Loss of TRAP1 causes mitochondrial defects in Parkinson's disease through an HtrA2 interaction that are reverted by metformin

Running title: TRAP1 in Parkinson's disease

Julia C. Fitzgerald[1], Alexander Zimprich[2], Daniel A. Carvajal Berrio[3], Kevin M. Schindler[1,4], Brigitte Maurer[1], Claudia Schulte[1], Christine Bus[1], Anne-Katrin Hauser[1], Manuela Kübler[1], Rahel Lewin[1,5], Dheeraj Reddy Bobbili[6], Lisa M. Schwarz[1,5], Evangelia Vartholomaiou[7], Kathrin Brockmann[1], Richard Wüst[1,8], Johannes Madlung[9], Alfred Nordheim[10] Olaf Riess[11], L. Miguel Martins[12], Enrico Glaab[6], Patrick May[6], Katja Schenke-Layland[3,13,14], Didier Picard[7], Manu Sharma [1,15], Thomas Gasser[1], Rejko Krüger[1,6, 16]

1. Department of Neurodegenerative Diseases, Center of Neurology and Hertie-Institute for Clinical Brain Research, University of Tübingen and German Centre for Neurodegenerative Diseases, Tübingen, Germany.
2. Medical University Vienna, Department of Neurology, Vienna, Austria.
3. Department of Women's Health, Research Institute for Women's Health, University of Tübingen, Tübingen, Germany.
4. University of Tübingen, Interfaculty Institute of Biochemistry, Tübingen, Germany.
5. Graduate Training Centre of Neuroscience, International Max Planck Research School, Tübingen, Germany.
6. Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Esch-sur-Alzette, Luxembourg
7. University of Geneva, Department of Cell Biology, Geneva, Switzerland
8. Department of Psychiatry and Psychotherapie, University Hospital Tübingen, Germany
9. Proteome Center Tübingen, Interfaculty Institute for Cell Biology, University of Tübingen, Tübingen, Germany.
10. University of Tübingen, Interfaculty Institute of Cell Biology, Unit of Molecular Biology, Tübingen, Germany.
11. University of Tübingen, Institute of Medical Genetics and Applied Genomics, Tübingen, Germany.
12. MRC Toxicology Unit, University of Leicester, Leicester, United Kingdom.

13. Department of Cell and Tissue Engineering, Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB Stuttgart, Germany
14. Department of Medicine/ Cardiology, CVRL, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States of America.
15. Centre for Genetic Epidemiology, Institute for Clinical Epidemiology and Applied Biometry, University of Tübingen, Germany.
16. Parkinson Research Clinic, Centre Hospitalier de Luxembourg (CHL), Luxembourg

Corresponding author: Julia Fitzgerald. Department of Neurodegeneration, Hertie Institute for Clinical Brain Research and DZNE, Otfried Müller Strasse 27, Tübingen, 72076, Germany. Julia.fitzgerald@uni-tuebingen.de

Abstract

The mitochondrial proteins TRAP1 and HtrA2 have previously been shown to be phosphorylated in the presence of the Parkinson's disease kinase PINK1 but the downstream signaling is unknown. HtrA2 and PINK1 loss of function causes parkinsonism in humans and animals. Here, we identified TRAP1 as an interactor of HtrA2 using an unbiased mass spectrometry approach. In our human cell models, TRAP1 overexpression is protective, rescuing HtrA2 and PINK1-associated mitochondrial dysfunction and suggesting that TRAP1 acts downstream of HtrA2 and PINK1. HtrA2 regulates TRAP1 protein levels, but TRAP1 is not a direct target of HtrA2 protease activity. Following genetic screening of Parkinson's disease patients and healthy controls, we also report the first *TRAP1* mutation leading to complete loss of functional protein in a patient with late onset Parkinson's disease. Analysis of fibroblasts derived from the patient reveal that oxygen consumption, ATP output and reactive oxygen species are increased compared to healthy individuals. This is coupled with an increased pool of free NADH, increased mitochondrial biogenesis, triggering of the mitochondrial unfolded protein response, loss of mitochondrial membrane potential and sensitivity to mitochondrial removal and apoptosis. These data highlight the role of TRAP1 in the regulation of energy metabolism and mitochondrial quality control. Interestingly, the diabetes drug metformin reverses mutation-associated alterations on energy metabolism, mitochondrial biogenesis and restores mitochondrial membrane potential. In summary, our data show that TRAP1 acts downstream of PINK1 and HtrA2 for mitochondrial fine tuning, whereas TRAP1 loss of function leads to reduced control of energy metabolism, ultimately impacting mitochondrial membrane potential. These findings offer new insight into mitochondrial pathologies in Parkinson's disease and provide new prospects for targeted therapies.

Key words

Mitochondrial diseases, Parkinson's disease: cellular mechanisms, Metabolic disease, Genetics: movement disorders, Parkinson's disease, Neuroprotection, Neurodegeneration: other, Neurodegeneration: experimental models, Unfolded protein response.

Abbreviations

ΔΨm	mitochondrial membrane potential
Ant A	antimycin A
COX	cytochrome *c* oxidase

CTRL	control
CTx	cortex
Cyto	cytoplasm
DA	dopamine
ECAR	extracellular acidification rate
ERK1/2	mitogen-activated protein kinase 1
EV	empty vector
HEK	human embryonic kidney cells
H_2O_2	hydrogen peroxide
Hsp90	heat shock protein 90
HtrA2	high temperature requirement protein A2
IB	immunoblot
IP	immunoprecipitation
KO	knock out
Mito	mitochondria
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
OA	oligomycin/antimycin A treatment together
OCR	oxygen consumption rate
PAGE	polyacrylamide gel electrophoresis
Phospho	phosphorylated
PINK1	PTEN-induced putative kinase 1
PPMI	Parkinson's Progression Markers Initiative
Rot	rotenone
SDH	succinate dehydrogenase
SNP	single nucleotide polymorphism
Starve	serum starvation
TRAP1	TNF receptor-associated protein 1
UT	untransfected
Val	valinomycin
WT	wild type

Introduction

Parkinson disease is an etiologically heterogeneous syndrome caused by a combination of genetic and environmental risk factors. At least 85% of cases are sporadic, and at present, there are only symptomatic treatments available, but the advancement of genetic testing and identification of patient endophenotypes has given hope for the emerging field of individualized medicine. Mitochondrial dysfunction, ensuing cellular energy failure and oxidative stress may be one important disease pathway in a subgroup of Parkinson's disease patients (Kruger et al., 2017). The aim is that these patients can be therapeutically targeted or serve as an entry point for precision medicine.

TRAP1 (tumor necrosis factor type 1 receptor associated protein, also known as HSP 75) is a chaperone that resides in the mitochondrial matrix (Altieri et al., 2012). It has a regulatory role in stress sensing in mitochondria allowing cellular adaption to the environment. TRAP1 is recognized as a potential effector protein in Parkinson's disease signaling, since it was found to be phosphorylated by the Parkinson's disease kinase PINK1 (Pridgeon et al., 2007). Loss of function mutations in *PINK1* and *PARK2* (encoding Parkin) cause familial Parkinson's disease (Valente et al., 2004, Kitada et al., 1998) and impair the elimination of damaged mitochondria (Narendra et al., 2010, Geisler et al., 2010). However, beyond mitophagy, there is relatively little known about the mitochondrial quality control pathways in Parkinson's disease.

Chaperones and proteases maintain mitochondrial proteostasis. Tight control of protein quality and turnover inside mitochondria is essential for the function of electron transport complexes which provide energy through oxidative phosphorylation. PINK1 has previously been shown to be required for the phosphorylation of the mitochondrial protease and Parkinson's disease-associated protein HtrA2 (Plun-Favreau et al., 2007). Here we highlight a signaling pathway involving PINK1, HtrA2 and TRAP1, where TRAP1 is the effector modulating mitochondrial chaperone activities and metabolic homeostasis.

The hypothesis that TRAP1 is an important downstream effector in mitochondrial signaling is underscored by reports that TRAP1 rescues mitochondrial dysfunction in neuronal models where PINK1 is silenced (Costa et al., 2013, Zhang et al., 2013). TRAP1 also protects cells from oxidative toxicity caused by respiratory complex I inhibition via an α-Synuclein variant known to induce a genetic form of Parkinson's disease (Butler et al., 2012). TRAP1 protects

mitochondria via its chaperone function (Altieri et al., 2012, Rasola et al., 2014) and by reducing reactive oxygen species (Hua et al., 2007, Im et al., 2007, Masuda et al., 2004).

TRAP1 also acts as a metabolic switch controlling the tumor cell's preference for aerobic glycolysis (Yoshida et al., 2013). ERK1/2 orchestrates the phosphorylation of TRAP1 controlling the metabolic switch (Masgras et al., 2017), which is reportedly via TRAP1 inhibition of succinate dehydrogenase (Masgras et al., 2017, Sciacovelli et al., 2013), although this remains controversial (Rasola et al., 2014). TRAP1 deficiency promotes mitochondrial respiration, accumulation of tricarboxylic acid cycle intermediates, ATP and reactive oxygen species (Yoshida et al., 2013). *TRAP1* deletion in mice does not affect viability and delays the appearance of tumors in a breast cancer model (Vartholomaiou et al., 2017).

Therefore the identification of TRAP1 as a novel HtrA2 interactor prompted us to further explore the PINK1-HtrA2-TRAP1 pathway related to neurodegeneration in Parkinson's disease. Here we show that TRAP1 takes an important role as downstream effector in this pathway and therefore provides an interface between Parkinson's disease and energy metabolism.

Materials and methods

Cell culture

Fibroblast culture from skin biopsies has been previously described by our laboratory (Burbulla and Kruger, 2012). All biopsies and DNA samples were obtained with patient's consent and approval of the local ethics committee and according to the Declaration of Helsinki. HeLa, SH-SY5Y, HEK293 cell culture has been described previously (Burbulla et al., 2014). TRAP1 knockout mouse adult fibroblasts (MAFs) and HtrA2 knockout mouse embryonic fibroblasts have been respectively described by (Yoshida et al., 2013) and (Kieper et al., 2010). Human induced pluripotent stem cells from a PINK1 knockout line generated in our laboratory and its isogenic control were used to generate small molecule neuronal precursor cells (smNPCs) according to (Reinhardt et al., 2013). smNPCs were cultivated in 1:1 DMEM/Ham's F12 (Biochrom, Harvard Bioscience) and Neurobasal (Gibco, Thermo Fisher) medium supplemented with 1% Pen/Strep, 1% Glutamax (Gibco, Thermo Fisher), B-27 Supplement (Gibco, Thermo Fisher), N2 (Gibco, Thermo Fisher), 200 µM ascorbic acid

(Sigma-Aldrich), 3 μM CHIR 99021 (Axon Medchem) and 0,5 μM Purmorphamine (Calbiochem, Merck Millipore) on Matrigel (Corning) coated cell culture dishes.

DNA constructs and RNAs

Human TRAP1 cDNA was cloned into the pIRES vector (Clontech, Takara). GST coupled wildtype HtrA2, A141S HtrA2, and G399S HtrA2 have been previously described (Martins, 2002). Cloning of Wildtype HtrA2 and S306A HtrA2 cDNA into the pcDNA3.1 vector was previously described (Strauss et al., 2005). siRNAs targeting HtrA2 were purchased from Sigma Aldrich (previously described (Fitzgerald et al., 2012) and targeting TRAP1 and non-targetting controls from Dharmacon. (siGENOME SMARTpool #D001206-13-05 POOL#1, Non-targetting siGENOME SMARTpool).

Mass spectrometry

We used recombinant, mature GST-HtrA2 (wildtype HtrA2, HtrA2-A141S, and HtrA2-G399S) as baits and lysates from SH-SY5Y cells. The supernatant contained the fusion proteins that were then bound to glutathione agarose (Molecular Probes, Thermo Scientific) and eluted with imidazole. Analyses were performed on 1D gel pieces of the eluates. The measurements of the peptides derived from tryptic in-gel digest were performed using a nano-HPLC-ESI-MS/MS system (Ultimate (LC Packings / Dionex, Germany) / QStar Pulsar i (Applied Biosystems / Sciex, Germany)), described by (Sauer et al., 2006). MS data were processed against the National Center for Biotechnology Information (NCBI) protein sequence database utilizing the search engine MASCOT (Matrix Science, UK)(Perkins et al., 1999).

Co-immunoprecipitation

HeLa and HEK293 cell lysates were prepared using a lysis buffer (1 % (v/v) Triton-X100, 1× protease inhibitor cocktail (Roche Complete, Roche), 1× phosphatase inhibitor (Roche PhosStop, Roche) and the nuclear material removed following homogenization. Where wild type HtrA2 was overexpressed, HtrA2 was transiently transfected (48h) using Effectine transfection reagent (Qiagen, according to the manufacturer's instructions). Mitochondrial enrichment was previously described (Fitzgerald et al., 2012). Brain tissue from TRAP1 knockout mice previously described (Vartholomaiou et al., 2017) was prepared by separating the cortices from the basal ganglia (mid brain) and cerebellum/brain stem (hindbrain) on ice. Brain tissue lysates were prepared according to (Casadei et al., 2016). Immunoprecipitation

was performed using HtrA2 (R and D Biosciences) or TRAP1 (BD biosciences) antibodies or bovine IgG coupled to protein A Sepharose beads (Sigma Aldrich P9424), according to (Fitzgerald et al., 2012).

SDS-PAGE and Western blotting

Cell lysates were prepared as described for co-immunoprecipitation and proteins electrophoresed on acrylamide gels and transferred to membranes, as previously described (Fitzgerald et al., 2012). Brain tissue lysates from non-transgenic and HtrA2 overexpressing mice previously characterized and described (Casadei et al., 2016) were prepared from whole brain and the total extracts (nuclear material removed) were prepared according to Casadei and colleagues, 2016. Total protein stain (copper pthalocyanine-3, 4', 4' 4'-tetra-sufonic acid tetra sodium salt in 12 mM HCL) and destain (12 mM NaCl). Antibodies against TRAP1 (BD Biosciences), β-Actin (Sigma Aldrich), GAPDH (Invitrogen, Thermo Scientific) Tom20 (Santa Cruz Biotechnology), Hsp60 (BioRad)), α-tubulin (Sigma Aldrich), rodent OXPHOS (#MS604 Mitosciences, AbCam), Hsp70 (Santa Cruz Biotechnology), Hsp90 (BD Biosciences), Human Total OXPHOS (all nuclear encoded subunits from Mitosciences, AbCam), ERK1/2 and P-ERK1/2 (Cell Signaling Technolgy) and mitobiogenesis antibody (containing SDH, GAPDH and COX, AbCam) were used. Secondary antibodies were purchased from GE Healthcare. Densitometry from Western blot was performed using the Image J 1.41o software (Wayne Rasband; National Institutes of Health, USA).

Live cell imaging

Mitochondrial morphology, mass and colocalization studies were visualized using 100 nM MitoTracker® Green FM (Thermo Scientific), lysosomes by 100 nM Lyostracker® Red DND-99 (Thermo Scientific) as previously decribed (Burbulla et al., 2014). Analyses were performed as described previously (Burbulla et al., 2014). The series of images were saved uncompressed and analyzed with AxioVision software (Zeiss) and Image J 1.41o software.

Fluorescence-activated cell sorting (FACS)

Cells were trypsinized and centrifuged at 300 g for 5 minutes and the cells incubated in dye, buffer only or dye plus a control. For early apoptosis, Annexin V-Pacific blue in Annexin V binding buffer (both from BioLegend) or Annexin V-Pacific blue plus Staurosporine was used. For mitochondrial membrane potential, 200 nM Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE, from Thermo Scientific) in Hanks buffer or TMRE plus Carbonyl

cyanide-p-trifluoromethoxyphenylhydrazone (CCCP) 10 μM was used. For mitochondrial reactive oxygen species, 2 μM MitoSox™ (Thermo Scientific) in Hanks buffer or MitoSox™ plus 10μM Rotenone was used. Cells were sorted using a MACSQuant® automated flow cytometer (Mitenyi Biotechnology) according to their mean average fluorescence signal. All mean average fluorescence values were divided by the background fluorescence in the same channel in the same unstained cells to account for auto-fluorescence.

Live Cell Kinetic Measurement of Mitochondrial Membrane Potential

Cells were seeded in Ibidi® dishes and the media exchanged for HBSS containing 200 nM TMRE stain (Thermo Scientific) for 15 minutes at 37°C with CO_2. The TMRE was removed and replaced with 360 μl Hanks buffer. The cells were imaged using a Zeiss Inverted Confocal microscope at Excitation HeNe1, 543 nm and Emission LP 560 nm and brightfield for 20 × 4s cycles. Followed by the addition of 360 μL (0.25 mg/ml Oligomycin), measured for 20 × 4s cycles, 180 μL (10 μM Rotenone), measured for 20 × 4s cycles and 100 μL (10 μM FCCP) and measured for 20-40 × 4s cycles. Using Image J, each transfected cell (detected using ZsGreen-TRAP1) in each frame was analysed for TMRE fluorescence intensity, mean fluorescence and total area. The corrected total cell fluorescence (CTCF) over time was calculated using the formula: CTCF = fluorescence intensity-(cell area × mean background fluorescence).

Genetic Screening by High Resolution Melting Analysis

Both PCR and high resolution melting analysis is performed in the presence of a saturating DNA binding dye. Mutations are detectable because heterozygote DNA forms heteroduplices that begin to separate in single strands at a lower temperature and with a different curve shape than homozygote DNA as described previously (Wust et al., 2016).

Whole Exome Sequencing and consanguinity analysis

Whole exome data were generated from 200 Parkinson´s disease patients from Vienna (100) and Tübingen (100). 3 μg of genomic DNA was fragmented into ~250 bp fragments, end-repaired, adaptor-ligated and sample index barcodes were included. Pooled libraries were enriched with SureSelect Human All Exon 50 Mb kit (AgilentTechnologies) to capture 50 Mb of exonic and flanking intronic regions. Sequencing of post-enrichment libraries was carried out on the Illumina HiSeq 2000 sequencing instrument (Illumina) as 2x100bp paired-end runs. On average, this yielded around 10 Gb of mapped sequences and a > 100x average coverage

for 90% of the targeted sequence per individual. Raw image files were processed by the Illumina pipeline. Reads were aligned to the human reference genome hg19 with the Burrows-Wheeler Aligner. SAM tools were used to identify single-nucleotide variants and small insertions and deletions. Patients were screened for consanguinity using an implemented algorithm of an analyzing tool of the Helmholtz Zentrum, München. Patients with homozygous regions encompassing a total of more than 20 Megabases were considered as likely consanguineous. Particularly, stretches of of >2Mb were surveyed for rare homozygous variants (missense, nonsense, frameshift and splice-site). Variants were further filtered for a minor allele frequency smaller than 1% in the in-house data set of approximately 10.000 control exomes from patients with other unrelated diseases and exomes and in public available databases (ExAC database and 1000 Genomes).

Computational Analysis of TRAP1 Genetic Variants

Computational analysis of TRAP1 variants can be found in the supplementary materials and methods.

Quantitative RT-PCR

Quantitative PCR reactions were performed using FastStart SYBR green Master mix (Roche) to amplify 1 µl of the 1:10 diluted cDNA using 5 µm of each primer h_TRAP1 5' UTR Forward: TTCCCATCGTGTACGGTCCCGC, h_TRAP1 Exon2 Reverse: GGCCCAACTGGGCTGTGGTCC, h_TRAP1 Spanning Exon2-3 Reverse: TGTTTGGAAGTGGAACCCTGC. Housekeeping gene GAPDH primers: Forward: CCATCACCATCTTCCAGGAGCGA, Reverse: GGATGACCTTGCCCACAGCCTTG. Standard curves of each amplified gene were created to calculate the PCR efficiency and relative expression using the efficiency corrected delta–delta Ct method. RNA was prepared from human fibroblasts using Qiashredder® and RNAEasy® preparation kits (Qiagen). 1 µg of RNA was reverse transcribed to cDNA using QuantiTECT® (Qiagen).

Oxygen Consumption and Extracellular Acidification Rate

Oxygen consumption rates (OCR) were measured in whole cells using a Seahorse™ XF96 Extracellular Flux Analyzer (Agilent) according to (Rogers et al., 2011). The concentrations of mitochondrial toxins used were optimized by titration in human fibroblasts according to the manufacturer's recommendations. The final concentration of all toxins used was 1µM and the volume of the toxin injected in each port was sequentially increased by several µl to maintain

the correct final concentration. Human fibroblasts were plated in Seahorse™ XF96 well plates 24h prior to measuring at a density of ~15,000 cells per well. The OCR for each well was corrected for cell number. Stained nuclei were counted using high content image capture and analysis using the BD Pathway 855 (BD Biosciences). Extracellular acidification rates (ECAR) from the same experiments provide an indication of glycolytic activity and were normalized to OCR/cell to account for the cell numbers in each well in each experiment.

Complex I Activity

Following isolation of crude mitochondria from approximately five million cells, described previously by (Burte et al., 2011), complex I activity was measured according to Hargreaves and colleagues (Hargreaves et al., 2007). The activity of complex I was normalized to citrate synthase activity, also according to (Hargreaves et al., 2007) and data expressed as a ratio of complex I/citrate synthase.

ATP

The concentration of ATP per µg of total protein was measured using the ATPLite™ Kit from Perkin Elmer. ATP standards are used to determine the concentration of ATP in a cell lysate replicated a minimum of three times in each experiment. µM concentration of ATP is expressed per µg of total protein in each well as measured by protein assay (BioRad).

Fluorescence Lifetime Imaging Microscopy (FLIM)

A detailed description of the FLIM method can be found in the supplementary information and is described in (Lakner et al., 2017).

Measurement of NAD^+/NADH levels

NAD^+ and total NAD^+ and NADH levels were measured using a fluorometric assay kit (Abcam). The levels of NAD^+ and NADH were quantified using standards and normalized to total protein in each sample according to protein assay (BioRad).

Statistics

Analyses of statistical significance were performed using GraphPad Prism 6.0 and the relevant statistical test. The statistical test used and the *p* values are shown in the figure legends. All cell culture experiments (including all imaging and FACS experiments) were performed a minimum of three times, using a different cell passage and on different days. In

the genetic studies, the initial screening by high temperature melt analysis was performed on two hundred and eighty German Parkinson's disease patients and a group of one hundred and ninety two healthy individuals. The exome sequencing was performed on the DNA from two hundred Parkinson's disease patients collected in Tübingen, Germany and Vienna, Austria.

Results

TRAP1 interacts with HtrA2 under physiological conditions

We have previously reported loss of function mutations in *HtrA2* in Parkinson's disease patients and therefore performed unbiased mass spectrometry on GST-HtrA2 baited SH-SY5Y lysates to identify novel interaction proteins (Figure 1A). We identified TRAP1 as an interactor of HtrA2 with the relevant controls.

To confirm the physical interaction, HtrA2 immunoprecipitations were performed in HeLa cells overexpressing HtrA2 or not. Immunoblotting revealed the presence of TRAP1 in the HtrA2 immunoprecipitation (endogenous and overexpressed HtrA2) but not in the IgG control (Figure 1B), enriched in the mitochondrial fraction. Knockdown of TRAP1 using siRNA reduced the amount of TRAP1 interacting with HtrA2, confirming the specificity of the immunoprecipitation (Figure 1C). The interaction between HtrA2 and TRAP1 occurs in mouse brain (cortex, midbrain and hindbrain) as demonstrated by the immunoprecipitation of TRAP1 with HtrA2 in extracts from wildtype mice and not TRAP1 knockout mice (Figure 1D).

To investigate the relevance of the HtrA2-TRAP1 interaction we monitored the amount of TRAP1 immunoprecipitated with HtrA2 under several stress conditions. We found that acute treatment with the mitochondrial toxins rotenone and antimycin A abolished the interaction in human HEK293 cells and this was due to reduced TRAP1 and not a global reduction of total protein (Figure 1E). We then assessed the influence of several other stressors, this time the concentrations of the toxins were titrated for HeLa cells and for the cells to survive a chronic treatment over a 24h period. We found that dopamine treatment had no effect on the interaction of HtrA2 and TRAP1, whereas, the TRAP1 inhibitor 17-AAG, hydrogen peroxide, the ionophore valinomycin and mitochondrial respiratory inhibitors oligomycin, antimycin A, and rotenone all largely reduced or abolished the interaction (Figure 1F). These data from two different human cell lines suggest that the interaction of HtrA2 and TRAP1 serves the mitochondria under normal physiological conditions, under starvation and dopamine toxicity but not respiratory inhibition.

TRAP1 rescues HtrA2 and PINK1 loss of function phenotypes but is not a proteolytic substrate of HtrA2

We hypothesized that HtrA2 interacted with TRAP1 to degrade it since HtrA2 is a key mitochondrial protease and the levels of TRAP1 appear to be a key factor in mitochondrial control (Zhang et al., 2015, Amoroso et al., 2016, Lv et al., 2016, Kang, 2012). Using PhosTag™ SDS-PAGE, we found a significant increase in the levels of phosphorylated and non-phosphorylated TRAP1 when we immunoprecipitated endogenous TRAP1 in the absence of HtrA2 (Figure 2A). We also found that stimulation of PINK1 kinase with the ionophore valinomycin (at concentrations known to induce accumulation of PINK1, (Rakovic et al., 2013)), increased the amount of phosphorylated TRAP1 in wildtype HtrA2 MEFs (Figure 2A). Phosphorylated TRAP1 levels were increased to the same extent in HtrA2 knockout MEFs, whether treated with valinomycin or not (Figure 2A). However, there was no significant effect of PINK1 knockout on TRAP1 phosphorylation status in neuronal progenitor cells (Figure 2A).

Overexpression of wildtype HtrA2 in human cells from four independent experiments (Figure 2B) or in mice (Figure 2C) results in reduced TRAP1 protein levels. However, overexpression of a protease dead form of HtrA2 (S306A), which is catalytically inactive but still targeted to the mitochondria (Martins et al., 2002) in human cells has the same effect on TRAP1 levels as the wildtype, indicating that the protease activity of HtrA2 is not important for the interaction between TRAP1 and HtrA2 (Figure 2D).

The HtrA2-TRAP1 interaction is not a protease-substrate interaction, yet TRAP1 is likely downstream of HtrA2 since the overexpression of TRAP1 rescues the HtrA2 knockdown-induced loss of mitochondrial membrane potential (Figure 2E), reduced basal oxygen consumption (Figure 2F), increased mitochondrial reactive oxygen species (Figure 2G) and sensitivity towards serum starvation induced apoptosis (Figure 2H). TRAP1 overexpression also rescues the reduced mitochondrial membrane potential observed in PINK1 deficient neuroprogenitor cells measured over a time course inclusive of mitochondrial toxin controls (Figure 2I).

TRAP1 loss of function in Parkinson's disease

Mutations in PINK1 cause early onset Parkinson's disease (Valente et al., 2004) and HtrA2 risk variants have been reported in German (Strauss et al., 2005) and Belgian (Bogaerts et al., 2008) Parkinson's disease cohorts. Therefore, we used the exome sequencing data from the Parkinson's Progression Markers Initiative (PPMI), to determine all non-synonymous TRAP1 single nucleotide variants with a minor allele frequency < 1% in the European Non-Finnish

population (ExAC, (Lek et al., 2016)) and an odds ratio > 1 in Parkinson's disease patients compared to controls. Variants (Suppl. Figure 1C) were further filtered by selecting those 'damaging', 'probably damaging' or 'likely damaging' and where crystal structures are available. All three resulting variants are located within known functional domains: S221P falls within a histidine kinase-like ATPase domain (HATPase_c, InterPro: IPR003594), and H311Q and R469C are positioned within an HSP90 domain (Pfam database: PF00183). However, none of these variants overlapped with known ubiquitination, acetylation or phosphorylation sites (Suppl. Figure 1A). The residues for variants S221P and R469C are largely buried (5% and 13% solvent accessibility,), whereas variant H311Q affects a residue that is partially accessible (25%) and could alter protein-protein interactions via residue size and charge alterations. In a multiple sequence alignment, high sequence conservation was observed for residues H311 and R469, but not for S211 (Suppl. Figure 1B). Correspondingly, a destabilizing effect was predicted by the majority of algorithms for H331Q and R469C, while the S221P variant was estimated to be neutral. Notably, R469C was also predicted to decrease the chaperone binding function of TRAP1 (LIMBO software, van Durme et al., 2009).

We used high resolution melting to screen for sequence variations in the TRAP1 gene in the genomic DNA from German Parkinson's disease patients and a group of healthy individuals. We detected several genetic variants, further identified as single nucleotide polymorphisms (listed in Suppl. Figure 1C). Burden analysis of *TRAP1* was performed using the Parkinson's Progression Markers Initiative (PPMI) dataset (summarized in Suppl. Figure 1D). The burden analysis showed that loss-of-function mutations in TRAP1 are rare, yet, there is an enrichment (p-values <0.05, not corrected) of rare (heterozygous) non-deleterious missense TRAP1 variants, and the effect is protective, which fits our functional data. In parallel, we analyzed two hundred exomes of Parkinson's disease patients from Austria and Germany for consanguinity. In addition to TRAP1 variants (listed in Suppl. Figure 1C), we found a moderate, but significant consanguinity of about 20 Mb in a German Parkinsons disease patient. One homozygous stretch encompasses 5 Mb at Chr.16, including the TRAP1 gene. Here we found a homozygous c.C158>T (R47X) mutation (Figure 3A). This mutation is not present in 10,000 control exomes of the Helmholtz database; however it occurs 12 times heterozygously in the ExAC database (60,000 controls). The R47X TRAP1 Parkinson's disease patient has no rare variant in any of the other established Parkinson's disease genes (See Supplementary materials and methods for the full list of genes).

The homozygous p.Arg47Ter single nucleotide exchange (R47X) in exon 2 of TRAP1 leads to a premature stop codon and truncation at the transit sequence of TRAP1 in a late-onset Parkinson's disease patient (Figure 3A). A TRAP1 antibody that binds at a region of TRAP1 encompassing amino acids 253-464 (shown in Figure 3B) was as expected unable to detect TRAP1 protein in fibroblasts biopsied from the R47X patient (Figure 3C). Using PCR primers upstream and downstream of the mutation to amplify patient cDNA, we found that TRAP1 RNA is present, suggesting no nonsense-mediated RNA decay (Figure 3D). The R47X TRAP1 patient was diagnosed with Parkinson's disease at age 70 years. There is no family history of Parkinson's disease but the mother of the index patient had dementia. The R47X patient has also been diagnosed with dilated cardiomyopathy, chronic pancreatitis, polyneuropathy and chronic kidney insufficiency (detailed in Figure 3E).

TRAP1 R47X Parkinson's disease patient mitochondria meet ATP demand but have reduced membrane potential

To understand the relevance of the R47X TRAP1 mutation, we assessed several readouts of mitochondrial form and function in patient-derived fibroblasts. There were no obvious differences in mitochondrial morphology between controls and the index patient under basal or serum starvation conditions (binary z-stack images shown in Figure 4A). Computational analysis of z-stack images revealed no difference in average mitochondrial size (Figure 4B) or mitochondrial branching (Figure 4C). However, after serum starvation there was a significant fragmentation of mitochondria in the R47X fibroblasts compared to controls (Figure 4D).

We measured the co-localization of mitochondria and lysosomes in patient and control fibroblasts and the results show similar co-localisation of mitochondria with lysosomes under normal physiological conditions. Following mild induction of autophagy by serum withdrawal, we found that mitochondria to lysosome translocation was more pronounced in TRAP1 R47X cells (Figure 4E), suggesting increased mitochondrial turnover.

Respiratory analysis of patient cells and controls, recorded oxygen consumption during a mitochondrial stress test, where minimal, maximal and inhibited respiration is induced by oligomycin, the uncoupler FCCP and antimycin A respectively (Figure 4F *left panel*). Basal respiration was significantly increased in R47X fibroblasts (Figure 4F *right panel)*. The extracellular acidification rate (an indicator of glycolysis) normalized to the rate of oxygen consumption per cell in R47X fibroblasts was generally higher than that of healthy controls (Figure 4G *left panel*). However, the calculated glycolytic shift after the addition of

oligomycin is similar between a patient and controls (Figure 4G *right* panel). Complex I enriched in mitochondrial extracts of R47X cells was significantly more active in oxidizing NADH given as a substrate along with decylubiquinone *in vitro* than healthy individuals (Figure 4H). Furthermore, significantly more ATP was produced in R47X fibroblasts compared to controls (Figure 4I), indicating that the complexes of the respiratory chain are not damaged and suggesting that mitochondria in R47X patient cells have increased respiratory activity.

TRAP1 deficiency is reported to promote increases in mitochondrial respiration, ATP levels and reactive oxygen species in mice (Yoshida et al., 2013). Therefore we identified the index patient in a previous study measuring mitochondrial reactive oxygen species in sporadic Parkinson's disease patient fibroblasts. The index patient has above average levels of mitochondrial reactive oxygen species, but are only mildly elevated in comparison to several other sporadic Parkinson's disease patients, genetic Parkinson's disease (PINK1, Parkin and DJ-1 patients) and Leigh syndrome patient fibroblast lines (Figure 4J). Finally, mitochondrial membrane potential was significantly reduced in TRAP1 R47X fibroblasts compared to controls (Figure 4K).

Metformin rescues the R47X phenotype via a mechanism involving mitochondrial biogenesis

In ovarian cancer, TRAP1 silencing causes resistance to chemotherapy drugs because oxidative phosphorylation is increased. Interestingly, the resistance to chemotherapy could be reversed by mild inhibition of mitochondrial respiration by the diabetes drug metformin or oligomycin (Matassa et al., 2016a). Therefore, we treated TRAP1 R47X patient fibroblasts with 10 mM metformin, sub-lethal concentrations of oligomycin and the antioxidant N-acetyl cysteine (NAC) to see whether we could rescue the patient phenotype. Metformin and oligomycin treatment restored the mitochondrial membrane potential observed in the patient, whereas the antioxidant NAC could not (Figure 5A).

We subjected human cancer cells to a range of toxins and stressors and measured their effect on mitochondrial membrane potential. Dopamine, hydrogen peroxide and the ionophore valinomycin greatly reduced mitochondrial membrane potential and this could not be rescued by metformin. Dopamine toxicity was protected by addition of the antioxidant NAC and metformin treatment alone does not reduce mitochondrial membrane potential (Figure 5B). Finally, reduced mitochondrial membrane potential induced by the Hsp90 family/TRAP1

inhibitor 17-AAG could be reversed by metformin (Figure 5B), suggesting there is a specific effect of metformin in paradigms related to TRAP1.

To investigate further the mechanism by which metformin is protective in our model, we measured the fluorescence lifetime of NADH in living cells from the TRAP1 R47X patient and healthy individuals with and without treatment with metformin. Bound NADH indicates usage in mitochondrial respiration, whereas free NADH is associated with glycolysis (Bird et al., 2005, Blacker et al., 2014). We found significantly reduced bound NADH and increased free NADH following metformin treatment in all cell types (Figure 5C). This finding supports the observation that metformin suppresses gluconeogenesis (Kim et al., 2008), inhibits complex I (Owen et al., 2000) and shift the balance between coupling and uncoupling reactions via the TCA cycle (Andrzejewski et al., 2014). We found a similar bound/unbound NADH ratio in the untreated R47X patient fibroblasts as in the metformin treated controls and the addition of metformin in the patient did not reverse the bound/unbound NADH ratio (Figure 5C top panel). These data although highly significant represent overall a very small shift in the total levels of bound versus unbound NADH levels (Figure 5C bottom panel). The data suggests that the protective mechanism of metformin in the R47X patient is not via the metabolic switch between oxidative phosphorylation (OXPHOS) and glycolysis. These data could mean that either glycolysis is favored in the R47X patient or mitochondrial turnover and/or the NAD^+/NADH pool are altered.

NAD^+ and combined NAD^+ and NADH levels are significantly increased in TRAP1 R47X patient cells compared to controls (Figure 5D). Metformin treatment in healthy individuals and in TRAP1 R47X patient cells lowers both NAD^+ and total NAD^+ and NADH levels in one control and the patient but not significantly (Figure 5D).

We observe a significantly reduced ratio of succinate dehydrogenase (SDH) to cytochrome c oxidase (COX mtDNA-encoded subunit) in both R47X patient fibroblasts and TRAP1 knockout MAFs compared to controls (Figure 5E left and middle panel), which indicates an imbalance between nuclear and mitochondrially encoded mitochondrial proteins (termed mitonuclear imbalance), likely induced by the increased NAD^+ and NADH pool and in agreement with the effect of NAD^+ boosters on the age associated metabolic decline and promotion of longevity in worms (Mouchiroud et al., 2013).

Metformin is able to reverse the mitonuclear imbalance in the TRAP1 R47X patient fibroblasts (Figure 5E right panel), indicating that the mitonuclear imbalance is the converging step in the survival pathway that can be targeted pharmacologically. Mitonuclear protein imbalance controls longevity in mammals via induction of the mitochondrial unfolded protein response (mtUPR) (Houtkooper et al., 2013, Mouchiroud et al., 2013). Therefore we monitored the levels of Hsp60, Hsp70 and mitochondrial Hsp90, three markers of the mtUPR. We found that on average both Hsp60 and mtHsp70 levels were higher in R47X TRAP1 patient fibroblasts compared to two healthy controls in three independent experiments (Figure 5F). Hsp90 levels were also elevated but the difference was not significant (Suppl. Figure 2C). These data suggest that in TRAP1 loss of function models, the mtUPR is upregulated. This is associated with increased turnover of mitochondria and the significant elevation of subunits of mitochondrial respiratory complexes I, II, III and IV, which is also rescued by metformin (Suppl. Figure 2B). Phosphorylated ERK1/2 orchestrates metabolic switching via TRAP1 (Masgras et al., 2017). Here we found that the levels of phosphorylated ERK1/2 are increased in the index patient fibroblasts and can be reversed by metformin (Suppl. Figure 2D).

Discussion

TRAP1 and HtrA2 are targets of the Parkinson's disease kinase PINK1 (Plun-Favreau et al., 2007, Pridgeon et al., 2007). However, how these three proteins act together in Parkinson's disease signaling still remains to be elucidated. One of the barriers to dissecting a pathway involving HtrA2 and TRAP1 was the lack of mechanistic evidence for the downstream mitochondrial function observed.

Here we have shown that HtrA2 and TRAP1 physically interact and regulate each other. The biochemistry of the interaction is non-canonical and does not involve the protease activity of HtrA2, leaving us to speculate that HtrA2 and TRAP1 perform in a common intramitochondrial chaperoning or quality control system. In this study, overexpression of the catalytically inactive HtrA2 S306A reduces TRAP1 protein levels to the same extent as wild type HtrA2. As in mnd2 mice carrying the S276C HtrA2 mutation, HtrA2 is catalytically inactive and as the mice phenocopy the mitochondrial dysfunction and neurodegeneration seen in HtrA2 knockout mice (Martins et al., 2004), S306A is also unlikely to rescue HtrA2 loss of mitochondrial function. However, HtrA2 possesses chaperone activity in its basal state (Li et al., 2002). Protease dead HtrA2 could still bind TRAP1 via its PDZ domain. The PDZ

domain of HtrA2 has a "YIGV" recognition pattern but also detects long hydrophobic stretches (Zhang et al., 2007), preferentially C-terminal peptides (Clausen et al., 2002). Interestingly, analysis of TRAP1 hydrophobicity shows a hydrophobic stretch at the C-terminal (Suppl. Figure 2A) and therefore an alternative mode of interaction by association should not be ruled out. Other mitochondrial proteases could also be influencing TRAP1 and loss of HtrA2 could trigger other proteases in order to maintain proteostasis, which displays some redundancy.

One concept that links HtrA2 and TRAP1 in the context of neurodegeneration is the mtUPR. The mtUPR is a highly conserved cellular response activated when the accumulation of unfolded or misfolded proteins goes beyond the chaperone capacity of the mitochondria (Pellegrino et al., 2013). The mtUPR activates transcription of nuclear-encoded mitochondrial chaperone genes to promote protein homeostasis within mitochondria. HtrA2 levels are increased during mtUPR (Spiess et al., 1999) and loss of HtrA2 contributes to transcriptional stress response (Moisoi et al., 2009). Overexpression of TRAP1 activates mtUPR and extends lifespan in *Drosophila* (Baqri et al., 2014) and TRAP1 inhibition promotes the mtUPR response in *Caenorhabditis elegans* (Munch and Harper, 2016).

TRAP1 gain of function rescues PINK1 (Zhang et al., 2013) and PINK1/Parkin loss of function in *Drosophila* (Costa et al., 2013) and here we can show that TRAP1 rescues HtrA2 and PINK1 loss of function in human cells. In addition to its role as a chaperone, TRAP1 is also involved in metabolic switching (Yoshida et al., 2013, Sciacovelli et al., 2013, Rasola et al., 2014, Masgras et al., 2017). Our data from R47X TRAP1 deficient Parkinson's disease patient fibroblasts provides a mechanism involving mitochondrial metabolism.

TRAP1 mutations could be important for our understanding of the underlying biological mechanisms that lead to Parkinson's disease. Our statistical analysis of TRAP1 genetic burden using the PPMI dataset showed that TRAP1 loss-of-function mutations in Parkinson's disease patients are rare. The role and influence of rare variants in complex diseases is a debated subject, and data generated so far indicated that common and rare variants are not mutually exclusive. The PPMI repository (with 300 Parkinson's disease cases), generated no significant association and further highlights the need for increased statistical power to investigate the impact of rare and low frequency variants in Parkinson's disease. Interestingly,

we found that there is an enrichment of rare (heterozygous), non-deleterious missense TRAP1 variants and the effect is protective, which fits the functional data.
In 2014, Luykx and colleagues hinted that TRAP1 variants are associated with neurotransmitter metabolism and Parkinson's disease. The authors performed a genome wide association study (GWAS) analyses and found a significant association of the ratio of HVA/ 5-HIAA, indicating enhanced monoamine turnover in variants of 6 genes, among them were PINK1 and TRAP1 (Luykx et al., 2014), further supporting the genetic contribution of TRAP1 to Parkinson's disease.

In the case reported here, a homozygous stop mutation in TRAP1 in a Parkinson's disease patient leads to complete loss of the TRAP1 protein. TRAP1 mutations have previously been associated with chronic pain, fatigue and gastrointestinal dysmotility (Boles et al., 2015), a recognized common dysfunction in Parkinson's disease (Pfeiffer, 2003). One highly conserved variant in this study (p.Ile253Val) was also identified in both German and Austrian Parkinson's disease patients. Furthermore, recessive mutations in TRAP1 were identified in two families with congenital abnormalities of the kidney and urinary tract (CAKUT) and VACTERL association (congenital abnormalities in multiple organs) (Saisawat et al., 2014). Interestingly, the late-onset Parkinson's disease patient with a homozygous stop mutation (R47X) in TRAP1 described here was also diagnosed with chronic pancreatitis and, chronic kidney insufficiency but not diabetes. The R47X patient also shows other symptoms related to mitochondriopathies such as cardiomyopathy, polyneuropathy, sleep apnea and cataracts. Studies in mice have shown that TRAP1 overexpression protects against cardiac hypertrophy (Zhang et al., 2011) and underscores the link between TRAP1 defects and mitochondriopathy.

In line with previous work performed in TRAP1 knockout mice (Yoshida et al., 2013), TRAP1 deficient patient fibroblasts show increased respiration, complex I activity and ATP output. We also found more unbound NADH, which indicates favoring of glycolysis. However, these changes although highly significant are overall very small, which might reflect the low metabolic demand in fibroblasts compared to neurons. Unbound NADH could also come from the NAD+/NADH pool, which is increased in mitochondrial biogenesis. NAD^+ metabolism engages key effectors of longevity, and interestingly modulating NAD^+ levels has become a focus for intervention in age-related diseases (Karpac and Jasper, 2013). NAD^+ signals mitochondrial biogenesis via the sirtuin pathway, this impacts mitonuclear protein balance and initiates the mtUPR, promoting longevity (Mouchiroud et al., 2013).

Altered stoichiometry between nuclear and mtDNA encoded proteins (mitonuclear protein balance) is a conserved longevity mechanism across many species. Mitonuclear protein imbalance is coupled with the activation of the mitochondrial unfolded protein response (mtUPR), activation of mitochondrial chaperones and longevity (Houtkooper et al., 2013). Mitochondrial biogenesis, normal aging, mitochondrial transcription and translation all influence the balance of nuclear and mtDNA encoded mitochondrial proteins (Houtkooper et al., 2013).

The diabetes mellitus type 2 drug metformin was investigated in this study because of its ability to reverse TRAP1-dependent chemotherapy resistance in ovarian cancer (Matassa et al., 2016b). The ability of metformin (and not an antioxidant) to rescue the reduced mitochondrial membrane potential phenotype is of particular interest since metformin has previously been shown to be protective in Parkinson's disease models (Perez-Revuelta et al., 2014, Patil et al., 2014) and there are significantly less cases of Parkinson's disease in diabetes mellitus type 2 patients taking metformin (Wahlqvist et al., 2012). We propose that loss of TRAP1 hinders the fine tuning of energy metabolism, proteostasis and the mtUPR response. It is this fine tuning that over time, when not available, pushes the cell in favor of meeting immediate energy needs, rather than energy restriction. Further work to generate induced pluripotent stem cells from the TRAP1 R47X patient fibroblasts and gene correct the mutation would confirm unequivocally cause of disease. In conclusion, loss-of-function mutations in TRAP1 are rare, yet analyses of the biological pathway involving TRAP1, show that TRAP1 is important for mitochondrial signaling in Parkinson's disease. These data also underscore the role of rare variants in the pathogenesis of Parkinson's disease and suggest that treatments other than antioxidants should also be considered for individualized therapies in etiologically heterogeneous syndromes such as Parkinsonism.

Acknowledgements

Data used in the preparation of this article were obtained from the Parkinson's Progression Markers Initiative (PPMI) database (www.ppmi-info.org/data). For up-to-date information on the study, visit www.ppmi-info.org.

Funding

The project underlying this publication was funded by the German Federal Ministry of Education (BMBF) and Research under the support code 031 A 430 A. Responsibility for the content lies with the author Rejko Krüger. This is also an EU Joint Programme - Neurodegenerative Disease Research (JPND) project. The study was supported by a grant of the German Research Council to RK (DFG KR2119/3-2) and the Fonds National de Recherche (FNR, PEARL) Luxembourg. E.G. acknowledges support by grants from the Luxembourgish Fonds Nationale de la Recherche (through the National Centre of Excellence in Research (NCER) on Parkinson's disease, I1R-BIC-PFN-15NCER, and grant C13/BM/5782168). Bioinformatics analyses presented in this paper were carried out in part using the HPC facilities of the University of Luxembourg (see http://hpc.uni.lu).
K.S.-L. acknowledges support by the Ministry of Science, Research and the Arts of Baden-Württemberg (33-729.55-3/214 and SI-BW 01222-91), and the Deutsche Forschungsgemeinschaft (INST 2388/30-1). EV and DP were supported by the Swiss National Science Foundation. PPMI – a public–private partnership – is funded by the Michael J. Fox Foundation for Parkinson's Research and funding partners, including Abbvie, Avid, Biogen, Bristol-Myers Squibb, Covance, GE Healthcare, Genentech, GlaxoSmithKline, Lilly, Lundbeek, Merck, Meso Scale Discovery, Pfizer, Piramal, Roche, Servier, Teva, UCB, and Golub Capital.

Supplementary Material

Supplementary Materials and Methods

Fluorescence lifetime imaging microscopy (FLIM)

A 5D multiphoton microscope system (JenLab) was employed in this study as described in detail before (Lakner et al., 2017). The femtosecond laser was used to excite the sample through a 40x oil immersion objective with a numerical aperture (NA) of 1.3 (EC Plan-

Neofluar 40x/1.30 Oil DIC – Zeiss). Coarse location was done using micrometer screws in all three axes, fine locating was achieved by dislocating the galvanometer scanners, and z axis fine focusing was executed by using a piezo-electrical control element. The TCSPC module (Becker & Hickl GmbH) was used to collect emission data pixel by pixel with photon arrival time as well as the location coordinates based on the position of the galvanometer scanners. After the region of interest was located with multiphoton imaging (25 mw for 23 seconds), the multiphoton microscope was switched to FLIM mode for the acquisition of the decay data. The emission maximum of NADH is located at ≈470nm (Huang et al., 2002); therefore, a spectral emission filter (425 to 509 nm) was used to isolate the NADH signal. All recording settings were adjusted in the software SPCM and DCC (both Becker & Hickl GmbH). The laser power was kept at 25 mW; however, FLIM measurements required a longer acquisition time when compared with the multiphoton imaging. Each FLIM measurement had a total recording time of 180 seconds and was controlled via the SPCM software. The photomultiplier tubes (PMTs) were enabled and managed with the DCC interphase. Similar to multiphoton imaging measurements, FLIM data acquisition is extremely sensitive to light; therefore, total darkness was also required to avoid unspecific noise and damage to the PMTs due to electrical overload. The resulting FLIM data, containing all pixels with their corresponding time correlated decays, were saved as SDT – files (a proprietary file extension of the SPCM software). Free and protein-bound NADH contributions were calculated with a biexponential decay fitting using the software SPCImage (Becker & Hickl GmbH). 30% of the maximum of photon numbers of the brightest pixel were used as a threshold. More detailed information on the methods is described in (Lakner et al., 2017).

Computational analysis and bioinformatics

Analysis of TRAP1 variants in the Tübingen-Vienna cohort

The investigated non-synonymous single nucleotide variants in TRAP1 were classified into having potentially damaging or neutral effects using the dbNSFP 3.3a (Liu et al., 2016) database and the software ANNOVAR (Wang et al., 2010). For non-synonymous variants, the following fifteen prediction methods were used: SIFT (Kumar et al., 2009), Polyphen2 (Adzhubei et al., 2010), LRT (Chun and Fay, 2009), MutationTaster (Schwarz et al., 2010), MutationAssessor (Reva et al., 2011), PROVEAN (Choi et al., 2012), CADD (Kircher et al., 2014), DANN (Quang et al., 2015), Eigen (Ionita-Laza et al., 2016), Revel (Ioannidis et al., 2016), M-CAP (Jagadeesh et al., 2016), MetaSVM (Liu et al., 2016), MetaLR (Liu et al.,

2016), fathmm-MKL (Shihab et al., 2014). For CADD variants are defined as 'damaging' if the CADD rawscore>5, for DANN if the score > 0.99, for VEST3 if the score >0.9, for Eigen if the score >0.8, for Revel if the score > 0.6, and for MutationAssessor if the prediction is "H" or "M". For all other tools the variant was considered as 'damaging' when assigned to the category "D. A variant is then defined as 'damaging', when predicted damaging by 12 or more tools, 'likely damaging' by 8 to 11 tools, 'possible damaging' by 4 to 7 tools and 'not likely damaging' by 1 to 3 tools. Additionally, conservation was scored by three tools: GERP_RS >3 (Cooper et al., 2005), SiPhy>10 (Garber et al., 2009) and phyloP>0.95 (Siepel et al., 2006). Conservation was classified into three categories: 'highly conserved' (3 tools), 'conserved' (1 or 2 tools), and 'not conserved' (no tool).

For the identified variants with potentially damaging effects located in protein regions covered by public TRAP1 crystal structures, the effects on protein structure stability were estimated with the prediction methods SDM (Worth et al., 2011), I-Mutant 3.0 (Capriotti et al., 2005), NeEMO (Giollo et al., 2014), SNPeffect 4.0 (De Baets et al., 2012), mCSM and DUET (Pires et al., 2014). Potential mutation effects on chaperone binding were predicted using LIMBO (Van Durme et al., 2009). Protein functional domains and post-translational modification sites in TRAP1 were determined using the public resources GeneCards (Safran et al., 2010), InterPro, Pfam (Finn et al., 2017, Finn et al., 2016), and PinSNPs (Lu et al., 2016). To generate a visualization of the protein structure with color-coded sequence conservation and the underlying multiple sequence alignment, using homologues of the TRAP1 sequence from the UNIREF90 database, the Consurf software was used (Ashkenazy, 2010).

Analysis of TRAP1 burden in Parkinson's disease using The Parkinson's Progression Markers Initiative (PPMI) dataset

The Parkinson's Progression Markers Initiative (PPMI) study is an effort to identify the biomarkers of PD progression. We used the whole exome sequencing data available as part of this project. Detailed information about this initiative and the data can be found on the website (http://www.ppmi-info.org/). Briefly, the variants were called following GATK (DePristo et al., 2011) best practices. The initial PPMI exome dataset comprised of 404 PD and 183 healthy controls.

Sample quality control (QC):

Samples with >3 standard deviation (SD) from the QC metrics (number of alternate alleles, number of heterozygotes, Ti/Tv ratio, number of singletons and call rate) that were calculated

by using PLINK/SEQ i-stats (https://atgu.mgh.harvard.edu/plinkseq/) were excluded from the analysis. Any sample that is >3 standard deviations from the center of the plot as generated from the first and the second principal component by PLINK (Chang et al., 2015) multi-dimensional scaling were considered as ethnicity outliers. Relatedness check up to second degree was performed by using both PLINK (Chang et al., 2015) and KING (Manichaikul et al., 2010) algorithms. From the identified related pairs of samples we randomly chose one sample to be included in the final analyses.

Variant QC:

GATK best practices (DePristo et al., 2011) were used to filter the low quality variants by the authors of PPMI study. In addition, we used GATK hard filtering to select only high quality variants. Individual genotypes with a read depth of <10 and GQ of <20 were converted to missing using bcftools (Li et al., 2009). Only variants with a call rate of >0.9 were kept for further analyses.

Variant annotation and filtering:

In the current study, we only focused on exonic variants (SNVs and indels) and variants affecting splicing. Splice variants were defined as variants falling into 2 base pairs from an exon boundary in both directions. Multi-allelic variants were decomposed by using variant-tests (Tan et al., 2015) and left normalized by bcftools (Li et al., 2009). Variants were annotated by using ANNOVAR (Wang et al., 2010) version '2016December05' using RefSeq gene annotations and the dbNSFP v3.0 (Liu et al., 2016) prediction scores including the CADD (Kircher et al., 2014) score. Only rare variants, as defined by variants with a MAF < 0.005 in the current dataset and also European population of public databases such as 1000 genomes (Altshuler et al., 2015), ExAC (release 0.3) (Lek et al., 2016), and the Exome variant server (http://evs.gs.washington.edu/EVS/) were selected. Variants were grouped according their exonic variant types into the following 8 variant groups: NONSYN (all missense mutations), SYN (all synonymous variants), LoF (Loss-of-Function mutations: stop gain, stop loss, frameshift, splicing), NONSYN+LoF, and different categories of missense variants grouped by their minimum CADD score (CADD10, CADD15, CADD20, CADD30). The higher the CADD score threshold the more likely a missense mutation is functional.

Burden tests:

To test the genome-wide differential burden of TRAP1 variants within the 8 different variant groups in cases compared to the controls the CMC Fisher Exact (Li and Leal, 2008) as well as the SKAT-O (Lee et al., 2012) test, both available as part of the rvtests (Zhan et al., 2016) software, with default parameters was used. For the CMC Fisher Exact test one sided and the

two-sided P-values were reported, for SKAT-O only a two-sided P-value. No corrections for multiple testing were applied.

Results:

PPMI Burden analysis:

After filtering the samples based on ethnicity, cryptic relatedness and quality parameters the final PPMI dataset comprised of 380 PD and 162 control samples. We could not find any enrichment in cases compared to controls. Only two variant groups (CADD10 and CADD15) showed a significant two-sided P-value using the CMC Fisher and the SKAT-O test (SKAT-O P-values: 0.025 (CADD10) and 0.017 (CADD15); ORs: 0.37 (CADD10) and 0.33 (CADD15); 95% CI:[0.15-0.93](CADD10) and [0.13-0.84] (CADD15); see Supplementary Figure 1D. Both variant groups also showed an enrichment of variants in the control group using the one-sided CMC Fisher test.

Despite the PPMI cohort not having enough power to reach significance after multiple testing, the results suggest that in sporadic Parkinson's disease cases there is evidence that rare, more benign (given by lower CADD thresholds), TRAP1 missense mutations are depleted in cases. The number of LoF mutations, like the nonsense mutation characterized in this study, in the PPMI cohort in TRAP1 is very small, only 1 frameshift variant was found present in two PD cases and not in controls, and no stop gain or other LoF variant was found in any control sample.

Established Parkinson's disease genes tested in exome sequencing

SNCA, LRRK2, VPS35, GBA, SMPD1, DNAJC13, PARK2, PINK1, Park7, ATP13A2, DNAJC6, SYNJ1, PLA2G6, DCTN1, FBXO7, PRKAR1B, COQ2, EIF4G1, ATP6AP2, GCH1, SCARB2, APP, MAPT, RAB39B, CHCHD2, VPS13C, TMEM230, PDE8B.

Supplementary References

ADZHUBEI, I. A., SCHMIDT, S., PESHKIN, L., RAMENSKY, V. E., GERASIMOVA, A., BORK, P., KONDRASHOV, A. S. & SUNYAEV, S. R. 2010. A method and server for predicting damaging missense mutations. Nat Methods, 7, 248-9.

ALTSHULER, D. M., DURBIN, R. M., ABECASIS, G. R., BENTLEY, D. R., CHAKRAVARTI, A., CLARK, A. G., DONNELLY, P., EICHLER, E. E., FLICEK, P.,

GABRIEL, S. B., GIBBS, R. A., GREEN, E. D., HURLES, M. E., KNOPPERS, B. M., KORBEL, J. O., LANDER, E. S., LEE, C., LEHRACH, H., MARDIS, E. R., MARTH, G. T., MCVEAN, G. A., NICKERSON, D. A., SCHMIDT, J. P., SHERRY, S. T., WANG, J., WILSON, R. K., GIBBS, R. A., BOERWINKLE, E., DODDAPANENI, H., HAN, Y., KORCHINA, V., KOVAR, C., LEE, S., MUZNY, D., REID, J. G., ZHU, Y. M., WANG, J., CHANG, Y. Q., FENG, Q., FANG, X. D., GUO, X. S., JIAN, M., JIANG, H., JIN, X., LAN, T. M., LI, G. Q., LI, J. X., LI, Y. R., LIU, S. M., LIU, X., LU, Y., MA, X. D., TANG, M. F., WANG, B., WANG, G. B., WU, H. L., WU, R. H., XU, X., YIN, Y., ZHANG, D. D., ZHANG, W. W., ZHAO, J., ZHAO, M. R., ZHENG, X. L., LANDER, E. S., ALTSHULER, D. M., GABRIEL, S. B., GUPTA, N., GHARANI, N., TOJI, L. H., GERRY, N. P., RESCH, A. M., FLICEK, P., BARKER, J., CLARKE, L., GIL, L., HUNT, S. E., KELMAN, G., KULESHA, E., LEINONEN, R., MCLAREN, W. M., RADHAKRISHNAN, R., ROA, A., SMIRNOV, D., SMITH, R. E., STREETER, I., THORMANN, A., TONEVA, I., VAUGHAN, B., ZHENG-BRADLEY, X., BENTLEY, D. R., GROCOCK, R., HUMPHRAY, S., JAMES, T., KINGSBURY, Z., LEHRACH, H., SUDBRAK, R., ALBRECHT, M. W., AMSTISLAVSKIY, V. S., BORODINA, T. A., et al. 2015. A global reference for human genetic variation. Nature, 526, 68-+.

ASHKENAZY, H., EREZ, E., MARTZ, E., PUPKO, T., & BEN-TAL, N. 2010. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. Nucleic Acids Research, 38 (suppl. 2), W529-W533.

CAPRIOTTI, E., FARISELLI, P. & CASADIO, R. 2005. I-Mutant2.0: predicting stability changes upon mutation from the protein sequence or structure. Nucleic Acids Res, 33, W306-10.

CHANG, C. C., CHOW, C. C., TELLIER, L. C., VATTIKUTI, S., PURCELL, S. M. & LEE, J. J. 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience, 4, 7.

CHOI, Y., SIMS, G. E., MURPHY, S., MILLER, J. R. & CHAN, A. P. 2012. Predicting the functional effect of amino acid substitutions and indels. PLoS One, 7, e46688.

CHUN, S. & FAY, J. C. 2009. Identification of deleterious mutations within three human genomes. Genome Res, 19, 1553-61.

COOPER, G. M., STONE, E. A., ASIMENOS, G., PROGRAM, N. C. S., GREEN, E. D., BATZOGLOU, S. & SIDOW, A. 2005. Distribution and intensity of constraint in mammalian genomic sequence. Genome Res, 15, 901-13.

DE BAETS, G., VAN DURME, J., REUMERS, J., MAURER-STROH, S., VANHEE, P., DOPAZO, J., SCHYMKOWITZ, J. & ROUSSEAU, F. 2012. SNPeffect 4.0: on-line prediction of molecular and structural effects of protein-coding variants. Nucleic Acids Res, 40, D935-9.

DEPRISTO, M. A., BANKS, E., POPLIN, R., GARIMELLA, K. V., MAGUIRE, J. R., HARTL, C., PHILIPPAKIS, A. A., DEL ANGEL, G., RIVAS, M. A., HANNA, M., MCKENNA, A., FENNELL, T. J., KERNYTSKY, A. M., SIVACHENKO, A. Y., CIBULSKIS, K., GABRIEL, S. B., ALTSHULER, D. & DALY, M. J. 2011. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet, 43, 491-8.

FINN, R. D., ATTWOOD, T. K., BABBITT, P. C., BATEMAN, A., BORK, P., BRIDGE, A. J., CHANG, H. Y., DOSZTANYI, Z., EL-GEBALI, S., FRASER, M., GOUGH, J., HAFT, D., HOLLIDAY, G. L., HUANG, H., HUANG, X., LETUNIC, I., LOPEZ, R., LU, S., MARCHLER-BAUER, A., MI, H., MISTRY, J., NATALE, D. A., NECCI, M., NUKA, G., ORENGO, C. A., PARK, Y., PESSEAT, S., PIOVESAN, D., POTTER, S. C., RAWLINGS, N. D., REDASCHI, N., RICHARDSON, L., RIVOIRE, C., SANGRADOR-VEGAS, A., SIGRIST, C., SILLITOE, I., SMITHERS, B., SQUIZZATO, S., SUTTON, G., THANKI, N., THOMAS, P. D., TOSATTO, S. C., WU, C. H., XENARIOS, I., YEH, L. S., YOUNG, S. Y. & MITCHELL, A. L. 2017. InterPro in 2017-beyond protein family and domain annotations. Nucleic Acids Res, 45, D190-D199.

FINN, R. D., COGGILL, P., EBERHARDT, R. Y., EDDY, S. R., MISTRY, J., MITCHELL, A. L., POTTER, S. C., PUNTA, M., QURESHI, M., SANGRADOR-VEGAS, A., SALAZAR, G. A., TATE, J. & BATEMAN, A. 2016. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res, 44, D279-85.

GARBER, M., GUTTMAN, M., CLAMP, M., ZODY, M. C., FRIEDMAN, N. & XIE, X. 2009. Identifying novel constrained elements by exploiting biased substitution patterns. Bioinformatics, 25, i54-62.

GIOLLO, M., MARTIN, A. J., WALSH, I., FERRARI, C. & TOSATTO, S. C. 2014. NeEMO: a method using residue interaction networks to improve prediction of protein stability upon mutation. BMC Genomics, 15 Suppl 4, S7.

HUANG, S., HEIKAL, A. A. & WEBB, W. W. 2002. Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. Biophys J, 82, 2811-25.

IOANNIDIS, N. M., ROTHSTEIN, J. H., PEJAVER, V., MIDDHA, S., MCDONNELL, S. K., BAHETI, S., MUSOLF, A., LI, Q., HOLZINGER, E., KARYADI, D., CANNON-

ALBRIGHT, L. A., TEERLINK, C. C., STANFORD, J. L., ISAACS, W. B., XU, J., COONEY, K. A., LANGE, E. M., SCHLEUTKER, J., CARPTEN, J. D., POWELL, I. J., CUSSENOT, O., CANCEL-TASSIN, G., GILES, G. G., MACINNIS, R. J., MAIER, C., HSIEH, C. L., WIKLUND, F., CATALONA, W. J., FOULKES, W. D., MANDAL, D., EELES, R. A., KOTE-JARAI, Z., BUSTAMANTE, C. D., SCHAID, D. J., HASTIE, T., OSTRANDER, E. A., BAILEY-WILSON, J. E., RADIVOJAC, P., THIBODEAU, S. N., WHITTEMORE, A. S. & SIEH, W. 2016. REVEL: An Ensemble Method for Predicting the Pathogenicity of Rare Missense Variants. Am J Hum Genet, 99, 877-885.

IONITA-LAZA, I., MCCALLUM, K., XU, B. & BUXBAUM, J. D. 2016. A spectral approach integrating functional genomic annotations for coding and noncoding variants. Nat Genet, 48, 214-20.

JAGADEESH, K. A., WENGER, A. M., BERGER, M. J., GUTURU, H., STENSON, P. D., COOPER, D. N., BERNSTEIN, J. A. & BEJERANO, G. 2016. M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. Nat Genet, 48, 1581-1586.

KIRCHER, M., WITTEN, D. M., JAIN, P., O'ROAK, B. J., COOPER, G. M. & SHENDURE, J. 2014. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet, 46, 310-5.

KUMAR, P., HENIKOFF, S. & NG, P. C. 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc, 4, 1073-81.

LAKNER, P. H., MONAGHAN, M. G., MOLLER, Y., OLAYIOYE, M. A. & SCHENKE-LAYLAND, K. 2017. Applying phasor approach analysis of multiphoton FLIM measurements to probe the metabolic activity of three-dimensional in vitro cell culture models. Sci Rep, 7, 42730.

LEE, S., EMOND, M. J., BAMSHAD, M. J., BARNES, K. C., RIEDER, M. J., NICKERSON, D. A., TEAM, N. G. E. S. P.-E. L. P., CHRISTIANI, D. C., WURFEL, M. M. & LIN, X. 2012. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. Am J Hum Genet, 91, 224-37.

LEK, M., KARCZEWSKI, K. J., MINIKEL, E. V., SAMOCHA, K. E., BANKS, E., FENNELL, T., O'DONNELL-LURIA, A. H., WARE, J. S., HILL, A. J., CUMMINGS, B. B., TUKIAINEN, T., BIRNBAUM, D. P., KOSMICKI, J. A., DUNCAN, L. E., ESTRADA, K., ZHAO, F., ZOU, J., PIERCE-HOFFMAN, E., BERGHOUT, J., COOPER, D. N., DEFLAUX, N., DEPRISTO, M., DO, R., FLANNICK, J., FROMER, M., GAUTHIER, L.,

GOLDSTEIN, J., GUPTA, N., HOWRIGAN, D., KIEZUN, A., KURKI, M. I., MOONSHINE, A. L., NATARAJAN, P., OROZCO, L., PELOSO, G. M., POPLIN, R., RIVAS, M. A., RUANO-RUBIO, V., ROSE, S. A., RUDERFER, D. M., SHAKIR, K., STENSON, P. D., STEVENS, C., THOMAS, B. P., TIAO, G., TUSIE-LUNA, M. T., WEISBURD, B., WON, H. H., YU, D., ALTSHULER, D. M., ARDISSINO, D., BOEHNKE, M., DANESH, J., DONNELLY, S., ELOSUA, R., FLOREZ, J. C., GABRIEL, S. B., GETZ, G., GLATT, S. J., HULTMAN, C. M., KATHIRESAN, S., LAAKSO, M., MCCARROLL, S., MCCARTHY, M. I., MCGOVERN, D., MCPHERSON, R., NEALE, B. M., PALOTIE, A., PURCELL, S. M., SALEHEEN, D., SCHARF, J. M., SKLAR, P., SULLIVAN, P. F., TUOMILEHTO, J., TSUANG, M. T., WATKINS, H. C., WILSON, J. G., DALY, M. J., MACARTHUR, D. G. & EXOME AGGREGATION, C. 2016. Analysis of protein-coding genetic variation in 60,706 humans. Nature, 536, 285-91.

LI, B. & LEAL, S. M. 2008. Methods for detecting associations with rare variants for common diseases: application to analysis of sequence data. Am J Hum Genet, 83, 311-21.

LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G., DURBIN, R. & GENOME PROJECT DATA PROCESSING, S. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics, 25, 2078-9.

LIU, X., WU, C., LI, C. & BOERWINKLE, E. 2016. dbNSFP v3.0: A One-Stop Database of Functional Predictions and Annotations for Human Nonsynonymous and Splice-Site SNVs. Hum Mutat, 37, 235-41.

LU, H. C., HERRERA BRAGA, J. & FRATERNALI, F. 2016. PinSnps: structural and functional analysis of SNPs in the context of protein interaction networks. Bioinformatics, 32, 2534-6.

MANICHAIKUL, A., MYCHALECKYJ, J. C., RICH, S. S., DALY, K., SALE, M. & CHEN, W. M. 2010. Robust relationship inference in genome-wide association studies. Bioinformatics, 26, 2867-73.

PIRES, D. E., ASCHER, D. B. & BLUNDELL, T. L. 2014. mCSM: predicting the effects of mutations in proteins using graph-based signatures. Bioinformatics, 30, 335-42.

QUANG, D., CHEN, Y. & XIE, X. 2015. DANN: a deep learning approach for annotating the pathogenicity of genetic variants. Bioinformatics, 31, 761-3.

REVA, B., ANTIPIN, Y. & SANDER, C. 2011. Predicting the functional impact of protein mutations: application to cancer genomics. Nucleic Acids Res, 39, e118.

SAFRAN, M., DALAH, I., ALEXANDER, J., ROSEN, N., INY STEIN, T., SHMOISH, M., NATIV, N., BAHIR, I., DONIGER, T., KRUG, H., SIROTA-MADI, A., OLENDER, T.,

GOLAN, Y., STELZER, G., HAREL, A. & LANCET, D. 2010. GeneCards Version 3: the human gene integrator. Database (Oxford), 2010, baq020.

SCHWARZ, J. M., RODELSPERGER, C., SCHUELKE, M. & SEELOW, D. 2010. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods, 7, 575-6.

SHIHAB, H. A., GOUGH, J., MORT, M., COOPER, D. N., DAY, I. N. & GAUNT, T. R. 2014. Ranking non-synonymous single nucleotide polymorphisms based on disease concepts. Hum Genomics, 8, 11.

SIEPEL, A., POLLARD, K. & HAUSSLER, D. (eds.) 2006. New Methods for Detecting Lineage-Specific Selection, Berlin, Heidelberg: Springer Berlin-Heidelberg.

VAN DURME, J., MAURER-STROH, S., GALLARDO, R., WILKINSON, H., ROUSSEAU, F. & SCHYMKOWITZ, J. 2009. Accurate prediction of DnaK-peptide binding via homology modelling and experimental data. PLoS Comput Biol, 5, e1000475.

TAN, A., ABECASIS, G. R. & KANG, H. M. 2015. Unified representation of genetic variants. Bioinformatics, 31, 2202-4.

WANG, K., LI, M. & HAKONARSON, H. 2010. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res, 38, e164.

WORTH, C. L., PREISSNER, R. & BLUNDELL, T. L. 2011. SDM--a server for predicting effects of mutations on protein stability and malfunction. Nucleic Acids Res, 39, W215-22.

ZHAN, X., HU, Y., LI, B., ABECASIS, G. R. & LIU, D. J. 2016. RVTESTS: an efficient and comprehensive tool for rare variant association analysis using sequence data. Bioinformatics, 32, 1423-6.

References

ALTIERI, D. C., STEIN, G. S., LIAN, J. B. & LANGUINO, L. R. 2012. TRAP-1, the mitochondrial Hsp90. *Biochim Biophys Acta,* 1823**,** 767-73.

AMOROSO, M. R., MATASSA, D. S., AGLIARULO, I., AVOLIO, R., LU, H., SISINNI, L., LETTINI, G., GABRA, H., LANDRISCINA, M. & ESPOSITO, F. 2016. TRAP1 downregulation in human ovarian cancer enhances invasion and epithelial-mesenchymal transition. *Cell Death Dis,* 7**,** e2522.

ANDRZEJEWSKI, S., GRAVEL, S. P., POLLAK, M. & ST-PIERRE, J. 2014. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer Metab,* 2**,** 12.

BAQRI, R. M., PIETRON, A. V., GOKHALE, R. H., TURNER, B. A., KAGUNI, L. S., SHINGLETON, A. W., KUNES, S. & MILLER, K. E. 2014. Mitochondrial chaperone

TRAP1 activates the mitochondrial UPR and extends healthspan in Drosophila. *Mech Ageing Dev,* 141-142**,** 35-45.

BIRD, D. K., YAN, L., VROTSOS, K. M., ELICEIRI, K. W., VAUGHAN, E. M., KEELY, P. J., WHITE, J. G. & RAMANUJAM, N. 2005. Metabolic mapping of MCF10A human breast cells via multiphoton fluorescence lifetime imaging of the coenzyme NADH. *Cancer Res,* 65**,** 8766-73.

BLACKER, T. S., MANN, Z. F., GALE, J. E., ZIEGLER, M., BAIN, A. J., SZABADKAI, G. & DUCHEN, M. R. 2014. Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. *Nat Commun,* 5**,** 3936.

BOGAERTS, V., NUYTEMANS, K., REUMERS, J., PALS, P., ENGELBORGHS, S., PICKUT, B., CORSMIT, E., PEETERS, K., SCHYMKOWITZ, J., DE DEYN, P. P., CRAS, P., ROUSSEAU, F., THEUNS, J. & VAN BROECKHOVEN, C. 2008. Genetic variability in the mitochondrial serine protease HTRA2 contributes to risk for Parkinson disease. *Hum Mutat,* 29**,** 832-40.

BOLES, R. G., HORNUNG, H. A., MOODY, A. E., ORTIZ, T. B., WONG, S. A., EGGINGTON, J. M., STANLEY, C. M., GAO, M., ZHOU, H., MCLAUGHLIN, S., ZARE, A. S., SHELDON, K. M., SKOLNICK, J. & MCKERNAN, K. J. 2015. Hurt, tired and queasy: Specific variants in the ATPase domain of the TRAP1 mitochondrial chaperone are associated with common, chronic "functional" symptomatology including pain, fatigue and gastrointestinal dysmotility. *Mitochondrion,* 23**,** 64-70.

BURBULLA, L. F., FITZGERALD, J. C., STEGEN, K., WESTERMEIER, J., THOST, A. K., KATO, H., MOKRANJAC, D., SAUERWALD, J., MARTINS, L. M., WOITALLA, D., RAPAPORT, D., RIESS, O., PROIKAS-CEZANNE, T., RASSE, T. M. & KRUGER, R. 2014. Mitochondrial proteolytic stress induced by loss of mortalin function is rescued by Parkin and PINK1. *Cell Death Dis,* 5**,** e1180.

BURBULLA, L. F. & KRUGER, R. 2012. The Use of Primary Human Fibroblasts for Monitoring Mitochondrial Phenotypes in the Field of Parkinson's Disease. *Jove-Journal of Visualized Experiments*.

BURTE, F., DE GIROLAMO, L. A., HARGREAVES, A. J. & BILLETT, E. E. 2011. Alterations in the mitochondrial proteome of neuroblastoma cells in response to complex 1 inhibition. *J Proteome Res,* 10**,** 1974-86.

BUTLER, E. K., VOIGT, A., LUTZ, A. K., TOEGEL, J. P., GERHARDT, E., KARSTEN, P., FALKENBURGER, B., REINARTZ, A., WINKLHOFER, K. F. & SCHULZ, J. B. 2012.

The mitochondrial chaperone protein TRAP1 mitigates alpha-Synuclein toxicity. *PLoS Genet,* 8**,** e1002488.

CASADEI, N., SOOD, P., ULRICH, T., FALLIER-BECKER, P., KIEPER, N., HELLING, S., MAY, C., GLAAB, E., CHEN, J., NUBER, S., WOLBURG, H., MARCUS, K., RAPAPORT, D., OTT, T., RIESS, O., KRUGER, R. & FITZGERALD, J. C. 2016. Mitochondrial defects and neurodegeneration in mice overexpressing wild-type or G399S mutant HtrA2. *Hum Mol Genet*.

CLAUSEN, T., SOUTHAN, C. & EHRMANN, M. 2002. The HtrA family of proteases: implications for protein composition and cell fate. *Mol Cell,* 10**,** 443-55.

COSTA, A. C., LOH, S. H. & MARTINS, L. M. 2013. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. *Cell Death Dis,* 4**,** e467.

FITZGERALD, J. C., CAMPRUBI, M. D., DUNN, L., WU, H. C., IP, N. Y., KRUGER, R., MARTINS, L. M., WOOD, N. W. & PLUN-FAVREAU, H. 2012. Phosphorylation of HtrA2 by cyclin-dependent kinase-5 is important for mitochondrial function. *Cell Death Differ,* 19**,** 257-66.

GEISLER, S., HOLMSTROM, K. M., SKUJAT, D., FIESEL, F. C., ROTHFUSS, O. C., KAHLE, P. J. & SPRINGER, W. 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol,* 12**,** 119-31.

HARGREAVES, I. P., DUNCAN, A. J., WU, L., AGRAWAL, A., LAND, J. M. & HEALES, S. J. 2007. Inhibition of mitochondrial complex IV leads to secondary loss complex II-III activity: implications for the pathogenesis and treatment of mitochondrial encephalomyopathies. *Mitochondrion,* 7**,** 284-7.

HOUTKOOPER, R. H., MOUCHIROUD, L., RYU, D., MOULLAN, N., KATSYUBA, E., KNOTT, G., WILLIAMS, R. W. & AUWERX, J. 2013. Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature,* 497**,** 451-7.

HUA, G., ZHANG, Q. & FAN, Z. 2007. Heat shock protein 75 (TRAP1) antagonizes reactive oxygen species generation and protects cells from granzyme M-mediated apoptosis. *J Biol Chem,* 282**,** 20553-60.

IM, C. N., LEE, J. S., ZHENG, Y. & SEO, J. S. 2007. Iron chelation study in a normal human hepatocyte cell line suggests that tumor necrosis factor receptor-associated protein 1 (TRAP1) regulates production of reactive oxygen species. *J Cell Biochem,* 100**,** 474-86.

KANG, B. H. 2012. TRAP1 regulation of mitochondrial life or death decision in cancer cells and mitochondria-targeted TRAP1 inhibitors. *BMB Rep,* 45**,** 1-6.

KARPAC, J. & JASPER, H. 2013. Aging: seeking mitonuclear balance. *Cell,* 154**,** 271-3.

KIEPER, N., HOLMSTROM, K. M., CICERI, D., FIESEL, F. C., WOLBURG, H., ZIVIANI, E., WHITWORTH, A. J., MARTINS, L. M., KAHLE, P. J. & KRUGER, R. 2010. Modulation of mitochondrial function and morphology by interaction of Omi/HtrA2 with the mitochondrial fusion factor OPA1. *Exp Cell Res,* 316**,** 1213-24.

KIM, Y. D., PARK, K. G., LEE, Y. S., PARK, Y. Y., KIM, D. K., NEDUMARAN, B., JANG, W. G., CHO, W. J., HA, J., LEE, I. K., LEE, C. H. & CHOI, H. S. 2008. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes,* 57**,** 306-14.

KITADA, T., ASAKAWA, S., HATTORI, N., MATSUMINE, H., YAMAMURA, Y., MINOSHIMA, S., YOKOCHI, M., MIZUNO, Y. & SHIMIZU, N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature,* 392**,** 605-8.

KRUGER, R., KLUCKEN, J., WEISS, D., TONGES, L., KOLBER, P., UNTERECKER, S., LORRAIN, M., BAAS, H., MULLER, T. & RIEDERER, P. 2017. Classification of advanced stages of Parkinson's disease: translation into stratified treatments. *J Neural Transm (Vienna)*.

LAKNER, P. H., MONAGHAN, M. G., MOLLER, Y., OLAYIOYE, M. A. & SCHENKE-LAYLAND, K. 2017. Applying phasor approach analysis of multiphoton FLIM measurements to probe the metabolic activity of three-dimensional in vitro cell culture models. *Sci Rep,* 7**,** 42730.

LEK, M., KARCZEWSKI, K. J., MINIKEL, E. V., SAMOCHA, K. E., BANKS, E., FENNELL, T., O'DONNELL-LURIA, A. H., WARE, J. S., HILL, A. J., CUMMINGS, B. B., TUKIAINEN, T., BIRNBAUM, D. P., KOSMICKI, J. A., DUNCAN, L. E., ESTRADA, K., ZHAO, F., ZOU, J., PIERCE-HOFFMAN, E., BERGHOUT, J., COOPER, D. N., DEFLAUX, N., DEPRISTO, M., DO, R., FLANNICK, J., FROMER, M., GAUTHIER, L., GOLDSTEIN, J., GUPTA, N., HOWRIGAN, D., KIEZUN, A., KURKI, M. I., MOONSHINE, A. L., NATARAJAN, P., OROZCO, L., PELOSO, G. M., POPLIN, R., RIVAS, M. A., RUANO-RUBIO, V., ROSE, S. A., RUDERFER, D. M., SHAKIR, K., STENSON, P. D., STEVENS, C., THOMAS, B. P., TIAO, G., TUSIE-LUNA, M. T., WEISBURD, B., WON, H. H., YU, D., ALTSHULER, D. M., ARDISSINO, D., BOEHNKE, M., DANESH, J., DONNELLY, S., ELOSUA, R., FLOREZ, J. C., GABRIEL, S. B., GETZ, G., GLATT, S. J., HULTMAN, C. M., KATHIRESAN, S., LAAKSO, M., MCCARROLL, S., MCCARTHY, M. I., MCGOVERN, D., MCPHERSON, R., NEALE, B. M., PALOTIE, A., PURCELL, S. M., SALEHEEN, D., SCHARF, J. M., SKLAR, P., SULLIVAN, P. F., TUOMILEHTO, J., TSUANG, M. T., WATKINS, H. C., WILSON, J. G., DALY, M. J.,

MACARTHUR, D. G. & EXOME AGGREGATION, C. 2016. Analysis of protein-coding genetic variation in 60,706 humans. *Nature,* 536**,** 285-91.

LI, W., SRINIVASULA, S. M., CHAI, J., LI, P., WU, J. W., ZHANG, Z., ALNEMRI, E. S. & SHI, Y. 2002. Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi. *Nat Struct Biol,* 9**,** 436-41.

LUYKX, J. J., BAKKER, S. C., LENTJES, E., NEELEMAN, M., STRENGMAN, E., MENTINK, L., DEYOUNG, J., DE JONG, S., SUL, J. H., ESKIN, E., VAN EIJK, K., VAN SETTEN, J., BUIZER-VOSKAMP, J. E., CANTOR, R. M., LU, A., VAN AMERONGEN, M., VAN DONGEN, E. P., KEIJZERS, P., KAPPEN, T., BORGDORFF, P., BRUINS, P., DERKS, E. M., KAHN, R. S. & OPHOFF, R. A. 2014. Genome-wide association study of monoamine metabolite levels in human cerebrospinal fluid. *Mol Psychiatry,* 19**,** 228-34.

LV, Q., SUN, H., CAO, C., GAO, B. & QI, Y. 2016. Overexpression of tumor necrosis factor receptor-associated protein 1 (TRAP1) are associated with poor prognosis of epithelial ovarian cancer. *Tumour Biol,* 37**,** 2721-7.

MARTINS, L. M. 2002. The serine protease Omi/HtrA2: a second mammalian protein with a Reaper-like function. *Cell Death Differ,* 9**,** 699-701.

MARTINS, L. M., IACCARINO, I., TENEV, T., GSCHMEISSNER, S., TOTTY, N. F., LEMOINE, N. R., SAVOPOULOS, J., GRAY, C. W., CREASY, C. L., DINGWALL, C. & DOWNWARD, J. 2002. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem,* 277**,** 439-44.

MARTINS, L. M., MORRISON, A., KLUPSCH, K., FEDELE, V., MOISOI, N., TEISMANN, P., ABUIN, A., GRAU, E., GEPPERT, M., LIVI, G. P., CREASY, C. L., MARTIN, A., HARGREAVES, I., HEALES, S. J., OKADA, H., BRANDNER, S., SCHULZ, J. B., MAK, T. & DOWNWARD, J. 2004. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol,* 24**,** 9848-62.

MASGRAS, I., CISCATO, F., BRUNATI, A. M., TIBALDI, E., INDRACCOLO, S., CURTARELLO, M., CHIARA, F., CANNINO, G., PAPALEO, E., LAMBRUGHI, M., GUZZO, G., GAMBALUNGA, A., PIZZI, M., GUZZARDO, V., RUGGE, M., VULJAN, S. E., CALABRESE, F., BERNARDI, P. & RASOLA, A. 2017. Absence of Neurofibromin Induces an Oncogenic Metabolic Switch via Mitochondrial ERK-Mediated Phosphorylation of the Chaperone TRAP1. *Cell Rep,* 18**,** 659-672.

MASUDA, Y., SHIMA, G., AIUCHI, T., HORIE, M., HORI, K., NAKAJO, S., KAJIMOTO, S., SHIBAYAMA-IMAZU, T. & NAKAYA, K. 2004. Involvement of tumor necrosis factor

receptor-associated protein 1 (TRAP1) in apoptosis induced by beta-hydroxyisovalerylshikonin. *J Biol Chem,* 279**,** 42503-15.

MATASSA, D. S., AMOROSO, M. R., LU, H., AVOLIO, R., ARZENI, D., PROCACCINI, C., FAICCHIA, D., MADDALENA, F., SIMEON, V., AGLIARULO, I., ZANINI, E., MAZZOCCOLI, C., RECCHI, C., STRONACH, E., MARONE, G., GABRA, H., MATARESE, G., LANDRISCINA, M. & ESPOSITO, F. 2016a. Oxidative metabolism drives inflammation-induced platinum resistance in human ovarian cancer. *Cell Death Differ*.

MATASSA, D. S., AMOROSO, M. R., LU, H., AVOLIO, R., ARZENI, D., PROCACCINI, C., FAICCHIA, D., MADDALENA, F., SIMEON, V., AGLIARULO, I., ZANINI, E., MAZZOCCOLI, C., RECCHI, C., STRONACH, E., MARONE, G., GABRA, H., MATARESE, G., LANDRISCINA, M. & ESPOSITO, F. 2016b. Oxidative metabolism drives inflammation-induced platinum resistance in human ovarian cancer. *Cell Death Differ,* 23**,** 1542-54.

MOISOI, N., KLUPSCH, K., FEDELE, V., EAST, P., SHARMA, S., RENTON, A., PLUN-FAVREAU, H., EDWARDS, R. E., TEISMANN, P., ESPOSTI, M. D., MORRISON, A. D., WOOD, N. W., DOWNWARD, J. & MARTINS, L. M. 2009. Mitochondrial dysfunction triggered by loss of HtrA2 results in the activation of a brain-specific transcriptional stress response. *Cell Death Differ,* 16**,** 449-64.

MOUCHIROUD, L., HOUTKOOPER, R. H., MOULLAN, N., KATSYUBA, E., RYU, D., CANTO, C., MOTTIS, A., JO, Y. S., VISWANATHAN, M., SCHOONJANS, K., GUARENTE, L. & AUWERX, J. 2013. The NAD(+)/Sirtuin Pathway Modulates Longevity through Activation of Mitochondrial UPR and FOXO Signaling. *Cell,* 154**,** 430-41.

MUNCH, C. & HARPER, J. W. 2016. Mitochondrial unfolded protein response controls matrix pre-RNA processing and translation. *Nature,* 534**,** 710-3.

NARENDRA, D. P., JIN, S. M., TANAKA, A., SUEN, D. F., GAUTIER, C. A., SHEN, J., COOKSON, M. R. & YOULE, R. J. 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol,* 8**,** e1000298.

OWEN, M. R., DORAN, E. & HALESTRAP, A. P. 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J,* 348 Pt 3**,** 607-14.

PATIL, S. P., JAIN, P. D., GHUMATKAR, P. J., TAMBE, R. & SATHAYE, S. 2014. Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience,* 277**,** 747-54.

PELLEGRINO, M. W., NARGUND, A. M. & HAYNES, C. M. 2013. Signaling the mitochondrial unfolded protein response. *Biochim Biophys Acta,* 1833**,** 410-6.

PEREZ-REVUELTA, B. I., HETTICH, M. M., CIOCIARO, A., ROTERMUND, C., KAHLE, P. J., KRAUSS, S. & DI MONTE, D. A. 2014. Metformin lowers Ser-129 phosphorylated alpha-synuclein levels via mTOR-dependent protein phosphatase 2A activation. *Cell Death Dis,* 5**,** e1209.

PERKINS, D. N., PAPPIN, D. J., CREASY, D. M. & COTTRELL, J. S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis,* 20**,** 3551-67.

PFEIFFER, R. F. 2003. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol,* 2**,** 107-16.

PLUN-FAVREAU, H., KLUPSCH, K., MOISOI, N., GANDHI, S., KJAER, S., FRITH, D., HARVEY, K., DEAS, E., HARVEY, R. J., MCDONALD, N., WOOD, N. W., MARTINS, L. M. & DOWNWARD, J. 2007. The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nat Cell Biol,* 9**,** 1243-52.

PRIDGEON, J. W., OLZMANN, J. A., CHIN, L. S. & LI, L. 2007. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. *PLoS Biol,* 5**,** e172.

RAKOVIC, A., SHURKEWITSCH, K., SEIBLER, P., GRUNEWALD, A., ZANON, A., HAGENAH, J., KRAINC, D. & KLEIN, C. 2013. Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1)-dependent ubiquitination of endogenous Parkin attenuates mitophagy: study in human primary fibroblasts and induced pluripotent stem cell-derived neurons. J Biol Chem, 288, 2223-37.

RASOLA, A., NECKERS, L. & PICARD, D. 2014. Mitochondrial oxidative phosphorylation TRAP(1)ped in tumor cells. *Trends Cell Biol,* 24**,** 455-63.

REINHARDT, P., GLATZA, M., HEMMER, K., TSYTSYURA, Y., THIEL, C. S., HOING, S., MORITZ, S., PARGA, J. A., WAGNER, L., BRUDER, J. M., WU, G., SCHMID, B., ROPKE, A., KLINGAUF, J., SCHWAMBORN, J. C., GASSER, T., SCHOLER, H. R. & STERNECKERT, J. 2013. Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. *PLoS One,* 8**,** e59252.

ROGERS, G. W., BRAND, M. D., PETROSYAN, S., ASHOK, D., ELORZA, A. A., FERRICK, D. A. & MURPHY, A. N. 2011. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS One,* 6**,** e21746.

SAISAWAT, P., KOHL, S., HILGER, A. C., HWANG, D. Y., YUNG GEE, H., DWORSCHAK, G. C., TASIC, V., PENNIMPEDE, T., NATARAJAN, S., SPERRY, E.,

MATASSA, D. S., STAJIC, N., BOGDANOVIC, R., DE BLAAUW, I., MARCELIS, C. L., WIJERS, C. H., BARTELS, E., SCHMIEDEKE, E., SCHMIDT, D., MARZHEUSER, S., GRASSHOFF-DERR, S., HOLLAND-CUNZ, S., LUDWIG, M., NOTHEN, M. M., DRAAKEN, M., BROSENS, E., HEIJ, H., TIBBOEL, D., HERRMANN, B. G., SOLOMON, B. D., DE KLEIN, A., VAN ROOIJ, I. A., ESPOSITO, F., REUTTER, H. M. & HILDEBRANDT, F. 2014. Whole-exome resequencing reveals recessive mutations in TRAP1 in individuals with CAKUT and VACTERL association. *Kidney Int,* 85**,** 1310-7.

SAUER, M., JAKOB, A., NORDHEIM, A. & HOCHHOLDINGER, F. 2006. Proteomic analysis of shoot-borne root initiation in maize (Zea mays L.). *Proteomics,* 6**,** 2530-41.

SCIACOVELLI, M., GUZZO, G., MORELLO, V., FREZZA, C., ZHENG, L., NANNINI, N., CALABRESE, F., LAUDIERO, G., ESPOSITO, F., LANDRISCINA, M., DEFILIPPI, P., BERNARDI, P. & RASOLA, A. 2013. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metab,* 17**,** 988-99.

SPIESS, C., BEIL, A. & EHRMANN, M. 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell,* 97**,** 339-47.

STRAUSS, K. M., MARTINS, L. M., PLUN-FAVREAU, H., MARX, F. P., KAUTZMANN, S., BERG, D., GASSER, T., WSZOLEK, Z., MULLER, T., BORNEMANN, A., WOLBURG, H., DOWNWARD, J., RIESS, O., SCHULZ, J. B. & KRUGER, R. 2005. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Hum Mol Genet,* 14**,** 2099-111.

UNAL GULSUNER, H., GULSUNER, S., MERCAN, F. N., ONAT, O. E., WALSH, T., SHAHIN, H., LEE, M. K., DOGU, O., KANSU, T., TOPALOGLU, H., ELIBOL, B., AKBOSTANCI, C., KING, M. C., OZCELIK, T. & TEKINAY, A. B. 2014. Mitochondrial serine protease HTRA2 p.G399S in a kindred with essential tremor and Parkinson disease. *Proc Natl Acad Sci U S A,* 111**,** 18285-90.

VALENTE, E. M., ABOU-SLEIMAN, P. M., CAPUTO, V., MUQIT, M. M., HARVEY, K., GISPERT, S., ALI, Z., DEL TURCO, D., BENTIVOGLIO, A. R., HEALY, D. G., ALBANESE, A., NUSSBAUM, R., GONZALEZ-MALDONADO, R., DELLER, T., SALVI, S., CORTELLI, P., GILKS, W. P., LATCHMAN, D. S., HARVEY, R. J., DALLAPICCOLA, B., AUBURGER, G. & WOOD, N. W. 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science,* 304**,** 1158-60.

VARTHOLOMAIOU, E., MADON-SIMON, M., HAGMANN, S., MUHLEBACH, G., WURST, W., FLOSS, T. & PICARD, D. 2017. Cytosolic Hsp90 alpha and its mitochondrial

isoform Trap1 are differentially required in a breast cancer model. *Oncotarget,* 8**,** 17428-17442.

WAHLQVIST, M. L., LEE, M. S., HSU, C. C., CHUANG, S. Y., LEE, J. T. & TSAI, H. N. 2012. Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. *Parkinsonism Relat Disord,* 18**,** 753-8.

WUST, R., MAURER, B., HAUSER, K., WOITALLA, D., SHARMA, M. & KRUGER, R. 2016. Mutation analyses and association studies to assess the role of the presenilin-associated rhomboid-like gene in Parkinson's disease. *Neurobiol Aging,* 39**,** 217 e13-5.

YOSHIDA, S., TSUTSUMI, S., MUHLEBACH, G., SOURBIER, C., LEE, M. J., LEE, S., VARTHOLOMAIOU, E., TATOKORO, M., BEEBE, K., MIYAJIMA, N., MOHNEY, R. P., CHEN, Y., HASUMI, H., XU, W., FUKUSHIMA, H., NAKAMURA, K., KOGA, F., KIHARA, K., TREPEL, J., PICARD, D. & NECKERS, L. 2013. Molecular chaperone TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis. *Proc Natl Acad Sci U S A,* 110**,** E1604-12.

ZHANG, B., WANG, J., HUANG, Z., WEI, P., LIU, Y., HAO, J., ZHAO, L., ZHANG, F., TU, Y. & WEI, T. 2015. Aberrantly upregulated TRAP1 is required for tumorigenesis of breast cancer. *Oncotarget,* 6**,** 44495-508.

ZHANG, L., KARSTEN, P., HAMM, S., POGSON, J. H., MULLER-RISCHART, A. K., EXNER, N., HAASS, C., WHITWORTH, A. J., WINKLHOFER, K. F., SCHULZ, J. B. & VOIGT, A. 2013. TRAP1 rescues PINK1 loss-of-function phenotypes. *Hum Mol Genet,* 22**,** 2829-41.

ZHANG, Y., APPLETON, B. A., WU, P., WIESMANN, C. & SIDHU, S. S. 2007. Structural and functional analysis of the ligand specificity of the HtrA2/Omi PDZ domain. *Protein Sci,* 16**,** 1738-50.

ZHANG, Y., JIANG, D. S., YAN, L., CHENG, K. J., BIAN, Z. Y. & LIN, G. S. 2011. HSP75 protects against cardiac hypertrophy and fibrosis. *J Cell Biochem,* 112**,** 1787-94.

Figure Legends

Figure 1. TRAP1 interacts with HtrA2 under physiological conditions

(A) A Coomassie stained gel of GST-HtrA2 eluates from SH-SY5Y cells for unbiased mass spectrometry. **(B)** Immunoblot (IB) of TRAP1 and HtrA2 in cytosolic (Cyto) mitochondrial (Mito) fractions from HeLa cells overexpressing wildtype HtrA2 or an empty vector control.

Input lysates (input), HtrA2 immunoprecipitates (IP HtrA2) and control immunoprecipitates using bovine IgG (IP IgG). **(C)** Immunoblot (IB) of TRAP1 and HtrA2 in lysates in HeLa cells transfected with TRAP1 siRNA or a non-targeting siRNA control. Input lysates (input), HtrA2 immunoprecipitates (IP HtrA2) and control immunoprecipitates using bovine IgG (IP IgG). **(D)** Immunoblot (IB) of TRAP1 and HtrA2 in wild type and TRAP1 knockout mouse brain lysates from cortex (CTx), Basal Ganglia/Midbrain (Mid) and Hindbrain (Hin). Input lysates (input), HtrA2 immunoprecipitates (IP HtrA2) and control immunoprecipitates using bovine IgG (IP IgG). **(E)** Immunoblot (IB) of TRAP1 and HtrA2 in total cell lysates from untreated HEK293 cells (UT) or HEK293 cells treated with serum free medium (starve), 1μM Rotenone (Rot) and 25nM Antimycin A (Ant A) for 24h. Input lysates (input), HtrA2 immunoprecipitates (IP HtrA2) and control immunoprecipitates using bovine IgG (IP IgG). **(F)** Immunoblot (IB) of TRAP1 in HeLa cell extracts either untreated (UT) or treated with 200μM dopamine (DA), serum free media (starve), 2μM Hsp90/TRAP1 inhibitor (17-AAG), 1μM oligomycin and 0.4μM Antimycin A (OA), 40μM Hydrogen peroxide (H_2O_2), 5μM Rotenone (Rot) or 100 nM valinomycin (Val) for 24h. Input lysates (input) and HtrA2 immunoprecipitates (IP HtrA2) are shown.

Figure 2. TRAP1 rescues HtrA2 and PINK1 loss of function phenotypes but is not a proteolytic substrate of HtrA2

(A) Immunoprecipitation of TRAP1 (IP TRAP1) from wildtype (WT) and knockout (KO) HtrA2 MEFs and PINK1 knockout (KO) and isogenic control (Ctrl) neuroprogenitor cells treated with or without 1μM valinomycin (Val) for 24h to activate PINK1. Input lysates (input) and TRAP1 immunoprecipitates (IP TRAP1) were run on SDS-PAGE (top three panels) and PhosTag™ SDS-PAGE gels (IP: TRAP1 PhosTag) to visualize all phosphorylated TRAP1 enriched by the TRAP1 pulldown. **(B)** Immunoblot (IB) of TRAP1, HtrA2 and mitochondrial marker citrate synthase (CS) in SH-SY5Y extracts from four experiments where wildtype HtrA2 (WT HtrA2) or an empty vector (EV) control is overexpressed. (**C**) Immunoblot (IB) of TRAP1 and HtrA2 in brain extracts from non-transgenic (NTG), overexpressing wildtype HtrA2 (WT HtrA2) and overexpressing G399S mutant HtrA2 (Mut HtrA2) mice. **(D)** Quantification of TRAP1 protein levels (normalized to GAPDH loading control) at 24h and 48h after transfection with wildtype HtrA2 (WT) or HtrA2 protease dead mutant (S306A). **(E)** Mitochondrial membrane potential (ΔΨm) in HeLa cells transfected with HtrA2 siRNA (HtrA2) or a non-targeting control (Ctrl) and overexpressing an empty vector or wildtype TRAP1 DNA construct. **(F)** Basal oxygen

consumption in HeLa cells transfected with HtrA2 siRNA (HtrA2) or a non-targeting control (Ctrl) and overexpressing an empty vector or wildtype TRAP1 DNA construct. **(G)** Mitochondrial reactive oxygen species (ROS) using MitoSox™ in HeLa cells transfected with HtrA2 siRNA (HtrA2) or a non-targeting control (Ctrl) and overexpressing an empty vector or wildtype TRAP1 DNA construct. **(H)** Early apoptosis measured by Annexin V in HeLa cells either untreated (UT) or treated with 1μM staurosporine (STS), serum free media (starve) for 24h and overexpressing an empty vector (EV) or wildtype TRAP1 DNA (+) construct. **(I)** Reduced ΔΨm in PINK1 knockout neuroprogenitor cells is rescued by overexpression of wild type TRAP1. TRAP1 (or empty vector control) transfected neuroprogenitor cells were identified using a ZsGreen tag. Confocal images were taken every 4s following incubation with TMRE, followed by washing (basal), oligomycin (oligo), rotenone (rot) and FCCP (fccp). All statistical tests were the student's T test assuming different standard deviation, except 2H, where two-way ANNOVA was used to compare groups and condition. Error bars show standard deviation and *=p<0.05, and **=p<0.01.

Figure 3. TRAP1 loss of function in Parkinson's disease

(A) A diagram showing the position of the c.C158_T (R47X) mutation within the amino acid sequence of TRAP1. **(B)** Diagram showing the position of the c.C158_T (R47X) mutation in the protein structure of TRAP1. The binding site of the TRAP1 antibody (amino acid sequence 253-464) is also shown. **(C)** Copper stain showing total protein loading of an immunoblot (upper panel) probed for TRAP1 (middle panel) and the loading control α-tubulin (lower panel). **(D)** Real time PCR amplified TRAP1 transcripts normalized to housekeeping gene GAPDH in healthy controls and in the TRAP1 R47X patient using primers amplifying a region starting at the 5' UTR and spanning until exon 2 of TRAP1 upstream of the R47X mutation and exon 3 downstream of the R47X mutation. **(F)** R47X patient information.

Figure 4. TRAP1 R47X Parkinson's disease patient mitochondria meet ATP demand but have reduced membrane potential

(A) Representative binary images of mitochondria in fibroblasts from a healthy individual (CTRL) and a Parkinson's disease (PD) patient carrying the R47X mutation (PD R47X) untreated or serum starved (Starve) for 24h. **(B)** Mitochondrial area **(C)** Form factor (mitochondrial branching) and **(D)** Aspect ratio (mitochondrial length) in fibroblasts from healthy individuals (CTRL) and a Parkinson's disease (PD) patient carrying the R47X mutation (PD R47X) untreated or serum starved (Starve) for 24h. **(E)** Mitochondrial-

lysosomal co-localisation expressed as Pearson's coefficient in fibroblasts from healthy individuals (CTRL) and a Parkinson's disease (PD) patient carrying the R47X mutation (PD R47X) untreated or deprived of serum (Starve) for 24h. **(F)** *Left panel*. Mean average Oxygen consumption rate (OCR) of two healthy control fibroblasts and PD patient carrying the R47X mutation over a time course. Measurement of basal OCR is followed by the addition of oligomycin (oligo) 1μM final concentration, FCCP (FCCP) 1μM final concentration and Antimycin A (Ant A, 1 μM final concentration) and Rotenone (AA) 1μM final concentration. *Right panel*. Statistical analysis showing increased mean average basal OCR in PD patient carrying the R47X mutation compared to the mean average OCR of two healthy control fibroblasts. **(G)** *Left panel*. Extracellular acidification rate (ECAR) normalized to OCR/cell to account for cell numbers of two healthy control fibroblasts and PD patient carrying the R47X mutation over a time course. *Right panel*. Statistical analysis showing no changes in ECAR/OCR under basal conditions, minimal OXPHOS (oligo) and the % shift from basal condition to minimal OXPHOS (glycolytic shift) in PD patient carrying the R47X mutation compared to the mean average OCR of two healthy control fibroblasts. **(H)** Complex I enzyme activity (normalized to citrate synthase enzyme activity) in isolated mitochondria is increased in PD patient carrying the R47X TRAP1 mutation compared to two healthy control fibroblasts lines. **(I)** ATP levels (normalized to total protein) are increased in PD patient carrying the R47X mutation compared to two healthy control fibroblasts lines. **(J)** Mitochondrial ROS levels in a PD patient carrying the R47X TRAP1 mutation (highlighted in red), compared to the mean average mitochondrial ROS measured in healthy controls (healthy), sporadic Parkinson's disease patients (PD), Leigh syndrome patients (Leigh) and familial Parkinson's disease patients (genetic PD, including PINK1, Parkin and DJ-1) in fibroblasts. **(K)** Mitochondrial membrane potential (ΔΨm) is significantly reduced in PD patient carrying the R47X mutation compared to two healthy control fibroblast lines. All statistical tests were the student's T test assuming different standard deviation. Error bars show standard deviation and *=p<0.05, **=p<0.01 and ***=p<0.001.

Figure 5. Metformin rescues the R47X phenotype via a mechanism involving mitochondrial biogenesis

(A) Mitochondrial membrane potential (ΔΨm) is reduced in PD patient carrying the R47X mutation (PD R47X) compared to the mean average of two healthy control (CTRL) fibroblast lines. All fibroblasts were treated with DMSO vehicle (Veh), 0.5mM antioxidant N-acetyl cysteine (NAC), 10 mM Metformin hydrochloride (MET) or 250 nM oligomycin (oligo) for

24h, of which Metformin and oligomycin reverted the phenotype. **(B)** Mitochondrial membrane potential (ΔΨm) is reduced in HeLa cells treated (Tx) with 500µM dopamine (DA), 400µM hydrogen peroxide (H202), 2µM 17-AAG, and 100 nM Valinomycin (Val) but not the DMSO vehicle control (Veh) for 24h. 0.5mM antioxidant N-acetyl cysteine (NAC) rescues the dopamine toxicity to some extent, whereas 10 mM Metformin hydrochloride (MET) rescued the inhibition of TRAP1 by 17-AAG. **(C)** *Upper panel*. The percentage of bound NADH in two healthy control fibroblast lines (CTRL) and in the PD patient carrying the R47X mutation (PD R47X), with or without treatment with 10 mM metformin for 24h. *Bottom panel*. The overall percentage of bound (green) and free (blue) NADH in two healthy control fibroblast lines (CTRL) and in the PD patient carrying the R47X mutation (PD R47X), with or without treatment with 10 mM metformin for 24h. **(D)** Levels of NAD^+ (*left panel*) and total NAD^+/NADH (*right panel*) measured in two healthy fibroblast lines and the D patient carrying the R47X mutation with or without treatment with 10 mM metformin for 24h. **(E)** (*Right panel*). **(F)** Immunoblots (IB) of Hsp60 and mtHsp70 in three independent extractions from two healthy fibroblast lines (CTRL) and the R47X Parkinson's disease patient (*left panel*), quantified for statistical analyses (*right panel*). The student's T test was used assuming different standard deviation to compare patient and control group. Two-way ANNOVA was used to compare cell types and treatments. Error bars show standard deviation and *=p<0.05, **=p<0.01 and ***=p<0.001.

Supplementary Figure 1. Statistical and bioinformatic analyses of TRAP1 Variants in Parkinson's disease patients

(A) Diagram of the TRAP1 amino acid sequence showing ATP binding domains and structural domains including the mitochondrial targeting sequence. The known TRAP1 amino acid modification sites (phosphorylation and acetylation (http://www.uniprot.org/uniprot/Q12931) are shown below indicated in black. The positions of TRAP1 variants identified in this study are shown above and indicated in red. **(B)** *Left panel.* Visualization of the TRAP1 protein structure (PDB: 4Z1F), highlighting the sequence conservation (see top right color code legend) of the investigated variants in the PPMI dataset. *Right panel*. Visualization of the TRAP1 protein structure (PDB: 4Z1F), highlighting the sequence conservation (see top right color code legend) and the investigated variants in the cohort of Tübingen and Vienna PD patients. **(C)** A table showing TRAP1 variants identified from German Parkinson's disease patients, where DNA was collected in Tübingen (Tü) and Austrian Parkinson's disease patients, where DNA was collected in Vienna (Wi) and variants

identified from the Parkinson's progressive markers initiative (PPMI) dataset. Homozygous TRAP1 mutations are shown and heterozygous mutations with low frequency according to the ExAC database http://exac.broadinstitute.org/. Conservation, functional effect prediction and structural predictions of each variant are summarized where possible. **(D)** Burden analysis for TRAP1 using the Parkinson's progressive markers initiative (PPMI) dataset. Controls without variants (co_wo_var), cases without variants (ca_wo_var), controls with variants (co_wi_var), cases with variants (ca_wi_var), odds ratio (OR), lower 95% confidence interval (lower CI), upper 95% confidence interval (upper CI). Significant findings are highlighted in grey.

Supplementary Figure 2. TRAP1 loss of function causes alteration of respiratory complex and P-ERK abundance

(A) A hydropathy plot of the TRAP1 amino acid sequence (from http://web.expasy.org/protscale/), where hydropathy scores closer to the threshold at approximately +1.8 (indicated by the red line) indicates a stretch of high hydrophobicity according to (Kyte and Doolittle, 1982). **(B)** Quantification of the abundance of respiratory complex subunits in fibroblasts from two healthy individuals (CTRL) and the Parkinson's disease patient carrying the TRAP1 R47X mutation (PD R47X) with or without treatment with 10 mM metformin for 24h. Antibodies targeted to nuclear encoded subunits of complex I (NDUFB8), complex II (SDH), complex III (UQCRC2), complex IV (COX-II) and complex V (ATP5A) using Western blotting and normalization to the loading control GAPDH. **(C)** Quantification of Hsp90 in fibroblasts from two healthy individuals (CTRL) and the Parkinson's disease patient carrying the TRAP1 R47X mutation (PD R47X) with or without treatment with 10 mM metformin for 24h and using Western blotting and normalization to the loading control GAPDH. (D) Quantification of phosphorylated ERK (Phospho-ERK) in fibroblasts from two healthy individuals (CTRL) and the Parkinson's disease patient carrying the TRAP1 R47X mutation (PD R47X) with or without treatment with 10 mM metformin for 24h and using Western blotting and normalization to the loading control total ERK. The student's T test was used assuming different standard deviation to compare patient and control group. Two-way ANNOVA was used to compare cell types and treatments. Error bars show standard deviation and *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$.