Parkinson's disease mutations in PINK1 affect synaptic function via Complex I activity in mitochondria

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Supplementary Material and Methods:

Morphometric analysis. Fibroblast cells were seeded at $5x10^5$cell/ml in plastic dishes containing a glass coverslips (Nunc), and after 24h a mitochondrial targeted yellow fluorescent protein (mtYFP) was tranfected with Lipofectamine2000 according to the supplier's protocol (Invitrogen). For epifluorescente imaging of the mitochondrial network, 24h post-transfection medium was replaced with Hanks balanced salt solution (HBBS) and 10mM HEPES and cells were placed on the stage of an Olympus IX81 inverted microscope equipped with a CellR imaging system (Olympus). Cells were excited using a 525± 20nm excitation filter and emitted light was collected using a 40x1.4 NA Plan Apo objective (Olympus). Morphometric analysis was performed as previously described (Cipolat et al., 2004).

Real-time imaging of mitochondrial membrane potential. For evaluation of membrane potential, fibroblast cells were grown in 3cm plastic dishes with glass coverslips (Nunc) and after 424h were loaded with 10nM TMRM (Molecular Probes) in the presence of 2mg/ml cyclosporine H (Sigma) or cyclosporine A, for 30 min at 37°C. Subsequently cells were placed on the stage of an Olympus IX81 inverted microscope equipped with a CellR Imaging system. Sequential images of TMRM fluorescence were acquired every 60s using exposure times of 40ms with a 40x, 1.4 NA Plan Apo objective (Olympus), a 525 ± 20 excitation filter and an emission 570 LP filter.

Flow cytometry. For evaluation of apoptosis, $5x10^5$ cells were grown in 12-well plates and after 24h cells were treated with increasing concentrations of H_2O_2 and Arachidonic acid in HBSS supplemented with 10mM HEPES buffer. After 2h incubation at 37°C cells were harvested and stained with propidium iodide (PI) and Annexin-V-FITC (Invitrogen) according to manufacturer's protocol. Cells were then analyzed by flow cytometry using with a FACSCalibur cytometer (Becton-Dickinson). Viability was measured as the percentage Annexin-V, PI negative cells.

Mitochondrial isolation. Mitochondria were isolated from fibroblast cells and from 2 month old mouse liver and brain by standard differential centrifugation and resuspended in Isolation buffer (IB: 0.2M sucrose, 10 mM Tris-MOPS pH 7.4, 0.1 mM EGTA-Tris pH 7.4) as previously described (Frezza et al., 2007).

Fly heads (brain enriched) and thoraxes (muscle enriched) were collected by vigorously shaking approximately 250 flies snap frozen in liquid nitrogen in a double sieve (top sieve 710 μm pore size, bottom sieve 300 μm pore size). Fly bodies are retained on the top sieve, wings and heads on the bottom sieve, and legs fall through. Thoraxes were manually separated from the abdomens, while wings were blown away gently. Thorax and head tissue was then homogenized and mitochondrial homogenates were purified as described by (Schwarze et al., 1998; Walker et al., 2006).

Cytochrome *c* release ELISA. Cytochrome *c* release in response to recombinant p7/p15 BID was determined as previously described (Frezza et al., 2006). Briefly, 50μg mitochondria were treated with recombinant p7/p15 BID (32 pMol/mg mitochondria) for the indicated times at 25°C. Cytochrome *c* release is reported as the percentage of cytochrome *c* in the supernatant over the total (pellet plus supernatant).

Respiratory Assays. Mitochondrial oxygen consumption was measured by using a Clarke-type oxygen electrode (Hansatech Instruments). Liver mitochondria were incubated in experimental buffer (EB: 125 mM KCl, 10 mM Tris-MOPS, 1 mM KPi, 10 μM EGTA-Tris, pH 7.4, 25°C) supplemented with 5 mM glutamate/2.5 mM malate for analysis of Complex I-driven respiration, 5 mM succinate in the presence of 2 μM rotenone for Complex II-driven respiration, or 3 mM ascorbate plus 150 μM TMPD in the presence of 0.5 μg/ml antimycin A for Complex IV-driven respiration. ADP and FCCP were added at a final concentration of 150μM and 10μM, respectively.

Oxidative phosphorylation complex measurements performed on mitochondrial homogenates from fibroblast cells, mouse brain, fly brain and muscle enriched tissue were analyzed by spectrophotometric assays as previously described (de Paepe et al., 2006). Briefly, measurements of Complex I (NADH:ubiquinone oxidoreductase, rotenone sensitive), Complex II (succinate:ubiquinone oxidoreductase, malonate sensitive), Complex III (ubiquinone:cytochrome *c* oxidoreductase, antimycine sensitive), Complex IV (cytochrome *c* oxidase), Complex V (ATPsynthase, oligomycine sensitive), and citrate synthase enzyme activities were performed. The protein concentration was in the range of 2–4 mg/ml.

Values were plotted according to the ratio between the specific complex's activity and citrate synthase acitivity. For statistical analysis, all measurements were analyzed using a Student's t test.

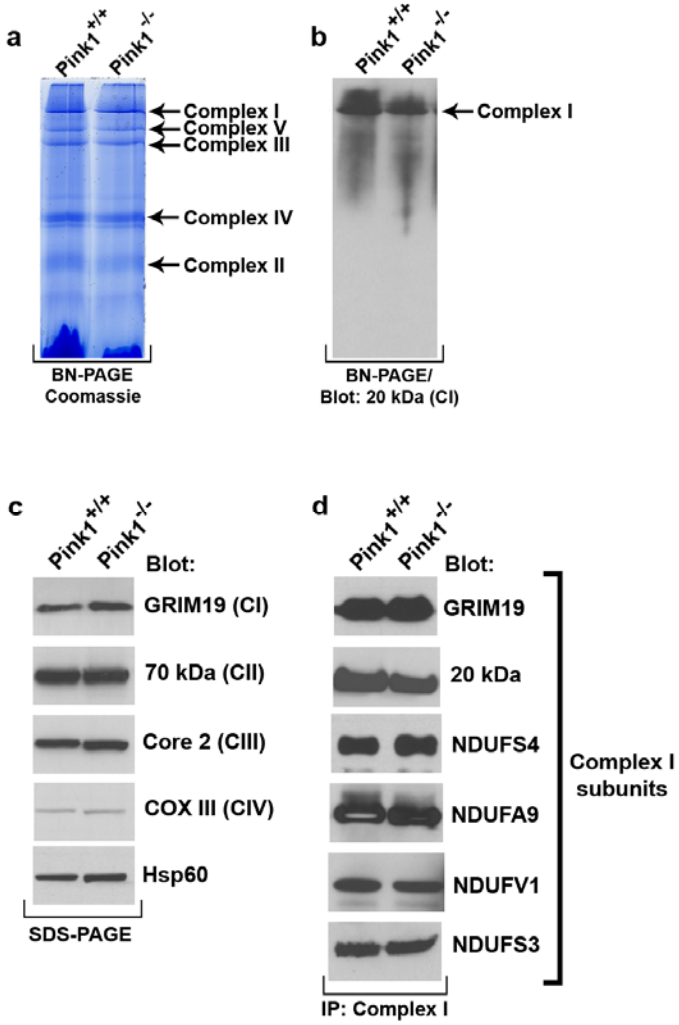
Blue Native Gel Electrophoresis. Mitochondria were isolated from 150mg skeletal muscle from *Pink1*$^{+/+}$ and *Pink1*$^{-/-}$ mouse. Samples were analyzed on a polyacrylamide gel (75µg mitochondrial proteins) and Complexes were separated by BN-PAGE using non-denaturing conditions as described previously (Van Coster et al., 2001). For further Western blot analysis, the gel was transferred onto polyvinylidene difluoride membranes. The blot was de-stained for 1h in distilled water/methanol/acetic acid (40/50/10) and probed with specified antibody.

Protein Analysis. Mitochondria were isolated from *Pink1*$^{+/+}$ and *Pink1*$^{-/-}$ mouse fibroblast cells. Mitochondria enriched fractions were prepared as previously mentioned, and 5µg total protein was analyzed on a 4–12% Bis-Tris NuPAGE gel (Invitrogen). The SDS-PAGE was followed by Western blot analysis using the specified primary antibody followed by the horse-radish peroxidase-conjugated secondary antibody. Visualization was performed using the Renaissance chemiluminescence detection system (Perkin-Elmer).

Antibodies. Monoclonal antibodies against the Complex II subunit 70 kDa, the Complex III subunit Core 2, the Complex IV subunit COXIII, the Complex I subunit GRIM19, 20 kDa subunit, NDUFS4, NDUFA9, NDUFS3 were from MitoSciences. The monoclonal antibody Hsp60, a mitochondria matrix protein, was from BD Biosciences and the monoclonal antibody against the Complex I subunit NDUFV1 was from Abcam. For detection of Complex I on BN-PAGE, antibodies against the 20 kDa subunit were used.

Co-immunoprecipitation. Complex I was co-immunoprecipitated from solubilised mitochondrial proteins as previously described (Keeney et al., 2006). Briefly, 500µg of mitochondrial fraction protein treated with n-dodecyl-β-maltoside, was incubated with Complex I Capture Matrix (MitoSciences). Immunocaptured proteins were eluted with 1% SDS and further analyzed by SDS-PAGE followed by Western blot using the mouse TrueBlot (eBiosciences) as secondary antibody.

Supplementary Figure:



Supplementary Figure 1 Absence of PINK1 does not disturb Complex I assembly

(**a**, **b**)- Solubilised mitochondrial proteins from *Pink1*$^{+/+}$ and *Pink1*$^{-/-}$ mouse skeletal muscle tissue were analyzed by BN-PAGE for (**a**) subsequent coomassie detection of respiratory chain complexes I, II, III, IV and Complex V and (**b**) further processed by Western blot against the 20 kDa Complex I subunit. Bands representing the different complexes are indicated by arrows. Note that in (**a**) different migration patterns were not observed, and in (**b**) different Complex I subunits were not detected.

(**c**)- Mitochondria enriched fractions from *Pink1*$^{+/+}$ and *Pink1*$^{-/-}$ mouse fibroblasts were analyzed by SDS-PAGE followed by Western blot against the following proteins: GRIM19, a Complex I protein; 70

kDa subunit, a Complex II protein; Core 2, a Complex III protein; COX III, a Complex IV protein; and Hsp60, a mitochondrial matrix protein. Hsp60 was used as a control. Note that no significant difference was observed.

(**d**)- Solubilised mitochondrial proteins were co-immunoprecipitated with Complex I immunocapture beads, followed by SDS-PAGE and Western blotting against the following Complex I subunit proteins: GRIM19 (19kDa), 20 kDa subunit, NDUFS4 (18 kDa), NDUFA9 (39 kDa), NDUFV1 (51 kDa) and NDUFS3 (30 kDa). Note that no significant difference was observed in the immunoprecipitated Complex I proteins.

Supplementary References:

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