Profound increases of nuclear DNAJA3 and cytosolic STAT1 with nucleic acid sensors underlie innate immunity activation in ClpP-null mouse

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Abstract: Mitochondrial dysfunctions, e.g. abnormal handling of mitochondrial DNA in TFAM mutants or in altered mitophagy, activate innate immunity. Recent reports also showed that deletion of mitochondrial matrix peptidase ClpP in mice transcriptionally upregulates inflammatory factors. Here, we studied ClpP-null mouse brain at two ages and embryonal fibroblasts, to identify which signaling pathways are responsible, employing mass spectrometry, immunoblotets, and reverse transcriptase polymerase chain reaction. Anomalies in the mitochondrial unfolded protein responses pathway were prominent for the co-chaperone DNAJA3, and for its known interactor STAT1. Their mitochondrial dysregulation affected also their extra-mitochondrial abundance, as possible innate immune modulators. Increased expression was observed not only for the transcription factors Stat1/2, but also for two interferon-stimulated genes (Ifit4, Ghp3). Inflammatory responses were strongest for RLR pattern recognition receptors (Ddx58, Ifih1, Oasl2, Trim25) and several cytosolic nucleic acid sensors (Ifit1, Ifit3, Oasl1, Ifi204, Muida). They can be explained by the accumulation of mitoribosomes and mitochondrial nucleoids in ClpP-null cells, which may act as damage-associated molecular patterns. The consistent dysregulation of these factors from early age might influence also human Perrault syndrome, where ClpP loss-of-function leads to early infertility and deafness, with subsequent widespread neurodegeneration.

Keywords: PRLTS3; Release of mtDNA and mtRNA; cGAS-STING; Leukodystrophy; Ataxia; mitochondrial amino acid tRNA synthetases; TWINKLE; POLG; MTRNR1.

1. Introduction

Mutations in the mitochondrial matrix peptidase ClpP in the human organism lead to Perrault syndrome type 3 (PRLTS3). This syndrome was initially described as autosomal recessive premature ovarian failure combined with sensorineural hearing loss, and was then shown to be accompanied by a growth deficit and a generalized neurodegenerative process with leukodystrophy upon neuroimaging, which manifests as late-onset progressive ataxia and neuropathy [1-5]. ClpP is highly conserved from bacteria to mammals, playing a crucial role for mitochondrial responses to unfolded protein stress (UPR<sup>mt</sup>) by the disaggregases ClpX and ClpB, chaperones of the Hsp70 and Hsp60 family, as well as co-chaperones of the DnaJ and the GrpE family [6]. Thus, ClpP deficiency leads to problems for the assembly of proteins with RNA in mitoribosomes and with DNA in mitochondrial nucleoids [7-9]. This mitochondrial pathology triggers innate immunity activation in the eukaryotic
host cell to a degree where altered skin microbiome defenses can modify the lifespan of ClpP-null mice [10, 11], mainly via abnormal mtDNA that activates cGAS-STING signaling to modulate type I interferon release [9].

Other genetic causes of Perrault syndrome include mutations in the mitoribosome chaperone ERAL1, the mitochondrial amino acid tRNA transferases HARS2 and LARS2, and the mitochondrial translation factor RMND1, highlighting the importance of mitoribosomal translation for fertility and neurodegeneration. Additional causes are mutations in the mitochondrial DNA helicase-primase TWNK and the mitochondrial transcription factor TFAM, emphasizing the relevance of mtDNA disassembly. Perrault syndrome was also reported to be caused by mutations in the peroxisomal factors HSD17B4 and PEX6, or in GGPS1 as an enzyme responsible for prenylation of proteins, which is a determinant of UPRmt [9, 12-14]. Also maternally inherited mutations in the mitochondrial DNA are known to cause progressive deafness via altered mitoribosome functions, and the chronic administration of aminoglycoside antibiotics can also lead to hearing deficits, presumably via their impact on mitoribosomal translation fidelity [15-17].

**Figure 1.** Innate immunity activation due to mutations in mitochondrial factors is mediated in the cytosol via the cGAS/STING pathway (when accumulated mtDNA is extruded to the cytosol) or the DDX58/IFIH1/MAVS sensors in the pattern recognition RLR pathway (when accumulated mitochondrial double-strand RNA is extruded). They stimulate the nuclear induction of interferon type I signaling. It is unclear if these established mechanisms also are prominent in ClpP-deficient cells, or additional pattern recognition receptors (TLR, NLR) and other cytosolic sensors play a relevant role. MRG = mitochondrial ribosomal granule; VDAC = PORIN; DDX58 = RIG-I; IFIH1 = MDA-5; STING = STING1, also known as TMEM173; ISRE = interferon-stimulated response element in the promoter of nuclear genes; other symbols are defined in the abbreviation list.

Particularly for TFAM mutations, the mechanism of innate immune activation has been elucidated in detail. Heterozygous TFAM deficiency results in abnormal packaging of mtDNA nucleoids and their extrusion into the cytosol, where TFAM-associated U-turn DNA will nucleate
cGAS dimers and activate STING/TBK1 to induce type I interferon. Similarly, deficiencies of the mitochondrial RNA helicase SUP3L1 or the mitochondrial polynucleotide phosphorylase PNPT1 lead to accumulation of double-stranded RNA that is released to the cytosol through the BAX/BAK1 and VDAC pores, then acting via DDX58/IFIH1/MAVS/TBK1 to upregulate interferon-stimulated gene expression (see schematic overview in Figure 1). These responses mediate resistance against microbial infections and enhance repair mechanisms that are also protective for the nuclear genome [18-24].

Not only does the rare Perrault syndrome show the combination of a primary mitochondrial dysfunction with subsequent sterile inflammation, but also the frequent neurodegenerative process in Parkinson’s disease (PD) has this dual characteristic. Mutations in the PINK1 and PARKIN genes are responsible for juvenile-onset autosomal recessive PD variants of relatively mild progression [25]. Both factors are responsible for the autophagic degradation of damaged mitochondrial fragments [26]. Loss-of-function in this pathway leads not only to abnormal turnover of mitochondria, but also affects the cellular susceptibility to invasion by bacterial pathogens such as *Mycobacterium Tuberculosis* [27]. Mice with PINK1 or PARKIN mutations show consistent innate immune activation even in a special-pathogen-free environment or upon cell culture, and the depletion of the cytosolic immune coordinator STING can prevent the neurodegenerative process in such mice [28, 29].

An additional pathway how mitochondrial dysfunction can modulate innate immunity was recently demonstrated in fumarate hydratase deficient cells that exhibit excessively succinylated proteins in the mtDNA replication machinery, with progressive accumulation of mis-assembled nucleoids. Both fumarate and succinate are metabolites that are known to modulate the immune status of cells [30, 31].

It is also known that the metabolites ATP and cardiolipin, reactive oxygen species (ROS), N-formylated peptides, and enzymes such as Cytochrome C and Carbamoyl phosphate synthetase-1, upon their release from bacteria/mitochondria, may be sensed by eukaryotic hosts as damage-associated molecular patterns (DAMPs) and trigger the innate immune responses [32]. In addition, nuclear transcription factors are used to govern intra-mitochondrial processes, and sustained nuclear efforts to compensate mitochondrial anomalies may modulate also the expression of cytosolic factors and produce side-effects throughout the cell over time. As examples, transcriptional activity in mitochondria is controlled (i) via autoregulation from mitochondrial TFAM (UniProt database entry P40630-1) to a nuclear isoform of TFAM (UniProt entry P40630-2) [33, 34], and via mitochondrial STAT1 that cross-talks with nuclear STAT1 [35]. It is well established that nuclear STAT1 has a massive impact on the innate immune response.

Our prior work has shown that the cGAS-STING pathway is primarily responsible for heightened activation of type I interferon production and downstream upregulation of interferon-stimulated genes in cells and tissues from ClpP-null mice [9]. However, it is possible that other alterations (i.e. accumulation of unfolded proteins in mitochondria with release of N-formyl peptides, a profoundly altered metabolite profile, and/or extruded mitochondrial RNA with hypomethylation, nucleotide chains in supercoil structure) could further enhance innate immune activation in ClpP-null mice. In view of clinical consequences like the resistance of ClpP-null mice against ulcerative dermatitis with lethal outcome [10], we tried to elucidate the molecular mechanisms how altered UPR can activate innate immunity, examining some interplay of mitochondrial / nuclear protein isoforms as well as specifying the most affected DAMP-sensing pathways. Our data document strong accumulations for DNAJA3 (also known as TIDI) and STAT1, intra-mitochondrially and extra-mitochondrially. These proteins are both known to modulate innate immunity, and were observed to interact physically [36, 37]. DNAJA3 was previously shown to be co-regulated with ClpP in the mitochondrial unfolded protein response pathway and might be a degradation substrate of ClpP or co-accumulate with such substrates in the mitochondrial matrix. It is possible that its impaired turnover would affect JAK-STAT signaling and modulate interferon signaling [22, 38]. In addition to the established inflammatory effects of mtDNA / mtdsRNA extrusion from mitochondria, this additional pathway might be ClpP-specific. The activation of innate immunity comprised a strong transcriptional induction of specific interferon-stimulated genes and cytosolic nucleic acid sensors
(higher number of RNA sensors than of DNA sensors), as well as several pattern recognition receptor families (number of elevated factors in RLR > TLR >> NLR).

2. Materials and Methods

2.1 Mouse breeding

Homozygous ClpP<sup>−/−</sup> and wild-type (WT) mice were generated as littermate offspring from heterozygous breeders. They were genotyped at postnatal day 10 by ear-punches, and immediately after weaning pairs of mutants with age-/sex-matched WT controls were housed together, aged and dissected as reported before [10]. The mice used were kept under FELASA-certified conditions at the Central Animal Facility (ZFE) of the Goethe University Medical Faculty in Frankfurt. All animal experiments were carried out in accordance with the German Animal Welfare Act and with approval of the local animal authorities (Regierungspräsidium Darmstadt, FK/1073).

2.2 Mouse Embryonic Fibroblast Generation and Culture

Generation and culture of mouse embryonic fibroblasts (MEFs) were done as previously described [11]. Following intercrosses of ClpP<sup>−/−</sup> mice, WT and ClpP<sup>−/−</sup> MEFs were prepared from individual embryos at 14.5 days post-coitus. Cells were cultivated in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% L-glutamine (Gibco, Thermo Fisher Scientific) at 37 °C and 5% CO<sub>2</sub> in a humidified incubator.

2.3 Mouse brain native gel electrophoresis and complexome profiling

Sample preparation [39] and high-resolution native electrophoresis (hrCNE) [40] of brain tissue were essentially done as described. Briefly: Brains were taken and further disrupted using a pre-cooled motor-driven glass/Teflon Potter-Elvehjem homogenizer at 2000 rpm and 40 strokes. Homogenates were centrifuged for 15 min at 600 g to remove nuclei, cell debris, and intact cells. Mitochondrial membranes were sedimented by centrifugation of the supernatant for 15 min at 22,000 g. Mitochondria-enriched membranes from 10 mg brain tissue were resuspended in 35 µl solubilization buffer (50 mM imidazole pH 7, 50 mM NaCl, 1 mM EDTA, 2 mM aminocaproic acid) and solubilized with 20 µl 20% digitonin (Serva, Heidelberg, Germany). Samples were supplemented with 5 µl 0.1% Ponceau S in 50% glycerol. Equal protein amounts of samples were loaded on top of a 3% to 18% acrylamide gradient gel (dimension 14x14 cm). After native electrophoresis in a cold chamber, native gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid, 10 mM ammonium acetate for 30 min and stained with Coomassie (0.025% Serva Blue G, 10% (v/v) acetic acid) or blotted onto PVDF membranes and used for antibody decoration using an antibody against NDUFB8 and MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (Mitosciences, Eugene, Oregon, USA). Coomassie-stained lanes were fractionated in 60 even pieces and digested with trypsin and subsequently analyzed by mass spectrometry. Experimental details and data were deposited to dataset identifier PRIDE: PXD025478.

2.4 Reverse Transcriptase Real-Time Quantitative PCR

As described in previous studies [10] and following manufacturers’ instructions, total RNA isolation from MEFs and brain tissues was performed with TRI reagent (Sigma-Aldrich, St. Louis, MO, USA), and reverse transcription by SuperScript IV VILO Master Mix (Thermo Fisher Scientific). RT-qPCR was carried out with TaqMan® Gene Expression Assays (Thermo Fisher Scientific) in cDNA from 10 ng total RNA in 10 µl reactions with 2x Master Mix (Roche, Basel, Switzerland and Thermo Fisher Scientific) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The data was analyzed with the 2<sup>−ΔΔCT</sup> method [41]. To verify the null mutation in used tissue and MEF samples, RT-qPCR assays of ClpP normalized to Tbp were performed, in
addition to the genotyping of each animal. The following TaqMan assays (Thermo Fisher Scientific) were employed to quantify the individual mRNA levels: Aim2- Mm01295719_m1, ClpP-Mm00489940_m1, Ddx58- Mm01216853_m1, Dnaja3- Mm00469723_m1, Eif2ak2- Mm01235643_m1, Gbp3- Mm00497606_m1, Ift204- Mm00492602_m1, Ift205b (=Mnda)- Mm04204353_m1, Ifi35-Mm00510329_m1, Ifi44- Mm00505670_m1, Ifih1- Mm00459183_m1, Ifit1 (=lg56)- Mm00515153_m1, Ifit3-Mm01704846_s1, Ifna1- Mm03030145_gf, Ifib1-Mm00439552_s1, Irgb-Mm00516784_m1, Mares (=lps-1)- Mm00523170_m1, Mbi21d1 (=Gas)- Mm01147496_m1, Njkb1- Mm00476361_m1, Nlrp3-m00840904_m1, Nlrx1-Mm00617978_m1, Oas1b-Mm00449297_m1, Oasl2-Mm00496187_m1, Rsad2-Mm00491265_m1, Stat1-Mm00439531_m1, Stat2-Mm00490880_m1, Supv3l-Mm00619586_m1, Tbp-Mm00446973_m1, Tlr3- Mm01207404_m1, Tlr9- Mm00446193_m1, Tmem173- Mm01158117_m1, Trim25-Mm01304226_m1, Trim30a- Mm00493346_m1, Trim56- Mm01207494_m1, Tspan6-Mm00451054_m1.

2.5 Quantitative Immunoblotting

Protein extraction and sample preparation from brain tissues and MEFs were carried out as described before [10, 42]. Samples of 20 µg of total protein were mixed with 2x loading buffer [250 mM Tris/HCl (pH 6.9), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.01% bromophenolblue, 5% MilliQ water], heated at 90 °C for 5 min and afterward separated in 8%, 10%, 12% or 15% tris-glycine polyacrylamide gels depending on the size of the investigated protein. Precision Plus Protein™ All Blue Standards (Bio-Rad, Hercules, CA, USA) was used as a size marker. Following the gel electrophoresis, protein transfer to nitrocellulose membranes (Bio-Rad) was done at 50 V over 90 min. The membranes were blocked in 5% BSA/TBS-T for 1 h at room temperature (RT) and incubated overnight at 4 °C with primary antibodies against ClpP (1:1000, Proteintech, Rosemont, IL, USA, 15698-1-AP), DDX58 (1:1000, Cell Signaling, Danvers, MA, USA, #37435), DNAJ A3 (1:500, Cell Signaling, #4775), IFIT3 (1:800/1:1000, Proteintech, 15201-1-AP), IKBα (1:1000, Cell Signaling, #4814), IκKα (1:500, Cell Signaling, #11930), IκKβ (1:1000, Cell Signaling, #8943), IRF3 (1:1000, Cell Signaling, #4302), IRF7 (1:1000, Abcam, Cambridge, UK, ab109255), ISG15 (1:1000, Invitrogen, PA5-17461), Nfkb P65 (1:1000, Cell Signaling, #8242), Phospho-IκKα/β (1:800, Cell Signaling, #2697), Phospho-IκBα (1:1000, Cell Signaling, #2859), STAT1 (1:1000, Cell Signaling, #9172), SUPV3L1 (1:800/1:1000, MyBioSource, San Diego, CA, USA, MBS9130033), TLR9 (1:800, Novus B, Centennial, CO, USA, NBP2-24729), TRIM25 (1:1000, Abcam, ab167154). As secondary antibodies, fluorescence-labeled anti-rabbit or anti-mouse antibodies (1:15,000, Thermo Fisher Scientific, Invitrogen) were used. The fluorescence was detected by using the Li-Cor Odyssey Classic Instrument and was densitometrically analyzed with Image Studio Lite Version 5.2 (Li-Cor Biosciences). Bands were normalized against β-ACTIN (= ACTB) (1:10,000, Sigma-Aldrich, A5441). For fractionation experiments GAPDH (1:10,000, Sigma-Aldrich, Taufkirchen, Germany, #CB1001), LAMIN A/C (1:1000, Abcam, #AB169532) or PORIN-1 (1:500, Cell Signaling, #4866) were used as loading controls.

2.6 Subcellular fractionation

Fractionation into nuclear, mitochondrial, and cytosolic fractions was done as follows: 4x10^6 MEF cells were collected with trypsin, centrifuged at 800 g for 3 min, washed with PBS, the centrifugation step was repeated, and the pellet was resuspended in 300 µl cytosol extract buffer (CEB). The suspension was shaken 5 min head-to-head at RT and centrifuged at 800 g for 3 min. The supernatant was stored as cytosolic fraction. The remaining pellet was washed once with CEB, centrifuged at 800 g for 3 min and then resuspended in 300 µl mitochondrial lysis buffer (MLB), shaken 10 min head-to-heat at RT and centrifuged at 800 g for 3 min. The supernatant was stored as mitochondrial fraction. The remaining pellet was washed once with MLB, centrifuged at 800 g for 3 min and then resuspended in 300 µl RIPA buffer. The suspension was sonicated, 10 min shaken head-to-head at RT and centrifuged at 800 g for 3 min. The supernatant was stored as nuclear fraction. Experiments were repeated with 3 WT and 3 ClpP−/− lines. Buffers were composed as follows:

CEB: (250 mM Sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, with freshly added 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 200 µg/ml digitonin)
MLB: (50 mM TRIS/HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X100, 0.3% NP40, with freshly added 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin)

RIPA buffer: [50 M TRIS/HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% triton, 0.5% sodium deoxycholate, 2 mM EDTA, protease inhibitor cocktail (Sigma Aldrich, St. Louis, Missouri, USA)]. All chemicals were purchased from Merck (Darmstadt, Germany) unless mentioned otherwise.

2.7 Statistical Evaluation

Statistical analysis of quantitative immunoblot and RT-qPCR results were conducted by using GraphPad Prism (Version 8.4.2, GraphPad, San Diego, CA, USA) with unpaired Student’s t-tests. Results including standard error of the mean (SEM) and p values (p= probability) were visualized in bar graphs, with the following significances illustrated by asterisks or symbols: * (or #/§) p < 0.05; ** (or ##/§§) p < 0.01; *** (or ###/§§§) p < 0.001; **** (or ####/§§§§) p < 0.0001; not significant (ns) p > 0.05; tendency (T) 0.05 < p < 0.1.

2.8 Visualization

Graphs were generated by using GraphPad Prism (Version 8.4.2).

3. Results

3.1. Protein mass spectrometry analysis of mitochondrial matrix unfolded protein response factors by complexomics reveals prominent assembly anomalies for DNAJA3 in ClpP−/− mouse brain.

To understand the cumulative effects of chronic ClpP deficiency on the UPR<sup>mt</sup> pathway in brain tissue with its postmitotic neurons during the aging process, dissected brains from 12-month-old ClpP−/− and sex-matched littermate control mice were homogenized and centrifuged to obtain mitochondria-enriched membranes. High-resolution native electrophoresis was used to resolve protein complex assemblies, and then their components were identified and quantified by mass spectrometry (Figure 2). A selective analysis of UPR<sup>mt</sup> factors demonstrated the absence of ClpP (monomer with size 29 kDa), as well as pronounced accumulations for the disaggregase ClpX (69 kDa), and the Hsp90-homologous molecular chaperone TRAP1 (size 80 kDa). Interestingly, accumulation with additional disperse migration at abnormally high molecular weights was evident for the Hsp70-homologous chaperone HSPA9 (Mortalin, 73 kDa), its GrpE-homologous co-chaperone GRPEL1 (24 kDa), and even more so for its DnaJ-homologous co-chaperone DNAJA3 (52 kDa for large isoform precursor, 49 kDa for small isoform precursors). DnaJ was originally identified in E. coli bacteria as a factor required for the replication of bacterial and viral DNA [43]. Given that DNAJA3 is known as protein interactor of STAT1 and as modulator of inflammatory responses also via NFκB [22, 36, 37, 44-47], we tested if STAT1 dysregulation also occurred in ClpP deficient tissue. Given that brain anomalies might be caused by the innate immune system or the adaptive immune system, we decided to perform further studies in primary fibroblast cultures taken at early embryonic age from ClpP−/− mice and their sex-matched littermate controls, comparing these results with data from brain samples at two ages.
3.2. Verification of ClpP<sup>−/−</sup> in MEFs, with analysis of mitochondrial DNA and RNA associated factors

First, the genetic ClpP-ablation was verified at mRNA and protein level by using immunoblots (Figure 3A) and RT-qPCRs (Figure 3B). Its deficiency was documented in MEFs and the brain samples used.

With quantitative immunoblots, a strong accumulation was again found for DNAJA3 (Figure 3C). This observation was not explained by transcriptional induction (Figure 3D), so it might simply represent a marker of protein complex assembly problems that result in slowed turnover and degradation of DNAJA3 targets.
Our previous work has demonstrated ClpP-null cells to display mitochondrial nucleoid assembly problems and extrude mtDNA into the cytosol [9]. In view of the parallel disturbance of mitoribosome assembly via dysregulation of the RNA chaperone ERAL1 in ClpP-null cells [8], it is conceivable that mtRNA gets also extruded into the cytosol. Although mtDNA was shown to be the main trigger of innate immunity activation in ClpP-cells, given that cGAS and STING deficiency reduced the induction of interferon-stimulated genes almost to WT levels [9], it is possible that other mtDAMPs could participate in the elevated innate immune responses of ClpP-null cells. The RNA helicase SUPV3L1, a protein component of the mitochondrial degradosome that was implicated in the mitochondrial release of mtdsRNA [24], showed a significant reduction of protein abundance (Figure 3E) in MEFs, while its transcripts were not reduced. Selectively in brain tissue of 11-19 month old mice the Supv3l1 mRNA levels were significantly diminished (Figure 3F). It is difficult to know if these slight deficits are meaningful, but clearly, a strong induction of this helicase in response to mtdsRNA pathology was not detected.
Figure 3. Verification of ClpP−/− genotype via (A) quantitative immunoblots and (B) RT-qPCRs in MEF and brain tissue of 3 and 11-19 month-old mice. Analysis of (C) DNAJA3 protein and (D) mRNA levels, as well as (E) SUPV3L1 protein and (F) mRNA levels, both in MEF and brain tissue of 3 and
11-19 month-old mice. Immunoblots were normalized to ACTB or GAPDH, RT-qPCRs to Tbp levels. Data are presented as mean ± SEM. WTs are shown as checked, ClpP−/− as plain colored bar graphs. Significances are illustrated by symbols: ** or §§ p < 0.01; ### p < 0.001; ###### p < 0.0001; not significant (ns) p > 0.05. Asterisks portray significant differences between WT and ClpP−/− MEF, hashtags show significant effects in 3-month-old brain tissue between WT and ClpP−/− mice, and section signs visualize significant differences in brain tissue from 11-month-old mice, between WT and ClpP−/− genotype. WT MEF: n= 4-5; ClpP−/− MEF: n= 3-5; WT brain 3 months: n= 3; ClpP−/− brain 3 months: n= 3; WT brain 11-19 months: n= 5; ClpP−/− brain 11-19 months: n= 6.

3.3. Cell fractionation demonstrates ClpP-deficiency to increase cytosolic STAT1 and nuclear DNAJA3

To elucidate how ClpP deficiency and UPRmt pathology signal retrogradely to dysregulate factors with nuclear function such as the transcription factor STAT1, already at embryonal stage in fibroblasts, the fractionation of different subcellular compartments by sequential protein extraction with appropriate detergents was done. Enrichment of the mitochondrial fraction was assessed by PORIN as component of the main outer mitochondrial membrane multimeric pore, the cytosolic fraction was assessed by GAPDH as glycolytic enzyme, and the nuclear fraction by LAMIN A/C as component of the matrix on the inner surface of the nuclear envelope. Immunoblot detection with DNAJA3 antibodies showed an accumulation for the mitochondrialy imported and cleaved small isoform not only in the ClpP−/− mitochondrial fraction, but also in the ClpP−/− nuclear fraction a band of very similar size was accumulated (Figure 4A). Accumulation of the DNAJA3 small isoform in the ClpP−/− mitochondrial fraction was also observed on membranes where more protein was loaded per lane, in a comparison to cytosolic fraction (Figure 4B), but DNAJA3 was below the detection threshold in the cytoplasm. In contrast, STAT1 immunoproduct of the predicted size accumulated in ClpP−/− mitochondrial fraction and in ClpP−/− cytosolic fraction (Figure 4B), but STAT1 was below the detection threshold in the nuclear fraction, possibly due to insufficient solubilization from DNA complexes (Figure 4A). It is known that ClpP-null cells have elevated levels of mis-assembled mtDNA [9, 10], so it seems appropriate to observe responses of a mammalian homolog of DnaJ chaperones which are known to control bacterial and viral DNA replication [43, 48], and of STAT1 that represses mitochondrially encoded transcripts [35]. Importantly, these responses involve not only intra-mitochondrial DNAJA3 and STAT1 but extend to extra-mitochondrial compartments.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Cell fractionation in MEFs, comparing mitochondrial with nuclear (A) and cytosolic (B) compartments. Immunoblotting was used to control the purity of the fractionation with the mitochondrial marker PORIN, the nuclear marker LAMIN A/C, and the cytosolic marker GAPDH, comparing littermate WT and ClpP−/− cells of matched sex. The detection of DNAJA3 revealed ClpP deficiency to cause accumulation of DNAJA3 short isoform (S) in mitochondria and nucleus, while STAT1 (87 kDa) accumulation occurred in mitochondria and cytosol.
3.4. Transcriptional analysis of induced ISGs, PRRs and associated factors

In view of published global transcriptome evidence of increased antiviral defenses in ClpP-null mice, we wanted to further characterize the signaling pathways involved [9-11]. Therefore, mRNA expression levels of relevant transcription factors, interferon-stimulated genes, cytosolic DAMP sensors, and pattern recognition receptors (PRRs) with associated factors were surveyed by RT-qPCR (Figure 5).

Several investigated inflammatory transcription factors and interferon-stimulated genes (Stat1, Stat2, Ifi35, Ifi44, Gbp3) showed significantly increased mRNA levels (Figure 5A). A very strong induction was detected especially for Ifi44, with effect sizes of 7.257-fold in the brain of 11-19 month-old ClpP-null mice and 11.11-fold in MEFs. The PRRs lead to an induction of interferon-stimulated genes (ISGs), via type-I interferons (IFN-I) and other pathways. Some of the ISGs are the PPRs themselves, which may lead to an effect of self-induction. Other ISGs also exert anti-pathogenic functions in the context of the innate immune system. The main IFN-I transcripts, Ifna1 and Ifnb1, showed only partially significant inductions (Error! Reference source not found.). While both were significantly upregulated in MEFs, only Ifnb1 was induced in the 11-19 month-old ClpP-null mice brain tissue samples.

Prominent significant upregulations were also found for a number of cytosolic nucleic acid sensors (Ifit1, Ifit3, Oas1b, Ifi204, Mnda, with lesser induction for Eif2ak2=Pkr and for Aim2=Pyhin4) (Figure 5B,C), and the retinoic acid inducible gene like receptors (RLRs) (Ddx58=Rig-I, Ifih1) with their associated factors (Oasl2, Trim25) (Figure 5D). No significant transcriptional changes were apparent in the cGAS-STING signaling pathway, with STING mRNA here referred to as Tmem173, consistent with previous observations that the regulation of this pathway occurs via dimerization and phosphorylation (Figure 5C). Nonetheless, Trim56 mRNA levels showed a 1.473-fold induction in 11-19 month-old ClpP-null mice. TRIM56 protein can induce cGAS and therefore the downstream signaling pathway [49]. Although a 2.261-fold induction of Ddx58 and a 2.016-fold induction of Ifih1 were detected in the brain tissue of 11-19 month-old ClpP−/− mice (Figure 5D), the downstream mitochondrial adapter Mavs was not significantly altered in expression. Interestingly, toll-like receptors (TLRs) showed fewer transcriptional inductions (Figure 5E), and nuclear oligomerization-domain like receptors (NLRs) almost none (Figure 5F). In young brains, only Trim30a levels were significantly increased (Figure 5E). In contrast to that, the interferons Ifna1 and Ifnb1, as well as Nfkb1 and Irf3 did not show transcriptional activation (Suppl. Figure S1A-C).
Figure 5. Analysis of mRNA expression by RT-qPCR in MEFs and brain tissue of 3 and 11-19 month-old mice, focusing on (A) induced interferon-stimulated genes, (B) cytosolic RNA sensors, (C) cytosolic DNA sensors, (D) RLRs with associated factors, (E) TLRs with associated factors, and (F) NLRs. RT-qPCRs were normalized to Tbp levels. Data are presented as mean ± SEM. WTs are shown as checked, ClpP−/− as plain colored bar graphs. Statistical tendencies and significances are illustrated by symbols: * or #/§ p < 0.05; ** or ###/§§ p < 0.01; *** or ####/§§§ p < 0.001; not significant (ns) p > 0.05; tendency (T) 0.05 < p < 0.1. Asterisks portray significant differences between WT and ClpP−/− MEF, hashtags illustrate significant effects in brain tissue between 3-month-old WT and ClpP−/− mice, and section signs visualize significant differences in brain of 11-19 month-old WT versus ClpP−/− mice. WT MEF: n= 3-9; ClpP−/− MEF: n= 3-8; WT brain 3 months: n= 3; ClpP−/− brain 3 months: n= 3; WT brain 11-19 months: n= 5; ClpP−/− brain 11-19 months: n= 5-6.

3.5 Quantitative Immunoblot of induced transcription factors, ISGs, nucleic acid sensors and PRRs

To validate the significant and strong transcript dysregulation at the protein level, quantitative immunoblots were employed whenever sufficiently specific and sensitive antibodies were available commercially. The significantly elevated abundances of STAT1, IFIT3, DDX58, TRIM25, and ISG15 in ClpP-null MEFs confirmed the activation of the innate immune defenses from embryonal stage, independent from the adaptive immune system (Figure 6). The significantly elevated abundances of STAT1, DDX58 and ISG15 in the brains of 3-month-old ClpP-null mice demonstrated this neuroinflammation to precede neural phenotypes, given that Perrault syndrome features such as hearing loss, ataxia and white matter degeneration in mice do not appear before ages around 12 months [10]. The even stronger increases of STAT1, IFIT3, and DDX58 abundance in brain of 11-19 month-old ClpP-null mice indicate that their levels correlate with the progression of sterile neuroinflammation. Other factors in the innate immune sensing pathways, such as the interferons IRF3 and IRF7, several NFκB-associated factors and TLR9 did not exhibit elevated abundance (Suppl. Figure S1C-E).
Figure 6. Analysis of protein expression by quantitative immunoblots for (A) the transcriptional immune modulator STAT1, (B) the cytosolic RNA sensor IFIT3, as well as the RLR pathway components (C) DDX58, (D) TRIM25 and (E) ISG15, in MEFs and brain from 3 versus 11-19 month-old mice. Immunoblots were normalized to ACTB or GAPDH. Data are presented as mean ± SEM. In bar graphs, WT is shown as checked, while ClpP−/− as plain colored. Statistical significances are illustrated by symbols: * or # p < 0.05; ** or §§ p < 0.01; *** or §§§ p < 0.001; **** or §§§§ p < 0.0001; not significant (ns) p > 0.05. Asterisks portray significant differences between WT and ClpP−/− MEFs, hashtags show significant effects in brain between 3-month-old WT and ClpP−/− mice, and the section sign visualizes significant differences in brain from 11-19 month-old WT versus ClpP−/− mice. n. s. b. = no specific bands. WT MEF: n= 3-5; ClpP−/− MEF: n= 3-5; WT brain 3 months: n= 3; ClpP−/− brain 3 months: n= 3; WT brain 11-19 months: n= 5; ClpP−/− brain 11-19 months: n= 6.

4. Discussion

Attempting to elucidate the molecular pathways how the deficiency of ClpP as a key molecule in the UPRmt triggers innate immune activation, the data obtained reflect a quite specific profile rather than a generic induction of interferon-stimulated genes, as is readily apparent by the comparison of the 2-fold induction of Ifi35 mRNA versus the >10-fold induction of Ifi44 mRNA (Figure 5A), or the comparison of the strong induction of many RLR factors with up to 7-fold effect size (Figure 5D) versus the singular induction of a NLR factor in MEFs (Figure 5F). This profile seems tuned to optimally detect immune-stimulatory dsDNA and dsRNA, by transcriptional activation of cytosolic DNA and RNA sensors, RLR pathway components, and ISGs. A detectable sensitization is also apparent towards ssRNA, while the sensing factors for muramyl dipeptides from Gram-negative bacteria appeared unchanged (Figure 7). We have previously shown by cGAS- and STING- depletion that the ISG induction depends mostly on the mtDNA [9], so the mRNA extrusion to the cytosol would make only a minor contribution. The observation that many RNA sensors are activated may simply reflect a general antiviral program in view of the large number of very diverse RNA viruses. This scenario is known from TFAM heterozygous knockout cells, appears similarly strong in ClpP homozygous knockout cells, while it appears considerably weaker in PINK1/PARKIN mutants, when the expression upregulation levels and the delay in pathology onset are compared.
Figure 7. Schematic overview of innate immunity signaling, with illustration of the results from this study. TLRs = outlined in pink; RLRs = outlined in red; cytosolic nucleic acid sensors = outlined in orange; NLRs = outlined in green. Significant RT-qPCR results are represented by highlighting the factors in red, green or yellow (red = induced; green = reduced; yellow = not significant/investigated). Significantly altered protein expressions were marked with red or green roofs (red = increased; green = reduced). Dashed lines indicate discussed functions of corresponding factors. += induction; P = phosphorylated; Ub = ubiquitinated; other symbols are defined in the abbreviation list.

Thus, the molecular mechanisms involved in this innate immune activation by endogenous, immunostimulatory nucleic acids from mitochondria are not only relevant in the rare ClpP mutants. So far, the concept prevailed that the Perrault syndrome late manifestations of neurodegeneration and deafness are explained by the impaired translation fidelity in mitochondria, which leads to bioenergetics deficits [8, 50]. If mitochondrial mishandling of nucleic acid / protein complexes triggers innate immune activation and if the neuroinflammatory process is responsible for deafness, ataxia, and leukodystrophy in the later course of Perrault syndrome, then the therapeutic injection of antisense oligonucleotides to deplete STING as integrator of inflammatory signaling might have neuroprotective value. Preventive benefits of STING depletion have already been demonstrated in PINK1 and PARKIN mutants where the neurodegenerative process could be mitigated [29]. The therapeutic efficacy of antisense oligonucleotide (ASO) injections is impressive in patients and animal models with motor neuron degeneration [51, 52]. Our data permit the identification of additional therapeutic targets in these inflammatory signaling pathways. The relevance of neuroinflammation due to nucleic acid toxicity might also hold true for related disorders, where neurodegeneration is triggered by mutations in mitochondrial factors such as TWNK, POLG, TFB1M, MTRNR1, TRMU and GTPBP3, or in components of the mitochondrial and nuclear DNA repair pathway such as RRM2B, or in the cytosolic ribosomal translation machinery amino acid tRNA synthetases. This notion is consistent with the existence of autoimmune disorders where the tRNA synthetases are targeted specifically by autoantibodies, resulting in early-onset myopathy, interstitial lung...
inflammation, skin rash, arthropathy and vasculitis [53-57]. Certainly in the autoimmune vasculitis variants known as Aicardi-Goutières syndrome, where altered degradation of cytosolic DNA or RNA is caused by mutations in TREX1 / SAMHD1 / RNASEH2A/B/C / ADAR / IFIH1 / PNPT1, the progressive immune activation leads to phenotypes of neuro-inflammation/-degeneration [58, 59].

Therefore, the late-onset progressive neuropathy, ataxia, and leukodystrophy in patients with ClpP mutations might be mediated by the innate immune activation. In contrast, the early-onset infertility could be due to meiosis defects, where the handling of nuclear DNA is impaired in similar ways as the handling of mitochondrial nucleoids in ClpP cells.

Not only the severity of inflammatory tissue destruction distinguishes the mutants in TFAM / ClpP / PINK1 / PARKIN, but also the selective massive affection of female and male meiosis upon ClpP loss-of-function, resulting in a complete infertility with azoospermia that is much more severe than the sperm motility impairment usually associated with bioenergetic respiratory or glycolysis failure. [55] Thus, there must be ClpP-specific effects extra-mitochondrially. One candidate seems to be the co-chaperone DNAJA3 that is mostly localized to the mitochondrial matrix, and particularly the short isoform normally has minimal cytosolic retention time, so physiologically only its long isoform would interact with the JAK-STAT pathway [37]. The findings above seem paradoxical, since DNAJA3 abundance increases in the nucleus, while STAT1 abundance is further enhanced in the cytosol. However, a scenario is conceivable where excessive amounts of DNAJA3 interfere with the correct disassembly of nuclear transcription factor complexes, thus overactivating Stat1 gene transcription, while leading to compensatory efforts to retain STAT1 protein in the cytosol and minimize its unwanted overexpression. It is interesting to note a very recent report that the dysfunction of another mitochondrial DnaJ homolog known as DNAJC30, which appears to have a crucial role for the assembly/disassembly and turnover of the respiratory complex N-module, results in a neurodegenerative process that selectively affects the optical nerve [60]. It has previously never been understood how different gene mutations that result in impairment of mitochondrial function would have vastly different tissue-specific consequences, if bioenergetics deficits are the underlying problem. If differently tuned inflammatory consequences of each gene mutation are responsible for the long-term clinical consequences, then the tissue-specificity variance of mitochondrial pathology may be much easier to understand.

5. Conclusions

Overall, our investigation of ClpP-null brains and MEFs detected UPRme anomalies prominently for DNAJA3. Enhanced levels and redistribution were also observed for its interaction partner STAT1. Both were altered in the intra-mitochondrial and extra-mitochondrial compartments. These anomalies may be ClpP-specific, but perhaps they might also be found in other variants of Perrault syndrome. These DNAJA3 anomalies are followed by early-onset deafness in ClpP-mutant patients, while a recent report showed anomalies of the mitochondrial matrix chaperone DNAJC30 to occur in hereditary optic nerve atrophy, so the tissue specific pattern of neurodegeneration in mitochondrial diseases could depend on the molecular profile of mitochondrial pathology and of immunological activation. An induction of Stat1 mRNA expression and widespread transcriptional activation of the innate immune defense was also observed in ClpP-null mice, fine-tuned to the detection of immunostimulatory nucleic acids particularly via cytosolic sensors and RLR signaling. These downstream pathways were similarly affected by ClpP homozygous deletion as in previous reports about TFAM heterozygous deletion, but more severe than findings in PINK1 and PARKIN mutants. Thus, the cytosolic sensors of DNA and RNA might constitute a general response network to dysfunctions in mitochondria and in the cytosolic translation pathway. For all such disorders, the documentation of their innate immune profile and a neuroprotective trial via STING depletion may be rewarding in the future.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1: Profile of innate immune signaling anomalies regarding mRNA expression and protein abundance levels.
Author Contributions: In this collaborative study between four departments in two cities, the individual contributions are defined subsequently: Conceptualization, A.M., J.K., I.W., S.G., S.T.-O., A.P.W. and G.A.; methodology, A.M., J.K., I.W., S.G., G.K, J.C.-P., S.T.-O. and A.P.W.; software, A.M., J.K. and I.W.; validation, A.M., J.K., S.G., G.K. and S.T.-O.; formal analysis, A.M., J.K., S.G., S.T.-O.; investigation, I.W., A.P.W. and G.A.; resources, I.W., S.G., A.P.W. and G.A.; data curation, I.W.; writing—original draft preparation, A.M., J.K. and G.A.; writing—review and editing, I.W., S.G., S.T.-O. and A.P.W.; visualization, A.M. and J.K.; supervision, I.W., A.P.W. and G.A.; project administration, G.A.; funding acquisition, I.W., A.P.W. and G.A.. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

| No. | Abbreviation | Description |
|-----|--------------|-------------|
| 1   | ACTB         | β-actin     |
| 2   | Aim2         | Interferon-inducible protein Absent in Melanoma 2, aka PYHIN4 |
| 3   | aka          | also known as |
| 4   | ASO          | antisense oligonucleotide |
| 5   | ATP          | adenosine tri-phosphate |
| 6   | BAK1         | BCL2-antagonist/killer 1, in OMM |
| 7   | BAX          | BCL2-associated X protein, in OMM |
| 8   | BCL2         | B cell leukemia/lymphoma 2, apoptosis regulator in OMM |
| 9   | BSA          | bovine serum albumin |
| 10  | cDNA         | Complementary deoxy-ribonucleic acid |
| 11  | CEB          | cytosol extract buffer |
| 12  | cGAS         | Cyclic GMP-AMP synthase, aka MB21D1 |
| 13  | ClpB         | Caseinolytic mitochondrial matrix peptidase ATP-binding subunit B |
| 14  | ClpP         | Caseinolytic mitochondrial matrix peptidase proteolytic subunit |
| 15  | ClpX         | Caseinolytic mitochondrial matrix peptidase AAA ATPase chaperonin |
| 16  | DAMP         | damage-associated molecular pattern |
| 17  | DDx58        | DExD/H-box helicase 58, aka RIG-I |
| 18  | DMEM         | Dulbecco’s modified Eagle medium |
| 19  | DNA          | Deoxyribonucleic acid |
| 20  | DnaJ         | E. coli protein J needed for DNA replication, aka Hsp40 |
| 21  | DNAJA3       | DnaJ heat shock protein family (Hsp40) member A3, aka TID1 |
| 22  | DNAJC30      | DnaJ heat shock protein family (Hsp40) member C30 |
| 23  | DPBS         | Dulbecco’s phosphate buffered saline |
| 24  | dsDNA        | double-stranded DNA |
| 25  | dsRNA        | double-stranded RNA |
| 26  | E. coli      | Escherichia coli bacteria |
| 27  | EDTA         | Ethylenediaminetetraacetic acid |
| 28  | EGTA         | Ethylene glycol-bis(β-aminoethyl ether)-N,N′,N,N′-tetraacetic acid |
| 29  | EIF2AK2      | Eukaryotic translation initiation factor 2 alpha kinase 2, aka PKR |
| 30  | ER           | endoplasmic reticulum |
| 31  | ERAL1        | Era-like 12S mitochondrial rRNA chaperone 1 |
| Term | Definition |
|------|------------|
| etc. | etcetera   |
| FBS  | Fetal bovine serum |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GBP3 | Guanylate binding protein 3 |
| GGPS1 | Geranylgeranyl diphosphate synthase 1 |
| GrpE | Gro-P like protein E, in *E. coli* bacteria |
| GRPEL1 | Guanylate binding protein 3, mitochondrial |
| GBP3 | Guanylate binding protein 3, mitochondrial |
| HARS2 | Histidyl-tRNA synthetase 2, mitochondrial |
| HSPB1 | Interferon-activated gene 204 |
| Hsp60 | Heat shock protein with 60 kiloDalton, aka chaperonin, homolog of GroEL |
| Hsp70 | Heat shock protein with 70 kiloDalton, aka DnaK |
| HSPA9 | Heat shock protein family A (Hsp70) member 9; aka Mortalin |
| HRBP | Heat shock protein with 17-kDa dehydrogenase 4 |
| HSPA9 | Heat shock protein family A (Hsp70) member 9; aka Mortalin |
| IFI204 | Interferon type 1 |
| IFI205b | Interferon-activated gene 205b, aka Mnda |
| IFI35 | Interferon-induced protein 35 |
| IFI44 | Interferon-induced protein 44 |
| IFI1H1 | Interferon-induced with helicase C domain 1, aka MDA-5 |
| IFIT1 | Interferon-induced protein with tetraicopeptide repeats 1 |
| IFIT3 | Interferon-induced protein with tetraicopeptide repeats 3 |
| IFN-1 | Interferon type 1 |
| IFNA1 | Interferon alpha 1 |
| IFNAR1 | Interferon (alpha and beta) receptor 1 |
| IFNAR2 | Interferon (alpha and beta) receptor 2 |
| IFNB1 | Interferon-beta 1 |
| IκBα | NF-kappa-B inhibitor alpha, aka NFKBIA |
| IKKα | Inhibitor of nuclear factor kappa-B kinase subunit alpha, aka CHUK |
| IKKβ | Inhibitor of nuclear factor kappa-B kinase subunit beta, aka IKBKB |
| IL18 | Interleukin 18, aka Interferon-gamma-inducing factor |
| IL1B | Interleukin-1 beta, aka Catabolin |
| IL6 | Interleukin-6, aka Interferon beta 2 |
| IRF3 | Interferon regulatory factor 3 |
| IRF7 | Interferon regulatory factor 7 |
| ISG15 | Interferon-induced 15 kDa protein, ubiquitin-like |
| ISGs | Interferon-stimulated genes |
| ISRE | Interferon-stimulated response element |
| JAK | Janus Kinase |
| kDa | kiloDalton |
| LAMIN A/C | Nuclear envelope protein LAMIN splice isoforms A and C |
| LARS2 | Leucyl-tRNA synthetase 2, mitochondrial |
| MAVS | Mitochondrial antiviral signaling protein |
| MEF | Mouse embryonic fibroblasts |
| MLB | Mitochondrial lysis buffer |
| MNDA | Myeloid nuclear differentiation antigen, aka Interferon-activated gene 205b |
| MRG | Mitochondrial ribosomal granule |
| mRNA | Messenger Ribo-nucleic acid |
| mtDAMP | Mitochondrial Damage-associated molecular pattern |
| mtDNA | Mitochondrial DNA |
| mttdsRNA | Mitochondrial double-stranded RNA |
| mtRNA | Mitochondrial RNA |
MTRNR1 mitochondrially encoded 12S rRNA  
NADH Nicotinamide adenine dinucleotide in its reduced form  
NDUFB8 NADH:ubiquinone oxidoreductase subunit B8  
Nfkβ1 Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, aka p105  
NFκB P65 Nuclear factor kappa-B P65 subunit, aka RELA  
NLR Nuclear oligomerization-domain like receptor  
Nlrl3 NLR family, pyrin domain containing 3  
NP-40 Nonyl phenoxypolyethoxylethanol, aka Nonoxynol-40  
ns not significant  
n.s.b. no specific bands  
OAS1B 2'-5' Oligoadenylate synthetase 1B  
OASL2 2'-5' Oligoadenylate synthetase-like 2  
OMM outer mitochondrial membrane  
OXPHOS oxidative phosphorylation  
PARKIN Parkinson juvenile disease protein 2, E3 ubiquitin protein ligase at OMM  
PCR Polymerase chain reaction  
PD Parkinson’s disease  
PEX6 Peroxisomal biogenesis factor 6  
PINK1 PTEN-induced kinase 1, protein in OMM  
PMSF Phenylmethylsulfonyl fluoride, a serine protease inhibitor  –  
PNPT1 Polymorphic origin nucleotide transferase 1  
POLG DNA polymerase gamma, catalytic subunit, mitochondrial  
PORIN mitochondrial outer membrane protein PORIN-1, aka VDAC1  
PRRT3 Perrault syndrome type 3  
PRR Pattern recognition receptor  
PTEN Phosphatase and tensin homolog  
RIPA radio-immune-precipitation assay buffer  
RIP Retinoic acid-inducible gene like receptor  
RMND1 Required for meiotic nuclear division 1 homolog  
RNA Ribo-nucleic acid  
ROS radical oxygen species  
rpm rotations per minute  
RRM2B Ribonucleotide reductase regulatory TP53 inducible subunit M2B  
rRNA ribosomal Ribo-nucleic acid  
RSAD2 Radical S-adenosyl methionine domain containing 2  
RT Room temperature  
RT-qPCR Reverse-transcriptase real-time quantitative polymerase chain reaction  
S. cerevisiae Saccharomyces cerevisiae yeast strain  
SDS Sodium dodecyl sulfate  
SEM Standard error of the mean  
ssRNA single-stranded RNA  
STAT1 Signal transducer and activator of transcription 1  
STAT2 Signal transducer and activator of transcription 2  
STING Stimulator of interferon response cGAMP interactor 1, aka TMEM173  
SUPV3L1 Suppressor of var1, 3-like 1 (S. cerevisiae)  
TANK TRAF family member-associated NF-kappa-B activator  
TBK1 TANK binding kinase 1  
Tbp TATA-box binding protein  
TBS-T Tris-buffered saline/Tween 20  
TFAM Transcription factor A, mitochondrial  
TFB1M Transcription factor B1, mitochondrial
|   | Symbol | Name                     |
|---|--------|--------------------------|
| 136 | TID1   | Tumorous imaginal discs protein Tid56 homolog, aka DNAJA3 |
| 137 | TLR    | Toll-like receptor       |
| 138 | Tlr3   | Toll-like receptor 3     |
| 139 | Tlr9   | Toll-like receptor 9     |
| 140 | Tmem173| Transmembrane protein 173, aka STING1 |
| 141 | TNF-α  | Tumor necrosis factor alpha |
| 142 | TRAF   | Tumor necrosis factor receptor associated factor |
| 143 | TRAP1  | TNF receptor-associated protein 1, aka mitochondrial HSP75 |
| 144 | TRIM25 | Tripartite motif-containing 25 |
| 145 | Trim30a| Tripartite motif-containing 30A |
| 146 | Trim56 | Tripartite motif-containing 56 |
| 147 | TRIS   | Tris(hydroxymethyl)aminomethane |
| 148 | TRMU   | mitochondrial tRNA mitochondrial 2-thiouridylase, aka MTO2 |
| 149 | tRNA   | transfer Ribo-nucleic acid |
| 150 | Tspan6 | Tetraspanin 6 |
| 151 | TWNK   | Twinkle mtDNA helicase |
| 152 | UPR^mt | mitochondrial Unfolded protein response |
| 153 | VDAC   | Voltage-dependent anion channel, aka PORIN, in OMM |
| 154 | v/v    | volume per volume |
| 155 | WB     | Western blot |
| 156 | WT     | Wild-Type |
| 157 | ZFE    | Central Animal Facility, University Hospital Frankfurt/Main |

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Supplementary Material
Figure S1. Profile of innate immune signaling anomalies regarding mRNA expression and protein abundance levels. RT-qPCR of (A) interferons Ifna1 and Ifnb1, (B) analysis of Nfkb1 mRNA expression via RT-qPCR, (C) RT-qPCR of IfN3 and quantitative immunoblots of IRF3 and IRF7 (D) Quantitative immunoblots of NFκB-associated factors, and (E) TLR9. Immunoblots were normalized to ACTB, RT-qPCRs to Tbp. Data are presented as mean ± SEM. WTs are shown as checked, ClpP-/- as plain colored bar graphs. Statistical significances are illustrated by symbols: * or # p < 0.05; ** or §§ p < 0.01; not significant (ns) p > 0.05. Asterisks portray significant differences between WT and ClpP-/- MEFs, hashtags show significant effects in brain of 3 month-old WT versus ClpP-/- mice, and section signs visualize significant differences in brain of 11-19 month-old WT versus ClpP-/- mice. n. s. b.= no specific bands. WT MEF: n= 4-5; ClpP-/- MEF: n= 3-5; WT brain 3 months: n= 2-3; ClpP-/- brain 3 months: n= 3; WT brain 11-19 months: n= 4-5; ClpP-/- brain 11-19 months: n= 4-6.

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