phosphatase (AP), an enzyme known to degrade polyP [81]. Results are expressed as mean \pm SEM of three experiments.

Figure 2. Heat maps and principal component analysis (PCA) showed that SH-SY5Y Wt and MitoPPX have different proteomics and metabolomics profiles. Heat maps identified the top 25 proteins **(A)** and metabolites **(C)** detected in MitoPPX cells with respect to Wt cells, some of them related to the status of mitochondrial physiology. Red colors depict proteins or metabolites

that were upregulated in the MitoPPX cells and blue colors depict those downregulated in the same samples. PCA identified clear separation between groups for both proteomics **(B)** and metabolomics **(D)** datasets. Shaded areas depict regions with at least 95% confidence.

Figure 3. Canonical pathways identified using IPA analysis, based on proteins (A) and metabolites (B) that were identified using label-free quantification proteomics ($\log_2FC \geq 1.5$ and $FDR < 0.05$) and metabolomics ($FDR < 0.05$). The bars represent the significance of the canonical pathway as calculated by a right-sided Fisher's exact test (threshold is showing raw p-value < 0.05 for proteomics and raw p-value < 0.001 for metabolomics). Therefore, the tallest bars represent the canonical pathways that are the least likely to have been identified due to molecules being in the canonical pathway by random chance. Canonical pathways which are likely activated (based on the pattern of differentially abundant proteins or metabolites) are presented in orange (positive z-score) and pathways that are likely inhibited are presented in blue (negative z-score).

Figure 4. SH-SY5Y MitoPPX cells showed alterations in OXPHOS and in TCA. A) Decreased and increased protein expression of distinct subunits of the ETC complexes in MitoPPX cells compared to Wt. Results are expressed as mean \pm SEM. **B)** Increased expression of different metabolites involved in mitochondrial metabolism in MitoPPX cells compared to Wt. Results are expressed as mean \pm SEM. **C)** Alterations in the levels of succinate and 2-oxoglutarate metabolites in MitoPPX cells as well as in the levels of ATP and NADH metabolites lead to a dysfunction of TCA.

Figure 5. Upstream analysis using proteomics and metabolomics datasets showed potential up-regulators mediating the effects of the enzymatic depletion of mitochondrial polyP in SH-SY5Y MitoPPX cells. A) The proteomics analysis showed that KDM5A (lysine-specific demethylase 5A) is one of the first 10 upstream regulators, which is predicted to regulate different mitochondrial proteins such as COX7A2 subunit of complex IV and NDUFA4 subunit of complex I. **B)** The metabolomics analysis identified creatine, SIX1 (homeobox protein SIX1) and HDAC 11 (histone deacetylase 11) as upstream

molecules predicted to regulate different mitochondrial metabolites such as ATP, AMP, phosphocreatine, succinic acid and 2-oxoglutaric acid.

Figure 6. Alterations in several metabolites identified impacts on different mitochondrial pathways in SH-SY5Y MitoPPX cells. Respiration of mitochondria, dysfunction of mitochondria and permeabilization of mitochondria (all of them FDR<0.001) were the top 3 significantly affected mitochondrial-related pathways shown by 'disease and function' analysis in IPA software from the metabolomics data.

Figure 7. Mitochondrial dysfunction is predicted in SH-SY5Y MitoPPX cells (meta-analysis). Predicted up- and downstream pathways of molecules participating in mitochondrial function were performed using the 'MAP' option in IPA software. The decrease of some of the subunits of ETC complex I and IV together with an increase of some of the subunits of ETC complex II lead to an unbalanced functioning of OXPHOS. The prediction states an increase in the glutathione (GSH) and NADP⁺ levels (due to increase in GSSG and TRX2, respectively), probably as an attempt to counteract the potential oxidative stress consequence of a potential mitochondrial dysfunction.

Figure 8. Western Blotting corroborated the proteomics and metabolomics results. Significant Western Blotting showing the expression of different subunits of the ETC complexes in SH-SY5Y Wt and MitoPPX samples.

REFERENCES

1. Kornberg, A., N.N. Rao, and D. Ault-Riche, *Inorganic polyphosphate: a molecule of many functions*. Annu Rev Biochem, 1999. **68**: p. 89-125.
2. Kulakovskaya, T.V., et al., *Inorganic polyphosphates in mitochondria*. Biochemistry (Mosc), 2010. **75**(7): p. 825-31.
3. Kumble, K.D. and A. Kornberg, *Inorganic polyphosphate in mammalian cells and tissues*. J Biol Chem, 1995. **270**(11): p. 5818-22.
4. Bayev, A.Y., P.R. Angelova, and A.Y. Abramov, *Inorganic polyphosphate is produced and hydrolysed in F0F1-ATP synthase of mammalian mitochondria*. Biochem J, 2020.

5. McIntyre, B. and M.E. Solesio, *Mitochondrial inorganic polyphosphate (polyP): the missing link of mammalian bioenergetics*. Neural Regen Res, 2021. **16**(11): p. 2227-2228.
6. Borden, E.A., et al., *Is there a link between inorganic polyphosphate (polyP), mitochondria, and neurodegeneration?* Pharmacol Res, 2021. **163**: p. 105211.
7. Paradies, G., et al., *Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging*. Free Radic Biol Med, 2010. **48**(10): p. 1286-95.
8. Armstrong, J.A., et al., *Oxidative stress alters mitochondrial bioenergetics and modifies pancreatic cell death independently of cyclophilin D, resulting in an apoptosis-to-necrosis shift*. J Biol Chem, 2018. **293**(21): p. 8032-8047.
9. Judge, S. and C. Leeuwenburgh, *Cardiac mitochondrial bioenergetics, oxidative stress, and aging*. Am J Physiol Cell Physiol, 2007. **292**(6): p. C1983-92.
10. Yoo, N.G., et al., *Polyphosphate Stabilizes Protein Unfolding Intermediates as Soluble Amyloid-like Oligomers*. J Mol Biol, 2018. **430**(21): p. 4195-4208.
11. Solesio, M.E., et al., *Inorganic polyphosphate is required for sustained free mitochondrial calcium elevation, following calcium uptake*. Cell Calcium, 2020. **86**: p. 102127.
12. Solesio, M.E., et al., *Contribution of inorganic polyphosphate towards regulation of mitochondrial free calcium*. Biochim Biophys Acta, 2016. **1860**(6): p. 1317-25.
13. Angelova, P.R., et al., *Signal transduction in astrocytes: Localization and release of inorganic polyphosphate*. Glia, 2018. **66**(10): p. 2126-2136.
14. Maiolino, M., et al., *Inorganic Polyphosphate Regulates AMPA and NMDA Receptors and Protects Against Glutamate Excitotoxicity via Activation of P2Y Receptors*. J Neurosci, 2019. **39**(31): p. 6038-6048.
15. Morrissey, J.H., S.H. Choi, and S.A. Smith, *Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation*. Blood, 2012. **119**(25): p. 5972-9.
16. Muller, F., et al., *Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo*. Cell, 2009. **139**(6): p. 1143-56.
17. Kim, S., et al., *Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase*. Cell Metab, 2011. **13**(2): p. 215-21.
18. Amodeo, G.F., M.E. Solesio, and E.V. Pavlov, *From ATP synthase dimers to C-ring conformational changes: unified model of the*

- mitochondrial permeability transition pore*. Cell Death Dis, 2017. **8**(12): p. 1.
19. Suess, P.M., et al., *Extracellular polyphosphate signals through Ras and Akt to prime Dictyostelium discoideum cells for development*. J Cell Sci, 2017. **130**(14): p. 2394-2404.
 20. Abramov, A.Y., et al., *Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death*. Proc Natl Acad Sci U S A, 2007. **104**(46): p. 18091-6.
 21. Pavlov, E., et al., *A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction*. Biophys J, 2005. **88**(4): p. 2614-25.
 22. Pavlov, E., et al., *Inorganic polyphosphate and energy metabolism in mammalian cells*. J Biol Chem, 2010. **285**(13): p. 9420-8.
 23. Solesio, M.E., et al., *Inorganic polyphosphate (polyP) as an activator and structural component of the mitochondrial permeability transition pore*. Biochem Soc Trans, 2016. **44**(1): p. 7-12.
 24. McCormack, J.G., A.P. Halestrap, and R.M. Denton, *Role of calcium ions in regulation of mammalian intramitochondrial metabolism*. Physiol Rev, 1990. **70**(2): p. 391-425.
 25. Picard, M., et al., *An energetic view of stress: Focus on mitochondria*. Front Neuroendocrinol, 2018. **49**: p. 72-85.
 26. Baltanas, A., et al., *The senescence-accelerated mouse prone-8 (SAM-P8) oxidative stress is associated with upregulation of renal NADPH oxidase system*. J Physiol Biochem, 2013. **69**(4): p. 927-35.
 27. Zheng, X., et al., *Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation*. Elife, 2016. **5**.
 28. Schon, E.A. and S. Przedborski, *Mitochondria: the next (neurode)generation*. Neuron, 2011. **70**(6): p. 1033-53.
 29. Lin, M.T. and M.F. Beal, *Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases*. Nature, 2006. **443**(7113): p. 787-95.
 30. Koopman, W.J., et al., *OXPHOS mutations and neurodegeneration*. EMBO J, 2013. **32**(1): p. 9-29.
 31. Kudryavtseva, A.V., et al., *Mitochondrial dysfunction and oxidative stress in aging and cancer*. Oncotarget, 2016. **7**(29): p. 44879-44905.
 32. Cai, Q. and P. Tammineni, *Alterations in Mitochondrial Quality Control in Alzheimer's Disease*. Front Cell Neurosci, 2016. **10**: p. 24.

33. Joshi, A.U. and D. Mochly-Rosen, *Mortal engines: Mitochondrial bioenergetics and dysfunction in neurodegenerative diseases*. *Pharmacol Res*, 2018. **138**: p. 2-15.
34. Singh, A., et al., *Oxidative Stress: A Key Modulator in Neurodegenerative Diseases*. *Molecules*, 2019. **24**(8).
35. Solesio, M.E., et al., *Depletion of mitochondrial inorganic polyphosphate (polyP) in mammalian cells causes metabolic shift from oxidative phosphorylation to glycolysis*. *Biochem J*, 2021. **478**(8): p. 1631-1646.
36. Xicoy, H., B. Wieringa, and G.J. Martens, *The SH-SY5Y cell line in Parkinson's disease research: a systematic review*. *Mol Neurodegener*, 2017. **12**(1): p. 10.
37. Solesio, M.E., et al., *3-Nitropropionic acid induces autophagy by forming mitochondrial permeability transition pores rather than activating the mitochondrial fission pathway*. *Br J Pharmacol*, 2013. **168**(1): p. 63-75.
38. Solesio, M.E., et al., *Characterization of mitophagy in the 6-hydroxydopamine Parkinson's disease model*. *Toxicol Sci*, 2012. **129**(2): p. 411-20.
39. Solesio, M.E., et al., *Carbonic anhydrase inhibition selectively prevents amyloid beta neurovascular mitochondrial toxicity*. *Aging Cell*, 2018. **17**(4): p. e12787.
40. Fossati, S., et al., *The carbonic anhydrase inhibitor methazolamide prevents amyloid beta-induced mitochondrial dysfunction and caspase activation protecting neuronal and glial cells in vitro and in the mouse brain*. *Neurobiol Dis*, 2016. **86**: p. 29-40.
41. Aschar-Sobbi, R., et al., *High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach*. *J Fluoresc*, 2008. **18**(5): p. 859-66.
42. Khong, M.L., et al., *Inorganic polyphosphate controls cyclophilin B-mediated collagen folding in osteoblast-like cells*. *FEBS J*, 2020. **287**(20): p. 4500-4524.
43. Castro, C.M., et al., *Adenosine A2A receptor (A2AR) stimulation enhances mitochondrial metabolism and mitigates reactive oxygen species-mediated mitochondrial injury*. *FASEB J*, 2020. **34**(4): p. 5027-5045.
44. Amodei, D., et al., *Improving Precursor Selectivity in Data-Independent Acquisition Using Overlapping Windows*. *J Am Soc Mass Spectrom*, 2019. **30**(4): p. 669-684.
45. Gessulat, S., et al., *Prosit: proteome-wide prediction of peptide tandem mass spectra by deep learning*. *Nat Methods*, 2019. **16**(6): p. 509-518.

46. Pino, L.K., et al., *Acquiring and Analyzing Data Independent Acquisition Proteomics Experiments without Spectrum Libraries*. Mol Cell Proteomics, 2020. **19**(7): p. 1088-1103.
47. Kall, L., et al., *Assigning significance to peptides identified by tandem mass spectrometry using decoy databases*. J Proteome Res, 2008. **7**(1): p. 29-34.
48. Mathon, C., et al., *Impact of sample preparation upon intracellular metabolite measurements in 3D cell culture systems*. Metabolomics, 2019. **15**(6): p. 92.
49. Meador, J.P., et al., *Metabolomic profiling for juvenile Chinook salmon exposed to contaminants of emerging concern*. Sci Total Environ, 2020. **747**: p. 141097.
50. Wei, R., et al., *Missing Value Imputation Approach for Mass Spectrometry-based Metabolomics Data*. Sci Rep, 2018. **8**(1): p. 663.
51. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic Acids Res, 2015. **43**(7): p. e47.
52. Smyth, G.K., *Linear models and empirical bayes methods for assessing differential expression in microarray experiments*. Stat Appl Genet Mol Biol, 2004. **3**: p. Article3.
53. Pang, Z., et al., *MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights*. Nucleic Acids Res, 2021. **49**(W1): p. W388-W396.
54. Wang, X., et al., *Oxidative stress and mitochondrial dysfunction in Alzheimer's disease*. Biochim Biophys Acta, 2014. **1842**(8): p. 1240-7.
55. Abou-Sleiman, P.M., M.M. Muqit, and N.W. Wood, *Expanding insights of mitochondrial dysfunction in Parkinson's disease*. Nat Rev Neurosci, 2006. **7**(3): p. 207-19.
56. Cui, H., Y. Kong, and H. Zhang, *Oxidative stress, mitochondrial dysfunction, and aging*. J Signal Transduct, 2012. **2012**: p. 646354.
57. Gonzalez-Casacuberta, I., et al., *Mitochondrial and autophagic alterations in skin fibroblasts from Parkinson disease patients with Parkin mutations*. Aging (Albany NY), 2019. **11**(11): p. 3750-3767.
58. Gonzalez-Casacuberta, I., et al., *Transcriptional alterations in skin fibroblasts from Parkinson's disease patients with parkin mutations*. Neurobiol Aging, 2018. **65**: p. 206-216.
59. Guzun, R., et al., *Systems bioenergetics of creatine kinase networks: physiological roles of creatine and phosphocreatine in regulation of cardiac cell function*. Amino Acids, 2011. **40**(5): p. 1333-48.

60. Cremers, C.M., et al., *Polyphosphate: A Conserved Modifier of Amyloidogenic Processes*. Mol Cell, 2016. **63**(5): p. 768-80.
61. Dahl, J.U., M.J. Gray, and U. Jakob, *Protein quality control under oxidative stress conditions*. J Mol Biol, 2015. **427**(7): p. 1549-63.
62. Gray, M.J. and U. Jakob, *Oxidative stress protection by polyphosphate--new roles for an old player*. Curr Opin Microbiol, 2015. **24**: p. 1-6.
63. Gray, M.J., et al., *Polyphosphate is a primordial chaperone*. Mol Cell, 2014. **53**(5): p. 689-99.
64. Lempart, J. and U. Jakob, *Role of Polyphosphate in Amyloidogenic Processes*. Cold Spring Harb Perspect Biol, 2019. **11**(5).
65. Xie, L. and U. Jakob, *Inorganic polyphosphate, a multifunctional polyanionic protein scaffold*. J Biol Chem, 2019. **294**(6): p. 2180-2190.
66. Rao, N.N., M.R. Gomez-Garcia, and A. Kornberg, *Inorganic polyphosphate: essential for growth and survival*. Annu Rev Biochem, 2009. **78**: p. 605-47.
67. Rao, N.N. and A. Kornberg, *Inorganic polyphosphate supports resistance and survival of stationary-phase Escherichia coli*. J Bacteriol, 1996. **178**(5): p. 1394-400.
68. Rao, N.N. and A. Kornberg, *Inorganic polyphosphate regulates responses of Escherichia coli to nutritional stringencies, environmental stresses and survival in the stationary phase*. Prog Mol Subcell Biol, 1999. **23**: p. 183-95.
69. Moran, M., et al., *Mitochondrial respiratory chain dysfunction: implications in neurodegeneration*. Free Radic Biol Med, 2012. **53**(3): p. 595-609.
70. Varaljai, R., et al., *Increased mitochondrial function downstream from KDM5A histone demethylase rescues differentiation in pRB-deficient cells*. Genes Dev, 2015. **29**(17): p. 1817-34.
71. Du, P., et al., *Sine Oculis Homeobox Homolog 1 Regulates Mitochondrial Apoptosis Pathway Via Caspase-7 In Gastric Cancer Cells*. J Cancer, 2017. **8**(4): p. 636-645.
72. Barbieri, E., et al., *Creatine Prevents the Structural and Functional Damage to Mitochondria in Myogenic, Oxidatively Stressed C2C12 Cells and Restores Their Differentiation Capacity*. Oxid Med Cell Longev, 2016. **2016**: p. 5152029.
73. Adihetty, P.J. and M.F. Beal, *Creatine and its potential therapeutic value for targeting cellular energy impairment in neurodegenerative diseases*. Neuromolecular Med, 2008. **10**(4): p. 275-90.

74. Smith, R.N., A.S. Agharkar, and E.B. Gonzales, *A review of creatine supplementation in age-related diseases: more than a supplement for athletes*. F1000Res, 2014. **3**: p. 222.
75. Hurtado, E., et al., *HDAC11 is a novel regulator of fatty acid oxidative metabolism in skeletal muscle*. FEBS J, 2021. **288**(3): p. 902-919.
76. Bhaskara, S., *Histone deacetylase 11 as a key regulator of metabolism and obesity*. EBioMedicine, 2018. **35**: p. 27-28.
77. Fedorenko, A., P.V. Lishko, and Y. Kirichok, *Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria*. Cell, 2012. **151**(2): p. 400-13.
78. Kazak, L., et al., *A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat*. Cell, 2015. **163**(3): p. 643-55.
79. Yang, Z., et al., *microRNA-488 inhibits chemoresistance of ovarian cancer cells by targeting Six1 and mitochondrial function*. Oncotarget, 2017. **8**(46): p. 80981-80993.
80. Chowdhury, S.R., et al., *Targeted apoptosis in ovarian cancer cells through mitochondrial dysfunction in response to Sambucus nigra agglutinin*. Cell Death Dis, 2017. **8**(5): p. e2762.
81. Lorenz, B. and H.C. Schroder, *Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase*. Biochim Biophys Acta, 2001. **1547**(2): p. 254-61.