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Polydatin reactivates mitochondrial bioenergetics and mitophagy while preventing premature senescence by modulating microRNA-155 and its direct targets in human fibroblasts with trisomy 21

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ABSTRACT

Mitochondrial dysfunction and redox dyshomeostasis are considered crucial factors causally linked to the pathogenesis of Down syndrome (DS), a human genetic anomaly currently lacking a cure, associated with neurodevelopmental deficits in children and early onset symptoms of aging in adults. Several natural plant-derived polyphenolic compounds, known for their neurostimulator, antioxidant and anti-inflammatory activities, have been proposed as dietary supplements to manage DS-linked phenotypic alterations. However, the poor bioavailability and rapid metabolism of these compounds have limited conclusive evidence regarding their clinical efficacy in individuals with DS. Polydatin (PLD), a natural polyphenolic glucoside precursor of resveratrol derived from *Polygonum cuspidatum*, is instead highly bioavailable and resistant to enzymatic oxidation. PLD supplementation has shown many therapeutic efficacies in several human diseases without side effects. In this study, we used fetal trisomy 21 human skin fibroblasts (DS-HSFs) to investigate, from a mechanistic point of view, whether PLD supplementation could prevent or counteract critical cellular alterations linked to both neurodevelopmental deficits and early aging in DS. Our findings demonstrate that PLD reactivates mitochondrial bioenergetics, reduces oxygen radical overproduction and prevents oxidative stress (OS)-induced cellular senescence and DNA damage in DS-HSF. Notably, we identified a novel mechanism of PLD action involving the chromosome-21-encoded microRNA-155 (miR-155) and its direct target genes casitas B-lineage lymphoma (CBL), BAG Cochaperone 5 (BAG5) and mitochondrial transcription factor A (TFAM). These proteins play pivotal roles in regulating mitochondrial bioenergetics, biogenesis and mitophagy. Given that the deregulation of miR-155/CBL axis is also implicated in acute leukemias, which frequently occur in children with DS, PLD emerges as a promising candidate for translational application. Its ability to enhance mitochondrial bioenergetics and address critical DS-associated phenotypic alterations highlights its therapeutic potential.

1. Introduction

Down syndrome (DS, OMIM #190685) due to the whole or partial third copy of human chromosome 21 (Hsa21) [1], is the most common genetic disease linked to neurodevelopmental abnormalities, neuroinflammation, neurobehavioral illnesses, early aging and

Abbreviations: BAG5, BAG Cochaperone 5; CBL, casitas B-lineage lymphoma; CREB, cyclic AMP response element binding protein; DCF, dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescein diacetate; 7,8-DHF, 7,8-dihydroxyflavone; DMSO, dimethyl sulfoxide; DS, Down syndrome; DS-HSFs, DS human skin fibroblasts; EGCG, epigallocatechin-3 gallate; DYRK1A, dual specificity tyrosine-phosphorylation-regulated kinase 1A; G6P-DH, glucose 6-phosphate dehydrogenase; HK, hexokinase; H_2O_2, hydrogen peroxide; Hsa21, human chromosome 21; HSFs, human skin fibroblasts; miR-155, microRNA-155; N-HSFs, normal human skin fibroblasts; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; OLIGO, oligomycin; OS, oxidative stress; OXPHOS, oxidative phosphorylation; PLD, polydatin; ROS, reactive oxygen species; RSV, resveratrol; SA-β-gal, senescence β-galactosidase; TFAM, mitochondrial transcription factor A.

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neurodegeneration (for refs see reviews [2–4]). According to the World Health Organization, the estimated DS incidence ranges from 1 in 1000 to 1 in 1100 live births globally. This translates to approximately 3000–5000 babies born with Down syndrome each year.

Dysfunctional mitochondrial bioenergetics represents a critical factor involved in DS-related both neurodevelopmental impairments in children and early aging and neurodegeneration in adults (for refs see Refs. [5–7]). Since fetal stage and in neonatal age, mitochondrial bioenergetic deficits have been reported to be broadly present in a variety of Hsa21 trisomy cells, including both peripheral cells such as fibroblasts and lymphocytes, and central nervous cells such as neurons and astrocytes [5–8], as well as brain tissues [9,10]. In adult population with DS, aberrant mitochondrial energy metabolism and oxidative stress (OS) lead to an increased vulnerability to a wide spectrum of diseases such as cardiomyopathy, Alzheimer disease and early aging [11–13]. In all cases, mitochondrial bioenergetic dysfunction is due to dysregulation of key pathways controlling mitochondrial function leading to severe shortage in oxidative phosphorylation (OXPHOS)-dependent ATP production and deficits in total brain energy status, as well as overproduction of reactive oxygen species (ROS) [2].

The plant kingdom is currently a large source of natural secondary metabolites with the potential to target multiple dysregulated pathways in various diseases. Among them, plant polyphenols are highly bioactive molecules with neurostimulator, antioxidant and anti-inflammatory activities (for refs see Refs. [14–16]), therefore, deeply investigated as potential therapeutics to correct or delay the pathological clinical symptoms associated with energy deficit, OS and neuroinflammation in DS [17]. Indeed, polyphenols have a multimodal action in targeting proteins and pathways, found to be affected by Hsa21 trisomy. Studies from our and other research teams have showed both in cellular and mouse models of DS that, the flavonoids epigallocatechin-3 gallate (EGCG), resveratrol (RSV) and 7,8-dihydroxyflavone (7,8-DHF), were able to target Sirt 1/PGC-1α/AMPK pathways [9,18,19], to modulate expression and activity of the dual specificity tyrosine-phosphorylation-regulated kinase 1 A (DYRK1A) [20], the cyclic AMP response element binding protein (CREB) [21] and the mitochondrial transcription factor A (TFAM) [19], restoring the cellular and brain levels of ATP and decreasing mitochondrial ROS production and OS, with a subsequent full restoring of cellular metabolism and hippocampal neurogenesis.

Remarkably, the polyphenol RSV has been characterized as natural modulator of a specific Hsa21-encoded microRNA, the miR-155–5p [22–24], and in DS, overexpression of the Hsa21-encoded miR-155–5p has been reported in several cell types [25–28]. The miR-155 regulates numerous genes, including some key genes related to mitochondria functions; indeed, the nuclear TFAM, a key regulator of mitochondrial biogenesis [29], the BAG cochaperone 5 (BAG5), involved in the mitophagy process [30] and the Casitas B-lineage lymphoma (CBL), recently shown to be essential for mitochondrial bioenergetic efficiency [31], are direct target genes of miR-155 [30–32]. Therefore, miR-155 normalization by RSV could be a promising approach to prevent mitochondrial alterations and other metabolic changes observed in DS. However, like most polyphenols, the RSV limited bioavailability and a quick metabolism and substantial modifications during its systemic absorption [33] are main disadvantages in taking supplements containing RSV, thus impacting its clinical efficacy in humans. Unlike RSV, its natural glucoside (trans-resveratrol-3-O-glucoside), the stilbenoid polydatin (PLD) results to be water-soluble and highly bioavailable, since penetrates cells by active mechanism mediated by the glucose carrier showing a higher antioxidant and anti-inflammatory activity compared to RSV [34]. Countless biological activities of PLD have been reported, including antitumor, cardioprotective, anti-inflammatory and neuroprotective effects (for refs see Refs. [34,35]). Recently, Emili and coworkers, at our suggestion, have tested the effect of PLD in a mouse model of DS showing a reversal of the neurogenesis impairment [36].

In this work, we evaluated the potential of PLD, the bioavailable form of RSV, to counteract mitochondrial energy deficit, oxidative stress and senescence in cultured human fibroblasts with trisomy 21. Our findings reveal novel pathogenic deregulations in DS involving the miR-155/BAG5/PINK1 and miR-155/CBL pathways and demonstrate a new pharmacological action of polydatin through the modulation of miR-155.

2. Materials and methods

2.1. Cell cultures and PLD treatment

Human phenotypically characterized fetal skin fibroblasts (HSFs), five lines for each genotype i.e. euploid and chromosome 21 trisomy, established from fetuses spontaneously aborted at a gestational age between 14 and 18 weeks, were obtained from the Biobank of the Laboratory of Human Genetics – IRCCS Istituto Gaslini, member of the Network Telethon of Genetic Biobanks and by Euro-BioBank Network [37]. The Gaslini genetic biobank operates in agreement with ethical guidelines stated in the Technical Governance Board (TGB) Network Charter with informed consent obtained from the guardians. The fibroblast cell lines were cultured using standard incubation conditions at 37 °C, 5 % CO_2, and 95 % relative humidity in RPMI 1640 medium supplemented with 15 % heat-inactivated fetal bovine serum (GIBCO/BRL), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/mL) (GIBCO/BRL). Adherent fibroblasts were subjected to a 1:3 split and used at a comparable number of passages between euploid normal (N-HSFs) and trisomic (DS-HSFs) human skin fibroblasts, ranging from 5 to 12 for all experiments, except for those of replicative senescence (>25 passage numbers).

Polydatin from *Polygonum cuspidatum* (≥95 % purity) obtained from Sigma-Aldrich was dissolved in dimethyl sulfoxide (DMSO) at 80 mM stock, stored at −20 °C and diluted in culture medium prior the use. An equal volume of DMSO was used for experiments considered as vehicle (veh). Through preliminary tryouts of cytotoxicity, performed incubating DS-HSF lines 48 h after seeding, with increasing concentration of PLD (in the range 5–100 μM) for 24 h (See Fig. 1A and B), we identified optimal the 20 μM PLD concentration and used for all experiments.

2.2. Cell proliferation and viability assay

Cell proliferation assay was performed by using 4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate reagent (WST-1, Cell Proliferation kit, Sigma-Aldrich). 6000 cells per well were seeded into a 96-well plate and incubated overnight at 37 °C in a CO_2 incubator to allow adherence. To assay cell proliferation, 100 μL of 1/10 WST-1 reagent diluted in the culture medium, were added to each well and the plate incubated at 37 °C for 2–4 h following the manufacturer instructions. Formazan dye accumulation produced by metabolically active cells was monitored by reading absorbance at 450 nm, with a reference wavelength at 600 nm, by using the microplate reader mod. 680 (Biorad).

2.3. Measurement of mitochondrial ATP production rate

The rate of mitochondrial ATP production by OXPHOS was determined in digitonin-permeabilized cells, essentially as previously described [19]. Briefly, aliquots of trypsinized fibroblasts (0.5 mg protein), washed with PBS, were incubated at 37 °C in 2 mL of respiratory medium consisting of 210 mM mannitol, 70 mM sucrose, 20 mM TRIS/HCl, 5 mM KH_2PO_4/K_2HPO_4, (pH 7.4), 3 mM $MgCl_2$ plus BSA (5 mg/mL) in the presence of the ATP detecting system (ATP-ds) consisting of glucose (2.5 mM), hexokinase (HK) (2 e. u.), glucose 6-phosphate dehydrogenase (G6P-DH) (1 e. u.) and $NADP^+$ (0.25 mM) and by adding glutamate plus malate (5 mM each) or 5 mM succinate in the presence of rotenone (3 μM) as energy sources, plus 10 μM diadenosine pentaphosphate (Ap5A), used to specifically inhibit adenylate kinase.

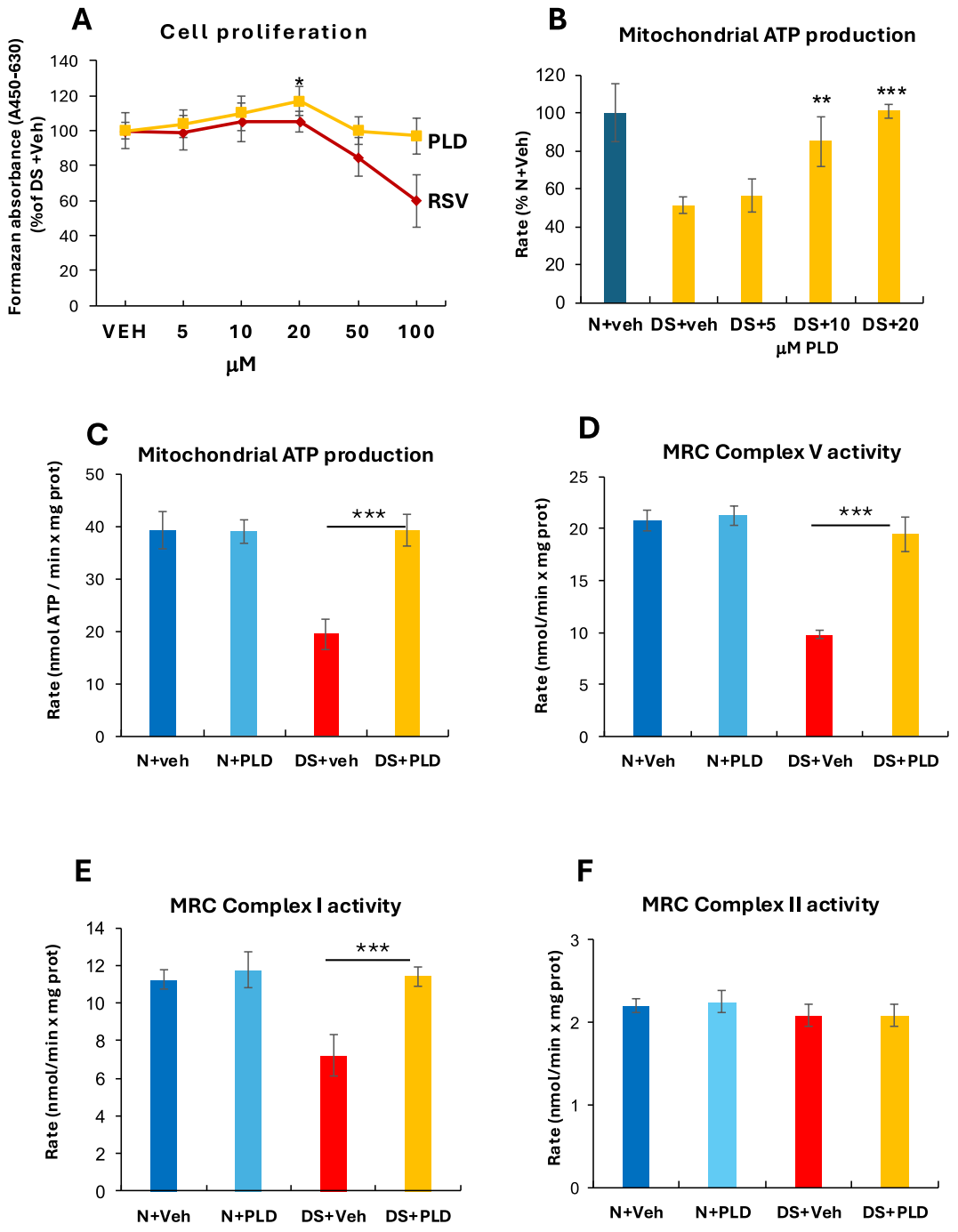

Fig. 1. PLD restores OXPHOS impairment in DS cells. Dose-dependence effect on (A) cell proliferation and (B) mitochondrial ATP production in euploid (N) and trisomic (DS) HSFs (n = 3) incubated for 24 h with increasing dose of PLD or the same volume of DMSO as vehicle (N + veh; DS + veh). (C) Mitochondrial ATP production rate, and MRC activity of (D) ATPase (complex V), (E) complex I, (F) complex II, measured in both euploid N- and DS-HSFs (n = 5) incubated for 24 h with 20 μM PLD or vehicle. Data are mean ± SEM. Significant differences, calculated with one-way ANOVA and Turkey's *t*-test, are indicated with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

After 5 min of incubation with 0.01 % (w/v) digitonin, the reduction of $NADP^+$ in the extramitochondrial phase, which reveals ATP formation from externally added ADP (0.5 mM), was monitored as an increase in absorbance at 340 nm. As a control, the ATP synthase inhibitor oligomycin (OLIGO, 5 μg/10 μL) was added in course of reaction to show the inhibition of the mitochondrial ATP production. Great attention was made to use enough HK/G6P-DH coupled enzymes to ensure a non-limiting ADP-regenerating system for the detection of ATP production.

2.4. Measurement of mitochondrial respiratory chain (MRC) complex activities

Aliquots of trypsinized fibroblasts (1–2 x 10^6 cells) were washed with ice-cold PBS and kept at −80 °C until use. The MRC complex activities were measured spectrophotometrically in mitochondrial membrane-enriched fraction obtained by suspending HSF pellets in 1 mL of 10 mM TRIS-HCl (pH 7.5) supplemented with 1 mg/ml BSA and exposed to ultrasound energy for 10 s at 4 °C (11 pulses 0.7 s on, 0.7 s off) at 20 kHz,

intensity 2. The ultrasound-treated cells were centrifuged (10 min at 600 g, 4 °C). The supernatant was collected and centrifuged again (10 min at 14000 g, 4 °C) to obtain a mitochondrial pellet that was suspended in 0.5 mL of the respiratory medium. The activities of NADH: ubiquinone oxidoreductase (complex I), ATPase-dependent ATP hydrolase (complex V) and succinate dehydrogenase (complex II) were measured by two assays which rely on the sequential addition of reagents to measure the activities of complexes I and V, and complex II, respectively, essentially as described in Refs. [38,39].

2.5. Measurements of intracellular ROS and OS status

Quantitative analysis of intracellular ROS was performed at basal condition or after hydrogen peroxide (H_2O_2)-induced OS. For OS induction, cells were plated in 5-cm cell dish, treated with 20 μM PLD or vehicle for 24 h followed by 50 μM H_2O_2 treatment for 1 h in incubator. Afterward, cell culture medium was removed, cell washed, added fresh cell culture medium and allowed to recover for 1 day. Cultured cells at basal condition or after OS induction, were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) for 30 min in growth conditions, washed and suspended in PBS. DCFH-DA is a non-fluorescent dye which is hydrolyzed in cells and reacts with multiple types of ROS, mainly H_2O_2, to give the fluorescent product, dichlorofluorescein (DCF) [40]. Quantitative analysis of intracellular ROS was measured by means of the LS50 PerkinElmer spectrofluorimeter using fluorescence recorded at λex 488 nm and λem 520 nm and through fluorescent cell counting by Countess Automated Cell Counter (ThermoFiscer Scientific) normalized to cell total number to determine the relative ROS production.

Cell OS status was measured through DNA oxidative damage examination in the culture medium by using DNA Damage Competitive ELISA Kit (Invitrogen, Carlsbad, CA, USA), which determines DNA damage by measuring the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a ubiquitous marker of oxidative stress, released in the culture medium. Cell culture media obtained from the different experiments used for measuring intracellular ROS, was transferred to an Eppendorf tube, centrifuged at 14,000×*g* and kept at −20 °C until assays. A 1:5 dilution in culture medium was used for the samples; standards ranging from 62.6 to 8000 pg/mL was prepared by serial dilution in culture medium. In brief, 50 μL of standard samples or cell culture media were added to appropriate wells of the 96 well plate pre-coated with goat anti-rabbit IgG, with 25 μL of 8-OHdG and 25 μL of 8-OHdG antibody. The plate was incubated for 2 h at room temperature with shaking, washed and incubated with 100 μL TMB substrate according to the manufacturer's instructions. Then, 50 μL of Stop Solution was added to each well and the absorbance at 450 nm was read by using the microplate reader. The concentration of 8-OHdG for each sample derived by the interpolation of 8-OHdG absorbance in the polynomial curve for the standards by using a four-parameter logistic regression model. The experiments were repeated at least three times.

2.6. In situ senescence-associated β-galactosidase assay

Cellular senescence was assessed in HSFs, both at basal (at 20–25 passage numbers) or after H_2O_2 induction of oxidative stress of HSFs at normal culture passages (ranging from 5 to 12), by using the senescence β-galactosidase (SA-β-gal) staining kit (Cell signaling, #9860). Briefly, 8 × 10^4 fibroblasts were seeded, cultured in 6-well plate, subjected to different treatments and allowed to recover for 2 days. After washing with PBS three times, HSFs were fixed and then incubated with the SA-β-gal staining solution at 37 °C overnight in a dry incubator without CO_2 following the manufacturer instructions. Finally, SA-β-gal-positive cells, stained blue, were randomly imaged. The percentage of senescent HSFs was evaluated by the ratio of SA-β-gal-positive cells to the total number of HSFs obtained from 20 different fields of view. The experiments were repeated at least three times.

2.7. Confocal imaging

Fibroblasts (10^5 cells/well) were seeded in Nunc™ Glass Bottom Dishes (ThermoFisher Scientific cat. #150682) and allowed to adhere overnight. Cells were treated with 20 μM PLD or DMSO vehicle for 24-h.

Mitophagy was monitored in live fibroblasts by co-label cells for at least 24-h at 37 °C with CellLight™ Mitochondria-GFP mitochondrial probe (ThermoFisher Scientific cat. #C10600) and PremoTM autophagy sensor LC3B-RFP (ThermoFisher Scientific cat. #P36236) by adding 30 μL of each BacMan 2.0 probes (3 μL per 10^4 cells) directly in the culture medium, following the manufacturer's instructions. The cells were imaged using a Leica Stellaris 5 laser scanning confocal microscope DMi8 (images collected using a 60 × objective). Mitochondria were visualized by exciting the sample setting the laser for FITC (λex 485 nm) and green fluorescence emitted; autophagy was visualized by exciting the sample with the laser for TRITC (λex 555 nm). Colocalization of green and red LC3 puncta fluorescence per cells, which gives a measure of mitophagy, were determined using JACoP Plug-In on FiJi (NIH) software.

2.8. Western blotting

HSFs cell lysate was obtained scraping cultured cells washed with PBS, with 0.1 % Triton in PBS plus a protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The lysate, placed in Eppendorf tube, is sonicated for 10 s at 4 °C (continuous pulses) at 20 kHz, intensity 2, then centrifuged at 14,000 g at 4 °C for 15 min to remove cellular debris. The total protein concentration of the supernatants was determined by the Bradford dye-binding method with BioRad Protein assay kit (BioRad Laboratories). 10 μg of proteins were resolved on a NovexTM 4–20 % or 10 % Tris-glycine Plus WedgeWell™ gel (Thermo Fisher Scientific) in Tris-Glycine SDS Running buffer (Novex, Life Thechologies). After electrophoresis, the proteins were transferred onto PVDF membranes *via* the iBlotTM 3 Western Blot Transfer System using iBlotTM 3 Transfer Stack (Thermo Fisher Scientific). Immediately after transfer, the membranes were stained with No-Stain™ Protein Labeling Reagent kit (Thermo Fisher Scientific) according to the manufacturer's instructions and then placed into a Chemi-Doc MP imaging System (Bio-Rad Laboratories) to collect total protein load images with Image Lab Software (Bio-Rad Laboratories). Membranes were blocked in TBS-T (50 mM Tris, 150 mM NaCl, 0.01 % Tween 20, pH 7.5) containing 3 % BSA and probed with the following primary antibodies overnight at 4 °C: *anti*-TFAM (1:1000 dilution, Abcam # AB47517); anti- BAG5 [N1N3] (1:1000 dilution GenTex); anti-PINK1 [D8G3] (1:1000 dilution, Cell Signaling). For CBL antibody, membranes were blocked in TBS-T containing 5 % nonfat dry milk and proved with *anti*-CBL antibody [YE323] (1:1000 dilution, Abcam). Immunoblot analysis was performed, using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1–20000 in TBS-T, Thermo Fisher Scientific) 1 h at room temperature. Signals were detected with SuperSignal West Pico substrate (Thermo Fisher Scientific) then acquired with Chemi-Doc MP imaging System and analyzed using Image Lab software (Bio-Rad, Laboratories). Densitometry value of immunoreactive bands for each sample was normalized versus the corresponding densitometry value of total protein lanes.

2.9. Quantitative Real-Time polymerase chain reaction (qRT-PCR) analysis

Total and small RNAs were extracted from euploid and T21 fetal skin fibroblasts (HSFs) using the protocol of Appendix D of the RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Purified RNA was quantified using NanoDrop 2000 Spectrophotometer and RNA quality was determined by running on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA.

cDNA synthesis of 500 ng of mRNA transcripts was obtained

according to the protocol of the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Reverse transcription of micro-RNAs was performed using the TaqMan Advanced miRNA Assays (ThermoFisher Scientific, Waltham, Massachusetts, United States) with 10 ng of total RNA per reaction, following the manufacturer instructions.

RT-qPCR experiments were performed in duplicate on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, United States) using the TaqMan™ Fast Advanced Master Mix. The TaqMan™ Gene Expression Assay (FAM)probes (Thermo Fisher) were used for the amplification of β-Actin (Hs99999903_m1), GADPH (Hs99999905_m1), RPL13 (Hs00744303_s1), HPRT1 (Hs03929098_m1), TFAM (Hs00273372_s1), CBL (Hs01011446_m1) and BAG5 (Hs01921361_s1) transcripts. The TaqMan™ Advanced miRNA Assay probes were used for the amplification of has-miR-155–5p (483,064_mir), has-miR-16–5p (477,860_mir), has-miR-191–5p (477,052_mir) and has-miR-24–3p (477,992_mir). Results were first analyzed in SDS 2.2.1 software and then the Reffinder tool [41] was used to identify among at least three different genes, the most stable housekeeping gene to normalize the target signal.

2.10. Statistics

Data were analyzed with the GraphPad Prism software 10.4.1 version. Datasets were analyzed for significance using either a one-way analysis of variance (ANOVA) or a two-way ANOVA with genotype (N-HSF vs DS-HSF) and treatment (vehicle vs PD) as independent variables. Post hoc comparisons were subsequently performed by Tukey's test. When comparing only two independent samples as genotypes or treatments separately, post hoc comparisons were performed by Student's T-test. A probability level of $p < 0.05$ has been considered to be statistically significant. Results are presented as mean ± standard error of the mean (SEM, bar plots).

3. Results

3.1. PLD restores mitochondrial OXPHOS deficit in DS- HSFs

We tested the potential of PLD, the bioavailable form of RSV, to counteract mitochondrial OXPHOS deficit found in DS cells [42,43]. We first checked the optimal concentration for PLD treatment of fibroblasts through dose-dependence experiments and found that treatment of DS human skin fibroblasts (DS-HSFs) for 24 h with the PLD concentration of 20 μM slightly but significantly increased (+17 %) cell proliferation (Fig. 1A), completely restoring the deficit of mitochondrial ATP production (Fig. 1B). Unlike RSV, its glucoside precursor, PLD was not cytotoxic, also at very high concentrations (Fig. 1A). PLD concentrations lower than 20 μM were not fully effective in the prevention of mitochondrial energy impairment in DS cells (Fig. 1B).

Treatment of DS-HSFs with 20 μM PLD for 24 h, beside the complete restoring of the efficiency of OXPHOS-dependent ATP synthesis similar to N-HSFs, (2-fold increase of mitochondrial ATP synthesis rate in DS + PLD *vs* DS + veh; Fig. 1C), significantly rescued the deficit of mitochondrial ATPase (complex V) (+97 % DS + PLD *vs* DS + veh) and complex I (+58 % DS + PLD *vs* DS + veh) activities (Fig. 1D and E) restabling values comparable than those measured in euploid N-HSFs. No significant differences were found when measuring the Complex II activities (Fig. 1F) and when comparing untreated and PLD-treated euploid normal cells for any mitochondrial activities (Fig. 1D–F).

3.2. PLD treatment significantly reduces mitochondrial ROS production and senescence and prevents damages due to H_2O_2-induced oxidative stress in DS-HSFs

We checked the capability of PLD to counteract ROS overproduction and oxidative stress in DS cells. 24 h cell treatment with 20 μM PLD strongly counteracted ROS overproduction in DS-HSFs restoring ROS levels comparable than measured in N-HSFs, DCF fluorescence being 40 % reduced in PLD-treated DS cells respect to DS-HSFs with vehicle (Fig. 2A). When 8-oxo-dG as marker of OS-induced DNA damage was assessed, just like the reducing of ROS accumulation by PLD, also DNA oxidative damage was completely abolished by PLD treatment of DS-HSFs (−58 % *vs* DS + veh; Fig. 2B). Since an increased ROS production and OS have been associated to premature senescence in fibroblasts [44] and in DS cells [45], we also checked whether PLD treatment, able to decrease ROS overproduction, cold prevent also the occurrence of premature senescence in DS-HSFs, assessed by SA-β-gal activity measurements. Fig. 2C showed, 2,7-fold higher percentage of SA-β-gal positive cells in DS-HSFs respect to euploid N-HSFs and that PLD treatment significantly reduced (−31 % *vs* DS + veh) the SA-β-gal positive number in DS-HSFs.

In a second set of experiments, we also explored the PLD capability to protect both DS- and N-HSFs against exogeneous induction of oxidative stress. 24 h PLD- or vehicle-pretreated HSFs were exposed for 1 h to 50 μM H_2O_2, proved to be a non-cytotoxic concentration by H_2O_2 dose dependent cell viability experiment (Fig. 2D). After removal of external added H_2O_2, ROS levels, DNA damage and premature senescence were measured in HSFs exposed to the H_2O_2-induced stress. DS-HSFs proved to be more susceptible respect to euploid N-HSFs to exogenous H_2O_2-induced oxidative damages (+141 %, +36 % and +65 % of ROS levels, DNA damage and premature senescence, respectively in DS + OS *vs* DS + veh; Fig. 2E–G). PLD treatments showed protective effects in preventing OS-induced ROS overproduction, DNA damage and premature senescence in DS-HSFs (−59 %, −55 % and −54 %, respectively in DS + PLD + OS *vs* DS + OS). Interestingly, also PLD pretreated N-HSFs resulted significantly protected from exogenous H_2O_2- induced OS (being −40 % and −20 % of ROS levels and premature senescence, respectively, in N + PLD + OS *vs* N + OS Fig. 2E and G).

3.3. PLD modulates the expression of miR-155 and its direct target TFAM, CBL and BAG5 genes and proteins

We explored the molecular mechanism of the PLD action, checking whether PLD, like RSV [46], was able to modulate the expression of the miR-155–5p and its direct target genes and proteins.

As previously reported [25–28], we confirm that the Hsa21-encoded miR-155 is upregulated in DS-HSFs respect to euploid N-HSFs being, the miR-155–5p expression in DS-HSFs in basal condition significantly higher (+20 %) than in N-HSFs (Fig. 3A) with a fold change of DS *vs* N of 1.3 ± 0.2 as assessed by qRT-PCR. To analyze the effect of PLD treatment on miR-155 expression in DS-HSFs, by time course experiments, we set 6 h as the optimal incubation time for the treatment of DS-HSFs with PLD. The analysis of miR-155–5p expression showed a significant reduction in PLD-treated DS-HSFs compared to DS cells incubated with vehicle (−20 %, Fig. 3A), giving evidence of a PLD-dependent modulation of miR-155 expression in DS-HSFs.

When transcriptional analysis of miR-155 direct targets was analyzed in basal condition, we found a significant reduction of mRNA levels of TFAM (−50 %, Fig. 3B) and CBL (−30 %, Fig. 3C) respect to N-HSFs; BAG5 mRNA expression in DS cells, albeit with a decreasing trend, was found not significantly different from N-HSFs (Fig. 3D). When comparing DS cells treated with PLD or vehicle, we found a significant increase of mRNA levels for all analyzed genes being gene expression 1,64-, 1,88- and 3-fold raised for TFAM, CBL and BAG5, respectively (Fig. 3B–D). All together these findings indicate the capability of PLD to modulate gene expression both by mi-R155 modulation or directly acting on gene expression, as in the case of BAG5.

When protein levels were evaluated by immunoblotting analysis in HSF homogenates obtained from both DS and normal samples treated with PLD or DMSO as vehicle, densitometric analysis revealed a decrease in either TFAM, (−37,6 %; Fig. 4A), CBL (−37.5 %; Fig. 4B) and BAG5 (−35 %; Fig. 5A) protein levels in DS + veh respect to N + veh

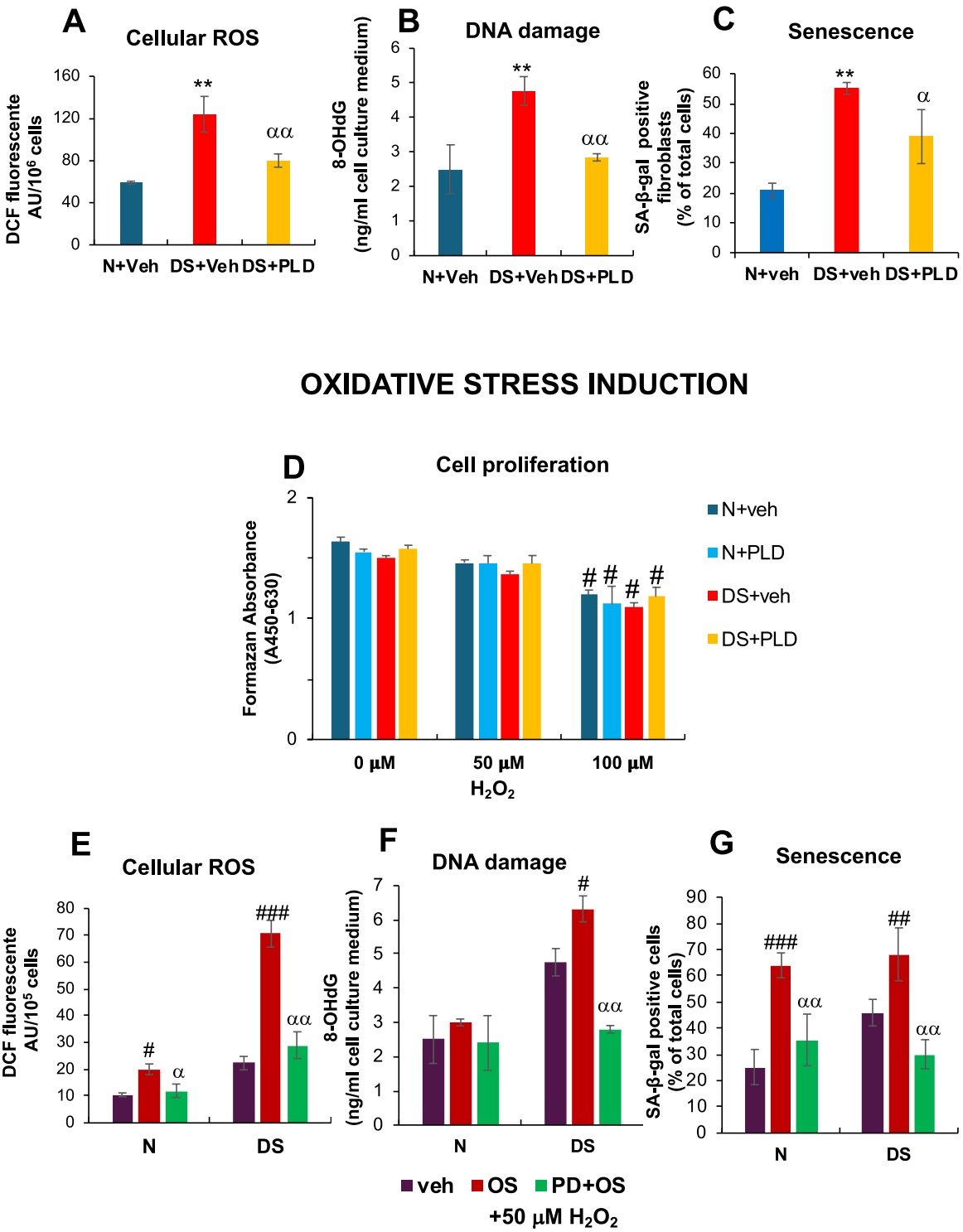

Fig. 2. PLD control of oxidative stress and premature senescence. Euploid (N) and trisomic (DS) cells (n = 3) were incubated for 24 h with 20 µM PLD (N + PLD or DS + PLD) or DMSO (N + veh or DS + veh). (A) Intracellular ROS reported as DCF fluorescence arbitrary units (A.U.); (B) DNA damage reported as 8-hydroxy-2′-deoxyguanosine (8-OHdG) released in the culture medium; (C) senescence reported as % of β-galactosidase (SA-β-gal) positive cells. OXIDATIVE STRESS INDUCTION: (D) Dose-dependence of H_2O_2 on cell proliferation in HSFs incubated with 20 µM PLD (+PLD) or DMSO (+veh), exposed for 1 h with 50 or 100 µM H_2O_2. Quantification of (E) cellular ROS, (F) DNA damage, and (G) cellular premature senescence measured in HSFs pretreated with PLD or vehicle and exposed for 1 h with 50 µM H_2O_2. N = 3. Data are mean ± SEM. Significant differences, calculated with one-way ANOVA and Turkey's *t*-test, are indicated as follow: N + veh *vs* DS + veh, **p < 0.01; H_2O_2 treated (OS)-induced *vs* no H_2O_2 treated HSFs, #< 0.05, ##p < 0.01, ###p < 0.001 HSFs + veh + OS vs HSFs + PD + OS, αα p < 0.01.

samples. PLD treatment for 24 h in DS-HSFs significantly raised protein levels of CBL (+27 %; Fig. 4B) and BAG 5 (+35 %; Fig. 5A) respect to DS + veh, showing a transcriptional regulatory mechanism of PLD modulation. Regarding TFAM protein level, although there was an increasing trend, it remained not significantly increased upon PLD treatment (Fig. 4A).

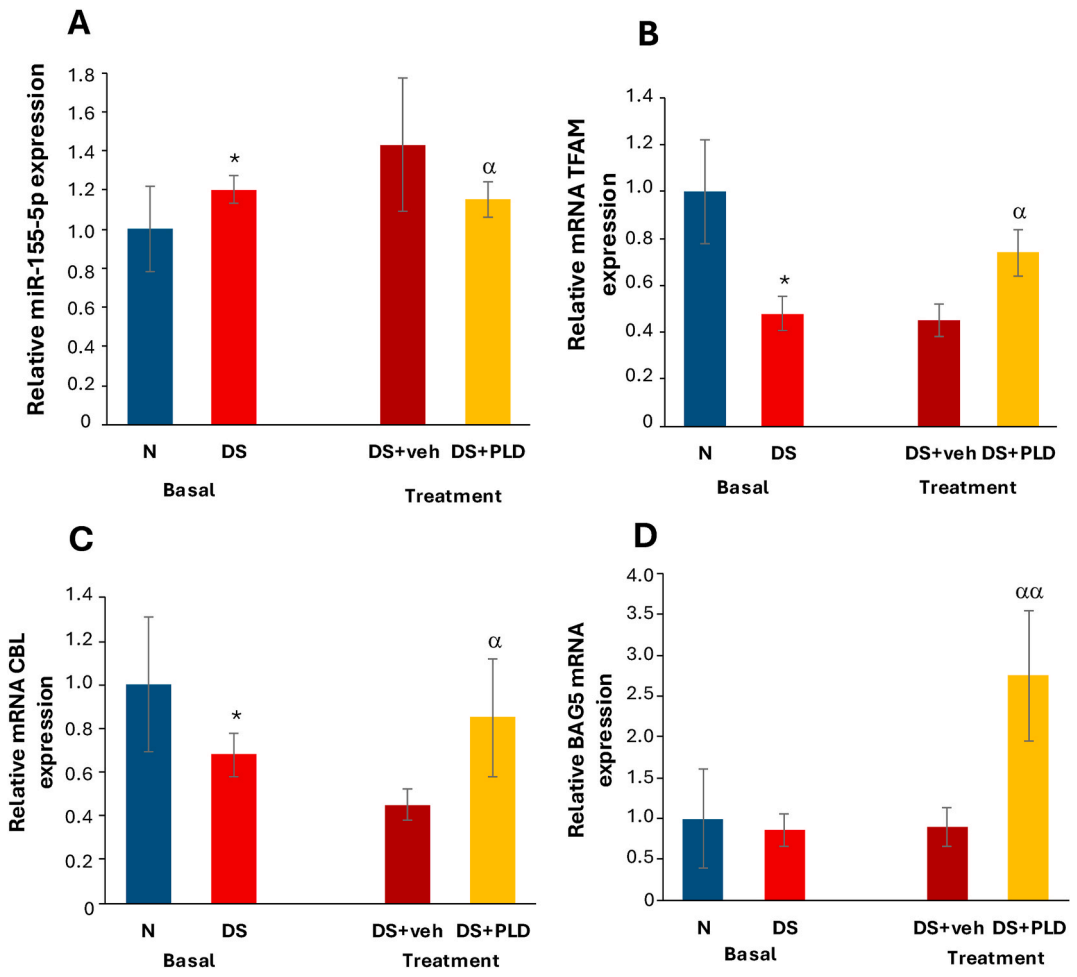

Fig. 3. PLD-dependent modulation of miR-155 and its target genes TFAM, CBL and BAG5. Levels of (A) miR-155–5p and mRNA levels of (B) TFAM, (C) CBL and (D) BAG5 evaluated by RT-qPCR in isolated total RNA from euploid (N) and trisomic (DS) fibroblasts (n = 3) (Basal) and in DS cells after incubation for 6 h with 20 μM PLD (DS + PLD) or DMSO (DS + veh) (Treatment). Data are mean ± SEM. Significant differences, calculated with Student's *t*-test, are indicated as follow: N *vs* DS, *p < 0.05; DS + veh *vs* DS + PLD, α p < 0.05 and αα p < 0.01.

3.4. PLD improves mitochondrial mitophagy in DS-HSFs

Deficit of mitophagy, a critical mechanism of quality control of mitochondria [47], has been reported in skin fibroblasts from DS children [48]. We explored whether PLD treatment could improve mitophagy in DS fetal HSFs.

We first investigated by immunoblotting analysis BAG5 and PINK1 protein levels. Indeed, it has been reported that a reduction of BAG5 destabilized PINK1 increasing its ubiquitination [32]. Consistently, we confirm a reduction of BAG5 at the protein level (−35 %; Fig. 5A) in DS-respect to N-HSFs, associated with a significant reduction (−47,5 %; Fig. 5B) of PINK1 protein accumulation. PLD treatment of DS-HSFs, increased BAG5 protein levels (+35 % DS + PLD respect to DS + veh; Fig. 5A) and raised the PINK1 protein accumulation (+29 % DS + PLD respect to DS + veh) to the level of N-HSFs. It is well known that PINK1, when associated on the mitochondrial outer membranes, initiates PARKIN-mediate mitophagy [47]. We confirm PLD-dependent mitophagy activation by evaluating the delivery of lysosomes to mitochondria by measuring colocalization of the lysotracher red (PremoTM autophagy sensor LC3B-RFP) and the mitotracker green (CellLight™ Mitochondria-GFP) thought confocal imaging analysis (Fig. 5C). We found in DS-HSFs (DS + veh) a reduction of the red LC3 fluorescence colocalization with the green mitotracker (Fig. 5D), consistent with a reduced capacity in DS cells to eliminate damaged mitochondria compared to N-HSFs. PLD treatment in DS-HSFs increased the colocalization of the LC3 cell fluorescence spots with the green mitotracker (+66 % in DS + PLD respect to DS + veh), suggesting a PLD-dependent mitophagy activation.

4. Discussion

This study sheds light on new pharmacological activities of the polyphenol polydatin in cells with T21 trisomy, discovering new targets in restoring mitochondrial bioenergetics and quality control and emphasizes PLD implications in improving and preventing critical phenotypic DS alterations.

In our previous studies we proposed trans-resveratrol as a possible natural diet integrator for the management of DS for its capability to improve adult hippocampal neurogenesis, through regulation of mitochondrial biogenesis and bioenergetics in progenitor cells from Ts65Dn mice model of DS [18]. However, RSV is rapidly metabolized in the intestine and liver resulting a very poor oral bioavailability in humans [49]. In addition, as we showed in this study (Fig. 1A), RSV at high concentrations appears to be cytotoxic for DS cells, raising a question mark for the dose that should be assumed for its therapeutic efficacy for DS population. Our attention has been then redirected to the natural pro-drug, glucoside of RSV, the stilbenoid PLD, for its capability to easily cross cell membranes both passively and by an active mechanism using the glucose carriers [34]. The glucose moiety of PLD triggers a higher resistance to enzymatic oxidation than RSV, also proving to this

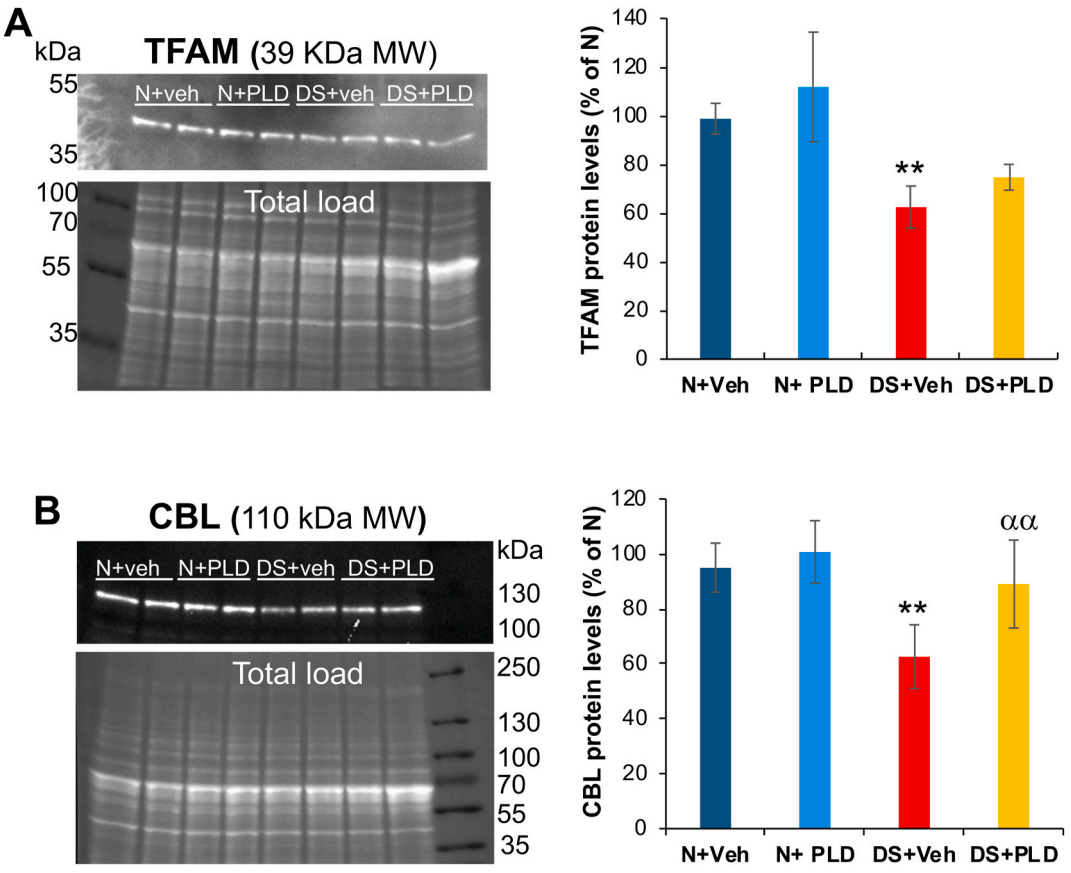

Fig. 4. Down-regulation of TFAM and CBL protein levels in DS-HSFs, effect of PLD treatment. Protein levels were evaluated in both euploid (N) and trisomic (DS) HSFs (n = 5) incubated for 24 h with 20 μM PLD (N + PLD or DS + PLD) or DMSO (N + veh or DS + veh). (A) TFAM and (B) CBL protein levels in representative immmunoblotting images (two different samples for each cell type lysates) and densitometric evaluation. Protein levels were normalized per total protein load. All densitometric values are given as percentage of N + veh set as 100 %. Data are mean ± SEM. Significant differences, calculated with one-way ANOVA and Turkey's *t*-test, are indicated as follow: N + veh *vs* DS + veh, **p < 0.01; DS + veh vs DS + PLD, αα p < 0.01.

polyphenol a greater hydro-solubility [50–52]. During the first passage metabolism, cellular β-glucosidases cleave off the PLD glucose molecule converting PLD into trans-resveratrol maximizing its cellular concentration [52]. Indeed, PLD, beside antioxidant and anti-inflammatory properties, exhibits many pharmacological effects reported for RSV, including neuroprotection in various neurodegenerative diseases [34,53] and carcer and multiple-organ protection [54–57]. PLD has been approved by the Food and Drug Administration (FDA) for human use both in adult and pediatric age and tested in clinical trials in a variety of disorders [58–61]. The immediate potential translation of PLD as natural drug to be tested in DS clinical studies, has prompted us to test the PLD efficacy and the mechanism of action in cells with Hsa21 trisomy.

In this study we performed experiments on fetal skin fibroblasts a widely used cellular model for studying DS-linked cellular alteration both in the early life and during aging, since fibroblasts premature senescence can be easily induced [62]. We showed the PLD capability not only to rescue the severe impairment of mitochondrial bioenergetics (Fig. 1), to scavenger ROS overproduction by mitochondria reducing oxidative stress in both cultured N- and DS-HSFs (Fig. 2A–B), but also to reduce the number of senescent-induced fibroblasts (Fig. 2C) and to prevent oxidative damages triggered by exogenous H_2O_2-treatment of both DS- and euploid fibroblasts (Fig. 2E–G). This is consistent with the reported PLD ability to scavenger ROS by blocking the radical cascade reducing OS markers both *in vitro* [63] and *in vivo* in patient with liver injury [61]. Interestingly, PLD appears effective in preventing OS and senescence not only in trisomic cells but also in normal euploid cells, suggesting that its supplementation could be considered not only for individual with DS to counteract oxidative- and senescence-related cellular decline, but also as potential strategy to mitigate aging-associated alterations in the general population. A recent study using a mouse model of DS, demonstrated that neonatal supplementation of mice with PLD was safe and effective in restoring neurodevelopmental and cognitive impairments [36]. Given that mitochondrial bioenergetics plays a critical role in brain neurodevelopment processes [5,9], we strongly recommend investigating the efficacy of PLD supplementation during infancy of subject with DS as strategy to improve mitochondrial metabolism and prevent mitochondrial dysfunction-associated DS features.

Our results further extend the knowledge about the molecular mechanisms of mitochondrial bioenergetics and mitophagy defects in DS cells identifying novel miR155-mediated pathways never explored in DS. MicroRNAs are a class of single-stranded non-coding RNAs constituted only by 18–24 nucleotides that control gene expression at the post-transcriptional level by binding the 3′-untranslated region (3′-UTR) of target mRNAs inhibiting their expression and thereby the translation of the coding mRNAs into proteins [64]. Several miRNAs were found overexpressed in DS cells, since encoded by Hsa21 resulting involved in neuronal developmental defects [27].

We focused on the Hsa21-encoded miR-155 since i) when deregulated, it plays an important role in both neurodevelopment and neurodegenerative diseases [65,66] ii) several miR-155 direct targets are linked to mitochondrial functions [29–32] iii) natural compounds, including RSV, have been shown to modulate its expression [46,67]. We confirmed in DS-HSFs upregulation of miR-155 respect to euploid cells and demonstrated that, like RSV, also PLD was able to normalize miR-155 levels (Fig. 3A). Since miR155 regulates a wide range of

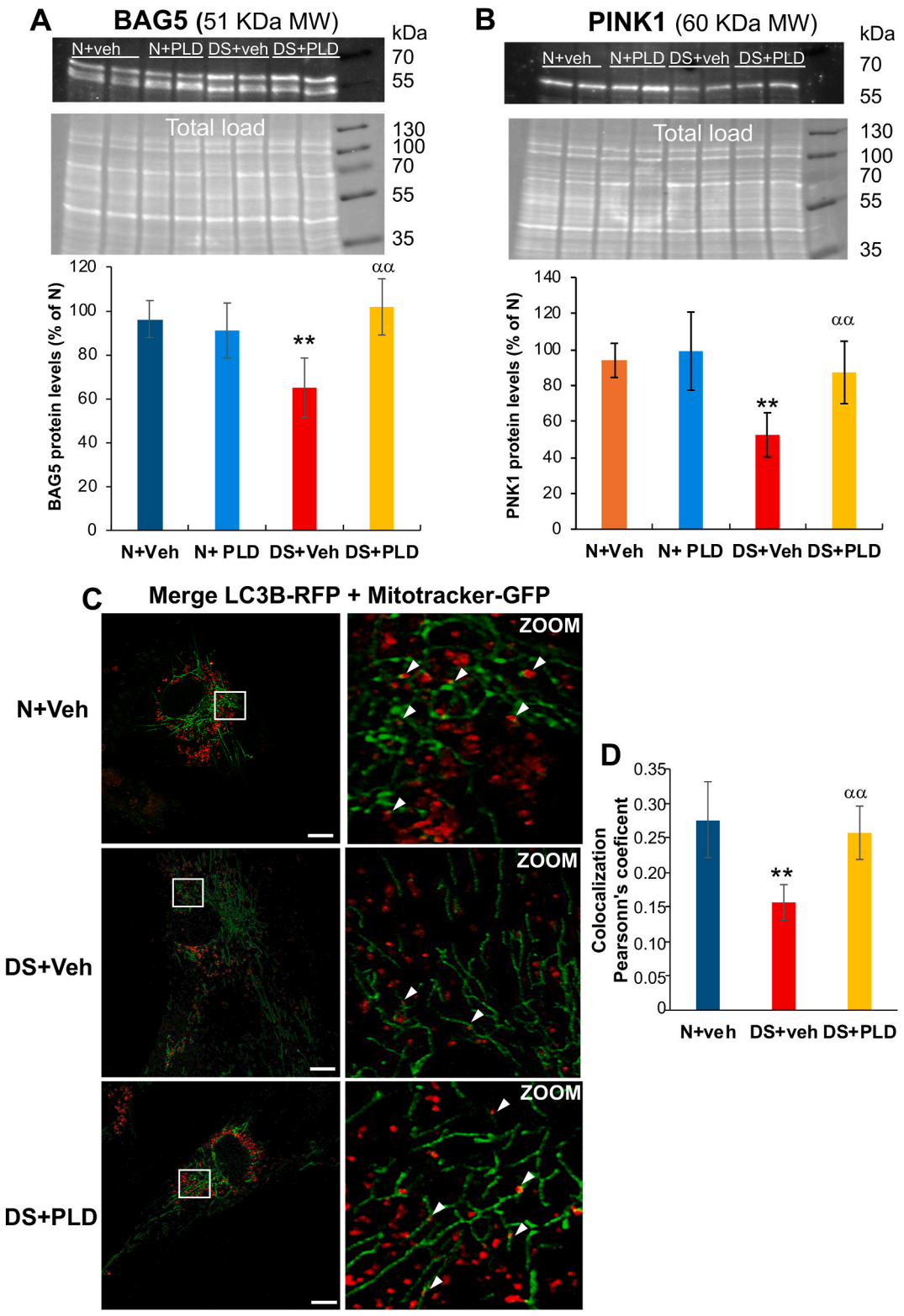

Fig. 5. PLD rescues mitophagy defects in DS cells. Protein levels were evaluated in both euploid (N) and trisomic (DS) fibroblasts (n = 5) incubated for 24 h with 20 μM PLD (N + PLD or DS + PLD) or DMSO (N + veh or DS + veh). (A) BAG5 and (B) PINK1 protein levels in representative immmunoblotting images (two different samples for each cell type lysates) and densitometric evaluation. Protein levels were normalized per total protein load. All densitometric values are given as percentage of N + veh set as 100 %. Data are mean ± SEM. Significant differences, calculated with one-way ANOVA and Turkey's *t*-test, are indicated as follow: N + veh *vs* DS + veh, **p < 0.01; DS + veh vs DS + PLD, αα p < 0.01. (C) Representative confocal images of co-stained with CellLight™ Mitochondria-GFP (green mitochondrial marker) and PremoTM autophagy sensor LC3B-RFP (red lysosomal marker) for 24 h upon vehicle or PLD treatment (20 μM for 24 h). Scale bar 10 μm. In the digital zoom of the boxed regions, the white arrows point to the co-localization of the red lysosomal puncta with green mitochondria. (D) Colocalization of mitotraker green/lysotracker red calculated by JACop plugin of FiJi softwear. Minimun 20 cell per condition were counted from 3 independent experiments. Significant differences, calculated with one-way ANOVA and Turkey's *t*-test, are indicated as follow: N + veh *vs* DS + veh, **p < 0.01; DS + veh vs DS + PLD, αα p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cellular functions and its deregulation is associated to various diseases (for refs see Ref. [68]), modulating miR-155 through the natural bioavailable PLD, could encourage further exploration of PLD's effects also in other diseases associated with miR-155 dysregulation.

Consistent with miR-155 upregulation, we found downregulation, at both mRNA and protein levels, of gene known to be direct targets of miR-155. We discovered for the first time two signaling pathways deregulated in DS cells i.e. miR-155/BAG5/PINK1 and miR-155/CBL axes (Fig. 6); furthermore, we confirmed in fetal fibroblasts impairment of miR-155/TFAM pathway. All these pathways are related to mitochondrial functions, and we show to be reactivated by PLD.

We confirm in DS HSFs, as already reported by Bordi et al. [48], the occurrence of impaired mitophagy (Fig. 5). Mitophagy regulates the control of quality of mitochondria by autophagic clearance of damaged mitochondria; the reduced mitophagy accumulates damaged mitochondria and it is dangerous for the cells [69]. We explored in DS cells a mechanism of miR-155-mediated mitophagy deficit through down-regulation of BAG5 and PINK1 (Fig. 6A). Indeed, BAG5 is both a direct target of miR-155 and a partner of PINK1, since protects him from proteasome-dependent ubiquitination [32]. The upregulation of miR-155 in mesenchymal cells, induced a downregulation of BAG5 at translational level and in turn a reduction of PINK1 protein, since more susceptible to degradation, with the consequent reduction of mitophagy [32]. PINK1 when stabilized on mitochondria activates PARKIN to initiate the autophagic clearance of damaged mitochondria [70]. Consistently, we demonstrated in DS cells, a link connecting upregulation of miR-155 and downregulation of PINK1 and mitophagy. Indeed, PLD treatment that restored miR155 expression to normal level, significantly increased BAG5 expression at both mRNA (Fig. 3) and protein level (Fig. 5A) and rescued both the level of PINK1 protein and the mitophagy process (Fig. 5B–D). The PLD action we found in DS cells, is consistent with the role of several polyphenols in promoting mitophagy by modulation of crucial signaling pathways and transcriptional regulators, as reviewed in Ref. [71]. Importantly, upregulation of miR-155 expression and reduced PINK1 were reported both in Alzheimer diseases and during aging [72,73]. We believe that the modulation of the molecular pathway miR-155/BAG5/PINK1, now we demonstrated in DS cells by PLD treatment, could prevent many DS-associated comorbidities and prompt further studies in the field of neurodegenerative diseases.

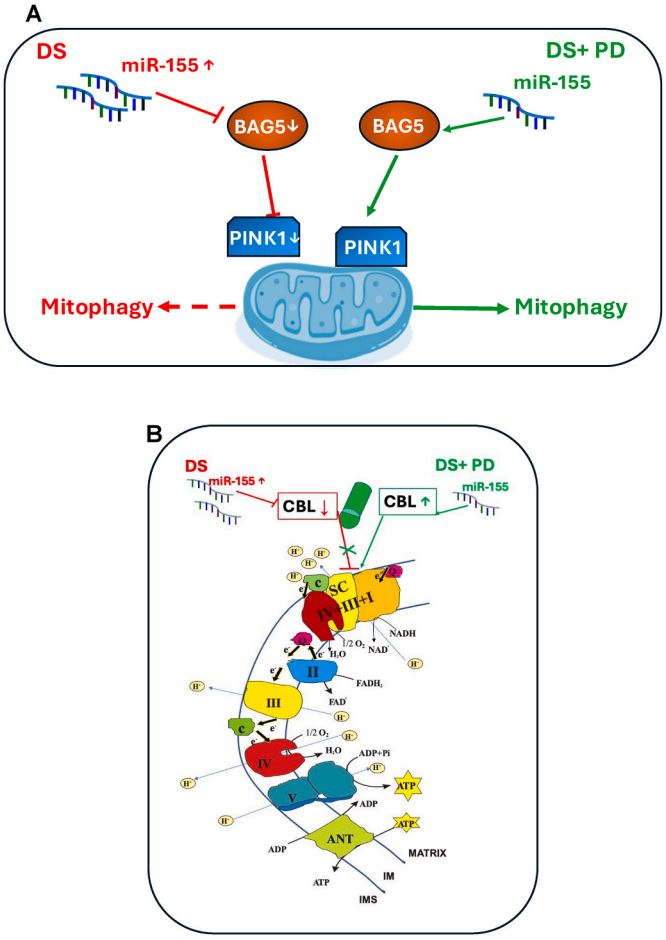

Fig. 6. Scheme of miR-155-dependent BAG5/PINK1 and CBL pathways down-regulated in DS and rescued by PLD. (A) MiR-155-mediated mitophagy deficit through down-regulation of BAG5 and PINK1 rescued by PLD. In DS cells, upregulation of miR-155 and downregulation of BAG5 and PINK1 together with mitophagy deficit was found. Indeed, PLD treatment normalized miR155 expression significantly increasing BAG5 expression and rescued both PINK1 protein levels and the mitophagy process. (B) In DS cells, miR-155 upregulation is related with CBL downregulation which could reduce the super complex CI + CIII + CIV assembly and impairs MRC activity and OXPHOS efficiency. The rescue of both miR-155 and CBL expression in PLD-treated DS cells associated with recovery of MRC complex I and V activities and OXPHOS efficiency may suggest the miR-155/CBL pathway as a novel mechanism for impaired mitochondrial bioenergetics in DS cells, found recovered by PLD action. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Regarding TFAM, another miR-155 direct target we analyzed in this study, of note is that the binding site for miR-155–5p in the 3′-UTR of TFAM has been found in DS fibroblasts [29]. In line, our study showed that both TFAM mRNA and protein expression was significantly lower in the DS cell than in normal fibroblasts under basal conditions. However, PLD treatment significantly increased TFAM mRNA level (Fig. 3C), but not the protein level, showing that TFAM is also regulated at the post-transcriptional/translational level in DS. Indeed, we have previously demonstrated in DS-HSFs impairment of AMPK-dependent pathways including the transcriptional coactivator PGC1α targeting TFAM protein and considered the master regulation of mitochondrial biogenesis [19]. Regulation of mitochondrial biogenesis also has been demonstrated occurring in DS fibroblasts through a mechanism involving the nuclear receptor interacting protein 1 (NRIP1) which exerted a repressive control on PGC-1α and its targets [74]. These findings and the results reported in this study point towards the involvement of multiple factors for the control of mitochondrial biogenesis in the DS.

Particularly interesting is the miR-155–5p/CBL axis, never analyzed in DS setting, that we found impaired in DS-HSFs, being miR-155 upregulation associated to a down regulation of CBL (Fig. 6B). CBL (known also as c-CBL) is a E3 ubiquitin ligase belonging to B-lineage lymphoma family proteins, traditionally known as a tumor suppression gene that, when mutated or down regulated, is involved in myeloid leukemias [75]. In addition, a very recent study has identified a new role for CBL in activating mitochondrial bioenergetics since essential in the mitochondrial assembly of the respiratory chain I, III and IV complexes in the super-complex namely respirasome, which increases mitochondrial bioenergetics efficiency; a down-regulation of CBL, reduces mitochondrial CI activity and assembly and induces mitochondrial dysfunctions [31]. The rescue of mitochondrial complex I activity and OXPHOS efficiency in PLD-treated DS cells, in which both miR-155 and CBL expression were restored to normal values, may suggest the miR-155/CBL pathway as a novel mechanism for impaired mitochondrial bioenergetics in DS identifying CBL as a new target (Fig. 6B).

Recently, it has been discussed a predominant role mitochondrial dysfunction in the occurrence of acute leukemia [76] and reported that in childhood acute lymphoblastic leukemia, upregulation of miR-155 and the inhibition of the direct target gene CBL promotes cell proliferation and inhibits apoptosis through the CBL/IRF4/CDK6 axis ([77]. MiR-155 deregulation has been reported in peripheral blood mononuclear cells (PBMCs) related to impaired B-cell response in children

with DS [78] playing a critical role in transient leukemia of DS [79]. Of note is that children with DS have an increased risk to develop both myeloid and lymphoid leukemias compared to the general pediatric population [80]. In addition to the information already reported [76–80], we provide new evidence of deregulation in the miR-155/CBL axis in Down syndrome which may open new avenues for exploring the molecular mechanisms underlying childhood leukemias in individuals with this condition.

5. Conclusions

The present study establishes a link between overexpression of miR-155 and downregulation of a new set of candidate proteins responsible for mitochondrial bioenergetics and mitophagy impairment in DS such as CBL and BAG5, discovering novel pathogenetic deregulated pathways in DS.

From this study PLD emerges as a treatment with a good translational potential for improving mitochondrial bioenergetics and critical bio metabolic alterations in DS. Beside classical antioxidant and anti-inflammatory activities of PLD, we determined an antiaging function of PLD and a new mechanism for the pharmacological action of this highly bioavailable polyphenol. PLD, targeting and reducing miR155 levels, induced the expression of crucial miR155 direct target genes counteracting mitochondrial dysfunctions, thus suggesting how modulation of miRNAs could be noticed as a novel pharmacotherapy approach in DS. Since PLD oral administration is well tolerated in humans (50–100 mg/die until 90 days in phase II clinical trials, as reviewed in Ref. [81]), we strongly suggest clinical studies to confirm its efficacy and safety in DS population. We further propose PLD as a potential prenatal pharmacological intervention to prevent DS-associated comorbidities related to mitochondrial dysfunction.

CRediT authorship contribution statement

Daniela Valenti: Writing – review & editing, Investigation, Formal analysis. **Daniela Isabel Abbrescia:** Writing – review & editing, Investigation, Formal analysis. **Flaviana Marzano:** Writing – review & editing, Formal analysis. **Giampietro Ravagnan:** Writing – review & editing, Validation. **Apollonia Tullo:** Writing – review & editing, Supervision. **Rosa Anna Vacca:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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